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Preface

This Ph.D. thesis, entitled "Deciphering the microbial ecology in biogas reactors for optimizing the anaerobic digestion process" comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from December 1st, 2014 to November 30th, 2017. Professor Irini Angelidaki was the main supervisor and Dr. Panagiotis Kougias and Dr. Laura Treu were the co-supervisors.

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

I **Zhu X**, Kougias PG, Treu L, Campanaro S, Angelidaki I (2017a) Microbial community changes in methanogenic granules during the transition from mesophilic to thermophilic conditions. Appl Microbiol Biotechnol 101:1313–1322.

II **Zhu X**, Treu L, Kougias PG, Campanaro S, Angelidaki I (2017b) Characterization of the planktonic microbiome in upflow anaerobic sludge blanket reactors during adaptation of mesophilic methanogenic granules to thermophilic operational conditions. Anaerobe 46:69–77

III **Zhu, X**., Treu, L., Kougias, P.G., Campanaro, S., Angelidaki, I. (2017). Converting mesophilic upflow sludge blanket (UASB) reactors to thermophilic by applying axenic methanogenic culture bioaugmentation. Chem Eng J 332:508–516.

IV **Zhu, X**., Campanaro, S., Treu, L., Kougias, P.G., Angelidaki, I. (2018). Microbial community in anaerobic digestion of saccharides: composition and functions revealed through metagenomics. (*Manuscript under preparation for submission)*

V Kougias, P.G., Campanaro, S., Treu, L., **Zhu, X**., Angelidaki, I. (2017). A novel archaeal species belonging to *Methanoculleus* genus identified via *de-novo* assembly and metagenomic binning process in biogas reactors. Anaerobe 46:23–32

In this online version of the thesis, paper **I-V** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk

In addition, the following publications, not included in this thesis, were also concluded during this PhD study:

Luo, G., De Francisci, D., Kougias, P.G., Laura, T., **Zhu, X**., Angelidaki, I. (2015) New steady-state microbial community compositions and process performances in biogas reactors induced by temperature disturbances. Biotechnol. Biofuels 8, 1.

Treu, L., Campanaro, S., Kougias, P.G., **Zhu, X**., Angelidaki, I. (2016) Untangling the effect of fatty acids addition at species level revealed different transcriptional responses of the biogas microbial community members. Environ. Sci. Technol 50(11): 6079-6090

Kougias, P.G., Treu, L., Campanaro, S., **Zhu, X**., Angelidaki, I. (2016) Dynamic functional characterization and phylogenetic changes due to Long Chain Fatty Acids pulses in biogas reactors. Sci. Rep. 6

Campanaro, S., Kougias, P.G., Treu, L., **Zhu, X**., Angelidaki, I. (2018) Do different high throughput sequencing techniques provide the same taxonomic results for the anaerobic digestion microbiome? *(submitted to Sci. Rep.)*

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This Ph.D. thesis is dedicated to everyone who was in my life in the last three years.

Summary

Anaerobic digestion (AD) is a microbial mediated process where organic compounds are degraded to biogas (CH_4 and CO_2). AD occurs in many natural anoxic environments and is an essential step for global carbon circle. Engineered AD systems, i.e. biogas reactors, enhance methanogenic activity by applying empirical operational conditions, in order to accelerate the methane production for energetic purposes. In Denmark, biogas produced from AD has a considerable share in renewable energy with the expectation to expand. Thus, the more effective operation of biogas plants will significantly benefit Denmark's sustainable development. As AD relies on complex microbial activity, a more comprehensive understanding of the AD microbial consortia and their activity provides the fundamental knowledge for process control and optimization.

In AD, the microbial metabolisms are mostly thermodynamically constrained and the obligatory syntrophy is an essential intermediary step. Thus, the majority of AD microbiota remains uncharacterized since in the past it was mainly investigated using cultivated-based methods. The advent of more powerful sequencing technology (i.e. next generation sequencing, NGS) and newly developed bioinformatic methods enable researchers to perform *in-situ* analyses on uncharacterized microbial communities. The applications of NGS technology were proved to be effective tools to reveal AD microbial ecology. However, the detailed mechanisms of microbial activity are still far from fully elucidated due to the intricacy of AD process.

This Ph.D. project relied on comprehensive investigations of microbial communities in order to optimize the AD process and elucidate the fundamental metabolisms. Specifically, in the case of process optimization, 16S rRNA amplicon sequencing was used to identify, analyse and solve the operational challenges during the start-up of thermophilic up-flow anaerobic sludge blanket (UASB) reactors. To elucidate the microbial metabolisms, genome-centric metagenomics was applied to characterize methanogenic communities degrading a set of defined substrates. In addition, the Ph.D. study also expands the understanding of AD microbial ecology by proposing and characterizing a novel *Candidatus* species ubiquitously present in AD systems.

The start-up of thermophilic UASB reactors was investigated in lab-scale reactors inoculated with mesophilic granules. After increasing the operational temperature from mesophilic to thermophilic, volatile fatty acids (VFAs) and

alcohols were found as the main digestion products. Methane production, on the other hand, only initiated after bicarbonate addition as external pH control. The dynamicity of microbial community composition in the granules during the temperature shift suggested that the majority of the mesophilic microbes could not tolerate the thermophilic conditions. Moreover, it was demonstrated that the fermentative thermophiles first evolved in the liquid phase of UASB reactor and then were encapsulated in the granular structure of the sludge. The growth of these bacteria rapidly restored the hydrolysis, acidogenesis and acetogenesis in the reactor. On the contrary, the thermophilic methanogens grew much slower than fermentative bacteria leading to severe process imbalance (i.e. accumulation of VFAs and alcohols). Thus, the evolvement of thermophilic methanogens was recognized as the biological 'bottleneck' during the temperature transition. To overcome the identified obstacle, bioaugmentation, i.e. provision of exogenous microbes, was proposed to accelerate the microbial community adaptation. The best strategy found to perform bioaugmentation was the injection of axenic methanogenic cultures. This practice significantly increased the thermophilic methane production rate by 40% compared with the control reactor (i.e. without bioaugmentation). The enhancement of methane production was attributed to the evolvement of exogenous *Methanothermobacter thermautotrophicus* and the concomitant growth of its syntrophic partners in the granular structure. The positive effects brought by bioaugmentation were persistent in UASB reactor due to the retention of the microbes in the granular sludge.

For the investigation of the basic microbial metabolism and ecology, methanogenic microbial communities were enriched in a lab-scale continuous stirred-tank reactor (CSTR) fed with synthetic feedstocks. In the experiment, the substrates used were stepwise simplified (i.e. polysaccharide, monosaccharide, short chain fatty acids, acetate) to mimic the four steps of AD process. During the continuous operation, the microbial community was substantially simplified, because the microbes that could not metabolize the specific compounds were washed out. The overall microbial community consisted of only 35 metagenome assembled genomes (MAGs) (31 bacterial and 4 archaeal). The abundance of these MAGs dramatically varied in the communities adapted to different substrates. The shifts in microbial community composition indicate that MAGs have specific functional roles in AD food chain and their roles cannot always be physiologically defined in accordance with 4 AD steps. Moreover, the explicit degradation pathways were reconstructed from the functional annotation of MAGs. It is notable that, a novel glucose degra-

dation model was proposed with the syntrophic activity of *Clostridiaceae* sp. and *Methanoculleus thermophilus*. In this model, acetate is not produced as intermediate compound.

The genome-centric metagenomics reveals a considerable number of MAGs that could not be taxonomically assigned to characterized species. A MAG extracted from co-assembly of 8 AD metagenomes was especially emphasized due to its ubiquity in AD system and its high abundance under specific conditions. From the functional annotation and gene expression profile, it is confirmed that this MAG performs hydrogenotrophic methanogenesis in AD system and is found dominant from the reactors where H_2 was added. This genome is present in 40 different samples from both full-scale and lab-scale AD reactors. The MAG was found in higher abundance during thermophilic reactor operations with relatively short hydraulic retention times. The phylogenetic assignment was based on 400 conserved genes and on 16S rRNA genes. The two methods concordantly showed that this MAG is closely related to *Methanoculleus bourgensis* MS2^T. However, the average nucleotide identity between *M. bourgensis* MS2^T and the selected MAG was only 89%, which is too low similarity to assign the MAG at the species level. Thus, we propose a novel *Candidatus* species inside the *Methanoculleus* genus. According to the metabolic traits, it is named as *Candidatus* Methanoculleus thermohydrogenotrophicum, sp. nov.

Overall, the results from this Ph.D. study bring new knowledge on the AD process based on NGS technology. Practically, the gained information regarding microbial community composition and dynamicity was directly used to solve technical challenges in AD operations. Fundamentally, deeper insights into the microbial metabolisms and ecology substantially expanded the current understanding of AD. The revealed knowledge provides pivotal prerequisites for future AD process control and optimization.

Dansk sammenfatning

Anaerob udrådning (AD) er en mikrobiel proces, hvor organiske materialer nedbrydes til biogas (CH_4 og CO_2). AD foregår i mange naturligt anaerobe miljøer og er en essentiel del af det globale kulstof-kredsløb. I konstruerede AD systemer, dvs. biogas-reaktorer, øges den metanogene aktivitet ved at anvende empirisk baserede operationelle forhold for at accelerere produktionen af metan til energiformål. I Danmark udgør biogas produceret ved AD en betragtelig del af den vedvarende energiproduktion, og denne andel forventes at øge yderligere. Således vil en mere effektiv drift af biogasanlæggene gavne Danmarks bæredygtige udvikling betydeligt. Da AD afhænger af kompleks mikrobiel aktivitet, vil en dybere indsigt i de mikrobielle konsortier og deres aktivitet udgøre fundamental viden om proceskontrol og optimering.

I AD er metabolismerne overvejende termodynamisk begrænset og den obligatoriske syntrofi er et essentielt led. Derfor er størstedelen af mikrobiotaen i AD processen ikke karakteriseret via kultiverings-baserede metoder. Udviklingen af mere tilgængelige sekventerings-teknologier (dvs. next generation sequencing, NGS) og bioinformatik-baserede metoder har gjort det muligt for forskere at udføre *in-situ* analyser på ikke-karakteriserede mikrobielle samfund. Anvendelsen af NGS-teknologien har vist sig at være et effektivt redskab til at afsløre den mikrobielle økologi i den anaerobe udrådningsproces. De detaljerede mekanismer er dog fortsat langt fra alle belyst, grundet den komplicerede proces.

Dette Ph.D.-projekt fokuserer på omfattende at undersøge de mikrobielle samfund involveret i AD, med det mål at kunne optimere processen og belyse de fundamentale metabolismer. Specifikt blev 16s amplicon sekventering, i forbindelse med procesoptimering, benyttet til at identificere, analysere og løse de operationelle udfordringer under opstart af en termofil UASB (up-flow anaerobic sludge blanket) reaktor. For at belyse de fundamentale metabolismer blev genom-centrisk metagenetik benyttet til at karakterisere metanogene mikrobielle samfund under nedbrydning af veldefinerede substrater. Dette Ph.D.-projekt bidrager med viden til forståelsen af den mikrobielle økologi i AD-processen ved at foreslå og karakterisere en ny *Candidatus* art, som er allestedsnærværende i AD-systemer.

Opstarten af UASB reaktorer blev undersøgt i laboratorie-skala reaktorer inokuleret med mesofile granulater. Efter at have øget temperaturen fra mesofil til termofil blev det observeret at flygtige fedtsyrer (VFAs) og alkoholer var

de dominerende nedbrydningsprodukter. Metanproduktionen påbegyndtes dog først efter yderligere bicarbonat blev tilsat, som ekstern pH-kontrol. Dynamikken i sammensætningen af de mikrobielle samfund i granulaterne ifm. temperaturskiftet indikerede, at hovedparten af de mesofile mikrober ikke kunne overleve de termofile forhold. De blev erstattet af termofile mikrober, som først indfandt sig i UASB reaktorens væskefase for derefter at blive indkapslet i granulatstrukturerne. Væksten hos disse bakterier genetablerede hurtigt hydrolysen, acidogenesen og acetogenesen i reaktoren. De termofile metanogener voksede derimod meget langsommere, hvilket resulterede i alvorlig ubalance i processen (dvs. akkumulering af VFA'er og alkoholer). Således kunne det slås fast, at væksten af de termofile metanogener var "flaskehalsen" ifm. temperaturskiftet. For at løse dette blev det foreslået at benytte bioaugmentation, dvs. tilsætning af exogene mikrober, for at accelerere tilpasningen af det mikrobielle samfund. Den bedste strategi til at udføre bioaugmentation var injektion af axeniske metanogene kulturer. Denne praksis øgede den termofile metanproduktion signikant med 40 % sammenlignet med kontrollen. Den øgede metanproduktion bidrog til udviklingen af exogene *Methanothermobacter thermautotrophicus*, samt dennes syntrofiske partnerorganismer, i den granulære struktur. De positive effekter ved bioaugmentation blev bibeholdt grundet tilbageholdelsen af granulaterne i UASB-reaktoren.

For at undersøge den fundamentale mikrobielle metabolisme og økologi, blev metanogene mikrobielle samfund beriget i en laboratorie-skala kontinuert omrørt tank-reaktor (CSTR) med syntetisk fødemateriale. Gennem eksperimentet blev substraterne gradvist forsimplet (hhv. polysakkarider, monosakkarider, kortkædede fedtsyrer, acetat) for at efterligne de fire dele af AD-processen. Gennem den kontinuere drift blev sammensætningen af det mikrobielle samfund væsentligt simplere, da de mikrober, der ikke metaboliserede de specifikke komponenter, blev vasket ud. Det samlede mikrobielle samfund bestod af kun 35 metagenome assembles genomer (MAGs; 31 bakterielle og 4 archaea). Tilstedeværelsen af disse MAGs varierede betragteligt i de mikrobielle samfund afhængigt af fødematerialet. De skift der blev observeret i de mikrobielle samfunds sammensætning indikerer, at disse MAGs har specifikke funktionelle roller i AD-fødekæden, og at deres roller ikke altid defineres fysiologisk gennem de 4 AD-skridt. Derudover var de omfattende nedbrydningsmønstre rekonstruerede fra den funktionelle annotation af MAG. En ny model for nedbrydning af glukose er desuden foreslået, baseret på syntrofisk aktivitet mellem *Clostridiaceae* sp. og *Methanoculleus thermophilus*. I denne model produceres ikke acetat som et mellemprodukt.

