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Technical report on suitability for PHA production of residual streams of different food processing companies

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AF	Anaerobic fermentation
CDW	Cell dry weight
COD	Chemical oxygen demand
COD_{sol}	Soluble chemical oxygen demand
COD_{tot}	Total chemical oxygen demand
COD_{VFA}	Chemical oxygen demand of the volatile fatty acids
FID	Flame ionisation detector
GC	Gas-chromatography
HB	Hydroxybutyrate
HRT	Hydraulic retention time
HV	Hydroxyvalerate
IC	Ion-chromatography
NH_4-N	Ammonia nitrogen
OLR	Organic loading rate
PO_4-P	Ortho-phosphate phosphor us
PHB	Polyhydroxybutyrate
PHA	Polyhydroxyalkanoates
PHV	Polyhydroxyvalerate
SRT	Solid retention time
STP	Sewage treatment plant
TEA	Techno-economic assessment
TS	Total solids
TSS	Total suspended solids
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids

1 Introduction

Our recent experience with the Covid-19 pandemic revealed the importance of creating a society based on values such as environmental protection, sustainable adaptation, and circular economy. It has been estimated that more than 8 million tons of microplastics are entering the oceans each year with this number estimated to increase even further by 2025 if the current plastic management does not improve (Briassoulis & Giannoulis 2018). Alternative renewable and biodegradable materials such as polyhydroxyalkanoates (PHAs) play a key role in replacing fossil-based plastics and are therefore a key topic in current research.

PHAs are biodegradable polymers that can be synthesized intercellularly by various microorganisms in the form of granules as energy storage. Commercial PHA is produced by pure or genetically modified microbial cultures and synthetic carbon feed. Such commercial PHAs are three times higher in price than fossil fuel-based plastic and are therefore still unable to compete in the market due to the high cost of the bacterial culture maintenance and expensive synthetic carbon feed. In the past few years, researchers have been mainly focused on the cost efficiency of PHAs (Rodriguez-Perez et al. 2018).

One possible and economically feasible alternative is the use of mixed microbial communities (MMC) cultivated from activated sludge under non-sterile conditions to produce PHA. The feedstock for the MMC can be derived from industrial side streams such as agricultural feedstock, food-derived side streams, and wastewater (Bengtsson et al. 2008, Carvalho et al. 2018). PHA can be produced in a three-step process. First, volatile fatty acids (VFAs) are produced from an organic carbon-containing side stream in an anaerobic fermentation process. Next, these VFAs are used as feedstock for the enrichment of an MMC with a high PHA production capacity. In the last step, this adapted MMC together with the VFAs as a substrate is used for PHA accumulation under aerobic conditions (Carvalho et al. 2014, Pittmann & Steinmetz 2017, Serafim et al. 2008). After this, the PHA is extracted from the accumulated biomass and the derived polymer can be further processed for bioplastic production.

Research for PHA production from sewage has recently reached large-scale demonstration projects. Such examples include the PHARIO project and the Interreg NWE project WOW!. Life cycle assessment (LCA) studies show that PHA produced as part of a sewage treatment plant (STP) value chain has a 70% lower environmental impact than other bioplastics currently available. However, there are still drawbacks. First, the yield of PHA is still significantly lower compared to PHA produced by pure microbial cultures on synthetic carbon feed with the maximum yield reaching 40% w/w per dry biomass. Furthermore, due to regulations on the usage of bioplastics coming from STP and the social image, such PHA still has limited applications in the market (Ajao 2020).

Side streams from the food processing industry could be applied as an alternative organic carbon source for PHA production by MMC. Food processing industries with complex organic side streams suitable to be turned into feedstock for the PHA production are for example sugarcane industry, dairy industry, meat processing industries, and all kinds of oil and fat residues (STP) (Ganesh Saratale et al. 2021). Sugar and molasses residual streams are interesting due to their abundance and low price with PHA yields up to 30%. Milk and cheese whey residual water consisting mainly of proteins and lactose has the advantage that besides high PHA production yield, valuable nutrients that are essential for bacterial growth are also present (Mirabella, Castellani & Sala 2014). Lipid-rich residual streams from edible oil processing industries, slaughterhouses, and oil mills have been reported as very promising as they can have higher PHA yields (~ 60-80%) (Talan et al. 2020). These examples all illustrate the potential of industrial side streams in the production of circular PHA (Figure 1).

While research has already explored the possibilities of PHA production from industrial side streams, the information on the quality, properties, and possible applications of PHA produced from different industrial

food processing side streams and the possible economic and environmental benefits of this PHA is still very limited (Morgan-Sagastume et al. 2020). This project will therefore investigate various side streams from the food processing industry within the Netherlands and Germany and report which of those streams are suitable for use as a carbon source and/or enrichment feed for PHA production. The influence of the yield and composition of the VFAs produced will be related to the PHA yield, composition and purity, and compared to PHA produced from other sources. Furthermore, the potential for and environmental impacts of PHA produced from industrial side streams will be investigated by means of a Techno Economic Assessment (TEA) and sustainability assessment. Based on the result of this research further research can be initiated for best yielding side stream(s) and optimization of process parameters for further up-scaling.

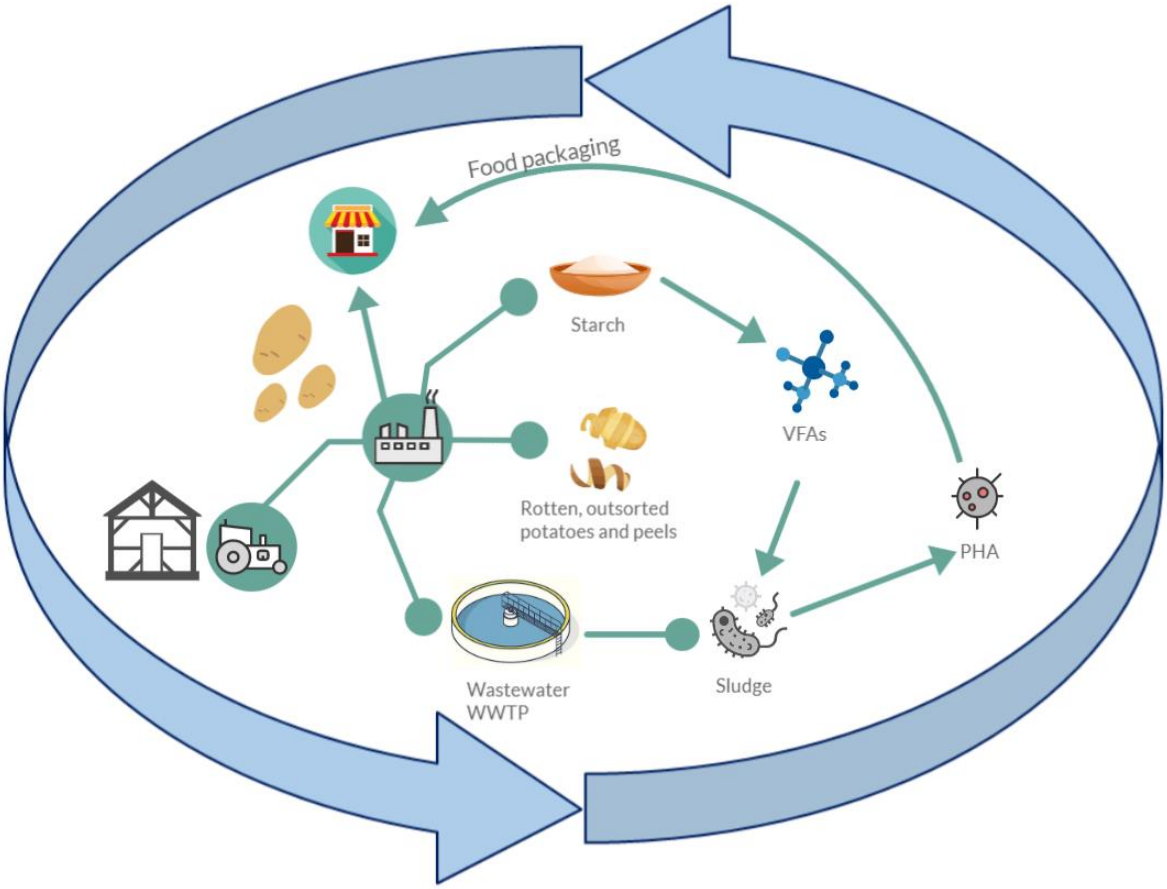

Figure 1: Schematic representation of integrated PHA production in potato processing industry.

1.2 Food processing residual streams availability

Rapid economic development and population growth have increased organic waste generation, which could serve as cheap carbon source for numerous microbial communities. Food waste contributes to approximately 50% of the loss of resources invested in food production. Globally nearly 1/3rd of the food grown is discarded as waste per year. By 2025, according to the World Bank Data, food waste from agricultural and industrial organic residuals will account for 60% of the waste exceeding 1.56 billion tons (FAO 2013).

Industrial companies and governments can drive value from these flows with positive effects on nature and climate. These flows are promising resources of natural fibers, proteins, and fermentable sugars to produce renewable products like biodegradable plastics, pharmaceutical products, adhesives, binding agents, and others.

Feedstock reaching industrial scales like lignocellulosic feedstock such as rice straw, husks, wheat straw, sugarcane bagasse, molasses, and corn stover are some of such well-recognized substrates with industrial scale production of recent microbial products (Raj et al. 2022).

