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## DEVELOPMENT OF BIOREFINERY SCHEMES FOR THE FOR THE PRODUCTION AND RECOVERY OF VOLATILE FATTY ACIDS FROM AGRO-INDUSTRIAL WASTES

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# Abstract

Volatile fatty acid (VFAs) are short chain aliphatic acids which represent the final product of anaerobic acidogenic fermentation of organic matter. Since VFAs can be exploited as precursors for the production of several added-value chemicals and materials, the biotechnological conversion of organic agro-industrial wastes into VFAs can be considered a valuable alternative to waste disposal. However, the productivity of processes, which refer to the carboxylation platform (*i.e.*, (bio)chemical reactions that convert carboxylic acids into target molecules), are affected by the purity of produced VFAs. Some industries, like Biosphere SRL (Forli, Italy), had started to valorize agro-industrial wastes to obtain energy in form of methane.

VFAs were produced using maize silage (MS), vegetable waste (VW) and cheese whey (CW) as feedstock. As first approach batch testes were carried out in microcosms experiments to get some knowledge about the potential of the substrates and the best mixture composition for the maximization of VFAs production. The co-digestion of VW (70%) and MS (30%) (VMW) under continuous operation with hydraulic retention time (HRT) of 6 days and a controlled pH of 6 and 7 resulted in a VFAs rich solution of 30 g.L$^{-1}$ mainly composed by acetic, butyric and propionic acids. The fermentation of CW was performed in a packed bed bioreactor where Vukuopor was used as supported material. The HRT, organic loading rate (OLR) and pH were parameters tested under continuous operation. By a HRT of 6 days, ORL of 4.2 g.L$^{-1}$.d$^{-1}$, and pH 5.8−6, 16 g·L$^{-1}$ of total VFAs were produced, with a yield higher than 75%.

After the production of VFAs from wastes and under biological processes a concentration step was performed in order to obtain a concentrated solution of VFAs suitable to employ in further processes such as the biopolymers (PHA) production.

Electrodialysis (ED) process was studied for the concentration of VFAs from the two acidogenic effluents resulted from the previous anaerobic acidogenic digestions mentioned before. With a membrane stack composed by 0.116 m$^2$ of total surface membrane area and under potentiostat operation at 10 V, different experiments were performed in a first phase with simulated solution and then with real acidogenic effluent rich in VFAs. The VMW concentration stream was concentrated from 29 g.L$^{-1}$ to 50 g.L$^{-1}$ in approximately 400 minutes of ED run. Thought it is expected that a concentration of 100 g.L$^{-1}$ can be achieved by performing sequential batch of ED within 1400 minutes. With CW acidified effluent the concentration achieved was 60 g.L$^{-1}$ in 2130 minutes since the initial solution was much more diluted (9 g.L$^{-1}$).

Experiments with Nanofiltration were performed for the separation of ferulic acid (FA) and sugars from an enzymatic hydrolysate of wheat bran. In total, eight different membranes, were screened at different applied pressures and solution pH. It was observed that the retention of charged molecules was due to the electrostatic interactions with the membrane surface area while for uncharged molecules was the molecular sieving. The best conditions for diafiltration and concentration step of FA was found be membrane NT7450 operated at pH 9 and 16 bar as applied pressure.

Keywords: Fermentation, VFAs, electrodialysis, nanofiltration

# Riassunto

Gli acidi grassi volatili (VFAs) sono acidi alifatici a catena breve che rappresentano il prodotto finale della fermentazione anaerobica acidogenica di materia organica. Poiché VFAs può essere sfruttato come precursori per la produzione di numerosi prodotti chimici e materiali a valore aggiunto, la conversione biotecnologica di rifiuti organici agro-industriale in VFAs può essere considerato una valida alternativa allo smaltimento dei rifiuti. Tuttavia, la produttività dei processi, che si riferiscono alla piattaforma carbossilica (vale a dire, (reazioni bio) chimici che convertono gli acidi carbossilici in molecole *target*), sono colpiti dalla purezza del prodotto VFAs. Alcuni industrie, come la Biosphere SRL (Forli, Italia), hanno gia iniziato a valorizare i rifiuti agro-industriali per ottenere energia in forma di metano.

VFAs sono stati prodotti utilizzando insilato di mais (MS), scarti vegetali (VW) e siero di latte (CW) come materia prima. Come primo approccio batch test sono stati effettuati esperimenti in microcosmi per ottenere la conoscenza del potenziale dei substrati e la migliore composizione della miscela per la massimizzazione della produzione di VFAs. La co-digestione del VW (70%) e MS (30%) (VMW) in operazioni continue con tempo di ritenzione idraulica (HRT) di 6 giorni e un pH controllato di 6 - 7 hanno risultato in una soluzione ricca in VFAs di 30 g.L$^{-1}$ composto principalmente da acido acetico, butirrico e propionico. La fermentazione di CW è stata eseguita in un bioreattore a letto impaccato dove Vukuopor è stato usato come materiale di immobilizzazione. Il HRT, il carico organico (OLR) e pH sono parametri testati in operazione in continuo. Con HRT di 6 giorni, ORL di 4.2 g.L$^{-1}$.d$^{-1}$, e pH 5.8-6, 16 g.L$^{-1}$ di VFAS totale sono stati prodotti, con una resa superiore al 75%.

Dopo la produzione di VFAS da rifiuti sotto processi biologici una fase di concentrazione è stata eseguita in modo da ottenere una soluzione concentrata di VFAs adatta da impiegare in ulteriori processi quali la produzione di biopolimeri (PHA).

Il processo di Elettrodialisi (ED) è stato studiato per la concentrazione di VFAs delle due effluenti acidogeni risultanti delle digestion anaerobica acidogenici menzionati prima. Con uno stack di membrana composta da 0.116 m$^2$ di superficie totale di membrana e sotto il funzionamento potenziostato a 10 V diversi esperimenti sono stati eseguiti in una prima fase con soluzione simulata e poi con reale effluenti acidogenica ricco di VFAs. Il flusso di concentrazione VMW si è concentrata da 29 g.L$^{-1}$ a 50 g.L$^{-1}$ in circa 400 minuti di corsa ED. Si prevede che una concentrazione di 100 g.L$^{-1}$ può essere ottenuta in 1400 minuti eseguendo batch sequenzilae di ED. Con CW acidificata la concentrazione ottenuta è stata di 60 g.L$^{-1}$ tuttavia la soluzione iniziale era molto più diluita (9 g.L$^{-1}$).

Esperimenti con Nanofiltrazione sono stati eseguiti per la separazione di acido ferulico (FA) e zuccheri da un idrolizzato enzimatico di crusca di grano. In totale, otto membrane differenti, sono stati studiate a diverse pressioni applicate e different pH della soluzione. È stato osservato che la ritenzione di molecole cariche è dovuta alle interazioni elettrostatiche con la superficie della membrana mentre per molecole non caricate era la setacciatura molecolare. Le migliori condizioni per diafiltrazione e fase di concentrazione di FA è stato trovato per la membrana NT7450 funzionare a pH 9 e 16 bar di pressione applicata.

Parole chiave: Fermentazione, VFAs, electrodialisis, nanofiltrazione

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# Abbreviations

| | |
|---|---|
| AFMC | Anaerobic fermentation mixed culture |
| COD | Chemical Oxygen Demand |
| Conc | Concentrated |
| CW | Cheese Whey |
| $CW_{acid}$ | Cheese Whey fermented broth |
| $CW_{acid}^{Sim}$ | Cheese Whey fermented broth simulated |
| Dil | Diluted |
| DiaNF | Dia-nanofiltration process |
| ED | Electrodialysis |
| EF | Enrichment Factor |
| EtOH | Ethanol |
| F | Faraday Constant |
| HRT | Hydraulic Retention Time |
| $Hydrol_{enzym}$ | Enzymatic hydrolysate |
| $Hydrol_{enzym}^{sim}$ | Enzymatic hydrolysate simulated |
| $i$ | Ion |
| IEP | Isoelectric Point |
| Jv | Flux |
| MS | Maize Silage |
| $N_i$ | Molar cumulative transport |
| PBBR | Packed bed bioreactor |
| PHA | Polyhydroxyalkanoates |
| rem | removal |
| $T_i$ | Transfer number |
| TMP | Transmembrane pressure |
| VFAs | Volatile Fatty Acids |
| VW | Vegetables Waste |
| $VMW_{acid}$ | Vegetable waste and maize silage fermented broth |
| $VMW_{acid}^{Sim}$ | Vegetable waste and maize silage fermented broth simulated |

# 1. Motivation and thesis outline

## 1.1 Motivations

The high production of waste is a problem worldwide specially because it is mandatory to perform some kind of treatment before through in the environmental. This treatment means to the industries spending money to avoid pollution. Due to the fast grow of the world population promotes the production of high amounts of food waste worldwide. However, these types of wastes are composed by organic matter that arouse the interest of transform what is not suitable for alimentation in something useful and valuable. One way to valorise residues is to take advantage of the organic matter content and transform it by into energy, e.g. biomethane. However nowadays methane has been lost the economic interest and so new add-value products are requested to keep valorising the wastes. This is one of the activities of Biosphere S.r.l., (Bertinoro, FC, Italy) an Italian enterprise which already active in agro-industrial waste management and valorisation. Biosphere S.r.l. together with an industrial partner, Ayrion (Bertinoro, FC, Italy), have been working in the anaerobic digestion from: vegetable wastes, maize silage and zootechnical animal effluent.

With the concern about the economical part and at the same time the generation of wastes, Biosphere S.r.l. asked: What kind of new add-value products can be obtained from the mentioned wastes?

Volatile fatty acids (VFAs) can be exploited as precursors for the production of several added-value chemicals and materials. The biotechnological conversion of organic agro-industrial wastes into VFAs can be considered a valuable alternative to waste disposal. However, the productivity of bioprocesses, which refer to the carboxylation platform (*i.e.*, (bio)chemical reactions that convert carboxylic acids into target molecules), are affected by the purity and concentration of produced VFAs.

All this considered the main goals of the present PhD thesis were:

a) Biotechnological anaerobic acidogenic processes dedicated to the production of VFAs from ago-industrial wastes currently used by Biosphere.

b) Physical-chemical strategies for the selective extraction of produced VFAs from the effluents of the acidogenic processes.

## 1.2 Thesis outline

The present thesis includes eight chapters describing the work developed.

The current chapter (Chapter 1) includes the motivations for the development of the present thesis and the respective main goals to achieve.

The chapter 2 presents the State of the art of the main processes reported in the literature about production of carboxylic acids, wastes production and recovery of carboxylic acids.

The chapter 3 and chapter 4 have in common being both about the anaerobic fermentation process to obtain the mixture of VFAs. Both have studies at small scale batch operation and then in continuous operation. Though, chapter 3 is by employing the same substrates currently employed in

the biogas plant held by Biosphere SRL and partner. Maize silage and vegetable wastes were used in co-digestion as substrate in the bioreactors with freely suspended cells. While chapter 4 is using cheese whey, a dairy waste, as substrate to fed a packed bed bioreactor.

After the collection of two different effluents rich in VFAs an electrodialysis process, presented and discussed in chapter 5, was studied to obtain a concentrated stream in VFAs suitable to employ in further biological processes.

In Chapter 6 is presented a Nanofiltration process study to recover ferulic acid from an enzymatic hydrolysate from wheat bran. This activity was carried out abroad was carried out in the Agro-Biotechnologies Industrielles (ABI) AgroParisTech, Reims – France.

Finally, the Chapter 7 contains all the bibliography consulted and used for discussion of the results obtained in the present work.

# 2. State of the art

## 2.1 VFAs production

Volatile fatty acids (VFAs) are chemically defined as short-chain length (C2 to C6) carboxylic acids, namely: acetic, propionic, butyric, valeric and hexanoic [1]. They are molecules which can be integrated through the carboxylation platform and potential precursors for the obtainment of a variety of compounds such as ketones, aldehydes, esters, alcohols and alkanes [2].

Nowadays VFAs are produced by chemical routes, e.g. acetic acid through Monsanto process [3]. There is a globalised concern about sustainability of production processes which prompted the development of alternative green technologies for replacing petrochemical-based production routes. The main target of this approach is to achieve economically-feasible and environmentally-friendly processes by making use of renewable resources as feedstock. This could lead to the development of new industrial production routes, e.g., polyhydroxyalkanoates and single esters. The last two decades, several studies have been conducted to develop a production scheme for VFAs by biological process using cheap, renewable carbon sources in an eco-friendly approach. A biological process, as anaerobic digestion, can be an alternative to the conventional production of VFAs. Anaerobic digestion is a collection of processes by which microorganisms convert biodegradable compounds in the absence of oxygen. After hydrolysis of organic polymers such as carbohydrates, during acidogenesis, acidogenic bacteria convert organic matter as sugars and amino acids into dioxide carbon, alcohol and organic acids[4,5]. The last step is methanogenesis in which organic acids are convert to methane and carbon dioxide. When methane production is inhibited (e.g., by setting low pH and/or high organic loading rates) VFAs tend to accumulate: VFAs related methanogenesis repression. This process is called acidogenic fermentation. Pure cultures of acidogenic strains can be employed for the production of organic acids, achieving high yields, remarkable reproducibility and final product titers (50-180 g.L$^{-1}$). Nevertheless, this approach is typically expensive due to the request of high quality substrates and reactor sterilization costs. Alternatively, mixed microbial consortia, composed by many different members of bacteria giving it robustness to support feed variations and synergies effect when extra supplements are needed [6] can make profitable use of undefined leftover material occurring in agro-industrial wastes without requiring sterilization. Scoma et al. [7] recently discussed some potential valorisation platforms for high-impact agro-industrial residues of the Mediterranean area, which include olive mill wastewater, tomato pomace, grape pomace and cheese whey among others. Adom et al.[8] reported valorisation strategies of dry-grind ethanol, which could supply the succinic acid and histidine industrial demands. Both these studies focus on the fact that many agro-industrial wastes are mainly composed of carbohydrates, proteins and lipids, which represent per se valuable compounds for many biotechnological processes. However, with few exceptions (e.g.: biodiesel glycerol, molasses and oils) where the substrate concentration is comparable to those of prepared feeding solutions, the organic content in these residues would not allow developing conventional pure culture fermentations with high productivities and titers. Nevertheless, they hold high chemical oxygen demands (COD; $30 < COD < 100$ gO2.L$^{-1}$), a strong acidic potential, possible anti-microbial activities and, therefore, slow

biodegradability. As crops are subjected to seasonality, leftovers are generated within 2-4 month periods, and their massive discharge prior to any treatment may lead to eutrophication of water bodies and/or reduced fertility of cultivable soils. Anaerobic fermentation carried out by mixed microbial consortia can play a strategic role in changing waste treatment into a biotechnological valorisation of carbohydrates, proteins and lipids hold by these residues, in order to generate added-value molecules such as VFAs.

The VFAs production is affected by operational parameters such as: pH, HRT and OLR [1,2,9,10]. According with the operational parameters, the microorganisms will produce different mixtures of VFAs. Consequently, the biological process must be operated according with the type of acid desired for the further applications.

## 2.2 Food wastes

Every day tones of wastes are produced around the world with, agriculture, industry or domestic source. Specifically, food wastes can be: fruits and vegetables (from agriculture of processed food), cheese whey (from dairy industry), olive mill waste water (OMW) (from olive oil production) or grape pomace (from winery production). The generation of food wastes has costs associated either economic and environmental. The economic cost includes not only the cost linked to the value of the products themselves, but also the costs linked to the production, transport and storage of the wasted products, as well as their treatment costs. From an environmental point of view, food waste represents a waste of the resources throughout the products' life cycle such as land, water, energy and other inputs, and the consequent increase in greenhouse gas emissions [11]. These wastes present harmful characteristics, which will be developed after. Anyhow it can be said that due to its characteristics, it is mandatory to perform some kind of treatment before discharged in the environment. It has been estimating that for each ton of food wastes there is an emission of about 2 tons of $CO_2$ [12]. Some efforts have been made to prevent the environmental problems. Italy is an example were some projects have been developed in this direction: a)The financing of a cereal storage silo which drastically reduced (from around 12 % down to 0.2 %) waste of cereals due to moulds and pollution by birds and rodents and b) Financing of investment in a dairy cow shed (moving from a tie-stall to a free stall with mattresses, scrapers, etc.) led to improved animal welfare and hygiene conditions, which in turn led to a reduction in the number of cows with mastitis and in the volume of milk wasted.

Much of these wastes have in their composition organic compounds which can be used in other productions such as materials or energy. And can be applied to the mentioned biological processes to produce the VFAs. Instead of doing and investment on a conventional treatment without valorization of all these residues, the situation could be interpreted as an opportunity to implement a technology with which the by-products are valorised by the production of added values [13]. In another words the situation for applying the bio-refinery concept: *maximization of each stream value*

### 2.2.1 Vegetables wastes

The food industry has developed very fast in the last decades as a response to the fast growing of world population and consequently, also the amounts of food waste have been increasing. This type of waste covers all the food life cycle: from agriculture, industrial manufacturing and processing (39%), retail and household (42%) [12]. According to FAOSTAT, between 2008 and 2015 in European Union countries about 1.8 million tonnes of fruit and vegetables were withdrawn from the market and 66% of these withdrawn products were wasted, and over 45,500 ha of land were harvested before maturity or have not been harvested [11]. In specific case of Italy, food wastes are generated along several stages of supply chain: 3.3% of agri-food products and 2.6% of final products from food industry are discarded before selling, while the loss in food retailers amounts to 250,000 ton/year (of which 40% are fruits and vegetables) [14]. Throw this waste in environmental without any treatment could cause serious damage to the environment such as cause an excess of oxygen depletion, reducing aquatic life, impermeabilization decreasing crop yield, eutrophication, toxicity, etc., in the receiving environments.

Despite all the harmful composition that compose these wastes it is also composed by around 75% of sugars [12] that can be used as only substrate or in co-digestion for the production of add-value products as had already reported in literature [15–21]. Biogas in form of methane and hydrogen are examples of the add-value products produced from vegetable wastes. To produce methane H. Bouallagui et al. [15] used fruits and vegetables wastes as substrate for the production of biogas which 64% was composed by methane in a tubular digester operated with an HRT of 20 days. The production of hydrogen has the advantage that use lower HRT respects to the methane production. With an HRT of only 2 days Tenca et al. [17] was able to produce biogas composed by 42% of hydrogen. Also by applying a shorter HRT, respects to methane production, it is possible to produce VFAs by using vegetable waste as substrate. Sans et al [18] successfully produced a VFAs-richer solution containing 19-24 g.L$^{-1}$ of VFAs where acetic acid was the main acid produced, followed by butyric and propionic acids.

### 2.2.2 Cheese whey

Dairy industry is practised all over the world for the production of milk, butter, yogurt, ice cream, cheese, and other milk derivate. From this activity, big amounts of high COD content waste are generated as effluents. Cheese is the milk derivate most produced around the world and Europe represents the higher producer with 57% of world production. From all wastes resulted from the dairy industry, cheese whey (CW) is the major and most contaminated waste generated in the cheese production. As rule of thumb it can be said that for production of 1 kg of cheese 10 kg of milk are used and 9 kg of CW are obtained as by-product [22]. According to FAO 10 M tons of cheese are produced on an annual base, resulting generation of 92 M tons of cheese whey. CW characteristics in general

terms is a green, due to riboflavin content, yellowish liquid resulting from the precipitation and removal of milk casein in cheese production process [22]. It is mainly composed by lactose, proteins, lipids and minerals [23]. The pH of CW depends on the procedure used for precipitation of casein protein. An acidic CW has a pH<5 and have a lower protein content while sweet CW has a pH 6-7 [22]. Cheese whey powder (CWP) consists in CW lyophilized and despite lyophilisation costs, this way of CWP is easily storage and transported since has a reduced volume and long-term stability [24].

Due to its characteristics CW is harmful for the environment it must be treated before being discharged in the ecosystems to prevent serious pollution problems for the surrounding environments. With a high COD content and hard biodegradability of lipids and proteins, a small variation on the content of these in the effluent can generate problems in a conventional treatment plant. From this it is difficult or almost impossible to treat it with other wastes. It is possible to take advantage of the sugars contained in the CW and use as substrate for an anaerobic digestion. In this process, microbial consortiums promote bioconversion of dissolved organic compounds present in the waste, like lactose, into valuable compounds; i.e: VFAs, lactic acid, methane, hydrogen, etc. [2]

In which respect to anaerobic digestion of CW is normally carried out at mesophilic conditions (25-38ºC). During this process lactose is bioconvert to lactic acid, VFAs, ethanol, among others. From proteins polypeptide, aminoacids and ammonia are produced. CW has a low quantity of proteins but a high content of lactose. This last is characterized as a disaccharide sugar derived from condensation of galactose and glucose. Microorganisms known species which can metabolize lactose are notably less then microorganisms able to perform the conversion of other simple sugars like glucose [23]. In the first group is, for example, *K. marxianus* specie of yeast capable to produce lactase enzyme and from it use lactose to produce ethanol. Ethanol production has been already reported in several studies in which were used diverse types of CW: raw CW, CWP solution [24], CW permeate from ultrafiltration and even CW deproteinized. However, from the reported studies it was seen that ethanol production using CW as substrate is not economically competitive respecting to when other wastes like cane sugar or corn starch are used as substrate. Biogas production is another possibility for valorization of CW by a biological process. Hydrogen is a clean energy and the production CW and is an economical viable option to produce it. After hydrogen production, the effluent generated has a significant COD value since is manly composed by VFAs, alcohols and carbohydrates not being possible to be discharge without an adequate treatment [22]. Thus, those compounds can be used in other fermentations and this way, after CW valorisation, also hydrogen effluent is valorised.

There are also some works in the literature which report some species able to bioconvert the lactose content in CW into lactic acid either as mixed cultures [25] as pure [26,27]. Lactic acid is a carboxylic acid with pKa value of 3.86, one unit less than acetic acid (4.75) and it is involved in several biochemical reactions and can be used in food or pharmaceutical industry [25]. Besides lactic acid, also VFAs production from CW has been study [10]. VFAs represent a group of carboxylic acids with perspectives for production of another add-value products.

A possible drawback, of production of add-value products from CW, is being a nutrient deficient and so, supplementation is needed to avoid slow microorganisms growth. It was observed that with nutrient supplementation or used of mixed cultures the lactic acid productivity increased significantly [22]. Bioreactor configuration can also contribute for increasing the productivity, for example the employ of immobilization.

## 2.3 Organic acids recovery

As it was mentioned before, the production of carboxylic acids, precisely VFAs, via anaerobic fermentation and specifically from wastes has the advantage of a low-cost process however one of the drawbacks of the later uses of these products is the low concentration and purity achieved. In order to be possible to use these low cost carboxylic acids in further processes the development of a competitive downstream process is critical since its costs are large, typically 30–40% of the total production costs [28]. Nowadays the recovery of acids can be performed by: liquid-liquid extraction [29,30], ion exchange [31], precipitation [28] and membrane technologies such as electrodialysis (ED) [32–42] and nanofiltration (NF) [42–44].

From the mentioned techniques, membrane processes had arouse very interest since they offer high productivity and low operational cost compared to other competing technologies [45], low energy consumption, unique separation properties and environmental friendly [46,47].

### 2.3.1 Electrodialysis

Electrodialysis (ED) is a separation process in which charged membranes are placed alternatively and an electrical field is applied, as driving force, to promote the separation of the molecules. The molecules positively or negatively charged (ions) will be affected by this electrical field however the uncharged molecules will not be affected. The operation of the ED unit at constant voltage (potentiostatic mode) is preferred compared to the operation at constant current (galvanostatic mode) due to safety reasons. During the ED process the concentration, and consequently electric conductivity, of the solution in diluted stream decreases resulting in the increase of electric resistance of the ED unit. In the case of the galvanostatic operating mode it could results in uncontrolled increase of voltage applied on the ED unit and potential collapse of electric current source or, in extreme cases, membrane stack due to Joule heating. The solutions flow through the ED unit upward to facilitate gas bubbles removal from the active space between the membranes or from the electrode compartments. The presence of the gas phase is undesirable because bubbles increase the electric resistance of the intermembrane space and block (reduce) active membrane surface and thus reduce the performance of the unit.

This technique has been reported in literature and applied at laboratory scale for the recovery and/or concentration of carboxylic acids such as lactic [32,48–50], succinic [28,42,51], fumaric and acids from the VFAs group either in separate or in mixture acetic [3,34,40,41,52–55].

In the literature, ED works have been reported from the last 30 years for carboxylic acids recovery however only in the last few years there are works with real acidogenic effluents coming from fermentation. Scoma and co-workers were one of the firsts to publish the concentration of a mixture of VFAs from a coming from an acidogenic fermentation process. Under galvanostatic mode (31 A.m$^{-2}$) Scoma et al. [41] was able to concentrate the VFAs mixture that composed the acidogenic fermentation broth of olive mill wastewater containing 14.5 g.L$^{-1}$ of a VFAs mixture, where acetic acid was the main acid, increased the concentration factor in 1.2, resulting in approximately 18 g.L$^{-1}$. Similarly to what was the aim of the present thesis Bing Tao [55] developed an integrated work in which propose the employment of the VFAs concentrated stream from ED for the production of PHA. After the production of VFAs, the acidified effluent was concentrated at constant voltage of 17 V and with a total membrane surface area of 1.28 m$^2$. After 17 hours of ED run the 6.15 g.L$^{-1}$ of VFAs were transfer from diluted to the concentrated compartment. Since the volume in the concentrated compartment was 2.5 times lower than the diluted compartments it was possible to achieve a final concentration of 19.81 g.L$^{-1}$. This concentrated solution was used as substrate for the PHA production were the PHA accumulation was increased 7 times compared when the diluted stream was used as substrate [55].

### 2.3.2    Nanofiltration

Pressure-driven membrane separation such as nanofiltration is the membrane technology promising for the downstream of the carboxylic acids. Respect to other membrane processes such as ED, have the advantage of high energy efficiency and easily to scale-up [56]. During NF process not only the charge of the molecule is important (electrostatic interactions) to separate but also the size (molecular exclusion) being one process efficient to separate acids from sugars [57]. The nanofiltration membranes have the particular characteristic of be able to have surface uncharged, positively or negatively charged according with the pH solution which are in contact. The fact that membrane surface can change not only the signal of the charge but also the intensity of the charge can influence the retention of charged molecules. The molecular sieving will influence particular the uncharged molecules which will be as retained as higher the difference between the molecular weight and the membrane pore size (cut-off).

By playing with this NF membranes characteristics it is possible to separate, e.g. sugars from acids as it was performed by Yu-Hsiang Weng et al. [44]. The work had the aim to study the best pH and pressure to separate acetic acid from xylose with one of the most used membranes DK from General Electrics (GE). The observed retention of xylose and acetic acid varied from 28% to 81% for xylose and 6.8% to 90% for acetic acid depending on the solution pH and the applied pressure. The retention of acetic acid was higher as higher the solution pH due to the electrostatic repulsions with the membrane surface which, as acetic acid, was also negatively charged at pH higher than 5.

There are few studies in the literature respects to the separation of carboxylic acids mixture with nanofiltration [45,56]. Recently Boya Xiong et al. [56] published a work in which studied two nanofiltration membranes (DK and DL from GE) at pilot scale (138 cm$^2$) for the separation of carboxylic acids (acetic, lactic, propionic, butyric and hexanoic acids) from a mix of sugars (xylose, glucose, galactose, manose and arabinose) which compose a willow wood digested liquor. It was tested the membrane flux, effect of the pH, ionic strength and feed pressure on solute rejection, the filtration in separate and integrated with the acidogenic digestion. It was observed that pH had higher influence in the retention of the acids than in the sugars. They were able to separate the sugars (retention higher than 90%) with the sugars (with retentions <40%) at acidic pH. Other membranes had already been studied to recover VFAs such as HL, also from GE, NF270 from Dow Chemicals e LF10 from Nitto Denko all of them used by Zacharof et al. [45].

Reverse osmosis (RO) is a membrane processes very similar with nanofiltration, in fact, the higher difference is the type of membrane used. Since the carboxylic acids are very small molecules, if the aim is to retain them it is possible to use wither nanofiltration or RO [58,59]. In which respects to a mixture of VFAs Sagne et al. [59] studied the purification of a condensate arising from distillery stillage concentration plant in a 2540 spiral wound RO pilot with a membrane surface area of 2.6 m$^2$. During the work the influence of different parameters, such as pH and applied pressure, in the separation of acetic, propionic, butyric, valeric, hexanoic, phenol and an alcohol were studied.  By changing the mentioned parameters during nanofiltration operation it was observed that acetic and propionic acid were the carboxylic molecules more affected by the changing of the pressure but also by the pH.

