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

PhD dissertation

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Abstract

The transition from a linear economy to a circular economy is the foundation of sustainable development. The bioprocessing of organic-rich waste during mixed culture fermentation has the potential to play a crucial role in the shift towards a circular economy by reducing dependence on non-renewable resources and minimizing waste. Mixed culture fermentation involves the use of diverse microorganisms to break down complex organic waste in a cascade of biochemical pathways, offering the potential for production of high-value bulk chemicals, biofuels and energy. Anaerobic digestion is commercialized and widespread technology based on mixed culture fermentation, but there is still a search for new bioprocesses to diversify bioproducts. One of the promising and growing technologies could be the bioproduction of medium chain carboxylates through the chain elongation. To enable the chain elongation, the fundamental metabolic requirement is the availability of compounds (electron donors) that supply the necessary energy, reducing equivalents (NADH), and intermediate acetyl-CoA to the reverse β-oxidation cycle (or occasionally, malonyl-CoA for fatty acid biosynthesis), wherein the carbon chain of the carboxylate is elongated by two carbon atoms in a series of enzymatic reactions, e.g. acetate is elongated to butyrate and butyrate to caproate. While various chemical compounds such as ethanol, lactate and sugars can serve as an electron donors for chain elongation, lactate-based bioconversion is receiving increasing attention. The research associated with lactate-based medium chain carboxylates production conducted so far has focused mainly on optimizing operational parameters of mixed culture fermentation such as pH, temperature, or hydraulic retention time, 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 research was to identify the influence of lactate-based substrate composition on medium chain carboxylates production. Three long-term continuous processes and two batch experiments were conducted and described in three scientific papers. Two main following hypotheses were stated:

1. The composition of lactate-based substrates, especially the lactate and acetate concentration, significantly influences the chain elongation 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.

It has been demonstrated that lactate-based feedstocks can be a promising substrates for the production of medium chain carboxylates, especially caproate. However, the process of converting lactate (as the sole carbon source) to caproate faced stability issues due to the limited availability of acetate, which needed to be produced first directly from lactate, and then elongated to butyrate, and caproate. Under high lactate loading rate conditions, the limited availability of acetate for carboxylate chain elongation resulted in lactate accumulation and fluctuations in caproate production. Therefore, it was crucial to control the lactate loading rate (or lactate concentration in the feedstock) for caproate production when using lactate as the sole carbon source.

Supplementation of acetate was found to change the lactate overloading limit, resulting in higher lactate consumption and stable caproate production (even under lactate overloading conditions). Moreover, acetate supplementation restored caproate production in lactate-overloaded bioprocess. Co-utilization of lactate and acetate increased caproate production, however controlling the relative concentrations of acetate in the feedstock was not an effective strategy for increasing caproate production. The concentration of lactate in the feedstock was found to be the main factor determining caproate production, rather than the concentration of acetate, but the excess of acetate in the system did not disturb caproate production. Interestingly, an unexpected competition between the production of butyrate and caproate was observed in long-term continuous processes.

A promising bioprocess for the simultaneous production of hydrogen and caproate from acid whey, which is a complex waste stream from dairy industry, was also proposed. During the process, an accumulation of ethanol and lactate, which are compounds acting as electron donors in the chain elongation, was observed. 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 implies that while during ethanol-based chain elongation it is a valuable electron donor for chain elongation, it was not considered a suitable electron donor for collaborating in lactate-based chain elongation.

The analysis of the microbiome structure showed that *Ruminococcaceae* bacterium CPB6, which was previously identified as a highly-efficient lactate-based caproate producer, and *Acinetobacter*, which was previously recognized as bacteria involved in chain elongation, were found dominant in our mixed culture fermentation systems. According to correlation network analysis, the acetate supplementation was not significantly correlated with the relative quantity of any taxons which suggested that the influence of acetate on the chain elongation may be more related to the thermodynamics and kinetics rather than to the microbial competition; however, changes in the relative abundance of *Ruminococcaceae* bacterium CPB6 in the microbiome structure were observed depending on the lactate consumption and caproate formation.

The findings from this dissertation demonstrate that not only operational parameters such as pH, temperature and hydraulic retention time affect lactate-based chain elongation, but also the composition of lactate-based substrates have an impact on the bioprocess.

Streszczenie

Podstawą zrównoważonego rozwoju jest przejście z gospodarki liniowej do gospodarki o obiegu zamkniętym. Bioprzetwarzanie odpadów bogatych w substancje organiczne w procesie fermentacji wykorzystującej kultury mieszane mikroorganizmów może odegrać kluczową rolę w przemianie gospodarki, przede wszystkim poprzez uniezależnienie od zasobów nieodnawialnych i minimalizację ilości odpadów. Fermentacja wykorzystująca mieszane kultury mikroorganizmów opiera się na wykorzystaniu różnorodności mikroorganizmów w strukturze mikrobiomu do rozkładania złożonych odpadów organicznych, co daje potencjał do produkcji wysokowartościowych związków chemicznych, biopaliw i energii. Produkcja biogazu jest skomercjalizowaną i szeroko rozpowszechnioną technologią opartą na wykorzystaniu mieszanych kultur mikroorganizmów w procesie fermentacji, jednakże poszukiwane są nowe rozwiązania w celu dywersyfikacji bioprodukcji. Jedną z obiecujących i rozwijających się technologii może być bioprodukcja średniołańcuchowych kwasów karboksylowych w procesie wydłużania łańcucha węglowego. Podstawowym wymogiem metabolicznym w celu przeprowadzenia procesu wydłużania łańcucha węglowego jest dostępność odpowiednich związków chemicznych (donorów elektronów), które dostarczają niezbędnej energii w postaci równoważników redukujących (NADH) i acetylo-CoA do odwrotnego cyklu β-oksydacji (lub rzadziej malonylo-CoA do szlaku biosyntezy kwasów tłuszczowych), w którym łańcuch węglowy kwasu karboksylowego jest wydłużany zawsze o dwa atomy węgla podczas szeregu reakcji enzymatycznych, np. kwas octowy jest wydłużony do kwasu masłowego, a kwas masłowy do kwasu kapronowego. Podczas gdy różne związki chemiczne, takie jak etanol, kwas mlekowy i cukry, mogą służyć jako donory elektronów do wydłużania łańcucha węglowego, biokonwersja oparta na kwasie mlekowym cieszy się coraz większym zainteresowaniem. Dotychczasowe badania nad procesem konwersji kwasu mlekowego do średniołańcuchowych kwasów tłuszczowych koncentrowały się głównie na optymalizacji parametrów operacyjnych fermentacji, takich jak pH, temperatura czy hydrauliczny czas retencji, jednakże wpływ składu substratu (stężeń donorów i akceptorów elektronów) nie zostały jeszcze w pełni zbadane. Głównym celem badań było określenie wpływu składu substratu na bazie kwasu mlekowego na produkcję średniołańcuchowych kwasów karboksylowych (przede wszystkim kwasu kapronowego). Przeprowadzono trzy długoterminowe procesy ciągłe i dwa eksperymenty wsadowe (okresowe), które opisano w trzech artykułach naukowych. Postawiono dwie główne hipotezy:

1. Skład substratu wpływa na proces wydłużania łańcucha węglowego na bazie kwasu mlekowego do średniołańcuchowych kwasów tłuszczowych (kwasu kapronowego).
2. Zastosowanie substratu zawierającego kwas mlekowy podczas fermentacji wykorzystującej mieszane kultury mikroorganizmów w warunkach fermentacji promujących proces wydłużania łańcucha karboksylowego doprowadzi do wzbogacenia struktury mikrobiomu w bakterie przeprowadzające proces wydłużania łańcucha karboksylowego na bazie mleczanu.

Wykazano, że surowce na bazie kwasu mlekowego mogą być obiecującymi substratami do produkcji średniołańcuchowych kwasów tłuszczowych, a zwłaszcza kwasu kapronowego. Proces konwersji kwasu mlekowego (jako jedynego źródła węgla) do kwasu kapronowego charakteryzował się niestabilnością produkcji. Wynikało to z ograniczonej dostępności kwasu octowego, który musiał być w pierwszej kolejności wytworzony bezpośrednio z kwasu mlekowego, a następnie mógł być wydłużony do kwasu masłowego i kwasu kapronowego. W warunkach wysokiego obciążenia bioreaktora kwasem mlekowym ograniczona dostępność kwasu octowego powodowała akumulację kwasu mlekowego i wahania w produkcji kwasu kapronowego. Stąd kluczową rolę podczas tego procesu miała kontrola dozowanego kwasu mlekowego do bioreaktora.

Ponadto wykazano, że suplementacja kwasem octowym „przesunęła" limit przeciążenia bioprocesu kwasem mlekowym, co skutkowało wyższym zużyciem kwasu mlekowego i stabilną produkcją kwasu kapronowego. Suplementacja kwasem octowym przywróciła również stabilną produkcję kwasu kapronowego w bioprocesie uprzednio przeciążonym kwasem mlekowym. Ko-utylizacja kwasu mlekowego i kwasu octowego wpłynęła na zwiększenie produkcji kwasu kapronowego, jednakże kontrolowanie względnego stężenia kwasu octowego w surowcu nie było skuteczną strategią zwiększenia produkcji kwasu kapronowego. Istotne jest jednak, iż nadmiar kwasu octowego w układzie nie zakłócał w żaden sposób produkcji kwasu kapronowego. Zaobserwowano, że głównym czynnikiem determinującym produkcję kwasu kapronowego było stężenie kwasu mlekowego w surowcu. Co ciekawe, w przeprowadzonych długotrwałych procesach ciągłych zaobserwowano również nieoczekiwaną konkurencję między produkcją kwasu masłowego i kwasu kapronowego.

Zaproponowano również obiecujący proces ko-produkcji wodoru i kwasu kapronowego z serwatki kwaśnej. Podczas procesu zaobserwowano akumulację związków będących podstawowymi donorami elektronów w procesie wydłużania łańcucha węglowego, tj. etanolu i kwasu mlekowego. Akumulacja kwasu mlekowego pod koniec procesu mogła być spowodowana toksycznym działaniem wytworzonych kwasów karboksylowych, które hamowały proces wydłużania łańcucha węglowego, zaś akumulacja etanolu w trakcie procesu może sugerować, iż współwykorzystanie etanolu i kwasu mlekowego może być nieefektywne w produkcji kwasu kapronowego, pomimo iż etanol jest powszechnie stosowanym donorem elektronów w procesie wydłużania łańcucha karboksylowego.

Analiza struktury mikrobiomu wykazała, że bakteria *Ruminococcaceae* CPB6, która została w innych badaniach zidentyfikowana jako wysoce wydajny producent kwasu kapronowego, oraz bakterie z rodzaju *Acinetobacter*, które również wykryto w innych strukturach mikrobiologicznych zaangażowanych w proces wydłużania łańcucha karboksylowego, okazały się dominujące w strukturze mikrobiomu. Analiza statystyczna wykazała, iż suplementacja kwasem octowym nie była istotnie skorelowana ze zmianami gatunkowymi mikroorganizmów, co sugeruje, że wpływ suplementacji kwasu octowego na wydłużenie łańcucha węglowego może być bardziej związany z termodynamiką i kinetyką reakcji, niż z konkurencją pomiędzy mikroorganizmami. Zaobserwowano

jednakże zmiany we względnej liczebności bakterii *Ruminococcaceae* CPB6 w strukturze mikrobiomu w zależności od konsumpcji kwasu mlekowego i wydajności produkcji kwasu kapronowego.

Wyniki zamieszczone w niniejszej rozprawie wykazują, że nie tylko parametry operacyjne, takie jak pH, temperatura i hydrauliczny czas retencji, wpływają na wydłużenie łańcucha karboksylowego, ale także skład substratu ma wpływ na przebieg wydłużania łańcucha karboksylowego.

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

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

   Sum of MEiN points: 540
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Scientific activity

Scientific activity which is not a part of the thesis, but it is relevant to the topic of the thesis.

Publications:

- Brodowski, F., Oleskowicz-Popiel, P., 2019. Caproic acid as an alternative product of bioconversion. Przem. Chem. 10, 1629-1634. https://doi.org/10.15199/62.2019.10.20

Oral conference presentations (presenting author):

- Brodowski, F., Lezyk, M., Gutowska, N., Duber, A., Oleskowicz-Popiel, P.: Competition between lactate-based and ethanol-based chain elongation: the influence of pH on product selectivity and microbiome structure. 2nd International Chain Elongation Conference, 2-4 November 2022, Bad Boll, Germany.
- Brodowski F., Zagrodnik, R., Lezyk, M., Gutowska, N., Duber, A., Oleskowicz-Popiel, P.: Chain elongation as a method for organic waste valorization. Workshop: Anaerobic biorefinery for resource recovery from waste feedstock - WasteValue, 11th IWA IWA International Symposium on Waste Management Problems in Agro–Industries, 26th October 2022, Gdansk, Poland.
- Brodowski, F., Lezyk, M., Gutowska, N., Oleskowicz-Popiel, P.: Lactate-based Chain Elongation in a continuously-fed mixed culture fermentation: Influence of external acetate and co-use of electron donor. 17th IWA World Conference on Anaerobic Digestion, 19-22 June 2022, Ann Arbor, Michigan, USA.
- Brodowski, F., Lezyk, M., Gutowska, N., Oleskowicz-Popiel, P.: Production of medium chain carboxylic acids from lactic acid - the effect of the concentration of electron donors and electron acceptors on the metabolic pathway selection. 2nd IWA Polish Young Water Professionals, 12-14 February 2020, Warsaw, Poland.
- Brodowski, F., Lezyk, M., Gutowska, N., Oleskowicz-Popiel, P.: Production of caproic acid from acid whey - foundations of modern waste bio-refineries. 3rd National Symposium on Bioorganic, Organic Chemistry and Biomaterials (BioOrg), 7th December 2019, Poznan, Poland.

Poster conference presentations (presenting author):

- Brodowski, F., Lezyk, M., Gutowska, N., Duber, A., Oleskowicz-Popiel, P.: Competition between lactate-based and ethanol-based chain elongation: the influence of pH on product selectivity and microbiome structure. 2nd International Chain Elongation Conference, 2-4 November 2022, Bad Boll, Germany.
- Brodowski, F., Lezyk, M., Gutowska, N., Oleskowicz-Popiel, P.: Lactate-based carboxylate platform: evaluation of the influence of feedstock composition on production efficiency. 11th IWA International Symposium on Waste Management Problems in Agro-Industry, 26-28 October 2022, Gdansk, Poland.
- Brodowski, F., Lezyk, M., Gutowska, N., Oleskowicz-Popiel, P.: Lactate overloading phenomenon in carboxylate platform: stable caproate and hydrogen

co-production. 43rd Symposium on Biotechnology for Fuels and Chemicals, 26th April 2021, online.

Conference co-authorship:

- Gutowska, N., Barski, P., Brodowski, F., Byliśnki, H., Cema, G., Dąbrowski, S., Dąbrowski, T., Duber, A., Estevez, M. M. R., Fudala-Książek, S., Kasinath, A., Kosek, K., Lidstaedt, A., Łężyk. M., Łuczkiewicz, A., Manamperuna, L. D., Pierpaoli, M., Pietrewicz-Kubicz, D., Surmacz-Górska, J., Szatkowska, B., Szopińska, M., Tomczak-Wandzel, R., Witt, D., Zabłotna, E., Zaborowska, E., Ziembińska-Buczyńska, A., Oleśkowicz-Popiel, P.: Anaerobic biorefinery for resource recovery from waste feedstock – WasteValue. 17th IWA World Conference on Anaerobic Digestion, 19-22 June 2022, Ann Arbor, Michigan, USA.
- Oleskowicz-Popiel, P., Lezyk, M., Duber, A., Brodowski, F., Jankowska E., Walkiewicz F., Gutowska, N., Zagrodnik R.: Caproic acid production: process development, product recovery, microbiome characterization and techno-economic analysis. 16th World Conference on Anaerobic Digestion Conference AD16, 23-27 June 2019, Delft, the Netherlands.
- Oleskowicz-Popiel, P., Lezyk, M., Duber, A., Brodowski, F., Gutowska, N., Zagrodnik, R.: Open culture fermentation for biofuels and biochemicals production - caproic acid production. 41st Symposium on Biotechnology for Fules and Chemicals, 27 April - 01 May 2019, Seattle, USA.
- Duber, A., Chwialkowska, J., Jaroszynski, L., Brodowski, F., Stodolny, M., Oleskowicz-Popiel, P.: From ethanol to caproic acid – evaluation of process re-integration. Challenges in Building a Sustainable Biobased Economy, ECO-BIO, 6-9 March 2016, Rotterdam, Netherlands.

Research projects:

- The National Centre for Research and Development, ‘Anaerobic biorefinery for resource recovery from waste feedstock’ (WasteValue), III edition of EEA and Norway grants, contract no. NOR/POLNOR/WasteValue/0002/2019-00, PI: Piotr Oleskowicz-Popiel, Filip Brodowski – Researcher, December 2020 – November 2023.
- National Science Centre, Poland, ‘Carboxylate chain elongation process during anaerobic mixed culture fermentation’ (C-Elong), SONATA BIS 7 programme, contract no. 2017/26/E/ST8/00007, 2018-2023, PI: Piotr Oleskowicz-Popiel, Filip Brodowski – Researcher, November 2018 – October 2022.

Other scientific activity:

- Researcher in SBMK 2020/2021 project ‘Preliminary study of nanopore sequencing potential as an accessible method for analyzing the composition of selected mixed cultures.’, project no.: 504101/0713/SBAD/0934, PI: Mateusz Lezyk.

List of abbreviations

ASV – amplicon sequence variant
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

Carboxylic acids refer to the neutral (undissociated) form of the molecule. When carboxylic acids are dissolved in water, they can dissociate, resulting in the formation of carboxylate ions. The carboxylic acid can exist in two forms simultaneously, depending on the pH of the solution. Low pH values favor neutral forms, while high pH values promote ionized forms. To simplify the nomenclature the name of ionized forms (i. e. medium chain carboxylates, acetate, propionate, lactate, butyrate, valerate, caproate, heptylate, caprylate) was used in the doctoral thesis.

The terminology "mixed culture fermentation" refers to the use of undefined mixed cultures in fermentation process. Another commonly used phrase for this type of fermentation is "open culture fermentation". For the sake of consistency in the PhD dissertation, the term "mixed culture fermentation" has been adopted; however, the phrase "open culture fermentation" appears in the Papers included in the dissertation.

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. Currently, bioethanol, biobutanol, biodiesel, biogas (biomethane), and hydrogen are the most popular second-generation bioproducts (Ubando et al., 2021), however, 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 to cultivate methanogen-free microbiome (De Groof et al., 2019). One effective method to stop methanogenesis is to adjust the pH level. For instance, a decrease in pH below 6.5 can help prevent the activity of methanogens, which have an optimal pH range of 6.8-7.2. However, it is crucial to prevent pH levels from dropping too low because it may negatively affect the performance of the other microorganisms in MCF. Another approach is to decrease the hydraulic retention time (HRT). This can help promote the growth of acidogenic bacteria, which produce SCCs that can be used by other microorganisms (e.g. by chain elongating bacteria for caproate production), while slowing down the methanogenesis.

Carboxylate platform has a potential to play a significant role in the bio-based products market such as drop-in biofuels, biochemicals, and bioplastics (Fig. 1). 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). Moreover, the latest research suggested that the medium chain carboxylic acids can be a precursor for further production of bioplastics, e.g. PHA (Iglesias-Iglesias et al., 2021; Vermeer et al., 2023). 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 and is struggling with economic viability, however, first projects and start-ups are already appearing on the market, e.g. the MixAlco^{TM} pilot project converting biomass into a range of chemical (including caproic acid), the ChainCraft B. V. producing fractionated fatty acids for animal nutrition and technical applications or Capro-X a spin-off business that converts Greek yogurt waste streams into bio-oils and currently working on scaling up its technology (De Groof et al., 2019).

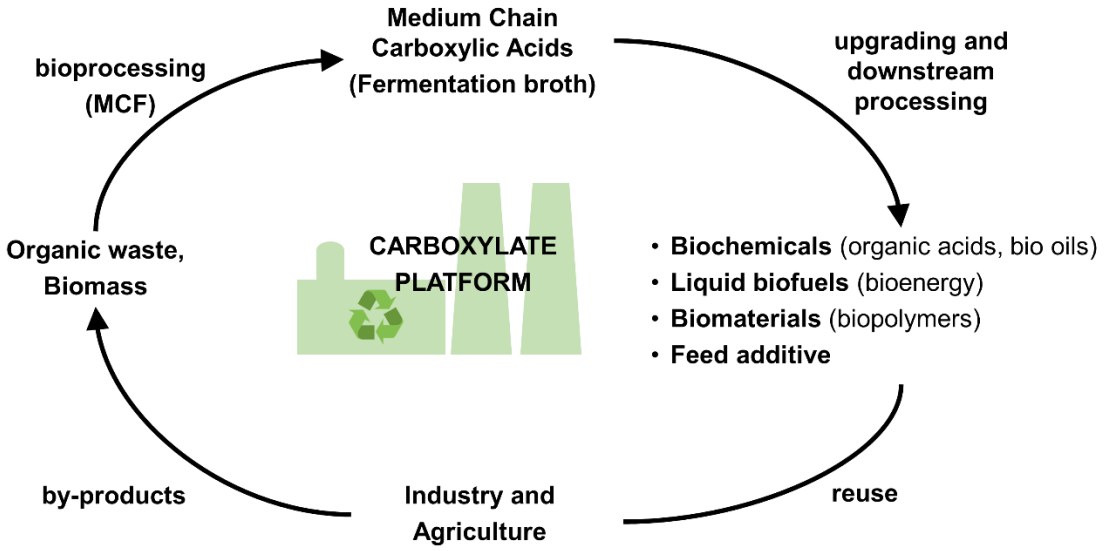

Figure 1. Carboxylate platform as a potential second-generation biorefinery

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). The graphical presentation of the caproate production from lactate-based feedstocks is presented in Fig. 2.

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.

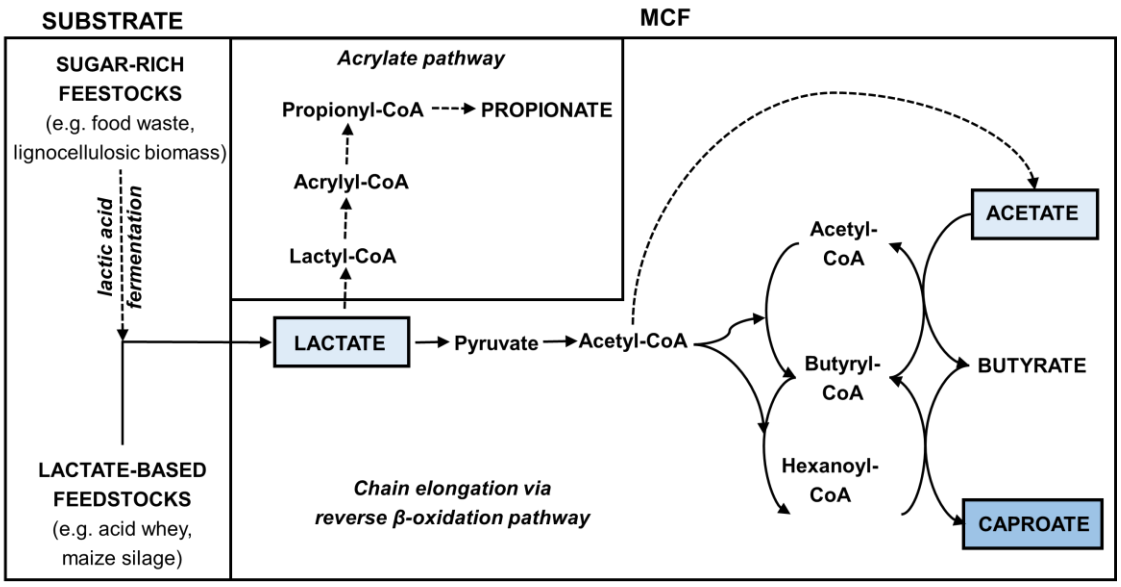

Figure 2. Chain elongation from lactate-based feedstocks.