1.3 Anaerobic Fermentation – VFAs as feed for PHA production

Anaerobic fermentation is a method to convert the myriad of compounds present in organic waste streams into just a few defined products that can be reused in agriculture. Anaerobic fermentation consists of three main processes converting the different components of the waste stream to methane and carbon dioxide: (1) hydrolysis of complex substrates, (2) fermentation of monomeric compounds to volatile fatty acids, and finally (3) methanogenesis. Usually, the aim of anaerobic fermentation is the production of methane as the main product. When methanogenesis is inhibited by a high dilution rate or at high or low pH the fermentation is directed into primarily an organic acids production process. Volatile fatty acids (VFAs) such as acetate, propionate, and butyrate are precursors of PHA production, converting organic waste streams into high-value application bioplastics (Tamis et al. 2015).

2 Objectives of the laboratory scale screenings of different residual streams of food processing companies

With the aim of widening the PHA production of residual streams beyond the scope of using primary sludge from municipal sewage treatment plants (STP), the main objective of the laboratory scale experiments was the screening of residual streams of different food processing companies for their fundamental suitability for the production of PHA. Furthermore, it was important to demonstrate the possibility to operate the PHA production without adding aid flows or chemicals to ensure a sustainable production process and to minimize operational costs. A special focus was placed on providing notes for an improvement of the acidification to obtain a VFA-rich substrate as well as on shortcomings which will be tackled during the pilot scale production using the two most promising residual streams, e.g. the nutrient balance within the substrate.

3 Materials and Methods

The following chapter comprises the materials and methods that were applied at the laboratories of Avans University of Applied Sciences in Breda, Netherlands and of RPTU University Kaiserslautern-Landau, Germany.

3.1 Laboratory scale experiments at Avans University of Applied Sciences

3.1.1 Preliminary tests

The residual waste streams used in this work were provided by LOOOP and PeelPioneers. Preliminary experiments were conducted to determine the most promising residual stream for VFA production and fermentation parameter regarding ratio of chemical oxygen demand (COD) of the residual stream to

microorganisms concentration as volatile suspended solids (VSS). According to Chen et al. (2017), Garcia-Aguirre et al. (2017), Silva et al. (2016) the amount of the substrates used was weighed to obtain 10.000 mg/L of COD. After the substrate mass was weighed, it was solubilized with deionized water. The temperature was kept at 37°C, the initial sludge concentration of VSS was 500 mg/L. The fermentation duration was 5 days.

A scheme of the apparatus used for these tests is shown in Figure 2. It is a 500 mL bottle, sealed with a metal lid and a rubber ring.

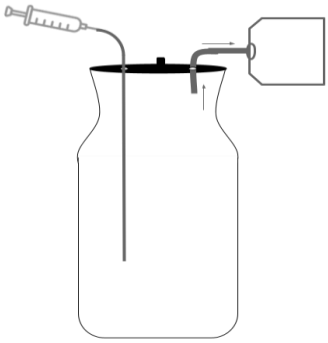

Figure 2: Scheme of the experimental apparatus in 500 mL borosilicate bottles

3.1.2 Anaerobic fermentation

Anaerobic fermentation experiments were conducted batch-wise in 2.5L bioreactors (Infors™ MINI-2.5-BACT, Bottmingen, Switzerland) with a working volume of 1L. The inoculum used in all the experiments was activated aerobic sludge collected at the STP of the water board Brabantse Delta in Bath, the Netherlands. The ratio of substrate to microorganisms (S/M) is adjusted to 20 g COD/g VS in all experiments. After the preliminary studies to select the most promising residual streams, a pH optimization experiment was performed. The goal was the optimal molar ratio of acetic and propionic acids combined with the higher g COD_{VFA} produced. Therefore, the solution of the diluted substrate and activated sludge was adjusted to pH 5.5, 5, and 4.5 in the bioreactors. The DO and pH probes are calibrated prior to the start of each fermentation.

The batch strategy is initiated with the preparation phase including nitrogen gas purging of the bioreactors until the DO is at 0% under constant stirring at 100rpm/min and at a constant temperature of 37°C. The pH is initially adjusted at the selected pH value by the addition of 1M NaOH. When the DO is 0% and the pH is adjusted the experiment goes to the anaerobic fermentation (AF) phase. During this phase, the gas flow is turned off and the reactors are left for acidification. The bioreactors are in a loop between the AF phase and pH adjustment meaning that every time the pH drops to 0.05 difference from the initially set pH, 0.5M of NaOH is added until the measured pH matches the set point. The pH of each experiment is kept at ± 0.05.

Each batch is running for a maximum retention time of 7 days. Samples for VFA yield and composition analysis are taken on day 0, 1, 2, 4, 7 and analysed with IC. Samples on day 0 and 7 were also analysed for COD_{tot}, COD_{sol}, total nitrogen, and phosphorus. Samples from days 0, 2, 4, and 7 were analysed for their bacterial community shift. Samples were collected daily and kept under a 5°C refrigeration until the IC analysis.

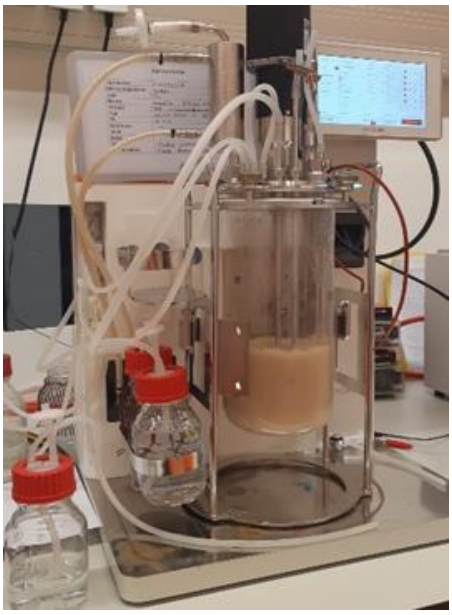

Figure 3: Bioreactor in anaerobic fermentation

3.1.3 Inoculum characterization

Activated sludge from Bath STP was used as inoculum in this work. The amount of inoculum used in the experiments was calculated based on the value of the VSS. To determine the TS and VSS, a glass fiber filter (GE Healthcare, Whatman™, diameter 70mm, CAT No.1820-070) was washed with demi water and dried overnight in an oven at 100°C. After that, the glass fiber filter was kept in a desiccator until ready to use. The filter was weighed (W_1) and used to filtrate a known volume of the inoculum. Demi water was used to wash the solids on the filter, to eliminate salts and other inorganic compounds. After the filtration, the filter with the cake was placed in an aluminum pan and dried overnight in the oven under the same conditions previously stated. The dried filter with the cake was then weighed (W_2). The filter was placed in a crucible and put in a muffle furnace at 550°C for 3 hours. The equations below were used to calculate the TS and VSS values of the inoculum.

$$Ashes = W3 - W1 \text{ [mg]} \quad (1)$$

$$TS = \frac{W2-W1}{V} \text{ [mg/L]} \quad (2)$$

$$VSS = \frac{TS-Ashes}{V} \text{ [mg/L]} \quad (3)$$

W_1: Filter [mg]

W_2: Filter + TS [mg]

W_3: Filter + Ashes [mg]

V: Inoculum volume [L]

3.1.4 COD analysis

To measure the total COD of the samples, a COD kit was used (LCK014, COD cuvette test 1,000-10,000 mg/L O_2, Hach). Using a pipet, 0.5 mL of the sample mixture was added to the vial and vigorously homogenizing. The vials were then put in a preheated dried bath at 148°C for 2 hours. The vials were allowed to cool down to room temperature prior the measurement of the COD using a spectrophotometer (Hach, DR6000 spectrophotometer).

3.1.5 VFA Analysis

To analyze the content of VFAs on the samples, the method used was Ion Chromatography (IC), on a Thermo Scientific Dionex ICS-1500 system. Before the analysis, all the samples were centrifuged for 5 minutes at 4.000 rpm. After that, the supernatant of the samples was placed in a 5 mL vial (Thermo Scientific, P/N 038009, 5mL Vials) and closed with the cap (Thermo Scientific, P/N 038009, 5mL Filter Caps). The eluents used were heptafluorobutyric acid (HFBA) 0.5mM and tetrabutylammonium (TBAOH) 5mM.

A calibration curve was done for the main four acids that are expected from the experiments: lactic, formic, acetic, propionic, butyric, and valeric acid (LA, FA, AA, PA, and BA, respectively). The result of the calibration curve is shown in Appendix 1. Quantification of lactic and valeric acids was only evaluated for the experiments varying the pH.

3.2 Laboratory scale experiments at RPTU University of Kaiserslautern-Landau

The setup of the laboratory screening experiment consisted of 7 process stages:

1. Anaerobic acidogenic fermentation of the residual stream to produce a VFA-rich stream.
2. Centrifuge to separate the non-acidified solids from the liquid VFA-rich stream.
3. VFA-storage tank to feed the following process stages.
4. Biomass enrichment tanks for selecting biomass with high PHA-production capacities.
5. PHA-accumulation tanks for producing biomass with a high intracellular PHA-content.
6. Centrifuge for dewatering the PHA enriched biomass.
7. Freeze-drying to dry the PHA-enriched biomass.

The process stages are described in more detail in the following subsections.

