Poznan University of Technology

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PhD thesis

Process operating parameters in open culture fermentation for short and medium chain carboxylic acids production

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I believe that results of my work will help to create healthy future for the young generation.

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List of publications chosen as the base for the PhD thesis

According to "Ustawa z dnia 14 marca 2003 r. o stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki (Dz. U. 2003 Nr 65 poz. 595)" Art. 13, ust. 2. „***Rozprawa doktorska może mieć formę*** *maszynopisu książki, książki wydanej lub spójnego tematycznie zbioru rozdziałów w książkach wydanych,* ***spójnego tematycznie zbioru artykułów opublikowanych lub przyjętych do druku w czasopismach naukowych****, określonych przez ministra właściwego do spraw nauki na podstawie przepisów dotyczących finansowania nauki, jeżeli odpowiada warunkom określonym w ust. 1".*

1. **Ewelina Jankowska**, Anna Duber, Joanna Chwiałkowska, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *Conversion of organic waste into volatile fatty acids – The influence of process operating parameters.* Chemical Engineering Journal 2018, 345: 395-403. 5-year Impact Factor 6.496, 45 MNiSW points, individual input: 60%.
   Ewelina Jankowska was responsible for planning process, chemical analysis, laboratory trials, data gathering and analyzing, preparation of tables and figures, writing the manuscript.

2. **Ewelina Jankowska**, Ashish K. Sahu, Piotr Oleśkowicz-Popiel, *Biogas from microalgae: Review on microalgae`s cultivation, harvesting and pretreatment for anaerobic digestion.* Renewable and Sustainable Energy Reviews 2017, 75: 692-709. 5-year Impact Factor 10.893, 45 MNiSW points, individual input: 85%.
   Ewelina Jankowska was responsible for reviewing scientific papers, data gathering and analyzing, preparation of tables and figures and writing the manuscript.

3. **Ewelina Jankowska**, Joanna Chwiałkowska, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *Volatile fatty acids production during mixed culture fermentation – The impact of substrate complexity and pH*. Chemical Engineering Journal 2017, 326: 901-910. 5-year Impact Factor 6.496, 45 MNiSW points, individual input: 65%.
   Ewelina Jankowska was responsible for planning process, chemical analysis, laboratory trials, data gathering and analyzing, preparation of tables and figures, writing the manuscript.

4. **Ewelina Jankowska**, Joanna Chwiałkowska, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *Effect of pH and retention time on volatile fatty acids during mixed culture fermentation.* Bioresource Technology 2015, 190: 274-280. 5-year Impact Factor 5.978, 45 MNiSW points, individual input: 40%.
   Ewelina Jankowska was responsible for planning process, chemical analysis, laboratory trials , data analyzing and writing the manuscript.

1. Abstract

The commercialized production of carboxylic acids is based on chemical routs that transform crude oil. The extensive use of the non-renewable fossil resources, which led to negative climate consequences, led to the idea of converting renewable resources into commodity chemicals. One of the most promising way to produce carboxylic acids is to convert organic waste or biomass via open culture fermentation.

The main aim of presented doctoral thesis was to analyze the impact of process operating parameters in open culture fermentation for short and medium chain carboxylic acids production. The impact of process parameters (initial pH, type of substrates, retention time and organic loading rate) on concentration and type of produced carboxylic acids, also the interaction between process performance and microorganisms in open culture were analyzed. Basing on obtained results the key parameters and process conditions were defined. Moreover, the calculation of process conversion efficiency i.e. yields of hydrolysis and acidification, allowed to determine the ability of open culture fermentation to transform organic wastes to carboxylic acids.

The results of fermentation of sludge mixture in wide range of initial pH and during three retention times were described in the first publication ***Effect of pH and retention time on volatile fatty acids production during mixed culture fermentation*** (**Jankowska E.,** Chwiałkowska J., Stodolny M., Oleśkowicz-Popiel P., Bioresource Technology, 2015, 190:274-280). Obtained results indicated that initial alkaline pH enhanced the production and accumulation of volatile fatty acids (VFAs) (mainly acetate). Initial alkaline pH enhanced the hydrolysis rate resulting with higher concentration of VFAs. However, mixture of VFAs produced at initial acidic conditions was more diverse. The methanogenesis and biogas production dominated when process run in neutral pH. The second analyzed factor was retention time, however its influence on fermentation was not as significant as the pH. Longer time of reaction enhanced the chain elongation to medium carboxylic acids.

The impact of substrates complexity and composition on concentration and variety of produced VFAs was verified during fermentation in different initial pH (acidic, neutral and alkaline) and three retention times. Based on the results presented in ***Volatile fatty acids production during mixed culture fermentation – The impact of substrate complexity and pH*** (**Jankowska E.,** Chwiałkowska J., Stodolny M., Oleśkowicz-Popiel P., Chemical Engineering Journal, 2017, 326: 901-910) was found that type and composition of applied substrates (i.e. acid whey, mixture of sludge, maize silage, microalgae biomass) had not significantly affected the type of produced VFAs. The process conversion efficiencies i.e. yields of hydrolysis, acidification and biomass were determined. It occurred that the key parameter that significantly influenced the performance of applied

fermentation process was pH. It had a direct influence on hydrolysis yield, higher diversity of VFAs produced in initial acidic conditions and their intensified production and accumulation in neutral conditions i.e. with inhibited methanogenesis. It was found that analyzed system had some buffering capacity resulting with self-maintenance of pH.

The results of fermentation of acid whey and sludge mixture in semi-continuous trials and initial acidic conditions were described in ***Conversion of organic waste into volatile fatty acids – The influence of process operating parameters*** (**Jankowska E.,** Duber A., Chwiałkowska J., Stodolny M., Oleśkowicz-Popiel P., Chemical Engineering Journal, 2018, 345: 395-403). Obtained results indicated that type of produced VFAs mainly depend on pH and microorganisms supplied within substrate added daily to the reactor. The microbial analysis revealed that retention time influenced the biodiversity of microbial community. In longer time microorganisms originated from substrate were able to adapt to the applied bioreactor conditions and to dominate its microbial community, while fermentation time was shortening. The application of simultaneous changes of retention time and organic loading rate had not direct impact on concentration and diversity of produced VFAs, but partly by involving changes in pH and in microorganisms biodiversity. Results of process conversion efficiency analyzing revealed that acidification yield decreased with increasing hydrolysis yield and in contrary, hydrolysis yield decreased while acidification was increasing. Moreover, that decrease in both yields and finally the process inhibition in short retention time is a result of bioreactor overloading.

The results of described research showed the possibility of application of organic waste as a natural and renewable carbon source to production of short and medium chain carboxylic acids in biological rout based on open culture fermentation. The efficiency of fermentation process depends on biodegradability of applied substrates, which is affected by the time of fermentation and pH - the key parameter that verifies quantity and variety of produced mixture of carboxylates.

The ***Biogas from microalage: Review on microalgae`s cultivation, harvesting and pretreatment for anaerobic digestion*** (**Jankowska E.,** Kumar A.K., Oleśkowicz-Popiel P., Renewable and Sustainable Energy Reviews, 75: 692-709) is a comprehensive review on biogas production from microalgae biomass. The limitations of the process implementation and commercialization were discussed and it occurred, that microalgae anaerobic digestion should be incorporated with production of other bioproducts such as bioethanol, biodiesel or volatile fatty acids. Thus, in the concept of biorefinery the anaerobic digestion should be the last step, that utilize the residues of biomass.

The presented results indicated that process of open culture fermentation can be used for effective production of short and medium carboxylic acids. Chemical and biological analysis

revealed, that it is possible to produce mixture of VFAs with similar composition despite the substrate type. The novelty of presented research is the analysis of open culture fermentation efficiency in wide range of initial pH without addition of significant amounts of chemicals to control the pH conditions of the process as well as application of maize silage, acid whey and microalgae biomass as substrates for the short and medium chain carboxylic acids production.

2. Streszczenie

Obecnie wytwarzanie kwasów karboksylowych opiera się na przetwarzaniu ropy naftowej. Intensywna eksploatacja nieodnawialnych źródeł energii wpływa negatywnie na środowisko wywołując znaczne zmiany klimatyczne. Najbardziej obiecującym rozwiązaniem jest wykorzystanie odnawialnych źródeł węgla (zawartego w odpadach organicznych i biomasie) i przetwarzanie go do kwasów karboksylowych w procesie fermentacji z użyciem otwartej kultury bakterii.

Celem niniejszej pracy była analiza wpływu parametrów operacyjnych procesu fermentacji z użyciem kultur otwartych na produkcję krótko- i średnio-łańcuchowych kwasów karboksylowych. Analizowano wpływ wybranych parametrów (pH, rodzaj substratu, czas trwania procesu oraz obciążenie materią organiczną) na ilość i rodzaj wytwarzanych kwasów karboksylowych oraz interakcję pomiędzy parametrami procesu a otwartą kulturą bakteryjną. Wyniki badań pozwoliły na określenie kluczowych parametrów procesu i zakresu wytwarzania poszczególnych kwasów karboksylowych oraz na określenie efektywności procesu degradacji odpadów organicznych do kwasów karboksylowych (poprzez określenie tempa hydrolizy i acydyfikacji).

W pierwszej publikacji ***Effect of pH and retention time on volatile fatty acids production during mixed culture fermentation*** (**Jankowska E.,** Chwiałkowska J., Stodolny M., Oleśkowicz-Popiel P., Bioresource Technology, 2015, 190:274-280) przedstawiono fermentację mieszaniny osadów wstępnego i nadmiernego w szerokim zakresie pH w trzech przedziałach czasowych. Uzyskane wyniki wykazały, że początkowe warunki zasadowe sprzyjały wytwarzaniu i akumulacji kwasów karboksylowych (głównie kwasu octowego). Co więcej, wstępne warunki zasadowe umożliwiły intensywną hydrolizę zastosowanego substratu, a to bezpośrednio przełożyło się na wysokie stężenie uzyskanych kwasów. Natomiast początkowe kwaśne środowisko pozwoliło na większe zróżnicowanie kwasów w wyprodukowanej mieszaninie. W środowisku neutralnym proces został zdominowany przez metanogenezę i produkcję biogazu. Drugim badanym parametrem procesu był czas retencji, jednak jego wpływ nie był tak widoczny, jak w przypadku zmiany pH. Dłuższy czas fermentacji pozwolił na wytwarzanie średnio-łańcuchowych kwasów karboksylowych.

Zweryfikowanie wpływu rodzaju substratu na ilość i rodzaj wytworzonych kwasów karboksylowych w trzech początkowych warunkach pH oraz trzech przedziałach czasowych stanowiły cel badań zaprezentowanych w drugiej publikacji ***Volatile fatty acids production during mixed culture fermentation – The impact of substrate complexity and pH*** (**Jankowska E.,** Chwiałkowska J., Stodolny M., Oleśkowicz-Popiel P., Chemical Engineering Journal, 2017, 326: 901-910). Na podstawie zrealizowanych badań stwierdzono, że rodzaj i skład zastosowanego substratu (serwatka kwaśna, mieszanina osadów ściekowych, kiszonka kukurydziana, biomasa mikroglonów) nie wpływają

znacząco na rodzaj wytwarzanych kwasów. Obliczono również wydajność procesu w tym tempo hydrolizy, wytwarzania kwasów oraz produkcji biomasy. Głównym parametrem sterującym przebiegiem procesu fermentacji był odczyn pH. Wpływał on bezpośrednio na tempo hydrolizy, większą różnorodność kwasów wytworzonych w początkowym środowisku kwaśnym oraz intensywną produkcję i akumulację w środowisku neutralnym (tj. przy inhibicji metanogenezy). Stwierdzono również zdolność układu do buforowania i regulacji pH.

Wyniki fermentacji serwatki kwaśnej oraz mieszaniny osadów ściekowych prowadzonych w trybie pół-ciągłym i początkowym środowisku kwaśnym zostały opisane w pracy ***Conversion of organic waste into volatile fatty acids – The influence of process operating parameters*** (**Jankowska E.,** Duber A., Chwiałkowska J., Stodolny M., Oleśkowicz-Popiel P., Chemical Engineering Journal, 2018, 345: 395-403). Otrzymane wyniki wykazały, że rodzaj produkowanych kwasów karboksylowych w niewielkim stopniu zależy od rodzaju i składu zastosowanego substratu, natomiast główną rolę odgrywają pH oraz mikroorganizmy dostarczane ze świeżą porcją substratu, które są w stanie zdominować kulturę zaszczepioną do reaktora. Analiza mikrobiologiczna wykazała wpływ czasu retencji na bioróżnorodność organizmów. Równoczesne zmiany czasu retencji oraz ilości materii organicznej dostarczanej codziennie do reaktora nie wpłynęły bezpośrednio na ilość i rodzaj wytwarzanych kwasów, ale pośrednio poprzez oddziaływanie na zmiany pH oraz zróżnicowanie mikroorganizmów. Na podstawie obliczonego tempa hydrolizy i formowania kwasów stwierdzono, że przy zwiększonym tempie hydrolizy maleje tempo wytwarzania kwasów i odwrotnie, wysokiemu tempu acydyfikacji towarzyszy mniej intensywna hydroliza. Co więcej, w przypadku przeciążenia reaktora ładunkiem organicznym i przy krótkim czasie retencji efektywność obu procesów maleje.

Otrzymane wyniki wykazały możliwość wykorzystania odpadów organicznych jako źródła węgla do produkcji krótko- i średnio-łańcuchowych kwasów karboksylowych w procesie biologicznej konwersji z użyciem kultur otwartych. Efektywność procesu wytwarzania kwasów zależy od podatności na biodegradację zastosowanych substratów, co bezpośrednio łączy się z czasem trwania fermentacji oraz zakresem pH będącym głównym czynnikiem warunkującym ilość i rodzaj wytwarzanych kwasów.

Publikacja ***Biogas from microalage: Review on microalgae`s cultivation, harvesting and pretreatment for anaerobic digestion*** (**Jankowska E.,** Kumar A.K., Oleśkowicz-Popiel P., Renewable and Sustainable Energy Reviews, 75: 692-709) stanowi kompendium wiedzy dotyczącej produkcji biogazu z biomasy mikroglonów. Wnikliwa analizy wyników badań oraz określenie ograniczeń przeciw wdrożeniu i komercjalizacji tego procesu, wykazały, że produkcja biogazu z biomasy mikroglonów nie powinna być głównym sposobem wykorzystania ich potencjału. Tak wartościowy

substrat powinien być przetwarzany w myśl koncepcji biorafinerii do wielu produktów (np. barwników, antyoksydantów, biodiesla, bioetanolu i kwasów karboksylowych), a produkcja biogazu powinna stanowić jej ostatni etap.

Uzyskane wyniki badań potwierdzają, że proces fermentacji z użyciem otwartej kultury bakterii może być zastosowany jako efektywna metoda produkcji krótko- i średnio-łańcuchowych kwasów karboksylowych. Ponad to, przeprowadzone analizy chemiczne i biologiczne wykazały możliwość otrzymania mieszaniny kwasów karboksylowych o podobnym składzie bez względu na rodzaj zastosowanego substratu. Wartością dodaną niniejszej pracy jest przede wszystkim analiza procesu fermentacji z użyciem otwartej kultury bakterii w szerokim zakresie pH początkowego bez stosowania ciągłej jego kontroli. Co więcej, zastosowanie jako głównego substratu kiszonki kukurydzianej, serwatki kwaśnej i biomasy mikroglonów oraz przeprowadzenie szerokiej analizy chemicznej wyprodukowanej mieszaniny kwasów pod względem zawartości atomów węgla (od dwóch do siedmiu) przyczyniło się do poszerzenia wiedzy z zakresu prowadzenia procesów fermentacyjnych z użyciem otwartych kultur mikroorganizmów.

3. Abbreviations

BOD – biological oxygen demand

COD – chemical oxygen demand

TS – total solids

VS – volatile solids

TCOD – total chemical oxygen demand

SCOD – soluble chemical oxygen demand

GC – gas chromatography

TN – total nitrogen

TP – total phosphorus

PCR-DGGE – polymerase chain reaction – denaturing gradient gel electrophoresis

CSTR – continuously stirred tank reactor

VFAs – volatile fatty acids

AD – anaerobic digestion

HRT – hydraulic retention time

OLR – organic loading rate

RT – retention time

4. Introduction

The rapid growth in the human population and the global economy has led to massive waste generation as well as increased demand and limited availability of natural resources. Proper waste management is crucial to minimize further degradation of the environment and to support the transition to a sustainable society. The conventional waste management hierarchy is treatment-oriented (Fig.1), the most preferred option is prevention, however the most promising is based on resource recovery. To achieve it, the non-renewable fossil resources should be systematically replaced with renewable and sustainable materials (i.e. biomass and waste) (Bastidas-Oyanedel et al., 2015).

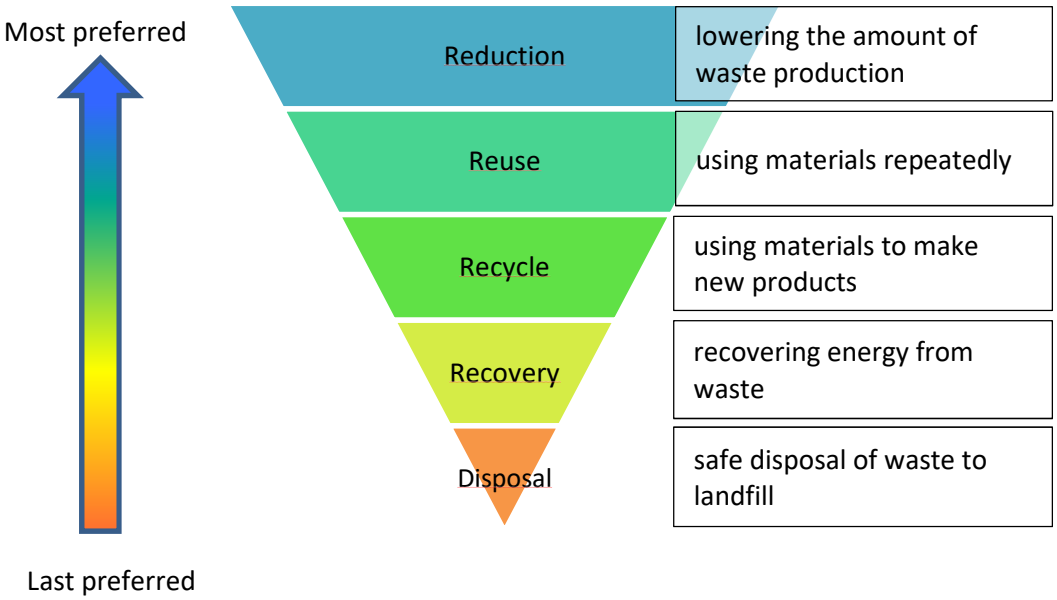

Fig. 1. The waste management hierarchy.

Biomass and organic wastes generated from industrial and agricultural sectors is a low-cost feedstock and a sustainable renewable resource for the production of value-added, carbon based products (e.g. chemicals, materials and liquid fuels) (Fava et al., 2015; Liang and Wan, 2015). The replacement of carbon from fossil fuels with carbon from biomass and/or wastes could be achieved by the biorefinery system (Fava et al., 2015). According to (Fava et al., 2015) the biorefinery is an integrated biobased facility that uses a variety of technologies to generate products such as chemicals, biofuels, food and feed ingredients, fibers, biomaterials, heat and power, tending to maximization of value-added products. It is built around three pillars of sustainability i.e. environment, economy and society. The biorefinery potential in Europe is tremendous, since it could be supplied with both organic waste and lignocellulosic biomass. Organic waste streams are mainly composed of agricultural waste, yard and forestry waste, sludge, food processing waste and organic

household waste (Fava et al., 2015). Their processing into valuable biocompound and/or bioproducts in biorefinery is based on integration of biological and chemical or physical processes. The biobased products could be further used in modern chemical, textile, energy, food/feed, pharmaceutical and cosmetic industry (Fig.2). The factors that influence their destination application are the origin, non-toxicity and biodegradability (Fava et al., 2015). The worldwide market of bioproducts is increasing remarkably. It was 77 billion € in 2005, 92 billion € in 2010 and 228 billion € in 2015 and it is anticipated that would increase to 515 billion € in 2020 (Festel, 2008).

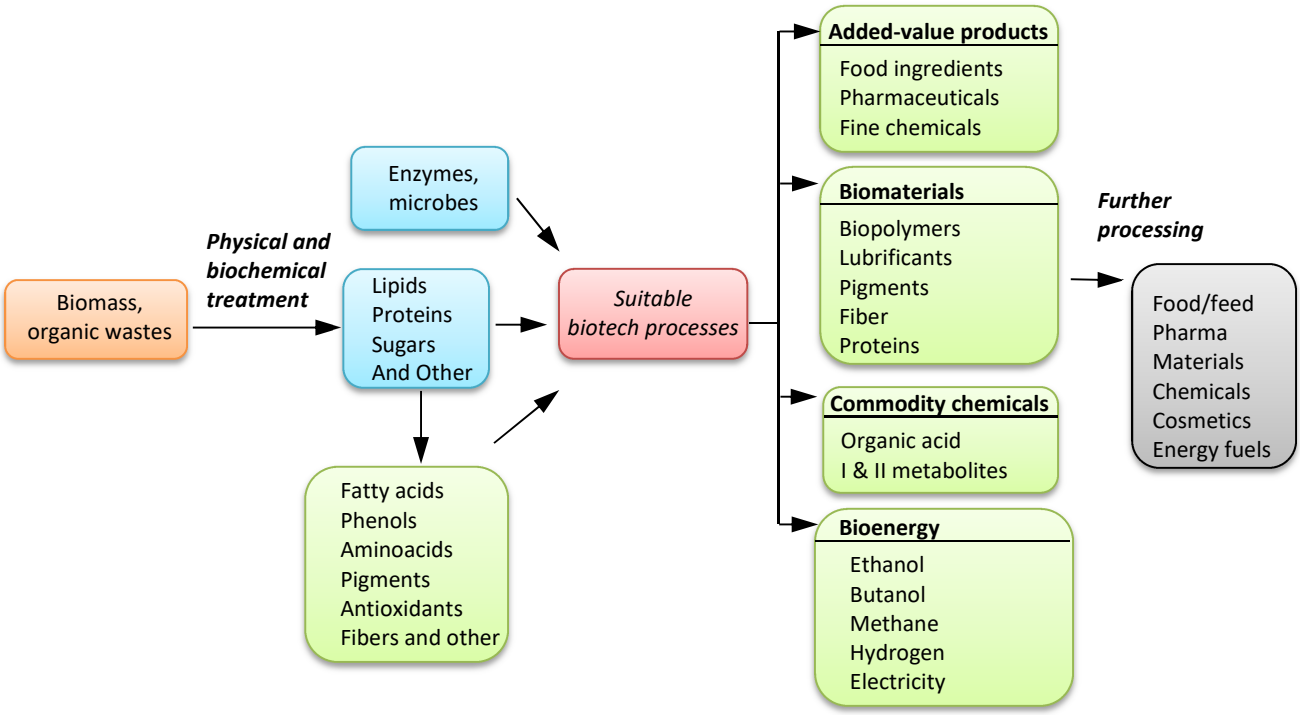

Fig. 2. The biorefinery approach: bioproducts and their application.

Independently of the applied substrate, the possible steps of biorefinery consist of: a) feedstock handling including enzymatic pretreatment, extraction and/or recovery of high value compounds, b) biotechnological conversion into tailored biocompounds, c) remaining effluents could be further processed into biofuels (e.g. ethanol, methane, or hydrogen), d) the final residues and effluents could be used as fertilizer in focus for returning the nutrients to the soil (Fava et al., 2015; Federici et al., 2009). Despite the variety of bioproducts, the constraints in commercialization of waste-based biorefineries are significant, i.e.: seasonality of available biomass, the necessity of biomass sorting, high costs of biotechnologies, energy balance depending on dilution of streams, costly and complex downstream processing (separation and purification of complex wastes) and extraction of products from heterogeneous outputs (Fava et al., 2015). The strategy of waste-based

biorefineries will become more attractive, but the research and development of fermentation process still need to be more intensive.

In this thesis, the author focused on the bio-based conversion of organic waste into short and medium chain carboxylic acids in open culture fermentation. The conventional and commercialized production of carboxylic acids is based on non-renewable crude oil conversion (i.e. chemical routs). The answer to depletion of non-renewable fossil fuels was the transition from chemical routs to biological routs, where renewable carbon sources might be used. One of the most promising new methods are the pure culture fermentation and open (mixed) culture fermentation. The production of carboxylic acids in pure culture fermentation requires sterile operating conditions and substrates of high quality and/or high purity. Proper and desired products of high quality can be extracted and used in pharmaceutical, food or cosmetic industry (Lu et al., 2011). However, to enhance the transformation into sustainable society, where the resources are recovered instead of wasted, the transition from conventional substrates (often competitive to food and feed production) into waste-origin substrate is needed. Recently, there has been strong research effort to develop bioprocess using open cultures of microorganisms that naturally co-exist in the environment. The open cultures can tolerate complex and variable substrates due to the metabolic flexibility (Agler et al., 2011). The other advantage over pure culture is that open cultures can grow under non-sterile and anaerobic conditions, thus the costs of sterilization and aeration can be eliminated (Liang and Wan, 2015).

5. Motivation and aim of the work

The aim of the presented thesis "Process operating parameters in open culture fermentation for short and medium chain carboxylic acids production" was schematically presented in Fig. 3; it was divided into three main themes:

1) analysis of several operating process parameters: pH, time, type of substrate, organic loading on the quantity and quality of produced mixture of carboxylic acids;
2) determination of the key process parameters;
3) determination of interdependence between the process operating parameters, the open microbial culture and the quality and quantity of carboxylic acids.

The main hypothesis of presented work is that by changing the operational parameters of open culture fermentation it will be possible to produce desired mixture of medium and short chain carboxylic acids from organic wastes and/or biomass.

Moreover, the scientific objective was to broaden the knowledge about the open culture fermentation process and to characterize the interdependence between substrates, microorganisms and final products. It would aid at creating a new concept of organic waste management by the recovering of the organic carbon and converting it into commodity biochemicals.

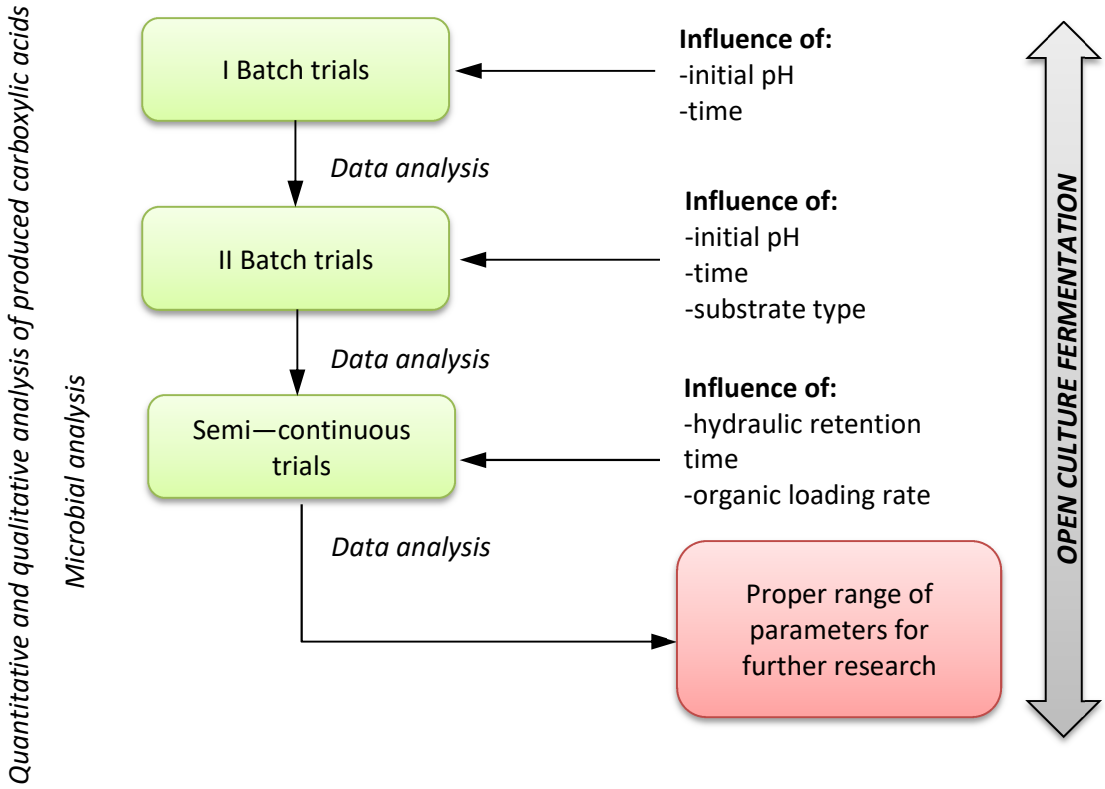

Fig. 3. Schematic representation of the objectives of PhD thesis.

6. Methodology

6.1. Substrates characterization

The seeding sludge (i.e. inoculum) used in all batch and semi-continuous trials was collected from full scale mesophilic anaerobic digester of municipal Wastewater Treatment Plant located in Kozieglowy (Poznan, Poland). Prior inoculation it was stored in mesophilic conditions for degassing and maintain the microbial activity (**Paper I**, **II** and **III**).

During the laboratory trials, five substrates were used: sludge mixture, acid whey, maize silage, microalgae biomass and glucose.

The sludge mixture (further named sludge) consisted of primary sludge and waste activated sludge mixed in ratio 1:1: (by weight). Both sludge were collected from local Wastewater Treatment Plant in Kozieglowy (Poznan, Poland) from primary and secondary settling tank, respectively. Prior usage sludge was stored at 4^{o}C. Sludge was used as a substrate in the first set of batch trials (**Paper I**) and in the semi-continuous trials (**Paper III**).

Acid whey was collected from Dairy in Dobczyca (OSM Kowalew–Dobrzyca, Poland) after the production of traditional quark. It was stored at -18^{o}C and prior usage was unfreezed and mixed. It was used in the second set of batch trials (**Paper II**) and in the semi-continuous trials (**Paper III**).

The maize silage was collected from the local agricultural farm and used in the second set of batch trials (**Paper II**). It was stored at -18^{o}C and before usage the required amount was unfreezed and milled in a mortar.

The microalgae biomass was collected from the pilot plant at the Wastewater Treatment Plan in Kozieglowy (Poznan, Poland). The effluent from the Anammox reactor was used as a cultivation medium for the photobioreactor. It was inoculated with microalgae collected from secondary settling tank. Biomass consisted mainly of *Scenedesmus quadricadua* and *Chlorella vulgaris.* Prior fermentation biomass was pretreated by thermal method in accordance to **Paper IV** and (Passos and Ferrer, 2014). Thermally pretreated microalgae biomass was used in the second set batch trials (**Paper II**).

The 1% glucose solution was used as a simple and model substrate in the second batch trials (**Paper II**). In the semi-continuous trials (**Paper III**), the glucose solution (4 g/L) supplied with nutrients and mineral compounds (according to Temudo et al., 2007) was used as a substrate in the bacterial community profiling test.

6.2. Analytical methods

The total solids (TS) and volatile solids (VS) were measured according to the Polish standard method (PN-EN-12879) in inoculum, substrates and effluent after fermentation (**Paper I**, **II**, **III**). The analysis was performed directly after sampling. The initial concentration of TS and VS was used to determine the amount of substrate (to obtain the same organic loading for all tested substrates) in the reactors and also to express the amount of carboxylic acids formed from 1 g of added VS (**Paper I, III**).

The total and soluble chemical oxygen demand (TCOD, SCOD) were measured according to the Polish standard method (PN-74/C-04578/03) in inoculum, substrates and effluent after fermentation (**Paper I**, **II**, **III**). The initial concentration of TCOD and SCOD were used to express the amount of carboxylic acids formed from 1 g of added COD (**Paper II**, **III**). It was also used for biomass yield, hydrolysis yield and acidification yield calculations (**Paper II**, **III**) in accordance to (Bengtsson et al., 2008; Wu et al., 2016).

The composition of short and medium chain carboxylic acids and produced gas were analyzed using the gas chromatography equipped with flame ionization detector and thermal conductivity detector, respectively (GC, Shimadzu, Japan). The description of liquid samples preparing prior analysis, gas sampling and chromatographs description and parameters of analyzing process with accordance to (Vasquez et al., 2014) were presented in **Paper I**, **II** and **III**. The following short and medium chain fatty acids were analyzed: acetic, propionic, butyric, i-butyric, valeric, i-valeric, caproic, heptanoic and caprylic. During gas analysis the concentration of methane, carbon dioxide and hydrogen was measured (**Paper II**). Results of chromatography analysis were shown in figures included in **Paper I**, **II** and **III** and were used for biomass yield, hydrolysis yield and acidification yield calculation (**Paper II**, **III**).

The concentration of total nitrogen (TN) and total phosphorus (TP) was measured according to a Merck procedure No. 1. 14763.0001 and No. 1. 14428.0002 for nitrogen and phosphorus, respectively. Those results were used for substrates characterization (**Paper I**, **II**) and for calculation their reduction during open culture fermentation (**Paper II**).

The microbial analysis contained DNA extraction, polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing, in accordance with (Nübel et al., 1996; Regueiro et al., 2015). The structural diversity was estimated by the Shannon-Weaver index (*H`*), in accordance with (Ciesielski et al., 2013). The range-weighted richness (Rr) was calculated in accordance with (Carballa et al., 2011) as the total number of bands in each line. The community

organization (Co) was calculated as the percentage of Gini coefficient (Marzorati et al., 2008; Wittebolle et al., 2009). Detailed description was presented in **Paper III**. Obtained results i.e. PCR-DGGE profiles of bacterial community and alignment of 16S rRNA gene sequences of DGGE bands were shown in figures and tables in **Paper III**.

The statistical analysis of variance (ANOVA) was applied to investigate the impact of pH and retention time on the concentration of produced medium and short carboxylic acids. The one-way (impact of pH and retention time alone) and two-way (impact of pH together with retention time) ANOVA was used. Results were shown on the figures and tables in **Paper I**.

6.3. Open culture fermentation process characterization

The open culture fermentation process was carried out in batch and semi-continuous modes. All processes were run in mesophilic conditions (35^{o}C). The batch trials (**Paper I**, **II**) (i.e. trials, where substrate is added only at the beginning of the process, were performed in OxiTop Control system (WTW, Germany) in 12 identical glass reactors. The working volume was 60 mL, where 30 mL was seeding sludge and the rest was substrate (calculation based on volatile solids loading) filled with distilled water in order to made up to 60 mL of the total working volume. The exact amounts of added substrates (corresponding with initial substrate concentration of 0.5 g VS/L) are described in the Materials and Methods sections in **Papers I** and **II**. All processes were carried out in triplicates with blank and control reactors. The initial pH was adjusted using 18% HCl and 3M KOH. In experimental reactors with neutral pH the specific methanogenesis inhibitor was added (**Paper II**) in accordance to (Zinder et al., 1984). To assure anaerobic conditions, each reactor was flushed with the mixture of carbon dioxide and nitrogen for 1 minute (20/80 vol/vol) according to (Angelidaki et al., 2009). The gas pressure in bottles was monitored by gas sensors (equipment of OxiTop Control system). After chosen time of fermentation i.e. 5, 10 and 15 days, the process was terminated and the samples for chemical analysis were taken. The rest of fermentation broth was frozen for further analysis. The gas samples for chromatography analysis were taken by gas-tight syringe with valve. The samples were immediately injected in the GC-TCD for analysis. The experimental design and characteristics of each fermentation trial was described in **Papers I** and **II**.

In a continuous process substrate (feed) constantly flows into the reactor and effluent (product) constantly flows out of the reactor. This transition is distributed in time and controlled by pumps. The feed is added from the top and product is removed from the bottom of the reactor. In the semi-continuous process the removal of a product and addition of a substrate is done manually and contrary to continuous process it occurred separately, thus at first proper amount of a product has to be removed, then corresponding amount of fresh substrate replaces it. The proper amount is an

amount of substrate/product resulted from hydraulic retention time (HRT). Longer HRT means lower amounts of product and substrate replaced at once, and higher amounts are required in short HRT (i.e. when the replacement is done manually). Moreover, this type of process is suitable for changing the rate of organic loading. As in batch trials the initial substrate concentration was equal for all retention times, in semi-continuous trials it depends on HRT. High OLR was achieved by decreasing the HRT, and low by increasing the HRT (Arslan et al., 2016). The exact amounts of added substrate and removed product were shown in **Table 2** in **Paper III,** and were calculated by dividing the working volume of reactor by days need for replacement of whole working volume**.** The semi-continuous trials run in two identical continuously stirred tank reactors (CSTR) (Bioprocess Control, Sweden) (**Paper III**). The total volume of the bioreactor was 7.28 L, while the working volume of each reactor was 4.5 L. One of the reactors was used as a control and the second one as an experimental. The initial experimental pH was adjusted to 5.2 by adding the 18% HCl and then, only monitored during the fermentation process. To ensure anaerobic conditions the reactors were flushed with mixture of carbon dioxide and nitrogen (20:80 vol/vol) for 15 minutes at the first day of the trial. Samples for chemical analysis were taken each day (from each feed out fermentation broth). The experimental design and characteristics of each fermentation trial was described in **Paper III**.

7. Production of short and medium carboxylic acids

The carboxylic acids are organic compounds that contain a carboxyl group *COOH* and the rest of the molecule *R*. Among carboxylic acids 3 groups of fatty acids can be identify: short, medium and long chained (Moss et al., 1995). The main aim of this work was to produce short and medium chain acids. The short chain carboxylic acids contain five or fewer carbon atoms, medium chain ones contain from six to twelve (Kannengiesser et al., 2016; Lee et al., 2014). In this study mostly short chain carboxylic acids were produced i.e. acetic, propionic, butyric, i-butyric, valeric and i-valeric. From here on they will be referred as volatile fatty acids (VFAs). Additionally, among medium chain caproic, heptanoic and caprylic acids were detected as well. As a renewable carbon sources all these acids have a wide range of application such as: production of bioplastics, biogas, biodiesel or electricity and also they could be applied in biosurfactants and bioflocculants synthesis (Hasan et al., 2015) or can be directly recovered as commodity chemicals (Angenent et al., 2004; Tamis et al., 2015). Moreover, VFAs produced during sludge fermentation can be used as an additional carbon source for enhanced biological removal of nutrients from wastewater (Obaja et al., 2004). In the biorefinery systems, carboxylic acids can serve as platform chemicals, in that case might refer to *carboxylate platform* or *VFA platform* (Agler et al., 2011; Holtzapple and Granda, 2009).

Currently, the commercial production of short and medium carboxylic acids is based on chemical routs, however in recent years the use of biological routs become more interesting due to increasing price of oil and high amount of easily accessible organic matter. The process of biological production is based on anaerobic digestion (AD). In traditional AD process four phases can be distinguished (Arslan et al., 2016; Lee et al., 2014):

I phase – the hydrolysis, where complex organic material composed of different polymers (lipids, proteins, polysaccharides) is broken down and solubilized into simpler monomers (such as monosaccharides, amino acids, long chain fatty acids or glycerol) by enzymes excreted from the hydrolytic microorganisms.

II phase – the acidogenesis, where monomers are converted into carboxylates (medium and short chain carboxylic acids, alcohols and inorganic compounds i.e. CO_2, H_2, NH_3 and reduced sulfur).

III phase - the acetogenesis, where the products of acidogenesis are converted into acetate.

IV phase – the methanogenesis, where methane is produced.

To enhance the production and accumulation of carboxylates, the methanogenesis phase needs to be inhibited to prevent short carboxylic acids consumption by methanogens (Lee et al., 2014). The

most common strategies are: heat shock, acid/alkali conditioning or addition of methanogenesis inhibitors (Arslan et al., 2016; Lee et al., 2014). In the presented work two last methods were used. The acid/alkali conditioning was obtained by addition of 18% HCl and/or 3M KOH to adjust the pH to the level outside the range suitable for methanogens i.e. 6.6 > pH > 8.0. Tested pH was 4.0, 5.0, 6.0 and 9.0, 10.0, 11.0, 12.0 (**Paper I**). In the second set of batch trials, fermentation was carried on in either acidic (5.0) or strong alkaline conditions (11.0). Moreover, to check the carboxylic acids production in the neutral pH, the inhibitor of methanogenesis was added ($BrCH_2CH_2SO_3$) (**Paper II**). In semi-continuous trial the pH of inoculum alone was adjusted to the initial pH of 5.2 (**Paper III**).

8. Factors determining the formation of short and medium carboxylic acids

The outcome of open cultures fermentation can be regulated by multiple factors, such as pH, temperature, type of inoculum, substrates type and concentration, hydrogen donor agents, nutrients availability, headspace partial pressure and headspace composition, bioreactor configuration, hydraulic retention time (HRT) and organic loading rate (OLR) (Arslan et al., 2016; Bastidas-Oyanedel et al., 2015; Liang and Wan, 2015). In the presented research the effect of the following process parameters were investigated: pH, substrate type, HRT together with OLR.

