

Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

IL FUTURO DELLA TERRA, CAMBIAMENTI CLIMATICI E SFIDE SOCIALI

Ciclo XXXV

Settore Concorsuale: 03/C2 – Chimica Industriale

Settore Scientifico Disciplinare: CHIM/04 – Chimica Industriale

**LOW-CARBON INTEGRATED BIOREFINERY FOR THE VALORIZATION OF
AGRO-FOOD WASTE AT A LOCAL SCALE**

Presentata da: Irene Maggiore

Coordinatore Dottorato

Prof. Dr. Silvana Di Sabatino

Supervisore

Dr. Leonardo setti

Esame finale anno 2023

*To my grandmother Anna,
the star inside my heart who always guides me.*

Summary

Climate emergence confronts us with the challenge of applying efficient mitigation and adaptation strategies as fast as possible in order to reach the Paris Agreement's goals. We have the moral obligation to take action at different levels, starting from leaving behind unsustainable development models based on the linear exploitation of fossil resources and moving toward a decarbonized society. In addressing the ecological transition toward a future beyond petroleum, the industry sector should rely on the use of waste biomass as a renewable resource to obtain (bio)products and (bio)energy while limiting the pressure on finite natural resources and reducing the amount of material to be disposed of and. In this regard, biorefineries are tools that make it possible if properly designed. Besides the origin of raw materials, it is their processing that mostly affects the level of greenhouse gas emissions. Considering that long distances are usually covered across an entire production chain, going from the feedstock supply to the product distribution, the shift from a global dimension to a local scale can help to comply with sustainable development criteria. The predilection of small-scale processing does not merely limit transportation and the use of external resources but also allows the application of a bio-based circular economy that can drive the ecological transition by reducing the amount of waste while ensuring profitability. The agri-food sector lends itself well to this approach due to its local connotations. The success of the deployment of small-scale processing lies in performing an efficient and cost-effective valorization of the feedstock into high values products desirable and marketable locally. For this purpose, the design of new conversion routes requiring low-energy demand processes and efficient valorizations of the input stream is crucial.

This Thesis provides an integrated biorefinery model that could encourage the chemical and energy valorization of agro- food wastes on a local scale. The one proposed is a cascade approach that starts with the production of enzymes as specific biocatalysts, continues with the chemical valorization of agro-food waste mediated by the enzymes, and ends with the conversion of the leftover residue into renewable electrical energy. The enzyme production has been carried out via a solid-state fermentation using *Pleurotus ostreatus* grown on a food-waste substrate (hemp bran). This allowed the obtainment of low-cost enzymes capable of hydrolyzing in a selective way a specific substrate, unlocking the potentiality of the (recalcitrant) lignocellulosic material. The produced enzymes have been then used as biocatalysts to mediate the proposed (bio)chemical conversion process of biomass, based on the disassimilation of the plant cell wall into an aqueous solution with the specific aim to extract functional ingredients such as emulsifier, lipid substances, antioxidant molecules which are then recombined in the form of a cosmetic emulsion prototype. Here three examples of agro-food waste are taken as cases of study: hemp oil seed cake, rice grain, and pomegranate pomace. Since the components from disassimilation are specific for each type of food waste, the final product is a high-value product with unique connotations suitable for creating new local markets. The residue obtained from these processes can be used as feedstock into the designed energy plant, currently pending patent. It is based on a new 3D-printed alkaline glucose fuel cell and comprises an upstream alkalization unit and a downstream neutralizer. The latter could give as final waste an exhausted organic residue with a potential soil improvement function, enabling the closure of the food cycle. The realized fuel cell operates at ambient conditions, both in static and

flux mode, has organic-based electrodes, and it is free from metallic catalysts. It uses sodium hydroxide to catalyze the formation of enediol species from biomass, which are then used as biofuel. This allows the sterility of the feedstock, ensuring low sensitivity toward the operative and environmental conditions and low selectivity toward the input stream. Indeed, the fuel cell was powered with both glucose solutions and with a real and complex matrix that is the residue from the pomegranate pomace (bio)chemical valorization. In the first case, it generates up to 1.45 mW/cm², in the second 0.77 mW/cm², corresponding respectively to 142 mW/unit and 75.5 mW/unit of power output. However, higher performances are reachable when more units are stacked into modules by series and parallel connections. Thus, effective integration of electrical energy can be provided to the upstream processes, making the whole biorefinery energetically sustainable.

Table of contents

1	Introduction	1
1.1	The current state of climate emergence	1
1.2	Circular economy to tackle climate change	4
1.3	Biorefinery and its sustainability	7
1.4	High-production scale vs. small-production scale	10
1.5	Biorefining practices for the valorization of agro-food waste	12
	Dissertation objectives	21
	Results and Discussion	22
	Dissertation outlines	
2	At the upstream of biorefinery	24
2.1	The context: valuable waste from agro-food chain	24
2.2	At the upstream of biorefinery	25
2.3	Information to support strategy: specific enzymes for specific substrates	26
2.3.1	A look inside the vegetable waste	26
2.3.2	Biotech biomass deconstruction	32
2.3.3	Biocatalyst production	39
2A	Case of study: Production of enzymes by SSF of <i>Pleurotus ostreatus</i> on Hemp Bran	44
	Materials and Methods Chp. 2	54
3	The biorefinery core	58
3.1	The context: conversion of agro-food waste into high-value products	58
3.2	The biorefinery core	59
3.3	Information to support strategy: from vegetable waste to biocosmetics	62
3.3.1	Plant adaptation capacity	62
3.3.2	Human skin adaptation capacity	65

3.3.3	A new waste-value chain perspective	67
3.3.4	Bio-based emulsions	68
3A	Case of study: biorefining of Cannabis sativa L. (hemp) seed cake	75
3B	Case of study: biorefinery of Oryza sativa (rice) grain	89
3C	Case of study: biorefining of Punica granatum (pomegranate) pomace	103
	Materials and Methods Chp. 3	133
4	The biorefinery integration	144
4.1	The context: conversion of agro-food waste biomass into electrical energy	144
4.2	The biorefinery integration	145
4.3	Information to support the strategy: customize fuel cell devices	151
4.3.1	The abiotic biofuel cell	151
4.3.2	Alkaline glucose biofuel cell	154
4.3.3	Fuel cell characterization	158
4.3.4	The 3D-printing	162
4A	Design studies for 3D-printed abiotic glucose biofuel cells	167
4B	3D-printed tubular fuel cell applications	201
	Materials and Methods Chp. 4	217
	Overall Conclusions	223
	References	227
	Acknowledgments	266

1 Introduction

1.1 The current state of climate emergence

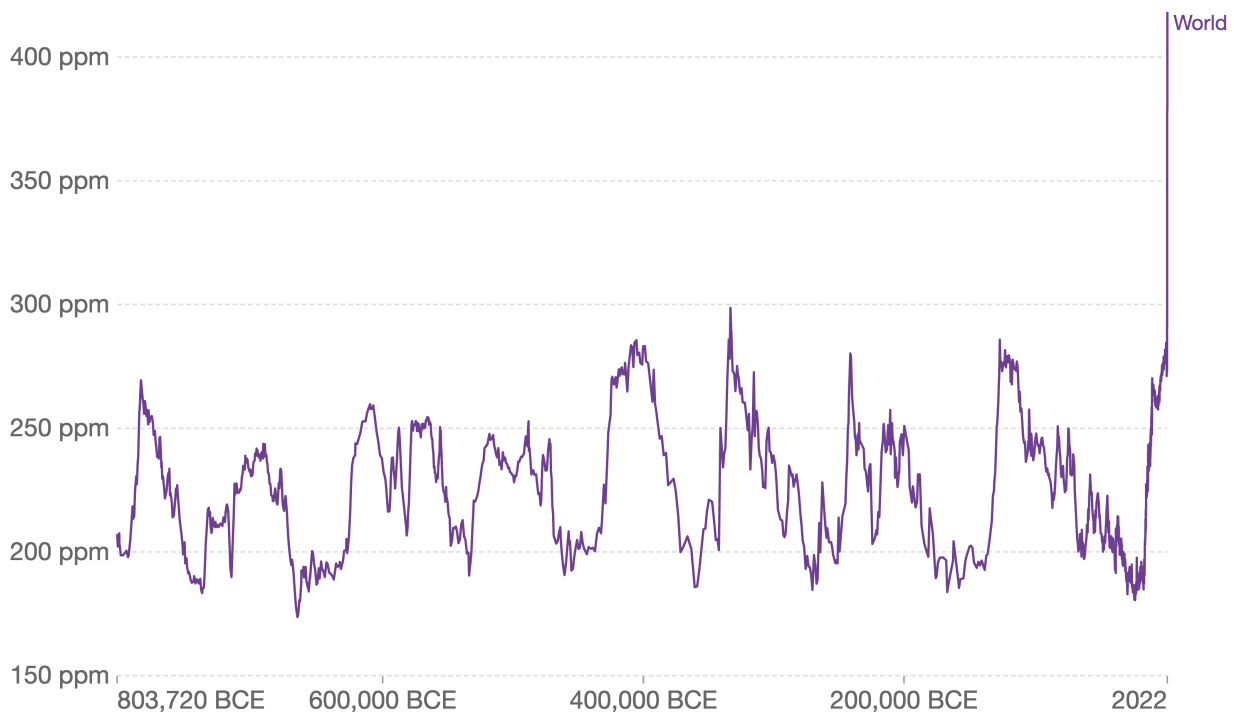
Climate change refers to changes in climatic conditions caused primarily by greenhouse gas emissions, which lead to global warming with consequences we are experiencing more every day. Indeed, greenhouse gas emissions cause heat retention in the Earth's atmosphere, which is the leading cause of global warming. The greenhouse gases include carbon dioxide (CO₂), methane (CH₄), nitrous oxide (N₂O), and fluorinated gases such as hydrofluorocarbons (HFCs), perfluorocarbons (PFCs) and sulfur hexafluoride (SF₆) (UNFCCC, 2008).

Greenhouse gases are released spontaneously during natural phenomena like forest fires, earthquakes, oceans, permafrost, swamps, mud volcanoes, and volcanoes (Xi-Liu & Qing-Xian, 2018), but nowadays is well known that anthropogenic activities are the main drivers of their excessive accumulation, especially in CO₂, causing uncontrolled global temperature rising rate (Fig.1)

Global atmospheric CO₂ concentration

Atmospheric carbon dioxide (CO₂) concentration is measured in parts per million (ppm). Long-term trends in CO₂ concentrations can be measured at high-resolution using preserved air samples from ice cores.

Our World
in Data



Source: National Oceanic and Atmospheric Administration (NOAA)

CC BY

Fig.1 Global atmospheric CO₂ concentration (ppm) (<https://ourworldindata.org/co2-and-other-greenhouse-gas-emissions>).

The Intergovernmental Panel on Climate Change (IPCC) stated in its AR5 assessment report (IPCC, 2014):

“Anthropogenic greenhouse gas emissions have increased since the pre-industrial era, driven mainly by economic and population growth, and are now higher than ever. This has led to atmospheric concentrations of carbon dioxide, methane and nitrous oxide that are unprecedented in at least the last 800,000 years. **Their effects, together with those of other anthropogenic drivers, have been detected throughout the climate system and are extremely likely to have been the dominant cause of the observed warming since the mid-20th century**”.

Human activities like energy production, industrial activities, forestry, and land use have exerted even more pressure on the natural system, compromising its capability to promptly restore the broken balances (Xi-Liu & Qing-Xian, 2018).

According to the emissions gap report prepared by the United Nations Environment Programme (UNEP) in 2019, total greenhouse gas emissions in 2018 amounted to 55.3 GtCO₂e, of which 37.5 GtCO₂ are attributed to fossil CO₂ emissions from energy production and industrial activities (Fawzy, Osman, Doran & Rooney, 2020).

The ongoing climate change affects our everyday life since we witness ever more extreme and disastrous events, including droughts, floods, hurricanes, severe storms, heatwaves, wildfires, cold spells, and landslides (UNCCS, 2019).

In addition, climate risks of temperature variations, precipitation unpredictability, shifting seasonal patterns, disease distribution changes, desertification, ocean effects, and degrading soil and coastal areas make many nations more vulnerable in various sectors (UNCCS, 2019).

Only in 2018, the world encountered 315 cases of natural disasters climate-related, and 68.5 million people were affected by natural disasters like floods, storms, and droughts. The Centre for Research on the Epidemiology of Disasters (CRED) speaks of 16 cases of drought, 26 cases of extreme temperature, 127 cases of flooding, 13 cases of landslides, 95 cases of storms, and 10 cases of wildfire. In terms of economic losses, this meant a total of \$131.7 billion lost in 2018 due to natural disasters, with storms (\$70.8B), floods (\$19.7B), wildfires (\$22.8B), and droughts (\$9.7B) accounting for approximately 93% of the total costs (Fawzy et al., 2020).

The chart in Fig.2 depicts future greenhouse gas emissions scenarios under various assumptions: if no climate policies were implemented; if current policies were maintained; if all countries met their current future pledges for emissions reductions; and the necessary pathways compatible with limiting warming to 1.5°C or 2°C this century (<https://ourworldindata.org/co2-and-other-greenhouse-gas-emissions>). It is almost clear that implementing climate and energy policies would reduce warming to a significant extent.

Global greenhouse gas emissions and warming scenarios



- Each pathway comes with uncertainty, marked by the shading from low to high emissions under each scenario.
- Warming refers to the expected global temperature rise by 2100, relative to pre-industrial temperatures.

Annual global greenhouse gas emissions
in gigatonnes of carbon dioxide-equivalents

150 Gt

100 Gt

50 Gt

Greenhouse gas emissions
up to the present

0

1990 2000 2010 2020 2030 2040 2050 2060 2070 2080 2090 2100

Data source: Climate Action Tracker (based on national policies and pledges as of November 2021).
OurWorldinData.org - Research and data to make progress against the world's largest problems.

Last updated: April 2022.
Licensed under CC-BY by the authors Hannah Ritchie & Max Roser.

No climate policies
4.1 – 4.8 °C

→ expected emissions in a baseline scenario if countries had not implemented climate reduction policies.

Current policies
2.5 – 2.9 °C

→ emissions with current climate policies in place result in warming of 2.5 to 2.9°C by 2100.

Pledges & targets (2.1 °C)
→ emissions if all countries delivered on reduction pledges result in warming of 2.1°C by 2100.

2°C pathways
1.5°C pathways

Fig.2 Global greenhouse gas emissions and warming scenarios (<https://ourworldindata.org/co2-and-other-greenhouse-gas-emissions>).

An assessment presented in 2018 in a special report by Intergovernmental Panel on Climate Change (IPCC, 2018), covered the impacts and projected risks associated with two levels of global warming, 1.5 °C, and 2 °C.

The paper investigated the detrimental effects of global warming on freshwater supplies, food security and production systems, ecosystems, human health, urbanization, poverty, and community change. It also looked at the effects of climate change on important economic sectors, including tourism, energy, and transport.

What results is that most of the impacts assessed have lower associated risks at 1.5 °C compared to 2 °C warming level. Water stress would carry double the risk under a 2 °C level compared to 1.5 °C. An increase of 70% in population would be affected by fluvial floods under the 2 °C scenario compared to 1.5 °C. Double or triple species extinction rates in terrestrial ecosystems are projected under the 2 °C level compared to 1.5 °C (IPCC, 2018).

We would reach 1.5 °C within the next three decades and keep the increase in warming levels beyond this point to not witness an amplified risk effect. However, the efforts carried out until now have not been sufficient in limiting the effects of climate change.

Acknowledgment of climate change realities started in 1979 when the first world climate conference was held in Geneva. Then, in 1988, the Intergovernmental Panel on Climate Change (IPCC) was set up to provide governments and official bodies with scientific knowledge and information that can be used to formulate climate-related policies. Then there was the adoption of the UNFCCC in 1992,

which went into force in 1994. It has been the most critical step and the main driving force of global climate action. The convention's main objective is stabilizing greenhouse gas concentrations in the atmosphere by engaging developed countries that were identified as the major ones responsible for producing anthropogenic emissions. The target was to reduce emissions by 2000 to the levels achieved in the previous decade (Fawzy et al., 2020).

During the third UNFCCC conference of the parties (COP-3) in 1997, the Kyoto Protocol was adopted and went into force in 2005. The protocol established monitoring and reporting systems and all related policies to achieve those targets. As a result, emission reduction has mainly been achieved through introducing renewable energy, energy efficiency, and afforestation/reforestation-related projects (Fawzy et al., 2020).

The Kyoto Protocol defines four emission-saving units, each representing one metric ton of CO₂ equivalent, and are all tradeable (UNFCCC, 2005). In 2012, the Doha amendment to the Kyoto protocol was adopted, mainly proposing a second commitment period from 2013 to 2020.

During the twenty-first UNFCCC conference of the parties (COP-21) held in Paris in 2015, the **Paris Agreement** was adopted and entered into force in 2016. It added further objectives, commitments, enhanced compliance and reporting regulations, and support mechanisms to the existing climate change combat framework. The agreement's principal goal is to pursue efforts to keep the rise in global temperature **to 1.5 °C and keep it to 2 °C by 2100** in order to achieve equilibrium between human-induced emission sources and greenhouse gas sinks and reservoirs as soon as possible. The agreement also included additional, legally enforceable obligations that required all parties to make and enforce national laws to carry out those commitments and try to go above and beyond them (Fawzy et al., 2020).

According to route modeling for a 1.5 °C warming scenario, anthropogenic greenhouse gas emissions must decrease by 45% from 2010 levels by 2030 and reach net zero by 2050.

In order to keep global warming below 2 °C by the end of the century, emissions must decrease by around 25% from 2010 levels in 2030 and reach zero emissions by 2070 (IPCC, 2018).

To meet the 1.5 °C target by the end of the century, the IPCC stated that by 2030 greenhouse gas emissions should be maintained at 25–30 GtCO₂e per year. In comparison, the current unconditional nationally determined contributions for 2030 are estimated at 52–58 GtCO₂e per year (Fawzy et al., 2020). Thus, to reach the temperature goals set by the Paris Agreement, coordinated and globally shared mitigation and adaptation actions are even more required.

1.2 Circular economy to tackle climate change

The globally shared climate mitigation targets point to reducing global warming below 1.5°C compared to the pre-industrial levels and reducing carbon emissions by 45% by 2030 until reaching carbon neutrality by 2050, as established by the Paris Agreement (Fawzy et al., 2020).

For these purposes, a great deployment of mitigation approaches has been discussed in the literature. First, there are the so-called conventional strategies, which include techniques to reduce CO₂ emissions, such as using and producing renewable energy, switching fuel from fossil-based to

renewable energy, and integrating renewable power technologies to reach efficiency gains. There are also more innovative so-called negative emissions technologies, which aim to capture and sequester CO₂ from the atmosphere and include, among others, bioenergy carbon capture and storage, biochar, direct air carbon capture and storage, ocean fertilization, ocean alkalinity enhancement, afforestation and reforestation. Finally, the most cutting-edge technologies go as far as managing solar and terrestrial radiation (Fawzy et al., 2020).

Mitigation efforts for climate change are necessary but frequently considered as expenditures.

In this regard, bioeconomy, which improves the utilization of raw resources to produce goods with added value while reducing waste, can ensure competitiveness and profitability, fostering mitigation actions.

A circular economy approach (Fig.3) can be used to front climate change because it prevents the consumption of resources and can reduce carbon emissions, optimizing the structure of the energy and material cycle by the principles of eliminating wastes, recirculating materials, and regenerating ecosystems (Yang et al., 2022).

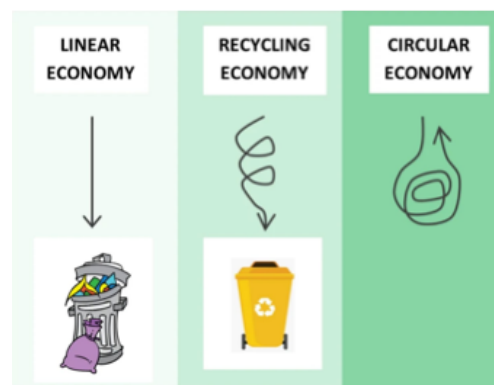


Fig.3 Transition from linear recycling to circular economy approach in relation to waste management (Yang et al., 2022).

The application of circular economy strategies in tackling the effects of climate change can be effective in different areas of applications, from industry to waste disposal, from energy use to building and transportation, and at various levels, from micro (consumers and enterprise) to macro levels (cities, regions, nations) (Rincón-Moreno, Ormazábal, Álvarez & Jaca, 2021). For example, if considered the food industry, a circular economy not only can improve waste management while boosting the value of by-products but also can drive a sustainable change in the supply chain, reducing the scale of food consumption and shortening the entire food chain through the reduction of nonrenewable resources, the increase of externally recyclable resources, and the recovery of internally generated waste. Furthermore, since generally food is wasted due to damage or spoilage caused by long supply chains and longer transportation distances, the local-processing approach can save food availability and optimize the use of resources in food processing, water first of all (Hoekstra & Chapagain, 2007), reducing the pressure on land and environment.

To improve industrial optimization, environmental protection, and economic benefits, the local-dimension perspective can be extended to all the industry sector and all sector's branches, as occurs in the **eco-industrial park** (Fig.4), in which waste recovery, treatment, production, packaging, and marketing are concentrated on one single site (Fan, Qiao & Fang, 2017).

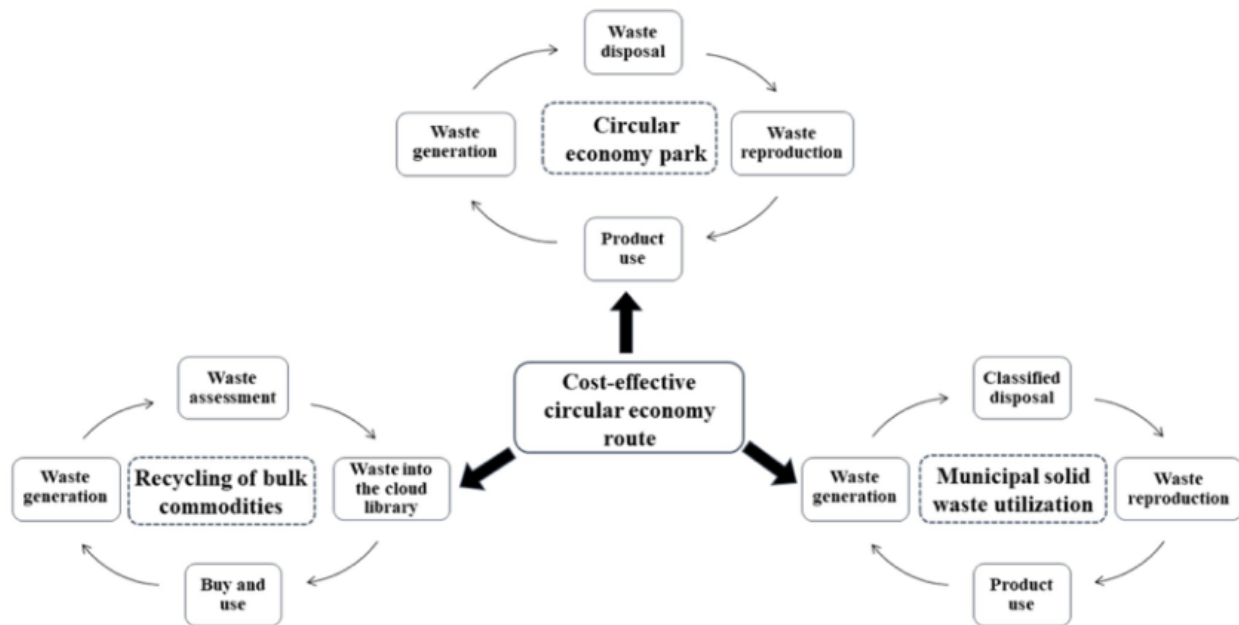


Fig.4 Cost-effective circular economy route map based on a circular economy industrial park, the recycling of bulk commodities, and a municipal solid waste utilization system (Yang et al., 2022).

To address the climate crisis, the European Union has established a new bioeconomy approach, called **circular bio-based economy (CBE)**, based on the principles of sustainability, circularity, and inclusivity (Fritsche & Iriarte, 2014) and centered on the use of aquatic and forestry biomass, including by-products and side-stream of other industries. It was made enforceable by a public-private partnership, the Bio-based Industries Joint Undertaking (BBI- JU) (<https://wayback.archive-it.org/12090/20221125105151/https://www.bbi.europa.eu/>), which created a new program of research and development based on sustainable technologies to convert biomass while preserving biodiversity. These technologies include biotechnology, enzyme applications, green-chemistry pillars, and advanced physical processing. Through **microbial-based systems**, it is possible to produce bio-actives and biomaterials not easily achieved by chemical methods and with a lower cost (Lange et al., 2021). Using properly **enzymatic reactions**, it is possible to fully exploit the lignocellulosic material potential as a chemical building block reservoir, overcoming the recalcitrant biomass conversion constraint. The production of a designed blend of enzymes is designated as a method to effectively disrupt the lignocellulosic structure while keeping the cost of enzymes down,

providing the basis for a new bio-based production of fuels, polymers, materials, feed additives, food ingredients, cosmeceuticals and pharmaceuticals (Lange, 2017).

Using green chemistry and combining chemical, biochemical and physical routes enables the most efficient feedstock conversions (EPA, 2017).

The application of these approaches contributes to meeting the UN Sustainable Development Goals (SDGs) (UN SDGs, 2020), especially:

- SDG #2 Zero Hunger, producing more food and feed from what is currently being wasted to feed a rising population.
- SDG #3 Good Health and Well-being, by developing biologically based health-promoting goods that boost gut health for increased human and animal resilience or decrease the demand for antibiotics in animal husbandry
- UNSDG #6 Clean Water, by develop of bio-based plant disease and pest management to replace chemical pesticides and ensure access to pure water
- SDG #14, Life Underwater and SDG #15, Life on Land, by preserving the world's terrestrial and marine biosphere by exploiting biological resources in an effective and responsible manner

And, more directly:

Clean Energy (SDG #7), Decent work and Economic growth (SDG #8), Industry Innovation and Infrastructure (SDG #9), Sustainable cities and communities SDG #11), Responsible Consumption and Production (SDG #12), and Climate Action (SDG #13).

The circularity and the efficiency of material flow that characterize the new bio-based economy can be expressed by the establishment of biorefinery, which is defined by Cherubini (2010) “facility (or network of facilities) that integrates biomass conversion processes and equipment to produce transportation biofuels, power, and chemicals from biomass. In particular, the circular bio-based economy represents a strong driver for developing decentralized and small biorefineries, in contrast to the initial phase of the bioeconomy, “where the low profit gave incentives for very large installments” (Lange et al., 2021).

1.3 Biorefinery and its sustainability

The biorefinery concept is able to condense the circular economy principle of an integrated supply chain, where a byproduct from one industry can become a resource for another.

According to Paini et al (2021), a biorefinery is a facility that allows the efficient conversion of biomass utilizing a variety of processes to produce a wide variety of products. This definition is conceptually similar to oil refineries, moving the product from a strictly biomass-to-energy route to the extraction of different compounds for diverse markets (ingredients for food and feed, fine

chemicals, materials, etc.). The main difference with respect to the oil refinery is that the fossil resources are concentrated in a few places around the world in a condensed form. This implies the establishment of a few big refineries occupying large sites, requiring huge investments. Instead, biomass is widespread worldwide, so more decentralized facilities are suitable for its valorization into higher-value products. As a result, improved use of biomass is achieved, producing valuable compounds from the whole feedstock while reducing the amount of waste to dispose of (Lange & Meyer, 2019). A sustainable resource cycle is possible if the biomass to be converted is the waste itself, starting from the food byproducts capable of providing a wide range of human-beneficial nutrients, besides restoring fertility in degraded land and optimizing the use of fertilizers (Paini et al., 2021).

However, sustainability should not be taken as a goal but also as a rule for designing processes to encourage a “development that meets the needs of the present without compromising the ability of future generations to meet their own needs” (Martin & Grossmann, 2013). Moreover, it is not easy to be pursued since it integrates economic, environmental, and social aspects that should be included and validated through robust calculations (Julio et al., 2017).

To accomplish a sustainable biorefinery, the design process should consider biomass as raw material to be exploited integrally and the product to be obtained as a real possibility for the specific market context.

Several authors have proposed different approaches to guide the design of a biorefinery. The optimization-based approach aims to maximize the yield or the minimum product cost through a preliminary analysis of conversion routes pointing to the critical steps of the process to be integrated with other technologies, to achieve the established purpose (Santibañez-Aguilar et al., 2014). The early-stage approach is similar to the oil-refinery one because it bases its assessment on a 100% product recovery assumption and avoids preliminary detailed process modeling. The **knowledge-based** approach focuses on the technical, economic, environmental, and preliminary social analysis by applying the concepts of hierarchy, sequencing, and integration (Fig.5) (Cardona-Alzate et al., 2020).

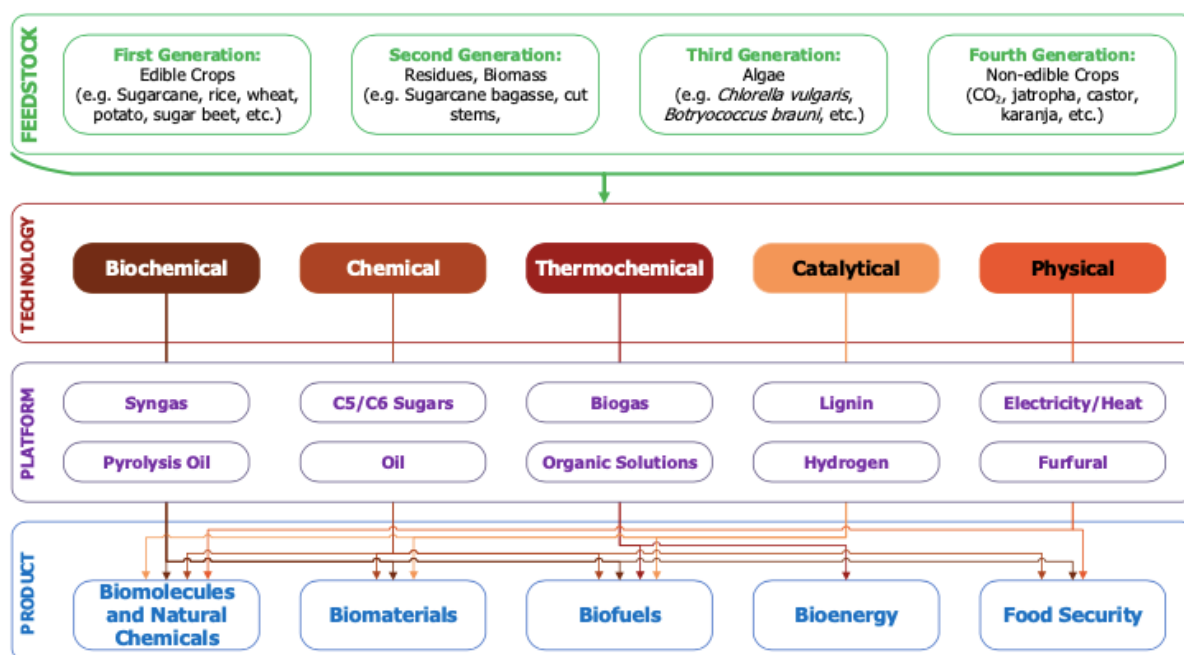


Fig. 5 Distribution of biomass types, building blocks (platforms), and possible products (Cardona-Alzate et al., 2020).

The hierarchy concept links the raw material components with the possible chemical building blocks to obtain and the specific products they can convert through a certain technology. Depending on the raw material origin, biorefineries are classified into the first (food crops), second (waste), third (algae), and fourth generation (not edible crops). The considerations about the choice of technologies to be used in the conversion should be centered on the step that most affects each process. The potential product categories are biofuels, bioenergy (e.g., power and heat), food products, biomaterials, and biomolecules. The sequencing step establishes a logical order and relation between technologies and products (Moncada, Tamayo & Cardona, 2014). Therefore, the first step must be selecting desired products, defining a main product and by-products, and the available technologies. Finally, the integration considers the mass and energy balances intending to optimize the use of reactants and the energetic efficiency of each stage in the biorefinery (Moncada, El-Halwagi & Cardona, 2013).

The biorefinery scale can be a crucial parameter to define the sustainability of a biorefinery since it can be related to the feedstock amounts to be processed and the products to be obtained. In addition, it is influenced by important factors like government, environmental policies, and market conditions (Daoutidis et al., 2013). However, the definition of scale is ambiguous because it is susceptible to different interpretations depending on the supply of raw materials, the flow of products, or the economic feasibility.

According to the supply of raw material, the scale could be assimilated to the ‘processing scale,’ which refers to the ‘amount of raw material processed in a period.’ Therefore, a biorefinery that processes raw materials such as sugarcane, palm, corn, and their respective wastes could be considered a large scale because the amount of feedstock available is very high.

This criterion is generally used to distinguish pilots from demonstrations and commercial plants, which can process 1-10 tons/day, 10-100 tons/day, and above 100 tons/day.

Other authors report that small-scale and pilot plants can have 1,500-7,500 tons/year of input of biomass and that at the commercial level; small-scale is within the range of 100-500 ton/day of input of biomass for bioenergy production, and 5,000-15,000 ton/year of product for biochemicals ([Cardona-Alzate et al., 2020](#)).

According to the flow of products, the scale could be assimilated to the 'production scale,' which refers to the 'amount of product obtained in a period.' Therefore, a biorefinery that produces bulk chemicals such as ethanol, sugar, butanol, and biodiesel could be considered as large scale, and one that produces lactic acid, polyhydroxybutyrate (PHB), and citric acid could be considered as small scale.

On the contrary, the economic approach defines the 'minimum processing scale at which the process presents a feasible economic performance.' It is based on an economic evaluation of the process and a sensitivity analysis of how the economic performance of the process [Net Present Value (NPV)] changes with the flow of raw material up to the equilibrium point (NPV at the end of the lifetime of the process equals to zero) is identified ([Cardona-Alzate et al., 2020](#)).

An example of a small-scale biorefinery is using fruit peels for the extraction of essential oils, colorants, and pectin. An example of a large-scale biorefinery is using the palm to produce biodiesel, ethanol, and electricity. In this case, both biorefineries include both processing and production scale perspectives (raw material and product). They depend on the amount of feedstock, which limits the production of a given product. This is why feedstocks such as sugarcane and palm are used in large-scale biorefineries to obtain bulk chemicals such as biodiesel, ethanol, and electricity. On the other hand, fruit peels and feedstocks in lower amounts are generally used for the production of specialty chemicals that have higher benefits and hence give more economic gains.

1.4 High-production scale vs. small-production scale

It is possible to obtain both bulk and specialty chemicals in both small and large-scale biorefineries ([Cardona-Alzate et al., 2020](#)). The difference relies on the factors that affect each production dimension, which can drive the decisions and investments in one direction or the other. Generally, large-scale are more affected by feedstock cost because, if considering, for example, fuel and bulk chemical productions, they require large quantities of raw materials as input flow and high uniformity in their chemical-physical composition. This can be easily achieved if feedstock from dedicated crops is used, which implies costs for transportation of raw materials from the fields to the factories and for forestry management, including the development of agriculture techniques, farmers' employment, the constant and massive use of natural resources to ensure the quality of the harvest. In large-scale facilities, these costs account for early 50% of the total cost of the finished product ([Bruins & Sanders, 2012](#)). However, they are charged by the principle of economy of scale, which ensures low utility costs because of the use of heat exchange networks and integrated systems. This makes the costs of buildings, infrastructure, and storage affordable and also lowers the costs for research and development, advertising, administration, and expensive skilled operational personnel. On the other hand, small-scale biorefineries instead resulted in being more affected by the utility costs because, while the costs for raw materials procurements can be lowered

using waste generated in the vicinity of the facility as feedstock, the cooling, heating, instrumentations air are generally fully supplied, thus resulting from being more expensive. In this regard, design implementation is crucial to contain the operational costs as much as possible, not to hinder the advantages of small-scale processing.

The main advantages of small-scale processing are related to transportation, local reuse of biomass resources, farmer benefits, and research innovations. For example, the possibility of pretreatments like densification or dewatering, as well as the separation of minerals and low-value organic materials that can be left directly on site to be used as soil fertilizer, reduce transportation movements for raw materials but also for products. Furthermore, local or decentralized pre-processing involves the integration of part of the agricultural value chain into the farm, leading to a redistribution of income in the chain (Bruins & Sanders, 2012), creating benefits also for the farmers, which could conduct the (pre)-processing by themselves.

A key factor for the success of small-biorefinery is the flexibility and robustness of the processes in response to the high variability of the feedstock, given that waste raw materials are affected mainly by seasonality or storage conditions. It follows the need to select the most suitable processes to convert local biowaste streams into locally desired products. Furthermore, considering the emerging self-sufficiency of local communities, the development of local products can be more appreciated because they can be sold for higher prices.

Bruins & Sanders (2012) provided a series of tools to drive the implementation of local processing design. They can be summarized into process splitting, low investments and heat exchanger costs, process combination, and energy integration. The separation of the processes can be driven by the season dependence and product deterioration so that one part can be carried out on-site, leading to intermediate products that could be stored for further use or process; the other part, preferably the more energy-intensive, can be carried out by more extensive and centralized factories. This expedient can lower the investment costs, reachable also by careful process design to minimize the energy requirements and heat transfers as much as possible. For this purpose, biotechnological processes like fermentation and enzymatic reactions should be preferred since they operate at low pressure and temperature; highly endothermic (for the energy to be supplied) and highly exothermic (for heat to be removed) should be avoided. If higher productivity wants to be reached, the use of more connected conversion units in place of larger ones can lead to better heat and mass transfer control and limit the expenditures for heat exchangers. Integrating the energy requirements with on-site energy production, for example using biogas / combined heat and power units, is a tool to make feasible small-scale operations. Lange et al. (2021) identify the small biorefinery as a hub around which small-medium enterprises and startups can develop to foster regional and local development via integration with primary production, innovative routes, and new value chains.

1.5 Biorefining practices for the valorization of agro-food waste

Second-generation biorefinery should be preferred in converting biomass into useful chemical and energy commodities and building blocks because waste biomass represents a sustainable and renewable source of feedstock. In addition, it is widely available and low cost, and its use limits the pressure over finite natural resources because it does not subtract land with a dedicated crop and does not compete with food production.

Food waste (FW) is one of the world's most generated bio-wastes. About 1.3 billion tonnes of food ([Paritosh et al., 2017](#)) and one-third of the total global food production is wasted each year, costing the world economy about US \$ 750 billion ([Gustavsson, Cederberg, Sonesson, van Otterdijk et Meybeck, 2011](#)).

According to the Food and Agriculture Organization (FAO), food losses refer to “the decrease in edible food mass throughout the part of the supply chain that specifically leads to edible food for human consumption” ([Gustavsson et al., 2011](#)). There are various stages in the food supply chain where waste is typically generated. These stages are post-production, handling and storage, manufacturing, wholesale and retail, and consumption. Pest infestations, spillage, spoilage, and loss of quality during storage can be the main reasons for the loss of agricultural production after harvesting. In the manufacture of products, waste is generated in the form of pomace or spent food material during processing stages such as peeling, washing, boiling, and slicing, or in the form of losses by process interventions, for example, for the plant shutdowns or washing ([Ravindran & Jaiswal, 2016](#)). Specifically, processing fruits and vegetables globally produces 16% of the food processing waste, with 48% of fruits and vegetable's edible mass discarded at the production stage ([Paini et al., 2021](#)).

Moreover, processed fruits and vegetables that are significantly wasted are organic waste that can negatively affect the environment if incorrectly disposed of ([Paini et al., 2021](#)). Either way, the whole agri-food sector contributes to environmental degradation and greenhouse gas (GHGs) emissions at all stages (i.e., production, processing, consumption, and disposal), emitting every year approximately 19–29% of the total GHGs ([Loboguerrero et al., 2019](#)). Moreover, the Agri-food industry is also a significant resource-depleting activity. For example, a study made by Kummu et al. ([2012](#)) estimated that almost one-fourth of resources (i.e., freshwater, cropland area, fertilizers) are spent on producing lost and wasted food crops ([Paini et al., 2021](#)). Therefore, integrating the agro-food supply chain with biorefinery facilities aims to extract value from sustainable resources and mitigate its environmental impact.

Fig. 6 provides a comprehensive overview of the various products that can be derived from the waste of the food supply chain.

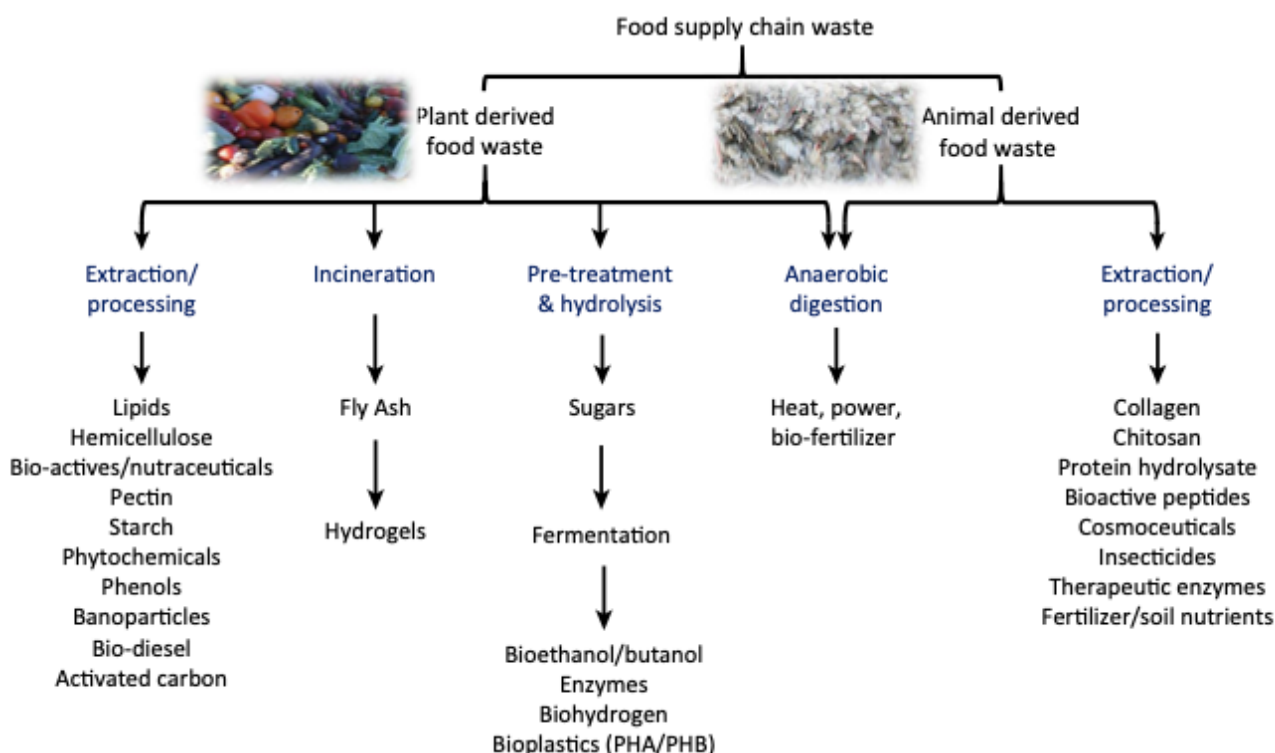


Fig.6 Possible Commercial Products from Food Supply Chain Waste (Ravindran & Jaiswal, 2016).

The biorefinery approach can result in various products like biofuels (H_2 , CH_4 , biohythane, biodiesel), platform chemicals (sugars, carboxylic acids, bioethanol, biobutanol), bioelectricity, biomass, biomaterials (biopolymers), biofertilizers, animal feed, etc., with enhanced waste treatment efficiency (Dahiya et al., 2018)

The conversion technology choice depends mainly on the feedstock's nature, which means the heterogeneity, biodegradability, and general physicochemical properties of the AFW (Lee et al., 2019), which are generally characterized by high moisture content. Other factors that determine the conversion technologies are the operational costs of the designed process, the management of by-products, and the end-use applications (Kassim, Thomas et Afolabi, 2022).

A strategy to extract high-value components with economic feasibility is the integration of individual approaches, capable of separating the components of interest, in order to use the effluent of one process as starting material for the subsequent or the energy produced in one step to power another process of the plant. This helps to achieve whole-waste exploitation by providing a range of components, from high value to low value, with reduced use of chemicals and energy.

The recovery of biomolecules from agro-food waste, such as carbohydrates, lipids, proteins, polyphenols, pigments, and other antioxidant molecules, is generally conducted with physical and chemical methods or their combination. Common **methods for the extraction of biomolecules** from agro-food waste are the following.

Solid-Liquid Extraction exploits the principle of solubility of the components contained in the solid waste to be extracted into a liquid solvent. Generally, it is an organic solvent alone or mixed with

water, representing the main disadvantage of the process: the use of hazardous chemicals, which are inflammable, explosive, and partially toxic (Baiano, 2014). On the other hand, it is easy to perform and can be optimized based on temperature, time, solid-to-liquid ratio, particle size, stirring rate, and solvent polarity.

Soxhlet Extraction relies on the same principle of solid-liquid extraction but uses less solvent recirculated through the solid matrix. It can process a low quantity of solid waste at a time, thus is generally used for analytical determinations and has limited application in food processing (extraction of essential oils and volatile compounds from botanical species). However, compared to the other methods, it has more advantages, such as industrial applications, reproducibility, and efficiency (Baiano, 2014).

Supercritical Fluid Extraction is similar to Soxhlet Extraction, but the solvents used are near their supercritical region. These conditions (high temperature and low pressure) enable higher solute solubility and higher diffusion rate into the solvent. Carbon dioxide is the most used supercritical fluid due to its moderate critical conditions (31.1 °C and 73.8 MPa), absence of toxicity, and chemical stability. Despite the high investment cost and the requirement of highly skilled workers, it is one of the most efficient green extraction techniques for polar compounds (Yu et al., 2018).

Ultrasound-Assisted Extraction occurs thanks to the propagation of the sound waves in the matter, with frequencies higher than 20 kHz, through expansion and compression cycles. This disrupts biological cell walls, thus facilitating the release of contents, and greater solvent penetration into cellular materials, improving mass transfer. The main disadvantages of this type of extraction are the ultrasound attenuation exerted by the presence of a dispersed phase and differences in compressibility, heat capacity, and thermal diffusion between the droplets of the dispersed phase and the continuous primary phase (Baiano, 2014).

Microwave-Assisted Extraction exploits the dielectric constant of the molecules, causing the material to heat up by ionic conduction and dipole rotation. For this reason, microwave-assisted extraction is suited for extracting compounds of medium to high polarity from a solid matrix. It is a promising technique in analytical laboratories, but its use for extracting natural compounds on an industrial scale still needs improvement (Proestos & Komaitis, 2008).

Pulsed Electric Field Extraction exploits the polarization of ions across the cell membrane of the material, induced by the application of a pulsed electric field above a trans-membrane potential of about 1 V. This results in the compression of the electrically non-conductive membrane to the point of rupture, leading to the formation of pores that enhance the membrane permeability, and hence the release of molecules.

Enzyme-Assisted Extraction is a valid alternative to extractive methods involving petroleum-based solvents. It is performed under mild processing conditions and in an aqueous solution. It is based on the ability of enzymes to catalyze reactions with specificity and regioselectivity in order to hydrolyze the plant cell wall and promote the release of bioactive molecules.

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin. The first two polymers form the main structure. Instead, the lignins act as a glue between the fibers, playing a significant role in structural stability and preservation by protecting the cellulosic material from decay, disintegration, and insect attacks (Maitan-Alfenas, Visser et Guimarães, 2015). Given the complexity of the biomass structure, the enzymatic destructuring of the lignocellulosic matrix is a

complex process due to the many factors involved: like any catalytic reaction, it involves the adsorption of the catalyst (enzyme) onto the substrate surface, the degradation of the substrate into its constituent monomers and the desorption of the enzyme ([Maitan-Alfenas et al., 2015](#)). Therefore, the choice of enzyme between cellulase, hemicellulase, and pectinase and their optimal combination is crucial and requires some know-how.

Furthermore, operational parameters such as substrate concentration, enzyme concentration, solution pH, and stirring speed can influence the rate of hydrolysis and thus favor the release of biomolecules into the solution ([Champagne & Li, 2009](#)). Enzymes can also be used in a raw material pre-treatment step to reduce extraction times, minimize the use of solvents and provide increased yield and quality. The technical limitations of this technique include the relative cost of processing large volumes of raw material, the current availability of enzyme preparations that cannot completely hydrolyze plant cell walls, the difficulty of scaling up enzyme-assisted extraction to an industrial scale because enzymes behave differently as the environmental conditions (percentage of dissolved oxygen, temperature and nutrient availability) change.

Well-established conversion **technologies for recovering energy** carriers from biowaste include thermal (incineration with energy recovery), biological (fermentation, anaerobic digestion), and thermochemical (torrefaction, hydrothermal processing, pyrolysis, gasification).

The choice of conversion technology depends, amongst other factors, on the nature of the feedstock, its moisture content, its heterogeneity, and biodegradability. Obviously, the balance between the operational costs and the benefits is also paramount.

Incineration with energy recovery is the primary thermal conversion technology most- often used in waste-to-energy plants for volume reduction and energy recovery. The process exploits the complete oxidative combustion of waste, occurring within 800–1450 °C, to generate heat for power or combined heat and power (CHP) generation. The energy recovery by incineration is directly related to the feedstock heating value or thermal potential ([Beyene, Werkneh et Ambaye, 2018](#)). It is suitable for treating material with relatively high calorific value and lower moisture content. However, it does not represent an efficient valorization practice. It has low conversion efficiency, ranging between 18 % and 25% ([Chen et al., 2020](#)). It generates toxic air emissions to be controlled, containing pollutants such as furans, dioxins, volatile organic compounds (VOC), and polyaromatic hydrocarbons (PAH). Finally, it produces ash which requires specific handling and management.

Pyrolysis processes operate in oxygen-depleted conditions, between 400 and 500 °C. During pyrolysis, a chain reaction including dehydration, depolymerization, decarboxylation, charring, isomerization, and aromatization thermally decomposes lignocellulosic waste. The main drawbacks of this technology are the low energy conversion efficiency (23-43%, [Tomczyk, Sokołowska, et Boguta, 2020](#)), the propensity to emit polluting fugitive gases ([Chen, Yin, Wang, et He, 2014](#)), and the requirement of low moisture content (<15%) in the feedstock. This implies drying the agro-food waste, with the related energy consumption and cost implication ([Yang, Heaven, Venetsaneas, Banks, et Bridgwater, 2018](#)).

Gasification occurs in the presence of air, steam, or oxygen at a relatively higher temperature between 800 and 900 °C, producing combustible gases (known as syngas, made of CO, H₂, and CH₄) and non-combustible gases (CO₂ and N₂) (McKendry, 2002; Susastriawan & Saptoadi, 2017). The combustible gases produced can be burnt directly to generate heat and power or used as an intermediate resource to produce gasoline, diesel, and jet fuel via the Fischer-Tropsch synthesis (Bosmans, Vanderreydt, Geysen, et Helsen, 2013). Concerning pyrolysis, it has a higher conversion efficiency (45-61%) and a higher tolerance toward moisture content (30%), but it requires the feedstock to be dried anyway. Other challenges associated with gasification are the management of the tar produced with syngas that fouls processing plants and the difficulty of separating the different syngas components for liquid fuel production (Watson, Zhang, Si, Chen, et de Souza, 2018).

Hydrothermal processes involve the treatment of biowastes in either subcritical or supercritical water medium in the absence of oxygen (Biller & Ross, 2016) to produce energy carriers with high energy density. They include Hydrothermal carbonization (HTC), Hydrothermal liquefaction (HTL), and Hydrothermal gasification (HTG). The first occurs under autogenous pressure in subcritical water conditions (temperature between 180 and 250 °C) and yields a solid fraction, called hydrochar, and an organic-rich liquid fraction, called process water. The second takes place at temperatures of 280–360 °C and pressures of 18–30 MPa, under sub- and supercritical water conditions to produce a liquid fuel called biocrude, a solid residue fraction containing an abundance of inorganic compounds, a gaseous product composed primarily of CO₂ and a liquid fraction containing a low amount of water-soluble organics (Hoffmann, Rudra, Toor, Holm-Nielsen, et Rosendahl, 2013). Finally, the third Hydrothermal gasification (HTG) occurs under supercritical water conditions, at a temperature above 375 °C, to produce syngas and a liquid fraction with a low amount of dissolved organics (Kruse, Bernolle, Dahmen, Dinjus, et Maniam, 2010).

All these processes are suited for wet feedstock with a moisture content greater than 50%. Hence, the energy-intensive pre-drying step here is not required.

Small-scale biorefinery of agro-food waste can be encouraged if low-cost and low-energy demanding processes are preferred. This could be ensured by adopting **bioconversion methods**, which, however, are more substrate dependent with respect to the methods listed above. We recall the main anaerobic fermentation and the direct bioelectricity generation. Biotechnologies like solventogenesis, which concerns the production of bio solvents like bioethanol and biobutanol, and oleaginous metabolism, employed in lipid synthesis mediated by yeast and fungi for biodiesel production, shall not be considered. The former generally requires the continuous processing of a high amount of feedstock; hence it is preferentially performed in large-scale biorefineries. The latter could be used to replace the supply of the limited feedstocks required by lipide transesterification in conventional biodiesel production (vegetable oils, waste cooking oils, and animal fat), but it is still an immature technology.

Anaerobic digestion (AD) is probably the most demanding process for the valorization of FW towards a wide range of biochemicals and biofuels. It is relatively inexpensive and consists of a sequential process (Fig.7) carried out by the action and syntrophic association of various bacteria of the types hydrolytic, acetogenic, hydrogen-producing, acetate-forming microbes, and methanogens

(Venkata Mohan, Mohanakrishna, Goud, et Sarma, 2009). Complex and long-chain polymers of organic waste are broken down in the absence of oxygen to produce biogas (a mix of CH_4 and CO_2) and digestate, a nutrient-rich by-product with high levels of N, P, and humic substances. Each stage of AD yields important bio-products: hydrolysis yields fermented sugars, acidogenesis yields volatile fatty acids (VFA) and H_2 , acetogenesis yields acetic acid and H_2 , while as the terminating process, methanogenesis yields CH_4 (Dahiya et al., 2018).

The biological process begins with the hydrolysis of complex organic substrates. Then, acidogenic and acetogenic bacteria take part in the fermentative stage. These bacteria belong to a large and diverse group of facultative and obligate anaerobes, which can convert biological building blocks (e.g., sugars and amino acids) into organic acids. The final step involves methanogens bacteria and hydrogenotrophic archaea, which convert acetic acid, H_2 , into CO_2 and CH_4 (Anukam, Mohammadi, Naqvi, et Granström, 2019).

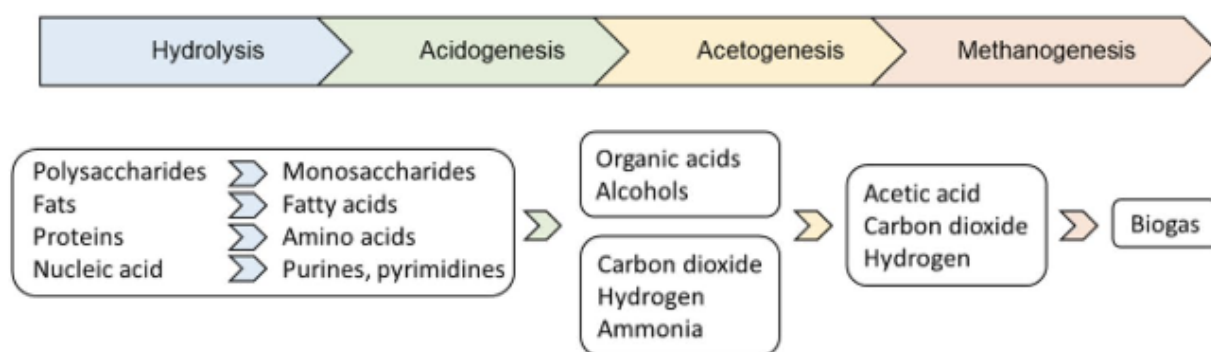


Fig.7 Schematic representation of the anaerobic digestion process (Paini et al., 2021).

Sugars undergoing fermentation, like glucose, fructose, galactose, and ribose, are mainly extracted during the hydrolytic pretreatment of the FW (Hafid, Shah, et Baharudin, 2015). Thus, the pre-treatment method is selected to achieve higher sugar yields, minimize the sugar-degraded compounds or losses, eliminate inhibitory by-product formation, and increase the economic viability of the process. Pre-treatment can be either done individually with physical, chemical, physicochemical, biological, and enzymatic methods or in a combination of the strategies (Hafid et al., 2015). The establishment of a comprehensive method is not easy due to the extreme variability in sugar content with respect to FW composition and origin, but using hydrochloric acid and sulphuric acid at low concentrations are commonly applied directly or in combination with an enzymatic method to avoid the formation of sugar degraded compounds (furfurals/hydroxyl methyl furfurals, Dahiya et al., 2018).

Volatile fatty acids have importance in pharmaceuticals, textiles, food and beverages, plastics, polymers, and many more industries. They include acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, and isovaleric acid. For their production, sterile fermentation conditions are not required. Still, the produced amount depends on many factors, such as the composition,

inoculum, reactor condition, temperature, organic loading rate, and redox conditions (Lee, Chua, Yeoh, et Ngho, 2014; Dahiya, Sarkar, Swamy, et Mohan, 2015).

Biohydrogen is considered a sustainable green fuel, and its production from waste has attracted the attention of researchers around the globe. Employing mixed consortia as a bio-catalyst and FW as feedstock for H₂ production has multiple advantages: economic viability, non-sterile operating conditions, and operational flexibility (Pasupuleti, Sarkar et Mohan, 2014; Venkata Mohan et al., 2009; Goud et al., 2017). However, the efficiency of the H₂ production in AF is directly dependent on the combination of various process parameters such as type/origin of inoculum, pretreatment procedure, inlet pH, co-substrate addition, reactor configuration, temperature, and feed composition (Venkata Mohan et al., 2009).

Biogas is produced at the end of the fermentation process. It is mainly composed of methane (CH₄), carbon dioxide (CO₂), nitrogen (N₂), moisture, and traces of hydrogen sulfide (H₂S). CH₄ production is the most explored technology for bio-energy generation through AD utilizing FW as substrate. Thousands of AD plants have been combined globally, mainly in Europe (>18,000 plants, European Biogas Association, 2020) and the USA (2,300, American Biogas Council, 2018). However, despite widespread adoption, AD's optimization and stable operation remain challenging because of several factors.

It depends on the various process parameters such as reactor design, inoculum type, temperature, pH, carbon/nitrogen ratio, volatile fatty acid, organic loading rate, co-digestion, etc. (Dahiya et al., 2018). The ideal ranges for optimum AD performance are 20–30 for carbon to nitrogen ratio, pH 6.8–7.4, and a retention time of 15–30 days. In addition, the ideal feedstock should have low recalcitrance and lignin content not to limit the rate-limiting step of microbial hydrolysis. However, the variability in food waste composition does not ensure it (Kassim et al., 2022).

To have a stable conversion of organic material into biogas, microbial growth conditions (e.g., temperature, pH, and organic loading rate) must be carefully monitored to prevent possible process failures due to an unbalance of the microbial community (Trabold & Babbitt, 2018; Fagbohunbe et al., 2017).

High concentrations of lipids, as well as proteins, limonene, furans, metals, pesticides, and antibiotics, can promote substrate-induced inhibition phenomenon (SII) AD (Fagbohunbe et al., 2017). In addition, the low pH value of certain vegetables can lead to an acidification of the anaerobic microbial community, blocking the optimum conversion (Paini et al., 2021).

Even the use of digestate as a fertilizer depends on the composition of the initial substrate, operating conditions, and compliance with environmental standards (Weiland, 2010). An important factor is the control of the rate of its disposal to agricultural land to prevent nutrient leaching, volatilization of ammonia, and GHG emissions (Möller & Müller, 2012; Sheets, Yang, Ge, Wang, et Li, 2015). This would result in considering the digestate a waste stream, requiring additional downstream management. The first step is solid-liquid separation, an energy-intensive operation that reduces the process's benefits from both environmental and economic perspectives (Paini et al., 2021).

Microbial fuel cell (MFC) is a sustainable technology capable of converting waste's chemical energy directly to bio-electrical energy (Goud & Venkata Mohan, 2011; Ishii et al., 2013). This process occurs when exoelectrogenic bacteria growing on the anode oxidize organic matter contained in the wastewater and transfer the electrons to the anode. A current is produced when electrons flow from the anode to the cathode through an external circuit. In contrast, the protons are delivered to the cathode via a membrane separating the anode and cathode. At the cathode, the electrons reduce an electron acceptor, typically oxygen, ultimately forming a reduced compound such as water (Fig.8) (Janicek, Fan, et Liu, 2014).

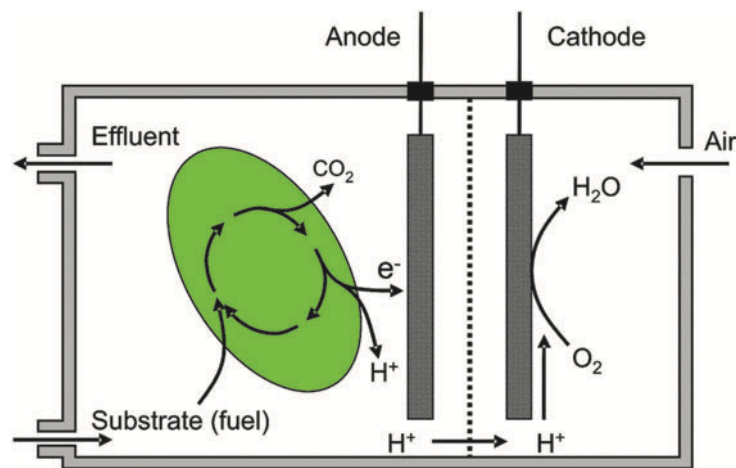


Fig.8 Schematized microbial fuel cell (Schröder, 2007).

MFC systems need to be able to treat large quantities of wastewater to guarantee their practical application. However, the scale-up process from the laboratory scale to the commercial level requires care since the MFC performance is affected by several factors. For example, the composition of the stream to be treated. In general, electrolyte conductivity is a crucial factor; reactors operating on buffered real waste streams produce higher power densities than unbuffered waste streams. Thus, integration and adjustments to waste streams, such as the addition of buffers and pH adjustments (Zhuang et al., 2012; Ge, Zhang, Grimaud, Hurst, & He, 2013; Clauwaert, Mulenga, Aelterman, & Verstraete, 2009), adjustments to increase conductivity (Zhuang et al., 2012), and the addition of carbon sources (Jiang et al., 2011), may be necessary to improve performance or reduce start-up times. However, these improvements will likely increase operating costs. Additionally, waste streams with high organic concentrations would likely generate higher performance and are the most suitable for energy generation. However, at higher organic loading rates, COD removal is typically lower.

Another essential feature in scale-up is the reactor design since it affects electrode configurations such as spacing, shape and orientation, size, and effective electrode surface area. In scaling up the reactor dimension, it is indeed crucial to maintain a small distance between electrodes to gain an increase in ionic diffusion rates and a reduced internal resistance, and a higher surface area-to-volume ratio. For this purpose, in recent years, tubular configurations have taken over the most

classical flat-plate type. They also have the advantage of being easily stacked in tubular modules, but their design is now limited to shapes and materials available on the market ([Janicek et al., 2014](#)). Another drawback of MFC is the possibility of clogging in situ applications due to wastewater composition or excess biofilm growth. Clogging can be reduced through the pretreatment of wastewater and the optimization of reactor design and operation. Excess biofilm growth can partially be controlled by modifying structural surfaces to prevent attachment. However, further research is needed in this area.

Dissertation objectives

The ruthless advance of climate change requires a series of actions to mitigate its impacts and to address the energy and ecological transition toward a future beyond petroleum. Any action, however, risks being in vain if not accompanied by a change of course from the mode of production and consumption to which we have become accustomed, and which we take for granted. From a global dimension, we need to move to a local dimension in both the supply and processing of resources, which can ensure the application of the principles of a circular bio-based economy: reduction of pressure on natural resources, sustainable production of products and energy, reduction of waste, followed by its reuse and recycling. Exploiting biomass as an alternative resource to fossil raw materials is a key driver to achieving the energy transition. Still, more is needed to ensure the minimal impact of production processes. To make this happen, it is necessary to shorten the various production chains, limiting transport and its environmental implications, and making them self-sustaining through the preference for biotech processes with low environmental impact, such as biorefineries, and the production of renewable energy to be integrated into the processes themselves.

The agro-food chain lends itself well to this new perspective because it can extract value from its waste through sustainable technologies and because its specificity already characterizes it on a local and territorial scale. Consequently, it can promote circular economy application on a local scale, bringing benefits to communities. The only limitation to the widespread of small-scale biorefineries is economic feasibility. Since it cannot rely on an economy of scale that dampens operating and utility costs, it must rely on the processes' high efficiency and low costs to be performed.

This research work aims to propose a second-generation cascade biorefinery model to be integrated into the food chain, capable of encouraging the valorization of agro-food waste on a local scale.

It unfolds through three more specific objectives, developed in dedicated sections:

- 1 carry out the production of enzymes on-site, exploiting the agro-food waste's components as nutrients.
- 2 design a method to recover specific chemicals based on the use of enzymes as selective biocatalysts, and establish a strategy to valorize byproducts with a local market application;
- 3 design an energy plant to convert solid residue and wastewater from chemical valorization plants to renewable electrical energy in a non-selective way.

Results and Discussion

Dissertation outlines

The present research work aims to propose a second-generation cascade biorefinery model capable of encouraging the valorization of agro-food waste on a local scale and is summarized in three more specific objectives, developed in dedicated sections, outlined in Fig. 1.

The first section (Chp. 2-At the upstream of biorefinery) concerns the description of the agro-food waste, the need for specific biocatalysts that can mediate its conversion into valuable products, and the opportunity to obtain it in a low-cost manner through solid-state fermentation processes that use the same waste as a growth substrate. In this specific case, the substrate taken into consideration is hemp bran.

The second section (Chp. 3-The biorefinery core) focuses on converting vegetable waste into high-value-added biomolecules through a low environmental impact process involving cell wall disassimilation mediated by enzymes, produced on-site or purchased. The strategy requires the deployment of a knowledge-based approach. It, therefore, presupposes the definition of the end products to be preferred and the sequential routes to obtain them, already in the preliminary process design phase. In this specific case, the cosmetic field is defined for the final application, and a series of examples are proposed (3 case studies) that show the possibility of obtaining diversified products on a territorial basis, thus capable of creating new local markets. The products are obtained through specific recombination techniques of the components previously extracted via biotechnological from three different raw materials, as reported in the case studies: hemp oilseed cake (Chp. 3A), rice grain (flour and husk from milling) (Chp. 3B), and pomegranate pomace (Chp. 3C).

The third section (Chp. 4-The biorefinery integration) concerns the development of an energy valorization plant for the conversion of residues from chemical valorization, which is the object of a pending patent on behalf of the University of Bologna. This is based on the operation of a new 3D-printed abiotic glucose alkaline biofuel cell capable of converting lignocellulosic biomass directly into electrical energy and in a non-specific way so that in such a way, the energy required by upstream processes could be effectively integrated. The cell design process is described in a dedicated section (Chp. 4A), as well as its applications, in which either glucose solutions or a real complex matrix, i.e., the solid residue from the conversion of pomegranate pomace, are used as biofuel (Chp. 4B). The biofuel cell has been designed to work both in batch and continuous modes, and it does not require metal catalysts or expansive electrodes. At the end of its operating cycle, an exhausted biofuel is produced, which, if neutralized with compounds containing the proper inorganic elements, could be used as a soil conditioner. This would favor the loop closure of the food chain in the same place from which it originated or transformed.

Each of the three main sections is structured as follows:

- A brief introduction aimed to define the specific context of research and the ensuing need to present a new proposal in the specific regard.
- The definition of the research proposal via the statement of specific objectives and key points of the strategy that is intended to deploy to achieve them.
- A literature section comprising information to support the research proposal
- At least a study case to explain the strategy put in place.
- Materials and Methods of the specific section.

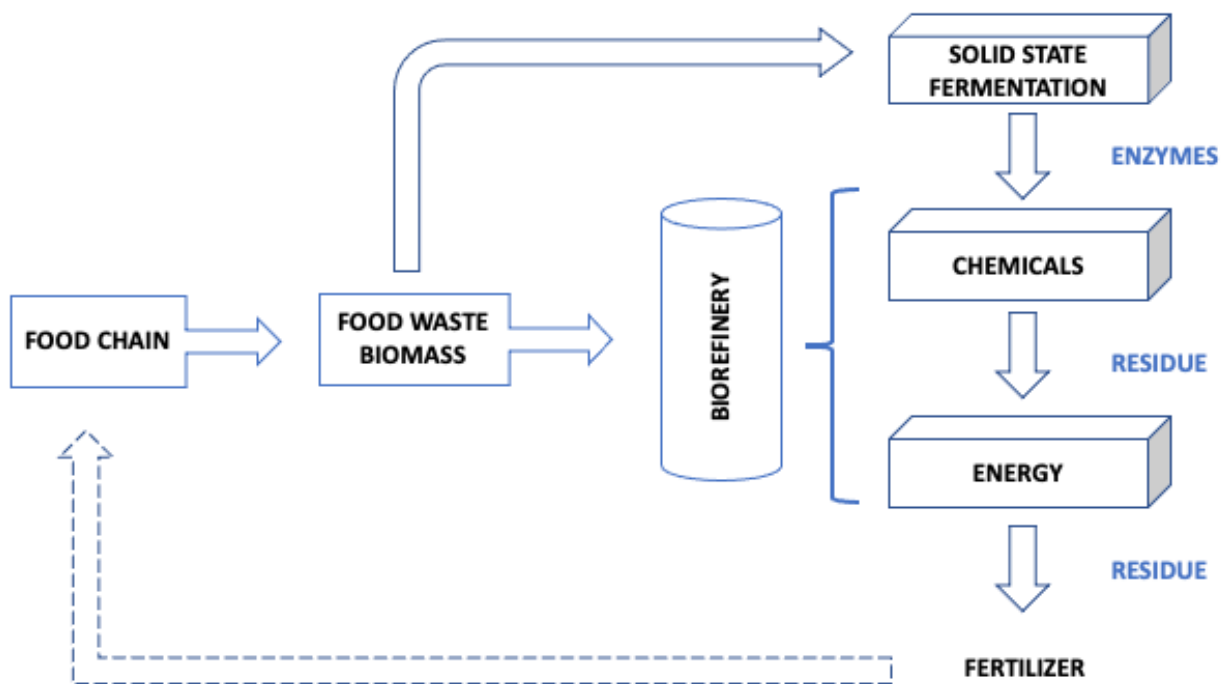


Fig.1 The structure of the cascade biorefinery process object of the Thesis.

2 At the upstream of biorefinery

The proposed biorefinery model begins with an upstream unit responsible for the production of low-cost enzymes, using agro food-waste as substrate. The specificity of enzyme action, depending on substrate growth, helps to unlock the potentiality of the (recalcitrant) plant cell wall as a reservoir of specific chemical building blocks.

2.1 The context: valuable waste from agro-food chain

According to the Food and Agriculture Organization (FAO) of the United Nations (UN), every year early, one-third of food is lost, generating $1.3 \cdot 10^9$ of food waste on the global scale (RedCorn, Fatemi, & Engelberth, 2018). Food loss occurs throughout the entire supply chain. It can be defined as the decrease in quality and quantities of edible material that prevent it from being consumed by humans. Food waste can be seen as the subset of food loss that is removed from the supply chain to be recovered or disposed of (Giroto et al., 2015). Food wastes and losses can be grouped into commodities and products of animal and vegetable origin. The animal ones comprise fish and meat trimming from the processing stage, their degradation products from post-harvest handling and storage, and milk and dairy products degradation. The vegetable includes scraps from harvest operations like threshing or fruit picking, losses due to spillage and degradation during domestic and industrial processing, e.g., washing, peeling, slicing, or boiling, along with residues from juice production canning and bread baking (FAO, 2011).

A schematic classification of vegetable wastes generated from the agro-food industry is given in Fig. 1

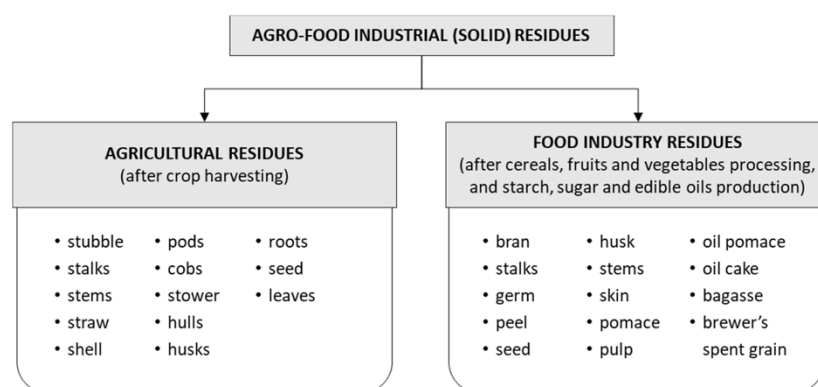


Fig.1 Classification of agro-food industrial (solid) residue (Šelo et al., 2021).

Food loss from industrial processing accounts for only 5% of the total food loss (RedCorn et al., 2018), but they are more suitable to be processed and valorized by biorefinery systems.

They are generally generated in high volumes at specific and non-distributing sites, which allows them to be easily collected; they are homogeneous, which allows for earlier identify the added value resulting from the bioconversion of each stream (RedCorn et al., 2018). The recovery of industrial

wastes can, on its own, mitigate approximately $9 \cdot 10^8$ of CO₂ equivalent greenhouse gas emissions globally (FAO, 2013). In addition, it could head the valorization of the non-edible fraction of food lost in harvest and post-harvest, resulting in 50% of the total, by providing the same facilities for the simultaneous conversion of upstream and downstream supply chain residues into valuable products (RedCorn et al., 2018)

Vegetable agro-food wastes, just like the vegetable resources of origin, are examples of lignocellulosic biomass and, thus, besides carbohydrates, contain lipids, proteins, and other molecules which render them suitable for their processing in second-generation biorefinery plants to produce fuels, and countless high-value chemicals and commodities (Arya, Yagnik, Rajput, Panchal & Raval, 2022), e.g., xylitol, xylose, glucose, furfural (Sánchez, C. 2009), biofibers (Reddy & Yang, 2005), biopulp (Scott, Akhtar, Swaney & Houtman, 2002), animal feed (Okano, Ohkoshi, Nishiyama, Usagawa, & Kitagawa, 2009) and enzymes (Hölker, Höfer, & Lenz, 2004).

2.2 At the upstream of biorefinery

The strength of plant biomass lies in its intricate diversity enclosed in a unique and well-defined hierarchy of structures that each plant organism has developed to face the environmental stress it has undergone due to its sessile nature. That has meant that different life forms would adopt new strategies to recognize and utilize plant components as metabolites for their growth, among them secreting enzymes capable of attacking the plant substrate selectively. Applying this natural process to recover molecules of interest from lignocellulosic biomass could be a helpful tool to reach the most effective valorization of agro-food waste.

- **The first thesis objective** is to carry out sustainable enzyme production that could act as selective biocatalysts for the conversion of agro-food wastes.
- **The strategy** concerns the application of **solid-state fermentation** processes for enzyme production, using the edible fungus **Pleurotus ostreatus** as an organism capable of secreting enzymes in a specific way, and using waste deriving from the agro-food chain as a growth substrate. Indeed, like all white-rot fungi, *Pleurotus ostreatus* manifests both hydrolytic and ligninolytic systems to disrupt the lignocellulosic matrix, depending on the specific type of growth material. Integrating an **upstream unit** for the production of enzymes into a second-generation biorefinery, using the same waste as growth substrate, could limit the costs for the downstream transformation of biomass and create added value, meanwhile reducing the food waste volumes to be chemically processed or finally disposed of (Fig.2). The case of study to explain the strategy is reported in Chapter 2A.

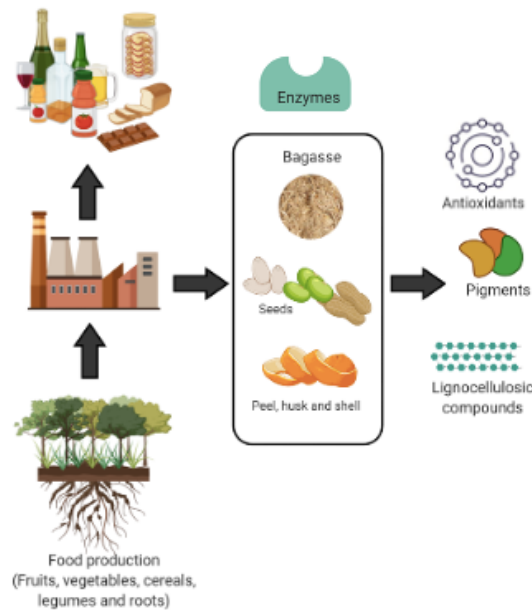


Fig.2 Simplified flow diagram of food from production to waste valorization (adapted from RedCorn et al., 2018).

2.3 Information to support strategy: specific enzymes for specific substrates

2.3.1 A look inside the vegetable waste

Vegetables obey five primary levels of structures, whose integrity is strongly interconnected. The outermost level includes the easily recognizable plant parts (fruit, leaf, seed, root), namely plant organs. It depends on the characteristics of the lower structures: plant tissue, plant cell wall, polysaccharides arrangement, and polysaccharide composition. Every change occurring in the aspect of the food appearance, for example, after undergoing thermal processing operations or during naturally occurring phenomena like the growing of the plant or the ripening of the fruit, is related to the alteration and the rearrangement of the polysaccharide units, the breaks of the plant cell (Waldron, Parker & Smith, 2003).

The plant cell is a complex and delocalized system that acts as a small engine able to metabolize nutrients producing energy to ensure the function and maintenance of the entire plant body. It comprises a cytoplasmatic matrix surrounding different compartments with specific functions (nucleus, vacuoles, Golgi bodies, and ribosomes), a plasm-membrane that responds to composition and pressure changes, and a plant cell wall that encloses it (Padayachee, Day, Howell, & Gidley, 2017). **The plant cell wall** (Fig. 3) is a highly ordered structure resulting from the layered deposition of polymeric components up to a maximum of three domains: the middle lamella, the primary cell wall, and the secondary cell wall (Waldron et al., 2003). Whereas all the plant tissues reach up to the primary cell wall, not all the plant organs exhibit a secondary cell wall. The first is assembled

during the plant growth, while the second is built onto the inner side of the primary cell wall and only once the plant growth is concluded, providing a thicker and lignified support to the cell (Cosgrove, D. J., 2005). Most fruit and leaf contain non-lignified parenchyma cells in their plant cell tissues, with some exceptions, e.g., in sclerenchyma fibers of mature asparagus, in sclereids of pears, or in the outer layer of some grains and bran (Harris & Smith, 2006). Instead, seed, root, and stem generally present secondary cell walls in their plant cells.

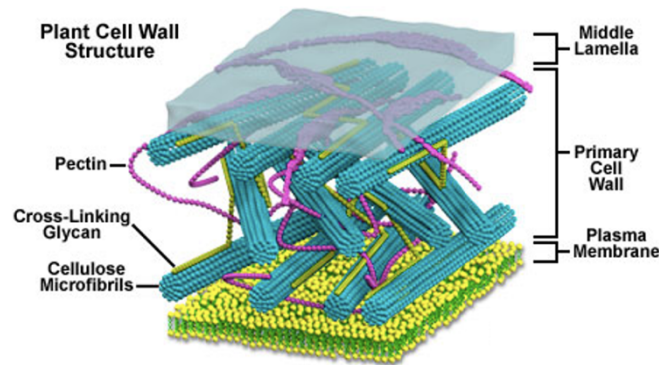


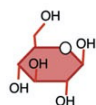
Fig.3 Plant cell wall structure (<https://micro.magnet.fsu.edu/cells/plants/cellwall.html>)

The primary plant cell wall polysaccharides are cellulose, hemicellulose, and pectin.

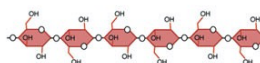
Cellulose (Fig. 4) is the most abundant plant-based polymer. It consists of D-glucose (Glc) molecules united by β (1-4) glycosidic linkage to create linear chains with a degree of polymerization (DP) between 4000 and 6000 (Waldron et al., 2003). Adjacent glucan chains self-associate via hydrogen bonds resulting in crystalline microfibrils, which associate into larger fibers capable of providing tensile strength and resistance to turgor pressure. The crystalline core is then surrounded by a paracrystalline layer of cellulose, followed by hemicellulose (Himmel et al., 2007).

Cellulose

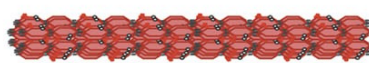
Glucose



Cellulose



Cellulose microfibril



Hemicelluloses

Glucose



Xylose



Galactose



Arabinose



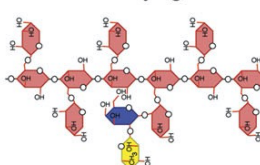
Mannose



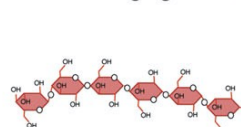
Fucose



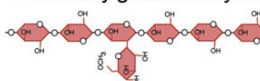
Fucoside xyloglucan



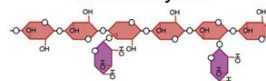
Mixed-linkage glucan



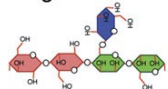
4-O-methylglucuronoxylan



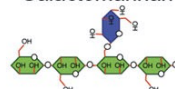
Arabinoxylan



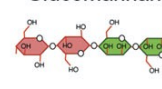
Glucogalactomannan



Galactomannan

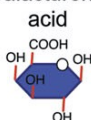


Glucomannan

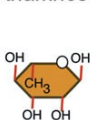


Pectins

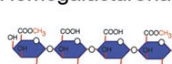
Galacturonic acid



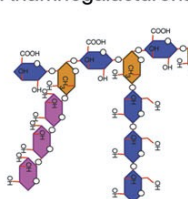
Rhamnose



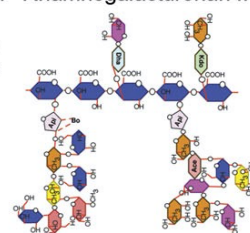
Homogalacturonan



Rhamnogalacturonan I



Rhamnogalacturonan II



Uronic acids

Glucuronic acid



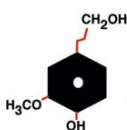
Mannuronic acid



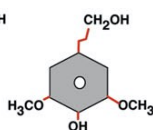
Unidentified polymers

Lignins

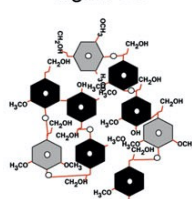
Guaiacyl unit



Syringyl unit



Lignin GS



Lignin G

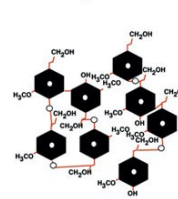


Fig.4 Chemical structure of the predominant building blocks of plant cell walls (Sarkar, Bosneaga & Auer, 2009).

Hemicelluloses (Fig. 4) may comprise monomers like glucose, xylose, mannose, rhamnose, and arabinose, comprising from 100 to 200 units linked to give branched and amorphous polymers:

xylans, xyloglucans, mannans, mixed linked β -glucans. **Xylans** are one of the most abundant hemicelluloses in biomass since they are in the main annual plants and hardwoods and can also be found in softwoods (Martínez-Abad, Ruthes & Vilaplana, 2016). They consist of β (1–4)-linked D-xylose (Xyl) backbone branched by α (1–2)-linked 4-O-methylglucuronic acid (GlcA), and α (1–2), α (1–3)-linked arabinose (Ara). Arabinoxylans contain xylose copolymerized with arabinose and can exhibit xylose residues acetylated at C-2 or C-3.

Xyloglucans are major hemicellulose constituents of the primary cell walls of plants and can also be found as storage polysaccharides in certain seeds and fruits (Martínez-Abad et al., 2016). They have β -(1-4) linked Glc backbone and side chains of α -(1-6)-linked Xyl residues, part of which may be further connected to other sugars like α (1-2)-linked D-galactose (Gal), and disaccharides of fucose giving to fucogalactoxyloglucans (Waldron et al., 2003).

Mannans constitute softwood's main hemicelluloses and are also abundant in seeds as non-starch energy-reserve polysaccharides (Martínez-Abad et al., 2016). They comprise a β (1-4)-linked mannose (Man) chain, with galactose (Gal) residues in galactomannans or include a non-repeating pattern of both mannose and glucose in the backbone, as in galactoglucomannans.

Hemicelluloses are interlocked to cellular fibers by noncovalent intermolecular interactions, modulated by their heterogeneous molecular structure, to give a specific arrangement for each plant organism, tissue, or developed stage (Martínez-Abad et al., 2016). This cross-linked cellulose-hemicellulose complex looks to be nestled into a pectin matrix (Waldron et al., 2003)

Pectins (Fig. 4) are complex macromolecules with high uronic acid content that involve different interconnected domains, including homogalacturonan (HG) linear chains and rhamnogalacturonan's branched chains. Homogalacturonan is made of D-galacturonic acid (GalA) units esterified to different extents, methyl esterified at C6, O-acetylated at O2, and O3. If substituted with β -(1-3)-linked D-xylose side chains, it is called xylogalacturonan (XGA). Rhamnogalacturonans are composed of alternated D-galacturonic acid and L-rhamnose (Rha) units and are distinguished into I and II types. The former can be substituted with galactosyl and arabinosyl residues at 4-O Rha or O-acetylated on O2 and O3 of GalA. The latter is characterized by a distinct region within HG, containing four different side chains with peculiar sugar residues. (Padayachee et al., 2017). Arabinan consists of α (1-5)-linked arabinose backbones partially substituted; arabinogalactans contain both rhamnose and arabinose residues. Apart from being mainly concentrated in the primary cell wall, pectins are also present in the middle lamella and have an essential role in ensuring the firmness and adhesion of cells and increasing their flexibility due to their ability to form a gel-like network.

The gelling properties come down to the degree of methyl esterification (DE), which represents the percentage of the galacturonic acid residues in uncharged methyl ester forms and varies between plant species and tissues (Prasanna, Prabha & Tharanathan, 2007). Depending on DE, pectins can self-associate through divalent cations and hydrophobic bonding (Oakenfull & Scott, 1984).

The specificity of the plant cell wall arises in the **primary cell wall**, in which polysaccharide backbones are associated with varying levels of proteins and phenolics and are organized in peculiar arrangements to give type I and type II distinction (Carpita & Gibeaut, 1993).

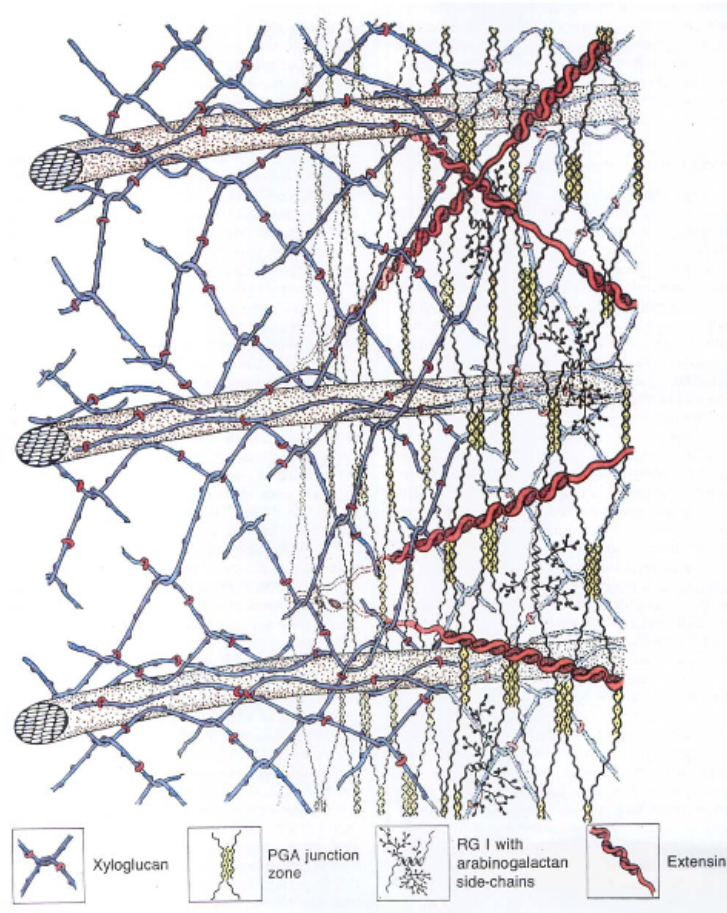


Fig. 5 Type I plant cell wall (Carpita & Gibeaut, 1993).

The type I primary cell wall (Fig. 5) is found in dicotyledon plants, most nongraminaceous monocotyledon plants, and gymnosperm. It comprises a cellulose-xyloglucan network by 50%, pectic polysaccharides by 30%, and structural proteins remaining (Carpita & Gibeaut, 1993). The structural proteins are mostly glycosylated and contain the amino acid hydroxyproline; they include extensins, arabinogalactan proteins (AGPs), proline/hydroxyproline-rich glycoproteins and solanaceous-lectins (Waldron et al., 2003).

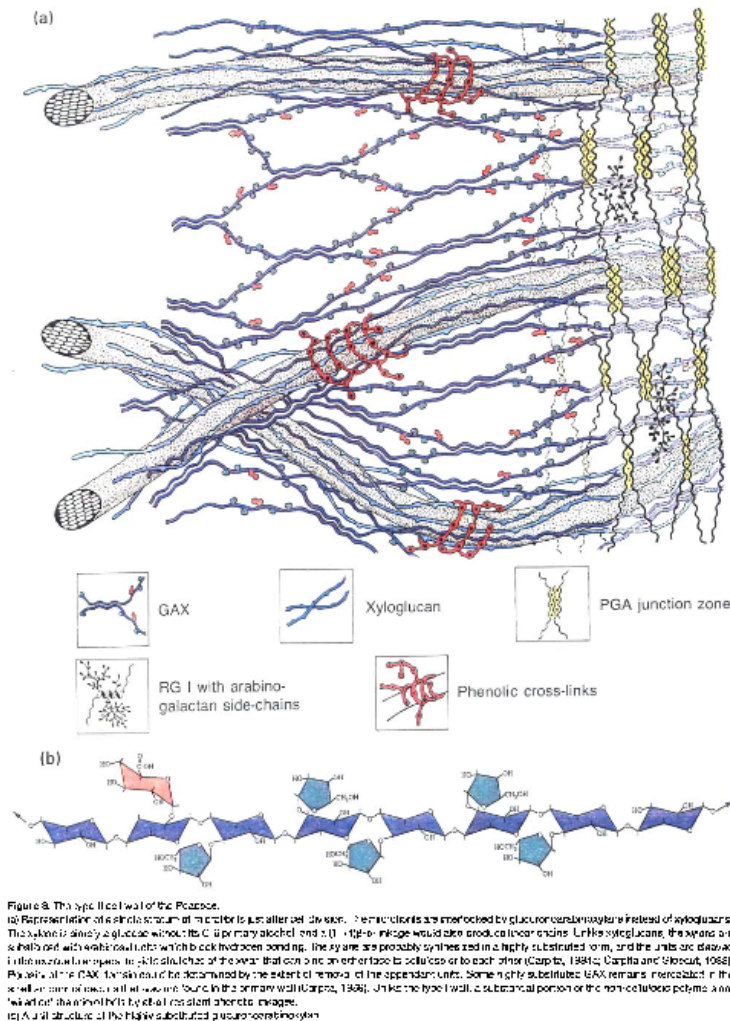


Fig. 6 Type II plant cell wall (Carpita & Gibeault, 1993).

The type II primary cell wall (Fig. 6) is found, instead, in monocotyledons (commelinid), like grasses and cereals. It exhibits a cellulose domain interlocked with heteroxylans, like glucuronoxylans (GAXs) and arabinoxylans, with low content of pectin (Voragen, Coenen, Verhoef & Schols, 2009) and variable portion of (1-3) (1-4) β glucans (Harris & Smith, 2006).

In addition to polysaccharides, primary cell walls show specific functionalization by ester moieties of cinnamic, ferulic, and dehydrodiferulic acids (Harris, 2005). In dicotyledon plants, they are esterified to the arabinan and the galactan side chains of the pectic polysaccharide rhamnogalacturonan I. In monocotyledons, they are linked to the C(O)5 hydroxyl of some GAXs arabinose residue (Smith & Harris, 2001).

The differentiation among the plant cells continues in the **secondary cell wall structure**. Here pectins are almost absent, and cellulose shows a higher polymerization degree, reaching more than 10000 (Delmer, 1987). However, the most differences can be encountered in the appearance of

lignin, which appeared to be covalently bound to underlying hemicellulose and pectin through different types of lignin-carbohydrate complexes (Lawoko, Henriksson & Gellerstedt, 2005), contributing to the tight and highly crosslinked architecture of the lignocellulosic biomass.

Lignin (Fig.4) is the third most abundant plant-based polymer (10-25%), after cellulose (30-35%) and hemicellulose (20-30%) (Arya et al.,2022). It is produced from the nonrepetitive polymerization of hydroxycinnamic acids like p-coumaryl, coniferyl, and sinapyl acids, which lead to a complex polyaromatic structure consisting of p-hydroxyphenyl, guaiacyl, and syngyl units (Vanholme, Demedts, Morreel, Ralph & Boerjan 2010). Its role in providing structural stability to the cell wall is well known, as well as in controlling water permeability, due to its hydrophobicity nature (Martínez-Abad et al., 2016). More specialized functions are, for the most part, related to the concentration and composition within plant cell walls since they are affected by environmental and developmental factors (Boerjan, Ralph, & Baucher, 2003; Crivellaro & Büntgen, 2020; Crivellaro, Piermattei, Dolezal, Dupree & Büntgen, 2022).

Table 1 below shows some examples of popular agro-food waste composition.

Table 1 Composition of common industry agro-food waste.

Food industry residues	Lignin %	Cellulose %	Hemicellulose %	Protein %	Ash%	Ref.
Pomegranate peel	15.3	36.4	28.2	15.6	11.4	[1]
Grape pomace	11.6-41.3	9.2-14.5	4-10.3	7-23.5	4.7-9.5	[2]
Apple pomace	14.8-22.4	47.5	27.8	6.0-7.0	1.1-5.1	[2]
Olive mill waste	13.3-15.8	24.8-33.8	13-16.3	6.7-7.2	2.5-8.9	[2]
Hemp oil cake	16.7	22.5	14.2	24.8	7.5	[3]
Wheat bran	5.0	10.7	39	18	0.05	[4]
Rice husk	20	35	25	3.0	17	[5]
Sugarcane bagasse	18.9-26.1	36.9-45.7	25.6-29.6	2.2	2.8	[2]

[1] = Chaudhary et al., 2021; [2] = Šelo et al., 2021; [3] = Budžaki et al., 2018; [4] = Cripwell et al., 2015; [5] = Ugheoke, 2012.

2.3.2 Biotech biomass deconstruction

The chemical complexity in plant cell walls has been developed to protect plant species in the course of evolution (Sarkar, Bosneaga & Auer, 2009); indeed, the significant variability in their composition is nothing but a form of adaptation to seasonal and geographic stress (Moore, Vitré-Gibouin, Farrant & Driouich, 2008), (Körner, 2016), (Ezquer, Salameh, Colombo & Kalaitzis, 2020). To respond to rapid

changes fielded by plant systems in defending themselves, microorganisms and animals have evolved specific strategies to weaken the recalcitrant structure of plant cell walls, to get the nourishment they needed. First, the ability to produce enzymes capable of disrupting plant cell wall integrity primarily through de-polymerization de-esterification reactions (Sarkar et al., 2009) (Mota, Oliveira, Marchiosi, Ferrarese-Filho & Santos, 2018). These strategies occurring in nature have been intensely studied and applied in several industry sectors to let specific plant-based chemicals be available either as extracted products or starting materials for commodities obtainment via chemical or biosynthetic routes (Martínez-Abad et al., 2016) (Arya et al., 2022) (Agustin et al., 2021) (Mayolo-Deloya, González-González & Rito-Palomares, 2020) (Espro et al., 2021) (Esteban & Ladero, 2018).

Thanks to this biomimetic approach nowadays, we can dispose of an enormous library of enzymes (<https://iubmb.qmul.ac.uk/>), obtainable through various fermentative processes (Kirk, Borchert & Fuglsang, 2002) (Howard, Abotsi, Van Rensburg & Howard, 2003) (Ergun & Urek, 2017) (Masutti, Borgognone & Setti, 2012).

According to the list provided by The International Union of Biochemistry and Molecular Biology (IUBMB), all enzymes may be classified into seven main groups and relative multi-level subgroups, depending on their mode of action (<https://iubmb.qmul.ac.uk/>). Each category is accurately labeled, and the list is constantly updated with new enzymes. Among these, the Hydrolases (EC 3) and the Oxidoreductases (EC 1) are arguably the most known and the most widely used in lignocellulosic biomass degradation.

Within the Hydrolases, the enzymes that modify complex carbohydrates are known as **Carbohydrate-Active Enzymes** (CAZ). A non-exhaustive list includes Glycosidases (EC 3.2), among them Amylase, Cellulases, Hemicellulases, and Pectinases, and enzymes active on ester bonds (EC 3.1) (Fig.7) comprising also various types of Lipases (EC 3.1.1.3). Proteases (EC 3.4) belong to another hydrolases subgroup. Instead, specific Ligninases like Laccase (EC 1.10.3.2) and Peroxidase (EC 1.11.1.7) belong to Oxidoreductases.