3.2.1 Acidification of the residual streams, solid/liquid-separation and VFA-storage tank

The residual stream was mixed with 10 % inoculum by volume to give the acidification a head start. Sludge from the anaerobic digester of the sewage treatment plant Kaiserslautern (STP KL) was collected and previously anaerobically stabilised referring to VDI 4630. Therefore, the anaerobic digested sludge was stored in a 60 L barrel for one week which was temperature controlled to 35.5°C using a heat exchanger and a temperature-controlled water bath (ME-12, Julabo) and constantly stirred using an overhead stirrer (RZR 1, Heidolph).

For the anaerobic acidogenic fermentation of the substrate two barrels with a working volume of 150 L each were used. During the screening process three different residual streams were acidified and used as substrate for the consecutive PHA production steps. The anaerobic acidogenic fermentation was

temperature controlled using a heat exchanger which was connected to a temperature-controlled water bath (ME-12, Julabo) with a set temperature of 35.5°C. The mixture of the residual stream and the inoculum was constantly circulated using an aquarium pump (Turbelle® Nanostream® 6015) with a flow rate of ca. 1,800 L/h. In the beginning of the experiment the initial pH was measured and adjusted to a pH < 6 using hydrogen chloride (HCl) with a concentration of 25 % (> 99 %, Carl Roth®). During the seven days of the acidification process, the pH was measured regularly on day 2, 5 and 7 and adjusted below pH 6 to prevent methane formation. However, to prevent the contamination of the surrounding air with hydrogen sulphide, methane or any other digester gases, the gases were directed to a gas washer.

The retention time was set to 7 days for an additional prevention of methane production and to allow a weekly working routine. After 7 days the reactor was emptied, and the content was pumped into a basket centrifuge (ZS21 EUR; Eurotec Innovation) with a 3.5 L basket and 2070 g to separate the VFA-rich liquid from the non-acidified solids.

After centrifugation, the VFA-rich solution was filled into 30 L cans and stored at -16°C, respectively 4°C until use for the PHA production. The storage tank used to feed the PHA production was only filled with the amount of substrate needed for 2 – 4 days to limit the volatilisation of the VFAs and constantly circulated using an aquarium pump (Turbelle® Nanostream® 6015) with a flow rate of ca. 1,800 L/h.

The experimental focus of the acidification was to examine and compare the composition of the VFAs produced from the different residual streams. Therefore, samples were taken in the beginning of the acidification as well as on day 2, 5 and 7. Due to the fact that the residual streams were taken as grab samples, it is likely that the VFA-concentration and nutrient composition varies during the future pilot scale production of VFA. Furthermore, the PHA-composition is likely to change as this is influenced by the VFA-composition of the feed.

3.2.2 Biomass enrichment, PHA-accumulation, dewatering and drying

The biomass enrichment and PHA accumulation were performed as triplicates, using three reactors with a working volume of 10 L for the biomass enrichment stage and three reactors for the PHA accumulation with a working volume of 5 – 7 L depending on the substrate used. Both the biomass enrichment as well as the PHA accumulation reactors were aerated using the compressed-air line from the laboratory which was connected to the reactors in a circular system with a pre-pressure of 1 bar, providing an air pressure of 0.1 bar to the reactors. Each reactor had a total of four stainless steel air diffusors which were cleaned once a week. Furthermore, overhead stirrers (RZR 1, Heidolph) were used to achieve a continuous mixing in the reactors. All of the reactors were provided with a pH- (SL 82-120 pHT, Xylem) and oxygen-measuring probe (MF39VP, Xylem) which recorded the data in the central process management system. pH was monitored but not controlled. The temperature in the reactors was controlled using an aquarium heater (Nano ThermoCompact 25 W, Dennerle). In the biomass enrichment reactors, the withdrawal of the supernatant as well as the feed of the substrate was controlled by level measuring (AquaBar (II) Pressure-suspension probe, Nivus). At the end of each cycle the volume was reduced to 7.5 L and subsequently filled with the VFA-feed until the level was back to 10 L (OEM-peristaltic pump M500, Verder). The pumps were controlled by a LabView based central process management system. The PHA accumulation reactors only comprised peristaltic pumps for time-dependent control of the VFA feed.

In the beginning of the PHA production experiments the biomass enrichment reactors were filled with 7.5 L of waste activated sludge with a content of total suspendend solids between 3.5 and 5.3 g/L from the STP KL. The biomass enrichment was operated in a 12 h feast and famine regime cycle. Therefore, in the

beginning of the first cycle, the VFA-rich substrate was added until a volume of 10 L was reached. After this feeding-phase the biomass entered the first feast and famine phase. Before every second VFA-feed after 11:15 h a sedimentation phase was implemented to retain the biomass (aeration and overhead stirrers were automatically stopped). Therefore, after 12 h either the mixed liquor or the supernatant was pumped out. The solid retention time (SRT) was 4 d and the hydraulic retention time (HRT) was set to 2 d, while the organic loading rate (OLR) was 1.2 (Brewery), 0.4 (first Milk batch), 0.2 (second Milk batch) and 0.3 (Pizza) g COD_{VFA}/(L·d) depending on the substrate used.

Once a week the mixed liquor from the biomass enrichment was used for PHA-accumulation. The accumulation was operated as a fed-batch reactor wherein over 24 h every 30 min a VFA-feed was pumped into the reactor (OEM-peristaltic pump M500, Verder). One feed consisted of about 100 mg VFA/(L starting volume). The 24 h duration of the accumulation was chosen for a better workability. Once the accumulation was finished, the untreated mixed liquor was prepared for analysation. To stop the accumulation and to prevent a degradation of the PHA until analysation concentrated sulfuric acid (95-97 %, EMSURE®, Merck KGaA) was added until a pH < 2 was reached. Afterwards the samples were washed with deionized water to remove the remains of the sulfuric acid. A centrifuge (SORWALL LYNX 600 380/400 V, Thermo Fisher Scientific™) was used for the washing and the solid-liquid separation of the PHA-rich biomass (3x for 1 minute at 4500g). The dewatered biomass was frozen at -20 °C and afterwards dried at -82 °C and 0.01 mbar for approximately one week in a freeze dryer (VaCo 5; ZIRBUS technology GmbH).

The focus of the biomass enrichment and PHA-accumulation was to examine the suitability of the different wastewaters for PHA-production, without adding nutrients or other chemicals e.g., for pH-control.

3.2.3 Analytical Methods

The analytical methods are in line with the previously applied methods of (Uhrig et al. 2022) and are specified in the following paragraphs for completeness.

3.2.3.1 Standard urban water management parameters

Standard urban water management parameters were measured either according to standard methods or with Hach cuvette tests. Table 1 summarizes the applied methods.

Table 1: Applied methods for determining standard urban water management parameter

Parameter	Method	Remarks
COD_{tot}	Photometric quick test	Hach cuvettes; LCK 514
COD_{sol}	Photometric quick test	Hach cuvettes; LCK 514
NH_4-N	Photometric quick test	Hach cuvettes; LCK 303 or 304
PO_4-P	Photometric quick test	Hach cuvettes; LCK 350
TSS and VSS	DIN 38 409- H 2-2	Black ribbon filter; 589/1, D = 100 mm, Fa. Whatman
TS	DIN EN 15934	-
VS	DIN EN 15935	-

To remove solids for the analyses of COD_{sol}, NH_4-N and PO_4-P samples were centrifuged for 10 min at 4500 g (SORWALL LYNX 600 380/400 V, Thermo Fisher Scientific™) and two-step filtrated through a folded filter (595½, D = 185 mm; Whatman) and a syringe filter (0.45 µm, Minisart, regenerated cellulose, D = 25 mm; Sartorius).

3.2.3.2 Volatile fatty acids

Before analysis, samples were prepared by centrifugation for 10 min at 4500 g (SORWALL LYNX 600 380/400 V, Thermo Fisher Scientific™) and a two-step filtration through a folded filter (595½, D = 185 mm; Whatman) and a syringe filter (0.45 µm, Minisart, regenerated cellulose, D = 25 mm; Sartorius). Afterwards, the pH of the samples was adjusted to a value of < 2 with sulfuric acid (95-97 %, EMSURE®, Merck KGaA) and they were subsequently frozen and stored at -20 °C until further procession (van Loosdrecht et al. 2016). The measurement of the VFA-concentration and -composition was done by ion chromatography (930 Compact IC Flex with Metrosep Organic Acids - 250/7.8 cation exchange column; Metrohm). The samples were analysed for formic-, lactic-, acetic-, propionic-, butyric-, iso-butyric-, valeric-, iso-valeric- and caproic-acid.

3.2.3.3 PHA

For evaluation of the PHA production, grab samples (3 x 45 mL per reactor) were taken directly after each accumulation batch and treated as described in chapter 2.3.

