

**Thesis for the Degree of Doctor of Philosophy**

**Volatile Fatty Acids as a Key to Sustainability and  
Circularity in Polyhydroxyalkanoates Production**

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UNIVERSITY  
OF BORÅS

Volatile fatty acids as a key to sustainability and circularity in polyhydroxyalkanoates production

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Digital version: <http://urn.kb.se/resolve?urn=urn:nbn:se:hb:diva-29578>

ISBN 978-91-89271-92-0 (printed)

ISBN 978-91-89271-93-7 (pdf)

ISSN 0280-391X, Skrifter från Högskolan i Borås, nr. 137

Cover photo: A redrawn picture presenting accumulated polyhydroxyalkanoates inside bacterial cells.

Borås, 2023

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## Abstract

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The negative consequences of plastic pollution on both environmental and socio-economic aspects have motivated the development of sustainable and renewable materials to replace the petroleum-based plastic. Polyhydroxyalkanoates (PHAs), which are bioplastics, having an outstanding biodegradability and rather comparable thermal and mechanical properties, are potential alternatives for the replacement of conventional plastics. However, one of the hurdles on the way to PHA commercial production is the cost of conventional feedstock, which can constitute up to 50% of the production cost. In this regard, volatile fatty acids (VFAs) derived from acidogenic fermentation of organic waste can be a promising substrate to increase the cost-competitiveness of PHA production. Therefore, in this thesis, VFAs were utilized and developed to be a key carbon feedstock for the sustainable and economically feasible production of PHAs.

The applicability of individual and mixed VFAs as potential substrates was initially investigated through the cultivation of two different PHA-bearing bacteria of *Bacillus megaterium* and *Cupriavidus necator*, providing an average PHA yield on biomass of 10 and 55%, respectively. Further thorough studies, in terms of VFA loading and inhibition thresholds and operating parameters, were conducted to improve the conversion efficiency of VFAs by *C. necator*. Consequently, a biomass yield on VFAs of up to 82% was obtained, rendering a PHA accumulation of 1 g/L using actual waste derived VFA effluent. In addition, in order to tackle the inherent issue of low productivity in batch and/or fed-batch cultivations under high VFA containing feed, a novel approach of immersed membrane reactor (iMBR) was introduced and applied in this thesis. With the assistance of membrane filtration, the PHA production was conducted in semi-continuous mode (up to 128 h), yielding a maximum biomass and PHA production of 6.6 and 2.8 g/L, respectively. The outcomes achieved, furthermore, were 32.1 and 28.5%, respectively, higher than that from a continuous stirred tank (CSTR), in which the cultivation was affected by the washout effect. Moreover, considering the insufficiency of the current recycling methods of PHA-based products in terms of resource recovery, a novel attempt of acidogenic fermentation has been conducted to valorize the PHA-based composites through the conversion into precursor VFAs. Afterwards, the recovered VFAs could be recirculated into PHA production, fulfilling the concept of a circular bioeconomy.

**Keywords:** bioplastics, food waste, polyhydroxyalkanoates, volatile fatty acids, immersed membrane bioreactor, acidogenic fermentation

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## List of Publications

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- I. **Danh H. Vu**, Dan Åkesson, Mohammad J. Taherzadeh, Jorge A. Ferreira (2020). Recycling strategies for polyhydroxyalkanoate-based waste materials: An overview. *Bioresource Technology*, 2020. 298: 122393.
- II. **Danh H. Vu**, Steven Wainaina, Mohammad J. Taherzadeh, Dan Åkesson & Jorge A. Ferreira (2021). Production of polyhydroxyalkanoates (PHAs) by *Bacillus megaterium* using food waste acidogenic fermentation-derived volatile fatty acids. *Bioengineered*, 12(1): 2480-2498.
- III. **Danh H. Vu**, Amir Mahboubi, Andrew Root, Ivo Heinmaa, Mohammad J. Taherzadeh, Dan Åkesson (2022). Thorough Investigation of the Effects of Cultivation Factors on Polyhydroxyalkanoates (PHAs) Production by *Cupriavidus Necator* from Food Waste-Derived Volatile Fatty Acids. *Fermentation*, 8(11): 605.
- IV. **Danh H. Vu**, Amir Mahboubi, Andrew Root, Ivo Heinmaa, Mohammad J. Taherzadeh, Dan Åkesson (2023). Integration of immersed membrane bioreactor in polyhydroxyalkanoates production. *Submitted*.
- V. **Danh H. Vu**, Amir Mahboubi, Jorge A. Ferreira, Mohammad J. Taherzadeh, Dan Åkesson (2022). Polyhydroxybutyrate-natural fiber reinforcement biocomposite production and their biological recyclability through anaerobic digestion. *Energies*, 15(23): 8934.

## **Statement of Contribution**

Danh Hoang Vu's contribution to the above-mentioned publications is as follows:

**Paper I:** Responsible for part of the idea, all literature review, data collection, writing and responsible for the manuscript revision.

**Paper II:** Responsible for all the experimental work, data processing, writing of the manuscript and revision.

**Paper III:** Responsible for part of the experimental work, data analysis, manuscript preparation and revision.

**Paper IV:** Responsible for part of the idea and designing the experiments, involvement in part of the experimental work, data analysis, writing of the manuscript and revision.

**Paper V:** Responsible for part of the idea and designing the experiments, all the experimental work, data analysis, manuscript preparation and revision.

## **Additional publication that is not included in this thesis**

Konstantinos Chandolias, Sudhanshu S. Pawar, **Danh H. Vu**, Steven Wainaina, Mohammad J. Taherzadeh (2023). Bio-hydrogen and VFA production from steel mill gases using pure and mixed bacterial cultures. *Submitted.*

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## Abbreviations

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AD	Anaerobic digestion	OECD	Organization for Economic Co-operation and Development
ATP	adenosine triphosphate	PBST	polybutylene succinate
BES	bromoethanesulfonate	PCL	polycaprolactone
bio-PA	bio-polyamide	PE	polyethylene
bio-PE	bio-polyethylene	PEF	polyethylene furanoate
bio-PET	bio-polyethylene terephthalate	PET	polyethylene terephthalate
bio-PP	bio-propylene	PGA	polyglycolic acid
bio-PU	bio-polyurethane	PLA	polylactic acid
CH <sub>4</sub>	methane	PHAs	polyhydroxyalkanoates
CO <sub>2</sub>	carbon dioxide	P3HB	poly(3-hydroxybutyrate)
CSTR	continuous stirred tank	P4HB	poly(4-hydroxybutyrate)
FS	flat sheet	P3HBV	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
GHGs	greenhouse gases	P3HBHHx	poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
HDPE	high-density polyethylene	PHV	polyhydroxyvalerate
HF	hollow fiber	PP	polypropylene
iMBR	immersed membrane bioreactor	PS	polystyrene
LDPE	low-density polyethylene	PTT	polytrimethylene terephthalate
LCA	Life cycle analysis	PVC	polyvinyl chloride
MF	microfiltration	RO	reverse osmosis
MMCs	mixed microbial cultures	TB	tubular
MSW	municipal solid waste	UF	ultrafiltration
NF	nanofiltration	USD	United States dollar

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## My journey

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My research journey has been like a flow as it moves forward without any preconceived plans. Here, I would like to share a brief narrative of motivations that have led me on this journey. I was raised in a typical Asian family, where the idea of becoming a doctor was ingrained in my mind from a young age. To be honest, it had such a profound impact on me that I have struggled to discover my true interests. The turning point in my life arrived when I failed the entrance exam to medical school and instead started a Bachelor's degree in the field of environmental science at Saigon University (SGU) in 2012. Without much consideration of other factors, I chose this field simply because it was trendy at the time. As the time went by, I found myself in the final year of my bachelor studies with the uncertainty about what I would like to pursue next. At that moment, the idea of studying abroad suddenly came to my mind with the hope of gaining a deeper understanding of myself in a new environment. Everything went on as if it was preordained, as I could swiftly ascertain my next destination. Based on the advice of one of my teachers at Saigon University, who had previously studied at the University of Borås, I decided to pursue a M.Sc. degree in resource recovery. Regardless of my background in biotechnology, my interest at that time was focused on the field of polymer technology. As a result, I chose to do my master's thesis on the development of 3D composite from orange waste. During the thesis study, I found myself increasingly drawn to research work, which motivated me to apply for a Ph.D. position in a cooperation project between the University of Borås and SGU. In 2019, I started my Ph.D. life with a project of polyhydroxyalkanoates (PHAs) production using volatile fatty acids (VFAs). It is fascinating that I have returned to the field of biotechnology after a long and winding journey. This comeback was filled with unexpected twists and turns, making it a truly memorable experience to me. Thanks to the exceptional support, invaluable guidance, and unwavering trust of my supervisors and colleagues, I am now on the verge of completing one of the most significant milestones in my life. I do not know what the future holds, but I am immensely grateful for everything that has led me to this path, as for the very first time in my life, I can feel a genuine enjoyment with what I am doing.



# INTRODUCTION

Together with overexploitation and excessive consumption, mismanagement of petroleum-based plastics has led modern society to a grand challenging issue of plastic pollution. Considering that plastics are cheap, lightweight, durable, and easy to process into various applications, they have been indispensable in various areas. The high durability of plastics, however, turns them into one of the worst materials when being released into the nature, imposing detrimental environmental and socio-economic issues. In order to alleviate the conventional plastic-dependency crisis and its related problems, bioplastics have been emerging as potential alternatives. In recent years, polyhydroxyalkanoates (PHAs) have been one of the bioplastics of interest due to their analogous thermal and mechanical properties and superiority in biodegradability compared to conventional plastics. PHAs can be produced in an environmentally friendly approach through microbial fermentation. One of the main factors hindering the industrial-scale production of bacterial PHAs has been the cost of conventional feedstocks (e.g., refined sugars or food-grade substrates) accounting for up to 30–50% of the production cost. The use of sugar- and starch-based feedstocks also raises concerns in terms of sustainability, as it directly competes with human food and animal feed regardless of the benefits gained in high productivity for commercial production. Organic municipal and industrial waste of negative or low value (e.g., food waste) contains nutrients that can support bacterial PHAs production; however, this mixed and rather recalcitrant substrate first needs to be homogenized into a compound suitable for bacterial assimilation. In this regard, volatile fatty acids (VFAs) generated from acidogenic fermentation of organic waste can be a potential economic substrate candidate to achieve the sustainable PHA production.

Although VFAs are well-known for their production from a variety of organic waste streams, like other low-cost feedstock, VFAs can face an inherent issue of low conversion efficiency mostly due to the effect of substrate inhibition at high concentration, leading to an inadequate productivity in the fermentation of PHA-bearing

microorganisms. Therefore, in this thesis, VFAs concentration thresholds have been determined together with the optimization in cultivation factors to improve the VFA assimilation, enhancing the yield in PHA accumulation using different microorganisms. In addition, in a novel attempt, PHA production using membrane bioreactors was introduced to pursue a semi-continuous PHA production, tackling the limitations of batch and fed-batch cultivations in terms of low productivity under high concentration of VFAs.

Lastly, to take advantage of the great characteristic of biodegradability, the end-of-life PHA-based products were subjected to acidogenic fermentation for their valorization into precursor VFAs. The recovered VFAs can then be used as a feedstock in PHAs production, fully adapting the circular economy concept to the sustainable production of PHAs.

## 1.1 Aims of the studies

The main goal of the research conducted in this thesis was to navigate the PHA production to a sustainable circular process by utilizing VFAs as a key carbon source. In order to achieve that concept, three objectives were pursued:

- Investigate the possibility of employing VFAs as an effective and economical substrate for the cultivation of two different bacterial strains of *Bacillus megaterium* and *Cupriavidus necator*. VFA concentration threshold and operating factors were thoroughly studied to improve the VFA bioconversion for efficient PHA production.
- Development of a semi-continuous PHA production with a novel approach using immersed membrane bioreactor (iMBR) to confront the negative impacts of high VFA concentration and overcome the low productivity of batch cultivation.
- Introduction of biological recycling of PHA-based products by using acidogenic fermentation.

## 1.2 Thesis outline

The thesis is divided into six main chapters:

- **Chapter 1** introduces the research topic and the main research objectives.
- **Chapter 2** presents the current mismanagement of conventional plastic wastes and how PHAs can be a potential solution to mitigate the related issues of plastic pollution.
- **Chapter 3** presents the possibility of VFA utilization to tackle the bottleneck of conventional feedstock, in terms of cost-competitiveness and substrate selectivity.
- **Chapter 4** includes the optimization of PHA production using VFA as the main substrate and the benefits of iMBR application for the semi-continuous production of PHAs.

- **Chapter 5** describes an establishment of a sustainable circular process of PHA production by valorizing the end-of-life PHA-based products into their precursors of VFAs via acidogenic fermentation.
- **Chapter 6** provides the main conclusions drawn from the thesis work and suggestions for future work.

### **1.3 Social, ethical, and sustainability reflections**

The development of modern society has resulted in significant social and environmental consequences. While rapid urbanization and globalization have propelled progress, marginalized groups have been left behind, exacerbating social inequality. Furthermore, the heavy reliance on fossil fuels and petroleum-based products has led to air and water pollution, deforestation, and climate change. Moreover, the growing human population has further added to the strain, increasing the demand for food, while significant amounts of food are wasted along the supply chain, creating more social and environmental challenges. To address these challenges, circular bioeconomy has emerged as a solution. Bioeconomy is an economic system using renewable biological resources, such as organic wastes, to produce valuable products, food, feed, and energy. By reducing the use of raw materials and increasing the efficiency of material use, waste generation can be minimized, leading to an improvement in the environmental and social outcomes.

Following this line of thinking, the present thesis is conducted with an ultimate goal of providing a close-cycle production of PHAs with VFAs as the core intermediates. In this regard, VFAs are initially produced from the valorization of food waste via anaerobic digestion, which is low in carbon print and energy demand. Afterwards, the VFAs obtained are utilized for the production of PHAs, which are potential alternatives for the reduction in petroleum-based plastics consumption. These bioplastics can be degraded into VFAs by anaerobic digestion, which can be recirculated back to the process of PHAs production. In this way, related issues from food waste and over-consumption of conventional plastics can be partly addressed in a sustainable way, corresponding to the United Nations Sustainable Development Goals numbers 6 (clean water and sanitation), 11 (sustainable cities and communities), and 12 (responsible consumption and production).



# A SOLUTION FOR PETROLEUM-BASED PLASTICS POLLUTION

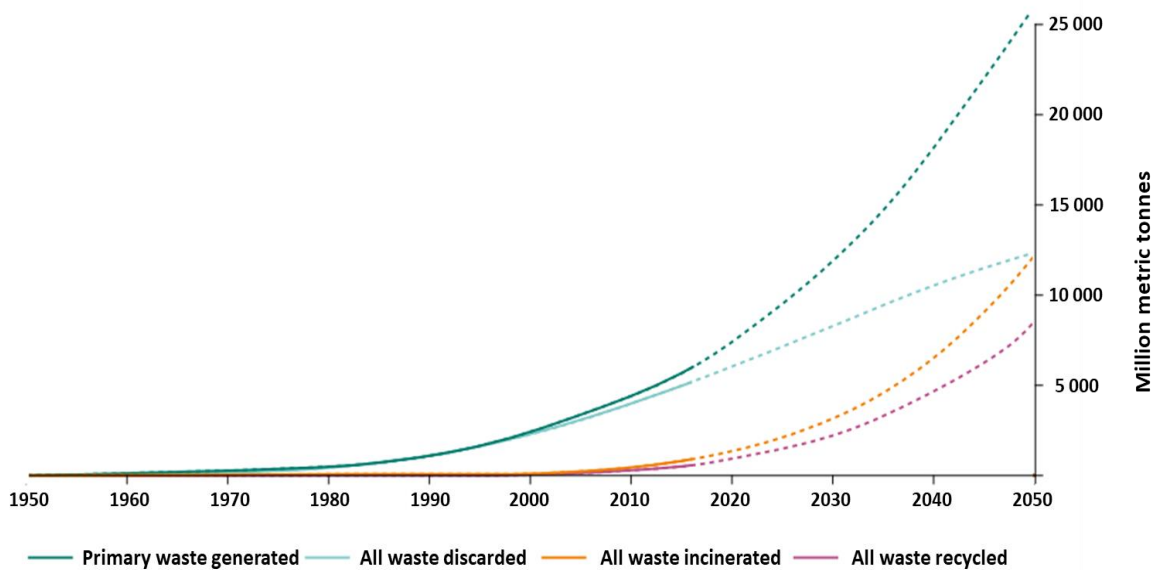
The versatility of petroleum-based plastics has led to the massive production of this material. However, the linear model adopted in production, application, and wastage of petroleum-derived plastics has long been recognized as its Achilles heel. The majority of plastic waste is poorly managed, resulting in extreme plastic pollution, stressing both the environment and socio-economy. Consequently, the development of sustainable materials for replacing conventional plastic to mitigate its negative impacts is necessary. All aforementioned aspects are thoroughly discussed in this chapter.