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.

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.

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.

Particular aims are linked to the objectives of experiments carried out and presented in Paper 1, Paper 2, and Paper 3 which are shown in the graphical scheme below (Fig. 3).

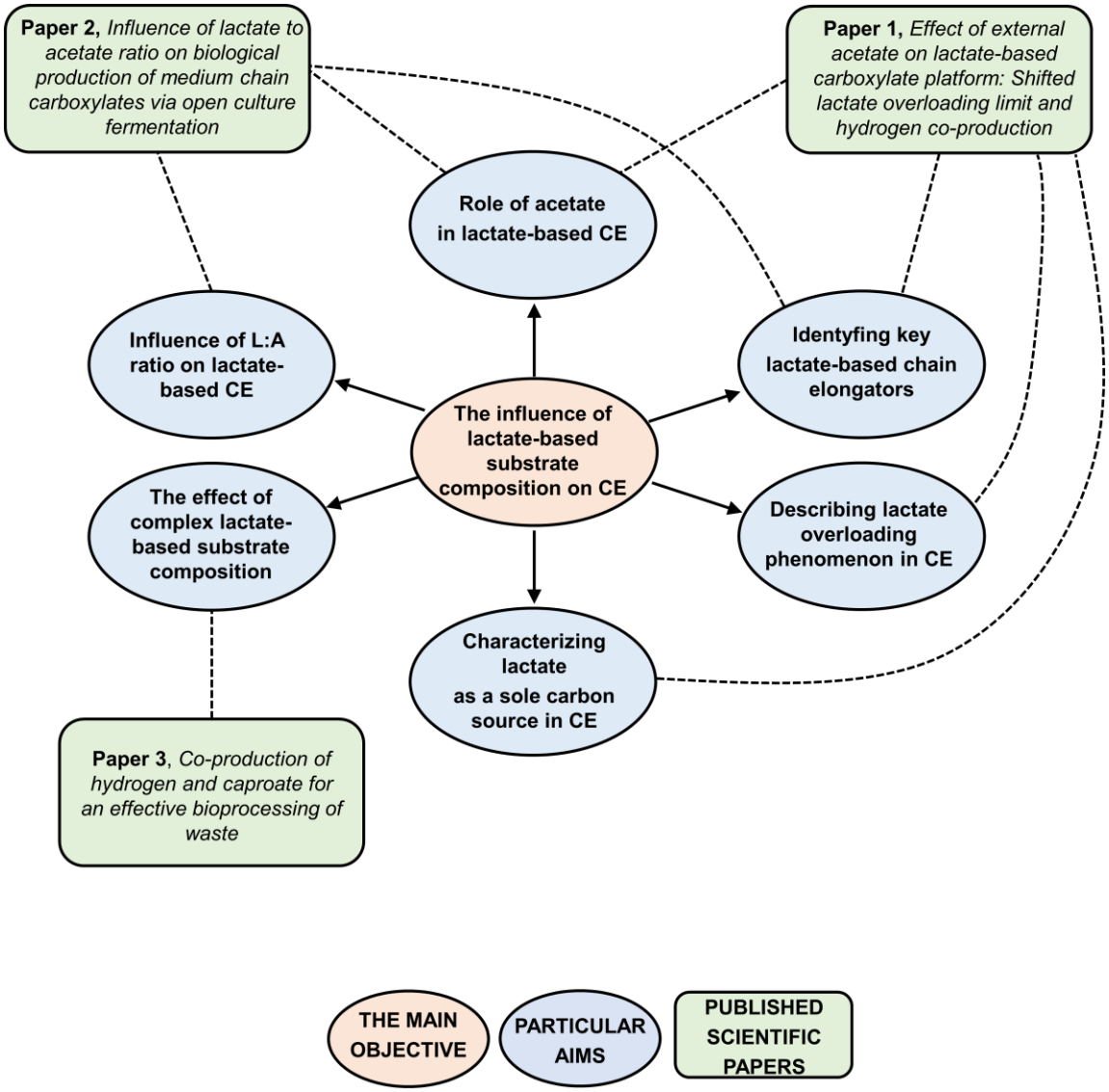

Figure 3. Schematic representation of the objectives of the Ph.D. thesis.

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 (A), vitamin solution (B), trace metal solution (C), chelating agent solution (D), reducing agent solution (E), buffer solution (F), yeast extract solution and carbon source solutions. Solutions were prepared as follows:

- Solution A: 100 g/L of NH_4Cl, 10 g/L of NaCl, 10 g/L of $MgCl_2 \cdot 6H_2O$, 5 g/L of $CaCl_2 \cdot 2H_2O$ were dissolved and then autoclaved;
- Solution B: 2 mg/L of biotin (vit. B7), 2 mg/L of folic acid, 10 mg/L of pyridoxine hydrochloride (vit. B6), 5 mg/L of riboflavin (vit. B2), 5 mg/L of thiamine (vit. B1), 0.1 mg/L of cyanocobalamine (vit. B12), 5 mg/L of nicotinic acid, 5 mg/L of p-aminobenzoic acid, 5 mg/L of lipoic acid, 5 mg/L of DL-pantothenic acid were dissolved and filter-sterilized;
- Solution C: 50 mg/L of H_3BO_3, 50 mg/L of $ZnCl_2$, 30 mg/L of $CuCl_2$, 50 mg/L of $MnCl_2 \cdot 4\ H_2O$, 50 mg/L of $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 50 mg/L of $AlCl_3$, 50 mg/L of $CoCl_2 \cdot 6H_2O$, 50 mg/L of $NiCl_2$, 100 mg/L of $Na_2SeO_3 \cdot 5H_2O$, 60 mg/L of $Na_2WO_4 \cdot 2H_2O$, 2000 mg/L of $FeCl_2 \cdot 4H_2O$ (previously dissolved in 1 mL of concentrated HCl and 15 mL of water) were dissolved and autoclaved;
- Solution D: 1 g/L of nitrilotriacetic acid was dissolved and autoclaved;
- Solution E: 25 g/L of $Na_2S \cdot 9H_2O$ was dissolved (using nitrogen for degassing) and filter-sterilized;
- Solution F: 1 M K_2HPO_4 and 1 M KH_2PO_4 were used for buffer preparation (pH of 5.5);
- Yeast extract solution: 25 g/L of yeast extract was dissolved and autoclaved;
- Carbon source solutions: 300 g/L lactate, acetate, and lactose were dissolved and autoclaved.

The medium was prepared by adding 10 mL/L of solution A, 10 mL/L of solution B, 1 mL/L of solution C, 10 mL/L of solution D, 10 mL/L of solution E, and 20 mL/L of yeast extract solution. Lactate, acetate, and lactose solutions were added according to the projected initial values presented in Tab. 1. Final pH was adjusted using 1M sodium hydroxide, then 50 mL/L of solution F was added to the medium.

The synthetic medium for continuous processes was prepared with the modifications as follows: 10 mL/L of the 1 M K_2HPO_4 solution and 15 mL/L of the 1 M KH_2PO_4 solution were added to the medium instead of 50 mL/L of solution F and the pH adjustment of the medium was not conducted (because pH was automatically controlled in CSTRs). 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), however, low concentrations of acetate and ethanol (about 1.1 g/L) were also detected.

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. The weighing module was used to maintain a constant weight of the vessel by controlling the effluent pump which was activated when the preset weight was exceeded. Base or acid was automatically pumped into the fermenters based on the set pH. IR radiation heater was used to maintain a constant temperature based on temperature measurments. A more detailed scheme of the Lambda Minifor fermenters configuration was presented in Paper 1, Fig. 1.

The continuous process in Paper 3 was carried out in a 1L UASB reactor made from plexiglass which was previously described and shown in (Duber et al., 2018). The recirculation installation equipped with a peristaltic pump ensured sludge suspension. The base was pumped automatically based on pH measurements (a pH probe was installed in the recirculation installation). 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 obtained from a 1L UASB reactor, which was used for MCCs production from AW as previously described in (Duber et al., 2018). The inoculum was prepared as follows: 7.5 mL of UASB sludge was centrifuged (3500 rpm for 10 min), washed with 0.9% w/v NaCl solution, then centrifuged again (3500 rpm for 10 min), resuspended in 2 mL of 0.9% w/v NaCl solution and added to each bottle (2mL per bottle). 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 for 5 min 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 obtained from an aforementioned 1 L MCC-producing UASB reactor. The inoculation sludge for both processes differed from each other as it was taken on a different day for the process in Paper 1 and Paper 2. The microbiome composition was identified by 16s rRNA gene sequencing. The inoculum was prepared as follows: 80 mL (per one bioreactor) of sludge was centrifuged, washed, and centrifuged again, then resuspended in 5 mL of 0.9% w/v NaCl solution and added to each bioreactor. Then, bioreactors were flushed with nitrogen gas to ensure anaerobic conditions. 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 made from plexiglass. The recirculation installation equipped with a peristaltic pump ensured sludge suspension. 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 (by heating the water jacket around the reactor) and pH at 5.5 with automatic correction (the pH probe was connected to a control unit which activated a peristaltic pump pumping 2M NaOH). 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). Sludge was stored at 30 °C for 48 hours for degasification and diluted to reach an initial total soluble solid concentration of about 5 g/L. Then 50 mL of AW was added and it was used for UASB reactor start-up. 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. Nitrogen at a flow rate of 15 mL/min was used as the carrier gas. The temperatures of the injector, column, and detector were 110 °C, 50 °C, and 80°, respectively. All gas volumes are reported at 1 atm and 273.15 K.

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 at a flow rate of 7.38 mL/min. In Paper 3, the ramp temperature program was set as follows: the initial oven temperature was 70 °C for 1 min, then was raised to 240 °C with a temperature ramp rate of 10 °C/min, and it was held for 3 min. The temperature of the FID and the injection port were 250 and 200 °C, respectively. In Paper 1 and Paper 2, the ramp temperature program was modified and was as follows: the initial oven temperature was 70 °C for 2.5 min, then was raised to 185 °C with a temperature ramp rate of 11 °C/min, and it was held for 10 min. The temperature of the injection port was also modified and set to 250°C.

The concentrations of lactate and lactose were monitored with high-performance liquid chromatography (Shimadzu LC-20, Rezex ROA-Organic Acid column, RI detector). For elution 5 mM aqueous sulfuric acid was used at the flow rate of 0.6 mL/min at 63 °C.

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 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.

The DNA was isolated using GeneMATRIX Soil DNA Purification Kit (EURx, Poland) according to the manufacturer's protocol. ZymoBIOMICS Microbial Community Standard D6300 (Zymoresearch, USA) was used as a mock microbial community to assess bias and errors in the analysis pipeline. Amplification of V3-V4 regions of 16S rRNA gene fragment was carried out with Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/μL) and primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 785R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) via PCR (25 cycles at an annealing temperature of 50°C). Afterward, samples were sent to Macrogen (Korea) for further sequencing according to 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, Part # 15044223, Rev. B). The Illumina Miseq instrument (300 bp paired-end sequencing, Miseq v3) was used to obtain reads.

The raw sequence data were collected for the following bioinformatic analyses. Firstly, USEARCH (Edgar, 2013) was used for trimming and merging paired reads. The

primer sequences were stripped off using cutadapt software (Martin, 2011). The USEARCH pipeline was used in quality filtering and clustering. In Paper 1, UPARSE algorithm was used for the picking of operational taxonomic units (OTUs). SINTAX was used for the taxonomy assignment (Edgar, 2016) using Silva LTPs v132 database (Yilmaz et al., 2014). In Paper 2, UNOISE2 algorithm was used for the generation of amplicon sequence variants (ASVs) and Qiime2 package (Bolyen et al., 2019) was used for the taxonomy assignment using Silva v138 database. Phyloseq package (McMurdie and Holmes, 2013) was used for the preparation of taxonomic distribution of reads mapping to OTUs or ASVs at genus level and non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity between microbial communities in samples analyzed in Paper 1 and Paper 2 (ASVs or OTUs with the total abundance of less than 0.1 % removed from the analysis).

Moreover, 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). OTUs that were present in less than three samples and OTUs with <0.1% relative abundance were not included in the analysis. Pearson, Spearman, and Kendall correlation coefficients were computed. *P*-values were determined by bootstrapping based on 1000 replications. Positive or negative correlations were considered if at least one method indicated a coefficient above 0.8 or below -0.8 and calculated after the Benjamini-Hochberg correction was less than 0.05.

3.6. Calculations

Carboxylates production rates (mmol C/L/d) for continuous processes in Paper 1, Paper 2, and Paper 3 were calculated based on (Xu et al., 2018) as a ratio of the concentration of carboxylate in the bioreactor (mmol C/L) to HRT (days). Specificities (%) for continuous processes in Paper 1, Paper 2, and Paper 3 were calculated as a ratio of the production rate of a specific carboxylate (mmol C/L/d) to the production rate of all detected carboxylates (mmol C/L/d). Lactate loading rate (LLR) and acetate loading rate (mmol C/L/d) for continuous process in Paper 1 were calculated as a ratio of lactate or acetate concentration in the influent (mmol C/L) to HRT (days). Carboxylate yields (mmol C of carboxylate per mole C of lactate) for continuous process in Paper 3 were calculated as the ratio of the production rate of a specific carboxylate (mmol C/L/d) to the LLR (mol C/L/d). Acetate consumptions (mmol C/L/d) for continuous process in Paper 3 were calculated as the difference between the acetate concentration in the feedstock and the concentration of the accumulated acetate in the bioreactor and it was divided by the HRT.

Carboxylate yields for batch processes in Paper 1 and Paper 3 were calculated differently depending on the units used. Carboxylate yields (mmol C of carboxylate per mole C of lactate) for batch trials in Paper 3 were calculated as the ratio of the final concentration of carboxylate (mM C) to the initial lactate concentration (mM C).

Carboxylate yield (mmol C of carboxylate per mole C initial) for batch trials in Paper 1 was calculated as the ratio of the final concentration of carboxylate (mM C) to the sum of the initial concentration of all metabolites (lactate, acetate, and ethanol) (mM C).

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 mmol of gaseous products in batch trials in Paper 3. Then gas production ($mmol/L_{medium}$) was calculated as a ratio of total gas production (mmol) to the working volume of batch trials (L).

4. 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 both pure culture (Zhu et al., 2017) and MCF (Kucek et al., 2016a; Zhu et al., 2015). The EA (acetate) was provided by the oxidation of lactate, and as a result, no external supply of acetate was required for CE. However, the use of lactate as the sole carbon source may have some limitations. Propionate production from lactate via the competing acrylate pathway can disturb the CE process (Nzeteu et al., 2022). It was reported that lactate overloading (residual lactate accumulation) in the continuously-fed bioreactor led to propionate production and CE inhibition (Kucek et al., 2016a). It was suggested that maintaining a low residual lactate concentration in the bioreactor was necessary for effective CE. However, monitoring residual lactate concentration in the bioreactor may pose a major challenge for future lactate-based carboxylate platform. Firstly, it would require the implementation of a continuous lactate concentration monitoring system. Secondly, the potential of the substrate might not be fully exploited, for instance Xu et al. (2018) diluted the lactate-rich influent to avoid lactate overloading in CE bioreactor.

In a long-term lactate-based CE (B1, days 0-118, Paper 1) it was demonstrated that caproate production from lactate as a sole carbon source is possible without the acrylate pathway activation despite the occurrence of lactate overloading, however, lactate overloading induced disturbances in caproate production. 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 such as butyrate, valerate, caproate, and heptylate. 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 (dominant) 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). Moreover, no *Megasphaera elsdenii* was found in the microbiome structure which could also contribute to the lack of acrylate pathway activation as a result of lactate overloading. *Megasphaera elsdenii* is a well-characterized lactate-based caproate producer, however, it is also

capable of propionate production mainly in the presence of excess lactate (Prabhu et al., 2012).

Although the production of propionate was not demonstrated as a result of lactate overloading, periodic fluctuations in caproate production were observed, i.e. alternately decrease in caproate production along with lactate accumulation and increase in caproate production along with lactate consumption 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. Butyrate may also act as a desirable electron EA in CE. It was successfully used as an EA in batch studies with isolated lactate-based chain elongator *Ruminococcaceae* bacterium CPB6 leading to an increase in caproate yields compared to the trial where acetate was used as an EA (Zhu et al., 2017). On the other hand, MCF studies indicated that butyrate was less efficient EA than acetate for lactate-based CE (Wu et al., 2018). Similarly in B1 in Paper 1, although lactate and butyrate were detected, the availability of acetate was crucial for lactate-based caproate production. 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 (and lactate consumption). 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 can be 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 compared to B1. The first symptoms of lactate overloading in B1 were already observed at LLR of 120 mmol C/L/d. In comparison, in acetated B2 no residual lactate accumulation was observed until LLR reached 270 mmol C/L/d. The increase of LLR by 50% in the acetated B2 in phases I and II boosted caproate production causing its improvement by 57% and 64%, respectively (Fig. 4, Paper 1). Interestingly, raising LLR affected only caproate production (Fig. 2, Paper 1). Lactate overloading was observed in the acetated B2 as an effect of increase of LLR in phase III and resulted in an initial decrease in caproate production and residual lactate accumulation. Continuous and stable caproate production was restored after a few days despite the lactate overloading, 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. 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). On the other hand, acetate supplementation provided a change in the LLR limit above which the lactate was accumulated.

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.

Moreover, hydrogen production was observed when acetate was supplemented at high LLR (in phase IV in B1 and in phase III in B2; Fig. 3, Paper 1). Hydrogen production is directly related to CE performance (Cavalcante et al., 2017), however, no hydrogen production (or insignificant amounts) were observed at lower LLRs and the gas mixture consisted mainly of carbon dioxide and methane. Hydrogen produced during CE could be consumed by the hydrogenotrophic methanogens (which were identified in B1 and B2 microbiome structure) or used as a co-electron donor for the CE. Hydrogenotrophic methanogens, which are capable to produce methane from hydrogen and carbon dioxide,

were also previously found in CE systems, because of no direct competition with chain elongators for the substrate (Grootscholten et al., 2014). Lactate and acetate were used for hydrogen production before (García-Depraect et al., 2019a; Tao et al., 2016), as well as a high organic loading rate also was recognized before as an important factor for successful lactate-driven hydrogen production (García-Depraect et al., 2019b).

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. Previous ethanol-based CE studies showed that the ethanol to acetate ratio in substrate influenced caproate production efficiency (Liu et al., 2016; Yin et al., 2017). Moreover, it was also shown that the ethanol to acetate ratio had an impact on carboxylate selectivity, resulting in an increase in butyrate specificity at lower ratios or an increase in caprylate specificity at higher ratios (Spirito et al., 2018). Furthermore, it also may determine the MCCs production strategy, i.e. two main strategies for MCCs production were proposed previously (Wu et al., 2019): one-stage production (production of MCC in one bioreactor) or two-state production (prior accumulation of EDs and/or EAs followed by MCC production). It has not yet been demonstrated whether a similar effect of ED to EA ratio can be observed in lactate-based CE. Scarborough et al. (2020) prepared a metabolic MCC-producing model (iFermCell215) simulating the co-utilization of lactate and acetate in various configurations. Modeling results predicted that various carboxylates including butyrate, caproate, and caprylate are produced at high L:A ratios, but as the L:A ratio was lowered, the MCCs production decreased along with an increase in butyrate production. Butyrate was predicted as the sole product at L:A ratios of 1.56 mM C/mM C and lower (own calculations based on acetate-to-lactate ratio provided by Scarborough et al. (2020)). 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). Moreover, in the first MCF studies describing the synthesis of caproate from lactate as a sole carbon source, the pH ranged between 6.0 and 6.5 (Zhu et al., 2015). 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, various carboxylates (from acetate to caprylate) were produced when lactate was used as a sole carbon source, but caproate production dominated (Fig. 2, Paper 2). Similar effect of conversion of lactate as a sole carbon source was presented in Paper 1. 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

Various waste containing lactate could be great sources of ED for CE, however other organic compounds present in a waste would also potentially affect the CE. One of the most popular lactate-based waste feedstock is AW from the dairy industry (Xu et al., 2018). There are two types of whey: sweet whey, which is rich in protein and has a pH of around 6-7 and is used as a food ingredient after being processed to whey powder. On the other hand, AW has a more acidic pH of around 4-5, and its drying for food/feed purposes is more challenging due to the conversion of lactose into its crystalline structure. Special additives could be added to AW for further processing, however, AW due to the high content of fermentable lactate and lactose and high COD, ranging from 60 000 to 80 000 mg/L (Duber et al., 2018; Lievore et al., 2015; Macwan et al., 2016), could be desirable feedstock for fermentation processes, e.g. CE processing (Rocha-Mendoza et al., 2021).

Firstly, batch studies with synthetic medium were conducted to examine the effect of lactose in substrate composition on lactate-based CE (Paper 3). For this purpose, several configurations of lactose, lactate and acetate concentrations in the feedstock were applied as it was previously presented in Tab. 1. 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. The impact of lactose on product selectivity was

also described in the latest CE studies (Duber et al., 2022) showing that higher initial lactose loading improved lactate-based CE.

The co-production of liquid and gaseous valuable compounds, such as caproate and hydrogen, can increase the economic viability of MCF, however, its generation mustn't be mutually exclusive. MCF is characterized by a large diversity of microorganisms that compete with each other for the substrate (Oleskowicz-Popiel, 2018). 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). As mentioned before, hydrogen production is associated with a lactate-based CE, however, hydrogen is mainly formed in a dark fermentation (DF) (Villanueva-Galindo et al., 2023). During DF some microbial communities can produce hydrogen and carbon dioxide from carbohydrates (e.g. glucose, lactose), proteins, and lipids. The combination of DF and CE could be a promising solution for biowaste valorization bringing mutual benefits for both processes. Previously, hydrogen has been successfully used as a co-substrate to improve the performance of CE, as demonstrated by Baleeiro et al. (2021). Acetate and butyrate which are by-products of DF are desirable EAs for caproate production in lactate-based CE (Wu et al., 2019). Furthermore, the co-occurrence of lactate-based CE and DF is microbiologically feasible, as lactate-based chain elongators and dark fermentation bacteria do not appear to compete for the same substrate. A recent study by Zagrodnik et al. (2022) demonstrated that efficient hydrogen production from a sugar mixture involved the coexistence of hydrogen-producing bacteria, such as *Clostridium beijerinckii* and *Clostridium guangxiense*, with *Caproiciproducens* sp. in a mixed microbial community structure. Moreover, both hydrogen producers and chain elongators have also a common "foe" in microbiome structure, i. e. propionate producer (Candry et al., 2020; Castelló et al., 2020).

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%). Odd-numbered carboxylates (mainly propionate and valerate) were also produced. 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, however, this cannot be confirmed because microbiome analysis was not performed in the trials presented in Paper 3. 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 and the production of odd-numbered carboxylates successively decreased until it was almost completely inhibited. Moreover, redirection in gaseous production was also observed. Methane production was almost completely inhibited and hydrogen production appeared. Previously, Grootscholten et al. (2013) also observed the inhibition of acetoclastic methanogenesis as an effect of reducing HRT. 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 first accumulation of lactate occurred at the beginning of stage II (the HRT was changed) and it could be attributed to the adjustment to the new LLR (a similar effect was previously observed in Paper 1). A second 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). It is worth noting that although the lactate-overloading was continuously observed, caproate was still the dominant product as in B2 in Paper 1.

4.5. Butyrate-caproate competition in lactate-based CE

It is very challenging to control the metabolic pathways in MCF because of large variety of microorganisms involved in bioprocess (Oleskowicz-Popiel, 2018). The most popular management strategy of MCF is to control the operational parameters such as pH, temperature, and HRT (De Groof et al., 2019). Moreover, initial mixed culture selection by choosing the inoculum enriched in desired microorganisms as well as a special inoculum pretreatment to eliminate specific groups of microorganisms is also often performed in MCF (Camargo et al., 2023; Luo et al., 2022). The use of the aforementioned techniques were also executed in the CE to avoid the main competitors of chain elongators, such as methanogens and propionate producers (Dong et al., 2023; Li et al., 2023; Zhu et al., 2015). However, 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 (after 65 days of process). 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. Therefore, the impact it might have on the future carboxylate platform-based biorefineries is yet to be determined.

