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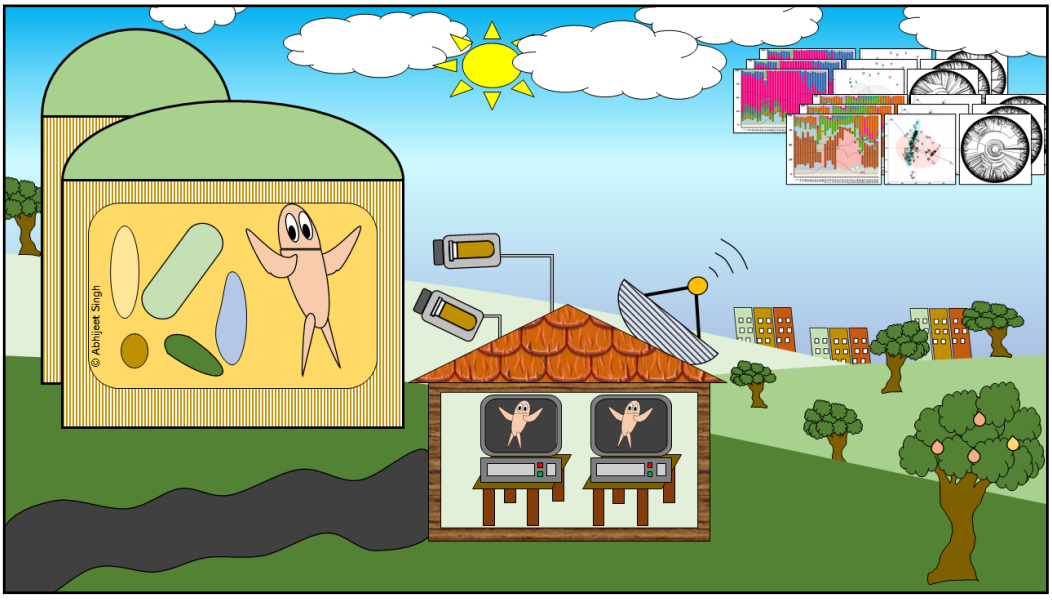
Microbiological surveillance of biogas plants

Focusing on the acetogenic community

Abhijeet Singh



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Cover: Microbiological surveillance strategy developed and used in this thesis for the acetogenic community surveillance in biogas plants.
(Photo: Abhijeet Singh)

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Microbiological surveillance of biogas plants

Abstract

Biogas process has great potential for reducing the current dependence on fossil fuels and for climate mitigation and sustainable development. In this process organic matter is decomposed under anerobic conditions by microorganisms to form biogas and a nutrient rich biofertiliser. For adequate use of the resources invested in commercial biogas production, constant monitoring and optimisation are extremely important. The biogas microbiome has been thoroughly studied, but remains a black box in terms of the microbe identity/diversity and functions/interactions in biogas production. Among known bacterial communities, acetogenic bacteria play a critical imperative role in the biogas process, so close monitoring or surveillance of the acetogenic community is important to ensure process stability and productivity.

This thesis presents a new microbiological surveillance strategy targeting the acetogenic community in biogas reactors and describes the underlying theory, tools and application. In the strategy, a database (AcetoBase) and a bioinformatics analysis pipeline (AcetoScan), developed within this thesis, are employed for surveillance of acetogenic communities in laboratory- and industrial-scale biogas facilities. Meticulous comparison of the surveillance strategy with conventional methods demonstrated its superiority in envisioning acetogenic community structure and dynamics. Acetogenic community surveillance using the strategy showed that acetogenic communities in biogas reactors are substrate-specific, diverse and dynamic. The dynamic response of acetogenic communities imparts strength in resisting disturbance and potential to recover post-disturbance. Future use of the acetogenic community surveillance strategy can greatly improve understanding of the acetogenic communities and their utilization for biogas process stability.

Keywords: AcetoBase, Acetogenesis, Acetogens, AcetoScan, Anaerobic digestion, Biogas, FTHFS, Monitoring, Surveillance, Wood-Ljungdahl pathway

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Microbiological surveillance of biogas plants. Focusing on the acetogenic community

Abstract

Biogasprocessen har stor potential att minska beroendet av fossila bränslen och att bidra till en hållbar utveckling. I denna process sönderdelas organiskt material i en syrefri miljö av mikroorganismer till biogas och biogödsel. För tillräcklig användning av de resurser som investeras i kommersiell biogasproduktion är processoptimering och konstant processövervakning extremt viktigt. Biogasmikrobiomet har studerats noggrant, men förblir en svart låda när det gäller både identitet/mångfald och funktioner/interaktioner. Bland kända bakteriesamhällen spelar acetogena bakterier en viktig roll i biogasprocessen, och noggrann övervakning av denna bakteriegrupp är viktig för att säkerställa processens stabilitet och produktivitet.

Denna avhandling presenterar en ny mikrobiologisk övervakningsmetod inriktad på acetogena bakterier i biogasreaktorer och beskriver den underliggande teorin, verktygen och tillämpningen. Metoden, som inkluderar en databas (AcetoBase) och en pipeline för bioinformatikanalys (AcetoScan), utvecklades inom denna avhandling och användes för analys av biogasanläggningar i laboratorie- eller industriell-skala. En noggrann jämförelse av den utvecklade övervakningsstrategin med konventionella metoder visade att den är överlägsen när det gäller att beskriva acetogen samhällsstruktur och dynamik. Analysen visade också att acetogena samhällen i biogasreaktorer är substratspecifika och olika och att ett dynamiskt svar ger styrka i att motstå störningar, och potential för återhämtning efter störningar. Framtida användning av den utvecklade övervakningsstrategin kan avsevärt förbättra förståelsen för acetogena bakterier och deras betydelse för biogasprocessstabilitet.

Keywords: AcetoBase, Acetogenesis, Acetogens, AcetoScan, Anaerobic digestion, Biogas, FTHFS, Monitoring, Surveillance, Wood-Ljungdahl pathway

Author’s address: Abhijeet Singh, Swedish University of Agricultural Sciences, Department of Molecular Sciences, Uppsala, Sweden

Preface

The purpose of this thesis is to introduce and demonstrate a new microbiological surveillance strategy for the acetogenic bacterial communities in biogas environments. The new strategy is based on the modern DNA sequencing approach and computer-assisted unsupervised analysis.

This thesis should be of interest to operators in decision making for the stable operation of biogas plants. It should also be of interest to environmental microbiologists in decoding the acetogenic community structure in different natural or artificial environments and to researchers in understanding the role of acetogenic community in human gut-brain physiology.

Dedication

To my parents and Pt. Shriram Sharma Acharya.

भूर्भुवः स्वः तत्सवितुर्वरेण्यं भर्गो देवस्य धीमहि धियो योनः प्रचोदयात् ||

May illuminate our intellect to guide us to the righteous path.

- Rig Veda (3.62.10)

सर्वे भवन्तु सुखिनः, सर्वे सन्तु निरामयाः।
सर्वे भद्राणि पश्यन्तु, मा कश्चिद्दुःखभाग्भवेत।

May all sentient beings be at peace, may no one suffer from illness.
May all see what is auspicious, may no one suffer.

- Brihadaranyaka Upanishad (1.4.14)

Contents

List of publications

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

I. Singh, Abhijeet, Bettina Müller, Hans-Henrik Fuxelius, and Anna Schnürer. 2019. "AcetoBase: A Functional Gene Repository and Database for Formyltetrahydrofolate Synthetase Sequences." Database 2019. doi: 10.1093/database/baz142.

II. Singh, Abhijeet, Johan A. A. Nylander, Anna Schnürer, Erik Bongcam-Rudloff, and Bettina Müller. 2020. "High-Throughput Sequencing and Unsupervised Analysis of Formyltetrahydrofolate Synthetase (FTHFS) Gene Amplicons to Estimate Acetogenic Community Structure." Frontiers in Microbiology 11(2066):1–13. doi: 10.3389/fmicb.2020.02066.

III. Singh, Abhijeet, Bettina Müller, and Anna Schnürer. 2021. "Profiling Acetogenic Community Dynamics in Anaerobic Digesters - Comparative Analyses Using next-Generation Sequencing and T-RFLP." BioRxiv 2021.01.26.427894. doi: 10.1101/2021.01.26.427894.

IV. Singh, Abhijeet, Moestedt, Jane, Berg, Andreas & Schnürer, Anna. (2021). Microbiological Surveillance of Biogas Plants. (Manuscript).

Papers I-II are reproduced with the permission of the publishers.

The contribution of Abhijeet Singh to the papers included in this thesis was as follows:

I. Co-created the study, performed all the data retrieval, curation and analysis and helped in the development of a web interface for the public database. Main author of the manuscript.

II. Participated in planning the study, conceptualised and was the main developer of the bioinformatics pipeline. Performed all the laboratory work, data analysis and visualisation. Main author of the manuscript.

III. Co-designed the study and performed all the laboratory work, data analysis and visualisation. Main author of the manuscript

IV. Was involved in planning the study and performed all the laboratory work, data analysis and visualisation. Main author of the manuscript.

In addition to paper I-IV Abhijeet Singh contributed to the following papers within the timeframe of the thesis work:

1. Ahlberg Eliasson, Karin, **Abhijeet Singh,** Simon Isaksson, and Anna Schnürer. (2018). "Co-substrate composition critical for efficiency during biogas production from cattle-manure" (Manuscript).
2. Brandt, Christian, Adrian Viehweger, **Abhijeet Singh**, Mathias W. Pletz, Daniel Wibberg, Jörn Kalinowski, Sandrina Lerch, Bettina Müller, and Oliwia Makarewicz. 2019. "Assessing Genetic Diversity and Similarity of 435 KPC-Carrying Plasmids." Scientific Reports 9(1):1-8. doi: 10.1038/s41598-019-47758-5.
3. Cunningham, Janet L., Ludvig Bramstång, **Abhijeet Singh**, Shishanthi Jayarathna, Annica J. Rasmusson, Ali Moazzami, and Bettina Müller. 2020. "Impact of Time and Temperature on Gut Microbiota and SCFA Composition in Stool Samples." PLOS ONE 15(8):e0236944.
4. Saheb-Alam, Soroush, **Abhijeet Singh**, Malte Hermansson, Frank Persson, Anna Schnürer, Britt-Marie Wilén, and Oskar Modin. 2017. "Effect of Start-Up Strategies and Electrode Materials on Carbon Dioxide Reduction on Biocathodes" edited by H. L. Drake. Applied and Environmental Microbiology 84(4). doi: 10.1128/AEM.02242-17.
5. **Singh, Abhijeet.** 2019. "FastA2Q." https://github.com/abhijeetsingh1704/fastA2Q. doi: 10.13140/RG.2.2.13695.15529.
6. **Singh, Abhijeet.** 2020a. "DupRemover: A Simple Program to Remove Duplicate Sequences from Multi-Fasta File". GitHub, DOI: 10.13140/RG.2.2.23842.86724. https://github.com/abhijeetsingh1704/Duplicate-remover.
7. **Singh, Abhijeet.** 2020b. "REDigest: A Python GUI for In Silico Restriction Digestion Analysis for Gene or Complete Genome Sequences". GitHub; https://github.com/abhijeetsingh1704/REDigest.
8. **Singh, Abhijeet**, Anna Schnürer, and Maria Westerholm. 2021. "Enrichment and Description of Novel Bacteria Performing Syntrophic Propionate Oxidation at High Ammonia Level." Environmental Microbiology 1462-2920.15388. doi: 10.1111/1462-2920.15388.
9. Westerholm, Maria, Bettina Müller, **Abhijeet Singh**, Oskar Karlsson Lindsjö, and Anna Schnürer. 2018. "Detection of Novel Syntrophic

Acetate-Oxidizing Bacteria from Biogas Processes by Continuous Acetate Enrichment Approaches." Microbial Biotechnology 11(4):680-93. doi: 10.1111/1751-7915.13035.

List of figures

Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
AD	Anaerobic digestion
FTHFS	Formyltetrahydrofolate synthetase
HRT	Hydraulic retention time
OLR	Organic loading rate
SAOB	Syntrophic acetate-oxidising bacteria
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
VFA	Volatile fatty acids
WLP	Wood-Ljungdahl pathway
qPCR	Quantitative polymerase chain reaction

1. Introduction

The 21st century is the century of technology and innovations. Standing tall on the shoulders of the 20th century, development is now proceeding at an unprecedented pace. Technological progress to date has brought humanity within one step away from being an interplanetary species. The ambition of becoming a species with a presence on multiple planetary objects is fuelled by the innate curiosity of human beings and the uncertainty of human existence on Planet Earth. For the first time in the history of existence, humans have changed the climate of an entire planet, which has created the risk of extinguishing life on Earth. Increases in the levels of greenhouse gases (*e.g.* carbon dioxide (CO_2), methane (CH_4)), mainly due to anthropogenic activities, have resulted in an increase in the average temperature on Earth, *i.e.*, global warming (Flannery, 2010). At the end of 2020, the United Nations vigorously appealed to all nations to declare 'climate emergency' (Deutsche Welle, 2020; The Guardian, 2020). To mitigate this drastic climate situation, global net carbon dioxide emissions must be curbed. Renewable and low-carbon energy is needed to alleviate the devastating climate situation, without impeding overall development of human society, especially in developing and under-developed countries.

Modern society is extremely technology-driven and energy demanding. Renewable energy types such as solar, wind, tidal energy *etc.* are ever-present and infinite sources of power. However, they are very expensive, require high technological infrastructure, have specific geographical prerequisites and also have some disadvantages (Capareda, 2013; Nelson & Starcher, 2015). This hampers wide-scale installation and use of renewable sources of energy. Bioenergy is an alternative source of power that can be

produced and used without a radical change in the current technological framework and is thus comparatively very economical (Robles *et al.*, 2018). Biofuels are the source of bioenergy and they have great potential to minimise dependency on fossil fuels, increase fuel security, mitigate climate change, enables sustainable development *etc*. There are different types of biofuels, *e.g.* biogas, biodiesel, biohydrogen, ethanol *etc*. (Mousdale, 2010). Biogas, or biologically produced methane is a unique fuel because it can easily be used in gaseous or liquid state and it is generated together with a co-product, biodigestate, which can be used as nutrient rich fertiliser (Koonaphapdeelert *et al.*, 2020; Ma *et al.*, 2017). Methane can also be extracted from methane hydrates, methane clathrates or methane ice, but is then considered an unconventional low-carbon fossil fuel which is not sustainable and will contribute to net carbon emissions (Reijnders, 2009; Stephenson, 2018). Therefore, this thesis focuses only on biomethane, the biologically produced and renewable form of methane. Biomethane is the upgraded/pure/refined product of biogas (Koonaphapdeelert *et al.*, 2020). It is considered to be the fuel of the future not only for Planet Earth but also for space missions, and is a perfect fuel for next-generation rocket and aviation engines (Dhoble & Pullammanappallil, 2014; Hiroyuki, 2018; Koonaphapdeelert *et al.*, 2020; Leucht, 2018; Newton, 2015; O'Callaghan, 2019; Ramesh, 2019; Reijnders, 2009)

Scientifically and commercially, the process of biogas production is called anaerobic digestion (AD) or the 'biogas process'. In the biogas process, almost any biodegradable material can be used as substrate for microbial decomposition to produce biogas and biofertiliser. This microbiological disintegration is performed by the cumulative action of complex anaerobic microbial communities. Anaerobic digestion is an ancient method, but throughout history has been used mainly for the purpose of sanitisation (Bond *et al.*, 2013; Lofrano & Brown, 2010). In the late 17th and early 18th century, it was realised that anaerobic digestion can be used for producing biogas as a renewable fuel source (Marchaim, 1992). Anaerobic digestion is a multipurpose process for the treatment of organic waste, sanitisation, production of renewable low-carbon energy, production of quality biofertiliser and reduction of methane emissions from biowaste (Marchaim, 1992; WBA, 2018) (***Figure 1***). The anaerobic digestion process

has potential to reduce global greenhouse gas emissions by ~20% to meet the commitments of UNFCCC Paris Agreement and contributes to at least nine of the 17 goals Sustainable Development Goals formulated by the United Nations (WBA, 2018).

