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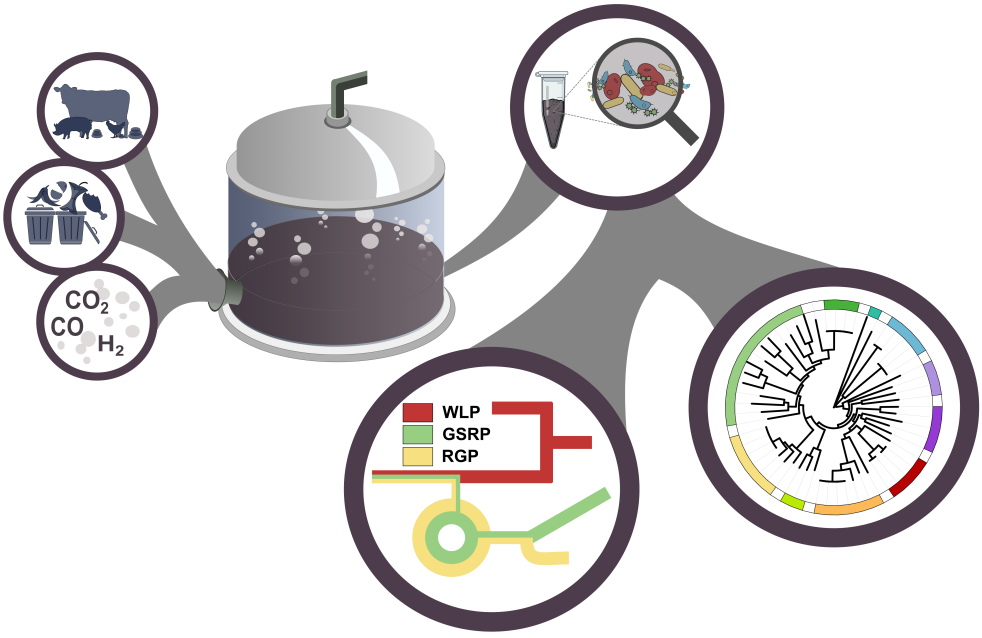
Exploration of biogas systems to unveil the acetogen community

Who are they and can we find them?

George B. Cheng



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Cover: A simple diagram of the thesis work in searching for acetogens in biogas systems (photo: George Cheng and Ebba Perman)

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Abstract

Biogas production through the anaerobic digestion of organic waste streams has great potential for reducing and replacing our dependency on fossil fuels, while also valorising organic wastes. Anaerobic digestion is a stepwise degradation process of organic material by a series of microorganisms. Among the groups of microorganisms involved, acetogens play a critical role. Their main product, acetate, is a crucial precursor for the methane producing microorganisms in the final degradation step. Acetogens are a ubiquitous group of microbes that can be found across many anoxic environments, from gastrointestinal systems to soil/sediments and biogas reactors. They have been well studied in other environments, but acetogens remain enigmatic in terms of identity and function in the biogas system. Unveiling novel acetogens and their functional pathways in such systems can provide tools for the optimisation of biogas systems. Moreover, acetogens are interesting due to their potential for use in carbon capture and biochemical production.

The aim of this thesis was to reveal knowledge on the acetogenic players responsible for the penultimate step of anaerobic digestion. Biogas environments were explored utilizing both molecular and cultivation-based methods. The investigation included 16S rRNA gene sequencing analysis, further supplemented with metagenome data analysis. The results showed the presence of several unknown/uncultured potential acetogens, supported by the possession of gene encoding for the enzymes of the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway, WLP). However, while investigating several unknown candidates, the pathway analysis revealed a lack of key WLP enzymes, such as carbon monoxide dehydrogenase and acetyl-CoA synthase, calling into question the definition of acetogens and requiring an exploration of alternative pathways.

Keywords: acetogen, biogas, anaerobic digestion, reductive acetyl-CoA pathway, Wood-Ljungdahl pathway, glycine cleavage system, glycine synthase reductase pathway, reductive glycine pathway

Utforskning av biogassystem för att avslöja acetogengemenskapen

Vilka är de och kan vi hitta dem?

Sammanfattning

Biogasproduktion genom anaerob nedbrytning (rötning) av organiskt avfall har stor potential att reducera och ersätta beroendet av fossila bränslen genom att använda avfall som en resurs. Rötning är en komplex nedbrytningsprocess där organiskt material bryts ner i flera steg genom ett samarbete av olika mikroorganismer. Bland dessa mikroorganismer spelar acetogenerna en central roll. Deras huvudsakliga produkt, acetat, är ett viktigt substrat för de metanproducerande mikroorganismerna i det sista nedbrytningssteget. Acetogener är en allmänt förekommande grupp av mikrober som kan hittas i många olika syrefattiga miljöer, från gastrointestinala system till jord/sediment och biogasreaktorer. Medan denna grupp är välstuderad i många miljöer är dess identitet och funktion i biogassystem mer okänd. En kartläggning av okända acetogener och deras metabola funktioner i sådana system kan ge värdefulla verktyg för att optimera biogasprocesser. Dessutom är acetogener av särskilt intresse med anledning av sin förmåga att fixera koldioxid och potential att användas vid tillverkning av biobaserade kemikalier.

Syftet med denna avhandling var att undersöka acetogena organismer i olika biogassystem, genom användning av både molekylära och odlingsbaserade metoder. Arbetet inkluderade isolering och karaktärisering av en acetatproducerande bakterie, analyser av 16S rRNA-gensekvenser samt metagenomik från olika biogassystem. Resultaten visade på förekomst av flera kända men också okända och ännu ej odlade potentiella acetogener, med gener som kodar för nyckelenzymer i den s k reduktiva acetyl-CoA-vägen (Wood-Ljungdahl pathway, WLP). Emellertid visade flera av dessa kandidater avsaknad av essentiella enzymer i WLP, såsom kolmonoxid-dehydrogenas och acetyl-CoA-syntetas, vilket väckte frågor om definitionen av acetogener och ledde till utforskande av alternativa reaktionsvägar.

Keywords: acetogen, biogas, anaerob nedbrytning (rötning), reductive acetyl-CoA pathway, Wood-Ljungdahl pathway, glycine cleavage system, glycine synthase reductase pathway, reductive glycine pathway

Dedication

To my family

至我的家人

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Cheng, G., Gabler, F., Pizzul, L., Olsson, H., Nordberg, Å., Schnürer, A. (2022). Microbial community development during syngas methanation in a trickle bed reactor with various nutrient sources. Applied Microbiology and Biotechnology, 106 (13), 5317-5333. doi.org/10.1007/s00253-022-12035-5

II. Cheng, G., Schnürer, A., Westerholm, M. *Microaceticoccus formicicus* gen. nov., sp. nov., an ammonia-tolerant formate utilizing bacterium originating from a biogas process. Submitted to International Journal of Systematic and Evolutionary Microbiology (Under revision)

III. Cheng, G., Bongcam-Rudloff, E., Schnürer, A. Metagenomic exploration uncovers several novel candidatus species involved in acetate metabolism in high-ammonia thermophilic biogas processes. Submitted to Microbial Biotechnology. (Manuscript)

IV. Cheng, G., Gabler, F., Nordberg, Å., Bongcam-Rudloff, E., Schnürer, A. Unveiling species involved in acetate metabolism within syngas biomethanation systems. (Manuscript)

Paper I is reproduced with the permission of the publisher.

The contribution of George B. Cheng to the papers included in this thesis was as follows:

I. Participated in the microbial laboratory work and performed bioinformatics work and analysis of the sequencing data. Main writer of the manuscript.

II. Participated in the microbial characterization laboratory work and compilation and analysis of data. Main writer of the manuscript.

III. Data compilation and performed bioinformatics work and analysis of the sequencing data. Main writer of the manuscript.

IV. Participated in the microbial laboratory work. Data compilation and performed bioinformatics work and analysis of the sequencing data. Main writer of the manuscript.

In addition to paper I-IV, George B. Cheng contributed to the following papers during the timeframe of the doctoral project, but not included in this thesis.

I. **Cheng, G**., Westerholm, M. & Schnürer, A. (2024). Complete genome sequence of Citroniella saccharovorans DSM 29873, isolated from human fecal sample. Microbiology Resource Announcements, 13 (4), e0001524. https://doi.org/10.1128/mra.00015-24

II. Gabler, F., **Cheng, G**., Janke, L., Pizzul, L., Schnürer, A. & Nordberg, Å. (Submitted). Suitability of various nutrient media for syngas biomethanation in fed-batch mode at mesophilic and thermophilic temperature.

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Abbreviations

acs$_{(r)}$	Acetyl-CoA synthase
acs$_{(o)}$	Acetyl-CoA synthetase
AD	Anaerobic digestion
AM	Acetoclastic methanogenesis
CODH	Carbon monoxide dehydrogenase
fdh	Formate dehydrogenase
fhs	Formyltetrahydrofolate synthetase
GCS	Glycine cleavage system
GSRP	Glycine synthase reductase pathway
HM	Hydrogenotrophic methanogenesis
LCFA	Long chain fatty acid
MAGs	Metagenome-assembled genomes
RGP	Reductive glycine pathway
SAO	Syntrophic acetate oxidation
SAOB	Syntrophic acetate oxidizing bacteria
TBR	Trickle bed reactor
VFA	Volatile fatty acid
WLP	Wood-Ljungdahl pathway

1. Introduction

The main instigator for the climate crisis is the release of anthropogenic carbon dioxide (CO_2) into the atmosphere, stemming from human reliance on fossil fuels for various purposes. The apparent consequences are environmental issues with increasing CO_2 emissions and the depletion of fossil fuels. There is a consensus that a crucial decision must be made either to limit the extraction of fossil fuels or to eliminate CO_2 emissions (Quadrelli et al. 2011; Gross 2014; Venkata Mohan et al. 2016). This can be achieved by using biological processes for carbon sequestration and fixation in various industries, such as the production of biobased energy or commodities (Venkata Mohan et al. 2016). The energy sector has increasing demands for energy that fulfils environmental concerns, which could be answered by using renewables and biobased energy. Global energy consumption in 2022 was 604.04 exajoules, an increase of 14.4% from 2012 (Energy Institute, 2023). Currently, the global energy supply is dominated by fossil-based energy (oil, coal, and natural gas), corresponding roughly to 80% of the total supply (Midilli & Dincer 2008; Muradov 2017; Gupta et al. 2023). Renewable energy sources, including solar, wind, geothermal and bioenergy (biomass), make up only 7% of the total supply, and the remaining 13% is supplied from nuclear energy and hydroelectricity (Solomon 2018; Sebestyén 2021; Energy Institute 2023). To reach total decarbonisation of the energy sector, the share of renewable energy sources must be increased to approximately 65% of the total energy infrastructure (Gupta et al. 2023). This can be achieved by increasing the share of bioenergy and creating a circular economy to transition from fossil-based chemical production to bio-based production (Hatti-Kaul et al. 2007; Gupta et al. 2023).

Among the bioenergy sources, biogas has proved to be a promising solution that can be utilized not only for renewable energy generation but also for valorisation of organic residues from waste streams. The primary composition of biogas is methane (CH_4) and carbon dioxide (CO_2), which is energy-rich and can be used as a replacement for fossil fuels and be implemented for electricity, heat production and vehicle fuel after removing gaseous impurities (*e.g.* hydrogen sulphide (H_2S)) (Weiland 2010; Aghel et al. 2022; Gupta et al. 2023). The biogas system is not only an energy generating process but also a waste management approach that closes the carbon cycle and recovers nutrients from industrial sectors by utilizing the digestate as bio-fertilizer during crop production (Ehmann et al. 2018). Biogas is produced via the anaerobic digestion (AD) of biodegradable material, such as household/food waste, sludge, manure, agricultural or industrial waste (Kougias & Angelidaki 2018). The anaerobic degradation of waste is mediated by a complex community of microbes that convert organic carbon through a series of oxidation and reduction reactions to the most oxidized and reduced forms of carbon, CO_2 and CH_4, respectively (Senneca 2017; Guerrero-Cruz et al. 2021). Importantly, in addition to the production of energy, the microbes within the system can also be used as cost-effective catalysts for carbon capture and utilization for bio-based commodity production (Jajesniak et al. 2014; Venkata Mohan et al. 2016). Anaerobic microorganisms provide a versatile closed loop system that can sequester organic and inorganic carbon/CO_2 and metabolize into "platform chemicals" that can be utilized as building blocks for other bioprocesses (Venkata Mohan et al. 2016). Additionally, the biogas process can be considered carbon neutral as the CO_2 generated during the production and consumption of biogas essentially poses no additional environmental concerns as it is created from the carbon pooled in biomass (Golmakani et al. 2022; Gupta et al. 2023). Each step of the AD process is crucial to the efficiency of the overall conversion. There are two groups of microorganisms of particular interest that contribute to the efficiency and stability of the process, acetogens and methanogens, both participating in the final steps of AD. Acetogens degrade various organic compounds while producing feed for the methanogens, such as acetate and H_2 (Drake 1994). While methanogens produce the most desirable product, CH_4, acetate is also an important intermediate and product as it can be used as a platform to produce other bio-based chemicals (Fast et al. 2015; Diender et al. 2021; Gong et al. 2022; Flaiz & Sousa 2024). Acetate can also be used as a feedstock for microbial cell

factories that produce different chemical and pharmaceutical compounds (Novak & Pflügl 2018). Additionally, acetogens have the capacity to utilize CO_2 and CO, which are valuable for carbon capture and utilization (Jajesniak et al. 2014). For this reason, there is presently an interest in further exploring this group of organisms, including the isolation of novel acetogens that contributes to the efficiency of the biogas process but also potentially could be used as sustainable producers for the building blocks of bio-commodities (Kutscha & Pflügl 2020; Gong et al. 2022). While acetogens have been well documented in most environments, only a few have been isolated and described from biogas processes (Drake et al. 2013; Kim et al. 2023) (Table 2). Furthermore, previous explorations into the bacterial community of biogas reactors indicate that there are many potential unknown/uncultured acetogens that remain to be uncovered (Müller et al. 2016; Singh et al. 2019; Singh 2021). Hence, uncovering novel acetogens and their pathways can aid better understanding of one phase of the process for optimization of the biogas system but are also appealing as an interesting prospect in the development process of biochemical production.

1.1 Aims of the thesis

The main aim of this thesis is to enhance the knowledge of microbial communities in biogas production systems and specifically to uncover insights into the acetate-producing/consuming bacteria communities involved in these processes. The investigation included both molecular and cultivation-based methods.

The thesis includes three different projects:

1. Exploration and scavenging of syngas biogas system for potential acetate-producers/consumers using 16S rRNA gene sequence analysis (paper **I**)
2. Unveiling potential acetogens in various biogas systems by creating metagenomic assembled genomes and screening for key enzymes of the Wood-Ljungdahl Pathway (Paper **III** and **IV**)
3. Characterization of an acetate producing novel genus (Paper **II**)

2. Microbiology of the Biogas Process

Anaerobic digestion (AD) is a microbial process that sequentially breaks down large molecules, like proteins, sugars, and fats, into methane and carbon dioxide, which is the desired biogas (Figure 1). AD is a sequence of four metabolic stages that are orchestrated by various obligate and facultative microorganisms mainly belonging to two biological kingdoms, bacteria and archaea. To achieve a stable and efficient biogas process, the group of microorganisms must work together, especially in the latter steps of the process (Angelidaki et al. 2011; Schnürer & Jarvis 2016). Many of the microbes in the final steps must function together in intricate and sometimes obligate relationships, where metabolically different organisms rely on each other to thrive and survive, for example, syntrophs (Worm et al. 2010). This consortium of microorganisms can be impacted by several factors, such as the source of inoculum and substrate, pH, temperature, and ammonia level, to name a few (Regueiro et al. 2012; De Vrieze et al. 2015; Liu et al. 2017).

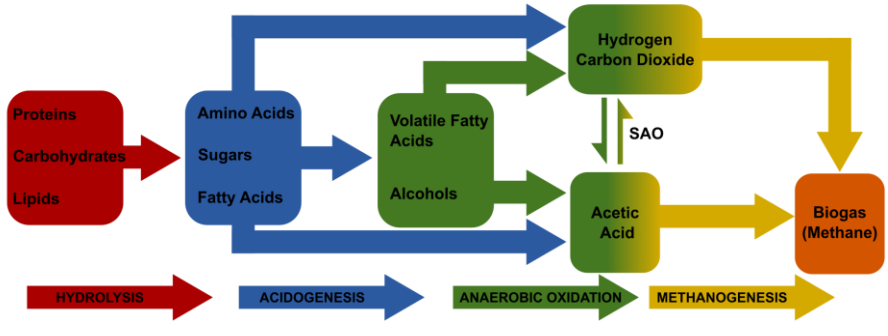

Figure 1. Steps in the anaerobic digestion process. Complex macromolecules are degraded into simpler molecules in a series of four steps: hydrolysis (red), acidogenesis (blue), anaerobic oxidation (green), and methanogenesis (yellow).

2.1 Hydrolysis and Acidogenesis

The first stage in AD is hydrolysis, an enzyme-mediated breakdown of insoluble organic polymers, such as polysaccharides, lipids, proteins, etc., into soluble derivatives (*i.e.* monosaccharides, long chain fatty acids (LCFA) amino acids and alcohols). This step is crucial for the initial breakdown of large molecules, since these would be too large to be directly utilized by microorganisms as a substrate or energy source. There are microorganisms that secrete various types of extracellular enzyme to break down the different types of macro molecules, and others that are specialized in targeting only one type of compound. Hydrolysis is one of two stages that determine the rate of the digestion process (Veeken & Hamelers 1999; Coelho et al. 2011). The rate of hydrolysis has been shown to be dependent on the composition of the biowaste, biodegradability, pH, ammonia levels and temperature (Pavlostathis & Giraldo-Gomez 1991; Veeken & Hamelers 1999; Perman et al. 2024). This step can be susceptible to inhibition from intermediary products from the subsequent steps, *e.g.*, volatile fatty acids (VFAs) (Veeken & Hamelers 1999; Siegert & Banks 2005; Cazier et al. 2015). The soluble derivatives are further utilized and transformed in the second stage, acidogenesis. This stage is typically responsible for the fermentation of monosaccharides and amino acids. The fermentation of these substrates produces various short chain organic acids (C_2-C_6), otherwise referred to as VFAs (acetic acid, butyric acid, propanoic acid, etc), alcohols, H_2 and CO_2 (Schnürer & Jarvis 2010; Angelidaki et al. 2011; Adekunle & Okolie 2015; Pan et al. 2021). Amino acid fermentation also generates ammonia and H_2S. Both hydrolysis and acidogenesis steps can be performed by a succession of microbes or at times by one and the same organism, such as some members the genus of *Defluviitoga*, which can metabolize carbohydrates into ethanol, acetate, and H_2/CO_2, providing substrates for the final step, methanogenesis (Maus et al. 2016; Kim et al. 2018; Westerholm et al. 2018). This and other bacterial species were found in the thermophilic high reactors R1 and R2 investigated in Paper III, potentially providing the necessary substrates directly to the final step.

2.2 Anaerobic Oxidation

During this third stage, acetogenesis or anaerobic oxidation, intermediary products (*i.e.* organic acids, H_2 and CO_2) from hydrolysis and acidogenesis are consumed as substrates by the next group of microorganisms. In this stage, these intermediates are converted to acetate, H_2 and CO_2. Acetate is a pivotal intermediate in this process and plays a major part in the production of methane (Aoyagi et al. 2020). Acetate metabolism in AD encompasses at least 3 reactions: acetogenesis, syntrophic acetate oxidation (SAO) and acetoclastic methanogenesis (see section 2.3). Depending on environmental conditions, acetate consumption can proceed either via SAO or acetoclastic methanogenesis (Sun et al. 2014; Westerholm et al. 2019). Both acetogenesis and SAO pathways help regulate AD through the production and consumption of acetate while competing or cooperating with methanogenesis (Pan et al. 2021).

2.2.1 Acetogenesis

Acetogenesis is performed by a group of microorganisms called acetogens. This group is composed of facultative autotrophs that can grow both as heterotrophs oxidising organic material (Table 1 reaction 2) or as chemolithotrophs, using inorganic substances (*i.e.* H_2 and CO_2) (Table 1 reaction 1) (Drake et al. 2013). Their flexible metabolism, allowing them to convert a variety of substrates to acetate, bridges the gap between fermentative bacteria and methanogens (Diekert & Wohlfarth 1994; Karekar et al. 2022). Acetogens can also utilize amino acids, and organic acids as part of acidogenesis to produce various reduced end products, like ethanol, CO, H_2, and lactate (Drake 1994; Pan et al. 2021). Having such a wide range of substrates, acetogens must compete with fermenters (Schuchmann & Müller 2016).

