Propionic acid production through anaerobic fermentation of food waste

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Abstract

The quest for minimization of waste coupled with resource recovery has focused attention on the use of food wastes as feedstocks for production of high-value products. About 1.3 billion tons of food waste are generated annually worldwide. These wastes are still dumped in landfill or incinerated leading to green-house gas emissions. Thus, bioconversion of food waste into value-added products such as propionic acid (PA) is a promising approach for developing a bio-based economy and reducing the dependence on non-renewable fossil resources. The aim of the present dissertation was to enhance propionic acid production from food waste through anaerobic fermentation. Accordingly, different batch and semi-continuous fermentation experiments were conducted at mesophilic temperature (30 °C).

Lab-scale batch fermentation tests were carried out to examine the influence of inoculum type, pH-value, and thermal pre-treatment of substrate. Vegan dog food as model of food waste was used as substrate. The selected inocula comprised a mixed bacterial culture selected over 24 months for growth on cellulose, milk, and soft goat cheese. The batch tests were performed at pH 4, pH 6, and pH 8 for both, untreated and pre-treated dog food. Results show that the production of PA and volatile fatty acids (VFAs) in general were clearly dependent on the chosen inoculum and adjusted pH value. The maximum PA production rates and yields were determined for the cheese inoculum at pH 6 using untreated and pre-treated dog food. PA concentration reached 10 g L^{-1} and 26.5 g L^{-1}, respectively. However, the highest VFA concentration of approximately 60 g L^{-1} was obtained when milk inoculum was used to ferment pre-treated dog food at pH 8.

The enhancement of PA production from dog food and food waste were also investigated in a 12 L semi-continuous anaerobic hydrolysis reactor. Three operational runs were carried out at a pH value of 6.0 ± 0.1 over more than 3 months each. Two of the three different types of inocula used for the batch tests, the mixed microbial culture and the culture contained in goat cheese were compared. The results showed that the goat cheese inoculum was more efficient for propionic acid production, resulting in an increase by about 50 %. The highest propionic acid concentration achieved amounted to 139 mmol L^{-1} and 105 mmol L^{-1} using dog food and food waste, respectively. Furthermore, it was observed that propionic acid production was enhanced by a combination of rather high hydraulic retention time (*HRT*) with rather low organic loading rate (*OLR*), ensuring sufficient time for complete processing of the complex organic substrates.

The pre-treatment of fermented dog food and food waste broths as a primary step in propionic acid recovery was evaluated. Two main procedures were involved: removal of large particles from the fermentation broth by using a separation unit followed by removal of the other suspended particles by a submerged microfiltration membrane system with continuous gas bubbling. The separation unit was able to remove more than 86 % of the total suspended solids from the fermentation broth. The microfiltration membrane was successfully employed for separation of particles in the hydrolysate. It has been demonstrated that using the microfiltration membrane with a pore size of 0.1 µm, 0.45 µm, and 0.8 µm allowed about 90 % VFA to pass through the membrane. Moreover, the membrane removed more than 85 % of the total suspended solids (TSS). The highest critical flux of approximately 14 L m^{-2} h^{-1} was observed for food waste broth with a pore size of 0.45 µm and a gas bubbling of 80 m^3 m^{-2} h^{-1}.

Zusammenfassung

Das Streben nach der Minimierung von Abfällen in Verbindung mit der Rückgewinnung von Ressourcen hat die Aufmerksamkeit auf die Verwendung von Lebensmittelabfällen als Ausgangsstoffe für die Herstellung hochwertiger Produkte gelenkt. Weltweit fallen jährlich rund 1,3 Milliarden Tonnen Lebensmittelabfälle an. Diese Abfälle werden immer noch auf Deponien abgeladen oder verbrannt, was zu Treibhausgasemissionen führt. Die biologische Umwandlung von Lebensmittelabfällen in Mehrwertprodukte wie Propionsäure (PA) ist daher ein vielversprechender Ansatz für die Entwicklung einer biobasierten Wirtschaft und die Verringerung der Abhängigkeit von nicht erneuerbaren fossilen Ressourcen. Ziel der vorliegenden Dissertation war es, die Propionsäureproduktion aus Lebensmittelabfällen durch anaerobe Fermentation zu verbessern. Dementsprechend wurden verschiedene Batch- und halbkontinuierliche Fermentationsexperimente bei mesophiler Temperatur (30 ° C) durchgeführt.

Batch-Fermentationstests im Labormaßstab wurden durchgeführt, um den Einfluss des Inokulums, des pH-Werts und der thermischen Vorbehandlung des Substrats zu untersuchen. Als Substrat wurde veganes Hundefutter als Modell für Küchenabfälle verwendet. Die ausgewählten Inokula umfassten eine gemischte Bakterienkultur, die über 24 Monate für das Wachstum auf Cellulose ausgewählt wurde, Milch und Ziegenweichkäse. Die Batchtests wurden bei pH 4, pH 6 und pH 8 sowohl für unbehandeltes als auch für vorbehandeltes Hundefutter durchgeführt. Die Ergebnisse zeigen, dass die Produktion von Propionsäure und anderen flüchtigen Fettsäuren deutlich vom gewählten Inokulum und dem eingestellten pH-Wert abhängt. Die maximale Propionsäure produktionsraten und Ausbeuten wurden für das Käse-Inokulum bei pH 6 unter Verwendung von unbehandeltem und vorbehandeltem Hundefutter bestimmt. Die Propionsäure Konzentration erreichte 10 g L^{-1} bzw. 26,5 g L^{-1}. Die höchste Konzentration an flüchtigen Fettsäuren von ungefähr 60 g L^{-1} wurde erhalten, wenn Milchinokulum verwendet wurde, um vorbehandeltes Hundefutter bei pH 8 zu fermentieren.

Die Verbesserung der PA-Produktion aus Hundefutter und Futterabfällen wurde auch in einem halbkontinuierlichen anaeroben 12 L-Hydrolysereaktor untersucht. Drei Betriebsläufe wurden bei einem pH-Wert von 6,0 ± 0,1 jeweils für mehr als 3 Monaten durchgeführt. Dabei wurden zwei der auch in den Batchtests untersuchten Inokula verglichen, die gemischte mikrobielle Kultur und die in Ziegenkäse enthaltene Kultur. Die Ergebnisse haben gezeigt, dass das Ziegenkäse-Inokulum für die Propionsäure Produktion effizienter war, was zu einer Erhöhung um 50 % führte. Die höchste Propionsäurekonzentration wurde mit 139 mmol L^{-1} unter Verwendung von Hundefutter und mit 105 mmol L^{-1} unter Verwendung von Küchenabfällen erreicht. Darüber hinaus wurde beobachtet, dass die Propionsäure Produktion durch eine Kombination einer relativ hohen hydraulischen Retentionszeit (*HRT*) mit einer relativ niedrigen organischen Beladungsrate (*OLR*) erhöht wurde, da dies eine ausreichende Zeit für die vollständige Verarbeitung der komplexen organischen Substrate sicherstellt.

Weiterhin wurde die Vorbehandlung der Fermentationsbrühen von fermentiertem Hundefutter und Küchenabfällen als erster Schritt im Propionsäurerückgewinnungsprozess untersucht. Hierbei wurden zunächst unter Verwendung einer Trenneinheit große Partikel aus der Fermentationsbrühe entfernt, in der Folge wurden die übrigen suspendierten Partikel durch ein getauchtes Mikrofiltrationsmembransystem abgetrennt. Es wurde gezeigt, dass die Trenneinheit ein effizientes Vorbehandlungsverfahren für den Mikrofiltrationsprozess ist. Die Einheit konnte mehr als 86 % der

gesamten suspendierten Feststoffe aus der Fermentationsbrühe entfernen. Die Mikrofiltrationsmembran wurde erfolgreich zur Abtrennung von Partikeln im Hydrolysat eingesetzt. Die Verwendung der Mikrofiltrationsmembran mit einer Porengröße von 0,1 µm, 0,45 µm und 0,8 µm führte zur Passage ca. 90 % der flüchtigen Fettsäuren einem. Darüber hinaus entfernte die Membran mehr als 85 % der gesamten suspendierten Feststoffe (TSS). Der höchste kritische Fluss von ungefähr 14 L m^{-2} h^{-1} wurde unter Verwendung des Küchenabfallhydrolysats, der Membran mit einer Porengröße von 0,45 µm und einer Begasung von 80 m^3 m^{-2} h^{-1} beobachtet.

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List of publication

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Chapter 1

1 Introduction

1.1 Motivation and scope of the thesis

Growing world population and the growth of the global economy in recent years have led to an exponential increase in consumption of non-renewable resources, depletion of fossil fuels and increase of global warming in addition to an increase in organic waste generation.

Therefore, many research efforts have been made to utilize renewable resources to produce high-value bioproducts such as platform chemicals and biofuels in order to replace non-renewable fossil resources (e.g. oil, coal and natural gas). Food waste which accounts for almost half of the total municipal wastes (Sindhu et al., 2019) is a promising renewable alternative to those conventional resources. According to the Food and Agriculture Organization of the United Nations approximately 1.3 billion tons of food waste are produced every year (FAO, 2011). These wastes which comprise a wide range of organic materials including fruits, vegetables, food residuals, meat etc. are discharged from various sources including households, restaurants and food industries and cause severe environmental pollution (Hafid et al., 2017). Despite of the wide range of disposal methods for food waste which include composting, animal feed, waste landfills, and biogas production (Kim et al., 2020). Unfortunately, these methods still lead to a major concern in tackling worldwide greenhouse gas emissions of carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and ammonia (NH_3), (Sindhu et al., 2019; Yang et al., 2019), while biogas remains a cheap product, due to the lower quality compared to natural gas. In this context, the production of platform chemicals with high value such as propionic acid through fermentation of available and cheap substrates as food waste can offer an efficient and competitive production process in comparison to the use of nonrenewable resources, and, at the same time, it minimizes the mass of food waste.

So far, none of the published researches were focused specifically on propionic acid (PA) production from complex renewable resources such as kitchen or food waste. There also has been no study on the optimization of fermentation process parameters. In addition, reviews on PA production methods are limited. Therefore, the aim of the present thesis was to develop an effective and environmental-friendly method for propionic acid production from food waste. To achieve this goal, different batch and semi-continuous fermentation experiments were conducted to identify the key process parameters for propionic acid production.

1.2 Thesis structure

The second chapter of the thesis provides a comprehensive review of propionic acid as an important platform chemical. The main properties, common uses, and production approaches, with a focus on chemical and biological methods including wild type and metabolically engineered strains inocula, the different substrates, and the best operational conditions for optimizing PA production as well as the recovery techniques for PA from the fermentation broth were reviewed.

The third chapter presents results on the impact of different operational conditions including inoculum type, pH, and thermal pretreatment of the substrate on the PA production in lab-scale batch fermentation tests.

In chapter four, an evaluation of propionic acid production from model and real food waste hydrolysis in a semi-continuous reactor is provided. In this chapter, the production of other VFA and the analysis of

microbial communities during the fermentation as well as the effect of hydraulic retention time (*HRT*) and organic loading rate (*OLR*) are discussed.

The fifth chapter assesses pretreatment methods for removal of large and suspended particles from the fermentation broth using a separation unit followed by a submerged microfiltration membrane system.

Finally, general conclusions are summarized in chapter six.

Chapter 2

2 Propionic acid: Properties, current uses, and production methods: An excellent example of waste bio-valorization

2.1 Introduction

According to estimations, the annual global municipal waste generation is around 2.01 billion tons, which is expected to reach 3.40 billion tons by 2050 (Gardiner & Hajek, 2020; Kaza, 2018). Biomass wastes generated from agriculture sectors are estimated to amount to 5358.54 million tons per year (Duque-Acevedo et al., 2020). These wastes are often disposed in landfill or sent to incineration with limited recovery of resources and high emission of greenhouse gases.

These wastes provide excellent raw material for exploitation and valorization to obtain products with an added value. Propionic acid (PA) is one of these products that has many industrial applications. According to the U.S. Department of Energy, PA is among the top 30 added value chemicals (Werpy, 2004). The acid and its derivatives have gained increasing interest in agriculture, food and pharmaceutical industries. At present, the production of PA is estimated to amount to approximately 500 thousand tons per year with an annual growth rate of 2.5 % (Du et al., 2015; Mohan & Sivaprakasam, 2016). The global market price of PA is valued at 2454 million USD in 2020, while it is expected to reach 2922 million USD by the end of 2026.

This chapter therefore focuses on PA as important platform chemical. PA is usually obtained through chemical synthesis of petrochemical substrates, while biological methods involve the partial oxidation of sugar. Thus, it provides a good example of how wastes with high carbohydrate contents could be exploited. The chapter also summarizes the main properties, uses and production methods for PA. In addition, the most common PA producing bacteria including wild type and metabolically engineered strains and their metabolic pathways are introduced. The so far known best operational conditions for optimizing microbiological PA production are also highlighted in this chapter.

2.2 Propionic acid properties

Propionic acid (PA) is a saturated short chain fatty acid, belonging to the carboxylic acid family. The acid is weak, non-volatile, colorless with unpleasant odor, soluble in water and alcohol (Xu et al., 2011). Its chemical and physical properties are listed in Table 2.1. PA is involved in the metabolism of a number of living organisms and naturally occurs on human skin and in human gut, plants, fruits and other foods such as milk, cheese, and yoghurts. At the same time, high dose of PA can be toxic and may pose risks to human health if absorbed into the body by inhalation or ingestion (Gad, 2014).

Table 2.1: Chemical and physical properties of propionic acid.

Molecular formula	Molar mass	Density	Melting point (°C)	Boiling point (°C)	pKa at 25 °C	Heat of combustion (kJ mol^{-1})
$C_3H_6O_2$	74.08	0.99	−22.4	141.1	4.88	1536

2.3 Current uses

PA is widely used as preservative in animal feed and human foods because of its bactericidal, fungicidal, insecticidal, and antiviral effects (Du et al., 2015). In 1984, the US Food and Drug Administration authorized the use of PA and its ammonium, sodium and calcium salts as preservatives for various foods. For example, it is commonly added to bread, dairy products, and cheese to preserve and enhance their properties (Fröhlich-Wyder et al., 2017). In parallel, FAO and the World Health Organization (WHO) have regulated the use of PA as food additive (Samel et al., 2018).

PA is also an important chemical intermediate for the synthesis of cellulose fibers, herbicides, perfumes, and pharmaceuticals. It can also be employed as precursor for production of value-added compounds, such as acetoin (Schmidt et al., 2018) and propylene (Stowers et al., 2014).

2.4 Production methods

At present, industrial production of PA is almost exclusively done by chemical synthesis using petrochemical feedstocks (Ahmadi et al., 2017). The market price for PA production from the petrochemical route is about 1.0 USD kg^{-1}, while the price for the PA production from the biotechnological route is about 1.5–2.0 USD kg^{-1} (Liu et al., 2012). However, biological methods gain more attention in recent years because of the necessity to reduce the dependence on petroleum and mitigate environmental impacts (Ahmadi et al., 2017). This section summarizes the different chemical and biological methods used so far for PA production.

2.4.1 Chemical methods

2.4.1.1 Carbonylation of Ethylene

The process for the synthesis of PA by carbonylation of ethylene was chemically described in 1941 by Walter Reppe (1892-1969), a professor of chemistry at BASF company, Germany. In Reppe's synthesis, ethylene reacts with carbon monoxide and water in the presence of Ni $(CO)_4$ as catalyst. The reaction occurs at high pressure (100–300 bar) and high temperature (250–320°C). This method is characterized by simple application, low raw material costs, high conversion, and high yield. However, the use of the highly toxic catalyst and the extreme operating conditions are still the major drawbacks. In 1960, BASF built a new large-scale plant that continues to produce propionic acid until today.

In recent years, the focus has shifted to facilitating the industrial adaptation of the previous procedures by using new catalytic methods and more inexpensive and environmentally benign reagents. For example, the carbonylation of ethylene, with a halide promoted Mo catalyst represents the first efficient carbonylation process using a Cr group metal as the active catalytic species at low to moderate pressure and temperature (Zoeller et al., 1997). Additionally, rhodium with hydroiodic acid, ethyl iodide and lithium iodide has been used as an alternative catalytic activity at low water content (< 4.6 wt %)(Hu et al., 2020).

2.4.1.2 Oxidation of propionaldehyde

Propionic acid also can be obtained through the Fischer-Tropsch process in which propionaldehyde, produced from the pyrolysis of fuel and wood under high pressure (200–280 bar) and temperature (130–150°C), is oxidized to propionic acid at very mild conditions of 40–50°C using rhodium as a catalyst (Samel et al., 2018). Although very pure PA is produced, this method is still less common and became obsolete.

2.4.1.3 Other methods

Two other methods are described for the chemical production of PA: (i) the direct oxidation of hydrocarbons, in this process Naphtha is preheated at 170°C and oxidized with air at 40–45 bar resulting in a mixture of crude acid in which propionic and other acids are obtained from this mixture by extractive dehydration followed by fractional distillation (Samel et al., 2018); (ii) the Larson process, in which ethanol and carbon monoxide are converted to propionate using boron trifluoride as catalyst (Boyaval et al., 1994).

2.4.2 Microbiological methods

Anaerobic fermentation is a promising alternative method for production of PA by the utilization of renewable resources such as organic waste (Atasoy et al., 2018; Sindhu et al., 2019). Commonly, PA is produced during the acidogenesis phase of anaerobic fermentation in which hydrolyzed organic compounds (e.g. sugars or amino acids) are transformed to short chain volatile fatty acids (e.g. formic acid, acetic acid, propionic acid, butyric acid and valeric acid), alcohols (e.g. methanol and ethanol), carbon dioxide and hydrogen (Kumar & Samadder, 2020; Li et al., 2019).

The first description of propionic acid was by Johann Gottlieb in 1844, while the first observation of PA that was derived from the fermentation of different substrates including sugars, alcohols, and organic acids was reported by Strecker in 1854, and subsequently by Pasteur and Fitz in 1879 (Xu et al., 2011).

Propionic acid can be synthesized by many microorganisms including *Propionibacterium*, *Veillonella* (Distler & Kröncke, 1981), *Clostridium* (Johns, 1952), and *Selenomonas* (Scheifinger & Wolin, 1973), which are able to produce PA from different carbon sources under anaerobic conditions (Boyaval et al., 1994). Primary candidate for the development of a biological production process of PA is the genus *Propionibacterium,* gram-negative, rod-shaped, nonmotile, none spore forming bacteria, and facultative anaerobes (Hsu & Yang, 1991), which can utilize a wide range of carbon sources to produce this acid as an end fermentation product. *Propionibacterium* belongs to the phylum *Actinobacteria*, and presently comprises approximately 16 species including pathogens associated with human and animal diseases. These species have been grouped as either classical or cutaneous *Propionibacteria* based on characteristic phenotypes and source of isolation (Table 2.2).

Table 2.2: Species of *Propionibacterium* genus and their niches

Cutaneous		
Species	**Niche**	**Reference**
P. acnes	Human skin oral cavity, and large intestine.	(Alexeyev et al., 2009)
P. avidum	Human skin moist area (e.g. sweat gland)	(Legaria et al., 2019)
P. propionicum	Human lacrimal duct	(Corvec, 2018)
P. granulosum	Human skin	(Branger et al., 1987)
P. lymphophilum	Human skin and urinary tract	(Ikeda et al., 2017)
P. acidifaciens	Human mouth	(Obata et al., 2019)
P. propionicus	Thoracic, abdominal, blood and the urinary tract.	(Pasic et al., 2004)
P. damnosum	Non-pasteurized Spanish green olives	(Lucena-Padrós et al., 2014)
P. namnetense	Human bone infection	(Aubin et al., 2016)
P. olivae	Non-pasteurized Spanish green olives	(Lucena-Padrós et al., 2014)
Classical		
Species	**Niche**	**Reference**
P. freudenreichii	Swiss-type cheeses	(El Soda & Awad, 2014)
P. acidipropionici	Dairy products	(Fröhlich-Wyder et al., 2017)

P. jensenii	Dairy products	(Fröhlich-Wyder et al., 2017)
P. thoenii	Dairy products	(Fröhlich-Wyder et al., 2017)
P. microaerophilum	Olive mill wastewater	(Koussémon et al., 2001)
P. australiense	Granulomatous Bovine Lesions	(Bernard et al., 2002)
P. cyclohexanicum	Spoiled orange juice	(Kusano et al., 1997)

Among these, in particular*, P. freudenreichii*, *P. jensenii, P. thoenii*, and *P. acidipropionici* species are of the most biotechnological interest for PA production, due to their enzymatic systems and the ability to utilize various carbon sources. Several batch and semi-continuous fermentations of different substrates using pure and mixed cultures have been investigated. Table 2.3 summarizes the most frequently applied methods for PA production found in literature. As can be seen, *P. acidipropionici* and *P. freudenreichii* were the most studied species as pure cultures for PA production from simple and complex substrates. However, the microbial propionic acid production is still not economically competitive to the petrochemical routes. In order to improve the competitiveness, methods for strain metabolic engineering have been proposed.

Although genomic information is available and several endogenous plasmids have been observed in *Propionibacterium*, metabolic engineering of these bacteria to enhance PA production is still in its infancy due to their thick cell walls, the restriction-modification systems, and high guanine-cytosine (GC) content (Liu et al., 2015).

To date, only a few studies are found in literature that report on improved PA production using metabolically engineered *Propionibacterium*. Several strategies have been tested including gene knockouts, in which acetate kinase gene (ack) was knocked out in *P. acidipropionici*, gene overexpression of glycerol dehydrogenase in *P. jensenii* (Navone et al., 2018), as well as the expression of heterologous genes in *P. freudenreichii.* Table 2.4 shows some of the metabolic engineering strategies performed in *Propionibacteria* to improve PA production as well as the achieved PA productions and yields.

Table 2.3: Some of methods applied for propionic acid production. Only the maximum PA concentrations, production rates, and yields are given.

Species	Substrate	Conditions	Concentration [g L^{-1}]	Productivity [g L^{-1} h^{-1}]	Yield [g g^{-1}]	References
Propionibacterium thoenii.[*]	Glycerol	5 L batch membrane bioreactor 30 °C and pH 7	40 ± 2	0.3	_	(Boyaval et al., 1994)
Propionibacterium acidipropionici.[*]	Lactose	2.2 L reactor 30 °C and pH 4.5 to 7.12	48	_	_	(Hsu & Yang, 1991)
Propionibacterium freudenreichii subsp. shermanii.[*]	Glucose & Glycerol	5-L batch stirred-tank reactor 32°C and pH 6.5	_	0.18–0.23	0.54–0.65	(Wang & Yang, 2013)
Propionibacterium acidipropi-onici.[*]	Lactose	2-L batch glass reactor 30°C and pH 7.1 and 5.0	_	Appx. 1	Appx. 0.66	(Jin & Yang, 1998)
Propionibacterium acidipropionici.[*]	Glycerol	7-L batch reactor 30°C and pH 7	44.62 ± 1.12	0.20 ± 0.0075	_	(Zhu et al., 2010)
Propionibacterium freudenreichii.[*]	Glucose	7.5 L batch fibrous-bed bioreactor 35 °C and pH 6	136.23 ± 6.77	0.47 ± 0.022	_	(Chen et al., 2013a)
Anaerobic sludge	Glycerol	2 L Fed-batch reactor (4 g L^{-1}) pH 7	22.6	0.45	_	(Chen et al., 2016)
Anaerobic sludge	Crude glycerol	Anaerobic fluidized bed reactor	_	4.09 ± 1.24	0.48 ± 0.06	(Nazareth et al., 2018)
Anaerobic sludge	Crude glycerol	Anaerobic fluidized bed reactor 30 °C and pH 4.5	_	1.35 ± 0.14	0.57	(Paranhos & Silva, 2020)
Propionibacterium freudenreichii.[*]	Syrup (containing: glucose, fructose, ash, and protein)	250 mL batch stirred-tank reactor 30 °C and pH 6.5 to 7	6.43	_	_	(Hashemi & Roohi, 2019)
Propionibacterium acidipropionici.[*]	Food waste & waste activated sludge	Two stages Immobilization fermentation by fibrous bed bioreactor 21 °C and pH 8.5	35.45	0.075	0.62	(Li et al., 2016)
Anaerobic consortium anaerobic reactor	Synthetic wastewater	0.25 L fed-batch reactor 30 °C and pH 7	1.22 ± 0.06	_	0.23	(Dahiya et al., 2020)
P. acidipropionici.[*]	Soy molasses (sucrose & stachyose)	1 L- Stirred-tank bioreactor	_	0.8	0.42	(Yang et al., 2018)

		32 °C and pH 6.5				
P. acidipropionici.[*]	Glucose	5 L- Fed batch stirred bioreactor 30 °C and pH 6	75.9	0.32	_	(Liu et al., 2016b)
Propionibacterium acidipropionici.[*]	Hemicellulose	5 L- Stirred bioreactor 30 °C and pH 6	71.8	0.28	_	(Liu et al., 2012b)
P. acidipropionici.[*]	Glucose or Glycerol	5 L- Anaerobic reactor 32 °C and pH 7	17.3	2.94		(Zhang et al., 2015)
Propionibacterium acidipropionici.[*]	Glycerol	3 L- Batch bioreactor 32 °C and pH 6.5	33.00	0.53	_	(Dishisha et al., 2015)
Propionibacterium freudenreichii.[*]	Molasses (sucrose, glucose, and fructose)	7.5 L- Stirred-tank reactor 35 °C and pH 6	91.89 ± 4.59	_	_	(Feng et al., 2011)

* Pure culture

Table 2.4: Examples of metabolic engineering strategies performed in *Propionibacteria* to improve the propionic acid production.

