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Short and medium chain fatty acids as carbon sources for polyhydroxyalkanoates production

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Krótko- i średniołańcuchowe kwasy tłuszczowe jako źródła węgla do produkcji polihydroksykwasów

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4.3. Publication 3. Szacherska K., Moraczewski K., Czaplicki S., Oleskowicz-Popiel P., Mozejko-Ciesielska J. 2022. Conversion of Short and Medium Chain Fatty Acids into Novel Polyhydroxyalkanoates Copolymers by *Aeromonas* sp. AC_01. Materials. 15, 4482. DOI: 10.3390/ma15134482. 26

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The doctoral dissertation "Short and medium chain fatty acids as carbon sources for polyhydroxyalkanoates production" consists of four articles published in 2021 and 2022:

I. **Szacherska K**., Oleskowicz-Popiel P., Ciesielski S., Mozejko-Ciesielska J. 2021. Volatile Fatty Acids as Carbon Sources for Polyhydroxyalkanoates Production. *Polymers*. 13, 321. DOI: 10.3390/polym13030321.

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[1]IF - Impact Factor of the journal in the year of publication
[2]MEiN - Number of points based on the announcement of the Minister of Education and Science on the list of scientific journals of December 2021

Abbreviations list

PHAs – Polyhydroxyalkanoates
PHA_{SCL} – Short-chain-length polyhydroxyalkanoates
PHA_{MCL} – Medium-chain-length polyhydroxyalkanoates
PHA_{LCL} – Long-chain-length polyhydroxyalkanoates
SMCFAs – Short and medium chain fatty acids
AD – Anaerobic digestion
CDM – Cell dry mass
MCF – Mixed culture fermentation
MMCs – Mixed microbial cultures
HAc – Acetic acid
HPr – Propionic acid
HBu – Butyric acid
HVa – Valeric acid
HCa – Caproic acid
Nd – Not determined
HPLC – High-performance liquid chromatography
μ_{max} – Maximum specific growth rate
P(3HB-*co*-3HV) – Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer
P(3HB) – Poly-3-hydroxybutyrate
3HV – 3-hydroxyvalerate
3HHx – 3-hydroxyhexanoate
3HDD – 3-hydroxydodecanoate
3HO – 3-hydroxyoctanoate
3HD – 3-hydroxydecaonate
DSC – Differential scanning calorimetry
T_g – Transition temperature
T_c – Crystallization temperature
T_m – Melting point
T_G – Thermogravimetry
T_{cc} – Cold crystallization temperature
ΔH_c – Change in enthalpy of the crystallization process
ΔH_{cc} – Change in enthalpy of the cold crystallization process
ΔH_m – Change in enthalpy melting process
X_c – Degree of crystallinity

ABSTRACT

The doctoral dissertation consists of four publications, one of which is a review article and three of which are original research articles. Biopolymers have attracted much attention in the last few decades as environmentally friendly alternatives to petrochemical polymers. Due to the unique material properties polyhydroxyalkanoates (PHAs) are particularly attractive. They are produced in biotechnological processes by various microorganisms as carbon and energy reserves under unbalanced growth conditions. PHAs have great potential in industry, nevertheless their production on a large scale is still limited due to the high production costs. Despite the fact that the fermentation technology and polymer extraction process have been improved over the last years, the microbial PHA production is still not economically feasible. Therefore, there is a growing need to develop novel microbial processes and to search for an efficient PHA producer capable of synthesizing these biopolymers using renewable carbon sources. In the literature there are many scientific reports on the microbial production of PHAs, however none of them describes the microbial production of unique PHAs from SMCFAs generated in the anaerobic mixed culture fermentation of acid whey. Also, there was a lack of studies that evaluated in details properties of the biopolyesters extracted in the cultivations with SMCFAs to assess their application potential. Therefore, the aim of the doctoral dissertation was to investigate the use of synthetic and acid whey derived short and medium chain fatty acids (SMCFAs) as feedstocks for PHA production by bacteria belonging to three different genera: *Paracoccus* sp., *Aeromonas* sp. and *Pseudomonas* sp..

The results revealed that all tested bacterial strains were able to grow and synthesize PHAs using individual synthetic SMCFAs, those derived from acidogenic anaerobic mixed culture fermentation or their synthetic mixtures as carbon sources in the growth media. Furthermore, the results indicated that the PHA content in bacterial cells depended on the type and concentration of the substrate, and applied culture conditions. The replacement of the individual SMCFAs by the SMCFAs-rich residual stream was found to be less beneficial for PHA productivity compared to the single, synthetic SMCFAs that suggested more inhibitory effect of waste-derived fatty acids. In the cultivation of *Paracoccus homiensis* quite low PHA productivity could be explained by the presence of butyric acid as the dominant component in the SMCFA-rich stream which as a individual fatty acid in the culture medium resulted in the lowest PHA production efficiency compared with others. Interestingly, a mixture of acetic acid and butyric acid supported better PHA synthesis and accumulation than individual acids in the cultivation of *Aeromonas* sp. AC_01. Moreover, unbalanced growth conditions positively

affected the PHA productivity. In the fermentation process of *Pseudomonas* sp. GL01 PHA productivity reached up to 4.5 mg/L·h when the ammonium was exhausted in the culture medium. Importantly, the monomeric composition of the extracted PHAs depended on the culture conditions used. The data showed that SMCFAs-rich stream used in the cultivation of *Paracoccus homiensis* could support the synthesis and accumulation of the P(3HB-co-3HV) copolymer. However, the 3HV content was low, therefore the induction of the acidogenic fermentation to produce more short and medium chain fatty acids with an odd carbon number has been suggested. Furthermore, *Aeromonas* sp. AC_01 cultured on the same carbon source was able to produce novel short- and medium-chain length (scl-mcl) copolymers of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), 3-hydroxytridecanoate (3HtriD) and/or 3-hydroxytetradecaonate (3HTD) with high 3HB and 3HV fractions. Also, *Pseudomonas* sp. GL01 showed the tendency to produce copolymers but with high amounts of 3-hydroxyhexanoate (3HHx) and 3-hydroxydodecanoate (3HDD) as the main monomers and lower content of 3-hydroxybutyrate (3HB), 3-hydroxyoctanoate (3HO), and 3-hydroxydecaonate (3HD). Furthermore, a differential scanning calorimetric, thermogravimetric and water contact angle analysis proved that the extracted biopolymers have useful properties. The melting temperature of the scl-mcl copolymer extracted from the *Pseudomonas* sp. GL01 was the lowest and unusual indicating its amorphous behavior and flexibility at ambient temperature. Also, the presence of the 3HB fraction could have caused its lower thermal resistance. The biopolyesters isolated from *Paracoccus homiensis* and *Aeromonas* sp. AC_01 cells possessed high thermal stability which is beneficial taking into account their processing technology. The obtained data are promising in the perspective of developing novel process of utilizing high SMCFAs content waste-derived streams without necessity for substrate pretreatment for production of value-added PHA copolymers.

STRESZCZENIE

Rozprawa doktorska składa się z czterech publikacji, wśród których jedna jest artykułem przeglądowym, a trzy to oryginalne prace badawcze. W ostatnich kilkudziesięciu latach biopolimery przyciągnęły wiele uwagi jako przyjazne środowisku substytuty polimerów pochodzenia petrochemicznego. Polihydroksykwasy (PHA) są szczególnie atrakcyjne ze względu na unikalne właściwości materiałowe. Są produkowane w procesach biotechnologicznych przez różne mikroorganizmy jako źródła węgla i energii w niezrównoważonych warunkach wzrostu. PHA mają duży potencjał w przemyśle, jednak ich produkcja na dużą skalę jest nadal ograniczona ze względu na wysokie koszty produkcji. Pomimo, iż technologia fermentacji i proces ekstrakcji polimerów zostały udoskonalone w ciągu ostatnich lat, mikrobiologiczna produkcja PHA nadal nie jest ekonomiczna. Dlatego też istnieje rosnąca potrzeba opracowania nowatorskiego procesu mikrobiologicznego oraz poszukiwania wydajnego producenta PHA zdolnego do syntezy tych biopolimerów z wykorzystaniem odnawialnych źródeł węgla. W literaturze można znaleźć wiele doniesień naukowych dotyczących mikrobiologicznej produkcji PHA, jednak w żadnym z nich nie opisano produkcji unikalnych PHA z SMCFA generowanych w beztlenowej fermentacji z wykorzystaniem kwaśniej serwatki. Brakuje również badań szczegółowo oceniających właściwości biopoliestrów wyekstrahowanych z hodowli z SMCFA w celu oceny ich potencjału aplikacyjnego. Dlatego też, celem rozprawy doktorskiej było zbadanie możliwości wykorzystania syntetycznych i pochodzących z kwaśniej serwatki krótko i średnio łańcuchowych kwasów tłuszczowych (SMCFA) jako źródeł węgla do produkcji PHA przez bakterie należące do trzech różnych rodzajów: *Paracoccus* sp., *Aeromonas* sp. i *Pseudomonas* sp..

Wyniki badań wykazały, że wszystkie badane mikroorganizmy były zdolne do wzrostu i syntezy PHA z wykorzystaniem pojedynczych, syntetycznych SMCFA, tych pochodzących z beztlenowej fermentacji mieszanych kultur drobnoustrojów lub ich syntetycznej mieszaniny jako źródeł węgla. Ponadto, uzyskane wyniki potwierdziły, że zawartość PHA w komórkach bakterii zależała od rodzaju i stężenia substratu oraz stosowanych warunków hodowli. Zastąpienie poszczególnych SMCFA przez te pochodzące z wykorzystaniem produktów odpadowych okazało się być mniej korzystne dla produktywności PHA w porównaniu z pojedynczymi, syntetycznymi SMCFA, co sugeruje hamujące działanie kwasów tłuszczowych pochodzących z procesu beztlenowej fermentacji. W hodowli *Paracoccus homiensis* stosunkowo niska wydajność PHA może wynikać z obecności kwasu masłowego jako

dominującego komponentu w strumieniu SMCFA, który stosowany jako jedyne źródło węgla w podłożu hodowlanym wpłynął na najniższą wydajność produkcji PHA w porównaniu z innymi badanymi kwasami. Co ciekawe, w hodowli *Aeromonas* sp. AC_01 mieszanina kwasu octowego i masłowego sprzyjała lepszej syntezie i kumulacji PHA aniżeli te kwasy stosowane osobno. Ponadto, niezrównoważone warunki wzrostu pozytywnie wpływały na produktywność PHA. W procesie fermentacji *Pseudomonas* sp. GL01 produktywność PHA osiągnęła 4.5 mg/L·h po wykorzystaniu azotu w podłożu hodowlanym. Co ważne, skład monomeryczny wyekstrahowanych PHA zależał od stosowanych warunków hodowli. Dane wykazały, że odpadowe SMCFA zastosowane w hodowli *Paracoccus homiensis* wpłynęły na syntezę i kumulację kopolimeru P(3HB-co-3HV). Jednakże zawartość 3HV była niska, dlatego zasugerowano indukcję fermentacji w kierunku produkcji wyższego stężenia krótko- i średniołańcuchowych kwasów tłuszczowych o nieparzystej liczbie atomów węgla. Ponadto, *Aeromonas* sp. AC_01 hodowany na tym samym źródle węgla był zdolny do syntezy nowatorskich kopolimerów scl-mcl składających się z 3-hydroksymaślanu (3HB), 3-hydroksywalerianu (3HV), 3-hydroksytridekanianu (3HtriD) i/lub 3-hydroksytetradekanianu (3HTD) o wysokiej zawartości frakcji 3HB i 3HV. Również *Pseudomonas* sp. GL01 wykazywał tendencję do wytwarzania kopolimerów, z dużą zawartością 3-hydroksyheksanianu (3HHx) i 3-hydroksydodekanianu (3HDD) jako głównych monomerów oraz niższym stężeniem 3-hydroksymaślanu (3HB), 3-hydroksyoktanianu (3HO) i 3-hydroksydekanianu (3HD). Ponadto, analizy różnicowej kalorymetrii skaningowej, termograwimetryczne i kątów zwilżenia wykazały, że wyekstrahowane biopolimery mają korzystne właściwości. Temperatura topnienia kopolimeru scl-mcl wyekstrahowanego z komórek *Pseudomonas* sp. GL01 była najniższa i nietypowa, wskazując na jego amorficzne zachowanie i giętkość w temperaturze otoczenia. Obecność frakcji 3HB mogła być również przyczyną jego niższej odporności termicznej. Biopoliestry wyizolowane z komórek *Paracoccus homiensis* i *Aeromonas* sp. AC_01 charakteryzowały się wysoką stabilnością termiczną, co wydaje się być korzystne biorąc pod uwagę technologię przetwarzania tych biopolimerów. Uzyskane dane są obiecujące w perspektywie opracowania nowatorskiego procesu wykorzystania odpadów o dużej zawartości SMCFA bez konieczności wstępnej ich obróbki do produkcji wartościowych kopolimerów PHA.

1. Introduction

Petroleum-based plastics are one of the most widely used materials due to their properties such as lightness, robustness, durability and resistance to degradation. Large scale dependence on synthetic polymers and their heavy overconsumption resulted in rapid reduction of fossil fuels and serious environmental pollution issues. Therefore, there is a growing interest in the environmentally friendly biopolymers that could replace petrochemical-derived compounds. Among various types of biopolymers, polyhydroxyalkanoates (PHAs) have become an attractive alternative to synthetic polymers due to their biodegradability, non-toxicity, biocompatibility and similarity of their properties to those of conventional polymers (Braunegg et al., 1998). In addition, these bacterial polymers have many advantages over petrochemical-derived polymers, such as hydrophobicity, thermoplasticity, relatively high melting point and optical purity (Zubairi et a., 2016). They are produced by various microorganisms as carbon and energy reserves under unbalanced growth conditions such as limitation of nitrogen, phosphorus or oxygen. The type of the produced PHAs depends on the carbon source used, the strain of bacteria engaged in the bioprocess and the applied culture conditions. Moreover, PHAs are produced n biotechnological processes, which allows controlling their chemical structure and physico-thermal properties that vary due to different sizes of the side chains in the repeat units of the biopolymer (Bonartsev et al., 2013). Based on the number of carbon atoms in the monomer, PHAs can be classified as short-chain-length PHAs (PHA_{SCL}; 3 to 5 carbon atoms), medium-chain-length PHAs (PHA_{MCL}; 6 to 14 carbon atoms) or long-chain-length PHAs (PHA_{LCL}; 15 or more carbon atoms). PHA_{SCL}, such as poly-3-hydroxybutyrate [P(3HB)] are rigid and brittle materials with a high degree of crystallinity, with low elasticity and oxygen permeability, and poor mechanical properties (Sudesh et al., 2000). The incorporation of other monomers into P(3HB) could improve its properties and applicability making this material to biodegrade faster than homopolymers due to the presence of the inherited amorphous region. Park et al. (2012) proved that the addition of the 3HV fraction in the copolymer molecule could decrease its crystallinity and in the consequence the biodegradation rate improves. PHA_{MCL} shows more beneficial and useful properties than PHA_{SCL}. They have elastomeric properties with low crystallinity, melting point, tensile strength and a high elongation to break. They are mainly synthesized by bacteria belonging to the *Pseudomonas* genus. PHA_{LCL} are uncommon and knowledge of its properties and applicability is still limited. PHAs have great potential in biomedical, agricultural and industrial applications (Tan et al., 2021), however their production on a large scale is still limited due to high production costs. To make this biotechnological

process economically feasible there is a need to explore new waste-derived streams as carbon sources and novel bacteria capable of metabolizing such organic wastes.

Utilisation of wastes into valuable products is a revolutionary waste management pathway and an environmentally beneficial process. Particular attention is focused on short and medium chain fatty acids (SMCFAs) generated by acidogenic anaerobic mixed culture fermentation (MCF). Bio-derived acids such as acetate, propionate, butyrate, valerate and capronate can be interesting alternatives to expensive ones. They are intermediate products in the anaerobic digestion (AD) of various wastes such as food, municipal and agricultural wastes, sludge, dairy wastewater, paper and pulp wastewater. The AD process basically consists of series of steps where organic matter is degraded by microorganisms under anaerobic conditions (Liu et al., 2018). Anaerobic fermentation with mixed microbial cultures (MMCs) leads to the transformation of organic molecules such as carbohydrates, proteins and fats. It has been previously shown that SMCFAs can effectively support the bacterial growth (Reddy et al., 2012). Furthermore, they are also the preferred substrates for PHAs production due to their role as direct metabolic precursors in the biosynthetic pathways. The synthetic SMCFAs have been successfully converted into bacterial biopolyesters by many microorganisms. *Ralstonia eutropha* ATCC 17699 was able to produce P(3HB) homopolymer using acetic, propionic and butyric acid. Higher productivity (0.037 g PHA/g substrate) was reached with the bacterial cells cultivated on butyric acid (Chakraborty et al., 2009). Also, *Bacillus megaterium* OU303A was found to be a good P(3HB) producer in the presence of propionic acid in a culture medium containing glycerol. This strain synthesized up to 62.4FF of CDM (Reddy et al., 2009). Reddy et al. (2017) reported that *Pseudomonas pseudoflava* (NBRC-102513) was capable of producing slightly lower content of PHAs (57% of CDM) in the cultivation supplemented with acetic and propionic acid. Moreover, the authors proved that the addition of propionic acid as a co-substrate stimulated the synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer. Furthermore, it was confirmed that *Alcaligenes eutrophus* NCIMB 11599 produced a higher P(3HB-co-3HV) copolymer yield in a two-stage fed-batch cultivation when using valeric acid than when supplementing propionic acid. Interestingly, the proportion of SMCFAs to co-substrates seems to have an impact on the PHAs productivity and biopolymer composition. It was reported that lower ratio of propionic acid to glucose led to lower concentration of 3HV fraction in the P(3HB-co-3HV) copolymer but higher P(3HB-co-3HV) productivity was observed. On the other hand, a high propionic acid to glucose ratio led to a high 3HV monomer content but a low P(3HB-co-3HV) concentration (Du et al., 2001).

There are only a few reports describing the potential of microorganisms to convert SMCFAs-rich streams into PHAs. The P(3HB-co-3HV) copolymer content of 55% of CDM was observed in the *Cupriavidus necator* cells when SMCFAs produced from purified olive mill wastewater (OMW) were used as the only substrate (Martinez et al., 2015). Furthermore, bacteria belong to *Pseudomonas* genus were tested towards PHA production using SMCFAs generated through AD of grass. The results revealed that the level of PHA concentration was dependent on the strain of *Pseudomonas* sp. The highest content of mcl-PHAs (about 44% of CDM) was detected in *Pseudomonas putid*a KT2440 cells cultivated on acetic and butyric acid. Similar values of this biopolymer was observed in the culture of *Pseudomonas putida* CA-3 supplemented with acetic, propionic or butyric acid. Moreover, gas chromatography analysis proved that the concentration of the monomers was dependent on the bacterial strain. The monomeric composition of the biopolyesters extracted from *P. putida* KT2440 and CA-3 cells was similar. These both strains showed a tendency to produce high levels of 3-hydroxydecanoate (3HD), lesser amounts of 3-hydroxyoctanoate (3HO) and 3-hydroxydodecanoate (3HDD), and trace amounts of 3-hydroxytetradecanoate (3HTD). Mcl-PHAs isolated from *Pseudomonas putida* GO16 contained higher levels of 3HDD monomer than those produced by the KT2440 and CA-3 strains (Cerrone et al., 2014).

In recent years, the amount of organic and biomass waste has been steadily increasing. Currently, sewage sludge is a burden for municipal wastewater treatment plants (Lad et al., 2022). Moreover, whey disposal is a global issue facing the dairy industry. Whey is a by-product of the cheese production process and contains lactose, milk proteins, fat, vitamins and mineral salts. Despite the fact that whey can be used in the production of lactose, casein and protein powder, it is estimated that around 50% of the whey produced is still disposed of in wastewater treatment plants (Pescuma et al., 2015). Furthermore, acid whey, a by-product of the production of Greek yoghurt, cottage cheese and cream cheese, poses a risk to the environment and its disposal is still expensive. Therefore, SMCFAs derived from sludge and acid whey are attractive carbon sources for the synthesis of biopolymers by bacteria, contributing to the reduction of waste and having a positive impact on the environment.

The microorganisms involved in the synthesis of PHAs are diverse, range from well-known defined bacterial strains to mixed microbial consortia. Strain selection is a fundamental aspect of biopolymer production that could lead to the discovery of new high-efficiency PHA producers. Up to date, the conversion of SMCFAs to PHAs using pure microbial cultures has

been poorly studied. Most research using SMCFAs are based on the use of mixed microbial cultures. Therefore, there is still a need to develop novel microbial processes and to search for an efficient PHA producer capable of synthesizing PHAs using renewable carbon sources such as SMCFAs. Due to robust growth and simple nutritional requirements, especially bacteria belonging to *Aeromonas* sp., *Paracoccus* sp. and *Pseudomonas* sp. genera are attractive targets for PHA production.

2. Aim and objectives

The aim of the doctoral dissertation was to investigate the use of synthetic and acid whey derived short and medium chain fatty acids (SMCFAs) as feedstocks for PHA production by *Paracoccus homiensis*, *Aeromonas* sp. AC_01, *Pseudomonas* GL01, *Pseudomona*s GL06 and *Pseudomonas antarctica*.

The scope of the thesis included:

- determination of the effect of individual synthetic SMCFAs (acetic acid, propionic acid, butyric acid, valeric acid, and caproic acid), synthetic mixtures of SMCFAs and SMCFAs derived from whey wastewater fermentation effluent on the bacterial growth and PHA concentration,
- determination of a type and a suitable concentration of SMCFAs that guarantee the effective growth of the bacteria and efficient PHAs synthesis,
- evaluation of PHA monomeric composition using gas chromatography coupled with mass spectrometry and spectroscopic analysis,
- detailed characterization of the extracted PHAs to assess their application potential.

3. Materials and methods

3.1 Microorganisms

Paracoccus homiensis (DSMZ 17862) and *Pseudomonas antarctica* (DSM 15318) were purchased from the German Collection of Microorganisms and Cells Cultures GmbH at the Leibniz Institute.

Aeromonas sp. AC_01 belongs to the Culture Collection of the Department of Microbiology and Mycology (University of Warmia and Mazury in Olsztyn, Poland). The strain was isolated from waste activated sludge collected in spring from an aeration tank at the municipal wastewater treatment plant in Olsztyn (Poland). The *16S rRNA* gene sequence of the tested microorganism showed that it possessed the highest similarity to *Aeromonas hydrophila* (Możejko-Ciesielska and Pokój, 2018).

Pseudomonas sp. GL01 and *Pseudomonas* sp. GL06 were isolated from mixed microbial communities and belong to the Culture Collection of the Department of Microbiology and Mycology at the University of Warmia and Mazury in Olsztyn (Poland). They were described as strains capable of synthesizing and accumulating PHA_{MCL} (Ciesielski et al., 2010).

3.2. Carbon sources

- Individual synthetic SMCFAs: acetic (HAc) (Chempur, Poland), propionic (HPr) (Chempur, Poland), butyric (HBu) (Aktyn, Poland), valeric (HVa) (Merck, Germany) and caproic acid (HCa) (Roth, USA);
- a mixture of HAc and HBu;
- a mixture of synthetic SMCFAs ($SMCFAs_{synthetic}$-rich stream) to simulate a real SMCFAs-rich stream ($SMCFAs_{extracted}$-rich stream): HAc (2.85 g/L), HBu (9.86 g/L), HVa (0.16 g/L) and HCa (3.05 g/L);
- a mixture of SMCFAs received from the acidogenic anaerobic mixed culture fermentation of acid whey obtained from a crude cheese production line ($SMCFAs_{extracted}$-rich stream).

Fermentation of acid whey to produce SMCFAs was conducted in a UASB bioreactor constructed of cylindrical plexiglass with recirculation to ensure biomass suspension as described by Duber et al. (2018). The process was conducted at a constant temperature of 30°C and pH 5.5 with automatic control using 2 M NaOH. $SMCFAs_{extracted}$-rich stream for PHA

production was harvested from a steady-state phase with dominant HBu production. The SMCFAs-rich stream sample was filtered and the supernatant was analyzed by high-performance liquid chromatography (HPLC) to evaluate its composition. $SMCFAs_{extracted}$-rich stream used in the research consisted of: HAc (2.85 g/L), HBu (9.86 g/L), HVa (0.16 g/L) and HCa (3.05 g/L), lactic acid (9.31 g/L) and ethanol (1.64 g/L).

3.3. Cultivations of *Paracoccus homiensis* towards PHA production

To prepare the bacterial inoculum, *P. homiensis* cells were transferred from glycerol long-term storage tubes at -80°C into 250 mL Erlenmeyer flasks containing Bacto Marine Broth. The analyzed strain was cultured at 28°C with 120 rpm shaking for 16 h. Bacto Marine Broth contained the following ingredients per liter: 5 g bacto peptone, 1 g bacto yeast extract, 0.1 g Fe(III) citrate, 19.45 g NaCl, 5.9 g $MgCl_2$ (anhydrous), 3.24 g Na_2SO_4, 1.8 g $CaCl_2$, 0.55 g KCl, 0.16 g $NaHCO_3$, 0.08 g KBr, 34 mg $SrCl_2$, 22 mg H_3BO_3, 4 mg Na-silicate, 2.4 mg NaF, 1.6 mg $(NH_4)NO_3$, 8 mg Na_2HPO_4. The pH of the medium was regulated to 7.6 by adding appropriate amount of 1 N NaOH and 1 N HCl. The medium components along with distilled water were autoclaved at 121°C.

Flask cultures were performed in triplicate in 250 mL Erlenmeyer flasks containing 100 mL of Bacto Marine Broth (composition described above) supplemented with varying concentrations of individual synthetic SMCFAs from 1 to 4 g/L and $SMCFAs_{extracted}$-rich stream from 5 to 30% v/v. Each substrate was added at the beginning of the cultivations. All experiments were carried out at 28°C on a rotary shaker at 110 rpm for 48 h.

3.4. Cultivations of *Aeromonas* sp. AC_01 towards PHA production

Before inoculation into fermentation shake flasks, cells of *Aeromonas* sp. AC_01 were transferred from glycerol stock maintained at -80°C to lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) and were cultured at 30°C for 16 h with 150 rpm shaking. To initiate cultivation for PHAs production, 250-mL Erlenmeyer flasks were inoculated with 5% (v/v) microbial seeds. *Aeromonas* sp. AC_01 were cultivated using two different mineral salt medium (MSM): non-limited medium (7 g/L KH_2PO_4, 3.5 g/L $Na_2HPO_4 \cdot 12H_2O$, 10 g/L $(NH_4)_2SO_4$) and nitrogen-limited medium (7 g/L KH_2PO_4, 3.5 g/L $Na_2HPO_4 \cdot 12H_2O$, 1 g/L $(NH_4)_2SO_4$). To each medium was added $MgSO_4 \cdot 7H_2O$ (1 g/L) and a trace element solution (2.5 mL/L) consisting of 20 g/L $FeCl_3 \cdot 6H_2O$, 10 g/L $CaCl_2 \cdot H_2O$, 0.03 g/L $CuSO_4 \cdot 5H_2O$, 0.05 g/L $MnCl_2 \cdot 4H_2O$, 0.1 g/L $ZnSO_4 \cdot 7H_2O$ dissolved in 0.5 N HCl.

All cultures were enriched (as the only carbon source) with: (A) different concentrations (1, 2, and 3 g/L) of individual synthetic SMCFAs: HAc, HBu, HVa and HCa; (B) different ratios of HAc and HBu (g/L): 1.6 : 0.4; 1.2 : 0.8; 0.8 : 1.2; 0.4 : 1.6 (C) different concentrations (10, 20 and 30% v/v) of the $SMCFAs_{synthetic}$-rich stream; (D) different concentrations (10, 20 and 30% v/v) of the $SMCFAs_{extracted}$-rich stream. The pH of the cultures medium was adjusted to 7.0 by adding 1M NaOH and 1M HCl. All cultures were incubated at 30ºC and 150 rpm for 48 h (with $SMCFAs_{synthetic}$-rich stream and $SMCFAs_{extracted}$-rich stream) or for 72 h (with single synthetic SMCFAs and different ratios of HAc and HBu) using a rotary shaker.

3.5. Cultivations of *Pseudomonas* sp. strains towards PHA production

The inocula were cultivated in lysogeny broth (composition described in section 3.4.) at 30°C with shaking at 150 rpm for 16 h. Subsequently, precultures were transferred to non-limited and nitrogen-limited mineral salt media (MSM): (*MSM 1*) 12.8 g/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 10 g/L NH_4Cl, 0.5 g/L NaCl (Poblete-Castro et al., 2012); (*MSM 2*) 12.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 10 g/L $(NH_4)_2SO_4$; (*MSM 3*) 3.5 g/L Na_2HPO_4, 5 g/L KH_2PO_4, 10 g/L $(NH_4)SO_4$. Nitrogen-limited MSM contained 1 g/L of nitrogen source in each medium. The media were additionally supplemented with 1 g/L $MgSO_4 \cdot 7H_2O$ and 2.5 mL/L of trace element solution consisting of (per liter): 20 g $FeCl_3 \cdot 6H_2O$, 10 g $CaCl_2 \cdot H_2O$, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.05 g $MnCl_2 \cdot 4H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$ dissolved in 0.5N HCl. The pH of each medium was adjusted to 7.0 by adding 1 N NaOH and 1 N HCl. All MSM ingredients were dissolved in water and autoclaved at 121°C. The $MgSO_4 \cdot 7H_2O$ solution was sterilized and added separately to each flask.

MSM media was supplemented with 20% (v/v) of $SMCFAs_{synthetic}$-rich stream or 20% (v/v) of $SMCFAs_{extracted}$-rich stream. *Pseudomonas* spp. strains were cultivated in 250-mL Erlenmeyer flasks on a rotary shaker at 150 rpm at 30°C for 48 h.

3.6. Fermentation of *Pseudomonas* sp. GL06 in a bioreactor

The cultivations in shake flasks (described in the section 3.5) enabled the selection of MSM consisting of 12.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, which promoted the best PHA productivity and *Pseudomonas* sp. GL06 as the most efficient PHA producer. The fermentation was conducted at 30°C with an aeration rate of 4 L/min in a 7 L bioreactor (Biostat A, Sartorius, Germany) equipped with a pH controller. The pH value was controlled at 7.0 by modulating addition of concentrated 1 N NaOH and 1 N HCl. The temperature was maintained

using a thermostatic jacket. Dissolved oxygen was continuously monitored during the whole cycle using an O_2 electrode (InPro 6800, Mettler Toledo GmbH, Switzerland) and 50% air saturation was maintained by automatically adjusting the agitation rate from 300 rpm to 1000 rpm. Total fermentation time was 48 h. The mineral salt medium was supplemented with total of 20% (v/v) $SMCFAs_{extracted}$-rich stream. The carbon source was added in two portions: first at the beginning of culture and then in 8 h of the fermentation. An A concentrate solution (Sigma Aldrich) was used as an antifoam in response to the antifoam controller.

3.7. Analytical procedures

Growth of bacterial cells was monitored by measuring absorbance at 600 nm (OD_{600}) using a spectrophotometer (Eppendorf, Germany). For more accurate information on the growth of the test strain, the maximum specific growth rate (μ_{max}) was determined as an equation for the linear trend line for the exponential phase.

To evaluate cell dry mass (CDM), 100 mL of culture broth was centrifuged at 9000 x g for 10 min and then lyophilized for 24 h using Lyovac GT2 System (SRK Systemtechnik GmbH). The biopolyesters were extracted by shaking the lyophilized cells in hot chloroform at 50^{o}C for 3 hours. Next, the mixture was filtered through Whatman No. 1 filter paper to remove cell debris. After filtration, the PHAs dissolved in chloroform were purified by precipitation with 70% cold methanol and then allowed to evaporate at room temperature. Precipitated PHA was quantified directly by gravimetric method. PHA content (% of CDM) was defined as the percentage of the ratio of the biopolymer concentration to the total bacterial cell concentration. PHA productivity was calculated as the concentration of PHA produced per hour.

Ammonium and phosphorus concentrations in the culture broth were determined spectrophotometrically using a Hach Lange DR 2800 spectrophotometer (Hach Lange, Düsseldorf, Germany) as well as the LCK338 and LCK350 kits according to the producer's instructions, respectively.

3.8. Analysis of the extracted PHAs

3.8.1 Gas chromatography coupled with mass spectrometry

To evaluate the monomeric composition of the produced PHAs, lyophilized cells were analyzed by gas chromatography coupled with mass spectrometry (GC-MS QP2010 PLUS, Shimadzu, Japan) according to the method described by Możejko-Ciesielska and Pokój (2018).

The first step was to analyze the samples suspended in the chloroform-methanol-sulfuric acid mixture (100/97/3, v/v/v) and the methylation was carried out by heating the vials at 100°C for 20 hours. Next, the sulfuric acid was neutralized with Na_2CO_3 and the resulting mixture was dried with anhydrous Na_2SO_4. After filtration methyl esters were analyzed with the use of a BPX70 (25m x 0.22mm x 0.25mm) capillary column (SGE Analytical Science, Victoria, Australia) with helium as a carrier gas at a flow rate of 1.38 mL/min. The column was uniformly heated from 80 to 240°C at the rate of 10°C/min. The interface and ion source temperatures of GC-MS was set at 240°C with the electron energy of 70 eV within the 45-500 m/z range. Pure standards of methyl 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxyhexanoate, -octanoate, -nonanoate, -decanoate, -undecanoate, -dodecanoate, -tetradecanoate, -hexadecanoate were purchased from Larodan Fine Chemicals (Sweden) to generate calibration curves for the methanolysis assay.

3.8.2 Spectroscopic studies

Biopolymer spectroscopic studies were performed using a Fourier Transform Spectrophotometer (FTIR) Nicolet iS10 (ThermoScientific, USA). Infrared spectroscopic spectra were recorded in the wave number range from 4000 to 650 cm^{-1} with a Fourier Transform Spectrophotometer (FTIR) Nicolet iS10 (ThermoScientific, USA) by attenuated total reflection method (ATR-FTIR) on samples without prior preparation. UV-Vis spectra were recorded on an spectrometer Evolution 220 (Thermo Scientific, USA) equipped with an integrating sphere. Each spectrum analyzed was the average of 16 measurements recorded in the wavelength range from 200 to 1100 nm.

3.8.3 Differential scanning calorimetry and thermogravimetry

Differential scanning calorimetry (DSC) studies were carried out using a Q200 differential scanning calorimeter (TA Instruments, USA) in a nitrogen atmosphere, in a temperature range from -70 to 230°C and a heating/cooling rate of 10°C/min. Material samples of approximately 1 mg were first rapidly heated to 230°C and conditioned for 1 minute to eliminate the thermal history of the material. The samples were then cooled to -70°C and re-heated to 230°C. The thermal analysis of the biopolymers was based on a second heating curve in which the lass transition temperature (T_g), crystallization temperature (T_c), change in enthalpy of the crystallization process (ΔH_c), cold crystallization temperature (T_{cc}), change in enthalpy of the cold crystallization process (ΔH_{cc}), melting point (T_m), change in enthalpy melting process (ΔH_m) and the degree of crystallinity (X_c) were registered. The Xc values were

calculated from the formula:

$$X_c = \frac{\Delta H_m - \Delta H_{cc}}{\Delta H_{m100\%}}$$

Where: $\Delta H_{m100\%}$ - change in melting enthalpy of a 100% crystalline sample – 109 J/g (Jost et al., 2017).

Thermogravimetric analysis (T_G) were conducted using a Q500 thermobalance (TA Instruments, USA) in a nitrogen atmosphere, with a temperature range from 25 to 600°C, with a heating rate of 10°C/min. On the basis of thermogravimetric curves, the values $T_{5\%}$, $T_{50\%}$ and $T_{95\%}$ corresponding to the temperature of 5, 50 and 95% loss of the initial mass of the PHA sample were determined. The differential thermogravimetric curve (DT_G) (first derivative of the T_G curve) was also used to determine T_{max} values, which determine the temperature of the fastest mass loss.