Acetogens have been the subject of many studies, including some within biogas processes, and have been proposed to be prevalent as key players in the AD process. Still, extensive investigation in biogas systems remains limited (Ryan et al. 2010; Kushkevych et al. 2019; Pramanik et al. 2020; Singh et al. 2020, 2021; Singh 2021). When looking for acetogens in thermophilic high ammonia biogas systems in the present thesis work, only a few acetogens were detected (Paper III). Perhaps the low number of

acetogens recovered in these reactors was the result of the extreme conditions (*e.g.* high ammonia and temperature), driving the microbial community structure (see Chapter 3). It is well known that microbial diversity, especially for the acetogenic and methanogenic community, is affected by high level of ammonia and high temperature and resulting in a lower diversity compared to low ammonia and mesophilic reactors (Levén et al. 2007; Guo et al. 2014; Westerholm et al. 2015; Braga Nan et al. 2020). However, in ex-situ syngas biomethanation systems comparably more acetogens were discovered, but in line with the results from the other thermophilic reactors, less acetogens seemed to have been enriched at thermophilic conditions compared to mesophilic conditions. Here several known acetogens, such as *Acetobacterium woodii* and *Sporomusa sphaeroides*, to name a few, were recovered (Paper IV). Additionally, there were several potential novel acetogens recovered (Paper IV).

2.2.2 Syntrophic acetate oxidation

Syntrophy is a mutualistic and interdependent relationship between two microorganisms that rely on one another for energetic and metabolic reasons; in the AD process, syntrophic organic acid oxidizing bacteria and methanogenic archaea form this syntrophic bond (Narihiro et al. 2015; Westerholm et al. 2019). Some acetogens, due to their versatile metabolism, may also be represented in this special group of syntrophic bacteria, such as, syntrophic acetate oxidizing bacteria (SAOB), which perform a thermodynamically unfavourable reaction (syntrophic acetate oxidation, SAO) at standard conditions, converting acetate to H_2/CO_2 (Table 1 reaction 4). Some of these acetogens include *Thermacetogenium phaeum* and *Schnuerera ultunensis* (Schnürer et al. 1996; Hattori et al. 2000); such a possibility makes the identification of acetogens complicated, as will be further discussed in section 4.4. SAO requires the presence of hydrogen consuming methanogens to maintain a low partial pressure of H_2 to proceed (Schink 1997; Westerholm et al. 2019). There are certain environmental conditions that can favour SAOBs, as will be discussed in Chapter 3.

Table 1 Stoichiometric reactions and change in Gibbs free energy of acetate metabolism during the final steps of anaerobic digestion.

Metabolic pathway	Reaction Equation	$\Delta G^{0'}$ (kJ)
Acetogenic		
Autotrophic/ Homoacetogenic	(1) $2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$	-104.6
Heterotrophic	(2) $C_6H_{12}O_6 \rightarrow 3CH_3COO^- + 3H^+$	-310.9
Propionate	(3) $CH_3CH_3COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2$	+76.2
SAO	(4) $CH_3COOH + 2H_2O \rightarrow 2CO_2 + 4H_2$	+104.6
Methanogenic		
Acetoclastic	(5) $CH_3COOH \rightarrow CH_4 + CO_2$	-31.0
Hydrogenotrophic	(6) $2CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-135.0

2.3 Methanogenesis

This final step is performed by a group of microorganisms belonging to the archaea domain, methanogens, producing methane and carbon dioxide from intermediate products (*i.e.* acetate, formate, H_2 and CO_2). This stage is the other rate-limiting step in the anaerobic digestion process due to the slow biochemical reaction. Methane is mainly produced, from either acetate (acetoclastic methanogenesis, AM, Table 1 reaction 5) or formate (H_2 and CO_2) (hydrogenotrophic methanogenesis, HM, Table 1 reaction 6). Occasionally, other substrates can be used, such as CO or methyl compounds, by carboxydotrophic or methylotrophic methanogenesis, respectively (Schöne & Rother 2019). However, neither of these two routes of methanogenesis are as common as AM or HM since the substrates are not as readily available (Conrad 2020). The dynamic between AM and HM is controlled by the growth rates of their respective microorganism, susceptibility to inhibitory compounds and temperature tolerances (De Vrieze et al. 2015; Wang et al. 2022). The methanogenic community typically experiences a shift from AM to HM under high ammonia stress and high temperatures (Westerholm et al. 2018; Westerholm & Schnürer 2019). AM is known to be more sensitive to the ammonia level, which is further exacerbated at higher temperatures (see section 3.3 and 3.4) (Rajagopal et al. 2013; Westerholm et al. 2016). There is a moderately ammonia-tolerant acetoclastic methanogen belonging to the genus *Methanosarcina* that can use both AM and HM that have been found in various high ammonia biogas

systems (De Vrieze et al. 2012; Lin et al. 2016; Liu et al. 2023; Paper III). The hydrogenotrophic methanogens typically enriched in thermophilic systems have a higher ammonia tolerance as compared to the acetoclastic methanogens (Angelidaki & Ahring 1993; Ho et al. 2016; Bu et al. 2018). Hydrogenotrophic methanogens are typically found partnered with SAOBs (Westerholm et al. 2012; Sun et al. 2014; Westerholm & Schnürer 2019; Paper III and IV).

3. Drivers of Microbial Communities

The variation in the microbial population responsible for the AD process can be attributed to several factors: substrate type, hydraulic retention time (HRT), organic loading rate (OLR), reactor type, temperature, pH, total ammonia, and VFA concentrations (Hori et al. 2006; Neumann & Scherer 2011; Zhang et al. 2014a; Moset et al. 2015; Westerholm & Schnürer 2019). Of these factors, the following will be touched upon below: inoculum/substrate source, pH, temperature, and total ammonia, as these were observed factors in the thesis work that seemed to have impacted the investigated acetogenic communities.

3.1 Inoculum/Substrate source

The types of organic material used in the biogas reactors can influence biogas production, the usability of the digestate and the microbial community. There are various types of substrates that could be used, spanning from dry fibrous to more wet materials, including household and industrial food waste, agricultural waste (crop residues and animal manure), industrial and municipal wastewater, and sludge and synthetic gas (syngas) generated from gasification of woody/recalcitrant materials (Schnürer & Jarvis 2016; Angelidaki et al. 2018). From a process standpoint, there are several logistical criteria that must be considered when selecting substrate source: the biomethane potential, the quality of the biofertilizer, physical accessibility, and profitability, to list a few (Feiz & Ammenberg 2017). The composition of macromolecules will be deterministic for the degradation rate as well as the methane yield; a high content of fat and protein has higher methane potential yield compared to carbohydrates (Kougias & Angelidaki 2018). The C/N ratio is the proportion of the amount of carbon and nitrogen

in the substrate, both of which are vital for microbial cell growth and function (Khanal et al. 2019; Tg et al. 2022). A substrate with a high C/N quota can be limiting for microbial growth due to a lack of nitrogen, while the opposite may result in process instability due to inhibition by ammonia (Lin & Lay 2004; Choi et al. 2020; Banerjee et al. 2022). Of equal importance to an efficient biogas process is the content of trace elements, which are typically iron, nickel, cobalt, molybdenum, selenium, and tungsten (Demirel & Scherer 2011; Fahlbusch et al. 2018; Tg et al. 2022). Trace elements are essential for the activity of various enzymes in the cell metabolism, and a deficiency in trace elements can lead to process instability and decreased methane yield (Zhang et al. 2014a; Moestedt et al. 2016). Among these elements iron, nickel, and cobalt are particularly crucial for both acetoclastic and acetogenic reactions (Glass & Orphan 2012; Choong et al. 2016; Ye et al. 2018; Mann et al. 2022). In Paper III, iron was added to all three reactors, with two of them further supplemented with additional other trace elements to help maintain process stability and boost the microbial activity. A balanced substrate, in terms of composition, C/N ratio and amount of trace elements in the substrate, is thus essential for proper growth and microbial activity.

The source of the inoculum has been shown to be important for starting the biogas system (Liu et al. 2017; Paper I). The inoculum can govern the initial microbial community, but the development of the core community is shaped by the substrates that are subsequently fed into the biogas reactors (Regueiro et al. 2012; Liu et al. 2017; Jeske & Gallert 2021). Zhang et al. (2014) compared the microbial communities from 78 different reactor samples spanning across 28 different studies and proposed that the source of the substrate drove the variation in the microbial community in the reactors. Given the similar operational conditions of the reactors, the differences observed in the microbial communities in Paper III could stem from the variation in the substrates used, suggesting that substrate type may be the primary factor influencing these community differences.

It has been shown that regardless of the starting community in the inoculum, the microbial community would “settle” into a core community based on the prevailing reactor conditions (Liu et al. 2017; Peces et al. 2018). For instance, several studies have reported well-functioning thermophilic processes that have been initiated from mesophilic inoculum, demonstrating

that the community will shift to be suitable to the environmental conditions (Kimmel et al. 1991; Grimalt-Alemany et al. 2020; Li et al. 2020b). But to avoid the time needed waiting for the community to conform to the condition, a well-adapted inoculum can be prepared to shorten the lag phase experienced at the start of a biogas process (Sposob et al. 2021). In Paper I, the inoculum was selected from a screening of three different sources. The inoculum with the highest syngas consumption and highest methane production was selected for the start-up of the reactors, assumed to be the most suitable for syngas conversion (Paper I). In these reactors the core microbial community was the same even through the operation changed over time. While some initial changes were observed in response to different nutrient medium and gas feeding rates, the core community always returned, illustrating the importance of the substrate as a driver for the community profile. This trend is in line with other microbial studies using continuously operated trickle bed reactors (TBR), which observed a complete change in syngas-enriched communities compared to the starting inoculum (Asimakopoulos et al. 2020; Grimalt-Alemany et al. 2020; Duan et al. 2021).

3.1.1 Food waste

Food waste, including slaughterhouse waste, is an attractive substrate due to the increasing amount in the world and its mixed nutrient composition and high methane yield; thus, it has been well-documented regarding the productivity, stability, and microbial communities in biogas processes (Braguglia et al. 2018; Westerholm et al. 2020; Paper III). Depending on the composition, anaerobic digestion of food waste alone may encounter process inhibitions due to the accumulation of long-chain fatty acids from fat degradation and ammonia (NH_3) and ammonium (NH_4^+) from protein degradation (Appels et al. 2008; Zhang et al. 2013; Pilarska et al. 2023; Luo & Pradhan 2024). While elevated levels of mineral nitrogen are positive for the value of the digestate as a fertilising agent (Koszel & Lorencowicz 2015; Ehmann et al. 2018), high concentrations can inhibit many microbes and lead to process inhibition (Chen et al. 2008). Potential acetogens have been found in food waste biogas reactors, primarily in mesophilic conditions (Levén et al. 2007; Li et al. 2016; Westerholm et al. 2018; Braga Nan et al. 2020; Pramanik et al. 2020). For example, Li et al. (2016) investigated the effects of overloading a mesophilic reactor with food waste, which induced the proliferation of acetogens. Unfortunately, the reactor spiralled into process failure as the methanogen failed to degrade the excessive acetate produced.

Interestingly, in the investigation of two thermophilic reactors with food waste in Paper III, only a small number of acetogens were identified, only one potential candidate, which may have been a consequence of temperature (see section 3.3). Amid the low recovery of acetogens, several novel potential SAOBs were found in thermophilic food waste reactors described in Paper III, identified based on the genomic content (see section 4.4). This is in line with a previously observed switch in metabolism from AM to SAO-HM in several food waste reactor studies (Chen et al. 2016; Westerholm et al. 2018; Pramanik et al. 2020; Li et al. 2022). As mentioned above, food waste reactors are at risk of having elevated ammonia, which could lower the abundance of acetogens (see section 3.4) (Peng et al. 2021; Wang et al. 2022).

3.1.2 Animal Manure

Manure is a bountiful and valuable substrate, with approximately 24 billion tons of animal manure being produced globally (Jain et al. 2019). If improperly managed, this can become a source of air and water contamination. However, integrating manure into a waste management chain and using it for anaerobic digestion can provide benefits such as replacing mineral fertilizers, reducing odours, limiting pathogens and reducing the greenhouse gas emissions that would otherwise be released during storage (Arthurson 2009; Holm-Nielsen et al. 2009; Chávez-Fuentes et al. 2017; Burg et al. 2018; Ahlberg-Eliasson et al. 2021; Farghali et al. 2022). As mentioned earlier, C/N ratio is important for proper microbial growth and activity; certain sources of manure, such as poultry and swine manure, contain urea, contributing to nitrogen content, which may cause ammonia inhibition just as in food waste (Bouallagui et al. 2005; Nasir et al. 2012; Abbas et al. 2023). Acetogens have been isolated from both rumen and animal faeces, so it would make sense to expect acetogens to be among the microbial community in the biogas digesters fed with manure (Rey et al. 2010; Yao et al. 2020; Wang et al. 2022; Kim et al. 2023). Indeed, acetogens have been observed in biogas reactors operating with manure, but very few have been isolated from those reactors (Siriwongrungson et al. 2007; Kushkevych et al. 2019; Singh et al. 2021; Kim et al. 2023). There seemed to be a low abundance of acetogens in the swine manure reactor in Paper III, with only one potential acetogen observed. SAOBs were seemingly more enriched. In fact, SAOBs have been observed to be present in manure

reactors, likely due to the dominant influence of the ammonia concentration (Hansen et al. 1998; Sun et al. 2014; Westerholm et al. 2016).

3.1.3 Synthetic Gas (Syngas)

A rising waste stream available for conversion comes from the gasification of recalcitrant matter into synthetic gas (syngas), and the off-gases/gaseous waste stream from industrial sectors are primarily composed of H_2, CO and CO_2, all of which can be converted by anaerobic microorganisms and incorporated within second- and third-generation biofuel technologies (Grimalt-Alemany et al. 2018; Paniagua et al. 2022). In recent years, research has been conducted on syngas conversion to methane. Syngas biomethanation presents a promising bioconversion method that combines the benefits of both thermochemical and biochemical processes (Grimalt-Alemany et al. 2018). By converting biomass into fermentable gas through gasification, it overcomes the challenge of biologically degrading complex substrates, such as agricultural and forestry residues or non-fermentable by-products from biorefineries (Grimalt-Alemany et al. 2018). One of the pertinent limitations during the syngas methanation process is the low gas-liquid mass transfer, the low solubility of the gases (H_2 and CO) in liquid, resulting in poor mass transfer into the anaerobic cells (Andreides et al. 2022; Paniagua et al. 2022). Additionally, syngas may contain impurities such as tars, H_2S, NO_x and NH_3, which may cause cell toxicity or inhibit enzymes found among the anaerobic microbes (Xu et al. 2011). Acetogens are not able to outcompete hydrogenotrophic methanogens for H_2, as they typically require a comparably higher partial pressure of H_2 (P_{H_2}) for autotrophic metabolism (Lee & Zinder 1988). Hydrogenotrophic methanogens has a lower requirement of P_{H_2} than acetogens but may have an upper limit to their H_2 consumption (c.a. 40% of total H_2) (Liu et al. 2016). The injection of exogenous H_2/CO_2 or syngas alters the levels of P_{H_2}, which increases the competitiveness of acetogens, hence likely explaining the observation of high abundances of acetogens in syngas reactors (Liu et al. 2017; Paper I and IV). However, acetogens are considered to have a flexible metabolism, which can offer many advantages for developing niche differentiation to avoid competition (Lever 2012; Schuchmann & Müller 2016). Interestingly, during syngas biomethanation there seems to be an enrichment of SAOBs as well, evidenced by the recovery of potential SAOBs in Paper IV and corroborated by other studies (Li et al. 2020a; Tsapekos et al. 2021; Spyridonidis et al. 2023; Kamravamanesh et al. 2024). This result appears

contradictory to the thermodynamic limitations in a high P_{H_2} environment and, in theory, SAOBs would not be able to compete with acetoclastic methanogens for acetate (Table 1) (Lee & Zinder 1988; Zinder 1994; Westerholm et al. 2019; Grimalt-Alemany et al. 2020). It is theoretically possible for some SAOBs to consume H_2 and act as acetogens under the right conditions (*e.g. T. phaeum*) but due to the potential competition with hydrogenotrophic methanogens for H_2, it is more likely for acetate oxidation to occur (Hao et al. 2011; Westerholm et al. 2019). Nevertheless, it cannot be ruled out that the H_2 consumption does not occur in SAOBs.

3.2 pH

Most microorganism prefer to operate at a neutral pH range between 7 and 7.5. Several studies have shown acidic or alkaline pH conditions can diminish diversity in the microbial community (Liu et al. 2012; Zhou et al. 2016). The optimal pH for the hydrolytic and acidogenic fermentation of protein and carbohydrates is around neutral pH; also, alkaline pH is more favourable to protein solubility and their degradability (Breure & van Andel 1984; Liu et al. 2012; Zhang et al. 2014a; Duong et al. 2019). Acetogenic bacteria are quite diverse in their phylogeny, and they have a wide pH range for growth, between 5.4 and 9.8 (Bengelsdorf et al. 2018). The methanogens operate most efficiently near neutral pH (7-7.5) (Mao et al. 2015). But methanogens are still active outside of this range, even though the methanogenic activity decreases at a higher or lower pH and their growth rates are greatly reduced at pH levels below 6.6 (Whitman et al. 2006; Zhang et al. 2009; Mao et al. 2015). The broader pH range of acetogens was also clearly seen in the study by Gabler et al. (submitted), where batch cultures showed higher relative abundance and activity of acetogens compared to methanogens under high pH levels (>9). However, as the pH decreased, there was an increase in relative abundance of known and potential SAOBs and increased activity of methanogens. But even so, alongside the increase in SAOBs, several known and potential acetogens were found (Paper IV). And yet, as mentioned earlier in section 3.1.1, the food waste reactors described in Paper III only recovered one potential acetogen, even though the reactors were all operating at high pH levels of about pH 8.

3.3 Temperature

Temperature is a strong driver of not only the biogas yield and process stability but also composition and diversity of the entire microbial population. In thermophilic (50-70°C) conditions, the diversity is lower than in mesophilic (30-37°C) conditions (Guo et al. 2014; Moset et al. 2015; Westerholm et al. 2018). Shifts in the microbial community were observed by Westerholm et al. (2018), when the temperature changed in a food waste biogas system from mesophilic to thermophilic. AM, along with heterotrophic and chemolithotrophic acetogenesis, is most likely to occur at lower temperatures, while HM is favoured at higher temperatures and high ammonia levels (see section 3.4), with SAO typically being enhanced under these conditions (Ryan et al. 2010; Westerholm et al. 2019; Conrad 2020; Paper III).

The effect of temperature is especially pronounced in methanogens, which in turn influences the SAOBs and acetogens. At thermophilic temperatures, acetoclastic methanogens are inhibited or become non-existent and hydrogenotrophic methanogens are typically enriched through utilizing the H_2 produced from fermentative metabolism and acetate oxidation (Lee & Zinder 1988; Liu & Conrad 2010; Sasaki et al. 2011; De Vrieze et al. 2015; Ho et al. 2016; Bu et al. 2018; Lim et al. 2020; Pan et al. 2021; Paper III). The hydrogenotrophic methanogens, order *Methanobacteriales* and genus *Methanoculleus*, are known to be partnered with SAOBs (Hattori 2008; Hao et al. 2011; Pan et al. 2021; Paper III). SAOBs play a crucial role in alleviating the accumulation of acetate in the reactors, since the acetoclastic methanogens are either inactive or missing in the community; in some thermophilic reactors, SAO has been seen to be the dominant acetate metabolism (Petersen & Ahring 1991; Nüsslein et al. 2001; Hori et al. 2006; Westerholm et al. 2018).

With hydrogenotrophic methanogens present, the acetogens have tough competition for H_2, which may be one explanation for the low turnout of acetogens in Paper III. Due to the thermodynamics of the autotrophic growth (Table 1 reaction 1) and requirement of high H_2 concentrations, acetogens are unable to compete with hydrogenotrophic methanogens using the more favourable HM and have a lower threshold of H_2 (Table 1 reaction 6) at higher temperature, keeping the acetogens low in abundance (Demirel &

Scherer 2008; Wegener Kofoed et al. 2021; Wu et al. 2021; Farghali et al. 2022). However, acetogens could maintain their competitive edge over methanogens via heterotrophic metabolism, once again owing their success to their metabolism flexibility (Schuchmann & Müller 2016). Acetogens may struggle to compete with methanogens at mesophilic and thermophilic temperatures, but they excel in psychrophilic temperatures, where the methanogens perform poorly (Kotsyurbenko et al. 2001). Not very many known acetogens to date have been isolated from thermophilic biogas reactors, let alone from thermophilic environments, instead many have been isolated from various animal guts (Karekar et al. 2022; Kim et al. 2023). While exploring the thermophilic reactors and batch samples in this thesis, there were no known acetogens recovered, except for handful of potential acetogen, one belonging to the family Dethiobacteraceae (R3.36), two belonging to the class Moorellia (DGT10.16, DGT10.21) (Table 2) (Paper III and IV). These findings reinforce the idea that acetogens are not as prevalent at high temperatures and that they are not able to compete against other microbes. Perhaps there is no need for acetogens in such environment, as in the case of the thermophilic reactors of Paper III, a known hydrolytic bacterium, *Defluviitoga tunisiensis*, was found to possibly take the place of acetogens. *D. tunisiensis* has often been observed in several thermophilic communities and proposed as a key hydrolytic bacterium capable of utilizing a plethora of substrates and produce acetate (Maus et al. 2016; Kim et al. 2018; Treu et al. 2018; Westerholm et al. 2018; Perman et al. 2024). With *D. tunisiensis* as the acetate producer, acetate must be consumed to prevent accumulation that will lead to inhibition. SAOBs can fulfil the role as the acetate consumer to provide H_2 for the hydrogenotrophic methanogens. Three potential novel SAOBs were found along with *Methanoculleus* and *Methanothermobacter* as the partnering hydrogenotrophic methanogens (Paper III). Similarly, the thermophilic samples in Paper IV (DGT, DMT), three potential novel SAOBs were found that belonged to the families Thermacetogeniaceae and Tepidanaerobacteraceae, known to have members that are SAOBs (Hattori et al. 2005; Westerholm et al. 2011b).

