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Preface

This Ph.D. thesis, entitled “Customized ammonia tolerant methanogenic inocula to alleviate ammonia toxicity in anaerobic digesters” presents the research performed at the Department of Environmental Engineering, the Technical University of Denmark from November 1st, 2017 to October 31st, 2020. The research was co-funded by the China Scholarship Council and the Technical University of Denmark. Professor Irini Angelidaki was the main supervisor and Associate Professor Ioannis Fotidis was the co-supervisor.

The thesis is organized into two parts: the first part puts into context the findings of the Ph.D. 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-VI**.

I **Yan, M**., Fotidis, I. A., Tian, H., Khoshnevisan, B., Treu, L., Tsapekos, P., & Angelidaki, I. (2019). Acclimatization contributes to stable anaerobic digestion of organic fraction of municipal solid waste under extreme ammonia levels: focusing on microbial community dynamics. Bioresource Technology, 286, 121376.

II **Yan, M**., Treu, L., Zhu, X., Tian, H., Basile, A, Fotidis, I. A., Campanaro S., Angelidakia I., (2020). Insights into ammonia adaptation and methanogenic precursor oxidation by genome-guided analysis. Environmental Science & Technology. 2020.

III **Yan, M**., Treu, L., Campanaro, S., Tian, H., Zhu, X., Khoshnevisan, B., Tsapekosa, P., Angelidakia, I., Fotidis, I. A., (2020) Effect of ammonia on anaerobic digestion of municipal solid waste: inhibitory performance, bioaugmentation and microbiome functional reconstruction. Chemical Engineering Journal, 126159.

IV **Yan, M**., Fotidis, I. A., Jéglot, A., Treu, L., Tian, H., Palomo, A., Angelidaki, I. (2020). Long-term preserved and rapidly revived methanogenic cultures: Microbial dynamics and preservation mechanisms. Journal of Cleaner Production, 121577.

In this online version of the thesis, paper **I-IV** 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.

Besides, the following publications, not included in this thesis, were also concluded during this Ph.D. study:

V **Yan, M**., Zhu, X., Treu, L., Ravenni. G., Ferrigno. R., Goonesekera, M., Angelidaki, I., Fotidis, I.A. (2020) Assessment of multiple strategies to alleviate ammonia inhibition (Manuscript under preparation for submission)

VI Tian, H., **Yan, M**., Treu, L., Angelidaki, I. and Fotidis, I.A. (2019) Hydrogenotrophic methanogens are the key for a successful bioaugmentation to alleviate ammonia inhibition in thermophilic anaerobic digesters. Bioresource Technology 293, 122070.

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Summary

Anaerobic digestion (AD) is a globally popular waste management technology where the organic matters are degraded into biogas (mainly CH_4 and CO_2) and digestate under oxygen-free conditions. To date, biogas has been widely promoted according to its following uses in electricity, other value-added products, transport, and heating. United Nations aims to achieve a greater share of the biogas in renewable energy for Sustainable Development Goals. The organic waste from industries and households are suitable feedstocks for biogas production, which is exactly following the important concept of "waste to energy". Despite the various benefits and great potential of AD, further development of AD is delayed by several difficulties, one of which is the sole degradation of N-rich substrates. The ammonia released from them (exceed 1.5 g NH_4^+-N/L) is an inhibitor to microorganisms in the AD, especially methanogens, which leads to less methane yield. Thus, approaches to recover methane production from ammonia inhibition are required. Furthermore, a balanced microbial composition is of utmost importance for the stable AD system process. Thus a deep insight into the microbial community provides the essential knowledge for process optimization. The continuous development of sequencing technology and bioinformatics methods is making it feasible to expand our understanding of complex microbial community composition and characteristics.

This Ph.D. project aimed to 1) develop methods to recover methane production from organic waste at high ammonia stress and 2) explore microbial synergistic networks and other functionalities determining stress resistance.

