Joana Margarida Bendada Domingos

B.Sc in Applied Chemistry

Acidogenic digestion of effluents of the cheese industry in packed bed biofilm reactors

Dissertation for the Master degree in Biotechnology

Supervisor: Fabio Fava, Professor, Università di Bologna

Co-Supervisor: Lorenzo Bertin, Assistant Professor, Universtià di Bologna

Jury:

President: Prof. Dr. Pedro Miguel Calado Simões

Examiner: Post-Doc Researcher Anouk Ferreira de Freitas Baptista Duque

Pascoal dos Santos Duque

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Resumo

O principal objectivo do presente trabalho foi o estudo de produção de ácidos gordos voláteis (VFAs) num bioreactor de leito empacotado (PBBR) utilizando soro de leite liofilizado (CW) num processo acidogénico.

Os primeiros ensaios foram realizados em frascos Pyrex de 100 mL de forma a realizar o estudo dos perfis do processo acidogénico, nomeadamente, consumo de lactose, produção de VFAs bem como de biogás e sua composição. Estes ensaios foram realizados com células em suspensão (controlo experimental) e com células imobilizadas utilizando como suportes carvão activado granular (AC) e cubos de cerâmica Vukopor S10. O inoculo utilizado – uma cultura mista acidogénica – pertence a um processo de digestão análogo em que um sistema de cultura diferente tem vindo a ser estudado. Assim, as condições de incubação foram as mesmas utilizadas nesse sistema de cultura: 20 g/L de CWP (que corresponde a 15 g/L de lactose), 37ºC e pH 6. Observou-se que o processo consistia no consumo de lactose, formação de ácido láctico (produto intermediário) e a através deste a produção de VFAs. O melhor rendimento foi obtido quando Vukopor foi utilizado (87% contra 30% no AC). Após de 9 dias de batch a composição em VFAs era (g/L) ácidos acético (1.6), propiónico (2.4); butírico (6.6).

Os estudos preliminares mencionados permitiram a selecção do tempo de retenção hidráulico (HRT) a ser operados nos bioreactores.

Dois PBBR de 1 L, um empacotado com Vukopor e outro com AC, foram desenvolvidos. A concentração de CWP, pH e temperatura foram as mesmas utilizadas nos ensaios em pequena escala. Ambos foram operados em batch e continuo. No primeiro batch realizado no PBBR-Vuko atingiu-se uma produção de 6 g/L de ácido propiónico. No entanto, a perda de capacidade de produção deste ácido foi observada durante a operação em continuo. Esta perda foi atribuída a um *wash-out* do microrganismo responsável pela produção. Com PBBR-Vuko foram testados dois HRT, 9 e 6 dias, enquanto que com PBBR-AC apenas um de 9 dias. Os rendimentos obtidos em PBBR-Vuko foram semelhantes aos obtidos anteriormente em menor escala, 80% para os diferentes HRT. Por outro lado, o rendimento PBBR-AC foi de apenas 20%. Este valor é a confirmação de que AC não é um suporte de imobilização adequado nem mesmo em escala de 1L.

Adicionalmente ao estudo de imobilização, foi ainda operado um reactor sem imobilização. Neste último, ao ser operado com HRT de 6 dias o rendimento, em VFAs, diminuiu para 44%.

Conclui-se então que o método de imobilização com material de cerâmica é uma vantagem para a produção de VFAs.

Termos chave: Ácidos gordos voláteis, cultura mista acidogénica, reactor de leito empacotado, soro de leite, suporte de cerâmica.

Abstract

The main goal of the present work was to study the production of volatile fatty acids (VFAs) from cheese whey powder (CWP) by employing a packed bed bioreactor (PPBR) for the anaerobic acidogenesis.

First experiments were performed in 100-mL Pyrex bottles to study the acidogenesis trends, namely: lactose consumption, VFAs and biogas production and composition. These tests were done with freely suspended-cells (control experiment) and with immobilized cells using granular activated carbon (AC) and ceramic cube Vukopor S10 supports. The utilized inoculum – an acidogenic mix consortium- belongs to an analogous CWP digestion process in which a different culture system is being studied. Therefore, the incubations conditions were the same as for that culture system: 20 g/L of CWP (corresponding to 15 g/L lactose), 37ºC and pH 6. The observed trend consisted on lactose consumption, lactic acid formation (as an intermediate product) and from this VFAs production. The best yield was obtained when Vukopor was used (87% against 30% for AC); after 9 days the VFAs was (g/L): acetic (1.6), propionic (2.4); butyric (6.6) acids.

The mentioned preliminary studies allowed selecting the operational hydraulic retention time (HRT) for the bioreactors.

Two recirculate 1-liter PBBR one filled with Vukopor and other with AC were developed. CWP concentration, pH and temperature were the same as in the microcosm experiment. Both were operated in batch and continuous. In first batch performed in PBBR-Vuko it was achieved 6 g/L of propionic. However a loss of capability of producing it was observed during continuous operation. It was ascribed to a wash-out of related strains. With PBBR-Vuko were tested two different hydraulic retention times (HRT), 9 and 6 days, instead for PBBR-AC only HRT of 9 days. The yields for PBBR-Vuko were the same as at the microcosms scale, 80% for both HRT. On the other hand, the yield for PBBR-AC was 20%, this is a confirmation that AC was not the proper support even at a 1-L scale.

Additionally to immobilization study, it was also set up a bioreactor with freely suspended cells. In this last mentioned bioreactor when a HRT of 6 days was set up it was observed a decrease in the VFAs yield to 44%. From this, it was concluded that the immobilization is an advantage for the VFAs production.

Keywords: Acidogenic mixed culture, Ceramic support, Cheese Whey, Packed-bed bioreactor, Volatile Fatty Acids

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Abbreviations

% w/w CWP – percentage mass of compound per mass of cheese whey powder

COD – chemical oxygen demand

CODin - COD content in CWP solution feed

CODout - COD content in bioreactor outlet

$COD_{in}^{supernatant}$ - COD content in inlet supernatant

$COD_{out}^{supernatant}$ – COD content in outlet supernatant

COD_{in}^{mix} - COD content in inlet mixture

COD_{out}^{mix} - COD content in outlet mixture

CO_2 – Dioxide Carbon

CW – Cheese whey

CWP – Cheese whey powder

GC – Gas chromatograph

H_2 – Hydrogen

HPLC - high-performance liquid chromatography

HRT – Hydraulic retention time

in mix – inlet mixture

in supernatant - inlet supernatant

Y_{VFAs} % (C-mol/C-mol) – yield of volatile fay acids respecting to lactose

FID - Flame Ionization Detector

MBR – Membrane Bioreactor

$m_{support}$ – mass of support

NaOH – Sodium hydroxide

out mix – outlet mixture

out supernatant - outlet supernatant of bioreactor

PBBR - packed bed bioreactor

PBBR-AC – packed-bed Bioreactor packed with granular activated carbon

PBBR-Vuko – packed-bed Bioreactor packed with Vukopor S10

PHA – pollyhydroxyalcanoates

µGC – micro-GC

VFAs – Volatile Fatty Acids

v_{inoc} - volume of inoculum

1 Introduction

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. With fast growth of human population and a respond to the nutrition needing brings to and increase of milk derivate production in the last years (Fig. 1.1). Cheese is the milk derivate most produced around the world (Fig. 1.2) and is in Europe where is observed the greatest production of cheese, representing 57% of world production.

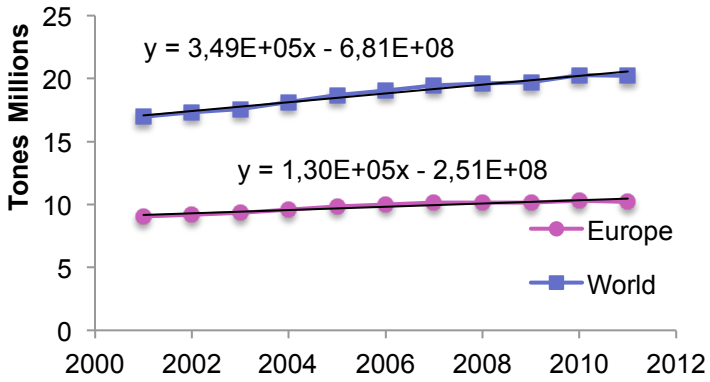

Figure 1.1. Cheese production registered in World and Europe in the last 10 years [data from FAO].

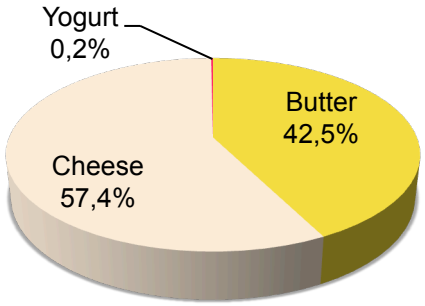

Figure 1.2. Milk-Production share by type of milk-derivate product in the world [data from FAO].

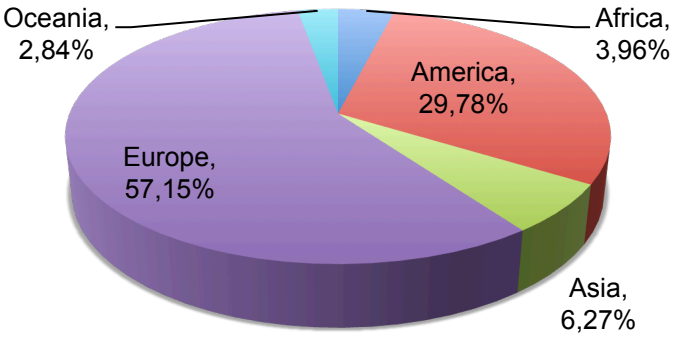

Figure 1.3. Contribution of each continent for the production of cheese [data from FAO].

Of 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 [Prazeres et al., 2012]. From this, 92 M tons of cheese whey are generated per year in Europe [FAO] since 10 M tons of cheese are produced on an annual base.

Since CW is harmful for the environment it must be treated before being discharged in the ecosystems. Otherwise it would cause serious pollution problems for the surrounding environments. CW can cause an excess of oxygen depletion, reducing aquatic life, impermeabilization, decreasing crop yield, eutrophication, toxicity, etc. in the receiving 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. Therefore, instead of doing and investment on a conventional biological treatment without valorization, 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 [Kosseva et al., 2009]. In other words, this is the situation for applying the bio-refinery concept: *maximization of each stream value.* In this way, one option for valorising the effluent is the anaerobic fermentation. In this process, microbial consortiums promote bioconversion of dissolved organic compounds present in the waste, like lactose, into valuable compounds; i.e: acetic, propionic, butyric, lactic, etc. These last so called Volatile Fatty Acids (VFAs) are by there on fine chemicals and also can be the substrates for others microbial conversions: methane, PHAs [Albuquerque et al., 2011; Bengtsson et al., 2010] among others.

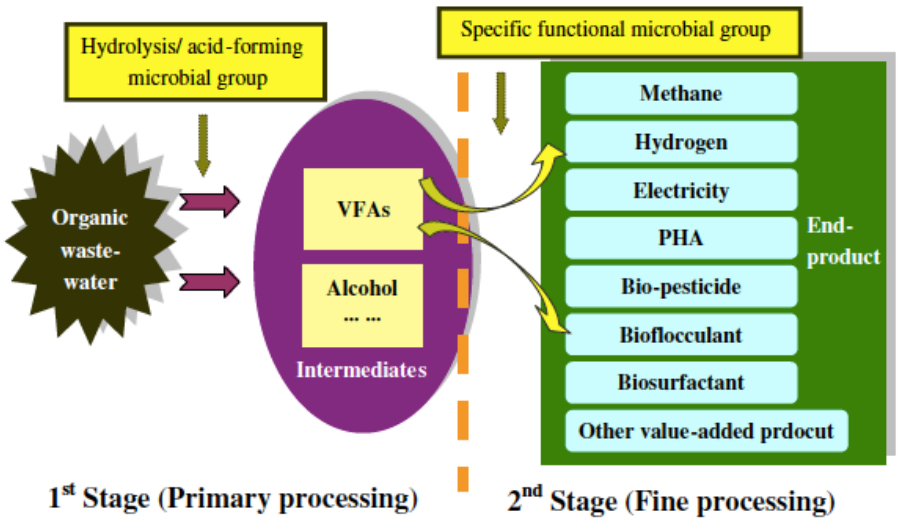

Figure 1.4. An example scheme of wastes valorization. Intermediates are generated, i.e.: with an anaerobic acidogenic digestion, and consumed/separated in a second stage for fine chemicals production [Li et al., 2011].

1.1 CW and its characterization

Cheese industry generates three types of effluents: CW which is the result of cheese production, from cottage cheese production results second CW and from washing water that contains different fractions of CW and/or second CW generates CW wastewater. The CW effluent can be in several forms: raw, diluted, pasteurized, deproteinized, powder, etc. [Prazeres et al., 2012]. Cheese whey powder (CWP) consists in CW lyophilized and despite lyophilization costs, this way of CWP is easily storage and transported since has a reduced volume and long-term stability [Kargi and Ozmihi, 2006].

CW characteristics depend on the milk used, which varies with: animal source (goat, cow, sheep and buffalo) and state (animal breed, feed, health and lactation station. It also depends on the process technic to remove the casein protein, present in the milk composition. However, in general terms it can be said that is a green, due to riboflavin (vitamin B2) content, yellowish liquid resulting from the precipitation and removal of milk casein in cheese production process [Prazeres et al., 2012]. In the following table (Table 1.1) is represented the range of values of CW composition. However, it is mainly composed by lactose, proteins (β-lactoglobulin (50%), α-lactalbumun (12%), immunoglobulins (10%), serum albumin (5%) and proteose peptones (0.23%), lipids and minerals. [Siso et al.,1996]. 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 [Prazeres et al., 2012].

Table 1.1. General characterization of CW [Prazeres et al., 2012]

Compound	Range of values
Lactose	39-60 kg/m^3
Minerals	0.46-10%
Total Suspended Solids	0.1-22 kg/m^3
pH	3.3-9.0
Phosphorus	0.006-0.5 kg/m^3
Total Kjeldahl Nitrogen	0.01-1.7 kg/m^3
Organic load	0.6-102 kg/m^3
COD	50-102 kg/m^3
BOD	27-60 kg/m^3

From previous statements it is obvious that CW must be treated before being discharged to the environment. For minimize the negative effects of CW discharge an alternative was promote the dilution with other wastewater, considered less polluted, like domestic wastewater [Gannoun et al., 2008]. There are several techniques to treat it like aerobic digestion. But, because of its high COD content, the implementation of a conventional sewage treatment plants is difficult; requiring in most cases a separated treatment plant.

Instead of doing and investment on a conventional biological treatment without valorization, 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 [Kosseva et al., 2009]. In another words the situation for applying the bio-refinery concept: *maximization of each stream value.*

1.2 Biological treatment of CW with valorization

Anaerobic digestion is a collection of processes by which microorganisms convert biodegradable compounds in the absence of oxygen. In Figure 1.5 is represented the different pathways which compose this complex process. 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 [Horiuchi et al., 2002; Itoh et al., 2012]. The last step is methanogeses in which organic acids are convert to methane and carbon dioxide.

The mentioned processes can be carried out by the implementation of a pure or mixed culture. The advantage, of a pure culture, is the high productivity achieved. In other hand, when a mixed culture is employed costs with energy are save due to no sterilization needs. These culture are composed by many different members of bacteria giving it robustness to support feed variations and synergies effect when extra supplements are needed [Agler et al., 2011].

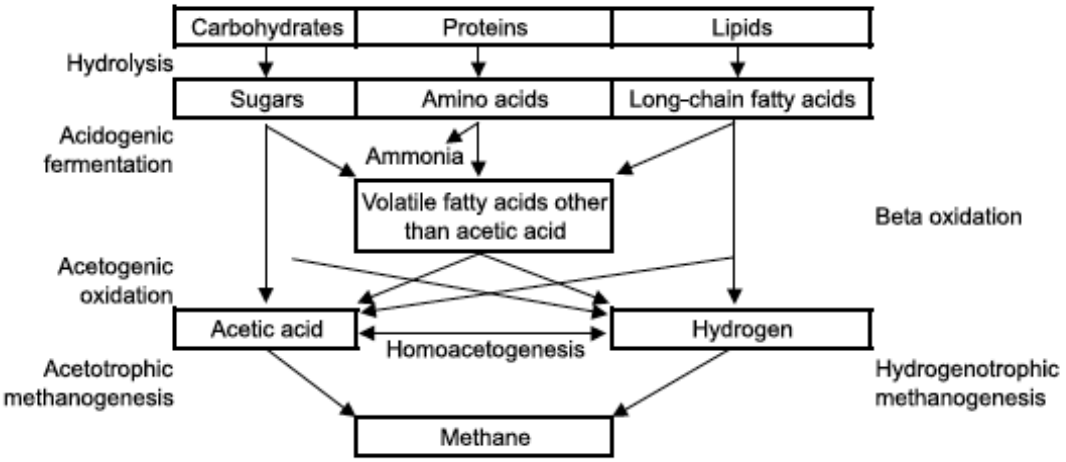

Figure 1.5.Scheme representing the different possible pathways during anaerobic digestion [Salminen et al., 2002].

In which respect to anaerobic digestion of cheese whey 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 species know which are able to metabolize lactose are notably less then microorganisms able to perform the conversion of other simple sugars like glucose [Siso, 1996]. 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 [Zafar and Owais, 2006], CWP solution [Kargi and Ozmihci, 2006], CW permeate from ultrafiltration [Domingues et al., 1999] and even CW deproteinized [Dragone et al., 2011]. However, from all of these studies it was seen that ethanol production using CW as substrate is not economically competitive respecting to when other wastes like cane sugar or cornstarch and also, when lactose is used as substrate, the HRT decreases from days to hours when the processes is aerobic instead of anaerobic. Biogas production is another possibility for valorization of CW with anaerobic digestion. Hydrogen is a clean energy and the production from wastes, like CW, is an economical viable option to produce it. After hydrogen production, the effluent generated has a significant COD vlaue since is manly composed by VFAs, alcohols and carbohydrates not being possible to be discharge without an adequate treatment [Prazeres et al. 2012]. Thus, those compounds can be used in other fermentations and this way, after CW valorization, also hydrogen effluent is valorize.

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 [Plessas et al. 2008] as pure cultures [Arasaratnam et al., 1996; Vasala et al., 2005]. 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 [Plessas et al., 2008]. Besides lactic acid, also VFAs production from CW has been study [Bengtsson et al., 2008]. 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 [Prazeres et al., 2012]. Bioreactor configuration can also contribute for increasing the productivity, for example the employ of immobilization. Few experiences about CW anaerobic acidogenic digestion for the production of ethanol and lactic acid with immobilized cells by employing a membrane-based cell recycle or fibrous-bed reactors were already described [Kosseva et al., 2009]. However, almost no experiments for VFAs production with immobilization in a packed-bed reactor, from CW, have been reported.

1.3 VFAs and its applications

As it was said before, with the continuous population growth an increase of alimentary production is needed. Because of that, the waste generated also increase representing a threat to the environment. However some wastes are composed by sugars with potential for valorization like glucose and xylose present in sugarcane bagasse [Zhu et al., 2012] or lactose that compose the CW. Microbial consortiums use those sources of energy to perform the conversion into other compounds, for example, VFAs.

VFAs are fatty acids with a carbon chain of six or less carbon atoms, which can be distillate at atmospheric pressure [Lee et al., 2013]. Acetic acid ($C_2H_4O_2$), propionic acid ($C_3H_6O_2$), isobutyric and butyric acids ($C_4H_8O_2$), isovaleric and valeric acids ($C_5H_{10}O_2$), isocaproic and caproic ($C_6H_{12}O_2$) acids are the molecules that compose the VFAs group. Actually the production of these compounds are by employing chemical processes and using non-renewable petrochemicals as raw materials [Lee et al., 2013]. In the last years “green technologies” have been studied intensely in order to be economical and environmental feasible in large-scale activity avoiding the use of non-renewable resources. Biological production of VFAs is an anaerobic process with conversion of sugars by microorganisms.

From those mentioned VFAs not all have the same application interest. During the fermentative process the operational parameters like pH, temperature, HRT or organic load, can influence the quality, quantity and yield of VFAs produced [Lee et al., 2013]. So, in order to favour the production into the most interest VFAs the process must be carefully controlled.

The optimal pH for VFAs production is usually in range 5.25-11 since in very acidic (pH 3) or alkaline (pH 12) values, acidogenesis cannot survive [Lee et al., 2013]. There are some studies about the influence of pH values in type of VFAs produced [Horiuchi et al., 2002; Bengtsson et al., 2008]. When CW is used it was observed that working with pH between 5.25 and 6 propionic increased its production while acetic and butyric acids decreased [Bengstonn et al., 2008]. Yet it was also observed higher production of propionic and acetic acids, respecting to butyric acid, when pH was increased from 6 to 8 but when a waste rich in glucose was use as substrate [Lee et al., 2013]. From all these, and others, researches it suggests that the optimal pH for a production of particular VFA depends also on the kind of waste used.