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. Both batch trials and long-term continuous processes were carried out. Concentrations of metabolites were monitored by analytical methods in line with the latest standards. To determine the relative microbial composition in reactors' microbiomes, a 16S rRNA gene sequencing for microbiome analysis was carried out. 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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PAPER 1

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Effect of external acetate on lactate-based carboxylate platform: Shifted lactate overloading limit and hydrogen co-production

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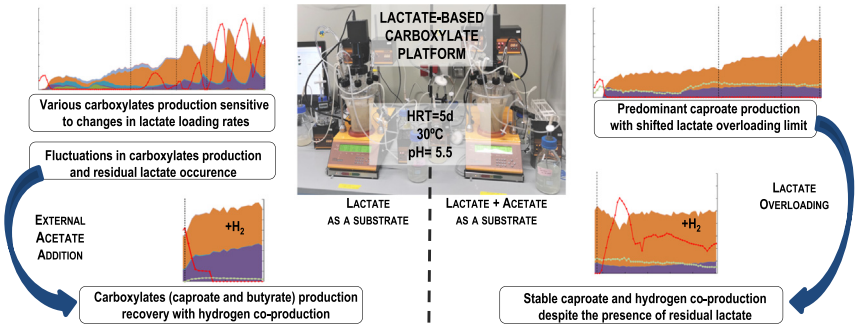
HIGHLIGHTS

- Co-production of caproate and hydrogen in the lactate-based chain elongation
- External acetate shifted lactate overloading limit and favored caproate production.
- Fluctuations in carboxylates production due to a low acetate availability
- Acetate addition to lactate-overloaded bioprocess recovered carboxylates production.
- Electron acceptor was identified as a limiting factor for caproate production.

GRAPHICAL ABSTRACT



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ABSTRACT

Chain elongation is an anaerobic biotechnological process that converts short chain carboxylates and an electron donor (e.g. ethanol, lactate) into more valuable medium chain carboxylates. Caproate production in lactate-based chain elongation is gaining popularity, however, the relation between lactate (electron donor) and acetate (electron acceptor) has not yet been fully elucidated. Herein, for the first time, the effect of an external acetate on the lactate-based chain elongation in a continuously-fed bioreactor was tested to verify how the external acetate would affect the product spectrum, gas production, as well as stability and efficiency of carboxylates production. Periodic fluctuations in caproate production were observed in bioreactor continuously fed with lactate as a sole carbon source due to the lack of an electron acceptor (acetate) and low chain elongation performance. The recovery of stable caproate production (68.9 ± 2.2 mmol C/L/d), total lactate consumption, and high hydrogen co-production (748 ± 76 mL_{H2}/d) was observed as an effect of the addition of an external acetate. The lactate conversion with the external acetate in the second bioreactor ensured stable and dominant caproate production from the beginning of the process. Moreover, despite the continuous lactate overloading in the process with external acetate, stable caproate production was achieved (71.7 ± 2.4 mmol C/L/d) and previously unobserved hydrogen production occurred (213 ± 30 mL_{H2}/d). Thus, external electron acceptor addition (i.e. acetate) was proposed as an effective method for stable lactate-based caproate production. Microbiological analysis showed the dominance of microbes closely related to *Ruminococcaceae* bacterium CPB6 and *Acinetobacter* throughout the process. Co-occurrence networks based on taxon abundances and process parameters revealed microbial sub-networks responding to lactate concentrations.

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1. Introduction

Population growth and consumerism affect the natural environment (Sanders and Langeveld, 2020). The response to global climate change, environmental pollution, and increasing consumption of fossil fuels is the development of a bio-based economy based on biotechnological processes (Van Schoubroeck et al., 2018). The traditional methods of dealing with organic-rich industrial wastewater lead to the loss of a valuable source of carbon. The utilization of agro-industrial wastewater during mixed (open) culture fermentation (MCF) ensures the production of added-value chemical compounds and environmentally friendly waste management. Commercialized and widespread technology based on MCF is anaerobic digestion (AD). AD is the process by which organic matter is converted into methane-rich biogas that can be used to generate heat and electricity (Karki et al., 2021). However, despite advances in the engineering of AD, the process has so far struggled with economic viability. It was estimated that 2 euro worth of compost or 76 euro worth of biogas could be obtained through AD from one ton of food waste (De Groof et al., 2019). Therefore, a new spectrum of products is being sought and more and more attention is paid to newly developed carboxylate platforms, leading to the formation of medium chain carboxylates (MCCs). Caproate, containing 6 carbon atoms in the molecule, due to its wide industrial application, is one of the most popular MCC. It can be used directly as a food additive and antibacterial agent, as well as in the production of lubricants, gums, dyes, pharmaceuticals and it can be a precursor for jet fuel production (Spirito et al., 2014).

Caproate in carboxylate platform can be formed by the CE through the fatty acid biosynthesis (FAB) pathway or more widespread cyclic reverse β-oxidation (RBO) pathway (Angenent et al., 2016; Han et al., 2018). Chemical compounds that are electron donors play a key role in the RBO pathway. An electron donor is oxidized providing acetyl-CoA to the RBO cycle, in which in a series of enzymatic reactions the carbon chain of the carboxylate (electron acceptor) is always elongated by two carbon atoms, e.g. acetate is elongated to butyrate and butyrate to caproate. So far ethanol is the most recognized electron donor for MCC production (Han et al., 2019). Nevertheless, many researchers are starting to turn their attention to other promising compounds like lactate, which is contained in waste from the food and agricultural industry, e.g. acid whey from the dairy industry, grass silage juice, and maize silage. Zhu et al. (2015) conducted the process of converting lactate to caproate for the first time. Lactate oxidation provided the necessary acetyl-CoA as well as acetate, thus there was no need to supply an external electron acceptor for caproate production. However, there are some limitations of lactate-based carboxylate platform affecting production stability, such as competitive metabolic pathways (methanogenesis and acrylate pathway) and lactate overloading as a result of the occurrence of residual (excess) lactate in the bioreactor, which may cause a decline in the MCC production and the acrylate pathway activation (Kucek et al., 2016a). Most of the research so far has focused on the effects of operational parameters (pH, temperature, and hydraulic retention time (HRT)) and the competition between microorganisms, but no studies have been reported on the influence of external acetate on the continuous lactate-based CE in MCF. Increased caproate production can be expected with the addition of external acetate, as well as lower lactate consumption per one mole of produced caproate. The addition of acetate in batch conversion of lactate to caproate with isolated *Ruminococcaceae bacterium* CPB6 resulted in a slight decrease (about 11%) in lactate consumption per one mole of produced caproate (Zhu et al., 2017). However, it is not known how the addition of external acetate would affect the product spectrum, the stability and efficiency of carboxylates production, and the residual lactate occurrence in continuous MCF.

Moreover, in our recent research, we have proposed product diversification by co-production of caproate and hydrogen which can be a mutually beneficial solution increasing economic viability of the carboxylate platform (Brodowski et al., 2020). Hydrogen is expected to play a key role in future energy systems and is considered to be one of the most promising energy carriers (Bundhoo and Mohee, 2016). Although lactate is not considered a suitable substrate for hydrogen production (Baghchehsaraee et al., 2009), it was shown that it can be produced from lactate as the sole carbon source (Ohnishi et al., 2012). Most of the research focused on producing hydrogen from lactate used acetate as an additional carbon source. Lactate acted as an electron donor and its presence determined the production of hydrogen, but the presence of acetate, which served as an oxidant agent, was shown to be essential to avoid hydrogen production inhibition (García-Depraect et al., 2019a; Tao et al., 2016; Wu et al., 2012). In addition, the main by-product of biological hydrogen production from lactate and acetate was butyrate (Tao et al., 2016), which can be used by chain elongators to produce caproate. Thus, the addition of external acetate may not only affect the conversion of lactate to MCC, but also the production of hydrogen.

The main objective of the study was to investigate how the addition of an external electron acceptor (acetate) would affect the conversion of lactate to MCCs and hydrogen co-production in a continuous process. The process was carried out in two continuous stirred-tank reactors (CSTR); the first one was fed only with lactate, whereas the second one was fed with lactate and acetate. Both were fed until they were overloaded. External acetate was added to the first bioreactor once overloading was achieved to examine external acetate addition on the lactate overloading. Microbial community analysis was conducted to shed a light on the external acetate influence on lactate-based chain elongation. Co-occurrence networks based on taxon abundances and process parameters and time were investigated for inferring the possible ecological interactions and potential metabolic functions.

2. Material and methods

2.1. Medium and inoculum

A synthetic medium was used in the experiment. The medium was prepared based on Grimalt-Alemany et al. (2018) with the following modifications: 10 mL/L of the 1 M K_2HPO_4 solution and 15 mL/L of the 1 M KH_2PO_4 solution were added to the medium. Lactate and acetate solutions were the carbon sources.

The sludge obtained from a 1 L upflow anaerobic sludge blanket (UASB) reactor producing caproate in the CE process from acid whey and located at the Poznan University of Technology was used as the inoculum (Duber et al., 2018). The sludge was prepared as follows: 80 mL (per one bioreactor) of UASB sludge was collected, then centrifugated, washed, resuspended in 5 mL of 0.9% *w*/*v* NaCl solution, and added to each bioreactor.

2.2. Bioreactor system

Two 1 L Lambda Minifor fermenters (LAMBDA Instruments GmbH, Baar-Switzerland) with automatic pH, temperature, agitation monitoring, and a control system were used (Fig. 1). The agitation system was based on the up-and-down movement of stirring discs. The weighing module was used to set and maintain a constant weight of the vessel (the outflow pump was activated when the preset weight was exceeded). IR radiation heater, which was placed at the bottom of the vessel, was used for temperature adjustment. The InPro 3253 pH electrode (Mettler-Toledo International Inc., UK) combined with a temperature sensor was used for in-line measurements. The reactor was equipped with four peristaltic pumps (medium inflow, effluent outflow, acid and base inflows). Hydrochloric acid (0.5 M) and sodium hydroxide (2 M) were used to maintain the constant pH. The pH was continuously monitored by an automatic pH control system (microprocessor) built into a bioreactor control unit. Based on the difference between the actual value and the set value, the base or acid was automatically pumped into the bioreactor. The gas production was quantified using a volumetric gas flow meter (Ritter, Germany).

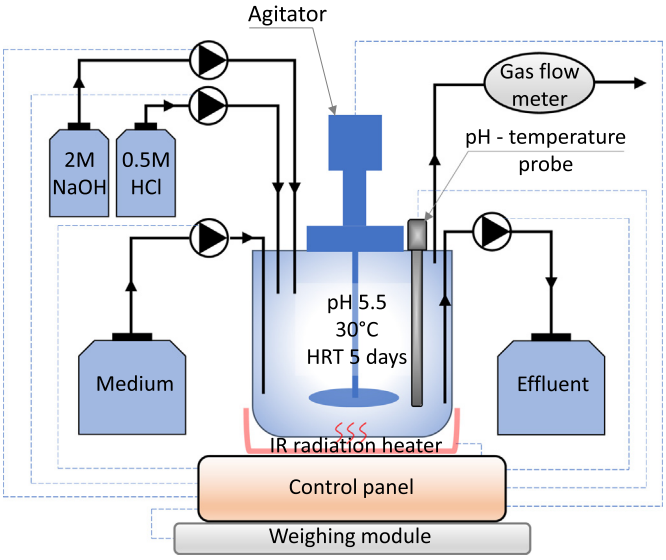

Fig. 1. Scheme of bioreactor system.

2.3. Operating conditions and process strategy

The process was divided into different phases based on the concentration of lactate and acetate in the feedstock as specified in Table 1. For the first bioreactor, denoted as B1, the process was divided into the start-up phase and phases from I to IV. The lactate concentration in the feedstock was 400 mM C in the start-up phase, and then, in each of the next phases (I-III), it was increased by 50% compared to the previous one. In the last phase (IV), where lactate concentration was maintained at the same level as in the phase III, the acetate in an amount of 200 mM C was added to the medium. In the second bioreactor, denoted as B2, the process was divided into a start-up phase and phases from I to III. Initially (start-up phase) the concentrations of lactate and acetate in the feedstock were 400 mM C and 200 mM C, respectively. Then (phases I- III) the lactate concentration in the feedstock was increased by 50% compared to the previous phase with a constant concentration of acetate. The duration of the phase III in B2 (days 89–140) corresponded to the duration of the phase III (89–118) and the phase IV (119–140) in the B1. There were no gas measurements at the beginning of the process due to technical problems with gas flow meters, on day 32 the gas flow meters (Ritter, Germany) were reconnected.

The process was initiated using a diluted medium with distilled water (the ratio of medium to water was 1:1 [vol:vol]) and the inoculum. The working volume of each of the bioreactor was 0.8 L. Nitrogen gas was used to flush fermenters to ensure anaerobic conditions (at the start of the process and on the day 32 after reconnecting the gas flow meters). The agitation frequency was set at 1 Hz. The process was carried out with a constant temperature of 30 °C and a constant pH of 5.5. The HRT was maintained at 5 days.

2.4. Analytical techniques and calculations

Analysis of gas composition (methane, carbon dioxide, and hydrogen) was performed using the Shimadzu GC-2014 gas chromatograph equipped with the Porapak N packed column and the TCD detector, under isothermal conditions. Nitrogen at a flow rate of 15 mL/min was used as the carrier gas. The temperatures of the injector, column and detector were 110 °C, 50 °C and 80°, respectively. Organic acids and alcohols concentrations were monitored by a gas chromatography with FID detector (Shimadzu GC-2014 equipped with Zebron ZB-FFAP column) using helium as the carrier gas and a ramp temperature program (initial temperature 70 °C for 2.5 min; temperature ramp 11 °C per min to 185 °C; final temperature 185 °C for 10 min). The injection port and detector were set to 250 °C. The concentration of lactate was monitored with a high-performance liquid chromatography (Shimadzu LC-20, Rezex ROA-Organic Acid column, RI detector). The detailed analytical procedure was described in (Zagrodnik et al., 2020). Equations for the analysis (i.e. carboxylates production rates and product specificities) were as described in Duber et al. (2020). Product-to-carboxylates specificities were calculated as the ratio of the production rate of a specific carboxylate to the production rate of all detected carboxylates.

2.5. Microbiome analysis

Collected biomass samples were centrifugated and stored frozen at −20 °C until processing. The denotation of the sample indicates the bioreactor (B1 and B2) and the day of the collection. On days 107 and 112, samples (B1d107 and B1d112) were collected only from a B1 to study the microbiome structure during fluctuations in the residual lactate concentration and the carboxylate production in the phase III. Total metagenomic DNA was isolated using GeneMATRIX Soil DNA Purification Kit (EURx, Poland) according to the manufacturer's recommendations. ZymoBIOMICS Microbial Community Standard D6300 (Zymoresearch, USA) was used as a mock microbial community to evaluate the analysis pipeline. Amplification of V3 and V4 region of 16S rRNA gene was carried out with Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/μL) and primers 341F (5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 785R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). The PCR reaction was carried out with 25 cycles and an annealing temperature of 50 °C. The resulting libraries were submitted to Macrogen (Korea) for index PCR and further processing according

Table 1
Process strategy with lactate and acetate concentrations in the feedstock.

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 in the feedstock [mM C]	400	600	900	1350	1350
Acetate concentration in the 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 in the feedstock [mM C]	400	600	900	1350
Acetate concentration in the 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

to 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, Part # 15044223, Rev. B) with Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 and sequencing using Illumina Miseq instrument (300 bp paired-end sequencing, Miseq v3).

The resulting paired reads were trimmed (the last 30 bp of forward and 60 bp of reverse reads) and merged using USEARCH (Edgar, 2013) and primer sequences stripped off (sequences that did not contain the primer sequences were filtered out) using cutadapt (Martin, 2011). USEARCH pipeline was subsequently employed for quality filtering, finding unique sequences, generation of OTUs by clustering with 97% identity (UPARSE algorithm), and OTU-table generation. Taxonomy was assigned to OTUs using SINTAX (Edgar, 2016) and Silva LTPs v132 database (Yilmaz et al., 2014) containing 13,899 curated 16S ribosomal RNA sequences (taxonomic ranks below 80% USINTAX bootstrap confidence threshold measure were considered unclassified). OTU table was rarified without replacement to the sample with the minimal count of mapped reads (49869). Downstream analyses were carried out using Phyloseq package (McMurdie and Holmes, 2013). Moreover, co-occurrence networks were inferred based on the microbial composition data and the abiotic parameters using CoNet App (v1.1.1 beta). OTUs with <0.1% relative abundance or presence in less than three samples were not included in the analysis. The following data and process parameters were included: concentrations of investigated extracellular metabolites, CO_2, H_2, and CH_4 content, gas amount, lactate, and acetate concentrations in the feedstock. Pearson, Spearman and Kendall correlation coefficients were computed and p-values determined by bootstrapping (1000). If at least one method featured a coefficient below −0.8 or above 0.8, an edge connecting the corresponding OTUs or abiotic parameters and q-value after Benjamini-Hochberg correction <0.05 threshold after Benjamini-Hochberg correction was added to the network. The network was visualized and analyzed for topological features in Cytoscape software (v 3.7.1) (Shannon et al., 2003).

Raw sequences obtained in this study were submitted to NCBI Sequence Read Archive (SRA) database and are available under BioProject ID PRJNA694955 (BioSample accessions SAMN18336565 -SAMN18336580).

3. Results and discussion

The carboxylates production along with the residual concentration of substrates (lactate and acetate) and the gas production during the process were presented in Figs. 2 and 3, respectively. In Fig. 4 the relationship between the caproate production and the lactate concentration in the feedstock during the process with external acetate (phase IV in B1, start-up phase and phases from I to III in B2) and without external acetate (start-up phase and phases from I to III in B1) was shown. Possible pathways and its stoichiometries were placed in Electronic Supplementary Material.

3.1. External acetate influenced the production of carboxylates from the beginning of the process

At the beginning of the process in B1, various carboxylates were produced with a chain length of 2 to 7 carbon atoms per molecule. The production of carboxylates with an odd number of carbon atoms per molecule (propionate, valerate and heptanoate) in B1 was predominant on days 14–36 (12.2 ± 2.2 mmol C/L/d) with a specificity of 61 ± 8%. Then, the production of caproate started to dominate and reached a value of 23.4 ± 1.3 mmol C/L/d on days 41–48 with a specificity of 63 ± 2%. In the meantime, the production of carboxylates with an odd number of carbon atoms per molecule decreased to 9.6 mmol C/L/d (specificity of 26%).

In B2, the stabilization of caproate and butyrate production was observed already after 10 days of the start-up phase. The caproate and

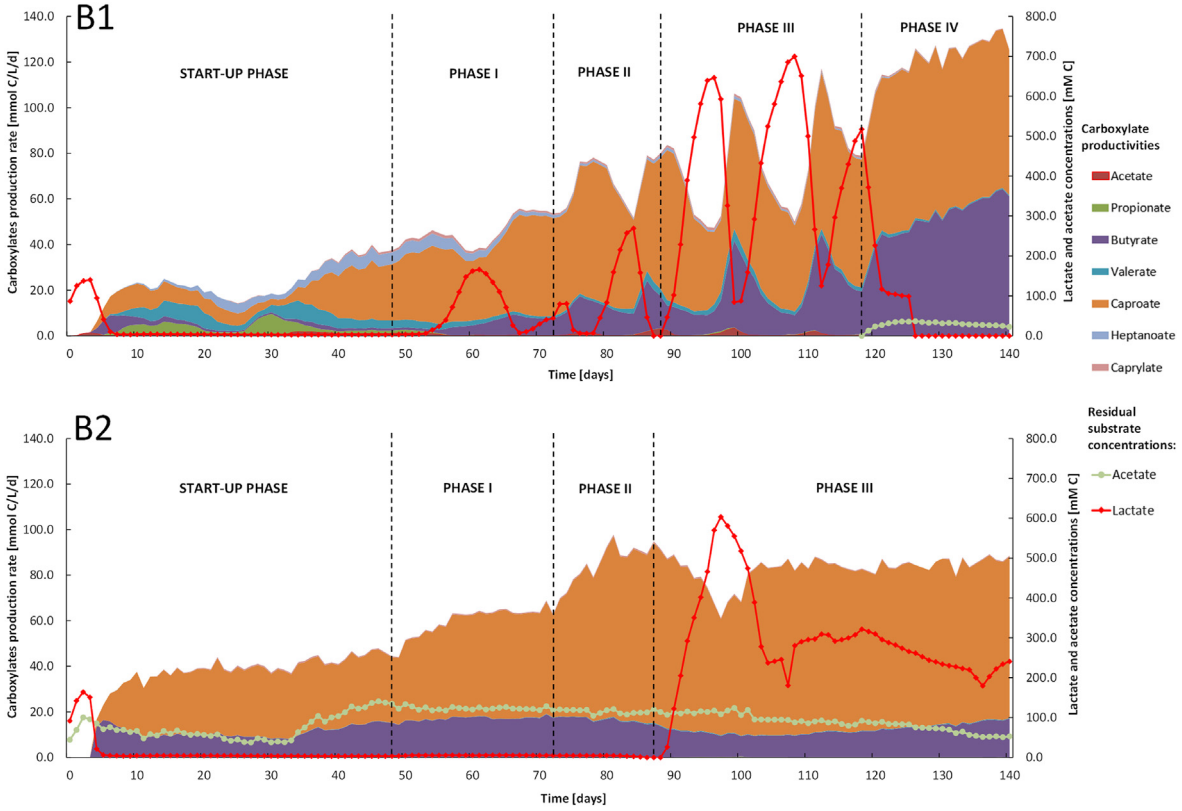

Fig. 2. The production rate profile of carboxylates with the residual substrate concentration in the bioreactor 1 (B1) and in the bioreactor 2 (B2). In B1, the concentration of acetate as the residual substrate concentration was shown only in the phase IV. In the earlier phases, acetate was not a substrate and was shown as a carboxylate product (its concentration in the feedstock was 0 mM C in start-up phase and phases I–III). In B2, lactate and acetate were listed as substrates throughout the process.