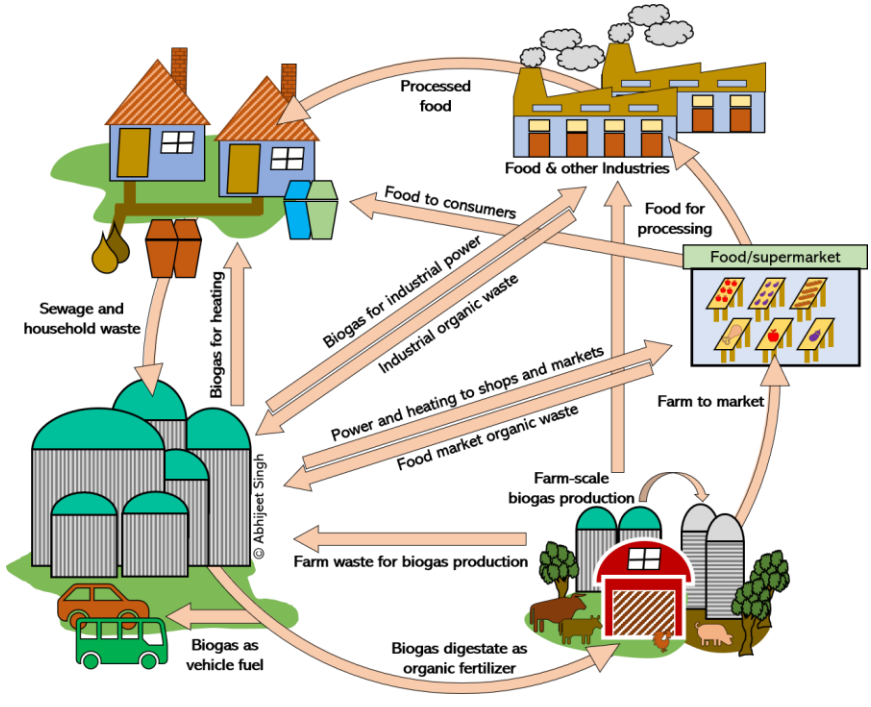


Figure 1. The ecological biogas process for recycling biodegradable organic waste to produce biogas as a fuel source and biogas digestate as a high quality organic biofertiliser.

Anaerobic digestion is a very versatile process serving multiple environmental goals, but the microbiological steps associated with the process (***Figure 2***) set limits on the extensive biogas production and efficient use of biogas reactor volume (Madsen *et al.*, 2011; Ward *et al.*, 2008; Wolf *et al.*, 2009). For adequate use of the resources invested in commercial biogas production, process optimisation and constant monitoring of the process are extremely important (Drosg, 2013; Madsen *et al.*, 2011; Schnürer *et al.*,

2016). The biogas process is a complex microbiological process involving interactions of thousands of known and unknown microbial species (Campanaro *et al.*, 2020; Ferguson *et al.*, 2014; Maus *et al.*, 2016; Treu *et al.*, 2016). It is thus very different from other industrial fermentation processes and it is difficult to automate, optimise and control, so it requires constant monitoring (Drosg, 2013; Madsen *et al.*, 2011; Wolf *et al.*, 2009; Yoshida & Shimizu, 2020). Several physical and chemical analysis technologies are currently available for monitoring the biogas process, but they are not completely reliable in assessing and predicting disturbances in the microbial communities (Ferguson *et al.*, 2018, 2014; Ni *et al.*, 2011; Ward *et al.*, 2008; Yoshida & Shimizu, 2020). Therefore, new methods are needed for constant monitoring of microbiological community structure and dynamics in biogas reactors (Drosg, 2013; Ferguson *et al.*, 2014; Fernández *et al.*, 1999).

An entire composite of diverse microbes in synergistic cooperation is required in the biogas process (Kleinsteuber, 2019; Schnürer, 2016) (***Figure 3***). Among these microbiomes, acetogenic bacteria are involved in synchronising and balancing the process and act as a link between the hydrolysing/fermenting microbial community and methanogenic archaea, so they play a crucial role in process stability (Kovács *et al.*, 2004) (***Figure 2***, ***Figure 3***). However, acetogenic bacteria are not very well studied and understanding of their functional role and community structure in biogas process is largely lacking (Theuerl, Klang, *et al.*, 2019). Therefore, microbial surveillance or close monitoring of these paramount sub-community can be used as a marker of the biogas process stability.

1.1 Aims of the thesis

The main aim of this thesis was to develop a microbiological surveillance strategy for acetogenic communities in biogas reactors, in order to enable acetogens to be used as a marker population of the biogas microbiome. In particular, the work in this thesis focused on assessment of acetogenic community structure in industrial biogas plants running on different feed

substrates and on identifying relationships between community dynamics and physico-chemical changes within biogas reactors. Specific objectives of the work described in Paper **I-IV** were:

1. Development of a public repository and database of the marker sequences of bacteria with potential for acetogenesis (Paper **I**).
2. Creation of a reliable bioinformatics analysis pipeline for high-throughput sequencing data and automated result visualisation (Paper **II**).
3. Comparative evaluation of the new high-throughput screening method with established conventional methods (Paper **III**).
4. Assessment of acetogenic community structure and its temporal dynamics in full-scale biogas reactors running on different substrates (Paper **IV**).

2. The microbiology of the biogas process

Biogas is a biologically produced mixture of gases mainly consisting of methane (60-70%) and carbon dioxide (30-40%) with small or trace amounts of hydrogen sulphide (0-4000 ppm), ammonia (0-100 ppm), nitrogen (0-10%), oxygen (0-2%), hydrogen (0-1%) and water vapour (0-10%) (Petersson & Wellinger, 2009; Ruan *et al.*, 2019; SGC, 2012). Biogas is produced during decomposition of organic matter by the cumulative interactions of complex anaerobic microbial communities (Borja & Rincón, 2017; Theuerl, Klang, *et al.*, 2019). These communities consist of bacteria, fungi and methanogenic archaea, which are involved in four main microbiological processes *i.e.*, hydrolysis, acidogenesis, anaerobic oxidation (including acetogenesis and syntrophic acid oxidation) and methanogenesis (***Figure 2***) (Angelidaki *et al.*, 2011; Dollhofer *et al.*, 2015; Hattori, 2008; Schnürer, 2016; Sun *et al.*, 2014; Thauer *et al.*, 2008; Vinzelj *et al.*, 2020; Westerholm, Müller, *et al.*, 2011; Westerholm *et al.*, 2016; Zhou *et al.*, 2002).

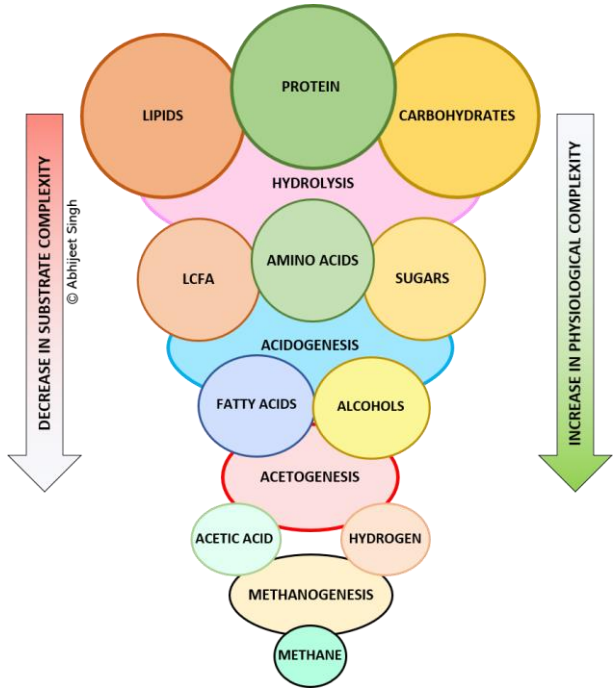


Figure 2. Simplified diagrammatic representation of the anaerobic digestion process, where complex biomolecules are degraded into simpler biomolecules in four complex interconnected microbiological events, hydrolysis, acidogenesis, anaerobic oxidation (including acetogenesis) and methanogenesis, which are carried out by bacteria together with fungi and methanogenic archaea.

2.1 Hydrolysis and acidogenesis

Hydrolysis and acidogenesis are the first two steps in the biogas process in which anaerobic bacteria and fungi degrade complex organic matter (***Figure 2***). Very diverse bacterial communities (phyla Firmicutes, Proteobacteria, Bacteriodetes, Chloroflexi, Actinobacteria, Spirochaetes, Synergistetes, Fibrobacteria, Thermotogae, Tenericutes *etc.*) and fungal communities (phylum Neocallimastigomycota including 18 genera) are responsible for hydrolysis and acidogenesis (Schnürer, 2016; Theuerl, Klang, *et al.*, 2019; Vinzelj *et al.*, 2020). These microbial groups secrete various extra-cellular hydrolysing enzymes which digest carbohydrates, proteins and fats into their soluble polymers, monomers, alcohols and carbon

dioxide, hydrogen (H_2), long- and medium-chain fatty acids *etc.* (***Figure 3***). The rate of hydrolysis is dependent on the structural and chemical complexity of organic material and hydrolysis can be a rate-limiting step if substrate is not easily digestible, for example plant-based materials (Borja, 2011; Borja & Rincón, 2017).

2.2 Anaerobic oxidation

The third microbial step in the biogas process is anaerobic oxidation, where polymeric and monomers molecules are further digested into short-chain fatty acids (C1-C6) or volatile fatty acids (VFA), carbon dioxide, ammonia (NH_3), hydrogen and alcohols (***Figure 3***). Anaerobic oxidation, including acetogenesis and syntrophic acid oxidation, is carried out by the bacterial phyla involved in previous steps, along with a special group of acetogenic bacteria (phylum Acidobacteria, Firmicutes Spirochaetes *etc.*) (Drake *et al.*, 2013; Küsel & Drake, 2011; Müller & Frerichs, 2013) (Paper **I**) and syntrophic acetate oxidising bacteria (SAOB) (genera *Schnuerera*, *Thermotoga*, *Thermoacetogenium*, *Tepidanaerobacter*, *Syntrophaceticus etc.*) (Balk, 2002; Hattori, 2008; Schnürer *et al.*, 1996; Westerholm *et al.*, 2010; Westerholm, Roos, *et al.*, 2011).

Acetogenesis is the process whereby acetogens produce acetic acid by reduction of carbon dioxide with hydrogen (***Figure 3***). However, due to the abundance of organic nutrients and VFA (Zakem *et al.*, 2021), acetogenesis is not the dominant pathway to produce acetate in biogas environment. Moreover, acetogenic bacteria do not always perform acetogenesis and grow as hydrogen producing anaerobic oxidative bacteria which utilize the products of hydrolysis/fermentation step to produce acetate, ammonia, carbon dioxide and hydrogen (Drake *et al.*, 2008). As acetogenic bacteria are metabolically very versatile they also represent a special group of bacteria *i.e.*, syntrophs/syntrophic bacteria, which can subsequently oxidise VFA to acetate and acetate to carbon dioxide and hydrogen (Zinder, 1994; Zinder & Koch, 1984). This oxidation has thermodynamics limitations and only feasible if hydrogen produced during oxidation is continuously removed (Hattori, 2008; Schink, 1997, 2002; Schink & Stams, 2006; Schnürer *et al.*,

1997; Stams, 1994). Some methanogens (hydrogenotrophs) can readily consume hydrogen being in the vicinity of these bacteria (Kovács *et al.*, 2004; Lettinga & Haandel, 1993; Thiele *et al.*, 1988; Thiele & Zeikus, 1988) (***Figure 3***). Thus, they establish a syntrophic relationship and are known as SAOB. Some acetogenic bacteria possess a special pathway which impart them the capability of intracellular hydrogen cycling. As they do not require a methanogen for syntrophic relationship, these acetogens are called intracellular syntrophs (Wiechmann *et al.*, 2020).

2.3 Methanogenesis

In the last step in the biogas process methane is produced mainly by cleavage of acetate (acetotrophic or methylotrophic) and reduction of carbon dioxide with hydrogen (hydrogenotrophic) by methanogenic archaea (***Figure 2, Figure 3***). Acetotrophic methanogens only belong to order Methanosarcinales (genera Methanosarcina and Methanosaeta), while hydrogenotrophic methanogenesis is carried out by member of order Methanobacteriales, Methanocellales, Methanococcales, Methanomicrobiales, Methanopyrales and Methanosarcinales (Garcia *et al.*, 2000; Liu & Whitman, 2008; Schnürer, 2016; Schnürer & Jarvis, 2017; Thauer *et al.*, 2008). In a normal/stable (mesophilic, low ammonia) biogas process approximately 50-75% of methane is produced by the acetotrophic methanogens which cleave acetate to produce methane and carbon dioxide (Jiang *et al.*, 2018). The remaining 50-25% of the methane production is carried out by hydrogenotrophic methanogens in syntrophy with syntrophic acetate oxidising bacteria (SAOB) and other syntrophic bacteria (Bryant *et al.*, 1967; Jiang *et al.*, 2018; McInerney *et al.*, 1979) (***Figure 3***). Process temperature, concentration of ammonia and concentration of VFA primarily are the decisive factors for the dominance of methanogenic pathways. Acetotrophic methanogenic pathway is the main pathway of methane production for manure or plant-based biogas reactors whereas in the case of protein rich substrate or under thermophilic conditions hydrogenotrophic methanogenic pathways dominates (Hattori, 2008; Karakashev *et al.*, 2006; Moestedt *et al.*, 2016; Schnürer & Nordberg, 2008; Sun *et al.*, 2014; Westerholm, Dolfing, *et al.*, 2011).