Den genom-centriske metagenetic afslører adskillige MAGs, som ikke taxonomisk kunne relateres til karakteriserede arter. Et MAG ekstraheret fra sammenblanding af 8 AD metagenomer var specielt interessant grundet dens udbredelse i AD-systemer og dens høje tilstedeværelse under specifikke forhold. Fra den funktionelle annotation og genekspressions-profil kan det bekræftes at dette MAG udfører hydrogenotrofisk metanogenese i AD-systemet. Dette genom er tilstede i 40 forskellige prøver, både fra laboratorie- og fuldskala AD-reaktorer. Prøverne viser desuden, at tilstedeværelsen af dette MAG øges under termofile forhold med relativt korte hydrauliske opholdstider. Rekonstruktionen af fylogenien, baseret på 400 bevarede gener, og på 16S rRNA gener viste begge, at dette MAG er tæt relateret til *Methanoculleus bourgensis* MS2^{T}. Den gennemsnitlige overensstemmelse mellem nukleotiderne i *M. bourgensis* MS2^{T} og det valgte MAG er kun 89%, hvilket er for lavt til at tilskrive dette MAG på arts-niveau. Vi foreslår derfor dette MAG som en ny *Candidatus* art i slægten *Methanoculleus*. Grundet de metaboliske egenskaber er den tildelt navnet *Candidatus* Methanoculleus thermohydrogenotrophicum, sp. nov.

Alt i alt demonstrerede resultaterne fra nærværende Ph.D.-studium forskellige anvendelser af NGS-teknologien i AD-systemer. Den opnåede indsigt i sammensætningen af de mikrobielle samfund og dynamikkerne blev direkte benyttet til at løse tekniske udfordringer i driften af AD-reaktorer. Dybere indsigt i den mikrobielle metabolisme og økologi har udvidet den nuværende forståelse af AD-processen væsentligt. Den opnåede viden udgør værdifulde forudsætninger for fremtidig AD proceskontrol og -optimering.

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Abbreviations

AD	anaerobic digestion
ARDRA	amplified rDNA restriction analysis
BMP	biomethane potential
CSTR	continuous stirred-tank reactor
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EU	European Union
FISH	fluorescence in-situ hybridization
g	gram
HRT	hydraulic retention time
L	liter
LCFA	long chain fatty acid
MAG	metagenome assembled genome
mRNA	messenger RNA
mtoe	million tonnes of oil equivalent
NGS	next generation sequencing
OLR	organic loading rate
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
SSCP	single strand conformation polymorphism
TGGE	temperature gradient gel electrophoresis
tRNA	transfer RNA
UASB	up-flow anaerobic sludge blanket
VFA	volatile fatty acid

1 Introduction

1.1 Background

The survival and development of humankind are based on the consumption of fuels. In 2016, oil was the dominant fuel followed by coal and natural gas. These three sources accounted for 86% of the global primary energy consumption (British Petroleum, 2017). The dependence on fossil fuels brings great challenges to sustainability due to resources limitations, global warming impacts, and pollution prone applications. In future pursuit, alternative energy sources should be developed to progressively replace the use of fossil fuels. Furthermore, initiatives and regulations should be adopted to motivate this transition. To address the challenges, European Union (EU) intends to increase the share of renewable energy to 20% by 2020. Denmark is one of the most ambitious countries for sustainable development and targets to completely replace fossil fuel from electricity and heating sectors by 2035 (European Commission, 2014; The Danish Government, 2011). In particular, biogas production was included as an integral part to achieve the 2035-goal. Additional subsidies were also planned to be implemented for biogas expansion in Denmark (Ministry of Climate Energy and Building, 2012).

Biogas, composed mainly of CO_2 and CH_4, is produced during a bio-mediated degradation process, namely Anaerobic Digestion (AD). In absence of oxygen, the organic matters are transformed into combustible biogas, which is subsequently utilized for heat and electricity production. The liquid residuals from AD, known as digestates, contain a mixture of minerals (N, P, K, Ca and etc.), which could be used as fertilizer on agriculture fields. A conspicuous advantage of AD is that it is an extremely robust process and can adopt heterogeneous substrates such as manure, sludge, organic waste, and wastewater. Therefore, biogas plants were implemented extensively to treat the organic wastes and recover energy simultaneously. In addition, AD is also widely used as the final step of biorefinery processes in order to scavenge organic residuals and to maximize the value derived from the feedstock. By October 2016, Denmark had installed 20 centralized biogas plants and 43 farm biogas plants. Those plants produced 6348 TJ energy in 2015, accounting for 4.1% of the total energy production obtained from the renewable sources (Danish Energy Agency, 2017).

1.2 Anaerobic digestion process

AD is mediated by a complex and specialized microbial community and initially occurs in natural anaerobic environments, such as marshes, sediments and animal rumens (Thauer et al., 2008). Because methane is a potential energy source, engineered systems, i.e. biogas reactors, were created to accelerate and scale up this bio-conversion process. Microorganisms that mediated the degradations are proliferated in the specific ecological niches provided by various reactor configurations and operational conditions. Consequentially, the microbial methanogenic activity is magnified in reactors to optimize the methane production rate and efficiency.

1.2.1 Anaerobic digestion steps

The entire AD process can be divided into four sequential steps, namely hydrolysis, acidogenesis (fermentation), acetogenesis and methanogenesis (Angelidaki and Batstone, 2011).

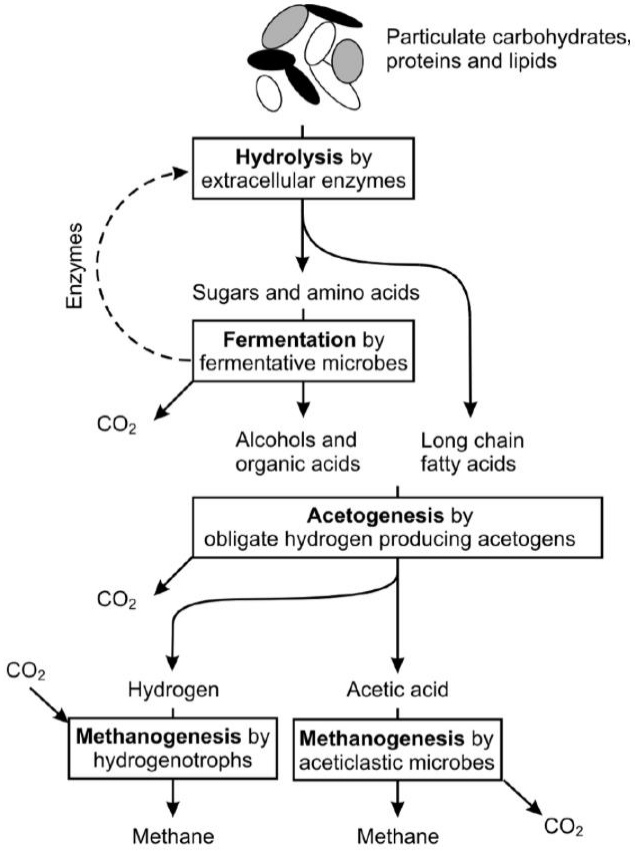

Figure 1: Schematic description of the AD process [Adapted from (Angelidaki and Batstone, 2011)].

The typical substrates used for biogas production are biopolymers such as carbohydrates, proteins, and lipids. Hydrolysis breaks down these macromolecules into monomers such as monosaccharides, amino acids, long chain fatty acids (LCFAs) and glycerol. Hydrolysis is an extracellular process catalysed by enzymes excreted by fermentative bacteria to allow the cellular uptake of the substrates. The soluble hydrolysed products are further catabolized by fermentative bacteria, also known as acidogenesis. During this step, volatile fatty acids (VFAs) (with more than two carbons) are produced together with acetate, carbon dioxide, and hydrogen. In acetogenesis step, volatile fatty acids can be further transformed into methanogenic substrates (i.e. acetate, CO_2 and H_2) by syntrophic oxidizers, with the presence of hydrogen consumers. Eventually, archaea scavenge the products from the previous steps via two main pathways, namely hydrogenotrophic and acetoclastic methanogenesis. It is notable that although both acetate and carbon dioxide can be used as carbon source for methanogenesis, the interconversion reactions, i.e. syntrophic acetate oxidation (SAO) and homoacetogenesis, were previously observed in several AD systems (Schnürer et al., 1999). The rationale behind these reactions is not fully elucidated, however, hydrogen pressure was considered as a key factor to manipulate the direction of the conversion (Hori et al., 2006).

1.2.2 Thermodynamics of AD reactions

The degradation of organic matters provides energy sources for the microbial community involvement and activity. Chemical and physical energy is released during the electron transition from the substrates to digestion products. Under aerobic conditions, the degradation process is driven by external electron acceptor, including O_2/H_2O ($\Delta G_0{}' = -474.5$kJ/mol), NO_3^-/NH_4^+ ($\Delta G_0{}' = -599.6$ kJ/mol), Fe^{3+}/Fe^{2+} ($\Delta G_0{}' = -114.3$ kJ/mol) and SO_4^{2-}/HS^- ($\Delta G_0{}' = -151.9$ kJ/mol). On the contrary, anaerobic condition lacks efficient external acceptors and the electrons have to be deposited in forms of digestion products such as alcohols, carboxylic products, and methane. Thermodynamically, a considerable number of AD reactions are unfavourable under the standard condition (0 ºC, 1 atm) and proceed close to equilibria at physiological conditions (Table 1). In order to achieve the energetically unfavourable reactions, obligatory synergistic microbial activities are required in all AD system.

Table 1. Thermodynamics of selected catabolic reaction in AD process under standard condition [Adapted from (Thauer et al., 1977)]

Substrates	Products	$\Delta G_0'$ (kj/reaction)
monomers		
Glucose+4H_20	2 Acetate$^-$ + 2HCO_3^- 4 H^++4 H_2	-206.3
Ribose	Acetate$^-$+ pyruvate- + H_2	-166.5
2 Glycine+4 H_20	Acetate$^-$+2 HCO_3^- + H^++ 2 $NH4^+$+2 H_2	-51.5
Glutamate-+3 H_20	2 Acetate$^-$+ HCO_3^- + H^++ $NH4^+$+ H_2	-33.9
Alanine	Acetate$^-$ + HCO_3^- + H^++ $NH4^+$+ H_2	+7.5
Leucine	Isovalerate$^-$+ HCO_3^- + H^++$NH4^+$+2 H_2	+4.2
Glycerol+2 H_20	Acetate$^-$+ HCO_3^- + 2 H^++3 H_2	-73,2
Carboxylic acids		
Acetate$^-$+4 H_20	2 HCO_3^- +4 H_2+ H^+	+104.6
Propionate$^-$+3 H_20	HCO_3^- + Acetate$^-$+ H^++3 H_2	+76.1
Butyrate$^-$+2 H_20	2 Acetate$^-$+ H^++2 H_2	+48.1
Succinate^{2-}	4 HCO_3^- + 2 H^++7 H_2	+160.2

$\Delta G_0'$, Standard free energy changes

1.2.3 Obligatory syntrophy in AD

AD metabolisms consist of extremely intricate sets of synergistic reactions executed by a specialized and complex microbial community. Different microbes have distinct roles to contribute to anaerobic carbon flow; however, the detailed characteristics of the individual members are far from being fully elucidated. Until now, AD microbial members can be categorised into four subgroups based on their functional involvement with each degradation step, namely hydrolytic bacteria, primary fermentative bacteria, secondary fermentative bacteria and methanogenic archaea. A small part of AD microbial community was characterized by cultivation-based methods, providing the knowledge to establish some taxon to functionality correlations. For example, *Clostridium thermocellum* was directly linked with hydrolysis of cellulose because of its specific cellulosome construction (Bayer et al., 1985). This knowledge was further used to develop process optimization practices in order to better utilize cellulosic substrates in AD (Tsapekos et al., 2017). However, the thermodynamic constraints of AD metabolisms hinder the cultivation of obligatory syntrophs (McInerney et al., 2009). Thus, the vast majority of the AD microbial members remained uncharacterized (Campanaro et al., 2016).

1.3 Sequencing technologies

1.3.1 Traditional molecular approaches

Molecular microbiology tools such as fluorescence in situ hybridization (FISH) and real-time polymerase chain reaction (qPCR) provide possibilities to explore the microbial community without cultivation (André et al., 2016; Ennouri et al., 2016). However, these methods demand prerequisites for gene markers/primers selection and could not be used for overall exploration of microbial community composition and dynamicity. Thus, the knowledge obtained with those methodologies was limited in hypothesis testing and verification. On the other hand, fingerprint analysis such as restriction fragment length polymorphism (RFLP), amplified rDNA restriction analysis (ARDRA), denaturing/temperature gradient gel electrophoresis (D/TGGE) and polymerase chain reaction single strand conformation polymorphism (PCR–SSCP), were used in the past to explore the diversity of AD microbial communities (Chackhiani et al., 2004; Huang et al., 2002; Klocke et al., 2007; Weiss et al., 2008). Combining with Sanger sequencing technology (Figure 2), it is possible to identify selective members in the microbial community. Nevertheless, the overall community description was hampered by the unaffordable efforts required for sequencing the entire community.