## **2.1 Current status of petroleum-based plastic wastes mismanagement**

Plastics are defined as synthetic materials consisting of large molecules composed of repeating monomers. The production of plastics is originated from the complex process of crude oil and natural gas refinery, which accounts for about 4% of the global oil consumption [1]. Due to the extraordinary properties and an incredible durability, plastics have become irreplaceable materials in any applications, from engineering sectors to daily life implements [2]. The important role of plastics is clearly depicted from its annual worldwide production, which has been growing at an extraordinary rate since 1950s and is expected to reach nearly 600 million tons by the end of 2050 [3]. In this regard, China, United States, and the Organization for Economic Co-operation and Development (OECD) in Europe account for half of the current plastics demand, with the specific amount of 20, 18, and 14%, respectively [4]. Based on the global plastic market in 2017, packaging, construction, and textiles are the dominant applications, accounting for a significant share of more than 60% of the plastic industry. Packaging, in particular, made up nearly 40%, which commonly has an extremely short lifespan due to the shift in the purpose of use from reusable to single-use item. The application of plastics and their lifespan are defining

factors in the generation of plastic waste. Packaging plastics contributed a significant share of about 59% in all plastic waste [5]. Plastic waste, in general, forms about 2 to 12% of the municipal solid waste (MSW), corresponding to an approximate amount of 150 million tons annually worldwide [6]. High production rates and the lack of consumer awareness are two of the main factors contributing to the huge amount of plastic waste generated, overloading the capacity of current recycling infrastructure [7].

Plastic waste has been handled in different manners, depending on local and national regulations and waste management capacities. Hence, Europe has implemented restrictions on landfilling to encourage the recycling and energy recovery from plastic waste. However, strict policy requirements for reuse and recycling have led to a rapid growth in the international trade of plastic waste, which has potentially exacerbated the current scenario of plastic wastes. Europe has been the greatest contributor to plastic waste export, by nearly one-third of all plastic waste exports, as reported in 2020 [8]. After a ban on plastic waste imports by China in 2017, the trade in plastic waste has been re-routed to developing countries in South East Asia (e.g., Malaysia, Thailand, Vietnam, etc.) [9]. Developing countries, however, send almost all their plastic waste to landfills, which can be attributed to the lack of capacity, technology, infrastructure and financial means to handle the waste. As a result, only 15% of all the global plastic waste was recycled in 2019, while the majority of plastic waste was incinerated (17%) and disposed of in landfills (46%) [4]. Ultimately, the mismanaged and uncollected litter contributed about 22% to the plastic waste, equating to 33 million tons of plastic materials being released into nature annually. If the current trend continues, in the next 30 years, the world will witness the presence of roughly 12,000 million tons of plastic waste in landfills or polluting the environment (See Figure 2.1).

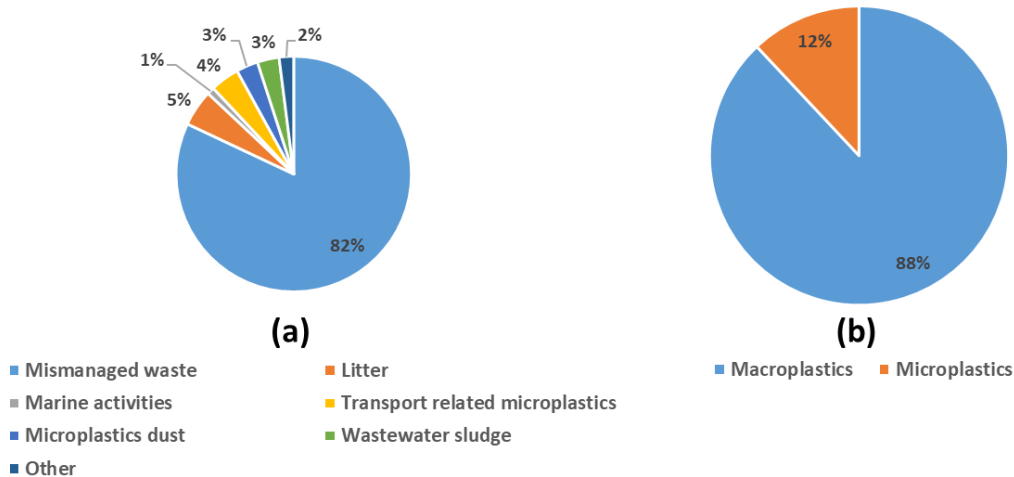


**Figure 2.1.** Current status of plastic waste generation/disposal and projections for 2050. Adapted from Geyer, et al. [3].



## 2.2 Plastic pollution

Mismanagement of plastic wastes was the main reason leading to the leakage of 22 million tons of plastic materials into the nature in 2019 (Figure 2.2a) [4]. In this context, the major issue faced is the exceptional durability of plastics to degradation in the nature. Petroleum-based plastics are known as non-biodegradable materials, which disintegrate very slowly by sunlight, oxidation, or friction into smaller fragments of macroplastics ( $\geq 25$  mm), mesoplastics ( $< 25$  mm – 5 mm), microplastics (5 mm – 1 mm), mini-microplastics (1 mm – 1  $\mu$ m), and nanoplastics ( $\leq 1$   $\mu$ m) [10]. It was estimated that a vast majority of up to 88% of the leaked plastics was in macro size with mismanaged waste, with marine activities being the main sources (Figures 2.2a & 2.2b). The share of microplastic particles, on the other hand, was only 12%, corresponding to 2.7 million tons in 2019. The amount of microplastic is expected to increase exponentially due to the substantial fragmentation of macroplastics. Regardless of the characteristics of the plastic (type, form, and size) and environmental conditions, the rate of plastic breakdown is extremely slow, prolonging its longevity. For instance, the half-lives of low-density polyethylene (LDPE) plastic bags are estimated to be 5 – 250 years on land, while this number was even up to 1,200 for high-density polyethylene (HDPE) pipes [11]. Therefore, if not properly handled, plastic wastes and its issues will be around for a long time.



**Figure 2.2.** The share of plastic leakage sources (a) and the distribution of macro- and microplastics leaked into the environment (b) in 2019 [4].

### 2.2.1 Aqueous pollution

Every year, over 14 million tons of plastics of different sizes are estimated to enter the ocean, creating a series of negative impacts on the aquatic ecosystem [12]. Entanglement and ingestion are the most visible and critical

issues associated with macroplastics derived from fishing gears (e.g. fishing nets, hooks, and ropes), causing lethal or sub-lethal outcomes to over 30,000 individuals from 243 species in marine wildlife [13, 14]. In addition, due to the similarity in size with planktons, marine organisms unintentionally ingest microplastics, which can be inherently incorporated with toxic chemicals (e.g. bisphenol-A, flame retardants, antibiotics, etc.) or contaminated by further absorption of persistent organic pollutants [15]. The contaminant-bearing organisms, consequently, become mobile pollutants, which not only pollute the surrounding water but also threaten human health via the food chain [16, 17].

### 2.2.2 *Atmospheric pollution*

In case of atmospheric environment, it is entirely dominated by microplastics which can trap air inside their hollow structure, allowing them to float in the air [4]. Aerial microplastic can be attached to the airborne bacteria or viruses, which further enter human bodies through breathing, causing health issues [18]. Macroplastics indirectly contribute to air pollution as their breakdown emits a considerable amount of greenhouse gases (GHGs), such as methane (CH<sub>4</sub>), carbon dioxide (CO<sub>2</sub>), and ethylene [19].

### 2.2.3 *Terrestrial pollution*

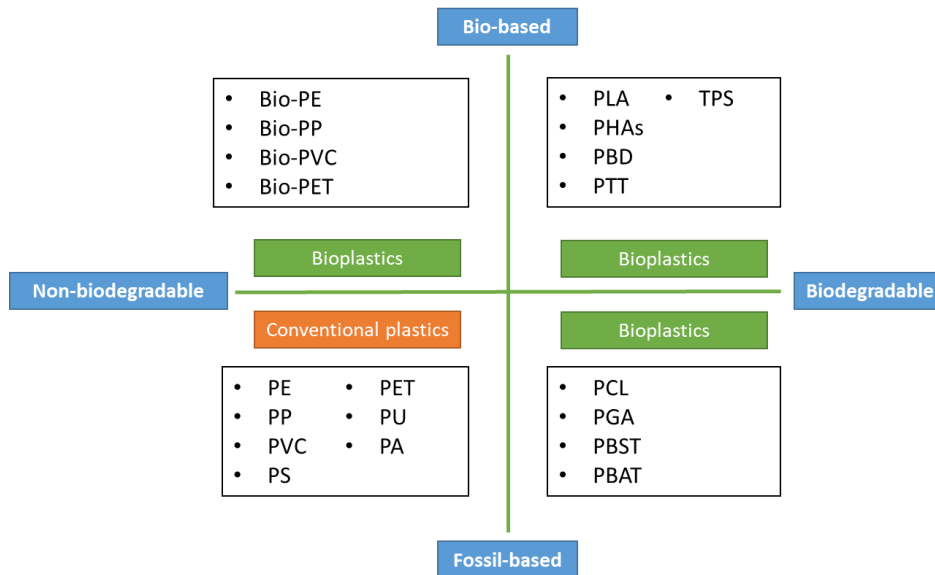
Terrestrial environment is the most plastic-polluted environment, receiving 4–23 times higher plastic waste than that released into the oceans [20]. Terrestrial ecosystem has encountered the same adverse impacts of macroplastics and microplastics ingestion as in the aquatic environment [21-24]. Plastic pollution does not only damage the environment; rather, the detrimental effects also concern the socio-economic systems. Extra funds have been allocated for the removal of plastic wastes to mitigate its consequences on commercial activities, such as fishery and tourism. Commercial fishery has been one of the most affected sectors with an annual loss of 250 million USD due to the adverse effects caused by ghost fishing [25, 26]. Ghost fishing is initiated by discard/abandoned or lost fishing gear (macroplastics), which also contributed to the transportation of invasive species (including algae), impairing the local ecosystem [27]. Together with an overload of plastic wastes, the damaged ecosystems, in terms of aesthetic value and natural beauty, will discourage tourism-related activities, eventually threatening the annual income of coastal communities heavily reliant on tourism [28]. According to an estimation by Beaumont, et al. [29], a ton of aquatic fabric released per year would cost the marine ecosystem services about 3,300 USD. Being part of the ecosystem, human exposure to plastic pollution through particle inhalation, tap water, and food ingestion is ultimately inevitable, resulting in adverse health effects [30].

Considering the above-mentioned issues related to plastic pollution, to address such issues systematically, a close collaboration between policymakers and researchers is needed. Strict regulations on plastic application and discarding, along with economic incentives on the plastic recycling system, are expected to reduce the production

of virgin plastic, gradually lowering the plastic footprint. Meanwhile, researches can potentially develop substitutes for conventional plastics. Thus, bioplastics, such as polyhydroxyalkanoates (PHAs), have emerged as promising alternatives.

### 2.3 Bioplastics

*Bioplastics* is a broad term used to describe plastic materials that are bio-based or biodegradable or fulfill both criteria. In this regard, bio-based standard refers to plastics that are made of renewable feedstock, such as corn, beet, cellulose, wheat, starch, vegetable fats and oils, and microbiota [31]. Meanwhile, biodegradable refers to the biodegradability of bioplastics, which can be decomposed by various microorganisms into CO<sub>2</sub>, H<sub>2</sub>O, and organic compounds without releasing any hazardous residues [32]. Based on that, available bioplastics are specifically divided into three major groups of (1) bio-based and non-biodegradable plastics, (2) bio-based and biodegradable plastics, and (3) fossil-based and biodegradable (Figure 2.3) [33].



**Figure 2.3.** Classification of bioplastics based on their origin and biodegradability. Adapted from [33]

Conventional plastics are typically produced from fossil resources; however, several of these plastics can currently be produced from renewable resources, rendering bio-polyolefins, such as bio-polyethylene (bio-PE), bio-polypropylene (bio-PP), etc. Those bio-polyolefins are known as ‘drop-in’ bioplastics, which possess chemically identical structures compared to conventional plastics but cannot be biodegradable [34]. The main application of bio-PE, bio-PP is packaging, while other members in this group, such as bio-polyamide (bio-PA), bio-polyurethane (bio-PU), are used for textiles fibers and in automotive sectors [35]. As presented in Figure 2.3,

fossil-based plastics, such as polycaprolactone (PCL), polyglycolic acid (PGA), polybutylene succinate (PBST), can be biodegradable and considered as bioplastics. The biodegradable fraction in those polyesters can be acquired from the addition of starch or biopolymers, which are included to enhance particular properties, e.g., physical and mechanical properties. This group, however, only constitutes a small share of less than 6% in the bioplastic market in 2022 (Table 2.1). The majority of those present in the bioplastic market today are innovative bioplastics, such as polylactic acid (PLA), polyhydroxyalkanoates (PHAs), and starch blends [36]. Excluding starch blends, which are a mixture of starch and plasticizers/additives, the building blocks of PLA and PHAs are produced from microbial fermentation of plant-based resources. Regardless of the great utility in various applications, the packaging sector is still the largest market segment of these innovative bioplastics [37]. Moreover, both PLA and PHAs are biodegradable, which can be further converted into the corresponding precursors, recirculating as feedstock for their production.

Compared to conventional plastics, the use of bioplastics generally offers several advantages, such as lowering carbon footprint, independence from non-renewable resources, and increasing the resource efficiency. In fact, bioplastics have been extensively employed in many sectors, including packaging, consumer goods, fibers, coating & adhesives, agriculture, automotive, electronic, etc. Therefore, the development of bioplastics is expected to increase gradually, with the volume being forecasted to reach 6.3 million tons by 2027. In this regard, Asia has been forecasted to produce 50% of the global bioplastics, which is almost two times higher than that produced in Europe (24.1%) [36]. However, the general share of bioplastics in the plastic industry is extremely modest, accounting for less than 1% in the total annual plastic production of 367 million tons [36].

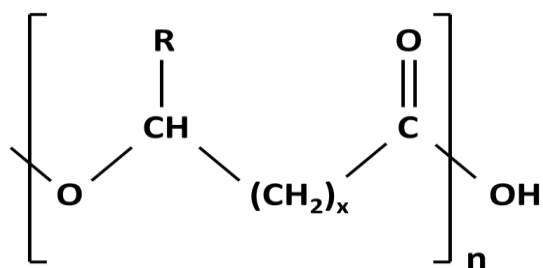
**Table 2.1** The production of bioplastics classified by material type in 2022 and forecasted production for 2027. Adapted from Bioplastics [36].

<b>Types of bioplastic</b>	<b>2022</b>	<b>2027</b>
Bio-polyethylene (bio-PE)	14.8%	11.8%
Bio-polyethylene terephthalate (bio-PET)	4.2%	1.8%
Bio-polyamide (bio-PA)	11.1%	18.7%
Bio-polypropylene (bio-PP)	3.9%	6.0%
Polyethylene Furanoate (PEF)	0.0%	0.1%
Polytrimethylene terephthalate (PTT)	13.3%	4.7%
Other (bio-based/non-biodegradable)	1.1%	0.4%
Polybutylene adipate terephthalate (PBAT)	4.5%	1.6%

Polybutylene succinate (PBST)	0.9%	0.3%
Polylactic acid (PLA)	20.7%	37.9%
Polyhydroxyalkanoates (PHAs)	3.9%	8.9%
Starch blends	17.9%	6.3%
Cellulose films	3.6%	1.5%
Total amount (million tons):	2.2	6.3

## 2.4 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) belong to the group of bio-based and biodegradable bioplastics, which can be accumulated by more than 300 species of microbes (including bacteria, algae, and fungi) as intracellular polyesters. The PHA accumulation is performed mostly under limiting conditions of excess carbon and deficit of nitrogen or phosphorus [38]. The role of PHAs includes energy storage, preventing cell autolysis, and supporting the survival of microorganisms during starvation conditions [39]. Generally, PHAs are constituted from (R)- $\beta$ -hydroxy fatty acid monomers, where the alkyl side chain (R) varies from a methyl (C1) to a longer chain of tridecyl group (C13) (Figure 2.4) [40]. Moreover, the alkyl side chain can be substituted by aromatic, epoxidized, halogenated, or even other branched monomers [41]. Depending on the number of carbon atoms in the backbone, PHAs are commonly classified into three groups of short chain length PHAs (3 – 5 carbons), medium chain length PHAs (6 – 14 carbons), and long chain length PHAs (over 14 carbons) [42]. Furthermore, different types of monomers belonging to the three mentioned groups can be processed together to generate various co- and terpolymers, diversifying the technical properties of PHAs, which ultimately leads to the wide range of PHA applications [42].