Strains	Genetic modification	Substrates	Conditions	Concentration [g L^{-1}]	Productivity [g L^{-1} h^{-1}]	Yield [g g^{-1}]	References
P. acidipropionici ATCC 4875	ack gene (encoding acetate kinase) knock-out (ACK-Tet)	Glycerol	Fibrous-bed bioreactor (FBB) 32 °C and pH 7	106	_	0.54–0.71	(Zhang & Yang, 2009)
P. freudenreichii subsp. shermanii DSM4902	Ps (pKCOA1)	Glucose/glycerol	5-L batch stirred-tank bioreactor pH 5	_	0.41	0.62	(Wang et al., 2015)
Propionibacterium jensenii ATCC 4868	pZGX04-gldA	Glycerol	3-L fed-batch bioreactor 32 °C and pH 5.9	27.31	0.152	_	(Zhuge et al., 2015)
P. acidipropionici ATCC 4875 and *P. acidipropionici* ATCC 55737	F3E8	Glucose	2 L fermenter 32 °C and pH 6.5	_	0.84 ± 0.02	0.55 ± 0.02	(Luna-Flores et al., 2017)
P. jensenii	Overexpression of ppc and deletion of ldh	Glycerol	Fed-batch anaerobic fermentation 32 °C and pH 5.9	33.21 ± 1.92	0.13 ± 0.01	_	(Liu et al., 2016a)
P. jensenii	Overexpression of malate dehydrogenase (MDH), and fumarate hydratase (FUM) (pZGX04-mdh-fumC)	Glycerol	Fed-batch anaerobic fermentation 32 °C and pH 5.9	39.43 ± 1.90	0.60 ± 0.03	0.16 ± 0.01	(Liu et al., 2015)
P. acidipropionici	Knockout of ack gene	Glucose	5-L stirred-tank fermenter 32 °C and pH 6.5	_	0.15 ± 0.01	0.45 ± 0.01	(Suwannakham et al., 2006)
Propionibacterium jensenii ATCC 4868	Overexpression of arginine deaminase and glutamate decarboxylase arcA, arcC, gadB , gdh , and ybaS)	Glycerol	Batch reactor 32 °C	10.81	_	0.56	(Guan et al., 2016)

2.5 Main pathways for propionic acid biosynthesis

2.5.1 Succinate pathway

The succinate pathway initiates with generation of metabolic intermediates such as glucose or glycerol. These molecules are converted to phosphoenolpyruvate, which is directly converted to oxaloacetate (OAA) by PEP carboxylation enzymes or to pyruvate, the latter functions as central metabolite for the production of other compounds such as lactate, alanine, acetate, or acetyl-CoA. Pyruvate is converted into oxaloacetate (OAA) which is further converted to succinate, and then to propionate through succinyl-CoA, and propionyl-CoA (Figure 2.1. a).

Two different mechanisms of how the microorganisms are utilizing this pathway were reported: the sodium pumping methylmalonyl-CoA and the transcarboxylase cycle (Wood-Werkman cycle). Bacteria such as *Bacteroides fragilis, Veillonella, Selenomonas ruminantium,* and *Propionigenum modestum* use the succinate pathway via methylmalonyl-CoA derived from succinate to propionyl-CoA with the pumping of two sodium ions across the cell membrane. In the Wood-Werkman cycle, the decarboxylation step is replaced by the methylmalonyl-CoA in *Propionibacterium acidipropionici* (e.g. *P. freudenreichii* and *P. shermanii*). In this step, a carboxyl group is transferred from methylmalonyl-CoA to pyruvate to generate propionyl-CoA.

2.5.2 Acrylate pathway

In this pathway, lactate is oxidized anaerobically to propionate, acetate and carbon dioxide with consumption of NADH. The key steps of the pathway are catalyzed by several enzymes as is depicted in Figure 2.1. b.

Only a few number of microorganisms are known to produce PA through this pathway including *Clostridium propionicum* (Akedo et al., 1983), *Megasphaera elsdenii* and *Prevotella ruminicola*. Lactate is not the only substrate utilized by these types of microorganisms, other substrates such as serine, alanine and ethanol can also be used for PA production via the acrylate pathway.

2.5.3 Propanediol pathway

The main steps of propanediol pathway are shown in Figure 2.1.c. Here, fucose, rhamnose or lactate are converted to 1,2-propanediol by several enzymes. 1,2-propanediol is converted to propionaldehyde by propanediol dehydratase. Subsequently propionaldehyde is either transformed to propanol or to propionyl-CoA which is further converted to propionate by phosphotransacylase and propionate kinase yielding one ATP. *Salmonella typhimurium* and *Roseburia inulinivorans* are the most common bacteria using this pathway to generate propionic acid from different substrates.

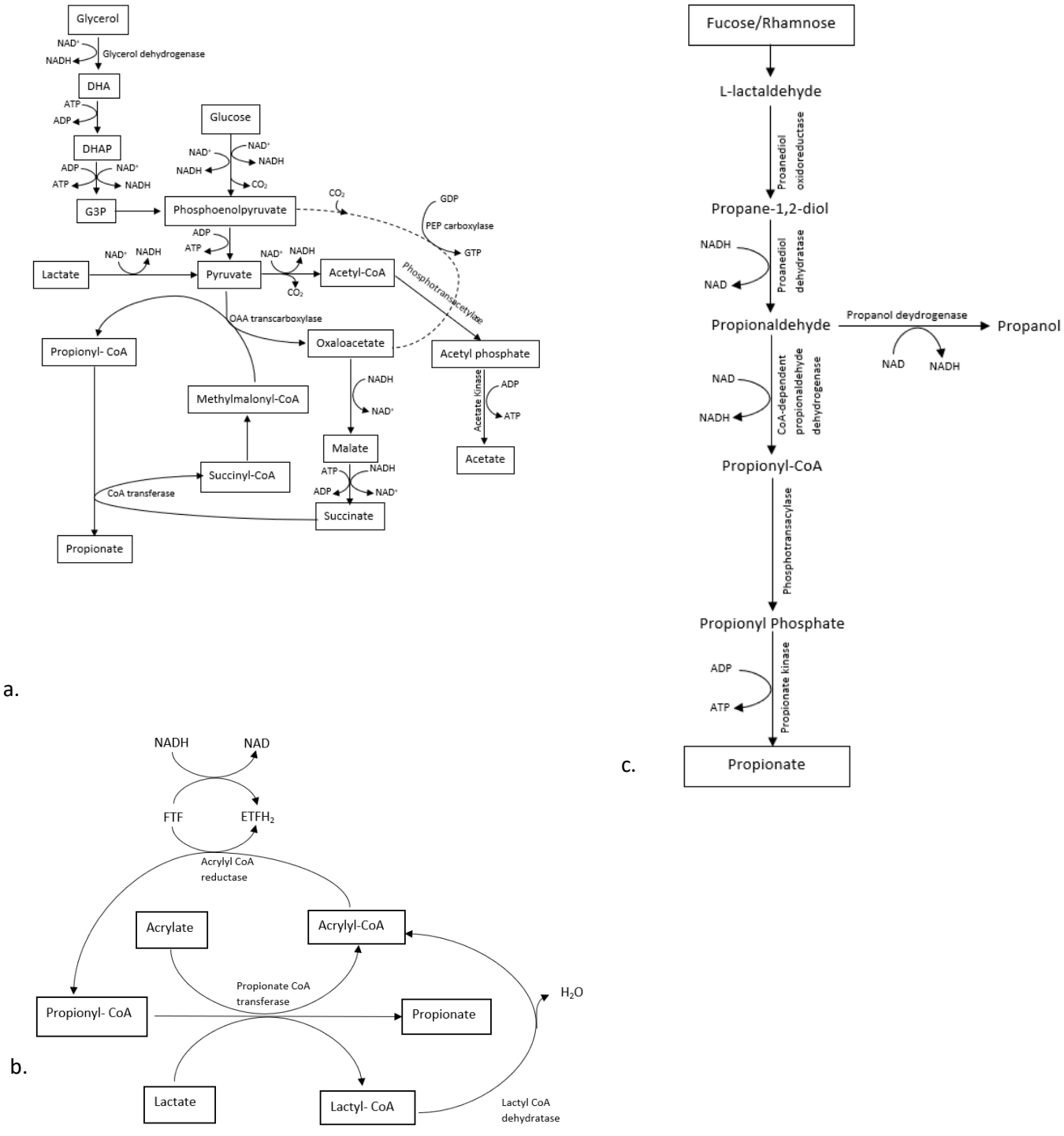

Figure 2.1:Propionic acid production metabolic pathways (Liu et al., 2016a; Liu et al., 2015), (a) Succinate pathway, (b) Acrylate pathway, and (c) Propanediol pathway.

2.6 Substrate for propionic acid biosynthesis

A variety of substrates, as presented in Table 2.3, have been studied for their potential for PA production ranging from simple (e.g. glucose, lactose, lactate, glycerol) to complex substrates generated from domestic and industrial wastes such as food waste, agriculture waste, molasses (Quesada-Chanto et al., 1994; Yang et al., 2018), and cheese whey (Jain et al., 1991)

Among them, glucose, lactose, and glycerol are the most investigated substrates for PA production. Several studies have shown that glycerol can be a suitable feedstock for PA production with a higher propionic acid yield and low acetic acid production. The large amounts generated from biodiesel industry also make glycerol a promising low-cost feedstock for this process (Zhu et al., 2010). However, glycerol has a high reduction degree, which leads to reduced cell growth and productivity, especially if it is used as sole carbon source. To overcome this problem, co-fermentation of glycerol with other carbon sources such as glucose or potato juice has been proposed by some researchers (Dishisha et al., 2013; Wang & Yang, 2013; Zhang et al., 2015). To improve PA production, further specific microorganisms such as propionic acid-tolerant *P. acidipropionici* (Zhu et al., 2010), and metabolically engineered *P. jensenii* (Zhuge et al., 2014) were used for glycerol fermentation.

Organic waste such as food and kitchen wastes appear to be suitable feedstocks for the production of PA due to their availability as renewable sources and high carbohydrate contents. Several studies reported that food waste could be a suitable substrate for value added products and energy generation (Hafid et al., 2017; Sindhu et al., 2019). However, only few studies were investigating the PA production from these wastes. For example, Chen et al. (2013b) used a new strategy to improve PA production from food waste by mixing food waste with sludge in a two-stage fermentation process. The study showed that a large amount of lactic acid was produced in the first stage which enhanced PA production in the second stage. Based on this result and the fact that *Propionibacteria* utilize lactate much faster than sugar (Tyree et al., 1991), the same strategy was used by Li et al. (2016) to produce PA from lactate delivered from the first fermentation stage using *Propionibacterium acidipropionici.*

Cheese whey is another waste commonly investigated for PA production due to its high lactose concentration, the readily fermentable organic content (Li et al., 2020), and the large quantity generated from the dairy industry (Sahoo et al., 2020). Several techniques including continuous fermentation with high cell retention (Gupta & Srivastava, 2001), enzyme inhibitors (Morales et al., 2006), metabolic engineered bacteria (e.g. enhanced trehalose synthesis mutant) (Jiang et al., 2015), and two stage fermentation (Aladár & Áron, 2017) have been employed to enhance the production of PA from cheese whey.

As the most abundant bioresource, cellulosic biomass also could be a suitable substrate for PA production. However, very few studies investigated this substrate, mostly due to its complex structure and the low hydrolysis rate. For example, a high concentration (71.8 g L^{-1}) of propionic acid was produced from a hemicellulose hydrolysate of corncob molasses in a study of Liu et al. (2012b), where the authors mention that hemicellulose can be a good substrate for efficient propionic acid production by the *P. acidipropionici* strain. Habe et al. (2015) reported that a concentration of 9 g L^{-1} of PA was observed in the fermentation of lignocellulose by *Rhodococcus hoagie* strain. Similar results were observed also by Li et al. (2018) who found that PA accumulated during anaerobic digestion of hemicellulose and lignocellulose.

2.7 Reactor types and operation modes

Like for other microbial production processes, two basic cultivation types are commonly used for PA production, comprising attached (immobilized-cell fermentation) and suspended growth (free-cell fermentation). Accordingly, different types of reactors have been developed, which can be operated in batch, fed-batch, or continuous fermentation mode. The most common reactor types and their operation modes used for PA production are listed in Table 2.3.

As an example of attached growth cultivations is the fibrous fixed bed reactor introduced by Lewis and Yang (1992), in which *Propionibacterium acidipropionici* cells were immobilized by natural attachment to fiber surface. A maximum PA productivity of approximately 40 g $L^{-1}d^{-1}$ was obtained at a dilution rate of 2.5 d^{-1} for four months without any clogging, degeneration, or contamination problems. The authors reported that this type of bioreactor could be suitable for industrial propionic acid production as the achieved productivity was four times higher than that of a conventional batch fermentation. However, in many cases clogging due to high concentrations of suspended solids is the main issue in this type of reactor. To avoid clogging, a fluidized bed reactor has been used. For example, Nazareth et al. (2018) used a fluidized bed reactor to produce PA from crude glycerol, the results showed that PA was the major acid produced during the process with a maximum productivity of 4.0 g L^{-1} h^{-1}.

Based on suspended growth, continuous stirred tank reactors (CSTR) were widely used for the production of propionic acid. Four different types of daily batch-fed single-stage CSTR, continuously fed single-stage CSTR, daily batch-fed two-phase CSTR, and daily batch-fed non-mixed single-stage reactors were evaluated by Kim et al. (2002) for process stability at mesophilic (35°C) and thermophilic anaerobic digestion (55°C). The results showed that all reactors except the non-mixed reactor showed increases in PA concentrations especially when the *OLR* increased.

Another example of a reactor that is based on suspended growth and was used for the production of propionic acid is the anaerobic membrane bioreactor (AnMBR). PA of high quality and with a productivity of 1 g L^{-1} h^{-1} was achieved by Boyaval et al. (1994) in a continuous fermentation of glycerol with a membrane bioreactor using *Propionibacterium thoenii.* The authors mention that this process could be of great interest for industries that need high-quality propionic acid.

2.8 Effect of functional and operational conditions

Like other microbiological processes, PA production is affected by several factors. In the literature, different functional (e.g. temperature, pH) and operational parameters (e.g. *OLR*, *HRT*) were reported as important. However, most of these researches investigated one condition at a time, there are only a few studies evaluating their interactive effects. The impact of different parameters on PA production are discussed in the following sections.

2.8.1 pH-value

pH plays a critical role in the PA production process, as it is directly affecting microbial activity by inhibiting enzymes if beyond the values tolerated by the organisms. Several studies concluded that the optimal pH for the propionic acid bacteria is in the range of 6-7 (Fröhlich-Wyder et al., 2017; Irlinger et al., 2017). However, the optimal pH range for the process vary from study to study depending on the type of feedstock, inoculum source and reactor operational conditions.

For example, The highest PA concentration range between 5.40 and 6.50 g L^{-1} was observed from the fermentation of food waste and anaerobic sludge at pH 6 in a study by Lim et al. (2008). Similar results

were also achieved by Jiang et al. (2013) who obtained about 6 g L^{-1} of PA as the highest concentration from a similar type of fermentation and at the same pH value. In a study by Li et al. (2013), the highest PA concentration was observed at pH 8 during the co-fermentation of food waste and sludge inoculated with *P. acidipropionici*, while production rapidly decreased with the decrease of pH and was severely inhibited at pH 10. Similar results were observed by Horiuchi et al. (2002) who found that the highest PA concentration was obtained at pH 8. Higher concentration of 1.9 gL^{-1} was observed at pH 9 (compared to the other pH values that applied to 5, 6, 7, 8, 10, and 11) from the fermentation of food waste using anaerobic sludge as inoculum by Dahiya et al. (2015), while the production rate was negligible at pH 6.

On the other hand, results by Wang et al. (2006) indicated that the optimal PA production occurred when the pH decreased to 5.5 in the fermentation of organic wastewater. Hsu and Yang (1991) also found that acidic pH improved PA production from lactose by *Propionibacterium acidipropionici*. The propionic acid yield increased from 46 % at pH 5.5 to 62 % at pH 4.5 for rich media and from 38 % at pH 5.5 to 47 % at pH 4.93 for low nutrient media.

2.8.2 Temperature

Temperature is another important factor influencing PA fermentation. Many studies reported that 30 °C is the optimal temperature for propionic acid bacteria (Hettinga & Reinbold, 1972; Seshadri & Mukhopadhyay, 1993). However, studies on the influence of temperature on PA production from various waste sources as substrate are limited and contradictory.

For example, Quesada-Chanto et al. (1994) found that the best temperature for PA production from molasses was 37°C. On the other hand, Jiang et al. (2013) determined 45 °C to be the most suitable temperature for the fermentation of food waste, while 30 °C was the optimal temperature in the study by Coral et al. (2008). They tested the PA production by *Propionibacterium acidipropionici* from different carbon sources including sugarcane molasses, glycerol, and lactate in small batch fermentations at 30°C and 36 °C. The accumulation of PA was reduced by increasing the temperature from 37 °C to 45 °C for 8 h in a continuously-stirred tank reactor (CSTR) fed with industrial wastewater in Sivagurunathan et al. (2014) study.

2.8.3 Hydraulic retention time (*HRT*)

HRT, defined as the ratio between the reactor volume and the flow rate, represents the time that substrate and microbial culture stay inside the reactor (David et al., 2019). Therefore, selecting proper *HRT* can avoid wash-out of slow-growing bacteria (e.g. propionic acid producing bacteria). An increase in *HRT* can enhance the process stability for PA production as the microorganisms have more time to process the substrate.

It had been reported that PA production increased with an increase in *HRT* in the acidic fermentation of synthetic wastewater (Kida et al., 1993). Similar results were observed by Dinsdale et al. (2000) who found that the increase of the *HRT* from 20 h to 95 h and from 11 h to 24 h led to increasing PA production during the acidogenic fermentation of whey and paper mill effluent, respectively.

On the contrary, Paranhos and Silva (2020) found that the production of PA increased by decreasing the *HRT* in the fermentation of glycerol. The results of (Elefsiniotis & Oldham, 1994) also showed higher PA yields and fraction at shorter *HRT* of 6 h compared to 9 h, 12 h, and 15 h in acidogenic fermentation of primary sludge.

The findings are contradictory and it was probably due to the different inocula, substrate, and operating conditions applied in these studies. Therefore, more research is needed to investigate the role of *HRT* on PA production, even more so as most of the mentioned studies focused on methane or hydrogen production rather than propionic acid.

2.8.4 Organic loading rate

Organic loading rate (*OLR*) which is calculated from the substrate concentration and hydraulic retention time, indicates the amount of organic substrate fed into the reactor daily per unit reactor volume (Lee et al., 2014). It can be expressed in terms of chemical oxygen demand (COD), Volatile solids (VS), volatile suspended solids (VSS) or dissolved organic carbon (DOC). The optimal range of *OLR* depends on the chemical characteristics of the organic substrates, therefore, long-term bench-scale studies are usually needed to determine the optimal *OLR* for a particular condition (Labatut & Pronto, 2018).

For a given *HRT*, a high *OLR* means high substrate concentration, hence the yield or production of PA increases with increasing *OLR* within a certain range. However, at higher *OLR* achieved through decreasing *HRT*, lower PA production may be obtained, due to lower hydrolysis efficiency and the washing out of slowly growing PA producers. Although the influence of *OLR* on PA was very limited according to literature, it has been observed that the increase in *OLR* improves the production of PA. For example, Yu et al. (2002) reported that the percentage of PA increased from 13 % to 41 % of the total VFA concentration, when the *OLR* increased from 4 to 24 kg COD $m^{-3}{\cdot}d^{-1}$ in an up-flow reactor operated with synthetic wastewater at mesophilic conditions. Bardi and Aminirad (2020) demonstrated that PA accumulated at *OLR* of 6.5 gL^{-1} more than at 9.5 g L^{-1} and 14 g L^{-1} in anaerobic co-fermentation of food waste and sewage sludge. The maximum PA concentration in the study of (Paranhos & Silva, 2020) also was obtained at higher *OLR* in a mesophilic (30 °C) anaerobic fluidized bed reactor (AFBR). The results showed that the maximum PA yield of 0.57 g g^{-1} glycerol was achieved at an *OLR* of 160.60 kg COD m^{-3} d^{-1}.

The difference of the optimal *OLRs* observed in the mentioned studies could be attributed to the type of inoculum, reactor configuration, the type of substrate and the other operational conditions used in those works, which may interfere with the metabolic pathways.

2.8.5 C/N ratio

The performance of anaerobic fermentation is significantly affected by feedstock total organic carbon (TOC), total nitrogen (TN), and their ratio (C/N) through influencing the microbial metabolism. Generally, C/N ratios ranging from 15 to 70 have been used for anaerobic fermentation, while a C/N ratio range of 20–30 is considered to be the optimum condition for anaerobic fermentation (Liu et al., 2008). However, most of these studies focused on the influence of C/N ratio on biogas and VFAs production in general, not on the production of PA specifically. It has been reported that the nitrogen content which is derived mainly from proteins in the substrate is necessary for *Propionibacteria* (Quesada-Chanto et al., 1998). Furthermore, high nitrogen contents can result in levels of ammonia toxic for the other microorganisms which are the main *Propionibacteria* competitors.

Dishisha et al. (2015) demonstrated that the PA production rate was significantly influenced by yeast extract concentration as nitrogen source when they studied the impact of C/N ratio on PA production in batch fermentation at pH 6.5. The maximum PA production rate of 0.53 g L^{-1} h^{-1} was achieved by using the optimum C/N ratio of 3:1.

In the contrary, Fu et al. (2012) who studied the effect of C/N ratio on butyric acid production from textile wastewater sludge by anaerobic digestion, the authors concluded that optimum butyric acid

production was found at a C/N ratio higher than 20, whereas PA was produced at higher concentrations at C/N ratio of 50 and 60 with approximately 9.27 and 9.75 g L^{-1}, respectively. Lin and Lay (2004) also found that PA fractions increased to 90 % when the C/N ratio increased to 130 in the sucrose fermentation and by using anaerobic sewage sludge as seeding material.

However, it is difficult to establish a clear pattern, due to the limited and inconsistent studies available in literature. More research is needed to understand the impact of C/N ratio on the PA production process from different feedstock.

2.8.6 Trace elements

Metals such as Zn, Co, Cd, Cu, Ni, Pb, and Cr play an important role in the anaerobic fermentation process. These elements at trace concentrations can function as co-factors for enzymatic reactions, and biomass stimulants. They can also serve as electron acceptors in heterotrophic or as electron donors in autotrophic pathways (Dahiya et al., 2020). While high concentration levels of these elements can have inhibitory effects on certain reactions or be toxic for some microorganisms.

Recently, elements such as cobalt, nickel, zinc and iron have been utilized to improve PA production. For example, Dahiya et al. (2020) studied the PA production at different concentrations of cobalt (Co) and zinc (Zn) in batch experiments. The authors found that the optimal concentrations for increased PA fraction were at 0.10 mM Co^{2+} and 0.16 mM Zn^{2+}. Kim et al. (2002) also reported that the PA production was enhanced by addition of Ca, Fe, Co, and Ni in a thermophilic anaerobic digestion.