1.3.2 Next generation sequencing

Next generation sequencing (NGS), also known as massively parallel sequencing, is the most important paradigm shift in the history of sequencing technology (Figure 2). Developed throughout 1980s and 1990s, NGS almost completely superseded traditional electrophoretic sequencing today, mainly because the multiplexing process significantly reduces the sequencing time and cost. The first commercial NGS platform was released by Roche 454 in 2005 and was followed by other platforms with slightly modification on library preparation and signalling, such as platforms from Solexa (acquired by Illumina), Agencourt (acquired by Applied Biosystems), Helicos (founded by Quake), Complete Genomics (founded by Drmanac) and Ion Torrent (founded by Rothberg). With emerging of new sequencing platforms, the per-base cost of DNA sequencing dramatically dropped (by four orders of magnitude) from 2007 to 2012 (Wetterstrand, 2017). Today, 454, Helicos and SOLiD platforms stopped their development and the market is dominated by Illumina platforms, although potential competitors still exist, such as Complete Genomics (Shendure et al., 2017). The cheaper and quicker sequencing technology enabled researchers to sequence the gene makers as well as the entire

metagenomes of the microbial community without pre-selection. Moreover, the advanced bioinformatics methods developed together with the sequencing technologies provide the possibilities to reconstruct microbial genomes (metagenomics) and their expression profiles (metatranscriptomics) without isolation.

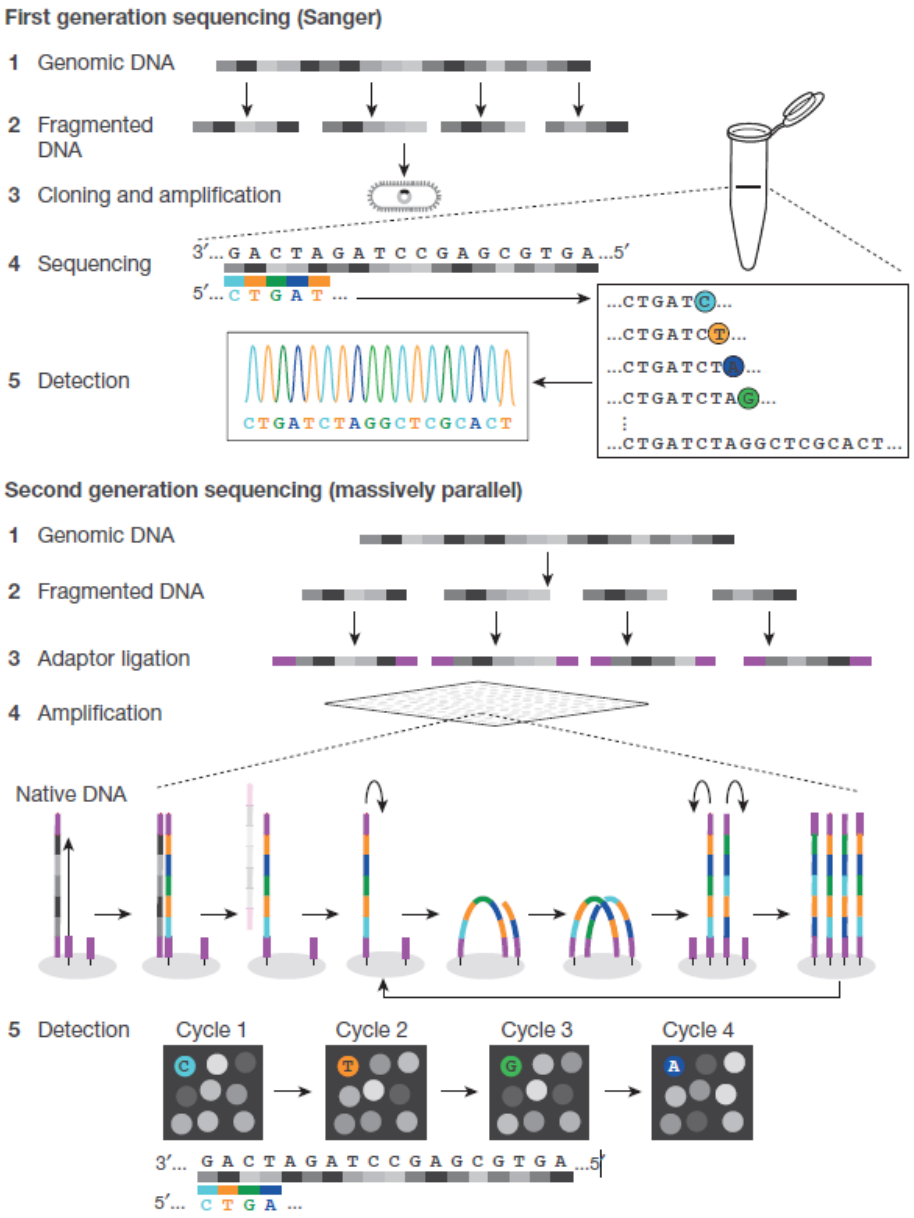

Figure 2. Schematic examples of first generation sequencing (Sanger sequencing) and second generation sequencing (NGS) [Adapted from (Shendure et al., 2017)].

1.4 Objectives and thesis structure

1.4.1 Objectives

The main aim of this Ph.D. project is to explore the AD microbial ecology with NGS technologies in order to provide fundamental microbiological insights that will lead to process control and optimization. In particular, the study has two main objectives: the first objective is to use NGS based methods to identify and solve technical challenges during the start-up of thermophilic UASB reactors. The second objective is to elucidate the fundamental metabolisms and the microbial interactions in order to deeper understand the AD process.

For the process optimization during start-up of thermophilic UASB reactors, the specific objectives are:

- Identify the biological 'bottleneck' in UASB granules during operational temperature shifts from mesophilic to thermophilic conditions (Paper I).
- Analyse the impact of microbial interaction between granules and the liquid phase of UASB reactor during the temperature shifts (Paper II).
- Propose and test bioaugmentation as technical solution to overcome the obstacles during microbial temperature adaptation (Paper III).
- Elucidate the microbial insights during the practice of bioaugmentation (Paper III).
- Evaluate the application of 16S rRNA amplicon sequencing to explore AD microbial ecology.

For elucidating the fundamental metabolisms and microbial interactions in AD, the specific objectives are:

- Identify microbial functional roles by gene profiles and metabolic traits in the reactor (Paper IV, V).
- Reconstruct degradation pathways for specific compounds according to gene presence in assembled genomes (Paper IV).
- Propose novel degradation models with syntrophic microbial activity (Paper IV).
- Characterize new *Candidatus* species having essential role in AD system (Paper V).

1.4.2 Structure of the thesis

In Chapter 2, the main methodologies used to explore the AD microbial ecology in this Ph.D. study are described, namely 16S rRNA amplicon sequencing, metagenomics, and metatranscriptomics. The theoretical background of those methods was present together with their applications in AD studies.

Chapter 3 presents a case study where 16S rRNA amplicon sequencing was used to explore the microbial community dynamicity during temperature transition in UASB reactors. First, the microbial obstacle during temperature transition was identified. In addition, the microbial interaction between granules and the liquid phase of UASB reactors were analysed. Finally, a technical solution, namely, bioaugmentation, was proposed and tested to overcome the operational challenges.

In Chapter 4, a genome-centric metagenomics study is presented to analyse the core microbial community during carbohydrates degradation. Four metagenomes were extracted from simplified microbial community adapted to specific substrates, namely avicel, glucose, VFAs and acetate. The study connects microbes to their functional roles with gene profiles as well as their abundance dynamics during the change of substrates.

Chapter 5 describes a novel species *Candidatus* Methanoculleus thermohydrogenotrophicum, having the genome sequence reconstructed from 6 AD metagenomes. The characteristics of this species were revealed through a combination of metagenomics, metatranscriptomics and FISH technology.

2 Explore AD microbiome with NGS

2.1 16S rRNA amplicon sequencing

Amplicon sequencing of gene markers containing functional or taxonomic information is one of the most popular sequencing strategies (Poretsky et al., 2014). Especially, the slow evolution rate of 16S rRNA gene in prokaryotes makes this gene ideal for phylogenies reconstruction. Moreover, the alternating structure of conserved and hypervariable regions also provides the universal binding sites for PCR primers. Thus, primers targeting at the conserved region in 16S rRNA gene are extensively used to explore the composition and dynamicity of the microbial communities in different environments and conditions. Bacteria 16S rRNA genes consist of nine hypervariable regions (V1-V9). Bacterial and archaeal universal primers targeting at V4 region (519f-802r) showed the best resolution of complex communities (Klindworth et al., 2013). Based on the sequences, the primer pair is able to catch 94% of Archaea and 95% Bacteria in SILVA database. The post sequencing analysis of 16S rRNA amplicon data typically consists of OTU clustering, alignment to database, phylogeny reconstruction and statistical comparison. The 16S rRNA gene pipelines are relatively more accessible when compared to comprehensive metagenomics because they require less computational expertise. The analyses can be easily achieved with commercial software such as CLC Workbench software, public available freeware such as RDP classifier (Wang et al., 2007) or user friendly web tools such as MG-Rast (Meyer et al., 2008).

In the context of AD system, 16S rRNA amplicon sequencing has been used extensively in the last years to characterize the microbial community composition (De Francisci et al., 2015; Luo et al., 2015; Ziganshin et al., 2013a).. This technology is also useful to reveal static microbial community composition. For example, Ziganshin et al., (2013) characterized the bacterial community during anaerobic digestion of agricultural waste materials. More commonly, according to the microbial dynamicity during operational shifts, specific taxa could be related to certain reactor behaviours, such as foaming, LCFA degradation and ammonia inhibition. (Kougias et al., 2014; Tian et al., 2018; Zhu et al., 2015). Luo et al. (2015) used 16S rRNA amplicon sequencing to successfully demonstrate that the overall microbial community composition shifted after temperature disturbance which leads to higher methane production efficiency. *Nocardia* and *Desulfotomaculum* were observed to

significantly increase in relative abundance during reactor foaming, suggesting their activity are responsible for the production of foams (Kougias et al., 2014). In this present Ph.D. study, 16S rRNA amplicon sequencing was used to provide the microbial insights into a specific case study: the start-up of thermophilic UASB reactors (Chapter 3). The microbial community composition and dynamicity during the start-up of the thermophilic UASB reactors were recorded. The gained knowledge was used to identify the biological challenges during the start-up of thermophilic UASB reactors (Paper I, II) and to develop specific engineering solutions to overcome the obstacles (Paper III).

It is known that amplicon sequencing has specific biases due to the amplification process and it is also known that the taxonomical results are still highly dependent on the availability of the public database. Nevertheless, the 16S rRNA amplicon sequencing first allowed the characterization of the microbial community without pre-selection and unveiled revolutionary microbial insights into AD system in the past decades. To date, 16S rRNA amplicon sequencing is still extensively used to record overall microbial community dynamicity in AD system and reveals valuable information for process optimization. In particular, 16S rRNA amplicon sequencing is user-friendly and cost-effective for engineering studies because it requires a relatively low number of sequences and a simpler post analysis when compared to total random sequencing.

2.2 Genome-centric metagenomics

Random shotgun sequencing on microbial community overcomes most of the limitations of amplicon sequencing and has the capacities to reveal much more information, including the sequences of protein coding genes which are directly related to metabolic potential. Especially, with the rapid development of bioinformatics tools, genome-centric metagenomics became an extremely powerful tool to elucidate the complex synergistic metabolism in AD (Vanwonterghem et al., 2016).

A typical workflow of a genome-centric metagenomics analysis for microbial community characterization starts from an experiment designed to solve a specific scientific question (Figure 3) and is followed by a series of computational analyses, i.e. *de-novo* assembly, metagenomics binning, MAGs quality control, gene annotation and community composition investigation.

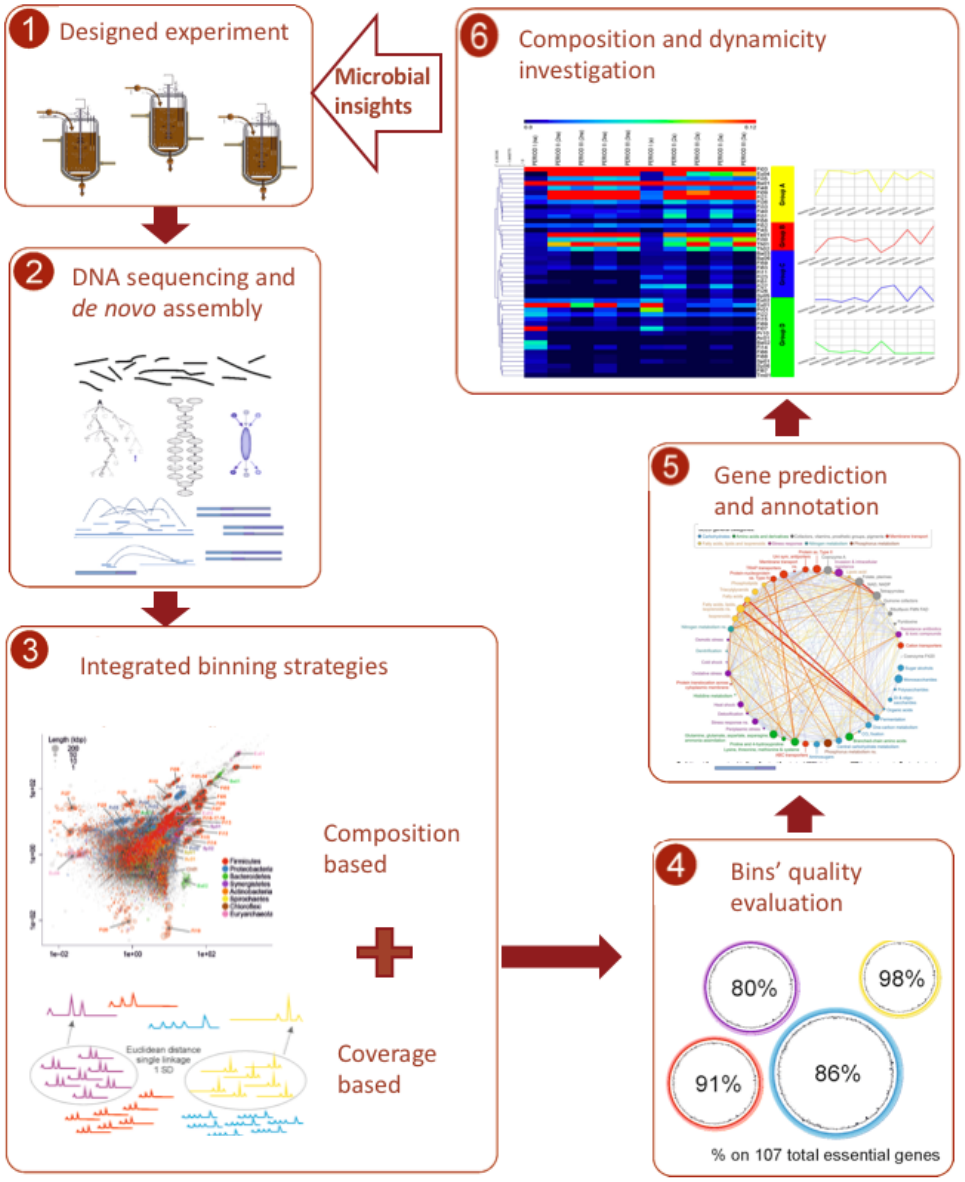

Figure 3. Workflow of genome-centric metagenomics analysis for microbial community characterization [Adapted from (Campanaro 2016)].