**Figure 2.4.** General structure of polyhydroxyalkanoates (PHAs), where R is mainly an alkyl side chain with different chain lengths and structures, n is the degree of polymerization ranging from 100 to 30,000 and x with values from 1 to 3.

In general, PHAs are water insoluble, have relatively good hydrolytic and ultraviolet resistance, are good barriers against oxygen, nontoxic, biocompatible and, especially, biodegradable under many environmental conditions [43]. Due to the variety in chemical structures, the properties of PHAs are various which could be partly comparable to some conventional plastics, such as polyethylene (PE), polypropylene (PP), polystyrene (PS), etc., in terms of the mechanical and physical properties [44]. For example, in comparison to PP, poly(3-hydroxybutyrate) (P(3HB)) has comparable tensile strength and modulus, while the elongation at break is clearly lower, namely below 10% (Table 2.2). Therefore, P(3HB) is also well characterized as a stiff and brittle material. In this regard, the brittleness of P(3HB) can be explained by the glass transition temperature, which is in the range of room temperature, leading to the secondary crystallization of the amorphous fraction at ambient temperature [43]. Furthermore, thermal stability of P(3HB) is also low, as the melting point is close to its thermal decomposition of around 180 °C. At high temperature, random chain scission of P(3HB) occurs, resulting in a significant decrease in the molecular weight.

Regarding the environmental impact, PHAs are more environmentally friendly than conventional plastics due to the biodegradability, leaving no harmful substances during the process. On the other hand, PHA production can be achieved through an eco-friendly approach of bacterial fermentation. According to a study by Kim, et al. [45], P(3HB) production from corn grain only consumed 2.5 – 9.2 MJ/kg, which is much lower than the minimum amount of energy required for the production of several conventional plastics, such as LDPE (64.6 MJ/kg), PP (64 MJ/kg), PS (70.8 MJ/kg), and PVC (52.4 MJ/kg) [46]. Regarding GHGs emissions, an approximate amount of 2.3 kg CO<sub>2</sub>/kg PHB was released, with corn grain production being the greatest contributor to the emissions. This number is predicted to decrease significantly as the conventional feedstock of PHAs production is gradually changed into economical and non-food substrates (discussed further in Section 3). Moreover, under specified conditions, the end-of-life PHAs can be fully biodegraded within a time span of two months into CO<sub>2</sub>, water, and biomass, leaving no toxic traces compared to petroleum-based plastics [47]. Altogether, PHAs are promising alternatives to replace conventional plastics, thereby reducing the plastic dependency and its related pollution issues.

**Table 2.2.** Comparisons between common polyhydroxyalkanoates (PHAs) and conventional plastics, in terms of the mechanical properties. Adapted from [48-50]

<b>Polymer</b>	<b>Melting temperature (T<sub>m</sub>) (°C)</b>	<b>Young's modulus (GPa)</b>	<b>Tensile strength (MPa)</b>	<b>Elongation at break (%)</b>	<b>References</b>
P(3HB)	175-180	3.5-4	40	3-8	[49]
P(4HB)	60	0.15	50	1000	[49]
P(3HB-co-3HV) (20% HV)	145	1.2	32	50-100	[49]
P(3HB-co-4HV) (3% HV)	166	Not available	28	45	[48]
P(3HB-co-4HV) (10% HV)	159	Not available	24	242	[48]
P(3HB-co-3HH) (11% HH)	124	Not available	29	263	[50]
PP	170-176	1.0-1.7	29.3-38.6	500-900	[48]
HDPE	112-132	0.4-1.0	17.9-33.1	500-700	[48]
LDPE	88-100	0.05-0.1	15.2-78.6	150-600	[48]
PS	80-110	3.0-3.1	50	3-4	[48]
Nylon-6,6	265	2.8	83	60	[48]
Polyurethane	195	0.004	38	550	[48]

## 2.5 Applications of PHAs and the current share in the bioplastic market

Poly(3-hydroxybutyrate) (P(3HB)) is the most produced polymer, with its accumulation being dominant in the majority of microorganisms [51]. However, the disadvantages of brittleness, narrow thermal-processing window, and secondary crystallization at room temperature have hindered the use of P(3HB) in engineering applications [52]. Therefore, the main applications of P(3HB) are only limited to raw materials and additives in different sectors of packaging (paper and cardboard coating), cosmetics (micro-powders), disposable items (bags, bottles), and biocomposites [53]. Several efforts have been made to overcome the drawbacks of P(3HB), whereby widening its practical applications. In this regard, incorporation of other longer hydroxyalkanoate acid

monomers, such as polyhydroxyvalerate (PHV) and polyhydroxyhexanoate (PHH), into P(3HB) backbone is one of the most effective solutions. These inclusions cause a defect in the morphology of the lamellae crystals, which further disrupts the crystallinity of P(3HB), improving its ductility and decreasing melting temperature [54]. This process renders co-polymers of poly(3-hydroxybutyrate-co-3hydroxyvalerate) (P3HBV) and poly(3-hydroxybutyrate-co-3hydroxyhexanoate) (P3HBH), with an increase in the polymer ductility (Table 2.2). This, in turn, extends the applications of PHAs in engineering sections, such as biomedical [55], pharmacy [56], agriculture [57], and other industrial sectors [58, 59] (Table 2.3).

However, high production cost has been the major drawback, hindering the commercialization of PHAs. The market price of these materials was reported to be around 1.5 – 5 €/kg in 2010, three times higher than the conventional plastic priced at 0.7 – 0.8 €/kg [60, 61]. Despite the current price difference between PHAs and traditional plastics, more PHA-derived products are anticipated to enter the market, ultimately increasing the share of PHAs due to the steady reduction in the PHA cost and the concomitant increase in the oil price. Therefore, the PHA fraction in the bioplastic market is predicted to extend, potentially reaching 8.9% by 2027 (Table 2.1).



**Table 2.3.** Commercial polyhydroxyalkanoate (PHA) products and their applications in 2015. Adapted from [53]

<b>Brand name and Company</b>	<b>PHA type</b>	<b>Class</b>	<b>Product</b>
Mirel (Metabolix, USA)	P(3HB)	Raw materials, Cosmetics, Packaging, Water treatment, Plasticizers	Injection molding, micro-powders, coating for paper and cardboard, denitrification for aquariums, additives for PVC and PLA
Nodax™ (MHG Bio, USA)	mcl PHAs	Raw materials (resins)	Bags, bottles, hygiene, mulch
Biocycle (Biocycles, Brazil)	P(3HB)	Raw materials (pellets)	Plastic sheet extrusion, injection, coating paper
MINERV-PHA™ (Bio-On, Italy)	PHAs (unclear)	Raw materials	Automotive, electronics, packaging
ENMAT (TianAn Biopolymer, China)	P(3HB), P(3HBV)	Raw materials (powder, pellets)	Thermoplastic, injection molding, extrusion, thermoforming, blown films, fiber and non-wovens, denitrification, water treatment
Sogreen™ (Tianjin GreenBio, China)	Ethyl 3-HB P(3,4HB)	Raw materials (resin, pellets)	
TephaFLEX (Tepha, USA)	P(4HB)	Medical materials	Suture, mesh, surgical film
AirCarbon™ (Newlight Technologies)	PHAs (unclear)	Raw materials	Extrusion, blown film, cast film, thermoforming, fiber spinning, and injection molding applications
VersaMer™ (Polyferm Canada)	mcl PHAs	Raw materials (pellets, latex)	Thermoelastomers



## **VOLATILE FATTY ACID<sub>s</sub> AS A POTENTIAL CARBON SOURCE FOR POLYHYDROXYALKANOATE PRODUCTION**

In response to the current crisis of plastic pollution, the development of PHAs has been one of the potential solutions of interest due to the high functionality in physical properties and low environmental impact. The commercialization of this material, however, has been hindered by its high production cost, which is mostly attributed to the cost of feedstock. Therefore, attempts have been made to use various negative or low value organic waste streams as economical substrates for PHA production. The heterogeneous, and rather recalcitrant or inhibitory, nature of waste streams, however, requires an initial breakdown, homogenization, and detoxification prior to bacterial consumption. In this regard, the volatile fatty acids (VFAs) generated as intermediate products of anaerobic digestion can act as a potential feedstock to attain a cost-competitive production of PHAs. This chapter will summarize the utilization of low-cost substrates, with an emphasis on VFAs, for PHA production.

### **3.1 Challenges in industrial-scale production of PHAs**

The production of PHAs is gradually commercialized after an increase in oil prices to over 100 USD/barrel in 2003 [62]. Afterwards, several enterprises producing PHAs were initiated worldwide, starting from small or pilot scale productions with a general capacity of 50 – 50,000 tons/year in 2010 (Table 3.1). Regardless of the significant increase in 2020, the capacity of PHA produced is still significantly lower than that of conventional plastics. For example, the annual production of PP alone is more than 60 million tons [63]. Moreover, the price of PP is less than 1 USD/kg, which is much lower than that of PHA (1.5 – 5 USD/kg) [64]. Therefore, high production cost has been considered being one of the most critical obstacles interfering with the widespread

industrial-scale production of PHAs. Both upstream and downstream processes are responsible for the high final price of PHA. In particular, the costly downstream operation, related to the recovery and purification procedures, consumes a huge amount of chemicals for solvent extraction of PHAs [65]. Besides the use of large amounts of chemicals, low PHA yield of around 33% (g PHA/g substrate) is ascribed to be one of the main factors generating the high downstream processing cost [63]. The insufficient PHA yield, however, is also associated upstream process of bacterial fermentation, which is influenced by various factors such as choice of substrate, operating conditions, productivity, and substrate conversion rate [66]. Fermentation substrate is found to be the most costly constituent, which can account for up to 50% of the total expenses in PHA production [67, 68].

**Table 3.1.** The currently active polyhydroxyalkanoate (PHA) manufacturers and their corresponding main substrates. Adapted from [53, 63]

Company	PHA type & trade name	Main substrate	Capacity in 2010 (tons/year)	Capacity in 2020 (tons/year)
Biomer Inc. (Krailling, Germany)	P(3HB) and P(3HB-co HV), Biomer	Sucrose	50	n/a
Bio-on (Bologna, Italy)	n/a, MINERV®-PHA	Beet sugar	10,000	n/a
Kaneka Co. (Takasago, Japan)	P(3HB-co3-HHx), Kaneka PHBH	Vegetable oil	1000	50,000
Tianjun Green Bio (Tianjin, China)	P(3HB), n/a	Sucrose	10,000	n/a
Tianan Biologic (Ningbo, China)	P(3HB-co HV), Enmat	Corn	10,000	50,000
PHB Industrial (Serrano, Brazil)	P(3HB), Biocycle	Sugarcane	50	10,000 (de-commissioned in 2015)
Danimer Scientific (USA)	mcl-PHAs, Nodax®-PHA	Cold pressed canola oil	n/a	272,000
Metabolix (Woburn, MA, USA)	P(3HB) and P(4HB), Mirel-PHA	Corn	50,000	n/a

n/a – not available

### 3.2 Conventional feedstock for PHA production

As presented in Table 3.1, sugar- and starch-based feedstocks, such as sugar beet, corn, and sugarcane, have been the major carbon sources used by PHA manufacturers. These materials can either be used directly or after being hydrolyzed for bacterial assimilation. For example, sucrose originated from sugar beet and sugarcane cannot be consumed directly by *Ralstonia eutropha* [69]. However, other bacteria, namely *Azotobacter vinelandii* [70], *Alcaligenes latus* [71], and *Hydrogenophaga pseudoflava* [72] are able to cleave the glycoside linkage in the sucrose structure, releasing glucose and fructose for further consumption. Another example is starch – the most extensively used feedstock in industrial-scale production of PHAs. Starch, derived mainly from corn, potato, and cassava, is usually hydrolyzed with the help of enzymes or acids to generate glucose for microbial fermentation [73].

However, the market price of the aforementioned feedstocks is within a range of €27 – 433/tons (Table 3.2). Furthermore, the utilization of sugar- and starch-bearing materials for bioplastics production directly competes with human food and animal feed applications, raising concerns in terms of sustainability, change of land use, and food security. For instance, it is estimated that approximately 0.67 kg of glucose can be produced from 1 kg of corn [74]. According to Gahlawat [63], at industrial scale, 1 kg of glucose can be converted into around 0.33 kg of PHAs. As a result, around 45,500 tons of corn is required, corresponding to an average price of €6.7 million for the production of 10,000 tons of PHAs. This emphasizes the need to exploit the low-cost and non-food/feed-based feedstock to achieve a sustainable and economically feasible production of PHAs.

**Table 3.2.** The annual production and market price of some conventional feedstock used for polyhydroxyalkanoate (PHA) production in Europe. Adapted from [75-77]

Substrates	Feedstock	Annual production (million tons)	Market price (€/ton)
Starch and sugar	Sugar beat	152	27 – 41
	Corn	123	75 – 220
	Wheat	207	393 – 433
	Potatoes	83	102 – 173
Vegetable oil	Canola	21	1018

### 3.3. Low-cost substrates for PHA production

Organic wastes, residues, and by-products originated from municipal activities and industrial processes, are abundant and rich in nutrients and could potentially be used for the cultivation of PHA-bearing microorganisms. A number of sustainable substrates used for PHA production are summarized below.

#### 3.3.1 Lignocellulose

Lignocelluloses are one of the most promising feedstocks for the sustainable production of PHAs. Lignocellulosic materials are abundant, cheap and rich in fermentable-sugars (up to 70% cellulose and hemicellulose) [78]. In particular, cellulose is a polysaccharide composed of glucose monomers linked together by  $\beta$ -1,4-glycosidic bond, existing mostly in a crystalline structure. Compared to cellulose, the structure of hemicellulose is more amorphous, composed of mixed polysaccharide of pentose (5 carbon-sugar of arabinose and xylose) and hexoses (6 carbon-sugar of glucose and mannose). However, in order to utilize the fermentable sugar fractions, an extensive pretreatment of de-lignification is required to separate the lignin component (10 – 35%), releasing hemicellulose and cellulose hydrolysate [79]. These hydrolysates contain their corresponding monosaccharides and a minor fraction of acetic acid, acid soluble lignin, alcohols, and volatile acids [80]. In general, the productivity of bacterial cultivation using cellulose-derived glucose is considerably higher than that of hemicellulose hydrolysate [81]. Among lignocellulosic materials, wheat straw could be the most promising substrate, which yielded a PHA accumulation of up to 72% in cell dry weight of *Burkholderia sacchari* DSM 17165 [82]. A considerable PHA content of 59 and 61%, respectively, was also obtained from the cultivation of *Bacillus* spp, using lignocellulosic materials of rice straw and sugarcane molasses [83, 84].

#### 3.3.2 Waste Animal Fats and Plant Oils

Lipid-rich wastes, such as animal fats, plant oils, and frying oils, originating from the agricultural and food industry, could be another potential substrate for PHA production. The composition of lipids is dominated by triacylglycerols, which are generally divided into two groups of saturated long-chain fatty acids and unsaturated short-chain fatty acids [85]. For the direct use of triacylglycerols, lipases-secreting bacteria, such as *Cupriavidus necator*, *Pseudomonas* spp, are normally employed to cleave the triacylglycerols into free fatty acids, which are subsequently directed to the  $\beta$ -oxidation cycle for further growth and PHA synthesis [81]. According to a life cycle analysis (LCA), reported in a study by Loo, et al. [86], plant waste-based oils such as olive, palm, sunflower, and corn oil are more preferable than conventional feedstock, in terms of sustainability, cost-competitiveness, and productivity in the PHA yield. Compared to plant oils, which are in liquid form and facilitate the fermentation, animal fats require an addition of emulsifier to obtain a homogeneous state prior to the bacterial cultivation [81]. Waste frying oil, on the other hand, was found to improve the PHA production due to the

existence of other nutrients [87], yielding a productivity of 0.14 g PHB/L.h [88]. Therefore, more genetically modified triacylglycerol-utilizing strains have been developed to fully utilize the advantages of lipid-rich wastes for offsetting the high production cost of PHA [89].