In contrast, in other studies also the consumption of PA was found to be faster when adding trace elements during the fermentation of food waste in a study by Capson-Tojo et al. (2018). In which (100 mgL^{-1}) Fe, (1 mgL^{-1}) Co, (5 mgL^{-1}) Mo, (5 mgL^{-1}) Ni, (0.2 mgL^{-1}) Se, (0.2 mgL^{-1}) Zn, (0.1 mgL^{-1}) Cu, and (1 mgL^{-1}) Mn were supplied to the reactor. Similar results were reported by Bardi and Aminirad (2020) who found that the PA concentration was reduced from 1500 mg L^{-1} to 500 mg L^{-1} when Fe (5000 mg L^{-1}), Ni (200 mg L^{-1}), Zn (320 mg L^{-1}), and Mo (2.2 mg L^{-1}) were added during the co-fermentation of food waste and sewage sludge. Jiang et al. (2017) observed that Se, at a concentration of 0.261 mg L^{-1}, has a key role in promoting the degradation rate of propionic acid in 250 mL batch experiments using digested food waste; while (0.33 mg L^{-1}) Mo and (1.035 mg L^{-1}) Co had a modest effect on increasing PA degradation rate.

However, it seems that the effects of these metals are highly dependent on the other process parameters, therefore, it is necessary to evaluate and clarify the effects of these elements more specifically for PA production process.

2.9 Downstream processes

Propionic acid recovery from the fermentation broth is a challenge, due to the complex mixture of various organics containing biomass, unhydrolyzed substrates, inorganic salts and by-products generated during fermentation. In the last years, several techniques for the propionic acid removal and recovery from different aqueous solutions and fermentation broths have been proposed comprising reactive extraction (Keshav et al., 2009b), membrane systems, electrodialysis (Zhang et al., 1993), adsorption, and distillation (Karp et al., 2018).

Reactive extraction is the technique for propionic acid recovery that has been studied most (Gu et al., 1998; Keshav et al., 2009b; Wang et al., 2009). Here, several organic solvents such as hexane, toluene, kerosene, ethyl acetate, and octanol are used. The separation yields achieved with this technique

depend on the concentration of the extracting agent, the type of diluent and pH of the solution. However, due to the high toxicity of these solvents, an alternative new solvent group is being investigated to replace the conventional solvents. For example, Ayan et al. (2020) used ionic liquids to extract PA from aqueous solutions with different concentrations of PA. In the study, mainly hexyl-3-methylimidazolium hexafluorophosphate ([HMIM][PF6]) and 1-hexyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide ([HMIM][Tf2N]) were utilized as diluents, and tributyl phosphate (TBP) was utilized as extractant. The results showed an extraction efficiency of 87.56 % and 88.16 % for [HMIM][PF6] and [HMIM][Tf2N], respectively. The authors mention that these solvents can be successfully be employed in the reactive extraction of PA.

Adsorption is another method applied for PA recovery, which based on physical interaction between the carboxylate group of the acid and the active site of the solid's matrix. A work by (Wang et al., 2012) demonstrated a novel in situ product removal (ISPR) process for the simultaneous production of PA and vitamin B_{12} with an expanded bed adsorption based bioreactor using unfiltered broth. PA concentration of 52.5 g L^{-1} and yields of 0.66 g g^{-1} were obtained by using the system.

Membrane technologies also have been proposed as promising alternative treatment for fermentation broths to reduce the overall recovery steps and improve the efficiency of the production. For example, microfiltration (MF) and ultrafiltration (UF) were used as pretreatment (clarification and removal of large particles) for reverse osmosis (RO), nanofiltration (NF), and electrodialysis (Jänisch et al., 2019; Tao et al., 2016; Thuy & Boontawan, 2017). Consequently, nanofiltration (NF), and reverse osmosis (RO) were used for VFA recovery. However, these techniques mostly need further purification steps to separate PA from the other acids.

Electrodialysis (ED) is another technique that could be applied to selectively recover charged components from mixed streams and obtain a high-quality PA. In a study by Zhang et al. (1993) PA and acetic acid were produced by fermentation of glucose using *Propionibacterium shermanii*. About 32 g PA was produced during the fermentation, where 27 g could be separated using electrodialysis.

Another less common way for PA recovery seems to be distillation. Karp et al. (2018) obtained a yield of 80 % and a PA purity of 98% by applying this method to hydrolysate from the fermentation of corn stover by *Propionibacterium acidipropionici*. Before distillation, the hydrolysate was pretreated first by cation exchange followed by activated carbon treatment. However, few researches have been conducted for optimization of PA recovery and most of the above presented technologies are still poorly tested using real fermentation broth.

Chapter 3

3 Propionic acid production from food waste in batch reactors: Effect of pH, types of inoculum, and thermal pre-treatment*

3.1 Introduction

Propionic acid (PA) is one of the most important and commercially valuable volatile fatty acids (VFA), which is extensively utilized in many industrial sectors such as food, pharmaceutical, medical, cosmetics and detergents (Border et al., 1987; Martínez-Campos & de la Torre, 2002; Morales et al., 2006). It can be obtained from a variety of sources and production methods (Ahmadi et al., 2017) but to date is mainly derived from fossil sources. Nevertheless, PA-production could be achieved in a sustainable manner if accomplished using renewable resources or even biomass waste and biological processes (Chen et al., 2013a; Li et al., 2016). Among the available biological methods, anaerobic digestion (AD) is relatively simple and was suggested to have a high potential for further development (Eryildiz et al., 2020; Esteban-Gutiérrez et al., 2018; Shi et al., 2019). However, the low productivity of PA produced through AD limit its commercialization as it competes with petrochemical production methods and entails increased costs of PA separation and recovery from the fermentation broth. However, several techniques for PA recovery from different aqueous solutions and fermentation broths have been proposed in the last years including reactive extraction, membrane systems, electrodialysis, adsorption, and distillation (Vidra & Németh, 2018).

Regardless of the type of substrate, full-scale implementation of PA production requires considerable and stable production rates. Process parameters such as pH, substrate concentration, organic loading rate, temperature, etc. have been reported to influence production rates significantly. Additionally, the type of inoculum is of great interest since it is known to be one of the most important factors affecting the fermentative pathways (De Gioannis et al., 2013). Different types of inocula have been used for anaerobic fermentation in other studies for either VFA or PA production, such as anaerobically digested sludge (Cappai et al., 2014; Karthikeyan et al., 2016) or bacterial isolates (Chen et al., 2016; Jin & Yang, 1998; Wang & Yang, 2013). Organisms that were mostly described to be involved in propionic acid production belong to the genus *Propionibacterium*. Although these microorganisms can grow with typical fermentation substrates such as glucose, they also have the ability to thrive as secondary fermenters converting the primary fermentation product lactate into propionate, acetate and carbon dioxide (3 $lactate^- \rightarrow$ 2 $propionate^-$ + $acetate^-$ + CO_2 + H_2O; $\Delta G^{0`}$= -162 kJ mol^{-1}). Consequently, the concerted action of lactic acid bacteria and *Propionibacterium* was revealed to increase propionate yields (Border et al., 1987; Tyree et al., 1991).

For accelerating the hydrolysis and improving substrate degradability, various types of pretreatment methods can be applied (Rajesh Banu et al., 2020). Autoclaving and thermal pretreatment were reported to enhance VFA and hydrogen yield (Hu et al., 2014) most probably by increasing the biological accessibility of proteins and carbohydrates (Abubackar et al., 2019). At the same time, it aims to inhibit hydrogen consumers and preserve hydrogen producers (Wang & Yin, 2017) in the substrate. Thus, possible competitors for hydrogen would be eradicated from the fermentation, which can lead to

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enhanced PA production, since hydrogen is needed by many PA producing bacteria (Kim et al., 2008; Ren et al., 2007; Vavilin et al., 1995). Finally, the pH-value is an essential and critical parameter that has an impact on the production of PA, since it affects the hydrolysis degree of the substrate, the activity of microorganisms, as well as the chosen metabolic pathways (Kim et al., 2011). In particular, highly acidic or basic pH values can negatively affect the activity of PA-producing bacteria (Ahmadi et al., 2017; Hsu & Yang, 1991; Inanc et al., 1996). As reported above, several factors were reported to affect the fermentation process. However, there is still a lack of knowledge regarding the effects resulting from combination of these factors on the PA production from food waste. Accordingly, this chapter describes the effect of type of inoculum, pH, and thermal pretreatment of the substrate on the production in batch tests. To investigate the effect of the inoculum, the influence of two typical sources of lactic acid bacteria to a third inoculum comprising a cellulolytic and a spontaneously developing consortium were compared.

3.2 Materials and methods

3.2.1 Inocula and substrate

Three types of inoculum were compared: a mixed bacterial culture that was selected for 24 months for growth on cellulose (I_1), fresh untreated milk (3.8 % fat) (I_2), and soft goat cheese (I_3). The mixed culture inoculum was prepared by cultivation at 30 °C in mineral medium containing cellulose particles (method described by Dolch et al. (2014)). Before inoculation, the culture was filtered through a paper filter of 25-µm pore size to remove the particles. The goat cheese was grinded into small particles using an iron grater.

Dry vegan grain-free dog food was used as model of food waste in this study because of their similar composition, while it provides standardized and reproducible experimental conditions (Kim et al., 2003; Nakasaki et al., 2004). The dog food was composed of dried potato, pea flour, potato protein, sunflower oil, beet fiber, and apple fiber, hydrolyzed vegetable protein, ground chicory root, herbs, fruits, and dried algae. Before addition to the reactors, the dog food was grinded to small particles using an electrical blender. The homogenized food material was applied in two different ways; it was used either directly without any further treatment or pretreated thermally at 121°C for 60 min. Thus, apart from assumingly changing some chemical properties of the substrate by thermal pretreatment, also the impact of microbiota potentially contained in the substrate on the fermentation process was diminished (Karthikeyan et al., 2016).

3.2.2 Design and operation of batch fermentation experiments

Six batch experiments were conducted using the Automatic Methane Potential Test System II (AMPTS II; Bioprocess Control Sweden AB) figure 3.1. The system consisted of 8 glass bottles to run four different experiments in duplicate, each bottle having a working volume of 1800 mL and 200 mL headspace. Each bottle was equipped with an individual mechanical plastic rod shaped stirrer (run at 60 revolutions per min; in intervals of 1 min mixing and 1 min resting). The produced gas in each bottle passed through a second bottle containing 3 M NaOH which adsorbs CO_2 while allowing CH_4 and H_2 to pass through. Thus, the unabsorbed gas was measured continuously through the water displacement using a flow measurement device. A more detailed description of the system can be found at https://www.bioprocesscontrol.com/products/ampts-ii/. To each of the 8 bottles, 250 g of dog food was added, followed by 500 mL of I_1 (cellulolytic bacterial culture), 500 mL of I_2 (milk), and 60 g of I_3 (cheese) for the first 2 bottles, the second 2 bottles, and the third 2 bottles, respectively. Thereafter, all bottles were filled with tap water up to 1800 mL. The remaining 2 bottles were set as the blank (dog food with tap water). The characteristic of the initial feed for every batch test is given in Table 3.1.

Experiments were done at a constant temperature of 30°C (± 1°C) in a water bath. According to many studies, 30 °C is the optimal temperature for propionic acid bacteria growth (Hettinga & Reinbold, 1972; Seshadri & Mukhopadhyay, 1993). Higher experiment temperatures were not included in this work, to keep the energy costs for the overall process as low as possible. Each experiment was performed two times at the same conditions using both untreated and pretreated dog food.

Experiments were carried out at 3 different pH values (4, 6, and 8), which were adjusted and controlled manually by adding either NaOH (5 M) or HCl (5 M). The specific pH values were chosen according to previous studies which concluded that the optimal pH for PA producing bacteria is in the range of 6 –7 with a maximum of 8.5 and a minimum of 4.6 (Fröhlich-Wyder et al., 2017; Irlinger et al., 2017). As the optimal pH range for the PA production expectedly varies depending on the other operational conditions (e.g. type of substrate and inoculum source), acidic (pH 4), slightly acidic and alkaline (pH 8) pH values were tested while pH 7 was excluded to alleviate possible methanogenic activity. No chemical methanogenesis inhibitor was added. The experimental details are provided in Table 3.2.

Samples were collected from each bottle every two days to analyze dissolved organic carbon (DOC) and volatile fatty acids (VFA) concentration. All the batch fermentation experiments were carried out for 20 days.

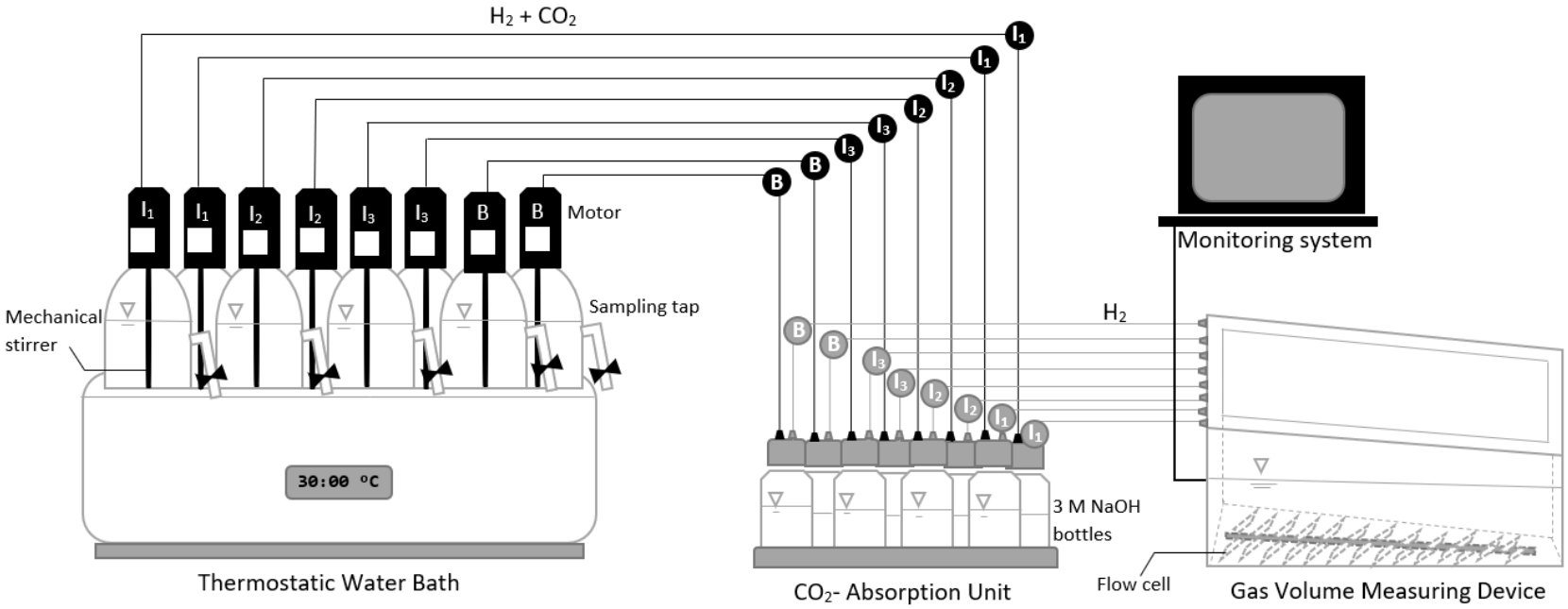

Figure 3.1 Schematic diagram of AMPTS II system. I_1 (mixed culture), I_2 (milk), I_3 (goat Cheese), and B (Blank).

Table 3.1: Characteristics of the fermentation broth at the beginning of each batch experiment (dog food (feed) and the inoculum).

	Untreated Dog food				**Pretreated Dog food**			
Parameter	Mixed bacterial culture (I_1)	Milk (I_2)	Soft goat Cheese (I_3)	Blank	Mixed bacterial culture (I_1)	Milk (I_2)	Soft goat Cheese (I_3)	Blank
TS (%)	11.6 ± 0.2	11.9 ± 0.5	12.8 ± 0.6	12.4 ± 0.4	11.0 ± 2.0	12.2 ± 0.4	12.2 ± 0.3	12.4 ± 1.5
VS (%)	10.5 ± 0.3	10.6 ± 0.4	11.8 ± 0.5	11.0 ± 0.4	9.8 ± 1.8	11.0 ± 0.2	11.0 ± 0.3	10.7 ± 1.1
TN (g L^{-1})	2.4 ± 0.7	4.4 ± 0.5	2.4 ± 0.1	1.6 ± 0.2	2.8 ± 0.5	4.4 ± 0.2	2.6 ± 0.3	1.9 ± 0.4
C:N	13.9 ± 1.8	11.8 ± 0.1	11.4 ± 0.1	14.0 ± 1.4	12.3 ± 0.5	11.6 ± 0.9	11.4 ± 0.7	14.6 ± 0.5
DOC (g L^{-1})	9.3 ± 0.1	23.6 ± 0.3	10.2 ± 0.2	7.5 ± 0.5	9.5 ± 0.5	18.7 ± 0.4	11.2 ± 0.4	8.4 ± 0.2
SO_4^{2-} (mg L^{-1})	95.9 ± 4.2	399 ± 3.5	120 ± 7.1	91.8 ± 4.5	90.1 ± 2.1	367 ±29.7	106 ± 0.0	101 ± 0.7
NO_3^- -N (mg L^{-1})	34.3 ± 1.4	58.3 ± 0.6	39.9 ± 0.8	42.3 ± 0.2	43.0 ± 1.7	48.9 ± 1.9	45.8 ± 4.4	43.1 ± 1.7
NO_2^- -N (mg L^{-1})	–	6.9 ± 0.3	0.31 ± 0.03	–	0.02 ± 0.00	5.6 ± 0.3	0.3 ± 0.1	0.04 ± 0.00
PO_4^{3-} (mg L^{-1})	18.6 ± 0.2	15.5 ± 0.1	18.6 ± 0.1	17.4 ± 0.3	14.8 ± 0.2	15.3 ± 0.1	17.4 ± 0.3	16.6 ± 0.5
NH_4^+ -N (mg L^{-1})	56.6 ± 0.5	53.5 ± 0.6	53.6 ± 0.5	43.5 ± 0.1	67.4 ± 0.8	55.5 ± 2.4	68.4 ± 0.2	50.0 ± 0.1

Table 3.2: Summary of experimental design and operation conditions of the batch fermentation experiments. Each experiment was performed two times under the same conditions using both untreated and pretreated dog food, repeated under different pH values (4, 6, and 8).

			Content of each bottle			
Experimental condition	Type of substrate	Batch test components	Substrate [g]	Type of inoculum	Inoculum (volume/weight)	Initial VS added [g L^{-1}]
- Mesophilic temperature (30 ± 1°C) - pH values of 4 ± 0.3, 6 ± 0.3, and 8 ± 0.3	Untreated dog food	Bottles 1 & 2	250	I_1 Mixed bacterial culture	500 ml	111
		Bottles 3 & 4	250	I_2 Milk	500 ml	139
		Bottles 5 & 6	250	I_3 Goat cheese	60 g	125
		Bottles 7 & 8	250	Blank (without inoculum)	-	111
- Mesophilic temperature (30 ± 1°C) - pH values of 4 ± 0.3, 6 ± 0.3, and 8 ± 0.3	Pretreated dog food (autoclaved at 121°C for 60 min)	Bottles 1 & 2	250	I_1 Mixed bacterial culture	500 ml	109
		Bottles 3 & 4	250	I_2 Milk	500 ml	136
		Bottles 5 & 6	250	I_3 Goat cheese	60 g	122
		Bottles 7 & 8	250	Blank (without inoculum)	-	109

3.2.3 Analytical methods

Analysis of total solids (TS) and volatile solids (VS) followed German Standard Methods for the Examination of Water, Wastewater and Sludge (DIN, 1989). Lactic acid and volatile fatty acids (VFA) concentrations were determined using IC analysis (Metrohm 881 Compact Pro, Herisau, Switzerland) using a Metrosep Organic Acids 250/7.8 column. Total organic carbon (TOC), total nitrogen (TN), and dissolved organic carbon (DOC) were measured with a Shimadzu TOC-$_{LCPH}$ analyzer (Duisburg, Germany). Before measurement, all samples were centrifuged at 8000 rpm for 10 minutes and filtered through a polyethersulfone (PES) membrane of 0.45 µm pore size. Gas samples from each bottle were collected for composition analysis using gas chromatography (Agilent 490 micro GC, Santa Clara, United States).

3.2.4 Data analysis

PA yield (Y_{PA}, given in g g^{-1}) was calculated from (Eq. 3.1) for all batch tests based on the amount of volatile solids initially added (VS added (g L^{-1})) and the amount of PA produced (as maximum concentration c_{max} (g L^{-1}), which was always also the final concentration achieved).

$$Y_{PA} = C_{max}/VS_{added} \qquad 3.1$$

The maximum propionic acid productivity (P_{PA}, given in g L^{-1} d^{-1}) was calculated according to:

$$P_{PA} = (dc/dt)_{max} \qquad 3.2$$

where the $(dc/dt)_{max}$ is the maximum gradient of PA concentration.

The VFA production efficiency (Y_{VFA}) (Eq. 3.3) was calculated as the ratio of the achieved total VFA concentration (as g L^{-1} DOC) compared to the total DOC (g L^{-1}).

$$Y_{VFA} = (VFA/DOC) \times 100\ \% \qquad 3.3$$

The yield of hydrogen (Y_{H2}) was calculated by relating the hydrogen volume to the amount of volatile solids added:

$$Y_{H_2} = \dot{V}_{H_2}/VS_{added} \qquad 3.4$$

Where $\dot{V}_{H2}$ is the final production of H_2 (NmL d^{-1}) and VS_{added} is the amount of volatile solids initially added to each batch test (g L^{-1}).

3.3 Results and discussion

3.3.1 VFA production and composition

During anaerobic fermentation of particulate substrates, organic compounds are hydrolyzed by microorganisms, resulting in higher amounts of dissolved organic carbon (DOC). Part of this DOC fraction is transformed to volatile fatty acids, which would be further converted in a conventional biogas process to methane and carbon dioxide. Overall, the produced gas in all experiments of this study contained mainly hydrogen (H_2) and carbon dioxide (CO_2) with negligible concentrations of nitrogen (N_2). CH_4 was not detected during any of the experiments. Furthermore, ethanol and methanol were not detected in any of the experiments. The maximum hydrogen production rate $\dot{V}_{H2}$ of 4.48 NL d^{-1} and yield of Y_{H2} 41.2 NmL g^{-1} were achieved in the tests conducted with inoculum I_1 and using treated dog food as substrate.

However, no obvious correlation was observed between the H_2 and the VFA production in the experiments.

Autoclaving and thermal pretreatment of complex substrates are known to enhance solubilization of complex organic compounds leading to an increase of DOC concentration (Liu et al., 2012a; Ma et al., 2011; Pagliaccia et al., 2016; Pecorini et al., 2016). However, in this work the experiments with vegan dog food the DOC concentrations did not change much after the pretreatment (compare Table 3.1). Yet, the lag phases between inoculation and onset of DOC increase due to hydrolysis were shortened by approximately two days in comparison to the application of untreated dog food. Regardless of which inoculum was used, in the experiments performed at pH 6 and 8, usually more than 80 % of the DOC concentration measured could be assigned to accumulating VFAs. Only at pH 4, DOC concentrations were generally lower of about 40 % for both untreated and pretreated dog food. Here, VFA/DOC ratios ranged between 9 % and 46 % for all inocula, and, as can be seen in Figure 3.1, VFA concentrations achieved did not exceed 12 g L^{-1}. The maximum VFA concentrations achieved in the batch experiments and the distribution of individual acids (also given as maximum concentrations) at different pH values are depicted in Figure 3.1. Lactic acid was excluded from this figure as it appeared only transiently as intermediate product during the experimental time course. The largest peaks of lactic acid concentration mostly appeared between day 5 and day 10 before it decreased significantly towards the end of the experiments. However, with more than 80 % of the total organic acids concentration it achieved the highest maximum concentrations in comparison to the other single VFA. Its production started right after the start of the experiments regardless of which type of inoculum was used. The sum of the VFA produced comprised mainly butyric, propionic and acetic acid. The highest total VFA concentrations obtained reached values of more than 60 g L^{-1} (Figure 3.2). These concentrations were achieved at pH 8 with the mixed culture (I_1) or milk (I_2) inoculum as well as at pH 6 with goat cheese as inoculum, and when using treated dog food as substrate. Interestingly, also the blank produced VFA concentrations in that range, when untreated dog food was processed at pH 6.

Figure 3.3 a & b, show the production of lactic acid plus all single VFA exemplified by the experiments conducted at pH 6 with untreated and pretreated dog food as substrate and goat cheese (I_3) as inoculum. Here, it can be suggested that a succession of fermentation reactions can be observed in which lactate is produced first and the final end products are a result of lactate conversion. This is, however, not valid for all experiments. The transformation of lactic acid to VFA has already been reported for the fermentation of dog food as model of food waste by Kim et al. (2003), as well as for fermentations of food waste (Tang et al., 2017), lactate (Grause et al., 2012), and tequila vinasse (García-Depraect et al., 2019). However, it cannot be proven by the data that lactic acid is the only source for VFA generation since several microorganisms also can use e.g. sugars as substrate to generate VFA (Reichardt et al., 2014), and preferred pathways depend on microorganisms available and fermentation conditions, too.