Another parameters with a huge impact in VFAs production is the HRT. Bengtsson and co-works [Bengtsson et al., 2008] performed an interesting study in which compared the influence of HRT by using two different wastewaters: CW and paper mill effluent. The increment in amount of propionic acid was obtained when HRT was increased from 20 h to 65 h when CW was substrate while in the case of paper mill effluent this increment was observed when the HRT was increasing from 10 to 20h. However in both cases when propionic acid increments its concentration, butyric acid decreased in the same proportions. This shift observed between butyric and propionic is a competition of those types of fermentations: butyrate type fermentation resulting in acetate and butyrate as main products and propionate type fermentation with acetate and propionate production [Cohen et al., 1984].

VFAs are compounds used as substrate in diversity applications (Figure. 1.4) being polyhydroxyalkanoates (PHAs) one of the principals. PHAs are polyesters biodegradable synthesizes

biologically [Albuquerque et al., 2011]. Numerous bacterial species produce these biodegradable polymers as intracellular carbon and energy reserves from renewable resources such as VFAs. PHAs have chemical and physical proprieties similar to conventional plastics but its biodegradable characteristic makes them a promising green alternative to the conventional plastics, which used petrochemical products as raw material Despite of industrial PHA production is already a realty [Fradinho et al., 2013] is still limited by the high production cost. High cost is related with carbon substrate, which represents 31% of the total process cost [Lee et al., 2013]. From this, VFAs produced from low-value substrates as dairy or paper wastewaters [Bengtsson et al., 2008] have been contribute to decrease cost of PHAs production.

The composition and proprieties of PHA is depended on type of VFAs which it has resulted. Acetic and butyric acids favour the production of 3-hydroxybutyrate (3HB) whereas propionic and valeric acids promote the synthesis of 3-hydroxyvalerate (3HV). Since some PHAs are too rigid, as Poly(3-hydroxybutyrate), the combination between polymers is an advantage. The incorporation of 3HV into P(3HB) leads to the formation of copolymer P(3HB-*co*-3HV) which is more stretchy and tougher. Besides is less permeable to oxygen as compared to the commercial polyethylene and polypropylene, making it a suitable food packing material [Lemos et al., 2006].

Those are the motives why is so important to control the operation parameters, during acidogenic digestion, in order to obtain the VFAs with most industry interest.

1.4 Immobilization

The immobilization of enzymes, animal cells or microorganisms, is a strategy used in several bioprocess. Is defined as "*the enzymes/microbial cells physically confined or localised in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously*". Nevertheless, in general, the objective of this practise is to promote the using of the cells for longer periods in continuous process. When is performed physically it calls entrapment, otherwise is attachment (Figure 1.6). Calcium alginate or membranes are the materials usually used for a physically immobilization. Since the cells are physically trapped in a matrix this method is employed in most of the times due to its facility performing spherical particles by dripping a polymer-cell suspension into a medium containing precipitate-forming counter ions or though thermal polymerisation. This method is suitable particularly for processes that the substrate is inhibitory since the cells are not in contact direct with the environment. A limitation can be the mass-transfer which effect negatively the speed of reactions promoting decrease in the velocity. Instead, attachment consists cells bound, forming the biofilm. Biofilm is defined as colonies of microbial cells encased in a porous matrix or attached to a surface with the polysaccharides segregated by the microorganisms [Brock]. Because of that, the huge advantage is the fact that is simple to carry out since only depends on microorganisms activity and the conformation applied to the bacterial consortium has few influence. As it only depends on the comportment of the microorganisms the major disadvantage is

related to the fact that binding forces, between the microorganisms and the surface, can be weak [Kosseva et al., 2009].

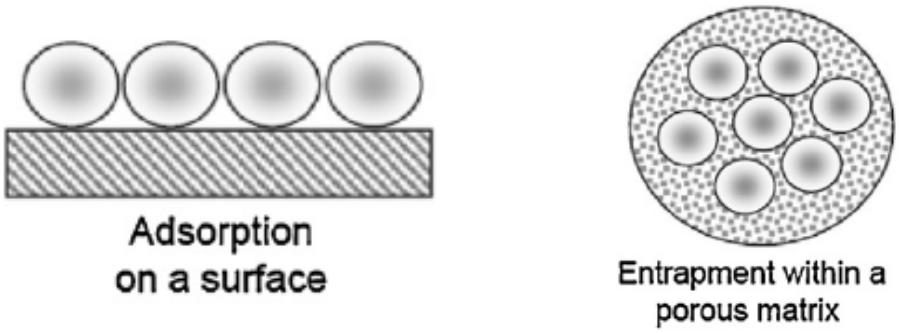

Figure 1.6 Scheme of two types of immobilization. On the left is represented adsorption - some cells are attached to a surface. On the right entrapment – the cells are physically inside of a material [Kosseva et al., 2009].

Despite all these referenced differences, in general, the immobilization method has several advantages respecting to suspended culture processes. The principal is the fact that it prevents the culture washout when continuous process is employed due to a higher cell density as well as its robustness formed by the consortium [Keskin et al., 2012]. With this stronger culture is also possible to work with lower hydraulic retention times (HRT). Another advantage is an easier downstream process since, during the bioconversion the mostly of the cells are separated from the liquid.

From all of this a several supports can be employed in a packed-bed bioreactor (PBBR), which the most common are porous materials composed by silica, ceramic or activated carbon.

Vukopor® S, produced by Igor-Lanik – Techservis Boskovic company, is a three-dimensional cube structure composed by a ceramic matrix and formed by interconnected system cells. This material, due to its ability to increase the degree of purity of the metals, is generally used in foundry industry for filter of molten material like iron, bronze or brass. Is characterized for having thermal conductivity, stability and resistance to sudden temperature changes. The ceramic structure is composed by high quality material of Silicon carbide (SiC) and Aluminium oxide (Al_2O_3) which gives an excellent stability at temperatures until 145°C [Igor-Lanik – Techservis Boskovic].

Activated Carbon (AC) is composed by microcrystals of graphite randomly arrange with an unclogged pours. There are some forms of AC: powder, granular (GAC), cloths, fibbers and felts [Da˛browski et al., 2005]. Its surface is nonpolar giving it an enormous capability of adsorption for organic compounds with a low molecular weight like VFAs or phenols. Furthermore is widely used in industry, for be a low-cost material being applied as organic acids recovery, phenols removal or even to absorb compounds, like lactic acid, which inhibits fermentative processes [Gao et al., 2011]. Besides its absorbent capacity of interest fermentation products, like VFAs, those compounds can be recover by employing a desorption technique.

2 Goals

The main goal of this thesis was the production of VFAs from CWP by employing a packed bed bioreactor, in which an acidogenic mixed culture was employed to bio-convert the lactose present in the CWP to produce these VFAs. In order to compare two different packing materials, and the influence of such a parameter in VFAs profile production, a ceramic material, Vukopor S10, and an activated carbon were employed in the formation of the reactor packed beds.

The present work was divided in two parts:

- Experiments carried out at a micro-scale, by developing microcosms prepared in 100mL-Pyrex bottles, for both immobilizing materials. This first approach was dedicated to study operational parameters to be used in the next step.
- Experiments carried out with two lab-scale (about 1 L of empty volume) PBBR, which were packed with Vukopor S10 and with Granular Activated Carbon, respectively, and which were operated by applying parameters values determined during microcosm experiments. Furthermore, a freely suspended cells bioreactor was also set up and used to compare the performance of freely suspended microorganisms, so to understand the role of the immobilization material during anaerobic acidogenic process using CWP as substrate.

Although the metabolic capability of the employed acidogenic mixed culture of biocoverting cheese whey into VFAs was already known, since the same culture was collected from a membrane bioreactor (MBR) employed for the same aim, the process configuration could significantly influence the microorganisms behaviour and thus the effluent features, mainly in terms of VFA relative concentration. In this respect, microcosm experiments were considered a determinant step to study the immobilized cell process parameters, especially HRT, to be employed in the PBBRs.

VFAs produced should be use as substrate for other processes, like PHA production. Due to the different characteristics and composition of VFAs some have more applicability than others. Propionic acid is one of the VFAs with more perspective in PHA industry and because of that along the development of the work, propionic acid production becomes a special goal.

3 Material and Methods

3.1 Materials

3.1.1 Inoculum and culture media

The inoculum used in the present work was an acidogenic mixed culture recovered from a membrane bioreactor (MBR) that was also producing VFAs from CWP (20 g/L) in a continuous production process at 37ºC. A concentrated sample of the consortia was cultured in a 500 mL-Pyrex bottle in order to generate enough active biomass for all experiments. The culture started by inoculating the culture media - consisting on 20 g/L of CWP as in the MBR - at 10% of the working volume 200 mL. It was maintained in the incubator at 37ºC (as in MBR) stirring 150 rpm and sampled every 2-3 days, as described in (see 3.2.1). In order to maintain the inoculum in activity, culture broth was changed on a week basis by centrifugation at 7,500 rpm and pellet suspension in 200 mL of a new culture media. In this way, lactose was added when it was exhausted.

The CWP used during the work was kindly provided by Lactogal[1] - Portugal. All experiments, except where mentioned, were done with the same culture media: a CWP solution (20 g/L) prepared with distilled water, corresponding to 15 g/L of lactose. Lactose quantity was decided in base of the quantity of lactose content in this CW before lyophilisation, which is 15 g/L. Since during lyophilisation process all the water is removed, all components became more concentrated. Knowing the characterization of the powder is possible to add more or less water according to final lactose concentration desired. The characterisation of CWP, provide by Lactogal, can be consulted in Table 3.1.

Table 3.1. Cheese Whey Powder characterisation. All these values were provide by Lactogal

Fat content (% w/w CWP)	1.21
Protein content (% w/w CWP)	13.62
Lactose content (% w/w CWP)	78.4
Acidity (cm^3 *per* 100 g CWP)	11.4
Moisture content (% w/w CWP)	1.8
Specific weight (g/L)	570
Insolubility index (cm^3)	< 0.1

3.1.2 Immobilization material

During the work, two different packing materials were study: Ceramic Cubes (Vukopor S10 product, Lanik, Boskovice, CZ) (Figure 3.1 a)) whose dimensions, porosity and density were 25 x 25 x 18 mm, 10 ppi and 2.38 g/mL, respectively; and Granular Activated Carbon (CP4-60 product,

[1] Portuguese dairy product company

Chemviron Carbon, Feluy, Belgium) (Figure 3.1 b)), consisting of cylinders of about 3 mm diameter and 10 mm length, whose density was 1.32 g/mL.

For the preliminary tests, which were carried out in microcosms Vukopor S10 supports were used in a smaller size, than that described before (Figure 3.1 a)). The original ceramic cubes were broken into a size sufficient enough to fit them inside the bottle. During the breaking step the weight and similarity shape between the pieces were took into consideration, in order to get supports with similar dimensions. In the case of the experiment with 1 L bottle the employed ceramic material was the original one, which was broke in half.

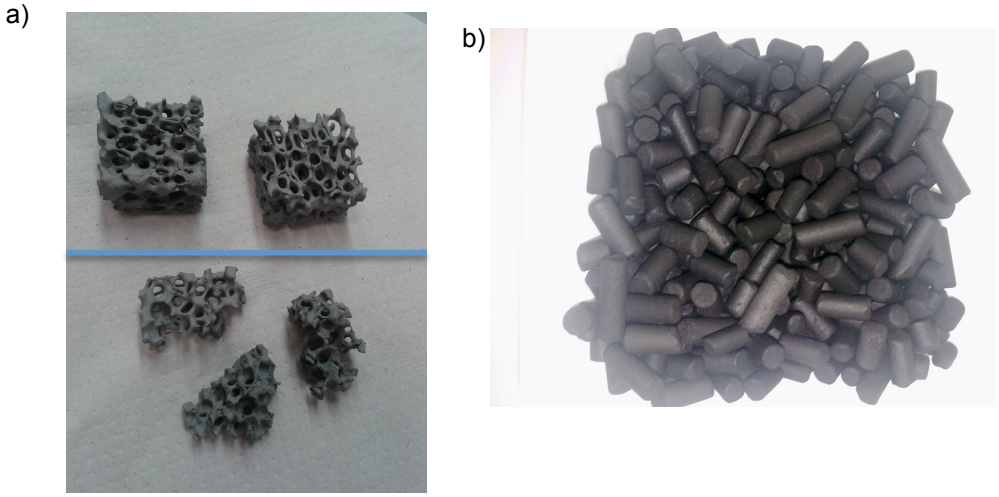

Figure 3.1. Immobilization supports used during the experiments. a) Comparison of size and shape between the supports used to pack the PBBR-Vuko (a.1) and the supports used in the microcosm experiments with immobilization (a.2). b) Granular Activated Carbon used as immobilizing support during the work, either PBBR-AC as microcosm experiments.

3.2 Experiments set-up

3.2.1 Batch trials at microcosm scale

The experiments were carried out in 100-mL Pyrex bottles (55 mL of working volume) equipped with two stacked silicone stoppers (thickness 2.5 mm each) tightly closed to the bottleneck through a modified Pyrex-cap to allow gas sampling. Cultures were started by inoculating with a 10% of the working volume. Incubation conditions were 37°C and 150 rpm. Sampling for biogas and metabolites production measurements were made every 2-3 days. After biogas sampling (see 3.3.2), the bottles were opened under nitrogen gas sparging, to keep anaerobiose and 1 mL of liquid phase was taken for analysing lactose, lactic acid and VFAs concentrations. Thereafter, pH was controlled and corrected to 6 by adding some drops of NaOH 10 M solution.

3.2.1.1 Freely suspended cells study

Microcosms with freely suspended cells were performed in order to study the bioconversion trends. This is lactose consumption, lactic acid production and consumption and VFAs production. The experiment was done in quadruplicate, using the CWP culture media – Experiment 1.

A preliminary experiment, also using freely suspended cells, was done to study the influence of the carbon source quantity on the VFAs composition profile. This experiment was done in quadruplicate, using 30 g/L of CWP - Experiment 5.

For both experiments it was performed two sequential batches. At the end of the first batch, all liquid was centrifuged 10 min at 7,500 rpms and pellet suspension in 55 mL of new CWP solution, with the respective concentration of each experiment. (20 or 30 g/L). The plateau of organic acids production determined the end of batch.

3.2.1.2 Batch trials at microcosm scale: immobilization supports study

The next experiments were prepared in order to determine the parameters for the set up of the PBBRs. The microcosm were prepared and treated in the same way as in 3.2.1, but utilizing a support to obtain immobilization. In order to be able to perform a comparison between the different supports, the packed and working volume were fixed. The packing volume, that corresponds to the bottle volume occupied by immobilization materials, was set by marking an horizontal line over the glass, corresponding to 45 mL of water, previously added. Then, the bottles were filled up, with water, to 55 mL level, which represents the working volume. This volume will comprise the liquid volume, CWP solution and inoculum, and the immobilization material volume. Then, empty and dried bottles were filled with each support, Vukopor S10 and Granular Activated Carbon, till completing the packing volume. Support accommodation and packing was obtained by giving smalls hits to the bottle. Since both supports have different specific volumes and the working volume was fixed, the liquid amount-comprising the CWP and inoculum necessary to add is different for Vukopor and AC. To determine that, water was added until reaching the 55 mL line. The quantity of added water in each bottle corresponds to the total liquid volume, comprising the inoculum and CWP solution, necessary to add to each bottle. This step was performed with the supports already wet in order to avoid the absorption phenomenon since it was previously observed that AC had a strong absorbent capacity and because during sequential batches the supports will be already wet in the beginning of each.

After setting up of all experimental volumes, cultures were started with the corresponding amounts of CWP and inoculum (Table 3.2), always bubbling nitrogen to maintain anaerobiose.

Table 3.2. Volumes determined for microcosm preparation as the final volumes added.

Microcosm	$m_{support}$ (g)	V_{liquid} (mL)	V_{CWPsol} (mL)	V_{inoc} (mL)	V_{total} (mL)
Vukopor S10	15.5 ± 0.1	49.0 ± 0.5	45.0	5.0	50.0
Granular Activated Carbon	22.0 ± 0.1	28.0 ± 1.0	25.0	3.0	28.0

3.2.1.2.1 Experiment 2 - Preliminary immobilization supports study

The aim was to study the bioconversion process when employing the immobilization supports. Being a preliminary activity, it was carried out just in double, for AC and Vukopor. Microcosm set-up parameters are presented in Table 3.2. The experiment was carried out for 98 days under the incubation conditions described in 3.2.1. In total eight consecutive batch were performed, six for biofilm formation - the first, second, fourth, fifth, sixth and seventh – and two for VFAs accumulation - third and eighth.

At the end of each batch, all liquid phase was took out and replaced with new CWP solution (20 g/L), leaving the supports inside the bottles. The total consumption of lactose was the parameter that determined the end of “biofilm formation batch”, while the end of VFAs production was determined when all organic acids concentration arrive to their *plateau*.

3.2.1.2.2 Experiment 3 - Improved immobilization supports study

With some gained knowledge from the preliminary test and in order to confirm those previous results, this test was carried out with four bottles for each support – AC and Vukopor. The preparation was as described in 3.2.1.2 with one added step: the bottles with the supports inside were autoclaved at 120ºC during 20 minutes before inoculation. This was because of the reutilization of support material that arrived from another process. Each bottle had the characteristics as described in Table 2.3. Along the 38 days of experiment, three sequential batches were performed for biofilm formation and the fourth batch was dedicated to VFAs production study.

Figure 3.2. Microcosms preparation. In this step it was already determine the packing volume line (green), which corresponds to 45 mL of water, and working volume line (red) which corresponds to 55 mL.

3.2.1.3 Experiment 4 - Batch production for consortia characterization

With the aim of characterizing the inoculum and the consortium which forms the biofilm and responsible for single metabolic activities, this experiment is a replica from previous experiment but only performed with ceramic material. In order to have enough liquid volume and support material for molecular analyses sampling, this experiment was carried, in double, in 1L-Pyrex bottles filled with Vukopor S10. Although the total volume of the bottles was different, the ratio between the packed and working volume were the same as in the previous immobilisation experiments, which results in 400 mL and 500 mL, respectively. The preparation was the same as described in 3.2.1.2, with the adjusted volumes.

In addition to the 1 mL sample, for analyse of VFAs, lactose and lactic acid, supports and liquid were taken out for molecular analyses. For the supports, 8 Vukopor cubes were removed from the bottle with a long clamp and placed in falcons of 50 mL, where were cleaned, very carefully, with physiologic solution NaCl 8 g/L. The cleaning step is important since the cells that form the biofilm will stay attached to the supports. For liquid, 5 mL were mixed with 5 mL of ethanol 98%. This sampling occurred 4 times along the experiment: 21, 27, 33 and 37 days from the beginning.

Table 3.3. Volumes determined for 1 L Pyrex bottles preparation and final volumes of CWP solution and incolum added.

nº Vukopor S10	m_{supp} (g)	V_{CWP} (mL)	$V_{inoculum}$ (mL)
48	127.9 ± 1.3	387	43

3.2.2 Continuous process

A freely suspended cells bioreactor and two identically configured anaerobic packed-bed biofilm reactors (PBBRs) were developed, similar to the bioreactor developed by [Bertin et al., 2010]. All of them were started in batch and then, when VFAs concentrations arrived to the plateau, changed to continuous operation. Each bioreactor consists of 1L-hermetically closed glass column (5 cm of diameter and 40 cm high) wrapped with a silicon tubing serpentine continuously recycling thermostated water, maintaining 37 ± 2ºC inside of the bioreactors, and equipped with a down flow recycle line. The liquid and the gas effluent were collected in a bottle, hydraulically connected to a 2.5 L “Marriotte” bottle through which the produced biogas volume was determined, as described in 3.3.2. All bioreactors had a working volume of 830 mL and were inoculated with 20% of liquid volume of CWP solution.

Before the inoculation and CWP solution addition, all volumes were determinate as it was performed for microcosm experiments (described in 3.2.1.2) but this time with the reactors. Also in this case all supports were autoclaved.

The PBBR with Vukopor (PBBR-Vuko) was operated in three batches and two continuous mode experiments: one with HRT of 9 days and other with HRT of 6 days. It was packed with 120.36 g of support and filled with 790 mL of liquid. After 14 days of initial batch operation, a continuous process was started with a HRT of 9 days for 38 days. Then, another two sequential batches were performed, each one with the durance of 10 days. In the first, in addition to the solution of CWP, 10% of the liquid corresponded to inoculum. In the second only solution of CWP was added. Once this latter batch ended, the continuous state was started again but with a HRT of 6 days having a durance of 41 days.

The recycle was maintained at 44 mL/min all along the 113 days, during which the PBBR-Vuko was working. Besides the change in the HRT the PBBR-Vuko was always operated in a CSTR equivalent.

Table 3.4. Stages in which PBBR-Vuko was operated with respective time as also the inoculation.