### 3.3.3 Crude Glycerol

With a share of 10 – 12% in the biodiesel produced, crude glycerol is the major by-product during the oil transesterification in biodiesel production. Depending on the type of feedstock and choices in production mechanisms, the purity of crude glycerol varies in a range of 55 – 90% compared to pure glycerol [90]. Regardless of the high value of pure glycerol, refinery of crude glycerol is not favorable due to the further requirements in capital investment, operating costs, and skillful operators. Therefore, applications without further processing are preferable for the use of crude glycerol, such as raw material in co-combustion or co-gasification for heat, power, and syngas production [91]. Crude glycerol, moreover, can be served as feedstock in microbial fermentation for the production of value-added metabolites, such as PHAs [92]. In a study by Cavalheiro, et al. [93], a promising PHB yield of 38% was obtained in PHA production using *C. necator*. However, it is recommended to keep the crude glycerol concentration at 3% (w/v) to prevent the high osmotic stress and deficiency in the bacterial enzymatic activity, which greatly affected the bacterial growth [94]. Further utilization of crude glycerol in the PHA production can be found in previous literature [95-97].

### 3.3.4 Industrial Waste Gases

Industrial waste gases, such as CO<sub>2</sub> and CH<sub>4</sub>, have been identified as major factors responsible for the GHG effect. The amount of such gases is expected to increase gradually due to the unceasing growth of industrialization and urbanization. However, attention has been drawn to the utilization of industrial waste gases as a potential substrate for PHA production. In this regard, type II methylotrophs have been able to convert methane into PHB with a conversion rate of up to 54% [73]. However, the use of waste gases is energy consuming compared to the aforementioned substrates due to the low solubility, which requires extensive stirring to ensure the mass transfer efficiency and availability for microbial consumption. Recently, the development of a two-phase partitioning bioreactor using silicone oil was found to increase the solubility of CH<sub>4</sub>, providing a specific productivity of 1.83 mg PHB/mg CH<sub>4</sub>.h [98]. Consequently, the improvement in diffusion rates and solubility of industrial waste gases could provide another option as a sustainable substrate for feasible commercialization of PHA in the future.

## 3.4. Volatile Fatty Acids (VFAs)

The use of organic municipal and industrial wastes could be beneficial for the cultivation of PHA-bearing microorganisms by the presence of advantageous nutrients. However, the concomitant existence of recalcitrant

and/or inhibitory compounds could generate negative variations in PHA production, in terms of PHA productivity, yields, and quality [99, 100]. Therefore, it is necessary to initially homogenize organic waste streams into suitable compounds for bacterial consumption. In this regard, volatile fatty acids (VFAs) derived from anaerobic digestion could be a potential candidate for solving the existing obstacles of PHA substrate toward the sustainable PHA production.

In general, VFAs are a group of linear aliphatic monocarboxylate compounds, consisting of two to six carbon atoms in the backbone. Conventionally, the production of VFAs is derived from the synthesis of petroleum-based resources via petrochemical pathways [101]. Regardless of the high efficiency of chemical synthesis, the production of VFAs by this method is energy intensive and not eco-friendly due to the over-exploitation of non-renewable resources [102]. Hence, biological treatment of anaerobic digestion has emerged as an economically feasible alternative to produce VFAs. Anaerobic digestion is one of the most effective and mature treatments to handle the complex organic wastes. VFAs are produced as intermediates of such processes, which are further converted into biogas as the final product [103]. However, attention has been more focused on VFAs due to the higher potential in market appeal and application range in comparison to biogas [104]. In fact, either in a form of individual or mixture molecules, VFAs can be utilized in numerous valuable applications, such as food additives, dyes, paints, perfumes, pharmaceuticals, resins, etc. [105]. Moreover, VFAs are found to be precursors in the synthesis of biodegradable bioplastics of PHAs [106]. Indeed, VFAs can be consumed by different types of microorganisms, such as *Bacillus*, *Pseudomonas*, *Methylocystis*, *Rhodococcus*, *Haloferax*, etc., yielding a significant PHA content of up to 78% [107-110]. Considering the availability and versatility of anaerobic digestion, which can cope with different kinds of organic wastes regardless of location because of the worldwide existence of anaerobic digesters, the VFA supply can be ensured to replace expensive conventional substrates for PHA production [111].

#### 3.4.1 VFA production from acidogenic fermentation of organic waste

Different types of organic waste streams originate from industrial activities, including agriculture, forestry, animal farming, meat processing, food supply chain, etc. on a daily basis in large quantities. In developing countries, due to the rapid urbanization and the lack of waste sorting and collection facilities, most municipal organic wastes are indiscriminately disposed in open dumping sites or landfills. Landfilling, however, has been known to be the least favorable treatment method, considering the emissions of strong odors, GHGs, and heavy metals containing leachates to the land and water resources [112, 113]. Regardless of the efforts made in sorting at the source, organic wastes are found to be mixed with other solid municipal wastes [114]. Therefore, in developed countries, organic wastes are used as feedstock in thermal treatment to reduce the use of landfills [115]. Thermal treatment is an effective technology to handle a large volume of heterogeneous wastes in an environmentally friendly and economically sustainable way. Depending on the oxygen requirement, thermal



waste treatment can be divided into three major methods, including incineration, gasification, and pyrolysis. In particular, incineration is one of the most common waste treatment methods, which involves a combustion process in a condition of sufficient oxygen to decompose organic- or fossil-based waste materials into ash, recovering energy in the form of electricity and heat [116]. Waste materials, on the other hand, are converted into syngas in gasification, which requires a low amount of oxygen, combined with an intense pressure for the heating process [117]. Lastly, pyrolysis is the only thermal treatment conducted in the absence of oxygen. Moreover, pyrolysis is commonly applied in organic waste treatment, in which the organic substances are decomposed into combustible gases and carbon-rich solid residue of bio-char and bio-oil [118]. However, organic wastes are high in moisture content, which could greatly reduce the efficiency of such thermal processes. This way, the organic fractions, which could be employed to produce high value biomaterials, and biochemicals, would end up as a source of energy and power. Thus, biological treatment by anaerobic digestion is a suitable method for recovering nutrients in the complex organic waste streams.

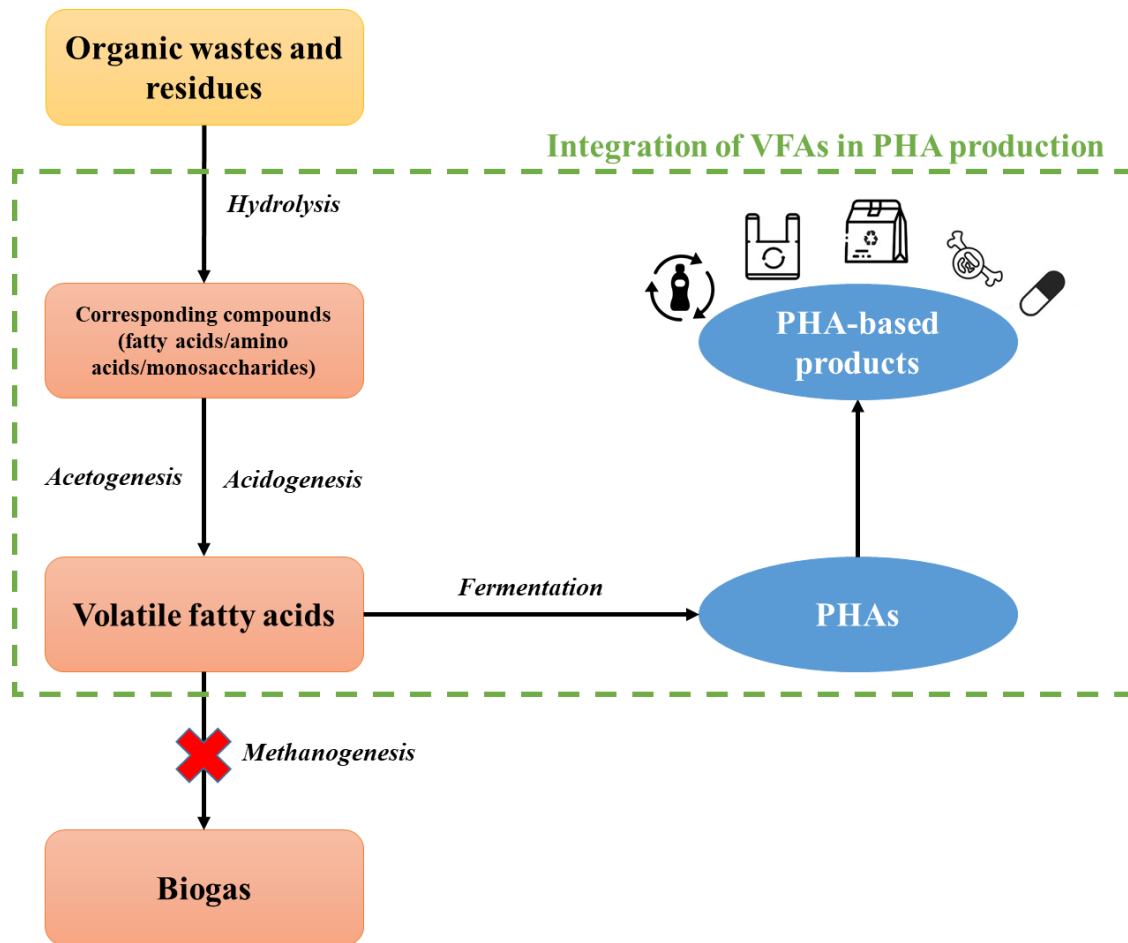
Anaerobic digestion is a sequential biological process in which organic wastes are decomposed under oxygen-deficient conditions [119]. In general, the process of anaerobic digestion can be classified into four major stages, including hydrolysis, acidogenesis, acetogenesis, and methanogenesis, with the presence of anaerobic and facultative anaerobic microbial consortia. These bacteria share a syntrophic relation, in which their metabolisms depend on each other to perform successive redox reactions throughout the process, converting organic wastes into biogas [120]. In particular, complex macromolecules, such as carbohydrates, proteins, and lipids, are initially hydrolyzed into their corresponding monomers in the form of simple soluble molecules by extracellular enzymes, such as amylases, lipases, and proteases. In the next stage of acidogenesis, the solubilized molecules are converted into VFAs, alcohols, lactate,  $\text{CO}_2$ , and  $\text{H}_2$  [121]. Subsequently, these low molecular weight metabolites are further oxidized to acetate and  $\text{H}_2$  by acetogenic bacteria in the third stage of acetogenesis. Lastly, the metabolites produced from acetogenesis are consumed by methanogenic archaea for the generation of biogas with a typical content of  $\text{CH}_4$  (50 – 75%),  $\text{CO}_2$  (25 – 50%), and a small amount of  $\text{N}_2$  (2 – 8%) [122]. The biogas obtained can be directly exploited for the generation of heat and power or used as bio-fuel after the removal of impurities, such as  $\text{H}_2\text{S}$  [119]. Besides the production of biogas, the nutrient-rich byproduct of the digestate can be utilized as an organic fertilizer or soil amendment. Moreover, the use of anaerobic digestion for organic waste treatment is beneficial in terms of volumetric reduction and pathogen removal [123]. Therefore, anaerobic digestion is the most effective technology to handle the organic wastes without wasting the organic fractions.

Regardless of the potential of biogas in the energy sector as a carbon-neutral energy source, the scale up in biogas production still faces some challenges, derived from both technical and economic barriers. From the technical aspect, low productivity is one of the major hurdles in biogas production. The insufficient biogas output can be attributed to the methanogenesis, which is considered as the limiting step due to the high sensitivity of methanogens to the fluctuations during the operation [124]. In a stable anaerobic digester, acidogenesis is considered as the fastest stage, in which the metabolites from the hydrolysis stage are consumed at an accelerating

rate, leading to the excessive production of VFAs [125]. This, in turn, leads to the sudden drop in pH, which could highly inhibit the activity of methanogenesis. Therefore, anaerobic digestion is generally performed at low loading rates with an addition of alkaline reagents, such as  $\text{NaHCO}_3$  and  $\text{CaO}$ , to ensure the stability [126, 127]. Such requirements will reduce the operation capacity, leading to a low efficiency in biogas production and unwanted aggregates in the digester [127]. Furthermore, methanogenic archaea have slow growth rates due to the selectivity in substrate consumption, leading to the further requirement of a long hydraulic retention time (>30 days) [128]. The expansion of biogas production, moreover, is less favorable because of the low profit of biomethane, which cannot compensate for the high investment capital for operation, storage, and refinery facilities [128].

Considering the aforementioned issues, a shift from biogas production to VFAs could be a feasible output for the anaerobic digestion of organic waste. As mentioned above, VFAs are the main intermediates synthesized in the stage of acidogenesis, which can be used in diverse applications of valuable biomaterials, providing the great market appeal than that of biogas [111]. As a result, the anaerobic digestion process has recently been manipulated to promote the production of VFAs by boosting the microbial activity of acidogenesis while the methane formation is inhibited [129]. Such an operating condition is known as acidogenic fermentation [105].

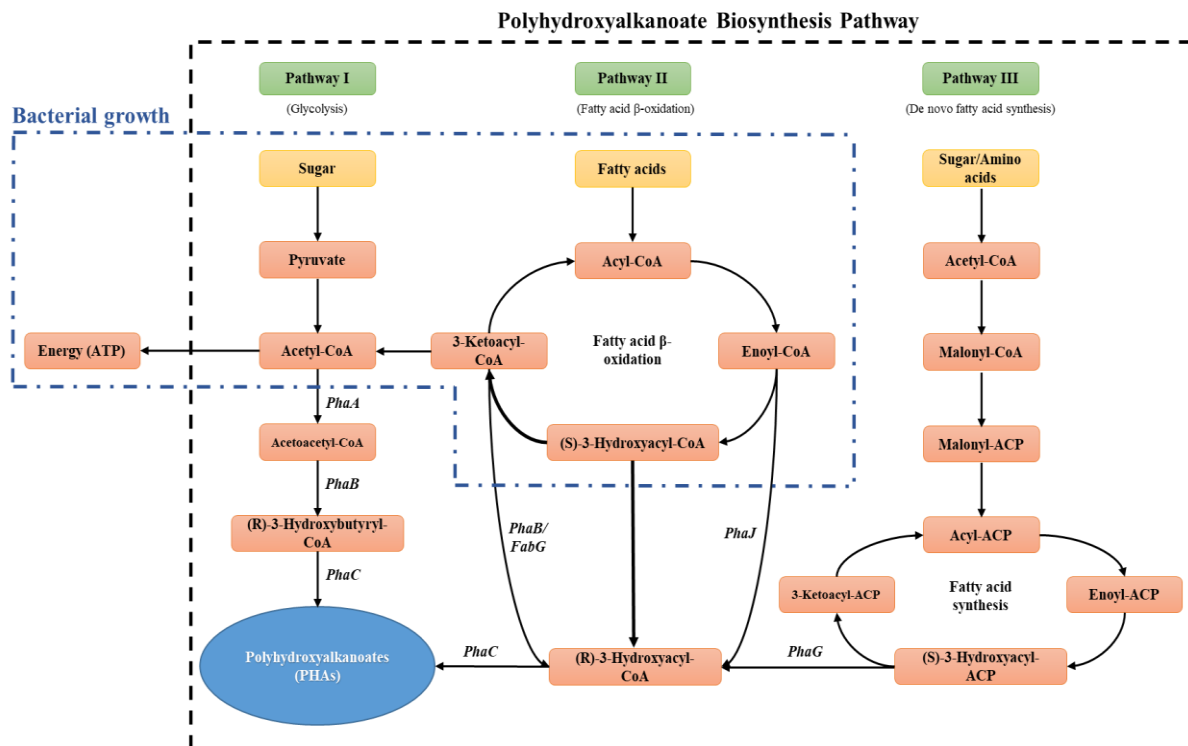
The prohibition of methanogenesis is one of the most crucial requirements to increase the accumulation of VFAs. Chemical agents, such as bromoethanesulfonate (BES), are commonly added to deactivate the coenzyme M in the methanogens, suppressing the methanogenesis activity [130]. Methanogens, moreover, are non-sporulating bacteria, which cannot tolerate high temperature conditions. Consequently, heat shocking of the inoculating microflora is also an effective method to deactivate methanogenesis [131]. Meanwhile, in order to increase the yield at the acidogenesis stage, a high feeding rate is a practical strategy. High organic loading rates would break the equilibrium between two stages of acidogenesis and methanogenesis, heading to the VFA accumulation [132]. Regardless of the great tolerance of acidogenic bacteria at very low pH conditions, a moderate acid level (around 5 to 6) is more favorable for the VFA production because of the better hydrolysis in such conditions [133].



**Figure 3.1.** An integration of recovered volatile fatty acids (VFAs) from the acidogenic fermentation of organic wastes for the production of high-value polyhydroxyalkanoate (PHA)-based products for packaging, bio-implants, encapsulation.