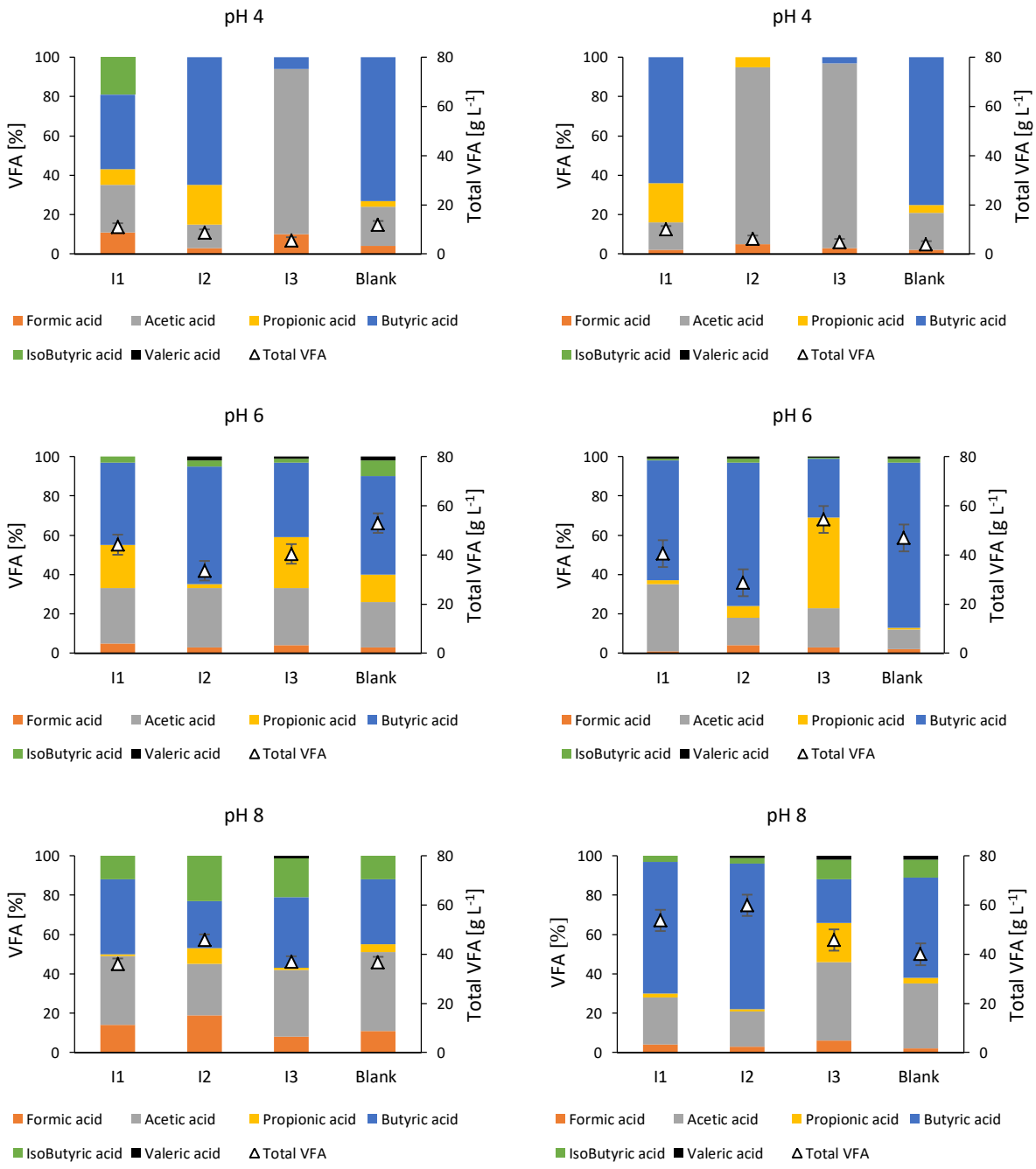

Figure 3.2: Total VFA concentrations and their relative composition (as maximum concentration of individual acids) in all experiments resulting from the fermentation of untreated (left column) and thermal pretreated dog food (right column) for each inoculum type (mixed bacterial culture (I_1), milk (I_2), and goat cheese (I_3)) and at different pH values. Lactic acid as intermediate product is excluded.

The evolution of the VFA revealed different fermentation patterns depending on inoculum type and pH of the process. As can be seen from Figure 3.1, butyric and acetic acid were the predominant products in all tests except for pH 6 when pretreated dog food and goat cheese (I_3) were used. At this condition, the dominant product was PA, which accounted for approx. 55 % of the total VFA. Besides pH 6, which seems to be the favorable condition for PA producing bacteria, a reason for this could be that the thermal pretreatment inhibited the bacteria present in the dog food which can be considered to be the main competitors for the inoculum bacteria (Hu et al., 2014; Wang & Yin, 2017). At the same time, it might have selected for spore-producing microorganisms that include hydrogen producing bacteria which stimulate the inoculum bacteria to produce PA (Kim et al., 2008; Koskinen et al., 2007; Ren et al., 2007; Vavilin et al., 1995). This might also explain the higher amount of butyric acid produced from thermally treated food in some tests. As Hu et al. (2014) and Kim et al. (2008)reported, some of the bacteria responsible for butyric acid production must have come with the dog food and were not destroyed by the pretreatment (e.g. spore-producing bacteria). This could be seen clearly in blank tests, where thermal pretreatment seems to have shifted the dominance towards this type of bacteria. Other acids such as formic acid, iso-butyric, and valeric acid appeared in very low concentrations. From the above results together with previous researcher's investigation, it can be concluded that the optimal pH for the production of a specific VFA is highly dependent on other parameters such as substrate and the type of inoculum used.

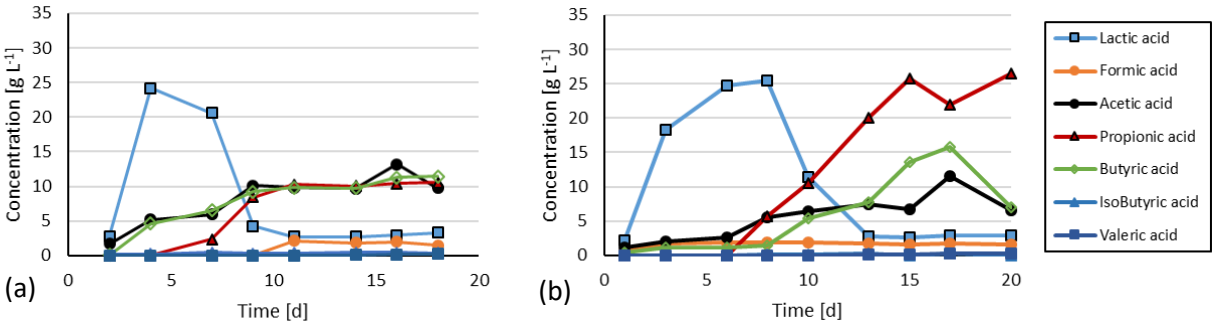

Figure 3.3: Course of lactic acid and VFA concentrations during the experiments of the fermentation of (a) untreated dog food and (b) thermal pretreated dog food at pH 6 using goat cheese (I_3) as inoculum (n=2).

3.3.2 Propionic acid

The PA production results indicated an obvious difference between the different inocula in terms of capability to produce PA at different pH values and with different substrate (untreated or pretreated food). In general, pH 6 was found to be the best pH value for PA production. At this pH and when soft goat cheese (I_3) was used as inoculum, the highest concentrations of all experiments were achieved in the fermentation of untreated and treated dogfood of approximately 10.5 g L^{-1} and 26.5 g L^{-1}, respectively.

To reveal the different impacts of inoculum type, pH value and type of substrate, the PA yields were compared (Figure 3.4. a & b). In the calculation, the maximum achieved PA concentrations were related to the total volatile solid (VS) initially added to each experiment, also considering the VS of the inoculum itself (e.g. cheese and milk). As can be seen in Figure 3.4, fresh goat cheese (I_3) showed the highest yield among the three inocula tested in this study. However, its performance was significantly affected by the thermal pretreatment of the substrate and the pH value. In particular, pH 6 was the optimal condition for both experiments with untreated and pretreated dog food. Thermal pretreated resulted in a significant yield increase by a factor of 2.6 (Y_{PA}=216.9 mg g^{-1}). Moreover, pretreated dog food proved to be quite suitable to goat cheese (I_3) inoculum for the production of PA also at pH 8.

In contrast, the above-mentioned conditions were not valid for the other inocula. For example, the optimal conditions for mixed bacterial culture (I_1) and the uninoculated reactors were at pH 6 when untreated dog food was used as a substrate. Approximately half of the PA yield was achieved at these conditions by I_1, compared to the yield of goat cheese (I_3) inoculum at the same pH and using the same substrate (untreated dog food). While the maximum yield of Milk (I_2) was achieved at pH 8 with untreated dog food. As discussed before, all inocula showed low VFA productivities at pH 4, and, thus, also achieved the lowest PA yields. Within the range of the applied conditions, the optimum for PA production was at pH 6 when goat cheese (I_3) was combined with pretreated dog food. Changing one of these variables would result in reduction of the PA yields.

Although, the relation between PA production and pH value has been mentioned in many studies, the optimal pH range reported varied depending on other process parameters such as substrate and type of inoculum.

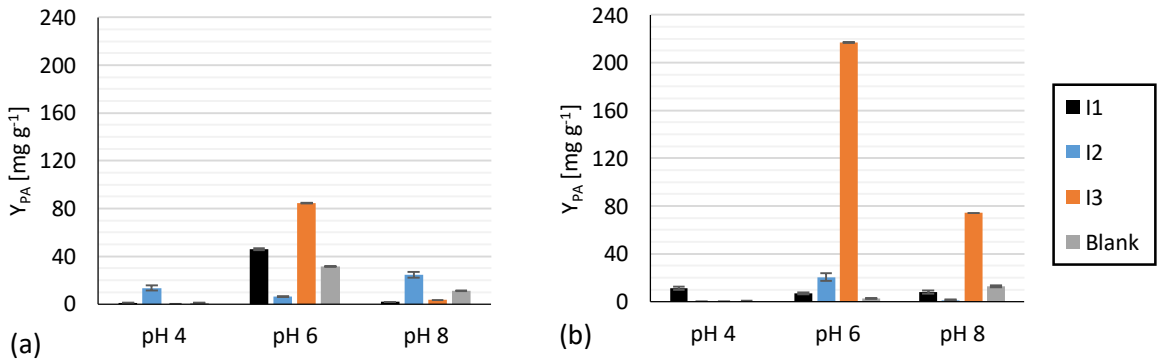

Figure 3.4: Propionic acid yields Y_{PA} achieved with different inocula (mixed bacterial culture (I_1), milk (I_2), and goat cheese (I_3)) at different pH values in the fermentation of, (a) untreated and (b) pretreated vegan dog food. Yields are given as mg g^{-1} propionic acid per g VS_{added}.

3.4 Conclusions

This chapter focused on revealing the effects of pH value, inoculum type, and thermal pretreatment of the substrate on propionic acid in the fermentation of vegan dog food as a model for organic food waste. The amount of VFA produced and their composition was compared related to these factors. Propionic acid production was highest for the fermentation of the treated dog food at pH 6 using soft goat cheese as inoculum. This approach resulted in a PA concentration of 26.5 g L^{-1} at a maximum production rate P_{PA} of 2.9 g L^{-1} d^{-1}, and a yield Y_{PA} of 217 mg g^{-1} propionic acid per VS_{added}. In this case, propionic acid was the dominant VFA produced. However, the highest total VFA concentration of almost 60 g L^{-1} was obtained when milk was applied as inoculum for the fermentation of pretreated dog food at pH 8. The evolution of the individual acids showed different fermentation patterns depending on inoculum type and pH value. In most cases, butyric acid was the dominant acid followed by acetic acid. Although the thermal treatment improved PA production, this pretreatment is still not commercially feasible for application to waste streams at large scale. Therefore, results from fermentation of untreated dog food as a model of food waste are more suitable to calculate scale-up options for PA production from real food waste. The corresponding PA concentration and yield, which were also achieved at pH 6 and with goat cheese as inoculum, amounted to 10 g L^{-1} and 84 mg g^{-1} propionic acid per VS_{added}; respectively, at a maximum PA production rate (P_{PA}) of 1.9 g L^{-1} d^{-1}.

Chapter 4

4 Enhanced production of propionic acid through acidic hydrolysis by choice of inoculum in a semi-continuous fermentation*

4.1 Introduction

Propionic acid (PA) and its salts are widely used in industries including agricultural, pharmaceutical and food industries as antifungal agents (Chen et al., 2013a; Jin & Yang, 1998). It can also be employed as precursor for the biotechnological production of value-added compounds, like e.g. acetoin (Schmidt et al., 2018) and, thus, is listed as an important platform chemical since the early 2000s (Werpy, 2004). Currently, most of the PA production around the world is done by chemical synthesis through the oxidation of petrochemicals like propane or propionaldehyde as raw material (Ahmadi et al., 2017). Acidic hydrolysis is an alternative method that gain more attention for PA production from available renewable sources, such as organic waste. It is increasingly applied with focus on biohydrogen production, a process known as dark fermentation, in which organic waste is utilized to generate renewable energy (Schmidt et al., 2018). However, the separation of single volatile fatty acids (VFA) from complex effluents such as the fermentation broth is still a challenge, due to the complex nature and the presence of various organics (Atasoy et al., 2018). Techniques such as electrodialysis (Weier et al., 1992), reactive extraction (Keshav et al., 2009a), reverse osmosis (Schlicher & Cheryan, 1990), nanofiltration (Xiong et al., 2015), and adsorption (Talebi et al., 2020) have been investigated to separate and concentrate these acids from aqueous solution and fermentation broth. This downstream processing has to be considered to make the hydrolytic process comparable to the petrochemical synthesis in terms of commercial feasibility. As a first step, it is however necessary to generally increase the portion of propionic acid in the sum of VFA usually produced in acidic hydrolysis. Accordingly, this chapter focused on the optimization of propionic production from a model and real food waste.

Many studies showed that despite the heterogeneous and nonstandard composition of kitchen and food waste, both could be utilized as suitable substrates for production of value added compounds and energy generation because of their constant availability, and high carbohydrate content (Chu et al., 2008). Sindhu et al. (2019) provided a review on the conversion strategies and different value added products that could be produced from kitchen and food wastes. Hafid et al. (2017) also gave an overview on utilization of kitchen wastes as substrate for bioethanol production. There are several publications presenting empirical studies on utilizing these wastes for the production of volatile fatty acids (Chen et al., 2013a; Zhang et al., 2020), bio-hydrogen (Slezak et al., 2017), biogas generation (Cappai et al., 2014; Karthikeyan et al., 2016; Sahu et al., 2017), bio-methane (Kaur et al., 2020) , ethanol (Tang et al., 2008), xanthan (Li et al., 2017), and enzymes (Bansal et al., 2012; Bhatt et al., 2020). However, none of these researches focused specifically on PA production from kitchen or food waste and the concentrations of PA produced were quite low. In general, process parameters like pH value, temperature, hydraulic retention time, organic loading rate, and type of inoculum are known to have strong impacts on PA production (Tang et al., 2016).

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Native propionic acid-producing bacteria have been the primary candidates for the development of a biotechnological process and several types of pure cultures and mixed cultures have been investigated. Species from the genera *Propionibacterium,* namely *P. acidipropionici* and *P. freudenreichii* were the most studied pure cultures for propionic acid production from simple substrates such as glucose (Chen et al., 2013a; Wang & Yang, 2013), lactose (Jin & Yang, 1998), and glycerol (Wang & Yang, 2013; Zhu et al., 2010). Limited studies have been reported that applied anaerobic sludge as mixed culture inocula for propionic acid production from glycerol (Chen et al., 2016), or crude glycerol (Paranhos & Silva, 2020).

During the fermentation process of waste, some types of lactic acid bacteria (e.g. *Lactobacilli*) have an important function in breaking down carbohydrates, amino acids, and monosaccharides into lactate, which is used by e.g. *Propionibacterium* to produce propionic acid as metabolic end product (Asunis et al., 2019; Dai et al., 2017; Zhou et al., 2018). The action of both microorganism types was reported to be important to increase the overall yield of propionic acid (Parker & Moon, 1982). Therefore, addition of a mixed culture of lactic and propionic acid producing microbial strains to the process seems to be promising. Few researchers investigated the species interaction in propionic acid production in detail, but none of their studies shows the impact of these microorganisms on the breakdown of complex substrates (e.g. food waste). Tyree et al. (1991) used a mixed culture of *Lactobacillus sp* and *Propionibacterium shermanii* to produce propionic acid from simple substrates like lactate, glucose, and xylose. Border et al. (1987) also produced propionic acid from wheat flour with a mixed culture of *Propionibacterium*, *Lactobacillus* and *Streptococcus.*

Acidic hydrolysis of complex substrates with a special focus on and optimization of propionic acid production has not been reported yet. Based on the results that obtained from chapter 3, the optimum operation conditions (pH 6 and soft goat cheese as inoculum) has been selected to evaluate the PA production in a semi-continuous mode using a 12 L hydrolysis reactor. For comparison, the reactor was also operated with a mixed microbial culture selected over 24 months for growth on cellulose. In this chapter, the production of other VFA and the composition of the microbial communities during the fermentation as well as the effect of hydraulic retention time (*HRT*) and organic loading rate (*OLR*) were also evaluated.

4.2 Materials and methods

4.2.1 Substrate characteristics

4.2.1.1 Dog food

Vegan grain-free dogfood (DF) was used as a model for organic food waste (Kim et al., 2003; Nakasaki et al., 2004). The food was composed of dried potato, pea flour, potato protein, sunflower oil, beet fiber, and apple fiber, hydrolyzed vegetable protein, ground chicory root, herbs, fruits, and dried algae.

4.2.1.2 Food waste

Although the dog food composition is similar to that of food waste, it was found important to additionally test real food waste to provide more realistic data with regard to envisaged large-scale application. In this context, and to maintain a standard composition throughout the period of the experiment, synthetic food waste was prepared by mixing 10 % cooked rice, 10 % cooked noodles, 10 % canned kidney beans, 35 % vegetables (lettuce, potato, tomato), 30 % fruits (apple, orange, banana skin). The waste was crushed using a mechanical mixer to obtain a homogenized texture.

4.2.2 Reactor configuration

A cylindrical stirred-tank reactor (BTP2, UIT Umwelt- und Ingenieurtechnik GmbH Dresden, Germany) was operated in this study Figure 4.1. The reactor was made of glass and had a total volume of 15 L (12 L working volume). The temperature was maintained at 30 °C by means of an electrical heating control unit, and the pH value was automatically controlled at 6 ± 0.1 (by adding 5M NaOH or 3M HCl solutions). The substrate was fed manually through a feeding funnel located at the top of the reactor. For biogas production rate measurement, a gas counter (MilliGascounter, Dr.-Ing. RITTER Apparatebau GmbH & Co. KG, Bochum, Germany) was connected to the top of the reactor to measure the biogas production rate. The gas produced during the fermentation process was periodically sampled by collecting it in a gasbag. The reactor was equipped with an internal agitator, which consisted of two parts, an upper U-shaped anchor-stirrer and a lower propeller shaped stirrer. The stirrer speed was set to 100 rpm to ensure homogeneous mixing of the digestate.

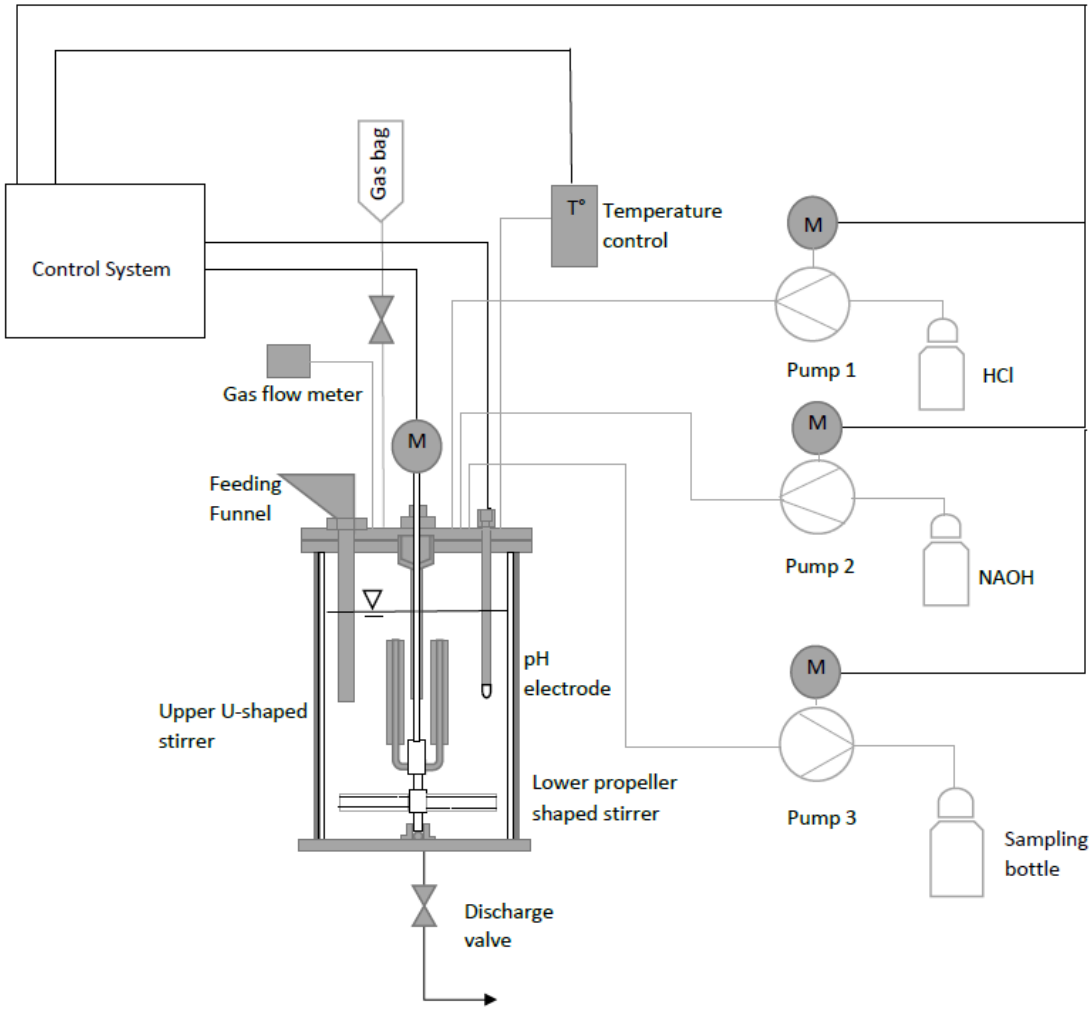


Figure 4.1: Schematic diagram of the reactor. M: motor.

4.2.3 Inoculation and operation of the reactor

In this work, three operational runs of the reactor are compared where temperature (30°C) and pH-value (6 ± 0.1) were fixed, but which differed with regard to the type of inoculum, substrate, organic loading rate, retention time, and substrate to water ratio of the feed. The reactor was operated for approximately 100 days.

In Run 1, the reactor was inoculated with a mixed microbial population that was selected for 24 months for growth on cellulose. The inoculum was chosen because the culture was produced significant levels of propionic acid from cellulose. To remove cellulose particles from the former feed of the culture, the inoculum was filtered through a paper filter of 25 µm pore size. The reactor was initially fed with 4 kg of dried dog food (3560 g Volatile Solids (VS); equivalent to 1760 g Total Carbon (TC) mixed with 4 L of bacterial culture and 4 L of tap water corresponding to 297 g L^{-1} VS in total. Thus, the substrate/water ratio was 1:2. Within the first 14 days, the reactor was operated in batch mode. After that, the operation mode was switched to three consecutive repeated fed-batch (semi-continuous) phases; where the reactor was fed daily with an organic loading rate (*OLR*) of 12.3 g L^{-1} d^{-1} VS for 27 days, 17.8 g L^{-1} d^{-1} VS for 30 days, and 29.7 g L^{-1} d^{-1} VS for 29 days (equivalent to 6.1, 8.8, and 14.7 g L^{-1} d^{-1} TC), respectively. This corresponds to hydraulic retention times (*HRT*) of 24 d, 16 d, and 10 d.

