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Lactate-based bioproduction of medium chain carboxylic acids via mixed culture fermentation

Self-reference of PhD dissertation

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List of articles included in the dissertation

The following publications form the basis of PhD thesis:

1. Brodowski, F., Łężyk, M., Gutowska, N., Oleskowicz-Popiel, P., 2022. Effect of external acetate on lactate-based carboxylate platform: Shifted lactate overloading limit and hydrogen co-production. Science of The Total Environment 802, 149885. https://doi.org/10.1016/j.scitotenv.2021.149885; **hereinafter as Paper 1**

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   Filip Brodowski, lead author: conceptualised and conducted the research, interpreted the results and wrote the manuscript.

2. Brodowski, F., Łężyk, M., Gutowska, N., Kabasakal, T., Oleskowicz-Popiel, P., 2022. Influence of lactate to acetate ratio on biological production of medium chain carboxylates via open culture fermentation. Science of The Total Environment 851, 158171. https://doi.org/10.1016/j.scitotenv.2022.158171; **hereinafter as Paper 2**

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3. Brodowski, F., Duber, A., Zagrodnik, R., Oleskowicz-Popiel, P., 2020. Co-production of hydrogen and caproate for an effective bioprocessing of waste. Bioresource Technology 318, 123895. https://doi.org/10.1016/j.biortech.2020.123895; **hereinafter as Paper 3**

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List of abbreviations

AW – acid whey
B1 – bioreactor no. 1
B2 – bioreactor no. 2
CE – chain elongation
CSTR – continuous stirred tank reactor
DF – dark fermentation
EA – electron acceptor
ED – electron donor
HRT – hydraulic retention time
L:A – lactate to acetate
LLR – lactate loading rate
MCC – medium chain carboxylates
NMDS – non-metric multidimensional scaling
OTU – operational taxonomic unit
MCF – mixed (open) culture fermentation
$r_{L:A}$ – lactate to acetate ratio
SCC – short chain carboxylates
16s rRNA – 16s ribosomal RNA
UASB – upflow anaerobic sludge blanket

1. Introduction

1.1. The role of biorefineries in the future biobased economy

Population growth and consumerism has increased primary energy consumption, depleted fossil fuel resources and impacted global climate change. Further economic transformation should be conducted without excessive environmental exploitation and degradation. The response to the challenges posed by economic growth is a sustainable development (Culaba et al., 2023). The sustainable development is defined as a development that meets the needs of the present without compromising the ability of future generations to meet their own needs (Brundtland Commission, 1987). Waste generation is an inseparable element of the economic development and it has become a major challenge for all societies. Popular landfilling and incineration can cause several threats, such as contamination of ground and surface waters, as well as pollution of soil and air ecosystems (Watson et al., 2018). Inadequate waste management is not only a problem for the environment but also causes economic losses due to the loss of valuable resources. Therefore, circularity in waste management may be crucial for the sustainable development concept (Halkos and Aslanidis, 2023).

The recovery of carbon from biomass and organic-rich waste can be a key approach in the transformation of waste management. The bioprocessing of biomass and waste into biofuels, bulk chemicals, and energy in microbial-based processes takes place in biorefineries (Van Schoubroeck et al., 2018). So far, the most popular are the first-generation biorefineries which use feedstock based on edible biomass such as wheat or corn starch. The development of this technology is very controversial because it requires large amounts of land and water resources leading to competition with the food and feed crops. These controversies have led to an increased focus on developing the second-generation biorefineries based on non-food biomass and organic waste as feedstock (Alalwan et al., 2019). The implementation of the second-generation biofuels production is still in the early stages of commercialization despite the high environmental benefits mainly because it faces the problem of economic viability. Thus new chemical biorefinery platforms have been developed in recent years (Pfleger and Takors, 2023).

1.2. Carboxylate platform concept

Microbial-based processes can be conducted through pure culture fermentation or mixed culture fermentation (MCF) (Oleskowicz-Popiel, 2018). Pure culture techniques are optimized for specific strains and provide consistent yields of the desired product, as well as repeatability and predictability of the bioprocessing. However, this type of fermentation requires sterile operating conditions and high purity of substrates. Therefore MCF, where a large diversity of microorganisms is used, is more suitable for the utilization of complex and nonsterile biowaste (Grimalt-Alemany et al., 2020). The activity of different groups of microorganisms and a wider range of enzymes provide better utilization of complex substrates. In addition, the diverse structure of microorganisms enables higher resistance to contaminants and inhibitors.