Stage	During time (days)	Inoculum add
1º batch	14	20 %
Continuous - HRT 9 days	38	-
2º batch	10	10 %
3º batch	10	-
Continuous - HRT 6 days	41	-

The PBBR with Granular Activated Carbon (PBBR-AC) was operated in one single batch, for 15 days, and one continuous experiment with a HRT of 9 days, along 33 days. The bioreactor was filled with 366.336 g of Granular Activated Carbon and 500 mL of total liquid, comprised CWP solution and inoculum.

The freely suspended cells bioreactor, without any support was operated in batch for 16 days and then in continuous state with HRT of 6 days.

a) b)

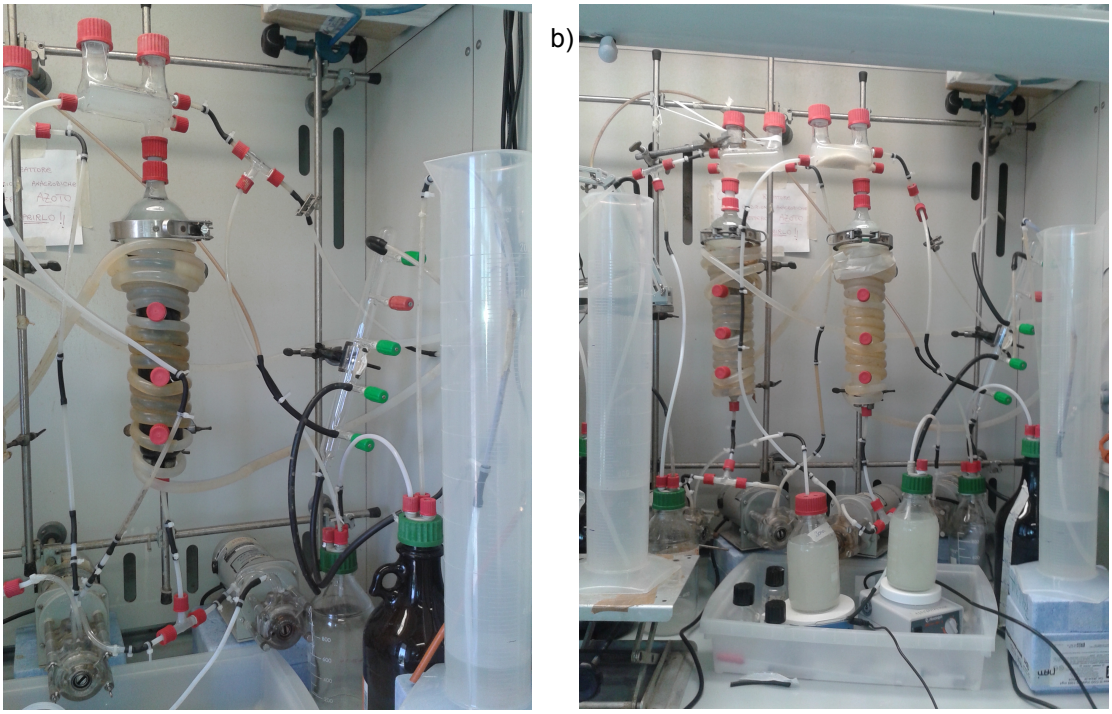

Figure 3.3. Pictures of the three bioreactors. a) PBBR-AC. b) The bioreactor on the left is PBBR-Vuko while on the right is the freely suspended cells bioreactor.

3.3 Analytical Methods

3.3.1 Liquid analysis

The VFAs concentrations were determined by gas chromatography using a GC (Agilent Technologies, Milano, Italy), which is coupled to a Flame Ionization Detector (GC-FID model 7890A) and equipped with a HP-INNOWAX column (length 30 m, diameter 0.250 mm film 0.25 μm) [Bertin et al., 2010]. Injection volume was 1 μl with injector temperature at 250°C; column head pressure 5 psi. The column was initially at 40 °C; 1 min isotherm; temperature rate 25°C/min; final temperature 150°C; 6 min isotherm; temperature rate 4°C/min; final temperature 180°C; temperature rate 25°C/min; final temperature 240°C; detector temperature 280°C). Nitrogen was the gas carrier with a flow rate of 17.6 mL/min. The samples were centrifuged at 14,000 RPM for 10 minutes; supernatant was diluted 2-4 times with oxalic acid solution 60 mM. The acidified mix contains all VFAs in their protonated form therefore these lasts are able to be separated by GC; finally samples were filtered (0.45 μm membrane).

The lactose and lactic acid concentrations were determined by HPLC using IR as detector and a Varian Hi-Plex H 300 x 7.7 mm column. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.6 mL/min and a 65°C operating temperature. The samples were centrifuged at 14,000 RPM for 10 minutes and filtered (0.45 μm membrane).

COD was determined using a Kit Aqua Lytic 420721. Calibration was done with glucose.

Total proteins was determinate - either for the liquid as for the liquid-solid mix - using the modified Lowry method [Lowry et al., 1951; Hartree et al., 1972]. Albumin serum albumin was used as standard. To determine the proteins in the liquid the samples were centrifuged at 14,000 rpm during 10 min. To determine of total proteins in the liquid-solid mix the samples were vortex. The fowling procedure is equal for both liquid and mix. 40 μL of sample were added to 960 μL water and 900 μL of Lowry solution (Appendix Solutions for total protein determination) and incubated 10 min at 50ºC. After that time 100 μL of Solution B (Appendix – Solutions for total protein determination) was add and left 10 min at ambient temperature. 3 mL of Follin solution were added leaving. During 10 min the preparation was left at 50ºC. In a 2 mL cuvvet the absorbance was read using a spectrophotometer (Cary 100 Scan UV-Visible, Varian) at 750 nm.

3.3.2 Biogas analysis

The biogas production was determined, either for microcosms experiments and bioreactors as described by [Bertin et al., 2004; Scoma et al., 2011].

The total gas production determination procedures were different for microcosm and bioreactors. In microcosms biogas volume was measured using a graduated glass syringe of 10, 50 or 100 mL, according with the estimated produced volume. When the biogas is produced, the bottle is in overpressure, when the syringe is inserted, the biogas produced enters inside the syringe until the system enters in equilibrium with atmospheric pressure. The quantity of biogas produced is the result

of the total headspace and the biogas measured with the syringe. For the bioreactors a “Mariotte” system was employed. The head of the bioreactor is connected to a 1 L Pyrex bottle where is collected the outlet. This bottle is the connection between the top of the bioreactor and the “Mariotte”. This system is composed by a bottle of 2.5 L filled with a known quantity of water. Its headspace is connected to the headspace of 1 L bottle by tube. It has also a tube immerse in the liquid phase that is connected to a graduated cylinder partially filled with water. When biogas is produced it will enter to the outlet bottle flowing to 2.5 L bottle headspace due to the increased pressure. This pressure impulses the water inside of the 2.5 L bottle into the cylinder. In this last the water will increase its level being possible read the volume level. The volume of biogas produced in the bioreactor is equal to the water volume transferred to the graduated cylinder, discounting the liquid collected in outlet bottle. After each bioreactor sampling, the system is leaving in equilibrium where the volumes of eater are ate the same level with known values.

Biogas composition in terms of H_2, O_2, CH_4 and CO_2, was measured by gas-chromatography using a µGC, model 3000 A (Agilent Technologies, Milano, Italy) under the following conditions: injector temperature 90°C; column temperature 60°C; sampling time 20 s; injection time 50 ms; column pressure 25 psi; run time is 44 s. and the carrier gas is nitrogen.

Since the microcosms are bottles which can be transported and had a silicone tap, it is possible to measure directly the composition by connecting the µGC to the bottle. This is done by inserting a needle until reach the headspace where 3 mL of gas sample are collected. In the case of the bioreactors, it is not possible to transport them for analysis. Therefore, using a syringe a gas sample of 3 mL was taken from each headspace of the system- three in total- and inserted in different vials of 11 mL. Each vial was cleaned before sampling, procedure to guarantee not having any other gas influence. The vials, closed with a rubber tap, were empty with a vacuum pump and filled with 11 mL of nitrogen using a syringe. After the cleaning and the gas collection, the composition was determined with the µGC.

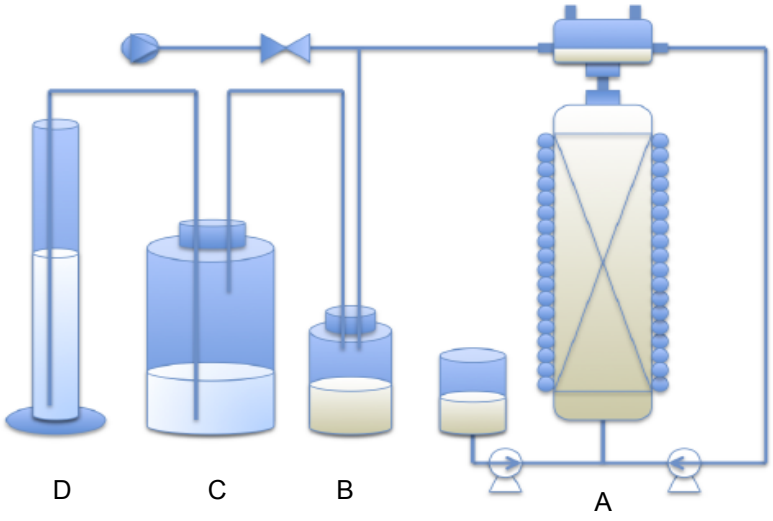

Figure 3.4. Scheme in which is represent a PBBR (A) connected to the outlet 1 L Pyrex bottle (B) which is connected to the “Mariotte” system (group of C and D).

3.3.3 Microbiological features of Vukopor-attached-cells analysis

The DNA isolation from the Vukopor-attached-cells of the microbial community was carried out by using a fixed number of carrier units for each kind of sample. Carrier units were first subjected to enzymatic cell lysis steps, with Lysozyme 160 µg/mL and Proteinase K 100 µg/mL, submerged in 5 mL of Tris–EDTAGlucose pH 8.0 solution for 30 min at 37°C, 120 rpm. Then, 1 mL of SDS 10% (w/v) and 4.5 g of sterilized quartz beads (0.5-1.0 mm diameter) were added, and tubes were secured horizontally on a vortex and mixed at maximum speed for 10 min. After centrifugation for 10 min at 8,000 rpm and recovery of the supernatant, proteins were precipitated with 3 mL of NaCl 5 M and DNA in supernatant was ethanol precipitated over night at -20°C. DNA was then resuspended in 0.7 mL of sterile deionized water and further purified on spin filter columns provided by the NucleoSpin® Soil (MACHEREY-NAGEL, Germany) kit for Genomic DNA from soil, according to the manufacturer's protocol.

DNA quality was checked on 1.0% (w/v) agarose gel stained with Ethidium bromide 0.5 µg/ml.

For Bacterial DGGE analysis, PCR amplification of one region of the genes coding for the 16S rRNA was performed with primers

GC-357f(5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTACGGGAGGCAGCAG-3') and 907r (5'-CCGTCAATTCCTTTGAGTTT-3') in 50 µL reaction mixtures containing 1× PCR buffer (Invitrogen, Paisley, UK), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 mM each primer, 1.0 U of Taq polymerase (Invitrogen, Paisley, UK) and 2 µL of template DNA. The reaction began with an initial 95°C denaturation for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 min and a final extension at 72°C for 7 min.

PCR products were resolved with a D-Code apparatus (Bio-Rad, Milan, Italy) on a 7% (w/v) polyacrylamide gel (acrylamide-N,N_-methylenebisacrylamide, 37:1) in 1 × TAE with a denaturing gradient from 40% to 60% denaturant, where 100% denaturant is 7 mol/L urea and 40% (v/v) formamide. The electrophoresis was run at 55 V for 16h at 60°C. The gel was stained in a solution of 1 × SYBR-Green (Sigma Aldrich, Milwaukee, WI) in 1 × TAE for 30 min and its image captured in UV transillumination with a digital camera supported by a Gel Doc apparatus (Bio-Rad, Milan, Italy). Bands with the highest intensities, together with those characteristic of a specific DGGE profile, were cut from the gel with a sterile scalpel and DNA was eluted in 50 µL of sterile deionized water at 4°C for 16 h. 4 µL of the solution were then used as template to re-amplify the band fragments using the same primers without the GC-clamp and the same PCR conditions described above.

4 Results

4.1 Batch trials at microcosm scale

Four experiments at microcosm scale were carried out in order to perform a first production study and therefore be able to define the process parameters for the production of VFAs at bioreactor scale.

4.1.1 Freely suspended cells study

Freely suspended cells systems were studied in order to gain the knowledge about the bioconversion trends, useful as “control experiment”. Two sequential batch experiments were performed. Both batches were carried out till achieving the VFAs concentrations *plateau*, allowing observing all expected bioconversions: (a) lactose to lactic acid, (b) and lactic acid to VFAs.

With respect to the first batch, results are shown in Figure 4.1 a). The conversion of lactose in lactic acid occurred in 7 days, with a yield (C-mol lactic acid/C-mol lactose) of 90%. It was also observed that until that day no biogas was produced. Biogas production started simultaneously with VFAs production. As soon as the lactic acid was completely consumed and VFAs started to achieve a constant concentration, also the biogas production decreased. At day 16, lactic acid was finished and a VFAs yield (C-mol/C-mol) of 81% was achieved, corresponding to a 9.0 ± 0.1 g/L. Biogas was composed by CO_2 and H_2 (Figure 4.1 b)) and achieved the maximum of 513 ± 94 mL/L of CO_2 and 539 ± 115 mL/L of H_2 at days 12 and 14, respectively.

The second batch experiment was performed to see if process rates could be improved by biomass accumulation and/or acclimation. This time butyric and acetic acids started to be produced during lactose consumption, this leading to a lower lactic acid maximum concentration and therefore yield (C-mol/C-mol) 62%. It was obtained 10.2 ± 0.2 g/L of total VFAs. The VFAs mixture was mainly composed by (in g/L): acetic (2.5 ± 0.2), propionic (2.3) and butyric (4.2 ± 0.2) acids with a yield of 82% (Table 4.3). With respect to biogas production, it was observed a peak of CO_2 (749 ± 208 mL/L), followed by a H_2 production (496 ± 71 mL/L) in the second days of batch, No CH_4 was detected during all experiment.

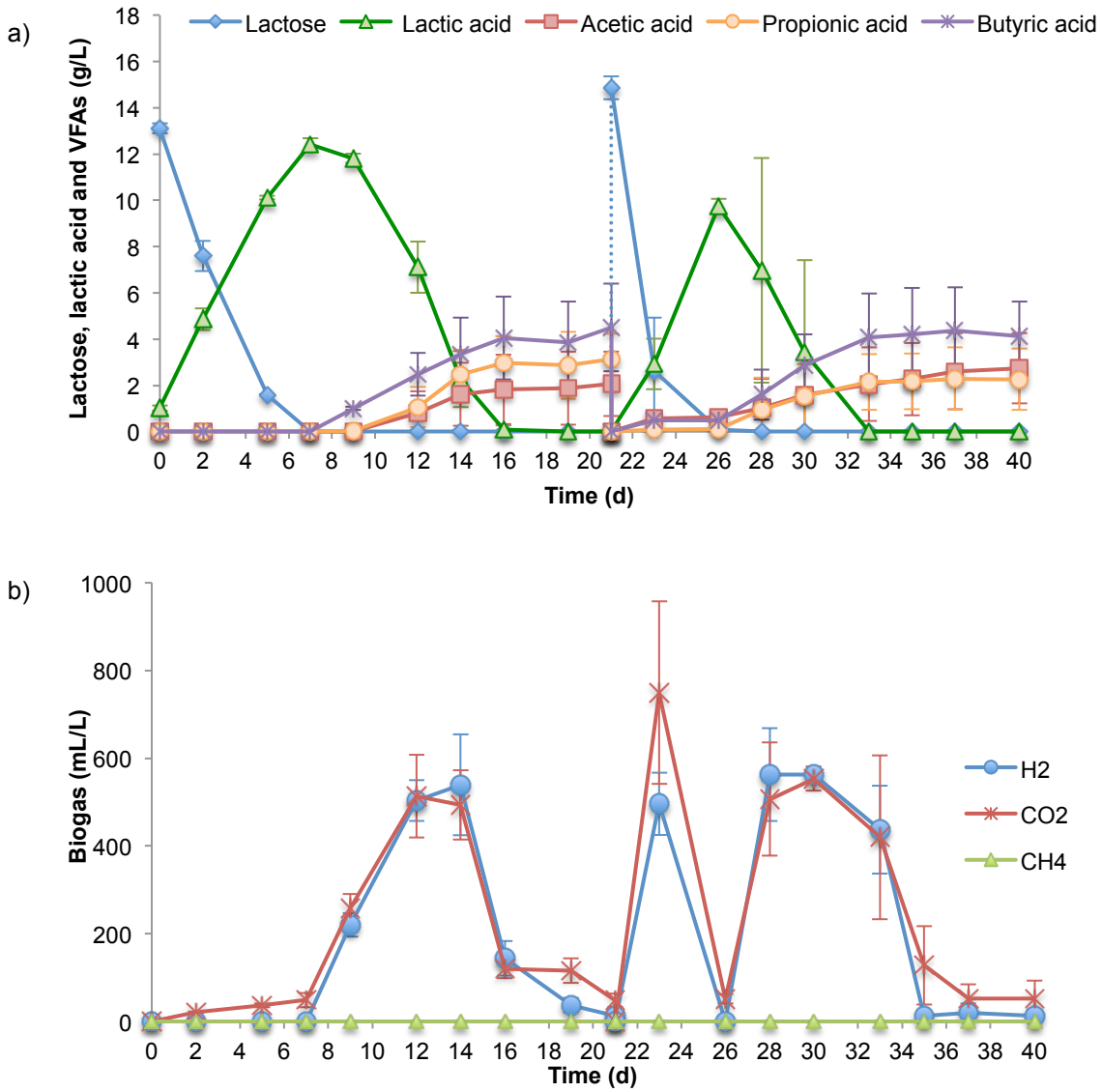

Figure 4.1. Concentration profiles of main monitored compounds during microcosm experiment with freely suspended cells a) Concentration of lactic acid and VFAs dissolved in liquid phase as a function of time. b) Biogas concentration as a function of time.

4.1.2 Immobilization supports study

Two different supports were tested at microcosm scale for the production of VFAs with immobilization, namely: Vukopor S10 and Granular Activated Carbon. The packed and working volumes were the same for both in order to be able to compare results between the immobilizing materials.

4.1.2.1 Experiment 2 - Preliminary Immobilization supports test

This was the first trial with support material in a small scale and it was carried out, for both support materials, along 98 days. The aim was to have a first approach with immobilized material and understand the impact that each material has in the metabolism.

No data is shown until day 28, which corresponds to the first batch time. The first 5 days only biogas was being monitored. During 19 days the microcosms remained at incubation conditions without being sampled, pH correction or even opened. In order to start new sequential batches, and so doing the adequate sampling for liquid and gas and pH correction (described in 3.2.1) at day 28 of the experiment, all the culture media was renewed, continue using the same supports with already grown biofilm.

Regarding the ceramic material, Figure 4.2 shows all batches performed during this experiment. A total of eight batches were carried out: six for biofilm formation and two for VFAs accumulation. Metabolites were not followed during the first batch, even though it was possible to see that at the end of this batch, which lasted 28 days, no VFAs were detected. All lactose was converted into lactic acid, which was not converted in any other metabolite; the pH was 4.

During the second batch, that lasted 7 days, it was possible to follow the consumption of lactose and production of lactic acid; the batch finished when lactose was depleted. After that, the third batch was started and ran for 17 days. During this batch, lactic acid was produced and after seven days it was started to be consumed, and VFAs started to be produced. The increasing concentration of VFAs followed lactic acid consumption. After 14 days of batch, which corresponds to day 49 in Figure 4.2, lactic acid was completely converted into a mixture of VFAs, mainly composed by acetic acid (1.6 ± 0.2 g/L), propionic acid (3.2 g/L) and butyric acid (4.8 g/L); the bioconversion yield (C-mol VFAs/C-mol lactose) was 84.6%.

For the next 25 days, between day 52 and 77, four sequential batch experiments were carried out with the aim of increasing the biofilm. The batches were stopped when lactose was depleted.

On day 77 the last batch (8^{th}) was began, corresponding to the second VFAs accumulation batch. During 21 days, between day 77 and 98, the process behaviour was similar as already happened in the previous VFAs accumulation batch: lactose consumption as lactic acid formation following by the VFAs production. However, some difference was observed for each phase length, which was shortened so that all the process concluded in 9 days. From this day until the end, the VFAs mix composition was stable along the time. VFAs concentration were: acetic acid 2.1 ± 0.2 g/L, propionic acid 2.3 ± 0.1 g/L and butyric acid 6.1 ± 0.1 g/L. The conversion yield (C-mol VFAs/C-mol lactose) was 88.8 ± 2.4 % (Table 4.3).