PHA-content and polymer composition were determined at least in duplicates according to (Braunegg, Sonnleitner & Lafferty 1978) with some minor changes. Approximately 5 mg of the sample was weighed into a 9 mL centrifuge DURAN®-glass tube (16 x 100 mm, conical bottom, PTFE coated GL 18 cap, Rettberg) and mixed with 1 ml of a methanol solution (LiChrosolv®, Merck KGaA) containing 5 % sulfuric acid, 0.8 ml of chloroform (>99 %, Fisher Scientific) and 0.2 ml of benzoic acid (99.5 %, Acros Organics) dissolved in chloroform (5 mg/ml) as an internal standard. Subsequently, the samples were heated in a thermo shaker (MHR 23, Hettich) for 6 h at 100 °C with continuous shaking at 450 rpm. After cooling to room temperature, 1 ml of 1 % NaCl solution (>99.5 %, Carl Roth GmbH + Co. KG, in ultrapure water) was added and samples were first shaken to promote phase separation and then centrifuged at 4500 g for 5 min (Hermle Z 206). This was followed by extraction of the lower solvent phase using a 1 ml-syringe. The solvent phase was measured using a GC-FID (Agilent 8860 design; Agilent Technologies™) with a HP 5 column (30 m x 0.32 mm, 0.2 µm), an injection volume of 0.5 µl, a flow rate of 20 mL/min helium, a split ratio of 1:50 with nitrogen. The Detector temperature was set to 250 °C with a heating rate of 10 °C/min after an initial temperature holding phase of 60 °C over 4 min. Evaluation was based on calibration using a PHBV standard (poly(3-hydroxybutyric aicd-co-3-hydroxyvaleric acid), 8 % PHV-content; Sigma-Aldrich Co.) and an internal benzoic acid standard (see above).

4 Results and Discussion

4.1 Laboratory scale experiments at Avans University of Applied Sciences

4.1.1 Preliminary tests

The aim of the preliminary tests was to better understand the influence of the different parameters during the AD, to standardize the ratio between COD of the residual streams and activated sludge concentration and fermentation duration, and select the two streams with the highest total VFA production for further experiments in lab scale bioreactors.

Table 2: Comparing VFA production of different residual streams.

Substrate	Concentration (mg/L)				
	Formic Acid	Acetic Acid	Propionic Acid	Butyric Acid	Total
Fibre	~0	83	9.4	3.8	96.2
Starch	~0	57.4	7.5	2.6	67.5
Spezial	~0	73.5	9.2	4	86.7
Futtermolke	~0	60.8	7.4	1.4	69.6

As shown in Table 2 Fibre and Spezial had the highest total VFA production with a high concentration of acetic and propionic acids. Comparing the composition of the different streams, Fiber and Spezial had the highest sugar and carbohydrate concentrations (Table 3). Carbohydrates and proteins provide different results in terms of fermentative products. Simple sugars are directly fermented while carbohydrates are easily converted through hydrolysis into glucose that is immediately used for VFA production through the glycolysis pathway (Rasi et al. 2022). On the contrary, proteins like lactose present in Futtermolke stream, exhibit lower biodegradability with respect to carbohydrates, due to their three-dimensional structure, which makes them less susceptible to degradation. The degradation of amino acids involves a hydrogen-consuming reaction that can be thermodynamically unfavourable and therefore makes protein-rich residual streams less prone to hydrolysis. For this reason, hydrolysis can be the rate-limiting step during fermentation (Strazzera et al. 2021).

Table 3: Residual stream characterization considering the dilution factor of experiment 1BT. Residual streams Fibre (FB), Spezial (SP), Starch (ST) and Futtermolke (FT). All units in g/kg of dry matter.

Parameter	RESIDUAL STREAMS			
	FB	ST	SP	FT
Dilution factor	11	62	29	10

COD measured	9,416	8,215	10,454	7,781
Dry matter	0.15	0.06	0.1	0.09
Sugar	2.22	0.4	7.0	~0
Starch	51.7	12.8	14.8	~0
Lactose	~0	~0	~0	79.5
Alcohol	0.80	0.15	0.07	~0
Protein	6.1	0.5	4.2	5.0
Fat	1.2	0.2	1.0	0.3
Lactic acid	8.0	0.6	3.0	11.8
Acetic acid	2.8	0.2	1.1	~0
Cl^-	~0	~0	0.1	2.2
K^+	0.3	~0	0.3	3.3
Na^+	0.6	~0	0.3	0.8
Ca^{2+}	0.1	~0	~0	1.0

**Unit: g/kg of dry matter.*

For the sugar comparison, it was noticed that by increasing 5 times the VSS from 100 to 500 mg/L, acetic acid production increased more than 32 times, and more than 92 times the propionic acid yield as shown in Table 4. In addition, although the butyric yield increased more than 10 times, the final yield and concentration was still low. From these results, it is noticed that by increasing the initial VSS and, consequentially, the number of active bacteria, it is possible to reach a considerably higher VFA production.

Table 4: Comparing the VFA production of sugar prepared with varying VSS concentrations after 4 days of AD.

COD (mg/L)	**VSS (mg/L)**	**Concentration (mg/L)**				
		FA	**AA**	**PA**	**BA**	**Total**
10 000	100	18.8	36.6	4.7	4.0	64.1
10 000	500	247.7	1190.1	462.3	45	1945.1

4.1.2 Bioreactor tests

For the preliminary experiments, the residual streams Fiber and Spezial were selected for lab-scale bioreactor tests. Moreover, the residual stream from orange peel processing, supplied by Peel Pioneers was tested as well. All bioreactor tests were prepared in 1L working volume with around 10,000 mg/L COD, 500 mg/L activated sludge inoculum, and pH controlled between 5 and 6. The results are shown in Table 5.

Table 5: Composition and results of tested food residual streams in bioreactors.

Substrate	Concentration (mg/L)							
	FA	AA	PA	BA	Total	$COD_{tot,in}$	$COD_{VFA,out}$	VFA-yield ($COD_{VFA,out}$/ $COD_{tot,in}$)
Fibre	175.4	1,741.4	145.2	51.1	2,113.1	10,410	2,236.08	0.21
Spezial	303.6	3,151.2	771.5	74.4	4,300.7	15,280	6,479.58	0.42
Orange peel	-	9,011.4	898.8	63.3	9,973.5	40,000	16,310.63	0.41

Analysing the yields obtained for each waste stream, it is noticed that the Orange peel and Spezial performed better regarding both the total yield and the acetic acid yield. Fiber and Spezial, do not present a huge difference in their compositions, but during this experiment, Spezial had around 8.7% higher VFA concentration. The difference in VFA yield between these streams can be explained as a result of an oxygen leak during the first 13h of fermentation. Literature shows that micro aeration conditions can improve the VFA production and influence the VFA composition during the fermentation however during the Fiber experiment more than 70% of dissolved oxygen was present in the bioreactor which could have caused the death of some strictly anaerobic organisms i.e., die within minutes in >0.5% oxygen, and aerotolerant bacteria i.e., tolerate 2% to 8% oxygen resulting in a lower VFA yield (Lim, Chiam & Wang 2014).

Acetic and butyric acids are preferred for polyhydroxybutyrate (PHB) production, while propionic acid is required when producing polyhydroxyvalerate (PHV) (Shen et al., 2014). In the accumulations conducted by the PHA team of Avans in the most recent project, a synthetic feed of acetic and propionic acid was given to the bacteria community in the process explained in 2.2. The acetic acid and propionic acid concentrations in this synthetic feed were 11,918 and 4,915 mg/L, respectively. These concentrations bring a molar ratio 3:1 of acetic acid to propionic acid aiming for the production of polyhydroxybutyrate-co-valerate (PHBV) with a molar ratio of 1:1 hydroxybutyrate (HB) to hydroxyvalerate (HV) monomers. The total COD of this feedstock was around 20 g/L. These values were used, theoretically, as the ideal concentrations and molar ratios of the feed for the accumulation process. The final concentrations of each VFA, the AA/PA molar ratio, and the total COD of the acidified products for the three best waste streams of OP, SP, and FB are shown in Table 6, together with the synthetic feed values.

Table 6: Comparison of VFAs COD for PHA production.

Substrate	FA concentration (mg/L)	AA concentration (mg/L)	PA concentration (mg/L)	BA concentration (mg/L)	Ratio AA/PA	COD (g/L)
Synthetic feed	0	11918	4915	0	3.0 : 1	20
FB	175	1741	145	51	14.8 : 1	2.2
SP	304	3151	771	74	5.0 : 1	4.8
OP	0	9011	899	63	12.4 : 1	11.1

As it is possible to see from Table 6, none of the waste streams could achieve the same molar ratio or COD as the synthetic feed. SP, however, was the substrate that presented the smallest molar ratio difference compared to the synthetic feed. This ratio is important because the VFA feedstock composition directly influences the final polymer obtained in the accumulation process. From this, it is possible to see that, even though the OP waste stream presented higher VFA content and higher yield, some adjustments in the fermentation parameters must be done to spike the propionic acid production once this acid is required to increase the PHV production. These modifications are important to increase the quality of the final PHA produced and open more possibilities for applications of this biodegradable polymer.

4.1.3 Anaerobic fermentation in varying pH

Anaerobic fermentation of Spezial and OP residual streams was done at different pH for seven days. The ratio between the residual stream to the inoculum was kept at 20 g COD/g VSS and the temperature of the reactors was maintained at 37°C with a working volume of 1L. The goal was to investigate how the pH influences VFAs' production and composition. Figure 4 shows the VFA yields expressed as COD concentration of VFAs produced per initial total COD for both residual streams tested. After 7 days of acidification, Spezial shows the highest VFA yield at pH 5.5 with around 39%. Orange peel reached its highest yield at pH 4.5 around 25%. The lowest yields for Spezial and Orange peel were obtained at pH 4.5 and 5.0 respectively. The varying results for each residual stream show that the optimal acidification parameters shift with the composition. These results can be explained as starch present in Spezial is more easily hydrolysed and converted than components, such as pectin, in Orange Peel that first have to be converted to lactic acid and later to VFAs.