3.8.4. Water related properties

Hydrophilicity of the biopolymers surface was assessed by measuring the water contact angle formed between the water drops and the surface of the samples using a DSA 100 goniometer (Krüss GmbH, Germany) equipped with an automatic dispensing system for measuring liquid drops. Measurements were carried out using water (polar liquid) and diiodomethane (dispersion liquid). A drop of the measuring liquid was placed on the surface of the tested biopolymer, its volume was continuously increased and at the same time the dynamic contact angle was determined, which in this case was the inflow angle. Following 12 measurements of contact angles of each measuring liquid, the smallest and largest values of these angles were discarded. The arithmetic mean was calculated from the remaining 10 values. Surface energy (SE) was calculated using Neumann method. The formula for the Neumann method applied to SE was in the following form:

$$cos\Theta_L = 2(\gamma_L/\gamma_S)^{0.5} exp[-\beta(\gamma_L - \gamma_S)^{0.5}]$$

Where: Υ_S- SFE of polymer sample, Υ_L– SFE of water, and β = 0.0001247 (equation parameters).

3.9. Statistical analysis

Statistical analysis of all data was conducted using STATISTICA v.13.1 (StatSoft, Inc,

Tulsa, OK, USA). To determine the significance of differences in biomass and amount of PHAs between experimental variants, the Mann-Whitney U test was used. Data were reported as median ± SD at a significance level of $P < 0.05$. Spearman correlation analysis was performed with a significance threshold of $\alpha=0.05$.

4. Results

4.1. Publication 1. Szacherska K., Oleskowicz-Popiel P., Ciesielski S., Mozejko-Ciesielska J. 2021. Volatile Fatty Acids as Carbon Sources for Polyhydroxyalkanoates Production. Polymers. 13, 321. DOI: 10.3390/polym13030321.

In recent years, significant research attention has been given to short and medium chain fatty acids since they can be used to generate high-value bioproducts in the biotechnological processes. This review article is an introduction to the subject of the potential of using SMCFAs as carbon sources to produce PHAs in microbial processes. In the publication the synthesis of SMCFAs by microorganisms and their characteristics were described. In addition, the article provides information about pure bacterial cultures and mixed microbial cultures capable of producing PHAs in satisfactory amounts using SCMFAs as the substrates. Moreover, the value chain for deriving PHAs from these fatty acids was described to direct the challenges and future perspectives in the synthesis of these biopolymers. In the introduction the environmental problem caused by excessive production and uncontrolled human consumption of plastics, which leads to irreversible changes affecting the environment and human health was presented. It was highlighted that biodegradable PHAs could be an attractive alternative to petrochemical-polymers. Furthermore, the drawbacks of using PHAs and their limitations in the commercialisation were discussed. It has been shown that the overall production costs of these value-added biopolyesters could be reduced by using waste feedstocks and their post-fermentation products such as SMCFAs (López et al., 2018). Converting wastes into innovative biopolymers is a modern approach guaranteeing the disposal of harmful substances and the generation of environmentally friendly materials. Furthermore, it has been demonstrated that the type of SMCFAs used in the microbial fermentation determines the monomeric composition of produced PHAs and have a strong effect on their properties (Moretto et al., 2019). Importantly, this publication presents the summary of scientific achievements that prove the ability of many microorganisms to convert SMCFAs into homopolymers and copolymers of PHAs, both pure bacteria cultures (Chakraborty et al., 2009; Reddy et al., 2009) and mixed microbial cultures (Serafim et al., 2004; Burniol-Figols et al., 2018). The collected and critically reviewed literature data made it possible to indicate challenges and further perspectives to increase the productivity of PHA using SMCFAs.

The main barrier to PHA commercialisation are too high overall production costs mainly because due to the price of the source of carbon and energy used in the microbial fermentations.

Processing the residual waste such as waste-derived SMCFAs into PHAs seems to be one of the solution. However, there are some difficulties that should be overcome to make the PHA production a reality through microbial fermentations using SMCFAs. First of all, toxic effects of these feedstocks on bacterial cells should be prevented, therefore there is a need to optimize the cultivations and evaluate the proper SMCFAs concentration that will be support the bacterial growth and PHA synthesis (Bhattacharyya et al., 2008). A significant factor will also be the selection of a microbial producer capable of maximizing the bioproduct yield. Furthermore, the co-metabolism could be proposed as an approach to produce PHA copolymers with desired repeat units composition. So far, only a few scientific reports describe the production of PHAs by pure bacterial cultures in the cultivations supplemented with SMCFAs as the only carbon sources. Considering the research results presented in this publication, there is still a need to develop novel biotechnological process and search for microbes that will be able to convert SMCFAs, especially those generated by acidogenic anaerobic mixed culture fermentation, into the valuable bacterial polyesters.

4.2. Publication 2. Szacherska K., Moraczewski K., Rytlewski P., Czaplicki S., Ciesielski S., Oleskowicz-Popiel P., Mozejko-Ciesielska J. 2022. Polyhydroxyalkanoates production from short and medium chain carboxylic acids by *Paracoccus homiensis*. Scientific Reports. 12, 7263. DOI: 10.1038/s41598-022-11114-x.

The aim of this study was to evaluate the effect of the individual synthetic SMCFAs and those derived from anaerobic mixed culture fermentation of acid whey on PHA synthesis by *Paracoccus homiensis*. The inspiration for this publication was the fact that there was a lack of studies focused on the PHA production from SMCFAs using species belong to *Paracoccus* genera even though these bacteria had substantial biotechnological potential. Moreover, none of the study determined the potential of microorganisms to convert SMCFAs-rich streams from MCF using acid whey and evaluated also in details properties of the extracted PHAs. Therefore, in this publication the effect of the type and concentration of SMCFAs on the biopolymer content of bacterial cells, their growth and the monomeric composition of the final bioproduct was determined. Moreover, the purified PHAs were thoroughly characterised considering their physico-thermal and water-related properties. The data proved that individual and waste-derived SMCFAs support the growth of *P. homiensis* and PHA production. The cultivation with 2 g/L of acetic acid showed both maximum μ_{max} (0.87 h^{-1}) and biomass concentration (1.6 g/L) suggesting that it was less toxic and was easier metabolized by the analyzed bacterium

compared to other tested fatty acids. However, significantly lower biomass values (0.15 g/L) were obtained by Chang et al. (2021) in *Bacillus* sp. CYR-1 culture supplemented with acetic acid derived from cheese whey fermentation. The differences in the growth rates were related to different metabolic pathways involved in the SMCFAs consumptions. Furthermore, the obtained results confirmed that with increasing the concentration of all SMCFAs to 4 g/L, μ_{max} and CDM value decreased. Also, the PHAs content in *P. homiensis* cells was dependent on the type and concentration of SMCFAs. The results showed that acetic acid at the concentration of 3 g/L guaranteed the highest PHA content of 26% of CDM. Furthermore, PHAs synthesis and accumulation by *P. homiensis* was inhibited with an increase of capronate concentration, reaching 20.26% and 13.7% of CDM at the level of 1 g/L and 4 g/L of this acid, respectively. It can be suggested that excessive acid concentrations could affect the inhibition of PHAs synthesis by the tested strain. Therefore, the selection of an appropriate substrate concentration is important to prevent the undissociated SMCFAs molecules not to enter the cytoplasm and in a consequence not to activated and utilized into CO_2. The lowest biopolymer content was observed during bacterial culture on butyric acid at all analyzed concentrations (from 9.65 to 10.1% of CDM). It suggested that butyric acid is not so productive source of energy for *P. homiensis* than other SMCFAs. Importantly, the replacement of the individual SMCFAs by a SMCFAs-rich residual stream was found to have a positive effect on the *Paracoccus homiensis* growth. The highest biomass concentration reached about 2.5 g/L with PHAs content of 17% of CDM. The relatively lower content of PHAs reported in the cultivation with the waste-derived SMCFAs compared to individual, synthetic fatty acids proved that the SMCFAs-rich stream showed a more inhibitory effect due to its complexity and the presence of components other than fatty acids. Moreover, the relatively low productivity of PHAs could be explained by the dominance of butyric acid in the SMCFAs-rich stream which as a single short fatty acid in the culture medium resulted in the lowest PHAs production efficiency compared to others. Also, Vu et al. (2021) obtained a twofold lower PHAs concentration during *Bacillus megaterium* culture with the addition of a stream containing SMCFAs derived from food waste fermentation.

Moreover, the results showed that the monomeric composition changed with the concentration of the SMCFAs and the PHA content was dependent on the culture time. The study showed that *P. homiensis* cultured on acetic and caproic acid synthesised a high level of 3HB monomer and a lower level of 3HV fraction. However, during cultivation supplemented with 2 g/L propionic acid and 4 g/L butyric acid, the analyzed bacterium was able to produce

the P(3HB) homopolymer. The predominant 3HV content was confirmed in the P(3HB-co-3HV) copolymer extracted from *P.homiensis* cells cultured on valeric acid. The data also showed that the use of bioprocess-derived SMCFAs led to the synthesis and accumulation of the P(3HB-co-3HV) copolymer. Considering the presence of high amounts of acetic and butyric acid in the SMCFAs-rich stream, the low concentration of valeric acid and the deficiency of propionic acid, the resulting copolymer contained a lower 3HV content than 3HB. In general, higher 3HV content was observed when higher concentrations of SMCFAs-rich stream were used in the cultures. The utilisation of waste to generate copolymers is an interesting approach due to the attractive physicochemical properties of the copolymers compared to pure P(3HB). The spectra of biopolymer samples produced from cultures supplemented with acetic, propionic, butyric, caproic acid and SMCFAs-rich stream were similar. Only for the P(3HB-co-3HV) sample from valeric acid some differences were observed, probably caused by the terminal -OH groups present at the ends of the polymer chains. These changes are also related to the high degree of crystallinity of this biopolymer. The UV-VIS absorbance spectra revealed three absorbance peaks with band structures similar to samples representing typical absorption bands for 3HB fraction. PHAs extracted from *P. homiensis* cells cultivated on acetic, propionic, butyric and caproic acid showed high 3HB content (above 93%) and low 3HV content. The only exception was the P(3HB-co-3HV) sample from valeric acid, for which only very slight absorption bands were identified in the spectrum. This could be due to the different chemical structure of these PHAs, for which the 3HB content was much lower (only about 17%). This study also investigated the effect of SMCFAs on thermal and water-related properties of the synthesized PHAs, which could help to determine the applicability of this biopolymers. The results showed that all biopolymers produced by *P. homiensis* possessed appropriate thermal stability. Nevertheless, P(3HB-co-3HV) extracted from the cultures supplemented with waste-derived SMCFAs had the highest thermal stability. These biopolymers showed a relatively wide thermal processing spectrum as the difference between melting point and degradation temperature is higher compared to polymers extracted from cultivated supplemented with individual, synthetic fatty acids. From a commercial point of view, these properties are important factors that determine the quality and final applications of the product (Karagöz, 2021). The obtained water contact angles and calculated value of surface energy of the extracted biopolymers showed that the highest hydrophobic properties of the PHAs extracted from the cultivation with butyric acid.

4.3. Publication 3. Szacherska K., Moraczewski K., Czaplicki S., Oleskowicz-Popiel P.,

Mozejko-Ciesielska J. 2022. Conversion of Short and Medium Chain Fatty Acids into Novel Polyhydroxyalkanoates Copolymers by *Aeromonas* sp. AC_01. Materials. 15, 4482. DOI: 10.3390/ma15134482.

The aim of the publication was to evaluate a new, sustainable production of PHAs using SMCFAs from whey wastewater biodigestion effluent, individual synthetic SMCFAs and their synthetic mixtures as the only substrates in the cultivation of *Aeromonas* sp. AC_01. This bacterium has the potential to synthesize PHA from various types of waste due to its simple nutritional requirements and adaptability to unfavourable environmental conditions (Możejko-Ciesielska and Pokój, 2018). In the first stage of the experiment, *Aeromonas* sp. AC_01 was evaluated towards its ability to synthesise the biopolyesters growing on single, synthetic SMCFAs: acetic, butyric, valeric and caproic acid. The results proved that the bacterial growth was dependent on the concentration of the individual SMCFAs. Only, acetic acid at all applied concentrations did not act as an inhibitor for *Aeromonas* sp. AC_01 growth. Whereas, in the cultivations with more than 1 g/L of other fatty acids a decreased cell concentration was observed. Interestingly, only in the culture medium supplemented with acetic acid pH value in all measured time-points was alkaline (pH >7), therefore the growth inhibition of *Aeromonas* sp. AC_01 could have been effectively alleviated even in the high acetic acid concentration. Also, Gao et al. (2017) proved that an alkaline environment could have affected positively the tolerance level to high SMCFAs concentration of *Yarrowia lipolytica* during lipid biosynthesis.

Moreover, the results confirmed that unbalanced growth conditions supported PHA synthesis and accumulation. The cultures with 1 g/L butyric acid and 2 g/L acetic acid conducted under nitrogen-limited conditions resulted in the highest biopolymer concentrations, 0.09 and 0.07 g/L, respectively. Lower PHAs yield (0.03 g/L) was determined by Shi et al. (2020) who cultured genetically modified *Aeromonas hydrophila* 4AK4 with yeast extract and acetate. The results showed that acetic and butyric acid were the most beneficial carbon sources that effectively promoted the growth and PHAs synthesis in *Aeromonas* sp. AC_01 cells. Thus, in the second step, the effect of the mixtures of both acids on the biomass concentration and PHA productivity was determined. The combinations of acetic and butyric acid promoted better PHA biosynthesis than the single acids. In the bioprocess with the mixture in the proportion of 1.2 acetic acid:0.8 butyric acid the highest PHA productivity (3.89 mg/L·h) was reported. Nevertheless, Gonzalez and Winterburn (2022) observed higher PHA productivity (12.8 mg/L·h) in the cultivation of *Haloferax mediterranei* grown on the mixture of butyric and

pentanoic acid. In the last stage of the experiment, the effect of the effluent from the anaerobic digestion of whey wastewater on the *Aeromonas* sp. AC_01 growth and PHA synthesis was explored. Due to the fact that the $SMCFAs_{extracted}$-rich stream was complex and besides SMCFAs consisted of other compounds such as lactic acid and ethanol, a simulated SMCFAs mixture was prepared identically with the SMCFAs components of the SMCFAs-rich stream. $SMCFAs_{extracted}$-rich stream was found to be less beneficial for PHA productivity than its synthetic mixture ($SMCFAs_{synthetic}$-rich stream). It could be suggested that the presence of ethanol or lactic acid in the $SMCFAs_{extracted}$-rich stream may adversely affect the growth and biosynthesis of PHAs by the tested strain. Moreover, the results proved that nitrogen-limited conditions supported the production of PHAs in the cultivations supplemented with $SMCFAs_{synthetic}$-rich stream, while in the cultures supplemented with $SMCFAs_{extracted}$-rich stream the PHAs was synthesized on the same level regardless the concentration of nitrogen source.

GC-MS and FTIR analyses proved that the monomeric composition of the extracted PHAs was dependent on the type of the carbon source and culture conditions used. Interestingly, the *Aeromonas* sp. AC_01 was able to synthesize novel scl-mcl copolymers of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), 3-hydroxytridecanoate (3HtriD) and 3-hydroxytetradecaonate (3HTD) with high 3HB and 3HV fractions. So far, no reports proved that bacteria have the ability to produce copolyesters contained 3HB, 3HV, 3-HTriD and 3-HTD fractions. Zhang et al. (2009) reported that *Aeromonas hydrophila* 4AK4 fed with a mixture of lauric and valeric acid synthesised the terpolyester P(3HB-co-3HV-co-3HHx). Importantly, it was shown that when $SMCFAs_{extracted}$-rich stream was supplied, the repeat-units composition depends not only on the substrate concentration but also on the culture condition used. The terpolyester consisted of 3HB, 3HV and 3HTD monomers was detected only in the cultivation with 10% of SMCFAs-rich stream under nitrogen starvation. Furthermore, the results indicated that the type of the carbon source and the monomeric composition of the extracted PHAs affected their thermal properties. The results of DSC and TG analyses proved that the presence of 3HtriD fraction in the PHA molecule lowered its melting temperature. The co-polymers extracted from the *Aeromonas* sp. AC_01 cells grown on the mixture that simulated the waste-derived SMCFAs possessed the highest thermal stability. The other analyzed co-polymers were reported to have lower $T_{5\%}$ values than the typical degradation temperatures of PHAs. Nevertheless, these values were high enough to conclude that these biomaterials could be used in commercial applications.

4.4. Publication 4. Szacherska K., Moraczewski K., Czaplicki S., Oleskowicz-Popiel P., Mozejko-Ciesielska J. 2022. Effect of short- and medium-chain fatty acid mixture on polyhydroxyalkanoate production by *Pseudomonas* strains grown under different culture conditions. Front. Bioeng. Biotechnol. 10, 951583. DOI: 10.3389/fbioe.2022.951583.

The aim of the study was to determine the potential of the *Pseudomonas* sp. strains to synthesize and accumulate PHAs grown on SMCFAs and different mineral salt media. In the first stage of the study, the cultivations with synthetic mixture of SMCFAs, which simulate a real SMCFAs-rich stream as the sole carbon source was conducted. Whereas in the second stage, the capability of the most productive strain to metabolize $SMCFA_{sextracted}$-rich stream from MCF of acid whey was tested. The results from the preliminary studies showed that the highest biomass concentration regardless the type of MSM medium was observed in the cultivation of *Pseudomonas* sp. GL06. Furthermore, the results indicated that PHA concentration and productivity increased under unbalanced growth conditions and depended on the type of MSM medium used. Only in *Pseudomonas antarctica* culture on MSM 3 medium, PHAs concentration was not increased by nitrogen limitation. The maximum biopolyesters concentration was detected in *Pseudomonas* sp. GL06 cells grown on MSM 2 medium under N-limited conditions (0.13 g/L) at the PHA productivity level of 2.7 mg/L·h. Moreover, the data suggested that the level of PHAs synthesis can be improved by controlling the nitrogen source. It was found that $(NH_4)_2SO_4$ was more beneficial for PHA yield than NH_4Cl. Furthermore, the data reported that the higher concentrations of the phosphate sources in the culture media ($Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4) resulted in improvement the PHAs productivity in *Pseudomonas* sp. GL06 and *Pseudomonas antarctica* cells. Based on the preliminary results described above, *Pseudomonas* sp. GL06 was the best PHA producer, therefore this strain was selected for further investigations in a 5-L bioreactor with $SMCFA_{sextracted}$-rich stream from the acidogenic anaerobic mixed culture fermentation of acid whey. The data from the conducted fermentation process showed that the biomass concentration increased up to 32 h achieving 1.3 g/L at the end of the bioprocess. Higher cell dry mass values were observed in the *Paracoccus homiensis* cultures using the same carbon source (Szacherska et al., 2022). Furthermore, the results confirmed that PHA productivity increased under nitrogen limitation, reaching a maximum value of 4.5 mg/L·h at 24 h of the process after depletion of ammonium in the growth medium. Comparable results (4.4 mg/L·h) were obtained by Kourmentz et al. (2009) in the cultivation of *Pseudomonas* sp. grown on a mixture of acetic, propionic and butyric acid under

nitrogen starvation. Moreover, PHA productivity was about 60% higher than that achieved in the shake flasks cultures using the same culture medium. The difference between $SMCFAs_{synthetic}$ – rich stream and $SMCFAs_{extracted}$ - rich stream was the presence of lactic acid and ethanol in the latter. Therefore, the results proved that neither of these components negatively influenced the PHA productivity.

The monomeric composition analyses of the extracted biopolymers differed depending on the bacterial strain and the type of growth medium used. The obtained data showed that *Pseudomonas* sp. GL01 and GL06 supplemented with $SMCFAs_{synthetic}$ - rich stream generated high amounts of 3-hydroxyhexanoate (3HHx) and 3-hydroxydodecanoate (3HDD) as major monomers and lower contents of 3-hydroxybutyrate (3HB), 3-hydroxyoctanoate (3HO), 3-hydroxydecaonate (3HD) in the biopolymer molecule. The results also showed that *Pseudomonas antarctica* was able to produce the homopolymer P(3HB) when cultured on MSM 1 and MSM 2 medium under non-limited growth conditions, whereas cultured on MSM 3 was able to produce scl-mcl copolymers. GS-MS analysis confirmed that also *Pseudomonas* sp. GL06 cultured in the bioreactor was capable of synthesizing scl-mcl copolymers consisted mainly of 3HD and 3HDD and smaller amounts of 3HB, 3HHx and 3HO. The thermal analyses showed that glass transition temperature (T_g) of the copolyester, extracted from the bacterial cells cultured in bioreactor, reached -45.4°C and low, untypical melting temperature (T_m=11.7°C). In most cases the reported T_m values of mcl-PHA are in the range from 40 to 55°C. The low T_m value could be related to the low degree of crystallinity of the extracted biopolymer and the predominance of the amorphous phase. The FTIR tests confirmed the low content of the crystalline phase in the biopolyesters. Also, this scl-mcl PHAs copolymer possessed lower degradation temperature (T_d =174.1°C) compared to the T_d values recorded for mcl-PHAs (above 200°C) (Basnett et al., 2017). It could be suggested that the presence of the 3HB monomer in the copolymer molecule could lowered its thermal resistance.

5. Conclusions

The conducted studies were focused on the evaluation of the ability of the selected bacteria to convert effectively synthetic SMCFAs and those derived from the anaerobic mixed culture fermentation of acid whey into value-added polyhydroxyalkanoates. The obtained results led to formulate the following conclusions:

- *Paracoccus homiensis*, *Aeromonas* sp. AC_01, *Pseudomonas* sp. GL01, *Pseudomonas* sp. GL06 and *Pseudomonas antarctica* have the ability to metabolize both synthetic and waste-derived SMCFAs into valuable polyhydroxyalkanoates.

- The type and concentration of the SMCFAs affected biomass concentration, PHA concentration and productivity, monomeric composition and material properties of the extracted biopolymers.
- The unbalanced growth conditions supported the PHA synthesis and accumulation.
- SMCFAs-rich residual stream was found to be less beneficial for PHA productivity compared to the synthetic SMCFAs in the cultivation of *Paracoccus homiensis* and *Aeromonas* sp. AC_01 that suggested more inhibitory effect of waste-derived fatty acids.
- The synthetic mixture of acetic acid and butyric acid was the most preferable carbon source that supported the highest PHA productivity in the *Aeromonas* sp. AC_01 cultivation.
- The PHA productivity reached in the cultivation of *Pseudomonas* sp. GL06 in the bioreactor with $SMCFAs_{extracted}$-rich stream was higher than that determined in the shake flask experiments using a mixture of SMCFAs that mimicked the effluent from whey wastewater anaerobic fermentation.
- The monomeric composition of the extracted PHAs varied depending on the bacterial strain, the type of the culture medium and the carbon source used.
- The novel copolymers of scl-mcl PHAs were extracted from the culture of *Aeromonas* sp. AC_01 and *Pseudomonas* sp. GL06.
- All extracted PHAs possessed promising thermal properties suggesting that they are beneficial for industrial applications.
- The P(3HB-co-3HV) copolymer extracted from the *Paracoccus homiensis* cells cultured with waste-derived SMCFAs had the highest thermal resistance compared to other tested biomaterials which is beneficial taking into account the perspective of these biopolymers processing technology.

- The thermal analyses of the PHAs extracted from the *Aeromonas* sp. AC_01 cells proved that the incorporation of 3HtriD monomer in the copolymer structure lowered its melting temperature.
- Due to the low degree of crystallinity and the predominance of the amorphous phase in the scl-mcl copolymer extracted from the *Pseudomonas* sp. GL06 cells, its melting point was low and unusual. The presence of 3HB monomer in the copolymer molecule could be the reason for its lower thermal resistance.

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Attachments

UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN
FACULTY OF BIOLOGY AND BIOTECHNOLOGY

Olsztyn, 11.08.2022

TO WHOM IT MAY CONCERN

I hereby certify that I am the co-author of the publications being a part of a series publications included in the doctoral dissertation of MSc Karolina Szacherska:

1. Szacherska K., Oleskowicz-Popiel P., Ciesielski S., **Mozejko-Ciesielska J.** 2021. Volatile Fatty Acids as Carbon Sources for Polyhydroxyalkanoates Production. Polymers. 13, 321. DOI: 10.3390/polym13030321.
2. Szacherska K., Moraczewski K., Rytlewski P., Czaplicki S., Ciesielski S., Oleskowicz-Popiel P., **Mozejko-Ciesielska J.** 2022. Polyhydroxyalkanoates production from short and medium chain carboxylic acids by *Paracoccus homiensis*. Scientific Reports. 12, 7263. DOI: 10.1038/s41598-022-11114-x.
3. Szacherska K., Moraczewski K., Czaplicki S., Oleskowicz-Popiel P., **Mozejko-Ciesielska J.** 2022. Conversion of Short and Medium Chain Fatty Acids into Novel Polyhydroxyalkanoates Copolymers by *Aeromonas* sp. AC_01. Materials. 15, 4482. DOI: 10.3390/ma15134482.
4. Szacherska K., Moraczewski K., Czaplicki S., Oleskowicz-Popiel P., **Mozejko-Ciesielska J.** 2022. Effect of short- and medium-chain fatty acid mixture on polyhydroxyalkanoate production by *Pseudomonas* strains grown under different culture conditions. Front. Bioeng. Biotechnol. 10, 951583. DOI: 10.3389/fbioe.2022.951583.

My contribution consisted of participation in the study's design, design of the methodology, coordination of the PhD student's work, supervision over the conducted analyses, participation in the data processing and analysis of results, writing and editing of the manuscripts, preparation of the manuscripts for the publishing procedure.

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OŚWIADCZENIE

Oświadczam, że jestem współautorką prac będących częścią cyklu publikacji wchodzących w skład rozprawy doktorskiej Pani mgr Karoliny Szacherskiej:

1. Szacherska K., Oleskowicz-Popiel P., Ciesielski S., **Mozejko-Ciesielska J.** 2021. Volatile Fatty Acids as Carbon Sources for Polyhydroxyalkanoates Production. Polymers. 13, 321. DOI: 10.3390/polym13030321.
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Mój wkład polegał na współudziale w opracowaniu koncepcji badań, tworzeniu metodyki badań, koordynowaniu pracy Doktorantki, merytorycznym nadzorze nad wykonywanymi analizami, współudziale w opracowaniu wyników badań, merytorycznym redagowaniu treści manuskryptów i przeprowadzeniu publikacji przez procedurę wydawniczą.

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I hereby certify that I am the co-author of a publication being a part of a series publications included in my doctoral dissertation:

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My contribution consisted of participation in the conceptualization, participation in writing and technical editing of the manuscript.

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Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji wchodzących w skład mojej rozprawy doktorskiej:

Szacherska K., Oleskowicz-Popiel P., Ciesielski S., Mozejko-Ciesielska J. 2021. Volatile Fatty Acids as Carbon Sources for Polyhydroxyalkanoates Production. Polymers. 13, 321. DOI: 10.3390/polym13030321.

Mój wkład polegał na współudziale w opracowaniu koncepcji, współudziale w przygotowaniu oraz technicznej redakcji manuskryptu.

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Mój wkład polegał na krytycznym redagowaniu treści manuskryptu.

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TO WHOM IT MAY CONCERN

I hereby certify that I am the co-author of a publication being a part of a series publications included in the doctoral dissertation of MSc Karolina Szacherska:

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Prof. dr hab. inż. Sławomir Ciesielski

Olsztyn, 09.08.2022

OŚWIADCZENIE

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji wchodzących w skład rozprawy doktorskiej Pani mgr Karoliny Szacherskiej:

Szacherska K., Oleskowicz-Popiel P., **Ciesielski S.,** Mozejko-Ciesielska J. 2021. Volatile Fatty Acids as Carbon Sources for Polyhydroxyalkanoates Production. Polymers. 13, 321. DOI: 10.3390/polym13030321.

Mój wkład polegał na krytycznym redagowaniu treści manuskryptu.

Sławomir Ciesielski

WYDZIAŁ GEOINŻYNIERII
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UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN
FACULTY OF BIOLOGY AND BIOTECHNOLOGY

Olsztyn, 10.08.2022

TO WHOM IT MAY CONCERN

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Karolina Szacherska

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WYDZIAŁ BIOLOGII I BIOTECHNOLOGII

Olsztyn, dn. 10 sierpnia 2022 r.

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Karolina Szacherska

(podpis)

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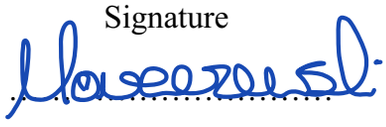
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(podpis)

Dr hab. PIOTR RYTLEWSKI, prof. uczelni

KIEROWNIK KATEDRY INŻYNIERII MATERIAŁÓW POLIMEROWYCH

UNIWERSYTET KAZIMIERZA WIELKIEGO w BYDGOSZCZY

Bydgoszcz, 08.08.2022 r.

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(podpis)

University of Warmia and Mazury in Olsztyn

Faculty of Food Sciences

Plac Cieszyński 1, 10-726 Olsztyn

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Mój wkład polegał na przeprowadzeniu analizy chromatografii gazowej sprzężonej ze spektometrią mas oraz współudziale w walidacji metody GC-MS.

Czaplicki S.

.....

(podpis)

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My contribution consisted of performing the statistical analysis and interpretation of the statistical data.

Sławomir Ciesielski

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Mój wkład polegał na przeprowadzeniu analizy statystycznej oraz interpretacji wyników tej analizy.

Sławomir Ciesielski

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Mój wkład polegał na współudziale w opracowaniu koncepcji, przeprowadzeniu analizy CAs, krytycznym redagowaniu treści manuskryptu.

.....

Prof. dr hab. inż. Piotr Oleśkowicz-Popiel

UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN
FACULTY OF BIOLOGY AND BIOTECHNOLOGY

Olsztyn, 10.08.2022

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Karolina Szacherska

UNIWERSYTET WARMIŃSKO-MAZURSKI W OLSZTYNIE
WYDZIAŁ BIOLOGII I BIOTECHNOLOGII

Olsztyn, dn. 10 sierpnia 2022 r.

OŚWIADCZENIE

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Karolina Szacherska

(podpis)

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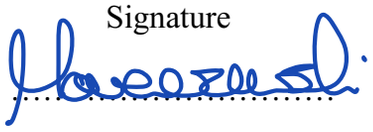
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(podpis)

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Prof. dr hab. inż. Piotr Oleśkowicz-Popiel

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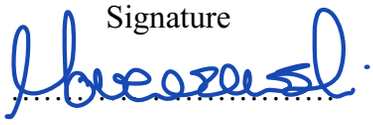
TO WHOM IT MAY CONCERN

I hereby certify that I am the co-author of a publication being a part of a series publications included in the doctoral dissertation of MSc Karolina Szacherska:

Szacherska K., **Moraczewski K.,** Czaplicki S., Oleskowicz-Popiel P., Mozejko-Ciesielska J. 2022. Effect of short- and medium-chain fatty acid mixture on polyhydroxyalkanoate production by *Pseudomonas* strains grown under different culture conditions. Front. Bioeng. Biotechnol. 10, 951583. DOI: 10.3389/fbioe.2022.951583.

My contribution consisted of performing the analyses of material properties and interpretation of the FTIR, differential scanning calorimetric, thermogravimetric data, participation in writing of the part of the manuscript.

Signature



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OŚWIADCZENIE

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Szacherska K., **Moraczewski K.,** Czaplicki S., Oleskowicz-Popiel P., Mozejko-Ciesielska J. 2022. Effect of short- and medium-chain fatty acid mixture on polyhydroxyalkanoate production by *Pseudomonas* strains grown under different culture conditions. Front. Bioeng. Biotechnol. 10, 951583. DOI: 10.3389/fbioe.2022.951583.

Mój udział polegał na przeprowadzeniu analiz właściwości materiałów oraz interpretacji wyników FTIR, skaningowej kalorymetrii różnicowej oraz analizy termograwimetrycznej, współudziale w przygotowaniu części manuskryptu.

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My contribution consisted of performing the analysis of gas chromatography coupled with mass spectrometry and participation in the GC-MS methodology validation.

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Mój wkład polegał na przeprowadzeniu analizy chromatografii gazowej sprzężonej ze spektometrią mas oraz współudziale w walidacji metody GC-MS.

Czaplicki S.

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My contribution consisted of participation in the study's design, performing waste derived SMCFAs analysis and critical revision of the written manuscript.

Signature

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Mój wkład polegał na współudziale w opracowaniu koncepcji, przeprowadzeniu analizy SMCFAs, krytycznym redagowaniu treści manuskryptu.

.....

Prof. dr hab. inż. Piotr Oleśkowicz-Popiel

polymers

MDPI

Review

Volatile Fatty Acids as Carbon Sources for Polyhydroxyalkanoates Production

Karolina Szacherska [1], Piotr Oleskowicz-Popiel [2], Slawomir Ciesielski [3] and Justyna Mozejko-Ciesielska [1,*]

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Abstract: Waste of industrial origin produced from synthetic materials are a serious threat to the natural environment. The ending resources of fossil raw materials and increasingly restrictive legal standards for the management of plastic waste have led to research on the use of biopolymers, which, due to their properties, may be an ecological alternative to currently used petrochemical polymers. Polyhydroxyalkanoates (PHAs) have gained much attention in recent years as the next generation of environmentally friendly materials. Currently, a lot of research is being done to reduce the costs of the biological process of PHA synthesis, which is the main factor limiting the production of PHAs on the industrial scale. The volatile fatty acids (VFAs) produced by anaerobic digestion from organic industrial and food waste, and various types of wastewater could be suitable carbon sources for PHA production. Thus, reusing the organic waste, while reducing the future fossil fuel, originated from plastic waste. PHA production from VFAs seem to be a good approach since VFAs composition determines the constituents of PHAs polymer and is of great influence on its properties. In order to reduce the overall costs of PHA production to a more reasonable level, it will be necessary to design a bioprocess that maximizes VFAs production, which will be beneficial for the PHA synthesis. Additionally, a very important factor that affects the profitable production of PHAs from VFAs is the selection of a microbial producer that will effectively synthesize the desired bioproduct. PHA production from VFAs has gained significant interest since VFAs composition determines the constituents of PHA polymer. Thus far, the conversion of VFAs into PHAs using pure bacterial cultures has received little attention, and the majority of studies have used mixed microbial communities for this purpose. This review discusses the current state of knowledge on PHAs synthesized by microorganisms cultured on VFAs.

Keywords: biodegradable polymers; bioproducts; polyhydroxyalkanoates; volatile fatty acids; waste stream

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1. Introduction

Nowadays, petroleum-based plastics, due to their properties, are one of the most common materials in modern society. Constant production of materials based on synthetic polymers and their intensive human overconsumption leads to irreversible changes that have a negative impact on the natural environment and human health. The growing accumulation of non-biodegradable polymer wastes and the exhaustive reserves of fossil fuels prompted scientists to develop new biopolymer production technologies. Biopolymers are gaining more interest due to not only their biodegradability/compostability, but also biocompatibility, and bio-based nature. Various types of biodegradable polymers are being used in various fields. They are grouped depending on their composition. Polymers

like starch and cellulose belong to the first group and are produced from agro-resources. The second group includes biopolymers synthesized by microorganisms during microbial processes, e.g., polyhydroxyalkanoates (PHAs). The third group consists of polymers that are conventionally and chemically synthesized (polylactic acid (PLA)). The fourth group contains blends of renewable resources with synthetic monomers (soy-based urethanes) [1]. However, until now, PLA and PHAs are the key players in the market for biodegradable polymers. PLA has gained much attention as the effective polymer for biomedical and packing composites. Nonetheless, due to fragility, reduced impact strength, and low thermal stability, PLA has become a hurdle for the application in engineering purposes [2]. Additionally, PHAs are attracting attention as the next generation of environmentally friendly materials and have become an alternative to synthetic polymers because they are completely biodegradable, biocompatible and can be produced from renewable sources [3]. The worldwide production of PHAs from commercial manufacturers reached up to 2.05 million tons in 2017 [4]. The interest in renewable, eco-friendly, and bio-based materials is still high, suggesting that the biopolymers market will be a serious player in the future. It is predicted that the global market value of PHA will grow from USD 57 million in 2019 to USD 98 million by 2024 [5].