8.1. pH

pH is an operational parameter that has a direct effect on biological activity and growth of microorganisms. According to work by (Temudo et al., 2008) each species (or bacterial enzymes) have different optimum range of pH and the selection of the most active organisms by pH is possible. This directly influence the product spectrum (Arslan et al., 2016; Temudo et al., 2008).

Batch trials

During the **first batch** process (**Paper I**) the wide range of pH was investigated (4.0-12.0). The fermented substrate was a mixture of primary sludge and waste activated sludge (1:1, vol/vol). **Figure 2** in **Paper I** shows the composition of the effluent. The production and accumulation of VFAs was observed at initial pH 4.0, 5.0, 10.0, 11.0 and 12.0. However, at the initial pH between 6.0 and 9.0 the accumulation did not occur due to pH near to the optimum for biogas production. The highest acidification yield occurred in the alkaline conditions (10.0-12.0), where the concentration of VFAs was the highest, and it was 0.62 g/g VS_{added}, 0.52 g/g VS_{added} and 0.67 g/g VS_{added}, respectively for initial pH 10.0, 11.0 and 12.0. For acidic conditions it varied between 0.21 g/g VS_{added} and 0.39 g/g VS_{added}. Similar results of higher production in alkaline pH was documented for fermentation of excess sludge (3-4 times higher VFAs production) (Hongying Yuan et al., 2006; Jie et al., 2014) as well as for waste activated sludge (Ma et al., 2016).

The composition of produced mixture of VFAs is shown in **Figure 3** (**Paper I)**. The acetic acid dominated among all acids produced at initial alkaline pH and its share reached up to 78%. However, in the acidic conditions it was not so significant (from 41% to 57%) and higher percentage of butyrate (10.5%-22%) and propionate (13%-25%) occurred. Propionate was also produced in initial alkaline pH (9%-26%), but butyrate share was minor, i.e. between 2% and 6%. As it was shown in **Figure 3** in **Paper I**, the acids composition differs between initial acidic and alkaline pH. However, there is no difference in composition of produced mixture of acids between pH in the same range (i.e. between

initial acidic pH 4.0 and 5.0, and between initial alkaline pH 10.0, 11.0 and 12.0). Only the share of acids is different. The noticeable changes occurred between pH 6.0 and 9.0, and the shift from butyrate to acetate was observed. It was also observed by Temudo et al. (2007) in similar range of pH (6.5-8). This shift is involved by pH changes. According to Jonke and Michal (2004) and Veeken et al. (2000) enzymes which are involved in hydrolysis have an optimum pH between 5 and 7. Moreover, pH near to neutral is suitable for methanogens. The conclusion is, that longer chain carboxylic acids (such as butyric) shifts to shorter ones (such as acetic), which are finally used for biogas formation. It was proved by intensive biogas production and minor VFAs concentration in the analyzed pH range (6.0-9.0). Moreover, when compared the acids composition obtained at initial acidic and alkaline conditions it might be said, that acidic conditions were more favorable for butyrate formation, however in alkaline its concentration decreased and the concentration of acetate doubled. This is in agreement with results of Temudo et al. (2008), where the highest butyrate production occurred between pH 4 and 5.5, whereas at high pH values concentration of acetate increased.

In the **second batch** process the fermentation also run at acidic (pH 5.0), neutral (pH 7.2-7.4) and alkaline (pH 11.0) initial conditions. The results of the previous test showed, that the highest concentration of VFAs among all analyzed acidic pH values occurred in pH 5.0. The choice of alkaline pH was based on trends in VFAs production and accumulation revealed during the first batch fermentation. The initial pH 12.0 was too high and inhibition of VFAs production occurred at the beginning of the fermentation process. However, when consider pH adjusted to 10.0, the risk of decrease to optimum for methanogens caused by acids accumulation was too higher. Based on that, in the second test pH 11.0 was applied. Considering that optimal pH for hydrolysis is between 5 and 7 (Jonke and Michal, 2004; Veeken et al., 2000) the trial in neutral conditions was conducted, but to prevent from biogas production the methanogenesis inhibitor was added (Zinder et al., 1984). The production and accumulation of VFAs was observed in all tested conditions (**Figure 1**, **Paper II**). During the fermentation at initial acidic conditions (**Figure 1A**, **Paper II)** the highest concentration of VFAs was obtained from maize silage (0.60 g/g SCOD), then from acid whey (0.49 g/g SCOD) and microalgae biomass (0.40 g/g SCOD). But the initial alkaline conditions (**Figure 1B**, **Paper II)** were beneficial for formation and accumulation of VFAs, and the highest concentration occurred during fermentation of microalgae biomass (0.83 g/g SCOD), then for maize silage (0.78 g/g SCOD). The initial alkaline conditions at the beginning inhibited the fermentation of acid whey, but the final concentration of VFAs was 0.71 g/g SCOD. The neutral initial conditions (**Figure 1C**, **Paper II)** were beneficial for microalgae biomass fermentation. The concentration of VFAs was 0.81 g/g VS (similar to obtained in initial alkaline pH), but at the beginning the process of fermentation was inhibited. However, the concentrations obtained during fermentation of maize silage (0.61 g/g VS_{added}) and acid

whey (0.57 g/g VS_{added}) are similar to the results of fermentation at initial acidic conditions. These results are related to the substrates types and their complexity and are described in section 8.2. "Substrate type". In the second batch trial the fermentation of simple substrate (1% glucose solution) was also analyzed. According to results presented on **Figure 1** and **Figure 4A** (**Paper II**), the initial alkaline conditions were more beneficial for fermentation of complex substrates, which resulted in higher VFAs concentration. These findings are similar to the ones presented in **Paper I** (sludge fermentation) and they are in accordance with the conclusion presented by Park et al. (2014) that alkaline conditions promote the hydrolysis of organic matter from complex substrates, which is the most crucial step in fermentation process. It was more effective due to the ionization of charged groups (e.g. carboxylic groups) which enhanced the solubilisation of carbohydrates and proteins (Mohanakrishna and Mohan, 2013; Noike et al., 1985).

The composition of produced mixture of carboxylic acids is shown in **Figure 3** in **Paper II**. The initial alkaline pH promoted acetic acid formation from all analyzed substrates (**Figure 3B**). Despite the decrease of pH to neutral and biogas production (**Figure 2**, **Paper II**) its share was high (67%-91%). However, in the initial acidic conditions the diversity of produced acids was higher and none of them clearly dominated (**Figure 3A**, **Paper II**) (despite the chosen substrate). Here, the highest share of acetate (48%) and butyrate (35%) occurred during maize silage fermentation. However, the share of acetate, propionate, butyrate and valerate produced from acid whey were similar to each other. The increase in pH from initial 5.0 to near to neutral, that occurred during microalgae biomass fermentation resulted with decrease in VFAs concentration. It was mostly visible in high drop of concentrations of acetate and butyrate. This could be the effect of biogas production (**Figure 2B**, **Paper II**) and/or chain elongation to propionate (increase from 19% to 38%), i-valerate (increase from 12% to 26%) and caproate (increase from 1% to 8%). In neutral initial conditions the pH of the process was the most stable for all analyzed substrates (**Figure 3C**, **Paper II)**, which was visible in the composition of produced acids. Similar to alkaline conditions, acetate was a dominant acid, but its share was not so high (46%-55%). Next dominant acids were propionic, which share varied between 18.5% and 29%, and butyric with share from 10% to 18%. The fermentation test based on glucose confirmed that initial pH influences the composition of produced VFAs. The open culture fermentation had a capability for production of acetate and butyrate, together with valerate and caproate as a main products in low pH (Bengtsson et al., 2008; Kim et al., 2016). However, in alkaline initial conditions it was possible to produce significant amount of acetate and propionate as a second dominant product (Jie et al., 2014). Moreover, it was proved that the shift from butyrate to acetate could be involved be increasing the pH from acidic to alkaline and that the type of applied substrate had a minor impact on VFAs composition (see **Figure 3** and **Figure 4B** in **Paper II**). Moreover, the

observed shift between butyrate and propionate in varied pH could be described as a competition between butyrate type fermentation (mainly produced are acetate and butyrate) and propionate type fermentation (with production of mainly acetate and propionate) (Bengtsson et al., 2008)

Both batch trails were carried out in unbuffered and unadjusted pH conditions. To improve the VFAs formation the initial pH was changed to acidic or alkaline, but during the fermentation process in was only monitored. And despite the initial conditions, the pH was tended to near to neutral. The drop of pH was accompanied by the accumulation of short chain fatty acids, which toxicity is higher in low pH (Hwang et al., 2004). According to this, the pH should decreased in both acidic and alkaline initial conditions, but it occurred only in the alkaline one. The explanation was the buffering capacity of fermentation system, which was strongly correlated with the VFAs production (Venkata Mohan, 2009). Higher buffering capacity in alkaline pH provided better conditions for VFAs production and accumulation (Dahiya et al., 2015). As for acidic conditions, the fermentation system prevented the drop of pH (despite high concentration of VFAs) by the production of *in situ* volatile buffers throughout the buffer effect of macromolecules` residues (Dinamarca et al., 2003). This self-maintenance of pH in unbuffered system was reported by Wu et al. (2016) in the range of 5.2-6.4 during the co-fermentation of food waste and excess sludge. Moreover, when bacteria consumed energy to maintain ion grades (i.e. undissociated acids, that passed through the cell membrane and dissociated inside the cell) and regulated pH inside the cell instead of using the energy for growth, the production of acids may finally drop (C. Zhang et al., 2009). This was noticed during acid whey and maize silage fermentation in initial neutral conditions (**Figure 1C**, **Paper II**). The concentration of VFAs decreased, despite inhibited methanogenesis and no biogas production (**Figure 2B, 2C**, **Paper II**).

Semi-continuous trial

The semi-continuous test was carried out to analyze the influence of hydraulic retention time and organic loading rate. Based on the results of previous tests the fermentation process was carried out at initial acidic conditions (here pH was adjusted to initial 5.2) to improve the diversity of produced acids. Moreover, the self-maintenance of pH was visible in the range between 5.2 and 5.7 (**Paper II**). The basic element that influence the choice of substrates used was their availability. In the future, the fermentation to VFAs could be used in full scale for utilization of municipal and agro-industrial wastewaters, which are produced in significant amounts and their utilization is problematic. For this reason, the sludge mixture (primary sludge and waste activated sludge in ratio 1:1, vol:vol) and acid whey were chosen. As the sludge after wastewater treatment is mainly used for biogas production and the acid whey is added to animal feed, the efficiency of these methods are not

suitable to the amount of organic matter that need to be recovered. Moreover, these sources of carbon can be effectively use as substrates for production of added value-products, commodity materials or other biocompounds.

The highest concentration of VFAs was generated from acid whey fermentation, precisely during its first phase and it was 11.8 g COD/L at the 10^{th} day (**Figure 1A**, **Paper III**). Here, the pH drop from the initial one (5.2) to 4.45 was correlated to acids production, but after second day of fermentation it stabilized and varied between 5.01 and 5.22 till the end of first analyzed HRT (20 days). The composition of produced acids was similar to the one described in **Paper II** i.e. similar share between acetate, butyrate, valerate and caproate in initial pH adjusted to 5.0. However, in the pH<5.3 the production of propionate was limited, but relatively high share of caproate occurred (27%-30%). In the next phases the HRT was shorten (i.e. 12, 8, 4 and 1 day). Its influence on the pH changes was not as significant as on the concentration and composition of VFAs. It was noticed (**Figure 2A**, **Paper III**) that concentration decreased and acetate started to be the main acid. In similar process conditions (i.e. 20-days HRT continuous process, pH 6.0, fermentation of cheese whey permeate) the share of acetate increased with RT shortening from 33% at RT 95 h to 45% at RT 8h (Bengtsson et al., 2008). However, Domingos et al. (2018) reported high concentration of caproate (4.13 ± 0.56 g/L) and caprytale (3.12 ± 0.94 g/L) during fermentation of cheese whey in controlled pH 6.0 and HRT 6 days. This results were similar to the one described in this work (**Figure 2B**, **Paper III**), where higher concentration of caprytale was detected in HRT 6 days and HRT 8 days.

The pH stability was also visible during the first phase of sludge fermentation, then it started to be irregular and rising, but it did not project on the concentration and composition of VFAs (**Figure 1B**, **Paper III**) due to higher amounts of added substrate (this is correlated with substrate type and time of fermentation, and is described in section 8.2. “Substrate type” and 8.3. “Retention time and organic loading rate”). The composition of produced mixture of acids characterized with higher share of acetate (3.4%-26%) and propionate (24%-32.5%). However, the share of butyrate (14%-16%), valerate (12%-17%) and i-valerate (13%-21.5%) were similar. These results are similar to one obtained in batch test and described in **Paper I** (a similar acids produced in initial acidic conditions despite applied retention time).

The fermentation test based on glucose (4 g/L glucose solution supplied with nutrients and mineral compounds according to Temudo et al. (2007)) confirmed faster start of the fermentation process for simple substrates (**Figure 1**, **Paper III**) and higher productivity of butyrate in acidic conditions (**Paper II**) (Tamis et al., 2015). Another conclusion was, that in continuous trials the influence of pH on production and composition of VFAs was more visible than in batch trials.

8.2. Substrate type

The type of substrate was the second analyzed factor that influenced total concentration and type of produced short and medium chain carboxylic acids. According to the composition of polymers, substrates can be grouped in three categories i.e. rich in carbohydrates, lipids or proteins. The hydrolysis rate and therefore carboxylate concentration depends on substrate complexity and its digestibility (Arslan et al., 2016).

Batch trials

The first analyzed substrate was a mixture of primary and waste activated sludge. The main produced were acetate, propionate, butyrate and caproate. The share of acids changed with pH of the process, but despite it, high concentration of acetate was obtained in both acidic (41%-57%) and alkaline conditions (33%-78%). This is related with applied substrate. Waste activated sludge is hard biodegradable and need to be pretreated before fermentation (Pang et al., 2015). According to Yu et al. (2008) and Zhang et al. (2009) hydrolysis of substrates with low digestibility is more effective in alkaline conditions (**Figure 2**, **Paper I**). In applied pH range the chemical pretreatment could occurred and concentration of solubilized compounds increased resulting with higher productivity of acetate. Moreover, the acetate inherent in primary sludge could influenced the distribution of VFAs. Other acids with relatively high share were propionate (20%-25% in initial acidic pH, 9%-16% in initial alkaline pH) and butyrate (11%-22% in initial acidic pH). The high share of propionate produced from sludge (primary, activated and their mixture) was reported by Ucisik and Henze (2008). During the first batch fermentation (**Paper I**) only one substrate was tested. Therefore to understand the effect of substrate type on fermentation process, it was necessary to analyzed substrates with different complexity and composition.

In the second batch process (**Paper II**) the fermentation of maize silage (lignocellulosic biomass), microalgae biomass (aquatic biomass) and acid whey (agro-food wastewater) was analyzed. To reveal the influence of substrates complexity on concentration and composition of produced acids, the conversion efficiency was specified (**Table 2**, **Paper II**). Moreover, prior the fermentation process, the effect of the initial pH on soluble fraction of COD (SCOD) was investigated (named solubilisation test).

The results of solubilisation test for acid whey and microalgae biomass did not revealed significant changes neither at pH 5.0. nor 11.0. However, for maize silage the slight increase in SCOD was noticed after 15 min (4.9%) and 24 h (9.4%) exposition for pH 5.0. But the pretreatment effect of pH was the most visible at the pH 11.0. Where, after 2 min of exposure the SCOD concentration increased by 27%, by 31% after next 13 min and by 39% after 24 h. The VFAs concentrations

presented in **Figure 1** (**Paper II**) and conversion efficiencies described in **Table 2** (**Paper II**) confirmed that, the most beneficial conditions for maize silage fermentation were initial alkaline, then acidic and neutral. The average acidification yield (the conversion of solubilized organic matter into VFAs) was higher in initial alkaline (71.5% ± 1.5) and acidic (61.0% ± 7.7) conditions, than in neutral (29.7% ± 6.0). This indicated that alkaline and acidic pH enhanced solubilisation of maize silage and obtained soluble organic matter was more available for acidifying microorganisms, which resulted in higher VFAs production. Moreover, the hydrolysis yield (the solubilisation of the initial organic compounds from solid substrate) in neutral conditions was higher than in acidic and alkaline pH. This confirmed more intensive activity of hydrolytic bacteria and lower of acidifying in neutral conditions. The calculated values of biomass yields (the formation of biomass from soluble compounds, but not from VFAs) proved the degradation of maize silage. In all three initial conditions biomass yield was lower than zero. This indicated that COD was not accumulated and/or the biomass content in maize silage was utilize to soluble compounds, especially at the beginning of the fermentation process carried out in initial alkaline pH (-5.1 g COD/g COD) (this confirmed results of "solubilisation test"). But considering the average values, it occurred that highest degradation of biomass was in neutral conditions (-4.28 g COD/g COD), which stays in accordance with hydrolysis yield (the highest in neutral conditions).

For the second analyzed substrate (microalgae biomass) the initial acidic conditions occurred to be unfavorable regarding the process performance and conversion efficiency. All three conversion factors decreased during fermentation process (**Table 2**, **Paper II**), so did the production of VFAs (**Figure 1B**, **Paper II**). It might be concluded, that at the beginning of the process, when pH was in acidic range, the solubilisation of microalgae biomass occurred and obtained organic matter was used for carboxylic acids production. Simultaneously the pH of the fermentation system increased to near to neutral and produced acetate was used for methane formation (**Figure 2B**, **Paper II**) (decrease in acidification yield). The process shifted to methane production. The initial alkaline conditions enhanced hydrolysis of microalgae biomass. Its yield was the highest and increased until the end of fermentation process (11.8%->16.0%). Simultaneously, the acidification yield decreased (33.8%->28.3%) and production of hydrogen occurred, but it not affected the production and accumulation of VFAs. The obtained results confirmed high complexity of microalgae biomass structure. The conversion efficiency of microalgae biomass depends on microalgae cell wall biodegradability. Since it consists mainly from glucose, mannose, and galactose, that can form cellulose and hemicellulose (hard biodegradable compounds) (**Paper IV**), it is necessary to apply pretreatment process that decreases the cell walls` resistance to microbial enzymes. This process was mostly visible in initial alkaline conditions (similar results to lignocellulosic maize silage), which

together with thermal pretreatment prior fermentation involved high solubilisation of microalgae biomass. During the fermentation carried out in neutral conditions, the hydrolysis yield was around 13.5% ± 0.3. Since at this trial the neutral pH was applied, the effect of pH pretreatment did not occurred and the boost in VFAs production occurred after intensive hydrolysis (**Figure 1C**, **Paper II**). This confirms, that the hydrolytic enzymes are most active in neutral pH (Jonke and Michal, 2004; Veeken et al., 2000). As for maize silage, the biomass degradation was also observed in all initial conditions, but the highest was at neutral pH (Y_x= -0.98 g COD/g COD over 5th days of fermentation).

Among all tested substrates acid whey had the most liquid form. The degradation of biomass did not occur, as the biomass yield was equal to zero in all analyzed pH conditions. The accumulation of COD in biomass produced from soluble COD occurred (comparable to results presented by Bengtsson et al. (2008)). At the beginning of the fermentation process carried out in highly alkaline pH, the acidogenesis was inhibited (η_a=7.4%). The intensive hydrolysis (14.47% and 15.81% after 5th and 10th day of fermentation) and pH decreased to less alkaline enhanced acidification yield to 61.9%, resulted with intensive production of VFAs (**Figure 1B**, **Paper II**), despite methane production (**Figure 2A**, **Paper II**). Initially the acidic pH enhanced VFAs production, but despite stable pH (5.30-5.48) and minor methane detection, the acidification yield (69.9%->53.45) and VFAs concentration decreased. However, the hydrolysis yield increased (11.1%->12.8%). This suggested the possibility of VFAs utilization by growing bacteria. According to obtained results, it occurred that most stable process conditions for acid whey fermentation (regarding concentration, not composition of acids) was in neutral conditions. Here, the hydrolysis yield (18.0%-20.8%) and biomass yield (0.39->0.45 g COD/g COD) were the highest among all tested substrates. Moreover, the acidification yield was lower, than in alkaline or acidic conditions, but despite it, the average VFAs concentration was around 0.54 g/g COD and comparable to achieved in acidic conditions (0.42 g/g COD).

It might be concluded, that to improve VFAs formation from complex substrates (i.e. microalgae biomass, maize silage) the process should be carried out at initial alkaline conditions (to increase biomass degradation and solubilisation of organic matter), but to achieve high concentration of VFAs the neutral pH is required. It was observed, that the reduction of hydrolysis yield occurred simultaneously with increase in the acidification yield and conversely, that significant rate of acidification lowers the hydrolysis yield (Wu et al., 2016). However, for complex substrates (such as maize silage or microalgae biomass) without pretreatment prior fermentation, the yields of hydrolysis and acidification might increase simultaneously. It was said, that pH is the factor that mainly determined the formation and composition of VFAs. However, the composition of polymers in substrates could enhanced the production of desired acids (Arslan et al., 2016). Therefore, it is possible to obtain high share of butyrate from substrates rich in carbohydrates i.e. cassava waste

water (22% at pH 5.9) (Hasan et al., 2015), wheat straw (33.3 g COD/kg TS from pH=5.2) (Motte et al., 2015), maize silage (26-35% at initial pH 5.0) (**Paper II**) and glucose (around 40% at initial pH 5.0) (**Paper II**; Tamis et al., 2015). However, substrates rich in proteins can be used for valerate production i.e. waste activated sludge (Chen et al., 2007; Feng et al., 2009), municipal solid waste (Okamoto et al., 2000) and whey (**Paper II**; Bengtsson et al., 2008).

Semi-continuous trials

In semi-continuous test the fermentation process of sludge, acid whey and glucose was described. The concentration and composition profile of produced VFAs are shown in **Figure 1** (**Paper III**) and the results of conversion efficiencies (yields of hydrolysis and acidification) calculation are presented in **Figure 3** (**Paper III**).

The most favorable conditions for VFAs production during acid whey fermentation occurred in the first phase of the process, when HRT was the longest (20 days) (**Figure 3A**, **Paper III**), pH was stable (between 5.01 and 5.22) and the acidification yield was the highest (over 73%). It was noticed that together with addition of substrate (225 mL/day; HRT=20 days) the hydrolysis yield increased (maximum was 48.5%) and since 17th day of the process was higher than acidification yield. This correlation was also observed during acid whey fermentation in initial acidic conditions (**Paper II**). In the second phase, when HRT was shortened to 12 days and amount of added substrate increased to 375 mL/d, the hydrolysis yield decreased to 27.6%. From 22nd day of fermentation it started to increase and varied between 33.2% and 45.9%. However, after shortening the HRT to 8 days (amount of added substrate increased to 563 mL/d) it decreased significantly and till the end of the test was under 10%.

The conversion efficiencies during sludge fermentation were not significantly affected by changing in HRT, OLR and pH (**Figure 3B**, **Paper III**). The acidification yield varied between 34.3% and 74.2%, however the hydrolysis yield did not exceed 17.0%. Therefore, the concentration and share of produced VFAs was rather stable. This stability could be an effect of similar bacterial community between inoculum and sludge (the same source) and lower concentration of soluble compound in added, fresh substrate.

The fermentation test based on glucose confirmed the results described in **Paper II**, that the increase in hydrolysis rate is accompanied with the decrease in acidification yield and conversely, the increase in acidification yield involved decrease in hydrolysis rate (**Figure 3C**, **Paper III**). Moreover, it was proved that, the decreasing HRT to achieve higher OLR can reduce the effectiveness of hydrolysis, resulting in lower concentration of carboxylic acids and reduction of products type

(Arslan et al., 2016). It occurred during acid whey fermentation, where share of acetate increase together with HRT shortening (**Figure 1A**, **Paper III**).

The composition and share of VFAs produced during fermentation of acid whey in initial acidic conditions, are similar in batch trial (**Figure 3A**, **Paper II**) and semi-continuous trial (for HRT 20 and 12 days) (**Figure 2A**, **Paper III**). However, the shortening of HRT in semi-continuous trial involved the shift to acetate production, due to washout of slower growing bacteria and retention of fast growing acetate produced bacteria (Arslan et al., 2016). The composition of VFAs obtained from sludge fermentation was diverse in all HRTs (**Figure 2B**, **Paper III**). However, the share of acetate increased with HRT shortening, but it was not the dominant acid. The much lower share of acetate between batch trial (40.7%-57.1% in initial acidic pH) and semi-continuous trial (3.4%-26.0%) is the main difference in the composition of produced VFAs. Similar to sludge fermentation, in glucose fermentation the acetate did not became a dominant acid due to HRT shortening, but its concentration decreased and dominant acid was butyrate (**Figure 2C**, **Paper III**). The results of batch trials and semi-continuous trial are also similar, so the production mainly of acetate and butyrate in pH lower than 5.2 (the butyrate type fermentation (Bengtsson et al., 2008)).

8.3. Retention time and organic loading rate

Another analyzed factor that determines open culture fermentation was retention time. From the economic point of view, the retention time influenced the reactor volume i.e. short HRT means fast conversion resulting in smaller reactor volumes (de Mes et al., 2003). Moreover, lower OLR means less frequent feeding resulting with easier reactor operation and less exploitation of the feed pumps by reduced the wear and tear (Nebot et al., 1995). From the operational point of view the HRT should be long enough for optimal hydrolysis and acidification (especially for complex and non-soluble substrates) and to provide the optimum conditions for growth of microorganisms. However, optimum OLR should prevent from process overloading and destabilization, and provide wide spectrum of products (Arslan et al., 2016).

Batch trials

In batch trials the applied operating time was 5, 10 and 15 days and the organic loading rate of substrates was 0.5 g VS/100 mL (**Paper I**, **Paper II**). Since the pH was adjusted only at the beginning of the process, it was noticed that during the fermentation the pH tended to neutral conditions. Simultaneously, changes in pH induced the shift in VFAs concentration and composition.

The first batch trial was based on sludge fermentation. The influence of retention time on process that run at initial acidic conditions was not substantial. The concentration was higher in

shorter RT (5 days; 0.25 g/g VS_{added} for pH_{in}=4.0 and 0.39 g/g VS_{added} for pH_{in}=5.0), but the difference between longer RT was minor (for 10 days long batch trial was 0.21 g/g VS_{added} for pH_{in}=4.0 and 0.23 g/g VS_{added} for pH_{in}=5.0; however for 15 days long batch trial was 0.25 g/g VS_{added} for pH_{in}=4.0 and 0.36 g/g VS_{added} pH_{in}=5.0). The effect of retention time was mainly visible in alkaline batch trials. In high alkaline initial pH (i.e. 11.0 and 12.0), the process of VFAs production was inhibited at the beginning due to pH regulation by microorganisms (Zhang et al., 2009a). Higher VFAs concentration was obtained at the 10^{th} day of the process i.e. 0.26 g/g VS_{added} for pH_{in}=11.0 and 0.67 g/g VS_{added} for pH_{in}=12 (**Figure 2B**, **2C**, **Paper I**). The retention time also influenced the composition of produced mixture of carboxylic acids (**Figure 3**, **Paper**). Longer retention time was beneficial for hydrolysis of hard biodegradable sludge and increased concentration of acetate in both acidic and alkaline conditions (Pang et al., 2015) (see section 8.2. "Substrate type"). Whereas, shorter RT in acidic conditions was beneficial for higher share of butyrate.

The results of maize silage, microalgae biomass and acid whey fermentation confirmed that retention time influenced on process performance (**Paper II**). As the hydrolysis rate (the slowest step of anaerobic digestion) depends on substrates complexity and amount of soluble compounds (Arslan et al., 2016), the production of acids from simple substrates (such as glucose rich substrates) required retention time of few hours (Horiuchi et al., 2002). However, for more complex and non-soluble substrates it should be higher. According to results described in **Table 2** in **Paper II** longer retention time enhanced the hydrolysis yield of microalgae biomass in initial alkaline pH i.e. 11.8% in RT=5 days, 14.4% in RT=10 days and 16.0% in RT=15 days. The increase of hydrolysis rate was also noticed during maize silage fermentation, but despite initial pH it was minor. However, for more-soluble acid whey, it was noticed in initial acidic and neutral conditions. The results of both batch trails revealed that different types of substrates implied different kinetics and required varied retention times (Lin and Jo, 2003; Liu and Fang, 2002; Min et al., 2005; Yu and Mu, 2006). Nevertheless, it is closely related with pH of the process. Similar to results of sludge fermentation (**Paper I**) the increase in concentration of VFAs with increasing RT was observed in initial alkaline pH during fermentation of maize silage, microalgae biomass and acid whey (**Figure 1B**, **Paper II**). However, in initial acidic and neutral pH the maximum concentration of VFAs occurred in different RT for different substrates. The retention time, not as significantly as pH, could affect the composition of produced carboxylic acids. It was observed during glucose fermentation (**Figure 4A**, **4B**) in initial alkaline and neutral conditions, where pH of the process was stable. Therefore, the shift from acetate to propionate or butyrate occurred.

Semi-continuous trial

In semi-continuous test the analyzed hydraulic retention time was 20, 12, 8, 4 and 1 day. The increase in organic loading rate was achieved by shortening the HRT (**Table 2**, **Paper III**). Obtained results differ between applied substrates.

During the fermentation of acid whey the shortening of HRT induced the decrease in VFAs concentration (**Figure 1A**, **Paper III**) and changes in share of acids (**Figure 2A**, **Paper III**). In the longest HRT (20 days) and the lowest OLR (3.6 g COD/L/d) the production of acetate was minor, however similar share of butyrate, valerate and caproate occurred. In longer retention time the products can shift from more oxidized compounds to more reduced e.g. from acetate or lactate to *n*-butyrate or *n*-caproate (Angenent and Kleerebezem, 2011), this was noticed in HRT 20 days and HRT 12 days. When HRT was shortened to 12 days and OLR increased to 6.0 g COD/L/d, the share of main acids (i.e. acetate, butyrate, valerate and caproate) was similar (as in batch fermentation described in **Paper II**). Since the HRT was shortened to 8 days, the acetate started to be a dominant acid, due to the washout of slower growing organisms (Arslan et al., 2016). Moreover, the OLR higher than 9.0 g COD/L/d (from HRT 8 days) had an inhibitory effect on the fermentation system. Significant reduction in yields of hydrolysis and acidification was noticed (**Figure 3A**, **Paper III**). Similar results were described by Azbar et al. (2009), where during the fermentation of cheese whey (thermophilic conditions 55^{o}C and pH controlled to 5.5) different OLR strategies were applied: (1) constant OLR in varying HRT and (2) varying OLR in constant HRT. It might be concluded that by changing the HRT and/or OLR it would be possible to produce mixture of carboxylic acids with different share of acids from one, particulate substrate while keeping the pH constant.

During the fermentation of sludge the reduction of HRT and increasing of OLR caused irregular and rising pH, due to high pH of supplied sludge (pH 7.6). Despite it, the concentration and composition of VFAs did not change significantly (**Figure 1B**, **2B**, **Paper III**). Similar observations were described in work by Banerjee et al. (1999), where neither the increase of HRT (18 h -> 30 h) nor the decrease of OLR (7 g TS/L/d -> 4 g TS/L/d) significantly affected the composition of carboxylic acids produced from primary sludge. According to (Maharaj and Elefsiniotis, 2001), the increase of HRT from 30 h to 60 h during primary sludge fermentation also had no significant effect on composition of short chain carboxylic acids. The OLR around 80 g COD/L/d had not inhibition effect on fermentation system. This is related with microorganisms and described in section 8.4 “Microorganisms”.

The results of semi-continuous fermentation of glucose are different. As the acetate was the main acid produced in the longest HRT of 20 days (what is similar to described in **Paper II**), the share

of butyrate doubled with decreasing of HRT and varied between 47% and 49%. This indicated that increasing substrate concentration (OLR) the product spectrum can shift to acetate as the major compound (like in acid whey fermentation) or can shift from acetate to butyrate as the major compound (Gómez et al., 2009). Similar to results of acid whey fermentation (**Figure 1A**, **Paper III**) the concentration of VFAs decreased due to HRT shortening (HRT 4 days and HRT 1 day).

To conclude, obtained results indicated that retention time is a factor that might influenced the production and concentration of short and medium chain carboxylic acids, but it should be considered with pH and type of substrate. Concerning the pH conditions, the retention time influenced on the pH neutralization and self-maintenance in unadjusted fermentation system. However, considering the substrate type, the range of RT (HRT) and OLR need to be established regarding substrate complexity, solubility, its pH and contained microorganisms.

8.4. Microorganisms

When the biological routs of carboxylates formation based on fermentation of organic wastes and wastewaters became more popular, the process was named *mixed culture fermentation*. It was defined as a microorganisms that naturally occur in the environment and cooperate with each other (Lu et al., 2011; Rodríguez et al., 2006). In time, the definition involved to *open culture fermentation*, due to the fact that the composition of microbial community is not adjusted, but is natural and can differ with time, process parameters or added substrates.

The results of batch trials revealed that product spectrum is strongly dependent on the pH. Five substrates with different composition and structure were tested. It occurred that the neutral conditions (i.e. initial pH neutral and as well as when pH decreased from initial alkaline to neutral pH) were beneficial for acids production and accumulation. Moreover, neutral pH is beneficial for growth of microorganisms and high efficiency of enzymes (Jonke and Michal, 2004; Veeken et al., 2000). It was proved by hydrolysis yield, which was higher in neutral conditions, than in initial alkaline and acidic pH (**Table 2**, **Paper II**).

Batch trial

Despite application of substrates with different complexity, composition of polymers and solubility, the composition and share of acids between substrates were similar in neutral (**Figure 3C**, **Paper II**) and alkaline (**Figure 3**, **Paper I** and **Figure 3B**, **Paper II**) pH conditions, where the dominant acid was acetic acid. Some differences in product spectrum occurred in the initial acidic conditions, where the composition of produced acids was more varied and a high share occurred not only for acetate, but also for propionate, butyrate, valerate, i-valerate and caproate (**Figure 3**, **Paper I** and

Figure 3A, **Paper II**). According to results of both batch trials (**Paper I**, **Paper II**) it might be concluded that increasing the pH of the process from acidic to neutral involves the shift from butyric acid producing bacteria to propionic acid producing bacteria resulting with higher share of butyrate in acidic conditions and higher share of propionate in pH close to neutral (Bengtsson et al., 2008; Horiuchi et al., 2002). Moreover, high alkaline pH (i.e. 11.0) could permanently inhibited methanogens from using the acetate to methane production, even when during the fermentation initial alkaline pH decreased to neutral (when pH was unadjusted during the fermentation process). As the concentration of acetate did not decrease together with methane production (**Figure 2** and **Figure 3B**, **Paper II**), it was probably produced from CO_2 and H_2. In work by Jie et al. (2014), the impact of different pH on microbial community in open culture fermentation of waste activated sludge was analyzed, but not straightforward conclusion on how exactly the microbial community change in different pH was drawn.

Semi-continuous trials

In semi-continuous trial the response of the fermentation system on changed HRT and OLR was analyzed (**Paper III**). Moreover, the fermentation run at initial acidic conditions to enhanced the diversity of produced mixture of short and medium chain carboxylic acids. To better understand and explain the response of microbial community on changing process parameters, the microbial analysis (based on PCR-DGGE and alignment of 16S rRNA gene sequences of DGGE bands) was performed.

The results of microbial analysis of acid whey fermentation are presented in **Figure 4A** and **Table 3A** in **Paper III**. The five main phyla were detected i.e. *Firmicutes*, *Cloacimonetes*, *Actinobacteria*, *Acidobacteria* and Uncultured Bacterium Clones (named *UBC*). The most detected organisms were classified as *Firmicutes* and UBC. Among detected *Firmicutes* were organisms closely related with *Bacillus* sp. which could produce enzymes (i.e. proteases and cellulases) and promote acidogenesis (Ariffin et al., 2006; Gupta et al., 2002). Other where closely related to *Lactobacillus* spp. that convert sugars into lactic acid. Microorganisms classified as *Cloacimnonetes* express propionate metabolism (Nobu et al., 2015) and are capable to produce H_2 and butyrate (Lykidis et al., 2011). This was proved by decreasing concentration of butyrate, when together with HRT shortening, the number of detected *Cloacimonetes* declined. The phylum *Actinobacteria* was represented by organisms closely related to *Rothia* sp. that produces lactic acid during fermentation of glucose (Giannino et al., 2009) and to *Bifidobacterium* sp. able to produce lactic acid, to ferment amino-acids and metabolize complex oligosaccharides to carbon and energy (Schell et al., 2002). However, band closely related to uncultured *Acidobacteria* would be able to ferment acetate and aromatic compounds (Hugenholtz et al., 1998). In the first stage of the process (HRT 20 days) the microbial richness and biodiversity were

the highest (**Figure 4C**, **Paper III**), what influenced the process stability and triggered the VFAs production and their diversity (**Figure 1A** and **Figure 2A**, **Paper III**). Since the HRT was shortening the microbial richness and biodiversity decreased, and from the HRT 8 days the most detected organisms were *Lactobacillus* spp. The microorganisms able to produce lactic acid were likely provided with acid whey.

In the fermentation of sludge apart from phyla *Firmicutes*, *Cloacimonetes* and UBC, a phylum *Proteobacteria* was detected (**Table 3B**, **Paper III**). The process of fermentation was not clearly dominated by any specific group of microorganisms. The phylum *Firmicutes* was represented by organisms related to *Bacillus* sp. and *Clostridia* sp. (characterized by strong hydrolytic ability, acceleration of polysaccharides hydrolysis and production of organic acids (Fu et al., 2015)). Two bands were classified as closely related to *Gammaproteobacteria* (phylum *Proteobacteria*) correlated with utilization of polysaccharides or fermentation of butyric acid (Lenin Babu et al., 2013). Together with HRT shortening the number of organisms classified as *Cloacimonetes* decreased and similar to fermentation of acid whey, were not detected from the third phase of the process (HRT 8 days). Moreover, bands *Cloacimonetes bacterium JGI 0000059-L07* and *JGI 0000039-M09* were also detected in microbial community carried on the fermentation of acid whey. The changes in HRT and OLR had no significant effect on the microbial community structure. This influenced the stable production and composition of carboxylic acids (**Figure 1A** and **Figure 2A**, **Paper III**). Contrary to acid whey fermentation, the washing out of slower growing bacteria and in consequence domination of acetate did not occur. The explanation is the same origin of inoculum and sludge. The inoculum used in batch and semi-continuous trials was collected from a full scale anaerobic digester, where digested substrate is the same mixture of primary sludge and waste activated sludge as used in the fermentation processes described in this thesis. Thus, the biodiversity was not affected by shortening of HRT, due to the application of microorganisms with supplied substrate i.e. sludge.

During the fermentation of glucose seven groups of microorganisms were detected *Firmicutes*, *Cloacimonetes*, *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, *Bacteroidetes* and UBC (**Table 3B**, **Paper III**). Similar to acid whey fermentation, the predominant identified species were members of phylum *Firmicutes*. Within this phylum *Clostridium* spp. and *Romboutsia* sp. were detected. According to (Wang et al., 2015) *Romboutsia* sp. is an anaerobic bacteria able to produce acetic acid, i-butanoic acid, i-valeric acid and ethanol in fermentation of glucose. Among bands aligned to *Cloacimonetes* were bands detected also during fermentation of acid whey (*Cloacimonetes bacterium JGI 0000059-L07*) and sludge (*Cloacimonetes bacterium QEDP3AB07*). Moreover, it was noticed that regardless the applied OLR the number of detected *Cloacimonetes* bacteria decreased with HRT shortening and after HRT 8 days they were not detected. The only exception is the

mentioned before band *Cloacimonetes bacterium QEDP3AB07* that was detected in all HRTs during the fermentation of glucose. The bands classified as *Actinobacteria* show high similarities to uncultured *Atopobium* sp. an anaerobic bacteria which in fermentation of glucose is able to produce lactic acid, acetic acid, formic acid and succinic acid (Clavel et al., 2014). The detected *Proteobacteria* was closely related to *Desulfovibrio desulfuricans* (an anaerobic sulfate-reducing bacteria usually presented in waste activated sludge (Kjeldsen et al., 2005)) and to *Eschericha* sp. (facultative anaerobic bacteria), which indicated the origin of applied inoculum. The phylum *Bacteroidetes* similarly to *Proteobacteria* and *Firmicutes*, had the ability to effective degradation of organic compounds such as proteins, lipids, celluloses, sugars and amino acids (Jaenicke et al., 2011) with simultaneously production of VFAs (Liu et al., 2014). However, *Acidobacteria* and *Actinobacteria* play a critical role in an anaerobic digestion (fermentation of acetate and aromatic compounds (Hugenholtz et al., 1998)) and in acidification process (Zheng et al., 2013). In all semi-continuous trials, the UBCs were significant of detected and identified microorganism. These organisms are unable to grow in the laboratory on standard media or the cultivation method had not yet been developed, but could play a critical role in the cycle of carbon, nitrogen or other elements and provide a stability of the reactor microbiome (Stewart, 2012).