### 3.4.2 VFAs for PHAs biosynthesis

For a better understanding of the role of VFAs in PHA biosynthesis, the general metabolic pathway of PHA-accumulating bacteria should be considered. Depending on the carbon feedstock, PHAs are synthesized via different pathways, as summarized in Figure 3.2. In general, during normal bacterial growth, carbon substrates are metabolized into acetyl-CoA, which afterward participates in the Krebs cycle to produce energy in adenosine triphosphate (ATP) form [134]. However, under stress conditions of limited nitrogen, phosphorus, oxygen, etc. and excess carbon, acetyl-CoA will be applied as a precursor for PHA accumulation. In particular, two molecules of acetyl-CoA are combined with 3-ketothiolase (PhaA) to form acetoacetyl-CoA, which is further converted into 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB). Lastly, monomers of 3-hydroxybutyryl-CoA are polymerized by PHA synthase (PhaC) to form PHB [135].

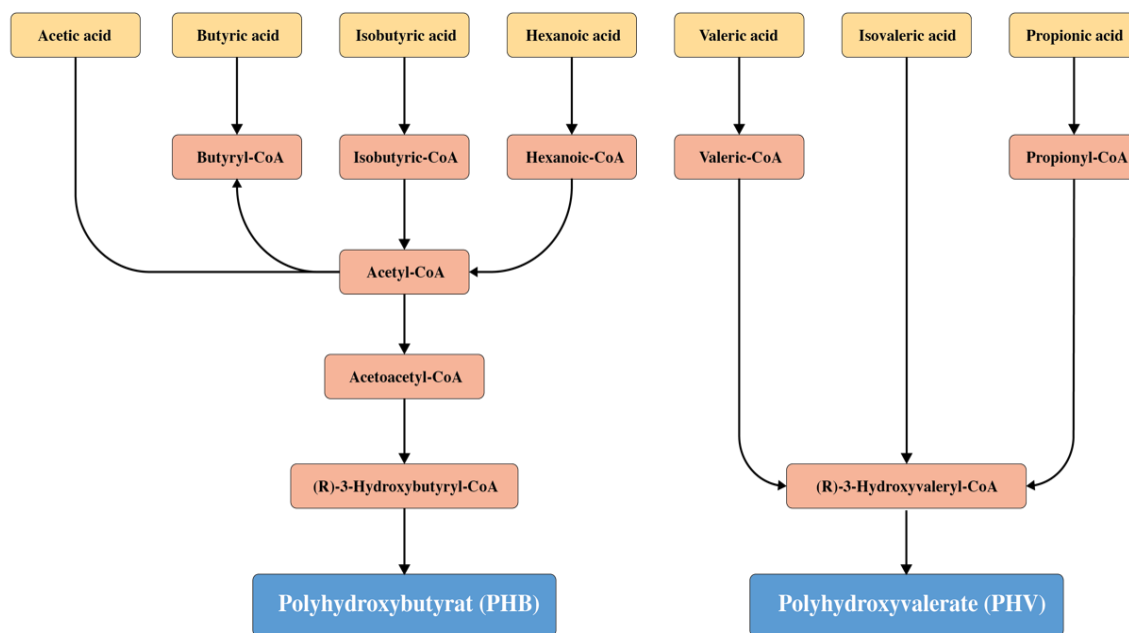


**Figure 3.2.** Metabolic pathway of polyhydroxyalkanoate (PHA)-accumulating microorganism during growth under balanced condition (blue dash dot) and PHA accumulation under stress conditions (black dash). Pha is  $\beta$ -ketothiolase; PhaB is acetoacetyl coenzyme A (CoA) reductase; PhaC is PHA synthase; PhaJ is enoyl-C; PhaG is acyl-acyl carrier protein (ACP)-CoA, and FabG is 3-ketoacyl ACP. Adapted from [135, 136].

VFAs were also found to be precursors that are directly involved in the PHA biosynthesis of PHA-bearing microorganisms (Figure 3.3). In order to participate in such a metabolic pathway, VFAs are originally subjected to fatty acid  $\beta$ -oxidation for the formation of corresponding derivatives of acyl-CoA, such as propionyl-CoA, butyryl-CoA, isobutyryl-CoA, valeryl-CoA, isovaleryl-CoA, and hexanoyl-CoA. However, only derivatives derived from VFAs with an even number of carbon atoms (C2, C4 and C6) are converted into acetyl-CoA, which is able to support both bacterial growth and accumulation of PHB [137, 138]. Odd number carbon atom VFAs, on the other hand, are transformed into (R)-3-hydroxyvaleryl-CoA for the production of PHV. Furthermore, PHV is favorable for incorporation with PHB to generate the copolymer of PHBV. The applications of PHBV are more diverse than that of PHB, which is limited by its inherent brittleness and stiffness [139]. In the last pathway of *de novo* fatty acid synthesis, the metabolism is regulated by two major enzymes of acetyl-CoA carboxylase and acyl-acyl carrier protein (ACP)-CoA (PhaG) [140]. The first enzyme is responsible for the production of malonyl-CoA by introducing a carboxyl group to acetyl-CoA. Malonyl-CoA is further conveyed into the *de novo* fatty acid synthesis by the acyl-acyl carrier protein in the form of malonyl-ACP. In this pathway, (S)-3-hydroxyacyl-

ACP is an important intermediate which is converted into (R)-3-hydroxyacyl-CoA for the polymerization into PHAs by PHA synthase [141].

The variety in the metabolic pathway of PHA biosynthesis is a great advantage to utilize various organic waste streams, extending the pool of carbon sources used for PHA-bearing microbial cultivation. In this regard, VFAs derived from acidogenic fermentation of organic waste can take advantage of the second pathway to be the potential feedstock for PHA production. With the large volumes of organic wastes produced worldwide, especially food waste with a yearly generation of 1.3 billion tons [142], the supply of food waste-based VFAs can be ensured to be an economical feedstock for PHA production.



**Figure 3.3.** The involvement of volatile fatty acids (VFAs) in the polyhydroxyalkanoate (PHA) biosynthesis. Adapted from [137, 138]



## POLYHYDROXYALKANOATE PRODUCTION USING ORGANIC WASTE DERIVED-VOLATILE FATTY ACIDS

Besides the availability and price of the carbon feedstock, the conversion efficiency of the substrate to a product is another crucial factor in the microbial production of PHAs. Consequently, similar to other low-cost, impure feedstocks, VFAs could pose a negative impact on the growth of PHA-accumulating bacteria due to their toxicity at high concentration, leading to the low PHA yield. Therefore, in this thesis (**Papers II and III**), the optimal VFA loading, and compositions were initially investigated for PHA production using two pure cultures of *Bacillus megaterium* and *Cupriavidus necator*. Moreover, in order to increase the economic competitiveness, a novel attempt of integrating membrane bioreactors with PHA production was introduced in **Paper V**, the utilization of which was to achieve a semi-continuous PHA production, overcoming the deficient productivity of batch and fed-batch cultivations under high VFAs concentration.

### 4.1 Production of PHAs by mixed microbial cultures

As described in the previous chapter, expensive conventional feedstocks have been the major bottleneck in the commercialization of PHAs. In an effort to reduce the PHA production cost, mixed microbial cultures (MMCs) have been studied to exploit the versatility in consuming complex substrates [143]. Compared to pure bacterial cultures, the application of mixed cultures is beneficial, in terms of energy consumption, because of the lower requirement in sterilization, equipment, or maintenance [144]. Activated sludge from wastewater treatment plants is a typical example of MMCs used for PHA production due to the ability of accumulating PHAs under transient conditions of intermitting feeding [145]. Prior to PHA production, MMCs are subjected to a stage of selective pressure to favor the PHA-accumulating bacteria [146]. In this regard, a feast-famine cycle is applied to enrich

PHA producers [147]. MMCs are primarily exposed to a condition of rich carbon (feast stage) for polyester accumulation by PHA-storing bacteria. The intracellular granules are subsequently used for microbial maintenance during the starvation, with no supply of nutrients (famine stage). Consequently, the bacteria that are incapable of producing PHA would be eliminated through the repetition of such a cycle. However, in order to obtain a high and stable PHA-storing microbial community, the famine period should be long enough, which could result in low volumetric productivity [148]. Different waste derived carbon feedstock, including VFAs, have been used for PHA production using MMCs. However, the obtained products are reported to be around 0.4 g PHA/L.h, which is 10-times less than that of a single culture, such as engineering *E. coli*, *C. necator*, *Bacillus* spp, etc.[149]. This, in turn, would require larger bioreactors to compensate for the low productivity, which means an increase in the capital investment and process footprint.

In addition, PHA extraction from MMCs is more challenging, affecting polymer recovery and purity [150]. The entanglement of sludge impurities and PHA-bearing microorganisms intensifies the downstream processing, affecting the overall cost of the process. The low purity of the recovered PHA, furthermore, could hinder the high-value PHA applications in the biomedical industry [150]. Therefore, considering all the aforementioned issues, pure cultures have been chosen in this thesis to study the PHA production using food waste-based VFAs.

## 4.2 Production of PHAs by pure microbial cultures

Over 300 microorganisms, including archaea, Gram-positive and negative bacteria, are capable of accumulating PHAs. However, only some of them, such as *Alicycobaculum* spp., *Cupriavidus necator*, *Pseudomonas* spp., *Haloferax mediterranei*, *Bacillus* spp., and recombinant *Escherichia coli*, are actually used for an economical production of PHAs. Such bacteria could utilize different types of substrates with a rapid growth and high bioconversion rate, providing significant PHA yields on biomass of up to 90% [151]. Among them, the Gram-negative bacteria called *Cupriavidus necator* is the most dominant PHA producer. Due to the exceptional metabolic versatility of chemoheterotroph, this bacterium could utilize different types of substrates from sugar-based to organic waste-based feedstock, yielding considerable PHA amounts of over 75% [152, 153]. Homopolymer of PHB is the major product accumulated in the cultivation of *C. necator* using a sugar-based substrate (e.g., glucose, fructose, sucrose, etc.). The highest productivity of 3.10 g/L.h was also reported, as glucose was used as the main carbon source for this bacterium [154]. While switching over to substrates such as vegetable oils, glycerol, or VFAs, co-polymers of PHBV and PHBHx were obtained, with the HV and HHx varying around 6 – 10% [155, 156]. In a study by Thinakaran, et al. [157], palm oil waste was used for the fed-batch cultivation of *C. necator* Re2058, yielding a considerable productivity of 1.06 g PHA/L.h. In the group of Gram-negative bacteria, *Pseudomonas* spp. are natural mcl-PHA producers [158]. A tripolymer of P(3HB-3HV-3HHx) was even achieved in the cultivation of *P. putida* and *Aeromonas hydrophila* GAK4, using a mixture of dodecanoate and gluconate [159, 160]. Regardless of the great metabolic versatility, *Pseudomonas* bacteria prefer



medium and long-chain-fatty acids as the main carbon sources. For example, in a fed-batch cultivation of *P. putida* CA-3 using decanoic acid, a co-polymer of P(HD-co-HO) was obtained with a productivity of 1.63 g/L.h [161]. Other PHA productions using other Gram-negative bacteria, such as *Azotobacter*, *Haloferax*, *Methylobacterium* and *Azohydromonas*, with mixed substrates, were described elsewhere [162-165].

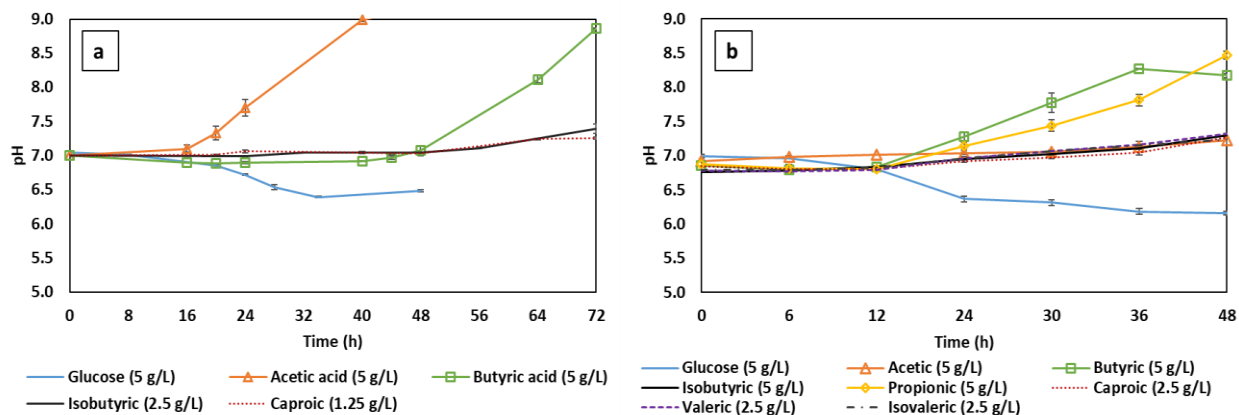
In the category of Gram-positive bacteria, *Bacillus* spp are the main PHA producers due to the unique metabolic characteristics. *Bacillus* species, such as *B. megaterium* OU303A and *B. sp.* 88D, could consume some of the complex organic wastes for the production of a co-polymer of PHBV with the HV content varying from 2.5 to 6.3%. In the cultivation of *Bacillus* sp. INT005 using a mixture of glucose and VFAs (1% v/v), a PHA yield on biomass of up to 64.5% was obtained with the HV content of 29 mol% [166]. Compared to other *Bacillus* spp, the maximum PHA accumulation obtained in the cultivation of *B. megaterium* ATCC 14945 using food waste based VFAs (5 g/L) was relatively low, at around 0.16 g/L (**Paper II**). Regardless of the difficulty in doing comparisons, in terms of PHA productivity and flexibility in substrate consumption, the PHA granules obtained from the Gram-positive bacteria are more preferable for medical applications [167]. The downstream process of PHA extraction, furthermore, could be facilitated due to the absence of lipopolysaccharides layers in Gram-positive bacteria [168].

### 4.3 The effect of cultivation conditions on PHA production from VFAs as the main carbon source

Besides the compatibility of the carbon source in bacterial metabolism, cultivation factors are important variables, defining the efficiency of PHA-accumulating bacterial fermentation. Any changes therein could directly affect the microbial activities in relation to carbon assimilation and bioconversion of secondary metabolites. Therefore, after proving the potential of using directly waste-based VFA effluent in **Paper II**, some of the fermentation parameters, e.g., pH, oxygen supply, C/N ratio, and nitrogen source are optimized in **Paper III** to enhance the VFA-to-PHA bioconversion.

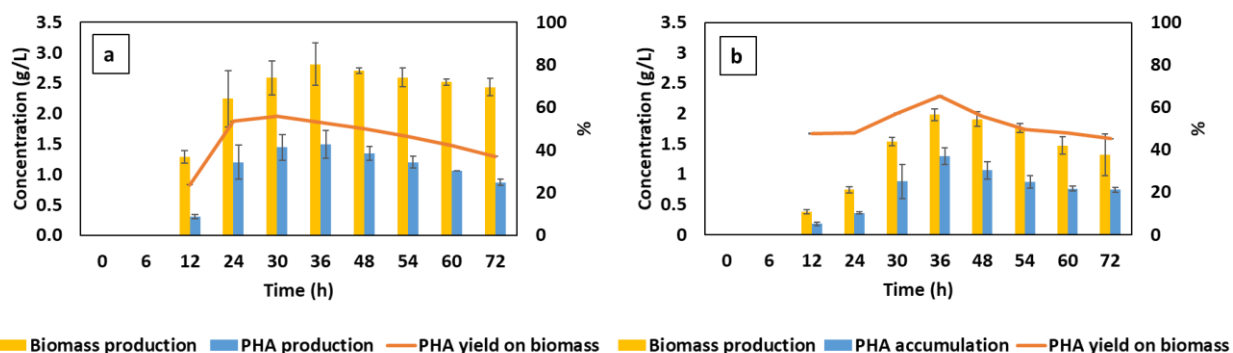
#### 4.3.1 The effect of pH

In bacterial cultivation, pH of the growth media is one of the most important factors, which directly affects the microbial growth via major influences on cell structure and intracellular metabolisms [169, 170]. Under optimal pH condition, the bacterial growth can be promoted, which is beneficial in PHA production due to the PHA-storing function of bacterial cells [171]. However, pH can be shifted away from the optimal level during microbial growth by the process of substrate consumption and metabolite excretion [172]. This phenomenon can be observed in **Papers II and III**, in which the assimilation of VFAs resulted in a pH increase of up to 9, while glucose consumption decreased the pH values (Figure 4.1).