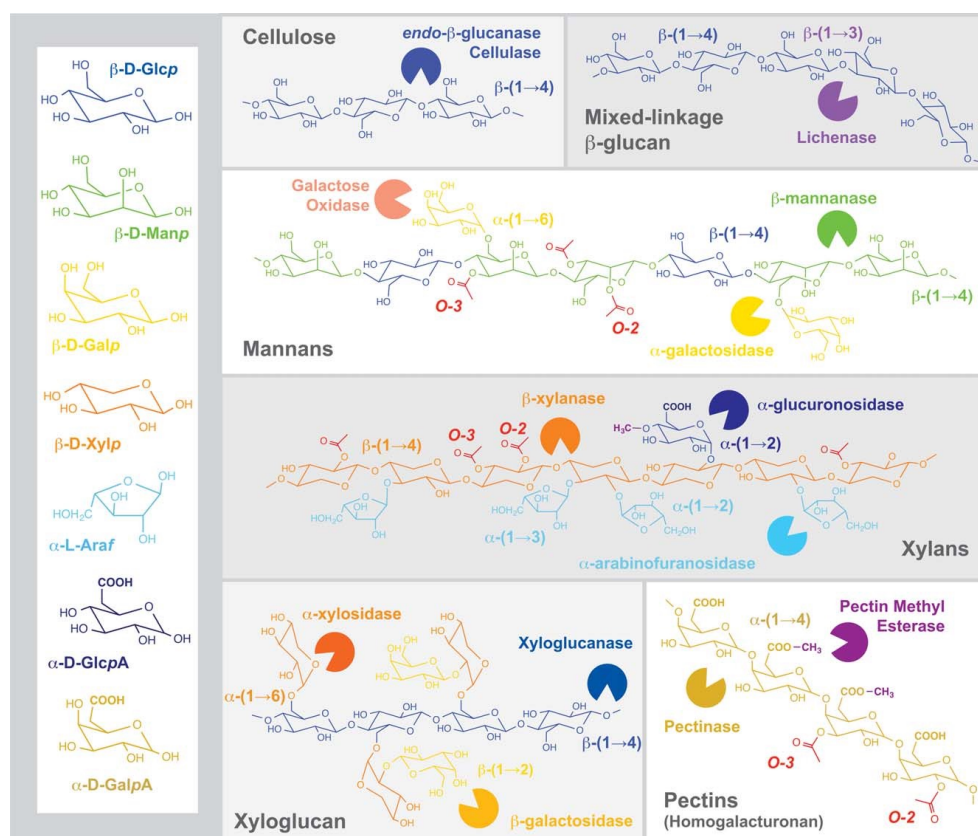


Fig. 7 Glycosidase enzymes and their action mode (Martínez-Abad et al., 2016).

Amylases are starch-hydrolyzing enzymes; they comprise α -amylases, which are endo-acting, β -amylases, and glucoamylases, which are exo-acting enzymes. α -amylases (EC 3.2.1.1) can be divided into liquifying and saccharifying depending on the content of the glucose units in the substrate, respectively more than fifteen or four. They cleave the α (1-4) glycosidic linkage on starch, glycogen, and related polysaccharides and oligosaccharides in a random manner to give oligosaccharide dextrin and reducing groups (<https://iubmb.qmul.ac.uk/>). β -amylases (EC 3.2.1.2) and Glucoamylases (EC 3.2.1.3) act on glycosidic linkages at non-reducing ends of amylose, amylopectin, glycogen molecules, providing low-molecular-weight carbohydrates in the β -anomeric form. The former generates maltose, while the latter generates glucose (Babbar & Oberoi, 2014).

Cellulases are responsible for cellulose deconstruction. It is achieved by the synergic activities of endoglucanases, cellobiohydrolases (and exoglucanases), and β -glucosidases. Endoglucanases (EC 3.2.1.4) hydrolyze the internal glycoside bonds of the non-crystalline cellulose portion (<https://iubmb.qmul.ac.uk/>). Cellobiohydrolases (EC 3.2.1.91) remove tetrasaccharides and disaccharides like cellobiose from the non-reducing end of chains generated by endocellulase (Sharma, Oberoi & Dhillon, 2016); exoglucanases (EC 3.2.1.176) hydrolyze β -(1-4)-D-glycosidic linkages from the reducing end β -glucosidases (EC 3.2.1.21) cleave the terminal, non-reducing β -D-glycosidic residues, e.g., cellobiose, with the release of β -D-glucose (<https://iubmb.qmul.ac.uk/>).

Hemicellulases include xyloglucanases, several types of xylanases and mannanases, and β -glucanase.

Xyloglucan endo β -(1-4)-glucanases (EC 3.2.1.151) hydrolyze the β -(1-4)-D-glucose backbone of xyloglucans with retention of the β -configuration of the glycosyl residues (<https://iubmb.qmul.ac.uk/>).

Xylanases are specific for any kind and degree of functionalization of xylan backbones. Endoxylanases (EC 3.2.1.8) cleave glycoside linkages from the xylan chain, releasing xylooligosaccharides. These can be hydrolyzed from the non-reducing end by β -xylosidases (EC 3.2.1.37), which also act on xylobiose. α -arabinofuranosidases (EC 3.2.1.55) and α -glucuronidases (EC 3.2.1.139) break respectively terminal non reducing α -L-arabinofuranoside residue in α -L-arabinosides, and D-glucuronate in D-glucuronoside chains (<https://iubmb.qmul.ac.uk/>).

Mannanases hydrolyze the β (1-4)-glucose-mannose chains; Endo- β -mannanases (EC 3.2.1.78) release randomly mannans, galactomannans, and glucomannans, Exo- β -mannosidases (EC 3.2.1.25) release β -D-mannosides from the non-reducing-ends-of β -D-mannose-polysaccharides (<https://iubmb.qmul.ac.uk/>).

β -glucanases can catalyze the degradation of mixed linked β -glucans in a specific way, depending on the type of linkage: endoglucanases (EC 3.2.1.4) are cellulases that hydrolyze β -(1,4) linkages in glucans also containing β -(1,3)-linkages; lichenases (EC 3.2.1.73) are β -(1-4)-glucanases acting in β -D-glucans containing both (1-3) and (1-4) bonds; laminarinases (EC 3.2.1.39) hydrolyze β -(1,3)-D-glycosidic linkages just in β -(1-3)-D-glucans, whereas β -1,3(4)-glucanases (EC 3.2.1.6) can hydrolyze β -(1,3) or β -(1,4) linkages in β -D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C-3 (<https://iubmb.qmul.ac.uk/>).

Pectinases are classified according to the mode of attack on the specific structural elements of pectin. Protopectinases solubilize protopectin forming highly polymerized soluble pectin (Favela-Torres, Volke-Sepúlveda & Viniegra-González, 2006). Depolymerizing enzymes include endo and Exopolygalacturonases. Endo-polygalacturonases (EC 3.2.1.15) broke randomly the α -(1-4)-D-galacturonan linkages preferentially in pectate and other galacturonans, showing different tolerance to methyl esterification of the substrate. Exopolygalacturonases named EC 3.2.1.67 and EC 3.2.1.82 hydrolyze the first and second α -(1-4)-glycosidic bonds from the non-reducing end of polygalacturonate. The action of Rhamnogalacturonan (3.2.1.171) on α -D-GalA-(1-2)- α -L-Rha glycoside bond release oligosaccharides with inversion of anomeric configuration as β -D-GalA at the reducing end (<https://iubmb.qmul.ac.uk/>).

Esterases comprise a large group of enzymes acting on the cleavage of ester bonds. Some examples are the pectin methyl esterases (3.1.1.11), which are responsible for the demethylation of side groups of the pectin backbone, leading to a reduction of molecular chain weight; feruloyl esterases (3.1.1.73) catalyzes the hydrolysis of the 4-hydroxy-3-methoxycinnamate (feruloyl) group from esterified sugar, which is usually arabinose. They are sometimes called hemicellulase accessory enzymes since they help xylanases and pectinases to break down plant cell wall hemicellulose.

Acetylxyylan esterases (3.1.1.72) act exclusively by the deacetylation of xylans and xylooligosaccharides, not f mannan or pectin (<https://iubmb.qmul.ac.uk/>).

Lipases are triacylglycerol acyl hydrolases (EC 3.1.1.3), which catalyze the hydrolysis of triacylglycerol with long beta fatty acids chains to glycerol and fatty acids. They often express other

activities, such as phospholipase, lysophospholipase, and cholesterol esterase (Svendsen, 2000). Specific esterases instead digest short-chain acylglycerols.

Proteases, also named peptidases, proteases, or proteinases, are proteolytic enzymes capable of hydrolyzing peptide bonds (<https://iubmb.qmul.ac.uk/>). Depending on the site of enzyme action, proteases (EC 3.4) can also be subdivided into exopeptidases or endopeptidases. Exopeptidases catalyze the hydrolysis of the peptide bonds near the N- or C-terminal ends of the substrate. Endopeptidases cleave peptide bonds within and distant from the ends of the polypeptide chain (Sharma et al., 2016). Additional subdivisions depend on the quantity and the types of released amino acids.

Ligninases comprise laccase, lignin peroxidase, and manganese peroxidase and can hydrolyze the lignin fraction. Laccases (EC 1.10.3.2) are copper-containing enzymes and catalyze the oxidative cleavage of phenolic compounds (both *o*- and *p*-quinols), and often acting also on aminophenols and phenylenediamine (<https://iubmb.qmul.ac.uk/>) producing radicals with the concomitant reduction of molecular oxygen to water. Lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) are produced from white-rot basidiomycete fungi; they present a heme group that requires hydrogen peroxide as an oxidant, in degradative reactions of aromatic compounds composing lignin. The lignin peroxidase cannot act directly on the lignin molecule, which is too large to fit into the active site, but it can act on a wide range of aromatic compounds (<https://iubmb.qmul.ac.uk/>).

Each sector of the **agro-food industry** uses a broad range of enzymes in its processing products to enhance their quality and aspect or sensorial characteristics. Starch manufacturers and sweeter producers perform liquefaction and saccharification by using amylases, glucoamylases, pullulanase, glucose isomerase, and other carbohydrate-degrading enzymes to obtain syrups made of monosaccharides like maltose, fructose, dextrose, sorbitol, etc. (Eshra, El-Iraki & Bakr, 2014).

The dairy industry uses lipases to help milk clotting and coagulation and enhance the taste and texture during cheese, butter, and bread manufacturing (Arya et al., 2022). In bread production, the employment of synergistic α - and β -amylases with other carbohydrate-degrading enzymes is common bakery industry to reduce dough viscosity and increase bread volume and crumb texture (Raveendran et al., 2018). Using protease to break down complex starch-gluten molecules into simpler ones improves the uniformity and rheology of dough. In brewery facilities, proteolytic, amylolytic, and hemicellulolytic enzymes are involved in the malting process, the flavoring, and quality improvement of finished goods (Arya et al., 2022). Wine industries use pectinases, hemicellulases, and α - and β -glycosidases to liberate molecules responsible for fragrance. The juice processing sector uses mainly endo- and exo-polygalacturonases to reduce the viscosity of the juice by hydrolyzing galacturonans to galacturonic acid. Fruit pulp processing uses polygalacturonases and pectin methyl esterases to encourage the peeling of fruits (Arya et al., 2022). Also, the waste and by-products from food processing, such as peels, seeds, and fibers, are excellent candidates for enzymatic processing to provide valorized derivatives.

Because of the heterogeneous composition of plant cell walls, the pursuit of their **efficient degradation** by enzymatic reactions requires expert knowledge of the substrate to be hydrolyzed. Cellulose is highly recalcitrant, pectins and xylans require a wide use of enzymes, and phenolic compounds are firmly cross-linked to the other components (Howard et al., 2003). Considering that

the synergic actions of multiple enzymes may be necessary, the understanding of the optimal conditions for each enzyme to work, as well as the proper sequence of their addition in the reaction mixture, may be needed, especially if the recovery of a specific molecule is to be reached, or the functionalization of a specific compound wants to be achieved (Martínez-Abad et al., 2016).

Consider, for example, pectin, a plant-based polysaccharide primarily used in food and beverage industries for its gelling and stabilizing properties. Its ability to form stable gel has been correlated to the degree of methylation (DM) of galacturonic acid units and to their distribution pattern, identified by the blocking degree (DB) parameter, together with the presence of divalent or monovalent ions that could or not stabilize the interactions among the chains, depending on the type and number of side substituents. The pectin gelling ability can be modulated by choosing the appropriate enzymes during the extraction process or in the functionalization of extracted pectin. In general, the use of backbone degrading enzymes causes a decrease in the viscosity of pectin gel, whereas the enzymatic modification of side chains leads to an increase in viscosity. Sometimes gelling process might be driven by the length of methylated galacturonic acid residues rather than by the methylation degree itself. If so, the action of endo-polygalacturonases has an important rule. At the same time, though, endo-polygalacturonases could not access the galacturonic acid backbone, unless the side chains have been removed by pectin methylesterase and acetyl esterases to a certain extent that changes on a case-by-case basis. Hence pectin with desired functional properties can be obtained only upon a balanced choice of enzymatic activities (Martínez-Abad et al., 2016).

A most explicative example of the targeted application of enzymes is the recovery of hydroxycinnamic acids, like ferulic and coumaric acids, from plant resources. In plant cell walls, ferulic acid is found to be covalently linked both to polysaccharides by ester bonds and to lignin components by ester and ether bonds, resulting in cross-linkages that change from plant to plant. Generally, in monocotyledon species, the feruloyl group is 5-O attached to L- α -arabinofuranosyl residues that functionalize the β -(1-4) xylans in arabinoxylans at C-2 and C-3 (Fig. 8).

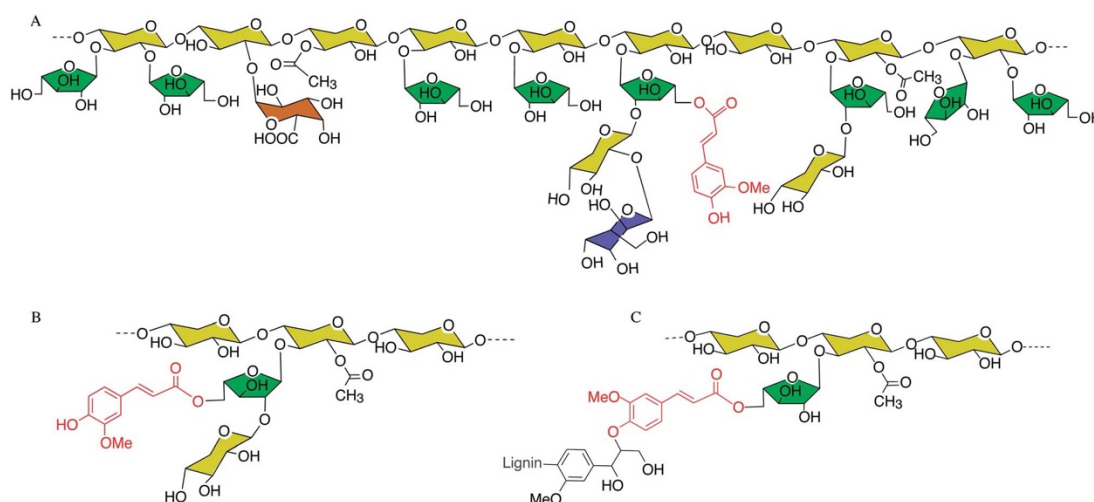


Fig. 8 Maize pericarp glucuronoarabinoxylan (GAX). Representative structure with ferulic acid (shown in red) esterified to arabinosyl residues (Mnich et al., 2020).

In dicotyledon species, ferulic acid is associated with pectin polymer to which it could be bound by either the C-2 hydroxyl group of arabinofuranose or C-6 hydroxyl group of galactopyranose residues of the α -(1-5) linked arabinan chains (Mathew & Abraham, 2004) (Fig. 9). This is a key factor to consider because it is amply shown that specific feruloyl esterases are not sufficient to extract ferulic acids from the plant cell walls, if not accompanied by the synergic action of accessory carbohydrates enzymes that release fragments more suitable for attack by esterase.

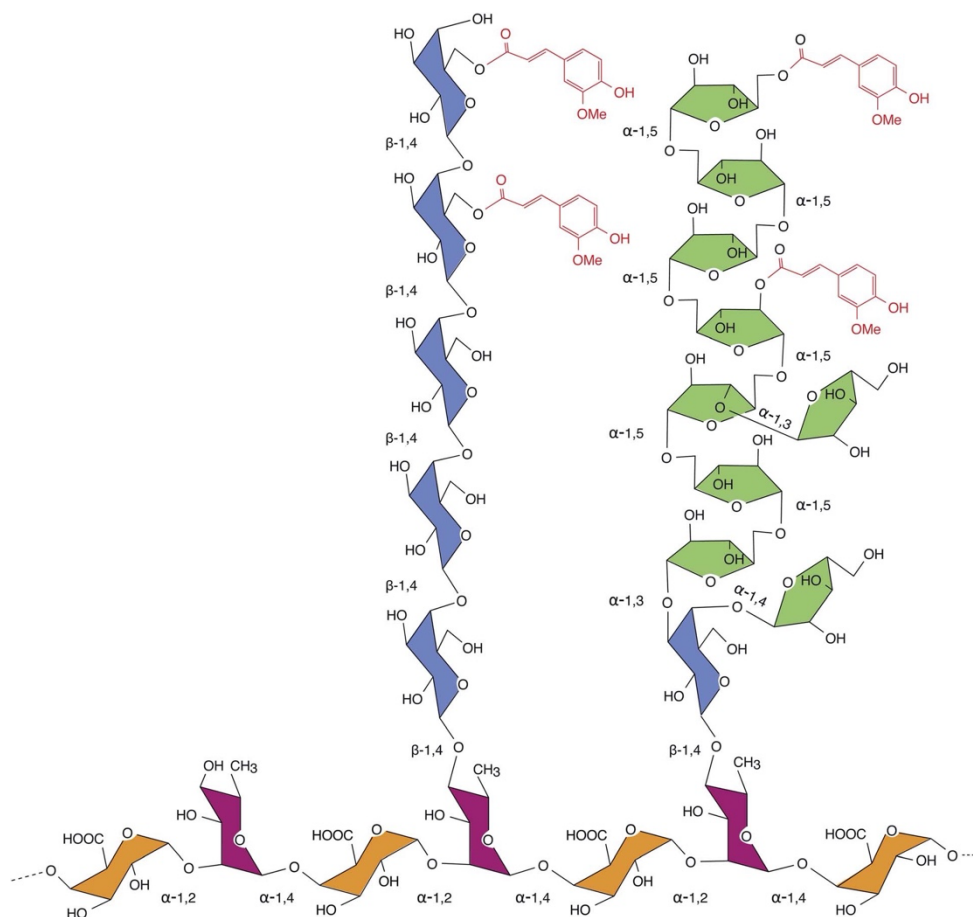


Fig.9 Simplified structure of RGI, a pectic polysaccharide with a back-bone of alternating rhamnosyl and galacturonosyl residues. Rhamnosyl residues may carry neutral side-chains esterified with ferulic acid (shown in red). Monosaccharides are color-coded: galacturonic acid, orange; rhamnose, dark pink; galactose, blue; arabinose, green (Mnich et al., 2020).

In monocot they are xylanases or β -xylosidases; in dicot are endo-arabinanases and α -L-arabinofuranosidases. Even within the same type of plant, there is a significant variability to take into account; for example, the treatment of de-starched wheat bran, barley spent grain, and maize bran with the same esterase and xylanase leads to different extraction yields of ferulic acid, even if it is linked to the same 5-O of arabinofuranosyl residues (Yu, McKinnon & Christensen, 2005). In this case, the branched degree of heteroxylans plays a big part because the substituents may restrict the accessibility of the esterase to the feruloyl groups (Yu et al., 2005). The complex features in

polymer arrangements and cross-linkages in lignocellulosic biomass originate a certain resistance toward the easy and efficacious decomposition by enzymes. Chemical, physical, and biological **pretreatments** have shown good effectiveness in increasing enzyme accessibility to the substrate by breaking various linkages, reducing particle sizes, and enhancing fiber porosity. These turn into a considerable reduction in the crystallinity of plant cell walls and an increase in the superficial area available for enzymatic reaction sites (Padayachee et al., 2017). Chemical methods include the usage of alkali or acid to improve the biodegradability of cellulose and the use of organic solvents to promote delignification. Physical methods comprise the steam explosion technique, microwave, and ultrasonic baths, as well as mechanical grinding to help the tissues rupture and milling to improve the porosity of plant material (Mota et al., 2018). Partial physical alteration of the native structures of fruit and vegetable can also derive from applying processes in the food industry such as blanching, boiling, cooking, pureeing, homogenization, juicing, and canning (Padayachee et al., 2017).

Ideally, the pretreatment steps should avoid the formation of enzyme inhibitors, such as furfural or other phenolic compounds, that could lower the yield of the subsequent enzymatic stages of bioconversion and avoid the usage of toxic substances that could entail additional costs in waste management (Martínez-Abad et al., 2016).

Biological pretreatments have some advantages over the previous because they are less energy-demanding and more environmentally friendly; they rely on the direct use of microorganisms, for example, brown, white, and soft rot fungi that produce hydrolytic ligninases enzymes, capable of weakening the secondary cell wall seal (Mota et al., 2018).

2.3.3 Biocatalyst production

Enzymes are found in nature as secreting by unicellular and pluricellular organisms to catch nutrients from complex natural resources and as specific mediators for some metabolic reactions to proceed. They work as biocatalysts lowering the activation energy required for reactions to occur. These features make them exploitable in many industrial sectors, mainly by green chemistry solutions to tailor well-established products, produce new ones, and make building blocks for innovative bioconversion platforms. There are several commercial-grade enzymes and enzyme cocktails that have been used in these contexts, mainly comprising polysaccharide backbone-cleaving enzymes, such as α -amylases (Fungamyl Super AX), endoglucanases (UltraFlo[®], FiberCarer, Accellerase BG[®]), cellulases (Celluclast 1.5L[®], Accellerase 1500[®]), xylanases (Pentopan Mono BG[®], Accellerase XY, NS50030, NS50014[®]), mannanases (Mannaway[®]), and pectinases (Pectawash, XPect[®], Rapidase C80 max[®]) (Martínez-Abad et al., 2016). Although sold for their main activities, they mostly exhibit side and concomitant activities because of the fermentation by which they are generated, which could be exploited for more comprehensive and effective enzyme usage (Giacinti, 2011). A restricting factor for the widespread application of enzymes is their high price, which can significantly contribute to overall manufacturing expenses (Barta, Kovacs, Reczey & Zacchi, 2010). Nevertheless, a valid and most economical alternative to benefit from enzyme capability and versatility is their on-site production by facilities that are willing to use them directly in their process. Enzymes can be biosynthesized by **fermentative process** upon the inoculation of proper organisms grown under aerobic conditions on specific substrates. Multiple factors can affect the fermentation

yield and thus must be taken under control: the chemical nature of the substrate and the producer microorganism, the inoculum size, C-source, N-source, O₂ dissolved, and moisture content in the medium (Chugh, Soni & Soni, 2016).

Natural organic wastes from agro-industrial and food sectors are sources of proteins and carbohydrates. Hence, they can be employed for the production of various hydrolytic enzymes. Many fungal species, among which different strains of *Aspergillus oryzae*, *A. niger*, and *Trichoderma reesei*, utilize organic wastes such as wheat and corn bran, rice husk, soybean fiber, sugarcane bagasse, fruit peels, and pomaces to produce cellulases, proteases, beta-glucosidases, xylanases, mannanases, pectinases, alpha-amylases, and glucoamylases (Arya et al., 2022).

In alpha-amylases and glucoamylases production, the yeast *Saccharomyces cerevisiae* is also notable (Blanco, Caballero, Barrios & Rojas, 2014). Likewise, various bacterial species (*Bacillus pumilus*, *Pseudomonas aeruginosa*) and actinomycetes (*Streptomyces* sp.) are found to produce amylase, xylanase, and alkaline protease from agricultural waste (Arya et al. 2022). White-rot fungi (WRT) like *Agaricus bisporus*, *Coriolus versicolor*, *Pleurotus ostreatus*, and *Trametes versicolor*, grown on various lignocellulosic substrates produced carbohydrase enzymes and the most specific ligninases, among which laccases and peroxidases (Mayolo-Deloisa et al., 2020), and for that are also employed in the pretreatment stage of biomass deconstruction (Isroi et al., 2011). According to Reshmy et al. (2022) (Arya et al., 2022), the cost of substrates for the growth of the enzyme accounts for 40% of the total fermentation process cost. In this way, the usage of agro-food wastes as alternative substrates can also contribute in a significant way to reducing process costs (Arya et al., 2022).

Moreover, the growth of enzymes on organic wastes is coupled with the release in the fermentation medium of bioactive compounds that were entrapped into the matrix, and different generated metabolites, which could better the economy of the process due to their high added value (Corrêa, Penha, Freitas-Silva, Luna & Gottschalk, 2021). It is well known that food by-products and wastes, besides vitamins and minerals, are unique reservoirs of anti-oxidant molecules, like polyphenols, flavonoids, phenolic acids, and carotenoids. What is more, some research shows that pomace residue deriving from some fruit and vegetable processing (i.e., skins, pulp, seeds, fruit stem) has more antioxidant content than the fresh matter (Waldron et al., 2003) or the processed product (Padayachee et al., 2017).

The growth of a bacteria or a fungus on a specific substrate leads to the production of enzymes able to selectively hydrolyze the constituents of the same kind of plant (Mathew & Abraham, 2004; Masutti et al., 2016); this can serve as an essential tool in recovering specific molecules. So, for example, the growth of *A. niger* on oat spelt xylan induces the production of FAE-III enzyme, which can release ferulic acid and methyl ferulate from wheat bran-derived feruloylated oligosaccharides, but is inactive on sugar beet pulp-derived feruloylated oligosaccharide (Mathew & Abraham, 2004). The enzymes produced by *Pleurotus ostreatus* hydrolyze efficiently the same agro-food wastes on which it grew (Masutti, Scardovi, Borgognone & Setti, 2016).

Enzyme production is achieved using both submerged fermentation (SmF) and solid-state fermentation (SSF) in aerobic conditions. **Submerged fermentation** was the most used method until some decades ago. It is based on the inoculation of the microbial culture in a liquid medium. It thus requires a series of precautions to ensure the optimal incorporation of oxygen without

compromising the strain resistance. At the industrial level, this is pursued by preferring airlift or bubble-column reactors (Kim, Kang & Lee, 1997), which have lower shear stress than stirred-tank reactors, the use of which may cause the rupture of mycelial cells and deactivate the enzymes (Wase, McManamey, Raymahasay, & Vaid, 1985).

Even if a practice well-known from ancient times, **Solid state fermentation** has only gained attention in enzyme production in the last decades. It is a static process involving microbial growth on a solid material that acts as physical support and a source of nutrients in the complete or almost complete absence of free liquid. The only moisture present is water in complexed form with the substrate, which is essential for microbial activities (Howard et al., 2003). Thus, the water activity and the substrate particle size can severely affect fermentation yield. Ideally, the proper substrate particle size for each process should be evaluated, considering the type of lignocellulosic material and the kind of microbes going to grow on it. Smaller particles provide a larger surface area for microbial colonization, but they may result in substrate agglomeration and poor strain growth; in contrast, larger particles provide better aeration but may limit the surface area for colonization (Pandey, Selvakumar, Soccol, & Nigam, 1999).

In comparison to SmF, SSF has many advantages. It provides higher volumetric productivity with less production and operative costs since it requires lower space and energy due also to the lack of mechanical handling (Viniegra-González, Favela-Torres & Aguilar, 2003). It requires lower medium complexity and shorter fermentation time to yield enzymes with a higher temperature and pH stability and less production of undesirable protease, which could degrade the produced enzymes (Toca-Herrera, Osma & Rodriguez Couto, 2007). The only relevant drawbacks are its limitation in the scale-up process due to heat and mass transfer (Sharma et al., 2016), biomass growth, and the control of parameters within the bioreactor, such as pH, aeration, and temperature, which are difficult to manage beyond the lab scale (Toca-Herrera et al., 2007). Although the production of enzymes at the commercial level is unfeasible by the solid-state fermentation process, it can be very suitable for the pretreatment of lignocellulosic biomass in small biorefinery or if used as a small enzymatic production unit to be integrated into facilities that want valorize the own wastes.

Solid-state fermentation can be convenient for the utilization of filamentous fungi, which require small quantities of water to grow and can penetrate the solid substrate through their mycelium extension (Toca-Herrera, Osma & Rodriguez Couto, 2007). They also grow by competing with other microorganisms, discouraging bacteria proliferation (Kertesz & Thai, 2018). White rot, brown rot, and soft-rot fungi are the most effective in degrading wood biomass. White-rot and brown-rot belong to Basidiomycetes, whereas soft-rot fungi belong to Ascomycetes (Isroi et al., 2011).

The **White-rot fungi** exhibit two different enzymatic systems: the first is a hydrolytic system, which produces hydrolases like amylase, hemicellulase, and cellulase, that are responsible for polysaccharide degradation; the second is a unique oxidative ligninolytic system which degrades lignin (Fig. 10) and opens phenyl rings (Lundell, Mäkelä, & Hildén, 2010) comprising laccases (EC 1.10.3.2), lignin peroxidases (EC 1.11.1.14) and manganese-dependent peroxidases (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16), and H₂O₂ forming enzymes like aryl alcohol (EC 1.1.3.7) (Isroi et al., 2011). To develop these specific abilities, white-rot fungi had to face with is three main challenges: the lignin polymer is large, therefore the lignocellulosic system must be exocellular; lignin comprises inter units of carbon-carbon and ether bonds, therefore the degradation system

must be oxidative that hydrolytic; lignin is stereo irregular and thus requires ligninolytic agents less specific than the degradative enzymes (Isroi et al., 2011).

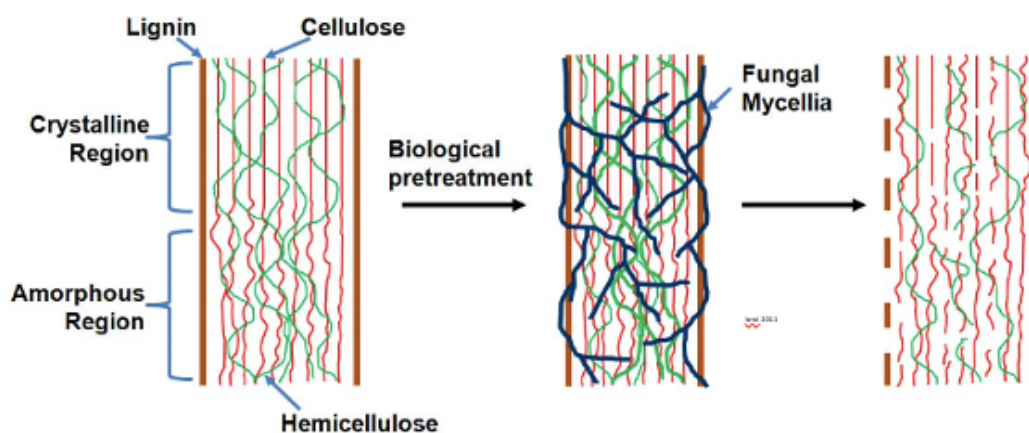


Fig.10 Degradation of lignocellulosic substrate by White-rot fungi.

Pleurotus ostreatus is a white rot fungus strain capable of efficiently metabolizing lignin in various lignocellulosic materials, acting preferentially on straws (Hatakka, 1983). It is edible; hence its use does not concern safety issues, and its growth does not require elaborate medium composition; thus, it is easy to perform. Table 2 reports some examples of its use on several kinds of substrates. Masutti et al. (2012) used *Pleurotus ostreatus* to conduct SSF on rice husk, wheat bran, grape stalks, and grape seed; they detected a wide range of enzymatic activities in the leachate produced by fungi, such as peroxidase, laccase, but also cellulase, xylanase, and arylesterase. They performed the fermentation on both individual and mixed substrates. They found that choosing the substrate makes it possible to modulate the inhibition or the induction of specific activities (Masutti, Borgognone, Scardovi, Vaccari, & Setti, 2015). Furthermore, they demonstrated the effect of close samplings in collecting enzymes from the fermentation bulk on the induction of higher enzymatic productivity (Masutti et al., 2012). Given the large number of small agro-food industries that use enzymes to improve their transformation process, SSF by *Pleurotus ostreatus* conducted on-site using their wastes or by-products as substrate represents a safe and low-cost alternative for having specific and effective biocatalysts to use for effective and green in-house conversion of biomass.

Table 2 Production of enzymes by *Pleurotus ostreatus* on food-waste substrates.

Substrate	Products	Ref
Tomato pomace	Laccase, xylanase, protease	Iandolo et al., 2011
Corn cobs	Laccase, cellulase, xylanase	Han et al., 2020
Poplar sawdust	Laccase, cellulase, xylanase	Han et al., 2020
Potato peel	Laccase, Peroxidase, Amylase, Protease	Ergun & Urek, 2017
Cassava waste	Laccase	Kumar et al., 2022
Wheat straw	Laccase, Peroxidase, Cellulase, Xylanase, Carreroyl esterase, Feruoyl esterase	Masutti et al, 2012
Rice husk	Laccase, Peroxidase, Cellulase, Xylanase, Carreroyl esterase, Feruoyl esterase	Masutti et al, 2012
Hemp Bran	Laccase, Peroxidase, Cellulase, Xylanase, Protease, Carreroyl esterase, Feruoyl esterase	Setti et al., 2020

2A Case of study: Production of enzymes by SSF of *Pleurotus ostreatus* on Hemp Bran

Introduction

Pleurotus ostreatus is able to produce hydrolytic and ligninolytic enzymes simultaneously through the fermentation of different solid lignocellulosic wastes ([Iandolo, Piscitelli, Sannia & Faraco, 2011](#); [Ergun & Ozturk, 2017](#); [Masutti et al., 2012](#); [Setti, Samaei, Maggiore, Nissen, Gianotti & Babini, 2020](#)). Under the SSF of potato peel, it is known to produce highly active laccase, but also to secrete exocellular alpha-amylase and protease enzyme ([Ergun et al., 2017](#)). It has also shown remarkable enzymatic activities from SSF on tomato skin ([Iandolo et al., 2011](#)) and more lignified matrices such as poplar sawdust and corncob wastes ([Han et al., 2020](#)), indicating that by using non-expensive substrates, it is possible to produce enzymes with high activities with the low-cost fermentative process. Many types of waste derived from agricultural scraps and the early stages of food processing have been used as substrate in solid-state fermentation; among these, hemp (*Cannabis sativa* L.) bran remains almost entirely underexploited, despite the hemp production and its processing have considerably increased in the last years. Indeed, there has been a surge of interest in it due mainly to its seed oil, rich in polyunsaturated fatty acid, its high protein content with antioxidant capacity, and its other bioactive and non-toxic compounds, which have been appreciated only recently, attracting the attention of food and cosmetic sectors ([Nissen, di Carlo & Gianotti, 2020](#)). The hemp bran (HPB) is the byproduct of hemp seed processing for the obtainment of hemp oil and flour. It represents the grossest part of hemp meal, the proteinaceous cake deriving from the mechanical pressing of defatted hemp seed, which is the initial processing step in the hemp food chain. Generally, hemp bran is discarded or used for animal feed, and although its important protein and polyphenols content ([Pojić et al., 2014](#)), there are very few examples in literature that explore the possibility of valorizing it, with only two studies about the application of biotechnological approaches ([Budžaki et al., 2018](#)). Setti et al. (2020) have proposed three different valorization strategies of hemp bran to be applied in a biorefinery context, among them the solid-state fermentation by *Pleurotus ostreatus* on hemp bran stripped of proteins. With the present study, I propose to conduct the same fermentation process with the only difference of using hemp bran-containing protein as substrate, and evaluate the produced enzymatic activities in comparison with those detected by our previous work ([Setti et al., 2020](#)).

Results and discussion

Different carbohydrase activities (xylanase, pectinase, alpha-amylase), together with protease, laccase, peroxidase, and esterase, has been estimated during three weeks of solid-state fermentation by *Pleurotus ostreatus* on hemp bran (Tab. 1). In addition, for the carbohydrase and protease, the optimum of pH has been evaluated (Fig. 1-4). To verify the effectiveness of the enzyme actions, three hydrolysis reactions of the hemp bran have been carried out by using the enzymes

collected each week as biocatalysts (Fig. 7), and reaction products have been characterized in terms of tyrosine and total phenols contents.

Tab. 1 Activities (U/L) and collected volume (mL) of enzymes produced during 3 weeks of SSF conducted using *Pleurotus ostreatus* on Hemp Bran (T= 30°C). n.d. stands for not detected.

Enzyme	Activity (U/L)		
	7 days	14 days	21 days
Xylanase	6702 ± 603	5401 ± 698	5081 ± 814
Amylase	3296 ± 485	2274 ± 98	2088 ± 267
Pectinase	8261 ± 1008	12300 ± 1379	10500 ± 2041
Protease	97.3 ± 14.1	n.d.	22.6 ± 2.7
Laccase	23.0 ± 0.9	398.5 ± 43.5	129.1 ± 26.8
Peroxidase	19.0 ± 1.9	405.8 ± 41.3	133.1 ± 28.9
Feruloyl esterase	7.32 ± 0.32	0.27 ± 0.02	3.09 ± 0.15
Caffeoyl esterase	3.81 ± 0.06	n.d	6.45 ± 0.54
Enzyme	Collected volume (mL)		
	7 days	14 days	21 days
	62	81	86

The carbohydrase expression follows the destructuring pathway typical of dicotyledon polysaccharides, in accordance with the nature of the *Cannabis Sativa* plant, showing important pectinase and xylanase activities since the beginning and for the whole duration of fermentation, with a peak in correspondence of the 14th (12300 ± 1379 U/L) and 7th (6702 ± 603 U/L) day respectively (Fig. 1-2). These are relevant results if compared to what emerges from Han et al. work, in which the xylanase activities derived from SSF of *Pleurotus ostreatus* on corncob waste and poplar sawdust are, respectively, 1305 and 1362 U/L (Han et al., 2020).

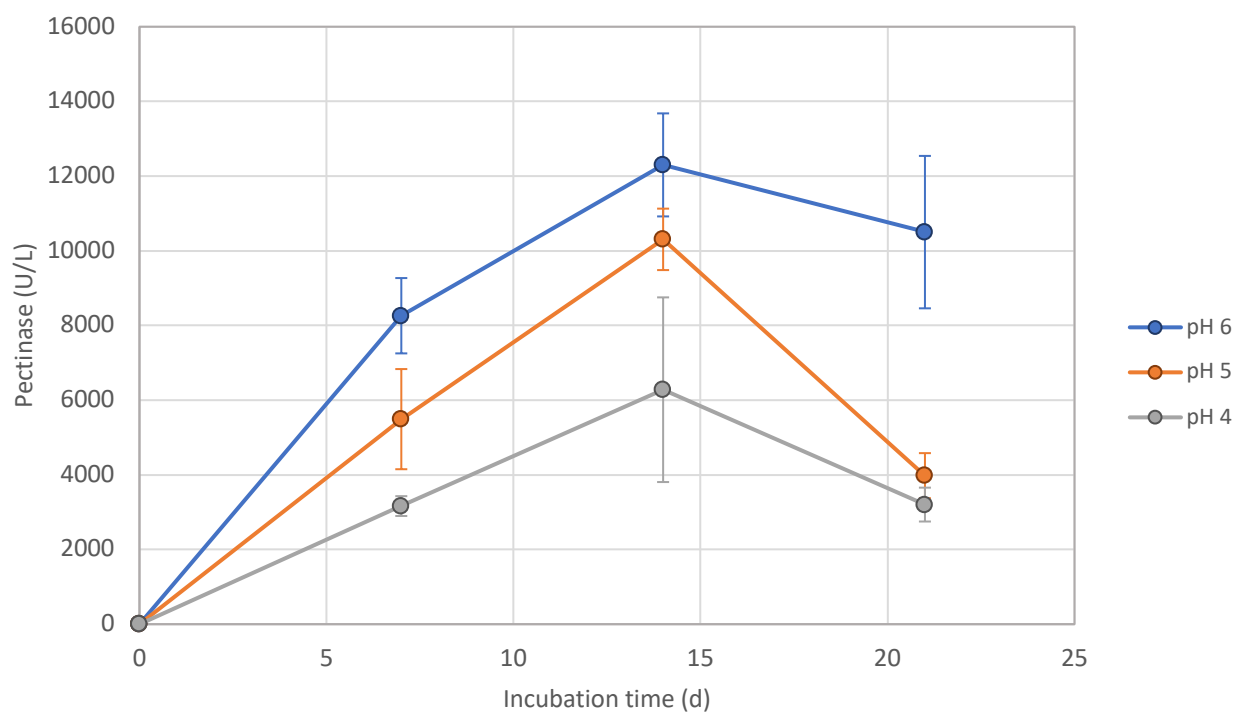


Fig. 1 Pectinase activities (U/L) with changes in pH detected during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran. The values are the mean for $\pm$ SD for three measurements in the same experiment.

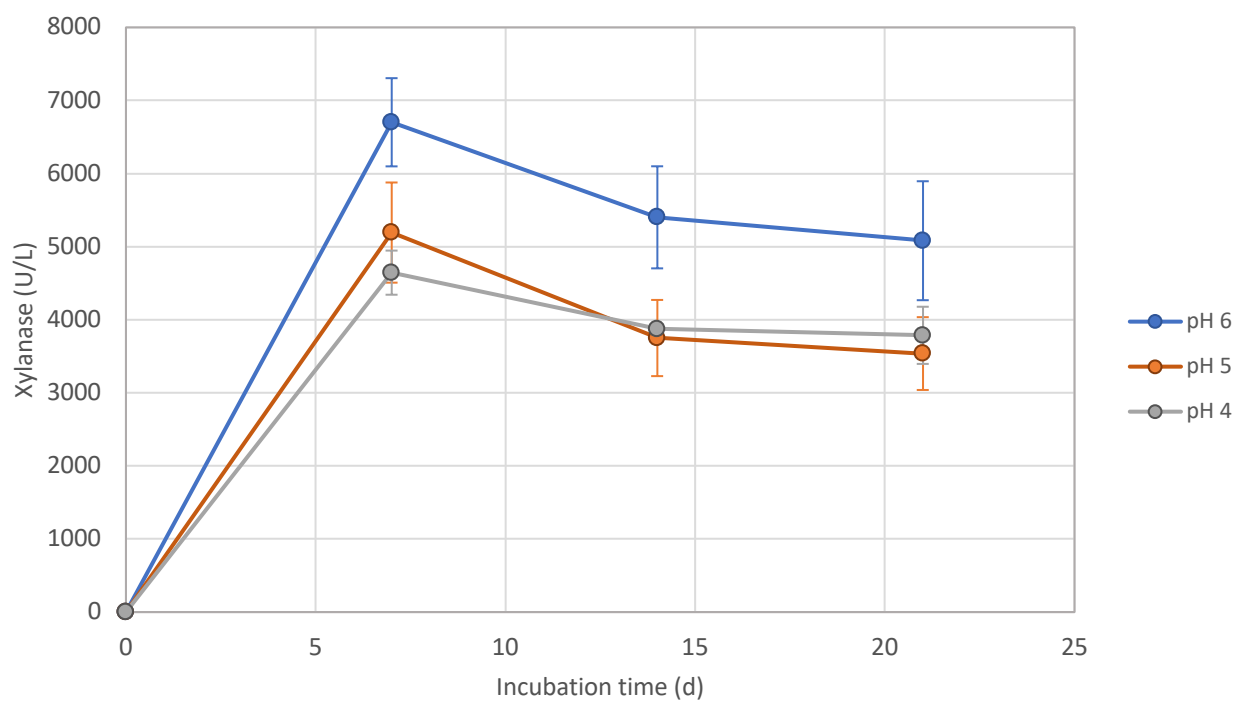


Fig. 2 Xylanase activities (U/L) with changes in pH detected during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran. The values are the mean for $\pm$ SD for three measurements in the same experiment.

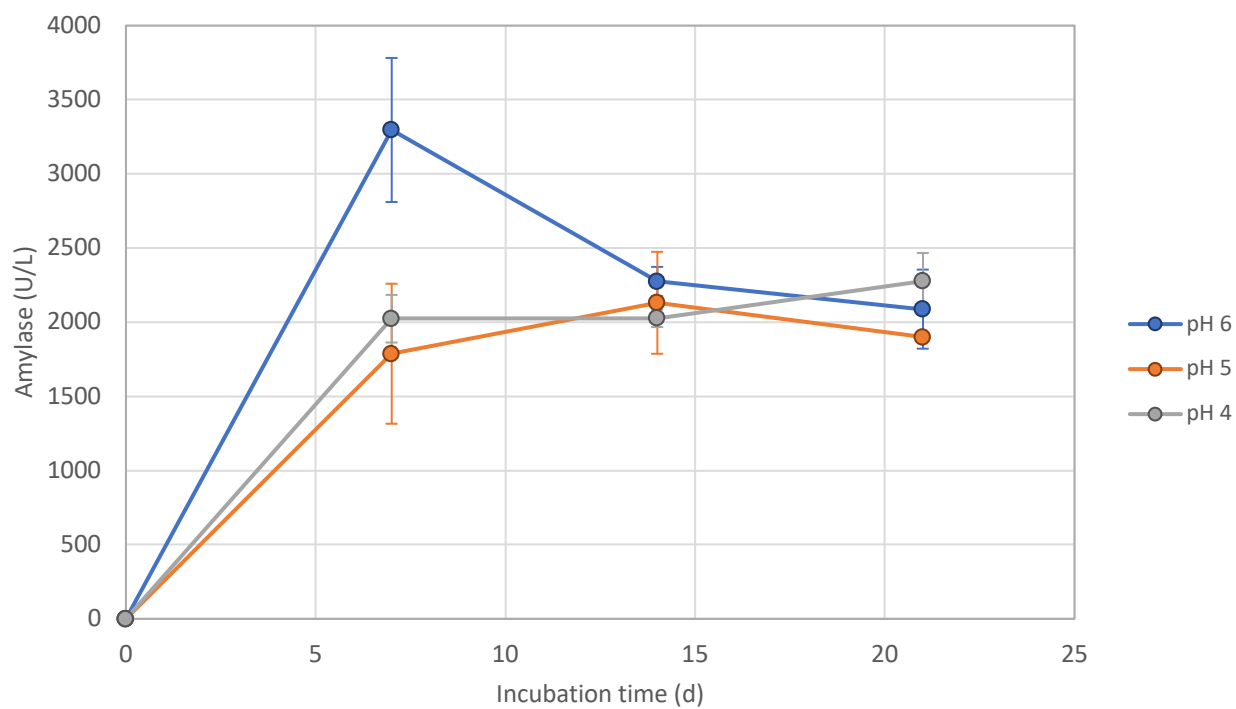


Fig. 3 Amylase activities (U/L) with changes in pH detected during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran. The values are the mean for $\pm$ SD for three measurements in the same experiment.

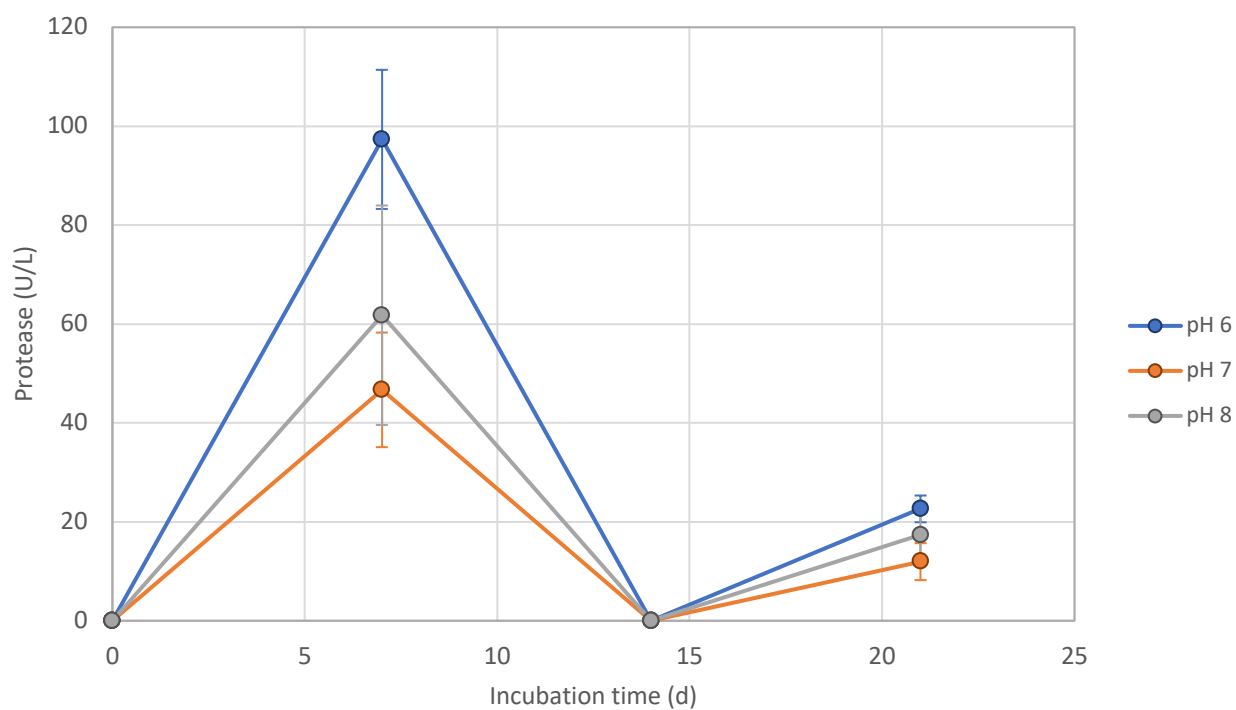


Fig. 4 Protease activities (U/L) with changes in pH detected during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran. The values are the mean for $\pm$ SD for three measurements in the same experiment.

In the first week, reducing sugars in enzyme production bulk has been detected in modest amounts, which decrease to 5 and 10-fold in the second and third week, respectively (Tab. 1), presumably because of the sugar consumption by fungus metabolism. The sugars metabolized could serve as a carbon source, inducing the synthesis of ligninolytic enzymes, which would explain the increase in Lacasse and Peroxidase activities observed in the second week (Fig. 5). Moreover, the two enzymes show the same trends and extent of activities during the SSF incubation period, indicating the relationship between their production by *P.ostreatus* and their synergic action in substrate colonization. In response to the sugar depletion observed in the second and third weeks, the fungus seems to modulate the production of enzymes involved in polysaccharide hydrolysis, as appears by the increase in pectinase and the sustained expression of xylanase (Fig. 1-2). The same behavior has been observed in a study about SSF by *P.ostr.* on tomato skin ([Iandolo et al., 2011](#)). The arylesterases detected on methyl ferulate and methyl caffeate substrates are very low (Fig. 6) stating the low amount of ferulic and caffeic acid residues in the specific plant cell wall. In dicotyledon plants, these kinds of phenolic acids are bound in the branches of pectin chains. Thus efficient hydrolysis of the pectic polysaccharides should release them to some extent. The low feruloyl esterase activity detected is instead due to the low content of total phenols in the seed of *Cannabis sativa*, compared to the inflorescence, the sprouts, and the aerial part of the plant ([Izzo et al., 2020](#)). Ferulic acid is a known inducer of laccase production in the degradation of lignin ([Karp et al., 2015](#)); hence its absence could partly explain the limited laccase activity.

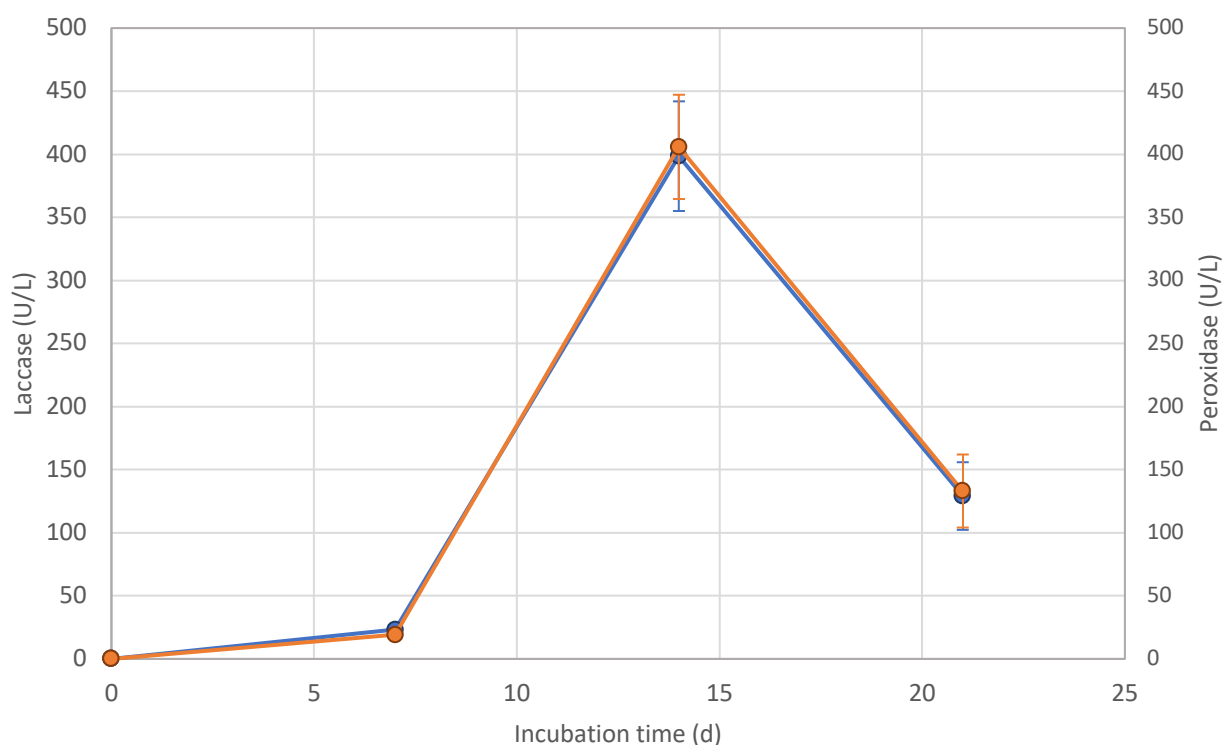


Fig. 5 Laccase (blue) and Peroxidase (orange) activities (U/L) detected during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran. The values are the mean for $\pm$ SD for three measurements in the same experiment.

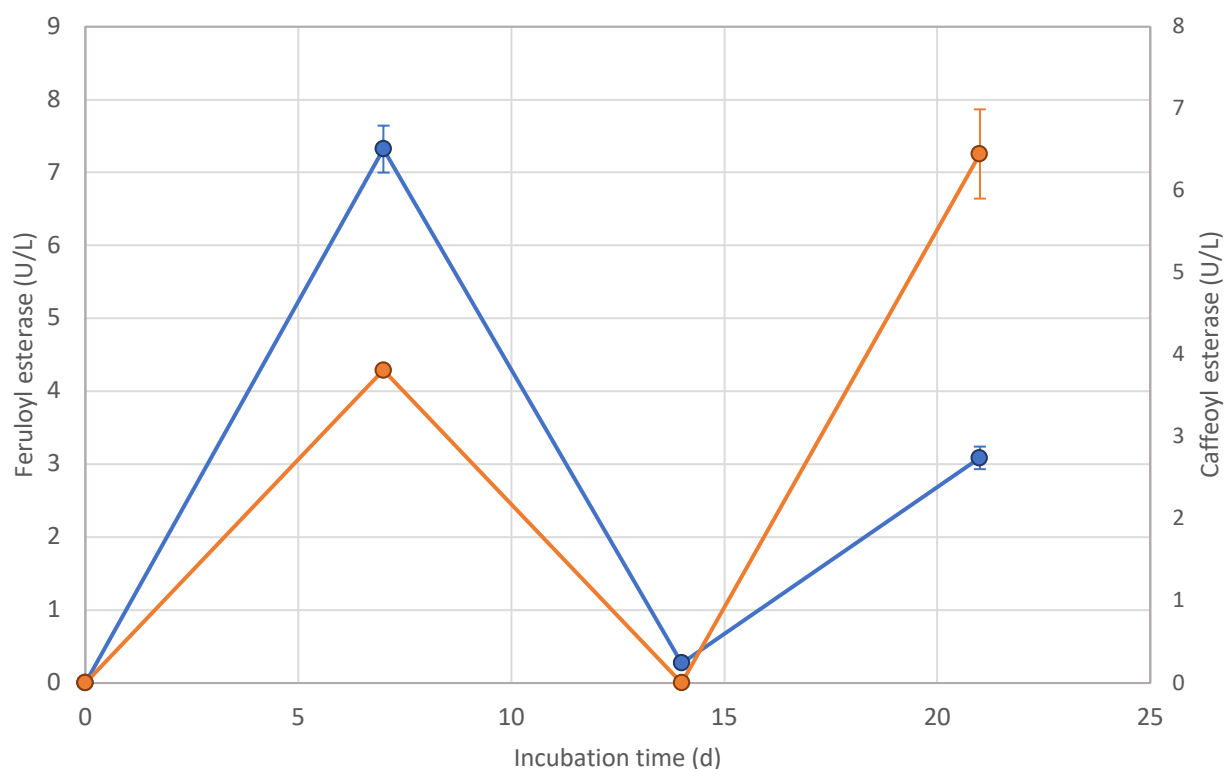


Fig. 6 Feruloyl (blue) and Caffeoyl (orange) activities (U/L) detected during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran. The values are the mean for $\pm$ SD for three measurements in the same experiment.

Surprises the notable production of amylase reaching 3296.56 U/L after one week of incubation (Fig. 3) since hemp seed has a very low amount of starch (<2%, [Schultz et al., 2020](#)). However, it is a validation of our previous finding ([Setti et al., 2020](#)). By comparison, the activity detected by Ergun et al. (2017) during SSF of *P. ostr.* on potato peel resulted in being 550-fold lower than the present study. However, potato is a well-known source of starch. This could be explained by both the pretreatment methods and autoclave cycles performed on potato peel, which could early extract, before the fermentation, a large part of the easily water-soluble potato starch.

The protease activity is very low and nearly suppressed in the second week of fermentation (Fig. 4), in correspondence with the higher amount of tyrosine residues detected in the enzymatic solution (Tab. 2). This could suggest the presence of protease inhibitors released by the plant in defending itself from the fungal attack, revealed by the absorption at 280 nm, as other proteins and glycoproteins ([Kunitz, 1947](#)). Dosoretz et al. (1990) postulated that white rot fungi could produce proteases to recycle nitrogen by breaking down secreted proteins or proteins released into the medium on cell autolysis.

The proteolytic process, resulting from tyrosine residue detection, might also signify phenol-oxidase released by the fungus ([Flurkey & Inlow, 2008](#)).

All the activity detected shows pH 6 as the optimum reaction condition (Fig. 1-4) Shall be excluded the influence of buffer pH (5.8) used in autoclaving sterilization stage and for the collection of enzymes since it is the same used in our group's previous work ([Setti et al., 2020](#)), in which instead,

each kind of activity manifests a proper and different optimum pH (data not shown). What differs in the present study is the lack of alkaline pretreatment of the substrate to extract hemp proteins. Respect the finding in our previous work, the present study shows the same trend of the carbohydrase activities, with a higher pectinase activity, probably because the alkaline pretreatment brings into solution a portion of the pectic polysaccharides of the substrate before it undergoes the fermentation. The same treatment affects the production of ligninolytic enzymes, which were up to 5-fold higher in our previous study (Tab. 3). This is because sodium hydroxide effectively attacks the linkage between lignin and hemicellulose, cleaving the ether and ester bonds and the ester and carbon-to-carbon (C–C) bonds in lignin molecules (Kim, Lee & Kim, 2016). Although it encourages the rupture of ester bonds, it could improve the accessibility of the enzyme to the residual ester linkages. This could justify the slight increase in arylesterase activities observed on the pretreated substrate, as well as the laccase and peroxidase. The pretreatment also alters the degree of polymerization of each component and leads to changes in the physical properties of treated solids. Such changes might involve surface area, porosity, and crystallinity due primarily to the removal of amorphous regions (lignin and hemicellulose) of the biomass (Kim et al., 2016). In our case, this is proved by the quantity of collected water for each fermentation step, which results be higher in the presence of the non-pre-treated substrate (Tab. 1; Tab. 3). This suggests a lower degree of coordination between the solution and the matrix less physically altered, potentially limiting enzymes' access to the solid.

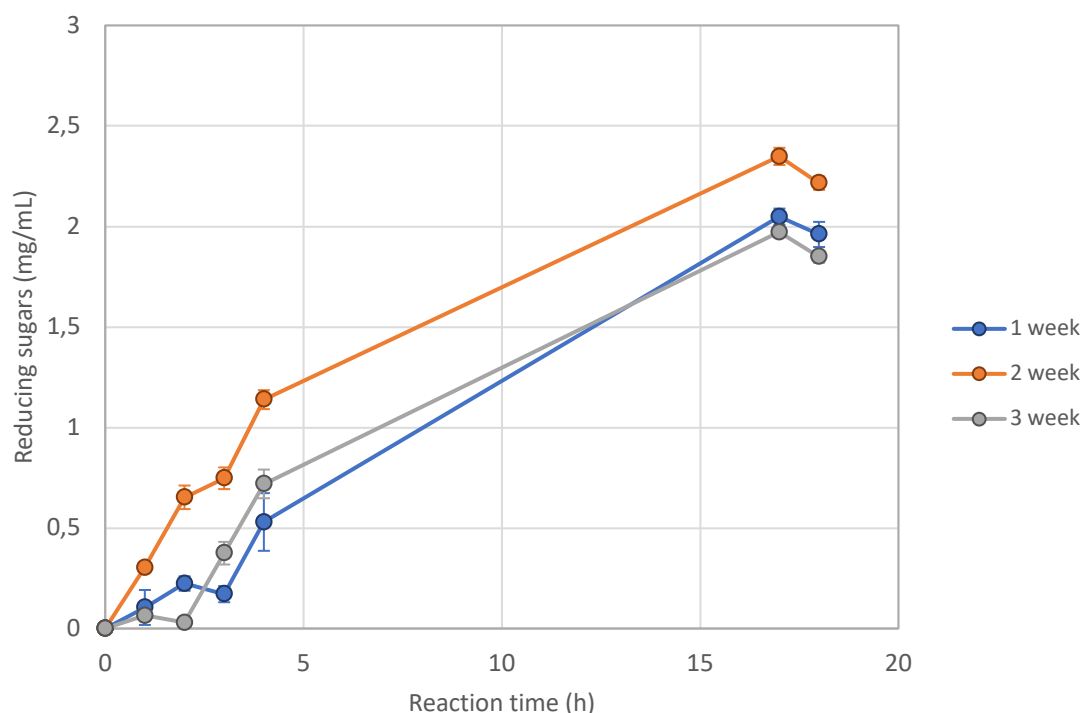


Fig. 7 Reducing sugars released by the hydrolysis of Hemp Bran by enzymes produced during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran.

To test the effectiveness of the enzymes produced by the solid-state fermentation, I performed three hydrolysis reactions of the hemp bran using the collected enzyme mixtures from each week

of fermentation as biocatalysts (Fig. 7). Reactions were monitored in terms of reducing sugars released during the time, indicating the depolymerization of polysaccharides that make up the hemp bran. The progress of reactions is comparable for each case, showing a slight advance when enzymes from the second fermentation week are used, in accordance with the higher pectinase activity. The total phenols calculated on the reaction products (Tab. 4) have a trend that in part confirms the hypothesis of phenol-oxidase presence, in correlation with a high amount of Tyr residues in the second week of fermentation (Fig. 8), which causes a decrease in phenolics released in solution by the disassimilation of the plant cell wall under enzymatic attack. The reactions carried out are susceptible to optimization by varying enzyme/substrate ratio and by including thermal pretreatment of the solid to hydrolyzed. Likewise, the enzymatic production by SSF could be improved by adding to the growth medium organic nitrogen sources or Fe^{3+} and Cu^{2+} ions to induce a higher laccase expression (Karp et al., 2015; Isroi et al., 2011).

Tab.2 Reducing sugars (mg/mL) and Tyrosine (mg/mL) detected on enzyme solutions produced during 3 weeks of SSF conducted using *Pleurotus ostreatus* on Hemp Bran ($T = 30^\circ\text{C}$).

Enzyme	Components detected (mg/mL)		
	7 days	14 days	21 days
Reducing sugars	0.315	0.133	0.118
Tyrosine	0.062	0.184	0.110

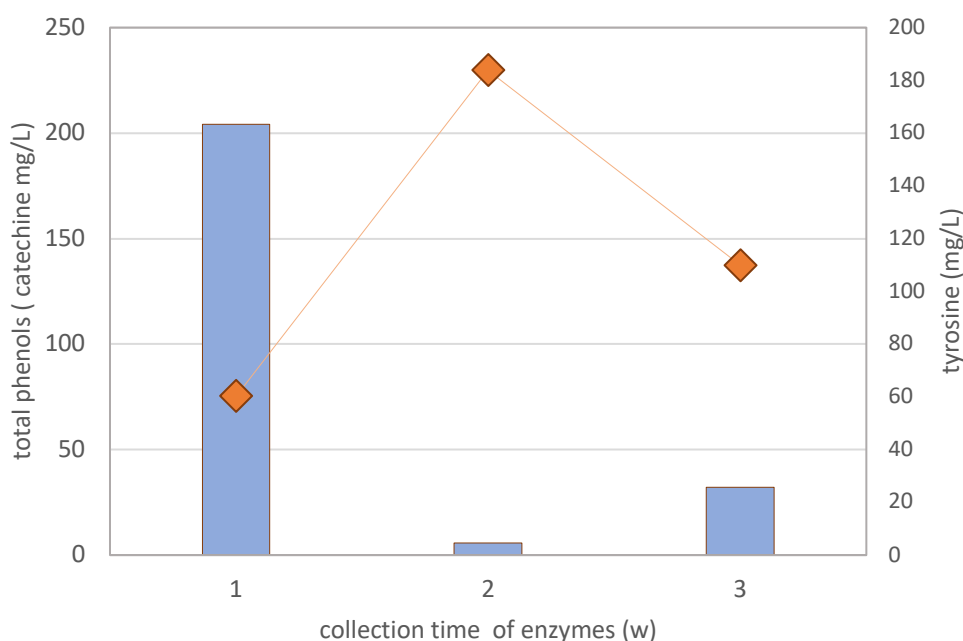


Fig. 8 Total phenols (catechine mg/L) detected in Hemp Bran hydrolysate and Tyrosine (mg/L) detected in the enzyme solutions used to carry out the hydrolysis, produced during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran.

Tab. 3 Activities (U/L) and collected volume (mL) of enzymes produced during 17 days of SSF conducted using *Pleurotus ostreatus* on Hemp Bran after protein extractions (T= 30°C). n.d. stands for not detected (adapted from Setti et al, 2020).

Enzyme	Activity (U/L)			
	4 days	9 days	13 days	17 days
Xylanase	2024 ± 235	9612 ± 316	6064 ± 497	9110 ± 400
Amylase	2058 ± 273	6240 ± 421	5155 ± 269	6470 ± 524
Pectinase	3418 ± 236	5444 ± 128	3556 ± 370	5916 ± 298
Protease	n.d.	120.9 ± 24.7	61.8 ± 8.1	105.5 ± 7.0
Laccase	96.1 ± 8.6	2084 ± 179	1413 ± 150	1914 ± 139
Peroxidase	848.7 ± 64.2	1060 ± 33	606.1 ± 59.3	973 ± 67
Feruloyl esterase	114.5 ± 18.4	168.9 ± 17.5	95.9 ± 13.6	111.3 ± 15.6
Cafferoyl esterase	81.8 ± 8.3	74.7 ± 16.5	71.4 ± 7.4	55.7 ± 5.9
Enzyme collected	Collected volume (mL)			
	4 days	9 days	13 days	17 days
	32.5	47.5	52.5	55

Tab.4 Total phenols (catechine mg/L) detected in Hemp Bran Hydrolysate

Hydrolysate	Total phenols (mg/L)		
	7 days	14 days	21 days
Catechine	204.11 ± 19.18	5.75 ± 7.84	32.21 ± 8.25

Conclusion

The usage of hemp bran as a substrate for lignocellulosic enzyme production through SSF by *P. ostreatus* was the main focus of this study. Among the determined activities, the xylanase and pectinase are remarkable and reach a maximum of 6702 and 12300 U/L after 7 and 14 days of fermentation. Even the alpha-amylase is noteworthy, showing a maximum activity of 3296 U/L after

one week of fermentation. The enzymes produced can be suitable to catalyze the hydrolysis reaction of lignocellulosic biomass at pH 6, as verified with the reaction carried out on hemp bran, which leads to the deconstruction of the plant cell wall into fermentable sugars and bioactive compounds. The results in enzymatic activities have been compared with our group's previous work, which considers as substrate the residual hemp bran from protein extraction by the alkalization method. This confrontation reveals the influence of alkaline pretreatment in enhancing the ligninolytic activities, whereas it discourages the evolution of pectinase activity, which reached the highest value in this work. The overall study provides an example of an efficient and cost-effective system for enzyme production from lignocellulosic biomass, which could apply to numerous types of waste matrices, as a valorization strategy of agro-food by-products, starting from the residual cake of each kind of oil seed processing for the obtainment of edible oil and flours. Any agro-food industries and small biorefineries might benefit from this model to extract value from a transformation residue, while ensuring the closure of the food chain on the same production and/or transformation site.

Abbreviations

Ara	Arabinose
DB	blocking degree
DE	degree of esterification
DM	degree of methylation
DP	Degree of polymerization
FAO	Food and Agriculture Organization
Gal	Galactose
GalA	D-galacturonic acid
Glc	Glucose
GlcA	Glucuronic acid
HG	homogalacturonan
HPB	Hemp Bran
Man	Mannose
Rha	L-rhamnose
SmF	Submerged fermentation
SSF	Solid state fermentation
UN	United Nations
WRT	White rot-fungi
XGA	xylogalacturonan
Xyl	Xylose

Materials and Methods Chp. 2

Reagents hemp bran was supplied by a local company (Hemp Positive World, Cesena, Italy). Original hemp variety was Futura 75

Instruments Coolsafe 55-4 freeze dryer (Scanvac, Lillerod, DK), Perkin Elmer UV-Vis Spectrophotometer, Gerhardt Bonn thermoshake incubator shaker, Allegra X30 refrigerated centrifuge, Forced ventilation stove – MAL.

SFF Process

50 g of hemp bran was placed in a 500-mL Pyrex bottle with a cotton cap, wetted with 15 mL of potassium phosphate buffer 0.1 M pH 5.8, and sterilized by autoclaving at 120 °C for 20 min. This substrate was inoculated with 6.5 g of *P. ostreatus* (commercial strain purchased from Azienda Agricola Funghi Mara, BO) grown on malt extract agar ([Masutti et al., 2015](#)). The fermentation was made at 27 °C, in the dark, for 21 days. The fermentation process was monitored sampling after 7, 14, and 21 days. The samples were recovered under sterile conditions by adding 100 mL of buffer in the fermenter, which was necessary to collect the enzymes produced during the SSF process. The “leachate,” the portion of the solution not adsorbed onto the substrate and containing the enzymes, was withdrawn by a sterile pipette. Each sample was centrifuged for 20 min at 4400 rpm to remove the solid fraction and lyophilized in a Coolsafe 55-4 freeze dryer (Scanvac, Lillerod, DK).

Enzymatic activities

Amylase, xylanase, and pectinase activities

The amylase, xylanase, and pectinase activities were determined as reducing sugars following the method described by Bailey et al. ([1992](#)); the substrates to hydrolyze were 1%w/w starch, 1% w/w of xylan from beechwood, 1%w/w of pectin from apple. The reactions were carried out at 30 °C for 3 min at variable pH (6-8) in citrate buffer 0.05 M. The glucose, xylose, and galacturonic acids released were detected by a UV-Vis spectrophotometer at 550 nm. Once the absorbances are obtained, they are converted into mg/mL of released monomer via the relative calibration curves:

Amylase:

$$y = 1.0239x - 0.0927$$

where y is the absorbance in a.u and x the glucose concentration in mg/mL, made with standard solutions at concentrations between 0.2 and 1 mg/mL

Xylanase:

$$y = 1.164x - 0.0094$$

where y is the absorbance in a.u and x the xylose concentration in mg/mL, made with standard solutions at concentrations between 0.2 and 1 mg/mL

Pectinase:

$$y = 0.9891x - 0.0333$$

Where y is the absorbance in a.u and x is the galacturonic acid concentration in mg/mL, made with standard solutions at concentrations between 0.2 and 1 mg/mL.

The activities are calculated via the formula

$$Activity \left(\frac{\mu\text{mol}}{\text{mL min}} \right) = y \frac{[\text{monomer}] \cdot 10^3 \cdot V_{tot}}{t \cdot V_{enz}}$$

Where [monomer] = mmol/mL

t = reaction time (min)

V_{enz} = volume of enzyme in the assay

V_{tot} = volume of the total solution in the assay

Alternatively, the [monomer]/t ratio can be derived from the slope of the line constructed by plotting the released monomers as a function of reaction time.

Varying the substrate concentration (0.2-1%), at a given enzyme concentration, temperature, and pH, the Michaelis Menten curve is derived. After the linearization of the curve through the Lineweaver-Burk equation, a more accurate reaction rate, and thus enzymatic activity, is estimated

The total activities were expressed as Units (μmol of monomer/min) per Liter.

Laccase activity

For the determination of laccase activity, the reaction between MBTH (3-methyl-2-benzothiazoline hydrazone) and ortho-methoxyphenol (guaiacol) is considered, which, in presence of enzyme catalysis, produces a red-colored azoic complex. Laccase activity is determined by adding 100 μL of sample containing the enzyme to 3 mL of phosphate buffer (25mM pH 6); the solution so prepared is kept stirring in an incubator at 30°C. After a few minutes, 50 μL of guaiacol (500 mM in ethanol) and 500 μL of MBTH (0.05% w/m) are mixed with vortex, and the time required for the formulation of the red-colored complex is recorded. Once the complex has formed the reaction is stopped by adding in rapid succession 500 μL of 1 N H₂SO₄ and 1 mL of acetone. Then, the spectrophotometer

reading at $\lambda=502$ nm is performed against the blank obtained following the procedure just described but substituting the sample containing the enzyme with 100 μ L of phosphate buffer (Setti et al., 1999)

Peroxidase activity

Peroxidase activity is determined by following a procedure very similar to that described for the determination of laccase activity, but, in this case, besides the guaiacol and MBTH, 40 μ L of H₂O₂ (250 mM) are added, being the latter the cosubstrate of the reaction. The procedure is carried out by keeping the samples in an inert environment created by insufflating nitrogen into the test tubes. Once noted the time required to observe the appearance of the red color, the reaction is stopped as previously described and the absorbance is read (Setti et al., 1998). The following formula is used to calculate laccase and peroxidase activity:

$$Activity (\mu\text{mol/mL min}) = \frac{Abs \times V_{tot}}{t \times \varepsilon \times b \times V_{enz}}$$

where:

- *Abs* is the absorbance at 502 nm
- V_{tot} , V_{enz} and V_{perc} are the volumes (mL) of total, enzyme (leachate) in the assay and.
- t is the reaction time (min)
- b is the cuvette thickness (=1 cm)
- ε is the molar extinction coefficient (mM⁻¹ cm⁻¹) which is:
 - for laccase activity: $\varepsilon = 8.355 \text{ mM}^{-1} \text{ cm}^{-1}$
 - for peroxidase activity: $\varepsilon = 15.5 \text{ mM}^{-1} \text{ cm}^{-1}$

Arylesterase activity

To determine arylesterase activity, the method of Giuliani et al. (2001) is applied with slight modifications. Through spectrophotometric measurements is evaluated the change in absorbance ΔAbs at 335 nm due to the formation of cinnamic, ferulic and caffeic acids from the respective methyl esters, methyl-ferulate and methyl-caffeate. The sample is prepared by adding 100 μ L of substrate, methyl-caffeate or methyl-ferulate, and 50 μ L of leachate to 900 μ L of phosphate buffer (pH 6, 100 mM). The reading of the sample at $\lambda=335$ nm against the blank is performed, which is prepared in the same way but substituting the leachate to the buffer. Further measurements are made to observe the decrease in substrate over an arbitrary time interval. Knowing the absorbance, the activity is derived through the following formula:

$$Activity (\mu\text{mol/mL min}) = \frac{\Delta Abs \times V_{tot}}{t \times \varepsilon \times b \times V_{enz}}$$

where:

- ΔAbs is the decrease in absorbance at 335 nm
- V_{tot} , V_{enz} and V_{perc} are the volumes (mL) of total, enzyme and leachate, respectively
- t is the reaction time (min)

- b is the cuvette thickness (=1 cm)
- ε is the molar extinction coefficient ($\text{mM}^{-1} \text{cm}^{-1}$) which is:
 - for methyl-caffeate substrate: $\varepsilon = 12.56 \text{ mM}^{-1} \text{cm}^{-1}$
 - for peroxidase activity: $\varepsilon = 12.21 \text{ mM}^{-1} \text{cm}^{-1}$

Protease activity

Protease activity is determined by the method of [Kunitz \(1946\)](#) with slight modifications. 0.2 mL of leachate is added to 1.8 mL of substrate, which consists of a 2% w/v casein solution, prepared in 0.1 M sodium-phosphate buffer solution at pH between 5 and 8. Each sample is placed in an incubator at 37 °C for 1 h, at the end of which the reaction is stopped by adding 2 mL of TCA 10% w/v (trichloroacetic acid). The samples are centrifuged at 130 rpm for 5 min, and the released tyrosine equivalents are detected by spectrophotometric measurements at 280 nm. From the spectrophotometric measurements the concentration of L-tyrosine is derived through the calibration curve

$$y = 0.0072x + 2e^{-16}$$

where y is the absorbance in a.u. and x the L-tyrosine concentration in $\mu\text{g/mL}$.

Having derived the concentration of L-tyrosine, the protease activity can be determined by the following equation:

$$\text{Activity } (\mu\text{mol/min}) = \frac{[L - \text{tyrosine}] \times 10^3 \times V_{\text{tot}}}{t \times V_{\text{enz}}}$$

where:

- $[L - \text{tyrosine}]$ is the L-tyrosine concentration (mmol/mL)
- V_{tot} , V_{enz} and V_{perc} are the volumes (mL) of total, enzyme (leachate)
- t is the reaction time (min)

For all the enzymatic activities calculation, two reaction controls are needed: the enzyme control (enzyme solution at the same concentration of reaction, in buffer without substrate) and the substrate control (substrate solution at the same concentration of the reaction, in buffer without enzyme). These controls undergo assay, too.

Hemp bran hydrolysis

see Materials and Methods Chp.3 -Hemp bran disassimilation-

3 The biorefinery core

The proposed biorefinery model continues with the (bio)chemical conversion of agro-food waste to high-value products. Thanks to the selectivity of the enzyme produced at the upstream unit, it is possible to extract the components of interest via a biotechnological way at low temperatures.

3.1 The context: conversion of agro-food waste into high-value products

Agri-food wastes often represent an economic and environmental burden for industries, especially for the small facilities, which, most of the time, are forced to rely on the bigger ones for their management. However, the opportunity to derive useful raw materials for several industries (e.g., cosmetics, nutraceuticals, and feed additives) from them through extractions and fractionations processes would improve the economic benefits while decreasing the negative environmental impacts.

Most food waste derives from the drink industry (26%), followed by the dairy and ice cream industry (21.3%), the production and preservation of fruits and vegetables (14.8%), the manufacture of grain and starch products (12.9%), the production, processing, and preservation of meat products (8%), the manufacture of vegetable and animal oils and fats (3.9%), the production and preservation of fish and fish products (0.4%) ([Baiano, 2014](#)).

These wastes include vegetable and fruit trimmings, peelings, stems, seeds, and shells; cereal residues; residual cake remaining after the defatting process; damaged, out-of-date, or returned products ([Baiano, 2014](#)).

In Italy, 30% of defatted olive pomace is currently used as biofuel through lipid transesterification ([Paini et al., 2021](#)). However, defatted olive pomace still contains important nutrients that can be extracted, such as proteins and phenolic compounds, representing 5–10% of the feedstock ([Akay, Kazan, Celiktaş, et Yesil-Celiktaş, 2015](#)).

Also, the wine-making process is a resource-depleting activity, producing many byproducts. Specifically, processing 1000 kg of grapes generates approximately 130 kg of marc, 60 kg of lees, 30 kg of stalks, and 1.65 m³ of wastewater ([Lucarini et al., 2018](#)). Grape marc is a mixture of grape seeds, skin, and pulp, containing polyphenols and anthocyanins with antioxidant activity. Apple pomace is another example of food processing residue. The main products of apple processing are juice, cider, jam, vinegar, and large amounts of residues produced. Among the bioactive compounds that can be extracted, apples contain 10–15% of pectin on dry weight and polyphenols with a recognized antioxidant capacity ([Panzella et al., 2020](#); [Ferrentino, Asaduzzaman, et Scampicchio, 2018](#)).

Protein and residual fibers can be extracted from the oilseed cake by processing oleaginous seeds for flour production. Likewise, grain and cereal processing residue like bran, hull, and husk are sources of extractable wax, fibers, and essential amino acids.

So, biomolecules potentially extractable from the targeted wastes include sugars, polysaccharides, proteins and derivatives, fibers, natural flavor compounds, and phytochemicals.

Conventional methods for biomolecule recovery are based on the use of organic solvents. However, nowadays, they are integrated or replaced by greener methods due to environmental and safety issues. The application of these alternative methods sometimes is restricted because of the operative costs due to more severe operation conditions, or because they are still unsuited to process high amounts of raw material. Rather than optimized stand-alone technologies, small biorefinery should point to process integration of physical and biochemical approaches to achieve whole-waste exploitation by providing a range of components, from high to low value, which can find application at the local level.

3.2 The biorefinery core

- **Second Thesis Objective**

Given the consideration above, the second thesis objective is to provide a strategy to let the small agro-food industries sustainably manage their waste and byproducts by processing them through a **knowledge-based biorefinery approach** that makes the recovery of high-value components economically feasible. **The proposal** will involve a **biotechnological approach** that could lead to a sequential release of components of interest in an aqueous solution. The **final application** of the extracted compounds will be used to drive the extraction processes. In the end, it will be proposed a solution to **valorize the agro-food waste on the local level**.

The agri-food sector lends itself well to the valorization of waste from its supply chain with a view to a circular bioeconomy. Agro-food waste is a reservoir of unique antioxidants in that it contains polysaccharides, proteins, lipids, polyphenols, and tannins. These are the building blocks of the plant cell, which arrange themselves in a specific way in response to the territorial stimuli to which they are subjected. So, the pull of chemicals that the plant synthesizes as a response changes from territory to territory. This gives greater value to the resulting product and the supply chain itself. This is why in recent years, much attention has been placed on local agri-food presidia and supply chains. Exists already a positive perception of the value of local communities that can be self-sustaining. Indeed, there are already examples of communities sharing energy dependence (e.g., comunitasolare.eu) or favoring small-scale distribution of both primary and secondary goods (e.g., www.campiaperto.org). This is encouraging from the perspective of developing innovative small-scale processes. The feasibility of small-scale valorization processes is linked to the use of conversion methods that are affordable and low-energy demanding.

- **The strategy** we propose for the biotechnological chemical valorization of food wastes is based on the **disassimilation of the cell wall** for the **top-down recovery** of the various components of interest through the **specific action of food-grade enzymes**. Once recovered, the specific component will be recombined into a final product in the form of a cosmetic emulsion prototype. The strategy is outlined in Fig.1 e Fig.2

Given the structure of the plant cell and its ability to store bioactive molecules in specific compartments, the extraction sequence becomes a critical factor in not restricting mass transfer across the cell membrane.

While polysaccharides and fibers are mostly contained between the primary and secondary cell walls, proteins, antioxidants, and lipids are mainly held in storage bodies like vacuoles or lipid vesicles within the cell cytoplasm. Therefore, damage of the primary cell wall layer is required in the first case. In contrast, in the second case, more intense or selective processing is needed, including physical disruption or permeabilization of inner membranes (Ferrari, Mustafa, & Donsi, 2017).

From each agro-food waste, it will be possible to recover, to a specific extent and in a specific manner, the **polysaccharide, lipid, protein, and antioxidant** components by enzymatic extractions. The enzyme cocktail will be chosen to hydrolyze the cell wall to release compounds of interest or with specific functionality. Thus, it is possible to directly extract protein-functionalized starches, pectins with a particular composition, and xylans with a certain degree of substitution.

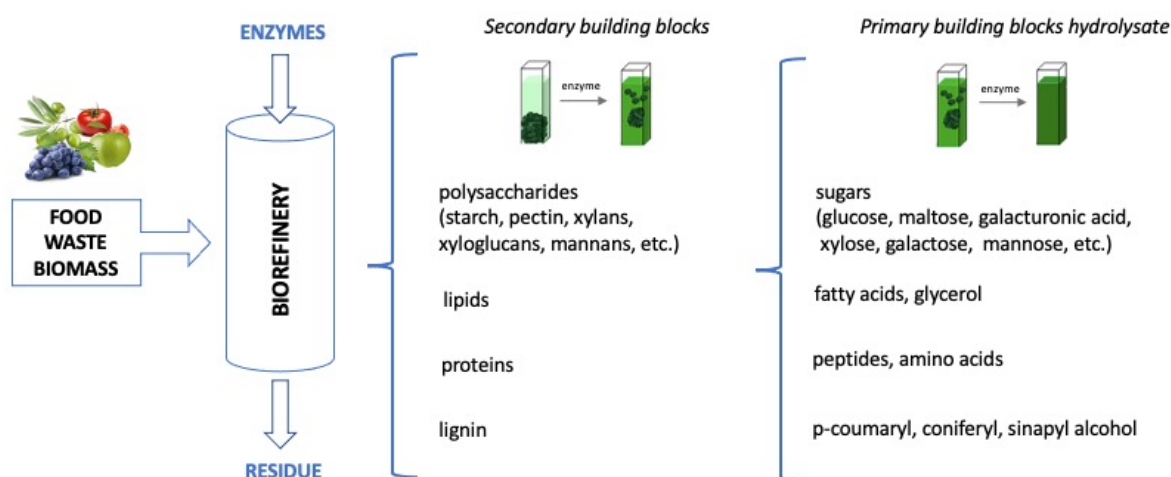


Fig.1 The proposed strategy: disassimilation mediated by enzymes.

These products are already secondary building blocks used directly in the food industries as **thickening agents, stabilizers, emulsifiers, and active substances**. Still, they are also intermediate products of the proposed process because they can be further hydrolyzed to recover useful primary building blocks or aqueous solutions containing a unique mixture of primary building blocks with antioxidant functions. Indeed, the strength of this method relies on the dissolution of the entire portions of plant cell walls releasing molecules previously bound to them. Not on the solubility of the targeted compound into a solvent, as for the conventional extractive methods. This requires specificity in the enzyme action that can be achieved through the **on-site production of enzymes** using the same food waste as the growth substrate (Chp. 2). This also makes their usage

economically feasible. The solution resulting from the complete dissimulation of a plant cell contains the liquefied plant cell wall's structure and manifests a broad spectrum of bioactive functions. Therefore, it represents by itself a product with added value and with specific geographical connotations. Consequently, it can be used as an additive to functionalize food or cosmetics.

The success of small-scale biomass processing also depends on the community's perception of the products. The proposed strategy aims to provide a **new waste-to-value perspective** to encourage the development of new local and sustainable communities. They will focus their growth on production and consumption channels alternatives to the global market's rules, preferring local resources and small distance supply to the exotic raw materials and the huge distances to cover to take them, at the expense of the ecosystems and humankind reservation. We propose a model to be applied to synthesize all the above challenges: creating a **cosmetic prototype** deriving from the biotechnological **deconstruction and recombination of agro-food waste** (Fig.2).

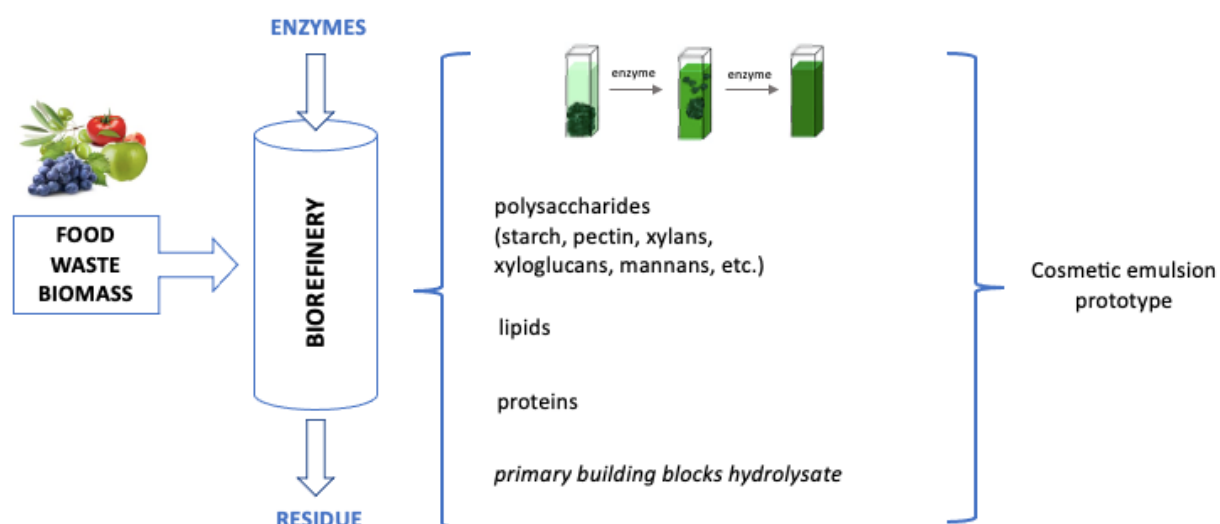


Fig.2 The proposed strategy: recombination of released components.

To show the feasibility of the proposed strategy, we applied the model displayed above to **three different study cases**. Each study represents the valorization of a food processing by-product type: a residue from oil extraction (hemp oilseed cake - **Chp. 3A**), an agro-industrial residue (rice grain residue from milling - **Chp. 3B**), and a fruit pomace (pomegranate pomace - **Chp. 3C**). Each valorization requires a specific enzyme mixture, depending on the type of raw material and the targeted function of the component to be extracted, and leads to three different oil-in-water emulsions with a proper emulsifying system and a specific final function.

3.3 Information to support strategy: from vegetable waste to biocosmetics

3.3.1 Plant adaptation capacity

Because of their sessile nature, plant organisms have evolved mechanical and biochemical functions as an adaptation to survive predation, unfavorable environmental conditions, and competition for resources. These functions are completed through the reinforcement and the rearrangements of the plant cell wall, whose specialization also determines the specificity of plant tissue properties (Graham, Cook & Busse, 2000). For instance, the cell walls of fruits and flowers do not have secondary walls and contain very low amounts of lignin. Instead, they contain a high level of pectins, which can be modified at different stages of fruit maturation, allowing a fine-tuning of cell wall properties during plant development (Rose, Catalá, Gonzalez-Carranza & Roberts, 2018). The general composition of primary cell walls in dicotyledons and non-grass monocotyledons is almost identical to that found in gymnosperms (Popper, 2008). They contain cellulose-glucuronoarabinoxylan framework, β -D-glucans, and pectins, including Rhamnogalacturonan II. Instead, the grass monocotyledon plant cell walls typically contain cellulose-xyloglucan domains, with a higher amount of cellulose and xylans and a lower amount of pectin (Carpita & Gibeaut, 1993). **Cell wall ultrastructure** has been observed to change in response to different environmental conditions, such as sudden flooding at warm temperatures or during leaf abscission in winter, but also in response to chemical exposure and pathogen attack (Sarkar, Bosneaga & Auer, 2009). For example, to increase the resistance to vascular plant pathogens, a severe reduction of the second plant cell wall thickness of fibers was observed, as well as less cellulose synthesis in xylem vessels (Miedes, Vanholme, Boerjan, & Molina, 2014). In *Arabidopsis* plants, enhanced levels of wall-bound xylose or alterations in the structure of xyloglucan and glucuronoxylan, such as a decrease in cell wall acetylation, resulted in enhanced resistance to several fungi (Miedes et al., 2014). Similarly, modification of the biosynthesis and structure (e.g., degree of methylesterification or acetylation) of wall pectins can affect pathogen resistance (Miedes et al., 2014).

Plant organisms rely on the secondary and lignified plant cell walls as a **passive physical barrier** (Fig.3), providing tensile strength to the plant body and defense against harsh biotic and environmental insults. Lignin is an aromatic polymer mainly deposited in secondary thickened cell walls during plant development and in response to biotic and abiotic stresses (Sarkar et al., 2009).

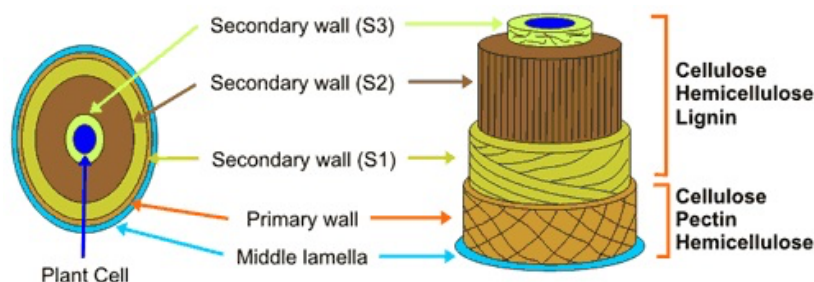


Fig.3 Plant cell wall structure (<https://onlinesciencenotes.com>)

In monocot and dicot plants, lignin is mainly made from the monolignols coniferyl and sinapyl alcohol that give rise to the guaiacyl (G) and syringyl (S) units in the lignin polymer, respectively. *p*-Coumaryl alcohol, which gives rise to the *p*-hydroxyphenyl (H) units in the lignin polymer, is a minor monolignol that is slightly more abundant in monocot than in dicot cell walls. The lignin of gymnosperms is typically composed of G units and low levels of H units but lacks S units. Syringyl units are hypothesized to be superior to guaiacyl units in their ability to strengthen cell walls (Li et al., 2001; Sarkar et al., 2009).

The perturbation in the amount of deposited lignin or its structure pathway modifies the physical barrier pathogens must overcome to invade the plant. This could also lead to the accumulation or decrease of soluble phenolics in second plant cell walls, such as stilbenes, coumarins, (neo-) lignans, phenylpropanoid conjugates, and flavonoids (Lo & Nicholson, 1998; Miedes et al., 2014). Many of these compounds are phytoalexins, i.e., antimicrobial compounds implicated in plant defense (Miedes et al., 2014), resulting in enhanced plant resistance to external threats.

Hence, plant cell walls also act as an **active biochemical barrier** and regulate signal transduction pathways by synthesizing secondary metabolites in response to pathogen attacks, environmental stresses, and growth and development stages (Sarkar et al., 2009). Antioxidant and anti-radical functions have been ascribed to the production of chemicals that includes lipoic and ascorbic acid, *o*-dihydroxy group-containing flavonoids like carotenoids, arylamines, quercetin, aliphatic and unsaturated fatty acids (Isah, 2019). Polyphenols and carotenoids are the most widely distributed compounds available from fruit and vegetables. Plant sterols, glucosinolates, and organosulfur compounds are less widely distributed (Padayachee, Day, Howell & Gidley, 2017). The production of sesquiterpenes is found in members of the family Solanaceae; the Brassicaceae members produce glucosinolates-myrosinase, stilbenes are associated with the defense response system of the Vitaceae, Fabaceae delivers isoflavones while limonoids are produced by Rutaceae and Meliaceae (Isah, 2019).

Plant species' production of secondary metabolites depends on environmental growth conditions and physiology. It performs specific stress response functions (Mazid, Khan & Mohammad, 2011), as reflected by type and concentration variations among taxonomic groups.

Specific plant behavior in cell wall arrangement has indeed been observed in **adaptation to abiotic stress**. Temperature change and climate conditions influence the lignification degree and the functionalization of plant cell walls. Less lignified, small plants are predominantly found in colder climates, especially where snow cover persists for most of the year. In contrast, under warmer temperatures at lower elevations and lower latitudes, the stem cell walls of trees and shrubs are always lignified to physically sustain the plant growth in height (Crivellaro, Piermattei, Dolezal, Dupree, & Büntgen, 2022). To respond to seasonal temperature changes, plants adjust their metabolic process to increase cryo-protectants levels (Isah, 2019). Cold stress can induce the production of phenolics and their incorporation in the plant cell wall. In some plants like beans and tobacco, the formation of polyamines has been observed as precursors of phenylamides that exhibit ROS-scavenging function in responding to stress (Edreva et al., 2008; Isah, 2019). Also the salinity stress can influence the plant wall physiology, inducing the synthesis of secondary metabolites like terpenoids and steroids, phenolics and flavonoids, and alkaloids (Syta, Mbarki, Zivcak, & Brestic,

2018). In *Olea europaea* cultivars salinity effect results in a stimulatory effect on the biosynthesis of oleuropein and phenols at higher levels in leaves (Petridis, Therios, Samouris, & Tananaki, 2012). The exposure of plants to a high level of UV-B (280-320 nm) radiation causes the biosynthesis of different levels of antioxidants and pigments as a response to the induced photooxidative stress (Bowler, Montagu & Inze, 1992). Among these molecules, flavonoids in the epidermis are responsible for the absorption of UV-B type rays and transmit nondamaging longer wavelengths (Kondo & Kawashima, 2000). Antioxidants such as superoxodismutase and catalase scavenge reactive oxygen species and protect plants from UV-induced damage. Pigments like carotenoids play an essential role in the light-harvesting process by quenching the triplet state of chlorophyll and scavenging singlet oxygen and other radical species (Zhang, Ervin & Schmidt, 2005). Among abiotic stresses that affect plant growth and development, drought exerts a great contribution. It is defined as the lack of available moisture in a particular region (Moore, Vitré-Gibouin, Farrant, & Driouich, 2008), and generally, it is associated with high photoinhibition and temperature stresses (Isah, 2019). To cope with water-deficit stress, plants have developed different strategies to improve water use efficiency and osmotic adjustment that mitigate damages associated with water loss (Moore et al., 2008). First of all, depending on the tissue's role, the capacity to either relax or tighten the cell wall by acting on polysaccharide compositions (Fig.4). When hydrated, polysaccharides are well separated; on the contrary, they are brought near each other in water's absence, causing a solid polymer adhesion through hydrogen bonds. This could lead to an irreversible rigidity of the plant cell wall, severely compromising its growth. A high concentration of arabinose, likely in the form of arabinans and arabinogalactan associated with the pectin matrix, can maintain the flexibility of plant cell walls due to its plasticizer effect that compensates alternation for the dehydration and rehydration status. It has also been observed that plants use sucrose synthesis to buffer the lost water and stabilize polymers to desiccation (Moore et al., 2008)

Also, the oxidative stress caused by the drought promotes the biosynthesis of various classes of SMs, including terpenes, complex phenols, and alkaloids (Quan et al., 2016; Isah, 2019).