In general, the reported works in which nanofiltration process is employed as step separation, recovery or concentration have in common the study the effect of: type of membrane (interaction between material/compounds hydrophilic or hydrophobics), pH (promote electrostatic interactions), temperature (viscosity of the solution and membrane fluidity) and applied pressure (influences the flux).

# 3. VFAs production from co-digestion

Summary: As an alternative for the valorisation of wastes for biogas production, volatile fatty acids (VFAs) were produced under anaerobic acidogenic fermentation using vegetable wastes (VW) and maize silage (MS) in co-substrate. Preliminary small scale batch experiments were performed by using these substrates in single and then in different proportions in co-digestion experiments in order to have some knowledge about the potential of substrates individually and in co-digestion for the maximum production of VFAs. A freely-suspended cells bioreactor was fed with 70% VW and 30% MS and operated in continuous mode with and hydraulic retention time (HRT) of 6 days. The effect of the operated pH, 5, 6 and 7, was studied in triplicate. Under pH continuously controlled was observed that at pH 5 lower VFAs concentrations were achieved (20 $g.L^{-1}$) higher concentrations (30 $g.L^{-1}$) were obtained pH 6 and 7. Moreover in which respects to the VFAs profile the butyric acid concentrations were similar between each pH conditions while propionic acid production increased with pH increasing.

## 3.1  Introduction

Fruit and vegetables are produced around the world and are the basis of human alimentation. With the fast population growth, the need to plant, harvest and process the food is increasing significantly resulting in the generation of millions of tonnes of waste. This type of waste could be valorising into add-new products instead of creating environmental pollution.

Maize is one of the crops more harvest and produced worldwide. According to FAOSTAT from 1994 until 2014 the maize production increased from 0.569 M tones to 1.04 M tones in other words, in 20 years an increased around 55%. Due to the fact that maize gives a high methane yield per hectare, in the last years, not all this harvest maize is directed to the alimentation but for energy generation, the so called dedicated cultures. In Europe there have been planted lands of maize which goes directly to biogas digesters for the methane production as Italy and Germany [60].

Nowadays the wastes such as fruit and vegetable wastes (FVW), cheese whey (CW), olive mill wastewater (OMW) and maize silage (MS) are mostly valorise by biogas production. However, it is also possible valorise this organic matter in stages. In a first phase produce add-value molecules such as the VFAs and then these VFAs or the leftover of VFAs-downstream step can be used for the biogas production (e.g. methane). In this way, the waste can be valorised twice. The major of the works available in literature in which VFAs are produced are reported as a by-product from other processes (hydrogen production) [10,61–64] and few as the main product to be obtained and reuse [2,55,65]

Sträuber et al. [60], reported the acidogenic fermentation to produce VFAs and lactic acid from MS. In parallel identified the most influenced microorganisms in each part of the fermentation process. In a 1.65 L glass column bioreactor, under batch operation, a solution rich in a VFAs mixture mainly composed by acetic, butyric and hexanoic acid was obtained after 8 days of fermentation.

In the literature, there are some reported studies for the valorization of fruits and vegetables wastes by biological processes. The majority of these fermentation are performed in a co-digestion with other agro-industrial wastes [15,17,66–71].

Bouallagui et al. [15] used fruit and vegetables as single substrate for the production of biogas. In attempt to increase the productivity by lowering the HRT in the bioreactor it was observed an accumulation of VFAs in the stream. From this he proposed the separation the acidogenic and methanogenic phase to valorise as much as possible the organic matter. Zhuang Zuo [71] divided the fermentation of vegetable wastes and performed the process in: 1 – acidogenic digestion were VFAs were produced reaching almost 9 g.L$^{-1}$ and 2 – biogas production where a possible VFAs accumulation was not a problem.

## 3.2    Materials and methods

### 3.2.1    Substrate

The substrates used in the present work were kindly supplied by Biosphere SRL: vegetables waste (VW), maize silage (MS) from a dedicated culture and zootechnical effluent (ZE). The substrates were all used individually. The co-digestion experiments were performed only mixing VW and MS. In Table 3.1 it is presented the average values, with the respective standard deviation, for total solids (TS) and volatile solids (VS) of the substrates employed.

Table 3.1 TS and VS average values with the corresponded standard deviation for the substrates used in continuous operation (MIX)

|  | TS (%) | VS (%) |
|---|---|---|
| VW | 10.35 ± 0.94 | 79.75 ± 10.00 |
| MS | 30.84 ± 3.38 | 96.94 ± 0.11 |
| VMW | 10.00 ± 0.69 | 87.50 ± 5.52 |

### 3.2.2    Inoculum

The initial inoculum was an acclimatized anaerobic mix culture from Ayrion plant (Forli, Italy), a Biosphere srl partner, dedicated to the biogas production of co-digestion MS (76%), ZE (15%) and VW (9%). As a first inoculum for the microcosms experiments, a liquid sample from the first anaerobic digestion phase was placed inside a 1000 mL Pyrex bottle, connected to a "Mariotte system", mixed at 270 rpm a wrapped with a water serpentine to maintain 37°C inside the bottle. No substrate or additional compounds were supplied. The bottle was daily controlled for biogas measurement and pH correction at 5.5 with a NaOH 10 M solution. The sampling was carried out until no biogas production was detected.

For the co-digestion experiments a different inoculum was used. An acidogenic mixed culture was developed in the laboratory starting from the industrial anaerobic mix culture mentioned before. Sequential batches (5) were performed to let exhaust the methanogenic component. The batches were ended when the VFAs production achieved its maximum or when methane was detected. When the batch was ended, all liquid was withdraw and centrifuged at 10,000 rpm. Then the pellet was ressuspended in new substrate (MS) and re-inserted in the respective bottle. In Table 3.2 is represented the composition of each batch.

Table 3.2 Caracteristcis of the beginning of ecah batch for the acidogenic mixed cultre development

| Batch | Inoculum (%) | Inoculum (mL) | Substrate (g) | Substrate (g) | VH2O (mL) | Working volume (mL) | Bottle Volume (mL) |
|---|---|---|---|---|---|---|---|
| 1st | 50 | 250 | 50 | 217 | 46 | 500 | 1000 |
| 2nd | 50 | 250 | 50 | 217 | 45 | 500 | 1000 |
| 3rd | 40 | 400 | 60 | 521 | 99 | 1000 | 2000 |
| 4th | 40 | 400 | 60 | 521 | 99 | 1000 | 2000 |
| 5th | 30 | 300 | 70 | 608 | 107 | 1000 | 2000 |
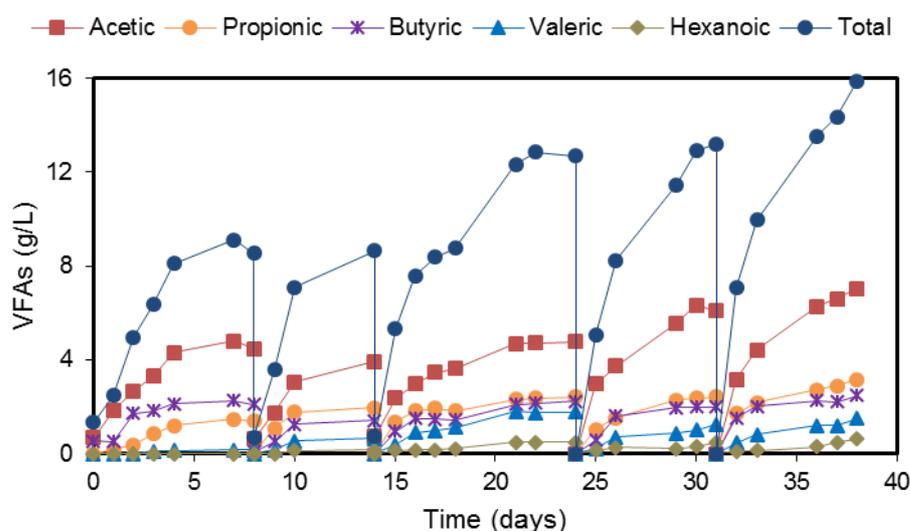


Figure 3.1 - VFAs profile of inoculum development along the 5 batches

### 3.2.3  Experiments at microcosm scale

Screening small scale batch experiments were performed to study the best substrate condition for the continuous operation. Two different batch experiments were performed: a) substrates used individually and b) substrates used in different proportions of VW and MS (Table 3.3). In addition, the influence of the added anaerobic mix culture was evaluated. The microcosms (50 mL working volume) with inoculum were inoculated with 10% (w/w) whereas 90% of working volume (45 g) corresponded to the added substrate: VW, MS, ZE or a substrate mixture, according with the condition. The microcosms without added inoculum were filled with 100% of substrate (50 g). All experiments were carried out in 100-mL Pyrex bottles (microcosms) in triplicate and as described in previous works [65,72]. Sampling for biogas and metabolites production measurements were performed frequently. After biogas sampling the bottles were opened under nitrogen gas sparging, to keep anaerobiose and 2 mL of liquid phase was taken for HPLC and GC analysis. Thereafter, pH was controlled and corrected to 6 by adding some drops of NaOH 10 M solution.

Table 3.3 - Co-digestion microcosms initial composition

| Substrate | Inoculum % (w/w) | VW% (w/w ) | MS% (w/w) |
|---|---|---|---|
| 100_0_I | 10 | 100 | 0 |
| 100_0_ | 0 | 100 | 0 |
| 50_50_I | 10 | 50 | 50 |
| 50_50_ | 0 | 50 | 50 |
| 80_20_I | 10 | 80 | 20 |
| 80_20_ | 0 | 80 | 20 |
| 20_80_I | 10 | 20 | 80 |
| 20_80_ | 0 | 20 | 80 |

### 3.2.4   Continuous process

After the experiments with microcosms, a continuous process was carried out in bioreactors designed by our research group (Appendix A) and manufactured by Acrilicos Gil (Lisboa, Portugal). The 500-mL vessels, operated with a working volume of 300 mL, were made in acrylic material and equipped with a water jacket. All bioreactors were connected in series to a thermostat bath (Julabo EH) maintaining a constant temperature of 40 ºC inside the bioreactors. The mixing was assured by a magnetic stirred (Heidolph, Hei-Standard) and a magnetic stirred bar (Ø 12x55 mm). The bioreactors were hydraulically connected to a "Mariotte system" for biogas collection and avoid creation of overpressure created by biogas production (mainly $H_2$ and $CO_2$).

Three different pH conditions were tested: 5,6 and 7. The pH was continuously controlled by a pH controller (BL 931700-0, Hanna Instruments) and a pH electrode (XS, Polymer HT Pro - S7). Three pH controllers were used to operate each pH condition in triplicate (Figure 3.2). The bioreactors were divided in 3 groups, each with a defined controlled pH value. Only one bioreactor from each triplicate group had the pH continuously measured (*main* bioreactor). The pH of the other two bioreactors, of each triplicate, was controller according to the behaviour of the *main* bioreactor. The *main* bioreactor changed frequently between the triplicates. Each pH controller, with a defined set point, was connected to a controller pump (Masterflex, CP07553-76) which regulates the flow of the peristaltic pump (Masterflex, CP07557-10). This pump was equipped with three pump heads (Masterflex, 7013-20) for the addition of NaOH solution (5 M) to all bioreactor according with the requirement.

The bioreactors were first operated in batch conditions till VFAs maximum production was achieved. The batches were stopped when the VFAs achieved its maximum and profile was similar in the last batches. After this first operation mode, the continuous operation was started with HRT of 6 days. Due to the substrate characteristics, it was difficult to operate at lab scale by continuously add the substrate and because of that, the bioreactors were fed manually. In order to disturb the less possible the system each 2 and 3 days, 100 mL or 150 mL respectively, were took out and replaced with an equivalent amount of fresh mixed substrate. Sampling for biogas and metabolites production measurements were performed with the same frequency as mentioned before. After biogas sampling

the bioreactors were opened under nitrogen gas sparging, to keep anaerobiose, temperature was measured and 2 mL of liquid was taken for dissolved metabolites analysis. Thereafter, pH was controlled and corrected. The "Mariotte system" was flushed with nitrogen and let come back to the equilibrium. The sampling was performed as described previous works [2].
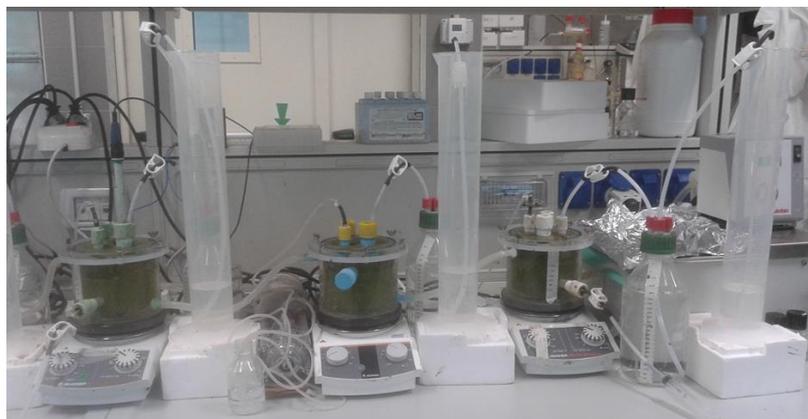


Figure 3.2 - A pH controlled triplicate bioreactors

### 3.2.5 Analytical procedures

The VFAs concentrations were determined by gas chromatography using a GC (Agilent Technologies, Milano, Italy) as described by Martinez et al. [65]. The samples were centrifuged at 14,000 RPM for 10 minutes. The supernatant was diluted with oxalic acid solution 60 mM and filtered (0.45 µm spin-filters).

The characterization of the substrates in total solids and volatile solids were analyse following Standard Measures Procedures (EPA).

Biogas production volume was measured using a graduated glass syringe in the microcosms experiments, while in PBBR a "Mariotte" system was connected to the bioreactors. Biogas composition was measured as described previously [65]: microcosms were directly connected to the µGC (model 3000, Agilent technologies, Milano, Italy) while for bioreactors biogas composition was determined by collecting biogas samples in a 10 mL vial previously flushed with nitrogen gas. The procedure was performed as described in Domingos et al. [2].

### 3.3 Results and discussion

### 3.3.1 Experiments at microcosm scale – Single substrate

During the present work, different *lots* of VW and MS were used as substrate for the experiments. It was observed the mixture of VW presented more variation in the composition according to the season of the year. The vegetables are seasonally which means that different

vegetables are harvest and processed at different times of the year. This variation in composition is identified visually, by distinguish the type of vegetables in the mixture, but more precisely, by the VS and TS characterization. In fact, the Table 3.1, presented previously in the substrate description, confirms this higher variation in VS content of VW than in MS.

The first experiments dedicated to the VFAs production were performed in 100-mL Pyrex bottles operated in batch. The goals of this first approach were: a) evaluation the potential of each different substrate for the obtainment of a VFA-rich effluent, b) influence of the initial total solids and c) find the best substrate mixture that leads to achieve the higher VFAs concentration to be employed in the continuous process.

In order to evaluate the potential of each waste used as substrate to the VFAs production all microcosm were inoculated with an anaerobic mix culture inoculum (see section 3.2.2) but for each quadruplicate a different substrate condition was studied, for each, was added VW or MS or ZE. Furthermore, in the same experiment the influence of initial substrate concentration for MS and VW, determined by TS content was studied. MS is the substrate that presents a higher TS content (about 30%) which is too high for the acidogenic fermentation while VW contains 10% of TS. The microcosms were set up with the maximum TS of VW, in this way the addition of water was minimized and 5% to study the impact of initial TS. In the case of ZE only a TS (7%) content was studied since its original content in TS is already diluted. In a preliminary study was already observed that reduce the TS to 2% was not worth due to the low VFAs final concentrations achieved (data not shown).

In Figure 3.3 it is represented the final profile of VFAs obtained at the end of fermentation, 10-15 days, of each substrate condition. Comparing the initial TS (5% and 10%) it was not observed inhibition since the VFAs production achieved its maximum concentration (in $g.L^{-1}$) and yield (in $gVFAs.gVS^{-1}$) after the same batch time (data not shown). For each substrate condition, it was observed higher TS leaded to a higher total VFAs production. When substrates are compared between them it was observed that with ZE a poorest VFAs mixture was achieved, then VW and with MS was the one which leaded to a higher VFAs production achieving concentrations of 30 $g.L^{-1}$ of VFAs. This result was expected since this last has higher VS and in fact the yield in VS was quite the same for VW (0.27 $gVFAs.gVS^{-1}$) and MS (0.3 $gVFA.gVS^{-1}$) while ZE remained in only 0.1 $gVFAs.gVS^{-1}$.

The organic wastes can carry in its composition some microbial communities. There are reports about the composition of these communities in agro-industrial wastes used in the acidogenic fermentation such as OMW, CW and MS [2,60,63] and all have in common the presence of bacteria able to hydrolysate complex molecules, e.g. *Lactobacillus*. Manure or ZE came from intestinal activity and because of that it is clearly that carry an important methanogenic component. From this reported information and since during anaerobic acidogenic fermentation the substrates are not sterilized it can be hypnotized that these microorganisms can participate and influence the fermentation process. At the same time, it was tested the influence of the potential and initial concentration of each substrate and also the effect of the mixed culture used as inoculum. This parameter was possible to observed

by the VFAs profile obtained in the microcosms where inoculum was added and the one obtained in microcosms with no added inoculum for each substrate condition. From the profiles obtained for all the microcosms with inoculum and all without inoculum it can be said the inoculum used was not effective since it was not observed a significance difference in the profile of VFAs obtained in the microcosms with and without inoculum. For the case of VW, for the 10% TS condition, it was even observed where inoculum was added the VFAs final concentration was lower than condition with no inoculum with 16.6 ± 2.9 g.L$^{-1}$ and 20.3 ± 1.0 g.L$^{-1}$ respectively. Nonetheless the inoculum was previously acclimatized to acidic pH to promote the inhibition of methanogenic activity, the methanogenic microorganisms had still some activity and able to consume the VFAs and reduce it into methane. Methane from VW was produced in higher quantity than from MS as soon as VFAs ruched it maximum concentration, by means, after 10 days of batch.
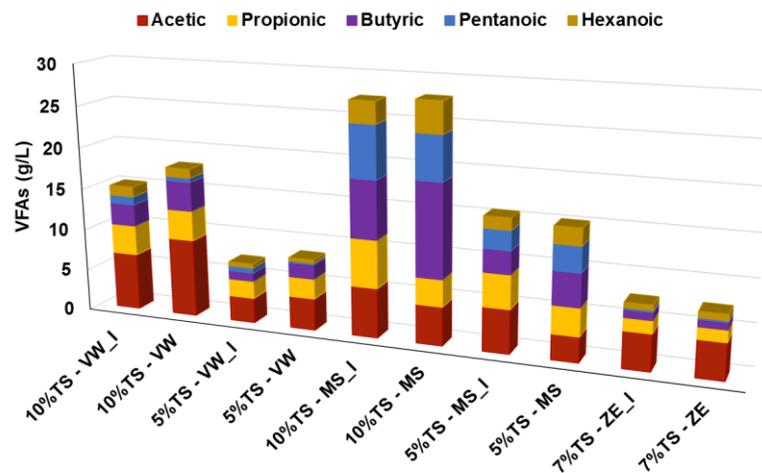


Figure 3.3 - Main VFAs profile obtained after 10 days of fermentation for each substrate with (_I) and without inoculum during microcosm batch experiments.

All wastes were used in single condition to evaluate the potential of each one for the VFAs production, as it was already mentioned. However, it was the interest of Biosphere SRL to use in co-digestion the available substrates for the VFAs production with a report between them that enhance a higher VFAs mixture composition. From the three substrates ZE is the substrate that presents lower TS and so is used in Ayrions' methane plant as a dilution component of the substrate mixture. The VFAs are realised in liquid phase and so the fermentation with low TS (TS<15%) enhance its accumulation. Since ZE has low TS (7%) it was hypostatized use ZE as a diluted component in a co-digestion fermentation instead of use water. Though, due to its strong methanogenic activity mentioned above it was observed that ZE leads a very low VFAs productions (Figure 3.3). Nevertheless, the experiment was conducted in acidogenic conditions (pH 6) it was observed methane production, even if low (data not shown). In fact, this type of waste is common used for the methane

production and not in acidogenic fermentation [73–77]. Since it is already proved that this substrate leads to methane production ZE was no longer use in further experiments during the present work.

### 3.3.2    Experiments at microcosm scale - Co-digestion

After the first evaluation about the potential of each available substrate, impact of the initial concentration (in terms of TS) and the inoculum for the VFAs production the next experiments were dedicated to the co-digestion. From the three substrates MS, VW and ZE this last was already set aside from the suitable substrates due to the observation of methane production. From now on only MS and VW were considered as wastes suitable for the employment in a acidogenic fermentation for VFAs production. The co-digestion experiment was conducted in the same type of bottles as for substrates used in single as well as the introduction or not an acidogenic mix culture. In a previous section (3.2.3) is presented the table (Table 3.3) with the substrate mixtures used for the each substrate condition considering only VW and MS (Figure 3.4).

Since in previous experiments it was observed that the inoculum was not appropriate, it was developed an acidogenic mixed culture acclimatized to MS (see section 3.2.2). It was chosen MS as the substrate to develop a strong acidogenic inoculum because it has a VS composition higher than VW and also because its proprieties are more consistent due to the origin from a dedicated culture. This inoculum was then used as in this experiment for the co-digestion. The condition which VW was used as single substrate, with and without inoculum, was repeated once again since this substrate has variability in composition depending in the season of the year as already referenced. The MS was not performed in parallel in this experiment since it was already performed in microcosms and was performed during inoculum development (Figure 3.1). The initial TS condition used for the co-digestion experiment was fixed at 10% for all fermentation conditions since it was previously observed that lead to a higher VFAs production either VW or MS.

In the next figure (Figure 3.4) is represented the main VFAs profile obtained at the end of batch for each co-digestion condition. As it was previously observed in the experiment with substrate in single, the microcosms that were composed by higher quantity of MS as substrate leaded to higher total VFAs production conditions 50_50 and 20_80 reaching 25.4 ± 3.4 g.L$^{-1}$ and 28.8 ± 3.5 g.L$^{-1}$ respectively. The VW used in single achieved higher total VFAs concentrations respects to the previous microcosm experiment. It is clearly that the inoculum used this time had an important influence in the VFAs profile. In all microcosms where inoculum was added is observed a significant production of hexanoic acid regardless the substrate mixture. This production is clearly due to the presence of the added inoculum since during its development the production of hexanoic acid was produced in the last batches. Sträuber et al. [60] concluded that the composition of the inoculum seems influence the VFAs profile achieved. Still about the profile of VFAs important notice that acetic acid is more produced in the conditions composed by MS while butyric acid from the digestion of VW. Although it was expected to have more production of propionic acid because during the inoculum

development and also in the last experiment. The propionic acid fermentation is not clearly explained [60] however its production could be enhance by the consumption of produced $H_2$ [78] which was not produced in high amounts (data not shown).
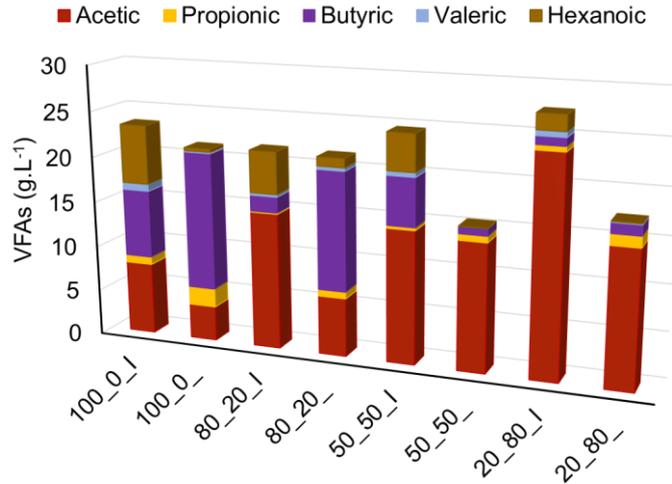


Figure 3.4 - VFAs profile obtained after 15 days of batch when VW and MS were used in co-digestion

According with these results the mixture with higher quantity of MS resulted in the substrate co-digestion condition to obtain the higher VFAs production. However, it is also necessary to consider the cost of the feedstock. In this case MS is provide by a dedicated culture representing a price of ~50€/ton while VW is the waste result from the frozen vegetables industry in which is cost is especially due to the transport (~4 €/ton). Moreover, the profile obtained may be not so interesting since more than 80% is acetic acid. The VW addition seems to contribute to a more diversify profile in the different VFAs.

To go in the same interest of Biosphere and at the same time produce higher VFAs amounts with a diversify profile it was decided to start the continuous operation in bioreactors with a co-digestion of 70% VW and 30% MS.

### 3.3.3 Continuous process

With the aim to have a scalable data the fermentation process was conducted in 500 mL bioreactors operated under continuous operation. It is well known the pH is a critical parameter during VFAs production since it influence the VFAs mixture composition [1,10,78,79]. However, there is still not a defined pH to obtained a specific mixture so, 3 different pH were studied during the continuous operation. Contrary to what happened with microcosms experiments in the bioreactors the pH was continuously controlled and adjusted. The feed used in all pH condition was the same – co-digestion of VW (70%) and MS (30%) as referred above.