Firstly, to achieve an effective continuous anaerobic degradation of N-rich substrates, the stepwise acclimatization process was performed in mesophilic reactors fed with organic fraction of municipal solid waste (OFMSW). The results showed the acclimatization could increase the continuous stirring tank reactors (CSTRs) robustness up to 8.5 g NH_4^+-N/L and the methane yields fluctuated less than 10%. Meanwhile, the shift of dominant methanogens was observed from *Methanosaeta concilii* at low ammonia levels (1.1-5 g NH_4^+-N/L) to *Methanosarcina soligelidi* at high ammonia levels (6-9.5 g NH_4^+-N/L), respectively.

Secondly, to investigate the microbial synergistic networks and other functionalities that regulate ammonia tolerance, the initial microbial inoculum were cultivated into four groups (i.e., $G_{methanol}$, $G_{H2/CO2}$, $G_{formate}$ and $G_{acetate}$)

under increased ammonia levels in batch reactors. Synthetic basal anaerobic (BA) medium containing a single carbon source (either methanol, or H_2/CO_2, or formate, or acetate) was used as feedstock. After several generations' cultivation, the microbial consortia in $G_{methanol}$ and $G_{H2/CO2}$ were robust up to 7.25 g NH_4^+-N/L, followed by $G_{formate}$ (5.25 g NH_4^+-N/L) and $G_{acetate}$ (4.25 g NH_4^+-N/L), respectively. The metabolic pathways of dominant microbes were reconstructed based on the genomics analysis coupling with intermediates measurement during the degradation process, thereby a possible mechanism of ammonia tolerance was proposed. Briefly, the existence of genes responsible for osmotic regulators (K^+, N^{ε}-acetyl-L-lysine, glutamine, glutamate, and glycine betaine) and energy conservation complexes (i.e. Ech and Eha) was proposed to be the main reason to assist the consortia against ammonia stress. Besides, the syntrophy (e.g., catabolic complementarity) between bacteria and methanogens supported them in overcoming bioenergetic barriers caused by ammonia inhibition.

Thirdly, the bioaugmentation with acclimatized ammonia tolerant *Methanoculleus* sp. was implemented to stimulate the under-performing AD process. A 21% increase in methane yields and 10% reduction in volatile fatty acids confirmed that the addition of *Methanoculleus sp.* successfully alleviated ammonia stress. The genome-centric metagenomics revealed that multiple energy regulating complexes and osmolytes uptake systems in *Methanoculleus* sp. might contribute to its remarkable robustness to ammonia. Meanwhile, the bioaugmentation of *Methanoculleus* sp. triggered the change in bacterial community composition. Regarding dominant bacteria, the genes involved in parallel degradation pathways of glucose and acetate possibly provided them with enough flexibility to overcome ammonia stress. Therefore, the resume of electron transfer between the syntrophic bacterial community and methanogen led to the successful bioaugmentation.

Finally, the microbial consortia preservation technology was developed to provide the ready-to-use inocula for future full-scale bioaugmentation applications. The ammonia tolerant methanogenic consortia were preserved in two carriers, namely, liquid BA medium and agar gel, at two different temperatures of 4 °C and 24 °C, respectively. The best strategy for long-term preservation was in agar gel up to 168 days at 24 °C, followed by a liquid BA medium within 84 days at 24 °C. Besides, high methanogenic activities of *Methanosarcina soligelidi* and *Methanoculleus palmolei* were observed during the revival test, indicating their high potential as ready-to-use inocula.

Overall, this Ph.D. project offered feasible methods to improve methane yields from the ammonia-stressed anaerobic reactors. Besides, the knowledge regarding microbial composition and metabolic pathways had practical guidance for AD process optimization.