2.2.1 *De-novo* assembly

For AD microbial community, the lack of known species hinders the direct reads alignment to reference genomes. Thus, *de-novo* assembly based on De Bruijn graph approach is commonly used to form contigs and scaffolds. The initial assembly strategies were restricted to the simple environment where the microbial community presents in low diversity (Tyson et al., 2004). However, remarkable efforts have been paid to improve assembly algorithms, in order to allow genome-centric metagenomics to be carried out in more diverse samples. Both free and commercial software has been developed to perform assembly with different computation capacity demands. MetaSPAdes

(Nurk et al., 2016), Megahit (Li et al., 2015) and CLC-working bench (used in Paper IV and V) have been evaluated to have the best assembly performances using metagenomic datasets obtained from complex microbial communities (Vollmers et al., 2017).

2.2.2 Metagenomic binning

Following the *de-novo* assembly, a further step namely binning is taken to extract metagenome assembled genomes (MAGs). The binning procedure groups the scaffolds obtained from the assemblies and assigns them to operation taxonomic units (OTUs). Up to now, all existing binning strategies are based on nucleotides composition and/or differential coverage. The former relies on the premise that each microbial member has a unique genomic composition such as dinucleotides and tetranucleotides frequency profile (Nakashima et al., 1998; Noble et al., 1998). While, the latter is based on the fact that contigs belonging to the same genome share similar coverage profiles among samples (Albertsen et al., 2013; Nielsen et al., 2014; Sharon et al., 2013). Manual binning procedures combining both criteria as proposed by Campanaro et al., (2016) showed better performance on MAGs recovery in terms of genome completeness and contamination when compared to the methods previously mentioned. In addition, automatic binning software such as MetaBAT (Kang et al., 2015) and Maxbin2 (Wu et al., 2015) are under development to adopt more complex community and larger datasets.

2.2.3 Quality control of metagenome assembled genomes (MAGs)

The genome quality of pure-strains is traditionally evaluated by assembly statistics, i.e. N50 (Salzberg et al., 2012). However, the same standards could not be applied to MAGs due to the potential incompleteness and contamination. The common strategy to assess MAG quality is to evaluate the presence of ubiquitous marker genes (Dupont et al., 2012; Wrighton et al., 2012). The results of this strategy are highly dependent on the gene sets chosen for evaluation and is potentially biased by the uneven distribution of marker genes, the potential absence of universal genes and the gene redundancy. In 2015, a more accurate approach was described with the launch of CheckM, which uses a dynamic gene set for evaluation, based on a genome's inferred lineage within a reference genome tree (Parks et al., 2015). In addition to CheckM, the presence of ribosomal RNA genes and transfer RNA (tRNA) genes in MGAs can also contribute to the estimation of completeness. Recently, the Genomic Standards Consortium set standards regarding the minimum infor-

mation required about a MAG of bacteria and archaea (Bowers et al., 2017) (Table 2).

Table 2. Genome reporting standards for MAGs[Adaped from (Bowers et al., 2017)].

Criterion	Description
Finished	
Assembly quality	Single contiguous sequence without gaps or ambiguities with a consensus error rate equivalent to Q50 or better
High-quality draft	
Assembly quality	Multiple fragments where gaps span repetitive regions. Presence of the 23S, 16S, and 5S rRNA genes and at least 18 tRNAs
Completion	>90%
Contamination	<5%
Medium-quality draft	
Assembly quality	Many fragments with little to no review of assembly other than reporting of standard assembly statistics
Completion	≥50%
Contamination	<10%
Low-quality draft	
Assembly quality	Many fragments with little to no review of assembly other than reporting of standard assembly statistics.
Completion	<50%
Contamination	<10%

2.2.4 Gene prediction and annotation

Without the combination of transcriptomic data, curation of genomic data is performed with *ab initio* gene finding depending on relatively well understood open reading frames in prokaryotes. Automated software based on machine learning algorithms, such as Prodigal (Hyatt et al., 2010) is extensively used to predict gene for MAGs. After the prediction, the functional annotation of coding DNA sequence (CDS) is typically achieved by homologies search (such as BLAST) against public databases. Commonly used protein sequences databases include the Clusters of Orthologous Groups of proteins (COGs) (Tatusov et al., 2003), Pfam database (Bateman et al., 2004) and KEGG Orthology (KO) database (Kanehisa et al., 2004). In addition, web-based annotation pipelines expert review version of the Integrated Microbial Genomes (ER IMG) is also under development in order to support systematic and efficient revision of microbial genome annotations (Markowitz et al., 2009).

2.2.5 Microbial community composition investigation

Besides the metabolic potential of individual MAGs, the composition of the entire microbial community is also a key parameter in AD context. The average coverage of MAGs can be calculated by sequences aligners such as BWA and Bowtie2 (Langmead and Salzberg, 2012; Li, 2013). Furthermore, the relative abundance of MAGs in each sample can be calculated through:

$$Relative\ abundance\ (MAG\ 1) = \frac{Coverage\ (MAG\ 1)}{Sum\ [Coverage\ (MAGs)]}$$

The relative abundance calculated from genome-centric metagenomic approaches is more accurate than 16S rRNA amplicon sequencing results because of the lack of PCR biases and normalization with genomes sizes.

2.2.6 Genome-centric metagenomics applications

Genome-centric metagenomics was progressively used to characterize complex microbial communities since the first near-complete genomes extract from metagenomics sequence reads by Tyson et al., (2004). With tremendous sequencing effort, the number of metagenome-assembled genomes (MAGs) exponentially increased since 2015 (Bowers et al., 2017) (Figure 4).

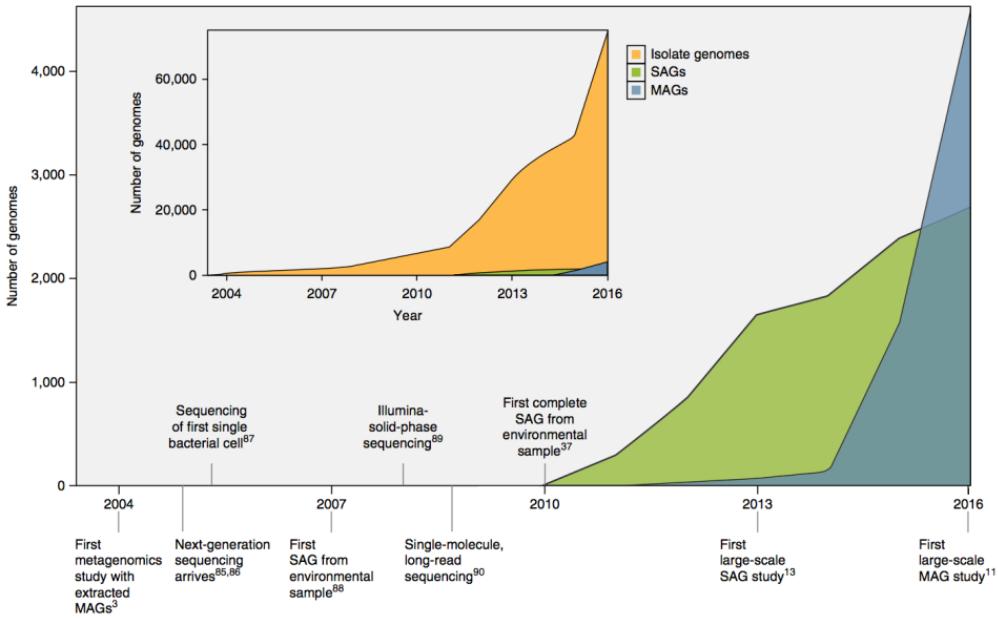

Figure 4. Sequencing of bacterial and archaeal genomes. SAG, single cell sequencing assembled genome. MAG, metagenome assembled genome. [Adapted from (Bowers et al., 2017)].

In AD context, genome-centric metagenomics could reveal static characterization of microbial community (Nobu et al., 2015; Treu et al., 2016b;

Vanwonterghem et al., 2016). Besides the investigations of fundamental microbial ecology, the experiments can also be designed to address operational challenges with realistic substrates, such as LCFA overloading during manure based digestion (Kougias et al., 2016). In this Ph.D. study, genome-centric metagenomics was used to explore the core microbial metabolism in simplified microbial community adapted to avicel, glucose, VFAs and acetates degradation (Paper IV). The gained knowledge led to the characterization of novel degradation pathways (Paper IV) and candidate species (Paper V). Moreover, the results provided novel insights into the competition and cooperation behaviours among members of the AD microbial community.

Compared with 16S rRNA amplicon sequencing, genome-centric metagenomics requires significantly more sequences and bioinformatics expertise for the analyses. Nevertheless, the random sequencing of the entire microbial community reveals much more information regarding functional potential. The additional information allows *de-novo* pathway reconstruction and characterization of novel microbial lineages. The independence of genome-centric metagenomics from references genome databases especially benefits the exploration of the uncharacterized microbial community, such as the one in AD.

2.3 Untangling microbial activities with metatranscriptomics

The functionality of a microbe is determined by the metabolic activity triggered in the specific environment. Thus, not only gene profiles but also the regulation of gene expression is essential to characterize the microbial metabolic traits. With the purpose of analysing gene regulation of MAGs under specific ecological conditions, the sequencing of the direct gene products (RNA) at community-wide level was performed (Frias-Lopez et al., 2008). In 2012, the first metatranscriptomic approach was used to analyse overall transcriptional activity in a production-scale biogas plant (Zakrzewski et al., 2012). Since then, the metatranscriptomics was progressively used to reveal keystone metabolic pathways in AD (Bremges et al., 2015; Embree et al., 2014). It is notable that by combining genome-centric metagenomics and metatranscriptomics, researchers were able to dissect the transcriptional activity of uncultivated microbes during AD process (Nobu et al., 2015; Treu et al., 2016a). In this Ph.D. study, metatranscriptomic approach was used to characterize the metabolic traits of a proposed *Candidatus* species, *Candidatus* Methanoculleus thermohydrogenotrophicum (Paper V).

Practically, it is still technically challenging to obtain high quality RNAs from AD bioreactors, especially from continuously operating reactors with complex substrates. Nevertheless, a metatranscriptomic study can determine which gene is expressed in MAGs under the specific environmental conditions and eventually elucidate the functional roles in AD microbial community.

3 Start-up thermophilic UASB reactors

Up-flow anaerobic sludge blanket (UASB) is a popular reactor type extensively used for anaerobic digestion of high strength wastewater (Kaparaju et al., 2010; Novak et al., 2013). In a typical UASB reactor, the microbial consortia reside in the granular sludge which is retained in the reactor due to its excellent settling properties (Lu et al., 2015). The organic matters are degraded as the wastewater flows up through the sludge bed and methane is produced simultaneously. Same as any biological process, the operational temperature of UASB reactors is a crucial operational parameter. UASB reactors are commonly operated under mesophilic or ambient temperature to reduce cost and potential inhibitions (Chong et al., 2012; Fang et al., 2011). Nevertheless, thermophilically operated UASB reactors were proved to be feasible in specific situations with a higher methane production rate and an analogous COD removal (Parawira et al., 2007). One of the obstacles of operating thermophilic UASB reactors is the lack of active granular sludge to initiate the process (start-up). Typically, granulation of suspended sludge is a time-consuming procedure which also requires elaborating control of many parameters including pH, organic load rates (OLRs) and hydraulic conditions (Vlyssides et al., 2008). In order to overcome this barrier, developed granular seeds were usually provided to facilitate the start-up of thermophilic UASB reactors. Mesophilic methanogenic granules are considered to be good candidates for the start-up seeds because they are present in similar habitat and harbour identical functionalities. It is postulated that temperature imposes remarkable selective pressure on microbial communities. Thus, a desirable dynamicity of microbial community composition is the prerequisite of a successful granules temperature adaptation.

The utilization of mesophilic methanogenic granules for starting up thermophilic UASB reactors was previously demonstrated in lab-scale reactors (Fang and Lau, 1996; Syutsubo et al., 1997). Previous investigations were able to conclude that several empirical practices, such as stepwise temperature increase, control of OLR and application of specific hydraulic conditions, would promote the temperature adaptation. However, the rationale behind those operations was never revealed. Moreover, although it is well known that microbial community adaptation is the key to this temperature transition, bioaugmentation was never attempted because the biological 'bottleneck' during temperature transition was never identified. In this Ph.D.

study, a series of experiments were designed to identify the biological obstacles during the start-up of thermophilic UASB reactors from mesophilic granules (Figure 5). In addition, the gained microbial insights were used to develop technical solutions directly targeting at the main obstacle of microbial community adaptation in order to achieve a rapid and smooth temperature transition.

