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BIOTECHNOLOGY FOR THE VALORIZATION OF DAIRY INDUSTRY BY-PRODUCTS: THE CASE OF THE LACTOBIONIC ACID

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GENERAL INDEX

INDEX OF TABLES

INDEX OF FIGURES

SOMMARIO

L'utilizzo delle biomasse per la produzione di bio-energia, bio-materiali e bio-molecole rappresenta una valida e sostenibile alternativa ai tradizionali processi chimici basati su combustibili fossili.

L'acido lattobionico (ALB) è un *fine chemical* largamente utilizzato nell'industria alimentare, chimica, cosmetica e farmaceutica. E' prodotto dall'ossidazione del lattosio, ma diversi problemi sono connessi alla sua sintesi chimica (processi costosi sia dal punto di vista energetico che dal punto di vista dell'impiego di catalizzatori metallici); l'attenzione dunque si è spostata verso l'impiego di processi biotecnologici, che utilizzano ceppi batterici in grado di utilizzare materiali di scarto per i loro processi metabolici. Questo approccio permette così di valorizzare dei sottoprodotti e reflui dell'industria agraria, producendo molecole di alto valore aggiunto.

Questo lavoro ha come obiettivo lo studio della capacità di ceppi batterici di utilizzare il lattosio non raffinato della 'scotta' per la produzione di ALB. La 'scotta' è il sottoprodotto ottenuto dai processi di produzione della ricotta, e attualmente viene utilizzato principalmente per l'alimentazione del bestiame sebbene presenti un alto contenuto di lattosio (4-5% p/p) e circa l'1% di proteine e peptidi che possono essere ulteriormente valorizzati.

In seguito a screening preliminari per la determinazione delle migliori condizioni operative, differenti strategie di scale up sono state investigate al fine di migliorare le rese, nell'ottica di un' implementazione industriale del processo.

Fermentazioni in reattori a perfetta miscelazione (STR) da 3 L, in condizioni batch, hanno permesso di raggiungere rese di produzione pari a 34,25±2,86 g/L e 30,18±0,3 g/L di ALB per due ceppi di *Pseudomonas taetrolens* (LMG2336 e DSM21104 rispettivamente) con rese di conversione intorno all'85%, dopo 48 ore .

Ulteriori prove in configurazioni fed-batch hanno permesso di raggiungere valori di circa 60 g/L di ALB, fornendo interessanti prospettive per la messa a punto di un processo in continuo.

E' stata anche valutata la possibilità di adoperare matrici ottenute in seguito a procedure di ultrafiltrazione e nanofiltrazione della 'scotta', finalizzate alla separazione di proteine e peptidi bioattivi, ottenendo risultati analoghi, ma promettenti nell'ottica della messa a punto di un concetto di bioraffineria integrata per la valorizzazione dei sottoprodotti dell'industria lattiero casearia.

Infine un approccio preliminare di downstrem per il recupero e la purifica dell'ALB prodotto dal processo fermentativo, utilizzando membrane ceramiche (idonee ad applicazioni

alimentari) in differenti condizioni di pH ha permesso di raggiungere un recupero del 32% a pH 9. Anche questo dato è molto promettente nell'ottica di un processo in continuo di produzione e purificazione dell'ALB al fine di abbattere i costi.

ABSTRACT

Utilization of biomass as raw material in biorefinery processes is a promising and renewable alternative to classic and costly fossil resources for producing bio-energy, bio-materials and bio-chemicals.

Lactobionic acid (LBA) is a fine chemical largely applied in food, chemical, cosmetic and pharmaceutical industries. It is obtained from lactose oxidation but several problems with chemical synthesis (energy-intensive processes requires the use of costly metal catalysts) have been shifted attention in biotechnological processes, employing bacteria strains which are able to use cheap raw material in their metabolism pathways. This approach allows to achieve two aims: valorisation of agro-industrial wastes producing bio-based fine chemicals. This work aimed to investigate bacteria capacity to use a unrefined lactose source as ricotta cheese whey (RCW) for LBA production. RCW is the main by-product obtained from ricotta cheese production process and is employed mainly for cattle feed, although still contains between 4 and 5% (w/w) of lactose and up to 1% of protein concentration that can be valorised.

Following preliminary screening to determine the best operating conditions, different scale-up strategies have been investigated in order to improve yields, in view of an industrial implementation of the process.

Fermentations in 3L-StirredTankReactors, in batch conditions, allowed to reach production yields of 34.25 ± 2.86 g / L and 30.18 ± 0.3 g / L for LBA for two strains of *Pseudomonas taetrolens* (LMG2336 and DSM21104 respectively) with conversion yields around 85%, after 48 hours.

Furthermore fed-batch configurations have allowed to reach values of about 60 g / L of ALB, providing interesting perspectives for the development of a continuous process.

It was also evaluated the possibility to use matrices obtained following procedures of ultrafiltration and nanofiltration of the RCW, aimed at the separation of proteins and bioactive peptides, obtaining similar results, but promising in view of the setup of a concept point of integrated biorefinery for the development of the dairy industry by-products.

Finally a preliminary approach to the downstream recovery and purifies LBA produced by the fermentation process, using ceramic membranes (suitable for food applications) in different pH conditions has allowed us to achieve a 32% recovery at pH 9. Even this figure it is very promising in view of a continuous process of production and purification Room Christmas Tree in order to cut cost

ABBREVIATIONS

ABTS: 2-2-azinobis-(3-ehtylbenzothiazoline-6-sulfonate)

ASGPR: ASialoGlycoProtein Receptors

BOD: Biochemical Oxigen Demand

CDH: Cellobiose Dehydrogenase

COD: Chemical Oxigen Demand

DCIP:2.6-DiChloroIndoPhenol

DMA: Dynamic Membrane Aeration

FAD: Flavin Adenine Dinucleotide

GalOS: Galacto-OligoSaccharides

GC: Galactosylated Chitosan

GFOR: Glucose-Fructose OxidoReductase

GL: Glucono-δ-Lactonase

GOOX: GlucOligosaccharide OXidase

HPTFF: High Performance Tangential Flow Filtration

LBA: Lactobionic Acid

MSM: Mineral Salt Medium

NF: NanoFiltration

PBR: Packed-Bed Reactor

PDH: Pyranose Dehydrogenase

Pd/C: Palladium on Carbon

Pd-Bi/C: Bismuth-promoted Palladium on Carbon

RCW: Ricotta Cheese Whey

STR: Stirred-Tank Reactor

UF: UltraFiltration

UW solution: University of Wisconsin solution

1. INTRODUCTION

Agriculture has always represents the main activity for humans support, but also the deeply connection with the Earth. Furthermore its importance is significant from the point of view of economic development and environmental impact, and increasingly attention is given to the necessity to consider aspects of economic, social and environmental sustainability.

All food production presents an environmental impact, in terms of energy request and wastes production, and the increase of the global demand of food in the last century, concomitant to a growing world population, represents an important problem for the institutions and agro-industrial systems. Minimize environmental impact, producing sufficient high-quality food by using available resources, is an important challenge for competitiveness and sustainability. (Capper et al., 2009)

A quantitatively significant of agro-industrial wastes are biodegradable and, therefore, susceptible of biochemical transformations which are associable problems and opportunities.

In general, the ability to recycle and exploit wastes allows reducing disposal costs, safeguarding raw materials and limiting environmental impacts. Specifically, the need to stabilize the biodegradable wastes is combined with the ability to valorize them from an energetic point of view.

Driven by the gradual depletion of fossil fuels and a growing demand for sustainability, chemical companies have seriously begun to consider the 'se of renewable and fermentation processes bio-based raw materials for the production of basic chemicals, which in the past they were synthesized via purely chemical. Recent developments in microbial genetics and in the understanding of cellular metabolic pathways have enabled researchers to develop and refine multiple enzymatic steps to convert renewable resources into high value-added products through very inexpensive procedures.

In this context, the global interest is directed towards the marketing of different microbial fermentation technology for chemical production.

Industrial biotechnology is therefore based on the exploitation of the metabolic activity of microorganisms, plants and animal cells to produce a wide range of compounds of interest in chemical, food, pharmaceutical and healthcare, from industry waste by-products (Parekh, et Win al., 2000). According to a market study from FESTEL CAPITAL it is estimated that the global share of biotechnological productions, representing approximately 3% in 2004 has risen more than 15% in 2015 (Festel Capital 2007).

Sustainability of agro-industrial systems represents not only an important solution for environmental problems concerned with food production (like emissions and energy security), but it play a key role for the economic competitiveness; valorized all the by-products and wastes in each production steps led to an increase of the added value of the company, avoiding landfill disposal and consumption of additional energy, and maximizing the economic value of the starter biomass (D'Avino et al., 2010).

Among the several definitions of biorefinery, the most exhaustive was recently formulated by the International Energy Agency Bioenergy Task 42 "Biorefinery" (IEA2008): "The sustainable processing of biomass into a spectrum of marketable products and energy" (Cherubini and Jungmeier, 2010). It is important that the biorefinery system is not only a new industrial ideology, but a new concept of integration between agriculture, industry and environment (Lazzari, 2012).

Avoiding, or at least reducing, the dependence from fossil fuels, which maintain market dominance, the production process must be reworked through greater integration between the different phases, minimizing transport costs and exploitation of the resources locally available (bio-fertilizers, additional residues, potential users of products) (Chen et al., 2005)

Animal products presents important environmental and social consequences, like: animal welfare; excessive use of antibiotics; the demand for scarce lands to produce the required feed; emission of greenhouse gases; liquids and solids wastes; intensive water consumption (Mekonnen and Hoekstra, 2012) and the necessity to reduce all these problems represents a significant cost for farmers and industries.

1.1DAIRY INDUSTRY

Dairy industry represents all the activities connected with milk production, transformation and distribution, and its contributes to worldwide food economy in undoubtedly one of the most important.

The 2007 annual milk yield averaged 9193 kg per cow (compared to 2074 in 1944), which is equivalent to 29.3 kg/d when adjusted for a 14-mo calving interval (426 d) and a 60-d dry period. In Table 1.1 a comparison between 1944 and 2007 is presented in terms of input, output and environmental. The increase in productivity in recent years is demonstrated by the percentage of the dairy population required to produce 1 billion kg of milk in 2007; only 21% of that required in 1944 (Capper et al., 2009).

Despite a decrease after the economic crisis of 2008, dairy production showed a rapid recovery, especially connected with high-quality products (Igp and Dop) which absorbed about 70% of national milk production. Indeed, in 2011, milk production was about 730 million tons, up 2.5% from a year earlier (Ismea, 2012).

Great importance in the international market are countries like India, China, New Zealand, Brazil and Argentina, thanks to a strong increase in production since 2009 and upward pressure on prices for the intensification of internal demand. Slowed growth for United States, Mexico, Canada and Russia (Ismea 2012).

Variable	1944	2007
Milk produced, billion kg	53.1	84.2
	Resources/waste per billion kg milk produced	
Animals, n		
Lactating cows, × 10^3	414.8	93.6
Dry cows, × 10^3	67.4	15.2
Heifers, × 10^3	429.2	90.3
Mature bulls, × 10^3	19.29	1.31
Adolescent bulls, × 10^3	17.17	1.08
Total population, × 10^3	948	202
Nutrition resources		
Maintenance energy requirement,[1] MJ × 10^9	16.66	3.87
Maintenance protein requirement,[1] kg × 10^6	165.4	48.4
Feedstuffs, kg of freshweight × 10^9	8.26	1.88
Land, ha × 10^3	1,705	162
Water, L × 10^9	10.76	3.79
Waste output		
Nitrogen excretion, kg × 10^6	17.47	7.91
Phosphorus excretion, kg × 10^6	11.21	3.31
Manure, freshweight, kg × 10^9	7.86	1.91
Gas emissions		
Methane,[2] kg × 10^6	61.8	26.8
Nitrous oxide,[3] kg × 10^3	412	230
Carbon footprint,[4] kg of CO_2 × 10^9	3.66	1.35

[1]Refers to nutrients required for maintenance (all animals), pregnancy (dry cows), and growth (heifers and adolescent bulls).
[2]Includes CH_4 emissions from enteric fermentation and manure.
[3]Includes N_2O emissions from manure (both years) and from inorganic fertilizer application (2007 only).
[4]Includes CO_2 emissions from animals, plus CO_2 equivalents from CH_4 and N_2O.

Table 1.1. Comparison of resource inputs, waste output, and environmental impact of dairy production systems in 1944 and 2007 (Capper et al., 2012).

The European Union is the largest production of milk area with almost 160 million tons (considering all species) with annual growth around 1-2% from 2009 onwards. In 2010 the old EU countries have provided to market 118.1 million tonnes of milk, while the new member countries only 18.1 million tons. This is the result of a 2.3% increase of Community

production compared to a year of downturn like 2009. But the gap regards dairy production only in terms of weights and volumes of production (over 7.2 million tons account for 1.2 million tonnes per year). Indeed for the rates of growth and response to the crisis the new EU member states are presented more reactive on the market, with a total increase of production between 2006 and 2010 that exceed 20%, with the exception of difficult situations for Romania and Bulgaria, which they have yet to complete a long process of restructuring and adaptation of its dairy industry (Pieri, 2010)

Italy is one of the best reality at European level for the production of milk occupying fifth place behind Germany, France, the United Kingdom and the Netherlands, while for cheese production is less in quantity only of Germany and France with a production of over one million tons per year. The national value of production achieved from the agricultural sector component in 2013 represented the 9.4% of the total of that made by the entire national primary sector. Even for the industrial component of the dairy supply chain were recorded significant performances. In 2012 dairy industry contributed for 16.5% to the total value of the turnover generated by the Italian food industry and for 2% to the entire national manufacturing industry. The dairy area was also protagonist of excellent results achieved in recent years for agro-food Italian exports. In 2013, in fact, the dairy sector has contributed for 9.5% to the total value of national agricultural and food exports. In 2012 the national dairy industry was consisted of 36000 companies with 68300 workers in terms of ULA (Annual Working Unit), 1871 dairy cows, 200000 buffaloes, 6.3 million sheep and 735 thousand goats, for a total of 10 million and 900 thousand tons of cow's milk delivered and a monetary value of € 3.5 billion. (D'Alessio, 2014). The most productive regions in terms of cow's milk are, in fact, in order, Lombardy, Emilia Romagna, Veneto and Piedmont. Italy mainly exports to European Union countries, especially products such as Parmigiano and Grana have seen a 25% increase in revenues in 2010. On the contrary, competitiveness' price of French and Dutch products led to a decrease in exports to the United States. An important data concerns the imported milk quantity, in particular from France, Austria and Hungary, with quantitatively +9% in 2010 (ISMEA, 2012).

1.1.1 Dairy industry product

MILK

Italian legislation defines drinking milk as 'the product obtained through regular, uninterrupted and complete from breast of animals in good health and nutrition'. The term

"milk" means the only milk cow origin, whereas for raw milk coming from different species is compulsory to present a specific indication (RD 994/29).

In cow's milk components, excluding water, breaks down as follows: 5% lactose, 3.5% fat, 3.3% protein, less than 1% of mineral salts, trace of enzymes, vitamins , pigments (carotenoids, xanthophylls, riboflavine) and dissolved gases (CO_2, O_2, N_2) (Fig. 1.1). Milk is a product subject to great variability, due to exogenous (environmental, climate, nutrition) and endogenous (breed and individual characteristics) factors, in particular for fats and proteins, opposed to lactose molecule that does not present great variation (Ramesh, 1997).

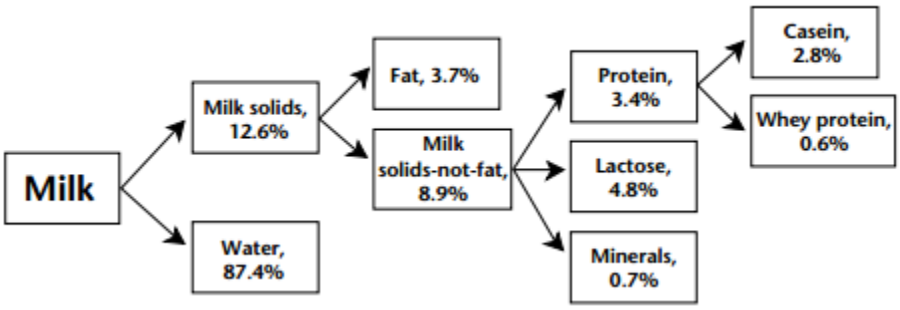


Fig. 1.1. Milk composition (Ramesh, 1997)

From a physic-chemical point of view milk presents, in addition to a great complex composition, a combination of different phases dispersed in aqueous phase which constitutes about 87%. Some substances are in solution, while others may be in emulsion, or in colloidal dispersion according to their different chemical nature. The components in solution are all those soluble in water (salts, lactose, whey protein, water-soluble vitamins), in emulsion we find the lipid fraction, while in colloidal suspension are caseins; also in milk are in suspension a variable number of cells of a different nature: microbial and somatic (Salvadori Del Prato, 1998).

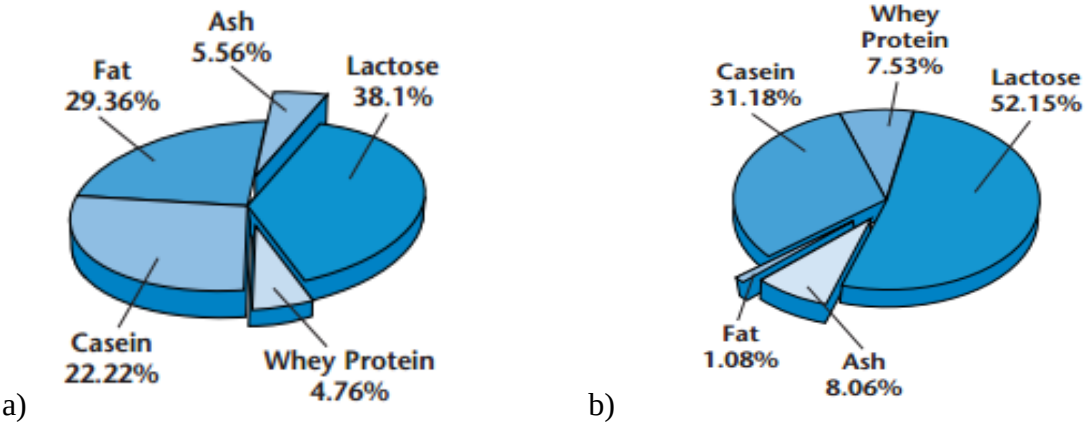


Fig. 1.2. Composition milk solids of whole (a) and skim milk (b) (Ramesh, 1997).

Milk contains about 3-3.5% of nitrogenous substances which include milk protein (casein and whey proteins, with high biological and nutritional role) and different nitrogenous molecules such as, urea, creatine, ammonia, free aminoacids, peptides and nucleic acids (about 5% of all nitrogenous substances). Caseins represent about 80% of milk proteins; they become insoluble when the milk is acidified (4.6) and exist in milk as spherical particles called micelles, which are made up of calcium phosphate and casein complexes stabilizes by surface binding. Caseins can be divided into five type with different chemical properties: αs1, αs2, β, k-fractions and a γ-fraction, that derived from the breakdown of β-casein by inherent proteolytic enzymes of milk. The whey proteins are water soluble protein and have a high content of sulphur-amino acids than the casein. They do not present in large protein aggregates but in simple forms such as monomers or polymers. They consist for the most part by albumin (70%), and to a lesser extent from globulins (15%), from protease-peptones (10%) and from metallic-proteins (5%). Despite casein precipitation occurs by enzymatic action, whey protein precipitate with heating or salt addition (Table 1.2) (Ramesh, 1997).

Caseins	Whey Proteins
Strong hydrophobic regions	Both hydrophobic and hydrophilic regions
Little cysteine content	Both cysteine and cystine present
Random coil structure	Globular structure with helical contents
Heat stable	Easily heat denatured and insolubilized
Precipitate in acidic conditions and insoluble at pH 4.6	Stable in mild acidic environment
Precipitated by di- and polyvalent ions	

Table 1.2. Physical and chemical differences in milk proteins (Ramesh, 1997).

Milk lipids represents a fraction between 3 and 4.6%, depending on race and feeding, and consist of 97–98% triacylglycerols, 0.2–1% phospholipids, 0.2–0.4% sterols, and traces of fatty acids, as well as vitamins A, D, E, and K. Lipids are presented in emulsion in globules form; if milk is rich in fat, and the globules are large, phenomena of rancidity are limited.

Lactose is the only carbohydrate presents in milk (4.5-5%), in two form (α and β) with different solubility and sweetness (α-form is less soluble and sweet than β-form). In addition to its nutritional role, lactose has a technology importance because is responsible for organoleptic changes in milk as a result of heating (Maillard reactions), and being the main substrate known to the bacteria in the fermentation of the milk and cheese.

295/5000

Lactose is the main energy and carbon source for fermentation during production of yogurt, fermented milks and cheeses with reactions as lactic, lacto-alcoholic, propionic but also butyric and propionic that led to bulges in late aged cheeses.

Furthermore milk presents a lot of minor constituents like mineral (<1% include chloride, PO_4^{2+}, and citrates of K^+, Na^+, Ca^{2+}, and Mg^{2+}), important in processing, nutritive value, and shelf life of dairy products; vitamins, acting as coenzyme factor, that can be divided in fat-soluble, water-soluble, thermolabile and thermostable; enzymes, especially oxidoreductases and hydrolases, carrying out important actions especially in the process of cheese maturing (protease and lipase), in thermal treatments of the milk because they have heat stability (alkaline phosphatase) and as indicators of hygienic quality of milk since their amount depends on the microbial load.

Milk at 15°C has a density of 1.028 to 1.033 g/mL with variations according to the title of fat and to its composition; to this parameter value contribute both the density of milky plasma and of lipid fraction; consequently a skimmed milk has a higher density value compared to whole milk. The surface tension of the milk at 15°C is 47-55 Dine /cm² much lower than water (72.8 Dine /cm²) which involve good foaming properties, thanks to the proteins that act as surfactants, with several implications in technology. Other important physical index is the freezing point, the temperature at which the two solid phases coexist and liquid. In this cow's milk it is lower than that of pure water and varies from -0.52 to -0.55°C, thanks to the solutes present in milk constituted by salts and lactose that being, as already stated, present in percentage constant mean that this index does not present big changes, constituting a possible indicator of watered down. The pH of the milk at 20°C has values around 6.6 to 6.8 which remain constant in the case of addition of small amounts of acids or bases, because milk is a buffered solution thanks to the action of proteins which are amphoteric compounds. The pH is

related to the percentage of protein in an inversely proportional way; mastitis milk in fact has a higher pH (≥7,0) because the content of total protein decreases while the colostrum milk has pH≤6,5 (Salvadori Del Prato, 1998).

CHEESE

Before the beginning of the processing processes, milk is subjected only to physical treatments, such as *pasteurisation*, which consists in bringing the milk to high temperature (70-72°C) for a short time (15-20 seconds) with a subsequent rapid cooling to temperatures of less than 6 °C; this treatment limits the presence of pathogenic micro-organisms which are sensible to heat. Pasteurization process is necessary especially for fresh cheeses, because for long-seasoning cheese the strictly conditions (pH acid, NaCl presence, natural antibiotics) limiting the presence of pathogenic microorganisms (Cappelli and Vannucchi, 2005).

During cheese making process k-casein molecule is cleaved by the enzyme *rennin*, determining destabilization of micelle and consequent curd formation. In the firs parts of the cheese making processes, fat does not take part in important activities, but influences structure and properties of casein structure, playing an important role in the process of maturation influencing decisively the organoleptic characteristics of the finished product (Remesh, 1997).

The production of cheese (Figure 1.3), called cheese making, consists in a first heating phase of the milk following by ferments addition as inoculum, if necessary; it takes place in heated boilers, with shape, sizes and materials depending on the type of processing. The second phase of the coagulation process is one of the most important steps of the entire process and consists in the gelation of casein fraction by precipitation of the micelles (lipoprotein particles with tendency to aggregate). Coagulation occurs or by pH reduction, acidifying the milk (acid coagulation), or through the intervention of rennet that modified enzymatically k-caseina (rennet or enzymatic coagulation). In common cheese making processes, however, it employ rennet coagulation, through addition of rennet to milk brought to temperatures of 30-37°C. The curd is a gelatinous mass of paracaseinato dicalcium that form a three-dimensional structure, trapping fat globules and the whey (liquid fraction), and that contract retaining the first and expelling the second. Production of lactic acid by the bacteria influence positively texture and elasticity of the clot, which contracts and ejects the whey. The lactic acid neutralizes the charges on the micelles and, at low levels, making the curd more permeable and facilitates the draining of whey attacking partially the paracaseinato dicalcium reticulum.

The next stage is the physical breaking of the curd (gelatinous mass of paracaseinato dicalcium obtained by the enzymatic action) for expelling the whey and concentrate fat. It is achieved through heated to temperatures ranging from 40 to 60°C, for times varying from a quarter of an hour up to two hours. After baking, the curd is removed from the coagulation baths serum and placed in forms, where it continues to release serum thanks to pressing, which also confers compactness and its shape of the cheese.

Finally, after the dry salting (repeated several times depending on the type of cheese), the obtained shape is matured for varying periods. In this stage are processed carbohydrates, proteins and lipids for the enzymes action. The curing times depends on the type of product required: can be a few days for fresh and soft cheese up to several years for the more mature cheese. (Kapoor and Metzger, 2008).

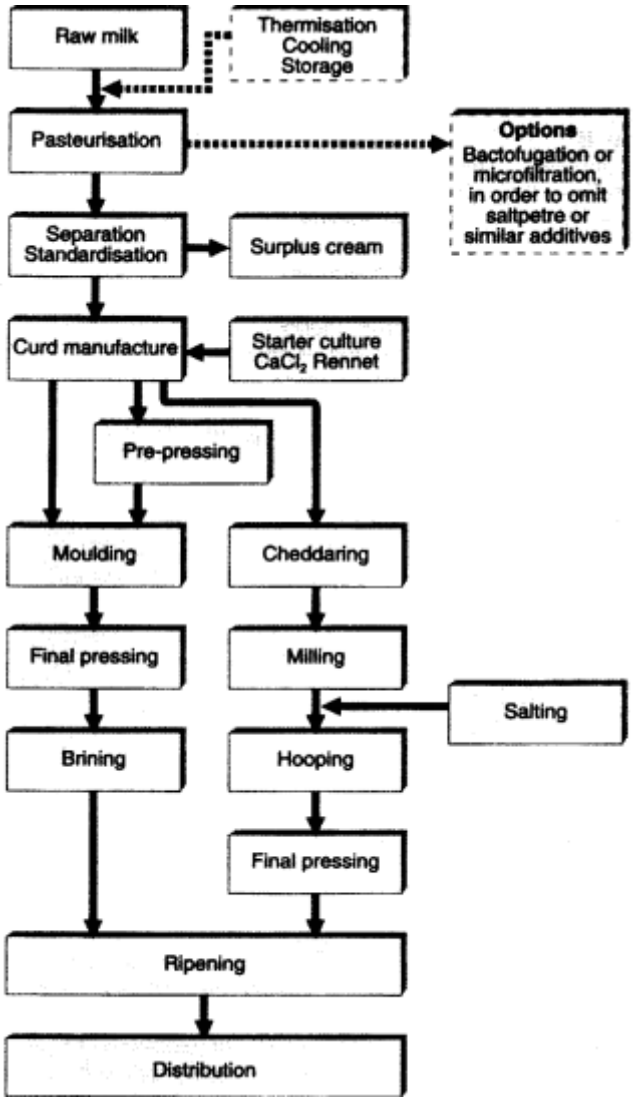


Fig. 1.3. Process flow in production of hard and semi-hard cheese (http://www.dairyprocessinghandbook.com/chapter/cheese).

RICOTTA CHEESE

Technology of ricotta cheese production take place through coagulation and precipitation of the whey proteins encouraged by acid environment. For this purpose it is, however, necessary to reach very high temperatures and over 85°C. Precipitation also involves cheese whey fat and lactose. With the traditional manufacturing, ricotta cheese is obtained from sweet whey, and excessive acidification would led to the precipitation of whey proteins before time. Whey heating occurs generally in the same tanks where cheese is made; it is quite common to add whole milk with percentages ranging from 5% to 25% of the total volume of the whey. This milk is added when the temperature reaches 60-70°C, and heating follow up to 80-90°C. Towards the end of the heating is added an acid whey, residue from previous processes, in order to lower the pH; the same result can be obtained with addition of citric acid, lactic acid or other acidifying agents. These acids are mixed when it reaches the minimum coagulation temperature, because a preliminary insertion may create serious damage to the coagulum with a not homogeneous precipitation. When whey proteins precipitate, incorporate air and steam, coagulating into a gelatinous mass that tends to surface. The clot is allowed to emerge and consolidate for about 5 minutes, suspending the heating simultaneously; subsequently starts the recovery of ricotta, which is placed in perforated plastic tanks in order to eliminate the liquid part. In many cases in the boiler is added salt, which functions both as a coagulation promoter that as real ingredient, to confer to the ricotta a stronger flavour. The addition of salt is normally approximately between 0.5% and 1.5% of the final weight. Consequently the product is left to dry for a few hours in a cool place and then sold immediately or, in industrial plants, subjected to packaging which allows longer storage (Salvatori del Prato, 1998).

1.1.2 Dairy industry by-product: Cheese Whey and Ricotta Cheese Whey

Dairy industry contributes significantly to the production of liquid waste with a high organic load. In 2012 in European Union countries they were produced 90.7 million tons. Italy is the third European cheese producer after Germany and France, with an annual output of 1.2 million tons. in 2012 (http://www.dairyindustries.com/).

CHEESE WHEY

Cheese production processes originate two main liquid waste streams: cheese whey and water wastewater resulting from cooling and cleaning of pipes, tanks etc .. Of these, cheese whey, aqueous fraction which is formed during the coagulation of the milk casein in cheese

manufacture, is considered the most problematic for its high values of biological (BOD) and chemical (COD) oxygen demand. The average specific production is about 0.9 L per litre of processed milk or per kilogram of cheese produced. Appropriate management of whey is necessary as consequent of the direct discharge prohibitions on land or into water bodies. (Guimarães et al., 2010).

Cheese whey world production of serum in 2008 was over 160 million tons per year, estimated at 9 times the production of cheese, and shows an annual growth rate of 1-2%; in 2010 the World serum production is over 180 million tons per year (estimated production of cheese in 9 times), and shows an annual growth rate of about 3-4% (Guimarães et al., 2010). In Italy whey production is estimated in about six million tonnes per year (Castilli, 2011).

Formerly, about 50% of the product cheese whey was disposed as wastewater by subjecting it to purification. The companies were therefore subject to the legal regulations relating to non-hazardous waste: temporary storage in refrigerated tanks, MUD, forms, loading-unloading registers. The situation has changed since the entry into force of Legislative Decree no. 4/2008, which transposition of Community Directive 2008/98 / EC, went to change the Legislative Decree no. 152/06 introducing into Italian law the definition of by-product (Legislative Decree 152/06 article 183 paragraphs 1 letter. N):

"A substance or object resulting from a production process, which primary aim is not the production of that item, it can't be considered as waste in accordance with Article 3, paragraph 1, but as by-product only if the following conditions are met:

a) it is certain that the substance or object will be further used;

b) the substance or object can be used directly without any further processing other than normal industrial practice;

c) the substance or object is produced as an integral part of a production process and

d) further use is lawful, that is the substance or object satisfies, for the specific use, all relevant requirements concerning the products and the protection of health and the environment and will not lead to overall adverse environmental or human health. "

In the same article, paragraph 1, letter. A, there is the definition of waste, already reported in the previous chapter:

"Any substance or object in Annex A (categories of waste - Q8 residues from industrial processes) Part Four of this decree, and which the holder discards or intends or is required to discard"

So the by-product is not and should not be considered a waste.

Cheese whey contains about 50% of the total solids of the milk origin, and its major components are lactose (44-52 g/L), proteins (6-8 g/L) and mineral salts (4-9 g/L) (Aktas et al., 2006). The type and the composition of whey mainly depend on the technique of process used for removing casein from milk. There are two main varieties of whey, derived from different technologies (Table 1.3) (Kosseva et al., 2009):

- sweet whey, which is derived from the production of pressed cheeses and cooked pasta, cheese, and casein obtained by the action of rennet. One litre of this whey contains 65 g of dry residue, to which 48 g of lactose, 8 g protein, 7 g of salt and about 1 g of fat, pH 6-7.
- acid whey, which is a by-product of the fresh paste cheeses, soft and lactic casein production. The acidification of the milk causes increased demineralization of the curd; consequently this serum is characterized by a higher mineral content of the sweet whey and lower pH if the coagulation rennet-induced casein occurs at a pH of about 6.5, the whey obtained is called sweet whey; the acid whey instead results from a process that uses fermentation or addition of organic or mineral acids to the coagulation of casein and which occurs at pH less than 5 (Adrian et al., 2009).

Components	Sweet whey ($g L^{-1}$)	Acid whey ($g L^{-1}$)
Total solids	63.0–70.0	63.0–70.0
Lactose	46.0–52.0	44.0–46.0
Protein	6.0–10.0	6.0–8.0
Calcium	0.4–0.6	1.2–1.6
Phosphate	1.0–3.0	2.0–4.5
Lactate	2.0	6.4
Chloride	1.1	1.1

Table 1.3. Typical composition for sweet and acid whey (Kosseva et al., 2009).

Because of its high level of organic substances, mainly lactose, the whey has a high BOD (Biological Oxygen Demand), about 30-60 g/L, depending on the cheese-making process used and an average value of COD (chemical oxygen demand) between 60 and 80 g/L. The recovery of protein reduces the COD of the serum of only about 10 g/L (Guimarães et al., 2010). Disposal treatment of whey is very costly for companies, so development of integral process for employing cheese whey as substrate for production of high- value added product, represent an important alternative, despite strong limitations due to storage and transport costs, related to limited lactose amount (Manzoni, 2006). A large part of treated whey is dried

to produce whey powder, which is commonly used as animal feed but even in small amounts in human food, for example in ice cream, cakes, milk derivatives, etc. Another important application of the cheese whey is the use of lactose contained in it as substrate for the production of compounds of value through fermentation: the classic examples are ethanol and single cell proteins (SCP) obtained from bioprocesses with yeasts, but may also include the production of biogas, organic acids, amino acids, vitamins, polysaccharides, oils, enzymes and other compounds (Guimarães et al., 2010).

RICOTTA CHEESE WHEY

Ricotta cheese whey (RCW) is a dairy by-product from ricotta cheese production process, after flocculation of soluble whey proteins as a consequent of acidification and heating of the cheese whey, which is widely product in Europe and especially in the Mediterranean area with 1.0 Mt per year only in Italy (Sansonetti et al., 2009). Because of lactose concentration between 4 and 5% (w/w), BOD of 50 g/L and COD of 80 g/L, RCW presents pollution problems and its disposable is an added cost for dairy industry. Its low protein concentration makes it unless for protein valorisation applications (like for cheese whey), and at the present it is employing just for cattle feed (Sansonetti et al, 2010). RCW valorisation can be achieved by its employment as a carbon source in biotechnological applications for lactose bioconversion into valuable product like lactic acid, ethanol and LBA (Secchi et al., 2012).

1.1.3 Lactose: problems and opportunities

Lactose, the only milk sugar, is a disaccharide consisting of a molecule of glucose and one of galactose, linked together by a glycosidic bond β 1-4. In aqueous solution at 20°C, lactose exists in two isomeric forms: for 62.7% as β-lactose and for 37.3% as α-lactose. The content of lactose in milk varies in different mammal: for example, cow's milk contains about 4.9% and the human milk approximately 6.7% lactose (Kuusisto et al., 2007). In fermented cow's milk, lactose content is about a third of which that is present in the milk, due to the lactose conversion work by lactic acid bacteria. During cheese production nearly all the lactose in the milk is transferred in whey. The lactose content in cheese whey is about 4.8 g per 100mL (G. Schaafsma, 2008). It is a crystalline solid, colour less, reductant and slightly soluble in water: its solubility limit is 22 g per 100 g of water at 25°C (Guimarães et al., 2010). The lactose plays a slight sweetening effect on the milk, even if much reduced compared to sucrose that is 6 times more sweetener. Lactose is hydrolysed in the two monomers which in this way may

be used by lactic acid bacteria to produce lactic acid, responsible for the acidification of the milk (Gigliotti and Verga, 2007).