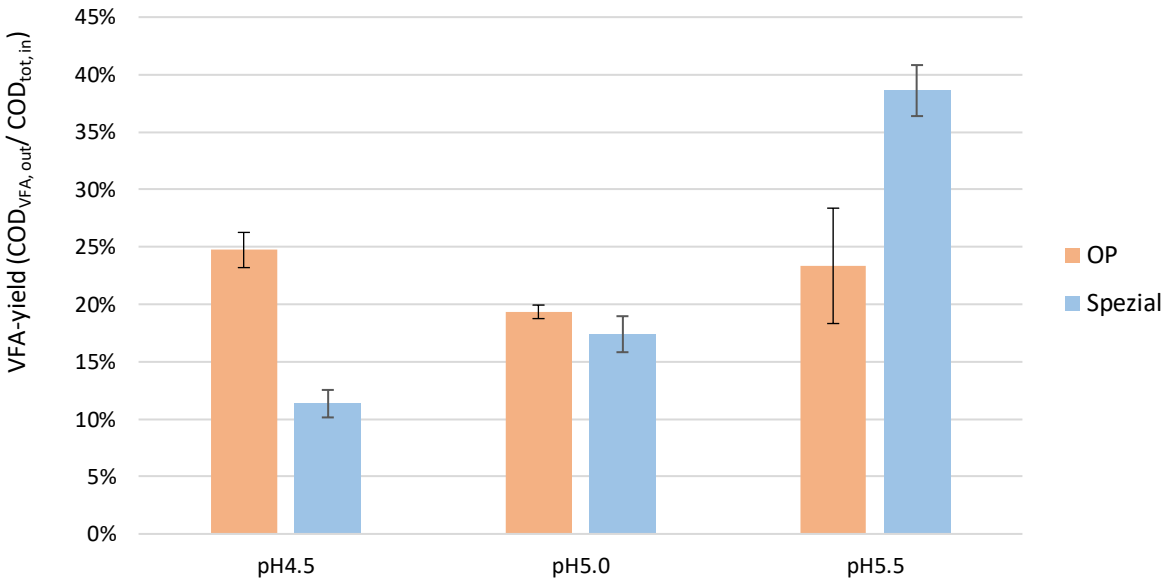

Figure 4: VFA yield comparison for ORANGE PEEL (OP) and SPEZIAL (SP) in different pHs at day 7.

Figure 5 shows the molar composition of Spezial residual stream for pH 4.5 which is divided among butyric (59%), acetic (30%) and propionic (11%) acid as the main acids produced after 7 days fermentation. The composition shifts gradually to a higher amount of acetic and propionic with the increase of the pH to 5.5. This shows that the production of acetic and propionic is favoured at pH 5.5. The ratio between acetic and propionic acids for each pH experiment can be compared in Table 6. At pH 4.5 and pH 5.5 the molar ratio is similar (2.8 and 2.8 respectively) but the presence of butyric acid is not preferential for further steps in the PHA production and therefore, at pH 5.5 is the considered the optimal for PHA production. The molar composition of Orange Peel for pH 4.5 and 5.0 presents a higher amount of lactic acid (80%) at the end of the 7 days of fermentation. The presence of propionic acid is shown when pH goes higher than 5.0 reaching 4% and 57% at pH 5.0 and 5.5 respectively. Acetic acid reaches a higher molar composition of 40% at pH 5.5.

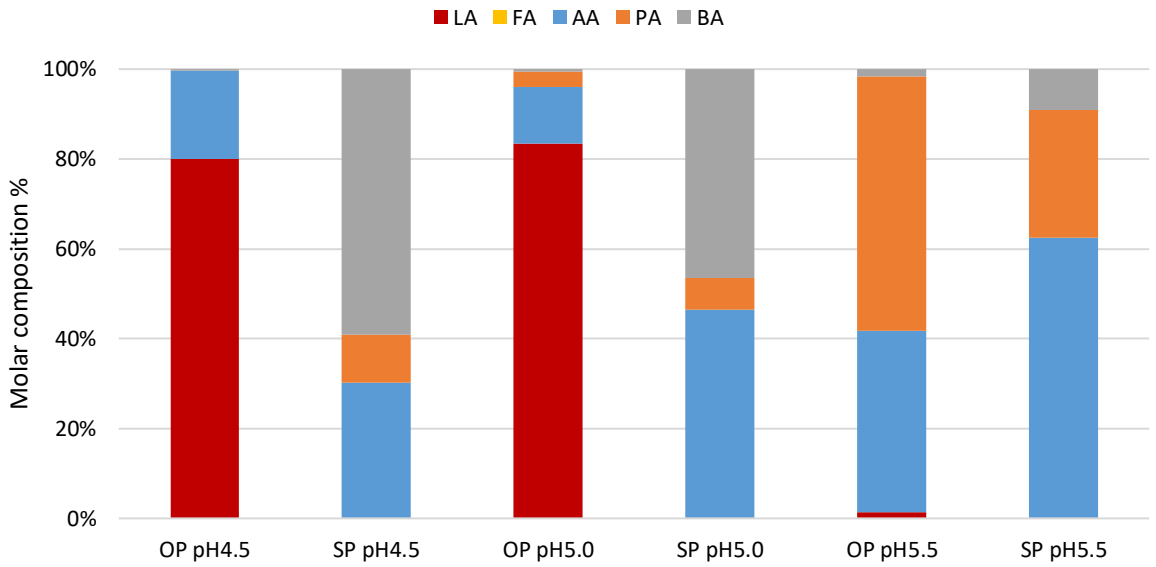

Figure 5: Molar composition of VFA production from ORANGE PEEL (OP) and SPEZIAL (SP) in different pH at day 7.

The profiles during the days (Figure 6 and Figure 7) show that for Spezial the concentration of lactic acid increases from day 0 to day 4 reaching its maximum, after day 4 lactic acid is decreased and completely consumed in day 7, with increasing concentration of acetic, propionic and butyric acid. On the other hand, for Orange Peel, lactic acid concentration reached its maximum already on day 2, and after that lactic acid is slowly consumed but not completely with a high amount still present on day 7. As a conclusion, even though Spezial shows a higher VFA yield, Orange Peel possibly needs more time to reach its optimal VFA yield as lactic acid will be later consumed and converted to VFAs.

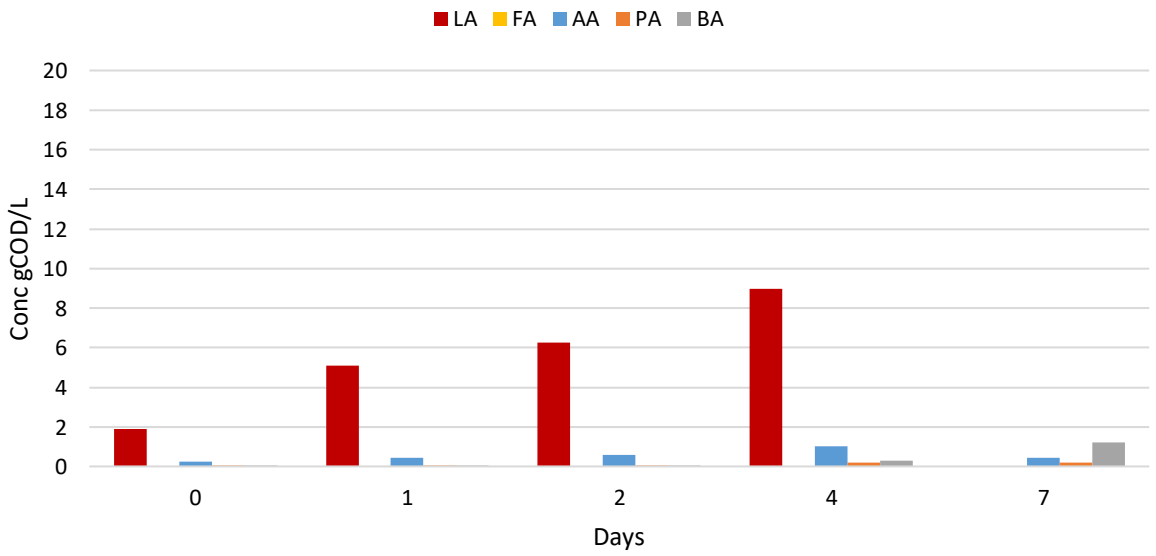

Figure 6: Profile of VFAs concentration during time for Spezial acidification.