Commercialized and widespread technology based on MCF is anaerobic digestion, in which organic waste is converted into methane-rich biogas that is used to directly generate heat and electricity or it is upgraded and injected to the gas grid (Karki et al., 2021). The new MCF

technologies such as the carboxylate platform have originated from the anaerobic digestion (Angenent et al., 2016). SCCs which are intermediates accumulated in the acidogenesis and acetogenesis during anaerobic digestion can be also converted into medium chain carboxylates (MCCs) through the chain elongation (CE) instead of forthgoing through methanogenesis to methane. Specific methanogenesis inhibitors (e.g. 2-bromoethylsulfonate) may be added to promote CE, however, a more popular method is to control the bioprocess by adjusting operating parameters such as pH, temperature and hydraulic retention time (HRT) to cultivate methanogen-free microbiome (De Groof et al., 2019).

Carboxylate platform has a potential to play a significant role in the bio-based products market such as drop-in biofuels, biochemicals, and bioplastics. Among medium chain carboxylic acids the most popular is caproic acid containing six carbon atoms in the molecule (Cavalcante et al., 2017). It can be used directly as a food additive, antibacterial agent, and plant growth promoter, as well as the intermediate component in the production of lubricants, gums, dyes, paints additives, or pharmaceuticals (Ren et al., 2022; Wu et al., 2019). It also can be further transformed, for instance, into 1-hexene which can be a component of liquid fuels (Harvey and Meylemans, 2014). The market value of caproic acid is estimated to reach more than $ 283.6M by the year 2027 (Global Industry Analysts, 2022), making the carboxylate platform an increasingly interesting concept. The biological production of medium chain carboxylic acids is at the early phase of technological readiness level, however, first projects and start-ups are already appearing on the market (De Groof et al., 2019).

1.3. Lactate-based carboxylate chain elongation

The basic metabolic requirement for the CE process is the presence of electron donors (EDs) providing required energy, reducing equivalents (NADH) and intermediate acetyl-CoA to the reverse β-oxidation cycle (or less often malonyl-CoA for fatty acid biosynthesis (Han et al., 2018)), wherein the carbon chain of the carboxylate (electron acceptor (EA)) is always elongated by two carbon atoms in a series of enzymatic reactions, e.g. acetate is elongated to butyrate and butyrate to caproate (Spirito et al., 2014). Various chemical compounds such as ethanol, lactate, and sugars can play the role of EDs in the CE (Dong et al., 2023). So far, ethanol is the best-recognized ED, however, more and more attention is being paid to lactate-based bioconversion. Lactate is commonly found in many waste streams as a byproduct of various industrial processes, e.g. food and beverage production. Acid whey (AW) from the dairy industry (Rocha-Mendoza et al., 2021) or maize silage as an agriculture by-product (Lambrecht et al., 2019) are very popular lactate-based feedstocks used in fermentation processes. Moreover, lactate can be also produced *in-situ* from sugars via lactic acid fermentation. Food waste or lignocellulosic biomass (e.g. corn stover, sugar cane bagasse) was used before to convert organic waste into lactate (Li et al., 2021; Tang et al., 2016).

Zhu et al. (2015) conducted lactate to caproate bioconversion for the first time using a unique microbiome selected from Chinese strong-flavor liquor production. In the research, lactate was used as the sole carbon source for CE providing necessary acetyl-CoA and acetate (EA). It was possible, because lactate was firstly oxidized to pyruvate, and then further oxidized to acetyl-CoA. Part of the acetyl-CoA was converted into acetate by substrate-level phosphorylation (Wu et al., 2019) and the rest was used in the reverse β-oxidation cycle. Further research showed that continuous conversion of lactate to caproate in MCF is possible (Kucek

et al., 2016a), but there were some limitations associated with the activation of competing microbial pathway (acrylate pathway of propionate production) which inhibited CE. The most important operational conditions for lactate-based CE were previously examined. In the study with isolated *Ruminococcaceae* bacterium CPB6, which was identified as an effective lactate-based chain elongator, it was indicated that the strain prefers acidic initial pH conditions (pH 5.0 - 6.5; however, at pH 5.5 and 6.0 shorter lag phases were observed) and the temperature ranging from 30°C to 40°C (Zhu et al., 2017). Other research (Candry et al., 2020) also confirmed that mesophilic temperatures and acidic pH of 5.0 - 6.0 stimulated lactate-based chain elongation. It was shown that pH above 6.0 promoted propionate producers growth which led to CE inhibition. Thermophilic conditions were also indicated to not be suitable for lactate-based chain elongators (Sakarika et al., 2023). However, the operational parameters of the MCF are not the only factors affecting lactate-based CE. For instance, it was suggested that excess lactate could activate the acrylate pathway leading to CE inhibition (Kucek et al., 2016a; Prabhu et al., 2012). Thus, the determination of the influence of the substrate composition on lactate-based CE is crucial for a better understanding of the bioprocess and for defining other important factors affecting the functioning of lactate-based chain elongators in mixed microbial culture.