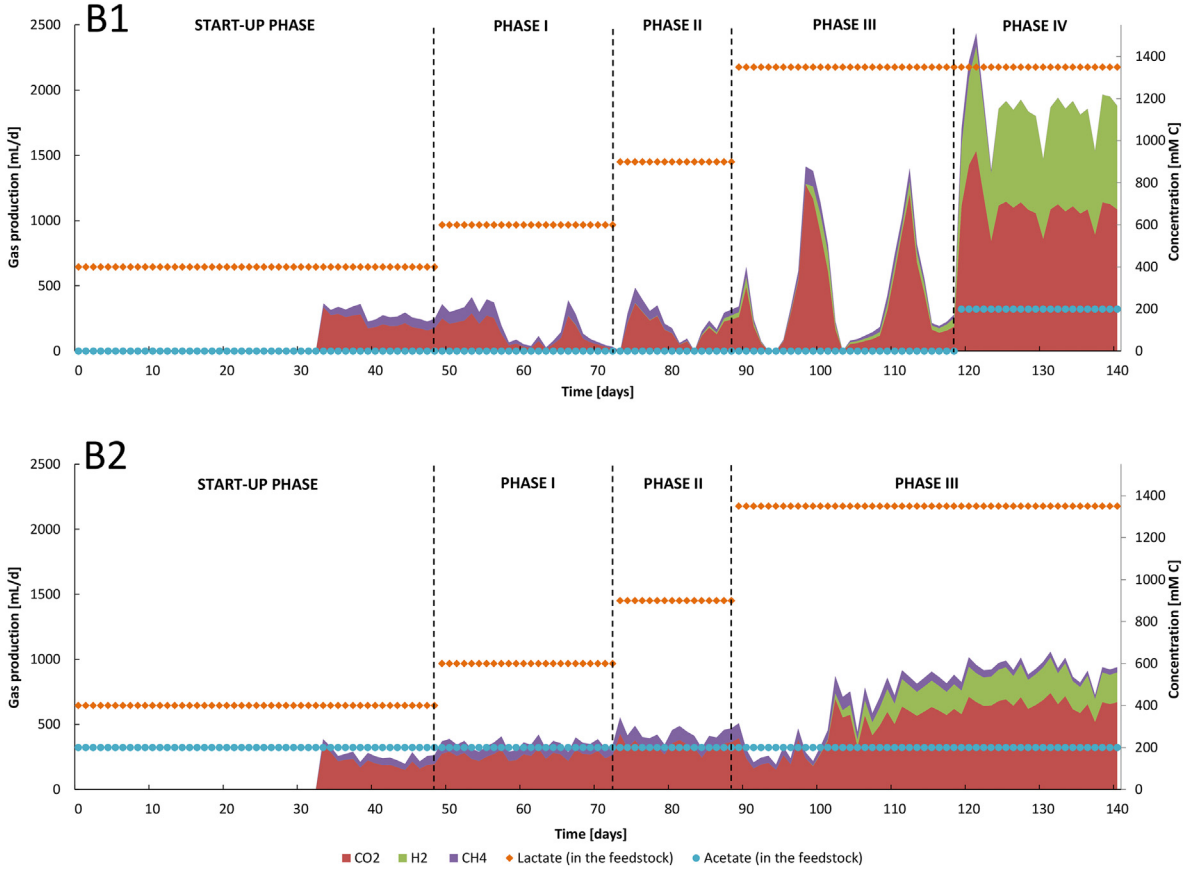

Fig. 3. Gas production with lactate and acetate concentrations in the feedstock in the bioreactor 1 (B1) and the bioreactor 2 (B2).

butyrate production on days 11–48 amounted to 29.1 ± 1.9 mmol C/L/d and 10.9 ± 2.4 mmol C/L/d, respectively. The acetate concentration in B2 on days 11–33 was 51.6 ± 9.7 mM, then it increased and at the end of the start-up phase it was 134.5 mM C. It is worth noting that carboxylates with an odd number of carbon atoms per molecule were not detected in B2.

The volumetric amounts of gaseous products in B1 and B2 in the start-up phase were 286 ± 46 mL/d and 263 ± 43 mL/d, respectively. The gas mixture consisted of carbon dioxide (77 ± 6% in B1 and 79 ± 5% in B2) and methane (23 ± 6% in B1 and 21 ± 5% in B2). Acetoclastic methanogens should not be active in pH of 5.5 but methane could be also produced in the carboxylate platform by hydrogenotrophic methanogens which were identified in microbiome structure (Electronic Supplementary Material). Hydrogenotrophic methanogens generating methane from H_2 and CO_2 and chain elongators can be present simultaneously in the carboxylate platform because there is no direct competition for the substrate (Grootscholten et al., 2014).

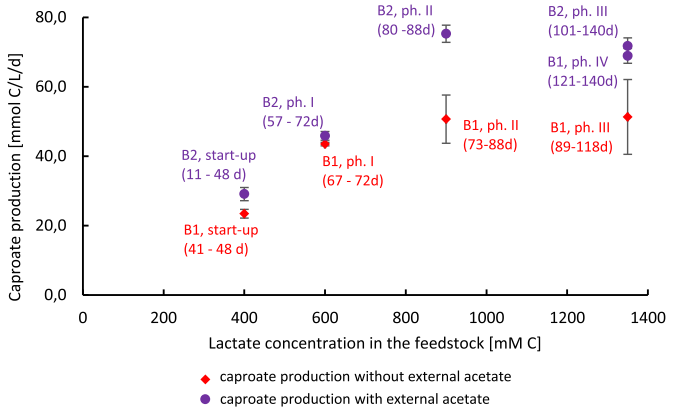

Fig. 4. The relationship between the caproate production and the lactate concentration in the feedstock. The denotation of the point indicates the bioreactor (B1 and B2), phase and the days of the caproate production under steady-state conditions (except for B1, ph. III and B1, ph. IV, where the entire duration of the phase was assumed because stable production of caproate was not achieved).

It is very challenging to control the metabolic pathways and final bioproducts in MCF (Oleskowicz-Popiel, 2018). The CE processes using mixed cultures of microorganisms are characterized by competition between chain elongators and propionate producers (Candry et al., 2020). Most of the studies focused on the lactate-based CE to caproate also showed the production of carboxylates with an odd number of carbon atoms in the molecule (Brodowski et al., 2020; Kucek et al., 2016a; Xu et al., 2018). However, the comparison of the results in the start-up phase between the B1 and B2 suggested that external acetate favored the production of carboxylates with an even number of carbon atoms in the molecule with the predominant caproate production. The lack of an external electron acceptor in the inflow to the B1 made it necessary to produce it directly from lactate. Acetate and propionate, which are electron acceptors in the CE, can be produced from lactate. Lactate can be oxidized to pyruvate with lactate dehydrogenase, then pyruvate can be further oxidized to acetyl-CoA, which can be finally converted to acetate. On the other hand, propionate can be also generated from lactate in the acrylate pathway with lactyl-CoA, acrylyl-CoA and propionyl-CoA as intermediates (Wu et al., 2019). These two metabolic pathways are competing with each other and the lack of an external electron acceptor may have triggered the activation of both competing pathways making the product spectrum more diverse in the B1.

The microbiome structure analysis shed more light on the changes during the carboxylates production in the start-up phase of B1, as well as the differences between the carboxylates production in B1 and B2

(no carboxylates with an odd number of carbon atoms in B2). Microbes closely related to *Ruminococcaceae* bacterium CPB6 and *Acinetobacter* were recognized as the dominant in periods of high caproate production. *Ruminococcaceae* bacterium CPB6 was previously identified as a lactate-based chain elongator and caproate producer (Zhu et al., 2017) and *Acinetobacter* was functional microorganisms for the CE (He et al., 2018; Kucek et al., 2016a). At the beginning of the start-up phase (day 22) in B1, when the production of carboxylates with an odd number of carbon atoms in the molecule was predominant, the relative abundance of *Ruminococcaceae* bacterium CPB6 and *Acinetobacter* were only 10.0% and 1.3%, respectively. However, at the end of the start-up phase (day 48), when caproate production had been already predominant, *Ruminococcaceae* bacterium CPB6 and *Acinetobacter* dominated the microbiome structure, reaching relative abundances of 23.1% and 36.9%, respectively. Acetate and propionate compete for the same enzyme system in the CE process but with the acetate dominance (Roghair et al., 2018) which could explain the shift in the production trend towards caproate at the end of the start-up phase in the B1. For comparison, in the B2, the relative abundance of *Ruminococcaceae* bacterium CPB6 and *Acinetobacter* was almost 50% in total already on the day 22, which may explain the phenomenon of predominant caproate production from the beginning of the process. Additionally, the presence of *Propionibacterium* capable of producing propionate was observed during the start-up phase only in the samples from B1 (the relative abundance was between 0.49%–0.55%; Electronic Supplementary Material), which could explain the production of carboxylates with an odd number of carbon atoms in the molecule only in the B1. However, the exact mechanism of complete propionate production inhibition in the process with external acetate remains elusive.

3.2. Lack of an external electron acceptor led to the occurrence of residual lactate and destabilization of the carboxylates production

At the beginning of phase I in the B1 (lactate concentration in the feedstock increased to 600 mM C), a gradual increase in the caproate production to 33.7 mmol C/L/d was observed; however, at some point (day 54), the reduction in the caproate production was observed and reached 26.0 mmol C/L/d on day 60 along with the residual lactate accumulation. Then the residual lactate consumption from day 61 translated into an increase in the caproate and butyrate production. The production of caproate and butyrate stabilized on day 67 and by the end of the phase I it was equal to 43.5 ± 0.5 mmol C/L/d and 7.7 ± 0.4 mmol C/L/d, respectively. Stable caproate and butyrate production were not achieved in the phase II in B1. Caproate production increased to 60.2 mmol C/L/d on day 78 but then decreased to 38.8 mmol C/L/d on day 84 along with an accumulation of the residual lactate. At the end of the phase II, the caproate and butyrate production increased to 57.5 mmol C/L/d and 14.3 mmol C/L/d, respectively, due to the observed residual lactate consumption on days 84–87. The production of carboxylates with an odd number of carbon atoms in the molecule gradually decreased in the phases I and II and it was 4.6 mmol C/L/d at the end of the phase II with a specificity of 6%. Gas production in the B1 was stable (227 ± 34 mL_{CO2}/d and 97 ± 15 mL_{CH4}/d) until the lactate accumulation occurred. From day 55, no stable gas production was achieved in the phases I and II in the B1.

The increase in lactate concentration in the feedstock in the phase III in the B1 resulted in periodic lactate accumulations combined with fluctuations in the carboxylates production. Three fluctuations were observed on days 89–99, 100–112 and 113–118, respectively. At the beginning of the phase III in the B1 a lactate overloading occurred (the residual lactate concentration increased up to 646.7 mM C on day 96) and the decrease in the caproate production from 65.8 mmol C/L/d (day 89) to 31.2 mmol C/L/d (day 97) was observed. Then the residual lactate consumption was observed and led to an increase in the caproate and butyrate production to 62.6 mmol C/L/d and 38.0 mmol C/L/d, respectively. On days 100–108, the caproate and butyrate production decreased to 37.7 mmol C/L/d and 8.5 mmol C/L/d, respectively, along with an increase in the residual lactate concentration to 699.9 mM C; however, the observed lactate consumption on days 109–112 led to another increase in the caproate and butyrate production to 68.6 mmol C/L/d and 43.4 mmol C/L/d, respectively. Another accumulation of the residual lactate and the decrease in carboxylates production were observed on days 113–118. Periodic carboxylates production and residual lactate accumulations also affected gas production. When an increase in the carboxylates production was observed, also a higher gas production was measured.

No such repeatable fluctuations in the residual lactate concentrations and the carboxylates production were observed in another CE process with a continuous lactate overloading (Kucek et al., 2016a). These periodic fluctuations could be attributed to a low CE performance due to the lack of an electron acceptor (acetate). The recovery of the carboxylates (caproate and butyrate) production was always observed after a slight accumulation of acetate (Fig. 2) and an increase in acetate accumulation affected high CE performance. Thus, herein the main limiting factor for CE was the limited availability of an electron acceptor. Moreover, in our study the residual lactate did not activate propionate production as it was previously observed (Kucek et al., 2016a). Some species are able to convert lactate to propionate via the acrylate pathway which can outcompete the acetyl-CoA formation under conditions of excess lactate (Prabhu et al., 2012), but also in the first fed-batch bioconversion of lactate to caproate authors did not report propionate production despite continued lactate excess (Zhu et al., 2015). Recent studies showed that a lower pH (5.0 and 5.5) favored the CE process, and chain elongators may be outcompeted by the propionate producers at pH above 6; however, it has been also suggested that pH was not solely responsible for community changes and the competition between chain elongators and propionate producers (Candry et al., 2020). Thus, the activation of the acrylate pathway is likely to be influenced by the unique composition of the microbial culture (Kucek et al., 2016a) and not always residual lactate may lead to the activation of this metabolic pathway. Interestingly, although no increased production of propionate was triggered, *Propionibacterium* was observed in B1 during lactate overloading (the relative abundance was between 0.44%–1.12%; Electronic Supplementary Material).

3.3. Shifted lactate overloading limit in B2: stable caproate and hydrogen co-production

Increasing the lactate concentration in feedstock in the phase I and phase II by 50% resulted in increases in the caproate production by 57% and 64%, respectively, despite the constant butyrate production and constant acetate presence in the bioreactor. The caproate production stabilized in the phase I and phase II at 45.8 ± 1.3 mmol C/L/d (days 57–72) and 75.3 ± 2.5 mmol C/L/d (days 80–88), respectively. Throughout both phases (days 49–88), the production of butyrate (16.7 ± 1.2 mmol C/L/d), as well as the concentration of acetate (119.8 ± 5.8 mM C) did not change significantly. The production of gases (CO_2 and CH_4) in the phase I and II was stable and amounted to 264 ± 28 mL_{CO2}/d, 86 ± 10 mL_{CH4}/d and 333 ± 43 mL_{CO2}/d, 97 ± 13 mL_{CH4}/d, respectively. No appearance of the residual lactate was noticed, although the lactate concentration in feedstock in the phases I and II in the B2 was the same as in the phases I and II in the B1.

Lactate accumulation was first observed in the B2 in the phase III. The maximum lactate concentration was 603.3 mM C and corresponded to the lowest caproate production in this phase (51.0 mmol C/L/d). On day 98, consumption of the residual lactate began, which resulted in an increase in the caproate production. The residual lactate concentration dropped to 180.5 mM C on day 107 and remained at the level of 179.5–315.5 mM C until the end of the process. Despite the presence of the residual lactate, the caproate production stabilized at 71.7 ± 2.4 mmol C/L/d on days 101–140. Along with the stable caproate

production recovery, the production of hydrogen in the bioreactor was observed and amounted to 213 ± 30 mL_{H2}/d on days 108–140.

Shifting the lactate overloading limit (compared to B1) was possible due to the presence of an external electron acceptor, as the carboxylates' production was dependent on the lactate concentration in the feedstock. In comparison, the limiting factor in the B1 was an availability of an electron acceptor, which had to be produced directly from the lactate and it caused the carboxylates' production fluctuations already in the phases II and III. After reaching the lactate overloading in the phase III in B2, the caproate production, although stabilized despite the presence of residual lactate, was slightly lower than in the previous phase (Fig. 4). Lactate concentration in the feedstock conditioned the increase in the caproate production in the B2 until the residual lactate occurred. Thus, the external acetate rather affected the process stabilization than the CE efficiency.

Interestingly, the production of hydrogen was observed in the B2 only under the lactate overloading conditions. Lactate-based CE reactions involve the release of hydrogen (Wu et al., 2019), however, if the reaction is coupled with hydrogenotrophic methanogenesis, produced hydrogen is immediately used (Duber et al., 2018; Grootscholten et al., 2014). In our previous work, hydrogen was also undetectable in the gas mixture (unlike methane) until HRT was shortened, which led to the inhibition of hydrogenotrophic methanogenesis and enabled hydrogen production (Brodowski et al., 2020). Moreover, recent studies showed that hydrogen could be used as a co-electron donor for the CE in the MCF (Baleeiro et al., 2021), as well as for the short-chain carboxylates production as precursors for the caproate production (González-Tenorio et al., 2020). It is possible that in our study, the sufficiently high lactate consumption led to the production of excess and non-consumed hydrogen which resulted in the hydrogen accumulation. In other studies, high organic loading rate also was recognized as an important factor for a successful lactate-driven hydrogen production (García-Depraect et al., 2019b).

3.4. The addition of external acetate to lactate-overloaded B1: carboxylates production recovery and hydrogen co-production

The increase in lactate conversion, as well as changes in the carboxylates and gas production were observed as a result of the addition of external acetate in the B1 in the phase IV. The decrease in residual lactate concentration to 117.1 mM C occurred at the beginning of the phase IV, then the residual lactate concentration was stable at 102.5 ± 2.9 mM C on days 122–125. From day 126 until the end of the process, the residual lactate was not present in the bioreactor. Stable caproate production in the phase IV was recovered and averaged 68.9 ± 2.2 mmol C/L/d on days 121–140. At the same time, the acetate concentration in the bioreactor stabilized at 30.6 ± 4.0 mM C (days 121–140). Changes in the lactate conversion also affected the butyrate production. A consumption of the residual lactate at the beginning of the process translated into an increase in butyrate production from 18.9 mmol C/L/d to 44.4 mmol C/L/d. Then, on days 122–125, a stabilization of the butyrate production was observed (44.2 ± 0.9 mmol C/L/d). From day 126, the butyrate production gradually increased and reached 64.3 mmol C/L/d on day 139. Tao et al. (2016), in studies with the newly isolated *Clostridium* cluster XIVa bacterium from the production of Chinese strong-flavored liquor, noticed that the external acetate may increase the lactate conversion at low pH. It was suggested that an external acetate addition may induce the expression of solventogenic enzymes, leading to an increased utilization of lactate and butyrate production. The effect of acetate on the increased activity of certain enzymes (coenzyme A transferase, acetate kinase and butyrate kinase) was also observed in studies with *Clostridium beijerinckii* NCIMB 8052 (Chen and Blaschek, 1999). Moreover, the use of an external electron acceptor may allow chain elongators to accumulate more ATP, thereby stimulating the CE (Contreras-Dávila et al., 2021).

As previously mentioned, hydrogen production is related to the CE performance, however, it can be completely consumed by the hydrogenotrophic methanogens and used as a co-electron donor for the CE. A slight hydrogen production was observed in the phase II (up to 31 mL_{H2}/d) and also in the phase III (up to 145 mL_{H2}/d). However, the addition of an external acetate in the phase IV caused the total lactate consumption and as a result, a high hydrogen production was observed, which on days 121–140 averaged 748 ± 76 mL_{H2}/d and accounted for about 41% of the total gas production. Moreover, a complete inhibition of methanogenesis was observed from day 123. Recent studies showed that high acetate concentrations may favor acidogens and the CE over methanogens (Cavalcante et al., 2020; Zhang et al., 2018) thereby the addition of an external acetate resulted in an acetate accumulation which could have an impact on the methanogenesis inhibition. Interestingly, the production of hydrogen and butyrate in the phase IV in the B1 and the phase III in the B2, despite the use of the same medium and operating parameters, was different. It can be a result of a different lactate consumption in both bioreactors. The microbiome in the B1, accustomed and adapted to operating with an excess lactate from day 54, achieved a higher lactate conversion efficiency which resulted in a higher production of butyrate and hydrogen than in the B2.

3.5. Summary of the effects of an external acetate on the lactate-based CE

Based on the results, the influence of an external acetate on the lactate-based carboxylate platform is presented in Fig. 5. The lack of an external acetate resulted in a variation in the carboxylates production at the beginning of the CE process (start-up phase, B1), as well as the need for electron acceptor generation directly from lactate, which made the CE process more sensitive to changes in lactate loading rates (the increase in lactate concentration in the feedstock resulted in the destabilization of carboxylates production; phases I – II, B1). Continuous lactate overloading of the process with lactate as a sole carbon source led to fluctuations in the carboxylates production dependent on the access (accumulation) of acetate (phase III, B1). The addition of an external acetate resulted in the residual lactate consumption and restored stable caproate production along with a stable hydrogen co-production occurrence (phase IV, B1). The lactate overloading phenomenon may pose a major challenge in future lactate-based carboxylate platforms. To avoid overloading, during the two-stage fermentation of acid whey to caproate, Xu et al. (2018) diluted the lactate-rich influent, but such actions may reduce the economic viability of the bioprocess. Herein, an effective method of recovering stable caproate production in lactate-based CE is provided – the use of an external acetate source.

In the lactate-based CE process with an external acetate, carboxylates with an even number of carbon atoms in the molecule were favored and the caproate production increased along with an increase in the lactate loading rate until the residual lactate occurred. Caproate production stabilized under continuous overloading conditions, however, its production was slightly lower in comparison to the previous phase with a lower lactate loading rate. Thus, the concentration of the electron donor in the inflow determined the effectiveness of the CE until reaching the limit value above which the lactate overloading appeared. Hydrogen co-production was only observed under high lactate consumption conditions. We presumed that the hydrogen produced in the earlier phases was immediately consumed by the hydrogenotrophic methanogens or could have been used as an a co-electron donor in the CE.

Summing up, the access to the external acetate (electron acceptor) ensured the stabilization of the CE process and its lack was a limiting factor for the lactate-based CE. However, the access to an external acetate caused only a slight increase in caproate production (Fig. 4) and a slight decrease in the lactate consumption per one mole of produced caproate. For comparison, in the phase I, 5.24 mol of lactate were required to produce 1 mol of caproate in the process with external acetate,

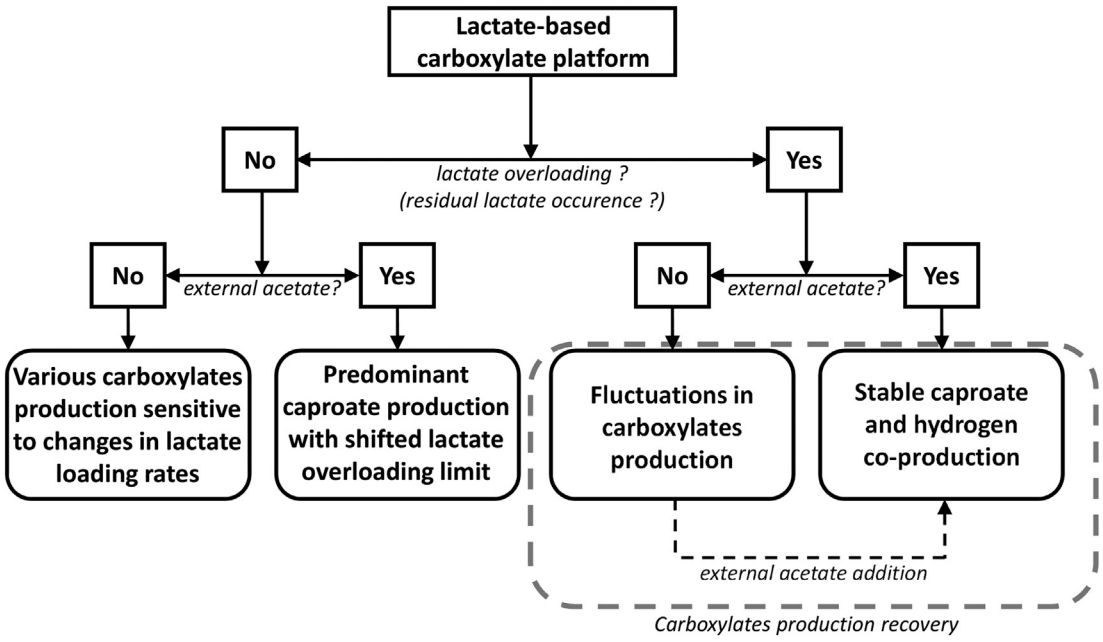

Fig. 5. Scheme of the influence of external acetate on lactate-based carboxylate platform.

while 5.52 mol of lactate were needed in the process without external acetate, which only resulted in a 5% lower lactate requirement per one mole of produced caproate. Similarly, in the batch tests with isolated *Ruminococcaceae bacterium* CPB6, the addition of external acetate caused a decrease from 4.29 to 3.83 mol of consumed lactate per 1 mol of produced caproate (decrease of about 11%) (Zhu et al., 2017). It is therefore desirable for the lactate-based CE process to provide access to acetate as an electron acceptor to avoid production fluctuations, however, the effectiveness of the CE is more dependent on the lactate (electron donor) loading rate.