In a first phase, all pH conditions begun under batch operation to develop a strong inoculum able to support possible variations in the characteristics of the feed. This control in the microbial community is very important to have a stable process where a mix culture is use and the substrate is not sterilized. In Figure 3.5A is represented the profile obtained during the sequential batches for the pH 6 condition. As it can be observed the time needed to achieve the maximum concentration of VFA decreased from 15 days, time need previous in microcosms experiments, to 6-8 days in bioreactors. The decrease in fermentation time is especially due to the constant pH control that was not performed in microcosms experiments. Before VFAs production the bacteria hydrolysate the complex matter into lactic acid. This acid has a very low pKa dropping the pH to values lower than 4 inhibiting the bacteria responsible for the VFAs production [78]. The profile obtained for the other pH conditions (5 and 7) was very similar (data not shown). During batch operation was not observed significant different in the acidogenic fermentation between the different pH conditions. The VFAs mixture was mainly composed by acetic, butyric and propionic acids. After 5 consecutive batches with a constant VFAs profile the continuous operation was started with a HRT of 6 days for all pH conditions (Figure 3.5 B, C and D). The HRT was chosen according with the time observed until it was achieved the maximum VFAs production during batch operation, which was 6 days.
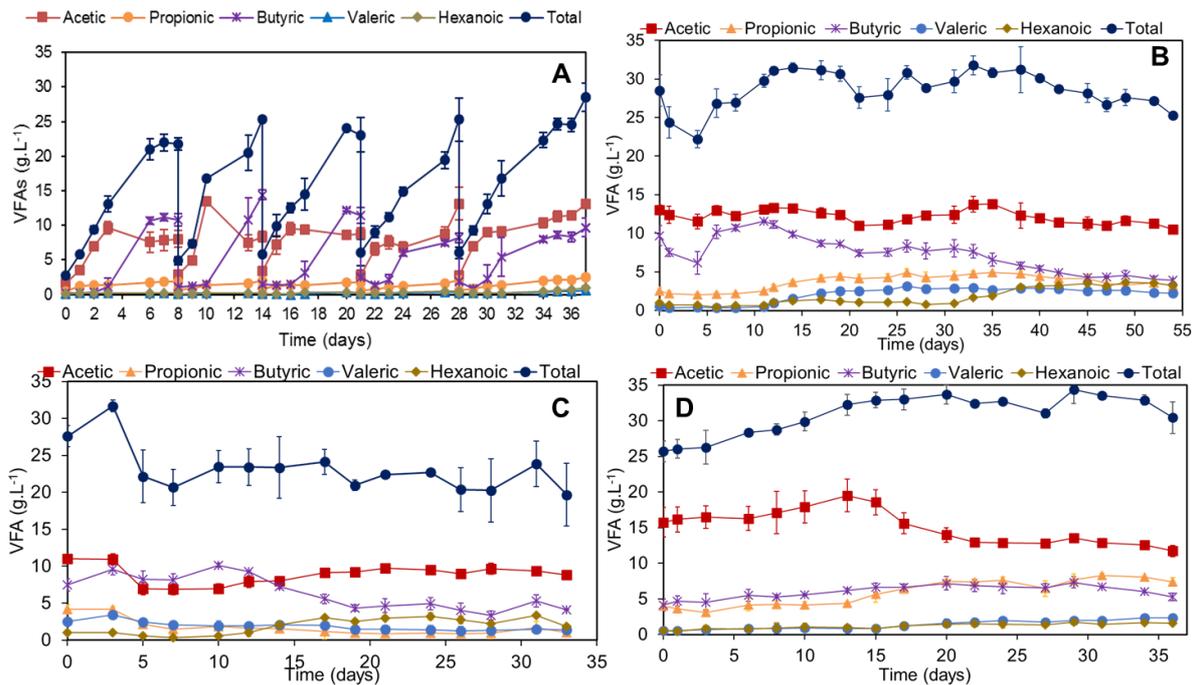


Figure 3.5 - VFAs production profile obtained during batch operation (A), continuous operation at pH 6 (B), 5 (C) and 7 (D)

The pH control is a critical parameter during acidogenic fermentation [10] but in the present study it can be said that the pH was well controlled during continuous operation. In Figure 3.6 is represented the pH profile along the days when bioreactors were in continuous operation: pH 4.9 ± 0.2, pH 6.0 ± 0.2 and pH 7.0 ± 0.3.
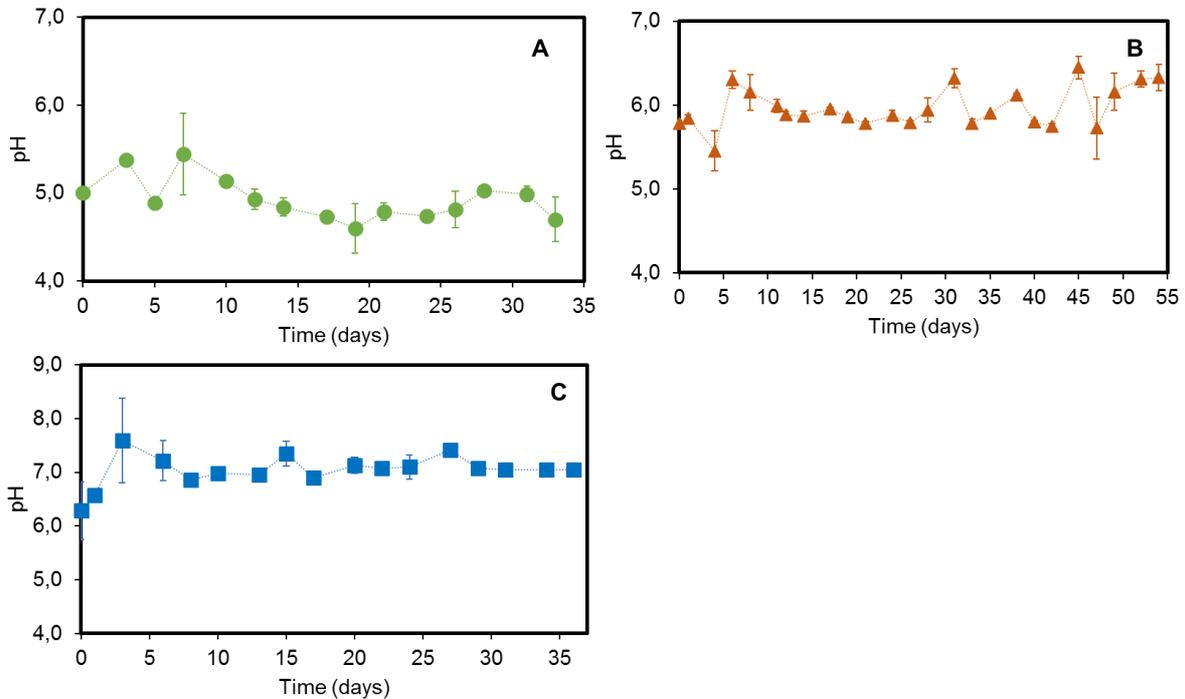


Figure 3.6 - pH profile during continuous operation for each pH condition: 5 (A), 6 (B) and 7 (C)

Under continuous operation the VFAs mixture composition changed according with the operated pH (Table 3.4). The pH 5 (Figure 3.6C) was the operated pH condition that leaded to a lower VFAs production decreasing from 28 g.L$^{-1}$, at the end of the last batch, to 22 g.L$^{-1}$ during steady state. This total VFAs production is similar to what was obtained with microcosms scale experiments where pH was manually controlled. At pH 6 and 7 the VFAs production slightly increased respect to batch operation. And in fact, increased in which respect to microcosms experiments. Notice that at these two pH values the total VFAs was always near 30 g.L$^{-1}$ being even higher (32 g.L$^{-1}$) at pH 7. Usually, independently on the substrate use, the optimal pH for the VFAs production is between 6 and 7 [2,10,78].

Regarding to the profile obtained for each pH condition under continuous operation the main difference is the production of propionic acid. From Figure 3.6 it can be observed that at pH 5 the propionic acid production was being lost along the operational time. In other hand for pH 6 and 7 the propionic acid concentration increased along the time. These results are in concordance with others already reported which mention the fact that for the propionic acid production the optimal pH is between 6 and 7 [2,10,78]. Propionic acid is an odd number carbon molecule and very desired for the

PHA production due the possibility to obtain a specific type of biopolymer with good characteristics [80]. From this, it can be said that the anaerobic acidogenic fermentation should be operated at pH 7 to enhance the propionic acid concentration in the fermented broth and use it after for the PHA production. However, the fermentation operated at pH 7 could promote the methanogenic bacteria and the risk to have methane production, even in traces, could happen and could lead to lower total VFAs concentrations. In the case that methane starts to be produce from VFAs, acetic acid should be the first to be consumed since is the most easily converted [6]. In fact, during the last days of continuous operation, traces of methane were detected, explaining why acetic acid decreased slightly its concentration. One way to avoid the methane production is by decreasing the HRT as it was observed by Bouallagui and co-workers [15]. Dareioti et al. [78] mentioned the fact that butyric acid production was not so affected by the operational pH as it was also observed in the present work.

In Table 3.4 it is resumed the main results obtained for the VFAs production during the co-digestion of MS and VW. All pH conditions were operated under continuous mode for more than 6 residence times. Comparing the total VFAs produced and respective yield ($gVFAs.gVS^{-1}$) as the operated pH increased also these two parameters increased.

Table 3.4 - Main results during continuous operation at three different pH: 5, 6 and 7

| pH | 5 | 6 | 7 |
|---|---|---|---|
| Operation time (days) | 33 | 54 | 36 |
| Acetic ($g.L^{-1}$) | 8.6 ± 1.1 | 12.1 ± 1.0 | 14.3 ± 2.6 |
| Propionic ($g.L^{-1}$) | 1.3 ± 0.4 | 4.0 ± 0.7 | 7.0 ± 1.2 |
| Butyric ($g.L^{-1}$) | 6.1 ± 1.7 | 7.0 ± 2.3 | 6.6 ± 0.6 |
| Valeric ($g.L^{-1}$) | 1.7 ± 0.4 | 2.4 ± 0.7 | 1.7 ± 0.6 |
| Hexanoic ($g.L^{-1}$ | 2.0 ± 1.1 | 2.0 ± 1.1 | 1.4 ± 0.3 |
| Total VFAs ($g.L^{-1}$) | 22.1 ± 1.6 | 29.3 ± 1.8 | 32.7 ± 1.1 |
| P ($g.L^{-1}.d^{-1}$) | 3.7 ± 0.2 | 4.9 ± 0.1 | 5.4 ± 0.1 |
| Yield ($gVFAs.gVS^{-1}$) | 0.257 ± 0.004 | 0.336 ± 0.004 | 0.372 ± 0.009 |

In conclusion, from the available substrates, MS, VW and ZE, for the VFAs production, ZE was excluded right after the small scale batch experiments as a potential substrate for the process due to the methane potential production. In both microcosm experiments either single substrate and co-digestion MS was the one which leaded to a higher VFAs concentration. Nevertheless it was the interest to the enterprise to have VW as major substrate due to economic reasons. Under continuous operation with HRT of 6 days, 3 pH conditions were tested – 5, 6 and 7. It was observed that the best pH condition was higher than 6 since more a higher VFAs concentration was achieved and the mixture was richer in propionic acid. A fermented broth with, approximately 30 $g.L^{-1}$, of VFAs was obtained to use in the ED experiments for the VFAs concentration.

# 4. VFAs production from CW



Cheese whey (CW) acidogenic fermentation using a packed bed bioreactor
- Support: ceramic Vukopor
- Productivity: 2.78 g.L$^{-1}$.d$^{-1}$
- Tot. VFAs titer: 16.7 g.L$^{-1}$
- Microbial characterization
- Richness/Evennes vs. Operational parameters

Taxonomic distribution

Deepest Taxon
- Lactobacillaceae
- Lachnospiraceae
- Marinilabiaceae
- Defluviitaleaceae
- Alcaligenaceae
- Veillonellaceae
- Coriobacteriaceae
- Prevotellaceae
- Others

**Summary:** Volatile fatty acids (VFAs) were produced using cheese whey as feedstock. A mixed culture packed bed bioreactor was set up to digest anaerobically, under an acidogenic condition, a water solution of a cheese whey powder. Batch tests pointed out that the whole VFAs production process occurred via two sequential phases: (a) conversion of lactose into lactic acid and (b) conversion of lactic acid into a mixture of VFAs. Furthermore, the same tests showed that the ceramic material Vukopor S10 can be used as an effective support for cell immobilization in anaerobic fermentation processes. The effect of the hydraulic retention time (HRT) and organic loading rate (OLR) were then studied in a benchtop bioreactor operated continuously. By a HRT of 6 days, ORL of 4.2 g/L/d, and pH $5.8 - 6$, 16 g·L$^{-1}$ of total VFAs were produced, with a yield higher than 75% (Cmol$_{VFAs}$·Cmol$_{lactose-1}$). Characterization with Illumina-based sequencing, performed by Center for Microbial Ecology and Technology – Faculty of Bioscience Engineering, Ghent University, suggested that high VFAs productivities were obtained when microbial community structures developed in the biofilm reactor were highly enriched in few genera.

## 4.1 Introduction

Volatile fatty acids (VFAs) are chemically defined as short chain length ($C_2$ to $C_6$) carboxylic acids, namely acetic, propionic, butyric, valeric and hexanoic.[1] They are potential precursors for the obtainment of a variety of compounds such as ketones, aldehydes, esters, alcohols and alkanes.[6] Many important compounds including traditional and new products could be obtained through a route involving their utilization [81] either directly (e.g., as reactants) or indirectly (e.g., as solvents) (Figure 4.1). As a result, VFAs are considered molecules of interest, some of them even as bulk products for the chemical industry. At the present time VFAs are produced by means of petrochemical processes,[82] but globalized concern about sustainability of production processes has prompted the development of alternative green technologies for replacing petrochemical-based ones. The main target of this approach is to achieve economically feasible and environmentally friendly processes by making use of renewable resources as feedstock. This could lead to the development of new industrial production routes, e.g., polyhydroxyalkanoates and single esters.
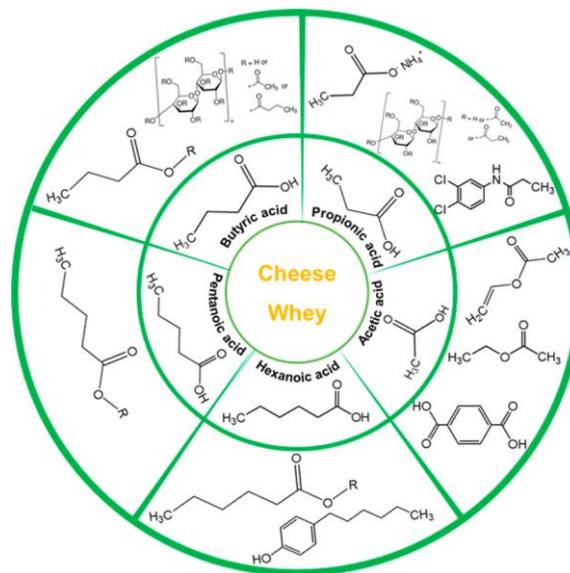


Figure 4.1 - VFAs related products.

Anaerobic digestion represents the core biotechnology for the transformation of renewable resources into VFAs. This is an oxygen-free bioprocess fed with organic substrates (e.g., agro-industrial wastes), in which a consortium of microorganisms first transforms complex molecules into simple intermediate products (such as VFAs) and finally mineralizes carbon into its most oxidized ($CO_2$) and reduced ($CH_4$) form.[83] When methane production is inhibited (e.g., by setting low pH and/or high organic loading rates) VFAs tend to accumulate, accentuating the methanogenesis repression.[84] This process is called acidogenic fermentation. Also pure cultures of acidogenic strains can be employed for the production of organic acids, achieving high yields, remarkable reproducibility

and high final product titers (50−180 g·L$^{-1}$).[85] Nevertheless, this approach is typically expensive due to the request of high quality substrates and reactor sterilization costs. Alternatively, mixed microbial consortia can make profitable use of undefined leftover material occurring in agro-industrial wastes8 without requiring sterilization. Scoma et al.[7] recently discussed some potential valorization platforms for high-impact agro-industrial residues of the Mediterranean area, which include olive mill wastewater, tomato pomace, grape pomace and cheese whey, among others. Adom et al.[8] reported valorization strategies of dry-grind ethanol, which could supply the succinic acid and histidine industrial demands. Both of these studies focus on the fact that many agro-industrial wastes are mainly composed of carbohydrates, proteins and lipids, which represent per se valuable compounds for many biotechnological processes. However, with few exceptions (e.g., biodiesel glycerol, molasses and oils) where the substrate concentration is comparable to those of prepared feeding solutions, the organic content in these residues would not allow developing conventional pure culture fermentations with high productivities and titers. Nevertheless, they hold high chemical oxygen demands (COD; 30 < COD < 100 gO2·L$^{-1}$), a strong acidic potential, possible antimicrobial activities and, therefore, slow biodegradability. As crops are subjected to seasonality, leftovers are generated within 2−4 month periods, and their massive discharge prior to any treatment may lead to eutrophication of water bodies and/or reduced fertility of cultivable soils. Anaerobic fermentation carried out by mixed microbial consortia (AFMC) can play a strategic role in changing waste treatment into a biotechnological valorization of carbohydrates, proteins and lipids held by these residues in order to generate added value molecules such as VFAs. Cheese whey (CW) is a common residue worldwide resulting from cheese production. As a rule of thumb, the production of 1 kg of cheese requires 10 kg of milk, 9 kg of which are set aside as CW byproduct.[22] This means that 191.7 M tons of CW are generated per year worldwide according to cheese productivities reported by FAO (21.3 M tons.year$^{-1}$). CW is composed of lactose (44−52 g·L$^{-1}$), proteins (6−10 g.L$^{-1}$, primarily β-lactoglobulin) and lipids (4−5 g.L$^{-1}$),[13,23], has a high COD (50−102 gO$_2$.L$^{-1}$), and contains some hardly biodegradable proteins which require CW to be treated before being discharged.[22] Several studies about CW treatment were reported,[22,86] including its valorisation through the production of H$_2$,[87−89] micronutrient fertilizers,[86] VFAs,[10,61,83,90,91] or biogas production using immobilized cells.[92] Regarding this last, cell immobilization has several advantages as compared to suspended cultures: (i) It prevents microbe washout in a continuous process by separating dilution rate and hydraulic retention time (HRT). (ii) It allows working at higher cell density. (iii) It confers an advantage for the microbial consortium against adverse conditions.[13] (iv) It helps downstream processes as cells are retained within the support material and separated from the liquid. Due to the many potential applications of VFAs, the present work aimed at studying their production through AFMC. A packed bed bioreactor (PBBR) was proposed as the culture system owing to the advantages it represents for industrial application due to the following: (a) Cell retention systems allow operating at small volumes [10] while still processing large amounts of noneasily biodegradable wastes such as CW. (b) Cell retention attained by immobilization is cheaper than by membrane bioreactors (MBR), which typically requires

much energy for cell recycling.[61] The ceramic material Vukopor was chosen as immobilization support due to its low cost and good performances in the AFMC of olive mill wastewater.[64,87] The operational parameters HRT and lactose feed concentration were tested during the continuous process, and the outcomes in terms of total VFAs, VFAs mixture profile, productivities and microbial community structure were studied. This allowed us to analyze how microbial community structures link to VFAs productivity and mixture profile.[93,94] Microcosm-scale experiments were first carried out to test CW bioconversion into VFAs and verify the possibility of employing the immobilization material. Afterward, experiments at bench scale with PBBR were performed in order to evaluate the culture system performance during continuous operations.

## 4.2 Materials and methods

### 4.2.1 Substrate and Inoculum

All tests were carried out using a liquid stream composed of water and cheese whey powder (CWP), courtesy of Lactogal−Produtos Alimentares S.A. (Porto, Portugal). The CWP was dried at the factory for animal feeding, composition reported by Duque et al.[61] Unless otherwise stated, CWP solutions were prepared by dissolving 20 g of powder in 1 L of distilled water (15 $g.L^{-1}$ of lactose);[61] no other mineral or organic supplement was added. The inoculum was a fully acclimated anaerobic acidogenic consortium obtained from a membrane bioreactor fed with the same CWP solution to generate VFAs at high yields.[61]

### 4.2.2 Experiment at Microcosms Scale

Small-scale batch experiments were carried out in quadruplicate with both freely suspended and immobilized cells in order to study lactose bioconversion and to verify the possibility of using the proposed ceramic support. Microcosms were prepared in 100 mL Pyrex bottles with a working volume of 50 mL (55 for immobilized condition), inoculated at 10% v/v in 45 mL of CWP solution. Twelve grams of Vukopor S10 (VK) (Lanik, Boskovice, CZ) porous cubes (12 mm × 12 mm × 9 mm) were used for cell immobilization.[87] Three preliminary sequential batches were carried out for the immobilized condition in order to allow biofilm formation. Each of them lasted until all organic acid concentrations reached their plateau (16−14 days). Afterward, liquid suspensions were centrifuged (8000 rpm, 6 °C, 10 min), pellets resuspended in 50 mL fresh CWP solution, and poured within the original corresponding bottles to start the consecutive batch. The former procedure was carried out maintaining anaerobic condition (nitrogen flux), and the packing material was not washed nor new inoculum added between runs. The final experimental batch run was started just by replacing the liquid with fresh CWP solution. Incubation conditions were 37°C, pH 6 and 150 rpm, as reported previously by the inoculum donor.[61] Biogas and other metabolites production monitoring and pH

control were carried out following the same procedure reported elsewhere.[65] VK samples were withdrawn from microcosms at 0, 5 and 12 days of the last incubation for microbial community characterization, and PCR-DGGE analysis was carried out according to Scoma et al.[72] Briefly, metagenomic DNA was extracted from liquid samples with the UltraClean Soil DNA kit (Mo Bio Laboratories, Carlsbad, CA, U.S.A.) by using approximately 250 mg of pellet. PCR-DGGE analysis of the bacterial and archaeal communities was performed after amplification of the 16S rRNA genes with the primer pairs GC-357f/907r and GC-344f/915r, respectively, as described elsewhere.[88]

### 4.2.3   Experiments with Bench Scale PBBR

An anaerobic acidogenic PBBR was set up, as described by Monti et al.,[64] for continuous process tests with immobilized cells. The PBBR consisted of a 1 L glass column (5 cm of diameter and 40 cm high) filled with $120 \pm 1$ g of VK (PBBR-VK) (Abstract figure and SI Figure S1). A serpentine silicon tubing (S) connected to a thermostat was used to maintain the temperature at $37 \pm 2$ °C. Peristaltic pumps (Masterflex) were used to feed CWP solution (P1) and recycle and mix the liquid phase (P2). The bioreactor effluent (transferred by overload) and gas were collected in a bottle (outlet), which was hydraulically connected to a "Mariotte system". The bioreactor had a working volume of 0.8 L (0.74 L liquid) and was inoculated with 20% v/v. It was operated initially under batch conditions for biofilm development (13−7 days) as in the experiment at the microcosm scale. Next to this, continuous operation start-up was done at a HRT of 6 days, high enough to guarantee an easy system adaption to changing from discontinuous to continuous operation. After 49 days, a third pump (P3) was implemented to continuously supply 10 M NaOH (flux manually tuned), which sensibly reduced pH variations (Appendix C, HRT6_15). Thereafter, AFMC experimental tests were carried out using the same CWP concentration as in the microcosms (15 $g_{lactose}^{-1}$) at HRT 6 and 4 days (hereafter referred to as HRT6_15 and HRT4_15, respectively). A third condition tested CWP at 25 $g_{lactose} \cdot L_{-1}$ and 6 days HRT (hereafter referred to as HRT6_25). Liquid samples from PBBRs were taken every 2−3 days to analyze the metabolites concentrations. While sampling, the pH was manually controlled. Steady state condition was considered achieved when variations of total acids concentration and yield were lower than 20%. After at least six residence times, PBBRs were sampled by taking out a VK sample. Cells were detached (VK sample, 20 mL ethanol, 25 °C, 150 rpm for 3 h), harvested (10,000 rpm, 6 °C for 10 min) and stocked (−20 °C) for the microbial community analysis by high throughput sequencing using the Illumina platform.[6]

### 4.2.4   Molecular Analysis

The total DNA was extracted from the pellets with 1 mL lysis buffer (pH 8.0) and 200 mg glass beads (0.11 mm, Sartorius) in a FastPrep-96 instrument (MP Biomedicals, Santa Ana, U.S.A.) by bead-beating two times for 40 s (1600 rpm). The beads were removed by centrifugation (5 min, max speed), the DNA extracted from the supernatant with phenolchloroform and precipitated with 1 volume

ice-cold isopropyl alcohol and 1:10 volume 3 M sodium acetate (1 h; −20 °C). Isopropyl alcohol was removed by centrifugation (30 min, maximum speed) and the DNA pellet dried and resuspended in 30 µL 1× TE buffer (10 mM Tris, 1 mM EDTA). Following the extraction procedure, the DNA samples were stored at −20 °C prior to further analysis. The quality of the DNA samples was assessed by 1% (w/v) agarose (Life TechnologiesTM, Madrid, Spain) gel-electrophoresis, and the DNA was quantified by a fluorescence assay (QuantiFluor dsDNA kit; Promega, Madison, WI, U.S.A.) and Glomax-Multi+ system (Promega, Madison, WI, U.S.A.). Samples were then normalized to contain 1 ng DNA·µL−1 and sent to LGC Genomics (Germany) for library preparation and sequencing on an Illumina Miseq platform. Further details on the Illumina platform procedure, on amplicon sequencing processing, sequences classification and collector curves are provided in the SI Section S2 and Figure S2. A search for type strains in highly productive PBBRs was carried out to check whether known bactéria were eventually enriched in the present study. Results are reported in SI Table S3 and made use of the database RDP (https://rdp.cme.msu.edu/), searching for isolated type strains with a sequence size ≥1200. A statistical analysis between high and low VFA-productive PBBRs was performed by using the results obtained during steady-state production phases (n = 10−15). The analysis stood on a 95% confidence interval (95% CI) calculated with a Student t-test with a two-sided distribution. The statistical significance was assessed using a nonparametric test (Mann Whitney test) which considered a two-sided distribution with 95% CI.

### 4.2.5  Analytical Procedures

The liquid samples were centrifuged (14000 rpm, 25 °C, 10 min); the supernatant was separated and diluted within distilled water (for HPLC) or oxalic acid (for GC) and finally filtered (0.45 µm cellulose acetate) prior to analysis. The lactose and lactic acid concentrations were determined by HPLC-IR (Agilent Technologies, Milano, Italy) according to what was previously reported.[95] The VFAs concentrations were determined by GC-FID analysis (Agilent Technologies, Milano, Italy) as described previously. [65] The standard VFAs mixture (Supelco) was purchased from Sigma−Aldrich (Milano, Italy). The biogas production volume was measured by using a graduated glass syringe in the microcosms experiments, while in PBBR a "Mariotte" system was connected to the bioreactor outlet (Abstract figure). The biogas composition was measured as described previously:[65] microcosms were directly connected to the µGC (model 3000, Agilent technologies, Milano, Italy), while for PBBR the biogas composition was determined by collecting biogas samples in a 10 mL vial previously flushed with nitrogen gas. The COD of the feeding and effluent solutions, both soluble (SCOD) and total (TCOD), were measured with a commercial kit (AQUALYTIC Vario MR).

### 4.2.6  Calculations

The total VFAs yield in C-mol basis ($Y_{VFAs;C-mol}$) was calculated both by dividing the total C-moles of VFAs by the C-moles of lactose occurring in the CWP solution and in terms of COD [10] ($Y_{VFAs;COD}$;

the total VFAs concentrations were calculated as COD equivalent by oxidation stoichiometry of the single acids ($COD_{TVFAs}$).

## 4.3   Results and discussion

Experiment at Microcosms Scale. The results of the AFMC of CW by freely suspended and immobilized cells are presented in Figure 4.2A and B and Figure 4.2C and D, respectively. The first difference recorded was that the support implementation shortened the microbial conversion of lactose into VFAs from 19 to 12 days; this was probably due to higher cell concentration in the immobilized condition. Two distinct phases were detected in both conditions. Initially, lactose was converted into lactic acid (primary fermentation), whose concentration peaked at days 7 and 5 in freely suspended and immobilized cells, respectively. Afterward, lactic acid was transformed into VFAs (secondary fermentation), with butyric, propionic and acetic acids being the prominent ones. The VFAs accumulation was accompanied by a prompt increase in the production of a biogas rich in $H_2$ and $CO_2$ (Figure 4.2B,D). No trace of $CH_4$ was detected even when the VFAs accumulation stopped. The $H_2$ maximum production was detected at days 12 and 9 in suspended and immobilized cells, respectively, finally accounting for $34 \pm 1$ and $36 \pm 1$ N mL $H_2 \cdot g^{-1}$ of lactose, respectively. As soon as all lactic acid was consumed, the H2 production dropped and the VFAs accumulation stopped (Figure 4.2A,B).
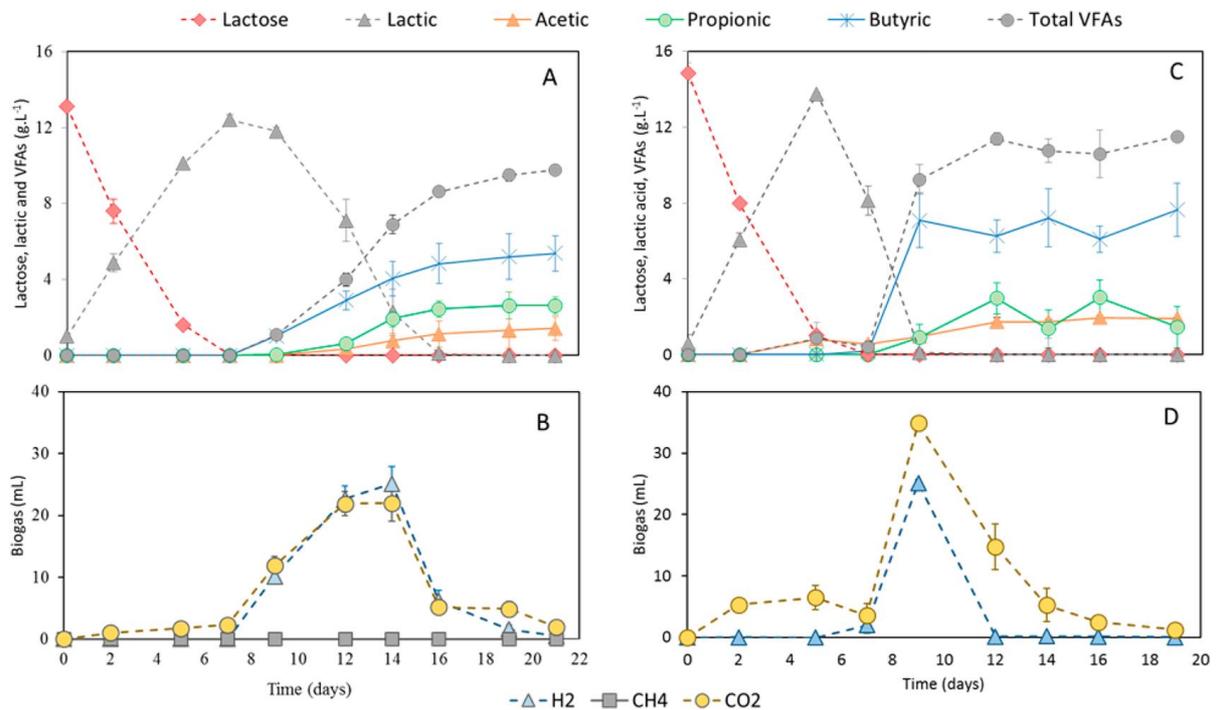


Figure 4.2 - AFMC of CW at microcosm scale. Lactose, lactic acid, VFAs concentrations, and biogas production trends for free suspended (A, B) and immobilized (C, D) cells conditions.