PHAs are water-insoluble biopolymers synthesized by microorganisms and collected intracellularly as granular supplementary substances. Microorganisms accumulate PHAs as a reserve material, serving as energy in the presence of excess carbon source and limited concentration of essential nutrients (nitrogen, phosphorus, magnesium, and oxygen) [6]. Excess carbon is used for cell growth and the PHA storage process, and after depletion of carbon source, the stored polymers are used by microorganisms as a source of carbon and energy. PHAs are synthesized by gram-negative bacteria and gram-positive ones, which belong to genera such as *Pseudomonas*, *Bacillus*, *Cupriavidus*, *Aeromonas* and *Rhodobacter*. Thus far, only a few species of bacteria have met the optimal PHA production criteria. The most important are *Cupriavidus necator*, *Alcaligenes latus*, *Pseudomonas oleovorans* or *Pseudomonas putida*. Microorganisms produce a number of PHAs differing in chemical structure. These polymers consist of 100 to 30,000 monomers that bind to each other with ester bonds. The type of the biopolymer synthesized depends on the carbon source, the PHA producer and the bioprocess conditions. Over 150 different types of PHAs are synthesized by various bacterial species under different growth conditions. PHAs are classified based on the number of carbon atoms present in the biopolymer molecule and they can be divided into short-chain-length (PHA_{SCL}), medium-chain-length (PHA_{MCL}) and long-chain-length (PHA_{LCL}) PHAs. PHA_{SCL} contains from three to five carbon atoms, e.g., poly-3-hydroxybutyric acid and poly-3-hydroxyvaleric acid. PHA_{SCL} is rigid and brittle thermoplastic biopolymer with a high degree of crystallinity (60%–80%) [7]. This type of PHA can be produced by a single species (e.g., *Cupriavidus necator*) as well as by mixed microbial cultures (MMC) derived from activated sludge. These polymers can be used in industry in the production of containers and packaging for everyday use. They can be the subject of investigation as food packaging materials due to their processing possibility to packaging films via thermoforming, a high water vapor barrier, a high barrier properties against CO_2, a high oxygen barrier hampered the growth of aerobic microbes and the oxidative spoilage of unsaturated fatty acids [8]. The low oxygen transmission rate and water and CO_2 barriers make PHAs an interesting basic materials for producing bottles for liquid foods, and also for CO_2-containing liquids [9]. As a major drawback of using PHAs as food packaging material is still not satisfying a cost-performance scenario mainly because of the high expenses of raw materials [10]. PHA_{MCL} contain from 6 to 14 carbon atoms in the molecule, e.g., poly-3-hydroxyoctanoic acid or poly-3-hydroxydecanoic acid. They have elastomeric properties and are flexible biopolymers with low crystallinity, low tensile strength, high elongation to break, low melting point and glass transition (below room temperature). PHA_{MCL} are mainly produced by bacteria of the genus *Pseudomonas*. Due to their properties, these polymers can be widely used in the production of paints and pigments, as well as in medicine and pharmacy. PHA_{LCL} contain more than 14 carbon

atoms in their structure, e.g., poly-3-hydroxyhexadecanoic acid [11]. This type of biopolyester is uncommon and knowledge about its properties and potential applications is still limited.

The main limitation in the production of PHA on a large scale is the high process costs, which are 15 times higher than in the case of conventional plastics [10]. Furthermore, the productivity of PHA during fermentations in terms of the amount of PHA formed per liquid volume per time (g PHA/L/h) is an important factor for techno-economical evaluation of this bioprocess. Therefore, it seems reasonable to use substrates that will not only be effectively used by microorganisms for growth and PHA synthesis, but can be obtained at prices that will guarantee the profitability of the process. These substrates could be waste materials such as organic industrial and food waste, and various types of wastewater [12–14]. The use of wastes for the production of biopolymers is a beneficial approach, ensuring the disposal of harmful substances and the creation of environmentally friendly materials [15]. Particular attention has been given to volatile fatty acids (VFAs), which are generated by anaerobic fermentation, such as acetic acid, propionic acid, butyric acid, valeric acid and caproic acid [16,17]. Previous results have shown that VFAs could be effectively used by microorganisms as a source of carbon and energy for the growth and production of PHAs [12,18].

For producing VFAs via anaerobic digestion, the organic fraction of municipal solid waste (OFMSW) is a promising potential substrate. The ever-growing amount of food waste in Europe, reaching about 90 million tons per year, requires the development of innovative solutions to the processing and disposing of it [19]. Therefore, OFMSW is subjected to anaerobic digestion to produce and extract VFAs, which can be further used as valuable sources of carbon for the production of value-added PHAs. Valentino et al. [20] conducted OFMSW fermentation for VFAs production at 55 °C using a 200 L continuous stirred-tank reactor (CSTR) with a hydraulic retention time (HRT) of 3.3 d and an average organic loading rate (OLR) of 20 kg VS/m^3·d. An acidic environment (pH 5.0–5.6) was maintained by recirculation of digestate from a parallel thermophilic anaerobic fermenter. VFA production reached its maximum (22.3 g COD_{VFA}/L) on day 9 (2.7 HRTs). In the fermented VFA stream, butyric acid (37.2%), acetic acid (23.4%), propionic acid (13.2%), valeric acid (10.9%) and caproic acid (9.6%) predominated. The authors also found smaller amounts of isobutyric acid (3.56%) and isovaleric acid (1.44%). Furthermore, it has been shown that pH can influence the rate of VFA production. Cheah et al. [21] revealed that a pH of nine can lead to a higher production of VFAs (high in acetic acid) than a pH of six. They demonstrated that the concentration of VFAs in wastewater increased up to 11.5 g/L.

The aim of this review is to present the challenges to bacterial PHA production using VFAs. Knowledge about the use of VFAs to synthesize PHAs is currently limited, so this review focuses on systematizing their potential in this field.

2. Characterization and Production of Volatile Fatty Acids

VFAs are linear short-chain fatty acids containing from two (acetic acid) to six (caproic acid) carbon atoms. Table 1 presents the characteristics and properties of VFAs.

Table 1. Properties of volatile fatty acids [22,23].

Volatile Fatty Acid	Boiling Point (°C)	Solubility (g/dm^3)	$COD_{equivalent}$ (g COD/g)	Vapor Pressure (mm Hg)	Smell
Acetic acid	117	Complete	1.066	20.9	Sharp smell of vinegar
Propionic acid	141	Complete	1.512	3.3	Sharp, rancid, acrid
Butyric acid	164	Complete	1.816	1.65	Rancid butter, sweat
Isovaleric acid	177	20	2.037	1	Rancid fat, blue cheese
Valeric acid	186	40	2.037	1	Sweat, valerian
Caproic acid	206	11	2.204	0.2	Sharp, sour

Recently, special attention has been paid to the production and recovery of VFAs due to their high potential as renewable carbon sources and their broad range of applications

[24]. VFAs can be used as sources in the production of biopolymers or biofuels (methane and hydrogen) and in the biological removal of nutrients from wastewater [25–27]. Due to the fact that VFAs contain a carboxylic group, they are also used in conventional organic chemistry as precursors of reduced chemical compounds and derivatives (aldehydes, alcohols, alkanes, ketones or esters) [28].

VFAs are intermediate products in the process of anaerobic digestion (AD) of various types of waste, such as food waste, municipal waste, agricultural waste, milk sewage, paper and cellulose sewage. Many microorganisms were reported to be capable of generating VFAs, such as acetic acid, propionic acid and butyric acid using a variety of substrates (Table 2). The AD process consists of a series of stages in which the organic matter is degraded by microorganisms under anaerobic conditions [29]. The synthesis of VFAs by mixed microbial cultures (MMC) is possible due to various metabolic pathways that occur inside cells, depending on the substrate used.

In many cases, co-fermentation of different wastes was employed, for example, agricultural waste and animal manure [30], or waste activated sludge and corn straw [31]. The purpose of co-fermentation is to balance the C/N ratio. Some experiments showed that an increase in the C/N ratio supported the growth of microorganisms responsible for acidogenesis [32,33]. In order to increase the efficiency of VFA production by MMCs, special attention should be paid to the conditions and parameters of the process. In particular, different hydraulic retention times (HRT), organic loading rates (OLR), temperatures and pHs can affect the productivity of VFAs. It is also possible that other by-products will be formed, e.g., long-chain fatty acids, alcohols, biohydrogen, biomethane, esters and other intermediates [34,35].

Bermúdez-Penabad et al. [36] studied the effect of different pH values (from 5.0 to 10.0) on the AD of tuna waste to produce VFAs. The authors showed that varying the pH influenced the production of VFAs. After 32 days of digestion of tuna waste, the highest total VFAs concentration at pH 8 was 30.611 mg COD/L. The resulting VFAs consisted mainly of acetic acid, propionic acid, butyric acid and valeric acid. Furthermore, acetic acid was the dominant product at all pH values tested. Den Boer et al. [37] used a co-culture consisting of *Klebsiella mobilis* and *Escherichia coli* to produce short-chain and medium-chain VFAs from kitchen biowaste and potato peels. The authors tested the effects of various feeding strategies to increase chain elongation under microaerobic conditions at pH 6.0–6.5. In the initial stages of the process, acetic acid and ethanol dominated, while in the later stages, an accumulation of propionic acid was observed, followed by accumulation of butyric and valeric acid. The highest level of final products, 325 mg/g TS, was obtained at pH 6.5, with a yield of 448 mg/L/h. Moretto et al. [38] optimized the fermentation process of urban organic waste to produce a stream rich in VFAs. The effects of different temperatures, pH values, HRTs and OLRs were studied in both batch and continuous processes. Using batch processes at a temperature of 37 °C and a pH of 9 ensured the production of VFAs using waste feedstock thermally pretreated for 76 h at 72 °C. At a later stage, these conditions were used in a continuous process, resulting in the efficient production of VFAs with high levels of VFAs (0.77 $COD_{VFA}/VS_{(0)}$ and 39 g COD_{VFA}/L).

VFAs converted anaerobically from various waste streams could be a promising platform for PHA production. The process of PHA synthesis by microorganisms using waste-derived substrate required expensive enzymes, sterilization processes, and high energy demand, which increases the overall production cost. The synthesis of PHAs using low-cost materials, such as VFAs as the feedstock, is considered to be an economical approach. Furthermore, PHA production from VFAs seems to be a good approach since VFAs composition determines the constituents of PHA polymer and has a great influence on the properties of PHAs [39].

Table 2. Production of volatile fatty acids by microorganisms.

Volatile Fatty Acid	Bacteria	Substrate	Concentration (g/L)	Productivity (g/L/h)	References
Acetic acid	*Acetobacter aceti*	cheese whey	96.9	4.060	[40]
	Clostridium acetium	mixed gas (4% H_2:18% Argon:78% CO)	1.3	nd	[41]
	Clostridium lentocellum SG6	paddy straw	30.9	nd	[42]
	Moorella thermoacetica	sugarcane straw hydrolysate	17.2	nd	[43]
	Saccharomyces cerevisiae + Acetobacter pasteurianus	glucose	66.0	0.367	[44]
Propionic acid	*Propionibacterium acidipropionici* (ATCC 4965)	lactate	15.1	0.113	[45]
		glycerol	6.8	0.051	
		sugarcane molasses	8.2	0.062	
	Propionibacterium acidipropionici (CGMCC 1.223)	glycerol	44.6	0.200	[46]
	Propionibacterium acidipropionici (ATCC 4875)	hemicellulose hydrolysate	18.0	nd	[47]
		chesse whey	19.7	0.980	[48]
	Propionibacterium freudenreichii CCTCC M207015	glucose	67.1	0.140	[49]
	Propionibacterium freudenreichii spp. *shermanii*	glycerol	9.0	0.180	[50]
Butyric acid	*Clostridium butyricum* S21	sucrose	20.0	0.210	[51]
	Clostridium butyricum ZJUCB	glucose	16.7	nd	[52]
	Clostridium thermobutyricum JW171K	glucose	18.4	2.400	[53]
	Clostridium tyrobutyricum	corn husk hydrolysate	20.8	0.420	[54]
		sugarcane bagasse hydrolysate	20.9	0.510	[55]

nd—not determine.

3. Synthesis of PHAs from VFAs by Pure Bacterial Cultures

In order to launch PHAs into the market, there is a need to reduce the costs involved in the production of these biopolymers. Therefore, the use of cheap substrates, including VFAs, may contribute to lowering the cost of obtaining the final bioproduct [56]. Numerous metabolic pathways are involved in VFA synthesis. VFAs are also the preferred carbon source for the synthesis of PHAs, because they are direct metabolic precursors in the PHA biosynthesis pathway (Figure 1). Several pure bacterial cultures are capable of producing PHAs in satisfactory amounts using VFAs as substrates (Table 3).

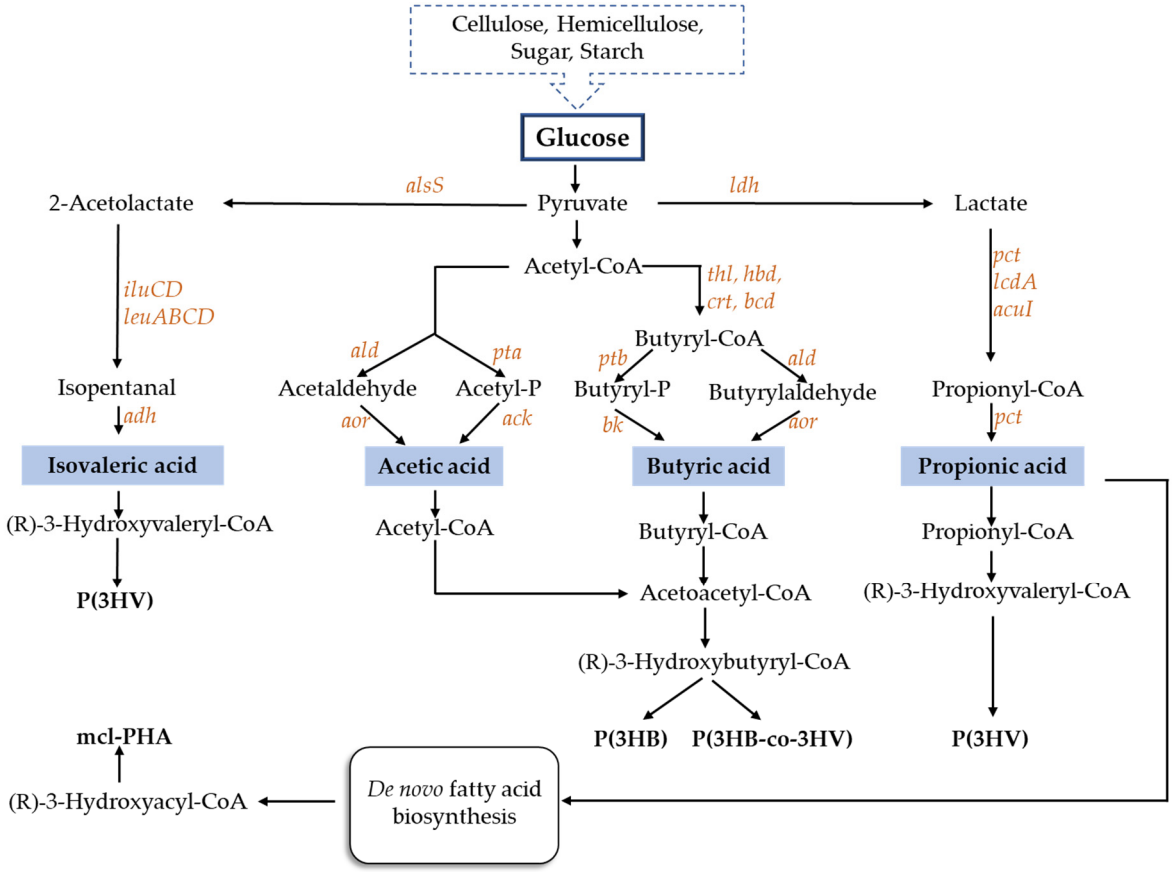


Figure 1. Scheme of metabolic pathways for volatile fatty acids production and polyhydroxyalkanoates synthesis. *ack*—acetate kinase, *acul*—acrylyl-CoA reductase, *adh*—alcohol dehydrogenase, *ald*—aldehyde dehydrogenase, *alsS*—acetolactate synthase, *aor*—aldehyde oxidoreductase, *bcd*—butyryl-CoA dehydrogenase, *bk*—butyrate kinase, *crt*—crotonase, *hbd*—hydroxybutyryl-CoA dehydrogenase, *ilvc*— keto-acid reductoisomerase, *ilvD*—dihydroxy acid dehydratase, *kivD*—2-keto acid decarboxylase, *lcdA*—lactoyl-CoA dehydratase, *ldh*—lactate dehydrogenase, *leuA*—2-isopropylmalate synthase, *leuB*—isopropylmalate dehydrogenase, *leuCD*—isopropylmalate isomerase complex, *pct*—propionyl-CoA transferase, *pta*—phosphotransacetylase, *ptb*—phosphotransbutyrylase, *thl*—thiolase.

Table 3. Production of PHAs by pure bacterial cultures using volatile fatty acids.

Bacteria	Type of PHA	Carbon Source	Biomass (g/L)	PHA Content (%)	PHA Productivity (g/L/h)	References
Ralstonia eutropha ATCC 17699	P(3HB)	acetic acid	5.4	30.8	0.013	[57]
		propionic acid	14.0	29.3	0.036	
		butyric acid	14.5	31.9	0.037	
Bacillus megaterium OU303A	P(3HB)	propionic acid + glucose	nd	62.4	nd	[58]
		propionic acid + glycerol	nd	57.2	nd	
Pseudomonas sp. ST2	P(3HB-co-3HV)	propionic acid + glucose	nd	34.0	nd	[59]
Bacillus sp. CS8		propionic acid + glucose	nd	24.0	nd	
Pseudomonas sp. ST2 + *Bacillus* sp. CS8		acetic acid+ propionic acid + glucose	nd	35.0	nd	
Haloferax mediterranei	P(3HB-co-3HV)	butyric acid + valeric acid + Tween 20	nd	58.9	0.010	[60]
Ralstonia eutropha ATCC 17699	P(3HB-co-3HV)	acetic acid + propionic acid + butyric acid	1.2	25.0	nd	[61]
Ralstonia eutropha KCTC 2658	P(3HB-co-3HV)	acetic acid + propionic acid + butyric acid	1.5	50.0	nd	
Cupriavidus necator	P(3HB-co-3HV)	olive mill wastewater effluent rich in acetic, propionic and butyric acid	2.0	55.0	0.022	[62]
Methylocystis hirsuta DSM 18500	P(3HB-co-3HV)	acetic acid	nd	2.4	nd	[18]
		propionic acid	nd	1.1	nd	
		butyric acid	nd	1.8	nd	
		valeric acid	nd	9.0	nd	
	P(3HB)	acetic acid + biogas	nd	52.3	nd	
	P(3HB-co-3HV)	propionic acid + biogas	nd	47.9	nd	
	P(3HB)	butyric acid + biogas	nd	52.2	nd	
	P(3HB-co-3HV)	valeric acid + biogas	nd	53.8	nd	
Ralstonia eutropha DSM 545	P(3HB-co-3HV)	glucose + propionic acid	52.1	78.3	0.74	[63]
Bacillus sp. INT005	P(3HB)	butyric acid	0.8	31.5	nd	[64]
	P(3HB-co-3HV)	valeric acid	0.7	18.8	nd	
Corynebacterium hydrocarboxydans	P(3HB-co-3HV)	acetic acid	nd	21.0	nd	[65]
Nocardia lucida	P(3HB-co-3HV)	acetic acid	nd	20.0	nd	
Rhodococcus sp.	P(3HB-co-3HV)	acetic acid	nd	29.0	nd	
	P(3HB-co-3HV)	valeric acid	nd	43.0	nd	
Pseudomonas putida KT2440	mcl-PHA	acetic acid	~0.5	26.0	nd	[66]
		propionic acid	~0.5	29.0	nd	
		butyric acid	~0.5	44.0	nd	
		valeric acid	~0.5	19.0	nd	

Pseudomonas putida CA-3	acetic acid	~0.6	~40.0	nd
	propionic acid	~0.6	~40.0	nd
	butyric acid	0.7	~40.0	nd
	valeric acid	~0.6	24.0	nd
Pseudomonas putida GO16	acetic acid	0.4	~10.0	nd
	propionic acid	0.1	~10.0	nd
	butyric acid	0.8	28.0	nd
	valeric acid	0.2	~10.0	nd

nd—not determined.

Poly(3-hydroxybutyrate) [(P(3HB)] is the most well-known and the most widely studied polyhydroxyalkanoic acid. It is a brittle biopolymer with low elasticity, high O_2 barrier, good thermoplastic properties and poor mechanical properties [67]. Chakraborty et al. [57] investigated the ability of *Ralstonia eutropha* ATCC 17699 to produce PHAs using acetic, propionic and butyric acid. This strain synthesizes homopolymer P(3HB) at a concentration of about 31% of cell dry weight (CDW) using acetic and butyric acid. However, higher productivity (0.037 g PHA/g substrate) was observed in a cultivation supplemented with butyric acid. According to López et al. [18], *Methylocystis hirsuta* was able to produce up to 52% of the CDW when grown on acetic and butyric acid as co-substrates during artificial biogas-based cultivation under nitrogen limiting conditions. Higher P(3HB) content was revealed in *Bacillus megaterium* OU303A isolated from municipal sewage sludge [58]. The authors found that this strain synthesized 62.43% of CDW in the presence of propionic acid in a medium containing glycerol. A slightly lower concentration (58.63% of CDW) was observed when the growth medium was supplemented with glucose. The obtained data suggested that the addition of propionic acid supported PHAs synthesis. The bacteria accumulated higher amounts of PHAs when this VFA was added, as compared to the use of individual carbon sources (glucose and glycerol). However, a two times lower P(3HB) content was reached with *Bacillus* sp. INT005 cells cultured on butyric acid [64].

In addition to homopolymers, pure bacterial cultures are able to biosynthesize copolymers using VFAs as carbon sources. Studies have shown that incorporating other PHA monomers into P(3HB) will significantly improve the mechanical properties of a final product, reduce crystallinity and melting temperature, and increase flexibility [68]. Kumar et al. [69] reported that bacteria belonging to the *Bacillus* genus are capable of synthesizing copolymers during cultivations supplemented with a specific mixture of VFAs obtained through the controlled hydrolysis of various wastes, such as pea shells, apple pomace, onion peels, and potato peels. The authors found that *Bacillus thuringiensis* EGU45 and *Bacillus cereus* EGU43 cultured on pea shells hydrolysates accumulated P(3HB-co-3HV) copolymer with a 3HV content of 1% *w*/*w*. Furthermore, it was observed that the efficiency of PHA synthesis could be improved by the addition of onion peel hydrolysate together with the supplementation with glucose. Interestingly, VFAs were not detected after the PHA production phase, suggesting that the applied waste hydrolysates supported efficient bacterial growth, and biopolyester synthesis and accumulation. Furthermore, the effect of combining cultures on PHA production was evaluated by Munir and Jamil [59]. The authors confirmed that a co-culture of *Pseudomonas* sp. ST2 and *Bacillus* sp. CS8 was capable of producing up to 35% P(3HB-co-3HV) copolymer when grown on acetic and propionic acids in combination with glucose. When a glucose and propionic acid mixture was used as a feedstock, the individual strains produced more PHAs than with glucose as the only carbon source. Furthermore, it was observed that *Alcaligenes eutrophus* NCIMB 11599 produced a higher P(3HB-co-3HV) copolymer yield and 3HV fraction of the copolymer in a two-stage fed-batch cultivation when using valeric acid than when using propionic acid. Additionally, *Ralstonia eutropha* KCTC 2658 has been identified as a PHAs copolymer producer when cultured on a mixture of acetic, propionic, and butyric acid [61]. It was reported that P(3HB-co-3HV) production reached 50% CDW using 5 g/L of acetic-acid:propionic-acid:butyric-acid at a proportion of 1:2:2. Furthermore, the authors confirmed that the applied conditions enabled the production of biopolyesters with a 3HV molar fraction of 21 mol%. Interestingly, *Ralstonia eutropha* ATCC 17699 synthesized the copolymer with a higher 3HV fraction (35 mol%), but the cell dry weight and biopolymers content in cells were lower (1.2 g/L and 25% of CDW, respectively). In addition, it was observed that the proportion of VFAs to co-substrates has an influence on the biomass, PHAs content and biopolymer composition. According to Du et al. [63], a low ratio of propionic acid to glucose led to a low concentration of 3HV fraction, but high dry cell

weight and P(3HB-co-3HV) productivity. In contrast, a high propionic-acid-to-glucose ratio led to a high 3HV concentration but a low P(3HB-co-3HV) content in *Ralstonia eutropha* cells.

In addition, Jeon et al. [70] determined that, by altering the levels of the acetyl-CoA pool, it is possible to improve the concentration of 3HV in P(3HB-co-3HV). They overexpressed acetyl-CoA acetyltransferase (*atoAD*) in a recombinant *Escherichia coli* YH090 carrying genes responsible for PHA synthesis (*bktB*, *phaB*, and *phaC*). Use of propionic acid as a carbon source stimulated copolymer synthesis, resulting in a 7.3-fold higher 3HV fraction (67.9 mol%) in the copolymer produced by the above-mentioned mutant than by the strain without the overexpression of *atoAD* genes. Further, *Hydrogenophaga pseudoflava* grown in a medium supplemented with non-hydrolyzed lactose whey and valeric acid was able to synthesize up to 40% P(3HB-co-3HV) with 5 mol% 3HV [71].

A substantial amount of P(3HB-co-3HV) copolymer (55% of CDW) was synthesized by *Cupriavidus necator* cultured on olive mill wastewater effluent rich in acetic, propionic and butyric acid (OMW_{Acid}) without adding any exogenous substrate. OMW_{Acid} contained not only different short chain VFAs, but also polyphenols (1.2 g/L), N-NH4 (60 mg/L), proteins (1.56 g/L), and lipids (3.24 g/L). Furthermore, it was observed that OMW_{Acid} at concentrations of 75% and 100% inhibited the synthesis and accumulation of the copolymer. This work shows that residues from the fermentation of wastes can be used for the biosynthesis of bacterial biopolymers [62]. Such approaches can potentially reduce the production costs of PHAs and offer environmental benefits through the reuse of wastes. It was demonstrated that the type II methanotroph *Methylocystis hirsuta* is capable of synthesizing P(3HB-co-3HV) copolymer using artificial biogas with the addition of VFAs [18]. The highest copolymer concentration was observed in a cultivation with valeric acid as a co-substrate (53.8% of CDW), and the highest 3-hydroxyvalerate content (13.5%) was found within the biocomposite. The same strain accumulated trace amounts of these biopolymers using pure VFAs as the only carbon sources (from 1.1 to 9.0% of CDW). Recently, Ferre-Guell and Winterburn [60] obtained increased P(3HB-co-3HV) copolymer concentrations in a cultivation of *Haloferax mediterranei* grown on a mixture of butyrate and valerate acids with the addition of surfactants to increase substrate bioavailability. The studied strain was able to synthesize up to 59% of the copolymer, resulting in a productivity rate of 10.2 mg/L/h in the fed-batch fermentation. These results demonstrate that a VFA mixture with the addition of Tween 80 supports the synthesis of polymers containing the desired 3HV fraction of 43 mol%.

Thus far, only one study reported the production of mcl-PHAs using VFAs. Cerrone et al. [66] studied the effect of nitrogen limitation on the growth, and synthesis and accumulation of mcl-PHAs by three *Pseudomonas putida* strains (KT2440, CA-3 and GO16) using VFAs produced from the anaerobic digestion of a lignocellulosic substrate. The effluent that was used as a carbon source to support growth and mcl-PHA synthesis consisted of 15.3 g/L of VFAs (from acetic acid to valeric acid) with a predominance of butyric acid (12.8 g/L). The results revealed that the analyzed strains differed in their ability to grow and accumulate mcl-PHAs. The highest mcl-PHA concentration (44% CDW) was detected in the cells of *Pseudomonas putida* KT2440 cultured on butyric acid and acetic acid. Similar levels of biopolyesters were extracted from *Pseudomonas putida* CA-3 grown on acetic, propionic or butyric acid. Furthermore, the production of mcl-PHAs was lower with the GO16 strain than with the KT2440 and CA-3 strains, regardless of the type of VFA. However, gas chromatography analysis revealed that the monomeric composition of the mcl-PHAs extracted from *P. putida* KT2440 and CA-3 cells was similar. The authors reported that 3-hydroxydecanoic acid (3-HD) was the major component. Furthermore, this biopolyester was found to contain lesser amounts of 3-hydroxyoctanoic acid (3-HO) and 3-hydroxydodecanoic acid (3-HDD), and trace amounts of 3-hydroxytetradecanoic acid (3-HTD). Interestingly, *Pseudomonas putida* GO16 produced mcl-PHAs containing higher levels of 3-HDD monomer than those produced by the KT2440 and CA-3 strains, suggesting that its level is dependent on bacterial strain.

4. Synthesis of PHAs from VFAs by Mixed Microbial Cultures

Recently, interest in using MMC for the production of PHAs has increased significantly among researchers. MMC are communities of microorganisms that have the ability to cooperate with each other by carrying out specific intracellular and extracellular reactions. The process of using MMC for the synthesis of PHAs can be economically competitive with the production of PHAs by pure bacterial cultures [72]. Using mixed cultures is simpler and requires less investment and operational expenses in terms of sterile conditions and a feedstock preparation. VFAs such as acetate, propionate, butyrate and valerate can be used efficiently by mixed microbial cultures for the synthesis of PHAs (Table 4).

Acetic acid is one of the best-studied carbon sources for the synthesis of PHAs by mixed cultures under feast and famine conditions. In the metabolic pathway, acetic acid is transformed into P(3HB). The efficiency and rate of polymer synthesis depend on the operating conditions of the bioreactor. Serafim et al. [73] found that aerobic dynamic substrate feeding (ADF) in a sequencing batch reactor (SBR) operated under ammonia limitation favored the selection of cultures with a high P(3HB) storage capacity. Furthermore, the authors observed that the P(3HB) specific storage rate could have been inhibited due to the acetic acid concentration. They confirmed that P(3HB) content reached 67.5% when acetic acid was supplied in one pulse at a concentration of 180 Cmmol/L. However, feeding the substrate pulse-wise caused an increase in the P(3HB) storage rate (78.5%). Beun et al. [74] reported that, during the feast period in an SBR reactor, the P(3HB) content reached only 40% CDW. It was also shown that, in the subsequent fed-batch, the enriched mixed culture was able to produce up to 89% CDW within 7.6 h of continuous feeding with acetic acid [75]. Furthermore, it was concluded that, the longer the cultivation in the feast-famine SBR, the higher the P(3HB) production rate. The average specific rate of PHB production over the first 7.6 h was 1.4 C-mol/C-mol/h (1.2 g/g/h). Moreover, it was proved that the presence of acetic acid stimulates the consumption of butyrate and propionate due to the reactivation of acetyl-CoA, and thus supports the internal activity of cellular metabolism. Thus, it can be assumed that acetic acid is one of the most appreciated VFAs in the production of PHAs by microorganisms [76].

Table 4. Production of PHAs by mixed microbial cultures using volatile fatty acids.

Carbon Source	Type of PHA	PHA (%)	References
Acetic acid	P(3HB)	40.0	[74]
	P(3HB)	78.5	[73]
	P(3HB)	89.0	[75]
Municipal wastewater + acetic acid	P(3HB)	30.0	[77]
Fermented paper mill wastewater (acetic acid, propionic acid, butyric acid, valeric acid)	P(3HB-co-3HV)	48.0	[78]
Fermented molasses (acetic acid, propionic acid, butyric acid, valeric acid)	P(3HB-co-3HV)	66.0	[14]
Fermented food waste (acetic acid, propionic acid, butyric acid, valeric acid) + dewatered sludge	P(3HB-co-3HV)	64.5	[79]
Fermented paperboard mill wastewater (acetic acid, propionic acid, butyric acid, valeric acid)	P(3HB-co-3HV)	67.4	[80]
Sludge hydrolysis liquid (acetic acid, propionic acid, butyric acid, valeric acid)	P(3HB-co-3HV)	24.1	[81]
Fermented crude glycerol	P(3HB-co-3HV)	76.0	[82]
Fermented wood waste (acetic acid, propionic acid, butyric acid)	P(3HB-co-3HV)	50.3	[83]
Fermented cheese whey (acetic acid, propionic acid, butyric acid, valeric acid)	P(3HB-co-3HV)	30.0	[84]

The substrate composition seems to be an important factor that strongly affects the diversity of PHA monomers. Fradinho et al. [76] evaluated the feeding of an acetate-enriched photosynthetic mixed culture (PMC) with mixed VFAs. The authors showed that acetic acid and butyric acid supported P(3HB) homopolymer synthesis, whereas propionic acid supported the formation of P(3HB-co-3HV) copolymers consisting of 51% 3HV. Furthermore, a low acetate concentration (<30 CmM) and specific light intensities around 20 W/gX were determined to be the optimal operating conditions that enable the improvement of P(3HB-co-3HV) content from 15% to 30% in less than 4 h [74]. Additionally, Lemos et al. [85] confirmed that, by manipulating the feed VFA profiles, polymers with different monomer compositions could be synthesized. The results indicated that sludge fed with acetate was able to produce a P(3HB) homopolymer, whereas sludge fed with propionate was capable of synthesizing a P(3HB-co-3HV) copolymer. Furthermore, switching the substrate feeds, i.e., feeding propionic acid to sludge adapted to acetate and acetic acid to sludge adapted to propionate, led to the production of a P(3HB-co-3HV-3HMV) terpolymer.

In the field of PHA biosynthesis, attention has focused on the possibility of using pretreated sludge as a possible carbon source for the synthesis of these polymers. Liao et al. [81] demonstrated that VFAs from heat-pretreated sludge hydrolyzed liquid could support PHA synthesis and accumulation. The authors observed that different heat pretreatment temperatures affect the composition of VFAs, and consequently, the biopolymer productivity rate. Hydrolyzed liquid that was heat pretreated at 60 °C was the optimal carbon source for P(3HB-co-3HV) synthesis, and the maximal copolymer content was 24.1% CDW. At that temperature, acetic acid was the predominant VFA in the hydrolyzed liquid, whereas propionic, butyric and valeric acid were present in smaller amounts. Chua et al. [77] noted that sludge acclimatized with municipal wastewater supplemented with acetic acid could accumulate PHAs up to 30% CDW, whereas sludge without this VFA accumulated 20% CDW.

Wastewater from waste-glycerol fermentation supplemented with different amounts of acetic and propionic acids improves PHA production capacity. In an SBR reactor, a mixed microbial community fed a substrate of organic acids supplemented with acetic acid produced P(3HB), while propionic acid supported P(3HB-co-3HV) synthesis. Genetic analysis revealed that *Thauera* sp. predominated in the reactor with acetate, while *Paracoccus denitrificans* predominated in the reactor with propionate [86]. Additionally, Burniol-Figols et al. [82], using fermented crude glycerol and mixed microbial consortia, confirmed the possibility of selectively converting VFAs into PHAs. The authors extracted up to 76% P(3HB-co-HV) from an SBR reactor where the dominant bacterial taxa were *Amaricoccus* and *Thauera*, which accounted for 56.3%–72.4% of the whole microbial community.