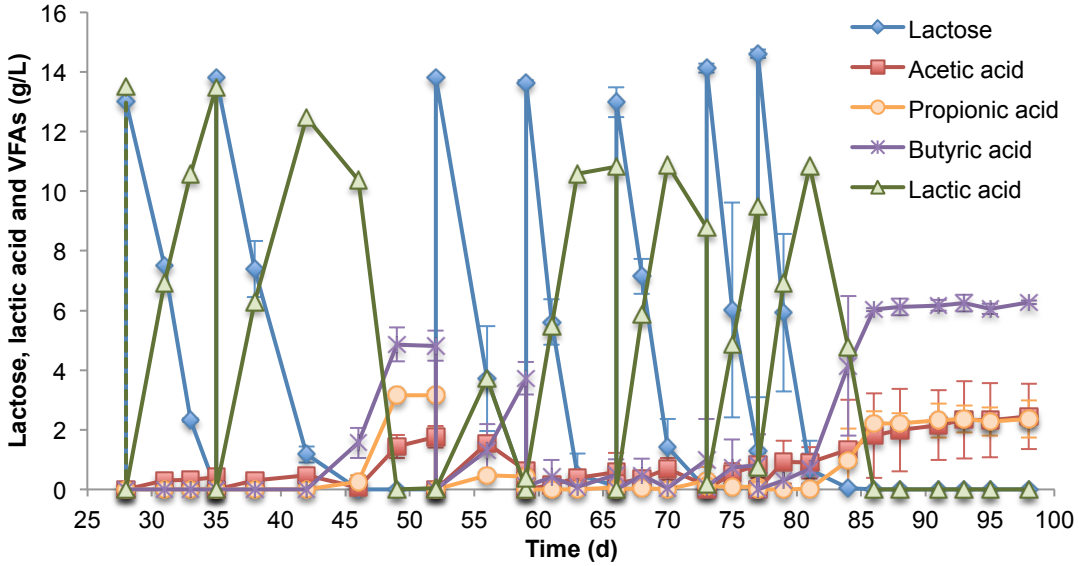

Figure 4.2. Concentration profiles of main monitored compounds during experiment 2 with Vukopor in which was performed eight sequential batches were performed: 2nd batch – days 28 - 35; 3rd batch – days 35 - 52; 4th batch – days 52 - 59; 5th batch – days 59 - 66; 6th batch – days 66 - 73; 7th batch – days 73 - 77; 8th batch – days 77 - 98.

In which respect to the granular activated carbon material (Figure 4.3), it was difficult to control the strategy for biofilm formation: a batch that lasted till lactose depletion. When activated carbon was used as immobilized support, lactose consumption was not possible to be followed along the experiment time. It was observed that 2-3 days after renewing the culture media – corresponding with the lactose addition – no lactose was detected.

Beyond the lactose at the beginning of each batch, acetic, propionic and butyric acids were the only metabolites detected along the experiment.

Acetic acid was detected along all experiment in amounts greater than 1 g/L, even during the batches dedicated for biofilm development. Butyric acid was also produced during the last batches for biofilm formation but in quantities below 1 g/L. For the last batch 2.6 g/L of butyric acid were detected after 19 days of bioconversion.

The yields obtained for both VFAs accumulation batches were in the order of 12% in the first with the only contribute of acetic acid, and 43% at the end of the last batch. In this last, besides acetic acid, butyric and propionic acids have been produced.

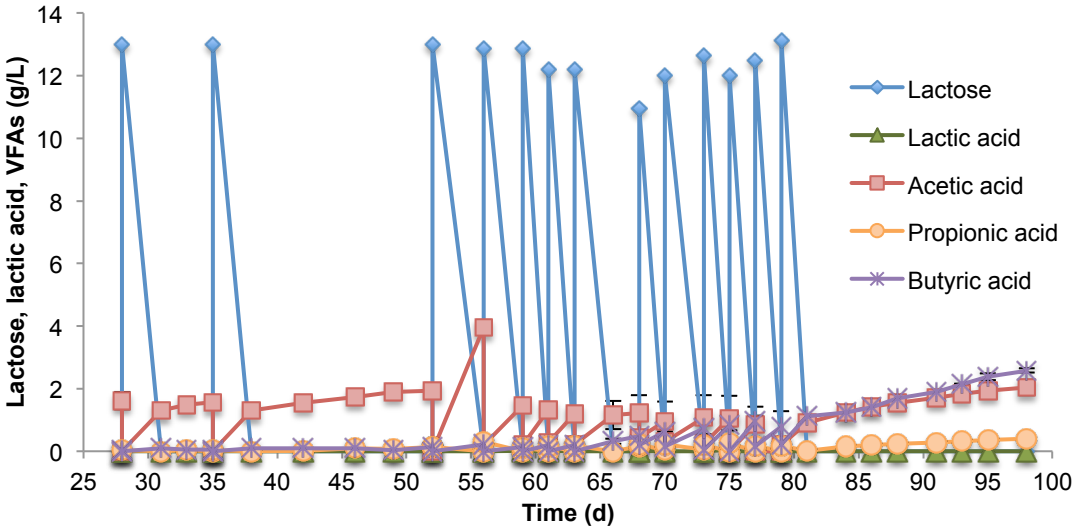

Figure 4.3. Concentration profiles of main monitored compounds during experiment 2 with AC in which was performed fourteen sequential batches.

4.1.2.2 Experiment 3 - Improved immobilization support test

This experiment was decided to perform more microcosm experiment in parallel with the characteristics described in 3.2.1.2.2, in order to confirm the previous results obtained and select the adequate HRT for the bioreactors. An essential information taken from the previous one was the necessity of sequential batches for biofilm formations and also that the VFAs production occurs after the lactose depletion. Consequently, this trial is composed of sequential batches, for biofilm formation, and thereafter a last batch for VFAs accumulation.

In the case of Vukopor S10, during the first 18 days, three sequential batches – for biofilm formation – were performed in which the consortium was only consuming the lactose for lactic acid production. Therefore, each batch lasted 6 days, at which the 14 g/L of lactose were converted into lactic acid with a yield (C-mol lactic acid/C-mol lactose) of 91%. The final batch, which began on day 19, was followed till VFAs concentration profiles arrived to the plateau. This time it was markedly observed the lactose conversion in lactic acid and thereafter VFAs production started.

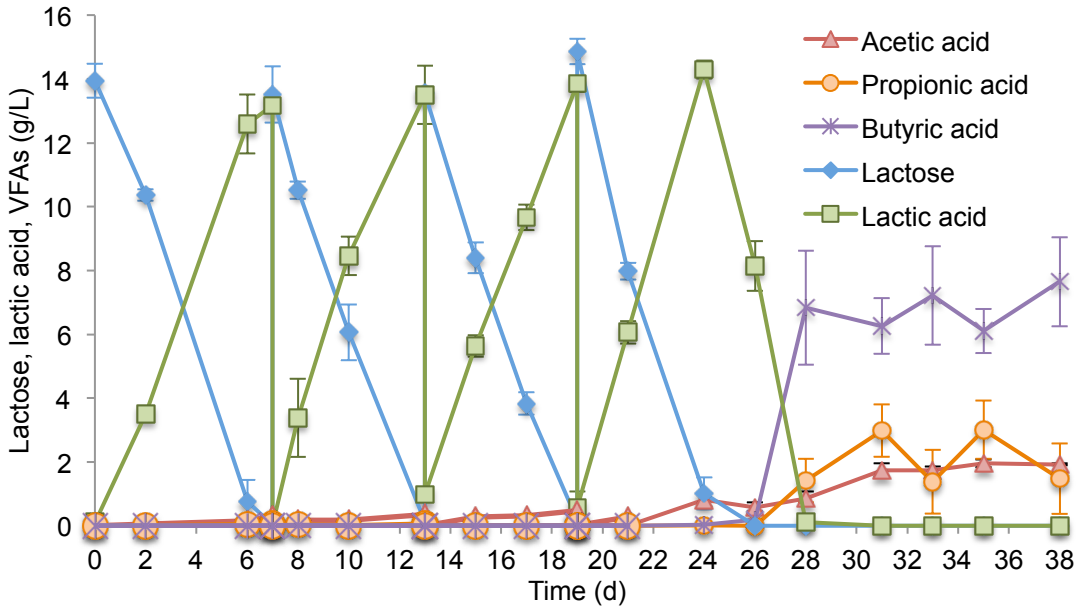

Figure 4.4. Concentration profiles of main monitored compounds during experiment 3 with Vukopor. Four sequential batches were done: three for biofilm formation and one last for VFAs accumulation.

In Figure 4.5 is represented only the VFAs accumulation batch experiment with its corresponding time. It was observed the typical behaviour, which respects to conversion of lactose in lactic acid. When lactic acid achieved the maximum production, VFAs started to be produced; therefore, their concentration increased till lactic acid was depleted. After 9 days, time that lactic acid was exhausted, the principal VFAs produced were, acetic, propionic and butyric acid with, with a VFAs yield (C-mol VFAs/C-mol lactose) of 77%. However the yield increased to 87 ± 7% after 12 days when acetic acid concentration increased, this corresponded to 11 g/L of total VFAs. The concentrations obtained (in g/L) were: acetic, 1.6 ± 0.5, propionic, 2.4 ± 0.8 and butyric, 6.6 ± 0.5 acids (Table 4.3).

In which respects to biogas production, during the first three sequential batches dedicated to biofilm formation, only CO_2 production was detected, with 52.2 ± 2.2 mL/L. A significant biogas production was observed during the VFAs accumulation batch, represented in Figure 4.5 b). Biogas production followed the conversion of lactic acid into VFAs, achieving its maximum at day 9 with 635 ± 51 and 535 ± 77 mL/L of CO_2 and H_2 respectively.

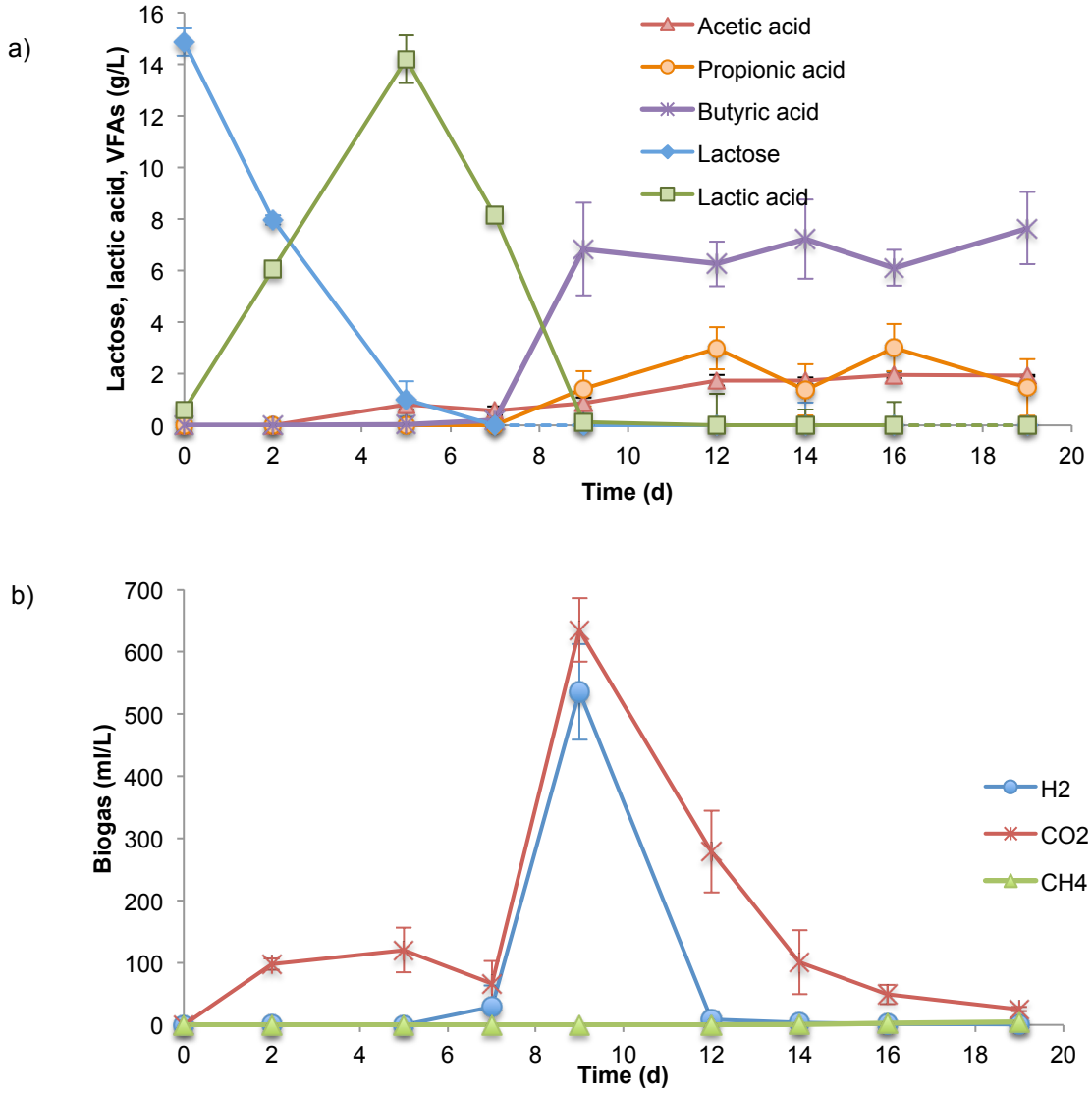

Figure 4.5: Concentration profiles of main monitored compounds during VFAs accumulation batch in experiment 3 with Vukopor as support material. a) Concentration of lactic acid and VFAs dissolved in liquid phase as a function of time. b) Biogas concentration as a function of time.

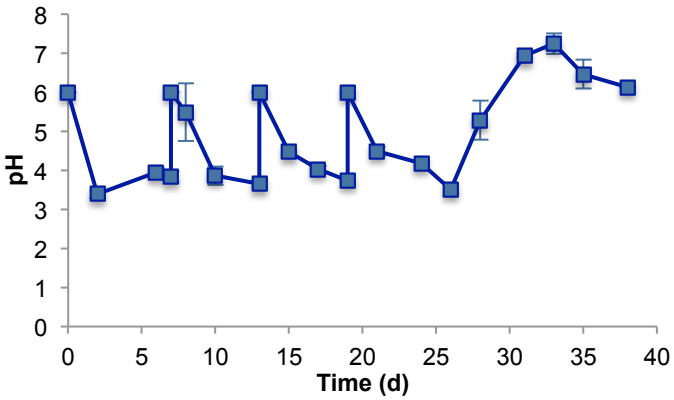

Figure 4.6. pH values obtained during experiment 3. Each sampling day pH value was correct to 6 as also in the beginning of each batch.

Also microcosm experiment packed with AC was repeated. The first seven batches were performed with the purpose of biofilm formation lasting till lactose depletion, corresponding to 2-3 days. Once more it was not possible to detect lactic acid production.

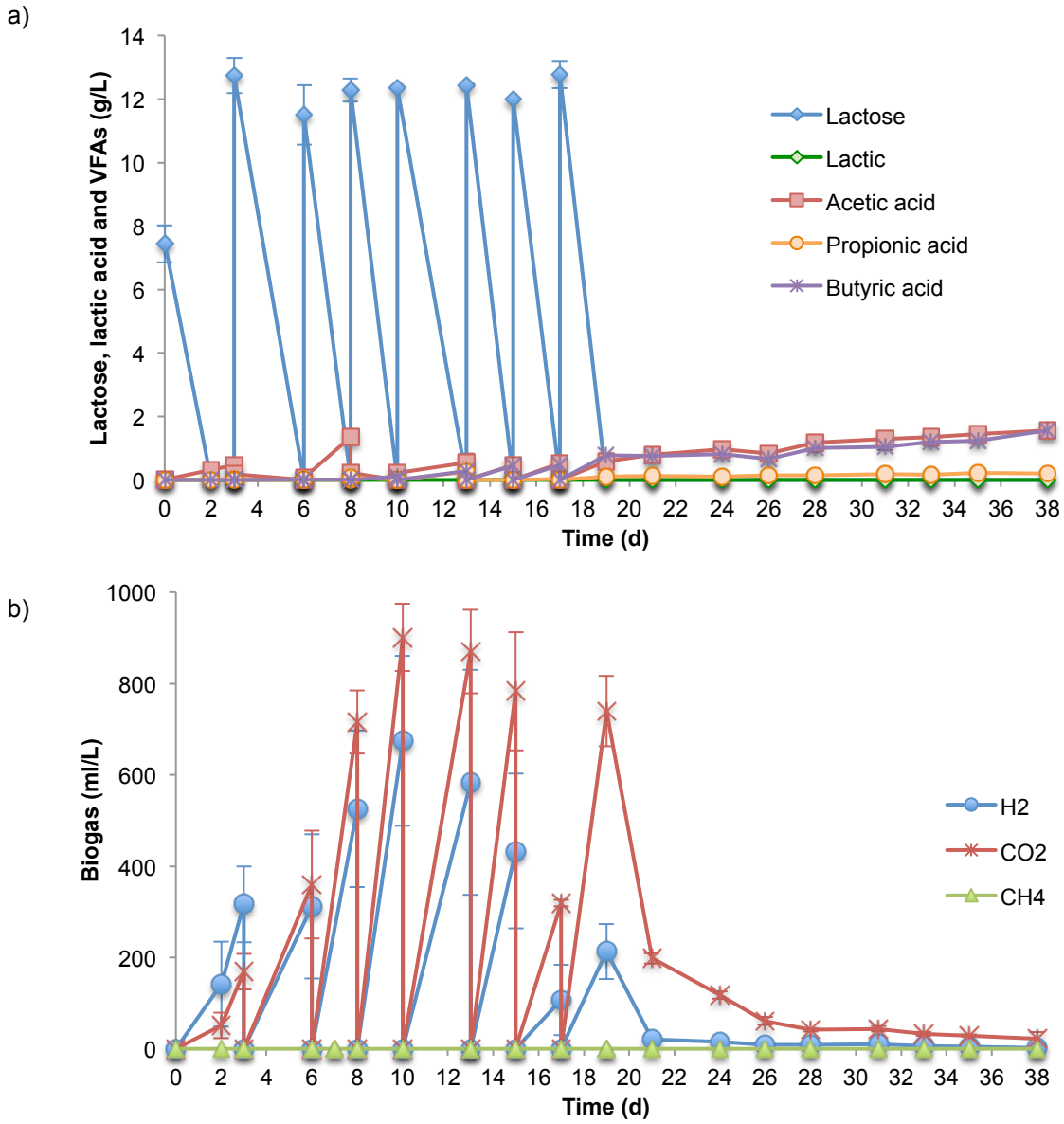

Figure 4.7. Concentration profiles of main monitored compounds during experiment 3 with AC. a) Concentration of lactose and main VFAs dissolved in liquid phase as a function of time. b) Biogas concentration as a function of time.

The last batch was dedicated to VFAs production. The final total VFAs concentration was 3.3 g/L and the VFAs yield (C-mol VFAs/C-mol lactose) was 29%. The VFAs profiles are presented in Figure 4.7 are represented the profile of VFAs which compose the VFAs mixture: acetic (1.11 ± 0.04 g/L), propionic (0.16 ± 0.02 g/L) and butyric (1.00 ± 0.05 g/L) acids.

In which respects to biogas production, hydrogen was produced even at the batches destinated to biofilm formation.

4.1.3 Experiment 4 - Batch production for consortia characterization

The aim of the experiment was to perform a molecular characterization of the consortium to identify the responsible of the single metabolic activities. It was a reproduction of the previous microcosm experiments with the ceramic support but carried out in 1L Pyrex bottle in order to have enough liquid and ceramic supports for sampling. The packed volume-working volume ratio was the same as in the microcosm experiments. It was also performed with three sequential batches for the biofilm formation and the last batch for the VFAs accumulation. It was obtained a yield (C-mol lactic acid/ C-mol lactose) of 93% in the three first sequential batches. During the VFAs accumulation batch (see Figure 4.8), when the sampling for molecular analyses was made, the principal VFAs produced were acetic, propionic and butyric acids with a yield of 82%, at day 11 of batch.