These results suggest that lactic acid was the major carbon source used for VFAs production during the secondary fermentation. Provided that butyric acid was the most prominent VFA, its production through lactate oxidation may have been the leading pathway at these conditions.[6] The alternative pathway entails lactate reduction to propionate, which also yields some acetate. The present work does not allow singling out precisely the leading pathway, the molecular balance between lactate oxidation and reduction cannot be entirely justified by the obtained results. However, the higher butyric rather than acetic and propionic acid concentrations coupled with the high $H_2$ production rates are in agreement with findings obtained by Davila-Vazquez et al.[62] for a condition with similar VFAs yield (0.75 gVFAs·glactose$^{-1}$) and four times more $H_2$. The total and individual VFAs concentrations were similar between the freely suspended and immobilized cell conditions (Figure 4.3A), as well as the yields $0.90 \pm 0.03$ and $0.95 \pm 0.01$ $Cmol_{lactic\ acid}·Cmol_{lactose-1}$ and $0.89 \pm 0.01$ and $0.86 \pm 0.05$ $Cmol_{VFAs}·Cmol_{lactose-1}$, respectively. These lactic acid production yields are comparable to those obtained under batch conditions with pure cultures and freely suspended cells (Lb. casei NRRL B-441, Lb. bulgaricus ATCC 8001, PTCC 1332),[96] but higher than those obtained with cocultures (Lb. Helveticus and K. marxianu, Lb. bulgaricus, and K. marxianus)[25] andimmobilized-cells (L. bulgaricus).[91] These comparisons confirm that lactic acid was produced from lactose and that the other minor fractions (i.e., proteins and lipids) did not contribute to lactic acid formation; otherwise, the yields would have been significantly higher. Concerning the total VFAs productivities, the values attained in both tests were higher (Figure 4.3B) than those recorded by Bengtsson et al.[10] for the AFMC of CW under batch conditions (0.32 g VFAs·L$^{-1}$·d$^{-1}$). Comparing lactose consumption and acids production rates, they were $1.3 - 1.9$ times higher for immobilized cells (Figure 4.3B), this showing that

the proposed ceramic support can represent an effective cell immobilization material. Nonetheless, the lactic acid productivity obtained in this work was lower than what previously reported,[25,91,96] likely due to low cell concentration (still requiring biofilm formation) and to the fact that no additional supplement was provided, such as manganese, which is a component of the lactate dehydrogenase;[22] both reasons can positively impact on specific rates of CW anaerobic fermentation.
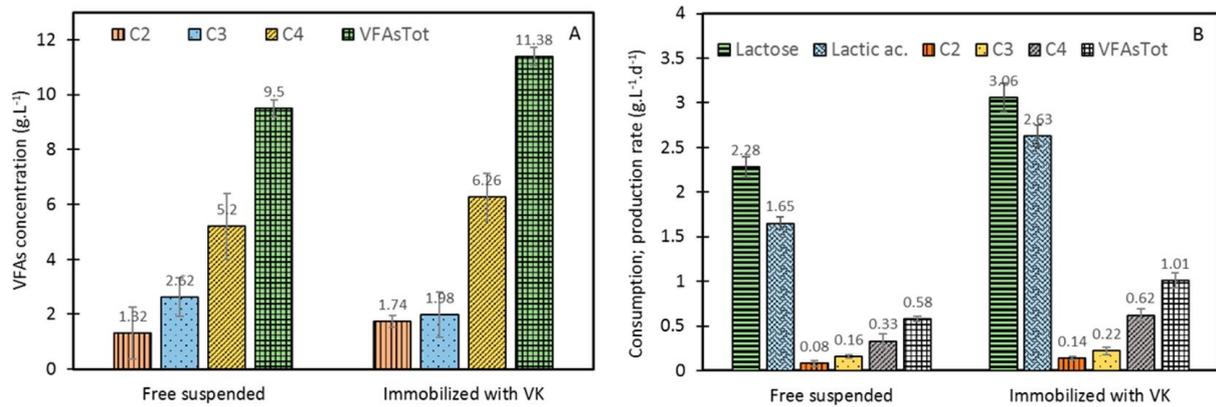
Figure 4.3 - Microcosm experiment comparison between free suspended cells and immobilized cells. (A) Final VFAs concentration and (B) lactose

Experiments with Bench Scale PBBR. Immobilized cells microcosm experiments indicated that lactic acid and total VFAs productivities were 2.8 and 4.6 $g·L^{-1}·d^{-1}$, thus requiring a hypothetical HRT of about 5 days. Therefore, the impact of HRT on continuous AFMC of CW in PBBRs was tested at HRT 6 and 4.

The results obtained for condition HRT6_15 are shown in Figure 4.4A and Table 4.1. From days 0 to 49, the VFAs concentration and yield varied concomitantly with the pH (Appendix C, HRT6_15). From day 49, the pH was better controlled; this allowed achieving steady state after 11 days (from day 60). From there on, butyric, acetic and propionic acids were equally produced, between 2.6 and 3.2 $g·L^{-1}$. Due to the relative low variations of acids concentrations and yields (<20%), the process was considered operating under steady state conditions. Neither lactose nor lactic acid were detected all along the experiment.
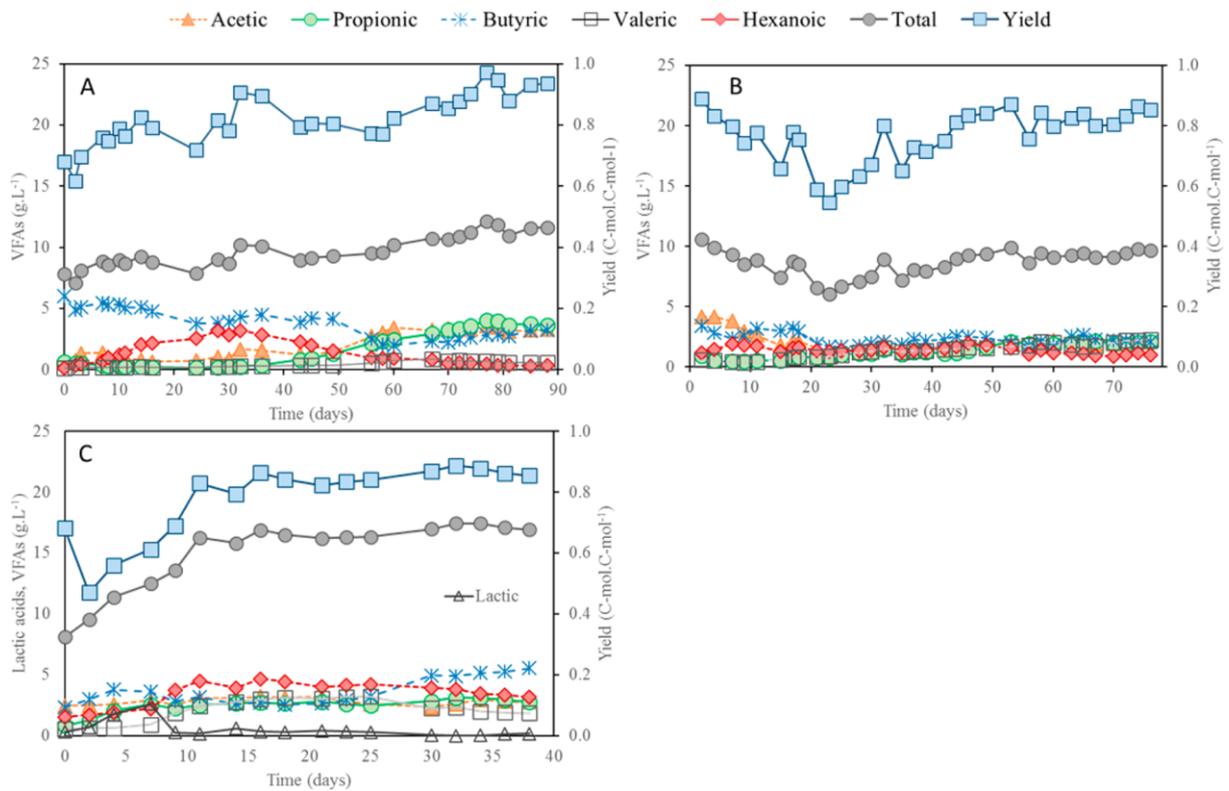
Figure 4.4 - Concentration profiles of main compounds during CW AFMC at conditions HRT6_15 (A), HRT4_15 (B) and HRT6_25 (C).

The HRT was then shortened to 4 days (HRT4_15, Figure 4.4B, Table 4.1). Steady state was achieved after 44 days. Yield and total VFAs concentration remained almost constant for the following 32 days (equivalent to 8 residence times). The single VFAs concentrations were more equally distributed, and an increase in productivity was attained as compared to HRT6_15. However, the total VFAs concentration was reduced (Table 4.1).

To enhance both productivity and VFAs concentration, a last test was performed where the CWP concentration was increased to 25 $g_{lactose} \cdot L^{-1}$ (equivalent to 4.2 $g_{lactose} \cdot L^{-1} \cdot d^{-1}$), and the HRT was set back to 6 days (HRT6_25). High VFAs concentration facilitates the application of these processed effluents as renewable feedstock, as in the case of PHAs production employing either mixed or pure cultures.[61,95] Steady state VFAs production was achieved within 10 days (Figure 4.4C) and yielded the highest total VFAs concentration of the present study (16.65 $g \cdot L^{-1}$, Table 4.1). This value is higher than the ones reported by Duque et al. (7.05 $g \cdot L^{-1}$) and Bengtsson et al. (3.08 $g \cdot L^{-1}$) [10] but lower than that attained by Davila-Vazquez et al. (25.62 $g \cdot L^{-1}$, with 46 $g \cdot L^{-1}$ of lactose and extra supplemented nutrients).[62] However, in the latter study the yield in VFAs per fed lactose was lower (0.56 $g_{VFAs} \cdot g_{lactose-1}$). This indicates that an improvement of VFAs concentration may be attained also in the present PBBR system by further increasing lactose concentration in the feed, although its conversion efficiency might be affected.

Table 4.1 - Main results describing PBBR performances at steady state

| | HRT6_15[b] | HRT4_15[c] | HRT 6_25[d] |
|---|---|---|---|
| C2 (g·L$^{-1}$) | 3.32 ± 0.13 | 1.89 ± 0.19 | 2.90 ± 0.28 |
| C3 (g·L$^{-1}$) | 3.45 ± 0.48 | 1.85 ± 0.29 | 2.77 ± 0.22 |
| C4 (g·L$^{-1}$) | 2.64 ± 0.40 | 2.23 ± 0.26 | 3.65 ± 1.15 |
| C5 (g·L$^{-1}$) | 0.68 ± 0.10 | 1.87 ± 0.25 | 2.66 ± 0.49 |
| C6 (g·L$^{-1}$) | 0.51 ± 0.21 | 1.27 ± 0.37 | 4.03 ± 0.41 |
| VFAs (g·L$^{-1}$) | 11.18 ± 0.61 | 9.27 ± 0.35 | 16.65 ± 0.53 |
| COD$_{Feed}$ | 21.43 ± 1.81 | 21.43 ± 1.81 | 40.28 ± 1.44 |
| COD$_{out}^{Mix}$ | 18.62 ± 1.01 | 19.27 ± 0.88 | 35.32 ± 3.54 |
| COD$_{out}^{liq}$ | 18.32 ± 0.77 | 17.59 ± 1.00 | 31.23 ± 4.68 |
| $Y_{VFAs/COD}$[f] | 0.85 ± 0.08 | 0.81 ± 0.09 | 0.80 ± 0.13 |
| $P_{C2}$ (g·L$^{-1}$·d$^{-1}$) | 0.55 ± 0.02 | 0.47 ± 0.05 | 0.48 ± 0.05 |
| $P_{C3}$ (g·L$^{-1}$·d$^{-1}$) | 0.58 ± 0.08 | 0.46 ± 0.07 | 0.46 ± 0.04 |
| $P_{C4}$ (g·L$^{-1}$·d$^{-1}$) | 0.44 ± 0.07 | 0.56 ± 0.07 | 0.61 ± 0.19 |
| $P_{C5}$ (g·L$^{-1}$·d$^{-1}$) | 0.11 ± 0.02 | 0.47 ± 0.06 | 0.44 ± 0.08 |
| $P_{C6}$ (g·L$^{-1}$·d$^{-1}$) | 0.09 ± 0.04 | 0.32 ± 0.09 | 0.67 ± 0.07 |
| $P_{VFAs}$ (g·L$^{-1}$·d$^{-1}$) | 1.86 ± 0.10 | 2.32 ± 0.01 | 2.78 ± 0.09 |
| $Y_{VFAs/Lactose}$[e] | 0.90 ± 0.05 | 0.82 ± 0.03 | 0.85 ± 0.03 |

[a]Acids concentrations (acetic, C2; propionic, C3; butyric, C4; valeric, C5; hexanoic, C6; and total acids, VFAs), productivities ($P_{Cx}$), COD of the feeding and effluent solutions (COD$_{Feed}$ and COD$_{out}^{liq}$) and total acids yield ($Y_{VFAs/Lactose}$). Unless otherwise stated, the reported results are the average of the replicates with standard deviation represented by ±. Average values for days. [b]60−88. [c]44−76. [d]11−38. [e](Cmol·Cmol$^{-1}$). [f]Deviation calculated by error propagation.

The consumption of proteins and lipids (the minor fraction, 25%, in CWP) could not be clearly determined on the basis of COD balances ([SCODIN − CODIN,lactose] − [SCODOUT − CODOUT,VFAs]) due to high uncertainty caused by error propagation. Nonetheless, it can be said that at least 10%−20% of that fraction was consumed in all tested conditions, probably for cell growth and acids production.[97] Regarding a potential inhibition from ammonia arising from protein digest, this was not considered because it was not observed in previous MBR fermentation test using the same CWP (on the contrary, lack of ammonia was reported) [61] and because it should have been noticed when increasing the CWP concentration for condition HRT6_25. Comparable YVFAs were obtained with respect to other VFAs production processes carried out under continuous operation mode. In the present study, the YVFAs values ranged between 0.79 and 0.90 g$_{COD}$·g$_{SCOD}$, while Duque et al.[61] obtained 0.65−0.74 g$_{COD}$·g$_{SCOD-1}$ with a membrane bioreactor, also comparable with what reported by Bengtsson et al.[10] (0.75−0.87 g$_{COD}$·g$_{SCOD-1}$ at pH 6 and HRT 0.3−2.1 d). The YVFAs obtained in the present work are higher with respect to those obtained in continuous fermentation processes using paper, wood, or olive mil effluents as renewable feedstock.[1]

Nonetheless, the present PBBR system needs to further improve its performance, especially in terms of immobilized biomass per reactor volume, since the obtained productivities were below those reported previously for the same CWP ($7.05 g \cdot L^{-1} \cdot d^{-1}$ [61]) and even more far away from an expected industrial productivity of 106 $g \cdot L^{-1} \cdot d^{-1}$,[62] which however was obtained by employing CW supplemented with salts. On the other hand, the proposed system allowed us to obtain hexanoic acid, which could represent a higher added value product and potentially easier to be separated.[84] In this respect, the remarkable relative increase of the hexanoic acid concentration with respect to the other VFAs in the HRT6_25 test suggests that higher lactose concentrations may yield interesting results also in terms of VFAs profile. Similarly, pH control may be considered to govern CW bioconversion pathways and steer microbial processes toward a different range of fermentation products. This was observed during the PBBR start up (HRT6_15, days 0 to 49, Figure 4.4A), although it was not a variable studied in the present work. Indeed, the reduction on pH variations to less acid values caused an increase in the relative abundance of propionic acid and, at a lower extent, valeric acid, as opposed to butyric acid. This is consistent with the results obtained by Bengtsson et al., who reported that acetic and butyric acids were mainly produced at pH 5 − 5.5, whereas a pH shift to a value of about 6 yielded propionic and valeric acids in a significant amount.[10] Propionic acid continued to be produced even at HRT4_15 (Figure 4.4B), suggesting that pH has more influence than HRT.

**Microbial Community**. All operating conditions shared a number of dominant genera (Figure 4.5). The potential relation in between the production of specific VFAs and the process' microbial community structures was investigated. When comparing the tested operating conditions in terms of the highest vs the lowest butyric and hexanoic acid productivities (HRT6_25 vs HRT6_15, $g_{VFAs} L^{-1} \cdot d^{-1}$, the high productivity of these VFAs was associated with a loss of richness and evenness (SI Table S4), i.e., when microbial communities were highly enriched in few genera. The microbial community that mediated high butyric and hexanoic acids production (HRT6_25, Table 4.1) was dominated by the genus Lactobacillus (OTU0001), which was identified as the type strain L. delbrueckii (SI Table S3). This strain was the most abundant in all reactors and was predominant in the CW feed (~98% of the sequences.[98] Other highly enriched genera were Olsenella (OTU0007), Actinomyces (OTU0010) and an unclassified Bacteria (OTU0015). All of them were not detected in the feed and inoculum (except Olsenella whose relative abundance was almost negligible) and were poorly associated with known type strains. When not related to type strains, they showed strong similarities with, respectively, O. uli, A. hyovaginalis and Clostridium colinum (≥0.99). This result is consistent with data from microcosms experiments loaded with the SI, where Lactobacillus was the predominant genera in the first 5 days of incubation, that is, during lactose to lactic acid conversion (Figure 4.2C), while the second phase associated with VFAs production (Figure 2C) was mostly enriched in Clostridium.

The highest propionic and valeric acids-producing PBBRs were also compared to the lowest producing ones, but no clear association in terms of richness or evenness was noted. However,

propionic acid productivity, which was the highest in HRT6_15 and comparable in HRT6_25 and HRT4_15 (0.46 g·L$^{-1}$·d$^{-1}$, Table 4.1), was associated with a reduction to 0.5% − 2.0% of the genera Lactobacillus, Actinomyces (both OTU0010 and OTU0017) and Olsenella (Figure 4.5), in concomitance with an increase in an unclassified Bacteroidetes (OTU0004), Sutterella (OTU0006), Clostridium XIVa (both OTU0005 and OTU0018), and unclassified Ruminococcaceae (OTU0020) and Oscillibacter (OTU0022) (Figure 4.5). Hence, all bacteria belonging to the class of Clostridia with the exception of the unclassified Bacteria (OTU0015) were associated with an increase in propionic acid productivity.



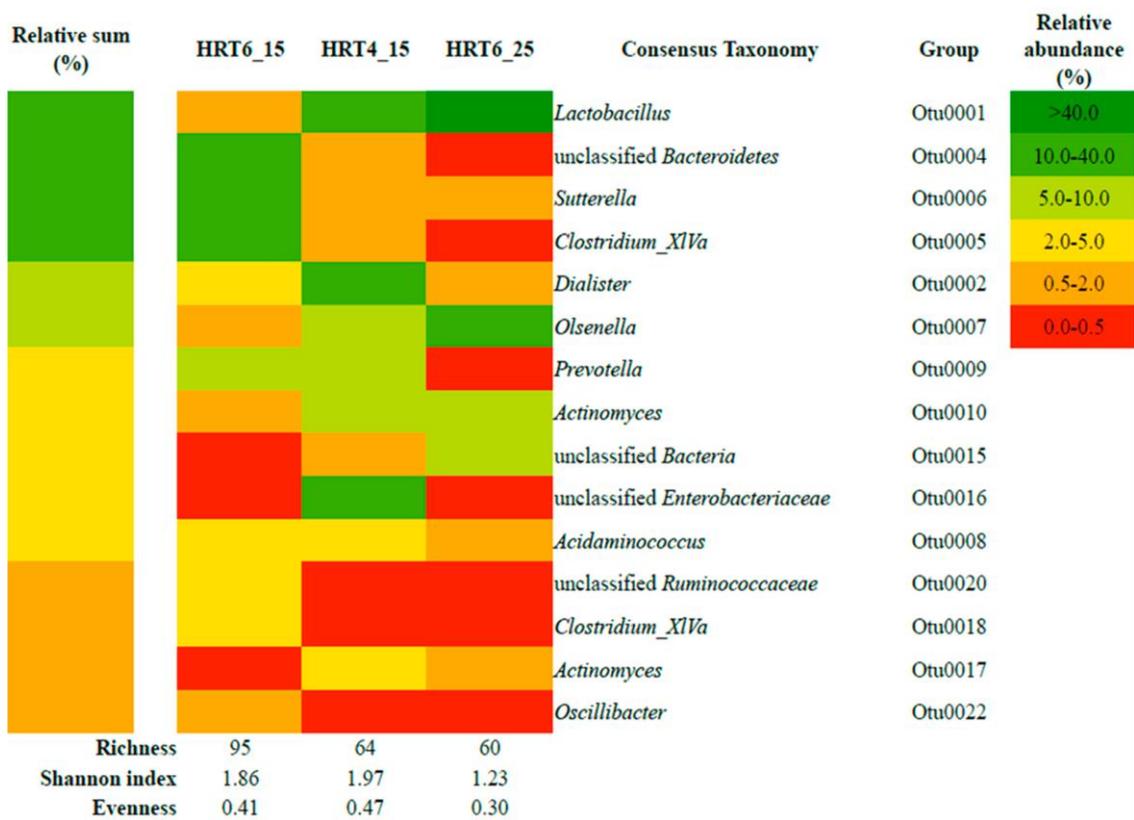| Relative sum (%) | HRT6_15 | HRT4_15 | HRT6_25 | Consensus Taxonomy | Group | Relative abundance (%) |
|---|---|---|---|---|---|---|
| | | | | Lactobacillus | Otu0001 | >40.0 |
| | | | | unclassified Bacteroidetes | Otu0004 | 10.0-40.0 |
| | | | | Sutterella | Otu0006 | 5.0-10.0 |
| | | | | Clostridium_XlVa | Otu0005 | 2.0-5.0 |
| | | | | Dialister | Otu0002 | 0.5-2.0 |
| | | | | Olsenella | Otu0007 | 0.0-0.5 |
| | | | | Prevotella | Otu0009 | |
| | | | | Actinomyces | Otu0010 | |
| | | | | unclassified Bacteria | Otu0015 | |
| | | | | unclassified Enterobacteriaceae | Otu0016 | |
| | | | | Acidaminococcus | Otu0008 | |
| | | | | unclassified Ruminococcaceae | Otu0020 | |
| | | | | Clostridium_XlVa | Otu0018 | |
| | | | | Actinomyces | Otu0017 | |
| | | | | Oscillibacter | Otu0022 | |
| Richness | 95 | 64 | 60 | | | |
| Shannon index | 1.86 | 1.97 | 1.23 | | | |
| Evenness | 0.41 | 0.47 | 0.30 | | | |

Figure 4.5 - Predominant OTU representatives and their relative abundance in PBBR converting CW into VFAs.

In conclusion, the PBBR turned out to be a robust culture system for the bioconversion of CW organic matter into VFAs. The process was monitored from both chemical and microbiological point of views. High bioconversion yields (>80%) and VFAs concentrations (up to 16 g·L$^{-1}$) were obtained by operating under different loading conditions and without nutrient supplement. The results obtained indicate that the process performance may be improved by increasing the OLR parameter (4 days < HRT ≤ 6 days; CWP ≥ 20 g·L$^{-1}$), which in turn will impact on the microbial community structures by reducing the number of microbial representatives (mainly Lactobacillus and Olsenella).

# **5.** VFAs concentration – Electrodialysis



Summary: In the present chapter, it is presented the concentration of organic acids by employing membrane processes an electrodialysis (ED) process. Two different effluents were used for the experiements: fermented broth from the VW and MS ($VMW_{acid}$), presented in the previous chapter and the fermented broth from cheese whey fermentation ($CW_{acid}$). With $VMW_{acid}$ a concentrated solution was obtained with 50 g.L$^{-1}$ of total VFAs within 400 minutes. However, experiments with simulated solutions showed the potential of achieving 100 g.L$^{-1}$, of total VFAs, in 1400 minutes. With $CW_{acid}$ it was possible to increase the VFAs concentration from 9 to 60 g.L$^{-1}$ in 2130 minutes.

## 5.1 Introduction

Electrodialysis (ED) is a membrane process in which the driven force is an electrical potential difference [99]. The current is usually promoted by an electrolyte that fill and continuous recirculate in electrodes compartment is the responsible for generate the current and at the same time clean the electrodes surface but does not participate in the ED separation. The electrolyte typically used is $Na_2SO_4$ solution in concentrations between 7 and 140 g.L$^{-1}$ [32,33,40–42,100,101] according to the composition of the feed solutions. This electrical field will promote the movement of the ions, electrically charged species, in solution where the cations (positive charge ions) will move in direction to the cathode (negative electrode) while the anions (negative charge ions) will move in the opposite direction by means to the anode (positive electrode) but uncharged molecules are not affected by the electrical field [99].

The ion exchange membranes, placed between the electrodes, have a very important role in the process since by disposing it alternately to form compartments resulting in a concentrated compartment where flows a ions-richer solution and a diluted compartment with a ions-poor solution [28]. These membranes are also responsible to control the migration of ions species: the anions are able to pass through the anion exchange membranes (AEM) but cannot pass through the cation exchange membrane (CEM) and *vice versa* for the cations. The most desired properties of the membranes required for the successful process are: high permselectivity, low electrical resistance, good mechanical stability and high chemical stability [102]. The spacers made by plastic material (polyethylene) are placed between the membranes and have the role of separate the concentrated and diluted compartments and improve the fluid distributions for better mass transfer [103]. Because of that, is crucial that during the assemble procedure the spacers are in the right orientation, otherwise the paths can be mixed or reversed. In Figure 5.1 it is represented a ED process scheme.
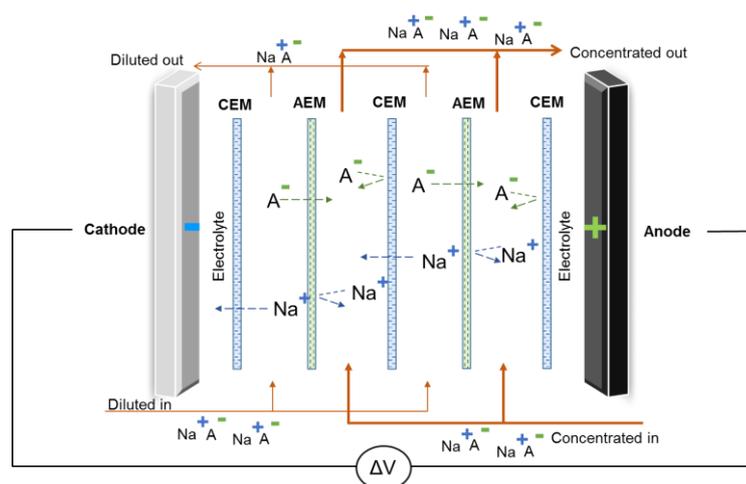


Figure 5.1 - Schematic diagram of membrane stack with membrane pairs: CEM – cationic exchange membrane, AEM – anionic exchange membrane, ΔV – potential difference.

The ED process can be applied in different fields, from demineralisation to concentration step of diluted compounds. Since ED is a process able to separate charged from uncharged molecules it can be used in separation of acids from sugars [28]. Specifically in the case of CW, which is composed mainly by lactose, acids, minerals and proteins, the removal of lactic acid (charged at basic pH) increase the efficiency in lactose purification (uncharged molecule) [48]. Lactic acid can be an inhibitory during fermentation and so, because of that, ED is used for its removal from bioreactors broth [28,32,104,105].  Also the recovery and concentration of succinate has been studied with ED process [28,42]. In which respect to the concentration or removal of volatile fatty acids (VFAs) there are only few reported studies. Rhys Jon Jones and co-works [39] performed ED to remove these acids, such as acetic and butyric, due to the inhibition effect during hydrogen production by fermentation. Recently Scoma et al. suggested the concentration of a VFAs mixture from the fermentation of olive mill wastewater (OMW) in order to obtain a concentration solution for later uses [41].