2. Motivation and aim of the research

The lactate-based CE research carried out so far has focused mainly on optimizing operational parameters of MCF such as pH, temperature, or HRT. However, the effects of substrate composition, specifically the composition of electron donors (EDs) and electron acceptors (EAs), have not been fully explored yet. The main objective of the study is to identify the influence of lactate-based substrate composition on CE. Three long-term continuous processes (two based on synthetic medium and one based on lactate-based model waste stream, i.e. AW), as well as two batch experiments, were conducted.

The particular aims and objectives of the research were:

- to characterize a process in which lactate is used as the sole carbon source in CE,
- to describe the lactate overloading phenomenon in CE systems,
- to identify the role of acetate in lactate-based CE,
- to investigate the influence of lactate to acetate (L:A) ratio on CE,
- to investigate the effect of complex lactate-based substrate composition on CE (using AW as a model lactate-based feedstock),
- to identify key microorganisms responsible for lactate-based CE.

Two main following hypotheses were proposed:

1) The composition of lactate-based substrates, especially the lactate (ED) and acetate (EA) concentration, will significantly influence the CE performance.

2) Utilizing a lactate-based substrate under CE-promoting conditions during MCF will lead to a microbiome enrichment in the lactate-based chain elongating bacteria.

3. Material and methods

3.1. Substrates characterization

3.1.1. Synthetic medium

A modified synthetic anaerobic growth medium was prepared based on (Grimalt-Alemany et al., 2018). The synthetic medium was used in batch trials (Paper 2, Paper 3) and continuous processes conducted in CSTRs (Paper 1, Paper 2). A synthetic anaerobic medium consisted of the following stock solutions: salt solution, vitamin solution, trace metal solution, chelating agent solution, reducing agent solution, buffer solution, yeast extract solution and carbon source solutions. The detailed method of preparing the solutions and the final medium has been described in the PhD dissertation. The concentrations of lactate and acetate in the medium were variable and presented in Tab. 1.

3.1.2. Waste feedstock

AW as a model complex lactate-based waste feedstock was used in the continuous experiment conducted in the UASB reactor in Paper 3. AW was obtained directly from the quark production line (Diary Plant OSM Kowalew – Dobrzyca, Poland). It was stored at 4 °C and used in the experiment without any pretreatment or preparation. It consisted mainly of lactose (about 30.7 g/L) and lactate (about 10.6 g/L).

3.2. Bioreactors set-up

Two 1 L Lambda Minifor fermenters (LAMBDA CZ, s.r.o., Brno, Czech Republic) were used for continuous processes in Paper 1 and Paper 2. Fermenters were equipped with a control unit connected to a pH-temperature electrode, an IR radiation heater, four peristaltic pumps (base pump, acid pump, feed pump, effluent pump), and a weighing module. A more detailed description and the scheme of the Lambda Minifor fermenters configuration was presented in Paper 1, Fig. 1 and in the PhD dissertation.

The continuous process in Paper 3 was carried out in a 1L UASB reactor made from plexiglass. The recirculation installation equipped with a peristaltic pump ensured sludge suspension. The base was pumped automatically based on pH measurements. The feed pump was set to maintain a constant HRT. The outflow was through a gravitational outlet. The temperature was maintained using a heating water jacket.

500 mL glass bottles capped with butyl rubber stoppers and aluminum caps were used in batch trials in Paper 2 and Paper 3. A laboratory incubator was used to maintain a constant temperature.

3.3. Mixed culture fermentation trials

All batch trials (Paper 2 and Paper 3) were performed in triplicates. The working volume (initial medium volume) was 150 mL. The sludge for inoculation was prepared as described in PhD dissertation. 16s rRNA gene microbiome analysis was performed for an inoculum sample in Paper 2. The initial pH was 5.50 ± 0.05 and was not adjusted during trials. Nitrogen gas was used to flash bottles to ensure anaerobic conditions. Bottles were incubated at 30 °C for 7 days (Paper 2) or 10 days (Paper 3). Microbiome analysis of the selected samples was performed for

batch trials in Paper 2. Details concerning initial concentrations of lactate, acetate, and lactose in each trial were shown in Tab. 1.

In the continuous processes carried out in CSTRs in Paper 1 and Paper 2 temperature was maintained at 30°C, pH at 5.5, and HRT at 5 days. The working volume was set at 0.8L in the CSTRs in Paper 1 and 1L in the CSTRs in Paper 2. The sludge for inoculation was prepared as described in PhD dissertation. Liquid samples were taken daily. Microbiome analysis of the selected samples was performed for both processes. Gas production was quantified using a volumetric gas flow meter (Ritter, Germany) in process in Paper 1. Gas production in a continuous process in Paper 2 was not measured. In the experiment in Paper 1, lactate conversion to caproate was carried out with and without the external acetate addition in two CSTRs simultaneously. The lactate concentration in the feedstock had been increased until lactate overloading occurred in both bioreactors. Then external acetate was supplemented to the lactate-overloaded CSTR without external acetate addition. In the experiment in Paper 2, different L:A ratios were applied in the continuous process. More details concerning the division into phases and their duration, as well as selected variables, are included in Tab. 1.