In Run 2, the reactor was inoculated with soft goat cheese grinded into small particles using an iron grater (see chapter 3). The reactor was initially fed with 3 kg of dried dog food (2670 g VS; equivalent to 1320 g TC) mixed with 1 kg of cheese (480 g VS) and 8 L of tap water, corresponding to 260 g L^{-1} VS in total with a substrate/water ratio of 1:3. It was also started as batch for 10 days. As in Run 1, the operation mode was then switched to semi-continuous where the reactor was fed every second day with an *OLR* of 11.1 g L^{-1} d^{-1} VS (5.5 g L^{-1} d^{-1} TC) for another 90 days. The retention time was maintained at 20 days. The substrate to water ratios of the feed remained unchanged during the above-mentioned reactor runs.

In Run 3, the reactor was operated again using the soft goat cheese inoculum but feeding synthetic food waste as substrate. By considering the physical differences in the nature of each food concerning e.g. degree of disintegration and water content, the reactor was operated at different *OLR*, *HRT*, and substrate to water ratio (S/W) ratio than in the previous runs. The reactor was initially fed with 4 kg of food (780 g VS) mixed with 1 kg of cheese (480 g VS) and 7 L of tap water, corresponding to 105 g L^{-1} VS in total, resulting in a substrate to water ratio of 1:1.4. Within the first 14 days, the reactor was operated in batch mode. After that, the operation mode was switched to three distinct and consecutive fed-batch (semi-continuous) phases, where the reactor was fed with an organic loading rate (*OLR*) of 3.2 g L^{-1} d^{-1} VS for 44 days, 5 g L^{-1} d^{-1} VS for 22 days, and again 3.2 g L^{-1} d^{-1} VS for the last 29 days, respectively, which corresponds to hydraulic retention times (*HRT*) of 30 d, 20 d, and 30 d.

4.2.4 Analytical methods

Samples were taken every 2 to 3 days to measure the concentrations of volatile fatty acids (VFA), dissolved organic carbon (DOC), total solids (TS) and volatile solids (VS). Before the quantitative analysis, the samples were pretreated by centrifugation for 10 minutes at 8000 rpm, and then the supernatant was filtered through a 0.45 µm polyethersulfone (PES) membrane filter. The amount of VFAs was determined by ion chromatography analysis (Metrohm 881 Compact Pro, Herisau, Switzerland) using a Metrosep Organic Acids 250/7.8 column. DOC concentration was measured with a Shimadzu TOC_{-LCPH} analyzer (Duisburg, Germany). TS and VS measurements were carried out according to the DIN 38 414

(DIN, 1985). Gas samples were collected every 2 to 3 days for composition analysis using gas chromatography (Agilent 490 micro GC, USA).

4.2.5 DNA Extraction and 16S Illumina MiSeq Sequencing

The bacterial diversity in the reactor was assessed via amplicon sequencing using the Bact_341F/Bact_805R primer pair (Herlemann et al., 2011). To this end, 200 to 300 mg samples were taken at different time points and extracted genomic DNA by applying the innuSPEED Soil DNA Kit (Analytic Jena) according the manufacturer's instructions. The microbial diversity was assessed via Illumina MiSeq sequencing (paired-end, 2 x 250 bp reads) conducted by IMGM Laboratories GmbH (Martinsried, Germany). The bioinformatic analysis was conducted with the CLC Genomic Workbench software 12.0.3 using the microbial genomic module 3.0 (Qiagen, Hilden) as described previously (Grießmeier & Gescher, 2018).

4.3 Results and discussion

4.3.1 VFAs concentration and composition

Time courses of VFA concentrations for Run 1, Run 2, and Run 3 are depicted in Figures 4.2 (a, b, & c). The main products of the fermentation in all runs were lactic, acetic, butyric acid, and the target acid of this study, propionic acid. While other acids such as formic acid, iso-butyric, and valeric acid were detected at very low concentrations. This is in line with what was reported in other studies on acidic hydrolysis of several substrates such as dog food (Kim et al., 2003), organic waste (Garcia-Aguirre et al., 2017), landfill leachate (Begum et al., 2018), and food waste (Tang et al., 2017). However, the concentrations reached in the broth are highly variable over time. Lactic acid was the main acid produced during the start-up batch period of Run 1 as the first detectable intermediate with a maximum concentration of 310 mmol L^{-1} at day 9. The concentration subsequently decreased significantly already towards the end of the batch phase, and resumed increasing once per adjusted retention time with maximum peaks being reached in intervals of approximately 23 days.

In general, there is a clear sequence of VFA appearance in the reactor broth. After lactic acid, concentrations of butyrate, propionate and acetate peak although at different maximum values. Acetic and butyric acid reach maximum concentrations in the range of 325 to 340 mmol L^{-1}, whereas maximum propionic acid concentrations reached only 77 mmol L^{-1}. It is noticeable that propionic acid concentrations, which were about 39 mmol L^{-1} on average, did not vary as much as the concentrations of the other acids. In addition, acetic acid, showing only one big peak during the course of the reactor run, remained at rather low but fairly constant concentrations of about 27 mmol L^{-1} on average from day 55 onwards. An important finding from the results of Run 1 was that a direct link between retention time/organic loading rate and VFAs concentrations could not be stated. Rather, it appears that the course of concentrations reached by one acid often is more dependent on the courses of the other acids, which act as precursors or develop as daughter products. The latter can for example be a result of a process called chain-elongation which entails a reverse b-oxidation that enables the partial usage of the substrate for energy generation (Agler et al., 2012). Chain elongation was for instance described for the conversion of ethanol and acetate or lactate and acetate to butyrate and could probably explain the depletion of acetate and production of butyrate between day 35 and 50. It also appears as if peaks in propionate production always occur after an increase in lactate productivity. The latter would be a logical consequence of secondary fermentation catalyzed by propionic acid bacteria. Using amplicon sequencing, to verify this and samples were taken from the reactor at days 76, 82 and 87, which correlate with a peak and following decrease in propionate concentration Figure 4.2 (a). The data reveals

the abundance of *Propionibacteria* but it also emphasizes the instability and the high variability of the microbial composition in the system. This high degree of instability is apparent in the fact that *Propionibacteria* were not detectable at day 76 and 87 but 40 % of the amplicon counts could be assigned to these organisms at day 82. Moreover, lactic acid bacteria were only detectable at day 76. The concentration of these organisms was probably higher at earlier time points of the Run corresponding to the lactate peak at day 64.

Still, although the occurrence of lactic acid consumption, propionic acid production and *Propionibacteria* is highly indicative of a Wood-Werkman-Cycle based fermentation of lactate to propionate, it should not be forgotten that lactate is not the only substrate for *Propionibacteria*. Sugars and alcohols are used as well, and other fermentation pathways leading to propionate also exist in other microorganisms (Reichardt et al., 2014). Still, the Wood-Werkman-Cycle is the thermodynamically most efficient fermentation pathway known so far (Gonzalez-Garcia et al., 2017). Other organisms known to produce propionate fermentatively belong typically to the genera *Clostridium, Bacteroidetes, Veilionella, Propionigenum, Selenomonas, Megasphera* and *Salmonella*. Some of these produce propionate also from lactate but substrates include also succinate, sugars, glycerol, amino acids and propanediol (Gonzalez-Garcia et al., 2017). Unfortunately, the phylogenetic diversity analysis conducted here does not allow to reveal whether these other organisms and their fermentation pathways might play a role as well.

Results of Run 1 show that lower organic loading rates (*OLR*) might be beneficial for propionic acid production. The detected PA concentration at an *OLR* of 12.3 g L^{-1} d^{-1} VS was higher compared to the concentration at other *OLR*s of 17.8 g L^{-1} d^{-1} VS and 29.7 g L^{-1} d^{-1} VS, respectively. Consequently, the second runs were operated with a rather low *OLR*.

In Run 2, which was inoculated with the soft goat cheese, acid concentrations generally showed smaller amplitudes at much lower average concentrations than in Run 1. Lactic acid, for example reached a maximum of 163 mmol L^{-1} during the start-up phase, which is roughly 50 % of the value reported for Run 1. It is butyric acid that showed both, highest variability over time (between 136 and 235 mmol L^{-1}) as well as the highest concentrations compared to all other acids. Interestingly, propionic acid was produced several days earlier than in Run 1 and reached the second highest concentrations of maximum 139 mmol L^{-1} and 78 mmol L^{-1} on average. This was twice as much as in Run 1. Accordingly, also the ratio of propionic acid concentration to total volatile fatty acids concentration (PA/VFA) was significantly higher ranging from 10 % to 62 % (26 % on average) whereas in Run 1 the range was between 4 % and 26 % (10 % on average). This result is also corroborated with 16S rRNA gene diversity data for three days at the end of reactor operation (day 72, 79 and 86) Figure 4.2 (b). The community seems to be more stable and *Propionibacteria* were detectable in all samples. Organisms belonging to the *Clostridium sensu stricto* group were not as common as in Run 1, while *Anaerotruncus* was the most abundant phylum. Although the information regarding these organisms is sparse, it seems that they produce acetic and butyric acid also as main fermentation end products (Lawson et al., 2004). The same is also true for organisms belonging to the *Peptoclostridium* group although lactic acid was also revealed to be a fermentation end product (Pereira et al., 2016). Regarding organisms that belong to the genus *Rubellimicrobium* it is not clear what the fermentation end products are. Interestingly, a very low abundance of *Lactobacilli* of below 1 % was observed in the three samples, which might suggest that the *Propionibacteria* thrive to a main extent on a different substrate than lactate.

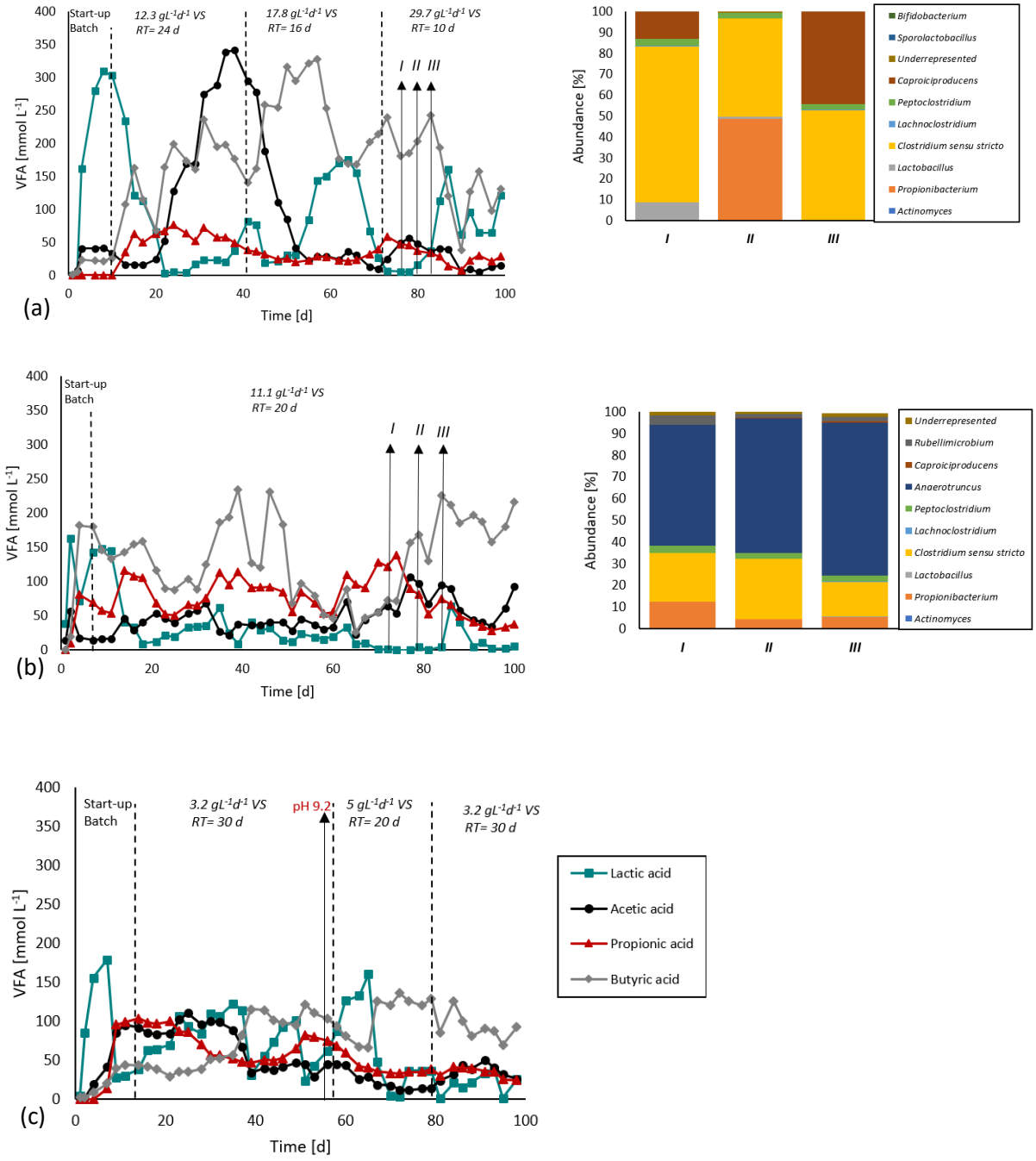


Figure 4.2: Courses of VFA concentrations during reactor run (a) 1, inoculated with a mixed culture, and (b) 2 & (c) 3, inoculated with soft goat cheese. In Run 1 and Run 2, three samples (I, II, and III) were taken for 16S analysis (marked by arrows). The results of the relative abundance of genera are shown on the right. No samples were analyzed from Run 3.

The VFA concentrations obtained in Run 3, which was fed with synthetic food waste, were generally lower than in the previous runs. However, similar trends as in Run 1 were observed for all VFA, but with a higher PA content. As it can be seen in Figure 4.2 c, lactic acid was the main acid produced during the start-up period with a maximum concentration of 179 mmol L^{-1}. The concentration always peaked when the reactor was loaded with new substrate (every three days), and peaked again when the *OLR* was increased to 5 g L^{-1} d^{-1} VS. The increase of lactic acid was always followed by an increase in propionic acid concentration. The highest concentration of PA was observed between day 15 and day 25 with 105 mmol L^{-1}, while the average PA concentration was 70 mmol L^{-1} in this first fed-batch phase (*OLR* of 3.2 g L^{-1} d^{-1} VS, *HRT* 30 d), which is similar to the concentration obtained in Run 2. However, by increasing the *OLR,* PA concentration decreased to 39 mmol L^{-1} on average, while it did not change much when *OLR* was decreased again. This could be explained by the fact that the pH value changed to 9.2 in the reactor at day 56 for a few hours due to a technical issue in the base pump. Thus, the alkaline condition will have limited growth and activity of *Propionibacterium* and consequently the production of propionic acid. Second, the *Propionibacterium* might have been diluted during the operation at high *OLR* and low *HRT*. Unfortunately, it is not clear, which one of the two reasons has a higher impact on the observed PA production as the results were not supported by microbial analysis.

A high presence of acetic acid was also observed between day 15 and day 32 of 95 mmol L^{-1} on average before it significantly decreased towards the end of the fermentation. This decrease occurred simultaneously with the increase in the concentration of butyric acid. As mentioned earlier, this could hint at the mechanism of chain elongation, in which the production of butyric acid results in a depletion of acetic acid.

In order to put the results into context, Table 4.1 lists achieved concentrations of propionic acid as reported in literature. Only those studies were considered, where food waste was used as feed and operation conditions were similar to the present study. As can be seen from the table, the concentrations of PA obtained in this study, especially in Run 2 and Run 3, are significantly higher than those obtained in other studies using mainly anaerobic sludge as inoculum. This indicates that the microbial communities contained in the soft goat cheese in Run 2 and Run 3 might have played an important role in improving propionic acid production throughout the fermentation period. However, in comparison to studies that use synthetic medium as substrate and a pure culture of a propionic acid producing bacterial strain as inoculum, the propionic acid production in this work cultivations was rather low. For example, Liu et al. (2016b) achieved a maximum concentration of about 1000 mmol L^{-1} of propionic acid during the batch fermentation of concentrated glucose solution (about 600 g L^{-1}) inoculated with a high density culture of *Propionibacterium acidipropionici* ATCC 4875. Chen et al. (2013a) obtained an even higher propionic acid concentration of about 1836 mmol L^{-1} in a fed batch fermentation of glucose (40 g L^{-1} as initial concentration) by using *Propionibacterium freudenreichii* CCTCC M207015 isolated from cheese.

Table 4.1: Comparison with other fermentation processes using food waste (FW) as substrate. In present work, vegan dog food (DF) was applied in Run 1 and 2 while food waste was applied in Run 3. Results from batch cultivations are representing the final concentrations reached. (W/S, water/substrate ratio)

Working volume (L)	Reactor operation mode	Duration of experiment	Substrate	W/S ratio	Inoculum	Working pH and temperature	Initial load (g L^{-1} VS)	*OLR* (g L^{-1} d^{-1} VS)	Propionic acid concentration (mmol L^{-1})	Reference
2	Batch mode	50 h	FW	2.5:1*	Anaerobic activated sludge	6 39 °C	40.0 70.2	_	50 40	(Cappai et al., 2014)
10	Batch mode	14 d	FW	2.3:1.5	Thermophilic anaerobic sludge	6 50°C	306.7*	_	15*	(Hussain et al., 2017)
6	Semi-continuous feeding mode	96 d	FW	2:1.5*	Anaerobic sludge	6 37°C	2432*	2	Ranged between 3 and 43*	(Karthikeyan et al., 2016)
20	Fed- batch mode	54 h	FW	_	Anaerobic culture from a bioreactor	Uncontrolled (6 – 6.5) 30°C	_	_	Appx. 12*	(Sarkar & Venkata Mohan, 2017)
12	Semi-continuous feeding mode	100 d	DF	2:1	Mixed culture	6 30°C	297	Batch 12.3 17.8 29.7	13 ± 24 59 ± 11 28 ± 6 31 ± 14	Present work Run 1
12	Semi-continuous feeding mode	100 d	DF	3:1	Soft goat cheese	6 30°C	260	Batch 11.1	54 ± 27 77 ± 30	Present work Run 2
12	Semi-continuous feeding mode	100 d	FW	1:1	Soft goat cheese	6 30°C	105	Batch 3.2 5 3.2	78 ± 42 70 ± 18 39 ± 8 33 ± 8	Present work Run 3

* calculated from the data published. Results include only the experiments that were conducted at pH 6.

4.3.2 Impact of *OLR* and *HRT* on propionic acid production and yield

For comparison of VFA production in dependence on the operation conditions, VFA production rates were calculated. This was only justified for the target product propionic acid, since fluctuations of the concentration were much lower than for the rest of the acids, especially in Run 1, and trends of stable, increasing or decreasing concentrations were deducible from the data for the single combinations of *HRT/OLR* (compare Figure 4.3). Moreover, concentrations of propionic acid do not seem to be significantly dependent on the concentrations of the other acids. These facts were considered prerequisites for the determination of a production rate that can be linked to the corresponding operation phases.

The average propionic acid production rate P_{PA} (mg L^{-1} d^{-1}) was calculated by the following equation (Eq. 4.1).

$$P_{PA} = (dc_{PA})/dt + Q/V \cdot c_{PA,\,avg} \qquad 4.1$$

Where the gradient dc_{PA}/dt represents the change of propionate concentrations with time for the time period of a single operation phase (*OLR* and *HRT*), *Q* represents the volumetric flow rate in L d^{-1} (given as the liquid reactor volume *V* divided by the *HRT*), and $c_{PA,\,avg}$ is the average propionic acid concentration of the corresponding operation phase.

Unlike Eq 3.1 in which PA yield was calculated according to the amount of VS initially added to each batch test. The yields of propionic acid Y_{PA} in semi-continuous reactor, given as mg g^{-1} propionic acid per VS_{added}, were calculated as average propionic acid production rate P_{PA} per corresponding *OLR* (Eq. 4.1).

$$Y_{PA} = P_{PA}/OLR \qquad 4.2$$

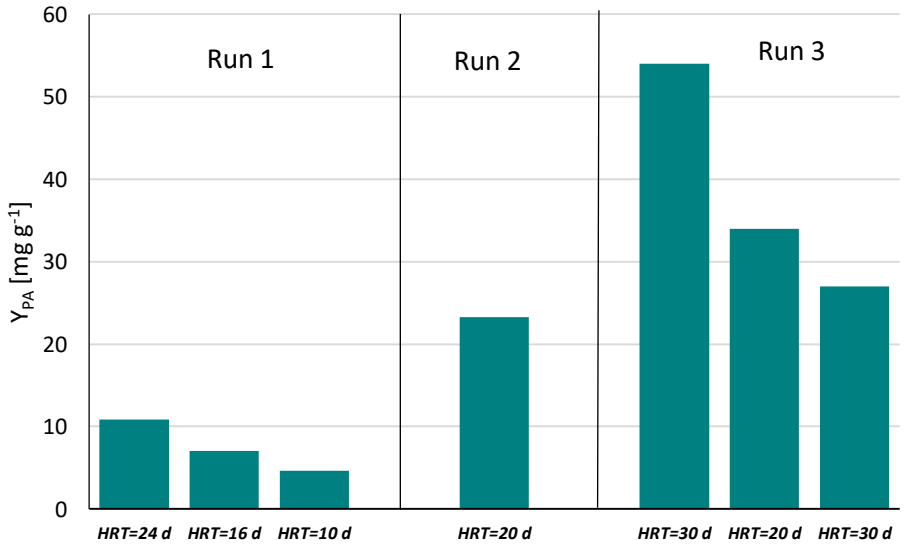


Figure 4.3: Yields of propionic acid Y_{PA} per VS added for different *HRT* and *OLR* in the semi-continuous operation mode.

The resulting propionic acid production rates and yields calculated for the different operation phases are given in Table 4.2. Since the VS concentration of the feed solution was constant in each Run, values of *HRT* and *OLR* are complementary in the semi-continuous feeding mode; an increase in the *OLR* is accompanied by a corresponding decrease in *HRT*.

The first finding that can be deduced from these values is that average propionic acid production rates were fairly constant during Run 1, and obviously not solely dependent on either *HRT* or *OLR* but their combination. Here, with regard to the production rate, a lower *HRT* seems to be compensated by a higher *OLR* within the ranges of *HRT* and *OLR* investigated.

However, the yields Y_{PA} of propionic acid per volatile solids added listed in Table 4.2 and plotted in Figure 4.3 indicate a much stronger dependency on the *HRT*, where the exploitation of the raw substrate gets worse with decreasing *HRT* and, thus, more substrate leaves the reactor before it can be converted to propionic acid. Consequences of lowering the *HRT* are clear, slow growing microorganisms might be washed out, and, thus, a shift in species composition and correspondingly the metabolic pathways realized by the biocoenosis will occur. At the same time, concentrations of intermediate products acting as precursors for VFA production might be affected.

According to many studies, applying longer *HRT* in general leads to increasing VFAs production as the microorganisms have more time to consume the substrate and process intermediate products. For example, Lim et al. (2008) obtained increasing total VFAs concentrations with increasing *HRT* in acidic fermentation of food waste. Bolaji and Dionisi (2017) reported similar results for the fermentation of vegetables waste. They found an increase of 13.3 % in propionate production by changing the *HRT* from 10 to 20 days. In contrast, other studies reported that increasing the loading rate at a certain point by decreasing the *HRT* could increase the VFA production by inhibiting the activities of hydrogen and methane-producing microorganisms, resulting in the accumulation of volatile fatty acids (Elbeshbishy et al., 2017; Mirmohamadsadeghi et al., 2019). Thus, the impact of *OLR* and *HRT* seems to depend significantly on the consortium of microorganisms at work and their specific growth and production rates.

Table 4.2: Average propionic acid production rates P_{PA} and yields Y_{PA} at different *HRT*s and *OLR*s in the three reactor runs.

	***HRT* [d]**	***OLR* [g L^{-1} d^{-1}]**	***P_{PA}* [mg L^{-1} d^{-1}]**	***Y_{PA}* [mg g^{-1}]**
Run 1	24	12.3	133	10.8
	16	17.8	126	7.1
	10	29.7	139	4.7
Run 2	20	11.1	259	23.3
Run 3	30	3.2	172	54
	20	5	171	34
	30	3.2	86	27

By considering the Y_{PA} obtained from the first run and the physical differences between the two substrates, it was decided to operate the reactor at low *OLR* of g L^{-1} d^{-1} VS $_{added}$ in Run 2, while choosing a rather low *OLR* in Run 3, which means that a significantly lower substrate concentration is offered in the reactor. Thus, basically the time available for acidification was increased, considering especially slower metabolic pathways including several intermediate products. As can be seen from Table 4.2, the highest propionic acid production rate was achieved of approximately 259 mg L^{-1} d^{-1} during the semi-continuous operation mode of Run 2.