In the present chapter, it was proposed the concentration of VFAs from different fermented broths by using ED process.

## 5.2    Materials and methods

### 5.2.1    Electrodialysis apparatus

The ED apparatus (Wortmann, Brecia - Italy) is represented in Figure 5.2 and it was equipped with 3 independent recirculation circuits – diluted and concentrate compartments and electrodes rinse solutions -  each equipped with a centrifugal pump and a liquid storage container. The membrane stack composed by: two electrodes, three membrane pairs, 1 mm thickness PTFE spacers and 1 mm thickness Viton gaskets per pair. The electrical current is supplied by a DC power supply connected to the anode and cathode electrodes.
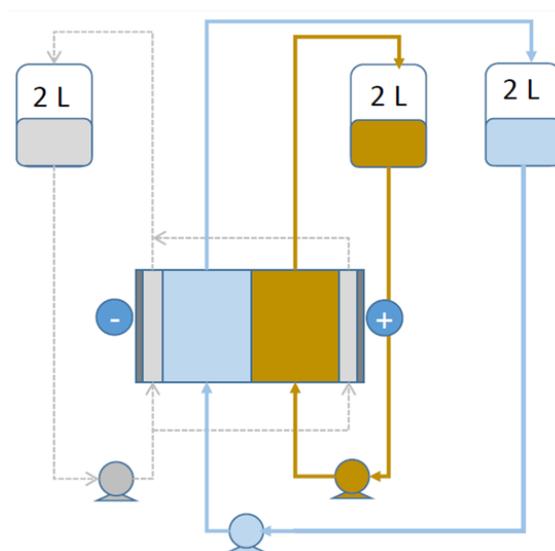
Figure 5.2 - ED system scheme

Electrode compartments consist of an electrode, an electrode water-flow spacer, and a cation membrane. Because of the corrosive nature of the anode compartments, the electrode for the anode was made of titanium and plated with platinum (Coated (RU-IR) TI) and where oxidation occurs and so anions movement is attracted in this direction. The cathode electrode was made of stainless steel (AISI 316 SS) and is where reduction occurs. Each electrode had an effective area of 0.016 $m^2$. A 80 ± 2 $g.L^{-1}$ $Na_2SO_4$ solution was used a electrolyte and it was continuously recirculating in both electrodes compartment. All experiments were performed at potentiostatic operation mode at fixed difference potential of 10 V.

During the present work, three membrane pairs were employed. In total 4 cation exchange membranes (CMX) and 3 anion-exchange membranes (AMX) which were displaced alternately – CMX, AMX, CMX. Each membrane sheet had an effective membrane area of 0.016 resulting in a total membrane area of 0.094 $m^2$ and the specific characteristics of the ion-exchange membranes are described in Table 5.1. Membranes were regenerate after each experiment by recirculating HCl 0.05 M for 30 min, then cleaned with distillate water, after recirculation NaOH 0.05 M for 30 min and in the end washed again with water [101]. In the end of each experiment or membranes regeneration a solution of NaCl 30 $g.L^{-1}$ was left inside the membrane stack. The electrodes were cleaned by reversing the cathode and anode and recirculating water in the membrane stack and electrolyte in the electrodes compartments. The membrane stack was never opened or change along all the experimental time.

Table 5.1. Characteristics of ion-exchange membranes used in ED experiments [32]

| Properties | AMX | CMX |
|---|---|---|
| Type | Strongly basic anion permeable | Strongly acidic cation permeable |
| Characteristics | High mechanical strength (Na-form) | High mechanical strength (Cl-form) |
| Material | Styrene-divinyl benzene copolymer | Styrene-divinyl benzene copolymer |
| Electric resistance (0.5 M NaCl) | $2.0 - 3.5 \Omega/cm^2$ | $2.0 - 3.5 \ \Omega/cm^2$ |
| Burst strength | 450 – 550 kPa | 500–600 kPa |
| Thickness | 0.14–0.18 mm | 0.16–0.20 mm |
| Temperature range allowance | 0 – 40 °C | 0 – 40 °C |
| pH range allowance | 0–10 | 0–10 |
| Area ($m^2$) | 0.016 | 0.016 |

### 5.2.2 ED experiments

Different types of solutions were used in ED experiments: a) desalting run of 10 g.L$^{-1}$ sodium chloride solution (blank experiment), b) simulated solutions (sim) with the composition as the fermented broths from co-digestion of vegetable waste and maize silage ($VMW_{acid}^{Sim}$) and from CW ($CW_{acid}^{Sim}$) and at last c) the real fermented broths ($VMW_{acid}$ and $CW_{acid}$).The blank experiment was performed intermittently to check for any deterioration in membranes performance. The time and trend of desalting was the measurement of performance. Sodium chloride is a frequently solution used for test membrane characteristics the manufactures.

*Sim* is a solution prepared in laboratory with deionised water and commercial solutions which compose the fermented broths. All acids and salts were purchase from sigma Aldrich. The pH was correct by adding NaOH solution (10 M).

The fermentation broths ($VMW_{acid}$ and $CW_{acid}$) are the outlet resulted from the anaerobic acidogenic bioreactors described in chapter 3 and 4 respectively. All liquid was previous centrifuged (12,000 rpm for 10 minutes) followed by vacuum filtration with a cellulose membrane 1.2 µm before ED experiments. No additional compounds were added to these solutions before ED experiments.

Table 5.2 - Main characteristics of simulated solution (SIM) and CW and VW fermented broth (acid) used during ED experiments

| pH | $VMW_{acid}^{Sim}$ | $VMW_{acid}$ | $CW_{acid}^{Sim}$ | $CW_{acid}$ |
|---|---|---|---|---|
| Acetic (g L$^{-1}$) | 10.23 ± 0.80 | 12.13 | 2.10 ± 0.17 | 1.29 ± 0.09 |
| Propionic (g.L$^{-1}$) | 3.51 ± 0.22 | 3.70 | 0.23 ± 0.02 | 0.40 ± 0.17 |
| Butyric (g.L$^{-1}$) | 7.42 ± 0.56 | 6.84 | 1.53 ± 0.11 | 1.53 ± 0.15 |
| Valeric (g.L$^{-1}$) | 2.05 ± 0.11 | 2.07 | 0.34 ± 0.03 | 0.27 ± 0.04 |
| Hexanoic (g.L$^{-1}$) | 1.65 ± 0.15 | 1.79 | 3.47 ± 0.25 | 4.15 ± 0.20 |
| Heptanoic (g.L$^{-1}$) | 0.88 ± 0.16 | 0.59 | - | - |
| Octanoic (g.L$^{-1}$) | - | - | 1.71 ± 0.21 | 1.34 ± 0.35 |
| Total VFAs (g.L$^{-1}$) | 27.14 ± 1.65 | 29.05 | 9.48 ± 0.73 | 9.28 ± 0.24 |
| Chloride (g.L$^{-1}$) | 1.43 ± 0.08 | 1.68 | 2.13 ± 0.17 | 1.09 ± 0.14 |
| pH | 6.16 ± 0.06 | 6.23 | 6.65 ± 0.14 | 7.24 ± 0.41 |
| Conductivity (mS.cm$^{-1}$) | 19.80 ± 0.29 | 25.7 | 12.21 ± 0.64 | 13.43 ± 0.13 |

The initial volume (2 L) was the same for diluted, concentrated and electrolyte compartments and all experiments. All solutions were in the recycle mode all experimental time. Before voltage apply all solutions were recirculated along 10 minutes inside the membrane stack. Sampling was performed every 10-20 minutes for liquid analysis: conductivity, pH, temperature, VFAs and anions concentrations. When conductivity in the diluted compartment achieved a value equal or below of 2 mS.cm$^{-1}$ the batch was stopped, therefore different experiment durations were obtained with respect to the solution studied. In the sequential batches experiments, when diluted solution was exhausted the next batch was begun by replace it by a fresh solution while in the concentrated compartment the initial solution was the result of previous batch. Fresh solution means the same solution used in the beginning of the first batch. In Figure 5.3 it is represented the scheme of the experiments where $X_{acid}$ represents $VMW_{acid}$ , $VMW_{acid}^{Sim}$ , $CW_{acid}$ or $CW_{acid}^{Sim}$ according with the experiment. While n is the repeated unit cell which can be reproduce as many times as many batches needed to achieve a desired concentration. The experiments with $CW_{acid}^{Sim}$ were performed in double as well the first batch of $CW_{acid}$. The required feed volume limited the number of sequential batch with fermented broths.
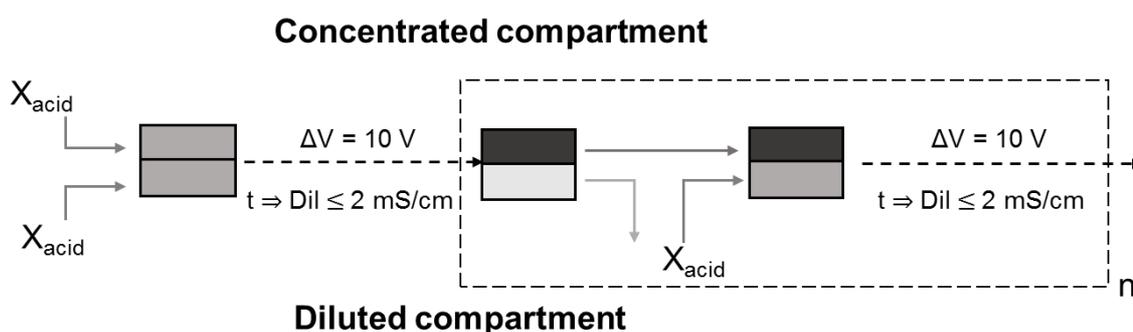
## Concentrated compartment



Figure 5.3 - Experimental procedure scheme for the ED sequential batches, where $X_{acid}$ means the effluent acidified and *n* the repeated cell unit.

### 5.2.3 Analytical procedures

The VFAs concertation and Chemical Oxygen Demand (COD) analysis were performed as previously described [65].

The inorganic anions were measured by using a Dionex DX-120 IC system equipped with an IonPac AS14 (4 × 250 mm) column and a conductivity detector in combination with an ASRS-Ultra conductivity suppressor system (Dionex Corp.). A solution of 8 mM $Na_2CO_3$ and 1 mM $NaHCO_3$ was used as eluent at 1 mL.min$^{-1}$ run ambient temperature.

Conductivity, pH and temperature were measured in simultaneous using a multiparameter (XS instruments)

### 5.2.4 Calculated parameters

Table 5.3 Ethanol and VFAs main features

| | Ethanol | Acetic | Propionic | Butyric | Valeric | Hexanoic | Heptanoic | Octanoic |
|---|---|---|---|---|---|---|---|---|
| **Chemical Formula** | $C_2H_6O$ | $C_2H_4O_2$ | $C_3H_6O_2$ | $C_4H_8O_2$ | $C_5H_{10}O_2$ | $C_6H_{12}O_2$ | $C_7H_{14}O_2$ | $C_8H_{16}O_2$ |
| **Molar mass (g.mol$^{-1}$)** | 46.07 | 60.04 | 74.08 | 88.11 | 102.13 | 116.16 | 130.18 | 144.21 |
| **COD (gO$_2$.gVFA$^{-1}$)** | 2.08 | 1.07 | 1.51 | 1.82 | 2.04 | 2.20 | 2.34 | 2.44 |

Ohm's Law where R (in Ω) is the resistance, V (in V) the voltage and I (in A) the current

$$R = \frac{V}{I}$$

(1)

Where R is the resistance (Ω), V the voltage (V) and I the electrical current (A).

Molar cumulative transport ($N$) in concentrate compartment, evaluated at the generic time t:

$$N_i\,(t) = \frac{n_i^{conc}\,(t) - n_i^{conc}\,(0)}{t}$$

(2)

Where $N_i$ (mol.s$^{-1}$), $n_i^{conc}$ the mol of each acid ($i$) in the concentrated compartment in a certain time (t) of at initial time (0).

Transfer number ($T$)

$$T\,(i) = \frac{N_i \times F}{n_c \times I}$$

(3)

Where $N_i$ (in mol.s$^{-1}$) is the molar cumulative transport, $n_c$ the number os membrane paris (in this case 3 pairs), I the electrical current (A) and F the Faraday's constant (96,485 C.mol$^{-1}$).

Enrichment factor ($EF$) of each single acid (i)

$$EF\,(i) = \frac{c_i(t) \big/ c_{i\_tot}(t)}{c_i(0) \big/ c_{i\_tot}(0)}$$

(4)

Where, in concentrated compartment, $c_i$ is the concentration (in mM) of each single acid in a certain time (t) or at the beginning (0) and $c_{i\_tot}$ the total concentration (in mM) of total acids.

Overall specific energy ($W$)

$$W = \frac{\int_0^{t_f} I \times V(t)dt}{N_{VFAs}(t_f) \times t_f}$$

(5)

Where $N_{VFAs}$ is the molar cumulative transport of total VFAs in the concentrated compartment, V the applied voltage (10 V) and $t_f$ the total time of batch.

The Current efficiency (CE) was calculated as followed:

$$CE = \frac{F \times (n_{i_{tot}}^{conc}(tf) - n_{i_{tot}}^{conc}(t0))}{n_c \int_0^{tf} I\ dt}$$

(6)

where F is the Faraday constant (96,485 C.mol^{-1}), $n_{i_{tot}}^{conc}(tf)$ the total moles in concentrated compartment at final time of each batch, $n_{i_{tot}}^{conc}(t0)$ the total moles in concentrated compartment at the beginning, $n_c$ the cell pairs (3), $I$ the current and $t$ the time.

## 5.3    Results and discussion

### 5.3.1    Blank experiments

Along thesis work, experiments with NaCl solution were performed frequently, the so called "blank experiments". These experiments were considered as a control to verify if membranes were damage during previous experiments resulting in fouling phenomena. A solution of NaCl 0.2 M was used in diluted and concentrated compartment as was also used and reported by Weier et al. [40] as way to check the membranes avoiding open the membrane stack. The manufactures of ED membranes use to use this type of solution to test membranes performance (NaCl 0.5 M). In Figure 5.4 it is represented the trends of conductivity in the concentrated (filled bullets) and diluted (empty bullets) and the electrical current observed during all blank experiments (from A to J).

The conductivity measure the ions in solution and so the profile along the time indicates if there is occurring, or not, the ions transport from one to the other compartment. As it can be observed in Figure 5.4 the conductivity obtained for diluted compartment was linearity decreasing and in concentrated compartment was linearity increasing along the experimental time for each Blank experiment. The profiles were quite similar between each running meaning that the membranes were still suitable for the followed experiments. However, the current profile showed some deviation in fact, trial A (the first to be done) begun with a value higher than the trial B to I. This was an indicator that

the electrodes may suffer some damage. Trial J was performed after some electrodes cleaning and so the current values become better.

Since the membrane stack showed repeatability in the profiles, it was never open or changed during all work development.
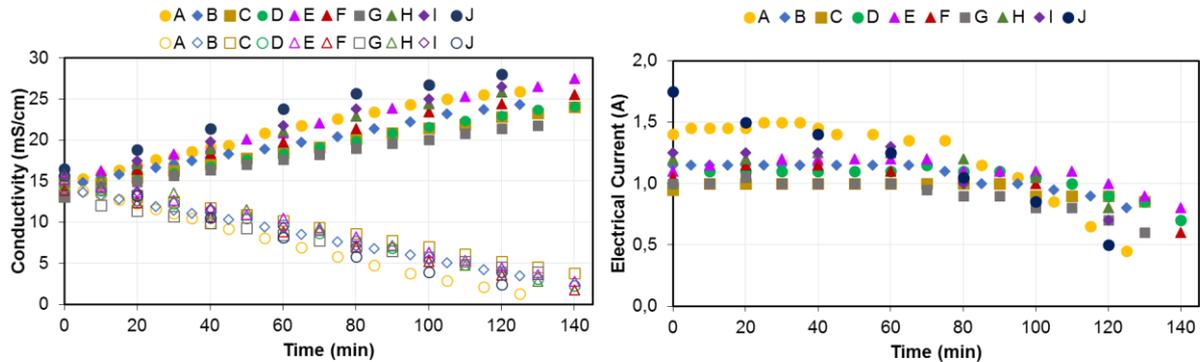


Figure 5.4 - Conductivity and current profile obtained during all experiments (A to J) performed with NaCl solution in diluted (empty bullets) and concentrated (filled bullets) compartments.

### 5.3.2 VMW$_{acid}$

Experiments with $VMW_{acid}^{Sim}$ were performed as first experiments in order to acquire some knowledge about the ED performance with a solution composed by this type of VFAs mixture.

The conductivity presented the same profile as Blank experiments, increases linearly in the concentrated and decreases linearity in diluted compartment (data not shown). The current had the same profile as verify previously for Blank experiments decreasing along the time either $VMW_{acid}^{sim}$ and $VMW_{acid}$ (Figure 5.5). By applying the Ohm's Law (equation ((1)) the resistance in the system increased from 6.9 to 22.2 Ω due to the unbalance of ions observed between diluted and concentrated compartments along the ED experimental time. The low concentrations of ions in diluted compartment leads to higher resistance in the system [106].
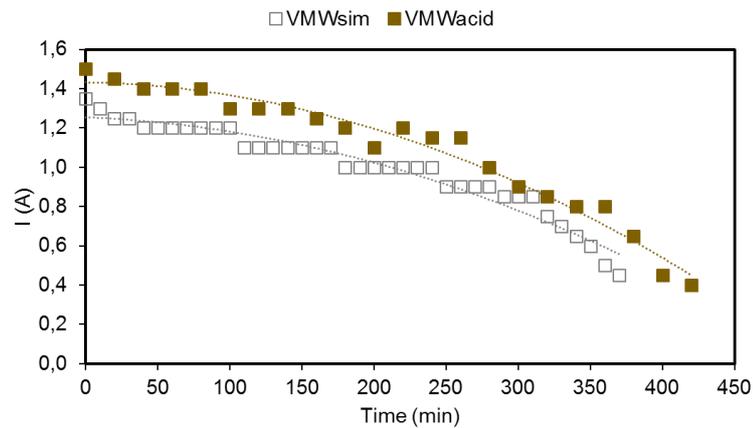
Figure 5.5 - Current profile for $VMW_{acid}^{Sim}$ and $VMW_{acid}$ experiments

In which respects to the VFAs concentration (Figure 5.6) it was observed concordance in the results between the $VMW_{acid}^{Sim}$ and $VMW_{acid}$ since in both experiments its concentration in concentrated compartment increased from 27.5 and 29.2 to 51.9 and 51 g.L$^{-1}$ respectively after approximately 420 minutes. The concentration in the concentrated stream stopped when, almost, all the acids were already transported from diluted to concentrated stream. It was not observed a significant preference for one acid in particular since the mass ration of each acid respects to the total VFAs concentration was the same during all ED experiment. As it has already been reported by several authors that during ED process, either for current or potential constant, water molecules are also transported through the ion-exchange membranes from diluted to concentrated compartment. This phenomena is occurs due to the osmosis promote by concentration gradient between the two fluxes (diluted and concentrated) [33,42,100]. Also during the present work was observed volume decreasing in diluted and increasing in the concentrated compartment. In an attempt, not so successfully, to avoid this volume difference and affect the final concentration the pump flows were changed during experiment. Because of this phenomena all the mass balance was performed in mol.
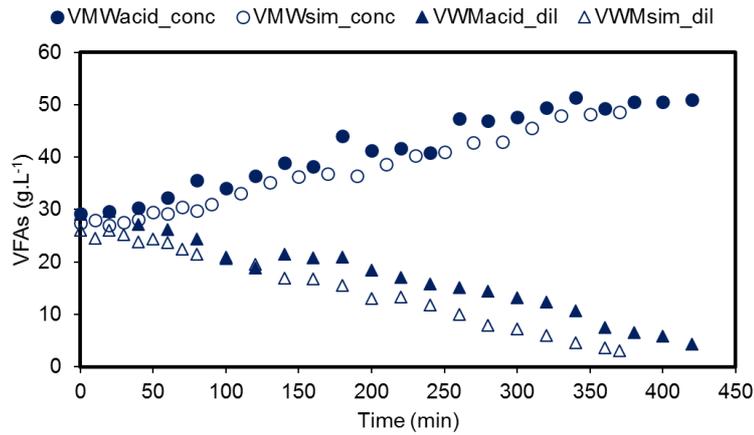
Figure 5.6 - Total VFAs in diluted and concentrated compartments in $VMW_{acid}^{Sim}$ and $VMW_{acid}$

Chloride was the ion in solution preferentially transported through the membranes and after 100 minutes of ED experiment with $VMW_{acid}$ the total initial concentration in diluted was completely transported to the concentrated compartment. This evidence is even confirmed by the EF (Figure 5.7) which achieved its maximum value of 1.48 after 100 minutes. For the acids, EF values are much lower being constant along the time and quite the same between each single VFA with a variation between 1.01 for acetic and 0.92 for butyric, but for chloride. Another evidence of completion between chloride and acids is the transfer number (Figure 5.8) that for chloride it was 0.38 right after 20 minutes of experiment while for the acids was almost 0. As chloride is transported to the concentrated compartment, the transfer number increased gradually for all the acids with bigger evidence for acetic acid. All this reported data are in concordance with Scoma and co-workers [41] which had already observed the competition between chloride and the acids during VFAs concentration which composed OMW fermented broth, with the same ED configuration.
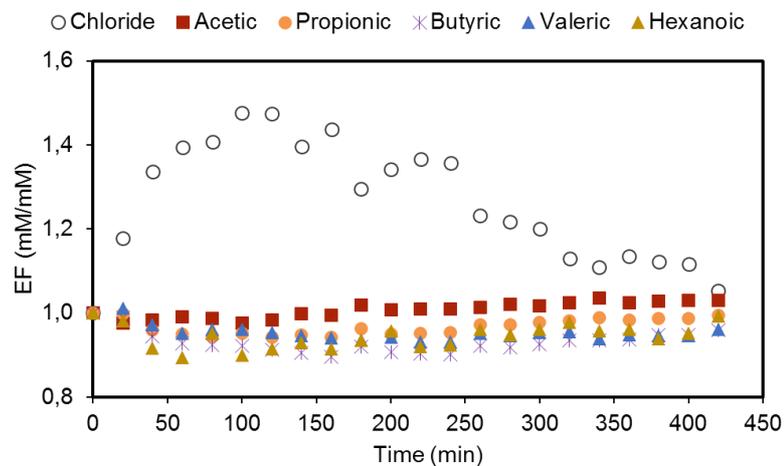


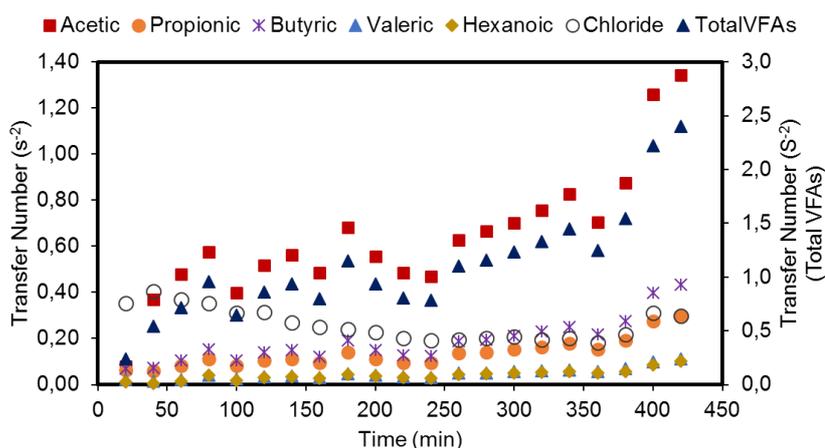Figure 5.7 - EF obtained for the ions in $VMW_{acid}$ solution

Figure 5.8 - Transfer number for each single acid and chloride for $VMW_{acid}$

Since $VMW_{acid}$ is the result of a fermented waste it is composed by organic matter besides VFAs and in consequence COD measurement allow to know the quantity of organic matter in diluted and concentrated streams before and after ED process. The initial COD was $54.99 \pm 0.35$ $gO_2.L^{-1}$ which $79.36 \pm 0.77\%$ was due to the VFAs concentration. After 410 minutes of experiment the COD in concentrated stream raised to $89.37$ $gO_2.L^{-1}$ which $85.65\%$ was due to the VFAs while in diluted stream COD content was $16.96$ $gO_2.L^{-1}$ and only $44.32\%$ due to the remain VFAs which were not transport through the membranes. In the end of the process, diluted stream becomes to be a waste and must be treated in some way before discharged due to its high COD content. Since it is composed by organic matter which can be: proteins, complex sugars not hydrolysate in previous fermentation and organic acids it is an opportunity to valorise this waste using as substrate for anaerobic digestion where methane and $CO_2$ can be produced.

Regarding to estimation about the ED process efficiency it must be considered: the overall specific energy required for the process by means the total electric power per mole of total VFA transported and the current efficiency that is defined as the ratio of the number of ions pass through the membrane and it is influenced by current density [37,41]. During this experiment was observed that the overall specific energy was similar for both solutions though for $VMW_{acid}^{sim}$ was slightly lower ($313$ $kJ.mol^{-1}$) respects to $VMW_{acid}$ ($367$ $kJ.mol^{-1}$). The values obtained in the present work are in the range as other reported works in literature as the OMW [41]. With OMW stream the specific energy was significant different for simulated and real fermented broth being the simulated solutions ($500$ $kJ.mol^{-1}$) higher than the present work but lower for OMW ($150$ $kJ.mol^{-1}$) respecting to $VMW_{acid}$. The current efficiency for this batch was $95\%$ for $VMW_{acid}^{sim}$ and $99\%$ for $VMW_{acid}$. This values were higher when compared to other works as for citric acid concentration (30-80%) [33], one batch for lactate (70% and 73%) [32,101], formic acid (59-70%) [100], succinic acid (29-22%) [42] and a mixture of acids as butyric, valeric and hexanoic (42-21%) [37].

The drawback of this technology for the concentration of molecules is the initial concentration of the stream and so it was proposed the perform sequential batch as represented in Figure 5.1 . This experiment was not possible to be performed with the $VMW_{acid}$ it was only performed with $VMW_{acid}^{sim}$.

In Figure 5.9 it is represented the profile obtained for the concentration of VFAs and chloride ions in concentrated and diluted streams along four sequential batches which had a durance of 1420 minutes in total. In the diluted compartment, every batch begun with the $VMW_{acid}^{sim}$ with the characteristics presented in Table 5.2. However contrary to the first batch where the initial solution in concentrated compartment was the same as diluted stream for the second, third and fourth batch the initial solution was the result of the concentrated stream resulted from the previous batch.
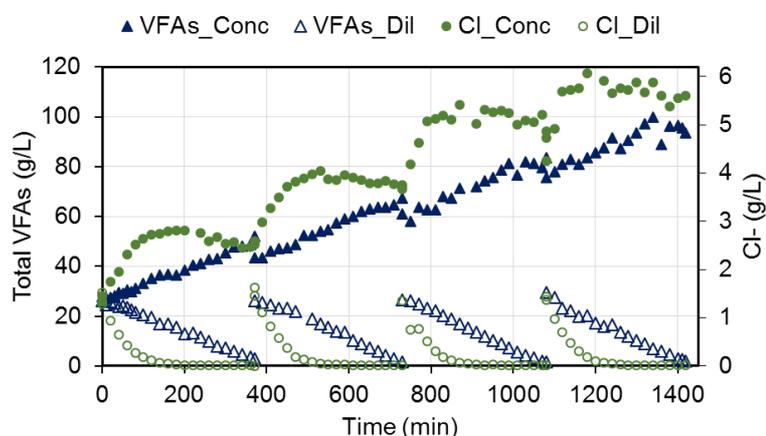


Figure 5.9 – VFAs and chloride concentrations profile during sequential batch with $VMW_{acid}^{sim}$

With the presented strategy was possible to increase the VFAs total concentration from 28 to 97 g.L$^{-1}$ in 1400 minutes (23.3 hours) resulting in a concentration rate of 0.05 g.L$^{-1}$.min$^{-1}$. However also the chloride was concentrated as it was observed in the first batch with both types of solutions.