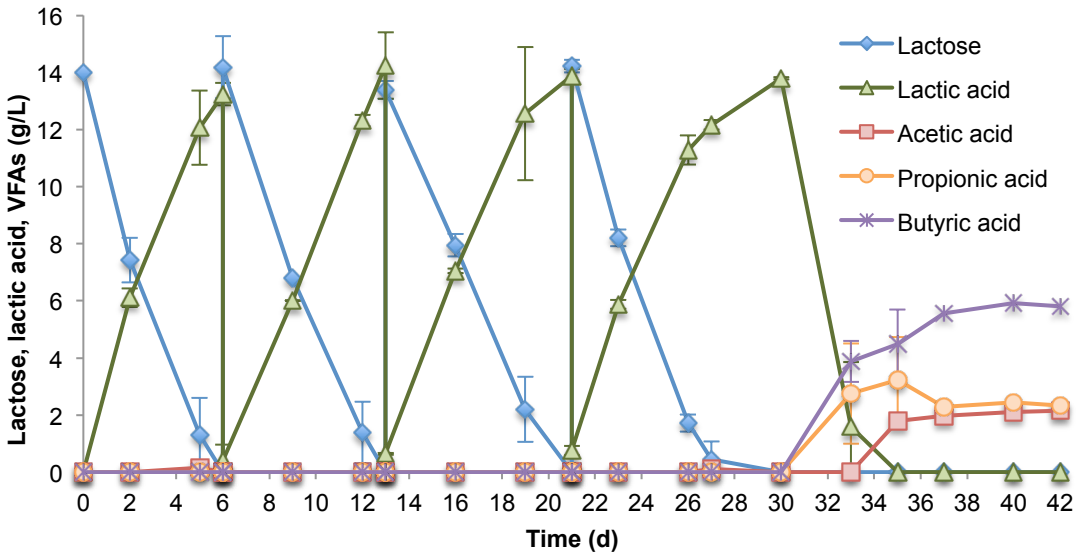

Figure 4.8. Concentration profiles of main monitored compounds during experiment 4 in which four batched were performed: three destinated to biofilm formation and one for VFAs accumulation. During this last sampling was performed for molecular analyses (days 21, 27, 33 and 37).

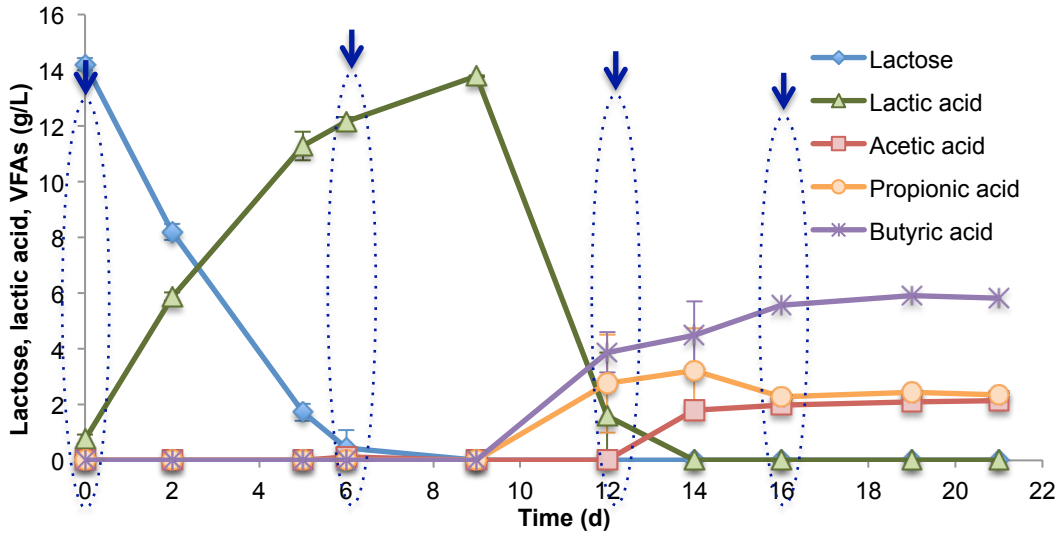

Figure 4.9. Concentration profiles of main monitored compounds during VFAs accumulation batch. Molecular analyses sampling was performed in days that are marked with an arrow and circle blue. Day 0 – biofilm formed during the previous three batches; day 6 – lactose exhausted; day 12 – lactic acid consumed with VFAs production; day 16 – VFAs production in steady state.

In which respect to biogas production, it was observed a typical trend with no significant biogas production during the first three sequential batches. At day 33 of experiment it was observed the maximum production of CO_2 and H_2 with values of 793 and 271 mL/L respectively. This occurred at the same moment as lactic acid was being converted into VFAs. No CH_4 was detected during all experiment.

Below, in Figure 4.10 and Table 4.1 are represented the results obtained after microbiological analyses of Vukopor-attached-cells samples. In DGGE picture is represented the profile at different times of the acidogenic process, days 0, 6, 12 and 16. Day 0 corresponds to the microbial community that compose the biofilm formed during the last three sequential batches. Between day 0 and 6, of batch, was not expected to have a significant difference in DGGE profile. The bioreaction occurred in these days was the same as in the previous three batches. Day 12 was the first VFAs production in this experiment because of that was expected to observe some differences, respecting to the previous two sampling days. Analysing the obtained profiles it can be seen some different layers along the time. The bands obtained in DGGE analysis were cut, the corresponded DNA was amplified with PCR and another DGGE will be carried. Finally the obtained bands are cut and sequentialized.

Values from dice coefficient are the result of similarity between the bands obtained for each sampling. The obtained values shows changes in the biofilm composition since the similarity decreases along the experiment time.

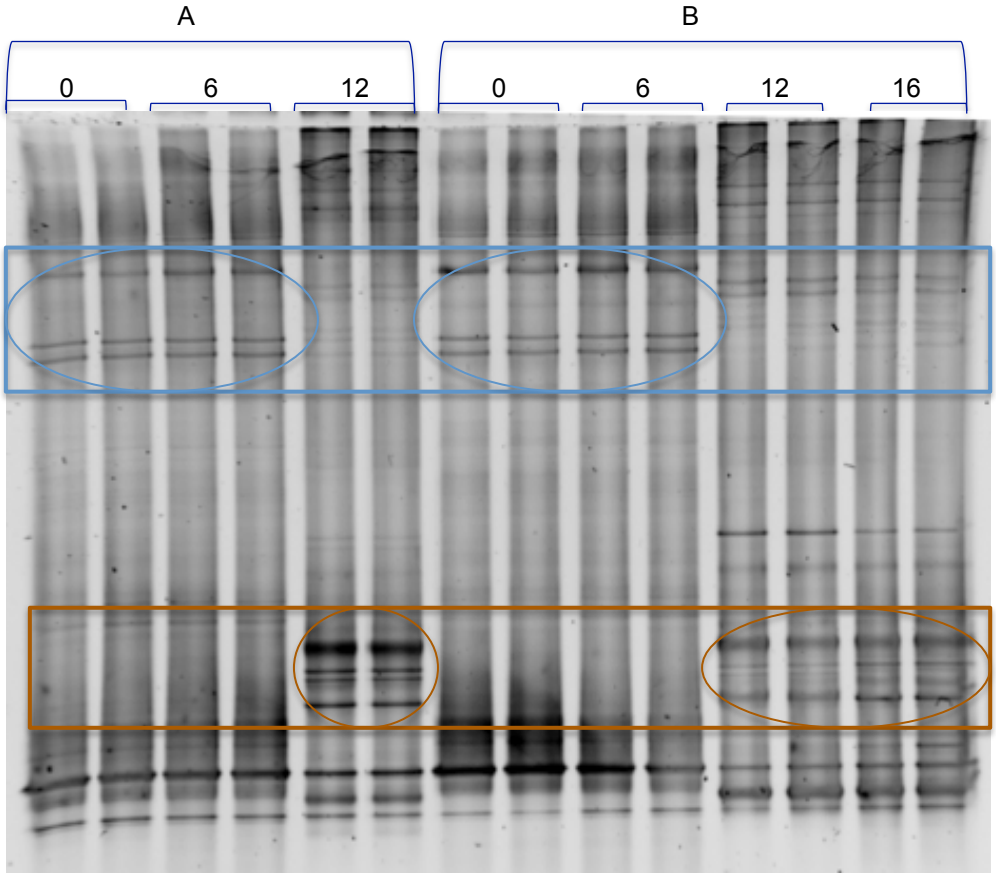

Figure 4.10. DGGE analysis of the bacterial communities that compose the biofilm attached to the ceramic material at different time in experiment 4. The analysis of the biofilm community was performed independently on duplicated samples. The letter (A or B) corresponds to the bottle and the number (0, 6, 12 and 16) corresponds to respective sampling day.

Table 4.1. Dice coefficient obtained from previous DGGE profile

	A0	A6	A12	B0	B6	B12	B16
A0	100.0	77.9	16.8	52.8	64.2	34.6	23.6
A6	77.9	100.0	23.9	57.3	71.5	43.2	32.2
A12	16.8	23.9	100.0	26.8	14.4	64.1	71.9
B0	52.8	57.3	26.8	100.0	68.1	41.1	32.0
B6	64.2	71.5	14.4	68.1	100.0	28.4	21.2
B12	34.6	43.2	64.1	41.1	38.4	100.0	75.0
B16	23.6	32.2	71.9	32.0	21.2	75.0	100.0

4.1.4 Experiment 5 - VFAs production with different initial substrate concentration

With this preliminary experiment it is intended to detect if the organic loading influence on the final VFAs composition. Until this experiment the quantity of lactose feeded was 15 g/L, here 22 g/L were tested.

In the first batch, lactose consumption took almost 8 days. During this time no biogas was produced, only lacti acid was detected which achieved the maximum concentration after 9 days with a yield (C-mol lacti acid/C-mol lactose) of 86%. It was verified a considerable biogas production composed by CO_2 (1461 ± 193 mL/L) and H_2 (1564 ± 42 mL/L) at day 12, this match with production of VFAs, specialy butyric acid. When VFAs concentrations achieved the *plateau*, tha mixture was composed essentially by butyric (8.7 ± 0.4 g/L) , acetic (1.0 ± 0.2 g/L) and propionic (2.5 ± 0.2 g/L) acids with a yield of 83% (Table 4.3).

In the second batch the lactose consumption and lactic acid production took seven days, achieving a yield (C-mol lactic acid/C-mol lactose) of 85%. It was observed a small production of VFAs at second day of batch, acetic acid 0.6 g/L and butyric acid 0.8 g/L. At day 14 it was achieved a yield (C-mol VFAs/C-mol lactose) of 86% (Table 4.3). Biogas production was verified two times: at day two with the small VFAs production and at day 12 with allying to a significant quantity of VFAs production.

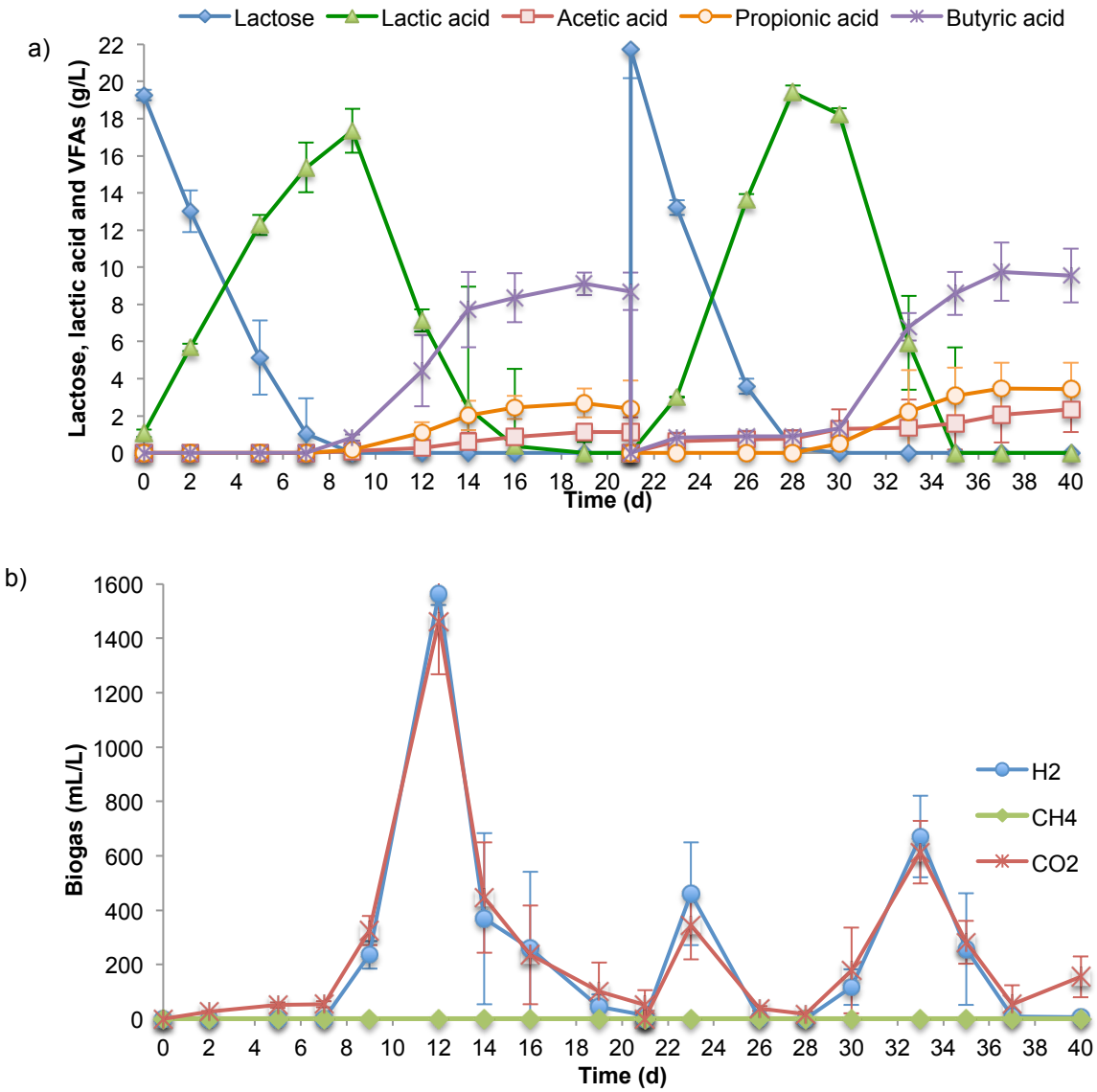

Figure 4.11. Concentration profiles of main monitored compounds during experiment 5 with freely suspended cells a) Concentration of lactose, lactic acid and main VFAs dissolved in liquid phase as a function of time. b) Biogas concentration as a function of time.

4.2 Bioreactors

After the microcosms study with the immobilizing material and freely suspended cells, three bioreactors were started.

4.2.1 PBBR-Vuko

After the microcosms study with the immobilizing material and freely suspended cells, three bioreactors were started with the purpose of studying the VFAs production at bench top scale.

For start-up of the PBBR-Vuko, on batch was performed till VFAs accumulation, this corresponded to 14 days. In the first six days, the acidogenic mixed culture converted the lactose into lactic acid, and during the next seven days transformed this last into the different VFAs. Due to a liquid loss, after the sampling on the third day, a solution of CWP was added in order to replace the lost liquid trying avoid influence in the system. From the Figure 4.12 is notice that the time for lactose conversion was not affected. However the maximum of lactic acid achieved was superior to the expected from previous experiment, corresponding to 17.4 g/L of lactic acid. Nevertheless, the yield (C-mol lactic acid/C-mol lactose) obtained was 93%. At batch end, and considering the total lactose which had entered in the system, it was obtained a yield (C-mol VFAs/C-mol lactose) of 84% corresponding to concentrations (g/L): acetic acid, 2.5; butyric acid, 3.8 and propionic acid, 5.9.

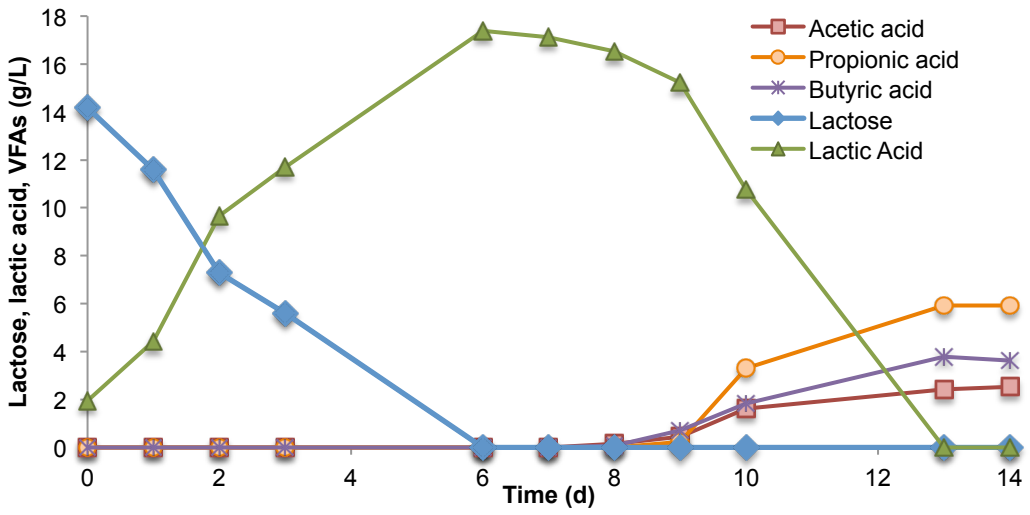

Figure 4.12. Concentration profiles of main monitored compounds during the first batch performed in PBBR-Vuko.

After 14 days the VFAs concentrations reached the plateau and the continuous operation - with HRT of 9 days - was started. It was operated at this condition for 38 days, shown in Figure 4.13. The VFAs concentration trends had some fluctuations during this time

During the first 10 days of continuous operation, VFAs mixture composition changed completely, acetic acid increased from 2.5 g/L to 5.7 g/L; propionic acid, that was the most concentrated at the end of the batch, decreased from 6 g/L to 1.3 g/L in 9 days of operation reaching 0.8 g/L at day 24. Butyric acid, maintained proximally the same concentration.

After those days of clearly instability, during the next 14 days (from day 10 to day 24), the VFAs yield (C-mol VFAs/C-mol lactose) was almost constant at 79 % (Table 4.3).

From day 24 till day 38, the VFAs profile changed once again and butyric acid started to be produce in higher amounts; achieving 5 g/L. At the same time acetic acid concentration decreased till 3.7 g/L (at day 38). Which respects to propionic acid, it continued decreasing till day 34; reaching the asymptotic value of 0.4 g/L.

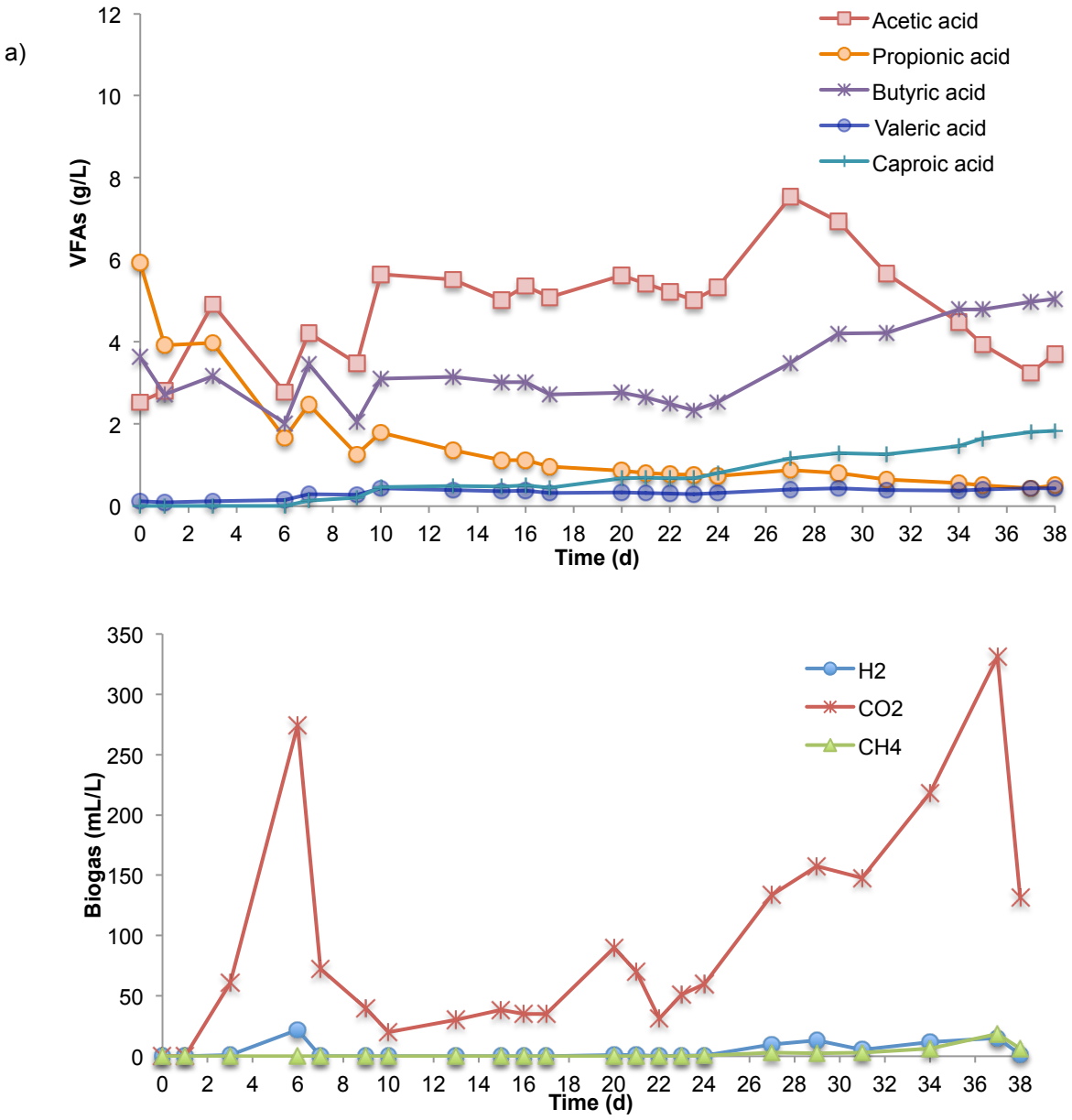

Figure 4.13. VFAs profile obtained during PBBR-Vuko operation with HRT 9 days.