3.6. Ruminococcaceae bacterium CPB6 and Acinetobacter were the dominant microbes throughout the process

In total, 847,773 reads from 17 samples analyzed by amplicon sequencing were assigned to 118 OTUs. The quality of the microbial analysis pipeline was evaluated using a mock community sample. Despite the use of Gram-positive-enabled DNA extraction protocol, certain positive biases can be observed for certain Gram-negative genera like *Listeria* and *Escherichia* (Electronic Supplementary Material). OTUs in a sample feature table were affiliated to 34 families and 54 genera, *Methanobacteriaceae* being the only representative family of Archaea. After the adaptation, the dominating genera were distinctly different from those in the inoculum, which indicates the conditions in our reactors were selective. The succession of microbial composition is presented in Fig. 6. In contrast to the strong community shifts during the initial phases of the fermentations in both bioreactors, data points representing microbial communities sampled after 70 days are less scattered in the NMDS plot. This indicates lower dissimilarity of the community structures within that period. Representative sequences from the two most abundant OTUs, unclassified with selected confidence threshold (0.8) against Silva LTPs v132 database – Otu2 and Otu3 (Table S1) were subsequently subjected to comparison with NCBI 16S ribosomal RNA and NCBI nucleotide collection databases using online BLAST with default parameters (Altschul et al., 1990). When compared with NCBI nucleotide collection close relation to several *Ruminococcaceae* bacteria was revealed (representative sequences of Otu2 and Otu3 exhibit 100% and 97% identity, respectively, with 16S RNA gene sequences of *Ruminococcaceae* bacterium CPB6). *Ruminococcaceae* bacterium CPB6 was previously identified as a powerful workhorse for a highly-efficient caproic acid production from lactate-containing waste streams (Zhu et al., 2017). Although representative sequences from Otu2 and Otu3 share 96,77% identity, Otu3 is not observed in bioreactor B2, suggesting they originate from two separate, albeit highly related microorganisms. Overall, OTUs assigned to taxa related to *Acinetobacter* and *Ruminococcaceae* bacterium CPB6 were dominant in both bioreactors (Fig. 7), with the exception of the samples from the 22nd day, where 75.9% and 36.8% reads mapped to OTUs affiliated with *Acetobacter* genus, for B1 and B2, respectively. At the end of the start-up phase (day 48), the relative abundance of reads mapped to *Acetobacter* OTUs decreased to 9.2% in B1 and 11.7% in B2, and then at the end of the phase I (day 72) their relative abundance in both bioreactors was less than 1%, which lasted until the end of the processes. On days 48, 72 and 88 in B1, the relative abundances of reads mapping to *Ruminococcaceae* bacterium CPB6 OTUs were 23.1%, 26.6% and 59.1%, respectively, while the abundances of *Acinetobacter* reads were 36.9%, 37.0%, and 23.0%, respectively. The lowest relative abundance of mapped to OTUs corresponding to *Ruminococcaceae* bacterium CPB6 (8.3%) along with the highest relative abundance of *Acinetobacter* reads (65.3%) in the B1 was observed at the end of the phase III (day 118). This corresponded to a period of continuous lactate overloading and fluctuations in caproate production. However, when sampled at the peak (day 107) and bottom (day 112) of fluctuating residual lactate concentration in phase III, only little change in the abundance of dominant microbes was observed. Recovering stability of caproate production with hydrogen co-production by adding external acetate in the phase IV in the B1 led to the observation of the maximum relative abundance of *Ruminococcaceae* bacterium CPB6 reads of 76.7% on day 140. The relative abundance of reads mapping to Otu3 and *Acinetobacter* in the B2 was between 23.9%–47.7% and 12.8%–58.1%, respectively. The highest relative abundance (47.7%) of Otu3 in the B2 was observed at the end of the process during the co-production of hydrogen and caproate with the presence of residual lactate in the bioreactor. No *Megasphaera elsdenii,* which was previously recognized as the main butyrate and caproate producer from lactate (De Groof et al., 2019; Prabhu et al., 2012), was found in the microbiome structure. Moreover, in another study of continuous lactate to caproate conversion, the absence of *M. elsdenii* was also observed and *Acinetobacter* spp. was predominant

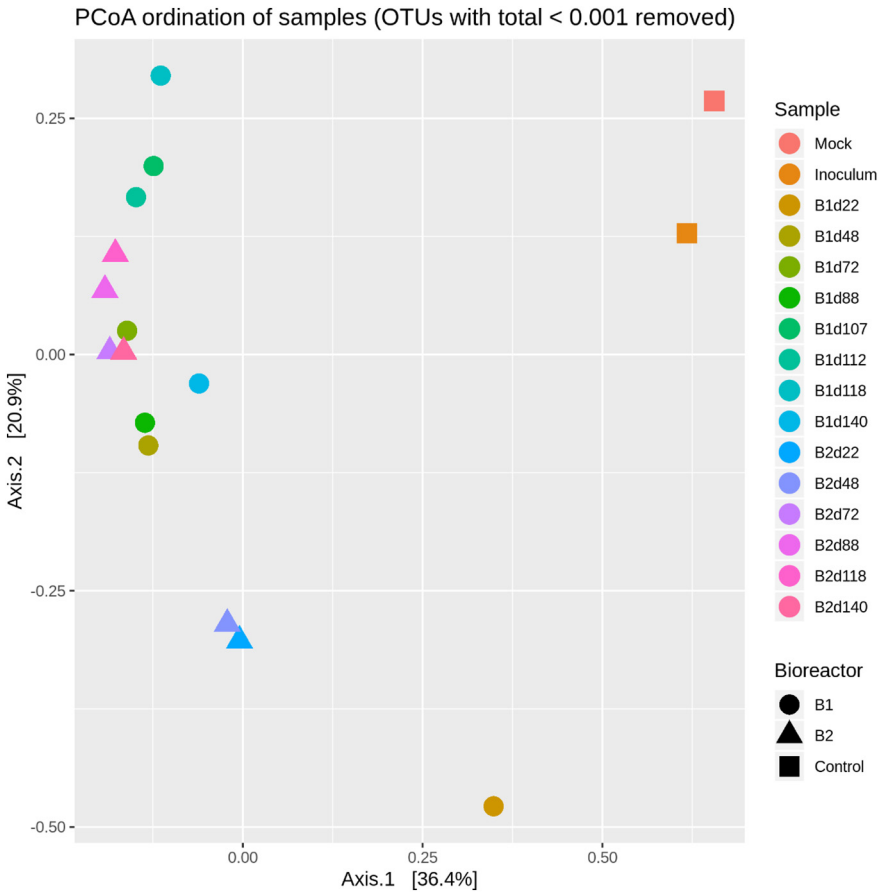

Fig. 6. Dynamics of microbial composition during the process illustrated by non-metric multidimensional scaling (NMDS). Dissimilarity between samples was computed using Bray-Curtis index. OTUs with less than 0.1% abundance were removed.

during the phase of high caproate productivity (Kucek et al., 2016a). Additionally, *Acinetobacter* spp. was found in other CE systems, i.e. with xylose as a feedstock (Qian et al., 2020), in the CO-based CE process (He et al., 2018) and ethanol-based CE process (Kucek et al., 2016b).

A small relative abundance (0.01%–1.12%) of reads mapping to OTUs related to *Propionibacterium*, capable of propionate production, was also observed in the B1 samples. This can explain the greater variation in the spectrum of produced carboxylates. In the B2, they were only detected

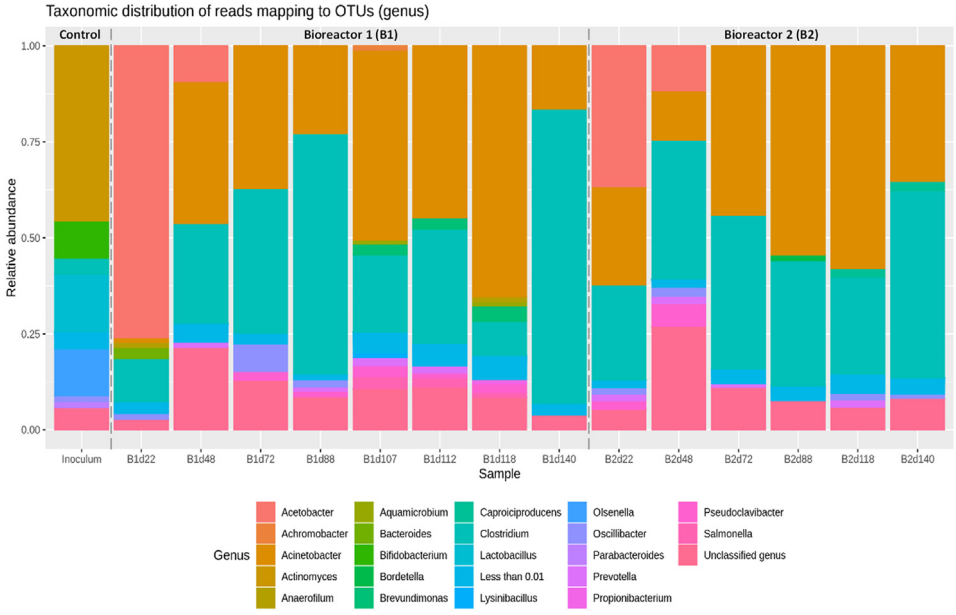

Fig. 7. Relative abundance of reads mapped to OTUs at genera level during operation of reactors B1 and B2. Less than 0.005 corresponds to reads mapped to OTUs assigned to genera with a percentage of reads below 1%. Otu2/Otu3 (close homology to 16S rRNA gene from *Ruminococcaceae* bacterium CPB6) are included here as Clostridium (USINTAX prediction with bootstrap confidence threshold above 50%).

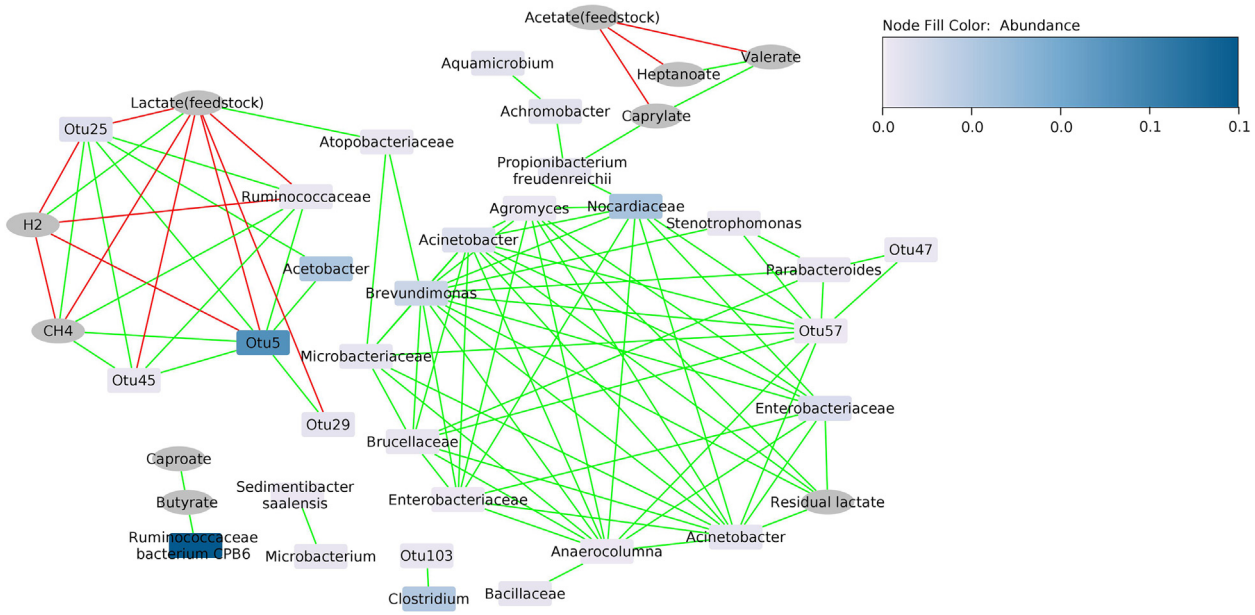

Fig. 8. Correlation networks for phase I–V. Pearson, Spearman and Kendall correlation coefficients were computed for each pair of OTUs (rectangular nodes) and abiotic parameters (elliptic nodes). Edges indicate a coefficient > 0.8 for positive correlations (green edges) and <0.8 for negative correlations (red edges), with a p-value <0.05 threshold after Benjamini-Hochberg correction. p-values were determined by bootstrapping. Shading of microbial taxon nodes indicates mean relative abundance in all samples. Node labels indicate the lowest classified taxonomic rank of each OTU. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in samples B2d118 and B2d140 with 0.04% and 0.11% mapped reads, respectively.

Moreover, we have constructed a correlation network to analyze the co-occurrences among microbial taxa and process parameters with the aim to reveal potential functions and ecological interactions within the microbial community in both bioreactors (Fig. 8). Co-occurrences between pairs of taxa may suggest bacterial cooperation such as mutualism (Faust and Raes, 2012). In our bioreactors, the inferred network consisted mainly of two sub-network modules. The first is related to the concentration of lactate in the feedstock, negatively correlated with a number of unclassified OTUs with various abundance. The second module consists of co-occurring bacteria belonging to *Enterobacteriaceae* and *Nocardiaceae* family, *Acinetobacter*, highly connected *Brevundimonas* and *Brucellaceae*, *Microbacteriaceae* and others. While the exact function of this bacterial cluster remains unknown it is significantly correlated with the residual lactate concentration in this study. Moreover, we identified more correlations, such as the co-occurring *Microbacterium* and *Sedimentibacter*. The presence of these microorganisms can be observed in microbial composition data from other chain elongation systems (Liu et al., 2020a; Qian et al., 2020). According to our analysis, the external acetate (acetate in the feedstock), as well as acetate concentration in the bioreactor is not significantly correlated with the change of any microbial species. This indicates the influence of acetate on the process may be more thermodynamic in nature rather than related to the kinetic competition of microbes for the substrate. The concentration of caproate and butyrate was found positively correlated with the presence of Otu3. This observation and the abundance of reads mapping to Otu2 and Otu3 with close homology to *Ruminococcaceae* bacterium CPB6 previously associated with efficient lactate-based caproic acid production, suggested that *Ruminococcaceae* were primary chain elongators in the investigated process.

No microbes previously implicated in biohydrogen production from lactate and acetate such as *C. acetobutylicum, C. tyrobutyricum, C. beijerinckii* and *B. methylotrophicum* (Wu et al., 2012) were identified in this study. However, it has been recently investigated (Liu et al., 2020c) that chain elongation core-genome harbors genes involved not only in the reverse β-oxidation and energy conservation but also the hydrogen formation. Genes related to hydrogen formation are present in *Ruminococcaceae* bacterium CPB6, similarly to other validated chain-elongating strains (Liu et al., 2020b). This includes energy-converting hydrogenase (Ech complex), which was proposed to generate hydrogen for maintaining the cytoplasmic redox balance caused by the oxidation of ferredoxin (Schuchmann and Müller, 2014). Hydrogen production is therefore likely to be related to energy conservation mechanisms in these bacteria and the excess of reducing equivalents obtained from lactate oxidation to acetyl-CoA.

4. Conclusions

For the first time, the effect of an external electron acceptor (acetate) on the lactate-based chain elongation in a continuously fed bioreactor was tested. The external acetate addition favored the production of carboxylates with an even number of carbon atoms in the molecule with predominant caproate production. We confirmed that lactate overloading in the process with lactate as a sole carbon source destabilized carboxylates production. In contrast to the selected previous study (Kucek et al., 2016a), long-term overloading did not result in propionate production, but periodic fluctuations in caproate and butyrate production dependent on acetate (electron acceptor) accumulation. The efficient method of stable caproate production in the lactate-based carboxylate platform has been demonstrated – the use acetate as an external electron acceptor. High hydrogen co-production was observed only under high lactate loading rate and with the external acetate addition. All microbes necessary for chain elongation from lactate (such as *Ruminococcaceae* bacterium CPB6) were enriched in both bioreactors. The presence of external acetate did not affect much the microbial composition of CE microbiome. However, two sub-networks of co-occurring bacteria either positively or negatively responding to residual lactate concentration were identified.

CRediT authorship contribution statement

Filip Brodowski: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Mateusz Łężyk:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Natalia**

Gutowska: Methodology, Writing – review & editing. **Piotr Oleskowicz-Popiel:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149885.

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PAPER 2

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Influence of lactate to acetate ratio on biological production of medium chain carboxylates via open culture fermentation

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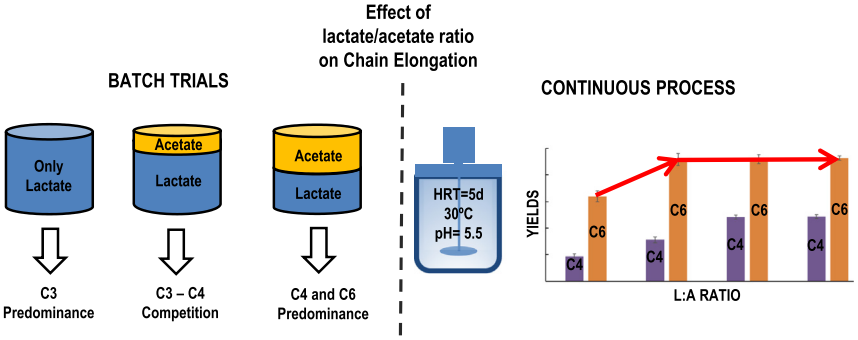
HIGHLIGHTS

- Lactate to acetate (L:A) ratio affected carboxylates selectivity in batch trials.
- Acetate as an additional carbon source to lactate affected carboxylates production.
- Controlling L:A ratio did not affect caproate yields in the continuous process.
- Caproate-butyrate production competition observed during long-term process
- *Caproiciproducens* was dominant throughout the continuous process.

GRAPHICAL ABSTRACT



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ABSTRACT

Waste valorisation via biological production of widely used in the industry medium chain carboxylates (MCCs) via open culture fermentation (OCF) could be a promising alternative to the commonly used anaerobic digestion. Lactate-rich waste streams are considered as valuable substrates for carboxylate chain elongation (CE), however, there are certain limitations related to the production efficiency. Acetate produced and accumulated in the acetogenesis plays an important role in CE, i.e. acetate is elongated to butyrate and then to caproate which is most popular MCC. Henceforth, it was investigated whether the ratio of lactate to acetate (L:A) affected carboxylates yields and product distribution in the lactate-based CE in OCF. The tested L:A ratios influenced carboxylates selectivity in batch trials. In the ones with lactate as the sole carbon source, propionate production was predominant but when a higher relative acetate concentration was used, the production of butyrate and CE to caproate was favored. The co-utilization of lactate and acetate in a continuous process increased the production of butyrate and caproate compared to the phase with lactate as the sole carbon source, however, controlling the relative concentration of lactate and acetate during co-utilization was not an effective strategy for increasing caproate production. 16S rRNA gene amplicon reads mapping to *Caproiciproducens* were the most abundant in samples collected throughout the continuous processes regardless of the L:A ratios.

1. Introduction

Over the last years, climate change and environmental degradation has posed a big threat to future generations (Sarkodie, 2022). Despite the development of the renewable energy market, fossil resources for energy (i.e. oil, coal and natural gas) still accounts for over 83 % of total primary energy

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consumption (BP, 2021). In order to ensure sustainable development, it is necessary to redirect the economy based on non-renewable energy sources to the biobased economy. The biorefinery sector, where organic wastes can be an alternative source for energy and valuable products (e.g. biofuels, industrial biochemicals, biomaterials), is crucial to the circular economy. Chain elongation (CE) process is a novel open culture fermentation (OCF) strategy, which has a great potential to serve as a biorefinery technology for efficient conversion of a various organic wastes into high-value medium chain carboxylates (MCC) (Fu et al., 2020). The CE has its roots in anaerobic digestion: short-chain carboxylates accumulated during acido- and acetogenesis are elongated into MCC instead of forthgoing through methanogenesis to methane (Hunter et al., 2021). The most common MCC is caproate which can be widely used in the chemical, pharmaceutical and food industry (Angenent et al., 2016). Moreover, it can be a precursor for the liquid fuels production in a subsequent chemical conversion processes (Harvey and Meylemans, 2014).

The basic metabolic requirement to carry out the CE is a presence of electron donors providing acetyl-CoA to the reverse β-oxidation cycle (or malonyl-CoA for fatty acid biosynthesis), in which in a series of enzymatic reactions the carbon chain of the carboxylate (electron acceptor) is elongated by two carbon atoms (Han et al., 2018). Various chemical compounds such as ethanol or methanol can play the role of electron donors in the CE (De Groof et al., 2019), however, recently more and more attention has been paid to a lactate-based CE. Lactate is present in many waste streams and feedstock such as acid whey (or cheese whey) from the dairy industry (Duber et al., 2020), grass silage juice (Khor et al., 2017) or maize silage (Lambrecht et al., 2019); it can also be synthesized in-situ in lactic acid fermentation of many wastes, including food waste (Tang et al., 2016) or lignocellulosic biomass (Li et al., 2021).

Lactate-based CE to caproate can be performed without external supply of electron acceptor (Zhu et al., 2015). It is possible because part of the acetyl-CoA can be also converted into acetate by substrate-level phosphorylation (Wu et al., 2019a). The acetate produced in this way acts as an electron acceptor and participates in the CE. However, the conversion of lactate to caproate is limited by lactate overloading and activation of the competitive acrylate pathway (propionate formation), resulting in a decrease in the MCC production (Kucek et al., 2016; Xu et al., 2018). In our previous study, it was indicated that the addition of external acetate to a lactate-overloaded continuously-fed bioreactor, where fluctuations in MCC production were observed, restored stable caproate production (Brodowski et al., 2022). In addition, external acetate provided stability of the CE to butyrate and caproate, moreover, no activity of the acrylate pathway was observed. However, how the acetate content (its concentration in the feedstock and bioreactor) affects the efficiency of the lactate-based CE has not been investigated so far. The concentration of electron donors and electron acceptors in the feedstock may affect the CE performance and the strategies for MCCs production (Wu et al., 2019a). Series of studies with the ethanol-based CE demonstrated that the ethanol to acetate ratio influenced the caproate and butyrate biosynthesis as well as spectrum of overall carboxylates produced (Liu et al., 2016; Spirito et al., 2018; Yin et al., 2017). The effect of lactate to acetate (L:A) ratios has not been yet recognized in the CE, however Scarborough et al. (2020) constructed a metabolic MCC-producing model and simulated lactate and acetate co-utilization in different acetate-to-lactate ratios. Modeling results predicted that low ratios of acetate to lactate produced variety of end products including butyrate, caproate and caprylate. Besides, as the acetate to lactate ratio increased, the production of MCC decreased accompanied by an increase in butyrate production.

In this study, the influence of the L:A ratio on the CE in the OCF was investigated. First, the evaluation of the influence of four L:A ratios on the spectrum of produced carboxylates as well as on competition between CE and acrylate pathway were performed in batch trials. Then, a continuous process was performed in two continuous stirred-tank reactors for 65 days. To the best of our knowledge, the aspect of the L:A ratio in a continuous process in OCF was raised for the first time. Results were compared to a metabolic MCC-producing model simulating lactate and acetate co-utilization in different L:A ratios (Scarborough et al., 2020). In addition, the impact of the residual acetate accumulation in the bioreactor on the CE process was examined. Microbial community analysis was conducted to explore how those ratios shape the microbiome structure.

2. Material and methods

2.1. Batch trials

Four different ratios of lactate to acetate ($r_{L:A}$) were assessed: $r_{L:A}$ = 0:1 (0 mM C of lactate and 300 mM C of acetate) denoted as R1; $r_{L:A}$ = 1:1 (150 mM C of lactate and 150 mM C of acetate) denoted as R2; $r_{L:A}$ = 4:1 (240 mM C of lactate and 60 mM C of acetate) denoted as R3; $r_{L:A}$ = 1:0 (300 mM C of lactate and 0 mM C of acetate) denoted as R4.

Batch trials were carried out in 500 mL serum bottle flasks with a working volume of 150 mL. All experiments were performed in triplicates. The medium and the sludge for inoculation were prepared as previously demonstrated in (Brodowski et al., 2020). The sludge was obtained from a 1 L caproate-producing upflow anaerobic sludge blanket (UASB) reactor (Poznan University of Technology, Poznan, Poland) fed with lactate-rich acid whey during the predominant production of caproate. The pH was initially set at 5.50 ± 0.05 and was not adjusted during trials. Anaerobic conditions were ensured by flushing nitrogen gas in the headspace for 5 min. After that, bioreactors were incubated in 30 °C for 7 days. Daily sampling and daily pH value measurements were performed. Gas composition was analyzed at the end of each trial.