It is clearly evidence the difference of concentration trend between chloride ions and total VFAs. The chloride transfer is much higher than any other carboxylic acid in solution, even compared with the less concentrated acids in solution. This could be attribute to the smaller molecular weight and Stokes radius of chloride compared to the other anions [48]. In the end, the total chloride concentration was almost 6 g.L$^{-1}$.

The EF profile (Figure 5.10) was the same as verified for the first batch (0-370 minutes) with the difference that even if EF of chloride was still higher than the acids its value (1.1) was lower than in the beginning.
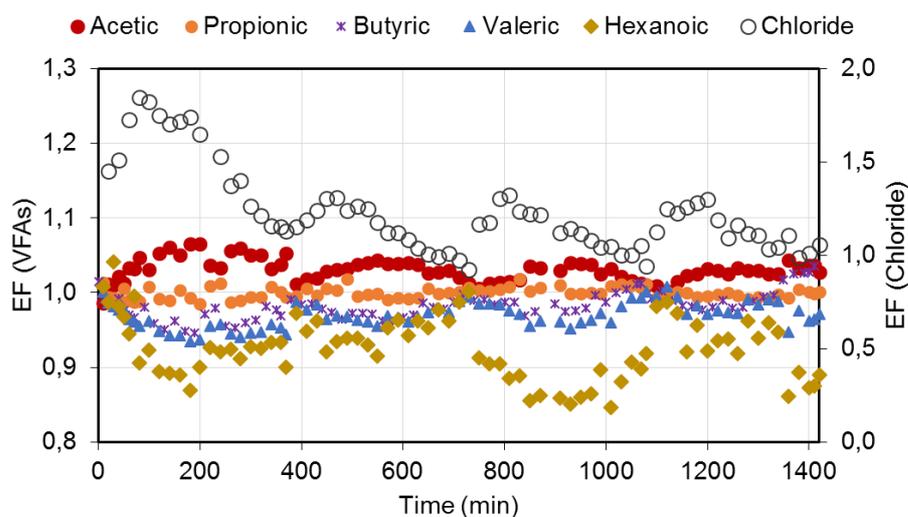
Figure 5.10 - EF profile obtained for each VFA and chloride during sequential batch with $VMW_{acid}^{sim}$

The overall efficiency of the process decreased with the number of batches performed. The specific energy increased from 313 kJ.mol$^{-1}$, in the first batch, to 603 kJ.mol$^{-1}$ in the fourth and last batch. In addition, it was observed a decrease in CE values: 94.72% (first batch), 98.7% (second batch), 39.30% (third batch) and 33.90% (fourth batch). Since the initial solution in the diluted compartment is always the same, with the increment in the sequential batches, the difference of initial ions concentration between diluted and concentrated compartments also increased which leads to lower CE values [33]. With the presented scheme of ED operation, it was possible to increase the VFAs concentrations in 2.5 times however also the costs verify being higher.

Since the batch performed with $VMW_{acid}$ had the same profile of the first batch of $VMW_{acid}^{sim}$ it is expected that also with sequential batch with the real fermented broth the results should be very similar.

### 5.3.3 CW$_{acid}$

For ED experiments, it was also performed experiments to concentrate VFAs from $CW_{acid}$. This solution is the fermented broth resulted from the CW fermentation which, as previous described for $VMW_{acid}$, was under anaerobic conditions and in continuous operation. The fermentation process was performed as described by Domingos et al. [2] with a slightly different VFAs profile obtained (Table 5.2).

The strategy to concentrate the VFAs with this effluent was the same as for $VMW_{acid}$ by means the performance of sequential batches. As first experiments a simulated solution was used ($CW_{acid}^{Sim}$) and then with the fermented broth ($CW_{acid}$). From Table 5.2 it can be observed that this effluent ($CW_{acid}$ and respective $CW_{acid}^{Sim}$) have completely different VFAs composition compared with the previous effluent ($VMW_{acid}$ and $VMW_{acid}^{sim}$). The initial solution was composed by 9.0 g.L$^{-1}$ of total VFAs

in which hexanoic acid was the main acid in the mixture composition. During the CW fermentation octanoic acid was also produced in considerable amounts (1.34 g.L$^{-1}$) and even if this acid does not belongs to the VFAs group, because is an carboxylic acid with eight carbon molecules, in the present work it was also considered for the total VFAs concentrations.

In the following figures (Figure 5.11 and Figure 5.12), it is represented the results obtained with $CW_{acid}^{Sim}$. Each batch had a duration of 180 minutes corresponding to half time need for the VFAs concentration in $VMW_{acid}^{Sim}$ and $VMW_{acid}$ though minor concentrations were obtained in concentrated stream. This can be explained by the fact that $CW_{acid}$ is an effluent much poor than $VMW_{acid}$. After 1080 minutes (18 hours) of ED it was possible to increase the total VFAs concentration from 8.78 ± 0.70 to 44.04 ± 3.56 g.L$^{-1}$ resulting in a concentration of 0.03 g L$^{-1}$ min$^{-1}$.
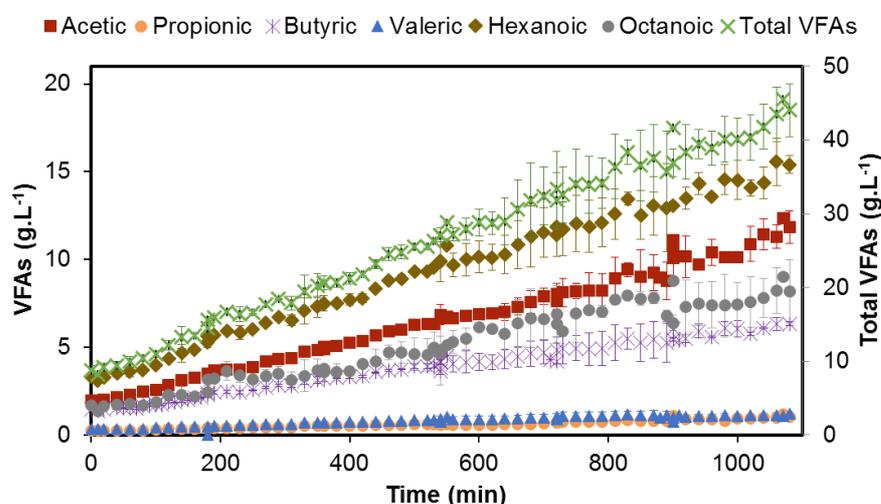


Figure 5.11 - Profile in concentrated compartment for each single VFA and the total VFAs during sequential batches with $CW_{acid}^{Sim}$

From Figure 5.11, where is represented the concentration profile obtained for each single acid, it can be said that the acids were affected equally by the electrical field. The concentrating profile for each acid, in particular hexanoic, acetic butyric and octanoic acid showed a linear profile. This can also be confirmed by the EF value of each acid, which is between 0.9 and 1.1. Tao et al. [55], as similar to the present work, reported the VFAs concentration of a thermally hydrolyzed activated sludge composed mainly by: acetic, propionic, butyric and valeric acids with a final concentration of 8.4 g.L$^{-1}$. Also in this case, the concentration characteristics were very similar between each acid with exception for valeric acid.  In the case of the other ions in solution, such as chloride it was observed a concentration increase from 2 till 15.1 g.L$^{-1}$. Also in this case the transfer of chloride ions was faster than the carboxylic acids in ion form, as it was discussed previously and observed by other researchers [41,48].

After get the knowledge the ED profile with simulated solution experiments were performed with the fermented broth $CW_{acid}$. The $CW_{acid}$ was the outlet of two 1 L PBBR operated in anaerobiose under continuous conditions with an HRT of 6 days as described in Chapter 4 though with a different VFAs composition (Table 5.2).

The duration of the ED experiment with $CW_{acid}$ was longer than the observed for $CW_{acid}^{Sim}$. Probably this loss in efficiency can be related to the lack of cleaning of the electrodes since some problems with the current have been observed. The first batch with $CW_{acid}$, performed in double, achieved the same results as obtained with $CW_{acid}^{Sim}$ however from the middle of second batch (minute 330) the movement of ions from the diluted to concentrated stream became much slower (Figure 5.12).
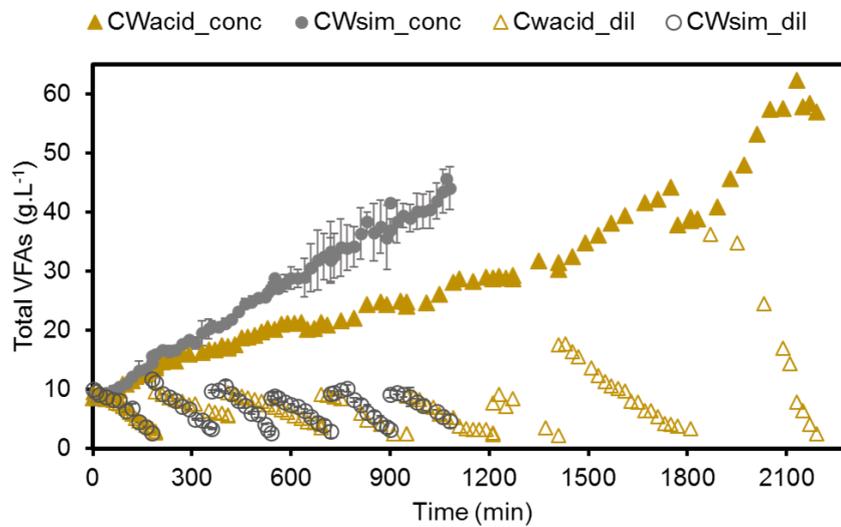


Figure 5.12 - Total VFAs concentration in diluted and concentrated compartment for $CW_{acid}$ and $CW_{acid}^{Sim}$
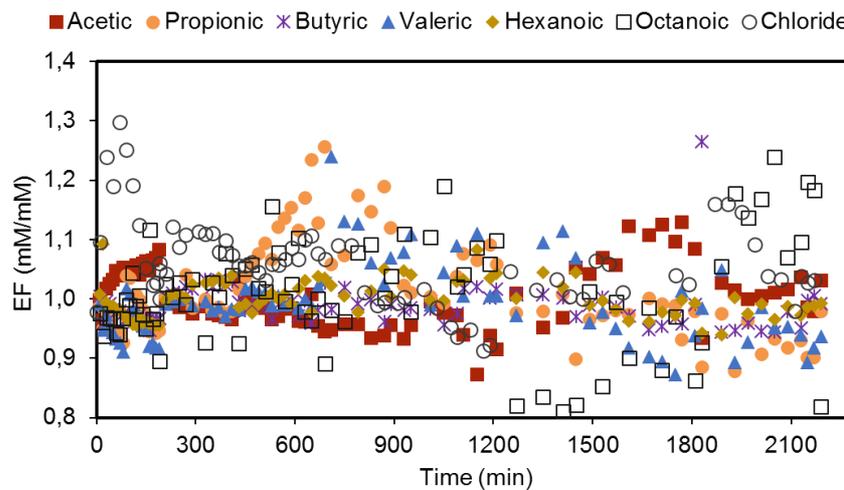


Figure 5.13 - Enrichment factor (EF) for each single acid for $CW_{acid}$

The $CW_{acid}$ was not only composed by acids and inorganic ions but also ethanol (EtOH). Since this molecule is uncharged is not influenced by the electrical field. In Figure 5.14 it can be observed that, as expected, EtOH concentration was constant in the concentrated compartment maintaining its initial concentration (around 1 g.L⁻¹) along all ED process. This confirms that the uncharged molecules are not affected by the electrical field.
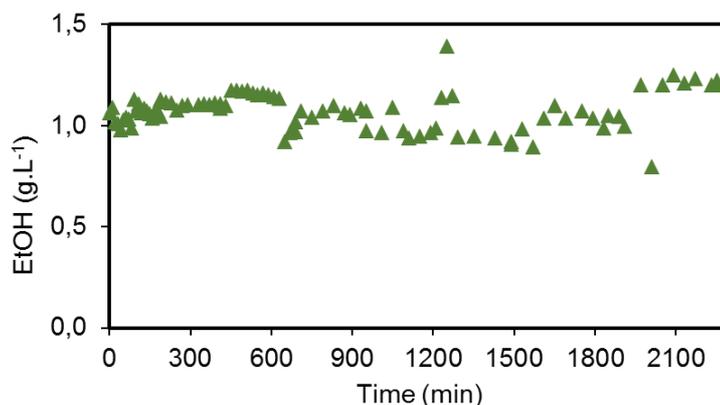


Figure 5.14 - EtOH concentration profile in the concentrated stream for $CW_{acid}$

As it was previously performed, also for $CW_{acid}$ the COD was analysed. This effluent was much poor in organic matter than $VMW_{acid}$ with an initial COD in the diluted compartment was 21.66 ± 3.45 gO₂.L⁻¹ but, as previous, 81.39 ± 9.33 % was due to VFAs content. At the end of each batch the COD content in the diluted compartment decreased to 9.01 ± 3.09 gO₂.L⁻¹ being 90.92 ± 13.74% VFAs. This last solution still has a considerable amount of COD but since it is not suitable to use in ED process it can employed as substrate for anaerobic digestion or used in other downstream process, such as nanofiltration, in attempt to recover the few VFAs that remained. Along the ED process in the concentrated compartment the COD increased to 136.39 gO₂.L⁻¹ due to the transfer of VFAs from diluted compartment. The ratio of COD from VFAs with the total COD was maintained quite constant during the process with 76.4 ± 14.1%.

Despite the different trends in the VFAs concentration between $CW_{acid}$ and $CW_{acid}^{Sim}$ ED experiments, the specific energy consumption was very similar however much higher than the $VMW_{acid}$ or respective simulated solution during sequential batches. This could be related to the fact that at the same applied voltage the $CW_{acid}$ is much poor in ions and respective low electrical conductivity.
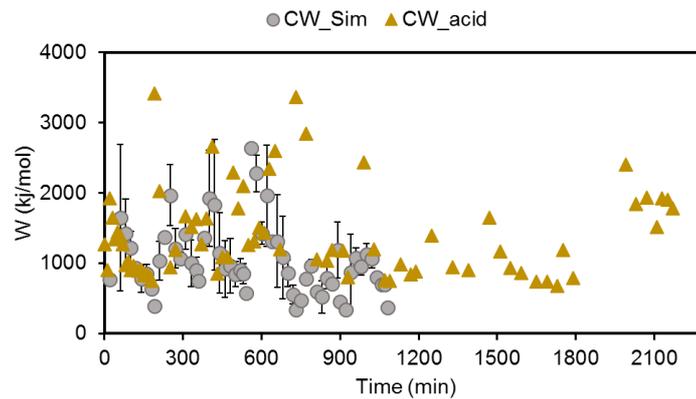
Figure 5.15 – Overall specific energy with $CW_{acid}$

The CE average of the sequential batches with $CW_{acid}$ ED experiment was 29.3 ± 7.2%. This value is considerable much lower respect to the CE observed with $VMW_{acid}$ leading to higher energy used. One reason for the so low value, even in the first batch, is the due to possible damage of the electrodes.

During the present work, it was proposed the performance of sequential batch, maintaining the same initial volume between diluted and concentrated compartment, to reach a higher concentration of VFAs. However, another strategy can be applied to achieved the same goal. If the initial volumes are different, by means, the volume in the concentrated compartment be much lower than the diluted compartment, less volumes will be need to achieve a concentrated stream in the end. This strategy was recently reported by Bing et al. [55] who begun with 2.5 times more volume in the diluted compartment with the acidogenic broth. Before the real fermented broth experiments, Bing, performed studied with synthetic solutions and same initial volumes in both compartments. These initial different volumes would allow to save volume however it was not verified decreased in the total time need to reach the same VFAs concertation beginning with same or different initial volumes.

From the presented and discussed results we can conclude that ED have the potential to obtain a concentrated stream rich in acids and chloride. Since good results were obtained by using different effluents with different characteristics and all experiments were carried out without change or even open the membrane module it can be said that the process is robust.

VFAs concentration – Electrodialysis

# 6. Acids separation - Nanofiltration

Summary: The present chapter is respect to the activity developed in CHAIRE ABI, Reims – France, where was proposed the recovery of ferulic acid (an important anti-oxidant) from a wheat bran enzymatic hydrolysate. The wheat bran is a lignocellulosic complex biomass rich in sugars and acids which need to be pre-treated, such as by an enzymatic digestion, in order to have these molecules in solution. At small scale eight different nanofiltration membranes were characterized and screened at different applied pressures and pH solutions in order to choose the best operating condition to recover and concentrate ferulic acid. After this, a scale up was performed by increasing membrane area in 10 times. Membrane 7450 (Nitto, Hydranautics) showed be the best membrane to separate FA from sugars at: pH 9, applied pressure of 16 bar and 25ºC in a DiaNF process.

## 6.1    Introduction

Nanofiltration (NF) is a pressure-driven process used in a wide range of industrial applications, in particular, for water purification. Due to its attractive advantages such as: low energy consumption, unique separation properties and environmental friendly [46,47] this process has arousing interest in the employment in the removal of inhibitors and concentration of fermentable molecules from biomass or fermented broths [107].

During the NF process two main characteristics of the membranes play a significant role: pore size and electrostatic interactions [57]. Molecules with a molecular weight larger than the pore sieving or exclusion proprieties of the NF membrane would be rejected. In addition, the separation of multi-valent ions by NF is high due to the Donnan effect [44]. The membrane surface charge can change according with the pH of the solution which is directly and this information is given by the ζ-potential value at each pH. When ζ-potential is zero the membrane surface is uncharged and at that pH is what is called isoelectric point (IEP), at pH values higher than the IEP the membrane is negatively charged, whereas for pH values lower than the IEP the surface is positively charged. According with the surface charge and the resulting ζ-potential of the membrane the electrostatic interactions will affect the rejections of the solutes, especially the charged molecules as acids. An acid is a substance that dissociates in aqueous solution and each one has pKa (acid dissociation constant) and presents a degree of dissociation as function of pH [44]. This characteristic of the acids can be explored and induce different electrostatic interactions with the membranes since both solutes and membranes can be positively or negatively charged or even uncharged.

Ferulic acid (FA) is a molecule widely used in cosmetic, food and medical industry due to its antioxidant proprieties and it can be found in lignocellulosic biomass as corn bran [108], wheat bran [109] and rice straw [46]. Since lignocellulosic biomass is complex, a pre-treatment step is necessary to perform before the fermentation and can be performed by enzymatic or alkaline hydrolysis. After the pre-treatment, a cocktail of sugars and acids is obtained and it can be used in fermentation processes however also inhibitors will be release such as the FA. Therefore, it is necessary to perform a purification step for the valorisation of sugars, in fermentative process for example, and valorise ferulic acid in pharmaceutical industry. Since FA, as VFAs, have a pKa (4.66 - 4.89 much lower than sugars (12.15 - 12.34), that means that the charge between these molecules is different for the same pH. From this, electrostatic interactions can be explored in NF process in order to separate sugars from acids.   There are a few works in literature were this was already tried [47,107,108,110] however few with real hydrolysates.
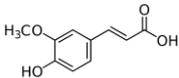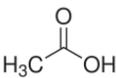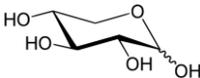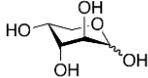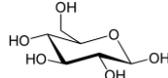
In the present chapter, it is proposed the purification of FA from wheat bran enzymatic hydrolysate by employing nanofiltration process.

## 6.2 Material and methods

### 6.2.1 Solutions and membranes

Simulated solutions ($Hydrol_{enzym}^{sim}$) were prepared by adding in de-ionized water the same amount of chemicals quantified in the wheat straw enzymatic hydrolysate in order to simulate it. The composition of the solutions is presented in Table 6.1. In addition to main solutes, $Hydrol_{enzym}^{sim}$ was also composed by other acids and salts (in g.L$^{-1}$): p-coumaric (0.002), propionic ac. (0.020), formic ac. (0.075), oxalic ac. (0.036), KCl (2.288), MgSO$_4$ (0.495) and NH$_4$H$_2$PO$_4$ (0.278). The pH was correct with NaOH 3 M. No other compounds were added to the enzymatic hydrolysate except the pH correction with NaOH 4 M. Before nanofiltration experiments with the hydrolysate it was performed an ultrafiltration step with membrane UP010 (Microdyn Nadir) with 10 000 Da a cut-off operated at 2.5 bar and ambient temperature.

Table 6.1 - Main characteristics of solutes in simulating solution and hydrolysate

| Name | Ferulic ac. | Acetic ac. | Xylose | Arabinose | Glucose |
|---|---|---|---|---|---|
| Molecular weight (Da) | 194.18 | 60.05 | 150.13 | 150.13 | 180.16 |
| Structure | | | | | |
| pKa | 4.66 | 4.76 | 12.15 | 12.34 | 12.28 |
| $Hydrol_{enzym}^{sim}$ (g.L$^{-1}$) | 0.055 | 0.043 | 9.000 | 0.200 | - |
| $Hydrol_{enzym}$ (g.L$^{-1}$) | 0.055 | 0.043 | 11.00 | 1.00 | 0.800 |

During the present work, eight membranes were screened to choose the best one and do a scale-up. In the following table (Table 6.2) it is represented the characteristics of each membrane.

Table 6.2 Membranes characteristics used in nanofiltration experiments

| Manufacturer | Membrane | Material | Cut-off (DA) | Max Temperature/Pressure | pH range |
|---|---|---|---|---|---|
| GE | DK | Proprietary thin film | 150-300 (on sucrose and glucose) | 80°C/40 bar | 2-10 |
| GE | DL | | | | |
| GE | HL | | | 50°C/40 bar | 3-9 |
| Dow | NF270 | Polyamide selective layer Polysulfone supporting layer | 400 | 45°C/41 bar | 2-11 |
| Dow | NF | Polyamide | 200-400 | - | 2-11 |
| Alfa Laval (AL) | NF | Thin film composite on polyester | 300 | 50°C/40 bar | 3-10 |
| Nitto - Hydranautics (NT) | 7450 | Sulfonated Polyethersulfone | 1000 | 60°C/41 bar | 2-11 |
| Nitto - Hydranautics (NT) | 7470 | Sulfonated Polyethersulfone | 720 | 70°C/41 bar | 1-13 |

### 6.2.2 Nanofiltration experiments

The first experiments were dedicated to the screening of the membranes in a closed-loop bench top unit (Evonik, Metcell). This unit was composed by a nitrogen bottle connected to a pressure regulator. The retentate and permeate were continuously recirculated in a 700-mL sealed reservoir. This last was connected to a Gilson 305 pump, by which the permeate was re-inserted and to a Micropump (Inc) which recirculate the solution in the system. A serpentine silicon tubing around the solution reservoir and the circulation pump was connected to a thermostat to maintain a constant temperature at 25 ºC. Each membrane had an effective area of 13 cm$^2$. This system allowed the study of the flux (Jv) for each applied pressure and respective permeability (Lp), considering all pressure range studied, and the solute rejection for all the membranes. The study was performed with $Hydrol_{enzym}^{sim}$ and $Hydrol_{enzym}$ at different pH values: before, after and at the IEP by applying different pressures: 10, 16, 20, 26 and 30 bar. In Table 6.3 it is represented the IEP of each membrane and the pH conditions experiments performed for each membrane. The Lp of each membrane was measured during experiments with the respective $Hydrol_{enzym}^{sim}$ or $Hydrol_{enzym}$, before (Lpb) and after (Lpa) each experiment using pure water. The experiments were started at higher pressure and was left 30 minutes stabilising before sampling. The sampling was performed from the retentate and permeate at each applied pressure.

Table 6.3 - Experiments performed (✓) or not (✗) for each membrane with the respective surface charge: positive (+), negative (-) or uncharged (0)

| | DK | DL | HL | NF270 | NF_DOW | NF_AL | NT50 | NT70 |
|---|---|---|---|---|---|---|---|---|
| **IEP** | 3.64 | 3.67 | 3.81 | 3.10 | 3.0 | - | - | - |
| $Hydrol_{enzym}^{sim}$ **pH 3.0** | ✓ (+) | ✓ (+) | ✓ (+) | ✗ | ✗ | ✗ | ✗ | ✗ |
| $Hydrol_{enzym}^{sim}$ **pH 3.1** | ✗ | ✗ | ✗ | ✓ (0) | ✓ (0) | ✓ (-) | ✗ | ✗ |
| $Hydrol_{enzym}^{sim}$ **pH 3.2** | ✗ | ✗ | ✗ | ✓ (-) | ✓ (-) | ✓ (-) | ✗ | ✗ |
| $Hydrol_{enzym}^{sim}$ **pH 3.8** | ✓ (-) | ✓ (-) | ✓ (0) | ✗ | ✗ | ✗ | ✗ | ✗ |
| $Hydrol_{enzym}^{sim}$ **pH 5.2** | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) |
| $Hydrol_{enzym}^{sim}$ **pH 7.0** | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) | ✓ (-) |
| $Hydrol_{enzym}^{sim}$ **pH 9.0** | ✗ | ✗ | ✗ | ✗ | ✗ | ✓ (-) | ✓ (-) | ✓ (-) |
| $Hydrol_{enzym}$ **pH 5.2** | ✓ (-) | ✗ | ✗ | ✗ | ✗ | ✗ | ✓ (-) | ✓ (-) |
| $Hydrol_{enzym}$ **pH 7.0** | ✗ | ✗ | ✗ | ✗ | ✗ | ✗ | ✓ (-) | ✓ (-) |
| $Hydrol_{enzym}$ **pH 9.0** | ✗ | ✗ | ✗ | ✗ | ✗ | ✗ | ✓ (-) | ✓ (-) |

After the membrane screening with the best membrane and operational conditions for FA retention it was performed a scale-up in a NF-pilot plant (SyvaLab, France). Figure 6.1 shows the schematic diagram of the system. The pilot scale apparatus consisted in a feed tank (5 L), piston pump, pressure gauge, thermostatic water bath for temperature control and a membrane module. This last (SEPA CFII) was a flat sheet module with a total surface membrane area of 140 cm$^2$.

In a recirculation mode, at 16 bar as applied pressure and 25 ± 1ºC, two different spacers were studied: 31 and 47 mil at 4 different feed velocities: 360, 404, 480 and 540 L.h$^{-1}$. For 31 mil the cross flow velocities were: 1.3, 1.5, 1.8 and 2.0 m.s$^{-1}$ while for 47 mil were 0.88, 0.99, 1.18 and 1.32 m.s$^{-1}$.

After choose the right spacer, dia-nanolfiltration process (DiaNF) step was performed in order to wash the solution as much as possible for the follow concentration step of the DiaNF resulted retentate. Along the process 4.5 DiaNF volumes (Vd) were collected. The volume in the reservoir that contained the retentate was maintained constant by adding denoised water as solution was permeate. To determined fouling of the membrane after DiaNF process, the pure water permeability of the used membrane was measured after had been cleaned with deionized water for several times. Then the permeability was measured again after membrane regeneration with NaOH 0.5 M solution for 1 h at 40ºC.
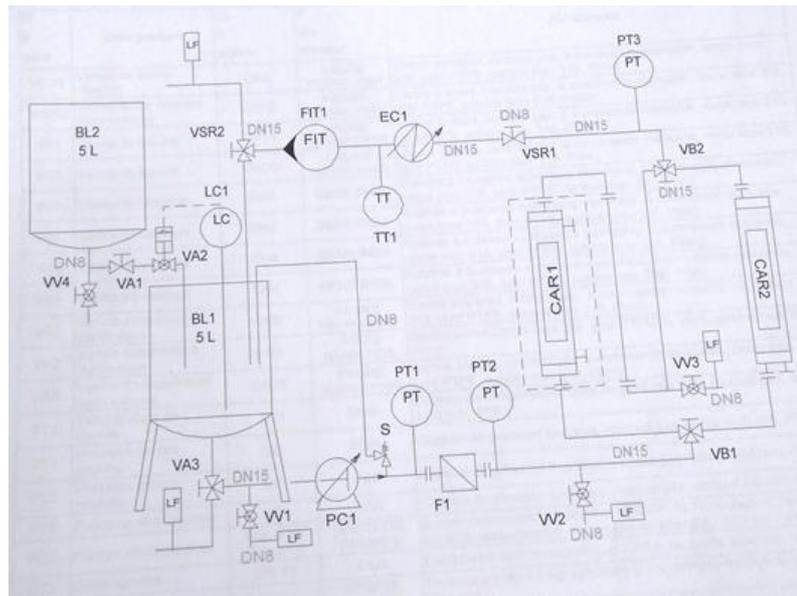


Figure 6.1 – NF-Pilot plant scheme

After DiaNF the resulted retentate was concentrated in the top-bench nanofiltration system with a total membrane surface area 39 cm$^2$, 24 ± 1ºC, applied pressure of 16 bar and the retentate was continuously recirculated. The initial retentate volume was 1538 mL and finished with 80 mL.