The continuous process in Paper 3 was carried out in 1L UASB reactor. The feed pump was set to maintain an HRT of 5 days or 2.5 days depending on the stage of the process. The temperature was maintained at 30°C and pH at 5.5 with automatic correction. The gas production was quantified using a volumetric gas flow meter (Ritter, Germany). The reactor was inoculated with anaerobic sludge from Central Wastewater Treatment Plant (Poznan area, Poland) and prepared as described in PhD dissertation. The process was divided into two stages depending on an HRT: stage I (0-44 days) when HRT was maintained at 5 days and stage II (45-127 days) when HRT was maintained at 2.5 days (Tab. 1).

Table 1. Process strategy: selected variables of processes.

PAPER 1 — Continuous proces (CSTR)

Bioreactor 1 (B1):					
Phase	start-up	phase I	phase II	phase III	phase IV
Duration [days]	0-48	49-72	73-88	89-118	119-140
Lactate concentration (feedstock) [mM C]	400	600	900	1350	1350
Acetate concentration (feedstock) [mM C]	0	0	0	0	200
Lactate loading rate [mmol C/L/d]	80	120	180	270	270
Acetate loading rate [mmol C/L/d]	0	0	0	0	40

Bioreactor 2 (B2):				
Phase	start-up	phase I	phase II	phase III
Duration [days]	0-48	49-72	73-88	89-140
Lactate concentration (feedstock) [mM C]	400	600	900	1350
Acetate concentration (feedstock) [mM C]	200	200	200	200
Lactate loading rate [mmol C/L/d]	80	120	180	270
Acetate loading rate [mmol C/L/d]	40	40	40	40

PAPER 2 — Batch trial

Batch no.	R1	R2	R3	R4
$r_{L:A}$ (mM C/mM C)	0:1	1:1	4:1	1:0
Initial lactate concentration (mM C)	0	150	240	300
Initial acetate concentration (mM C)	300	150	60	0

PAPER 2 — Continuous proces (CSTR)

Bioreactor 1 (B1):	
Phase	phase I
Duration (days)	0-65
$r_{L:A}$ (mM C/mM C)	0.6
Lactate concentration in susbtrate (mM C)	300
Acetate concentration in susbtrate (mM C)	500

Bioreactor 2 (B2):				
Phase	phase I	phase II	phase III	phase IV
Duration [days]	0-27	28-39	40-52	53-65
$r_{L:A}$ [mM C/mM C]	-	2.4	1.2	0.6
Lactate concentration in susbtrate (mM C)	300	300	300	300
Acetate concentration in susbtrate (mM C)	0	125	250	500

PAPER 3 — Batch trial

Batch no.	1	2	3	4	5	6	7	8	9
Initial lactate concentration (mM C)	0	216	238.2	108	119.1	216	238.2	108	119.1
Initial acetate concentration (mM C)	0	54	31.8	27	15.9	54	31.8	27	15.9
Initial lactose concentration (mM C)	135	0	0	0	0	135	135	135	135

PAPER 3 — Continuous proces (UASB)

UASB reactor		
Stage	Stage I	Stage II
Duration (days)	0-44	45-127
HRT (days)	5	2.5

3.4. Analytical methods

Analysis of gas composition (methane, carbon dioxide, and hydrogen) was performed with gas chromatograph Shimadzu GC-2014 equipped with the Porapak N packed column and the TCD detector, under isothermal conditions as described in PhD dissertation. Organic acids and alcohols (i. e. acetate, propionate, i-butyrate, butyrate, i-valerate, valerate, caproate, heptylate, caprylate, methanol, ethanol, propanol, i-propanol, butanol, and i-butanol) concentrations were monitored by a gas chromatograph (GC) with flame ionization (FID) detector (Shimadzu GC-2014 equipped with Zebron ZB-FFAP column) using helium as the

carrier gas. The concentrations of lactate and lactose were monitored with high-performance liquid chromatography (Shimadzu LC-20, Rezex ROA-Organic Acid column, RI detector). All the details concerning the measurements' conditions were described in PhD dissertation.

All liquid samples were centrifuged for 15 minutes at 13 000 rpm before analysis. Then, to prepare samples for gas chromatography, supernatants were acidified with H_3PO_4 and filtered with 0.45 μm syringe filters. In preparing samples for high-performance liquid chromatography, supernatants were filtered with 0.25 μm syringe filters.