3.4 Ammonia

Ammonia is another strong driver of the microbial population that goes hand in hand with temperature (De Vrieze et al. 2015; Westerholm & Schnürer 2019). High temperatures can accelerate the inhibitions triggered by the ammonia level, shifting the equilibrium of the different forms of ammonia to the more toxic form (Angelidaki et al. 1993; Westerholm & Schnürer 2019). Elevated concentrations of total ammonia can lead to microbial inhibitions and reduced microbial diversity in the reactors (Müller et al. 2016; Westerholm et al. 2018). The methanogenic community experiences a shift from acetoclastic methanogens to hydrogenotrophic methanogens at high ammonia concentrations (Schnürer & Nordberg 2008; Zhang et al. 2014b; Lim et al. 2020; Westerholm et al. 2020). Typically, acetoclastic methanogens are sensitive to high total ammonia concentrations and become inhibited, and under such conditions, methanogenesis from acetate can continue but via another acetate degrading pathway (*i.e.* SAO) (Zhang et al. 2014b; De Vrieze et al. 2015; Müller et al. 2016; Westerholm et al. 2020; Paper III). As the acetoclastic methanogens begin to fail, continued acetate consumption is critical for preventing its accumulation, which could lead to an acidification of the process (De Vrieze et al. 2015; Westerholm et al. 2020). SAOBs can take on that role as long as there is a hydrogenotrophic methanogen partner present to consume the H_2 that is produced (De Vrieze et al. 2015; Westerholm et al. 2020). Interestingly, one acetoclastic methanogenic group has been shown to be present and dominate over other acetoclastic methanogens at elevated total ammonia concentrations, belonging to *Methanosarcinaceae* (Conklin et al. 2006; Vavilin et al. 2008; Ziganshin et al. 2013; Paper III). Even though *Methanosarcinaceae* can tolerate the ammonia stress, hydrogenotrophic methanogens, such as *Methanoculleus* and *Methanothermobacter*, have also been shown to be the dominating methanogen in high ammonia conditions (Pan et al. 2021). These three genera of methanogens can be seen in the investigation of high ammonia reactors in Paper III. Either *Methanoculleus* or *Methanothermobacter* were present in all three reactors and dominating in the food waste reactors, while *Methanosarsinca* was recovered in only the manure reactor as the dominating methanogen (Paper III). *Methanosarcina* has been shown to dominate in other manure reactors as well (Kouzuma et al. 2017), perhaps explained by recent research observing a switch back to

AM from SAO-HM after a period of adaptation to high levels of ammonia (Pan et al. 2021). The candidates recovered in the high ammonia reactors described in Paper III, showed the potential to be either acetogens or SAOBs. But considering the high ammonia condition and the lack or low level of acetoclastic methanogens, these appear likely to be SAOBs in charge of oxidizing acetate.

4. Acetogens

With the mention of acetogens in the sections above, it is now time to dive into who these really are. Acetogens are involved in acetogenesis, which may be one of the most primordial processes. Critical to the beginning of life, they use a thermodynamically favourable reaction to sustain growth (Peretó et al. 1999; Russell & Martin 2004; Drake et al. 2008; Küsel & Drake 2011). Across all the anoxic environments globally, acetogens play a role in the cycling of carbon through the production of roughly 10^{13} kg of acetate (Drake et al. 2006). Acetate plays a crucial part in anaerobic digestion as the central intermediate during the degradation of organic matter. Acetate is an especially important intermediate in the biogas process as it is the precursory substrate for methane production; the acetate can be used directly by acetoclastic methanogens or oxidized by SAOBs and further processed by hydrogenotrophic methanogens (Demirel & Scherer 2008; Westerholm et al. 2016).

4.1 Definition

Acetogens are chemolithoautotrophic heterotrophs that are strictly anaerobic, performing reductive carbon fixation (*i.e.* acetogenesis) (Fuchs 1986). The "classical" definition of acetogens, as described by Drake (1994), are anaerobes that can utilize the reductive acetyl-CoA pathway (Wood-Ljungdahl Pathway, WLP) for three purposes:

1) reducing CO_2 to synthesize acetyl-CoA,

2) acting as a terminal electron-accepting, energy-conserving process,

3) fixating CO_2 to synthesize cell carbon.

However, the terms acetogen, “homoacetogens” and “acetogenic bacteria” have often been used interchangeably, which causes confusion. Acetogens have occasionally been used simply as a term for microorganisms that produce acetate, regardless of the pathway. This usage can be problematic when trying to collect information about known acetogens as it includes organisms that produce acetate via fermentation and not utilizing the WLP. For example, Fuchs (1986) uses the term acetogenic bacteria to refer to bacteria restricted to fermentation of purines and amino acids (*e.g. Coprothermobacter proteolyticus* (formerly *Thermobacteroides proteolyticus*) and syntroph strain PA-1) (Ollivier et al. 1985b; Brulla & Bryant 1989). The adjective form, acetogenic bacteria, is often used to describe microorganism that utilize acetogenesis, which is the process of acetate production via the reduction of CO_2 or from organic acids or sugars (Angelidaki et al. 2011; Drake et al. 2013). According to this definition, the novel species isolated in the present thesis work, *Microaceticoccus formicicus,* could have been given the name “acetogenic” bacteria (Paper II). However, this would give the wrong impression, suggesting the production of acetate via the acetyl-CoA pathway. *M. formicicus* does not have a complete WLP but may perform glycine-mediated acetate synthesis (see section 4.2.2). Another term, “homoacetogens”, has typically been used to distinguish between WLP users and non-WLP users and implies that acetate is the sole product that is formed (Drake et al. 2013). However, in addition to acetate production, WLP users can produce butyrate (Lynd & Zeikus 1983), ethanol (Buschhorn et al. 1989), lactate (Misoph & Drake 1996), and other compounds depending on the cultivation conditions.

Based on the classical definition, the fate of acetyl-CoA becoming acetate is not as critical as the process by which acetyl-CoA is formed. Schuchmann and Müller et al. (2016) appended the definition with the requirement of having the WLP coupled to energy conservation, such as *Rnf* or *Ech* complex, to distinctly identify genuine acetogens. It has also been proposed that the usage of the term should be restricted to the process by which two molecules of CO_2 are used to form one molecule of acetate (Wood & Ljungdahl 1991; Drake et al. 2013). However, in doing so, two other known acetate metabolism pathways could be included, the glycine synthase-dependent pathway and the reductive citric acid cycle (Bar-Even et al. 2012b).

4.2 Diversity and applicability

Acetogens have been isolated from a wide variety of environments, ranging from marine sediments, soil, wetlands and gastrointestinal systems, proof of their metabolic versatility (Müller 2019; Kim et al. 2023). Acetogens are phylogenetically diverse, spanning across over 30 different bacterial genera (Drake et al. 2013; Singh & Schnürer 2021; Kim et al. 2023) (Table 2). They are well studied in many environments, including lake sediments, gut systems, sludge, etc (Kim et al. 2023). Considering the wide taxonomic span of acetogens, acetogenesis does not seem to be a phylogenetic trait but rather a physiological attribute. Acetogen versatility is in part due to their usage of the WLP for energy conservation and autotrophic carbon assimilation. Acetate is a key intermediate for methanogenesis in biogas systems; however, still only a few acetogens have been isolated from anaerobic digesters: *Moorella mulderi*, *Sporanaerobacter acetigenes*, *Sporomusa acidovorans* and *Thermacetogenium phaeum* (Ollivier et al. 1985a; Hattori et al. 2000; Hernandez-Eugenio et al. 2002; Balk et al. 2003) (Table 2). Still, previous studies have indicated the presence of acetogenic bacteria in various biogas communities using various genomic-based methods (*e.g.* 16S rRNA (see section 5.1) and *fhs* gene detection with high-throughput sequencing and T-RFLP). These studies have found acetogenic genera, *Acetoanaerobium*, *Acetobacterium*, *Clostridium*, *Oxobacter, Marvinbryantia*, *Peptococcaceae*, *Sporomusa* and *Thermoanaerobacter,* to list just a few (Duan et al. 2021; Singh et al. 2021; Singh & Schnürer 2021; Paper I). However, these cannot be confirmed as an acetogen without at least a pathway analysis to determine the presence of the WLP as per the definition. Presented in this thesis work, known acetogens were also recovered in syngas biomethanation systems, such as *Acetobacterium wieringae*, *Alkalibaculum bacchi*, *Blautia coccoides*, and *Sporomusa sphaeroides* (Paper IV). Alongside these, several potential novel acetogens were also recovered from different biogas systems, some belonging to the same known families, presented in Table 2 (Paper III and IV). These recovered potential acetogens are spread across various known acetogenic genera and are not placed into any specific genus, with some potentially classifying as novel genera (Paper III and IV) (Figure 2).

Acetate is a valuable chemical platform that can be applied in various applications, from flavouring agent and food preservatives to vinyl acetate

used in paints. Acetogens have been used in biogas upgrading systems to remove CO_2 and produce acetate (Zhou et al. 2024). Additionally, aside from acetate, acetogens can act as biocatalysts in syngas fermentation systems to produce other compounds of industrial value, such as ethanol, methanol, isopropanol, etc (Berzin & Tyurin 2012; Yang et al. 2021; Flaiz & Sousa 2024; Thunuguntla et al. 2024). The acetogens harnessed for syngas fermentation have mainly been isolated from different environments other than biogas systems, such as *Acetobacterium woodii* from sediments and *Clostridium autoethanogenum* from rabbit faeces (Balch et al. 1977; Abrini et al. 1994; Bengelsdorf et al. 2018). While many acetogens have been isolated and cultivated in laboratory conditions, their acetogenic capabilities may be obscured by other metabolic pathways. Acetogens have been isolated from a range of environments, cultivated under laboratory conditions and shown to operate well in different bioprocesses. However, potential acetogens isolated from biogas systems may offer distinct advantages for various applications as they are already adapted to such environments. This potential benefit is suggested by the results from the syngas biomethanation system investigated in paper IV, which identified several promising candidates.

Table 2 List of known acetogens to date. Additional potential acetogens from this thesis work are included (adapted from Kim et al. 2023).

Strain	Temp (°C)	pH	Source of isolate	Type of energy conservation	Product
Homoacetogenic strain					
Acetoanaerobium noterae	37	7.6–7.8	Sediment	Rnf	Acetate
Acetobacterium bakii	20	6.5	Sediment	Rnf	Acetate
A. dehalogenans	25	7.3–7.7	Sludge	Rnf	Acetate
A. fimetarium	30	7.5	Sediment	Rnf	Acetate
A. paludosum	20	7.0	Sediment	Rnf	Acetate
A. tundrae	20	7.0	Tundra	Rnf	Acetate
A. wieringae	30	7.6	Sludge	Rnf	Acetate
A. woodii	30	7.0–7.4	Mud	Rnf	Acetate
Acetohalobium arabaticum	37	7.6–8.0	Lake	Rnf	Acetate
Blautia hydrogenotrophica	35–37	6.6	Faeces	Rnf	Acetate
B. schinkii	39	6.5–7.0	Rumen	Rnf	Acetate
B. producta	37	6.5–6.8	Sludge	Rnf	Acetate
Caloramator fervidus	68	7.0–7.5	Thermal spring	–	Acetate
Clostridium bovifaecis	30	7.0	Faeces	Rnf	Acetate
C. magnum	30–32	7.0	Freshwater	Rnf	Acetate
C. scatologenes	25–30	5.8–6.9	Sediment	Rnf	Acetate
Desulfosporomusa polytropa	28	6.1–8.2	Sediment	–	Acetate
Fuchsiella alkaliacetigena	40	8.8–9.3	Lake	Rnf	Acetate
F. ferrireducens	30–37	9.8	Lake	–	Acetate
Holophaga foetida	28–32	6.8–7.5	Mud	–	Acetate
Marvinbryantia formatexigens	37	7.0	Faeces	Rnf	Acetate
Moorella glycerini	58	6.3–6.5	Mixed sediment–water	Ech	Acetate
M. mulderi	65	7.0	Sludge	Ech	Acetate
M. thermoacetica	55–60	5.7–6.8	Faeces	Ech	Acetate
Natroniella acetigena	37	9.7–10.0	Mud	Rnf	Acetate
N. sulfidogena	35	9.8–10.0	Lake	Rnf	Acetate
Natronincola histidinovorans	37–40	9.4	Sediment	–	Acetate
Schnuerera ultunensis	37	7.0	Faeces	Rnf	Acetate

Strain	Temp (°C)	pH	Source of isolate	Type of energy conservation	Product
Sporanaerobacter acetigenes	40	7.4	Sludge	Rnf	Acetate
Sporomusa acidovorans	35	6.5	Wastewater	Rnf	Acetate
S. aerivorans	30	7.0	Gut	–	Acetate
S. intestinalis	35–37	7.0	Gut	–	Acetate
S. malonica	28–32	7.3	Sediment	Rnf	Acetate
S. ovata	34–39	5.3–7.2	Animal feed	Rnf	Acetate
S. paucivorans	35	7.0	Sediment	–	Acetate
S. silvacetica	30	6.8	Soil	Rnf	Acetate
S. termitida	30	7.2	Gut	Rnf	Acetate
Terrisporobacter mayombei	33	7.3	Gut	Rnf	Acetate
Thermacetogenium phaeum	58	6.8	Wastewater	–	Acetate
Thermoanaerobacter kivui	66	6.4	Lake	Ech	Acetate
Tindallia californiensis	37	9.5	Lake	Rnf	Acetate
Treponema primitia	30	7.2	Gut	Rnf	Acetate
Non-homoacetogenic strain					
Acetitomaculum ruminis	38	6.8	Rumen	Rnf	Acetate, propionate, isobutyrate, butyrate, isovalerate, valerate
Acetonema longum	30–33	7.8	Gut	–	Acetate, butyrate
Alkalibaculum bacchi	37	8.0–8.5	Soil	Rnf	Acetate, ethanol
Butyribacterium methylotrophicum	37–40	7.5	Sludge	–	Acetate, butyrate
Clostridioides difficile	37	7.0	Faeces	Rnf	Acetate, ethanol
Clostridium aceticum	30	8.3	Soil	Rnf	Acetate, ethanol
C. autoethanogenum	37	5.8–6.0	Faeces	Rnf	Acetate, ethanol, butanediol
C. carboxidivorans	38	6.2	Lake	Rnf	Acetate, ethanol, butyrate, butanol
C. drakei	25–30	3.6–6.8	Peat	Rnf	Acetate, butyrate
C. formicaceticum	37	7.2	Sludge	Rnf	Acetate, formate
C. ljungdahlii	37	6.0	Animal waste	Rnf	Acetate, ethanol, butanediol
C. ragsdalei	35	6.0	Sediment	Rnf	Acetate, ethanol,butanediol

Strain	Temp (°C)	pH	Source of isolate	Type of energy conservation	Product
Eubacterium aggregans	35	7.2	Wastewater	Rnf	Acetate, formate
E. limosum	37	7.0	Anaerobic digester	Rnf	Acetate, butyrate
E. maltosivorans	35–37	7.0–7.5	Faeces	Rnf	Acetate, butyrate
Natranaerofaba carboxydoora	48–50	9.5	Sediment	Rnf	Acetate, formate
Oxobacter pfennigii	36–38	7.3	Rumen fluid	Rnf	Acetate, butyrate
Proteocatella sphenisci	29	8.3	Guano	Rnf	Acetate, ethanol, butyrate
S. sphaeroides	35–39	6.4–7.6	Mud	Rnf	Acetate, ethanol
Terrisporobacter glycolicus	22–37	7.4–7.6	Mud	Rnf	Acetate, ethanol
Treponema azotonutricium	30		Gut	Rnf	Acetate, ethanol
Candidate acetogens from thesis work					
Ca. "Thermotepidanaerobacter aceticum" (R1.8)	52	8.1	Anaerobic digester	Rnf	–
Ca. "Thermosyntrophaceticus schinkii" (R2.32)	52	8.1	Anaerobic digester	Ech	–
Ca. "Thermodarwinisyntropha acetovorans" (R3.10)	52	7.9	Anaerobic digester	Rnf	–
Limnochordia sp (DGT9.28)	55	8.5	Anaerobic digester	Rnf	–
Natronincolaceae sp (DGT9.20)	55	8.5	Anaerobic digester	Rnf	–
Moorellia sp (DGT10.16)	55	8.2	Anaerobic digester	Rnf	–
Moorellia sp (DGT10.21)	55	8.2	Anaerobic digester	Rnf	–

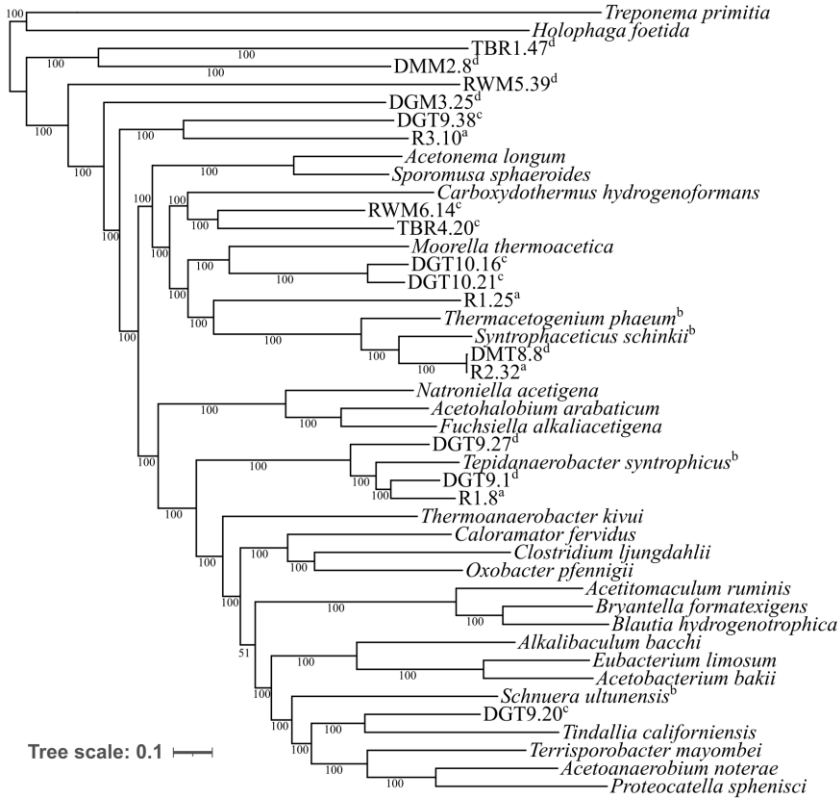

Figure 2 The whole genome phylogenetic tree of known acetogens and SAOBs and potential novel acetogens SAOBs (Paper III, IV). One representative species from each known acetogen genera was used in this tree. The novel acetogens are recovered from Paper III and IV. Values at the nodes are bootstrap percentages based on 1000 iterations. Bar, 0.1 substitutions per nucleotide position. [a]- MAGs that are recovered from thermophilic biogas digesters (Paper III). [b]- known SAOB representatives. [c]- potential acetogen MAG recovered from syngas batch cultures (Paper IV). [d]- potential SAOB MAG recovered from syngas batch cultures (Paper IV).

4.3 Pathways

The WLP is a core part of the identity of acetogens, based on Drake's (1994) definition. Recent research has proposed an alternative, or rather a co-functioning pathway, the glycine synthase reductase pathway (GSRP) (Song

et al. 2020). The GSRP was initially thought not to be used for autotrophic growth but has recently been shown to allow certain bacteria to grow autotrophically (Bar-Even et al. 2012a; Song et al. 2020; Sánchez-Andrea et al. 2020). While there are other types of carbon fixation pathways (Bar-Even et al. 2012b), the WLP and the GSRP will be the main spotlight of this thesis.

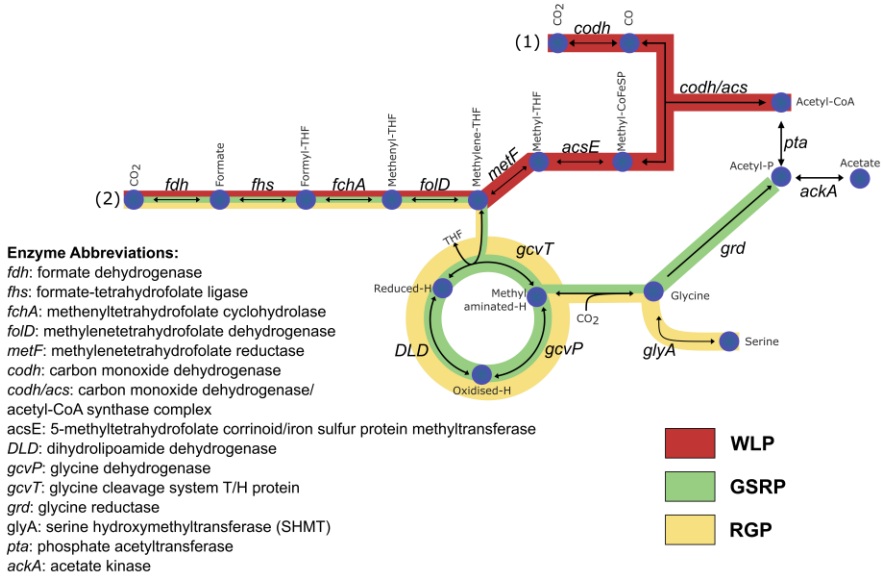

Figure 3. Pathway diagram of the Wood-Ljungdahl pathway (WLP), the glycine synthase reductase pathway (GSRP), and the reductive glycine pathway (RGP). (1) Carbonyl branch of the WLP, (2) Methyl branch of the WLP.