In Figure 6.2 is represented the schemes for the DiaNF and concentration steps.

For all experiments sampling was performed frequently for permeate and retentate for pH, conductivity and osmotic pressure measurements and HPLC analysis.
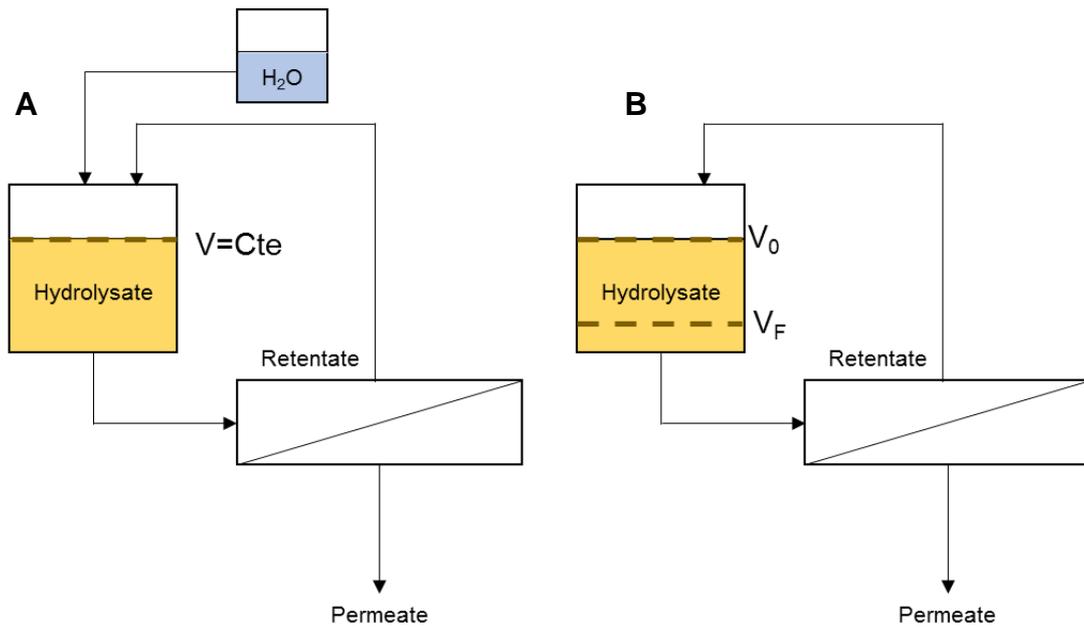
Figure 6.2 - DiaNF (A) and Concentration (B) processes schematic diagram

### 6.2.3 Analytical procedures

For all membranes used a SurPASS® (Anton Paar GmbH, Graz, Austria) electrokinetic analyzer was used to measure the streaming current as a function of pH. A 1 mM KCl solution was used as the background electrolyte and HCl (0.1 M) was used to automatically adjust the pH. The streaming current data was recorded at an applied pressure ranging from 0 to 300 mbar. The ζ-potential was given by the instrument as well the respective IEP. For each pH value, 4 measurements were performed and an average value was obtained.

Ferulic acid concentrations were quantified by HPLC-UV (Thermo Scientific) equipped with BetaMax Neutral column (150 x 4.6 nm x 5 μm) (Thermo Scientific). The elution was performed at 1 mL.min$^{-1}$, 40°C, with 20% acetonitrile and 80% formic solution 0.1% (v/v) as mobile phase. Ferulic acid was detected at 322 nm. The sugars and acetic acid concentrations were determined by HPLC-IR (Thermo Scientific) equipped with column Aminex HPX-87H (300 x 7.8 mm x 9 μm) (Aminex, Biorad). The elution was performed with 4 mM $H_2SO_4$ with a flow of 0.5 mL.min$^{-1}$ at 35°C.

The osmotic pressure was directly measured with an automatic osmometer (Roebbling).

### 6.2.4 Calculations

$$TMP = P_{applied} - \Delta\pi \tag{7}$$

$$\Delta\pi = [\pi_{retentate} - \pi_{permeate}] \tag{8}$$

The flux ($J_v$ in L.h$^{-1}$.m$^{-2}$) for each membrane and each applied pressure was calculated as following equation where $V_p$ (in L) is the volume permeate in a known time ($t$, in hours) considering the membrane surface area ($A$ in m$^2$).

$$J_v = \frac{V_p}{t \times A} \tag{9}$$

With the different flux is possible to measure the permeability if the membrane ($L_p$ in L.h$^{-1}$.m$^{-2}$bar$^{-1}$):

$$L_p = \frac{J_v}{TMP} \tag{10}$$

The fouling (F) of the membrane can be evaluated by comparing the permeability obtained before and after experiments. If F is higher than 10% is considered that membrane

$$F\ (\%) = \frac{L_{pb} - L_{pa}}{L_{pb}} \times 100 \tag{11}$$

The observed retention ($R_{obs}$) of each single compound is determined by the difference of each compound concentration observed in the retentate ($C_R$) and permeate ($C_P$).

$$R_{obs}\ (\%) = \frac{C_R - C_P}{C_R} \times 100 \tag{12}$$

A mathematical model was used to predict the performance of each membrane in a DiaNF process and was calculated as followed:

$$C_i = C_{0,i} \times e^{(V_D \times (1 - R_{obs}))} \tag{13}$$

Where $C_i$ is the solute concentration, $C_{0,i}$ is the solute initial concentration in the feed solution and $R_{obs}$ is the retention observed for the solute in a specific pH and pressure conditions. It was considered a constant volume and no pH changes during the process.

## 6.3 Results and discussion

The Lp$_b$ and Lp$_a$ were measured with pure water allowing to evaluate, by applying equation (11) if membrane fouling had occurred. During experiments at different pH it was also measured the Lp since it is an important parameter to consider to choose the best membrane for the process. In Table .6.4 it is represented the Lpb as well Lp measured for each experienced pH. These values are in concordance with other reported works in the literature [56].

Table .6.4 - Permeability obtained for pure water before (Lpb) $Hydrol_{enzym}^{sim}$ experiments with the respective standard deviation and the permeability (Lp) obtained for each membrane and each pH solution experiment

| | DK | DL | HL | NF270 | NF_DOW | NF_AL | NT7450 | NT7470 |
|---|---|---|---|---|---|---|---|---|
| | Lm$^{-2}$h$^{-1}$bar$^{-1}$ | | | | | | | |
| Lpb | 3.5±0.2 | 8.3±0.4 | 11.0±0.7 | 14.7±1.4 | 7.1±0.5 | 4.3±0.8 | 6.6±1.9 | 3.5±0.6 |
| $Hydrol_{enzym}^{sim}$ pH 3.0 | 3.14 | 6.89 | 8.38 | - | - | - | - | - |
| $Hydrol_{enzym}^{sim}$ pH 3.1 | - | - | - | 9.62 | 6.13 | 3.86 | - | - |
| $Hydrol_{enzym}^{sim}$ pH 3.2 | - | - | - | 10.23 | 5.58 | 4.37 | - | - |
| $Hydrol_{enzym}^{sim}$ pH 3.8 | 3.79 | 7.43 | 9.25 | - | - | - | - | - |
| $Hydrol_{enzym}^{sim}$ pH 5.2 | 3.59 | 7.50 | 9.18 | 11.77 | 6.06 | 4.55 | 3.28 | 1.86 |
| $Hydrol_{enzym}^{sim}$ pH 7.0 | 3.68 | 7.57 | 8.98 | 10.20 | 5.63 | 3.79 | 8.71 | 3.78 |
| $Hydrol_{enzym}^{sim}$ pH 9.0 | - | - | - | - | - | 2.61 | 6.88 | 3.67 |
| $Hydrol_{enzym}$ pH 5.2 | 2.24 | - | - | - | - | - | 1.80 | 1.15 |
| $Hydrol_{enzym}$ pH 7.0 | - | - | - | - | - | - | 1.14 | 0.99 |
| $Hydrol_{enzym}$ pH 9.0 | - | - | - | - | - | - | 2.18 | 1.51 |

After the enzymatic hydrolysis in solution remains a cocktail of sugars, acids and the FA. The separation of sugars (not charged molecules) from FA can be performed with nanofiltration by applying one from two strategies:

- Strategy 1 – retained the FA, e.g. by promoting electrostatic repulsions with the membrane surface, and permeate the sugars.
- Strategy 2 – retained the sugars (by molecular sieving) and permeate the ferulic acid (by avoiding electrostatic interactions).

Simulated solutions with similar composition to the wheat bran hydrolysate were prepared at different pH values in order to study the influence of the pH in the retentions of FA and the other compounds in solution, mainly sugars and acetic acid. The pH values were chosen according with the IEP of the membranes since the pH could change the charge of the membrane surface and affect the interactions with the compounds and respective retentions.

FA and the sugars have similar molecular weight, 194.18 and 150.13 g.mol$^{-1}$ respectively but very different pKa value, 4.66 for FA and 12.15-12.34 for sugars. The molecular weight allows to select the suitable membranes according with the cut-off and in this way the retention is affected by the molecular sieving. With the pKa information it is possible to predict degree of ionization of each

specie and from that choose the best pH for the solutions to retain the compounds by promoting more or less electrostatic interaction. From Figure 6.3 it can be observed that between pH 2 and 10 Xylose, the main sugar in solution, is a neutral molecule while FA is uncharged at low pH and from pH 7 is negatively charged. As it was mentioned before, not only the species are affected by the pH but also the membranes itself. One of the nanofiltration membranes characteristics is the different membrane surface charge as function to pH solution which is in contact [57]. The IEP is the pH value in which the membrane surface is neutral, at a pH below the IEP membrane surface is positive while above it membranes have surface negatively charge. Because of this phenomena IEP was measured for the 8 membranes used in the present work and can be consulted in Table 6.2. The IEP of the membranes is in the range of pH 3-4 except of membranes NT7450, NT7470 and AL that are always negatively charged.
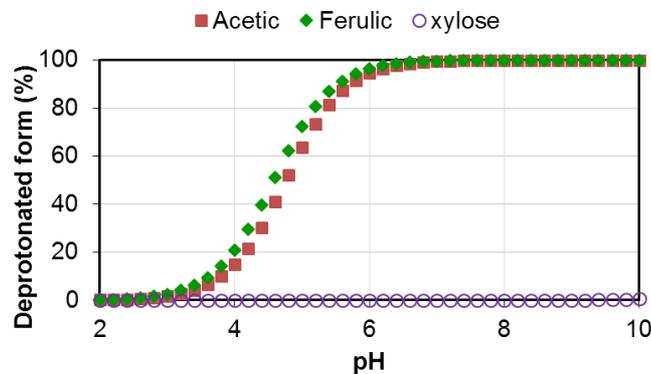


Figure 6.3 - Speciation of Xylose, ferulic and acetic acids in function of pH

The ζ-potential profile for each membrane (can be consulted from Appendix D to Appendix K) shows the surface membrane charge according with the pH. The studied membranes can be divided in two mains groups: membranes which have an IEP (DK, DL, HL, NF270 and NF_Dow) and the membranes which are negatively charged independent of the solution pH (AL, NT7450 and NT7470). Important notice that from the zeta-meter profile these last are always negative but with different zeta-potential value which can promote more or less repulsions with the charged molecules

The results of the retentions obtained for the main compounds (total sugars FA and acetic acid) that compose the $Hydrol_{enzym}^{sim}$ are presented from Figure 6.4 to Figure 6.11. For membranes from the first referenced group (Figure 6.4 - Figure 6.8) it was observed a slightly decrease in the retention of sugars as increasing the initial $Hydrol_{enzym}^{sim}$ solution pH. At lower pH sugars retentions were about 98% while for pH 7 retentions decreases to around 82%. Xiong et al. [56] performed a very complete study for the separation of carboxylic acids and sugars, also with simulated solutions, by employing nanofiltration process with two membranes which were also used in the present study: membranes DK and DL. Xiong also observed and reported the decrease in the sugars retention as solution pH

was increased. In fact, this phenomena can be explained by the fact that at higher pH the membranes carry more negatively charged molecules, confirmed by zeta-potential profiles (Appendix D-Appendix H), resulting in a membrane swelling and consequently increased in pore size [56]. This increasing in the cut-off affects specially the neutral molecules since these molecules do not promote electrostatic interactions with membrane surface.

The acids in solutions (FA and acetic) showed a complete different trend from the sugars as was expected. These molecules were rejected either by molecular sieving - FA has a molecular weight similar to the sugars and smaller than membrane cut-off - and electrostatic repulsions – at higher pH higher retentions were obtained. At a pH higher than 7 all compounds presented high retention (>80%) while at lower pH (<7) acids the retentions were lower.
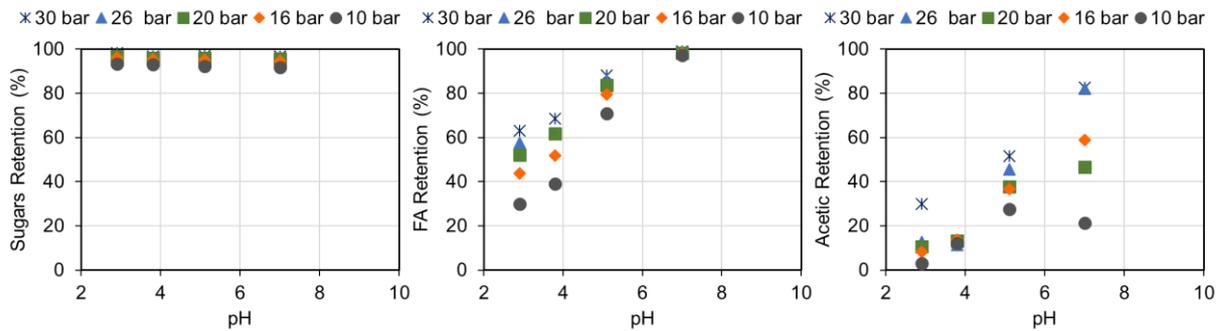


Figure 6.4 – Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}^{sim}$ for membrane DK at different pH and pressures
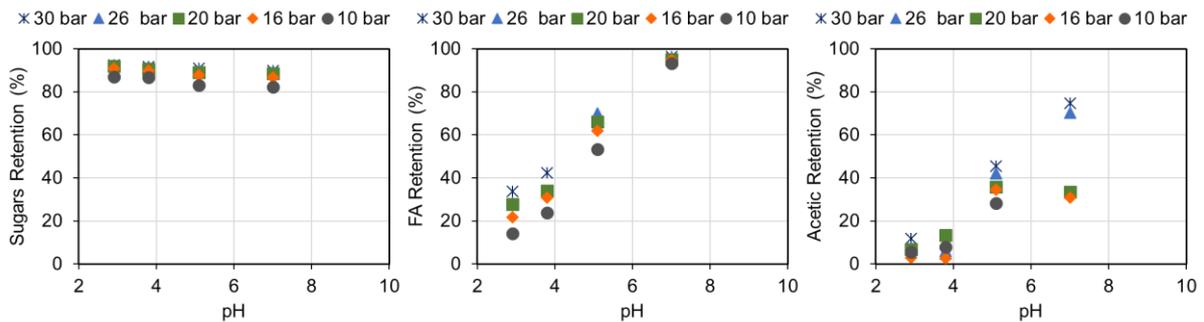


Figure 6.5 - Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}^{sim}$ for membrane DL at different pH and pressures
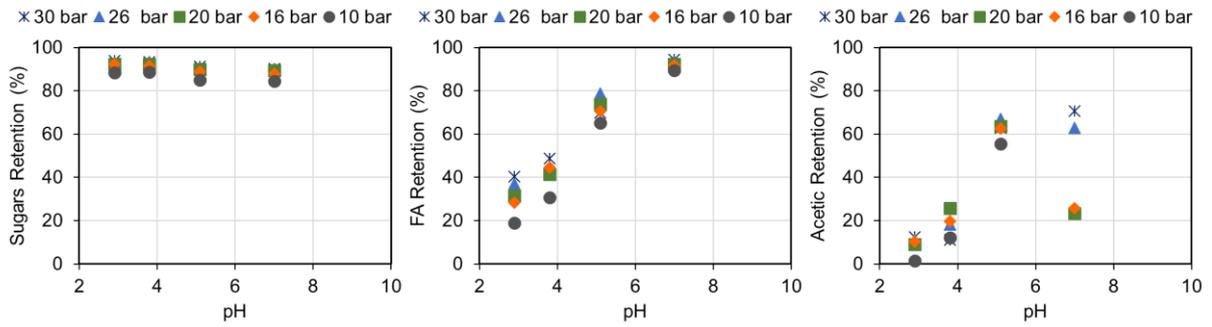
Figure 6.6 - Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}^{sim}$ for membrane HL at different pH and pressures
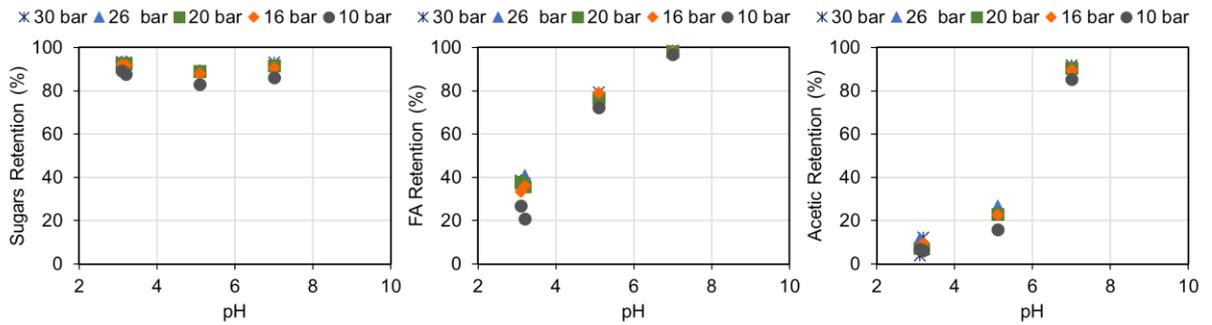


Figure 6.7 - Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}^{sim}$ for membrane NF270 at different pH and pressures
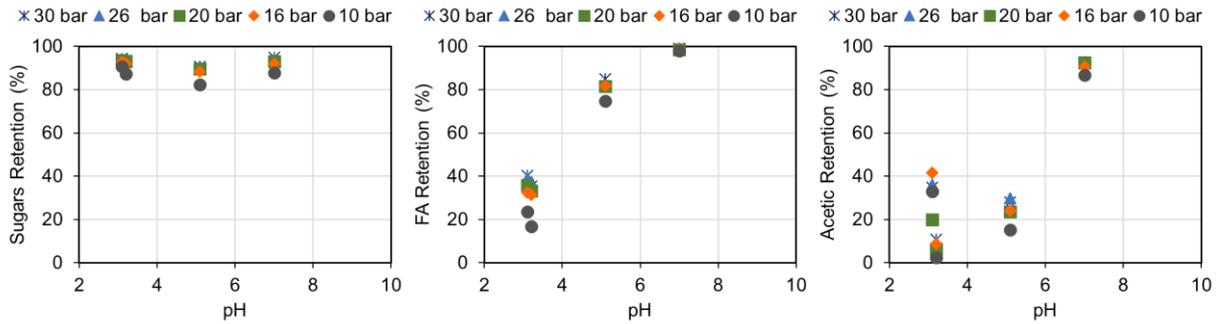


Figure 6.8 - Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}^{sim}$ for membrane NF_Dow at different pH and pressures
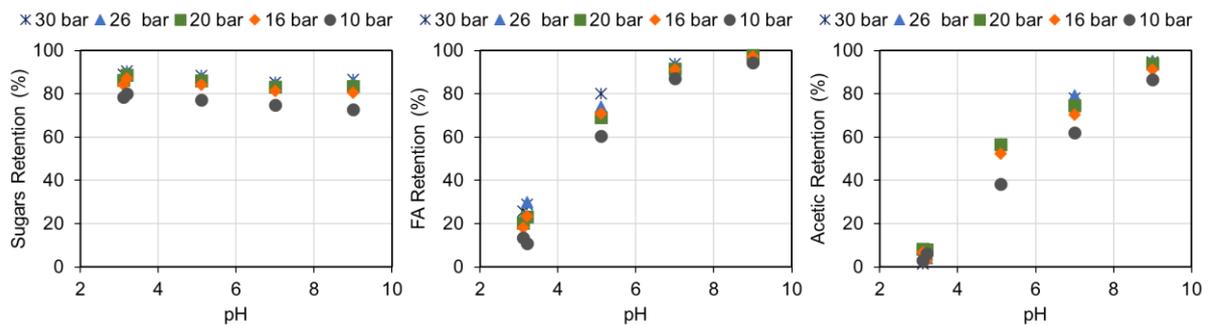
Figure 6.9 - Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}^{sim}$ for membrane AL at different pH and pressures

For all the mentioned membranes as pH increases also the deprotonated form of acetic acid and FA increases, promoting the electrostatic repulsion with the membrane surface resulting in increasing in the retentions. Furthermore, not only the pH influenced the retention of the acetic acid and FA but also the pressure. For acetic acid at higher pressure, at any pH solution, retentions were 5 a 10% higher than at lower pressures.

In the other hand, more interesting results were obtained for the membranes from the group of membranes (AL, NT7450 and NT7470) which are always negatively charged independent on pH solution. With these membranes, the sugars retentions were lower especially to NT7450 (Figure 6.10) and NT7470 (Figure 6.11) and retention changed as pH increases either for sugars and acids. Despite AL (Figure 6.9) being always negatively charged in the range of pH experienced, it presented a similar trend as the membranes referenced before. Even if with lower retentions were achieved with AL, the retentions were as high as the previous explained membranes (74%-92%).

The membranes NT7450 and NT7470 were tested only for pH higher than 5 since Luo and co-workers [107] had already shown that better results were obtained for FA retention and sugars permeation at basic pH (>7). The retention of the acids was high as expected (80-95%) since, as it was referred before, from pH higher than pKa (4.66 - 4.76) FA and acetic acid are deprotonate and important refer that all membranes studied in present work have negative charge surface above pH 3 (Appendix D - Appendix K). The interaction of FA and acetic acid in deprotonated form promotes a strong electrostatic repulsion with membrane surface leading to higher retentions [111]. Once again, FA is more retain than acetic acid, probably, due to size exclusion, FA has a molecular weight higher than acetic acid. The sugars retentions were much lower than the previous described membranes by the fact that NT7450 and NT7470 have higher pore size (720<Da<1000) allowing the sugars pass more easily through the membrane respect to the other membranes group and AL (150<Da<400). Even if it was expected that sugars could be less retain it was also observed that even being a neutral molecule in the pH range studied the retention was affected by the pH stream and in specific case of xylose the retention decrease from 80% (pH 5) to 30% (pH 9). The sugars retentions decreased with pH increase can be attribute to the pore swelling at alkaline pH [107,111] as it was already mentioned

above for the other membranes. For these membranes the swelling effect caused by the pH is more evidence in the retentions since the cut-off is also higher.
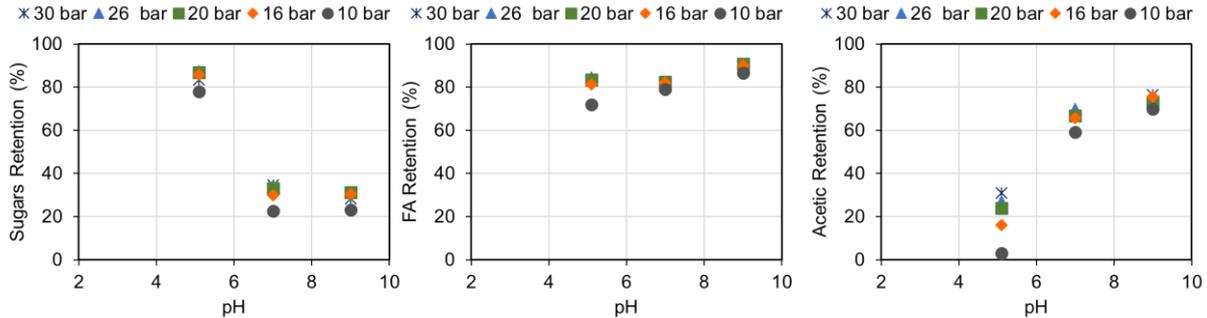


Figure 6.10 – Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}^{sim}$ for membrane NT7450 at different pH and pressures
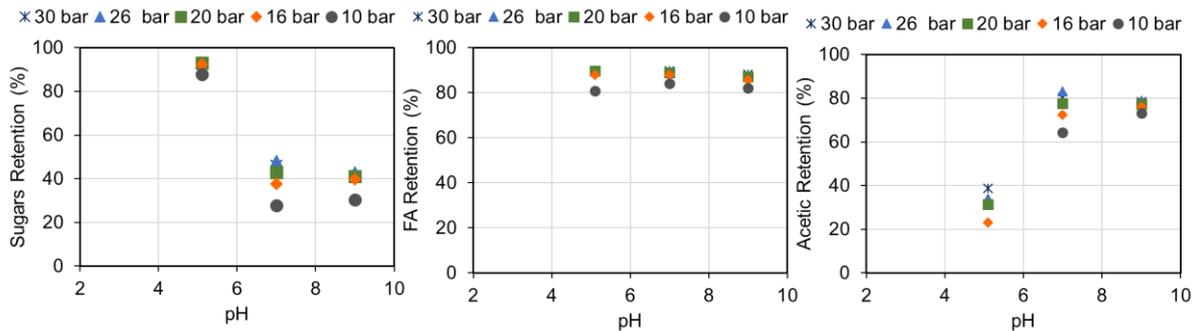


Figure 6.11 - Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}^{sim}$ for membrane NT7470 at different pH and pressures

From all reported retentions for sugars and FA with simulated solutions, it can be said that the membranes DK, DL, HL, NF270, NF_Dow and AL did not show good profiles to separate them. The retentions for FA were satisfactory (>90%) at basic pH however also for sugars. Even though it is possible to use one of these membranes for a strategy in which sugars are retain but no FA. By using an acidic pH (2-3) the FA have low retentions (>20%) because the electrostatic interactions are low (2-3). In this way, sugars and FA can be separate. For the case scenario of this strategy a mathematical modulation for DiaNF process was performed. The model was build employing the equation (13) and considering, for each membrane: retention of each molecule at different pH, applied pressures and permeability observed during the experiments with $Hydrol_{enym}^{sim}$. The criteria for the operated parameters selection was the condition in which the FA loss was lower than 10%. Moreover, to select the best membrane was considered the one which the retentate stream contained the highest percentage of initial sugars concentration. The referred conditions would allow to obtain, in a

DiaNF process, a retentate stream richer as maximum as possible of sugars, a permeate stream with the maximum FA where to achieved that a minimum of DiaNF volumes were needed at lower pressure. With all this considered and from these 6 membranes, the model showed DK membrane as the best option for the strategy to separate sugars from FA. Though, the disadvantage of this strategy is the difficulty to obtain a concentrated FA solution.