Run 3, on the other hand, showed the highest overall PA yields compared to Run 1 and Run 2 at lower *OLRs* and higher *HRT*s. The highest yield of PA of 54 mg g^{-1} was achieved at an *OLR* of only 3.2 g L^{-1} d^{-1} VS $_{added}$ in the first fed-batch phase, which is more than twice as much as achieved in Run 2, and even 5 times higher than the highest of obtained in Run1. This yield decreased by 50 % at the same *OLR* during the final phase. Compared to Run 2, production rates were lower in Run 3 and remained unchanged when the ORL was increased, in the final phase the rate was decreased by 50 % by decreasing the *ORL* again. However, as mentioned before it was not clear if the reduction in the PA production rates and yields were due to the change in pH value or if they were affected by the changing of *OLR* and *HRT*.

Thus, it can be concluded that the propionic acid production rates and yields of acidic hydrolysis of the two substrates used in this work cannot be generally predicted from neither the single parameters of *OLR* and *HRT* nor their combination. This might indicate that the biocoenosis itself has a critical role in the ultimate performance of the reactor in this study, and that the propionic acid production might depend to a larger extent on the inoculum than on operation conditions.

4.3.3 Gas production and composition

The gas produced in this study was comprised of mainly hydrogen (H_2) and carbon dioxide (CO_2) with a very low concentration of nitrogen (N_2), whereas CH_4 was not detected in all reactor runs.

In this study, the total volumetric production rate of gaseous compounds ranged between 0.5 and 21 NL d^{-1}day in Run 2 and 0.9 to 8 NL d^{-1}day in Run 3, while it was not quantified in Run 1. The H_2 to CO_2 ratio in the produced gas was similar between all runs. The highest content of H_2 in the gas phase was 52 % (28 % on average), 45 % (27 % on average), and 52 % (40 %) in Run 1, Run 2, and Run 3, respectively. CO_2 contents amounted to a maximum of 94 % (68 % on average) in Run 1, 84 % (65 % on average) in Run 2, and 44 % (58 % on average) in Run 3.

The production of butyric acid and/or acetic acid are usually accompanied by hydrogen production under controlled lab conditions (e.g. use of a monoculture and glucose as substrate), while propionic acid production consumes hydrogen. Thus, it is often reported, that the increase in H_2 concentration stimulates PA production (Dahiya et al., 2020; Koskinen et al., 2007; Sivagurunathan et al., 2014). In contrast, the accumulation of propionic acid was not always linked to the production rate of H_2 in anaerobic treatment of wastewater as stated by Wang et al. (2006). Similar results were also observed by Inanc et al. (1999) showing that a lower H_2 pressure did not affect the accumulation of propionic acid and other VFA. However, no obvious correlation was observed between H_2 and propionic acid or other VFA production in this study, probably due to the variations in the composition and performance of the microbial communities and the wide metabolic diversity associated with the different species.

4.3.4 Acidification yield

Acidification yield is an important indicator of how much soluble organic matter is converted into VFA and, thus, how successful the VFA production process is. The acidification yield was calculated as the ratio of the average VFA and average DOC concentrations (VFA/DOC).

The variation of the average DOC concentrations in the reactors, the VFA/DOC ratios as well as the PA/DOC ratios achieved at different *HRT* and *OLR*s are shown in Figures 4.4 (a, b, & c) for the three reactor runs. In Run 1, it can be seen that the average DOC concentration was 48 g L^{-1}, and rather constant despite different *OLR*s. In Run 2, the values fluctuated more and only reached 24 g L^{-1} DOC on average. The DOC concentration was only 18 g L^{-1} on average in Run 3. The latter was expected due to the lower organic loading rate.

The higher DOC concentrations found in Run 1 indicate that much of the organic matter originating from the dog food released high levels of DOC and supplied an adequate amount of organic substrates to produce VFAs. However, the acidification attained by this Run was lower compared to Run 2 and Run 3. The highest values ranged between 33 % and 62 % at *HRT* of 16 days. By decreasing the *HRT* to 10 days, the ratio of VFA/DOC was the lowest and ranged between 10 % and 46 %, which showed that the fermentation was to some extent delayed at this *HRT* due to the higher *OLR*.

Although, the ratio was also low at *HRT* of 24 d, it seems probable that the acidification might not have been completed by the end of this phase of the fermentation, and it could have been increased further by maintaining the retention time at 24 days. As can be seen in Figure 4.4 (a), the VFA/DOC ratio increased to 60 % in last few days of the fermentation at this *HRT*.

The same is true for Run 2, the longer *HRT* of 20 days led to a higher acidification yield. The highest VFAs conversion ratio ranged between 40 % and 90 % (55 % on average) and was observed during the semi-continuous feeding mode. While the ratio was ranging between 48 % and 88 % (62 % on average) in Run 3.

More importantly, a high PA/DOC ratio of 14 % and 13 % on average was observed, respectively, in Run 2 and Run 3 compared to 4 % on average in Run 1. This indicates that the microbial communities in Run 2 and Run 3 were more efficient in acidification and, thus, achieved a higher yield per DOC offered.

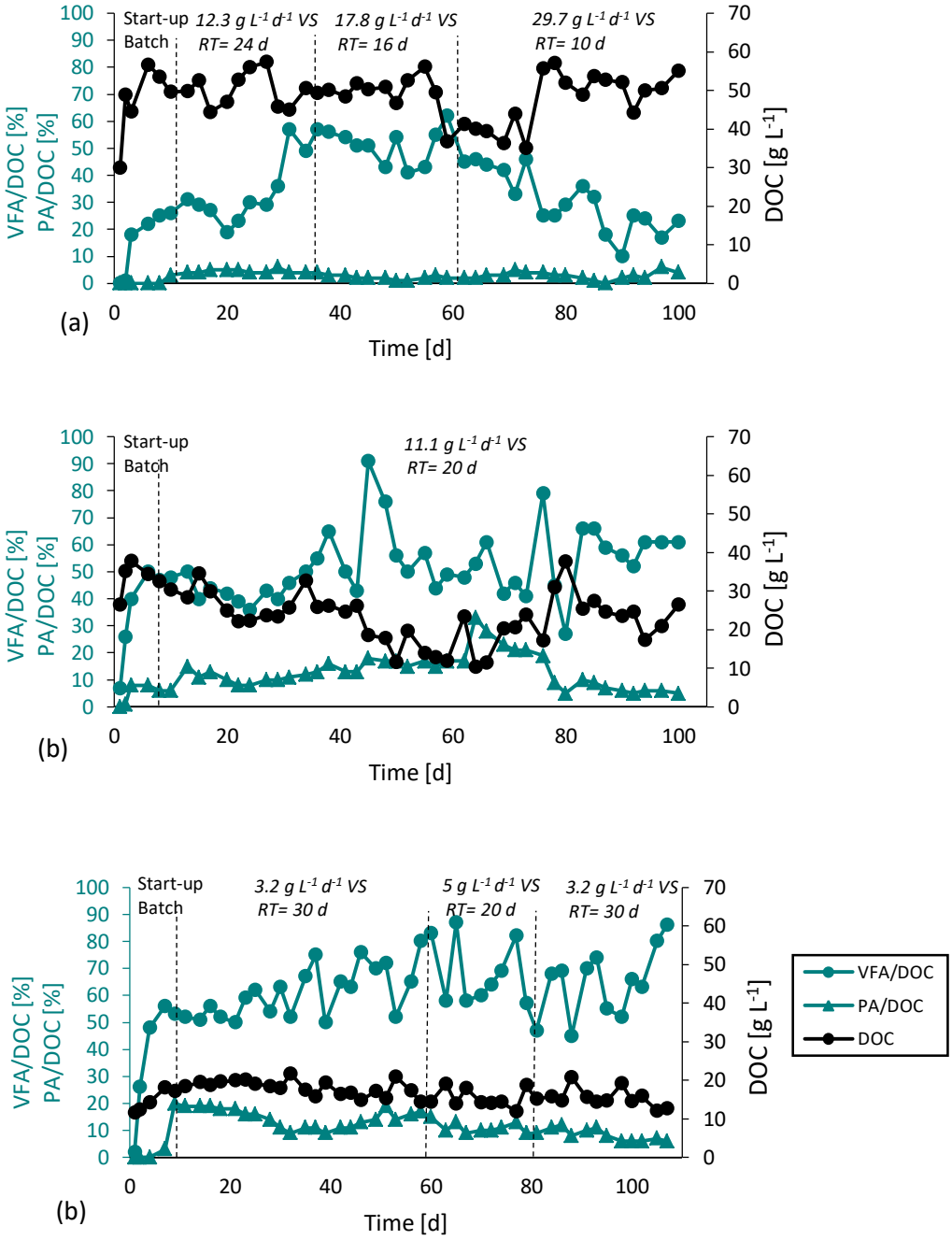


Figure 4.4: Variation of the average DOC concentration and VFAs/DOC ratios at different *OLRs* and *HRTs*. (a) Run 1, (b) Run 2, and (c) Run 3.

4.4 Conclusion

Soft goat cheese was successfully used as inoculum to drive the propionic acid production fermentation process. A maximum PA concentration of 139 mmol L^{-1} and 105 mmol L^{-1} at a yield of 23.3 mg g^{-1} and 54 mg g^{-1} VS were obtained from dog food and food waste, respectively. The fermenter could be kept in a stable process of propionic acid production at *HRT* of 30 days and a rather low *OLR* of 3.2 g L^{-1} d^{-1} VS. The different inocula proved to have a significant impact on the absolute and relative production of the individual VFA, which could be supported by microbial community analysis. 16S rRNA gene diversity data showed that the community was more stable in run 2 inoculated with goat cheese, in which *Propionibacteria* were detectable in all samples, even after 86 d of cultivation (corresponding to 3.6 times the *HRT*). Results show that a high propionic acid production is possible, applying optimized process parameters and selecting the adequate microbial community for inoculation.

4.5 Evaluation of propionic acid production and yield in both batch and semi-continuous experiments

The results of chapters 3 and 4 verifying that propionic acid production and yield were affected by the operational parameters including pH, *HRT*, and *OLR* together with inoculum type in both batch and semi-continuous fermentation. To reveal the difference between the PA production and yields in both cases, the results were compared at pH 6 based on the *OLR* that applied in each experiment as the main variable (Figure 4.5 a & b). The *OLR* for batch mode during the start-up of the hydrolysis reactor as well as the lab-scale batch AMPTs experiments was calculated according to the *HRT* of the maximum PA production reached in both cases.

It is possible to notice that the *OLR* has a higher impact on the production and yields of PA in both batch and semi-continuous operation modes. The production rate of PA was almost higher in batch than it is in the semi-continuous mode, however, the highest productivity was observed at *OLR* of around 8 g L^{-1} d^{-1} VS in both cases. On the other hand, the highest PA yields were achieved at *OLR* of approximately 5 g L^{-1} d^{-1} VS in both batch and semi- continuous fermentation mode of the hydrolysis reactor. While it tends to decrease with increasing the *OLR* in semi-continuous mode due to the higher concentration of substrate and washout of bacteria. The latter was not possible in the batch experiments (AMPTs) leading to the highest production and yields especially when goat cheese inoculum and pre-treated food were used at *OLR* of 10 g L^{-1} d^{-1}.

Based on the comparison of all experiments, it can be concluded that PA production from food waste was successfully performed in both batch and semi-continuous mode. The process in both cases was enhanced by using goat cheese as inoculum and further by pretreatment of the substrate. Results of the semi-continuous operation are promising to apply for commercial and large scale indicating that the reactor can be operating over a long time and under stable conditions with low *OLR*. While the batch operation mode can offer useful information to the functionalities of the parameters affecting the PA production process.

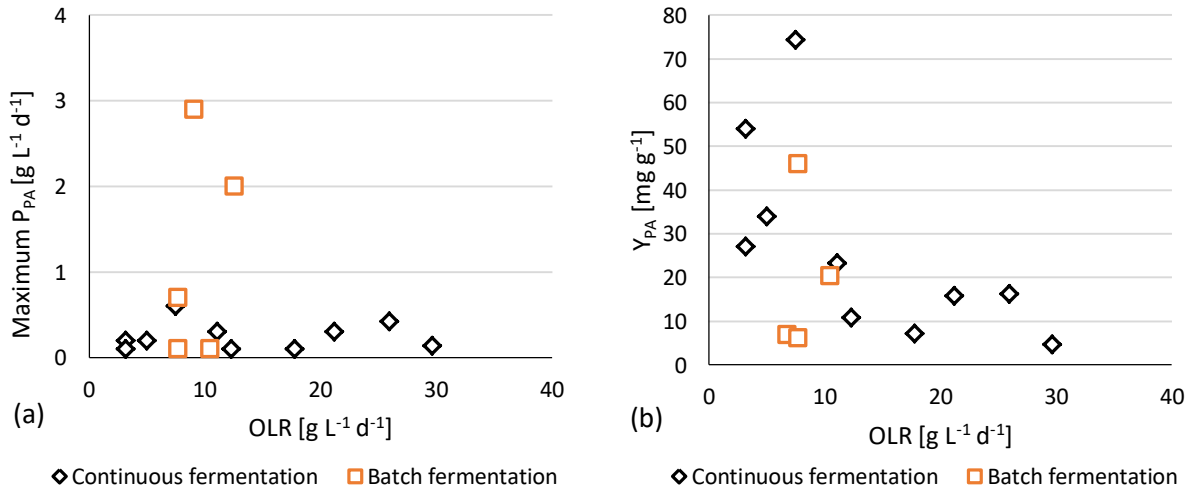


Figure 4.5: The maximum PA (a) production rates and (b) yields as a function of the *OLR* applied in both batch and semi-continuous fermentation experiments.

Chapter 5

5 Treatment of fermentation broth with high VFA content using microfiltration

5.1 Introduction

As a not straightforward process, separation of VFA from complex effluents such as fermentation broths is a challenge, due to the complex mixture that contains several impurities including unhydrolyzed substrate particles, fibers, biomass, inorganic salts, and by-products generated during the fermentation (Aghapour Aktij et al., 2020). Electrodialysis (Pan et al., 2018), reverse osmosis (Schlicher & Cheryan, 1990), nanofiltration (Xiong et al., 2015), adsorption (Talebi et al., 2020), forward osmosis (Garcia-Aguirre et al., 2020), ion exchange (Rebecchi et al., 2016), and liquid–liquid extraction (Alkaya et al., 2009; Mostafa, 1999) are common techniques used for the separation and recovery of VFAs from aqueous solutions and fermentation broth. However, pretreatment of the effluents is needed to make these techniques applicable.

Within this context, microfiltration (MF) has been proven to be an effective pretreatment method for different aqueous waste streams and fermentation broths, due to its ability to remove various particles, colloidal organics, and microorganisms, at high flux and low pressure and with high scaling up potential. Systems in which microfiltration was combined with other methods such as reverse osmosis (RO), electrodialysis, and nanofiltration have been reported in many publications on VFA separation (Jänisch et al., 2019; Tao et al., 2016; Thuy & Boontawan, 2017).

Microfiltration membranes are commercially available in diverse modules made of different materials (Chae et al., 2009), which are usually employed to separate the fine particles in the size range of 0.1–10 µm. However, fouling issues are still the main obstacles in the MF process.

Membrane fouling is normally caused by particle deposition (cake formation), adsorption of solute, biological film growth (biofouling), and deposition of biopolymers such as proteins and polysaccharides (organic fouling) on pores and membrane surfaces, which decreases the membrane efficiency and increases the operating costs. Different strategies have been developed to overcome the membrane fouling problems and to increase the filtration flux by physical (back washing, gas bubbling, relaxation) and chemical cleaning. Membrane fouling can also be reduced by using other pretreatment methods before MF is applied such as addition of chemicals and electrocoagulation (Gamage & Chellam, 2011; Huang et al., 2017; Sari & Chellam, 2013), flocculation and adsorption (Guo et al., 2005).

In this work, the treatment of the fermentation broth consisted of two processes: 1) a separation unit with a pore size of 60 µm was used as a pretreatment for the removal of big particles and fibers and 2) a submerged MF system with a high flow rate of nitrogen gas which creates a cross flow velocity and scrubs the membrane surface.

The focus of this chapter is to evaluate the treatment of fermentation broths by microfiltration. Within this context two different hydrolysates from the fermentation of dog food (Run 2) and food waste (Run 3) have been tested. Evaluation comprises the (i) the separation properties of the membranes including TSS removal as well as the concentration of DOC and VFA in the permeate and (ii) the membrane performance including the impact of membrane pore size on the permeate flux.

5.2 Materials and methods

5.2.1 Separation units

The effluents from the different hydrolysis reactor runs were rich in solids and contained some large particles of unhydrolyzed food waste. To remove these particles a small separation unit was connected directly to the hydrolysis reactor. The unit consists of two detachable cylinder halves made of stainless steel and has an internal capacity of 172 cm^3 in total. The first half contains a rotating brush, which is driven by a motor and rotates at a controlled speed ranging from 0 to 100 rpm, while the second half includes a 60 µm pore size sieving mesh with an area of 60.8 cm^2. The unit was designed in such a way that the brush facing the mesh when the two halves are combined (Figure 5.1).

The reactor effluents were pumped into the unit using a peristaltic pump. Filtrated liquid flowed through an outlet to the collecting tank. The concentrated effluent which was removed by the brush was recirculated back to the reactor to avoid the accumulation of these particles on the mesh.

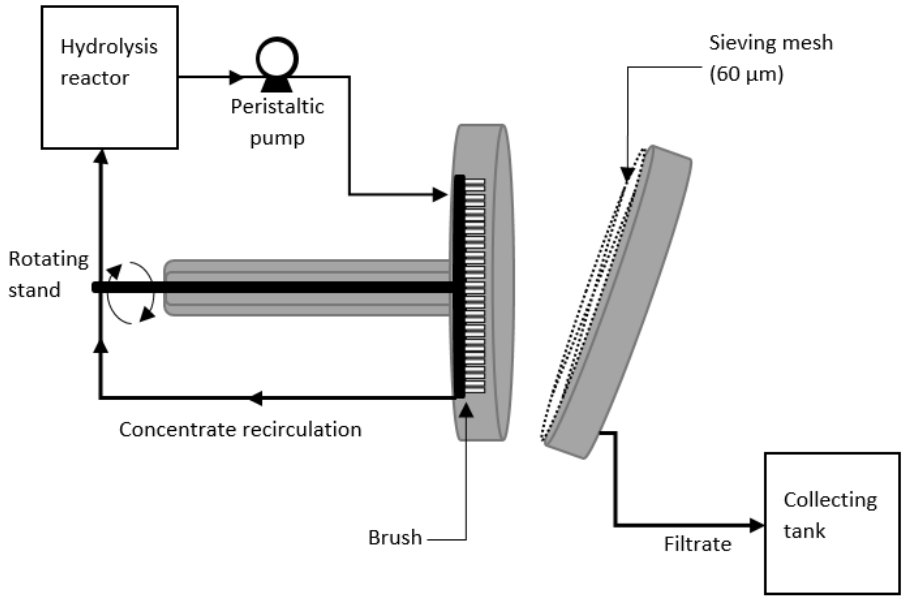


Figure 5.1: Schematic diagram of the separation unit (SU).

5.2.2 Submerged membrane system

A schematic of the submerged membrane filtration system is shown in Figure 5.2. The system consisted of a feed tank of 1 L working capacity, membrane chamber, plate stirrer (for feed mixing), nitrogen gas supplier, gas volume flow meter, pressure meter, peristaltic pump, filtrate collecting bottle, and

electronic balance. The membrane chamber was fixed on one side of the feed tank. The membrane with a filtration area of 12.6 cm^2 was oriented in such a way that the active layer of the membrane faced the feed solution (Saravia, 2009). Since the experiments should be conducted under anaerobic conditions, nitrogen gas bubbles were supplied to the membrane surface through a small diffuser located in the membrane chamber. Nitrogen bubbling with a flow rate of 80 m^3m^{-2} h^{-1} was adopted to reduce membrane fouling. A peristaltic pump was used to create an under pressure on the permeate side to suck the effluent through the membrane. A pressure meter was employed to measure the transmembrane pressure (TMP) generated by the suction pump. Experiments were carried out without backwashing or relaxation times.

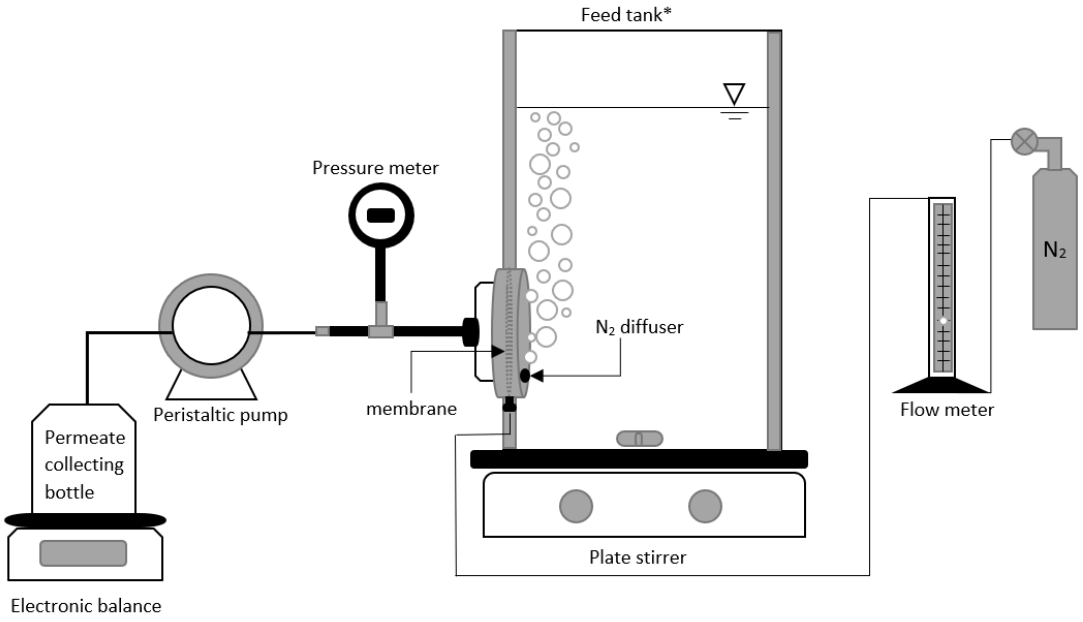


Figure 5.2: Schematic diagram of the submerged membrane system. * Filtrate from separation unit (SU).

5.2.3 MF membrane characteristics

Flat sheet of Polyethersulphone (PES) membranes with different pore sizes of 0.1 µm, 0.45 µm, and 0.8 µm were employed in this work. Table 5.1 shows the characteristics of the three membranes used.

Table 5.1: Membranes characteristics according to the manufacturer.

Characteristics	**0.1 µm**	**0.45 µm**	**0.8 µm**
Company	MEMBRANA GmbH - A Polypore Company (USA)	Pall Corporation (Dreieich, Germany)	MEMBRANA GmbH - A Polypore Company (USA)
Material	Polyethersulfone	Polyethersulfone	Polyethersulfone
Membrane type	Flat sheet	Flat sheet	Flat sheet
Hydrophilicity	Hydrophilic	Hydrophilic	Hydrophilic
Contact angle (°)*	40°	30°	50°

Thickness	110 ± 10 µm	140 µm	110 ± 10 µm
Clean water permeability	≥ 2.4 L / (h m^2 bar)	≥ 60 L / (h m^2 bar)	≥ 102 L / (h m^2 bar)

*Measured with the water drop adhesion protocol using the contact angle system (OCA) from DataPhysics, Filderstadt, Germany.

5.2.4 Experimental design

5.2.4.1 Hydrolysate characteristics

The feed solutions (hydrolysates) used in this study were taken from reactor Run 2 (fermentation of dog food) and Run 3 (fermentation of food waste). More details of each run and the characteristics of the hydrolysate were discussed in chapter 4.

5.2.4.2 Filtration experiments

All the experiments were conducted at room temperature. Temperature and the pH of the feed were measured at the beginning and the end of each experimental run. The temperature of the feed ranged between 20° C and 25° C, while the pH ranged between 5.8 and 6.0, which is the same as the pH during the fermentation process. Thus, pH adjustment was not necessary. The permeate volume was measured every two minutes using a balance. All the experiments were carried out for 30 minutes.