**Figure 4.1.** Changes in the pH values during the substrate consumption of glucose and volatile fatty acids (VFAs) by two bacteria of *B. megaterium* ATCC 14945 (a) and *C. necator* DSM 545 (b). (Adapted from **Papers II & III**)

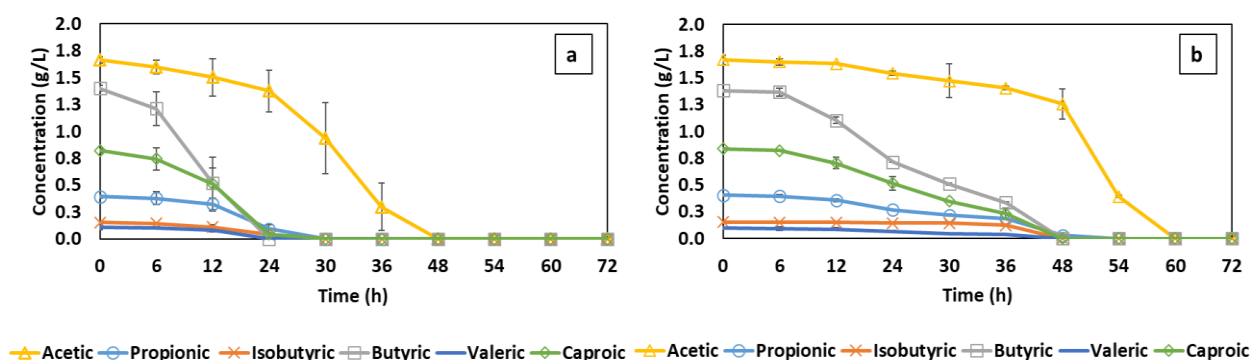
The change in pH, however, negatively affected the cultivation of *C. necator*, resulting in a decrease of biomass production and PHA accumulation (**Paper III**). Similar results were obtained in a study by Kourmentza, et al. [173], in which a shift of pH from 7.25 to 9.1 provided a lower PHA productivity of 0.5 g/L.d compared to that obtained with a change from 6.9 to 7.9 (1.36 g/L.d). This can be attributed to the microorganism's response to the change of external pH, as suggested by Chen, et al. [174]. In order to maintain the cytoplasmic pH for optimal cell functioning, cell maintenance could be activated. This further interrupts the bacterial growth, adversely affecting the PHA content. The results upon investigation of such a hypothesis is elaborately presented in **Paper III**, where a higher biomass production and PHA accumulation were obtained in the cultivation of *C. necator* under a controlled pH at a neutral condition of 7 (Figure 4.2). In fact, a neutral pH was found to be optimal for the activity of PHA-synthesizing enzymes, providing a productive PHA accumulation [175]. In another study by Wei, et al. [176], a pH value of 7 rendered the highest PHA content of 43% compared to acidic (30%) and alkaline (31%) conditions. Unfavorable pH conditions, on the other hand, could be considered as a limiting factor that hinders microbial growth, consequently activating the PHA accumulation sooner. As a result, an alkaline pH was found to be favorable for PHA production by MMCs in the studies by Montiel-Jarillo, et al. [177] and Villano, et al. [178].



**Figure 4.2.** Comparisons of biomass production, polyhydroxyalkanoate (PHA) accumulation, and PHA yield on biomass in two conditions of pH control (a) and no pH control (b). (Adapted from **Paper III**).

#### 4.3.2 The effect of aeration

With an extraordinary versatility of facultative chemolithotroph, *C. necator* can utilize a wide range of carbon sources for growth under both aerobic and anaerobic conditions [179]. However, in order to employ VFAs for the PHA production, *C. necator* requires sufficient oxygen to conduct the fatty acid  $\beta$ -oxidation process, producing energy and building blocks for the biosynthesis of PHA [180, 181]. Insufficient oxygen availability, therefore, would hinder the uptake process of carbon sources, which consequently affects the bacterial growth and metabolite production [182]. Indeed, a slow assimilation of VFAs was observed under oxygen deficiency of 0.25 vvm (Figure 4.3), leading to an inferior rate in the PHA accumulation compared to 1 vvm (**Paper III**).

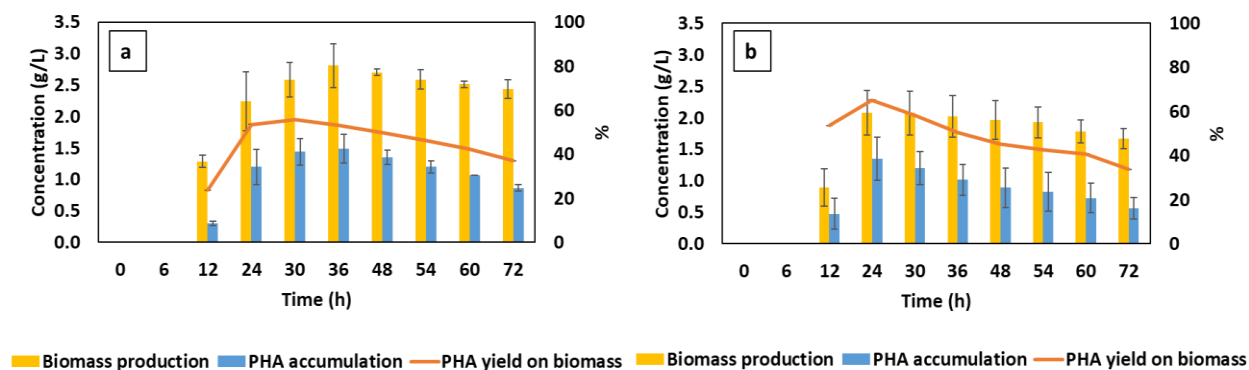


**Figure 4.3.** Changes in volatile fatty acid (VFA) consumption during the cultivation of *C. necator* DSM 545 in two aeration conditions of 1 vvm (a) and 0.25 vvm (b). (Adapted from **Paper III**).

The same behavior was seen in a study by Gobi, et al. [183], as the PHA-accumulating phase was accelerated at intensified aeration. At the same aeration rate of 1 vvm, a higher PHA content of 64% was obtained as well, compared to that of at 0.5 vvm (52%) in the cultivation of the same bacterial specie of *C. necator* [184]. The important role of sufficient oxygen supply was also presented in a study by Kulpreecha, et al. [185], in which a PHA yield obtained at an elevated saturated dissolved oxygen (DO) level of 60% was 14% higher than that at 40% DO (47%). The oxygen demand, moreover, is increased overtime due to the augmentation of the cell densities. Under oxygen deficiency, high-density cultures were almost terminated, significantly lowering the PHA accumulation as revealed by Follonier, et al. [186]. Additionally, the generation of exopolysaccharides during the bacterial cultivation could increase the viscosity in the system, which adversely affects the oxygen distribution, inversely hindering the bacterial growth [187]. Therefore, optimal aeration should be well provided and maintained throughout the cultivation to ensure metabolic activity of the PHA-bearing microorganisms.

#### 4.3.3 *The effect of carbon to nitrogen ratio*

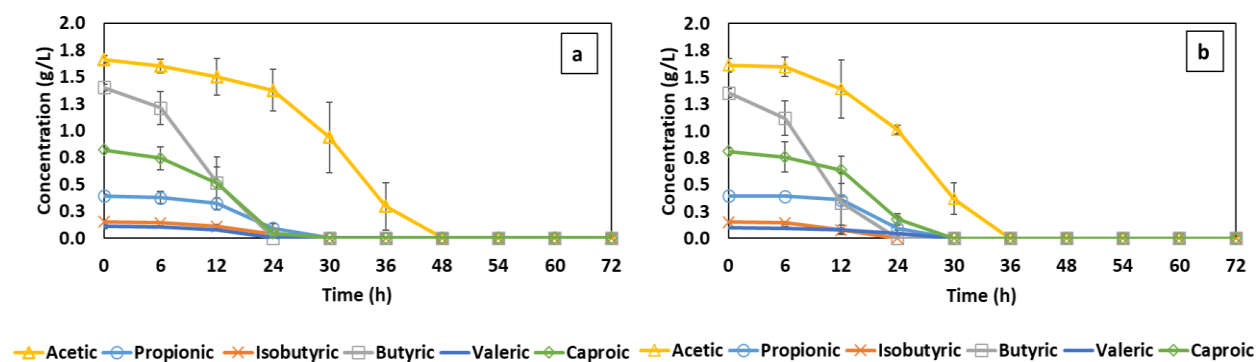
PHA-accumulating bacteria can be divided into two groups of growth-associated and non-growth-associated PHA producers [188]. Besides engineered *Escherichia Coli*, non-growth-associated microorganisms, such as *Bacillus* spp, *Pseudomonas* spp, and *Ralstonia eutropha*, are bacterial strains used for PHA production by common PHA manufacturers [152]. This microbial group requires restricted conditions of deficient nitrogen, phosphorus, sulfur and excess carbon to trigger PHA synthesis. Among them, limitation in nitrogen has been pointed out to be the most defining in promoting PHA accumulation [189]. In fact, under the condition of nitrogen scarcity, acetyl-CoA is hindered from entering the Krebs cycle and directed into PHA synthesis [190]. Different studies on C/N ratios within a range of 0.15 – 100 revealed that PHA yields were highly promoted with the increasing degree of nitrogen deficit conditions [191]. In the cultivation of *C. necator* ATCC 17696 using 20 g/L of glucose, the maximum PHA content obtained at the C/N ratio of 36 was 38% higher than that at a lower ratio of 3.6 (20%) [191]. However, in some cases of extremely high or low C/N ratio, such as 100 and 0.15, the PHA contents were significantly reduced from 70% to 57% and 73% to 6.7%, respectively [192, 193]. Although nitrogen-insufficient condition is beneficial for PHA synthesis, this circumstance concomitantly suppresses the bacterial growth, which partly reduces the number of microbial cells for PHA storage. Indeed, when the C/N ratio was doubled from 6 to 12, a decreasing tendency in biomass and PHA production was observed from 2.8 to 2.1 and 1.6 to 1.4 g/L, respectively (Figure 4.4). The result was consistent with the cultivation of *Rhodobacter sphaeroides* using the same C/N ratios of 6 and 12 [193].



**Figure 4.4.** Comparisons of biomass production, polyhydroxyalkanoate (PHA) accumulation, and PHA yield on biomass in two C/N ratios of 6 (a) and 12 (b). (Adapted from **Paper III**).

#### 4.3.4 The effect of type of nitrogen source

Type of nitrogen bearing compound present in a fermentation media can affect microbial performance and secondary metabolite synthesis, such as PHAs. Different kinds of nitrogen derived from both organic and inorganic sources have been studied, showing various effects on the PHA production. Overall, organic nitrogen feedstock, such as tryptone, peptone, beef extract, yeast extract, etc., provided higher PHA productivities because of the presence of extra amino acids and vitamin contents [194-197]. On the other hand, ammonium sulfate was found to be one of the most effective sources of inorganic nitrogen for PHA production. It has been tested in various concentrations of 0.01 to 20 g/L, depending on the bacterium used and initial carbon concentrations [83, 193, 195, 198, 199]. At a glucose concentration of 5 g/L, a nitrogen concentration of 0.5 g/L was proven to be optimal for the biomass production (2.1 g/L), corresponding to the highest PHA content of 1.6 g/L [198]. A similar PHA accumulation of 1.5 g/L was obtained at a higher concentration of ammonium sulfate (3 g/L) as glucose was replaced by VFAs at the same concentration of 5 g/L (**Paper III**). However, when it comes to large scale production of microbial products, such as PHAs, the availability and cost of nitrogen source are also a matter of consideration. In this regard, the use of economical nitrogen sources, such as hydrolysates of cheese whey [200], chicken feather [201], or silage juice [202] have been considered to alleviate further expenses on the overall production. Moreover, urea could be another economic choice to complete the fermentation substrate in terms of quantity and quality of nitrogen content, which not only ensures the PHA yield but also reduces the lag phase of PHA-bearing bacterial cultivation (Figure 4.5).



**Figure 4.5.** Changes in volatile fatty acid (VFA) consumption during the cultivation of *C. necator* DSM 545 in two nitrogen sources of  $(\text{NH}_4)_2\text{SO}_4$  (a) and Urea (b). (Adapted from **Paper III**).

#### 4.3.5 The effect of VFA concentration and distribution

As previously mentioned, the bioconversion of organic wastes and residuals into VFAs via acidogenic fermentation is advantageous, in terms of waste management and obtaining cost competitive PHA. Acidogenic fermentation of waste could result in the removal of inhibitory compounds and homogenize mixed organics into fermentable compounds for PHA-bearing microbial cultivation. The application of VFAs as a carbon source in the PHA production, however, still possesses drawbacks, in terms of bacterial growth due to substrate inhibition at high concentrations [203]. As observed in this thesis work (**Papers II & III**), the maximum concentration of individual VFAs that *B. megaterium* and *C. necator* could tolerate was in the range of 1.5 – 5 g/L. Moreover, at the VFA concentration of above 10 g/L, the bacterial growth was found to be hindered or completely halted, as reported by Agustín Martínez, et al. [204] and Yun, et al. [205]. In addition to substrate inhibition, the synergistic effect of VFA components is another considering hindrance of this substrate. The generation of different short-chained carboxylates creates a condition for selective and prioritized substrate consumption throughout the bacterial cultivation. In particular, butyric acid was found to be a favorable compound that is commonly consumed in prior to other VFAs [205, 206]. While the assimilation of acetic acid, on the other hand, was only conducted after the depletion of the rest [206]. This is in line with the results obtained in **Paper IV**. Furthermore, the selectivity in VFA consumption combined with the washout effect have led to the accretion of acetic acid, which ultimately resulted in a failure of semi-continuous PHA production after 80h (**Paper IV**).

#### 4.4 The effect of fermentation mode in PHA production

The cultivation of PHA-bearing microorganisms has been conducted in three common modes of batch, fed-batch, and continuous fermentation. Each fermentation strategy benefits the PHA production in different ways, which will be described in the following sections.

##### 4.4.1 Batch cultivation

In batch cultivation, the bacteria was fully provided with a rich-nutrient medium at the beginning, without further addition or removal of medium during the process [207]. After achieving desirable cell densities, the biomass was harvested for PHA extraction. Regardless of the simplicity, the productivity obtained in this mode was quite low due to the limitation in feeding concentration. Both the carbon and nitrogen contents were restricted at the typical concentration of 1 – 30 and 0.2 – 5 g/L, respectively, due to the consideration of substrate inhibition [208]. This, in turn, resulted in the low PHA productivity of 0.14 – 0.17 g/L.h in batch cultivations of *C. daeguensis* TAD<sub>1</sub>, *C. necator* H16, and *A. australica* DSM 1124 [209-211]. The low efficiency could be severe in the case of VFAs, as the bacterial tolerance to these compounds is much lower compared to sugar-based substrates. In a batch cultivation of *C. necator* using VFAs (5 g/L), the PHA productivity obtained was only around 0.015 g PHA/ g biomass (**Paper III**). This result was in line with the study by Yun, et al. [205], who conducted the cultivation of *R. eutropha* KCTC 2658 using the same VFA concentration of 5 g/L in batch mode. Therefore, the batch cultivation is further developed into the sequence batch cultivation to remedy the competition in carbon content between two phases of biomass production and PHA accumulation. Hence, the processes of biomass production and PHA synthesis are optimized and performed separately in two or more reactors to increase the process efficiency [212, 213].

##### 4.4.2 Fed-batch cultivation

In biotechnological processes, fed-batch cultivations are the most common strategy, in which a pulse feeding is applied to maintain the nutrient contents in desirable concentrations throughout the cultivation [214]. In PHA production, both carbon and nitrogen sources are supplied at time intervals, based on the specific growth rate and nutrient consumptions of the bacteria [215]. Such conditions significantly increase cell densities of up to 159 and 221 g/L with the PHA yield of 51 and 45% in the cultivation of *P. putida* and *B. sacchari*, respectively [216, 217]. Higher PHA productivities of 1.91 and 1.44 g/L.h were also achieved in other fed-batch cultivations of *P. putida* KT2240 growing on octanoic and nonanoic acid, respectively [216, 218]. However, fed-batch cultivation could face a drawback of concomitant dilution in fermentation substrates because of the gradual increase in the fermentation volume from the addition of the feeding solution [211]. Therefore, concentrated substrates of up to 100 g/L are recommended to cope with the dilution rate [211]. However, the substrate concentration in organic

residue streams was relatively low, at around 40 – 50 g/L, which requires further processes such as evaporation or ultrafiltration to concentrate the medium [219]. These extra steps, which are energy and time consuming, could greatly intensify the upstream processing stage, increasing the overall cost of PHA production.