The results of glucose fermentation confirmed that shortening of HRT induced the wash out of slower growing organisms (as in acid whey fermentation) and that higher biodiversity enhanced the production of VFAs and higher diversity of acids within produced mixture (as in fermentation of sludge). Moreover, that microbial community originating from the substrate might be able to adapt to the applied process conditions and together with HRT shortening might dominate over microorganisms community of inoculum.

9. Biorefinery concept

The current industrial systems are unsustainable. The production efficiency is less than 10%, whereas 90% of used material resources end up as wastes (Gavrilescu, 2014). Together with continuous World development and increasing population there is a high pressure on Earth biocapacity. The depletion of natural resources and fossil fuels is alarming. On the other hand, the continuously increasing energy demand requires higher extraction of natural resources. To provide smart and sustainable supply, the production systems need to be based on renewable raw materials. In this context, the use of a biological feedstock and promotion of biotechnology is a sustainable alternative to various production sectors (Gavrilescu and Chisti, 2005).

9.1. Categories of biomass

The biological feedstock could be referred as biomass, which traditionally defines all organic materials originated from plants. The development of biomass utilization processes expands the biomass definition on waste biomass such as industrial residues, agricultural residues or municipal residues (Yang et al., 2015).

The first generation of bfeedstock was based on starch-rich and oily agricultural crops used for production mainly of bioethanol and biodiesel (Fatih Demirbas, 2009). The main disadvantage is the competition between food and fuel production, that involved the increase of food prices (Naik et al., 2010). Moreover, the negative carbon balance of biodiesel production did not provide reduction of greenhouse gases (Chang et al., 2010).

The second generation feedstock was based on lignocellulosic biomass such as trees, grass species, agricultural or industrial residues (Gnansounou and Dauriat, 2010). The production of ethanol and synthetic biofuels (mainly biodiesel) from second generation of biomass reduces carbon dioxide (good carbon balance) and other pollutants such as SO_x and NO_x (Fang et al., 2010), it also do not compete with food production and some types of biofuels provides better engine performance (Naik et al., 2010). The main disadvantages are long time of cultivation, application of pretreatment methods and high energy demand of production processes i.e. thermo-chemical processes of gasification and pyrolysis (Yang et al., 2015). The solution to decrease the costs of the bioconversion processes is the utilization of waste biomass. Moreover, the costs of bio-production might be lowered by application of fast breeding techniques that reduces the time of biomass cultivation (Faraco and Hadar, 2011; He et al., 2008). The recent developments in plant biology and achievements in processing of biomass conversion increases the potential of biofuels production

from second generation biomass. According to the World Energy Council the biofuels based on lignocellulosic biomass might replace 40% of petroleum by 2050 (Gírio et al., 2010).

The third generation of feedstock includes engineered crops, plant residues and algae biomass. The engineered crops are crops improved by genetic technologies to increase carbohydrates or lipid content and to fulfill the requirements of bioconversion technologies i.e. improvement of bioconversion efficiency for single target product (Yang et al., 2015). However, this concept is still under research. According to Weiland (2006) the ideal third generation biomass should contain high concentration of substrate (i.e. carbohydrates, lipids or proteins), its cultivation time should be lowered to minimum and it should be able to thrive in critical conditions (e.g. arid or acidic soil). The microalgae biomass can fulfill this requirements, due to high lipid, carbohydrates or proteins content (the percentage of each depends on the microalgae specie), fast and easy cultivation and high biomass productivity (**Paper IV**). Moreover, the application of microalgae biomass is environmentally efficient due to the ability of cultivation in nutrient rich wastewaters (wastewater treatment) and reduction of CO_2 emission by the uptake of carbon (**Paper IV**). It was commonly applied as a substrate for biodiesel, bioethanol and biogas production, but each conversion process had to be preceded by pretreatment and/or extraction. The complexity and energy demand of these processes together with problematic harvesting influenced the high costs of microalgae processing for single products. The solution is to apply multistep production from extraction of valuable compounds (i.e. proteins, antioxidants, vitamins, enzymes) to biofuels and fertilizers production (**Paper IV**).

The fourth feedstock generation was named “carbon storage biomass” and should be characterized by higher yields, easier bioconversion processes and less energy demanding for cultivation than previous ones (i.e. first, second and third generation of biomass) (Yang et al., 2015). The carbon and mass balance consists of capturing and storing of carbon sources from atmosphere or soil, which is further converted into other target products. This ultra clean application is still in the phase of research. However, according to (Kim et al., 2011) and (McKendry, 2002) the organic wastes could be defined as a feedstock of fourth generation.

9.2. Biorefinery platforms

The first industrial conversion of renewable resources is dating on 6000 BC, and it was an utilization of sugar cane (Kamm and Kamm, 2004) whereas, the ethanol was produced by distillation (China, 9000 BC) or by fermentation of vegetal material (ancient Egypt) (Demirbas and Demirbas, 2010) long before that. The current biorefineries are based on several integrated conversion strategies i.e. mechanical, chemical, thermochemical and biological (the microorganism platform) (Gavrilescu, 2014). The mechanical conversion is based on separation, drying, pelleting and

extraction. Acid hydrolysis, supercritical conversion and solvent extraction are basic processes of chemical conversion. The main processes in thermochemical conversion are gasification, pyrolysis, torrefaction and combustion. Whereas, the anaerobic digestion, fermentation and enzymatic hydrolysis are main methods of biological conversion. Through the combination of above mentioned conversion methods the obtained bioproducts are economically feasible (Kamm and Kamm, 2007). Each classical biorefinery is based on following conversions: 1) separation of biomass by physical methods; 2) obtained main products and by-products are applied to chemical or microbiological conversion methods; 3) the obtained follow-up products can be further converted or applied in conventional refinery (Kamm and Kamm, 2007).

As the biomass used in biorefinery must neither compete with food/feed production, nor be cultivated in prime arable land, the integrated biorefinery model is required (Gavrilescu, 2014). Such integration provides valorization of streams of organic wastes e.g. effluents, agrofood by-products, resulting with production of new value-added chemicals, biomaterials, biofuels and water (Laufenberg et al., 2003; Liu et al., 2010; Visvanathan, 2010). The integration of biorefinery platforms would provide incorporation of various industrial systems. The application of material closed cycle, cascading utilization and recycling would ensure utilization of raw materials and products, reduction of resources together with waste minimization (preventing from resources loss) (De Jong and Marcotullio, 2010). Nevertheless, the constraints in commercialization of waste-based biorefinery are connected with high costs of integration of the system, methods of conversion and production platforms (Fava et al., 2015). Next one is the lack of knowledge of functionality of such biorefinery in the full scale, as current research are mainly carried on in lab or pilot scales regarding only single processes(Fava et al., 2015). All the limitations and possible solutions were summarized in Table 1.

Table 1

Sum –up of the limitations and solutions for waste-based biorefinery.

Limitations	Solutions	Advantages
Scaling-up	Integration of processes i.e. existing processes of biomass treatment with technologies of bioconversion. Cooperation between food/feed companies and chemical companies. Developing a full biomass value chain.	Diversified production for different markets provides economical sustainability. Avoidance of residues production provides environmental sustainability.
Availability of biomass feedstock	Storage of feedstocks and/or mid-products. Use of multiple feedstocks. Year-round cultivation of microalgae as a third generation biomass.	Sustainable and feasible biowaste biorefinery. Effective use of the equipment and continuous supple of products to the market.
Procedures of substrates collection, stabilization and homogenization	Automatic sorting and characterization of collected substrates.	Fractionation of substrates in terms of biomolecules to be recovered or converted into more sophisticated compounds.
High capital costs	Intensify development of integrated processes. Low/no cost of primary substrates.	Lower investments costs. Biowaste disposal instead of landfilling. Environmental benefits.
High energy demand for substrate pretreatment and mixing	Energy production from biogas and/or use of waste heat from CHP.	Lower energy losses.
Large variety of quality of available feedstock	Developing of processes depending on the type of feedstock and the desired products. Combination of existing and new technologies applied to existing biomass streams.	Continuous production of sufficiently homogenous products.
Social acceptance	Analysis of carbon and water footprints, nutrient cycles and water management. Analysis of competition between food, feed and fuel production.	Comparison of bio-based and fossil-based products. Comparison of commercialized biological routs and biological routs based on biowastes. Public access to information to stakeholders and society.

The full-scale system should be eco-efficient. It means that all production processes should be energy and resource efficient, obtained products and goods should have a long lifetime, all by-products and auxiliaries should be consumed, and the negative impact on the environment should be avoided (Gavrilescu, 2014). The proposition of integrated biorefinery is shown on Figure 5.

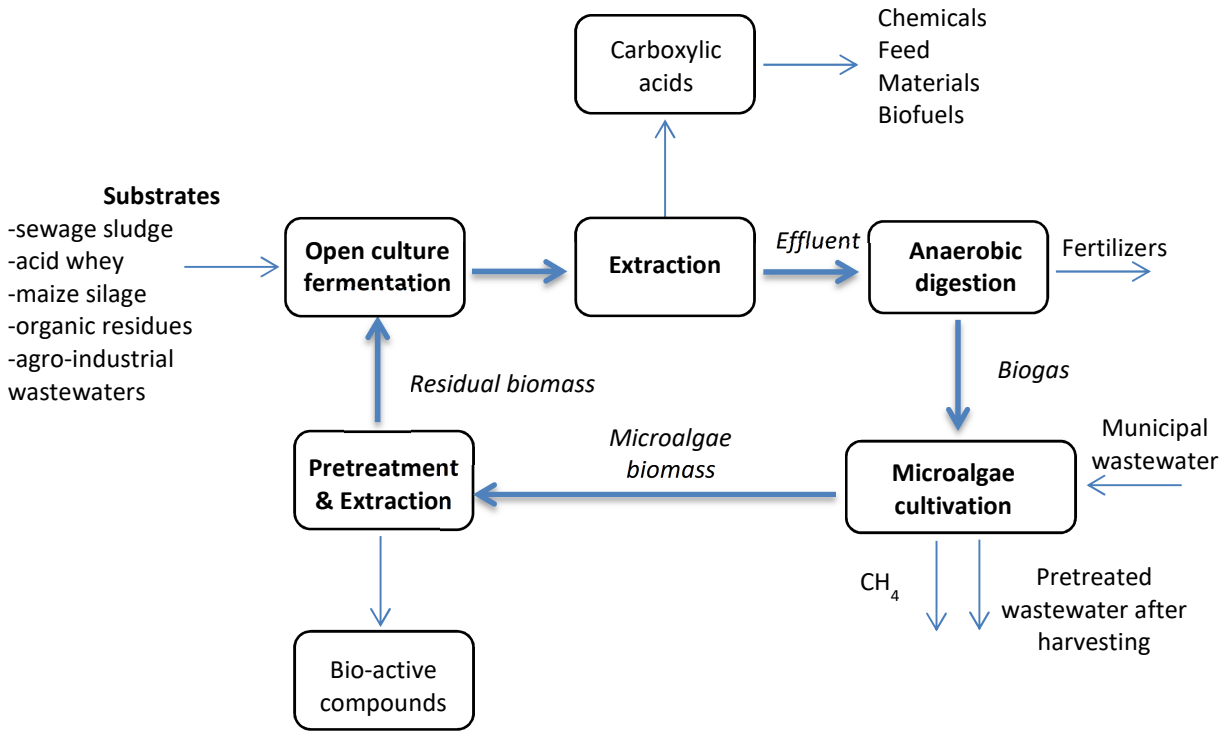

Fig. 5. Biorefinery concept based on wastewater treatment plant and reuse of organic wastes and wastewaters.

At first substrate is converted in microbiological process i.e. open culture fermentation/carboxylate platform. The output is extracted and obtained carboxylic acids are used as substrates for production of more sophisticated, added value products. The valuable effluent after extraction is digested to biogas. The second main part of the biorefinery is microalgae cultivation, where biogas could be upgraded by CO_2 capturing. Moreover, wastewaters used as cultivation medium provides nutrients required for growth of microalgae biomass. After extraction of valuable bio-active compounds e.g. antioxidant, pigments, enzymes, the residual microalgae biomass is used in open culture fermentation for carboxylates production. This closed loop provides effective utilization of many types of biomass, together with utilization of existing facilities (anaerobic digester and facilities in WWTP). Moreover, the effective recycling of nutrients i.e. phosphorus, nitrogen, carbon and CO_2 fixation could be achieved.

The commercialization of biorefineries based on alternative, organic waste sources of biomass is possible. The high content of organic compounds, easy to obtain and at low cost makes it a new,

promising feedstock in the production of valuable bioproducts. Moreover, the bioconversion of organic wastes allows to solve environmental problems. Despite the economic and ecological advantages the most methods of organic waste utilization are still performed in the lab-scale. Since the upstream and midstream of the process is mostly examined, it would be difficult to create a full-scale process without downstream one. Moreover, the development of effective methods of products extraction is a key element for commercialization waste-based biorefineries. Nevertheless, to make commercialization possible, the standardization of conversion processes for each product by using varied advanced technologies should be carried out.

10. Summary

The presented doctoral thesis was focused on the use of organic waste streams as a substrates for short and medium chain carboxylic acids production in open culture fermentation. The dependency between used substrates and range of process parameters was investigated in details. The quantitative and qualitative analysis of produced mixture of carboxylic acids, as well as microbial profiling of bacterial open culture and evaluation of process performance were performed.

Analysis of the results confirmed the effectiveness of open culture fermentation of biomass to short and medium carboxylic acids. Influence of several factors on fermentation efficiency was analyzed. Among the time of fermentation, organic loading rate, substrates complexity and microbial community of inoculum and substrates, the initial pH was the crucial factor that determines the performance of fermentation process. Obtained results suggested that interaction appeared between pH, structure of substrates and microbial community. The selection of pH conditions of the process should be related with the expected effect of fermentation. It was proved, that the initial alkaline conditions enhanced the solubilisation of complex substrates (i.e. sludge, maize silage, microalgae biomass) and production of acetic acid. The neutral conditions enhanced the growth and enzymatic activity of microorganisms resulting with higher rate of hydrolysis. Moreover, to achieve high concentration of VFAs from complex and non-soluble substrates, the process of fermentation should be carried out in neutral conditions preceded by alkaline conditions (to enable pretreatment of substrates by pH). However, production of diverse mixture of carboxylic acids is possible by lowering the pH to acidic conditions (i.e. acetate, butyrate i-valerate, valerate, caproate). The results of batch trials revealed, that despite applied substrate the composition of produced acids strongly depends on pH conditions. As the share of acetate increased with pH increase from initial acidic to alkaline, the share of butyrate was the highest in acidic conditions and for propionate it was the highest when the pH was around neutral. The shift between butyrate type fermentation to propionate type fermentation was induced by the changes in pH conditions, which influenced on microbial community. This indicated, that different type of microorganisms and/or bacterial enzymes could be more efficient in different pH conditions. Nevertheless, the composition of substrate could also affect the type of produced acids (but not so significant as pH). It was revealed that higher share of butyrate was produced from substrates rich in carbohydrates and more valerate might be produced from proteins rich substrates. Moreover, despite the pH conditions, the higher share of i-valerate was detected during fermentation of microalgae biomass. The retention time is the second factor that needs to be considered in designing of the process. Obtained results indicated that the retention time should be considered with pH and the type of substrate. During the fermentation in batch mode it was observed that longer RT in initial alkaline pH enhanced the productivity and

accumulation of VFAs. Moreover, longer time enhanced chain elongation and formation of medium chain carboxylic acids. Microbial analysis preformed for trials that run in semi-continuous mode, revealed that the longer time of fermentation was beneficial for higher biodiversity of microbial community, what enhanced the production and variety of VFAs. However, shortening of HRT to achieve higher organic loading rate lead to wash out of slower growing organisms and domination of acetate in produced mixture of VFAs. Another conclusion is that this system is able to achieve self-maintenance of pH in proper conditions not only in batch mode, but also in semi-continuous mode with constantly added substrate and increasing concentration of VFAs. The chemical and biological analysis revealed, that it is possible to produce mixture of VFAs with similar composition despite the substrate type.

From the scientific point of view the determination of interaction between factors and VFAs production was essential (Fig. 4). The novelty of presented research is the analysis of the open culture fermentation efficiency in wide range of initial pH without addition of significant amounts of chemicals (buffers, acid and alkali) to control the pH of the process. The open culture fermentation of maize silage, acid whey and microalgae biomass was barely presented in the literature before. Moreover, the chemical analysis of produced carboxylates included more acids i.e. C2-C7 than previously presented in literature i.e. C2-C5 (Arslan et al., 2016).

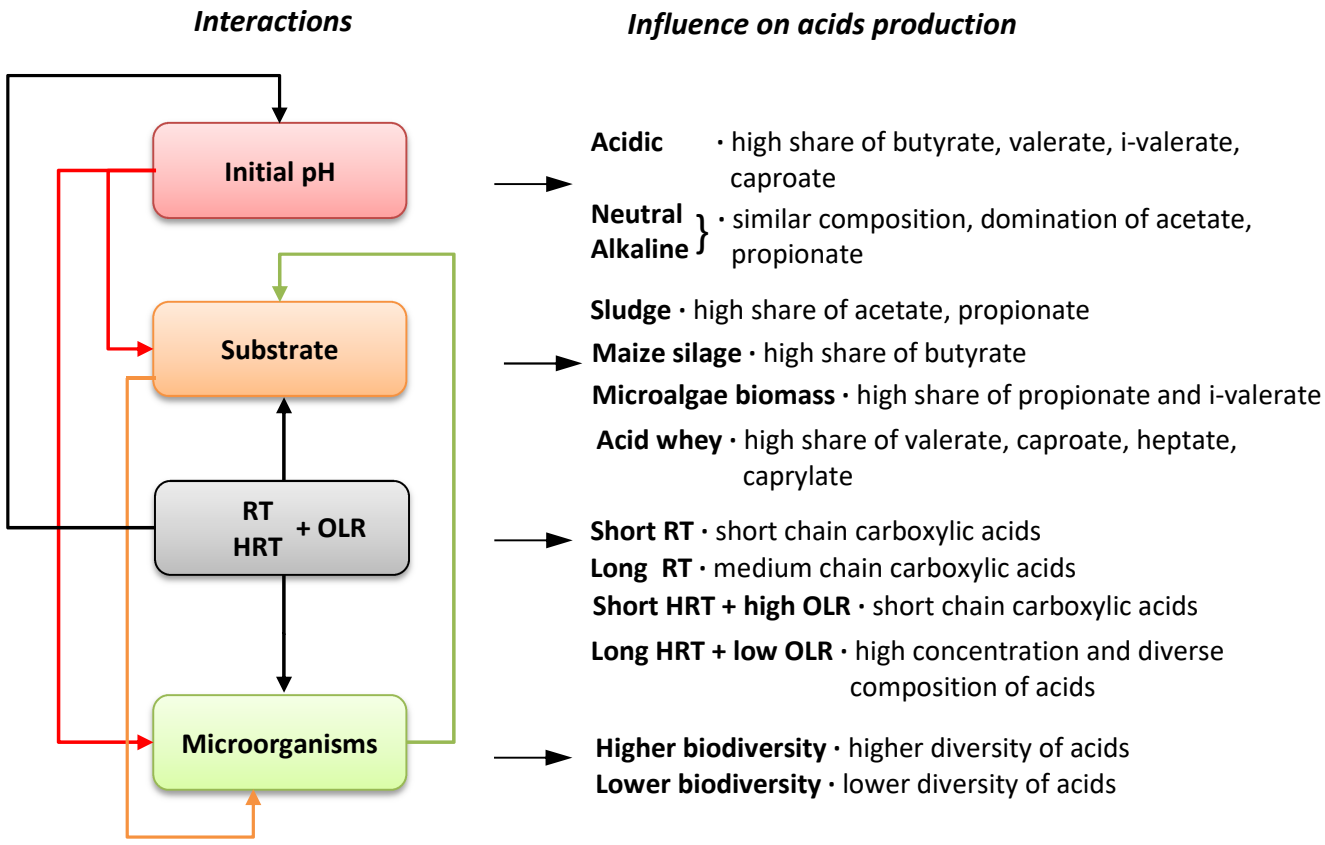

Fig. 4. Interactions between factors affecting open culture fermentation.

Described results showed the novel, sustainable and natural approach of utilization of organic wastes, thus biological conversion to valuable bioproducts, instead of landfilling or incineration as main methods. In the future, the open culture fermentation could be used for utilization of significant amounts of organic wastes and wastewaters as one of the internal process within the biorefinery system.

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Effect of pH and retention time on volatile fatty acids production during mixed culture fermentation

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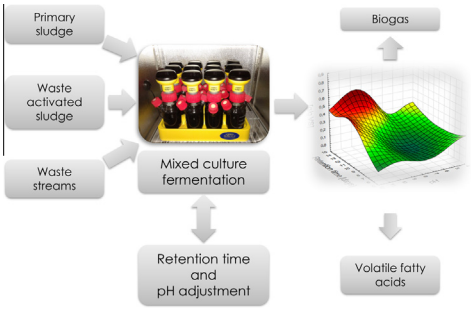
HIGHLIGHTS

- The highest total VFAs concentration at RT = 5 days was achieved at acidic environment.
- Alkaline environment was favorable at long RT due to WAS disintegration.
- Acetate dominated all of the VFAs most likely due to WAS disintegration.
- Caproate concentration doubled with prolonging RT from 5 to 15 days.

GRAPHICAL ABSTRACT



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ABSTRACT

Mixed culture fermentation consists of stable microbial population hence waste could be potentially used as a substrates. The aim of the work was to investigate the impact of pH and retention time on the anaerobic mixed culture fermentation. Trials at different pH (4–12) in unbuffered systems were conducted for 5, 10 and 15 days. The highest VFAs concentration was achieved after 15 days at pH 10 (0.62 g/g VS_{added}), promising results were also achieved for pH 11 (0.54 g/g VS_{added}). For pH 4 and short retention time propionic acid was the major product instead of acetic acid. For batches run at 15 days (besides pH 6) caproic acid presence was noticed whereas at pH 11 occurrence of succinic was quantified. Significant correlation between operational factors and fermentation's effluents was proved. Throughout changing simple operating parameters one could design process to produce desirable concentration and composition of VFAs.

1. Introduction

Fermentation processes can produce valuable chemicals and biofuels. Most of the biotechnological processes use pure culture fermentation because process parameters can be optimized for specific strains of microorganism. However, pure culture fermentation has fundamental disadvantage – it requires sterile operating conditions and high quality (and very often, high purity) raw material. Mixed culture fermentation (MCF), on the other hand, does not rely on specific microorganism's strain and can be operated in non-sterile conditions without significant risk of contamination (Lu et al., 2011). Moreover, MCF is able to consume wide spectrum of substrates containing diverse organic chemical compounds. Due to those characteristic, MCF can be fed with municipal, agricultural or food-production waste streams (Rodriguez et al., 2006). Agriculture by-products and municipal waste streams are produced in vast amounts; their utilization into useful products would bring great advantages. First of all it would decrease the amount of waste generated, secondly it would provide sustainable products (in contrast to fossil fuels based product) while cutting

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down green-house gasses emission. If optimized, MCF processes have a great chance to outcompete pure culture fermentations.

Anaerobic digestion (AD), best example of MCF, is a biological process where most organic matter (carbohydrates, lipids, proteins) except for lignin components, in the absence of oxygen, is degraded into methane and carbon dioxide. The process consists of series of reactions and it is a natural process which takes places in several anaerobic environments (Gujer and Zehnder, 1983; Angelidaki et al., 2003). Typically, AD provides renewable energy source (biomethane) and it also delivers highly efficient natural fertilizer. Biological conversion such as AD that converts wet biomass waste into biogas is a well-established technology (Holm-Nielsen et al., 2009), whereas conversion of biomass waste to high-value biochemicals is still only in the exploratory research phase (Agler et al., 2010, 2014; Hoelzle et al., 2014).

Volatile fatty acids (VFAs) are intermediate products in anaerobic digestion, nevertheless they could become valuable substrates for more advanced final products (Fu and Holtzapple, 2010). VFAs could be generated from various waste streams such as presented by Sans et al. (1995), Yu and Fang (2002), Kim et al. (2006), Chen et al. (2007) or Alkaya and Demirer (2011). Different types substrates for VFAs production were summarized and reviewed by Lee et al. (2014). Many studies which investigated VFAs production, focused on generation of polihydroxyalkanoates (PHA) (e.g. (Albuquergue et al., 2007; Bengtsson et al., 2008) because the composition of VFAs determines the composition of the main products i.e. acetate and butyrate usually result in formation of hydroxybutyrate monomers whereas propionate leads to increased concentration of hydroxyvalerate monomers (Bengtsson et al., 2008). Furthermore, the VFAs composition also has impact on hydrogen (Aguilar et al., 2013) or even lipids (Fontanille et al., 2012) production, and, as recently investigated, as well on medium and long chain fatty acids (Agler et al., 2012; Grootscholten et al., 2013a,b).

Understanding the impact of several operating conditions on this complex but widely occurring process would significantly extent knowledge about it. It was suggested that the operational conditions could determine which catabolic product would be dominant allowing at the same time more efficient growth in MCF systems (Rodriguez et al., 2006; Temudo et al., 2007). In a longer perspective, it would help utilizing various waste streams generated by nowadays societies not only into methane but also into high-value chemicals. In our work we refocused on the influence of pH and retention time (RT) on the VFAs productivity and composition. Other studies investigated the impact of pH but they used glucose (Horiuchi et al., 2002; Temudo et al., 2007, 2008; Lu et al., 2011) or synthetic wastewater (Infantes et al., 2011). To the knowledge of the authors there are no studies investigating the full spectrum of pH and RT impact on VFAs productivity and composition using actual waste streams (in our case: mixture of primary sludge (PS) and waste activated sludge (WAS)). The aim of the work was to investigate whether by changing simple operating parameters one could design process in order to produce desirable composition of VFAs while using mixture of the most abundant waste streams: PS and WAS.

2. Methods

2.1. Raw materials

Substrates were PS and WAS obtained from the Central Wastewater Treatment Plant in Kozieglowy (Poznan area, Poland) and were stored at −20 °C prior usage. Mixture of PS and WAS (in 1:1 ratio, by volume) was applied for each MCF trial. Inoculum for MCF trials originated from the same wastewater treatment plant and was collected in increments from full-scale mesophilic anaerobic digestion reactor. Inoculum was stored at 35 °C for 48 h prior usage. The characteristics of the inoculum, PS, and WAS are shown in Table 1.

2.2. Batch tests

The batch trials were run at mesophilic conditions (35 °C) in sets of 12 batches in 400 mL glass bottles with gas pressure detectors (OxiTop Control, Germany). Mixing was assured by magnetic stirrers. The trials run at 5, 10 and 15 days in the pH range of 4–12 (adjusted with 18% HCl and 3 M KOH solutions) in unbuffered systems with control and blank samples. Each experiment was performed in triplicate. The loading of substrate was 0.5 g VS/100 mL. Headspace of each bottle was flushed with nitrogen and carbon dioxide (80/20) before the trials in accordance with (Angelidaki et al., 2009).

2.3. Analytical methods

Total solids (TS) and volatile solids (VS) were measured using standard method PN-EN-12879: for TS determination samples were dried in 105 °C over night, for VS determination samples were ashed in 550 °C for 3 h. The pH value was measured by pH meter (Mettler Toledo FiveEasy™ pH bench meter, FE20).

Chemical oxygen demand (COD) was determined according to the Polish Standard (PN-74/C04578/03) with dichromate method which measures the amount of oxygen needed for oxidizing chemical compounds in the acidic environment created by potassium dichromate. Samples were diluted with demineralized water 1:50 and 1:100. Absorbance was measured in the photometer at wavelength 605 nm and the COD values were determined from the calibration curve.

C/N ratio was measured as the total content of organic carbon (TOC) (according to the Merck procedure No. 1. 14879.0001, range: 50–800 C/L) and of total nitrogen (TN) (according to the Merck procedure No. 1. 14763.0001, range: 10–150 N/L) (Merck Millipore, Billerica, MA, USA). Samples were diluted with demineralized water in the 1:10 and 1:20 ratio for C and N, respectively. Concentration of the TN and the TOC were measured in a photometer.

Qualitative and quantitative analysis of particular VFAs and alcohols including: formate, malate, acetate, propionate, lactate, butyrate, i-butyrate, valerate, i-valerate, caporate, methanol, ethanol, butanol, and i-butanol, were determined through HPLC Dionex Ultimate 3000 (Dionex Corporation, Sunnyvale, USA). The HPLC was equipped with Shodex 1011 column (300 × 8 mm) (Showa Denko, Japan), on-line degassing and refractive index detector. It was eluted with 5 mM aqueous sulfuric acid at the flow rate 1 ml/min at 60 °C. Aqueous and solid phases were separated by centrifugation before being analyzed with the HPLC.

2.4. Statistical analysis

Analysis of variance (ANOVA) was applied to investigate pH and retention time and their two-way interactions on the composition of MCF effluents. All ANOVA were run using Statistica software v.10.

3. Results and discussion

In order to investigate the impact of the pH and RT on the VFAs productivity and distribution during fermentation of sludge by an undefined mixed microbial culture, trials were carried out at pH values ranging from 4 to 12 for RT of 5, 10 and 15 days. The total VFAs productivity based on the obtained results is given on Fig. 1.

Table 1
Characteristic of inoculum, primary and waste activated sludge.

	Inoculum	Primary sludge	Waste activated sludge
TS [%]	4.0	4.2	6.1
VS [%]	2.6	3.1	4.7
COD [mg/L]	5127	2052	3306
C/N	6.0	21.5	9.7
Malate [g/gTS]	0.003	0.024	0.008
Formate [g/gTS]	0.005	0.017	0.023
Acetate [g/gTS]	0.019	0.196	0.048
Propionate [g/gTS]	0.005	0.105	0.036
Succinate [g/gTS]	0.003	0.007	0.017
Butyrate [g/gTS]	0.009	0.046	0.006
i-Butyrate [g/gTS]	0.001	0.005	0.003
Valerate [g/gTS]	0.001	0.003	0.001
i-Valerate [g/gTS]	0.000	0.000	0.000
Caproate [g/gTS]	0.002	0.004	0.002
Ethanol [g/gTS]	0.002	0.001	0.007
Methanol [g/gTS]	0.000	0.000	0.000
Propanol [g/gTS]	0.000	0.000	0.000
Butanol [g/gTS]	0.000	0.000	0.000

3.1. Biogas and VFAs productivity

Small volumes of biogas was read even in pH values outside the range of optimal for methanogens (6.5–8.5). Those readings might be caused by pH changes in micro-spots, however the values were insignificant. The obtained concentration of VFAs in unchanged pH (7.5) after 5 days trials was (0.037 g/g VS_{added}) and lower for 15 days long trials 0.025 g/g VS_{added}. The highest concentration was noticed for 10 days long trials 0.052 g/g VS_{added}. In unadjusted pH trials, biogas production was increasing with longer RT, i.e. 319 mL/g VS_{added}, 409 mL/g VS_{added} and 480 mL/g VS_{added} for 5, 10 and 15 days, respectively. The concentration of the remaining VFA was insignificant.

In batch trials with initial pH of 4 the increase in pH levels was noticed (around 1.25 on average). The VFAs concentration was in the same range for all RT, thereby, 0.239 g/g VS_{added}, 0.228 g/g VS_{added} and 0.267 g/ VS_{added} for 5, 10 and 15 RT, respectively. In batch trials with initial pH of 5, the increase in pH level was observed by 0.48, 1.16 and 1.25 for RT of 5, 10 and 15 days, respectively. The increase in pH values in unbuffered systems resulted in slight biogas production. The biogas production during 5 days trials reached 5.5 mL/g VS_{added} and was higher for longer batch trials: 82 mL/g VS_{added} for 10 days and 238 mL/g VS_{added} for 15 days. The VFAs concentration after 5 days batch fermentation was at the level of 0.390 g/g VS_{added}, it was lower for processes running longer: 0.230 g/g VS_{added} for 10 days and 0.207 g/g VS_{added} for 15 days. Possible adaptation of methanogens occurred at micro-spots with higher pH level, which allowed biogas production. During the batch trials with pH adjusted to 6, the pH did not changed significantly during 5 days trials. For the processes conducted for 10 and 15 RT, pH increased by 0.96 on average. The obtained biogas production was 225 mL/g VS_{added} for 5 days trials and 381 mL/g VS_{added} for 10 days. For 15 days trials it was much higher compared to 10 days trials (642 mL/g VS_{added}). The analysis of VFAs concentration showed the gradually decrease with time: 0.067 g/g VS_{added}, 0.016 g/g VS_{added}, 0.005 g/g VS_{added}, for 5, 10, and 15 days long trials, respectively.

During the MCF batch trials in alkali conditions, for the process run at pH 9, reduction of the pH was observed regardless of the duration of the trials: about 1.33–1.40. The biogas production was negligible during 5 days trials – only 29 mL/g VS_{added} but for 10 days trials increased till 462 mL/g VS_{added} whereas for 15 days trials it achieved 534 mL/g VS_{added}. The biogas production was caused by decreased pH from the initial value and activation of methanogens. The VFAs concentration was very low for all trials: 0.012 g/g VS_{added},0.017 g/g VS_{added} and 0.012 g/g VS_{added}, for 5, 10 and 15 days respectively. In more alkali environment (pH 10), the decrease in pH level was more noticeable: 2.4 on average for all RT. No biogas production was noticed for RT 5 but for longer RT it reached 111 mL/g VS_{added} and 154 mL/g VS_{added} for RT 10 and RT 15, respectively. Again, in unbuffered systems, the production of VFAs caused decrease of the initial pH values in consequence small biogas production. Linear increase with time in VFAs concentrations was noticed (0.277 g/g VS_{added}, 0.420 g/g VS_{added} and 0.621 g/g VS_{added}, for 5, 10 and 15 RT, respectively). The pH decrease was also observed in experimental set-ups with pH 11. It declined by 2.81 on average for all RT. For all trials the biogas production was not observed. The VFAs concentration was increasing with prolonging of the fermentation time: 0.190 g/g VS_{added} (5 days), 0.261 g/g VS_{added} (10 days), and 0.540 g/g VS_{added} (15 days). The changes in batch processes carried out at pH 12 showed similar trends like in the ones at pH 10 and 11 but the drop was more significant (3.31 on average). There was no biogas production at highly alkaline environment. The VFAs concentration at trials run at 5, 10 and 15 RT was as follows: 0.039 g/g VS_{added}, 0.629 g/g VS_{added} and 0.461 g/g VS_{added}.

The RT had noticeable effect on both, VFAs productivity and biogas generation. In general, during long RT more VFAs were produced which in consequence overcompensated the unfavorable pH, which might had led to reach optimum pH for methanogens. Additionally, some of the WAS might have been disintegrated releasing additional amounts of acetate. On 5 days RT batch processes, the highest amount of VFAs was at pH 5 (0.390 g/g VS_{added}). The lowest was observed in assays with pH 9 (only 0.012 g/g VS_{added}). No biogas production was observed for 5 days RT and highly alkaline environment because such short RT was not sufficient enough for produced acids to compensate high pH values. On 10 an 15 days RT batch processes intensified biogas production was observed at pH 9 (462 mL/g VS_{added} and 534 mL/g VS_{added}, respectively). The initiated pH was just outside the optima for methanogens (6.5–8.5), so during the 10 and 15 days of the process, the produced VFAs buffered the system and consequently methanogens became active again. For 10 days RT, the highest VFA concentration was observed at pH = 12 (0.629 g/g VS_{added}). On 15 days RT batch processes intensified VFAs production was observed. The biggest volume of biogas was obtained at pH 6 (642 mL/g VS_{added}) and pH 9 (534 mL/g VS_{added}), higher than the one achieved in unadjusted assays (470 mL/g VS_{added}) due to disintegration of WAS. The highest VFAs concentration was achieved at pH 10 (0.621 g/g VS_{added}). For pH 11 and pH = 12 concentration of 0.540 and 0.461 g/g VS_{added} was achieved, respectively.

3.2. Impact of pH and RT on the VFAs

From Fig. 1 the effect of pH on VFAs productivity can be seen. It was observed that higher rate was achieved at higher pH values, similar trend was observed in (Infantes et al., 2011). Temudo et al. (2008) stated that pH determines the fraction of undissociated acids in the broth which were able to permeate cell membranes. On the Fig. 2 yields of particular VFAs depending on pH and RT are shown. As explained in (Rodriguez et al., 2006) and (Infantes et al., 2011), the rate of the fermentation is related to the inhibition effects caused by the permeability of undissociated acids through the cellular membrane. At lower pH, more energy is needed for transport of undissociated acid whereas at higher pH, energy can be gained from transport of the free form of the acid (Rodriguez et al., 2006). The distribution of produced VFAs is important and useful information regarding the degree of hydrolysis and fermentation. pH can affect the type of VFA produced during MCF: particularly acetate, propionate and butyrate (Bengtsson

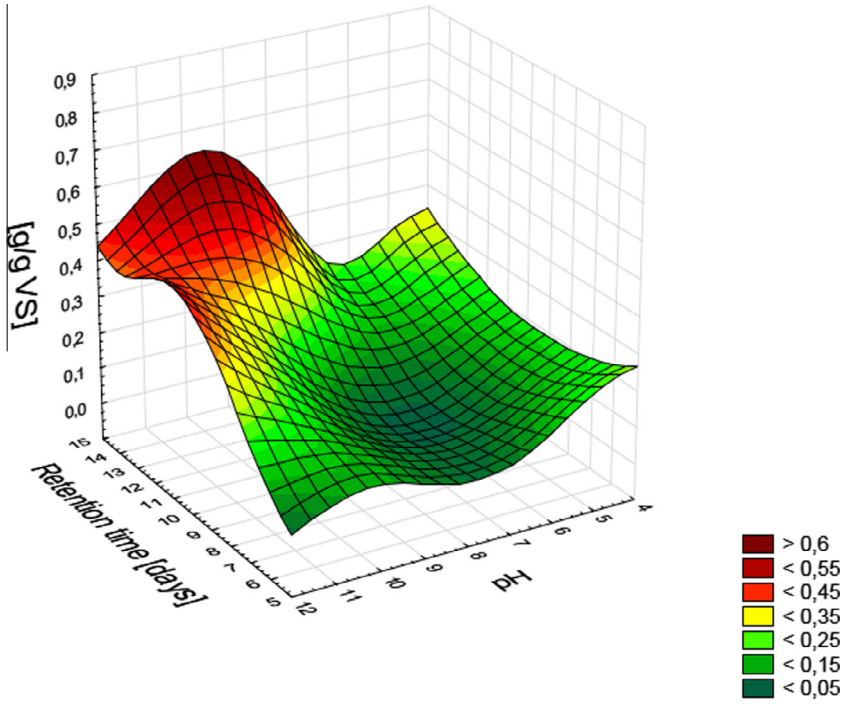

Fig. 1. Total VFAs changes at trials conducted at different pH (4–12) for 5, 10 and 15 retention times.

et al., 2008). Parameters such as the growth rate, the utilization of the carbon source or the efficiency of substrate conversion are also affected by the pH (Russell, 1992; Temudo et al., 2008). Moreover, Temudo et al. (2008) documented that depending on the pH range different groups of microorganisms become dominant which has direct effect on the spectrum of generated products. It has been reported previously that acetate, propionate and butyrate could be formed directly from the fermentation of soluble proteins, carbohydrates and lipids (Horiuchi et al., 2002), while iso-valerate and n-valerate were mainly produced from proteins degradation (McInerney, 1998). In the conducted trials, VFAs production rate increased with RT because more soluble proteins and carbohydrates were available at longer RT. Additionally, disintegration of WAS occurred with prolonging RT. Substrates used in the conducted trials (mixture of PS and WAS) are known to be not as easily fermentable as for instance glucose, that is why longer RT was needed to achieve high conversion yields. With the increase of RT from 5 to 15 days, especially the concentration of acetate increased significantly but also other compounds which were produced in lower concentrations such as caproate (Fig. 2).

3.3. VFAs distribution

Fig. 3 shows the VFAs percentage distribution in the fermentation broths. At the RT of 5 days and the pH 4 the prevalent VFAs generated were acetate, propionate and butyrate, which accounted for 40.7%, 20.0% and 22.4% of the total, respectively. The composition of VFAs was relatively stable between pH 4 and 5. However, at pH 6, propionate and caproate were the dominant products because most of the acetate was consumed by methanogens. The overall VFAs concentration was low at batch assays at pH 6, 9 and unadjusted (ca. 7.5) due to optimal condition for the methanogens and consequent biogas generation. Total VFAs content was much higher at the pH values of 10 and 11. Distribution of particular VFAs was similar for both alkaline conditions with acetate as the dominant compound. At highly alkali environment (pH 12) at RT = 5 the microbial activity was inhibited and only small concentration of VFAs was detected with mainly acetate and iso-butyrate (32.6% and 28.2%, respectively). Additionally small concentrations of formate and malate were detected. Similarly to RT = 5, at RT of 10 days the lowest VFAs concentration was at the pH values between 6 and 9 due to near optimal pH range for methanogens (between 6.5 and 8.5). In those effluents small amounts of malate, butyrate and iso-butyrate were detected. Additionally, succinate presence was noticed. At alkaline environment (pH of 11 and 12) higher percentage of acetate and lower butyrate was observed. Prolonging RT to 15 days led to significant shift towards acetate production in alkaline environments. Whereas at the acidic environment (pH 4 and 5) noticeable amount of butyrate was detected. Again in conditions close to the optima for the methanogens almost all of the VFAs were converted into biogas, within the remaining VFAs malate was detected.