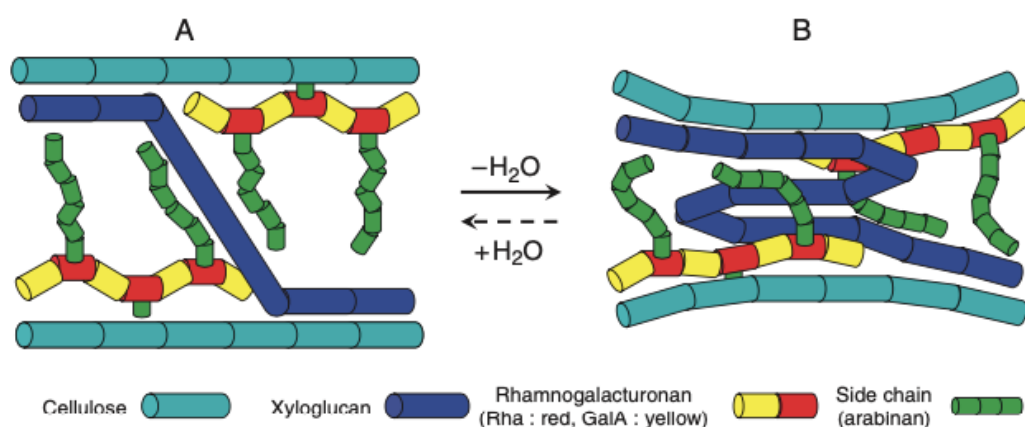


Fig.4 A model representing the effect of water loss on plant cell wall structure. (A) Hydrated plant cell wall and (B) dehydrated cell wall (Moore et al., 2018)

3.3.2 Human skin adaptation capacity

The epidermis is a stratified epithelium that protects the human body and provides a barrier against the chemical and physical agents in the external environment (Costin & Hearing, 2007). It comprises several distinct cell types arranged in layers with specific functions, among which keratinocytes and melanocytes occupy 95% (Fig. 5). Keratinocytes differentiate from the basal layer to the most external stratum corneum, in which they result be adherent and cornified. Melanocytes are the cell responsible for melanin biosynthesis, which occurs through the transfer of melanosomes to the surrounding keratinocytes, where they play an important role in photoprotection (Fitzpatrick & Szabo, 1959). The size, concentration, and distribution of the melanosomes within keratinocytes vary among human beings as a response to different types of intrinsic and extrinsic factors, including age, ethnicity/gender differences, variable hormone responsiveness, genetic defects, UV rays, climate/season, toxin, pollutants, chemical exposure and infestations (Costin & Hearing, 2007).

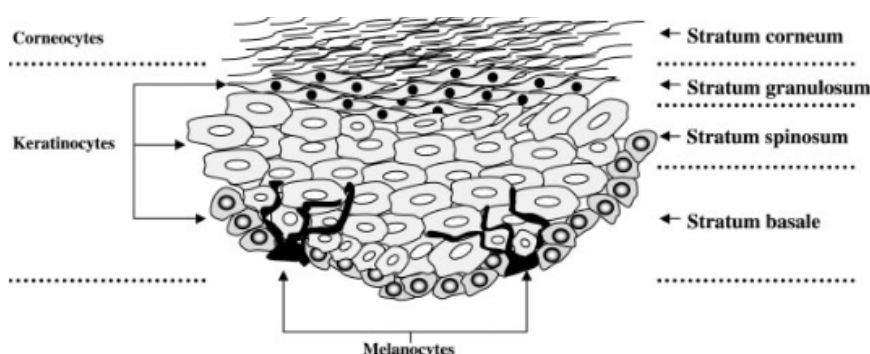


Fig. 5 Layers of the epidermis in human skin (Costin & Hearing, 2007).

The skin responds to UV-R exposure by developing two defensive barriers: the thickening of the stratum corneum and the elaboration of a melanin filter in cells of the epidermis. Indeed, cutaneous pigmentation, occurring either in the tanning process or in the color variations among the races, is due to the capacity of melanin granulates in keratinocytes to absorb harmful UV rays before they can reach the nucleus and damage the DNA (Jimbow, Quevedo, Fitzpatrick & Szabo, 1976). UV radiation is divided into UV-A (320 – 400 nm), UV-B (280 –320 nm), and UV-C (200–280 nm); the latter is usually screened by the ozone layer and does not reach the Earth's surface, like most wavelengths 280 nm. UV-A rays penetrate deep into the dermis and are responsible for the alteration of the skin photoaging process involving the disorganization of collagen fibrils and elastin (Fisher et al., 1996); 19 –50% of UV-A reach the depth of melanocytes and for this are ascribed to manifest potential carcinogenic effects (Garland C., Garland F. & Gorham, 1993).

On the contrary, only 9–14% of solar UV-B reaches these cells, mainly absorbed by the epidermis and upper dermis. This induces erythema phenomena and sunburn reactions (Garland et al., 1993). The major cellular chromophores that absorb in the UV-B range are nucleic acids (mainly the pyrimidine and purine bases) and proteins (mostly tryptophan and tyrosine). Melanin interacts with

harmful oxygen species such as $O_2\cdot$, $\cdot OH$, and 1O_2 produced by UV-A, minimizing ROS's toxic effects and preventing DNA damage (Pathak & Stratton, 1968). The stratum corneum is characterized by a structural cohesiveness thanks to a balance between lipid content (ceramides, cholesterol, fatty acids), lectins, desmosomes, and retained water for which natural moisturizing factor (NMF) plays a central role (Rawlings & Harding, 2004). NMF consists primarily of amino acids or their derivatives, such as pyrrolidone carboxylic acid (PCA) and urocanic acid, together with lactic acid, urea, citrate, and sugars. Amino acids are produced by the hydrolysis of filaggrin, which is generated during the corneocyte maturation process (Rawlings & Harding, 2004).

The NMF compounds are present in high concentrations within the corneocytes. They can absorb atmospheric water and retain it as a humectant allowing the outer layers of the stratum corneum to remain hydrated, despite the desiccating action of the environment (Rawlings, Scott, Harding & Bowser, 1994). Low humidity, temperature, and seasonal changes negatively affect skin barrier functions. Activation of filaggrin proteolysis is indeed dependent on the drop-in external water activity, so a drastic decrease in the environmental humidity results in a reduced total free amino acid generation (and thus the level of NMF and the capacity of the SC to maintain hydration), and consequently, induce the so-called transepidermal water loss (TEWL), and skin surface dryness in the SC (Engebretsen, Johansen, Kezic, Linneberg & Thyssen, 2016) (Fig. 6). The water content in the SC upper layer seems to be more affected by environmental exposure than the deeper ones. Indeed, seasonal changes do not influence the depth water profile of the SC. During winter, a decrease in SC lipids and an increase in unsaturated fatty acids have been identified.

In contrast, during summers, sebum production is higher, and thus TEWL is reduced, and skin hydration is increased. The seasonal effects may be biased by ultraviolet (UV) irradiation. The exposure to UVA and UVB irradiation is higher in summer, and that results in epidermal thickening (Pearse, Gaskell & Marks, 1987) and activation (regarding the UV-B component) of the cutaneous vitamin D-system, with an increased expression of antimicrobial peptides, lipids, and skin barrier proteins including filaggrin and involucrin (Engebretsen et al., 2016).

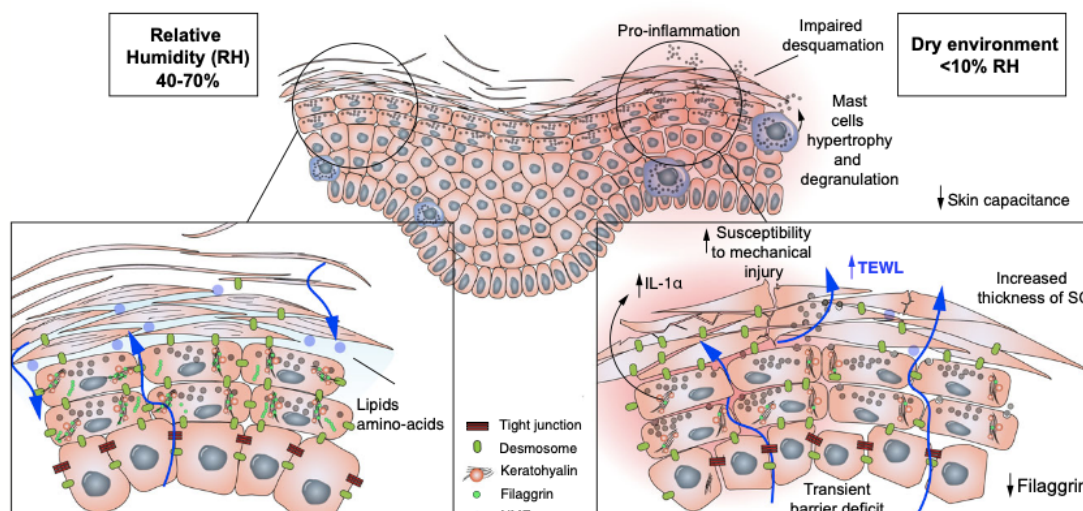


Fig. 6 The short-term effect of dry environment (RH<10%) on skin barrier function and composition (Engebretsen et al., 2016).

3.3.3 A new waste-value chain perspective

Although it could look speculative, the comparison of the structure of the plant cell wall with the structure of the human skin epidermis reveals a remarkable similarity. Both separate the organism from the external environment, using physical and biochemical mechanisms to protect it. Also, the adaptation and defense strategies toward environmental threats are the same, comprising adhesion of the cell's structural components, synthesis of molecules as humectants to retain the water content and allow the ordinary cell functions, and generation of molecules with conjugated bonds capable of absorbing harmful UV-rays. Either in plants or humans, the biochemical response to external factors depends on the kind of exposure and the kind of stimuli that changes on seasonality, latitudes, and temperatures, so that, for each boundary condition, a specific pool of molecules is generated. The strength of this principle lies in the correspondence between humans and plants, just as manifest forms of the repeated but never equal Nature's patterns. Each living cell of evolved beings is made up of carbohydrates, lipids, and proteins interacting with each other, constituting membranes and creating interconnecting compartments that ensure and preserve vital functions. The ability to see these correspondences can guide a new sustainable approach toward natural resources, based not on exploitation but rather on the appreciation of the chemical potential built up by Nature and enclosed in the powerful plant organisms. Starting from these considerations, the following thesis section intends to provide **a new perspective on a waste-value chain**: the total recovery of components from vegetable wastes useful to make up a biocosmetic product for human skin protection and preservation. This is not simply biomimesis; this is a deconstruction of a plant material that, even though is underutilized, is still able to represent a specific territory because it encloses chemicals originating from the unique combination of external stimuli and conditions, and specific reactions to them. Then, recombining these molecules into a prototype of biocosmetic is a strategy to benefit local communities and favor the development of small-scale and more sustainable markets. Bruins and Sanders (2012) pointed out that to reach the transition toward a future beyond petroleum as soon as possible, the envelopment of second-generation and small-scale biorefinery should be encouraged. They also provided some guidelines to achieve this objective, comprising the development and the use of low-energy processes to convert waste biomass into chemicals and energy commodities meanwhile containing utility and operative costs, the minimization of transports from the sources of the raw materials to the processing plant and along the distribution chains, and the creation of high-value products marketable on a local scale to benefit local communities.

The strategies developed and presented in the following thesis sections (Chp. 3A-3C) have been thought of as integrating part of the food-production chain to allow the original on-site valorization of agro-food waste by biorefinery models. They are based on the biotechnological processes for the disassimilation of the plant cell wall into an aqueous solution to recover useful chemical compounds. These reactions involve a series of enzymatic hydrolysis capable of unleashing bioactive molecules bound to the cell wall's polysaccharide chains. Given the distinct features of each plant organism and the ensuing waste, the choice of specific biocatalysts is required, acting as selective scissors for the fragmentation of the cell wall's structure into molecules with the function of emulsifiers, emollients, and antioxidants. These are essential components for the realization of a biocosmetic

emulsion. According to Segen's Medical Dictionary (2012), “biocosmetic is a cosmetic product containing the biotechnology-based product(s) or a cosmetic that has a mechanism of action based on biologic principles.”

With regard to a waste-derived product, Novak, Sidney & Soccol (2014) defined a **biocosmetic** as:

a product that uses, in at least one step of its production (or production of one of its raw materials), some industrial residue or waste from food, feed, or other kinds of industry. In order to be considered as an interesting material for the production of biocosmetics, the waste must have at least one important and desired property, such as high-antioxidant potential, high amounts of phenolic compounds, capacity to improve sensorial properties, act in the product smoothness or lightness, and being skin softener or hair shiner. Furthermore, as a common cosmetic, it must pass through tests to guarantee its physical, chemical, and microbiological safety, according to international rules and regulations (p.391).

Since 11 July 2013, a European Regulation (1223/2009) has been in force, stating the key features for cosmetic product commercialization. It defines a cosmetic product as follows:

any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odors. (EU Regulation 1223/2009, Article 2.1.a)

3.3.4 Bio-based emulsions

Depending on the intended use and contained ingredients, cosmetic products may vary in physicochemical forms. They are available in the form of multiphase systems, such as emulsions (creams, lotions, hair conditioners), suspensions (toothpaste, facial masks, make-up cosmetics), and foams, or the form of single-phase systems, i.e., solutions, (tonics, toilet waters) (Sikora, 2019).

Among the various cosmetic forms, biphasic emulsions are the most widespread. They are systems consisting of two immiscible liquids in which liquid droplets (the disperse phase) are dispersed in a liquid medium (the continuous phase) (Tadros, 2013). Depending on the nature of each phase, they can be distinguished in oil in water (o/w), water in oil (w/o), and oil in oil (o/o).

The formation of a dispersion state (II), starting from a non-disperse one (I, Fig.7), is accompanied by a change in free energy ΔG^{form} that is described by a certain interfacial tension (γ_{12}), coupled with a variation in surface energy ΔA , and entropy of dispersion ΔS^{conf} (Eq. 1).

$$\Delta G^{\text{form}} = \Delta A \gamma_{12} - T \Delta S^{\text{conf}} \quad \text{Eq.1}$$

Both terms ΔA and ΔS are positive, with generally $\Delta A \gamma_{12} \gg -T \Delta S^{\text{conf}}$ indicating that the formation of the emulsion is not spontaneous, and the system is thermodynamically unstable.

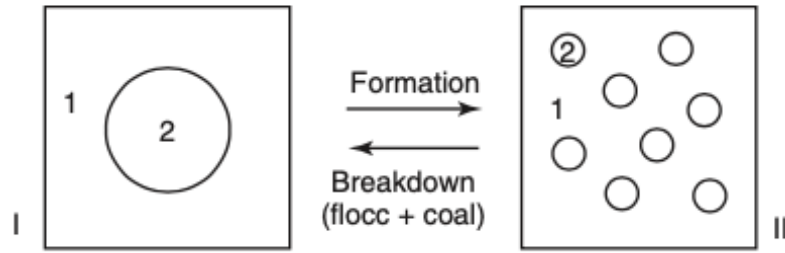


Fig.7 Schematic representation of emulsion formation and breakdown (Tadros, 2013).

A way to stabilize the system is by making it kinetically stable. This can be pursued by supplying external energy to the system, as occurs during the emulsification process, but is a short-term solution, or by adding an emulsifier that creates an energy barrier between the droplets avoiding the easy transition from the disperse phase to the separate phase. The phase separation can be driven by reversible and irreversible phenomena, distinct by their energy contribution (Fig.8).

The emulsifier action requires the fast adsorption of the surfactant molecules at the liquid/liquid interface, which leads to an interfacial tension reduction $-d\gamma$ (Tadros, 2013). It depends on the emulsifier activity a (concentration), as given by the Gibbs adsorption equation (Eq.2)

$$-d\gamma = RT\Gamma d\ln a \quad \text{Eq.2}$$

Where R is the gas constant, T is the absolute temperature, Γ is the surface excess (number of moles adsorbed per unit area of interface).

Thus, the chemical nature of the emulsifier, its affinity with the molecules to be dispersed, and the interaction between them should be considered since all these factors affect the droplet's surface coverage and stabilization (Ozturk & McClements, 2016).

The adsorption capacity of surfactants at the interface oil/water relies on their amphiphilic character given from the co-presence of hydrophobic (tail) and hydrophilic portions (head) in the same molecule.

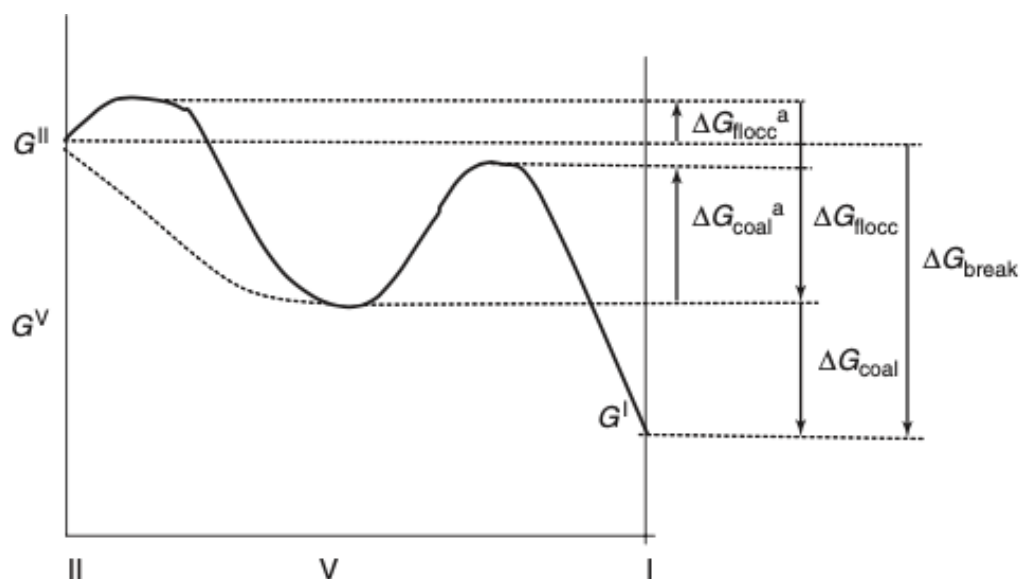


Fig.8 Schematic representation of free energy path for breakdown (flocculation and coalescence) for systems containing an energy barrier (Tadros, 2013).

The polar portions orient toward the aqueous phase, and the apolar portions toward the oil phase, forming a film at the interface o/w driven by the higher solubility of the emulsifier in one of the two phases (Bancroft rule). This imparts a difference in the interfacial tension of the two phases, causing a change in film conformation that results in curved and concave orientation at the phase boundary at the higher interfacial tension originating o/w or o/w emulsions by the formation of the so-called micelles (Fig.9)

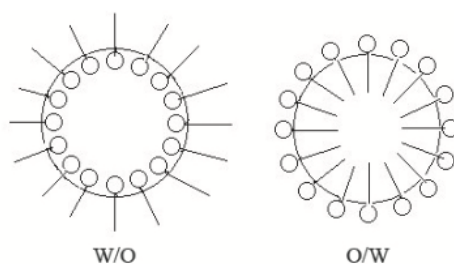


Fig. 9 Orientation of emulsifier molecules at an interfacial surface in forming micelles (Sikora, 2019).

The polar portions usually contain fragments that form salts like acidic groups (carboxylic (-COOH), sulfate (-OSO₃H), sulphonic (-SO₃H), or phosphatic (-OPO(OH)₂), or alkaline group (primary, secondary and tertiary amine groups and quaternary ammonia groups [-NR₃]⁺); but also alcohol groups (-OH), ethoxy groups (-CH₂CH₂O-) or propoxy groups (-CH(CH₃)CH₂O-), as well as ester groups (-COOR-), amide groups (-CONH-), or ether groups (-O-). The hydrophobic portion usually is a long,

straight, or branched chain of hydrocarbons (with 8 to 18 atoms of carbon) (Sikora, 2019). An empirical method for the choice of the proper emulsifier is based precisely on the so-called Hydrophilic Lipophilic Balance (HLB), a scale that takes into account the relative percentage of hydrophilic to lipophilic (hydrophobic) groups in the surfactant molecule(s) (Griffin, 1954).

The emulsifier can have an ionic or non-ionic nature, acting respectively by electrostatic and steric effects in the dispersion mechanism.

The stability of the dispersion is indeed given by the combination of the interaction energies (forces) occurring between the emulsion droplets. From one side, there is the van Der Waals attraction energy G_A that comprises three contributions: dipole–dipole (Keesom), dipole-induced dipole (Debye), and dispersion (London) interactions. They are short-range forces and are inversely proportional to the separation distance between the molecules. On the other side, there are the electrostatic R_{el} repulsive forces. The total energy G_T is thus given by the sum of each contribution (Eq. 3) with an extent dependent on the distance between particles, according to DLVO theory (Fig.10). To these can be added the steric repulsive forces (Tadros, 2013).

$$G_T = G_{el} + G_A \quad \text{Eq.3}$$

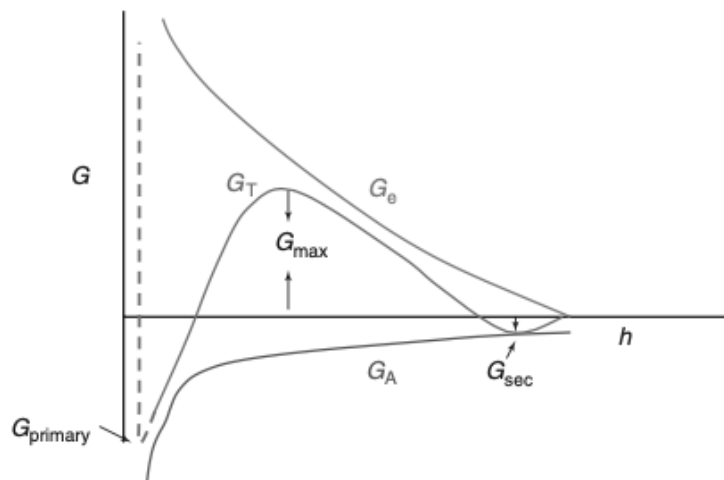


Fig.10 Total energy–distance curve according to the DLVO theory (Tadros, 2013).

Fig.11 represents various **instability phenomena** occurring in emulsion systems. Creaming and sedimentation are instability processes due to the formation of two distinct layers within the volume of an emulsion, with one of the layers containing a more dispersed phase. Depending on the density difference between the two phases and the mass of globules, the globules accumulate in the upper layer (which essentially is creaming) or in the lower layer (which is sedimentation). These phenomena can be described with an equation derived from the law of hydrodynamics, namely the

Stokes law (Eq.4) that relates the velocity of motion of the drop flow with the viscosity of the medium

$$V = \frac{2a^2 (\rho_D - \rho_C)g}{2\eta} \quad \text{Eq.4}$$

Where:

V is the velocity of motion (m/s),

a is droplet radius (m),

ρ_D is internal phase density (kg/dm³),

ρ_C is continuous phase density (kg/dm³),

g is apparent gravity (m/s²)

η is continuous phase viscosity (Pas).

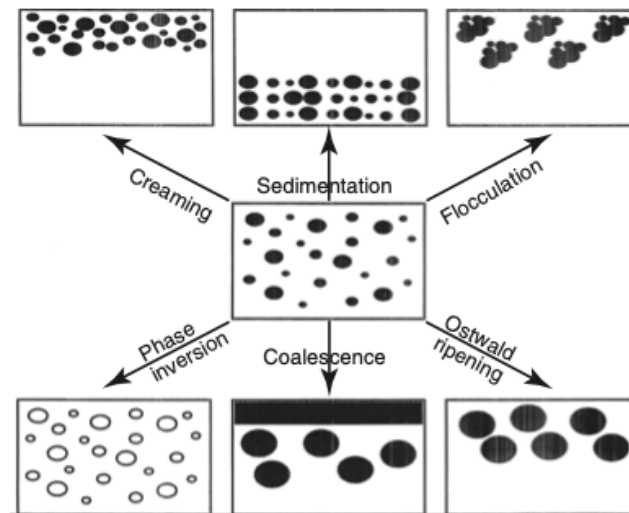


Fig.11 Schematic representation of the various breakdown processes in emulsions (Tadros, 2013).

Breaking (or cracking) is an irreversible emulsion destabilization process driven by the van der Waals forces, Brownian motions, and the Ostwald ripening, and comprises two stages (Sikora, 2019). The first is flocculation, during which the internal phase droplets begin forming larger aggregates. The aggregation process is still reversible, and the sedimentation of the aggregates can be avoided by changing the emulsifier or increasing the emulsifier concentration. The second stage is coalescence. Here the aggregates are transformed irreversibly into large, isolated droplets (Sikora, 2019).

Among the countless classes, **food-grade emulsifiers** are the greener and safer ones. They can be distinguished into proteins, polysaccharides, and saponins. Each of them acts by specific modes, depending on its chemical nature and structures (Fig.12).

Proteins are usually relatively small molecules (10–50 kDa) that contain a mixture of hydrophilic and hydrophobic amino acids along their polypeptide chains (Lam & Nickerson, 2013). The adsorption of protein on lipid droplets leads to stabilization against aggregation through a combination of long-range electrostatic interactions and short-range steric repulsion. The former occurs because of the content in amino acids that possess negative ($-\text{COO}^-$) or positive ($-\text{NH}_3^+$) charges. They are more important for protecting droplets against flocculation. The latter occurs by forming thick interfacial layers or having carbohydrate moieties attached (Ozturk & McClements, 2016). They are more important for protecting droplets against coalescence. Several external factors may promote the undesirable aggregation of the coated droplets in systems emulsified by the protein action. When the solution pH is close to the protein isoelectric point, droplets tend to flocculate because of the reduced magnitude of the charge (Delahaije, Wierenga, van Nieuwenhuijzen, Giuseppin, & Gruppen, 2013). High salt levels (particularly multivalent counter-ions) promote flocculation by screening the electrostatic repulsion between charged droplets. Temperatures above the denaturation of globular proteins may promote flocculation due to an increased hydrophobic attraction when non-polar surface groups are exposed (Ozturk & McClements, 2016).

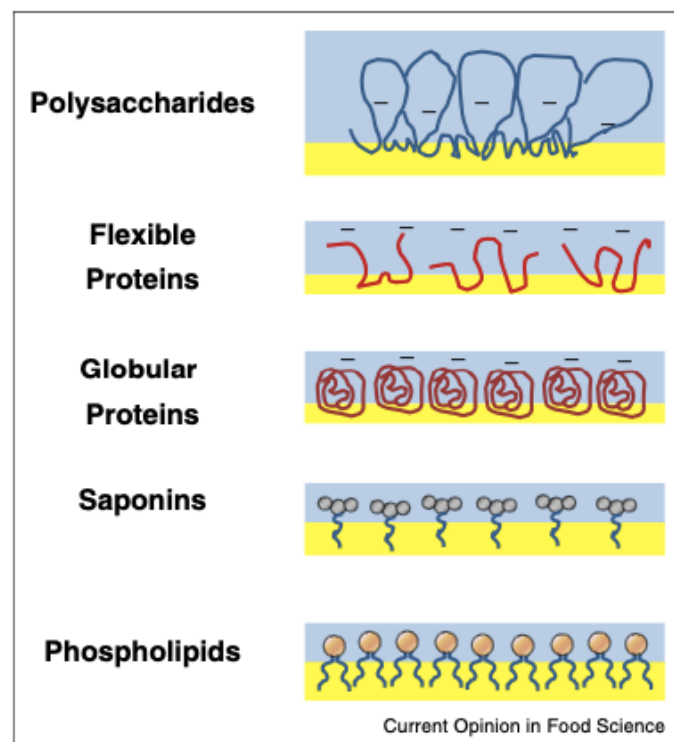


Fig.12 proposed interfacial structures of different kinds of natural emulsifiers (Ozturk & McClements, 2016).

Polysaccharides are usually relatively large molecules (100–1000 kDa) that adsorb relatively slowly to droplet surfaces and form thick hydrophilic interfacial layers (Ozturk & McClements, 2016). They are not particularly surface-active but stabilize droplets by steric repulsion because they have large hydrophilic groups that protrude into the aqueous phase (Dickinson, 2003). Consequently,

polysaccharide-stabilized emulsions tend to be relatively stable to changes in pH, ionic strength, and temperature. Polysaccharides like starches also stabilize emulsions by increasing aqueous phase viscosity and inhibiting droplet movement. Others like gum arabic, pectin, and galactomannans have good emulsifying properties because they already have non-polar groups or proteins attached to their hydrophilic carbohydrate chains ([Dickinson, 2003](#)).

Saponins tend to rapidly adsorb to droplet surfaces and form thin interfacial layers because they have relatively low molecular weight (1.67 kDa) and can pack more efficiently at oil–water interfaces, producing smaller droplets during homogenization than biopolymers. In addition, they tend to have higher surface activities because of the higher proportion of non-polar to polar groups. Saponin-coated droplets are also mainly stabilized against flocculation due to their electrical charge, mainly due to glucuronic acids ($pK_a = 3.25$) ([Yang, Leser, Sher & McClements, 2013](#)). The strong electrostatic repulsion between them allows high stability to incubation at high temperatures. At low ionic strengths, they are stable to droplet aggregation from pH 8 to 3 due to their high charge, but they flocculate at pH 2 due to the loss of charge. They tend to flocculate at high salt concentrations at neutral pH due to electrostatic screening effects ([Ozturk & McClements, 2016](#)).

Phospholipids (like lecithin) exhibit hydrophobic fatty acid tail groups and hydrophilic head groups containing phosphoric acid esterified with glycerol and other substitutes ([Kralova & Sjöblom, 2009](#)). They show behavior somewhere between saponins and polysaccharides despite their relatively low molecular weights (0.760 kDa). This may be because they tend to form large supramolecular structures in solution, such as bilayers or vesicles, rather than individual molecules or small micelles. At low ionic strengths, lecithin-coated droplets tend to be stable at neutral pH due to their high negative charge but unstable under acidic conditions due to the reduction in surface charge and electrostatic repulsion ([Ozturk & McClements, 2016](#)). The addition of high levels of salts may also promote extensive droplet aggregation through electrostatic screening effects. The dispersed droplets are instead relatively stable at high temperatures due to the strong electrostatic repulsion between them ([Ozturk & McClements, 2016](#)).

3A Case of study: biorefining of *Cannabis sativa* L. (hemp) seed cake

Introduction

During food processing, the generation of a high amount of biological-specific waste is inevitable. Due to their characteristics like high water content, high enzymatic activity, biological instability, and potentially pathogenic nature, the disposal of food wastes should require particular attention because of their potential environmental impact. To limit the risk of environmental pollution, a strategy to reduce waste generation is advisable, apart from their appropriate management. For example, the development of various bioprocess to convert starting materials to novel products ([Hadnađev et al., 2018a](#)). Among the others, the oil industry produces large quantities of underutilized by-products, primarily oilcake and oil meal deriving from the pressing of raw materials during the oil extraction processes. The most common use of these by-products is in animal nutrition or organic fertilizers ([Ramachandran, Singh, Larroche, Soccol & Pandey, 2007](#)). However, their high content of proteins and other numerous biologically active compounds (vitamins, minerals, phenols) makes them suitable for a most specific valorization as high-added products.

The hemp oil seed processing residue falls into this category since hemp seed is composed of over 30% of oil and 25% of high-quality protein. The hemp resources used in the food and feed industry originate from the species *Cannabis sativa* L., which unlike marijuana (*Cannabis indica*), contains only traces of δ -9-tetrahydrocannabinol (THC) ([Hadnađev et al., 2018a](#)). The FAO estimates that 32 square kilometers (km²) of hemp are harvested globally, including 143 metric tons (MT) of hempseed. Their processing produces 50 MT of seed oil, 93 MT of cake, and 331 MT of leaves, but these are underestimated quantities since Canada and USA, the major hemp producers, are not considered ([Semwogerere, Katiyatiya, Chikwanha, Marufu & Mapiye, 2020](#)).

The fiber composition of hemp seed, as well as the proteins and oil content, varies from the heart, the inner part, to the hull, the outer part.

Generally, the seed heart contains mainly xyloglucan and pectin and very low cellulose and lignin. The seed hull contains mainly lignin (16-19 %), crystalline cellulose (22-36%), and xylans as the most abundant polysaccharides ([Schultz et al., 2020](#)). Some of the valorization processes of hempseed by-products are the extraction of bioactive and polyphenol ([Teh, Niven, Bekhit, Carne & Birch, 2014](#)), the hydrolysis of the fiber component for fuel usage ([Marcolongo, La Cara & Ionata, 2021](#)), the obtainment of hemp bran protein isolates and hydrolysate ([Hadnađev et al., 2018a](#)) and the solid-state fermentation for enzyme production ([Setti et al., 2020](#)).

Hemp seed oil is a niche market product, mainly produced in small-scale artisan-type plants by mechanical pressing with a screw press. Although this type of oil production is relatively simple and does not involve any refining procedures, the disposal of waste generated throughout the processing can pose several problems to the processors if they do not have enough resources to cope with it ([Burton, Andres, Cole, Cowley & Augustin, 2022](#)). Thus, it should be advisable to reduce

the waste to be disposed by valorizing it in such a way that implies simple operation, low operative costs, and the realization of marketable products.

In this regard, the present study intends to propose a solution to meet the need of small food industries. It is based on an innovative biorefinery process to be integrated into the hemp food chain and provides a new use of hemp byproducts by realizing the first food-based cosmetic prototype. The developed process may provide guidelines for the food-chain valorization of each food seed with high protein content and all the oleaginous seeds and leguminous sources.

Results and discussion

The integrated process for valorizing hemp seed byproducts is presented in the flowchart in Fig.1. The starting material of our process is defatted hemp flour, whose nutritional composition is reported in Table 1. It likely derives from the milling of hemp seed and the pressing of the ensuing hemp flour, from which the majority of hemp oil is obtained, as the first marketable product of the hemp seed food chain. The ulterior recovery of hemp oil leaves behind the hemp seed cake, which then undergoes protein extraction. Once the flour is stripped of proteins and oil, the last and poorest byproduct of the food chain is obtained. Its crudest component, the hemp seed bran, has been used as a substrate for enzyme production by solid-state fermentation, as described in detail in the previous thesis section, “Production of enzymes by SSF of *Pleurotus ostreatus* on Hemp Bran” (Chp. 2A). The produced enzymes are used to hydrolyze the same hemp bran, giving a bioactive hydrolysate with a significant amount of carbohydrates and phenols. The deconstruction of hemp flour leads to the innovative valorization of the extracted compounds in the form of a biocosmetic emulsion made of hemp bran oil as the fat phase and hemp protein solution as the emulsifiers and aqueous phase.

Flowchart guidelines

In the flowchart, the bold squares indicate the commodities used as starting materials and the products obtained. The solid lines show the conversions of food materials carried out by chemical (black line) or biochemical (green line) routes, and their combination into the proposed final product (red line). The dotted lines respond to the same color legend but indicate the suggested pathways yet to be achieved. The bold numbers stand for the masses in weight of raw materials (black) and products (red).

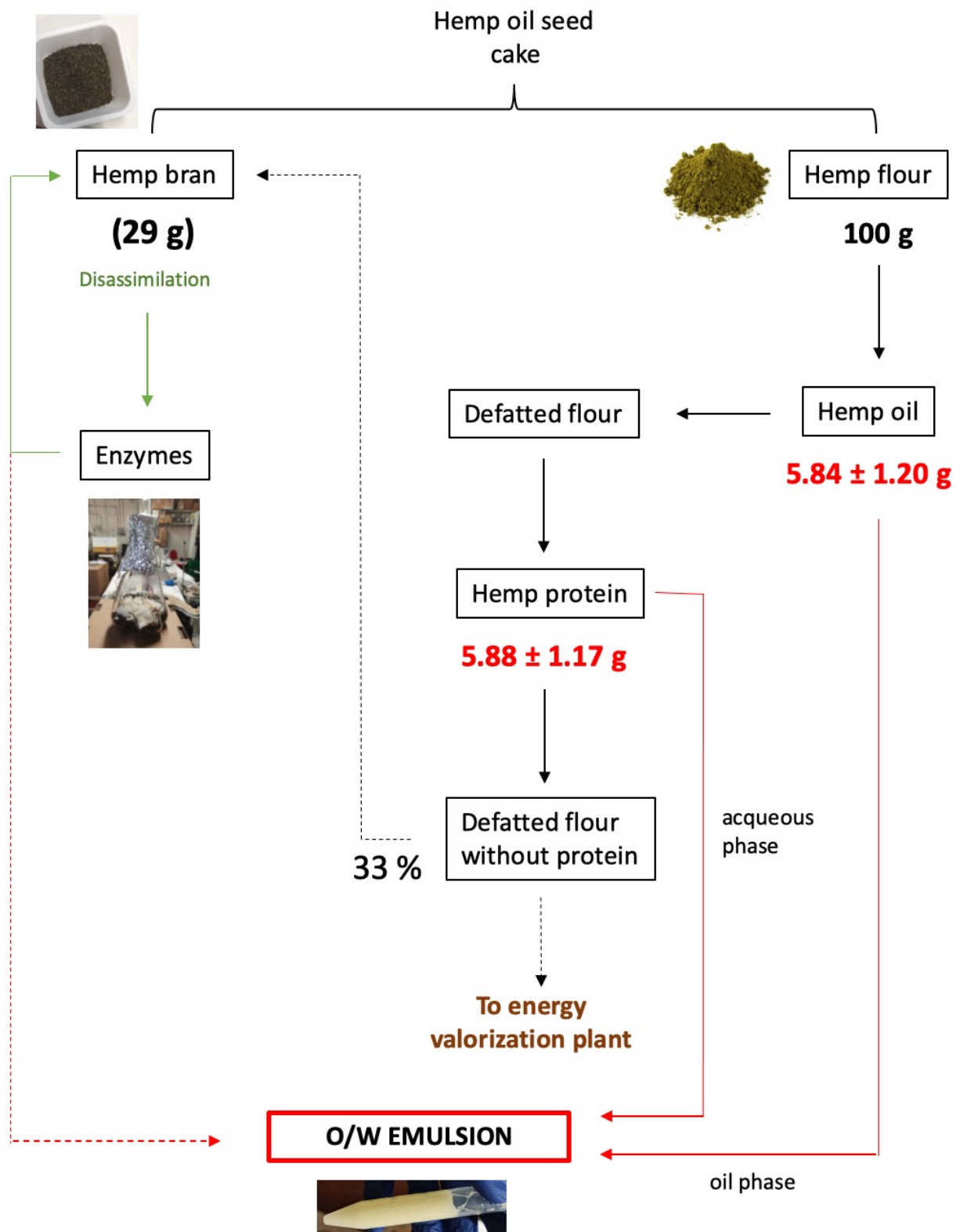


Fig.1 Flowchart of the whole deconstruction and recombination of Hemp Oil Seed Cake.

Table 1 Nutrient composition (%) of hemp seed and hemp flour (Fior di Loto brand).

Composition %		
	Hemp seed	Hemp flour
Fat	44	9.4
Carbohydrates	0	12.8
Fiber	8.3	35.7
Protein	33	30.3

Deconstruction of hemp seed flour

The deconstruction of hemp seed flour is outlined in Fig.2.

In the present study, we assumed that the hemp flour used as starting material is assimilable to a hemp cake depleted of the seed hulls due to its oil content (Table 1). Indeed, the residue obtained from the mechanical treatment of the hemp seed for the extraction of oil, namely seed cake, has 8.4-15.5 oil content ([House, Neufeld & Leson, 2010](#); [Burton et al., 2022](#)). Besides, the oil content in our flour is 78% less than that of hemp virgin seed produced by the same retailer (Table 1), which corresponds to the oil extraction yield on average by screw pressing ([Matthäus & Brühl, 2008](#)), confirming that flour has been depleted of fat.

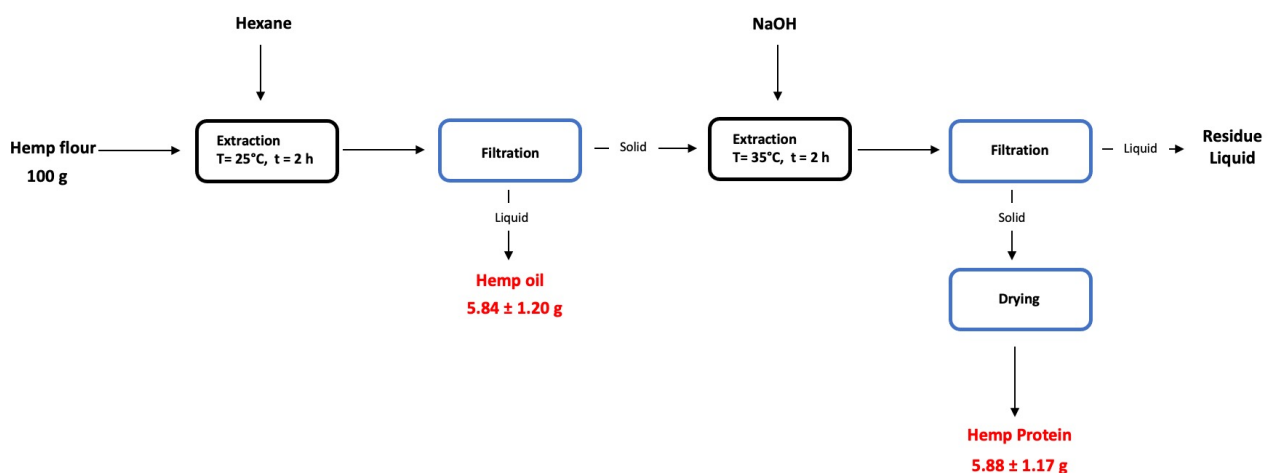


Fig.2 Simplified hemp flour deconstruction process.

The residual content of hemp oil in hemp seed cake is abundant in polyunsaturated fatty acids (PUFAs), then its recovery could be beneficial (Moliterni, Pojić & Tiwari, 2022). The residual oil has been extracted with hexane because, when the amount to be recovered is low, the extraction with organic solvent could lead to a higher yield in a reasonable time (Kostić et al., 2013). The extraction leads to 5.85 ± 1.20 g oil /100 g four, with an extraction efficiency of 65%. The separation of biomass components aims to exploit specific functionalities of target molecules to set up new products. However, it is also a strategy to enhance the accessibility of the plant cell wall to subsequent treatments (Moliterni et al., 2022). To recover hemp proteins that will act as an emulsifier of the final product, alkaline extraction with sodium hydroxide followed by isoelectric precipitation was performed on the defatted cake, leading to a protein yield of 6.25 ± 1.28 %. The pH of extraction was over 10 because of the tightly compacted conformation of native hempseed proteins, which may be integrated with other components such as phenolic compounds (Wang & Xiong, 2019). Hemp seed protein (HSP) has been reported to contain 65% of globulin, edestin, and 33% of albumin (Malomo & Aluko, 2015). The former has a closed and non-flexible structure and is typically salt-soluble; the latter has an open, flexible structure which allows its solubilization in water solution. The method based on alkaline extraction and isoelectric precipitation should favor the isolation of globulin fraction, because of the higher solubility of hemp seed albumins (75%) at pH 5, compared to hemp seed globulins (<10%) (Hadnađev et al., 2018b). This could enhance the oil dispersion during the emulsification due to the high content of hydrophobic amino acids in globulins (Malomo & Aluko, 2015). In addition, using alkali can cause the partial unfolding of proteins, increasing surface hydrophobicity and allowing stronger interactions between proteins with the oil core (Wang & Xiong, 2019). Alkaline pretreatment with diluted NaOH also allows the cleavage of ester and ether bonds between lignin and cellulose or hemicellulose, increasing plant matrix accessibility to enzymes (Moliterni et al., 2022), as our experiments of flour hydrolysis will confirm. After isolating the hemp proteins, we evaluated their emulsifier capacity by dispersing them at 1% w/w in sodium phosphate buffer at different pH (Fig. 3). pH 4 is the most suitable for obtaining a stable emulsion, in accordance with the amino acid composition of hemp protein isolate (Table 2), which is soluble mainly for pH <5. After one hour of stirring, the dispersions at different pH resulted in different solubility degrees (Table 3). Emulsifying properties of proteins are related to many factors, like the rate of protein adsorption at the oil-water interface, the amount of protein adsorbed, the conformational rearrangement at the interface, the extent of interfacial tension reduction, and the rheology of the cohesive film (Wang & Xiong, 2019). In particular, protein-lipid interactions occur through hydrophobic attractions of the aliphatic chains in fatty acids and nonpolar side chains in some amino acids.

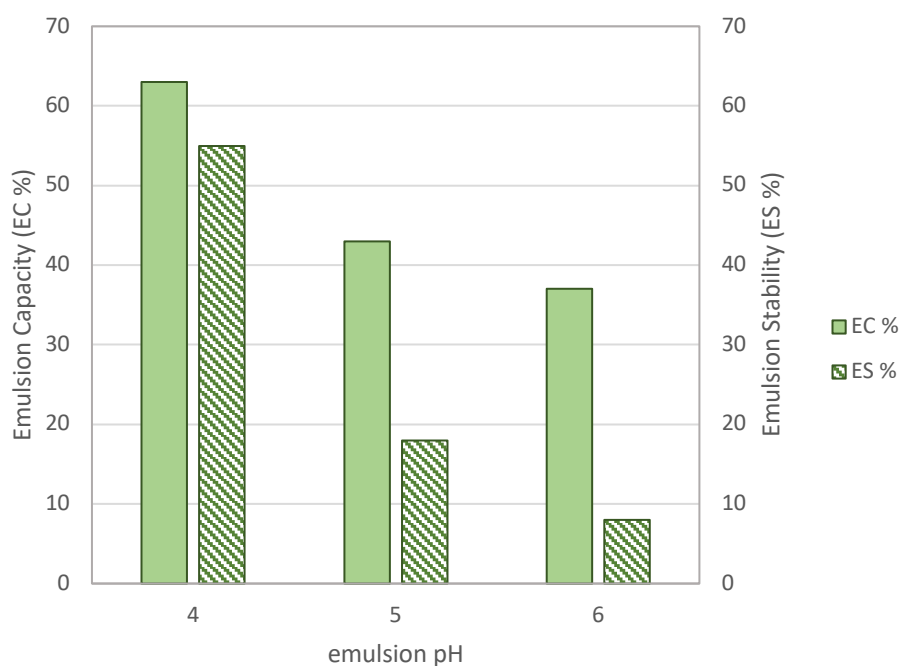


Fig. 3 Emulsion capacity (EC %) (left) and Emulsion stability (ES %) (right) of hemp proteins isolate solubilized at different pH.

Table 2 Amino acids composition (%) of hemp protein isolate (HPI), and their isoelectric point.

Amino acid	Asp + Asn	Thr	Ser	Glu + Gln
Content ^(a)	11.81 ± 0.69	3.54 ± 0.22	4.78 ± 0.26	22.39 ± 2.23
Isoelectric point ^(b)	2.77 / 5.43	5.6	5.7	3.22 / 5.65
Amino acid	Gly	Ala	Cys	Val
Content ^(a)	4.38 ± 0.22	4.14 ± 0.21	1.49 ± 0.04	4.14 ± 0.14
Isoelectric point ^(b)	6.06	6.11	5.15	6.02
Amino acid	Ile	Leu	Tyr	Phe
Content ^(a)	3.67 ± 0.35	5.51 ± 1.23	3.09 ± 0.44	3.66 ± 1.10
Isoelectric point ^(b)	n.d	6.04	5.63	5.76
Amino acid	Met	His	Arg	Pro
Content ^(a)	2.36 ± 0.02	2.65 ± 0.23	13.91 ± 0.55	4.13 ± 0.54
Isoelectric point ^(b)	5.74	7.64	10.76	6.3

(a) = Girgih et al., 2011; (b) = Liu et. al, 2004

Table 3 Emulsion Capacity (EC %) and Emulsion stability (ES%), and soluble protein expressed as BSA proteins (mg/mL) of 1% w/w hemp protein isolate dispersed in water solution containing 0.5% w/w of xanthan gum, with respect to changes in pH.

Protein 1% w/w	pH		
	4	5	6
EC %	63	43	37
ES %	55	18	8
BSA protein (mg/mL)	0.62 ± 0.04	0.28 ± 0.06	0.27 ± 0.03

Disassimilation of Hemp bran

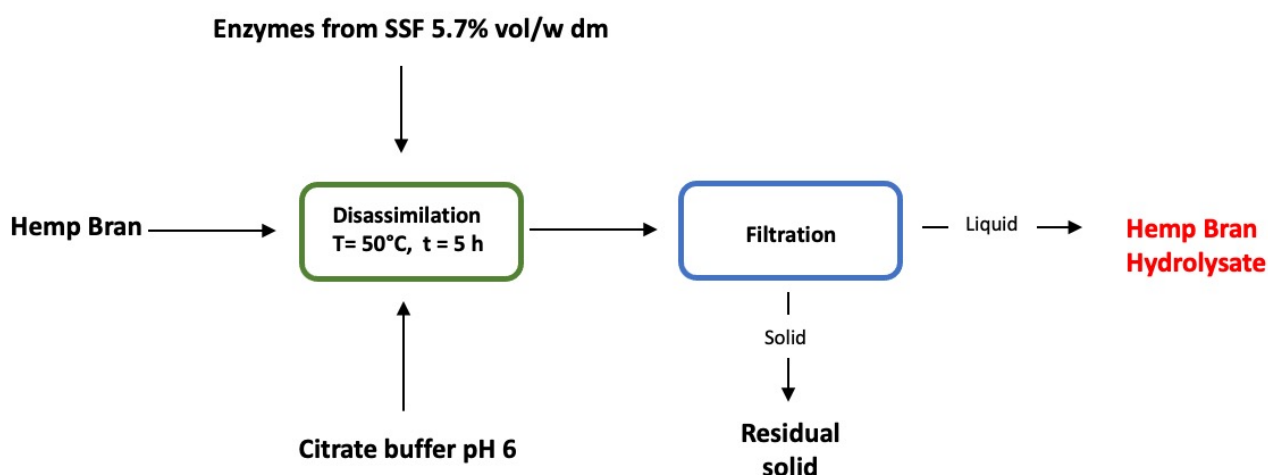


Fig.4 Simplified hemp bran deconstruction process.

After the two sequential extractions, 88.27 ± 1.95 g of residual hemp meal is obtained. In the oil seed meal, two groups of fractions with different sizes could be identified, which also differ in fiber content and bioactive activities. Generally, the coarse fractions of particle sizes > 350 and > 250 μm contain hull particles, whereas the fine fraction of particles > 180 and < 180 μm contain predominantly cotyledon particles (Pojić et al., 2014). The coarsest part of hemp seed meal, which accounts for 33% of the total (Pojić et al., 2014), is richer in fiber (29.4%, Pojić et al., 2014) and exhibits higher radical scavenging activity. The finest part in the range 180-250 μm instead, has the

highest yield (39.4%, [Pojić et al., 2014](#)) and is rich in carbohydrates (18.6%, [Pojić et al., 2014](#)) and proteins (44.3%, [Pojić et al., 2014](#)) but contains more amounts of antinutrient substances like phytic acid that could limit or prevent the bioavailability of beneficial components by food ingestion ([Pojić et al., 2014](#)). This could encourage the reutilization of hemp-by products in other sectors like cosmetics or pharmaceuticals. In our previous study ([Setti et al., 2020](#)), we applied three different methods to assist the hemp bran valorization, the coarsest part of hemp cake, finding that its hydrolysis mediated by commercial protease gives hydrolysate with important biological activities. In the present study, I performed the hydrolysis of hemp bran using as biocatalysts the enzymes produced by three weeks of solid-state fermentation (SSF) on the same hemp bran, as reported in the previous section of the Thesis (Chp. 2A). They exhibit a different trend in xylanase, pectinase, amylase, protease, laccase, peroxidase, and arylesterase, depending on the fermentation period.

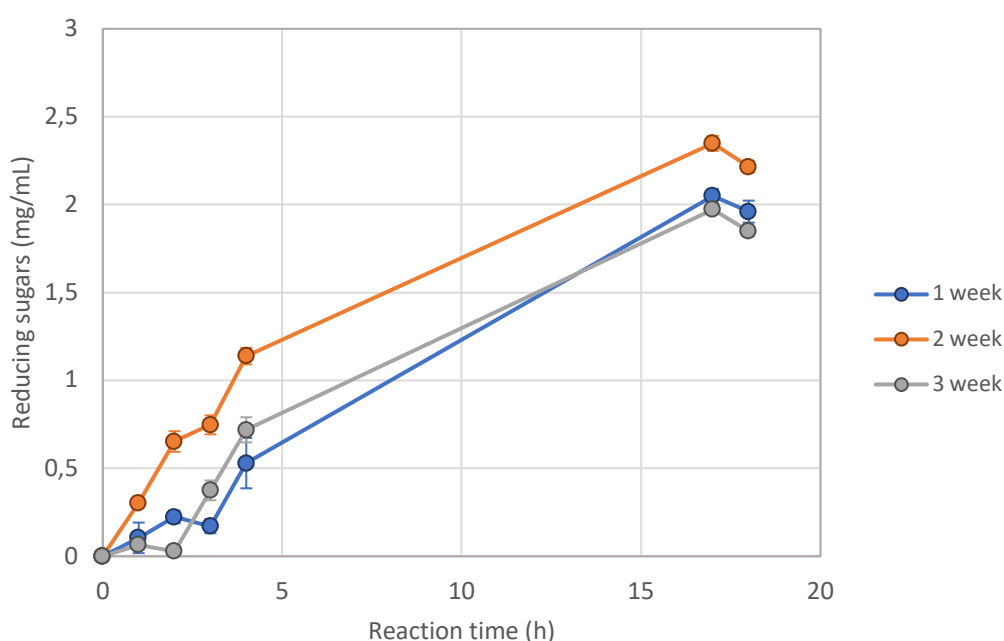


Fig.5 Reducing sugars released after the hydrolysis of Hemp Bran by enzymes produced during 3 weeks of SSF by *Pleurotus ostreatus* on Hemp Bran (from Chp. 2A)

The hydrolysis reaction products were characterized in terms of reducing sugars, total carbohydrates, total phenols, and antioxidant capacity (Table 4). Enzymes collected after two weeks of fermentation encourage the release of reducing sugars from hemp bran (Fig.5), probably due to the higher pectinase activity detected (Chp. 2A), up to 2.35 ± 0.04 mg/mL after 17 h or reaction at 37°C. Instead, the use of enzymes from the first week of fermentation led to a higher release of phenols (3.95 ± 0.53 mg catechine/mL), pointing out that they were not predominantly directly bound to the polysaccharide chains since they do not follow the reducing sugar trend.

As expected, the phenols trend is in accordance with the arylesterase activities trend revealed during fermentation. The antioxidant activity follows the phenols trend, as they are generally responsible for it. However, in the case of hemp bran, peptides could contribute significantly ([Setti](#)

et al., 2020), as well as looking at laccase produced during the fermentation (Chp. 2A), the compounds deriving from the lignin oxidation. Some studies showed that the ability of protein isolates to scavenge radicals (DPPH•) was associated with the high hydrophobicity of the protein themselves (Hadnađev et al., 2018a) and with the protein size since the smaller ones possessed a higher portion of hydrophobic amino acids (Li, Jiang, Zhang, Mu & Liu, 2008). The production of enzymes on hemp bran and its subsequent hydrolysis brought the respective valorization of 24 g and 5 g of the starting hemp seed cake. All the processes described until now valorized 40.86 % of the starting raw material. Afterward, I performed a further valorization of the remaining hemp seed cake by conducting the hydrolysis of the finest part of it. In this case, I used the commercial enzyme cocktail Celluclast BG, produced from *Aspergillus* and exhibiting endocellulase, xylanase, and pectinase activities (Zanichelli, 2008). Different enzymes concentration was considered, as shown in Fig.6, and Celluclast BG, 1% w/w, led to the maximum amount of reducing sugars, equal to 3.97 ± 0.15 mg/mL, after six hours of reaction at pH 5 and 50°C. The same reaction conditions were applied to the hemp flour residue before the extraction of proteins, on which the reactions seem to be more hindered (Fig. 7), leading to the full release of reducing sugars of 3.01 ± 0.09 . The comparison between the progress of the two reactions confirmed that the alkaline pretreatment enhances the enzyme access to the plant cell wall.

Table 4 Reducing sugars (RS), Total Carbohydrates (TC), Total phenols (TP) and Trolox equivalent antioxidant capacity (TEAC) on Hemp Bran hydrolysate after 17 h of reaction at 37°C with enzymes from SSF of Hemp Bran.

Hydrolisate	Component detected		
	7 days	14 days	21 days
RS (mg/mL)	2.04 ± 0.04	2.35 ± 0.04	1.97 ± 0.02
TP (cathechine mg/mL)	0.204 ± 0.019	0.006 ± 0.008	0.032 ± 0.008
TC (mg/mL)	3.95 ± 0.53	2.85 ± 0.25	3.56 ± 0.75
TEAC (μ mol trolox/mL)	2.44 ± 0.27	1.81 ± 0.12	2.04 ± 0.28

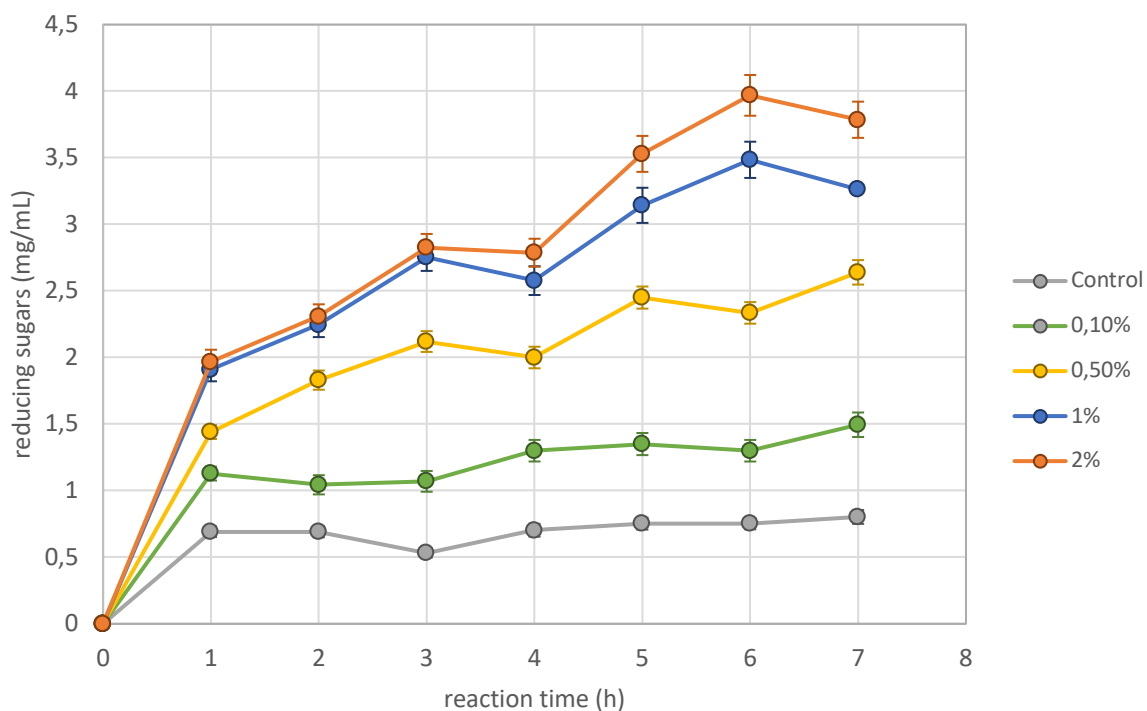


Fig. 6 Enzymatic hydrolysis of hemp flour (after protein extraction) carried out with different concentrations of Celluclast BG, pH 5, 50°C. The values are the mean for $\pm$ SD for three measurements in the same experiment.

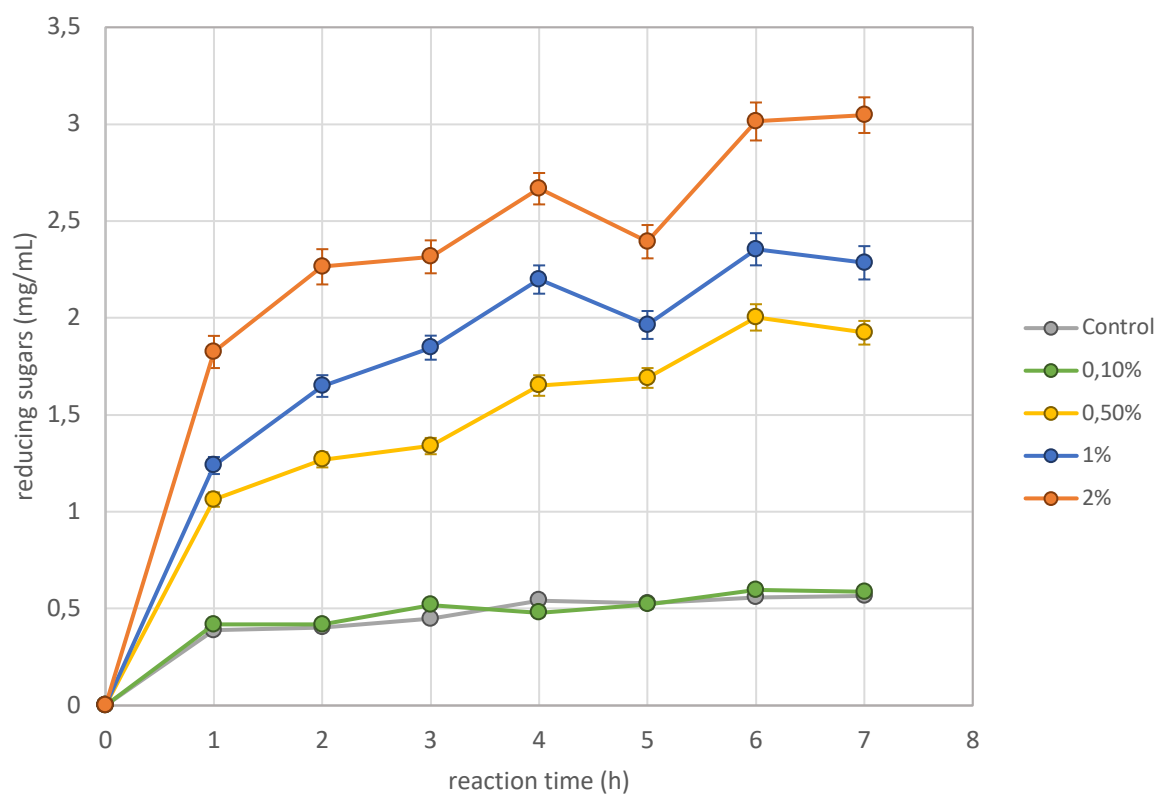


Fig. 7 Enzymatic hydrolysis of hemp flour (before protein extraction) carried out with different concentrations of Celluclast BG, pH 5, 50°C. The values are the mean for $\pm$ SD for three measurements in the same experiment.

Combination of hemp byproducts into cosmetic emulsion

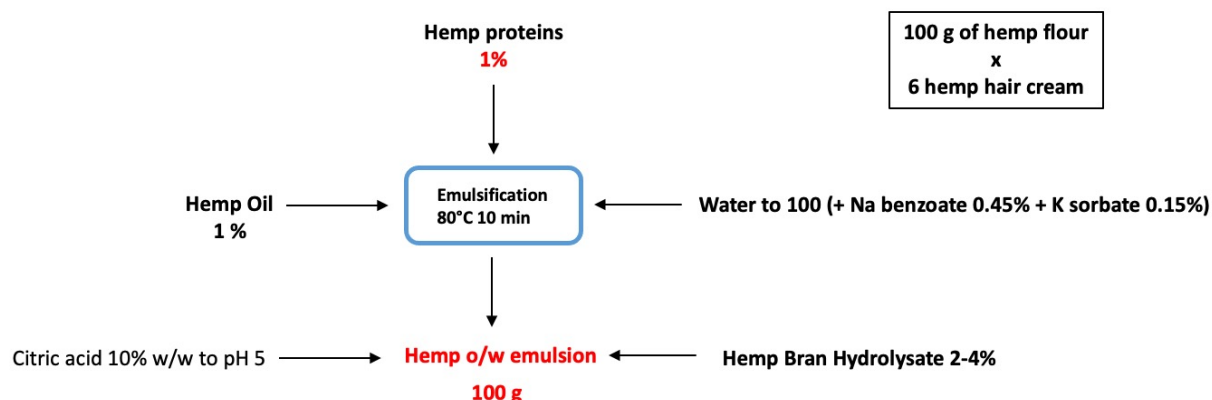


Fig. 8 Simplified representation of the emulsification process to obtain hemp-based emulsion. Scenario to obtain six emulsions from 100 g of raw material.

The byproducts from the hemp seed cake have been extracted as target compounds for the realization of oil in water food-derived emulsion (Fig. 8) with cosmetic function with a suggested application in hair care. The emulsion was realized, as pointed out in the Materials and Methods section, by dispersing 5% of extracted hemp oil in 95% of 1% w/w hemp protein aqueous solution as an emulsifier, containing xanthan gum as a thickening agent and sodium benzoate and potassium sorbate as preservatives (Table 5). The pH of the emulsion was adjusted to 4 by adding 30% w/w citric acid solution. Xanthan gum is a biopolymer produced through the fermentation process by Gram-negative bacteria of the genus *Xanthomonas* and is used to increase viscosity and enhance the system's stability (Petri, 2015). The quantity of oil was chosen to ensure the seal of system stability as much as possible without giving the formula a persistent and unpleasant odor. The amount of thickening agent has been evaluated after performing emulsion capacity (EC) and emulsion stability (ES) tests (Fig. 9), which reveal a maximum value of 95%, with 2% w/w of xanthan gum. The emulsion showed a viscosity equal to 1150 Cp, measured by the R5 spindle. The pH 4, which ensures maximum stability, suggests the most appropriate final use of the emulsion, which is hair care, since it is most compatible with the hair shaft's pH (Dias, de Almeida, Cecato, Adriano & Pichler 2014).

Table 5 Emulsion Capacity (EC %) and Emulsion stability (ES%) of 1% w/w hemp protein isolate dispersed in water solution at pH 4, with respect to xanthan gum concentration (% w/w)

pH 4	Xanthan gum % (w/w)		
	0.5	1	2
EC %	63	75	95
ES %	55	70	95

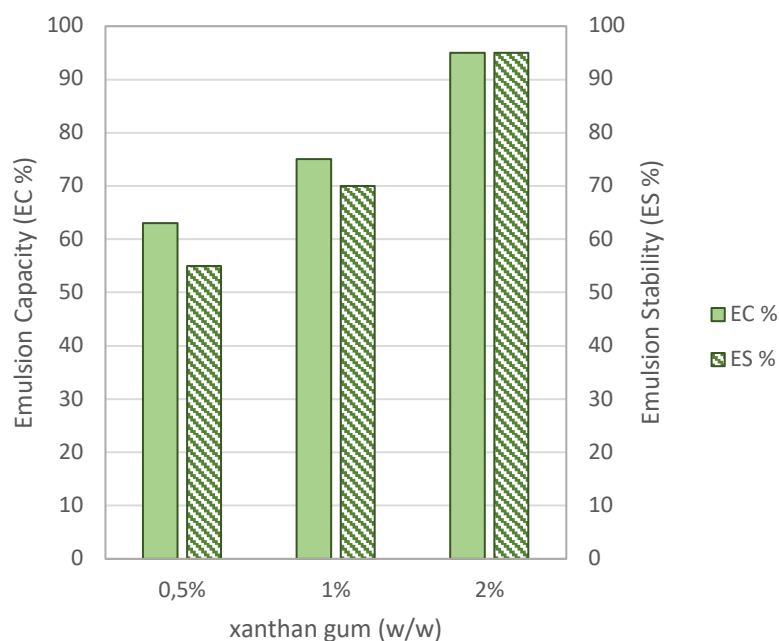


Fig.9 Emulsion capacity (EC %) (left) and Emulsion stability (ES %) (right) of hemp emulsion containing different xanthan gum concentrations.

The hemp seed oil is a rich source of polyunsaturated fatty acids (> 70%), particularly the essential fatty acids linoleic acid (C18:2) and α -linolenic acid (C18:3). It contains a high amount of total tocopherols (56–97 mg/100 g of oil) and phytosterols (279 mg/100 g of oil) (Burton et al., 2022), which prevent oxidation phenomena, ensuring the preservation of the formulation itself (Matthäus & Brühl, 2008). Linoleic acid sustains hair growth (Ryu et al., 2021). It helps the scalp absorb other ingredients faster and more efficiently, besides maintaining the health of cell membranes, improving nutrient use, and establishing and controlling cellular metabolism (Dias, de Almeida, Cecato, Adriano & Pichler, 2014). Considering the final use of the prototype, the proteins and peptide content is of great importance. It was indeed demonstrated that proteins have a high chemical affinity to hair keratin and the ability to be absorbed on the hair surface and even penetrate the hair cortex, improving the tensile strength and elasticity of the hair and protecting it from adverse effects of the chemical treatments (Cruz, Ribeiro, Martins & Cavaco-Paulo, 2017). Peptides and hydrolyzed proteins improve conditioning properties, smoothness, luster, repair, and protection and reduce the effect of volume change at high relative humidity conditions (Cruz et al., 2017). Thus, the functionalization of the emulsion with the hydrolysate from hemp bran or hemp flour, for example, from 1 to 3 % w/w, gives high added value and specificity to the final product.

Limitations of the study and Perspectives

In the presented process, the hemp bran and the hemp flour are considered as deriving from the same seed cake; instead, the hemp bran and the hemp flour have been purchased separately. This assumption brought to consider the hemp bran present in the amount found in the literature (33%) to estimate a mass balance of process for the conversion of the starting hemp seed cake. In evaluating the feasibility of the proposed process, the extraction yield of hemp proteins should be considered the limiting factor since its content in the emulsion determines the stability of the product. The yield of hemp oil is not so important, as its amount in the final product depends on the value to be added, up to the maximum tolerated by the system before causing a decrease in emulsion capacity and emulsion stability. After a first experimental evaluation, the quantity of raw material destined for hydrolysate production should be established to avoid the devaluation of the recovered high-value substance in accordance with the volume/price pyramid's law. The enzyme price factor could limit the conversion if commercial enzymes are used in the hydrolysis reaction. The waste that could not be further chemically valorized should be then sent to an energy plant capable of processing biomass in an effective way. Bearing in mind the above, improving the protein extraction as much as possible is recommended, also considering lower-impact methods like micellization ([Hadnađev et al., 2018b](#)). For oil extraction, a greener method is advisable, involving, for example, the use of enzymatic-assisted reactions. Regarding the final product, the assessment of its safety by microbiological tests and its efficacy is required to validate the usage of the prototype.

Conclusion

Hemp seed oil is a product for niche markets driven by small producers, who should cope with managing the produced waste, which exhibits an important biological activity limiting its disposal. A good strategy to reduce the environmental risk of food waste is to reduce the content of unstable organic matter by valorizing it before disposing. Nevertheless, valorization processes generally require equipment and utility costs that are not even affordable for small industries. In addition, a market for the commercialization of the products should be guaranteed. The presented study aimed to provide an integrated and innovative biorefinery process for the cost-effective valorization of hemp seed cake on a small scale by its chemical and biochemical deconstruction into high-value components that are then recombined in the form of a cosmetic emulsion prototype, suitable for hair care applications. It could simultaneously offer experimental data helpful to evaluate the feasibility options of the entire process.

From the chemical processing of 100 g of hemp seed cake, hemp oil and hemp proteins have been obtained in 5.85 ± 1.20 g and 5.88 ± 1.17 g, respectively. The coarsest part of the residual flour, namely the hemp bran, has been used as a substrate for enzyme production valorizing 24 g of the starting material. The produced enzymes were used to make a hemp bran hydrolysate valorizing 5 g of it. An example of hydrolysis of the finest residual flour using a commercial enzyme cocktail was

also provided. Every component has been united in a cosmetic emulsion using a water solution at pH 4 containing 1% hemp protein as an emulsifier and 5% hemp oil as the dispersed phase. The oil amount was chosen to have a stable final product with no unpleasant odor and color, but a lower amount could be considered to increase the yield of high-value products. Finally, 1-3% hydrolysate was suggested to functionalize the emulsion. The final product has been thought to be used as a hair mask, capable of nourishing the scarp and reducing the volume of hair exposed to humidity, imparting smoothness and luster to them.

Following the proposed process, from the processing of 100 g of raw material, up to six emulsions could be made, containing 1% of hemp protein, 1 % of hemp oil, and 2% of hemp hydrolysate, valorizing early 41% of starting material. Otherwise, if emulsions with higher oil content are wanted to be realized, the proteins in excess could be sold or distributed on other market channels since their solubility limits their concentration in emulsions.

The proposed model could be applied to each oleaginous seed byproduct and high protein-content food resources, like the leguminous ones.

Abbreviations

EC	Emulsion Capacity
ES	Emulsion Stability
FAO	Food Agricultural Organization
HPI	Hemp Protein Isolate
RS	Reducing Sugars
TC	Total Carbohydrates
TP	Total Phenols

3B Case of study: biorefinery of *Oryza sativa* (rice) grain

Introduction

Cereal production and processing are one of the most important sectors of the agri-food industry (Belc et al., 2019). They generate a vast quantity of loss and waste, accounting for approximately 35 % of the total production in North America, Europe, and industrialized Asia (Gustavsson, Cederberg, Sonesson, Van Otterdijk, & Meybeck, 2011), which was equal to 2719 million tons in 2019, as estimated by FAO. (Fraterrigo Garofalo, Tommasi & Fino, 2021). Of the global waste generated along the food chain, the cereal industry's byproducts correspond to 12.9 % (Baiano, 2014), with 40000-45000 tonnes/year produced only in Europe (Belc et al., 2019). Cereal processing waste consists of effluent and solids rich in organic and inorganic harmful substances, with high BOD (1050–7950 mg/L), COD (1400–29000 mg/L), and pH between 3.5 to 8.5, causing the potential ground and water pollution (Karunaratne, 2010). According to the International Finance Corporation's (IFC) environmental guidelines, the permitted limits for discarding wastewater are pH comprised between 6 and 9, BOD up to 30 mg/L, COD to 125 mg/L, suspended solids (SS) up to 50 mg/L, total phosphorous up to 2 g/L, total nitrogen and oil and grease equal to 10 mg/L. (Simate et al., 2011). Thus, one of the aims of waste management in the agri-food industry is to find strategies to reduce waste or transform it into new raw materials, to improve resource efficiency while protecting the environment (Hassan et al., 2021). Among the produced cereals worldwide, rice is the most important staple food for the world's population and the third most important in production (FAO,2022). The US Department of Agriculture (USDA) estimated that the world's rice production should reach 503.27 million tons in 2022 (<http://www.worldagriculturalproduction.com>). After the harvesting step and the separation of straw from the crop, the “paddy” or rough rice grain is obtained. It is generally composed of hull or husk (~ 20%), bran (~ 9%), rice kernel or starchy endosperm (~ 70%), and rice germ or embryo (~ 1%) (Fig. 1) (Garofalo et al., 2021).

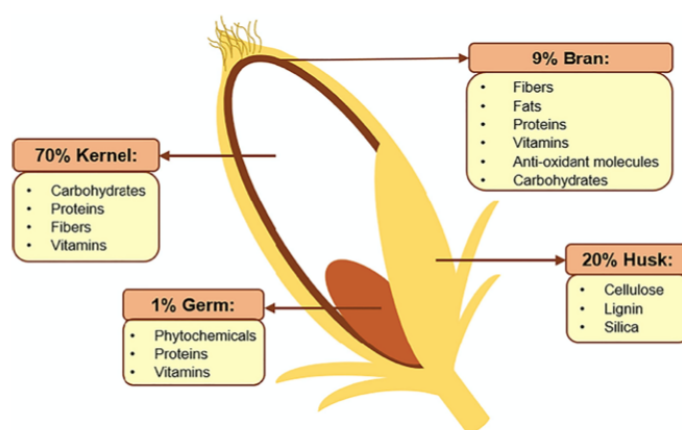


Fig.1 Rice grain components (Garofalo et al., 2021).

The remotion of the husk, the outer layer, leads to the brown rice, whereas the remotion of the second outer layer, the bran, is the first step of the milling process, which at the end gives the white and polished rice kernel that is the most commercialized food product. In the end, the whole milling process generates more than 30% of by-products, which are still rich in nutritive substances and high-value molecules and thus are susceptible to valorization practices (Garofalo et al., 2021). The rice bran contains around 65% of the nutrients of the whole rice grain (Begum et al., 2015). One of the most common uses of rice bran is the extraction of rice bran oil (RBO), an essential source of γ -oryzanol, a mixture of ferulic acid and phytosterol esters (Garofalo et al., 2021). Rice husk is primarily used for energy production by thermal processes, such as gasification and combustion, but it is also an essential source of dietary fiber and silica (Rafiee, Shahebrahimi, Feyzi & Shaterzadeh, 2012). The embryo contains proteins and vitamins. The damaged or broken rice from the mechanical stress induced by the milling system is usually transformed into flour and employed in the food industry (Bodie, Micciche, Atungulu, Rothrock Jr & Ricke, 2019).

The present work intends to provide an innovative process to valorize the rice milling by-products through the biotechnological deconstruction of rice flour and rice husk designed to realize the first agro-food-based cosmetic emulsion, with the suggested function of nutrient body cream.

Results and Discussion

The present study concerns the biotechnological valorization of rice milling processing residues, as shown in the flowchart reported in Fig.2, in which the mass balance of the solids is also pointed out. It starts with the enzymatic deconstruction of brown rice flour into rice starch, rice fiber, and rice proteins partially bound to leftover flour, which is susceptible to further valorization. The process carries on with the dissassimilaton of rice husk mediated by enzymes, which occurs in water solution and causes the release of hydrolyzed plant cell wall polymers and bioactive molecules with antioxidant properties that were bound to them. In the end, a cosmetic emulsion prototype with the function of body cream is realized by dispersing commercial rice bran oil into extracted starch solution. The emulsion is then functionalized by adding the rice husk hydrolysate.

Flowchart guidelines

In the flowchart, the bold squares indicate the commodities used as starting materials and the products obtained. The solid green lines from them show the conversions of the agro-food materials carried out by biochemical routes; the red lines point to their combination into the final product. The lines from the not bolded squared text box show processes not developed in the present study. The dotted lines respond to the same color legend but indicate the suggested pathways yet to be achieved. The brackets state the components which made up the material under discussion. The bold numbers stand for the masses in weight of raw materials (black) and products (red). The percentages refer to composition data from the literature.

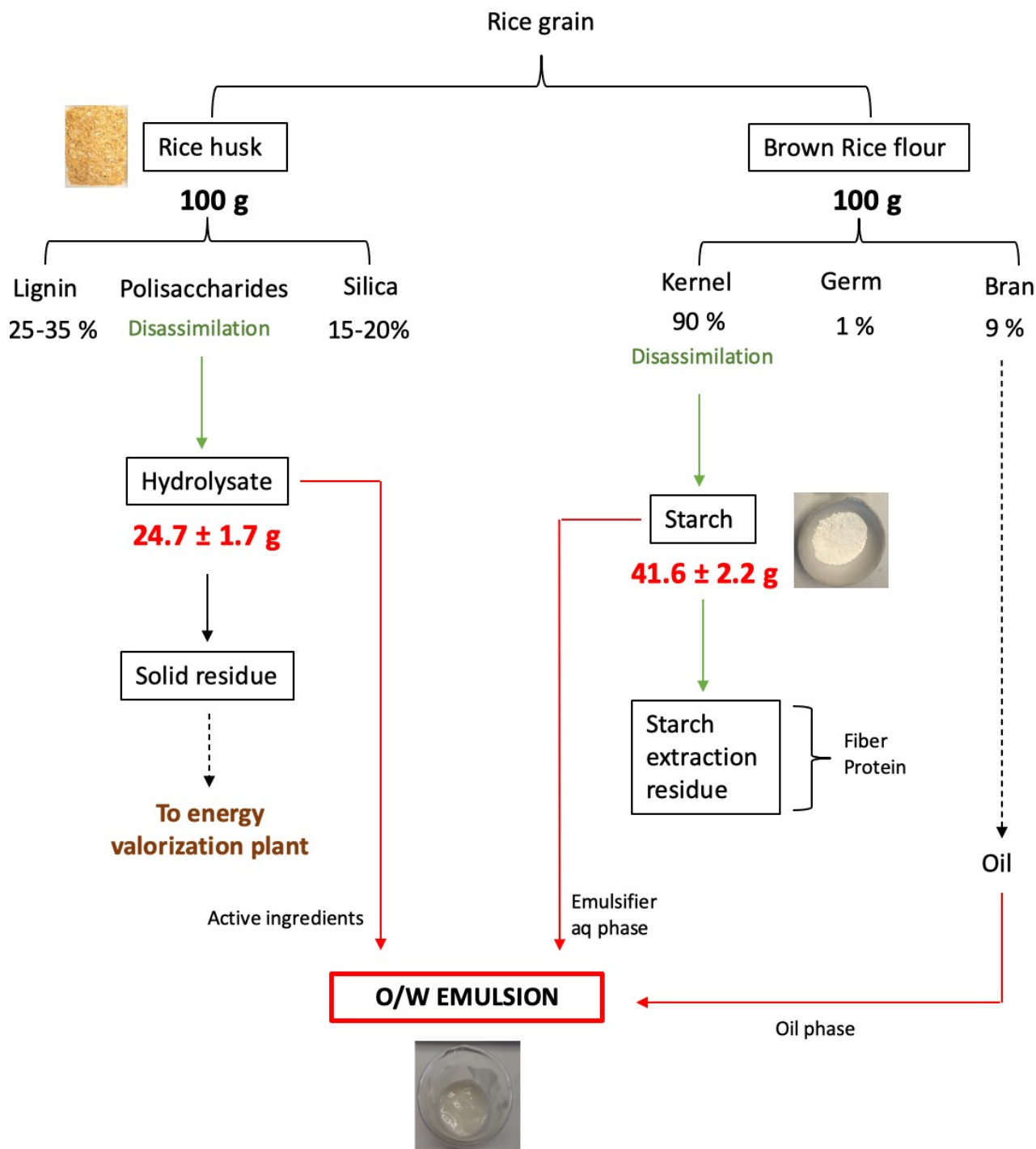


Fig.2 Flowchart of the whole deconstruction and recombination of rice grain.

Deconstruction of rice flour

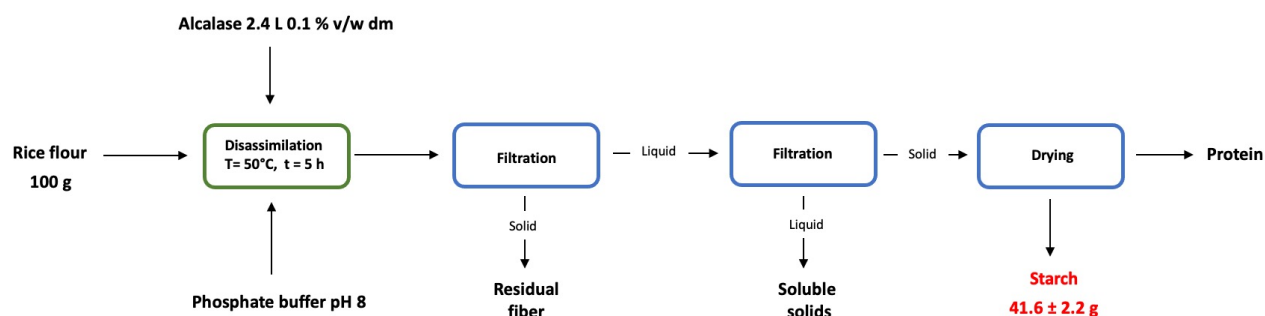


Fig.3 Simplified rice flour deconstruction process.

The deconstruction process of rice flour is reported in Fig. 3. The composition of rice flour used as starting material is reported in Table 1.

Table 1 Nutrient composition (%) of brown rice flour (Pam Panorama brand)

Composition %	
	Brown rice flour
Fat	3.2
Carbohydrates	77
Fiber	3.1
Protein	6.8

Rice flour is a primary source of starch used in the food sector due to its excellent swelling and gelling properties, even if the commercial applications are currently limited owing to the low yield and high cost of industrial production (Choi et al., 2018). In cereal grains, it occurs as small semicrystalline granulates in the amyloplast of the starchy endosperm and consists of amylose (25%) and branched amylopectin (75%). In the grain endosperm, water-extractable arabinogalactans-peptide is also observed husk (Taina Simoinen & Maija Tenkanen, 1999). Starch is generally recovered by extraction mediated by alkali or detergents followed by purification and whitening steps to meet commercial standards, even if greener extraction methods have been developed in the last years to reduce the environmental impacts of the generated effluents (Lumdubwong & Seib, 2000). The enzymatic method exploits the action of proteases on hydrolyzing the protein bodies (PB), made by 20% of prolamin and 60% of glutelin, that link starch granulates (Lumdubwong & Seib, 2000). In the present study, the *Bacillus subtilis* alkaline proteinase, namely

Alcalase 2.4 L, has been used as a biocatalyst in starch extraction, followed by a physical separation step based on the fiber sieving and centrifugations, as reported in the Materials and Methods section. Different enzyme concentrations were investigated to ensure the obtainment of rice starch in a good yield and with a reasonable extent of bound protein moieties. (Table 2).

Table 2 Amino acids composition (%) brown rice protein concentrate.

Amino acid	Asp	Thr	Ser	Glu
Content ^(a)	8.7	3.6	4.9	17.4
Isoelectric point ^(b)	2.77	5.6	5.7	3.22
Amino acid	Gly	Ala	Cys	Val
Content ^(a)	4.4	5.6	2.1	5.8
Isoelectric point ^(b)	6.06	6.11	5.15	6.02
Amino acid	Ile	Leu	Tyr	Phe
Content ^(a)	4.4	8.0	8.0	5.3
Isoelectric point	n.d	6.04	5.63	5.76
Amino acid	Met	His	Arg	Pro
Content ^(a)	2.9	2.2	7.9	4.6
Isoelectric point ^(b)	5.74	7.64	10.76	6.3

(a) = Kalman., 2014; (b) = Liu et. al, 2004

The use of 0.01 % vol/ w dry flour of Alcalase 2.4 L solution at pH 8 led to 41.55 ± 2.16 g of rice starch after 5 hours of reaction at 50°C, leaving as solid residues fiber and proteins, which are further susceptible to valorization by using, for example, amylase enzymes to hydrolyze the leftover starch partially bound to them. The reaction pH was chosen to allow the main part of rice proteins to migrate into the solution unleashing starch molecules on the basis of the optimum pH for protease activity and the rice flour amino acid composition (Table 3). The digestion temperature was set below the gelatinization temperature of rice starch (Bhattacharya, 1979) to make the enzymatic reaction and the subsequent separation steps possible. Unlike the food application, using starch functionalized with proteins could be advantageous in the cosmetic field because it may enhance the emulsifying property of the system since it summarizes the abilities of both a polysaccharide and proteins in dispersing the oil droplets effectively in a water solution. Polysaccharides stabilize emulsions by increasing aqueous phase viscosity, inhibiting droplet movement (McClements, 2004). They are usually relatively large molecules (100–1000 kDa) that adsorb relatively slowly to droplet surfaces and form thick hydrophilic interfacial layers which stabilize the emulsion by steric repulsions (Bouyer, Mekhloufi, Rosilio, Grossiord & Agnely, 2012). Proteins are active surface molecules because they contain a mixture of hydrophilic and hydrophobic amino acids along their

polypeptide chains (Lam & Nickerson, 2013). They generally stabilize droplets from aggregation by electrostatic repulsion since they contain amino acids that possess negative ($-\text{COO}^-$) or positive ($-\text{NH}_3^+$) charges (Ozturk & McClements, 2016). This has been confirmed by our emulsion capacity and stability studies carried out of 1% w/w solutions of extracted starches exhibiting different protein coordination degrees (Fig.3). The extraction by the crescent concentration of enzymes brought a decrescent starch functionalization degree and affected the stability of the emulsion (Table 4). This confirmed that the lower Alcalase 2.4 L concentration performs the adequate reaction for our purpose.

Table 3 Rice Starch yield (%) and residual protein (%), fiber (%), and soluble protein (BSA protein mg/mL) in the extraction supernatant, as a function of Alcalase 2.4 L concentration (% vol/w dm).

Component	Enzyme concentration (% vol/w dm)			
	Control	0.01	0.03	0.05
Starch %	9.70	40.1	33.3	32.9
Protein %	4.40	8.20	9.60	11.5
Fiber %	47.7	11.8	9.50	5.60
BSA protein (mg/mL)	1.10	0.67	0.62	0.58

Table 4 Emulsion Capacity (EC %) and Emulsion stability (ES%) of brown rice starch for starch concentration (w/w) and Alcalase 2.5L concentration (% vol/w dm).

Starch 1% w/w	Enzyme concentration (% vol/w dm)			
	Control	0.01	0.03	0.05
EC %	47.2	69.2	62.8	58.7
ES %	44.3	51.3	51.3	50.7
BSA protein (mg/mL)	0.11	0.47	0.13	0.12
Starch 2% w/w	Enzyme concentration (% vol/w dm)			
	Control	0.01	0.03	0.05
EC %	58.7	98.6	80.2	73.4
ES %	56.0	94.6	67.9	59.2
BSA protein (mg/mL)	0.14	0.88	0.26	0.17

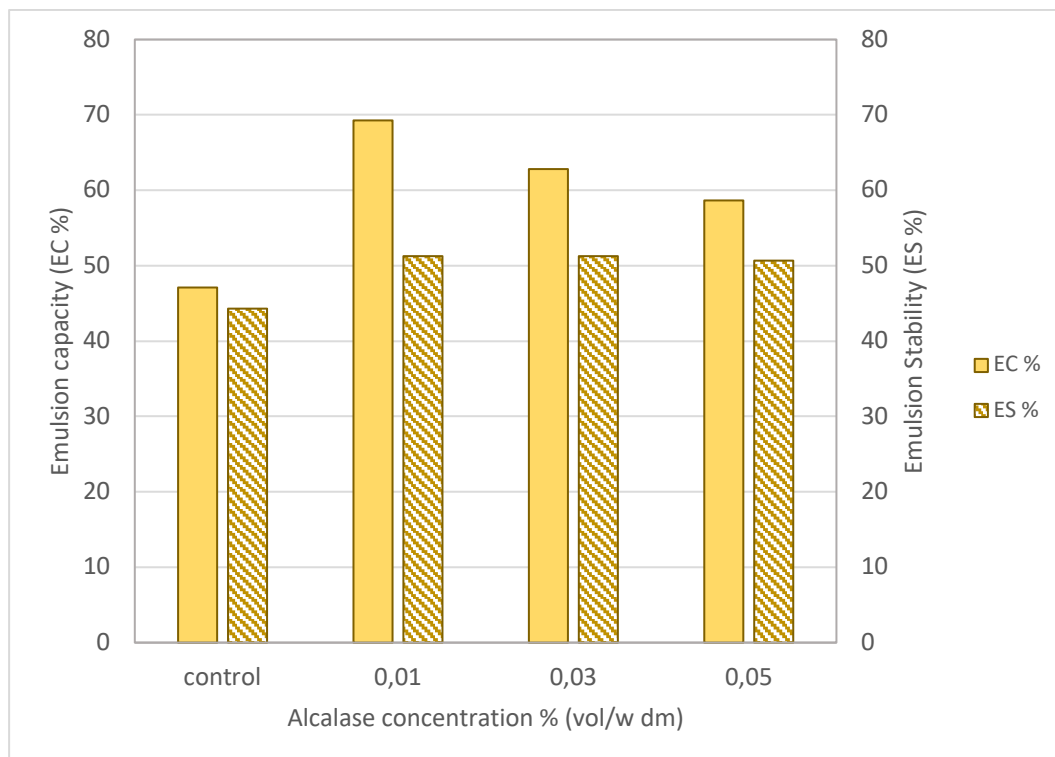


Fig. 3 Emulsion capacity (EC %) (left) and Emulsion stability (ES %) (right) of rice starch extracted at different Alcalase concentrations.

Disassimilation of rice husk

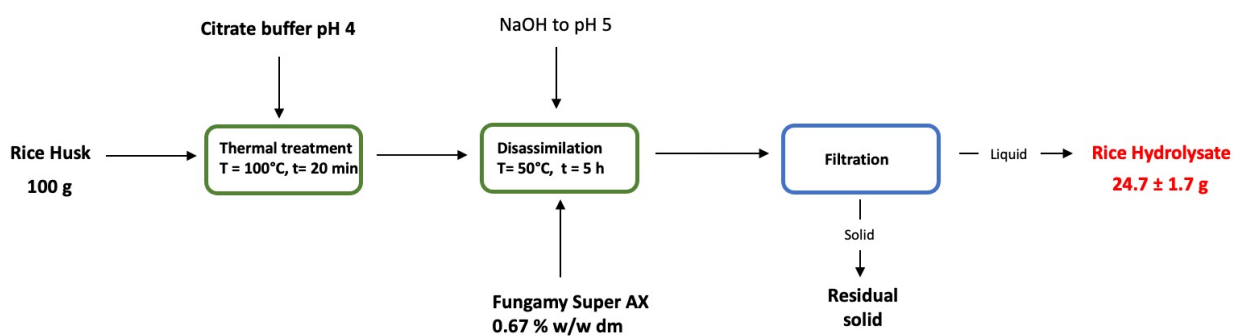


Fig.4 Simplified rice husk deconstruction process.

The deconstruction process of rice husk is outlined in Fig.4. Rice belongs to monocotyledon-type plant material. Its plant cell wall consists of cellulose, β -glucans, and arabinoxylan partially substituted with phenolic acids such as ferulic and p-coumaric acids. They are detected in grains endosperm, but their content increases gradually until reaching the maximum in grain husk (Taina Simoinen & Maija Tenkanen, 1999). Rice husk represents approximately 23 % of total harvested rice (Santos et al., 2017), corresponding to 800 million tons per year globally (Belc et al., 2019). It comprises around 25–35 % cellulose, 18– 21 % hemicelluloses, 26–31 % lignin, 15–17 % silica, 2–5 % solubles, and around 7.5 % moisture (Santos et al., 2017). Due to its high content of lignin and silica, rice husk is a very recalcitrant plant material, so its treatment generally requires severe reaction conditions. However, its polysaccharide components can be easier hydrolyzed by resorting to enzymatic reactions. In the present study, the commercial enzyme cocktail Fungamyl Super AX has been used to carry out the disassimilation of the husk through mechanical and thermal pretreatment followed by a hydrolysis reaction. The thermal treatment aims to encourage the coordination of enzymes to the plant cell wall, according to the procedures reported in the Materials and Method section. It was conducted in sodium citrate buffer at pH 4 after a first trial to evaluate the pH that could lead to a higher reducing sugar release into the solution (data not shown). Afterward, the reaction conditions capable of ensuring a high hydrolysis degree have been researched by evaluating enzyme concentration (Fig.5), solid-to-solvent ratio (Fig.6), and reaction pH (Fig.7). The optimal reaction conditions resulted in being: 0.67% w/w of the enzyme, solid-to-solvent ratio equal to 0,45 and reaction pH 5, which led to the release of 16.7 mg/mL of reducing sugars, after 4 hours of reaction at T= 50°C. At the end of the reaction, the disassimilation of an early one-quarter of the starting material occurred, leading to the valorization of 24.7 ± 1.7 % of the starting rice husk, which represents almost half of the hydrolyzable material. The Fungamyl Super AX has been chosen as a biocatalyst because it exhibits mainly xylanase and amylase activities and a significant arylesterase side activity that is useful to release phenolic acids from esterified heteroxylans. This conferred interesting biological properties to the hydrolysate, as shown in Table 5. In cereals grains, esterases have been identified, suggesting a wide range of functions occurring in nature, starting from a role in plant pathogen defense to a role in plant cell wall structure affecting both the properties and the processability of the grain components (Taina Simoinen & Maija Tenkanen, 1999). The characterization of the final hydrolysate reveals that the hydrolysis of polysaccharides by enzymes carries an increase of bioactive substances into the solution, in particular to 7-fold higher reducing sugars (17.16 ± 0.67 mg/mL), one and a-half higher total phenols (0.22 ± 0.02 mg catechin/mL), and an increase in antioxidant capacity equal to 15-fold Trolox equivalent (2.16 ± 0.09 TEAC/mL) with respect to the control.