Chen et al. [79] showed the effect of VFAs obtained from co-fermentation of municipal wastewater and food waste on the production of PHAs, and revealed that the feeding regimes affect the rate of PHA synthesis. Continuous pulsed feeding with fermentation fluid containing VFAs (acetic acid, propionic acid, butyric acid, and valeric acid) was most effective, leading to the highest P(3HB-co-3HV) synthesis rate of 64.5%. A similar copolymer content (66% of CDW) was observed when mixed cultures were supplemented with VFAs from fermented molasses [14]. The use of a continuous feeding strategy seems to be more efficient than a pulse feeding strategy and results in higher biopolymer productivity. Furthermore, a continuous feeding regime increased the 3HV content by 8% compared to one using pulse-wise feeding. Lower copolymer content (50.3% of CDW) was observed by Li et al. [83] when VFAs obtained from the co-fermentation of pretreated wood waste and sewage were used as a feedstock. The authors showed that the PHA production rate reached 0.237 g COD PHA/L/h. Monomeric composition analysis revealed that mixed microbial consortia were able to produce P(3HB-co-3HV) copolymer that contained only the 6 mol% 3HV fraction. Promising results were described by Bengtsson et al. [78], who

demonstrated that activated sludge cultivated on VFAs produced by acidogenic fermentation of paper mill wastewater was capable of synthesizing 48% CDW of P(3HB-co-3HV). A higher PHA content was obtained using acetate-enriched bacteria growing on the same fermented effluent [80]. The final biopolymer content was 58.57%, while the PHA yield reached 0.46 g PHA/g VFA. High throughput sequencing of the bacterial *16S* rRNA gene confirmed that *Proteobacteria* and *Bacteroidetes* spp. increased during the enrichment and accumulation phases from 37.4% to 77.6% and from 2.49% to 17.66%, respectively. In the production of PHAs from waste streams, the presence of salts and compounds that inhibit biopolymer productivity should be taken into account. Palmeiro-Sánchez et al. [87] showed that the concentration of sodium chloride has an effect on biopolymer synthesis and accumulation. A mixed microbial culture enriched on a mixture of VFAs containing sodium chloride produced PHAs less efficiently than a culture without sodium chloride (53% of CDW).

5. Value Chain for Deriving Polyhydroxyalkanoates from VFAs

Among many different biodegradable polymers, PHAs deserve special attention due to their properties, which are similar to those of conventional plastics, as well as the fact that they are completely biodegradable to water and carbon dioxide in different environmental conditions [88]. Many efforts have been made in the past decades to increase PHA performance on both the laboratory and pilot levels. The main challenge in the commercialization of PHAs is the high production cost, which results from the low efficiency of the microbial processes.

PHAs derived from microbial processes are much more expensive than those produced using petrochemical processes. The cost of producing disposable products with P(3HB) is higher (7–10 EUR/kg) compared to production with synthetic polymers [89]. The final production costs of PHAs depend mainly on the price of the substrates used as carbon sources for microbial growth. Ensuring that the optimal carbon source is present in the microbial growth medium usually accounts for about 50% of the total cost of the PHA production process, which is why low-cost waste materials that can be effectively used to accumulate PHAs are constantly sought [90]. The conversion of waste materials to PHAs seems to be a solution to make the production of these biopolymers feasible (Figure 2) [91].

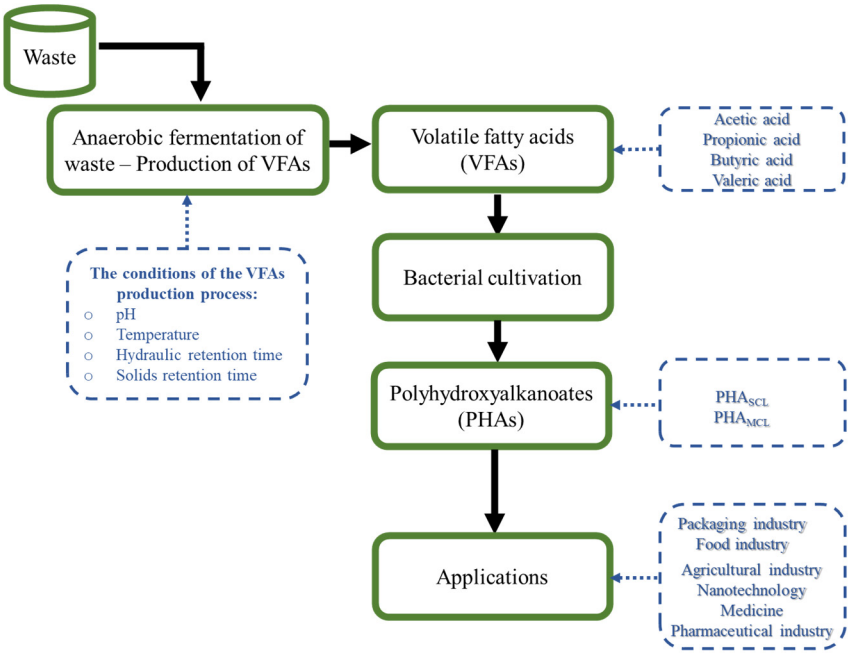


Figure 2. PHAs process value chain using volatile fatty acids.

Many reports show that VFAs could be good carbon sources for PHA synthesis. However, most studies on PHA synthesis are conducted using petrochemical-derived VFAs as carbon sources. In order to reduce the overall costs of PHA production to a more reasonable level, it will be necessary to design a bioprocess that maximizes VFA production, which will be beneficial for the PHA synthesis. While the generation of large amounts of VFAs is desired, it is also necessary to prevent the toxic effect of VFAs on bacterial cells [92]. Mixed-culture PHA production offers the possibility of using renewable VFAs from wastes and industrial effluents. Utilizing fermented wastes or waste sludge as useful resources for VFA production is a promising alternative for reducing PHA production costs.

Additionally, a very important factor that affects the profitable production of PHAs from VFAs is the selection of a microbial producer that will effectively synthesize the desired bioproduct. In recent years, many microorganisms with the ability to synthesize various types of PHA have been studied. The PHA productivity of a microorganism is dependent on many factors, such as the type of carbon source, the temperature, the pH and the concentration of macro and microelements. For PHA production on a large scale, microorganisms should be able to utilize wastes as carbon sources, achieve a good growth rate and produce large amounts of PHAs. However, the concentration of fatty acids may result in toxicity for bacterial cells, that, up to a point, can be overcome by the fast growth of cells [93]. To enhance PHA productivity using VFAs, efforts can be made to use genetically recombined microorganisms. Furthermore, different types of PHAs are suitable for different applications. In particular, mcl-PHAs have gained much attention in recent years. Due to their thermoplasticity and biocompatibility, they can be used for medical or pharmaceutical applications. Thus far, only one study on the biosynthesis of mcl-PHAs has focused on substrates such as VFAs [66]. After bacterial cultivation, the downstream processing of PHA plays a crucial role in the production process. The separation of PHAs from non-PHA cell mass and their purification is technically challenging, especially considering the PHAs extraction that are formed in a wastewater treatment facility. Some downstream processing strategies have recently been comprehensively discussed by Surendran et al. [94].

Many companies have tried to increase PHA productivity and to commercialize them on the market. Currently, the bacterial polymer market is small. Scientists are making every effort to optimize the production process, e.g., by using low-cost and waste materials. To successfully implement the widespread commercial production of PHAs, the culture conditions, the microbial growth conditions and the method of PHA extraction will need to be taken into account.

6. Challenges and Future Perspectives

Biopolymers have received considerable attention in recent years as consumer preferences are shifting toward the use of biodegradable commodities. Among the various types of bio-based polymers, PHAs seem to be the next generation of environmentally friendly materials since their global market size is expected to considerably grow. Due to their useful properties, they are one of the most investigated class of biopolymers estimated to replace some of the today's synthetic polymers. However, the major limitation for PHAs wide industrialization is the high overall cost of their production, mainly because of the price of the carbon substrate used in the microbial cultivation process. Therefore, there is still a need to find efficient substrates to maintain economic feasibility of PHAs commercialization. VFAs are promising carbon sources for PHA production. So far, the conversion of VFAs into PHAs using pure bacterial cultures has received little attention, and the majority of studies have used MMC for this purpose. Indeed, the production of PHAs by MMCs capable of effectively accumulating these polymers can be a cost-effective alternative to their production by pure bacterial cultures, which require sterile process conditions. PHA fermentation using MMC can be continuously conducted in unsterile conditions. Despite this, cultivation with a pure culture is often preferred because it usually leads to reproducible results.

To make PHA production a reality through fermentation using VFAs, there is a need to focus on a certain area, as discussed here. Additional investigations are needed to overcome some difficulties in the use of VFAs for producing PHAs. There are a number of challenges in microbial PHA synthesis from VFAs (Figure 3).

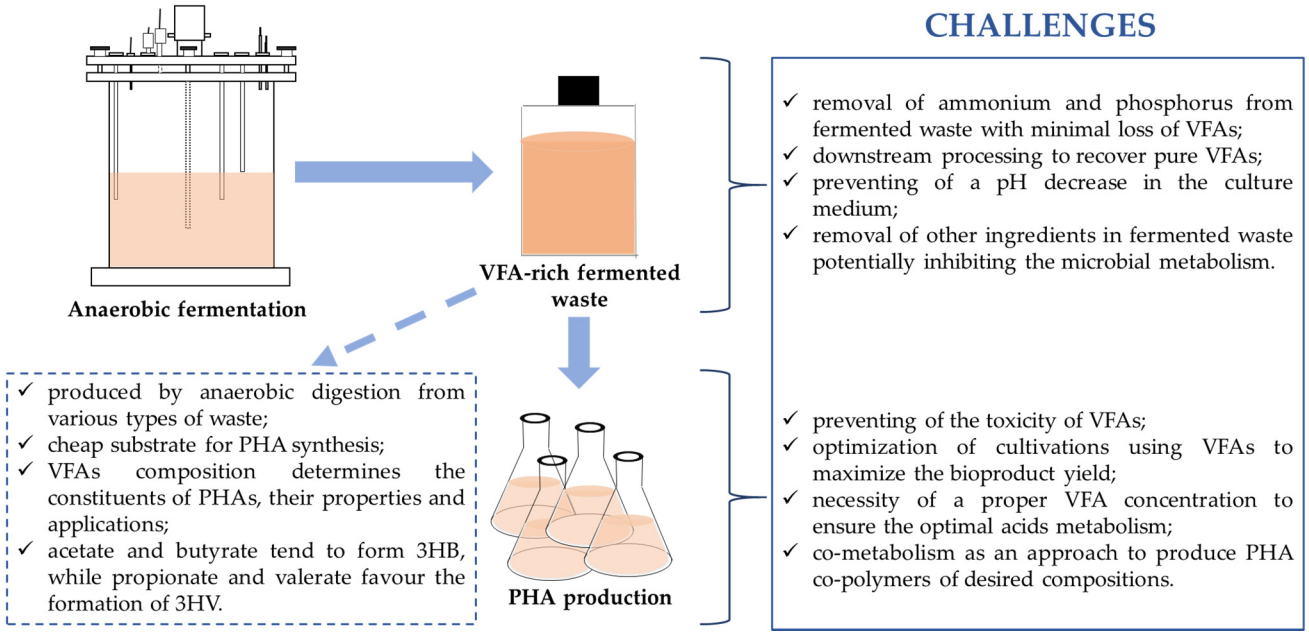


Figure 3. Challenges in producing polyhydroxyalkanoate biopolymers using VFAs.

One of the main challenges is the regulation of ammonium and phosphorus in the VFA-rich fermented waste. It is known that the excess of these nutrients would promote the growth of microorganisms and reduce the PHA yield. The limited nitrogen and phosphorus conditions are of major importance for the effectiveness of the PHA synthesis process [95]. Struvite precipitation was proved to be effective in the rapid removal of these components from fermented waste with a minimal loss of VFAs [96]. Furthermore, the fermented waste should be filtered, especially in the case of PHA production by pure microbial culture [97]. Moreover, one major drawback is the toxicity of VFAs that could inhibit bacteria growth rates; therefore, optimization of the fermentation process using VFAs towards PHA production is crucial to maximize the bioproduct yield [98]. It seems to be necessary to know a proper concentration of VFAs which ensures the optimal acids metabolism to support a bacterial growth to a maximum level and high PHA productivity. Moreover, it was determined that the optimum level of VFAs available to bacteria increases the undissociated VFAs concentration inside their cytoplasm. The higher concentration of VFAs the higher dissociation that influences on the cytoplasm acidity. The reduction in a proton gradient across the membrane has an impact on the rising osmotic pressure that reduces the VFAs uptake inhibiting not only cell activity but also PHAs synthesis and accumulation. VFAs in undissociated form can penetrate the bacterial cell membrane and dissociate inside the cell, which may contribute to blocking ATP synthesis and disturbing the physiological balance [99]. Therefore, some energy must be used to restore the physiological balance in the cell, which contributes to the reduction of energy used for the growth and accumulation of PHAs by microorganisms. However, if the dissociated form of VFAs is present in high concentrations in the fermentation system then can cause an increase in ionic strength and in a consequence cell lysis [39]. Furthermore, VFAs may aggravate a decrease of pH in the culture medium and in a consequence may cause the overall bioprocess failure. In addition, VFAs-rich fermented waste may also contain ethanol, which can lead to high permeability of the cell membrane as well as suppressing the metabolism [100].

It is known that the chain-length of the VFA influences on the composition, properties and application of the synthesized PHAs. The data suggested that the feeding of mixed microbial culture with acetic and butyric acids results in the production of 3HB whereas propionic and valeric acids favor the synthesis and accumulation of 3HV [95]. Due to the poor properties, P(3HB) has limited applications; therefore, the production of PHA co-polymers with better properties is a good approach to overcome the limitations of P(3HB). By the appropriate combination of substrates and supplements, it is possible to produce PHA co-polymers of desired compositions. PHA co-polymers can be achieved by supplementing the feed with volatile fatty acids or through hydrolysates of different biowastes [101,102]. Some strategies of co-utilization were described above (Table 3).

Extensive research and development are still needed to optimize the synthesis of PHAs using VFAs. Only few microbes have been explored for their potential to produce PHAs using VFAs. Isolation and identification of new microbial cultures for a higher productivity is required. The PHAs productivity using VFAs generated in the process of anaerobic digestion of various types of waste is reported to be still too low. The major bottleneck is a downstream processing. There is a need to focus the attention in the area to recover VFAs with a higher productivity and purity. VFAs could also hamper the growth and PHAs synthesis by bacteria. Microbe engineering to tolerate VFAs as feedstocks may be an appropriate approach. Moreover, the great efforts should be made to separate VFAs from a fermentation broth using the cost-efficient method to ensure that the process of PHAs production will be economically feasible.

Advances in bioinformatics and metabolic engineering, along with expanding knowledge of microbial genomes may open many opportunities for the introduction of new metabolic pathways and modification of these pathways in pure bacterial cultures. A better understanding of genetic and metabolic regulation could lead to optimization of PHA production processes and the efficient utilization of VFAs as a source of carbon. In addition, the use of genetically modified microorganisms should be investigated as a potential method of improving PHAs production in the bioprocesses using VFAs. From a practical point of view, it is essential to construct a bacterial platform that will provide a consistent PHA molecular structure, thus ensuring stable material properties, which are essential for further applications of these polymers.

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Polyhydroxyalkanoates production from short and medium chain carboxylic acids by *Paracoccus homiensis*

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The aim of this study was to evaluate an effect of short and medium chain carboxylic acids (CAs) rich stream derived from acidogenic mixed culture fermentation of acid whey on polyhydroxyalkanoates (PHAs) synthesis by *Paracoccus homiensis* and compare it with the impact of individual synthetic CAs. The obtained results confirmed that the analyzed bacterium is able to metabolize synthetic CAs as the only carbon sources in the growth medium with maximum PHAs production yields of 26% of cell dry mass (CDM). The replacement of the individual CAs by a CAs-rich residual stream was found to be beneficial for the *Paracoccus homiensis* growth. The highest biomass concentration reached about 2.5 g/L with PHAs content of 17% of CDM. The purified PHAs were identified as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by applying gas chromatography coupled with mass spectrometry, Fourier transform infrared spectroscopic spectra and UV–Vis spectra. Furthermore, a differential scanning calorimetric, thermogravimetric and water contact angle analysis proved that the extracted copolymers have useful properties. The obtained data are promising in the perspective of developing a microbial PHAs production as a part of an integrated valorization process of high CAs content waste-derived streams.

Due to the dependence of synthetic polymers on fossil fuels and the sustainability issues related to plastic waste, biopolymers have attracted much attention in the last few decades[1]. Polyhydroxyalkanoates (PHAs) are especially attractive because being biodegradable, non-toxic and biocompatible they can replace conventional petroleum-based polymers in the near future[2]. Furthermore, they are produced in biotechnological processes, which enables to control their chemical structure and physico-thermal properties during cultivations of microorganisms[3]. PHAs are accumulated in bacterial cells as carbon and energy reserves under environmental stress in the form of granules with a diameter of 0.2–1.0 mm[4]. They are classified according to the number of carbon atoms presents in the biopolymer molecule. There are short-chain-length PHA (scl-PHA) containing 3–5 carbon atoms in the molecule, medium-chain-length PHA (mcl-PHA) which contains 6–14 carbon atoms in the molecule, and long-chain-length PHA (lcl-PHA) with more than 15 carbon atoms[5]. Moreover, because of their unique properties they have great potential in biomedical, agricultural and industrial applications[6]. However, their production on the commercial scale is still limited due to their high production costs compared to their synthetic alternatives. Thus, there is a need for the optimization of novel fermentation processes using renewable, low value carbon sources. Furthermore, to improve physicochemical properties of biopolymers, there is a need to study the biosynthesis process in the context of the possibility to synthesize not only poly(3-hydroxybutyrate) homopolymer [P(3HB)] but also co-polymers with different content of the monomers. So far, various low value substrates have been studied as potential carbon sources for PHAs production, but most of them required additional pretreatment steps that further increased the PHA production costs[7,8].

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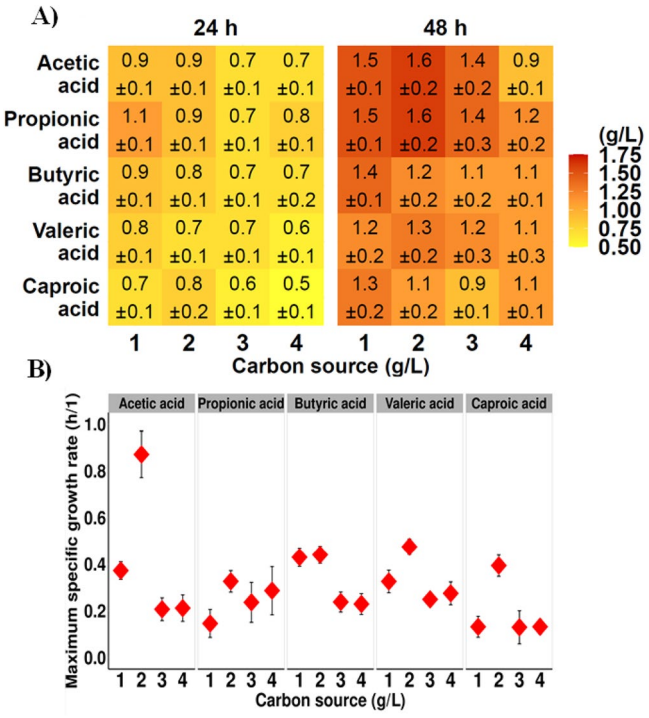


Figure 1. Profiles of *P. homiensis* growth in the medium supplemented with individual short and medium chain carboxylic acids as the only carbon source. (**A**) Biomass concentration (g/L) in 24 h and 48 h of the cultivations; (**B**) maximum specific growth rate (μ_{max}).

A promising substrate for PHAs production are short and medium chain carboxylic acids (CAs) generated by acidogenic anaerobic mixed culture fermentation (MCF). Significant research attention is being given to CAs since they can be converted into high-value bioproducts such as PHAs[9]. The utilization of CAs for PHAs synthesis will not only make cost-effective PHAs production feasible but also will be beneficial for the environment since CAs are produced through degradation of organic wastes largely available worldwide. The synthesis of PHAs from synthetic CAs has been conducted by employing bacteria such as: *Pseudomonas* sp. ST2[10]; *Ralstonia eutropha*[11] or *Haloferax mediterranei*[12].

To the best of our knowledge, only in a few publications the potential of microorganisms to convert CAs-rich streams from MCF using organic wastes into value-added PHAs was evaluated[13–16]. None of this study evaluated in details properties of the extracted biopolyesters. Moreover, there is still a lack of studies that consider the biopolyesters production from CAs using species belong to *Paracoccus* genera, even though these bacteria have substantial biotechnological potential[17]. Hence, in this study, the individual synthetic CAs and those derived from MCF of acid whey were used as feedstocks to determine the possibility of producing PHAs by *Paracoccus homiensis*. Additionally, the impact of the type of CAs and CAs-rich stream concentration on the content of biopolymers in bacterial cells, the growth of the bacterial producer and the monomeric composition of the final bioproducts were determined. Furthermore, the extracted PHAs were comprehensively characterized taking into account their physico-thermal and water related properties.

Results and discussion

Growth and PHAs production using individual synthetic CAs. To determine a type and a suitable concentration of individual CAs that guarantee the effective growth of the *Paracoccus homiensis* and efficient PHAs production, the culture medium was supplemented with the different concentrations of acetic acid, propionic acid, butyric acid, valeric acid and caproic acid. The same growth medium conditions and the amount of inoculum were maintained in all cultivations.

As shown in Fig. 1A,B all tested CAs were utilized to support cell growth and PHAs synthesis and accumulation. During all cultivations, the growth rate depended on the individual CAs concentration. In all experiments, the maximum specific growth rate (μ_{max}) reached a higher value than 0.1/h (Fig. 1B). Using 2 g/L of acetic acid, both maximum μ_{max} (0.87/h) and biomass concentration (1.6 g/L) were obtained. It was previously suggested that acetic acid is less toxic and could be easier metabolized by bacteria than other tested CAs[18]. Saraphirom et al.[19] revealed that when acetic acid is present in the medium microorganisms prefer to utilize acetic acid than butyric acid. Our study showed that the addition of 2 g/L of propionic acid or valeric acid also resulted in higher biomass concentration compared to other analyzed concentrations of these CAs. For butyric and caproic acids the most preferable concentration to support the bacterial growth was 1 g/L, at this level the CDM values reached 1.4 and 1.3 g/L, respectively. Moreover, the results confirmed that with an increase of all CAs concentration to 4 g/L, μ_{max} and CDM value decreased. However, the CDM concentration was higher at 48 h of all treatments

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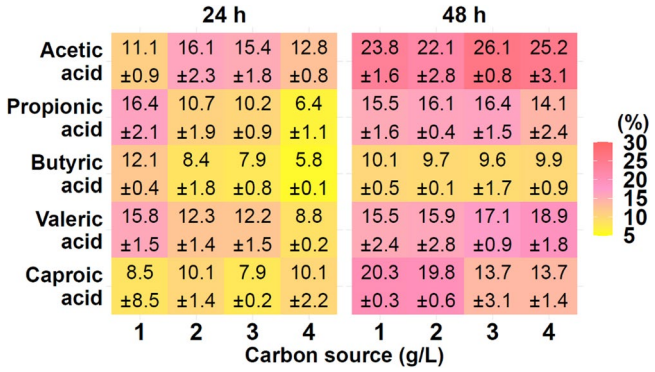


Figure 2. PHAs content (% of CDM) in *P. homiensis* cells in 24 h and 48 h during growth in the medium supplemented with individual short and medium chain carboxylic acids as the only carbon source.

compared with 24 h. The reported differences in the μ_{max} values were related to different metabolic pathways that were involved in the utilization of CAs. Acetic acid, present in the culture medium can be initially converted to acetyl-CoA which is one of an intermediate in PHA synthesis. Then, two acetyl-CoA moieties are transformed to acetoacetyl-CoA which is needed for the synthesis of 3HB monomer. This monomer could be directly synthesized via acetoacetyl-CoA when butyric acid is present in the growth medium. For propionic acid the carbonyl carbon of propionyl-CoA is eliminated that enable to synthesize both 3HB and 3HV units[20].

Our results indicated that the PHAs content in *P. homiensis* cells was dependent on the type and concentration of individual CAs. The analyzed bacteria were able to produce PHAs in all conducted cultivations with CAs. As expected, no PHAs were synthesized in bacterial cells grown on Bacto Marine Broth (treated as a control). As may be observed from data reported in Fig. 2 the highest PHAs production was obtained when acetic acid was added as a single carbon source. However, the statistically significant difference ($P = 0.014$) was found only between cultivations supplemented with acetic and butyric acid. The best accumulation of the PHAs from this CA was achieved at 3 g/L (26.1% of CDM). Also, this concentration was ideal for PHA production by *P. homiensis* grown on propionic acid, however, about 1.6 fold lower PHA accumulation value was observed compared with acetic acid. The bacteria could efficiently metabolized valeric acid at 4 g/L to produce higher content of the biopolymer (18.9% of CDM) compared to other concentrations of this substrate. PHA synthesis and accumulation by *P. homiensis* was inhibited as the caproic acid concentration increased reaching 20.3% and 13.7% of CDM at 1 g/L and 4 g/L, respectively. This could be due to inhibitory effect of the substrate for the accumulation mechanisms of the analyzed bacterial cells. Too high CAs level could have reduced the proton gradient across the cell membrane and increased osmotic pressure of the cells due to their need to pump out anions to survive under acidic pH conditions in the growth medium. Moreover, the presence of organic acids in cytoplasm results in releasing protons that caused its acidification and more energy is used to maintain the gradient inside the cell[14]. Thus, higher CAs concentration used in our study may have resulted in low acid utilization and PHA concentration. Therefore, the optimization conducted within our studies seems to be essential as the undissociated CAs molecules could enter the cytoplasm and in a consequence could be activated and utilized into CO_2, bacterial cell growth or PHA synthesis only when CAs are present at appropriate levels[21,22]. Thus, CAs concentration should be carefully regulated during bacterial cultivations. In general, the lowest biopolymer content in all treatments was observed when the bacteria were cultured on butyric acid at all analyzed concentrations (from 9.7 to 10.1% PHAs of CDM). Kedia et al.[23] have also revealed the inhibitory effect of butyric acid for PHAs storage ability by *Cupriavidus necator,* a lower PHA concentration was observed when this acid was used as the only substrate compared to acetic acid. Nevertheless, these results are not in accordance with the data reported by Chakraborty et al.[24] for *Ralstonia eutropha* which grown on butyric acid and yielded the best results of PHA concentration compared to acetic acid. It could be suggested that for *P. homiensis* butyric acid is not energetically efficient as other CAs for biopolyesters synthesis and accumulation. Furthermore, the cell mass concentration could be the primary factor that affect this acid tolerance of the analyzed bacteria[25]. During the *P. homiensis* growth in the medium supplemented with butyric acid, the lowest CDM level was achieved.

Growth and PHAs using CAs-rich stream. Studies on CAs utilization for the PHA biosynthesis by *P. homiensis* have not been described in literature so far. The production of these biopolyesters by other well-known microorganisms are limited to the use of individual synthetic CAs. Therefore, to determine the effect of CAs derived from acid whey in more detail, CAs-rich stream was added to the Bacto Marine Broth at varying concentrations (from 5 to 30% v/v). During all cultivations, an increase in the absorbance of the cultures was observed. The highest μ_{max} (0.16/h) and CDM (2.5 g/L) value was determined when 30% of CAs-rich stream was added to the culture broth (Fig. 3A,B). PHA production was observed under all CAs-rich stream concentrations. At 24 h, the PHAs content decreased when the carbon source concentration increased (from 7.7 to 2.7% of CDM). However, at the end of the experiments, the PHAs content was the highest in *P. homiensis* cells cultivated in culture medium containing 25% CAs-rich stream, about 16.7% of CDM. As may be observed in Fig. 3C, the further increase of the concentration of this waste carbon source resulted in a decrease of PHAs

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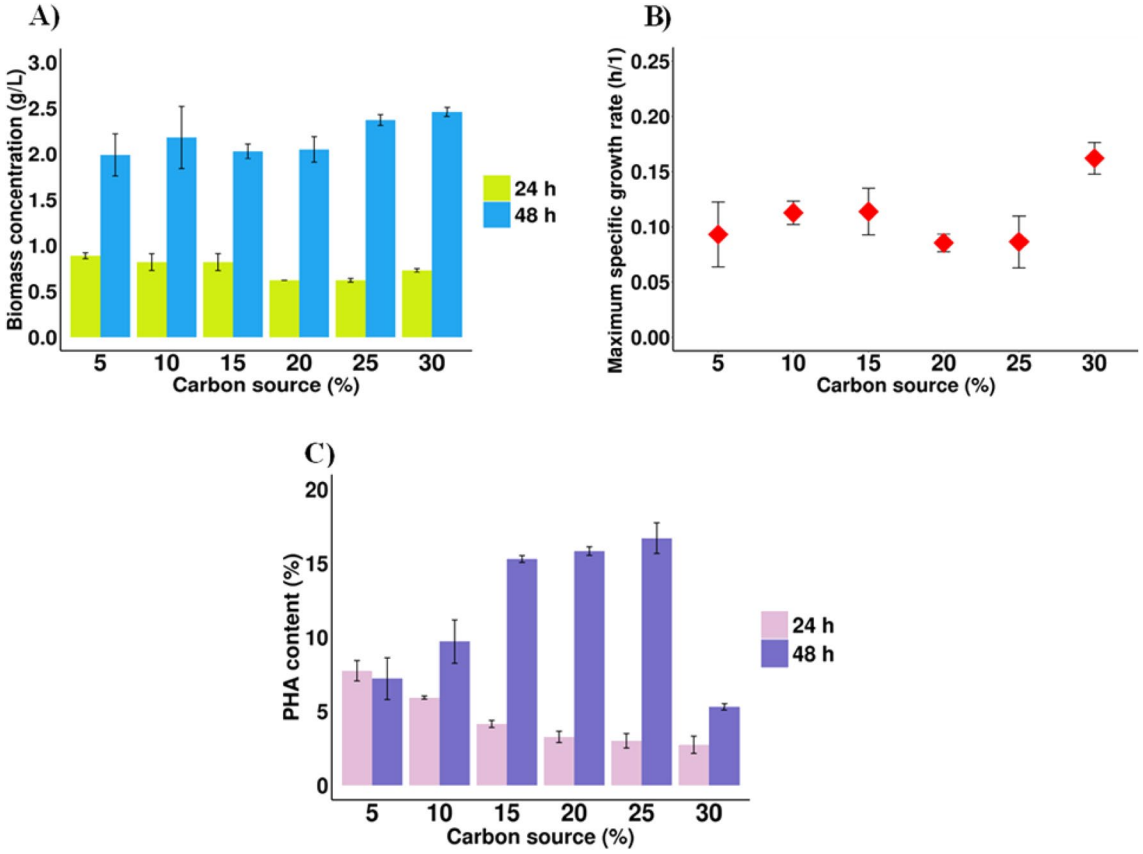


Figure 3. Profiles of *P. homiensis* growth and PHAs content in the medium supplemented with different concentrations of CAs-rich stream from acidogenic mixed culture fermentation of acid whey as the only carbon source. (**A**) Biomass concentration at 24 h and 48 h of the cultivations; (**B**) Maximum specific growth rate (μ_{max}); (**C**) PHAs content in *P. homiensis* cells at 24 h and 48 h. Mean values are calculated from triplicate measurements.

content. PHAs were detected in a trace amount in the bacterial cells cultured in the medium supplemented with 30% CAs-rich stream (5.3% of CDM). Relatively lower PHA content from CAs-rich stream compared to the individual CAs proved that CAs-rich stream had more inhibitory effect due to its complexity. The inhibition of PHA biosynthesis could have been caused by the CAs-rich stream which may interfered with the proton gradient mechanism[15]. Furthermore, quite low PHA productivity could be explained by the presence of butyric acid as the dominant component in the CAs-rich stream which as a single carboxylic acid in the medium resulted in the lowest PHAs production efficiency compared with others. It should be also noted that the presence of high CAs concentration can reduce growth rate, therefore most researchers add such carbon sources after reaching by bacteria its maximum growth, whereas in our study CAs-rich stream was added at the beginning of the cultivations. Furthermore, the experiments were conducted in 250-mL shake flasks in which a control of the parameters like aeration rate, pH value is not possible. It could be presumed that the above mentioned factors might have affected achievement of higher PHA concentration. However, the obtained PHA content was about twofold higher than that reported by Vu et al.[16] who cultivated *Bacillus megaterium* also on the CAs-rich stream from the acidogenic fermentation of food waste. To improve the PHA yields, CAs feeding adjustment, optimization of oxygen, pH control is needed.

Characterization of extracted PHA copolymers. *PHA biopolymer repeat-units composition.* The extracted PHAs from all performed cultivations fed with individual CAs and CAs-rich stream were subjected to gas chromatography analysis to provide information about their repeat-units composition (Table 1). Our data indicate that the monomeric composition varied with the carbon sources concentration and the monomers content was changeable at different process time.

The obtained results proved that *P. homiensis* cultured on acetic acid and caproic acid showed a tendency to synthesize the biopolymer consisted of high 3HB monomer and lower 3HV monomer for all concentrations and both time intervals. The 3HV content increased with prolonging the process time. The data revealed that the *P. homiensis* fed at 2 g/L of propionic acid and 4 g/L of butyric acid produced P(3HB) homopolymer. At other concentrations of those carboxylates the 3HB and 3HV monomers were detected. However, higher 3HV content was observed in the cultivations with propionic acid at both 24 h and 48 h of the processes. The highest 3HV content was confirmed in the P(3HB-co-3HV) copolymer extracted from the cells grown on valeric

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Carbon source	Substrate concentration (g/L; %*)	PHA monomeric composition (mol%)			
		24 h		48 h	
		3HB	3HV	3HB	3HV
Acetic acid	1	99.2	0.8	98.8	1.2
	2	99.4	0.6	98.8	1.2
	3	99.3	0.7	98.7	1.3
	4	99.5	0.5	99.1	0.9
Propionic acid	1	93.2	6.8	91.2	8.8
	2	100.0	0.0	92.7	7.3
	3	94.3	5.7	90.9	9.1
	4	93.6	6.4	91.3	8.7
Butyric acid	1	99.2	0.8	99.0	1.0
	2	99.3	0.7	99.1	0.9
	3	99.4	0.6	99.0	1.0
	4	100.0	0.0	99.3	0.7
Valeric acid	1	40.1	59.9	37.6	62.4
	2	17.5	82.5	33.9	66.1
	3	18.2	81.8	16.6	83.4
	4	14.7	85.3	13.0	87.0
Caproic acid	1	98.9	1.1	98.1	1.9
	2	99.0	1.0	98.8	1.2
	3	98.8	1.2	98.5	1.5
	4	98.9	1.1	98.6	1.4
CAs—rich stream	5*	98.6	1.4	98.5	1.5
	10*	97.6	2.4	98.8	1.2
	15*	97.3	2.7	98.3	1.7
	20*	92.5	7.5	98.5	1.5
	25*	95.9	4.1	98.4	1.6
	30*	95.1	4.9	98.1	1.9

Table 1. Monomeric composition of PHAs produced by *P. homiensis* grown on synthetic and waste derived short and medium chain carboxylic acids. 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; CAs, carboxylic acids. %* - an unit of the concentration of CAs-rich stream

acid. The 3HV content was higher than 3HB content at both 24 and 48 h and this fraction showed higher share with increase in valeric acid concentration, from 59.9 to 85.3 mol% at 24 h and from 62.4 to 87.0 mol% at 48 h.

Our data showed that the utilization of CAs-rich stream led to the synthesis and accumulation of the P(3HB-co-3HV) copolymer. Due to the presence in the CAs-rich stream high amounts of acetic acid and butyric acid, low concentration of valeric acid, and the absence of propionic acid the extracted copolymer consisted of the lower 3HV content than 3HB. Furthermore, the GC results proved that the 3HV content was higher at 24 h compared to 48 h ($P = 0.045$). At 24 h of cultivation, the 3HV content was positively correlated with the CAs-rich stream concentrations (Spearman's correlation coefficient = 0.83). Surprisingly, the highest 3HV content (7.5 mol%) was observed when 20% of CAs-rich stream was added. At 48 h correlation between the 3HV content and the CAs-stream concentrations was not observed. Generally, higher 3HV content was observed when higher CAs-stream concentrations were applied. The possibility of the copolymer production using CAs-rich stream is very attractive since pure P(3HB) has limited applicability due to similar melting and degradation temperatures[26]. However, the 3HV content in the extracted polymers was not sufficiently high, therefore to increase the level of this monomer the induction of the acidogenic fermentation to produce more CAs with an odd carbon number would be beneficial[15].