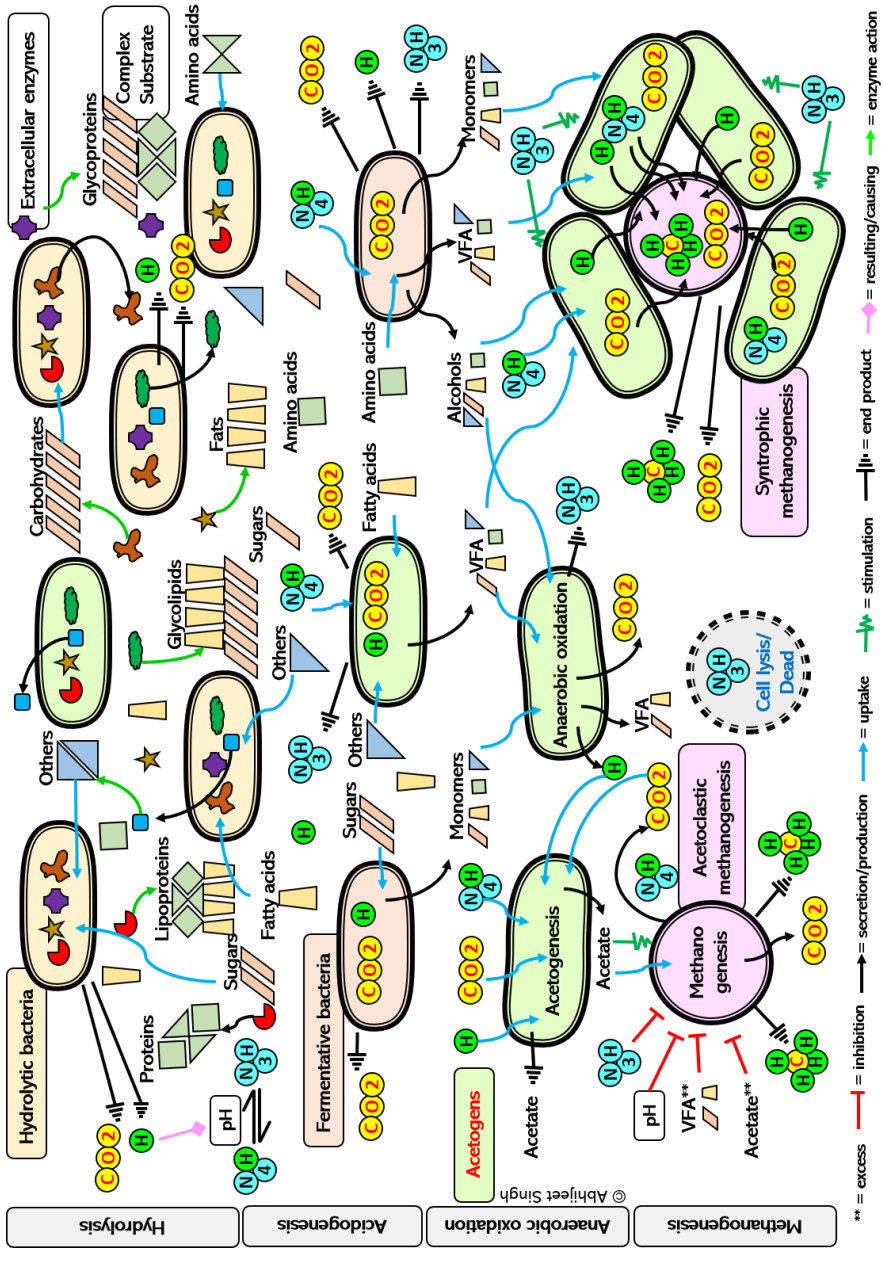


Figure 3. Descriptive graphical representation of the biogas process microbiological steps hydrolysis, acidogenesis, anaerobic oxidation and methanogenesis in the biogas process.

3. Acetogens

Acetogens, or acetogenic bacteria are chemolithoautotrophic bacteria performing reductive carbon fixation, *i.e.* acetogenesis, under anaerobic conditions (Fuchs, 1986; Zeikus, 1983). Acetogenesis is one of the most ancient and primitive biological processes responsible for the generation of one of the first organic molecules on Planet Earth (Peretó *et al.*, 1999; Russell & Martin, 2004). Acetogenesis involves the formation of acetate by biological fusion of carbon dioxide and hydrogen by the acetyl-coenzyme A (acetyl-CoA) pathway, also referred to as the Wood-Ljungdahl pathway (WLP), a characteristic of acetogens. Acetogenic bacteria were critical in the origination of life on early Earth, where reductive acetogenesis provided enough thermodynamic potential to sustain the first biological and reproducing (binary fission) life forms (Peretó *et al.*, 1999; Russell & Martin, 2004). In the present world, acetogens are essential for environmental carbon cycling, with production of at least 10^{13} kg of acetate in different anaerobic environments globally (Drake, 1994b; Drake *et al.*, 2013; Lovell & Leaphart, 2005; Müller, 2003; Ragsdale, 2007; Ragsdale & Pierce, 2008). They also produce industrial compounds such as ethanol, butyrate, lactate *etc.* (Das & Ljungdahl, 2003; Hügler & Sievert, 2011; Lovell & Leaphart, 2005; Wu *et al.*, 2019). Acetogenic bacteria are highly versatile in their metabolic potential and diverse in phylogeny, representing over 23 genera in bacterial classification (without any acetogen formyltetrahydrofolate synthetase (FTHFS) sequence specific clustering) (Drake *et al.*, 2013; Müller & Frerichs, 2013) (***Figure 3***, ***Figure 9***). Acetogens include SAOB, which use a reverse acetyl-CoA pathway for oxidation of acetate to carbon dioxide and hydrogen (Lee & Zinder, 1988a, 1988b; Schnürer *et al.*, 1997). Acetogenesis is a physiological attribute of acetogenic bacteria and there is no scientific

consensus on the genome construction which can define their phylogeny. Therefore, taxonomic markers like 16S rRNA gene are not very helpful in the identification and classification of acetogens (Drake, 1994b; Lovell, 1994) (Paper **III**). Thus, for the purposes of identification and classification of acetogens, presence of WLP is a prerequisite (Papers **I** and **II**).

3.1 Wood-Ljungdahl pathway

The Wood-Ljungdahl pathway is named after Harland G. Wood and Lars G. Ljungdahl who first proposed the complete biochemical pathway of autotrophic growth of acetogenic bacteria using carbon dioxide and hydrogen (Drake, 1994b; Schuchmann & Müller, 2014; Wood & Ljungdahl, 1991) (***Figure 4***). Biochemically, WLP is called the acetyl-CoA pathway of energy conservation for acetogenic growth, where hydrogen as an electron donor and two moles of carbon dioxide as an electron acceptor are converted to one mole of a precursor molecule acetyl-coenzyme A (CoA) (Fuchs, 1986; Ljungdahl, 1986; Wood, 1986, 1991). Thus, bacteria which: **i**) use WLP for energy conservation **ii**) generate acetyl-CoA by reduction of carbon dioxide, **iii**) may or may not produce acetate as the main end-product and **iv**) are obligate anaerobes, with tolerance to periods of aerobiosis, are defined as acetogenic bacteria or acetogens (Drake *et al.*, 2013; Schuchmann & Müller, 2016; Seifritz *et al.*, 2003; Singh *et al.*, 2020; Wagner *et al.*, 1996).

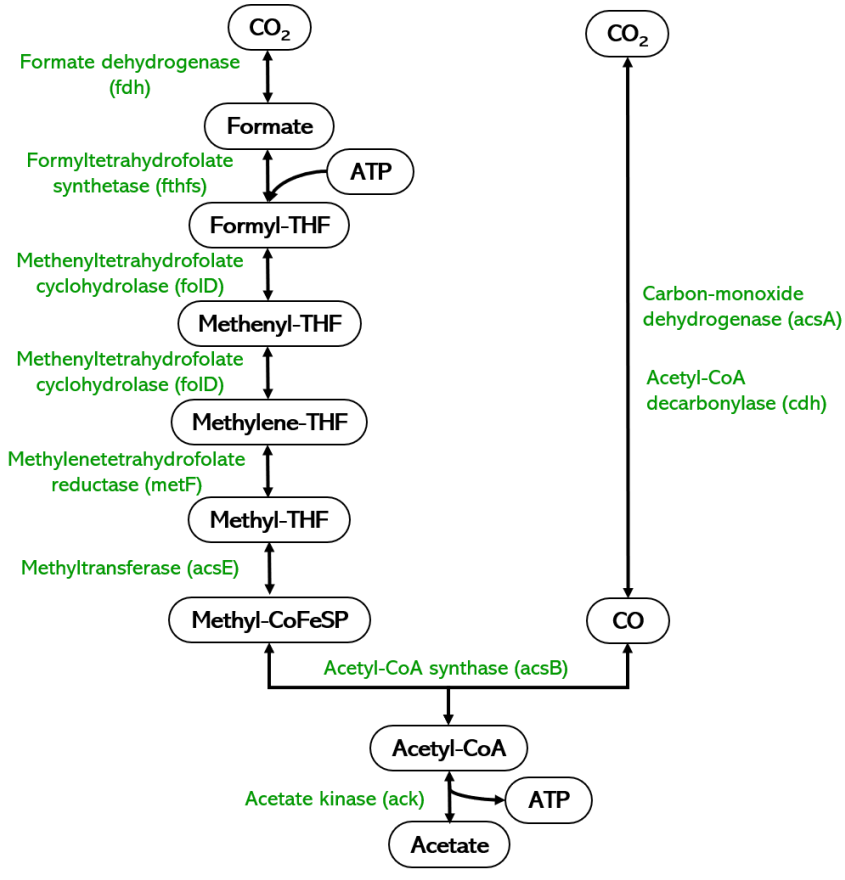


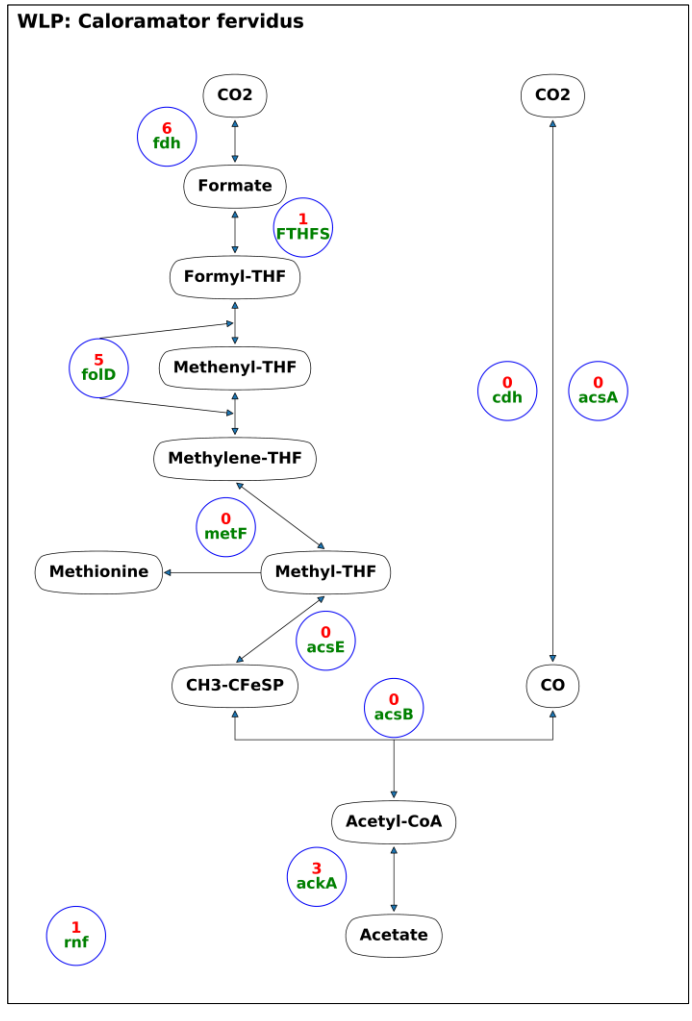
Figure 4. Diagrammatic representation of the Wood-Ljungdahl pathway/acetyl-CoA pathway of acetogenic bacteria.

Acetogenesis is a conglomerate physiological process which occurs under particular favourable conditions and thus cannot be restricted to a special genomic or phylogenetic construction (Drake, 1994a; Drake *et al.*, 2002; Küsel *et al.*, 2001; Schink, 1994; Schuchmann & Müller, 2016; Tanner & Woese, 1994) (Paper **I**) (***Figure 5***). Although presence and utilisation of WLP is a primary requirement for acetogenesis, many of the known acetogens lack a complete acetyl-CoA pathway or its genes in their genome or these genes cannot be detected due to unavailability of complete genome sequences (Paper **I**) (***Figure 5***). Nevertheless, the main enzymes in WLP, *i.e.*

formyltetrahydrofolate synthetase (FTHFS), acetyl-CoA synthase/carbon monoxide dehydrogenase complex (*acsA*/CODH complex) and acetate kinase (*ackA*), are the most critical and necessary enzymes for acetogenesis (Drake, 1994b; Hattori *et al.*, 2005; Zinder, 1994). Therefore, for decades FTHFS and *acsA*/CODH complex genes have been used as a marker for the identification of acetogenic bacteria (Gagen *et al.*, 2010; Lovell & Leaphart, 2005; Matsui *et al.*, 2011, 2008; Moestedt *et al.*, 2016; Müller *et al.*, 2016; Westerholm *et al.*, 2018; Westerholm, Müller, *et al.*, 2011; Yang, 2018) (Papers **I**, **II**, **III** and **IV**).

Figure 5 presents the WLP of two known acetogens *Caloramator fervidus* and *Thermoacetogenium phaeum* (Drake *et al.*, 2013) and their count of WLP genes. Complete genome/genome assembly of *C. fervidus* strain DSM 5463 (NZ_FNUK01000046.1) and *T. phaeum* strain DSM 12270 (NC_018870.1) was obtained from NCBI (Sayers *et al.*, 2012) and automatic pathway reconstruction was done using software AcetoPath developed within this thesis (Abhijeet Singh, unpublished). AcetoPath uses whole genome/assembly sequence, searches WLP genes based on homology and produces a WLP diagram with counts of respective genes. If multiple genome sequences are used, a heatmap of genomes used and constituent WLP gene is also generated. Use of AcetoPath in future analyses will allow exploration of organisms which harbour WLP or its major genes for acetogenic potential.

A)


WLP: Caloramator fervidus
CO2
6
fdh
Formate
1
FTHFS
Formyl-THF
5
folD
Methenyl-THF
Methylene-THF
0
metF
Methionine
Methyl-THF
0
acsE
CH3-CFeSP
0
acsB
CO2
0
cdh
0
acsA
CO
Acetyl-CoA
3
ackA
Acetate
1
rnf

B)

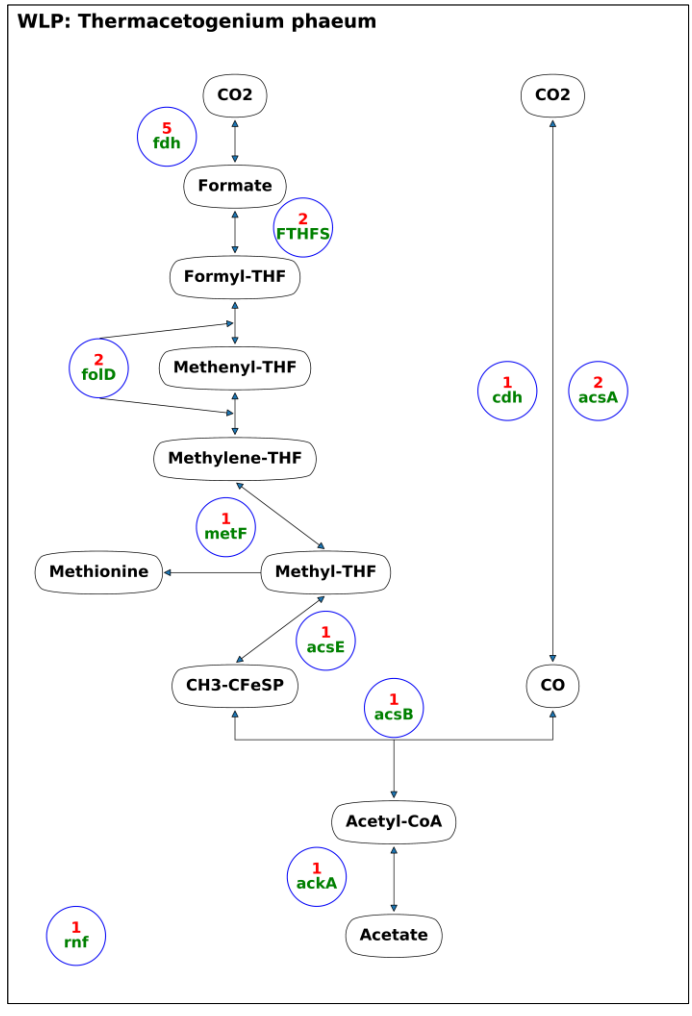


Figure 5. Diagrammatic representation of the Wood-Ljungdahl pathway (WLP) showing absence and presence of acetyl-CoA pathway genes in the known acetogens **A)** *Caloramator fervidus* (DSM 5463; NZ_FNUK01000046.1) and **B)** *Thermoacetogenium phaeum* (DSM 12270; NC_018870.1). Pathway reconstructions were made with the software AcetoPath (Abhijeet Singh, unpublished). The numbers above gene names represent number of gene copies detected within the genome sequence.