Table 5 Reducing sugars (RS), Total Carbohydrates (TC), Total phenols (TP) and Trolox equivalent antioxidant capacity (TEAC) on Rice Husk hydrolysate after 4 h of reaction at 50°C with Fungamyl Super AX 0.67% w/w dm.

Rice Husk Hydrolysate	Component detected	
	Control	Fungamyl Super AX
RS (mg/mL)	2.32 ± 0.44	1.16 ± 0.67
TP (cathechine mg/mL)	0.15 ± 0.03	0.22 ± 0.02
TC (mg/mL)	23.7 ± 0.7	26.5 ± 1.1
TEAC (μmol trolox/mL)	0.14 ± 0.02	2.16 ± 0.09

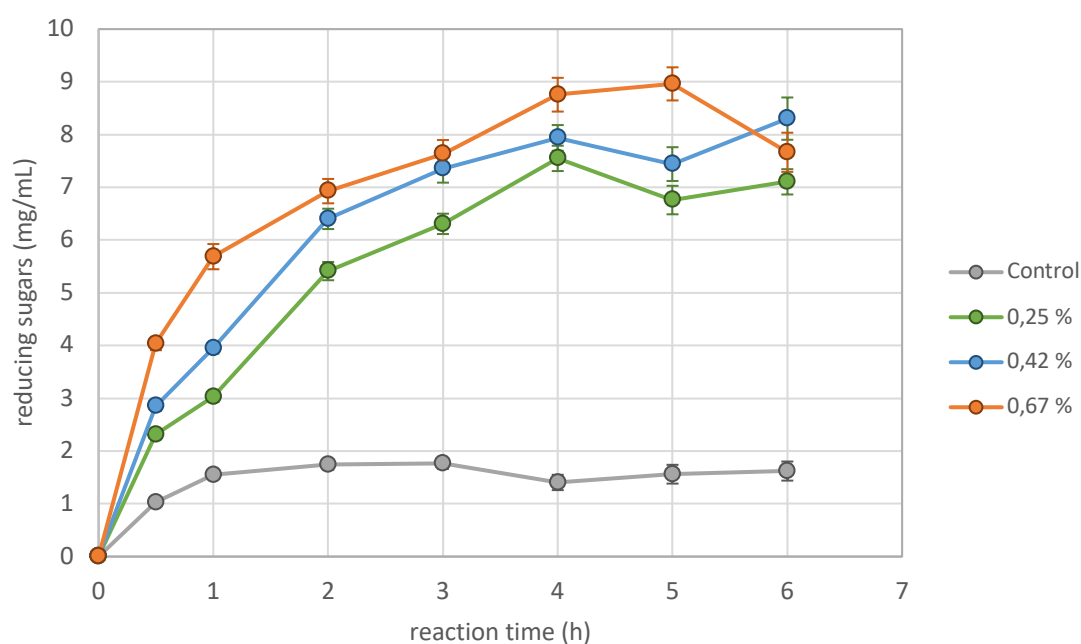


Fig. 5 Enzymatic hydrolysis of rice husk carried out with different concentrations of Fungamyl Super AX (% w/w), pH 4, 50°C. The values are the mean for ± SD for three measurements in the same experiment.

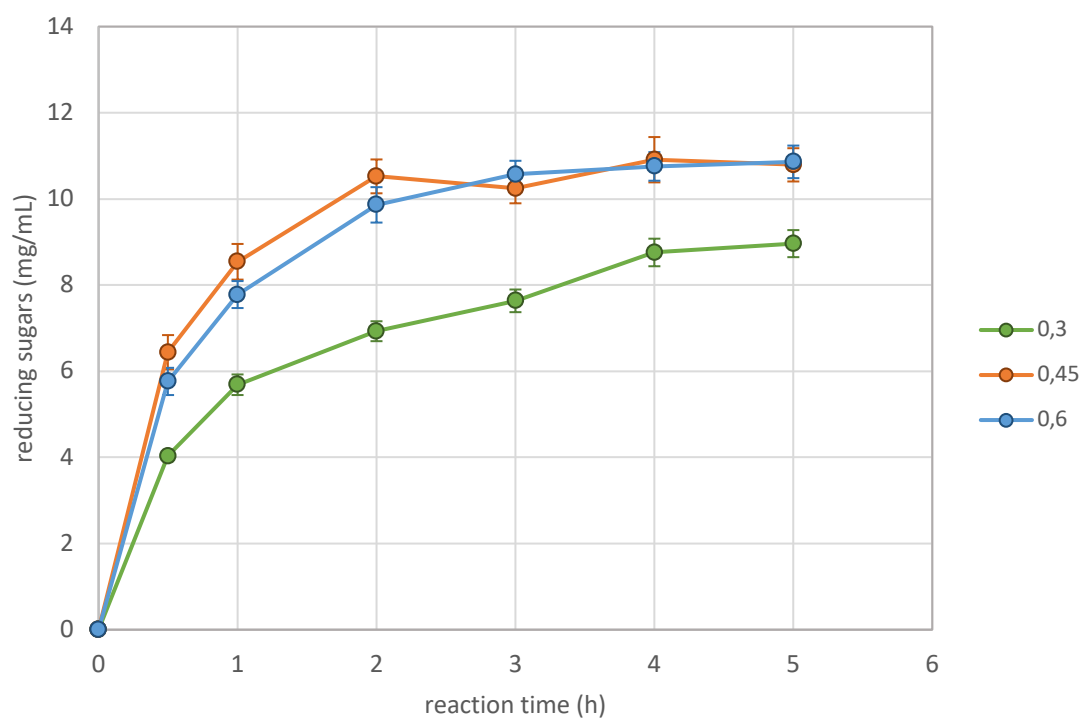


Fig. 6 Enzymatic hydrolysis of rice husk carried out with different solid/solvent ratios with Fungamyl Super AX 0.67% (w/w), pH 4, 50°C. The values are the mean for $\pm$ SD for three measurements in the same experiment.

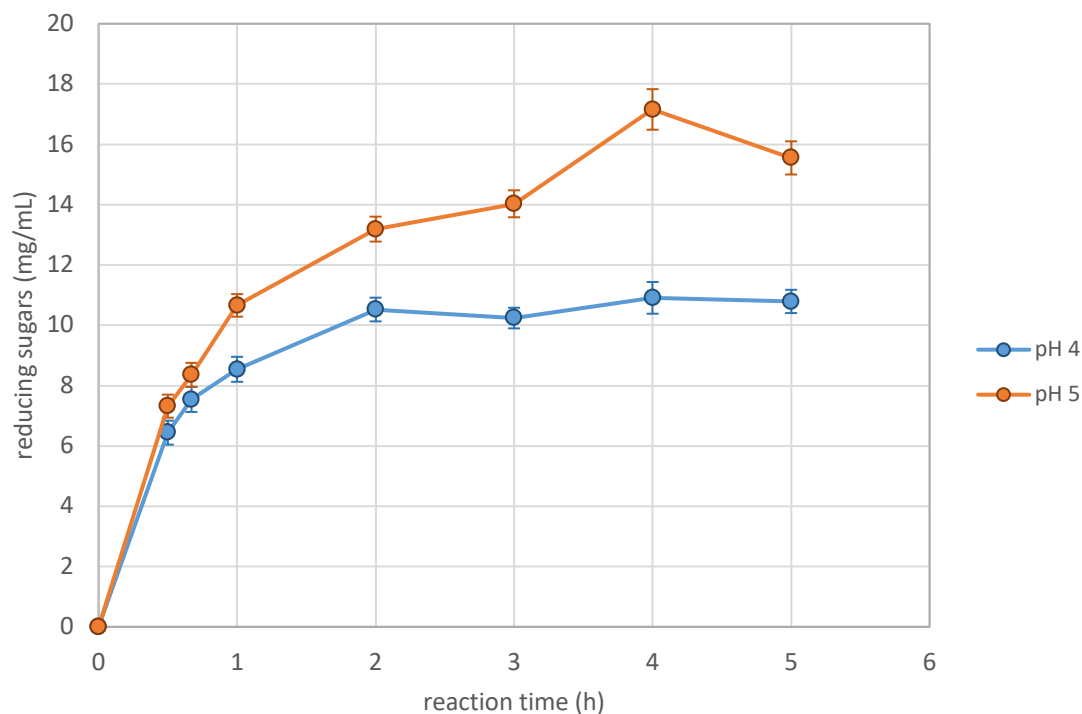


Fig. 7 Comparison of enzymatic hydrolysis of rice husk carried out at pH 4 and pH 5, with 0.45 solid/solvent ratio with Fungamyl Super AX 0.67% (w/w), 50°C. The values are the mean for $\pm$ SD for three measurements in the same experiment.

Recombination of rice flour by-products into emulsion

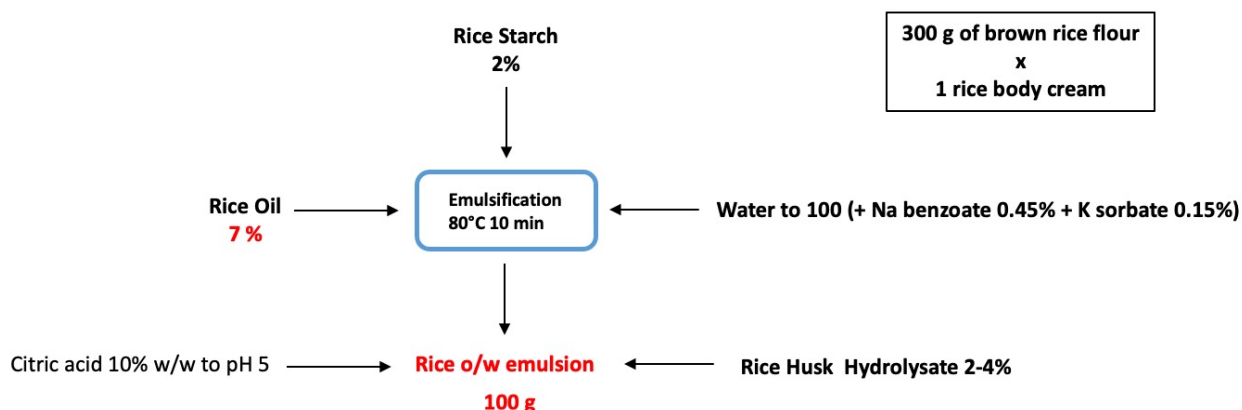


Fig. 8 Simplified representation of the emulsification process to obtain a rice-based emulsion. Scenario to obtain one emulsion from 300 g of raw material.

The extracted byproducts from the rice milling process have been recombined into an oil-in-water emulsion, with the potential function as a cream for body care applications, as reported in Fig. 8. 10% w/w of rice bran oil was dispersed into a solution of 2% w/w rice starch as an emulsifier, containing food-grade preservatives and 1% of xanthan gum as a thickening agent (Table 6). The mixture is held at 80°C before homogenization. The elevated processing temperature helps reduce the viscosity of the system and facilitates the mixing and dispersion of the phases. The system is cooled down to 40 to 50°C before adjusting the final pH to 5 with a citric acid solution. The choice of starch concentration came from the comparison between 1% and 2% starch solution in emulsion capacity (EC %) and emulsion stability (ES %) (Fig.9). Rice starch has a unique combination of several desirable characteristics, including hypoallergenicity, smooth texture, bland flavor (Mitchell, 2009), and small particle size (8 mm or less), with a very narrow size distribution compared to those of other starches (Choi et al., 2018) which make makes it especially suitable for cosmetic applications. Like all polysaccharides, it exhibits an intrinsic ability to preserve the moisture of the human skin by forming a film onto the epidermis, which limits the transepidermal water loss (TEWL) (Camargo Junior, Gaspar & Campos, 2012). The choice of oil quantity comes from the evaluation of the proper emollient degree for the desired product. Rice bran oil contains 2.5–3.2% of an unsaponifiable fraction rich in functional components, such as tocopherols (vitamin E), γ -oryzanol, tocotrienols, and phytosterols, responsible for the antioxidant activity of the oil. The ferulic acid ester content in γ -oryzanol is a powerful antioxidant, anti-free radical, and a strong radical scavenger (Garofalo et al., 2021). Rice bran oil also contains other micronutrients, such as squalene and phospholipids, which are naturally present in the stratum corneum of the epidermis (Lampe et al., 1983). Besides enhancing the skin's moisturizing activity, rice bran oil may prevent skin aging and protect it from lipidic peroxidation induced by UV light (Kushwaha, 2018). In addition, anti-inflammatory properties have been observed.

The realized emulsion has a viscosity of 1450 Cp, recorded by the R5 spindle, and shows an antioxidant capacity equal to 1075 ± 45 μmol of Trolox equivalent/ 100 g of emulsion. It was then functionalized with the rice husk hydrolysate by 3% w/w, responsible for the increased antioxidant capacity of 5%, resulting in 1130 ± 92 Trolox equivalent/100 g of emulsion.

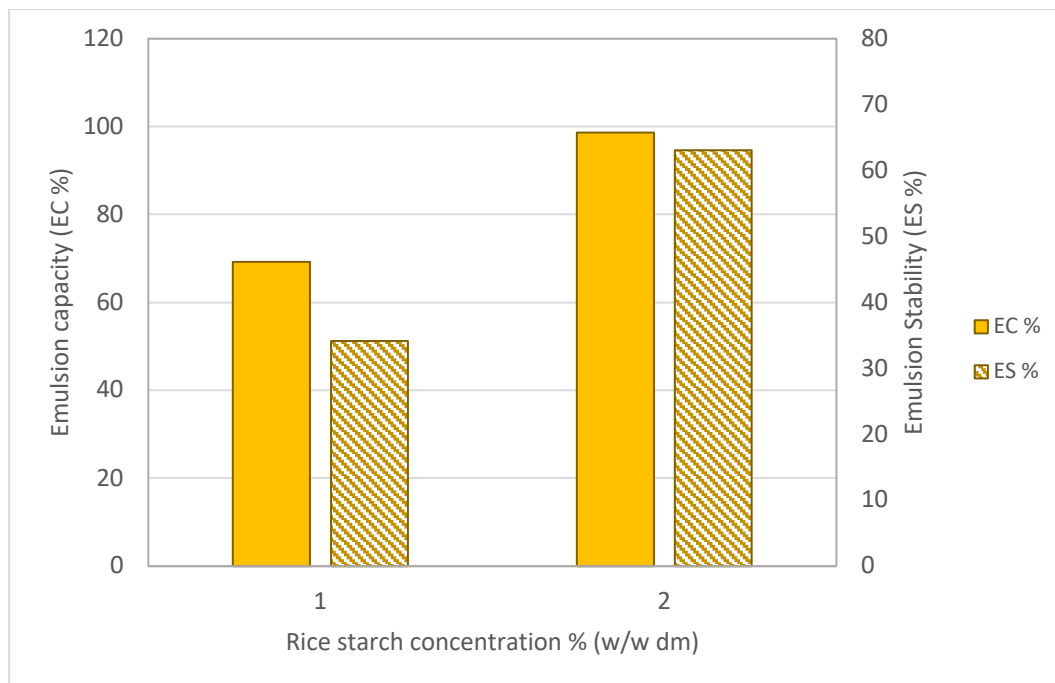


Fig. 9 Emulsion capacity (EC %) (left) and Emulsion stability (ES %) (right) of rice emulsion at different concentration of rice starch.

Table 6 Composition (%) of the rice-derived emulsion

Rice-derived emulsion		
Phase	Component	Quantity (%)
aqueous	Water	86.4
	Rice starch	2.00
	Xanthan gum	1.00
	Sodium benzoate	0.45
	Potassium sorbate	0.15
oleous	Rice Bran oil	10.0

Limitations of the study and Perspectives

In the present study, virgin brown rice flour has been used as raw material for rice starch extraction. However, the one derived from the broken rice milling could represent a valid option because of its higher value as recovered waste. Meanwhile, using a raw material with the poorest fiber content could enhance starch extraction since fewer materials enclosing the soluble polysaccharides should be retained in the sieving step. Commercial rice bran oil has been used as the dispersed phase of the emulsion instead of extracting it from the brown flour used as starting material for the starch extraction. On the contrary, defatting is advisable if oil-containing flour is used since it could encourage extraction yield. In addition, the lack of oil yield information in the current process brings us to make extrapolations from the literature to asset the mass balance and hence the efficiency in terms of emulsion produced per 100 g of raw material. Thus, since rice bran is an early 9% rice grain and contains up to 25% of rice bran oil ([Garofalo et al., 2021](#)), starting from 100 g of rice grains, early 2.3 % rice bran oil may be obtained. Although it would be reasonable to take the emulsifier (rice starch) as the limiting component to evaluate the maximum quantity of emulsion to be manufactured, in the provided example it is produced in significant excess with respect to the oil phase.

Consequently, hemp bran oil extraction yield should be considered the limiting component of the cosmetic prototype. This consideration brings us to estimate that to obtain 100 g of hydrating cream with early 7 % of oil inside, the processing of 300 g of rice flour is needed. However, depending on the desired emollient function, it could decrease by up to one-third. The hydrolysate production can be set as a result of this evaluation, considering a potential functionalization degree varying from 2 to 4 %. The starch produced in excess could be sold as an active cosmetic ingredient, especially if the industry that deals with rice valorization has a medium/large processing scale. However, if broken rice is used as starting material instead of virgin brown flour, a lower processing material should be required, giving a lower amount of high-value product. That also allows the small-scale biorefinery to take advantage of it.

Conclusions

Cereal production covers an important sector of the agro-food industries. Their processing generates a considerable amount of solid waste and effluents rich in organic and inorganic harmful substances, which should be valorized to reduce the environmental threats due to their uncontrolled discharge. The milling phase in the rice production chain is an example of the generation of diversified byproducts such as broken rice, rice bran, and rice husk. The present work provided an innovative way to valorize them through a biorefinery model based on their deconstruction into rice starch, rice bran oil, and rice husk hydrolysate containing active ingredients, which then have been mixed up into an agro-food derived cosmetic prototype with the function of moisturizing body cream. The designed process led to the valorization of 25% of rice husk and 41 % of rice flour, corresponding to almost 36% of the whole rough rice grain, providing a model valid for

all the monocotyledon plant species, like wheat, barley, rye, and oak, but also the starchy dicotyledon grains like amaranth. Depending on the starting material to be processed, varying from rough brown flour to broken rice flour, and the functionalization degree of the final emulsion, ranging from 2 to 7 % in rice bran oil, the proposed model can fit both small and large-scale biorefinery system's need in valorizing the waste generated by the agro-industrial production chain.

Abbreviations

IFC	International Finance Corporation's
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
USDA	US Department of Agriculture
RBO	Rice Bran Oil
EC	Emulsion Capacity
ES	Emulsion Stability
RS	Reducing Sugars
TC	Total Carbohydrates
TP	Total Phenols
TEWL	Trans Epidermal Water Loss

3C Case of study: biorefining of *Punica granatum* (pomegranate) pomace

Introduction

The processing of fruits into dried and canned/frozen slices, powders, wines, and juices, generates considerable quantities of solid waste, estimated at around 20–50% of the processed fruit weight (Banerjee et al., 2017). The common method of disposing of fruit processing waste (FPW) is landfilling or incineration, which, if not properly managed, can cause groundwater contamination and generate methane, carbon dioxide, and pollutants (Manhongo, Chiphango, Thornley & Röder, 2021). In response to the need to minimize waste disposal impacts, FPW is currently used for producing animal feed, compost, and biogas (Sawatdeenarunat et al., 2016). However, its high potential as a bioactive compound source opens the doors to more valuable ways of valorization (Deng et al., 2012). Among the most popular fruits consumed worldwide, citrus, banana, pineapple, and pomegranate generate a higher amount of waste % per processed fruit weight (Manhongo et al., 2011; Banerjee et al. 2017).

Pomegranate is a widespread fruit, with a global production amounting to 1,500,000 tonnes in 2014 (Farag, Abdel-Latif, Emam & Tawfeek, 2014). The edible part forms 38-65% of the weight (Mazaheri, Orooji, Mazaheri M., Moghaddam & Karimi-Maleh, 2021), and the remaining is non-edible. The former comprises the arils, containing the pulp (87-93%, Wetzstein, Zhang, Ravid & Wetzstein, 2011) surrounding the seed. The latter consists of the mesocarp in which arils are grouped. The pericarp (peel) that wraps the whole fruit is made of a thin inner mesocarp, namely albedo, and an outer lignified exocarp (Fig.1).

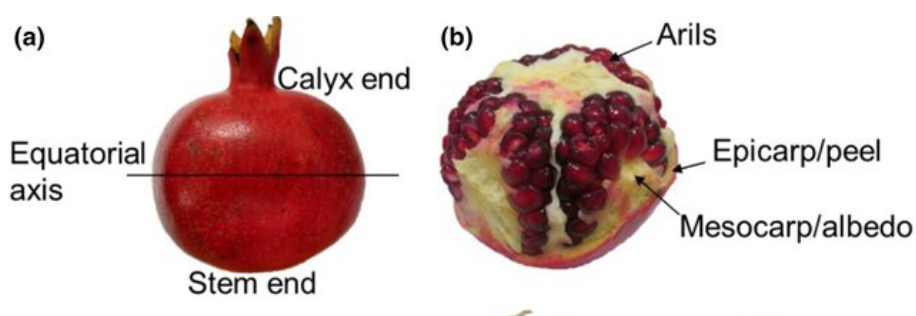


Fig.1 Pomegranate fruit and its main component. (a) outer view, (b) inner section (Mukama et al., 2019).

The processing of the fruit within the food chain is almost wholly destined for juice production by pulp pressing, generating 500–550 kg of waste per each ton of fresh treated pomegranates, namely pomace. The pomegranate pomace consists of pomegranate peel for 75-80% and seeds (El-Shamy & Farag, 2021) and has high water content (around 70–75%) and high organic matter content (about

96% of total solids) (Qu et al., 2009). According to European regulation (Directive 2008/ 98/EC, 2008), this makes it unsuitable for landfills or on-land disposal because of the risk of uncontrolled greenhouse gas production and contamination of watercourses, causing managing issues for the processing factories (Talekar, Patti, Vijayraghavan & Arora, 2018a). Moreover, the waste pomegranate peel (WPP) is also a reservoir of valuable compounds such as phenolics (10-20% w/w), pectin (20-25% w/w), and sugars (30-35% w/w) (Shinde, Mandavgane & Karadbhajane, 2020). Consequently, in the last years, several strategies for the effective valorization of pomegranate waste have been proposed, both by individual processing and integrated ones, to recover functional compounds that could be used for disparate industrial applications (El-Shamy & Farag, 2021). Green and efficient methods have been developed to extract oil and polyphenols from pomegranate seeds, including ultrasound and microwave-assisted reactions (Goula, 2013), (Đurđević et al., 2017), supercritical CO₂ usage (Silva, Ranquine, Monteiro & Torres, 2019), and aqueous enzymatic extraction (Talekar, Patti, Singh, Vijayraghavan & Arora, 2018b). To make full use of pomegranate peel, eco-friendly techniques have been proposed to extract pectin, such as enzyme-assisted reactions (Zhai et al., 2018), green solvents (Pereira et al., 2016), hydrothermal processing (Talekar et al., 2018a). The same applies to the extraction of the phenolics, which was optimized by ultrasound-assisted extractions (Foujdar, Bera & Chopra, 2020) and enzymatic reactions (Nag & Sit, 2018). Biorefinery approaches have been proposed to integrate each pomegranate components processing to recover high-value products while saving energy usage, operational costs, and environmental impact (Talekar et al., 2018a; Pathak, Mandavgane & Kulkarni, 2017; Shinde et al., 2020)

The present work intends to provide a new biorefinery model for the pomegranate waste valorization, based first on the biotech deconstruction of the juicing residue into components of interest and followed by their recombination in the form of an emulsion, which can act as an innovative waste-based cosmetic prototype. The deconstruction process involves entirely enzymatic-assisted extractions (EAE) using commercial enzyme cocktails that hydrolyze specific plant cell wall structures, releasing molecules with cosmetic function into an aqueous solution. The study on pomegranates may represent a model to be applied to each fruit and vegetable residue from juicing, jam manufacturing, and canning.

Results and discussions

The biorefinery approach for the entire pomegranate pomace deconstruction into high-value products and their innovative recombination is summarized in the flowchart (Fig.2), in which mass balances on the dry basis of the processed solids are pointed out. 100 g of dry raw material corresponds to 317 ± 44 g of fresh material. The process begins with the manual separation from the waste pomace of the arils stuck to the pomegranate mesocarp and endocarp from the juicing. Firstly, arils undergo disassembling into pulp and seeds for pomegranate oil recovery. Then, the leftover pomegranate peel is processed by separating it into the inner part mesocarp, and the outer part, namely the pericarp. From the mesocarp, pomegranate pectin is extracted by an enzymatic-assisted reaction; from the pericarp, a solution enriched by bioactive compounds is obtained by

enzymatic disassimilation of the plant cell wall into aqueous solution. Ultimately, all the recovered chemicals are combined into an oil-in-water emulsion that acts as the first biocosmetic prototype waste-derived, with the possible functions of scrubber fluid emulsion and antiaging facial serum.

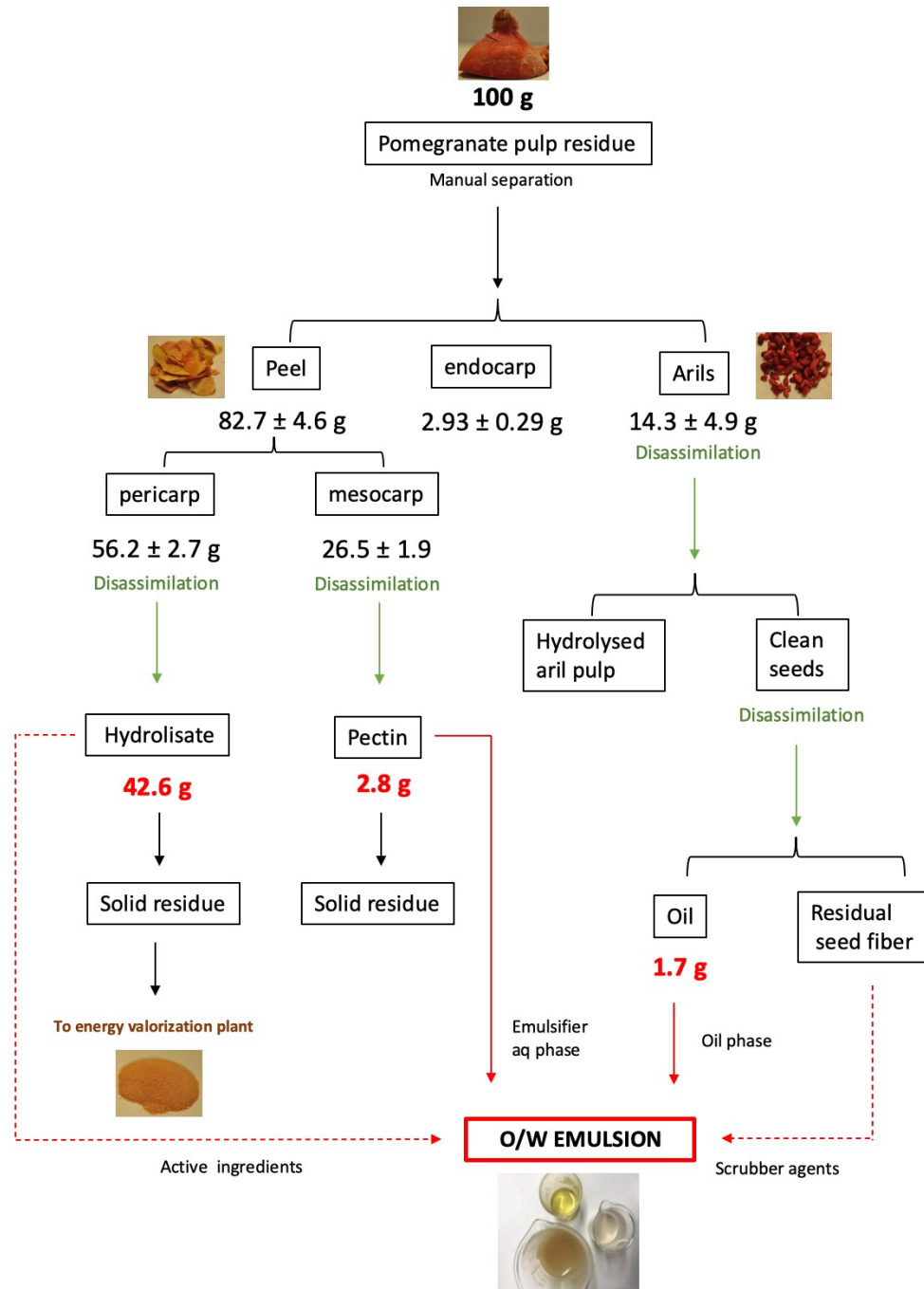


Fig.2 Flowchart of the whole deconstruction and recombination of pomegranate pomace from juicing.

Flowchart guidelines

In the flowchart, the bold squares indicate the commodities used as starting materials and the obtained products in the proposed biorefinery. The brackets state the components which made up the material under discussion. The bold numbers stand for the masses in weight of raw materials (black) and products (red). The solid lines indicate the conversions of food materials in byproducts carried out by biochemical (green line) routes, and their combination into the proposed final product (red line). The dotted lines respond to the same color legend but state the suggested pathways yet to be achieved.

Arils deconstruction

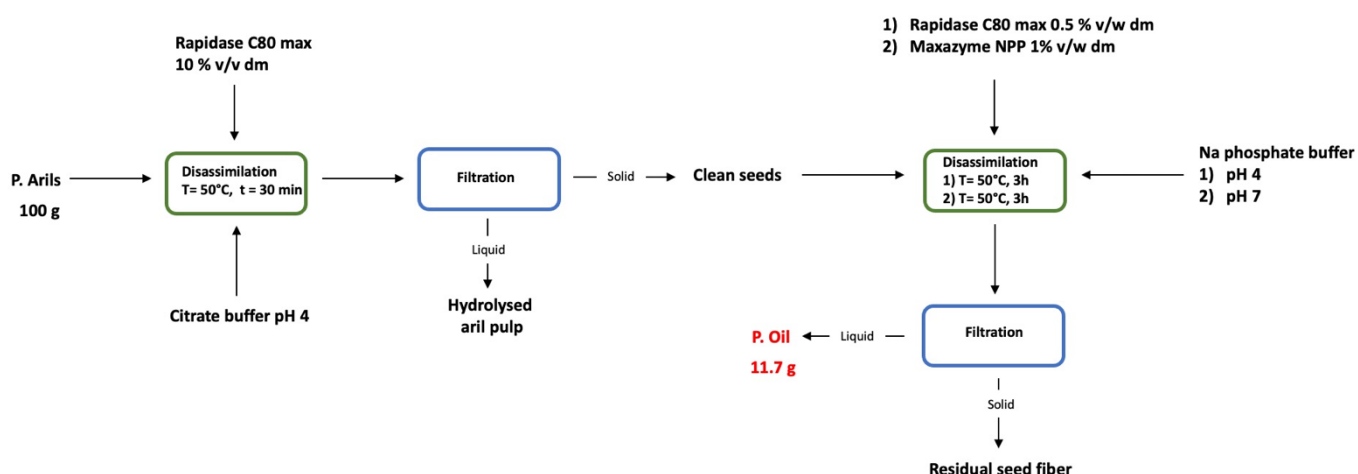


Fig.3 Simplified pomegranate arils deconstruction process.

The arils deconstruction is outlined in Fig. 3. Arils are contained in 20.62 ± 7.03 % w/w of total pomegranate pomace. Once removed from the endocarp and mesocarp, they are disassembled into juice and seeds by hydrolyzing the pulp surrounding the seeds using a 1:10 solution of Rapadase C80 max, a commercial enzyme from *Aspergillus niger* that exhibits a series of pectolytic activities. The enzymatic action causes a loss of turgidity and promotes the detachment of the pulp from the seed. This kind of process occurs naturally during the ripening process of many fruits, involving the synergic effects of polygalacturonase (PG), pectin methyl esterase (PME), and pectate lyase (PL) enzymes (Wang et al., 2013). This reveals the pectic polysaccharide in the tissue surrounding the pomegranate seed, whose rupture leads to pomegranate juice. However, the only literature references are low quantities of arabinose residues in pomegranate juice, in contrast with the high amount of glucose and fructose (Hasnaoui, Wathelet, & Jiménez-Araujo, 2014). Even a prolonged boiling of the arils leads to naked seeds, but the addition of Rapadase C80 max at pH 4 (optimum pH) allows a better cleaning in less time and at a lower temperature. After the seeds cleaning, an

estimation of their content in the arils was possible, resulting in 34.26 ± 11.40 %. The clean kernel then undergoes biotech destructuration into pomegranate oil, soluble proteins, and residue fibers by aqueous enzymatic extraction (AEE). This method allows oil to migrate into an aqueous solution by disrupting the cytoplasmatic membrane of the seed but generally also implies the emulsification of a portion of the extracted oil because of the co-releasing of other surface-active molecules, such as proteins or carbohydrates. A good extraction strategy aims to encourage free oil formation as much as possible (Yusoff, Gordon & Niranjan, 2015) since the emulsion destabilization for oil recovery comes with additional energy use and costs. Talekar et al. (2018b) showed that oil bodies in pomegranate seeds are entrapped into a proteinaceous matrix, which represents a barrier for oil extraction, proving the effectiveness of protease action in breaking down the cytoplasmatic membrane (Talekar et al., 2018b). In the present study, the oil extraction is carried out using a solution of Maxazyme NNP from *Bacillus amyloliquefacien*. This protease exhibits maximum activity at pH 6, which is also close to the isoelectric point (IP) of the majority of amino acids present in pomegranate seeds (Table 1). This could limit oil diffusion into an aqueous solution since proteins would not be soluble, justifying the choice to work at pH 7. The reaction was carried out at different times (Fig.4) at a constant enzyme concentration (1% vol/w). Then the effect of enzyme concentration on the free oil release was evaluated (Fig.5), confirming that the increase in both protease concentration and extraction time decreased the emulsion formation (Wu, Johnson & Jung, 2009).

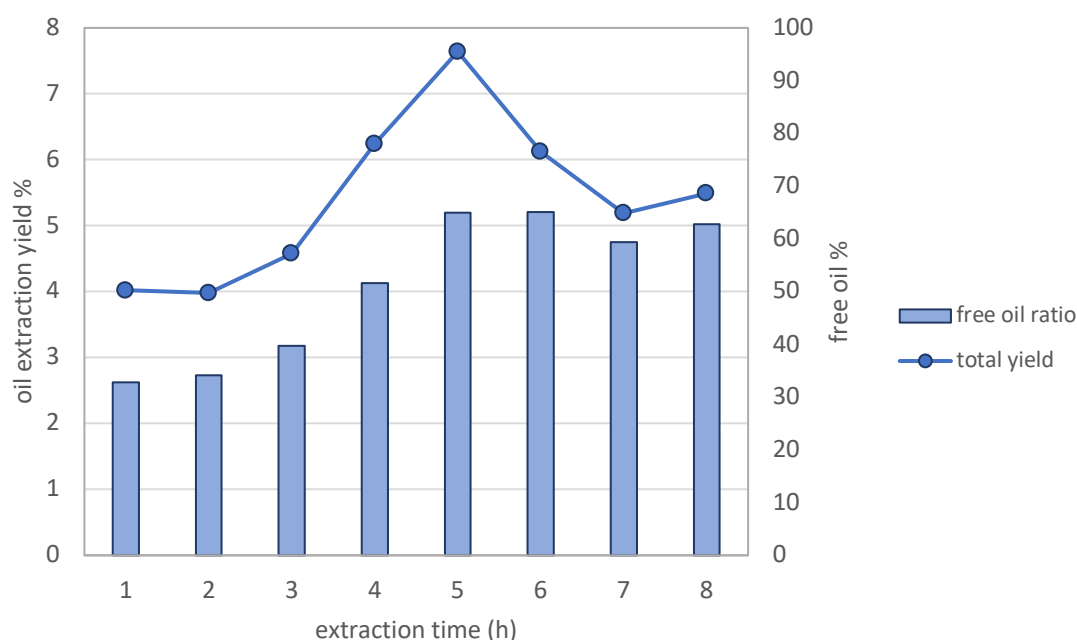


Fig.4 Pomegranate oil extraction % (left) and free oil ratio (right) as a function of extraction time (h) after reaction with Maxazyme NNP 1% vol/w, pH 7, 50°C.

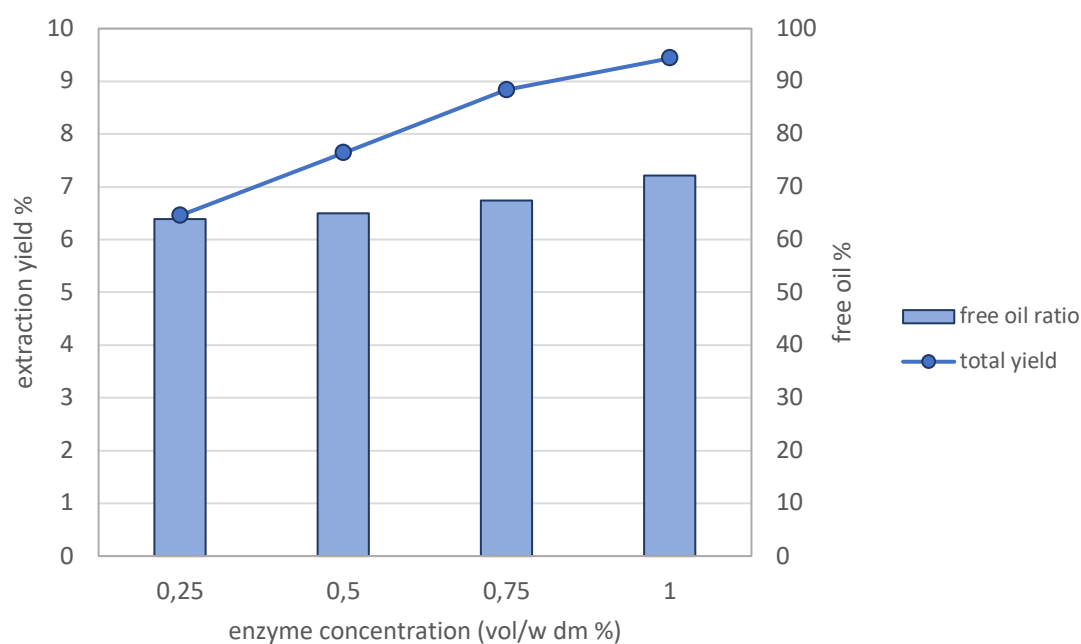


Fig.5 Pomegranate oil extraction % (left) and free oil ratio (right) as a function of Maxazyme NNP concentration (vol/w %) after 5 h of reaction, pH 7, 50°C.

Table 1 Amino acids composition (mg/100 g DW seed) and isoelectric point of pomegranate seeds.

Amino acid	Asp	Thr	Ser	Glu
Content ^(a)	918.08 ± 205.71	345.50 ± 92.75	578.85 ± 145.20	2433.91 ± 701.26
Isoelectric point ^(b)	5.43	5.60	5.70	5.65
Amino acid	Gly	Ala	Cys	Val
Content ^(a)	726.20 ± 163.72	447.44 ± 109.56	389.43 ± 70.79	341.28 ± 76.80
Isoelectric point ^(b)	6.06	6.11	5.15	6.02
Amino acid	Ile	Leu	Tyr	Phe
Content ^(a)	205.72 ± 78.12	723.49 ± 197.49	287.18 ± 83.05	380.35 ± 124.12
Isoelectric point ^(b)	n.d	6.04	5.63	5.76
Amino acid	NH3	His	Arg	Pro
Content ^(a)	166.53 ± 25.85	259.94 ± 57.85	1183.00 ± 352.89	439.72 ± 64.58
Isoelectric point ^(b)	n.d.	7.64	10.76	6.30

(a) = Elfalleh et al., 2012; (b) = Liu et. al, 2004

Maxazyme NNP 2% vol/w dm leads to the maximum yield of 9.4%, 73% of which is in free oil, after five hours of reaction at 50°C. This is relatively lower than what was found by Talekar et al. (22.9 %)

for the same S/L ratio and pH but using protease from *Aspergillus oryzae* (Talekar et al., 2018b). In addition to the different protease origins, the significant variability in the composition of the pomegranate fruit from other regions should be considered (Schwartz et al., 2009); indeed, a content of oil varying from 11 to 24 % was reported (El-Shamy & Farag, 2021). Moreover, since our study lacks a comparison with hexane extraction, we don't have information about the actual oil content or the maximum extraction yield achievable for the treated seeds. To improve our system's extraction yield, we evaluated the additive effect of protease and a carbohydrase enzyme. The choice fell to the pectinase Rapidase C80 max for two reasons. First, to limit the process costs, since it has been already used in the previous step; second, because, belonging to dicotyledon plant species, the plant cell wall arrangement of pomegranate seed could contain a pectic matrix which surrounds and entraps the proteins to hydrolyze (Carpita & Gibeaut, 1993).

Furthermore, Goula et al. showed that the action of pectinase on pomegranate seed leads to higher oil extraction than cellulase (Goula, Papatheodorou, Karasavva & Kaderides, 2018). Before combining the enzymes, we evaluate the contribution extent of the only Rapidase at different times of reaction and enzyme concentration (Fig. 6-7). It is comparable to the protease one, giving a maximum yield of 8.2 g %, with a 72 % free oil ratio for an enzyme concentration of 0.5 % vol/w dm and three hours of reaction. The trend of free oil ratio in function of enzyme concentration, instead, is opposite to the one noticed with protease, showing a linear lowering with the increase of enzyme in the reaction bulk (Fig. 6). After five hours of reactions with protease and three hours with pectolytic enzyme, the extraction yield differs considerably from the controls but shortly afterward both the enzymes seem to be partially inhibited by hydrolysis products (Fig.4, Fig.6).

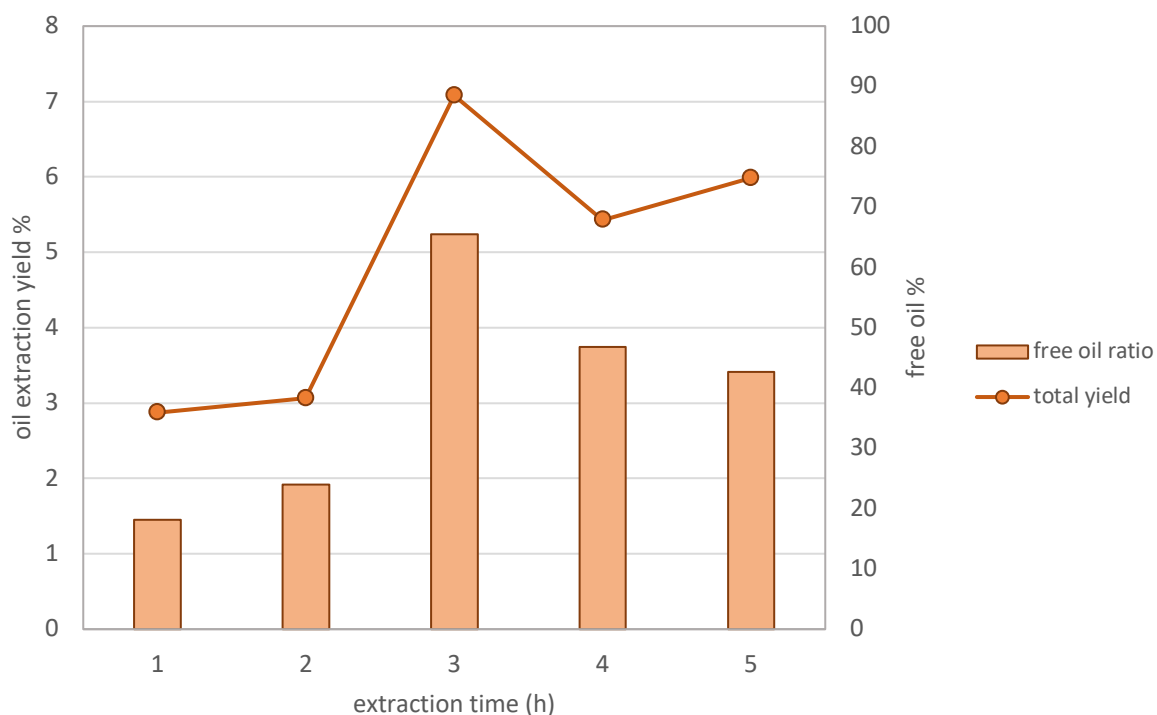


Fig.6 Pomegranate oil extraction % (left) and free oil ratio (right) as a function of extraction time (h) after reaction with Rapidase C80 max 0.5 vol/w, pH 4, 50°C.

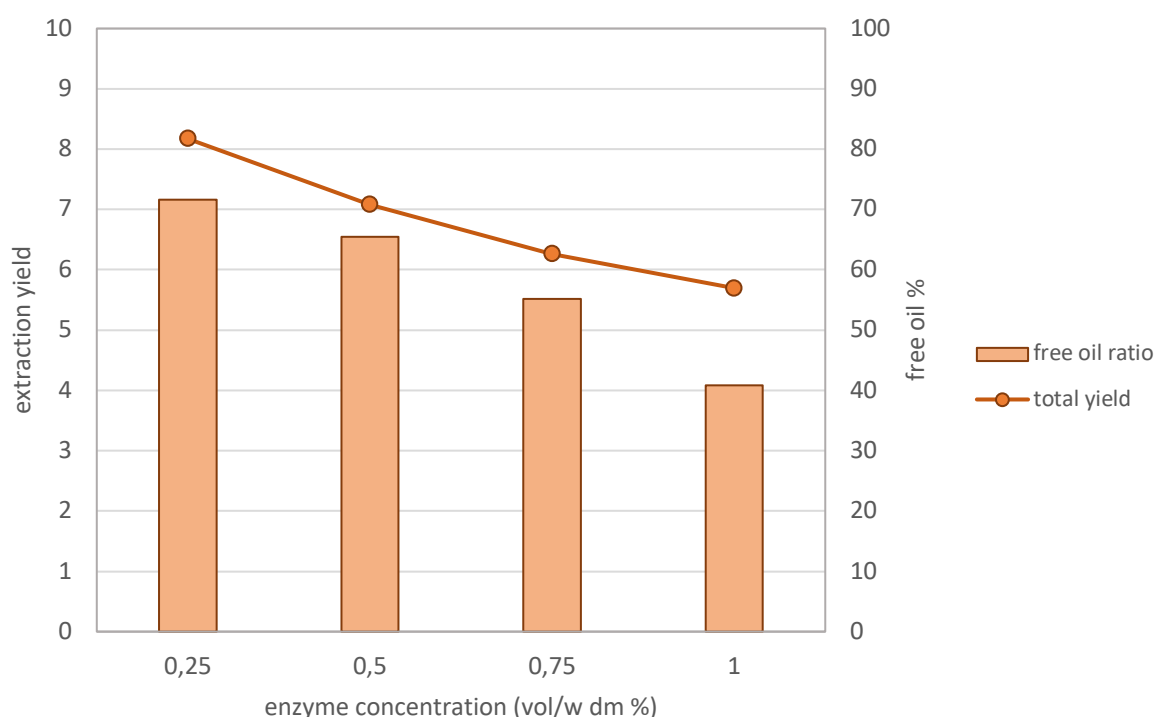


Fig. 7 Pomegranate oil extraction % (left) and free oil ratio (right) as a function of Rapidase C80 max concentration (vol/w %) after 3 h of reaction, pH 7, 50°C.

Looking at the proportion of emulsified oil in the controls, both enzymes seem to encourage the release of free oil from the seeds (Table 2-3). Then the effect of the combined enzymes was evaluated. Interestingly, the sequential addition of the enzymes in the optimum reaction condition previously figured out (Rapidase C80 max 0.5 % vol/w for 3 hours followed by Maxazyme NNP 2% vol/w for 5 hours) leads to a maximum yield of 10.3 % after eight hours of reaction and a decrease in free oil amount (58%) (Fig.8). Instead, diminishing the Maxazyme NNP to 1% vol/w dm in the mixture, causes the reaching of a better result, yielding 11.7 % pomegranate oil (72% of which in free form), after six hours of reaction (Fig.9) pointing that a higher amount of protease added to a solution containing carbohydrates residues, previously released by Rapidase C80 max, could enhance the emulsifying capacity of the solution, at the cost of less efficient oil recovery. The oil extracted will be used as the fat phase of the cosmetic emulsion, whereas the residual exhausted seeds, which accounted for 59.9 % of the clean seeds, could act as a natural scrubbing agent to be added into.

Table 2 Oil extraction yield after reaction with Maxazyme NNP 1% vol/w d.m.

Maxazyme NNP			Control	
Time (h)	Total oil yield %	Free-oil ratio %	Total oil yield %	Free-oil ratio %
1	4.02	32.8	3.77	20.5
2	3.98	34.2	3.69	17.8
3	4.58	39.7	3.32	13.7
4	6.24	51.6	4.02	11.6
5	7.64	64.9	4.09	17.2
6	6.12	65.0	4.37	17.6
7	5.19	59.3	4.28	16.9
8	5.49	62.8	4.29	17.9

Table 3 Oil extraction yield after reaction with Rapidase C80 max 1% vol/w d.m.

Rapidase C80 max			Control	
Time (h)	Total oil yield %	Free-oil ratio %	Total oil yield %	Free-oil ratio %
1	2.87	18.1	3.30	38.1
2	3.06	24.0	3.50	30.8
3	7.08	65.5	4.24	35.5
4	5.42	46.8	4.10	36.1
5	5.98	42.7	4.63	29.8

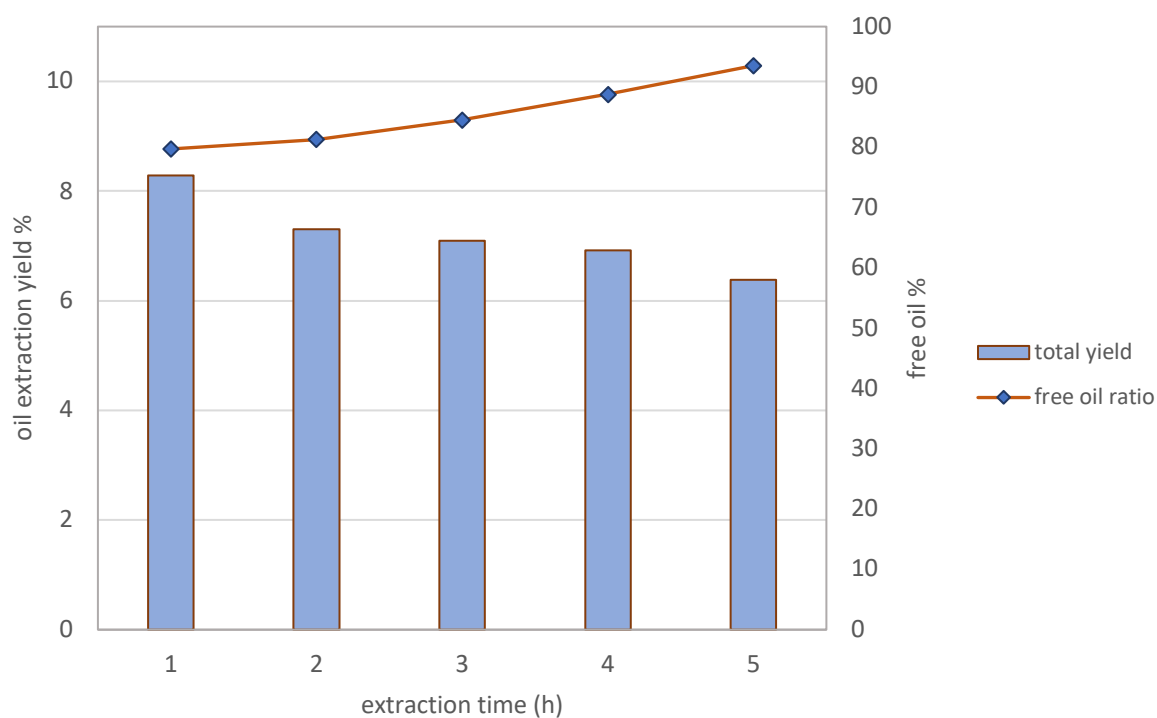


Fig. 8 Pomegranate oil extraction % (left) and free oil ratio (right) as a function of extraction time (h) with Maxazyme NNP 2% vol, pH 7, 50°C, after 3h reaction with Rapidase C80 max 0.5 vol/w, pH 4, 50°C.

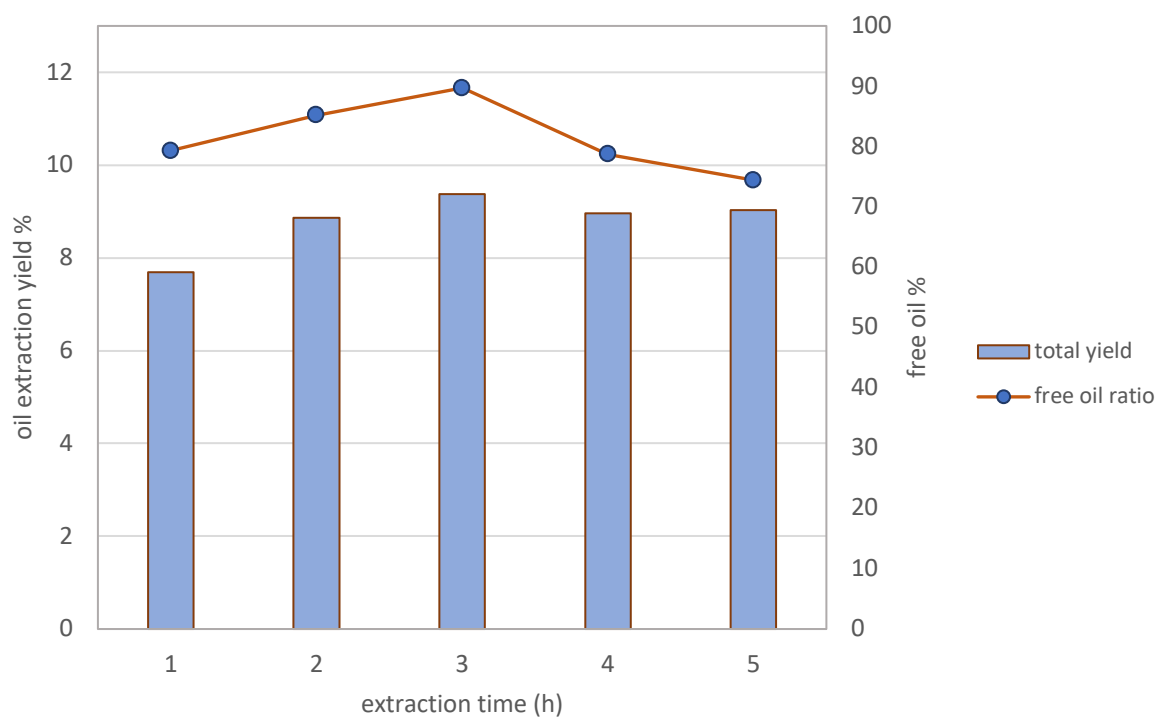


Fig. 9 Pomegranate oil extraction % (left) and free oil ratio (right) as a function of extraction time (h) with Maxazyme NNP 1% vol, pH 7, 50°C, after 3h reaction with Rapidase C80 max 0.5 vol/w, pH 4, 50°C.

Mesocarp processing

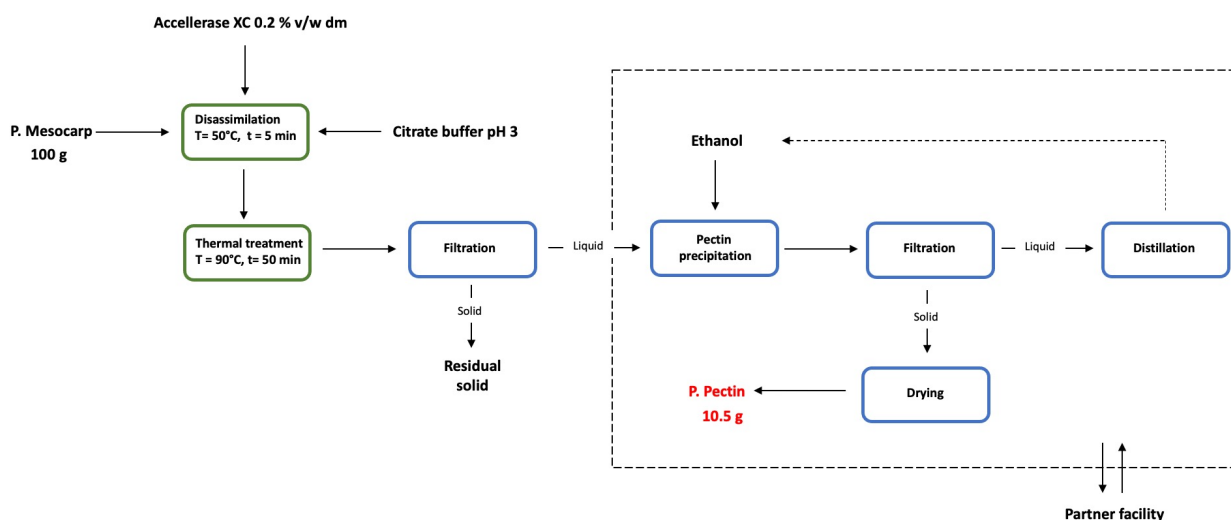


Fig.10 Simplified pomegranate mesocarp deconstruction process

The deconstruction of pomegranate waste goes on with the pomegranate peel processing (Fig. 10). The inner part of the peel, namely the mesocarp, was manually separated from the outer part. It was used as a pectin source, which will act as the emulsifier of the cosmetic emulsion prototype due to its ability to form stable gels and to stabilize emulsions by steric and electrostatic interactions (Ngouémazong, Christiaens, Shpigelman, Van Loey & Hendrickx, 2015). The mesocarp turns out to be 39.57 ± 2.88 % by weight of our sample peels.

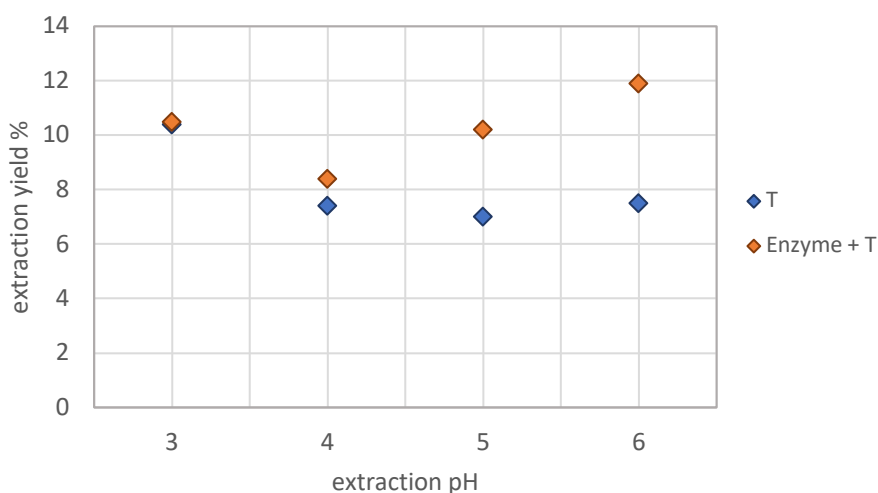


Fig. 11 Pectin extraction from pomegranate mesocarp by thermal treatment, 50 min, 90°C, and enzymatic reaction with Accellerase XC 0.2 % vol/ w dm, 50°C followed by thermal treatment as a function of pH extraction.

The pectin extraction was carried out initially by thermal treatment and sodium citrate buffer as solvent at different pH (3-6). The solid/solvent ratio, reaction time, and temperature were chosen after preliminary trials to have the maximum viscosity of the resulting solution and were kept constant. As pointed in Fig.11, pH 3 seems to improve the pectin extraction, being the more acidic among those tested. Although, in this case, the acid strength is not a parameter that can be taken into account, simply the pH solution can exert a certain influence on increasing pectin yield, as reported by Pereira et al. in the extraction of pectin from the citrus peel with citric acid (Pereira et al., 2016). The diluted salt aqueous solution leads only to the extraction of the soft soluble pectin (Babbar, 2016). To further improve the pectin extraction, we added, five minutes before the thermal treatment, 0.2 % vol/w dm of solution Accellesare XC, an enzyme complex produced by *Penicillium funiculosum* sold mainly for its cellulose and xylanase activity. The small amount of enzyme and the low reaction time were sufficient to cause an increase in extraction yield, especially for pH5 and pH6 (Fig.11). This is ascribable to the diffusion of protopectin into solution, upon the action of the enzymes in hydrolyzing cellulose components of the plant cell wall. In reactions involving enzymes without thermal treatment, pectin detected was lower, especially for higher enzyme concentration and reaction time at pH 3 and 4 (Table 4). This was due not only to the prevalent cellulose and xylanase activity for pH 5 and 6 but also to a side polygalacturonase activity that grows at pH 3 and 4 (Fig. 12). Hence the choice to work with low time and concentration to prevent the reduction of pectin molecular weight and the subsequent less precipitation in the ethanol extraction phase (Faravash & Ashtiani, 2007). The maximum yield was 11.9 % at pH 6 and was achieved with 5 min of reaction with Accellerase XC 0.2 % vol/w dm at 50°C, followed by thermal treatment at 90°C for 50 min. For comparison, Yang et al. obtained 8.5% pectin from pomegranate peel after 80 min of extraction at 86°C and pH 1.7 (Yang et al., 2018).

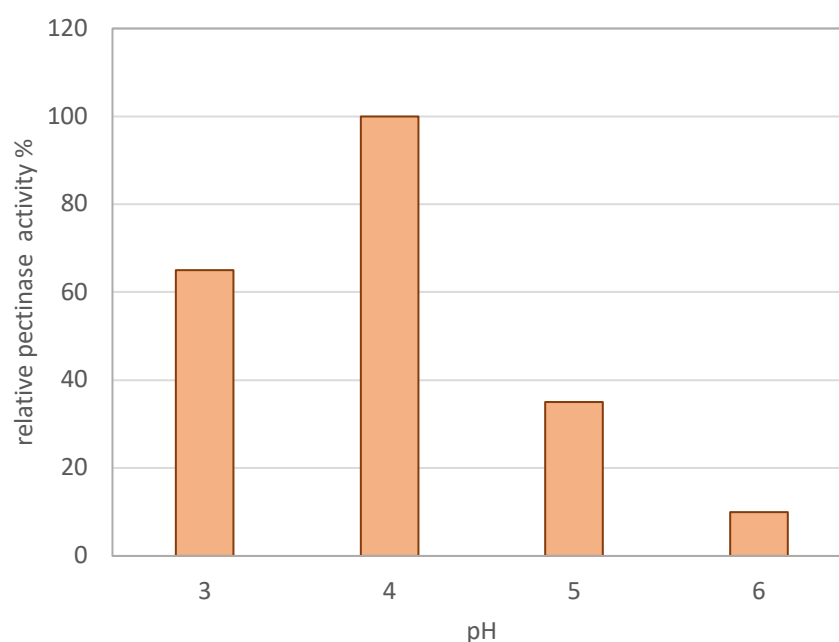


Fig.12 Relative pectinase activity % of Accellerase XC as a function of pH.

Table 4 Pectin extraction yield after reaction with Accellerase XC

Enzyme (vol/w %)	Time (h)	Pectin yield %			
		pH 3	pH 4	pH 5	pH 6
0.02	5	6.3	6.3	6.1	6.9
0.04	5	4.6	5.6	5.5	5.9
0.06	5	3.2	4.7	6.1	5.7
0.02	10	3.6	4.4	6.0	5.6

Considering pectin's final and specific use in this study, the emulsion capacity (EC %) and emulsion stability (ES %) of each extracted pectin were evaluated before choosing it as an emulsifier. Among the pectins obtained at different pH, the one at pH 3 exhibits higher EC % and higher ES %, whereas the one at pH 6 the lower (Fig.13). The interpretation of these results could rely on the impact of the pH of extraction on the pectin functional groups, such as the amount of galacturonic acid residue, which increases at acidic pH (Yapo, Robert, Etienne, Wathelet, & Paquot, 2007). This is reflected in the pH of each extracted pectin solution, which is 4.1, 4.2, 5.2, and 4.9 going up from pH 3 to pH 6 of extraction (Table 5).

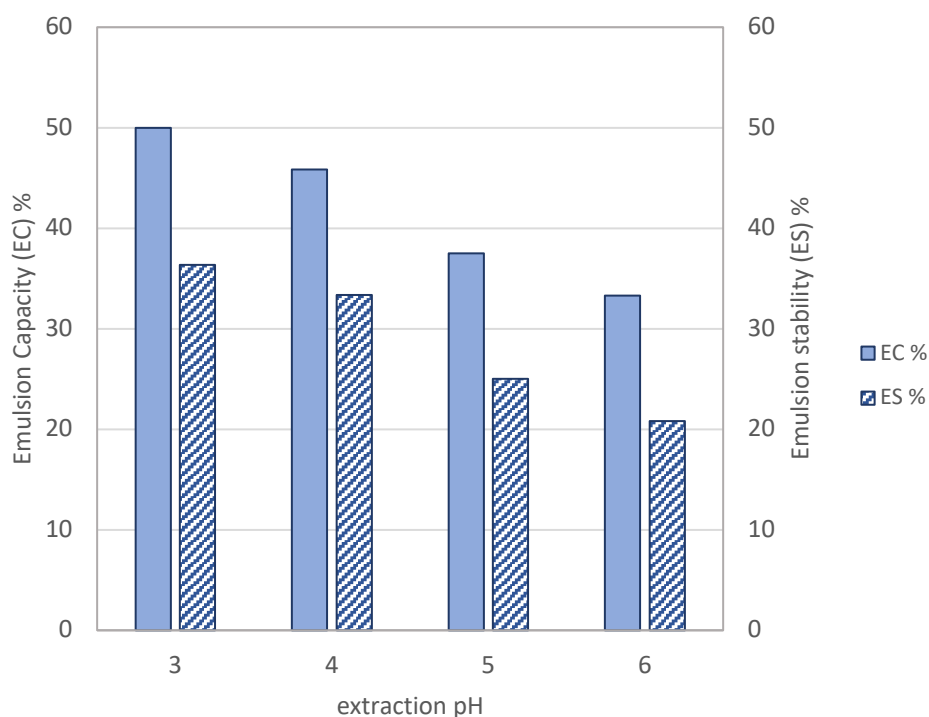


Fig. 13 Emulsion capacity (EC %) (left) and Emulsion stability (ES %) (right) of pomegranate pectin extracted at different pH.

Table 5 Pectin characterization: Emulsion Capacity (EC%), Emulsion Stability (ES %) esterification degree (DE %), pH of 1% w/w pectin solution for each pH of extraction. Comparison between the extraction methods: thermal treatment and enzymatic reaction followed by thermal treatment.

Thermal treatment	Extraction pH			
	pH 3	pH 4	pH 5	pH 6
Pectin yield %	10.5	7.4	7.0	7.5
EC %	8.34	n.d.	n.d.	n.d.
ES %	3.34	n.d.	n.d.	n.d.
Enzyme + thermal treatment	Extraction pH			
	pH 3	pH 4	pH 5	pH 6
Pectin yield %	10.5	8.40	10.2	11.9
EC %	50.0	45.8	37.5	33.3
ES %	36.4	33.3	25.0	20.8
DE %	75.0	75.6	86.0	74.0
pH	4.10	4.20	5.20	4.90

From the estimation of the degree of esterification (DE), the extracted pectins resulted be highly esterified, showing DE of 75%, 75.6%, 86%, and 74% for each extraction pH (Table 5), confirming the investigations on the pomegranate peel pectin by other research groups ([Talekar et al., 2018a](#), [Yang et al., 2018](#)). The extraction methods proposed in the present study also led to DE>75%, which is not reported for pomegranate pectin extracted by hydrothermal process or strong acids ([Talekar et al., 2018a](#), [Yang et al., 2018](#)), probably because of the milder operative conditions used here, which causes a lower degradation degree of pectin chains. The greater emulsion capacity and stability reached by the pectin solution at pH 4.10 and 4.20 can be ascribed to the more balanced distribution of negative surface charges due to the dissociation of the galacturonic acid's carbonyl groups, which occurs for $\text{pH} > \text{pka}$ (3.48). Thus, the electrostatic repulsion could be enough to prevent the oil droplet aggregation ([Ngouémazong et al., 2015](#)) but not so large as to cause intramolecular repulsion between pectin chains, turning into the unfolding of their structure. Indeed, the compactness maintenance in pectin chain conformation could ensure more uniform coverage of oil droplets, stabilizing their dispersion ([Bindereif, Karbstein, Zahn & van der Schaaf, 2022](#)). Pectin at pH 6 is affected by a lower DE and a supposed larger magnitude of zeta potential due to the higher dissociation degree of galacturonic acid ([Bindereif et al., 2022](#)), which turns into a smaller EC and ES (Table 5). These evaluations led to ruling out the use of pectin extracted at pH 6, although it is

obtained with a higher yield. Considering the pectin extracted at pH 3, the one obtained by the enzyme addition was extracted with a yield of 10.5% and showed better EC and ES than the one derived from the only thermal treatment. This can be due to the enzyme action in releasing more hydrophobic portions of pectins, such as acetyl groups, or higher content of protein moieties in galacturonic acid chains, upon the hydrolysis of structural components of the peel cell wall. Both these features enable the greatest pectin absorption on the oil droplets, thereby decreasing the interfacial tension at the oil/water interface (Ngouémazong et al., 2015). Further comparisons were based on the pectin concentration by varying it from 1% to 2% w/w (Fig. 14), and by ranging the ethanol volume (EV) used in pectin precipitation from 1:1 to 2:1 ratio to pectin solution (Fig. 15). The former stabilizes the system by increasing the viscosity of the gel-dispersing medium (Ngouémazong et al., 2015); the latter may cause a more diversified distribution of molecular mass since higher ethanol concentration in the final solution could encourage the complete precipitation of the lower molecular size polysaccharides, in addition to the higher ones. (Xu et al., 2014). The prevalence of high molecular mass could limit the approach of the pectin chain to the oil droplets due to the steric hindrance and intermolecular interactions.

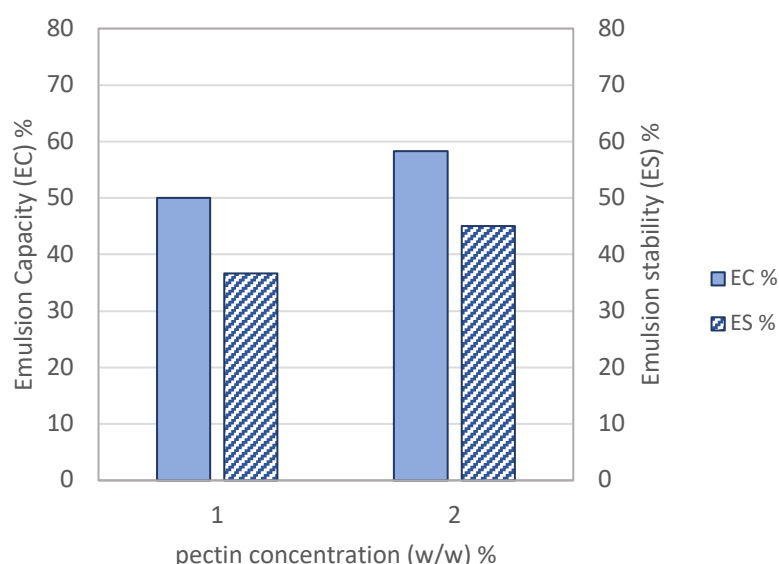


Fig. 14 Emulsion capacity (EC %) (left) and Emulsion stability (ES %) (right) of pomegranate pectin extracted at different pectin concentration.

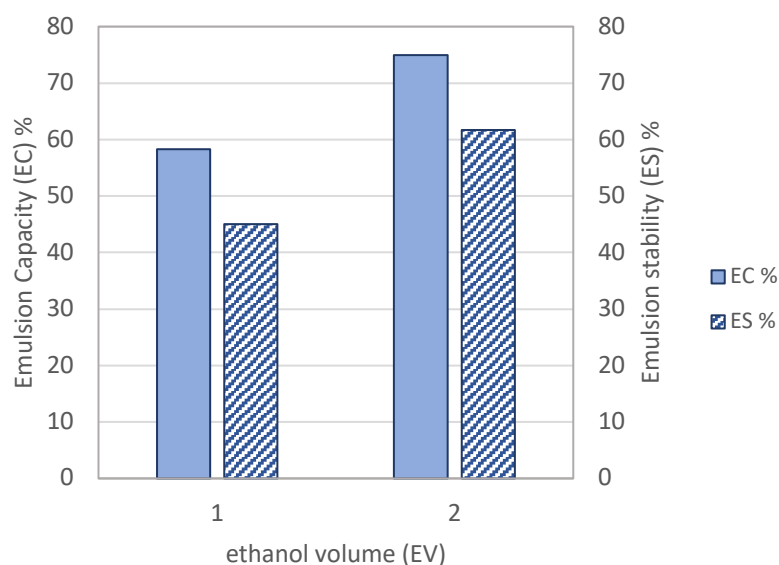


Fig. 15 Emulsion capacity (EC %) (left) and Emulsion stability (ES %) (right) of pomegranate pectin extracted at different ethanol volumes used in the precipitation step.

Table 6 Emulsion Capacity (EC %) and Emulsion stability (ES%) of pomegranate pectin for pectin concentration (w/w) and ethanol volume (EV).

Pectin 1% w/w	Ethanol Volume		
	0	1	2
EC %	50.0	50.0	54.2
ES %	20.0	36.7	41.7

Pectin 2% w/w	Ethanol Volume		
	0	1	2
EC %	n.d.	58.3	75.0
ES %	n.d.	45.0	61.7

The better system that guarantees an efficient emulsion capacity and stability resulted in the 2% w/w solution of pomegranate pectin extracted at pH 3 by enzymatic reaction followed by thermal treatment and precipitated with two volumes of 95% ethanol (Table 6). The extraction of pectin left a solid residue of 18.3 % w/w on a dry basis of the initial pomegranate mesocarp. [Shinde et al. \(2020\)](#) performed the life cycle assessment of the biorefinery process for the sequential recovery of valuable compounds from pomegranate peel and attributed the highest contribution in the overall environmental indicators to the extraction of pectin and precisely to the precipitation step with ethanol. In addition ([Ortiz-Sanchez, Solarte-Toro, Orrego-Alzate, Acosta-Medina & Cardona-Alzate,](#)

2021), in their study on the economic assessment of orange peel biorefinery, showed that pectin separation is one of the most cost-effective operations, contributing to the total process costs by 36%. To provide an alternative solution for the small processing facilities, we evaluated the emulsion capacity and stability of pomegranate pectin solution from the extraction process, bypassing the separation step by precipitation with ethanol. The EC value is comparable to the precipitated pectin (EC = 50%), whereas the ES decreases moderately to 20% (Table 6). Although emulsion stability is an important factor in the assessment of a potentially marketable emulsifier, this evaluation opens the doors to a new perspective for biocosmetic products, which could be “fresh” that is with a short shelf life, if producers are empowered to process the raw material and to market the transformed product on the same site. Another possibility to overcome the impact of the pectin separation step and its economic costs could be to rely on a bigger and closer facility already equipped to carry out this process (dotted square in Fig. 10). This could encourage deploying a local network of industries that collaborate to create new opportunities for the local people and new value for the territory.

Fig.16 shows the viscosity characterization of a 2% w/w pomegranate pectin solution adjusted to pH 5, the one appropriate for the final use. The solution exhibits typical shear thinning with a decreasing trend when the shear rate increases. This decrease is reversible because when the shear rate diminishes up to the starting point, the viscosity backs to the initial value (data not shown). This behavior is a critical factor in not compromising the viscosity of the final product during the emulsification process, in which a shear rate is applied to the fluid.

Pomegranate peel is known to manifest high antioxidant capacity, even more than pomegranate pulp, due to the relevant content of different polyphenols and flavonoids, especially anthocyanidins and hydrolyzable tannins (Pathak, Mandavgane & Kulkarni, 2017). Only the mesocarp is found to contribute from 18% to 30% to the antioxidant capacity of the whole fruit (Skrt et al. 2022). Therefore, in the present study, its extent in the 2% solution of the extracted pectin was evaluated, that was 12352 μmol Trolox equivalents/L, correspondent to 617,6 μmol Trolox/g pectin and 205 μmol Trolox/g peel mesocarp. These are remarkable results respect as found by Skrt et al. 2022 in both water and ethanol extract of pomegranate mesocarp. This finding makes the pectin solution more suitable to act as a biocosmetic emulsion's high-value dispersing and emulsifier phase.

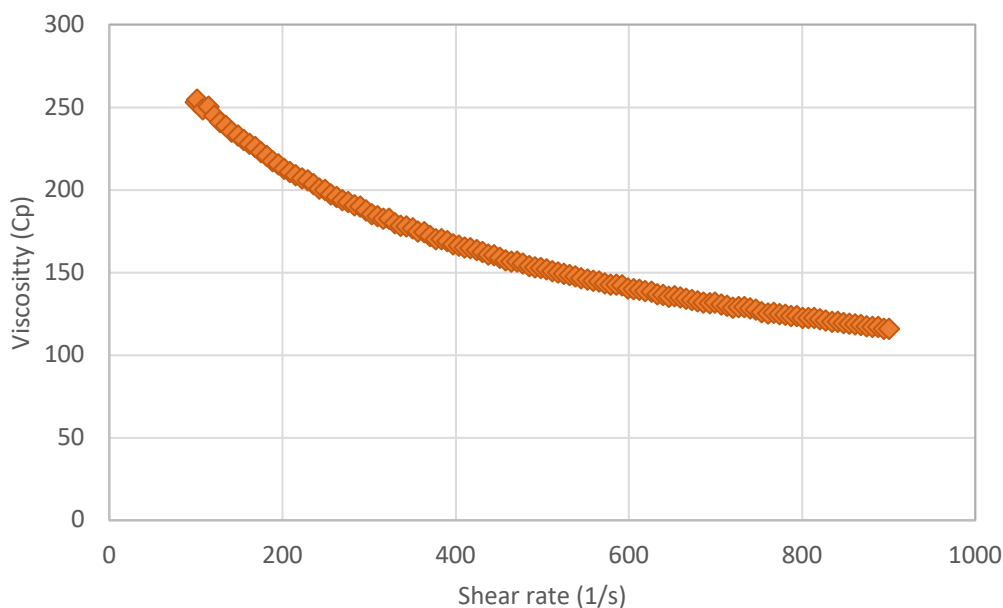


Fig. 16 Viscosity (Cp) as a function of Shear rate (1/s) of pomegranate pectin 2% w/w solution.

Pericarp disassimilation

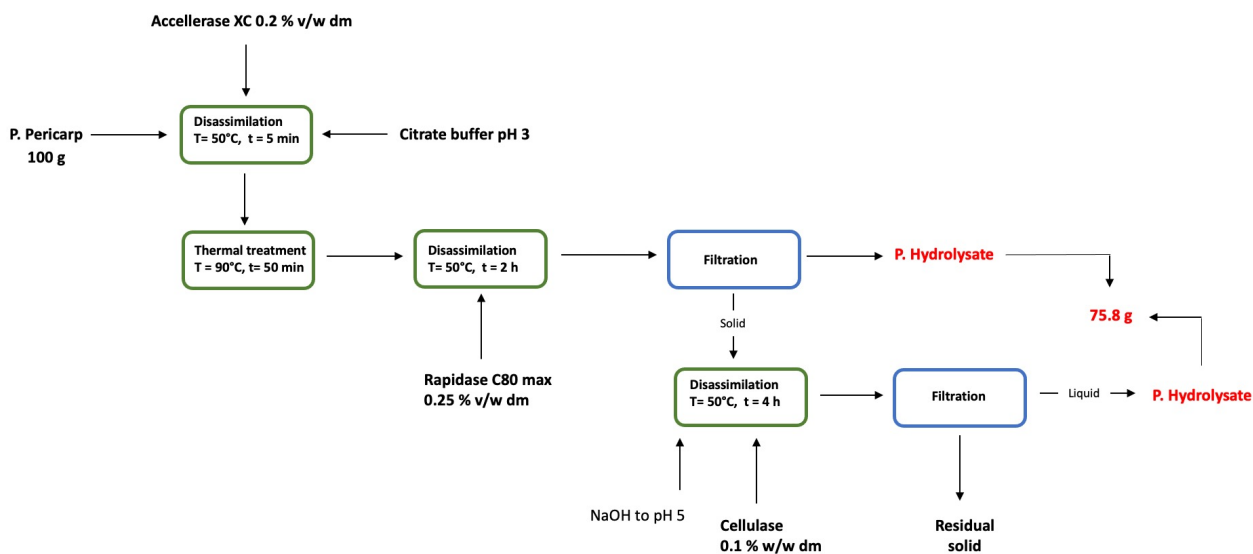


Fig. 17 Simplified pomegranate pericarp deconstruction process.

The last step of the proposed biorefinery process is the enzymatic disassimilation of the pomegranate pericarp (Fig. 17). Pericarp accounts for 60.43 ± 2.88 % of our juicing pomace, comprising an inner part made of a thin layer of mesocarp and an outer layer of exocarp. Its content of fibers in lignin, cellulose, and hemicellulose is reported to vary considerably among the pomegranate species, ranging from 12.2 to 15 %, 11 to 25 %, and 11.6 to 20 % respectively (Skrt et al. 2022).

The first operation is the dispersion of pomegranate peel 6% w/w into sodium citrate at pH 3, following the same method used previously in the pectin extraction, that is, the reaction with Accelerase XC 0.02 % vol/w for 5 minutes at pH 3 and 50°C dm and the subsequent thermal treatment at 90°C for 50 minutes. This pretreatment is finalized to make available the soluble components of the peel and to help the coordination of enzymes to the insoluble components. It leads to the solubilization of early 60% of the treated peel, leaving behind early 40% of solid residue. Presumably, the solution contains pectins and part of hydrolyzed hemicellulose, whereas the solid residue comprises lignin, cellulose, and non-hydrolyzed hemicellulose. Once this two-phase solution is obtained, enzymatic disassimilation of pomegranate peel is conducted to hydrolyze both the soluble and insoluble compounds by sequential enzymatic reactions on the same bulk. The first was performed by adding to the solution Rapidase C80 max at a crescent concentration (Fig. 18) after evaluating the optimum hydrolysis pH (data not shown). The optimal reaction time was found by the maximum amount of reducing sugars released in the solution, indicating the highest hydrolysis degree. The best reaction conditions were achieved using Rapidase C80 max 0.25 % vl/w dm at pH 3 and 50°C for 2 hours, which releases 34.2 ± 2.44 mg/mL of reducing sugars. The resulting solution was then adjusted to pH 5 and underwent the subsequent enzymatic reaction by adding Cellulase from *Trichoderma reesei* 0.1% w/w dm to encourage the hydrolysis of the solid matrix (Fig. 19). The enzyme concentration was chosen after preliminary trials (data not shown). At the end of the process, a solid residue equivalent to 40% of the starting pericarp peel was obtained. Still, as appears from Fig.19, the second enzymatic reaction seems to not proceed properly, probably due to the cellulase inhibition by the reaction products given by Rapidase C80 max. Indeed, after the cellulase reaction, the amount of reducing sugars in the solution doesn't increase over time (33.8 ± 0.5 mg/mL).

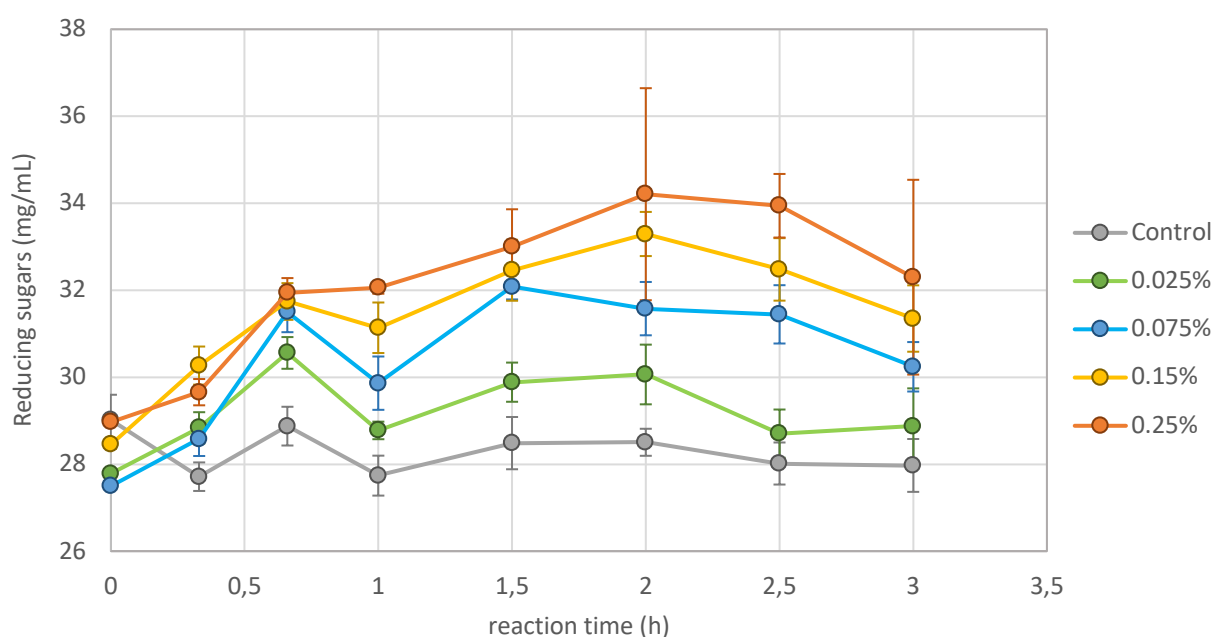


Fig.

18 Enzymatic hydrolysis of soluble solids of pomegranate pericarp carried out with different concentrations of Rapidase C80 max, pH 3, 50°C. The values are the mean for $\pm$ SD for three measurements in the same experiment.

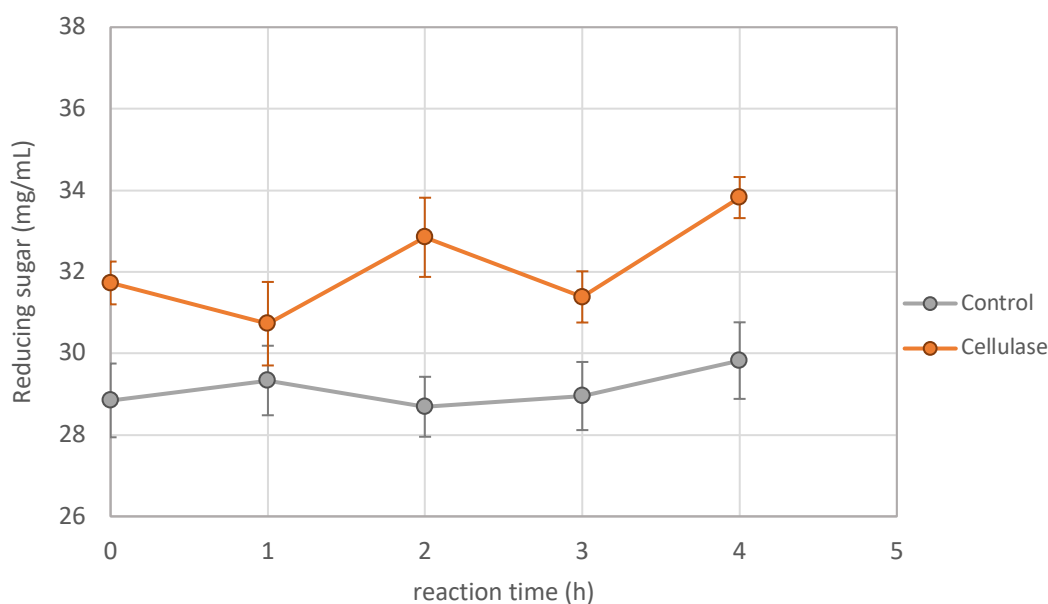


Fig. 19 Enzymatic hydrolysis of insoluble solids of pomegranate pericarp carried out with cellulase 0.1 %w /w dm, 50°C, pH 5, after reaction with different Rapidase C80 max 0.25 % vol/w dm, pH 3, 50°C. The values are the mean for $\pm$ SD for three measurements in the same experiment.

In response to this consideration, two distinct enzymatic reactions were conducted upon the physical separation by centrifuging the bulk deriving from the pretreatment step into a solution and the solid residue. The solution enriched in pectin content was hydrolyzed with Rapidase C80 max, searching for a new optimal concentration for the reaction to occur (Fig.20), which turned out to be 0.25 % vol/w dm as in the previous reaction and leads after 2 hours of reaction at pH 3 and 50° to 39.90 ± 1.01 mg/mL of reducing sugars. This is in accordance with what was found by Pocan et al. (2018). After being dispersed into a buffer solution at a 1:25 S/L ratio, the solid was hydrolyzed by the Cellulase 0.1 % w/w dm (Fig21). As clearly appears from the hydrolysis trend in Fig. 21, the reaction is not inhibited and proceeded to a greater extent, compared to the combined one, leading to 16.48 ± 0.39 mg/mL of reducing sugars after four hours or reaction at pH 5 and 50°C. At the end of the two reactions carried out separately, a final solid residue equal to 24.2 % on a dry basis of the starting pericarp was obtained. Although this method allowed more efficient hydrolysis of pomegranate peel, the additional energy consumption due to the centrifugation operation and the extra use of water required by the second reaction should be considered in the mass and energy balance evaluation of the entire process. To compare the effectiveness of the plant cell wall disassimilations in realizing bioactive compounds, the solution from the combined reaction and the ones derived from the separate hydrolysis of the soluble and insoluble solids were characterized in terms of total carbohydrates content (TC), total phenols (TP), and antioxidant capacity (TEAC) (Table7-8). Pomegranate peel is an essential source of phenolics and flavonoids (e.g., kaempferol, quercetin, luteolin, and proanthocyanidin), complex polysaccharides, hydrolyzable tannins (ellagitannin, punicalin, punicalagin, and pedunculagin), proanthocyanidin compounds, and minerals (Pathak, Mandavgane, & Kulkarni, 2017).

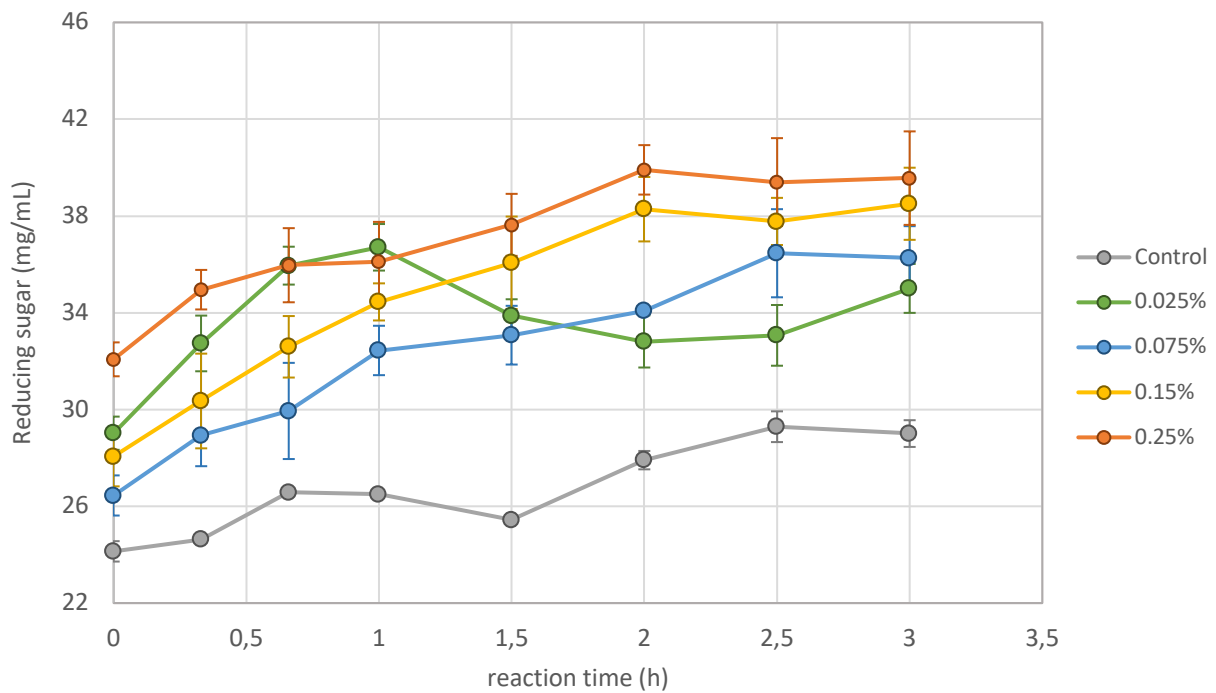


Fig. 20 Enzymatic hydrolysis of soluble solids of pomegranate pericarp carried out with different concentrations of Rapidase C80 max, pH 3, 50°C, after centrifugation of solution derived from pretreatment phase. The values are the mean for $\pm$ SD for three measurements in the same experiment.

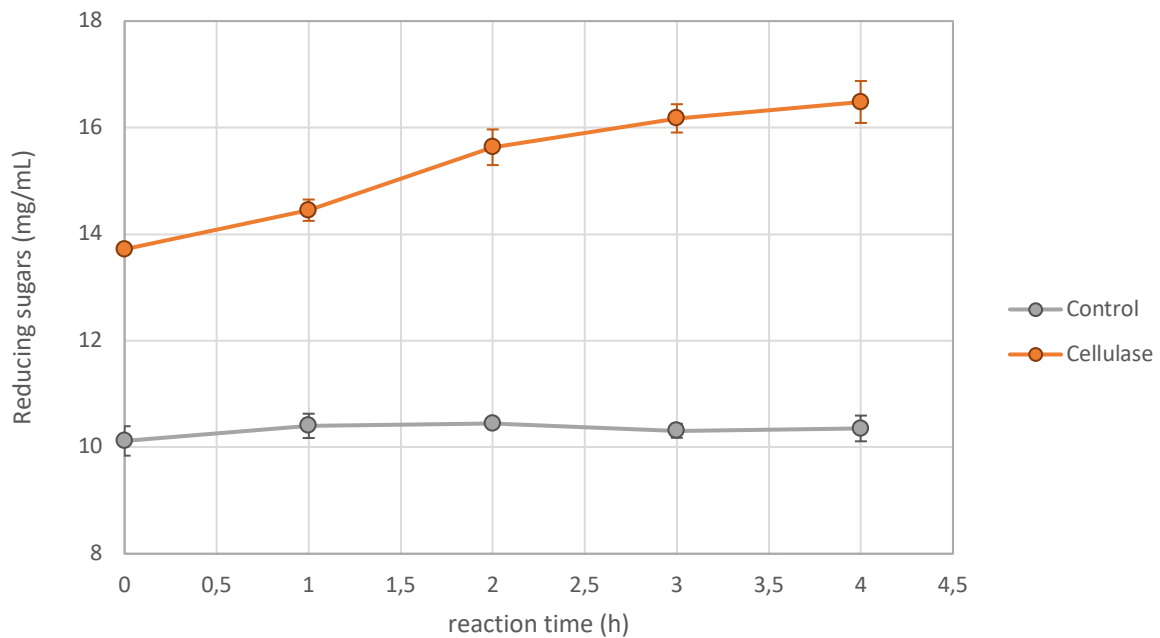


Fig. 21 Enzymatic hydrolysis of insoluble solids of pomegranate pericarp carried out with cellulase 0.1 %w/w dm, 50°C, pH 5, after centrifugation of solution derived from pretreatment phase. The values are the mean for $\pm$ SD for three measurements in the same experiment.

Table 7 Reducing sugars (RS), Total Carbohydrates (TC), Total phenols (TP) and Trolox equivalent antioxidant capacity (TEAC) on pomegranate pericarp (PP) hydrolysate from combined reactions with Rapidase C80max and Cellulase

PP Hydrolysate from combined reactions			
	Control	After Rapidase C80 max	After Cellulase
RS (mg/mL)	28.5 ± 0.3	34.2 ± 2.4	33.8 ± 0.5
TC (mg/mL)	33.7 ± 4.6	39.5 ± 4.4	41.5 ± 4.2
TP (catechine mg/mL)	5.8 ± 0.5	10.6 ± 0.6	10.5 ± 0.6
TEAC (μmol trolox/mL)	35.9 ± 1.1	40.1 ± 1.7	50.4 ± 1.6

Table 8 Reducing sugars (RS), Total Carbohydrates (TC), Total phenols (TP) and Trolox equivalent antioxidant capacity (TEAC) on pomegranate pericarp (PP) hydrolysates from reactions with Rapidase C80max on supernatant and with Cellulase on solid residue.