3.4. Composition of effluents

Temudo et al. (2007) reported formate formation at high pH (8.5) at short retention time (20 h). In our study we noticed formate formation at pH 9 but only at RT = 5 (however the concentration was low due to active methanogens). Formate was also noticed at pH 12 and RT 5. At longer RT formate, most likely, was converted into other metabolites. At the low pH, formate could be cleaved into H_2 and CO_2 by the formate hydrogenlyase (Temudo et al., 2007), so its presence was not noticed. In all our results the dominant product was acetate which could be in disagreement with other studies. This was caused by the characteristic of the substrate: WAS is hardly fermentable substrate. Highly acidic or alkali environments served as pretreatment e.g. (Pang et al., 2015) which could have revealed higher concentration of acetate compared to the MCF run on glucose or synthetic media. Additionally, initial content of acetate in PS also influenced the distribution of the VFAs in the batch tests effluents.

Typically, at low pH ranges dominant products should be butyrate and acetate, whereas at high pH ranges, butyrate production should decrease due to dominant presence of genus *Klebsiella*

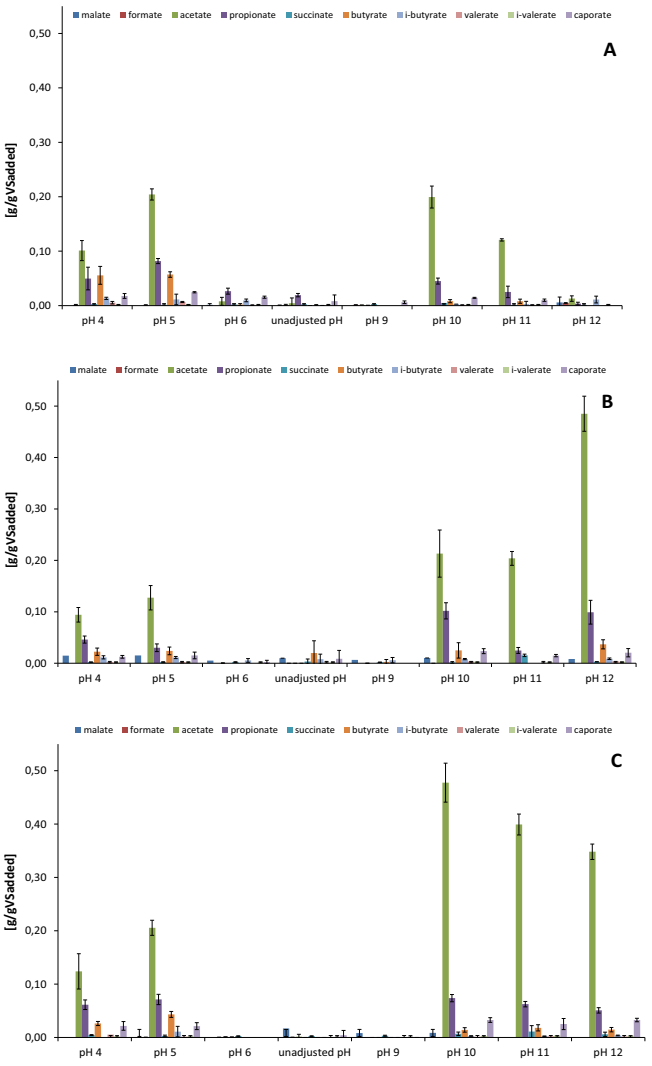

Fig. 2. Composition of the effluents after MCF batch trials conducted at different pH (4, 5, 6, 9, 10, 11, 12) and in unadjusted pH for different RT: 5 (A), 10 (B), and 15 (C) days.

which lacks the enzymes involved in butyrate production (Temudo et al., 2008). In the agreement with the theory, Temudo et al. (2008) reported that at the low pH values (4–5.5) main products were butyrate and acetate whereas at high pH values (7.5–8) the production shifted to acetate and ethanol. Oddly, Infantes et al. (2011) achieved slightly higher yield of butyrate in pH of 6 compared to 4. In our study (similarly to (Temudo et al., 2007)) such a trend was not observed. Temudo et al. (2007) observed the shift from butyrate to acetate/ethanol production in the pH range between 6.5 and 8, similar trend was noticed for our mixed cultures, where the acetate participation doubled for alkaline conditions. The exact process conditions when the shift from butyrate to acetate/ethanol occurs will depend on the specific mixed culture applied as inoculum. In our case, it could be seen between pH 6 and 9. More precise experiments would be necessary in order to specify those conditions. Additionally, during the trials the methanogens were only inhibited by the pH changes, therefore in the range from 6.5 to 8.5 significant amount of biogas was generated.

We noticed ethanol presence at trials with RT of 15 days at pH of 9 and 10 (data not shown). Additionally, ethanol was detected at pH 5 (RT = 15) (data not shown) which is with agreement with model developed by (Rodriguez et al., 2006) but it is not consistent with study described in (Temudo et al., 2007). In either our case the concentration was not significant, probably due to long RT. Significant ethanol presence could be achieved with shorter RT (i.e. 20 h (Temudo et al., 2007) or 6 h (Lu et al., 2011)). Other alcohols (methanol, butanol, i-butanol) were not detected in effluents from conducted batch trials. In the aforementioned study (Temudo et al., 2007), authors reported succinate mainly at pH value of >5.5, suggesting that its presence depended on the bicarbonate concentration (it involves a carboxylation reaction). On the Fig. 3C, one can see succinate dominance at pH 6. During that trials all of the VFAs were converted into biogas, only small concentration of succinate remained, hence those results should not be read as permanent pattern.

Horiuchi et al. (2002) proved that spectrum of products during the acidogenic fermentation was strongly dependent on the pH. The authors stated that it was caused by shift of the microbial population from butyric acid producing bacteria to propionic acid producing bacteria. Horiuchi et al. (2002) also suggested that due to strong pH dependency of organic acids production, there would be possibility for selective production of acids from various organic waste streams. Chen et al. (2007) noticed that alkaline pH is favourable for VFAs production from WAS because it supports hydrolysis of sludge, whereas Yu and Fang (2002) underlined that acidic conditions are favourable for maximizing the VFAs productivity. In the presented study, we could confirmed both results. For short RT, the highest total VFAs concentration was achieved at acidic environment (pH 5), however prolonged RT favoured alkaline conditions due to disintegration and hydrolysis of WAS.

To summarize, at high pH values (>7) acetate production increased, moreover longer RT promoted disintegration of WAS which resulted in significantly higher acetate concentration. On average at increasing pH values butyrate yields decreased. Valerate and iso-valerate concentration were insignificant at any process operating condition and caproate was present in almost all MCF broths and its concentration doubled with prolonging RT from 5 to 15 days.

The changes in effluents' composition were most likely caused by the shifts in the dominant microbes within mixed microbial cultures. Jie et al. (2014) characterized, through phylogenetic analysis, the dominant species while fermenting WAS at different pH levels. During 10 days trials, authors noticed *Pseudomonas* sp., *Clostridium* sp., *Variovorax* sp., and uncultured bacteria (Jie et al., 2014). However, not straightforward conclusion was drawn how exactly the community changed at different pH. Gou et al. (2014), on the other hand, investigated impact of the temperature and organic loading rate on the microbial community during anaerobic digestion process. It was concluded that temperature had stronger effect on the richness and diversity of microbiome compared to the organic loading rate. No specific microbial species were determined during that analysis. Similar conclusion were drawn by Maspolim et al. (2014), where no significant change of the microbial community was noticed in the acidogenic reactor while decreasing hydraulic retention time. The review of microbial community changes, in samples originating from 78 different anaerobic digesters, was presented by Zhang et al. (2014). The authors concluded that even though the reactor configuration, pH and temperature has impact on the structure of microbiomes, it was the substrate type which mostly influenced the phylogenetic structure.

3.5. Statistical analysis

Analysis was performed in two stages: in the first, one-way ANOVA was applied to investigate the influence of pH and retention time on the composition of MCF effluents. In the second stage the effect of pH and retention time interactions on the composition was measured in the two-way ANOVA test. For all calculations significance level equal to 0.05 was assumed. The results are shown in

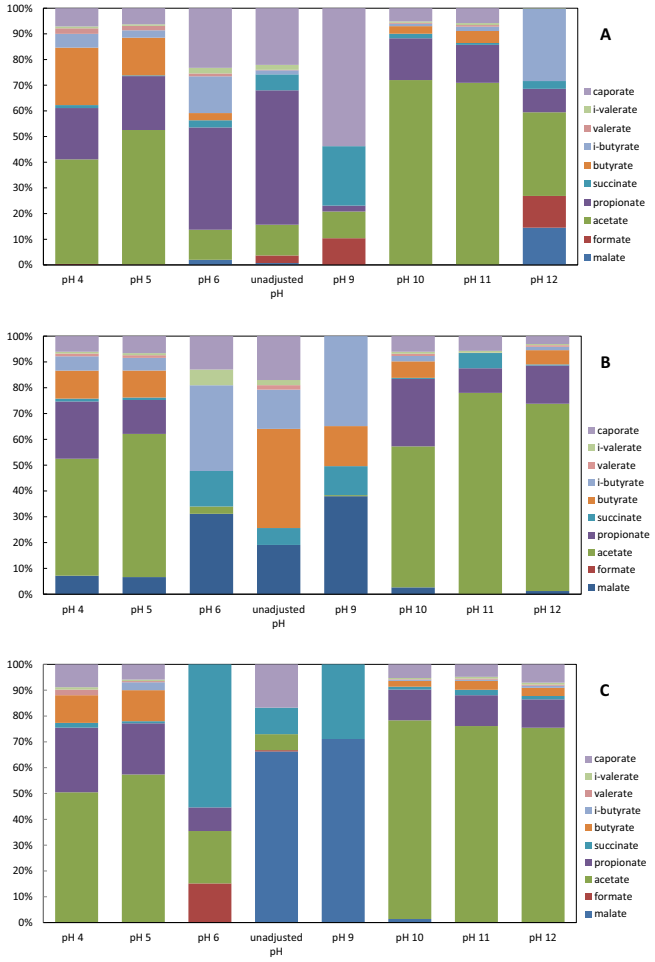

Fig. 3. Percentage distribution of VFAs in batch trials conducted at different pH (4, 5, 6, unadjusted, 9, 10, 11, 12) for different period of time (5 (A), 10 (B), and 15 (C) days).

Table 2. It was noticed that malate concentration was neither correlated to pH nor RT. Production of propionate, butyrate, valerate, i-valerate did not depend on RT but the correlations were significant for pH. In ANOVA of two-way interaction strong significance was noticed for formate, acetate, propionate, butyrate, valerate, i-valerate, and caproate concentrations. For caproate two-way interaction was more significant than for these two factors separately. For most VFAs pH was more significant than RT, except for formate, which is typically quickly converted to other metabolites. Butyrate, on the other hand, appeared almost no significance in correlation with RT but very significant correlation with pH. Additionally ANOVA indicated significant correlation between ethanol concentration and pH and RT.

The results also indicated the strong correlation between VFAs distribution and substrate applied in the process. Using WAS significantly increased concentration of acetate due its disintegration in highly acetic or alkaline environments. Lastly, MCF could become attractive process for biochemicals production, however in order to produced desired composition of VFAs one might have to design two-three steps process e.g.: (1) long RT and alkaline pH for disintegration of WAS, (2) acidic pH for butyrate (short RT) or caproate (longer RT) production, finally, (3) biogas generation at near neutral pH from the rest of the organic compounds.

Table 2
Analysis of variance for MCF effluents.

Compound	pH	Retention time	pH and retention time
	One-way ANOVA		Two-way ANOVA
	p		
Malate	0.532565	0.676678	0.997647
Formate	0.025576	0.000020	0.000000
Acetate	0.000000	0.006420	0.000000
Propionate	0.000000	0.252675	0.000000
Succinate	0.000557	0.043630	0.001017
Butyrate	0.000000	0.972542	0.000001
i-Butyrate	0.000236	0.022025	0.034108
Valerate	0.000000	0.701541	0.000000
i-Valerate	0.000004	0.094889	0.000000
Caproate	0.000719	0.034789	0.000006
Ethanol	0.002660	0.004933	0.002802
Total VFAs	0.000000	0.001984	0.000000

4. Conclusions

Throughout prolonging fermentation RT, higher VFAs concentration was possible to achieve, however, the risk of contamination by methanogens existed in unbuffered systems. Promising results were achieved for both acidic and alkaline conditions: for short RT (5 days) the highest total VFAs concentration was achieved at pH of 4 whereas the alkaline conditions were more favorable for longer RT. Shit from butyrate to acetate production was noticed between pH of 6 and 9, unfortunately it was corrupted by high concentration of acetate originating from disintegrated WAS. MCF could become attractive process for biochemicals production but in two or three steps process.

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Volatile fatty acids production during mixed culture fermentation – The impact of substrate complexity and pH

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HIGHLIGHTS

- Different substrates will lead to a similar spectrum of products in the MCF process.
- In the acidic pH there is higher diversity of volatile fatty acids.
- Pre-adjusted alkaline pH is favorable for hydrolysis of complex organic matter.

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ABSTRACT

Volatile fatty acids (VFAs) are obtained during chemical routes from non-renewable petrochemicals. Intensive exploitation of oil resources have renewed the idea of VFAs production during biological routes, mainly throughout anaerobic mixed culture fermentation (MCF). We carried out MCF trials at initial acidic (5.0), neutral (7.0, with addition of specific methanogenesis inhibitor) and alkaline (11.0) pH conditions for four different substrates i.e. maize silage, cheese whey, microalgae biomass and glucose. The goal of the study was to investigate the impact of the substrate complexity on the produced VFAs' quality and quantity. The highest VFAs concentration occurred in neutral pH proceeded by initial alkaline pH (0.83 gVFAs/gSCOD for microalgae biomass, 0.78 gVFAs/gSCOD for maize silage and 0.71 gVFAs/gSCOD for cheese whey, respectively). In the fermentation of glucose, the highest VFAs concentration was achieved in neutral pH. We demonstrated that the alkaline pH was favorable for hydrolysis of complex organic matter (acidification yield over 71% for maize silage fermentation), while the neutral pH was beneficial for the acidogenesis and the overall VFAs production. Our findings showed that it was possible to carry out efficient and stable MCF process without using a large amounts of acid or base for pH adjustment and that the distribution of VFAs only merely depended on the substrate type.

1. Introduction

Volatile fatty acids (VFAs) are short-chain fatty acids that contain from 2 to 5 carbon atoms. Nowadays, VFAs are obtained during chemical routes from non-renewable petrochemicals. Intensive exploitation of oil resources have renewed the idea of VFAs production during biological routes. VFAs have wide range of applications i.e. as a carbon source for biogas production, biopolymers (PHA), biofuels precursors, chemical building blocks or utilized as a carbon source for the biological removal of phosphorus or nitrogen [1,2]. The biological production of VFAs can be carried out by anaerobic acidogenic mixed culture fermentation, also known as dark fermentation [3]. Earlier work indicated that the VFAs production could effectively run on sludge, organic fraction of municipal solid waste, food waste, organic residues from food production or microalgae biomass [4,5,6] but there have not been a clear answer on how different substrate types influence the composition of the VFAs.

There is a great potential in Europe for VFAs production from biomass and biowaste. Only the EU food sector generates about 250 mil Mton/year of by-products and waste of which only a part is reused and recycled. Most of the generated food production waste is spread on land, used as an animal feed or composted;

Abbreviations: VFAs, Volatile fatty acids; VS_{added}, Added volatile solids; WWTP, Wastewater treatment plant; MCF, Mixed culture fermentation; VS, Volatile solids; TS, Total solids; RT, Retention time; TCOD, Total chemical oxygen demand; SCOD, Soluble chemical oxygen demand; TN, Total Nitrogen concentration; η_h, Hydrolysis yield; η_a, Acidification yield; Y_X, Yield of biomass.

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unfortunately the largest fraction is disposed as wastes [7]. Other substrates could be terrestrial or aquatic biomass. According to Chang et al. [8] the potential of global exergy flow for terrestrial and ocean biomass is 65 and 25 TW, respectively. However, less than 0.2% of it is used as a biofuels. Substrate considered as a valuable feedstock for the VFAs production should be characterized by a high carbon content, good biodegradability, as well as it should be available in sufficient amount (especially in case of substrates which are only available seasonally) [6].

Through the acidogenic fermentation of organic residues and biomass, the sustainability of a food sector could be significantly improved, especially by the possible production of valuable chemicals such as acetic, propionic, butyric or valeric acids. VFAs can be further used for the production of pharmaceuticals [9,10], chemicals and polymers [11], esters (used in food and cosmetics industries) [12] and fuels [13] or solvents [14]. The market size of acetic acid is the highest (3,500,00 ton/year), next to propionic acid (180,000 ton/year), the smallest market is for butyric and caproic acids (30,000 ton/year and 25,000 ton/year, respectively) [12]. On the other hand, caproic and butyric acid have the highest market prices (2500 USD/ton), followed by lactic acid (2100 USD/ton), propionic acid (1700 USD/ton) and the lowest price for acetic acid (800 USD/ton) [12]. The world market of the bio-based products is increasing exceedingly. It had a value of 92 billion € in 2010 and it is expected to achieve 515 billion € in 2020 (values reported without the biofuels and pharmaceuticals) [15]. The minimum selling price of dark fermentation products was estimated for 382 USD/ton VFAs (cost of the VFA separation/purification was not detailed) [16]. Work of Bonk et al. [17] indicated that VFAs obtained from dark fermentation would be a cost effective if the operation cost of the separation/purification did not exceed 15 USD/$m^3_{effluent}$.

To produce high concentrations of the desired products, the process of mixed culture fermentation should be properly designed and controlled. Main factors affecting the fermentation, that have an impact on a proportion of the produced organic acids, solvents and gases, are: microbial population, inoculum source, substrates' complexity, nutrients' availability, pH, temperature, head space partial pressure, the gases composition and a bioreactor configuration [3]. It was reported that the pH for the VFAs production lays between 5.25 and 11, however it depends on the type of the substrate used [6]. Moreover, the optimal pH range is different for the different VFAs produced [3,18]. All aforementioned investigations were based on the controlled pH, thus adjusting to the assumed alkaline or acidic pH level by addition of large amount of base or acid. Whereas it is a good strategy for the laboratory trials, it might fail in a full scale operation, adding unnecessary operating costs.

The microbial ecology of mixed culture fermentation is still not fully understood. According to Wu et al. [19] the phyla associated with metabolizing of proteins, lipids, celluloses, sugars and amino acids are mainly *Proteobacteria, Bacteroidetes, Firmicutes* and *Chloroflexi*. *Clostridia* (phylum *Firmicutes*) had a high hydrolysis efficiency of polysaccharide and could generate organic acids [20]. Other one, *Sporanaerobacte,* degrade proteins and carbohydrates [21]. The *Bacteroidetes* (especially class *Bacteroidia*) showed a positive correlation with acetic and propionic acids production [22]. Class *Parabacteroides* could degrade proteins and polysaccharides [23] and according to Babu et al. [24] *Proteobacteria* were responsible for polysaccharides utilization or fermentation of butyric acid whereas *Chloroflexi* (*Bacilli* and *Anaerolineae*) enhanced the hydrolysis and decreased methane production [25]. The characterization of shifts in the microbial community was not a purpose of this work, our main aim to investigate only abiotic factors and to understand the process response regardless the mixed microbial community structure. It was documented that the VFAs were successfully produced from the variety of solid and liquid wastes [26]. However, the impact of the substrate type on the quality and quantity of the generated VFAs has not been clearly identified. Therefore, we took this crucial matter and investigated representatives of the most probable feedstock i.e. maize silage (lignocellulosic biomass), cheese whey (agro-food waste product) and microalgae (aquatic biomass). Additionally, we checked those feedstocks against glucose as a model substrate. The objectives of the study were (1) to investigate the differences between fermentation of simple and more complex substrates, (2) to reveal the influence of retention time on pH fluctuations during acidogenesis and (3) to explore the mechanism of how partly controlled (initial pH of 5.0 or 11.0) and neutral pH (with methanogenesis inhibitor addition) influence the VFAs production. We aimed at identifying the correlation between VFAs production without pH control and providing valuable information for the development of VFAs production by anaerobic mixed culture fermentation.

2. Materials & methods

2.1. Inoculum and substrates

The anaerobic mixed culture consortium used as a seed sludge (inoculum) was collected from the full scale mesophilic anaerobic digestion reactor used for primary and waste activated sludge treatment (WWTP Kozieglowy, Poland). Prior inoculation, seed sludge was stored in 35 °C for 48 h for degasification. The substrates used were: maize silage, cheese whey, microalgae biomass and 1% glucose solution (as a model substrate). The maize silage was stored at −18 °C and before usage the required amount was unfreezed and milled in mortar to homogenize it. No other pretreatment process was applied to prepare maize silage for acidogenic fermentation. The cheese whey was collected from the Dairy in Dobrzyca (Poland) and stored at −18 °C. Before usage, the required amount was unfreezed and mixed. The microalgae biomass was collected from a photobioreactor (WWTP in Kozieglowy), where it was cultivated in the effluent from the Anammox process and among all detected species the majority were *Scenedesmus quadricadua* and *Chlorella vulgaris*. Prior the anaerobic fermentation, the microalgae biomass was pretreated for 12 h at 90 °C in accordance with [27]. The 1% glucose solution was prepared from the powder glucose (Sigma-Aldrich). The main characteristics of substrates and seed sludge are shown in Table 1.

2.2. Reactor and operation – batch fermentation

The mixed culture fermentation (MCF) experiments were conducted in batch mode in twelve identical anaerobic reactors (Oxi-Top Control, WTW) equipped with two stub pipes and gas measuring sensor. The glass bottles had the working volume of 60 mL and the headspace capacity of 242 mL. The reactors were operated in the mesophilic temperature condition (35 °C). Each experimental batch set was operated with blank and control batches. 30 mL of inoculum and mass of substrate equivalent to the volatile solids loading of 0.5 gVS/100 mL was added to each reactor. The reactors were fed only at the beginning of the experiment. The initial substrate concentration (corresponding to 0.5 gVS/100 mL) was 5.33 gSCOD/L, 7.23 gSCOD/L, 5.16 gSCOD/L and 3.73 gSCOD/L for glucose, cheese whey, maize silage and microalgae biomass, respectively. Prior to the process, pH in the experimental reactors was adjusted to the initial values of 5 or 11 using 18% HCl and 3 M KOH, respectively. In the trials with neutral (unadjusted) pH, the methanogenesis was inhibited by a specific inhibitor ($BrCH_2CH_2SO_3$). To completely inhibit the methanogens, the concentration of BES (50 μmol/mL) was added

Table 1
Characteristics of the inoculum and substrates.

	Inoculum	Maize silage	Cheese whey	Microalgae biomass
TS [%]	3.45 ± 0.32	33.00 ± 2.44	5.20 ± 0.16	5.94 ± 0.09
VS [%]	1.97 ± 0.29	31.27 ± 2.50	4.48 ± 0.19	5.42 ± 0.09
pH	7.6 ± 0.26	7.01 ± 0.1	3.45 ± 0.2	7.3 ± 0.3
TCOD [g/L]	43.45 ± 5.21	883.31 ± 1.15	66.31 ± 0.61	89.31 ± 0.46
SCOD [g/L]	2.13 ± 0.70	150.71 ± 0.23	72.31 ± 0.40	38.11 ± 0.61
TN [mg/L]	1360 ± 68	–	1 220 ± 6110	5 500 ± 275.15
TN [mg/L] (soluble)	1000 ± 53	830 ± 41.50	980 ± 49.15	2025 ± 16.2
PO_4^{-3} [mg/L] (soluble)	153.1 ± 10.89	936 ± 31.11	636 ± 31.81	790 ± 39.6

according to the results presented in [28]. To assure the anaerobic conditions, each reactor was flushed with the mixture of carbon dioxide and nitrogen (20:80 vol/vol) gas for 1 min. After that, all the bottles were incubated in an appropriate process temperature and protected from the light. The mixing was assured by the magnetic stirrers, all the trials were run in triplicates.

2.3. Analytical methods

Composition of the volatile fatty acids was analyzed using the gas chromatography (GC-2014, Shimadzu, Japan), equipped with flame ionization detector (FID) and a silica capillary column (Zebron ZB-FFAP, 30 m × 0,53 mm × 1.00 μm). The VFA were analyzed according to the methods described in [29] and it included detection of acetate, propionate, iso-butyrate, butyrate, iso-valetarte, valerate, caproate, heptate and caprylate. The initial column temperature of the column was 70 °C for 3 min followed with a ramp of 10 °C/min and the final temperature was 240 °C. The temperature of the injector and the detector were 200 °C and 250 °C, respectively. Helium was used as the carrier gas at a flow of 7.4 mL/min. Prior the injection, the samples were centrifuged (13,000 rpm, 17 min) to separate the liquid phase from the solids and filtered (the syringe filters fabricated from the regenerated cellulose, 25 mm diameter, pore size 0.45 μm) then 2 mL of sample was injected.

Composition of the gas produced (methane, hydrogen and carbon dioxide) was analyzed using gas chromatography (GC-2014, Shimadzu, Japan), equipped with thermal conductivity detector (TCD) and a stainless steel column (80–100 Porapak, 2 m × 1/8 inch). The oven holding temperature was kept constant at 50 °C for 3 min. Nitrogen was used as a carrier gas at flow of 15 mL/min. The gases were analyzed according to standard gaseous mixture consisting of hydrogen, carbon dioxide and methane. 0.2 mL of standard gaseous mixture was injected three times prior the analyzed gas samples.

Total solids (TS) and volatile solids (VS) were measured according to the Polish standard method (PN-EN-12879) (105 °C over night for the TS determination and 550 °C for 3 h for the VS determination, respectively).

The total chemical oxygen demand (TCOD) and the soluble chemical oxygen demand (SCOD) was measured according to the PN-74/C-04578/03 method. The SCOD was measured in the supernatant after centrifugation (13,000 rpm, 5 min).

The total nitrogen (TN) was measured according to a Merck procedure No. 1. 14763.0001 (10–150 mg/L N) and it was quantified also in the supernatant. The concentration of ammonium was measured according to a Merck procedure No. 1. 14428.0002 (0.025–0.40 mg/L NH_4^+) and it was measured in the supernatant (13,000 rpm, 5 min). The concentration of phosphate was measured according to a Merck procedure 1.00673.0007 (3.0–100.0 mg/L PO_4-P). The pH was measured by the pH-meter (Mettler Toledo FiveEasy™ pH bench meter, FE20).

2.4. Calculation methods

The yield of biomass (Y_X) (Eq. (1)) was defined as the ratio of the amount of SCOD produced to the amount of substrate consumed [30]. The hydrolysis yield (η_h) (Eq. (2)) was calculated as the ratio of SCOD in the leachate to the initial TCOD of the substrate [19]. The acidification yield (η_a) (Eq. (3)) was calculated as the ratio of the cumulative VFA and final concentration of SCOD in the leachate [19]:

$$Y_X = \frac{(TCOD_{Out} - SCOD_{Out}) - (TCOD_{In} - SCOD_{In})}{(SCOD_{In} - TVFA_{In}) - (SCOD_{Out} - TVFA_{Out})} \frac{mgCOD/L}{mgCOD/L} \quad (1)$$

$$\eta_h = \frac{SCOD_{Out}}{TCOD_{In}} \times 100\% \quad (2)$$

$$\eta_a = \frac{VFA_{Out}}{SCOD_{Out}} \times 100\% \quad (3)$$

where $TVFA_{in}$ and $TVFA_{out}$ are the initial and cumulative total final VFA concentration (g COD); $SCOD_{in}$ and $SCOD_{out}$ is the initial and final soluble COD concentration (g COD); $TCOD_{in}$ and $TCOD_{out}$ are the initial and final total COD concentration (g COD).

3. Results and discussion

3.1. VFAs production - effect of pH and retention time

The VFAs accumulation during anaerobic digestion is the result of a series of complex biological reactions. The VFAs are generated during acidogenesis step including the primary fermentative acidogenesis, syntrophic acetogenesis and homoacetogenesis [31]. The results of the experiments illustrated a shift in the total VFAs concentration in relation to the initial pH, the retention time (RT) and the inhibitor addition. The detailed results are shown in Table 2 and the VFAs production profiles in all anaerobic fermentation reactors from day 5 to 15 in different initial pH are shown in the Fig. 1.

Maize silage is among the most popular feedstock in the agricultural biogas plants. It can be seen that the concentration of VFAs (Fig. 1A), at the initial pH adjusted to 5.0, increased together with time and it reached 0.40 g/gSCOD, 0.44 g/gSCOD and 0.60 g/ gSCOD for RT of 5, 10 and 15 days, respectively. Moreover, the pH level also increased with time and it stabilized at 5.25, 5.16 and 5.70, respectively. Due to acids production, it stayed in unfavorable pH for methanogens, thus further VFAs accumulation was possible. The second tested substrate was cheese whey (rich in sugars and protein substrate). Obtained results indicated that VFAs concentration increased from 0.41 g/gSCOD (RT = 5 day) to 0.49 g/gSCOD, then it decreased to 0.37 g/gSCOD at RT of 15 days. During the fermentation of cheese whey, the pH level was the most stable. After the first 5 days of the process, the pH increased from the initial 5.0 to 5.30, then it steadily increased till 5.35 (RT = 10 days) and it achieved 5.48 at the longest RT (15 days). The VFAs concentration

Table 2
Process parameters and total VFAs concentrations in effluents.

Substrate	Trial	RT [day]	ΔpH [−]	η_h [%]	η_a [%]	Yx[gCOD/gCOD]
Maize silage	Experimental pH = 5	5	−0.22	0.68	60.77	−4.13
		10	−0.18	0.82	53.43	−4.16
		15	−0.74	0.83	68.85	−4.11
	Experimental pH = 11	5	3.50	0.78	73.11	−5.053
		10	3.70	0.87	70.99	−4.11
		15	3.61	0.89	70.24	−4.11
	BES addition	5	0.95	1.57	26.05	−4.27
		10	0.98	1.60	36.60	−4.25
		15	1.16	1.68	26.38	−4.33
Microalgae biomass	Experimental pH = 5	5	−0.83	5.77	42.603	−0.64
		10	−1.48	5.21	43.91	−0.36
		15	−1.70	4.98	38.33	−0.20
	Experimental pH = 11	5	2.69	11.78	33.81	−0.75
		10	2.84	14.35	30.92	−0.53
		15	3.29	16.02	28.32	−0.34
	BES addition	5	0.15	13.75	4.09	−0.98
		10	0.29	13.25	32.54	−0.76
		15	0.27	13.41	35.11	0.062
Whey	Experimental pH = 5	5	−0.31	11.05	69.89	0.31
		10	−0.28	12.80	70.05	0.26
		15	−0.49	13.20	53.38	0.27
	Experimental pH = 11	5	1.32	14.47	7.41	0.34
		10	1.38	15.81	12.40	0.35
		15	2.85	14.94	61.91	0.36
	BES addition	5	0.55	17.97	48.49	0.39
		10	0.62	20.45	38.49	0.39
		15	0.39	20.82	39.86	0.45

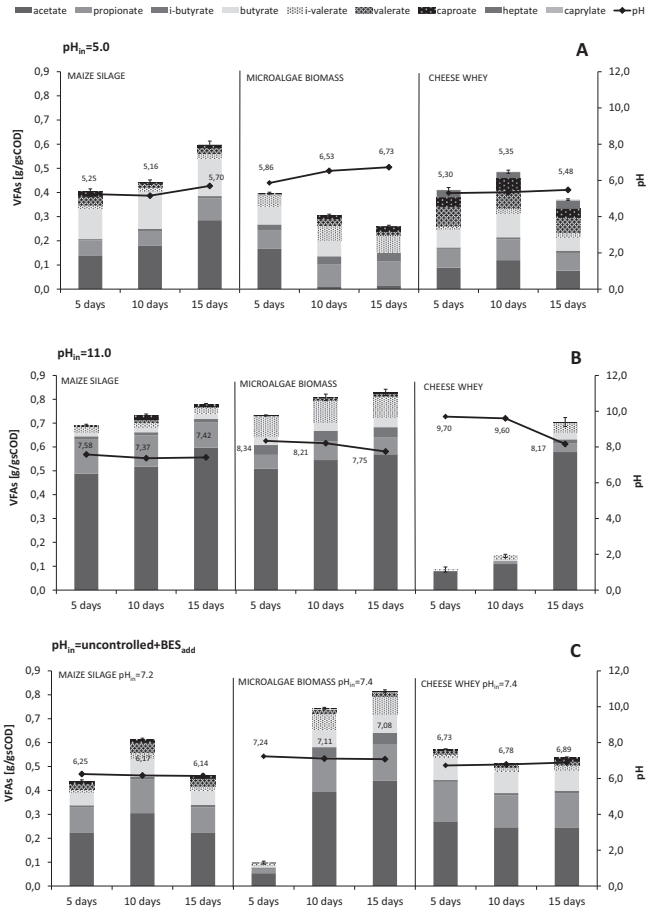

Fig. 1. The concentration of VFAs obtained during MCF of maize silage, microalgae biomass and whey at initial pH of 5.0 (A), 11.0 (B) and neutral with BES addition (C). The lines indicate the changes in the pH during the course of the trials.

obtained from microalgae biomass (a complex substrate containing proteins, lipids and carbohydrates) was the lowest and it decreased during the fermentation time: from 0.40 g/gSCOD, through 0.31 g/gSCOD till 0.26 g/gSCOD, respectively for 5, 10 and 15 RT. Adversely to the other substrates, during the acidic fermentation of microalgae, the pH increased during the course of the process from 5.0 to 5.85, then to 6.53 at the 10th day of the process and finally to 6.73 at the 15th day.

Fig. 1B shows the VFAs production profiles at the initial pH of 11.0. The VFAs concentration obtained from maize silage increased during fermentation time and it reached 0.70 g/gSCOD, 0.73 g/gSCOD and 0.78 g/gSCOD for 5, 10 and 15 RT, respectively. The pH remained steady during the trials, but it decreased drastically from the initial 11.0 to 7.58 during the first days of the process, and then it remained constant around 7.5. In the both described processes, for the initial pH of 5.0 and 11.0, the VFAs concentration increased simultaneously with time of the process and the pH tended to adjust to the neutral level. In the fermentation of cheese whey, the concentration of VFAs in the first days of the process was low (between 0.09 g/gSCOD and 0.14 g/gSCOD) moreover, the pH remained at high level: 9.7 and 9.6. for 5 and 10 RT, respectively. But for the RT of 15 days it increased 5 folds to 0.71 g/gSCOD (at pH 8.17). Production of VFAs from microalgae biomass was steady, similarly to maize silage, and it achieved 0.40 g/gSCOD at 5 RT and around 0.30 g/gSCOD for RT of 10 and 15 days. The pH dropped from the initial 11.0 (day 0) to 8.34 (day 5), then to 8.21 (RT 10 days) and achieved 7.75 (RT 15 days).

Obtained results indicated that the initial alkaline pH was beneficial for VFAs production from complex substrates (maize silage, microalgae biomass). However, the alkaline pH did not maintain for a long time and it always tended to the neutral level. The initial pH had a strong effect on the fermentation process of substrate rich in carbohydrates: in case of lignocellulosic biomass (maize silage) the pH drop was the quickest and the VFAs production was stable for all three RT. The same trend occurred in the microalgae biomass fermentation (other complex substrate) but the pH decline was slower and also the VFAs concentration was lower than for maize

silage. The fermentation of cheese whey behaved differently due to the very slow pH decline and low VFAs concentration at the beginning of the process. This could be the effect of higher concentration of soluble organic matter, which could have caused organic overloading and consequently a lag phase of biological process. After an adaptation period, the VFAs production increased drastically. It has to be mentioned, that gas production (Fig. 2) (mainly H_2 and CH_4 in varied percentage) was detected in all trials, however the CH_4 formation was only minor.

Park et al. [32] indicated that the alkaline pH improved the hydrolysis of organic matter and provided substrates for the acidogenic microorganisms for the production of the VFAs. Similar trend was observed in our trials. The initial alkaline conditions promoted the disintegration and hydrolysis of organic matter which in consequence was beneficial for the VFAs production. Similar trend was observed for the sewage sludge [18], where the highest VFAs concentration was obtained in the initial pH 10 and 11 (0.62 g/gVS_{added} and 0.54 g/gVS_{added}, respectively), as well as for the excess sludge (3–4 times higher VFAs production at pH = 10 compared to the acidic conditions) [33,34] or waste activated sludge (higher hydrolysis rate in alkaline pH resulted in higher VFAs production) [35].

The other option to promote the VFA accumulation is the addition of methanogenesis inhibitor. In order to investigate product formation during the MCF trials in neutral pH, BES with concentration of 50 μmol/mL was added. Zinder et al. [28] reported that after 2 h of exposure to concentration of 50 μmol/mL the methanogenesis during sludge digestion was completely inhibited. Addition of 1 μmol/mL already resulted in a reduction of methane production by 60% and the increase in the acetate accumulation [28]. In our work, BES was used to prevent the methane production in the trials without the pH adjustment. Obtained results (Fig. 3) showed that for trials with maize silage the course of VFAs production proceeded differently compared to the trials with initial pH 5 and 11. The increase was observed during the first days of the fermentation: from 0.44 g/gSCOD (RT = 5 days) to 0.61 g/gSCOD and then a slight decline till 0.46 g/gSCOD (RT = 15 days). At the beginning of the process the pH decreased from the initial 7.2 to 6.25 and it remained more or less stable at that level, i.e. 6.17 and 6.14 at the RT of 10 and 15 days, respectively. Similar results were depicted by Lai et al. [36].

The fermentation in the neutral pH performed better for microalgae biomass. The highest VFAs concentration among all tested pH conditions was achieved i.e. 0.81 g/gSCOD for RT of

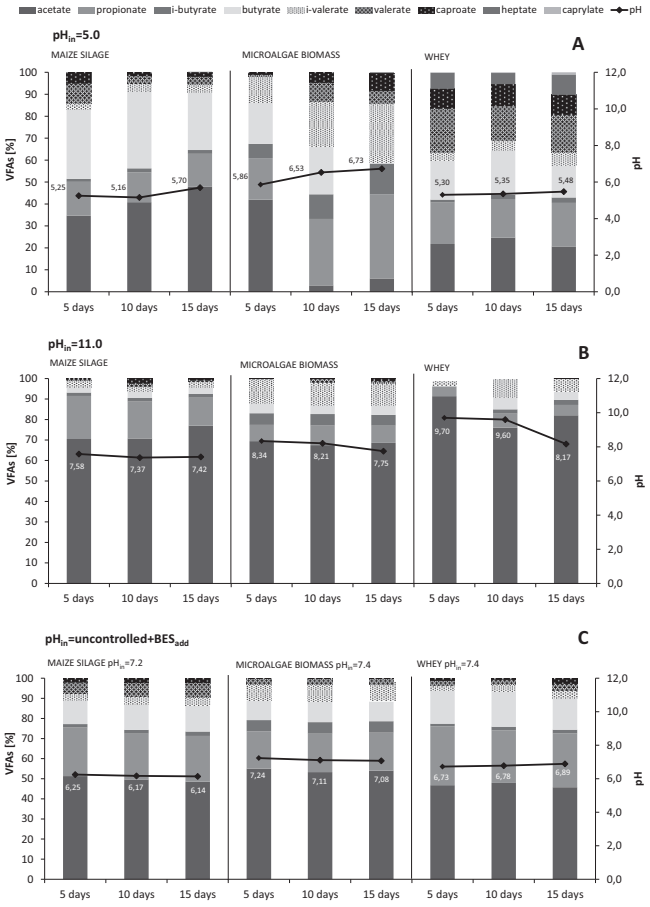

Fig. 3. The composition of VFAs obtained during MCF of maize silage, microalgae biomass and whey at initial pH 5.0 (A), 11.0 (B) and at neutral with BES addition (C).