FTIR spectra. In the FTIR spectra, several bands have been observed that can be assigned to functional groups characteristic for P(3HB-co-3HV) copolymer[27]. The spectra of PHAs samples extracted from the cultivations with acetic, propionic, butyric, caproic acid and CAs-rich streams were similar and only in the case of P(3HB-co-3HV) sample from valeric acid some differences were observed. FTIR spectra of the analyzed samples were recorded in the range from 4000 to 700 cm^{-1} (Supplementary materials 1). A band with a maximum of ~ 3333 cm^{-1} was observed only for the PHA sample produced by *P. homiensis* grown on valeric acid. It probably originates from terminal –OH groups present at the ends of the polymer chains. The presence of this band confirms earlier assumptions about the large number of shorter macromolecules (oligomers). The band in the range 3100–2700 cm^{-1} is associated with the presence of the $-CH_3$ and $-CH_2$ groups. Clearly larger peaks with a maximum of ~ 2925 and ~ 2852 cm^{-1} in this material are due to the higher content of 3HV units, which con-

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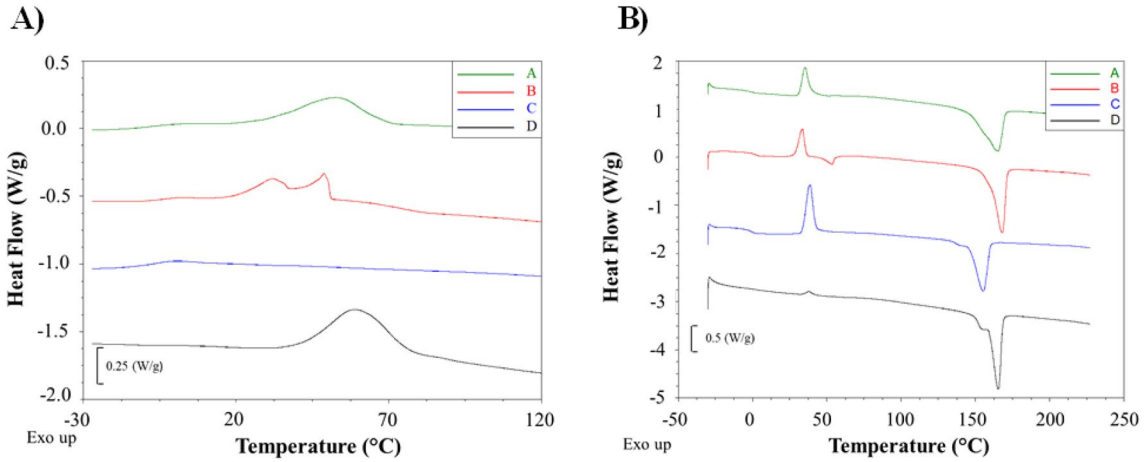


Figure 4. DSC curves (**A**) cooling, (**B**) second heating of selected samples: A—PHAs from acetic acid; B—PHAs from valeric acid, C—PHAs from caproic acid, D—PHAs from 15% of CAs-rich stream.

tain additional $-CH_3$ and $-CH_2$ groups. The single peak band with a maximum of ~ 1722 cm^{-1} is derived from C=O carbonyl groups present in macromolecules. In the spectra range from 1500 to 800 cm^{-1}, which is a so-called fingerprint region, several bands with numerous peaks were recorded. Peaks ~ 1460, ~ 1379, ~ 980, ~ 900 and ~ 825 cm^{-1} are associated with the presence of the $-CH_3$ and $-CH_2$ groups, as well as C–C bonds. Whereas, peaks ~ 1278, ~ 1228, ~ 1180, ~ 1132, ~ 1101 and ~ 1053 cm^{-1} are associated with bonds in the C–O–C region. Unlike the other extracted biopolyesters in the produced copolymer using valeric acid, instead of the ~ 1278 and ~ 1228 cm^{-1} peaks, one high intensity peak derived from C–O–C bonds was recorded with a maximum at 1263 cm^{-1}. The observed changes are related to the high crystallinity degree of this biopolymer. Peaks ~ 1278 and ~ 1228 cm^{-1} are very sensitive to the content of the crystalline phase[28]. In the amorphous materials in the 1330–1230 cm^{-1} range two peaks are observed, while for semi-crystalline materials only one[29].

UV–VIs absorbance spectra. The spectra of the UV–VIs absorbance revealed three absorbance peaks attributed to the wavelength range from about 200 to about 400 nm (Supplementary Materials 1). The absorption bands structure is similar to the samples representing typical absorption bands for 3HB. The polymers, extracted from the bacterial cells cultured on acetic, propionic, butyric and caproic acid, showed high 3HB content (above 93%) and low 3HV content. Except for the P(3HB-co-3HV) sample from valeric acid, for which only very insignificant absorption bands were identified in that spectra region. This resulted most likely from the different chemical structure of this biopolymer for which the 3HB content is significantly reduced (only about 17 mol%).

Physico-thermal properties of extracted PHAs. The extracted PHAs properties determine which applications these materials are best suited for and how they can be processed. Therefore, in this work, the influence of the CAs on the thermal properties of the accumulated PHAs was also analyzed (Fig. 4; Table 2; Supplementary materials 1).

Cooling curves, and therefore the nature of changes in the material as a result of cooling, of individual materials followed one of four types. In the first type, only the inflection related to the glass transition of the tested biomaterial was observed on the cooling curve. This type was determined for PHAs extracted from the bacteria cells grown on propionic and caproic acid. The T_g values determined from the cooling curves of these materials were − 3.9 and − 6.3 °C, respectively.

In the second type of cooling curve, in addition to a clear glass transition inflection, an exothermic peak associated with the crystallization of the biomaterial was also observed. This type occurred in PHAs from the cultivation supplemented with acetic and butyric acid, with the crystallization peak being much larger for the PHAs produced using acetic acid, which indicates a more intense crystallization process and more crystalline phase formation. The determined T_g and T_c values were − 6.6 and 52.9 °C for the biopolymer synthesized using acetic acid and − 5.6 and 46.6 °C using butyric acid.

A third type of cooling curve pattern was observed for the PHAs extracted from the bacterial cells grown on valeric acid. The T_g of this biopolymer was − 3.1 °C. Also, for this biomaterial an exothermic crystallization peak was revealed on the cooling curve. However, the crystallization peak was bimodal with clearly visible two arms of similar intensity with a maximum at 32.3 and 48.9 °C. The reason for the bimodal peak of crystallization may be the high content of 3HV mers, which significantly affected the nature of this process or the presence of a large number of macromolecules with a much lower molecular weight (oligomers). As a result, two crystalline phases with different structure and/or composition of crystallites having different crystallization temperatures may be formed during cooling.

The fourth type of cooling curves was observed for polymers obtained from CAs-rich stream. The course of the cooling process was very similar to the course of polymers obtained from acetic and butyric acid. However, the temperatures of the crystallization process and the intensity of the process were much higher. The highest crystallization temperature for this group of polymers was 65.4 °C and the enthalpy value of the crystallization

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Carbon source	PHA composition	Cooling			2nd Heating						Thermogravimetric analysis			
		T_g [°C]	T_c [°C]	ΔH_c [J/g]	T_g [°C]	T_{cc} [°C]	ΔH_{cc} [J/g]	T_m [°C]	ΔH_m [J/g]	X_c [%]	$T_{5\%}$ [°C]	$T_{50\%}$ [°C]	$T_{95\%}$ [°C]	T_{max} [°C]
Acetic acid	P (99.2% HB-co-0.8% HV)	− 6.6	52.9	31.2	− 0.8	35.5	47.2	165.0	78.8	29.0	232.4	258.4	296.3	262.0
Propionic acid	P (93.6% HB-co-6.4% HV)	− 3.9	nd	nd	0.8	38.0	60.2	158.9	62.5	2.1	228.7	252.6	366.6	253.0
Butyric acid	P (99.4% HB-co-0.6% HV)	− 5.6	46.6	9.0	1.9	34.2	68.9	165.8	72.1	2.9	215.9	243.2	452.3	240.2
Valeric acid	P (17.5% HB-co-82.5% HV)	− 3.1	48.9 (32.2)[a]	32.7	2.2	33.4	17.0	168.1 (53.6)[a]	72.8 (6.3)[a]	51.2	184.0	248.6	439.5	253.9
Caproic acid	P (98.8% HB-co-1.2% HV)	− 6.3	nd	nd	− 1.4	38.6	59.3	155.4	60.4	1.0	218.9	262.5	406.4	262.9
5% CAs—rich stream	P (98.5% HB-co-1.5% HV)	nd	57.2	32.7	1.5	39.7 (89.6)	31.4	165.4 (152.3)[a]	71.4	36.8	238.0	257.4	339.4	259.1
10% CAs—rich stream	P (98.8% HB-co-1.2% HV)	nd	65.4	44.5	2.0	38.2 (90.4)[b]	20.1	166.0 (155.5)[a]	66.9	42.9	234.5	251.9	527.4	253.5
15% CAs—rich stream	P (98.3% HB-co-1.7% HV)	nd	59.2	43.8	2.5	38.1 (85.4)[b]	24.6	165.4 (155.0)[a]	79.2	50.2	232.2	251.4	266.5	253.8
20% CAs—rich stream	P (98.5% HB-co-1.5% HV)	nd	59.2	51.9	2.3	38.5 (86.1)[b]	17.5	162.0 (150.3)[a]	74.9	52.7	231.2	250.2	397.2	251.9
25% CAs—rich stream	P (98.4% HB-co-1.6% HV)	nd	65.4	54.1	1.9	41.8 (86.5)[b]	21.9	163.4 (152.9)[a]	75.3	49.0	230.9	252.6	335.1	255.0
30% CAs—rich stream	P (98.1% HB-co-1.9% HV)	nd	61.1	28.9	1.0	45.0 (86.2)[b]	38.6	161.4 (149.6)[a]	65.9	25.0	221.9	247.4	409.4	247.3

Table 2. Thermal properties of PHAs using short and medium chain carboxylic acids as the only carbon sources. nd, not detected. [a]A distinct and separate, second crystallization and melting peak. [b]A second cold crystallization peak.

process was 54.1 J/g. However, it was not possible to determine the glass transition for these samples due to the low intensity of this process.

The heating curves of the analyzed biopolymers were similar. The inflections and exothermic or endothermic peaks typical for the occurrence of glass transition, cold crystallization and melting of the crystalline phase processes were observed on all curves. T_g values determined from the second heating curves of individual biomaterials ranged from − 1.4 (PHAs from caproic acid) to 2.5 °C (PHAs from 15% CAs-rich stream), which agrees with the literature values that have been shown for a P(3HB-co-3HV) copolymer[30,31].

Regardless of the nature of the crystallization during cooling, all biopolyesters showed a tendency to cold crystallization, which was the cause of the exothermic peak observed on the curves. T_{cc} values were ranged from 33.4 (PHAs from valeric acid) to 45.0 °C (PHAs from 30% CAs-rich stream). An interesting two-stage course of the cold crystallization process was observed for polymers obtained from CAs-rich stream. In the initial phase of cold crystallization there was a slight peak at about 40 °C, then the process was less intensive in a wide temperature range, which was reflected in an exothermic peak of low intensity ranging from 50 to 120 °C with a maximum around 90 °C. More significant changes were observed in the intensity of the cold crystallization process, which resulted from the course of the crystallization during cooling.

The lowest ΔH_{cc} value of 17.0 J/g was obtained for PHAs extracted from the analyzed strain grown on valeric acid, which is probably due to the different composition of this sample (high content of 3HV units) and its negative impact on susceptibility to cold crystallization. The remaining samples had significantly higher ΔH_{cc} values ranging from 47.2 (PHAs from acetic acid) to 68.9 J/g (PHAs from butyric acid). The course of the cold crystallization process depended on the course of the crystallization during cooling. The lower the intensity of the crystallization process during cooling, the greater the peak of cold crystallization, and therefore the intensity of this process.

The last phase change observed in the studied temperature range was the melting of the crystalline phase seen as an endothermic peak. The highest T_m value of 168.1 °C was obtained for PHAs from valeric acid, which were also characterized by a high value of ΔH_m. This data proved the high intensity of this process and the high content of the crystalline phase. The degree of crystallinity of this sample was 51.2% and was the highest among all tested biomaterials. In addition, a second endothermic melting peak, not found in other samples, with a maximum at 53.6 °C and ΔH_m of 6.3 J/g was observed on the heating curve of this biopolymer. The low melting point of this peak suggests that the most likely reason for its occurrence is the melting of lower molecular weight macromolecules (oligomers), which at the same time suggests that they are the cause of the bimodal crystallization peak during cooling.

Similar melting temperatures were obtained for PHAs extracted from the cultivations supplemented with acetic acid and butyric acid. The T_m values of these biopolymers were 165.0 and 165.8 °C, respectively. Although the difference in ΔH_m was only about 7 J/g, they differed significantly in the degree of crystallinity. The calculated X_c values were 29.0 and 2.9%, for PHAs from acetic acid and butyric acid, respectively. These differences resulted from the crystalline phase formation stage. In the case of PHAs from butyric acid, most of the crystalline phase is formed during the cold crystallization process, so it is practically non-existent in the starting material suggesting it is amorphous. Whereas, in PHAs from acetic acid, most of the crystalline phase is formed during the cooling of this material, so the crystalline phase already occurs before it is heated, indicating that it is semi-crystalline.

The melting temperatures of the polymers obtained from CAs-rich stream ranged from 161.4 to 166 °C. However, the biggest difference compared to other polymers was the presence of a distinct second melting peak

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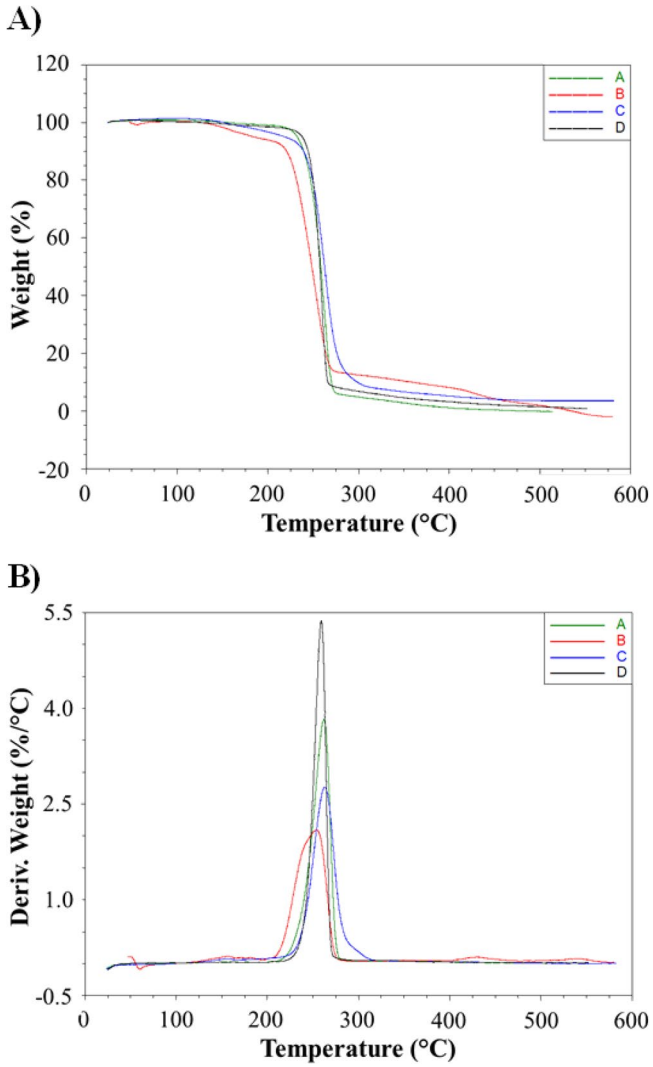


Figure 5. Curves (**A**) thermogravimetric and (**B**) derivative thermogravimetric of selected samples; A—PHAs from acetic acid; B—PHAs from valeric acid, C—PHAs from caproic acid; D—PHAs from 5% of CAs-rich stream.

around 150 °C. The appearance of the second melting peak resulted from the two-stage process of cold crystallization, in which two crystalline phases with distinctly different melting points were formed. The degree of crystallinity of the polymers obtained from the cultivations with waste derived CAs ranged from 25.0 to 52.7%.

Lower melting temperatures and melting enthalpy changes were obtained for PHAs extracted from the cultivations with propionic and caproic acid. The T_m and ΔH_m values were 158.9 °C and 62.5 J/g, and 155.4 °C and 60.4 J/g, respectively. The intensity of the melting process of these samples was therefore similar, which with a similar course of the crystallization and the cold crystallization processes resulted in a similar degree of their crystallinity. The X_c values of these biopolymers were less than 3%, indicating that the starting materials had an amorphous structure.

Figure 5 shows the TG and DTG curves of selected samples (PHAs from acetic acid, valeric acid, caproic acid and 5% of CAs-rich stream, the remaining ones are shown in Supplementary materials 1). The curves of the remaining samples are similar and correspond to one of the presented, so they were not added. The numerical results of TG measurements of all samples are shown in Table 2. In the analysis of test results, as the temperature of material degradation enabling determination of its thermal resistance, a temperature of 5% loss of the mass of the tested sample was assumed ($T_{5\%}$). The $T_{5\%}$ depended on the characteristic of the weight loss process and ranged from 238.0 (PHAs from 5% CAs-rich stream) to 184.0 °C (PHAs from valeric acid).

The biopolymer extracted from the cultivation supplemented with CAs-rich stream had the highest thermal resistance among all tested biomaterials. The $T_{5\%}$ of those polymers ranged from 221.9 to 238.0 °C. The highest crystalline phase content among these tested biopolyesters, characterized by greater thermal resistance than the amorphous phase, was probably the reason for the highest thermal resistance of this materials. However, as the concentration of CAs-rich stream increased, the thermal resistance of the extracted biopolymers decreased,

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Carbon source	PHA composition	Water contact angle [°]	Surface energy [J/cm²]
Acetic acid	P (99.2% HB-co-0.8% HV)	29.5 ± 5.4	64.0
Propionic acid	P (93.6% HB-co-6.4% HV)	nd	nd
Butyric acid	P (99.4% HB-co-0.6% HV)	103.6 ± 0.8	20.4
Valeric acid	P (17.5% HB-co-82.5% HV)	68.2 ± 1.3	42.2
Caproic acid	P (98.8% HB-co-1.2% HV)	78.5 ± 1.3	35.9
5% CAs—rich stream	P (98.5% HB-co-1.5% HV)	81.4 ± 0.4	34.1
10% CAs—rich stream	P (98.8% HB-co-1.2% HV)	71.3 ± 1.1	40.3
15% CAs—rich stream	P (98.3% HB-co-1.7% HV)	98.4 ± 1.1	26.6
20% CAs—rich stream	P (98.5% HB-co-1.5% HV)	64.9 ± 1.7	44.2
25% CAs—rich stream	P (98.4% HB-co-1.6% HV)	85.5 ± 0.8	31.5
30% CAs—rich stream	P (98.1% HB-co-1.9% HV)	65.5 ± 2.4	43.9

Table 3. Average water contact angles and surface energy of the analyzed PHAs films. nd, not determined; HB, hydroxybutyrate; HV, hydroxyvalerate.

despite even higher degree of crystallization. $T_{5\%}$ dropped to 221.9 for PHAs obtained from 30% of CAs-rich stream.

Similar high thermal resistance was observed for the biopolymer extracted from the cultivation supplemented with acetic acid. The $T_{5\%}$ was 232.4 °C, and the highest rate of weight loss occurred at T_{max} 262.0 °C. High thermal resistance, as in the case of PHAs obtained from CAs-rich stream, resulted from the high degree of crystallinity of this material. Noteworthy is the high thermal resistance of PHAs obtained from the cultivations using propionic acid. It is similar to the resistance of PHAs extracted from the cultures with acetic acid and CAs-rich stream, despite a much lower degree of crystallinity. These polymers are characterized by a higher content of 3HV units and this may be the reason for the observed increase in thermal resistance.

The P(3HB-co-3HV) produced in the bacterial cultivations with butyric acid and caproic acid had lower thermal resistance ($T_{5\%}$ values were 215.9 and 218.9 °C, respectively) suggesting that the onset of thermal degradation of these materials was similar. However, these biopolymers differed significantly in the rate of degradation process. The T_{max} value of PHAs from caproic acid was 253.9 °C, while the value of PHAs from butyric acid was lower by more than 20 °C and was the smallest among all tested biomaterials indicating that it has the lowest thermal stability. Low $T_{5\%}$ of the above mentioned copolymers were due to the characteristic of the weight loss process. Based on the TG curves, it can be seen that the degradation is a two-step process. In the first step, a slight decrease of about 5% of the sample mass were observed, followed by a second step with rapid mass loss. The cause of the initial slight weight loss may be small-molecule compounds characterized by lower degradation temperatures contained in the biomaterial, which are residues after the synthesis process or macromolecules with shorter polymer chains. For the PHAs from valeric acid a loss of 5% of sample weight occurred already at 184.0 °C because of a high content of smaller macromolecules (oligomers). It was also confirmed by DSC analysis as well as observed evident two-step characteristic of weight loss, which in the first stage was as much as 8%. However, further rate of the mass change was similar to the other samples, which is proved by the T_m value of 253.9 °C.

Water related properties of extracted PHAs. The obtained water contact angles and calculated value of surface energy (SE) of the extracted biopolymers were summarized in Table 3. For the PHA-copolymer from propionic acid water droplet was rapidly dispersed on the surface, thus the results were not presented. As shown in Table 3 the smallest water contact angles was detected for the PHAs extracted from the *P. homiensis* cells cultured on acetic acid. The average contact angle of 29.5° suggested that this sample had strong hydrophilic properties and a high surface energy value. Whereas, the highest average water contact angle was observed for the PHAs from butyric acid indicating their high hydrophobic properties.

Conclusion

Inexpensive carbon sources are essential to make the PHA production process economically feasible. Our results indicated that *P. homiensis* has the potential to convert CAs into PHAs which have not been reported before. As the use of pure CAs would be costly, we propose the novel idea of utilizing CAs-rich stream derived from acidogenic mixed culture fermentation of agro-industrial waste without necessity for substrate pretreatment for production of P(3HB-co-3HV) copolymer. The structure of the copolymer was analytically verified and we found that the extracted biopolymers have properties that are beneficial for industrial applications. The obtained results proved that all copolymers produced by *P. homiensis* possessed good thermal stability. However, the P(3HB-co-3HV) extracted from the cultivations supplemented with CAs-rich stream had the highest thermal resistance. These polymers show a relatively wide processing thermal window, since the difference between melting temperature and degradation temperature is higher compared to the polymers extracted from the cultivations with synthetic CAs. However, to maximize PHA production, it would be essential to extend the knowledge on metabolic pathways in *P. homiensis* that are engaged in the biopolymers synthesis.

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Methods

Bacterial strain and inoculum preparation. The *Paracoccus homiensis* (DSMZ 17862) used in the study was purchased from the German Collection of Microorganisms and Cells Cultures GmbH in Leibniz Institute. Before inoculation into the Erlenmeyer flasks, *P. homiensis* cells were transferred from glycerol deep-frozen stock at − 80 °C to 250 mL Erlenmeyer flasks where the analyzed strain was cultured in Bacto Marine Broth at 28 °C with 120 rpm shaking for 16 h. The pre-culture medium contains the following components per liter: 5 g bacto peptone, 1 g bacto yeast extract, 0.1 g Fe(III) citrate, 19.45 g NaCl, 5.9 g $MgCl_2$ (anhydrous), 3.24 g Na_2SO_4, 1.8 g $CaCl_2$, 0.55 g KCl, 0.16 g $NaHCO_3$, 0.08 g KBr, 34 mg $SrCl_2$, 22 mg H_3BO_3, 4 mg Na-silicate, 2.4 mg NaF, 1.6 mg $(NH_4)NO_3$, 8 mg Na_2HPO_4. The pH-value of the medium was adjusted to 7.6 through the addition of an appropriate amount of 1 N NaOH or 1 N HCl. The medium components, together with water, were autoclaved at 121 °C.

Carbon sources and culture conditions for PHA production. The carbon source in the culture medium were individual CAs (acetic acid, propionic acid, butyric acid, valeric acid and caproic acid) and CAs-rich stream from MCF of acid whey. Acid whey was collected from a crude cheese production line, upon usage, feedstock was stored at 4 °C. The CAs production was carried out in a 1 L working volume UASB bioreactor constructed from cylindrical plexiglass with recirculation ensuring biomass suspension as described before in Duber et al.[32]. The process was carried out at constant temperature of 30 °C and constant pH of 5.5 with automatic control using 2 M NaOH. The effluent for PHAs production was taken from a steady-state phase with high butyrate production and its characteristics were as follows: acetic acid (2.85 g/L), butyric acid (9.86 g/L), valeric acid (0.16 g/L), caproic acid (3.05 g/L), lactic acid (9.31 g/L), and ethanol (1.64 g/L).

The flask experiments were conducted in triplicate in 250 mL Erlenmeyer flasks containing 100 mL of Bacto Marine Broth (the composition described above) supplemented with varying concentration of individual CAs from 1 to 4 g/L and CAs-rich stream from 5 to 30% v/v. The CAs were added at the beginning of the cultivations. Fermentation shake flasks were inoculated with 5% v/v of the seed and were subsampled for PHA production. The cultivation with Bacto Marine Broth was treated as a control. All experiments were conducted in a rotary shaker at 110 rpm and the bacteria were incubated at 28 °C for 48 h. The cells were harvested to determine the optical density value, cell concentration, PHAs content and physico-chemical analysis of the purified biopolymers.

Analytical procedures. Cell growth was monitored by measuring absorbance at 600 nm (OD_{600}) using a spectrophotometer (Eppendorf, Germany). To obtain more accurate information on the growth of the test strain, the maximum specific growth rate (μ_{max}) was determined as an equation for the linear trend line for the exponential phase. Linearization was made in which the slope of the linear regression gives μ_{max} in 1/h. To estimate cell dry mass (CDM), culture samples were harvested at 24 and 48 h and centrifuged at 9000 × *g* for 10 min. Next, the supernatant was removed and the biomass pellet was lyophilized for 24 h using Lyovac GT2 System (SRK Systemtechnik GmbH, Riedstadt, Germany). PHAs were extracted from lyophilized cells by shaking in hot chloroform at 50 °C for 3 h. Then, the mixture was filtered through No. 1 Whatman filter paper to remove cellular debris. Biopolymers dissolved in chloroform were purified by precipitation with 70% cold methanol and then allowed to evaporate at room temperature. The biopolymer content (% of CDM) was defined as the percentage of the ratio of PHAs concentration to total cell concentration.

PHAs analysis. The monomeric composition of produced PHAs was determined by gas chromatography coupled with mass spectrometry (GC–MS QP2010 PLUS, Shimadzu, Japan) according to the method described by Możejko-Ciesielska and Pokój[33]. Firstly, the analyzed samples were suspended in mixture: chloroform–methanol-sulfuric acid (100/97/3, v/v/v) and methylation was done by heating the vials at 100 °C for 20 h. Then, sulfuric acid neutralize with Na_2CO_3 addition and obtained mixture was dried with anhydrous Na_2SO_4. After filtration methyl esters were analyzed with the use of a BPX70 (25 m × 0.22 mm × 0.25 mm) capillary column (SGE Analytical Science, Victoria, Australia) with helium as a carrier gas at a flow rate of 1.38 mL/min. The column was uniformly heated from 80 to 240 °C at the rate of 10 °C/min. The interface and ion source temperatures of GC–MS was set at 240 °C, and the electron energy was set at 70 eV. The total ion current (TIC) mode was used in 45–500 m/*z* range. Known quantities of pure 3-hydroxyacids standards (Larodan, Sweden) were used in quantitative analysis.

Infrared spectroscopy studies were performed using a FTIR Nicolet iS10 spectrophotometer (Thermo Scientific, USA). Fourier transform infrared spectroscopic spectra (FTIR) were obtained by attenuated total reflection (ATR-FTIR). Each analyzed spectrum was the average of 16 measurements recorded in the wave number range from 4000 to 650 cm^{-1}. UV–Vis spectra were recorded on a spectrometer Evolution 220 (Thermo Scientific, USA) equipped with an integrating sphere. Each analyzed spectrum was the average of 16 measurements recorded in the wavelength from 200 to 1100 nm.

Differential scanning calorimetry (DSC) tests were carried out in a nitrogen atmosphere using a Q200 scanning calorimeter (TA Instruments, USA). Samples of approximately 1 mg were tested in the temperature range from − 30 to 210 °C. Due to the nature of the samples and the need to use several layers of material in one measuring crucible, the samples were heated to 210 °C in the initial stage of the test and kept at this temperature for 1 min. The sample was then cooled to − 30 °C and reheated to 210 °C. In both cases, the temperature change rate was 10 °C/min. From the cooling and second heating curves, glass transition temperature (T_g), crystallization temperature (T_c), change in enthalpy of the crystallization process (ΔH_c), cold crystallization temperature (T_{cc}), change in enthalpy of the cold crystallization process (ΔH_{cc}), melting point (T_m), change in enthalpy melting process (ΔH_m) and the degree of crystallinity (X_c) were determined. The X_c values were calculated from the formula:

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$$X_c = \frac{\Delta H_m - \Delta H_{cc}}{\Delta H_{m100\%}}$$

where $\Delta H_{m100\%}$—change in melting enthalpy of a 100% crystalline sample—109 J/g[34].

Thermogravimetric analysis was carried out in a nitrogen atmosphere using the Q500 thermal balance (TA Instruments, USA). Samples of approximately 1 mg were tested in the temperature range from room temperature to 600 °C with a temperature change rate of 10 °C/min. Based on thermogravimetric curves, the values of $T_{5\%}$, $T_{50\%}$ and $T_{95\%}$ corresponding to the temperature of 5, 50 and 95% loss of the initial sample mass were determined. From the differential thermogravimetric curve (DTG) (first derivative of the TG curve), the T_{max} values determining the temperature of the fastest mass loss were also determined.

The hydrophilicity of biopolymers surface was evaluated by measuring the water contact angle formed between water drops and the surface of the samples using a DSA 100 goniometer (Krüss GmbH, Germany) equipped with an automatic dosing system for measuring liquid drop was used. Measurements were carried out using water (polar liquid) and diiodomethane (dispersion liquid). A drop of measuring liquid was placed on the surface of the test sample, its volume was constantly increased and at the same time the dynamic contact angle was measured, which in this case was the character of the inflow angle. After 12 measurements of contact angles with each measuring liquid, the smallest and largest values of these angles were rejected. The arithmetic mean was calculated from the remaining 10 values. Surface energy (SE) was calculated using Neumann method. The formula for the Neumann method applied to SE was in the following form:

$$cos\theta_L = 2(\gamma_L/\gamma_S)^{0.5} exp\left[-\beta(\gamma_L - \gamma_S)^{0.5}\right]$$

where Υ_S- SE of polymer sample, Υ_L- SE of water, and $\beta = 0.0001247$ (equation parameters).

Statistical analysis. Statistical evaluation of all data was conducted using the STATISTICA v.13.1 (StatSoft, Inc, Tulsa, OK, USA). All samples were analyzed in triplicate. The Mann–Whitney U-test was used to determine the significance of differences in PHAs yield and biomass between experimental variants. Data were reported as the median ± SD at the significance level of $P < 0.05$. Spearman's correlation analysis was carried out with a significance threshold of $\alpha = 0.05$.

Data availability

All data generated or analyzed during this study are present in the paper (and its Supplementary Information files).

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Author contributions

K.S. participated in the study's design, performed all cultivations and PHA extractions. K.M. and P.R. performed and interpreted a FTIR, differential scanning calorimetric, thermogravimetric and water contact angle analyses. S.Cz. performed and interpreted gas chromatography coupled with mass spectrometry analysis. S.C. conducted and interpreted statistical analysis. P.O.P performed waste derived CAs analysis and participated in the study's design. J.M.C. participated in the study's design and supervised in study coordination. K.S. and J.M.C wrote the paper with input from all the authors. All authors revised and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Conversion of Short and Medium Chain Fatty Acids into Novel Polyhydroxyalkanoates Copolymers by *Aeromonas* sp. AC_01

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Abstract: Polyhydroxyalkanoates (PHAs) production by *Aeromonas* sp. AC_01 was investigated using synthetic and waste derived short and medium chain fatty acids (SMCFAs). The obtained results revealed that the analyzed bacterial strain was able to grow and synthesize PHAs using SMCFAs. The highest PHA productivity was observed in the cultivation supplemented with a mixture of acetic acid and butyric acid (3.89 mg/L·h). Furthermore, SMCFAs-rich stream, derived from acidogenic mixed culture fermentation of acid whey, was found to be less beneficial for PHA productivity than its synthetic mixture, however the PHA production was favored by the nitrogen-limited condition. Importantly, *Aeromonas* sp. AC_01 was capable of synthesizing novel scl-mcl copolymers of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), 3-hydroxytridecanoate (3HtriD) and/or 3-hydroxytetradecaonate (3HTD) with high 3HB and 3HV fractions. They were identified with alterable monomers composition depending on the culture conditions used. Moreover, in-depth thermal analyses proved that they are highly resistant to thermal degradation regardless of their monomeric composition. The obtained results confirm that *Aeromonas* sp. AC_01 is a promising candidate for the biotechnological production of PHAs from SMCFAs with thermal properties that can be tuned together with their chemical composition by the corresponding adjustment of the cultivation process.

Keywords: *Aeromonas* sp.; biopolymers; medium chain fatty acids; PHAs; PHA copolymers; polyhydroxyalkanoates; short chain fatty acids

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1. Introduction

In the last few decades, plastics have become an integral part in our daily life. Due to their persistence and accumulation in the ecosystems as a harmful waste, it is crucial to replace petrochemical-derived compounds with biodegradable, biobased and environmentally friendly polymers derived from renewable resources. Among biopolymers, polyhydroxyalkanoates (PHAs) are particularly a promising alternative to conventional plastics. They are produced by different microorganisms from a range of substrates under nutrient-limited conditions such as nitrogen, phosphorus or oxygen [1]. They are classified into short-chain-length PHA (scl-PHA) containing 3–5 carbon atoms per monomer, medium-chain-length PHA (mcl-PHA) with 6–14 carbon atoms per monomer, and long-chain-length (lcl-PHA) which contains more than 15 carbon atoms [2]. The best studied molecules are poly(3-hydroxybutyrate) homopolymer [P(3HB)] and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer [P(3HB-co-3HV)] [3]. The potential usage of P(3HB) is limited due to its high crystallinity, stiffness, and brittleness. The

incorporation of 3-hydroxyvalerate (3HV) monomer into P(3HB) improves its thermo-mechanical properties making this material more robust and flexible [4]. Furthermore, copolymers are known to biodegrade faster than homopolymers due to the presence of the inherited amorphous region. It was proved that as the 3HV content in the copolymer increases, the crystallinity decreases and the biodegradation rate improves [5]. One of the key challenges in microbial PHA production is to make this biotechnological process economically feasible. High production costs could be reduced by using waste-derived streams as organic substrates and novel microbial strains able to metabolize such carbon sources into the valuable biopolymers. Transforming waste into valuable products is a revolutionary waste management path and an environmentally friendly process [6]. In recent years, a continuous increase in organic and biomass waste has been observed. It was estimated that sewage sludge from wastewater treatment contains around 50–80% of organic, toxic, and perishable contaminants that in high quantity poses a risk to the ecosystem [7,8]. Furthermore, acid whey constitutes also a serious environmental problem and is estimated that its global production reaches 180–190 million tons. Moreover, its worldwide volume is expected to rise in the coming years [9]. Sweet whey, a by-product from hard cheese production, is turned into valuable products such as protein powder. Acid whey, on the other hand, a by-product from the manufacture of Greek yogurt, cottage cheese, and the like, constitutes a serious environmental problem and its disposal is associated with a fee [10]. Therefore, SMCFAs that are produced from sludge and acid whey, and could be a feedstock for biopolymers synthesis by microorganisms make the bioprocess of PHA production more sustainable and eco-friendlier. It was previously shown that waste organic compounds from a wide range of waste streams can be used to produce short and medium chain fatty acids (SMCFAs) by acidogenic anaerobic mixed culture fermentation (MCF) as a platform towards PHA production [11,12]. So far, the synthetic SMCFAs have been successfully used as carbon sources in the cultivations of *Pseudomonas oleovorans* [13]; *Pseudomonas* sp. ST2 [14]; *Ralstonia eutropha* [15,16] and *Haloferax mediterranei* [17]. However, there are only a few reports describing the ability of pure bacteria cultures to convert waste-derived SMCFAs into PHAs [6,18–20].