5.2.4.3 Critical flux concept and determination

The concept of critical flux has been firstly described in an empirical approach by Field et al. (1995), who defined the critical flux as "a flux below which a decline of flux with time does not occur; above it fouling is observed". In other words, there is always a flux, in which there is no (or little) flux decline, independent of water composition. Therefore, operation below critical flux is usually assumed when the transmembrane pressure is steady and does not increase. However, the critical flux concept is related to short time fouling and flux behavior, such as cake formation or adsorption. Fouling issues related to biofilm formation cannot be evaluated using the concept of critical flux.

To determine the critical flux in this work, the flux was increased stepwise with a step length/time of 30 min. The increase of the flux leads to an increase in the TMP. At a certain flux, the TMP is not constant during the step length indicating that a fouling layer has formed on the membrane and that the membrane is being operated under conditions above the critical flux.

5.2.4.4 Calculation

The volumetric permeate flux (J) in terms of liter per square meter per hour (L m^{-2} h^{-1}) was calculated by Eq. (5.1):

$$J = V/\Delta t\ A \qquad 5.1$$

where A is the effective membrane area, and V is the volume of permeate collected over a time interval Δt.

The rejection (R) of DOC and VFA concentrations and TSS removal were calculated using Eq. (5.2):

$$R\ \% = \left[1 - \left(\frac{C_p}{C_f}\right)\right] x\ 100 \qquad 5.2$$

where C_f and C_p represent the concentrations of DOC, VFA, or TSS in feed and permeate, respectively.

5.2.4.5 Analytical methods

Analysis of total solids (TS), volatile solids (VS), and total suspended solids (TSS) were carried out according to the German Standard Methods for the Examination of Water, Wastewater and Sludge (DIN, 1989). Volatile fatty acids (VFA) concentration was determined using IC analysis (Metrohm 881 Compact Pro) using a Metrosep Organic Acids 250/7.8 column. Total organic carbon (TOC), total nitrogen (TN), and dissolved organic carbon (DOC) were measured with a Shimadzu TOC-$_{LCPH}$ analyzer.

5.3 Result and discussion

5.3.1 Separation unit performance

Figure 5.3 shows photographs of hydrolysate samples before and after the separation unit (SU) from both reactor runs (Run2 and Run 3). Most of the large particles obviously were removed from the hydrolysates. However, the sample color did not change much and filtrate samples still have a high suspended solids concentration due to the presence of particles smaller than 60 µm.

The composition of hydrolysates and filtrates are presented in Table 5.2. The TSS and TS concentrations were higher in the hydrolysate from Run 2 (hydrolysis of dog food) than in the hydrolysate from Run 3 (hydrolysis of food waste) probably due to the differences in the composition of the feeds. This is also attributed to different feeding rate in both reactor runs during the fermentation process. Comparison of the separation unit (SU) feed and permeate from both reactor runs in Table 3.2 shows a good performance of the SU concerning the removal efficiency of TSS and TS. TSS, which mostly represents unhydrolyzed food particles were removed by 86 % and 95 % from the hydrolysates of Run 2 and Run 3, respectively.

As expected, most VFA passed through the mesh due to the large pore size, only slight differences in DOC and VFA concentration before and after the separation unit were observed. Elimination of VFA can be ascribed to the separation of particles and the VFA adsorbed on them. This was confirmed by the results of (Tuczinski et al., 2018) who observed 15 % reduction in VFA concentration after filtration by a 0.45 µm pore size membrane. The authors assumed that some of the VFA were adsorbed on the surface of the particulate matter of the hydrolysate and eventually removed by the membrane. Differences in the concentration of the acids in the SU permeate compared to the feed could be also due to measurement inaccuracies and VFA degradation before analytics.

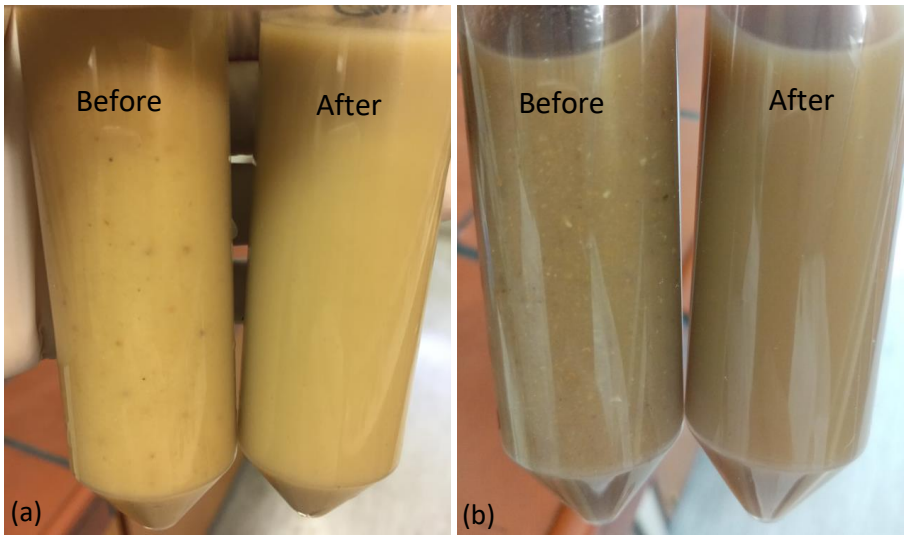


Figure 5.3: visual observations of samples from (a) Run 2 and (b) Run 3, before and after separation unit.

Table 5.2: Hydrolysates characteristics before and after separation unit.

	Run 2*		**Run 3 ***	
Parameter	Hydrolysate	After SU (Feed for MF)	Hydrolysate	After SU (Feed for MF)
TSS [g L^{-1}]	248.1 ± 21.2	34.1 ± 0.2	150.6 ± 3.5	8.1 ± 0.7
TS [g L^{-1}]	141 ± 32	112 ± 44	110 ± 0.7	83 ± 3
VS [g L^{-1}]	100 ± 39	73 ± 38	53 ± 1.8	25.6 ± 1.9
DOC [g L^{-1}]	17.8 ± 0.6	17.5 ± 0.8	15.9 ± 1.4	16.1 ± 0.7
Lactic acid [g L^{-1}]	1.3 ± 0.6	1.1 ± 0.5	10.2 ± 0.5	9.8 ± 0.5
Acetic acid [g L^{-1}]	2.4 ± 0.6	2.0 ± 0.7	4.0 ± 0.5	3.6 ± 0.5
Propionic acid [g L^{-1}]	6.2 ± 1.2	5.8 ± 0.9	3.5 ± 0.7	3.2 ± 0.7
Butyric acid [g L^{-1}]	16.1 ± 3.2	16.8 ± 2.6	7.2 ± 0.9	7.1 ± 0.9

*The samples were taken at days 49 and 37 from Run 2 and Run 3, respectively.

5.3.2 Membrane filtration performance

5.3.2.1 Separation properties

5.3.2.1.1 TSS removal

As mentioned earlier, two different hydrolysates were used as a feed for the MF system after the pretreatment by the separation unit (Table 5.2).

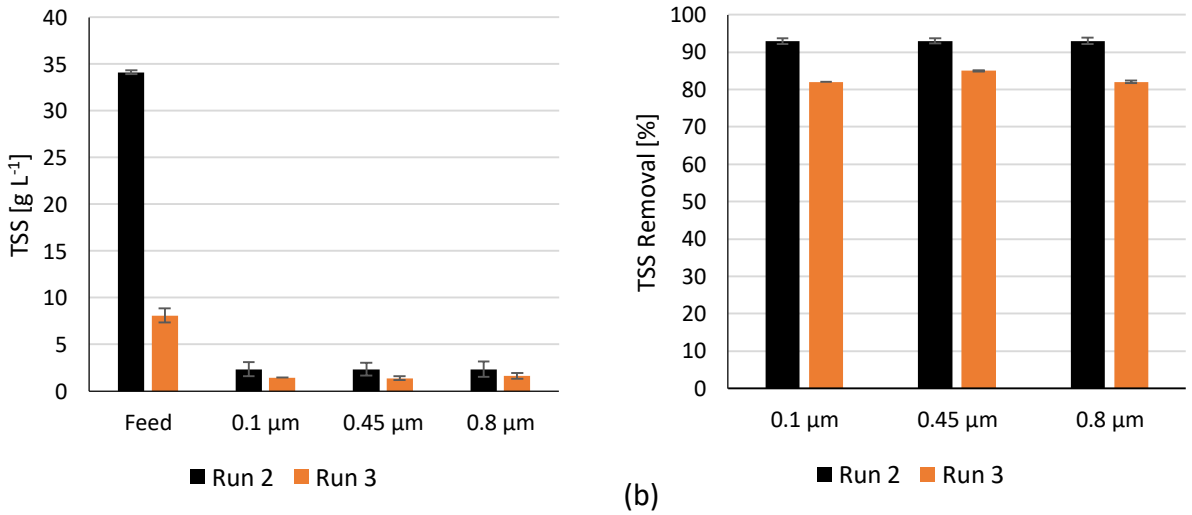
Figure 5.4 shows the TSS concentrations in the feed and the permeate for both hydrolysates for microfiltration experiments using membranes with different pore sizes. Regardless of the pore size, the membranes were able to remove 93 ± 0.8 % of the TSS concentration of the feed from Run 2 (dog food). Using the feed from Run 3 (food waste), a lower removal of TSS while quite similar for all membrane pore sizes was observed with a reduction of 82 ± 0.3 % for both, 0.1 µm and 0.8 µm pore sizes, and a reduction of 85 ± 0.2 % for the 0.45 µm pore size membrane.

The differences of the removal of suspended particles from the hydrolysate is mostly linked to the different pore sizes of the membranes and the different sizes of the suspended particles, which forces the particles to be removed differently (Waeger et al., 2010). This can be seen in the different percentages of TSS removal between the two permeates, which indicates that the hydrolysate from Run 3 had a smaller size of suspended particles than the hydrolysate from Run 2, which could be due to the difference in the characteristics of the dog food and food waste.

Similar results of TSS removal of 94 % were obtained by Madaeni et al. (2012) during the treatment of oily wastewater using ceramic microfiltration membrane with pore size of 0.2 µm. Umaiyakunjaram and Shanmugam (2016) also observed a 95 % reduction of TSS using a membrane with 0.4 µm pore size in submerged anaerobic membrane bioreactor (SAMBR) treating high suspended solids raw tannery wastewater for biogas production.

However, the results achieved in this work showed that some particles still passed through the membrane in both cases and agglomerated in the permeate. This was confirmed by the results of Jänisch et al. (2019) who observed presence of suspended particles after MF treatment of hydrolysates (sugar beet, grass cut and grass-corn hydrolysate) from biogas plant. This phenomenon might also be associated with the high calcium ion concentrations in both hydrolysates with 807.0 ± 15.7 and 188.3 ± 3.9 mg L^{-1} in Run 2 and Run 3, respectively. The presence of calcium ions together with organic components such as humic substances or natural organic matter (NOM) can form insoluble complexes which eventually precipitate (Swift et al., 1988). Additionally, the permeate has a very high concentration of VFA and high potential of biomass production. This was clearly observed in a permeate sample a few days after the filtration. Figure 5.5 shows a permeate sample from Run 3 with precipitates at the bottom of the tube.

From these results and considering the studied literature, it can be concluded that TSS removal is highly dependent on the type of hydrolysate and the particle sizes as well as the types of membrane and depending on the feed and permeate composition, precipitates may be formed after filtration.



(b)

Figure 5.4: (a) Concentrations of TSS in the feed and permeate for both hydrolysates (b) TSS removal expressed as concentrations in MF permeate compared to feed concentrations.

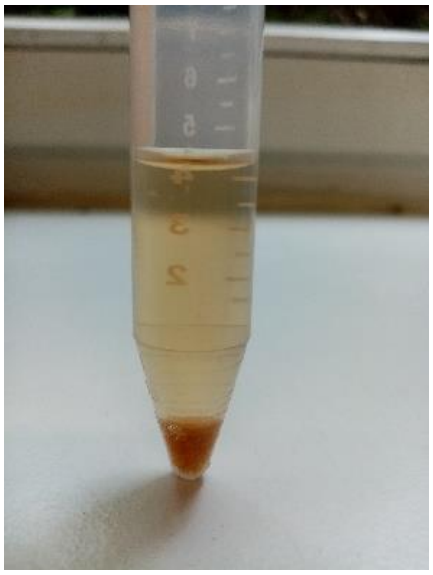


Figure 5.5: Permeate sample from Run 3.

5.3.2.1.2 DOC and VFA concentrations

The DOC concentrations of the feed and permeate were measured to investigate the elimination of DOC during filtration (Figure 5.6). As can be seen, hydrolysate (feed) from Run 3 had a higher DOC concentration than the hydrolysate (feed) from Run 2. However, in both cases, membranes were permeable for most of the DOC with slight changes in the concentration. Less than 3 % of DOC reduction was observed in both permeates with all membranes, which is consistent with VFA concentrations observed throughout the experiments.

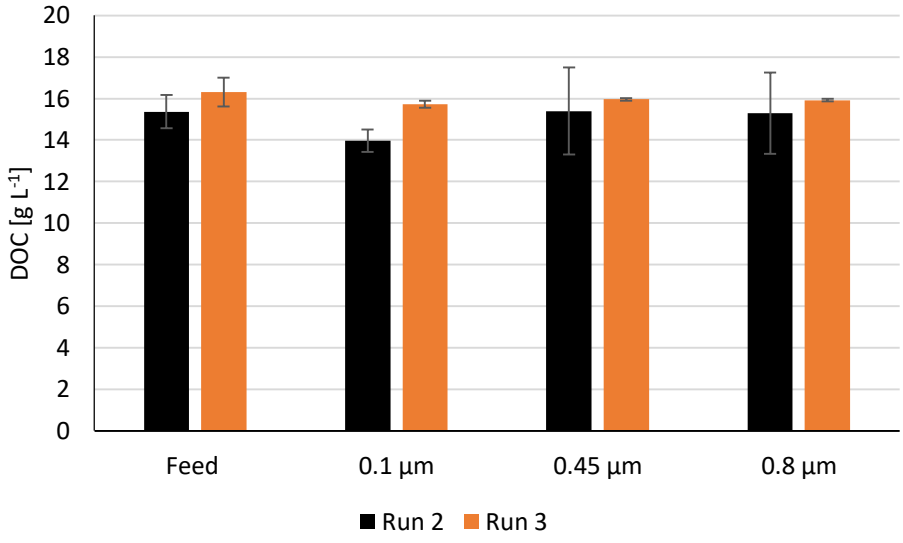


Figure 5.6: DOC concentrations in feed and permeate for both hydrolysates before and after MF with membranes of different pore sizes.

The tested membranes were almost completely permeable to all VFA regardless of which membrane pore size was used. This was expected as all MF membrane pore sizes are much larger than the acids´ molecular size (Jänisch et al., 2019). The rejection of VFA by MF membranes can only be explained the retention of VFA adsorbed on particles. Figure 5.7. a, shows an example of the concentration of VFA (lactic, acetic, propionic, and butyric acid), as well as the rejection characteristics of the membrane with 0.45 µm pore size for both reactor hydrolysates (Run 2 and Run 3). Overall, the retention rate of VFA by the membrane was relatively low (lower than 13 %). This is in line with the study by Tao et al. (2016) who reported that 80 % of VFA were found in the permeate after MF of a thermally hydrolyzed waste activated sludge.

However, the retention of total and individual VFA was higher in the filtration of the hydrolysate from Run 3. These differences between the two hydrolysates might be attributed to the different characteristics of each effluent and its particle size. In addition to that, VFA concentration in total was higher in Run 3 hydrolysates than in Run 2. As mentioned earlier, it was assumed that Run 3 had different shapes and generally smaller particles sizes than in Run 2. That may be also associated to the adsorption mechanism of VFA on particles, in which smaller particles offer bigger surface for adsorption, and thus for VFA adsorption and removal via MF.

The rejection characteristics of the single acids slightly varied. Membrane showed very similar rejection characteristics with the hydrolysate from Run 2 for acetic, propionic, and butyric acid whereas the rejection of lactic acid was slightly higher. However, this was not the case in the filtration of Run 3 hydrolysate, only acetic acid was highly rejected by the membrane in comparison to the other acids (lactic, propionic and butyric acid). This high acetic acid rejection might be due to the presence of microorganisms in the permeate and the storage of the sample before measurement.

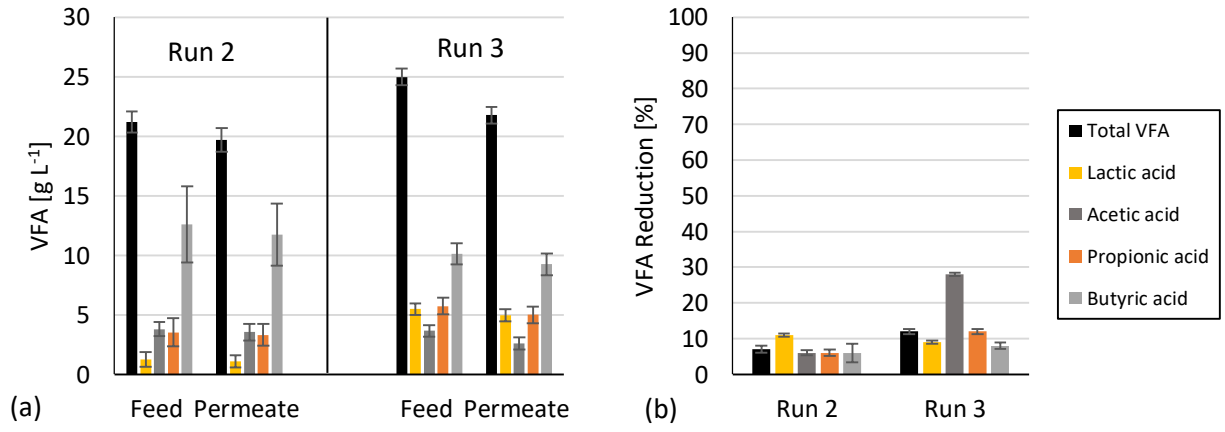


Figure 5.7: Composition of feed and permeate for both hydrolysates, before and after MF with a membrane of 0.45 µm pore size (a) concentration of total and individual VFA and (b) rejection of the total and individual VFA.

5.3.2.1.3 Critical flux

Figure 5.8 shows the critical flux determination for the MF membranes, a visual observation of the membrane surface after use, as well as feed and permeate samples of both hydrolysates. Particle free permeates were achieved in both cases regardless of the membrane applied.

The flux results suggest that the membrane fouling depends of hydrolysate composition and membrane properties, principally pore size. In the experiment with the hydrolysate from Run 2, the critical flux of 0.1 µm and 0.8 µm pore size membranes was approx. 9 L m^{-2} h^{-1}, whereas the one of the 0.45 µm pore size membrane was about 13 L m^{-2} h^{-1}. For the filtration of hydrolysate form Run 3, the results indicated that the critical fluxes were approx. 8 $Lm^{-2}h^{-1}$, 11 $Lm^{-2}h^{-1}$, 14 $Lm^{-2}h^{-1}$, with a pore size of 0.1 µm, 0.8 µm, and 0.45 µm, respectively. It can be also seen that for all three membranes pores sizes, the TMP could be maintained constant when the permeate flux was less than 7 L m^{-2} h^{-1} regardless of which hydrolysate was filtered. The hydrolysate from Run 2 had a higher TSS concentration compared to the one from Run 3 resulting in a lower critical flux for Run 3 (food waste). This was expected as the cake formation would be faster by using the hydrolysate from Run 2.

Regarding to the membrane pore size, the results indicated that the membrane with a pore size of 0.45 µm displayed the highest permeate flux and the highest critical flux among the other membranes.

The reasons of the differences between the membranes can be explained by the following parameters:

1) the different characteristics of hydrolysates and the size and amount of particles, which blocked the membrane faster in the filtration of the hydrolysate from dog food fermentation (Run 2) especially with the pore size of 0.8 µm. It seems that the particles of the hydrolysate from Run 2 are bigger than 0.8 µm, therefore the critical flux for 0.1 µm and 0.8 µm membranes (membranes from the same producer and similar properties) is very similar and related to cake formation. The hydrolysate from Run 3 probably contains particles of around 0.1 µm which induce pore blocking and a lower critical flux in the experiments with the 0.1 µm membrane.

2) the fact that the membrane of 0.45 µm pore size had different characteristics, namely a different structure and higher hydrophilicity than the other membranes, which have a similar structure and approximately similar hydrophilicity because they are produced in a similar way (see Table 5.1). The scanning electron microscope (SEM) images in Figure 5.9 show cross sectional cuts through the membranes and membrane surfaces of the used three PES microfiltration membranes at the end of each filtration experiment. The images also support the assumed fouling mechanisms identified above. The large areas where the membrane is covered in case of the 0.1 µm and 0.8 µm pore size membranes provides further evidence of deposition of particles on and inside the pores causing an increase of membrane fouling. As it can also be seen in Figure 5.9, the membrane with 0.45 µm pore size had different structure and pore shapes in comparison to the other two membranes. The higher fouling percentage on the membrane surfaces of 0.1 and 0.8 µm pore size membranes suggest that these membranes interact with the hydrolysate and more fouling was produced during the filtration.

It was very difficult to compare the achieved critical fluxes in this work to other studies, due to the various approaches and operational conditions used found in literature. Most of the studies on submerged membranes and filtration of hydrolysate used synthetic feed medium, while limited studies used real fermentation broth.

Nevertheless, the critical fluxes achieved in this work were higher than those achieved in other studies using hydrolysate from fermentation broths. For example, a critical flux of 7 L m^{-2} h^{-1} was obtained by Tuczinski et al. (2018) during the treatment of hydrolysate from a hydrolysis reactor operated with corn silage at thermophilic conditions (55 °C) and pH range of (5.6–6.0) using submerged ceramic membranes at thermophilic conditions with different membrane pore sizes. A critical flux of 7 L m^{-2} h^{-1} was also achieved by Martinez-Sosa et al. (2011) in an anaerobic submerged membrane bioreactor (AnSMBR) for municipal wastewater treatment. In both studies, backwash and relaxation times were applied during the process but with a lower gas flow rate of approximately 2 m^3 m^{-2} h^{-1} compared to 80 m^3 m^{-2} h^{-1} of this study. However, in comparison to studies that use synthetic medium feed and an aerobic submerged system, the critical flux achieved in this study is still low (Lu et al., 2008).

It could be noted, from the above results together with previous studies that the permeate flux does not only depend on the pore size but is also influenced by both membrane and feed characteristics as well as the operational conditions of the filtration process.

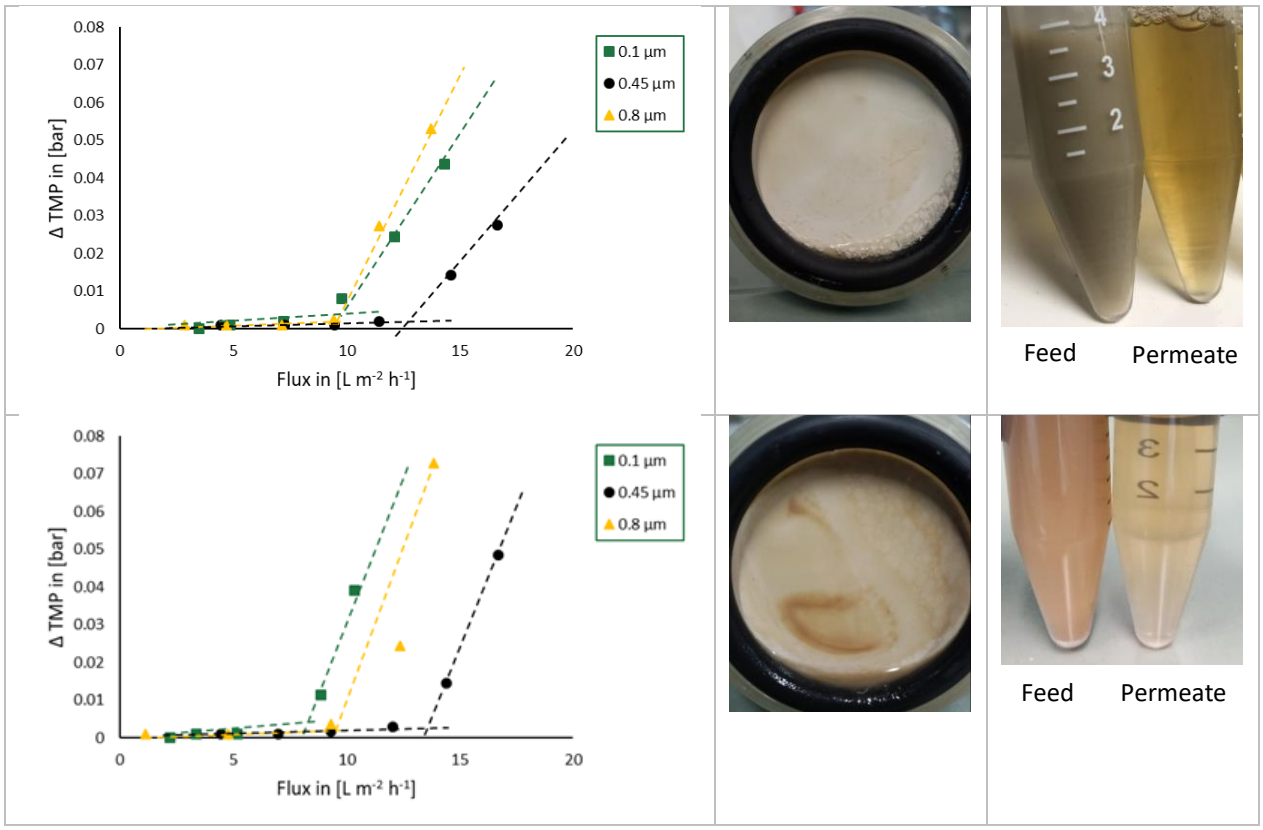


Figure 5.8: Membrane performances in MF of (a) hydrolysate from Run 2, and (b) hydrolysate from Run 3. The first column displays the critical fluxes of the three membranes, the second and third columns show visual observations of the used membranes as well as feed and permeate samples, respectively.