15 days. However, during the first days of the fermentation, the production was at a very low level (0.1 g/gSCOD). The pH decrease was minor but steady: from 7.4 to 7.24 during the first 5 days of the fermentation, then to 7.11 and finally to 7.08. The average VFAs production in the neutral pH was lower compared to the ones initiated in the alkaline conditions. It clearly indicated that alkaline

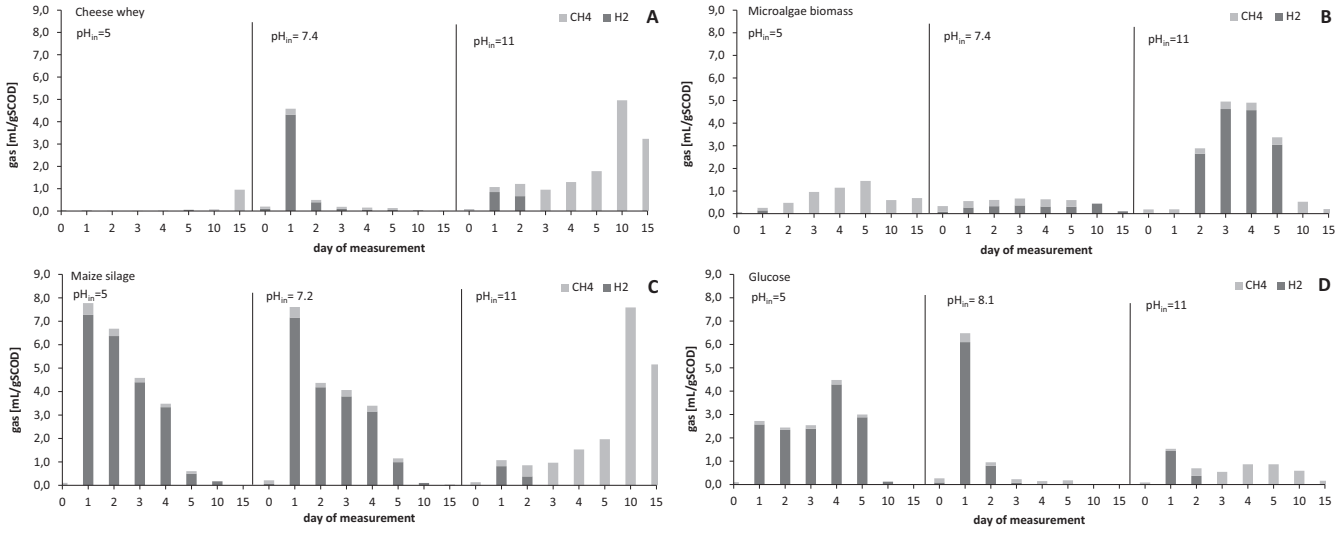

Fig. 2. The composition of gas produced during MCF of cheese whey (A), microalgae biomass (B), maize silage (C) and glucose (D).

conditions were more favorable for more complex substrates fermentation due to the enhanced hydrolysis. In the fermentation of cheese whey the highest concentration of VFAs was detected after the first period of the process (0.57 g/gSCOD), when the pH was the lowest and dropped from the initial 7.4 to 6.73, then it remained steady at around 0.52 g/gSCOD. Moreover, the pH changes were different from the ones observed in the fermentation of maize silage or microalgae biomass. Sudden drop of the pH was followed by a slow increase towards the neutral conditions. From the presented results it could be concluded that in order to enhance the VFAs production from complex substrates, the fermentation process should be carried out in neutral pH (beneficial for growth of acidogenic microorganisms [35]), however, for achieving best yield it should be preceded by alkaline conditions. Those conditions boosted the hydrolysis and increased the amount of solubilized organic compounds available for the microorganisms.

The gas production was observed in some of the tests with the top production of 7.6 mL/gSCOD·d (Fig. 2). During the fermentation of cheese whey (Fig. 2A), at initial acidic pH, the production was minor (less than 1.0 mL/gSCOD·d of CH_4 observed at the 15th day of the process). The production of H_2 occurred in neutral conditions with BES addition (4.3 mL/gSCOD·d at the 1st day) but during following days the gas production was minor. Small production of H_2 was observed at the beginning of the fermentation in alkaline conditions. Together with pH neutralization, the increased production of CH_4 was observed (5.0 mL/gSCOD·d at the 10th day), most likely due to activity of autotrophic methanogens. The production of CH_4 was also observed during the fermentation of microalgae biomass at initial pH = 5 (Fig. 2B). It increased at the 5th day (1.4 mL/gSCOD·d) then it decreased and it remained stable (on average 0.65 mL/gSCOD·d). At neutral pH, the gas production was less than 1.0 mL/gSCOD·d and at the beginning of the process the only trace amount of CH_4 was detected. Then at the 3rd hour (data not shown) of the process, H_2 occurred and their proportion was stable until the 10th day when only H_2 production was detected. The high production of H_2 occurred between the 2nd and the 5th day of fermentation in the initial alkaline conditions and it was between 2.65 mL/gSCOD·d and 4.62 mL/gSCOD·d. In the process with maize silage (Fig. 2C) high production of gas was detected in all the examined conditions. At the initial neutral pH and the one adjusted to 5.0 intensive H_2 production occurred after the 1st day of the process (7.27 mL/gSCOD·d and 7.16 mL/gSCOD·d for acidic and neutral initial conditions, respectively). Then it decreased and at the end of the process it was not detected. The concentration of CH_4 was minor in both processes. At the initial alkaline conditions, the production of H_2 also started after the 1st day of the fermentation but then it immediately decreased and after the 2nd day it was not detected any further. The decrease in pH (to neutral range) caused the production of CH_4. The highest concentration occurred on the 10th day of the process (7.59 mL/gSCOD·d). The main produced gas from glucose was H_2 (Fig. 2D). In acidic conditions the average production remained between 2.34 mL/gSCOD·d and 4.29 mL/gSCOD·d. In neutral conditions high production of H_2 (6.1 mL/gSCOD·d) was detected during the first day. Then it immediately decreased and remained minor or not detected. After the 1st day of the process run at the initial alkaline conditions the production of H_2 started. Then the production of CH_4 began and it remained at the level of 1.0 mL/gSCOD·d till the end of the trial.

To summarize, the CH_4 and H_2 production in low amounts was noticed from all substrates and it occurred in all the examined pH conditions. The intensive production of hydrogen was detected during the first 24 h of the fermentation whereas the constant production was observed in the fermentation of microalgae biomass (pH_{in} = 11) and glucose (pH_{in} = 5). The highest volume was detected during the fermentation of the most complex substrate (maize silage). The most significant production of CH_4 (cheese whey, maize silage) was observed when pH reached neutral range after the initial value of 11.0.

3.2. VFAs composition – effect of pH and retention time

3.2.1. Initial pH 5 and 11

The composition of the produced VFAs at the initial pH 5.0 is illustrated on the Fig. 3A. During the maize silage fermentation the dominant acids were acetate (35%, 41%, 48%), butyrate (32%, 35%, 26%), propionate (15%, 14%, 15%) and valerate (9%, 4%, 4%), for RT 5, 10 and 15, respectively. Other detected acids were iso-butyrate, iso-valerate, and caproate. Results of our work are in accordance with work on the VFAs production from cassava wastewater (pH = 5.9) [1] or wheat straw (pH = 5.1) [37].

During the acidic fermentation of cheese whey the acids distribution was similar regardless the RT, however the dominant acid in the produced mixture was not observed. Among all detected acids were acetate (22%, 25%, 21%), propionate (19%, 18%, 20%), butyrate (18%, 20%, 15%) and valerate (20%, 16%, 17%), also minor concentrations of iso-butyrate, respectively for RT 5, 10 and 15. For both, maize silage and cheese whey, there was no evident shift in the produced acids, however, significant concentrations of medium chain carboxylic acids were detected during the cheese whey fermentation. The caproate (9%, 10%, 9%) and heptate (7%, 5%, 9%) accumulated quickly and remained stable during all the time. Less than 1% of caprylate was detected at the RT of 15 days.

The results obtained from the fermentation of microalgae biomass were slightly different. During the first days of process the acetate was foremost product (42%), then propionate (19%), butyrate (19%) and *iso*-valerate (12%) whereas the concentration of valerate and caproate were minor. When the pH increased to 6.53 (RT = 10 days), the shift in acids distribution was noticed. The acetate concentration rapidly dropped and the concentration of other acids increased. During the next 5 days of the process, the pH was still increasing (up to 6.73) and the shift in butyrate production was detected. Its percentage dropped from 22% to less than 1%, whereas the amounts of other acids increased, especially propionate (30%–>38%) and *iso*-valerate (20%–> 27%). At the RT of 15 days, the small amount of heptate was detected. It was obviously noticed that pH strongly influenced the VFAs distribution. When the pH was stable, like in processes with maize silage and cheese whey, the qualitative and quantitative amount of acids was stable as well and it changed only slightly (no significant shift was detected). On the other hand, when the pH increased the changes in the VFAs distribution occurred quickly.

It was mentioned before that alkaline conditions enhanced the hydrolysis of complex organic matter. For all tested substrates (Fig. 3B) the acetate was the dominated acid. Its average share in the fermentation of maize silage was around 70–77%, whereas the share of propionate stabilized between 14 and 21%. Other acid such as *iso*-butyrate, butyrate, iso-valerate, valerate and caproate appeared in minority. The distribution of VFAs produced from microalgae biomass was stable in all the RT. The amount of acetate (around 70%) was similar to results obtained from maize silage but the quantity of propionate was lower (between 8 and 10%). However, the share of iso-butyrate (around 5.5%), butyrate (around 5%) and iso-valerate (around 11%) were much higher than the ones measured in the maize silage trials. Caproate and valerate acids were also detected, but their quantity was minor. In the process with microalgae biomass, the pH varied between 8.34 and 7.75, thus it could have caused higher percentage of iso-valerate. Similar results were obtained from the fermentation of cheese whey. The acetate was the major acid produced during the first days of the process (over 91%), then propionate (5%) and minor concentration of butyrate and iso-valerate. Simultaneously with pH decline, the

shift from acetate to iso-butyrate, butyrate, propionate and iso-valerate occurred. When pH dropped to 8.17 (RT = 15 days) the VFAs distribution was similar to the one achieved in batches with maize silage and microalgae biomass.

The retention time and pH of the process showed significant impact on the composition of the fermentation broth, however the pH was the major parameter. The concentration of the acetate, followed by propionate appeared to be the highest in the alkaline conditions for all tested substrates. Moreover, together with the pH increase the concentration of butyrate decreased. Longer RT was favorable for chain elongation and accumulation of medium chain fatty acids. Thus in the RT of 15 days, presence of caproate and caprylate was noticed. The pH, rather than RT, influenced the composition of produced mixture of acids. These observations agreed with previous studies that reported acetate and butyrate as the dominant acids under pH < 5.0 or near to neutral (pH > 5.5) [30,38]. It was observed that VFAs concentration tended to increase and acetate and propionate were the major acids obtained in the alkaline conditions [34]. Strong alkaline pH (≥10) could have prevented the growth of methanogens thus the VFAs were not converted to methane by methanogens [39] and their accumulation was possible [39,40].

3.2.2. Neutral pH-BES addition

Fig. 3C shows the qualitative composition of carboxylate mixture produced during anaerobic fermentation carried out at the neutral pH. The acids composition did not vary much during the process and their percentage share in the fermentation broth was not changing rapidly together with the pH decrease. The main produced acid was acetate and its highest share amounted for microalgae biomass (54.1%), followed by maize silage (49.8%) and whey (46.7%). In case of propionate, the highest concentration was obtained from protein rich cheese whey (27.3%), followed by maize silage (23.2%) and microalgae biomass (18.8%). The detected concentration of butyrate occurred in the same order as propionate i.e.: 16.8% for cheese whey, 12.5% for maize silage and 9.9% for microalgae biomass. Other detected acids were iso-butyrate (minor amount detected in maize silage and cheese whey batch tests but over 5% in trials with microalgae biomass), iso-valerate (8–9% from microalgae biomass) and valerate (5–7% in microalgae biomass batch test). The caproate and longer chain acids were also detected but only in small concentrations. Even though, the microbial community structure in the mixed culture microbiome is substrate dependent [41], according to our results the spectrum of the produced VFAs did not depend much on the substrate type. This could lead to a conclusion that the mixed culture community functional redundancy could accommodate different substrate types and it would project to generation of specific products spectrum dependent only on the process operating parameters and not on the substrate type.

3.2.3. Acidogenic fermentation with glucose

Glucose was used as a simple model substrate to further explore the pH impact on the hydrolysis of organic matter and the VFAs production. When fermented at the initial pH adjusted to 5.0, the concentration of VFAs increased from 0.24 g/gSCOD to 0.30 g/gSCOD for RT of 5 days and it reached 0.50 g/gSCOD for RT of 15 days (Fig. 4A). At the beginning of the process, the pH decreased from the initial 5.0 to 4.7 (RT = 5 days), then at the RT of 10 days, it increased to 5.6 but over the next 5 days it dropped to 5.2. Nevertheless, this pH fluctuation had no noticeable influence on the VFAs concentrations. The highest VFAs concentration was observed when the initial pH was adjusted to 11.0. Though, the pH decreased rapidly to 7.8 over the first 5 days of fermentation process and then it stabilized around 7.5. During the fermentation in the neutral pH with BES addition, it decreased to mildly

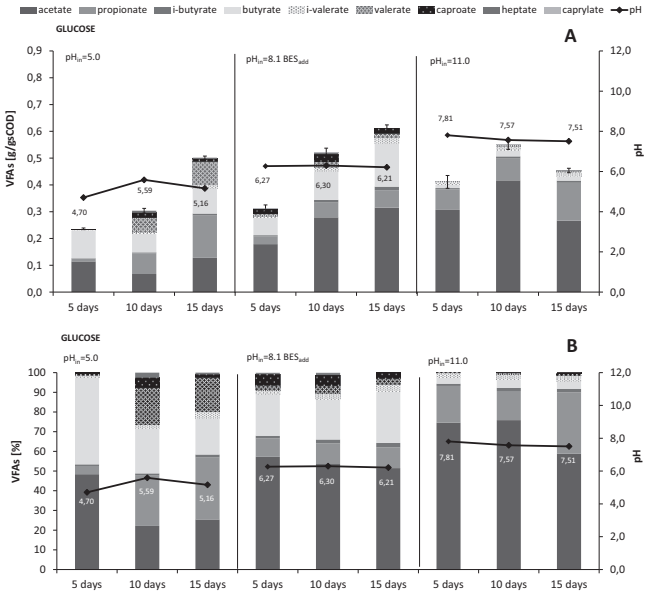

Fig. 4. The concentration (A) and composition (B) of VFAs obtained during MCF of glucose at initial pH 5.0, 11.0 and neutral with BES addition, respectively.

acidic conditions (6.27) over the first 5 days of the process. Those pH conditions occurred to be beneficial for the VFAs accumulation. Their concentration was constantly increasing from 0.31 g/gSCOD to 0.61 g/gSCOD during 15 days long batch trials.

The composition of fermentation broth (Fig. 4B) was similar to results of all tested substrates described above. Thus in the acidic conditions the main obtained acids were acetate and butyrate with presence of valerate and caproate. Moreover, the shift from butyrate to propionate and the accumulation of acetate was observed with the pH increase. Results presented in our work were with agreement to the theoretical studies on the prediction of mixed culture glucose fermentation [2,42]. The shift from butyrate to acetate and propionate occurred with the pH increase from the acidic to alkaline conditions. Mixed culture fermentation had a capability for significant production of acetate in the alkaline pH with propionate as second dominant product, whereas acetate and butyrate were the main products in low pH (4.0–6.5) conditions.

It can be concluded that the VFAs concentrations of fermentation at the neutral pH were higher than those at alkaline and acidic ones only for simple substrate such as glucose. The initial alkaline conditions enhanced the hydrolysis of complex substrates. The average concentration of VFAs obtained at initial alkaline conditions was higher by 31% and 34% (for maize silage) and by 30% and 59% (for microalgae biomass) compared to the neutral and initial acidic conditions, respectively. For the fermentation of cheese whey it was 73% and 35% lower than at the initial pH 5.0 and neutral conditions, respectively.

According to Ghosh et al. [43] the HRT longer than 2 days was not preferable for fermentation processes due to methanogens growth. Moreover, De Mes et al. [44] stated that fast conversion resulted in smaller reactor volumes, which would be desirable from an economic point of view. The hydrolysis determines the HRTs needed to maximize VFAs production, because it is the slowest step in the anaerobic digestion. On the other hand, the hydrolysis rate depends on the complexity of substrate and amount of soluble compounds. The acids production from glucose-rich substrates require retention time of a few hours [45] however, for more complex and non-soluble substrates it should be longer (Figs. 1 and 4). Our findings showed that different types of sub-

strates implied different kinetics and required varied HRT [46–49]. Moreover, the HRTs could also affect the composition of acids produced. Longer retention time causes the shift of product type from more oxidized compounds (acetate or lactate) towards more reduced (n-butyrate or n-caproate) [50].

The initial concentrations of substrates applied in the experimental tests (corresponding to 0.5 g VS/100 mL) were 5.33 gSCOD/L, 7.23 gSCOD/L, 5.16 gSCOD/L and 3.73 gSCOD/L for glucose, cheese whey, maize silage and microalgae biomass, respectively. In such a low substrate loading, the negative effect of the substrate overloading could have been avoided. According to the work [26], the most typical range of initial substrate concentration varied between 5 and 40 gCOD/L. At higher feed levels (above 40 gCOD/L) the concentrations of carboxylates did not increase further above 20 g/L due to the overloading or inhibition. The OLR could have a double effect on the VFAs production: 1/ in high OLR caused by high substrate loading, the product concentration increases but the type of products becomes less varied; 2/ in high OLR caused by decreasing of HRT, the hydrolysis becomes less efficient, the concentration of total carboxylates is lower and/or the production is directed mainly towards acetate formation [51].

3.3. Redox condition

The critical parameter that most influenced the VFAs production was the initial pH. Typically, the pH drop is caused by the accumulation of short chain volatile fatty acids, which toxicities are higher when pH is below 7.0 [52]. The enzyme activities and consequently the metabolic activities of the biocatalyst could depend on the pH changes during the fermentation process [6] .

Prior the process start up, the pH was adjusted either to 5 or 11 and no buffer was added. The pH was measured in the intervals at the 5^{th}, 10^{th} and the final 15^{th} day of the each process (Table 2). The most significant drop of pH was observed in the trials with the initial alkaline pH. The most noticeable one occurred in the batch tests with maize silage followed by the microalgae trials and the slowest decline was observed in tests with cheese whey. Typically, the pH stabilized at the range of 7.4–9.7 for alkaline tests, 6.1–7.1 for the tests with BES addition and at 5.2–6.7 for the acidic tests. Wu et al. [19] studied the co-fermentation of food waste and excess sludge in uncontrolled pH and indicated the self-maintenance of pH in the beneficial range for VFAs accumulation between 5.2 and 6.4. We noticed that this was depended not only on the initial pH value but also on the complexity of the substrate.

Higher concentration of VFAs in the alkaline condition was caused by more efficient hydrolysis which was the first and the most crucial step in the mixed culture fermentation of complex substrates. During the hydrolysis, the complex compounds were broken down to the simpler molecules, which then became available to the microorganisms. The hydrolysis in the alkaline environment was more effective due to the ionization of charged groups (e.g. carboxylic groups) thereby enhanced solubilisation of carbohydrates and proteins [53,54]. According to Venkata Mohan et al. [55] the buffering capacity of the fermentation system was strongly correlated to the production of fatty acids. Results illustrated by Dahiya et al. [6] also confirmed that buffer capacity was higher in the alkaline conditions. Moreover, Dinamarca et al. [56] reported that it was possible to keep the pH stable by the production of *in situ* volatile buffers in the anaerobic system throughout the buffer effect of the macromolecules' residues. This could be probable explanation of the pH increase in the trials with initial pH adjusted to 5, i.e.: the fermentation system prevented the pH drop, even when the obtained concentration of VFAs was high.

Additionally, the effect of the initial pH on the soluble fraction of COD was investigated. No significant changes were observed for cheese whey and microalgae biomass neither at pH 5 or 11. For maize silage, the slight increase was noticed i.e. 4.9% and 9.4% increase in SCOD after 15 min and 24 h exposition for pH = 5, respectively. The effect of pH pretreatment was most visible at the pH = 11, where after 2 min of exposure the SCOD concentration increased by 27%, then by 30.7% during the next 13 min and in total by 38.7% after 24 h. This indicated that alkaline pH could boost the hydrolysis of complex substrates.

3.4. Conversion efficiency and process performance

The hydrolysis yield (η_h) defines the solubilization of the initial organic compounds from solid substrates [19]. As shown in Table 2, the lowest hydrolysis yield was obtained for the most complex substrate i.e. maize silage. It slowly increased within processing time till the values of 0.78% ± 0.08, 0.84% ± 0.06 and 1.61% ± 0.06 for pH_{in} = 5, pH_{in} = 11 and neutral conditions with BES addition, respectively. The average acidification yield (the conversion of solubilized organic matter into VFAs) in maize silage fermentation reached 61.02% ± 7.71 at initial acidic conditions and 29.68% ± 6.00 at neutral, whereas at the alkaline conditions it was the highest among all substrates (71.45% ± 1.49). Moreover, at the acidic conditions the yields of both parameters increased simultaneously. This indicated faster rate of SCOD transformation to VFAs in comparison to the hydrolysis rate. Henceforth, hydrolyzed organic matter was immediately used for acids formation.

The second substrate in terms of hydrolysis yield was microalgae biomass. The relation between hydrolysis and acidification yield observed during the fermentation run at the initial acidic conditions was significantly different compared to the other substrates. The values of both yields decreased with increasing HRT: hydrolysis yield from 5.77% to 4.98% and acidification yield from 43.91% to 38.33%. Moreover, the concentration of VFAs also decreased and the production of CH_4 and H_2 was barely detected (less than 1.0 mL/gSCOD). It indicated that soluble matter and VFAs produced during the first 5 days could have been used for other processes than analyzed i.e. accumulation of organic compounds within microalgal cells. At the initial alkaline conditions hydrolysis yield increased until the end of the fermentation process and reach 16.02%. Acidification yield decreased from the initial 33.81% to 28.32% at the 15^{th} day of fermentation. Due to high rate of hydrolysis, production of VFAs obtained from microalgae biomass was significant. At neutral pH, the hydrolysis rate decreased (till 14.41%) and simultaneously the acidification took place (significant increase from 4.09% till 35.11%).

Results obtained from cheese whey fermentation indicated that in the initial acidic and neutral conditions enhanced the hydrolysis yield (from 11.05% to 13.20% and from 17.97% to 20.82% – the highest rate among all analyzed testes). It was accompanied by a decrease in the acidification rate (from 70.05% to 53.38%). When the process run in the alkaline pH, the hydrolysis yield initially increased to 15.81% but then it decreased to 14.94%. The reduction of hydrolysis yield was minor but the increase in the acidification yield was noteworthy – almost 55%.

The yield of biomass (Y_x) shows the formation of biomass from soluble compounds (without VFA soluble COD). For the most complex substrate (maize silage and microalgae biomass) reduction of biomass occurred. During maize silage fermentation it was −4.13 gCOD/gCOD, −4.09 gCOD/gCOD and −4.28 gCOD/gCOD (on average) at initial pH 5, 11 and neutral, respectively. The biomass reduction of microalgae biomass was −0.40 gCOD/gCOD, −0.54 gCOD/gCOD and −0.56 gCOD/gCOD (on average) at initial pH 5.0, 11.0 and neutral, respectively. Contrary, positive values characterized the fermentation of cheese whey – 0.28 gCOD/gCOD, 0.35 gCOD/gCOD and 0.41 gCOD/gCOD (average) at the initial acidic, alkaline and neutral conditions, respectively.

To summarize, our findings indicated that the reduction of hydrolysis rate occurred simultaneously with increase in the acidification yield. Conversely, significant rate of acids production lowers the hydrolysis yield. Our results confirmed those achieved by Wu et al. [19]. For the very complex substrates without pretreatment prior fermentation, the hydrolysis rate might be insufficient, thus the increased yield of hydrolysis and acidification simultaneously might occurre. Values of biomass yield lower than zero indicated that COD was not accumulated and/or the biomass content in substrate was utilized to soluble compounds. Accumulation of COD in biomass produced from soluble COD occurred in the fermentation of cheese whey, comparable results were obtained by Bengtsson et al. [30].

The reduction of nutrients (measured as TN and PO_4^{3-}) was also monitored and it was calculated as the difference between initial content in raw substrate and final concentration after the fermentation. During the fermentation of microalgae biomass the reduction of TN was: at the initial acidic conditions 94.92%, 97.98%, 99.06, at the alkaline conditions 51.60%, 57.05%, 71.85% and the neutral conditions 51.60%, 56.05%, 59.01% for the 5th, 10th and 15th day of the process, respectively. The reduction of PO_4^{3-} reached 92.18%, 89.37%, 88.53% at the initial acidic conditions, 95.89%, 94.70%, 88.81% at the alkaline conditions and 93.44%, 94.58%, 88.78% at the neutral conditions for the 5th, 10th and 15th day of the process, respectively. The reduction rates during the fermentation of cheese whey were lower than for microalgae biomass. At the initial acidic pH it reached 10.20%, 18.37% and 26.53% of TN reduction and 75.25%, 78.27% and 78.71% of PO_4^{3-} reduction. However, at the alkaline conditions 12.25%, 4.08% and 17.35% of TN reduction and 86.42%, 87.14% and 78.49% of PO_4^{3-} reduction was achieved for 5, 10 and 15 days long RT, respectively. Significant reductions were obtained also in the neutral pH i.e. 38.78%, 24.49% and 31.63% of TN reduction and 90.54%, 91.76% and 91.10% of PO_4^{3-} reduction. The presence of ammonium was detected only in trials with cheese whey at the 5th day of the fermentation. The amounts of detected ammonium equaled to 0.75 mL NH_4^+/L, 0.25 mL NH_4^+/L and 0.50 mL NH_4^+/L for the initial acidic, alkaline and neural conditions, respectively. Those low values did not present any risk of ammonia inhibition. For the most complex substrate (maize silage), the nitrogen reduction values were as follows: 100%, 97.95% and 98.80% at the initial pH 5.0, 95.78%, 99.04% and 97.23% at the initial pH 11.0 and 33.74%, 36, 15% and 31.33% in the neutral conditions, respectively. Reduction of PO_4^{3-} remained also significant: 90.09%, 96.67%, 91.56% at pH_{in} = 5.0, 97.22%, 96.86% at pH_{in} = 11.0 and 94.21% at the neutral pH.

Obtained results indicated that pH played a major role in the volatile fatty acids production and it was more significant than the substrate type. Several trends in the VFAs production could be noticed independently of the substrate type. In the acidic conditions the major products were acetate and butyrate whereas in the alkaline conditions the major products were acetate and propionate. In the acidic conditions, in contrast to the alkaline ones, higher diversity of volatile and medium chain fatty acids was observed. The highest concentration of propionate was obtained from carbohydrates rich substrate (maize silage) whereas protein-rich substrates enhanced the production of valerate, isovalerate and caproate (microalgae biomass and whey). When the process time was prolonged, the chain elongation was promoted and slow accumulation of caproate and caprylate occurred. It was noticed that complex substrates (i.e. carbohydrate-rich and protein-rich microalgae biomass) enhanced the production of isobutyrate. If the process operating parameters were kept similar, different substrates would lead to a similar spectrum of products. On top of that, throughout simple alkaline pre-adjustment, it was possible to boost hydrolysis rate. In the mixed culture fermentation, the phenomena of self-maintenance of pH prevented from the pH drop to unfavorable range for microorganisms even if the acidic conditions had been initiated. Therefore, we conclude that it would be possible to carry out the fermentation process without pH control and keep the process stable.

4. Conclusions

Mixed culture fermentation is a complex process and it depends on various parameters such as pH, retention time, but only merely on the substrate type. That is crucial for designing bioprocesses fed with waste streams. The variations in the composition of the substrate will have only minor impact if the other process parameters are kept constant. Besides, the initial alkaline environment was favorable for VFAs generation from complex substrates (such as maize silage) due to the enhanced hydrolysis and effectively inhibited activity of methanogens. Alkaline environment promoted hydrolysis and subsequently enhanced the process efficiency. The MCF process run with pre-adjusted pH could be a good alternative for the VFAs production from complex substrates, especially beneficial would be the alkaline conditions. Better understanding of the interaction between substrate type, retention time, pH, and hydrolysis and acidogenesis yields helps to develop industrially feasible volatile fatty acids production from waste streams.

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Publication 3

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Conversion of organic waste into volatile fatty acids – The influence of process operating parameters

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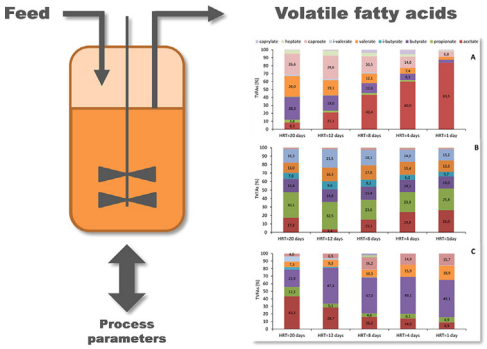
HIGHLIGHTS

- Decreasing the HRT to achieve high OLR reduces the hydrolysis yield.
- Shortening of HRT resulted in decreasing of TVFAs produced from cheese whey.
- Changes in HRT and OLR did not affect production of TVFAs from sludge.
- TVFAs composition was impacted by substrate type and microbial biodiversity.
- Microbial community from substrate tended to dominate bioreactor.

GRAPHICAL ABSTRACT



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Hydraulic retention time

ABSTRACT

The objectives of this study were to investigate the effect of hydraulic retention time (HRT) and organic loading rate (OLR) on the volatile fatty acids (VFAs) production from cheese whey and sludge, and to find the relation between total volatile fatty acids (TVFAs) accumulation at different HRT and OLR and the corresponding bacterial community. The highest concentrations of butyrate (5.0 g COD/L), valerate (3.8 g COD/L), caproate (4.2 g COD/L) and heptate (0.8 g COD/L) were obtained from whey fermentation, where proportion of acetate increased with HRT shortening (from 8.3% to 83.5% for HRT of 20 to 1 day, respectively). The highest concentrations of acetate (1.6 g COD/L), propionate (2.9 g COD/L), iso-butyrate (0.8 g COD/L) and iso-valerate (1.8 g COD/L) were obtained from sludge fermentation, where proportion of acids was independent of HRT. Bacterial communities consisted mostly of the phyla *Firmicutes*, *Cloacimonetes*, *Proteobacteria* and uncultured bacterium clones. The bacterial community originating from the substrate was able to adapt to the applied bioreactor conditions and it had an immense impact on the process performance.

1. Introduction

Biogas production via anaerobic digestion (AD) has become a commercial way of organic waste utilisation and energy production. However, the intermediate products, carboxylic acids, produced during the anaerobic fermentation, have also a great potential and they could be used as a carbon source for further processing [1] or as a building blocks to produce commodity chemicals [2].

Short chain carboxylic acids or volatile fatty acids (VFAs) are formed as an intermediates in acidogenesis and acetogenesis steps of

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the AD process. During the mixed culture fermentation (MCF) a variety of organic compounds (such as lipids, proteins and carbohydrates) are broken down to simple molecules and the effluent consist of mixture of volatile and medium chain carboxylic acids such as acetic, propionic, butyric, valeric or caproic acid. The key advantage of the MCF process is the ability to ferment organic waste in non-sterile condition. The organic waste are produced in abundant amounts and are significantly lower-priced than pure substrates. Both aspects are important from the economic and ecologic point of view [2]. The MCF is a complex process, which is determined by many competing microorganisms, biokinetics, catalysts and intermediate syntrophic reactions [2]. To enhance the production of VFAs, the hydrolysis and acidogenesis must be accelerated and at the same time the methanogenesis must be inhibited to prevent the VFAs consumption by methanogens [3]. There are several strategies to promote the production of VFA such as [3]: pretreatment, optimization of key process parameters or addition of methanogenesis inhibitor.

The crucial aspect in VFAs production during MCF process is the relation between the variety of generated VFAs, the process operation parameters and the structure of mixed microbial community. Even though, recently metagenomics analysis has become the analysis of choice for determination of changes in microbial community in the course of the fermentation process [4], the PCR-DGGE fingerprinting analysis gives advantage of being simple and robust. In last decades PCR-DGGE was proved to be reliable and powerful tool and become a routine fingerprinting method for comparative biodiversity analysis between samples collected over time [5], henceforth serving as bioprocess monitoring tool. It facilitates to determine and discern the microbial structure and dynamics under changing environmental conditions, such as different substrate, HRT, OLR, pH or temperature [6,7]. Thus, this approach let to monitor microbial shifts and follow the succession of microbial populations during the fermentation process and calculation of environmental indices let to explain how changes of operational conditions influenced the microbial population and VFAs formation.

In previous work [8], we studied the effect of pH and the various substrates on the total volatile fatty acids (TVFAs) production in the batch mode. However, it is necessary to optimize and study the process under conditions that would make it more scalable in the future. In this work we studied the semi-continuous process of acidic fermentation at initial pH of 5.2. In this study we focused on the analysing of the effect of different HRT and OLR on TVFAs production, as they mainly affect the capital and maintenance costs. According to [3] the size of a reactor is related to HRT (the higher HRT the larger the required reactor). However, easier operation and reduced wear and tear of the pumps with less frequent feeding could be achieved at lower OLR. From the operational point of view the relation between HRT, ORL and VFAs formation is not clearly defined. HRT should be adequate to determine the conditions for microbial population growth and long enough for optimal hydrolysis and acidification. ORL should be adequate to prevent from the process destabilization, but high enough to provide wide spectrum of produced acids [2,3]. The systematic investigation of operating parameters in real waste fermentation processes would help to determine the specific effect of these parameters, however they should be consider together rather than as single parameters for better process optimisation.

The main objective of presented study was to reveal the influence of HRT and OLR (increasing the ORL by decreasing the HRT) on the VFAs production and distribution. The fingerprinting-based approach to analyse the microbial community was applied to better understand and explain the process.

2. Materials and methods

2.1. Seeding sludge and substrates

The seeding sludge was collected from the full scale mesophilic anaerobic digester of municipal wastewater treatment plant (WWTP, Kozieglowy, Poznan area, Poland). Prior reactor inoculation, the seeding sludge was stored in mesophilic temperature (35 °C) for 48 h.

During the laboratory trials, three types of substrates were used: cheese whey, mixture of primary sludge and waste activated sludge (further named as sludge) and glucose solution (4.0 g/L) that was used as a model substrate in the bacterial community profiling test. The sludge were collected from the primary and secondary settling tanks located in the same WWTP, then mixed in ratio 1:1 (by volume) and stored in 4 °C. The cheese whey was collected from the Dairy in Dobrzyca (Dobrzyca, Poland) and stored at −18 °C prior usage. The glucose solution was supplemented with the following compounds (g/L): NH_4Cl 1.34; KH_2PO_4 0.78; NaCl 0.292; $Na_2SO_4{\cdot}10H_2O$ 0.130; $MgCl_2{\cdot}6H_2O$ 0.120; $FeSO_4{\cdot}7H_2O$ 0.0031; $CaCl_2$ 0.0006; H_3BO_3 0.0001; $Na_2MoO_4{\cdot}2H_2O$ 0.0001; $ZnSO_4{\cdot}7H_2O$ 0.0032; $CoCl_2{\cdot}H_2O$ 0.0006; $CuCl_2{\cdot}2H_2O$ 0.0022; $MnCl_2{\cdot}4H_2O$ 0.0025; $NiCl_2{\cdot}6H_2O$ 0.0005; EDTA 0.050 [9]. The main characteristics of substrates and seed sludge are shown in Table 1.

2.2. Semi-continuous trials

Two jacketed glass continued stirred tank reactors (CSTRs) with a working volume of 4.5 L (Bioprocess Control, Sweden) were used for three subsequent continuous experiments, each with different substrate. The temperature was maintained at 35 °C by circulation of water from a water bath through the reactors jackets. The reactors were stirred by mechanical stirrers and the gas production was monitored by volumetric gas flow meters (Ritter, Germany). During the MCF trials, the pH was monitored, but not controlled. It was adjusted only at the beginning of the MCF process. The control reactor was operated at initial pH of 7, while the experimental one at initial pH of 5.2 (adjusted by addition of 18% HCl) in order to inhibit methanogeneis. No other specific inhibitor was added. At the start of each run the reactors were inoculated with the mixture of mesophilic seed sludge. Each substrate was supplied to the reactor manually in amount corresponding to the designed HRT by replacing the fermentation broth with fresh substrate once a day. During the fermentation experiments samples were collected daily from each reactor for the analysis. Table 2 shows the experimental design and characteristics of each fermentation trial.

2.3. Analytical methods

The total solids (TS) and volatile solids (VS) were measured according to the Polish standard method (PN-EN-12879). TS and VS were analyzed directly after sampling. 10 mL of fresh sample was dried over night at 105 °C to determine the TS, and then ashed at 550 °C for 3 h for VS determination.

Total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) were analyzed according to the Polish standard method (PN-74/C-04578/03). Prior TCOD and SCOD analyzes samples were stored in 4 °C. To measure the SCOD the samples were first centrifuged at 13,000 rpm for 5 min, then the supernatant was diluted with demineralized water. The absorbance was measured with the photometer (Spectroquant® Prove100, Merck KGaA, Darmstadt, Germany) at

Table 1
Characteristics of substrates and seeding sludge.

Parameters	Seed sludge	Cheese whey	PS:WAS
Total solids (TS) (g/L)	30.9 ± 3.6	58.2 ± 1.6	44.9 ± 0.7
Volatile solids (VS) (%)	1.9 ± 0.4	5.1 ± 0.1	3.3 ± 0.1
Total chemical oxygen demand (TCOD) (mg/L)	50,865 ± 215	71,923 ± 215	79,896 ± 120
Soluble chemical oxygen demand (SCOD) (mg/L)	1541 ± 37	70,705 ± 124	8185 ± 0.00
pH	7.5 ± 0.3	4.7 ± 0.2	7.6 ± 0.4
TVFA [g/L]	0.11 ± 0.00	0.14 ± 0.00	1.45 ± 0.07

the wavelength of 600 nm and the TCOD and SCOD concentrations were calculated from calibration curve. The pH was measured by the pH-meter (Mettler Toledo).

For VFAs analysis 2 mL samples were centrifuged (13,000 rpm for 17 min) directly after sampling and obtained supernatants were analyzed by gas chromatography (GC) according to [10] with slight modifications as described below. The supernatants were acidified with 85% H_3PO_4 to pH < 2.0, centrifuged (13,000 rpm, 17 min) and filtrated (0.45 μm pore size regenerated cellulose syringe filters) for carboxylates concentration determination. The concentration of acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, caproate, heptate and caprylate were assessed by a GC (GC-2014, Shimadzu, Japan), equipped with flame ionization detector (FID) and a silica capillary column (Zebron ZB-FFAP, 30 m × 0.53 mm × 1.00 μm). The column temperature was maintained at 70 °C initially and held for 3 min. After that the temperature was increased by 10 °C per minute until it reached 240 °C at the final step. The temperatures of injection port and detector were 200 °C and 250 °C, respectively. The helium was used as a carrier gas with the flow rate of 7.4 mL/min. The determined amount of volatile fatty acids (VFAs) (C1–C5 acids) and medium-chain fatty acids (MCFA) (C6–C8) was given as a sum and named total volatile fatty acids (TVFAs).

2.4. Microbiological analysis

2.4.1. DNA extraction, PCR-DGGE and sequencing

About 20 mg aliquots of previously frozen biomass samples from all reactors were used for bacterial genomic DNA extraction using GeneMATRIX Soil DNA Purification Kit (Eurx®, Poland), according to the manufacturer's instructions.

The V6 - V8 hypervariable regions of 16S rRNA gene were amplified with PCR primers U968-f with GC-rich clamp (5′-CGCCCGGGGCGCGC CCCGGGCGGGGCGGGGGCAC GGGGGG AACGCGAAGAACCTTAC-3′) and L1401-r (5′-CGGTGTGTACAAGACCC-3′) [11] using the OptiTaq DNA Polymerase (EURx, Poland) with 10× Pol Buffer C, according to the manufacturer's instructions. The primers were synthesised commercially (GENOMED, Poland). The final volume of each PCR mixture was 50 μl. The fragments were amplified in T100 Thermal Cycler (BioRad) using the following programme: initial denaturation for 2 min at 94 °C; 34 cycles, each consisting of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C; and extension of incomplete products for 7 min at 72 °C. Prior to DGGE, 5 μl of each PCR product was verified in 1% agarose gel stained with SimplySafe™ (EURx, Poland) and visualised in UV light.

Bacterial amplicons were separated in 8% polyacrylamide gel with urea/formamid gradient of 40–70% (100% denaturants is a mixture of 7 M urea and 40% (vol/vol) formamide). DGGE analysis was performed in 1x TAE buffer at 85 V and 60 °C for 16 h using kuroGel Verti 2020 (VWR international). DGGE gels were stained with 1× TAE buffer containing Sybr®Gold (Thermo Fisher Scientific) for 45 min in dark and visualised in UV light. Differentiating bands were excised with a sterile razor, suspended in 50 μl of pharmaceutical water, stored at 4 °C overnight and reamplified using the same primers but without GC-rich clamp [7]. Positive PCR amplicons were cleaned and concentrated into a final volume of 40 μl using a Clean-Up kit (A&A Biotechnology, Poland). Obtained products were sequenced (Macrogen Europe, The Netherlands) and aligned via NCBI BLASTn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4.2. Microbial community analysis

For interpretation of DGGE profiles, all gels were analysed using KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company, USA). Microbial community was analysed on the following levels: microbial diversity, richness, dynamics and organisation. The structural diversity was estimated by the Shannon-Weaver index (*H'*) basing on band intensities [12]. Range-weighted richness (Rr) was calculated as the total number of bands in each lane [13]. Banding profiles were compared using band matching algorithms to generate a binary data matrix representing a presence or absence of bands. Profile similarities were obtained by the determination of Jaccard coefficient and clustering of patterns were constructed using UPGMA algorithm with DGGEstat software (Erik van Hannen; the Netherlands Institute of Ecology). Community organisation (Co) was calculated as the percentage of Gini coefficient, describing a specific degree of evenness of a microbial community by measuring the normalised area between a given Pareto-Lorenz curve and the perfect evenness line [14,15].