Bacteria belonging to *Aeromonas* genera are Gram-negative, heterotrophic rods that can produce PHAs even under toxic environmental conditions [21]. Due to their robust growth and simple nutritional requirements, they are attractive targets for potential PHA production using different waste. *Aeromonas* sp. AC_01 was originally isolated from waste activated sludge in Poland. We previously published a brief report indicating that *Aeromonas* sp. AC_01 was capable of synthesizing PHAs using crude glycerol from biodiesel production [22]. Up to date, no effort has been made to investigate the ability of any *Aeromonas* strains to produce PHAs from waste-derived SMCFAs. However, *Aeromonas* sp. have been used for PHA synthesis using other substrates [23]. Therefore, the aim of this study was to explore a new, sustainable production of PHAs using SMCFAs by this highly interesting bacterium. *Aeromonas* sp. AC_01 was assayed to evaluate their capability to biosynthesize PHA while growing on SMCFAs from whey wastewater biodigestion effluent as well as single synthetic pure acids and their synthetic mixtures as carbon sources in the growth media.

2. Materials and Methods

2.1. Microorganism

Aeromonas sp. AC_01 was obtained from the Culture Collection of the Department of Microbiology and Mycology (University of Warmia and Mazury in Olsztyn, Olsztyn, Poland). It was isolated from waste activated sludge collected in spring from the aeration tank at the municipal sewage treatment plant "Łyna" in Olsztyn (Poland). The genetic analysis based on the 16S *rRNA* gene sequence revealed that this isolate exhibited the highest similarity with *Aeromonas hydrophila* [22].

2.2. Culture Conditions for PHA Biosynthesis

Before inoculation into the fermentation shake flasks, cells of *Aeromonas* sp. AC_01, maintained as a cryo-conserved culture at −80 °C in a glycerol stock, were grown in lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) at 30 °C for 16 h with 150 rpm shaking. For PHA production, 250-mL Erlenmeyer flasks were inoculated with 5% (*v*/*v*) of the bacterial seed. The analyzed strain was cultivated in two different mineral salt media (MSM): non-limited MSM (7 g/L KH_2PO_4, 3.5 g/L $Na_2HPO_4 \cdot 12H_2O$, 10 g/L $(NH_4)_2SO_4$) and nitrogen-limited MSM (N-limited MSM) (7 g/L KH_2PO_4, 3.5 g/L $Na_2HPO_4 \cdot 12H_2O$, 1 g/L $(NH_4)_2SO_4$). Each medium was supplemented with $MgSO_4 \cdot 7H_2O$ (1 g/L) and trace element solution (2.5 mL/L) consisting of 20 g/L $FeCl_3 \cdot 6H_2O$, 10 g/L $CaCl_2 \cdot H_2O$, 0.03 g/L $CuSO_4 \cdot 5H_2O$, 0.05 g/L $MnCl_2 \cdot 4H_2O$, 0.1 g/L $ZnSO_4 \cdot 7H_2O$ dissolved in 0.5 N HCl. The experiments were conducted using (as the only carbon source): (A) various concentrations (1, 2 and 3 g/L) of single synthetic SMCFAs: acetic (HAc), butyric (HBu), valeric (HVa) and caproic acid (HCa); (B) different ratios of HAc and HBu (g/L): 1.6:0.4; 1.2:0.8; 0.8:1.2; 0.4:1.6 (C) various concentrations (10, 20 and 30% *v*/*v*) of a mixture of synthetic SMCFAs ($SMCFAs_{synthetic}$-rich stream) to simulate a real SMCFAs-rich stream: acetic acid (2.85 g/L), butyric acid (9.86 g/L), valeric acid (0.16 g/L), caproic acid (3.05 g/L; (D) various concentrations (10, 20 and 30% *v*/*v*) of a mixture of SMCFAs received ($SMCFAs_{extracted}$-rich stream) from the acidogenic anaerobic mixed culture fermentation of acid whey obtained from a crude cheese production line. The SMCFAs production was conducted in an up-flow anaerobic sludge blanket (UASB) bioreactor constructed from cylindrical plexiglass with recirculation ensuring biomass suspension as described by Duber et al. [9]. The effluent for PHAs production was taken from a steady-state phase with a dominant butyrate production. The sample of the effluent was filtered and the supernatant was analyzed by high-performance liquid chromatography (HPLC) to evaluate its composition (Table 1).

Table 1. Composition of $SMCFAs_{extracted}$ -rich stream used in this study.

Components	[g/L]	[%]
Acetic acid	2.85	10.61
Butyric acid	9.86	36.70
Valeric acid	0.16	0.60
Caproic acid	3.05	11.35
Lactic acid	9.31	34.65
Ethanol	1.64	6.10

The pH of the growth media was adjusted to 7.0 by addition of 1M NaOH and 1M HCl. All cultivations were incubated at 30 °C and 150 rpm using rotary shaker. The cultivations were conducted for 48 h (with $SMCFAs_{synthetic}$-rich stream and $SMCFAs_{extracted}$-rich stream) or for 72 h (with single synthetic SMCFAs and different ratios of HAc and HBu). The samples for analyses were harvested at 24 h, 48 h and 72 h (only from cultivations with single synthetic SMCFAs and different ratios of HAc and HBu). All cultivations were conducted in triplicate.

2.3. Bacterial Growth and PHA Quantification

Cell growth was controlled by estimating cell dry mass (CDM). The culture samples were centrifuged at 9000× *g* for 10 min. The supernatant was removed and the biomass pellet was lyophilized for 24 h using Lyovac GT2 System (SRK Systemtechnik GmbH, Riedstadt, Germany). PHAs were extracted by shaking lyophilized cells in hot chloroform (50 °C) for 3 h. Next, the prepared mixture was filtered through No. 1 Whatman filter paper to remove cell debris. After filtration, the biopolyesters dissolved in chloroform were precipitated with 70% cold methanol and then allowed to evaporate at room temperature. The precipitated PHAs were quantified directly by the gravimetric method. The PHAs

content was defined as the percentage of the ratio of PHAs concentration to total bacterial cells concentration.

2.4. Determination of Monomeric Composition by Gas Chromatography/Mass Spectrometry Analysis

To evaluate the PHAs composition, the lyophilized cells were analyzed by gas chromatography coupled with mass spectrometry (GC-MS QP2010 PLUS, Shimadzu, Kyoto, Japan) according to the method described previously by Możejko-Ciesielska and Pokój [22]. Shortly, the freeze-dried bacterial cells were suspended in a chloroform-methanol-sulfuric acid mixture (100/97/3, *v*/*v*/*v*). The methanolysis was conducted by heating the vials at 100 °C for 20 h. Then, the sulfuric acid was neutralized with Na_2CO_3 and the resulting mixture was dried with anhydrous Na_2SO_4. After filtration, the methyl esters were analyzed using a BPX70 (25 m × 0.22 mm × 0.25 mm) capillary column (SGE Analytical Science, Victoria, Australia) with helium as a carrier gas at a flow rate of 1.38 mL/min. The column was heated from 80 to 240 °C at a rate of 10 °C/min. The interface and ion source temperatures of GC-MS was set at 240 °C with the electron energy of 70 eV within the 45–500 *m/z* range. Identification of the mass signals were analyzed by comparison with pure 3-hydroxyacids standards (Larodan, Solna, Sweden): 3-hydroxyhexanoate (3HHx), -octanoate (3HO), -nonanoate (3HN), -decanoate (3HD), -undecanoate (3HuD), -dodecanoate (3HDD), -tridecanoate (3HtriD), tetradecanoate (3HTD).

2.5. PHAs Spectroscopic Studies

The PHAs spectroscopic studies were performed using a Fourier Transform Spectrophotometer (FTIR) Nicolet iS10 (ThermoScientific, Waltham, MA, USA) by attenuated total reflection method (ATR-FTIR) on samples without prior preparation. FTIR spectra were recorded in the range from 4000 to 650 cm^{-1}. Each spectrum analyzed was the average of 16 recorded measurements.

2.6. Analysis of PHAs Thermal Properties

The thermal properties of the extracted PHAs were determined using differential scanning calorimetry (DSC) and thermogravimetry (TG). DSC tests were conducted using a Q200 differential scanning calorimeter (TA Instruments, New Castle, DE, USA) in a nitrogen atmosphere, temperature range from −70 to 230 °C and heating/cooling rate of 10 °C/min. Samples of approximately 1 mg were first rapidly heated to 230 °C and conditioned for 1 minute to remove the thermal history of the material. Pre-heating was also aimed at homogenizing the sample, which due to its characteristics consisted of several layers of the same material. The samples were then cooled to −70 °C and re-heated to 230 °C. Thermal analysis of the PHAs was based on the second heating curve, from which the glass transition temperature (T_g), cold crystallization temperature (T_{cc}), change in enthalpy of cold crystallization (ΔH_{cc}), melting point (T_m) and change in enthalpy of melting process (ΔH_m) were evaluated.

TG tests were performed using a Q500 thermobalance (TA Instruments, New Castle, DE, USA) in a nitrogen atmosphere, temperature range from −70 to 230 °C and heating rate of 10 °C/min. The mass of the analyzed samples was approximately 1 mg. From the thermogravimetric curve 5% mass loss temperature ($T_{5\%}$) was determined. The value of $T_{5\%}$ was used as a parameter determining the start of thermal degradation of the material, taken as the thermal resistance of the biomaterial. The TG analysis was also completed with the determination of the mass loss onset temperature (T_{ons}) of the main stage of degradation of the tested PHAs.

2.7. Statistical Analysis

STATISTICA v.13.1 (StatSoft, Inc., Tulsa, OK, USA) was used to conduct statistical analysis of all data. All samples were analyzed in triplicate. The Mann–Whitney U-test was used

to determine the significance of differences in PHAs concentration between experimental variants. Data were reported as the median ± SD at the significance level of $p < 0.05$.

3. Results and Discussion

3.1. Biomass Concentration and PHAs Production Using Individual Synthetic SMCFAs

In the first stage of the study, typical SMCFAs products of anaerobic acidification (acetic acid, butyric acid, valeric acid, and caproic acid) were used separately to investigate the ability of *Aeromonas* sp. AC_01 strain to metabolize them into PHAs. They were tested at the initial concentrations of 1.0, 2.0, and 3.0 g/L. High initial carbon source concentrations were speculated to be beneficial to achieve high cell densities and PHAs accumulation [24]. However, SMCFAs could be toxic for bacterial cells. At higher concentrations (over 3.0 g/L) these synthetic SMCFAs were acting as inhibitors for *Aeromonas* sp. AC_01 growth (data not shown). Also, Zhang et al. [25] reported a strong inhibition of *Aeromonas hydrophila* 4AK4 growth when the culture medium was supplemented with valerate at the concentration over 3 g/L.

As it is shown in Figure 1A,B and Table 2, all tested SMCFAs in all concentrations supported the bacterial growth and PHA production. The results indicated that SMCFAs concentration considerably impacted the growth of bacteria. Only the increase in acetic acid concentration caused an increase in the biomass production, whereas in the cultures with more than 1 g/L of butyric acid, valeric acid or caproic acid a decreased cell concentration was revealed.

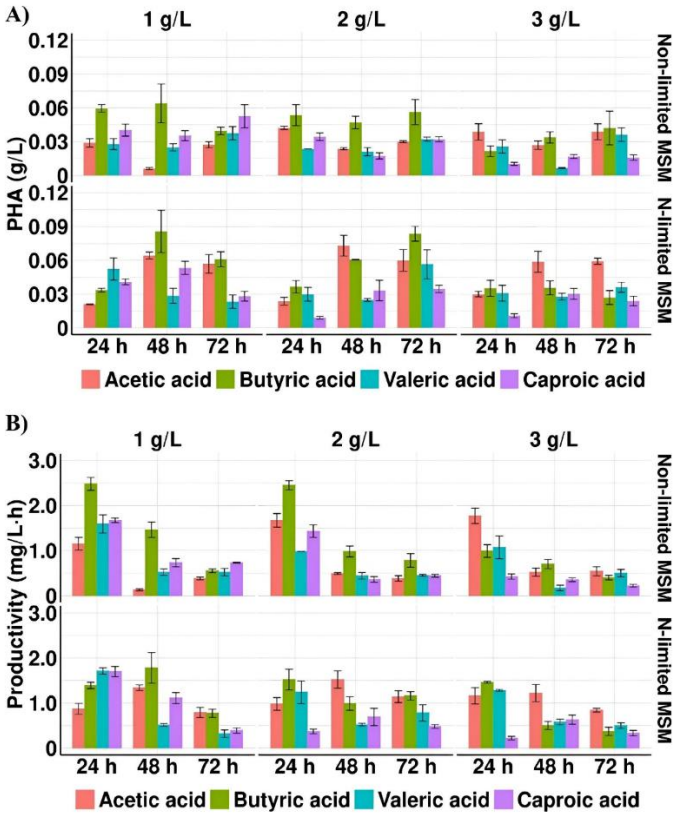


Figure 1. (**A**) PHA concentration and (**B**) PHA productivity profiles of *Aeromonas* sp. AC_01 cultured in the medium supplemented with individual short and medium chain fatty acids at the concentration of 1 g/L, 2 g/L and 3 g/L Abbreviations: non-limited MSM, non-limited mineral salt medium; N-limited MSM, nitrogen limited mineral salt medium.

Table 2. Biomass concentration and PHA production from individual short and medium chain fatty acids by *Aeromonas* sp. AC_01.

Medium	Substrate	Substrate Concentration [g/L]	Biomass [g/L]			PHA [% of CDM]			pH Value		
			24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Non-limited MSM	Acetic acid	1	0.61 ± 0.07	0.55 ± 0.14	1.48 ± 0.08	4.76 ± 0.05	1.11 ± 0.12	1.79 ± 0.10	7.66 ± 0.00	7.52 ± 0.01	7.75 ± 0.01
		2	0.85 ± 0.04	1.87 ± 0.09	1.73 ± 0.16	4.95 ± 0.05	1.27 ± 0.07	1.80 ± 0.24	7.54 ± 0.01	7.42 ± 0.00	7.74 ± 0.00
		3	0.51 ± 0.04	1.89 ± 0.24	1.99 ± 0.07	7.60 ± 0.84	1.41 ± 0.02	1.94 ± 0.39	7.43 ± 0.00	7.31 ± 0.01	7.68 ± 0.01
	Butyric acid	1	0.39 ± 0.04	0.67 ± 0.004	1.56 ± 0.07	14.90 ± 1.07	9.51 ± 0.63	2.53 ± 0.09	6.65 ± 0.01	6.68 ± 0.01	6.93 ± 0.01
		2	0.37 ± 0.08	0.27 ± 0.03	1.09 ± 0.09	13.68 ± 0.44	17.25 ± 0.82	5.02 ± 0.44	6.56 ± 0.01	6.52 ± 0.01	6.86 ± 0.01
		3	0.18 ± 0.04	0.25 ± 0.03	0.54 ± 0.13	13.15 ± 0.75	13.48 ± 0.34	13.04 ± 1.76	6.44 ± 0.01	6.51 ± 0.01	6.81 ± 0.01
	Valeric acid	1	0.39 ± 0.01	0.79 ± 0.11	0.91 ± 0.05	8.57 ± 0.42	3.51 ± 0.37	4.81 ± 0.61	6.65 ± 0.02	6.47 ± 0.01	6.83 ± 0.02
		2	0.24 ± 0.03	0.30 ± 0.09	0.39 ± 0.03	10.08 ± 1.24	5.58 ± 0.82	8.20 ± 0.10	6.56 ± 0.01	6.34 ± 0.04	6.65 ± 0.04
		3	0.24 ± 0.04	0.29 ± 0.00	0.45 ± 0.03	10.07 ± 1.01	3.09 ± 1.06	7.24 ± 0.30	6.41 ± 0.01	6.32 ± 0.02	6.45 ± 0.02
	Caproic acid	1	0.15 ± 0.02	0.53 ± 0.02	0.94 ± 0.07	23.87 ± 2.90	6.79 ± 0.69	5.59 ± 1.08	6.53 ± 0.10	6.48 ± 0.08	6.57 ± 0.08
		2	0.15 ± 0.02	0.37 ± 0.06	0.30 ± 0.03	23.36 ± 1.63	4.83 ± 0.24	10.70 ± 0.61	6.54 ± 0.02	6.36 ± 0.08	6.51 ± 0.16
		3	0.04 ± 0.01	0.28 ± 0.05	0.26 ± 0.06	28.17 ± 0.56	5.90 ± 0.46	6.10 ± 0.76	6.44 ± 0.04	6.23 ± 0.03	6.44 ± 0.15
N-limited MSM	Acetic acid	1	0.37 ± 0.02	0.46 ± 0.06	0.82 ± 0.07	5.86 ± 1.22	15.35 ± 0.43	7.10 ± 0.68	7.65 ± 0.01	7.55 ± 0.01	7.56 ± 0.01
		2	0.42 ± 0.02	0.82 ± 0.04	1.63 ± 0.21	5.66 ± 0.55	8.73 ± 0.51	3.45 ± 0.01	7.59 ± 0.01	7.46 ± 0.05	7.52 ± 0.01
		3	0.48 ± 0.04	0.99 ± 0.12	1.63 ± 0.25	6.63 ± 0.76	5.74 ± 0.06	3.37 ± 0.72	7.48 ± 0.01	7.43 ± 0.01	7.45 ± 0.02
	Butyric acid	1	0.24 ± 0.01	0.46 ± 0.10	0.56 ± 0.02	14.23 ± 1.88	17.93 ± 0.73	7.54 ± 1.06	6.65 ± 0.00	6.52 ± 0.01	6.67 ± 0.01
		2	0.24 ± 0.01	0.27 ± 0.03	1.00 ± 0.06	14.93 ± 1.65	17.67 ± 1.00	8.23 ± 0.01	6.65 ± 0.01	6.51 ± 0.01	6.72 ± 0.01
		3	0.13 ± 0.01	0.19 ± 0.03	0.18 ± 0.01	18.56 ± 0.54	13.19 ± 0.98	17.91 ± 0.16	6.64 ± 0.02	6.52 ± 0.01	6.67 ± 0.01
	Valeric acid	1	0.42 ± 0.06	0.60 ± 0.10	0.67 ± 0.13	11.01 ± 0.54	4.80 ± 0.89	3.47 ± 0.23	6.73 ± 0.01	6.52 ± 0.02	6.76 ± 0.11
		2	0.26 ± 0.08	0.28 ± 0.01	0.85 ± 0.02	13.94 ± 2.04	8.80 ± 0.57	12.38 ± 0.16	6.65 ± 0.04	6.53 ± 0.03	6.83 ± 0.03
		3	0.19 ± 0.05	0.29 ± 0.01	0.31 ± 0.03	12.97 ± 0.94	10.34 ± 0.48	12.52 ± 0.16	6.62 ± 0.02	6.53 ± 0.03	6.75 ± 0.03
	Caproic acid	1	0.22 ± 0.05	0.57 ± 0.04	0.70 ± 0.01	16.83 ± 0.24	9.38 ± 0.86	4.59 ± 0.36	6.72 ± 0.03	6.62 ± 0.14	6.91 ± 0.03
		2	0.10 ± 0.01	0.17 ± 0.04	0.20 ± 0.01	9.21 ± 0.36	19.84 ± 0.28	18.31 ± 0.81	6.70 ± 0.02	6.52 ± 0.02	6.80 ± 0.04
		3	0.13 ± 0.00	0.15 ± 0.02	0.18 ± 0.03	9.61 ± 0.36	20.38 ± 0.92	13.36 ± 0.61	6.63 ± 0.01	6.41 ± 0.02	6.75 ± 0.03

Non-limited MSM, non-limited mineral salt medium; N-limited MSM, nitrogen limited mineral salt medium; PHA, polyhydroxyalkanoates; CDM, cell dry mass.

The results showed that only in the cultures with acetic acid pH of the medium in all measured time-points was alkaline (pH > 7) (Table 2). In these cultivations where the highest biomass concentration was observed, it could suggest that the high consumption of acetate caused a pH increase [26]. Whereas in the cultivations with other acids the CDM values were lower due to their inhibitory effect, thus bacteria were not able to consume them effectively. Moreover, it was assumed that an alkaline growth medium could positively affect the tolerance level of *Aeromonas* sp. AC_01 to high acetic acid concentration. Whereas the pH values of the media with other tested SMCFAs were below 7 in all measured time-points, therefore the severe effect of the high concentrations of butyric, valeric and caproic acids may not be eliminated. The same observation was made by Gao et al. [27] who proved that an alkaline environment could effectively alleviate growth inhibition of *Yarrowia lipolytica* during its cultivations with SMCFAs towards lipid biosynthesis. The highest biomass concentration (2.0 g/L) was evaluated in the culture with 3.0 g/L of acetic acid under non-limited condition at 72 h. Lesser value (about 1.6 g/L) was detected when *Aeromonas* sp. AC_01 was grown on 1.0 g/L of butyric acid under the same condition. The results indicated that in all cultivations supplemented with valeric and caproic acid, regardless of the culture condition used, the biomass concentration was below 1.0 g/L.

The results suggested that nitrogen limitation affected positively PHA concentration. The highest PHA concentration was obtained with 1 g/L of butyric acid and 2 g/L of acetic acid under N-limited conditions (MSM with 1 g/L $(NH_4)_2SO_4$)), 0.09 and 0.07 g/L, respectively. The statistically significant difference ($p = 0.017$) was reported between cultures with acetic and butyric acid under non-limited conditions (MSM with 10 g/L $(NH_4)_2SO_4$). The reported observations were similar to those achieved by Anburajan et al. [21] who reached higher PHA yield cultivated *Aeromonas hydrophila* ATCC7966 in the famine phase compared to feast phase. Moreover, our data revealed that when valeric acid or caproic acid were used as the sole carbon source, PHAs were synthesized at lower concentrations (below 0.06 g/L) compared with acetic and butyric acid ($p = 0.042$ and $p = 0.022$, respectively). Lower PHA yield (0.03 g/L) was also achieved by Shi et al. [28] who cultured engineered *Aeromonas hydrophila* 4AK4 harboring overexpressed genes encoding β-ketothiolase and acetoacetyl-CoA reductase grown with yeast extract and acetate. However, further metabolic engineering through overexpression of *pta-ackA* gene was proved to strengthen the utilization of acetic acid and consequently PHA yield increased to 0.57 g/L.

The highest PHA productivity was observed in the cultures under non-limited conditions at 24 h when the growth medium was also supplemented with butyric acid (2.48 mg/L·h) and acetic acid (1.77 mg/L·h). The PHA productivity decreased about 23% and 30% at 72 h compared to 24 h in the cultures with butyrate and acetate, respectively. Also, *Ralstonia eutropha* ATCC 17699 showed the capability of producing PHAs on acetic, propionic and butyric acid, however the biopolymer productivity reached only 0.013, 0.036, and 0.037 mg/L·h, respectively [15]. The addition of glucose in the cultivation of this bacterial strain with propionic acid enhanced the PHA productivity to 0.74 mg/L·h [29].

3.2. Cell Dry Weight Concentration and PHA Production Using a Mixture of Acetic and Butyric Acid

As it was described above, acetic and butyric acids were the most preferable carbon sources that effectively supported *Aeromonas* sp. AC_01 growth, PHA concentration and productivity. Therefore, in the second stage of the study, the combinations of both acids were prepared to determine their effects as carbon sources on the biomass production and PHA biosynthesis level. Biomass concentration, PHA concentration and productivity were improved (Figure 2A–C) compared to the results obtained in the cultures with single acetic or butyric acid.

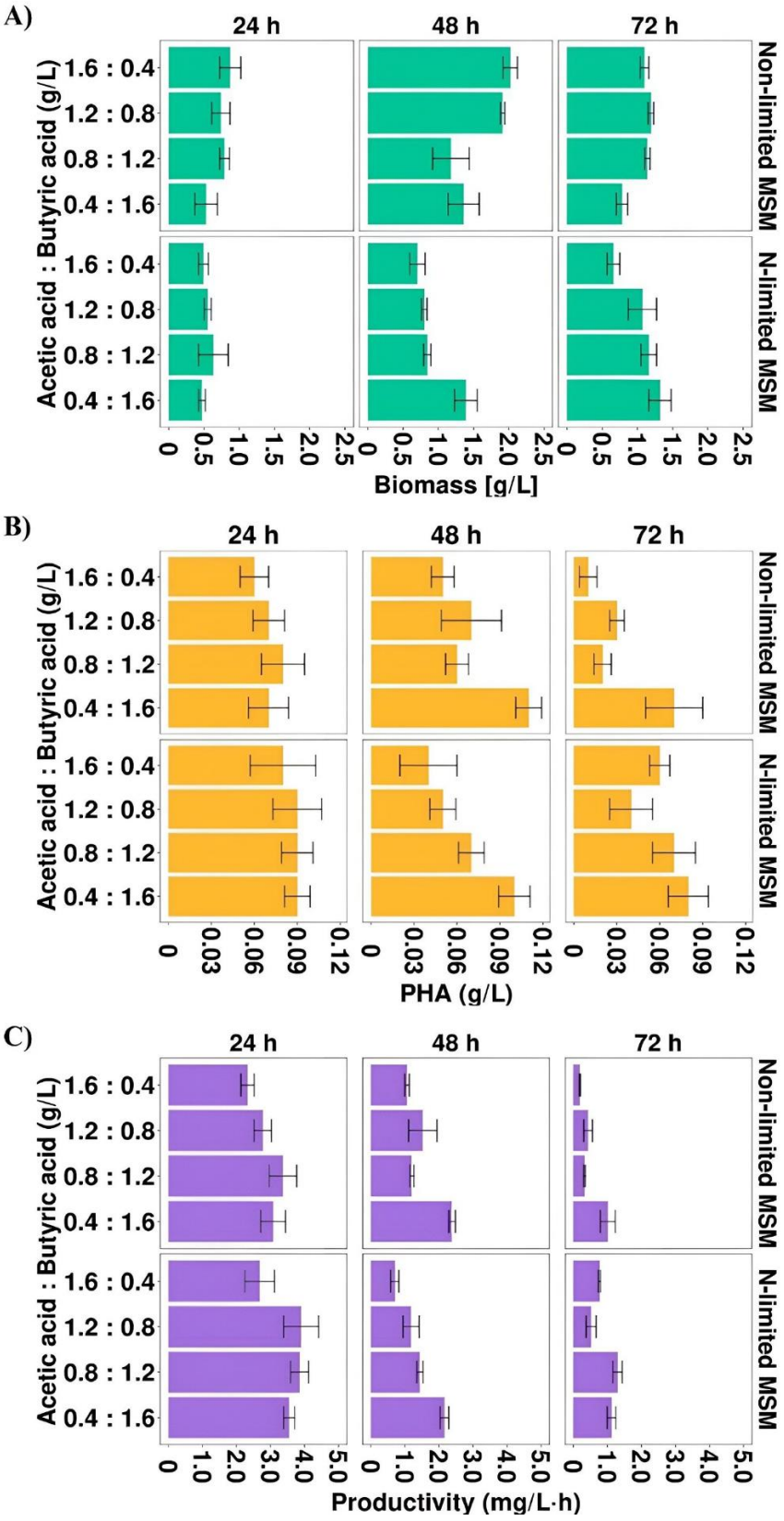


Figure 2. (**A**) Biomass concentration, (**B**) PHA concentration and (**C**) PHA productivity profiles of *Aeromonas* sp. AC_01 cultivated on the mixture of acetic and butyric acid. Abbreviations: non-limited MSM, non-limited mineral salt medium; N-limited MSM, nitrogen limited mineral salt medium.

The results confirmed that the highest biomass concentration (2.02 g/L) was observed in the cultivation under non-limited conditions at 48 h with the highest proportion of acetic acid (1.6 HAc:0.4 HBu). This value was higher compared to those received from the cultures at the same time point with single acetic acid and butyric acid, the maximum biomass concentration reached 1.9 and 0.7 g/L, respectively. In addition, the results indicated that the mixture of both acids supported better PHA production than individual acids. The PHA concentration and productivity depended on the ratio of these SMCFAs (Figure 2B,C). Nevertheless, the highest PHA concentration was observed at 48 h in the cultivations under non-limited and N-limited conditions with the highest proportion of butyric acid (0.11 and 0.10 g/L, respectively). These values were also higher than those obtained in the cultures with single acids. As can be observed in Figure 2C, nitrogen limitation positively influenced on PHA productivity. In the cultivations with the mixture in the proportion of 1.2 HAc:0.8 HBu the PHAs were produced in the highest efficiency, reaching 3.9 mg/L·h at 24 h. At 48 and 72 h, in all experimental variants, PHA productivity decreased. Higher PHA productivity was reported by Gonzalez and Winterburn [30] who observed that when in the fermentation of *Haloferax mediterranei* the mixture of butyric and pentanoic acid was used at the constant concentration of 0.25 M the process productivity was improved and reached up to 12.8 mg/L·h. The same bacterial species produced PHA with lower productivity (0.010 mg/L·h) when was cultured on the mixture of butyric acid, valeric acid and Tween 20 [17].

3.3. Growth and PHA Production Using a SMCFAs$_{extracted}$-Rich Stream and SMCFAs$_{synthetic}$-Rich Stream

Most of the previous studies described individual SMCFAs as carbon sources towards PHA production. However, the effluent from whey wastewater anaerobic fermentation used in this study was complex and, except for SMCFAs, consisted of other compounds (lactic acid and ethanol). To investigate whether the non-SMCFAs compounds could have impact on *Aeromonas* sp. AC_01 growth and its PHA productivity, the simulated SMCFAs mixture was prepared identically with the SMCFAs components of the SMCFAs-rich stream. Due to the fact that the proper SMCFAs concentration is essential to achieve satisfying bacterial growth and consequently higher PHA yield, three different concentrations of the carbon sources were tested (10, 20, and 30% *v*/*v*). Figure 3A–C showed that biomass concentration, PHA concentration and PHA productivity were dependent on the amount of the carbon source used.

In all experimental variants the values of these parameters were higher in the cultivations with simulated SMCFAs-rich stream compared to SMCFAs-rich stream derived from anaerobic fermentation. The biomass value was the highest in the cultivation under both applied conditions supplemented with 30 g/L of the effluent indicating high tolerance of the *Aeromonas* sp. AC_01 to SMCFAs-rich stream. Nevertheless, the highest PHA concentration and PHA productivity was lower than that for the simulated SMCFAs mixture. The maximum PHA yield and efficiency was achieved with 10% of SMCFAs-rich stream, i.e., 0.03 g/L and 0.54 mg/L·h, respectively. It could suggest that there are inhibitors in the extracted SMCFAs mixture that due to its complex composition may adversely affect PHA biosynthesis process. Based on the chemical characterization, the main difference between the simulated SMCFAs solution and SMCFAs-rich stream was the presence of ethanol and lactic acid in the latter. Those components had inhibition effects on the *Aeromonas* sp. AC_01 growth and in a consequence on the PHA concentration and productivity. These results were inconsistent with a study of Obruca et al. [31] who proved that *Cupriavidus necator* exposed to ethanol at the beginning of the stationary phase intensified P(3HB) yields about 30%. Moreover, Martinez et al. [6] reported lower PHA productivity (0.022 mg/L·h) compared to the above-described data in the cultivation of *Cupriavidus necator* supplemented with olive mill wastewater effluent rich in acetic, propionic and butyric acid. However, the effluent consisted of not only different short chain fatty acids, but also polyphenols (1.2 g/L), N-NH_4 (60 mg/L), proteins (1.56 g/L), and lipids (3.24 g/L). Furthermore, in our study, nitrogen-starvation conditions were beneficial to PHA production process using synthetic mixture, whereas in the culture supplemented with SMCFAs-rich stream PHA synthesis was on the same level.

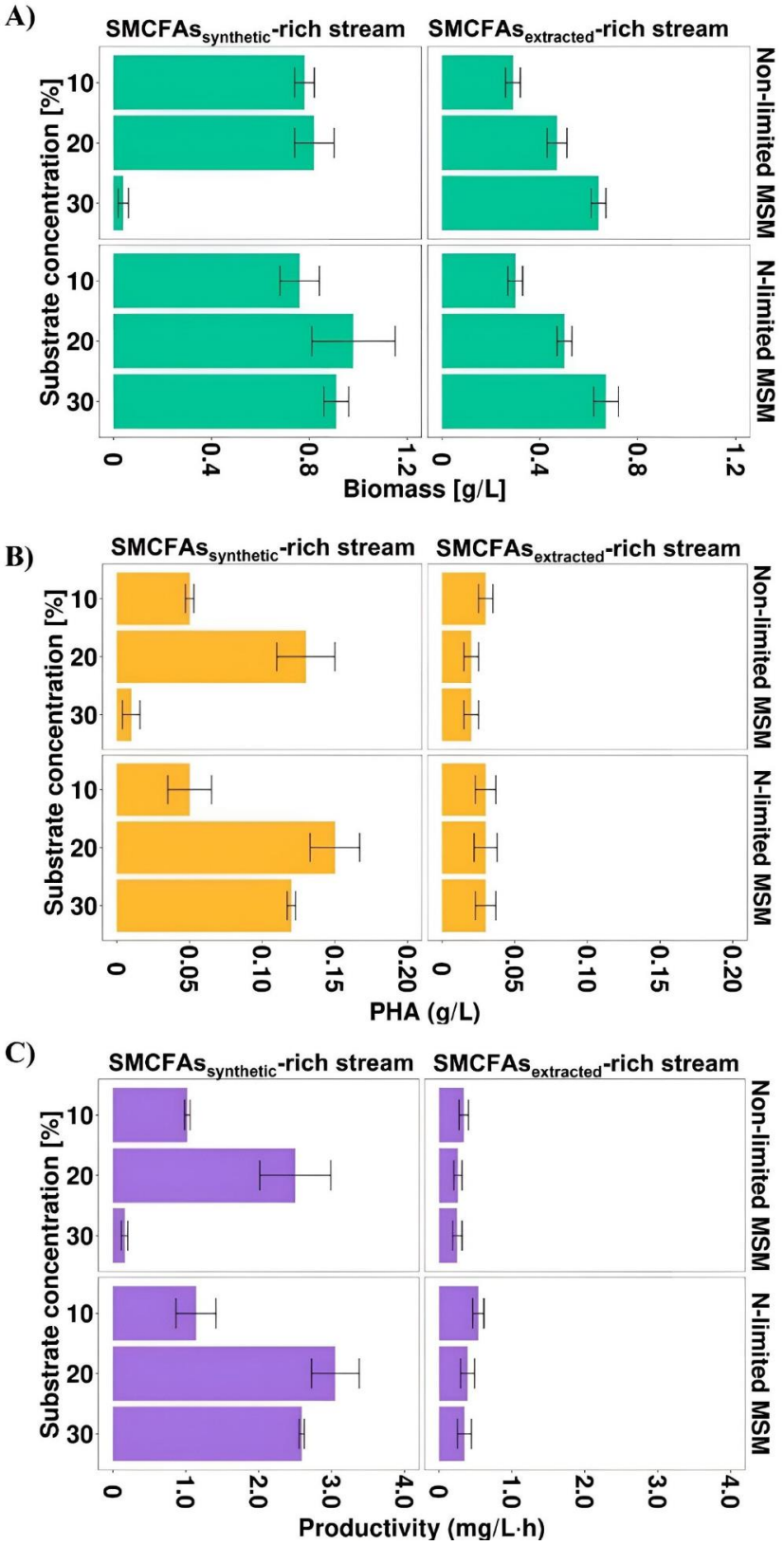


Figure 3. (**A**) Biomass concentration, (**B**) PHA concentration and (**C**) PHA productivity profiles of *Aeromonas* sp. AC_01 at 48 h of the cultivations supplemented with SMCFAs$_{synthetic}$-rich stream and SMCFAs$_{extracted}$-rich stream. Abbreviations: non-limited MSM, non-limited mineral salt medium; N-limited MSM, nitrogen limited mineral salt medium.