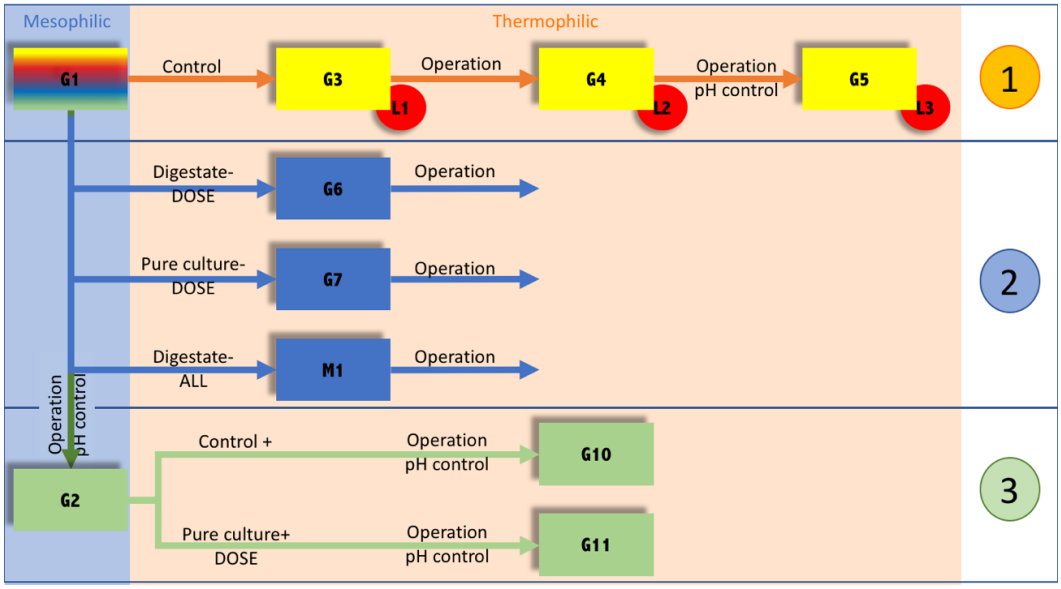

Figure 5. The experimental scheme to investigate the start-up of thermophilic UASB reactors from mesophilic granules

Firstly, Experiment 1 used mesophilic methanogenic granules as start-up seeds for thermophilic operation. The reactor was first operated without any deliberate control. In the following experimental period, additional sodium bicarbonate was added into the reactor for pH control. The microbial community composition of the granules and of the liquid phase of the UASB reactor was followed throughout the experiment. From the microbial community dynamicity, the biological challenges during the adaptation were identified. Based on the results of experiment 1, an engineering solution, namely bioaugmentation, was tested with three different strategies in Experiment 2. Both the microbial modifications and the performance of the reactors after bioaugmentation were recorded in order to establish the best practice. Finally, in Experiment 3, the best bioaugmentation strategy was tested under optimal condition, i.e. with external pH control, to evaluate the significance and persistence of the positive effect on methanogenesis. During all experiments, 16S rRNA amplicon sequencing technology was used to explore the microbial community composition and technical triplicates were taken for all granule samples to allow statistical evaluation.

3.1 Microbial dynamics during temperature transition

3.1.1 Microbial “bottleneck” in granular microbiota

The results of the first experiment showed that the mesophilic granules could not immediately start performing methanogenesis at thermophilic conditions without deliberate process control (Paper I). When the OLR of 3.97±0.19 gVS/(L-reactor.d) was applied after 6 day-adaptation, the reactor showed significant pH drop and no methane was produced in the reactor. The main digestion products were VFAs and alcohols, corresponding to 63.1% and 9.31% of total methane potential (based on methane equivalent). After introducing external pH control by adding sodium bicarbonate, the methane production was initiated, the alcohol production was eliminated and VFA production was enhanced. By the end of the operation periods, the methane production rate was 92 ±5 mL-CH_4/g VS, corresponding to 31.37% of total methane potential.

The microbial community dynamicity was analysed by comparing the community compositions in the mesophilic granule (G1), the granules shortly after adaptation(G2), the granules before the external pH control (G3) and the granules after the external pH control (G4) (Figure 6).

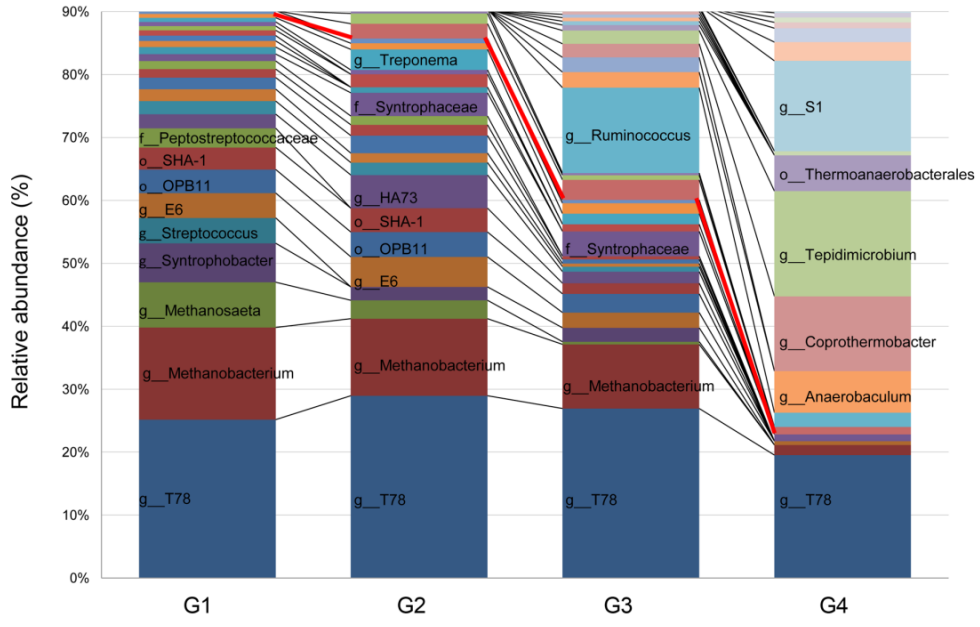

Figure 6. Relative abundance of OTUs in the community. The redline indicate the changing of initial community [Adapted from Paper I].

Overall, the granules gradually lost changed their microbial community composition. Specifically, the initial OTUs accounted for 85.75% in G2, 59.53% in G3 and 22.82% in G4. A significant decrease of essential bacteria and archaea was observed in all thermophilic samples, including members belonging to *Streptococcus*, *Syntrophobacter*, *Methanosaeta* and *Methanobacterium*. The OTUs decreased in abundance were found to be mesophilic microbes whose metabolism potentially covered the entire AD pathways from hydrolysis to methanogenesis. The inhibited microbes were replaced by members of *Ruminococcus*, *Coprothermobacter* and *Tepidimicrobium*, which have the metabolic potential for fermentation at the thermophilic condition. The proliferation of those thermophilic bacteria quickly restored the hydrolysis and acidogenesis in the reactor, leading to the accumulation of VFAs. However, methanogenesis could be slowly initialized only with external pH control, evidenced by the minor but significant increase in abundance of *Methanoculleus*. It is notable that microbes belonging to genus *T78*, member of family *Anaerolinaceae*, remained highly abundant throughout the experiment. Previous studies based on cultivation showed that *Anaerolinaceae* members share multicellular filamentous morphology and were considered as important bacteria for granulation and the maintenance of granular structure (Satoh et al., 2007; Yamada et al., 2005). Thus, we hypothesize that the tight bonds between *Anaerolinaceae* members and granular structure prevented the bacteria from washing out together with the liquid phase of the reactors.

In summary, the remarkable difference recorded in microbial community composition suggested that the temperature adaptation of mesophilic methanogenic granules to thermophilic conditions requires a major 'turn-over' of the microbial consortium. Among AD steps, methanogenesis was the last to be restored and could only be initialized with external pH control. Thus, the involvement of thermophilic methanogens was identified as the biological "bottleneck" of the temperature adaptation.

3.1.2 Microbial interaction between granules and surrounding liquids

Considering the typical UASB operation, it is easy to assume that the vast majority of the active microbes reside in the granular structures. This assumption would be valid in case of stable operations, where the hydraulic retention times (HRTs) applied to the reactor are too short to host a robust microbial community in the liquid phase. However, the HRTs were sometimes prolonged to avoid overloading, especially when relatively complex substrates are used or during the start-up/ adaptation periods (Fang et al., 2011).

During this situation, the liquid phase of UASB reactors would potentially nourish active microbial activities that are significant to overall degradation and imposes great impacts on granular microbial community composition. In the Ph.D. study, the planktonic microbial community composition (L2, L3, and L4) were analyzed in order to reveal their functionality and potential impacts on granular microbial consortium (Paper II).

The profile of microbial community suggested that the planktonic microbes are mainly 1) the microorganisms that could not survive thermophilic conditions and thus released from the granules, and 2) the bacteria that grow spontaneously during the increase of temperature and given substrates (Figure 7).

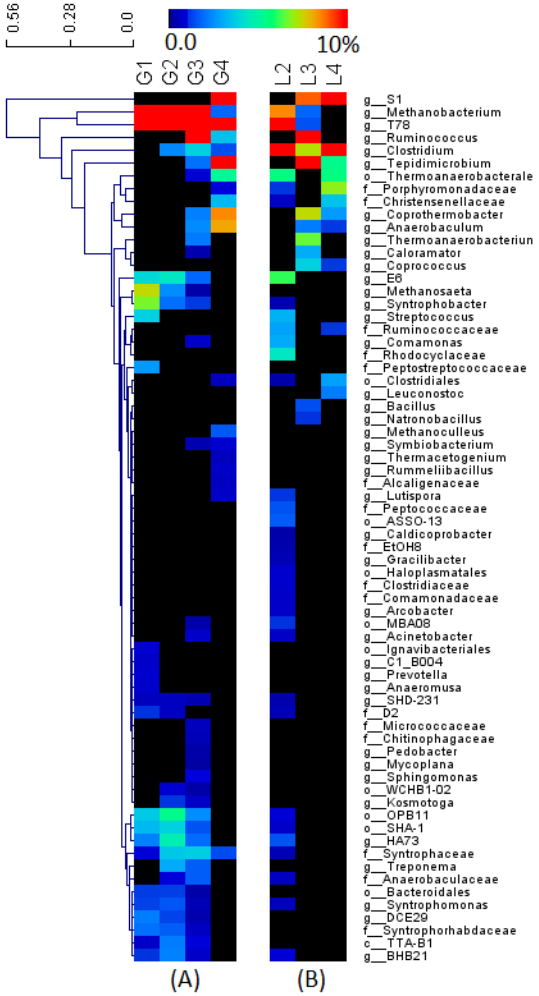

Figure 7. Hierarchical clustering performed on the most abundant OTUs of the community. Samples collected in the granules are presented in column (A), those collected in liquid phase are in column (B). Colour scale on top of the heatmap indicates the relative abundance of the OTUs [Adapted from Paper II].

L2 consisted of the most microbes released from the granules including members of *Methanobacterium*, *Betaproteobacteria* and *I* genus *T78*. The 16S rRNA amplicon sequences of those inhibited microorganisms were assigned to obligatory mesophilic strains. Simultaneously, *Clostridium* and *Thermoanaerobacterales* species were present at high relative abundance in the liquid microbiota because of their involvement in the thermophilic fermentation of the substrates. Before the deliberate pH control, members of *Ruminococcus* became the most abundant bacteria in the liquid phase due to a possibly higher acid tolerance. Other thermophilic fermentative bacteria, including *Tepidimicrobium*, *Coprothermobacter* and *Thermoanaerobacterium* species, were also observed in high relative abundance. Finally, the planktonic microbial composition changed significantly after introducing external pH control. During the operation with pH control, genus *S1* (belonging to family *Thermotogaceae)* became the most abundant taxon in the liquid microbiota, accounting for 47.7% of the total community.

Results also showed that the liquid in the UASB reactor hosted a more specialized microbial community, which is also more sensitive to operational conditions, such as HRT, pH, and temperature. A number OTUs were found present in both liquid and granule of the reactor suggesting a probable microbial interaction between the two phases. Specifically, the impact of granule to liquid mainly attributed to the disintegration and wash out of microbes from granule structure during long HRT operation. While, the impact from the other direction (i.e. from liquid to granule) was more significant during relatively shorter HRT, evidenced by the facts that the highly abundant planktonic OTUs (74.9% of L3 and 86.0% of L4) were also found in the corresponding granule phase (G3 and G4). The interaction did not only allow the organic matter transfer between liquid and granular phase but also provided the possibility for planktonic microbes to attach and evolve in granule structures.

In summary, the planktonic microbiota was mainly composed of hydrolytic and fermentative bacteria and was more sensitive to operational conditions compared to granules. The interaction between the microbial community in the granules and in the surrounding liquid led to a rapid restoration of hydrolysis and acidogenesis in the reactor. However, the slow growth rate of archaea and their more specific growth requirement prevented thermophilic archaea to evolve in the planktonic microbial community and to be encapsulated in granules structure.

3.2 Bioaugmentation is a promising method for thermophilic UASB start-up

After identifying the biological limitation of the temperature adaptation as the presence/absence of thermophilic methanogens, a technical solution directly targeting at this obstacle was developed in Paper III. The method used to accelerate the microbial community adaptation is known as "bioaugmentation" or "microbial community invasion". This practice relies on the provision of exogenous microbial consortia into certain ecosystems to enhance the microbial community adaptation to new or extreme conditions. Bioaugmentation was recently proposed to solve series operational challenges in AD system such as to increase hydrolysis of cellulose, to overcome ammonia inhibition and to restore methane production from the overloaded reactor (Fotidis et al., 2014; Tale et al., 2015; Tsapekos et al., 2017). The positive bioaugmentation effects were reported to occur for a limited time, due to the washout of the exogenous strains (Tsapekos et al., 2017). However, UASB operation would potentially overcome this limitation as active microbes can be immobilized into the granules, which are retained in the reactor for longer periods.