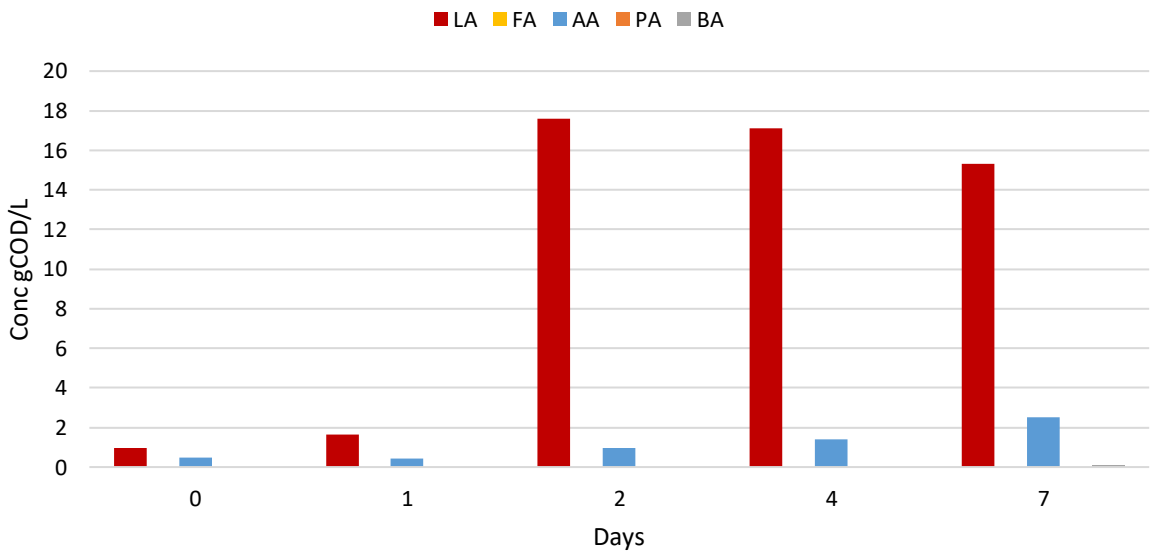

Figure 7: Profile of VFAs concentration during time for OP acidification

Table 7 shows the overview of the results for both residual streams. Comparing the ratio AA/PA to the synthetic feed aiming to produce PHBV with a 1:1 monomer ratio the residual stream Spezial is most promising after 7 days of acidification at pH 4.5 and Orange Peel at pH 5.0.

Table 7: Comparison between COD-Fractions of Spezial and OP residual streams at different pH

		Spezial			Orange Peel		
Parameters	**Unit**	**pH 4.5**	**pH 5.0**	**pH 5.5**	**pH 4.5**	**pH 5.0**	**pH 5.5**
$COD_{tot,in}$	g/L	15.93	12.20	12.21	72.73	71.24	40.52
$COD_{VFA,out}$	g/L	1.79	2.12	4.71	17.96	13.77	9.31
VFA-yield ($COD_{VFA,out}$/ $COD_{tot,in}$)	-	0.11	0.17	0.39	0.25	0.19	0.23
$COD_{sol,out}/COD_{tot,in}$	-	0.51	0.69	0.52	*	*	*
$COD_{VFA,out}/COD_{tot,out}$	-	0.20	0.20	0.32	0.96	0.26	0.37
$COD_{VFA,out}/COD_{sol,out}$	-	0.22	0.25	0.74	*	*	*
$COD_{sol,out}/COD_{tot,out}$	-	0.89	0.80	0.43	*	*	*
Ratio AA/PA		2.8	6.5	2.2	N/A	3.6	0.7

*Data on analysis review

N/A No propionic acid was produced in this batch

4.2 Laboratory scale experiments at RPTU University of Kaiserslautern-Landau

4.2.1 Acidification of residual streams

During the lab scale experiments a total of four different residual streams from three different industries (brewery, milk factory, pizza factory) were acidified and tested for their suitability for PHA production. The main evaluation parameters for the acidification are the VFA-composition, which influences the PHA-properties, the VFA-yield and the achievable VFA-concentration as they determine the PHA production potential. An additional factor is the nutrient composition. The VFA-composition of the different acidifications after seven days expressed as the COD_{VFA}-concentration is displayed in Figure 8. When comparing the different batches, it is obvious that both the overall amount of VFAs as well as the composition of the substrates differ significantly. The brewery wastewater achieved the highest VFA-concentrations with 2.334 g COD_{VFA}/L compared to 0.727 g COD_{VFA}/L respectively 0,373 g COD_{VFA}/L from the residual stream of the milk factory and 0.546 g COD_{VFA}/L from the pizza factory's wastewater. Regarding the different batches of the residual stream of the milk factory it is striking, that the concentration of the first batch is nearly twice as high as of the second batch. The main reason for this significant difference can be found in a process failure during the acidification which probably led to a loss of active biomass, as the temperature in one of the acidification reactors exceeded 42°C for several hours. During the acidification of the pizza factory's wastewater, it is likely that a loss of VFA occurred as the pH was not successfully kept below 6, probably resulting in the formation of methane and carbon dioxide.

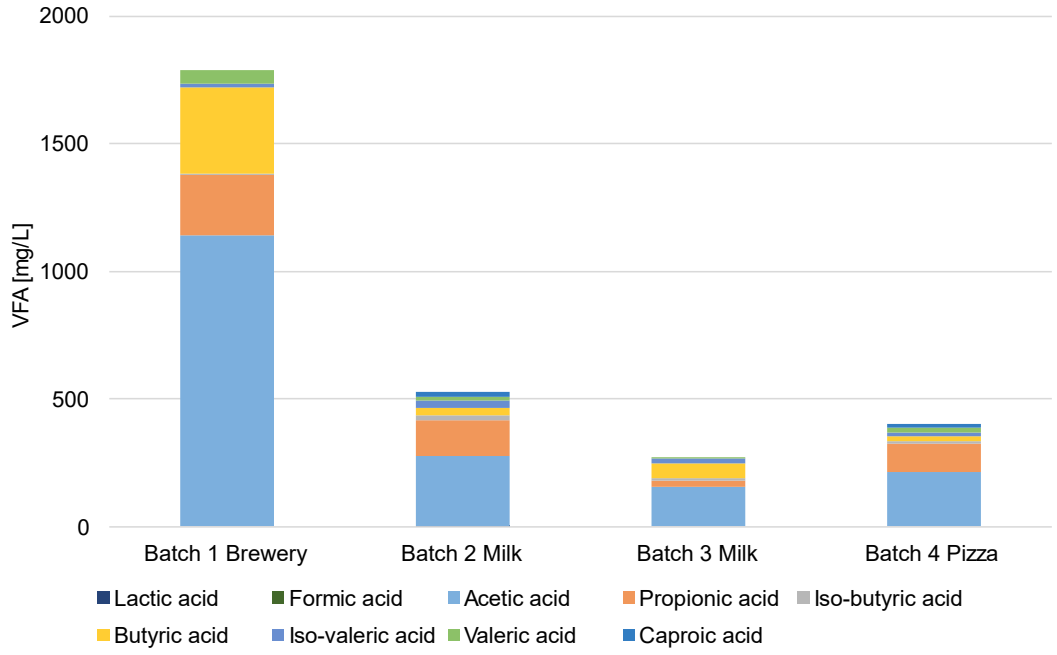

Figure 8: VFA concentrations after acidification of three different industrial streams for seven days

Figure 9 shows the VFA-composition and the percentage of each volatile fatty acid of the different acidification batches. Due to the fact that only the brewery wastewater achieved a promising COD_{VFA} concentration after the acidification, the following paragraph will only discuss the composition of this substrate. Acetic acid is the main component accounting for 71% of the substrate's COD_{VFA}. Propionic acid accounts for 12% of the COD_{VFA}, butyric acid makes up 14% and valeric acid accounts for 2%.

VFAs with an even number of carbon atoms (acetic, butyric, isobutyric, caproic acid) tend to be processed to PHB and VFAs with an odd number (propionic and valeric acid) to PHV (Albuquerque et al. 2007, Lemos, Serafim & Reis 2006). In the substrate derived from the brewery wastewater, the given C-molar ratio of 81 % to 19 % of even to odd carbon atoms, the majority of the produced PHA is expected to be PHB with only a minor amount of PHV produced. These results can be used for a first indication which applications of the produced biopolymers are possible. Moreover, regarding the individual substrates it is also important to ensure that the PHA composition stays stable in order to meet market needs for stable product properties.

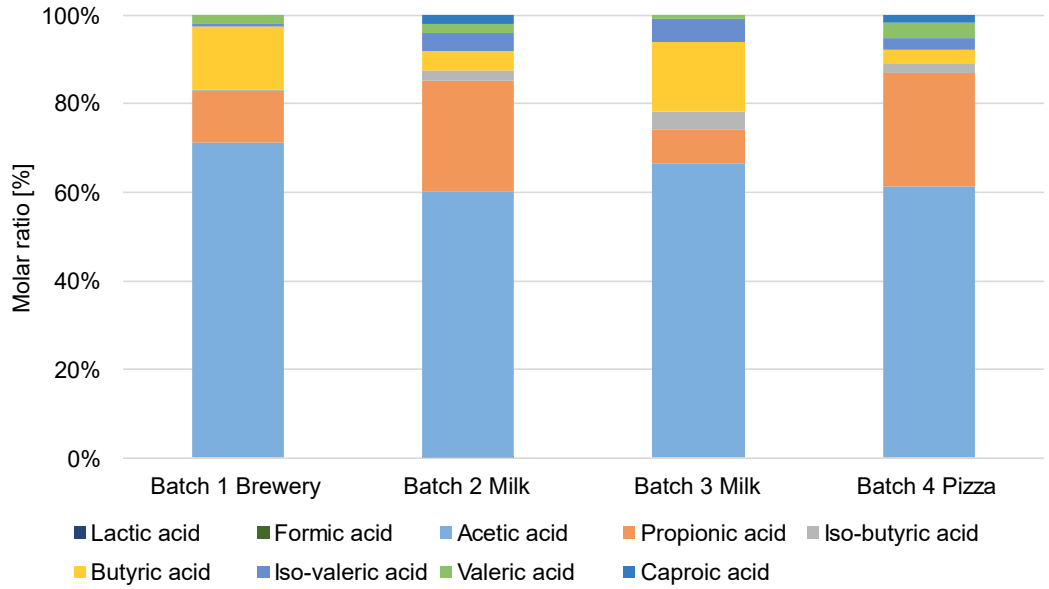

Figure 9: Molar ratio of the different VFAs of three different industrial streams after an acidification for seven days

Acidification of brewery wastewater leads to a VFA-concentration of 2.33 g COD_{VFA}/L (Table 8). Bengtsson & Ab (2017) stated that the VFA-concentration should be at least higher than 2 g COD_{VFA}/L or rather in the range of 5-30 g COD_{VFA}/L for the PHA-accumulation. A study by Chen et al. (2017) also showed that a substrate concentration of 2.52 g COD/L was optimal. The mentioned OLRs from the literature would lead to a necessary VFA-concentration range of at least 2.0 g COD_{VFA}/L, which was only achieved by the acidified brewery wastewater, which is discussed in Chapter 3.2 PHA production.