2.2. Continuous processes

Two continuous stirred tank reactors (Lambda Minifor laboratory bioreactors; LAMBDA CZ, s.r.o., Brno, Czech Republic) with working volume of 1 L was used in the experiment. Fermenters were equipped with a temperature and pH control unit as well as with four peristaltic pumps for feed, effluent, base and acid. A more detailed description of the bioreactors was previously shown (Brodowski et al., 2022). The pH and the temperature was measured by the InPro 3253 probe (Mettler, Columbus, OH, USA). The pH was maintained at 5.5 by the addition of sodium hydroxide (2 M) or hydrochloric acid (0.5 M). The temperature was maintained at 30 °C by IR radiation heater. Bioreactors were operated for 65 days with a hydraulic retention time (HRT) of 5 days. The basal medium was prepared as in batch trials with modification of lactate and acetate concentrations. The process was initiated and inoculated using the sludge obtained from a 1 L UASB reactor fed with lactate-rich acid whey and producing MCC as previously described (Brodowski et al., 2022) during the predominant production of butyrate. Anaerobic conditions were ensured using nitrogen gas which was bubbled into the medium and the headspace of bioreactors for 5 min.

The control bioreactor, denoted as B1, was operated for 65 days with 300 mM C of lactate and 500 mM C of acetate in the feedstock ($r_{L:A}$ = 0.6 mM C/mM C). The process in the second bioreactor, denoted as B2, was divided into four operating phases (phase I-IV) based on the acetate content in the feedstock and $r_{L:A}$. During the phase I, the B2 was operated for 27 days and lactate was used as a sole carbon source (its concentration in the feedstock was 300 mM C). Phase II lasted from day 28 to 39 and the acetate was added into the feedstock as an additional carbon source (300 mM C of lactate and 125 mM C of acetate; $r_{L:A}$ = 2.4 mM C/mM C). In phase III (days from 40 to 52) and phase IV (days from 53 to 65) acetate concentration in the feedstock was increased to 250 mM C ($r_{L:A}$ = 1.2 mM C/mM C) and 500 mM C ($r_{L:A}$ = 0.6 mM C/mM C), respectively. During phase IV in B2 lactate and acetate concentrations were the same as in the B1. Liquid samples for carboxylates and alcohols analysis were collected daily.

2.3. Analytical techniques and calculations

The concentrations of the analytes were monitored using an HPLC (Shimadzu Prominence with a RID-20A) equipped with a Rezex ROA-Organic Acid H+ (8 %) column and gas chromatograph (Shimadzu

GC-2014) equipped with FID and a capillary column - Zebron ZB-FFAP (30 m × 0,53 mm; 1 μm). All the details concerning the measurements' conditions were previously described in (Brodowski et al., 2022).

The composition of the gaseous samples was measured using the gas chromatograph (Shimadzu GC-2014) equipped with the TCD and Porapak N packed column as described before (Brodowski et al., 2022).

Equations for the substrate loading rate (mmol C/L/d), carboxylates production rate (mmol C/L/d), product-to-carboxylates specificity (% mol C) were described in Supplementary material of previous work (Duber et al., 2020). Equations for calculations of gas amount for batch trials was described in Supporting Information of (Zagrodnik et al., 2020). Carboxylate yields in mmol C of carboxylate per mole C of lactate for batch trials were calculated as the ratio of the final concentration of carboxylate (mM C) to the initial lactate concentration (mM C). Carboxylate yields in mmol C of carboxylate per mole C of lactate for continuous process were calculated as the ratio of the production rate of a specific carboxylate (mmol C/L/d) to the lactate loading rate (mol C/L/d). Acetate consumptions (mmol C/L/d) for continuous process were calculated as the difference between the acetate concentration in the feedstock and the concentration of the residual (accumulated) acetate in the bioreactor and it was divided by the HRT.

2.4. Microbiome analysis

At day 6, towards the end of the batch trials 1 mL of culture broth was sampled in triplicates and cell biomass separated by centrifugation at 4 °C. Similarly, biomass from 1 mL of starting inoculum and at the end of each phase during continuous processes was collected. Biomass samples were stored frozen at −20 °C until further processing. Total metagenome DNA was extracted using GeneMATRIX Soil DNA Purification Kit (Eurx, Poland) according to manufacturer's recommendations. ZymoBIOMICS Microbial Community Standard D6300 (Zymoresearch, USA) was used for validation of analysis pipeline (data not shown). Moreover, batch samples were spiked with 2ul of ZymoBIOMICS Spike-in Control I (High Microbial Load) and corresponding ASVs filtered out from the final feature table. 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, Part # 15044223, Rev. B) was used for library preparation and sequencing. V3 and V4 region of 16S rRNA gene was amplified from the community DNA with primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGCCTACGGGNGGCWGCAG) and 785R (5′-GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) and Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/μL). The libraries were indexed and sequenced by Macrogen (Korea). The reads were obtained using Illumina Miseq instrument (300 bp paired-end sequencing, Miseq v3).

Trimming of paired ends (last 30 bp of forward and 60 bp of reverse reads) and merging was carried out using USEARCH (Edgar, 2013). Primer sequences were stripped off (sequences that did not contain the primer sequences were filtered out) using cutadapt (Martin, 2011). USEARCH pipeline was then employed for quality filtering, identifying unique sequences and generation of ASVs with UNOISE2 algorithm. Qiime2 (Bolyen et al., 2019) package was used for ASV-table generation and taxonomy assignment using Qiime's hybrid vsearch-sklearn classifier and Silva v138 database (Quast et al., 2013). ASV table was rarified without replacement to the sample with minimal count of mapped reads. Phyloseq package was used for carrying out the downstream analyses (McMurdie and Holmes, 2013).

Raw read sequences obtained in this study were uploaded to NCBI Sequence Read Archive (SRA) database and can be accessed under BioProject ID PRJNA833049.

3. Results and discussion

3.1. L:A ratio affected carboxylates selectivity in batch trials

In the R1 batch trial, with an acetate as a sole carbon source ($r_{L:A}$ = 1:0 mM C/mM C), no carboxylates production was recorded (Table 1). The pH remained constant at initial value of around 5.5 (Fig. 1). The lack of carboxylates production in R1 is understandable as the electron donor was not provided and there was no acetyl-CoA generation for reverse β-oxidation pathway (or malonyl-CoA for fatty acid biosynthesis). Even though acetate could be a precursor to the methane generation (Welte and Deppenmeier, 2014), no gas production was observed indicating lack of active acetoclastic methanogens.

Table 1
The initial L:A ratios, final carboxylates yields and gas productions for batch trials.

	R1	R2	R3	R4
Initial conditions				
$r_{L:A}$ [mM C/mM C]	0:1	1:1	4:1	1:0
Lactate [mM C]	0.0	150.0	240.0	300.0
Acetate [mM C]	300.0	150.0	60.0	0.0
Carboxylates yields [mmol C per mol C of lactate]				
Acetate	NC	−88.8 ± 0.5[a,b]	27.5 ± 0.6[a]	139.3 ± 19.6[a]
Propionate	NC	42.8 ± 3.6	242.1 ± 1.7	333.2 ± 5.9
Butyrate	NC	529.8 ± 22.5	385.8 ± 22.1	215.2 ± 33.8
Valerate	NC	ND	14.9 ± 2.3	26.0 ± 6.3
Caproate	NC	128.3 ± 4.6	12.1 ± 1.2	12.8 ± 0.6
Gas production [mmol/L_{medium}]				
H_2	ND	1.4 ± 0.3	1.1 ± 0.1	2.1 ± 0.1
CO_2	ND	18.7 ± 0.3	19.4 ± 0.3	21.2 ± 0.8
CH_4	ND	ND	ND	ND

ND - production not detected.
NC - production not calculated (lactate in the feedstock was 0 mM C).
[a] Yields calculated as the difference between the final concentration and the initial concentration.
[b] Negative values indicate consumption of the initial acetate supplied to the process.

The production of short chain carboxylates (mainly butyrate and propionate) dominated in the other batch trials, however, the distribution of carboxylates varied (Fig. 1). In the trial R2 ($r_{L:A}$ = 1:1 mM C/mM C), the CE process outcompeted propionate formation (Table 1) and the highest butyrate and caproate yields per 1 mol C of added lactate were recorded (529.8 ± 22.5 mmol C per mol C of lactate and 128.3 ± 4.6 mmol C per mol C of lactate, respectively). The final propionate yield was 42.8 ± 3.6 mmol C per mol C of lactate, but no CE to valerate or heptanoate was observed. Despite the high CE yields to butyrate and caproate in R2, a final acetate concentration remained comparable high i.e. 136.7 ± 0.7 mM C (Fig. 1) indicating consumption of only about 13.3 mM C of acetate. The stoichiometric calculations based on the CE equations (Table 2) showed that the consumption of acetate should be much higher during the process. According to the stoichiometric equation for the CE from lactate to butyrate (Eq. (2)) and from lactate to caproate (Eq. (3)), 46.2 mM C acetate would be needed to achieve 79.5 mM C butyrate and 19.2 mM C caproate in R2. Although, acetate could be also formed as a by-product of propionate formation according to Eq. (4) but based on stoichiometric calculations only 2.1 mM C acetate would be formed by this pathway in R2 (assuming final propionate concentration of 6.4 mM C). Such a low external acetate consumption in our trial (compared to the theoretical calculated acetate consumption from stoichiometric equations) indicated that, despite providing the external acetate for the chain elongators, it was most likely produced from lactate (via acetyl-CoA). In the batch trials R3 ($r_{L:A}$ = 4:1 mM C/mM C) the competition between CE and the acrylate pathway was more pronounced. Although high propionate yield was observed (242.1 ± 1.7 mmol C per mol C of lactate), the CE to butyrate was still dominant (385.8 ± 22.1 mmol C per mol C of lactate). Moreover, low yields of valerate (14.9 ± 2.3 mmol C per mol C of lactate) and caproate (12.1 ± 1.2 mmol C per mol C of lactate) were observed. The final acetate concentration increased by around 6.6 mM C and reached 66.6 ± 1.3 mM C. As mentioned before, acetate could be produced as a by-product of propionate formation, as well as directly from lactate, however, similar to trial R2, the theoretical consumption of acetate for the butyrate and caproate formation (based on stoichiometric calculations: 47.6 mM C acetate would be needed for butyrate and caproate formation in R3) suggested that, despite the availability of acetate in the feedstock,

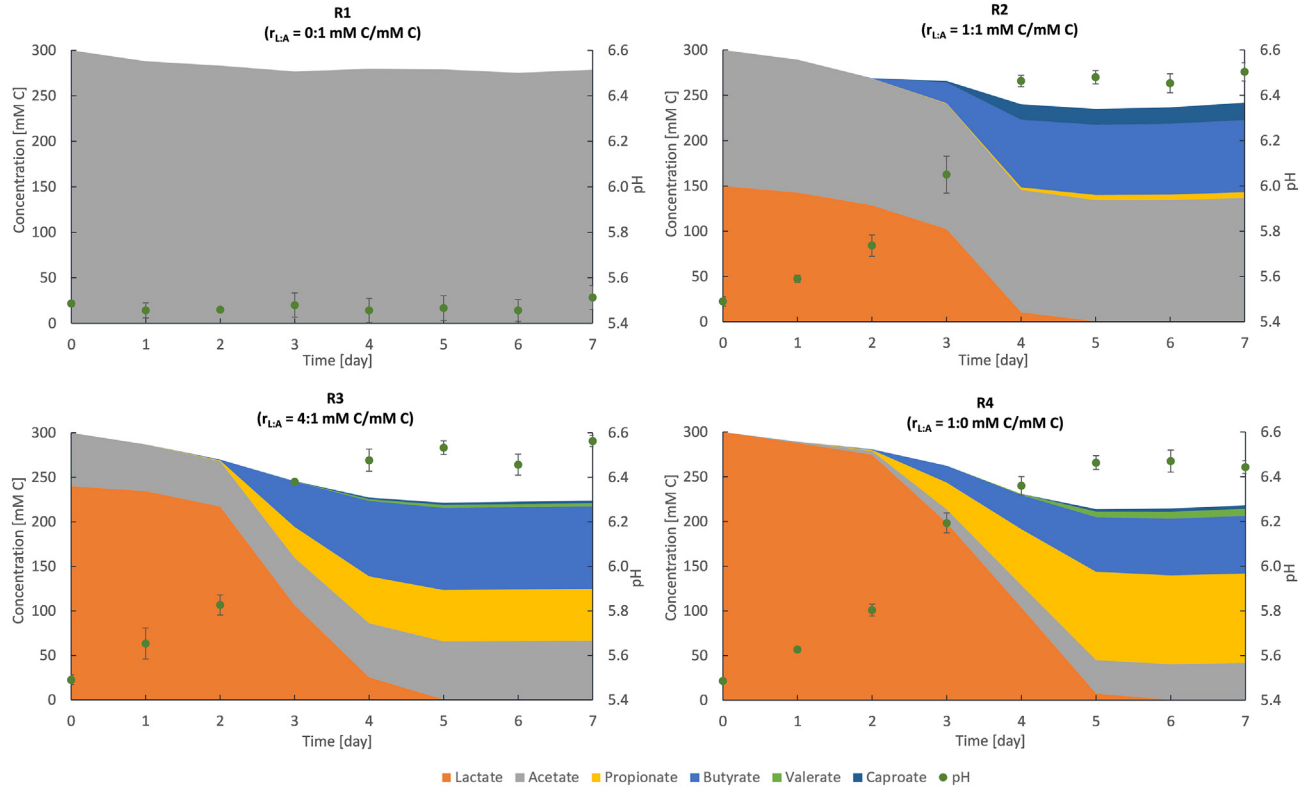

Fig. 1. Carboxylates concentrations profiles and pH values in the conducted batch trials. Metabolites with a concentration below 3.0 mM C are not shown.

its intracellular production from acetyl-CoA was highly active. Moreover, it is interesting that despite the acetate accumulation in both trials R2 and R3, the spectrum of the products (and the competition between CE and propionate formation) was different, and the L:A ratio influenced product differentiation even though low acetate consumption was observed in both trials. Therefore, the acetate could have a higher impact on carboxylates selectivity rather than on the amount of produced carboxylates. The amount of produced carboxylates could be more determined by the concentration of lactate, which was fully consumed in all trials. In the trial R4 with lactate as a sole carbon source ($r_{L:A}$ = 1:0 mM C/mM C), carboxylates with a chain length of 2 to 6 carbon atoms per molecule were produced. Propionate yield was predominant and amounted to 333.2 ± 5.9 mmol C per mol C of lactate. Similarly, in other batch studies with lactate as sole carbon source, a dominant propionate production was achieved (Nzeteu et al., 2022; Wu et al., 2018). Butyrate and caproate in R4 were produced directly from lactate without external acetate addition. As mentioned before, it was possible because acetate, which acts as an electron acceptor in the reverse β-oxidation cycle or fatty acid biosynthesis pathway, can be directly produced from lactate. Despite a high propionate yields, the CE to valerate (26.0 ± 6.3 mmol C per mol C of lactate) was significantly less efficient than the CE of carboxylates with an even number of carbon atoms in the molecule (yields of butyrate and caproate were 215.2 ± 33.8 mmol C per mol C of lactate and 12.8 ± 0.6 mmol C per mol C of lactate, respectively). That is because acetate and propionate compete for the same enzyme system in the CE process but, as described by (Roghair et al., 2018), with the dominance of acetate.

Caproate was not a dominant product in any of the trials, although the inoculum was enriched in bacterium belonging to *Caproiciproducens* genus which had 100 % ASV identity to *Ruminococcaceae* bacterium CPB6 previously recognized as a lactate-based caproate producer. The relative abundance of reads mapping to this genus was 67.9 % in the inoculum. The range of initial pH from 5.0 to 6.5 was very favorable for cell growth of *Ruminococcaceae* bacterium CPB6 (Zhu et al., 2017), however, none or only up to 2.0 % of these bacterium was found in the samples at the end of R2, R3 and R4 trials. In the first recognized studies with lactate to caproate conversion batch trials were conducted at pH manually controlled between 6.0 and 6.5 (Zhu et al., 2015) but recent studies showed that pH value had a major influence on the competition between lactate-based chain elongators and propionate producers in OCF indicating that mildly acidic pH (lower than 6) was favorable for the lactate-based chain elongators, and higher pH favored other microorganisms which caused dominance of propionate and acetate production (Candry et al., 2020). The final pH values for R2, R3, and R4 were 6.50 ± 0.04, 6.56 ± 0.02, and 6.44 ± 0.03, respectively. The large diversity of microorganisms in OCF along with the lack of pH adjustment could have affected the decrease of relative abundance of ASV2 corresponding to *Ruminococcaceae* bacterium CPB6, although the range of pH in batch trials was favorable for its cell growth. However, despite the lack of pH adjustment, pH changed in a similar manner in R2, R3 and R4 trials. It is also worth noting that selectivity of produced carboxylates could also be affected by substrate (lactate and acetate) concentrations (Wu et al., 2022). It was observed that low substrate concentrations (total concentrations of 100 mM C and 200 mM C) favored butyrate over caproate production. The highest selectivity of caproate was obtained for substrate concentration of 400 mM C; further increase of substrate concentration led to a decrease in a caproate selectivity.

Table 2
Possible pathways of lactate utilization in batch trials based on (Isipato et al., 2020; Wu et al., 2019a; Zhu et al., 2017).

No.	Pathway	Equation
(1)	Lactate oxidation	$Lactate + H_2O \rightarrow Acetate + 2\,H_2 + CO_2$
(2)	Lactate-based chain elongation to butyrate[a]	$Lactate + Acetate + H^+ \rightarrow Butyrate + CO_2 + H_2O$
(3)	Lactate-based chain elongation to caproate[a]	$Lactate + Butyrate + H^+ \rightarrow Caproate + CO_2 + H_2O$
(4)	Propionate formation from lactate	$3\,Lactate \rightarrow Acetate + 2\,Propionate + CO_2 + H_2O$

[a] The same equations for odd-carbon carboxylates, e.g. when even-carbon butyrate as electron acceptor are replaced by odd-carbon valerate, odd-carbon heptanoate can be generated.

No methane production was detected in any of the trials, which proved lack of methanogens activity. Hydrogen and CO_2 are by-products of lactate-based CE. The main gaseous product was CO_2 and its production for R2, R3 and R4 was 18.7 ± 0.3 mmol/L_{medium}, 19.4 ± 0.3 mmol/L_{medium} and 21.2 ± 0.8 mmol/L_{medium}, respectively. Hydrogen production for R2, R3 and R4 was 1.4 ± 0.3 mmol/L_{medium}, 1.1 ± 0.1 mmol/L_{medium} and 2.1 ± 0.1 mmol/L_{medium}, respectively. Indeed, hydrogen and CO_2 can have a significant impact on CE (Wu et al., 2019a). First of all, hydrogen can be used as an electron donor to synthesize carboxylates. González-Cabaleiro et al. (2013) verified that CE to butyrate and caproate using hydrogen and acetate is thermodynamically infeasible, on the other hand, propionate could be formed from hydrogen and acetate (Yin et al., 2017), and hydrogen can also favor the conversion of propionate into MCC (Wu et al., 2019b). Moreover, controlling the hydrogen partial pressure is crucial for CE because it thermodynamically inhibits competing pathways such as anaerobic oxidation of MCC (Angenent et al., 2016; Wu et al., 2019a). In addition high hydrogen partial pressure is necessary to achieve high caproate selectivity (Nzeteu et al., 2018). Moreover, hydrogen and CO_2 could be used by a homoacetogens to produce acetate and further MCC (Guo et al., 2015). It was also reported that a combination of CO_2 and hydrogen in the headspace can affect carboxylate selectivity (Arslan et al., 2012).

3.2. Limited influence of L:A ratios on MCC production in a continuous process

Carboxylates production along with the residual concentration of substrates (lactate and acetate) is presented on Fig. 2. During the phase I in the B2, carboxylates of even and odd carbon numbers were produced with a predominant caproate production from the very beginning. Although total carboxylates production from day 7 in the phase I was stable (from 27.1 mmol C/L/d to 32.2 mmol C/L/d), there were variations in the carboxylate distribution. At the beginning of the process in the B2 (until day 12), in addition to a predominant caproate production, mainly butyrate and valerate were produced (on day 12 production of butyrate and valerate was 8.6 mmol C/L/d and 2.0 mmol C/L/d, respectively). Subsequently, the production of butyrate and valerate decreased, resulting in an increase in the production of longer MCC, mainly caproate (up to 24.6 mmol C/L/d on day 17), but also heptanoate (1.8 mmol C/L/d on day 18) and caprylate (1.0 mmol C/L/d on day 18). The overall specificity of a longer MCC (caproate, heptanoate and caprylate) in the mixture of carboxylates in the B2 reached maximum of 86.6 % (with a predominant caproate specificity of 79.7 %) on day 16, demonstrating a high CE performance. To the best of our knowledge, a higher specificity of caproate (81.3 %) in a continuous lactate-based CE was only achieved recently by (Kim et al., 2022) but no production of longer carboxylates was recorded at that time. In the B2, the production of longer MCC decreased from day 18 (mainly caproate which decreased to 17.9 mmol C/L/d on day 23) causing an increase in a production of shorter carboxylates such as butyrate and valerate. Similar phenomenon of periodic fluctuations in a caproate production depending on the accumulation of acetate in the lactate-based CE was observed in our previous study (Brodowski et al., 2022). Thus, the limited availability of acetate (electron acceptor), which had to be produced in-situ, could have resulted in a decrease in the caproate production. Moreover, the limited access to acetate could have caused a competition between the CE to butyrate/caproate and a propionate formation (along with its further CE to valerate and heptanoate). However, as mentioned earlier, acetate dominated the competition with propionate in the CE, which resulted in the predominance of carboxylates with an even number of carbon atoms in the molecule in the phase I in the B2. Interestingly, although the external electron acceptor (acetate) was supplied in other phases, the highest caprylate production and the highest caproate specificity were achieved in the phase I in the B2.