3.2.1 Comparison and selection of bioaugmentation strategy

Initially, three bioaugmentation strategies were examined in Paper III. The practice of each strategy is listed in Table 3.

Table 3. Operation details of all reactors during bioaugmentation period.

Reactor	Mesophilic granules	Liquid phase	Daily augmentation	Daily feed
Control	600mL	BA medium	na.	40 ml potato juice
Strategy 1		AD digestate	na.	
Strategy 2		BA medium	40 ml AD digestate	
Strategy 3		BA medium	40ml axenic culture	

Results showed that all the granules could be maintained integer except from Strategy 1. The disintegration of granule in the reactor where Strategy 1 was applied could attribute to the high density of the AD digestates. The dense liquid phase of the UASB reactor hampered a proper settlement of the granules and could not provide the hydraulic conditions to maintain the granular structure. The methane production accounted only for 9% of total digestion products in the reactor where bioaugmentation was performed according to Strategy 2. The overall performance of the reactor in which Strategy 2 was

applied was similar to the performance of the control reactor. In both cases accumulation of VFAs, production of alcohols and a concomitant pH drop were recorded. Strategy 3, where the axenic methanogenic culture was used as the bioaugmentation source, showed the most significant improvement of methanogenesis. During the operation period, the methane production yield was observed as 240.1±29.5 CH_4 mL/g VS, accounting for 62% of the total digestion products.

The microbial community compositions of granules before and after the bio-augmentation strategies (G1, G2, G5, and G6), were recorded and compared (Figure 8).

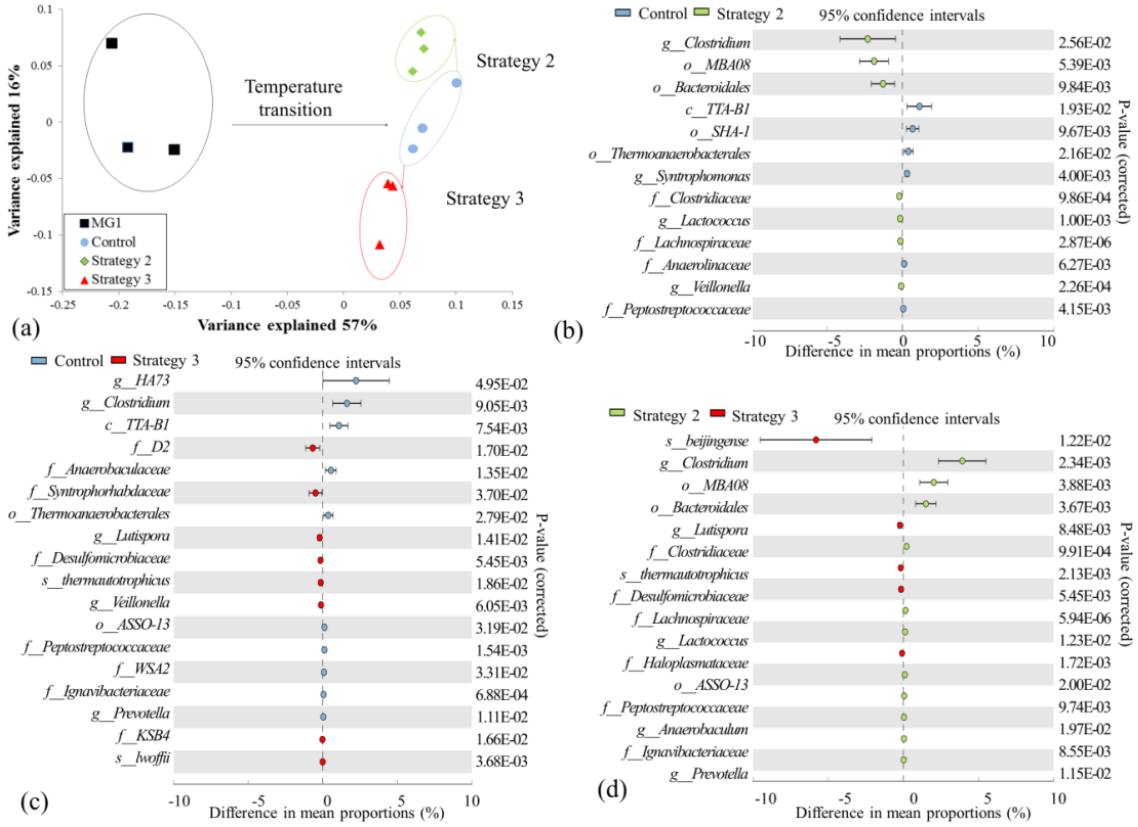

Figure 8. Microbial community composition shifts after the different bioaugmentation strategies. a) PCoA based on Bray-Curtis dissimilarity distance showed distinct communities in mesophilic granules (MG) and thermophilic granules (Control, Strategy 1 and Strategy 2. b-d) Taxa presenting in significantly different relative abundance under the applied conditions (t-test) [Adapted from Paper III].

The results of the principal coordinate analysis showed that the microbial community composition of all granules after bioaugmentation strategies was dramatically different from mesophilic granules. In addition, relatively minor but still significant differences were recorded between Control and each bio-augmentation strategy. Seven bacterial taxa were found in higher relative

abundance in granules where bioaugmentation was performed according to Strategy 2 compared to the Control. It is suggested that the injection of digestates affected the granule microbial community composition. However, the metabolic potential for all increased taxa was mainly related to thermophilic fermentation, which could be spontaneously restored even without external enhancement. Thus, although with a slightly different microbial community composition, the final methanogenesis process was still not restored by with applying Strategy 2. On the other hand, a significant increase in abundance of *Methanothermobacter thermautotrophicus* was observed in the reactor where Strategy 3 was applied, in comparison with the Control. In addition to *M. thermautotrophicus*, seven bacterial taxa were recorded to increase in relative abundance. The most significant increase was found in the OTU assigned to family *D2* whose 16S rRNA amplicons were identical to *Thermanaerovibrio velox*. A potential synergic reaction between *T. velox* and *M. thermautotrophicus* was proposed due to *T. velox*'s H_2 forming property during sugar fermentation.

Among all bioaugmentation strategies, Strategy 3 showed the most significant microbial enhancement. Thus, axenic culture used as bioaugmentation source was chosen for further investigations.

3.2.2 Enhanced methane production with bioaugmentation

As the following step, the Strategy 3 was further tested in optimized conditions where pH, OLR and HRT were carefully controlled. The results showed the methanogenic activity was significantly enhanced by applying bioaugmentation with thermophilic methanogenic cultures. Specifically, the methane production rate in the bioaugmented reactor was 30% higher than the Control during HRT of 6 days and 40% higher than the Control during HRT of 3 days. Differently from what reported in literature, the positive bioaugmentation effect persisted until the end of the experiment for over 2 months (8 HRTs). Moreover, no significant wash out of the exogenous methanogens was observed.

The microbial community composition of granules during stable mesophilic operation (MO), stable thermophilic operation without bioaugmentation (Control 2) and stable thermophilic operation with bioaugmentation (TA) was followed. All present microbes could be clustered into four groups (Figure 9) according to the dynamicity of their relative abundance in different samples.

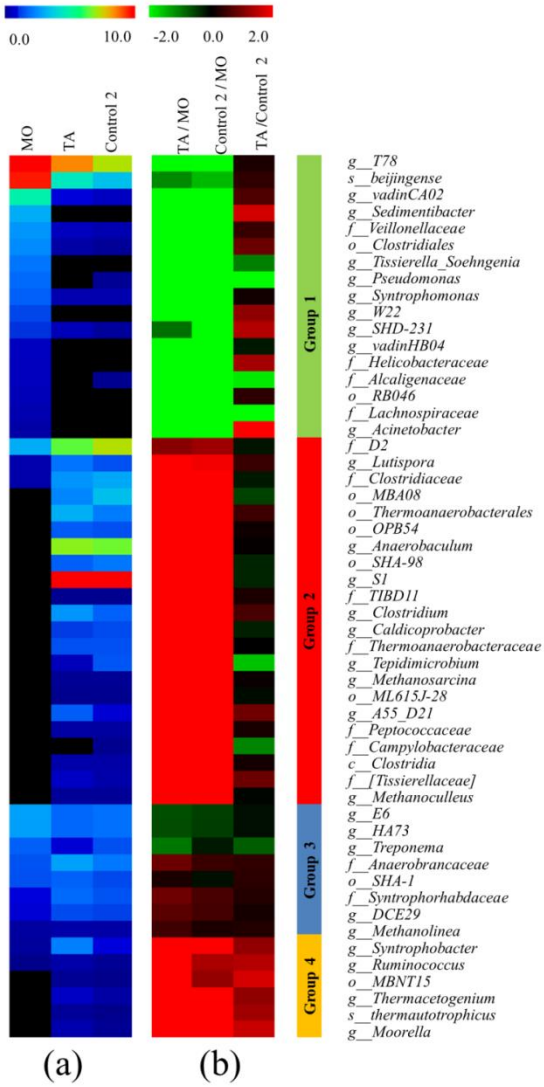

Figure 9. Microbial community composition in mesophilic granules (MO), thermophilic granules with bioaugmentation (TA) and thermophilic control granules (Control 2). Correspondence between colours and relative abundance/ folds change is reported in the top scale (a) Relative abundance of selected taxa identified in top deciles of the total community. (b) Fold change (log_2) of relative abundance for selected taxa [Adapted from Paper III].

Group 1 consisted of mesophiles whose relative abundance was decreased regardless of bioaugmentation. This group included the most abundant methanogen at the mesophilic condition, namely *Methanobacterium beijingense*. Group 2 is made by thermophiles which increased in relative abundance regardless of bioaugmentation. The most significant increase in abundance was observed for genus *S1*, which was previously found to be dominant in the liquid phase of UASB reactor in Paper II. The proliferation of this specific taxon can probably attribute to its metabolic potential of degradation of given substrates (i.e. potato juice) and its rapid involvement in liquid phase at

thermophilic condition. In addition, *Methanoculleus* and *Methanosarcina* species increase their relative abundance regardless of bioaugmentation which can be considered as the spontaneous microbial adaptation towards thermophilic conditions. A small group of microbes were found abundant both under mesophilic and thermophilic operations (Group 3) due to their capability to survive in a wide temperature range. In this group, archaeal members closed to *Methanolinea tarda* probably played an important role due to metabolic potential for methanogenesis. Moreover, the filamentous property of *Methanolinea* spp. also increased their possibilities to be retained in the granular structures (Imachi et al., 2008). Lastly, the most important microbial group is composed by the microbes proliferated due to bioaugmentation (Group4). *M. thermautotrophicus* was found 2.5 folds higher in relative abundance compared with the Control. Besides, *Thermacetogenium* sp. and *Syntrophobacter* sp. also increased by 2.2 folds compared to the Control due to their potential syntrophic relationship with *M. thermautotrophicus* (Boone and Bryant, 1980; Hattori et al., 2000).

After bioaugmentation with axenic methanogenic culture, the exogenous *M. thermautotrophicus* could be encapsulated in granular structure, performing hydrogenotrophic methanogenesis. Concomitantly, H_2 producing bacterial increased their relative abundance in bioaugmented granules due to their syntrophic activity with *M. thermautotrophicus*. The enhancement of methanogenic members in the granular microbial community led to a significant increase of methane rates and yields. Thus, bioaugmentation with axenic methanogenic culture in UASB reactor is an effective method to restore the methanogenesis during operational temperature shifts from mesophilic to thermophilic conditions.

4 Unveiling AD metabolism by metagenomics

As discussed in Chapter 2.1, the results of 16S rRNA amplicon sequencing cannot directly reveal the metabolic potential of the microbial community. For this reason, the interpretation of the results relies on the phylogeny construction and taxonomy assignment. Moreover, in the context of AD, early metagenomics investigation suggested that the microbial community mainly consisted of uncultivated and uncharacterized taxa, whose metabolic potentials were not revealed with traditional cultivation-based methods (Campanaro et al., 2016). It is also known that AD process requires massive syntrophic activity between microbes and this further hampers the elucidation of the digestion pathways, and consequentially, the microbial functional roles (Nobu et al., 2015; Stams et al., 2012). With the purpose to further decipher the microbial metabolisms of AD process, the present Ph.D. study used genome-centric metagenomics. This technique was chosen to unravel the simplified microbial communities during the degradation of carbohydrates (Paper IV).

4.1 Enrichment of the AD microbial community with synthetic substrates

The majority of the investigations on AD microbial communities were performed on realistic systems where manure, wastewater, and organic wastes were used as substrates (Kougias et al., 2016; Luo et al., 2016; Treu et al., 2016b). The degradation of those heterogeneous substrates typically involves extremely diverse microbial communities in order to mediate the degradation of different organic compounds. However, the functional roles of individual AD microbes are difficult to be determined due to synchronized mutualistic interaction and functional redundancy of the microbes in the communities (Campanaro et al., 2016; Carballa et al., 2015; Vanwonterghem et al., 2014). In order to bypass these limitations, simplified methanogenic microbial consortia were obtained by operation of lab scale continuous tank reactor with synthetic substrates. Stepwise simplified organic carbons were used to enrich the core microbial community adapted to specific substrates, namely avicel (polysaccharide), glucose (monosaccharide), VFAs mixture (Acetate: propio-

nate: butyrate = 2.6:1.1:3.8 in mass) and acetic acid. In this way, only the microbes that were able to metabolize the specific influent compounds (and the following products) were maintained in the reactor, while the rest was washed out.