4.3.1 Wood-Ljungdahl Pathway

The WLP is an ancient pathway that seems to be the first pathway used for the production of biomass by CO_2 fixation; the pathway is the most widely utilized C1 carbon fixation pathway (Bar-Even et al. 2012b; Poehlein et al. 2012). This pathway is used by organisms that perform acetogenesis. The WLP can also be utilized in the reverse direction for syntrophic acetate oxidation, and some acetogens have been characterized to also be SAOBs (*e.g. T. phaeum* and *S. ultunensis*) (Schnürer et al. 1996; Keller et al. 2019). WLP is made up of two branched "linear" pathways, reducing two molecules of CO_2 as an electron acceptor and with hydrogen as the electron donor into one molecule of acetyl-CoA, a metabolic intermediate molecule (Fuchs 1986; Ljungdahl 1986:198; Shi & Tu 2015). The methyl branch handles the

reduction of CO_2 to tetrahydrofolate (THF) using the enzymes encoded by formate dehydrogenase (*fdh*), formyltetrahydrofolate synthetase (FTHFS/*fhs*), methylenetetrahydrofolate dehydrogenase (*folD*), and methylenetetrahydrofolate reductase (*metF*) (Figure 3). The second, carbonyl branch, reduces another CO_2, causing the carbon monoxide dehydrogenase (CODH) and acetyl-CoA synthase ($acs_{(r)}$) to combine with the first branch to form acetyl-CoA. That is the end of the WLP, but the acetyl-CoA can be further synthesized to acetate via a two-step reaction involving the phosphate acetyltransferase (*Pta*) and acetate kinase (*AckA*). The key enzymes of the WLP (*fhs*, CODH/$acs_{(r)}$ complex and *AckA)* are essential for acetogenesis (Drake 1994; Hattori et al. 2005). When the WLP is run in the oxidative direction, converting acetate to H_2 and CO_2, *Pta* and *AckA* are proposed to be used to oxidize acetate, which can be substituted with acetyl-CoA synthetase ($acs_{(o)}$) (Hattori 2008; Ragsdale & Pierce 2008; Dyksma et al. 2020; Li et al. 2022). Several known SAOBs have been observed to utilize the $acs_{(o)}$ and not the *Pta-AckA* (*e.g. S. ultunensis*) (Manzoor et al. 2018). However, even though SAOBs have the WLP, not all encode enzymes required for $acs_{(o)}$ (*e.g. Tepidanaerobacter acetoxydans*) (Müller et al. 2015; Dyksma et al. 2020; Li et al. 2022). The SAOBs that lack $acs_{(o)}$ have been proposed to instead use the *Pta-AckA* route or, alternatively, continue via the glycine cleavage system (Li et al. 2022; Puchol-Royo et al. 2023; Zeng et al. 2024).

4.3.2 Glycine Synthase Reductase Pathway and Reductive Glycine Pathway

The GSRP is another C1 carbon fixation pathway, where glycine is the intermediate formed, which is then further reduced to acetate or acetyl-P for biomass production (Fuchs 1986; Bar-Even et al. 2012b). The core of the GSRP, the glycine cleavage system (GCS), is a multi-protein complex that catalyses the reversible synthesis of glycine encoded by gene *gcvT* and *gcvH* (Kikuchi 1973; Bysani et al. 2023). Energy is not conserved when solely using GSRP due to the pathway not utilizing the enzyme methylenetetrahydrofolate reductase, which is typically coupled with a Na^+ translocation protein across the membrane to generate energy (Fuchs 1986; Bar-Even et al. 2012b). GSRP had not been shown to be used for autotrophic growth (Bar-Even et al. 2012b), until recently, demonstrated in the acetogen *Clostridium drakei* to co-utilize the WLP with the GSRP and RGP for

autotrophic growth (Song et al. 2020). The GSRP and RGP use the GCS, sharing the same enzymes, *gcvT*, *gcvH*, glycine dehydrogenase (*gcvPA* and *gcvPB*), and dihydrolipoyl dehydrogenase (*DLD*); the difference is that GSRP uses glycine reductase (*grd*) and RGP uses serine hydroxymethyltransferase (SHMT, *glyA*) to convert glycine to acetyl-P or serine, respectively (Song et al. 2020). Typically, the GSRP is mainly used as part of the recycling of reduced electron carriers generated during the fermentation of purines and amino acids (Fuchs 1986; Schneeberger et al. 1999; Fonknechten et al. 2010). The RGP had recently been shown to have the capability of fixing CO_2, supporting autotrophic growth (Figueroa et al. 2018; Sánchez-Andrea et al. 2020). In SAOBs, when the GSRP and RGP are used in tandem with the WLP, it has been referred to as glycine-mediated acetate oxidation (Li et al. 2022; Zeng et al. 2024). GSRP has also been proposed for use by another potential family (Darwinibacteriaceae) of SAOBs, determined by Puchol-Royo et al. (2023). The proposal of utilizing GSRP and RGP as alternatives to the WLP in the oxidative direction may seem promising but should be taken with a grain of salt as there is no definitive evidence to support this claim.

4.4 Pathways in known acetogens and SAOBs

Studies on acetogens and their pathway facilities have mainly investigated the acetyl-CoA pathway, which is well conserved between related species within their respective genus (Kamlage et al. 1997; Ragsdale & Pierce 2008; Esposito et al. 2019). The GSRP or the RGP is not typically mentioned in the description of the acetogen. It was initially thought that acetogens that harbour the WLP would not have the GSRP present (Fuchs 1986). However, Song et al. (2020) presented *C. drakei* as being able to functionally co-utilize WLP, GSRP and RGP. Several acetogens belonging to Clostridia (*e.g. C. ljungdahlii* and *C. aceticum*) can harbour the GCS, but not all do (*e.g. C. pasteurianum*) (Hong et al. 2021). Comparing the list of known acetogens belonging in genus *Clostridium* (Table 2) to the list of *Clostridium* described in Hong et al. (2021), nearly all harbour the GCS and *grd*, except for *C. bovifaecis*. The presence of the glycine synthase and GCS in *C. drakei* and in an engineered strain of *C. pasteurianum* was shown to increase the amount of C1 fixation (Song et al. 2020; Hong et al. 2021). The RGP has been shown to be used for autotrophic growth, although not in an acetogen but by

sulphate-reducing bacteria (Sánchez-Andrea et al. 2020). *A. woodii*, one of the most studied model acetogen, possesses the complete WLP and the RGP but lacks a glycine reductase for a complete GSRP (Neuendorf et al. 2021). Growing on formate, the GCS in *A. woodii* was upregulated, which could theoretically indicate the use of RGP for carbon assimilation (Neuendorf et al. 2021). However, more investigation is required to fill in the information gap about which known acetogens harbour and can utilize the GSRP/RGP for carbon assimilation. The novel potential acetogens that were recovered in this present study were shown to possess both the WLP and the GCS, some completing the RGP or GSRP (Paper IV). Of the recovered known acetogens, *S. sphaeroides* had both the GSRP and RGP, and *A. wieringae* only had RGP; the other two known acetogens, *A. bacchi* and *B. coccoides*, did not possess either of these two pathways (Paper IV). In the thermophilic reactors studied, there was a lack of complete WLP genes observed in the recovered genomes, but the GCS component of GSRP and RGP was recovered (Paper III). De Bernardini et al. (2022) have also recovered genomes without a complete WLP but complete GSRP and RGP and proposed these genomes as being able to utilize the pathways to sustain the CO_2 reduction and fixation for biomass growth. It appears that acetogens recovered from the biogas systems can have various combinations of all three pathways.

Identification of genes encoding for both GSRP and RGP in several genomes recovered from various biogas processes operating at high ammonia conditions has motivated the proposal of this as an alternate path for SAO (Zhu et al. 2020; Li et al. 2022; Singh et al. 2023; Weng et al. 2024). It has been speculated in several studies that the presence of GCS could be a potential route to connect acetate to the methyl branch of the WLP; however, there is no experimental evidence of such activity as this system can be used by many organisms for amino acid biosynthesis (Kieft et al. 2023; Singh et al. 2023; Weng et al. 2024). In some instances, the support for these genomes to function in SAO was the simultaneous increase in abundance with the dominance of hydrogenotrophic methanogens after acetate feeding combined with the prevailing environmental conditions (Zhu et al. 2020; Li et al. 2022). This is however still an indication of these specific MAGs could be SAOBs but still not enough proof as acetogens can have a similar genetic repertoire. Thermophilic and high ammonia conditions are known to push the community into a specific niche that is more favourable to SAO and hydrogenotrophic methanogenesis, as mentioned before in section 3.3 and 3.4 (Paper III). Under such conditions, in the present thesis work, two

potential novel SAOBs were recovered that had WLP as well as GCS, utilizing them in either GSRP or RGP (Paper III and IV, Table 3). Two candidatus species were recovered ("*Ca*. Thermotepidanaerobacter aceticum" and "*Ca*. Thermosyntrophaceticus schinkii"). "*Ca.* T. aceticum" was considered as the true acetogen using the classical definition, possessing the WLP. "*Ca* T. schinkii" was proposed as a SAOB due to the presence of the gene *PntAB,* encoding for NAD(P) transhydrogenase, which has been proposed to be a gene that acetogens lack and SAOBs possess (Nobu et al. 2015; Zeng et al. 2024). The positioning as SAOB was additionally supported by the close similarity to *Syntrophaceticus schinkii*, which is a known SAOB (Westerholm et al. 2010; Paper III). Also found in the high ammonia and thermophilic reactors were members of MBA03 and Dethiobacteria, which have previously been proposed as having SAO capability, however these did not possess the *PntAB* genes (Sorokin & Merkel 2022; Puchol-Royo et al. 2023; Paper III). These members of Dethiobacteria found in Paper III had the key genes of the WLP, *fhs* and CODH, and the GCS, but lacking the *PntAB*, suggesting that they could be either acetogens or SAOBs. Although the pathway genes were identified, the operational direction remains unclear and the only evidence supporting their classification as SAOBs is their taxonomic history.

Exploration into the syngas biomethanation system established the potential presence of acetogens as well as SAOBs (Paper I). Analysis of their genomes demonstrated the presence of genes for the WLP with various levels of completeness of the GSRP/RGP in the genome (Paper IV). The known acetogens included *Acetobacterium wieringae*, *Alkalibaculum bacchi*, *Sporomusa sphaeroides*, and *Blautia coccoides* (Braun & Gottschalk 1982; Möller et al. 1984; Allen et al. 2010; Niu et al. 2024; Paper IV). As mentioned in section 3.1.3, the amount of H_2 available may direct the metabolism of acetogens. Acetogens are restricted to heterotrophic growth at low P_{H_2} conditions, but once P_{H_2} reaches their minimum limit, acetogens can switch to autotrophic metabolism (Lee & Zinder 1988). As the investigated TBRs were feed with syngas composed of 56% H_2, it may be a plausible explanation for recovering known and potential novel acetogens (Paper IV). The novel acetogens found all harbours the complete WLP, except for one belonging to Natronincolaceae (DGT9.20). DGT9.20 was only missing *fdh* from the pathway, but it has been observed that other acetogens can lack *fdh* and still be capable of utilizing the WLP for acetogenic growth (Zhuang et al. 2014; Trischler et al. 2022; Guan et al. 2023). Two other novel acetogens

were identified, belonging to class Moorellia (DGT10.16, DGT10.21) and had the full WLP along with the GSRP and RGP and the energy conservation *Rnf* gene.

There was one known SAOB recovered from the TBR, *S. schinkii*, which is partnered with hydrogenotrophic methanogen, *Methanoculleus bourgensis* (Westerholm et al. 2010; Manzoor et al. 2016a; b; Paper IV). This was rather surprising, considering that the conditions were not "typical" for SAO driven methanogenesis (low ammonia and mesophilic temperature) (Westerholm et al. 2016). Both acetoclastic and hydrogenotrophic methanogens were recovered from these samples (Paper IV). Among the potential novel SAOBs harbouring the complete WLP and *PntAB* genes, two (DGT9.1, DGT9.27) belonged to the family Tepidanaerobacteraceae, which is includes members that are syntrophic acid oxidizers, such as the SAOB, *Tepidanaerobacter acetatoxydans* (Westerholm et al. 2011b). Two other potential SAOBs were found, one belonging to the class Dethiobacteria (DGM3.25) and the other belonging to Thermacetogeniaceae (DMT8.8). These two potential SAOBs had a complete WLP along with the *PntAB* gene. DMT8.8 was classified as the provisional species DTU068 sp001513545, which had been previously found and suggested to be a SAOB in other studies; one enriched in a high ammonia propionate oxidising culture (Singh et al. 2023), another from a thermophilic wastewater treatment plant enriched for syntrophic acetate-oxidising consortia (McDaniel et al. 2023) and in Paper III (R2.32). An additional candidate belonged to the family Anaerolineae (RWM5.39) possessing all the WLP but did not have the *PnatAB* gene, however, the family is known to include members that have been shown to be capable of SAO (Zeng et al. 2024). Several of these genomes also possess the RGP, and as mentioned above, that could be utilized in an alternative acetate oxidation route via glycine (section 4.3.2).

Table 3 The recovered genomes that are potential acetogens and SAOBs from high ammonia thermophilic biogas processes (Paper III) and mesophilic and thermophilic syngas biomethanation systems (Paper IV). The MAGs that are denoted with * are from Paper III, otherwise they are all from Paper IV.

Next highest classification level	Species	MAGs	WLP	RGP	GSRP	ATPase	Cytochrome C oxidase	NADH:quinone oxidoreductase	Phosphate acetyltransferase- acetate kinase	Rnf	Ech	NAD(P) transhydrogenase (*PntAB*)
Potential novel acetogens												
Pelotomaculaceae (f)	Pelotomaculum_C sp003489345	**R4.20**	x	-	-	F	-	-	-	x	x	-
Carboxydocellales (o)	JAJZSN01 s___	**RWM6.14**	x	-	-	F	-	x	x	-	x	-
Natronincolaceae (f)	JAAYNB01 s___	DGM3.17, DGM4.26, **DGT9.20**, DGT10.18	x[a]	-	-	F	x	-	x	x	-	-
Limnochordia (c)	JAAZLZ01 sp012799075	**DGT9.38**	x	x		F	-	-	x	x	-	-
Moorellia (c)	JAAZDD01 sp012512955	DGT9.13, **DGT10.21**	x	x	x	F	-	x	x	x	-	-
Moorellia (c)	JAAZDD01 sp012520135	DGTT9.32, **DGT10.16**	x	x	x	F	-	x	x	x	-	-
Potential novel SAOBs												
Anaerolineae (c)	JAAYZQ01 s____	**RWM5.39**	x	x		F	x	x	x	x	-	-
Syntrophorhabdaceae (f)	MWEV01 sp002071245	TBR2.34, **TBR1.47**	x[a]	-	-	F				x	-	-
Syntrophobacteraceae (f)	JABUEY01 sp013314815	DMM1.28, **DMM2.8**, RWM5.20	x	-	-	F	-	x	x	x	-	-
Tepidanaerobacteraceae (f)	DUNY01 s___	DGM3.1, DGM4.21, **DGT9.1**, DGT10.1	x[a]	x	-	V/A	-	x	x	x	-	x
Tepidanaerobacteraceae (f)	Tepidanaerobacteraceae s___	**DGT9.27**	x	x	-	F, V/A	-	-	-	x	-	x
Dethiobacteria (c)	DTU022 sp001512835	**DGM3.25**	x	-	-		-	-	x	x	x	x
Thermacetogeniaceae (f)	DTU068 sp001513545	**DGT8.8,** DGT9.5, DGT10.2	x	x[c]	x[c]	F	-	-	x	x	x	x
Tepidanaerobacteraceae (f)	DTU063 sp001512695	**R1.8***	x[a]	-	-	V/A	-	-	x	x		x
Thermacetogeniaceae (f)	DTU068 sp001513545	**R2.32***	x	-	-	F	x	x	x	-	x	x
Limnochordia (c)	DTU010 sp002391385	**R3.10***	x[d]	x	x	F	x	x	x	x	-	x

x[a]-Missing one module- formate dehydrogenase
x[b] – Missing two modules- acsE and acsBCD
*- MAGs from Paper III
x[c] - found with only the grd
x[d]-only the methyl branch recovered

5. Exploring the systems

Over the years, many studies have used various techniques to understand and pinpoint the presence of acetogens in different environments, ranging from quantitative polymerase chain reaction (qPCR), 16S ribosomal RNA gene sequencing, *fhs* database curation, metagenomics, and isolation and characterization. Throughout this thesis, so far recovery of acetogens and SAOBs have been discussed. These were recovered through several methods: utilizing 16S ribosomal RNA gene sequencing (Paper I), metagenomics study/analysis (Paper III and IV), and isolation and characterization (Paper II).

5.1 Quantification of key genes

There have been several studies that have utilized qPCR to quantify acetogenic communities targeting *fhs*, one of the key genomes of the WLP (Xu et al. 2009; Westerholm et al. 2011a; Delgado et al. 2012; Aydin et al. 2015; Sagheddu et al. 2017). However, there are limitations to this method when investigating the *fhs* gene; one is that the gene is a functional marker, not a taxonomic marker (Singh & Schnürer 2021). It has, however, been shown to result in similar microbial community profiles compared to 16S rRNA gene profiling (Singh et al. 2021). Additionally, the need for high specificity primers to target the *fhs* gene proves difficult, as the optimal size of the target gene to run qPCR is between 200-300 base pairs (bp), and the size of the gene is around 1100 bp, well over the reliable size limit (Leaphart & Lovell 2001; Xu et al. 2009; Müller et al. 2016). Another limitation is that the *fhs* gene is also found in non-acetogenic bacteria and some archaea, leading to potential inflation in quantification since there is no taxonomic information that can be used to filter these out (Lovell & Leaphart 2005; Borrel et al. 2016). To relieve some of these complications, a repository of

fhs genes from all available known acetogens has been created (AcetoBase) as a platform to assist in designing suitable primers to target diverse populations with the *fhs* gene (Singh et al. 2019). While successful identification of the *fhs* gene will reveal the *fhs* gene-harbouring bacteria, it cannot determine if the targeted bacteria is a classical acetogen or a SAOB. Another key gene, CODH, has also been used to screen for potential acetogens. However, this gene has so far only been quantified in environments other than the biogas systems (Matson et al. 2011; Hoshino & Inagaki 2017; Omae et al. 2021).

5.2 16S ribosomal RNA gene sequencing

The 16S rRNA gene is a taxonomic marker that has been used in countless studies to uncover the microbial communities in environmental samples (Werner et al. 2011; Niu et al. 2015; Nobu et al. 2015; Paper I). The analysis uses the Silva database, a comprehensive dataset of rRNA sequences, to identify the taxonomy (Quast et al. 2013). This technique will always be required for the phylogenetic placement of the bacteria when completing the isolation and characterisation. 16S rRNA analysis can give a rough idea of the taxonomy of known acetogens that may be present in the biogas system if they belong to one of the 30 genera of proposed acetogens. As mentioned in section 4.2, acetogenesis is a physiological trait rather than a phylogenetic characteristic, making it difficult to say whether their placement next to a known acetogen would make them an acetogen. Analysing 16S rRNA can provide an overview of the community structure, but it has its limitations as it cannot be solely used to identify acetogens. 16S rRNA gene analysis is confined to a short sequence within the whole genome and provides limited taxonomic fidelity at the species level (Janda & Abbott 2007). The sequences can be compared against the NCBI nucleotide database to add support to the taxonomic identity. However, this does not offer sufficient evidence in determining whether the query species is the same as the reference species. When proposing *Microaceticoccus formicicus* as a novel genus and species, the requirement to delineate between novel and known species included the 16S rRNA gene similarity along with the whole genome Average Nucleotide Identity (ANI), Average Amino-Acid Identity (AAI), and digital DNA-DNA hybridization (dDDH) comparisons (Paper II). The 16S rRNA gene analysis determined the closest relatives was *Citroniella saccharovorans* with a 92.52% similarity, which is below the proposed genus (94.5%) and species

(98.65%) delineation boundary (Janda & Abbott 2007; Kim & Chun 2014; Yarza et al. 2014; Paper II). Even though the 16S rRNA gene analysis already indicated that *M. formicicus* was a novel genus, the poor discriminatory power of the analysis cannot guarantee that the whole genome relatedness confers the same similarity.

The 16S rRNA analysis of the syngas biomethanation reactors in the present thesis revealed several genera that belonged to genera of known and potential acetogens and SAOBs (Paper I). This is a good starting point in identifying unknown species, but as mentioned, the poor species level fidelity requires a comparison of the whole genome to confer with the 16S gene analysis (Janda & Abbott 2007). Thus, this information would need to be supplemented with additional genomic work (metagenomics, see section 5.2) and isolation and characterization (see section 5.3) to pinpoint whether the bacteria is a true acetogen.