Table 8: Comparison of the different batches of acidified residual streams after centrifugation and mixing of the two 150 L batches

Parameters	Unit	Brewery	Milk_Batch 1	Milk_Batch 2	Pizza
$COD_{tot, in}$	g/L	4.67	3.15	2.92	4.53
$COD_{VFA, out}$	g/L	2.33	0.72711	0.37	0.55
VFA-yield ($COD_{VFA, out}$/ $COD_{tot, in}$)	-	0.50	0.23	0.13	0.12
$COD_{sol, out}/COD_{tot, in}$	-	0.69	0.47	0.44	0.37
$COD_{VFA, out}/COD_{tot, out}$	-	0.67	0.27	0.14	0.21
$COD_{VFA, out}/COD_{sol, out}$	-	0.73	0.49	0.47	0.32
$COD_{sol, out}/COD_{tot, out}$	-	0.93	0.55	0.47	0.64
NH_4-N	mg/L	<0.015	265.2	252.50	161.45
PO_4-P	mg/L	62.1	54.9	54.9	37.7
COD_{VFA} : NH_4-N : PO_4-P	-	1:0.00:0.03	1:0.36:0.08	1:0.68:0.15	1:0.30:0.07
COD_{sol} : NH_4-N : PO_4-P	-	1:0.00:0.02	1:0.18:0.04	1:0.20:0.03	1:0.10:0.02

The VFA-yield of the different batches of acidified residual streams is shown in Table 8 and amounts to 0.5 for the brewery wastewater. It is obvious that a high VFA-yield results in higher PHA production capacities, but besides a high VFA-content, the feed for the following process steps should also meet requirements for the nutrient ratio (C:N:P), aiming for a ratio of 100:5:1. The C:N:P-ratio shows a shortage of nitrogen for the brewery derived substrate, independently of the calculation method, as shown in table 8. On the one hand, a continuously running biomass enrichment needs enough nutrients for biomass growth. But on the other hand, a high nutrient supply during the PHA accumulation process can lead to a decreased PHA storage as cell growth is favoured by the bacteria (Nguyenhuynh et al. 2021). For this reason, nutrients are often added to the substrate during the enrichment step (Chen et al., 2017; Matos et al., 2021a; Matos et al., 2021b; Cruz et al. 2021, Pittmann & Steinmetz, 2014). For an up-scaling this would lead to additional process costs if external nutrients need to be dosed. Therefore, it would be more appropriate to use the produced VFA-feed without further adaptations. However, a nutrient limitation in the substrate may lead to insufficient biomass growth or even biomass loss during the enrichment process which would have a negative impact on the overall PHA production. In this case, higher production volumes would be needed which would lead to higher investment costs. A sufficient nutrient supply in the substrate is therefore favourable.

4.2.2 PHA production

During the biomass enrichment and accumulation experiments, the produced VFA from the three industrial streams were investigated for their ability to select a PHA-producing microbial community and consequently for their suitability for PHA production. Accumulation essays were done on different days of biomass enrichment. The results are shown in Table 9.

Table 9: PHA content and composition during enrichment and accumulation experiments with VFA produced from three different industrial streams

	Brewery		Milk 1		Milk 2		Pizza	
Day	**PHA [%VSS]**	**HB/HV [%]**	**PHA [%VSS]**	**HB/HV [%]**	**PHA [%VSS]**	**HB/HV [%]**	**PHA [%VSS]**	**HB/HV [%]**
0	3.2 ± 0.5	100/0	1.9 ± 0.1	0/100	4.3 ± 0.6	100/0	2.0 ± 0.2 (n=2)	0/100
7	-	-	2.9 ± 2.7	22/78	5.6 ± 0.8	100/0	0	0/0
11	-	-	-	-	3.2 ± 0.6	100/0	-	-
13	20 ± 1	73/27	-	-			-	-

The very low VFA content achieved during the first acidification batch of the condensed Milk stream 1 and the acidification of the residual stream from the Pizza factory (chapter 4.2.1) resulted in a very low content of PHA or no PHA production, respectively (Table 9). In addition, both experiments had to be stopped after 7 days of biomass enrichment due to some technical problems. A second acidification batch with the condensed Milk stream 2 in combination with a second biomass enrichment experiment was conducted because this stream showed better VFA results in a previous screening experiment. The use of this substrate for biomass enrichment and accumulation resulted in a maximum PHA content of 5.6 ± 0.8 % on day 7. However, the achieved PHA content declined to day 11. The highest PHA content with a value of 20 ± 1 % was reached after 13 days of biomass enrichment with the acidified brewery residual stream. On day 7 of this experiment no accumulation experiment could be performed due to a power failure. The composition of the maximum PHA produced with the brewery stream showed a higher amount of PHB with 73 % in comparison to PHV with a value of 27 % which exhibits the same tendency as the substrate composition (chapter 4.2.1). Table 9 furthermore summarises the maximum PHA contents achieved during all experiments with different industrial streams. The best performing substrate was achieved using the residual stream of the Brewery and suggest that a PHA production using this stream is possible. Regarding the Milk batch 2, the low produced PHA content of 5.6 ± 0.8 % may be a result of the low VFA concentration that could be achieved during the acidification experiments (chapter 4.2.1). With the Brewery substrate the highest OLR of 1.2 g COD_{VFA}/(L·d) could be applied whereas with the other substrates only an OLR of 0.2-0.4 g COD_{VFA}/(L·d) could be reached during the enrichment. Furthermore, this substrate was the only one of the tested which exceeded a COD_{VFA}/COD_{tot}-ratio of 65 %, as suggested in the report on optimised PHA production process layout (Uhrig et al. 2022), with a value of 67 % (Table 9). The other substrates showed lower COD_{VFA}/COD_{tot}-ratios with 27 % for the first Milk and 14 % for the second Milk batch as well as 21 % for the acidified Pizza stream. This may have led to a better selection of PHA producing organisms in the biomass fed with acidified Brewery wastewater which in turn would result in a higher PHA production capacity. However, in other studies using Brewery wastewater higher PHA contents of 39 % (g/gVSS) (Ben et al. 2016) and 45 % (g/gCDW) (Tamang et al. 2019) could be reached in comparison to this study. Though, in these two studies the biomass enrichment was conducted using a synthetic feed whereas only the accumulation assays were performed under the addition of acidified Brewery wastewater as a substrate. This may lead to the assumption that a more complex substrate or a substrate with a higher non-VFA share in the enrichment phase results in a reduced selection of organisms with the ability to produce PHA, which also could be shown in the previous project phase (Uhrig et al. 2022). This circumstance may be overcome by a better solid-liquid-separation after the acidification or a longer enrichment phase to promote the acclimatisation of the microorganism to a more complex substrate.

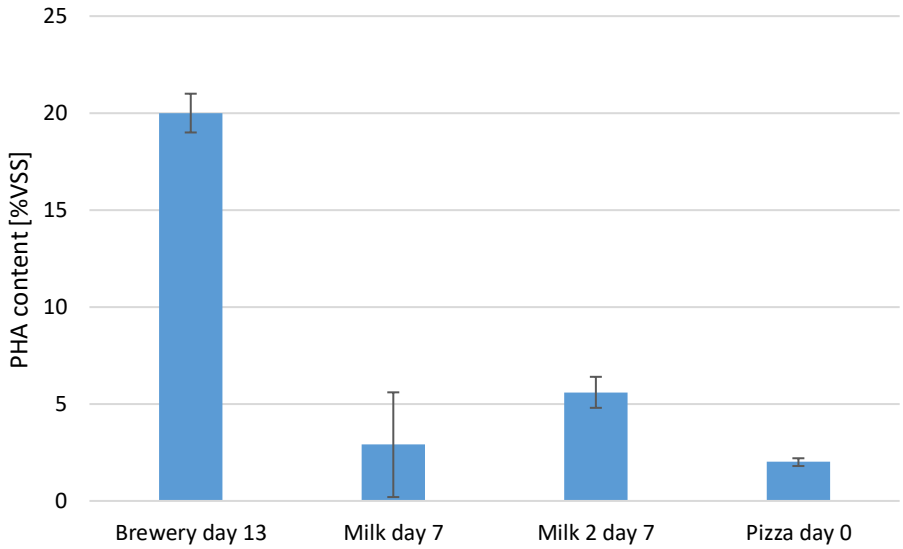

Figure 10: Maximum PHA contents during enrichment and accumulation experiments with VFA produced from three different industrial streams