Lactose is therefore an important product of cheese and ricotta cheese manufacture, whose production was estimated at 1.2 million tons a year, and whose global market will reach 1.4 billion dollars by 2015 (Gutiérrez et al., 2012). It is mainly used as an ingredient in food, drink and sweet products, and it has been widely used as a diluents in pills and as a support drug for pharmaceutical industry. Nevertheless, its use is limited in many applications due to its low sweetness and solubility, and for the intolerance of the population; finally only a small amount of lactose is used as a raw material for fine chemicals productions (Gutiérrez et al., 2012).

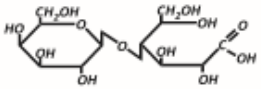
From the feed stock milk, skimmed whey is generated after casein precipitation and lipids removal. After removing 80% of water from the skimmed whey, the concentrate whey is ultra filtrated to generate whey permeate (the lactose fraction) and whey retentate (the protein fraction with considerable lactose residues). Certain components of the retentate such as lactoalbumin and lactoferrin fractions are of pharmaceutical interest, instead whey permeate (which contains 81% of the original lactose in milk) is a potential carbon source (Povolo et al., 2010). Lactose can easily be separated from natural matrix (milk or whey) and purified for food (baby food, cakes, biscuits, chocolate, soups and sauces) feed and pharmaceutical applications; but it can also be used as a precursor of lactose-derived bioactive substances (Schaafsma, 2008).

A lot of studies were focused on the opportunity to valorised lactose as a source for producing polyhydroxyalkanoates (Pantazaki et al., 2009; Povolo et al., 2010), ethanol (Sansonetti et al., 2009; Guimarães et al., 2010), hydrogen, lactic acid (Secchi et al., 2012), and the conversion into lactobionic acid has been investigated as a new and promising strategy (Alonso et al., 2013). Lactose can be used directly by lactose-consuming microorganisms or pre-hydrolysed for lactose-negative microorganisms, and its employment as a renewable and cheap feedstock for fermentative processes to obtain value-added products is an interesting alternative to chemical synthesis processes, within the general framework of the rapid advances in microbial biotechnology (Guimarães et al., 2010).

1.2 LACTOBIONIC ACID

LactoBionic Acid LBA (molecular weigh: 358.3 g/mol and pKa: 3.6) is a versatile complex polyhydroxy acid ($C_{12}H_{22}O_{12}$) composed of a galactose molecule linked to a gluconic acid

unit by an ether-like linkage (Table.1.4). Notable is its high water solubility; calcium salt of LBA is 40000 times higher water soluble than calcium carbonate and 10 times higher than calcium lactate (Nakano et al., 2010).

Structural formula	
Systematic name	4-*O*-*β*-D-galactopyranosyl-D-gluconic acid
Molecular formula	$C_{12}H_{22}O_{12}$
Molecular weight (g/mol)	358.30
Physical status	Solid
Appearance	White powder
Melting point (°C)	128–130 °C
Solubility	Freely soluble in water, slightly soluble in anhydrous ethanol and methanol
$[\alpha]^{20}_{D}$	+53° to +22.6°
pKa [a]	3.6

[a] : 25 °C.

Table 1.4. Physiochemical properties of LBA (Alonso et al., 2013).

It is obtained via lactose oxidation and presents a lot of interesting application in food, cosmetics, pharmaceutics and chemical industries thanks to its antioxidants, chelating and humectants properties (Gutiérrez et al., 2012).

Recently the biotechnological industry has moved from commodity bulk carboxylic acids to value-added organic acids which display outstanding applications, and LBA has emerged as a promising and interesting substance, with a significant improvement of the commercial rate in recent years (Alonso et al., 2013). Actually LBA market is between 15000 and 17000 tonnes per year with an annual rate of about 5% growth (Gutiérrez et al., 2012). The majors LBA industrial manufacturers are Solvay (Germany), FrieslandCampina Domo (Netherlands), Sandoz (Germany), Reliable Biopharmaceutical Corporation (USA) and the US Dairy Ingredient Company (USA). LBA is increasingly taking on a predominant role in the market, so that companies like Solvay opened a plant in Germany in the late 1990s with the aim of achieving an annual production of 1000 t. (Alonso et al., 2013).

1.2.1 Properties

Thanks to its particular chemical structure with a lot of hydroxyl groups, LBA presents antioxidant, ion-chelating and humectants properties which make it biodegradable, biocompatible and non-cytotoxic (Selim et al., 2009; Alonso et al., 2013; Tasic-Kostov et al.,

2012). It is a hygroscopic compound with a high water solubility of its salts and forms gels contain about 14% of water with atmospheric moisture (Nakano et al., 2010; Gutiérrez et al., 2012). Also it presents pH-reducing effects, combining with sweet taste (2 kcal/g) and competes with lactose for binding to intestinal beta galactosidase. LBA presents a strong mineral-complexing properties and a good resistant to digestive enzymes, exercising prebiotic effects (Schaafsma, 2008).

1.2.2 Use and applications

LBA excellent physiochemical properties make it suitable for a lots of interesting commercial applications, as can be resume in Fig. 1.7.

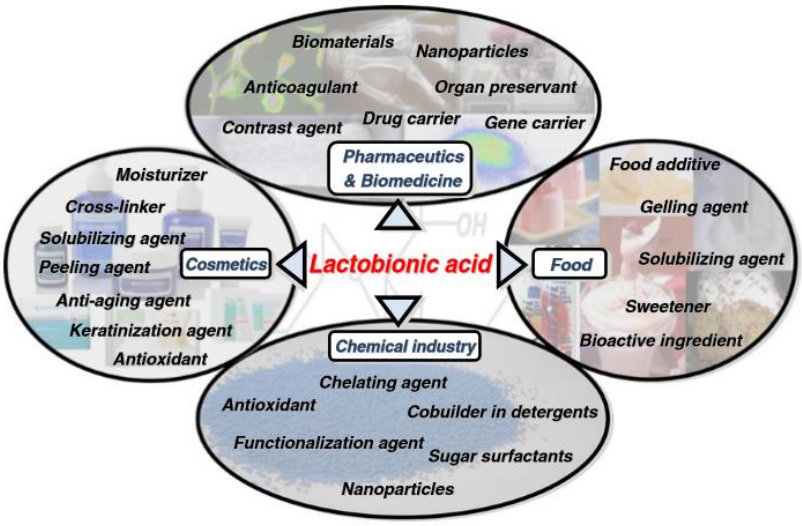


Fig. 1.4. Summary of LBA applications (Alonso et al., 2013)

LBA is widely used in pharmaceutical industry, as a ligand molecule in drug-delivery systems, thanks to its unique properties such as biocompatibility, biodegradability and ion-chelating ability (Alonso et al., 2013). According to the development of novel therapeutic treatments based on hepatic-targeted drug delivery system for clinical therapy (i.e. chemotherapy), LBA-based drug delivery system are able to target efficaciously hepatocytes; in fact LBA is a successfully ligands for asialoglycoprotein receptors (ASGPR) located on hepatocytes (hepatocytes targeted cells), and these receptors are the favoured targets for potential hepatic interventions (Lin et al., 2009). Bio-functionalization of gene carriers with LBA may improve their hepatocyte specificity; for example chitosan has been widely investigated as a drug delivery carrier and gene delivery vector for its several biological

advantages (biodegradability and low toxicity), and LBA-coupled chitosan (GC) binds to hepatocytes presents better efficacy (Fig. 1.8) (Kim et al., 2006).

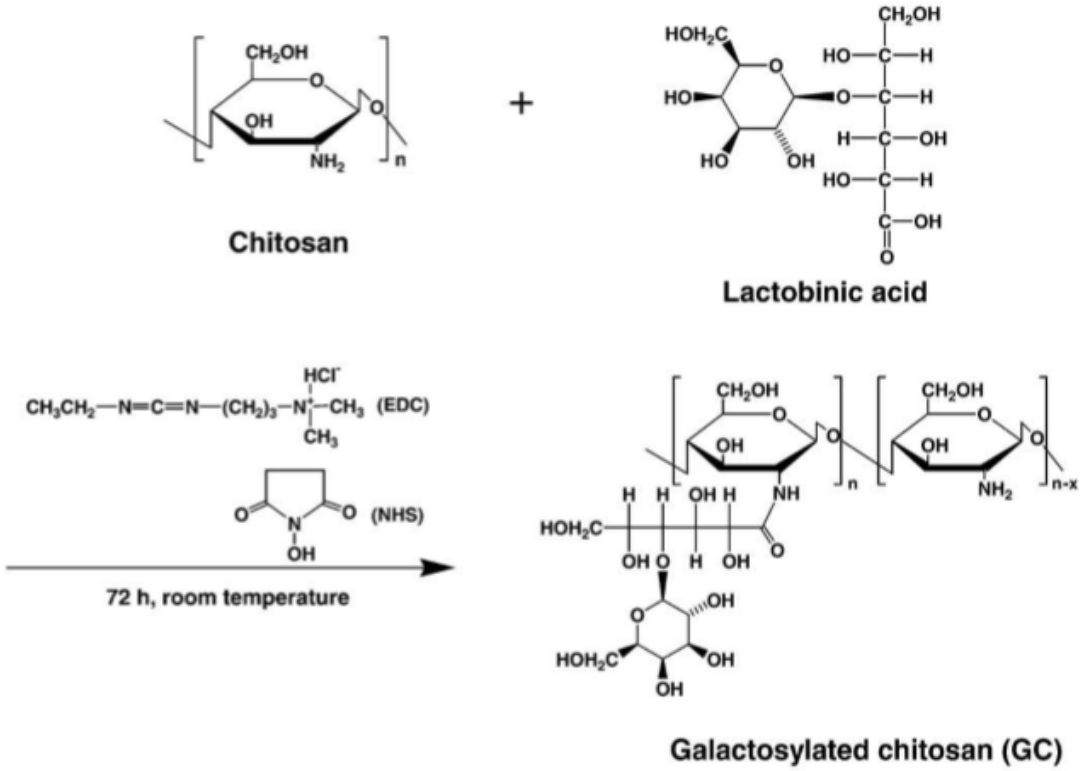


Fig. 1.5. Synthesis scheme of GC (Kim et al., 2006)

It can also be used as a platform for chemical biosynthesis of antioxidants carriers (Ortial et al., 2006) or as a carrier shielding from enzymatic attack for peptides and proteins delivery (Meng et al., 2012).

Another LBA application for novel diagnostic tools in biomedicine, is as coating material surface of nanoparticles used for biomedical applications such as biodetection, cancer therapies, magnetic resonance imaging or bio-labeling, contributes to physicochemical stability (Chung et al., 2002; Fa et al., 2010). In bioimaging applications, for example, LBA bound to nanoparticles fluorescent has showed an efficient and promising means of survey in the discovery and identification of liver cancer cells in a complex cellular matrix such as blood. The organic nanoparticles functionalizing have presented several advantages compared to traditional therapeutic tools (Fig. 1.9), such as biodegradability, biocompatibility, sensitivity detection, solubility in water, long-term stability and low cytotoxicity (Kekkonen et al., 2009; Selim et al., 2007).

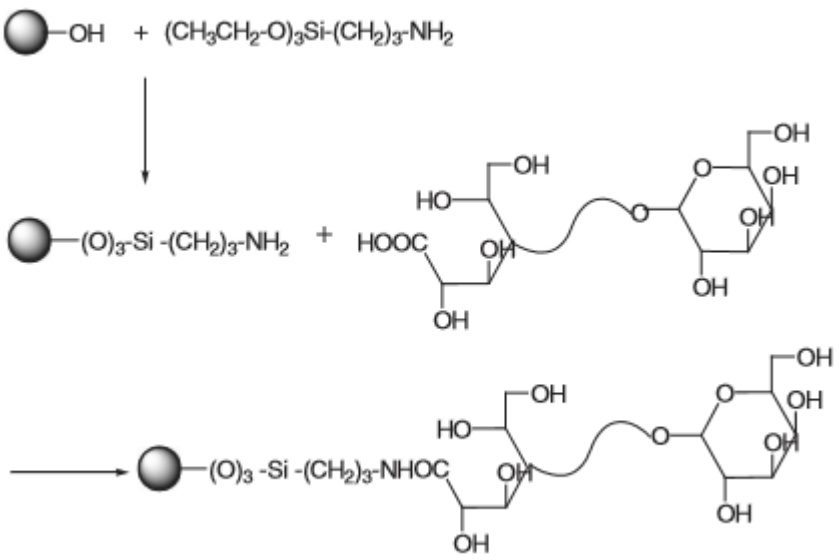


Fig. 1.6. Synthesis of lactobionic acid-grafted magnetite nanoparticles (Selim et al., 2007)

In tissue engineering, aimed at developing new fabrics functional to replace, repair or regenerate damaged tissues or organs, LBA does not contribute directly to the growth of tissues and biomaterials, but it can facilitate the attachment and the entrapment of hepatocytes: LBA is entrenched in a polymer matrix through functional groups such as the galactose molecule, enhancing functional capacities and biocompatibility of tissue engineering materials such as scaffolds generated from natural polymers or synthetic polyesters. These form the links that are able to increase the adhesion and aggregation of hepatocytes and to maintain long term specific functions of the liver and the mechanical stability (Kim et al., 2008; Kim et al., 2011). Furthermore, LBA addition has a key role in antibiotic formulations like erythromycin for treating bacteraemia and in formulation containing chlorhexidine as antiseptic substance, increasing their water solubility and stability (Nakano et al., 2010). A recent European Patent from Cavallo et al. (2012) was concerned on the possibility to employ LBA as a therapeutic agent for treatment of corneal edema and inflammation, overcoming the problems exhibited by conventional hyperosmotic current formulation (Cavallo et al., 2012).

LBA also has an important role in preservative solutions for organ storage avoiding oxidative damage, because of its metal ions chelating capacity (Hart et al., 2002), and in anticoagulant and antithrombotic drugs (Gutiérrez et al., 2012). Efficacy organ preservation is based on the minimizing the metabolic rate of the organ and the inactivation of most enzyme reactions protects the cell and drops the use of high-energy purine nucleotides. The University of Wisconsin solution (UW solution), called Via Span or Belzer solution as commercial name,

was development by Belzer et al. (1992) for cold storage of organs before transplantation and actually it has been used worldwide thanks to its efficiently prevention of ischemia and reperfusion injury, playing a key role in organic cytoprotection and viability prior to transplantation. LBA is an important component of UW solution, acting as a cell impermeant agent during the organs cold storage, providing osmotic support and preventing cell swelling; it also has antioxidant effect, scavenging free hydroxyl radicals generated in the medium as well as cryptic inhibitor of matrix metalloproteinases (Belzer et al., 1992; Hart et al., 2002; Alonso et al., 2013).

In cosmetics products, due to its anti-aging and regenerative skin-care effects added to a trong anti-oxidant effect, it represents an important niche in the market as new and essential component in formulations for skin care, generating US sales of about $1 billion. Currently, LBA is used in cosmetic industry as a key component in the new anti-aging products and like keratinization agent formulations cosmeceutical; in fact, the acid inhibits the collapse of the enzyme matrix metalloproteinase due to metal chelation, thus reducing the presence of photoaging and wrinkles. LBA is also used in therapeutic treatments for skin diseases such as atopic dermatitis and rosacea, or even anti-acne treatments (Tasic-Kostov et al., 2012). Recently, polyhydroxyacids such LBA have offered similar therapeutic effect of alpha-hydroxyacids reducing irritant effects, suggesting the possibility of an employment for sensitive skin treatments. In addition to LBA employment as an anti-aging and keratinization agent in cosmeceutical formulations, it also exhibits moisturizing, exfoliative and humectant properties, which increase its commercial role in cosmetic field (Green et al., 2008; Yu and Van Scott, 2004).

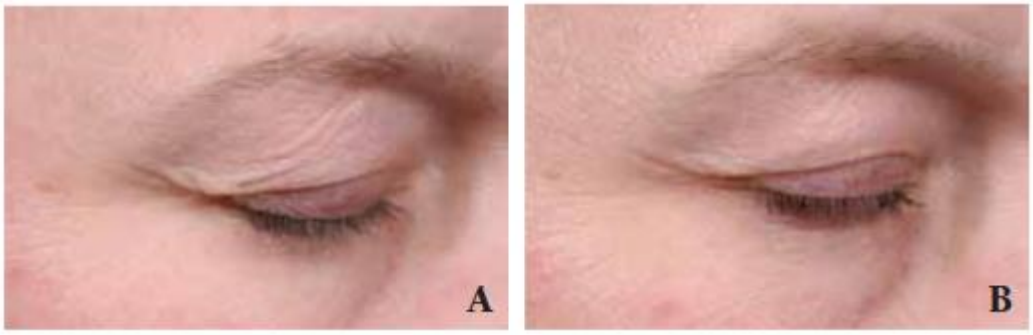


Fig. 1.7. Eyelid skin before (A) and after (B) 12 weeks of treatment with lactobionic acid 8% Diminished periocular fine lines and improved eyelid skin texture are shown (Green et al., 2008).

In chemical applications, thanks to its iron-chelating properties and emulsifying properties, LBA contribution is significant for the production of biodegradable detergents and sugar-based surfactants (Oskarsson et al., 2007) improving physical-chemical properties reducing environmental impact. Others fundamental applications of LBA in chemical products are as an active element in a novel antibacterial agent composition, which confers excellent preservation stability on consumers goods (Araki et al., 2006), and as a building block for the biocatalytic synthesis of novel polymers, as reported for example in Fig. 1.11, where GL was used as non-derivatized co-monomer, but other derivatives of sugars (like LBA) could were also appropriate (Kakasi-Zsurka et al., 2011).

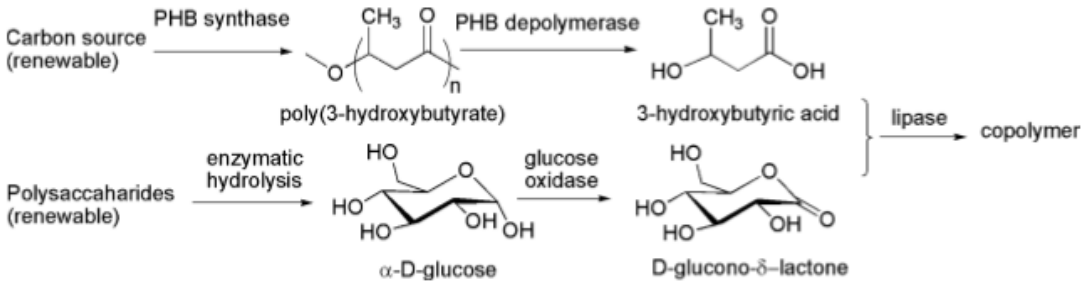


Fig. 1.8. Biocatalytic route for the synthesis of sugar-containing copolymers of 3-HBA based on renewable resources (Kakasi-Zsurka et al., 2011).

In food industry LBA has received growing attention as food additive: for its antioxidant, stabilizer and gelling effect in dessert products; as acidifier agent in fermented milk products; and as aging inhibitor for bread. Additionally LBA may stimulate calcium absorption avoiding health disorders associated to this mineral shortage. In particular LBA may stimulate intestinal Ca^{2+} or mineral absorption, exerting health-promoting influence for preventing and treating health disorders connected with calcium insufficiency, and recently were appeared on commercial market dairy beverages containing this polyhydroxy bionic acid as functional ingredient (Nielsen and Hoeier, 2009). Furthermore LBA was studied as a feed additive for laying hens reinforcing eggshell qualities by boosting calcium absorption (Kimura, 2006). Recently a lot of novel dairy-making processes were performed including LBA as a key element, provides extra-functional properties and sensory attributes through the reduction of undesirable Maillard browning reactions in cooking products (Merrill and Singh, 2011). Anti-oxidant effect and water-holding capacity of LBA were recently investigated in meat products submitted to thawing and cooking processes, reducing the water loss upon freezing (Nielsen, 2009). The importance of LBA as a functional food have been demonstrated by its approval

as a food additive by FDA in the USA, thanks to its prebiotic effects, low-calorie sweetener (2 kcal/g), gelling, stabilizing and aging inhibitor effects. LBA was also described as an active bifidus promoter molecule for different functional food and beverages (Alonso et al., 2013; Schaafsma, 2008; Oe and Kimura, 2011). Kiryu et al. (2009) isolated LBA from "Caspian Sea yogurt", estimating an indivual intake of 0.5-1.0 g of LBA per year when consuming 100 g of the yogurt per day (Kiryu ert al., 2009).

1.2.3 Production methods

Conversion of lactose to LBA proceeds through the oxidation of free aldehyde group of glucose, in lactose molecule, to carboxylic acid group. LBA was firstly synthetized by Fisher and Meyer (1889) through a chemical oxidation of lactose with bromine (Fisher and Meyer, 1889). Nevertheless currently chemical procedures (electrochemical and heterogeneous catalytic oxidations) involving refined-lactose oxidation (Fig. 1.20) and harmful and costly catalysts with undesirable side-reaction products (Yang and Montgomery, 2005; Kuusisto et al., 2007; Tokarev et al., 2009) have been shelved in favour of more interesting and sustainable biocatalytic processes, that lead to LBA through lactobiono-δ-lactone intermediate (Fig. 1.21). They comprises enzymatic synthesis, expensive processes due to the presence of redox mediators and cofactors need to be regenerated (Maischberger et al., 2008; Van Hecke et al., 2009), and microbial production employing bacteria strain able to oxidize lactose in their metabolism producing LBA.

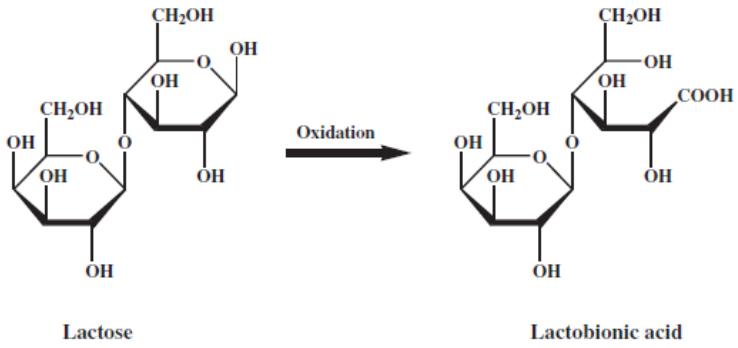


Fig. 1.9. Schematic oxidation of lactose to LBA (Gutiérrez et al., 2012).

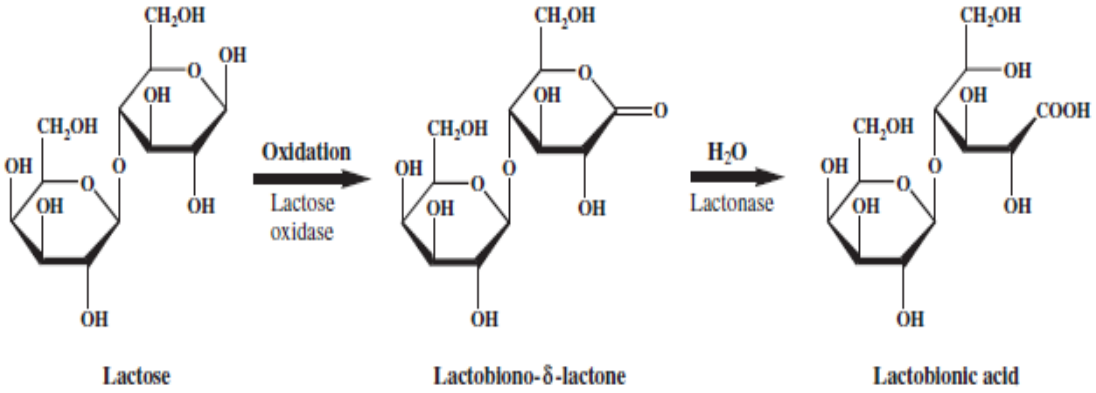


Fig. 1.10. Schematic biocatalytic oxidation of lactose to LBA (Gutiérrez et al., 2012).

ELECTROCHEMICAL OXIDATIONS

Electrochemical oxidation of lactose, separating calcium lactobionate from the products, was development by Isbell (1934) in an US Patent, in the presence of calcium carbonate and a bromide, separating the calcium lactobionate first in the form of a crystalline calcium bromide double salt and then treating this with an excess of lime to cause the precipitation of a basic calcium lactobionate from which the normal salt is prepared by carbonation (Isbell, 1934). This procedure led to an aqueous solution and acid isolate in a solid form was very difficult, hindering an efficient industrial implementation. So Magariello and Islip (1956) improved this process for production of high quantity of LBA via electrolytic lactose oxidation using graphite electrodes subjecting lactose to electrolysis in an aqueous solution in the presence of iodine and bromine catalysts, and an alkaline substance to maintain the pH above 5.2, avoiding decomposition of any unoxidized lactose. After this the crude electrolysis reaction solution was treated with cation and anion exchange resins to remove inorganic ions, and spray-dried the effluent aqueous lactobionic acid solution to convert the lactobionic acid therein into a dry mixture of acid and δ-lactone with a global yield of about 98% of LBA (Magariello and Islip, 1956). Subsequently different studies were carried out by Druliolle's group demonstrating how high yields and selectivity rates were achieved employing noble metal electrodes (platinum, platinum-modified and gold electrodes) in alkaline media (Druliolle et al., 1994; Druliolle et al., 1995; Druliolle et al., 1997), but the high cost of the process obstacle global interesting for this procedure (Gutiérrez et al., 2012).

HETEROGENEOUS CATALYTIC OXIDATIONS

It is a very promising field for the possibility of integration with others transformation technologies (Gutiérrez et al., 2012). LBA is the main product but, as reported in Fig. 1.22, lactulose and 2-keto lactobionic acid are undesired by-products of the reaction, depending on different operating conditions; metal (platinum or palladium modified with bismuth), pH (between 8.0 and 9.0, since isomerization of lactose to lactulose is greatly favoured), pressure (atmospheric pressure), temperature (from 50 to 70°C) and O_2 concentration presents importance influence on LBA selectivity. Lactose catalytic oxidation on palladium an bismuth-palladium, for LBA production, was firstly investigated by Hendriks, Kuster and Marin (1990), where Bi promotion of a commercial Pd-C catalyst resulted in 100% selectivity to sodium lactobionate up to conversions of 95% in the pH range 7–10 and at temperatures up to 333 K (Hendriks et al., 1990). Since then, different studies concerning the improvement of lactose oxidations on palladium and bismuth-promoted palladium, have been investigated (Gutiérrez et al., 2011), but the problems connected with the poisoning and deactivation of the catalysts by oxygen (Belkacemi et al., 2007) and the limited opportunity to use bismuth for the food and pharmaceutical productions, for its tendency to leaching, led to shift attention on more stable catalysts, such as supported gold catalysts. Particularly is the case of Au/CeO_2, where the consecutive oxidation step to 2-keto-lactobionic acid did not take place at all, resulting in 100% conversion and 99% selectivity for lactobionic acid over 3 h reaction time, while on Pd/C catalyst 89% selectivity at 93% lactose conversion was achieved (Climent et al., 2011). Furthermore, according with others studies, supporting gold nanoparticle on Al_2O_3, TiO_2, and CeO_2 led to over than 80% of conversions with selectivity up to 95%, using Au loadings in the range of 0.4-2.0% (Kuusisto et al., 2007).

Gold catalysts supported on silica materials mesoporous were synthetized by Gutiérrez et al., (2012) , which made possible to achieve 100% of the conversion lactose and 100% selectivity for LBA after only 10 min reaction (Gutiérrez et al., 2012). Despite the interesting LBA yield results obtained, chemical production employing metal catalysts also presents negative aspects related to security of the substances used and to the increasing interests of the consumer, which tends to prefer to source products "bio" (Alonso et al., 2013).

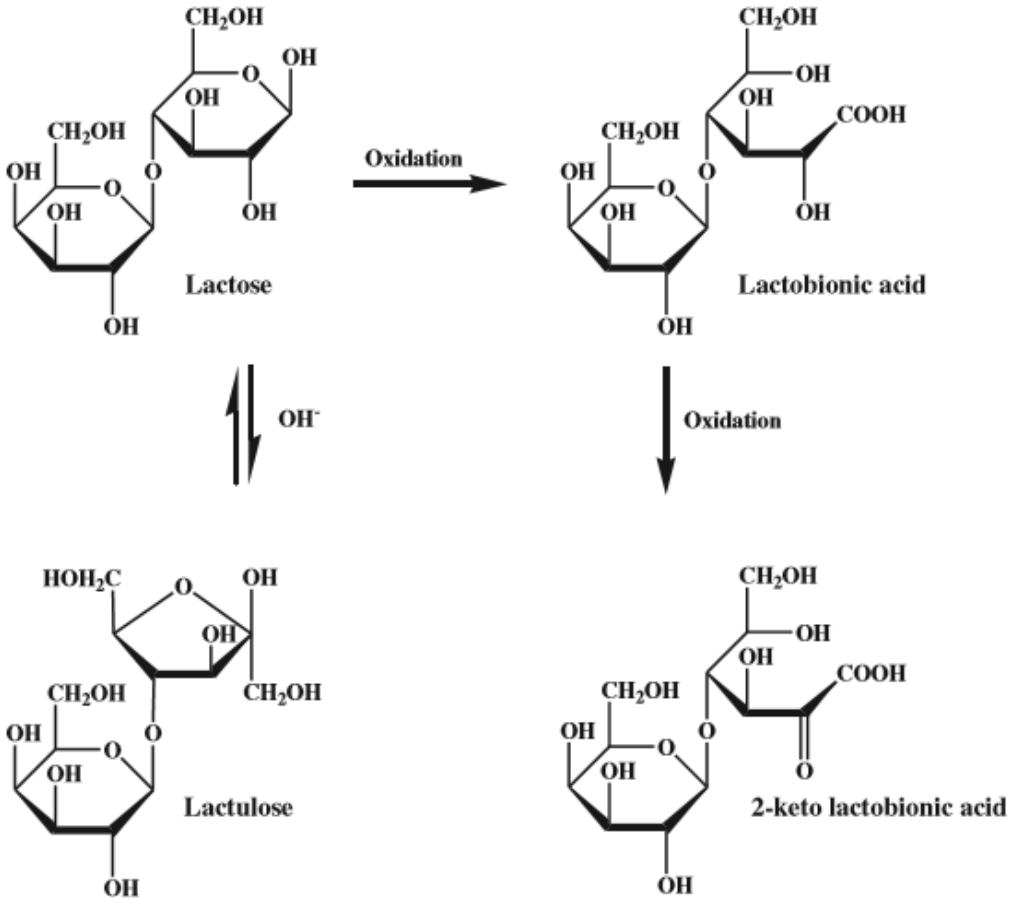


Fig. 1.11. Schematic catalytic oxidation of lactose to LBA under alkaline conditions (Gutiérrez et al., 2012).

BIOCATALYTIC OXIDATION: ENZYMATIC SYNTHESIS AND MICROBIAL PRODUCTION

LBA biocatalytic production approach involves lactose oxidation of lactose thanks to specific enzymes or microorganisms used as biocatalysts. The general scheme of reaction (Fig. 1.23) leads to the formation of an intermediate product, the lactobiono-δ-lactone, which is subsequently hydrolysed to lactobionic acid. Usually the process takes place in medium temperatures (25-50 ° C) and, since the pH must be maintained constant by addition of bases of Na-, Ca- or K-, the final product can be LBA or a salt (Gutiérrez et al., 2012). From an industrial point of view, the yield obtained through any biotechnological approach must be at least 50-100 g/L in order to achieve product concentrations that are comparable to those obtained with chemical processes (S. Alonso et al., 2013), and a comparison of production costs is essential to evaluate the advantage of biotechnology.

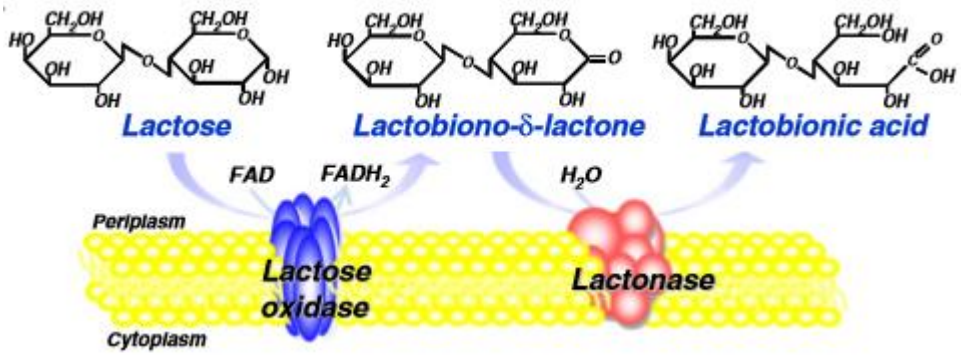


Fig. 1.12. Biocatalytic oxidation of lactose molecule to LBA in bacteria strains (Alonso et al., 2013).

- **Enzymatic production** of LBA proceeds through refined lactose oxidation employing enzymes (such as oxidoreductases) (Fig. 1.24).

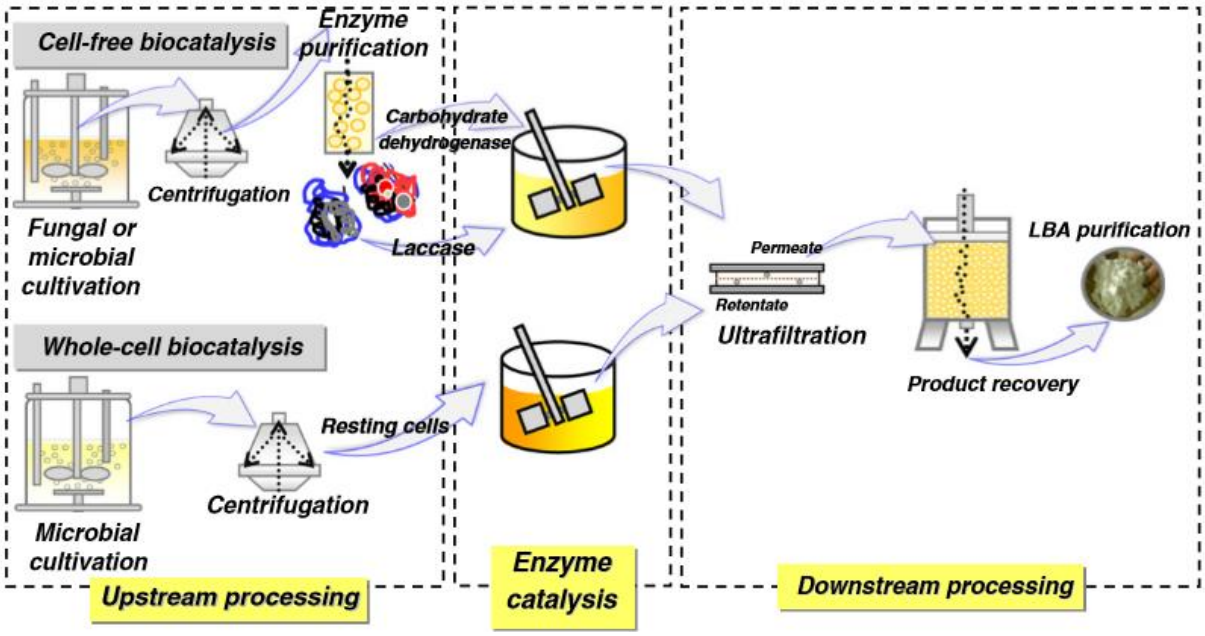


Fig. 1.13. Schematic processes for LBA production through enzymatic synthesis (Alonso et al., 2013)

Cellobiose dehydrogenase (CDH) is an extracellular hemoflavoenzyme produced by wood-degrading fungi (i.e. *S. rolfsii),* that oxidise lactose to the corresponding lactones. A combination of an electron acceptor, 2-2-azinobis-(3-ehtylbenzothiazoline-6-sulfonate) (ABTS), and a regenerating enzyme (laccase) was studied by Dhariwal et al. (2006) in an electrochemical process using electricity instead oxygen as a final acceptor of electrons, for the oxidation of lactose to LBA. This biocatalytic system led

to achieved 1.8g/L*h of LBA in 25 ml electrolysis cell with ABTS as the redox mediator, removing the need of the second enzyme (laccase) in the regeneration system. This new system has the advantage of operating at the optimum pH value for CDH activity and of controlling mediator concentration, but the lower LBA titres obtained, in comparison with others biocatalytic approaches represented a drawback to an industrial implementation (Dhariwal et al., 2006).