5.3 Metagenomics

Metagenomics combines genomic technologies and bioinformatics to directly analyse genetic content from environmental samples. Metagenomics accesses the functional gene composition of the microbial communities, giving more information than phylogenetic analysis alone (*e.g.* 16S rRNA gene analysis) (Thomas et al. 2012). This technique can elucidate information about uncultured organisms that could not be classified at a lower taxonomic rank in the 16S rRNA analysis. The metagenomic data can be further processed by assembling the genetic data into genomes to create metagenome-assembled genomes (MAGs). These MAGs provide a better understanding of the microbial populations and can propose their potential capabilities in the environment. Most MAGs that are recovered in a variety of environments belong to uncultured novel species; this technique would aid in the uncovering of the so-called microbial dark matter (Setubal 2021).

For the metagenomic work presented in this thesis, the short reads were assembled using MEGAHIT, a de novo assembler optimized for metagenomes (Li et al. 2015). The assembled contigs were then grouped into bins using the unsupervised binning tools, MetaBat2 and MaxBin2 (Wu et al. 2016; Kang et al. 2019). The bins were then passed through CheckM to determine the quality and to select the bins and designate as MAGs (cutoff completeness 90% and contamination 5%). The prediction of protein-coding

genes was completed using Prokka (Seemann 2014) and used EggNOG-mapper (Cantalapiedra et al. 2021) for functional annotation of the metagenomic sequences and then assessed with KEGG Mapper Reconstruct tool to reconstruct metabolic pathways. GTDB-Tk (Chaumeil et al. 2020) was used to assign taxonomy to the bins. There was a deviation in the workflow between the methods in Paper III and IV, where the nf-core/mag pipeline was used in Paper IV (Krakau et al. 2022). All the tools used at each step were the same except for the use of DASTool (Sieber et al. 2018) in Paper IV. DASTool refines the bins from each of the binning methods to generate a non-redundant set of bins from the assembly. One potential limitation to the set of bins generated in Paper III, is that it may not have been the optimized for the metagenome, which may also explain the lack of acetogens as mentioned in previous chapters. Another potential constraint is the need to have high quality MAGs, high completeness and low contamination, to be able to reliably find and annotate genes, resulting in a smaller set of MAGs to explore (Howe et al. 2014; Campanaro et al. 2016).

In this thesis work, microbial community exploration via 16S rRNA analysis provided the basis for a selection of some samples for metagenomics analysis to recover known and potential acetogens (Paper I and IV). Metagenome data was collected from the samples that were used to generate the 16S rRNA gene analysis. To identify potential acetogens, the *fhs* gene was used as a starting point to filter out non-relevant MAGs. The presence of this gene alone cannot immediately determine the identity of acetogens, the rest of the WLP would need to be present as well (Paper III and IV). Another key enzyme of the WLP is the CODH/$acs_{(r)}$ complex, which brings the two branches of the WLP together to create acetyl-CoA (Figure 3). Many of the MAGs recovered in this thesis work had the *fhs* gene; however, the MAGs from the thermophilic high ammonia reactors were notably missing the genes that code for CODH/$acs_{(r)}$ complex (Paper III and IV). This led to the rest of the bins to be analysed as well, but to no avail, CODH/$acs_{(r)}$ was still missing. There were, however, sequences that were not assembled into MAGs and remained unbinned and left separate from the MAGs. Investigating the unbinned data revealed several fragmented CODH/$acs_{(r)}$ orthologs. The displaced fragments may be due to low abundance of genomic material, leading to incomplete assemblies of sequences, which results in a lack of information to accurately place them into bins. In addition, the $acs_{(o)}$ was also missing, which brought into question how acetate metabolism happens. Both CODH/$acs_{(r)}$ complex and $acs_{(o)}$ being missing from the

MAGs led to a search for the genes responsible for coding GSRP and RGP, as mentioned earlier, to be a potential co-utilized path for autotrophic growth or alternative path for SAO (Song et al. 2020; Li et al. 2022; Puchol-Royo et al. 2023; Zeng et al. 2024). This opened the possibility that some of the recovered MAGs could be players in acetate metabolism and perform SAO. The genes for GSRP and RGP were recovered in many of the MAGs, but it cannot, as mentioned before, be said for certain that these participate in SAO.

The syngas metagenomic samples were collected from both a continuously fed TBR and from batch tests (Gabler et al. submitted; Paper I). The 16S analysis identified known acetogens and SAOBs, belonging to the genera *Acetobacterium*, *Alkalibaculum*, *Blautia*, *Sporomusa* and *Syntrophaceticus* (Gabler et al. submitted; Paper I), and these were later recovered as MAGs and classified as *Acetobacterium wieringae*, *Alkalibaculum bacchi*, *Blautia coccoides Sporomusa sphaeroides*, and *Syntrophaceticus schinkii* (Paper IV). The metagenomic analysis, in this case, corroborated the 16S rRNA analysis of the presence of the known acetogens and SAOBs. However, the metagenomic analysis was able to provide more clarity on the species level taxonomy compared to the 16S rRNA gene analysis. The 16S also suggested the presence of potentially novel acetogens and SAOBs, which were tentatively recovered in at least 13 MAGs. There is a small caveat when evaluating the taxonomy assignments between the two methods, which variation in names used for certain taxonomic ranks. For example, the 16S analysis observed the presence of a potential SAOB belonging to the family Spirochaetaceae and order Spirochaetales. The GTDB classification used the synonymous name of the order, Treponematales, which is not incorrect, per se. However, during the initial metagenomic analysis, it was thought that Spirochaetales was not recovered.

The gene annotation analysis of the MAGs revealed potential acetogens that possessed both a full WLP and coupled with *Rnf* complex for energy conservation (Table 3), which follows the classical definition, and the addendum found in Schuchmann and Müller (2016) about energy conservation coupling. Some of the genera proposed to be acetogens and SAOBs in the 16S analysis were recovered in MAGs classified to a provisional species name. *Natronincola*, as an example, was seen at high relative abundance in one of the sampling points during increased consumption of CO_2 and accumulation of acetate, suggesting this bacterium

to grow as an autotrophic acetogen (Gabler et al. submitted). The 16S was revealed to belong to *Natronincola peptidovorans*, which has been found in other anaerobic digestions with high H_2 partial pressure (Braga Nan et al. 2022). However, *N. peptidovorans* was not recovered. Instead, a MAG belonging to the same family, Natronincolaceae, was recovered under the provisional genus JAAYNB01, without a species classification (Paper IV). The genome annotation revealed that this MAG was only missing a formate dehydrogenase for a complete WLP pathway, indicating that the bacterium that was recovered in the 16S analysis could be a CO utilizing bacterium. However, this only brings the identification one step closer to determining whether it is an acetogen that was observed. The next challenge would be to isolate and characterize the bacterium to determine its metabolism.

5.4 Isolation and Characterisation

Using both the taxonomic marker analysis (16S rRNA) and the metagenomic analysis will point towards potential acetogens and SAOBs. However, to confirm the role of the query, isolation and physiological characterization of the bacteria must be performed (Paper II).

Microaceticoccus formicicus was isolated from a propionate enrichment culture from a high-ammonia biogas digester. The goal was to isolate an acetogen and to expand knowledge about the ammonia-tolerant microbial populations that plays a key role in degradation processes (Paper II). *M. formicicus* produced acetate as one of the main products from various substrates, including formate, glucose, and various amino acids. However, this bacterium was not able to utilize H_2/CO_2, which was confirmed with genomic characteristics, lacking a full WLP, missing the CODH/$acs_{(r)}$ complex. Thus, *M. formicicus* could not be defined as an acetogen using the classical definition. However, the first four steps of the WLP were present, along with genes encoding GCS/GSRP/RGP (Paper II). As mentioned in chapter 4, the RGP was suggested to be used for assimilating CO_2 and, additionally, formate, which is particularly relevant for this formate-utilizing isolate (Sánchez-Andrea et al. 2020; Claassens et al. 2022). Formate is an important intermediate in the biogas process as it can be produced from the reduction of CO_2 or consumed for carbon fixation (Crable et al. 2011).

There are several challenges with isolation and characterization. One of the difficulties to face is ensuring the isolation of a clean colony of the

bacterium from the environmental sample following anaerobic techniques. To isolate the bacterium, the selection of suitable substrates will be challenging to determine, as it may or may not grow on similar substrates to its closest relative. Repeated sequencing tests may be necessary to determine the purity of the isolated cultures before proceeding with the physiological tests. The physiology work of the close relatives must be repeated when completing it for the isolate. Physiology characterization is one of the most time-consuming parts of this process, particularly if the isolate has a slow growth. As part of the physiology work, cell wall analysis is required to compare between the isolate and close relatives. However, it can be difficult to gather enough cells to perform such analysis. Additionally, to have a comprehensive comparison of the cell wall composition, the cultivation conditions must be identical for both the isolate and closest relatives prior to the analysis (Tindall et al. 2010; da Costa et al. 2011). However, it may not always be possible to grow them under the same conditions adding complications to characterization. As mentioned above (section 5.2), analysing with the 16S rRNA gene can provide the closest relatives and compare the similarity of the genes, giving phylogenetic placement. However, recent requirements for phylogenetic placement have changed to also require whole genome placement and comparisons. The reference genome of the closest relative is not always available, putting the onus on the researchers to retrieve the genome themselves to complete the placement (Cheng et al. 2024). The closest relative, based on the 16S rRNA gene, for *M. formicicus* was *Citroniella saccharovorans*. The whole genome had not been assembled and was not available for whole genome comparison and phylogenetic placement. *C. saccharovorans* was ordered, cultivated, sequenced and assembled into the genome ready to be used for comparison (Cheng et al. 2024). The comparison between *M. formicicus* and *C. saccharovorans* can be then completed to reveal better taxonomic information for the isolate. However, after all the characterisation work of *M. formicicus*, the behaviour of the isolate observed on the lab bench can be different to what occurs in the natural habitat of the biogas reactor.

6. Conclusions and Perspectives

Acetogens are prevalent in many environments and can also be found in the biogas system if conditions are primed for them. Acetogens, in most circumstances, provide a bridge to methanogenesis. In this thesis work, efforts to recover novel acetogens were performed utilizing various techniques. This work illustrates that a combination of techniques is necessary to pinpoint and identify acetogens. For a quick scan of the microbial community, 16S rRNA can be used to assess the potential presence of acetogens and can then be followed up with metagenome analysis to determine the genomic characteristics and to obtain more in-depth taxonomic information. However, this does not address the biological reality of these microorganisms; isolation and characterisation are required to reveal their true capabilities.

The classical definition of acetogen has generally remained the same to reflect bacteria that utilize the WLP, first and foremost, to reduce CO_2 to synthesize acetyl-CoA. However, the GSRP/RGP has been shown do the same and, in some instances, co-utilized with the WLP. Exploring the different reactors, obvious acetogen candidates were recovered from the syngas reactors, while from the food waste and manure biogas reactors, some potential candidates were recovered. Due to the nature of acetogen's metabolic flexibility, the potential acetogen candidates from the food waste and manure reactors, may function in a different capacity as heterotrophs rather than lithoautotrophs from the syngas reactors and batch cultures. Perhaps the definition of acetogens should be revisited to also include those possessing GSRP and/or RGP. Since the methyl branch of the WLP can be used to fuel the GSRP and RGP pathway, it does eventually end up producing acetyl-CoA, albeit via a roundabout path. Some GSRP/RGP users can utilize the pathway for autotrophic growth. The GSRP/RGP still fulfils the requirements of being able to: 1) reduce CO_2 to synthesize acetyl-CoA, 2)

act as a terminal electron-accepting, energy-conserving process, and 3) fix CO_2 to synthesize cell carbon. The difference when amending the definition with the GSRP/RGP is that the full WLP is not used. Perhaps the combination of the two pathways may help expand the possibility to recover more acetogens. Even though this could help uncover less obvious acetogens, the three pathways are reversible and can be utilized by SAOBs, which further obfuscates the identification of acetogens. To compound the difficulty even more, some acetogens have SAO capability. Differentiating between acetogens and SAOBs will require more analyses related to functions (*e.g.* proteomics and transcriptomics).

6.1 Future perspectives

Three types of exploration techniques were used for this thesis; however, there are more that can be utilized to further describe and identify acetogens that are in the biogas systems. While isolation and characterization can determine substrate utilization and product formation of the organism in a controlled environment, which does not necessarily reflect their activity in the natural environment. To understand the microorganism's response in the biogas systems would require utilizing another metagenomic method, metatranscriptomics. This involves the study of RNA transcripts that are present in the reactor to gain insight into the actively expressed genes and functional activities at a specific time point. Metatranscriptomics can reveal which enzymes may be involved in the consumption of a substrate. Such a technique has been used on several isolated SAOBs and acetogens from biogas systems (*e.g. S. schinkii*, *A. woodii*, *T. acetatoxydans*) (Müller et al. 2015; Manzoor et al. 2016a; Song et al. 2021). Additionally, in combination with metagenomics and metatranscriptomics, studying proteins and their cellular structures and reactions (proteomics) can provide more information to understand the molecular mechanisms essential to the process (Coorssen 2022). This technique, along with protein-stable isotope probing (protein-SIP), has been used to identify and describe the activity of known acetogens and SAOBs (Mosbæk et al. 2016; Valgepea et al. 2022). These techniques open opportunities to explore the metabolism characteristics of acetogens and SAOBs under different treatments in the reactor and how the microbial community responds. These techniques could also be used to characterize enzymes encoded by unidentified/hypothetical genes from MAGs in biogas reactors. Reflecting on the high-ammonia thermophilic reactors in this work,

perhaps there are unknown and uncharacterized genes that would be highly expressed, but since they are not properly described, it is uncertain if they could be tied to the capabilities of acetogens, especially thermophilic species. Using these methods could provide additional support in identifying novel acetogens and reveal how they fit metabolically into the complex process of anaerobic digestion in biogas systems.

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Popular science summary

Climate change continues to be a challenge that humanity is struggling to contain as the average temperature of Earth continues to increase. To mitigate this issue, one of the drivers of the climate crisis must be reduced, that is the release of anthropogenic carbon dioxide (CO_2). This driver is primarily created by human reliance on using fossil fuels. Energy production is one of the sectors contributing most to the increase of CO_2 emissions. Production of renewable and low-carbon energy can alleviate the dependence in fossil-based energy sources (oil, coal, and natural gas), while also increasing energy security and mitigating environmental impact. These renewable energy sources include solar, wind, geothermal, hydropower, and bioenergy.

Among renewable energy sources, biogas (a mixture of methane and carbon dioxide) offers great potential for reducing dependence on fossil-based products. Biogas production is not only a great renewable energy source, but also contributes as a sustainable waste management strategy that brings value to various waste streams. Biogas is produced through a microbial process called anaerobic digestion, breaking down organic material in a series of four steps. In this process, a complex community of microbes degrades organic waste in an oxygen-free environment. This microbial community can be divided into four groups, each playing a crucial role. Of the microbial groups responsible to anaerobic digestion, acetogens (“makers of vinegar”) are arguably one of the most interesting.

Acetogens are a versatile group of bacteria that can degrade various types of organic compounds to produce the precursory compound acetate, which is crucial for methane-producing microorganisms. Acetogens can also capture CO_2, important for carbon capture and utilization as well as for producing carbon-efficient bio-based commodities. Despite the great interest

in and the potential of acetogens, the group has not been well studied in the biogas system until recently, even though previous explorations into biogas systems have indicated the presence of these acetogens. Hence, in this thesis, various techniques are used to uncover the specific acetogens that are present in different biogas systems and to reveal their potential function in better aiding the understanding of their role in the process.

Populärvetenskaplig sammanfattning

Klimatförändringarna utgör en betydande utmaning för mänskligheten då medeltemperaturen på jorden fortsätter att stiga. För att lindra detta problem måste en av de drivande faktorerna bakom klimatkrisen minskas, nämligen utsläppen av antropogen koldioxid (CO2), som främst beror på människans användning av fossila bränslen (olja, kol och naturgas). Energiproduktion är en av de sektorer som bidrar till ökningen av CO2-utsläpp. Produktion av förnybar energi och energi med låga utsläpp kan minska beroendet av fossila bränslen, minska miljöpåverkan och även öka energisäkerheten. Förnybara energikällor inkluderar solenergi, vindenergi, geotermisk energi, vattenkraft och bioenergi.

Biogas (en blandning av metan och koldioxid) är en förnybar energikälla med stor potential att bidra till minskat beroende av fossila produkter. Biogas är inte bara en utmärkt energikälla, utan produktionen av biogas bidrar också till hållbar avfallshantering genom att ta vara på resurser i organiska rester från olika avfallsströmmar. Biogas produceras från nedbrytning av organiskt material genom en mikrobiologisk process som kallas anaerob nedbrytning eller rötning. Rötningsprocessen sker i flera olika steg där olika grupper av organismer är ansvariga för olika nedbrytningssteg.

Bland de organismer som bidrar till rötningsprocessen kan acetogenerna (”framställare av vinäger”) sägas vara en av de mest intressanta. Detta är en mångsidig grupp av bakterier som kan bryta ner olika typer av organiska föreningar för att producera ättiksyra (acetat), en viktig energi- och kolkälla för de metanproducerande mikroorganismerna. Acetogener kan också binda CO_2, vilket gör gruppen intressant att använda för att fånga in koldioxid och för att producera olika klimatsmarta biobaserade produkter. Detta har skapat ett stort intresse för acetogenernas potential.

Tidigare undersökningar har indikerat närvaro av denna grupp av mikroorganismer i olika biogassystem, men trots detta har de fram till nyligen inte studerats särskilt väl i dessa system. I arbetet som presenteras i denna avhandling har olika tekniker använts för att ta fram ny information om acetogener i olika typer av biogassystem med målet att bättre förstå deras funktioner och den roll de spelar i nedbrytningsprocessen.

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ENVIRONMENTAL BIOTECHNOLOGY

Microbial community development during syngas methanation in a trickle bed reactor with various nutrient sources

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Abstract

Microbial community development within an anaerobic trickle bed reactor (TBR) during methanation of syngas (56% H_2, 30% CO, 14% CO_2) was investigated using three different nutrient media: defined nutrient medium (241 days), diluted digestate from a thermophilic co-digestion plant operating with food waste (200 days) and reject water from dewatered digested sewage sludge at a wastewater treatment plant (220 days). Different TBR operating periods showed slightly different performance that was not clearly linked to the nutrient medium, as all proved suitable for the methanation process. During operation, maximum syngas load was 5.33 L per L packed bed volume (pbv) & day and methane (CH_4) production was 1.26 L CH_4/L_{pbv}/d. Microbial community analysis with Illumina Miseq targeting 16S rDNA revealed high relative abundance (20–40%) of several potential syngas and acetate consumers within the genera *Sporomusa*, *Spirochaetaceae*, *Rikenellaceae* and *Acetobacterium* during the process. These were the dominant taxa except in a period with high flow rate of digestate from the food waste plant. The dominant methanogen in all periods was a member of the genus *Methanobacterium*, while *Methanosarcina* was also observed in the carrier community. As in reactor effluent, the dominant bacterial genus in the carrier was *Sporomusa*. These results show that syngas methanation in TBR can proceed well with different nutrient sources, including undefined medium of different origins. Moreover, the dominant syngas community remained the same over time even when non-sterilised digestates were used as nutrient medium.

Key points

- *Independent of nutrient source, syngas methanation above 1 L/L_{pbv}/D was achieved.*
- *Methanobacterium and Sporomusa were dominant genera throughout the process.*
- *Acetate conversion proceeded* via *both methanogenesis and syntrophic acetate oxidation.*

Keywords Methanation · Syngas · Microbial community · *Sporomusa* · *Methanobacterium* · Trickling bed reactor

Introduction

Anaerobic digestion is a well-established and well-known process-based technology for treatment of different types of organic waste streams, such as sewage sludge, manure or food waste, while producing renewable energy (biogas) and a nutrient-rich digestate that can be used as fertiliser (Kougias and Angelidaki 2018). Among possible substrates for biogas production, plant biomass residues, such as straw, represent a huge global resource with great potential (Paul and Dutta 2018). However, such materials are currently rather under-used in biogas reactors due to limited applicability within the conventional digestion process (Hendriks and Zeeman 2009; Paul and Dutta 2018). One way to tap the potential of such materials can be thermal gasification to syngas, followed by conversion to methane (Ren et al. 2020).

The composition of syngas varies depending on biomass type and gasification conditions, but it mainly consists of methane (CH_4), carbon dioxide (CO_2), hydrogen (H_2) and carbon monoxide (CO), with very low concentrations of nitrogen (N_2) and oxygen (O_2) and trace gases in varying amounts (Ciliberti et al. 2020). Although syngas can be

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combusted directly, conversion to an established biofuel, such as methane, offers synergies with existing infrastructure for energy storage and distribution (Ren et al. 2020). Methanation of syngas can be performed by chemical catalytic methods or biologically (biomethanation) using methanogenic archaea (Ren et al. 2020). Biomethanation has the advantage that it can be carried out at ambient operating conditions (low pressure, low temperature) (Asimakopoulos et al. 2020b; Aryal et al. 2021; Wegener Kofoed et al. 2021). Moreover, compared with the chemical conversion process biomethanation is more robust to impurities, such as tar or hydrogen sulphide (H_2S) (Grimalt-Alemany et al. 2018). Based on this, biomethanation is estimated to be more cost-efficient than chemical catalytic conversion (Benjaminsson et al. 2013).