The metagenomes in each experimental condition were dissected with a genome-centric strategy. Overall, this experiment successfully reduced the microbial community diversity resulting in a much more specialized community consisting of 11 high quality MAGs, 13 medium quality MAGs, 9 low quality MAGs and 2 meta-MAGs (contamination higher than 80%) (Figure 10). Excluding the ubiquitous methanogens, the bacterial community could be clustered into 4 groups based on their catabolic traits: G1, avicel adapted bacterial community; G2, glucose adapted bacterial community; G3, VFA adapted bacterial community and G4, acetate adapted bacterial community.

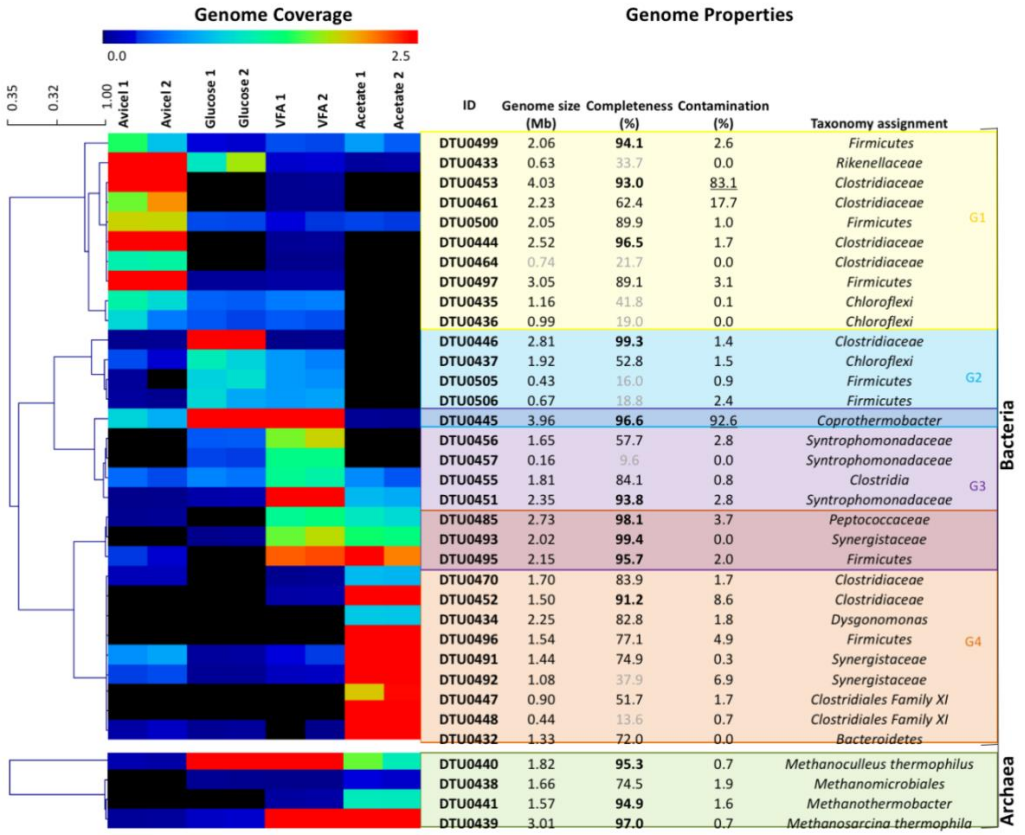

Figure 10. The coverage of 35 MAGs, properties and taxonomy assignment. On the left the coverage of 35 MAGs was presented in a heat map. The correspondence between colours and coverage is reported at the top of each panel. The basic PG properties (genome size, completeness and contamination) are listed on the right and the high quality PGs are highlighted [Adapted from Paper IV].

4.2 Remapping the AD pathways

One of the advantages of using genome-centric metagenomics is the possibility to analyse the metabolic potentials based on the gene profiles. In addition, the simplified community composition provides useful information to aid the manually *de-novo* pathway reconstruction. By exploring 92 enzymes spanning among 8 KEGG metabolic maps, AD catabolic pathways in the high quality MAGs were manually asserted. In general, the hydrolysis step was mainly mediated by extracellular enzymes found in G1 during avicel degradation. In most of MAGs the glucose oxidation step was performed through Embden–Meyerhof–Parnas (EMP) pathway (Romano and Conway, 1996). PFOR-driven pyruvate oxidation was assessed through the presence of pyruvate ferredoxin oxidoreductase genes. In addition, acetogenesis from propionate and butyrate were mapped through methylmalonyl-coenzyme A pathway and β-oxidation pathway respectively (Müller et al., 2010). The potential of performing syntrophic acetate oxidation was evaluated by the assertion of the conventional Wood-Ljungdahl pathway and by a novel pathway through glycine cleavage system and tetrahydrofolate pathway (Nobu et al., 2015). Finally, both hydrogenotrophic and acetoclastic methanogenesis were reconstructed from the gene profiles of the 4 archaeal MAGs.

It is notable that a novel glucose degradation model was proposed in Paper IV with the syntrophic microbial activity of *Clostridiaceae* sp. (DTU0446) and *Methanoculleus thermophilus* (DTU0440) (Figure 11). Briefly, the glucose is firstly fermented by DTU0446 to pyruvate through Embden–Meyerhof–Parnas (EMP) pathway and then to CO_2/ H_2 through glycine cleavage system and tetrahydrofolate pathway (Nobu et al., 2015). The model was asserted into the two most abundant MAGs in glucose adapted community, accounting for 36.5% and 33.0% respectively. Interestingly, acetate is not produced as an intermediate compound in this model, which could explain the absence of acetoclastic methanogenesis during AD with cellulose as substrates (Lü et al., 2014).

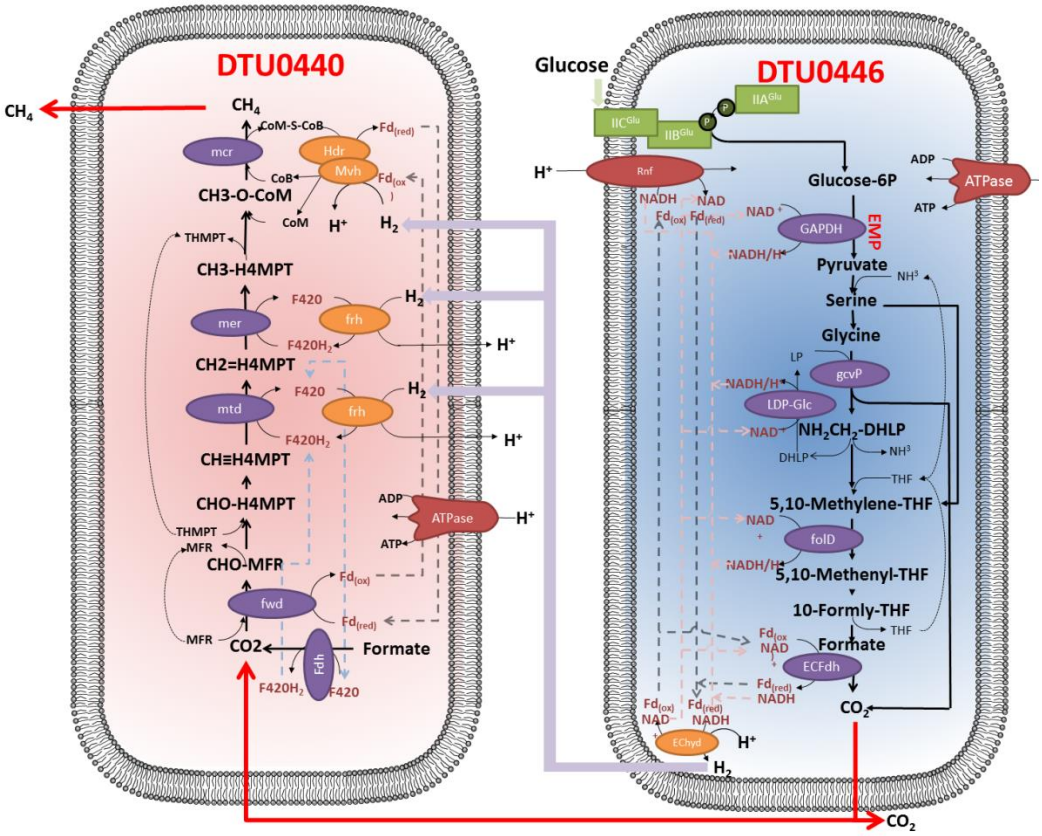

Figure 11. Obligate syntrophic glucose degradation pathway asserted in DTU0446 and DTU0440. Glucose is first transported into the cell of DTU0446 through phosphotransferase system (PTS) and oxidized by a series of oxidoreductases indicated in oval boxes. First step is the oxidation to pyruvate with glyceraldehyde phosphate dehydrogenase (GAPDH) in Embden–Meyerhof–Parnas (EMP) pathway. Pyruvate is further oxidized to CO_2 through gcvP (Glycine dehydrogenase), dihydrolipoyl dehydrogenase (LDP), folD (Methylenetetrahydrofolate dehydrogenase-cyclohydrolase) and Energy-conserving formate dehydrogenases complex (ECFdH). The released electrons are disposed in form of H_2 with *Rhodobacter* nitrogen fixation complex (Rnf) and electron-confurcating hydrogenase complex (EChyd). The CO_2 and H_2 are transferred to DTU0440 and metabolized to CH_4 through fwd (formylmethanofuran dehydrogenases), methylene-H4MPT dehydrogenase (Mtd, Mer) and methyl-CoM reductase (Mcr). The electron used for the redox reactions were extracted from H_2 through F420-reducing hydrogenase (Frh), F420- non-reducing hydrogenase (Mvh) and heterodisulfide reductase (Hdr). The utilization of formate for interspecies electron transfer can be supported by Foc (formate transporters) and Fdh (formate dehydrogenase). V-type adenosine triphosphate synthase (ATPase) are found in both PGs for ATP synthesis. Co-reactants and co-products include oxidized/reduced ferredoxin (Fd_{ox} and Fd_{red}), lipoprotein (LP), tetrahydrofolate (THF), oxidized/reduced nicotinamide adenine dinucleotide (NAD+ and NADH), methanofuran (MFR), Tetrahydromethanopterin (THMPT, H_4MPT) and oxidized/reduced coenzyme F420(F420/ $F420H_2$) [Adapted from Paper IV]

4.3 Microbes in competition and cooperation

From the metabolic potential of the MAGs and overall pathway reconstruction, Paper IV concluded that the functional roles of individual microbes cannot always be physiologically defined in accordance with "4-step" concept. It is known that frequently microbes perform more than one step of AD process. Interestingly, although many bacterial MAGs contain genes for versatile metabolisms, the dramatic variations of community composition during the degradation of different substrates suggested that these microbes are mainly specialized on specific metabolisms. The current work points out that the metabolic traits of microbes could be attributed to their unique systems for substrates uptake. For example, bacteria with genes encoding glucose permease of phosphotransferase system (PTS) dominate the microbial community during glucose degradation. This could happen because PTS favours the growth of microbes when extracellular free glucose is available. Furthermore, considering microbial cooperation, besides the generally accepted catabolic complementarity, the energy conservation system also plays an important role. The capability to mediate reactions in limited energy margins (i.e. fatty acids oxidation) needs to be facilitated by genes encoding efficient energy conservation systems, such as ion-translocation and electron-confurcation.

5 Identify novel *Candidatus* species

5.1 Genome characteristics

Recently, large scale sequencing efforts have been performed to elucidate uncultivated microbes. MAGs recovered by several genome-centric metagenomics studies reveal a considerable number of novel lineages in the tree of life (Parks et al., 2017). In AD context, 106 MAGs were extracted from a combined assembly of 6 metagenomic samples from lab scale biogas reactors (Campanaro et al., 2016). One specific high quality archaeal MAG (DTU006) was emphasized due to its ubiquitous presence and high abundance in all analysed biogas reactors. The characteristics of this MAG are shown in Table 4.

Table 4. Genomic characteristics of DTU006 [Adapted from Paper V]

NCBI ID	GCA_001512375.1
Genome size [bp]	2.15 Mbp
GC content	59.20%
Number of Scaffolds	287
Scaffold N50 [bp]	17178
Average scaffold length [bp]	7527
Number of protein-encoding sequences (SEED subsystem)	2177
Number of protein-encoding genes (Prodigal)	2297
Total number of essential genes	32
Univocal number of essential genes	31
Estimated contamination level	3.20%
Estimated completeness % (CheckM)	92.70%
Estimated contamination level % (CheckM)	2.30%

5.2 Taxonomical assignment

Based on the analysis of 400 highly conserved marker genes, DTU006 was assigned to *Methanoculleus* genus using PhyloPhlAn software (Segata et al., 2013). Moreover, the phylogenetic positioning of this MAG within all *Methanoculleus* reference genomes showed that DTU006 is more closely related to *Methanoculleus bourgensis* MS2^T (isolated from a sewage sludge digester) (Maus et al., 2012) and to *Methanoculleus* sp. MAB1 (isolated from a mesophilic methanogenic system) (Schnürer et al., 1999). However, the highest average nucleotide identity result was found with *M. bourgensis* MS2^T

(89%), but is substantially lower than the threshold suggested for species level assignment (95%) (Varghese et al., 2015). In addition, the similarity between the 16S rRNA genes of DTU006 and *M. bourgensis* is 96%, result that does not support the taxonomical assignment at the species level (Kim et al., 2014).

Thus, based on the preliminary taxonomical assignment and on the metabolic traits (described in 5.3.2), we proposed the *Candidatus* status for DTU006 and named it as *Candidatus* Methanoculleus thermohydrogenotrophicum, sp. nov.