	PP Hydrolysate from supernatant		PP Hydrolysate from solid	
	Control	After Rapidase C80 max	Control	After Cellulase
RS (mg/mL)	27.9 ± 0.4	39.9 ± 1.0	10.3 ± 0.2	16.5 ± 0.4
TC (mg/mL)	36.3 ± 3.9	48.9 ± 4.9	19.3 ± 0.6	23.7 ± 3.9
TP (catechine mg/mL)	5.9 ± 0.4	12.4 ± 0.4	3.6 ± 0.1	5.8 ± 0.2
TEAC (μmol trolox/mL)	38.4 ± 1.4	43.9 ± 1.4	22.8 ± 0.8	30.8 ± 1.5

As shown in Fig 22-25. each reaction leads to an increase of bioactive molecules as against the relative controls. Among the solutions analyzed, the hydrolysate from supernatant disassimilation shows the highest content of total phenols, equivalent to 12.43 ± 0.41 mg catechine/mL, which corresponds to 316.4 ± 10.44 mg catechine/g pericarp. The product from the sequential enzymatic reactions shows the highest antioxidant capacity, equal to 50.36 ± 1.59 TEAC /mL of hydrolysate, corresponding to 609 ± 19.24 μmol Trolox eq./g peel, early 1000-fold higher than Skrt et al. found in their water extract (Skrt et al. 2022). Such a significant difference could be explained by the efficiency of our disassimilation method in bringing into solution a wide range of antioxidants, being less selective than other solvent extraction, by exploiting not only the chemical affinity but the breaking of the plant cell wall coordinated to the most varied molecules. Amongst the pomegranate peel phytochemicals, high molecular weight polyphenols (e.g., ellagitannins and the pomegranate-peculiar punicalagin) are likely to have protective effects against a wide range of oxidative and inflammatory disorders (Akhtar, Ismail, Fraternale & Sestili, 2015). This property could render the hydrolysate suitable for topical application in treating human skin disease, making it an active ingredient in cosmetics. Further all, thanks to the high content of hydrolyzable tannins and the

ensuing free radical scavenging properties, PP extract can efficiently inhibit lipid oxidation (Akhtar et al., 2015), sustaining lipid preservation also in cosmetic formulation.

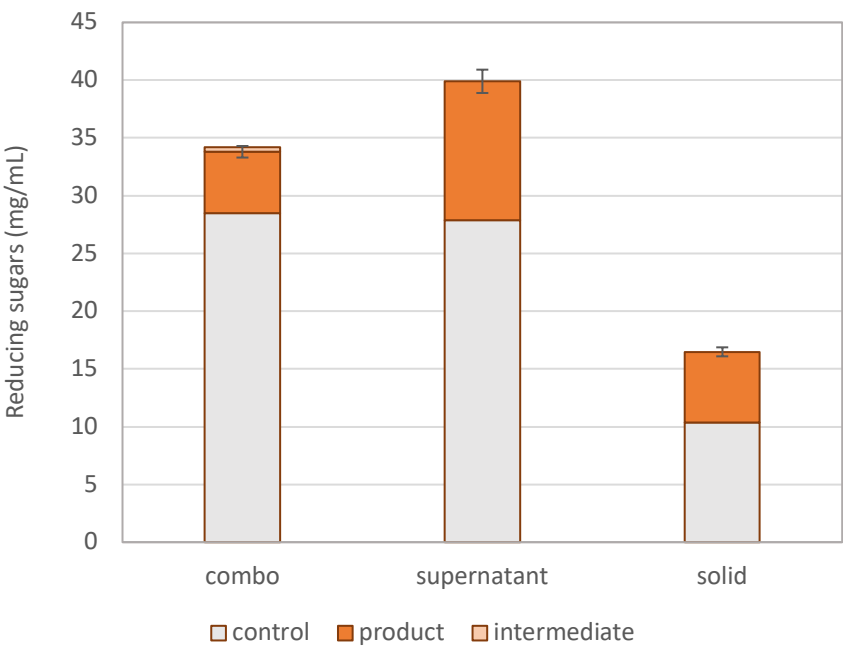


Fig. 22 Reducing sugars (mg/mL) in pomegranate pericarp hydrolysate. Comparison between consecutive reaction with Rapidase C80 Max and Cellulase on the same bulk (combo) and on separated bulk (supernatant and solid). The values are the mean for $\pm$ SD for three measurements in the same experiment.

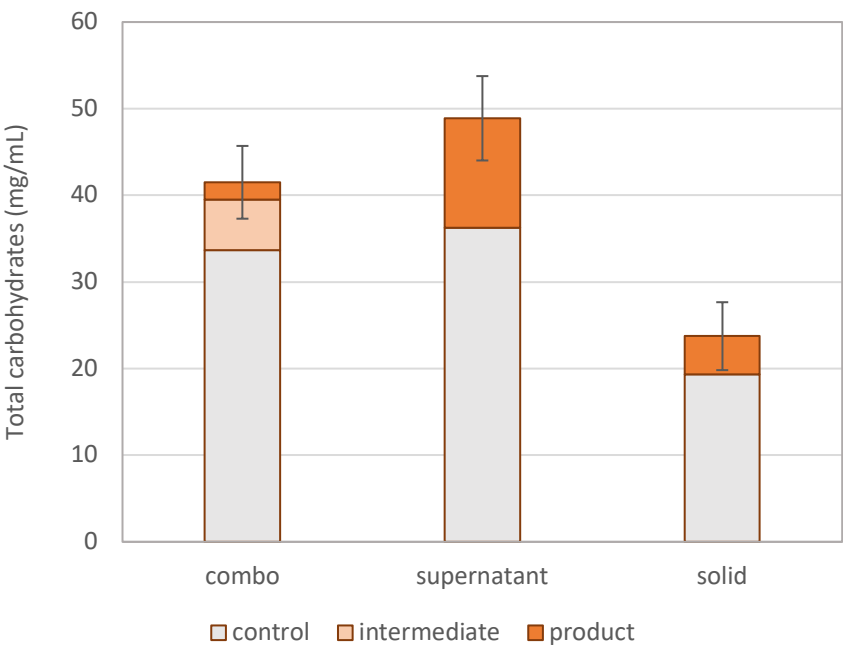


Fig. 23 Total carbohydrates (mg/mL) in pomegranate pericarp hydrolysate. Comparison between consecutive reaction with Rapidase C80 Max and Cellulase on the same bulk (combo) and on separated bulk (supernatant and solid). The values are the mean for $\pm$ SD for three measurements in the same experiment.

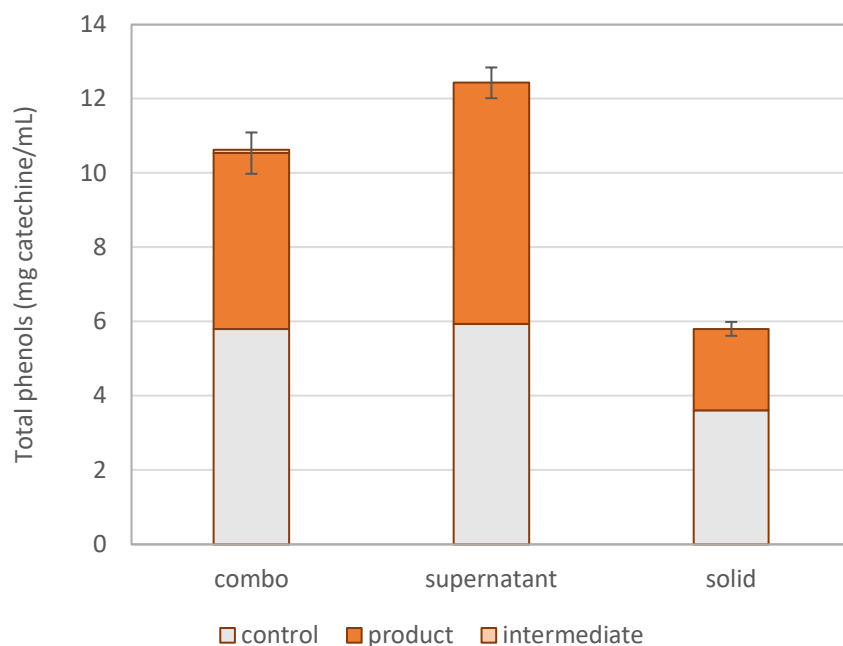


Fig. 24 Total phenols (mg catechine/mL) in pomegranate pericarp hydrolysate. Comparison between consecutive reaction with Rapidase C80 Max and Cellulase on the same bulk (combo) and on separated bulk (supernatant and solid). The values are the mean for $\pm$ SD for three measurements in the same experiment.

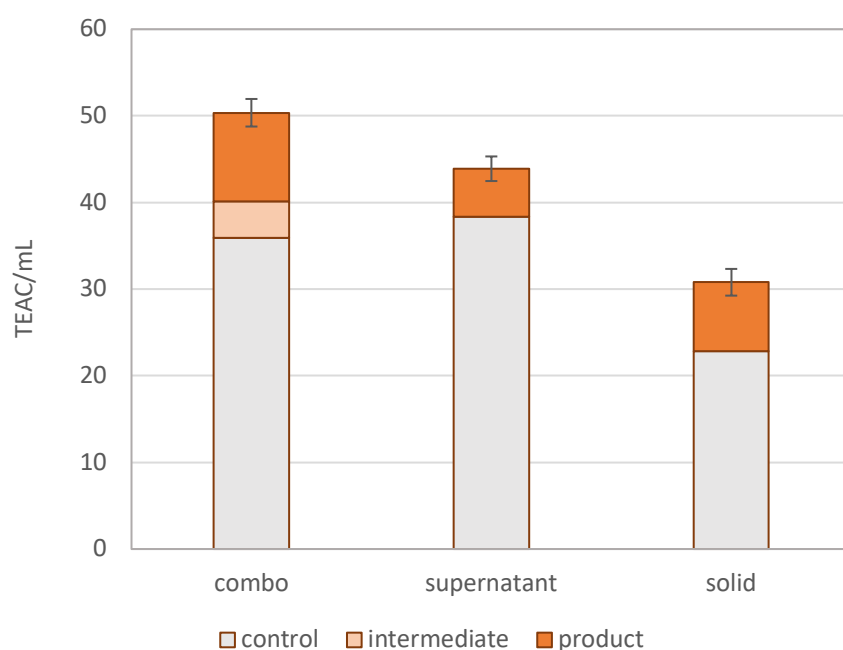


Fig. 25 Antioxidant capacity TEAC Trolox equivalent /mL in pomegranate pericarp hydrolysate. Comparison between consecutive reaction with Rapidase C80 Max and Cellulase on the same bulk (combo) and on separated bulk (supernatant and solid). The values are the mean for $\pm$ SD for three measurements in the same experiment.

As belonging to the available hydrolyzable tannins, acid ellagic was detected by HPLC analysis at 254 nm wavelength with a retention time of 3.9 min and quantified (Fig. 26). It resulted in being present

in 1.90 ± 0.38 mg /g peel, which is higher than what was encountered in the exocarp methanol extract by Fisher et al. (0.64 mg/g) (Fischer, Carle & Kammerer, 2011). Still, it does not represent a remarkable result compared to other studies on the whole non-edible peel extract: 12.1 - 28.3 mg/g (Talekar et al., 2018a), 18 mg/g (Ibrahim, 2010). As the spectrum shows, other compounds are also detected at 254 nm but haven't been identified in this study.

From the more efficient deconstruction of 100 g of dry waste, the early 47.2 % are recovered as solids, 22.9 % are soluble solids lost in the various extraction steps but susceptible to further recovering, reaching a potential valorization of 70% of the starting material, whereas 30 % are solid wastes from the entire process, made mainly of insoluble fibers that could act as biomass for energy recovery.

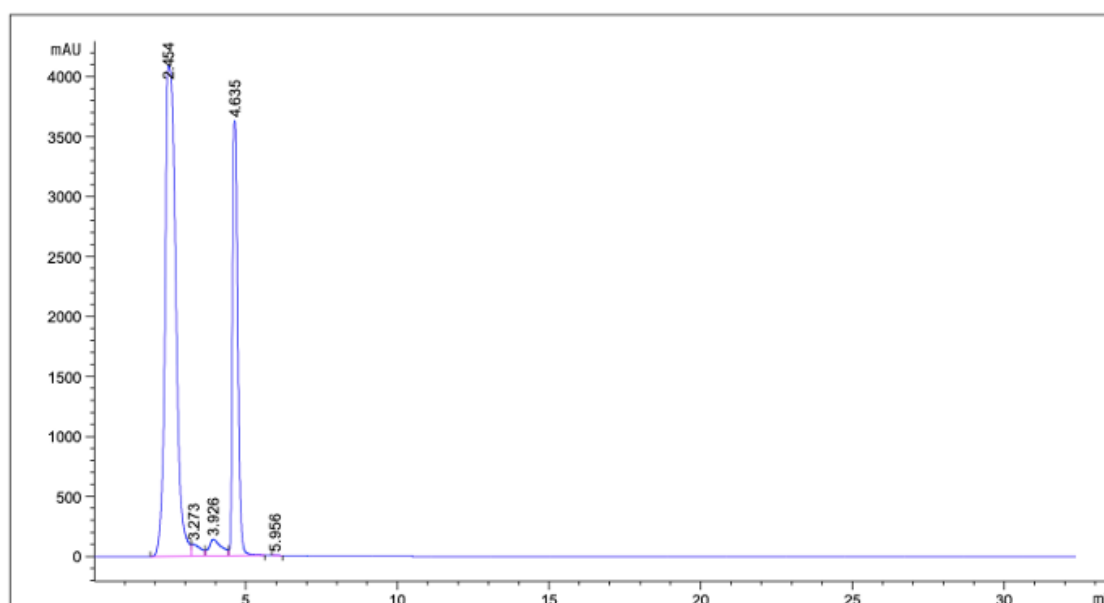


Fig.26 HPLC spectrum of ellagic acid. Retention time 3.93 min. Detection wavelength 254 nm.

Recombination into cosmetic prototype

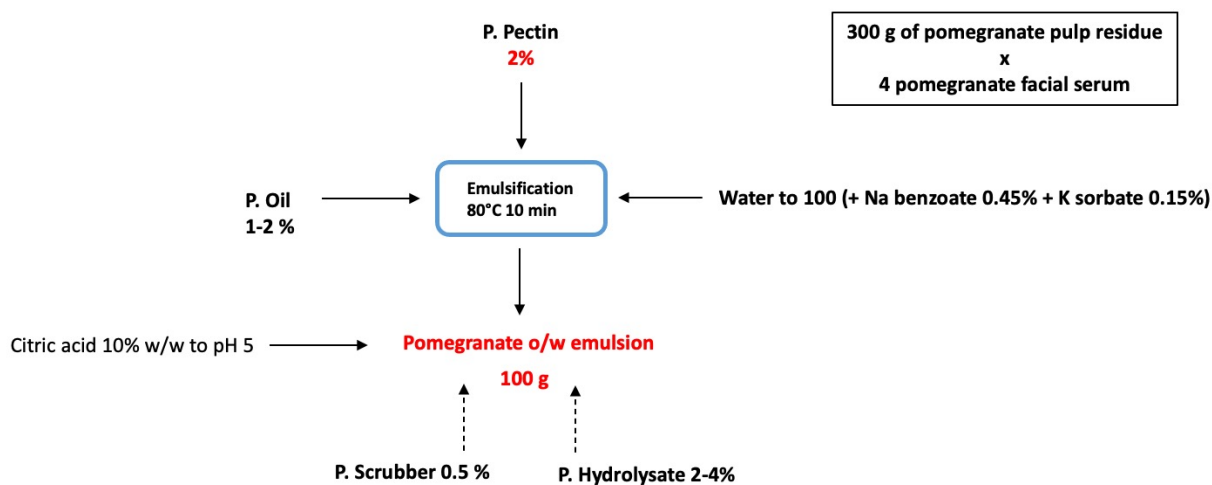


Fig. 27 Simplified representation of the emulsification process to obtain pomegranate-based emulsion. Scenario to obtain four emulsions from 300 g of raw material.

All the processes listed above underlie the new perspective presented by the present study: deconstructing plant cell wall waste into compounds with cosmetic function by the biotechnological process to recombine them into a new cosmetic emulsion prototype able to biomimicry the plant cell wall structures and defenses (Fig. 27). To give an example of the feasibility of this project, I realized an oil-in-water emulsion with cosmetic properties dispersing 10% w/w of pomegranate oil into 2% w/w pectin solution as the emulsifier phase. The emulsion has been spiked with food preservatives and with a low amount of xanthan gum (Table 9), a biopolymer produced through the fermentation process by Gram-negative bacteria of the genus *Xanthomonas*, and used to increase the viscosity and enhance the system's stability (Petri, 2015). The amount of pectin was chosen to confer maximum stability to the system, according to the solubility constraint. The amount of oil was chosen to test the maximum possible seal of the one-emulsifier system. The viscosity of the final emulsion was 1320 Cp, which meets the current standard to be marketed in the form of face serum. For comparison, the same system with commercial emulsifier Tego Care CG 90 (Cetearyl Glucoside) instead of pectin requires 3-fold higher xanthan gum to reach a viscosity of 1040 Cp. The pomegranate-based emulsion was characterized in terms of antioxidant capacity and compared with an emulsion realized by using cold-pressed commercial pomegranate oil rather than the one obtained by the enzymatic treatments described in this study. The emulsion including the oil extracted by enzymes, shows a 3-fold higher antioxidant capacity (Fig 28) than the control, exhibiting 2085.57 ± 75.75 Trolox μmol equivalent / g of emulsion, against 762.76 ± 31.35 TEAC/g of emulsion. This confirms the better ability of the enzymatic method to extract oil components with higher bioactive properties. The reason is that the protease-mediated extraction is based on the diffusion in the aqueous medium of soluble components of the oil seed, which was previously bound in the original structure rather than on the dissolution of the oil (Rosenthal, Pyle & Niranjana, 1996), resulting in different oil composition enriched of phenolics compounds that were bound to

the proteinaceous matrix of the plant cell wall (Yusoff et al., 2015). Pomegranate oil has predominantly C18:3 unsaturated fatty acids such as the α conjugated linolenic acids α -eleostearic and punicic acid, but also C18:2 (linoleic acid) and C18:1, and it is naturally rich in tocopherols and phytosterols (Melo et al., 2016). α -eleostearic and punicic acid exhibit considerable antioxidant power, especially in contrast to lipid peroxidation (Saha & Ghosh, 2009), and anti-inflammatory effects (Costantini et al., 2014); they are used in topical application as lightening agents to contrast hyperpigmentation induced by UV-rays (Ando, Ryu, Hashimoto, Oka & Ichihashi, 1998). γ -tocopherol traps and neutralizes existing free radicals (Michalak, 2022). Phytosterols promote skin barrier recovery (Puglia & Bonina, 2008). Besides these benefits, the cream formulated in this work also contains pomegranate pectin extracted by the enzymatic method, which exhibits its antioxidant capacity, as the current research proves. Pectin is also a polysaccharide capable of forming a protective layer on human skin, preventing water loss from the inner part of the epidermis and ensuring a balanced moisture level.

To conclude, the final emulsion could be functionalized by the addition of exhausted seed fibers 1% w/w to give a facial scrub cream, or the hydrolysate obtained from the disassimilation of pomegranate peel could be added by 2-4 % w/w to give an antiaging serum due to the extract's potential activity as an antimicrobial, scavenger (Akhtar et al., 2015) as well as an antiaging agent. Indeed, oligogalacturonides obtained from pectin enzymatic hydrolysis promote epidermal growth, improving cell-cell interactions and, therefore, dermal-epidermal cohesion (Lebreton-Decoster et al., 2011).

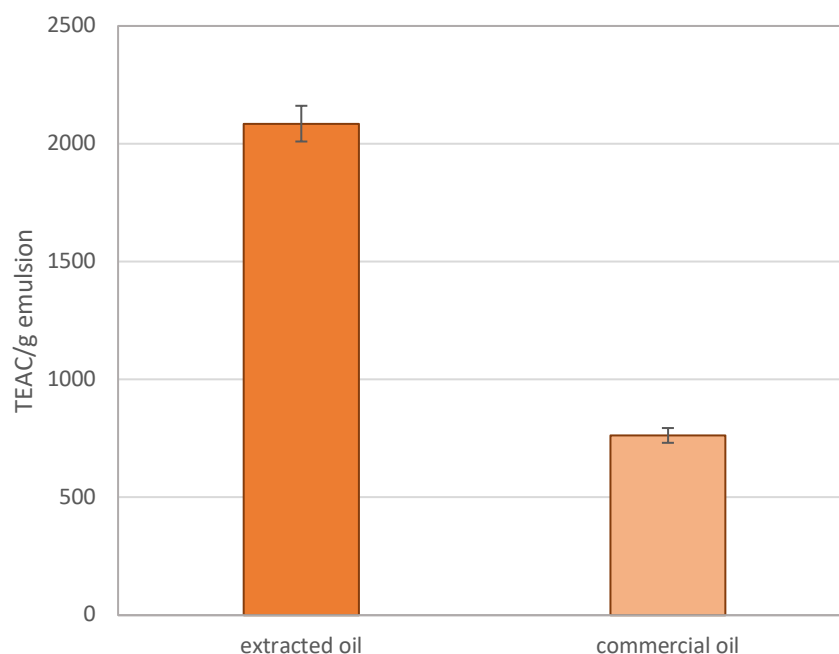


Fig. 28 Comparison of antioxidant capacity (TEAC/g emulsion) between pomegranate emulsion containing extracted pomegranate oil and commercial pomegranate oil. The values are the mean for $\pm$ SD for three measurements in the same experiment.

Table 9 Composition (%) of the pomegranate-derived emulsion

Pomegranate-derived emulsion		
Phase	Component	Quantity (%)
aqueous	Water	86.9
	Pomegranate pectin	2.00
	Xanthan gum	0.50
	Sodium benzoate	0.45
	Potassium sorbate	0.15
oleous	Pomegranate oil	10.0

Limitations of the study and Perspectives

The process proposed in the present study was thought to maximize the conversion of raw materials. However, it is a flexible scheme to be applied at some levels of biorefinery. Some operations, like pectin extraction, could be less affordable for small facilities and may require joint ventures between companies. For the small biorefinery, the disassimilation process of most of the pomegranate peel could be disadvantageous for the expenses and the surplus hydrolysate for the quantity to be used in the final emulsion. Bigger biorefinery, on the contrary, could take advantage more from the hydrolysate production, feeding a specific market, rather than from the low-yield pectin and the oil extractions. Considering the final aim of the study, pectin production is the limiting step since the emulsifier is the component that guarantees the seal of the final product. Therefore, its optimization could lead to higher product per processing starting material. In fact, from the processing of 300 g of dry raw material, equivalent to early 951 g of fresh pomegranate pomace, up to four emulsions could be made, containing 2% of pomegranate pectin and 1.25 % of hemp oil. The oil production could be less constant due to the variable amount of seeds leftover from the juicing, but also small quantities (1-2%) are suitable for an emulsion to be applied on facial skin. The quantity of the hydrolysate to be added to functionalize the serum could be set at will since it is produced in excess respect the needed. In realizing the final product, the preservative system common in the food sector is used, even if it works for a pH equal to or below 5. Instead, the appropriate pH for a facial serum is equal to 5.5, or just over. From this point of view, an alternative and not pH-dependent preservation system should be considered, such as some essential vegetable oil ([Mellencamp, Koppien-Fox, Lamb & Dvorak, 2011](#)).

The emulsion is chemically and physically stable over a long time without showing significative color and odor variation or loss in firmness (data not shown), but to ensure its safety, we consider it essential to perform challenge and patch tests in verifying the preservative system and the compatibility with human skin applications.

Conclusions

The disposal of waste pomegranate peel is becoming a major problem for many processing factories due to the high produced amount per ton of fruit juiced. The present work provides an innovative approach to letting small agro-food industries valorize their wastes. Through the biorefinery deconstruction of pomegranate pomace into potential cosmetic ingredients and their recombination in oil in water emulsion, it is possible to create a cosmetic prototype that effectively biomimicry the plant defense developed in a specific territory by recovering from 47 % to 70 % of the starting waste pomegranate peel. A series of hydrolysis reactions were carried out using commercial enzyme cocktails to mediate the disassimilation of the pomegranate plant cell wall. This led to the recovery of pomegranate oil (1.67 /100 g dry waste), pomegranate pectin (2.77/100 g dry waste), and pomegranate hydrolysate (42.72 /100 g dry waste), which then acted respectively as oil, emulsifier phase, and bioactive ingredient of a face serum prototype, with potential scrubbing, calming, repairing, and anti-aging properties.

The present study offers even a new perspective in proposing a cosmetic that, besides being highly effective and sustainable because a waste-derived product, could be fresh and with a short shelf life, which means fewer energy-intensive processing steps and theoretically fewer preservatives. This is because its value lies in the small-chain processing of local natural resources and its opportunity to deploy a territory-based network between producers and consumers rather than in exploiting even far and exotic resources to favor the profitability of a process and in sustaining a global market that most of the time enlarges at the expense of those who in a territory live and work.

The pomegranate pomace deconstruction and recombination presented in this research is an innovative biorefinery model applied on a dicotyledon plant species that can be employed to valorize every kind of fruit and vegetable processing residue containing oil seeds, pectin, and peels with high concentrations of antioxidants. Other examples are the waste derived from apples, pears, plums, peaches, apricot, pumpkin, etc. Although in the presented case, the disassimilation of the plant cell wall was performed by using commercial cocktail enzymes as biocatalysts, the opportunity to include in the bioconversion plant an upstream unit to produce specific enzyme mixtures, for example, by solid-state fermentation of edible white-rot fungi on the same wastes, opens the way for a more integrated, effective, and affordable process for the small biorefinery to extract value from waste biomass. To conclude, a final opportunity to entirely complete the on-site valorization of agro-food wastes relies on using the residue of chemical valorization, which in this specific case represents 22.9 % of the processed waste, as feedstock in a downstream energy plant by exploiting its high content in reducing sugars monomers. The present thesis also intends to provide an efficient solution for the energy valorization of lignocellulosic biomass, as reported in the following section.

Abbreviations

EAE	Enzymatic assisted extraction
EC	Emulsion Caacity
ES	Emulsion Stalbiity
EV	Ethanol Volume
FPW	Fruit Processing Waste
PG	Polygalacturonase
PL	Pectate Lyase
PME	Pectin Methyl Esterase
PP	Pomegranate Pericarp
RD	Reducing Sugars
TC	Total Carbohydrates
TP	Total Phenols
WPP	Waste Pomegranate Peel

Materials and Methods Chp.3

Reagents: hemp bran was supplied by a local company (Hemp Positive World, Cesena, Italy). Original hemp variety was Futura 75. The Hemp Flour Fior di Loto was purchased from Natura Si (Verona, Italy). Pomegranate pomace was purchased from a private house. Rice flour was purchased from Pam (Pam Panorama, Italy), and rice husk was purchased from <https://www.mr-malt.it/>. Dinitrosalicylic acid (ADNS), Bradford reagent, Folin & Ciocalteu reagent, sodium carbonate, methanol, and sulphuric acid 1N were purchased from Merck KGaA (Darmstadt, Germany). Sulphuric acid 0.1 N and 0.01N were obtained by dilution of sulphuric acid 1N. Xylan from birchwood (Fluka), pectin from apple (Fluka); Galacturonic acid, xylose, starch were purchased from Merck KGaA (Darmstadt, Germany). 2,2-diphenyl-picrylhydrazyl (DPPH) and 6 - hydroxy - 2,5,7,8 - tetramethylchroman - 2 -carboxylic acid (C₁₄H₁₈O₄ ; Trolox) from Thermo Fisher Scientific (Waltham, United States); end-capped C18 cartridge, from Phenomenex srl,(Milan, Italy). Fungamyl Super AX, Accellerase XC, Alcalase 2.4 L, Rapidase C80 Max, Cellulase from *Aspergillus niger*, Maxazyme NNP

Instruments Perkin Elmer UV-Vis Spectrophotometer, Gerhardt Bonn thermoshake incubator shaker, Allegra X30 refrigerated centrifuge, Forced ventilation stove – MAL, Ultra Turrax T25 Homogenizer Basic, Brookfield viscometer, Coolsafe 55-4 freeze dryer (Scanvac, Lillerod, DK).

Citrate buffer solutions 0.05 M

The 0.05 M citrate buffer solutions at various pHs are obtained from different combinations of citric acid monohydrate and trisodium citrate dihydrate. For 1 L of solution the proportions are given in Table 3.1.

Table 3.1: Citrate buffer solutions 0.05 M

pH	Citric acid monohydrate (g)	Trisodium citrate dihydrate (g)
3	8.619	2.642
4	6.168	6.073
4.8	4.529	7.769
5	3.716	9.505
6	1.264	12.937

Phosphate buffer solutions 0.1 M

The 0.1 M phosphate buffer solutions at various pHs are obtained from different combinations of disodium hydrogen phosphate and sodium dihydrogen phosphate. For 1 L of solution, the proportions are given in Table 3.2.

Table 3.3: Phosphate buffer solutions 0.05 M

pH	Disodium hydrogen phosphate (g)	Sodium dihydrogen phosphate (g)
4	0.036	13.781
5	0.360	13.614
6	3.218	12.143
7	15.466	5.836
8	24.970	0.942

ADNS solution

To prepare 250 ml of solution, 75 g of sodium potassium tartrate is dissolved in 50 ml of NaOH 2M and 100 ml of distilled water is added. To this solution 0.25 g of ADNS are added: the dissolution is slow but there is no need to heat or even using the ultrasonic bath. When the ADNS is perfectly dissolved, it is brought to volume up to 250 ml with distilled water and the stored at 4°C and protected from light.

Colorimetric assays (quantitative determinations)***Reducing sugars (RS)***

The method of Bailey et al. (1992) require that 400 µL of sample, eventually diluted in water, are added to 600 µL of dinitrosalicylic acid (ADNS) to reveal the presence of reducing sugars. ADNS reacts 1:1 with the reducing sugar functionality, turning into its reduced form that absorbs at 550 nm. Once ADNS is added, the samples are boiled for 5 minutes, centrifugated for 5 minutes at 13000 rpm, and cooled for 5 minutes before measuring the absorbance.

Total proteins (Bradford)

The Bradford (1976) method for quantitative measurement of the proteins in solution is based on the observation that Coomassie Brilliant Blue G-250, contained in Bradford reagent, exists in two different colors: red and blue. Red color is converted to blue by binding to proteins, and the resulting complex absorbs at the wavelength of 595 nm. To perform the test, 1.5 mL of Bradford's reagent is added to 50 µL of sample; as Bradford's reagent is photosensitive, it should be kept in the dark for 5 minutes after vortex shaking. Then, a spectrophotometer measurement is made at $\lambda=595$ nm against the blank, prepared according to the same procedure but using distilled water in place of the sample. Once the absorbances are obtained, it is converted into mg/mL of released monomer via the relative calibration curves:

$$y = 0.661x + 0.036$$

where y is the absorbance in a.u. and x the BSA concentration of protein standard in mg/mL made with standard solutions at concentrations between 0.25 and 1.5 mg/mL

Total phenols (TP)

For the determination of total polyphenols, the method developed by [Folin & Ciocalteu \(1927\)](#) is considered. It uses as reagent the so-called Folin & Ciocalteu reagent, consisting of a mixture of phosphotungstic acid and phosphomolybdic acid. This determination is carried out in a basic environment to let the redox reaction between polyphenols and the FC reagent occurs. This leads to the formation of tungsten oxides and molybdenum (W_8O_{23} and Mo_8O_{23}) of blue color that exhibit an absorption maximum at $\lambda=700$ nm.

However, the reagent is not specific. Indeed, it interacts also with other analytes such as proteins and nitrogen-containing compounds, which is why interferents are removed by using an end-capped C18 cartridge.

The cartridge is then washed with 2 mL of methanol and conditioned with 5 mL of 0.01 N sulfuric. 1 mL of sample diluted in sulfuric acid 0,1 N is adsorbed. Hence, the cartridge is connected to the 20 mL flask and the elution is carried out with 2 mL of methanol and 5 mL of distilled water. 1 mL of FC reagent is added, into the flasks, and after 5 min, 4 mL of sodium carbonate (10% w/v) is added. Distilled water is used to make up the volume, and then the reaction is allowed to develop for 60 minutes at 30 °C. Finally, the solutions contained in flasks are centrifuged to sediment the carbonate precipitate. The spectrophotometric measurement is made against the blank consisting of only methanol.

To determine the concentration of phenolic compounds, the calibration line of the catechin is used

$$y = 0.0044x + 0.0646$$

where y is the absorbance in a.u. and x is the catechin concentration in mg/L, which was obtained by using standard solutions with concentrations between 20 and 300 mg/L. The concentration of polyphenols is then expressed as mg/L of catechin.

Total carbohydrates (TC)

Determination of total carbohydrates is carried out by the phenol/sulfur method developed by [Dubois, et al. \(1951\)](#), which exploits spectrophotometric analysis following the reaction between carbohydrates, phenol and sulfuric acid. Following this method, phenol in excess is first added so that it complexes the glucose molecules. Then, sulfuric acid is added, which breaks the formed bonds, producing furfural derivatives.

In a reaction tube, 0.2 mL of sample, diluted 1 to 200, and 0.2 mL of 5% w/v aqueous solution of phenol are added. Then, 1 mL of sulfuric acid concentrated at 98% w/w is further added. The tube is closed and shaken vigorously. The reaction is kept running for 30 min at 30 °C until a brown color is obtained. Next, spectrophotometric measurements are made at $\lambda=490$ nm against a blank made with 200 μ L of distilled water to replace the sample.

Once the absorbances are obtained, the total carbohydrate concentration is derived via the glucose calibration curve

$$y = 11.609x - 0.0837$$

where y is the absorbance in a.u. and x is the glucose concentration in mg/mL, made with standard solutions at concentrations between 0.02 and 0.1 mg/mL.

Antioxidant capacity (TEAC)

To determine the antioxidant power, the reaction with the free radical 2,2-diphenyl-picrylhydrazyl (DPPH) in a methanol solution is considered. The reduction of this compound is monitored as absorption reduction at a specific wavelength; in fact, when DPPH reacts with antioxidant species or radicals, absorption is reduced (Brand- Williams, Cuvelier, & Berset, 1995).

The sample is centrifuged and properly diluted. 1.5 mL of 0.1 mM DPPH solution in methanol is added to 0.5 mL of diluted sample. A control is prepared by adding 1.5 mL of DPPH to 0.5 mL solution of distilled water as a reference without antioxidant activity. Once the samples have been prepared, they must be closed quickly so as to prevent evaporation of the solvent, they are shaken and placed in a dark environment for 30 minutes. The spectrophotometric measurements are made at $\lambda=517$ nm against a blank prepared with 1.5 mL methanol and 0.5 mL distilled water: the first at zero time (t_0), the second after 30 minutes of reaction (t_{30}).

By means of such spectrophotometric measurements, the antioxidant power expressed in TEAC units, corresponding to $\mu\text{mol/L}$ of Trolox equivalent as antioxidant compound, is derived.

The absorbance measurements taken at t_0 and t_{30} are converted to DPPH concentrations by exploiting the respective calibration line

$$y = 0.0118x - 0.0522$$

where y is the absorbance in a.u. and x the DPPH concentration in μM .

The residual DPPH (rem) is evaluated as the ratio of final to initial concentration, and converted to Trolox concentration through the calibration line

$$y = -0.0345x + 0.98$$

where y is the absorbance in a.u. and x the Trolox concentration in μM .

The μmol of Trolox equivalent are then derived by multiplying μmol of Trolox from the curve by the total volume (2 mL) and, finally, by calculating the units TEAC/L according to the equation:

$$\text{unit (TEAC/L)} = \frac{\mu\text{mol of Trolox equivalent}}{\text{mL of sample (0.5 mL)}} \times \text{dilution} \times \text{conversion factor} \frac{\text{mL}}{\text{L}}$$

Emulsion characterization

o/w emulsion preparation

The aqueous phase and the oil phase are prepared in two different beakers. The components of each phase, except the xanthan gum, are mixed. The aqueous solution is placed under stirring and

the xanthan gum is added gradually to the center of the vortex. Stirring is continued until a uniform gel without lumps is generated. The gel is heated up to 75°C. The components of the oil phase are all mixed together, the solution is stirred up to the complete dissolution of the substances until a uniform and clear oil phase is obtained. The oil is heated to 70°C. When both solutions have reached the desired temperature, the aqueous phase is placed under vigorous agitation with an immersion blender and the oil phase is added wire type. Once a stable emulsion is reached, the manual stirring is kept until completely cooled.

At this point, it can be functionalized with hydrolysates or fragrances from 1 to 3 %. The pH is raised to the desired pH with citric acid 30% w/w, or with NaOH 10% w/w.

Emulsifying capacity (EC)

Emulsifying capacity is defined as the ratio of emulsion phase volume to total volume expressed as a percentage EC%. The evaluation of the emulsifying capacity of proteins extracted from hemp flour is done through a slightly modified version of the method documented by [Bayar, et al., \(2016\)](#). This method requires that the emulsion, created by the addition of commercial seed oil to the aqueous phase containing hemp sativa protein, is homogenized by using the homogenizer for 1 minute at 19000 rpm. After homogenization is performed, the emulsion is centrifugated at 4400 rpm for 10 min. Then, the volume of emulsion (V_e) is measured against the total volume (V_{tot}):

$$EC\% = \frac{V_e}{V_{tot}} \times 100$$

Emulsifying stability (ES)

The emulsion created is subjected to thermal stress to evaluate its stability under such conditions. After being in a centrifuge for EC% determination, it is placed in an oven for 1 hour at 80 °C, and the again in a centrifuge at 4400 rpm for 10 minutes ([Bayar, et al., 2016](#)). Afterward, the volume of emulsion (V_{es}) is measured again against the total volume (V_{tot}):

$$ES\% = \frac{V_{es}}{V_{tot}} \times 100$$

Viscosity Measurement

The viscosity of the emulsion was measured because this parameter is an indication of the stability of the cream; in fact, the higher the viscosity the more stable the emulsion is over time. Viscosity was measured by means of the HAAKE Viscotester 7 plus using the R5 spindle and at the angular speed of 200 rpm. Three samples of the emulsion were made; one was placed in a refrigerator in order to provide a reference, the second was placed in an oven at 40 °C to promote the occurrence of possible undesirable phenomena such as separation or loss of consistency, the third was placed under light at T amb, to promote photo-induced phenomena. The measurements of viscosity are taken for comparison once a week over two months.

Chp-3A-Case study: biorefining of *Cannabis sativa* L. (hemp) seed cake

Hemp oil extraction

The hemp flour defatting process is done with the method of [Hadnavdev, et al. \(2018\)](#), which involves hexane extraction with a flour-hexane ratio of 1:3 (w/v) for 2 hours. At the end of the two hours, the solution is filtered through a Buchner funnel connected to the vacuum pump. After the evaporation of hexane via a rotavapor, the hemp oil is recovered. The mass was taken after one night in an oven at 40°C.

Hemp protein extraction

The defatted flour is suspended in a water solution adjusted to pH 10 with a solution of NaOH 1 M, in a 1:20 w/v ratio. The solution is kept stirring for 2 hours at 35 °C, then the solid fraction remaining in solution is separated by centrifugation for 20 min at 4400 rpm. The precipitate is brought to pH 7.5 by washing with water, while the supernatant is adjusted to pH 5.0 with a solution of HCl 1M to precipitate proteins. The suspension is left in the refrigerator overnight to facilitate the precipitation. The precipitated proteins are then recovered by centrifugation (4400 rpm, 20 min) and left to dry in an oven ([Hadnavdev, et al. 2018](#)).

Hemp Bran disassimilation

Prior to the hydrolysis, a mechanical pretreatment is performed consisting of grinding the matrix to obtain a fine powder, to which a citrate buffer pH 6 is added in a 1:10 ratio (20 g of bran and 200 mL of buffer) and then placed in an autoclave for 20 minutes at 120 °C This treatment was found to be ideal in promoting the release of the components of interest, as confirmed by [Zanichelli \(2008\)](#). At the end of the heat treatment, the enzymatic reaction is carried out by using the enzymes from the leachates of 1, 2, and 3 weeks of fermentation. To the conical flask containing the substrate and the solvent, the leachate is added. The quantities used are as follows:

- citrate buffer volume pH 6: 10 mL
- substrate mass: 5 g
- leachate volume: 4 mL

Three different conical flasks are prepared containing the leachate from weeks 1, 2 and 3 of fermentation. The respective blanks are also made, three with only the leachates and buffer and another with only the substrate and buffer. The samples are placed in a shaking incubator at 30 °C, at 130 rpm, from one to 17 hours. At the end of the reaction, the hydrolysate is separated from the nonhydrolyzed solid residue by centrifugation, brought to pH 3.5 with citric acid, and added sodium benzoate 0.2% w/V and potassium sorbate 0.1% w/V as preservatives. The hydrolysate is characterized via colorimetric assays to determinate reducing sugars, total carbohydrates, total phenols, antioxidant capacity, as described in-Colorimetric assays-.

To evaluate the progress of hydrolysis, the amount of reducing sugars released into the solution is assessed. To determine the reducing sugars, the supernatant is taken from each sample and placed in 1.5 mL test tubes to make it boil for 10 minutes in order to stop the hydrolysis process. These are centrifuged for 5 minutes at 13000 rpm. The supernatant is diluted with distilled water in a ratio of 1 to 5. Of this, 400 μ L are taken as sample for the reducing sugar assay (-Materials and Method Chp 3, colorimetric assay-).

Chp-3B-Case study: biorefinery of *Oryza sativa* (rice) grain

Rice starch extraction

Starch extraction in this study is performed with protease Alcalase 2.4 L, conducted for 5h at T= 50°C, 200 rpm.

To evaluate the action of the enzyme, several reaction conical flasks are placed containing:

- 50 g of rice flour dispersed in 100 mL of 0.1 M potassium phosphate buffer pH 8 and
- increasing amounts of Alcalase 2.4 (0.1, 0.3, 0.5 mL)

At the end of the reaction, the contents of the conical flasks are filtered through sieves with pores of 75 and 32 micrometers. The solid residue that remains above the sieves is the fibrous part of the flour. The filtrate contains more or less purified "free" starch and starch bound to protein bodies. The two are separated by centrifugation and weighed. The precipitated starch is returned to the solution and subjected to several washes with water until neutral pH. The three separated components are dried in an oven at 40°C and weighed.

Rise husk disassimilation

Pretreatment phase

15 g of rice husk, dried and sieved to 75 microns, were put in decoction for 20 min at 100°C in 100 mL citrate buffer 0.05 M pH 4

Hydrolysis reaction

Substrate: 4-6-9 g solid residue from pretreatment (wet; 1 g wet = 0.3 dry) in 20 mL of solvent from pretreatment

Enzyme: 0.25-0.42-67% vol/g dry matrix of 0.2% w/v solution of Fungamyl Super AX

Reaction: T=50°C, t = 4h, pH = 5, 150 rpm

At the end of the reaction, the hydrolysate is boiled for 10 minutes to deactivate the enzyme. Then, it is separated from the nonhydrolyzed solid residue by centrifugation, brought to pH 3.5 with citric acid, and sodium benzoate 0.2% w/V, and potassium sorbate 0.1% w/V are added as preservatives.

Chp-3C-Case study: biorefining of Punica granatum (pomegranate) pomace

Aril deconstruction

As a first step, the arils were manually separated from the rest of the pomegranate pomace. Complete cleaning of the seed from the pulp was performed by placing arils inside an Erlenmeyer flask with citrate buffer pH4 (in a 1:10 ratio) and 10% v/v Rapidase C80max enzyme (diluted 1:100). The reaction was conducted in a water bath at a constant temperature of 50°C for 30 minutes, keeping the solution under continuous stirring. Upon completion, the enzyme was deactivated by boiling it for 10 minutes. The seeds are dried in an oven at 50 °C. After drying, the seeds were coarsely crushed so as to break up the outer layer enclosing the oil

Seed pre treatment

For each extraction reaction, an initial heat treatment is performed by taking an amount of crushed seeds to have a matrix/solvent ratio of 10% (2.5 g for 25 mL). Then, are added (depending on reaction)

- the phosphate buffer pH 7 in anticipation of the use of Maxazyme NNP enzyme
- the citrate buffer pH 4 in anticipation of the use of Rapidase C80max enzyme.

This solution is placed inside the incubator at 50°C and kept under constant stirring for 15 minutes.

Pomegranate oil extraction

Each solution undergoing enzymatic reaction is previously subjected to heat treatment. At this point, the necessary amount of enzyme is added namely Maxazyme NNP.

The reaction is conducted at 50°C and 110 rpm, in 0.1 M sodium phosphate buffer at pH7 for varying times (1-8 hours and 16 hours) and at varying amounts to have the desiderated concentration (0.25-0.50-0.75-1.0 % vol/w dm). At the end of each reaction the enzyme is deactivated by boiling the sample for 10 min.

Pomegranate oil recovery

When the enzymatic reaction is finished, the solution is transferred inside falcon tubes, then subjected to centrifugation at 4200 rpm, 18 °C for 20 minutes. The presence of three phases is now noted: oily phase at the surface, an intermediate aqueous phase containing the hydrolyzed proteins, and a solid phase at the bottom. Afterward, the falcon tubes are immediately placed in a freezer at -20 °C for 12 h; this makes it easier to physically separate the oily phase from the aqueous phase. Using a spatula, the solidified oil is taken out, placed in test tubes type Heppendorf then again in centrifuge and freezer for 4h. The solidified oil on the surface is removed and placed in a previously calibrated Heppendorf tube. This amount of oil corresponds to the *free oil* extracted from pomegranate seeds.

At this point, the extraction of *stress oil* is performed, which was obtained in the form of emulsion by the enzymatic extraction in the mixture within the falcon tube. The falcon tubes then undergo the following procedure, to promote the emulsion rupture:

1. Incubator for 30 minutes at 50 °C, under stirring
2. Freezer for 30 minutes at -20 °C
3. Incubator for 30 minutes at 50 °C, under stirring
4. Centrifuge for 20 minutes at 18 °C and 4200rpm
5. Freezer for 12 h at -20°C

After the 12 hours in the freezer, the solidified oil is removed with a spatula and placed in Heppendorf tubes then again in centrifuge and freezer for 4h. The solidified oil at the surface is taken and placed in a previously calibrated heppendorf tube. This amount of oil corresponds to the oil present in the emulsion.

Extraction yield

For each oil sample extracted, its yield is calculated as shown below:

$$\text{Yield of free oil \%} = \frac{\text{mass of free oil (g)}}{\text{total mass of pomegranate seeds (g)}} \times 100$$

$$\text{Yield of oil in emulsion \%} = \frac{\text{mass of oil in emulsion (g)}}{\text{total mass of pomegranate seeds (g)}} \times 100$$

$$\text{Total yield of oil \%} = \frac{\text{mass of free oil (g)} + \text{mass of oil in emulsion (g)}}{\text{total mass of pomegranate seeds (g)}} \times 100$$

Mesocarp deconstruction

The pomegranate pomace, already devoid of arils, was separated manually into mesocarp end pericarp. Then the mesocarp was used as a pectin source.

Pectin extraction

Heat treatment

Each heat treatment extraction reaction is performed by preparing a suspension of 6% pomegranate peel matrix/solvent in 0.05 M citrate buffer at different pHs (3-6). This solution is placed in a balloon in a thermal bath at 90°C and allowed to run for 50 minutes.

Enzymatic reaction

Every solution to be subjected to enzymatic reaction is thermostated at 50°C for 15 minutes. The reaction is conducted at 50°C and 110 rpm, in 0.05 M citrate buffer at the different pHs (3,4,5,6) at varying enzymatic concentrations: 0.02, 0.04 and 0.06% vs. solid matrix for 5 minutes and 0.02% for 10 minutes. At the end of each reaction, the enzymes are inactivated by boiling the samples for 5 minutes.

Heat treatment and enzymatic reaction

For every solution at different pHs, the two previous methods are combined: heat treatment followed by enzymatic reaction and enzymatic reaction followed by heat treatment.

In the former case, the solution is placed in a thermal bath at 90°C for 50 minutes under stirring, then, after cooling down to 50°C, the 0.02% enzyme is added and the reaction is let running for 5 minutes. In the second case, the suspension is subjected to enzymatic reaction with enzyme at 0.02% with respect to the solid at 50°C for five minutes, followed by heat treatment at 90°C for 50 minutes.

Following each type of extraction process, the solution is cooled to ambient temperature and centrifuged for 20 min at 4400 rpm to remove the solid residue. The supernatant containing the pectin dissolved in solution is added to the same volume of 95% ethanol, under vigorous stirring to precipitate the pectic component extracted. The solution is left overnight at 4°C to promote greater precipitation. The precipitated gelatinous component is separated by centrifugation, filtered and washed with 95% ethanol three times. The filtered sample is placed in an oven at 30°C until constant weight. Then ground in a mortar and in an electric grinder.

Extraction yield

The % pectin yield is calculated via alcohol precipitation method (95 % ethanol). The pretreated sample is cooled and centrifuged at 4400 rpm for 20 min to separate the supernatant, containing the largest percentage of dissolved pectin, from the solid residue. A known volume of supernatant is added under agitation, drop by drop, into a conical flask containing the same amount by volume of ethanol. After a night in the refrigerator, the obtained solution is centrifuged, and the precipitated solid is filtered, washed with ethanol and dried in an oven at 45 °C to constant weight. The % yield of pectin is calculated as in Equation:

$$\text{Pectin yield \%} = \frac{\text{g of pectin}}{\text{g of initial mesocarp}} \times 100$$

Degree of esterification (DE)

The degree of esterification (DE) is defined as the ratio of galacturonic acid groups esterified and galacturonic acid groups present. To determine this degree of esterification in pectin, an acid-base titration is employed using phenolphthalein as an indicator (USP NF 21, 2003).

50 mg of pectin are dissolved in 10 mL of distilled water; once obtained a homogeneous solution, a couple of drops of phenolphthalein are added. The solution is titrated with a volume of 0.05 M NaOH until the turning of indicator (V_1). Next, 10 mL of 0.05 M NaOH is added and the solution is stirred for 1 h to allow saponification. Then, 10 mL of HCl 0.05 M is added and titrate again with NaOH 0.05 M (V_2) until the turning of indicator. The degree of esterification (DE) is defined by the following formula:

$$DE = \frac{V_2}{V_1 + V_2} \times 100$$

Pericarp disassimilation

The pericarp leftover from the pomegranate pomace deconstruction is grinded, dried in an oven at 45°C up to a constant weight, and sieved to 75 µm to reduce it to a powder.

Heat treatment

A 6% w/V matrix/water ratio suspension is prepared consisting of dried pomegranate pericarp (75 µm powder) and 0.05 M citrate buffer at various pH 3. The sample undergoes pectin extraction as described in -Mesocarp deconstruction-.

Enzymatic hydrolysis

Hydrolysis reactions of polysaccharide structures were conducted by using specific enzyme preparations and monitored in terms of the release of reducing sugars, by performing 0.5-mL samplings every 20 min up to the first hour and every 30 min for the following hours. All reactions with pectin-acting enzymes were conducted:

1. In 0.05 M citrate buffer and pH ranging from 3 to 5;
2. At different enzyme concentrations from 0.025 to 0.25 % V/g dry solid;
3. At T = 50°C, 110 rpm, monitoring the reaction for a time t= 0-4 h.

First case: separation of the supernatant and residual solid

After heat treatment, the solution is cooled and the supernatant and solid residue are separated by centrifugation at 4400 rpm for 20 min. On the supernatant, hydrolysis of the pectic component is conducted at pH 3 with Rapidase C80 Max 0.25% V/g dry solid for 2.5 hours; on the solid residue of the cellulase component, at pH 5 with Cellulase from *Aspergillus Niger* 0.1% V/g dry solid for 4 hours. The resulting bioliquefacts are stored as they are and also combined in a ratio of 1:1.

Second case: combined supernatant and residual solid

After heat treatment, the solution is cooled and the enzymatic reactions are conducted in the same operating conditions as described before in the hydrolysis on the supernatant and residual solid. On the same sample was first performed hydrolysis with Rapidase C80 Max and, after deactivation by boiling the solution, with Cellulase from *Aspergillus niger*.

At the end of the reaction, the hydrolysate is separated from the nonhydrolyzed solid residue by centrifugation, brought to pH 3.5 with citric acid, and added sodium benzoate 0.2% w/V and potassium sorbate 0.1% w/V as preservatives. The hydrolysate is characterized via colorimetric assays to determine reducing sugars, total carbohydrates, total phenols, and antioxidant capacity, as described in-Colorimetric assays-.

4 The biorefinery integration

The proposed biorefinery ends with an energy plant capable of processing the leftover residue from the chemical valorization to renewable electrical energy. This allows the integration of the energy demand of the upstream processes and makes the whole biorefinery energetically sustainable.

4.1 The context: conversion of agro-food waste biomass into electrical energy

Various food industry processes generate a high volume of waste during their processing, packaging, transportation, and storage stages. These wastes are mainly organic in nature and characterized by high biological oxygen demand (BOD) and chemical oxygen demand (COD) and variations in composition and pH owing to seasonal variations and handling processes (Ravindran & Jaiswal, 2016). Such wastes lead to bacterial contamination due to the high-water content and accumulation rates, which entails disposal management problems and the associated cost (Pfaltzgraff, Cooper, Budarin, et Clark, 2013). Biochemical conversion technologies (e.g., anaerobic digestion and fermentation) are generally preferred in treating wet waste, like most agro-food waste. Instead, it is more convenient to treat waste with lower moisture content by dry thermochemical processes (i.e., combustion, pyrolysis, and gasification - Trabold, & Babbitt, 2018; Izydorczyk et al., 2020). Even if the biomass is partially depleted of organic matter, for example, because it underwent a chemical or biochemical process to extract biomolecules, treatments for energy recovery are recommended before composting to extract much value as possible from it, limiting the environmental issue associated with the disposal on land. However, a significant share of the by-products produced during the processing of fruit and vegetables is used as animal feed. At the same time, the remainder is usually delivered to anaerobic digestion plants to produce biogas. This operation often results in a cost for the agri-food company rather than a profit. The handling and processing of these by-products and waste produced in the same production context also have a significant environmental impact regarding the production of greenhouse gases, particularly carbon dioxide.

The biochemical routes are also more affordable for the small agro-food industry that intends to valorize its waste due to their relatively low operative costs. Still, their operative parameters should be calibrated on the waste type because the activity of the diversified bacterial consortium involved in the processes is affected by several factors.

Due to the high content of glucose, biomass can be converted into heat, gas, or direct electrical energy in glucose fuel cell devices. In this way, the chemical energy of glucose can be directly changed to electrical energy with less energy loss. Much work has been done over the past decades (e.g., Calabrese Barton, Gallaway, et Atanassov, 2004; Ghasemi et al., 2013) on glucose as a fuel for direct oxidation fuel cells, including enzymatic and microbial fuel cells (MFC). In MFCs, the chemical energy of glucose is converted to electrical energy by the catalytic reaction of microorganisms, which mediates the oxidation of wastewater at the anode. MFCs have relatively long-term lifetimes

and complete oxidation of glucose (Kim, Zhang, et Heller, 2004). However, serious application problems have prevented their industrial deployment, such as the microbial contamination of the bacterial families responsible for fuel cell operation by the microorganisms already present in the waste materials fed to them. A possible solution to prevent this problem is to sterilize the waste materials fed to the bioreactor, but this would entail costs incompatible with the economic sustainability of the treatment itself. Another limit is the poor ability of microbial species to metabolize all the organic species present, such as high molecular weight polysaccharides. Finally, the scale-up of microbial fuel cells for commercial use presents several challenges. Experimental microbial fuel cells are currently small and must be much larger to compensate for the low power density. However, increasing the size would significantly increase the distance between the anode and cathode, decreasing the efficiency.

Because of the above-described problems of enzymatic and microbial fuel cells, direct oxidation glucose fuel cells that use metal catalysts and alkaline medium have been explored (Chan et al., 2009; An, Zhao, Shen, Wu, & Chen, 2011; Li, Y. S., Zhao, T. S., & Liang, 2009), including alkaline fuel cells (AFCs) or alkaline anion exchange membrane fuel cells (AAEMFCs). Furthermore, the usage of noble metal as a catalyst and alkaline solution instead of the enzymes and microbes revealed the possibility of improving the performance of the glucose fuel cell (Fujiwara et al., 2009; Chan et al., 2009) by enhancing the kinetics of the glucose oxidation reaction (GOR) and oxygen reduction reaction (ORR) (Zhao, Chen, Yang, et Xu, 2009) and overcoming the constraints related to microbic contaminations.

For a direct oxidation glucose fuel cell, the membrane separating the cathode and the anode can be either proton exchange membranes (PEM, typically Nafion) or anion exchange membranes (AEM) (An et al., 2011; Li et al., 2009; Apblett et al., 2005; Zhao et al., 2009). Some reports showed that the performance of the glucose fuel cell could be remarkably improved when an acidic electrolyte is replaced by an alkaline one, i.e., an anion-exchange membrane (Fujiwara et al., 2009). However, its long-term stability in an alkaline environment could be compromised. The main drawbacks of the alkaline fuel cell developed up to now are the high cost of noble metal catalysts and their susceptibility toward poisoning, besides their excellent electro-catalytic activity for glucose oxidation and oxygen reduction (Tan et al., 2009; Ei-Deab, Sotomura, et Ohsaka, 2005; Asazawa et al., 2009).

4.2 The biorefinery integration

- **The Third Thesis Objective** is to provide an efficient method for the direct and non-specific conversion of food waste biomass into electrical energy with low operative costs. The proposal will be displayed by the set-up of a **glucose abiotic biofuel cell** having **carbon-based electrodes**, an alkaline solution as a **catalyst active indistinctly** toward different biomass components, and a design that ensures **continuous electrolyte feeding** with a variable volumetric capacity to reach the **easy scaling up of the system**. The fuel cell will be realized

by **3D printing**, giving the opportunity to progressively adapt the bioreactor to the experimental needs until satisfactory performed are reached.

- **The strategy** content has been the object of a **pending patent on behalf of the University of Bologna**, whose formal request was sent on Dec 1, 2022.

The object of the invention ([Setti & Maggiore, pending patent](#)) concerns the set-up of a new energy plant that allows a low-cost and non-specific conversion of waste biomass into electrical energy. This requires the design of a **new abiotic glucose biofuel cell** and its realization by **3D printing**. The summary of the invention is reported below.

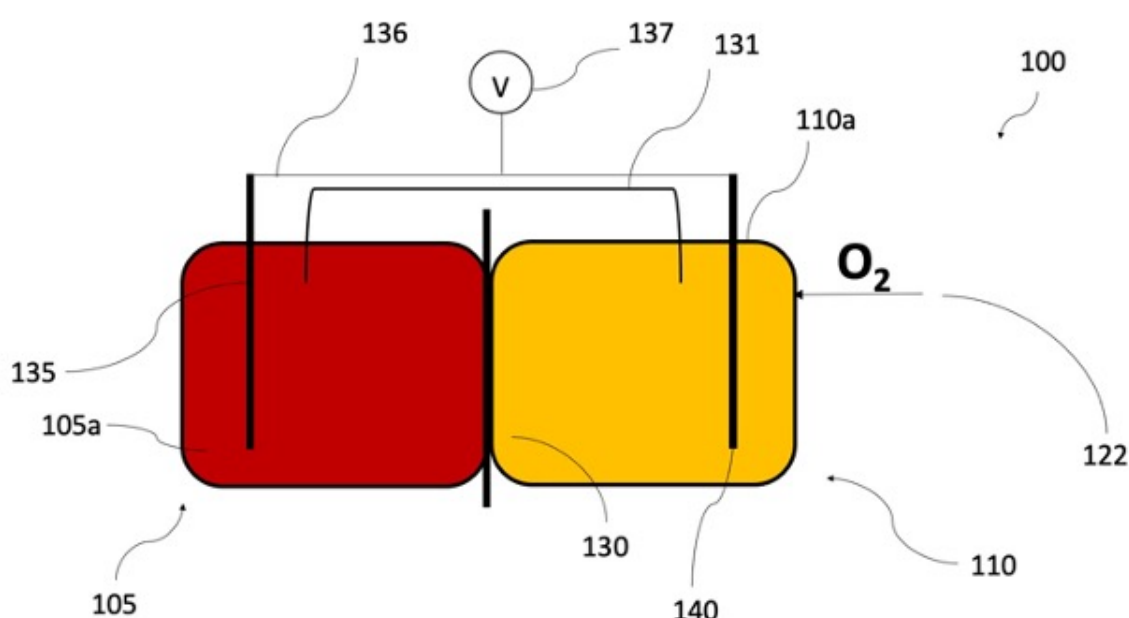


Fig.1 Simplified representation of the proposed abiotic glucose biofuel cell (adapted from [Setti & Maggiore, pending patent](#)).

The abiotic fuel cell presented in Fig.1 (labeled as 100), and in more detail in Fig.3, is the object of the invention under patent request evaluation ([Setti & Maggiore, pending patent](#)). It comprises an anodic half-cell 105 and a cathodic half-cell 110, constituting two tanks (105a and 110a) containing electrolytes. They can be separated by an ion-selective membrane 130 and/or by a salt bridge 131 and comprise two electrodes 135 and 140 immersed into the electrolytic solutions and linked to an electric circuit 136 in which passes the current generated by the cell, and to a voltmeter 137 that

measures the potential difference. Thus, the electrons released by the oxidation of the biofuel occurring in the anode are collected by the electrodes and flow from the anode to the cathode through the low-resistance circuit. At the same time, the ions can move across the ion-selective means between the chambers to establish the system electroneutrality.

In the current invention, biofuel refers to non-fossil recovery organic matter, such as waste plant or animal-derived and/or industrial or urban wastewater. It preferentially derives from the agro-food and agro-industrial supply chain. Preferably it is enriched in carbohydrates comprising monosaccharides, disaccharides, and polysaccharides, but it can also include lipids, preferentially triglycerides, fatty acids and/or their derivatives, and proteins. Among the carbohydrates, glucose, fructose, arabinose and sorbose are preferred.

The anode contains an alkaline solution with a pH between 11 and 14, preferably between 12.5 and 14, in which biofuel is dispersed to give a concentration between 250 g/L and 1000 g/L. The alkaline solution comprises an inorganic base, Arrhenius or Brønsted-Lowry type, that is a strong base with pK_b equal to or lower than 5. It is preferentially an oxide or hydroxide like sodium or/and potassium hydroxide with a concentration comprised between 0.5 and 8 N, or better between 0.7 and 3.5 N. This alkaline environment allows the utilization of any biofuel-containing substituents susceptible to the basic solution.

The cathode comprises at least a Fe (III) specie, as a Fe (III) salt or its solvated or hydrated forms, chosen among iron (III) sulfate, iron (III) phosphate, iron (III) sulfide, iron chloride, or ferric cyanide, or their combination. Preferably it is ferric chloride hexahydrate $FeCl_3(H_2O)_6$ in a concentration between 0.5 N and 1.5 N, most preferably between 0.8 N and 1.2 N, and a pH between 1 and 5. The versatility of this Fe (III) salt as a universal electron acceptor allows the non-selective reduction at the cathode.

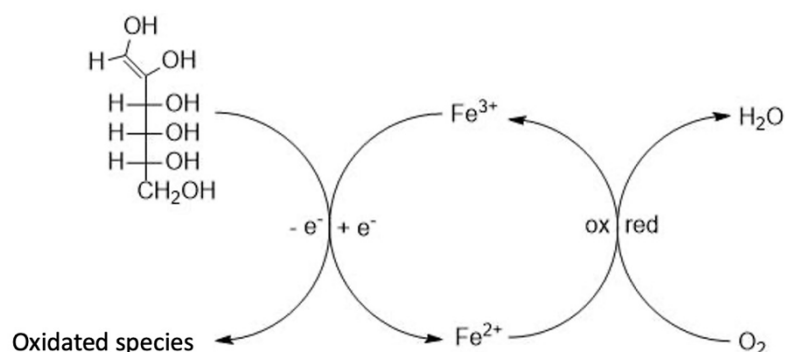
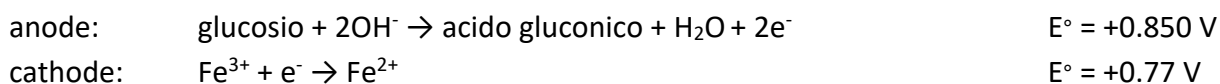


Fig.2 Schematic representation of reactions occurring in the proposed abiotic glucose biofuel cell.

The electrochemical reaction by which the fuel cell works (Fig. 2) occurs upon the oxidation of glucose from the alkaline biofuel contained in the anode and the reduction of Fe (III) to Fe (II) in the cathode, which establishes a potential difference, according to the following reactions:



The abiotic fuel cell also includes means for Fe (III) regeneration, consisting of an O₂ bubbling line 122 entering the cathodic chamber 110a. Here the Fe (II) generated by the cell reaction can be again oxidated to Fe (III), which becomes available for a new reaction with carbohydrates.

The ion-selective means are preferably a Nafion as membrane and/or a salt bridge made of agar gel and potassium chloride, which can act as a filter retaining potentially suspended solids from moving between the chambers.

Electrodes are graphite rods and/or electronic grade graphite, or carbon felts.

The fuel cell object of the invention has a specific tubular geometry, as simplified in Fig.3, which is realizable by 3D printing. The following thesis section describes the detailed design process (Chp. 4A)

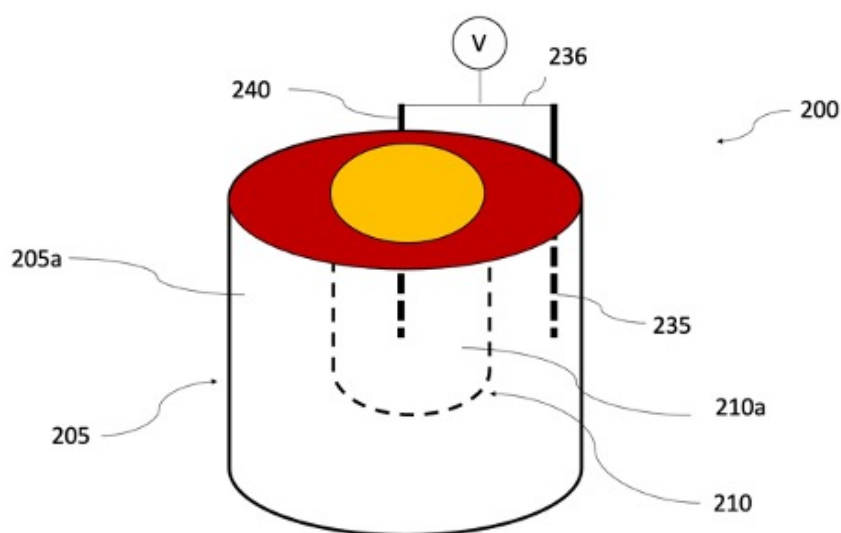


Fig.3 Simplified representation of the proposed abiotic glucose biofuel cell with tubular design (adapted from [Setti & Maggiore, pending patent](#)).

The components reported in Fig.3 are the same as presented in Fig. 1 and are labeled by adding one hundred units to each corresponding number. Thus, the anode 205 comprising the anodic chamber 205a and the cathode 210 comprising the cathodic chamber 210a are separated by ion-selective means (not shown in Fig.3) and linked to an external circuit 240 via electrodes 235 and 236, which are immersed in the respective electrolytes. The ion selective membrane and/or salt bridge are allocated into a cavity with a circular section placed between 205a and 210a, which are circular shaped too. 210a has a lower capacity than 205a. In particular, the 205a volume should be big enough to contain the anodic chamber, the cathodic chamber, and the space between them, originating a so-called crown-circular section. It also presents a perimeter with equidistant cylindrical cavities in which graphite rods can be inserted. All these features can be easily realized by 3D printing. The printing process occurs by deposition of a fused thermoplastic polymer chosen

among acrylonitrile butadiene styrene (ABS), polyethylene terephthalate (PET), polypropylene (PP), polytetrafluoroethylene (PTFE), ethylene tetrafluoroethylene (ETFE) or their combination. They can be virgin or recycled polymers and have the advantages to be chemically resistant to the anolyte and catholyte pH while conferring lightness and rigidity to the printed device. Concerning the printing settings, when ABS polymer is used, the setting of plate temperature to 110°C, nozzle temperature to 235°C, and printing rate to 60 mm/s are recommended. Once completed the printing, the 3D-printed fuel cell is left to cool at ambient temperature.

The fuel cell tubular design allows multiple units to be stacked in modules by series and parallel connections. This lets the system reach higher power output and current intensity for the same volume of electrolytes, with respect to a single unit having a volumetric capacity equal to the sum of the volumes of the units constituting the stack.

Each 3D-printed fuel cell unit is equipped with inlet and outlet pipes. Thus, both the operations in continuous and in static modes can be performed. Furthermore, the effective conversion of biofuel into electrical energy can be accomplished by establishing an energy plant based on the 3D-printed and comprising an upstream alkalization unit and a downstream neutralizer, as pointed out in the scheme in Fig.4.

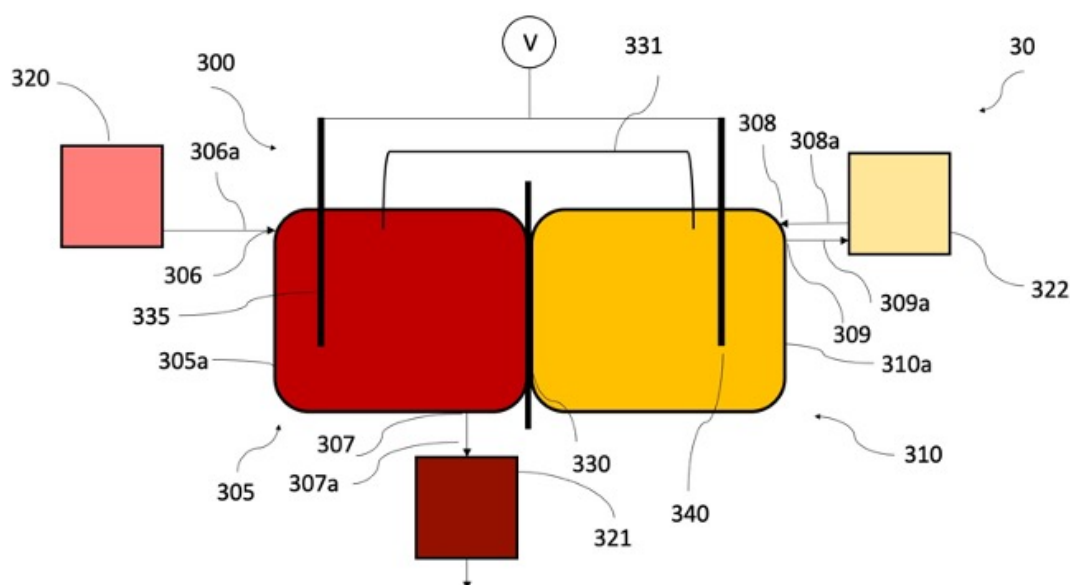


Fig.4 Simplified representation of the proposed energy plant, including the abiotic glucose biofuel cell (adapted from [Setti & Maggiore, pending patent](#)).

For simplicity, the constituents of the abiotic fuel cell jet presented and explained in Fig.1 are labeled in Fig.4 by adding two hundred to the corresponding number in Fig.1.

The anodic chamber 305a has an inlet 306 for loading an anolyte solution through a feeding line 306a and an outlet line 307 for its discharge through a discharge line 307a once the anolyte is oxidated and exhausted. The same scheme is applied to the cathodic chamber 310a, which has an inlet 308 and outlet 309, linked respectively to lines 308a and 309a. The anode and cathode feeding

lines can be predisposed to work with continuous and/or semicontinuous flux in the presence or absence of recirculation.

When the fuel cell works in continuous mode, the process conditions are maintained constant, and electrolytes can be fed with a constant flow rate. However, when it works in a semi-continuous way, the plant can run under cyclic operations and with an intermittent flow rate.

The cathodic chamber 310 is linked to downstream regeneration unit 322 by the feeding and discharge lines 308a and 309a. Once occurring the fuel cell reaction, the catholyte depleted of Fe (III) is sent to the regeneration unit 322 by line 309a, where Fe (II) is re-oxidated to Fe (III) by reaction with molecular oxygen. The new solution enriched by Fe (III) is sent to cathode 310 by 308a line. In particular, the regeneration unit 322 can be a chemical reactor equipped with tubular or disc air diffusers suppliers to ensure contact between the oxygen and the exhausted catholyte.

The energy plant comprises an alkalization unit 320 responsible for the activation and the pretreatment of the biofuel, thereby placed upstream of the anode 305 and linked to it by line 306a. 320, 305, and 306a work in adiabatic conditions and operate under different modalities, as explained in detail in the Chp. 4B. The 320 unit includes a tank in which the alkaline solution is mixed with the raw material for biofuel generation. If fatty esters and triglycerides enrich the biofuel, saponification is carried out. The 320 unit can also be linked to a filtration (or ultrafiltration unit) placed between the alkalization unit and the anodic chamber, responsible for the filtration of the electrolyte and its separation from solid particles and microorganism before entering the fuel cell.

The process also contains a neutralizer 321, placed downstream of anodic chamber 305 and linked to it by line 307a coming out from outlet pipe 307. Here the exhausted anolyte solution can be mixed up with a neutralizer solution containing an organic acid or inorganic acid like phosphoric acid to obtain a fertilizer in the form of slush. The slush should have a pH comprised between 9 and 4, or better, between 8 and 5. In addition, the plant can include a drying unit (not shown in Fig.4) downstream of the neutralizer 321, such as a sprat-drying unit, suitable to convert the slush into a dried fertilizer powder.

In addition, the energy plant can be equipped with an external battery suitable for the storage of the electrical energy produced by the abiotic fuel cell.

The entire designed energy plant allows the processing of high volumes of agro-food waste to produce electrical energy and obtain a soil improver for agriculture. It is easily scalable, and it is realizable with low investments as well as maintenance costs.

Energy plant operation phases:

- 1) Generate electrical energy by the oxide-reduction reaction occurring in the 3D-printed biofuel cell between the biofuel in the anodic chamber and Fe (III) in the cathodic chamber and obtaining the oxidated biofuel and Fe (II) species.
 - 1a) The generation of anolyte (biofuel) occurs upon the reaction of the raw materials with a base like sodium hydroxide. It can be accomplished in an alkaline pretreatment unit, placed upstream of the fuel cell, operating between 25°C and 90°C, better between 25°C and 65°C. The heat released by

the dissolution of the biofuel into the basic solution can be used to carry out the reaction instead of an external heating supply. The pretreatment occurs between 5 and 45 minutes but preferentially between 10 and 30 minutes. After the pretreatment, the solution is cooled to a temperature between 25 and 65°C and maintained until the fuel cell is feeding. Before that, the solution can be properly filtered, passing through a filter unit.

- 2) Bring to contact at least a portion of the catholyte with oxygen to generate Fe (III) species by the oxidation of Fe (II) species contained in the exhausted catholyte.
- 3) Discharge the anodic chamber of the abiotic fuel cell.
- 4) Bring the discharged and exhausted anolyte to the neutralizer.
- 5) Neutralize the anolyte with an acid like phosphoric acid, obtaining a final product as slush that can act as fertilizer. The slush can then be powdered after entering a drier unit.

4.3 Information to support the strategy: customize fuel cell devices

4.3.1 The abiotic biofuel cell

A fuel cell is an electrochemical device that converts chemical energy directly into electricity. Like batteries, fuel cells exploit a redox reaction, but in the case of batteries, the reactants are inside the reactor, while in the case of fuel cells, they flow, from an external tank, inside the device where the reaction occurs (Breeze, 2017). In general, the reaction in a fuel cell is like combustion, where oxidizing and fuel are divided, exploiting the thermodynamically favored reaction to generate current. The overall reaction is the same as in internal combustion, but there are no in-between energy transformations, resulting in higher efficiency. The classic fuel cell utilizes hydrogen as fuel and oxygen as oxidizing, producing only water as the product (Bagotsky, 2011). A schematic picture of a fuel cell's operating mechanism is shown in Fig.5.

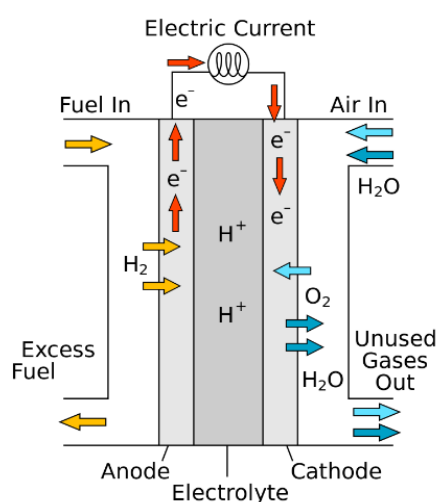


Fig.5 Operating scheme of a hydrogen/oxygen fuel cell (https://en.wikipedia.org/wiki/Fuel_cell).

The origin of fuel cells is linked to the reverse process, i.e., water electrolysis by which hydrogen and oxygen gas are generated by splitting water molecules through a passage of current.

The main advantages are low environmental pollution, flexibility in terms of power output, the opportunity to use different fuels, and improving efficiencies in cogeneration systems. Also, the long lifetime, if compared to the batteries, and the low cost required for the utilization and installation ([Gharehpetian & Mousavi, 2017](#)). However, there are still several disadvantages that limit these new technologies: the high cost of catalysts used in the reactions, the decomposition of the electrolyte and the poisoning of the catalyst, also the expensive steam reforming process from which hydrogen is derived if hydrogen-fuelled, and the cost of the device, if conductive and corrosion-resistant noble metals are used. Moreover, the current generated is a direct current (DC) that must be converted to an alternating current (AC) via an inverter ([Gharehpetian & Mousavi, 2017](#)).

Fuel cells that use fuel derived from renewable raw materials are called biofuel cells. Electricity is generated by oxidation biofuels such as glucose, glycerol, or ethanol, coupled with oxygen reduction in the water. Biofuel cells fully oxidize carbohydrates and other organic molecules to CO₂ and H₂O, using relatively low-cost renewable catalysts that exhibit high specificity toward the used organic fuel ([Watt, 2014](#)).

There are different criteria for classifying biofuel cells. Depending on the type of catalysts used, biofuel cells can mainly be classified as enzymatic, microbial, or abiotic. The most common fuel is glucose.

The enzymatic biofuel cells use isolated enzymes such as glucose oxidase or glucose dehydrogenase as catalysts at the anode and laccase, bilirubin oxidase, or copper oxidase at the cathode ([An et al., 2011](#); [Cosnier, Le Goff, & Holzinger, 2014](#); [Falk et al., 2013](#); [Katz, & MacVittie, 2013](#)). This type of fuel cell has the advantage that it can operate without electrode compartmentalization because the catalysts are selective. Thus, their design can be very simple and can be easily miniaturized. Indeed, the main application fields are biosensors or implants such as pacemakers ([Sue & Tsai, 2012](#); [Pankratov et al., 2015](#); [Du Toit et al., 2016](#)). However, the main disadvantage of enzymes for long-term applications is their progressive deactivation due to the degradation of their complex protein structure over time.

The microbial fuel cells use electroactive microorganisms that can oxidize glucose and transfer the resulting electrons to an electrode. Microorganisms can also be used to reduce oxygen. Unlike enzymes, microorganisms are less susceptible to poisoning and loss of activity under normal operating conditions, leading to greater stability and longevity of the devices ([Chen et al., 2012](#)). Another reason for the longer life is that in optimal conditions, microbial populations reproduce themselves; thus, the rate at which the catalytically active species decreases is relatively slower. However, despite the increase in catalyst life, microbial biofuel cells suffer from a limited efficiency of the electro-transfer process from the microorganisms to the electrode's surface, which results in low generated power ([An et al., 2011](#); [Chen et al., 2012](#)).

In contrast to the microbial fuel cell, in an **abiotic fuel cell**, the electron transfer is mediated by a species that does not reside directly on the electrode. This allows reaching higher power output because there are no issues related to the microorganism charge transfer capacity or deactivation due to the operative condition. The abiotic fuel cell uses abiotic catalysts, usually metals or their alloys, to catalyze the reactions fueling it. This catalysis offers several advantages over the previous ones (Kerzenmacher, Ducreé, Zengerle, et Von Stetten, 2008; Li et al., 2019). They have great long-term stability, which gives them a long lifetime. In addition, catalysts can be selected to ensure adequate biocompatibility (Cosnier et al., 2014; Sue & Tsai, 2012).

Typically, abiotically catalyzed glucose-based biofuel cells use platinum as a catalyst for glucose oxidation. However, platinum has the major disadvantage of poisoning (Tao, Miao & Chu, 2012; Rao et al, 1976; Cheng et al., 2007; Schmittinger & Vahidi, 2008). Moreover, its high price limits its range of applications. For these reasons, researchers investigated new materials and catalyst structures to improve glucose's electro-oxidation and the lifetime of catalysts. Therefore, they determined that Pd and its alloys, transition metals, and their oxides in an alkaline environment can catalyze glucose oxidation even better than platinum (Kung, Cheng, et Ho, 2014; Chen, Lin, et Chen, 2015; Jiang et al., 2015).

For years, studies have been focused on various biofuel cell systems that use a wide variety of noble metal-based catalysts and metal alloy composites for the oxidation of carbohydrates (Chen et al., 2012; Joh et al., 2008). However, three main problems have hindered their development: 1) incomplete fuel oxidation, which limits overall energy production. The reaction is typically glucose/gluconic acid, with only two of the twenty-four electrons initially present being used for energy generation; 2) the poisoning of metal catalyst by-products, such as CO and other organic compounds, which lower the availability of the catalyst; and 3) the high cost and limited availability of these noble metals.

The first of these problems results in an electrical energy production that corresponds to only 8% of the theoretically available electrical energy from the oxidation of carbohydrates. The second problem is being addressed by forming metal composites and formulations to prevent catalyst inhibition and corrosion from side reactions (Chen et al., 2012; Chin et al., 2011). Nanostructured materials are also being investigated (Chen et al., 2012), but catalyst poisoning and degradation pose complex challenges. Only finding efficient, abundant, and cheaper catalysts can solve the third problem.

If containing mediators of electron transfer, the fuel cells are called mediated fuel cells. Some types of mediated cells can be the recent photo fuel cells that use photoinitiators for biomass oxidation (solar-induced direct biomass-to-electricity hybrids) and redox biofuel cells, in which redox mediators transport electrons from the biofuel into the cell, thus promoting the passage of a current (Slaughter & Sunday, 2014). Biofuel cells with redox operation are insensitive to most organic and inorganic pollutants and do not need noble metals as catalysts (Leonov, 2011). The improvement sought in new fuel cell prototypes is an increase in energy efficiency while keeping costs low (Falk et al., 2013), thus attempting to use more efficient catalysts without using precious metals and uneconomical materials.

4.3.2 Alkaline glucose biofuel cell

An alkaline glucose biofuel cell is represented in Fig.6.

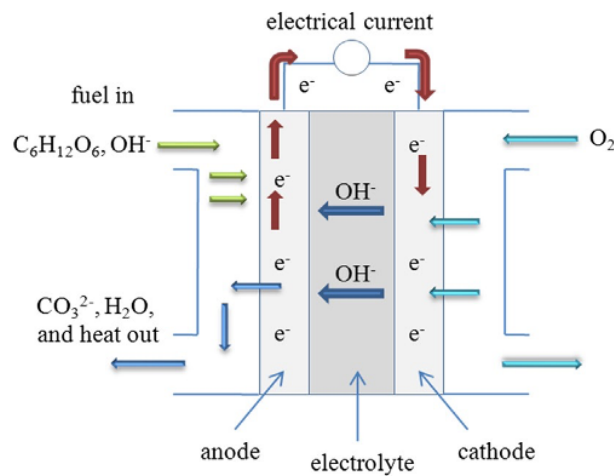


Fig.6 An alkaline biofuel cell using a basic solution of glucose as anolyte and oxygen as catholyte (Watt, 2014).

The oxidation reaction that takes place in a fuel cell between glucose at the anode in basic solution, and an oxidant at the cathode as oxygen gas, without a redox mediator, can be described as follows (Mor, Rubin, et Schechner, 2008):

Reaction at the cathode:



Reaction at the anode:

Glucose reacts with NaOH producing enediol ion



The enediol ion is oxidated at the electrode, releasing an electron



The radical reacts instantly with water to give a molecule of gluconic acid



The sum of (2), (3), (4) gives the reaction at the anode:



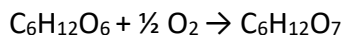
The hydrogen radical reacts subsequently to release an electron to the anode



Thus, the net reaction at the anode is:



The cell overall reaction results:



(8)

In a fuel cell based on biomass, mainly glucose, an alkaline electrolyte performs better than an acid electrolyte. In an alkaline environment, glucose is easily oxidized, being structurally a polyhydroxyaldehyde, via the enediol intermediate species, to gluconic acid or gluconolactone (Chen et al., 2012). The reactions that can take place are varied and complex. First of all, monosaccharides, and sugars in general, undergo a degradation process at non-neutral pH; in an alkaline environment, as opposed to an acid environment, degradation takes place in such a way that the primary degradation products are also involved, with a concomitant change in the color of the solution, which is remarkably rapid above 40°C (Chaudhuri & Lovley, 2003). After the so-called 'yellowing point,' the thermally catalyzed complex of degradation reactions produces countless species of products irreversibly, changing from a straw-yellow to a dark red to brown color. For reactions involving monosaccharides in basic solutions, the color change is associated with the production of long-chain organic acids (>C6), which are regarded as polymeric species, even though they are primarily oligomers or high-molecular-weight species with a complex structure (Sowden & Schaffer, 1952). The interconversion reaction among the three main monosaccharides in solution, D-glucose, D-fructose, and D-mannose, is known as Lobry de Bruyn-Alberda van Ekenstein rearrangement (De Bruijn, 1986), shown in Fig.7

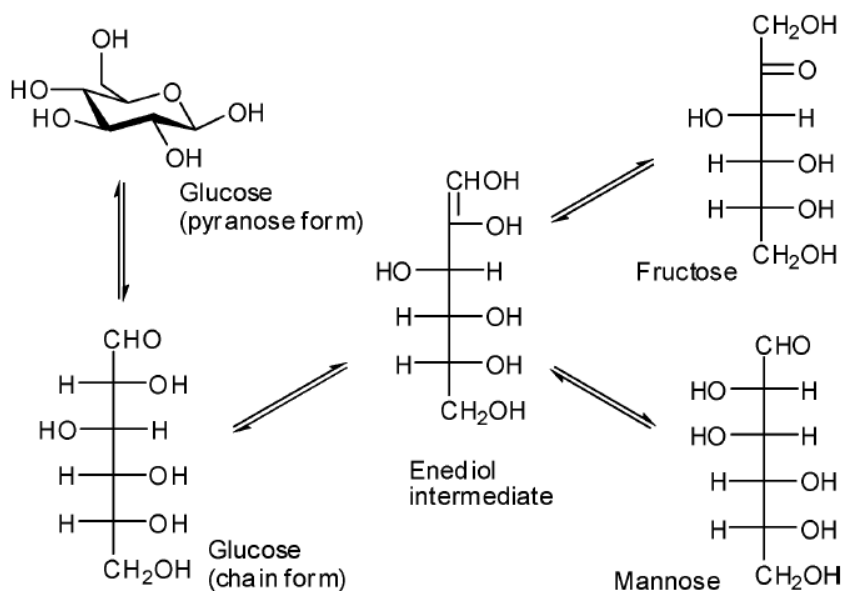


Fig.7 Lobry de Bruyn-Alberda van Ekenstein rearrangement (Kobayashi et al., 2011).

In solution, glucose can occur mainly in five forms (mutarotation): linear aldehydic, the cyclic forms α -glucopyranose and β -glucopyranose, and α -glucofuranose and β -glucofuranose (Yei, Beden, et Lamy, 1988). But the most prevalent form, due to the greater thermodynamic stability of the six-membered ring, is the pyranose one (Brown, Iverson, Anslyn, et Foote, 2013), with the β -anomer present for 64% and the α -anomer for 36% in solution at the steady-state (Tester & Karkalas, 2003). Acid and base catalysis favor mutarotation, while isomerization and enolization are mostly favored at basic pH, compared to an acid or neutral environment where equilibria are slower (Caballero, Finglas, et Toldrá, 2015). In an acid environment, glucose is relatively stable (Shallenberger & Mattick, 1983). In an alkaline environment, glucose is unstable, undergoing a series of reversible and irreversible reactions that transform it into a more reactive species (Kato, Yamamoto, et Fujimaki, 1969). In particular, the enediol species at equilibrium appear to be more easily oxidized; furthermore, OH^- ions help to neutralize the protons from dehydrogenation reactions by forming the catalytic species OH_{ads} adsorbed on the electrode, which takes part in the oxidation (Cui et al., 2006). It is also reported that the glucose anomeric pyranose form β is more present in an alkaline solution. It is more easily oxidizable (Moggia, Kenis, Daems, et Breugelmans, 2020) due to the axial position of the anomeric hydrogen atom, which results in being more easily approachable by the oxidant species than in the α -pyranose form. Moreover, electrocatalytic processes are generally favored in the alkaline environment due to the absence of adsorbable ions in a specific way (Fujiwara et al., 2009).

The possible transformations involving monosaccharides in solutions with basic pH are shown in Fig.8 (Sirisangsawang, R., & Kitchaiya, 2019; De Bruijn, 1986):

- 1) enolization/isomerization reactions (Lobry de Bruyn-Alberda van Ekenstein rearrangement)
- 2) β -elimination reactions
- 3) benzyl acid rearrangement
- 4) bond breaking between carbonyl carbons in α -dicarbonyls
- 5) aldolisation and retroaldolisation
- 6) extensive aldolization and retroaldolization of dicarbonyl compounds

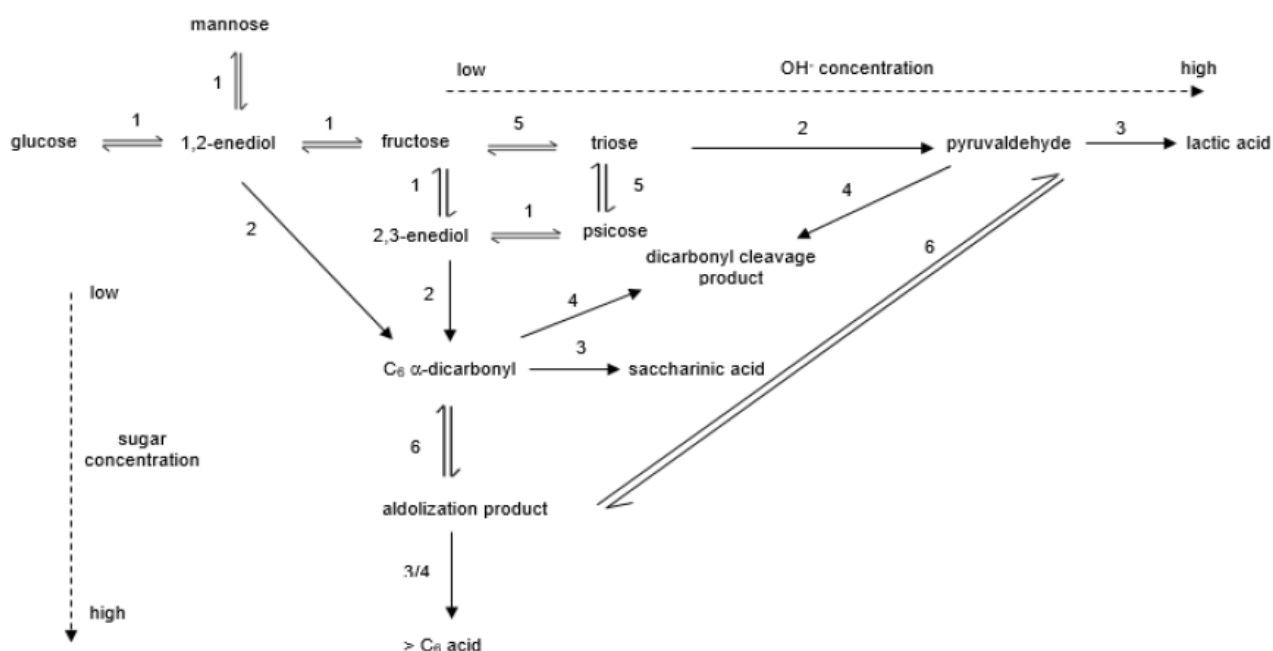


Fig.8 Possible reactions of monosaccharides in basic solution (Sirisangsawang & Kitchaiya, 2019).

The reactions in Fig.8 are valid for each type of monosaccharide, dissolved in a basic solution formed via the initial Lobry de Bruyn-Alberda van Ekenstein rearrangement. The fundamental reactions can be divided into two distinct groups: the first includes the initial transformations in which the structures of the monosaccharides are conserved, i.e., mutarotations, enolizations, isomerizations, and ionizations; the second groups the reactions that lead to degradation of the starting molecules. The OH^- species act as a catalyst in the first group of reactions, while they are consumed in the reactions of the second group (Moulik, Basu, et Bhattacharya, 1978).

The whole system of reactions is characterized by fast kinetics, each with a low activation energy, since consistent temperature variations do not lead to a different concentration distribution of the substances obtained (De Bruijn, Kieboom, & Van Bekkum, 1986). This is instead influenced by a different initial concentration of the reactants, i.e., glucose and NaOH, and by the nature of the base used. For example, an increase in initial glucose concentration leads to more $>\text{C}_6$ and a decrease in $\leq\text{C}_6$ acids, including saccharin and lactic acid; the concentrations of some secondary acids, such as glycolic, acetic, and formic acids, remain unchanged. An increase in the concentration of the base, instead, affects all the species in the solution. Specifically, considerable increases of acids $\leq\text{C}_6$, and in particular of lactic acid, are reported as well as a more significant presence of saccharin acids; and a decrease of $>\text{C}_6$ acids, with a slighter decrease of glycolic, formic, and acetic acids (De Bruijn, Kieboom, & Van Bekkum, 1986). Furthermore, the complex of reactions, including isomerization and thermoxidative degradation, is related to the reaction conditions, such as the operating temperature and duration (Moggia et al., 2020).

4.3.3 Fuel cell characterization

Open-Circuit Voltage (OCV)

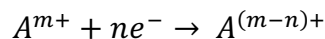
Open-Circuit Voltage, or potential, is the difference in electrical potential between two terminals in a device when no current flows. It is the variable that takes into consideration the thermodynamic of the system and is directly related to the Gibbs free energy, thus to the spontaneity of the process, by the following equation:

$$OCV = E = - \frac{\Delta G}{n_e F}$$

Where n_e is the moles of electrons, and F is the Faraday constant ($\approx 96485 \text{ C}\cdot\text{mol}^{-1}$). If we consider the standard conditions ($T=298 \text{ K}$; $p=1 \text{ atm}$), the equation becomes:

$$E^\circ = - \frac{\Delta G^\circ}{n_e F}$$

The free energy, and thus the potential, depends on the amount and the nature of the species in the system. The Nernst equation expresses this dependency. For a half-reaction like the following one:



With the m^+ charge of the reactant and n number of electrons gained in the reduction, the Nernst equation is:

$$E = E^\circ - \frac{RT}{nF} \ln \frac{a(A^{(m-n)+})}{a(A^{m+})}$$

Where a is the activity of the species and is proportional to the concentration.

As expressed, the OCV depends on the reacting species at the electrode; however, all the reactions, even if not in direct contact with the electrode's surface, that occur in the half-cells affect the potential by varying the species concentration.

The OCV is the highest potential value measurable. When a load is applied to the circuit, the OCV is reduced by a term V_{loss} in the function of the mean current density $J=I/A$, where I is the current and A the electrode surface. So, the measured potential of the cell will be:

$$V_{cell} = OCV - V_{loss}$$

Short-Circuit Current (ISC)

The electric current (I) is defined as the amount of charge (dq) crossing the internal section of a conducting material in a time interval (dt):

$$I = \frac{dq}{dt}$$

It is one of the fundamental parameters; in the I.S., its measure unit is Ampere (A). The Short-Circuit Current (ISC) is the value of current obtained when the device is short-circuited, so the electrodes are connected without applying any load. It is the maximum value of the current measurable.

Polarization and power curves

A method to characterize electrochemical systems, such as batteries and fuel cells, is the polarization curve (or V-I curve), which is drawn by making varying current or voltage inside the system, reading back the variation on the other parameter. This simple experiment helps to understand the chemical-physical properties of the cell and allows us to point out the best conditions of use and the maximum energetic efficiency, and more to obtain the nature and the importance of internal resistances.

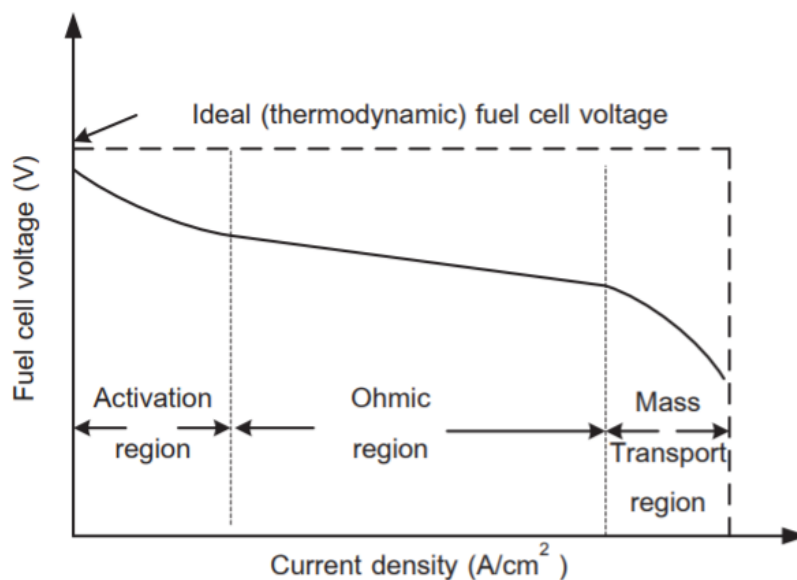


Fig. 9 V-I curve of a fuel cell. Three regions are highlighted based on the resistance process that occurs ([Chanpeng et al., 2010](#))

From a polarization curve, different regimes can be identified (Fig.9). An ideal infinite resistance (or load) is applied in open-circuit conditions. Thus, no current is allowed to flow, and the maximum potential of the cell can be measured; on the contrary, in short-circuit conditions, no resistance is applied, and the maximum current flow is measured. In between these two limit regimes are all the chemical and physical phenomena typical of all the electrochemical devices: depending on the load, one may prevail over the others ([Benzinger et al., 2006](#)). The term “polarization curve” is referred to the distancing from the equilibrium potential (or reversible potential), the thermodynamic one, when a current is allowed to flow. The difference between the equilibrium potential and the

potential measured when the current flows is called overpotential and is the cause of the lowering of the potential in polarization curves (Sundén, 2019). The overpotential lowers the efficiency of every electrochemical device. For example, in electrolytic cells, we have to apply a higher voltage than the equilibrium one to make it work; in galvanic cells, we can obtain a lower voltage than the equilibrium one.