3.2 Formyltetrahydrofolate synthetase

Formyltetrahydrofolate synthetase, also known as formate--tetrahydrofolate ligase, is a characteristic and one of the main enzymes for acetogenesis in WLP (Drake, 1994b; Zinder, 1994). It is structurally and functionally very conserved and, due to high thermo-oxidative stability, relative ease of isolation and reliability, it has been preferred over *acsA*/CODH in earlier enzymological studies (Drake *et al.*, 2013; Ragsdale, 1991). FTHFS is a marker enzyme of WLP and is present in all acetogenic bacteria. It can also be present in SAOB, sulphate-reducing bacteria and some archaea/methanogens (Drake, 1994b; Drake *et al.*, 1997; Poehlein *et al.*, 2012; Ragsdale & Pierce, 2008; Sakimoto *et al.*, 2016). It can even be found in yeasts, plants, mammals and humans (Christensen & MacKenzie, 2006; MacFarlane *et al.*, 2009; Meiser & Vazquez, 2016). However, to meet the essential conditions for acetogenesis, only acetogenic bacteria can utilise the FTHFS gene as part of WLP for autotrophic growth. For this reason, FTHFS is widely used to identify acetogenic bacteria in different environments, like anaerobic digesters, human/animal and insect gut, paddy fields, lake and marine sediments, oilfields *etc*. (Fu *et al.*, 2018; Henderson *et al.*, 2010; Hori *et al.*, 2011; Leaphart *et al.*, 2003; Leaphart & Lovell, 2001; Lovell & Hui, 1991; Matsui *et al.*, 2008; Moestedt *et al.*, 2016; Müller *et al.*, 2016; Westerholm *et al.*, 2018) (Papers **I**; **II**, **III** and **IV**). There has been an overall increase in the study of acetogens/acetogenesis in the past two decades, particularly within the field of biogas/AD environments (***Figure 6***). Metagenomics studies have contributed to identification of WLP in metagenomics data, but studies focusing on the FTHFS gene have not gathered pace due to the lack of a suitable analytical strategy (Gagen *et al.*, 2010; Henderson *et al.*, 2010; Hori *et al.*, 2011; Leaphart & Lovell, 2001; Lovell & Hui, 1991; Xu *et al.*, 2009) (Papers **I**, **II** and **III**) (***Figure 6***).

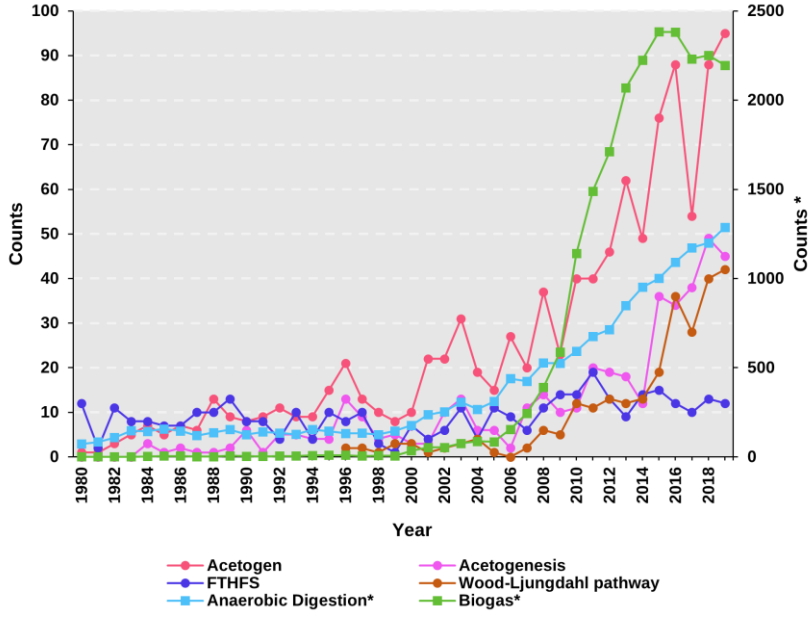


Figure 6. Line graph representing the increase in number of PubMed indexed studies published related to the respective topic published 1980-2019. The graph is based on a keyword (acetogen, acetogenesis, FTHFS and Wood-Ljungdahl pathway, anaerobic digestion and biogas) search in the PubMed database, accessed December 2020. The secondary y-axis in the graph is marked with asterisk and the values on the secondary y-axis are shown as squares.

4. Factors affecting the biogas process

The amount and composition of the biogas, and the efficiency and stability of the process, are dependent on several parameters such as feedstock composition, reactor technology, operating parameters and the structure and activity of the microbiological community engaged in the process (Angelidaki *et al.*, 2011; Herrmann *et al.*, 2012; Horváth *et al.*, 2016; Lebuhn *et al.*, 2015; Pöschl *et al.*, 2010; Schnürer, 2016; Schnürer *et al.*, 2016; Schnürer & Jarvis, 2017; Wellinger *et al.*, 2013). Each biogas installation has its own specific operating strategy and parameters (Drosg, 2013; Schnürer, 2016; Schnürer & Jarvis, 2017). Thus the microbiome associated with every biogas reactor is unique and specific to its physical and chemical properties (Calusinska *et al.*, 2018; Theuerl *et al.*, 2018; Theuerl, Klang, *et al.*, 2019) (Paper **IV**). As a generalisation, the process parameters can be classified into two categories 1) direct and 2) derived parameters. Direct parameters are under the direct control of the biogas plant operator and can be modulated. These parameters include substrate characteristics, carbon/nitrogen (C/N) ratio, temperature, organic loading rate (OLR), hydraulic retention time (HRT), stirring, additives *etc*. Derived parameters are parameters are important for the process which originate from the interaction between direct parameters and microbial communities. They include pH, alkalinity, ammonia/ammonium nitrogen (NH_4^+-N), VFA concentration, methane content, carbon dioxide content *etc*.

The substrate is the direct source of nutrition for the biogas microbiome. For efficient biological functioning of microbes, balanced availability of nutrients is necessary and an imbalance in the nutrient ratio could result in disruption of the microbial synergy and biogas yield (Chan, 2003; Theuerl,

Klang, *et al.*, 2019). Typically, hydrolysis is a slow process if substrate contains complex organic compounds which are not readily digested, such as lignocellulosic materials (Azman *et al.*, 2015; Lynd *et al.*, 2002; Taherzadeh & Karimi, 2008). In the case of substrates rich in easily digestible compounds, hydrolysis and acidogenesis can promptly produce intermediate products like alcohols, hydrogen, ammonia, VFA *etc*. (Bouallagui *et al.*, 2005; Schnürer, 2016; Schnürer & Jarvis, 2017). If the rate of production of intermediate products exceeds the rate of their uptake for anaerobic oxidation, this can cause accumulation of VFA, a drop in pH and consequently inhibition of methanogenesis (Yang *et al.*, 2015) (see ***Figure 3***). Since hydrolysis is primarily carried out by extra-cellular enzymes and fermentation is performed by very diverse bacterial and fungal groups, these steps are less susceptible to inhibition caused by excess VFA (formate, acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate *etc*.) as compared to methanogenesis. The optimum range of C/N ratio in substrate is reported to be 15:1 to 25:1 (Esposito *et al.*, 2012). A ratio higher than this range (in the case of easily accessible carbon) can cause excess VFA production, a decrease in pH and slow cellular growth, due to scarcity of nitrogen for microbial growth/protein synthesis (Resch *et al.*, 2011). A ratio lower than this range can result in excess availability of nitrogen and thus production of excess ammonia (Rajagopal *et al.*, 2013; Schnürer, 2016; Theuerl, Klang, *et al.*, 2019). Most of the studies conducted in biogas reactors with different substrates have identified organic loading rate and ammonia as major causes of disturbance or inhibition of microbial processes (Wu *et al.*, 2019) (Paper **III**). High levels of free ammonia often result in significant inhibition of methanogenesis, and sometimes also hydrolysis and fermentation (Czatzkowska *et al.*, 2020; Franke-Whittle *et al.*, 2014; Gerardi, 2003; Schnürer, 2016; Schnürer & Jarvis, 2017; Siegert & Banks, 2005; Wang *et al.*, 2009; Westerholm *et al.*, 2016) (***Figure 3***). Consequently, accumulation of VFA occurs, especially of acetate and propionate, followed by a drop in pH, which can enhance inhibition or even cause complete process failure (Frank *et al.*, 2016; Moestedt *et al.*, 2016; Rajagopal *et al.*, 2013; Schnürer, 2016; Schnürer & Nordberg, 2008).

Another important parameter which affects the biogas process is temperature. Fluctuations in temperature can result in instability of

enzymatic processes, especially methanogenesis, whereas hydrolysis/fermentation and acidogenesis are relative less sensitive to temperature fluctuations (Robles *et al.*, 2018). Furthermore, if the substrate is rich in nitrogen, an increase in temperature can result in higher ammonia production, which is the most common cause of methanogenesis inhibition (Fotidis *et al.*, 2013; Khalid *et al.*, 2011; Schnürer, 2016; Schnürer & Jarvis, 2017; Schnürer & Nordberg, 2008; Wu *et al.*, 2019). For a stable biogas process, mesophilic temperature (30-40 °C) is preferred, as the microbial communities at this temperature are more diverse and relatively less susceptible to disturbance. However, bio-conversation rate is higher at thermophilic temperature (50-60 °C), which can permit higher organic loading rate or shorter hydraulic retention time and higher biogas yield (Ge *et al.*, 2016; Li *et al.*, 2011). Nevertheless, thermophilic systems are relatively more susceptible to disturbance due to their lower microbial diversity and higher chances of ammonia inhibition (Levén *et al.*, 2007; Zhao & Kugel, 1996).

The 'inhibition triangle' illustrates the relationship of hydrolysis/acidogenesis, anaerobic oxidation (including acetogenesis and syntrophic acid oxidation) and methanogenesis to the main internal process parameters temperature, ammonia/ammonium and pH, and to external influencing parameters like organic loading rate and process speed (***Figure 7***). The inhibition triangle can be interpreted as follows: In general, a normal biogas process is in equilibrium (represented by green broken line) with the interconnected microbiological process (red smooth line). An increase in the temperature or organic loading rate (brown dotted line) can cause a higher risk of elevated ammonia levels eventually resulting in VFA accumulation and a drop in pH (blue broken line). Methanogens are susceptible to changes in these parameters and variations outside the optimum cause stress in the biogas process, reduced activity or inhibition of methanogenesis (brown broken line). During these events, the acetogenic community plays an important role in VFA production/oxidation, balancing the pH and overall functioning of the biogas process (Kovács *et al.*, 2004; Zeeman & Lettinga, 1999) (***Figure 3***, ***Figure 7***). Due to this special characteristic of acetogenic bacteria, they can act as a marker for the process stability and health of biogas reactors (Papers **II**, **III** and **IV**).

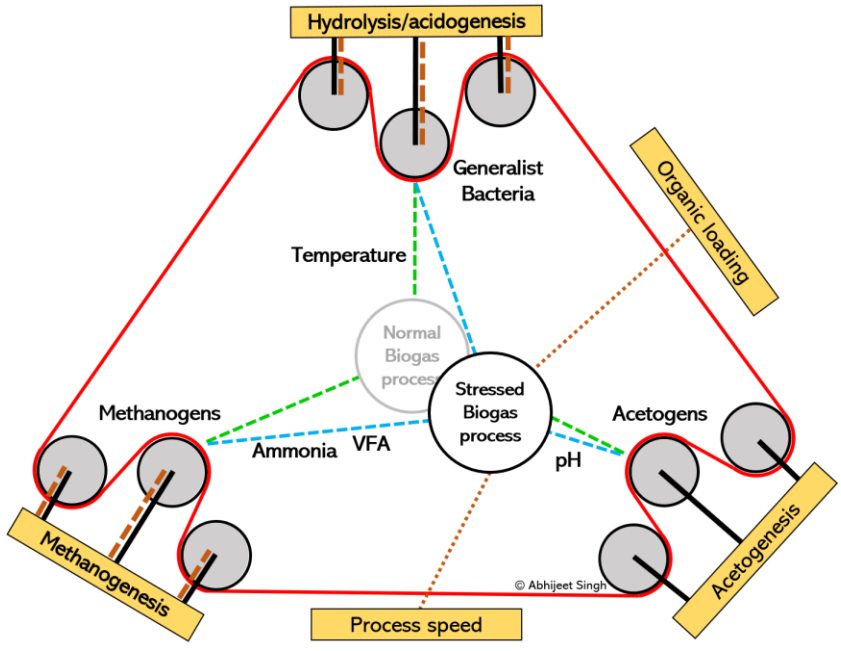


Figure 7. "Inhibition triangle" of the biogas stress system, showing the interrelationships between microbiological processes and internal and external parameters in the biogas system.

By continuous monitoring of direct and derived parameters, any imbalance/disturbance in the process can be detected in time, which provides an opportunity to take corrective action and ensure maximum efficiency (Drosg, 2013). Biogas process involves various parameters and disturbance can be caused by unknown parameters, therefore, biogas plants uses consequential parameters such as produced total gas volume (cu.m./day), content of methane and carbon dioxide (%) , hydrogen sulphide (ppm), pH (A.U.), volatile fatty acids (VFA) (g/L), NH_4^+-N (g/L), volatile solids (VS) (g L^{-1} day^{-1}), temperature (°C), alkalinity (mg/L) *etc*. to monitor the process (Drosg, 2013; Schnürer *et al.*, 2016).

5. Monitoring the biogas process

In the past few decades, there was a rapid increase in the research for the development of reliable monitoring strategy for biogas reactors. Studies to date have proposed monitoring based on early warning indicators for physico-chemical parameters, such as alkalinity ratios (Martín-González *et al.*, 2013), CH_4/CO_2 ratio, VFA/alkalinity ratio (D., Li *et al.*, 2017; Li *et al.*, 2014, 2018), stability and auxiliary index (Dong *et al.*, 2011), VFA/calcium concentration (Kleyböcker *et al.*, 2012), stable isotope signature (Lv *et al.*, 2014; Polag *et al.*, 2015), isotope fractionation (De Vrieze, De Waele, *et al.*, 2018), total volatile acids/total inorganic carbon ratio (Voß *et al.*, 2009) *etc*. Other studies have used advanced technologies like near-infrared (NIR) spectroscopy (Bruni *et al.*, 2013), fluorescence spectroscopy (Palacio-Barco *et al.*, 2010), electronic nose/tongue (Peris & Escuder-Gilabert, 2013), proportional-integral-derivative (PID) controller (Marsili-Libelli & Beni, 1996) and artificial neural networks (Holubar, 2002; Holubar *et al.*, 2000, 2003) *etc*. for identification and rapid detection of process disturbances. Advanced technologies and instruments are therefore available for monitoring and analysis of these parameters in real time or within few hours. However, they have some methodological/technical limitations, are not highly reliable and they need to be interpreted in combination with other parameters (Drosg, 2013; Ferguson *et al.*, 2014; Guebitz *et al.*, 2015; Lebuhn *et al.*, 2014; Ward *et al.*, 2008; Wu *et al.*, 2019).

Application of modern molecular and microbiological techniques to monitor the anaerobic digestion process has the advantage that these techniques can detect changes significantly earlier than is possible by conventional chemical and physical parameters (Lebuhn *et al.*, 2014, 2015).

They involve the monitoring of microbiological composition, dynamics and health (Lebuhn *et al.*, 2015; Schnürer *et al.*, 2016). Microbiological communities involved in the biogas process are highly diverse (Calusinska *et al.*, 2018; Campanaro *et al.*, 2020; Maus *et al.*, 2016) and dynamic, with changes over time even without any disturbances (Fernandez *et al.*, 2000; Fernández *et al.*, 1999; Theuerl *et al.*, 2015, 2018). However, microbiome and microbiological processes in biogas reactors continues to be a black box (Kleinsteuber, 2019; Rivière *et al.*, 2009; Theuerl, Klang, *et al.*, 2019; Treu *et al.*, 2016) as there is incomplete understanding of their functional potency and redundancy (Langer *et al.*, 2015; Moya & Ferrer, 2016). Therefore, research into microbiological processes is currently the focus as regards anaerobic digestion processes (Lebuhn *et al.*, 2014, 2015; Theuerl, Herrmann, *et al.*, 2019).