In order to try to recover the production of propionic acid in high quantity, as happened during the first batch, it was decided to interrupted the continuous operation and repeat the batch. This time, two sequential batches were made instead of one; trends are represented in Figure 4.14. Altogether, these were the second and third batch performed in this PBBR.

The second batch was started with a re-inoculation. In this way the biomass was composed by the inoculum added plus the possible biofilm already formed during the previous 52 days that the PBBR was operated (batch and continuous at HRT of 9 days). The objective was to increase the biofilm content. The lactose was consumed in 4 days, time that lactic acid achieved its maximum with a yield (C-mol lactic acid/C-mol lactose) of 92%. Thereafter, it was consumed in two days for the production of VFAs, corresponding to sixth day of batch. Between days 7 and 10 the yield (C-mol VFAs/C-mol

lactose) was 79% and the three principal VFAs, acetic, propionic, butyric, had the same concentration, approximately 3.5 g/L. Since the production of propionic was recovered, once again, another batch was made. The aim was to detect if the propionic acid was produced by the cells that forms the biofilm or by freely cells present because of inoculation. For this, contrary with what was done for the previous batch, no inoculum was added, only the CWP culture media. It means that during this batch only the cells that previous formed the biofilm will participate in the bioconversions.

In this batch (the 3rd batch) lactose was consumed in less than three days but the yield (C-mol lactic acid/C-mol lactose) was only 42%. This time, VFAs production started from the beginning, achieving a yield (C-mol VFAs/C-mol lactose) of 80% in seven days. –The final VFAs concentrations were (g/L): acetic acid (1.7), propionic acid (0.8) and butyric acid (6.3 g/L).

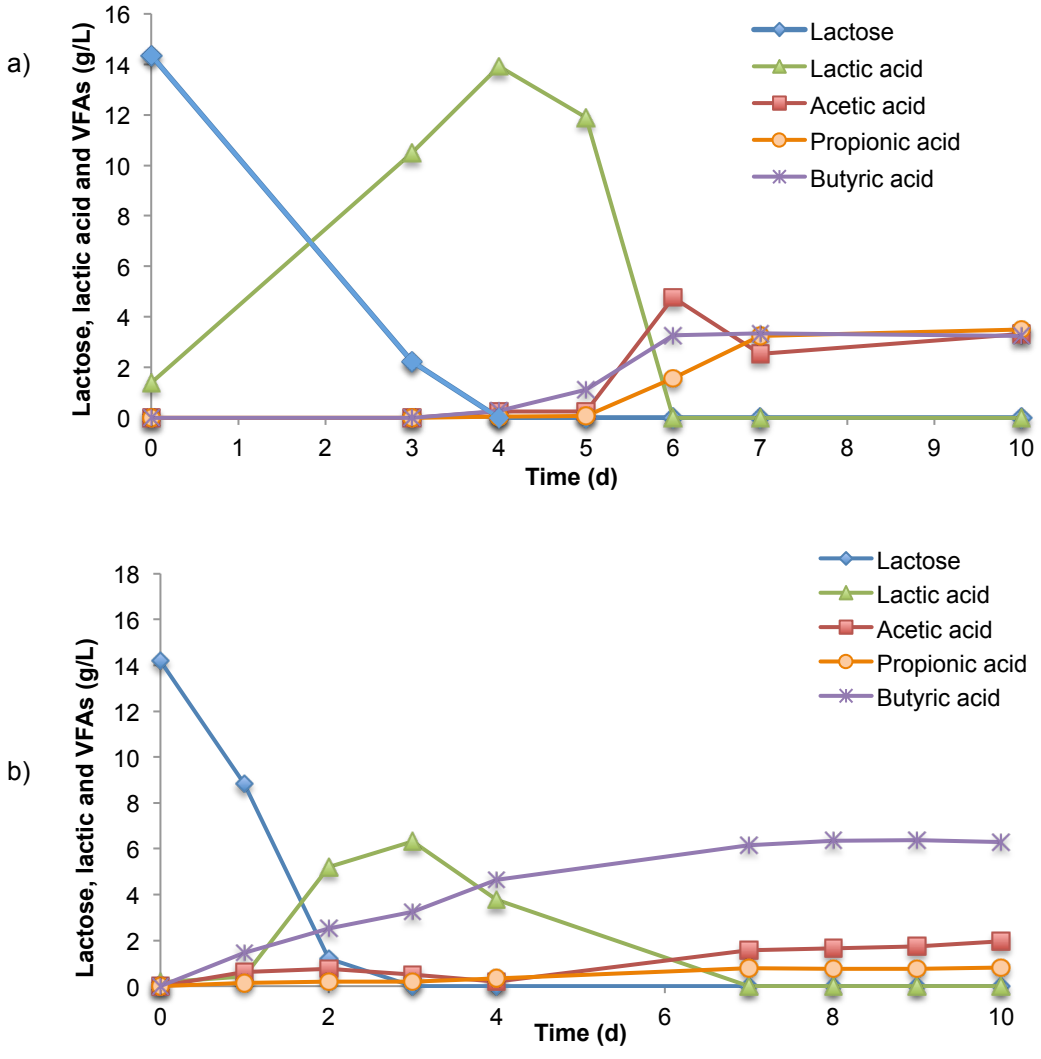

Figure 4.14. Concentration of lactose, lactic acid and mainly VFAs during 10 days of a) 2nd and b) 3rd batches performed in PBBR-Vuko.

After the third batch the PBBR-Vuko was operated in continuous but this time with a HRT of 6 days, since the 2nd and 3rd batches arrived to the maximum VFAs concentration after 6 days.

It can be said that the transition period lasted till day 27, where the bioreactor showed instability and lactic acid was detected at days 6 (0.8 g/L), 13 (4 g/L) and 22 (2 g/L). A rising in the concentration of caproic acid was observed from the beginning, arriving to 1.1 g/L on the day 11. Then it decreased slowly till 0.5 g/L on day 25. The VFAs yield had variations between 51% (day 13) and 80% (day 27). Butyric acid has the highest concentration along the whole experiment.

From day 27 till 41 the profile seems to start to stabilize, VFAs concentrations varies in 1g/L for the butyric acid and as much as 0.3 g/L for the others VFAs.

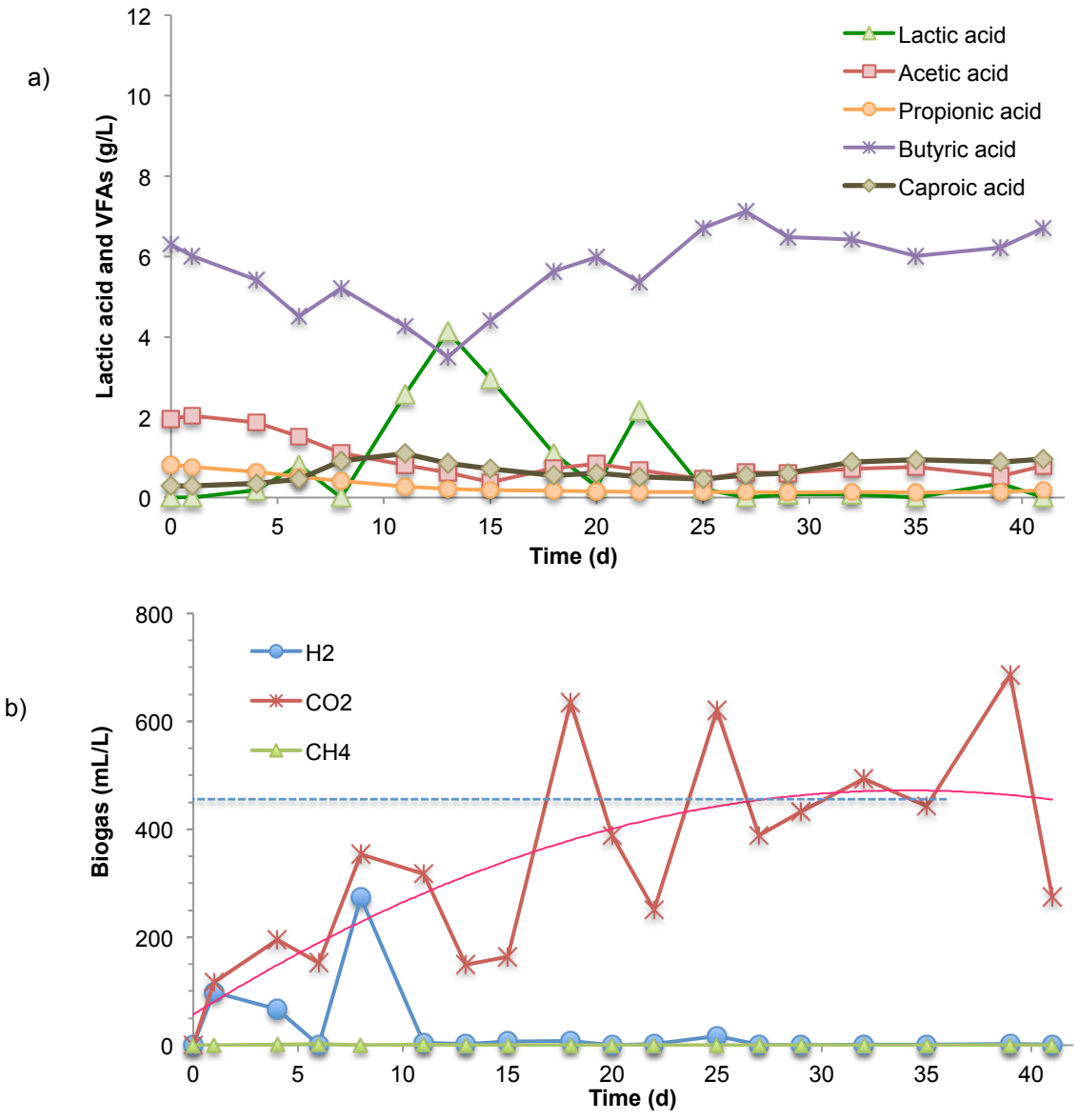

Figure 4.15. Concentration profiles of main monitored compounds during PBBR-Vuko operation with HRT 6 days. a) Concentration of lactic acid and VFAs dissolved in liquid phase as a function of time. b) Biogas concentration as a function of time.

In order to perform a general material balance, COD analysis was performed for the inlet stream (the CWP culture media) and for the outlet stream.

The inlet stream can be described as a yellowish solution with suspended particles. From this the COD analysis was done for the supernatant and for the complete solution, in order to identified soluble and suspended COD. The same rasoning for the outlet stream, which in composed by the suspended cells and the metabolites.

The results of the analysis for part of the continuous operation (the last of the transition state and the continuous state) is shown in Figure 4.16. It can be seen that the inlet stream contain all its COD in solution, since the supernatant and the mix analysis gave the same COD value (21.5 ± 0.1 gO_2/L).

In which respect to the outlet stream, the mix COD average was 20.51 ± 1.1 gO_2/L and the supernatant COD average value was 16.72 ± 0.57 gO_2/L.

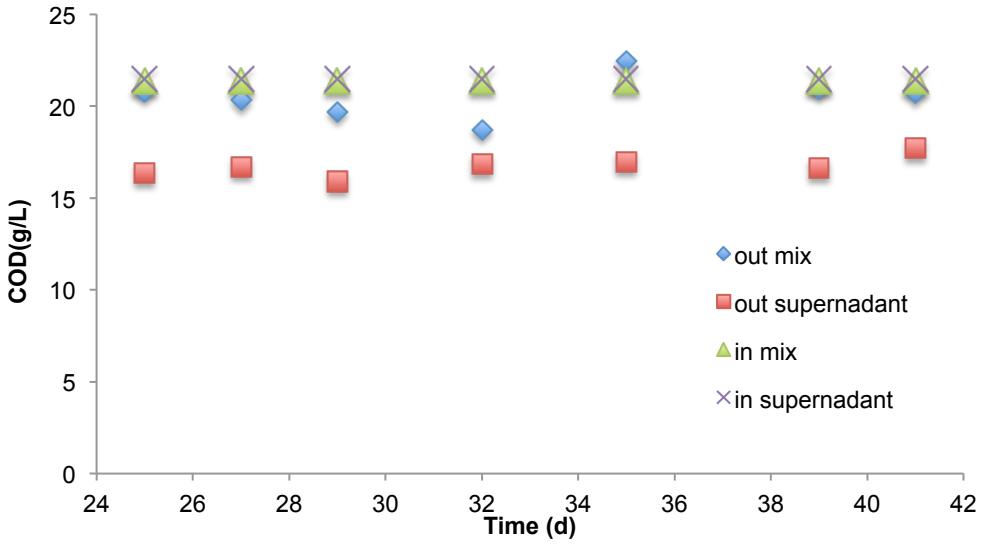

Figure 4.16. Overall COD values CWP solution (in) and outlet (out) in PBBR-Vuko as a function of time during steady state when operated with HRT of 6 days. It was measure in the mixture (mix) and in the liquid phase (supernatant). In-mix 21.4 In-supernatant 21.5 gCOD/L; Out-mix- 21.5 ± 1.1 gCOD/L; Out-supernatant 16.7± 0.16 gCOD/L.

The whole COD balance in the bioreactor is:

$$COD_{in} - COD_{out}^{mix} = 21.4 - 20.5 = 0.9 \frac{gO_2}{L}$$

The COD balance in the bioreactor for the liquid phase is:

$$COD_{in} - COD_{out}^{supernatant} = 21.4 - 16.7 = 4.7 \frac{gO_2}{L}$$

This means that 4.7 gO_2/L are consumed from the liquid phase.

The solid component of the outlet has a COD of:

$$COD_{out}^{mix} - COD_{out}^{supernatant} = 20.5 - 16.7 = 3.8 \frac{gO_2}{L}$$

From the obtained results a general material balance was done in order to identify if the process is consuming another carbon source apart from the lactose.

For the inlet, the COD of the CWP was theoretically calculated in order to compare it with the value measured.

Lactose into COD:

$$C_{12}H_{22}O_{11} + 12\ O_2 \rightarrow 12CO_2 + 11H_2O$$

$$\frac{gO_2}{g\ Lactose} = \frac{12mol\ O_2}{1mol\ Lactose} * \frac{1mol\ Lactose}{342.3g\ Lactose} * \frac{32gO_2}{1mol\ O_2} = 1.12\frac{gO_2}{gLactose}$$

Therefore the CWP has a COD component from the lactose of:

$$\frac{15gLactose}{L} * 1.12\frac{gO_2}{gLactose} = \mathbf{16.8}\frac{gO_2}{L} \quad \textbf{(A)}$$

Protein into COD:

It was assumed that the all the protein was β-lactoglobulin. It composition was publish by Brand et al. and it is known that the molecular mass is two folds the value given by Brand et al.

$$C_{800}H_{1292}O_{200}N_{248} + 843\ O_2 \rightarrow 800CO_2 + 274H_2O$$

$$\frac{gO_2}{g\ Protein} = \frac{843mol\ O_2}{1mol\ Protein} * \frac{1mol\ Protein}{19000g\ Protein} * \frac{32gO_2}{1mol\ O_2} = 1.42\frac{gO_2}{gProtein}$$

Therefore the CWP has a COD component from the proteins of:

$$\frac{3gProteins}{L} * 1.42\frac{gO_2}{gProtein} = \mathbf{4.3}\frac{gO_2}{L} \quad \textbf{(B)}$$

Lipids into COD:

The following composition on lipids was assumed: palmitic (31%), mystiric (12%), stearic acid (11%), oleic acid 24%, others 22%. From this and using the typical stechiometric formula for oxidation:

$$C_nH_aO_bN_c + \left(n + \frac{a}{4} - \frac{b}{2} - \frac{3}{4} * c\right)O_2 \rightarrow nCO_2 + \left(\frac{a}{3} - \frac{3}{2} * c\right)H_2O + cNH_4$$

The COD for this acids are:

Name	Formula	PM	Demanded mol O_2	gO_2/gFatty acid
Palmitic acid	$C_{16}H_{32}O_2$	256.40	23.00	2.87
Mystiric acid	$C_{14}H_{28}O_2$	228.40	20.00	2.80
Stearic acid	$C_{18}H_{36}O_2$	284.50	26.00	2.92
Oleic acid	$C_{18}H_{34}O_2$	282.00	25.50	2.89

This 78% of the lipids have an average COD of 2.87gO_2/gLipids. Considering this, the CWP has a COD component from the lipids of:

$$\frac{20gCWP}{L} * \frac{1.2}{100} * 2.87 \frac{gO_2}{gLactose} = \mathbf{0.69} \frac{gO_2}{L} \quad \textbf{(C)}$$

Considering all components:

$$\boldsymbol{COD_{in} = A + B + C = 16.8 + 4.3 + 0.69 = 21.79 \frac{gO_2}{L}}$$

The measured value was 21.4 ± 0.1 gO_2/L

For the outlet, the COD was theoretically calculated to compare with the obtained value by measurement.

VFAs into COD:

Once again the general formula for oxidation was applied for all the VFAs present in the outlet stream and the COD was calculated: 16.2 gO_2/L.

$$COD_{out;VFAs}^{supernatant} = \mathbf{16.2} \frac{gO_2}{L} \quad \textbf{(A)}$$

The same was done for proteins, but for this it was necessary to measure its concentration in the outlet stream. Figure 4.17 shows the results for the same period as for COD analysis. From this, the supernatant of the outlet stream has a COD component from the proteins:

$$COD_{out;Proteins}^{supernatant} = 0.7 \frac{gProteins}{L\,supernatant} * 1.42 \frac{gO_2}{gProteins} = \mathbf{1} \frac{gO_2}{L} \quad \textbf{(B)}$$

The overall COD content in the supernatant should be:

$$\boldsymbol{COD_{out}^{supernatant} = A + B = 16.2 + 1 = 17.2 \frac{gO_2}{L}}$$

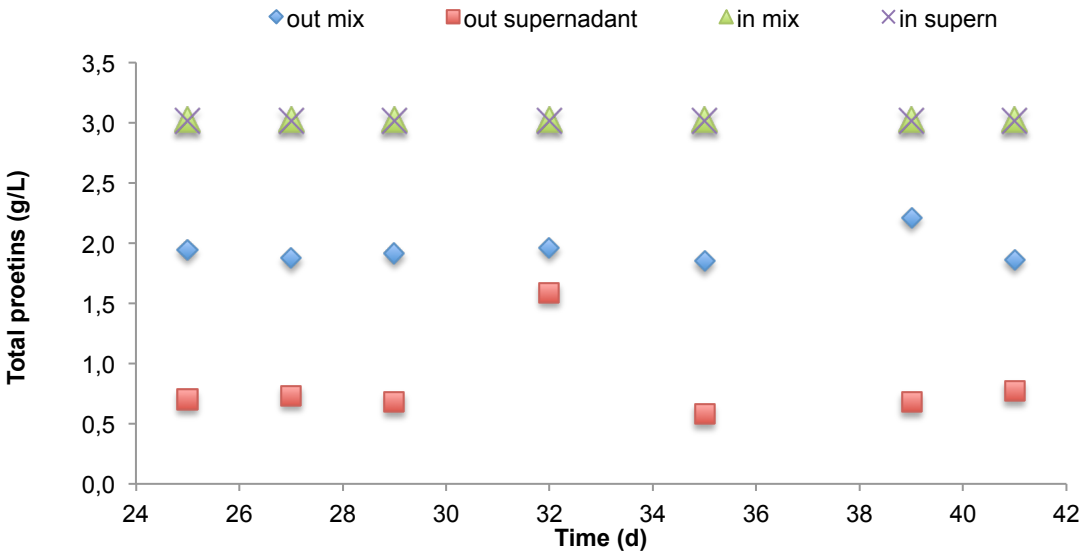

Figure 4.17. Total protein content in CWP solution (in) and outlet (out) in PBBR-Vuko as a function of time during steady state when operated with HRT of 6 days. It was measure in the mixture (mix) and in the liquid phase (supernatant). In-mix 3.01 In-supernatant 3.03 g/L; Out-mix- 1.95 ± 0.13 g/L; Out-supernatant 0.69 ± 0.07 g/L.

The same protein content was detected for the mix and for the liquid phase which means that all proteins are in solubilized.