Dansk sammenfatning

Anaerob nedbrydning (AD) er en populær global affaldshåndteringsteknologi, hvor de organiske stoffer nedbrydes til biogas (hovedsageligt CH_4 og CO_2) under iltfrie forhold. Til dato er biogas blevet promoveret i henhold til følgende anvendelser: elproduktion, andre merværdiprodukter, transportbrændstof og opvarmningsformål. En større andel af biogas i vedvarende energi forventes at nå de Forenede Nationers mål for bæredygtig udvikling. Det organiske affald fra industrier og husholdninger er egnetsom råmateriale til produktion af biogas, hvilket er god i overensstemmelse med konceptet om "affald til energi". På trods af de forskellige fordele og det store potentiale ved AD forsinkes udviklingen af AD grundet en række udfordringer, hvoraf den ene er nedbrydningen af N-rige substrater. Den frigjorte ammoniak (over 1,5 g NH_4^+ -N/L) hæmmer vækst af mikroorganismer i AD, især methanogener, hvilket fører til mindre methanudbytte. Derfor er metoder til at opretholde metanproduktion på trods af ammoniakhæmning påkrævet. Desuden er en afbalanceret mikrobiel sammensætning af stor betydning for en stabil AD-proces. Således giver en dyb indsigt i den mikrobielle sammensætning væsentlig viden for procesoptimering. Udvikling af sekventeringsteknologi og bioinformatiske metoder gør det muligt at udvide vores forståelse af komplekse mikrobielle sammensætning og egenskaber.

Dette ph.d. projekt har til formål at 1) udvikle metoder til at opretholde metanproduktion fra organisk affald med højt ammoniakindhold og 2) udforske mikrobielle synergistiske netværk og andre funktioner, der påvirker den mikrobielle stressmodstand.

For at opnå en effektiv kontinuerlig anaerob nedbrydning af N-rige substrater blev den trinvise akklimatiseringsproces udført i mesofile reaktorer fodret med den organiske fraktion af husholdningsaffald (OFMSW). Resultaterne viste, at akklimatisering kunne øge procesrobustheden i fuldomrørte reaktorer (CSTRs), med en ammoniakbelastning op til 8,5 g NH_4^+-N/L. Methanudbyttet varrierede mindre end 10%. I mellemtiden var der et skift af methanogener, der var dominerende i processen, fra *Methanosaeta concilii* ved lave ammoniakniveauer (1,1-5 g NH_4^+-N/L) til *Methanosarcina soligelidi* ved høje ammoniakniveauer (6-9,5 g NH_4^+-N/L).

For at undersøge de mikrobielle synergistiske netværk og andre funktionaliteter, der regulerer ammoniak-tolerancen, blev podematerialet dyrket i fire grupper (dvs. $G_{methanol}$, $G_{H2/CO2}$, $G_{formiat}$ og G_{acetat}) under øgede

ammoniakniveauer i batchreaktorer. Syntetisk basalt anaerobt (BA) medium indeholdende en enkelt carbonkilde (methanol, H_2/CO_2, formiat og acetat) blev anvendt som råmateriale. Under akklimatiseringsprocessen var konsortierne i $G_{methanol}$ og $G_{H2/CO2}$ robuste op til 7,25 g NH_4^+-N/L efterfulgt af G_{format} og G_{acetat} til henholdsvis 5,25 og 4,25 g NH_4^+-N/L. Omsætningsveje for dominerende mikrober blev rekonstrueret baseret på en genomanalyse kombineret med målinger af mellemprodukter under nedbrydningsprocessen, hvorved den mulige mekanisme for ammoniak-tolerance blev foreslået. I korte træk skulle eksistensen af gener, der er ansvarlige for osmotiske regulatorer (K^+, Nε-acetyl-L-lysin, glutamin, glutamat og glycinbetain) og energibesparelseskomplekser (dvs. Ech og Eha), være hovedårsagen til at hjælpe konsortier mod ammoniak stress. Desuden understøttede syntrofi (fx katabolisk komplementaritet) mellem bakterier og methanogener dem i at overvinde bioenergiske barrierer forårsaget af ammoniakhæmning.