5.1 Microbiological monitoring and surveillance

Microbiological monitoring and surveillance, although similar, have some fundamental differences that mainly relate to the aims and principle of the underlying strategy employed in the respective method (Artois *et al.*, 2009; Doherr & Audige, 2001; Salman, 2003). The same set of techniques can be applied with different aims and objectives, and thus surveillance can include monitoring but not *vice versa*. With relation to the anaerobic digestion process, the definitions used within this thesis for microbiological monitoring and surveillance are as follows:

Microbiological monitoring: *Systematic, continuous or periodical, active or passive collection of data to detect any changes and their influence on microbiological community.*

Microbiological surveillance: *Active, systematic, dynamic and intensive investigation of a specific microbial group to detect any changes in its composition or abundance within certain threshold limits, which can indicate a further course of action.*

Etymologically, microbiological means a defined microbial group in its natural environment, while surveillance means quantitative analysis of temporal dynamics. A microbiological surveillance strategy for detection or prediction of changes in the dynamic profile of acetogenic bacterial communities present in biogas reactors was developed in this thesis (***Figure 8***). The prerequisites for microbiological surveillance formulated in this thesis were:

1. Target microbial group: acetogenic bacterial community.
2. Reliable analysis method: high-throughput sequencing and bioinformatics data analysis pipeline.
3. Threshold limit: increase or decrease in relative abundance of respective members of acetogenic community.
4. Reclamation proceedings: depending on type of biogas system and nature of variation in acetogenic community.

5.1.1 The theory of microbiological surveillance in biogas plants

The theory, hypothesis, empirical consequences and auxiliary assumptions applied in development of the microbiological surveillance strategy for biogas plants in this thesis were as follows:

Theory: Acetogens/acetogenic bacteria are very important members of the anaerobic microbial community, imperative for balance and synergy in biogas process and can be used for microbiological surveillance in biogas reactors.

Hypothesis (H): The community dynamics and abundance of acetogenic bacteria influence the stability of the methanogenic process, so microbiological surveillance of the acetogenic population can help in assessment and prediction of process stability.

Empirical consequence (E):

i. A reduction in abundance and/or activity of a certain population (P1) of the acetogenic community under the influence of an external stress factor.
ii. An increase in abundance and/or activity of a fraction (P2) of acetogenic community under the influence of external stress factor.
iii. The activity of P2 can also be responsible for increasing the degree of stress caused by the external factor.
iv. The remaining population (P3) of the acetogenic community may or may not change in its abundance or activity under the influence of the external stress factor.

Auxiliary assumptions (A):

i. Acetogens produce volatile fatty acids (mainly acetate) in the biogas process.
ii. Acetogens include organic acid-oxidising bacteria which degrade volatile fatty acids in the biogas process.
iii. Acetogens may not always perform acetogenesis.

If H and A, then E
E false

Either H or A is false

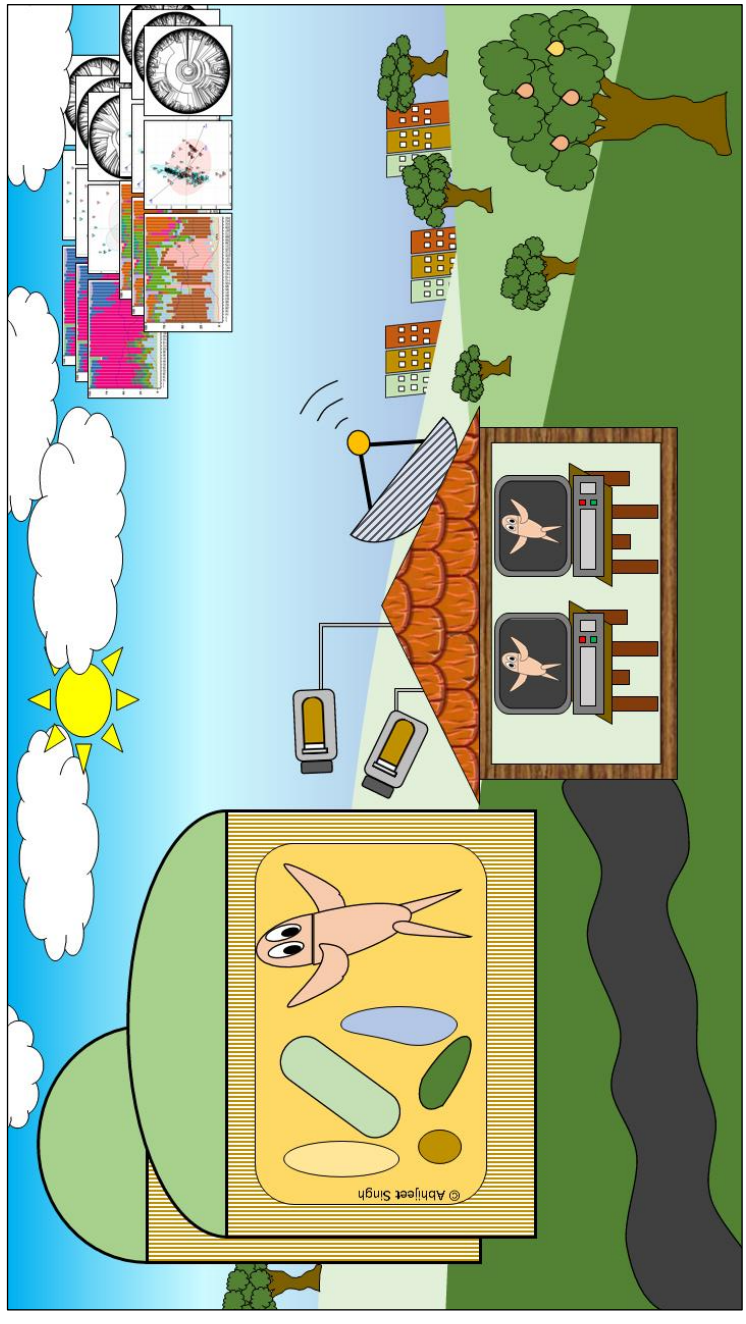


Figure 8. Diagrammatic representation of acetogens targeted in microbiological surveillance of biogas plants, as envisioned in this thesis.

6. Microbial community analysis in anaerobic digesters

Advances in microbiological techniques have led to extensive and elaborate investigations on biogas reactors to identify the microbiological processes, community structure and interactions within the unknown world of environmental microbiomes. Metagenomics techniques have demonstrated that the biogas microbiome is highly diverse and that each process develops its own unique microbial community based on its substrate and operating parameters (Campanaro *et al.*, 2016, 2020; Güllert *et al.*, 2016; Luo *et al.*, 2016; Maus *et al.*, 2016; Ortseifen *et al.*, 2016; Schlüter *et al.*, 2008; Treu *et al.*, 2016). Detailed metaproteomics/metatranscriptomics have also been applied in some studies, in attempt to get in-depth knowledge of the active microbiome and pathways for the biogas microbiome (Hanreich *et al.*, 2012; Heyer *et al.*, 2013, 2016; Kohrs *et al.*, 2014). Although very extensive and detailed, such studies have some major limitations. For example, they are exploratory and based on few samples which are restricted in number, replicates and time series of samples, and thus only give snapshot information. They produce big data that are often dependent on diversity and accuracy of reference databases, analysis duration, analytical software, computational resources, skillset of the user *etc*. (Fan *et al.*, 2014; Heyer *et al.*, 2015, 2017; Kleinsteuber, 2019; Najafabadi *et al.*, 2015; Prosser, 2015; Stephens *et al.*, 2015). In addition, the results must be interpreted in correlation with findings obtained using other omics techniques to fully understand the diversity, interaction and functions of microbiomes (Heyer *et al.*, 2015, 2017). Unfortunately, none of the large omics-centred studies performed previously in biogas reactors focuses on or describes acetogens or

the acetogenic community, which was thus main focus of this thesis (Papers **II**, **III** and **IV**).

6.1 Analysis of the acetogenic community

Acetogenic bacteria are one of the most versatile groups of anaerobic bacteria studied to date (Müller, 2003; Schink, 1994; Schuchmann & Müller, 2014). Acetogens have been studied for past few decades and are now attracting increasing attention because of their importance in modern sustainable biomanufacturing and electrochemical processes (Liew *et al.*, 2016; Müller, 2019; Nevin *et al.*, 2011; Saheb-Alam *et al.*, 2017; Wiechmann & Müller, 2019) (see ***Figure 6***). Most previous studies on acetogenic bacteria have been conducted using conventional methods, *i.e.* isolation and physiological characterization. Isolation, pure culturing and physiological analysis will always be the best method for characterisation of particular acetogenic bacteria. Metagenomics/metaproteomics applications have also contributed and have revealed new acetogenic/syntrophic candidates, *e.g.* acetogenic bacteria in the phylum Cloacimonodota, genus *Candidatus* Syntrophopropionicum or phylotype unFirm_1 *etc*. (Frank *et al.*, 2016; Lucas *et al.*, 2015; Pelletier *et al.*, 2008; Singh *et al.*, 2021). However, these candidate organisms have not yet been isolated and physiologically characterised because of limitations in culturing techniques and lack of knowledge about the correct method and growth characteristics. Moreover, in an ecological monitoring/surveillance perspective, isolation and pure culturing is not feasible, practical and applicable. Therefore, ecological studies targeting acetogens are mostly performed with molecular biological techniques, such as quantitative polymerase chain reaction (qPCR), clone library, terminal restriction fragment length polymorphism (T-RFLP) *etc*.

6.2 Acetogenic community analysis with qPCR and clone libraries

For quantitative analysis of microbial communities in environmental samples, qPCR is a very powerful and accurate method and that has been

used in multiple studies (Aydin *et al.*, 2015; Delgado *et al.*, 2012; Ouwerkerk *et al.*, 2009; Parameswaran *et al.*, 2011; Sagheddu *et al.*, 2017; Westerholm, Müller, *et al.*, 2011; Xu *et al.*, 2009; Yang, 2018). However, this method has the limitations that it requires high specificity of primers, is likely not efficient in targeting FTHFS sequences from a diverse bacterial population (Xu *et al.*, 2009), and the amplicon size for the target gene should be around 200-300 base pairs (bp) for efficient quantitative assay (Sharma *et al.*, 2007). Thus, it is surprising that several studies (Aydin *et al.*, 2015; Ouwerkerk *et al.*, 2009; Sagheddu *et al.*, 2017) have used FTHFS primers from Leaphart and Lovell (2001) or Lovell and Leaphart (2005) which generate amplicons of ~1100 bp and are not suitable for qPCR. In addition, many acetogens have multiple copies of FTHFS genes (see examples in ***Figure 5***), and hence, quantitative assumptions that FTHFS gene copies correspond to the bacterial cell in soil (Xu *et al.*, 2009) do not seem to be reliable. Further, in the study by Xu *et al.* (2009), the amplicon size generated by FTHFS was over the reliable limits for a quantitative assay. An added complication is, that non-acetogenic bacteria and some archaea also harbour FTHFS genes (Borrel *et al.*, 2016; Lovell & Leaphart, 2005; Whitman, 1994). This is not desirable in a qPCR assay and unavailability of taxonomic information will hamper filtering and removal of quantitative data of non-acetogenic bacteria and archaea. Due to these technical complications, qPCR assay is not the best method for the study of acetogenic communities. Due to lack of an acetogen-specific database (Küsel *et al.*, 2001; Xu *et al.*, 2009), FTHFS sequences from many acetogenic groups have not been available for the design of new primers which can target broader diversity than the primers from Leaphart and Lovell (2001), Lovell and Leaphart (2005) and Xu (2009) (Paper **I**). Therefore, within this thesis, a new FTHFS gene repository and database called AcetoBase, which can assist in designing new primers to target a diverse population of FTHFS gene-harbouring bacteria, was developed (Paper **I**). ***Figure 9*** shows the diversity of bacterial FTHFS protein sequences present in AcetoBase. Furthermore, qPCR quantification of the FTHFS gene harbouring community lacks taxonomic information and for quantitative of specific acetogenic bacteria, species-specific primers are required (Müller *et al.*, 2016).

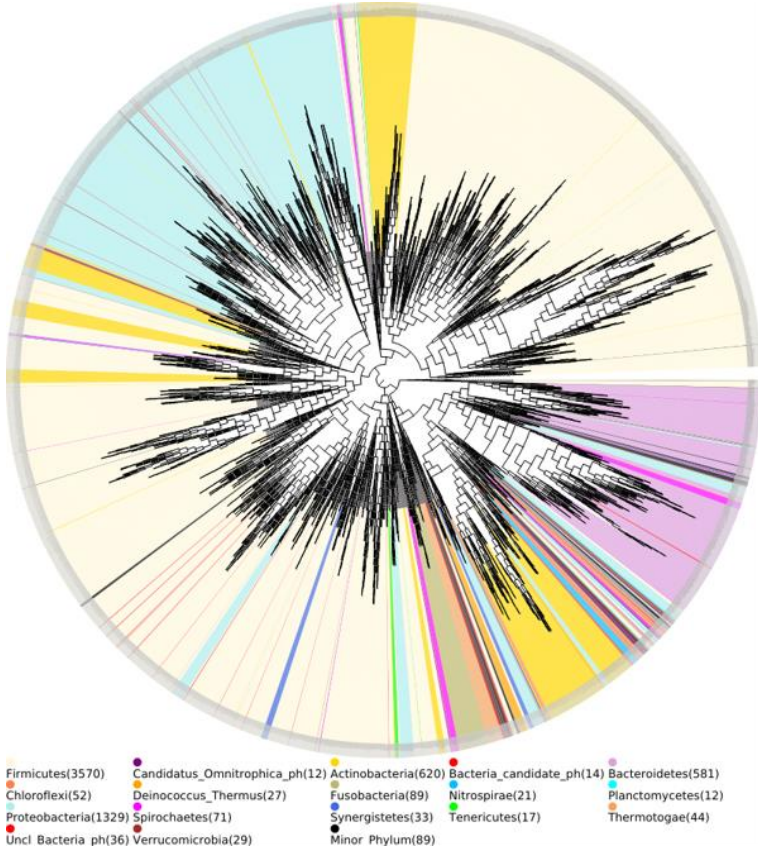


Figure 9. Phylogenetic tree showing formyltetrahydrofolate synthetase (FTHFS) amino acid sequence diversity in AcetoBase (Paper **I**). Phlya with less than 10 sequences were merged in the group Minor_phyla during tree annotation and visualisation.

Due to the limitations in acetogen-targeted qPCR analysis clone library construction/sequencing is widely used for environmental samples. Cloning of the FTHFS gene and sequencing is a frequently used method for identification of acetogenic bacteria in environmental samples (Gagen *et al.*, 2010, 2014; Henderson *et al.*, 2010; Leaphart & Lovell, 2001; Moestedt *et al.*, 2016; Müller *et al.*, 2016; Westerholm *et al.*, 2018). Sequencing of clones generally yields long sequence reads with good quality, which is very useful in sequence analysis and establishing phylogenetic relationships. However,

this method has a technical shortcoming deriving from the process of clone library generation, which can be biased in ligation, transformation and colony selection and may not represent the whole microbial diversity present in any sample. The analysis in Paper **I** supported this notion of selective targeting of FTHFS primers in clone library construction. It also showed that the clone library is limited to few hundreds of clones (maximum) which are redundant. The phylogenetic tree constructed for all published and publicly available FTHFS clone sequences indicated dominance of certain taxa (Paper **I**) (***Figure 10***).