3.5. Microbiome analysis

Microbiome (16s rRNA gene microbiome) analysis was performed in Paper 1 and Paper 2. The sample of inoculum and other selected samples were analyzed. Collected biomass samples were centrifuged at 4 °C and stored frozen at −20 °C until further processing. All the details concerning the DNA isolation and the bioinformatic analyses were described in PhD dissertation. Co-occurrence networks were prepared in Paper 1 based on microbial composition data and abiotic parameters (i.e. concentrations of metabolites in a bioreactor, $CO_2/H_2/CH_4$ content in a gas mixture, gas production, as well as lactate and acetate concentrations in the feedstock) using Cytoscape software (v 3.7.1) (Shannon et al., 2003) as described in PhD dissertation.

3.6. Calculations

All the details concerning the calculations of carboxylates production rates (mmol C/L/d), specificities of carboxylate (%), lactate loading rate (LLR) and acetate loading rate (mmol C/L/d), acetate consumptions (mmol C/L/d) and carboxylate yields (mmol C of carboxylate per mole C of lactate; mmol C of carboxylate per mole C initial) were described in PhD dissertation.

The ideal gas law ($pV = nRT$, where: p – pressure, V – volume, n – number of moles, R – ideal gas constant, T – temperature) was used for the calculation of gas production as described in PhD dissertation.

4. Selected Results and Discussion

4.1. Lactate as a sole carbon source in CE

Lactate was used successfully as a sole carbon source for CE in a long-term process in B1, days 0-118, Paper 1. The acrylate and CE pathways competed with each other at the beginning of the process (Fig. 2, Paper 1). Both acetate and propionate were produced, and then elongated into longer chain carboxylates. As the process progressed caproate production began to dominate, and the production of odd-numbered carboxylates was almost completely inhibited. Acetate and propionate compete for the same enzyme system in the CE process, however, acetate was previously recognized as a more favorable EA (Roghair et al., 2018). Interestingly, subsequent lactate overloading did not activate the acrylate pathway (propionate production) as was suggested before (Kucek et al., 2016a). The latest research showed that pH is a very important factor in the competition between chain elongators and propionate producers (Candry et al., 2020). It was demonstrated that acidic pH (5.0 and 5.5) favored chain elongators, but pH above 6.0 promoted propionate producers. Continuous pH maintenance at 5.5 in B1 in Paper 1 ensured a small relative abundance of propionate producers (0.11% – 1.12% of OTUs assigned to *Propionibacterium*) and a high relative abundance of OTUs assigned to *Ruminococcaceae* bacterium CPB6, which was previously identified as a highly-efficient lactate-based caproate producer (Zhu et al., 2017), and *Acinetobacter* which was previously recognized as bacteria involved in CE (He et al., 2018; Kucek et al., 2016b, 2016a; Qian et al., 2020).

Although the production of propionate was not demonstrated as a result of lactate overloading, periodic fluctuations in caproate production were observed (B1, phases I-III, Fig. 2, Paper 1). These periodic fluctuations could be attributed to the low CE performance due to the low availability of acetate (EA). The recovery of caproate production was always observed after a slight accumulation of acetate (Fig. 2, Paper 1). Therefore, the limiting factor for the caproate production was the availability of acetate affecting lactate consumption.

A unique microbiome enriched in the lactate-based chain elongating bacteria was developed during the process, however, the relative abundance of OTUs assigned to *Ruminococcaceae* bacterium CPB6 was varying depending on the occurrence of lactate overloading, e.g. from 8.3% (day 118) during lactate accumulation period up to 59.1% (day 88) during caproate-producing and lactate-consuming period (Electronic Supplementary Material, Paper 1). Although the relative abundance of microbial composition varied due to the lactate overloading, samples were closely located on the non-metric multidimensional scaling (NMDS) ordination plot which indicated low dissimilarity of the community structures (Fig. 6, Paper 1).

4.2. The role of acetate in lactate-based CE

The external EA is not essential for lactate-based CE, however, as it was demonstrated, the low availability of acetate can be a limiting factor for CE. The long-term transformation of lactate to caproate in MCF with external acetate supplementation was carried out in B2 in Paper 2. The role of acetate in the lactate-based CE was described by comparing lactate conversion to caproate with and without acetate supplementation (B1 days 0-118 and B2 days 0-140, respectively, in Paper 1).

Caproate production dominated in both processes, but acetate supplementation promoted caproate production from the very beginning (B2, Fig. 2, Paper 1). Unlike the process based on lactate as a sole carbon source, no propionate production was observed in the acetated B2. OTUs assigned to *Propionibacterium* were only detected in two samples in B2 on days 118 and 140, but their relative abundance was only 0.04% and 0.11%, respectively. The lack of competition between EAs (acetate and propionate) in B2 caused that only even-numbered carboxylates (butyrate and caproate) were produced.