5 Summary and conclusion

Aiming to investigate the suitability of various residual streams from the food processing industry as a carbon source and/or enrichment feed for PHA production, in total seven different residual streams from the Netherlands and Germany were examined. The residual streams were acidified anaerobically and their yield and composition of the produced VFAs were analysed. At Avans between four residual streams, Hamimo Special, a co-product released during the wet processing of wheat flour, provided by LOOOP had the highest VFA production of 39% total VFA yield after seven days of acidification at pH 5.5. The VFAs produced have a high yield of acetic and propionic acid that can be utilised for the production of PHA. At RPTU the acidified residual stream from a brewery was the best performing of the three substrates with a concentration of 2.334 g COD_{VFA}/L after six days of anaerobic fermentation. In this substrate acetic acid is the main component accounting for 71% of the substrate's COD_{VFA}, while propionic acid accounts for 12% of the COD_{VFA} and butyric acid makes up 14%. Using this substrate it was possible to achieve a PHA content of 20±1 % PHA. The composition of the maximum PHA produced with the brewery stream showed a higher amount of PHB with 73 % in comparison to PHV with a value of 27 % which exhibits the same tendency as the substrate composition. Therefore, the residual stream of the brewery will be used in further pilot scale experiments.

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7 APPENDIX 1 – VFA calibration curve

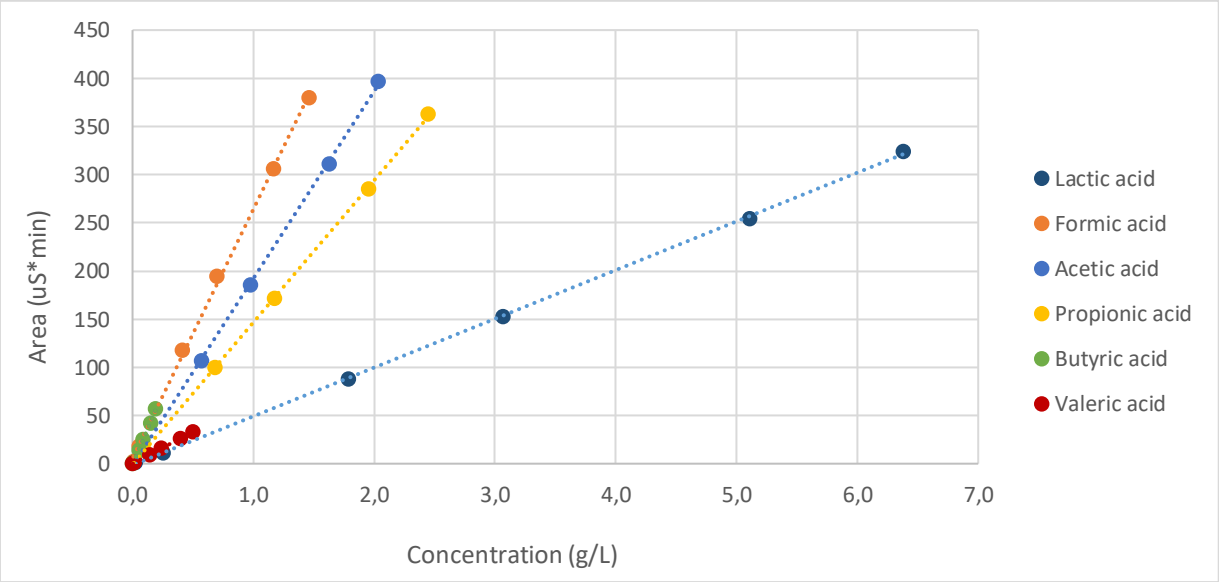

Figure 11 Calibration curves for organic acids at Avans Hogeschool

Table 10. Equations of the calibration curves of FA, AA, PA, and BA

Organic acid	Equation	R^2
Lactic acid	y = 50.6x-1.39	0.9998
Formic acid	y = 258.21x+5.81	0.9987
Acetic acid	y = 194.40x-2.23	0.9998
Propionic acid	y = 147.44x-0.78	0.9999
Butyric acid	y = 296.11x-0.84	0.9961
Valeric acid	y = 66.51x-0.11	0.9994

8 APPENDIX 2 – VFA concentration in g COD/L summary

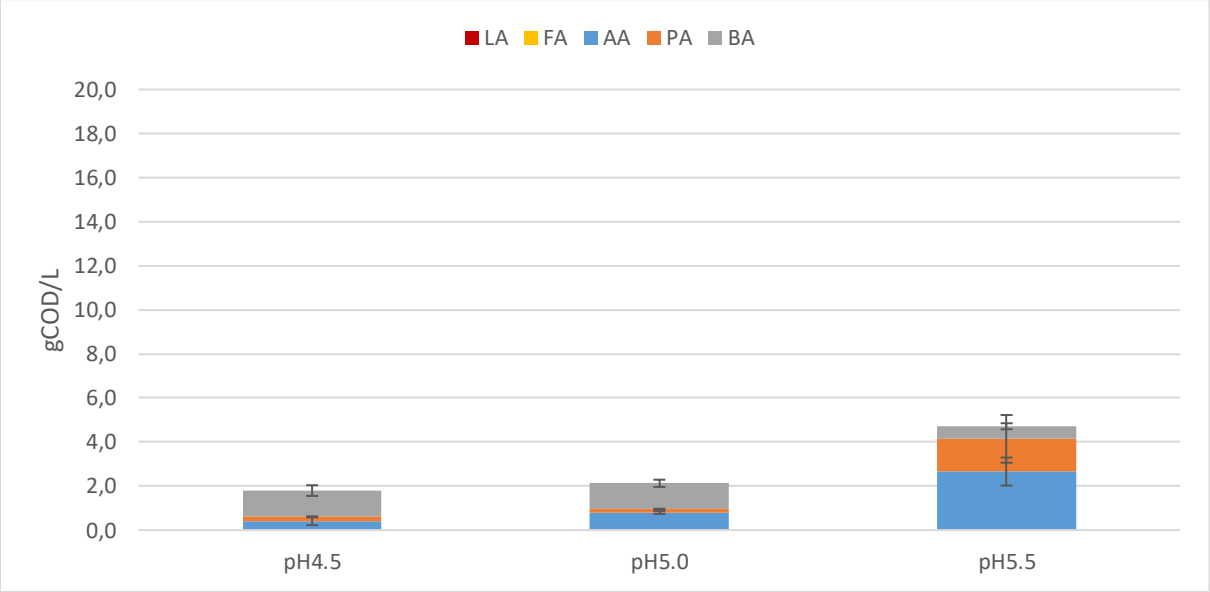

Figure 12: VFA concentration in g COD/L after acidification of SPEZIAL for 7 days

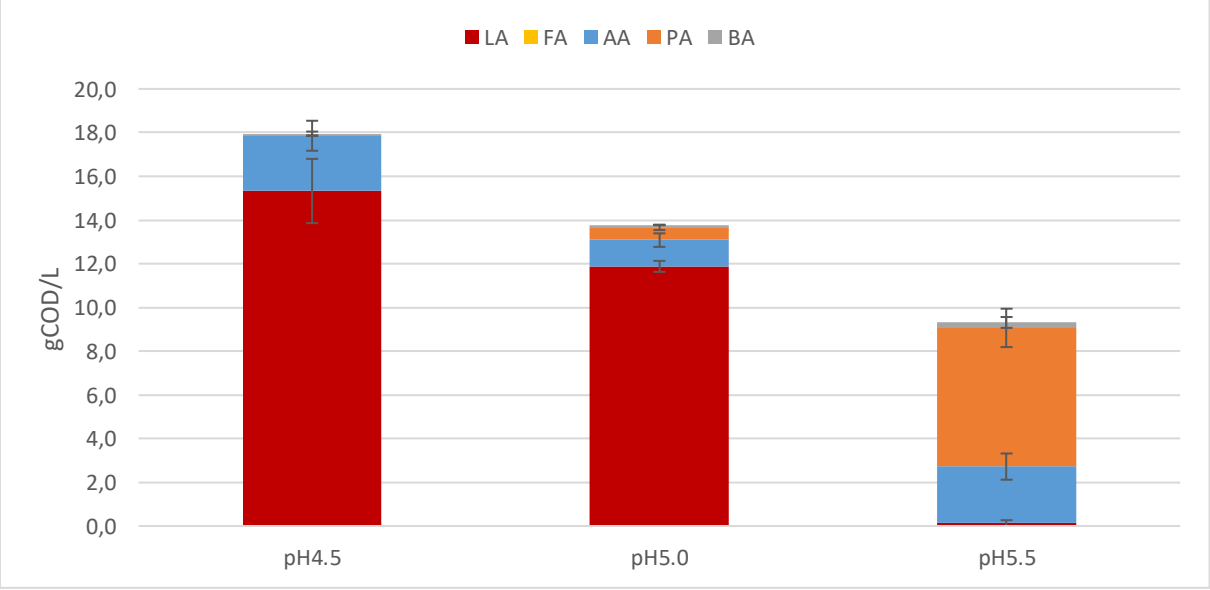

Figure 13: VFA concentration in gCOD/L after acidification of ORANGE PEEL for 7 days

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