A biocatalytic approach for LBA production from refined lactose by CDH, with ABTS as electron acceptor and laccase as regenerating enzyme, was also carried out by Baminger et al. (2001). A novel concept of enzymatic regeneration of electron acceptors was introduced (Fig. 1.25). A complete lactose bioconversion with a LBA volumetric productivity of 18 g/L*h in 50-mL batch reactor with DCIP (2.6-dichloroindophenol) as redox mediator (Baminger et al., 2001).

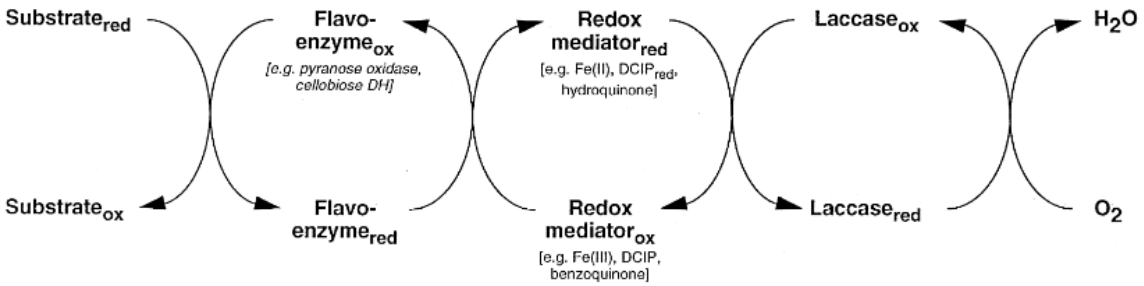


Fig. 1.14. Continuous, laccase-catalyzed regeneration of electron acceptors (redox mediators) that are employed in oxidation reactions of flavoproteins (Baminger et al., 2001).

Inactivation of the enzymatic system with growing of dissolved oxygen is a drawback of enzymatic biocatalytic approach for lactose oxidation to LBA by CDH and laccase, and Ludwing et al. (2004) were displayed the need to remove redox mediators from the system after biocatalysis by means of adsorption due to their toxicity. In the biotransformation system, featuring the continuous laccase-catalyzed regeneration of the electron acceptors, 7g/L*h of LBA were achieved after 10 h with complete lactose bioconversion (Ludwig et al., 2004).

The beneficial effect of membrane facilitated, diffusive (bubble-less) oxygenation on enzyme stability through prevention of enzyme inactivation at the gas/liquid interface has been observed by several authors for various enzymatic oxidation reactions (Van Hecke et al., 2009). These authors also performed a 20L-reactor to model and study this process using the dynamic membrane aeration (DMA) reactor coupled to an

ultrafiltration module for discontinuous and continuous operation; the conversion was catalyzed by an enzyme cascade consisting of cellobiose dehydrogenase as synthesizing enzyme and laccase as regenerating enzyme coupled by a redox mediator, achieved 3.21 g/L*h fo LBA with 97% of lactose conversion yield (Van Hecke et al., 2011).

Excessive lactose from a transgalactosylation process for galacto-oligosaccharides (GalOS)production was removed efficiently through a biocatalytic approach coupled with a chromatographic steps: lactose was firstly oxidized to LBA using CDH, and removed by ion-exchange and size-exclusion chromatography. Two different CDH, originated from *S.rolfsii* and *M.thermophilum* were compared with respect to their applicability for this process: CDH from *S.rolfsii* showed higher specificity for lactose substrate, ranging from 6 to 16 g/L*h of product LBA, with 96-100% lactose bioconversion (Maischberger et al., 2008).

The use of a glucose-fructose oxidoreductase (GFOR) enzyme from *Zymomonas mobilis* has been employed as a biocatalyst to oxidize lactose and fructose, for LBA and sorbitol synthesis under batch, fed-batch and continuous conditions. The apparent K_m of GFOR for lactose is 1.2±0.1 M, which corresponds to a 80-fold reduction of affinity for substrate binding, relative to the physiological substrate glucose. Thanks to the presence of reaction stabilizers, the GFOR presented constant biocatalytic activity up to 150 h, that led to a productivity of 4.6 g/L*h of LBA in continuous ultrafiltration membrane reactors (1.8-fold higher than batch conditions) (Satory et al., 1997).

Oxidation of lactose to LBA was also studied by Nordkvist et al. (2007) through *Microdochium nivale* carbohydrate oxidase activity, considering as safe for use in the food industry. This oxidise, contained one FAD per protein with relative molecular mass of 55000 Da, is able to oxidize several mono-, oligo- and polymeric saccharides and transfer their electrons to molecular oxygen, producing peroxide hydrogen which can be removed through catalase addition. This approach allows to achieve a LBA volumetric productivity of 4.9 g/L*h with 98% yield in batch enzymatic reaction performed at 38°C and 0.2 Lpm continuous gas supply (mixture N_2/O_2); but after 6h the system has showed a decrease in productive efficiency due to the inactivation of the enzyme resulting from the addition of NaOH for maintained constant pH 6.4 (Nordkvist et al., 2007)

A pilot-scale reactor (1L-STR) of 600L working volume, using a rotary jet head for improving mixing and mass transfer, led to an higher LBA titre (49 g/L after 12h), avoiding oxidases enzymes deactivations and demonstrating the scalable potential of the biocatalytic process (Fig. 1.26) (Hua et al., 2007).

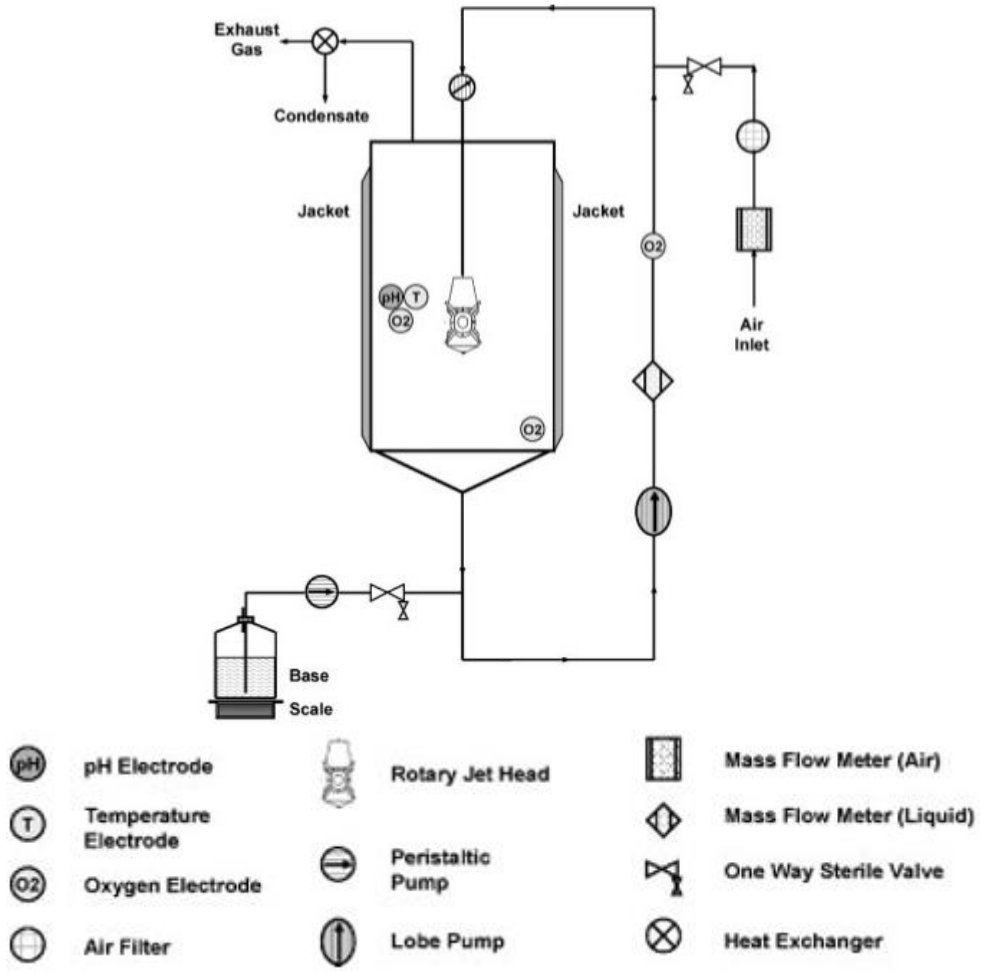


Fig. 1.15. Experimental set-up of the rotary jet head system (Hua et al., 2007).

A complete lactose (10-20% w/v) conversion to calcium lactobionate was carried out in a 500 mL reactor under aeration, agitation and pH 5.5, with a lactose-oxidizing enzyme obtained from culture supernatant of a fungal strain of *Paraconiothyrium* sp. KD-3 at 40°C; from 100-150 g/L of lactose a rate of 9-11 g/L*h were achieved after 10-20 h (Murakami et al., 2008).

An approach with immobilized purified lactose-oxidizing enzyme from *Paraconiothyrium* sp. KD-3 on cation exchange resin allowed to oxidized up to 185 g/L of lactose, despite the efficient of the process after different batch process slow down for the formation of hydrogen peroxide during the reaction, which inactivated

the immobilized enzymes (Nakano et al., 2010). Hydrogen peroxide, which was formed in concomitant to lactose oxidation by carbohydrate oxidases (from *Microdochium nivale*) or lactose-oxidizing enzymes (from *Paraconiothyrium* sp. KD-3) as a consequent of oxygen employment as electron acceptor, has an inactivated effect on enzymes and this drawback can be overcome by addition of catalase. Also glu-cooligosaccharide oxidase (GOOX) from *Acremonium strictum* was also tested as an efficient sugar oxidizing enzyme which displays a high optimum at pH 10 (Lee et al., 2005).

Substrate	Relative activity[a]		K_m[b] (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
	0.2 mM maltose	10 mM maltose			
Glucose	0.2	3.5	8.12 ± 0.16	546 ± 16	67
Lactose	6.4	3.8	0.066 ± 0.008	819 ± 25	12,400
Maltose	1.0	6.0	2.47 ± 0.05	531 ± 16	215
Maltotriose	1.1	6.2	1.11 ± 0.02	385 ± 12	348
Maltotetraose	0.4	4.3	2.51 ± 0.05	388 ± 12	155
Maltopentaose	0.2	2.6	10.6 ± 0.12	618 ± 18	58
Maltohexaose	0.2	3.1	2.60 ± 0.05	276 ± 11	106
Maltoheptaose	0.2	2.3	6.95 ± 0.15	415 ± 13	60
Cellobiose	7.7	2.8	0.048 ± 0.08	322 ± 11	6,710
Cellotriose	7.4	2.1	0.026 ± 0.002	776 ± 16	29,800
Cellotetraose	7.5	2.0	0.012 ± 0.002	200 ± 10	16,700
Cellopentaose	7.6	1.3	0.026 ± 0.003	239 ± 11	9,190
Cellohexaose	7.2	1.1	0.069 ± 0.007	222 ± 10	3,220

[a] The enzyme activity with 0.2 mM maltose was defined as 1.
[b] The kinetic parameter data are means ± standard errors for three independent experiments.

Table 1.5. Substrates specificity of native GOOX (Lee et al., 2005).

Despite high LBA productivity and selectivity and simplified downstream processes, the costs of the process, connected with redox mediator, cofactor regeneration and arduous upstream steps, and limitations related to the enzymes activity loss during the process (Alonso et al., 2013).

- LBA **microbial production** strains was firstly reported by Stodola and Lockwood in the middle of 19th century; they screened *Pseudomonas* species and identified *Pseudomonas graveolens* 14 as the best producer of LBA from lactose oxidation (75% yield after 165 hours in shake flasks) (Stodola and Lockwood, 1947). Several microorganisms have been investigated for LBA production (*Pseudomonas fluorescens, Pseudomonas myxogenes, Burkholderia cepacia, Acetobacter orientalis,*

Streptococcus lactis, Zymomonas mobilis) but the process proceeds slowly with low yield (Fig 1.27; 1.28) (Alonso et al., 2013).

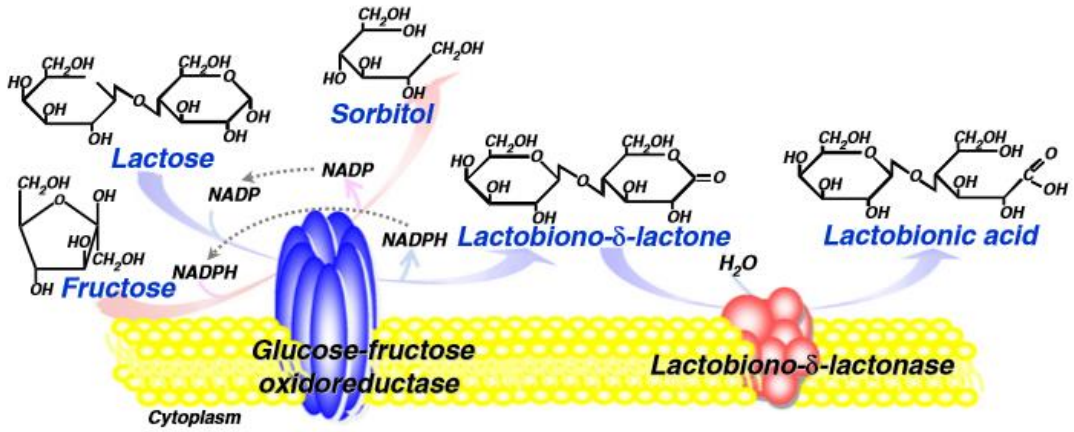


Fig. 1.16. Reaction schemes of a bi-substrate enzymatic system carried out by glucose-fructose oxidoreductase in *Zymomonas mobilis* for LBA and sorbitolo productions (Alonso et al., 2011).

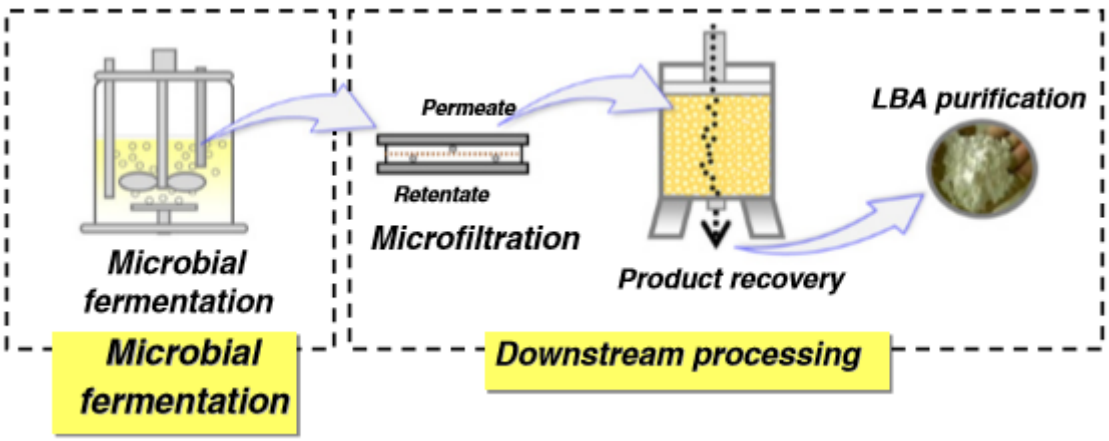


Fig. 1.17. Schematic process for LBA production through microbial activity (Alonso et al., 2011).

Miyake and Sato also reported aldonic acids production from sugars through a two-stages process, comprising a first growth phase of *Pseudomonas* species and a second enzymatic synthesis of the interested compounds by permeabilized cells. *Pseudomonas* species are the ideal platforms for lactose oxidation producing LBA, thanks to the absence of the genes encoding the β-galactosidase enzyme, which divide lactose molecule in glucose and galactose components (Miyake and Sato; 1975). In *Pseudomonas* species LBA is formed via lactose oxidation pathway, in which a

membrane-bound dehydrogenase system catalyzes this single biotransformation (Fig. 1.23); a lactose dehydrogenase enzyme first catalyzes lactose oxidation t a lactone intermediate (lactobiono-δ-lactone) which is consequently hydrolyzed into LBA by a lactonase enzyme. Lactose dehydrogenase does not use oxygen as a direct acceptor of electrons and presents an optimum pH at 5.6 with K_m value for lactose of about 11 mM; while lactonase presents an optimum pH at 6.5-6.7, with K_m of 20 mM, although the specific enzyme activities vary with growth conditions (Nishizuka and Hayaishi, 1962).

According to Vakil and Shahani (1969) lactobionate constituted an initial intermediate in the lactose metabolism of *Streptococcus lactis* UN; the lactobionate thus formed was further hydrolyzed to gluconate and galactose through enzyme lactobionase (*β*-galactosidase) action and then metabolized. During the lactobionate and glucose fermentations, the organism produced varying amounts of lactic, formic, and acetic acids, carbon dioxide, ethanol and glycerol, and their concentrations were comparable to those produced during the lactose fermentation (Vakil and Shahani, 1969).

LBA production from lactose oxidation by filamentous fungi (*Penicillium chrysogenum*) was investigated by Bucek et al. (1956) in shake-flasks cultures where 50% of LBA yield was achieved after 120h (Bucek et al., 1956).

It was observed in the course of a study of the carbohydrate metabolism of the marine red alga, *Iridophycus flaccidum*, that cell-free extracts of this plant had the ability to oxidize D-glucose to D-gluconic acid, thanks to carbohydrates with an optimum pH of 5.0. Further investigation revealed that this preparation will also oxidize several reducing disaccharides, like maltose, lactose, and cellobiose, to their corresponding aldobionic acids (Bean and Hassid, 1956). *Pseudomonas taetrolens* is a non-pathogenic wild-type microorganisms taxonomically belongs to the *Pseudomonas choloraphis* group (Sugiyama et al., 2005) able to oxidize lactose producing LBA with production yields, titres and productivity values comparable with LBA bio-production carried out by other bacteria strains like *Burkholderia cepacia* and *Zymomonas mobilis* (Murakami et al., 2002, 2003, 2006; Pedruzzi et al., 2011). Alonso et al. (2011, 2012) studied the positive effect of the size of the inoculum in order to reduce the onset of the stationary phase, where LBA molecule is obtained, and the importance of a pH-shift an high-cell density conditions with the aim of overcome drawbacks which limited LBA productivity by *P. taetrolens* cells (Alonso et al., 2011, 2012). Negative effects on LBA bio-production yield of both high aeration and

agitation rates were also investigated; mild agitation rates (350 rpm) increase LBA productivity 1.2-fold compared to high agitation rates (1000 rpm) and the best value of oxygen supply was 0.5 Lpm. Another important feature of the bio-production of LBA by *P. taetrolens* is the strongly relation with the physiological status of the cells used as inocula, like seed culture age and microenvironmental pH. In fact prolonged-time seed cultures (>12h), resulting in the presence of lag-phases along with reduced fermentation efficiencies in term of LBA productivity; furthermore pH values lower than 6.0 led to stressed environmental conditions for the cells physiological response (Alonso et al., 2012). All these aspects are very important in order to an industrial implantation and scale up processes.

High-level production of LBA can be obtained by *Pseudomonas* sp. LS13-1 mutants which, under fed-batch fermentation coditions supplemented with 15 g/L of peptone as nitrogen source, achieved a LBA titre of 290g/L with 1.87 g/L*h of volumetric productivity (Miyamoto et al., 2000). But an industrial implementation with mutant strains presents a lot of difficult for their genetic instability.

Mutant strains of *Bulkolderia cepacia* (No. 24) exhibited higher lactose-oxidizing activity: 5.55 g/L*h in shake flask cultivations, and better performance can be obtained through metabolic engineering approach for the identification and modification of the target genes directly or indirectly involved in LBA formation (Murakami et al., 2002, 2003, 2006).

The problems connected with the pathogenicity of *B. cepacia* have overcome through biotechnological production of LBA by an acetic acid microorganism, *Acetobacter orientalis*, which was isolated from “Caspian sea yogurt” and oxidized 2-10% of lactose at a yield of 97-99% with resting cells, nutrient-rich media and shake flasks conditions, thanks its membrane-bound dehydrogenase oxidize monosaccharides and disaccharides (Kiryu et al., 2009, 2012).

Another acetic acid bacteria was investigated by Oe et al. (2008); *Gluconobacter cerinus* UTBC-427 showed strong lactose-oxidizing activity and has been recently selected by Unitika (Japan) as microbial platform for LBA industrial bio-production under resting cells conditions (Oe et al., 2008; Kimura 2012).

In addition different studies employed permeabilized cells have been carried out in order to achieve higher LBA titres. *Zymomonas mobilis* is able to produce LBA in a bi-substrate system in which lactose oxidation was coupled with the reduction of fructose to sorbitol (Fig. 1.27). The two products results from GFOR enzymatic

system contained in the permeabilized cells, at pH 6.4 and temperature from 39 to 45°C, despite the low affinity of GFOR for both lactose and fructose required high level of substrate concentration to promote high productivity. High LBA titres (125-182 g/L) and yields (78-100%) were achieved with permeabilized cells of *Z. mobilis* with 5.8-7.6 g/L*h of production rates. Also the possibility of cells immobilization in calcium-alginate beads (with diameter of 1.2 mm) was approached and 14.9 g/L*h LBA was achieved, but additional studies should be performed to verify the long-term mechanical stability of calcium alginate beads (Malvessi et al., 2013; Pedruzzi et al., 2011).

Biocatalyst	**Conversion yield (%)**	**Selectivity for LBA (%)**	**Reaction time (min)**	**Space-time yield for LBA (g/L*h)**	**References**
GFOR/GL enzymes from *Zymomonas mobilis* ATCC 29191	~85	100	1500-4500	5.6	(Borges da Silva et al., 2011; Pedruzzi et al., 2011)
GFOR from permeabilized cells of *Zymomonas Mobilis* CP4 (ATCC 31821)	~90-95	100	3600	0.8-2.3	(Severo Junior 2008)
GFOR from *Zymomonas mobilis* DSM 473	90-95	100	4200	2.6-4.6	(Satory et al., 1997)
GFOR from *Zymomonas mobilis* DSM 3580 CDH (EC 1.1.99.18) from *Sclerotium rolfsii* (SrCDH; 420 U L^{-1}) Þ laccase (EC 1.10.3.2; 2030 U L^{-1})	95-98	100	720	3.1	(Vn Hecke et al., 2011)
CDH (EC 1.1.99.18) from *Sclerotium rolfsii* CBS 191.62 þ laccase (EC 1.10.3.2) from *Trametes pubescens* MB 89	100	~100	360	-	(Van Hecke et al., 2009)
CDH from *Sclerotium (Athelia) rolfsii*	~100	100	600	-	(Meischberger et al., 2008)

CDH from *Sclerotium rolfsii* CBS 191.62 Þ laccase from *Trametes pubescens* MB 89	99	100	150	15.9	(Splechtna et al. 2001)
CDH (EC 1.1.99.18) from the basidiomycete *Sclerotium rolfsii* CBS 191	100	100	420	3.0	(Van Hecke et al., 2009)
CDH from *Athelia (Sclerotium) rolfsii* CBS 191.62 þ laccase from *Trametes pubescens* MB 89	~100	100	300-1350	3.0-21.0	(Ludwig et al., 2004)
CDH from *Athelia (Sclerotium) rolfsii* CBS 191.62 þ laccase from *Trametes pubescens* MB 89	100	100	150-250	7.2-27.0	(Baminger et al., 2001)
CDH from the ascomycete *Myriococcum thermophilum*	100	100	420	3.3	(Van Hecke et al., 2009)
CDH from *Myriococcum thermophilum*	~100	100	600	-	(Mainschberger et al., 2008)
CDH from *Microdochium nivale* Þ catalase (Catazyme® 25 L from Novozymes A/S)	98	-	~600	-	(Norkvist et al., 2007)
CDH from *Microdochium nivale* Þ catalase (Catazyme® 25 L from Novozymes A/S)	100	100	300-380	7.9-11.5	(Budtz et a., 2005
CDH from *Termitomyces clypeatus*	~100	100	300	0.01	(Saha et al., 2008)
CDH from *Sporotrichum thermophile*	-	-	-	-	(Canevascini et al., 1982)
CDH from white mold Þ laccase from *Trametes versicolor*	-	-	-	1.8	(Dhariwal et al., 2006)

Carbohydrate oxidase from *Microdochium nivale* Þ catalase (Catazyme® 25 L from Novozymes A/S)	>98	100	720	~4.0	(Hua et al., 2007)
Carbohydrate acceptor oxidoreductase from *Paraconiothyrium* sp.	100	100	420-900	14.3	(Kiryu et al., 2008)
Cellooligosaccharide oxidase from *Sarocladium Oryzae* F137 Þ catalase	90	100	720	2.1	(Lee et al., 2006)
PDH from various *Agaricus* species	-	83-95	960-2880	-	(Volc et al., 2004)
Hexose oxidase from *Chondrus crispus*	-	-	-	-	(Savary et al., 2001)
Glucooligosaccharide oxidase from *Acremonium Strictum* T1-38 Þ catalase	98	100	240	6.7	(Lin et al., 1993)
Lactose-oxidizing enzyme from *Paraconiothyrium* sp. KD-3 þ catalase	75-100	100	600-3000	7.4-11.5	(Murakami et al., 2008)
Lactose dehydrogenase from *Pseudomonas Graveolens* ATCC-4683 Þ lactonase	100	100	-	-	(Nishizuka & Hayaishi, 1962)
Pseudomonas graveolens ATCC-4683	-	-	-	-	(Wright & Rand, 1973)
Pseudomonas graveolens 14	100	96	9900	~0.5	(Stodola & Lockwood, 1947)
Pseudomonas taetrolens LMG 2336	66-100	100	2880-4800	0.59-1.12	(Alonso et al., 2011)
Pseudomonas taetrolens LMG 2336	6-100	100	3600	0.04-1.3	(Alonso et al., 2012)
Pseudomonas sp. LS13-1	>90	100	10800	0.46-1.0	(Miyamoto et al., 2000)
Pseudomonas sp. LS13-1	>90	100	7200	1.5-1.9	(Miyamoto et al., 2000)
Pseudomonas calco-	85-90	-	11520	~0.5	(Kluyver et al.,

acetica					1951)
Pseudomonas quercito-pyrogallica	~90	-	~46000	~0.5	(Kluyver et al., 1951)
Burkholderia cepacia No. 24	~100	100	900-1620	5.6-10.01	(Murakami et al., 2003; 2006)
Burkholderia cepacia No. 216	100	100	3000	0.2	(Murakami et al., 2002)
Acetobacter orientalis KYG22	36	-	4320	0.1	(Kiryu et al., 2009)
Acetobacter orientalis KYG22	~100	-	4320	~0.4	(Kiryu et al, 2012)
Paraconiothyrium Sp.	-	-	-	-	(Nakano et al., 2006)
Halobacterium saccharovorum	97	100	2880	-	(Tomlinson et al., 1978)
Paracolon bacteria B1x urease-positive and C6 urease-negative	100	100	2880	~0.4	(Eddy, 1958)
Penicillium chrysogenum	~6	-	7200	0.04-0.07	(Cort et al., 1956)

Table 1.6. Overview of LBA production by biocatalytic oxidation of lactose (Gutiérrez et al., 2012).

Although a lots of studied were concerned on the LBA microbial production through oxidation of refined lactose, recent interesting were focused on the possibility to employ inexpensive feedstock as fermentation broth for *Pseudomonas* species, like dairy industry by-product suggests a promising strategy to achieve cost-effective upgrading of these fractions. This approach also provides an environmentally-friendly and competitive alternative to costly and harmfully upstream processing step for LBA production through chemical or enzymatic oxidation of refined lactose (Alonso et al., 2011).

Improvements in microbial processes for LBA production can be obtained using inexpensive feedstock as a lactose source, like dairy industry wastes; several studies have been focused on whey use but few ones are with ricotta cheese whey (Alonso et al, 2011).

1.3 INDUSTRIAL IMPLEMENTATION STRATEGIES

In recent years, bio-based production of platform chemicals blocks, basic starting materials for producing chemical intermediates and building compounds, from renewable resources has attracted increasing attention as an alternative to petrochemical production methods, due to

the limited nature of fossil oil, increasing oil prices, and global concerns on sustainability and the environment. The techniques and strategies for developing microbial strains for chemicals production have advanced rapidly (Jang et al., 2012).

Interesting features of biological systems include flexibility, selectivity of the substrate, regioselectivity, chemoselectivity, enantioselectivity and catalysis ambient temperature and pressure. However, the challenge is the bioprocess competitiveness from the point of view of costs with assets of existing capital chemical processes associated with the production of chemical raw materials. The chemical industry serves nearly all sectors of the manufacturing industry and is often indicated as a key industry. The range of markets served and volume of generated product resulting in worldwide consumption of 270 million tons of oil and gas a year. Only in the US chemical industry it converts $ 27 billion of raw materials in 419 billion dollars of products each year, with industry revenues globally 1.5 billion dollars, and consumes 7% of the annual energy production. The biggest challenge the chemical industry is the long-term sustainability: the issue is often addressed by focusing on the use of renewable energy reserves rather than fossil fuels. In this regard, it is pushing towards the exploration of bioprocesses for the production of chemicals (Green chemistry): recently there has been a renewed interest in the use of conversion of biomass for the production of valuable products (Thomas et al., 2002).

1.3.1 Cells Immobilization

Biofilm formation is a natural process where microbial cells attach to a support (inorganic or organic material) or form aggregates, without chemicals use, forming a high-density cells layers called “biofilm” (Qureshi et al., 2005).

Immobilization of microorganisms in a polymeric matrix exhibits great potential, especially, in a packed bed or fluidized bed reactors, with positive effects in terms of particle size control, biomass regeneration and reutilization, easy separation of cells from fermentative broth and high cells density (Vijayaraghavan et al., 2007).

Interesting application in food industry and treatment of food industry wastes were investigated concerning the immobilization cells strategy; but the strictly rules on food safety led to choose immobilization cells supports with precise characteristics of inalterability and absence of reactivity of the material (Kosseva et al., 2009).

Reactor Type	Comments
Membrane reactor	
Advantages	High productivities, high cell concentration can be achieved inside the reactor, clear permeates for further separation
Disadvantages	Fouling with cells, cost prohibits their use in low cost large volume chemical production
Immobilized cell reactors	
Covalent bond formation	
Advantages	High cell concentration may be achieved, high productivity
Disadvantages	Cell growth inside matrix may be restricted, cells leach out of the matrix and hence centrifugation of effluent may be required, chemical may affect the cells
Entrapment	
Advantages	High cell concentration may be achieved, high productivity
Disadvantages	Matrix often starts disintegration with time, cells leach out of matrix, centrifugation of reactor effluents is required for further separation
Biofilm	
Advantages	Comparatively high reactor productivities and high cell concentrations are achieved, reactors run longer and are economic to operate
Disadvantages	Effluent centrifugation is required

Table 1.7. Comparison of different types of reactors with biofilm reactors (Qureshi et al., 2005).

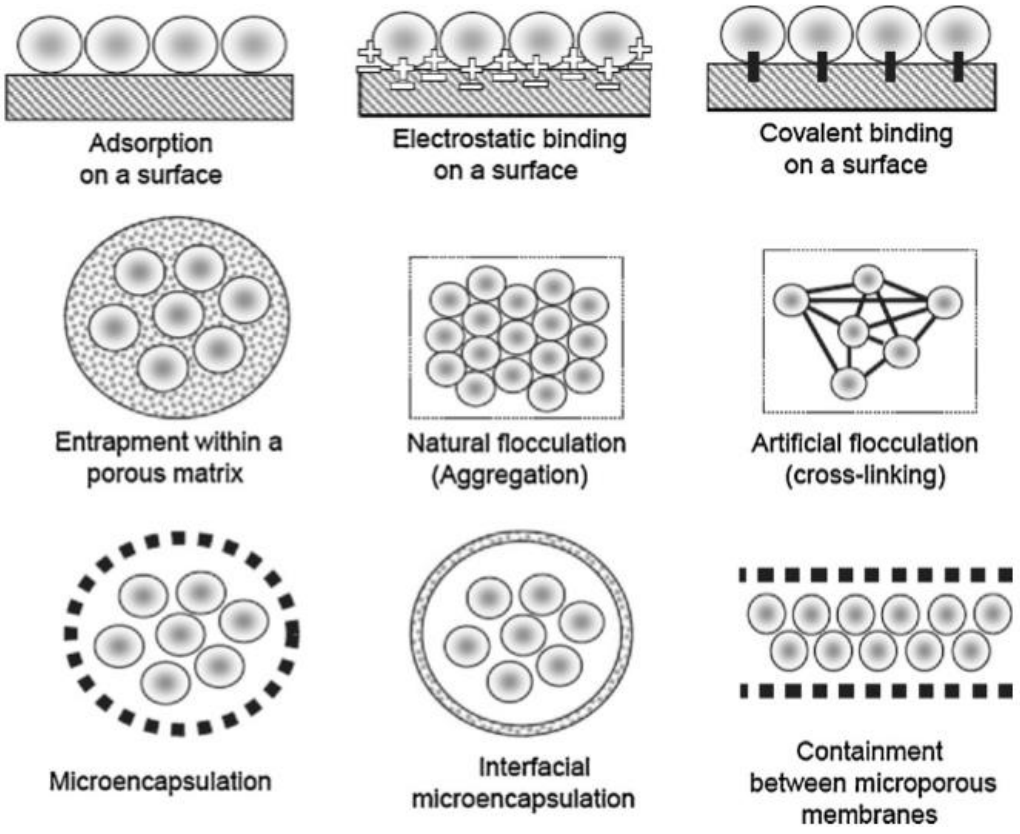


Fig. 1.18. Basic methods for biocatalyst immobilization (Kosseva et al., 2009)

1.3.2 Downstream process

The key factors for the advance of the bio-based production of platform chemicals include the development of natural or engineered microorganisms for efficient production, access to cost-competitive and sustainable resources, and optimization of downstream processes, to compete

against the classical petrochemical processes and guarantee industrial implementation (Jang et al., 2012).

In the last few years new approaches for LBA recovery and purification have been performed (Table 1.6); in particular several attempts have been made to separate and purify LBA after bioconversion processes and the techniques adopted depends on the nature of the matrix employed for LBA production, because metabolites from the fermentative broth could represent a drawback for the outcome of purification process in comparison with downstream processing for LBA recovery after catalytic production from refined lactose (Alonso et al., 2013).

Approach	Matrix	LBA concentration (g/L)	Recovery (%)	Reference
Crystallization + ion exchange chromatography	Synthetic solution	200	79	Jones and Ho (2002)
Ion exchange chromatography	Synthetic solution	50	100	Pedruzzi et al. (2008)
Ion exchange chromatography	Reaction solution	40	100	Splechtna et al. (2001)
Simulated moving bed technology	Bioconversion broth	125	100	Borges da Silva et al. (2011)
Electrodialysis	Bioconversion broth	20	38.7	Peretti et al. (2009)

Table. 1.8. Overview of downstream processes for LBA purification (Alonso et al., 2013).

The first attempt for LBA purification after microbial fermentation was suggested by Lockwood and Stodola (1950), which performed a precipitation step through ethanol addition to the culture supernatant after fermentation (Lockwood and Stodola, 1950). Magariello et al. (1956), after an electrolytic reaction for lactose oxidation to LBA, proposed a vacuum evaporation of LBA solution to a syrup and a dehydration by distillation with dioxane and toluene for obtaining a dry residue; this is added with dioxane and toluene again and the process repeated. The finally dry residue obtained was further crystallized (Magariello et al., 1956). Alternatively the proposed processed included treating the crude electrolysis reaction solution with cation and anion exchange resins to remove inorganic ions, and spray-drying the effluent aqueous solution to convert LBA in a dry mixture of acid and δ-lactone (Magariello et al., 1956). Jones and Ho (2002) proposed an optimization of the method for LBA recovery thanks to a first evaporation step (45 min at 70°C) followed by a crystallization through a precipitation method and a final ion-exchange process. Because calcium lactobionate is cheaper compared to the acid form, an object of the patent is to prepare LBA using calcium lactiobionate as a starting material. A final freeze-drying process allowed to achieve a LBA recovery rate of 79% (Jones and Ho, 2002).