Three phenomena cause losses in potential: activation potential loss (η_{act}), ohmic loss (η_{ohm}), and concentration polarization loss (η_{conc}), which affect the functional potential of the cell V:

$$V = OCV - \eta_{act} - \eta_{ohm} - \eta_{conc}$$

These dissipative phenomena must be reduced to enhance the efficiency of the chemical energy conversion into electricity.

The activation potential loss – η_{act} , is a loss due to overcoming the activation energy at the electrode surface. It occurs at low current densities and determines the first steep drop in potential. The equation that describes this effect is the Tafel equation:

$$\eta_{act} = \left(\frac{RT}{\alpha F}\right) \ln \left(\frac{j + j_{loss}}{j_0}\right)$$

Where α is an activity coefficient, j_{loss} is the current loss, j_0 is the exchange current density for the reaction, and j is the applied current density.

The Ohmic loss – η_{ohm} is loss due to internal resistance to the motion of charged species by the materials in the cell. They can be electron transport resistance (R_{elec}) by conductive components, ion transport resistance (R_{ionic}) by the electrolyte, and contact resistance ($R_{contact}$) at the interface of different materials or phases. The curve is the central part and causes the linear drop. The equation for this phenomenon is Ohm's law:

$$\eta_{ohm} = j(R_{ohmic})$$

Where $R_{ohmic} = R_{elec} + R_{ionic} + R_{contact}$.

Polarization of concentration – η_{conc} is due to mass transport and fluid dynamics, usually associated with the depletion of active species near the electrode. It is evident at high current density and causes the second steep drop of the potential curve. The equation is:

$$\eta_{conc} = \left(\frac{RT}{nF}\right) \ln \left(\frac{i_L}{i_L - i}\right)$$

Where i_L is limiting current density (constant) (Vincent, C., & Scrosati, 1997).

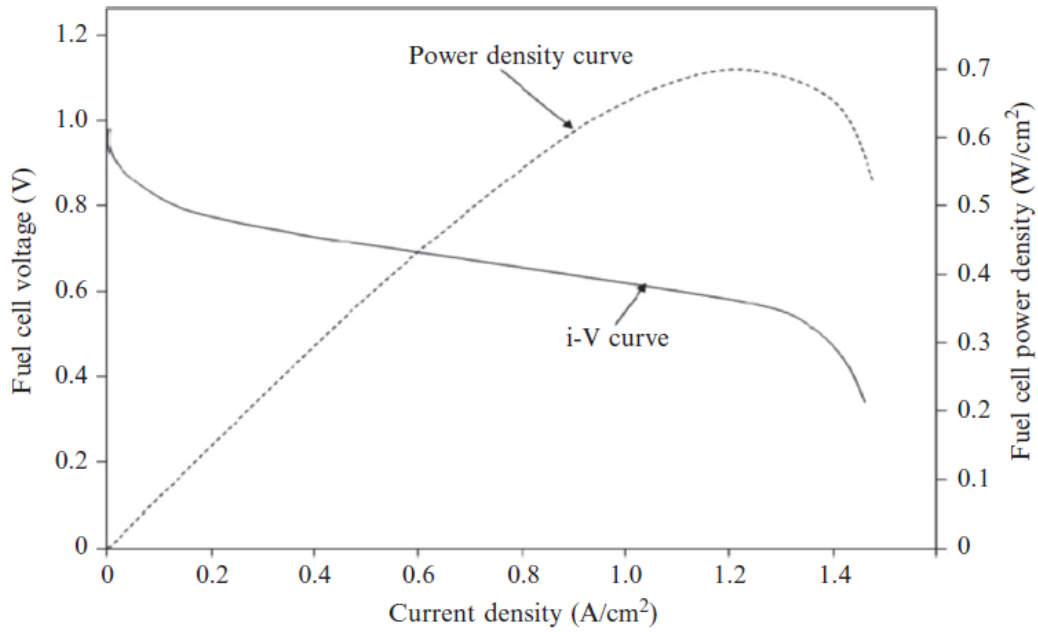


Fig.10 Power and V-I curves generated by a fuel cell (Gharehpetian & Mousavi, 2017).

From the polarization curve, the power curve (Fig.10) can be calculated by multiplying each point V for the current i (A), as the definition of power:

$$P = V \cdot i$$

Usually, this equation is normalized by the area of the electrode, and to do so, we have to introduce the current density j ($\text{A}\cdot\text{m}^{-2}$):

$$P_{density} = V \cdot i$$

Where the power density $P_{density}$, is in $\text{W}\cdot\text{m}^{-2}$.

Looking at the power curve, we can find out the range of utilization of the cell according to the peak. Power is zero when we are in short-circuit or open-circuit, while it is maximum for an external load impedance that matches the cell's internal resistance, that is, in the ohmic region of the V-I. This is known as the maximum power transfer theorem. It can be demonstrated by considering a closed

circuit with a direct current source (fuel cell), the internal resistance r , and the external load resistance R . The power of the load P_L is:

$$P_L = i^2 \cdot R = \left(\frac{V}{R + r} \right)^2 \cdot R$$

Deriving by R , the equation becomes:

$$\frac{dP_L}{dR} = V^2 \left[\frac{(R + r)^2 - 2R(R + r)}{(R + r)^4} \right]$$

To maximize the power, we have to equal the derivative to 0:

$$(R + r)^2 = 2R(R + r)$$

We obtain that P_L is maximum when $R=r$. However, it is important to underline that the conditions that maximize power are not the same that maximizing efficiency. Maximum efficiency can be obtained when the potential is equal to the OCV.

4.3.4 The 3D printing

3D printing is a fast-growing technology with enormous potential across various sectors, such as engineering, art, education, medicine, and aerospace. The flexibility in design provided by this technique offers many opportunities to produce sophisticated 3D devices. This method is highly versatile, with the ability to print various sizes (from micrometer to metric scale) and multi-material, multi-functional 3D structures. In addition, several advanced properties of the polymers used for printing are exploited, such as mechanical strength, conductance, self-repair, and other integrated properties.

This technology has numerous advantages, such as operational simplicity, customizable design, reliability, affordability, and diversity of compatible materials, making it very promising for various technological applications in biotechnology, energy/environmental, robotics, and aerospace (Gross et al., 2014; Derakhshanfar et al., 2018; Murphy & Atala, 2014; Pekkanen et al., 2017; Jang et al., 2018; Chisholm et al., 2014).

3D printing converts a computer-generated model into a real, physical object. The model is first converted into a series of spatial coordinates (x , y , z) that can be read by a printer and then transformed into a series of sequential layers of material. Next, optical lasers or ink-based printheads are moved under computer control according to the designed pattern to manufacture objects one layer at a time.

Extrusion-based printing is widely used due to its simple printing mechanism and low manufacturing cost. The first step is the extrusion of materials through a nozzle using mechanical force. The extruded and deposited polymer then undergoes solidification through crystallization, chain rearrangement, the formation of non-covalent bonds, or chemical cross-linking. After the completion of a single layer, the extrusion head moves up, or the printing platform moves down for the deposition of the next layer.

Printable materials or 'inks' are crucial components. They are specific to the printing method and determine the performance of the final 3D-printed products. Due to their processability and low cost, polymeric materials are the most widely used materials for 3D printing ([Cheng, Liu, et Logan, 2006](#)). Improvements in 3D printable polymeric materials are facilitating growth in this field. Many types of polymers, including thermoplastics, thermosets, elastomers, hydrogels, functional polymers, polymer blends, and composites, can be processed by 3D extrusion printing ([Ligon et al., 2017](#); [Li, Lin, Tang, Duncan, et Ke, 2019](#)).

3D extrusion printing comprises two main types of techniques: fused filament fabrication (FFF) and direct ink writing (DIW) ([Ligon et al., 2017](#)). FFF printing is generally used for the 3D extrusion of thermoplastic polymers, which are mechanically extruded as thin filaments through a heated nozzle in the printhead ([Scheithauer, Schwarzer, Richter, et Moritz, 2015](#)). To facilitate flow and deposition, the thermoplastic material is heated above the melting temperature for semi-crystalline polymers or above the glass transition temperature for amorphous polymers. Once the filament has exited the nozzle, it cools, and the viscosity of the polymer increases sharply, retaining its shape. The DIW system is used to form 3D materials from viscoelastic polymers. This process has been used to produce a variety of objects with a wide range of geometries, sizes, and small details ([Lewis, 2006](#)). During the DIW process, viscoelastic polymer ink is dispensed from a small nozzle a few hundred micrometers in diameter by external pressure and then deposited along digitally defined paths to fabricate the desired 3D structures.

For polymers to be printable via the extrusion method, the following properties are required: 1) polymers must be highly thixotropic and exhibit shear thinning behavior, 2) viscosity must be appropriate so that the material can extrude properly and form continuous filaments to ensure the shape of the structure, 3) need to have a rapid increase in viscosity after extrusion to maintain the 3D printed shape, 4) sufficient mechanical strength is required after exiting the nozzle to support the subsequently printed structures and to prevent delamination during and after printing ([Jiang et al., 2020](#)).

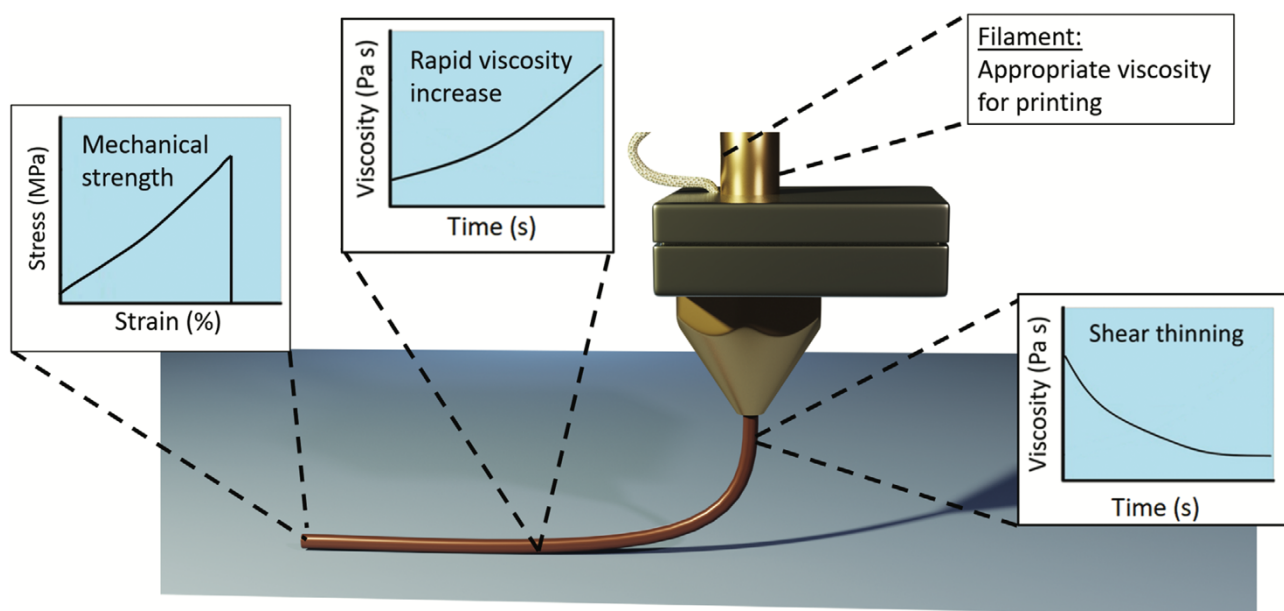


Fig.11 Key properties required for 3D extrusion printing of polymers (Jiang et al., 2020).

Thermoplastic polymers are very suitable for FFF. The significantly reduced viscosity after heating facilitates extrusion through a nozzle. Using 3D extrusion techniques, several commercially used filaments have been formed. However, extruded filaments of some polymers could easily suffer shrinkage, compromising the integrity of 3D-printed structures. Furthermore, some of these polymers are semi-crystalline and have a low melt viscosity that may hinder solid dispersion in the filaments. Therefore, the design and development of new thermoplastics with improved 3D printability are particularly attractive (Jiang et al., 2020).

Historically, one of the most widely used polymers for FFF 3D printing has been acrylonitrile-butadiene-styrene (ABS) copolymers. Incorporating reinforced nanomaterials such as carbon fibers (Ning, Cong, Qiu, Wei, et Wang, 2015), glass fibers (Zhong, Li, Zhang, Song, et Li, 2001), graphene (Wei et al., 2015), or carbon nanotubes (Dul, Pegoretti, et Fambri, 2018) into thermoplastic materials to form composites is one of the most widely used methods to improve dimensional stability due to the increased toughness that these materials contribute when added. The addition of reinforced nanomaterials and small plasticizer molecules has also been shown to improve the 3D printability of other important and widely used thermoplastics, such as polyurethane (TPU) (Christ, Aliheidari, Ameli, et Pötschke, 2017) and polylactic acid (PLA) (Prashantha & Roger, 2017). In contrast, polyvinyl alcohol (PVA) is traditionally complicated to print due to the high amount of hydrogen bonds. Therefore, glycerol, water (Wu, Chen, et Wang, 2018), and ionic liquids (Chen, Chen, et Wang, 2018) have been introduced to reduce its melting point.

In summary, incorporating nano-fillers or other polymers improves structural integrity and offers additional multifunctionality, such as biocompatibility (Chen et al., 2017) and conductivity in 3D-printed devices. However, anisotropy and poor adhesion between layers are the main challenges limiting further practical applications of FFF technology.

In contrast to thermoplastics, thermosetting polymers are important materials used in various high-tech applications due to stable thermomechanical properties such as excellent chemical and thermal resistance. Unlike thermoplastic polymers, thermosetting polymers cannot be remolded after thermal curing due to irreversible chemical cross-linking. They generally cannot flow under applied pressure, making it extremely difficult to use extrusion methods to manufacture thermoset 3D objects.

Compared to traditional microfabrication technologies, 3D printing enables the direct fabrication of materials in 3D geometries and the combining of different functional materials using a single printing tool. It is therefore expected that the 3D-printed object will exhibit improved properties.

Many polymeric materials with various modular and fascinating properties are being developed. However, it is still a very young field, and the polymeric materials designed for 3D printing still need to be improved. Nevertheless, there will be a considerable improvement from the constant and innovative development of functional polymer inks with improved printability and material properties.

With 3D printing, however, there is a potential risk of further increasing the use of plastics. Therefore, sustainable and renewable materials must be designed to not exacerbate the already significant environmental issues associated with plastic use.

Abbreviations

AAEMFCs alkaline anion exchange membrane fuel cells

ABS acrylonitrile butadiene styrene

AC alternating current

AEM anion exchange membranes

AFCs alkaline fuel cells

BOD biological oxygen demand

COD chemical oxygen demand

DC direct current

DIW direct ink writing

FFF fused filament fabrication

GOR glucose oxidation reaction

ISC Short-Circuit Current

MFC microbial fuel cells

OCV Open-Circuit Voltage

ORR oxygen reduction reaction

PEM proton exchange membranes

PET polyethylene terephthalate

PLA polylactic acid

PP polypropylene
PTFE ethylene tetrafluoroethylene
PTFE polytetrafluoroethylene
PVA polyvinyl alcohol
TPU polyurethane

4A Design studies for 3D-printed abiotic glucose biofuel cells

Our study aims to develop a fuel cell design that effectively converts agro-food waste into electrical energy at ambient operative conditions, using organic-based electrodes and low-cost catalysts. Although fuel cells are consolidated technologies on a commercial scale, the opportunity to model new designs could facilitate the scale-up process, especially from laboratory to pilot scale. Indeed, whereas modular units make the passage from pilot to plant scale easy, the development of new configurations can ease the passage from laboratory to pilot scale by ensuring uniformity in the mass transfer process, which is generally affected by the cell sizes characteristics (Goodridge & Scott, 1995). Goodridge & Scott (1995) identified five criteria to be accomplished in new electrochemical cell designs. They correspond to the main key cell parameters. They are: 1) good current efficiency and 2) chemical conversion yield, which is both dependent on mass transfer characteristics, uniformity of electrode potential, and electrode material; 3) the space-time yield, which takes account of the cell size and the effective electrode area; 4) the energy yield and the 5) the energy consumption which depend on current efficiency and cell voltage. 3D printing is a helpful tool for creating and realizing new fuel cell designs since it allows fine-tuning cell size and geometrical parameters like the distance between electrodes and their configuration. Even the ability to withstand a wide range of electrolytes' pH and working temperatures can be modulated by simply acting on the choice of material to be used in printing the cell body and components. In addition, electrodes with new shapes and materials can support the functionality of the 3D printed object since they can adapt easier to uncommon forms and geometry while achieving high conductivities (Percin, Zoellner, Rall, & Wessling, 2020).

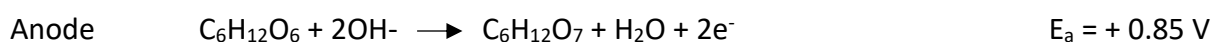
Before the 3D-printing

The reaction taken as a model for biomass conversion into electrical energy in an alkaline fuel cell is the oxidation of glucose solution. It has been carried out for the first time in two Woulfe bottles, constituting the two distinct anode and the cathode semi-chambers, linked by an agar salt bridge (Fig.1).



Fig.1 Fuel cell reaction in Woulfe Bottles. Anode (left) contains the product of the reaction between glucose 1M and NaOH 0.8M ($T = 65^{\circ}\text{C}$, $t = 15$ minutes) as an electrolyte. Cathode (right) contains FeCl_3 (aq.) as an electrolyte.

The system was set up, as reported in the Materials and Methods section, to allow the generation of electrical energy upon reducing sugars oxidation at the anode and the reduction of ferric chloride at the cathode, as shown below:



The solubilization of ferric chloride into an aqueous solution generated the catholyte. The anolyte was generated by the reaction between glucose and sodium hydroxide at 65°C for 15 min, after preliminary evaluation of the reaction times that could maximize the formation of enediol species (Fig.2).

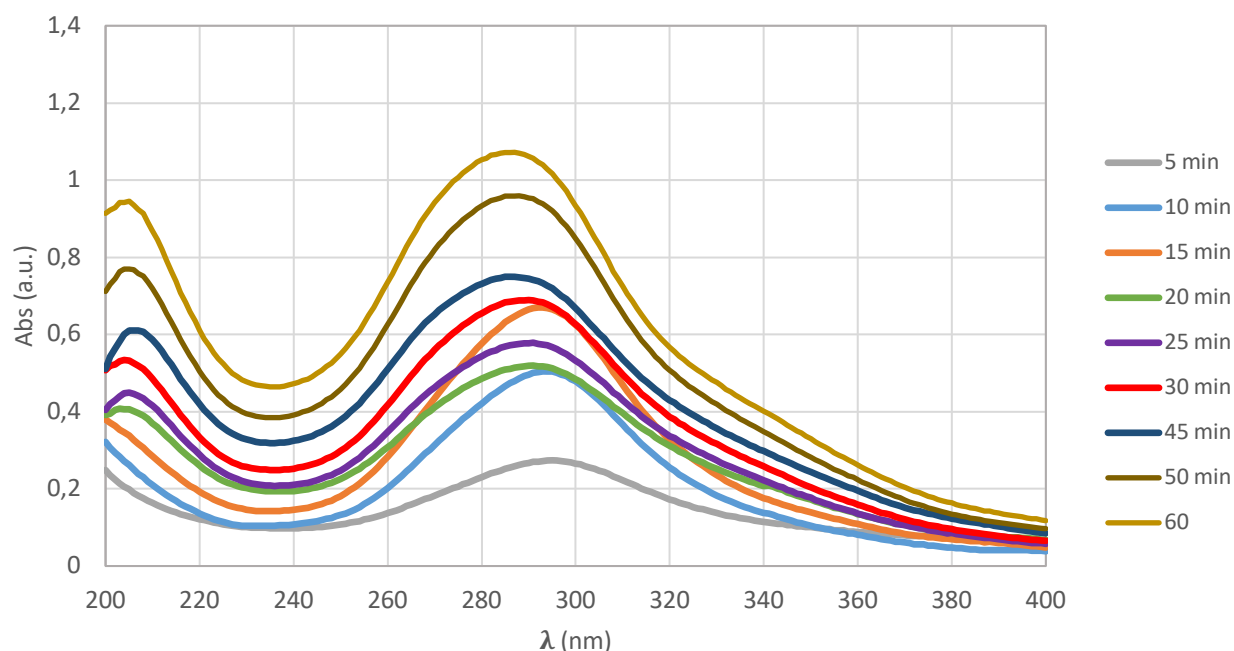


Fig.2 UV-spectrum of the reaction between glucose 0.7 M and NaOH 0.8 M, carried out at 65°C and monitored over time.

The absorption peak at 290 nm is indeed ascribable to the formation of enediols occurring during the glucose isomerization and degradation in an alkaline environment and in the presence of oxygen. The shift of this peak toward lower wavelengths could indicate the decrease in solution pH and the transformation of the enediol anion into the protonated form (Simonov, Pestunova, Matvienko & Parmon, 2005). After the first 15 minutes of reaction, a slight shifting of the absorption at 290 nm is observed, together with the appearance of a second peak at 203 nm and a band formation toward 265 nm, with the latter ascribed to the formation of glucose degradation products (Simonov et al., 2005), as well as the brownish of the solution (Yei, Beden & Lamy, 1988). The reaction was stopped after 15 min from the addition of NaOH by cooling down the solution before setting up the cell. The cell reaction conditions (T, t, reactant concentration) were chosen after our group's preliminary investigations (Maggiulli, 2020). The power and polarization curves generated

under a crescent voltage load are reported in Fig.3. This is the starting point of our study. Every change applied in fuel cell body design and the set-up of electrolytes and electrodes has been aimed to improve the system's performances, which initially were remarkably low, up to reach power and current output interesting for commercial purposes. The Woulfe system has OCV = 1.41 V, ISC = 2.43 mA, and generates 0.027 mW/cm² maximum power output under 0.63 V.

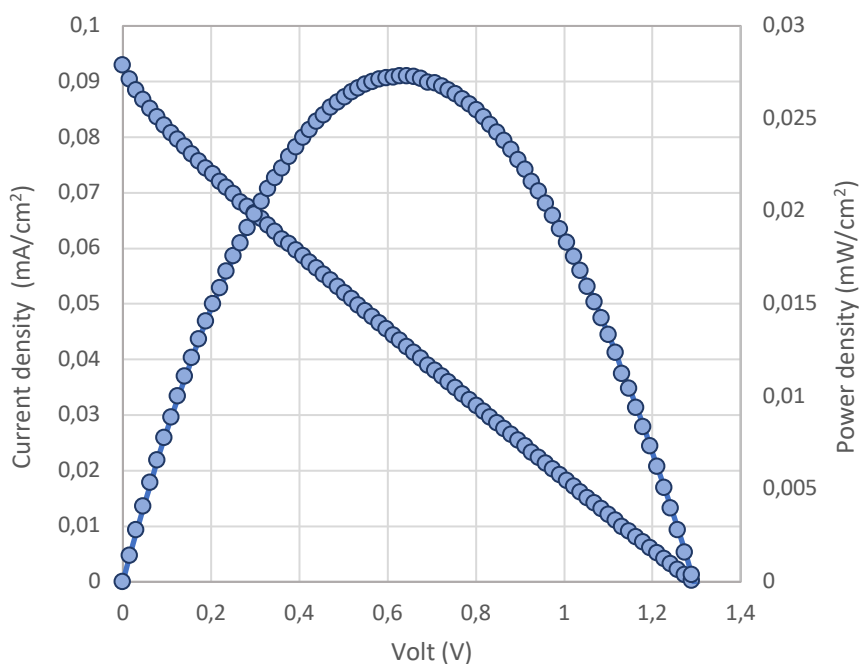


Fig.3 I-V (left axis) and Power curves (right axis) recorded on a fuel cell in the Woulfe bottles system. Anolyte: glucose 1M +NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode: graphite rods. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

3D-printed cuboid design

The first 3D-printed glucose biofuel cell design is reported in Fig.4. It derives from the concept of uniting the two chambers of the Woulfe bottles into a single device but maintaining the electrolytes separated by a salt bridge and the electrodes not in contact. These purposes brought to insert into the cell design two channels located at the opposite extremities of the cell body, which allocate the electrodes, and to print two additional and removable walls, which will be used to shape and narrow the salt bridge that is intercalated at the center of the cell body (Fig.5). The final 3D printed object has a cuboid geometry (Fig.6) and has an intermediate form between the Woulfe bottle and the commercial planar fuel cell. It has an ion-selective separator between a positive and a negative electrode. It works in a batch mode, which requires the loading of the reactants, the reaction under constant stirring, and the discharging of the exhausted electrolytes once the reaction is terminated. A PAN-derived activated carbon felt, AvCarb 100, replaced the graphite electrodes used in the starting system. It is an example of material commonly used in redox vanadium batteries ([Ressel et](#)

al.,2017), which should improve the current density in response to the penetration of the electrolyte by diffusion or convection (Goodridge & Scott, 1995).

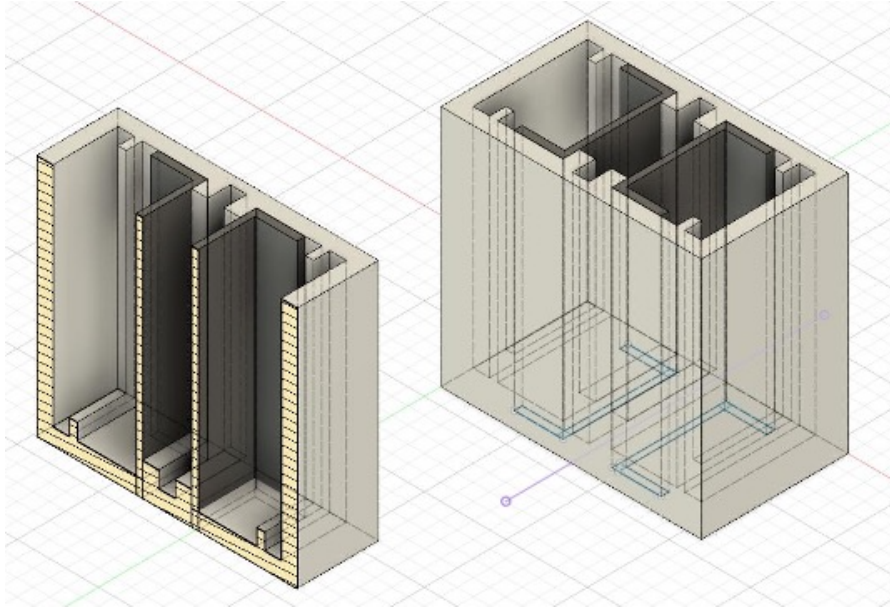


Fig. 4 Fuel cell cuboid design for 3D printing. Entire object and its section. The picture includes the fuel cell body (grey) and the supports for the salt bridge (black). Project realized with AutoDesk Fusion 360® software.

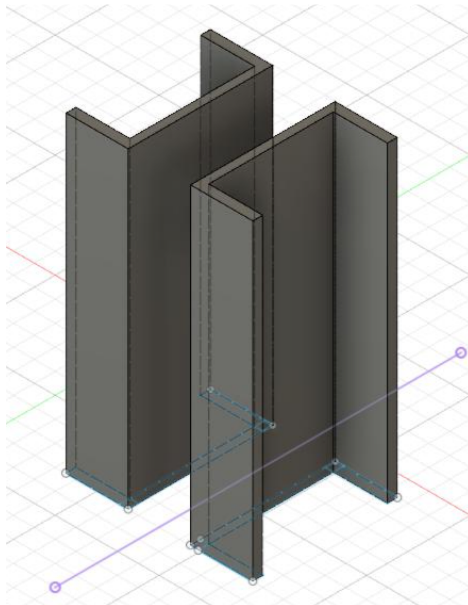


Fig.5 Design of removable supports for the salt bridge in the cuboid 3D-printed fuel cell. Project realized with AutoDesk Fusion 360® software.

Design characteristics

Material: ABS (weight = 40 g)

Geometry: cuboid

Dimension: 80 mm x 45 mm (base) x 75 mm (height). Capacity of the anodic and the cathodic chambers: 67 cm³. Salt bridge thickness: 15 mm.

Printing parameters

T plate = 110°C, T nozzle = 235°C, printing speed = 60 mm/s, printing time = 8.40 h

Slicing features

ABS polymer is an amorphous material, and its usage requires some care because, during its 3D-printing cooling phenomena can bring an inhomogeneous deposition of the printed layer, creating breaks or holes in the final object. A high plate temperature must be settled with the plate fan turned off to avoid this. Then, the closing of the printing chamber is suggested so that the drafts and temperature changes in the external environment cannot affect the printing process. Moreover, this precaution limits the escape of toxic fumes into the air.

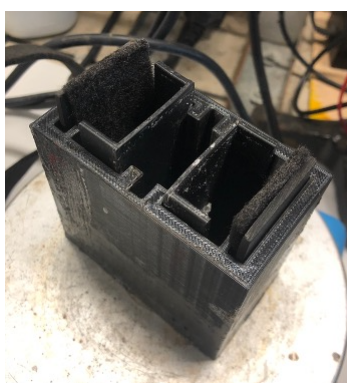


Fig. 6 Picture of the 3D-printed fuel cell with cuboid design. The picture includes the fuel cell body, the removable supports, and the electrodes (carbon felt and graphite plate).

- *Effect of glucose concentration*

To study the new system's configuration, the effect of glucose concentration on power and current generation was evaluated (Fig.7a-7b). It revealed that a solution of glucose 0.25 M is sufficient to generate 0.76 mW/cm² of power density, which is 20-fold higher than the power generated by the Woulfe bottle system (0.025 mW/cm²). These performances are maintained with glucose 0.5 M (0.77 mW/cm²) and decrease for higher glucose concentrations up to 0.64 mW/cm² (Table 1).

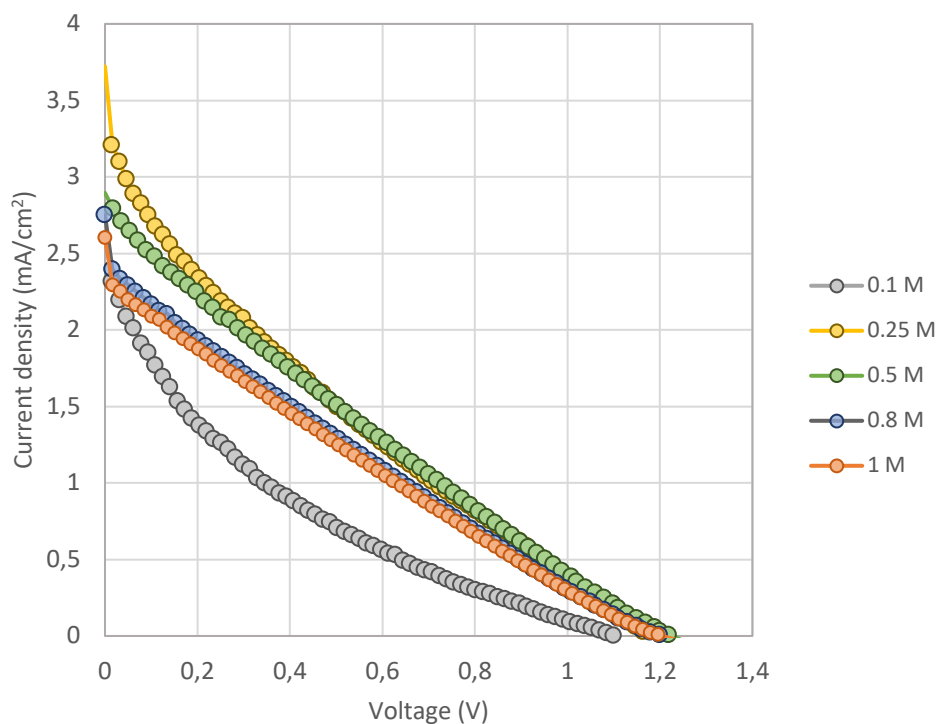


Fig.7a Effect of glucose concentration on I-V curves recorded on a 3D-printed fuel cell with the cuboid design. Anolyte: glucose at variable concentration + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl_3 1M. Electrode: activated carbon felt AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

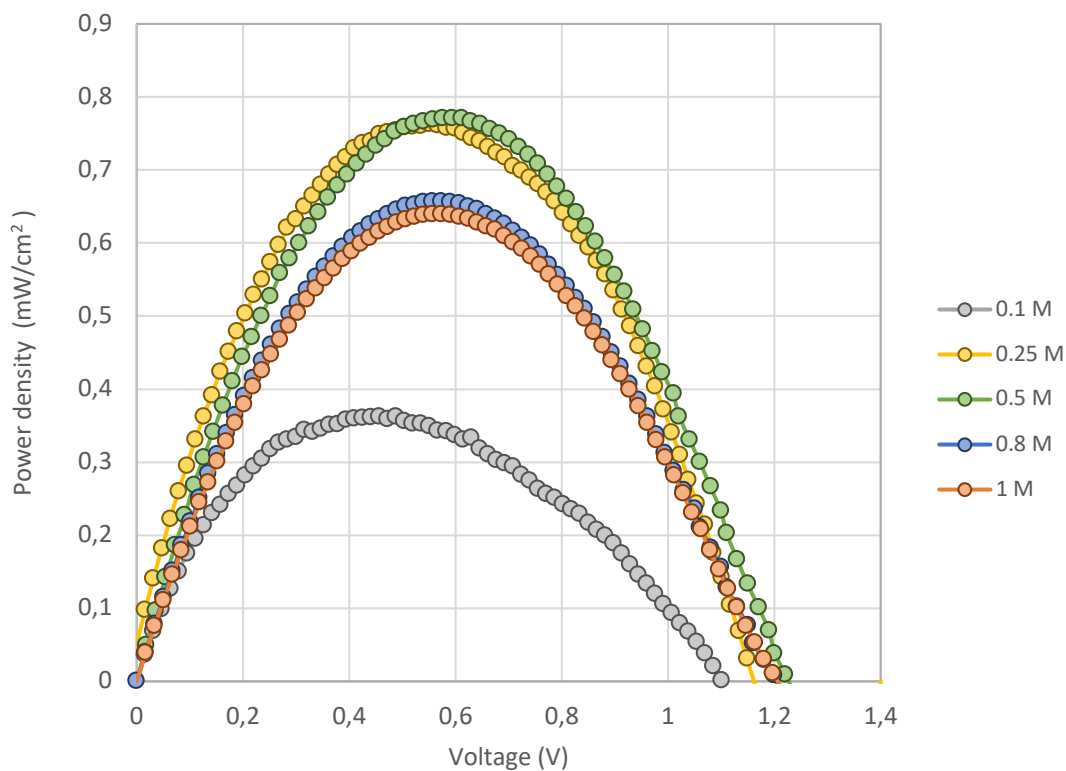


Fig.7b Effect of glucose concentration on power curves recorded on a 3D-printed fuel cell with the cuboid design. Anolyte: glucose at variable concentration + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl_3 1M. Electrode: activated carbon felt AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

Table 1 Effect of the glucose concentration on the fuel cell performance (cuboid design). Open Circuit Voltage (OCV), Short Current Intensity (ISC), Power density (P), and peak voltage (V). OCV and ISC are mean values over 5 minutes of measurement. Electrode felt: AvCarb 100.

Cuboid design				
Glucose (mol/L)	OCV (V)	ISC (A)	P max (mW/cm²)	Peak Voltage (V)
0.1	1.32	0.09	0.36	0.45
0.25	1.37	0.15	0.76	0.55
0.5	1.40	0.12	0.77	0.57
0.8	1.39	0.11	0.65	0.54
1	1.39	0.11	0.64	0.55

The I-V profiles (Fig.7a) indicate that the current density increased up to 0.25 M glucose concentration and decreased at higher concentrations. The Ohmic resistance (V/I), extrapolated by the curves' slope, decreased from 0.1 M to 0.25 M and then slightly increased from glucose 0.5 M to 1 M. Using activated carbon felt as an electrode ensures a good charge transfer and does not show significant potential losses. However, its proper usage in our system requires the addition of an electron collector between the felt and the electronic junctions of the measurement station to avoid the ascent of the electrolyte solutions by capillarity up to the electronic junctions. Indeed, the extreme solution's pH (14 in anolyte and 3 in catholyte) could lead to oxidation and corrosion of the electronic components. To overcome such problems, a graphite plate has been placed as an electron collector, in tight contact with the carbon felt electrode (Fig.6).

- *The effect of graphite plate*

The effect of the presence of the intercalated graphite plate is shown in Fig.8-10, which were compared the two electrode configurations (carbon felt with and without graphite plate), in terms of fuel cell performances OCV, ISC and power and polarization curves. The fuel cell is fueled by glucose 1M. In the presence of both felt and plate, the system (OCV = 1.20V; ISC = 0.07 A) generates 0.44 mW/cm² of power output at 0.40 V, in contrast to 0.64 mW/cm² at 0.56 V produced in the plate absence (OCV = 1.39V; ISC = 0.11 A).

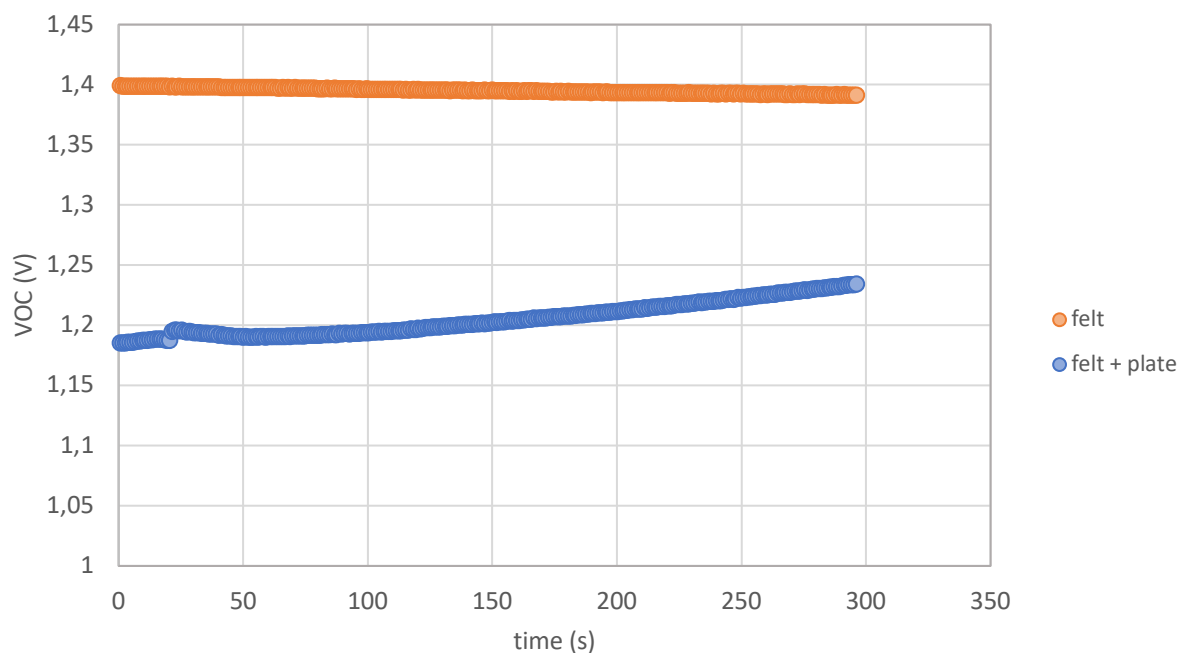


Fig.8 OCV (V) recorded on a 3D-printed fuel cell with the cuboid design. Comparison between electrode configurations. Anolyte: glucose at variable concentration + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode: activated carbon felt AvCarb 100 and graphite plate. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

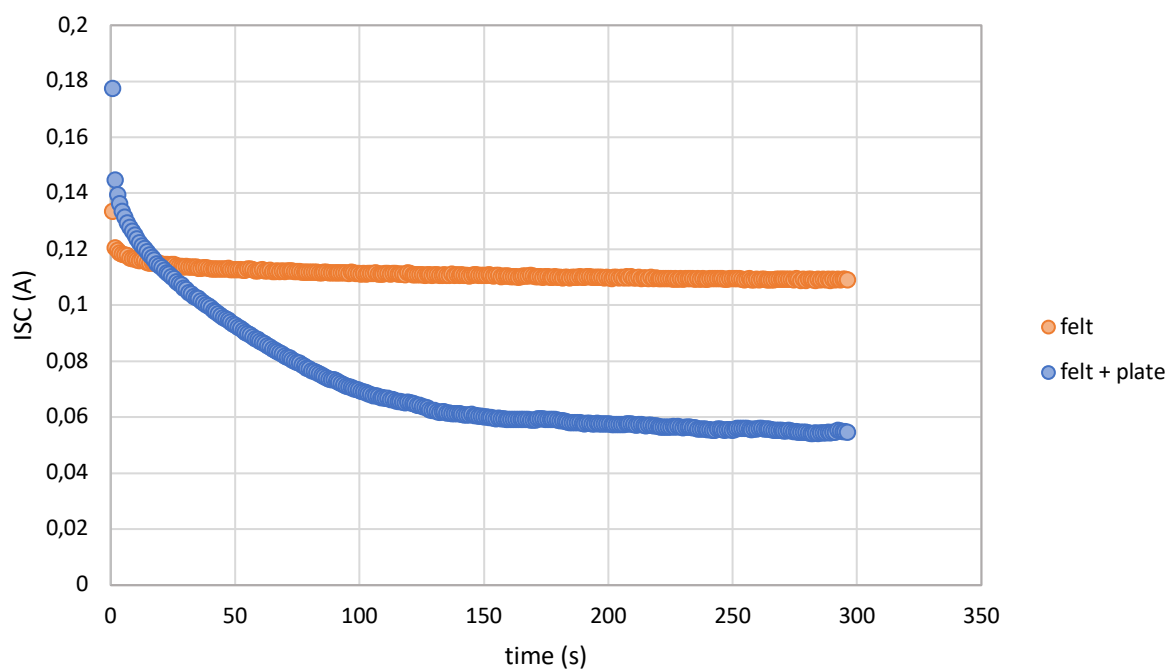


Fig.9 ISC (A) recorded on a 3D-printed fuel cell with the cuboid design. Comparison between electrode configurations. Anolyte: glucose at variable concentration + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode: activated carbon felt AvCarb 100 and graphite plate. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

The charge transfer process appears to be more hindered in the presence of a graphite plate (Fig.10), probably for an inefficient adhesion to the carbon felt. Likewise, the recorded OCV and ISC seem to be affected by the placement of the graphite plate, causing a poor interaction between reactant species in the bulk and the electrode surface. This could also be due to the lack of surface uniformity of the graphite plate, which was obtained by cutting a larger plate into two smaller pieces generating irregular grooves on the surface (Fig. 11), and different paths for electrons flow.

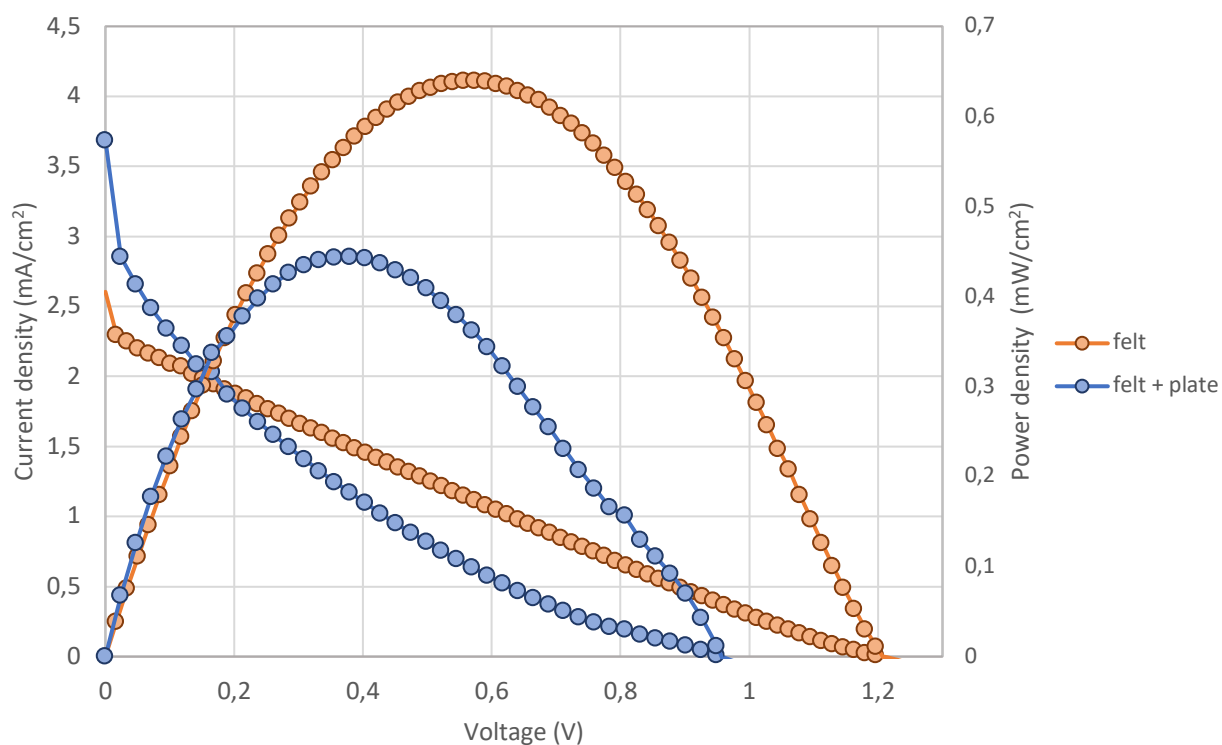


Fig.10 I-V (left axis) and Power curves (right axis) recorded on a 3D-printed fuel cell with the cuboid design. Comparison between electrode configurations. Anolyte: glucose 1M +NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode: activated carbon felt AvCarb 100 and graphite plate. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

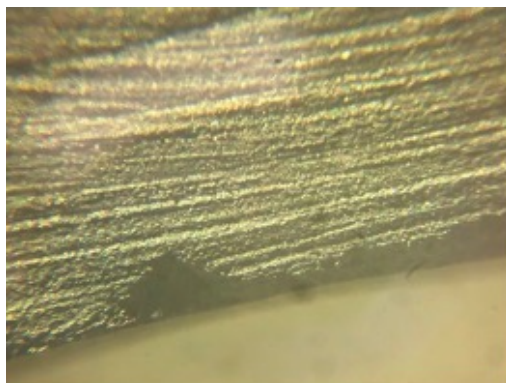


Fig.11 Picture of a graphite plate after cutting (detail).

The current fuel cell configuration places limitations not only in ensuring a good charge transfer but also in letting the fuel cell operate in a continuous way. Indeed, suppose an inlet feed pipe is inserted into a reactor channel with a square cross-section. In that case, the flow expands into larger channels, producing longitudinal variation of mass transfer and spanwise distribution of the mass transfer coefficient. In the recirculating flow region, mass transfer coefficients are highest at the center of the electrode and decrease toward the edges (Goodridge & Scott, 1995). That may cause problems related to current distribution: primary current distribution occurs in the absence of electrode reaction when the surface overpotential is neglected; secondary current distribution appears when an electrochemical reaction occurs, and the electrode surface can no longer be considered an equipotential boundary. Finally, tertiary distribution exists when a shift in potential causes significant concentration changes so that mass transport limits, to some extent, the current flow (Goodridge & Scott, 1995).

The cylindrical design

The first change in 3D-printed fuel cell design led to a cylindrical geometry configuration (Fig. 12). A first gain of this kind of system is given by the opportunity to have a more extensive anodic section suitable to contain larger volumes of anolyte solution for the same catholyte quantity. This choice is driven by the need to process variable volumes of agro-industrial waste to obtain electrical energy. The new configuration is suitable for a fuel cell operating in batch mode. It has a circular crown section at the center, constituted by a salt bridge that separates the catholyte (inner section) from the anolyte (outer section). These features require printing a sort of cage (Fig. 13) to be inserted in an anode chamber designed to accommodate the electrodes, suspended from the bottom, to ensure the proper stirring of the solution by stir bars. The electrode system comprises graphite rods encircled by a stripe of activated carbon felt AvCarb 100. In addition, also the printing of two concentric and removable cylinders is needed for the salt bridge realization (Fig.14). The picture of the 3D-printed object is in Fig. 15.

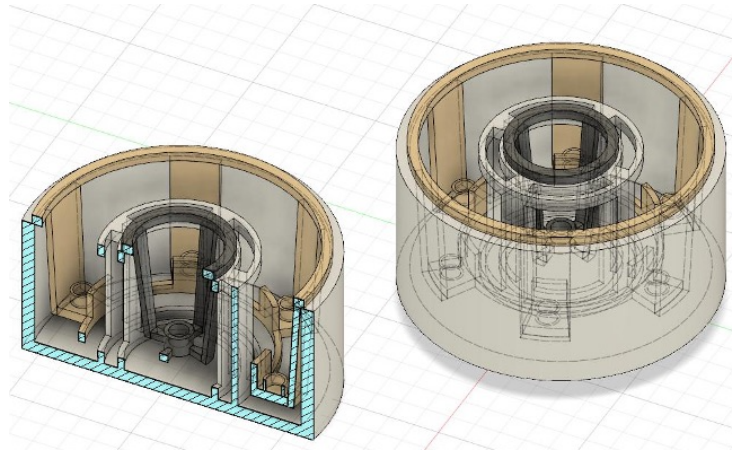


Fig. 12 Fuel cell cylindrical design for 3D printing. Entire object and its section. The picture includes the fuel cell body (grey), supports for the electrodes at the anode (brown), and supports for the electrodes at the cathode (black). Project realized with AutoDesk Fusion 360® software.

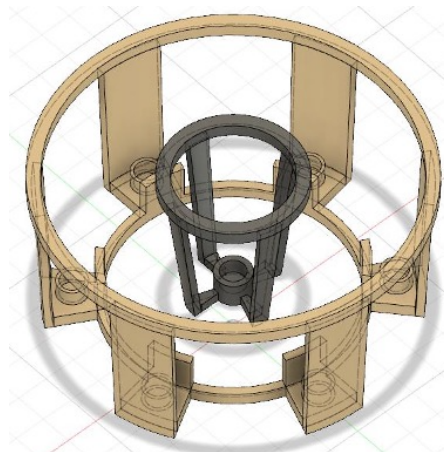


Fig.13 Design for the electrode supports in the cylindrical 3D-printed fuel cell. Anode support: brown. Support for the cathode: black. Project realized with AutoDesk Fusion 360® software.

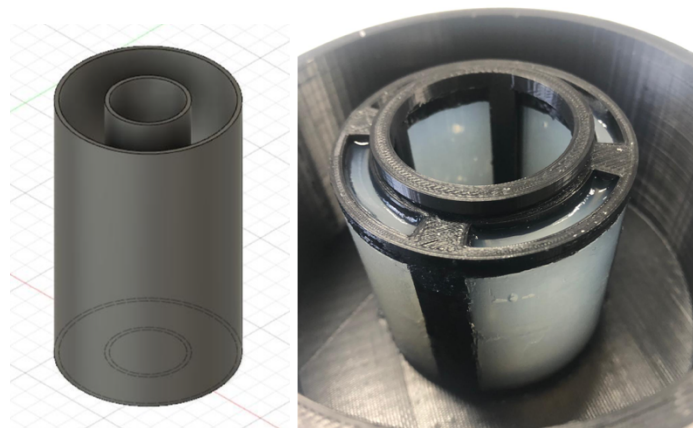


Fig. 14 Design of removable supports for the salt bridge in the cylindrical 3D-printed fuel cell. Project realized with AutoDesk Fusion 360® software (left). Picture of the 3D-printed cylindrical fuel cell containing salt bridge after gelification (right).

Design characteristics

Material: ABS (weight = 94 g)

Geometry: cylindrical

Dimension: 20 mm (inner radius), 55 mm (outer radius), 100 mm (height). Capacity of the anodic chamber: 367 cm³. Capacity of the cathodic chamber: 69 cm³. Salt bridge thickness: 10 mm.

Printing parameters

T plate = 110°C, T nozzle = 235°C, printing speed = 60 mm/s, additional supports generation, printing time = 12 h

Slicing features

Given the geometrical complexity of this design with respect to the previous cuboid one, the parameter “generate support” is set up. It is used when the 3D printing of raised components without a base plate is required to avoid object deformation by pouring. It is possible to set the support pattern (such as zig-zag, grides, concentric, etc.), the distance between them and the first deposited layer, and the overlap degree among them. The printer generates autonomously these supports that can be removed at the end of the printing.



Fig.15 Picture of the 3D-printed fuel cell with cylindrical design. The picture includes the fuel cell body, the removable supports, and the electrodes: 3 rods encircled by a felt ring at the anode and 1 rod encircled by a felt stripe at the cathode.

- *Effect of felt sonication*

The first study on the new design concerns the effect of an ultrasound bath on the coordination capacity of the material with the aqueous solutions. It has been demonstrated that the macro / micro streaming processes and cavitation events arising from the application of ultrasound can significantly influence electrochemical processes (Casimero, Hegarty, McGlynn & Davis, 2020). For example, it can impact the anodization processes at the carbon fiber by increasing the penetration of the electrolyte through the network, removing bubbles, and expediting delamination of mechanically weak carbonaceous layers (Casimero et al., 2020). The comparison between the performances of the fuel cells containing not pretreated and pretreated activated carbon felt by sonication are shown in Fig. 16.

From the curved recorded results that the system with the sonicated felt reaches higher maximum power which is 0.45 mW/cm^2 (peak voltage = 0.44 V), in contrast to 0.26 mW/cm^2 generated without sonication on felt. Furthermore, as a result of the sonification process, the increase of mean OCV (from 1.20 V to 1.31 V) and the ensuing increase in ISC (from 0.104 A to 0.196 A) show a higher degree of coordination with the solution, attributable to the higher hydrophilicity and wetting capability due to the pretreatment (Casimero et al., 2020). Thus, this procedure is repeated for each of the following experiments.

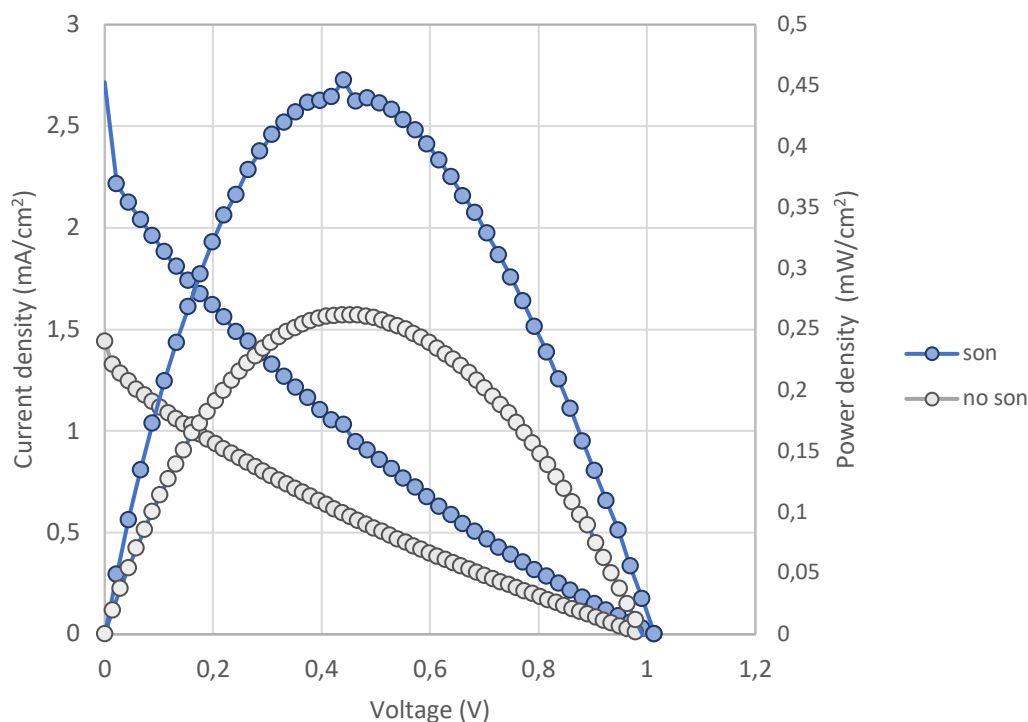


Fig.16 I-V (left axis) and Power curves (right axis) recorded on a 3D-printed fuel cell with the cylindrical design. Comparison between electrode pretreatment (with and without sonication). Anolyte: glucose 0.25 M +NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl_3 1M. Electrode configuration: one rod encircled by a carbon felt stripe at the cathode, and one encircled by a carbon felt ring at the anode. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

- *Effect of rod number in the anode*

Another investigation conducted on this fuel cell design concerns the effect of the crescent number of rods in the anodic chamber to find the proper condition to ensure the maximum collection capacity of the electrons generated from the reaction (Table 2). The power and polarization curves of the system, including from 1 to 4 rods (Fig. 17a-17b), show that the higher power density is generated with three equidistant rods at the anode. For the same configuration, a plateau in mean ISC values is reached (Fig.18), indicating it is the necessary condition to collect all the electrons released.

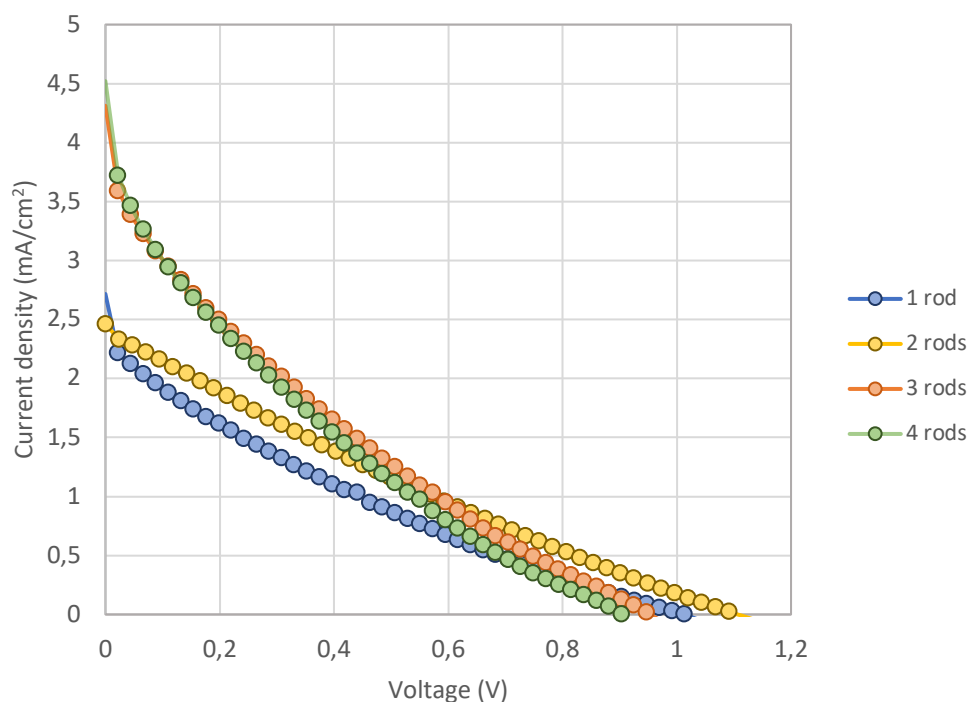


Fig.17a Effect of rod number at the anode on I-V curves recorded on a 3D-printed fuel cell with the cylindrical design. Anolyte: glucose 0.25 M + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode configuration: one rod encircled by a carbon felt stripe at the cathode, variable rod encircled by a carbon felt ring at the anode. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

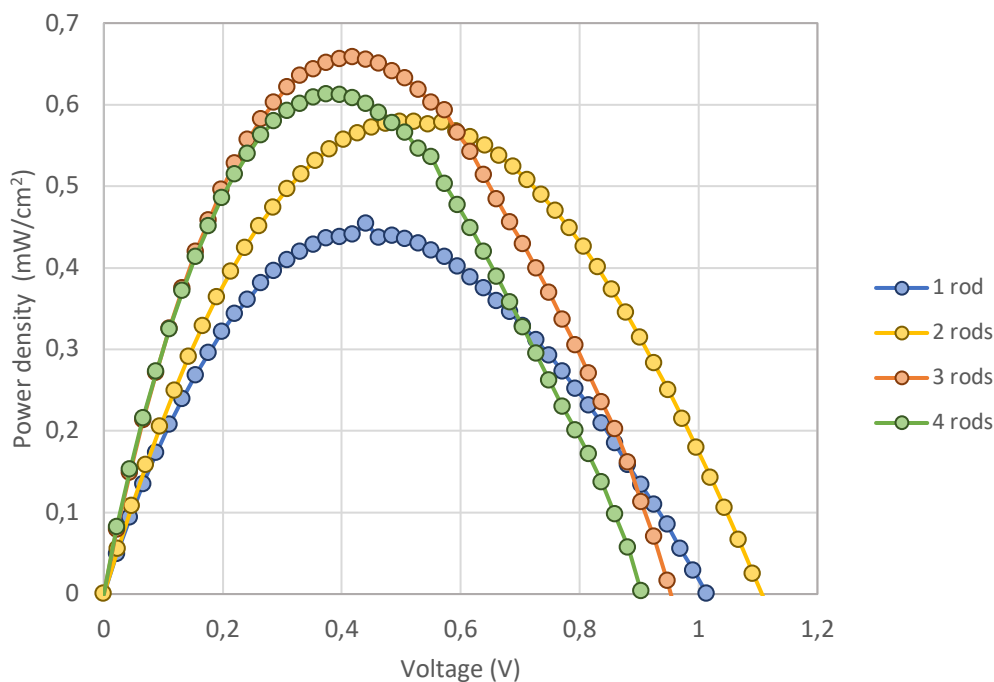


Fig.17b Effect of rod number at the anode on power curves recorded on a 3D-printed fuel cell with the cylindrical design. Anolyte: glucose 0.25 M + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode configuration: one rod encircled by a carbon felt stripe at the cathode, variable rod encircled by a carbon felt ring at the anode. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

Table 2 Effect of the rod number at anode on the fuel cell performance (cylindrical design) in terms of Open Circuit Voltage (OCV), Short Current Intensity (ISC), Power density (P), and peak voltage (V). Anolyte: glucose 0.25 M + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode configuration: rods encircled by a continuous ring felt stripe. OCV and ISC are mean values over 5 minutes of measurement. Electrode felt: AvCarb 100.

Cylindrical design				
Rod number In the anode	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)
1	1.25	0.19	0.45	0.45
2	1.39	0.23	0.58	0.50
3	1.14	0.29	0.66	0.40
4	1.22	0.29	0.61	0.40

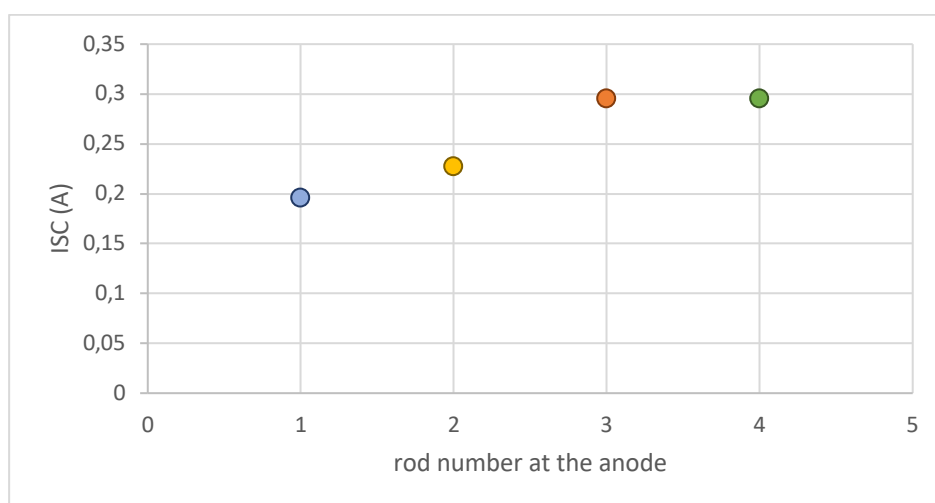


Fig.18 Mean values of ISC (A) as a function of rod number at the anode in the 3D-printed fuel cell with the cylindrical design. Anolyte: glucose 0.25 M + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M.

- *Effect of electrode configuration*

Finally, the influence of the electrode arrangement was evaluated (Table 3) by comparing the characterization curves of the fuel cells realized with the anode configuration: 1) three graphite rods encircled by three felt stripes; 2) three rods surrounded and linked by only a long carbon felt ring; 3) three graphite rods encircled by three felt stripes and connected by a carbon felt ring. As appears from Fig.19, the continuous connection among the rods ensures a higher power output.

Table 3 Effect of the electrode configuration at anode on the fuel cell performance (cylindrical design) in terms of Open Circuit Voltage (OCV), Short Current Intensity (ISC), Power density (P), peak voltage (V), and Current density (J) under peak voltage load (20 min). Anolyte: glucose 0.25 M + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode configuration: 3 rods encircled by a continuous felt ring (felt ring); 3 rods encircled by separated felt stripes (felt stripes); 3 rods encircled by separated felt stripes and connected with felt ring (felt stripes + ring). OCV and ISC are mean values over 5 minutes of measurement. J is expressed as the mean value over 20 minutes. Electrode felt: AvCarb 100.

Cylindrical design					
Electrode configuration	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)	J load (mA/cm ²)
Felt ring	1.14	0.29	0.65	0.42	1.26
Felt stripes	1.15	0.16	0.62	0.37	1.16
Felt stripes + ring	1.38	0.39	0.68	0.41	1.46

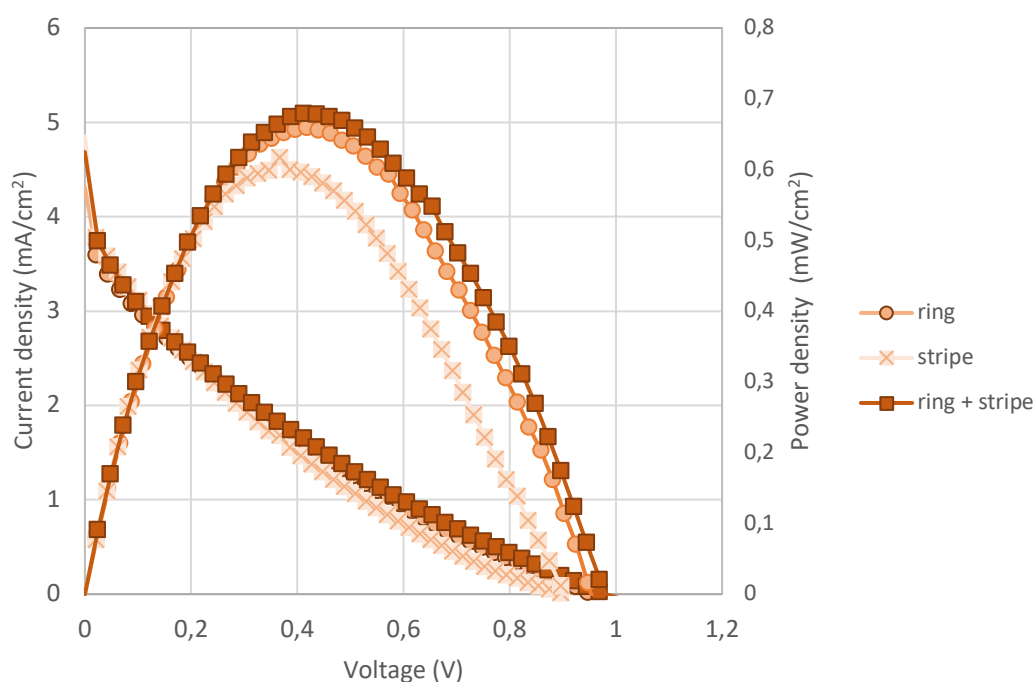


Fig.19 I-V (left axis) and Power curves (right axis) recorded on a 3D-printed fuel cell with the cylindrical design. Comparison between electrode configurations. felt ring = 3 rods encircled by a continuous felt ring; felt stripes = 3 rods encircled by separated felt stripes; felt ring + stripes = 3 rods encircled by separated felt stripes and connected with felt ring. Anolyte: glucose 0.25 M + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

By applying a constant load equal to the peak voltage extrapolated from the power curves of each system, the trend of the current density generated over time has been recorded (Fig.20). The curves reported in Fig.20 are normalized with respect the current density at the initial measurement time

and show a more constant trend for the system including both felt stripes surrounding each rod and a longer felt stripe closed loop ring around them, which can generate 1.46 mA/cm² under 0.4 V over 30 min of reaction.

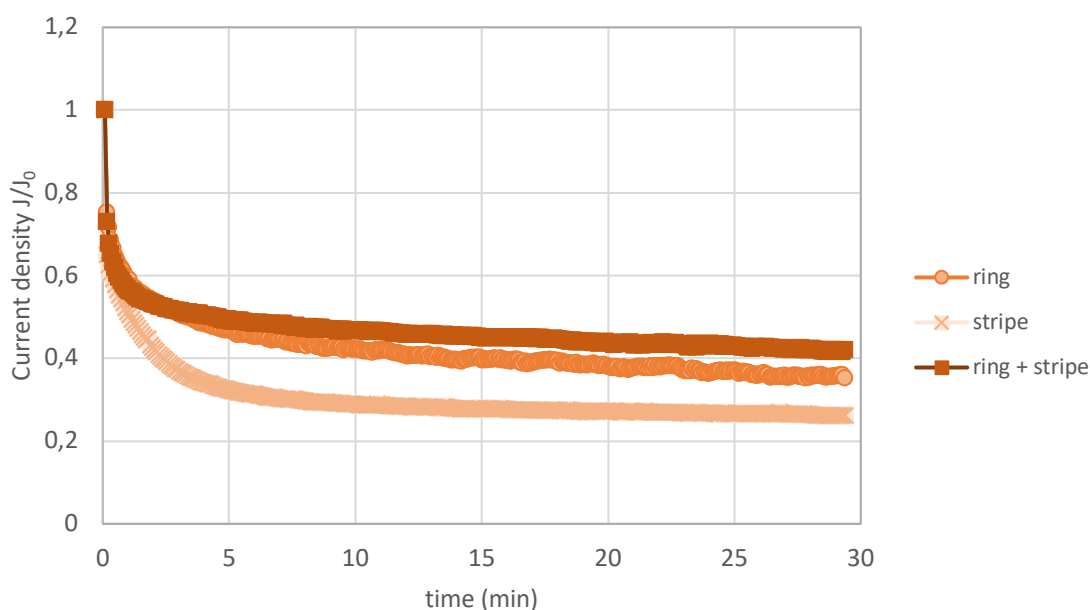


Fig.20 Current density (mA/cm²) normalized with respect to the value recorded at time zero. 3D-printed fuel cell with the cylindrical design. Comparison between electrode configurations. felt ring = 3 rods encircled by a continuous felt ring; felt stripes = 3 rods encircled by separated felt stripes; felt ring + stripes = 3 rods encircled by separated felt stripes and connected with felt ring. Anolyte: glucose 0.25 M +NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

The cylindrical fuel cell design allowed the processing of a larger volume of anolyte (200 cm³) compared to the first 3D printed design (50 cm³). However, the current and power density generated are comparable to the ones reached in the cuboid design, pointing out that in the batch cylindrical design, there is no gain in volumetric power density, and thus in the efficiency in terms of current generated per cm³ of processed solution.

Furthermore, although convenient for initial experimentation, the cell design's simplicity limits the stirred reactor's applicability for up-scaling (Perry, de León & Walsh, 2020). The use of magnetic stirrer bars gives a limited mass transport range with poor reproducibility due to the variable stirrer position, non-standardized stirrer bar size, and interaction of stirred solution with the beaker wall (Perry et al., 2020). These considerations introduced further modifications in the fuel cell design to obtain a more compact system, less affected by the mass transport process, capable of being fed in continuous mode, and easily scaled up to commercial scale.

The tubular design

The tubular design consists of two circular coaxial sections (the anode and cathode chambers) separated by a circular crown in which the salt bridge is allocated as a selective ion medium. It has the same shape as the cylindrical fuel cell, but it has half the diameter. It is equipped to work with a flux feeding through the inlet and outlet pipes entering in and coming out from the anodic and cathodic chambers (Fig. 21). The anode can hold up to 37 cm^3 of electrolytes (50 cm^3 if operating in batch mode); the cathode has a capacity of 16 cm^3 . The boundary of the anodic chamber contains six cylindrical cavities, which can hold up to six graphite rods, placed in tight contact with the activated carbon felt running along the cell perimeter. Instead, the cathode can allocate one rod at its center, encircled by the carbon felt rolled up on itself to fill the entire chamber (Fig. 22).

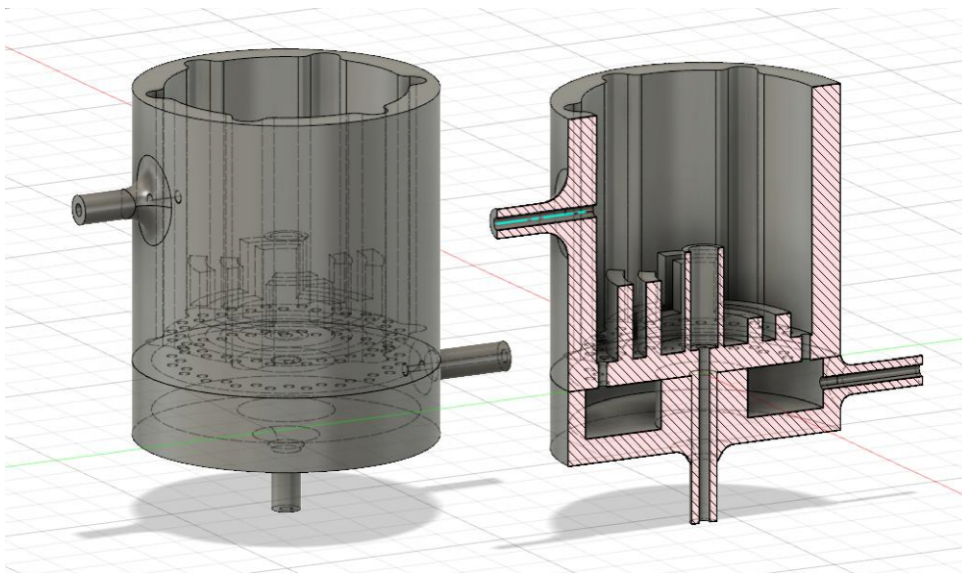


Fig.21 Fuel cell tubular design for 3D printing. Entire object and its section. The picture shows the fuel cell body (grey), including the pre-loading chamber at the anode (at the base of the cell). Project realized with AutoDesk Fusion 360® software.

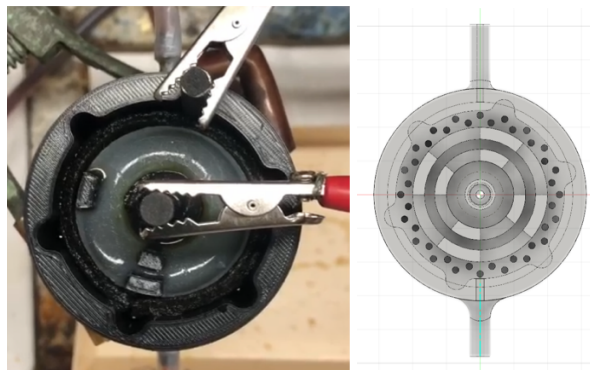


Fig.22 Picture of the 3D-printed tubular fuel cell (left) and project showing the cavities for the rod's accommodation at the anode (right). Top view.

The electrolytes can be inserted in counter-flow with a peristaltic pump. The feeding can occur with or without recirculation. The inlet of the anode is placed in correspondence with an inner perforated pre-chamber to allow the filling of the anodic chamber by the electrolyte, avoiding preferential paths. All the experiments reported in this section have been performed in semi-batch mode, with the cathodic electrolyte loaded before the reaction and unloaded at the end. The anolyte is supplied in a continuous way making the solution recirculate from and in the same tank by a peristaltic pump (Fig.23).

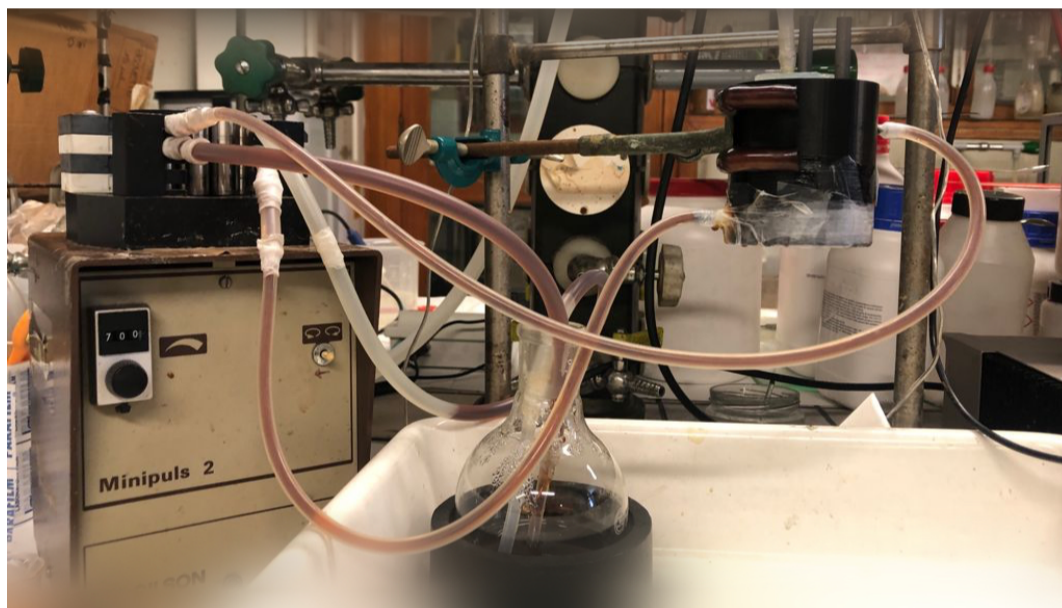


Fig.23 Picture of the 3D-printed tubular fuel cell (right) fed at the anode by a peristaltic pump (left), with the recirculating anolyte from and into a single tank (below).

As for the cylindrical design, 3D-printed additional removable supports are needed to contain the salt bridge under preparation.

On the contrary, no support for electrodes is needed because the rods and the carbon felt are placed directly on the fuel cell basis since mechanical stirring is absent. Furthermore, in the new design, the opportunity of acting on the felt electrode compression has been taken into account since it has been widely proven to favor lower resistance and higher battery efficiency ([Brown et al., 2016](#)). However, due to geometrical limitations, realizing sufficient compression in a tubular system is more challenging than in a planar system ([Percin et al., 2020](#)).

An advantage of tubular systems is that it enables a higher surface-to-volume ratio than planar systems. Therefore, when their geometry is scaled up, the volume increase of the tubular shape would be smaller than the one required in the cuboid geometry. Further, a smaller volume per surface area of tubular shape would be advantageous in terms of the power density of the electrochemical reactor because the volumetric power density (power per volume) of the systems can be improved ([Percin et al., 2020](#)). Therefore, having a cylindrical reactor for an electrochemical process may be more versatile in terms of scaling up the modules without increasing the volume as great as it is for the planar systems ([Percin et al., 2020](#)). This could help control heat exchange

changes on scale-up (Goodridge & Scott, 1995), even if, in our specific case, these potential problems are minimized since our fuel cell has been designed to operate at environmental temperature and atmospheric pressure. A modular approach to reactor design is common in electrochemical process engineering because electrodes have to occur in pairs (one anode and one cathode) and because there must be external electrical connections (Goodridge & Scott, 1995). Design features required for a modular configuration are narrow gaps between electrodes, good mass transfer characteristics, relatively easy replacement of electrodes, and relatively low-pressure drops. The newly designed tubular fuel cell system that has been thought to be stacked into a tube bundle configuration can quickly satisfy these requirements.

Design characteristics

Material: ABS (weight = 52 g)

Geometry: tubular

Dimension: 10 mm (cathode chamber radius), 25 mm (anode chamber radius), 93 mm (diameter including inlet and outlet nozzles), 75 mm (height), 85 mm (height including the outlet nozzle). Capacity of the anodic chamber: 35 cm³. Capacity of the cathodic chambers: 16 cm³. Salt bridge thickness: 10 mm.

Printing parameters

T plate = 110°C, T nozzle = 235°C, printing speed = 30-60 mm/s, additional supports generation, additional plate adhesion tools generation, printing time = 7 h

Slicing features

The slicing features considered in the previous designs are maintained. In addition, the printer speed parameter is optimized, by decreasing it for the initial layers to 3 mm/s and by setting the height of the extruded layers to a low value (0.2 mm) to ensure a better printing resolution, despite the possible abrupt changes in printer nozzle direction. Another important implementation is the addition of a plate adhesion support (brim, raft, or skirt), which ensures the lack of detachments of the object under printing.

- *Effect of catholyte regeneration*

The first study on this fuel cell design evaluated the influence of catholyte regeneration by molecular oxygen. The polarization curves of the fuel cell fed by glucose 0.25 M in the presence and in the absence of oxygen bubbling in the FeCl₃ catholyte solution are reported in Fig.24.

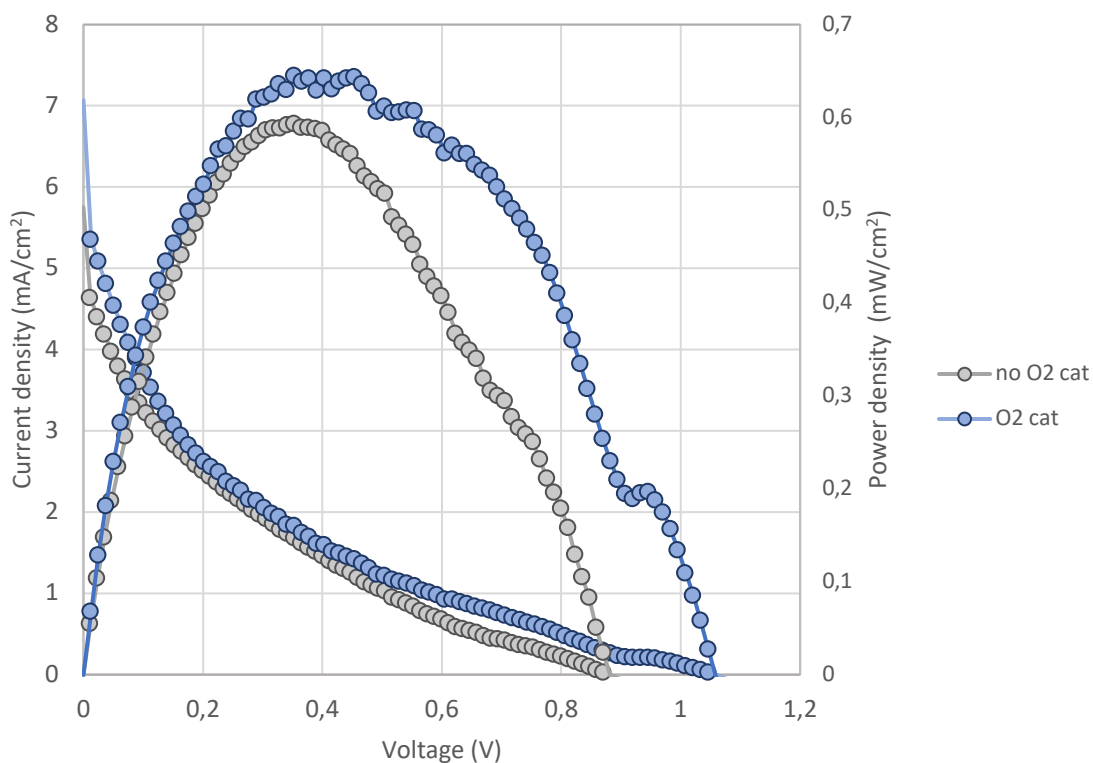


Fig.24 I-V (left axis) and Power curves (right axis) recorded on a 3D-printed fuel cell with the tubular design. Effect of bubbling O_2 at the cathode. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Anolyte: glucose 0.25 M + NaOH 0.8 M (15 min at 65°C). Catholyte: $FeCl_3$ 1M. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

Fig 24 does not show a significant difference in the maximum power output reached by the two systems (0.59 mW/cm² without bubbling O_2 , 0.65 mW/cm² bubbling O_2). This is because the regeneration effect could be significant over the long reaction term. Anyway, the condition of bubbling oxygen to promote the continuous formation of the active specie Fe^{3+} from the reduced Fe^{2+} has been maintained for most of the experiments.

- *Effect of glucose concentration*

The effect of the glucose concentration (Fig.25a-25b) in the new system has been evaluated by pumping the anolyte at constant velocity (20 mL/min) and by bubbling O_2 in the cathode. With the new conditions, the system generates crescent power and current density going from glucose 0.25 to glucose 1M, which reaches 1.20 mW/cm². At a constant load of 0.5 V (peak voltage), the system generates up to 1.86 mA/cm² over 30 min of reaction by recirculating the same anolyte (Table 4).

Table 4 Effect of the glucose concentration on the fuel cell performance (tubular design) in terms of Open Circuit Voltage (OCV), Short Current Intensity (ISC), Power density (P), and peak voltage (V). OCV and ISC are mean values over 5 minutes of measurement. Electrode felt: AvCarb 100. O₂ bubbling at the cathode.

Tubular design				
Glucose (mol/L)	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)
0.25	1.36	0.32	0.3	0.60
0.5	1.31	0.36	0.98	0.55
0.75	1.43	0.39	1.11	0.55
1	1.35	0.50	1.20	0.50

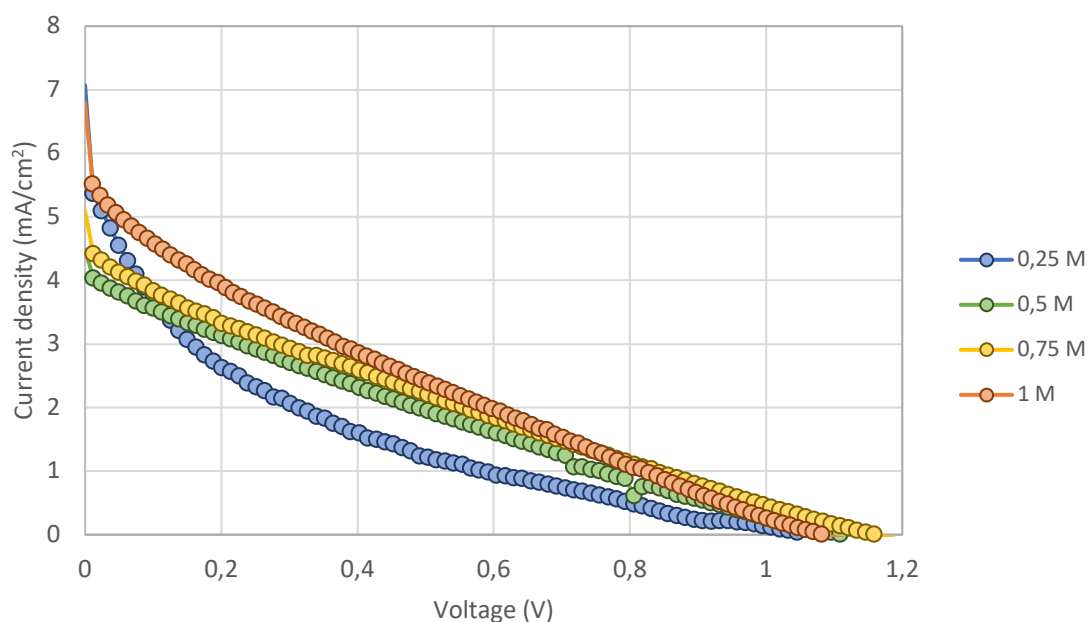


Fig.25a Effect of glucose concentration on I-V curves recorded on a 3D-printed fuel cell with the tubular design. Anolyte: glucose at variable concentration + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Bubbling O₂ at the cathode. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

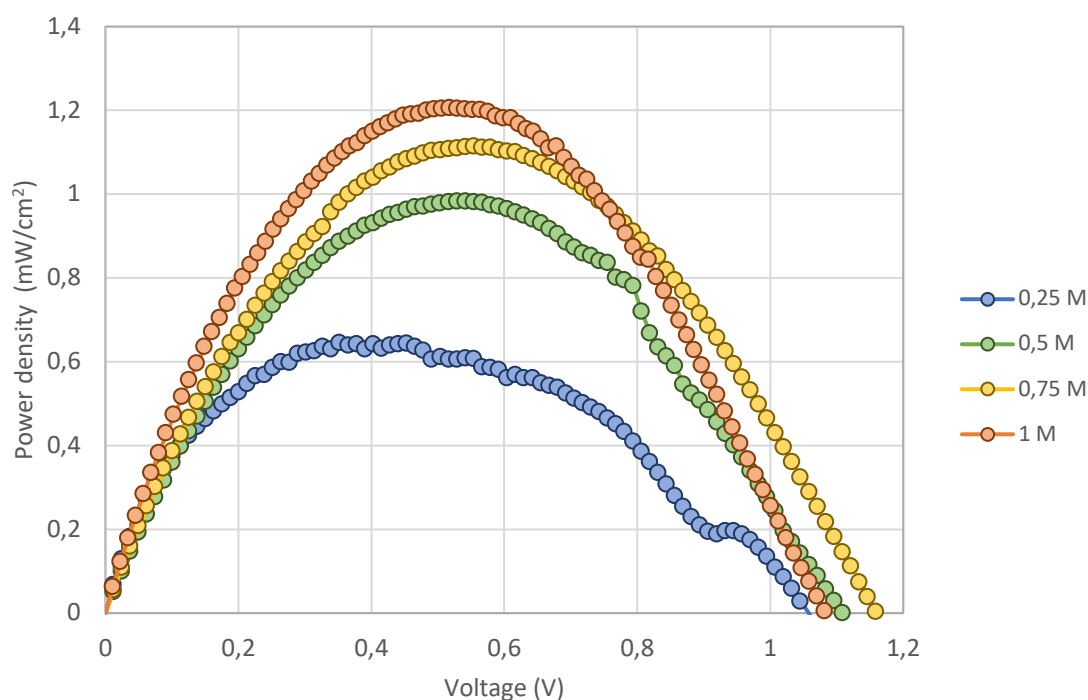


Fig.25b Effect of glucose concentration on power curves recorded on a 3D-printed fuel cell with the tubular design. Anolyte: glucose at variable concentration + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Bubbling O₂ at the cathode. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

As for the batch cylindrical design, the effect of rod number at the anode has been evaluated (Table 5). The reactor dimension is low enough to let the collection of electrons by only one graphite rod.

- *Series and parallel connections*

To verify the effectiveness of the modularity, two tubular fuel cells have been connected in series and parallel (Fig.26). For each configuration, the characterization curves have been recorded (Fig. 27-28).

Table 5 Effect of the rod number at anode on the fuel cell performance (tubular design) in terms of Open Circuit Voltage (OCV), Short Current Intensity (ISC), Power density (P), and peak voltage (V). OCV and ISC are mean values over 5 minutes of measurement. Electrode felt: AvCarb 100.

Tubular design				
Rod number In the anode	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)
1	1.38	0.50	1.10	0.45
2	1.33	0.46	1.11	0.45

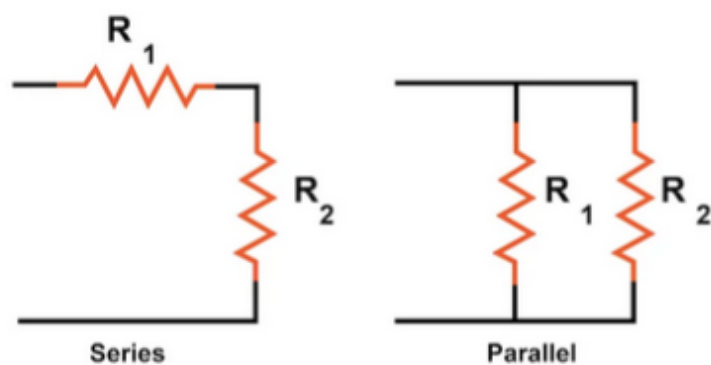


Fig.26 Schematic representation of series and parallel circuit connections.

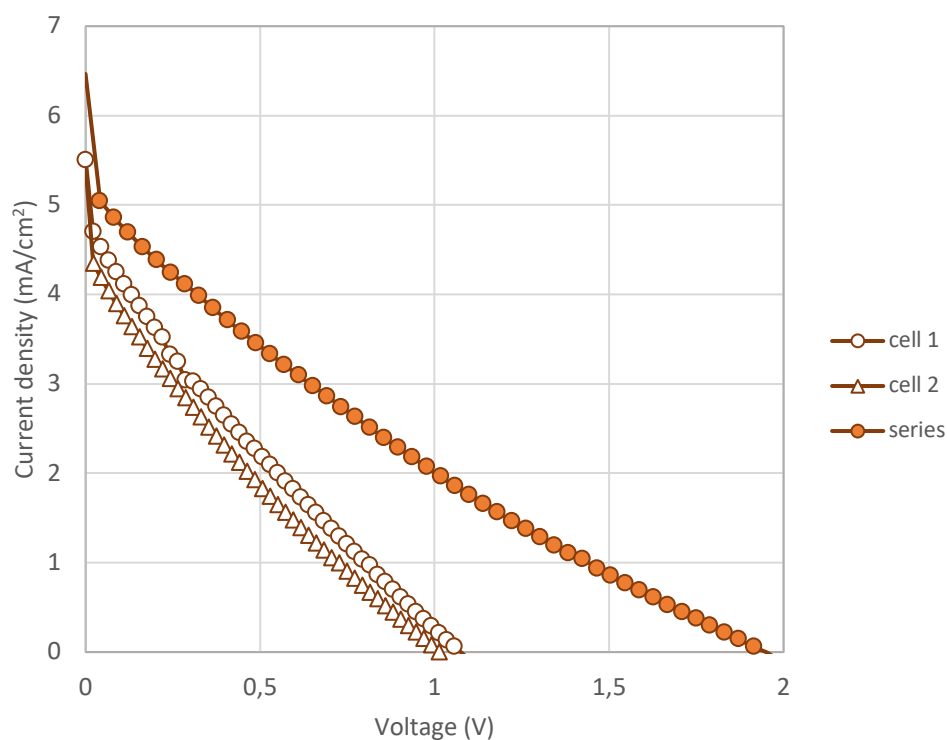


Fig.27a I-V curves recorded on two 3D-printed fuel cells with the tubular design, stacked in a series configuration. Comparison with the single cells. O_2 bubbled at the cathode. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Anolyte: glucose 1M +NaOH 0.8 M (15 min at 65°C). Catholyte: $FeCl_3$ 1M. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

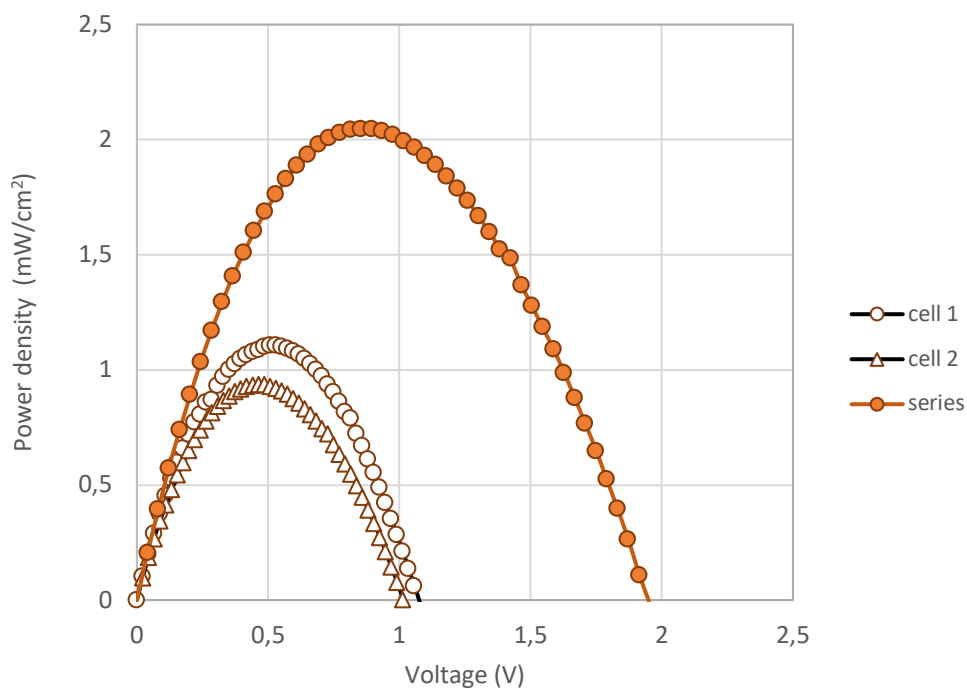


Fig.27b Power curves recorded on two 3D-printed fuel cells with the tubular design, stacked in a series configuration. Comparison with the single cells. O_2 bubbled at the cathode. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Anolyte: glucose 1M +NaOH 0.8 M (15 min at $65^\circ C$). Catholyte: $FeCl_3$ 1M. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at $30^\circ C$, p atm.