2.5. Calculation methods

The hydrolysis yield (η_h) (Eq. (1)) was calculated as the ratio of final SCOD concentration measured in leachate to the initial TCOD concentration of the substrate [19]. The acidification yield (η_a) (Eq. (2)) was calculated as the ratio of cumulative TVFAs and final concentration of SCOD measured in the leachate [16]:

$$\eta_h = \frac{SCOD_{out}}{TCOD_{in}} \times 100\% \quad (1)$$

$$\eta_a = \frac{VFA_{out}}{SCOD_{out}} \times 100\% \quad (2)$$

where VFA_{out} is the cumulative TVFAs concentration (g COD/L); $SCOD_{out}$ is the final soluble COD concentration (g COD/L); $TCOD_{in}$ is the initial total COD concentration (g COD/L).

3. Results and discussion

3.1. Effect of HRT and OLR

The pH plays a major role in TVFAs formation during acidogenic fermentation. Results of our previous work [8] indicated the ability of pH self-regulation in acidic conditions (the pH stabilized at the range of 5.2–6.4), without a need of adjustment. It is known that both HRT and OLR affect the stability and productivity of the MCF process; however, the issue on how precisely HRT and OLR impact the process has not been fully revealed yet [3]. The TVFAs concentration and composition profiles from cheese whey and sludge fermentations are shown in Table 2 and Fig. 1.

The TVFAs concentration profile obtained from cheese whey acidic fermentation (initial pH 5.2) from day 0 to 45 is shown on Fig. 1A. It was noticed that the TVFAs concentration increased rapidly after the 2nd day and started accumulating reaching the maximum concentration of 11.8 g COD/L at the 10th day. It remained at the level between 8.0 and 10.0 g COD/L till the HRT was shortened. The qualitative analysis (Fig. 2A) revealed that the main produced acids were butyrate (28.3%), valerate (26.0%) and caproate (26.6%). Higher OLR, achieved by shortening HRT, caused the decrease in concentration of carboxylates and pH drop in next phases. When the HRT was shortened to 12 days (pH 5.1 → 3.4) the percentage of acetate started to increased. The short HRT (HRT ≤ 8 days; pH 3.5 → 3.8) favoured mainly acetate formation. Other detected acids were propionate, iso-butyrate, iso-valerate, caprylate and heptate, but in insignificant amounts. In other work conducted under similar process conditions (cheese whey permeate, 20-days long continuous process, pH 6.0) main produced acids were acetate (45%, 33%), butyrate (17%, 2%) and propionate (29%, 61%) for RT 8 h and 95 h, respectively) [1]. Domingos et al. [17] documented that during the continuous fermentation of cheese whey at controlled pH (6.0) and HRT of 6 days the main produced acids were acetate (3.01 ± 0.45 g/L), butyrate (1.83 ± 0.27 g/L), caproate (4.13 ± 0.56 g/L) and caprylate (3.12 ± 0.94 g/L) and it is similar to results described in this work. Azbar et al. [18] monitored the TVFAs formation in two different OLR strategies: (i) constant OLR (47 g COD/L/d) and varying HRT (3.5, 2, 1 day) and (ii) varying OLR (47, 35 and 21 g COD/L/d) and constant HRT (1 day). The process was run in

Table 2
The experimental design and components of the fermentation substrates.

Process stages	HRT [days]	OLR			Feed in/out amount [mL/d]	pH		VFAs		
			[gVS/L/d]	[gCOD/L/d][1]		Initial	Final	[gCOD/L]	[g/gVS$_{added}$]	[g/gCOD$_{added}$][1]
W1	20	Cheese whey	2.55	3.60	225	5.2	5.1	8.41 ± 1.02	1.77 ± 0.20	1.27 ± 0.14
W2	12		4.25	5.99	375	4.4	3.5	4.90 ± 0.54	0.67 ± 0.01	0.48 ± 0.06
W3	8		6.38	9.00	563	3.4	3.4	2.01 ± 0.27	0.21 ± 0.04	0.15 ± 0.03
W4	4		12.75	17.98	1125	3.4	3.6	1.63 ± 0.31	0.10 ± 0.02	0.07 ± 0.02
W5	1		50.99	71.92	4500	3.7	3.8	1.22 ± 0.33	0.25 ± 0.07	0.17 ± 0.05
S1	20	PS + WAS	1.65	3.99	225	5.2	5.8	4.69 ± 0.47	1.80 ± 0.20	0.74 ± 0.08
S2	12		2.74	6.66	375	5.9	5.4[2]	7.01 ± 0.78	1.47 ± 0.18	0.61 ± 0.07
S3	8		4.12	10.00	563	4.9	5.3	6.97 ± 0.60	1.03 ± 0.10	0.43 ± 0.04
S4	4		8.23	19.97	1125	5.9	5.7	6.20 ± 0.60	0.49 ± 0.07	0.20 ± 0.02
S5	1		32.9	79.90	4500	6.0	5.9	5.07 ± 0.51	0.41 ± 0.05	0.16 ± 0.02
G1	20	Glucose	0.20	0.24	225	5.2	4.9	0.77 ± 0.11	2.77 ± 0.50	2.29 ± 0.41
G2	12		0.33	0.40	375	5.0	4.8	0.58 ± 0.09	1.13 ± 0.19	0.94 ± 0.16
G3	8		0.50	0.61	563	4.8	4.5	1.19 ± 0.18	1.40 ± 0.21	1.15 ± 0.17
G4	4		1.00	1.21	1125	4.4	4.0	0.48 ± 0.08	0.28 ± 0.04	0.23 ± 0.04
G5	1		4.00	4.85	4500	4.0	3.9	0.10 ± 0.02	0.17 ± 0.03	0.14 ± 0.02

[1] VFAs concentration was expressed in TCOD.
[2] 18% HCl addition for pH adjustment to acidic conditions.

thermophilic conditions (55 °C) and in controlled and adjusted pH (pH 5.5). The composition of produced TVFAs (when the process was run at the highest OLR and constant HRT) was similar to our study. Moreover, changing of HRT with constant or variable OLR revealed similar shifts in acids composition. This indicated that by changing the HRT and/or OLR it would be possible to produce different mixture of TVFAs from a particulate substrate while keeping pH constant.

The concentration of TVFAs from sludge mixture increased slowly at the beginning of the process (Fig. 1B) and the highest accumulation was observed on the 30th day (8.5 g COD/L) at pH 5.5. In the first phase of the process, the pH was stable and in the range of 5.2–6.2 (despite acids accumulation). However, the increase of OLR and shortening HRT to 12 days caused a rapid increase in pH to 6.9 due to high pH of supplied sludge (pH 7.6). Irregular and rising pH did not project on TVFAs concentration and acids composition (Fig. 2B). The main produced acids were acetate, propionate, butyrate, valerate and iso-valerate. Similarly to Ucisik [19], large share of propionate was observed in the sludge fermentation. The share of the acids remained similar during the process regardless the HRT. Comparable observation was done by Banerjee et al. [20], where neither the increase of HRT from 18 h to 30 h nor the decrease of OLR from 7 g TS/L/d to 4 g TS/L/d

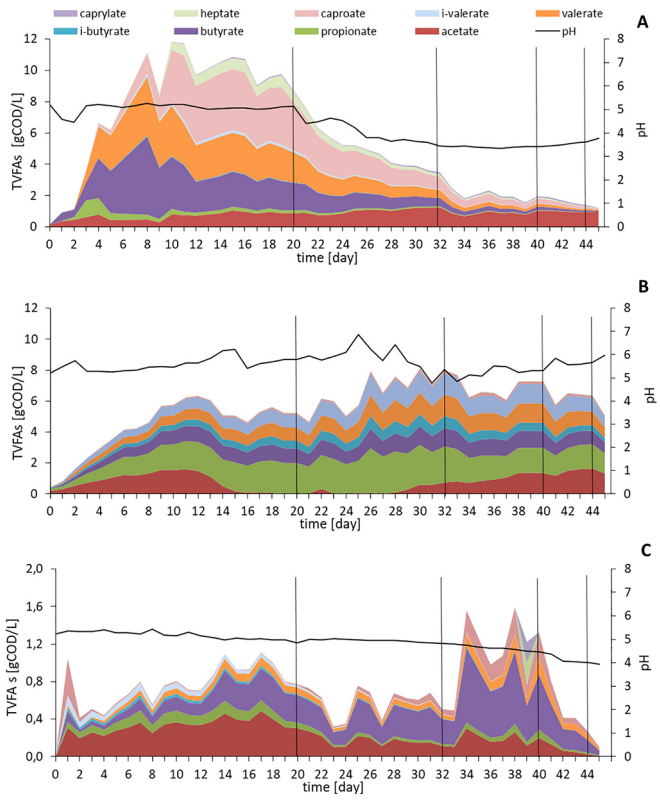

Fig. 1. The production of VFAs obtained during MCF of cheese whey (A), sludge (B) and glucose (C).

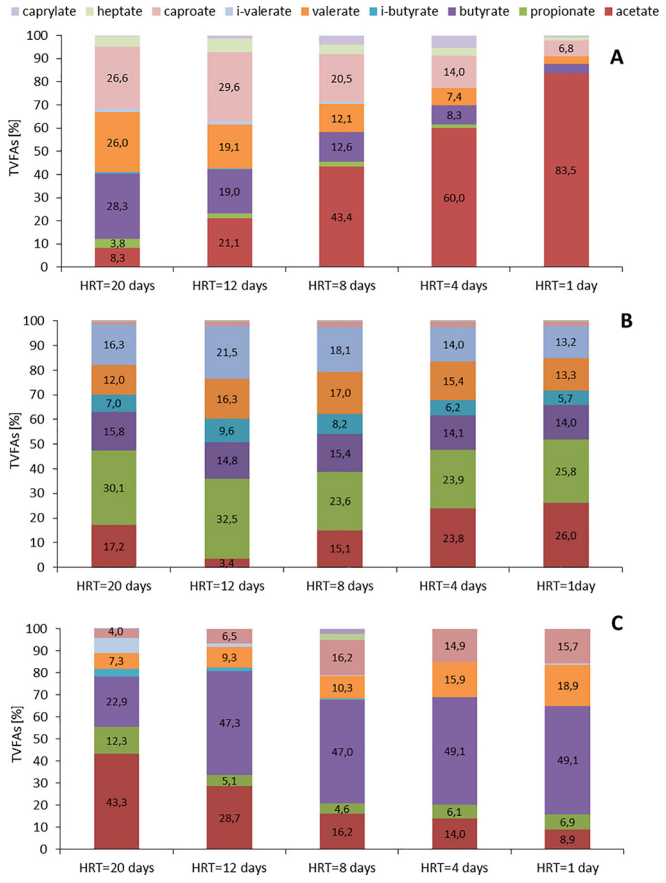

Fig. 2. The composition of VFAs obtained during MCF of cheese whey (A), sludge (B) and glucose (C).

significantly affected the composition of acids. This was further documented by Maharaj and Elefsiniotis [21] that increasing the HRT (30 h → 60 h) had no significant effect on acids composition formed from primary sludge (pH 6.9). In this study, we additionally proved that in case of sewage sludge acidogenic fermentation significant changes in HRT (20 days → 1 day) have only minor effect on TVFAs productivity.

On the other hand, simultaneous change of OLR and HRT had an impact on the fermentation process, with regards to the pH and TVFAs concentration. Since the pH had not been adjusted during the course of the process, the production of in situ volatile buffers (the buffer effect of macromolecules' residues) could cause the pH stabilization [22]. This occurred especially in the first phase of cheese whey fermentation with the longest retention time (20 days) and the lowest OLR (3.6 g COD/L/d), where pH was stable and around 5, despite the high acids concentration (over 8 g COD/L/d) and low pH of fed cheese whey. On the other hand, during the sludge fermentation, the pH increased (due to high pH of added substrate) and tended to 6.0 despite acids formation. The composition of acids depended on the type and the complexity of the substrate. The composition and the proportion of acids produced from the sludge under investigated process conditions did not show significant differences, however it differed during cheese whey fermentation. This could be a consequence of change in the microbial community structure caused by changes in HRT and OLR. Nevertheless, different production profiles were observed and the highest concentration of acetate, propionate, iso-butyrate and iso-valerate were obtained from sludge fermentation, whereas butyrate, valerate, caproate and heptate were produced from cheese whey fermentation.

3.2. Acidogenic fermentation with glucose

Glucose was used as a model, simple substrate to investigate the bioreactor response over changing HRT and OLR conditions, where glucose was the only carbon source and there was no input of additional microorganisms. In low organic loading rate (OLR = 0.24 g COD/L/d), the response of the microorganisms in TVFAs production from simple substrate was immediate. The production of TVFAs started from the beginning of the process (Fig. 1C), unlike the trials with cheese whey or sludge, and the highest product accumulation occurred at the 38th day (1.59 g COD/L at pH 4.6). Fig. 2C shows the results of qualitative analysis. At the beginning of the process acetate was the dominant acid (43.3%), then its share decreased. After 20 days of fermentation, butyrate become a dominant acid (its share was almost 50%). Similar results concerning production of acetate and butyrate from glucose in strong acidic conditions were documented by other authors e.g. Tamis et al. [23], where during fermentation of glucose at pH 4.5, 5.0 and 5.5 acetate and butyrate were dominant acids (acetate of around 15% on COD basis and butyrate of around 45% on COD basis).

3.3. Conversion efficiencies and process performance

The results of conversion efficiencies calculations are shown on Fig. 3. The most favourable process conditions for acids formation during the cheese whey fermentation occurred in the first phase, when the HRT was the longest (Fig. 3A) and the acidification yield (η_a) was the highest (over 73%). The hydrolysis yield (η_h) increased during the trials and it was higher than η_a from the 17th day of the process (maximum η_h was 48.5%). Decreasing HRT and increasing OLR caused the slowdown of the process most likely due to organic overloading combined with microbial washout.

The results of conversion efficiency during sludge fermentation are presented on Fig. 3B. During the process the acidification yield was significantly higher than hydrolysis yield and the maximum and average acidification values were 79% and 62%, respectively. Contrary to the cheese whey fermentation, no sudden drops or increases in acidification yield were recorded. The average hydrolysis yield was 7.2% and the highest reached 17%. Changing OLR and HRT did not significantly affected the conversion efficiencies of sludge fermentation and the activity of hydrolysing and acidifying microorganisms was rather stable.

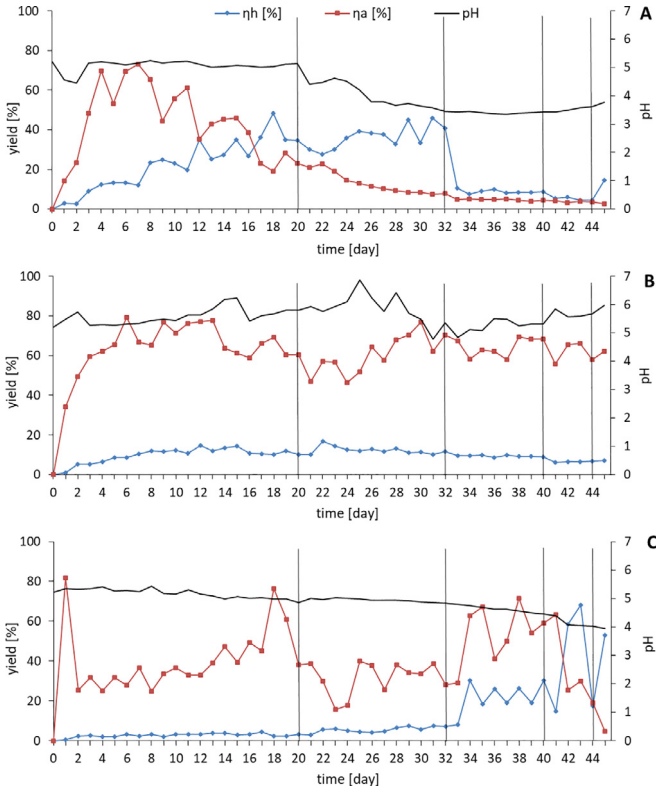

Fig. 3. Hydrolysis and acidification yields during cheese whey (A), sludge (B) and glucose (C) fermentation. The vertical lines indicate the stages of the fermentation.

The conversion efficiencies during the first (HRT 20 days) and the second (HRT 12 days) phase of glucose fermentation were analogous to those calculated for sludge fermentation (high yield of acidification and low yield of hydrolysis). From Fig. 3C we could notice that at the beginning of the process, the dose of organic matter was so small, that it was immediately processed by bacteria. Together with decreasing HRT to 12 days, the acidification yield decreased from average 41% to 31.5%, but the increase in hydrolysis yield was almost imperceptible (3% → 6%). In the third phase both yields increased and the highest production of TVFAs occurred. The shortening of reaction time to 4 days caused sudden decrease in acidification (63% → 25.5%) and increase in hydrolysis yield (15% → 58.5%).

The results of our work confirmed that the increase in hydrolysis yield was accompanied with the decrease in acidification yield. We noticed that in most cases during large acids production, the hydrolysis yield was kept at low values. Any disturbance in the acidification process was resulting in accumulation of the hydrolysis products. Longer period of high hydrolysis yield and minor TVFAs production led to process termination. Moreover, it was proved that the decreasing HRT to achieve higher OLR reduced the effectiveness (yield) of hydrolysis. Consequently, it resulted in lower concentrations of acids but favoured only acetate production. It was due to the fact, that only fast growing acetic bacteria were able to retain in the reactor [2].

3.4. Microbial analysis

Based on DGGE profiles, microbial community was analysed in terms of its structure, dynamics and functional organisation. To investigate changes in the bacterial community in each bioreactor during the fermentation process Shannon-Weaver index (H') was calculated

from fingerprinting data. Additionally, to determine the carrying capacity of each reactor range-weighted richness (Rr) was assessed. For the reactors fed with whey and glucose, the H' biodiversity and Rr showed temporal dynamics in experimental reactors with initial pH of 5.2 and the mean values were $H' = 2.43 \pm 0.4$; $Rr = 15 \pm 6$ and $H' = 2.59 \pm 0.25$; $Rr = 18 \pm 5$ for whey and glucose fermentation, respectively. In the control reactors, at pH 7 the diversity values obtained slightly higher level for both substrates indicating that these reactor microbiomes were only able to survive in the higher pH, but were not able to efficiently produce TVFAs in applied conditions. The indexes drastically decreased reaching the H' value below 2.3 and Rr below 13 for whey and H' value below 2.5 and Rr below 16 for glucose, when the pH dropped below 5 (HRT = 12 days) and it went in accordance with decreasing TVFAs concentration in bioreactors. The very high Rr at the last stage in both processes (HRT = 1) might been the result of accumulation of new portion of microorganisms delivered with each feeding. In reactors fed with sludge the diversity was quite stable through the fermentation processes regardless the HRT, OLR or pH and the mean H' and Rr values were 2.85 ± 0.13 and 24 ± 3 for control trial and 3.10 ± 0.17 and 28 ± 4 for the experimental one, respectively.

The Community organisation coefficient (Co) was calculated for each sample to assess the evenness of the community. The higher the Co the more uneven and functionally organised the community is. On average, low Co values were obtained for all reactors (< 0.45) with somewhat higher values for the experimental trials. Considering clustering analysis for each reactor (data not shown) the samples were grouped into two main clusters and then divided into smaller clusters clearly as a result of HRT and OLR change. It suggests that shortening the HRT resulted in removing some groups of microorganisms that needed more time for growing. It was in favour to other groups of microbes that resisted the conditions and were grouped into distanced clusters. These results implies that even though the communities were quite diverse, they did not manage to functionally organise in applied environmental conditions resulting in lower TVFAs production.

3.5. Bacterial community characterisation

Bacterial communities based on PCR-DGGE analysis were shown by distinguishing the DGGE bands at each fermentation condition (Fig. 4, Tables 3A–C). In the MCF of cheese whey, the identified microorganisms belonged to phylum *Firmicutes*, the rest were members of *Cloacimonetes*, *Actinobacteria* and *Acidobacteria* (Table 3A). A large group of microorganisms has been classified as an Uncultured bacterium clones (named UBC). The first stage (HRT 20 days) of the process was the most habitable and triggered the TVFAs production. The most of the dominated organisms were classified as *Firmicutes* (mostly *Lactobacillus* spp. provided by cheese whey) and UBC, followed by *Cloacimonetes* and *Acidobacteria*. Together with HRT shortening the microbial richness and biodiversity decreased and from the third phase (HRT 8 days) mostly detected microorganisms were *Lactobacillus* sp. Band B1 and B2 were closely related to *Bacillus* sp. which could produce enzymes such as proteases and cellulases and promote acidogenesis [24,25]. Bands A1, A3 and A2 showed high similarity to *Cloacimonetes bacterium* and uncultured *WWE1* (taxa *Cloacimonetes*), respectively, being capable of producing H_2 and butyrate [26] and also express propionate metabolism [27]. Our results indicated that production of butyrate could be related to the presence of phylum *Cloacimonetes* (lower concentration of butyrate occurred simultaneously with lower number of detected *Cloacimonetes* phylum). Bands J2, O1, J3, J4, H1, C2, C3, P1, P2, P3, P4, P5 were aligned to *Lactobacillus* spp. (lactic acid bacteria that convert sugars into lactic acid) and were likely provided with cheese whey. The bands related to *Lactobacillus amylovorus* (J2, O1, J3, J4) were detected in all phases of the process. This suggested that these bacteria might adapt well to studied process conditions. Bands C2, C3 (*Lactobacillus* sp. *JCM 8676* and *JCM 8674*) and H1 (*Lactobacillus pontis*) were not detected from the third phase (HRT = 8 days). However, at the end of the process new species were detected and were closely related to *Lactobacillus frumenti* (P1, P2, P3), *Lactobacillus oris* (P4) and uncultured *Lactobacillus* (P5). Band K3 showed high similarity to uncultured *Rothia* sp., anaerobic bacteria that could be present in a raw cow milk and had ability to produce mainly lactic acid from glucose fermentation [28]. Band F1 was closely related to uncultured *Acidobacteria* that could ferment aromatic compounds and acetate [29]. Band B3 showed high similarity to *Bifidobacterium strain JCM 11342*, which is an anaerobic bacterium producing lactic acid and is able to metabolize complex oligosaccharides to carbon and energy, and to ferment amino-acids [30].

Relatively decreased biodiversity at shorter HRT could be inferred from the fact that the amount of fresh substrate added in each phase was increasing, so the number of microorganisms added with cheese whey displaced the microorganisms delivered with seeding sludge. Under applied processes conditions (varying HRT and OLR, low pH of added substrate and not controlled pH) there was no opportunity for the microorganisms contained in seeding sludge and cheese whey to create a strong consortia for the effective production of TVFAs. However, greater biodiversity of microorganisms in the first phases of the fermentations influenced the process stability, thus better process

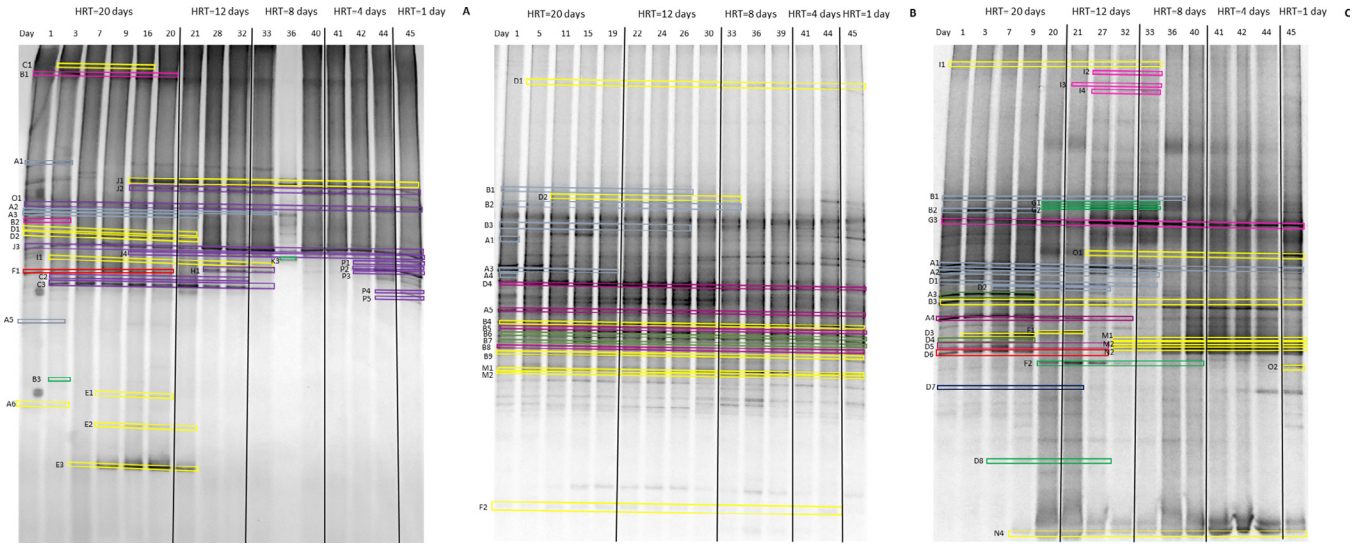

Fig. 4. PCR-DGGE profiles of bacterial community in fermentation process of cheese whey (4A), sludge (4B) and glucose (4C).

Table 3A
Alignment of 16S rRNA gene sequences of DGGE bands- cheese whey fermentation.

Band	Closest relative	Phylum	Identity (%)	Accession No.
C1, D1	*Uncultured bacterium clone OUT_62*		83–94	KT796673
B1	*Bacillus cereus strain TN7*	Firmicutes	83	JQ415980
A1	*Cloacimonetes bacterium 0000039-M09*	Cloacimonetes	91	KJ535423
J1	*Uncultured bacterium clone RRH_aaa01f02*		98	EU474977
J2, O1	*Lactobacillus amylovorus LGM7-3*	Firmicutes	99–100	KU612254
A2	*Uncultured WWE1 bacterium clone QEDP3AB07*	Cloacimonetes	99	CU924714
A3	*Cloacimonetes bacterium JGI 0000059-L07*	Cloacimonetes	100	KJ535434
B2	*Bacillus* sp. *SSM 65*	Firmicutes	82	JQ068830
D2	*Uncultured bacterium clone NBBSO0508_68*		92	JQ072760
J3	*Lactobacillus amylovorus strain 56LAB3*	Firmicutes	99	KR055506
J4	*Lactobacillus amylovorus strain 56LAB2*	Firmicutes	99	KR055505
K3	*Uncultured Rothia* sp. *clone ACH-14L-245*	Actinobacteria	100	KM873083
I1	*Uncultured bacterium clone DC104*		96	HM107020
H1	*Lactobacillus pontis strain M17-5*	Firmicutes	99	KF030785
F1	*Uncultured Acidobacteria bacterium clone QEEB3BF05*	Acidobacteria	94	CU917923
C2	*Lactobacillus* sp. *JCM 8676*	Firmicutes	99	AB911517
C3	*Lactobacillus* sp. *JCM 8674*	Firmicutes	99	AB911515
P1, P3	*Lactobacillus frumenti strain 2.1*	Firmicutes	99	JX272061
P2	*Lactobacillus frumenti strain TMW 1.666*	Firmicutes	100	NR_025371
P4	*Lactobacillus oris strain NS13-bA1*	Firmicutes	99	LC122285
P5	*Uncultured Lactobacillus* sp. *clone 148*	Firmicutes	99	KF998498
A5	*Uncultured bacterium isolate DGGE gel band 8*		84	KM505006
B3	*Bifidobacterium longum subsp strain: JCM 11,342*	*Actinobacteria*	99	LC306854
E1	*Uncultured bacterium clone NBBEQ0409_62*		100	JQ072222
A6	*Uncultured bacterium clone OUT_830*		99	KT251976
E2	*Uncultured bacterium clone BD17604*		100	JQ190603
E3	*Uncultured bacterium clone TS51_a01e11*		99	FJ369523

Table 3B
Alignment of 16S rRNA gene sequences of DGGE bands- sludge fermentation.

Band	Closest relative	Phylum	Identity (%)	Accession No.
D1,D2	*Uncultured bacterium clone OTU_368*		86–96	KT796977
B1, B3, A1, A3	*Cloacimonetes bacterium JGI 0000059-L07*	Cloacimonetes	93–99	KJ535434
B2	*Cloacimonetes bacterium JGI 0000039-M09*	Cloacimonetes	99	KJ535423
A4	*Cloacimonetes bacterium JGI 0000039-P09*	Cloacimonetes	98	KJ535424
D4	*Uncultured Clostridia bacterium clone O1-21*	Firmicutes	97	AB936412
A5	*Uncultured Firmicutes bacterium clone Hyd-55-101025-10*	Firmicutes	99	KJ590266
B4	*Uncultured bacterium clone OTU_5*		91	KU648411
B5	*Bacillus* sp. *DV9-59*	Firmicutes	76	GQ407203
B6	*Uncultured Gammaproteobacteria bacterium clone QEEB1BB08*	Proteobacteria	99	CU918408
B7	*Uncultured Gammaproteobacteria bacterium clone QEEB2CH11*	Proteobacteria	89	CU917787
B8	*Uncultured Firmicutes bacterium clone QEDQ2BC11*	Firmicutes	98	CU923016
B9	*Uncultured bacterium isolate DGGE gel band 18*		97	JX627832
M1	*Uncultured bacterium clone OTU_2598*		94	KU651004
M2	*Uncultured bacterium clone IAN11*		84	KF428077
F2	*Uncultured bacterium clone OTU_1601*		99	KT798203

parameters (η_h and η_a) and finally higher production of TVFAs.

In the fermentation of sludge four main phyla of microorganisms were detected *Firmicutes*, *Cloacimonetes*, *UBC* and *Proteobacteria* (Table 3B). Fig. 4B shows that together with HRT shortening the number of *Cloacimonetes* decreased and similarly to cheese whey fermentation, they were not detected from the third phase of the process. Other microorganisms (*Firmicutes, Proteobacteria* and UBC) were observed in all HRTs. Bands B1, B3, A1, A3, B2 and A4 were closely related to *Cloacimonetes* sp. Moreover, bands *Cloacimonetes bacterium JGI 0000059-L07* and *Cloacimonetes bacterium JGI 0000039-M09* were also detected in cheese whey fermentation. Band D4 showed high similarity to *Clostridia* sp. that had a strong hydrolytic ability and could accelerate the hydrolysis of polysaccharides and production of organic acids [31]. Uncultured *Firmicutes* bacterium (bands A5 and B8) and *Proteobacteria* sp. (bands B6 and B7) had the ability to effectively degrade organic compounds such as proteins, lipids, celluloses, sugars and amino acids [32] and to simultaneously produce TVFAs [33]. Moreover, bands B6 and B7 were related to class *Gammaproteobacteria*, that was correlated with utilization of polysaccharides or fermentation of butyric acid [34]. Band B5 showed high similarity to *Bacillus* sp. Contrary to cheese whey fermentation no *Acidobacteria* and *Actinobacteria* were detected during sludge fermentation.

Quite high biodiversity of microorganisms during sludge fermentation process could have an impact on the constant hydrolysis yield and high acidification yield which was reflected in stable production and composition of acids. In contrary to cheese whey fermentation, the process was not clearly dominated by any specific group of microorganisms although the retention time was shortened. Similar community structure in sludge digestion was documented by Yuan et al. [35]. One might conclude that regardless the location of the process and the origin of the sludge, the community structure will be similar. Henceforth, we confirmed that the community structure strongly depends on the substrate type and process operating parameters.

The results of the microbiological analysis from the glucose trials revealed the presence of 7 groups of microorganisms, namely *Firmicutes, Cloacimonetes, Actinobacteria, Acidobacteria, Proteobacteria, Bacteroidetes* and UBCs (Table 3C), however, predominant identified species were members of phylum *Firmicutes*. Bands I2 and G3 were

Table 3C
Alignment of 16S rRNA gene sequences of DGGE bands- glucose fermentation.

Band	Closest relative	Phylum	Identity (%)	Accession No.
I1	*Uncultured bacterium clone nbw109a03c1*		96	GQ007726
I2	*Uncultured Clostridium* sp. *clone FecD015*	Firmicutes	96	KM244808
I3,I4	*Romboutsia* sp. *DR1/DR2*	Firmicutes	99–100	LN998074
B1, A2	*Cloacimonetes bacterium JGI 0000059-L07*	Cloacimonetes	99	KJ535434
G1,G2, F2, D8	*Uncultured Atopobium* sp.*clone SS1-9*	Actinobacteria	88–99	AB936325
B2, D1, D2	*Uncultured WWE1 bacterium clone QEDP3AB07*	Cloacimonetes	97–99	CU924714
G3	*Uncultured Clostridium* sp. *clone 12 m0215073*	Firmicutes	99	JX548178
O1	*Uncultured bacterium clone C20*		99	EU234250
A1	*Uncultured WWE1 bacterium clone QEDQ2BH07*	Cloacimonetes	100	CU922996
A3	*Escherichia* sp. *UIWRF0504*	Proteobacteria	99	KR189686
B3	*Uncultured bacterium clone 285ZH12*		96	JN093487
A4	*Uncultured Firmicutes bacterium clone Hyd-55-101025-10*	Firmicutes	99	KJ590266
F1	*Uncultured bacterium clone C4*		98	GU595092
D3	*Uncultured bacterium clone OUT_276*		86	KU648682
D4	*Desulfovibrio desulfuricans strain E9*	Proteobacteria	98	KJ459868
M1	*Uncultured bacterium clone NBBSP0409_85*		100	JQ072839
M2	*Uncultured bacterium clone inf39*		100	JN245805
D5	*Uncultured Firmicutes bacterium clone QEDQ2BC11*	Firmicutes	99	CU923016
N2	*Uncultured bacterium clone C20*		99	EU234250
D6	*Uncultured Acidobacteria bacterium clone QEEB3CG12*	Acidobacteria	98	CU917622
O2	*Uncultured bacterium clone OTU_1847*		98	KT798442
D7	*Uncultured Bacteroidetes bacterium clone TP_7*	Bacteroidetes	84	JF681267
N4	*Uncultured bacterium clone NBBEQ0409_62*		99	JQ072222

closely related to uncultured *Clostridium* spp. The *Clostridia* class was also detected during sludge fermentation. Bands I3 and I4 showed high similarity to *Romboutsia* spp., an obligatory anaerobic bacteria able to produce acetic acid, ethanol, iso-butanoic acid and iso-valeric acid from glucose [36]. Bands B1, A2, B2, D1, D2 and A1 were aligned to *Cloacimonetes* and uncultured *WWE1* (taxa *Cloacimonetes*), respectively. *Cloacimonetes bacterium JGI 0000059-L07* and *Uncultured WWE1 bacterium clone QEDP3AB07*, were also detected in sludge and cheese whey fermentations, respectively. In all fermentation tests (regardless the OLR) the amount of *Cloacimonetes* bacteria decreased as the retention time decreased and they were not detected after shortening HRT to 8 days. However, in glucose fermentation *Uncultured WWE1 bacterium clone QEDQ2BH07* (band A1) was detected at each stage of the fermentation (regardless the HRT). Our results indicated, that the HRT had a strong impact on the presence of *Cloacimonetes* bacteria. Bands G1, G2, F2 and D8 showed high similarities to uncultured *Atopobium* spp. strictly anaerobic bacteria (phylum Actinobacteria, family *Coriobacteriaceae*), able to produce mainly lactic acid, acetic acid, formic acid and trace amount of succinic acid in glucose fermentation [37]. Other *Actinobacteria* (playing critical role in anaerobic digestion and acidification process [38] were detected during cheese whey fermentation. Band A3 was aligned to facultative anaerobic *Escherichia* sp. Bands A4 and D5 aligned to uncultured *Firmicutes* bacteria. Moreover, *Bacillus* sp. were present in fermentation of cheese whey and sludge but not in glucose. Band D4 showed high similarity to *Desulfovibrio desulfuricans*, which is an anaerobic sulfate-reducing bacteria usually present in activated sludge from wastewater treatment plant [39]. Band B6 was closely related with uncultured *Acidobacteria* that were also detected during cheese whey fermentation. Uncultured *Bacteroidetes* (D7 band), similarly to *Proteobacteria* and *Firmicutes*, could effectively degrade organic compounds.

The fermentation of glucose indicated that in shorter HRT the bacterial community was washed out from the reactor (Fig. 4C), hence the lower diversity of microorganisms could be noticed. In glucose fermentation, similarly to sludge fermentation, higher biodiversity of microorganisms resulted in higher hydrolysis or acidification yields. Among all detected phyla, only *Bacteroidetes* were not identified during the fermentation of cheese whey and sludge. Moreover, in all processes the UBCs were a significant part of the identified microorganisms. According to Stewart [40] the uncultured bacterium clones are organisms unable to grow in the laboratory on standard media or the cultivation methods have not been developed yet. These bacteria could play a critical role in carbon, nitrogen and other elements cycles and to maintain the stability of the reactor microbiome.

4. Conclusions

We examined the effect of varying HRT and OLR on the production of TVFAs and bacterial community structure during the acidogenic fermentation. The shortening of HRT together with increasing of OLR had a significant effect on the performance of acidogenic fermentation, which also depended on the complexity of the substrates and its inherent microbial population. Obtained results from whey fermentation revealed that shortening of HRT caused a decrease in TVFAs concentration and lower microbial biodiversity of detected microorganisms. On the other hand, the fermentation of sludge indicated that HRT shortening and OLR increase influenced the TVFAs productivity, but not the microbial community and consequently, not the VFAs composition. The bacterial community originating from the substrate was able to adapt to the applied bioreactor conditions and it tended to dominate the microbial community in the bioreactor while shortening the HRT.

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Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion

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ABSTRACT

The goal of this work was to give a comprehensive review on biogas production from microalgae biomass. Different process parameters were summarized in tables which could become comprehensive compendium of operation conditions of microalgae preparation for biogas production. Further,, the limitations of the process implementation and commercialization (e.g. high costs of implementation and maintenance, low biomass productivity, limited methane yield due to specific structure of microalgae cell wall) were discussed. It was concluded that the microalgae anaerobic digestion should be incorporated with production of other bioproducts such as biodiesel, bioethanol or volatile fatty acids. Such a biorefinery would open possibility to improve both wastewater treatment and generate valuable products from waste streams.

1. Introduction

Limiting resources of fossil fuels and progressing climate change confronted the World's population with a need for searching for a renewable and sustainable energy source. The superiority of biofuels over commonly used fossil fuels consists in greater energy security, reducing the negative impact on the environment, and savings in fuels export [1–3]. The first generation of biofuels are obtained from food crops causing undesirable competition between food, feed and energy. The second generation biofuels aim at being produced from lignocellulosic crops, but several obstacles occur, such as low productivity of biomass, requirement for excessive amounts of water and arable land as well as need for efficient and low energy demand pretreatment [4]. During the last years, a new perspective for biofuels generation appeared – the third generation biofuels from microalgae cultures. In comparison with land-based feedstock, algae have certain advantages: they grow 5–10 times faster in beneficial conditions and also have higher production rate compared to the terrestrial biomass [5]. Additionally, the microalgae can be cultivated in barren areas like deserts or coastal land (which are much less or even not competitive to food production) [6]. Moreover, microalgae can be grown using nutrient rich wastewater or reject water [7], which would provide all the necessary nutrients needed for their growth [8–10]. Alternatively, they could also be grown in saline/brackish water [11]. During the growth throughout the photosynthesis process, they reduce CO_2 emission by carbon uptake and remove nutrients from wastewater [9,12–14]. Moreover, some microalgae's consortia can be used for industrial wastewater treatment e.g. black oil biodegradation and detoxification [15]. All of those advantages shortly described above, place microalgae as an alternative and promising feedstock for energy production.

This review is intended to describe the biogas production from microalgae biomass, however some additional fundamental information have been included in order to give a reader a view of the whole value chain, that is: the definition and characteristic of algae, the current state of the art of algae cultivation and cell harvesting, since these processes have an effect on the overall value chain for microalgae anaerobic digestion. Additionally, authors included the limitation of the microalgae applications for energy production. Finally, examples of co-digestion of microalgae with different waste streams as well as suggestion of microalgae AD based biorefineries were described. Lastly, possible solutions to overcome obstacles towards scaling-up and commercialization were summarized at the end of this paper..

2. Definition and characteristic of algae

Algae can be classified into microalgae and macroalgae. Microalgae are unicellular, autotrophic organisms, which use sunlight, water and

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Table 1
Experimental conditions of microalgae cultivation, harvesting and obtained methane yields.