Also electrodialysis technique have been used for separate LBA from a solution contained lactose, fructose and sorbitol too, resulting from a biotechnological process catalysed by the

enzymes GFOR and GL of *Zymomonas mobilis*. However, the low yield obtained (38.7%) and the new trends in food industries (Daufin et al., 2001) are focused on the use of green processes (Peretti et al., 2009). Cation exchange resins purification can be considered a green process and Pedruzzi et al. (2007; 2008) studied different chromatographic systems to perform the separation of the quaternary mixture (lactose, fructose, LBA and sorbitol) from an enzymatic process carried out through GFOR and GL activity. But the resins need sometimes to be changed because of that they can represent an additional cost for the industry (Pedruzzi et al., 2008). In the last years, membrane filtration processes were presented as a valuable green technique in order to obtain purified extracts (Mirabella et al., 2014). For several years membrane processes have been presented with more promising application in food industry and the mail application are in dairy industry, beverages and egg products, and wine industry, limiting their environmental impact (Daufin et al., 2001)

Tangential cross-flow filtration is a physics-mechanical separation procedure. While the medium flows through the membrane tangentially the filtrate (permeate) is carried away at a right angle. This filtering method is also called “dynamic filtration”. The compounds to be separated do not undergo any chemical modification which means that they can be recycled or recovered. By choosing an appropriate pore size low-molecular substances are able to pass through the membrane while macro molecules are being held back.

Inorganic membranes manufactured by atech innovations gmbh are made of alumina oxide (Al_2O_3). These are composite membranes which are produced by applying a coat to a highly porous carrier material. This coat makes up the actual membrane coat.

The supporting material is made of an extruded mix of silicon carbide which is sinter-fused to its defined mean pore by using an exact control of temperature and moisture. The layer is then sinter-fused to the supporting material which results in a structure with a very high stability.

Atech membranes are characterized by high thermal stability, resistance to chemicals, high mechanical stability (hammering), high filtrate yield, easily washed and regenerated steps, low operating costs due to long service life (Ref: ‘membranes protocol’ by atech innovations gmbh).

2. AIM OF THE WORK

Cheese Whey (CW) and Ricotta Cheese Whey (RCW), obtained after cheese and ricotta cheese manufacture respectively, are the most important dairy industry wastes (80–90% of the volume of milk transformed) (Pantazaki et al., 2009); and their significant lactose amount (4-5% w/w) represent a disposal problem for agro-industrial companies. Currently, about 30% of annual world cheese whey production (about 177 million tonnes in 2006) (Alonso et al., 2011) and

RCW is a high pollution dairy industry waste. It is characterized by BOD and COD values of 50 g/L and 80 g-L, respectively, a lactose concentration of about 40-45 g/L, without significant concentration of proteins and lipids. It is a by-product obtained after ricotta cheese production, which consists of the following steps: acidification of raw cheese whey and heating up to 85–90ºC for about 25 min, to promote the precipitation of most of whey proteins to get the cottage cheese, known as ricotta cheese. The liquid solution remaining after cheese separation is actually the so-called Ricotta Cheese Whey, which, due to both the severe thermal treatment and the addition of acid salts, has different characteristics with respect to raw cheese whey (Sansonetti et al., 2009).

Although about 30% of world production of cheese whey remains underutilized, destined to become waste or animal feed. The environmental and economic interests that require a greater amount of serum is converted into high value-added products (S. Alonso et al., 2011).

Lactobionic acid (LBA) is an aldonic acid obtained from lactose oxidation, which chemical structure comprises a galactose molecule bound to a molecule of gluconic acid via an ether linkage (Gutiérrez et al., 2012). This acid presents a lot of recently discovered biological activities, and a great therapeutic potential due to its excellent biocompatibility, biodegradability and non-toxicity, but also its chelating properties, amphiphilic and antioxidants (S. Alonso et al., 2013). LBA has important commercial use as an ingredient in solutions used to stabilize the organs prior to transplantation, but is also used as a new ingredient in care products skin and in food industry as acidulante with sweet taste and functional beverages fortified with essential minerals such as iron and copper. It also has an important role as functional supplementation of calcium (Gutiérrez et al., 2012). To date, the lactobionic acid is produced in greater part through chemical synthesis in a high-energy-intensive process, which requires the use of expensive metal catalysts and which may also lead to the formation of products from unwanted side reactions. Its biotechnological

production has not been developed so intensely until now, in comparison to that of other organic acids such as

lactic, succinic or citric. However, recently, the bio-production of LBA has emerged as a promising and flexible approach to satisfy the growing demand for this bio-product. In addition, the environment friendly and cost efficient production of bio-LBA may be carried out using dairy industry by-products as cheap raw materials: in fact, although they represent a natural source of protein and lactose, the increase of their production and their treatment remain two of the major problems for the dairy industry. Therefore, the search for innovative solutions disposal and management of this flow of high strength waste is become the driving force behind the development of new sustainable processes (S. Alonso et al., 2013).

Thus, the aim pursued with this project is the design and development of an integral process, using membrane filtration, to produce a purified extract of LBA from the fermentation broth with Ricotta Cheese Whey as substrate, and *Pseudomonas* and *Acetobacter* sp. as bacteria strains. Improvement of bioconversion performance were performed with different strategies (Stirred-Tank and Packed-Bed reactors), in order to achieved an industrial implementation of the process. A finally approach of downstream process with high performance tangential flow filtration was investigated for a purification of LBA from fermentative broth.

3. MATERIALS AND METHODS

3.1 MICRORGANISMS, MEDIA AND SUBSTRATES EMPLOYED

Seven bacterial strains were used in this study. *Pseudomonas taetrolens* LMG 2336 obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium)- -and six (*Pseudomonas taetrolens* DSM 21104; *Pseudomonas fluorescens* DSM 50106; *Pseudomonas chlororaphis* DSM 50083; *Pseudomonas citronellolis* DSM 50382; *Acetobacter orientalis* DSM 15550; *Acetobacter syzygii* DSM 15548) obtained from DSMZ culture collection. The strains were maintained frozen (-80°C) in their corresponding growth media containing 20% v/v glycerol. The *Pseudomonas* sp. and *Acetobacter* sp. strains were grown on nutrient broth and acetic acid bacterium media, respectively.

The composition of bacterium media and the optimal conditions of growth (temperature and pH) was reported in Table 3.1, as indicated by LMG and DSMZ bacteria collections.

Microrganism	**Media, Temperature**	**Composition (g/L)**
• *Pseudomonas taetrolens* LMG 2336	M2, 28°C	KH2PO4 0.45 Na2HPO4 x 12 H2O 2.39 Beef extract 1 Yeast extract 2 Peptone 5 NaCl 5 Agar 15 pH 6.8
• *Pseudomonas taetrolens* DSM 21104	M535, 28°C	Trypticase Soy Broth 30 Agar 15 pH 7.3
• *Pseudomonas fluorescens* DSM 50106 • *Pseudomonas chlororaphis* DSM 50083 • *Pseudomonas citronellolis* DSM 50382	M1, 30°C	Peptone 5 Meat extract 3 Agar 15 pH 7.0

• *Acetobacter orintalis* DSM 15550 • *Acetobacter syzygii* DSM 15548	M989, 30°C	Meat extract 5 Yeast extract 5 Glucose 5 MgSO4 x 7 H2O 1 Agar 15 pH 6.6-7.0

Table 3.1. Composition of bacterium media and the optimal conditions of growth

Experiments for testing lactose oxidation ability of the bacteria strains were performed with different substrates as lactose source as reported in Table 3.2.

Substrates	**Composition (g/L)**
S1. Fermentative broth+Synthetic Lactose (from Sigma-Aldrich)	D-Lactose monohydrate 98% 10-50 Peptone 2 Yeast extract 1 K2HPO4 0.2
S2. Mineral Salt Medium+Sigma Lactose (from Sigma-Aldrich)	D-Lactose monohydrate 98% 50 Glucose 0.1 KH2PO4 0.7 Na2HPO4 0.9 NaNO3 2 MgSO4 x 7 H2O 0.4 CaCl2 x 2 H2O 0.1 Trace Element Solution 2mL NaCl 5
S3. Ricotta Cheese Whey	Ricotta Cheese Whey from bovine milk (provided by Granarolo, Bologna, Italy)
S4. Permeate X	Permeate X (obtained after a RCW ultrafiltration process-500 KDa)
S5. Permeate 4	Permeate 4 (obtained after a permeate X ultrafiltration process-30 KDa)
S6. Fermentative broth+Concentrated Lactose (obtained after a RCW ultrafiltration and nanofiltration processes)	Concentrated Lactose 50 Peptone 2 Yeast extract 1

	K2HPO4 0.2
S7. Mineral Salt Medium+Concentrated Lactose (obtained after a RCW ultrafiltration and nanofiltration processes)	Concentrated Lactose 50 Glucose 0.1 KH2PO4 0.7 Na2HPO4 0.9 NaNO3 2 MgSO4 x 7 H2O 0.4 CaCl2 x 2 H2O 0.1 Trace Element Solution 2mL NaCl 5

Table 3.2. Substrates employed as lactose source

Permeate X and 4, and Concentrated Lactose were obtained after Ultrafiltration and Nanofiltration processes, respectively, of RCW for proteins recovery and valorization as bioactive compounds (project S.O.F.I.A.), as reported in Fig.3.1.

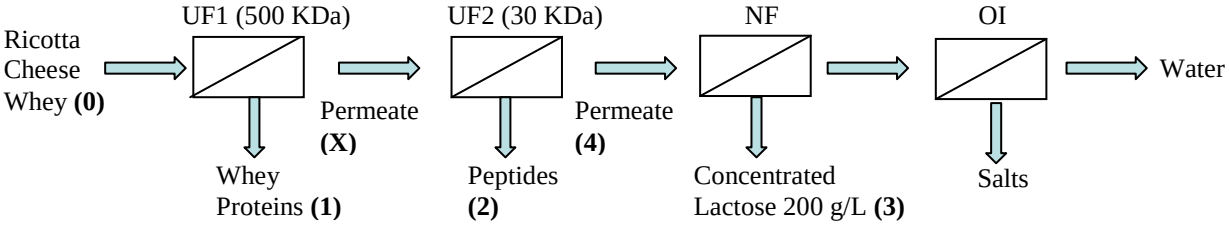


Fig 3.1. Biorefinery process of Ricotta Cheese Whey

Composition and chemical characterization of the RCW employed were presented in Table 3.3 (data provided by project S.O.F.I.A.'s partners).

						Sugars		
pH	**Acidity (°SH/100mL)**	**Density (g/mL)**	**Dry (g/100g)**	**Ashes (g/100g)**	**Lipids (g/100g)**	**Lactose (g/100g)**	**Glucose (g/100g)**	**Galactose (g/100g)**
5.31	3.70	1.029	6.32	1.29	0.14	3.95	0.004	0.014

Acids			Whey Proteins			
Citric (mg/100g)	**Lactic (mg/100g)**	**Total Proteins (g/100g)**	**Ala (mg/100g)**	**BlgB (mg/100g)**	**BlgA (mg/100g)**	**Soluble Peptides (g/100g)**
127	90	0.37	1.27	1.55	1.71	0.096

Table 3.3. Biorefinery process of Ricotta Cheese Whey

3.2 INOCULUM PREPARATION

A loopful of each strain grown on agar plate was used to inoculate a 250 mL flask containing 100 mL of nutrient broth (DSM or LMG medium as reported in Table 3.1), acetic acid bacterium medium (Table 3.1) or synthetic reach medium supplemented with lactose at a final concentration of 10 g/L (substrate S1-Table 3.2). The preculture flasks were incubated on an orbital shaker at 150 rpm and 30°C for 12h and 48h for *Pseudomonas* and *Acetobacter* strains, respectively. Active cells from precultures were then employed as inoculum in shake flasks or in bioreactors (STR and PBR) containing synthetic medium with lactose (substrates S1, S2, S6, S7-Table 3.2), sterile RCW (substrate S3-Table 3.2) from bovine milk (kindly provided by Granarolo, Bologna, Italy), or permeate X and 4 (substrates S4, S5-Table 3.2) adjusted to appropriate pH (7 for *Pseudomonas* species and 6.6 for *Acetobacter*) by adding NaOH 2M before autoclaving.

3.3 BATCH EXPERIMENTS WITH SYNTHETIC LACTOSE (from Sigma-Aldrich) IN SHAKE FLASKS

3.3.1 Screening with 10 g/L of synthetic lactose in different conditions

Bioconversion experiments were performed, for the 7 bacteria strains selected, in 500 mL flasks containing 250 mL of fermentative reach broth with 10 g/L of synthetic lactose purchased from Sigma-Aldrich (substrate S1-Table 3.2), sterilized by autoclaving at 121°C for 20 minutes. The flasks were seed with 2 different inoculum size (5% vol/vol or 10 g/L of wet biomass) grown for 12 h at 30°C and 150 rpm on an orbital shaker, on 2 different preculture media (bacterium media from Table 3.1 or substrate S1 from Table 3.2), as reported in paragraph 3.2.

The cultures were incubated on an orbital shaker at 150 rpm and 30°C for up to 144 h. Samples were aseptically withdrawn after 0, 3, 6, 24, 48, 72 and 144 h of incubation for growth monitoring and quantification of produced LBA and residual lactose. The tests were conducted in duplicate as independent experiments.

3.3.2 Batch experiments with 50 g/L of synthetic lactose in different conditions

After the first screening in fermentative reach broth with 10 g/L of synthetic lactose purchased from Sigma-Aldrich (S1-Table 3.2), a bioconversion experiments with highest synthetic lactose concentration (50 g/L) were performed with the best producers strains. As culture media were employed a synthetic reach medium and a mineral salt medium (MSM), both supplemented with 50 g/L of synthetic lactose from Sigma-Aldrich (S1, S2-Table 3.2). 500 mL flasks containing 250 mL of fermentative broth were seed with 5% vol/vol as inoculum grown for 12 h at 30°C and 150 rpm on an orbital shaker, on bacterium media (Table 3.1) as preculture media, as reported in paragraph 3.2.

The cultures were incubated on an orbital shaker at 150 rpm and 30°C for up to 144 h. Samples were aseptically withdrawn after 0, 3, 6, 24, 48, 72 and 144 h of incubation for growth monitoring and quantification of produced LBA and residual lactose. The tests were conducted in duplicate as independent experiments.

3.4 BATCH EXPERIMENTS WITH LACTOSE (from Ricotta Cheese Whey) IN SHAKE FLASKS

3.4.1 Screening with Ricotta Cheese Whey

A second screening was performed with the 7 selected bacteria strains, in 500 mL flasks containing 250 mL with Ricotta Cheese Whey (RCW) (S3-Table 3.2) as substrate. The flasks were seed with 5% vol/vol as inoculum grown for 12 h at 30°C and 150 rpm on an orbital shaker, on bacterium media (Table 3.1) as preculture media, as reported in paragraph 3.2.

The cultures were incubated on an orbital shaker at 150 rpm and 30°C for up to 144 h. Samples were aseptically withdrawn after 0, 3, 6, 24, 48, 72 and 144 h of incubation for growth monitoring and quantification of produced LBA and residual lactose. The tests were conducted in duplicate as independent experiments.

3.4.2 Batch experiments with permeate X (UF-500 KDa), permeate 4 (UF-30 KDa) and concentrated lactose (NF) in different conditions

Bioconversion experiments for the best producers strains were performed with different RCW permeate as substrate: permeate X obtained after RCW ultrafiltration process (500 KDa) (S4-Table 3.2), permeate 4 obtained after permeate X ultrafiltration process (30 KDa) (S5-Table 3.2), a synthetic reach medium and a mineral salt medium (MSM), both supplemented with concentrated lactose obtained after a RCW ultrafiltration and nanofiltration processes (S6 and S7-Table 3.2), according with Fig. 3.1. 500 mL flasks containing 250 mL of fermentative broth were seed with 5% vol/vol as inoculum grown for 12 h at 30°C and 150 rpm on an orbital shaker, on LMG and DSM bacterium media (Table 3.1) as preculture media, as reported in paragraph 3.2.

The cultures were incubated on an orbital shaker at 150 rpm and 30°C for up to 144 h. Samples were aseptically withdrawn after 0, 3, 6, 24, 48, 72 and 144 h of incubation for growth monitoring and quantification of produced LBA and residual lactose. The tests were conducted in duplicate as independent experiments.

3.4 SCALE UP IN PACKED-BED REACTORS (PBR)

3.4.1 Preliminary tests for cells immobilization

Batch experiments in 500 mL shake flasks containing 250 mL of working volume (100 mL of packing volume and 150 mL of fermentative medium) were performed with the selected strains with 3 different inorganic supports: ceramic material (Vukopor), sintered glass (Biopearl) and polypropylene (Sagm500) (Fig. 3.2).

1. VUKOPOR (Lanik, Boskovice, CZ):

Technical Specification

Chemical Composition:	SiC, Al_2O_3 ,SiO_2
Bond:	Ceramic
Colour:	Grey
Porosity:	10, 20, 30 ppi
Application Temperature:	max. 1480°C
Basic Shapes:	Square, circle, rectangle
Special Shapes:	According to request from customer
Tolerance of Dimensions: Note: measured with callipers equipped with flat contact elements (46x30 mm)	+ 0,5 / - 1,5 mm (up to 99 mm) + 0,5 / - 2,5 mm (above 100 mm)

Vukopor
(Ceramic Material)

2. BIOPEARL (Haquoss):

sintered glass
molecular structure porous and adsorbent
diameter about 5mm
colour: white

Biopearl
(Sintered Glass)

3. SAGM 500 (Refill - Tech Solutions Srl. Biassono, MB):

polypropylen virgen
suspended attached growth media: 500 m^2/m^3
specific weight: 0.95 Kg/dm^3
colour: white

SAGM500 (Polypropylene)

Fig 3.2. Supports employed in the preliminary shake flasks experiments for cells immobilization

In a first batch with RCW (S3-Table.3.2) as substrate (Batch 1), 3 shake flasks with supports and 1 of positive control (without supports) were seed with 10% vol/vol as inoculum grown for 6-8 h at 30°C and 150 rpm on an orbital shaker, on 500 mL shake flasks containing 250 mL of RCW (S3-Table.3.2) as starter culture. The starter culture was seed with 5% vol/vol as inoculum grown for 12 h at 30°C and 150 rpm on an orbital shaker, on LMG and DSM bacterium media (Table 3.1) as preculture media, as reported in paragraph 3.2.

The cultures were incubated on an orbital shaker at 80 rpm and 30°C for up to 10 days. Samples were aseptically withdrawn after 0, 24, 48, and 240 h of incubation for biofilm formation monitoring and quantification of produced LBA and residual lactose.

A second batch with synthetic reach medium supplemented with concentrated lactose obtained after a RCW ultrafiltration and nanofiltration processes (S6-Table 3.2) as substrate (Batch 2), was performed using supports with biofilm from Batch 1 as inoculum.

Cultures incubation and sampling were carried out after 0, 24, 48 and 96 h as reported previously. The tests were conducted in duplicate as independent experiments.

3.4.2 PBR

Scale up in 1L-Packed Bed Reactors (PBRs) were set up for the selected strains employing Vukopor (ceramic material) and Biopearl (sintered glass) as inorganic supports, at 30°C, pH 7 and 40 mL/min of recycle rate.

PBR 1 (Vukopor):
- Nominal Volume: 500 mL;
- Working Volume: 790 mL;
- Packing Volume: 420 mL;
- Supports Volume: 40 mL;
- Total Liquid Volume: 800 mL;
- Dry weight of supports: 124,5 g;
- Wet weight of supports: 148,9 g.

PBR 2 (BIOPEARL):
- Nominal Volume: 500 mL;
- Working Volume: 790 mL;
- Packing Volume: 420 mL;
- Supports Volume: 230 mL;
- Total Liquid Volume: 600 mL;
- Dry weight of supports: 400,6 g;
- Wet weight of supports: 442 g.

Fig 3.3. PBRs data

In a first batch (Batch 1) PBRs with RCW (S3-Table.3.2) were seed with 25% vol/vol as inoculum grown for 6-8 h at 30°C and 150 rpm on an orbital shaker, on 500 mL shake flasks containing 250 mL of RCW (S3-Table.3.2) as starter culture. The starter culture was seed with 5% vol/vol as inoculum grown for 12 h at 30°C and 150 rpm on an orbital shaker, on LMG and DSM bacterium media (Table 3.1) as preculture media, as reported in paragraph 3.2. Samples were aseptically withdrawn after 0, 24, 48, 96, 120, 144, 168, 192, 240 h of incubation for biofilm formation monitoring and quantification of produced LBA and residual lactose. The tests were conducted in duplicate as independent experiments.

A second batch with synthetic reach medium supplemented with concentrated lactose obtained after a RCW ultrafiltration and nanofiltration processes (S6-Table 3.2) as substrate (Batch 2), was performed using supports with biofilm from Batch 1 as inoculum.

Cultures incubation and sampling were carried each 24 h from initial time up to 17 days of incubation. The tests were conducted in duplicate as independent experiments.

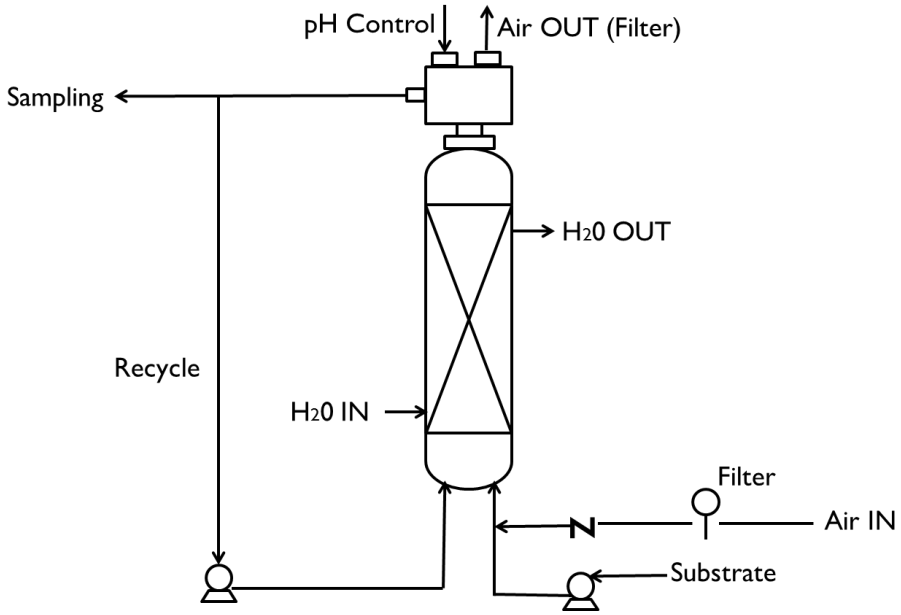


Fig 3.4. Layout of Packed Bed Reactor (PBR)

3.5 SCALE UP IN STIRRED-TANK REACTORS (STR)

3.5.1 Batch fermentations in 3L-STR

LBA production by the most promising strains was performed in a 3liter bioreactor (BIOSTAT® B Sartorius) with a working volume of 1.5 L at 30°C, thanks to thermostatically controlled water in reactor jacket, and pH 6.8-7, thanks to a computer-controlled peristaltic pumps via automatic addition of 8M NaOH and 2M H_2SO_4. Dissolved oxygen tension was maintained above 20% through a cascade control between agitation rate (200-800 rpm) and aeration rate (0.12-0.3 Lpm). Excessive foam formation was limited by automatic addition of 1:10-diluited Y-30 emulsion (Sigma-Aldrich). Fermentative broth (RCW 'S3', permeate 4 'S5' and synthetic reach medium supplemented with concentrated lactose from RCW UF and NF 'S6'-Table 3.2), were seed with 5% vol/vol as inoculum grown for 12 h at 30°C and 150 rpm on an orbital shaker, on LMG and DSM bacterium media from Table 3.1 as preculture media, as reported in paragraph 3.2.

Samples were aseptically withdrawn after 0, 3, 6, 8, 24, 30, 48, 54, 72 and 80 h of incubation for growth monitoring and quantification of produced LBA and residual lactose. This test was conducted in duplicate as independent experiments.

3.5.2 Fed-Batch fermentations in 3L-STR

A fed-batch experiments were performed in 3L-STR with permeate 4 as substrate (Table 3.2-S5), supplemented with 250 mL of Concentrated Lactose (obtained after a RCW ultrafiltration and nanofiltration processes) after 80 h of incubations. Inoculum and withdraws are similar to those reported previously (par. 3.5.1).

3.5.3 Batch fermentations in 15L-STR

Batch experiments were performed in a 15-L Stirred-Tank Reactor (BIOSTAT ® CTplus & Cplus from Sartorius) employing Pseudomonas taetrolens LMG 2336 bacteria strain, with two different working volumes (5L and 7L of sterilized RCW). The system is equipped with the measurement and control systems for steam heating (including automatic in-situ sterilization), agitation, pH-value, pO2-value, gassing of the culture vessel, foam control, high foam alarm, and level control. The connection to computer systems is possible about the built-in serial RS 422 interface. The output measured signals to a printer can be made by the built-in serial RS 232 interface.

Oxygen partial pressure (pO2) was maintained at 20% through an agitation cascade (from 200 to 800 rpm) and an aeration cascade (from 0.12 to 0.3 Lpm). pH was maintained up to 7 thanks to a computer-controlled peristaltic pumps via automatic addition of 8M NaOH and 2M H2SO4. Temperature was constantly maintained at 30°C by means of the presence of a thermostatically controlled water in the reactor jacket. Excessive foam formation was limited by automatic addition of 1:10-diluited Y-30 emulsion (Sigma-Aldrich). Inoculum levels of 10% (v/v), corresponding at 10 g/L of wet biomass, were employed from overnight precultures growth in 4 500 mL-shake flasks contained 250 ml of reach medium each one (for a total of 1 L), in an orbital shaker at 30°C and 150 rpm. Samples were aseptically withdrawn after 0, 24 and 48 h of incubation for quantification of produced LBA and residual lactose. 15L-STR Bioreactor cultivations with ricotta cheese whey medium RCW was conducted in duplicate as independent experiments.

3.6 DOWNSTREAM PROCESS FOR LBA RECOVERY

3.6.1 High-Performance Tangential Flow Filtration

Coltural broth, after fermentation process for lactose oxidation to LBA, has been pretreated for removing residual cell by centrifugation step (8000 rpm, 10 min, 4˚C).

The bench-scale cross flow unit (from PS Prozesstechnik GmbH) is designed for testing membranes and membrane filtration applications in the laboratory. Two membrane housing are installed:

- Housing for 1,8" spiral wound membranes (min. working volume =1000 ml);
- Housing for 200 cm2 flat sheet membranes (min. working volume=250 ml).

The tested solution is contained in a double walled tank, and is pumped either through the one or the other membrane module with a diaphragm pump (pump controlled with a frequency converter; flow range: 1,8-12 L/min (=15-100% on the regulation knob); pressure range: max 40 bar; as it is a volumetric pump, the flow rate Q [L/min] is proportional to the knob position KP [%]: Q=0.12xKP). The pressure is adjusted with a spring loaded pressure regulating valve. The operating temperature is regulated automatically, via a PID temperature controller and a magnetic valve in the heating/cooling medium line. The unit works batchwise. The operating limits are 0-40 bar and 0-60˚C. The main process parameters (pressure, temperature) are measured and displayed. All the components are fixed on a stainless steel base plate, and the unit has a stainless steel control cabinet with the electronics, power supply of pump and instruments. The system is protected against overpressure and overtemperature. The unit is compatible with aqueous systems and alcohols. All medium wetted metallic parts are made of stainless steel 316L or equivalent (1.4435; 1.4404). All parts and pipes are fixed on a stable stainless steel frame (304-1.4301).

There are three ways to operate the unit:

- OPERATION MODE "RECYCLING": the permeate hose goes back to the feed vessel , that means the product concentrations remain constant over time. In this way, one determines the influence off different operating parameters on permeate flux and retention at a given constant concentration. After the start up, the unit is in the recycling mode.
- OPERATION MODE "CONCENTRATION": the permeate is collected in a separate vessel. The level in the feed vessel decreases and the concentration of the retained soluble components increases. Starting from the recycling mode, the end of the permeate hose is put into an external vessel (on a scale and tare) for collecting permeate and when the desired mass of permeate has been removed, the concentration is stopped by putting the permeate hose back to the feed vessel (recycling mode).
- OPERATION MODE "DIAFILTRATION": the permeate leaves the membrane module (collected in a separate vessel) and is replaced manually with pure water (or solvent) so that the level in the feed vessel remains constant. This procedure is used to wash out components with low retention (small molecular weight). The diafiltration is stopped when the desired

quantity of water has been added, by putting the permeate hose back to the vessel (recycling mode).

Table 3.3 shows the characteristics of the tubular ceramic membrane employed (atech Al_2O_3-membrane; single-duct design, type 10/6)

SUPPORT MATERIAL	MEMBRANE MATERIAL	TYPE	OVERALL LENGTH	OUTER DIAMETER	DIAMETER OF DUCT	AMOUNT OF CHANNELS
α-alumina oxide	UF:TiO_2	Single-duct design	1.000 mm	10±0.5 mm	Approx. 6 mm	1

FILTERING SURFACE	MAXIMUM PRESSURE	pH value	MEAN PORE DIAMETER
Approx. 0.019 m^2	15 bar	0 to 14 with 80-90˚C	1 kDa

Table 3.4. Characteristics of HPTFF membrane

Three tests have been performed at 30°C and 14-15 bar at different pH values: pH 3 (test 1), pH 9 (test 2) and pH 5.44 (test3) with 1L of raw material initial volume. For each test a cycle of filtration and a cycle of diafiltration have been performed; bringing to a final volume of filtrate (500 mL), diafiltrate (500 mL) and concentrate (500 mL) and monitoring the volume of permeate with time and the concentration in the feed, retentate and accumulated permeate of LBA and lactose.

3.7 ANALYTICAL METHODS

1. Bacterial growth was monitored by cell counts using the drop plate method (Herigstad et al., 2001) and by the optical density measurement after growth in synthetic medium or RCW, respectively.

2. Specific growth rate (μ) was estimated from CFU/ml values using the following equation:

$$\mu = \ln \frac{CFU/ml_t}{CFU/ml_{t-1}} * \Delta t^{-1} \qquad \mu = \ln \frac{OD600_t}{OD600_{t-1}} * \Delta t^{-1}$$

where CFU/ml_t and CFU/ml_{t-1} are the CFU/ml values at two time intervals, $OD600_t$ and $OD600_{t-1}$ are the OD600 values at two time intervals and Δt is the time interval between the two measurements.

3. The pH was measured using a pH meter (Thermo Orion Model 720A, Research Inc., Beverly, MA, USA) and a pH probe (Orion 81-04).

4. Quantification of the produced lactobionic acid and residual lactose was performed by high performance liquid chromatography (HPLC). The samples were previously centrifuged to remove cell debris (14.000 rpm for 10 min) and filtered through a cellulose acetate membrane 0,22µm-25 mm filter (GVS Filter Technology). HPLC analyses were carried out in a liquid chromatography system (Agilent 1260 Infinity) equipped with an Agilent Hi-Plex Ligand Exchange Columns (H+) 300 x 7,8 mm set at 75°C, coupled to a refractive index detector at 40°C. The mobile phase consisted of a 0.1 mM H_2SO_4 (pH 3.4) solution at a flow rate of 0.5 mL/min. Samples were quantified according to HPLC-grade external analytical standards, lactose and lactobionic acid, obtained from Sigma-Aldrich (Milan, Italy). Data acquisition and analysis were performed with ChemStation software (Agilent).

5. The operating parameters for High-Performance Tangential Flow Filtration must be chosen according to the medium to be filtered.

$c=V/A$ [m/s] where:

c=Velocity inside membrane ducts (approx. 4-6 m/s); V=Feed volume flow; A=Surface of ducts flown past.

$\Delta p= [(P1+P2)/2] – Pp$ [bar] where:

Δp=Trans-membrane pressure (approx. 1.5-3 bar); P1=Pressure at entry of module; P2=Pressure at exit of module; Pp=Pressure of permeate.

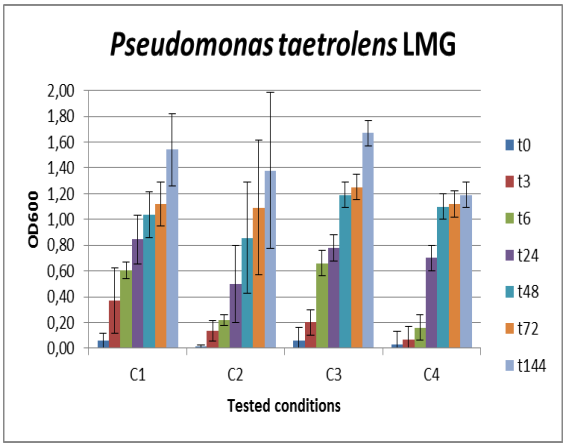
4. RESULTS AND DISCUSSIONS

4.1 BATCH EXPERIMENTS WITH SYNTHETIC LACTOSE (from Sigma-Aldrich) IN SHAKE FLASKS

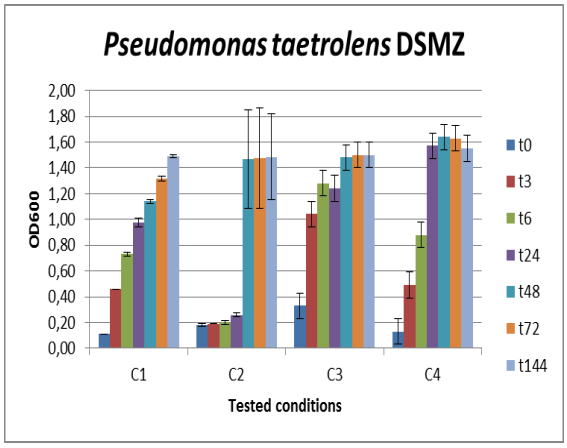
4.1.1 Screening with 10 g/L of synthetic lactose in different conditions

In the first step, the ability of the seven strains tested in this study to grow and perform the oxidation of the lactose to LBA was evaluated on synthetic medium containing 10 g/L of chemically pure lactose purchased from Sigma-Aldrich. Four conditions were tested: C1 (DSMZ or LMG medium as preculture medium and 5% vol/vol of inoculum), C2 (DSMZ or LMG medium as preculture medium and 10 g/L of wet biomass as inoculum), C3 (synthetic reach medium containing 10 g/L of lactose purchased from Sigma-Aldrich as preculture medium and 5% vol/vol of inoculum), C4 (synthetic reach medium containing 10 g/L of lactose purchased from Sigma-Aldrich as preculture medium and 10 g/L of wet biomass as inoculum). The microbial growth, evaluated by drop-counting and optical density at 600 nm, revealed that all *Pseudomonas*' strains were able to grow in synthetic medium (Fig. 4.1a, 4.1b, 4.1c, 4.1d, 4.1e). In particular maximum OD600 values of 1.6±0.1-1.8±0.1 were reached after 72-144 h, corresponding to a maximum cell density of up to 10^9 CFU/mL starting from a cell concentration of 10^7 CFU/ml , and no significant differences were revealed between the 4 conditions.

a)



b)



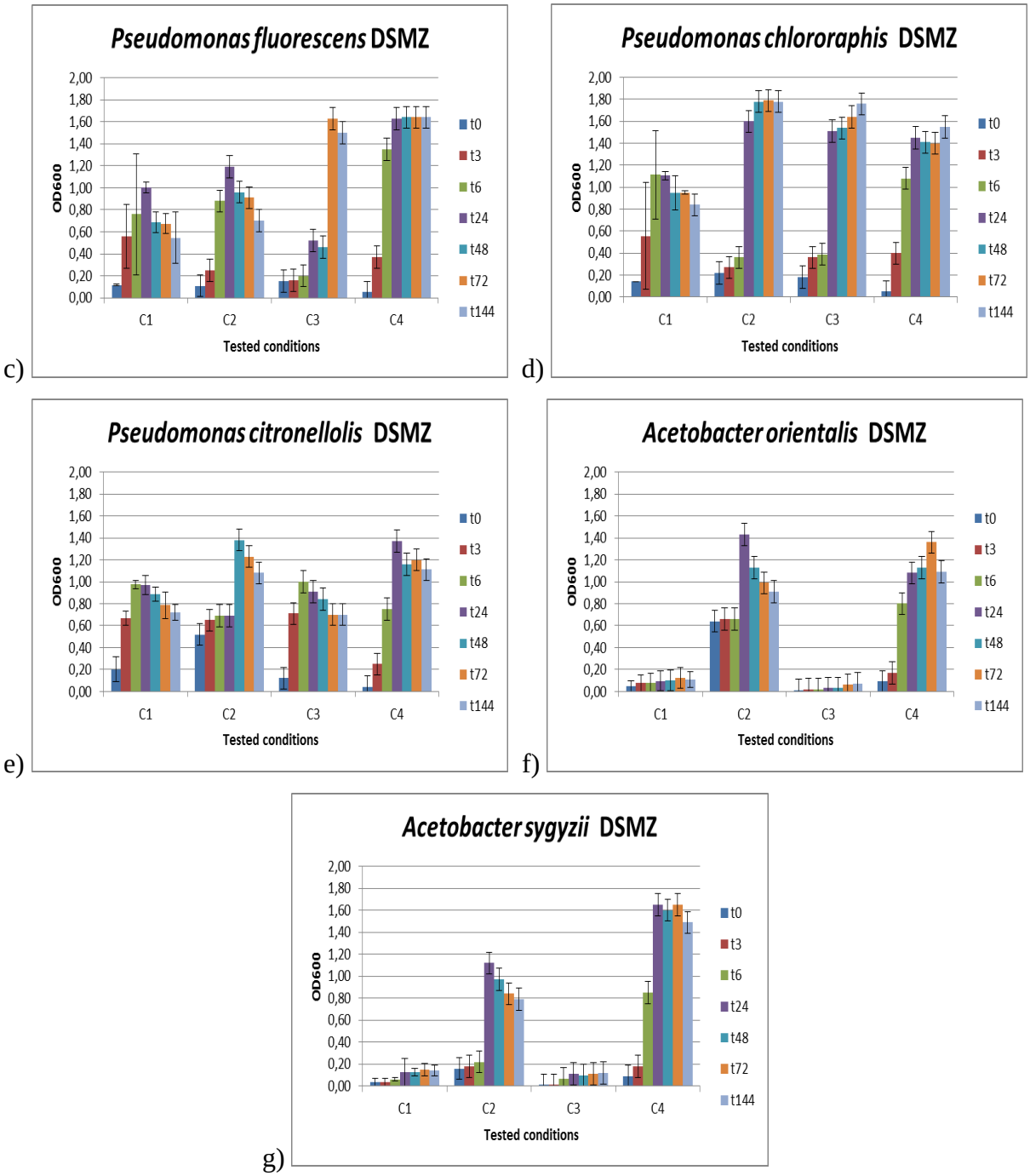


Fig. 4.1. Growth values (OD600 nm) of *P. taetrolens* LMG (a), *P. taetrolens* DSM (b), *P. fluorescens* DSM (c), *P. choloraphis* DSM (d), *P. citronellolis* DSM (e), *A. orientalis* DSM (f), *A. sygyzii* DSM (g) in synthetic reach medium with 10 g/L of chemically pure lactose (Sigma) as substrate, in 4 different conditions of preculture media and inoculum (C1, C2, C3, C4).