General Protein balance is calculated:

$$\boldsymbol{PROT_{in} - PROT_{out}^{mix} = 3 - 2 = 1 \frac{gProtein}{L}}$$

$$\boldsymbol{PROT_{in} - PROT_{out}^{surnatant} = 3 - 0.7 = 2.3 \frac{gProtein}{L}}$$

From this it can be seen that 2.3 g/L of protein are consumed in the liquid phase.

Table 4.2. Results obtained during PBBR-Vuko operation

	Y (C-mol lactic acid/C-mol lactose)	Y (C-mol VFAs/C-mol lactose)	Acetic acid (g/L)	Propionic acid (g/L)	Butyric acid (g/L)	Lactose consumption (days)	Stage Durance (days)
First batch	93%	84%	2.5	5.8	3.8	6	14
Second batch	92%	79%	3.2	3.5	3.2	4	10
Third batch	42%	80%	1.6	0.8	6.1	3	10
HRT 9 days	-	79%	5.3	1.0	2.8	-	38
HRT 6 days	-	78%	0.6	0.1	6.5	-	41

4.2.2 PBBR-AC

The PBBR-AC was settled as described in 3.2.2 and operated for 48 days. The first 15 days corresponded to batch operation (data not shown). During the first days of batch no significant VFAs production was detected. The VFAs concentration trends arrived to the plateau after 7 days, with a poor VFAs content (g/L): acetic acid (1.4), propionic acid (0.1) and butyric acid (0.1).

Continuous operation was started with of 9 days, being operated at the same time that PBBR-Vuko was operated with HRT of 9 days (Figure 4.18). After 4 days, acetic acid concentration was stable at 2.1 ± 0.3 g/L, propionic and butyric acid slightly increased till 0.7 g/L and 0.3 g/L (at day 22) respectively; the yield (C-mol VFAs/C-mol lactose) in this period was 18%. After day 22 the acetic acid concentration decreased, propionic and butyric acid concentration continued the slightly increase; the yield (C-mol VFAs/C-mol lactose) for this period was 10%. Due to these results, it was decided to not continue with PBBR-AC operation.

Methane production was detected in the biogas mixture, it increased along the continuous operation; achieving a maximum of 234 mL/L. No hydrogen was detected during this period time.

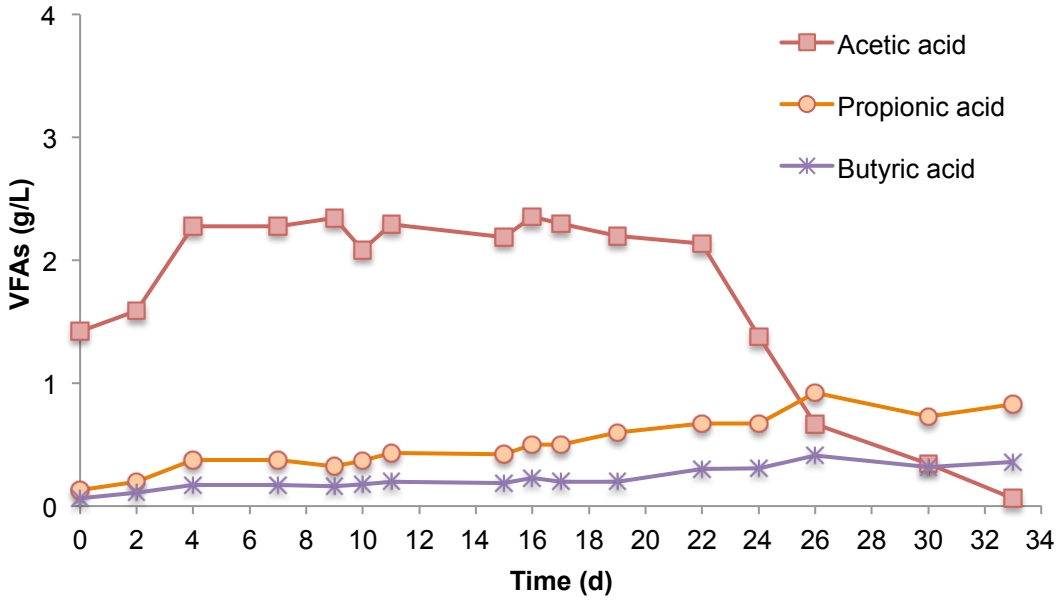

Figure 4.18. Principal VFAs concentration in function of time during PBBR-Vuko operation with HRT 6 days. Only the continuous operation mode is shown.

4.2.3 Freely suspended cells

Two different support materials – Vukopor S10 and Granular Activated Carbon- were tested in PBBRs configuration. From this it was interesting to study the behaviour of the acidogenic mixed culture in a continuous system culture without immobilization. This would help to conclude about the advantages, or disadvantages, of having an immobilization in a continuous system. In this order of idea, a freely suspended cells reactor was performed which was first operated in batch biomass generation/acclimation and then in continuous. It was chosen a HRT of 6 days since it works for the PBBR-Vuko.

The bioreactor was operated in batch for 16 days (data not shown). During the first four days, the 14 g/L of lactose were clearly consumed while lactic acid (5.82 g/L) and some VFAs - butyric and propionic acids - were produced. At day 9 the VFAs production was finished, with a yield of 88% and a VFAs mixture composed by acetic (1.36 g/L), butyric (5.60 g/L) and propionic (1.79 g/L) acids. Since for the last 8 days of batch the concentration of VFAs was stable, the operation in continuous was started. Although it was in continuous mode for 15 days, it was possible to observe (see Figure 4.17 a) that the production of VFAs decreased as lactic acid increased its concentration. Due to a drop on the bioconversion, the yield of VFAs decreased to 44%.

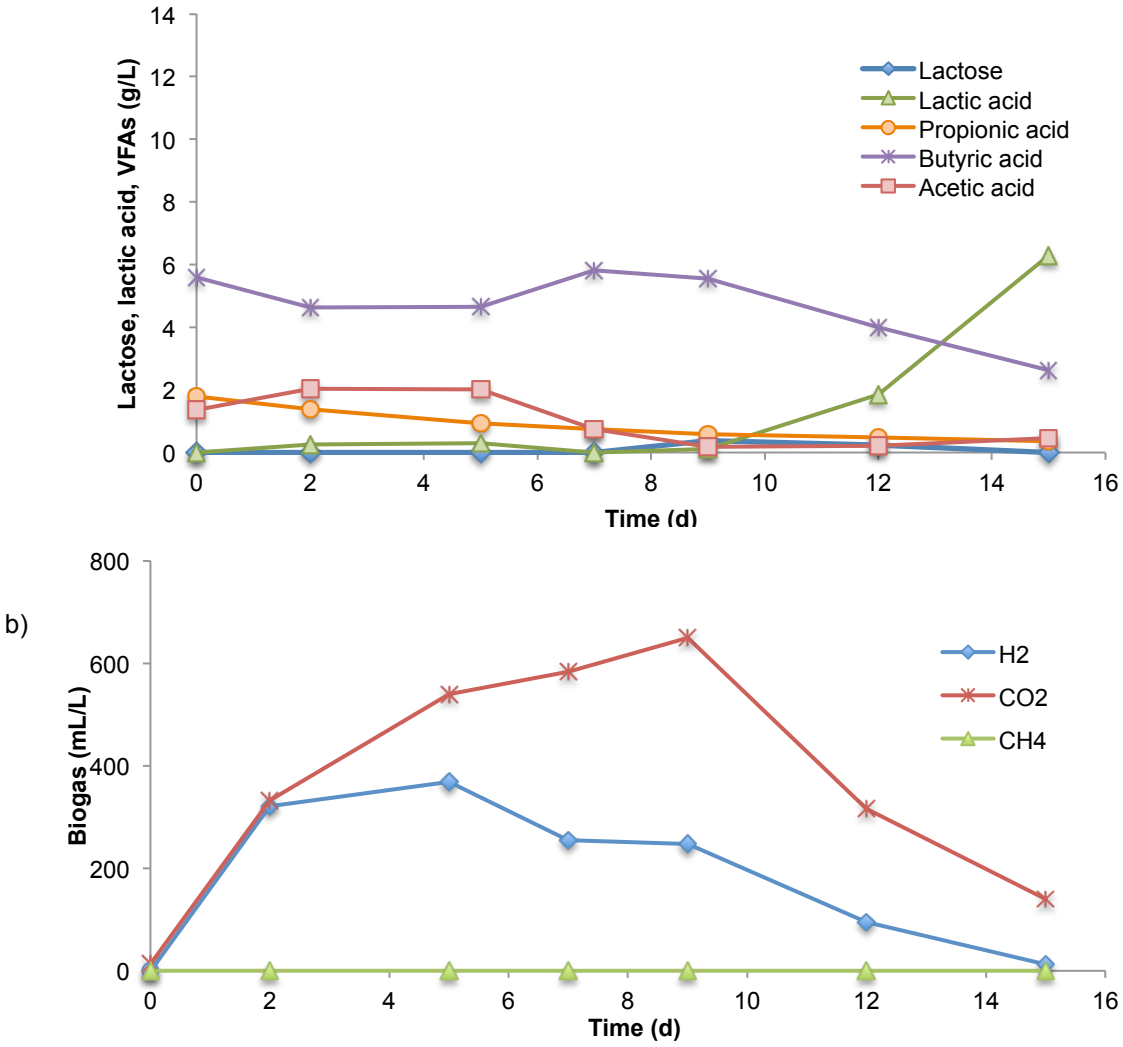

Figure 4.19. Concentration profiles of main monitored compounds during freely suspended cells bioreactor operated with HRT 6 days. a) Concentration of lactic acid and VFAs dissolved in liquid phase as a function of time. b) Biogas concentration as a function of time.

4.3 Results resume for ceramic material and freely suspended cells

Table 4.3. Results from acidogenic fermentation in microcosm experiments and bioreactors with ceramic material and freely suspended cells.

Experiment	Experiment Phase	Acetic acid	Propionic acid	Butyric acid	Total VFAs	Y_{VFAs}
		g/L				%(C-mol/C-mol)
Preliminary Immobilization supports study	1st VFAs acc. batch	1.6	3.2	4.8	9.9	84.6
	2nd VFAs acc. batch	2.1	2.3	6.1	13.1	88.8
Improved immobilization supports study	VFAs acc. batch	1.6	2.4	6.6	10.9	87.1
PBBR-Vuko	1st batch	2.4	5.9	3.8	12.7	89.6
	HRT 9 days	5.3	1.0	2.8	10.5	78.9
	2nd batch	3.3	3.5	3.2	11.3	87.6
	3rd batch	1.7	0.8	6.3	9.7	84.0
	HRT 6 days	0.6	0.1	6.5	9.0	77.3
Freely suspended cells bioreactor	1st batch	1.3	1.8	5.8	10.0	88.6
Freely suspended cells microcosms	1st batch	1.9	3.0	4.1	9.3	84.1
	2nd batch	2.5	2.3	4.2	10.2	78.8
Freely suspended cells microcosms CWP 30 g/L	1st batch	1.0	2.5	8.7	12.8	82.8
	2nd batch	2.0	3.3	9.3	15.2	85.7

4.4 VFAs mixture obtained in batch experiments and bioreactor with ceramic material and freely suspended cells

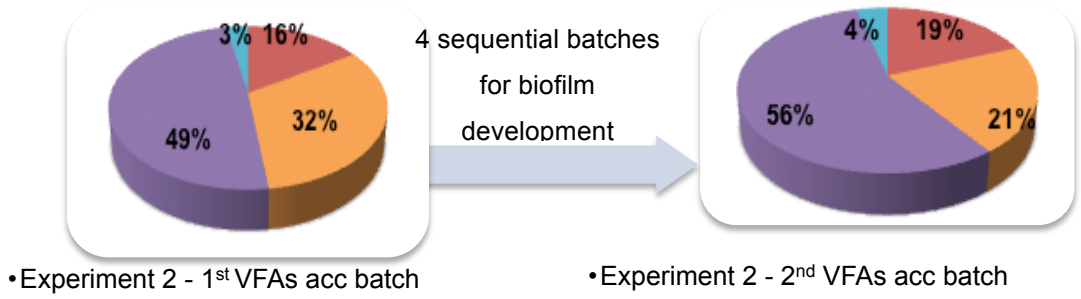

Figure 4.20. VFAs mixture composition in %(gVFA/gTotalVFAs) obtained in experiment 2 a) first batch b) second batch. **Purple** – Butyric acid; **Orange** – Propionic acid; **Red** –Acetic acid; **Blue** – Other acids

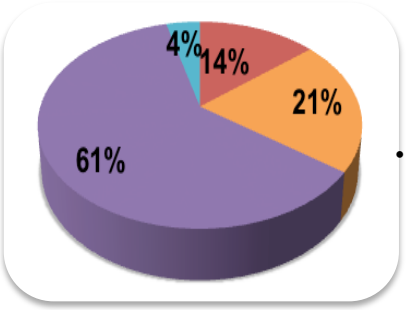

Figure 4.21. VFAs mixture composition in %(gVFA/gTotalVFAs) obtained in VFAs accumulation batch in experiment 3. **Purple** – Butyric acid; **Orange** – Propionic acid; **Red** –Acetic acid; **Blue** – Other acids

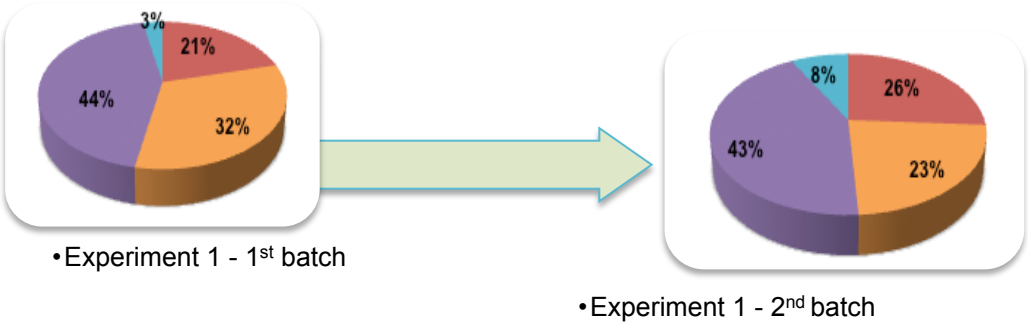

Figure 4.22. VFAs mixture composition in %(gVFA/gTotalVFAs) obtained in experiment 1 – freely suspended cells with CWP 20 g/L in a) First batch b) second batch. **Purple** – Butyric acid; **Orange** – Propionic acid; **Red** – Acetic acid; **Blue** – Other acids

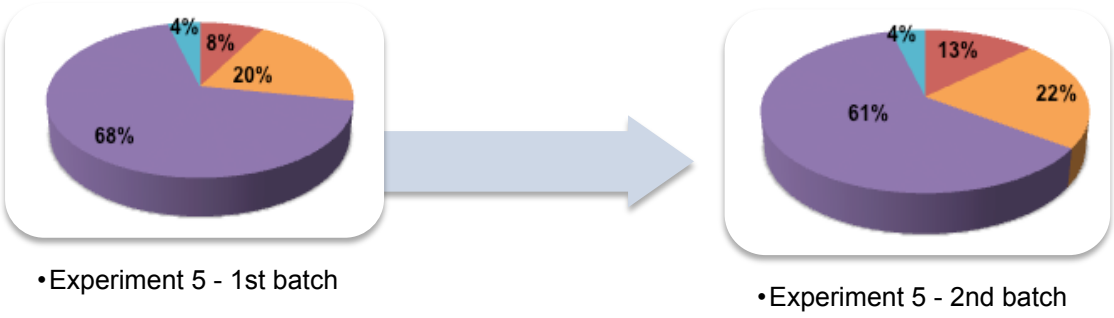

Figure 4.23. VFAs mixture composition in %(gVFA/gTotalVFAs) obtained in experiment 5 – freely suspended cells with CWP 30 g/L in a) first batch b) second batch. **Purple** – Butyric acid; **Orange** – Propionic acid; **Red** – Acetic acid; **Blue** – Other acids

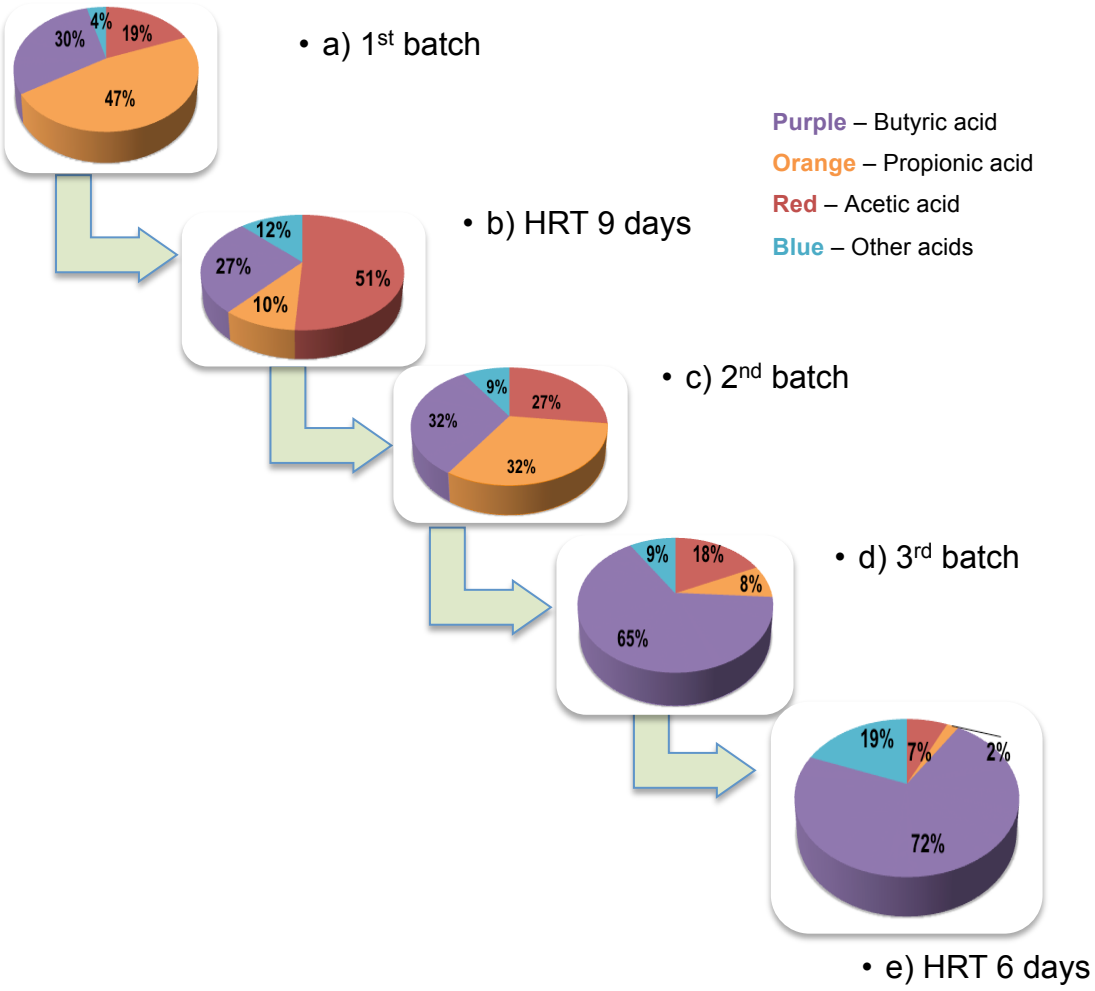

Figure 4.24. VFAs mixture profile obtained in the different operative stages in PBBR-Vuko. a) other acids are refer to isocaproic (0.2 g/L) and isovaleirc and valeric (0.1 g/L). b) Other acids refers to isobutyric (0.2 g/L), isovaleric (0.2 g/L), valeric (0.3 g/L) and caproic (0.6 g/L). c) other acids refers to isovaleric, and caproic (0.1 g/L) and valeric and isobutyric (0.2 g/L). d) other acids refers to valeric and caproic (0.2 g/L) and isovaleric (0.3 g/L). e) other acids refers to isobutyrc (0.7 g/L), isovaleric and valeric (0.1 g/L) and caproic (0.7 g/L).

5 Discussion

The scope of the present work was to study the production of VFAs from CWP by employing a PBBR as an alternative to other culture system that are being tested (i.e MBR). Two different materials were studied: Vukopor S10 and Granular Activated Carbon. The choice of these two materials was due to its different characteristics that could induce different effects in the microorganisms, which compose the acidogenic mixed culture. These two materials were already study also for the production of VFAs with an acidogenic mixed culture but using olive mill wastewater as substrate [Bertin et al., 2010].

The first part of the study was carried out in small scale, through experiments prepared in microcosms 100 mL-Pyrex bottles in which was possible to get the first knowledge about the metabolism of the mixed culture with and without packing material. It was already knew its capacity for VFAs production at pH 6 and at 37ºC since this inoculum was taken from a MBR in which was already producing VFAs. From two microcosm experiments with immobilization, experiments 2 and 3, it was possible to notice the different impact that each material had in the process by detecting different results in terms of VFAs production amount and composition of the mix. Those two experiments were fundamental to define the parameters for the bioreactors, particularly the HRT.