#### 4.4.3 Continuous production

Compared to batch and fed-batch cultivations, continuous production of PHA was conducted in a steady condition, in which the fermentation medium is continuously added together with the removal of the same amount to maintain the bioconversion rate of the active biomass [220]. Consequently, the efficiency of the continuous processes is defined by a decisive parameter of the dilution rate ( $D$ ), which is described by the ratio of the flow rate and working volume. This parameter should be harmonized with the specific growth rate ( $\mu$ ) to maintain the productive cultivation. Too low or too high  $D$  values will result in an insufficient supply of substrates or washout, which negatively influences the overall productivities [211]. An example of a continuous process was executed on the cultivation of *C. necator* using a one-stage setup, providing a PHA yield on biomass of 0.3 g/g at a  $D$  of 0.15 1/h [221]. In order to obtain higher productivity, continuous processes are also upgraded into two-stage setups to optimize the two fractions of biomass concentration and intracellular PHA mass separately [211]. The two-stage continuous processes were conducted for the first time in the same study by Ramsay, et al. [221], in which the PHA fraction in biomass, at the same  $D$  of 0.15 1/h, was significantly increased to 0.5 g/g. The role of the dilution rate was demonstrated in a study by Du, et al. [222], where a higher PHA productivity of 1.43 g/L.h was achieved at an overall  $D$  of 0.12 1/h compared to that of 1.24 g/L.h in a lower  $D$  of 0.075 1/h. Dilution rate, furthermore, could be optimized for each phase of the microbial growth (0.21 1/h) and PHA biosynthesis (0.16 1/h) to achieve the maximum volumetric productivity of 1.06 g/L in the cultivation of *P. putida* GPo1 [223]. Regardless of the consistent production of PHAs, in terms of both quantities and qualities, the long lasting continuous cultivation could face issues related to contamination and changes in bacterial genetics [224]. Thus, the use of extremophile organisms, such as *Halomonas* spp or MMCs, which are less demanding on sterilized conditions, could be suitable choices for exploiting the benefits of continuous PHA production. For example, a continuous cultivation of *Halomonas campaniensis* LS21 was conducted with non-sterile substrate mimicking kitchen waste, yielding a productivity of 0.7 g PHA/g biomass throughout the process [225]. With regard to MMCs, an excellent PHA production capacity of 0.85 g/g biomass was obtained in a study by Marang, et al. [226], who conducted the cultivation of enriched PHA-accumulating bacteria in two sequencing batch reactors using continuous substrate feeding.

#### 4.5 Semi-continuous production of PHAs using immersed membrane bioreactor

Membrane and membrane technology have been extensively applied in many biotechnological processes to assist in the recovery of high-value metabolites without interfering with the bio-process. The use of membrane,



furthermore, can enhance the volumetric productivity of the process by facilitating the fermentation at a high dilution rate and cell concentration [227]. In this thesis, an integration of membrane bioreactors in PHA production was introduced as a novel attempt to address the washout effect in semi-continuous fermentation, remediating the low productivity in batch PHA production using feed with high VFA concentrations.

#### 4.5.1 Membrane technology and membrane bioreactors

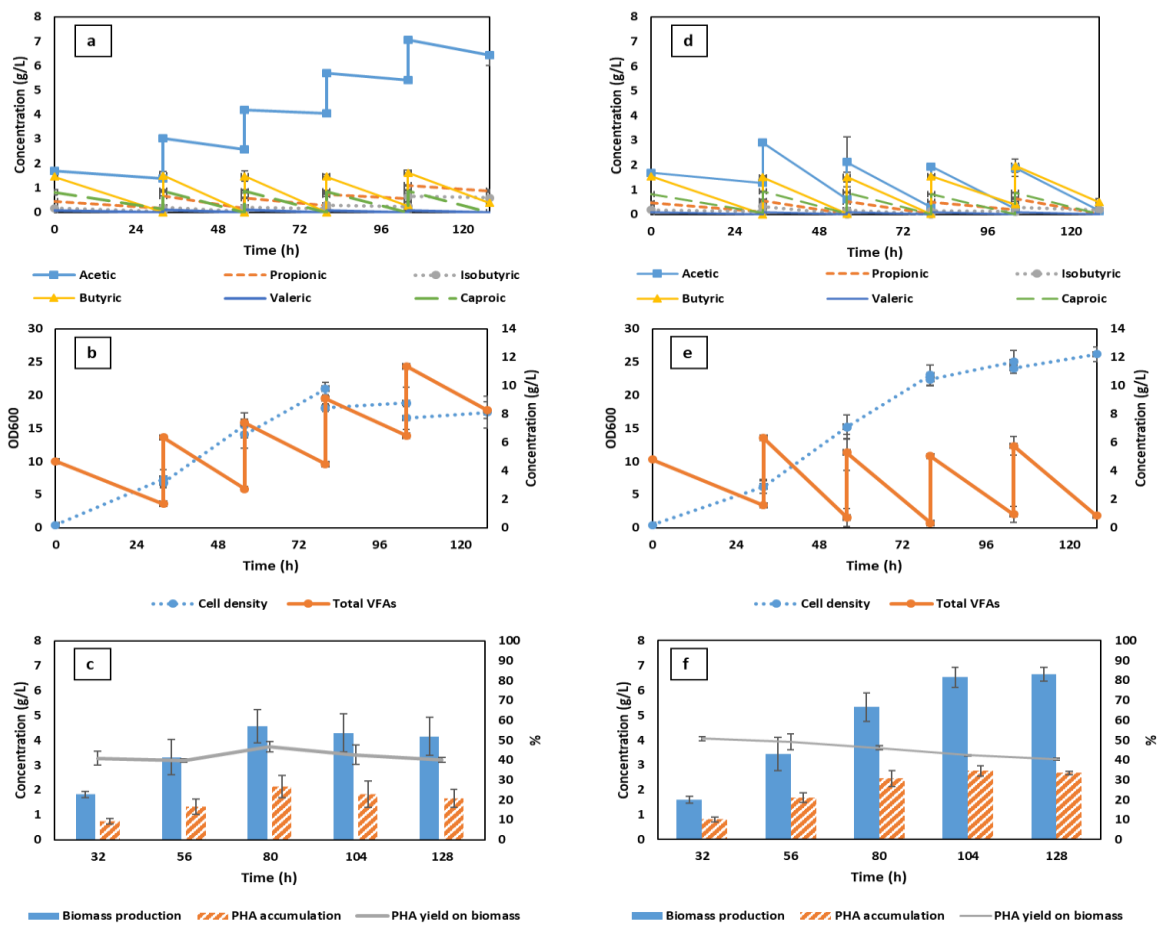
In biological processes, membranes have been defined as discontinuous interfaces, allowing for selective permeation of specific components based on their physical and/or chemical properties [228]. The permeation process is driven by the differences in pressure, concentration, temperature, or electrical potential between two phases over the membrane surface [229]. In general, synthetic membranes can be made of different materials, such as ceramics, metals, polymers, rendering various formations of symmetric or asymmetric, and homogenous or heterogeneous [230]. Depending on the pore size, pressure-driven membranes can be divided into different groups of macro- (10  $\mu\text{m}$  – 50 nm), meso- (2 – 50 nm), micro- (0.1 – 2 nm), and non-porous membranes corresponding to microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO), respectively [230]. Moreover, membranes can be further classified into three major groups based on their configurations: flat sheet (FS), tubular (TB), and hollow fiber (HF).

Depending on the ultimate purposes of the biological process, different membrane modules and their configurations have been integrated into bioreactors to optimize the synergistic effect of selective separation and bio-chemical reactions [231]. In general, membrane modules can be placed either internally or externally to the bioreactors, resulting in two typical types of MBR configuration of the immersed membrane bioreactor (iMBR) and side-stream membrane bioreactors. Due to their outstanding separation abilities, membranes with different modules and configurations have been applied in diverse industrial applications such as production of beverage, dairy, food, pharmaceuticals, and water and wastewater treatment [232-234].

#### 4.5.2 Immersed membrane bioreactor for semi-continuous production of PHAs

Microbial washout is a critical issue in semi-continuous PHA production (**Paper IV**). The microbial loss during interval feedings reduces the capacity of VFA assimilation in the system, resulting in an accumulation of acetic acid, which ultimately halted the fermentation (Figure 4.6a). In this regard, the presence of high cell density in the fermentation system could be a solution, in which the bioconversion rate can be improved, boosting the assimilation of VFAs [235]. With the great ability in cell retention, an immersed MBR was applied to eliminate the washout effect in semi-continuous fermentation, providing high cell density to increase the bacterial tolerance at high VFA concentration. Considering the average diameter of *C. necator* (0.4 – 0.6  $\mu\text{m}$ ), a flat sheet membrane with an average pore size of 0.3  $\mu\text{m}$  was selected for an MBR for the PHA production (**Paper IV**).

The comparison of semi-continuous production of PHAs in CSTR and iMBR is presented in Figure 4.6. With the assistance of iMBR, the cell densities could be increased over time, reaching a peak of 26 (OD600) at the end of the cultivation (Figure 4.6e). This amount was 1.25 times higher than that obtained in the semi-continuous reactor without membrane (Figure 4.6b). The cell concentrations in CSTR, furthermore, started to decrease after 80h due to the washout effect, leading to the aggregation of acetic acid. On the other hand, the VFA assimilation in the iMBR benefits from the higher cell densities, resulting in the presence of low VFA concentration during the process. In this way, the fermentation could be extended for up to 128 h, yielding a maximum biomass and PHA accumulation of 6.6 and 2.8 g/L, respectively (Figure 4.6f). Afterwards, the VFA concentration in the following feeding cycles became insufficient to support further bacterial growth.



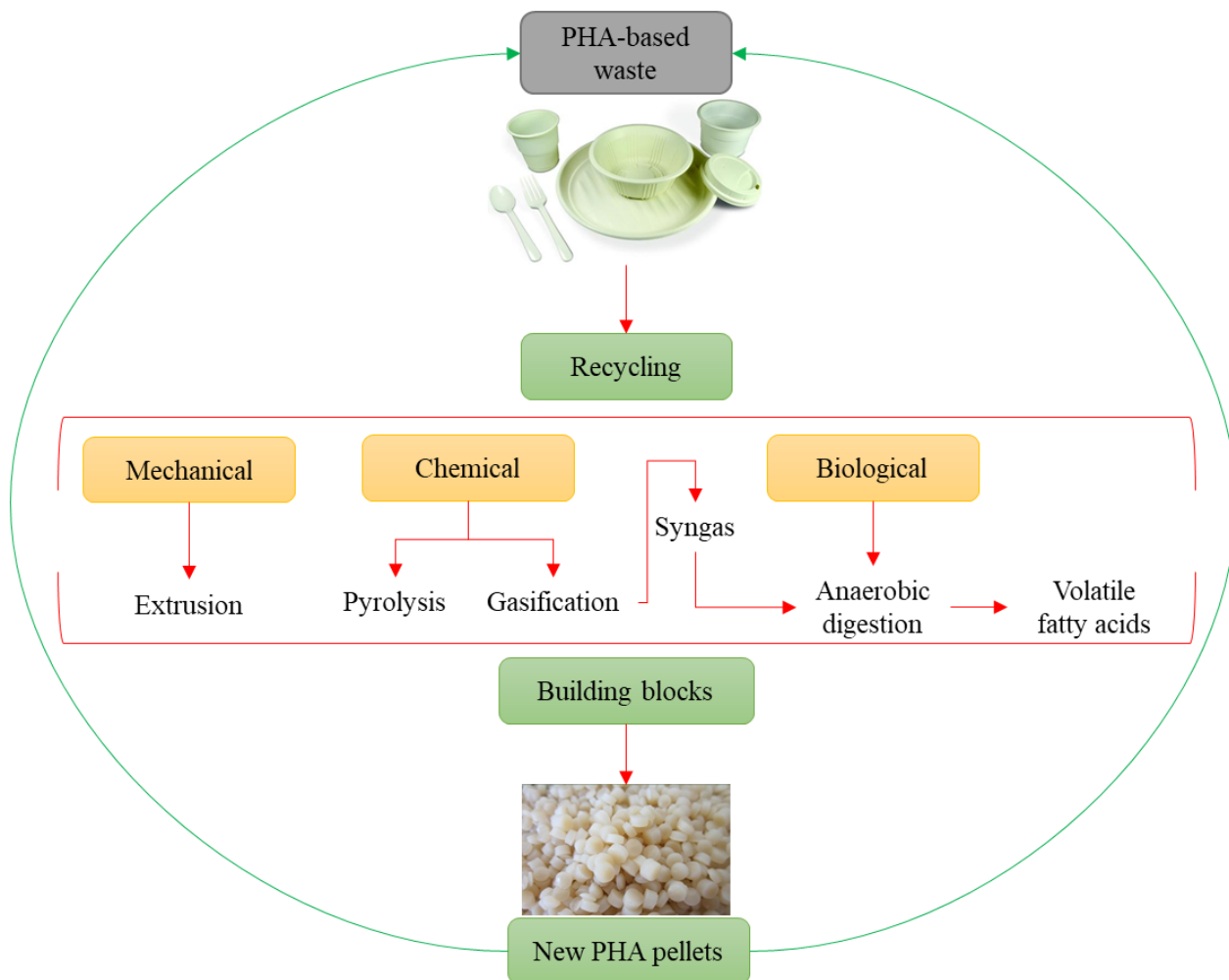
**Figure 4.6.** Comparison of semi-continuous production of polyhydroxyalkanoate (PHA) by *C. necator* DSM 545 using synthetic volatile fatty acids (VFAs) in continuous stirred-tank reactor (CSTR) and immersed membrane bioreactor (iMBR), in terms of VFA composition (a and d), total VFA concentration and cell density (b and e), and biomass production and PHA accumulation (c and f) (Adapted from Paper IV).

## **BIOLOGICAL RECYCLING OF POLYHYDROXYALKANOATE-BASED MATERIALS**

Considering the renewable character and mechanical performance, great attention has been drawn to PHAs with an increase in both domestic and industrial applications. Regardless of the biodegradability, PHA-based products could face the same current scenario of conventional plastic wastes. The production of PHAs is predicted to increase exponentially, with the main use being single-use products, such as bottles, trays, and packing materials. Therefore, a competent recycling method is essential to prevent the potential mismanagement and accumulation of PHA-based product wastes. This chapter presents different methods for recycling PHAs, with the focus on bioconversion approaches to comply with the sustainable circular bioeconomy concept.

### **5.1 Recycling PHA-based materials**

PHA-based products are superior to conventional plastics as they are biodegradable in nature and can be produced from renewable materials. PHA-based wastes can be recycled and be valorized into its building blocks for the production of either original precursors or new products, whereby, reducing the feedstock use and offsetting the overall production costs [236]. Similar to conventional plastics, PHA-based products can be recycled by conventional mechanical, chemical, and biological techniques. The advantages of each recycling approach are outlined in **Paper I**, where their potential contributions to closing the loop on PHA processing and recycling are presented (Figure 5.1).



**Figure 5.1.** Corresponding dominant methods in each recycling method for a closed-loop process of polyhydroxyalkanoate (PHA) recycling (**Paper I**).