Strain	Cultivation					Harvesting	Anaerobic digestion process conditions	Methane/biogas yield	Ref.
Scenedesmus obliquus	Open pond	20°C	Laboratory scale (200 L plastic bag)	Synthetic medium Z −8	Continuous illumination fluorescent lamp (Edison 58 W)	Natural sedimentation	33 days (35 °C, batch reactor)	0.076 L CH_4/g COD	[4]
Chlamydomonas reinhardtii	–	–	Laboratory scale	TAP medium	Continuous white light (40 μmol/m^2/s)	Centrifugation 3.100x*g*, t=6 min	32 days (38 °C, batch test)	0.587 L biogas/gVS (66% CH_4) (0.387 LCH_4/gVS)[a]	[34]
Chlorella kessleri								0.335 L biogas/gVS (65% CH_4) (0.218 L CH_4/g VS)[a]	
Scenedesmus obliquus				ProF medium				0.287 L biogas/gVS (62% CH_4) 0.178 L (CH_4/gVS)[a]	
Chlorella vulgaris	Suspended polyethylene bag	–	Laboratory scale	Estuarine	–	Centrifugation at room temperature	30 days (35 °C, batch test	0.337 L CH_4 g VS fed	[36]
Nannochloropsis sp.	Outdoor raceway pond			Marine				0.357 L CH_4 /g VS fed	
Nanofrustulum sp.	Outdoor raceway pond			Marine				0.507 L CH_4 /g VS fed	
Nannochloropsis salina	Outdoor photo-bioreactor (PBR)			Marine				0.557 L CH_4 /g VS fed	
Phaeodactylum tricornutum	GreenHouse pond replete conditions			5% carbon dioxide sparge	Natural daylight			0.337 L CH_4 /g VS fed	
Algae biomass: *Stigeoclonium sp., Monorraphidium sp., Nitzchia sp., Amphora sp.,*	HRAP (High Rate Algal Pond)	–	Pilot scale	Wastewater	–	Secondary settlers (natural sedimentation, HRT 9 h) Imhoff cone 24 h (thickened to 2.0–2.5% (w/w))	15 days HRT (37 °C, continuous test)	0.10 L CH_4/g VS	[38]
							20 days HRT (37 °C, continuous test)	0.18 L CH_4/g VS	
							20 days HRT (37 °C, continuous test	0.18 L CH_4/g VS	
Chlorella vulgaris	PBR batch process	25 °C	Laboratory scale	Modified Z–8 medium	Continuous light (6 25 W daylight neon lamps)	Natural sedimentation	16days and 28days HRT (35 °C)	0.110 LCH_4/g COD_{in} and 0.180 LCH_4/g COD_{in}	[43]
Spirulina maxima	Flat tank PBR	–	Laboratory scale (64 L)	–	–	Centrifugation	20 days HRT (35 °C, batch reactor)	0.190 L^L CH_4/g VS	[44]
Scenedesmus obliquus	PBR bubble column (semi-continuous mode)	25 °C	Laboratory scale	Fresh water	Continuous light (metal halide lamps 250 μmol/m^2/s)	Centrifugation (10 000/ $_{min}$)	30 days batch (33 °C)	0.13 L CH_4/gVS	[45]
							30 days batch (54 °C)	0.17 L CH_4/gVS	
Phaeodactylum tricornutum	Serpentine PBR (semi-continuous mode)			Artificial sea water	Continuous light (fluorescent lamp 100 μmol/m^2/s)		30 days batch (33 °C)	0.27 L CH_4/gVS	
							30 days batch (54 °C)	0.29 L CH_4/gVS	
Scenedesmus sp.	Open pond	20 °C	Pilot scale (500 L)	Synthetic medium Z −8	Continuous illumination fluorescent lamp (Edison 58 W)	Natural sedimentation	34 days (35°C, batch reactor)	0.0818 L CH_4/g COD	[86]
Chlorella vulgaris	From WWTP	25 °C	Laboratory scale	Synthetic inorganic medium	Constant illumination (fluorescent lamp)	Centrifugation	29 days (35 °C , batch reactor)	0.156 L CH_4/g COD	[87]
Chlorella vulgaris	PBR	21 °C	Laboratory scale	Non-sterile effluent from UASB (swine manure)	–	Centrifugation (3500 rpm, t=5 min)	24 days (37 °C, batch reactor)	0.364 $L_N$biogas /gVS (62.6% CH_4) (0.228 L_N CH_4/gVS)[a]	[88]
H.reticulatum (filamentous algae)	Bench-scale raceway pond	Indoor conditions	Bench-scale	Secondary wastewater	LED (120 μmol/m^2/s, 12 light and dark cycle	Manually one a day	25 days (35 °C , batch reactor)	0.170 L CH_4/g VS	[89]
Rhizoclonium	Open pond	–	Full scale	Drainage water	–	Manually	28 days (53 °C, batch reactors)	0.23–0.24 L CH_4/g TS	[90]

(continued on next page)

Table 1 *(continued)*

Strain	Cultivation					Harvesting	Anaerobic digestion process conditions	Methane/biogas yield	Ref.
Pediastrum sp., Micractinium sp., Scenedesmus sp.	HRAP	–	Laboratory scale	Primary settled sewage	–	Gravity-settling cone	128 days SRT(20 °C, AVR reactor) 128 (175) days SRT (37 °C, AVR reactor 91 (130) days SRT (ambient temperature, AVR reactor) 14–16 days HRT (37 °C, CSTR reactor)	0.101–0.103 L CH_4/g VS_a 0.225 L CH_4/g VS_a 0.083 L CH_4/g VS_a 0.179 L CH_4/g VS_a	[91]
Microalgal biomass	HRAP	–	Pilot scale	Primary treated wastewater	–	Secondary settler, Imhoff cone-natural sedimentation	15days and 20 days HRT (35 °C, continuous test)	0.13 L CH_4/g VS and 0.17 L CH_4/g VS	[92]
Nannochloropsis salina	PBR	–	Large scale	Modified f/2	–	Continuous clarifier	19 days (35 °C, batch test	0.70 L biogas/g VS (0.43 L CH_4/gVS)	[96]
Algae biomass	High -rate Sewage Stabilization Pond	–	Full scale	Sewage	–	Sedimentation: (I stage to 10 g/kg VS), (II stage to 2.0–2.5% solid algae); Centrifugation (6000 rpm, t=20 min)	28 days (38 °C), batch reactor)	0.34 g MSGP g/L[b]	[97]
Chlorella vulgaris	–	–	Laboratory scale (Batch process)	Swine wastewater, nitrified secondary effluent	–	–	25 days (mesophilic conditions, batch reactor)	0.366 L biogas/g VS (62.5%CH_4) (0.229 L CH_4/gVS)[a]	[98]
Algae biomass: *Scenedesmus obliquus, Chlorella vulgaris*	HRAP	20 °C	Pilot scale (500 L)	Primary treated urban wastewater	–	Settling tank	46 days (35 °C, batch reactor)	0.172 L biogas/g VS (68.2%CH_4) (0.117 L CH_4/gVS)[a]	[99]
Chlorella	Erlenmeyer flasks – batch reactor	20 °C	Laboratory scale	Modified Zarrouk medium	24 h continuous light (5000 lx, 6 40-W fluorescent lamps)	Centrifugation 3000 rpm, t=5 min	45 days (37 °C, batch reactor)	0.123 L CH_4 /g $VS_{fed.}$	[100]
Chlamydomonas reinhardtii *Chlorella vulgaris*	–	25 °C	Laboratory scale	Mineral salt medium	Continuous illumination (fluorescent lamp)	Centrifugation (5000 rpm, t=15 min)	22 days (35 °C)	0.263 L CH_4/gCOD 0.191 L CH_4/gCOD	[101]
Chlorella vulgaris	PBR	25 °C	Laboratory scale	Mineral salt medium	Continuous illumination (fluorescent lamp)	–	30 days (35 °C)	0.139 L CH_4/$gCOD_{in}$	[102]
Isochrysis galbana	Batch reactor	–	Laboratory scale	Modified f/2 medium	–	Centrifugation (4000 rpm, t=10 min,20 °C)	15 days (30 °C, 1 atm)	0.022 L (79.4%CH_4) (0.0175 L CH_4)[a]	[103]

AVR – Accumulating-volume reactor.

[a] Recalculated by authors.

[b] MSGP – Methane Specific Gas Production.

atmospheric CO_2 for growth [5,16,17]. Throughout the process of photosynthesis CO_2 absorbed from the atmosphere is converted into valuable products - lipids, which can be further used as an energy source [6]. The microalgae have a wide range of size: from nano- to milli- meter: they exist as independent organisms or in chains/groups [6]. Moreover, the structure of microalgae is not differentiated - has no roots, stem and leaves and the estimated number of identified species is approx. 100 000 [5]. From all, only about 35000 species have been characterized so far [18]. Their population can be divided into filamentous and phytoplankton and further categorized into classes: diatoms, green, blue-green and golden algae [19,20]. Several studies have shown the use of microalgae species for biogas production (Table 1). On the other hand, the macroalgae are multicellular, eukaryotic organisms, that consist of branches, roots and leaves. Macroalgae can be classified into brown, red and green algae [21]. This review is limited to microalgae and further in the text term "algae" is referred to microalgae, if not specified differently.

The microalgae biomass constitutes at high quantity of lipids (7–23%), proteins (6–71%) and carbohydrates (5–64%). Those proportions depend on algae species and growth conditions [22–25]. The most valuable microalgal products for biofuels production are lipids. Various species of microalgae, in beneficial and optimum conditions, can synthesize up to 50–70% of oil/lipid per dry weight and produce from 58 700 L_{oil}/ha to 136 900 L_{oil}/ha for microalgae with 30% and 70% of oil content (by wt), respectively [26]. By contrast, the oil yields of agricultural crops are as follows: corn 172 L_{oil}/ha, soybean 446 L_{oil}/ha, coconut 2 689 L_{oil}/ha, oil palm 5 950 L_{oil}/ha. Based on the oil yields, the content of oil in soybean and palm oil, compared with microalgae, is less than 5% of total biomass [27]. However, Moody et al. [28] argued that current life cycle, techno-economic and resource assessments are significantly overestimated for microalgae lipid yields. Thus, all production systems based on laboratory-scale simplistic growth models overestimate the possible productivity potential and ignore significant factors e.g. biological effects, geographical location, cultivation architecture. Through the analysis of microalgae biomass (*Nannochloropsis*) productivity in large-scale outdoor photoreactors and historical meteorological data from 4,388 global locations, the authors estimated the maximum annual average lipid yield range between 24 and 27 $m^3\ ha^{-1}\ y^{-1}$, corresponding to 13–15 $g\ m^{-2}\ d^{-1}$ of biomass yield. The results were obtained for Australia, Brazil, Colombia, Egypt, Ethiopia, India, Kenya and Saudi Arabia. The analysis also indicated that the largest average annual lipid yields occurred in locations with low climate variability.

The structure of the cell wall is important from the point of view of microalgae vulnerability to digestion. According to Takeda [29] the structure of microalgae cell wall (e.g. *Scenedesmus obliquus)* consist of glucose, mannose and galactose. Those compounds can form cellulose and hemicellulose, which cause a high resistance of cell walls to enzymatic hydrolysis and limit their availability for anaerobic digestion. Several authors have highlighted that the resistance of microalgae cell walls depends on the presence of biodegradable polymers. Often mentioned are sporopollenin and algaenan [30–32]. The sporopollenin has high stability and can appear in two forms, which depends on the oxidative polymerization of lipids: an oxygenated aromatic building block and an aliphatic biopolymer [30]. The algaenan is an aliphatic molecule with resistance to treatment by acids and alkalis [32]. The presence or absence of any of these biopolymers in microalgae cell wall has an impact on the efficiency of whole methane production process [33]. A closer examination showed that even easily degradable cell wall did not ensure an efficient methanisation [34], which suggests a need for a pretreatment method. The pretreatment methods for microalgae biomass for decreasing the cell walls' resistance are elucidated further in the text.

3. Cultivation of microalgae

The most frequently applied systems for cultivation of microalgae used for biogas production are open ponds, photo-bioreactors (PBR) and hybrid systems currently used in research (pilot and laboratory) scale and industrial scale.

Open pond systems (OPR) are reactors open to the environment [5]. The commonly used types are raceway ponds [35,36] and High Rate Algal Pond (HRAP) used for wastewater treatment [37,38]. Open pond reactors have relatively low cost of construction, installation and maintenance and are easy to operate [5,39]. The drawbacks of OPR are: the contamination with other algae species and predators, vaporization and the lack of control of the growth parameters [5,40]. The biomass concentration is relatively low [35,41], according to Borowitzka [42] it is around 10–25 g dry matter of algae biomass per day per m^2.

The second method for microalgae cultivation is in photo bioreactors (PBRs), which are closed systems. Frequently used types are tubular [43], flat-tank [44], bubble column and serpentine [45]. The main advantage of PBRs is the control of algae growth, which leads to high productivity of algal biomass. Additionally, by optimization and control of the culture environment conditions, the contamination with other algae species can be avoided [6,46]. Nevertheless, PBRs have some drawbacks: higher operating and maintenance costs than open systems. The biomass concentration is between 20 and 100 g dry matter of algae biomass per day per m^2 [47].

The last type of algae cultivation systems is a combination of OPR systems and PBRs. In those, the role of the bioreactor (closed reactor) is to cultivate preferred algal species, which subsequently inoculate the OPR as in the example described in [36].

3.1. Factors determining the microalgae cultivation

The main factors determining the growth of microalgae's are: concentration and quality of nutrients, CO_2 concentration, water supply, temperature (16–27 °C), exposure to light (1 000–10 000 lx), pH levels (4–11) [48,49], culture density, salinity (12–40 g/L) [48], turbulence [6,50], biological factors [48,51], presence of toxic compounds, heavy metals and synthetic organisms, as well as bioreactor operating conditions [20,48].

3.1.1. Nutrients

The most important nutrients during the microalgae growth are inorganic carbon (some microalgae species are able to utilize organic carbon from wastewater [10]) and inorganic nitrogen (ammonium or nitrate) [52,53] and phosphorus [54]. The microalgae source of carbon is CO_2 uptaken from the atmosphere, industrial exhaust gases and soluble carbonates. Today the whole purpose of creating biogas is to extract energy using methane as it has high calorific value. The combustion process of methane results in CO_2 emission and part of which could be used for recirculation for microalgal biomass growth. According to Doušková et al. [55] the use of biogas as a source of carbon dioxide could reduce the costs of biomass production, moreover, biogas does not contain harmful compounds. Work by Sumardiono et al. [56] indicated that use of biogas and air in switch-cycle (240 min biogas and air from 240 min to 600 min) increased the growth rate of *Spirulina platensis* to $0.39{\cdot}10^{-3}$/min (from $0.21{\cdot}10^{-3}$/min, biomass aerated with air continuously). This result was confirmed by kinetic model, where kinetic constant of maximum value of *Spirulina platensis* A=0.744 and maximum specific growth rate $\mu=0.588{\cdot}10^{-3}$/min were also higher for cycle-switching operation.

Another important element required for microalgal nutrition is nitrogen [57]. Microalgae species with fast growth rate require ammonium as a primary source [52], however, when the growth medium is out of nitrate, the intermittent nitrate feeding can enhance the growth of microalgae [53]. The microalgae reaction to stress conditions, thus insufficiency of nitrogen, is a lower growth rate and

a lower productivity, but high production of lipids (reserve compounds) [58]. The results obtained by Klassen et al. [59] indicated that progressing nitrogen limitation (processing starvation) during microalgae cultivation leads to efficiency degradation of algal cells during fermentation process (35–100% of cell degradation). The biogas production from *Scenedesmus obliquus* biomass increased by 106% due to the so-called nitrogen limitation treatment. It is worth to underline that no other (enzymatic, chemical or physical) pretreatment was performed [59].

The third most important nutrient for growth of microalgae biomass is phosphorus. It should be applied in significant excess in form of phosphates, because not all phosphorus compounds are available for biodegradation (e.g. phosphorus combined with metal ions) [54]. Nevertheless, to achieve effective cultivation (especially in laboratory scale cultivation, examples of synthetic mediums are presented in Table 1) the trace species like metals (Mg, Ca, Mn, Zn, Cu, Mb) and vitamins need to be added [57].

3.1.2. Toxic compounds

The toxic compounds that may affect the stable microalgae growth rate are heavy metals, however the changes in concentration of various gases (CO_2, NO_x, SO_x, O_2 and NH_3) could significantly influence the microalgae growth as well. Different microalgae species require different optimal concentration of CO_2. The concentration higher than 5% adversely affects growth rate of most freshwater microalgae (e.g. *Chlamydonomas reinhardtii, Chorella pyrenoidosa, Scenedesmus obliquus*) due to changes in photosynthetic characteristics, thus lower affinity to CO_2, higher photosynthetic sensitivity to O_2 or higher point of CO_2 compensation and lower activity of carbonic-anhydrase [60,61]. On the other hand, some species of green microalgae (e.g. *Scenedesmus*, *Chlorella)* were able to tolerate the concentration of CO_2 up to 50% and the growth rate remained high [60]. In case of other gases, minor addition of NO_x and SO_x had no effect on the *Chlorella* [62]. Moreover, results of work of Yoshihara et al. [63] indicated that *Nannochloropsis* sp. was able to grow under 100 ppm concentration of NO. Other work [64] showed, that *Tetraselmis* sp. could grow with 185 ppm SO_x and 125 ppm NO_x. Moreover, even high concentration of NO (~300 ppm) had not directly influenced microalgal growth, because of fact that NO was absorbed by the cultivation medium and changed to NO_2^-, which could be further used as a source of nitrogen. Nevertheless, the high concentration of SO_2 (> 400 ppm) could involve the decrease of medium pH, thereby occurred lower productivity [64]. The major inhibitor of algae metabolic processes is excessive dissolved oxygen (DO) [36,60]. The high concentration of DO could cause photo-oxidative damage on the microalgal cells, thereby the reduction of treatment efficiency [65,66]. The results showed by Matsumoto et al. [67] indicated that DO increase from 0 to 29 mg O_2/L inhibited process of photosynthesis (98% decrease in O_2 production). During microalgae cultivation, produced O_2 was continuously consumed by heterotrophic bacteria, thus low DO concentration (~0 mg O_2/L) when pollutants (salicylate) were used by microalgae as nutrients and rapid growth after completely depletion of pollutants [68,69].

3.1.3. Bioreactor operating conditions

The bioreactor operating conditions might affect the CO_2 availability, share rates and light exposure. Important factor is bioreactor gas transfer, that supplies CO_2, NO_x and SO_x (sources of C, N and S), provides internal mixing and control of pH by appropriate concentration of CO_2. The internal mixing prevented nutrients concentration gradients, also reduced the excessive DO and promoted light exposure for all cells [20].

3.1.4. Light provision

Other important factor is light provision. The crucial point in microalgal bioreactors were intensity and utilization efficiency of light supplied and appropriate ratio of light to dark or light to low-intensity light periods [19]. In high-density cultures the light intensity could decrease within depth, thus causing mutual shading. To avoid light attenuation and improve light absorbance, the bioreactors should have high surface area-to-volume ratio and short light path [70]. Moreover, the condition of cultivation medium could also influence light supply. Growing algae in wastewater or reject water might have the right nutrients, however light transmittance becomes a limiting factor. Cho et al. [71] investigated the microalgae growth in effluent waters (from an aeration tank of municipal WWTP) with different pretreatments. The obtained results showed that in raw effluent the growth of microalgae biomass was inhibited by a great amount of bacteria, due to limiting light penetration and nutrients consumption. Moreover, during cultivation with autoclaved effluent occurred problem of light utilization in photosynthesis caused by some content of remaining suspended solid. The best growth rate was obtained from cultivation with filtered medium (0.2 μm-pore size filter) and achieved biomass content was 0.67 g/L [71]. Other works [72–76] confirmed that higher biomass growth could be obtained from pretreated medium. Another important factor is light intensity. The work of Hu et al. [77] indicated that good microalgal growth was obtained under light intensity of 4000 $\mu mol\ m^{-2}\ s^{-1}$, however light above saturation point could cause light inhibition, that could be counterbalanced by application of short-cycles of light and darkness [78,79]. The light/dark cycles might provide that microalgae achieve the maximum efficiency of photosynthesis. However, the second type of light period (light with low intensity) indicated better results than light/dark cycles, thus, significant increase of algae growth, assimilation of CO_2 and production of lipids [36,80]. The source of light has also significant impact on the growth rate. However, different microalgal species requires different light wavelength, thereby before startup of microalgae cultivation each individual case should be investigated to choose the best operational parameter [19]. The recent study by Yan et al. [81] indicated that using of LED light (red and blue light in ratio 5:5) during *Chlorella* cultivation resulted in increased biogas slurry nutrient purification effects and higher microalgae growth than that of the monochromatic. The results showed by Das et al. [82] using blue LEDs improved the specific growth rate of *Nannochloropsis* sp. and was 0.64 and 0.66 d^{-1} in photrophic and mixotrophic cultures. It was also proved that using LED light (especially blue) improved production of extracellular polysaccharides [79], fatty acids methyl ester (from intracellular lipid of *Nannochloropsis* sp.) [82] and astaxanthin (from *Haematococcus pluvialis*, under flashing light illumination) [83].

3.2. Cultivation strategy

According to Lodi et al. [84] there are several cultivation strategies, which could be applied in biotechnological processes: batch (discontinuous), fed-batch, repeated fed-batch, semi continuous and continuous. For the laboratory scale and research purposes, due to its advantages, the most suitable seems to be the continuous model. The key advantages of the continuous set-ups are: no need of post-processing preparation of the reactor systems (obligatory in batch systems prior and after each fermentation), the possibility of higher automation, constant production of fresh and active algal cells, and high productivity of algae biomass over extended period of time. Furthermore, when steady state is reached, the biomass and products are produced constantly in regular quality and quantity [40]. The major drawback of cultivation systems with continuous biotechnological processes is insufficient information in literature for constructing efficient and robust full-scale systems [85] and additionally the complexity and high costs of the system installation and running [40].

4. Microalgae harvesting

After the cultivation, the microalgae biomass need to be separated from the bioreactor effluent. The concentrations reached after harvest-

ing process are between 2–7% of algae in slurry material [6]. The harvesting costs comprise about 20–30% of total cost of microalgae biomass production [20,48]. The choice of appropriate method is depended on microalgae specie, cell density and culture condition [51]. The basic microalgae harvesting methods prior anaerobic digestion in laboratory scale processes are sedimentation [4,86], centrifugation [36,87,88] as well as manual techniques [89,90]. Natural sedimentation is typically carried out in settlers (clarifiers) and Imhoff cones [38,91,92]. The choice for proper harvesting procedure should be adjusted to the desired product quality [93]. For the low value products gravity sedimentation, sedimentation enhanced by flocculation or settling ponds (especially for harvesting of biomass cultivated in sewage) might be used. However, for high-value products, like for food or aquaculture applications, continuously operating centrifugation should be used. What is more, not all the methods of harvesting are suitable for all microalgae species e.g. filtration only for large microalgae (*Scenedesmus platensis*), but centrifugation can be used for any type of microalgae. Moreover, maintenance of centrifugation devices is easy, especially cleaning and sterilization [93]. Other basic criterion is its potential to adjust the biomass to the subsequent process, thus adjustment of density or proper moisture [93,94]. To achieve the optimal parameters of biomass, methods of harvesting should be combined, e.g. pre-concentration (mechanical dewatering-centrifugation) and then post-concentration (screw centrifugation or thermal drying). Nevertheless, the choice of proper method or methods of biomass harvesting should be also adjusted to the economic requirements [93,94]. In this paper only methods used for harvesting biomass dedicated for anaerobic digestion were mentioned. The information about harvesting, thickening and dewatering of microalgae biomass suitable for research scale (pilot and laboratory scale) and industrial scale were reviewed by Pahl et al. [95].

5. Industrial applications

High potential of microalgae made them desirable substrate for several commercial applications: livestock feed as a source of protein for fish farming, feeding of cattle, pigs and poultry [5,104]; chlorophyll for cosmetic purposes [105]; bioactive compounds like antioxidants, antibiotics, toxins for pharmaceutical industry [106] and in health industry for weight control [107] as well as (due to high contents of vitamins, polysaccharides and proteins) as a nutrient supplement for human consumption [108]. In addition, they are used as fertilizers or even pigments [109]. Nowadays the potential of microalgae is also expanding into combining them with biofuels industry.

To produce biofuels from microalgae, the biomass need to be transformed by biochemical or thermochemical conversion processes [110,111]. Through the thermochemical methods such as gasification, pyrolysis, hydrogenation and liquefaction, gaseous or liquid biofuels could be generated [112–114]. Alternatively, microalgal biomass could be used in combined heat and power (CHP) facilities to generate electricity [115]. The biochemical approach consists of the fermentation and anaerobic digestion processes. By biochemical transformation, microalgae could be converted into ethanol, methane, hydrogen, SNG (synthetic natural gas), alcohols and alkanes [6,10,112]. The major chemical process is the extraction and transesterification of lipids for biodiesel production [26,116]. Other chemicals, which can be obtained from microalgae biomass, are: alkanes, butanol and acetone [117–119]. A microalgae biomass value production chain is shown in Fig. 1. The anaerobic digestion of microalgae will be described in details below.

6. Anaerobic digestion

Anaerobic digestion (AD) is a biochemical process, where specialized anaerobic bacteria decompose organic matter and produce biogas, which contains methane (55–75%) and carbon dioxide (25–45%) [35]. This process consists of multiple steps [120]: hydrolysis, fermentation, acetogenesis and methanogenesis [121]. The AD of microalgae was firstly studied by Golueke et al. [122]. In latter studies Golueke and Oswald [123] proposed a completely closed system of biological conversion of light energy to chemical energy, that consisted of three main parts: microalgae biomass cultivation, aerobic bacterial growth unit and anaerobic digester. Obtained results indicated that it was possible to convert light energy into chemical energy in short time together with liquid recovery and maintenance of gaseous environment for a longer period of time. However it is worth to underline, that microalgae biomass as a fuel source has been proposed by Meier already in 1955 [124]. The biogas/methane yields obtained from microalgae are listed in Table 1.

Main benefit of the microalgae AD is much higher energy efficiency in comparison to biofuels briefly described above. It is mainly due to the fact that oil and lipids extraction is not required [4] and the main product – methane – is captured in the gaseous phase. During the methanogenic fermentation all of the macromolecules (proteins, lipids and sugars - all parts of the microalgae structure) are utilized [87]. Additionally, the nutrients such as organic nitrogen or phosphorus could be mineralized and later recycled for algae cultivation [125]. Produced biomethane could be burnt in CHP unit to produce heat and electricity or it could be upgraded and injected into natural gas grid or used as a car fuel [8,26,97,121]. Both, raw microalgae biomass and residuals from other biofuels production could be utilized as substrates for the AD process. Using residuals would bring one more benefit, i.e. it would help to reduce the amounts of algal waste and the requirement for its landfilling [89,126].

The methane yield depends on the specie of microalgae, the pretreatment of algae biomass (due to the impact of algae cell wall structure on the volume of produced biomethane) [34] and on the presence or absence of the inhibitors of methanogenesis [127]. The main drawbacks of AD of single celled species (e.g. *Chlorella*) are economic and energetic costs of algae biomass cultivation and harvesting [125]. Nevertheless, applying wastewater as nutrient source for microalgae cultivation could drive the costs down [128]. It is worth to highlight that the residuals after the AD of microalgae could be further utilized as natural fertilizer [35,40].

The AD process is conditioned by a number of factors, which are: organic loading, retention time, temperature, pH level, quality of the substrates (the cell wall characteristic and substrate pretreatment) and the presence of methanogenesis inhibitors [6,33,35,129]. All of those are described below.

6.1. Organic loading and retention times

The high rate of organic compounds increases the methane yield, similarly to long solid retention time [42,130]. In a conventional biogas plant the hydraulic retention time (HRT) is about 30–50 days [129]. The closer examination showed that in microalgae AD in the laboratory scale, the HRT range should be between 15 [131] and 28 days [43] or even 30 days [121].

6.2. Temperature

The temperature range includes mesophilic (30–38°C) and thermophilic (50–55°C) conditions. However, the production of methane from microalgal biomass in AD process can be enhanced by increasing the temperature process [33], due to reduction of microalgae photosynthesis activity together with temperature increase. Results of previous work by Foree and McCarty [132] concluded that the increase in the rate of stabilization and utilization rate of the volatile fatty acids by methane bacteria occurred with increase of temperature (from 15°C to 25°C) . On the other hand, the results obtained by Kinnunen et al. [91] indicated that the efficiency of microalgae biomass digestibility was similar at 20°C (SRT=128 d) and ambient temperature (SRT=91 d)

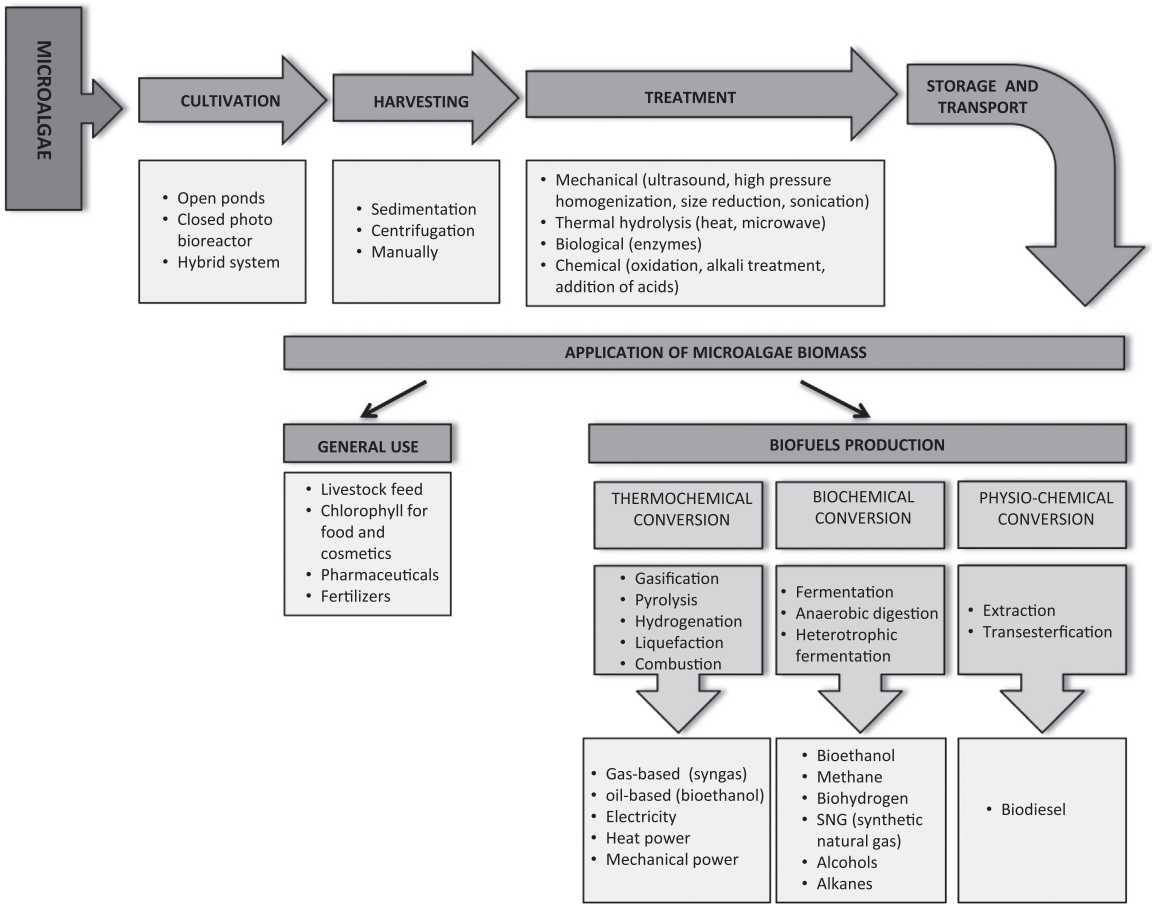

Fig. 1. Microalgae process value chain.

(unless it dropped below 16°C).

6.3. pH level

The optimum pH level for methanogenesis is between 6.5 and 8.5 [133]. The work of Wang et al. [100] indicated that the final pH of microalgae digested alone was 6.5 and co-digested with waste activated sludge was between 6.8 and 7.0. Other study [35] confirmed that the final pH values of digested microalgae ranged from 6.94 to 7.28. Kinnunen et al. [91] reported the pH of raw microalgal biomass between 6.4 and 6.5. Other noticed pH values were 7.5 [99] or 8.2 [134]. In many examples, pH values were adjusted to values between 7 and 8 [4,86,102,135] to meet optimum conditions for methanogenesis.

6.4. Inhibitory influence of ammonia and C/N ratio

The potential inhibitor that could decrease the rate of methanogenesis is a high concentration of ammonia (NH_4) dissolved during the degradation of proteins [136,137]. To increase to low algae C/N ratio and simultaneously to reduce the inhibitory effect of ammonia, the carbon rich substrates should be added to the digesters. Such substances could be primary sludge, secondary sludge, oil-greases, waste paper and various food wastes [19,100,138]. The optimum C/N ratio AD process is between 26 and 31 [139].

6.5. Inoculum to substrate ratio

Another factor which has an influence on the methane yield in microalgae AD is inoculum to substrate ratio (ISR). According to work by Zeng et al. [140] the examined ISR value decreased from 2.0 to 0.5 and obtained methane yield decreased from maximum 140.48 to 94.42 mL/g VS. The highest methane yield calculated from Ørskov equation was 153.66 mL/g VS and it was obtained for ISR value of 1.0. Zhao et al. [36] confirmed that during the biomethane potential test the viable ISR was 1.0 (VS/VS). It was possible to overcome the drop of pH and inhibition of long-chain fatty acids, consequently the specific methane productivity and the VS reduction were more effective.

6.6. Other factors

Besides aforementioned factors, there are other ones, which might affect the methane production. The most important among the operational parameters are: nitrogen deficiency as well as harvesting and storage of microalgal biomass.

The nitrogen deficiency occurred during the microalgae cultivation caused increased production of intracellular lipids [141]. Starvation strategy resulted in decreasing of microalgae productivity and inhibited the digestibility by two different ways. The first one was connected to the change in cell morphology i.e. storing of lipids, proteins and carbohydrates inside the cell, thereby the cell volume and wall thickening was increasing [142,143]. The second type of observed behavior was the secretion of exudates. These substances could accumulate in the external part of cell wall and protect it against enzymes [144,145].

Time of microalgae harvesting is also important for the AD process since it affects the distribution of intracellular macromolecules changes during the microalgae growth. Therefore, the microalgae biomass, harvested in appropriate stage of growth, contains prevalence one or other intracellular macromolecule [146,147]. The other hypothesis is that cysts formed during the maturation could hinder the algae digestibility [148]. On the other hand, the harvesting methods (centrifugation, filtration or flocculation) had no influence on macromole-

cular distribution [149]. Moreover, due to application of specific operating conditions it was possible to achieve higher concentration of favorable macromolecule [33].

The techniques of storage have strong impact on biochemical composition of microalgae. Several authors have mentioned that storage temperature influenced the quality of microalgae e.g. during freezing the content of carbohydrates and proteins decrease [150], in temperature range of 40 °C to 60 °C the macromolecular distribution also changed [151]. The decrease in content of organic compounds was observed due to bacterial degradation, chemical oxidation or the presence of the protease enzymes [152,153].

7. Microalgae pretreatment

The quality of the microalgal biomass depends on the cell wall digestibility, which could be enhanced by physical, chemical or biological pre-treatment [34]. The cell wall resistance to degrading bacterial is dependent on the content of the cellulose, hemicellulose and hardly biodegradable biopolymers. In order to increase the digestibility of the microalgae cell wall (hence boost methane potential), the microalgae biomass need to be pretreated prior the process [99]. State of the art pre-treatment methods include: mechanical (ultrasounds [98], high pressure homogenization [17], size reduction and sonication [90]), thermal hydrolysis (heat, microwave) [98], biological (enzymes) [90,98] and chemical (oxidation, alkali treatments [98], addition of acids [104], ionic liquids [154]). Summary of different methods for microalgae pretreatment regarding biogas/biomethane production is shown in Table 2. This table includes microalgae strain, applied pretreatment method, operating conditions and enhancement of methane yield or biogas potential.

7.1. Mechanical pretreatment

The size reduction consists mainly of cutting and blending [90]. For the sonication process, the low frequency ultrasound horn was used [90] which resulted in higher supplied energy than in e.g. thermal treatment [4]. The performed studies indicated that to improve the microalgae biomass digestibility, the size reduction should be carried on in combination with other pretreatment method e.g. size reduction with homogenization and ultrasounds resulted in methane yield increase by range of 82–106% [89]. On the other hand the work by Park et al. [98] indicated that ultrasound pretreatment could increase the methane yield up to 91%. The quantum energy of microwave radiation is not able to break down the chemical bonds [155], but the induction heating and dielectric polarization could cause the cell hydrolysis by changing the structure of proteins in the cell wall [99]. The microwave pretreatment of microalgae depends on the microwave frequency, time of radiation, concentration of biomass, and depth of penetration [156]. The main disadvantage of this method is its high energy consumption. Improvement of biogas production reached 79% [99]. Mechanical pretreatment methods are mainly applied prior biodiesel production [157–160].

7.2. Thermal hydrolysis

According to Mendez et al. [87] the impact of heating time on the thermal pretreatment was negligible, when it was compared with applied temperature. The heat is typically provided by water baths, autoclaves or heat chambers. This method is said to give the best results in microalgae pretreatment [17,97]. The results achieved by González-Fernández et al. [4] confirmed that, the thermal pretreatment resulted in almost 123% increase in methane yield. The other kind of thermal treatment of algae biomass is freezing and thawing. It improved methane/biogas production, but not as much as heating [91]. The thermal processes have high energy demands [161], hence some authors combined it with AD in low temperatures (below 20°C) in order to effectively decrease input energy [162].

7.3. Biological pretreatment

Biological methods are sensitive for any process disturbances, hence precise process control might be crucial [163,164]. For instance, inappropriate pH level and too high temperature could deactivate the enzymes leading to the denaturalization of proteins [165]. Other important factor is the enzyme/microalgae ratio. The high loading (% w/w dry mass) of microalgae may cause a high viscosity, because of insoluble matter releasing. The effect might be similar to the one occurring during the enzymatic hydrolysis of lignocellulosic biomass: viscosity is increasing which results in decreasing the activity of enzymes [166]. The most popular studied enzymes are cellulases [167–169] because the cellulose is present in cell walls of most microalgae e.g. *Chlorella sorokiniana, Chloroccum* sp., *Chloerlla pyrenoidosa* [33]. Fu et al. [170] used the cellulase enzyme to hydrolyze *Chlorella pyrenoidosa* biomass, the obtained conversion range was 62%. Additionally, the lipid extraction efficiency increased rapidly from 32% to 56% due to cell wall disruption. The main drawback of the enzymatic pretreatment is the high cost of enzymes. In the technical scale the costs are increasing relatively with the amounts of digested biomass.

7.4. Chemical pretreatment

So far, the chemical methods were mostly applied prior bioethanol or biohydrogen production [35,171–173]. The chemical pretreatment consists of addition of acids (most popular is H_2SO_4) or alkaline (e.g. NaOH). If temperature is elevated, then it is often referred to as thermo-chemical pretreatment. Similarly to pretreatment of lignocellulosic biomass, the addition of acid or base in high temperature to the microlalgal biomass results in the organic compound release. According to work by Nguyen et al. [172] and Harun and Danquah [171] addition of sulphuric acid to algae biomass (*Chlamydomonas reinhardtii* and *Chlorococcum humicola,* respectively) enhanced the bioethanol production 2-fold and 4.5-fold, respectively. The improvement was similar with the addition of NaOH to algae biomass (*Chlorococcum infusionum*) [35]. Alkali pretreatment of *Scenedesmus* biomass also resulted in significant enhancement of efficiency in case of biohydrogen production (3-fold) [173]. Other authors reported improved methane yield during anaerobic digestion, for instance Penaud et al. [174] indicated that addition of NaOH improve the chemical oxygen demand (COD) solubilization and the total solids (TS) elimination rate. Sukias and Craggs [104] achieved over 3-fold increase in methane yield by acid addition.

7.5. Ionic liquids

New chemical “green” solvents [175,176] such as ionic liquids (ILs) also have been applied for microalgae pre-treatment [154]. Ionic liquids are organic salts composed of organic cations and organic or inorganic anions. The major advantage is the ability to dissolve a wide range of organic and inorganic compounds. Additionally, ILs are recyclable thereby the utilization requires low equipment and energy costs however high recycling rate is necessary due to their high costs [177]. According to Zhou et al. [178] the ionic liquids-based chemical hydrolysis could dissolve over 75% of *Chlorella* biomass and simultaneously release over 88% of total sugars contained. However, the results obtained by Fujita et al. [179] indicated that wet and saliferous marine microalgae could be completely dissolved by addition of polar ionic liquids. Obtained results confirmed that ILs could enhance the biodegradability of algae cells (consist of sugars) by removing the cellulose and simultaneously increasing the susceptibility to cellulase enzymes. Nevertheless, application of ILs as a method for microalgae pretreatment need further studies e.g. ecological impact, effective

Table 2
The review of different methods of microalgae pretreatment for biogas production.