One challenge for the biomethanation process is gas–liquid transfer. Among different reactor systems available, the trickle bed reactor (TBR) can be considered an efficient technology for producing biomethane from syngas (Sposob et al. 2021). The TBR consists of a column packed with carrier material with high surface area, for immobilisation of microbial biomass. A liquid nutrient medium to support microbial growth and activity is sprinkled at the top of the TBR and trickles over the carrier material to the bottom of the reactor, while input gas flows in a counter-current or co-current direction. Rate-limiting mass transfer of gases is circumvented in TBR, since the carrier material provides a large surface area for interactions between gas, liquid and biofilm (Sposob et al. 2021). However, combining TBR and biomethanation is a relatively new concept and parameters indicating a stable continuous process and high output have not yet been identified (Grimalt-Alemany et al. 2017).

Acetogenesis and methanogenesis are two main essential microbial routes during methanation of syngas or H_2/CO_2 (Grimalt-Alemany et al. 2018), although syntrophic acetate oxidation (SAO) can also be part of the process (Sancho Navarro et al. 2016) (Fig. 1). Hydrogenotrophic methanogens convert H_2 and CO_2 to CH_4, but the same substrates can be used by acetogens for the production of acetate, creating competition for H_2. In addition, CO can be used by acetogenic bacteria (Arantes et al. 2020) but can also be converted by some methanogens (Ferry 2010; Sancho Navarro et al. 2016). Based on thermodynamics and substrate affinities, methanogens have an advantage over acetogens as they can use lower levels of dissolved hydrogen (reviewed in Wegener Kofoed et al. 2021). However, acetogens become more competitive at lower operating temperatures (Fu et al. 2019), higher hydrogen levels (Liu et al. 2016) and high or low pH values, which inhibit methanogens (Voelklein et al. 2019). Acetate can be consumed by acetoclastic methanogens to produce methane or oxidised to H_2/CO_2 by syntrophic acetate-oxidising bacteria. The latter process is preferentially operating under low partial pressures of hydrogen

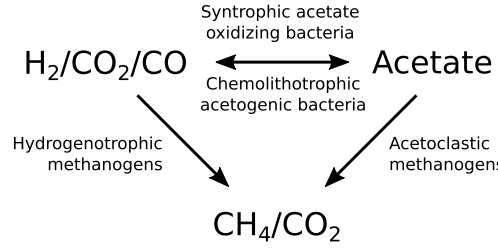

Fig. 1 Groups of microbes involved in methanation of syngas. Hydrogenotrophic methanogens converting H_2/CO_2 to methane (CH_4) also compete with chemolithotrophic acetogenic bacteria that consume H_2/CO_2 to produce acetate. Acetate is consumed by either acetoclastic methanogens for CH_4 production or syntrophic acetate oxidising bacteria to produce H_2/CO_2

and carbon dioxide and thus are likely to have a negligible role during methanation of syngas. However, under certain conditions syntrophic acetate oxidation can be enabled and compete with acetoclastic methanogenesis, such as during low P_{CO2} levels (< 0.01 atm), which improves the thermodynamics of this metabolic route (Grimalt-Alemany et al. 2020a), and high P_{CO} concentrations, which inhibits acetate utilising methanogens (Sancho Navarro et al. 2016).

To ensure high microbiological activity and growth during conversion of gases, such as syngas or H_2/CO_2, it is important to supply sufficient amounts of nutrients with the liquid medium (Wegener Kofoed et al. 2021). It is well known that, in addition to carbon and energy sources represented by the process gases, microorganisms (including methane producers) also need other macronutrients and micronutrients, such as nitrogen (N), phosphorus (P), sulphur (S) and various salts and trace metals (Jarrell and Kalmokoff 1988). Several previous studies have examined syngas methanation in laboratory-scale TBR operating with different defined nutrient media (Burkhardt et al. 2015; Asimakopoulos et al. 2020a, b). For full-scale application, there is a need for more accessible and economically feasible nutrient sources, such as manure, digestate or reject water from sludge processing in wastewater treatment plants (WWTP). The use of such undefined nutrient media has mainly been evaluated for biomethanation of H_2/CO_2, during operation in batch or continuous mode (Kougias and Angelidaki 2018; Sieborg et al. 2020; Tsapekos et al. 2021), and for biomethanation of syngas in a TBR in batch mode (Aryal et al. 2021). To our knowledge, only one previous study has used a non-defined nutrient source during continuous operation of a TBR for syngas methanation (Figueras et al. 2021).

The biological processes involved in syngas conversion have been investigated in many studies and have been shown to be influenced by different parameters, such as type of reactor and carrier, composition of the gas, environmental

parameters such as temperature, and liquid recirculating rates and nutrient composition (for reviews, see Grimalt-Alemany et al. 2017; Aryal et al. 2021; Li et al. 2021; Sposob et al. 2021; Tsapekos et al. 2021). Other determinants of process efficiency, such as microbial community structure and abundance as influenced by operating parameters, have been less thoroughly investigated and the links between operational settings and performance and the microbial community are not fully understood. The few microbiological analyses that have been performed have mainly focused on methanation from H_2/CO_2 (Sposob et al. 2021; Tsapekos et al. 2021) and less on syngas, and most studies have been performed at thermophilic temperatures, using defined medium (Li et al. 2020a, 2021; Andreides et al. 2021; Aryal et al. 2021). The aim of the present study was to extend knowledge on the microbiology of syngas methanation and, more specifically, to investigate the influence of different nutrient media on microbial community development during long-term operation of a mesophilic TBR. The work formed part of a larger research project and was performed over 3 years in a succession of periods utilising defined and complex nutrient medium, represented by digestate from a food waste–based biogas plant and reject waster from a WWTP. The syngas used in the study was designed to mimic the expected composition of syngas according to an industrial partner, Cortus Energy Ltd.

Material and methods

Screening and selection of inoculum

Initial screening of syngas consumption capacity, using digestate from three different mesophilic biogas reactors (a, b, c), was performed to select a suitable microbial inoculum for the start-up of the TBR. Reactors a and b were mesophilic laboratory-scale reactors, operating with cow manure and mixed food waste, respectively. Operation and performance of these reactors have been described elsewhere, as manure-based Reactor A_{ref} in Ahlberg-Eliasson et al. (2021) and food waste reactor GR2 in Westerholm et al. (2015), respectively. The third inoculum was taken from a full-scale reactor (c), located at Uppsala WWTP, operating with a mix of secondary and primary sludge at mesophilic temperature (37 °C), using an organic load of 2 g volatile solids/L day and a hydraulic retention time of 18 days. The volatile solid concentration (% of wet weight) in inoculum from reactor a, b and c was 6.1, 2.9 and 1.5%, respectively. Each inoculum was incubated at 37 °C for 7 days to remove excess gas from endogenous material and then 200 mL of inoculum was transferred to each of 12 serum bottles (539.5 mL) under flushing with nitrogen gas. The bottles were sealed with butyl rubber stoppers and aluminium caps and filled with different gases to one of the following concentrations (%) at a final pressure of 1.5 atm: (i) CO/N_2 (15/85%); (ii) $CO/H_2/CO_2/N_2$ (15/28/7/50%); (iii) H_2/CO_2 (28/72%); (iv) N_2 (100%), with three replicates per gas composition. The gases used were synthetic mixtures supplied by Air Liquide (Paris, France). The bottles were then incubated at 37 °C for 10 days on a shaking table at 200 rpm (Orbitron, Infors, Bottmingen, Switzerland). Gas compositional analyses were performed after 1, 2, 3, 7, 8 and 10 days. On each sampling occasion, the pressure was measured (model GMH3111; Greisinger Electronics, Regenstauf, Germany) and a 5 mL gas sample (at normal pressure) was taken with a plastic syringe. The gas was analysed by gas chromatography (see below). Based on the initial screening of syngas consumption (Fig. S1), inoculum from the manure-based reactor (reactor a) was selected as the inoculum source.

Digestate collected from reactor a (inoculum A) was filtered through a 2-mm mesh to remove large particles, after which 1.25 L was diluted with defined mineral medium (Westerholm et al. 2010) to reach a final volume of 5 L. The diluted digestate was transferred under flushing (N_2) to two plastic containers (20 L), each filled with 17.5 L plastic carrier (Hiflow® ring 15–7 plastic; height 15 mm, specific area 313 m^2/m^3, density 80 kg/m^3, void fraction 91%). The containers were closed and incubated anaerobically at 37 °C for 7 days, with manual shaking twice every day. This incubation was intended to reduce the level of organic matter in the inoculum, decrease background CH_4 production and initiate biofilm development on the carrier before filling the TBR.

Source of nutrients

During operation of the TBR, three different nutrient sources were used: defined medium (M1); digestate from a thermophilic biogas plant (Uppsala, Sweden) operating with mixed food waste (Grim et al. 2015) (M2); and reject water from dewatered digestate from a biogas unit at a WWTP (Höganäs, Sweden) operating with mixed primary and activated sludge (M3). Medium M1 was prepared as described previously (Westerholm et al. 2010) and consisted of phosphate/bicarbonate buffer supplemented with salt, trace metals, vitamins and reducing agents (e.g. cysteine-hydrochloric acid (HCl) and sodium sulphide (NaS_2)). These reducing agents also represented the only S source in the medium, with a total S concentration of 135 mg/L. The N source in the medium was ammonium chloride (NH_4Cl), with an ammonium-N concentration of 400 mg/L. The pH of M1 was 7.2–7.4. Medium M2 was prepared by mixing one volume unit of digestate with two volume units of deionised water. The resulting liquid was passed through a cloth to remove particles. No sterilisation of the liquid was performed. This diluted solution had a pH of 8.5–8.6, total alkalinity of 2249–3057 mg/L and an ammonium-N

concentration of 300–670 mg/L. Sulphur was analysed as sulphate and the concentration was 50–60 mg/L. The reject water medium (M3) was dewatered but not sterilised at the WWTP and no further treatment was done before its use as nutrient medium for the TBR. The pH was 7.6–7.9, the total alkalinity was 2676–3871 mg $CaCO_3$/L and the concentration of ammonium-N and S was 560–800 mg/L and 107 mg/L, respectively. Additional supplements were added to the media as described in section TBR Operation

TBR and anaerobic filter

The TBR was constructed from acid-proof stainless steel (Fig. 2) and was placed in a movable container together with all associated equipment (Fig. S2). The reactor had a total volume of 49 L, including head space and liquid reservoir, an inner diameter of 215 mm and a total height of 1344 mm. The reactor was filled with the inoculated carrier to a total packed bed volume (pbv) of 35 L. A grid plate spreader (5 mm pores) was placed 80 mm above the bottom of the reactor, creating a reservoir in which a volume of nutrient medium (max. 8 L) could be contained and collected. Liquid from this reservoir was manually removed on a regular basis and replaced with fresh nutrient medium. The liquid was recirculated with a hose pump (FPSH 15, 0.37 kW; Valisi, Rozzano, Italy) from the reservoir to the top of the reactor, where it was sprinkled over the packed bed and trickled back to the liquid reservoir. The pump was operated in semi-continuous batch mode with 5 s of pumping followed by 37 s of stop time, giving an average flow of approximately 14.3 L/h. At the top of the reactor, two stainless steel grid plate spreaders were positioned to distribute the nutrient liquid (Fig. 2, Fig. S2d). Syngas was added through a port in the lower part of the reactor (Fig. 2) to meet the liquid coming from the top, thus operating in a counter-current manner. At the top of the reactor, the gas was collected and the volume was measured by a drum meter (TG 0.5; Ritter, Germany). Samples for chemical and microbiological analyses of the liquid were taken at position 6 in Fig. 2. The composition of the outgoing gas (CH_4, CO_2, CO, O_2, H_2) was analysed by a ETG MCA 100 Syn Biogas Multigas Analyzer (ETG Risorse e Tecnologia, Chivasso, Italy). The reactor was heated to 37 °C by a water jacket and the temperature in the reactor was logged using a temperature probe (Tinytag View 2; Gemini Data loggers, Chichester, United Kingdom). The temperature was 36–38 °C during the entire operating period of the TBR. After around 200 days of operation, an additional reactor (anaerobic filter, AF) was installed, through which nutrient liquid from the bottom of the TBR was recycled in an upflow manner (Fig. 2, Fig. S2d). The aim was for the AF to prolong the retention time for the nutrient liquid recirculate and by doing so allow more time for degradation of accumulated volatile fatty acids (VFA) in the nutrient liquid. This reactor was made of plastic and had a total/active volume of 1.5 L (height 190 mm, diameter 100 mm). The AF was filled with the same type of inoculum and carrier as the TBR and the same procedure as for TBR inoculation was used, with an incubation period of 14 days before filling the AF. The gas from this reactor was not collected.

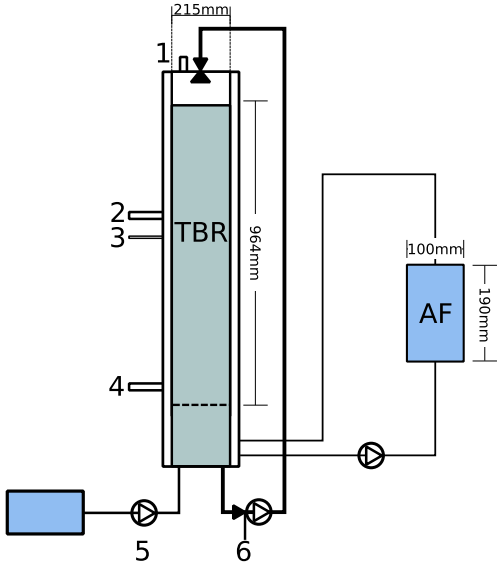

Fig. 2 Schematic diagram of the trickle bed reactor (TBR) and anaerobic filter (AF). 1: Outlet for product gas. 2: Carrier sampling port. 3: Position of temperature probe. 4: Syngas inflow. 5: Inflow of liquid nutrient medium. 6: Sampling port for microbial analysis

TBR operation

The TBR was operated for a total of 862 days. The initial 129 days of operation were devoted to start-up and acclimatisation of the process. Thereafter, TBR operation was divided into different main periods based on the nutrient medium used (Table 1). Each main operating period was in turn divided in two sub-phases (A and B) based on major operational changes, installation of the AF or changes in flow or composition of the nutrient medium (Table 1). The gases used throughout TBR operation were synthetic mixtures (Air Liquide, Paris, France). The different periods are described briefly below and summarised in Table 1 and Supplementary Material.

Start-up and acclimatisation (129 days) During this period, the biomass was allowed to adjust to the prevailing conditions in the reactor and to the syngas. Defined nutrient

Table 1 Description of the different operating periods in the trickle bed reactor (TBR) process

Phase name	Period dates	Days of operation[a]	Nutrient solution[b]	Feed rate[c] (mL/day)	Gas composition[d] (%)	Description
Start-up	2018/06/01–2018/07/26	− 129 (56)	M1[e]	140	CO: 15 N_2: 85	Initiation of reactor and enrichment of CO-utilising microorganisms
Acclimatisation	2018/07/27–2018/10/07	− 73 (73)	M1[e]	140	CO: 15 CO_2: 7 H_2: 28 N_2: 50	Change of gas mixture towards industrial-like gas composition and acclimatisation
Period 1A	2018/10/08–2019/04/04	0–179 (179)	M1[e]	140	CO: 30 CO_2: 14 H_2: 56	Change of gas mixture to simulate syngas produced by Cortus Energy. This gas mixture was used in the following periods
Period 1B	2019/04/05–2019/06/05	180–241 (62)	M1[e]	140	CO: 30 CO_2: 14 H_2: 56	Addition of a small anaerobic filter (AF) reactor to alleviate accumulating VFA levels. The small reactor was used to the end of the process
Period 2A	2019/06/06–2019/08/27	242–324 (83)	M2[f]	1000	CO: 30 CO_2: 14 H_2: 56	Change in liquid nutrient feed stabilisation and feed rate
Period 2B	2019/08/28–2019/12/22	325–441 (117)	M2[f]	1000→200	CO: 30 CO_2: 14 H_2: 56	Gradually decrease in liquid feeding throughout phase
Period 3A	2019–12-23– 2020/05/19	442–590 (149)	M3[g]	400	CO: 30 CO_2: 14 H_2: 56	Change in liquid nutrient feed and feed rate
Period 3B	2020/05/20–2020/07/29	591–661 (71)	M3[g]	200	CO: 30 CO_2: 14 H_2: 56	Nutrient feed rate reduced to 200 mL

[a]Operating time set to zero at the start of period 1; days in brackets represent the number of operation days for each period

[b]Recirculated liquid nutrient solution

[c]Feeding rate of liquid nutrient medium

[d]Composition of ingoing gas mixture

[e]Defined nutrient medium

[f]Digestate from a co-digestion plant, operated under thermophilic conditions, digesting sorted household food waste with organic food waste from larger kitchens, stores and food distributors

[g]Digestate from a wastewater treatment plant, operated under mesophilic conditions, digesting sludge from the wastewater treatment process and minor fractions of different sludges from the food processing industry

medium (M1) was used at a low flow rate (140 mL/day) and the inlet gas composition was initially CO/N_2 (15/85), to enrich CO-consuming bacteria. Thereafter, the gas composition was changed to $CO/CO_2/H_2/N_2$, representing 15, 7, 28 and 50%, respectively, in order to add CO_2 and H_2 while maintaining the same partial pressure of CO.

Period 1 (241 days) The start of this operating period was defined as Day 0. In this period, the reactor was fed syngas with the target composition expected by the industrial partner (Cortus Energy), which was 30% CO, 14% CO_2 and 56% H_2. The defined nutrient medium (M1) at an average inflow rate of 140 mL/d was used throughout. During operation, VFA were produced quickly in response to increasing syngas inflow, so an AF was installed (phase 1B) and process liquid from the TBR reservoir was recirculated through the TBR (Fig. 1). To evaluate possible nutrient limitation as a cause of VFA accumulation and decreasing gas consumption, additional N (NH_4Cl; Fisher Chemicals, Göteborg, Sweden) and S (NaS_2 and cysteine-HCl; Merck, Darmstadt, Germany) were also added at the end of period 1A and during period 1B (see Supplementary Material).

Period 2 (200 days) In this period, the nutrient medium was changed to digestate from the industrial food waste biogas plant (M2). In the initial phase (2A), a high flow of

nutrients was supplied (1000 mL/day), while in the second phase (2B), this was gradually reduced to 200 mL/day. In phase 2B, additional S (NaS_2 or $NaSO_4$, Merck, Darmstadt, Germany) was added (Table 1, Supplementary Material) in an attempt to mitigate decreasing syngas consumption rate.

Period 3 (220 days) In this period, the nutrient medium was changed to reject water from dewatered digested WWTP sewage sludge (M3)**,** initially at a flow rate of 400 mL/day (phase 3A) and later reduced to 200 mL/day (phase 3B). In addition, extra S (NaS_2 or $NaSO_4$, Merck, Darmstadt, Germany) was added throughout the whole operating period (Supplementary Material) and in phase 3B, sodium bicarbonate (Na_2CO_3) was added to enhance the alkalinity and mitigate a trend of decreasing pH (Supplementary Material).

Analytical methods

In the screening experiments with different inocula, the gas composition was analysed by gas chromatography according to Westerholm et al. (2012). Short-chain VFA (C2-C6) were quantified by ion-exclusion chromatography according to Westerholm et al. (2012). Process pH was measured with a Hanna instrument HI83141 (Woonsocket, Rhode Island, United States). Ammonium and sulphate were analysed with a spectrophotometer (Spectroquant® Nova 60A photometer; MilliporeSigma, Burlington, MA, USA) with reagent test kits from the series Supelco (Merck, Darmstadt, Germany). The total alkalinity was calculated as the amount of acid required to bring the sample to pH 4.4. Titration was carried out with an automatic titrator (TitraLab® AT1000 series; Hach, Düsseldorf, Germany).

Microbial analysis

Samples for DNA extraction were withdrawn from the recirculated liquid on a weekly basis from sampling port 6 as shown in Fig. 2, and on a few occasions, samples of carrier were taken from the TBR and microbial material was scraped off the carrier using a small spatula. DNA was extracted from 200 µL of liquid sample using the FastDNA Spin Kit for Soil (MPBiomedicals, Illkirch-Graffenstaden, France) according to the manufacturer's protocol with an additional cleaning step with guanidine thiocyanate (Danielsson et al. 2017). DNA was also extracted from the ingoing nutrient medium by concentrating 4 mL of sample by centrifugation and dissolving the cell pellet obtained in 200 µL of the supernatant. The samples were initially extracted in triplicate but, after preliminary sequence analysis showing no significant variations between triplicate extractions, single extractions were done in order to allow analysis of more samples. Sequencing libraries were generated by SciLifeLab, in Stockholm, Sweden, using Illumina MiSeq (2 × 300 bp) targeting 16S rDNA. To cover both bacteria and archaea, the amplification was done using the forward primer 515′F and reverse primer 806R, as described previously (Westerholm et al. 2018). The paired end reads were processed with Cutadapt version 1.13, removing the aforementioned primers and adapters on forward and reverse reads (GTGBCAGCMGCCGCGGTAA and GACTACHVGGGTATCTAATCC, respectively) and filtering based on quality and trimming reads to 300 bp. The trimmed reads were processed with Division Amplicon Denoising Algorithm2 (DADA2) version 1.16.0 in Rstudio running R version 4.1.1, as described by Westerholm et al. (2018), with forward and reverse reads truncated at positions 240 and 160, respectively. Microbial classification was performed using the SILVA reference database v. 132. The data were organised with *phyloseq* v1.32.0 (McMurdie and Holmes 2013) into a single data object that was subsequently used for graphic generation in RStudio v1.4.1717 (RStudio Team 2020) running R v4.1.1. The following R packages were used for visualisation of the microbial data: ggplot v2 3.3.5, data.table v1.13.4, plotly v4.9.2.1, lattice v0.20.45, permut v0.9.5, vegan v2.5.7, readxl v1.3.1, plyr v1.8.6, grid v4.1.1 and ggtext v0.1.1. The amplicon sequence variants (ASV) were submitted to the Basic Local Alignment Search Tool (BLAST) algorithm provided by the National Center for Biotechnology Information (NCBI), The sequences obtained by Illumina sequencing are too short (~250–300 bp) to clearly reveal the identity of the engaged microorganisms on species level. However, ASVs showing 100% identity with a known organism are in the presentation referred to the putative species name. Raw sequence data have been deposited in NCBI PRJNA796200.