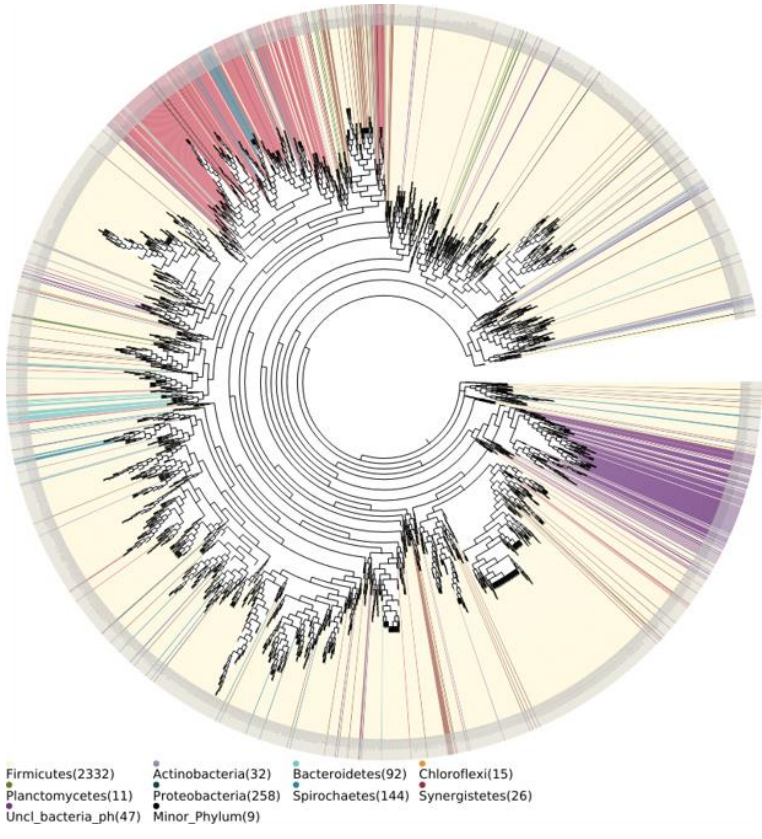


Figure 10. Phylogenetic tree representing formyltetrahydrofolate synthetase (FTHFS) clone sequence diversity in AcetoBase (Paper **I**). Predicted phlya with less than 10 sequences were merged in the group Minor_phyla during tree annotation and visualisation.

Before the work presented in Paper **I**, researchers tended to use the homoacetogen similarity (HS) score proposed by Henderson *et al.* (2010) to predict the phylogeny and physiological characteristics of clone sequences (Akuzawa *et al.*, 2011; Gagen *et al.*, 2010, 2014, 2015; Z., Li *et al.*, 2017; Matsui *et al.*, 2019; Mitsumori *et al.*, 2014). The HS score is based on the hypothesis of positional conservation of FTHFS sequences of acetogenic bacteria. However, diligent and elaborate analysis has shown that FTHFS sequences may have positional conservation in acetogens, but that this it is not universal (Lovell, 1994) (Paper **I**). With this hypothesis HS score cannot help in identification of acetogens or their physiological characteristics (Paper **I**). The limitations of HS score were pointed out by developers themselves (Henderson *et al.*, 2010). Besides, the term 'homoacetogen' is a misnomer and its use is discouraged by several experts in the field (Drake, 1994b; Drake *et al.*, 2013; Müller & Frerichs, 2013).

6.3 Acetogenic community profiling with T-RFLP

Typically, phylogenetic analysis is performed with clone sequences to visualise clustering of FTHFS sequences from acetogens among non-acetogenic bacterial sequences (Ohashi *et al.*, 2007; Pester & Brune, 2006). However, the phylogenetic and cluster analyses performed in Paper **I** indicated that this assumption is not entirely true, due to the fact that there is no positional conservation in the FTHFS sequences of acetogenic and non-acetogenic bacteria (Lovell, 1994) (Paper **I**). Thus, although clone library construction is a very useful method, it needs detailed analysis to be connected to taxonomy and be useful. Additionally, the method is low-throughput, time- and resource-intensive, requires laboratory/technical skills and data analysis is difficult to automate. Therefore, for cost-/resource-efficient analysis of large numbers of samples and effortless data analysis for microbiological surveillance, clone library sequencing cannot be a method of choice (Dunbar *et al.*, 2000; Talbot *et al.*, 2008) (Paper **II**).

For fast screening of environmental samples, T-RFLP is a very popular and established method (Lebuhn *et al.*, 2015; Robles *et al.*, 2018). In T-RFLP, microbial community analysis is based on the restriction digestion of marker gene amplicons, where length heterogeneity of the terminally

labelled restriction fragment (T-RF) represents the diversity of the microbial population in a sample (Liu *et al.*, 1997). T-RFLP has been widely used for analysis of microbial community structure and diversity in environmental samples (Blackwood *et al.*, 2003; Brugger *et al.*, 2012; Dickie & FitzJohn, 2007; Klang *et al.*, 2019; Osborn *et al.*, 2000). It has also been used for analysis of acetogenic populations in environmental and biogas samples (Akuzawa *et al.*, 2011; Hori *et al.*, 2011; Moestedt *et al.*, 2016; Müller *et al.*, 2016; Saheb-Alam *et al.*, 2017; Westerholm *et al.*, 2018; Westerholm, Müller, *et al.*, 2011) (Paper **III**). However, this method has some technical and methodological limitations which reduce its overall efficiency (Dunbar *et al.*, 2000; Prakash *et al.*, 2014). Furthermore, one T-RF can be represented by many different microorganisms, and hence relating T-RF to exact bacterial taxonomy is not possible (Paper **III**). Although the T-RFLP method can effectively show microbial community dynamics in environmental samples, this method alone is not able to associate T-RF to any bacterial lineage (Dunbar *et al.*, 2000; Nikolausz *et al.*, 2005; Osborn *et al.*, 2000). Thus, a prior exploratory study with a combination of T-RFLP and cloning is necessary to assign T-RF and probable taxonomy (Nikolausz *et al.*, 2005; Osborn *et al.*, 2000). However, with the help of AcetoBase and the REDigest software, *in silico* analysis can be performed to estimate the probable taxonomy of a particular T-RF (Singh, 2020) (Papers **I** and **III**).

6.4 16S ribosomal RNA gene sequencing

The 16S rRNA gene has been used in countless studies focusing on decoding the taxonomy of microbial dark matter in environmental samples (Janda & Abbott, 2007; Johnson *et al.*, 2019; Nobu *et al.*, 2015; De Vrieze, Ijaz, *et al.*, 2018). However, since acetogenesis is a physiological property and cannot be revealed by the taxonomy of the respective bacteria, 16S rRNA gene sequencing cannot serve the purpose of identifying acetogenic bacteria in an environmental perspective (Lovell, 1994; Tanner & Woese, 1994) (Paper **III**). However, during isolation of bacteria and their characterisation, 16S rRNA gene sequencing will always be a necessity in phylogenetic placement of the bacteria. 16S rRNA gene sequencing can be used for the microbiological surveillance of acetogenic bacteria, if species-

specific primers are used. Species-specific 16S rRNA primers have been used *e.g.* by Westerholm *et al.* (2011a) for the detection of some acetogens in qPCR analysis. To date, no 16S rRNA-based, high-throughput sequencing or data analysis for acetogenic bacteria has been performed and published. In Paper **III**, an alternative approach was proposed, where a 16S rRNA gene sequence database (RibocetoBase) was developed for the FTHFS harbouring bacteria present in AcetoBase. Thus, an indirect assessment of the FTHFS-possessing bacterial population can be performed with 16S rRNA gene amplicon sequencing (AmpSeq) data (Papers **III** and **IV**). However, this indirect method has some limitations and cannot be used as a replacement for FTHFS gene AmpSeq (Papers **III** and **IV**).

6.5 High-throughput FTHFS gene-based analysis of acetogenic bacteria

Since the 16S rRNA gene cannot be used for high-throughput identification and quantification of acetogenic communities, this created a need for a FTHFS gene database and high-throughput analysis method (Gagen *et al.*, 2010; Henderson *et al.*, 2010; Hori *et al.*, 2011; Leaphart & Lovell, 2001; Xu *et al.*, 2009). Therefore, in this thesis the database AcetoBase (Paper **I**) (***Figure 11***) and a new method AcetoScan (Paper **II**) were developed and successfully used for the high-throughput analysis of acetogenic bacteria (Papers **III** and **IV**). In most sequencing-based scientific studies, complex analysis of big sequence data and visualisation procedures are the most common limitations to wider application of high-throughput sequencing methods (Kulkarni & Frommolt, 2017; De Vrieze, Ijaz, *et al.*, 2018).

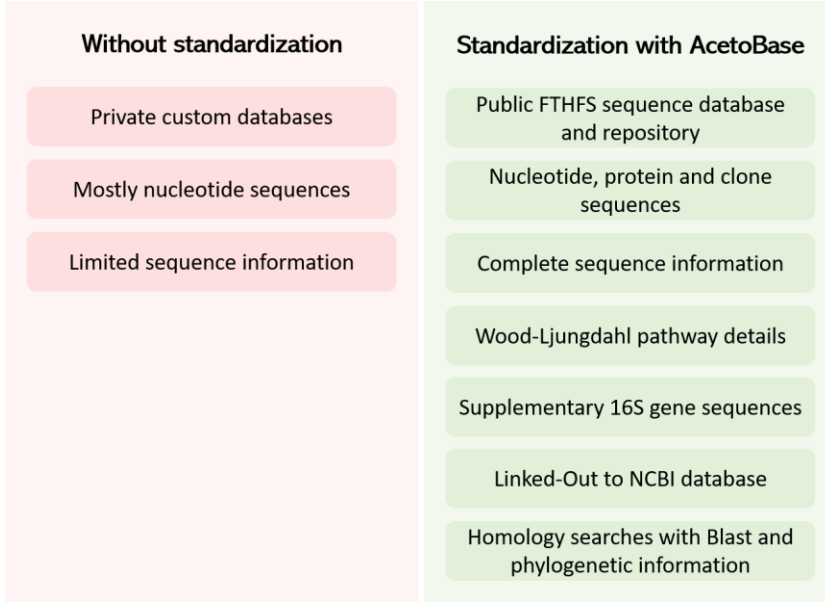


Figure 11. Comparative visualisation of the pre-existing scenario and benefits from establishment of a database and repository for formyltetrahydrofolate synthetase (FTHFS) sequences, *i.e.* AcetoBase (Paper **I**).

AcetoScan is a bioinformatics pipeline developed for rapid and accurate analysis of FTHFS AmpSeq data with minimum user input (Paper **II**). It does not require a high-performance computing cluster and can even work on any modern desktop computer/laptop (Paper **II**) (***Figure 12***). Unsupervised analysis of FTHFS AmpSeq data and automated result visualisation make AcetoScan a fast and reliable method (Paper **III**) (***Figure 13***). These qualities mean that the tools and strategy developed in this thesis are suitable for acetogenic community-focused microbiological surveillance of biogas plants (Paper **IV**) (***Figure 14***).

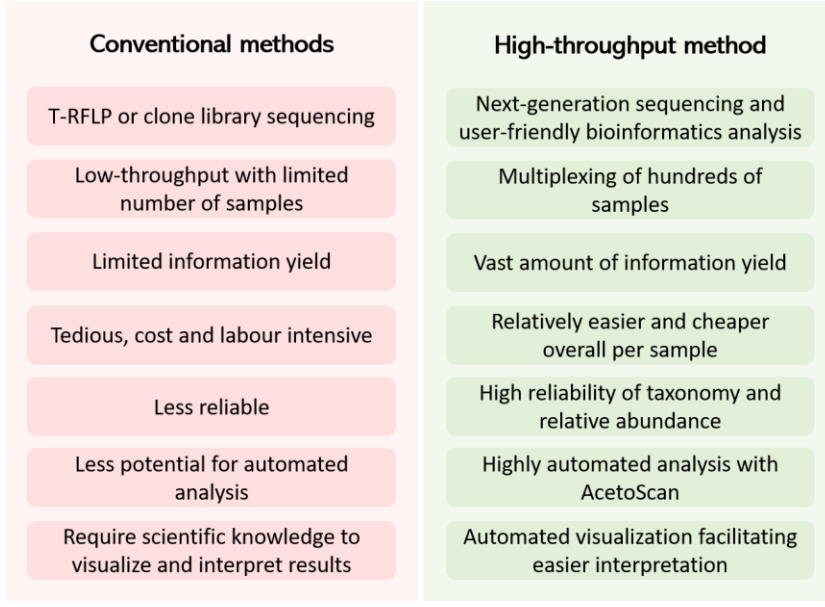


Figure 12. Comparative visualisation of the advantages of the new AcetoScan method for high-throughput sequencing and data analysis conventional methods used for formyltetrahydrofolate synthetase (FTHFS) gene based acetogenic community profiling (Paper **II**).

To determine the accuracy, reliability and utility of high-throughput FTHFS AmpSeq and AcetoScan analysis method, comparative analyses were conducted with the FTHFS amplicon-based T-RFLP and 16S rRNA AmpSeq methods (Paper **III**). The results showed that FTHFS Ampseq and AcetoScan analysis is a reliable method for detection of community disturbance and taxonomy identities. It is more sensitive in targeting the low abundance members of communities which are otherwise not covered in 16S rRNA gene survey/monitoring (Papers **III** and **IV**).

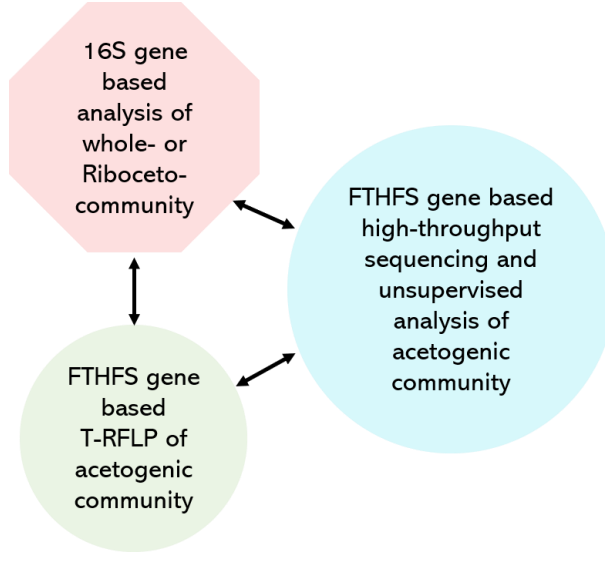


Figure 13. Comparison of different methodological approaches for analysis of the acetogenic community using the established methods (FTHFS T-RFLP and 16S rRNA gene) and the new high-throughput FTHFS gene sequencing and unsupervised AcetoScan analysis method (Paper **III**). The shape of objects represents the target community, where T-RFLP and AcetoScan target the acetogenic community with FTHFS sequences and 16S rRNA gene analysis targets the whole microbial community. Object colour indicates the desirability of the method in acetogenic community analysis, where pink means less desirable, green is intermediate and blue is most desirable. Object size indicates overall usability of the method in acetogenic community analysis.