Strain	Reactor type & conditions	Method of pretreatment	Operating conditions	Biogas/methane production		Ref.
				before pretreatment (raw material)	after pretreatment	
Scenedesmus sp.	33 days (35 °C, batch reactor	Thermal	70 °C, 15 min in water bath 90 °C, 15 min in water bath	0.076 L CH_4/g COD	0.085 L CH_4/g COD 0.170 L CH_4/g COD	[4]
Nannochloropsis salina	40 days (38 °C, batch test)	Freezing overnight Thermal Microwave heating Ultrasound French press (high pressure homogenization)	−15 °C, overnight 100 °C, 8 h 5x until boiling at 600 W, 2450 MHz 3×45 s at 200 W, 30 kHz 2×10 MPa	0.347 L biogas/g VS[a)]	0.233 L biogas/g VS[a)] 0.549 L biogas/g VS[a)] 0.487 L biogas/g VS[a)] 0.274 L biogas/g VS[a)] 0.460 L biogas/g VS[a)]	[17]
Chlamydomonas reinhardtii	32 days (38 °C, batch test)	Drying	105 °C (24 h)	0.587 L biogas/gVS (66% CH_4) 0.387 L CH_4/g VS[b)]	0.464 L biogas/gVS (0.306 L CH_4/gVS)[b)]	[34]
Chlorella kessleri				0.335 L biogas/gVS (65% CH_4) 0.218 L CH_4/g VS[b)]	0.258 L biogas/gVS (0.168 L CH_4/gVS)[b)]	
Scenedesmus obliquus				0.287 L biogas/gVS (62% CH_4) 0.178 L CH_4/g VS[b)]	0.0574 L biogas/gVS (0.0353 L CH_4/gVS)[b)]	
Algae biomass: *Stigeoclonium sp., Monorraphidium sp., Nitzchia sp., Amphora sp.,*	15 days HRT (37 °C, continuous test)	Thermal	95 °C, t=10 h	0.10 L CH_4/gVS	0.12 L CH_4/gVS	[38]
	20 days HRT (37 °C, continuous test)		95 °C, t=10 h	0.18 L CH_4/gVS	0.31 LCH_4/gVS	
	20 days HRT (37 °C, continuous test		75 °C, t=10 h		0.30 L CH_4/gVS	
Spirulina maxima	60 days (35 °C, semi-continuous test)	Ultrasound Thermochemical[c)]	10 min (Polytron Generator) 50 °C (1 h in water bath) 100 °C (1 h in water bath) 150 °C (1 h in steam sterilizer)	0.19 L CH_4/g VS	0.17 L CH_4/g VS 0.21 L CH_4/g VS[d)] 0.22 L CH_4/g VS[d)] 0.24 L CH_4/g VS[d)]	[44]
Scenedesmus sp.	34 days (35 °C, batch test)	Thermal Ultrasound	70 °C, 25 min 80 °C, 25 min 130 MJ/kg, 30 min	0.082 L CH_4/g COD	0.089 L CH_4/g COD 0.129 L CH_4/g COD 0.154 L CH_4/g COD	[86]
Chlorella vulgaris	29 days (35 °C, batch test)	High pressure heating	140 °C, (10 and 20 min 3 bar) 160 °C, (10 and 20 min, 6 bar) 180 °C, (10 and 20 min 10 bar)	0.156 L CH_4/g COD (0.091 L CH_4/g VS[m)b)]	0.226 L CH_4/g COD[e)] (0.131 L CH_4/g VS[m)b)] 0.259 L CH_4/g COD[e)] (0.151 L CH_4/g VS[m)b)] 0.232 L CH_4/g COD[e)] (0.135 L CH_4/g VS[m)b)]	[87]
H.reticulatum (filamentous alga)	45 days (35 °C, batch test)	Size reduction (ultrasound homogenization), ultrasound	50–5 000 J/mL	0.170 L CH_4/g VS	0.310–0.350 L CH_4/g VS	[89]
Rhizoclonium (filamentous alga	28 days (53 °C, batch reactors)	Size reduction (cutting up) Size reduction (blending) Sonication Enzymes	5 cm, 1 cm < 0.1 mm 20 kHz, 10 min Combination of: lipase, xylanase, α-amylase, protease, cellulose	0.23–0.24 L CH_4/g TS digested	0.093–0.100 L CH_4/g TS 0.100–0.113 L CH_4/g TS 0.113–0.127 L CH_4/g TS 0.143 L CH_4/g TS	[90]
Algae biomass: *Pediastrum* sp., *Micractinum* sp. *Scenedesmus* sp.	91 (130) days SRT (20 °C, batch test, AVR)[f)]	Thermal	−20 °C, 24 h, and meltdown in room temperature (20 °C) 57 °C, 3.8 h	0.103 L CH_4/g VS_a	0.155 L CH_4/g VS_a 0.136 L CH_4/g VS_a	[91]
	CSTR (37 °C, HRT 14–16 days)		−20 °C, 24 h, and meltdown in room temperature (20 °C)	0.179 L CH_4/g VS_a	0.205 L CH_4/g VS_a	
Microalgal biomass	HRT 15 (35 °C, batch test)	Microwave	900 W, t=3 min, 110,200 kJ/kgVS	0.13 L CH_4/gVS	0.17 L CH_4/gVS	[92]
	20 days (35 °C, batch test)			0.17 L CH_4/gVS	0.27 L CH_4/gVS	
Algae biomass	28 days (38 °C), batch reactor)	Thermal	100 °C for 8 h,	0.34 g MSGP g/L[n]	0.45 g MSGP g/L[n]	[97]

(continued on next page)

Table 2 *(continued)*

Strain	Reactor type & conditions	Method of pretreatment	Operating conditions	Biogas/methane production		Ref.
				before pretreatment (raw material)	after pretreatment	
Chlorella vulgaris	25 days (35 °C, batch test)	Ultrasound	200 J/mL	0.230 L CH_4/g VS (algae with sewage sludge)	0.440 L CH_4/g VS[g)]	[98]
Algae biomass: *Scenedesmus obliquus, Chlorella vulgaris*	46 days (35 °C, batch test)	Microwave	21 800 kJ/kg TS[h)]	0.172 L biogas/g VS (68.2% CH_4) 0.117 L CH_4/g VS	0.2120 L biogas/g VS (68.5% CH_4)[i)] (0.14 L CH_4/gVS)[b)]	[99]
			43 600 kJ/kg TS[h)]		0.245 L biogas/g VS (68.4% CH_4)[i)] (0.16 L CH_4/g VS)[b)]	
			65 400 kJ/kg TS[h)]		0.307 L biogas/g VS (68.4% CH_4)[i)] (0.21 L CH_4/g VS)[b)]	
Chlorella vulgaris	3 weeks (23 days) (35 °C, batch test)	Thermal with enzymes	75 °C, t–30 min[j] (T)	0.191 L CH_4/gCOD	0.191 L CH_4/gCOD	[101]
			Carbohydralase 0.3 mL/gDW, pH=5.5, water bath (50 °C, t=5 h), 75 °C t=15 min (V)		0.217 L CH_4/gCOD	
			Protease 0.2 mL/gDW, pH=8, water bath (50 °C, t=5 h), 75 °C t=15 min (A)		0.287 L CH_4/gCOD	
			75 °C, t–30 min; Protease 0.2 mL/gDW, pH=8, water bath (50 °C, t=5 h), 75 °C t=15 min (T+A)		0.271 L CH_4/gCOD	
			Carbohydralase 0.3 mL/gDW, pH=5.5, water bath (50 °C, t=3 h) and Protease 0.2 mL/gDW, , water bath (50 °C, t=2 h), 75 °C t=15 min (V+A)		~0.287 L CH_4/gCOD	
			75 °C, t–30 min; Carbohydralase 0.3 mL/gDW, pH=5.5, water bath (50 °C, t=3 h) and Protease 0.2 mL/gDW; water bath (50 °C, t=2 h), 75 °C t=15 min (T+V +A)		0.300 L CH_4/gCOD	
Chlamydomonas reinhardtii			Carbohydralase 0.3 mL/gDW, pH=5.5, water bath (50 °C, t=5 h), 75 °C t=15 min (V)	0.263 L CH_4/gCOD	0.256 L CH_4/gCOD	
			75 °C, t–30 min; Carbohydralase 0.3 mL/gDW, pH=5.5, water bath (50 °C, t=5 h), 75 °C t=15 min (T +V)		0.279 L CH_4/gCOD	
			Protease 0.2 mL/gDW, pH=8, water bath (50 °C, t=5 h), 75 °C t=15 min (A)		0.289 L CH_4/gCOD	
			Carbohydralase 0.3 mL/gDW, pH=5.5, water bath (50 °C, t=3 h) and Protease 0.2 mL/gDW, , water bath (50 °C, t=2 h), 75 °C t=15 min (V+A)		0.312 L CH_4/gCOD	
			75 °C, t–30 min; Carbohydralase 0.3 mL/gDW, pH=5.5, water bath (50 °C, t=3 h) and Protease 0.2 mL/gDW; water bath (50 °C, t=2 h), 75 °C t=15 min (T+V +A)		~0.340 L CH_4/gCOD	
Chlorella vulgaris	30 days (35 °C, batch test)	Thermochemical	120 °C, t=20 min	0.139 mLCH_4/gCOD_{in}	0.180 L CH_4/gCOD_{in}	[102]
			120 °C, t=40 min		0.268 L CH_4/gCOD_{in}	
			120 °C, t=40 min+4 M H_2SO_4 in pH=2		0.229 L CH_4/gCOD_{in}	
			120 °C, t=40 min+4 M NaOH in pH=10		0.241 L CH_4/gCOD_{in}	
			4 M H_2SO_4 in pH=2		0.113 L CH_4/gCOD_{in}	
			4 M NaOH in pH=10		0.120 L CH_4/gCOD_{in}	
Isochrysis galbana	15 days (30 °C, batch test)	Mechanical	Stirring with 1 g of glass beads, t=1 min	0.022 L biogas (79.4% CH_4) 0.017 L CH_4/g VS[b)]	0.0127 L biogas (79.0% CH_4) (0.0100 L CH_4)[b)]	[103]
		Chemical	40 °C, 0.2%v/v acid, t=16 h		0.0260 L biogas (63.1% CH_4) (0.0164 L CH_4)[b)]	
		Thermal	60 °C, t=16 h		0.0037 L biogas (77.6% CH_4) (0.00287 L CH_4)[b)]	
		Thermal	40 °C, t=16 h		0.0030 L biogas (76.3% CH_4) (0.00229 L CH_4)[b)]	
Fresh algae solids	7 months (20 °C, semi-continuous)	Ultrasound	600 W, 90 min	0.23 L CH_4/g VS removed	0.21 L CH_4/g VS removed	[104]
Algal Settling Pond solids		Ultrasound	600 W, 90 min	0.014 m^3 CH_4/kg VS	0.016 LmCH_4/g VS removed	

(continued on next page)

Table 2 *(continued)*

Strain	Reactor type & conditions	Method of pretreatment	Operating conditions	Biogas/methane production		Ref.
				before pretreatment (raw material)	after pretreatment	
		Acid addition	HCl to pH < 2.0, after 60 min neutralization with NaOH	removed	0.046 L CH_4/g VS removed	
		Thermal	> 80 °C, 30 min		0.054 L CH_4/g VS removed	
Nanochloropsis oculata	12 days (37 °C batch test)	Thermal	90 °C, 4 h[k)]	0.28 L biogas/g VS_a° (74% CH_4) (0.21 L CH_4/g VS_a°)[b)]	0.39 L biogas/g VS_a° (74%CH_4) (0.29 L CH_4/g VS_a°)[b)]	[180]
			90 °C, 12 h[l)]	0.32 L biogas/g VS_a° (75% CH_4) (0.24 LCH_4/g VS_a°)[b)]	0.44 L biogas/g VS_a° (75.5% CH_4) (0.33 L CH_4/g VS_a°)[b)]	
Scenedesmus	35 days (38 °C, batch test)	Lipid extraction	In hexane, Soxhelt apparatus, t=6 h	0.18 L CH_4/g VS	0.33 L CH_4/g VS	[181]
		High pressure thermal hydrolysis	170 °C, 800 kPa, t=30 min		0.24 L CH_4/g VS	
		LE and HPTH	–		0.38 L CH_4/g VS	
Algae biomass A: *Chlamydomonas, Scenedesmus, Nannochloropsis.*	60 days (35 °C, batch test)	Ultrasound	10,000 kJ/kg TS	A=0.272 L CH_4/g VS_{algae}	A=0.310 L CH_4/gVS_{algae}(U1)	[182]
			27,000 kJ/kg TS	B=0.198 L CH_4/g VS_{algae}	B=0.223 L CH_4/ gVS_{algae}(U3,U4)	
Algae biomass B: *Acutodesmus obliquus, Oocystis sp., Phormidium, Nitzschia sp.*			40,000 kJ/kg TS	C=0.255 L CH_4/g VS_{algae}	C=0.314 L CH_4/gVS_{algae} (U1)	
			57,000 kJ/kg TS			
Algae biomass C: *Microspora*		Thermal hydrolysis	110 °C, 1 bar		A=0.398 L CH_4/gVS_{algae} (T3)	
			140 °C, 1.2 bar		B=0.307 L CH_4/gVS_{algae} (T3)	
			170 °C, 6.4 bar		C=0.413 L CH_4/gVS_{algae}(T1)	
			t=15 min			
		Microaerobiological	55 °C		A=0.262 LCH_4/gVS_{algae} (B1)	
			12 h		B=0.193 L CH_4/gVS_{algae} (B1)	
			24 h in the dark		C=0.266 L CH_4/gVS_{algae} (B1,B2)	
Scenedesmus	32–40 days (37 °C, batch test)	Mechanical, thermal and chemical	Extraction of aminoacids	0.140 L CH_4/gVS	0.273 L CH_4/gVS	[183]
			Extraction of lipids		0.212 L CH_4/gVS	

CSTR – continuously stirred tank reactor.
LE – Lipid extracred/
HPTH – High pressure thermal hydrolysis/
[a] Specific biogas production.
[b] Recalculated by authors.
[c] Used pH=1.0, 3.0, 11.0, 13.0.
[d] The highest increase in methane production achieved by trials with pH=11.0.
[e] The highest increase in methane production after 20 min of high pressure heating.
[f] Data from experiment 2.
[g] The highest increase in methane production achieved at an energy dose of 200 J/mL.
[h] Output power: 300 W, 600 W, 900 W.
[i] The highest increase in biogas yield achieved by trials with output energy 900 W.
[j] Thermal pretreatment before enzymatic hydrolysis.
[k] Constant time with variable temperature.
[l] Constant temperature with variable time.
[m] COD/VS=1.72 g/gAVR- accumulating-volume reactor.
[n] MSGP – Methane Specific Gas Production.
[o] VS_a – Volatile Solids added.

methods of IL recovery and IL recycling, biocompatibility or toxicity to enzymes and microorganisms [154]. Moreover, its economic effectiveness for enhancing methane potential should also be investigated.

8. Other methods for increasing biogas yield

Apart from the pretreatment methods, there are other possible ways for enhancing biogas production, that is: combining it into biorefinery or co-digesting microalgae biomass with sewage sludge or other carbon rich waste streams.

8.1. Biorefinery concepts

In the biorefineries, efficient use of microalgae could be achieved by process integration. Mussgnug et al. [34] presented one example, where obtained biogas yield increased by 123% through AD of algae biomass after biohydrogen production. In other work [184], lipid extracted biomass of *Scenedesmus* was used in two-stage process of hydrogen and methane production. The two-stage process (subsequent hydrogen and methane production) was more productive when compared with one-stage process (methane production alone). Authors explained that increase in biogas production and methane yield could be achieved by starch and lipid storage during production of biohydrogen. The second hypothesis was that strong pretreatment of biomass prior to hydrogen production was sufficient to improve methane production to the same yield without previous biohydrogen production. Ramos-Suárez and Carreras [183] indicated that methane production from *Scenedesmus* residues after extraction of amino acids and lipids was higher than from raw biomass, thus 272.8 L CH_4/kgVS, 212.3 L CH_4/kgVS and 140.3 L CH_4/kgVS, respectively. On the other hand the results obtained by Quinn et al. [96] showed that methane production from lipids extracted from algae biomass was three times lower (140 cm^3 CH_4/gVS) when compared with the whole microalgae (430 cm^3 CH_4/gVS). Those results were confirmed by work of Zhao et al. [36]. Hernández et al. [185] studied the AD of microalgae residues after lipid extraction. The improvement in methane production was observed for all tested lipid exhausted algae biomass, the highest methane production was obtained for *Tetraselmis* sp. (236 mL CH_4/gVS_{added}).

8.2. Co-digestion of microalgae

Co-digestion of microalgae with carbon rich substrates is important due to low C/N ratio of microalgae biomass (C/N = 6–9) [33,137]. Additionally, this might help to avoid ammonia inhibition effect in nitrogen rich substrates. The addition of glycerol (low price and easily available co-product of biodiesel production) to lipid-extracted *Chlorella* biomass increased the methane production marginally from 4% to 7% [186]. Further examination by Ehimen et al. [131] showed that addition of glycerol to algae biomass was favorable for carbon to nitrogen ratio (C/N=12.4) and consequently to methane yield (50% increase). Yen and Brune [137] applied waste paper to digestion of microalgae biomass consisted of *Chlorella sp.* and *Scenedesmus*. The results indicated that addition of 50% (VS basis) of waste paper doubled methane production. Authors highlighted that further addition of waste paper (75% VS basis) decreased the methane production rate because of the negative impact of low content of nitrogen and its inhibitory effect on cellulase enzymes activity. Other examined substrate was soybean oil presented by Salerno and co-workers [11]. The addition of 0.5 mL of soybean oil increased the methane yield by 95.6% and 49.7% in samples with 9 mL and 18 mL of algae biomass, respectively. The addition of maize silage increased the biogas production from 439 L/kg to 628 L/kg [187]. In work by Zhong et al. [188] the co-digestion of *Taihu* blue algae with corn straw gave the best results at a C/N ratio of 20. The methane yield increased by 62%, to 0.33 L CH_4/gVS from 0.20 L CH_4/gVS (of algae digested alone). According to Fernández-Rodríguez et al. [134] addition of olive mill solid waste (OMSW) to *Dunaliella salina* biomass in the ratio of 75% (OMSW) to 25% (*D.Salina*) resulted in the improvement in methane yield from 63 mL CH_4/gVS_{added} (raw algae biomass) to 330 mL CH_4/gVS_{added}.

Several authors highlighted that co-digestion of microalgae and waste activated sludge or sewage sludge could improve the production of biogas. Wang et al. [100] used *Chlorella* biomass to co-digest it with waste activated sludge. The highest biogas yield was obtained in samples with 41% of microalgae addition (468 mL/g VS). The biogas production was almost 2-fold higher in comparison to control samples (microalgae alone). The results of other study [189] also confirmed, that the co-digestion increased the biogas production, in this case the co-substrate was undigested wastewater sludge (a mixture of primary sludge, biosludge and chemical sludge). After 35 days of AD the biogas production increased by 12% in samples with 12% of microalgal biomass. Surprisingly with higher microalgal content (25% and 37%) the biogas production was lower when compared to samples with sludge alone. The results obtained by LeDuy and Therien [190] showed that addition of sewage sludge (50% of sludge content in mixture) improved the C/N ratio of *Spirulina maxima* and simultaneously doubled the methane production. Rusten and Sahu [7] co-digested the *Chlorella* sp. biomass and wastewater sludge (pretreated sludge liquor). The specific methane gas production (mL CH_4/g VS fed) was not increased when compared to AD of wastewater sludge alone. It achieved between 65% and 90% of specific methane gas production for sludge liquor (depending on the HRT, temperature of incubation and pretreatment of algae biomass). However, this co-digestion indicated, that tested microalgae could be cultivated in pretreated reject water and removed nitrogen and phosphorus from the sludge liquor, thereby, increasing the efficiency of the overall WWTP performance.

The co-digestion of microalgae biomass and waste activated sludge has economic and ecological advantages for WWTPs. Microalgae could effectively grow in nutrient-rich environment like wastewater and simultaneously they could utilize organic carbon and inorganic phosphorus, nitrogen and metals. Thereby both, the costs of wastewater treatment and the costs of algae cultivation may decrease [10,40,117,128,191]. It is worth mentioning that algae-based treatment has low energy requirement and it provides reduction in sludge formation [192–194]. Other advantages of algae production at WWTP are the reduction of CO_2 emission to the atmosphere. The possible ways of using of microalgae in WWTP are shown in Fig. 2.

9. Discussions

Microalgae biomass is a valuable source of many products. Nowadays the main attention is given to production of biofuels, but the scaling-up is a major bottleneck for commercialization of microalgae production for biofuels and bioenergy. The limitations and solutions are summarized in Table 3.

The first obstacle is low energy ratio – the ratio of the energy contained in microalgal oil to energy from fossil fuels required for microalgal oil recovery - which is a crucial for oil production profitability [195]. The oil production is cost-effective when the energy ratio is at least 7 [196]. The energy ratio estimated for algal diesel is only 1.4 [197]. However, it could be improved by e.g. application of used algae biomass for production of methane in the AD process, which could be further used for algae cultivation and harvesting processes, recovery of N and P fertilizers in the effluents of anaerobic digesters, and finally the minimization of input of fossil energy in biomass production, biomass recovery from water and oil extraction [195,198,199].

Other barrier is the limitation of the microalgae biomass production technologies. According to work by Weyer et al. [200] the estimated theoretical maximum productivity of algal crude oil was 354 000 L/ha-yr, but the best results came in a range from 40 700 L/ha-yr to 53 200 L/ha-yr. Microalgae growth is limited by light, thereby the existing systems of microalgae biomass cultivation and systems of lipids

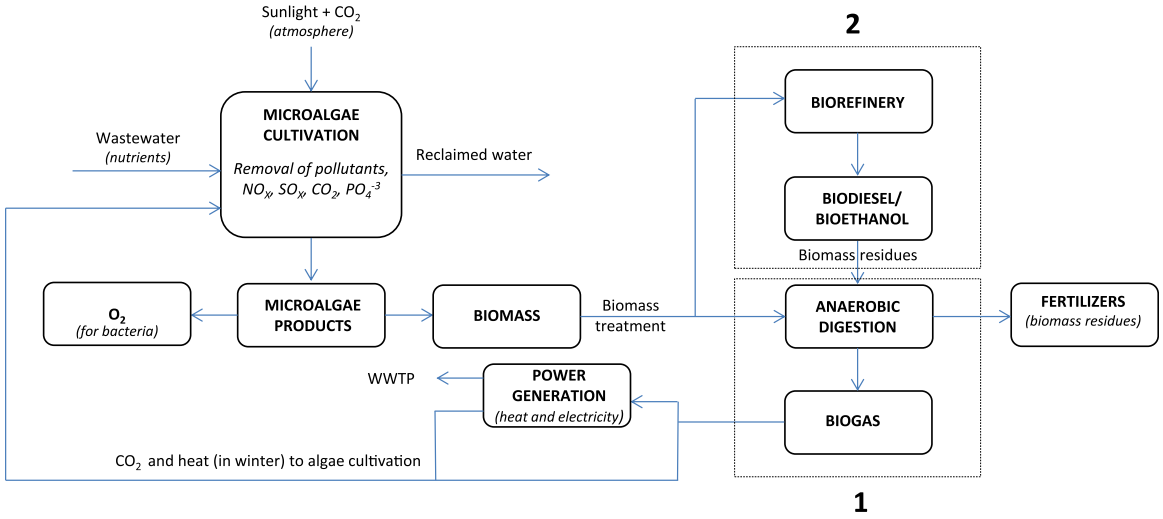

Fig. 2. Possible use of microalgae at the Wastewater Treatment Plant – 1. Anaerobic digestion, 2. Biorefinery.

extraction cannot achieve the biological limits of productivity [196]. The work by Cooney et al. [201] indicated that the content of lipids have an influence on the algae biomass productivity - it is lower when lipids content is high e.g. for 40% of lipids content by weight the theoretical algae productivity was approximately 0.095 kg/m²-d and for 60% of lipids content by dry weight it resulted in 0.080 kg/m²-d.

The last bottleneck is the cost of production. Major challenge is to develop a technology, which could provide sustainable production of large microalgae quantities with low cost at the same time. The estimation provided by Chisti [202] suggested that the production cost of algae biomass with oil content of 40% by weight should be no more than $0.25/kg. Nowadays the production cost is even 10 times higher. There are however, ways to improve it. By simplifying of the production technology and adjusting the operating scale (in that case: 200 t/year), Acién et al. [203] achieved the cost reduction from €69.00/kg dry biomass to €12.60/kg dry biomass. Likewise, the results presented by Norsker et al. [204] indicated that the optimization of production conditions could reduce the production costs from €4.95/kg to €0.68/kg. However, the cost of energy obtained from methane based on microalgae biomass was estimated to be between 0.087 and 0.170 €/kW h (microalgae cultivated with fresh water, nutrients, sunlight and in 400 ha raceway pond; biogas reactor conversion 60%) [205]. Biofuels production from microalgae still faces several difficulties and further process improvements to reduce the costs of microalgae cultivation, processing, and product purification should be done in order to make microalgae-originated products market competitive [39,196,206]. Moreover, it is important to remember that term microalgae includes many different species with very different characteristics. As pointed by Klein-Marcuschamer et al. [207] without proper characterization of the microalgae specie one cannot develop highly efficient process for biofuels production. This is crucial issue, very often omitted in research and it has to be always taken into account.

Microalgae as a sole source for AD process is not economic feasible and consequently it would not be possible to commercialize it. On the other hand, microalgal biomass is considered as a valuable feedstock

Table 3
Sum-up of the limitations and solutions for microalgae anaerobic digestion.

Limitations	Solutions	Advantages
High capital costs	Utilization of existing infrastructure	Lower investment costs, utilization of oversized equipment
High energy demand	Use of waste heat from CHP	Lower energy losses
High costs of cultivation	Use of natural sun light instead of artificial or use of LED lamps to intensify the growth of biomass	Lower energy consumption
High costs of harvesting	Immobilization of microalgae biomass prior and during cultivation;	Natural sedimentation of biomass
	Enzymatic hydrolysis to unlock biochemical molecules from complex microalgal cell wall	No need for biomass condensation/drying
Limited biomass productivity in open cultivation reactors	Cultivation in closed photo-bioreactors	Controlled conditions, prevention of contamination
High construction and operating cost of closed photo-bioreactors	Multiple usage of biomass (for other biofuels production prior anaerobic digestion)	Improved exploitation of microalgae biomass, utilization of residual biomass
	Use of high-rate ponds for microalgae cultivation	Wastewater treatment, source of nutrients for microalgae, source of dissolved hydrogen for bacteria
Low biomass concentration	Optimization of biofuel producing strains through metabolic engineering	Improved microalgal growth, maximization of cell density and lipid content in microalgal cells
Water and nutrients consuming cultivation process	Using wastewater as a growth medium and source of nutrients	Wastewater treatment, source of nutrients for microalgae, biomass residues recirculation
Methane potential is limited by the complex structure of microalgal cells	Pretreatment of microalgae biomass	Increase of solubilized compounds, enhancement of biogas productivity
	Integration into biorefinery	Co-production of biofuels and biogas, usage of waste heat for microalgal biomass pretreatment
Lower biogas yield due to high ammonia concentration during microalgae digestion	Co-digestion with substrates with high C/N ratio e.g. organic fraction of municipal solid wastes, waste biomass after biodiesel or bioethanol production, sewage sludge, manure, maize silage etc.	Organic wastes utilization, higher methane yield

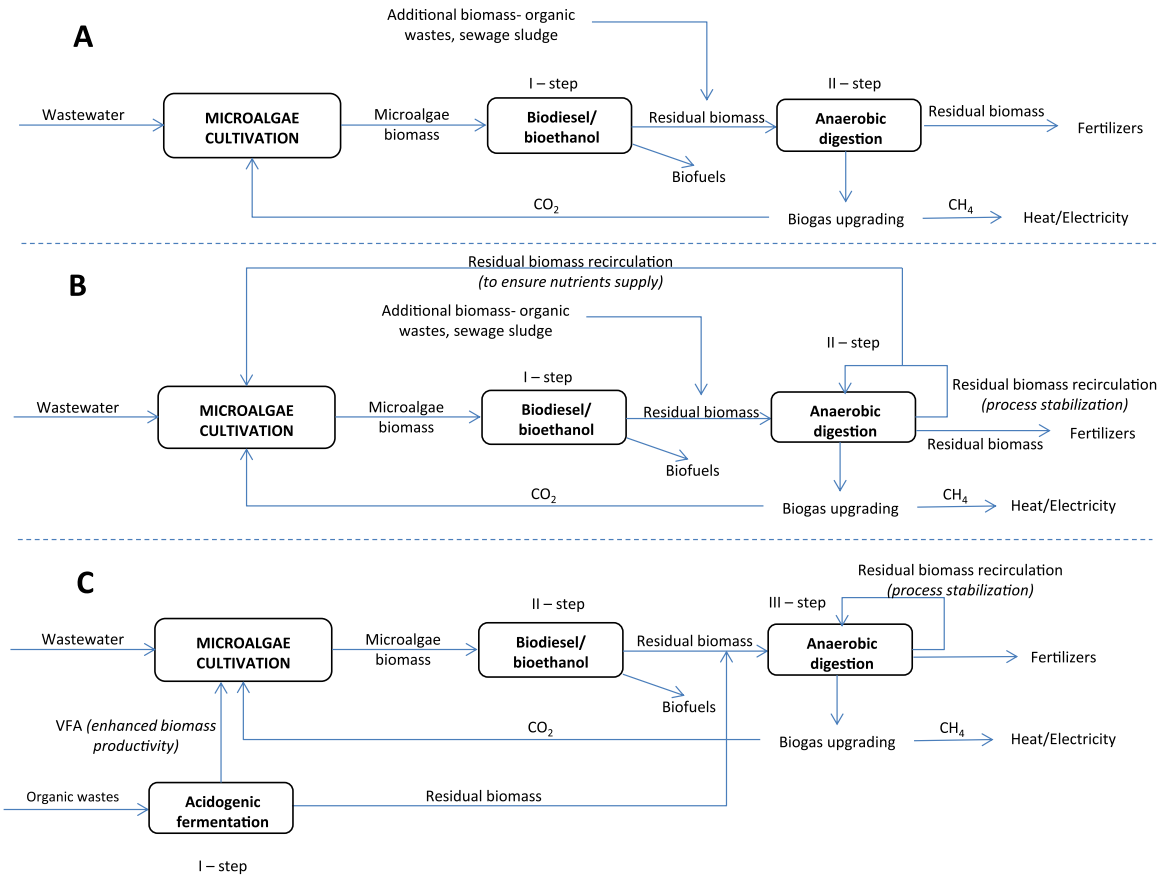

Fig. 3. Microalgae biorefinery concepts: A – two-step biorefinery for biodiesel, bioethanol and biogas production, B – two-step biorefinery with recirculation for enhanced process stability, C – three-step biorefinery with acidogenic fermentation for enhanced microalgae production.

for bioprocessing. Integration of microalgae into biorefineries seems to be a solution to overcome the aforesaid limitation and a way to improve the feasibility of the microalgae AD (Fig. 3). The primary source for microalgae cultivation should be wastewater: a source of water and nutrients for microalgae growth. At the same time it would serve as wastewater treatment. In order to boost the economic efficiency, the microalgae biomass could be used for more valuable products (for instance biodiesel or bioethanol) and only the residue biomass (with additional external organic biomass) should be directed to the AD process (Fig. 3A). The generated biogas could be upgraded to high methane content. Stripped CO_2 could be used for microalgae cultivation, whereas the heat and electricity from methane CHP unit could be utilized directly at the wastewater treatment facilities. The digestate could be both a valuable fertilizer or source of nutrient to be partly recirculated for microalgae cultivation (Fig. 3B). The recirculation would increase the process stability and enhance the overall biorefinery yields. Alternatively, the additional extremal organic biomass would be firstly directed to the acidogenic fermentation for volatile fatty acids production (Fig. 3C), which would increase the organic loading in the wastewater stream.

The goal of the review was to give a compendium of knowledge for microalgae AD. We described each of the process steps and indicated the obstacles and limitations in commercializing it and we suggested that the microalgae AD could be part of wastewater/organic-wastes based biorefinery for co-production of biofuels and energy while treating the waste streams. It would bring closer the scaling up of the microalgae processing.

10. Conclusions

Microalgae could be source of many products, biofuels being the most promising ones, however there are still several obstacles that needs to be overcome. The existing technologies are not efficient enough to gain all of the microalgae energy potential. The costs of microalgae cultivation, harvesting and processing are high which in consequence causes the price of microalgal biofuels greater than fossil fuels. The anaerobic digestion of microalgae seems to be the most promising solution mainly because the process itself is less complex, than the production of bioethanol or biodiesel. The promising solution would be to integrate the microalgae AD into a biorefinery which would not only generate valuable products but it would also treat wastewaters and organic waste streams. Microalgae could substantially reduce the C, N and P loadings and the grown biomass could be further used for biogas production. The required pretreatment of microalgae biomass could be integrated with biofuels production (e.g. biodiesel or bioethanol). Such a solution would add one more valuable stream into the biorefinery and would significantly increase the chance of the microalgae processing commercialization.

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Statement of co-authorship

I confirm participation in the following publications:

1. Ewelina Jankowska, Anna Duber, Joanna Chwiałkowska, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *Conversion of organic waste into volatile fatty acids – The influence of process operating parameters.* Chemical Engineering Journal 2018, 345:395-403.

2. Ewelina Jankowska, Ashish K. Sahu, Piotr Oleśkowicz-Popiel, *Biogas from microalgae: Review on microalgae`s cultivation, harvesting and pretreatment for anaerobic digestion.* Renewable and Sustainable Energy Reviews 2017, 75: 692-709.

3. Ewelina Jankowska, Joanna Chwiałkowska, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *Volatile fatty acids production during mixed culture fermentation – The impact of substrate complexity and pH*. Chemical Engineering Journal 2017, 326: 901-910.

4. Ewelina Jankowska, Joanna Chwiałkowska, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *Effect of pH and retention time on volatile fatty acids during mixed culture fermentation,* Bioresource Technology 2015, 190: 274-280.

5. Piotr Oleśkowicz-Popiel, Ewelina Jankowska, *Application of fermentation process in biorefineries.* Przemysł Chemiczny 2014, 93 (3): 351-354.

My participation in publications 1 is estimated at **4%** and includes scientific support and sustentative care.

My participation in publications 2 and 3 is estimated at **10%** and includes scientific support and substantive care.

My participation in publications 4 is estimated at **15%** and includes scientific support and substantive care.

My participation in publications 5 and is estimated at **50%** and includes data analyzing, paper preparation, scientific support and sustentative care.

P.O. Mgr

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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:

1. Katarzyna Antecka, Jakub Zdrada, Katarzyna Swiwińska-Stefańska, Grzegorz Sztuk, **Ewelina Jankowska**, Piotr Oleśkowicz-Popiel, Teofil Jesionowski, *Synergistic Degradation of Dye Wastewaters Using Binary or ternary Oxide System with Immoblilzed Laccase.* Catalysts 2018, 8 (9): 402.

2. Piotr Oleśkowicz-Popiel, **Ewelina Jankowska**, *Application of fermentation process in biorefineries.* Przemysł Chemiczny 2014, 93 (3): 351-354.

Conference presentations:

1. **Ewelina Jankowska**, Joanna Chwiałkowska, Anna Duber, Piotr Oleśkowicz-Popiel, *Conversion of organic wastes and wastewaters to carboxylic acids*. Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, 16-17 September 2018, Sitges, Spain (poster).

2. **Ewelina Jankowska**, Joanna Chwiałkowska, Łukasz Jaroszyński, Bjarne Paulsrud, Beata Szatkowska, Piotr Oleśkowicz-Popiel, *Microalgae biomass for wastewater treatment and volatile fatty acids production in the mixed culture fermentation.* 1st IWA Conference on Algal Technologies for Wastewater Treatment and Resource Recovery, 16-17 March 2017, Delft, The Netherlands (poster).

3. **Ewelina Jankowska**, Beata Szatkowska, Łukasz Jaroszyński, Grzegorz Cema, Agata Karło, Aleksandra Ziembińska-Buczyńska, Joanna Surmacz-Górska, Bjarne Paulsrud, Piotr Oleśkowicz-Popiel, *Produkcja biogazu z mikroglonów kultywowanych na odcieku z procesu ANAMMOX*. HydroMicro, 13-16 September 2015, Gliwice, Poland (oral presentation).

4. **Ewelina Jankowska**, Joanna Chwiałkowska, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *The influence of external factors on mixed culture fermentation of primary and waste activated sludge*. IWA Balkan Young Water Professionals, 10-12 May 2015, Thessaloniki, Greece (oral presentation).

5. **Ewelina Jankowska**, Beata Szatkowska, Łukasz Jaroszyński, Grzegorz Cema, Agata Karło, Aleksandra Ziembińska-Buczyńska, Joanna Surmacz-Górska, Bjarne Paulsrud, Piotr Oleśkowicz-Popiel, *Produkcja biogazu z mikroalg uprawianych na ścieku*. Budmika Ogólnopolska Studencka Konferencja Budowlana, 22-24 April, 2015, Poznań, Poland (oral presentation).

6. **Ewelina Jankowska**, Joanna Chwiałkowska, Piotr Oleśkowicz-Popiel, *Platforma karboksylowa do produkowania biochemikaliów i biopaliw z odpadów i ścieków.* Wpływ Młodych Naukowców na Osiągnięcia Polskiej Nauki, 3 November 2013, Poznan, Poland (oral presentation).

Conference co-authorship (presenter is underlined):

1. Ewelina Jankowska, Piotr Oleśkowicz-Popiel, Łukasz Jaroszyński, Grzegorz Cema, Agata Karło, Aleksandra Ziembińska-Buczyńska, Joanna Surmacz-Górska, Bjarne Paulsrud, Beata Szatkowska, *Integration of microalgae biomass for sustainable wastewater treatment plant operation.* 3rd Specialized International Conference Ecotechnologies for Wastewater Treatment, 27-30 June 2016, Cambridge, UK (poster).

2. Ewelina Jankowska, Joanna Chwiałkowska, Anna Janicka, Agnieszka Szuster-Janiaczyk, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *Volatile fatty acids production - effect of pH, retention time and substrate type.* XIV World Conference on Anaerobic Digestion, 15-18 November 2015, Vina del Mar, Chile (oral presentation).

3. Ewelina Jankowska, Joanna Chwiałkowska, Mikołaj Stodolny, Piotr Oleskowicz-Popiel, *Impact of pH and retention time changes on the metabolites composition during anaerobic digestion*. 7th European Meeting on Chemical Industry and Environment, 10-12 June 2015, Tarragona, Spain (oral presentation).

4. Ewelina Jankowska, Beata Szatkowska, Joanna Chwiałkowska, Łukasz Jaroszyński, Grzegorz Cema, Aleksandra Ziembińska-Buczyńska, Joanna Surmacz-Górska, Bjarne Paulsrud, Piotr Oleśkowicz-Popiel, *Biogas production from microalgae grown on primary sludge and anammox effluents.* 7th European Meeting on Chemical Industry and Environment, 10-12 June 2015, Tarragona, Spain (poster).

5. Joanna Chwiałkowska, Ewelina Jankowska, Mikołaj Stodolny, Piotr Oleśkowicz Popiel, *Biologiczne przetwarzanie osadów ściekowych do lotnych kwasów tłuszczowych*. 1st International Symposium for Students and PhD Students, 21-22 May 2015, Gliwice, Poland (oral presentation).

6. Ewelina Jankowska, Joanna Chwiałkowska, Anna Janicka, Mikołaj Stodolny, Piotr Oleśkowicz-Popiel, *Impact of the external factors on the catabolic pathways in mixed-culture fermentation*. Environmental Technology for Impact conference, 29-30 April 2015, Wageningen, The Netherlands (poster, short speech).

Research projects:

1. National Science Centre Poland, Nr NCN 2012/05/D/ST8/02289, *Wpływ czynników zewnętrznych (temperatury, składu i stężenia substratów, pH, stosunku węgla do azotu) na katabolizm w procesach fermentacji przy użyciu kultur mieszanych (MixFerment)*. Piotr Oleśkowicz-Popiel - PI, Ewelina Jankowska – investigator. 02.2013-02.2016.

2. The National Centre for Research and Development, Nr NCBR 197025/37/2013, *Integrated technology for improved energy balance and reduced greenhouse gas emissions at municipal wastewater treatment plants (BARITECH), WP 3 - Enhanced biogas generation in the sludge digestion process.* Ewelina Jankowska – investigator in WP 3. 09.2013-08.2016.

Internship and Workshops:

1. Second Training School *"Microalgae processes: From fundamentals to industrial scale".* COST Action EUAlgae (European Network for Algal-Bioproducts (ES1408)), Almería, Spain, 13-15.09.2017.

2. 1st Training School *"Energy Recovery On Anaerobic Processes in Wastewater And Sludge Processing"*. COST Action Water2020 (Conceiving Wastewater Treatment in 2020: Energetic, environmental and economic challenges (ES1202)), Gliwice, Poland, 24-27.09.2013.

3. Internship in Aalborg University in Esbjerg, Esbjerg, Denmark. 07-09.2016.