Results

Selection of inoculum

Evaluation of different sources of inoculum for methane production from different gas mixtures (H_2/CO_2, CO/N_2 or $H_2/CO_2/CO$; see Fig. S1) before the start-up of the TBR process revealed that the consumption/production patterns of the different inocula did not differ significantly. However, CO consumption rate, with or without H_2 and CO_2, was highest for the inoculum from the manure-based biogas reactor (reactor a; see Fig. S1), and therefore, inoculum A was chosen for the TBR.

TBR operation: process and microbiology

Period 1

Throughout period 1, the syngas inflow fluctuated between 1.11 and 3.33 $L/L_{pbv}/d$, depending on the consumption

efficiency (Fig. 3a). During periods of high syngas gas outflow, the inflow was decreased to match the rate of consumption. In line with the variation in inflow, the total biogas outflow for the period ranged from 0.041 to 1.86 $L/L_{pbv}/d$, with higher values towards the end of the period (Fig. 3a). The CH_4 content in the gas was around 50%, resulting in an output range of 0.06–0.91 L CH_4 $/L_{pbv}/d$ (mean 0.60 L $CH_4/L_{pbv}/d$) for the period (Fig. 3b). VFA accumulation and pH declines were observed early in operation (Fig. 3c) and the gas inflow was temporarily stopped/lowered on a number of occasions to mitigate further accumulation and decreasing pH. Once VFA were consumed, normal syngas inflow was resumed. However, with an increase in the gas inflow rate, it was no longer possible to control VFA levels, which increased continuously to values as high as 3.7 g/L by the end of period 1B (Fig. 3c, Supplementary Material). The VFA present were mainly represented by acetic and propionic acid, with propionic acid initially making up 71–96%, but with a higher proportion of acetic acid in the later phase (59–99%) (Supplementary Material).

The microbial community in the starting inoculum was characterised by high relative abundance of phylum *Firmicutes* (57.0%), dominated by uncultivated members of genus *MBA03* (15.5%) and genus *Sedimentibacter* (7.7%) and phylum *Bacteriodetes* (24.0%), mainly dominated by unknown members of family *Rikenellaceae* (11.9%) (Fig. 4, Fig. S3a). During the stabilisation phase, these two phyla continued to show high relative abundance, accompanied by emergence

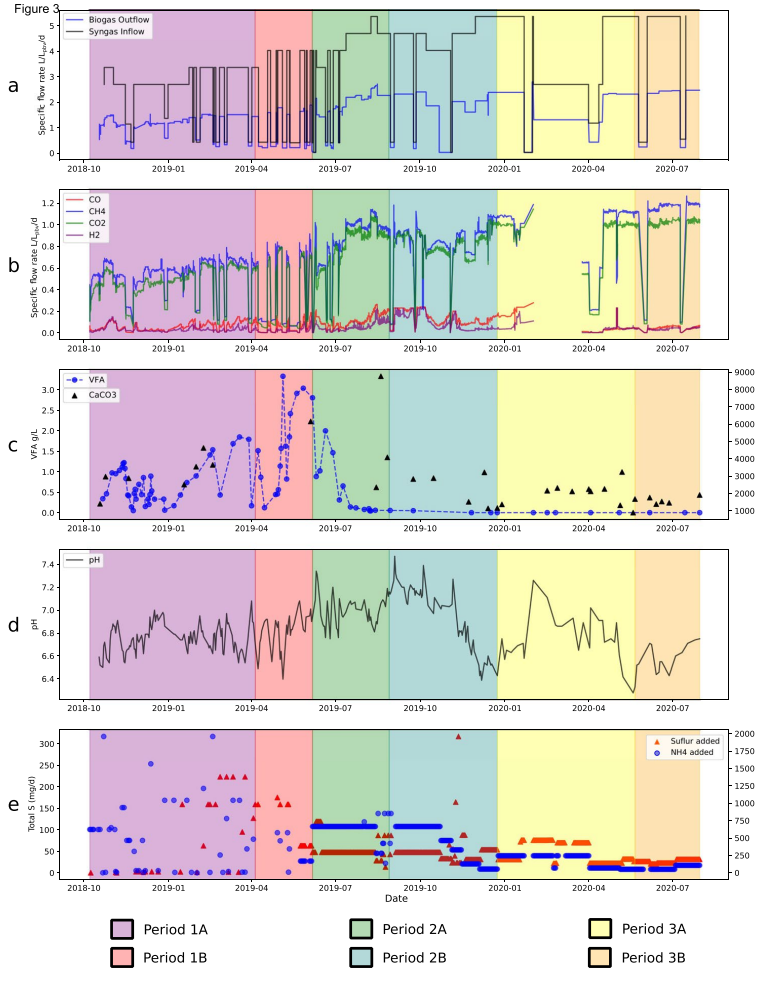

Fig. 3 Process data from trickle bed reactor (TBR) operation during three periods (1–3) operating with different nutrient medium: 1) defined medium (M1) 2) dewatered digestate from a thermophilic biogas plant operating with food waste (M2) and 3) reject water from a biogas plant at a wastewater treatment plant (M3). Each period was further divided into two sub phases (A,B) based on major changes in operating parameters, such as flow rate of nutrient medium (see Table 1). **a** Specific syngas inflow (black) and biogas outflow rate (blue). **b** Specific outflow gas rate. The gap seen in period 3A was due to gas analyser malfunction. **c)** Total volatile fatty acids (VFA) concentration and alkalinity. **d** pH. **e** Total amount of sulphur (S) and ammonium nitrogen (NH4-N) added via nutrient medium and by additional supply via external source. For details, see Supplementary Material

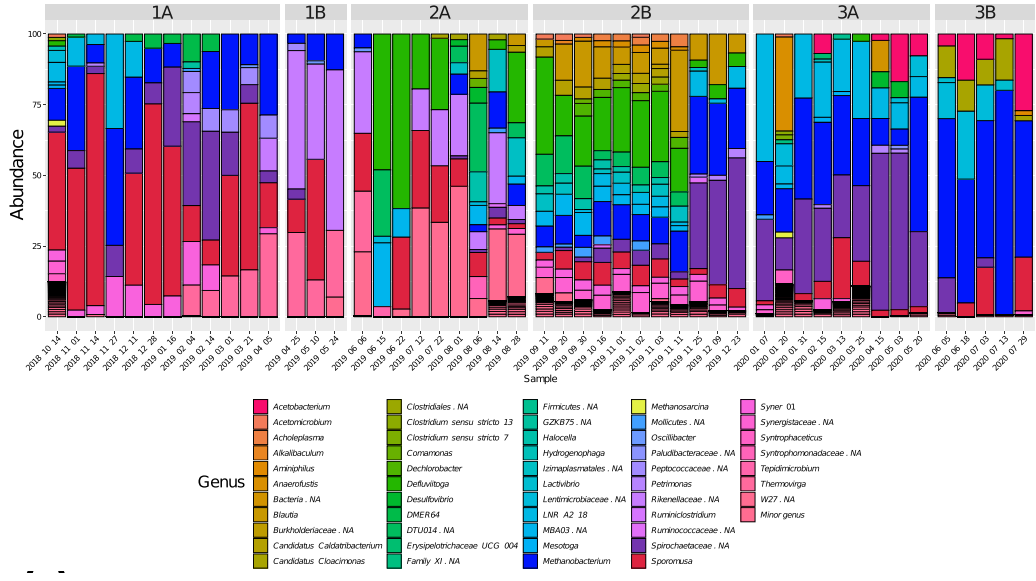

Fig. 4 Microbial community structure at genus level during trickle bed reactor (TBR) operation in three periods (1–3) operating with different nutrient medium: (1) defined mineral medium (M1) (2) dewatered digestate from a thermophilic biogas plant operating with food waste (M2) and (3) reject water from a biogas plant at a wasterwater treatment plant (M3). Each operating period was further divided into two sub-phases (A,B) based on major changes in operating parameters, such as flow rate of nutrient medium (see Table 1)

of phylum *Syngergistetes* (31.6–50.2%), primarily composed of unknown members of family *Synergistaceae*. The abundance of methanogens (phylum *Euryarchaeota*) was very low in this initial phase of operation and they represented less than < 1% of the total community. However, in period 1, when the H_2 level in the syngas was increased, the relative abundance of *Euryarchaeota* increased rapidly and for most samples the value was between 10 and 35% (Fig. S3a). Phylum *Euryarchaeota* was represented mainly by one amplicon sequence variant (ASV), which according to a BLAST search in NCBI corresponded to the putative species *Methanobacterium bryantii* (100% similarity). The bacterial community during period 1 also changed compared with that in the stabilisation/acclimatisation phase. Relative abundance of phylum *Bacteriodetes* was initially low but increased when the VFA content was high, represented by two genera within family *Rikenellaceae*, genus DMER64 (1–7%) in period 1A and an unknown genus in period 1B (11–50%). Genus *DMER64* was mainly abundant when propionate represented a major part of the VFA, while the unknown genus was more abundant when propionate level decreased. Phylum *Firmicutes* and phylum *Syngergistetes* showed significant shifts compared with the stabilisation/acclimatisation phase. Throughout period 1, *Firmicutes* was dominated by a member within genus *Sporomusa*, reaching values between 8 and 90% (Fig. 4). A BLAST search of this ASV showed 100% similarity to the putative species *Sporomusa sphaeroides*. Within *Syngergistetes*, genus *Thermovirga* showed an increasing trend over the period. In addition, phylum *Cloacimonetes* and phylum *Spirochaetes* were present during this period, represented mainly by genus *LNR_A2-18* and an unknown member within genus *Spirochaetaceae*, respectively. Genus *LNR_A2-18* initially increased to a high level (~ 23%) in the beginning of period 1A but showed a significant drop just before the start of VFA accumulation (late December 2018) and thereafter remained at low abundance throughout the rest of period 1. *Spirochaetaceae* showed increased relative abundance with a concurrent decrease in relative abundance of *LNR_A2-18*, and represented up to 35% of the community in period 1A, after which this ASV also decreased in abundance. At the time of the decrease, a shift in VFA composition towards a higher level of acetate relative to propionate was seen.

Period 2

In period 2, the recirculated nutrient solution was changed from the defined medium (M1) to the digestate from the thermophilic biogas plant operating with mixed food waste (M2) and the nutrient solution feeding rate was increased to

1000 mL/day. The syngas inflow rate in period 2A was initially kept at the same level as in period 1B but, as the VFA level was significantly lower than in period 1, the rate was gradually increased to around 4 L/L_{pbv}/d. In line with this increase, the volume of outgoing methane also increased, to reach values of around 0.9–1.10 L CH_4/L_{pbv}/d by the end of period 2A (Fig. 3b). However, towards the end of period 2A, a rise in the outflow levels of CO and H_2 was observed (Fig. 3b), resulting in a decrease in production of CH_4. The decreasing trend in CH_4 production continued during the beginning of period 2B, although VFA were not detected, and the average pH was around 7.2. From the middle to the end of period 2B, the nutrient solution feeding rate was gradually decreased to 200 mL/day, which led to a gradual decrease in NH_4-N and S supply (Fig. 3c). In an attempt to improve syngas conversion efficiency, which was assumed to be limited by S availability, S was added to the process (Fig. 3e, Supplementary Material). This strategy improved syngas conversion, allowing syngas inflow rate to be increased to 5 L/L_{pbv}/d and resulting in an increase in CH_4 production to around 1.1 L/L_{pbv}/d without any accumulation of VFA, although with a decrease in pH to ~ 6.5 (Fig. 3d).

The change of nutrient solution (from M1 to M2) had a major effect on microbial community composition. At the beginning of period 2A, members from the thermophilic phylum *Thermotogae* appeared in high relative abundance, along with *Firmicutes* (Fig. S4a). This community composition mainly reflected the composition of the dewatered digestate used as the nutrient source (Fig. S3a). Phylum *Thermotogae* was represented by genus *Defluviitoga* within the family *Petrotogaceae* (48–56%) and *Firmicutes* were represented by unknown members within two main orders, *DTU014* (6.28–23.49%) and MBA03 (12.36–23.61%). These families stayed in the system until the end of period 2A (Fig. S4b), when the feeding rate of the nutrient medium was reduced to 200 mL/day. In addition, period 2A showed high abundance of phylum *Cloacimonetes* (Fig. S4a), which was not observed in the nutrient solution. This phylum was represented by family *W27* and reached relative abundance values of 17–42% by the end of period 2A (after the decrease in VFA), after which it quickly decreased in period 2B. Moreover, *Sporomusa sphaeroides*, established in period 1 and not present in the digestate, maintained its presence throughout period 2, initially at high relative abundance (~ 20%) but stabilising at lower levels (1.6–6.6%) from the middle of period 2A to the end of period 2B. *Methanobacterium bryantii*, the only methanogen identified in period 2, as in period 1, was present throughout but with increasing relative abundance values towards the end, representing 21–27% of the microbial community (Fig. 4). The community at the end of period 2B was also characterised by high abundance of a member within the genus *Spirochaetaceae* (30–46%), the same species that was dominant in the late stages of period 1.

Period 3

In period 3, the nutrient source was switched to reject water from the mesophilic wastewater treatment plant (M3). The feed rate was 400 mL/d during period 3A and decreased to 200 mL/day during period 3B (Table 1). During the whole of period 3, the process was supported with additional S. In addition, sodium hydrogen carbonate ($NaHCO_3$) was added to mitigate decreasing pH values, as low as 6.4 during period 3B (Fig. 3e, Supplementary Material). In period 3A, the syngas inflow was initially continued at the same rate as in the previous period, i.e. 5 L/L_{pbv}/d, resulting in biogas and methane production of around 2 and 1 L/L_{pbv}/d, respectively (Fig. 3a). However, due to problems with the gas analyser, no data were obtained for the outgoing gas for some time and therefore the syngas inflow was decreased to 2.67 L/L_{pbv}/d in order to avoid the risk of overloading. When the functionality of the gas analyser was restored, the syngas inflow rate was again set to the previous level, which resulted in similar CH_4 production values as before. In period 3B, syngas conversion was maintained at a high level, which led to an average CH_4 production rate of 1.15 L/L_{pbv}/d. No VFAs were observed during operation in period 3 (Fig. 3c).

The use of the new medium (M3) in period 3 had little or no influence on the community composition compared with that in period 2. The relative abundance of *Methanobacterium bryantii*, the dominant methanogen, increased in period 3 compared with period 2 and reached values of 21–45% for most of the samples analysed (Fig. 4). For bacteria, the relative abundance of the previously observed member within the genus *Spirochaetaceae* was initially maintained at a similar level as observed in period 2B, but it decreased gradually when the nutrient flow rate decreased in period 3B, to reach values of 2–3% at the end (Fig. 4). *Sporomusa sphaeroides* was initially high in period 3 but decreased during the pH decrease and then recovered towards the end of period 3B, reaching values of around 10%. In addition, at the end of period 3B, the relative abundance of a member within genus *Acetobacterium* increased and it became one of the most abundant species at the end, representing ~ 31% (Fig. 4). A BLAST search in NCBI showed 100% similarity with the putative species *Acetobacterium wieringae*. In addition, genus LNR_A2-18 (phylum *Cloacimonetes*) was present in high relative abundance (5–26%) throughout period 3. This ASV was the same as that previously identified in high relative abundance in period 1. Another unknown member within this phylum was also identified in period 3B and showed increasing abundance towards the end, representing 12% of the total community. This increase in abundance was the opposite of the decreasing trend seen for *Sporomusa sphaeroides* during the stage of decreasing pH.

Microbial analysis of carrier samples

Carrier samples were taken on only four occasions and only from one position (Fig. 2), due to the difficulty in removing the carrier without causing process disturbance. Independent of sampling time, *Firmicutes* was one of the most highly abundant phyla on the carrier samples, with relative abundance ranging from 37 to 92%, primarily represented by *Sporomusa* at the genus level (Fig. 5). *Euryarchaeota* was present in all carrier samples (Fig. S5), represented mainly by *Methanobacterium* (3–36%), but at the final carrier sampling point in the process (phase 2B), *Methanosarcina* (4%) was also detected (Fig. 5). Phylum *Synergistetes* (4–30%) maintained a presence on the carrier through the four sampling points (Fig. S5) and was comprised mainly of genus *Syner-01* (3–19%) and *Thermovirga* (5–10%) at the first three sampling points. At the final sampling point in period 2B, an unknown genus in *Syngeristaceae* (0.21%) was observed together with *Acetomicrobium* (2.2%) and *Aminobacterium* (0.49%) (Fig. S5). At the final sampling point, there was also comparatively high relative abundance of Bacteroidetes (15%), represented by genus *Fermentimonas* (3%, family *Dysgonomonadaceae*) and a genus belonging to family *Lentimicrobiaceae* (7%) (Fig. 5, Fig. S5b). The ASV belonging to the *Sporomusa* found on all carrier samples was identified as *Sporomusa sphaeroides*, with 99.2% similarity in a BLAST search. The ASV for *Methanosarcina* showed 99.2% similarity with *Methanosarcina flavescens.*

Discussion

Selection of start-up inoculum

The inoculum for the TBR was chosen from an initial screening of three different digestates. The selection of digestate was based on the processes having different microbial communities and dominance of different methane-producing pathways, potentially influencing syngas consumption capacity. It is known that microbial community structure in processes operating with sludge, manure and food waste is different and statistically distinct, mostly driven by differences in ammonia levels (Sundberg et al. 2013; De Vrieze et al. 2015). The food waste reactor (reactor b), operating at a high ammonia level (0.5–0.9 g NH_3/L), was shown to have high abundance of the hydrogentrophic methanogen *Methanoculleus bourgensis* (genus *Methanomicrobiales*) and different known syntrophic acetate-oxidising bacteria, and dominance of methane production via SAO (Westerholm et al. 2015). In the reactor operating with manure (reactor a), quantification of methanogens by qPCR illustrated dominance of order *Methanosarcinaceae* and *Methanobacteriales* and lower levels of *Methanomicrobiales*, suggesting a mix of methylotrophic and hydrogenotrophic methanogenesis. Analysis of the inoculum from the WWTP plant (reactor c) in a previous study showed it to be dominated by the acetoclastic genus *Methanosaeta,* a strict acetoclastic methanogen (Liu et al. 2017). The highest methane production from syngas was seen for inoculum A (from the manure-based

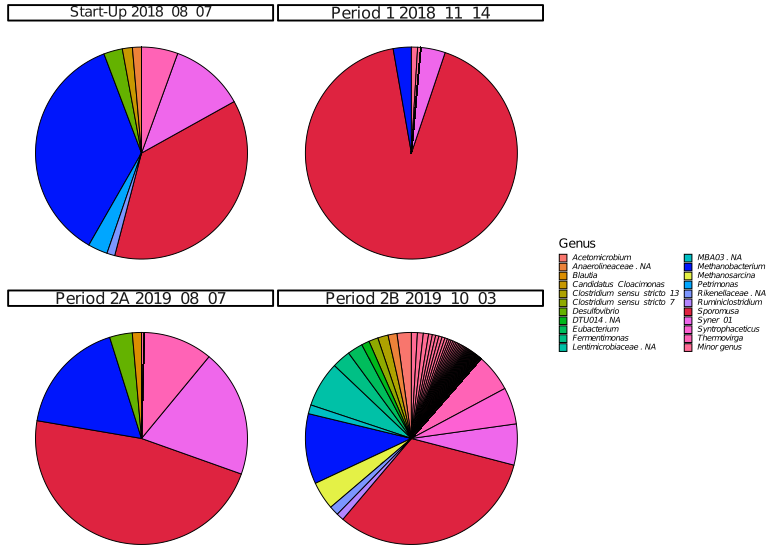

Fig. 5 Microbial community structure on genus level in biofilm recovered from plastic carriers in the trickle bed reactor (TBR) in the start-up phase and in operating periods 1 and 2. Carrier samples taken in period 2B were sequenced in triplicate. Carrier samples taken in start-up, period 1 and period 2A were sequenced without replicates, due to lack of extracted material

reactor), which could have been caused by factors such as (i) the presence of both methylotrophic and hydrogenotrophic methanogenesis, since hydrogenotrophic methanogens mainly utilise hydrogen during syngas methanation but acetate can be produced via acetogenesis from CO, requiring acetoclastic methanogens for further conversion to methane and (ii) high abundance of order *Methanobacteriales*, as several studies on biomethanation in TBR have shown enrichment of methanogens within this order, such as genus *Methanobacterium* and *Methanothermobacter*, indicating importance for biomethanation in such reactors (Aryal et al. 2021; Sposob et al. 2021). Previous studies on syngas methanation in TBR have used inoculum from biogas processes operating with manure (Aryal et al. 2021) and sludge (Grimalt-Alemany et al. 2020; Figueras et al. 2021; Li et al. 2021), or a mix of both (Asimakopoulos et al. 2020a), as well as syngas- or H_2-enriched cultures (Asimakopoulos et al. 2020b, 2021; Sieborg et al. 2020) and defined cultures comprising just a few organisms (Kimmel et al. 1991; Klasson et al. 1992). No obvious trends have emerged that some inocula are more suitable than others. Some thermophilic processes have even been initiated with mesophilic inocula but have still resulted in well-functioning processes (Kimmel et al. 1991; Grimalt-Alemany et al. 2020; Li et al. 2020b). However, for methanation of CO_2 with H_2, inoculation with enriched culture is reported to shorten the lag phase during start-up (Sposob et al. 2021). Microbiological studies of continuously operated TBR have observed a complete change in syngas-enriched communities compared with the inoculum and high adaptive capacity, likely due to intrinsic biological diversity (Asimakopoulos et al. 2020a; Grimalt-Alemany et al. 2020). Such a change was also observed in the present study and is discussed further below in the section 'Microbial communities in the trickle bed reactor'.