The availability of an external acetate in phases II-IV influenced an increase in total carboxylates production during the CE process. Total

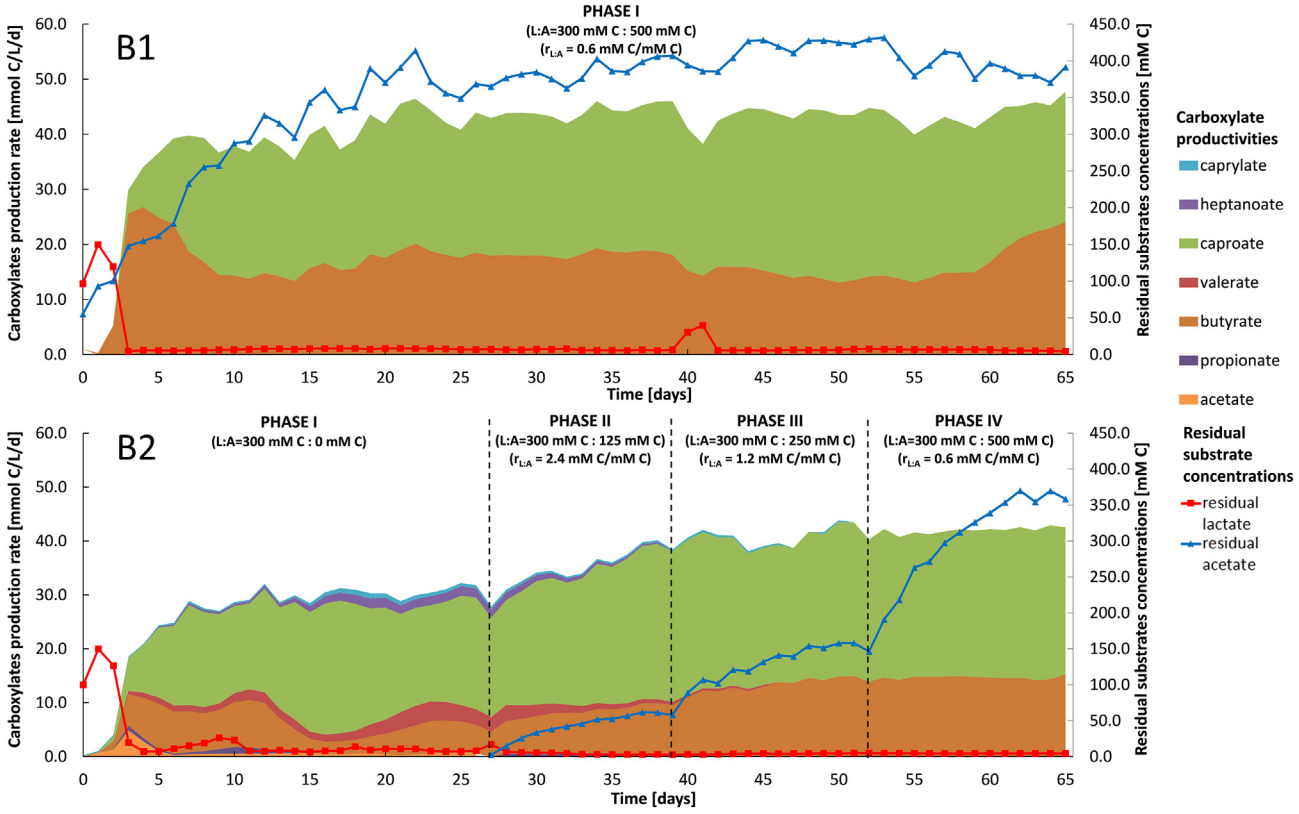

Fig. 2. Production rate profile of carboxylates with the residual substrate concentrations (lactate and acetate) in the bioreactor 1 (B1) and in the bioreactor 2 (B2). In B2, the concentration of acetate as the residual substrate concentration was shown in the phases II-IV. In the phase I, acetate was not a substrate and was shown as a carboxylate product (its concentration in the feedstock was 0 mM C). In B1, lactate and acetate were listed as substrates throughout the process.

carboxylates production increased from an average of 29.8 ± 1.5 mmol C/L/d (days 7–27; phase I) to 38.4 ± 1.7 mmol C/L/d (days 35–39; phase II). This was mainly due to an increase in the production of butyrate (9.4 ± 0.6 mmol C/L/d on days 35–39) and caproate (27.5 ± 1.3 mmol C/L/d on days 35–39). In addition, displacement of the production of carboxylates with an odd number of carbon atoms in the molecule by carboxylates with an even number of carbon atoms in the molecule was observed. On day 39 (end of phase II) 98.5 % of produced carboxylates corresponded to carboxylates with an even number of carbon atoms in the molecule. Lactate was completely consumed by microorganisms, however residual acetate accumulated from the beginning of the phase II, reaching a concentration of 58.1 mM C at the end of the phase II. In the phase III ($r_{L:A}$ = 1.2 mM C/mM C) minor changes were observed. Although the production of caproate did not change significantly and it was 27.6 ± 1.0 mmol C/L/d (days 48–52), the production of butyrate increased to 14.5 ± 0.5 mmol C/L/d (days 48–52). In addition, residual acetate accumulation increased, reaching 146.3 mM C on day 52. In the phase IV ($r_{L:A}$ = 0.6 mM C/mM C) no significant changes in carboxylate production were observed. Only the residual acetate concentration increased, which at the end of the process was 358.4 mM C.

The yields of butyrate and caproate expressed in mmol C per mol C of lactate in the tested L:A ratios in B2 are shown on Fig. 3 to summarize the impact of the L:A ratio on lactate transformation. Although the addition of acetate in the feedstock in the phase II in the B2 increased the caproate yields, for all tested trials with acetate, caproate yields were similar and amounted to 458.1 ± 22.0 mmol C per mol C of lactate ($r_{L:A}$ = 2.4 mM C/mM C), 459.3 ± 16.4 mmol C per mol C of lactate ($r_{L:A}$ = 1.2 mM C/mM C) and 463.5 ± 8.1 mmol C per mol C of lactate ($r_{L:A}$ = 0.6 mM C/mM C). Thus, the results suggested that the L:A ratio in the feedstock did not play a significant role in the efficiency of lactate to caproate transformation, but external acetate influenced an increase of lactate to caproate conversion efficiency by about 40 %. Moreover, in our previous study (Brodowski et al., 2022) we investigated lactate-overloading phenomenon in CE by increasing lactate concentration in the feedstock (from 400 mM C to 1350 mM C) while keeping constant concentration of acetate in the feedstock (200 mM C) which resulted in an increase in the caproate production until lactate-overloading occurred. Increasing lactate concentration to 600 mM C and to 900 mM C resulted in an increase in the caproate production by about 57 % and 64 %, respectively. However, there were no significant changes in yields per mol C of lactate, i.e. for $r_{L:A}$ = 2:1 mM C/mM C (400 mM C lactate and 200 mM C acetate), $r_{L:A}$ = 3:1 mM C/mM C (600 mM C lactate and 200 mM C acetate) and $r_{L:A}$ = 4.5:1 mM C/mM C (900 mM C lactate and 200 mM C acetate). Calculated yields were 381 ± 10 mmol C of caproate per mol C of lactate, 387 ± 12 mmol C of caproate per mol C of lactate and 416 ± 13 mmol C of caproate per mol C of lactate, respectively (avg. calculated from the last 5 days of steady-state of each phase). Much lower caproate yields were observed for the last phase (1350 mM C lactate and 200 mM C acetate) as an effect of lactate-overloading and it was 261 ± 3 mmol C of caproate per mol C of lactate. Thus, increasing the concentration of lactate in the feedstock determined the production of caproate, however, it did not significantly affect the efficiency of lactate to caproate conversion (along with increase of lactate concentration in the feedstock and L:A ratio, an increase in caproate yields per mol C of lactate of only 9 % were observed) until lactate overloading occurred.

In the batch tests with the newly isolated *Ruminococcaceae* bacterium CPB6, identified as a highly efficient lactate-based chain elongator (Zhu et al., 2017), an increase in caproate yields was observed as an effect of acetate supplementation. The butyrate yields of 466.2 mmol C per mol C of lactate in the trial with lactate as sole carbon source and 522.2 mmol C per mol C of lactate in trial with additional acetate supplementation were observed (own calculations based on the data presented in Zhu et al. (2017)). Competitive metabolic pathways and predicting culture's product response are the main limitation of OCF (Oleskowicz-Popiel, 2018), however, in our research favorable operating parameters and substrate composition led to the suppression of competitive pathways such as methanogenesis or acrylate pathway and ensured similar caproate yields as in fermentation with isolated specialized pure culture *Ruminococcaceae* bacterium CPB6. The maximum caproate yields of 482.4 mmol C per mol C of lactate was reported on day 41, which is just about 8 % less compared to the studies with pure *Ruminococcaceae* bacterium CPB6 culture (Zhu et al., 2017). Moreover, we observed a significantly higher butyrate yields (Fig. 3) compared to the studies with isolated *Ruminococcaceae* bacterium CPB6 (59.2 mmol C butyrate per mol C of lactate was achieved in trial with acetate supplementation by (Zhu et al., 2017)), although the effect of the L:A ratio was limited. The butyrate yields were higher for lower L:A ratios and were 241.7 ± 7.5 mmol C per mol C of lactate ($r_{L:A}$ = 1.2 mM C/mM C) and 243.4 ± 6.8 mmol C per mol C of lactate ($r_{L:A}$ = 0.6 mM C/mM C). In conclusion, a supplementation with an external acetate increased the butyrate and caproate yields as well as inhibited the production of carboxylates with an odd number of carbon atoms in the molecule. However, the tested L:A ratios with acetate supplementation did not influence the caproate yields (similar yields for three tested $r_{L:A}$) and the butyrate yields were affected only to a limited extent (higher butyrate

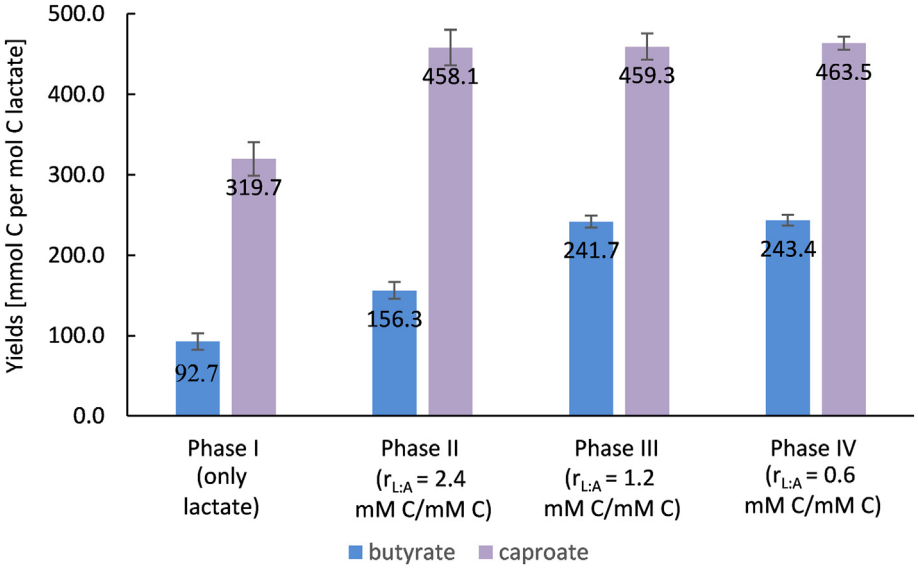

Fig. 3. Comparison of the butyrate and caproate yields in B2 depending on the L:A ratio. Carboxylate yields in mmol C of carboxylate per mole C of lactate were calculated for last 5 days of every phase.

yields was achieved for $r_{L:A}$ = 1.2 mM C/mM C and $r_{L:A}$ = 0.6 mM C/mM C in comparison to yields achieved for $r_{L:A}$ = 2.4 mM C/mM C). Metabolic model (iFermCell215) simulation carried out by Scarborough et al. (2020) also suggested that in lactate-based CE, controlling the relative amounts of acetate and lactate may not be an effective strategy for increasing caproate production. Interestingly, an excess amount of acetate was predicted by iFermCell215 to increase butyrate production and decrease MCC (caproate and caprylate) production. Butyrate was predicted as the sole product at L:A ratios of 1.56 mM C/mM C and lower (own calculations based on acetate-to-lactate ratio provided by Scarborough et al. (2020)). The results derived from our study also demonstrated an increase in butyrate production with acetate excess, however, no major changes in caproate production were observed as iFermCell215 model predicted.

Importantly, a high accumulation of residual acetate in the B2 did not cause a significant disturbance in caproate and butyrate production, although acetate could be a precursor to the methane production by acetoclastic methanogens (Welte and Deppenmeier, 2014). Despite the accumulation of residual acetate, some of the supplied acetate was consumed, e.g. the calculated consumptions of the supplied acetate for the last days of the phases II, III and IV were 13.4 mmol C/L/d, 20.7 mmol C/L/d and 27.8 mmol C/L/d, respectively.

3.3. Competition between caproate and butyrate production in a long-term continuous process under steady conditions

Carboxylates only with an even number of carbon atoms in the molecule were produced in the B1 ($r_{L:A}$ = 0.6 mM C/mM C; as in the phase IV in the B2) from the very beginning; however no CE to caprylate and longer carboxylates was reported. During the first 5 days, butyrate production was dominant and reached 24.9 mmol C/L/d. Subsequently, the CE performance improved causing an increase of caproate production at the expense of butyrate production. Caproate production reached the value of 24.6 mmol C/L/d (410.0 mmol C per mol C of lactate) on day 12 and then averaged 26.2 ± 2.3 mmol C/L/d (436.7 ± 38.4 mmol C per mol C of lactate) by the end of the process. The production of butyrate was slightly lower and it was 16.8 ± 2.6 mmol C/L/d (280.0 ± 43.1 mmol C per mol C of lactate) under steady-state conditions (days 12–65). Lactate was completely consumed in the B1, except on days 40–41 where residual lactate appeared as a result of technical problems with the feedstock dosing pump. Despite the brief interruption, it did not significantly affect further production of carboxylates. Acetate accumulated in the B1 from the very beginning. Its concentration in the bioreactor gradually increased and from day 22 till the end of the process it amounted to 391.3 mM C (with maximum concentration of 431.7 mM C on day 53). At the end of the process, a decrease in the CE performance to caproate was observed, which translated into an increase in the production of butyrate. The production of butyrate (24.1 mmol C/L/d) was slightly higher on the last day of the process compared to the caproate production (23.7 mmol C/L/d). Competition between caproate and butyrate production was previously observed by (Liu et al., 2020). During the long-term bioreactor operation predominated butyrate production was achieved instead of MCC production. Moreover, as mentioned before, the metabolic model provided by Scarborough et al. (2020) predicted low L:A ratios to displace MCC production by butyrate production. This suggests that operating the bioreactor at low L:A ratios in a long-term process may result in butyrate-caproate production competition and the reduced CE efficiency. Despite the use of the same substrate composition and operating conditions in the B2 in the phase IV, no similar phenomenon of displacing caproate production was noted. This might suggest that in our research, as in (Liu et al., 2020), competition between butyrate and caproate production occurred as a result of a long-term reactor operation.

3.4. Caproiciproducens was the dominant throughout the continuous processes regardless of the L:A ratios

Inocula for both batch and continuous processes were sampled from caproate-producing UASB reactor process operating with acid whey as a substrate. Although microbial composition of inocula differed due operating conditions at the time of sampling, both samples were located closer to each other than to other samples on NMDS ordination plot (Fig. 4). Moreover, clear grouping of samples in batch and continuous reactors can be observed, suggesting the mode of cultivation having the largest effect on the microbial community. ASVs in a samples featured table were affiliated with 29 bacterial families and 38 genera (ASVs with at least 0.005 corresponding abundance in one sample). No Archaea was detected in the analyzed samples.

We have identified bacterium belonging to *Caproiciproducens* genus (ASV2) as dominant throughout the continuous processes carried out in this study, regardless of L:A ratio applied (Fig. 5). The first identified member of this genera - *Caproiciproducens galactitolivorans* gen. nov., sp. nov. - was isolated from the activated sludge of an anaerobic digestion reactor during a study of bacteria utilizing galactitol as the carbon source (Kim et al., 2015). *C. galactitolivorans* produces H_2, CO_2, ethanol, acetic acid, butyric acid and caproic acid as metabolic end products of anaerobic fermentation (Kim et al., 2015). ASV2 corresponding to *Caproicidproducens* has a sequence 100 % identical to the one we have identified in the previous study (Brodowski et al., 2022) where lactate and acetate were used as fermentation co-substrates. It is also closely related (100 % ASV identity) to *Ruminococcaceae bacterium* CPB6, so far the only isolated lactic acid chain elongator (Zhu et al., 2017). Differences at various acetate loading investigated in this study included mainly relative abundance of microbes with yet unknown function in chain-elongating communities such as *Acetobacter* and *Prevotella*.

Testing lactate and acetate co-fermentation in batch reactors resulted in markedly different microbial consortia. In contrast to high initial relative abundance of *Caproiciproducens* genus (*Ruminococcaceae* family) in batch reactors after inoculation (sample Inokulum1: 67.9 %), none or small portion of corresponding 16S reads were identified after 6 days of cultivation. pH at the end of batch fermentations reached 6.5 ± 0.1 (Fig. 1). *Caproiciproducens galactitolivorans* gen. nov., sp. nov., similar to other model chain-elongating bacteria grows optimally at pH values at pH 6.0–8.0. However so far, this genera has been rather isolated and identified in consortia at mildly acidic pH. Moreover, *Ruminococcaceae bacterium* CPB6 was also shown to grow best at pH 5.5–6.0 pH which has been already identified as important parameter in steering both the competition between lactate-consuming propionic acid producers and chain elongators (Candry et al., 2020) as well as butyrate fermenters and chain elongating bacteria (Liu et al., 2020). This effect is attributed to both kinetic and thermodynamic factors as with lower pH the advantage shifts towards chain elongators. Unlike in continuous process carried out at pH 5.5, at the end of batch fermentations (at higher pH) we observed a higher relative abundance of reads corresponding to *Clostridiaceae* family (from 2.9 % in Inokulum1 to 17.8–28.8 % after 6 days in batch cultivation). These were almost exclusively genera *Clostridium* sensu stricto *1, 2, 7, 10, 12, 13* and *18* – previously associated with high butyrate production (Candry and Ganigué, 2021; Liu et al., 2020) and corresponded with an increased output towards short-chain fatty acid production in the batch fermentations in this study. Moreover, both in continuous and batch processes, increased residual concentration of acetate was accompanied by higher abundance of microbes from *Clostridiaceae* family. In batch reactors less abundant representatives from *Firmicutes* (so far the only phylum with known chain-elongating microbes) included also *Oscillospiraceae*. Genomic analyses of metagenome from human gut samples suggested they had a fermentative metabolism and ability to produce butyrate (Gophna et al., 2017). The majority of 16S reads in the samples at the end of batch cultivations mapped to ASVs corresponding to genera *Acetobacteraceae* (more than 36 % in R2 and R3) as well as *Enterobacteriaceae* (45 % corresponding reads in R4). While these *Proteobacteria* are frequently identified in chain-elongating communities their role remains largely unknown and it is likely that they are not directly involved in CE of carboxylates. It is plausible that secondary functions of these organisms confer the essential interactions to the microbial community.

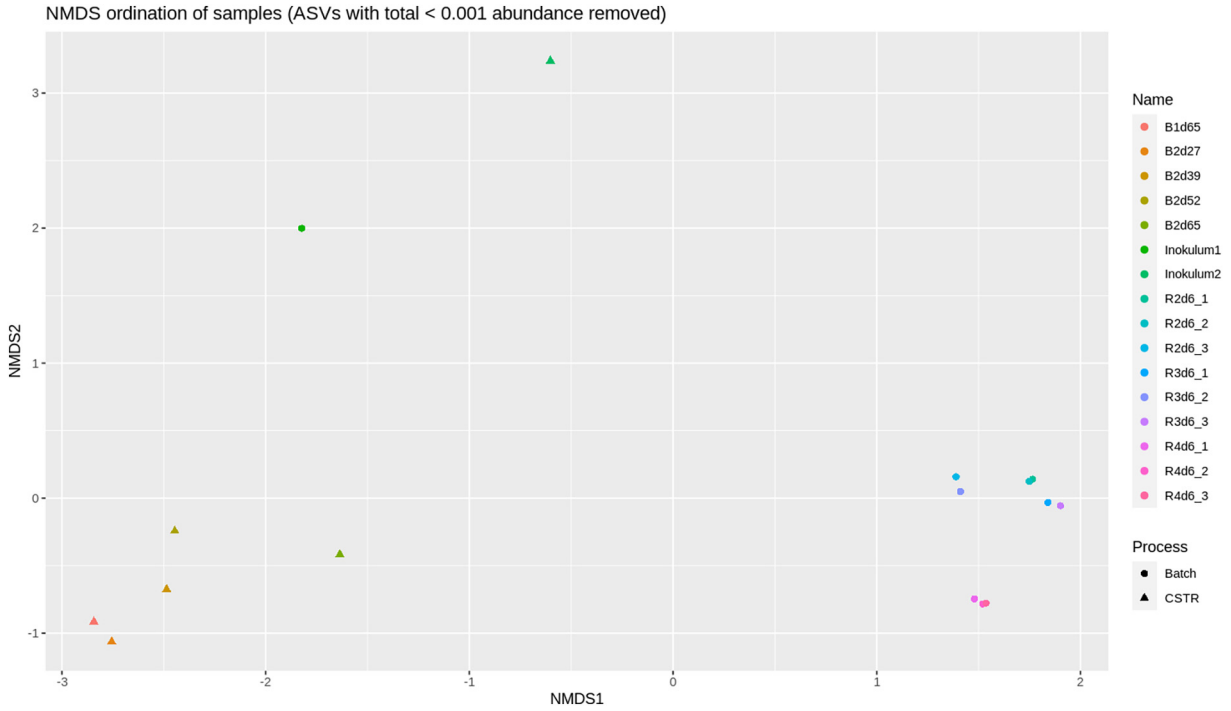

Fig. 4. Nonmetric multidimensional scaling (NMDS) ordination of Bray–Curtis dissimilarity between microbial communities in samples analyzed in this study. ASVs with the total abundance of less than 0.1 % removed from the analysis. Final stress 0.0534.

4. Conclusions

Different effects of L:A ratios on the CE were observed depending on the configuration of the process. The tested L:A ratios in the batch trials affected carboxylates production: a higher relative acetate concentration favored the production of butyrate and the CE to caproate. In the continuous process, differences in the carboxylates production profile were observed between the co-utilization of lactate and acetate and the use of only lactate as the sole carbon source, however, the strategy of controlling the L:A ratios during lactate and acetate co-utilization did not affect caproate yields.

CRediT authorship contribution statement

Filip Brodowski: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.

Mateusz Łężyk: Conceptualization, Methodology, Formal analysis, Writing – review & editing.

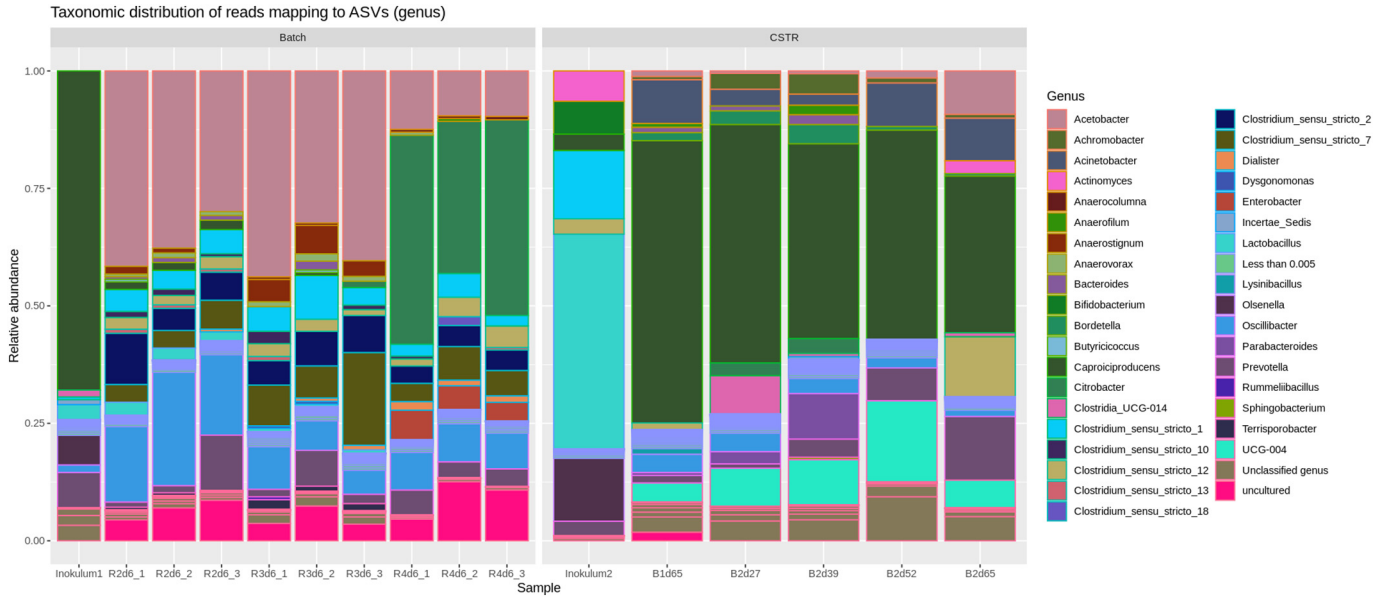

Fig. 5. Relative abundance of reads mapped to ASVs classified at genus level. 'Less than 0.005' corresponds to the sum of all genera with relative abundance below 0.5 % in individual samples. 'Unclassified genus' corresponds to all reads mapped to ASVs not classified at genera level. Batch trial samples are denoted as R2d6 ($r_{L:A}$ = 1:1 mM C/mM C), R3d6 ($r_{L:A}$ = 4:1 mM C/mM C) and R4d6 ($r_{L:A}$ = 1:0 mM C/mM C). Continuous process samples are denoted as B1d65 ($r_{L:A}$ = 0.6 mM C/mM C), B2d27 (lactate as a sole carbon source), B2d39 ($r_{L:A}$ = 2.4 mM C/mM C), B2d52 ($r_{L:A}$ = 1.2 mM C/mM C) and B2d65 ($r_{L:A}$ = 0.6 mM C/mM C); "Inokulum1" corresponds to batch trials and "Inokulum2" corresponds to continuous process.