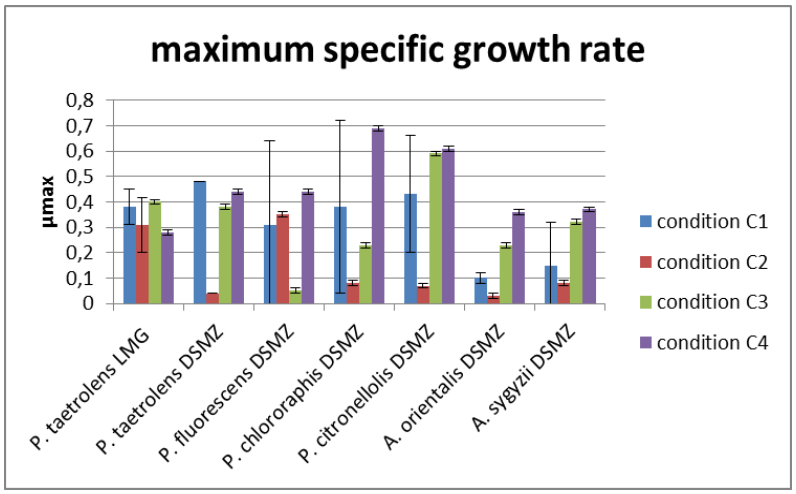


Fig. 4.2. Growth rate (µmax) [h-1] of the seven bacterial strains after 3-6 hours of incubation shake flasks with synthetic reach medium with 10 g/L of chemically pure lactose (Sigma) as substrate, in 4 different conditions of preculture media and inoculum (C1, C2, C3, C4).

The maximum specific growth rate (µmax) was reached after 3-6 hour of incubation (Fig. 4.2), with the highest of 0.69±0.01/h exhibited by *P. chlororaphis* DSM. A slower growth was observed for *A. orientalis* which showed a µmax of 0.03±0.01/h reached between 6h and 24h of incubation.

The pH-measurements revealed a significant rise of the broth pH upon growth of *P. citronellolis* and the two *Acetobacter* reaching values of up to 8.8±0.1-9.26±0.1, starting from 7±0.1 and 6.8±0.1 respectively; while for *P. chlororaphis* was observed an increase in the case of condition C1 (DSMZ medium as preculture medium and 5% vol/vol of inoculum), from 6.8±0.1 to 8.8±0.1. (Fig. 4.3).

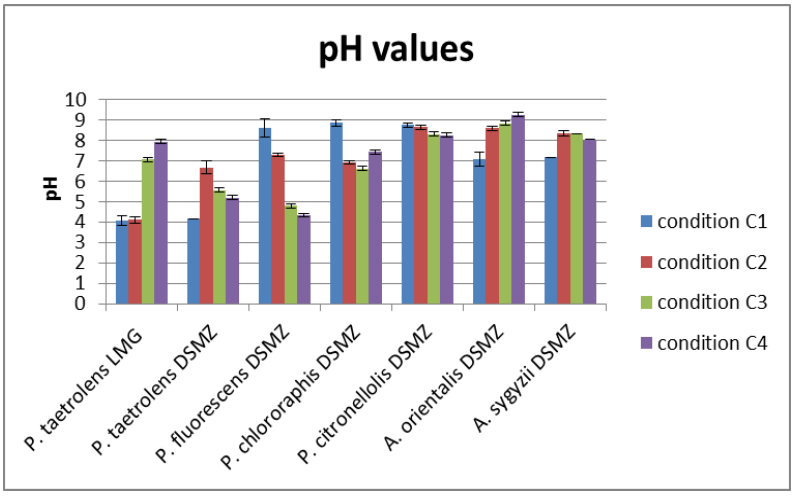


Fig. 4.3. pH measurement of the seven bacterial strains after 72-144 hours of incubation in shake flasks with synthetic reach medium with 10 g/L of chemically pure lactose (Sigma) as substrate, in 4 different conditions of preculture media and inoculum (C1, C2, C3, C4).

The appearance of nitrogen by-products from metabolized proteins during bacterial growth could be the reason of the increase of the pH. The rise of the broth pH was observed also with *P. taetrolens* LMG 2336 by Alonso et al. (2011) employing 0.64 g/L of biomass (about 30% v/v) as inoculum into protein-rich sweet cheese medium in shake flasks (Alonso et al., 2011). However, under the experimental conditions applied here, in the case of *P. taetrolens* strains we did not observe a significant rise of the broth pH which remained almost constant during the growth (around 7). The broth acidification was observed after 24 h of incubation reaching the lowest values of pH 4.07±0.23 and pH 4.15±0.01 for the strain *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104, respectively, after 48-72 h of incubation.

The HPLC-measurement performed on cell-free culture supernatants revealed important difference between the tested conditions and the selected strains, confirming the pH decrease. It showed how the two *Pseudomonas taetrolens* strains, when DSM or LMG medium as preculture medium and 5% vol/vol of inoculum were used (condition C1), were able to produce 6.73±1.9 (conversion yield=76±6%) and 7.69±0.04 g/L (conversion yield=90±1%) of LBA by strains LMG 2336 and DSM 21104, respectively, after 72h and 48h of incubations (Fig.4.4). These values remained constant after 144h of incubation.

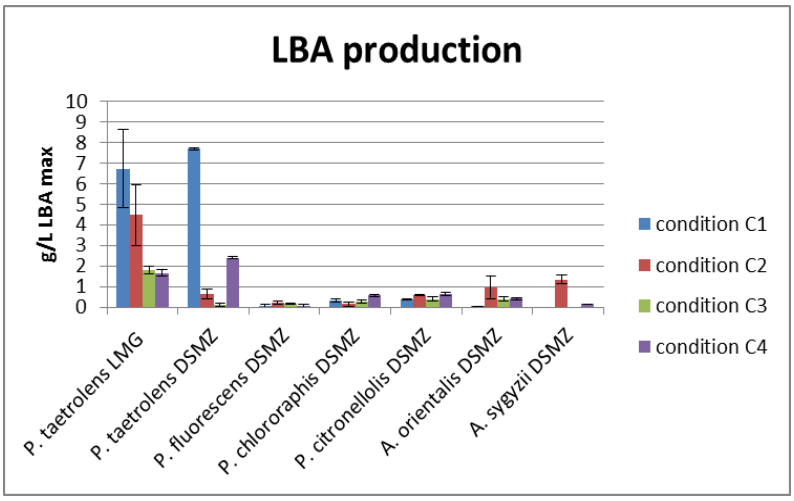


Fig. 4.4. Maximum LBA product [g/L] of the seven bacterial strains after 48-72 hours of incubation in shake flasks with synthetic reach medium with 10 g/L of chemically

pure lactose (Sigma) as substrate, in 4 different conditions of preculture media and inoculum (C1, C2, C3, C4).

Although the other *Pseudomonas* and *Acetobacter* species were able to grow, no lactose consumption and LBA production were detected under the experimental conditions applied here.

P. fluorescens presented a particular behaviour when synthetic reach medium containing 10 g/L of lactose purchased from Sigma-Aldrich was used as preculture medium (conditions C3 and C4), because the pH decreased up to 4.8±0.1 and 4.3±0.1, respectively, but no LBA production was detected, probably due to the formation of others acids compounds.

The obtained results confirm the ability of bacteria from the species *P. taetrolens* to produce lactobionic acid from lactose and are in agreement with those previously reported for *P. taetrolens* where a conversion yield of 0.75 in shake-flask culture was observed after 165 h (Stodola and Lookwood, 1947).

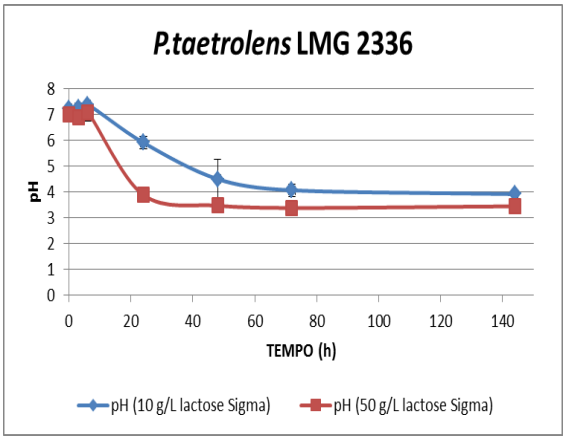
This preliminary screening led to the identified of the best conditions in the use of DSM or LMG medium as preculture medium and 5 % vol/vol as inoculum (condition C1), probably due to a reduction of cellular stress in the process of biomass recovery and limitation of competitive phenomena.

4.1.2 Batch experiments with 50 g/L of synthetic lactose in different conditions

Based on these results, the two *P. taetrolens* strains were tested for growth and LBA production in the presence of 50 g/L of pure lactose in order to mimic the concentration in the RCW.

With synthetic reach medium, for both strains, the higher lactose amount had not a consistent influence on maximum specific growth rate μmax (evaluated by OD600) that is less than the values of previously experiments (with 10 g/L Sigma lactose). Indeed the maximum specific growth rate, achieved after 3 hours of incubations, of 0.36±0.21 h^{-1} and 0.40±0.07 h^{-1} for *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104, respectively, are similar to the results obtained with little amount of lactose (0.38±0.07 h^{-1} and 0.48±0.01 h^{-1} for *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104, respectively). Similar considerations can be made for pH values since it has been revealed a significant reduction of pH from 7±0.15 up to 3.44±0.08 and 3.2±0.06 was observed in the case of *P. taetrolens* LMG 2336 and of *P. taetrolens* DSM 21104, respectively (Fig. 4.5a and 4.5b). The drop of the pH value led to a decrease of cells vitality, as can be seen with the cells density; the maximum values were reached after 24

hours (10^7 CFU/mL from initial 10^6 CFU/mL and 10^8 CFU/mL from initial 10^7 CFU/mL for *P.taetrolens* LMG 2336 and *P.taetrolens* DSM 21104, respectively). Afterwards, it progressively fall down and no growth, on agar plates for drop counting, were observed until up to 144 h of incubation.

a) 

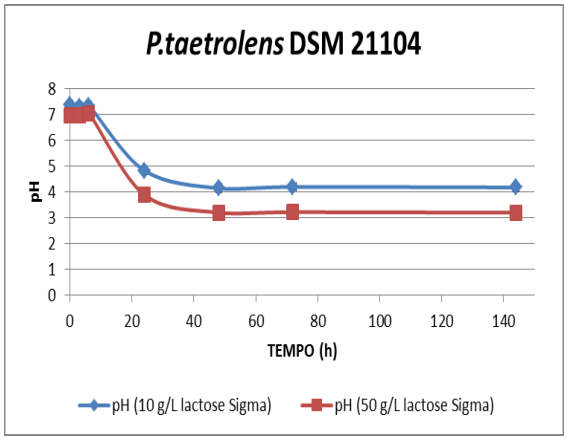
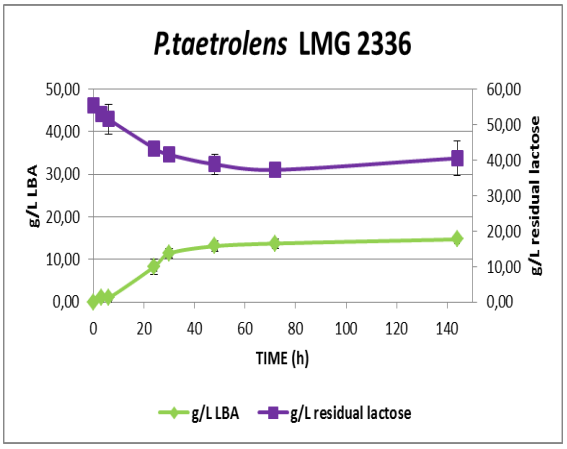
b) 

Fig. 4.5. pH values for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with synthetic reach medium with 10 and 50 g/L of chemically pure lactose (Sigma) as substrate, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

But the results showed an inhibitory effect of high lactose quantity on oxidized ability of *P. taetrolens* strains; after 48 hours of incubations only 13.16±1.25 g/L (conversion yield=80±31%) and 18.61±0.01 g/L (conversion yield=67±3%) of LBA have been detected for *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104, respectively. An increase of incubation time did not led to an improve of the yield and a large quantity of lactose remains unused from initial 55 g/L; in particular 40.56±4.95 and 32.86±1.18 g/L of lactose for *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104, respectively, after 144 hours of incubations (Fig. 4.6a and 4.6b).

a) 

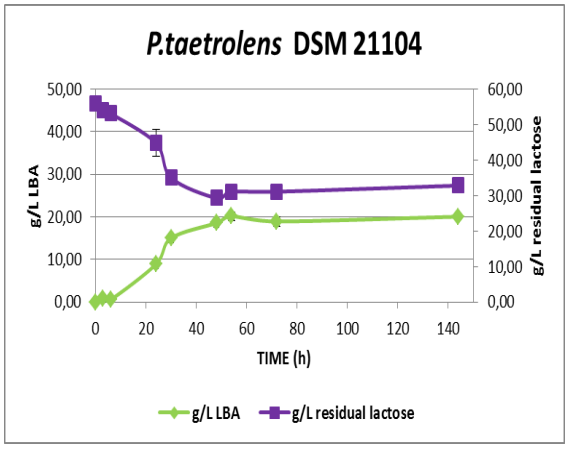
b) 

Fig. 4.6. LBA product and residual lactose [g/L] for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with synthetic reach medium with 50 g/L of chemically pure lactose (Sigma) as substrate, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

This effect may be due to the pH drop that resulted in the decrease of the cell viability and the inhibition of the enzymes (lactose oxidase and lactonase) involved in the process of LBA production from lactose. Alonso et al. (2012) reported indeed, that in shake-flask fermentation, a progressive loss of cellular metabolic activity, membrane polarization and integrity was observed during the lactobionic acid production phase (stationary phase). This phase involved a morphological changes in Gram negative bacteria (Navarro et al., 2010) that combined with higher stressing level conditions led to a drop in bioconversion performance (David et al., 2011).

When mineral salt medium (MSM) added with 50 g/L of synthetic lactose (Sigma) was employed, the selected strains showed a sharp fall of the growth and production capabilities, although the pH values were very low. In particular for *P. taetrolens* LMG 2336 after 144 hour of incubations at 30°C and 150 rpm, 2.03±0.1 g/L of LBA were produced (conversion yield=23±1.6%), corresponding to 3.24±0.1 of pH and 0.19±0.01 of OD600 (Fig. 4.7a). Instead *P. taetrolens* DSM 21104, in the same conditions, presented a higher value of OD600 (0.67±0.04) and similar value of pH (3.27±0.14), without significant LBA production (0.71±0.03 g/L with 31±2.4 of conversion yield) (Fig. 4.7b). The pH decrease without LBA suggested that probably others acid compounds were produced, like gluconic acid, as reported by Alonso et al., 2015, when *P. taetrolens* LMG 2336, in a substrate with both lactose and glucose, produced both LBA and gluconic acid (Alonso et al., 2015).

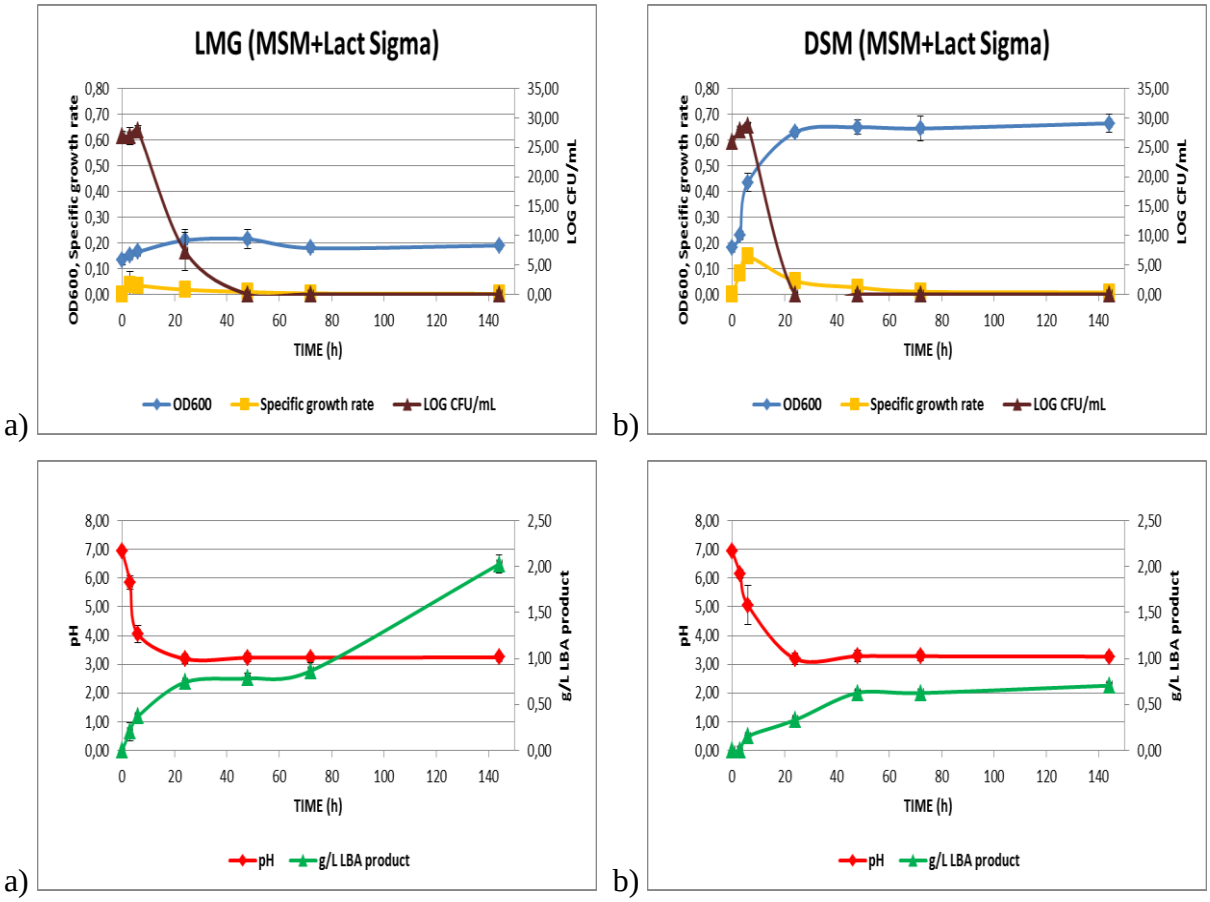


Fig. 4.7. OD600, specific growth rate (μ) [h^{-1}], LOG CFU/mL, pH values and LBA product [g/L] for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with mineral salt medium and 50 g/L of chemically pure lactose (Sigma) as substrate, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

4.2 BATCH EXPERIMENTS WITH LACTOSE (from Ricotta Cheese Whey) IN SHAKE FLASKS

The first screening led to the identification of the best preculture and inoculum conditions (bacterium medium preculture and 5%vol/vol of inoculum), and the selection of the most promising bacteria strains for LBA production via lactose oxidation (chemically pure lactose from Sigma-Aldrich). After this, different experiments were performed in shake flasks employed lactose from dairy industry wastes as substrates for LBA production, in order to investigate the effects of a natural matrix, compared to a synthetic one, on cells vitality and bioconversion performances.

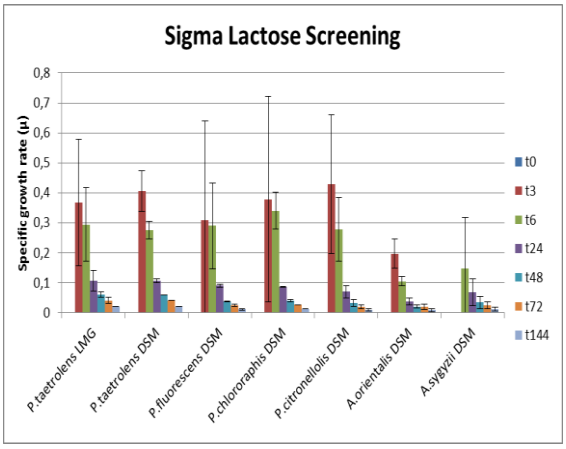
4.2.1 Screening with Ricotta Cheese Whey

Although the first screening in fermentative broth added with chemically pure lactose (Sigma) showed how only *P. taetrolens* strains (LMG 2336 and DSM 21104) were able to produce LBA, a second screening with the 7 bacteria strains were performed in shake flasks with RCW, having a lactose content of about 40-45 g/L, to confirm or disprove the pervious results.

With the exception of the two *P. taetrolens* strains, the other three *Pseudomonas* strains (*Fluorescens* DSM, *Choloraphis* DSM and *Citronellollolis* DSM) and *Acetobacter sygyzii* DSM presented higher values of growth rate (μmax) after 3 hours of incubations when fermentative broth with 10 g/L of chemically pure lactose (Sigma) was employed as substrate, probably as a consequence of substrate inhibition phenomena when RCW with higher lactose concentration was employed. Instead a slow improvement in specific growth rate value was observed for *Acetobacter orientalis* DSM after 6 hours of incubation with RCW as substrate: 0.17±0.12 h^{-1} in comparison to 0.10±0.02 h^{-1} with 10 g/L of chemically pure lactose (Sigma) (Fig. 4.8a and 4.8b).

However a similar increase in cell density was observed for the same five strains, in both two screening: from 10^7 to 10^8 CFU/ml and from 10^6 to 10^7 CFU/ml for the three *Pseudomonas* and the two *Acetobacter* stains, respectively, in the first screening (Sigma Lactose); and from 10^8 to 10^9 CFU/ml for all five strains with the second screening (RCW), probably thanks to a positive effect, on cells membrane integrity, of microelements present in a natural matrix.

a)



b)

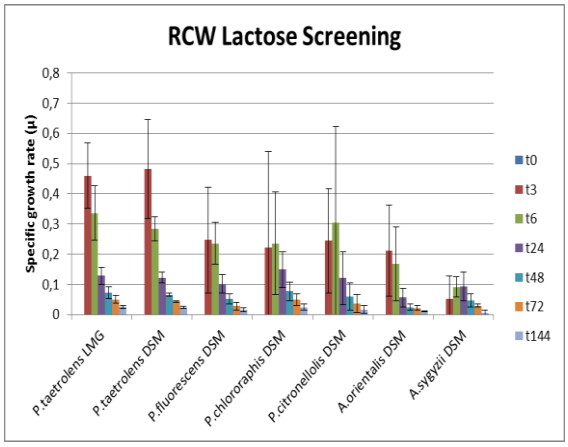
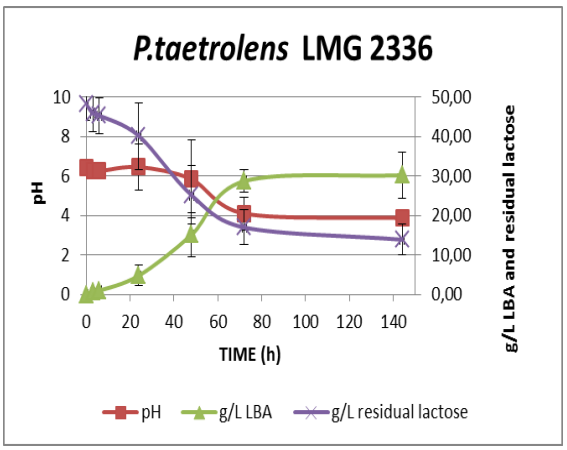


Fig. 4.8. Comparison of specific growth rate values (μ) [h^{-1}] between first screening with synthetic reach medium with chemically pure lactose (Sigma) as substrate (a) and second screening with RCW as substrate (b), in shake flasks, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

Instead a slight increase on growth (µmax) of *P. taetrolens* species was detected after 3 hours of incubation at 30°C and 150 rpm in shake flask with RCW: 0.46±0.11 h^{-1} and 0.48±0.16 h^{-1} for *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104, respectively, in comparison 0.37±0.21 h^{-1} and 0.41±0.07 h^{-1} in fermentation broth added with 50 g/L of chemically pure lactose (Sigma).

Both *Pseudomonas* taetrolens strains reached stationary growth phase based on cell counts ($1.75x10^9$ and $1.15x10^9$ CFU/mL from $1.9x10^7$ and $1.99x10^7$ initial CFU/mL for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively). The cell density remains constant up to 48h of incubation at 30°C and 150 rpm, after which a drop in the cell counts from 10^9 to 10^6 CFU/mL was observed. The pH of the culture medium remains almost constant during the first 24 h of incubation for both strains. Afterwards, it progressively drops from 6.42±0.01 to 3.87±0.35 and from 6.4±0.01 to 4.51±0.55 for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively, after 72 h of incubation (Fig. 4.9a and 4.9b). Evaluation of LBA production revealed the detection of low concentrations of LBA after 24h of incubation that increases with increasing the incubation time to reach 28.7±2.8 g/L and 27.87±1.12 g/L for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively, after 72h of incubation (Fig. 4.9a e 4.9b).

a)



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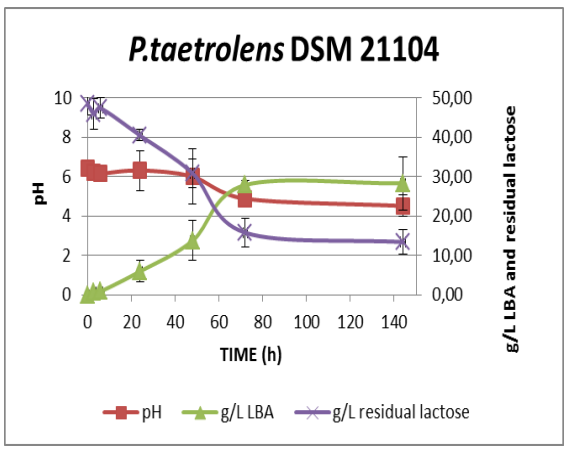


Fig. 4.9. pH values and LBA product and residual lactose [g/L] for *P.taetrolens* LMG 2336 (a) and *P.taetrolens* DSM 21104 (b) in shake flasks with RCW as substrate, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

In terms of yield (defined as the percentage of lactose converted into lactobionic acid), the highest values of 87.8±2.75% and 77.5±1.56% were obtained after 72 h of incubation for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively. Further prolongation of the incubation time to 144h did not result in significant improvement of the productivity but in a decrease of the yield up to 84±2.75% and 72±2.22% for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively.

It is interesting to highlight that, using a similar concentration of chemically pure lactose (Sigma 61345 D-Lactose monohydrate), lower titres of LBA were achieved by both strains. Indeed, as it can be appreciated from figure Fig. 4.6a and 4.6b (50 g/l of chemically pure lactose from sigma with *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSMZ 21104); from an initial concentration of 55 g/l of lactose, maximal fermentation yields of 14 and 20 g/l of LBA were obtained from *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104, respectively with a residual lactose concentration of 40 and 32 g/L. This could be due to the presence in RCW of enzymatic cofactors or compounds that enhance the bioconversion rate and/or protect the bacterial cells from the effects of low pH, as can be seen from the improve in cell density (CFU/mL), in particular at low pH values. With 50 g/L of Sigma Lactose both *P. taetrolens* LMG 2336 and DSM 21104 were showed no cellular growth after 48 hours, when the pH value was less than 3.5; but with RCW medium the buffer effect due to the presence of particular components led to a gradual pH decrease restricting cellular stress conditions (Alonso et al. 2013).

4.2.2 Batch experiments with permeate X (UF-500 KDa) and permeate 4 (UF-30 KDa)

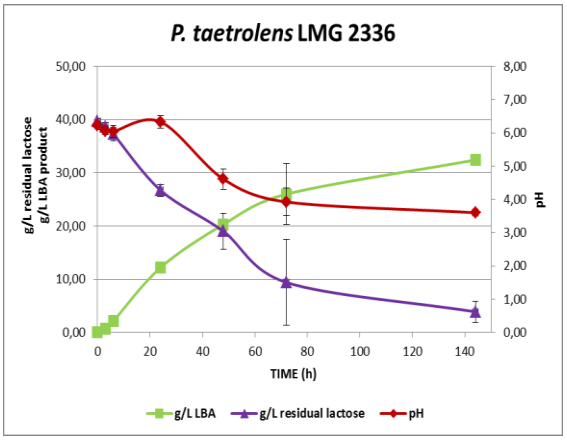
The second screening with RCW confirmed how *P. taetrolens* strains were the most promising LBA producers employing lactose from a dairy industry waste.

The lowly amount of whey proteins and peptides presented in the RCW (Table 3.3) suggested the possibility of developing an integrated biorefinery process (Fig. 3.1), based on ultrafiltration processes, for the enhancement of these bioactive molecules. Therefore, bioconversion experiments using the permeated fractions were carried out in shake flasks to evaluate the ability of the selected strains to produce LBA: permeate X, obtained from the UF-500KDa of RCW for the removal of serum proteins, and permeate 4, obtained from the UF permeate of 30KDa-X for the peptides removal.

With permeate X, both *P. taetrolens* LMG 2336 and DSM 21104 achieved 4.72 $x10^9$ and $6.03x10^9$ CFU/mL after 48 h of incubation at 30°C and 150 rpm, but *P. taetrolens* DSM 21104 was presented higher maximum specific growth rate (μmax) of 0.48±0.04 h^{-1} in

comparison of 0.17±0.02 h^{-1} for *P. taetrolens* LMG 2336 after 3 h. The longer exponential growth phase of *P. taetrolens* DSM 21104, also reported by a constant value of pH up to 48 h compared to 24 h for *P. taetrolens* LMG 2336, had a mild negative effect on bioconversion yield: 29.22±4.04 g/L (83±0.6% conversion yield) and 32.43±0.35 g/L (87±3% conversion yield) after 144h of incubation for DSM 21104 and LMG 2336 strains respectively (Fig. 4.10a e 4.10b).

a)



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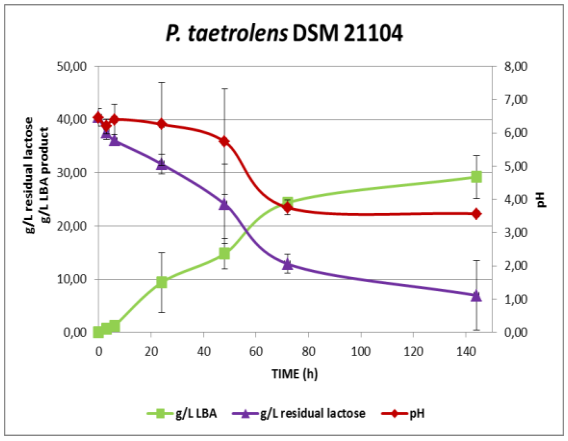
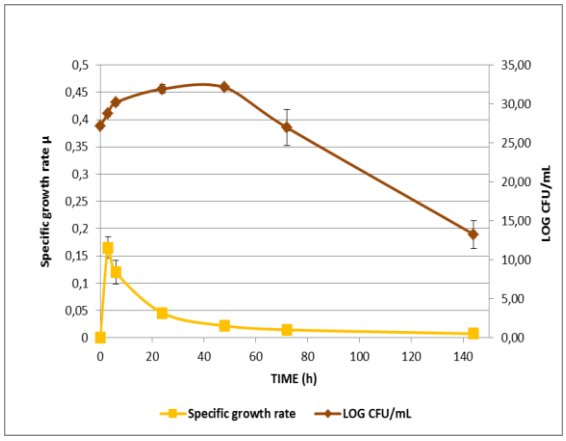


a)



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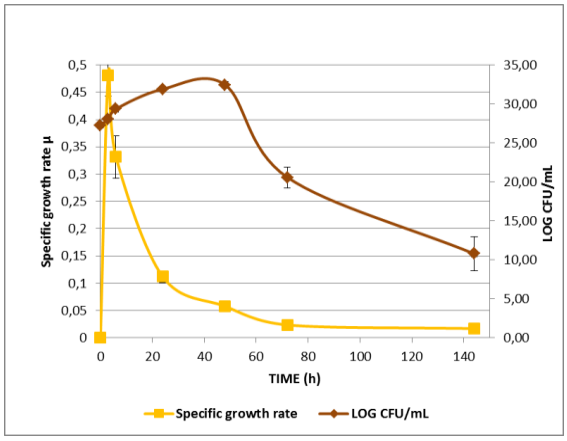


Fig. 4.10. Specific growth rate (µ) [h^{-1}], LOG CFU/mL, pH values, LBA product and residual lactose [g/L] for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with permeate X (UF-500KDa) as substrate, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

Similar behaviur was detected for *P. taetrolens* DSM 21104 also with permeate 4 as substrate: 4.79x10^9 CFU/mL after 48 h of incubation at 30°C and 150 rpm, starting from 1.13x10^8 CFU/mL; maximum specific growth rate (µmax) of 0.4±0.04 h^{-1}, in comparison of 0.16±0.01 h^{-1} for *P. taetrolens* LMG 2336 after 3 h, and 27.4±4.04 g/L of LBA product after 144 h of

incubation with 0.86±0.08 of conversion yield. While the growth of *P. taetrolens* LMG 2336 was similar between the employment of permeate X and permeate 4 as substrates, the LBA production presented significant improvements in the second case: 36.57±4.17 g/L (89±11% of conversion yield) after 144 h of incubation (Fig. 4.11a and 4.11b). This is probably due to a macromolecules removal with an ultrafiltration at 30KDa step, that affected enzymes involved in lactose oxidations (paragraph 1.2.3; page 24).