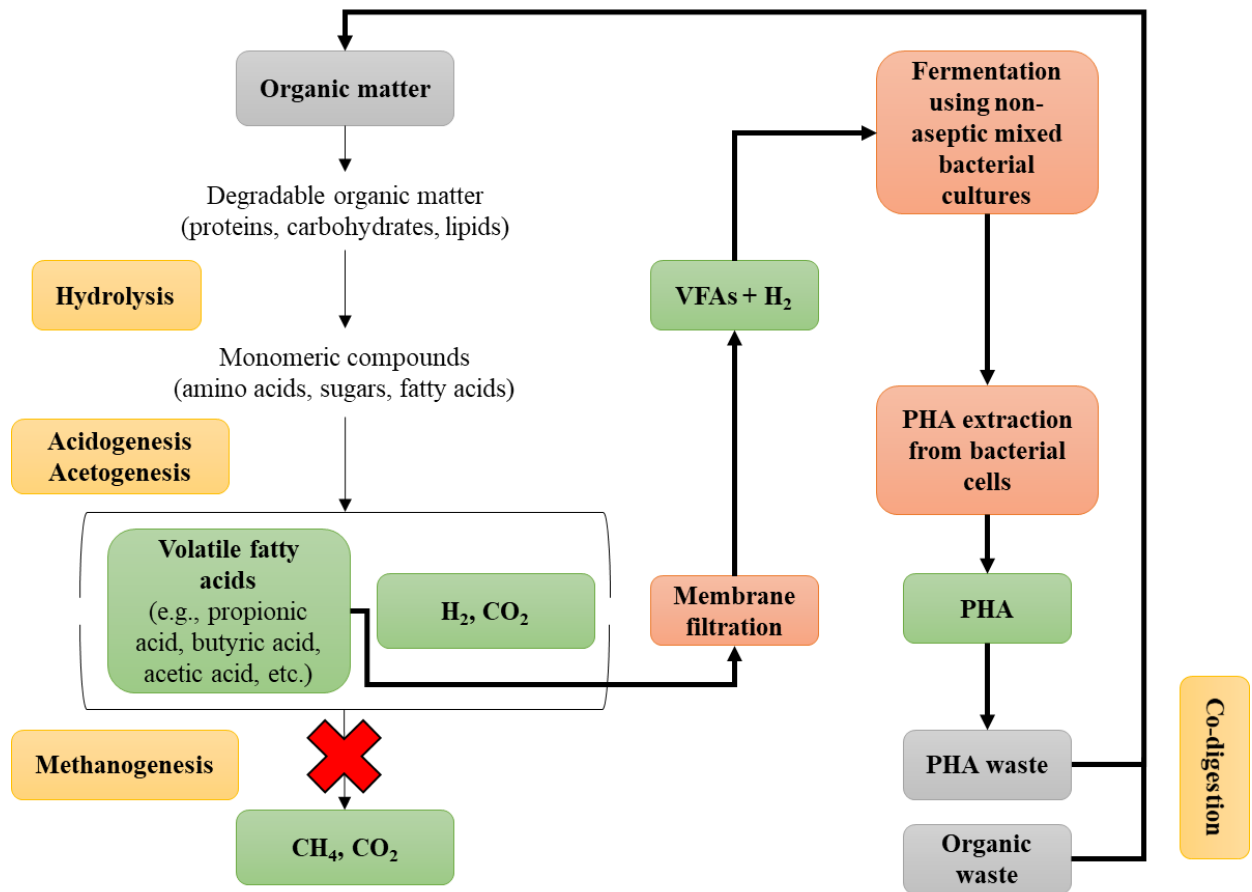
PHA-based wastes can be converted into original materials or blends by mechanical recycling for use as raw materials or additives in the production of the same or different products [237]. Among PHAs, PHB is the most commonly produced polymer; however, recycling PHB using a mechanical method is deemed challenging [238, 239]. On the other hand, PHA blends and co-polymers present positive results, which can be recycled up to 5 – 6 re-processing of extrusion or/and compression molding with a slight reduction in the mechanical properties [240, 241]. PHA-based products, however, are expected to exist in a heterogeneous form with other kinds of solid wastes, requiring an additional costly step of separation, which subsequently limits the use of mechanical recycling on a large scale. In this regard, chemical recycling appears to be a preferable approach to depolymerization and refining as the dominant options [242]. In chemical recycling, thermal degradation through pyrolysis has been studied the most, which can be applied to both pure PHB and PHB blends, providing crotonic acid and crotonate end-group compounds as the main final products. Those secondary products can be used for

the production of poly(crotonic acid) and its co-polymer (e.g., vinyl acetate) [243]. Furthermore, the generated chemicals are potential platforms for the production of valuable compounds, such as dental cement, cosmetic products, herbicides, and plasticizers [244].

Biodegradability is one of the most fascinating characteristics of PHAs. Biological recycling, therefore, appears to be a suitable method for utilizing the outstanding intrinsic property of this material. PHA decomposition, moreover, depends, to a high extent, on the activities of microbial communities. The microbial community used for biodegradation is greatly affected by important factors, such as polymer characteristics and environmental conditions. In general, the biodegradation of PHAs can be conducted in both aerobic [245] and anaerobic conditions [33], releasing CO<sub>2</sub>, H<sub>2</sub>O, and CH<sub>4</sub> as the final products. Composting could be used to recycle PHA-based products into fertilizer. However, most of the carbon is converted into CO<sub>2</sub> by this method. While energy recovery also converts the material into CO<sub>2</sub>, the energy can be recovered. Recently, acidogenic fermentation has become a dominant approach for recycling biodegradable materials. This approach produces VFAs as intermediates, which can channel the whole process into PHA production, fitting the circular concept proposed in this thesis.

## **5.2 Acidogenic fermentation of PHA-based composites**

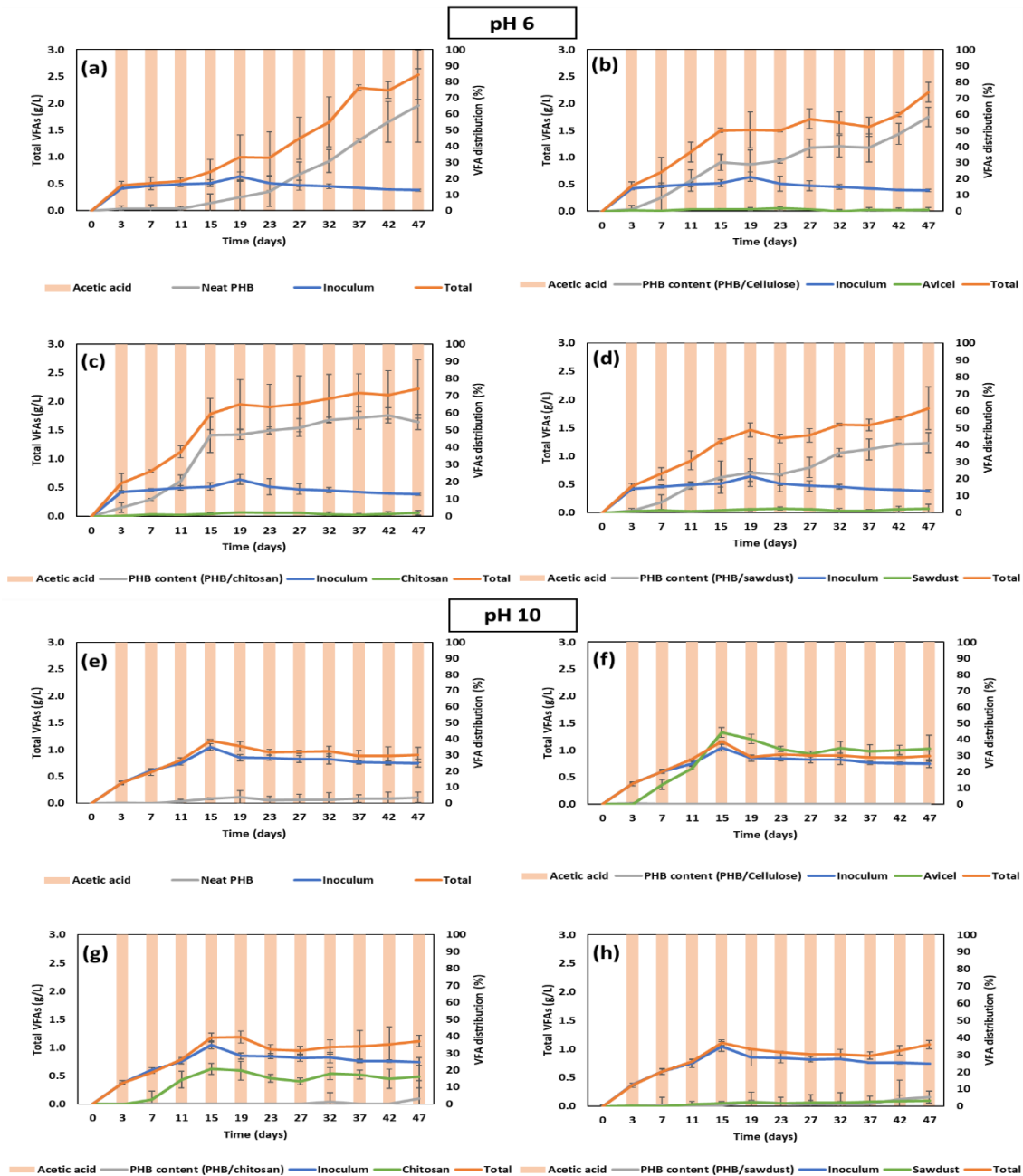
Bioconversion through anaerobic digestion (AD) is the preferable method for recycling PHAs. PHAs can be co-digested with the organic fraction from MSW for biogas production. The biogas obtained afterwards can be utilized in various applications, such as biofuels in transportation, cooking, electricity, and heat [246]. However, attention has recently been drawn to the production of VFAs, which can be obtained from the acidogenic fermentation as a result of hindrance in the last stage of the anaerobic digestion [247]. Comprehensive studies have proven the benefits of VFAs, as potential platform chemicals for the production of various higher value-added bio-products [248]. VFAs have been well discussed in Chapters 3 and 4 as an economical feedstock for the sustainable production of PHAs. Therefore, valorization of PHA-based products into VFAs not only increases the economic appeal of biological waste treatment but also supplies sustainable, low-cost, renewable precursors for re-production of PHAs (Figure 5.2).



**Figure 5.2.** Circular process of the polyhydroxyalkanoate (PHA) production by utilizing volatile fatty acids (VFAs) derived from acidogenic fermentation of PHA-based wastes.

Under acidogenic fermentation, PHAs will be extracellularly degraded by the microbial activity in the sludge. Overall, the PHA degradation is initially conducted with the hydrolysis process, at which time the bacteria attach to the material surfaces to facilitate the secretion of depolymerases and oligomer hydrolase, converting PHAs into corresponding monomers [249]. The hydrolysis stage, however, is a time-consuming process with a lag phase of around 11 days (**Paper V**). This is congruent with the results obtained in the study by Shin, et al. [250], where the biodegradation of 89% PHBV only accelerated after the first 10 days under anaerobic digestion. In this regard, the presence of natural fillers included in the PHA composites was found to shorten the lag phase to 3 days (Figures 5.3b,c,d). After the stage of hydrolysis, the monomeric components are oxidized into acetoacetyl-CoA, which is ultimately converted into acetyl-CoA by the support of  $\beta$ -ketothiolase [251]. Under anaerobic conditions, acetyl-CoA is the building block for the production of acetic and butyric acid [105]. Therefore, those VFAs were the main products obtained in the anaerobic digestion of accumulated PHA in the activated sludge [252]. The accumulation of VFAs, furthermore, resulted in a pH drop from 8 to below 6 in the same study. The same behavior was also observed in **Paper V**; however, acetic acid was the only product obtained in this study.

The changes in pH, moreover, potentially affected the activities of the microbial enzyme systems, inversely influencing the biodegradation [253, 254]. This concern is reinforced by the higher VFA yield obtained in a study by Zhang, et al. [255], in which the pH levels were controlled throughout the anaerobic digestion. Moreover, the initial pH values were also found to have a critical impact on the PHA biodegradation of PHAs, in which the PHA products were highly degraded at pH 6 compared to an alkaline level of 10 (**Paper V**).



**Figure 5.3.** Comparisons of the changes in the volatile fatty acid (VFA) production during acidogenic fermentation of neat polyhydroxybutyrate (PHB) (a) vs (e) and PHB composites of PHB/cellulose (b) vs. (f), PHB/chitosan (c) vs. (g) and PHB/sawdust (d) vs. (h) at two pH levels of 6 and 10.





## CONCLUDING REMARKS AND FUTURE SUGGESTIONS

### 6.1 Conclusions

The results in this thesis have presented a possibility of producing PHAs in a close-loop cycle by using food waste-originated VFAs as a key carbon source, fully complying with the concept of circular economy. The general conclusions can be drawn as follows:

- Waste-based VFAs are potential carbon feedstock to enhance the economic feasibility of the PHA production.
- With PHAs as the core intermediates, PHA production can be integrated into the biorefinery concept, in which the organic waste can be utilized to obtain cost-competitive PHA, increasing the sufficiency in the use of resources and addressing issues related to conventional plastics.
- Food waste-derived VFAs can be used directly for PHA-bearing microorganisms, yielding a maximum PHA yield on biomass of 9 – 55%. Acetic and butyric acid were favorable compounds, while the assimilation of other VFAs was highly dependent on the capacity of individual bacteria. As cultivated in mixture VFAs, the consumption of butyric acid was dominant, while acetic acid was used at the end after the depletion of other VFAs.
- Under optimal conditions, the maximum biomass yield on real VFA effluent (5 g/L) can be up to 72%, providing maximum biomass and PHA concentration of 2.26 and 1.06 g/L, respectively.
- With the assistance of iMBR, substrate inhibition and washout effect were addressed, leading to a success in the PHA production using semi-continuous mode. After 128h of cultivation, a maximum PHA content of 2.8 g/L was obtained. The application of iMBR in this thesis proved to be a potential

approach that can be utilized in different strategies of PHA production without intensifying the overall process.

- PHA-based composites can be biologically recycled by acidogenic fermentation. pH 6 was a favorable condition for PHA degradation, yielding a maximum VFA production of 2 g/L. At the same pH level, the addition of natural fillers significantly improved the bio-composite's overall deterioration. While at alkaline pH of 10, the VFA recovery was obtained based on the degradation of natural fillers. The recovered VFA can be recirculated into the PHA production, fulfilling the concept of bioeconomy.

## **6.2 Future directions**

Based on the results achieved in this thesis, the following research works are proposed to approach closer to the use of food waste based VFAs and iMBRs into PHAs production in industrial scale.

- An effect of higher VFAs concentrations (above 10 g/L) on PHA-storing bacteria cultivation should be performed.
- Optimization of biomass and PHA in relation to yield and quality from food waste-derived VFAs in scaled up bioreactors.
- Techno-economic analysis of the iMBR application in semi-continuous production of PHA should be performed.
- The application of iMBR should be further studied in PHA production, using one-stage cultivation for growth associated PHA producers and two-stage cultivation for non-growth associated PHA producers.
- A profound study of the fermentation factors in acidogenic fermentation should be performed in order to optimize the VFA production from the degradation of PHA-based products.
- Bio-compatilizer or surface modification can be added to improve the interfacial adhesion of PHA and natural fillers, whereby improving the tensile strength and maintaining the biodegradation of the composites.

## Acknowledgements

During the Ph.D. study, I have received a lot of company and support from many people that have helped me to accomplish this thesis. Here, I would like to express my appreciation to all of them.

I would like to start with my most sincere gratitude to my main supervisor, Dan Åkesson, for his expert guidance and mentorship throughout the Ph.D. study.

I am grateful to my former co-supervisor, Jorge Ferreira, for introducing me to the field of biotechnology. It is unquestionable that you are one of the very first to put the first brick on my journey. Thank you for your patience, encouragement, and guidance to make the start of my Ph.D. easier.

I would like to express my greatest thankfulness to my co-supervisor, Amir Mahboubi, for his guidance and support during my up and down Ph.D. days. Thank you for all the constructive discussions, encouragement, and trust, which has made me improve in many aspects. I have learned a lot from you.

I would like to express my appreciation to my thesis examiner, Mohammad Taherzadeh, for his guidance through the ISP meeting and keeping me on track in my research work. I am also thankful to Tomas Wahnström and Ilona Sárvári Horváth for your support, and for providing me with the best conditions during my study. I gratefully acknowledge all the efforts from our devoted administrative colleagues, Louise Holmgren, Jonas Edberg, Pernille Sjölyst Lunde, and Lina Färm. I would like to thank all of my previous teachers and supervisors at the University of Borås for all the things they have taught me.

Furthermore, I would like to sincerely thank the laboratory supervisors, Thomas Södergren, Jonas Hansson, Marlen Kilberg, Kristina Laurila, Ville Skrifvars, and Andreas Nordin, for your invaluable assistance during my lab work.

Also, I would like to thank all my friends in Borås: Amir, Abas, Andreas, Clarisse, David, Ebo, Faranak, Ghasem, Gulru, Hanieh, Jason, Jorge, Kanishka, Kamran, Kin, Kostas, Madumita, Mahdi, Mahtab, Mohsen, Mohsen B, Milad, Naemieh, Najmeh, Neda, Negar, Pedro, Pooria, Ricky, Raouf. Sabrina, Shahab, Samira, Sajjad, Steven, Sunil, Supri, Taner, Tugba, Umarin, and Vahid. I am so blessed to have you here to make my years of studies and research memorable.

I cannot be more thankful to my dearest friend, Linh, for enduring my nagging and continuing to support me. I also want to thank all of my friends at home: Nhung, Ngáo nì, Janie, Ngọc, Nga, Tú, Trang, Hiếu, Nguyễn, Bảo, Dương, Anh, Dung nhi, Luna, and Phuong, for being on my side during my sunny and rainy Ph.D. days, with loads of patience and kindness.

To my wonderful family, no words can describe how thankful I am to everyone. I could not have come this far without your love and support. I believe I can keep going further when I have everyone here to get my back.

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