The other two membranes (NT7450 and NT7470), in another hand, showed satisfactory results, as the others, for retention of FA at basic pH but lower retentions for sugars. By using those the strategy of separate FA and sugars by favouring the retention of FA could be possible. Yet since the scale-up will be performed for only one membrane being mandatory choose only one from these two. The mathematical model mentioned above was used also for these two membranes in order to choose the best one. For the strategy to retain FA, the model had not shown a preferable membrane between NT7450 e NT7470. Because of that, experiments, still at small-scale, were carried out with the $Hydrol_{enzym}$ for NT7450 (Figure 6.12) and NT7470 (Figure 6.13) at pH 5, 7 and 9.
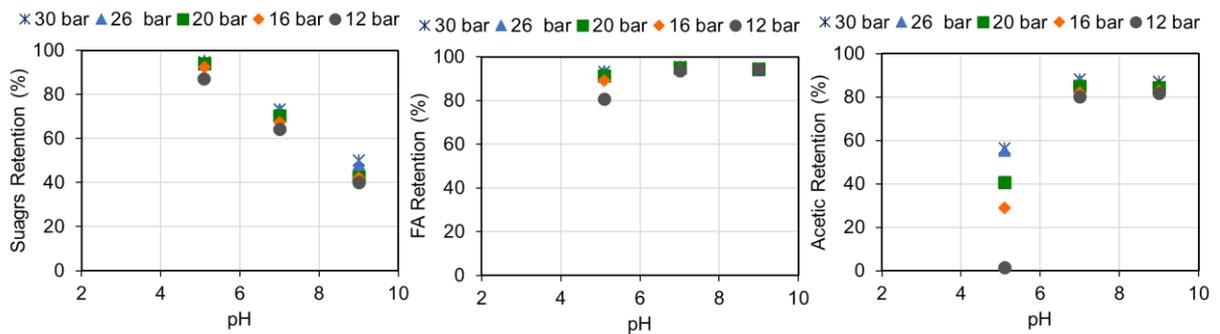


Figure 6.12 - Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}$ for membrane NT7450 at different pH and pressures
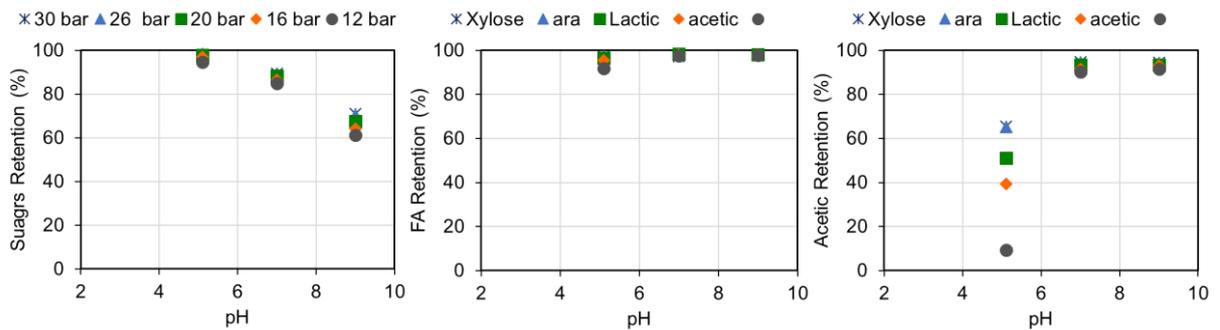


Figure 6.13 - Retentions profile for sugars, FA and acetic acid with $Hydrol_{enzym}$ for membrane NT7470 at different pH and pressures

The results obtained with the hydrolysate were as expected: sugars retention decreasing as pH increases and the contrary for the acids. Even if slightly higher retentions were obtained when compared with $Hydrol_{enzym}^{sim}$. This increasing in the retentions between $Hydrol_{enzym}$ and $Hydrol_{enzym}^{sim}$ could be related to the less permeability for the $Hydrol_{enzym}$ as Xiong et al. explained in his reported work [56].

Comparing both membrane performance it was observed the membrane NT7470 presents higher retentions for FA thought also higher retentions for sugars. With this data, it was also applied, once again, a mathematical model as for model solutions and NT7450 is the membrane which needs less DiaNF volumes and less time to obtain the FA separated, at least, from the sugars.

After the membranes screening a scale-up of the process was performed were the membrane area increased from 13 to 140 cm$^2$. At pilot scale, it was studied the influenced of crossflow velocity and different spacers dimensions - 31 and 47 mil - in order to investigate the effect of these two components in the FA and sugars retentions. The Figure 6.14 shows the retentions obtained of each spacer and respective crossflow velocities. It was not observed significant influence between the two different spacers or the crossflow velocity applied, result that was already observed by Weng and co-workers [44]. From this, the DiaNF step was performed with the spacer 31 mil because it is the most common used in industry and at low crossflow velocity (1.5 m.s$^{-1}$) in order to minimize the energy consumption.
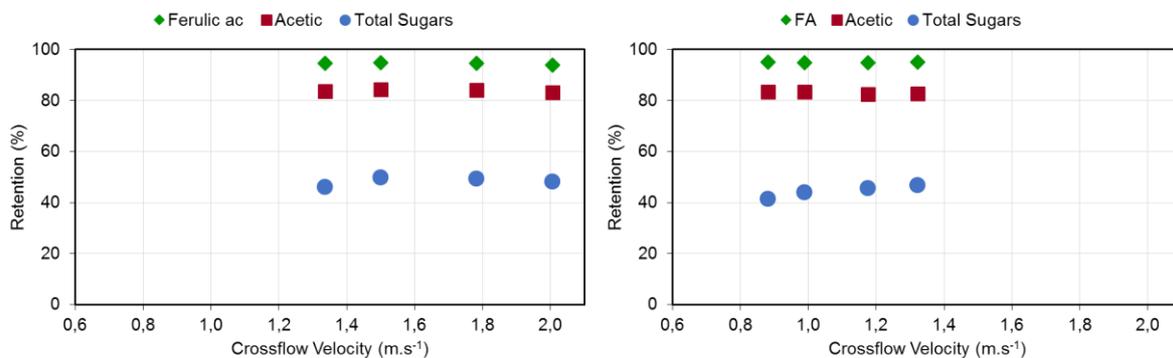
Figure 6.14 – Retention profile with different spacers 31 (a) 47 (b) mil

With the membrane selected (NT7450), the optimal operating pH (9.0) determined, the spacer (31 mil) and crossflow velocity (1.5 m.s$^{-1}$) study, a DiaNF step was performed with the aim to wash as much as possible the sugars and increase FA purity.

In Figure 6.15 it is represented the sugars removal from the retentate and the pH profile it can be observed that after Vd 4.5, 56% of the sugars was removed. However, the result was not good as estimated with the mathematical model. For the model, it was considered that pH was constant during the process but it was observed a gradual decreasing during DiaNF which affected considerable the process. In fact, until Vd 2 and pH 7.4 the sugars removal was linear and 40% of it was removed.

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After this point to the end (Vd 4.5) the pH was maintained at 7 and only 10% more was removed. It is clearly evidenced the loss of about 20% in the process efficiency due to the pH drop which is in concordance to the membrane screening, in the present work, and the previous work of Jianquo Luo [107] who had already evidenced that in order to separate the sugars from the FA the pH is a critical parameter and should be 9 or higher.

In which respect to the FA, contrarily with which was expected and predicted by the mathematical model, its retention was not affected during the process. It was not observed negative influence due to the pH drop since pH was maintained to a value were FA is completely deprotonated and consequently negatively charged.
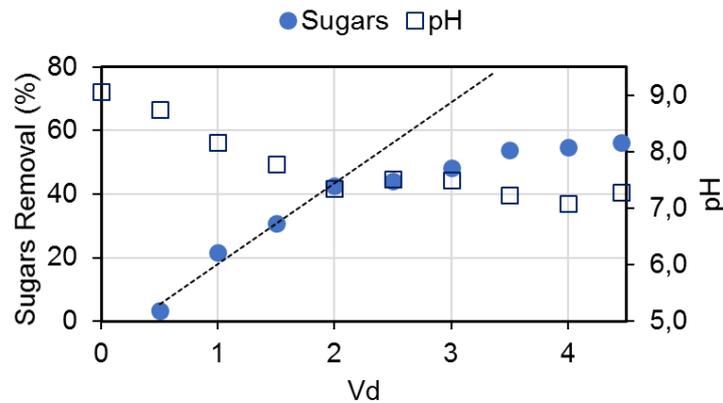


Figure 6.15 - Retentions and pH in retentate as functional of DiaNF volumes

After DiaNF process it was performed an evaluation about the possibility of membrane fouling. The permeability was measured after DiaNF and compared with the permeability obtained with pure water before DiaNF. The permeability of the membrane dropped from 6.673 $L.m^{-2}.h^{-1}.bar^{-1}$ ($Lp_b$) to 1.479 $L.m^{-2}.h^{-1}.bar^{-1}$ ($Lp_a$) which means a 75% permeability loss indicating that fouling had occurred. A regeneration step was performed to evaluate if the fouling was reversible or irreversible by recirculating NaOH solution. With this treatment, the permeability was recover to 5.047 $L.m^{-2}.h^{-1}.bar^{-1}$ demonstrating the fouling was reversible.

Since the solubility of FA is 0.78 $g.L^{-1}$ [112] which is much lower than the other acids and sugars in solution a concentration step was performed after the DiaNF. Nevertheless, even if FA was concentrated 7.6 times it was not enough to achieve the saturation.

After the screening of 8 different nanofiltration membranes with different characteristics respects to IEP and cut-off is possible to conclude that this downstream process can be employed in recovery of small molecules as sugars and acids. Nevertheless, NF being a pressure-driven process it was observed that the pH have more influence in the separation of FA and sugars than the applied pressure. In order to separate sugars and acids the pH should be or very acid (around 2) or very basic (higher than 8). The membrane 7450 (Nitto, Hydranautics) showed better performances to retained

FA (96%) and permeate the sugars. From de DiaNF experiment it was confirmed the importance of maintaining a pH in the retentate near 9 in order to permeate the sugars and not retain them.

Even if this activity was performed with an enzymatic hydrolysate which resulted in a solution richer in sugars and FA, and completely different from the fermented broths mentioned in previous chapters, revelled potential to future experiments for the concentration of VFAs and eventually select them according with the electrostatic interactions that each single acid can perform with the membrane surface.

# 7. General conclusions and future work

In the present thesis, the development of biorefinery schemes for the for the production and recovery of volatile fatty acids from agro-industrial wastes was studied. The results will contribute towards the improvement of fermentation and, specially, the downstream processes to concentrate the VFAs for later uses such as the PHA production.

From the available wastes for the present study – vegetable wastes, maize silage, zootechnical animal and cheese whey – either by itself or in co-digestion all showed be favourable for the production of VFAs by an anaerobic process except for zootechnical animal. It was observed that the type of substrate, inoculum and pH were the principal parameters that affect the VFAs production. The co-digestion of VW and MS was the substrate combination that leaded to a higher VFAs production being achieved approximately 30 $g.L^{-1}$ of total VFAs in which acetic acid was the most abundant (8.6-14.3 $g.L^{-1}$) independently of the operating pH (5,6 or 7). Propionic acid was the acid which was more affected by the operating pH since its concentration increased with the increase of the pH either for the co-digestion experiments and when the pH was changed in the acidogenic fermentation performed in PBBR where CW was employed as substrate.

In which concerns to the most changeling part of the work which was the separation/concentration of the VFAs from the outlet of the bioreactors it can be concluded that electrodialysis process revealed be effected in the concentration of acids however not selective between them. The profile obtained with simulated solutions and the real fermented broth were very similar. The strategy to perform the sequential batches showed be a good strategy to improve the VFAs concentration even if the initial solution is poor. By using two different acidogenic broth (from CW fermentation and co-digestion of MS and VW) with complete VFAs composition it was observed that ED is a process that even with different energy requirements is capable to achieve a high VFAs concentration. The maximum VFAs concentration which can be achieved and the energy required for the process is limited by the initial concentration of the initial solution. Important result is that from a CW fermented broth contain initial 8 $g.L^{-1}$ of VFAs a VFAs concentrated solution in 55 $g.L^{-1}$ was achieved. With the FMW it was concentrated from 30 to 50 $g.L^{-1}$ however a final potential concentration of 100 $g.L^{-1}$ would be possible to achieve if applied the strategy of sequential batches.

With the other membrane process that presents a high potential to obtain a high VFAs concentrated mixture which is nanofiltration experiments from an enzymatic hydrolysate of wheat bran were performed in order to separate ferulic acid. From all the obtained results, it was concluded that electrostatic interaction between charged molecules and membrane surface is the principal parameter that influence the retention. The molecular weight influenced majority the uncharged molecules. After screening experiments were pH and applied pressure was studied, from eight different membranes NT7450 (Nitto, Hydranautics) showed better results in which respects to the separation of ferulic acid from a mixture of sugars. A scale-up was performed where membrane surface area was increased 10 times it was possible to remove 60% of the total sugars with a diafiltartion step and after this, obtain a concentrated solution in which ferulic acid was concentrated 7 times.

Considering the positive results obtained during the thesis in fermentation and recovery process some activities are proposed as future work:

A. In which respects to the VFAs production:
   a. Scale-up the continuous anaerobic acidogenic digestion using VW and MS as co-substrate;
   b. Decrease the HRT during continuous process in order to avoid the methane production in case of operation at pH 7.
B. In which respects to the VFAs separation/concentration
   a. Perform more experiments with electrodialysis with the aim to change some operational parameters such as applied voltage, increase membrane area and clean the electrodes more often;
   b. Employ the nanofiltration process for the fermented broth riches in VFAs
   c. Use the concentrated streams from ED and, eventually, NF for the production of PHA

# 8. Bibliography

Summary: This chapter contains all the bibliography consulted are used in the previous 7 chapters.

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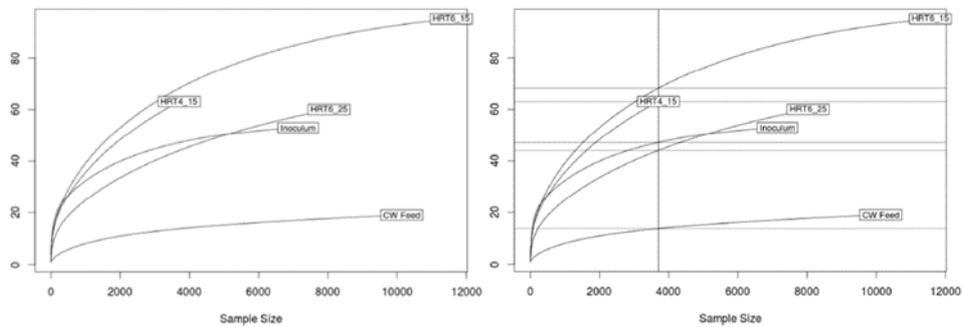# 8 Bibliography

# Appendices

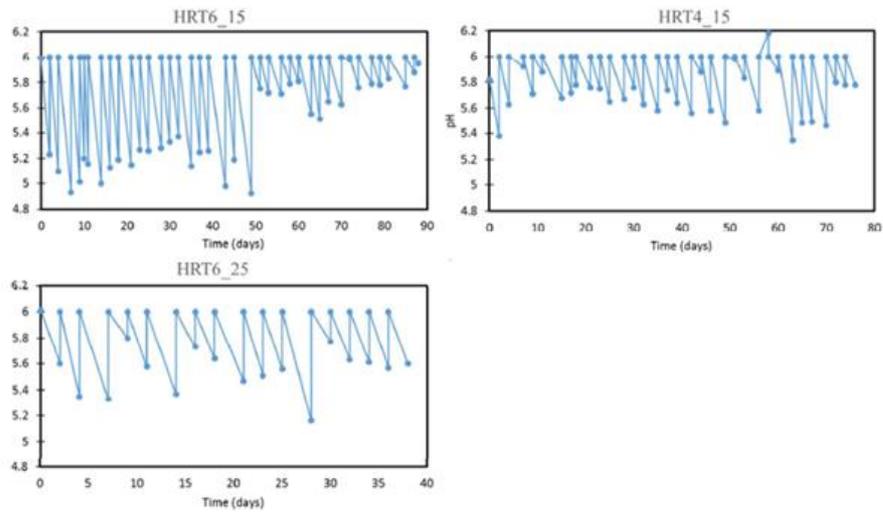0 Appendices

**Chapter 3**
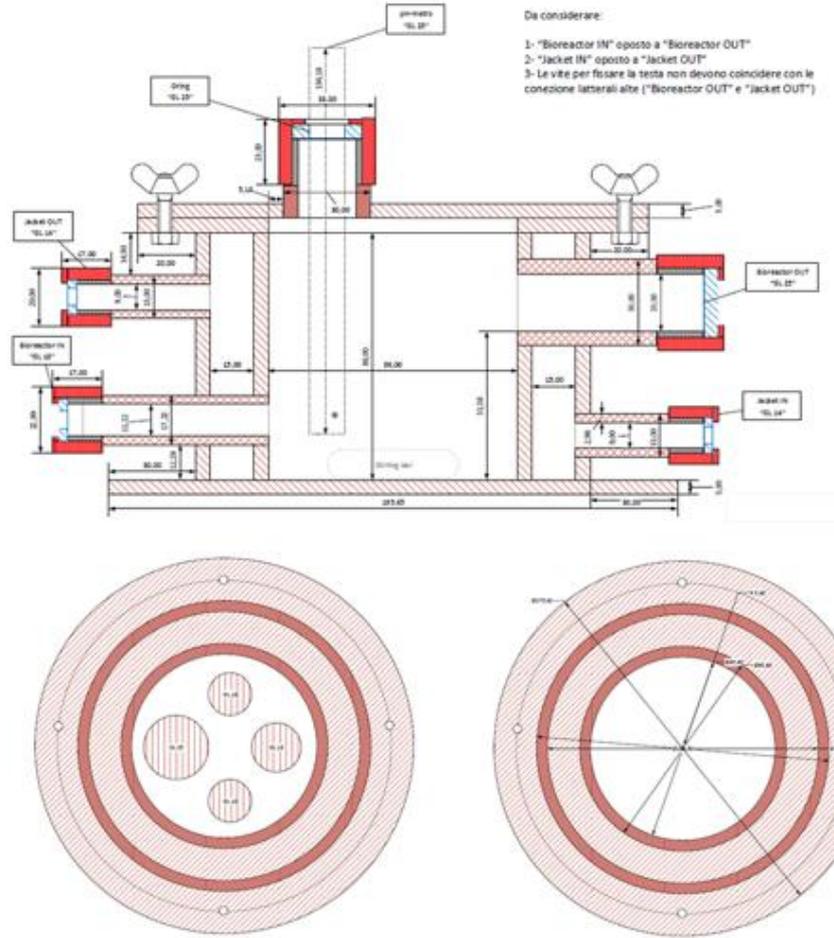


Appendix A - Bioreactors design

**Chapter 4**



Appendix B - Rarefaction curves of the dataset without singletons. (A) overview of the rarefaction curves (B) Intersection indicating subsampling to the lowest sequence count with the rarefaction curves.
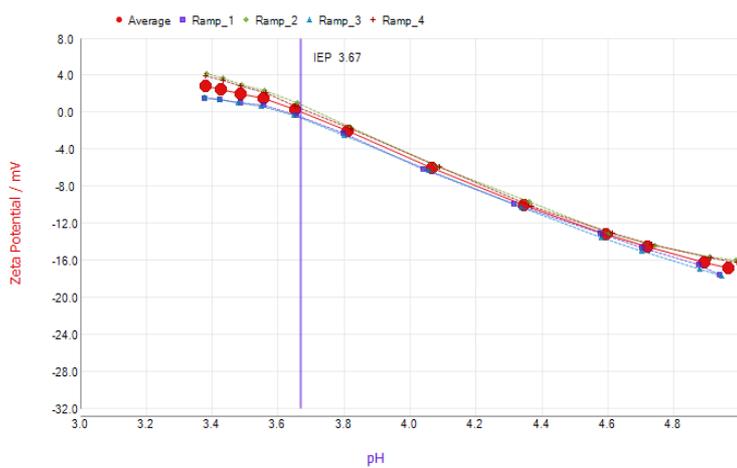
Appendix C - pH trends during continuous digestion in PBBR. For HRT6_15, the start-up was considered finished at day 49 when the base supplying pump was implemented
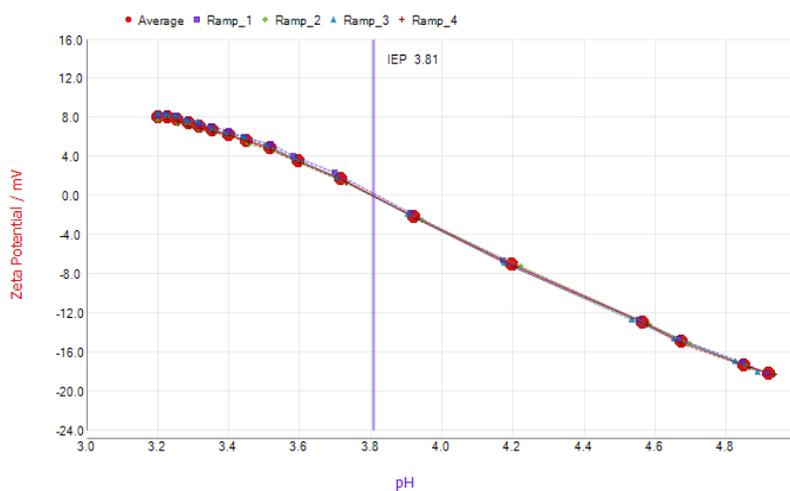
**Chapter 6**
Zeta-Potential profiles
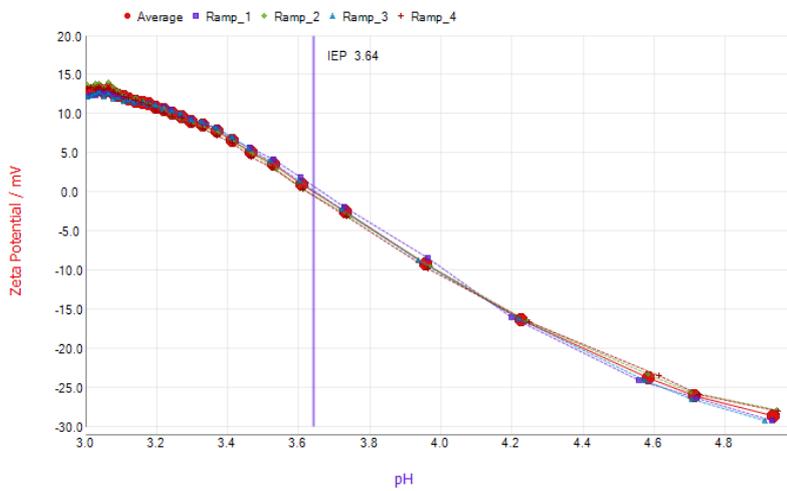


Appendix D - Zeta-Potential profile of membrane DK

Appendix E - Zeta-Potential profile of membrane DL



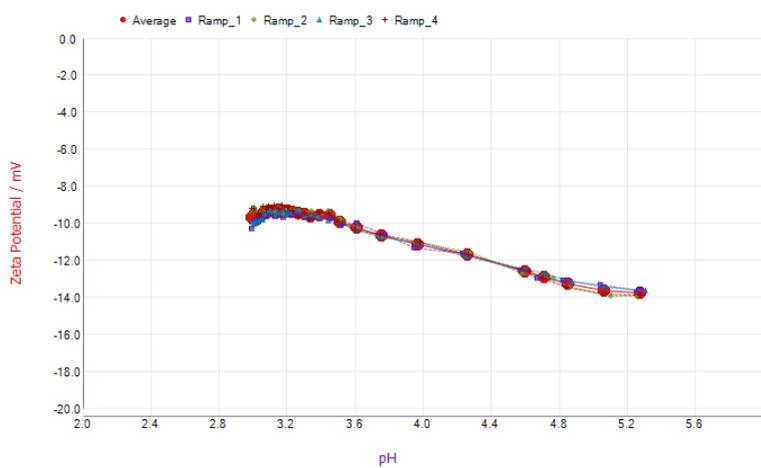Appendix F – Zeta-Potential profile of membrane HL
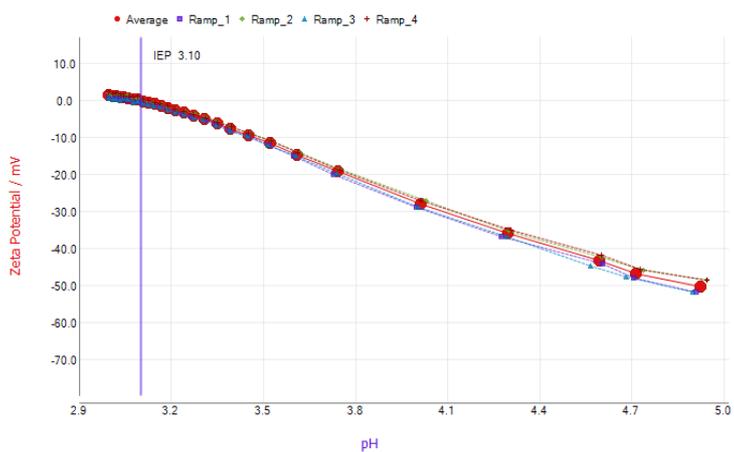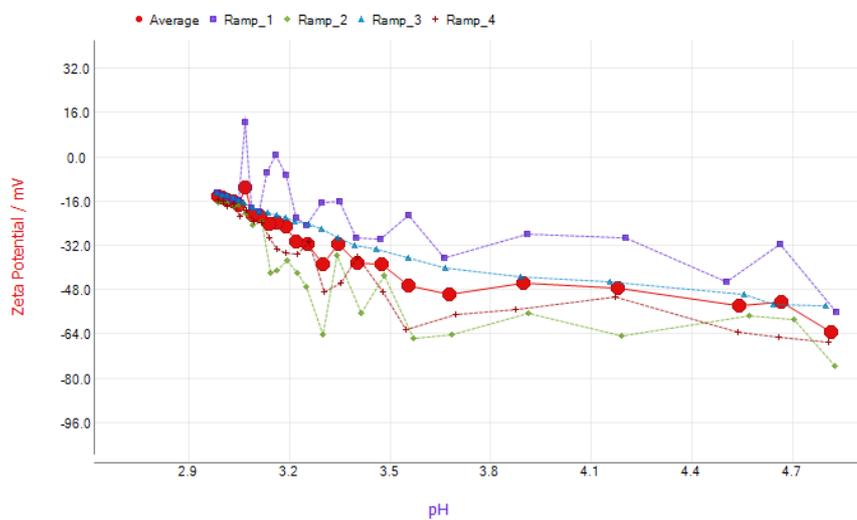
Appendix G - Zeta-Potential profile of membrane NF270
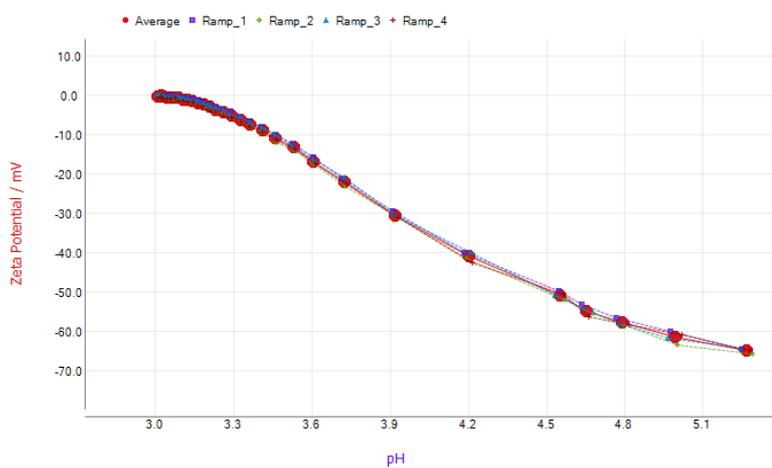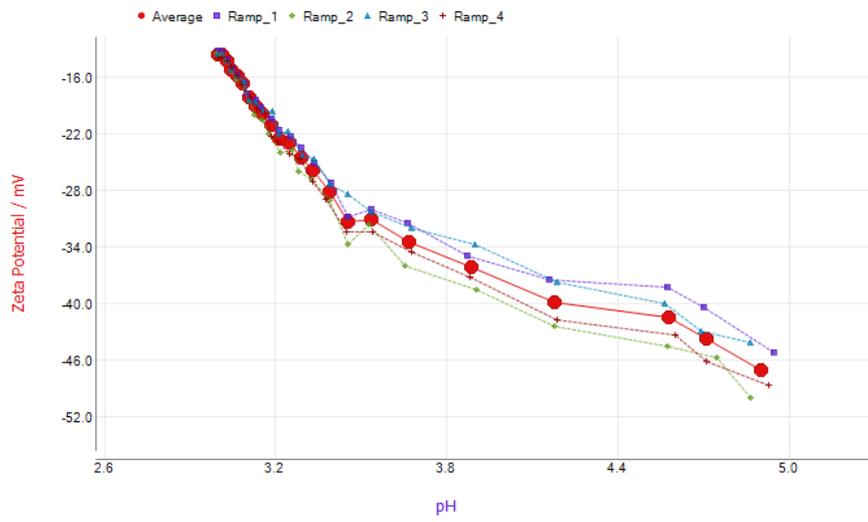


Appendix H – Zeta-Potential profile of membrane NF

Appendix I - Zeta-Potential profile of membrane AL



Appendix J - Zeta-Potential profile of membrane NT7450

Appendix K - Zeta-Potential profile of membrane NT7470