Natalia Gutowska: Methodology, Writing – review & editing.
Tugba Kabasakal: Formal analysis, Investigation.
Piotr Oleskowicz-Popiel: Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.158171.

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PAPER 3

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Co-production of hydrogen and caproate for an effective bioprocessing of waste

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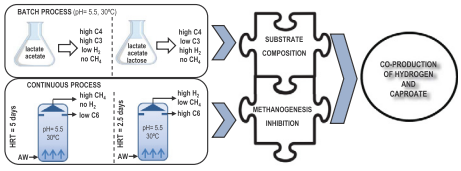
GRAPHICAL ABSTRACT



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ABSTRACT

The objective of the study was to valorize waste stream for the co-production of hydrogen and caproate via open culture fermentation (OCF). Batch studies confirmed that the use of sugar (lactose) together with carboxylates (lactate and acetate) may allow mutual coexistence of chain elongation and dark fermentation processes. During the continuous test in an upflow anaerobic sludge blanket reactor (UASB), acid whey was used as a model feedstock due to a high concentration of lactose and lactate. Shortening hydraulic retention time (HRT) to 2.5 days allowed the co-production of hydrogen and caproate with almost complete methanogenesis inhibition. During the 50 days period, the average hydrogen and caproate production were 1.78 ± 0.75 L_{H2}/L/d and 133.4 ± 17.9 mmol C/L/d, respectively.

1. Introduction

The constantly growing demand for primary energy and depleting fossil fuel resources are conducive to the search for new energy solutions based on the renewable resources. Biorefineries producing energy and marketable bioproducts from biomass and organic waste are becoming increasingly popular (Dragone et al., 2020). The production of bulk chemicals from organic waste, however, faces the problem of economic viability, which is why a new spectrum of products is being sought. Therefore, more and more attention is paid to new solutions such carboxylate platform, i.e. open culture fermentation (OCF) leading to the formation of medium chain carboxylic acids (MCCAs) containing from 6 to 12 carbon atoms in the molecule in the process of carboxylic chain elongation (CE). The CE process is based on the oxidation of the electron donor to acetyl-CoA and then combining it with another CoA derivative, e.g. acetyl-CoA in combination with acetyl-CoA leads to the formation of butyrate, and the combination of acetyl-CoA with butyryl-CoA leads to caproate (Spirito et al., 2014). Large species diversity in OCF systems enables fermentation in non-sterile conditions, which translates into a reduction in the capital and operating costs compared to the axenic systems. Conducting the CE via OCF competes with the methanogenesis, to overcome this issue, specific methanogenic inhibitors can be added to promote the CE. However, the high cost of chemicals makes it unprofitable. Another strategy is to run the OCF at a controlled mildly acidic pH of 5.5, which leads to the inhibition of acetoclastic methanogens (Angenent et al., 2016). The production of methane is still possible by hydrogenotrophic methanogens; however, theoretically it does not cause competition for the substrate with the

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CE-conducting microorganisms (Grootscholten et al., 2014). The most popular MCCA described in the literature is caproate, containing 6 carbon atoms in the molecule. Caproate can be a precursor for the production of liquid fuels in the chemical conversion processes and together with other MCCAs can be widely used in various sectors of the industry (Wu et al., 2019). The extensive use of MCCAs could promote a carboxylate platform over a methane production.

However, there are many restrictions on caproate production that affect production stability. The biggest problems are fluctuations in the structure of the microbiome, competitive metabolic pathways and a product toxicity (toxic effect of undissociated forms of MCCAs) (De Groof et al., 2019). Therefore, the CE process is characterized by relatively high instability, which translates into reduced economic viability of the process. The solution might be a diversification of products during the OCF; however, their generation should not be mutually exclusive.

Co-production of caproate and biohydrogen may be a promising solution. Hydrogen is considered to be one of the most promising energy carriers of the future energy systems (Bundhoo and Mohee, 2016). During a dark fermentation (DF) microorganisms can produce hydrogen with acetate and butyrate as main by-products. The main obstacle in the biohydrogen production is the presence of hydrogen-consuming microorganisms such as methanogenic archaea, homoacetogenic bacteria or propionic fermenters (Castelló et al., 2020). Inhibition of methanogenic archaea is also a key to enhance the CE process. Hydrogen consumption for propionate production followed by its elongation to valerate and heptanoate is also unfavourable for caproate production because it affects the consumption of carbon that could be used to produce caproate. Another common microorganisms, which negatively affect biohydrogen production during the DF, are lactic acid bacteria (LAB). Their presence in the DF is typically associated with substrate competition between them and hydrogen producers. However, in a caproate-hydrogen co-production system, formation of lactate might be desirable due to its positive impact on the CE. In addition, the coexistence of CE and DF may enable the use of DF by-products to further form caproate. Therefore, the co-production of both products is not only possible but can also be mutually beneficial. Unintentional caproate production, during research related to hydrogen production using glucose as the main substrate, has been observed previously in (Ding et al., 2010). Nevertheless, the co-production of caproate and hydrogen has not been demonstrated so far as the two main OCF products. Their efficient co-production may prove to be a more cost-effective solution than seeking to maximize MCCAs generation, which often leads to destabilization of the process.

The main objective of this study was to conduct long-term co-production of hydrogen and caproate as the two main anaerobic OCF products. First, batch tests were carried out using a synthetic medium consisting of nutrients and various lactate, acetate and lactose concentrations in order to investigate the potential of presence of these compounds for hydrogen and caproate co-production. Then a continuous process was carried out in the upflow anaerobic sludge blanket reactor (UASB) using acid whey as a model feedstock due its high concentration of lactose and lactate. Furthermore, it was hypothesized that shortening of a hydraulic retention time (HRT) would inhibit methanogenesis and homoacetogenesis, which would enable mutual coexistence of CE and DF as leading processes in the bioreactor.

2. Materials and methods

2.1. Batch processes with a synthetic medium

2.1.1. Inoculum and substrate

Modified basal medium was prepared based on (Grimalt-Alemany et al., 2018) with the modification of carbon source solutions as indicated in Table 1.

The sludge was obtained from a 1 L UASB reactor located at the Poznan University of Technology used to conduct the CE process to MCCAs from AW as previously described in (Duber et al., 2018). Each bottle was inoculated as follows: 7.5 mL of UASB sludge was taken, centrifuged and washed with 0.9% w/v NaCl solution, then centrifuged again, resuspended in 2 mL of 0.9% w/v NaCl solution and added to the bottle.

2.1.2. Operating conditions and process configuration

The batch processes were carried out in triplicate in 500 mL serum bottles capped with butyl rubber stopper and aluminium cap. The working volume was 150 mL. Initial pH was set to 5.50 ± 0.05. Nitrogen was used to flush bottles to ensure anaerobic conditions. The bottles were kept in an incubator at 30 °C for 10 days. Liquid samples were taken daily for organic acids, alcohols and lactose analysis. Gas samples for analysis of gas composition were taken after 10 days at the end of the process. The tests were carried out in two lactate to acetate ratios (4:1 and 7.5:1 mM C/mM C) for two sums of carbon source (270 and 135 mM C). All tests were executed with or without lactose (135 mM C). A control fermentation of lactose at a concentration of 135 mM C was also carried out. Detailed initial conditions are listed in Table 1.

2.2. Continuous processes

2.2.1. Inoculum and substrate

The anaerobic sludge from Central Wastewater Treatment Plant (Poznan area, Poland) was used as an inoculum and prepared as in (Duber et al., 2018). The substrate was AW obtained from traditional quark production at the diary plant (OSM Kowalew – Dobrzyca, Poland). AW was obtained directly from the production line before reaching the collecting tank. After transporting, the AW was stored at 4 °C and used as a substrate without prior preparation. The average concentrations of lactose, lactate, acetate and ethanol in AW were 1077 mM C, 352 mM C, 37 mM C and 46 mM C, respectively. The substrate was fed into the bioreactor from a tank stored at 4 °C.

2.2.2. Operating conditions and process configuration

The process was carried out in a 1 L working volume bioreactor UASB made from cylindrical plexiglass with recirculation ensuring sludge suspension as described before in (Duber et al., 2018). The process was carried out while maintaining constant temperature of 30 °C and constant pH of 5.5 with automatic correction using 2 M NaOH. The gas production was quantified using a volumetric gas flow meter (Ritter, Germany). The process was divided into two periods depending on a HRT. During the stage I, the bioreactor was operated for 44 days and the HRT was maintained at 5 days. On day 44, HRT was shortened by half. Stage II lasted from day 45 to 127.

2.3. Analytical techniques

Details of metabolites analysis were described before (Zagrodnik et al., 2020). Briefly, analysis of gas composition (methane, carbon dioxide and hydrogen) was performed with gas chromatography with TCD detector (Shimadzu GC-2014 equipped with Porapak N column). Organic acids and alcohols concentrations were monitored by gas chromatography with FID detector (Shimadzu GC-2014 equipped with Zebron ZB-FFAP column). Concentrations of lactate and lactose were monitored with high performance liquid chromatography (Shimadzu LC-20, Rezex ROA-Organic Acid column, RI detector). Methodology and equations for the analysis were described before (Duber et al., 2018; Zagrodnik et al., 2020).

Table 1
Initial concentrations and yields of carboxylates and gases during the batch process.

Batch no.	1	2	3	4	5	6	7	8	9
Initial conditions [mmol C/L]:									
lactate to acetate ratio	–	4 : 1	7.5 : 1	4 : 1	7.5 : 1	4 : 1	7.5 : 1	4 : 1	7.5 : 1
lactate	0.0	216.0	238.2	108.0	119.1	216.0	238.2	108.0	119.1
acetate	0.0	54.0	31.8	27.0	15.9	54.0	31.8	27.0	15.9
lactose	135.0	0.0	0.0	0.0	0.0	135.0	135.0	135.0	135.0
Carboxylates yield [mmol C/mol $C_{initial}$][a]:									
acetate	118.9 ± 9.4	45.8 ± 1.0	104.1 ± 2.7	54.8 ± 19.2	117.3 ± 21.1	−86.0 ± 12.2[b]	−49.6 ± 12.1[b]	−45.5 ± 1.0[b]	−5.3 ± 6.7[b]
propionate	3.9 ± 0.0	214.0 ± 2.9	291.1 ± 1.2	247.3 ± 53.2	276.3 ± 12.4	20.9 ± 10.5	61.4 ± 2.9	9.1 ± 2.3	23.8 ± 15.7
butyrate	202.9 ± 25.0	340.9 ± 21.0	292.7 ± 45.4	323.0 ± 12.6	278.3 ± 39.2	603.0 ± 11.6	482.9 ± 28.4	585.8 ± 4.5	551.8 ± 9.6
valerate	ND.	15.2 ± 1.5	17.0 ± 0.5	17.2 ± 3.3	24.3 ± 3.8	6.0 ± 3.1	15.2 ± 0.8	ND.	ND.
caproate	ND.	11.9 ± 1.6	7.7 ± 2.2	7.7 ± 6.5	16.1 ± 8.8	31.2 ± 15.6	90.4 ± 0.4	10.4 ± 2.0	28.2 ± 18.3
Gas yield [mL/mol $C_{initial}$]:									
H2	622.9 ± 38.7	23.3 ± 2.7	29.3 ± 3.2	40.4 ± 5.4	62.3 ± 5.5	514.7 ± 14.1	466.8 ± 11.2	631.4 ± 30.9	627.7 ± 15.1
CO2	574.3 ± 25.4	368.9 ± 13.3	461.2 ± 1.4	510.5 ± 43.7	462.1 ± 5.6	553.4 ± 6.0	516.7 ± 36.6	617.0 ± 4.0	590.2 ± 18.8
CH4	ND.	ND.	ND.	ND.	ND.	ND.	ND.	ND.	ND.

ND. - not detected
[a] - yield calculated as the difference between the final concentration and the initial concentration per 1 mol C of initial substrate
[b] - negative values indicate an overall consumption of the acetate

3. Results and discussion

3.1. The composition of substrate affects the choice of metabolic pathway

Initial conditions as well as gas and carboxylates yields are presented in Table 1. Various carboxylates were produced in the batch trials with lactate and acetate as substrates. The presence of butyrate and the detectability of hydrogen at the end of the process indicated the activity of CE. Despite the use of acetate in the CE, the final acetate concentration in the trials was higher compared to the initial one. This is due to the propionate production from lactate during which acetate was formed as a by-product (27.0 mmol C acetate per 100 mmol C propionate) (Seeliger et al., 2002). Lack of methane production indicated the inactivity of methanogens during the OCF. The change in the lactate to acetate ratio slightly affected the distribution of carboxylates in the mixture. For the 7.5: 1 ratio, propionate yields were approximately equal to the butyrate yields, while for the 4: 1 ratio, butyrate prevailed in the carboxylates mixture. Changes in the amount of total initial carbon sum did not affect the product spectrum.

OCF of the control batch, containing only lactose, showed high hydrogen yields as well as the presence of butyrate and acetate which confirmed a high activity of microorganisms responsible for the hydrogen production. In addition, lactate was also detected in the effluent, indicating the activity of LAB. Low final pH (3.42 ± 0.05) probably inhibited further lactate fermentation processes. In the gas mixture methane was undetectable, which confirmed the lack of activity of methanogens during the OCF.

The use of lactose as an additional carbon source, in addition to lactate and acetate, resulted in high yields of hydrogen (from 466.8 to 631.4 mL/mol $C_{initial}$) and butyrate (from 482.9 to 603.0 mmol C/mol $C_{initial}$). Again, no methanogen activity was observed. Interestingly, in the tests with lactose as an additional carbon source, carboxylates with an even number of carbon atoms in the molecule were promoted. Propionate yields were marginal and ranged only from 9.1 to 61.4 mmol C/mol $C_{initial}$ compared to the propionate yields between 214.0 and 291.1 mmol C/mol $C_{initial}$ for the trials, in which lactate and acetate were the only carbon sources. Change of carbon sum of lactate and acetate from 270 mM C to 135 mM C, while maintaining the same lactate to acetate ratio and the same concentration of initial lactose, did not change the spectrum of products in any case, which also suggested that the process redirection was affected by the lactose addition and not by the total available initial carbon. The decrease in the activity of propionate formation contributed to the elimination of the acetate accumulation. The observed lower final acetate concentration compared to the initial one suggested that the acetate supplied to the process was used in the CE process.

The trials proved that hydrogen co-production with compounds that enabled the CE to caproate was possible. Lack of caproate production in the batch processes could be explained by short fermentation times or rapid lactate consumption to form butyrate. Lactose as an additional carbon source, besides lactate and acetate, not only influenced the production of hydrogen but also directed the production to carboxylates with an even number of carbon atoms in the molecule. It was shown that not only the presence of electron donors and acceptors affected the selection of the metabolic pathway, but also the availability of other compounds i.e. lactose. Thus, the composition of the substrate might affect the selection of metabolic pathway for the co-production of bioproducts. Waste streams such as AW which consist mainly of lactose and lactate may be a real promising substrates for the co-production of caproate and hydrogen.

3.2. Inhibition of methane production in the carboxylate platform led to co-production of caproate and hydrogen during continuous process

Stage I lasted from day 0 to 44. The first 15 days constituted the start-up during which the total carboxylates concentration raised to 777.8 mM C (Fig. 1A). In the following days the production of total carboxylates stabilised and their concentration ranged from 777.8 mM C to 939.5 mM C. The production of short chain carboxylic acids was predominated throughout the entire period with butyrate and acetate as the main products. Caproate was also produced from the beginning of the process, which proved the activity of microorganisms responsible for CE. However, its concentration and specificity were relatively low (maximum concentration and specificity were 144.7 mM C and 15%, respectively). The gaseous products detectable in the gas mixture were carbon dioxide and methane (the maximum production rate was 3.91 L_{CO2}/L/d and 1.99 L_{CH4}/L/d, respectively) (Fig. 1B). Hydrogen produced during the OCF could be immediately consumed by hydrogenotrophic methanogens as well as homoacetogenic bacteria, which could also be responsible for a high acetate concentration (Saady, 2013). Carboxylates with an odd number of carbon atoms in the molecule were also produced, which indicated the activity of propionic

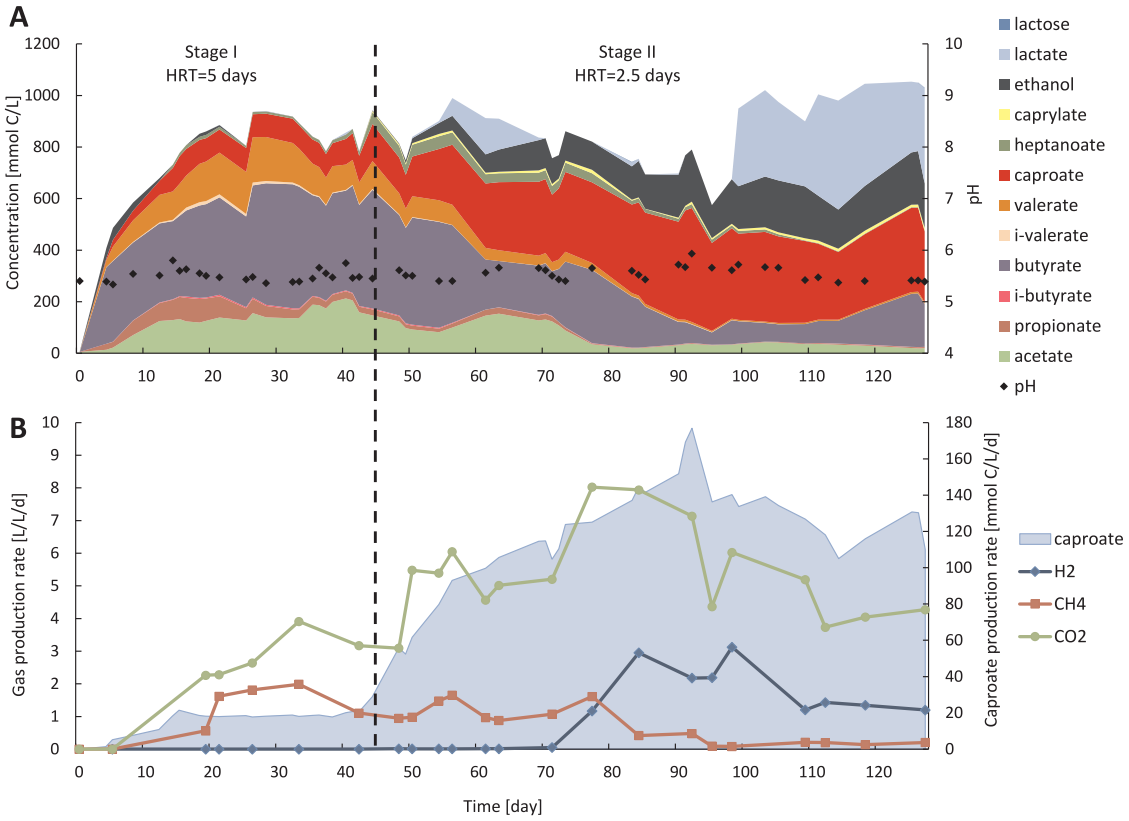

Fig. 1. The concentration profile of carboxylates, ethanol, lactose as well as pH (A) and gas production rates together with a caproate production rate (B) in the UASB reactor.

fermentation, followed by the CE of propionate to valerate and heptanoate. In order to achieve high H_2 yield, it is necessary to inhibit the microorganisms responsible for hydrogen consumption. One way to eliminate the effects of hydrogenotrophic methanogens is to wash them out of the system by reducing HRT (Grootscholten et al., 2013). Therefore, on day 44 the HRT was reduced to 2.5 days.

Stage II lasted from day 45 to 127. The change in a HRT resulted in a redirection of the process to MCCAs. Butyrate production began to decline, while caproate production increased. The MCCAs specificity increased starting from 21% on day 45 and reaching 80% on day 95. On days 71 to 77, a temporary increase in butyrate production from 69.7 to 113.9 2 mmol C/L/d was observed. It could be due to a higher use of acetate in CE, which translated to decrease in acetate production on those days from 49.5 to 14.0 mmol C/L/d. From day 77, acetate production averaged only 11.9 ± 3.2 mmol C/L/d. Along with the increased production of caproate, ethanol production appeared in the bioreactor. In addition, the production of carboxylates with an odd number of carbon atoms in the molecule decreased (from 58.0 mmol C/L/d on day 45 to 11.7 mmol C/L/d on day 83 and 4.4 mmol C/L/d at the end of the process). From day 71, hydrogen begun to be detectable in the gas mixture. It could have been influenced by the decreased activity of hydrogenotrophic methanogens, which translated into a decrease in the consumption of hydrogen and CO_2 (Fig. 1B). On the 84th day, methane production was almost completely inhibited (0.42 L_{CH4}/L/d). Washing out methanogens during caproate production has been observed in the literature at much lower HRT than in our studies (Grootscholten et al., 2013); however, the methanogens growth may depend on the substrate used, reactor type and operational parameters. In the following days, methane production averaged 0.24 ± 0.15 L_{CH4}/L/d. The reduction of HRT and conducting the process at the acidic pH led to almost complete inhibition of of both acetoclastic and hydrogenotrophic methanogens. On the 92nd day, the highest productivity of caproate was achieved (176.9 mmol C/L/d) along with high hydrogen production (2.18 L_{H2}/L/d). The electron recovery for caproate and hydrogen for day 92 of the process was 45.8%. On that day, the increase in ethanol production also ended and amounted to 80.5 mmol C/L/d. The decrease in total carboxylates and caproate production later in the process as well as the accumulation of lactate might have been affected by the toxic effect of the accumulated products. During the 50 days period (from 78 to 127 day) the average hydrogen and caproate production of 1.78 ± 0.75 L_{H2}/L/d and 133.4 ± 17.9 mmol C/L/d was achieved, respectively.

Co-production of MCCA and hydrogen from AW in one bioreactor can be an alternative to the recently proposed solution for the production of MCCA from AW by using two-stage fermentation with maximization of lactate production under thermophilic conditions and CE under mesophilic conditions (Xu et al., 2018). However, further research, in particular regarding downstream processing, is needed to achieve detailed economic viability.

4. Conclusions

The presence of lactose in the medium along with lactate and acetate enabled the co-production of hydrogen and compounds involved in CE as well as reduced the activity of propionic fermentation. In addition, trials proved that the presence of lactose influenced CE to butyrate selection. Promising co-production of hydrogen and caproate in an OCF system was achieved from AW in the UASB reactor after shortening the HRT, during 50 days of co-production, almost no methane formation was observed.

CRediT authorship contribution statement

Filip Brodowski: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Anna Duber:** Conceptualization, Investigation, Formal analysis, Writing - review &

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.123895.

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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

- Filip Brodowski, lead author: conceptualised and conducted the research, interpreted the results and wrote the manuscript.
- Mateusz Łężyk: conceptualised the research, performed the microbial composition analysis, contributed to the interpretation of the results and reviewed the manuscript.
- Natalia Gutowska: performed chromatographic analyses, contributed to the interpretation of the results and reviewed the manuscript.
- Piotr Oleskowicz-Popiel: supervised the study, supported the interpretation of the results and reviewed the final version of the article.

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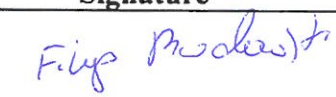
Filip Brodowski	
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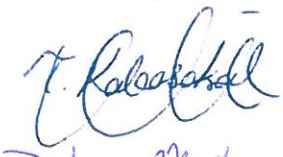
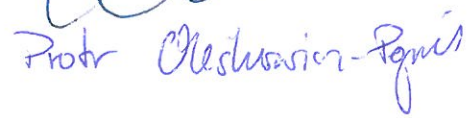
Tugba Kabasakal	
Piotr Oleskowicz-Popiel	

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