3.4. GC-MS Analysis

The monomer composition of the accumulated PHAs varied with the carbon source and culture conditions used (Table 3). The highest synthesis efficiency of 3HV monomer was determined in the cultivation supplemented with valeric acid. It was previously confirmed that acids with an even number of carbon on the molecule are more favorable to the synthesis of 3HB, while acids with odd number of carbon on the molecule tends to produce 3HV [32]. To produce 3HB monomers only acetyl-CoA is required which is formed during metabolism of acids with an even number of carbon in the molecule. Whereas, to synthesize 3HV, bacteria need to produce acetyl-CoA and propionyl-CoA. The latter is produced from pyruvate and valeryl-CoA formed from valeric acid. Interestingly, when valeric acid was used as the only carbon source, PHA composed of uneven carbon numbered monomers (3-hydroxytridecanoic acid) was accumulated by *Aeromonas* sp. AC_01, with the highest content (23.6 mol%) detected in the PHAs extracted from the bacterial cells cultured on 1 g/L of valeric acid under non-limited conditions. Moreover, a concentration of 3HB molar fraction in the P(3HB-co-3HV-co-3HTriD-co-3HTD) copolyester seems to be dependent on the valerate concentration and culture condition. The highest 3HB content (32.9 mol%) was detected in the cultivation with 1 g/L of valerate under non-limited conditions. Except for 3HB, 3HV and 3HTriD monomers in the extracted copolymers 3HTD was also detected, however in trace amount (from 1.58 to 8.31 mol%). So far, no data has been reported about the ability of bacteria belong to *Aeromonas* genera to produce copolyester consisting of 3HB, 3HV, 3-HTriD and 3-HTD. Terpolyester P(3HB-co-3HV-co-3HHx) was reported to be accumulated by *Aeromonas hydrophila* 4AK4 cultivated on mixed carbon sources of lauric acid and valerate [25]. The authors confirmed that 3HV content was affected by valerate concentration added to the culture medium.

Table 3. Monomeric compositions of PHAs extracted from *Aeromonas* sp. AC_01 cells.

Carbon Source	Substrate Concentration [g/L; % *]	PHA Composition [mol%]			
		3HB	3HV	3HtriD	3HTD
Non-limited MSM					
Acetic acid	1	nd	nd	nd	100.0 ± 0.0
	2	nd	nd	nd	100.0 ± 0.0
	3	nd	nd	nd	100.0 ± 0.0
Butyric acid	1	83.4 ± 0.9	nd	nd	16.6 ± 0.9
	2	95.6 ± 0.3	nd	nd	4.4 ± 0.3
	3	93.3 ± 0.6	nd	nd	6.7 ± 0.6
Valeric acid	1	32.9 ± 0.3	35.1 ± 0.4	23.7 ± 0.1	8.3 ± 0.6
	2	4.6 ± 0.1	88.6 ± 0.0	5.1 ± 0.1	1.6 ± 0.0
	3	6.2 ± 0.2	87.1 ± 0.0	2.9 ± 0.2	3.9 ± 0.0
Caproic acid	1	89.6 ± 0.2	5.2 ± 0.1	nd	5.2 ± 0.1
	2	92.2 ± 0.5	3.5 ± 0.1	nd	4.3 ± 0.4
	3	92.2 ± 0.4	3.5 ± 0.4	nd	4.3 ± 0.0
Acetic acid:Butyric acid	0.4:1.6	92.0 ± 0.0	nd	nd	8.0 ± 0.0
	0.8:1.2	80.8 ± 0.5	nd	nd	19.3 ± 0.5
	1.2:0.8	77.1 ± 0.0	nd	nd	22.9 ± 0.0
	1.6:0.4	77.1 ± 0.7	nd	nd	22.9 ± 0.7
$SMCFAs_{synthetic}$-rich stream	10 *	74.5 ± 0.5	nd	nd	25.5 ± 0.5
	20 *	95.5 ± 0.1	nd	nd	4.5 ± 0.1
	30 *	94.7 ± 0.1	0.9 ± 0.0	nd	4.4 ± 0.1
$SMCFAs_{extracted}$-rich stream	10 *	95.6 ± 0.0	nd	nd	4.4 ± 0.0
	20 *	100.0 ± 0.0	nd	nd	nd
	30 *	71.7 ± 3.1	nd	nd	28.3 ± 3.1

Table 3. *Cont.*

Carbon Source	Substrate Concentration [g/L; % *]	PHA Composition [mol%]			
		3HB	3HV	3HtriD	3HTD
N-limited MSM					
Acetic acid	1	48.9 ± 0.7	nd	nd	51.1 ± 0.7
	2	nd	nd	nd	100.0 ± 0.0
	3	nd	nd	nd	100.0 ± 0.0
Butyric acid	1	92.2 ± 0.2	nd	nd	7.8 ± 0.2
	2	94.6 ± 0.1	nd	nd	5.4 ± 0.1
	3	93.6 ± 0.2	nd	nd	6.4 ± 0.2
Valeric acid	1	7.8 ± 0.1	73.4 ± 0.1	13.8 ± 0.1	5.1 ± 0.0
	2	4.9 ± 0.0	83.9 ± 0.0	8.5 ± 0.0	2.6 ± 0.0
	3	2.8 ± 0.1	92.9 ± 0.1	2.8 ± 0.1	1.6 ± 0.0
Caproic acid	1	80.7 ± 0.2	nd	nd	19.3 ± 0.2
	2	82.3 ± 0.1	13.4 ± 0.1	nd	4.3 ± 0.0
	3	78.7 ± 0.0	18.1 ± 0.0	nd	3.2 ± 0.0
Acetic acid:Butyric acid	0.4:1.6	92.7 ± 0.2	nd	nd	7.3 ± 0.2
	0.8:1.2	86.1 ± 0.0	nd	nd	13.9 ± 0.0
	1.2:0.8	77.8 ± 0.3	nd	nd	22.2 ± 0.4
	1.6:0.4	65.4 ± 0.5	nd	nd	34.6 ± 0.5
$SMCFAs_{synthetic}$-rich stream	10 *	88.4 ± 0.1	nd	nd	11.6 ± 0.1
	20 *	94.9 ± 0.2	0.6 ± 0.0	nd	4.5 ± 0.2
	30 *	90.6 ± 0.0	5.5 ± 0.0	nd	3.9 ± 0.1
$SMCFAs_{extracted}$-rich stream	10 *	90.4 ± 0.2	2.5 ± 0.1	nd	7.1 ± 0.1
	20 *	90.3 ± 2.0	nd	nd	9.7 ± 2.0
	30 *	100.0 ± 0.0	nd	nd	nd

nd, not detected; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HtriD, 3-hydroxytridecanoate; 3HTD, 3-hydroxytetradecanoate; non-limited MSM, non-limited mineral salt medium; N-limited MSM, nitrogen limited mineral salt medium; SMCFAs, short and medium chain fatty acids; $SMCFAs_{synthetic}$-rich stream, a mixture of synthetic SMCFAs to simulate a real SMCFAs-rich stream; $SMCFAs_{extracted}$-rich stream, a mixture of SMCFAs received from the acidogenic anaerobic mixed culture fermentation of acid whey. *—an unit of the concentration of $SMCFA_{Ssynthetic}$-rich stream and $SMCFA_{Sextracted}$-rich stream.

Furthermore, the 3HV monomers were also detected in biopolymers extracted from the tested bacterium grown with caproic acid but to a lesser extent (from 3.52 to 18.05 mol%) in comparison to that determined with valeric acid. Furthermore, when caproic acid was supplied 3HB monomer was also detected as a main constituent and trace amounts of 3HTD were found in all cultivations. It may be assumed that caproate or its intermediates involved in metabolism are less efficient substrates to produce monomers consisting of higher numbers of carbon. The same observations was made by Cerrone et al. [19] who cultivated *P. putida* strains with propionate and also observed an uneven carbon chain in extracted PHA. However, its content was lesser compared to that revealed with valeric acid.

When acetic acid and butyric acid were used as co-substrates, the synthesis of 3HB and 3HTD was observed. Along with the increase of acetic acid ratio in the mixture, the content of 3HB fraction in the copolymer decreased whereas the 3HTD content increased under both applied culture conditions. Furthermore, the data obtained from the cultivations with these single acids indicated that acetic acid supported the synthesis of 3HTD monomer whereas in the cultivation with butyric acid the 3HB monomers were detected and were dominant.

As can be seen in Table 3, the biopolyester composition in the cultivations with $SMCFAs_{synthetic}$-rich stream depends on the carbon source concentration used. The obtained results proved that the analyzed strain showed a tendency to accumulate P(3HB-co-3HV-co-3HTD) terpolyester in the cultivations with the highest concentration of the simulated mixture. With decreasing concentration of this substrate, *Aeromonas* sp. AC_01 synthesized P(3HB-co-3HTD) copolymer with large amounts of 3HB and lesser content of 3HTD. Interestingly, when SMCFAs-rich stream was supplied, the monomeric composition of PHAs depends not only on the substrate concentration supplied but also on the

culture condition used. The P(3HB-co-3HV-co-3HTD) terpolyester was detected only in the cultivation with 10% of SMCFAs-rich stream under N-limited condition.

3.5. PHAs Spectroscopic Analysis

FTIR spectra of selected representative samples recorded in the range from 4000 to 600 cm^{-1} are shown in Figure 4 and Supplementary Materials Figure S1. Due to the similar structure of individual 3HV, 3HtriD, and 3HTD units, the obtained spectra were similar regardless of the obtained monomeric composition of the polymer. Any observed differences in the absorbance of individual peaks resulted mainly from the characteristics of the sample, e.g., thickness.

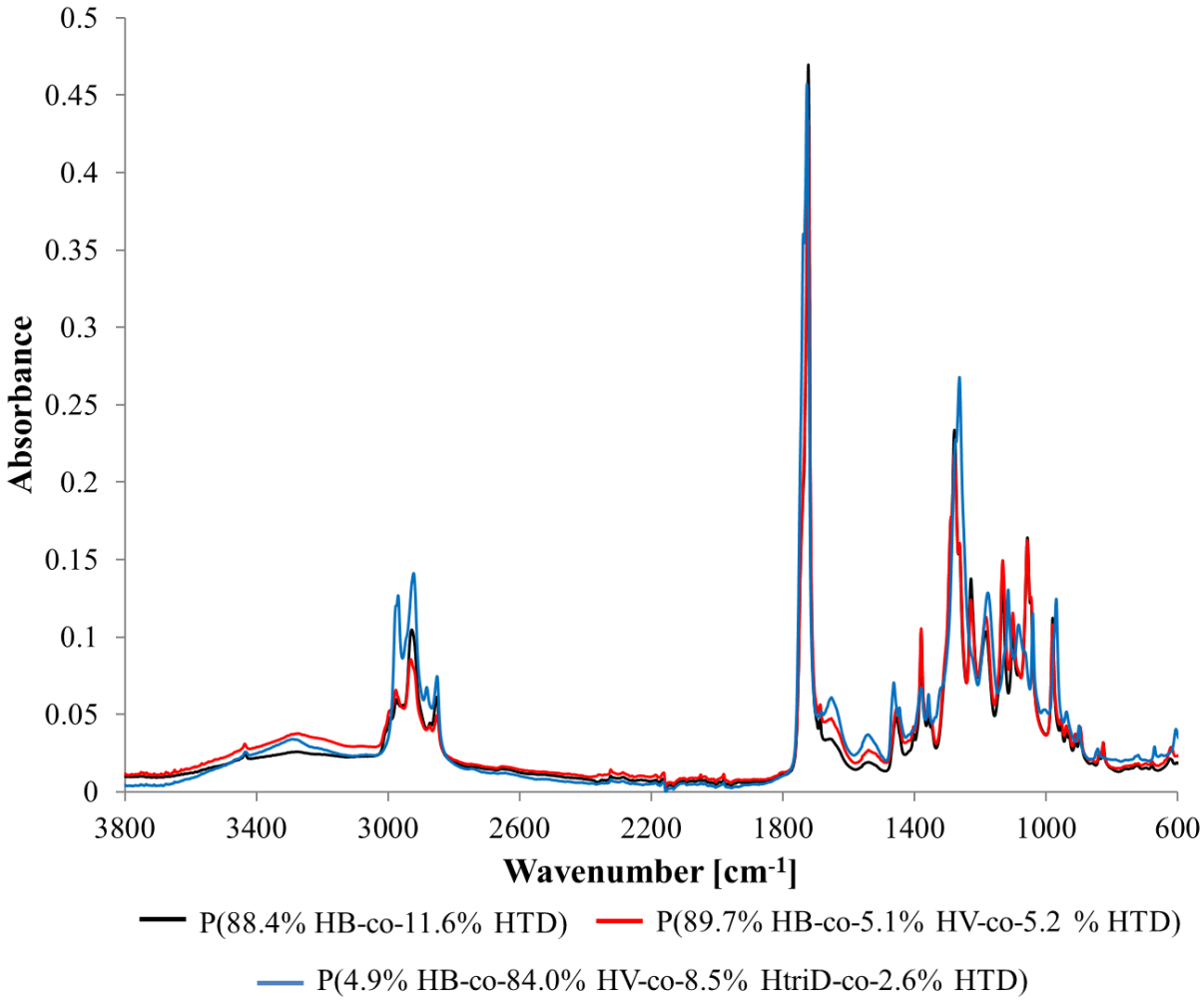


Figure 4. FTIR spectra of selected samples: (**black line**) P(88.4% HB-co-11.6% HTD) extracted from the culture supplemented with 10% $SMCFAs_{synthetic}$-rich stream under nitrogen-limited condition (**red line**) P(89.7% HB-co-5.1% HV-co-5.2% HTD) extracted from the culture supplemented with 1 g/L of caproic acid under non-limited condition, (**blue line**) P(4.9% HB-co-84.0% HV-co-8.5% HtriD-co 2.6% HTD) extracted from the culture supplemented with 2 g/L of valeric acid under nitrogen-limited condition.

Several bands have been observed that can be assigned to the functional groups characteristic for PHAs [33,34]. Table 4 presents the wavenumbers of individual peaks with associated functional groups and/or bonds. The values given in the table are averaged and the deviations for individual samples did not exceed a few units.

Table 4. Functional group and its quantified frequencies of extracted PHAs.

Wavenumber [cm^{-1}]	Assignment
~3300	-OH, terminal
~2976	CH_3, asymmetrical stretching
~2928	CH_2, C-H asymmetrical stretching
~2852	CH_3, C-H symmetrical stretching
~1723	C=O, stretching
~1658	C-O, stretching
~1539	N-O asymmetrical stretching
~1455	CH_3, C-H asymmetrical scissoring
~1380	CH_3, C-H symmetrical scissoring
~1279	C-O-C, asymmetrical stretching
~1229	C-O-C, asymmetrical stretching
~1183	C-O, asymmetrical stretching
~1133	C-O-C
~1101	C-O-C
~1057	C-O, asymmetrical stretching
~980	C-C, stretching
~900	CH_2, rocking
~825	CH_3, rocking

For some biopolymers (extracted from the cultivations with valeric acid, caproic acid, 0.4 HAc:1.6 HBu and 10% $SMCFAs_{synthetic}$-rich stream under N-limited condition) a slight band with a maximum of ~3300 cm^{-1} was observed. It probably originates from terminal -OH groups present at the ends of the polymer chains. Their presence may suggest that extracted polymers have high number of oligomers. The band in the range of 3100–2700 cm^{-1} is associated with the presence of the -CH_3 and -CH_2 groups. The single peak band with a maximum of ~1723 cm^{-1} is derived from C=O stretching vibrations of the carboxyl groups present in macromolecules. In the spectra range from 1500 to 800 cm^{-1}, which is a so-called fingerprint region, several bands with numerous peaks were recorded. Peaks ~1455, ~1380, ~980, ~900, and ~825 cm^{-1} are associated with the presence of the -CH_3 and -CH_2 groups, as well as C-C bonds which are representative of chemical structures of 3HB monomers [20]. Peaks ~1279, ~1229, ~1183, ~1133, ~1101, and ~1057 cm^{-1} are associated with bonds in the C-O-C region. Also, one nitrogen-related band at ~1539 cm^{-1} was identified in the extracted biomaterials that was related to the N-O asymmetrical stretching bond.

3.6. PHAs Thermal Properties

The thermal properties of PHAs are important for their processing and applications. Determination of the thermal parameters of the produced biopolymers allows for the selection of appropriate processing temperatures and determination of their characteristics at operating temperatures. Our results revealed that the type of the substrate and in a consequence monomeric composition of the extracted PHAs influenced their thermal properties (Table 5).

Table 5. Differential scanning calorimetry (DSC) and thermogravimetry (TG) results of the produced PHAs extracted from *Aeromonas* sp. AC_01 cells grown on butyric acid, valeric acid, caproic acid, a mixture of acetic and butyric acid, $SMCFAs_{synthetic}$-rich stream and $SMCFAs_{extracted}$-rich stream.

Substrate and Culture Condition	PHA Composition	DSC					TGA	
		T_g [°C]	T_{cc} [°C]	ΔH_{cc} [W/g]	T_m [°C]	ΔH_m [W/g]	$T_{5\%}$ [°C]	T_{ons} [°C]
Butyric acid, non-limited MSM	P(84.1% HB-co-15.9% HTD)	−0.3	39.1	19.1	165.5	44.4	155.8	269.8
Butyric acid, N-limited MSM	P(92.1% HB-co-7.9% HTD)	2.6	37.7	23.7	172.2	41.1	226.6	267.5
Valeric acid, non-limited MSM	P(4.7%HB-co-88.6% HV-co-5.1% HtriD-co-1.6% HTD)	−18.7	58.3	28.1	104.4	34.6	182.5	266.6
Valeric acid, N-limited MSM	P(4.9% HB-co-84.0% HV-co-8.5% HtriD-co-2.6% HTD)	−22.7	52.8	26.9	103.4	33.0	183.1	261.3
Caproic acid, non-limited MSM	P(89.7% HB-co-5.1% HV-co-5.2% HTD)	1.8	46.4	43.9	161.0	56.4	174.8	272.3
Caproic acid, N-limited MSM	P(80.8% HB-co-19.2% HTD)	0.7	40.8	11	161.9	27.4	183.5	265.5
Acetic acid:Butyric acid, non-limited MSM	P(92.0% HB-co-8.0% HTD)	2.6	41.0	26.9	172.8	78.1	183.8	271.5
Acetic acid:Butyric acid, N-limited MSM	P(93.0% HB-co-7.0% HTD)	1.7	51.7	39.8	166.9	57.7	181.4	267.4
20% $SMCFAs_{synthetic}$-rich stream, non-limited MSM	P(95.5% HB-co-4.5% HTD)	3.3	47.3	13.5	167.3	61.7	225.2	269.7
20% $SMCFAs_{synthetic}$-rich stream, N-limited MSM	P(94.7% HB-co-0.6% HV-co-4.7% HTD)	2.9	42.2	5.6	168.2	55.1	243.6	267.7
10% $SMCFAs_{synthetic}$-rich stream, N-limited MSM	P(88.4% HB-co-11.6% HTD)	2.6	39.2	15.7	167.8	32.8	215.7	266.4
30% $SMCFAs_{synthetic}$-rich stream, N-limited MSM	P(90.6% HB-co-5.5% HV-co-3.9% HTD)	2.0	46.7	14.4	156.6	29.0	235.7	266.2
10% $SMCFAs_{extracted}$-rich stream, non-limited MSM	P(95.6% HB-co-4.4% HTD)	0.2	65.4	16.3	151.0	21.9	168.1	261.4
10% $SMCFAs_{extracted}$-rich stream, N-limited MSM	P(90.6% HB-co-2.4% HV-co-7.0% HTD)	2.7	53.4	40.2	155.9	56.2	174.2	262.3

T_g, glass transition temperature; T_{cc}, cold crystallization temperature; ΔH_{cc}, change in enthalpy of cold crystallization; T_m, melting point; ΔH_m, change in enthalpy of melting process; $T_{5\%}$, 5% mass loss temperature; T_{ons}, mass loss onset temperature; non-limited MSM, non-limited mineral salt medium; N-limited MSM, nitrogen limited mineral salt medium; SMCFAs, short and medium chain fatty acids; $SMCFAs_{synthetic}$-rich stream, a mixture of synthetic SMCFAs to simulate a real SMCFAs-rich stream; $SMCFAs_{extracted}$-rich stream, a mixture of SMCFAs received from the acidogenic anaerobic mixed culture fermentation of acid whey.

Regardless of the monomeric composition of the macromolecule, phase changes related to glass transition, cold crystallization and melting were observed on the DSC curves of the tested polymers (Figure 5; Supplementary Materials Figure S2).

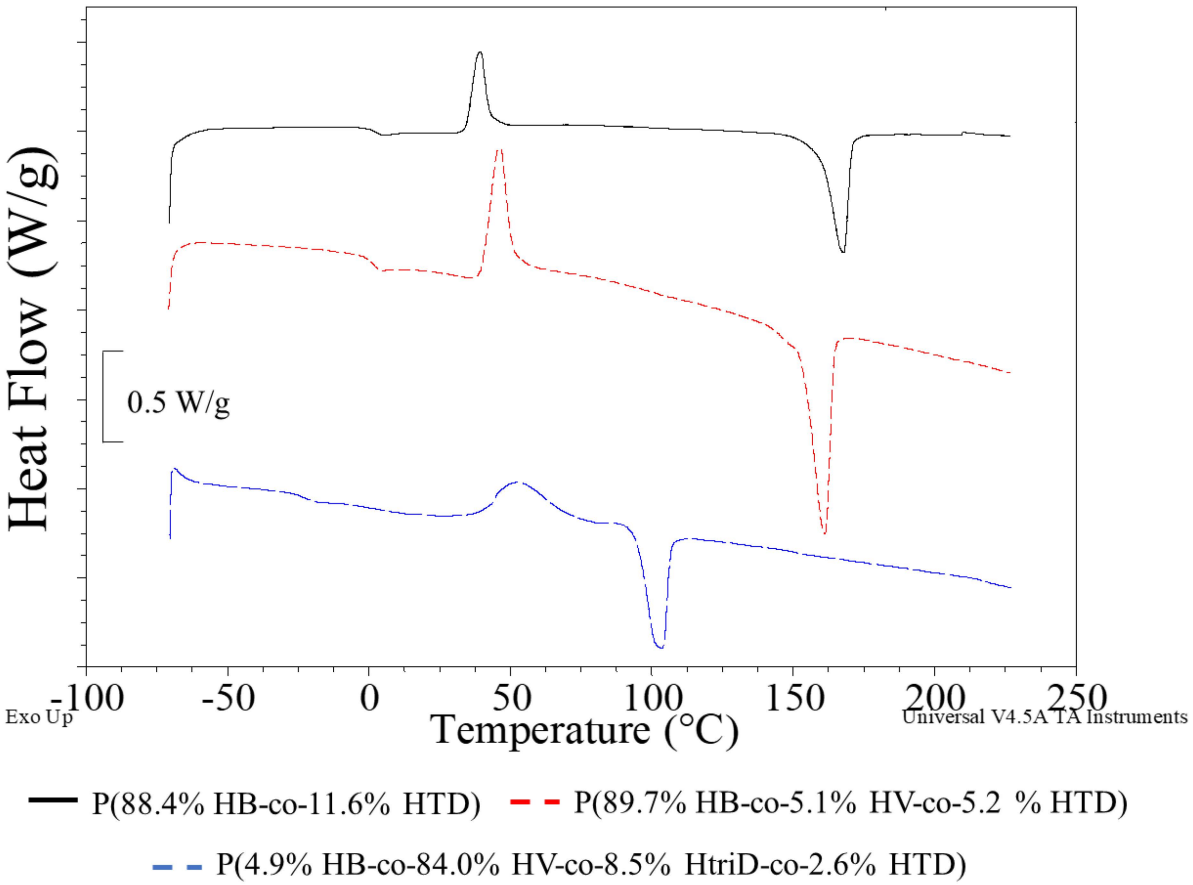


Figure 5. DSC curves of selected samples: (**black line**) P(88.4% HB-co-11.6% HTD) extracted from the culture supplemented with 10% $SMCFAs_{synthetic}$-rich stream under nitrogen-limited condition (**red line**) P(89.7% HB-co-5.1% HV-co-5.2% HTD) extracted from the culture supplemented with 1 g/L of caproic acid under non-limited condition, (**blue line**) P(4.9% HB-co-84.0% HV-co-8.5% HtriD-co 2.6% HTD) extracted from the culture supplemented with 2 g/L of valeric acid under nitrogen-limited condition.

The glass transition temperature (T_g) of pure P(3HB) was estimated to be 5 °C and its melting point (T_m) was determined at the level of 170–180 °C [35,36]. In our study, the lowest T_g value was observed in the PHAs extracted from the cultivations supplemented with valeric acid under non-limited and N-limited condition. The 3HtriD monomer seems to affect the thermal properties of the extracted copolymers. The presence of this unit caused a decrease in T_g to −18.7 or −22.7 °C depending on the mer content (the higher content, the lower T_g values). Also, the T_m decreased compared to the other extracted biopolymers. The recorded T_m values of the P(3HB-co-3HV-co-3HtriD-co-3HTD) co-polymer were 104.4 or 103.3 °C (i.e., they were lower by more than 50 °C compared to the P(3HB-co-3HTD) and P(3HB-co-3HV-co-3HTD) copolymers). Furthermore, T_m values determined for the copolymers extracted from bacterial cells grown on SMCFAs-rich stream derived from acidogenic fermentation were slightly lower compared to the melting temperature for PHAs produced by *Aeromonas* sp. AC_01 cultured on synthetic SMCFAs-rich stream. However, these values were high enough to suggest that these biomaterials could be used in commercial purposes. It was proved that petrochemical derived polymers utilized for

general purpose, engineering and high temperature specialty need to possess melting temperatures around 100, 150, and 300 °C, respectively [37].

TGA thermograms were recorded to evaluate the thermostability of the PHAs produced by *Aeromonas* sp. AC_01 (Figure 6, Supplementary Materials Figure S3).

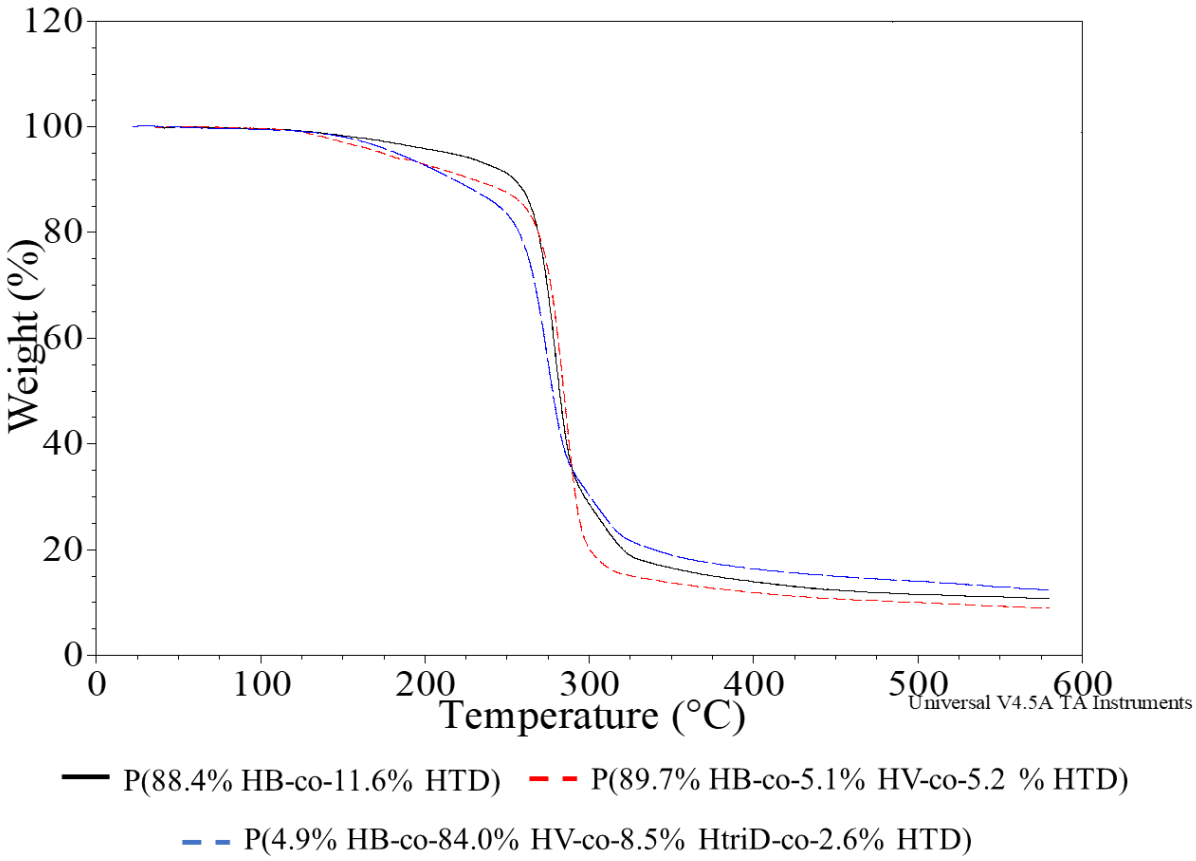


Figure 6. TGA thermograms of selected samples; (**black line**) P(88.4% HB-co-11.6% HTD) extracted from the culture supplemented with 10% $SMCFAs_{synthetic}$-rich stream under nitrogen-limited condition (**red line**) P(89.7% HB-co-5.1% HV-co-5.2% HTD) extracted from the culture supplemented with 1 g/L of caproic acid under non-limited condition, (**blue line**) P(4.9% HB-co-84.0% HV-co-8.5% HtriD-co 2.6% HTD) extracted from the culture supplemented with 2 g/L of valeric acid under nitrogen-limited condition.

The degradation temperature of PHAs, determined on the basis of the 5% weight loss temperature ($T_{5\%}$), ranges from 220 to 300 °C depending on the monomeric composition, with the most popular P(3HB) having a degradation temperature of about 225 °C [34,38]. The recorded $T_{5\%}$ values of the tested biopolymers ranged from 155.8 to 243.6 °C, with no clear relationship between their monomeric composition and $T_{5\%}$ value. The highest thermal stability was observed for the co-polymers extracted from the *Aeromonas* sp. AC_01 cells cultured on $SMCFAs_{synthetic}$-rich stream. The other co-polymers were characterized to possess lower $T_{5\%}$ values than the typical degradation temperatures of PHAs. This was due to an additional minor degradation step ranging from 100 to 200 °C and preceding the main degradation process of the polymer. An additional degradation step may result from the presence of much lower molecular weight compounds in the polymer (non-polymer compounds or oligomers), which are left over from the polymer production process or are formed during polymer synthesis and are characterized by low degradation temperatures. However, FTIR studies indicate the latter, as polymers with higher $T_{5\%}$ values had no or a smaller peak from the terminal -OH groups. In the case of these co-polymers, no additional degradation stage was observed.

An additional parameter (i.e., onset temperature of the main stage of the sample decomposition (T_{ons})) was determined which did not take into account the decrease in the temperature value due to the first stage of degradation. Most of the polymers had very similar T_{ons}, as the recorded values ranged from 261.3 to 272.3 °C. Therefore, it can be concluded that their thermal resistance is similar regardless of the obtained monomeric composition.

4. Conclusions

Our study confirmed that *Aeromonas* sp. AC_01 represents a highly promising candidate for the biotechnological production of PHA being able to utilize SMCFAs for the production of unique short-chain-length–medium-chain-length (scl-mcl)PHAs copolymers. Furthermore, we revealed that SMCFAs-rich stream was not toxic for *Aeromonas* sp. AC_01 and could support bacterial growth and PHA biosynthesis. The composition of SMCFAs could be regulated by the type of feedstock and operation conditions. In the SMCFAs-rich stream used in this study, butyric acid was the dominant product. The GC-MS and FTIR analyses confirmed that the monomeric composition of the extracted PHAs was dependent on the type of the carbon source used and its concentration. Our study proves that *Aeromonas* sp. AC_01 has the ability to change the repeat-units composition of the produced PHAs according to the culture conditions. Furthermore, the results of DSC and TG analyses revealed that the incorporation of 3HtriD monomer in the PHA structure lowered its melting temperature. All extracted PHAs possess high thermal stability which is beneficial taking into account the perspective of these biopolymers processing technology. However, to make this process more efficient, optimization is needed. The PHA productivity should be improved by feeding adjustment and optimization of nutrients and oxygen as well as initial inoculum in the fermentation system. Moreover, the obtained data should be supplemented with additional material analysis especially mechanical properties of the biopolymers that could provide information about their usefulness in particular application areas.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ma15134482/s1.

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Effect of short- and medium-chain fatty acid mixture on polyhydroxyalkanoate production by *Pseudomonas* strains grown under different culture conditions

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Short- and medium-chain fatty acids (SMCFAs) derived from the acidogenic anaerobic mixed culture fermentation of acid whey obtained from a crude cheese production line and their synthetic mixture that simulates a real SMCFA-rich stream were evaluated for polyhydroxyalkanoate (PHA) production. Three individual *Pseudomonas* sp. strains showed different capabilities of growing and producing PHAs in the presence of a synthetic mixture of SMCFAs. *Pseudomonas* sp. GL06 exhibited the highest SMCFA tolerance and produced PHAs with the highest productivity (2.7 mg/L h). Based on these observations, this strain was selected for further investigations on PHA production in a fed-batch bioreactor with a SMCFA-rich stream extracted from the effluent. The results showed that PHA productivity reached up to 4.5 mg/L h at 24 h of fermentation together with the ammonium exhaustion in the growth medium. Moreover, the PHA monomeric composition varied with the bacterial strain and the type of the growth medium used. Furthermore, a differential scanning calorimetric and thermogravimetric analysis showed that a short- and medium-chain-length PHA copolymer made of 3-hydroxybutyric, -hexanoic, -octanoic, -decanoic, and -dodecanoic has promising properties. The ability of *Pseudomonas* sp. to produce tailored PHA copolymers together with the range of possible applications opens new perspectives in the development of PHA bioproduction as a part of an integrated valorization process of SMCFAs derived from waste streams.