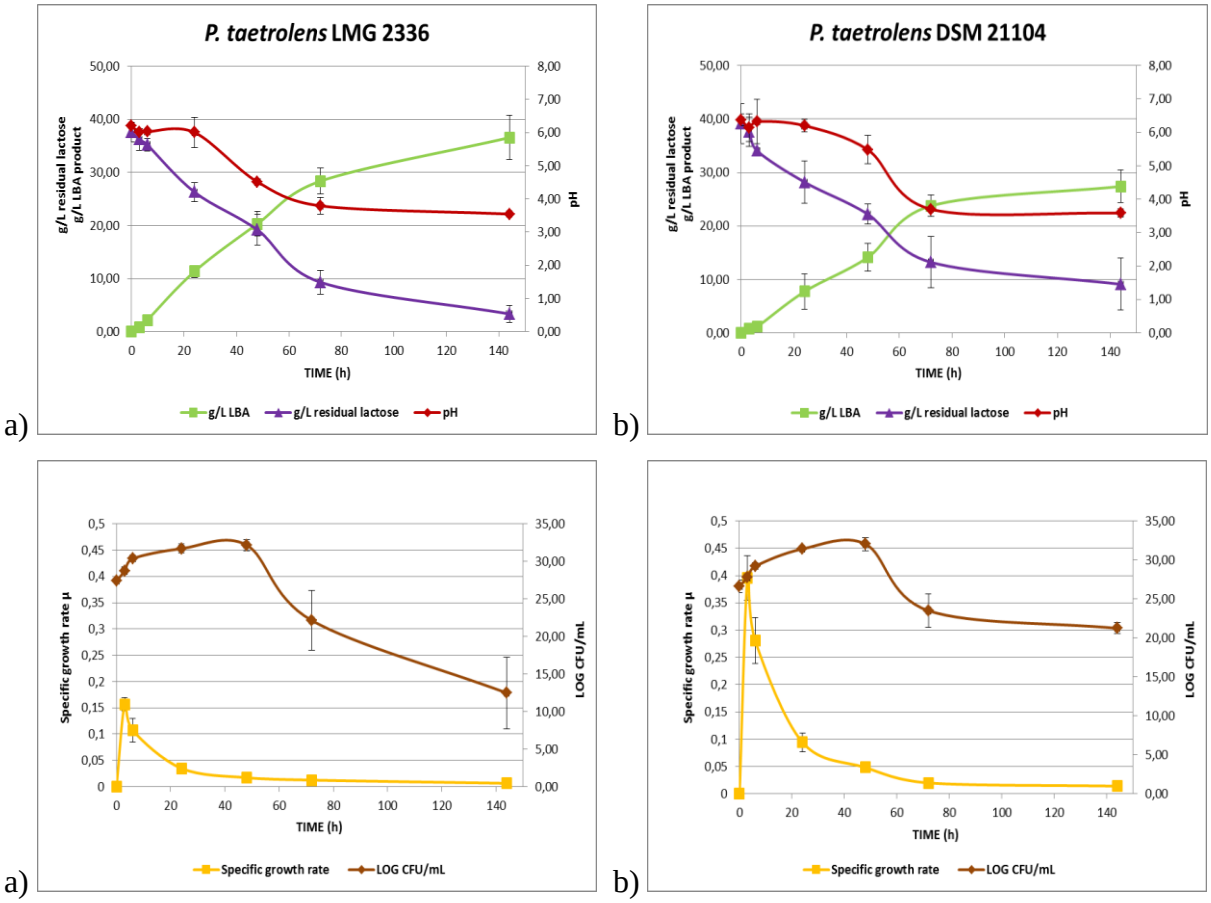


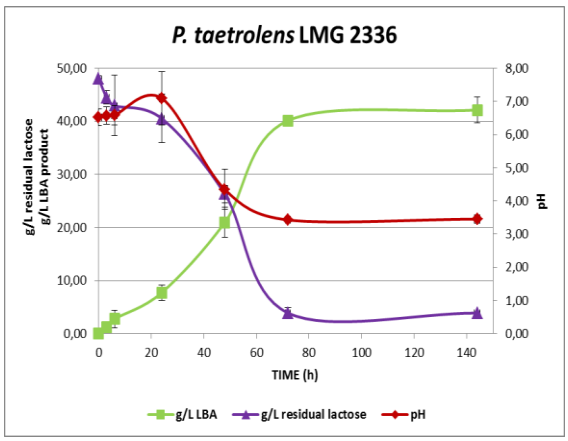
Fig. 4.11. Specific growth rate (μ) [h^{-1}], LOG CFU/mL, pH values, LBA product and residual lactose [g/L] for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with permeate 4 (UF-30KDa) as substrate, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

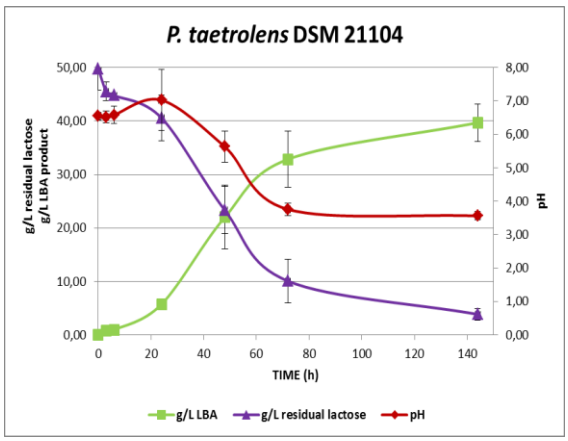
4.2.3 Batch experiments with 50 g/L of concentrated lactose (NF) in different conditions

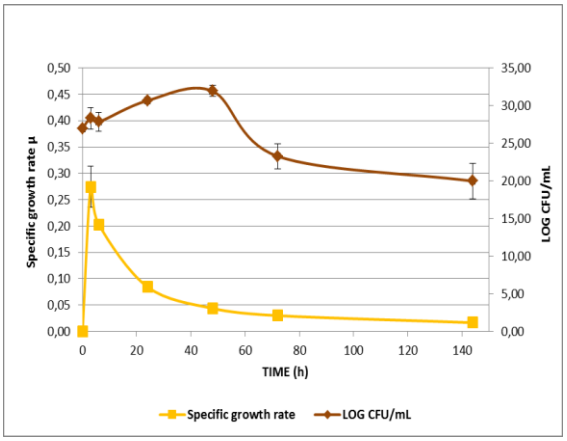
A final nanofiltration step of the biorefinery process (Fig. 3.1) was performed on permeate 4 for achieving a concentrated lactose (250 g/L). In order to evaluate difference between chemically pure lactose and bio-based lactose bioconversion experiments were carried out, in different conditions: synthetic reach medium and mineral salt medium (MSM) employing

concentrated lactose as substrate for LBA production up to a final concentration of 50 g/L, in order to mimic the lactose concentration in RCW, permeate X, and permeate 4 (paragraph 4.2.1 and paragraph 4.2.2).

When synthetic reach medium supplemented with 50 g/L of concentrated lactose was used, maximum cell density of $2.15x10^9$ CFU/mL after 48 h of incubation at 30°C and 150 rpm, was achieved for *P. taetrolens* LMG 2336, starting from $1.39x10^8$ CFU/mL. Instead for *P. taetrolens* DSM 21104 the maximum cell density was reached just after 24 h: in particular $4.35x10^9$ CFU/mL, starting from $6.84x10^7$ CFU/mL. Different growth performance for both *P. taetrolens* strains, in comparison with shake flasks experiment with RCW, permeate X and permeate 4, was showed with the maximum specific growth rate (µmax) values: 0.27±0.04 h^{-1} and 0.19±0.02 h^{-1} for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively, as also reported in Table 4.1. LBA detection presented high value for *P. taetrolens* LMG 2336, in particular 40.14±0.86 g/L with 91±6% of conversion yield, after 72h of incubation at 30°C and 150 rpm, consuming all the lactose initially presents in the medium, and a prolongation of incubation time up to 144h did not presents significant improvement in bioconversion performance. Also *P. taetrolens* DSM 21104 produced 39.68±3.5 g/L of LBA (83±2% of conversion yield) after 144h of incubation in the same condition. pH, starting from 7 for both strains, achieved very low values after 144h of incubation: 3.55±0.02 and 3.59±0.12 for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively (Fig. 4.12a and 4.12b). This low values of pH affected cell integrity and the bioconversion performance, so a constant value of pH may be improved LBA production, both as time and as yield.





a) 

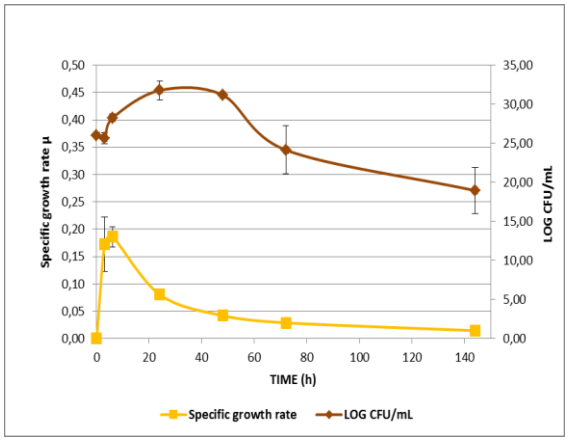
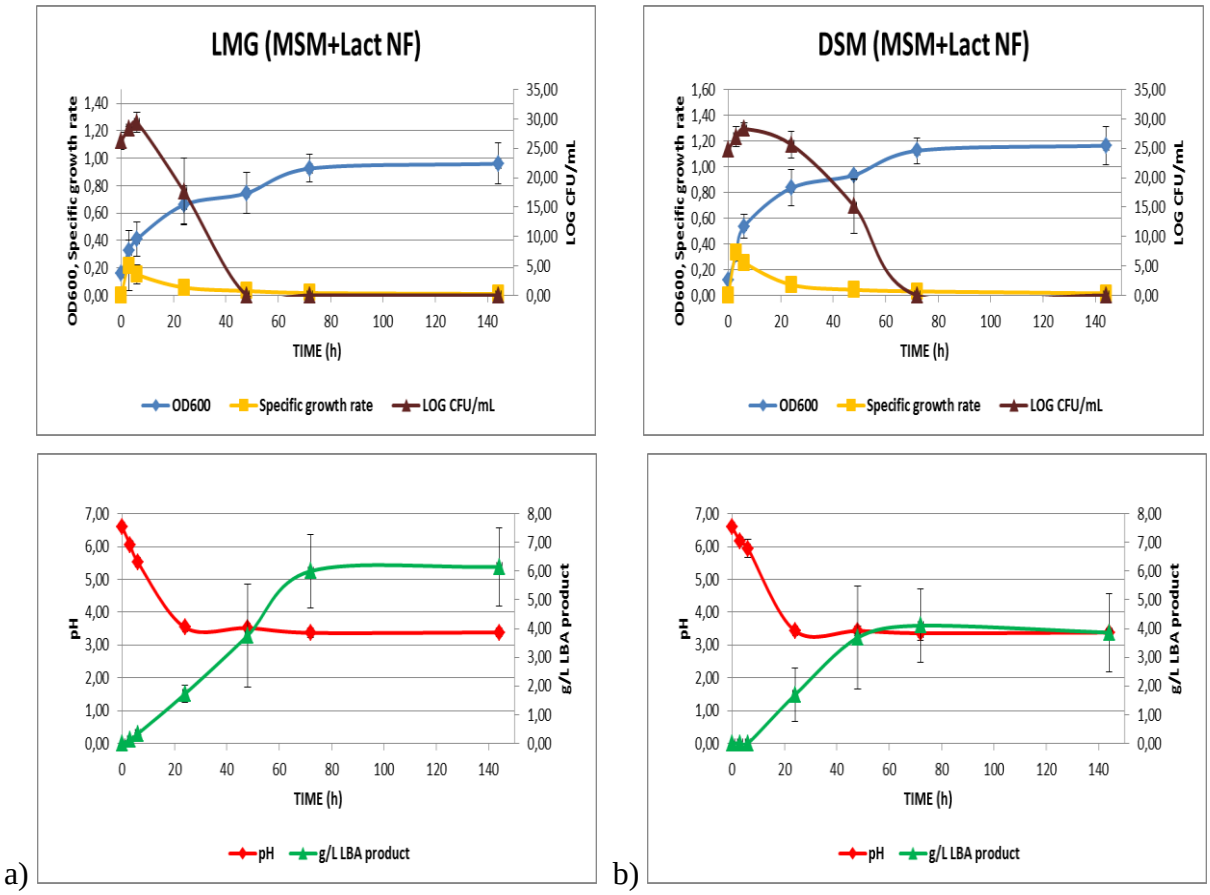
b) 

Fig. 4.12. Specific growth rate (µ) [h^{-1}], LOG CFU/mL, pH values, LBA product and residual lactose [g/L] for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with synthetic reach medium with concentrated lactose (50g/L of final concentration), obtained after UF and NF processes on RCW, as substrate, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

The possibility to use a cheaper medium (MSM) in comparison to synthetic reach medium for lactose oxidation was evaluated, as reported previously with chemically pure lactose from Sigma (paragraph 4.1.2). Despite the use of concentrated lactose, obtained from a natural matrix as RCW, presented positive effect on LBA production (6.15±1.36 and 4.1±1.28 g/L of LBA for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively after 144h of incubation at 30°C and 150 rpm) in comparison to the employed of Sigma lactose. But the low values of bioconversion yield (52±1.8 and 61±2,6 for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively, after 144h of incubation) and maximum specific growth rate µmax (0.22±0.19 and 0.61±0.26 h^{-1} for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively, after 3h of incubation), suggested how a reach medium was important for cells growth, which can produced in better condition and oxidized all the lactose initially present (50 g/L) (Fig. 4.12a and 4.12b).

Peptone and yeast extract represent two important sources of nitrogenous substance for the growth medium (Alonso et al., 2011; 2013). If the cells need micronutrients and microelements, they use the lactose for LBA production as carbon source, encouraging the expression of enzymes used to the lactose molecule breakage into glucose and galactose.



a) b)

Fig. 4.13. OD600, specific growth rate (μ) [h^{-1}], LOG CFU/mL, pH values and LBA product [g/L] for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with concentrated lactose (50g/L of final concentration), obtained after UF and NF processes on RCW, as substrate, inoculated with 5%vol/vol from bacterium medium preculture (condition C1).

4.2.4 Comparison between shake flasks experiments with chemically pure lactose from Sigma, RCW, permeate X, permeate 4 and synthetic reach medium supplemented with concentrated lactose, as substrates

SHAKE FLASKS		Ferm Broth +50 g/L Lactose Sigma	MSM +50 g/L Lactose Sigma	Cheese whey	Permeate X (UF-500 KDa)	Permeate 4 (UF-30 KDa)	Ferm Broth +50 g/L Concentrated Lactose (NF)	MSM +50 g/L Concentrated Lactose (NF)
LMG 2336	μmax (h^{-1})	0.36±0.21 (3h)	0.04±0.01 (3h)	0.46±0.11 (3h)	0.17±0.02 (3h)	0.16±0.01 (3h)	0.27±0.04 (3h)	0.22±0.01 (3h)
	LBA max (g/L)	13.16±1.25 (48h)	2.03±0.1 (144h)	28.7±2.8 (72h)	32.43±0.35 (144h)	36.57±4.17 (144h)	40.14±0.86 (144h)	6.15±1.36 (144h)
	pH	3.47±0.07 (48h)	3.24±0.1 (144h)	3.87±0.35 (72h)	3.6±0.08 (144h)	3.55±0.02 (144h)	3.55±0.02 (144h)	3.39±0.12 (144h)
	Conversion yield (%)	80±31 (48h)	23±1.6 (144h)	88±3 (72h)	87±3 (144h)	89±11 (144h)	91±6 (144h)	52±1.8 (144h)
DSM 21104	μmax (h^{-1})	0.40±0.07 (3h)	0.15±0.01 (6h)	0.48±0.16 (3h)	0.48±0.04 (3h)	0.4±0.04 (3h)	0.19±0.02 (3h)	0.33±0.03 (3h)
	LBA max (g/L)	18.61±0.01 (48h)	0.71±0.03 (144h)	27.87±1.1 2 (144h)	29.22±4.04 (144h)	27.4±3.00 (144h)	39.68±3.5 (144h)	4.1±1.28 (144h)
	pH	3.2±0.01 (48h)	3.27±0.14 (144h)	4.51±0.55 (144h)	3.57±0.07 (144h)	3.59±0.12 (144h)	3.59±0.12 (144h)	3.38±0.11 (144h)
	Conversion yield (%)	67±3 (48h)	31±2.4 (144h)	78±2 (72h)	83±0.6 (144h)	86±8 (144h)	83±2 (144h)	61±2.6 (144h)

Table. 4.1. Summary of maximum specific growth rate (μmax) [h^{-1}], maximum LBA product [g/L], pH and conversion yield values for *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104 in shake flasks experiments with chemically lactose from Sigma and with natural lactose from RCW (RCW, permeate X, permeate 4 and concentrated lactose after NF).

In Alonso et al., 2011 high quantity of lactobionic acid production (35-40 g/L after 48 hours of incubation in shake flasks) by *P.taetrolens* LMG 2336 have been achieved employing a more nutrients rich raw material (cheese whey) and optimal conditions (inoculum size, nitrogen source supplementation and seed culture age).

The importance of keep pH at constant values was also reported by Pedruzzi et al. 2007; indeed during the oxidation of lactose to LBA there is an intermediate structure, lactobionolactone molecule, which peak appears in HPLC chromatogram at low pH values. So a pH adjustment (alkaline hydrolysis) was necessary also for a high quality quantification analysis.

As can be seen in Table 4.1 both tested *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104 in shake flasks experiments, show interesting bioconversion performance when

fermentative broth supplemented with concentrated lactose was employed as substrate. In an economic optimization noteworthy is the results with permeate 4, because it is obtained after separation of bioactive proteins and peptides (through UF processes), but before NF process for obtaining concentrate lactose.

Note how P.taetrolens LMG 2336 has two stages of growth

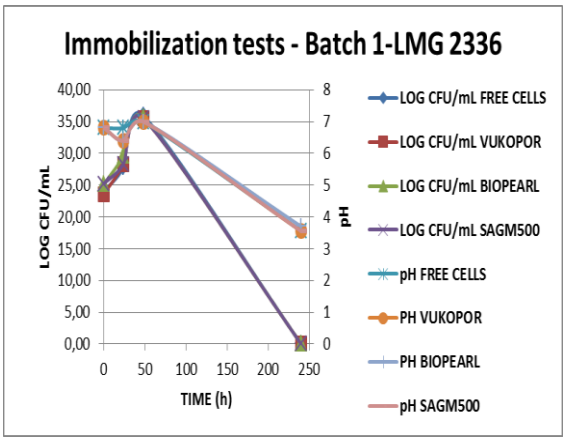
4.3 SCALE UP IN PACKED-BED REACTORS (PBR)

4.3.1 Preliminary tests for cells immobilization

After different bioconversion experiments in shake flasks, with different kind of inoculum and substrates, were selected the best bacteria strains and culture conditions for performing reactors scale up.

The importance of cells immobilization for improving growth and bioconversion performances, presented in a lot of papers, suggested the possibility to performed a Packed-Bed Reactor (PBR) with the selected *P. taetrolens* strains (LMG 2336 and DSM 21104), but preliminary tested with different inorganic supports were needed previously. Three supports were investigated (Vukopor, Biopearl and SAGM500) for their material stability, that making them suitable for food applications, and, based on the observations reported in paragraph 4.2.4, active cells in exponential phase growth in shake flasks RCW and inoculated from bacterium medium preculture (condition C1-paragraph 4.1.1) were used as inoculum for shake flasks with RCW and selected supports (Batch 1) and, after a long time of incubation (10 days) for promoting biofilm formation, supports with cells were used as inoculum in shake flasks with synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW) (Batch 2).

As reported in Fig. 4.15, in Batch 1 experiments were not detected significative difference in growth and pH values for both *P. taetrolens* strains between free cells colture (positive control) and the three supports coltures; maximun cell density of about 10^{10} CFU/mL starting from 10^{7} CFU/mL, was achieved after 48 h of incubation at 30°C and 80 rpm, lowest than 150 rpm employed in previus screening experiments in order to encouraged cells attachment to the tested supports. Prolongation of incubation time and the starting of lactose oxidation to LBA, led to a decrease of pH values that, concurrently with substrate consuption, facilitated the creation of an adverse environment for growth, stimulating the cells to create a biofilm to guarantee their survival (Qureshi, 2005).

a) 

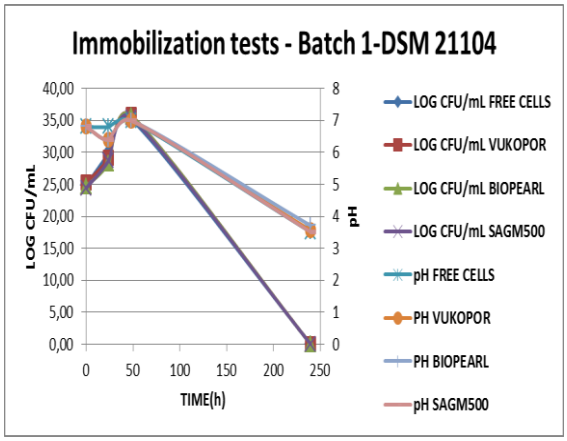
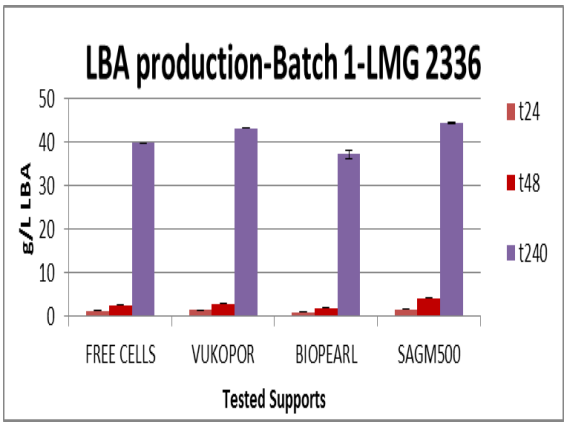
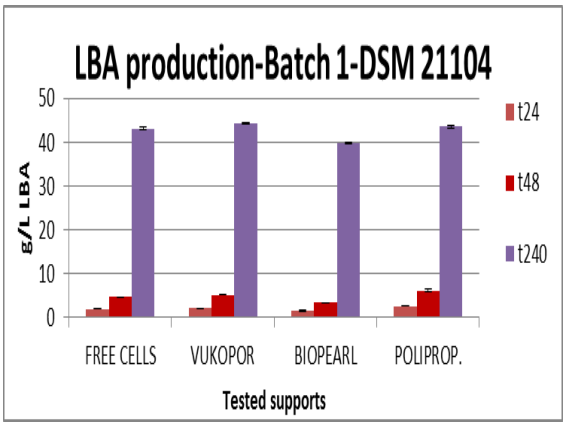
b) 

Fig. 4.14. LOG CFU/mL and pH values for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with RCW in 4 conditions (free cells, Vukopor supports, Biopearl supports and SAGM500 supports), as substrate, inoculated with 10%vol/vol of cells growth in RCW shake flasks up to the exponential phase.

No significant differences for LBA production, in comparison to free cells cultures, were also detected for the supports coltures in Batch 1 experiments, (Fig. 4.15a, 4.15b and 4.15d); and the conversion yield (Fig. 4.15d) confirmed the results obtained in bioconversion experiments for bacteria screening in shake flasks with RCW, as reported in Table 4.1: 0.88±0.03 and 0.78±0.02 for *Pseudomonas taetrolens* LMG 2336 and *Pseudomonas taetrolens* DSM 21104 respectively, after 72h of incubation at 30°C and 150 rpm.

a) 

b) 

BATCH 1	*Pseudomonas taetrolens* LMG 2336		*Pseudomonas taetrolens* DSM 21104	
	FINAL LBA CONCENTRATION (g/L) (240 h)	VARIANCE %	FINAL LBA CONCENTRATION (g/L) (240 h)	VARIANCE %
FREE CELLS	39.75±0.03	-	43.11±0.34	-
VUKOPOR SUPPORTS	43.12±0.02	+8.5%	44.41±0.21	+3%
BIOPEARL SUPPORTS	37.17±1.01	-6.5%	39.86±0.15	-7.55%
SAGM500 SUPPORTS	44.24±0.18	+11.28%	43.54±0.36	+1%

c)

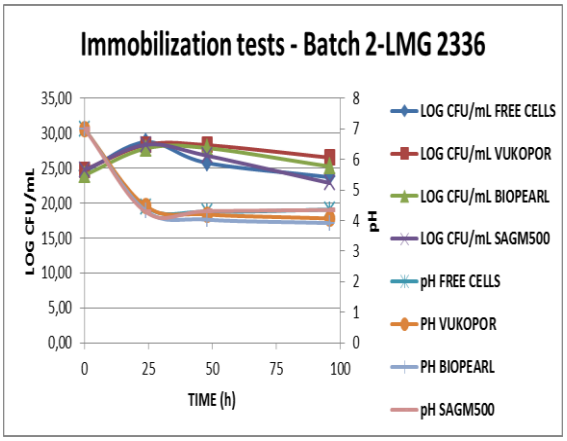
BATCH 1	*Pseudomonas taetrolens* LMG 2336	*Pseudomonas taetrolens* DSM 21104
	FINAL LBA CONVERSION YIELD (240 h)	FINAL LBA CONVERSION YIELD (240 h)
FREE CELLS	0.74±0.01	0.77±0.01
VUKOPOR SUPPORTS	0.83±0.02	0.83±0.01
BIOPEARL SUPPORTS	0.81±0.03	0.82±0.01
SAGM500 SUPPORTS	0.8±0.01	0.87±0.01

d)

Fig. 4.15. LBA product [g/L] for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b), percentage variance in comparison to free cells cultures (c), and conversion yield values (d) in shake flasks with RCW with 3 selected supports (Vukopor, Biopearl and SAGM500), as substrate, inoculated with 10%vol/vol of cells growth in RCW shake flasks up to the exponential phase.

After 10 days of incubations at 30°C and 80 rpm, the tested supports with cells biofilm were employed as inoculum in shake flasks with synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW) (Batch 2) and incubated again in the same condition reported previously for Batch 1 (30°C and 80 rpm).

Despite cell density (CFU/mL) and pH values were not presented differences between positive control (free cells shake flasks) and shake flasks with the free selected supports (Fig. 4.16a and 4.16b), g/L of LBA product for both *P. taetrolens* strains were presented little changes for the three selected supports (Fig. 4.17). In particular *P. taetrolens* LMG 2336 was achieved 10.52±0.13 g/L of LBA, after 96 h of incubation, when Biopearl was employed as support, with 0.54±0.03 of conversion yield. Also *P. taetrolens* DSM 21104, with the same supports, was showed a little LBA production, even after 96 h of incubation: 3.67±0.14 g/L of LBA (0.25±0.02 of conversion yield).

a) 

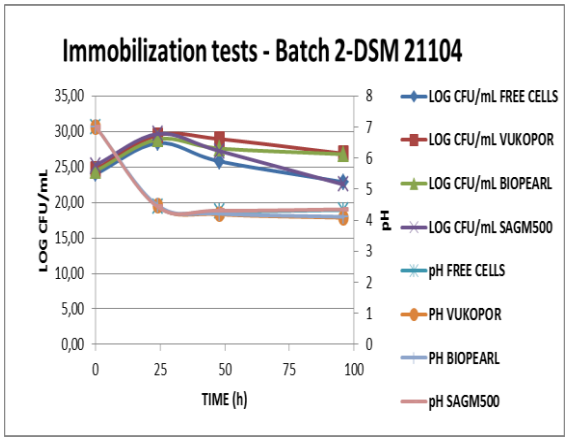
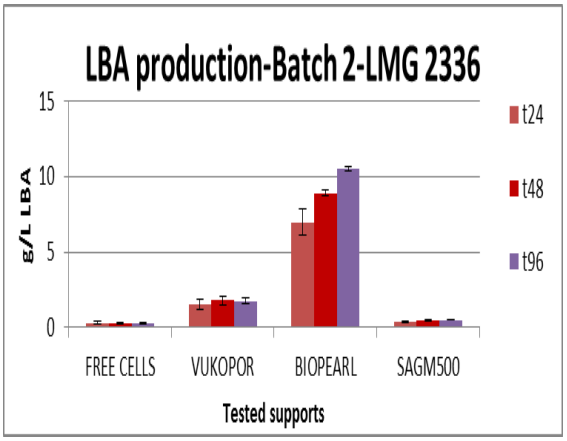
b) 

Fig. 4.16. LOG CFU/mL and pH values for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW) in 3 conditions (Vukopor supports, Biopearl supports and SAGM500 supports), as substrate, inoculated with biofilm formed on supports during Batch 1.

a) 

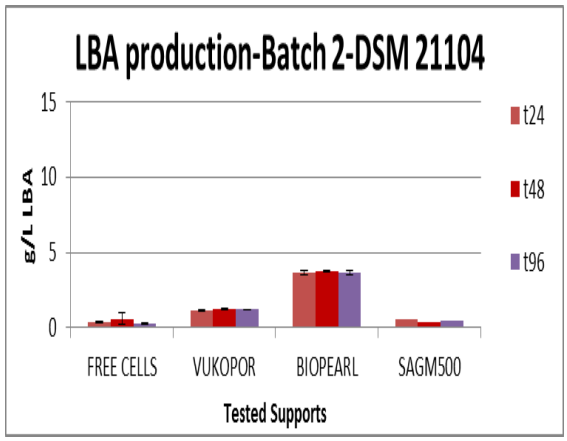
b) 

Fig. 4.17. LBA product [g/L] for *P. taetrolens* LMG 2336 (a) and *P. taetrolens* DSM 21104 (b) in shake flasks with synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW) in 3 conditions (Vukopor supports, Biopearl supports and SAGM500 supports), as substrate, inoculated with biofilm formed on the same supports during Batch 1.

Despite only with Biopearl supports a little production of LBA was detected in batch 2, thanks to biofilm formation, also Vukopor supports were considerate for PBR setting up, for their particular structure with high specific surface that encouraged cells attachment. Indeed at the end of Batch 1 and Batch 2 both Vukopor and Biopearl supports were presented a visible biofilm on their surface, for the two tested *P. taetrolens* strains

4.3.2 Packed Bed Reactor (PBR)

Preliminary experiments for biofilm formation on three selected supports (Vukopor, Biopearl and SAGM500), for *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104 strains growth on RCW and synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW), were led to the identification of the most promising supports and inoculum condition for PBR setting up. Two parallel reactors were performed with Vukopor and Biopearl (Fig. 4.18)

a)  b) 

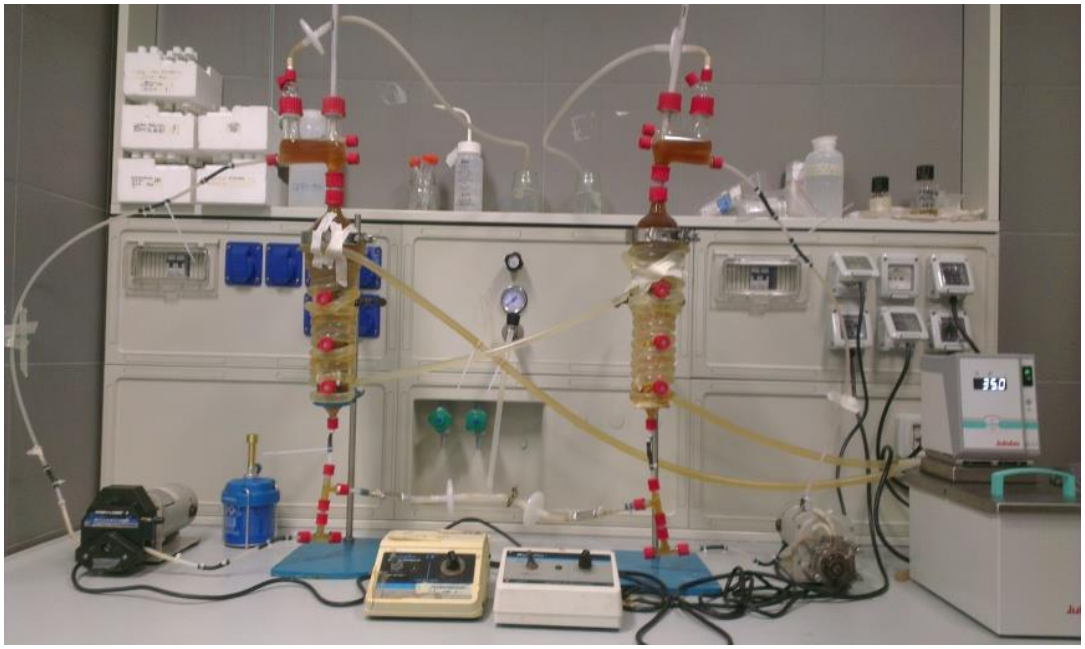
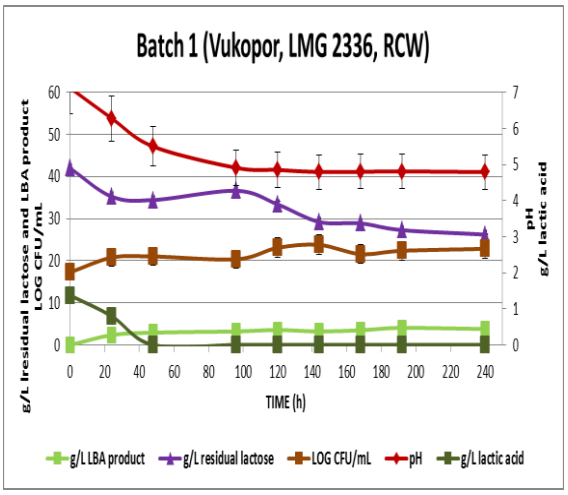
c) 

Fig. 4.18. Packed-Bed Reactor with Vukopor supports (a), Biopearl supports (b) and setting up of the two configurations (c), for *P. taetrolens* LMG 2336 and *P. taetrolens* DSM 21104 with RCW and synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW).