Supplementation of external acetate shifted the lactate overloading limit (B2, Fig. 2, Paper 1), i.e. residual lactate accumulation was observed at higher LLRs in B2 (270 mmol C/L/d) compared to B1 (120 mmol C/L/d). Moreover, despite the continuous lactate overloading in the last phase of process with external acetate, stable caproate production was achieved; however, lactate was not fully consumed and production of caproate did not increase compared to the previous phase despite a 50% higher LLR (Fig. 2 and Fig. 4, Paper 1). It showed that the effect of lactate overloading on caproate production was different in both bioreactors depending on acetate supplementation. The limited availability of acetate for CE during lactate overloading in B1 (when lactate was used as a sole carbon source) made it necessary to produce it directly from lactate causing fluctuations in caproate production. On the other hand, acetate supplementation provided a change in the LLR limit above which the lactate was accumulated. It was indicated that LLR (ED loading rate) was the main factor determining the increase in caproate production until lactate overloading occurred (Fig. 4, Paper 1).

It was also shown that the external acetate supplementation to the lactate-overloaded non-acetated bioreactor (in phase IV in B1, Paper 1) restored stable caproate production and affected chain elongators' relative abundance. Microbiome structure analysis indicated that the relative abundance of OTUs assigned to *Ruminococcaceae* bacterium CPB6 increased from 8.3% to 76.7% as an effect of external acetate supplementation (Electronic Supplementary Material, Paper 1).

Previously recognized in B1 OTU2 assigned to *Ruminococcaceae* bacterium CPB6 and OTU1 assigned to *Acinetobacter* were also dominant in acetated B2. The relative abundance of OTU2 was between 23.9% and 47.7%, while the relative abundance of OTU1 was between 12.8% and 57.2% during the process in B2. According to correlation network analysis (Fig. 8, Paper 1), the external acetate was not significantly correlated with the microbial species changes. It is possible that the influence of acetate supplementation on the CE may be more related to the thermodynamics and kinetics rather than to the microbial competition.

4.3. Lactate to acetate ratio in substrate composition

The impact of acetate supplementation on lactate-based CE was presented in Paper 1, however, whether the concentration of acetate (in the feedstock or bioreactor) can affect CE, was not shown. The concentrations of EDs and EAs, as well as the ratio between the concentrations of ED and EA in the feedstock, may affect the carboxylate selectivity and CE performance as it was indicated in ethanol-based CE (Liu et al., 2016; Yin et al., 2017). Batch trials and continuous processes in Paper 2 were carried out to investigate the influence of the L:A ratio on the CE in MCF. Different effects of L:A ratio were observed in both processes.

In batch trials, in Paper 2, the L:A ratios affected carboxylates selectivity. Higher relative acetate concentrations promoted even-numbered carboxylates, but as the relative

concentration of acetate decreased the competition between the production of even- and odd-numbered carboxylates became more expressed (Table 1 and Fig. 1, Paper 2). Interestingly, caproate was not a dominant product in any batch trial despite the inoculum was enriched in *Ruminococcaceae* bacterium CPB6. The *Ruminococcaceae* bacterium CPB6 did not remain dominant in the microbial community and its relative abundance decreased from 67.9% in the inoculum to less than 2% at the end of the batch trials. The microbial composition could have been affected by the lack of pH control in batch trials. The pH increased during the trials from the initial 5.5 to approximately 6.5, however, this range of pH was still recognized as favorable for cell growth of isolated *Ruminococcaceae* bacterium CPB6 (Zhu et al., 2017). On the other hand, the latest research indicated that pH above 6.0 in MCF could be characterized by the competition between chain elongators and other microorganisms such as propionate producers (Candry et al., 2020). Thus, the maintenance of lactate-based chain elongators such as *Ruminococcaceae* bacterium CPB6 in the MCF could be an individual issue depending on the unique microbiome structure involved in bioprocess.

In a continuous process in Paper 2, the simultaneous lactate and acetate utilization (B2, Fig. 2, Paper 2) led to the production of even-numbered carboxylates, resulting in higher caproate yields; however, controlling and adjusting L:A ratios during the continuous process did not influence caproate yields (Fig. 3, Paper 2). It is also worth noting that the excess of acetate, which accumulated in the process, did not negatively affect caproate production. Unlike the batch trials, *Ruminococcaceae* bacterium CPB6 was the dominant microbe during the continuous process and its relative abundance increased from 3.4% in the inoculum to even 60.1% during the process indicating that operating conditions were favorable for its growth.