7. Surveillance of acetogenic communities: Opportunities and obstacles

Acetogenic communities are important ecological entities and play a paramount role in the biogas microbiome, but are still a neglected bacterial group in most omics studies (Lebuhn *et al.*, 2015; Robles *et al.*, 2018; Theuerl, Klang, *et al.*, 2019). Additionally, without a proper understanding of acetogenic community structure and dynamics, a microbiology oriented predictive mathematical model for biogas process cannot be developed (Fernandez *et al.*, 2000; Ni *et al.*, 2011). In this chapter, the overall practicality, usability and reliability of acetogenic community surveillance are discussed in relation to its practical application in commercial biogas installations. Physical and chemical analyses are not sufficiently reliable for use in optimizing and monitoring a biogas reactor, and therefore microbial community analysis is necessary (Ferguson *et al.*, 2014; Wu *et al.*, 2019). Several methods based on different principles have been proposed for assessment of microbial dynamics and health. However, there is still no single method that can be used independently and reliably for this purpose (Ferguson *et al.*, 2014; McMahon *et al.*, 2007). This is due to the inbuilt complexity and diversity of the biogas microbiome and to the absence of a core community which can represent all the variability in anaerobic digestion processes (Ferguson *et al.*, 2014; Fernandez *et al.*, 2000; Sundberg *et al.*, 2013) (Paper **IV**).

Different monitoring parameters have been proposed for monitoring of the bacterial community in biogas reactors. for example, the ratio of Firmicutes to Bacteroidetes (F/B) has been suggested as a performance

indicator in biogas reactors (Chen *et al.*, 2016). However, conflicting results have also been reported, with unexpected stability observed between these two phyla in reactors with different substrates (Kampmann *et al.*, 2012). Therefore, F/B ratio can work as an indicator in certain situations, but it cannot be used as a universal ratio affecting biogas reactor health. Moreover, Firmicutes and Bacteroidetes are among most dominant phyla in biogas reactors running on different substrates (Regueiro *et al.*, 2012; Schlüter *et al.*, 2008; Sundberg *et al.*, 2013), and the range of F/B ratio (16S rRNA gene 3:1-10:1, metagenomic 4:1-10:1) as an indicator is not reliable (Ferguson *et al.*, 2014; Güllert *et al.*, 2016). Further, a phylum-level comparison might have a risk of missing the community dynamics and variations at the lower taxonomic levels (family-genus) (Paper **III**).

Advanced microscopic methods have also been developed and employed in bacterial and archaeal visual quantification, *e.g.* fluorescence *in situ* hybridisation (FISH), confocal/electron microscopy and flow cytometry (Dhoble *et al.*, 2016; Karakashev *et al.*, 2005; Kinet *et al.*, 2016; Krakat *et al.*, 2010; Lebuhn *et al.*, 2015). However, these methods have limitations in biogas environments. In particular, they are too sophisticated and sensitive for dirty biogas samples, employ expensive instruments or require specific probes (mostly 16S rRNA gene) for targeting the bacterial community. Since methanogenic archaea harbour a methanogenic redox cofactor F_{420} in their cell membrane, visual detection is relatively easy under ultra-violet light (Schnürer & Jarvis, 2017). However, this cofactor is also present in bacterial phylum Actinobacteria (Ney *et al.*, 2017), which might interfere with visual quantification of methanogens. Thus, reliable and viable visual monitoring or surveillance is not a practical option. Further, no scientific studies specifically employing these microscopy/spectroscopy methods for monitoring the acetogenic community have been reported. In fact, there has been a complete lack of acetogen-specific studies employing FISH and microscopic/spectroscopic techniques.

A rapid cytometric histogram image comparison (CHIC) method has been developed and used by Koch and co-workers for rapid monitoring of microbial community dynamics (Koch, Fetzer, Harms, *et al.*, 2013; Koch, Fetzer, Schmidt, *et al.*, 2013). This method involves whole microbial

community profiling based on fluorescent staining with DAPI (4',6-diamidino-2-phenylindole), a stain which binds to the A-T rich region of DNA (Gomes *et al.*, 2013). This is the fastest method for microbial profiling in biogas environments presented (claimed) to date, with high resolution. However, this method has several drawbacks for the anaerobic digester samples. The major drawbacks are i) the type of samples which can be used and ii) DAPI as fluorescent stain. Koch and co-workers demonstrated the method with samples from an enrichment reactor using distillers' dried grain with solubles as substrate. In practice, flow cytometry is very sensitive to the quality of samples and any impurity can interfere with the assay or can even damage the instrument. The methodology cannot not be used for dirty biogas samples, which contain all sorts of impurities and inhibitory substances. Further, DAPI stains all living (less efficiently) or dead cells, prokaryotic or eukaryotic cells (Gomes *et al.*, 2013), and therefore the resulting profile is based on all living or dead bacterial, archaeal and fungal cells. Fluorescence staining and microscopy/cytometry of cells (eukaryotic or prokaryotic) is a sensitive process and any unknown parameter (impurities, inhibitors, inefficient staining *etc*.) can negatively affect the assay. Koch and co-workers claim that the method can be performed within few hours, but failed to mention the overnight incubation step in sample preparation. Thus, although the CHIC method could be very potent in quantifying community dynamics in biogas reactors, the complex environment of anaerobic digester is highly incompatible for cytometric analysis.

Quantitative analysis by qPCR is very powerful, sensitive and reliable methodology for analysis of whole bacterial or methanogenic communities. Since methanogens are very sensitive to changes in organic loading rate, hydraulic retention time, temperature changes, ammonia concentration, pH, VFA concentration *etc*., change in their abundance and activity can be very helpful in assessing the health of biogas reactors (Lebuhn *et al.*, 2015). However, methanogens are less diverse than whole bacterial communities (Sundberg *et al.*, 2013), respond less dynamically to changes in the reactor, and changes in methanogenic pathways without significant changes in process performance have been reported (Dearman *et al.*, 2006; Ferguson *et al.*, 2014; Fernandez *et al.*, 2000; Lebuhn *et al.*, 2015; Lv *et al.*, 2019). Therefore, use of cDNA/DNA ratio to analyse methanogen activity might

not provide very conclusive results (Lebuhn *et al.*, 2015). Moreover, qPCR can be used for quantification of gene copy numbers. This method has been widely used for bacterial and methanogens based on 16S rRNA or methanogen-specific *mcrA* genes (Bartell *et al.*, 2015; Bergmann *et al.*, 2010; Lebuhn *et al.*, 2015; Steinberg & Regan, 2009; Traversi *et al.*, 2011). However, there have been only a few attempts to target the acetogenic community in qPCR assays. This is due to the requirement for acetogen-specific qPCR primers. As discussed previously in this thesis, currently published FTHFS primers are not suitable for quantitative analysis of whole acetogenic communities (Paper **III**) and species-specific (16S rRNA or FTHFS gene) primers need to be designed, as demonstrated by Westerholm *et. al*. (2011a; 2012) and Müller *et al.* (2016). Although qPCR assay can be very powerful tool in accurate quantification of acetogenic bacteria, the limitations discussed hamper its widespread use in microbiological surveillance of acetogenic communities.

A new approach for calculating the metabolic quotient of methanogens was developed by Munk *et al.* (2012), based on relating methane production to the expression and count of *mcrA*/*mrtA* genes. It has been proposed as an important eco-physiological parameter to assess the health of biogas reactors, but the method still needs to be refined and calibrated, followed by continuous evaluation in a production-scale biogas reactor (Lebuhn *et al.*, 2015). Wider application of this method has not yet been achieved, but if it could be integrated with FTHFS gene-based acetogenic community dynamics and structure, it could be of extreme importance for biogas process optimisation.

The strategy in this thesis for surveillance of the acetogenic community based on the FTHFS gene in biogas reactors was developed, meticulously tested and compared with conventional methods and applied to samples from different laboratory-scale and commercial biogas reactors (Papers **III** and **IV**) (***Figure 13***, ***Figure 14***). In-depth analyses of acetogenic communities in samples from laboratory-scale or commercial biogas reactors revealed that the acetogenic communities (potential) in biogas reactors are very diverse, but have not previously been visualised and described (Papers **III** and **IV**). There is only one published article on high-throughput sequencing of FTHFS

amplicons, by Planý *et al.* (2019), but the approach they used is highly questionable. They do not describe the analysis method and have not submitted sequencing data to any public repository, and thus their results cannot be reproduced or verified.

Furthermore, the acetogenic communities are very dynamic regarding the relative abundance of different groups within these communities (Paper **IV**). It has been reported in countless studies that microbial community structure is very specific to the substrate and parameters used. The study reported in Paper **IV** described the acetogenic community structure and its temporal dynamics in full-scale biogas reactors running on different substrates, which had not been attempted before. The strategy employed in the surveillance described in Paper **IV** is visually depicted in ***Figure 14***. The surveillance results in Paper **IV** revealed that the acetogenic community is also dependent on the substrate and reactor operating conditions. Time series sample analysis of full-scale commercial plants indicated that changes in acetogenic community structure can occur with apparently no or minimum changes in VFA profiles (Paper **IV**). Some indicator genera and species that can be used as a marker or indicator of disturbance prior to any disturbance in VFA profile were identified in the thesis (Papers **III** and **IV**). However, detailed and descriptive FTHFS surveillance data are needed to validate these findings. Further, multiple biogas reactors running on different feed substrates need to be analysed to understand feed-specific acetogenic community structure and its temporal dynamics.

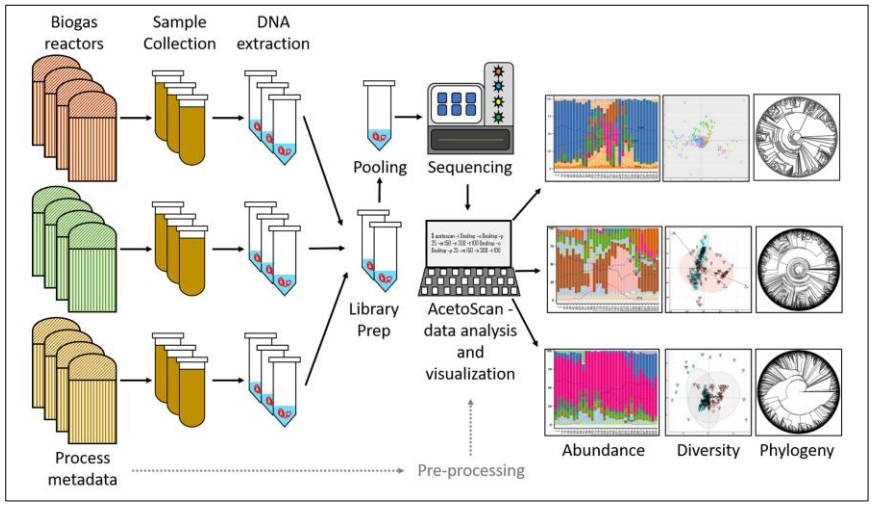


Figure 14. Diagrammatic visualisation of the microbiological surveillance carried out in Paper **IV**, where time-series samples from different biogas reactors were subjected to DNA isolation, library preparation and Illumina sequencing. The unsupervised data analysis and visualisation were done by AcetoScan.

8. Conclusions and perspectives

A new microbiological surveillance method targeting the acetogenic community in biogas reactors was developed. Thorough evaluation of the method indicated good potential for use in assessing the dynamics of acetogenic community in biogas reactors. However, the microbiological knowledge obtained must be integrated with technical advances for optimisation of the biogas process. Methanogens and hydrolysing/fermentative bacteria are very important in the biogas process and have been extensively studied. A good understanding of the community structure and dynamics of the acetogenic community is also needed so that a predictive mathematical model can be developed.

Swot analysis of the FTHFS gene-based microbiological surveillance method for biogas plants showed that accuracy, relative ease of application to a large number of samples, fast data analysis and visualisation are the main strengths of the surveillance method (***Figure 15***). Some technical and practical limitations of the method were also identified in this thesis. Overall, the method is good enough to expand the knowledge base on acetogenic communities in biogas reactors and can be also applied to other environments where acetogenic communities are involved. The method enables the most descriptive study to date of FTHFS gene-harbouring and potential acetogenic bacteria. The methodology for acetogen-focused studies in biogas reactors could be further improved in future by incorporating a functional activity-based approach.

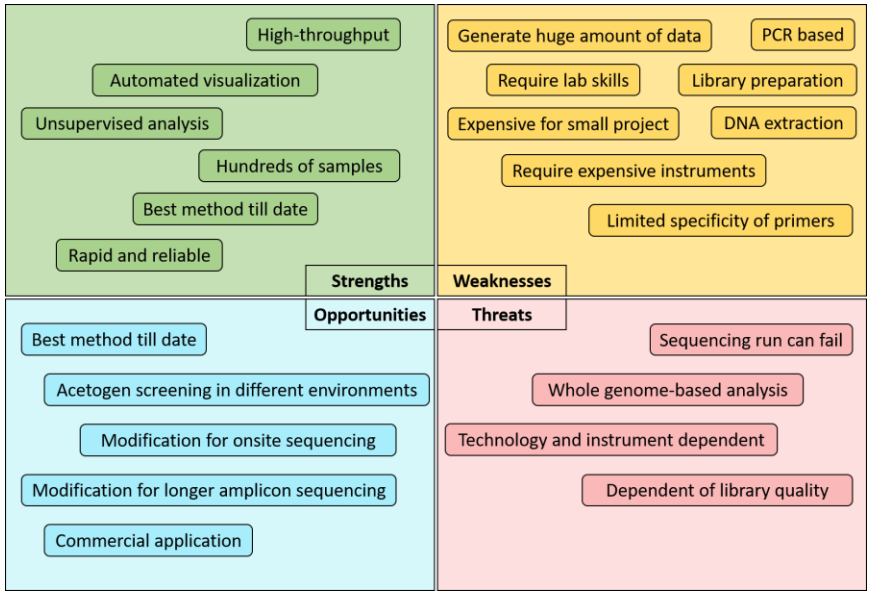


Figure 15. A swot analysis diagram describing the strengths, opportunities, weaknesses and threats of the FTHFS gene based microbiological surveillance of biogas plants.

8.1 Future perspectives

The tools and strategies presented in this thesis can help in achieving a greater understanding of acetogenic bacteria in ecosystem. Acetogenic bacteria are not only important in biogas systems, but are also present in abundance in human and animal/insect gut, where they play a critical role in gut physiology and gut-brain interactions (Breznak, 1994; Gibson *et al.*, 1990; Laverde Gomez *et al.*, 2019; Leclerc *et al.*, 1997; Mackie & Bryant, 1994; Ohashi *et al.*, 2007; Rey *et al.*, 2010). Acetogens have also been found to have an intricate relationship with plants (Küsel, Pinkart, *et al.*, 1999; Ohkuma *et al.*, 2015; Pester & Brune, 2006) and to play an important role in ecological carbon cycling in marine and sub-surface environments (soil/lake/marine sediments, hypersaline water bodies, rice fields, oilfields, deep subsurface sediments) (Conrad, 1986; Kotsyurbenko *et al.*, 1996, 2001; Küsel, Wagner, *et al.*, 1999; Liu & Conrad, 2011; Liu & Suflita, 1993;

Marcelis *et al.*, 2003; Nozhevnikova *et al.*, 1994; Ollivier *et al.*, 1994; Rosencrantz *et al.*, 1999; Sokolova *et al.*, 2020). Acetogens are highly diverse organisms, are very versatility metabolically and can grow heterotrophically at the thermodynamic borderlines in different environments (Lever, 2012; Schuchmann & Müller, 2014; Seifritz *et al.*, 2003). Modern circular bio-economy trends to mitigate climate change and sustainable industrial processes are now using acetogenic bacterial communities for production of biochemicals, modern biofuels/syngas and biohydrogen (Liew *et al.*, 2016; Müller, 2019; Nevin *et al.*, 2011; Oren, 2012; Parameswaran *et al.*, 2011; Saheb-Alam *et al.*, 2017; Scott & Yu, 2015; Wiechmann & Müller, 2019). Acetogens are ubiquitously found in almost all anaerobic environments and thus elaborate acetogenic community studies are needed to decode their role in environmental ecology (Ni *et al.*, 2011).

9. Glossary of definitions

For any subject or scientific study, it is important to formulate definitions in relation to the theme of the main topic, since definitions can differ in different perspectives. The following definitions were used in this thesis.

16S rRNA gene - a highly conserved gene encoding 16S ribosomal RNA, which is widely used as a taxonomic marker for prokaryotes.