Derefter blev bioaugmenteringen med akklimatiseret ammoniak-tolerant *Methanoculleus* sp. implementeret for at stimulere den begrænsede AD-proces. En stigning på 21% i methanudbytte og 10% reduktion i flygtige fedtsyrer bekræftede, at tilsætningen af *Methanoculleus* sp. lykkedes med at lindre ammoniakstress. Metagenomics analysen viste, at flere energiregulerende komplekser og osmolytter optager systemer i *Methanoculleus* sp. og kan bidrage til dets bemærkelsesværdige robusthed over for ammoniak. I mellemtiden forårsagede bioaugmenteringen af *Methanoculleus* sp. ændringer i bakteriesamfundets sammensætning. Med hensyn til dominerende bakterier gav generne, der var involveret i parallelle omsætningsveje for nedbrydning af glucose og acetat, muligvis tilstrækkelig fleksibilitet til at overvinde ammoniakstress. Derfor førte genoptagelsen af elektronoverførsel mellem det syntrofiske bakteriesamfund og methanogen til en vellykket bioaugmentering.

Endelig blev den mikrobielle konserveringsteknologi udviklet til at give en klar-til-brug podning til fremtidige fuldskala bioaugmenteringsapplikationer. De ammoniak-tolerante metanogene konsortier blev konserveret i to medier, nemlig flydende basisk anaerobt medium og agargel ved to forskellige temperaturer på henholdsvis 4 °C og 24 °C. Den bedste strategi til langvarig konservering var i agargel op til 168 dage efterfulgt af et flydende basisk anaerobt medium inden for 84 dage ved 24 °C. Derudover blev der observeret høje metanogene aktiviteter af *Methanosarcina soligelidi* og *Methanoculleus palmolei* under genoplivningstesten, hvilket indikerer deres høje potentiale som inokula klar til brug.

Samlet set har dette Ph.D. projekt udviklet mulige metoder til forbedring af methanudbyttet fra ammoniak-stressede anaerobe reaktorer. Desuden har den nye viden om mikrobiel sammensætning og omsætningsveje under ammoniumstress givet ny forståelse, som kan anvendes til procesoptimering af ammonium belastede AD-processer.

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Abbreviations

AD	Anaerobic digestion
SDGs	Sustainable Development Goals
LCFA	Long-chain fatty acids
EMP	Emben-Meyerhof-Parnas
ED	Entner Doudoroff pathway
VFA	Volatile fatty acid
FAN	Free ammonia nitrogen
TAN	Total ammonia nitrogen
OFMSW	Organic fraction of municipal solid waste
GHG	Greenhouse gas
BA	Basal anaerobic medium
VS	Volatile solid
PCA	Principal component analysis
TS	Total solid
MAG	Metagenome assembled genome
PTA-ACKA	Phosphotransacetylase-acetate kinase pathway
ACS	Forming acyl-CoA synthetase
acs	Acyl-CoA synthetase gene
ack	Acetate kinase gene
mcrA	Methyl coenzyme M reductase gene
Mta/b	Coenzyme M methyltransferase genes
coo	Carbon monoxide dehydrogenase gene
gcv	Glycine decarboxylase genes
grd	Glycine reductase gene
Vho	Methanophenazine-reducing hydrogenase
Frh	Coenzyme F420-reducing hydrogenase
Ech	Energy converting hydrogenase
Fpo	F420H2 dehydrogenase
hdr	Membrane-bound heterodisulfide reductase gene
Ehb	Energy-conserving hydrogenase B
WL	Wood-Ljungdahl
Rnf	Rhodobacter nitrogen fixation complex

1 Introduction

1.1 Background

The human population is predicted to reach over 9 billion by 2050; meanwhile, the demand for energy and food is expected to increase by over 50%. Consequently, the increasing amount of organic waste and disposal from the human is estimated as a global problem (Ferroukhi et al. 2015). If there is no proper treatment, the massive waste generated may worsen the water, soil, and air quality (Minelgaitė and Liobikienė 2019). To achieve the United Nations' Sustainable Development Goals (SDGs) by 2030, a circular economy concept should be performed by integrating organic waste treatment with bioenergy production and nutrients recovery (Schroeder et al. 2019, Favaro et al. 2019). European Union has banned the application of biodegradable organic wastes in landfills (Briassoulis et al. 2019). As a consequence, many countries have introduced incineration for waste treatment and energy production (Tozlu et al. 2016). However, incineration is not preserving nutrients and seems to be an unsustainable approach from economic and environmental perspectives (Tozlu et al. 2016, Tian et al. 2020).