4.4. Complex lactate-based substrate composition

One of the most popular lactate-based waste feedstock is AW from the dairy industry (Xu et al., 2018) mainly consisted of lactate and lactose. Firstly, batch studies with synthetic medium were conducted to examine the effect of lactose in substrate composition on lactate-based CE (Paper 3). It was demonstrated that the composition of a substrate affected carboxylates and hydrogen yields. Co-utilization of lactose with lactate and acetate promoted even-numbered carboxylates (butyrate and caproate) and significantly increased hydrogen yields in comparison to the trials where lactate and acetate were used as a substrate (Table 1, Paper 1). It is also worth noting that no methane production was observed in all trials.

Based on the batch studies, it was assumed that the use of lactose- and lactate-rich substrate should significantly improve hydrogen production as well as lactate-based caproate production. Hydrogen is a valuable gaseous product considered to be one of the most promising energy carriers (Bundhoo and Mohee, 2016) and is mainly formed in a dark fermentation (DF) (Villanueva-Galindo et al., 2023). AW was used as a promising substrate for simultaneous CE and DF in a UASB reactor described in Paper 3. At the beginning of the process (stage I) SCCs were mainly produced with butyrate dominance (Fig. 1, Paper 3). The concentration of caproate and its specificity were relatively low (up to 144.7 mM C which corresponded to a specificity of 15%). Methane and carbon dioxide were gaseous products. It is probable that the use of sludge from the Wastewater Treatment Plant, which is most often enriched in methanogens, influenced the adaptation of chain elongators in the microbiome structure. The redirection of an MCF from SCCs to MCCs was observed in stage II as an effect of shortening the HRT.

Caproate was the dominant product. Moreover, redirection in gaseous production was also observed. Methane production was almost completely inhibited and hydrogen production appeared. On the other hand, ethanol and lactate accumulations were observed in stage II. It was previously observed that although ethanol can be an ED for CE, it may have a neutral effect on lactate-based CE and it can accumulate (Carvajal-Arroyo et al., 2021) or could be even an obstacle for lactate-based CE (Duber et al., 2020). The lactate accumulation occurred on day 91 and continued until the end of the process. Its occurrence corresponded to the highest production of caproate, so it was assumed that it could be affected by the toxic effect of the produced carboxylates, which was previously reported (Agler et al., 2012; Angenent et al., 2016).

4.5. Butyrate-caproate competition in lactate-based CE

In the last phase in B1 in Paper 1 and at the end of the process in B1 in Paper 2 a trend of unexpected raising butyrate production was observed. The competition between chain elongators and butyrate producers was previously noticed (Liu et al., 2020) as an effect of long-term CE. Similarly, at the end of process in B1 in Paper 2 after 60 days of process butyrate production increased causing caproate production decrease. It could be associated with the presence of *Clostridium sensu stricto 12* genera (Electronic Supplementary Material, Paper 2) which was previously correlated with high butyrate production (Candry and Ganigué, 2021; Liu et al., 2020). Also in the last phase in B1 in Paper 1, a trend of raising butyrate production was reported, however, the origin of the phenomenon could be different. Interestingly, both CSTRs in Paper 1 (B1 and B2) were operated with the same operational conditions and fed with the same medium from day 118 to day 140. The production of caproate was very similar in both bioreactors, however, in contrast to B2, all lactate was consumed in B1 and it corresponded to an increase in butyrate production. 16s rRNA gene sequencing for samples corresponding to day 140 revealed that both B1 and B2 microbiome structures were enriched in bacteria associated to CE (OTU2 assigned to *Ruminococcaceae* bacterium CPB6 at the relative abundance of 26.3% and 47.7%, respectively, and OTU1 assigned to *Acinetobacter* genus at the relative abundance of 16.4% and 35.2%, respectively); however, OTU3 was dominant in B1 and its relative abundance was 50.3%, while the OTU3 was not detected in B2. OTU3 showed 97% identity of representative sequences with *Ruminococcaceae* bacterium CPB6 and OTU2, which suggested that OTU3 originated from separate, but closely related to OTU2 microorganism. It is also worth noting that both microbiome structures (from B1 and B2 on day 140) evolved from the same inoculum and were closely located on NMDS analysis indicating low dissimilarity of the community structures. Moreover, no OTU3 was found during the whole process in B2, while in B1 OTU3 was first identified in the sample corresponding to day 72 when first symptoms of lactate overloading were observed because of the limited availability of acetate and its relative abundance varied between 4.3% and 11.8% up to day 118. Therefore, it is possible that lactate overloading in B1 and limited availability of acetate for CE induced the growth of a selected microorganism (assigned to OTU3) which affected further lactate transformation and butyrate production. To sum up, while the competition between butyrate production and caproate production (as well as chain elongators and propionate producers) has been identified, the precise factors controlling it, remain to be elucidated.