AcetoBase - a repository and database for FTHFS sequences.

Acetogens - anaerobic bacteria which use the acetyl-CoA pathway and reduce two moles of carbon dioxide to one mole of acetyl-CoA, while conserving energy in an autotrophic mode of growth.

AcetoScan - an automated and unsupervised data analysis pipeline for next-generation sequence data analysis for FTHFS amplicon sequencing.

Anaerobic digestion - an anaerobic microbiological process where a complex consortium of interdependent bacteria, fungi and methanogenic archaea degrade organic substrate to biogas and biofertiliser.

Biogas - a mixture of gases, comprising mostly of methane and carbon dioxide, produced by microorganism during the anaerobic digestion of biodegradable substrates.

Carbon dioxide - an inorganic molecule composed of one carbon and two oxygen atoms which acts as an electron acceptor in the process of

acetogenesis. A gaseous metabolic by-product of microbiological processes in anaerobic digesters.

ELR - economic loss risk, a risk factor of economic losses on a scale from 1 to 10 predicted for all biogas installations together for a Swedish county. It is a non-standard parameter formulated in this thesis for the aim of visualising county-wise Swedish biogas installations (see Appendix).

FTHFS - formyltetrahydrofolate synthetase, an important enzyme of the acetyl-CoA pathway which is structurally and functionally conserved and its coding gene is a marker for acetogenic bacteria.

Methane - a gaseous metabolic product of methanogenic archaea in the anaerobic digestion process which is flammable and used as a fuel.

Methanogens - a member of the domain archaea, which use the methanogenic biochemical pathway to generate methane.

Microbial - a property of a microorganism related to its physical construction, genome and phylogeny.

Microbiological - a property of a microorganism related to its physiology and interaction with its environment.

Microbiological monitoring - systematic, continuous or periodical, active or passive collection of data to detect any changes and their impacts within a microbiological community.

Microbiological surveillance - active, systematic, dynamic and intensive investigation of a specific microbial group to detect any changes in its composition or abundance within a certain threshold limit, which can indicate a further course of action.

Renewable energy - energy generated from renewable resources, which may or may not be entirely carbon neutral or aesthetically pleasing.

SAOB - syntrophic acetate-oxidising bacteria, which produce carbon dioxide and hydrogen by oxidation of acetate and have a hydrogen-based interdependent relationship with hydrogen-consuming methanogenic archaea.

Syntrophy - a mutualistic and interdependent relationship between organic acid-oxidising bacteria and methanogenic archaea where bacteria and methanogens act as producer and consumer of metabolic products.

T-RFLP - terminal restriction fragment length polymorphism, a method for analysing microbial identity and diversity by the restriction enzyme digestion of marker gene amplicons from an environmental sample followed by size detection of terminally labelled restriction fragments.

VFA - volatile fatty acids, are short-chain derivatives of fatty acids, mainly contains acetate and propionate, produced during anaerobic digestion process.

Wood-Ljungdahl pathway - also known as acetyl-CoA pathway, of autotrophic growth used by acetogenic bacteria to conserve energy during the reduction of two moles of carbon dioxide to one mole of acetyl-CoA.

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Popular science summary

Increases in atmospheric levels of greenhouse gases (carbon dioxide, methane), mainly due to human activities, have resulted in an increase in the average temperature of the Earth, *i.e.* global warming. To mitigate the drastic climate situation, net carbon dioxide emissions world-wide must be reduced. Production of renewable, low-carbon energy can alleviate the devastating climatic impacts of global warming without impeding the development of human societies world-wide. Biogas has great potential to minimise the current dependence on fossil fuels, increase fuel security, climate mitigation impacts and enable sustainable development. Biogas is produced in a microbiological process called anaerobic digestion, where biodegradable material undergoes microbial decomposition, yielding biogas and biofertiliser. Anaerobic digestion is a very versatile process and can serve multiple environmental goals, but the microbiological steps involved in the process can restrict large-scale biogas production and efficient use of biogas reactor volume. For adequate use of the resources invested in commercial biogas production, process optimisation and continuous monitoring of the process are essential.

Biogas microbiology is not fully understood, in particular regarding the microbes present and their specific roles in the biogas process. Current scientific information indicates that acetogenic bacterial communities play a very important role in the process. Acetogenic bacteria are a special group which are functionally versatile and act as an important link between two key microbiological steps. Acetogenic group of bacteria also help in equilibration of compounds, which is important for methane-producing microorganisms in the biogas process. Therefore, microbiological surveillance or close

monitoring of the acetogenic community can be used to assess biogas process stability. In this thesis, the new methods for assessment of acetogenic community structure in biogas processes were developed and a surveillance strategy based on bacterial DNA sequencing and computer-assisted methods was devised.

The surveillance strategy was carefully tested and compared against existing methods. The results showed that the method developed in this thesis was more helpful in analysis and interpretation of the acetogenic communities than existing methods. In further testing, the surveillance method was used to study acetogenic bacterial community structure and dynamics in full-scale commercial biogas reactor operated with different feed substrates, such as household food waste, sludge, manure, green waste *etc*. This revealed that the structure of the acetogenic community was specific for the feed substrate used in the reactor for biogas production.

Thus the tools and acetogenic community surveillance strategy developed within this thesis can be used reliably in microbiological surveillance of commercial biogas plants. Furthermore, the overall approach used in this thesis can be of great help in uncovering the role of the acetogenic community in other environments, such as the gut of insects, animals and humans, marine sediments, soil *etc*.

Populärvetenskaplig sammanfattning

Ökningar i atmosfäriska nivåer av växthusgaser (koldioxid, metan), främst på grund av mänskliga aktiviteter, har resulterat i en ökning av jordens medeltemperatur, dvs. global uppvärmning. För att mildra den drastiska klimatsituationen måste nettokoldioxidutsläppen över hela världen minskas. Produktion av förnybar energi med låga koldioxidutsläpp kan lindra den globala uppvärmningen utan att hindra utvecklingen av mänskliga samhällen över hela världen. Biogas har stor potential att minimera det nuvarande beroendet av fossila bränslen, försäkra bränsletillförsel, ge klimatreducerande effekter och möjliggöra en hållbar utveckling. Biogas produceras i en mikrobiologisk process som kallas anaerob rötning, där biologiskt nedbrytbart material genomgår mikrobiell nedbrytning i en syrefri miljö. Processen ger utöver biogas också ett biogödsel. Anaerob rötning är en mycket mångsidig process som kan uppfylla flera miljömål, men de mikrobiologiska stegen som är involverade i processen kan begränsa storskalig biogasproduktion och effektiv användning av biogasreaktorvolym. För adekvat användning av de resurser som investeras i kommersiell biogasproduktion är processoptimering och kontinuerlig övervakning av processen avgörande.

Mikrobiologi i en biogasprocess är ännu inte helt förstådd. Särskilt fattas kunskap med avseende på de närvarande mikroberna och deras specifika roller i processen. Aktuell vetenskaplig information tyder på att acetogena bakteriesamhällen spelar en mycket viktig roll i processen. Acetogena bakterier är en speciell grupp som är funktionellt mångsidiga och fungerar som en viktig länk mellan två viktiga mikrobiologiska steg. Den acetogena gruppen av bakterier bidrar också till att skapa jämvikt mellan olika kemiska

föreningar i biogasprocessen, vilket är viktigt för de metanproducerande mikroorganismer. Därför kan mikrobiologisk övervakning eller noggrann övervakning av de acetogena bakterierna användas för att bedöma biogasprocessens stabilitet. I denna avhandling utvecklades en ny metod för analys av den acetogena samhällsstrukturen i biogasprocesser och en övervakningsstrategi baserad på bakteriell DNA-sekvensering och datorassisterade metoder utformades.

Övervakningsstrategin testades noggrant och jämfördes med befintliga analysmetoder. Resultaten visade att metoden som utvecklats i denna avhandling var mer användbar vid analys och tolkning av de acetogena samfunden än befintliga metoder. Vid ytterligare tester användes övervakningsmetoden för att studera samhällsstruktur och dynamik av acetogener i flera fullskaliga kommersiella biogasreaktorer som drevs med olika material, såsom hushållsavfall, slam, gödsel, grönt avfall *etc.* Analysen visade att strukturen hos det acetogena samhället var specifikt för det material som användes i reaktorn för produktion av biogas.

Sammantaget visade studierna att verktyg och analysmetoder som utvecklats inom denna avhandling kan användas på ett tillförlitligt sätt för mikrobiologisk övervakning av kommersiella biogasanläggningar. I förlängningen kan också det övergripande tillvägagångssättet som används i denna avhandling vara till stor hjälp för att analysera acetogena bakterier i andra miljöer, såsom tarmen av insekter, djur och människor, marina sediment, jord *etc*.

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If you are reading this thesis text and understanding it in correct meaning and sense, for that, I want to thank Mary McAfee for her great and fast assistance in linguist corrections and modification.

Appendix

To mitigate climate change and reduce the greenhouse gas emissions, global partnership and cooperation is needed. Sweden is an environmental pioneer and leads the world in the area of climate change and its prevention (Naturvårdsverket, 2004; SI, 2020). Sweden has become the first Nordic country to enter the climate emergency movement (CED, 2020) and is the fourth-ranked country (first three places unassigned) on the climate change performance index (Germanwatch e.V., 2020). The Swedish government has set the sustainability goal of being a 100% fossil-free, renewable energy-driven country by the year 2045 (SI, 2020). This is a very ambitious goal. Sweden excels as a global leader in sustainable biogas production and use (up to 78% of biogas for transport fuel) (Koonaphapdeelert *et al.*, 2020; Price, 2011). Biogas production in Sweden is mainly based on animal and agricultural waste, sewage sludge and municipal solid waste, with some use of energy crops, which makes Swedish biogas very sustainable. However, in 2019, Sweden imported almost half of its total biogas demand (Klackenberg, 2020). A Swedish government report clearly state that more biogas is needed and recommends policies to boost production of more biogas and biofertiliser (co-product) (SOU, 2019).

Due to the high demand and support from government, the biogas market in Sweden is growing and several national and multinational companies are focusing on establishing biogas plants. Commercial biogas production is a lucrative business, but a constant and stable supply of biogas is needed for it to be profitable. Although anaerobic digestion is a simple process, in commercial applications it is complex and sensitive. This complexity and sensitivity are associated with the large volumes of substrates used as a feedstock. As anaerobic digestion is a microbiological process where different microorganisms work together, biological homeostasis inside the digester is important. Any disturbance in the microbial community can result in unstable biogas production or sometimes even failure of the biogas

reactor. Thus microbiological associations with reactor disturbance were investigated in this thesis.

With Sweden's ambitious aim of fossil-free transport by 2030, its biogas market is growing at a fast pace. In 2019, there were 280 biogas plants with a cumulative volume of 741,655 m^3 and producing about 1970 GWh of biogas (Klackenberg, 2020). However, they will not be enough to meet the growing demand for biogas in future unless they can achieve stable high-level operation. To ensure balanced and steady production of biogas, constant monitoring of process operations is required (Drosg, 2013). This is done using physical and chemical analysis of different parameters. In commercial biogas plants, huge capital is invested in reactors and stable operation of the process and there is always a risk of economic losses. The theoretical economic loss risk (ELR) describes the risk of economic losses on a scale from 1 to 10. Different companies own the biogas plants in Swedish counties, but for the ELR calculation in this thesis a county was considered the owner of the biogas plant and would bear the economic losses in the case of biogas process failure.

$$ELR = round\left(\frac{\left(\frac{Cumulative\ volume\ (cu.m.)}{Number\ of\ reactor}\right)}{1000\ (cu.m.)}\right) \ldots\ldots\ldots \textbf{(equation 1)}$$

To calculate the ELR for the individual county, **equation 1** was used. The resulting ELR of 21 Swedish counties is presented in ***Figure A1***. The results indicated that Gotland and Stockholm county (2 and 17 reactors, respectively) have a high risk of economic losses, while Västra Götaland county (44 reactors) has a low risk of economic losses. This theoretical ELR of county-wise biogas reactor indicates the probability of economic losses, but all biogas reactors, irrespective of high or low ELR, need constant and careful monitoring. Due to the fear of process failure, most biogas plants do not operate their biogas reactors to full capacity. This reduces the overall biogas production and profitability of the whole facility.

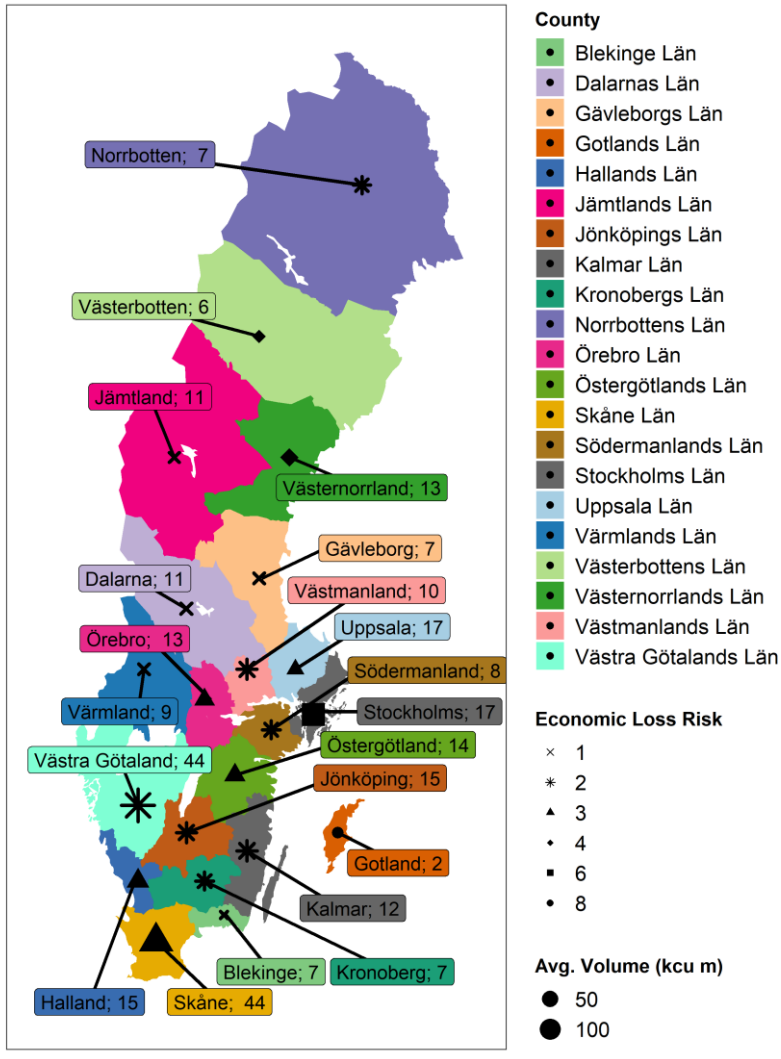

Figure A1. - Map (©Abhijeet Singh) of Sweden showing the county-wise number of biogas reactors in Sweden with their cumulative reactor volume and economic loss risk (ELR), calculated using equation 1. The raw data for the calculations was taken from Klackenberg (2020).

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Acetogens are ubiquitously found in anaerobic environments, thus elaborate studies are needed to decode their role in environmental ecology. Especially in biogas environments, acetogenic bacteria help in equilibration of compounds, which is important for methanogens. This thesis focused on establishment of a microbiological surveillance strategy for acetogenic communities in industrial biogas reactors using different substrates. The strategy reported in this thesis will advance understanding of acetogenic communities in anaerobic environments.

Abhijeet Singh received his PhD education at the Department of Molecular Sciences, SLU, Uppsala. He received his M.Sc. in AgriGenomics at Christian Albrechts Universität zu Kiel, Germany and B.Sc. in Agriculture at Anand Agricultural University, Anand, India.

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