In a first batch fermentations (Batch 1) each tested *P. taetrolens* strains (LMG 2336 and DSM 21104) was inoculated in two parallel PBRs setting up with Vukopor or Biopearl supports and autoclaved RCW as bioconversion medium, with cells in exponential phase growth in RCW shake flasks, as previously reported in paragraph 4.3.1, in order to encouraged biofilm formation.

a)



b)

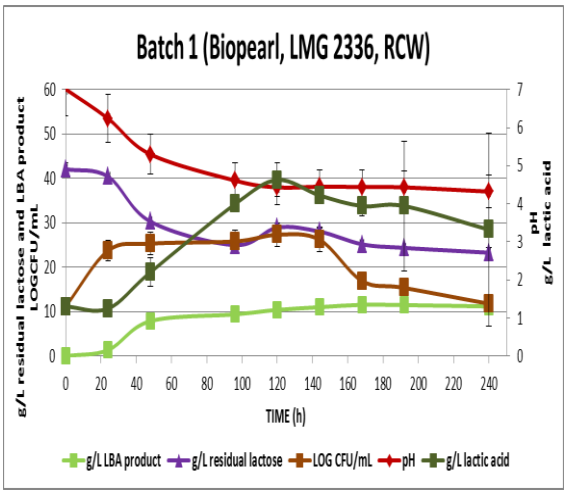
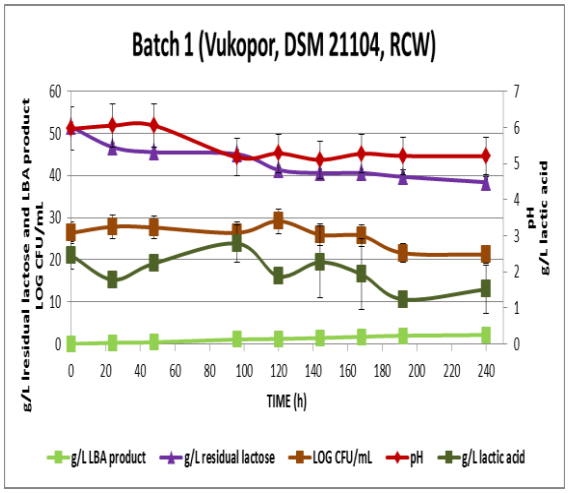


Fig. 4.19. Cellular growth [LOG CFU/mL], pH and bioconversion values [g/L] of residual lactose, LBA and lactic acid in PBR with Vukopor supports (a) and Biopearl supports (b) for *P. taetrolens* LMG 2336 with RCW (Batch 1).

a)



b)

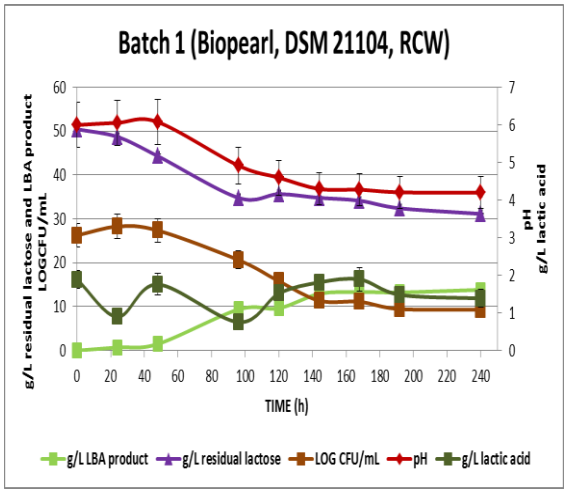
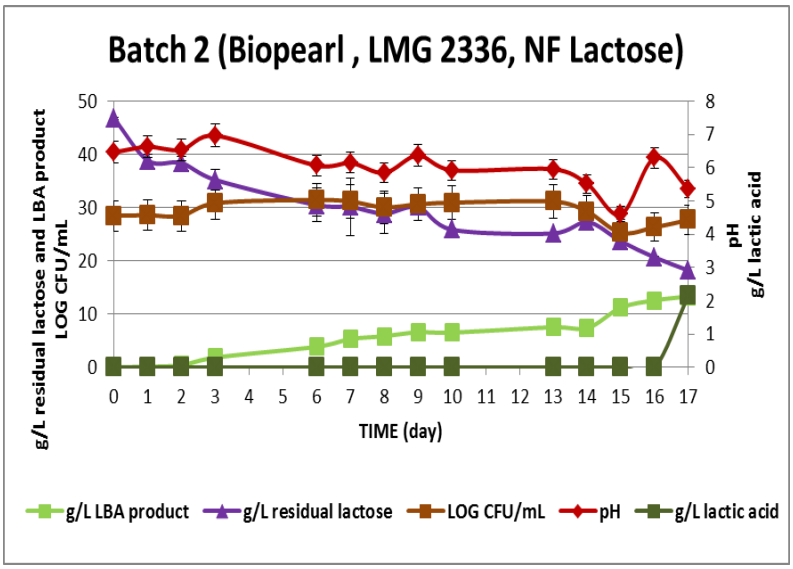


Fig. 4.20. Cellular growth [LOG CFU/mL], pH and bioconversion values [g/L] of residual lactose, LBA and lactic acid in PBR with Vukopor supports (a) and Biopearl supports (b) for *P. taetrolens* DSM 21104 with RCW (Batch 1).

a)



b)

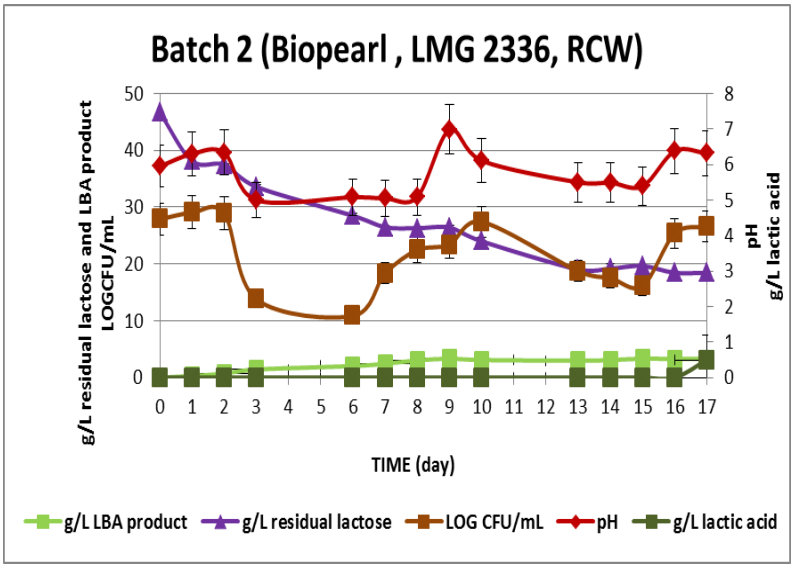


Fig. 4.21. Cellular growth [LOG CFU/mL], pH and bioconversion values [g/L] of residual lactose, LBA and lactic acid in PBR with Biopearl supports for *P. taetrolens* LMG 2336 with synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW) (a) and RCW(b) (Batch 2).

In batch 1 pH was not kept constant in order to stimulate the development of a challenging environment to incentivized the strains to adhere to media; in the batch 2 instead it was kept on values included between 5.5±0.55 E 6.5±0.65.

In PBR experiments was important a preliminary phase of removal of deposits resulting from the precipitation of the proteins in an autoclave which can cause blockages in the reactor piping.

4.4 SCALE UP IN STIRRED-TANK REACTORS (STR)

The complications that were observed in PBR setting up, as reported previously, suggested the possibility to performed a different scale up in Stirred-Tank Reactors (STRs), in order to guarantee a constant pH value and an uniformly oxygen dissolved .

4.4.1 Batch fermentations in 3L-STR

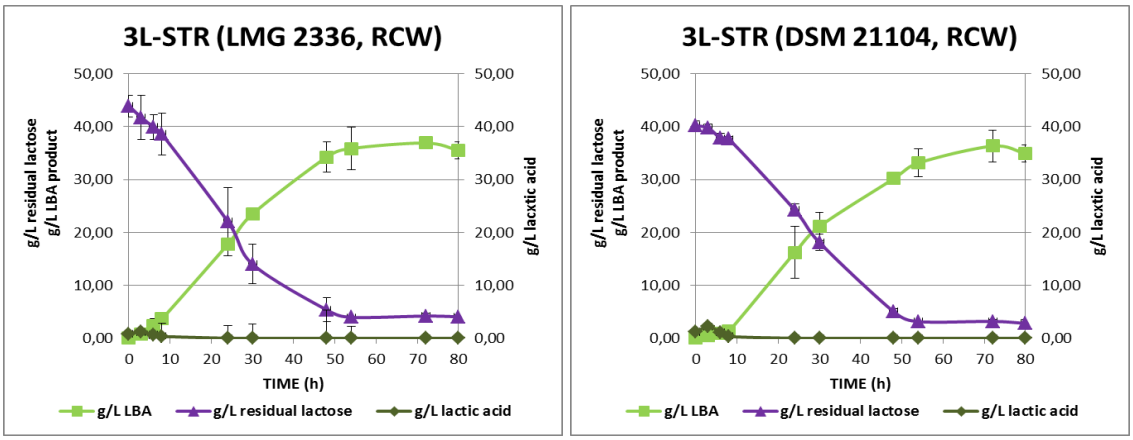
a)  b) 

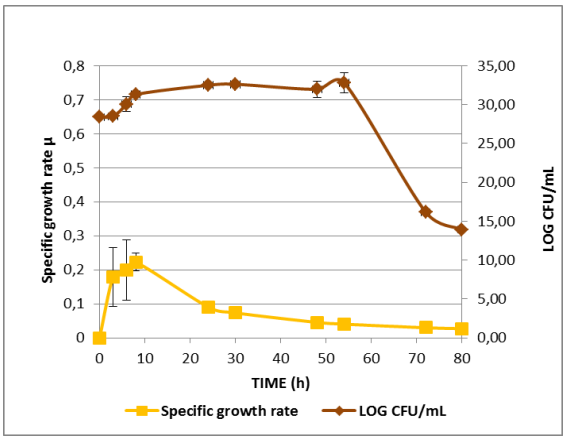
Fig. 4.22. 3L-STR with autoclaved synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW) (a) and autoclaved RCW (b) as substrates, inoculated with *P.taetrolens* LMG 2336 and *P.taetrolens* DSM 21104, in batch configuration.

The bioreactors were inoculated with 5% (v/v) of overnight precultures grown on nutrient broth medium, in order to minimized cellular stress during biomass recovery processes. When autoclaved RCW was employed as substrate, After 6-8 h of incubation, the cultures reached the maximum cell count ($9.15x10^9$ CFU/ml) with a maximum specific growth rate of 0.25/h. The oxygen uptake shows the onset of growth phase (until 6-8 hours), with low values of pO2, and the start of stationary and LBA production phase. After 48 hours of incubations *P. taetrolens* LMG 2336 produced 34,25±2,86 g/L of LBA (with 85±7% of conversion yield) (Fig. 4.23a). Interesting yields were also achieved with *P. taetrolens* DSM 21104 (Fig. 4.23b), producing 30,18±0,3 g/L LBA (with 84±7% of conversion yield) after 48 hours of batch fermentation. For both strains, prolongation of the incubation up to 80 h did not results in improving the productivities and a residual lactose concentration of about 4 g/l was observed starting from an initial concentration of 40 g/l. This could be due to the cell death as a result of increasing concentration of LBA and or the depletion of cofactors affecting the enzymatic activities.

The bioreactor results were comparable to those obtained in shake flasks experiments, but it could be noticed an important reduction of the time required for a total lactose consumption (from 120 to 48 hours), thanks to a constant parameters control. This aspects is very important for an improvement in bioconversion performance through fed-batch and continuous configurations.

The yields obtained from RCW are slightly lower compared to those obtained by Alonso et al. (2011). In that study, a concentration of 42.4 g/L of LBA and a bioconversion yield of 100% were obtained, after 32 hours, from sweet cheese whey using the strain P. taetrolens LMG2336 and an inoculum of 30% v/v. The higher LBA concentration and bioconversion yield observed could be due to the higher cell density used as inoculum and or to the higher protein content of the cheese whey compared to RCW (Alonso et al., 2011). The yields obtained here are lower compared to those obtained by Pedruzzi et al. (2011) where a LBA concentration of 125.4 g/L was achieved. However, in that study the process was based on the use of enzymes and pure lactose solution. Higher LBA titres were also achieved by Alonso et al. (2013) obtaining 164g/l of lactobionic acid. However, the experimental conditions were different from those applied here i.e. the process was a fed-batch and a co-feeding of concentrated lactose solution and highly concentration cheese whey solution supplemented with yeast extract and peptone was adopted.



a) 

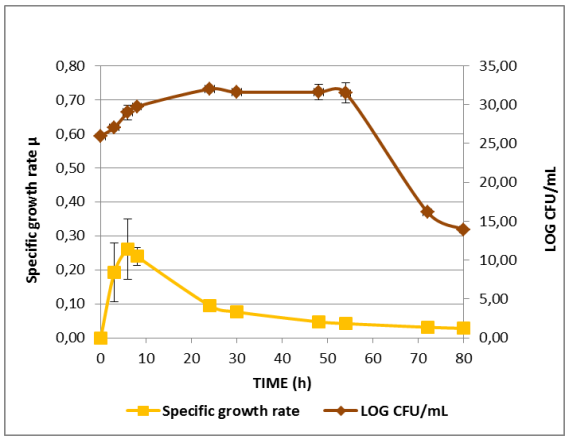
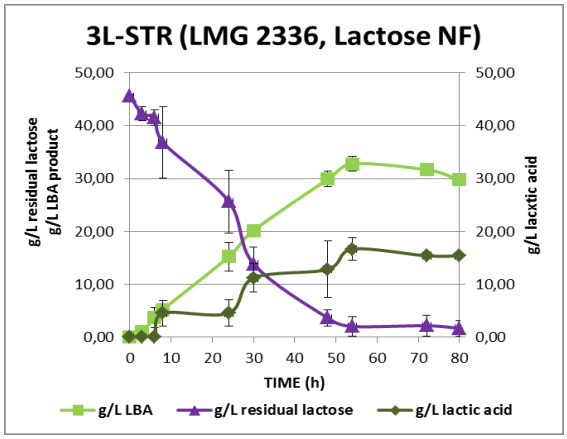
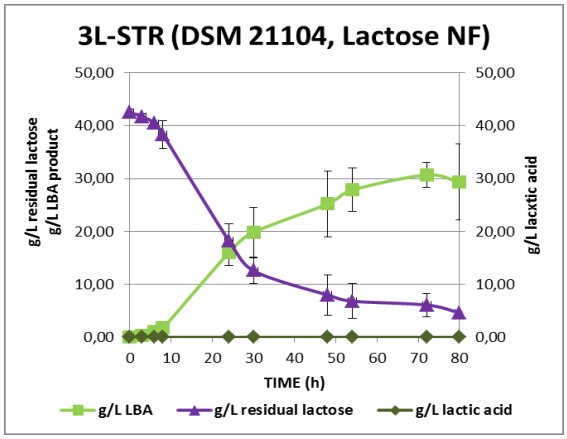
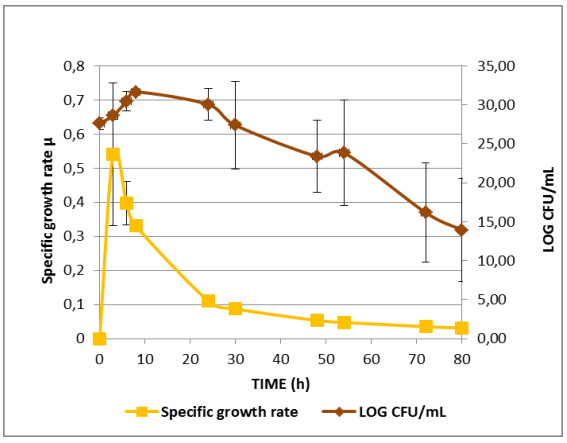
b) 

Fig. 4.23. Cellular growth [LOG CFU/mL], specific growth rate [h^{-1}] and bioconversion values [g/L] of residual lactose, LBA and lactic acid in 3L-STR with autoclaved RCW as substrate for *P.taetrolens* LMG 2336 (a) and *P.taetrolens* DSM 21104 (b), in batch configuration.





a) 

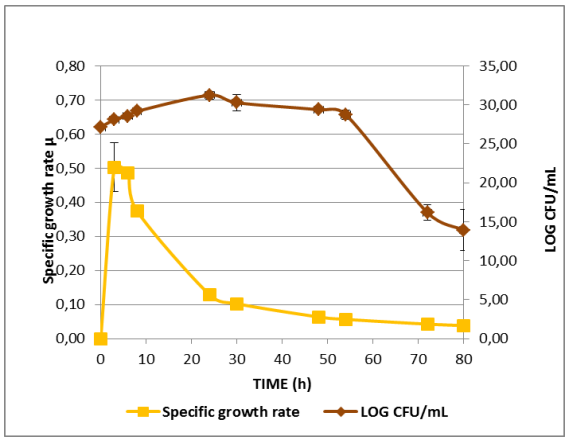
b) 

Fig. 4.24. Cellular growth [LOG CFU/mL], specific growth rate [h^{-1}] and bioconversion values [g/L] of residual lactose, LBA and lactic acid in 3L-STR with autoclaved synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW) as

substrate for *P.taetrolens* LMG 2336 (a) and *P.taetrolens* DSM 21104 (b), in batch configuration.

When autoclaved synthetic reach medium added with 50 g/L of concentrated lactose (UF+NF of RCW) (Fig 4.24) and permeate 4 (30KDa-UF) (Fig. 4.25) as substrates for *P.taetrolens* LMG 2336 and *P.taetrolens* DSM 21104, in batch configuration, growth rate are better in comparison with RCW 3L-STR experiments, but the LBA titres were not presented significate improvement.

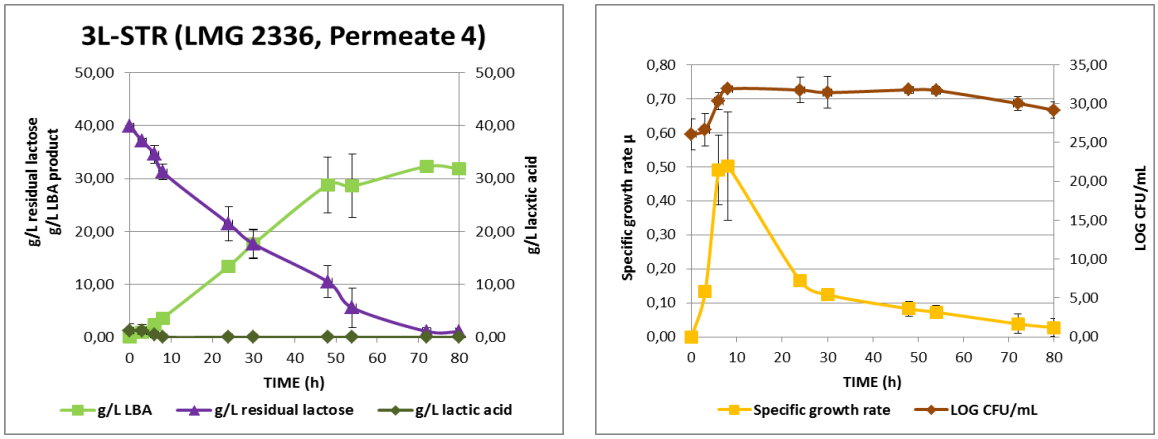


Fig. 4.25. Cellular growth [LOG CFU/mL], specific growth rate [h^{-1}] and bioconversion values [g/L] of residual lactose, LBA and lactic acid in 3L-STR with autoclaved permeate 4 as substrate for *P.taetrolens* LMG 2336, in batch configuration.

4.4.2 Fed-Batch fermentations in 3L-STR

Fed batch fermentations, employed *P.taetrolens* LMG 2336 (Fig. 4.26) with RCW, supplemented after total lactose consumption (80h) with concentrated lactose led to achieved a 60 g/L of LBA, demonstrating how a continue supplemented of lactose can improve the performance of the process.

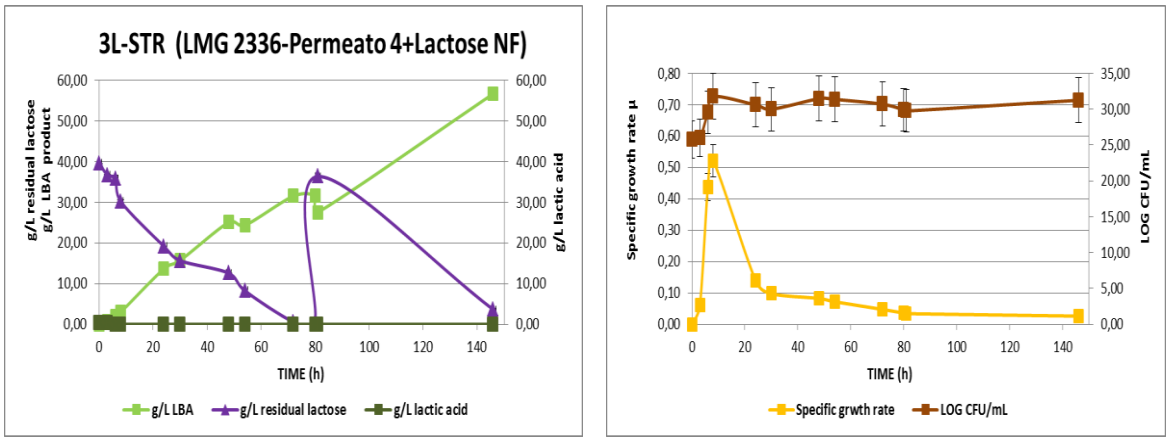


Fig. 4.26. Cellular growth [LOG CFU/mL], specific growth rate [h^{-1}] and bioconversion values [g/L] of residual lactose, LBA and lactic acid in 3L-STR with autoclaved permeate 4 as substrate for *P.taetrolens* LMG 2336, in fed-batch configuration with concentrated lactose (UF+NF of RCW).

4.4.3 Batch fermentations in 15L-STR

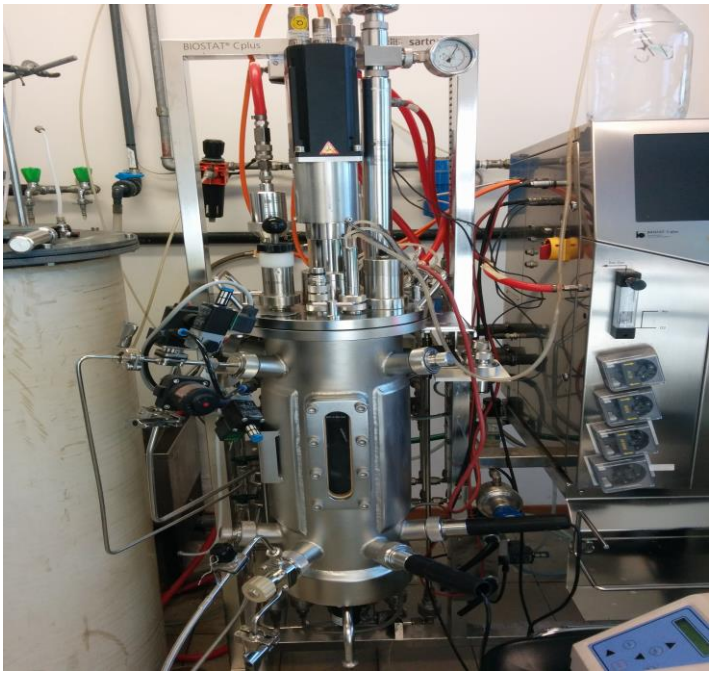


Fig. 4.27. 15L-STR with autoclaved RCW as substrate, inoculated with *P.taetrolens* LMG 2336, in batch configuration.

STR cultivations were inoculated with 10% (v/v) from overnight precultures in order to avoid contaminations and cell stress. The oxygen uptake shows the onset of growth phase (until 6-8 hours), with low values of pO2, and the start of stationary and production phase (from 12 to

48 hours), without oxygen consumption. Two different working volumes have been tested (5L and 7L) to evaluate the influence of headspace on fermentation yield (66.6% and 53% respectively).

Pseudomonas taetrolens LMG (Fig.4.28) shows better performance with a lower headspace (53% corresponding to 7L of working volume), producing 46.69±3.63 g/L LBA (with 0.89±0.05 of conversion yield) after 48 hours using all the initial lactose substrate. In the same conditions a 66.6% of headspace has been led to a reduction of 28.5% in the LBA production and of 18% in the conversion yield (33.37±0.65 g/L LBA with 0.73±0.02 of conversion yield).

The LBA production is a non-growth related product and the starter of the stationary (coincides with the production phase) was indicated by the maximum specific oxygen consumption, as reported by Alonso et al. (2012). So a lower headspace, and a consequent lower oxygen presence in the reactor, during the fermentation phase has been a positive effect in cells oxidation performance.

The influence of dissolved oxygen availability on cell growth and LBA production from cheese whey by Pseudomonas taetrolens LMG has been investigated by Alonso et al. (2012) and the results have been showed how the use of high aeration rates neither stimulated cell growth nor lactobionic acid production due to a prolonged proliferation phase that delayed the onset of the production phase.

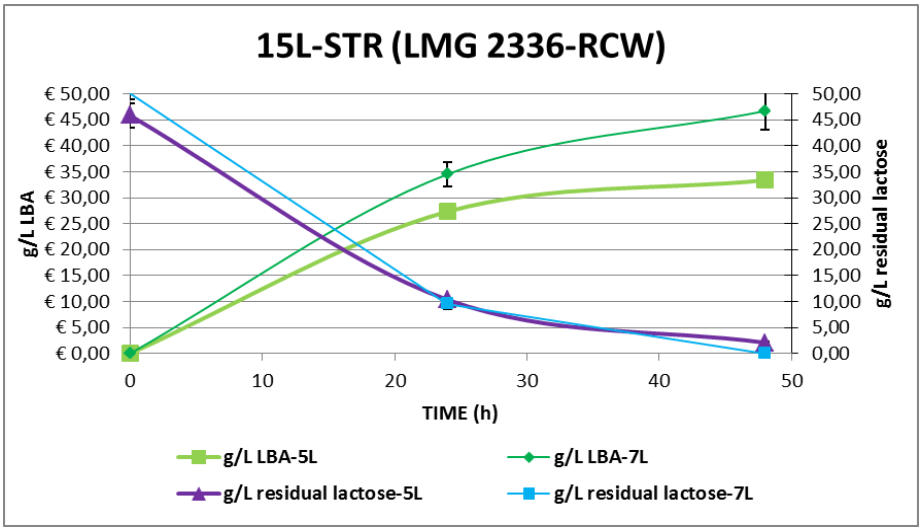


Fig. 4.28. Production of LBA and residual lactose for P. taetrolens LMG in Stirred-Tank Reactor-15L with autoclaved RCW (5L and 7L of working volume)

From the various screening in shake flasks is known as LMG 2336 is little influenced by the variability of the substrate, instead DSM 21104 has poor reproducibility in the growth and production values (as evidenced by the much higher standard deviations).

4.5 DOWNSTREAM PROCESS FOR LBA RECOVERY

4.5.1 High-Performance Tangential Flow Filtration

Fig. 4.29. Bench-scale cross flow unit (from PS Prozesstechnik GmbH)

The membrane employed has been characterized by a calibration line between flow (ml/sec) and pressure (bar) with demineralized water (Fig.4.29)

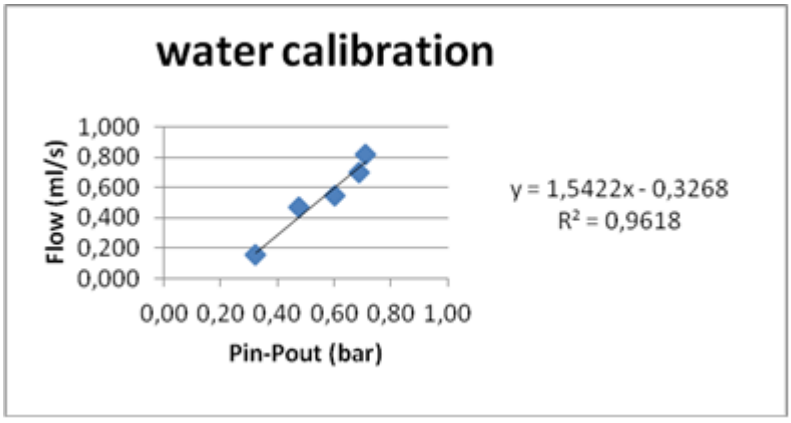


Fig.4.30: Membrane calibration

For each test has been performed a product calibration (Fig.4.31, Fig.4.32, Fig.4.33)

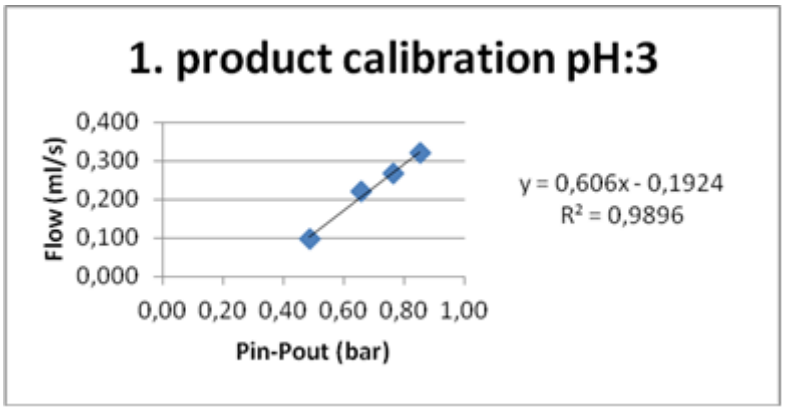


Fig.4.31: Product calibration (test 1: pH3)

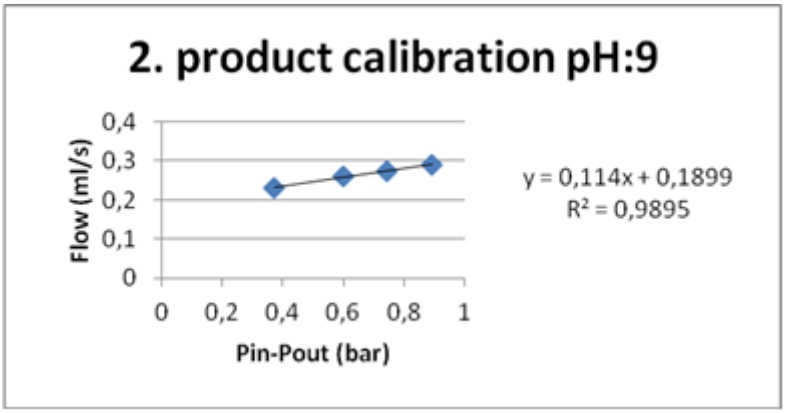


Fig.4.32: Product calibration (test 2:pH9)

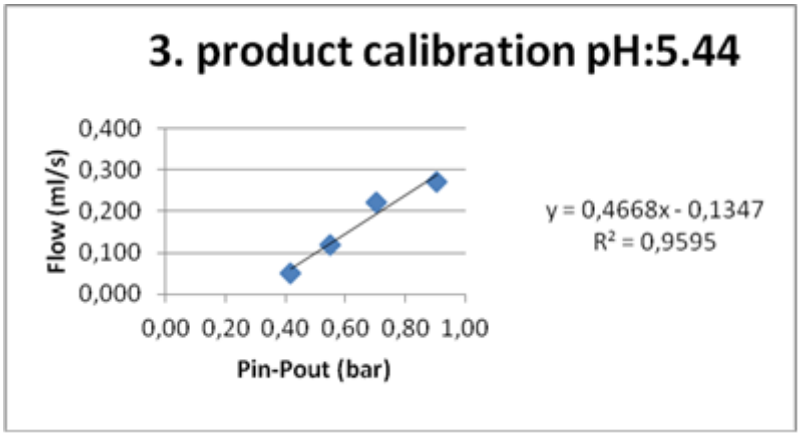


Fig.4.33: Product calibration (test 3: pH5.44)

As can we see low pH values led to a high flow rate thanks to the minimized membrane pores fouling.

For each test the filtration and diafiltration flow have been reported in the figures below (Fig.4.34, 4.35, 4.36, 4.37, 4.38, 4.39).

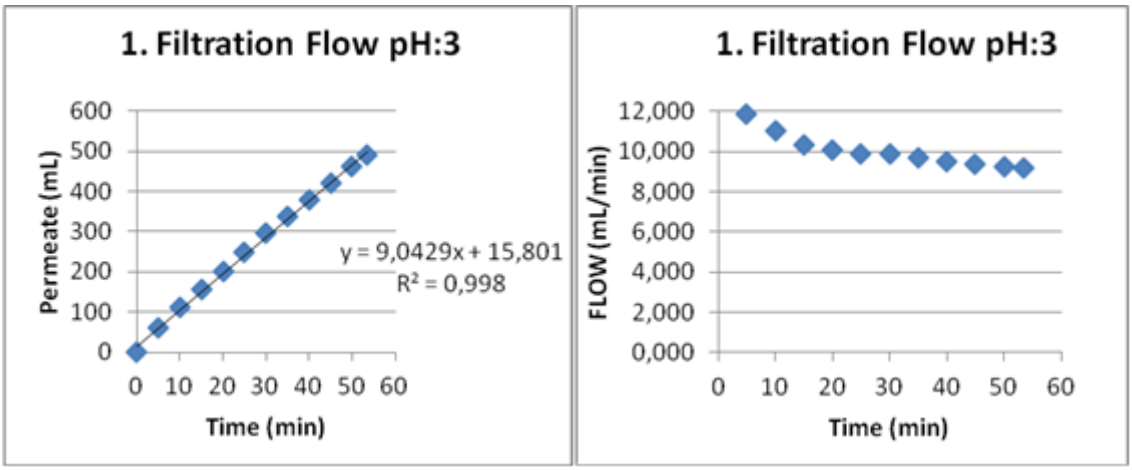


Fig.4.34: Filtration Flow (test 1: pH 3)

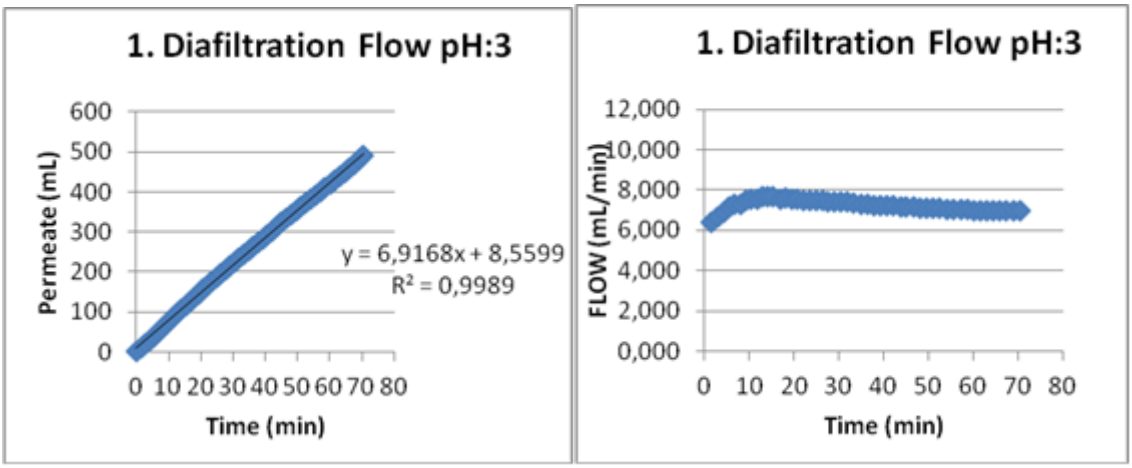


Fig.4.35: Diafiltration Flow (test 1:pH3)

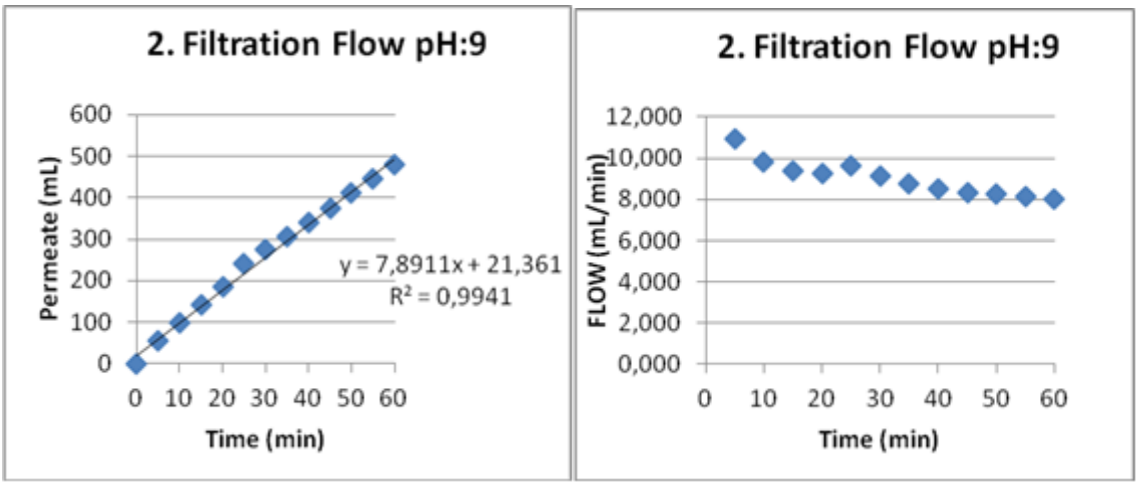


Fig.4.36: Filtration Flow (test 2: pH 9)

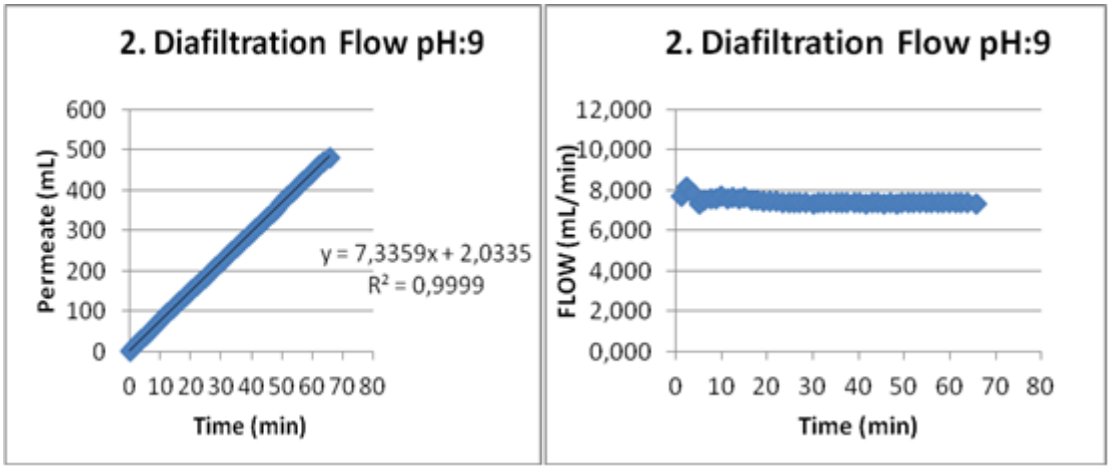


Fig.4.37: Diafiltration Flow (test 2: pH 9)

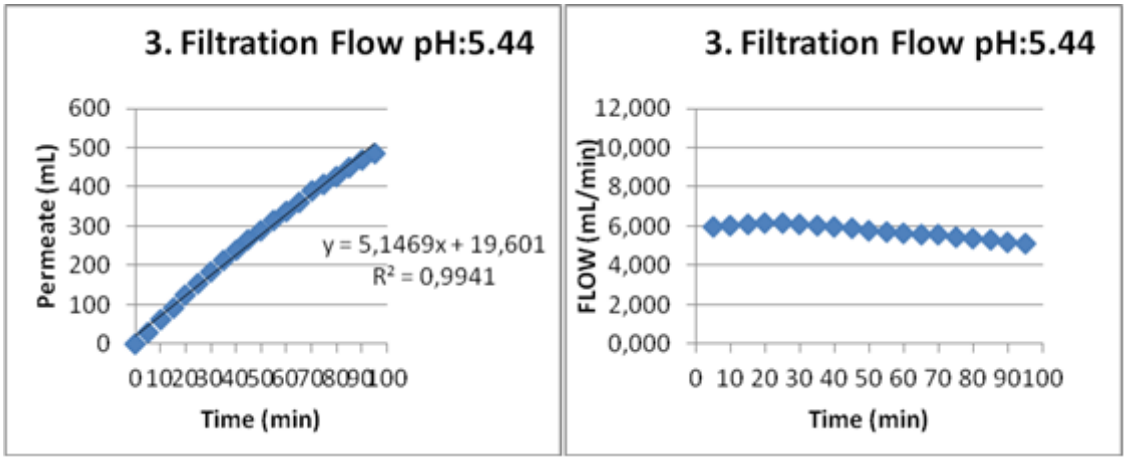


Fig.4.38: Filtration Flow (test 3: pH 5.44)

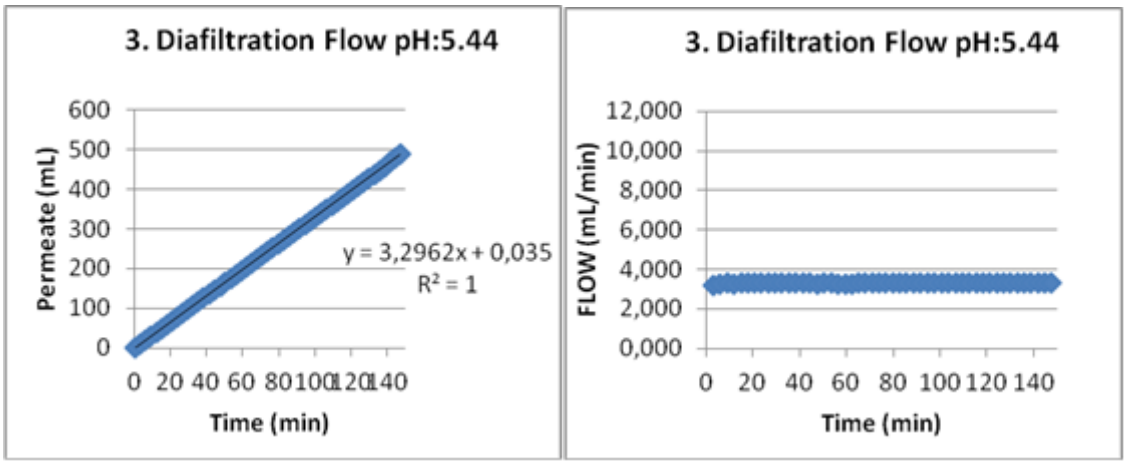


Fig.4.39: Diafiltration Flow (test 3: pH 5.44)

The evolution of flow with time (min) at 30°C and different pH values shows a diminution of filtration flow at extreme pH values (3 and 9) probably for pores fouling; but this effect was not relevant at pH 5.44, where the flow was smaller but constant with time.