5. Summary

The results presented in the doctoral dissertation focused on the bioprocessing of lactate to caproate. Caproate is the most popular MCC due to its high potential for industrial applications. Since the most of research so far has focused on the effects of operational parameters on lactate-based CE, herein research focused on the influence of the lactate-based substrate composition on the CE. Nevertheless, the most important operational parameters affecting lactate-based CE were also verified. It was confirmed that mesophilic condition (30 °C) and the acidic pH of 5.5 favored caproate production and the growth of chain elongators such as *Ruminococcaceae* bacterium CPB6 in MCF (Paper 1, Paper 2). It was noted that HRT may also be an important factor influencing the competition between methanogens and chain elongators, as well as determining the efficiency of CE (Paper 3). However, the main purpose of the research was to indicate that not only the operational parameters could affect the lactate-based CE, but also the composition of the substrate. The hypothesis that the composition of the lactate-based substrate, particularly the concentration of lactate (ED) and acetate (EA), affects the CE performance, has been confirmed.

It was shown that lactate as a sole carbon source could be a promising feedstock for caproate production in MCF, however, there were some limitations (Paper 1). The limiting factor for the bioprocessing of lactate to caproate turned out to be access to acetate (EA) which had to be produced directly from lactate. Too high LLR resulted in lactate accumulation (lactate overloading) and caused fluctuations in the caproate production because of the limited availability of acetate for the CE. Therefore, the controlling of LLR was crucial for the caproate production when lactate was used as a sole carbon source. The activation of the competitive acrylate pathway as a result of lactate overloading was not reported as it was suggested by Kucek et al. (2016a).

Acetate supplementation promoted the production of even-numbered carboxylates (butyrate and caproate), shifted the lactate overloading limit which resulted in higher lactate consumption, ensured stable caproate production even despite lactate overloading, as well as restored caproate production in a lactate overloaded bioreactor. Nevertheless, the main factor determining the production of caproate was not the concentration of acetate, but the concentration of lactate (ED) in the feedstock.

Despite these positive aspects of lactate and acetate co-utilization, controlling the relative concentrations of lactate and acetate in the feedstock was not an effective strategy to increase caproate production, but importantly the excess of acetate also did not disturb the caproate production (Paper 2). On the contrary, the L:A ratio determined the selectivity of carboxylate production in batch trials affecting the competition between propionate and butyrate production; however, probably the lack of pH control had an impact the formation of the microbiome (although the inoculum was enriched in chain elongators, they were barely detectable in the microbiome structure) which corresponded to the carboxylates' production.

The co-production of hydrogen (DF) and caproate (CE) from the complex waste feedstock was also proposed as a promising bioprocess configuration (Paper 3). AW containing mainly lactate and lactose was used as a model feedstock. First, batch studies showed that the co-utilization of lactate and lactose boosted hydrogen production and affected carboxylate selectivity by promoting even-numbered carboxylates. Then, in the long-term conversion of

AW it was confirmed that the simultaneous production of hydrogen and caproate was feasible. However, the process encountered challenges related to the accumulation of EDs (ethanol and lactate). Lactate accumulation at the end of the bioprocess could be attributed to the toxic effect of produced carboxylates which inhibited chain elongation, however, the accumulation of ethanol implied that, it was not considered a suitable ED for contributing to the lactate-based CE.

As an additional observation from the conducted research, it is worth noting that an unexpected competition between the production of butyrate and caproate was observed in the long-term continuous processes (Paper 1, Paper 2). The competition between the butyrate fermenters and the chain elongators was observed before (Liu et al., 2020); however, it exact factors allowing butyrate fermenters take control of the process, remains to be investigated.

The conducted trials confirmed the hypothesis that utilizing a lactate-based substrate under CE-promoting conditions during MCF will lead to a microbiome enrichment in the lactate-based chain elongating bacteria. The analysis of the microbiome structure revealed that *Ruminococcaceae* bacterium CPB6 and *Acinetobacter* were the predominant microorganisms in mixed culture fermentation systems. *Ruminococcaceae* bacterium CPB6 was previously known to be an effective lactate-based caproate producer (Zhu et al., 2017), while *Acinetobacter* was recognized as bacteria involved in chain elongation (He et al., 2018; Kucek et al., 2016b, 2016a; Qian et al., 2020). Acetate supplementation, although it influenced the chain elongation, did not significantly affect the formation of the microbiome community, however, changes in the relative abundance of *Ruminococcaceae* bacterium CPB6 in the microbiome structure were observed depending on the lactate consumption and caproate formation.

In summary, MCF is an evolving technology that has a promising future in the bio-based economy. The findings from the carried out research contribute towards the development of an advanced carboxylate platform based on the lactate-based feedstock. Described results provided a broader view of lactate-based CE in MCF, which led to a better understanding of the bioprocess indicating that not only the operational parameters affected the lactate-based CE, but also the composition of the feedstock.

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