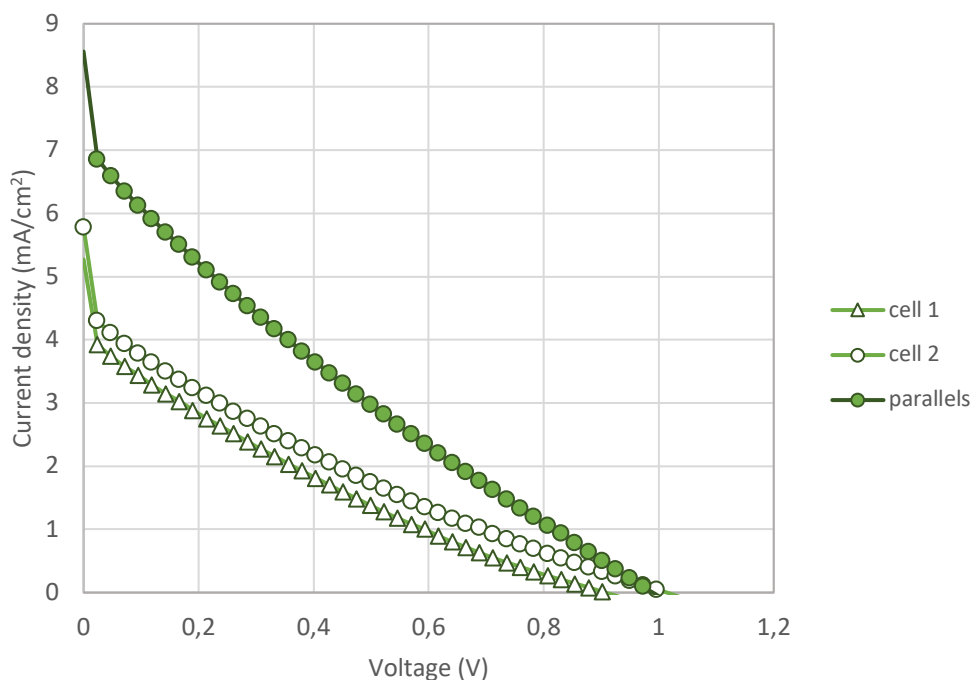


Fig.28a I-V curves recorded on two 3D-printed fuel cells with the tubular design, stacked in a parallel configuration. Comparison with the single cells. O_2 bubbled at the cathode. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Anolyte: glucose 1M +NaOH 0.8 M (15 min at $65^\circ C$). Catholyte: $FeCl_3$ 1M. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at $30^\circ C$, p atm.

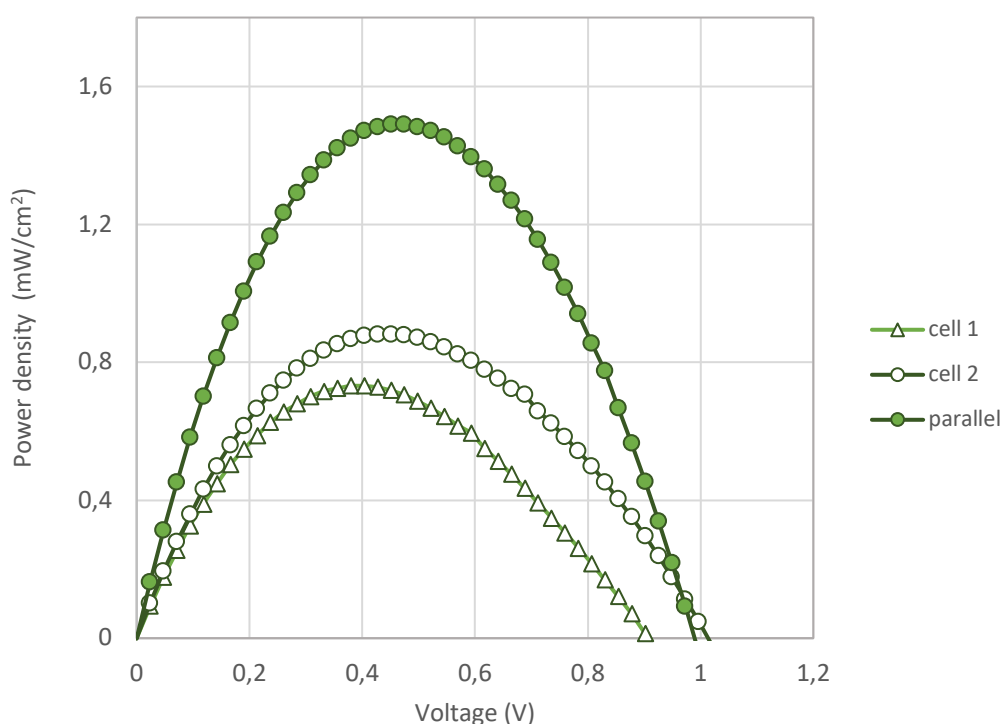


Fig.28b Power curves recorded on two 3D-printed fuel cells with the tubular design, stacked in a parallel configuration. Comparison with the single cells. O₂ bubbled at the cathode. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Anolyte: glucose 1M +NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Felt: AvCarb 100. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

Table 6 Comparison in modular cell configuration: two tubular fuel cells connected in series and parallel. Performance in terms of Open Circuit Voltage (OCV), Short Current Intensity (ISC), Power density (P), peak voltage (V), and Current density J under peak voltage load (20 min). OCV and ISC are mean values over 5 minutes of measurement. J is expressed as the mean value over 20 minutes. Electrode felt: AvCarb 100. O₂ bubbling at the cathode.

Tubular Design _modular configuration					
Series	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)	J load (mA/cm ²)
Cell 1	1.29	0.43	1.10	0.50	n.d.
Cell 2	1.27	0.42	0.93	0.46	n.d.
Series	2.33	0.48	2.05	0.85	2.09
Parallel	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)	J load (mA/cm ²)
Cell 1	1.35	0.43	0.73	0.40	n.d.
Cell 2	1.31	0.43	0.88	0.45	n.d.
Parallel	1.20	0.67	1.49	0.45	2.54

As expected, the two units stacked in parallel exhibits the early double short current circuit values for the same open voltage circuit, and the ones united in series present the double OCV for the same ISC. As a result, for each configuration early, the double power output is generated, respecting the one produced by the single unit, even if the series connections ensure a more reproducible performance since they are less affected by the current dependent overpotentials, including ohmic, activation and concentration/mass transport losses (Table 6).

Compared with the curves recorded on the previous designs, the ones of the tubular fuel cell exhibit a drift for high current densities, corresponding to concentration overpotentials. These overpotentials cause a potential loss due to mass transport limitations of electroactive species reaching, or leaving, the electrode surface and can be minimized by employing high surface area electrodes, high mass transport flow regimes, or turbulence promoters (Perry et al., 2020).

In the presence of 3D and porous structure of the electrodes, as the activated carbon felt, the mass transport is controlled by the capability of the electrolyte to penetrate the three-dimensional structure, whose thickness should be established to ensure that all the covered electrode surface is electrochemically active and with the same potential (Perry et al., 2020). When the cell is fueled in batch mode, the whole electrode surface is equally coordinated with the solution in a short time. On the contrary, when it is fueled in a continuous way, the coordination degree depends on the flow velocity, the time in which the solution is pumped, and the intrinsic characteristic of the 3D structure. In order to continue the flow, the liquid must move through the 3D electrode structure itself. This increases electrolyte-electrode interaction and a faster reaction rate (Perry et al., 2020). Our studies used two Activated Carbon Felt types: AvCarb 100 and AvCarb 280. Both the materials are sold by the same retailer with the same technical datasheet (<https://www.fuelcellearth.com/fuel-cell-products>), but exhibit different abilities in coordination with the electrolytes solutions, as verified by saturation measurements conducted on the felts inserted in fuel cells operating either in batch or flux mode (Table 7).

Table 7 Felt mass (g) as a function of coordination saturation time (min) with anolyte. Comparison between AvCarb100 and AvCarb280 after sonication, in batch and flux feeding mode.

Felt type	Feeding type	Felt coordination time (min)					
		10	20	30	40	50	60
AvCarb 100	Batch	17.3	17.5	17.7	17.6	17.6	17.6
	Flux	13.3	14.6	15.4	16.2	16.9	17.3
AvCarb 280	Batch	12.8	13.7	13.7	13.5	13.6	13.6
	Flux	9.1	11.4	11.9	12.7	13.4	13.5

Given such a discrepancy in the results, we decided to find a standard condition to let the electrodes be equally equilibrated with the electrolytes before the beginning of the fuel cell operation. The condition was the coordination of carbon felt by immersing them into the respective electrolyte solution for 30 min at 30°C before placing the same felts in the cell for the set-up.

- *Effect of the inlet flow rate*

Once standardize the coordination condition, the effect of the inlet flow rate has been estimated by varying it from 8 to 26 cm³/min (Fig. 29a-29b). The challenge in flow field design is to provide optimal flow conditions for reactant supply and product removal from the electrode surface while minimizing the pressure drop between the inlet and outlet (Perry et al., 2020).

Pressure drops in a tubular system can be related to the electrolytes' flow profile and the degree of felt compression (Percin et al., 2020). Electrolytes can flow -by or -through the electrode. Flow-by configurations flow electrolytes within a channel over the surface of the porous structure, usually assisted by turbulence promoters to maximize interaction between the electrolyte and the inner porous structure. Flow-through instead fills the channel with the porous structure, requiring the electrolyte to permeate the whole structure during the flow (Perry et al., 2020). In our tubular design, the catholyte flows by the electrode, whereas the anolyte flows through the electrode.

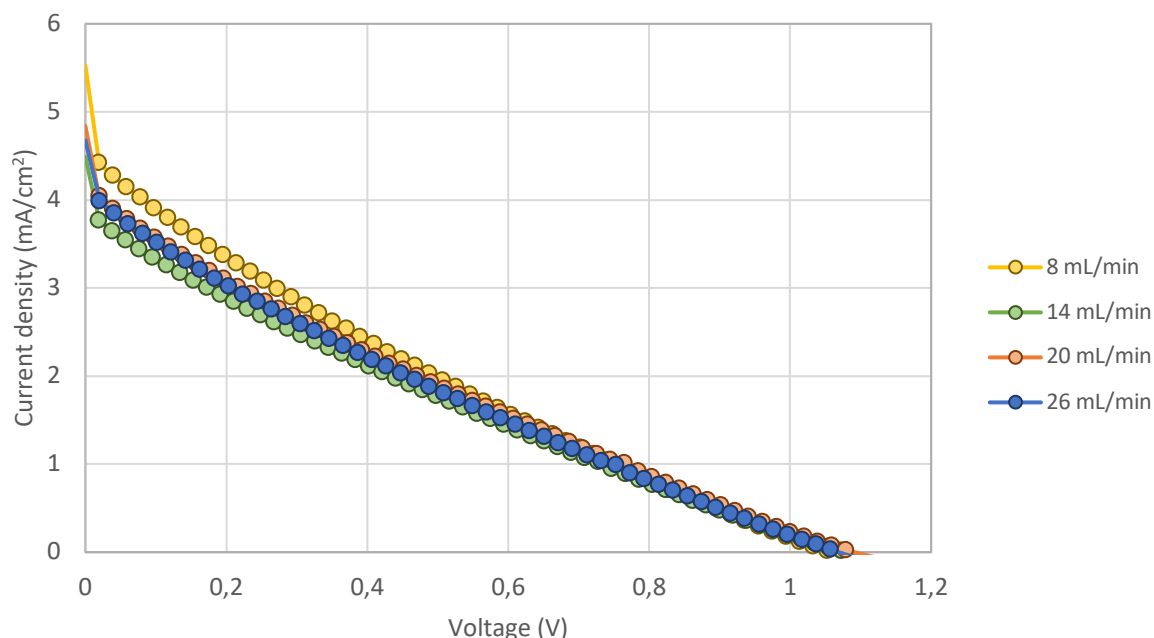


Fig.29a Effect of anolyte flow rate on I-V curves recorded on a 3D-printed fuel cell with the tubular design. Anolyte: glucose 1M + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 280. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

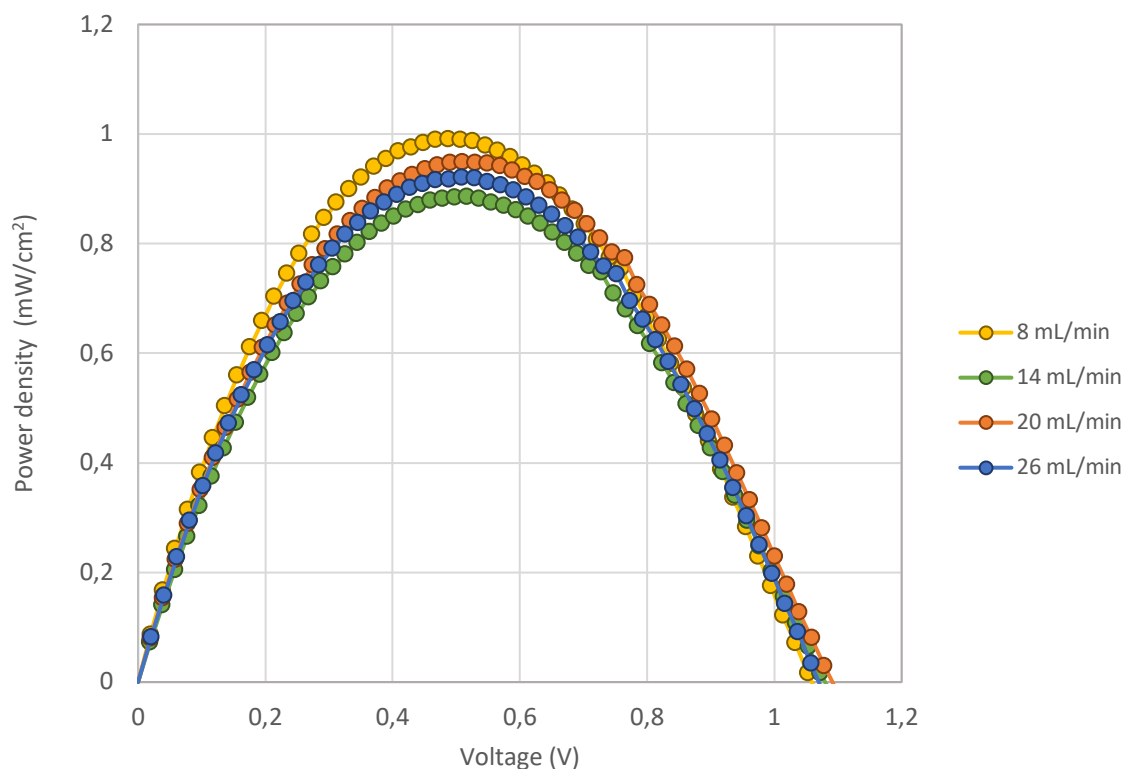


Fig.29b Effect of anolyte flow rate on power curves recorded on a 3D-printed fuel cell with the tubular design. Anolyte: glucose 1M + NaOH 0.8 M (15 min at 65°C). Catholyte: FeCl₃ 1M. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 280. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

Table 8 Effect of anolyte flow rate (mL/min) on the fuel cell performance (tubular design) in terms of Open Circuit Voltage (OCV), Short Current Intensity (ISC), Power density (P), and peak voltage (V). Anolyte: from the reaction between glucose 1M and NaOH 0.8 M. OCV and ISC are mean values over 5 minutes of measurement. Electrode felt: AvCarb 280.

Tubular design				
Anolyte flow rate (mL/min)	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)
8	1.30	0.39	0.99	0.50
14	1.34	0.33	0.88	0.50
20	1.35	0.38	0.96	0.50
26	1.38	0.40	0.92	0.50

Looking at the characterization curves at the crescent inlet flow rate, relevant variations are not observed (Table 8). This suggests that the current in the considerate system is governed by the reaction rate rather than by the diffusion of fuel to the electrode surface, which is not advantageous if the power output in a flow system wants to be tuned. The maximum power density and corresponding cell voltage for each experiment confirmed what was observed in our studies carried out so far. In more detail, the newly designed tubular fuel cell powered by glucose 1M solution, previously activated by reaction with NaOH 0.8 M ($T=65^{\circ}\text{C}$, 15 min), generated up to $1.2\text{ mW}/\text{cm}^2$ of power per electrode surface, corresponding to $120\text{ mW}/\text{unit}$. The tubular fuel cell generates up to $3.20\text{ mW}/\text{cm}^3$ of anolyte processed, in contrast to $0.61\text{ mW}/\text{cm}^3$ of maximum power output generated by the cuboid design, confirming that the tubular design gives higher efficiency, producing significantly higher volumetric power density.

The last experiments' results show the need to operate under the diffusion-limited regime. Thus, the conduction of a study in this sense is suggested. A first evaluation can be obtained by conducting the cell reaction for crescent glucose concentration in static mode, which excludes other effects beyond the reaction. In addition, before introducing the flow feed, a change in anodic flow configuration is advisable by favoring the flow-by over the flow-through one since the latter is limited due to pressure drop across the porous electrode material. Other potential improvements may concern the activation of graphite rods by chemical methods to enhance their hydrophilicity ([Shanahan et al., 2021](#)), as well as their replacement with a hollow tube of graphite that can be totally in tight contact with the felt electrodes. In such a case, a further narrowing of the fuel cell dimension could be considered.

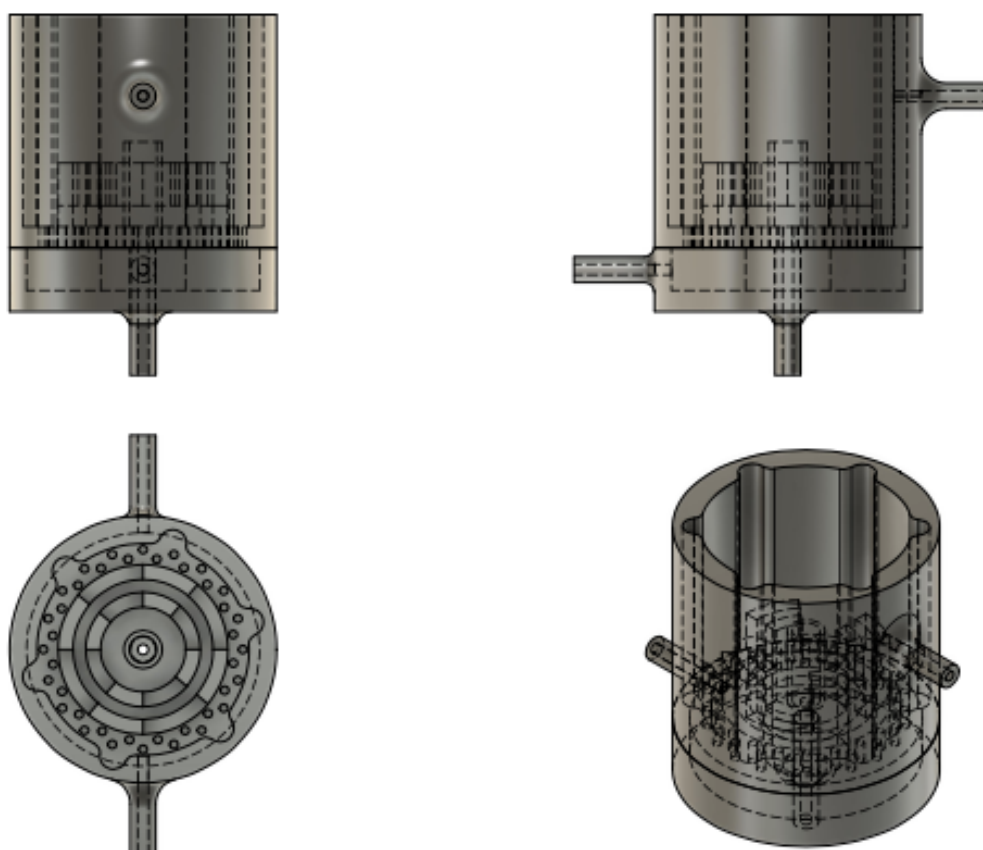


Fig. 30 Design project (projection) of the tubular 3D-printed biofuel cell.

At the end of the design study, the fuel cell depicted in Fig. 30 has been realized and suggested as the base unit for the energy plant proposed in this thesis ([Setti & Maggiore, pending patent](#)). It has been designed to be easily stacked in modules and to operate under a continuous flow of electrolytes, but it is also suitable to work in static mode. The tubular fuel cell was 3D-printed by fused filament deposition of ABS polymer by setting fine slicing and printer parameters, with respect to the first printing setting, to obtain a high-quality device, as reported below. This implies a higher printing time (12 h) and a higher polymer consumption (59 g) but ensures the obtainment of an object without any defects.

Fig.31 reported the phases involved in the 3D printing of the specific device.

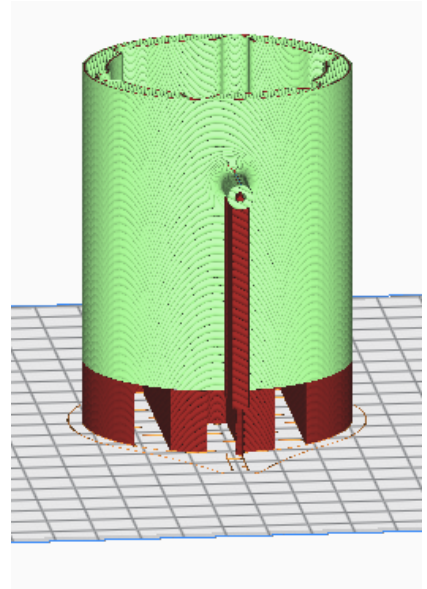
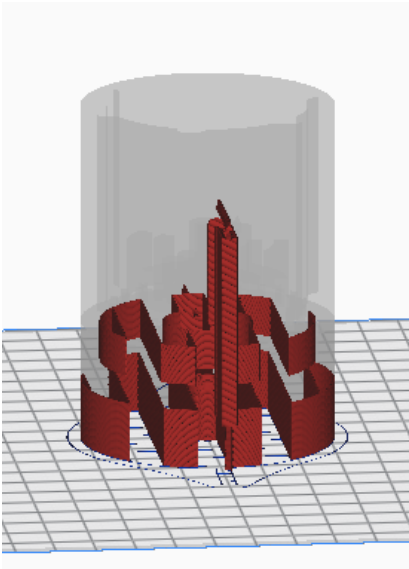


Fig. 31. 3D-printing phases in tubular fuel cell: printing of supports (red) and fuel cell body (green). The skirt-type adhesion feature can be noticed on the base (plate surface). The images are generated by Ultimaker Cura 4.9 software.

Definitive printing parameters:

T plate = 110°C
 T nozzle: 235°C
 Print T start: 230°C
 Print T end: 210°C
 Height of the layer: 0.1 mm
 Initial height of the layer: 0.15 mm
 Line width: 0.4 mm
 Wall width: 0.8 mm
 Filling density: 20%
 Filling pattern: grid
 Print speed: 60 mm/s
 Travel speed: 120 mm/s
 Initial layer speed: 30 mm/s
 Initial travel speed: 60 mm/s
 Print supports: everywhere
 Support overlapping: 50%
 Support pattern: zig zag
 Support density: 5%
 Plate adhesion: skirt
 Skirt distance: 3 mm

Abbreviations

OCV	Open Circuit Voltage
ISC	Short Current Intensity
P	Power density
J	Current density
Jload	Current density under peak voltage load

4B 3D-printed tubular fuel cell applications

The objective of designing a new energy capable of converting agro-food biomass into energy is to let the small-scale biorefinery and facilities effectively manage their waste while producing electrical energy to integrate the energy supply needed for the transformation and/or valorization of agro-food materials. A way to encourage the development of small-scale biorefinery processes is to promote the processing of raw materials close to the production site, minimizing the transport distance and expenditures, the usage of low-energy demand processes and low-cost equipment, the creation of high-value products expendable on local markets ([Bruins & Sanders, 2012](#)). Our new 3D-tubular glucose biofuel cell has been designed to accomplish these tasks ([Setti & Maggiore, patent pending](#)). Its body is realized with low-cost material, which is ABS polymer or other recyclable thermoplastic polymers; it includes no precious metals in electrode systems, which is totally carbon-derived and hence organic-based; it operates at ambient conditions ($T=30^{\circ}\text{C}$, $p = 1 \text{ atm}$) and does not require expensive catalysts because they are contained in the same biofuel solution as alkaline electrolyte. The biofuel is generated in the upstream alkalization unit by the hydrolysis of lignocellulosic biomass mediated by alkali, which produces reactive enediols from the glucose, and other reducing sugars. Glucose molecules are the building blocks of lignocellulosic biomass, and they can also be released by the direct hydrolysis of solid biomass mediated by NaOH. Thus, the solution derived from its alkaline pretreatment is then used as anolytes in powering the 3D-printed fuel cell. The opportunity to use NaOH also has the advantage of ensuring the sterility of the organic feedstock, making abiotic the fuel cell condition. At the end of biomass conversion into energy, exhausted organic materials are discarded, and their neutralization can generate organic soil improver for agriculture, thus providing a value-added product.

Fuel cell powered by Biofuel from glucose

The alkalization step is usually carried out by providing a heating supply in order to favor the obtainment of biomass hydrolysis products. In the first stage of our experiments (Chp. 4A), we performed the reaction to generate the anolyte solution at 65°C for 15 min, using commercial glucose as a model molecule for biomass and sodium hydroxide as a model for low-cost catalyst. The reaction conditions have been chosen after performing preliminary tests intended to find the temperature ([Romanello, 2019](#)) and time (Chp. 4A) to reach the highest formation of enediols with reasonable energy costs. The enediol formation in an alkaline environment is promoted by heat. It is ascribable to a transitory stage of the glucose chain rearrangement, which can be followed by isomerization, β -elimination and sugar degradation pathways, depending on the sugar and base concentration ([Sirisangsawang & Kitchaiya, 2019](#)). Once overcoming the so-called “yellow point,” these degradative processes become irreversible, forming brown-red colored solutions.

To save more energy needed during the conversion process, we implemented the alkalization unit by overcoming the external heating supply. So, we performed a series of hydrolysis to find the reaction conditions that allow the progress of the reaction above the “yellow point” just by exploiting the heat released by the exothermic dissolution of NaOH into an aqueous solution.

First, we evaluated the effect of NaOH concentration on the quantity of heat released by reaction with glucose 1M (Fig.1).

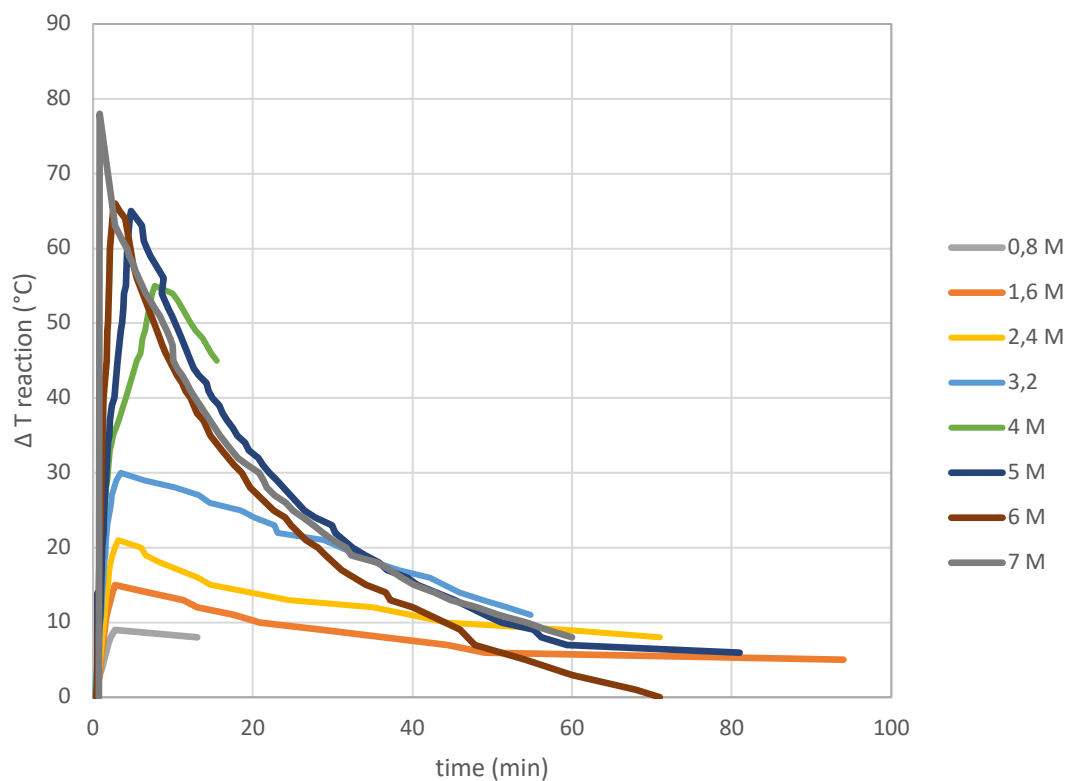


Fig.1 ΔT of glucose 1M and NaOH reaction at a crescent NaOH concentration (mol/L). Reactions carried out without heating supply.

This study identified the NaOH threshold concentration to reach a temperature above 40°C ascribable to a rapid formation of the glucose degradative products. It resulted in being 3.2 M, as shown in Fig.2, in which the maximum temperature reached by the dissolution process is correlated with the temperature to which the reddish solution is observed (Table 1).

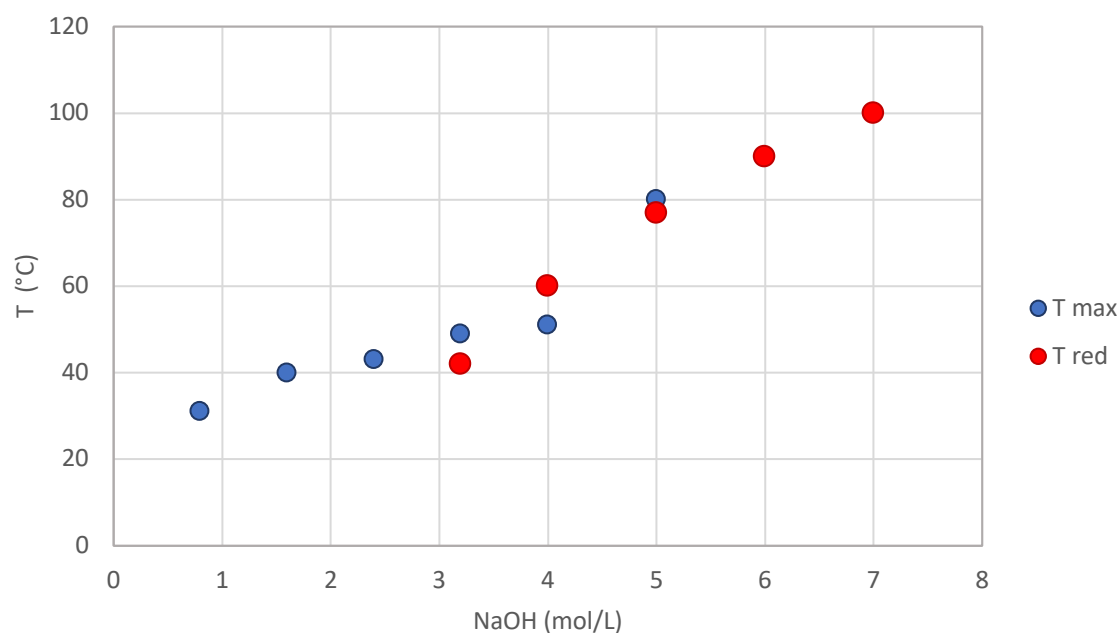


Fig.2 T max (°C) reached for each NaOH concentration and the Tred (°C) at which solutions turn red during the reaction with glucose 1M, without an external heating supply.

Table 1 Effect of NaOH concentration (mol/L) on the temperature profile of the reaction with glucose 1M. T max (°C) points to the maximum T reached at the time t max (min). T red points to the T at which the reddish solution is observed at the time t red (min). n.d. stand for not detected.

NaOH (mo/L)	0.8	1.6	2.4	3.2	4	5	6	7
Tmax (°C)	31	40	43	49	51	80	90	100
t max (min)	2.7	2.8	3.2	3.5	7.7	4.8	2.8	0.8
T red (°C)	n.d.	n.d.	n.d.	42	60	77	90	100
t red (min)	n.d.	n.d.	n.d.	28	3	3	2	1

Then we evaluated the effect of glucose concentration on heating released over time, keeping constant the NaOH concentration at 3.2 M (Fig.3). This study reveals that also exists a glucose threshold concentration to turn the solution into a reddish color, corresponding to 1M (Fig.4) (Table 2).

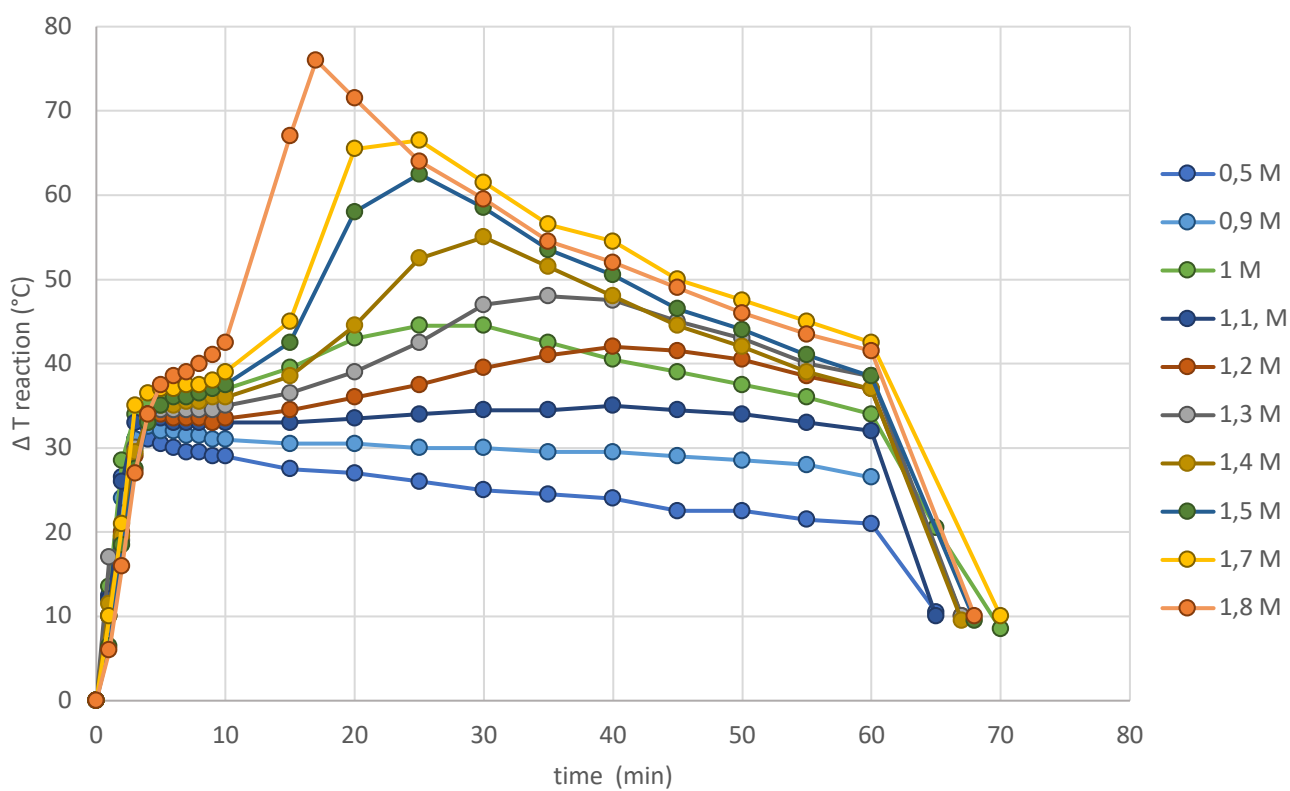


Fig.3 ΔT of the reaction between glucose at a crescent concentration (mol/L) and NaOH 3.2 mol/L. Reactions carried out without heating supply in an insulated beaker.

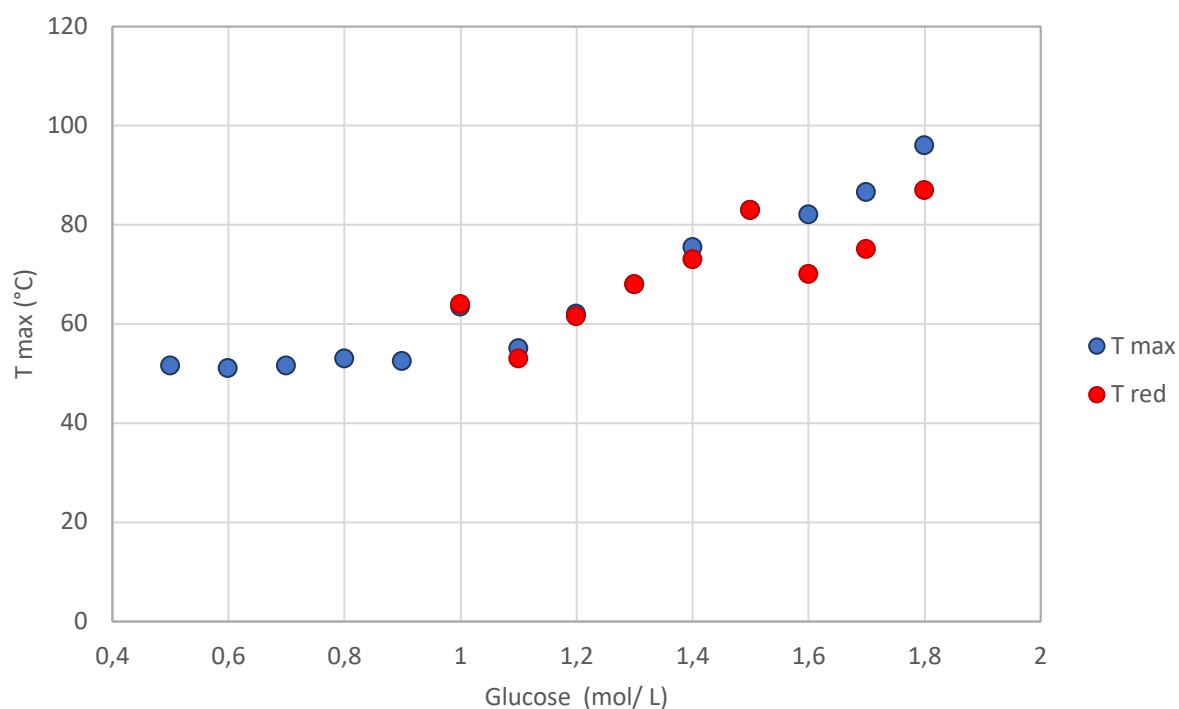


Fig.4 T max (°C) reached for each glucose concentration and the Tred (°C) at which solutions turn red during the reaction with NaOH 3.2 M, without an external heating supply.

Table 2 Effect of glucose concentration (mol/L) on the temperature profile of the reaction with NaOH 3.2M. T max (°C) points to the maximum T reached at the time t max (min). T red points to the T at which the reddish solution is observed at the time t red (min). n.d. stand for not detected.

Glucose (mol/L)	0.5	0.6	0.7	0.8	0.9	1	1.1
Tmax (°C)	51.5	51	51.5	53	52.5	63.5	55
t max (min)	3	4	4	4	4	25	40
T red (°C)	n.d.	n.d.	n.d.	n.d.	n.d.	64	53
t red (min)	n.d.	n.d.	n.d.	n.d.	n.d.	30	55

Glucose (mo/L)	1.2	1.3	1.4	1.5	1.6	1.7	1.8
Tmax (°C)	62	68	75.5	83	82	86.5	96
t max (min)	40	35	30	25	25	25	17
T red (°C)	61.5	68	73	83	70	75	87
t red (min)	45	35	25	25	20	17	15

For glucose 1M and NaOH 3.2M, at least ten minutes at T above 60°C are required to observe it, corresponding to 30 min of reaction since the addition of NaOH. If the heat released by the reaction is not stripped away, for glucose concentration above 1.8 M, there has been an uncontrolled temperature rise above 100°C. For glucose concentration above 2 M, the viscosity of the solution increased significantly, hindering the sodium hydroxide tablet dissolution. Thus, any significant increase in temperature ($T > 60^{\circ}\text{C}$) is observed.

We also identified the threshold temperature of the ambient and of the solution to trigger the reactions listed above, which are respectively $18 \pm 0.5^{\circ}\text{C}$ and $20 \pm 0.5^{\circ}\text{C}$.

Looking at Fig. 3, it can be noted that within the first 15 minutes of the reaction, the temperature trend follows the same profile for each glucose concentration. After the first 15 minutes of reaction time, a second exponential and chain reaction seem to be triggered, but just in the presence of glucose concentration above 1 M. For these reaction conditions, the formation of a second contribution (right side of the graph in Fig. 3) is observed, which becomes more important and faster for a more concentrated glucose solution. High base and glucose concentrations promote reactions that lead to aldolization products or $>\text{C}_6$ acids (Fig.5) (Sirisangsawang & Kitchaiya, 2019).

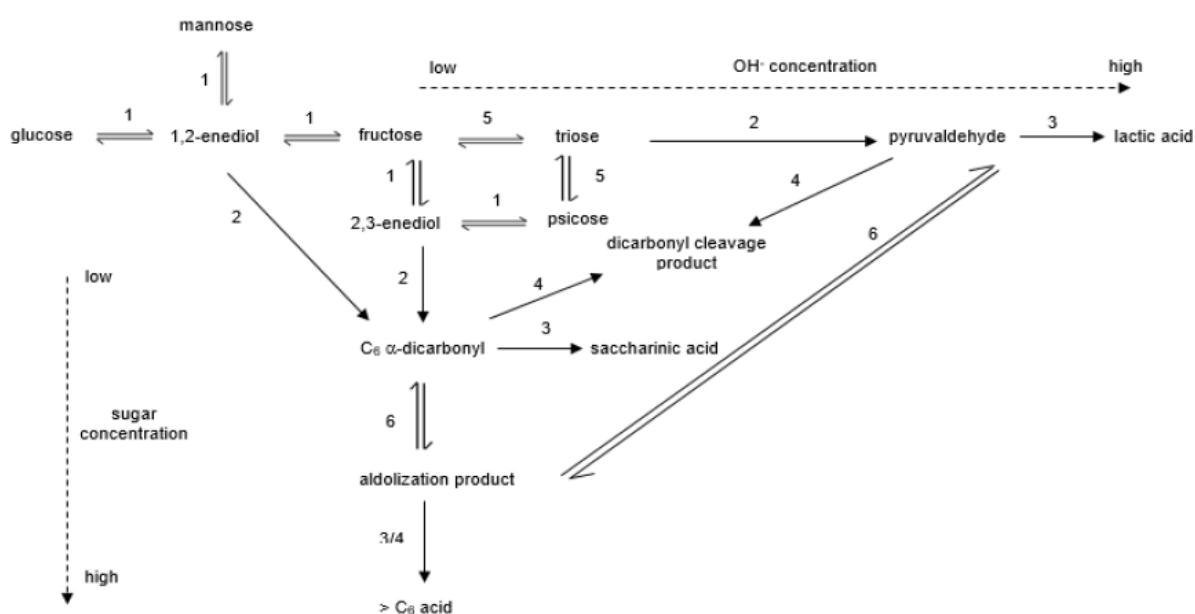


Fig.5 Reactions of glucose in the alkaline environment: 1 enolization/isomerization; 2 β eliminations, 3 benzylic acid rearrangements, 4 cleavages of bonds between carbonylic carbons, 5 aldolization and retro aldolization, 6 aldolization and retro aldolization of carbonylic compounds (Sirisangsawang & Kitchaiya, 2019).

If we look at Fig.6, showing the UV spectrum of the solution produced by the reaction between glucose 1M and NaOH 3.2M, we can see correspondence with the abovementioned phenomena. Indeed, a difference can be observed in the band shape and peak before and after 15 minutes of reaction. After 15 min, a shift of the peak at 290 toward lower wavelengths is observed, as well as the appearance of a peak toward 210 nm and a band at early 265 nm are observed. The same features are found in a lower quantity of NaOH (Chp. 4A), with the difference that for NaOH 3.2 M, the contribution at 210 nm prevails over the others. The peak at 210 nm could be referred to as

lactic acid (<https://sielc.com/compound-lactic-acid>), in accordance with the reaction pathways occurring for higher NaOH concentration (Fig.5). Also, the lactic acid formation reaction is in equilibrium with the aldolization reactions occurring for a high concentration of glucose, which is previously suggested to be responsible for the second contribution observed in Fig.3.

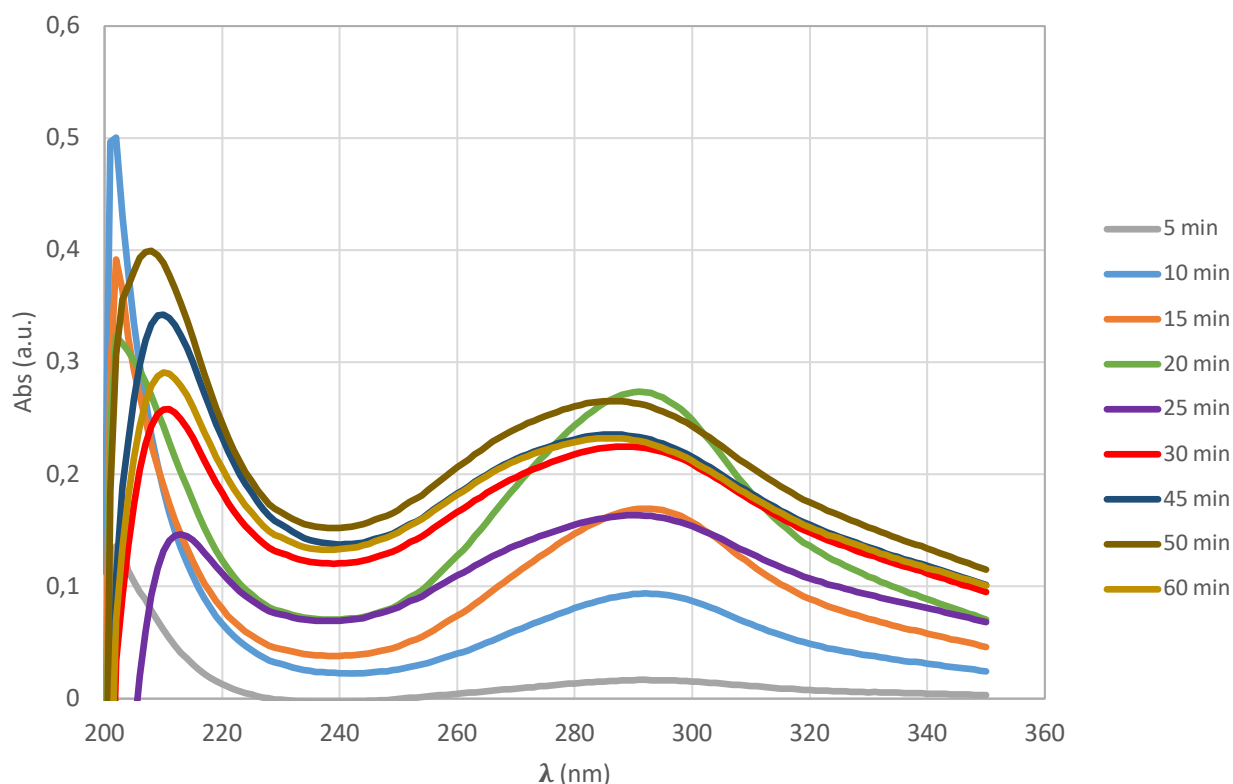


Fig.6 UV spectrum of the reaction between glucose 1M and NaOH 3.2M without heating supply, monitored over the time.

- *Effect of heating supply in the anolyte generation*

To test the possibility of overcoming the heating supply in the upstream alkalization unit of the designed energy plant, we fueled a flow tubular 3D-printed fuel cell with the anolyte coming from 30 min of reaction between glucose 1M and NaOH 3.2M without any external heating and, for comparison, we fueled another cell with the anolyte obtained after 15 min of reaction with NaOH 0.8 M at 65°C.

Table 3 compares the characterization of the so-realized 3D-printed fuel cell; Fig. 7-8 their power and polarization curves, and the current generated under constant voltage.

Table 3 Effect of the anolyte generation conditions (with and without heating supply in alkalization unit) on the fuel cell performance (tubular design) in terms of Open Circuit Voltage (OCV), Short Current Intensity (ISC), Power density (P), peak voltage (V), and Current density (J) under peak voltage load (50 min). Heating supply conditions: 15 min at 65°C for the reaction between glucose 1M and NaOH 0.8 M. No heating conditions: heat released from the reaction between glucose 1M and NaOH 3.2 M. OCV and ISC are mean values over 5 minutes of measurement. J refers to the mean value over 50 minutes. Electrode felt: AvCarb 280 after equilibration for 30 min.

Tubular design					
Alkalization phase	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)	J load (mA/cm ²)
Heating supply	1.35	0.41	1.02	0.5	1.30
No heating	1.47	0.40	1.14	0.5	1.61

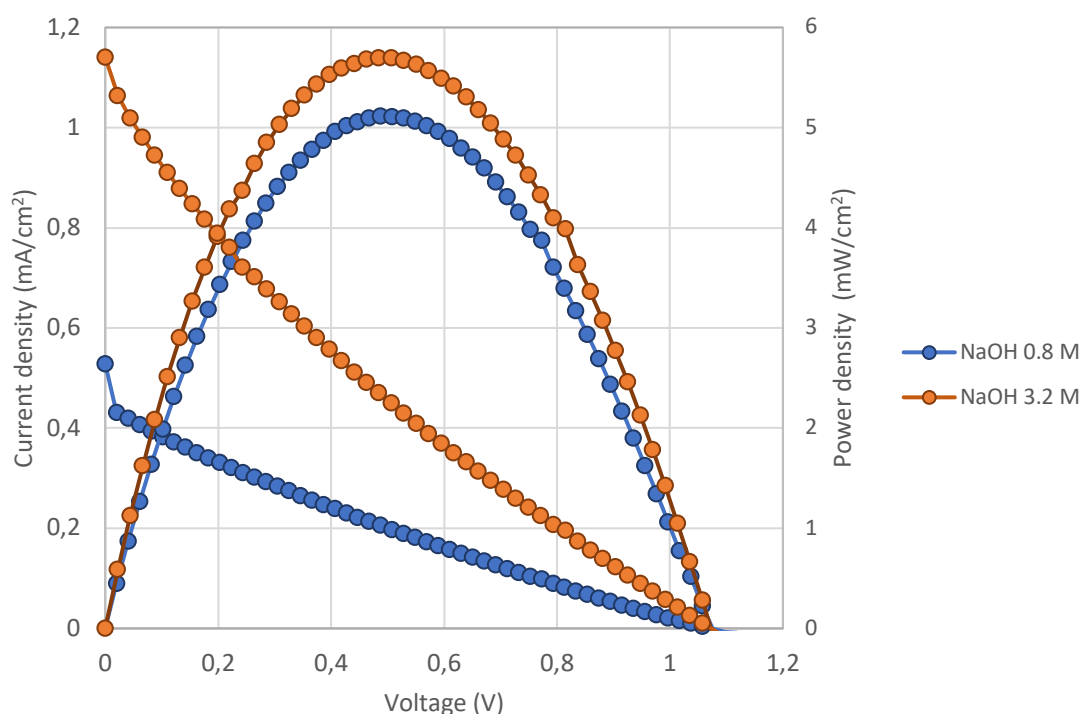


Fig. 7 Effect of NaOH concentration (NaOH 0.8M for alkalization step with heat supply, NaOH 3.2M without heat supply) on I-V (left) and power curves (right) recorded on a 3D-printed fuel cell with the tubular design. Anolyte: glucose 1M + NaOH 0.8 M (15 min at 65°C), and glucose 1M + NaOH 3.2 M (no heat supply). Catholyte: FeCl₃ 1M. Anolyte feeding: flux with recirculation with 20 mL/min flow rate. Catholyte feeding: batch. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 280 after 30 min of saturation. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

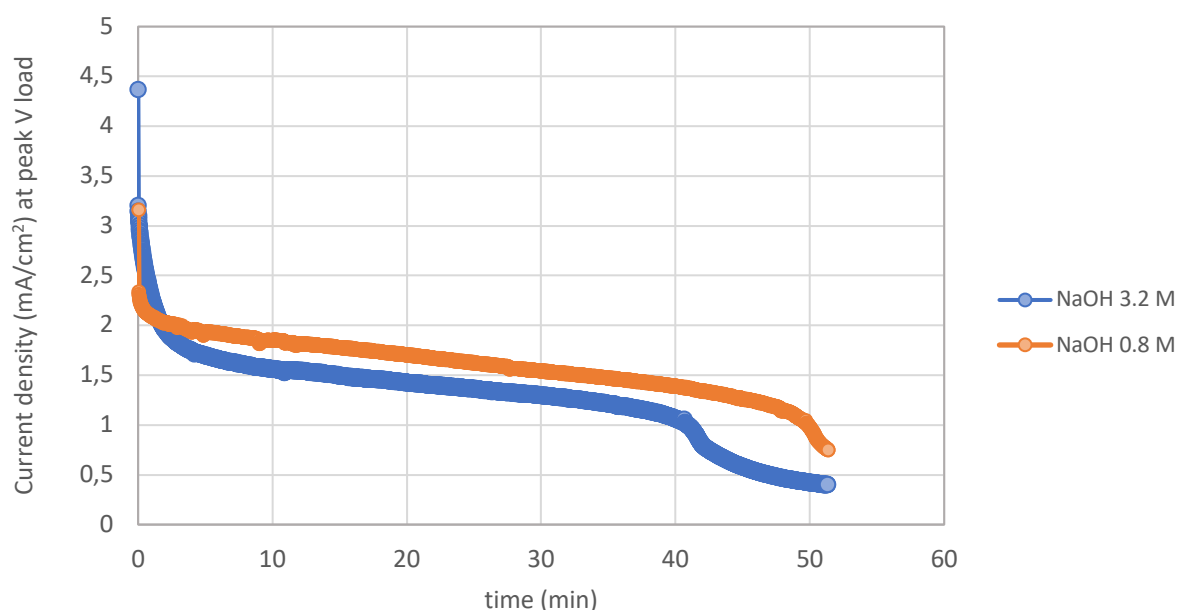


Fig. 8 Effect of NaOH concentration (NaOH 0.8M for alkalization step with heat supply, NaOH 3.2M without heat supply) on Current density at peak voltage recorded on a 3D-printed fuel cell with the tubular design. Anolyte: glucose 1M + NaOH 0.8 M (15 min at 65°C), and glucose 1M + NaOH 3.2 M (no heat supply). Catholyte: FeCl₃ 1M. Anolyte feeding: flux with recirculation with 20 mL/min flow rate. Catholyte feeding: batch. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 280 after 30 min of saturation. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

Looking at OCV values, the reaction carried out with NaOH 3.2 M seems to generate more species electrochemically active even if, looking at ISC values, not able to generate a higher current. The higher OCV could be due also to a higher coordination degree of the electrode with the electrolyte. This could result from the saturation step in which the carbon felt has been immersed for 30 minutes into a 4-time more concentrated NaOH solution with respect to the previous experiments.

Since the electrode is PAN-derived activated carbon felt, its structure could include nitrogen functionalities as trace components incorporated within the graphitic planes during the carbonization process used to form the fibers (Huang, 2009). Furthermore, these nitrogen moieties could be susceptible to alkaline hydrolysis to some extent when in contact with aqueous NaOH, enhancing the hydrophilicity of the fiber surface due to the formation of -CONH₂ and -COOH groups. Looking the Fig.8 appears that after 40 minutes of reaction, there is a prominent decrement in current density. This could be ascribed to the consumption of anolyte whose oxidation products increase over time, since the electrolyte feeding occurs with recirculation, or to the partial quenching of the active species due to the meeting of anolyte and catholyte migrating within the salt bridge. At the end of the cell reaction, the exhausted anolyte solution (pH 9) was neutralized by adding H₃PO₄ until neutrality to enrich the oxidated organic matter with inorganic elements that could promote soil fertilization. Although the neutralizer unit was not the object of study in this thesis, future investigations about the production of soil fertilizer from fuel cell waste, its environmental impact, and its applicability are strongly recommended.

- *Effect of reaction time in the anolyte generation*

Then, to investigate the formation of the different products in the alkalization phase on the fuel cell performance, we powered the 3D-printed device with the anolyte solution derived from 10 minutes and 30 minutes reaction between glucose 1.7 M and NaOH 3.2 M (Table 4). The choice of glucose concentration was due to the possibility of efficiently controlling the rising reaction temperature with lab equipment ($T_{\text{max}} = 66.5\text{ }^{\circ}\text{C}$) and the clear distinction between the two contributions. The experiment results are shown in Fig. 10 -11.

Table 4 Effect of reaction time for the anolyte generation in alkalization unit (10 and 30 minutes) on the fuel cell performance (tubular design) in terms of Open Voltage Circuit (OCV), Short Current Intensity (ISC), Power density (P), peak voltage (V), and Current density (J) under peak voltage load (40 min). Alkalization unit without heating supply for the reaction between glucose 1.7 M and NaOH 3.2M. OCV and ISC are mean values over 5 minutes of measurement. J refers to the mean value over 40 minutes. Electrode felt: AvCarb 280 after equilibration for 30 min.

Tubular design					
Alkalization time (min)	OCV (V)	ISC (A)	P max (mW/cm ²)	Peak Voltage (V)	J load (mA/cm ²)
10	1.47	0.41	1.13	0.55	1.77
30	1.47	0.51	1.44	0.48	1.90

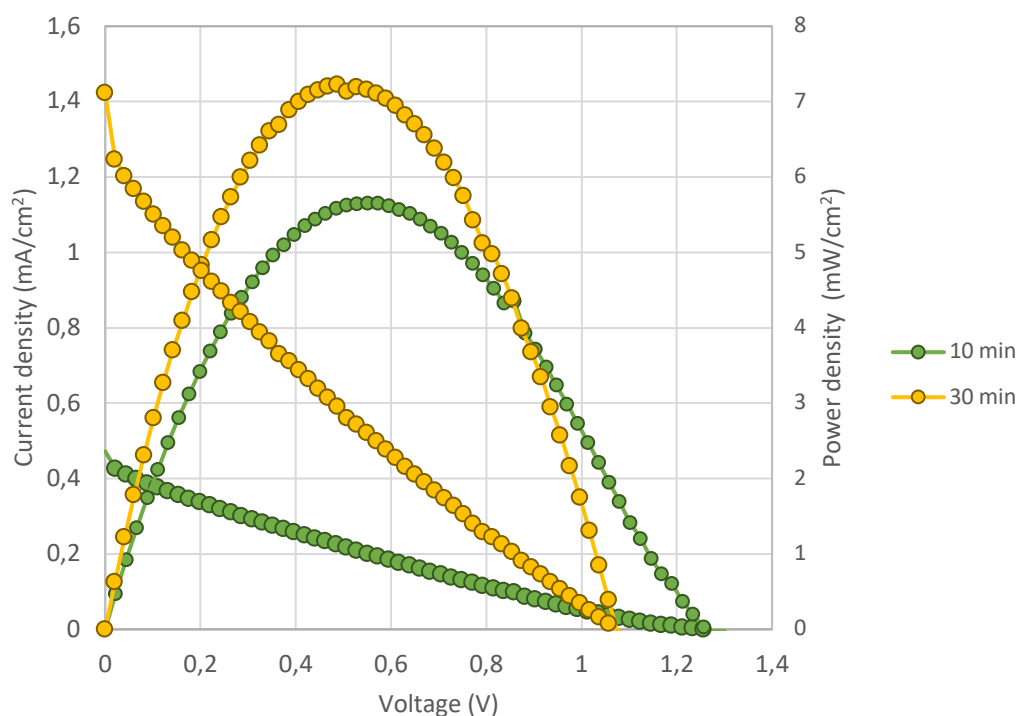


Fig.10 Effect of reaction time in the alkalization step on I-V (left) and power curves (right) recorded on a 3D-printed fuel cell with the tubular design. Anolyte: glucose 1M + NaOH 0.8 M (15 min at 65°C), and glucose 1M + NaOH 3.2 M (no

heat supply). Catholyte: FeCl_3 1M. Anolyte feeding: flux with recirculation with 20 mL/min flow rate. Catholyte feeding: batch. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 280 after 30 min of saturation. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

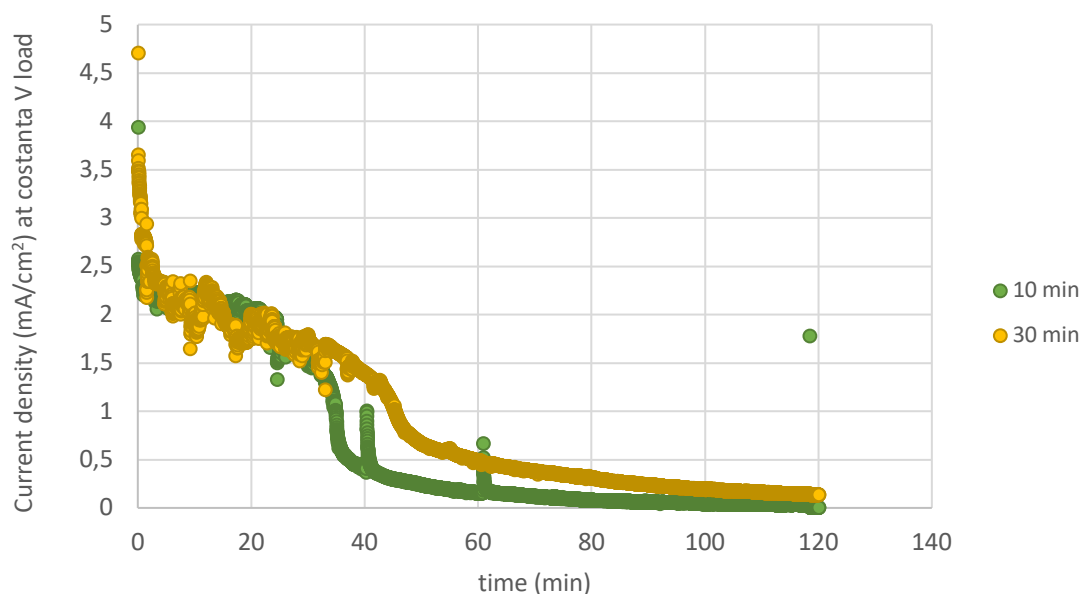


Fig.11 Effect of reaction time in the alkalization step on the current density at peak voltage recorded on a 3D-printed fuel cell with the tubular design. Anolyte: glucose 1M + NaOH 0.8 M (15 min at 65°C), and glucose 1M + NaOH 3.2 M (no heat supply). Catholyte: FeCl_3 1M. Anolyte feeding: flux with recirculation with 20 mL/min flow rate. Catholyte feeding: batch. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 280 after 30 min of saturation. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

The fuel cell powered by the anolyte after 30 minutes of reaction generates higher current under load and power densities, corresponding to 2 mA/cm² under 0.48 V over 30 minutes and 1.45 mW/cm² at 0.48 V, corresponding to 196 mA/unit and 142 mW/unit. The other cell fueled by anolyte solution after ten reaction minutes with NaOH gave 2mA/cm² current density under 0.55 V and 1.13 mW/cm² at peak voltage 0.55 V power density over 30 minutes, corresponding to 111 mW/unit. The increase in the power output may be related to some reducing species preferentially formed for longer-time reactions in the alkalization unit or unstable enediols formed as intermediate products. Understanding this aspect could allow tuning the fuel cell power output by acting on the reaction time in the alkalization unit. Although potentially less effective due to its low solubility, using $\text{Ca}(\text{OH})_2$ instead of NaOH as a basic catalyst could promote the stabilization of reactive enolate intermediates by interacting with Ca^{2+} (Jönsson, Ariksson & Nilvebrant, 2013). This could reveal the enediol's contribution to generating electrical energy. In addition, once $\text{Ca}(\text{OH})_2$ is neutralized upon adding HNO_3 , it generates $\text{Ca}(\text{NO}_3)_2$, which is directly used as fertilizer in soil pH control.

Fuel cell powered by biofuel from Pomegranate pomace residue

Our designed energy plant (Chp. 4) works effectively with reducing sugars solution, as demonstrated by the experiment carried out with glucose solution at different concentrations as biofuel. In the case of a complex lignocellulosic matrix, the availability of free reducing sugars can be implemented by enzymatic pre-treatment aid to hydrolyze the polysaccharide component of the waste biomass. This thesis has reported examples of enzymatic production (Chp. 2) and their utilization in biomass conversion (Chp. 3). The disassimilation of waste biomass mediated by enzymes can also be employed in the energy conversion process based on the 3D-printed fuel cell since it releases reducing sugars as hydrolysis products, which can directly act as biofuel precursors. Indeed, to generate the anolyte of the 3D-printed fuel cell, the alkalization step is required. Using NaOH ensures the activation of the electrolyte solution. It has the advantage of acting on solid matrices breaking down the primary components of the lignocellulosic biomass, which are cellulose, hemicellulose, and lignin. During the treatment, two main reactions occur hydrolytic cleavage of polysaccharides, which leads to new reducing ends that can be easily degraded, and the formation of alkali-stable end groups (Xu & Sun, 2016). It could be effective even when enzyme usage is limited due to the matrix structure or the cost requirement, leading to the valorization of the most recalcitrant biomass. That could be, for example, the exhausted organic matter generated from the chemical valorization process of agro-food waste.

The alkaline pretreatment on lignocellulosic biomass aims to disrupt the physical structure of biomass by breaking the lignin barriers, disrupting cellulose crystallinity, and removing non-cellulose components (Xu & Sun, 2016). It can be carried out at ambient conditions, even if it is encouraged by higher temperatures. Calcium, potassium, sodium, barium, lithium, and ammonium hydroxides are reactants effective in alkaline pretreatment. Among these, NaOH is the most used, followed by $\text{Ca}(\text{OH})_2$, due to its inexpensive, secure use, and easily recoverable (Xu & Sun, 2016).

At strong alkali concentrations, dissolution, end-group peeling, alkaline hydrolysis, degradation, and decomposition of the dissolved polysaccharides can occur (Wen et al., 2011). Besides the glycosidic hydrolysis, alkaline hydrolysis implicates the cleavage of alpha-ether linkages between lignin and hemicellulose and the ester bonds between lignin and/or hemicelluloses and hydroxycinnamic acids (Xu & Sun, 2016). Acting on NaOH concentration and operative conditions, it could be possible to favor the release of different chemical components from the biomass stream. With low NaOH concentration (0.5-4 %) and severe conditions (high temperature and pressures), the disruption and solubilization of hemicellulose and lignin take place. At high NaOH concentrations (6-20%) and moderate conditions (ambient pressure and low temperatures), the dissolution of cellulose is the main phenomenon (Xu & Sun, 2016).

After using several biofuels produced from the alkaline treatment of glucose solution, we tested the 3D-printed fuel cell performance by powering it with a biofuel produced from a real and complex matrix. We chose as an example the solid biomass leftover from the chemical valorization of pomegranate waste (Chp. 3C). It has been subjected to thermic pretreatment, and enzymatic reactions aimed at hydrolyzing pectic and cellulose components, before being dried. Thus, the biomass to be energetically valorized was composed mainly of lignin, hemicellulose, and residual cellulose. The reaction to produce the reducing species that will constitute the anolyte was carried out by dissolving the dried waste pomegranate powder into an aqueous solution (5% w/w) and

adding NaOH 5M. The reactants concentration was chosen after preliminary tests to find the condition to reach 60°C (data not shown) without supplying external heating. The reaction conditions used in the alkalization phase are intermediate between the severe conditions required for the optimum degradation of lignin and hemicellulose and moderate conditions necessary for advanced cellulose dissolution. The spectrum UV-Vis of the solution obtained at the end of the reaction is shown in Fig. 12. It shows the presence of the distinct band at 270 nm, as observed with the glucose solutions.

The performance of the tubular fuel cell powered by pomegranate waste is shown in Fig.13.

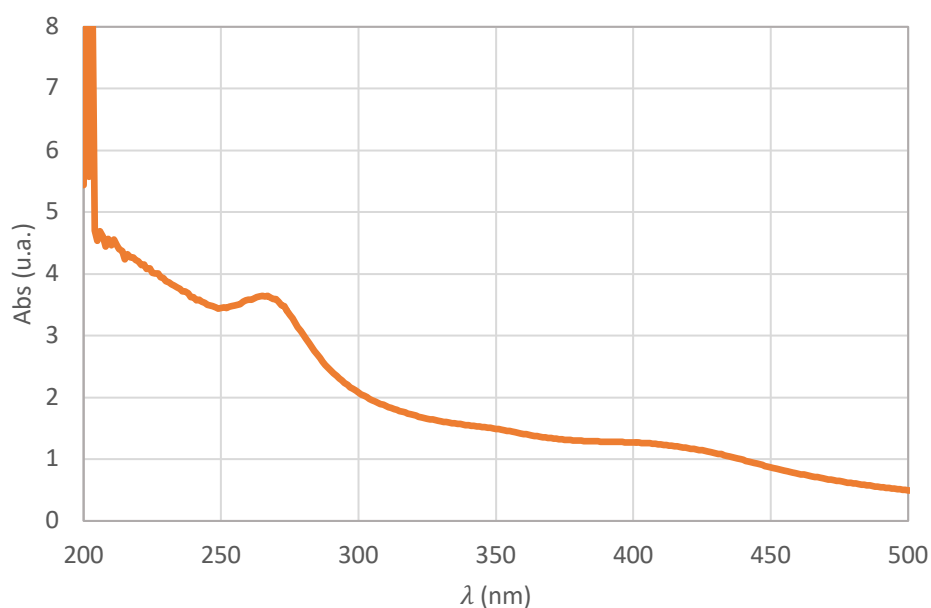


Fig.12 UV-Vis spectrum of the solution produced by the reaction between pomegranate waste 5% w/w and NaOH 5M without a heating supply.

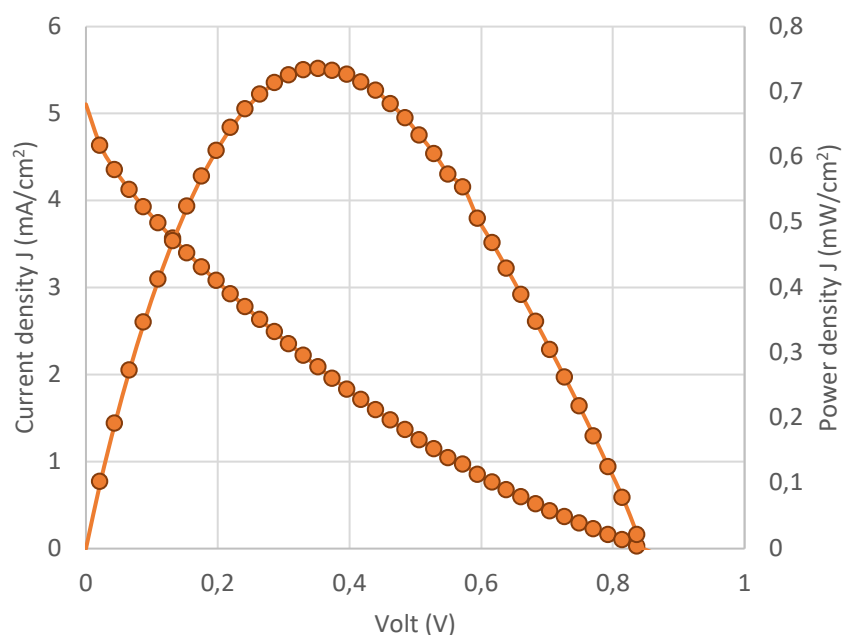


Fig.13 I-V (left) and power curves (right) recorded on a 3D-printed fuel cell with the tubular design. Anolyte: pomegranate waste 5% w/w + NaOH 5M (no heat supply). Catholyte: FeCl_3 1M. Anolyte and catholyte feeding: batch. Electrode configurations: one rod encircled by carbon felt at the cathode; one rod in tight contact with carbon felt enveloping along the anode perimeter. Felt: AvCarb 280 after 30 min of saturation. Salt bridge: agar potassium chloride gel. Measurements are taken at 30°C, p atm.

It operated in static mode and in the absence of catholyte regeneration.

It generates 0.77/cm² mW of maximum power output at 0.5 V (OCV = 1.22 V; ISC = 0.48 A), corresponding to 75.5 mW/unit. Although these results do not seem so remarkable with respect to the previous cells tested, they could be improved by acting on the upstream step of anolyte production by modulating temperature and time reaction, or on the fuel cell operation, by adding the flux feeding and the catholyte regeneration. Therefore, the outcome of this application is considered satisfactory because it provides an example of the direct conversion of solid food waste into electrical energy.

50 cm³ of the anolyte made of pomegranate waste have been enough to power a Monster Truck toy car's engine for 30 minutes. Thus, the 3D-printed fuel cell replaced in an effective way the machine Mg/air battery, whose power curve is reported in Fig.14.

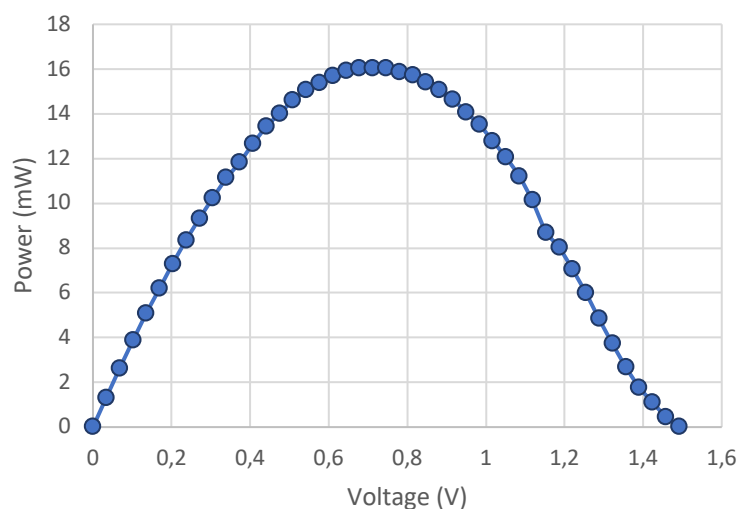


Fig.14 Power curve of the MgO/air battery powering the Monster Truck's engine.

Table 5 resumes the main examples of fuel cell applications studied in the present thesis.

Table 5 Resume of the tubular fuel cell performances recorded for different types of anolytes and reaction conditions for anolytes generation. Anolytes feeding: flux at 20 mL/min flow rate, and batch. Catholyte feeding: batch. Electrode felt: AvCarb 280 after equilibration for 30 min. No O₂ bubbling at the cathode.

Tubular design						
Glucose (mol/L)	NaOH (mol/L)	OCV (V)	ISC (A)	P max (mW/cm ²)	Anolyte feed	Anolyte generation
1	0.8	1.35	0.41	1.02	flux	15', 65°C
1	3.2	1.47	0.40	1.14	flux	30', no heat
1.7	3.2	1.47	0.51	1.44	flux	30', no heat
1.7	3.2	1.47	0.41	1.13	flux	10', no heat
Pomegranate % (w/w)	NaOH (mol/L)	OCV (V)	ISC (A)	P max (mW/cm ²)	feed	Anolyte generation
5	5	1.22	0.48	0.73	batch	15', no heat

Conclusions

The experiments carried out and reported in this thesis section have demonstrated the versatility of the new 3D-printed glucose biofuel cell. Its design ensures the flux fuel cell operation and its feeding in a continuous way, but it is also suitable for reactions conducted in static mode. It is suitable to process homogeneous solutions (commercial glucose) and real waste matrices from a biorefinery chemical valorization (solid pomegranate peel). Thus, its capacity to produce electrical energy resulted in being independent of the type of biofuel. Each 3D-printed fuel cell could generate from 76 to 142 mW of power output in the investigated conditions. However, higher performance is reachable by a modular approach, as demonstrated in our study (Chp. 4A), since the units have also been designed to be easily stacked. These aspects and its simplicity in components, material, and operation characteristics make it applicable and effective for agro-food waste treatment in small-scale biorefineries. Further studies about the formation and stabilization of reducing species are recommended to standardize the fuel production process into the proposed alkalization unit. To allow the utilization of this technology at the industrial level, the replacement of the agar salt bridge with an electrolytic membrane is a requirement.

Abbreviations

OCV	Open Circuit Voltage
ISC	Short Current Intensity
P	Power density
J	Current density
Jload	Current density under peak voltage load

Materials and Methods Chp. 4

Reagents D-Glucose, FeCl_3 , NaOH, H_3PO_4 65%, Agar agar, potassium chloride, were purchased from Merck KGaA (Darmstadt, Germany). ABS was purchased from <https://www.paperdifferent.com>; AvCarb 100 and AnCarb 280 was purchased from <https://www.fuelcellearth.com/>

Instruments Multimeter: SourceMeter Keithley 2400 controlled by LabTracer software; Ultra sound sonicator: Elmasonic S30h; 3D-printer: Geeetech A10; CAD software: AutoDesk Fusion 360; Slicer Software Ultimaker Cura 4.11

3D-printing

After having manually calibrated the 3D-printer plate, the plate temperature (110°C) and the nozzle temperature (235°C) are set. Once the temperature are reached the print is launched from the printer control panel, and the chamber containing the 3D-printed is closed, under constant aspiration in a fume hood.

Salt bridge solution

40 g of KCl are placed inside a 250 mL beaker with a magnetic stirring bar. 200 mL of distilled water is added, and the clear solution is heated to. Once the dissolution of the salt is complete, and 90°C is reached, 5.4 g of agar is added, initially making the solution turbid. Then it turns clear when the complete dissolution of the agar is reached. At room temperature, the solution solidifies to the consistency of a jelly.

Anolyte generation

The anolyte solutions are generated by the reaction of the anolyte precursor (glucose or real matrix) with NaOH, both in the presence and absence of a heating supply.

Presence of a heating supply: put the reagent used as anolyte precursor in a Becker and disperse it in water to give the desired concentration. Put the beaker containing the solution in a water bath at 65°C for 10 minutes. Add NaOH to the solution under stirring to give a final concentration of 0.8 M. Carry out the reaction at 65°C for 15 min. In the end, put the solution into a fridge at 4°C for 10 min to let the solution cool down to 30°C .

Absence of a heating supply: put the reagent used as anolyte precursor in an insulated beaker and disperse it in water to give the desired concentration. Put the beaker containing the solution in an insulated chamber with a water bath at 20°C for 10 minutes. Add NaOH to the solution under stirring to give a final concentration of 3.2 M. Carry out the reaction for the time required to have the increase of solution temperature at least up to 65°C and then carry out the reaction for 15 min. In the end, add cooling water to the water bath to let the solution cool down to 30°C .

Catholyte generation

Dispersing and solubilizing $\text{FeCl}_3(\text{H}_2\text{O})_6$ into an aqueous solution to give a concentration of 1 mol/L.

Electrode's preparation and cleaning

Cut the graphite rods, and the activated carbon felt in pieces with proper dimensions, depending on the fuel cell design. Before the usage, sonicate the electrodes for 10 min. After the usage, clean the electrodes by sonication in a water ultrasonic bath until the neutrality of solutions is reached.

Woufle bottles:	graphite rods with 16 mm height, 6 mm diameter
Cuboid design:	graphite rods with 8 mm height, 6 mm diameter felt: 80 x 30 x 3 mm
Cylindrical design:	anode: graphite rods with 8 mm height, 6 mm diameter 1) felt stripes: 100 x 10 x 3 mm 2) long felt ring: 490 x 10 x 3 mm 3) short felt ring: 300 x 10 x 3 mm cathode: graphite rods with 8 mm height, 6 mm diameter felt stripe: 100 x 10 x 3
Tubular design:	anode: graphite rods with 8 mm height, 6 mm diameter felt: 140 x 35 x 3 mm cathode: graphite rods with 8 mm height, 6 mm diameter felt stripe: 80 x 20 x 3 mm

Fuel cell set-up

The 3D-printed fuel cells have geometry reported in Fig.4, 12, and 21 in Chp.4A

Once the salt bridge solution is prepared, it is intercalated still hot in the proper cavity at the center of the fuel cell body, enclosed by the proper removable supports. Next, the fuel cell is placed in the fridge at 4°C to promote the salt bridge's cooling and gelation. After that, the supports are removed, and the agar salt bridge is obtained.

The electrodes are cleaned and allocated in the dedicated spaces with the proper configuration:

Woulfe bottles:	graphite rods. Contact area: 31 cm ²
Cuboid design:	1) activated carbon felt alone; 2) activated carbon felt in contact with graphite plate. Contact area: 48 cm ² . A graphite plate is considered just a collector.
Cylindrical design:	
anode:	1) three graphite rods encircled by three felt stripes Contact area: 60 cm ² ; 2) three rods surrounded and linked by a long carbon felt ring: 98 cm ² ; 3) three graphite rods encircled by three felt stripes and connected by a shorter carbon felt ring. Contact area: 120 cm ² . Graphite rods are considered just as collectors.

cathode: one graphite rod encircled with an activated carbon felt stripe. Contact area: 48 cm². A graphite rod is considered just a collector.

Tubular design:

anode: one graphite rod inserted in one cavity in tight contact with activated carbon felt stripe that envelops the entire anode chamber perimeter. Contact area: 98 cm². A graphite rod is considered just a collector.

cathode: one graphite rod encircled with an activated carbon felt stripe. Contact area: 32 cm². A graphite rod is considered just a collector.

The electrolytes solutions are loaded into the fuel cell in the proper mode (see Fuel cell operation).

Woulfe bottles: 50 cm³ in batch mode, with a stirring bar for each half cell.

Cuboid design: 50 cm³ in batch mode, with a stirring bar for each half cell.

Cylindrical design: 200 cm³ (anode) and 50 cm³ (cathode) in batch mode, with a stirring bar for each half cell.

Tubular design:

anode: 37 cm³ in continuous mode for flux operation by a peristaltic pump at the inlet and the outlet, settled to 20 cm³/min constant flow rate. 50 cm³ in batch mode for static operation.

cathode: 16 cm³ in batch mode and static operation.

The electrodes immersed into the electrolytes are then connected to the power supply unit, and the cell is left to operate in batch mode (each design) and in continuous mode with recirculation of the electrolyte solution (only tubular design) to record the characterization curves.

Fuel cell operations

Chp.3A

Cuboid design

- *Effect of glucose concentration*

The anolyte was generated from a solution of glucose 0.25, 0.5, 0.75, 1 M by adding NaOH to have 0.8 M, in the presence of a heating supply, as described in -Anolyte generation-. 50 cm³ of electrolytes are loaded in each chamber, containing AvCarb 100 as electrode end electrons collector. The fuel cell is left operating in a batch mode, under constant stirring, for 15 minutes. OCV, ISC and Power has been recorded.

- *Effect of graphite plate*

The anolyte was generated from a solution of glucose 0.25 M by adding NaOH to have 0.8 M, in the presence of a heating supply, as described in -Anolyte generation-. 50 cm³ of electrolytes are loaded in each chamber, containing AvCarb 100 as electrode and graphite plate as electrons collector. The fuel cell is left operating in a batch mode, under constant stirring, for 15 minutes. OCV, ISC and Power has been recorded.

Cylindrical design

- *Effect of felt sonication*
- *Effect of rod number in the anode*

The anolyte was generated from a solution of glucose 0.25 M by adding NaOH to have 0.8 M, in the presence of a heating supply, as described in -Anolyte generation-. 200 cm³ of anolyte and 50 cm³ of catholyte are loaded in the proper chamber, containing AvCarb 100 as electrode and graphite rods as electrons collector. The fuel cell is left operating in a batch mode, under constant stirring, for 15 minutes. OCV, ISC and Power has been recorded.

- *Effect of electrode configuration*

The anolyte was generated from a solution of glucose 0.25 M by adding NaOH to have 0.8 M, in the presence of a heating supply, as described in -Anolyte generation-. 200 cm³ of anolyte and 50 cm³ of catholyte are loaded in the proper chamber, containing AvCarb 100 as electrode and graphite rods as electrons collector. The fuel cell is left operating in a batch mode, under constant stirring, for 35 minutes. OCV, ISC, Power and Current Intensity generated under constant load (V peak) has been recorded.

Tubular design

- *Effect of catholyte regeneration*
- *Effect of glucose concentration*

The anolyte was generated from a solution of glucose 0.25 M (0.25, 0.5, 0.75, 1 M in the study at variable glucose concentration) by adding NaOH to have 0.8 M, in the presence of a heating supply, as described in -Anolyte generation-. 37 cm³ of anolyte are pumped from a tank containing 100 mL by a peristaltic pump in the anodic chamber and left recirculating as long as the characterization. 16 cm³ of catholyte are loaded in the cathodic chamber, containing AvCarb 100 as electrode and graphite rods as electrons collector. The fuel cell is left operating in a flux mode, with 20 mL/min flow rate, for 15 minutes. OCV, ISC, and Power have been recorded.

- *Series and parallel connections*

Two fuel cells are connected to each other by parallel hydraulic connections (anode feeding) and both series and parallel electrical connections. The anolyte was generated from a solution of glucose 1 M by adding NaOH to have 0.8 M, in the presence of a heating supply, as described in -Anolyte generation-. 37 cm³ of anolyte are pumped from a tank containing 200 mL of the solution by a peristaltic pump in the anodic chamber and left recirculating as long as the characterization. 16 cm³ of catholyte are loaded in the cathodic chamber, containing AvCarb 100 as electrode and graphite rods as electrons collector. The fuel cell is left operating in a flux mode, with 20 mL/min flow rate, for 35 minutes. OCV, ISC, Power, and Current Intensity generated under constant load (V peak) have been recorded.

- *Effect of the inlet flow rate*

The anolyte was generated from a solution of glucose 1 M by adding NaOH to have 0.8 M, in the presence of a heating supply, as described in -Anolyte generation-. 37 cm³ of anolyte are pumped from a tank containing 100 mL of the solution by a peristaltic pump in the anodic chamber and left recirculating as long as the characterization. 16 cm³ of catholyte are loaded in the cathodic chamber, containing AvCarb 280 as electrode and graphite rods as electrons collector. AvCarb felts saturated with the electrolyte solutions for 30 minutes at 30°C, before being put into the cell. The fuel cell is left operating in a flux mode, with a variable flow rate, from 8 to 20 mL/min, incrementing by 3 mL/min at a time for 35 minutes. OCV, ISC, and Power have been recorded.

Chp. 3B

- *Effect of heating supply in the anolyte generation*
- *Effect of reaction time in the anolyte generation*

The anolyte was generated from a solution of glucose 1 M and 1.7 M by adding NaOH to have respectively 0.8 M, or 3.2 M, in the presence of, and without heating supply, as described in -Anolyte generation-. For the study at variable time: glucose 1.7 M and NaOH 3.2 M reacted for 10 and 30 minutes). 37 cm³ of anolyte are pumped from a tank containing 100 mL of the solution by a peristaltic pump in the anodic chamber and left recirculating as long as the characterization. 16 cm³ of catholyte are loaded in the cathodic chamber, containing AvCarb 280 as electrode and graphite rods as electrons collector. AvCarb felts saturated with the electrolyte solutions for 30 minutes at 30°C, before being put into the cell. The fuel cell is left operating in a flux mode, with a 20 mL/min flow rate for 65 minutes. OCV, ISC, and Power Current Intensity generated under constant load (V peak) have been recorded.

Pomegranate pomace residue as biofuel

The anolyte was generated from a dispersion of dried pomegranate pomace residue (75 micrometers) into water at 5 %w/w by adding NaOH to have 5 M, or 3.2 M, without heating supply, as described in -Anolyte generation-. Reaction carried out for 30 minutes from the adding of NaOH. 50 cm³ of electrolytes are loaded in each chamber, containing AvCarb 280 as electrode and graphite rods as electrons collector. AvCarb felts saturated with the electrolyte solutions for 30 minutes at 30°C, before being put into the cell. The fuel cell is left operating in static mode for 35 minutes. OCV, ISC, and Power Current Intensity have been recorded.

Overall Conclusions

The object of my thesis project has been the proposal of a biorefinery model to be applied on a small scale, enabling the agro-food chain to valorize its waste in an effective way. The aim was to propose low costs and sustainable processes to make waste valorization efficient and economically feasible. In such a way, local supply chains could be promoters of new communities based on a bio-based circular economy to tackle the ecological transition to which we are morally obligated. The proposed approach is a cascade valorization of vegetable agro-food waste, consisting of:

- 1) a unit upstream of the biorefinery for the production of low-cost industrial biocatalysts, which uses waste as growth substrates;
- 2) a unit for the valorization of the components contained in the waste through biorefinery processes involving the breakdown of the waste cell wall mediated by enzymes and its recombination into a prototype cosmetic with territorial connotations
- 3) a unit to be integrated into the biorefinery for the conversion of the resulting residue into renewable electrical energy by the work of a 3D printed abiotic glucose biofuel cell, whose design is currently patent-pending, was conceived ex novo and adapted following experimental evaluations.

Each strategy deployed was supported by case studies conducted on a laboratory scale.

- 1) Enzyme production was conducted through solid-state fermentation of the fungus *Pleurotus ostreatus* on hemp bran as a substrate, and it was carried out for three weeks. At the end of each week, the enzymes produced were collected, and various activities were determined: pectinase, xylanase, amylase, laccase, protease, peroxidase, and arylesterase. The activities showed different trends during fermentation, reaching significant values in the case of xylanase and amylase activities after one week of fermentation (3296 U/L and 60702 U/L, respectively) and pectinase activities after two weeks (12300 U/L). Furthermore, the activity spectrum was compared with that resulting from our previous work in which the same hemp bran was used as a substrate after basic pretreatment for protein extraction. Enzymes were then used as biocatalysts to hydrolyze the hemp bran itself, from whose reactions three hydrolysates with different contents of bioactive substances, such as polyphenols, were obtained. This demonstrates not only the possibility of getting specific enzymes at low cost and in a safe way, using edible fungi as organisms and lignocellulosic material derived from agro-food waste as the source, but also shows the possibility of modulating enzyme activities by acting on the fermentation time, pH, matrix pre-treatment.

The biorefinery strategy put in place to obtain high-value-added components from food waste was knowledge-based. Thus, in the design phase, sequential operations to be carried out were defined depending on the raw material considered. The aim was to obtain products that could have applicability on the same territory from which the raw materials originate, guaranteeing the applicability of circular economy principles on a small scale. The proposed final products were oil-

in-water emulsions with a potential cosmetic function. They were obtained by breaking down the residue into ingredients functional to the purpose, such as lipids, emulsifiers, and antioxidants, and recombining them in the form of a cosmetic prototype with its own functionality.

To explain the strategy, we used three case studies representing three different categories of agro-food waste to be valorized: hemp oilseed cake as an example of a residue from vegetable oil extraction, rice flour and husk as an example of residues from cereal processing, pomegranate pomace as an example of juicing or canning residue.

For each case studied, mass balances were established to assess the degree of conversion achieved and to estimate how much raw material would be needed to make the targeted product through the proposed process.

The processes proposed in each case study involved the breakdown of the waste by plant cell wall disassimilation techniques. These were preferentially carried out by specific and sequential enzymatic reactions that led to the top-down extraction of secondary building blocks, which can be used as such for their emulsifying, thickening, stabilizing, and preservative properties. In addition, the residues from extractions have been further hydrolyzed using a proper enzyme cocktail to obtain an aqueous mixture of bioactive substances containing primary building blocks specific to the treated matrix.

- 2) From the breakdown of the hemp oil seed cake, hemp oil (5.8 %), hemp protein (5.6 %) (via chemical extraction), and hydrolysates of hemp bran and residual flour were obtained via enzymatic hydrolysis. The enzymes used were, respectively, the ones produced previously via solid-state fermentation and a commercial one (Celluclast). If the valorization of the hemp bran via SSF is also considered, the whole process results in a raw material conversion of 41%. The recombination of the extracted ingredients resulted in an emulsion suitable for hair care (nourishing mask), obtainable with a maximum feedstock: product ratio of 1:6.

From the deconstruction of rice grain (flour and husk), protein-functionalized starch (40 %) was obtained via enzymatic extraction mediated by the protease Alcalase 2.4L, and a husk hydrolysate (24%) containing reducing sugars, carbohydrates, polyphenols, via a disassimilation process mediated by the enzymatic cocktail Fungamyl Super AX. Considering the two raw materials deriving from the same bulk, the process valorizes 36 % of the initial feedstock. In this specific case, commercial rice bran oil was used to make the emulsion, but if considered as extracted from the same processed residue, it is estimated that a body care emulsion (moisturizing) can be realized with a feedstock: product ratio of at least 3:1.

The decomposition of pomegranate pomace was carried out entirely by a specific enzyme-mediated breakdown of the plant cell wall. Pomegranate oil (12%) was obtained from the deconstruction of the fruit arils by the sequential use of pectinase (Rapidase C80 max) and protease (Maxazyme NNP) enzymes. From the mesocarp, pomegranate pectin (10.5%) was extracted by heat treatment followed by rapid enzymatic extraction with hemicellulase (Accellerase XC), also exhibiting action towards protopectin. The disassimilation of the pericarp was performed by two distinct combinations of pectinase (Rapidase C80 max) and cellulase. They resulted in two hydrolysates (with conversion up to 76%) with remarkable antioxidant activities (TEAC up to 50.40 mmol Trolox/mL). The whole process resulted in converting 70% of the starting raw material into ingredients of interest that, once

recombined, yielded a specific facial care emulsion (anti-age serum) with a feedstock: product ratio of 3:4.

This case study also offers several perspectives for making more energy-intensive processes, such as pectin extraction, economically feasible and viable on a small scale: from the possibility of not purifying the pectin by ethanol precipitation to the opportunity of making joint ventures with larger companies that can bear the more expensive part of the process.

Each example showed how enzyme choice and reaction sequences are fundamental in reaching an efficient waste valorization. They also showed how to obtain products that, although in the same physical form and structure, differ remarkably in their composition and final function, as they have different origins. This can open the way to new local markets that can add value to the specific food chain.

The development of the energy unit is crucial because it enables the integration of the energy required by the various valorization processes, making the deployment of small-scale and low-environmental impact biorefineries economically viable.

- 3) The proposed plant, currently patent-pending, was based on a 3D-printed abiotic glucose biofuel cell capable of directly processing agro-food waste in a non-specific way to generate electrical energy. The fuel cell was preceded by an alkalization unit responsible for generating the anolyte from the food feedstock and ensuring its sterility. Downstream of the cell, a unit for neutralizing the spent organic residue has been proposed, which can give, at the end of the entire biorefinery, a final waste with soil conditioner functions so that the food cycle can be closed.

The 3D-printed biofuel cell works by oxidation of organic matter at the anode after undergoing pretreatment in an alkaline environment (NaOH) to generate highly reducing enediols, and the simultaneous reduction of FeCl_3 at the cathode, which can be easily regenerated by direct contact with molecular oxygen. The cell is made of ABS polymer, a low-cost and recyclable material chemically resistant to both half-cells' pH conditions. It is free of metal catalysts, which could be susceptible to poisoning and deactivation phenomena, and contains carbon-based electrodes such as graphite rods and activated carbon felt. To fine-tune the electrochemical system, a design study was conducted, which, starting from the system with a cuboid configuration (static), and passing through an intermediate cylindrical configuration (static), led to the realization of the final device. It presents a tubular flow configuration, operates at ambient T in both continuous and discontinuous modes, and can be easily stacked in modules composed of several units electrically and hydraulically connected to each other, in order to generate higher power output.

The implementations progressively applied to the design resulted from experimental evaluations such as the effect of anolyte concentration and its feed rate, the effect of catholyte regeneration, the study of the electrode capacity to collect the generated charge, the study of the felt coordination with the electrolytes, and the effect of the electrical connection between two cells in series and parallel. For each of these variables, the cell's

performances in terms of OCV, ISC, polarization curves, and power produced under variable load were tested.

Once the final design was realized, the 3D printed cell was fueled with model solutions (glucose 1M and 1.7 M) and a real complex matrix (pomegranate pomace residue dispersed in 5% w/w of basic solution).

To further reduce operating costs, the alkaline pre-treatment step was implemented by overcoming the external heat supply and exploiting the heat generated by the dissolution of the sodium hydroxide to carry out the reaction itself. The phase thus implemented was applied to generate the biofuel used to power the fuel cells. The power values generated by the various systems ranged from 1.1 mW/cm² (at 0.5 V) to 1.45 mW/cm² (at 0.5 V) for 1M glucose and 1.7M (pretreated with NaOH 0.8 M and 3.2M), corresponding respectively to 109 mW/unit and 142 mW/unit, and settled at 0.77 mW/cm² for the 5% w/w pomegranate solution pretreated with 5M NaOH, corresponding to 75.5 mW/unit. Since the anolyte is generated in the alkalization phase, its operating conditions, including reagent concentrations, were crucial for the generation of the active species. Extensive studies in this regard are therefore recommended.

What emerged from the study highlighted the ability of the designed system to process agro-food waste in a non-specific way for the low-cost and continuous production of renewable electrical energy.

The entire integrated biorefinery model resulting from my studies could open the way for new, efficient, and low-cost processes for converting agro-industrial waste into materials and energy on a small and local scale. This would mitigate the impact of the production processes themselves and, at the same time, provide a viable alternative to the current conversion processes based on fossil resources, providing feasible solutions to address the ecological transition in the short term.

References

Chp. 1 Introduction

(Text)

American Biogas Council (2018), Biogas Opportunities Roadmap (USDA, EPA, DOE, 2014), EPA AgSTAR 2016, EPA LMOP 2017, Water Environment Federation “Enabling the Future”.

<https://americanbiogascouncil.org/biogas-market-snapshot/>. (Accessed 22 January 2023).

Anukam, A., Mohammadi, A., Naqvi, M., & Granström, K. (2019). A review of the chemistry of anaerobic digestion: Methods of accelerating and optimizing process efficiency. *Processes*, 7(8), 504.

Baiano, A. (2014). Recovery of biomolecules from food wastes—A review. *Molecules*, 19(9), 14821-14842.