Methane productivity and VFA accumulation

Previous studies on productivity during biomethanation in TBR have reported different values, influenced by different parameters such as reactor design, carrier material, inflow gas composition and rate, nutrient composition and loading rate, gas injection, operating time and inoculum source (Asimakopoulos et al. 2020a; Grimalt-Alemany et al. 2020b; Aryal et al. 2021; Sposob et al. 2021). For methanation of gas composed of only H_2 and CO_2, values of around 1.17–3.1 L_{CH4}/L/d during mesophilic (38–40 °C) operation have been reported (Burkhardt and Busch 2013; Burkhardt et al. 2015; Rachbauer et al. 2016). During thermophilic operations, considerably higher values, up to 8.85–15.4 L_{CH4}/L/d, have been reported (Strübing et al. 2017; Lemmer and Ullrich 2018). For methanation from syngas, values between 0.21–1.90 and 1.88–9.46 L_{CH4}/L/d for mesophilic (Grimalt-Alemany et al. 2018) and thermophilic conditions, respectively, have been reported. The higher productivity at higher temperatures is suggested to relate to higher conversion efficiencies resulting from increased methanogenic activity and abundance (Lemmer and Ullrich 2018; Asimakopoulos et al. 2020b). The TBR in the present study was operated under mesophilic conditions, and methane production was in line with that in previous studies operating at this temperature, reaching maximum values of 0.9–1.2 L_{CH4}/L_{pbv}/d. Production efficiency was lowest at the beginning of the process (period 1), mainly caused by difficulties in increasing the load of syngas due to accumulation of organic acids and low pH values. Accumulation of VFA indicates imbalances between the microbiological steps in digestion, with acid formation rate exceeding methanogenesis. Instances of acid accumulation have been observed previously during methanation of syngas, particularly in response to increasing levels of H_2 in the syngas, and it is believed to be caused by inhibition of syntrophic acid conversion (Li et al. 2021). The acids produced in the present study were initially composed of both acetate and propionate but shifted towards a higher fraction of acetate at the end of the period. Acetate is the main product of acetogenesis, but none of the acetogenic carboxydotrophs isolated to date can produce propionate. However, in addition to acetate, acetogens can also produce ethanol and small amounts of butyrate, butanol and 2,3-butanediol, which in turn can be converted by other bacteria to propionate (Moreira et al. 2021). Organic acids were degraded in the second period of operation in this study (period 2), which allowed a higher syngas load and initially resulted in slightly increased methane productivity. Degradation of propionate proceeds via syntrophic collaboration and results in formation of acetate and hydrogen, which if present in high levels can block further degradation (Westerholm et al. 2021). Hydrogen is used by methanogens, but acetate can be converted via acetoclastic methanogens or via SAO (Westerholm et al. 2021; Sancho Navarro et al. 2016). No acetoclastic methanogens were detected in period 1, which might explain why acetate accumulated, although accumulation could also have been caused by decreased hydrogenotrophic methanogenic activity, causing problems for acetate degradation via SAO. Methanogenic activity can be inhibited by CO, VFA and low pH (Luo et al. 2013; Sancho Navarro et al. 2016; Grimalt-Alemany et al. 2018), which all appeared at the same time in period 1. The relative abundance of methanogens was lowest in periods 1B and 2A, which could have resulted in less efficient acid degradation. The improved VFA conversion observed in period 2 could have been caused by factors such as (i) acclimatisation of the methanogenic community to inhibiting conditions; (ii) installation of the anaerobic filter, prolonging the time for degradation and/

or (iii) the change of nutrient medium, providing buffering capacity and additional nutrients for improved microbial growth or providing new microbes, including acetate-degrading microorganisms.

Effect of nutrient source

Among the various parameters influencing the methanation processes and the activity of the microorganisms involved, the nutrient source is of crucial importance. For economic feasibility of full-scale applications, finding a cheap nutrient source is essential (Wegener Kofoed et al. 2021). Ideally, the nutrient medium should supply macronutrients and micronutrients, as well as buffering agents that can help to stabilise the pH in the event of acid formation (Sposob et al. 2021). For biological CO_2 methanation, including TBR, several different non-defined cheap nutrient sources, such as digestate from different biogas processes and manure, have been evaluated and have been shown to be economically feasible for both mesophilic and thermophilic operation (for reviews, see Sposob et al. 2021; Wegener Kofoed et al. 2021). However, many previous studies have used defined nutrient medium for syngas methanation (Grimalt-Alemany et al. 2018; Asimakopoulos et al. 2020a, 2021; Grimalt-Alemany et al. 2020) and only a few have evaluated non-defined nutrients sources, mostly in batch systems (Ács et al. 2019; Aryal et al. 2021). To our knowledge, only one previous study has used a undefined nutrient source during continuous operation of a TBR for syngas methanation (Figueras et al. 2021). The batch reactors were operated under mesophilic conditions and with digestate from manure and wastewater processes and methane production reached only 0.15–0-22 L CH_4/L/d. However, during continuous operation with dewatered WWTP digestate for more than 70 days under thermophilic conditions (55 °C), methane production values of 6.8 mmol $CH_4/L_{reactor}$/h, corresponding to 3.65 L L_{CH4}/L_{pbv}/d, have been reported (Figueras et al. 2021). This is three-fold the value obtained in the present study when using a similar nutrient source (in period 3 of operation). A likely explanation for the higher productivity is thus the higher temperature rather than the nutrient source, and possibly the higher pressure (~4 atm) applied in the previous study (Figueras et al. 2021) than in the present study (1.5 atm).

There is currently no consensus on the medium composition and origin that represent the best nutrient source for a biomethanation process (Wegener Kofoed et al. 2021). Nutrients suggested to be of specific importance for methanation from both H_2/CO_2 and syngas include macronutrients such as N, S and P and various trace elements, all of importance for methanogenic activity (Strübing et al. 2017; Li et al. 2020b; Figueras et al. 2021; Wegener Kofoed et al. 2021). According to the results in this and other studies, undefined nutrient sources of different origins can work well for biomethanation, offering the possibility to establish full-scale sustainable processes using cheap nutrient sources. A possible drawback/limitation with such nutrient sources is the need for pre-treatments to remove particles and to prevent growth of unintended microorganisms potentially also producing biogas from additional carbon sources (Sposob et al. 2021). In the present study, a shift was made from a defined nutrient medium to two different types of digestate. Neither of these digestates was sanitised before use, but the digestate from the food waste biogas plant (M2) had to be filtered before use. The process showed better performance during operation with the undefined nutrient sources than with the defined medium, with no acid accumulation and with the possibility for higher syngas loads. However, the defined medium was used in the initial phase of TBR operation and it cannot be concluded that this medium was less beneficial for the process, since process stabilisation and biofilm development may not have been complete. The periods with undefined medium occasionally suffered from low pH (with no VFA accumulation), particularly when the nutrient flow rate was lowered, leading to additional need for buffering capacity. Moreover, during most of the TBR operating time, the process was supported by additional S, in all phases, indicating a need for additional nutrients. During methanation of syngas in the study by Figueras et al. (2021), supplementation with additional S (Na_2S) was found to be beneficial for the process. Similar findings were made in a previous study on biomethanation from H_2/CO_2 at thermophilic temperature using a TBR (Strübing et al. 2017). Strübing et al. (2017) suggested that sulphur deficiency could be caused by the loss of sulphide in the off-gas due to trickling of the liquid. These previously observed positive effects of sulphide addition were confirmed in the present study, where supplementation with additional sulphide was found to be beneficial for the conversion efficiency in periods 1A, 1B and 3A. Methanogens mainly use sulphide as a sulphur source, but some can also assimilate cysteine (Liu et al. 2012), and both were present in the defined medium (M1) in this study. In periods 2B and 3B, addition of sulphate instead was evaluated (Supplementary Material). Sulphate is not used directly by methanogens but can be converted to sulphide by sulphate-reducing bacteria present in the recycled nutrient medium. No obvious difference in methane productivity related to S source was however seen in this study.

Microbial communities in the TBR

Methanogenic community

Throughout the process, independent of nutrient medium, one dominant methanogen was observed, represented by one ASV showing 100% similarity with *Methanobacterium*

bryantii. Even though the digestate-based nutrient sources (M2 and M3) were not sterilised, methanogens from these sources did not establish in the process. Nutrient medium M2 was derived from a thermophilic biogas process, which might explain why no methanogens from this medium established in the mesophilic TBR. However, transition from thermophilic to mesophilic conditions with inoculum from the same biogas plant as M2 has been shown to be possible, illustrating the presence of mesophilic methanogenic species in this biogas plant (Westerholm et al. 2018). Nutrient medium M3 was derived from a mesophilic biogas plant, but both M2 and M3 showed little to no abundance of methanogens (representing less than 1% of the whole community), possibly also explaining the low contribution of methanogens to the methanation process in the TBR. The dominant methanogen, identified as *Methanobacterium bryantii*, is a hydrogenotrophic methanogen using H_2/CO_2, but not formate (Benstead et al. 1991). It is unclear whether this bacterium can use CO, but other members within this genus, e.g. the thermophilic *Methanobacterium thermoautotrophicus*, can grow with CO as the sole energy source, although at very low growth rates (Ferry 2010). Members within the genus *Methanobacterium* have been found to dominate in several other studies on biomethanation in TBR at both mesophilic and thermophilic temperatures (Rachbauer et al. 2017; Porté et al. 2019) and in other processes during ex situ and in situ biomethanation of H_2 and syngas (Li et al. 2020a; Aryal et al. 2021; Jiang et al. 2021; Braga Nan et al. 2022). In line with results in the present study, this genus has also been found on carrier biofilm (Rachbauer et al. 2017; Thapa et al. 2021). Moreover, in several studies it has been observed under mesophilic conditions together with the hydrogenotrophic genus *Methanoculleus* and is suggested to be more crucial of the two in restoring efficiency after starvation periods and VFA accumulation, while also being decisive for efficient biomethanation due to high hydrogen consumption rates (Logroño et al. 2021; Braga Nan et al. 2022). *Methanoculleus*, a known partner during SAO and prevailing under low hydrogen concentrations (Westerholm et al. 2016), was not detected in the present study. In addition to *Methanobacterium*, genus *Methanosarcina* was observed in low relative abundance on the carrier biofilm in period 2B. In BLAST searches, this ASV was identified as *Methanosarcina flavescens*, a methanogen utilising both acetate and H_2/CO_2 for growth, and also methanol and methylamines (Kern et al. 2016). Establishment of this genus in period 2 could have occurred via the nutrient medium, as it has been identified previously at low abundance in the biogas plant from which the digestate originated (Westerholm et al. 2018). However, the genus could not be detected in the nutrient solution M2 (Fig. S3). In the process, *Methanosarcina* sp. could have used either acetate or H_2/CO_2, or both, but its enrichment in period 2B after the observed decrease in acetate concentration suggests that it acted as an acetoclastic methanogen. However, members of this genus, including *M. flavescens*, may be able to shift their metabolism from acetate to H_2 in response to increasing partial pressure of H_2, making them more competitive for hydrogen (Thapa et al. 2021). Thus, it is possible that *M. flavescens* acted as a hydrogenotrophic methanogen in the present study, together with *Methanobacterium*. Moreover, it is possible that *M. flavescens* used CO, as several species within genus *Methanosarcina* have been demonstrated to have this ability (Oelgeschläger and Rother 2008). In line with the results in this study, *M. flavescens* was recently identified at higher abundance in biofilm than in the liquid phase during in situ biogas upgrading in an anaerobic TBR treating thermal post-treated digestate (Thapa et al. 2021).

Bacterial community

The AVS identified as *Sporomusa sphaeroides* showed high abundance throughout the operation. This genus is known to utilise H_2 and CO_2, why it seems likely that this acetogen competed with the methanogen for its substrate. Some species within genus *Sporomusa* can also utilise CO, however, not *S. sphaeroides* (see review by Bengelsdorf et al. 2018). The end product of *Sporomusa* is mainly acetate, but *S. sphaeroides* can also produce small amount of ethanol (Möller et al. 1984). In addition to *Sporomusa*, one unknown member of *Spirochaetaceae* was highly abundant in the first phase of period 1. Members within *Spirochaetaceae* have been proposed to be involved in syntropic acid degradation, specifically acetate, together with methanogens (Wang et al. 2019). The abundance of this genus decreased/increased with observed acetate accumulation/consumption, indicating involvement in syntrophic acetate degradation also in this study. In the period with high VFA levels, increasing abundance of family *Rikenellaceae* and genera *Thermovirga* was also observed. Members within *Thermovirga* cannot use H_2/CO_2 or fatty acids but utilise proteinaceous substrate and amino acids, including cysteine, while producing acetate as the end product (Dahle and Birkeland 2006). This bacterium likely contributed to production of acetate, using yeast extract and/or the cysteine used as a reducing agent in nutrient medium M1. The abundance of this genus decreased to < 1% when the nutrient medium was changed in periods 2 and 3, but it was still found in biofilm on carrier samples from period 2, suggesting that also the digestates supported growth of this genus. Family *Rikenellaceae* contains several different genera, and species isolated so far can ferment carbohydrates or proteins and grow on yeast extract, while producing acetate and propionate and also H_2 and CO_2 and other acids (Krieg et al. 2010; Abe et al. 2012; Graf 2014; Su et al. 2014). Members of this family have been found previously in batch reactors fed with syngas (Aryal et al. 2021). In

the present study, family *Rikenellaceae* was represented by two different genera, one of which (genus DMER64) is suggested to be a potential syntrophic propionate degrader (Lee et al. 2019). This is in line with the decreased abundance of this genus with decreasing propionate concentration. This genus possibly took over the role of propionate degrader from genus LNR_A2_18, within family *Cloacimonadaceae*, that was initially present in the TBR. This family is suggested to act also as a syntrophic propionate degrader and its disappearance has been shown to be accompanied by an increase in propionate (as in the present study) and to be an indicator of process disturbance (Klang et al. 2019; Singh et al. 2021).

In period 2A, a drastic change in microbial community composition was seen. An immediate rise in several well-known thermophilic microorganisms was observed, i.e. genus *Defluviitoga* (phylum Thermotogae) and order MBA03 and DTU014 (phylum Firmicutes). This composition was very much influenced by the microbial composition of the nutrient medium, which changed in period 2 to thermophilic food waste digestate at a high flow rate. The observed organisms were highly abundant in the nutrient medium per se, and is also common in thermophilic biogas reactors (Westerholm et al. 2018; Dyksma et al. 2020). It has been suggested that members within these taxonomic groups perform carbohydrate fermentation and do not have the ability to use gaseous substrates, so most likely, they did not contribute to the methanation process. However, in contrast, genus W27, within family *Cloacimonadaceae*, was highly abundant in period 2A and not detected in the nutrient medium. As mentioned earlier, members within this taxonomic group are suggested to be involved in propionate degradation (Westerholm et al. 2021). However, it is difficult to predict the role of genus W27 in the present study, as it was highly abundant in period 2A after VFA had been degraded and disappeared in phase 2B. The high nutrient flow rate may have supplied the process with substrates for bacteria producing propionate, but kept to a low level by the genus W27. In period 2B, when the nutrient flow was reduced, there might not have been enough substrate to maintain the growth of this organism at a high level. In period 2B, no accumulation of acids was seen and the process appeared to be more stable than in period 1 (with defined nutrient medium). Degradation of propionate, and acetate, via syntrophic reactions, only proceeds at low P_{H2}, and thus, the observed improved VFA degradation could potentially have been caused by a more efficient H_2 turnover in period 2B as compared to 2A. However, looking at the hydrogen level in the gas out flow illustrated small differences in this regard between the periods. The more complex medium M2 may instead have allowed for more efficient acetate turnover by enrichment of the potential acetate oxidiser *Spirochaetaceae*. The improved acetate conversion might also be explained by the establishment of this organism on the carrier biofilm, which was observed in period 2B, but not 2A. However, in periods 1 and 2A, other members within phylum *Synergistetes*, also representing potential acetate oxidisers, were observed.

In period 3, when the nutrient solution was changed to digestate from a mesophilic wastewater biogas system, the microbial community initially maintained the same composition as at the end of period 2B, with dominance of *Methanobacterium*, *Sporomusa* and *Spirochaetaceae*. Approaching the end of period 3A, the *Spirochaetaceae* genus even became dominant in the community (55.5%). In addition, the potential propionate-degrading LNR_A2-18 (phylum *Cloacimonetes*) reappeared in the community. The high abundance of these two organisms likely contributed to the low acid level in this period of operation. However, on entering period 3B, *Spirochaetaceae* decreased in abundance (12.4–3.3%), possibly coinciding with a drop in pH since it is suggested to be favoured by slightly basic conditions (Lee et al. 2013). Moreover, in this period, an increased abundance of *Acetobacterium wieringae* was observed. This species can grow and produce acetate while consuming CO_2 and H_2 (Braun and Gottschalk 1982; Poehlein et al. 2016). A recently isolated novel strain of *Acetobacterium wieringae* is also able to grow on carbon monoxide (100% CO), producing mainly acetate as the end product (Arantes et al. 2020). Thus, in this phase of TBR operation, CO might have been used by this organism. The CO-utilising organisms in operating periods 1 and 2 were not identified, but the member within *Sporomusa* found in both periods could have been a CO utiliser.

In conclusion, methanation of syngas (56% H_2, 30% CO, 14% CO_2) in a TBR during long-term operation (862 days) was possible using different nutrient sources (defined nutrient medium, dewatered digestate from a thermophilic biogas plant treating food waste and reject water from a biogas plant at a wastewater treatment plant). The process reached maximum methane production levels of 0.9–1.15 $L/L_{PBV}/d$, with some variation during operation, which corresponded to similar production levels as observed before at mesophilic conditions. The process showed some imbalance with accumulation of VFA in period 1, when the defined nutrient medium was used. However, concentrations declined in later operating periods with undefined medium and after introduction of an anaerobic filter to prolong nutrient recycling time.

For the microbial community, the overall trend within each period with different nutrient medium was stabilisation towards the same dominant species by the end of the period. Thus, the community was altered at the start of each period with the change in nutrient source, but after some time, it returned to the composition established prior to the change in nutrient medium. The main microbes observed included *Methanobacterium*, as the dominant methanogen, and the acetogen *Sporomusa*, as a dominant bacterial genus, both in liquid and on the carriers. These are both using hydrogen

and carbon dioxide, while producing mainly methane and acetate, respectively, and have commonly been detected in various biomethanation processes before. Acetate was likely mainly converted via syntrophic acetate oxidation by an abundant representative within the genus *Spirochaetaceae* but could also have been directly converted to methane via *Methanosarcina*, present on the carriers. *Acetobacterium* also appeared later in the process and represent a potential CO-consuming acetogen.

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Author contribution ÅN and AS contributed to the study conception and design. Material preparation, data collection and analysis were performed by FG, LP, HO and GC. The first draft of the manuscript was written by AS and GC and all authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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Data availability The sequence data generated and analysed from this study is made available in NCBI repository in BioProject PRJNA796200.

Declarations

Ethics approval No human or animal participants were involved in this study.

Consent for publication All authors have read and approved the final manuscript.

Conflict of interest The authors declare no competing interests.

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Acetogens are ubiquitous and well-studied in many anaerobic environments. However, they remain enigmatic in terms of identity and function in the biogas systems. Unveiling novel acetogens in such environments can provide crucial information for optimising biogas systems. This thesis aimed to expand the knowledge of acetogenic players involved in the penultimate step of anaerobic digestion in different biogas systems, using both molecular and cultivation-based methods. During the investigation of several unknown candidates, the definition of acetogens was called into question.

George Cheng received his graduate education at the Department of Molecular Sciences, SLU, Uppsala. He received his M.Sc. in Biology at Department of Ecology at Uppsala University, Uppsala and B.Sc. in Biology at the University of Illinois, Urbana-Champaign.

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