5.1 Microcosm experiment employing freely suspended cells

In microcosm experiments with freely suspended cells it was possible to know about the sequential reactions which involve VFAs production through lactose content in cheese whey. In the first part, the acidogenic mixed culture, performed the bioconvertion of lactose into lactic acid. This conversion took 7 days, resulting in a productivity of 1.8 g/L/day a lower value when compared with other works in literature about lactic acid production from CW [Plessas et al., 2008]. However in the present work the yield (lactic acid/lactose) is higher since all lactose is consumed, while in Plessas study, some sugar remained in culture media. The slowly conversion observed can be due to nutrients deficient since during this work CWP solution was the only substrate used, without any addition of nutrients. When nutrients supplementation is given, the cells have de opportunity to grow, and in this way increase the conversion velocity [Prazeres et al., 2012].

In which respect to VFAs production it was observed that starts as soon the previous reaction is complete. It was obtained a good yield (more than 80%) in which butyric acid was the main VFA produced with 44% of total VFAs mix composition, corresponding to 4 g/L. The mixture obtained is an advantage for PHA production since a good quantity of butyric acid, substrate for PHB production, and low content of acetic acid (approximately 2 g/L). VFAs mixtures with low quantities of acetic are an advantage since this acid is a strong inhibitor for PHAs accumulation. Furthermore it was produced 3 g/L of propionic acid, quantity higher than other reports, also using the CW [Bengstsson et al., 2008]. This acid is one of the most interesting acids for PHA production because can result into 3HV which can be incorporate into other polymers improving their characteristics.

5.2 Microcosm experiments employing Vukopor S10 as support material

Experiment 2 was the first trial with immobilized cells but, as said before, the first batch was not monitored; nevertheless some interesting observations were made after those 28 days of batch. Analyses showed that lactose was completely converted into lactic acid and no VFAs were produced, but 28 days should be more than enough time to produce VFAs, after lactic acid production, as it was observed in the free cells experiments. The pH was not corrected for a long period, lactose was converted into lactic acid so the pH went down (in fact it was measured a pH 4 at day 28). From this, it is thought that this non-conversion could be assigned to the incapacity of the consortium to produce VFAs that also lowers the pH value. This was confirmed during the next batches.

The second batch, in which pH was controlled, began with culture media renovation and without adding new inoculum. Lactose was converted into lactic acid, proving the presence of biofilm inside of microcosms.

In general, when using the ceramic material as biofilm support, the obtained trends were similar to those obtained at freely suspended cells in terms of bioconversion steps: 1- Lactose consumption and Lactic acid formation and 2- Lactic acid consumption and VFAs production. Analysing the Figures 4.2 and 4.4 where is represented the trend of substrate consumption and metabolites production obtained in experiments 2 and 3 is possible to see repeatability in the results and so robustness in the process. This is shown by considering the average value of the final concentration of each VFA in each experiment.

It seems that sequential batches were an important step to decrease the VFAs production time. Analysing the Figure 4.2 it is possible to observe that in the first VFAs accumulation batch, performed after two biofilm development batches, in which the first were not monitored, 14 days were needed for reaching the VFAs concentrations plateaus. When the second VFAs accumulation batch was performed, after several biofilm batches, VFAs were produced in 9 days with a similar yield respect to the previous VFAs accumulation batch. This is confirmed with Figure 4.5 that represents the VFAs accumulation batch in the improved immobilisation test (experiment 3). In this experiment only one batch for VFAs production was performed but also in this case, after 3 sequential batches for biofilm growth, the VFAs production took 9 days, as the second VFAs accumulation batch in experiment 2.

Between those three referenced batches, the VFA yields are very similar and high since is over 85%. VFAs compositions are also similar and it was composed by acetic, propionic and butyric acids.

5.3 Consortia characterization

To understand the development of the biofilm along the experiments microbiological analysis were performed for liquid and support. The samples are still in process not being possible to present neither microorganisms characterization composition responsible for biofilm nor presented in liquid phase as function of time. However the preliminary results, comprising DGGE image (Figure 4.10) and dice coefficients (Table 4.1), of Vukopor-attaced-cells show some biofilm evolution along the experiment time.

In DGGE the profiles correspondent to time 0 and 6 days is possible to observe that both are similar and have some lanes, in the top, which not figured in times 12 and 16 days. Since these first samplings correspond only to lactose conversion in lactic acid those lanes should correspond to the microorganisms responsible for this specific bioconversion. Is notice that in the next part of the process, times 12 and 16, appear some lanes, which were not present in previous samplings. Those should correspond to the microorganisms responsible for the VFAs production.

From the similarity values obtained between the samples (Table 4.1) the differences between the biofilm presented during the first bioconversion - lactose in lactic acid - and in the second – lactic acid into VFAs are evident. Between A0 and A6, which corresponds 0 and 6 days samplings, the coefficient is high (77.9%) which means that the biofilm did not change significantly during the first 6 days. At day 12, another sampling was performed corresponding to sample A12 and here was noticed, a considerable change since the value decreases to 23.9%, respecting to day 6. Furthermore, the similarity between time 0 (A0) and 12 (A12) days after is of only 16.8%.

Bioconversion times were already known from the previous results obtained in experiments 2 and 3. After the sequential batches for biofilm development the reproducibility observed in the conversion of lactose in lactic acid (6 days), and the conversion into VFAs (9 days) allowed to plan the sampling day/time for microbiological analysis. However along this experiment 4 was observed an increasing conversions time. Each batch dedicated only for lactose conversion has a durance nearly 6 days. The first sampling, for microbiological characterization, was made at day 21 and corresponds to the beginning of fourth sequential batch. It was made at planned time. It was expected at day 9, of this last batch, all the process was complete and VFAs were already produced. VFAs production only occurred 3 days later than expected, day 12 of running batch. The explanation for that can be due to amount of cells took out from the system. In total, at day 0 and 6, 16 ceramic supports and 14 mL of liquid were remove for analyse.

5.4 Microcosm experiments employing Granular Activated Carbon as support material

In microcosms packed with ceramic support and freely suspended cells experiments it was observed that lactic acid is an intermediary of the bioconversion. Lactic acid is the result of lactose conversion and it could be an indicator of metabolism activity, however it was never detected in solution during all experimental work with AC. Therefore it was very difficult to study the utilization of AC as immobilized support. One of the possible reasons is its high affinity for organic compounds as

previously reported by [Gao et al. 2011, Silva et al., 2013]. In this way it was impossible to distinguish between the produced/consumed and absorbed/desorbed metabolites.

As previously explained lactose depletion was what determined the end and the beginning of each batch. However, when using the AC, this strategy could not be applied and so more biofilm formation batches were perform in order to assure biomass content: fourteen in the experiment 2 and nine for the experiment 3; while for the Vukopor: eight for experiment 2 and four in experiment 3. Nevertheless was observed that the yield (C-mol VFAs/C-mol lactose) increases with the number of batches performed with AC. This increasing can be due to gradual desorption of the compounds from the support. The existence of microorganisms inside of microcosms or in the bioreactor is evident because VFAs, despite slowly, are being detected.

Gao and co-works [Gao et al., 2011] observed that the AC effect decreases with repeated use of support during lactic acid fermentations due to the quantity which was already adsorbed onto the material. This could be the case for the present study since an increase of the final VFAs concentration is observed during the sequential batches. Especially in experiment 2 in which more sequential batches were performed.

During this work neither adsorption isotherm studies nor analytical or biological analyses of the AC have been done. It was just consider the final results of the process. These procedures could help to understand the real effect of AC on this biological system. It can be speculated that the results of these analysis would outcome in good VFAs production yield. For the “natural” desorption of VFAs observed along the time means that the AC could have reached a “saturation” point. Because of that desorption of acetic, propionic and butyric acids were detected (Figures 4.3, 4.7, 4.15). Allying/combining this VFAs release to Gao and Silva reports is expected to have a considerable amount of VFAs adsorbed in AC.

From all of this it can be said that the inoculum was entrapped/attached/adsorbed into AC support and was performing the sequential reaction of its metabolism. The adsorption of lactose on AC was not considered a disadvantage since the microorganisms still had access to the carbon source. The draw back of this material is the difficulty desorption of the produced VFAs, without organic resources like ethanol or n-propanol [Silva et al., 2013].

5.5 Packed bed bioreactors

Comparing the PBBR-Vuko 1st batch with experiments 2 and 3, it was observed that the yields for lactic acid production were similar (about 90%) and that the same VFAs were produced with yields about 85-90%. But the concentration profiles were different. The fact that at the end of the PBBR-Vuko batch 6 g/L of propionic acid - against 2 g/L for the microcosm experiment - caught the attention because of being an isolated phenomenon and at the same time because propionic acid could be an attractive compound for polyhydroxyalkanoates production; specifically for the production of polihydroxyvalerate. At the moment the only hypothesis is that high propionic production could be due to the lactose addition made on the second day, increasing the carbon source available in the system.

When tests with higher carbon source content were done the final propionic concentration was just increased from 2 g/L (experiment 2 and 3) to 3 g/L (experiment 5).

Now comparing between the three batches performed at the PBBR-Vuko, it was observed that the propionic production capacity was lost: 6, 4 and 1 g/L for each batch. This could be attributed to the phenomenon of biofilm formation and so microbial competition. In other words, during the batches biofilm growth but it seems that the microorganisms responsible of propionic production growth slower, losing the competition with the others that achieve immobilization better. When using 30 g/L of CWP (experiment 5) this capacity was not lost meaning that the amount of carbon source is enough for the consortia. Until now no answer was already found to justify what led to this high propionic acid production in the PBBR-Vuko first batch.

After the VFAs concentration reached the plateau in the first batch, on PBBR-Vuko and PBBR-AC, the continuous operation began in both PBBRs. The HRT for the bioreactors was based on the microcosm experiments with immobilizing support. The microcosms, with ceramic support, shown that in 9 days all the lactose is consumed and VFAs are produce with a yield (C-mol VFAs/C-mol lactose) near 80%. Conversely, in the microcosms experiments with AC, was not possible to follow the lactose conversion and after 9 days the maximum VFAs yield achieved was only 30% (experiment 2). Therefore, in order to be able to compare the productivities between both PBBRs, a HRT of 9 days was utilized at both bioreactors; that were operated at the same time.

Comparing the behaviour between the PBBRs along the steady state period, besides the principals VFAs produced were the same (acetic, butyric, and propionic acids) the results evidence that PBBR-Vuko worked much better since produced more VFAs in quantity (10.5 g/L), than in PBBR-AC (2.7 g/L), reflecting also the yield (C-mol VFAS/ C-mol lactose) with 79% and 20% for PBBR-Vuko and PBBR-AC respectively. Bertin and co-worked [Bertin et al., 2010] had already tried VFAs production with a PBBR packed with AC but also for them the results were not satisfactory since the yield achieved was lower than 13%. From all of this, is clearly seen that AC is not adequate to produce VFAs.

If PBBR-AC did not offered good acceptable effects, PBBR-Vuko revealed versatile and interesting results.

During the operation with HRT of 9 days the profile presented some instability with a changing VFAs profile but, it can be said that an almost stationary state condition was reached after 9 days, which means after the bioreactor changed 1 time its liquid phase. During 14 days the concentrations of butyric and acetic were stable but the same not occurred with the other acids; in particular, propionic acid concentration decreased drastically from 6 g/L at day 0, until 0.8 g/L. Because of that is not possible to say that the bioreactor was being operated in a continuous steady state. To affirm that should be no variation of concentrations with time and a constant reactions rate should be observer during 36 days, which corresponds to 4 times of HRT [Chemical Reactors E. Heinzle, Technische Biochemie]. The significantly lost of propionic acid could be attributed to a wash out of the

microorganisms that produces it or, another hypothesis, could be that the microorganisms need a higher HRT to produce propionic acid in a higher concentration.

During the 38 days, that the PBBR-Vuko, was operated in continuous, it was clearly visible variations in the VFAs mixture composition despite the yield being always high. It is possible that reason of a high yield is related with protein consumption but this was not checked. This instability could be attributed to defined variation on the microbial consortium composition attached to Vukopor. This phenomenon did not happen in the microcosm experiments. In experiment 2 is possible to observe that in the two VFAs accumulation batches the VFAs mixture composition was similar, where butyric is always the most produced, then propionic and acetic acid the less (Figure 4.20). But in experiment 2 and 3, sequential batches dedicated to biofilm formation were made. It can be said that the sequential batches, for biofilm development, are a determinant step to get stability in the VFA mixture composition along the operation time. Perhaps if before starting the continuous operation, more sequential batches were performed, like in microcosm experiments, during the continuous operation the VFAs mixture should be more constant.

Previously it was shown that a HRT of 9 days could be not enough for the propionic acid production, but in order to increase the total VFAs productivity a continuous operation was tried with a HRT of 6 days. This time, before the continuous operation, two batches were performed to promote biofilm development. In this way it was expected a formation of a stronger biofilm which could result in a more stable VFA mixture composition.

In the first, of those two batches, more inoculum was added to increase cells concentration and to give an opportunity to the propionic producers to recover. It was verify the return of propionic production but in minor concentrations (3.5 g/L). In the second, after the liquid phase replace, only remained the microorganisms that formed the biofilm during the previous days (62 days). Almost no propionic was produced (0.8 g/L). This is another suggestion that the microorganisms responsible for the propionic production could be in liquid phase and do not contribute for the biofilm in the ceramic material. In the last batch it was detected that the VFAs production started since the first day -along with lactose consumption- this could be due to the existence of a stronger biofilm developed in the previous sequential batches.

Six days was the time observed, in microcosm experiments, for the total conversion of lactose into lactic acid, previous to the VFAs production phase. Knowing this and adding to the fact that was observed VFAs production started earlier in the last two batch in PBBR-Vuko (6-7 days), an operation with HRT of 6 days was started.

Decreasing the HRT from 9 to 6 days it was expected not produce propionic acid -due to the reasons already explain above- but a higher VFAs productivity. During the operation with HRT of 6 days, it was confirmed the necessity of increase the HRT for propionic acid production. During the operated time with HRT 6 days propionic acid did not achieved even 1 g/L. The yield was never constant and the system never achieved the steady state until day 27. Between day 27 and 41 the

bioreactor seems to enter in steady state, however another 10 days of operation were need to confirm this state.

These 14 days of apparently steady state indices of steady state was an opportunity to make a general material balance between all compounds which enter into the bioreactor (inlet) and the bioconverted products (outlet) resulted from the acidogenic process.

Since the COD of the liquid phase is the same as the COD for the whole inlet stream (21 ± 2 gO_2/L) it can be inferred that all COD is from compounds that were solubilized.

In order to check the consistence of the measurement, a theoretical COD value for the inlet stream was calculated (21.8 g O_2/L). Compared with the value obtained by measurement, it can be said that is enough consistent; with an error that represent less than 4% of the theoretical value.

Regarding the outlet, the obtained value for the whole stream was 21 ± 1 g O_2/L. This guarantee that no COD accumulation exists, and so, being in accordance with the continuous state condition.

In the case of the outlet stream, the COD for the liquid phase was 17 ± 1 g O_2/L, which means that 3 gO_2/L are from the solid component. It is high probable that this COD corresponded to suspended cells (biofilm regeneration).

A theoretical COD for the liquid phase of the outlet stream was also calculated in order to check the consistence of the measurement. The obtained value was 17.2 g O_2/L which is in accordance with the measured value. It is important to mention that for obtaining the previous theoretical value it was necessary to measure the protein content in the liquid phase of the outlet stream, and so taking the opportunity to measure also the protein content in the inlet (mix and liquid phase) and for the mix of the outlet.

From this last, it was identified that all proteins were solubilized since the values obtained for the mix and the liquid were identical. Then protein consumption was detected in the liquid phase, 2.3 gProt/L. Thereafter it was observed that if the 3 g O_2/L that correspond to the solid component of the outlet stream (and thought to be cells) are converted into proteins (3 gO_2/L * 1 gProt/1.42 g O_2) it turns a value of 2.1 gProt/L which is highly in accordance with the detected protein consumed value.

5.6 Freely suspended cells bioreactor

Also a freely suspended cells bioreactor was set up and it was first operated in batch. During btach operation butyric acid was the VFA mainly produced.

To operate the bioreactor in continuous it was chosen HRT of 6 days. In microcosm experiment it was observed that for achieve a good VFAs yield it was needed more than 10 days, however the bioreactor was operated with the lowest HRT operated in PBBR-Vuko.

During the continuous operating time it was observed a loss of capacity by the mixed culture to produce VFAs. After 12 days in continuous, lactic acid started to be detected in the bioreactor as the same time that VFAs stopped to be produced, however no lactose was detected. This means that the first conversion (lactose into lactic acid) was occurring but not the next stage.

In PBBR-Vuko, when operated with the same HRT it was also detected lactic acid, however in a lower concentration. These results show that for this process, immobilization is an advantage.

6 Conclusions

Cheese Whey is the waste from dairy industry with more pollution impact for the environment. A conventional treatment is difficult to implement due to its high COD content, in which lactose is the majority responsible. Lactose is a carbon source for some species of microorganisms making the CW a potential substrate for biotechnological processes, like the anaerobic digestion. During this process an acidogenic culture promotes the bioconversion of sugars into other compounds, like VFAs. These products can be use as substrates to other biological processes, like PHA. In this way, a valorization of a waste like CW was proposed.

From preliminary experiments it was possible to study the metabolism of the acidogenic mixed culture used for VFAs production from CW. It was observed the process is composed by sequential reactions: 1 - lactose is converted into lactic acid; 2 – lactic acid is converted into the different VFAs.

An immobilization study was performed in order to see the impact that different materials as its advantages, respecting to a freely suspended cells process, in VFAs production. Two different immobilization supports were study: Vukopor S10 and Granular Activated Carbon. For both supports small scale experiments (100-mL Pyrex bottles) and bioreactors at lab scale (1 L of empty volume) were carried out.

Sequential batches were performed in microcosm experiments in order to allow biofilm development. This set shown be an important in the process in order to have a stable biofilm as constant VFAs production.

Activated carbon has a huge absorbent capacity, especially for organic compounds. Its characteristic did not allow a complete study. In microcosm experiments it was achieved a VFAs yield of 30%. While in continuous operation in the bioreactor it was achieved only 20%.

On the other hand, ceramic material (Vukopor S10) it was achieved, during all microcosm experiments and in the bioreactors, yields above 80% with VFAs production between 9.0 and 13.1 g/L. In microcosm experiments the profile was always mainly compose by butyric acid (5-6 g/L), propionic acid (2.3 - 3.2 g/L) and acetic acid (1.6 - 2.1 g/L). In the bioreactor, different profiles were obtained. In the first batch propionic acid was produced with 6 g/L, a considerable amount of an important acid. When operated in continuous, a wash-out of microorganisms responsible for this production was observed. It was tried to recover this production by performing more batches. However, this high production was not achieved anymore. With HRT of 9 days, acetic acid was the principal VFA produced (5.3 g/L), while with a decrease HRT to 6 days, VFAs mixture was mainly composed by butyric acid (6.5 g/L).

Despite of not being possible produce propionic acid in a continuous process, Vukopor S10 was choose the best immobilizing support due to its good VFAs yield.

A freely suspended bioreactor was also set up. During continuous operation with the lower HRT operated in PBBR-Vuko, it was observed that 6 days were a period to short to perform the two sequential reactions.

From all the results obtained, it can be said the immobilization process is an advantage for VFAs production in an acidogenic process with a mixed culture using cheese whey as carbon source.

7 Future work

To complete this study about VFAs production with immobilized cells, despite during this thesis work several experiments were performed, there are yet some experiments to do:

- Another support material should be tested, like plastic material. Has a good surface in which the microorganism could attach to form the biofilm and it is cheap material.

- Test different concentrations of CWP solution.

- Microbiological analyses along the continuous operation in the bioreactors.

- With PBBR-Vuko should continue work until achieve a steady state and after that test different HRT until is possible to decrease HRT through days to hours.

- Study different pH values and different HRT in order to produce more propionic acid.

8 Publications

- Poster contribution for ECOMONDO conference (November 2013);

- Extended Abstract to be published in, Environmental Engineering Management Journal with:

Domingos J.M.B, Martinez G.A., Scoma A., Bertin L., Reis M.A.M., Fava F., (2013), Production of Volatile Fatty acids from cheese whey with immobilized cells.

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10 Appendix

Solution for Total proteins determination

Reagent A

2 g sodium potassium tartrate 4 H_2O

100 g sodium carbonate

500 mL 1 N NaOH, H_2O to 1 liter (that is, 7 nM NA-K tartrate, 0.81 M Sodium Carbonate, 0.5 N NaOH final concentration)

Reagent B

2 g Sodium Potassium tartrate x $4H_2O$

1 g Cooper Sulphate ($CuSO_4$) x $5H_2O$

90 mL H_2O

10 mL 1 N NaOH (final concentration 70 mM Na-K tartrate, 40 mM cooper sulphate)