The quantification analyses of the samples, performed by HPLC system for LBA detection, have been reported in Table 4.2 and Table 4.3, for pH 3 and pH 9 respectively.

TEST 1	CONCENTRATE			PERMEATE				
t DIAF	VOL (L)	[LBA] (g/L)	LBA (g)	VOL (L)	[LBA] (g/L)	LBA (g)	TOT LBA (g)	%REC LBA
0	0,5	45,3	22,65	0	0	0	22,65	0
1/4	0,5	46,02	23,01	0,125	3,16	0,395	23,405	0
1/2	0,5	46,56	23,28	0,25	3,28	0,82	24,1	0
3/4	0,5	47,07	23,535	0,375	2,71	1,01625	24,55125	0
1	0,5	45,88	22,94	0,5	2,71	1,355	24,295	0

Table 4.2: Evolution of LBA amount during the diafiltration cycle at pH 3, 30˚C and 14-15 bar (test 1: pH 3)

TEST 2	CONCENTRATE			PERMEATE				
t DIAF	VOL (L)	[LBA] (g/L)	LBA (g)	VOL (L)	[LBA] (g/L)	LBA (g)	TOT LBA (g)	%REC LBA
0	0,5	34,03	17,015	0	0	0	17,015	0,0
1/4	0,5	29,71	14,855	0,125	10,43	1,30375	16,15875	12,7
1/2	0,5	26,29	13,145	0,25	9,58	2,395	15,54	22,7
3/4	0,5	24,96	12,48	0,375	8,36	3,135	15,615	26,7
1	0,5	23,02	11,51	0,5	7,78	3,89	15,4	32,4

Table 4.3: Evolution of LBA amount during the diafiltration cycle at pH 9, 30˚C and 14-15 bar (test 2: pH 9)

It is cleary how the pH value affects the LBA recovery; after 1 cylce of diafiltration process the LBA recovery was 32% at pH 9. No recovery was observed at pH 3.
Finally a comparison with test 3 (pH 5,44 - intermediate between 3 and 9) has been presented in Table 4.4.

TEST	[LBA] initial (g/L)	LBA initial (g)					
1	34,23	34,23					
2	20,68	20,68					
3	31,93	31,93					
TEST	time of filtration cycle (min)	Permeate Final volume (L)	[LBA] permeate (g/L)	LBA final permeate (g)			
1	53	0,5	2,58	1,29			
2	60	0,5	10,48	5,24			
3	95	0,5	3,63	1,815			
TEST	time of diafiltration cycle (min)	Permeate Final volume (L)	[LBA] permeate (g/L)	LBA final permeate (g)	Concentrate Final volume (L)	[LBA] concentrate (g/L)	LBA final concentrate (g)
1	70	0,5	2,71	1,355	0,5	45,88	22,94
2	65	0,5	7,78	3,9	0,5	23,02	11,51
3	147	0,5	5,69	2,845	0,5	55,61	27,805

Table 4.4: Comparison of test 1 (pH 3), test 2 (pH 9) and test 3 (pH 5,44) at 30˚C and 14-15 bar (1KDa)

Results confirm that pH 9 encourages LBA flow through the ceramic membrane (1KDa) and additional diafiltration cycles could lead to further increases in LBA recovery from fermentative broth.

Furthermore it can be seen how at low pH the material balance between initial and final LBA was not respected, probably due to a conformational change of the molecule, as reported previously by Pedruzzi et al. (2007).

5. CONCLUSION

Disposal and treatment of dairy industry by-products (cheese whey and ricotta cheese whey) is a crucial challenge for dairy industry both as environmental and economicic point of view.
Microbial production of LactoBionic Acid, a novel and interesting high-value added molecule for industry, may overcome the major drawbacks associated with chemical and enzymathic approaches (costs and harmful operative conditions) and a complete transition to industrial implemetation needs improvment three important aspects: high level cells density and stability (metabolic engineering strategies), efficient scale up configuration and optimization of downstream process with the aim of achieving an integrated continue biorefinery process for LBA bioproduction from inexpensive dairy industry feedstock.
RicottaCheeseWhey, the byproduct of ricotta cheese manufacturing currently used mainly for feed preparation, and different permeates were used as a raw material for growth and LBA production by *P. taetrolens* strains. After shake flasks screening, in order to individuate the best condition of fermentation, in 3L-STR batch processes, fermentation and bioconversion yields of about 36g/L of LBA with 85% of conversion yield were achieved after 48 h. Furthermore fed-batch configuration and scale up in 15L-STR allowed to achieve better performance in term of LBA production and time of reaction, respectively.
To the best of our knowledge, this study shows for the first time that bovine RCW, a substrate completely different from cheese whey, can be used as substrate for the growth and production of lactobionic acid with results comparable to those obtained using cheese whey (Alonso et al., 2011). Moreover, it is worthy to stress that RCW is a dairy waste having high COD and BOD values and its disposal without treatment could create environmental problems.
The use of lactose from a natural matrix from dairy industry waste, like RCW or permeates after filtration processes for recovering of bioactive proteins and peptides, improve bioconversion capacity of *Pseudomonas taetrolens* species for producing LBA via lactose oxidation reaction. Also interesting to note that despite the matrix is subject to fluctuations in the composition and properties arising from the initial raw material (milk and cheese-making processes), the strains of P. taetrolens are affected in a manner of little relevance.
The proposed biotechnological process offers an interesting approach for the valorization of RCW into highly valuable fine chemical. A more detailed investigation on the effects of feeding strategy, inoculum size or the agitation on LBA productivity was beyond the

objective of the present preliminary screening. Optimization of the bioconversion process through response surface methodology, a fed-batch process and the evaluation of the effect of cell immobilization on LBA yields is advisable. Moreover, set up of the downstream process for the recovery of LBA remains necessary for industrial exploitation/implementation of this biorefinery process.

REFERENCES

Adrian J, Frangne R, Potus J (2009). Dizionario degli alimenti. Scienza e tecnica. II edizione. Translation from French edition by Valentina Guani. Editore tecniche nuove: 462-463

Aktas N, Boyacı IH, Mutlu M, Tanyolac A (2006) "Optimization of lactose utilization in deproteinated whey by Kluyveromyces marxianus using response surface methodology (RSM)". Bioresource Technology 97: 2252–2259.

Alonso S, Rendueles M, Díaz M (2011) Efficient lactobionic acid production from whey by *Pseudomonas taetrolens* under pH-shift conditions. Bioresource Technology; 102: 9730-9736.

Alonso S, Rendueles M, Díaz M (2012) Physiological heterogeneity of *Pseudomonas taetrolens* during Lactobionic acid production. Applied Microbiology and Biotechnology; 96: 1465-1477.

Alonso S, Rendueles M, Díaz M (2013) Bio-production of lactobionic acid: Current status, applications and future prospects. Biotechnology Advances 31(8): 1275-1291.

Alonso S, Rendueles M, Díaz M (2015) Simultaneous production of lactobionic acid and gluconic acid in cheese whey/glucose co-fermentation by *Pseudomonas taetrolens*. Bioresource Technology; 196: 314-323.

Araki K, Fujii K, Ueno H, Fujii N, Takahane M (2006) New antibacterial agent composition. Japan Patent Apllication Pub. No.: JP2006104164.

Baminger U, Ludwig R, Galhaup C, Leitner C, Kulbe KD, Haltrich D (2001) Continuous enzymatic regeneration of redox mediators used in biotransformation reactions employing flavoproteins. Journal of Molecular Catalysis B: Enzymatic 11: 541–550

Bean RC, Hassid WZ (1956) Carbohydrate oxidase from A red alga, *Iridophycus flaccidum.* The Journal of Biological Chemistry; 218(1); 425-436.

Belkacemi K, Vlad MC, Hamoudi S, Arul J (2007) Value-Added Processing of Lactose: Preparation of Bioactive Lactobionic Acid Using a Novel Catalytic Method. International Journal of Chemical Reactor Engineering 5; Article A64.

Belzer FO, D'Alessandro AM, Hoffman RM, Knechtle SJ, Reed A, Pirsch JD (1992) The use of UW solution in clinical transplantation. A 4-year experience. Annals of Surgery; 215: 579-585.

Borges da Silva, Pedruzzi I, Rodrigues AE (2011) Simulated moving bed technology to improve the yield of the biotechnological production of lactobionic acid and sorbitol. Adsorption; 17: 145-158.

Bucek W, Connors WM, Cort WM, Roberts HR (1956) Evidence for the formation and utilization of lactobionic acid by *Penicillium chrysogenum*. Archives of Biochemistry and Biophysics; 63: 477-478.

Budtz P, Vindeløv J, Nielsen P, Ashie I, Nordkvist M (2005). Enzymatic process for obtaining increased yield of lactobionic acid. Canadian Patent WO104859; A3.

Canevascini G, Etienne K, Meier H (1982). A direct enzymatic lactose assay using cellobiose-(lactose-)dehydrogenase from *Sporotrichum thermophile*. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung; 175: 125-129.

Cappelli P, Vannucchi V (2005). Chimica degli alimenti, Chapter: Formaggio. Zanichelli.

Capper JL, Cady RA, Bauman DE (2009) The environmental impact of dairy production: 1944 compared with 2007. Journal of Animal Science; 87(6): 2160-2167.

Carvalho F, Prazeres A R, Rivas J (2013) "Cheese whey wastewater: Characterization and treatment". Science of the Total Environment 445–446: 385–396.

Cavallo G, Stagni M, Sodo E (2012) Ophthalmic composition based on lactobionic acid useful for reducing corneal edema and inflammation. European Patent Application Pub.No.: EP2494954A2.

Castelli S (2011). Biomasse per la produzione di energia. Maggioli S.p.A.

Chen S, Wen Z, Liao W, Liu C, Kincaid R, Harrison J, Elliott D, Brown M, Stevens D (2005) Studies into using manure in a biorefinery concept. Applied Biochemistry and Biotechnology 124 (1), 999–1015.

Cherubini F, Jungmeier G (2010) LCA of a biorefinery concept producing bioethanol, bioenergy, and chemical from swithgrass. The International Journal of Life Cycle Assessment; 15: 53-66.

Chung TW, Yang J, Akaike T, Cho KY, Nah JW, Kim SI, Cho CS (2002) Preparation of alginate/galactosylated chitosan scaffold for hepatocyte attachment. Biomaterials 23: 2827-2834.

Climent MJ, Corma SA, Iborra S (2011) Converting carbohydrates to bulk chemicals and fine chemicals over heterogeneous catalysts. Green Chemistry 13; 520-540.

Cort WM, Connors WM, Roberts HR, Bucek W (1956). Evidence for the formation and utilization of lactobionic acid by *Penicillium chrysogenum.* Archives of Biochemistry and Biophysics; 63: 477-478.

D'Alessio M (2014) Filiera lattiero-casearia: caratteri strutturali e andamenti congiunturali. Economia della produzione: 15-24.

D'Avino L, Croce B, Lazzeri L (2010). ISPRA Rapporti 128/2010 - Multifunzionalità dell'azienda agricola e sostenibilità ambientale - La chimica verde e il concetto di Bioraffineria.

Daufin G, Escudier JP, Carrère H, Bérot S, Fillaudeau L, Decloux M (2001) Recent and emerging applications of membrane processes in the food and dairy industry. Transactions of the Institution of Chemical Engineers; 79: Part C.

David F, Berger A, Hänsch R, Rohde M, Franco-Lara E (2011) Single cell analysis applied to antibody fragment production with *Bacillus megaterium*: development of advanced physiology and bioprocess state estimation tools. Microbial Cell Factories; 10: 23.

Dhariwal A, Mavrov V, Schroeder I (2006) Production of lactobionic acid with process integrated electrochemical enzyme regeneration and optimisation of process variables using response surface methods (RSM) Journal of Molecular Catalysis B: Enzymatic 42; 64–69.

Druliolle H, Kokoh KB, Beden B (1994) Elecrooxidation of lactose on platinum and on modified platinum-electodes in alaìkaline-emdium, Electochimica Acta 39: 2577-25

Druliolle H, Kokoh KB, Beden B (1995) Selective oxidation of lactose to lactobionic acid on lead-adatoms modified platinum-electrodes in $Na_2CO3 + NaHCO_3$ buffered medium. Journal of Electroanalytical Chemistry 385: 73-83.

Druliolle H, Kokoh KB, Hahn F, Lamy C, Beden B (1997) On some mechanistic aspects of the electrochemical oxidation of lactose at platinum and gold electrodes in alkaline medium. Journal of Electroanalytical Chemistry 426: 103-115.

Eddy BP (1958) Bacterial oxidation of lactose and melibiose. Nature, 181, 904-905.

Fan J, Shang Y, Yang J (2010) Preparation and characterization of chitosan/galactosylated hyaluronic acid scaffolds for primary hepatocytes culture. Journal of Material Science: Materials in Medicine 21: 319-327.

Fisher W, Mejer J (1889) Oxydation des milchzuckers. Berichte der Deutschen Chemischen Gesellschaft; 22:361-361-364.

Gigliotti C, Verga R (2007). Cap. Principali tecniche di microbiologia e enzimologia nel settore alimentare (author Verga R.), cap. Latte e derivati (authors Verga R e Gigliotti C) , cap. Alimenti ad uso zootecnico (Autore Marcozzi G.) in: Biotecnologie alimentari. Piccin Nuova Libraria S.p.A.

Green BA, Edison BL, Single ML (2008) Antiaging effects of topical lactobionic acid: Results of a controlled usage study. Cosmetic Dermatology; 21(2): 76-82

Guimarães PMR, Teixeira JA, Domingues L (2010) Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnology Advances; 28: 375-384.

Gutiérrez L-F, Hamoudi S, Belkacemi K (2011) "Selective production of lactobionic acid by aerobic oxidation of lactose over gold crystallites supported on mesoporous silica". Applied Catalysis A: General 402: 94–103.

Gutiérrez L-F, Hamoudi S, Belkacemi K (2012) "Lactobionic acid: A high valueadded lactose derivative for food and pharmaceutical applications". International Dairy Journal 26: 103-111.

Jang YS, Kim B, Shin JH, Choi YJ, Choi S, Song CW (2012) Bio-based production of C2-C6 platform chemicals. Biotechnology and Bioengineering 2012;10:2437–59.

Jones WE, Ho Y (2002) Novel techniques for the preparation and crystallization of 4-o-Beta-D-galactopyranosyl-D-gluconic acid. United State Patent Application Pub. No.: US 2002/0006884 A1.

Hart NA, Leuvenink HGD, Ploeg RJ (2002) New solutions in organ preservation. Transplantation Reviews 16 (3): 131-141.

Hendriks HEJ, Kuster BFM, Marin GB (1990) The effect of bismuth on the selective oxidation of lactose on supported palladium catalysts. Carbohydrate Research 204; 121-129.

Herigstad B, Hamilton M, Heersink J (2001) How to optimize the drop plate method for enumerating bacteria. Journal of Microbiological Methods Mar 1; 44(2):121-9.

Hua L, Nordkvist M, Nielsen PM, Villadsen J (2007) Scale-up of enzymatic production of lactobionic acid using the rotary jet head system. Biotechnology and Bioengineering; 97(4): 842-849.

Kakasi-Zsurka S, Todea A, But A, Paul C, Boeriu CG, Davidescu C, Nagy L, Kuki A, Keki S, Peter F (2011) Biocatalytic synthesis of new copolymers from 3-hydroxybutyric acid and a carbohydrate lactone. Journal of Molecular Catalysis B: Enzymatic; 71(1-2): 22-28.

Kapoor R, Metzger LE (2008) Process cheese: scientific and technological aspects-a review. Comprehensive Reviews in Food Science and Food Safety; Vol.7:194-214.

Kekkonen V, Lafreniere N, Ebara M, Saito A, Sawa Y, Narain R (2009) Synthesis and characterization of biocompatible magnetic glyconanoparticles. Journal of

Magnetism and Magnetic Materials 321: 1393-1396.

Kim EM, Jeong HJ, Kim SM, Sohn MH, Nah JW, Bom HS, Park IK, Cho CS (2006) Asialoglycoprotein-receptor-targeted hepatocyte imaging using 99mTc galactosylated chitosan. Nuclear Medicine and Biology 33: 529-534.

Kim IY, Seo SJ, Moon HS, Yoo MK, Park IY, Kim BC, Cho CS (2008) Chitosan and its derivatives for tissue engineering applications. Biotechnology Advances; 26(1): 1-21.

Kim BS, Park IY, Hoschiba T, Jiang HL, Choi YJ, Akaike T, Cho CS (2011) Design of artificial extracellular matrices for tissue engineering. Progress in Polymeric Science; 36(2): 238-268.

Kimura T (2006) Feed additive for laying hens and feed containing the additive. European Patent Application Pub. No: EP 1731042 A1.

Kimura T (2012) Development of a new functional oligosaccharides from lactose lactobionic acid. Seibutsu-kogaku Kaishi; 90: 595-597.

Kiryu T, Nakano H, Kiso T, Murakami H (2008). Purification and characterization of a carbohydrate: acceptor oxidoreductase from *Paraconiothyrium* sp that produces lactobionic acid efficiently. Bioscience Biotechnology and Biochemistry; 72: 833-841.

Kiryu T, Kiso T, Nakano H, Ooe K, Kimura T, Murakami H (2009) Involvement of *Acetobacter orientalis* in the production of lactobionic acid in Caucasia yogurt ("Caspian Sea yogurt") in Japan. Journal of Dairy Science; 92: 25-34.

Kiryu T, Yamauchi K, Masuyama A, Ooe K, Kimura T, Kiso T (2012) Optimization of lactobionic acid production by *Acetobacter orientalis* isolated from Caucasian fermented milk, "Caspian Sea yogurt". Bioscience Biotechnology and Biochemistry; 76: 361-363.

Kluyver AJ, de Ley J, Rijven A (1951) The formation and consumption of lactobionic and maltobionic acids by *Pseudomonas* species. Antonie Van Leeu-wenhoek; 17: 1-14.

Kosseva MR, Panesar PS, Kaur G, Kennedy JF (2009) "Use of immobilised biocatalysts in the processing of cheese whey". International Journal of Biological Macromolecules 45: 437-447.

Kuusisto J, Tokarev AV, Murzina EV, Roslund MU, Mikkola JP, Murzin DY, Salmi T (2007) "From renewable raw materials to high value-added fine chemicals—Catalytic hydrogenation and oxidation of D-lactose". Catalysis Today 121: 92–99.

Isbell HS (1934) Process for the preparation of calcium lactobionate. US Patent 1.980.996.

ISMEA (2012) Rapporto Annuale: 26-28.

Lazzeri L (2012). Presentation "Modelli di bioraffineria in agricoltura: le strategie europee".

Lee MH, Lai WL, Lin SF, Hsu CS, Liaw SH, Tsai YC (2005) Structural Characterization of Glucooligosaccharide Oxidase from *Acremonium strictum*. Applied and Environmental Microbiology ;71(21): 8881-8887.

Lee MH, Lai WL, Lin SF, Liu Y, Hsu YH, Tsai YC (2006). Purification and characterization of a novel cellooligosaccharide oxidase from rice pathogen *Sarocladium oryzae*. Enzyme and Microbial Technology; 39: 85-91.

Lin WL, Chen TD, Liu CW (2009) Synthesis and characterization of lactobionic acid grafted pegylated chitosan and nanoparticle complex application. Polymer 50: 4166-4174.

Lin SF, Hu HM, Inukai T, Tsai YC (1993) Production of novel oligosaccharide oxidase by wheat bran solid-state fermentation. Biotechnology Advances; 11: 417-427.

Lockwood LB, Stodola FH (1950) Process of culturing bacteria. United States Patent Application Pub. No.: US2496297.

Ludwig R, Ozga M, Zámocky M, Peterbauer C, Kulbe KD, Haltrich D (2004) Continuous Enzymatic Regeneration of Electron Acceptors Used by Flavoenzymes: Cellobiose Dehydrogenase-Catalyzed Production of Lactobionic Acid as an Example. Biocatalysis and Biotransformation 22; 97-104.

Maischberger T, Nguyen TH, Sukyai P, Kittl R, Riva S, Ludwing R, Haltrich D (2008) Production of lactose-free galacto-oligosaccharide mixtures: comparison of two cellobiose dehydrogenases for the selective oxidation of lactose to lactobionic acid. Carbohydrate Research 343: 2140-2147.

Magariello ER, Islip NY (1956) Production of lactobionic acid and its δ-lactone. US Patent 2746916.

Malvessi E, Carra S, Pasquali FC, Kern DB, da Silveira MM, Ayub MAZ (2013) Production of organic acids by periplasmic enzymes present in free and immobilized cells of *Zymomonas mobilis*. Journal of Industrial Microbiology & Biotechnology; 40(1): 1-10.

Manzoni M (2006). Cap. Terreni colturali e materie prime e cap. Etanolo in: Microbiologia industriale. Casa Editrice Ambrosiana. Pagg. 72-73, 77, 220, 249-253.

Mekonnen MM, Hoekstra AJ (2012) A Global Assessment of the Water Footprint of Farm Animal Products. Ecosystem 15: 401-415.

Meng Q, Tian L, Wang J (2012) Random amphiphilic copolymeric sub-micro particles as a carrier shielding from enzymatic attack for peptides and proteins delivery. Journal of Materials Science: Materials in Medicine; 23(4): 991-998.

Merril RK, Singh M (2011) Food ingredients and food products treated with an oxidoreductase and methods for preparing such food ingredients and food products. United State Patent Application Pub. No.: US 8021704.

Mirabella N, Castellani V, Sala S (2014) Current options for the valorization of food manufacturing waste: a review. Journal of Cleaner Production; 65: 28-41.

MiyakeT, Sato Y (1975) Process for producing aldonic acids and starch sugars containing aldonic acids. United States Patent Application Pub.: US 3862005.

Miyamoto Y, Ooi T, Kinoshita, S (2000). Production of lactobionic acid from whey by Pseudomonas sp LS13-1. Biotechnology Letters; 22: 427-430.

Murakami H, Kawano J, Yoshizumi H, Nakano H, Kitahata S (2002) Screening of lactobionic acid producing microorganisms. Journal of Applied Glycoscience, 49, 469-477.

Murakami H, Seko A, Azumi M, Ueshima N, Yoshizumi H, Nakano H (2003) Fermentative production of lactobionic acid by *Burkholderia cepacia*. Journal of Applied Glycoscience; 50: 117-120.

Murakami H, Seko A, Azumi M, Kiso T, Kiryu T, Kitahata S (2006) Microbial conversion of lactose to lactobionic acid by resting cells of *Burkholderia cepacia* no. 24. Journal of Applied Glycoscience; 53: 7-11.

Murakami H, Kiryu T, Kiso T, Nakano H (2008) Production of calcium lactobionate by a lactose-oxidizing enzyme from *Paraconiothyrium* sp. KD-3. Journal of Applied Glycoscience; 55: 127-132.

Nakano H, Kiryu T, Kiso T, Murakami H (2006) Lactobionic acid and its biocatalytic production. Foods and Food Ingredients Journal of Japan, 211, 874-881.

Nakano H, Kiryu T, Kiso T, Murakami H (2010) Biocatalytic production of lactobionic acid. In: Hou CT, Shaw JF, editors. Biocatalysis and biomolecular engineering. New Jersey: Jonh Wiley & Sons Inc.: 391-404.

Navarro LLorens JM, Tormo A, Martínez-García E (2010) Stationary phase in gram-negative bacteria. Microbiology Reviews; 34: 476-495.

Nielsen PM, Hoeier E (2009) Food products containing aldobionic acid. International Patent Application Pub. No.: WO 007398 A1.

Nielsen PM (2009) Meat based food product comprising lactobionic acid. United States Patent Application Pub. No.: US 0214752 A1.

Nishizuka Y, Hayaishi O (1962) Enzymic Formation of Lactobionic Acid from Lactose. The Journal of Biological Chemistry; 237: 2721-2728.

Nordkvist M, Nielsen PM, Villadsen J (2007) Oxidation of lactose to lactobionic acid by a *Microdochium nivale* carbohydrate oxidase: kinetics and operational stability. Biotechnology and Bioengineering 97; 694-707.

Oe K, Kimura T (2008) Mineral absorption promoter. Japan Patent Application Pub. No.: JP2008303208.

Oe K, Kimura T (2011) Aging inhibitor for bread. Japan Patent Application Pub. No.: JP2011177121.

Ortial S, Durand G, Poeggeler B, Polidori A, Pappolla MA, Böker J, Hardeland R, Pucci B (2006) Fluorinated Amphiphilic Amino Acid Derivatives as Antioxidant Carriers: A New Class of Protective Agents. Journal of Chemical Chemistry; 49(9): 2812-2820.

Oskarsson H, Frankenberg M, Annerling A, Holmberg K (2007) Adsorption of Novel Alkylaminoamide Sugar Surfactants at Tailor-made Surfaces. Journal of Surfactants and Detergents 10: 41-52.

Pantazaki AA, Papaneophytou CP, Pritsa AG, Liakopoulou-Kyriakides M, Kyriakidis DA (2009) Production of polyhydroxyalkanoates from whey by *Thermus thermophilus* HB8. Process Biochemistry; 44: 847–853

Parekh S, Vinci VA, Strobel LJ (2000) Improvement of microbial strains and fermentation processes. Applied microbiology and biotechnology 54 (3): 287-301.

Pedruzzi I, Malvessi E, Mata VG, Borge da Silva EA, Silveira M, Rodrigues AE (2007) Quantification of lactobionic acid and sorbitol from enzymatic reaction of fructose and lactose by high-performance liquid chromatography. Journal of Chromatography; 1145: 128-132.

Pedruzzi I, Borges da Silva EA, Rodrigues AE (2008) Selection of resins, equilibrium and sorption kinetics of lactobionic acid, fructose, lactose and sorbitol. Separation and Purification Technology 63: 600-611.

Pedruzzi I, Borges da Silva EA, Rodrigues AE (2011) Production of lactobionic acid and sorbitol from lactose/fructose substrate using GFOR/GL enzymes from *Zymomonas mobilis* cells: a kinetic study. Enzyme and Microbial Technology; 49: 183-191.

Peretti FA, Silveira MM, Zeni M (2009) Use of electrodialysis technique for the separation of lactobionic acid produced by *Zymomonas* mobilis. Desalination 2009; 246: 253-257.

Pieri R (2010) Il mercato del latte. Rapporto 2011. Osservatorio sul mercato dei prodotti zootecnici. Franco Angeli editore.

Povolo S, Toffano P, Basaglia M, Casella S (2010) Polyhydroxyalkanoates production by engineered *Cupriavidus necator* from waste material containing lactose. Bioresource Technology; 101: 7902–7907.

Prazeres AR, Carvalho F, Rivas J (2012) "Cheese whey management: A review". Journal of Environmental Management 110: 48-68

Qureshi N, Annous BA, Ezeji TC, Karcher P, Maddox IS (2005) Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. Microbial Cell Factories; 4 (24): 1-21.

Ramesh C (1997) Dairy-Based Ingredients. Eagan Press Handbook Series. Chapter 1,3 and 4.

Saha T, Ghosh D, Mukherjee S, Bose S, Mukherjee M (2008) Cellobiose dehydrogenase production by the mycelial culture of the mushroom *Termito-myces clypeatus*. Process Biochemistry, 43, 634-641.

Salvadori Del Prato O (1998) Trattato di tecnologia casearia. Edagricole-Bologna.

Sansonetti S, Curcio S, Calabrò V, Iorio G (2009) Bio-ethanol production by fermentation of ricotta cheese whey as an effective alternative non-vegetable source. Biomass and Bioenergy; 33: 1687-1692.

Satory M, Fürlinger M, Haltrich D, Kulbe KD, Pittern F, Nidetzky B (1997) Continuous enzymatic production of lactobionic acid using glucose-fructose oxidoreductase in an ultrafiltration membrane reactor. Biotechnology Letter 19 (12): 1205–1208.ù

Savary BJ, Hicks KB, O'Connor JV (2001) Hexose oxidase from Chondrus crispus: improved purification using perfusion chromatography. Enzyme and Microbial Technology; 29: 42-51.

Schaafsma G (2008) "Lactose and lactose derivatives as bioactive ingredients in human nutrition". International Dairy Journal 18: 458–465.

Secchi N, Giunta D, Pretti L, García MR, Roggio T, Mannazzu I, Catzeddu P (2012) Bioconversion of ovine scotta into lactic acid with pure and mixed cultures of lactid acid bacteria. Journal of Industrial and Microbiological Biotechnology 39: 175-181.

Selim KMK, Ha YS, Kim SJ, Chang Y, Kim TJ, Lee GH, Kang IK (2007) Surface modification of magnetite nanoparticles using lactobionic acid and their interaction with hepatocytes. Biomaterials 28: 710-716.

Selim KMK, Xing ZC, Guo H, Kang IK (2009) Immobilization of Lactobionic acid on the surface of cadmium sulphide nanoparticles and their interaction with hepatocytes. Journal of Material Science: Materials in Medicine 20:1945-1953.

Severo Junior JB (2008) Síntese biocatalítica do sorbitol e ácido lactobiônico com separação simultânea por eletrodiálise. MSc. thesis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

Splechtna B, Petzelbauera I, Bamingera U, Haltricha D, Kulbea KD, Nidetzky B (2001) Production of a lactose-free galacto-oligosaccharide mixture by using selective enzymatic oxidation of lactose into lactobionic acid. Enzyme and Microbial Technology 29 (6-7): 434-440.

Stodola FH, Lockwood LB (1947) The oxidation of Lactose and Maltose to Bionic Acids by *Pseudomonas*. The Journal of Biological Chemistry; 171: 213-221.

Sugiyama M, Watanabe K, Funakoshi N, Amino Y, Kawahara S, Takemoto T (2005) Process of producing glutamate derivatives. United States Patent Application Pub. No.: US 0009153; A1.

Tasic-Kostov M, Pavlovic D, Lukic M, Jaksic I, Arsic I, Savic S (2012) Lactobionic acid as antioxidant and moisturizing active in alkyl polyglucoside-based topical emulsions: the colloidal structure, stability and efficacy evaluation. International Journal of Cosmetic Science 34: 424-434.

Thomas SM, DiCosimo R, Nagarajan V (2002) Biocatalysis: applications and potentials for the chemical industry. Biotechnology; 20 (6): 238-242.

Tokarev AV, Murzina EV, Eränen K, Marcus H, Plomp AJ, Bitter JH, Mäki-Arvela P, Murzin DY (2009) Lactose oxidation over palladium catalysts supported on active carbons and on carbon nanofibres. Research on Chemical Intermediates 35: 155-174.

Tomlinson GA, Strohm MP, Hochstein LI (1978) The metabolism of carbohydrates by extremely halophilic bacteria: the identification of lactobionicacid as a product of lactose metabolism by *Halobacterium saccharovorum*. Canadian Journal of Microbiology; 24: 898-903.

Vakil JR, Shahani KM (1969) Carbohydrate metabolism of lactic acid cultures. V. Lactobionate and gluconate metabolism of *Streptococcus lactis* UN. Journal of Dairy Science; 52: 1928-1934.

Van Hecke W, Bhagwat A, Ludwing R, Dewulf J, Haltrich D, Van Langenhove H (2009). Kinetic Modeling of a Bi-Enzymatic System for Efficient Conversion of Lactose to Lactobionic Acid. Biotechnology and Bioengineering 102: 1475-1482.

Van Hecke W, Haltrich D, Frahm B, Brod H, Dewulf J, Van Langenhove H, Ludwig R (2011) A biocatalytic cascade reaction sensitive to the gas–liquid interface: Modeling

and upscaling in a dynamic membrane aeration reactor. Journal of Molecular Catalysis B: Enzymatic 68: 154–161.

Vijayaraghavan K, Han MH, Choi SB, Yun YS (2007) Biosorption of Reactive black 5 by *Corynebacterium glutamicum* biomass immobilized in alginate and polysulfone matrices. Chemosphere; 68: 1838-1845.

Volc J, Sedmera P, Kujawa M, Halada P, Kubatova E, Haltrich D (2004). Conversion of lactose to beta-D-galactopyranosyl-(1/4)-D-arabino-hexos-2-ulose-(2-dehydrolactose) and lactobiono-1,5-lactone by fungal pyranose dehydrogenase. Journal of Molecular Catalysis B & Enzymatic; 30: 177-184.

Wright DG, Rand AG (1973). Direct enzymatic conversion of lactose to acid lactose dehydrogenase. Journal of Food Science; 38: 1132-1135.

Yang BY, Montgomery R (2005) Oxidation of lactose with bromine. Carbohydrate research 340: 2698-2705.

Yu RJ, Van Scott EJ (2004) Alpha-hydroxyacids and carboxylic acids. Journal of Cosmetic Dermatology; 3(2): 76-87.

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