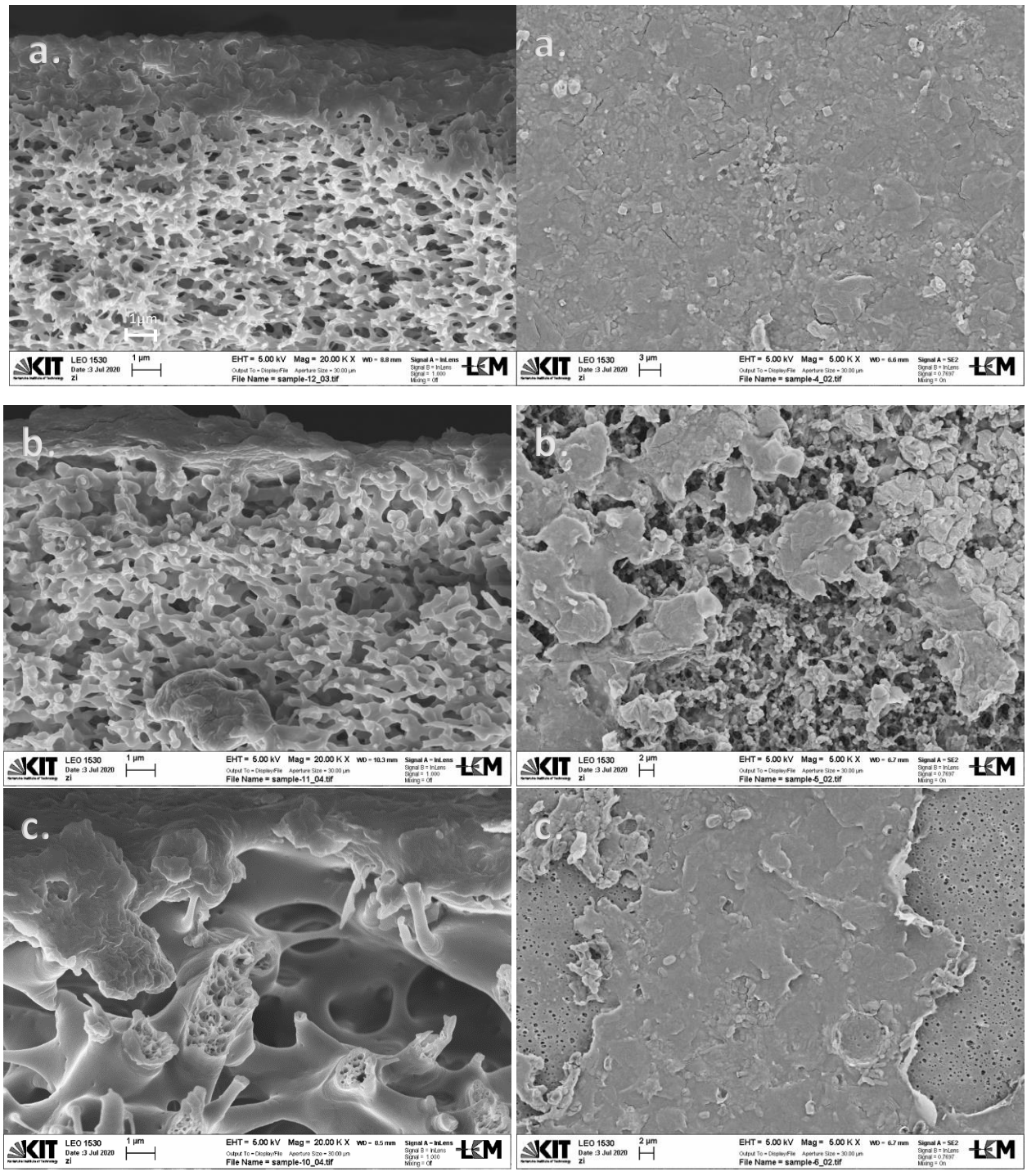


Figure 5.9: SEM images of cross sections (left column) and surface (right column) of the membranes of (a) 0.1 µm, (b) 0.45 µm, and (c) 0.8 µm pore size.

5.4 Conclusion

An integrated downstream process was developed involving two main steps: removal of large particles from the fermentation broth by using a separation unit (SU) followed by removal of the smaller suspended particles by a submerged microfiltration membrane system. The separation unit was shown to be an efficient pretreatment method for microfiltration processes. The unit was able to remove more than 86 % of the total suspended solids (TSS) from the fermentation broth, which represents all particles larger than 60 µm. The microfiltration membrane was successfully employed for separation of particles in the hydrolysate after the SU. Microfiltration is a necessary step before further procedural steps (e.g. nanofiltration (NF) and microbial electrolysis cell (MEC)) can be pursued for PA recovery and purification. It has been demonstrated that using microfiltration membranes with pore sizes of 0.1 µm, 0.45 µm, and 0.8 µm allowed about all 90 % VFA to pass through the membrane. Moreover, the membrane removed more than 85 % of the remaining TSS. The results show that MF performance is strongly affected by the characteristics of the membrane and hydrolysate. The highest critical flux of approximately 14 L m^{-2} h^{-1} was observed for hydrolysate from Run 3 (fermentation of food waste) with a pore size of 0.45 µm and a gas bubbling rate of 80 m^3 m^{-2} h^{-1}.

Chapter 6

6 Summary and conclusions

Propionic acid (PA) is one of the most important and commercially valuable volatile fatty acids (VFA), which is extensively utilized in many industrial sectors such as food, pharmaceutical, medical, cosmetics, and detergents. PA production by anaerobic fermentation is a promising approach for developing a bio-based economy and reducing the dependence on non-renewable fossil resources. This thesis has mainly focused on the enhancement of PA production from food waste through anaerobic fermentation. For that purpose, batch and semi-continuous fermentation experiments were conducted at mesophilic temperature (30 °C) using dog food as a model feedstock mimicking food waste.

The batch fermentations were carried out in 2 L lab-scale tests to optimize the process parameters for PA production including inoculum type, pH-value, and thermal pre-treatment of the substrate. Three types of inocula (a mixed microbial culture selected over 24 months for growth on cellulose, milk, and soft goat cheese) and three pH values (pH 4, pH 6, and pH 8) were chosen to apply for both, untreated and thermally pre-treated dog food.

Based on the results obtained from lab-scale fermentation experiments, the optimum operational conditions (pH 6 and goat cheese inoculum) were transferred to a 12 L hydrolysis reactor to evaluate the long-term process of PA production in a semi-continuous mode. For comparison, the reactor was also operated with a mixed microbial culture. The impact of *OLR* and *HRT* on the PA production process was also evaluated in three runs operated for approximately 100 days. In order to provide more realistic data with regard to envisage large-scale applications, real food waste was additionally tested.

The pre-treatment of fermented dog food and food waste broths as a primary step in PA recovery was evaluated. In this context, an integrated downstream process was developed involving two main steps: removal of large particles from the fermentation broth by using a separation unit (SU) with a 60 µm pore size sieving mesh followed by removal of the smaller suspended solids by a submerged microfiltration membrane system. Three different membrane pore sizes of 0.1 µm, 0.45 µm, and 0.8 µm were also compared.

The results from both fermentation types show clearly that the food waste has a high potential as a cheap renewable resource to produce a large amount of VFA with a high PA fraction. More importantly, methane was not detected during any of the experiments. The main conclusions of each experiment are outlined as follows:

6.1 Optimization of process parameters for PA production in lab-scale batch reactors

The results showed that the production of PA and other acids, in general, were dependent on the chosen inoculum and adjusted pH value. Moreover, high PA production is possible through applying optimized process parameters and selecting the adequate microbial community for inoculation. The optimal PA production for both untreated and thermally pre-treated dog food was obtained at pH 6 and when soft goat cheese used as inoculum, with concentrations and yields respectively of 10 g L^{-1} and 84 mg g^{-1} for untreated and 26.5 g L^{-1} and 217 mg g^{-1} for pre-treated dog food. However, the productions and yields in both cases exceed those obtained by other studies under similar conditions.

The highest total VFA concentration of 60 g L^{-1} was obtained when milk was applied as inoculum for the fermentation of pre-treated dog food at pH 8. The evolution of the individual acids showed different

fermentation patterns depending on inoculum type and pH value. In most cases, butyric acid was the dominant acid followed by acetic acid. While pre-treated food and pH 8 were the optimal conditions for both acids resulting in 35 g L^{-1} of butyric acid by milk inoculation and 19 g L^{-1} of acetic acid when goat cheese used as inoculum.

The results of this section provided practical guidance for the optimal operational process parameters needed to achieve satisfactory performance not only for PA production but also for other acids in a batch reactor. Hence, it could be possible to obtain one dominant acid type in the broth of fermented food waste either by manipulating operational conditions or by selecting the suitable inoculum.

6.2 propionic acid production in a semi-continuous fermentation

The result from the hydrolysis reactor presented the possibility of using goat cheese as inoculum to enhance PA production from food waste at pH 6 in a long-term process. The highest propionic acid concentration achieved amounted to 10 g L^{-1} and 8 g L^{-1} using dog food and food waste, respectively. Moreover, it was observed that propionic acid production was enhanced by a combination of rather high hydraulic retention time (*HRT*) going along with a low organic loading rate (*OLR*), ensuring sufficient time for complete processing of the complex organic substrates. The highest yield of PA of 54 mg g^{-1} was achieved at an *OLR* of only 3.2 g L^{-1} d^{-1} VS added from the food waste, which is more than twice as much as achieved from dog food, and even 5 times higher than the highest that obtained when mixed culture used. Furthermore, the microbial analysis data showed that the community was more stable during the fermentation inoculated with goat cheese, in which *Propionibacteria* were detectable in all samples, even after 86 days of cultivation.

PA produced in the three semi-continuous fermentation runs was higher than those achieved so far in the literature, in which food waste and mixed bacterial culture were used. This makes semi-continuous fermentation as a promising cost-effective for PA production and recovery due to continuous high acid production.

6.3 Treatment of fermentation broth using microfiltration

The separation unit was shown to be an efficient pre-treatment method for microfiltration processes with 86 % of TSS removal. The microfiltration membrane showed a good performance for the fermentation broth treatment, resulting in VFA rich and particle free solution. The highest critical flux of approximately 14 L m^{-2} h^{-1} was observed for hydrolysate from the fermentation of food waste with a pore size of 0.45 µm and a gas bubbling rate of 80 m^3 m^{-2} h^{-1}. Furthermore, it has been demonstrated from the results that microfiltration is an appropriate step before further procedural steps (e.g. nanofiltration (NF) and microbial electrolysis cell (MEC)) can be pursued for PA recovery and purification.

To sum up, the PA production from food waste can be enhanced by using a mixed bacterial culture from soft goat cheese. Specifically, anaerobic fermentation of food waste using this culture was associated with stable PA production of more than 8 g L^{-1} during long term operation of the semi-continuous fermentation reactor. The present findings can be exploited for sustainable bio-based chemical production and food waste treatment. Moreover, the results of both batch and semi-continuous experiments provided useful information on the experimental process. Thus, the semi-continuous operation mode could be successfully used to produce PA on large scale.

7 References

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8 Appendices

Table A. 1: Maximum propionic acid production rates P_{PA} and yields Y_{PA} from untreated and pretreated dog food during batch experiments.

Exp.	Type of inoculum	Initial VS added [g L^{-1}]	PA [g L^{-1}]	P_{PA} [g $L^{-1}d^{-1}$]	Y_{PA} [mg g^{-1}]	P_{H_2} [NL d^{-1}]	Y_{H_2} [NmL g^{-1}]
(pH 4 – untreated)	I_1 Mixed bacterial culture	111	0.1 ± 0.3	0.0 ± 0.0	0.9 ± 0.4	0.1 ± 0.1	0.6 ± 0.8
	I_2 Milk	139	1.5 ± 1.2	0.3 ± 0.2	13.4 ± 2.1	0.1 ± 0.2	0.9 ± 1.4
	I_3 Goat cheese	125	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
	Blank (without inoculum)	111	0.1 ± 0.1	0.0 ± 0.0	1.0 ± 0.2	0.0 ± 0.0	0.1 ± 0.2
(pH 4 –pretreated)	I_1 Mixed bacterial culture	109	1.2 ± 1.3	0.2 ± 0.2	11.2 ± 1.5	0.2 ± 0.4	1.9 ± 4.0
	I_2 Milk	136	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2
	I_3 Goat cheese	122	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
	Blank (without inoculum)	109	0.1 ± 0.1	0.0 ± 0.0	0.6 ± 0.1	0.0 ± 0.0	0.3 ± 0.7
(pH 6 – untreated)	I_1 Mixed bacterial culture	111	5.1 ± 3.1	0.7 ± 0.6	46 ± 0.7	0.7 ± 1.2	5.9 ± 0.8
	I_2 Milk	139	0.8 ± 0.3	0.1 ± 0.0	6.1 ± 0.2	1.6 ± 2.4	11.5 ± 1.1
	I_3 Goat cheese	125	10.5 ± 0.3	2.0 ± 0.1	84.3 ± 0.3	0.9 ± 1.3	7.5 ± 0.7
	Blank (without inoculum)	111	3.5 ± 0.3	0.4 ± 0.4	31.6 ± 0.3	1.0 ± 1.5	8.7 ± 3.7
(pH 6 –pretreated)	I_1 Mixed bacterial culture	109	0.7 ± 0.7	0.0 ± 0.0	6.8 ± 0.8	4.5 ± 3.9	41.2 ± 6.7

	I_2 Milk	136	2.8 ± 2.0	0.1 ± 0.3	20.3 ± 3.2	1.3 ± 2.0	9.2 ± 14.8
	I_3 Goat cheese	122	26.5 ± 0.0	2.9 ± 0.7	216.9 ± 0.1	0.1 ± 0.2	0.9 ± 1.8
	Blank (without inoculum)	109	0.3 ± 0.2	0.0 ± 0.0	2.6 ± 0.3	3.1 ± 3.0	28.5 ± 8.5
(pH 8 – untreated)	I_1 Mixed bacterial culture	111	0.2 ± 0.1	0.0 ± 0.0	1.9 ± 0.0	1.0 ± 3.3	9.1 ± 2.2
	I_2 Milk	139	3.6 ± 0.4	0.5 ± 0.6	24.4 ± 2.4	0.9 ± 1.4	6.4 ± 0.2
	I_3 Goat cheese	125	0.4 ± 0.1	0.1 ± 0.0	3.5 ± 0.1	2.5 ± 5.1	19.9 ± 1.0
	Blank (without inoculum)	111	1.3 ± 0.2	0.3 ± 0.1	11.3 ± 0.2	1.7 ± 0.4	15.6 ± 3.7
(pH 8 –pretreated)	I_1 Mixed bacterial culture	109	0.9 ± 0.3	0.1 ± 0.1	8.1 ± 1.4	1.3 ± 1.9	12.3 ± 1.8
	I_2 Milk	136	0.2 ± 0.3	0.0 ± 0.0	1.2 ± 0.6	0.0 ± 0.0	0.1 ± 0.1
	I_3 Goat cheese	122	9.1 ± 0.3	1.1 ± 0.6	74.1 ± 0.0	0.7 ± 1.3	6.0 ± 1.0
	Blank (without inoculum)	109	1.4 ± 0.7	0.3 ± 0.1	12.5 ± 0.8	0.9 ± 1.6	8.8 ± 4.7

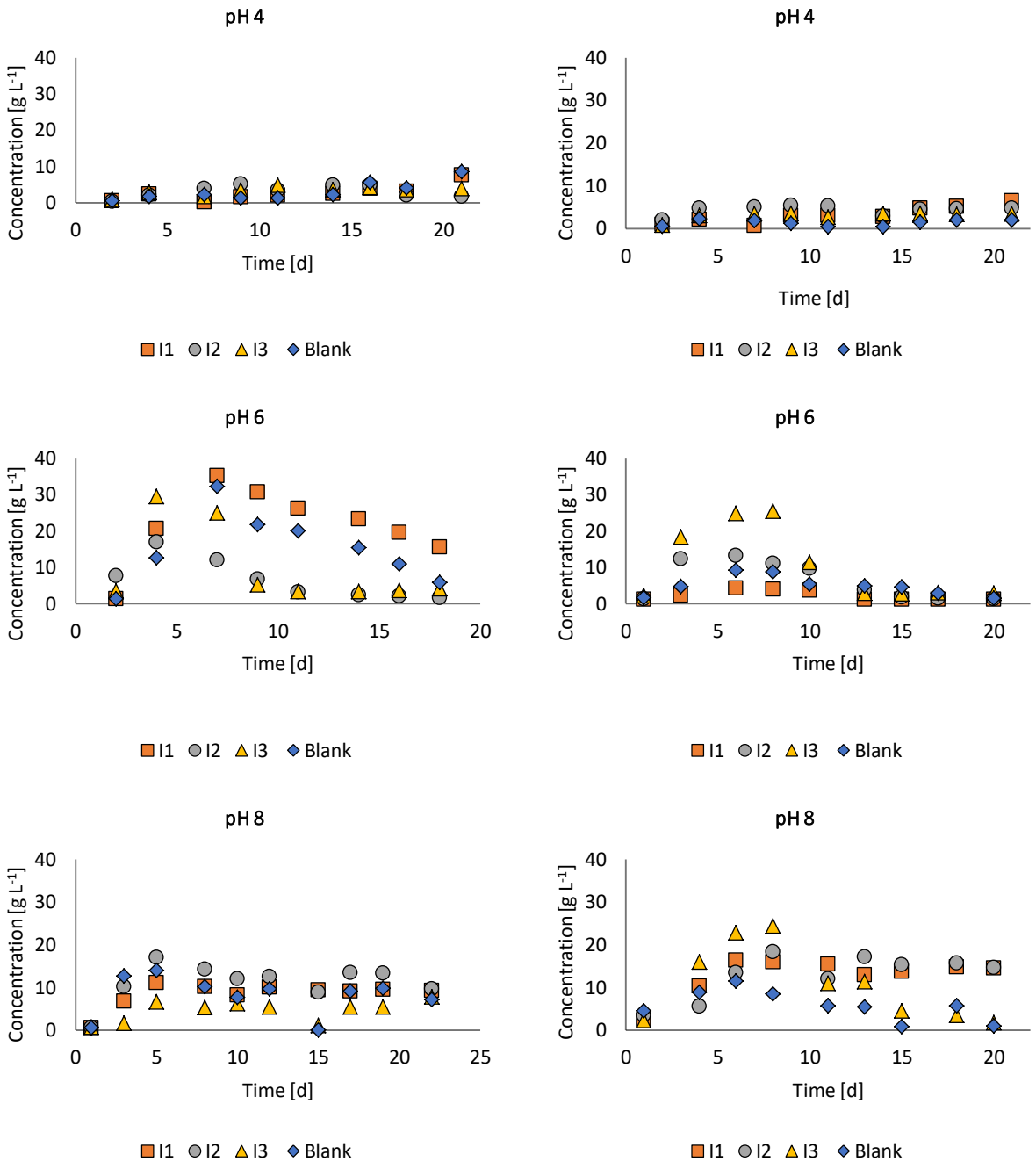

Figure A. 1: Courses of lactic acid concentration in the fermentation of untreated (left column) and pretreated dog food (right column) for each inoculum (mixed bacterial culture (I_1), milk (I_2), and goat cheese (I_3)) and at different pH values.

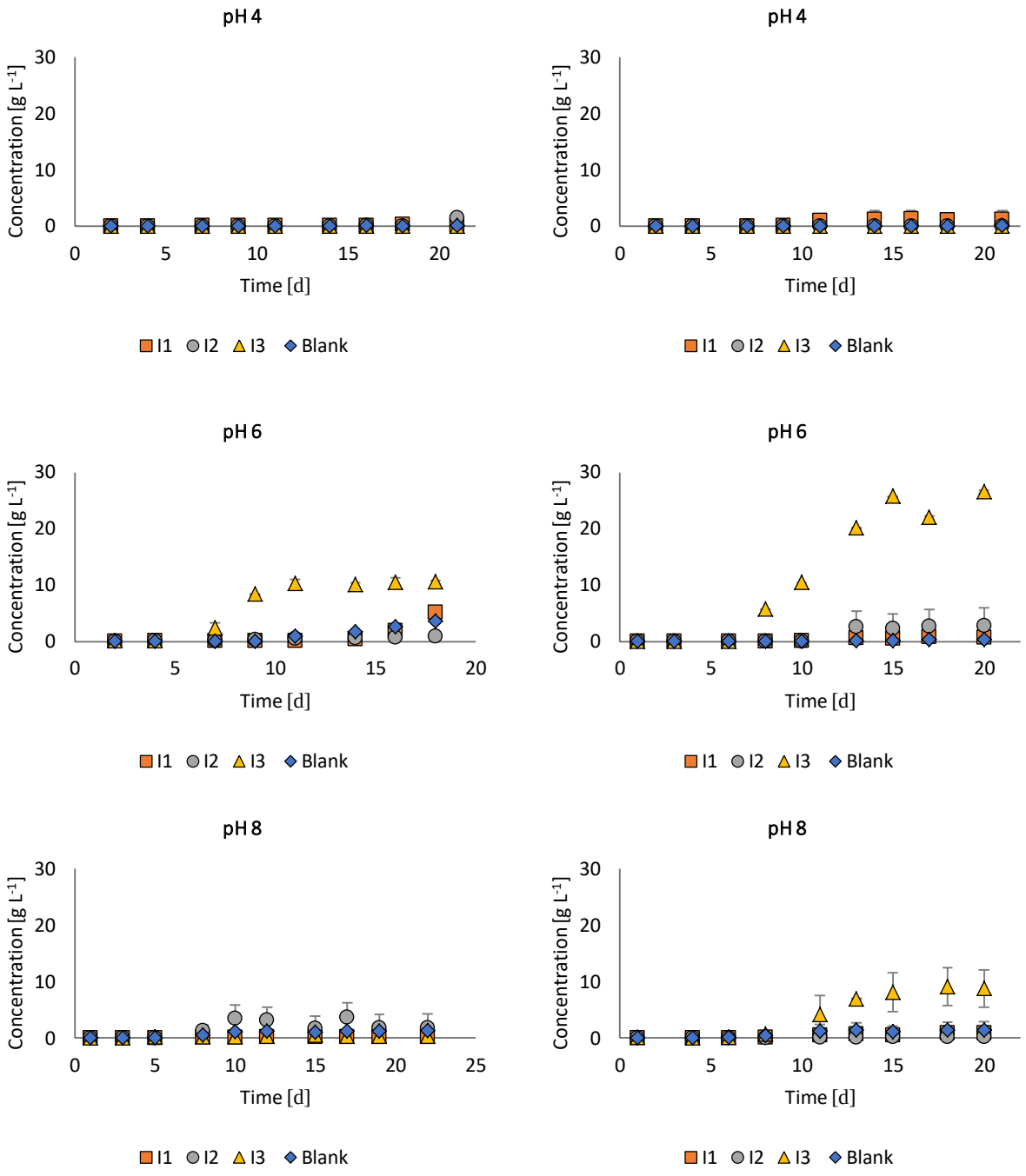

Figure A.2. Propionic acid concentration produced from untreated (left column) and pretreated dog food (right column) in batch experiments in dependence on inoculum (mixed bacterial culture (I_1), milk (I_2), and goat cheese (I_3)) and pH value.