5.3 Description of *Candidatus* Methanoculleus thermohydrogenotrophicum

5.3.1 Chromosome reconstruction

The chromosome of *Candidatus* M. thermohydrogenotrophicum was reconstructed by organizing scaffolds derived from the binning using *M. bourgensis* MS2^T as reference genome (Figure 12). The functional annotation results showed that *Candidatus* M. thermohydrogenotrophicum harbours a lower number of genes in most SEED categories such as “RNA metabolism”, “nucleosides and nucleotides”, “protein folding”, “protein biosynthesis”, “DNA metabolism”, “isoprenoids”, “respiration” and “stress response” when compared with *M. bourgensis* MS2^T. Moreover, RNA-seq data from a previous metatranscriptomic study was aligned to *Candidatus* M. thermohydrogenotrophicum genome in order to reconstruct the transcriptome (Treu et al., 2016a). The genome with gene annotation (COG) and structure of operons that include important genes for methanogenesis (such as formylmethanofuran dehydrogenase subunit (fwd) genes, coenzyme F420 hydrogenase subunit (frh) genes, methyl-coenzyme M reductase (mcr) genes and tetrahydromethanopterin S-methyltransferase (mtr) genes) were shown in Figure 12.

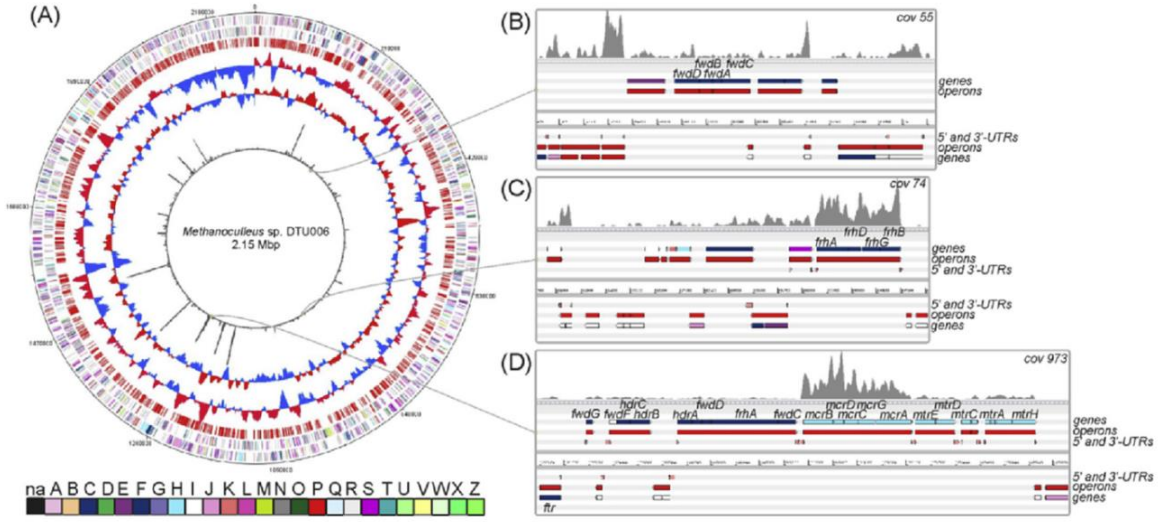

Figure 12. Tentative reconstruction of the *Candidatus* M. thermohydrogenotrophicum chromosome. (A) The scaffolds organized using the *M. bourgensis* MS2^{T} genome as a reference. From the inner to the outer concentric circles; circle 1, coverage determined using RNA-seq data; circle 2, GC skew; circle 3, GC content; circle 4, operons predicted from RNA-seq coverage data; circles 5 and 6, predicted protein-coding sequences (CDS) transcribed clockwise (outer part) and counter clockwise (inner part) and coloured according to the COG association (legend on the bottom part of the figure). (B-D) Coverage profiles determined for three genes clusters encoding proteins involved in methanogenesis [Adapted from Paper V].

5.3.2 Abundance and metabolic traits

From the functional annotation and the gene expression profile reconstruction, it was confirmed that *Candidatus* M. thermohydrogenotrophicum preforms hydrogenotrophic methanogenesis in AD context. The presence of *Candidatus* M. thermohydrogenotrophicum was proven in 40 different samples, including 9 samples from full-scale biogas plants in Denmark (highlighted in green), 9 samples from full-scale biogas plants in Germany (highlighted in blue) and 22 samples from laboratory-scale biogas reactors (Figure 13). The abundance of *Candidatus* M. thermohydrogenotrophicum was extremely variable ranging from 0.01% in the mesophilic Nysted biogas plant to nearly 20% in lab-scale biogas reactor where addition lipids were added. Nevertheless, the presence of this archaea was proven to be ubiquitary. Statistical analyses also showed that *Candidatus* M. thermohydrogenotrophicum would proliferate during thermophilic condition and is favoured by external H_2 addition (Bassani et al., 2016) These Its metabolic traits suggested that it is an important methanogen responsible for biological biogas upgrading process under thermophilic conditions.

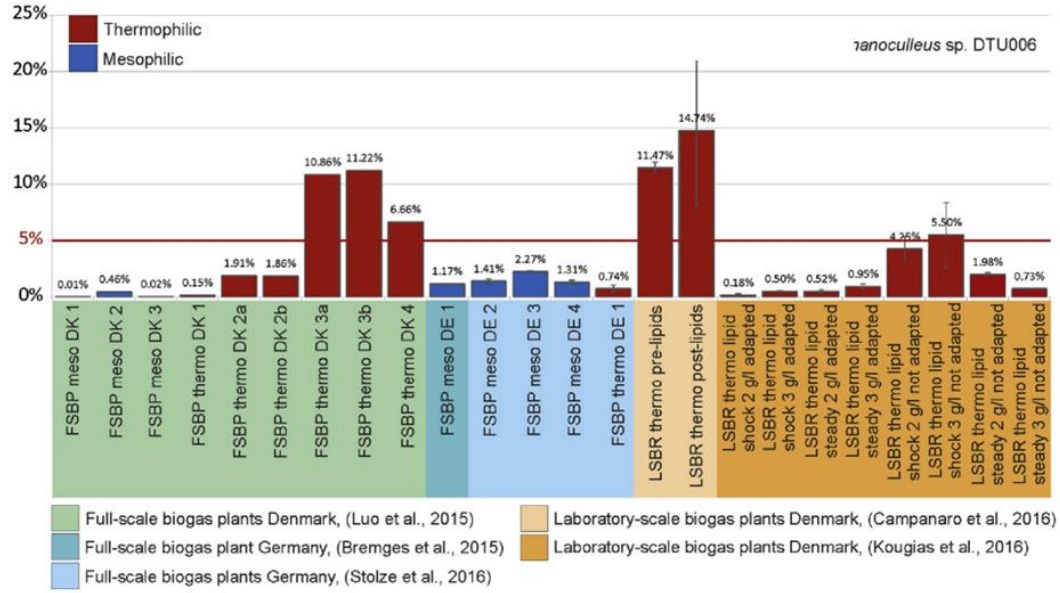

Figure 13. Relative abundance of *Candidatus* M. thermohydrogenotrophicum in 40 different samples. The SD is reported for the experiments where multiple samples have been collected from the same reactor in the same conditions [Adapted form Paper V].

5.3.3 Morphotype

Finally, fluorescence in situ hybridization (FISH) using *Candidatus* M. thermohydrogenotrophicum specific probe was performed in order to visualize the morphotype of the cells (Figure 14). From the micrograph, the recognized general archaea, that are maked with green, is represented by different morphotypes (irregular cocci and bacilli). While, cells of *Candidatus* M. thermohydrogenotrophicum (red) are represented by irregular cocci with the diameter ranging in size from 1 to 2 µm.

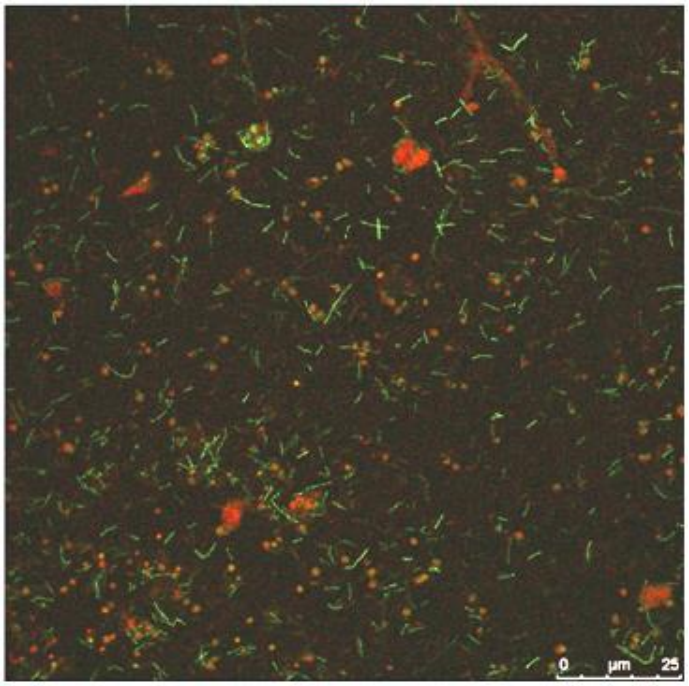

Figure 14. FISH micrograph of *Candidatus* M. thermohydrogenotrophicum. General probe (ARC915) designed on 16S rRNA of general archaea shows green signal and specific probe (METHY470) designed on 16S rRNA of *Candidatus* M. thermohydrogenotrophicum shows red signal [Adapted from Paper V].

6 Conclusions

This Ph.D. study focused on exploring the anaerobic digestion (AD) microbial ecology with next generation sequencing based tools. Firstly, the main biological obstacle during the start-up of thermophilic Up-flow anaerobic sludge blanket (UASB) reactors has been successfully addressed with 16S rRNA amplicon sequencing technology. Then, genome-centric metagenomics was applied to reveal the fundamental microbial roles in an AD context. Additionally, the Ph.D. project also contributed to the discovery of a novel *Candidatus* methanogenic species.

The major findings of the Ph.D. thesis are summarized below:

- In UASB reactors, the shifts of operational temperature from mesophilic to thermophilic impose dramatic changes in the microbial community composition of granular sludge. The difficult adaptation of thermophilic methanogens is the biological 'bottleneck' during the start-up of thermophilic UASB reactors from mesophilic granules.
- Planktonic microbiota in UASB reactors is more sensitive to operational conditions and contributes to restoring fermentation process during temperature adaptation. The microbial interaction between granular sludge and the liquid phase of a UASB reactor affects the final microbial community composition in the granules.
- The bioaugmentation with axenic methanogenic cultures, i.e. *Methanothermobacter thermautotrophicus,* was proven to be an effective practice to accelerate the start-up of thermophilic UASB reactors. The bioaugmented reactor showed up to 40% higher methane production rate compared with the Control. The methanogenesis enhancement persisted in the augmented reactor for over 8 hydraulic retention times.
- The 16S rRNA amplicon sequencing is an effective approach to identify, analyse and solve technical challenges during AD operation.
- The diversity of AD microbial communities was significantly reduced using synthetic substrates and continuous operation. The functional annotation of MAGs extracted from such simplified community allowed *de-novo* pathways reconstruction.

- A novel glucose degradation model was proposed with the syntrophic microbial activity of *Clostridiaceae* sp. and *Methanoculleus thermophilus*. It is notable that acetate is not produced as intermediate in this model which was never reported for glucose metabolism.
- Although many MAGs harbour genes for versatile metabolism, they have specific functional roles in the AD food chain and their roles cannot always be physiologically defined in accordance with the "4-step" concept.
- The genome-centric metagenomics reveals comprehensive insights into the AD microbial ecology. It allowed the characterization of novel species, pathways, and microbial cooperation models.
- *Candidatus* Methanoculleus thermohydrogenotrophicum is proposed as a new *Candidatus* species belonging to *Methanoculleus* genus. It is ubiquitary and plays an important role during biological biogas upgrading process under thermophilic conditions.

7 Future Perspectives

The present PhD study showed that the NGS technology has potential to play an important role for further investigation of the AD microbial ecology. Further metagenomic and metatranscriptomic analysis could reveal more microbial insights into the AD system. Moreover, the obtained results could be further used as fundamental knowledge for AD process control and optimization.

More specifically, from the fundamental perspectives:

- Extensive metagenomic investigations are needed to systematically characterize the uncultivated microbes found in high abundance in AD systems. These newly characterized MAGs could be a key to expand current understanding of AD microbial ecology.
- Further metatranscriptomic investigations on simple methanogenic microbial communities would be a potentially effective method to further reveal gene expression regulations in AD system.
- Other '-omics' investigations such as proteomics and metabolomics would be potentially useful to fully describe the AD microbial activity.

Applicable investigations:

- The cost-effectiveness of bioaugmentation should be analysed in order to evaluate the feasibility of this solution.
- The newly proposed degradation model could be tested and eventually implemented in AD simulation models.

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9 Papers

I Zhu, X., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I. (2017). Microbial community changes in methanogenic granules during the transition from mesophilic to thermophilic conditions. Appl. Microbiol. Biotechnol.

II Zhu, X., Treu, L., Kougias, P.G., Campanaro, S., Angelidaki, I. (2017). Characterization of the planktonic microbiome in upflow anaerobic sludge blanket reactors during adaptation of mesophilic methanogenic granules to thermophilic operational conditions. Anaerobe.

III Zhu, X., Treu, L., Kougias, P.G., Campanaro, S., Angelidaki, I. (2017). Converting mesophilic upflow sludge blanket (UASB) reactors to thermophilic by applying axenic methanogenic culture bioaugmentation. Chem. Eng. J.

IV Zhu, X., Campanaro, S., Treu, L., Kougias, P.G., Angelidaki, I. (2018). Microbial community in anaerobic digestion of saccharides: composition and functions revealed through metagenomics (*Manuscript under preparation*)

V Kougias, P.G., Campanaro, S., Treu, L., Zhu, X., Angelidaki, I. (2017). A novel archaeal species belonging to *Methanoculleus* genus identified via de-novo assembly and metagenomic binning process in biogas reactors. Anaerobe.

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