Beyene, H. D., Werkneh, A. A., & Ambaye, T. G. (2018). Current updates on waste to energy (WtE) technologies: a review. *Renewable Energy Focus*, 24, 1-11.

Biller, P., & Ross, A. B. (2016). Production of biofuels via hydrothermal conversion. In *Handbook of biofuels production* (pp. 509-547). Woodhead Publishing.

Bosmans, A., Vanderreydt, I., Geysen, D., & Helsen, L. (2013). The crucial role of Waste-to-Energy technologies in enhanced landfill mining: a technology review. *Journal of Cleaner Production*, 55, 10-23.

Bruins, M. E., & Sanders, J. P. (2012). Small-scale processing of biomass for biorefinery. *Biofuels, bioproducts and biorefining*, 6(2), 135-145.

Cardona-Alzate, C. A., Serna-Loaiza, S., & Ortiz-Sanchez, M. (2020). Sustainable Biorefineries: What was learned from the design, analysis and implementation. *Journal of Sustainable Development of Energy, Water and Environment Systems*, 8(1), 88-117.

Champagne, P., & Li, C. (2009). Enzymatic hydrolysis of cellulosic municipal wastewater treatment process residuals as feedstocks for the recovery of simple sugars. *Bioresource technology*, 100(23), 5700-5706.

Chen, D., Yin, L., Wang, H., & He, P. (2014). Pyrolysis technologies for municipal solid waste: a review. *Waste management*, 34(12), 2466-2486.

Chen, H., Zhang, M., Xue, K., Xu, G., Yang, Y., Wang, Z., ... & Liu, T. (2020). An innovative waste-to-energy system integrated with a coal-fired power plant. *Energy*, 194, 116893.

Cherubini, F. (2010). The biorefinery concept: using biomass instead of oil for producing energy and chemicals. *Energy conversion and management*, 51(7), 1412-1421.

Clauwaert, P., Mulenga, S., Aelterman, P., & Verstraete, W. (2009). Litre-scale microbial fuel cells operated in a complete loop. *Applied microbiology and biotechnology*, 83(2), 241-247.

Dahiya, S., Sarkar, O., Swamy, Y. V., & Mohan, S. V. (2015). Acidogenic fermentation of food waste for volatile fatty acid production with co-generation of biohydrogen. *Bioresource technology*, 182, 103-113.

Dahiya, S., Kumar, A. N., Sravan, J. S., Chatterjee, S., Sarkar, O., & Mohan, S. V. (2018). Food waste biorefinery: Sustainable strategy for circular bioeconomy. *Bioresource technology*, 248, 2-12.

Daoutidis, P., Kelloway, A., Marvin, W. A., Rangarajan, S., & Torres, A. I. (2013). Process systems engineering for biorefineries: new research vistas. *Current Opinion in Chemical Engineering*, 2(4), 442-447.

EPA (2017). *Basics of Green Chemistry*. Available online at: <https://www.epa.gov/greenchemistry/basics-green-chemistry> (accessed December 12, 2022).

European Biogas Association. (2020). European Biogas Association Statistical Report: 2019 European Overview. *EBA: Brussels, Belgium*.

Fagbohunbe, M. O., Herbert, B. M., Hurst, L., Ibeto, C. N., Li, H., Usmani, S. Q., & Semple, K. T. (2017). The challenges of anaerobic digestion and the role of biochar in optimizing anaerobic digestion. *Waste management*, 61, 236-249.

Fan, Y., Qiao, Q., & Fang, L. (2017). Network analysis of industrial metabolism in industrial park—A case study of Huai'an economic and technological development area. *Journal of cleaner production*, 142, 1552-1561.

Fawzy, S., Osman, A. I., Doran, J., & Rooney, D. W. (2020). Strategies for mitigation of climate change: a review. *Environmental Chemistry Letters*, 18, 2069-2094.

Fritsche, U. R., & Iriarte, L. (2014). Sustainability criteria and indicators for the bio-based economy in Europe: State of discussion and way forward. *Energies*, 7(11), 6825-6836.

Gallaspy, D. T., & Sears, R. E. (2010, January). Application of Regional Bio-Refining to Increase the Sustainability and Energy Self-Sufficiency of Rural and Agricultural Communities. In *Energy Sustainability* (Vol. 43956, pp. 997-1007).

Ge, Z., Zhang, F., Grimaud, J., Hurst, J., & He, Z. (2013). Long-term investigation of microbial fuel cells treating primary sludge or digested sludge. *Bioresource Technology*, 136, 509-514.

Goud, R. K., & Mohan, S. V. (2011). Pre-fermentation of waste as a strategy to enhance the performance of single chambered microbial fuel cell (MFC). *International Journal of Hydrogen Energy*, 36(21), 13753-13762.

Goud, R. K., Arunasri, K., Yeruva, D. K., Krishna, K. V., Dahiya, S., & Mohan, S. V. (2017). Impact of selectively enriched microbial communities on long-term fermentative biohydrogen production. *Bioresource technology*, 242, 253-264.

Gustavsson, J., Cederberg, C., Sonesson, U., van Otterdijk, R., & Meybeck, A. (2011). Global food losses and food waste: Extent. *Causes and prevention*, 29. FAO, Rome.

Hafid, H. S., Shah, U. K. M., & Baharudin, A. S. (2015). Enhanced fermentable sugar production from kitchen waste using various pretreatments. *Journal of environmental management*, 156, 290-298.

Hoekstra, A. Y., & Chapagain, A. K. (2007). Water footprints of nations: water use by people as a function of their consumption pattern. *Water resources management*, 21, 35-48.

Hoffmann, J., Rudra, S., Toor, S. S., Holm-Nielsen, J. B., & Rosendahl, L. A. (2013). Conceptual design of an integrated hydrothermal liquefaction and biogas plant for sustainable bioenergy production. *Bioresource technology*, 129, 402-410.

IPCC (2014): [Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change](#) [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151.

IPCC (2018) Global warming of 1.5 °C. In: Masson-Delmotte V, Zhai P, Pörtner H-O, Roberts D, Skea J, Shukla PR, Pirani A, Moufouma-Okia W, Péan C, Pidcock R, Connors S, Matthews JBR, Chen Y, Zhou X, Gomis MI, Lonnoy E, Maycock T, Tignor M, Waterfield T (eds) An IPCC special report on the impacts of global warming of 1.5 °C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty. https://www.ipcc.ch/site/assets/uploads/sites/2/2019/06/SR15_Full_Report_High_Res.pdf. Accessed 2 Dec 2022.

Ishii, S. I., Suzuki, S., Norden-Krichmar, T. M., Wu, A., Yamanaka, Y., Nealson, K. H., & Bretschger, O. (2013). Identifying the microbial communities and operational conditions for optimized wastewater treatment in microbial fuel cells. *Water research*, 47(19), 7120-7130.

Janicek, A., Fan, Y., & Liu, H. (2014). Design of microbial fuel cells for practical application: a review and analysis of scale-up studies. *Biofuels*, 5(1), 79-92.

- Jiang, D., Curtis, M., Troop, E., Scheible, K., McGrath, J., Hu, B., ... & Li, B. (2011). A pilot-scale study on utilizing multi-anode/cathode microbial fuel cells (MAC MFCs) to enhance the power production in wastewater treatment. *International Journal of Hydrogen Energy*, 36(1), 876-884.
- Julio, R., Albet, J., Vialle, C., Vaca-Garcia, C., & Sablayrolles, C. (2017). Sustainable design of biorefinery processes: existing practices and new methodology. *Biofuels Bioprod Biorefin* 11: 373–395.
- Kassim, F. O., Thomas, C. P., & Afolabi, O. O. (2022). Integrated conversion technologies for sustainable agri-food waste valorization: A critical review. *Biomass and Bioenergy*, 156, 106314.
- Kruse, A., Bernolle, P., Dahmen, N., Dinjus, E., & Maniam, P. (2010). Hydrothermal gasification of biomass: consecutive reactions to long-living intermediates. *Energy & Environmental Science*, 3(1), 136-143.
- Kummu, M., De Moel, H., Porkka, M., Siebert, S., Varis, O., & Ward, P. J. (2012). Lost food, wasted resources: Global food supply chain losses and their impacts on freshwater, cropland, and fertiliser use. *Science of the total environment*, 438, 477-489.
- Lange, L. (2017). Fungal enzymes and yeasts for conversion of plant biomass to bioenergy and high-value products. *Microbiology spectrum*, 5(1), 5-1.
- Lange, L., & Meyer, A. S. (2019). Potentials and possible safety issues of using biorefinery products in food value chains. *Trends in Food Science & Technology*, 84, 7-11.
- Lange, L., Connor, K. O., Arason, S., Bundgård-Jørgensen, U., Canalis, A., Carrez, D., ... & Vieira, H. (2021). Developing a sustainable and circular bio-based economy in EU: by partnering across sectors, upscaling and using new knowledge faster, and for the benefit of climate, environment & biodiversity, and people & business. *Frontiers in bioengineering and biotechnology*, 1456.
- Lee, S. Y., Sankaran, R., Chew, K. W., Tan, C. H., Krishnamoorthy, R., Chu, D. T., & Show, P. L. (2019). Waste to bioenergy: a review on the recent conversion technologies. *Bmc Energy*, 1(1), 1-22.
- Lee, W. S., Chua, A. S. M., Yeoh, H. K., & Ngoh, G. C. (2014). A review of the production and applications of waste-derived volatile fatty acids. *Chemical Engineering Journal*, 235, 83-99.
- Loboguerrero, A. M., Campbell, B. M., Cooper, P. J., Hansen, J. W., Rosenstock, T., & Wollenberg, E. (2019). Food and earth systems: priorities for climate change adaptation and mitigation for agriculture and food systems. *Sustainability*, 11(5), 1372.
- Maitan-Alfenas, G. P., Visser, E. M., & Guimarães, V. M. (2015). Enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products. *Current Opinion in Food Science*, 1, 44-49.

- Martin, M., & Grossmann, I. E. (2013). On the systematic synthesis of sustainable biorefineries. *Industrial & Engineering Chemistry Research*, 52(9), 3044-3064.
- McKendry, P. (2002). Energy production from biomass (part 2): conversion technologies. *Bioresource technology*, 83(1), 47-54.
- Möller, K., & Müller, T. (2012). Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. *Engineering in life sciences*, 12(3), 242-257.
- Moncada, J., El-Halwagi, M. M., & Cardona, C. A. (2013). Techno-economic analysis for a sugarcane biorefinery: Colombian case. *Bioresource technology*, 135, 533-543.
- Moncada, J., Tamayo, J. A., & Cardona, C. A. (2014). Integrating first, second, and third generation biorefineries: Incorporating microalgae into the sugarcane biorefinery. *Chemical Engineering Science*, 118, 126-140.
- Paini, J., Benedetti, V., Ail, S. S., Castaldi, M. J., Baratieri, M., & Patuzzi, F. (2021). Valorization of wastes from the food production industry: A review towards an integrated agri-food processing biorefinery. *Waste and Biomass Valorization*, 1-20.
- Paritosh, K., Kushwaha, S. K., Yadav, M., Pareek, N., Chawade, A., & Vivekanand, V. (2017). Food waste to energy: an overview of sustainable approaches for food waste management and nutrient recycling. *BioMed research international*, 2017.
- Pasupuleti, S. B., Sarkar, O., & Mohan, S. V. (2014). Upscaling of biohydrogen production process in semi-pilot scale biofilm reactor: evaluation with food waste at variable organic loads. *International journal of hydrogen energy*, 39(14), 7587-7596.
- Proestos, C., & Komaitis, M. (2008). Application of microwave-assisted extraction to the fast extraction of plant phenolic compounds. *LWT-food science and technology*, 41(4), 652-659.
- Ravindran, R., & Jaiswal, A. K. (2016). Exploitation of food industry waste for high-value products. *Trends in biotechnology*, 34(1), 58-69.
- Ravindran, R., & Jaiswal, A. K. (2016). Exploitation of food industry waste for high-value products. *Trends in biotechnology*, 34(1), 58-69.
- Rincón-Moreno, J., Ormazábal, M., Álvarez, M. J., & Jaca, C. (2021). Advancing circular economy performance indicators and their application in Spanish companies. *Journal of Cleaner Production*, 279, 123605.
- Santibañez-Aguilar, J. E., González-Campos, J. B., Ponce-Ortega, J. M., Serna-González, M., & El-Halwagi, M. M. (2014). Optimal planning and site selection for distributed multiproduct biorefineries involving economic, environmental and social objectives. *Journal of cleaner production*, 65, 270-294.

Sheets, J. P., Yang, L., Ge, X., Wang, Z., & Li, Y. (2015). Beyond land application: Emerging technologies for the treatment and reuse of anaerobically digested agricultural and food waste. *Waste Management*, 44, 94-115.

Susastriawan, A. A. P., & Saptoadi, H. (2017). Small-scale downdraft gasifiers for biomass gasification: A review. *Renewable and Sustainable Energy Reviews*, 76, 989-1003.

Tomczyk, A., Sokołowska, Z., & Boguta, P. (2020). Biochar physicochemical properties: pyrolysis temperature and feedstock kind effects. *Reviews in Environmental Science and Bio/Technology*, 19(1), 191-215.

Trabold, T., & Babbitt, C. W. (2018). *Sustainable food waste-to-energy systems*. Elsevier, Cambridge

UN SDGs (2020). *Transforming Our World, the 2030 Agenda for Sustainable Development*. Available online at: <https://sdgs.un.org/2030agenda> (accessed December 12, 2022).

UNCCS (2019) Climate action and support trends, United Nations Climate Change Secretariat. https://unfccc.int/sites/default/files/resource/Climate_Action_Support_Trends_2019.pdf. Accessed 02 Dec 2022

UNFCCC (2005) Report of the convergence of the parties serving as the meeting of the parties to the kyoto protocol on its first session. <https://unfccc.int/resource/docs/2005/cmp1/eng/08a02.pdf>. Accessed 3 Dec 2022.

UNFCCC (2008) Kyoto protocol reference manual on accounting of emissions and assigned amount. https://unfccc.int/sites/default/files/08_unfccc_kp_ref_manual.pdf Accessed 02 Dec 2022

Venkata Mohan, S., Mohanakrishna, G., Goud, R. K., & Sarma, P. N. (2009). Acidogenic fermentation of vegetable based market waste to harness biohydrogen with simultaneous stabilization. *Bioresource Technology*, 100(12), 3061-3068.

Watson, J., Zhang, Y., Si, B., Chen, W. T., & de Souza, R. (2018). Gasification of biowaste: A critical review and outlooks. *Renewable and Sustainable Energy Reviews*, 83, 1-17.

Weiland, P. (2010). Biogas production: current state and perspectives. *Applied microbiology and biotechnology*, 85(4), 849-860.

Xi-Liu, Y., & Qing-Xian, G. (2018). Contributions of natural systems and human activity to greenhouse gas emissions. *Adv Clim Chang Res* 9: 243.

Yang, Y., Heaven, S., Venetsaneas, N., Banks, C. J., & Bridgwater, A. V. (2018). Slow pyrolysis of organic fraction of municipal solid waste (OFMSW): Characterisation of products and screening of the aqueous liquid product for anaerobic digestion. *Applied energy*, 213, 158-168.

Yang, M., Chen, L., Wang, J., Msigwa, G., Osman, A. I., Fawzy, S., ... & Yap, P. S. (2022). Circular economy strategies for combating climate change and other environmental issues. *Environmental Chemistry Letters*, 1-26.

Yu, I. K., Attard, T. M., Chen, S. S., Tsang, D. C., Hunt, A. J., Jérôme, F., ... & Poon, C. S. (2018). Supercritical carbon dioxide extraction of value-added products and thermochemical synthesis of platform chemicals from food waste. *ACS Sustainable Chemistry & Engineering*, 7(2), 2821-2829.

Zhuang, L., Zheng, Y., Zhou, S., Yuan, Y., Yuan, H., & Chen, Y. (2012). Scalable microbial fuel cell (MFC) stack for continuous real wastewater treatment. *Bioresource technology*, 106, 82-88.

(Figures)

Cardona-Alzate, C. A., Serna-Loaiza, S., & Ortiz-Sanchez, M. (2020). Sustainable Biorefineries: What was learned from the design, analysis and implementation. *Journal of Sustainable Development of Energy, Water and Environment Systems*, 8(1), 88-117.

Paini, J., Benedetti, V., Ail, S. S., Castaldi, M. J., Baratieri, M., & Patuzzi, F. (2021). Valorization of wastes from the food production industry: A review towards an integrated agri-food processing biorefinery. *Waste and Biomass Valorization*, 1-20.

Ravindran, R., & Jaiswal, A. K. (2016). Exploitation of food industry waste for high-value products. *Trends in biotechnology*, 34(1), 58-69.

Schröder, U. (2007). Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. *Physical Chemistry Chemical Physics*, 9(21), 2619-2629.

Yang, M., Chen, L., Wang, J., Msigwa, G., Osman, A. I., Fawzy, S., ... & Yap, P. S. (2022). Circular economy strategies for combating climate change and other environmental issues. *Environmental Chemistry Letters*, 1-26.

Cp.2 At the upstream of biorefinery

(Text)

Agustin, M. B., de Carvalho, D. M., Lahtinen, M. H., Hilden, K., Lundell, T., & Mikkonen, K. S. (2021). Laccase as a Tool in Building Advanced Lignin-Based Materials. *ChemSusChem*, 14(21), 4615-4635.

Arya, P. S., Yagnik, S. M., Rajput, K. N., Panchal, R. R., & Raval, V. H. (2022). Valorization of agro-food wastes: ease of concomitant-enzymes production with application in food and biofuel industries. *Bioresource Technology*, 127738.

Babbar, N., & Oberoi, H. S. (2014). Enzymes in value-addition of agricultural and agro-industrial residues. *Enzymes in value-addition of wastes*, 29-50.

Barta, Z., Kovacs, K., Reczey, K., & Zacchi, G. (2010). Process design and economics of on-site cellulase production on various carbon sources in a softwood-based ethanol plant. *Enzyme research*, 2010.

Bailey, M. J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of biotechnology*, 23(3), 257-270.

BIO-Intelligence Service, Food and Agriculture Organization of the United Nations (FAO). (2013). Food Wastage Footprint - Impacts on Natural Resources – Summary report. Food and Agriculture Organization of the United Nations (FAO)

Blanco, C. A., Caballero, I., Barrios, R., & Rojas, A. (2014). Innovations in the brewing industry: Light beer. *International journal of food sciences and nutrition*, 65(6), 655-660.

Boerjan, W., Ralph, J., & Baucher, M. (2003). Lignin biosynthesis. *Annu. Rev. Plant Biol.*, 54,519–46

Budžaki, S., Strelec, I., Krnić, M., Alilović, K., Tišma, M. and Zelić, B. (2018), Proximate analysis of cold-press oil cakes after biological treatment with *Trametes versicolor* and *Humicola grisea*. *Eng. Life Sci.*, 18: 924-931. <https://doi.org/10.1002/elsc.201800033>

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, 3(1), 1-30.

Chugh, P., Soni, R., & Soni, S. K. (2016). Deoiled rice bran: a substrate for co-production of a consortium of hydrolytic enzymes by *Aspergillus niger* P-19. *Waste and biomass valorization*, 7(3), 513-525.

- Corrêa, C. L., Penha, E. M., Freitas-Silva, O., Luna, A. S., & Gottschalk, L. M. (2021). Enzymatic technology application on Coffee co-products: a review. *Waste and Biomass Valorization*, 12(7), 3521-3540.
- Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nature reviews molecular cell biology*, 6(11), 850-861.
- Crivellaro, A., & Büntgen, U. (2020). New evidence of thermally constrained plant cell wall lignification. *Trends in plant science*, 25(4), 322-324.
- Crivellaro, A., Piermattei, A., Dolezal, J., Dupree, P., & Büntgen, U. (2022). Biogeographic implication of temperature-induced plant cell wall lignification. *Communications Biology*, 5(1), 1-10
- Delmer, D. P. (1987). Cellulose biosynthesis. *Annual review of plant physiology*, 38(1), 259-290.
- Dosoretz, C. G., Chen, H. C., & Grethlein, H. E. (1990). Effect of environmental conditions on extracellular protease activity in lignolytic cultures of *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*, 56(2), 395-400.
- Ergun, S. O., & Urek, R. O. (2017). Production of ligninolytic enzymes by solid state fermentation using *Pleurotus ostreatus*. *Annals of Agrarian Science*, 15(2), 273-277.
- Eshra, D. H., El-Iraki, S. M., & Bakr, A. (2014). Performance of starch hydrolysis and production of corn syrup using some commercial enzymes. *International Food Research Journal*, 21(2).
- Espro, C., Paone, E., Mauriello, F., Gotti, R., Uliassi, E., Bolognesi, M. L., ... & Luque, R. (2021). Sustainable production of pharmaceutical, nutraceutical and bioactive compounds from biomass and waste. *Chemical Society Reviews*.
- Esteban, J., & Ladero, M. (2018). Food waste as a source of value-added chemicals and materials: a biorefinery perspective. *International journal of food science & technology*, 53(5), 1095-1108.
- Ezquer, I., Salameh, I., Colombo, L., & Kalaitzis, P. (2020). Plant cell walls tackling climate change: Biotechnological strategies to improve crop adaptations and photosynthesis in response to global warming. *Plants*, 9(2), 212.
- FAO. 2011. *Global food losses and food waste – Extent, causes and prevention*. Rome
- Favela-Torres, E., Volke-Sepúlveda, T., & Viniegra-González, G. (2006). Production of Hydrolytic Depolymerising Pectinases. *Food Technology & Biotechnology*, 44(2).

Flurkey, W. H., & Inlow, J. K. (2008). Proteolytic processing of polyphenol oxidase from plants and fungi. *Journal of inorganic biochemistry*, 102(12), 2160-2170.

Giacinti, Agnese (2011) *Valorizzazione del Pisum Sativum L. tramite tecniche di bioliquefazione: studio e caratterizzazione*. [Laurea magistrale], Università di Bologna, Corso di Studio in [Chimica industriale \[LM-DM270\]](#)

Giroto, F., Alibardi, L., & Cossu, R. (2015). Food waste generation and industrial uses: A review. *Waste management*, 45, 32-41.

Giuliani, S., Piana, C., Setti, L., Hochkoeppler, A., Pifferi, P. G., Williamson, G., & Faulds, C. B. (2001). Synthesis of pentylferulate by a feruloyl esterase from *Aspergillus niger* using water-in-oil microemulsions. *Biotechnology Letters*, 23, 325-330.

Han, M. L., An, Q., He, S. F., Zhang, X. L., Zhang, M. H., Gao, X. H., ... & Bian, L. S. (2020). Solid-state fermentation on poplar sawdust and corncob wastes for lignocellulolytic enzymes by different *Pleurotus ostreatus* strains. *BioResources*, 15(3), 4982-4995.

Harris, P. J. (2005). Diversity in plant cell walls. *Plant diversity and evolution: genotypic and phenotypic variation in higher plants*, 201-227.

Harris, P. J., & Smith, B. G. (2006). Plant cell walls and cell-wall polysaccharides: structures, properties and uses in food products. *International journal of food science & technology*, 41, 129-143.

Hatakka, A. I. (1983). Pretreatment of wheat straw by white-rot fungi for enzymic saccharification of cellulose. *European journal of applied microbiology and biotechnology*, 18(6), 350-357.

Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., & Foust, T. D. (2007). Biomass recalcitrance: engineering plants and enzymes for biofuels production. *science*, 315(5813), 804-807.

Hölker, U., Höfer, M., & Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied microbiology and biotechnology*, 64(2), 175-186.

Howard, R. L., Abotsi, E. L. J. R., Van Rensburg, E. J., & Howard, S. (2003). Lignocellulose biotechnology: issues of bioconversion and enzyme production. *African Journal of biotechnology*, 2(12), 602-619.

Iandolo, D., Piscitelli, A., Sannia, G., & Faraco, V. (2011). Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace. *Applied biochemistry and biotechnology*, 163(1), 40-51.

- Isroi, I., Millati, R., Niklasson, C., Cayanto, C., Taherzadeh, M. J., & Lundquist, K. (2011). Biological treatment of Lignocelluloses with white-rot fungi and its applications. *BioResources*, 6(4), 5224-5259.
- Izzo, L., Castaldo, L., Narváez, A., Graziani, G., Gaspari, A., Rodríguez-Carrasco, Y., & Ritieni, A. (2020). Analysis of phenolic compounds in commercial Cannabis sativa L. inflorescences using UHPLC-Q-Orbitrap HRMS. *Molecules*, 25(3), 631.
- Karp, S. G., Faraco, V., Amore, A., Letti, L. A. J., Thomaz Soccol, V., & Soccol, C. R. (2015). Statistical optimization of laccase production and delignification of sugarcane bagasse by *Pleurotus ostreatus* in solid-state fermentation. *BioMed research international*, 2015.
- Kertesz, M. A., & Thai, M. (2018). Compost bacteria and fungi that influence growth and development of *Agaricus bisporus* and other commercial mushrooms. *Applied microbiology and biotechnology*, 102(4), 1639-1650.
- Kim, J. S., Lee, Y. Y., & Kim, T. H. (2016). A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresource technology*, 199, 42-48.
- Kim, S. W., Kang, S. W., & Lee, J. S. (1997). Cellulase and xylanase production by *Aspergillus niger* KKS in various bioreactors. *Bioresource technology*, 59(1), 63-67.
- Kirk, O., Borchert, T. V., & Fuglsang, C. C. (2002). Industrial enzyme applications. *Current opinion in biotechnology*, 13(4), 345-351.
- Körner, C. (2016). Plant adaptation to cold climates. *F1000Research*, 5.
- Kunitz, M. (1947). Crystalline soybean trypsin inhibitor: II. General properties. *The Journal of General Physiology*, 30(4), 291-310.
- Lawoko, M., Henriksson, G., & Gellerstedt, G. (2005). Structural differences between the lignin-carbohydrate complexes present in wood and in chemical pulps. *Biomacromolecules*, 6(6), 3467-3473.
- Lundell, T. K., Mäkelä, M. R., & Hildén, K. (2010). Lignin-modifying enzymes in filamentous basidiomycetes—ecological, functional and phylogenetic review. *Journal of basic microbiology*, 50(1), 5-20.
- Martínez-Abad, A., Ruthes, A. C., & Vilaplana, F. (2016). Enzymatic-assisted extraction and modification of lignocellulosic plant polysaccharides for packaging applications. *Journal of Applied Polymer Science*, 133(2).

Masutti, D., Borgognone, A., & Setti, L. (2012). Production of enzymes from rice husks and wheat straw in solid state fermentation. *Chemical engineering transactions*, 27, 133-138.

Masutti, D. C., Borgognone, A., Scardovi, F., Vaccari, C., & Setti, L. (2015). Effects on the enzymes production from different mixes of agro-food wastes. *CHEMICAL ENGINEERING*, 43.

Masutti, D., Scardovi, F., Borgognone, A., & Setti, L. (2016). Agro-food wastes for the release of phyto-chemicals and the production of enzymes by solid state fermentation using *pleurotus ostreatus*. *Chemical Engineering Transactions*, 49, 139-144.

Mathew, S., & Abraham, T. E. (2004). Ferulic acid: an antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. *Critical reviews in biotechnology*, 24(2-3), 59-83.

Mayolo-Deloisa, K., González-González, M., & Rito-Palomares, M. (2020). Laccases in food industry: Bioprocessing, potential industrial and biotechnological applications. *Frontiers in bioengineering and biotechnology*, 8, 222.

Mayolo-Deloisa, K., González-González, M., & Rito-Palomares, M. (2020). Laccases in food industry: Bioprocessing, potential industrial and biotechnological applications. *Frontiers in bioengineering and biotechnology*, 8, 222.

Moore, J. P., Vicié-Gibouin, M., Farrant, J. M., & Driouich, A. (2008). Adaptations of higher plant cell walls to water loss: drought vs desiccation. *Physiologia plantarum*, 134(2), 237-245

Mota, T. R., Oliveira, D. M., Marchiosi, R., Ferrarese-Filho, O., & Santos, W. (2018). Plant cell wall composition and enzymatic deconstruction. *AIMS Bioengineering*, 5(1), 63-77.

Nissen, L., di Carlo, E., & Gianotti, A. (2020). Prebiotic potential of hemp blended drinks fermented by probiotics. *Food Research International*, 131, 109029.

Oakenfull, D., & Scott, A. (1984). Hydrophobic interaction in the gelation of high methoxyl pectins. *Journal of Food Science*, 49(4), 1093-1098.

Okano, K., Ohkoshi, N., Nishiyama, A., Usagawa, T., & Kitagawa, M. (2009). Improving the nutritive value of madake bamboo, *Phyllostachys bambusoides*, for ruminants by culturing with the white-rot fungus *Ceriporiopsis subvermispora*. *Animal Feed Science and Technology*, 152(3-4), 278-285.

Ozcirak Ergun, S., & Ozturk Urek, R. (2017). Production of ligninolytic enzymes by solid state fermentation using *Pleurotus ostreatus*. *Annals of Agrarian Science*, 15(2), 273-277.
<https://doi.org/10.1016/j.aasci.2017.04.003>

- Padayachee, A., Day, L., Howell, K., & Gidley, M. J. (2017). Complexity and health functionality of plant cell wall fibers from fruits and vegetables. *Critical reviews in food science and nutrition*, 57(1), 59-81.
- Pandey, A., Selvakumar, P., Soccol, C. R., & Nigam, P. (1999). Solid state fermentation for the production of industrial enzymes. *Current science*, 149-162.
- Pojić, M., Mišan, A., Sakač, M., Dapčević Hadnađev, T., Šarić, B., Milovanović, I., & Hadnađev, M. (2014). Characterization of byproducts originating from hemp oil processing. *Journal of Agricultural and Food Chemistry*, 62(51), 12436-12442.
- Prasanna, V., Prabha, T. N., & Tharanathan, R. N. (2007). Fruit ripening phenomena—an overview. *Critical reviews in food science and nutrition*, 47(1), 1-19.
- Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., ... & Pandey, A. (2018). Applications of microbial enzymes in food industry. *Food technology and biotechnology*, 56(1), 16.
- RedCorn, R., Fatemi, S., & Engelberth, A. S. (2018). Comparing end-use potential for industrial food-waste sources. *Engineering*, 4(3), 371-380.
- Reddy, N., & Yang, Y. (2005). Biofibers from agricultural byproducts for industrial applications. *TRENDS in Biotechnology*, 23(1), 22-27.
- Reshmy, R., Philip, E., Madhavan, A., Sirohi, R., Pugazhendhi, A., Binod, P., ... & Sindhu, R. (2022). Lignocellulose in future biorefineries: Strategies for cost-effective production of biomaterials and bioenergy. *Bioresource Technology*, 344, 126241.
- Sánchez, C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnology advances*, 27(2), 185-194.
- Sarkar, P., Bosneaga, E., & Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *Journal of experimental botany*, 60(13), 3615-3635.
- Schultz, C. J., Lim, W. L., Khor, S. F., Neumann, K. A., Schulz, J. M., Ansari, O., Skewes, M. A., & Burton, R. A. (2020). Consumer and health-related traits of seed from selected commercial and breeding lines of industrial hemp, *Cannabis sativa* L.. *Journal of Agriculture and Food Research*, 2, 100025. <https://doi.org/10.1016/j.jafr.2020.100025>

Scott, G. M., Akhtar, M., Swaney, R. E., & Houtman, C. J. (2002). Recent developments in biopulping technology at Madison, WI. *Progress in biotechnology-amsterdam-*, 61-72.

Setti, L., Scali, S., Degli Angeli, I., & Pifferi, P. G. (1998). Horseradish peroxidase-catalyzed oxidative coupling of 3-methyl 2-benzothiazolinone hydrazone and methoxyphenols. *Enzyme and Microbial Technology*, 22(8), 656-661.

Setti, L., Giuliani, S., Spinozzi, G., & Pifferi, P. G. (1999). Laccase catalyzed-oxidative coupling of 3-methyl 2-benzothiazolinone hydrazone and methoxyphenols. *Enzyme and Microbial Technology*, 25(3-5), 285-289.

Setti, L., Samaei, S. P., Maggiore, I., Nissen, L., Gianotti, A., & Babini, E. (2020). Comparing the effectiveness of three different biorefinery processes at recovering bioactive products from hemp (*Cannabis sativa* L.) byproduct. *Food and Bioprocess Technology*, 13(12), 2156-2171.

Sharma, R., Oberoi, H. S., & Dhillon, G. S. (2016). Fruit and vegetable processing waste: renewable feed stocks for enzyme production. In *Agro-industrial wastes as feedstock for enzyme production* (pp. 23-59). Academic Press.

Smith, B. G., & Harris, P. J. (2001). Ferulic acid is esterified to glucuronoarabinoxylans in pineapple cell walls. *Phytochemistry*, 56(5), 513-519.

Svendsen, A. (2000). Lipase protein engineering. *Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular Enzymology*, 1543(2), 223-238.

Toca-Herrera, J. L., Osma, J. F., & Rodriguez Couto, S. (2007). Potential of solid-state fermentation for laccase production. *Communicating current research and educational topics and trends in applied microbiology*, 10, 391-400.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., & Boerjan, W. (2010). Lignin biosynthesis and structure. *Plant physiology*, 153(3), 895-905.

Viniegra-González, G., Favela-Torres, E., & Aguilar, C. N. (2003). S. d. J. Romero-Gomez, G. Díaz-Godínez and C. Augur. *Biochem. Eng. J*, 13, 157-167.

Voragen, A. G., Coenen, G. J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry*, 20(2), 263-275.

Waldron, K. W., Parker, M. L., & Smith, A. C. (2003). Plant cell walls and food quality. *Comprehensive reviews in food science and food safety*, 2(4), 128-146.

Wase, D. J., McManamey, W. J., Raymahasay, S., & Vaid, A. K. (1985). Comparisons between cellulase production by *Aspergillus fumigatus* in agitated vessels and in an air-lift fermentor. *Biotechnology and bioengineering*, 27(8), 1166-1172.

Yu, P., McKinnon, J. J., & Christensen, D. A. (2005). Hydroxycinnamic acids and ferulic acid esterase in relation to biodegradation of complex plant cell walls. *Canadian journal of animal science*, 85(3), 255-267.

(Figures)

Šelo, G., Planinić, M., Tišma, M., Tomas, S., Koceva Komlenić, D., & Bucić-Kojić, A. (2021). A comprehensive review on valorization of agro-food industrial residues by solid-state fermentation. *Foods*, 10(5), 927.

RedCorn, R., Fatemi, S., & Engelberth, A. S. (2018). Comparing end-use potential for industrial food-waste sources. *Engineering*, 4(3), 371-380.

<https://onlinesciencenotes.com/differences-between-primary-and-secondary-cell-wall-in-plants/>

Sarkar, P., Bosneaga, E., & Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *Journal of experimental botany*, 60(13), 3615-3635.

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, 3(1), 1-30.

Martínez-Abad, A., Ruthes, A. C., & Vilaplana, F. (2016). Enzymatic-assisted extraction and modification of lignocellulosic plant polysaccharides for packaging applications. *Journal of Applied Polymer Science*, 133(2).

Mnich, E., Bjarnholt, N., Eudes, A., Harholt, J., Holland, C., Jørgensen, B., ... & Ulvskov, P. (2020). Phenolic cross-links: building and de-constructing the plant cell wall. *Natural product reports*, 37(7), 919-961.

(Tables)

Chaudhary, A., Hussain, Z., Aihetasham, A., El-Sharnouby, M., Abdul Rehman, R., Azmat Ullah Khan, M., Zahra, S., Saleem, A., Azhar, S., Alhazmi, A., El Askary, A., Sayed, S., Ali El Enshasy, H., Zulaiha Hanapi, S., & Qamer, S. (2021). Pomegranate peels waste hydrolyzate optimization by Response Surface Methodology for Bioethanol production. *Saudi Journal of Biological Sciences*, 28(9), 4867-4875. <https://doi.org/10.1016/j.sjbs.2021.06.081>

Šelo, G., Planinić, M., Tišma, M., Tomas, S., Koceva Komlenić, D., & Bucić-Kojić, A. (2021). A comprehensive review on valorization of agro-food industrial residues by solid-state fermentation. *Foods*, 10(5), 927.

Budžaki, S., Strelec, I., Krnić, M., Alilović, K., Tišma, M. and Zelić, B. (2018), Proximate analysis of cold-press oil cakes after biological treatment with *Trametes versicolor* and *Humicola grisea*. *Eng. Life Sci.*, 18: 924-931. <https://doi.org/10.1002/elsc.201800033>

Cripwell, R., Favaro, L., Rose, S. H., Basaglia, M., Cagnin, L., Casella, S., & van Zyl, W. (2015). Utilisation of wheat bran as a substrate for bioethanol production using recombinant cellulases and amylolytic yeast. *Applied Energy*, 160, 610-617. <https://doi.org/10.1016/j.apenergy.2015.09.062>

Ugheoke, I.B. (2012). A critical assessment and new research directions of rice husk silica processing methods and properties. *Maejo International Journal of Science and Technology*, 6, 430-448.

Iandolo, D., Piscitelli, A., Sannia, G., & Faraco, V. (2011). Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace. *Applied biochemistry and biotechnology*, 163(1), 40-51.

Han, M. L., An, Q., He, S. F., Zhang, X. L., Zhang, M. H., Gao, X. H., ... & Bian, L. S. (2020). Solid-state fermentation on poplar sawdust and corncob wastes for lignocellulolytic enzymes by different *Pleurotus ostreatus* strains. *BioResources*, 15(3), 4982-4995.

Ergun, S. O., & Urek, R. O. (2017). Production of ligninolytic enzymes by solid state fermentation using *Pleurotus ostreatus*. *Annals of Agrarian Science*, 15(2), 273-277.

Kumar, V. V., Venkataraman, S., Kumar, P. S., George, J., Rajendran, D. S., Shaji, A., ... & Rathankumar, A. K. (2022). Laccase production by *Pleurotus ostreatus* using cassava waste and its application in remediation of phenolic and polycyclic aromatic hydrocarbon-contaminated lignocellulosic biorefinery wastewater. *Environmental Pollution*, 309, 119729.

Setti, L., Samaei, S. P., Maggiore, I., Nissen, L., Gianotti, A., & Babini, E. (2020). Comparing the effectiveness of three different biorefinery processes at recovering bioactive products from hemp (*Cannabis sativa* L.) byproduct. *Food and Bioprocess Technology*, 13(12), 2156-2171.

Chp. 3 The biorefinery core

(Text)

Akay, F., Kazan, A., Celiktaş, M. S., & Yeşil-Celiktaş, O. (2015). A holistic engineering approach for utilization of olive pomace. *The Journal of Supercritical Fluids*, 99, 1-7.

Akhtar, S., Ismail, T., Fraternali, D., & Sestili, P. (2015). Pomegranate peel and peel extracts: Chemistry and food features. *Food chemistry*, 174, 417-425.

Ando, H., Ryu, A., Hashimoto, A., Oka, M., & Ichihashi, M. (1998). Linoleic acid and α -linolenic acid lightens ultraviolet-induced hyperpigmentation of the skin. *Archives of dermatological research*, 290(7), 375-381.

Babbar, N., Roy, S. V., Wijnants, M., Dejonghe, W., Caligiani, A., Sforza, S., & Elst, K. (2016). Effect of extraction conditions on the saccharide (neutral and acidic) composition of the crude pectic extract from various agro-industrial residues. *Journal of agricultural and food chemistry*, 64(1), 268-276.

Baiano, A. (2014). Recovery of biomolecules from food wastes—A review. *Molecules*, 19(9), 14821-14842.

Bailey, M. J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of biotechnology*, 23(3), 257-270.

Banerjee, J., Singh, R., Vijayaraghavan, R., MacFarlane, D., Patti, A. F., & Arora, A. (2017). Bioactives from fruit processing wastes: Green approaches to valuable chemicals. *Food chemistry*, 225, 10-22.

Bayar, N., Kriaa, M., & Kammoun, R. (2016). Extraction and characterization of three polysaccharides extracted from *Opuntia ficus indica* cladodes. *International journal of biological macromolecules*, 92, 441-450.

Begum, A., Sarma, J., Borah, P., Moni Bhuyan, P., Saikia, R., Hussain Ahmed, T., ... & Chowdhury, P. (2015). Microwave (MW) energy in enzyme deactivation: stabilization of rice bran from few widely consumed indigenous rice cultivars (*Oryza sativa* L.) from Eastern Himalayan range. *Current Nutrition & Food Science*, 11(3), 240-245.

Belc, N., Mustatea, G., Apostol, L., Iorga, S., Vlăduț, V. N., & Mosoiu, C. (2019). Cereal supply chain waste in the context of circular economy. In *E3S Web of Conferences* (Vol. 112, p. 03031). EDP Sciences.

Bhattacharya, K. R. (1979, February). Gelatinization temperature of rice starch and its determination. In *Chemical Aspects of rice Grain Grain Quality". Proceedings of the Workshop, International Rice Research Institute, Manila, Philippines* (pp. 231-269).

Bindereif, B., Karbstein, H. P., Zahn, K., & van der Schaaf, U. S. (2022). Effect of Conformation of Sugar Beet Pectin on the Interfacial and Emulsifying Properties. *Foods*, 11(2), 214.

Biocosmetics. (n.d.) *Segen's Medical Dictionary*. (2011). Retrieved December 23 2022 from <https://medical-dictionary.thefreedictionary.com/biocosmetics>

Bodie, A. R., Micciche, A. C., Atungulu, G. G., Rothrock Jr, M. J., & Ricke, S. C. (2019). Current trends of rice milling byproducts for agricultural applications and alternative food production systems. *Frontiers in Sustainable Food Systems*, 3, 47.

Bouyer, E., Mekhloufi, G., Rosilio, V., Grossiord, J. L., & Agnely, F. (2012). Proteins, polysaccharides, and their complexes used as stabilizers for emulsions: alternatives to synthetic surfactants in the pharmaceutical field?. *International journal of pharmaceutics*, 436(1-2), 359-378.

Bowler, C., Montagu, M. V., & Inze, D. (1992). Superoxide dismutase and stress tolerance. *Annual review of plant biology*, 43(1), 83-116.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. L. W. T. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food science and Technology*, 28(1), 25-30.

Bruins, M. E., & Sanders, J. P. (2012). Small-scale processing of biomass for biorefinery. *Biofuels, bioproducts and biorefining*, 6(2), 135-145.

Burton, R. A., Andres, M., Cole, M., Cowley, J. M., & Augustin, M. A. (2022). Industrial hemp seed: from the field to value-added food ingredients. *Journal of Cannabis Research*, 4(1), 1-13.

Camargo Junior, F. B. D., Gaspar, L. R., & Campos, P. M. B. G. M. (2012). Immediate and long-term effects of polysaccharides-based formulations on human skin. *Brazilian Journal of Pharmaceutical Sciences*, 48, 547-555.

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, 3(1), 1-30.

- Choi, J. M., Park, C. S., Baik, M. Y., Kim, H. S., Choi, Y. S., Choi, H. W., & Seo, D. H. (2018). Enzymatic extraction of starch from broken rice using freeze-thaw infusion with food-grade protease. *Starch-Stärke*, 70(1-2), 1700007.
- Costantini, S., Rusolo, F., De Vito, V., Moccia, S., Picariello, G., Capone, F., ... & Volpe, M. G. (2014). Potential anti-inflammatory effects of the hydrophilic fraction of pomegranate (*Punica granatum* L.) seed oil on breast cancer cell lines. *Molecules*, 19(6), 8644-8660.
- Costin, G. E., & Hearing, V. J. (2007). Human skin pigmentation: melanocytes modulate skin color in response to stress. *The FASEB journal*, 21(4), 976-994.
- Crivellaro, A., Piermattei, A., Dolezal, J., Dupree, P., & Büntgen, U. (2022). Biogeographic implication of temperature-induced plant cell wall lignification. *Communications Biology*, 5(1), 1-10.
- Cruz, C. F., Ribeiro, A., Martins, M., & Cavaco-Paulo, A. (2017). Effect of a peptide in cosmetic formulations for hair volume control. *International Journal of Cosmetic Science*, 39(6), 600-609.
- Delahaije, R. J., Wierenga, P. A., van Nieuwenhuijzen, N. H., Giuseppin, M. L., & Gruppen, H. (2013). Protein concentration and protein-exposed hydrophobicity as dominant parameters determining the flocculation of protein-stabilized oil-in-water emulsions. *Langmuir*, 29(37), 11567-11574.
- Deng, G. F., Shen, C., Xu, X. R., Kuang, R. D., Guo, Y. J., Zeng, L. S., ... & Li, H. B. (2012). Potential of fruit wastes as natural resources of bioactive compounds. *International journal of molecular sciences*, 13(7), 8308-8323.
- Dias, M. F. R. G. (2015). Hair cosmetics: an overview. *International journal of trichology*, 7(1), 2.
- Dias, M. F. R. G., de Almeida, A. M., Cecato, P. M. R., Adriano, A. R., & Pichler, J. (2014). The shampoo pH can affect the hair: myth or reality?. *International journal of trichology*, 6(3), 95.
- Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food hydrocolloids*, 17(1), 25-39.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. A. J. N. (1951). A colorimetric method for the determination of sugars. *Nature*, 168, 167-167.
- Đurđević, S., Milovanović, S., Šavikin, K., Ristić, M., Menković, N., Pljevljakušić, D., ... & Bogdanović, A. (2017). Improvement of supercritical CO₂ and n-hexane extraction of wild growing pomegranate seed oil by microwave pretreatment. *Industrial Crops and Products*, 104, 21-27.
- Edreva, A., Velikova, V., Tsonev, T., Dagnon, S., Gürel, A., Aktaş, L., & Gesheva, E. (2008). Stress-protective role of secondary metabolites: diversity of functions and mechanisms. *Gen Appl Plant Physiol*, 34(1-2), 67-78.

- El-Shamy, S., & Farag, M. A. (2021). Novel trends in extraction and optimization methods of bioactives recovery from pomegranate fruit biowastes: Valorization purposes for industrial applications. *Food chemistry*, 365, 130465.
- Engelbrechtsen, K. A., Johansen, J. D., Kezic, S., Linneberg, A., & Thyssen, J. P. (2016). The effect of environmental humidity and temperature on skin barrier function and dermatitis. *Journal of the European Academy of Dermatology and Venereology*, 30(2), 223-249.
- Farag, R. S., Abdel-Latif, M. S., Emam, S. S., & Tawfeek, L. S. (2014). Phytochemical screening and polyphenol constituents of pomegranate peels and leave juices. *Agric Soil Sci*, 1(6), 86-93.
- Faravash, R. S., & Ashtiani, F. Z. (2007). The effect of pH, ethanol volume and acid washing time on the yield of pectin extraction from peach pomace. *International journal of food science & technology*, 42(10), 1177-1187.
- Ferrari, G., Mustafa, W., & Donsì, F. (2017). Use of agri-food residues for oil structuring and functionalization. *Chemical Engineering Transactions*, 57, 1831-1836.
- Ferrentino, G., Asaduzzaman, M. D., & Scampicchio, M. M. (2018). Current technologies and new insights for the recovery of high valuable compounds from fruits by-products. *Critical Reviews in Food Science and Nutrition*, 58(3), 386-404.
- Folin, O., & Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. *J. biol. Chem*, 73(2), 627-650.
- Fischer, U. A., Carle, R., & Kammerer, D. R. (2011). Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MSn. *Food chemistry*, 127(2), 807-821.
- Fisher, G. J., Datta, S. C., Talwar, H. S., Wang, Z. Q., Varani, J., Kang, S., & Voorhees, J. J. (1996). Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature*, 379(6563), 335-339.
- Fitzpatrick, T. B., & Szabo, G. (1959). Part III: general considerations of skin pigmentation: the melanocyte: cytology and cytochemistry. *Journal of Investigative Dermatology*, 32(2), 197-206.
- Food and Agriculture Organization of the United Nations (FAO). Cereal Supply and Demand Brief. <http://www.fao.org/worldfoodsituation/csdb/en/> Accessed Dec 2022
- Foujdar, R., Bera, M. B., & Chopra, H. K. (2020). Optimization of process variables of probe ultrasonic-assisted extraction of phenolic compounds from the peel of *Punica granatum* Var. Bhagwa and it's chemical and bioactivity characterization. *Journal of Food Processing and Preservation*, 44(1), e14317.

Fraterrigo Garofalo, S., Tommasi, T., & Fino, D. (2021). A short review of green extraction technologies for rice bran oil. *Biomass Conversion and Biorefinery*, 11(2), 569-587.

Garland, C. F., Garland, F. C., & Gorham, E. D. (1993). Rising trends in melanoma an hypothesis concerning sunscreen effectiveness. *Annals of epidemiology*, 3(1), 103-110.

Goula, A. M. (2013). Ultrasound-assisted extraction of pomegranate seed oil–Kinetic modeling. *Journal of Food Engineering*, 117(4), 492-498.

Goula, A. M., Papatheodorou, A., Karasavva, S., & Kaderides, K. (2018). Ultrasound-assisted aqueous enzymatic extraction of oil from pomegranate seeds. *Waste and Biomass Valorization*, 9(1), 1-11.

Graham, L. E., Cook, M. E., & Busse, J. S. (2000). The origin of plants: body plan changes contributing to a major evolutionary radiation. *Proceedings of the National Academy of Sciences*, 97(9), 4535-4540.

Griffin, W. C. (1954). Calculation of HLB values of non-ionic surfactants. *J. Soc. Cosmet. Chem.*, 5, 249-256.

Gustavsson, J., Cederberg, C., Sonesson, U., Van Otterdijk, R., & Meybeck, A. (2011). Global food losses and food waste.

Hadnađev, M., Dapčević-Hadnađev, T., Lazaridou, A., Moschakis, T., Michaelidou, A. M., Popović, S., & Biliaderis, C. G. (2018 b). Hempseed meal protein isolates prepared by different isolation techniques. Part I. physicochemical properties. *Food Hydrocolloids*, 79, 526-533.

Hadnađev, M., Dizdar, M., Hadnađev-Dapčević, T., Jovanov, P., Mišan, A., & Sakač, M. (2018 a). Hydrolyzed hemp seed proteins as bioactive peptides. *Journal on Processing and Energy in Agriculture*, 22(2), 90-94.

Hasnaoui, N., Wathelet, B., & Jiménez-Araujo, A. (2014). Valorization of pomegranate peel from 12 cultivars: Dietary fibre composition, antioxidant capacity and functional properties. *Food Chemistry*, 160, 196-203.

Hassan, G., Shabbir, M. A., Ahmad, F., Pasha, I., Aslam, N., Ahmad, T., ... & Aadil, R. M. (2021). Cereal processing waste, an environmental impact and value addition perspectives: A comprehensive treatise. *Food Chemistry*, 363, 130352.

House, J. D., Neufeld, J., & Leson, G. (2010). Evaluating the quality of protein from hemp seed (*Cannabis sativa* L.) products through the use of the protein digestibility-corrected amino acid score method. *Journal of agricultural and food chemistry*, 58(22), 11801-11807.

- Ibrahim, M. I. (2010). Efficiency of pomegranate peel extract as antimicrobial, antioxidant and protective agents. *World Journal of Agricultural Sciences*, 6(4), 338-344.
- Isah, T. (2019). Stress and defense responses in plant secondary metabolites production. *Biological research*, 52.
- Jimbow, K., Quevedo Jr, W. C., Fitzpatrick, T. B., & Szabo, G. (1976). Some aspects of melanin biology: 1950–1975. *Journal of Investigative Dermatology*, 67(1), 72-89.
- Karunaratne, H. W. G. I. (2010). *Removal of pollutants in parboiled paddy waste water* (Doctoral dissertation).
- Kondo, N., & Kawashima, M. (2000). Enhancement of the tolerance to oxidative stress in cucumber (*Cucumis sativus* L.) seedlings by UV-B irradiation: possible involvement of phenolic compounds and antioxidative enzymes. *Journal of Plant Research*, 113(3), 311-317.
- Kostić, M. D., Joković, N. M., Stamenković, O. S., Rajković, K. M., Milić, P. S., & Veljković, V. B. (2013). Optimization of hempseed oil extraction by n-hexane. *Industrial crops and products*, 48, 133-143.
- Kralova, I., & Sjöblom, J. (2009). Surfactants used in food industry: a review. *Journal of Dispersion Science and Technology*, 30(9), 1363-1383.
- Kushwaha, R. (2018). Pharmacognosy of rice bran oil-A review. *International Journal of Green Pharmacy (IJGP)*, 12(04).
- Lam, R. S., & Nickerson, M. T. (2013). Food proteins: a review on their emulsifying properties using a structure–function approach. *Food chemistry*, 141(2), 975-984.
- Lam, R. S., & Nickerson, M. T. (2013). Food proteins: a review on their emulsifying properties using a structure–function approach. *Food chemistry*, 141(2), 975-984.
- Lampe, M. A., Burlingame, A. L., Whitney, J., Williams, M. L., Brown, B. E., Roitman, E., & Elias, P. M. (1983). Human stratum corneum lipids: characterization and regional variations. *Journal of lipid research*, 24(2), 120-130.
- Lebreton-Decoster, C., Rousselle, P., Laperdrix, C., Lubrano, C., Robin, J. R., & Coulomb, B. (2011). Oligogalacturonides improve tissue organization of in vitro reconstructed skin. *International journal of cosmetic science*, 33(5), 455-461.
- Li, L., Cheng, X. F., Leshkevich, J., Umezawa, T., Harding, S. A., & Chiang, V. L. (2001). The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. *The Plant Cell*, 13(7), 1567-1586.

- Li, Y., Jiang, B., Zhang, T., Mu, W., & Liu, J. (2008). Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food chemistry*, 106(2), 444-450.
- Lo, S. C. C., & Nicholson, R. L. (1998). Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls: implications for a compensatory role in the defense response. *Plant Physiology*, 116(3), 979-989.
- Lucarini, M., Durazzo, A., Romani, A., Campo, M., Lombardi-Boccia, G., & Cecchini, F. (2018). Bio-based compounds from grape seeds: A biorefinery approach. *Molecules*, 23(8), 1888.
- Lumdubwong, N., & Seib, P. A. (2000). Rice starch isolation by alkaline protease digestion of wet-milled rice flour. *Journal of Cereal Science*, 31(1), 63-74.
- Malomo, S. A., & Aluko, R. E. (2015). A comparative study of the structural and functional properties of isolated hemp seed (*Cannabis sativa* L.) albumin and globulin fractions. *Food Hydrocolloids*, 43, 743-752.
- Manhongo, T. T., Chimphango, A. F. A., Thornley, P., & Röder, M. (2021). Current status and opportunities for fruit processing waste biorefineries. *Renewable and Sustainable Energy Reviews*, 111823.
- Marcolongo, L., La Cara, F., & Ionata, E. (2021). Hemp waste valorization through enzymatic hydrolysis for biofuels and biochemicals production. *Chemical Engineering Transactions*, 86, 127-132.
- Matthäus, B., & Brühl, L. (2008). Virgin hemp seed oil: An interesting niche product. *European Journal of Lipid Science and Technology*, 110(7), 655-661.
- Mazaheri, D., Orooji, Y., Mazaheri, M., Moghaddam, M. S., & Karimi-Maleh, H. (2021). Bioethanol production from pomegranate peel by simultaneous saccharification and fermentation process. *Biomass Conversion and Biorefinery*, 1-9
- Mazid, M., Khan, T. A., & Mohammad, F. (2011). Role of secondary metabolites in defense mechanisms of plants. *Biology and medicine*, 3(2), 232-249.
- McClements, D. J. (2004). *Food emulsions: principles, practices, and techniques*. CRC press.
- Mellencamp, M., Koppien-Fox, J., Lamb, R., & Dvorak, R. (2011). Antibacterial and antioxidant activity of oregano essential oil.
- Melo, I. L. P. D., Carvalho, E. B. T. D., Silva, A. M. D. O., Yoshime, L. T., Sattler, J. A. G., Pavan, R. T., & Mancini-Filho, J. (2016). Characterization of constituents, quality and stability of pomegranate seed oil (*Punica granatum* L.). *Food Science and Technology*, 36, 132-139.

Michalak, M. (2022). Plant-derived antioxidants: Significance in skin health and the ageing process. *International journal of molecular sciences*, 23(2), 585.

Miedes, E., Vanholme, R., Boerjan, W., & Molina, A. (2014). The role of the secondary cell wall in plant resistance to pathogens. *Frontiers in plant science*, 5, 358.

Mitchell, C. R. (2009). Rice starches: production and properties. In *Starch* (pp. 569-578). Academic Press.

Moliterni, V. M. C., Pojić, M., & Tiwari, B. (2022). Industrial hemp by-product valorization. In *Industrial Hemp* (pp. 301-340). Academic Press.

Moore, J. P., Vitré-Gibouin, M., Farrant, J. M., & Driouich, A. (2008). Adaptations of higher plant cell walls to water loss: drought vs desiccation. *Physiologia plantarum*, 134(2), 237-245.

Nag, S., & Sit, N. (2018). Optimization of ultrasound assisted enzymatic extraction of polyphenols from pomegranate peels based on phytochemical content and antioxidant property. *Journal of Food Measurement and Characterization*, 12(3), 1734-1743.

Néjib Hasnaoui , Rania Jbir , Messaoud Mars , Mokhtar Trifi , Afaf Kamal-Eldin , Pablo Melgarejo & Francisca Hernandez (2011) Organic Acids, Sugars, and Anthocyanins Contents in Juices of Tunisian Pomegranate Fruits, *International Journal of Food Properties*, 14:4, 741-757, DOI: 10.1080/10942910903383438

Ngouémazong, E. D., Christiaens, S., Shpigelman, A., Van Loey, A., & Hendrickx, M. (2015). The emulsifying and emulsion-stabilizing properties of pectin: A review. *Comprehensive Reviews in Food Science and Food Safety*, 14(6), 705-718.

Novak, A.C., Sydney, E.B., Soccol, C.R. (2014). Biocosmetics. In: Brar, S., Dhillon, G., Soccol, C. (eds) *Biotransformation of Waste Biomass into High Value Biochemicals*. Springer, New York, NY. https://doi.org/10.1007/978-1-4614-8005-1_16

Ortiz-Sanchez, M., Solarte-Toro, J. C., Orrego-Alzate, C. E., Acosta-Medina, C. D., & Cardona-Alzate, C. A. (2021). Integral use of orange peel waste through the biorefinery concept: an experimental, technical, energy, and economic assessment. *Biomass conversion and Biorefinery*, 11(2), 645-659.

Ozturk, B., & McClements, D. J. (2016). Progress in natural emulsifiers for utilization in food emulsions. *Current Opinion in Food Science*, 7, 1-6.

Ozturk, B., & McClements, D. J. (2016). Progress in natural emulsifiers for utilization in food emulsions. *Current Opinion in Food Science*, 7, 1-6.

- Padayachee, A., Day, L., Howell, K., & Gidley, M. J. (2017). Complexity and health functionality of plant cell wall fibers from fruits and vegetables. *Critical reviews in food science and nutrition*, 57(1), 59-81.
- Paini, J., Benedetti, V., Ail, S. S., Castaldi, M. J., Baratieri, M., & Patuzzi, F. (2021). Valorization of wastes from the food production industry: A review towards an integrated agri-food processing biorefinery. *Waste and Biomass Valorization*, 1-20.
- Panzella, L., Moccia, F., Nasti, R., Marzorati, S., Verotta, L., & Napolitano, A. (2020). Bioactive phenolic compounds from agri-food wastes: An update on green and sustainable extraction methodologies. *Frontiers in nutrition*, 7, 60.
- Pathak, M. A., & Stratton, K. (1968). Free radicals in human skin before and after exposure to light. *Archives of Biochemistry and Biophysics*, 123(3), 468-476.
- Pathak, P. D., Mandavgane, S. A., & Kulkarni, B. D. (2017). Valorization of pomegranate peels: A biorefinery approach. *Waste and biomass valorization*, 8(4), 1127-1137.
- Pearse, A. D., Gaskell, S. A., & Marks, R. (1987). Epidermal changes in human skin following irradiation with either UVB or UVA. *Journal of investigative dermatology*, 88(1), 83-87.
- Pereira, P. H. F., Oliveira, T. Í. S., Rosa, M. F., Cavalcante, F. L., Moates, G. K., Wellner, N., ... & Azeredo, H. M. (2016). Pectin extraction from pomegranate peels with citric acid. *International journal of biological macromolecules*, 88, 373-379.
- Petri, D. F. (2015). Xanthan gum: A versatile biopolymer for biomedical and technological applications. *Journal of Applied Polymer Science*, 132(23).
- Petri, D. F. (2015). Xanthan gum: A versatile biopolymer for biomedical and technological applications. *Journal of Applied Polymer Science*, 132(23).
- Petridis, A., Therios, I., Samouris, G., & Tananaki, C. (2012). Salinity-induced changes in phenolic compounds in leaves and roots of four olive cultivars (*Olea europaea* L.) and their relationship to antioxidant activity. *Environmental and Experimental Botany*, 79, 37-43.
- Pocan, P., Bahcegul, E., Oztop, M. H., & Hamamci, H. (2018). Enzymatic hydrolysis of fruit peels and other lignocellulosic biomass as a source of sugar. *Waste and Biomass Valorization*, 9(6), 929-937.
- Pojić, M., Mišan, A., Sakač, M., Dapčević Hadnađev, T., Šarić, B., Milovanović, I., & Hadnađev, M. (2014). Characterization of byproducts originating from hemp oil processing. *Journal of Agricultural and Food Chemistry*, 62(51), 12436-12442.

- Popper, Z. A. (2008). Evolution and diversity of green plant cell walls. *Current opinion in plant biology*, 11(3), 286-292.
- Puglia, C., & Bonina, F. (2008). In vivo spectrophotometric evaluation of skin barrier recovery after topical application of soybean phytosterols. *Journal of Cosmetic Science*, 59(3), 217-224.
- Qu, W., Pan, Z., Zhang, R., Ma, H., Zhu, B., Wang, Z., & Atungulu, G. G. (2009). Integrated extraction and anaerobic digestion process for recovery of nutraceuticals and biogas from pomegranate marc. *Transactions of the ASABE*, 52(6), 1997-2006.
- Quan, N. T., Anh, L. H., Khang, D. T., Tuyen, P. T., Toan, N. P., Minh, T. N., ... & Xuan, T. D. (2016). Involvement of secondary metabolites in response to drought stress of rice (*Oryza sativa* L.). *Agriculture*, 6(2), 23.
- Rafiee, E., Shahebrahimi, S., Feyzi, M., & Shaterzadeh, M. (2012). Optimization of synthesis and characterization of nanosilica produced from rice husk (a common waste material). *International nano letters*, 2(1), 1-8.
- Ramachandran, S., Singh, S. K., Larroche, C., Soccol, C. R., & Pandey, A. (2007). Oil cakes and their biotechnological applications—A review. *Bioresource technology*, 98(10), 2000-2009.
- Rawlings, A. V., & Harding, C. R. (2004). Moisturization and skin barrier function. *Dermatologic therapy*, 17, 43-48.
- Rawlings, A. V., Scott, I. R., Harding, C. R., & Bowser, P. A. (1994). Stratum corneum moisturization at the molecular level. *Journal of investigative dermatology*, 103(5), 731-740.
- Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. <http://data.europa.eu/eli/reg/2009/1223/oj>
- Rose, J. K., Catalá, C., Gonzalez-Carranza, Z. H., & Roberts, J. A. (2018). Cell wall disassembly. *Annual Plant Reviews online*, 264-324.
- Rosenthal, A., Pyle, D. L., & Niranjana, K. (1996). Aqueous and enzymatic processes for edible oil extraction. *Enzyme and microbial technology*, 19(6), 402-420.
- Ryu, H. S., Jeong, J., Lee, C. M., Lee, K. S., Lee, J. N., Park, S. M., & Lee, Y. M. (2021). Activation of hair cell growth factors by linoleic acid in *Malva verticillata* seed. *Molecules*, 26(8), 2117.
- Saha, S. S., & Ghosh, M. (2009). Comparative study of antioxidant activity of α -eleostearic acid and punicic acid against oxidative stress generated by sodium arsenite. *Food and Chemical Toxicology*, 47(10), 2551-2556.

- Santos, F., Machado, G., Faria, D., Lima, J., Marçal, N., Dutra, E., & Souza, G. (2017). Productive potential and quality of rice husk and straw for biorefineries. *Biomass Conversion and Biorefinery*, 7(1), 117-126.
- Sarkar, P., Bosneaga, E., & Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *Journal of experimental botany*, 60(13), 3615-3635.
- Sawatdeenarunat, C., Nguyen, D., Surendra, K. C., Shrestha, S., Rajendran, K., Oechsner, H., ... & Khanal, S. K. (2016). Anaerobic biorefinery: current status, challenges, and opportunities. *Bioresource technology*, 215, 304-313.
- Schultz, C. J., Lim, W. L., Khor, S. F., Neumann, K. A., Schulz, J. M., Ansari, O., ... & Burton, R. A. (2020). Consumer and health-related traits of seed from selected commercial and breeding lines of industrial hemp, *Cannabis sativa* L. *Journal of Agriculture and Food Research*, 2, 100025.
- Schwartz, E., Tzulker, R., Glazer, I., Bar-Ya'akov, I., Wiesman, Z., Tripler, E., ... & Amir, R. (2009). Environmental conditions affect the color, taste, and antioxidant capacity of 11 pomegranate accessions' fruits. *Journal of agricultural and food chemistry*, 57(19), 9197-9209.
- Semwogerere, F., Katiyatiya, C. L., Chikwanha, O. C., Marufu, M. C., & Mapiye, C. (2020). Bioavailability and bioefficacy of hemp by-products in ruminant meat production and preservation: A review. *Frontiers in Veterinary Science*, 7, 572906.
- Setti, L., Samaei, S. P., Maggiore, I., Nissen, L., Gianotti, A., & Babini, E. (2020). Comparing the effectiveness of three different biorefinery processes at recovering bioactive products from hemp (*Cannabis sativa* L.) byproduct. *Food and Bioprocess Technology*, 13(12), 2156-2171.
- Shinde, P. N., Mandavgane, S. A., & Karadbhajane, V. (2020). Process development and life cycle assessment of pomegranate biorefinery. *Environmental Science and Pollution Research*, 27(20), 25785-25793.
- Sikora, E. (2009). *Cosmetic Emulsions: Monograph*. Krakow: Wydawnictwo PK.
- Silva, L. D. O., Ranquine, L. G., Monteiro, M., & Torres, A. G. (2019). Pomegranate (*Punica granatum* L.) seed oil enriched with conjugated linolenic acid (cLnA), phenolic compounds and tocopherols: Improved extraction of a specialty oil by supercritical CO₂. *The Journal of Supercritical Fluids*, 147, 126-137.
- Simate, G. S., Cluett, J., Iyuke, S. E., Musapatika, E. T., Ndlovu, S., Walubita, L. F., & Alvarez, A. E. (2011). The treatment of brewery wastewater for reuse: State of the art. *Desalination*, 273(2-3), 235-247.

Skrť, M., Albrecht, A., Vovk, I., Constantin, O. E., Răpeanu, G., Sežun, M., ... & Poklar Ulrih, N. (2022). Extraction of polyphenols and valorization of fibers from Istrian-grown pomegranate (*Punica granatum* L.). *Foods*, 11(18), 2740.

Sytar, O., Mbarki, S., Zivcak, M., & Brestic, M. (2018). The involvement of different secondary metabolites in salinity tolerance of crops. In *Salinity Responses and Tolerance in Plants, Volume 2* (pp. 21-48). Springer, Cham.

Tadros, T. F. (2013). Emulsion formation, stability, and rheology. *Emulsion formation and stability*, 1, 1-75.

Taina Simoinen & Maija Tenkanen (Eds.). (1999). 2nd European Symposium on Enzymes in Grain Processing. Helsinki: VTT Biotechnology.
<https://www.vttresearch.com/sites/default/files/pdf/symposiums/2000/S207.pdf>

Talekar, S., Patti, A. F., Singh, R., Vijayraghavan, R., & Arora, A. (2018b). From waste to wealth: High recovery of nutraceuticals from pomegranate seed waste using a green extraction process. *Industrial Crops and Products*, 112, 790-802.

Talekar, S., Patti, A. F., Vijayraghavan, R., & Arora, A. (2018a). An integrated green biorefinery approach towards simultaneous recovery of pectin and polyphenols coupled with bioethanol production from waste pomegranate peels. *Bioresource technology*, 266, 322-334.

Teh, S. S., Niven, B. E., Bekhit, A. E. D. A., Carne, A., & Birch, E. J. (2014). The use of microwave and pulsed electric field as a pretreatment step in ultrasonic extraction of polyphenols from defatted hemp seed cake (*Cannabis sativa*) using response surface methodology. *Food and Bioprocess Technology*, 7(11), 3064-3076.

USP NF 21. The United States pharmacopeia – The national formulary. Rockville, MD: United States Pharmacopeial Convention., pp. 1401–1402 , 2003.

Wang, D., Zhang, H., Wu, F., Li, T., Liang, Y., & Duan, X. (2013). Modification of pectin and hemicellulose polysaccharides in relation to aril breakdown of harvested longan fruit. *International journal of molecular sciences*, 14(12), 23356–23368. <https://doi.org/10.3390/ijms141223356>

Wang, Q., & Xiong, Y. L. (2019). Processing, nutrition, and functionality of hempseed protein: A review. *Comprehensive Reviews in Food Science and Food Safety*, 18(4), 936-952.

Wetzstein, H. Y., Zhang, Z., Ravid, N., & Wetzstein, M. E. (2011). Characterization of attributes related to fruit size in pomegranate. *HortScience*, 46(6), 908-912.

World Rice Production 2022/2023 World Agricultural Production.
<http://www.worldagriculturalproduction.com/crops/rice.aspx>. Accessed Dec 2022

- Wu, J., Johnson, L. A., & Jung, S. (2009). Demulsification of oil-rich emulsion from enzyme-assisted aqueous extraction of extruded soybean flakes. *Bioresource Technology*, 100(2), 527-533.
- Xu, J., Yue, R. Q., Liu, J., Ho, H. M., Yi, T., Chen, H. B., & Han, Q. B. (2014). Structural diversity requires individual optimization of ethanol concentration in polysaccharide precipitation. *International journal of biological macromolecules*, 67, 205-209.
- Yang, X., Nisar, T., Hou, Y., Gou, X., Sun, L., & Guo, Y. (2018). Pomegranate peel pectin can be used as an effective emulsifier. *Food Hydrocolloids*, 85, 30-38.
- Yang, Y., Leser, M. E., Sher, A. A., & McClements, D. J. (2013). Formation and stability of emulsions using a natural small molecule surfactant: Quillaja saponin (Q-Naturale®). *Food Hydrocolloids*, 30(2), 589-596.
- Yapo, B. M., Robert, C., Etienne, I., Wathelet, B., & Paquot, M. (2007). Effect of extraction conditions on the yield, purity and surface properties of sugar beet pulp pectin extracts. *Food chemistry*, 100(4), 1356-1364.
- Yusoff, M. M., Gordon, M. H., & Niranjana, K. (2015). Aqueous enzyme assisted oil extraction from oilseeds and emulsion de-emulsifying methods: A review. *Trends in Food Science & Technology*, 41(1), 60-82.
- Zanichelli, Dario (2008) *Processi di biorefining per l'estrazione di secondary chemical building blocks da sottoprodotti dell'agro-industria*, [Dissertation thesis], Alma Mater Studiorum Università di Bologna. Dottorato di ricerca in Biocatalisi applicata e microbiologia industriale, 20 Ciclo. DOI 10.6092/unibo/amsdottorato/641.
- Zhai, X., Zhu, C., Li, Y., Zhang, Y., Duan, Z., & Yang, X. (2018). Optimization for pectinase-assisted extraction of polysaccharides from pomegranate peel with chemical composition and antioxidant activity. *International journal of biological macromolecules*, 109, 244-253
- Zhang, X., Ervin, E. H., & Schmidt, R. E. (2005). The role of leaf pigment and antioxidant levels in UV-B resistance of dark-and light-green Kentucky bluegrass cultivars. *Journal of the American Society for Horticultural Science*, 130(6), 836-841.

(Figures)

Costin, G. E., & Hearing, V. J. (2007). Human skin pigmentation: melanocytes modulate skin color in response to stress. *The FASEB journal*, 21(4), 976-994.

Engebretsen, K. A., Johansen, J. D., Kezic, S., Linneberg, A., & Thyssen, J. P. (2016). The effect of environmental humidity and temperature on skin barrier function and dermatitis. *Journal of the European Academy of Dermatology and Venereology*, 30(2), 223-249.

Moore, J. P., Vitré-Gibouin, M., Farrant, J. M., & Driouich, A. (2008). Adaptations of higher plant cell walls to water loss: drought vs desiccation. *Physiologia plantarum*, 134(2), 237-245.

Mukama, M., Ambaw, A., & Opara, U. L. (2019). Thermal properties of whole and tissue parts of pomegranate (*Punica granatum*) fruit. *Journal of Food Measurement and Characterization*, 13(2), 901-910.

Ozturk, B., & McClements, D. J. (2016). Progress in natural emulsifiers for utilization in food emulsions. *Current Opinion in Food Science*, 7, 1-6.

Sikora, E. (2009). *Cosmetic Emulsions: Monograph*. Krakow: Wydawnictwo PK.

Tadros, T. F. (2013). Emulsion formation, stability, and rheology. *Emulsion formation and stability*, 1, 1-75.

(Tables)

Elfalleh, W., Hannachi, H., Guetat, A., Tlili, N., Guasmi, F., Ferchichi, A., & Ying, M. (2012). Storage protein and amino acid contents of Tunisian and Chinese pomegranate (*Punica granatum* L.) cultivars. *Genetic resources and crop evolution*, 59(6), 999-1014.

Girgih, A. T., Udenigwe, C. C., & Aluko, R. E. (2011). In vitro antioxidant properties of hemp seed (*Cannabis sativa* L.) protein hydrolysate fractions. *Journal of the American Oil Chemists' Society*, 88(3), 381-389.

Kalman, D. S. (2014). Amino acid composition of an organic brown rice protein concentrate and isolate compared to soy and whey concentrates and isolates. *Foods*, 3(3), 394-402.

Liu, H., Zhang, R., Yao, X., Liu, M., Hu, Z., & Fan, B.T. (2004). Prediction of the Isoelectric Point of an Amino Acid Based on GA-PLS and SVMs. *Journal of chemical information and computer sciences*, 44 1, 161-7 .

Chp. 4 Biorefinery integration

(Text)

Apblett, C., Schubert, K., Kelley, B., Walker, A., Simmons, B., Meserole, S., ... & Casalnuovo, S. (2005). Bio Micro Fuel Cell Grand Challenge Final Report. <http://www.prod.sandia.gov/techlib/accesscontrol.cgi/2005/055734.pdf>

An, L., Zhao, T. S., Shen, S. Y., Wu, Q. X., & Chen, R. (2011). Alkaline direct oxidation fuel cell with non-platinum catalysts capable of converting glucose to electricity at high power output. *Journal of Power Sources*, 196(1), 186-190.

Asazawa, K., Sakamoto, T., Yamaguchi, S., Yamada, K., Fujikawa, H., Tanaka, H., & Oguro, K. (2009). Study of anode catalysts and fuel concentration on direct hydrazine alkaline anion-exchange membrane fuel cells. *Journal of The Electrochemical Society*, 156(4), B509.

Bagotsky, V. S. (2011). Fuel cells, batteries, and the development of electrochemistry. *Journal of Solid State Electrochemistry*, 15(7), 1559-1562.

Benziger, J. B., Satterfield, M. B., Hogarth, W. H., Nehlsen, J. P., & Kevrekidis, I. G. (2006). The power performance curve for engineering analysis of fuel cells. *Journal of power sources*, 155(2), 272-285.

Breeze, P. (2017). *Fuel cells*. Academic Press.

Brown, W. H., Iverson, B. L., Anslyn, E., & Foote, C. S. (2013). *Organic chemistry*. Cengage Learning.

Brown, L. D., Neville, T. P., Jervis, R., Mason, T. J., Shearing, P. R., & Brett, D. J. (2016). The effect of felt compression on the performance and pressure drop of all-vanadium redox flow batteries. *Journal of Energy Storage*, 8, 91-98.

Bruins, M. E., & Sanders, J. P. (2012). Small-scale processing of biomass for biorefinery. *Biofuels, bioproducts and biorefining*, 6(2), 135-145.

Caballero, B., Finglas, P., & Toldrá, F. (2015). *Encyclopedia of food and health*. Academic Press.

Calabrese Barton, S., Gallaway, J., & Atanassov, P. (2004). Enzymatic biofuel cells for implantable and microscale devices. *Chemical reviews*, 104(10), 4867-4886.

Casimero, C., Hegarty, C., McGlynn, R. J., & Davis, J. (2020). Ultrasonic exfoliation of carbon fiber: electroanalytical perspectives. *Journal of Applied Electrochemistry*, 50(3), 383-394.

Chan, K. Y., Zhan, X., Lam, C. M., Chung, M., Tseung, A. C. C., Shen, P. K., & You, J. K. (2009) US 7,419,580 B2, Methods and apparatus for the oxidation of glucose molecules, September 15, 2009.

Chaudhuri, S. K., & Lovley, D. R. (2003). Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. *Nature biotechnology*, 21(10), 1229-1232.

Chen, G., Chen, N., & Wang, Q. (2018). Preparation of poly (vinyl alcohol)/ionic liquid composites with improved processability and electrical conductivity for fused deposition modeling. *Materials & design*, 157, 273-283.

Chen, Q., Mangadlao, J. D., Wallat, J., De Leon, A., Pokorski, J. K., & Advincula, R. C. (2017). 3D printing biocompatible polyurethane/poly (lactic acid)/graphene oxide nanocomposites: anisotropic properties. *ACS applied materials & interfaces*, 9(4), 4015-4023.

Chen, C. C., Lin, C. L., & Chen, L. C. (2015). A binary palladium–bismuth nanocatalyst with high activity and stability for alkaline glucose electrooxidation. *Journal of Power Sources*, 287, 323-333.

Chen, J., Zhao, C. X., Zhi, M. M., Wang, K., Deng, L., & Xu, G. (2012). Alkaline direct oxidation glucose fuel cell system using silver/nickel foams as electrodes. *Electrochimica acta*, 66, 133-138.

Cheng, X., Shi, Z., Glass, N., Zhang, L., Zhang, J., Song, D., ... & Shen, J. (2007). A review of PEM hydrogen fuel cell contamination: Impacts, mechanisms, and mitigation. *Journal of Power Sources*, 165(2), 739-756.

Cheng, S., Liu, H., & Logan, B. E. (2006). Power densities using different cathode catalysts (Pt and CoTMPP) and polymer binders (Nafion and PTFE) in single chamber microbial fuel cells. *Environmental science & technology*, 40(1), 364-369.

Chin, K. L., H'ng, P. S., Wong, L. J., Tey, B. T., & Paridah, M. T. (2011). Production of glucose from oil palm trunk and sawdust of rubberwood and mixed hardwood. *Applied energy*, 88(11), 4222-4228.

Chisholm, G., Kitson, P. J., Kirkaldy, N. D., Bloor, L. G., & Cronin, L. (2014). 3D printed flow plates for the electrolysis of water: an economic and adaptable approach to device manufacture. *Energy & Environmental Science*, 7(9), 3026-3032.

Christ, J. F., Aliheidari, N., Ameli, A., & Pötschke, P. (2017). 3D printed highly elastic strain sensors of multiwalled carbon nanotube/thermoplastic polyurethane nanocomposites. *Materials & Design*, 131, 394-401.

Cosnier, S., Le Goff, A., & Holzinger, M. (2014). Towards glucose biofuel cells implanted in human body for powering artificial organs. *Electrochemistry Communications*, 38, 19-23.

- Cui, H. F., Ye, J. S., Liu, X., Zhang, W. D., & Sheu, F. S. (2006). Pt–Pb alloy nanoparticle/carbon nanotube nanocomposite: a strong electrocatalyst for glucose oxidation. *Nanotechnology*, 17(9), 2334.
- De Bruijn, J. M. (1986). Monosaccharides in alkaline medium: isomerization, degradation, oligomerization.
- De Bruijn, J. M., Kieboom, A. P. G., & Van Bekkum, H. (1986). Alkaline degradation of monosaccharides III. Influence of reaction parameters upon the final product composition. *Recueil des Travaux Chimiques des Pays-Bas*, 105(6), 176-183.
- Derakhshanfar, S., Mbeleck, R., Xu, K., Zhang, X., Zhong, W., & Xing, M. (2018). 3D bioprinting for biomedical devices and tissue engineering: A review of recent trends and advances. *Bioactive materials*, 3(2), 144-156.
- Du Toit, H., Rashidi, R., Ferdani, D. W., Delgado-Charro, M. B., Sangan, C. M., & Di Lorenzo, M. (2016). Generating power from transdermal extracts using a multi-electrode miniature enzymatic fuel cell. *Biosensors and Bioelectronics*, 78, 411-417.
- Dul, S., Pegoretti, A., & Fambri, L. (2018). Effects of the nanofillers on physical properties of acrylonitrile-butadiene-styrene nanocomposites: Comparison of graphene nanoplatelets and multiwall carbon nanotubes. *Nanomaterials*, 8(9), 674.
- Ei-Deab, M. S., Sotomura, T., & Ohsaka, T. (2005). Oxygen Reduction at Electrochemically Deposited Crystallographically Oriented Au (100)-like Gold Nanoparticles. *Electrochem Commun*, 7, 29-34.
- Falk, M., Narváez Villarrubia, C. W., Babanova, S., Atanassov, P., & Shleev, S. (2013). Biofuel cells for biomedical applications: colonizing the animal kingdom. *ChemPhysChem*, 14(10), 2045-2058.
- Fujiwara, N., Yamazaki, S. I., Siroma, Z., Ioroi, T., Senoh, H., & Yasuda, K. (2009). Nonenzymatic glucose fuel cells with an anion exchange membrane as an electrolyte. *Electrochemistry communications*, 11(2), 390-392.
- Gharehpetian, G. B., & Mousavi, M. (Eds.). (2017). *Distributed generation systems: design, operation and grid integration*. Butterworth-Heinemann.
- Ghasemi, M., Ismail, M., Kamarudin, S. K., Saeedfar, K., Daud, W. R. W., Hassan, S. H., ... & Oh, S. E. (2013). Carbon nanotube as an alternative cathode support and catalyst for microbial fuel cells. *Applied Energy*, 102, 1050-1056.
- Goodridge, F., & Scott, K. (1995). Electrolytic Reactor Design, Selection, and Scale-up. In *Electrochemical Process Engineering* (pp. 177-244). Springer, Boston, MA.

Gross, B. C., Erkal, J. L., Lockwood, S. Y., Chen, C., & Spence, D. M. (2014). Evaluation of 3D printing and its potential impact on biotechnology and the chemical sciences.

Huang, X. (2009). Fabrication and properties of carbon fibers. *Materials*, 2(4), 2369-2403.

Jönsson, L. J., Alriksson, B., & Nilvebrant, N. O. (2013). Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for biofuels*, 6(1), 1-10.

Izydorczyk, G., Skrzypczak, D., Kocek, D., Mironiuk, M., Witek-Krowiak, A., Moustakas, K., & Chojnacka, K. (2020). Valorization of bio-based post-extraction residues of goldenrod and alfalfa as energy pellets. *Energy*, 194, 116898.

Jang, J., Park, J. Y., Gao, G., & Cho, D. W. (2018). Biomaterials-based 3D cell printing for next-generation therapeutics and diagnostics. *Biomaterials*, 156, 88-106.

Jiang, T., Yan, L., Meng, Y., Xiao, M., Wu, Z., Tsiakaras, P., & Song, S. (2015). Glucose electrooxidation in alkaline medium: Performance enhancement of PdAu/C synthesized by NH₃ modified pulse microwave assisted polyol method. *Applied Catalysis B: Environmental*, 162, 275-281.

Joh, H. I., Hwang, S. Y., Cho, J. H., Ha, T. J., Kim, S. K., Moon, S. H., & Ha, H. Y. (2008). Development and characteristics of a 400 W-class direct methanol fuel cell stack. *International Journal of hydrogen energy*, 33(23), 7153-7162.

Kato, H., Yamamoto, M., & Fujimaki, M. (1969). Mechanisms of browning degradation of D-fructose in special comparison with D-glucose-glycine reaction. *Agricultural and Biological Chemistry*, 33(6), 939-948.

Katz, E., & MacVittie, K. (2013). Implanted biofuel cells operating in vivo—methods, applications and perspectives—feature article. *Energy & Environmental Science*, 6(10), 2791-2803.

Kerzenmacher, S., Ducrée, J., Zengerle, R., & Von Stetten, F. (2008). An abiotically catalyzed glucose fuel cell for powering medical implants: Reconstructed manufacturing protocol and analysis of performance. *Journal of Power Sources*, 182(1), 66-75.

Kim, H. H., Zhang, Y., & Heller, A. (2004). Bilirubin oxidase label for an enzyme-linked affinity assay with O₂ as substrate in a neutral pH NaCl solution. *Analytical chemistry*, 76(8), 2411-2414.

Kung, C. W., Cheng, Y. H., & Ho, K. C. (2014). Single layer of nickel hydroxide nanoparticles covered on a porous Ni foam and its application for highly sensitive non-enzymatic glucose sensor. *Sensors and Actuators B: Chemical*, 204, 159-166.

- Leonov, V. (2011). Simulation of maximum power in the wearable thermoelectric generator with a small thermopile. *Microsystem technologies*, 17(4), 495-504.
- Lewis, J. A. (2006). Direct ink writing of 3D functional materials. *Advanced Functional Materials*, 16(17), 2193-2204.
- Li, L., Lin, Q., Tang, M., Duncan, A. J., & Ke, C. (2019). Advanced polymer designs for direct-ink-write 3D printing. *Chemistry—A European Journal*, 25(46), 10768-10781.
- Li, Z., Li, G., Wu, Z., Jiao, S., & Hu, Z. (2019). Cobalt sulfides/carbon nanohybrids: a novel biocatalyst for nonenzymatic glucose biofuel cells and biosensors. *RSC advances*, 9(56), 32898-32905.
- Li, Y. S., Zhao, T. S., & Liang, Z. X. (2009). Performance of alkaline electrolyte-membrane-based direct ethanol fuel cells. *Journal of Power Sources*, 187(2), 387-392.
- Ligon, S. C., Liska, R., Stampfl, J., Gurr, M., & Mülhaupt, R. (2017). Polymers for 3D printing and customized additive manufacturing. *Chemical reviews*, 117(15), 10212-10290.
- Maggiulli, Luca (2020) *Studio di celle a biocombustibile a base di glucosio e composti di ferro*. [Laurea magistrale], Università di Bologna, Corso di Studio in [Chimica industriale \[LM-DM270\]](#).
- Moggia, G., Kenis, T., Daems, N., & Breugelmans, T. (2020). Electrochemical oxidation of d-glucose in alkaline medium: Impact of oxidation potential and chemical side reactions on the selectivity to d-gluconic and d-glucaric acid. *ChemElectroChem*, 7(1), 86-95.
- Mor, L., Rubin, Z., & Schechner, P. (2008). Measuring open circuit voltage in a glucose alkaline fuel cell operated as a continuous stirred tank reactor. *Journal of fuel cell science and technology*, 5(1).
- Moulik, S. P., Basu, D., & Bhattacharya, P. K. (1978). Effects of various conditions on the alkaline degradation of d-fructose and d-glucose. *Carbohydrate Research*, 63, 165-172.
- Murphy, S. V., & Atala, A. (2014). 3D bioprinting of tissues and organs. *Nature biotechnology*, 32(8), 773-785.
- Ning, F., Cong, W., Qiu, J., Wei, J., & Wang, S. (2015). Additive manufacturing of carbon fiber reinforced thermoplastic composites using fused deposition modeling. *Composites Part B: Engineering*, 80, 369-378.
- Pankratov, D., Sundberg, R., Sotres, J., Maximov, I., Graczyk, M., Suyatin, D. B., ... & Shleev, S. (2015). Transparent and flexible, nanostructured and mediatorless glucose/oxygen enzymatic fuel cells. *Journal of Power Sources*, 294, 501-506.

- Pekkanen, A. M., Mondschein, R. J., Williams, C. B., & Long, T. E. (2017). 3D printing polymers with supramolecular functionality for biological applications. *Biomacromolecules*, 18(9), 2669-2687.
- Percin, K., Zoellner, O., Rall, D., & Wessling, M. (2020). A tubular electrochemical reactor for slurry electrodes. *ChemElectroChem*, 7(12), 2665-2671.
- Perry, S. C., de León, C. P., & Walsh, F. C. (2020). The Design, Performance and Continuing Development of Electrochemical Reactors for Clean Electrosynthesis. *Journal of The Electrochemical Society*, 167(15), 155525.
- Pfaltzgraff, L. A., Cooper, E. C., Budarin, V., & Clark, J. H. (2013). Food waste biomass: a resource for high-value chemicals. *Green Chemistry*, 15(2), 307-314.
- Prashantha, K., & Roger, F. (2017). Multifunctional properties of 3D printed poly (lactic acid)/graphene nanocomposites by fused deposition modeling. *Journal of Macromolecular Science, Part A*, 54(1), 24-29.
- Ravindran, R., & Jaiswal, A. K. (2016). Exploitation of food industry waste for high-value products. *Trends in biotechnology*, 34(1), 58-69.
- Rao, J. R., Richter, G. J., Von Sturm, F., & Weidlich, E. (1976). The performance of glucose electrodes and the characteristics of different biofuel cell constructions. *Bioelectrochemistry and Bioenergetics*, 3(1), 139-150.
- Ressel, S., Laube, A., Fischer, S., Chica, A., Flower, T., & Struckmann, T. (2017). Performance of a vanadium redox flow battery with tubular cell design. *Journal of Power Sources*, 355, 199-205.
- Romanello, Rosa Sofia (2019) *Studio per il design e la caratterizzazione di celle a biocombustibile destinate al trattamento di acque e automotive*. [Laurea magistrale], Università di Bologna, Corso di Studio in [Chimica industriale \[LM-DM270\]](#).
- Scheithauer, U., Schwarzer, E., Richter, H. J., & Moritz, T. (2015). Thermoplastic 3D printing—an additive manufacturing method for producing dense ceramics. *International journal of applied ceramic technology*, 12(1), 26-31.
- Schmittinger, W., & Vahidi, A. (2008). A review of the main parameters influencing long-term performance and durability of PEM fuel cells. *Journal of power sources*, 180(1), 1-14.
- Setti L. & Maggiore I. (2022) - “Cella a biocombustibile abiotica” – “Abiotic biofuel cell”: Italian patent pending n°102022000024867 by ALMA MATER STUDIORUM – UNIVERSITA’ DI BOLOGNA.

- Shallenberger, R. S., & Mattick, L. R. (1983). Relative stability of glucose and fructose at different acid pH. *Food Chemistry*, 12(3), 159-165.
- Shanahan, B., Seteiz, K., Heizmann, P. A., Koch, S., Büttner, J., Ouardi, S., ... & Breitwieser, M. (2021). Rapid wet-chemical oxidative activation of graphite felt electrodes for vanadium redox flow batteries. *RSC advances*, 11(51), 32095-32105.
- Simonov, A. N., Pestunova, O. P., Matvienko, L. G., & Parmon, V. N. (2005). ¹³C NMR studies of isomerization of D-glucose in an aqueous solution of Ca (OH) 2. The effect of molecular oxygen. *Russian chemical bulletin*, 54(8), 1967-1972
- Sirisangsawang, R., & Kitchaiya, P. (2019). Concentrated Lactic Acid Production from Invert Sugar in Alkaline Solution. *Chemical Engineering Transactions*, 75, 613-618.
- Slaughter, G., & Sunday, J. (2014). A membraneless single compartment abiotic glucose fuel cell. *Journal of Power Sources*, 261, 332-336.
- Sowden, J. C., & Schaffer, R. (1952). The reaction of d-Glucose, d-Mannose and d-Fructose in 0.035 N Sodium Hydroxide at 35. *Journal of the American Chemical Society*, 74(2), 499-504.
- Sue, C. Y., & Tsai, N. C. (2012). Human powered MEMS-based energy harvest devices. *Applied Energy*, 93, 390-403.
- Sundén, B. (2019). *Hydrogen, batteries and fuel cells*. Academic Press.
- Tan, C., Wang, F., Liu, J., Zhao, Y., Wang, J., Zhang, L., ... & Endo, M. (2009). An easy route to prepare carbon black–silver hybrid catalysts for electro-catalytic oxidation of hydrazine. *Materials Letters*, 63(12), 969-971.
- Tao, B., Miao, F., & Chu, P. K. (2012). Preparation and characterization of a novel nickel–palladium electrode supported by silicon nanowires for direct glucose fuel cell. *Electrochimica acta*, 65, 149-152.
- Tester, R. F., & Karkalas, J. (2003). Carbohydrates: classification and properties. V: Encyclopedia of Food Sciences and Nutrition. Caballero B., Trugo LC, Finglas P. M.(eds.).
- Trabold, T., & Babbitt, C. W. (2018). *Sustainable food waste-to-energy systems*. Elsevier, Cambridge
- Vincent, C., & Scrosati, B. (1997). *Modern batteries*. Elsevier.
- Watt, G. D. (2014). A new future for carbohydrate fuel cells. *Renewable Energy*, 72, 99-104.

Wei, X., Li, D., Jiang, W., Gu, Z., Wang, X., Zhang, Z., & Sun, Z. (2015). 3D printable graphene composite. *Scientific reports*, 5(1), 1-7.

Wen, J. L., Xiao, L. P., Sun, Y. C., Sun, S. N., Xu, F., Sun, R. C., & Zhang, X. L. (2011). Comparative study of alkali-soluble hemicelluloses isolated from bamboo (*Bambusa rigida*). *Carbohydrate research*, 346(1), 111-120.

Wu, J., Chen, N., & Wang, Q. (2018). Preparation of novel thermoplastic poly (vinyl alcohol) with improved processability for fused deposition modeling. *Polymers for Advanced Technologies*, 29(5), 1447-1455.

Xu, J. K., & Sun, R. C. (2016). Recent advances in alkaline pretreatment of lignocellulosic biomass. *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery*, 431-459.

Yei, L. E., Beden, B., & Lamy, C. (1988). Electrocatalytic oxidation of glucose at platinum in alkaline medium: on the role of temperature. *Journal of electroanalytical chemistry and interfacial electrochemistry*, 246(2), 349-362.

Zhao, T. S., Chen, R., Yang, W. W., & Xu, C. (2009). Small direct methanol fuel cells with passive supply of reactants. *Journal of Power Sources*, 191(2), 185-202.

Zhong, W., Li, F., Zhang, Z., Song, L., & Li, Z. (2001). Short fiber reinforced composites for fused deposition modeling. *Materials Science and Engineering: A*, 301(2), 125-130.

References (Figures)

Setti L. & Maggiore I. (2022) - “Cella a biocombustibile abiotica” – “Abiotic biofuel cell”: Italian patent pending n°102022000024867 by ALMA MATER STUDIORUM – UNIVERSITA’ DI BOLOGNA.

Watt, G. D. (2014). A new future for carbohydrate fuel cells. *Renewable Energy*, 72, 99-104.

Gharehpetian, G. B., & Mousavi, M. (Eds.). (2017). *Distributed generation systems: design, operation and grid integration*. Butterworth-Heinemann.

Chanpeng, W., Khunatorn, Y., & Plangklang, B. (2010). Model and experiment analysis of 1.2 kW PEMFC electrification.

Sirisangswang, R., & Kitchaiya, P. (2019). Concentrated Lactic Acid Production from Invert Sugar in Alkaline Solution. *Chemical Engineering Transactions*, 75, 613-618.

Kobayashi, H., Ito, Y., Komanoya, T., Hosaka, Y., Dhepe, P. L., Kasai, K., ... & Fukuoka, A. (2011). Synthesis of sugar alcohols by hydrolytic hydrogenation of cellulose over supported metal catalysts. *Green Chemistry*, 13(2), 326-333.

Jiang, Z., Diggle, B., Tan, M. L., Viktorova, J., Bennett, C. W., & Connal, L. A. (2020). Extrusion 3D printing of polymeric materials with advanced properties. *Advanced Science*, 7(17), 2001379.

Acknowledgments

I really wish to thank

my beautiful mother Mina, the star on Earth lighting my way every day;
my reserved father Antonio, always present behind the lines;

my lovely partner and comrade Antonio, who accompanies me in the pleasures of everyday life, and support me in the constant and beneficial critique of capitalism, which is the only source of pain for the humanity and the Earth;

my unique and fantastic friend Francesco, ever by my side, even when are separated by many miles.

my wonderful friends who never left me alone during the last three years, both in moments of discouragement and happy sharing: Elia, Carla, Andrea, Beppe, Stella and all “the girl”;

my supervisor Leonardo, who always encouraged me in carrying out my research, and shared with me his point of views, helping me to understand how I’d like and I’d not like to be;

my lab partner Sofia, who has been a shoulder to rely-on;

the entire Departmet of Industrial Chemistry Toso Montanari, who saw me grown, and all the students I followed in the last three years in the role of thesis and internship co-supervisor, to have given me new and interesting perspectives: Florita, Federica, Arianna, Ilaria, Filippo, Sofia, Yoanis, Zachee, Mattia, Alessandra.

Special thanks go to

the research group of Prof. Largus Angenent of GEO and Enviromental Center of University of Tübingen, DE, who hosted me as visiting researcher for three months during my thesis writing, showing me what does it means doing high quality and collaborative scientific research;

Prof. Gianluigi De Gennaro and Dr. Ruggero Rossi to have evaluated and commented my PhD Thesis;

all the PhD’s committee in Future Earth, Climate Change and Societal Challenges of University of Bologna, to have given me the possibility of conducting a research project of high scientific and social responsibilities.