KEYWORDS

biopolymers, medium-chain fatty acids, polyhydroxyalkanoates, *Pseudomonas* sp., short-chain fatty acids

1 Introduction

The negative impact of petrochemical plastics on the environment has prompted a growing interest in environmentally friendly alternative materials. Polyhydroxyalkanoates (PHAs) are especially attractive because of their unique material properties such as biodegradability, non-toxicity, and biocompatibility and the similarity of their physical properties to those of synthetic polymers (Zubairi et al., 2016). Moreover, PHAs have many advantages over petrochemical-derived polymers, such as hydrophobicity, thermoplastic processability, relatively high melting point, and optical purity. They are natural polyesters synthesized by various microorganisms under unbalanced growth conditions as intracellular energy compounds in the form of granules in the cytoplasm (Akaraonye et al., 2010). Generally, PHAs are classified according to the carbon-chain-length of the constituent monomers into short-chain-length (PHA_{SCL}) containing 3–5 carbon atoms in the molecule, medium-chain-length (PHA_{MCL}) with 6–14 carbon atoms in the molecule, and long-chain-length (PHA_{LCL}) which contains more than 15 carbon atoms (Tan et al., 2014). It is known that the monomeric composition of PHAs depend mainly on the carbon source used for bacterial culture and the bacterium that is able to synthesize and accumulate PHAs (Koller and Braunegg, 2015). The applicability of bacterial PHAs is directly related to their properties. PHA_{SCL}, such as poly-3-hydroxybutyrate [P(3HB)], shows high crystallinity, stiffness, and brittleness with poor elastic properties, limiting its use as a stable material. PHA_{MCL} shows more favorable and useful properties. They are elastomers with a low degree of crystallinity, melting point, and tensile strength as well as high elongation to break. They are synthesized mainly by bacteria of the *Pseudomonas* genus. Due to their useful properties, PHAs have great potential in biomedical, agricultural, and industrial applications (Tan et al., 2021). Nevertheless, the commercialization of PHAs in the world market is currently limited, mainly due to the high production costs compared to synthetic polymers. Despite the fact that the fermentation technology and polymer extraction process have considerably improved over the past decade, the cost of the carbon source still accounts for half of the cost of producing PHAs. Consequently, there is a growing need to develop novel microbial processes using renewable and inexpensive carbon sources. Short and medium chain fatty acids (SMCFAs) generated by acidogenic anaerobic mixed culture fermentation (MCF) are an interesting alternative to the expensive substrates. Recycling waste into desirable and valuable products is a revolutionary path in waste management and an environmentally friendly process (Martinez et al., 2015). Various renewable wastes converted into SMCFAs such as sugarcane molasses (Albuquerque et al., 2007), paper mill wastewater (Bengtsson et al., 2008), olive oil wastewater (Beccari et al., 2009), food waste (Venkateswar Reddy and Venkata Mohan, 2012; Aremu et al., 2021; Thomas et al., 2022), and pea shells (Patel et al., 2012) have been used to produce environmentally friendly PHAs. In contrast, few scientific publications have evaluated the potential of pure bacterial cultures to convert SMCFA-rich streams from MCF using organic waste into PHAs (Venkateswar Reddy et al., 2012; Cerrone et al., 2014; Martinez et al., 2015; Vu et al., 2021). It should also be mentioned that none of these studies evaluated, in detail, the properties of the extracted PHAs.

An important aspect in the cost-effective production of PHAs is also the selection of a microorganism that has the ability to efficiently intracellularly accumulate this biopolymer using renewable carbon sources. Bacteria belonging to the *Pseudomonas* genus meet these criteria by transforming post-fermentative carbon sources into multipurpose biopolymers. They are also characterized by fast biomass growth, low production requirements, easy adaptation, and tolerance to oxidative stress (Mozejko-Ciesielska et al., 2019). *Pseudomonas* sp. Have been characterized as the producers, usually producing mcl-PHA rather than scl-PHA (Solaiman and Ashby, 2005). The synthesis of PHAs from SMCFAs has been described for several *Pseudomonas* spp., including: *Pseudomonas* sp. ST2 (Reddy et al., 2008; Munir and Jamil, 2018), *Pseudomonas* sp. H9 (Liu et al., 2021), *P. putida* KT2440 (Kourmentza et al., 2009; Cerrone et al., 2014; Yang et al., 2019), *P. otitidis* (Venkateswar Reddy et al., 2012), *P. pseudoflava* (Venkateswar Reddy et al., 2017), *P. palleronii* (Venkateswar Reddy et al., 2017), and *P. oleovorans* (Aremu et al., 2021). To the best of our knowledge, none of these studies described the properties of the extracted scl-mcl-PHA copolymers.

In recent years, the amount of organic and biomass waste has been steadily increasing. Currently, sewage sludge is a burden for municipal wastewater treatment plants (Lad et al., 2022). Moreover, acid whey, a by-product of Greek yoghurt, cottage cheese production, and the like, poses a risk to the ecosystem and its disposal is associated with a fee (Erickson, 2017). Therefore, SMCFAs derived from sludge and acid whey are attractive raw materials for biopolymer synthesis by microorganisms, and this makes the bioprocess more environmentally friendly. This is the first study that reports about SMCFAs from the anaerobic mixed culture fermentation of acid whey, which is available worldwide, to produce unique scl-mcl PHAs. Three strains belonging to *Pseudomonas* genera were first evaluated to study their capability of producing PHAs while they grew on different media supplemented with a synthetic mixture of SMCFAs, that simulates a real SMCFA-rich stream, as the only carbon sources. Then, the most effective *Pseudomonas* sp. strain was cultured with SMCFAs derived from whey wastewater fermentation effluent. The extracted PHAs were characterized using GC/MS, FTIR, DSC, and TG to assess the application potential of these materials.

2 Materials and methods

2.1 Microorganisms and inoculum preparation

Pseudomonas sp. Gl01 and *Pseudomonas* sp. Gl06 strains were isolated from mixed microbial communities and belong to the Culture Collection of the Department of Microbiology and Mycology, University of Warmia and Mazury in Olsztyn. They were previously described as strains capable of synthesizing and accumulating PHA_{MCL} (Ciesielski et al., 2010). The *Pseudomonas antarctica* (DSM 15318) used in the study was purchased from the German Collection of Microorganisms and Cell Cultures GmbH at the Leibniz Institute.

2.2 Culture condition and carbon sources

Seed cultures were grown in lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 30°C, shaking at 150 rpm for 16 h. Then, the precultures were transferred to three different non-limited and nitrogen-limited mineral salt media (MSM): (MSM 1) 12.8 g/L $Na_2HPO_4{\cdot}7H_2O$, 3 g/L KH_2PO_4, 10 g/L NH_4Cl, 0.5 g/L NaCl (Poblete-Castro et al., 2012); (MSM 2) 12.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 10 g/L $(NH_4)_2SO_4$; (MSM 3) 3.5 g/L Na_2HPO_4, 5 g/L KH_2PO_4, 10 g/L $(NH_4)SO_4$. Nitrogen-limited MSM consisted of 1 g/L of nitrogen source in each media. The media were supplemented with 1 g/L of $MgSO_4{\cdot}7H_2O$ and 2.5 ml/L of trace element solution consisting of (per liter): 20 g $FeCl_3{\cdot}6H_2O$, 10 g $CaCl_2{\cdot}H_2O$, 0.03 g $CuSO_4{\cdot}5H_2O$, 0.05 g $MnCl_2{\cdot}4H_2O$, and 0.1 g $ZnSO_4{\cdot}7H_2O$ dissolved in 0.5N HCl. The pH of each culture was adjusted to 7.0 by adding 1N NaOH and 1N HCl. All MSM components were dissolved in water and then autoclaved at 121°C. An $MgSO_4{\cdot}7H_2O$ solution was sterilized and added separately. The MSM were supplemented with 20% (v/v) of a mixture of synthetic SMCFAs ($SMCFA_{synthetic}$-rich stream) or 20% (v/v) of SMCFAs extracted from mixed culture fermentation of acid whey ($SMCFA_{extracted}$-rich stream). $SMCFA_{synthetic}$-rich stream is a mixture of synthetic carboxylic acids composed of 2.85 g/L of acetic acid, 9.86 g/L of butyric acid, 0.16 g/L of valeric acid, and 3.05 g/L of caproic acid. The effluent for PHA production was taken from the acidogenic anaerobic mixed culture fermentation of acid whey obtained from a crude cheese production line (Duber et al., 2018). It was taken from a steady-state phase and contained (except for organic acids mentioned previously) 9.31 g/L lactic acid and 1.64 g/L ethanol.

The *Pseudomonas* spp. strains were cultured in 250-ml Erlenmeyer flasks in a rotary shaker at 150 rpm, at 30°C for 48 h. Fermentation shake flasks were inoculated with 5% v/v of the precultures. The carbon source was added at the beginning of the cultivations. Three replicate cultivations were carried out. The bacterial cells were harvested at 48 h to determine the cell dry mass, PHA concentration, and composition.

2.3 Bioreactor fermentation

The preliminary cultivations in shake flasks led to the selection of MSM, consisting of 12.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$ that supported the best PHA productivity, and *Pseudomonas* sp. GL06 as the most effective PHA producer.

The PHA production of the selected *Pseudomonas* sp. strain was conducted at 30°C with an aeration rate of 4 L/min in a 7 L bioreactor (Biostat A, Sartorius, Germany) equipped with a pH controller. The pH-value was maintained at seven through the modulated addition of concentrated 1N NaOH and 1N HCl. The temperature was maintained by a thermostatic jacket. The dissolved oxygen was monitored during the whole cycle with an O_2 electrode (InPro 6800, Mettler Toledo GmbH, Switzerland) and was maintained at 50% air saturation by adjusting the agitation rate from 300 to 1,000 rpm automatically. Total fermentation time was 48 h. The inoculation size was 10% (v/v). The mineral salt medium was supplemented with a total of 20% (v/v) SMCFAs-extracted. The substrate was added in two portions: first at the beginning of the cultivation and then after 8 h of fermentation. A concentrate solution (Sigma Aldrich) was used as an antifoam in response to the antifoam controller. The samples of the culture broth were removed at several time intervals for analysis of biomass concentration, ammonium and phosphate concentration, PHA yield, the composition, and concentration of PHA monomers.

2.4 Analytical procedures

To measure cell dry mass (CDM), 100 ml of culture broth was centrifuged at 11.200 × g for 10 min and then lyophilized for 24 h using a Lyovac GT2 System (SRK Systemtechnik GmbH). Ammonium and phosphorus concentration were determined spectrophotometrically using a Hach Lange DR 2800 spectrophotometer (Hach Lange, Düsseldorf, Germany) as well as the LCK338 and LCK350 kits according to the manufacturer's instructions, respectively. Quantitative and qualitative analysis of the obtained biopolymers was performed. Biopolyesters were extracted by shaking lyophilized cells in chloroform at 50°C for 3 h. Then the mixture was filtered through No. 1 Whatman filter paper to remove the cellular debris. Biopolymers dissolved in chloroform were purified by precipitation with a 70% cold methanol solution and then allowed to evaporate at room temperature. The PHA content (% of CDM) was defined as the percentage of the ratio of biopolymer concentration to total cell concentration.

2.5 PHA analyses

2.5.1 Gas chromatography coupled with mass spectrometry

To evaluate the PHA composition, the lyophilized cells were analyzed by gas chromatography coupled with mass spectrometry (GC-MS QP2010 PLUS, Shimadzu, Japan) according to the method described by Możejko-Ciesielska and Pokój (2018). As a first step, analyzed samples were suspended in a chloroform-methanol-sulfuric acid mixture (100/97/3, v/v/v), and the methylation process was performed by heating the vials at 100°C for 20 h. After that, the sulfuric acid was neutralized with Na_2CO_3 and the resulting mixture was dried with anhydrous Na_2SO_4. After filtration, the methyl esters were analyzed using a BPX70 (25 m × 0.22 mm × 0.25 mm) capillary column (SGE Analytical Science, Victoria, Australia) with helium as a carrier gas at a flow rate of 1.38 ml/min. The column was heated from 80 to 240°C at a rate of 10°C/min. The interface and ion source temperatures of GC-MS were set at 240°C, and the electron energy was set at 70 eV. The total ion current (TIC) mode was used in 45–500 m/z range. Pure standards of methyl 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxyhexanoate, -octanoate, -nonanoate, -decanoate, -undecanoate, -dodecanoate, -tetradecanoate, and -hexadecanoate were purchased from Larodan Fine Chemicals (Sweden) to generate calibration curves for the methanolysis assay.

2.5.2 Fourier-transform infrared spectroscopy

The infrared spectra were recorded in the range from 4,000 to 650 cm^{-1} with a Fourier Transform Spectrophotometer (FTIR) Nicolet iS10 (ThermoScientific, United States) by the attenuated total reflection method (ATR-FTIR) on samples without prior preparation. Each spectrum analyzed was the average of 16 recorded measurements.

2.5.3 Differential scanning calorimetry and thermogravimetry

Differential scanning calorimetry (DSC) tests were carried out in a nitrogen atmosphere with a Q200 differential scanning calorimeter (TA Instruments, United States). The temperature ranged from −70 to 230°C with a heating/cooling rate of 10°C/min. Samples of approximately 1 mg were first rapidly heated to 230°C and conditioned for 1 min to remove the thermal history of the material. The samples were then cooled to −70°C and re-heated to 230°C. Thermal analysis of polymers was based on the second heating curve, where the glass transition temperature (T_g), melting point (T_m), and change in enthalpy of the melting process (ΔH_m) were recorded.

Thermogravimetry (TG) tests were performed in a nitrogen atmosphere with a Q500 thermobalance (TA Instruments, United States). The temperature ranged from 25 to 700°C with a heating rate of 10°C/min. The mass of the analyzed samples was approximately 1 mg. From the thermogravimetric curve, 5% mass loss temperature ($T_{5\%}$) was determined and used as a parameter determining the onset of thermal degradation of the material, taken as the thermal resistance of the material (T_d). The differential thermogravimetric curve (DTG) (first derivative of the TG curve) was also presented, and the T_{max} values determining the temperature of the fastest mass loss were also presented.

3 Results and discussion

3.1 Capability of *Pseudomonas* sp. strains to grow and produce PHAs in mineral salt media containing $SMCFA_{synthetic}$-rich stream

A preliminary study of the *Pseudomonas* sp. GL01, *Pseudomonas* sp. GL06, and *P. antarctica* was conducted to determine their ability to grow and synthesize PHAs in the presence of a mixture of SMCFAs ($SMCFA_{synthetic}$-rich stream) that mimicked the effluent from whey wastewater anaerobic fermentation as the carbon source.

As can be seen in Figure 1 all tested bacteria could grow in the medium supplemented with $SMCFA_{synthetic}$-rich stream under all culture conditions used. In particular, the *Pseudomonas* sp. GL06 showed the highest biomass concentration among all tested MSM media, achieving the maximum biomass value in MSM 1 (1.8 g/L of CDM). A slightly lower amount of CDM (1.7 g/L) was achieved in the cultivation of *Pseudomonas* sp. GL01 grown on MSM 2 under non-limited conditions. A 3-fold lower CDM of all *Pseudomonas putida* strains (KT2440, CA-3, and GO16) was determined by Cerrone et al. (2014) ,who cultured them using fatty acids derived from anaerobic digestion of grass. In our study, low growth was detected in the cultivation of *P. antarctica* in culture media under non-limited as well as nitrogen-limited conditions, that could suggest the inhibitory effect of the $SMCFA_{synthetic}$-rich stream used. However, higher CDM values were observed in the cultures under non-limited conditions.

In general, adequate nitrogen source is essential for bacterial cell growth. On the other hand, PHA synthesis and accumulation by bacteria belonging to *Pseudomonas* genera is induced by nitrogen limitation in the culture medium (Hoffmann and Rehm, 2005). Our results showed that PHA concentration and productivity increased under nitrogen starvation and depended on the type of MSM used. Only in the case of *P. antarctica* grown on MSM 3, PHA yield was not triggered by nitrogen limitation (Figures 2A,B). The best PHA concentration was achieved in the culture with *Pseudomonas* sp. GL06 grown on MSM 2 under N-limitation (0.13 g/L) at the PHA productivity level of 2.7 mg/L h. Aremu et al. (2021) reported a similar PHA value (0.12 g/L) in *Pseudomonas oleovorans* culture supplemented with acetic acid as the carbon source in a nitrogen-limited medium.

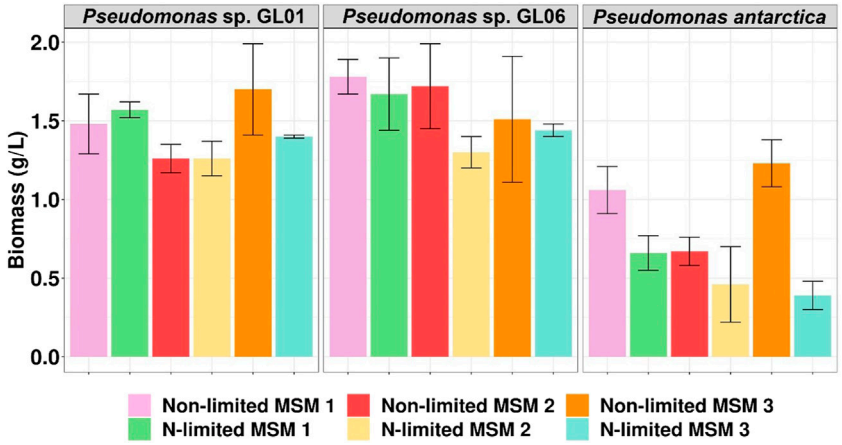


FIGURE 1
Biomass concentration (g/L) of *Pseudomonas* sp. Gl01, *Pseudomonas* sp. Gl06, and *P. antarctica* at 48 h cultured on three different growth media (MSM 1, MSM 2, and MSM 3) in shake flask experiments under non-limited and N-limited conditions using $SMCFA_{synthetic}$-rich stream.

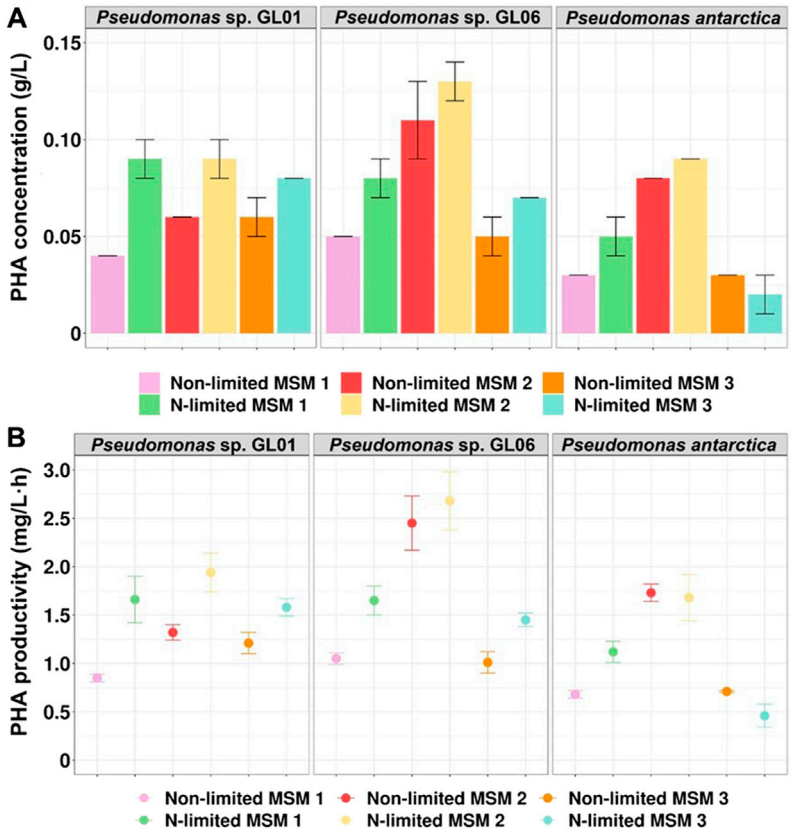


FIGURE 2
PHA production from $SMCFA_{synthetic}$-rich stream by *Pseudomonas* sp. GL01, *Pseudomonas* sp. GL06, and *P. antarctica* at 48 h cultured on three different growth media (MSM 1, MSM 2, and MSM 3) in shake flask experiments under non-limited and N-limited conditions. **(A)** PHA concentration (g/L) and **(B)** PHA productivity (mg/L h).

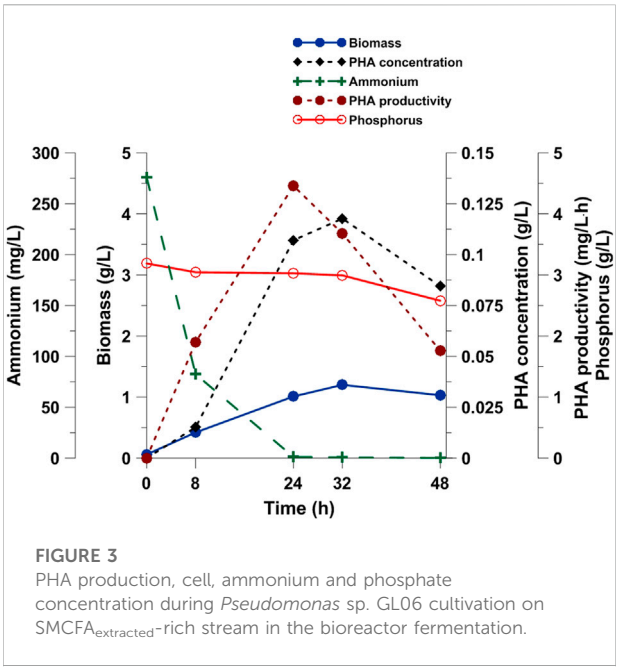


FIGURE 3
PHA production, cell, ammonium and phosphate concentration during *Pseudomonas* sp. GL06 cultivation on $SMCFA_{extracted}$-rich stream in the bioreactor fermentation.

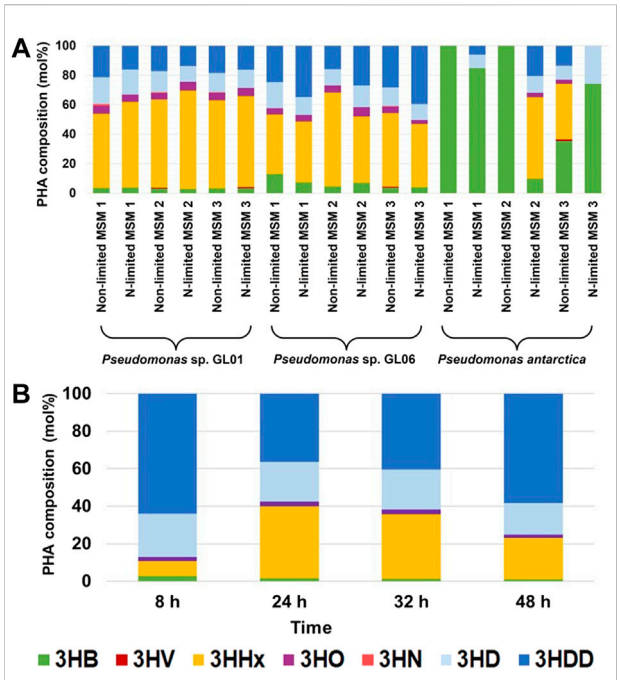


FIGURE 4
Monomeric composition of extracted PHAs from shake flasks **(A)** and bioreactor **(B)**.

Furthermore, our results suggests that PHA production could be enhanced by controlling the nitrogen source. In all tested *Pseudomonas* strains, higher PHA efficiency was observed when MSM 2 [contained $(NH_4)_2SO_4$] was used under both growth conditions compared to MSM 1 (consisted of NH_4Cl). Moreover, our data revealed that when phosphate sources ($Na_2HPO_4{\cdot}7H_2O$ and KH_2PO_4) were used in a higher concentration (MSM 2 compared to MSM 3), PHAs were synthesized and accumulated at higher yields, in particular by *Pseudomonas* sp. GL06 and *P. antarctica*. It could be concluded that the PHA concentration and productivity were dependent on the culture conditions used.

3.2 $SMCFA_{extracted}$-rich stream derived from anaerobic fermentation as the carbon source for biomass growth and PHA production

On the basis of the previously described results that *Pseudomonas* sp. GL06 produced PHAs with the highest efficiency, it was selected for further investigations into PHA production with $SMCFA_{extracted}$-rich stream from the acidogenic anaerobic mixed culture fermentation of acid whey obtained from a cheese production line. The fermentation process in a bioreactor led us to precisely monitor the bioprocess due to larger volume for sampling. As may be observed from the data reported in Figure 3, biomass concentration had been increasing for up to 32 h, and then reached 1.03 g/L at the end of the bioprocess. Comparable biomass data (1.03 g/L) were reported in *P. oleovorans* culture supplemented with volatile fatty acid streams from chicken manure where acetate was the predominant acid (Aremu et al., 2021). Higher biomass concentration was determined by our research group by supplementation of the culture medium with the same $SMCFA_{extracted}$-rich stream in the cultivation of *Paracoccus homiensis* (Szacherska et al., 2021). Moreover, our results confirmed that PHA productivity increased under nitrogen limitation, reaching a maximum value of 4.5 mg/L h at 24 h of the fermentation after ammonium exhaustion in the growth medium was reached. Similar results (4.4 mg/L h) were achieved by Kourmentza et al. (2009), who cultivated *Pseudomonas* sp. on a mixture of acetic, propionic, and butyric acids under nitrogen-limiting conditions. Goh and Tan (2012), by feeding of glucose and glycerol in the cultivation of arctic *Pseudomonas* sp. UMAB-40, determined a much lower productivity, i.e., 2.6 mg/L h and 1.6 mg/L h, respectively. Exponential nonanoic acid-limited growth of *P. putida* KT2440 resulted in a PHA content of 75.4%, giving a high cumulative PHA productivity of 1.11 g/L h (Sun et al., 2007). It should be emphasized that in our study, the PHA yield did not increase more after 24 h. The observed reduction in PHA synthesis can be explained by the consumption of the accumulated PHAs as energy reserves to extend the survival of bacteria after depletion of the carbon and nitrogen sources (Kourmentza et al., 2017). The same observation was made by Venkateswar Reddy et al. (2012) who reported that after reaching the maximum PHA concentration, the production capacity of

Pseudomonas otitidis decreased up to the end of the experiment. The authors suggested that due to the famine phase, the stored PHAs were consumed by the bacteria to maintain their cell activity in the absence of essential nutrients. Furthermore, the PHA productivity was about 60% higher than that determined in the shake flask experiments using the same culture medium. It should be noted that the difference between $SMCFA_{synthetic}$-rich stream and $SMCFA_{extracted}$-rich stream was the presence of lactic acid and ethanol in the latter. Thus, our data suggested that neither lactic acid nor ethanol affected the PHA productivity negatively.

Furthermore, quite low PHA productivity was reported in the cultivation of *P. homiensis* grown on the same (as in our study) $SMCFA_{extracted}$-rich stream, suggesting that the presence of butyric acid as the dominant component had more inhibitory effect due to the fact that a single short-chain-fatty acid in the medium resulted in the lowest PHA production efficiency compared with others (Szacherska et al., 2021).

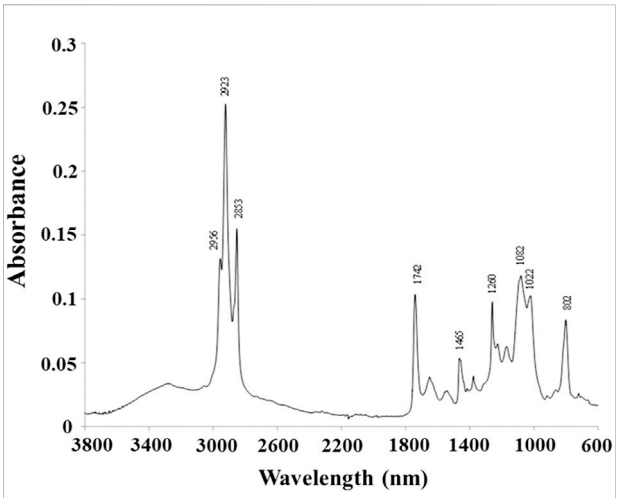


FIGURE 5
FTIR spectra of P(3HB-co-3HHx-co-3HO-co-3HD-co-3HDD) extracted from the *Pseudomonas* sp. GL06 cells at 48 h cultured in the bioreactor.

3.3 The characterization of PHAs

3.3.1 GC-MS

As shown in Figure 4, the PHA monomeric composition varied with the bacterial strain and the type of the growth medium used. Our results proved that in the cultivations supplemented with $SMCFA_{synthetic}$-rich stream that simulated a real SMCFA-rich stream, *Pseudomonas* sp. GL01 and GL06 showed the tendency to produce high amounts of 3-hydroxyhexanoate (3HHx) and 3-hydroxydodecanoate (3HDD) as the main monomers and lower content of 3-hydroxybutyrate (3HB), 3-hydroxyoctanoate (3HO), and 3-hydroxydecaonate (3HD). Interestingly, only in the cultivations of *Pseudomonas* sp. GL01 performed under non-limited growth conditions a trace amounts of 3-hydroxynonanoate was detected. This monomer was found mainly at the end of the fermentation of the same bacterial strain using saponified waste palm oil (Możejko and Ciesielski, 2013). The level of 3HB in the extracted PHAs seems to be strain dependent. Our results indicated that *P. antarctica* is able to synthesize P(3HB) homopolymer grown on MSM1 and MSM2 under non-limited conditions, whereas this bacterium cultured on MSM 3 under both conditions applied was capable of producing scl-mcl copolymers. *P. otitidis* also prefers to produce copolymers, however, consisting of 3HB and 3HV monomers (Venkateswar Reddy et al., 2012). The authors reported that the copolymer showed higher 3HB monomer content (91 mol%) when the bacteria were cultured on synthetic acids (acetate, propionate, and butyrate) compared to acidogenic effluent from the biohydrogen reactor. Furthermore, it was observed that *P. putida* KT2440 grown in the medium supplemented with acetate as the sole carbon source produced P(3HO-*co*-3HD-*co*-3HDD) terpolymer with 3HD as the principal monomer (Yang et al., 2019).

The GC/MS analysis confirmed that at the beginning of the cultivations of *Pseudomonas* sp. GL06 in the bioreactor, no PHAs were detected. The repeat-unit composition of the purified biopolymers extracted from the bioreactor at 8 h of the fermentation was found to consist mainly of 3HD and 3HDD and lesser amounts of 3HB, 3HHx, and 3HO. From 24 to 48 h of the cultivation higher content of 3HHx fraction was observed from 38.5 mol% to 22.1 mol%, respectively. 3HB and 3HO monomers were detected as minor components in extracted biomaterials. Similarly, when glucose, glycerol, and fructose were supplied as carbon sources to *Pseudomonas* sp. B14-6 by Choi et al. (2021), scl-mcl copolymer was detected. However, *P. putida* KT2440 and CA-3 synthesized mcl-copolymers cultured on the concentrated fatty acid mixture derived from the anaerobic digestion leachate. Cerrone et al. (2014) showed that these strains produced 3HD as the main constituent and 3HO and 3HDD as minor components.

3.3.2 FTIR

The FTIR spectra of the polymer produced is presented in Figure 5. The spectrum displays the typical bands for PHAs (Shamala et al., 2009; Rebocho et al., 2020; Meneses et al., 2021). Peaks at 2,956, 2,923, and 2,853 cm^{-1} can be assigned to the stretching vibration of C-H bonds of methyl (CH_3) and methylene (CH_2) groups of the PHA molecule. The high intensity of the 3,000–2,700 band (the highest in the entire spectrum) results from the presence of a large amount of medium chain length monomers in the tested polymer. The absorbance band located at 1740 cm^{-1} is attributed to the stretching vibration of the C=O group (ester carbonyl) in the

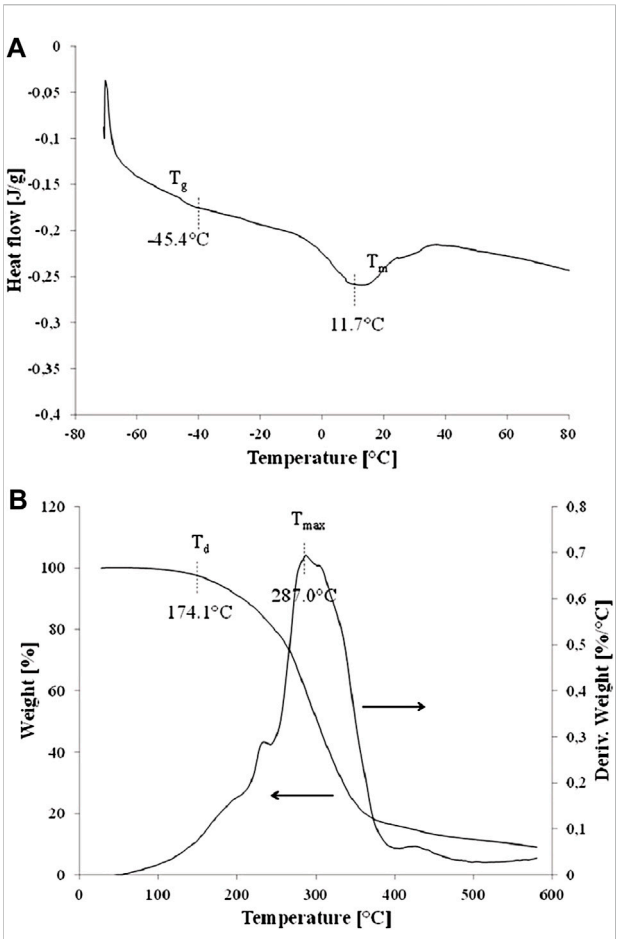


FIGURE 6
DSC curves **(A)** and TG curves **(B)** of P(3HB-co-3HHx-co-3HO-co-3HD-co-3HDD) extracted from the *Pseudomonas* sp. GL06 cells at 48 h cultured in the bioreactor.

PHA polyester. The bands between 1,022 and 1,260 cm^{-1} are related to the crystallinity of the material, with the 1,082 cm^{-1} peak attributed to C–O bonds. The band appearing at 802 cm^{-1} represents the C-C stretching bond and is also characteristic of PHA.

3.3.3 Thermal analysis

As can be shown in Figure 6A, DSC studies proved that glass transition temperature (T_g) of P(3HB-co-3HHx-co-3HO-co-3HD-co-3HDD) produced by *Pseudomonas* sp. GL01 reached −45.4°C. Furthermore, a small melting peak with a melting temperature (T_m) of 11.7°C and enthalpy change (ΔH_m) of 6.6 J/g was observed. The recorded T_g value is typical for mcl-PHAs and corresponds to the values reported in the literature (Simon-Colin et al., 2012; Basnett et al., 2017). The obtained melting point (11.7°C) is low and unusual, as in most cases the recorded T_m mcl-PHA values are in the range from 40 to 55°C. Lower T_m values are related to the low degree of crystallinity of this polymer and the predominance of the amorphous phase. The low content of the crystalline phase in the tested material is confirmed by the FTIR tests (low absorbance of peaks related to the crystalline phase) and the very low ΔH_m value obtained in the DSC tests. Nevertheless a P(3HO-*co*-3HD-*co*-3HDD-*co*-3HTD) copolymer produced by *Yarrowia lipolytica* ThYl_1,166 also possess low T_g (−39°C) and T_m (19°C) values indicating its amorphous behavior and flexibility at ambient temperature (Rigouin et al., 2019).

Moreover, the degradation temperature (T_d) of the tested scl-mcl copolymer was observed at 174.1°C (Figure 6B) and was lower than the typical T_d values recorded for mcl-PHAs (above 200°C) (Simon-Colin et al., 2012; Basnett et al., 2017) due to its low degree of crystallinity. Additionally, TG analysis showed that the beginning of the degradation process already started just above 100°C and proceeded over a fairly large temperature range with the maximum intensity (T_{max}) at 287.0°C (Figure 6B). The course of the degradation process may have been caused by a large number of macromolecules of shorter length (lower molecular weight), which are characterized by lower degradation temperatures. The fact that 3HB monomer was present in the extracted copolymer could be the reason for its lower thermal resistance.

4 Conclusion

Supplementation of synthetic SMCFAs in bacterial cultures would be expensive. Therefore using SMCFA-rich stream from the fermentation of agro-industrial waste to produce PHAs can significantly reduce the cost of the process. In this study, both variants of SMCFAs were proved to be potential substrates for PHA production by *Pseudomonas* strains. The highest PHA productivity was achieved in *Pseudomonas* sp. Gl06 cultivation in the bioreactor under N-limited with MSM 2 using $SMCFA_{extracted}$-rich stream derived from MCF using food waste. The structure and thermal properties of the PHAs were analytically verified and we proved that the extracted scl-mcl copolymers possess lower melting point and degradation temperature compared to the PHAs described in the literature. These findings indicate the possibility of feeding the *Pseudomonas* sp. GL06 with cheap $SMCFA_{extracted}$-rich stream to produce PHA copolymers with improved properties.

Data availability statement

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Author contributions

KS participated in the design of the study and performed the cultivations and PHA extractions. KM performed and interpreted FTIR and differential scanning calorimetric and thermogravimetric analyses. SC performed and interpreted gas chromatography coupled with mass spectrometry analysis. PO-P performed waste-derived SMCFA analysis and participated in the design of the study. JM-C participated in the design of the study and supervised in study coordination. KS and JM-C wrote the manuscript with input from all the authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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