Anaerobic digestion (AD) is a promising approach where involves varieties of microbes in converting organic waste (e.g., municipal solid waste, industrial wastewater, agriculture waste, algae waste, etc.) to biogas and digestate (Ambaye et al. 2020, Satchwell et al. 2018). Biogas, one of the essential renewable energy, is expected to partially substitute fossil fuels for the supply of electricity, heat, and transportation in Denmark (Figure 1) (McAnulty et al. 2017). Furthermore, the digestate is rich in various types of nutrients, which can improve soil quality in agriculture sector (Tambone et al. 2010). Thus over 163 biogas plants are widely implemented in Denmark to maximize the energy recovery from the organic waste (European biogas association).

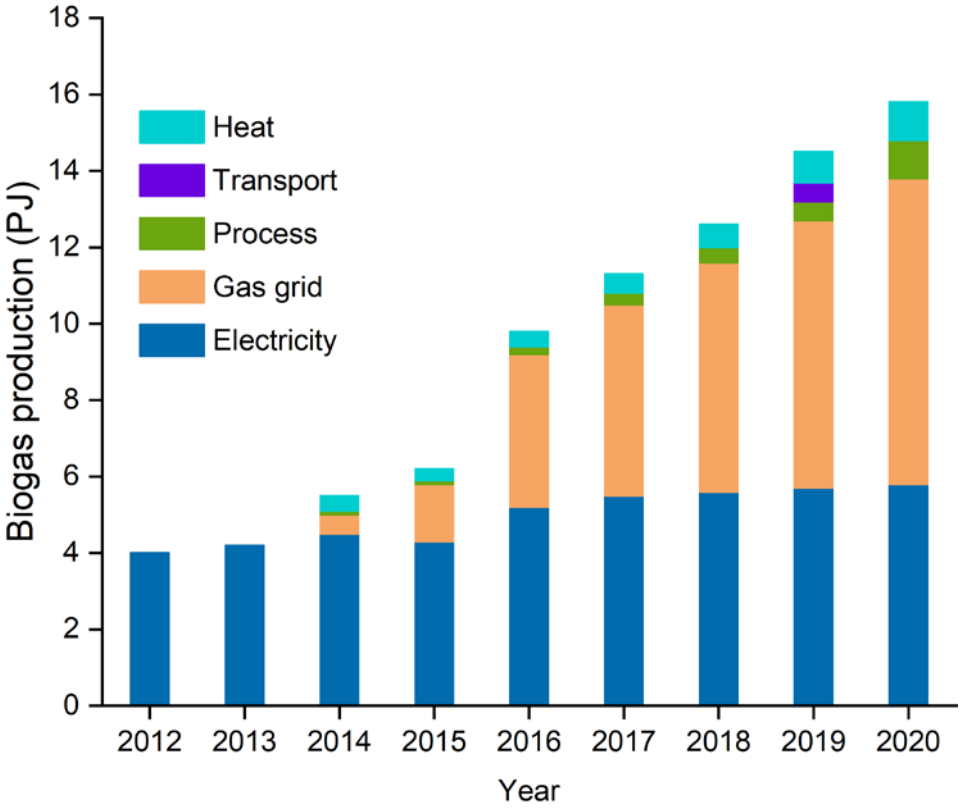

Figure 1. Biogas production and its application in Denmark 2012-2020. [Adapted from Danish Energy Agency]

1.1.1 Anaerobic digestion

The AD is a biological process, where organic matters are converted into methane (50-70%), carbon dioxide (30-50%), H_2 (0-1%), and H_2S (0-3%), etc., under oxygen-free conditions (Angelidaki et al. 2011a). However, the percentage of the biogas is determined by several factors, e.g., feedstock composition, digester retention time and temperature (Karube et al. 1980, Lin et al. 2019, Uçkun Kiran et al. 2016, Chen et al. 2008). AD process mainly occurs in four steps, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Ponsá et al. 2008) (Figure 2). Bacteria are involved in the first three steps, and archaea responsible for the final stage (Campanaro et al. 2018).

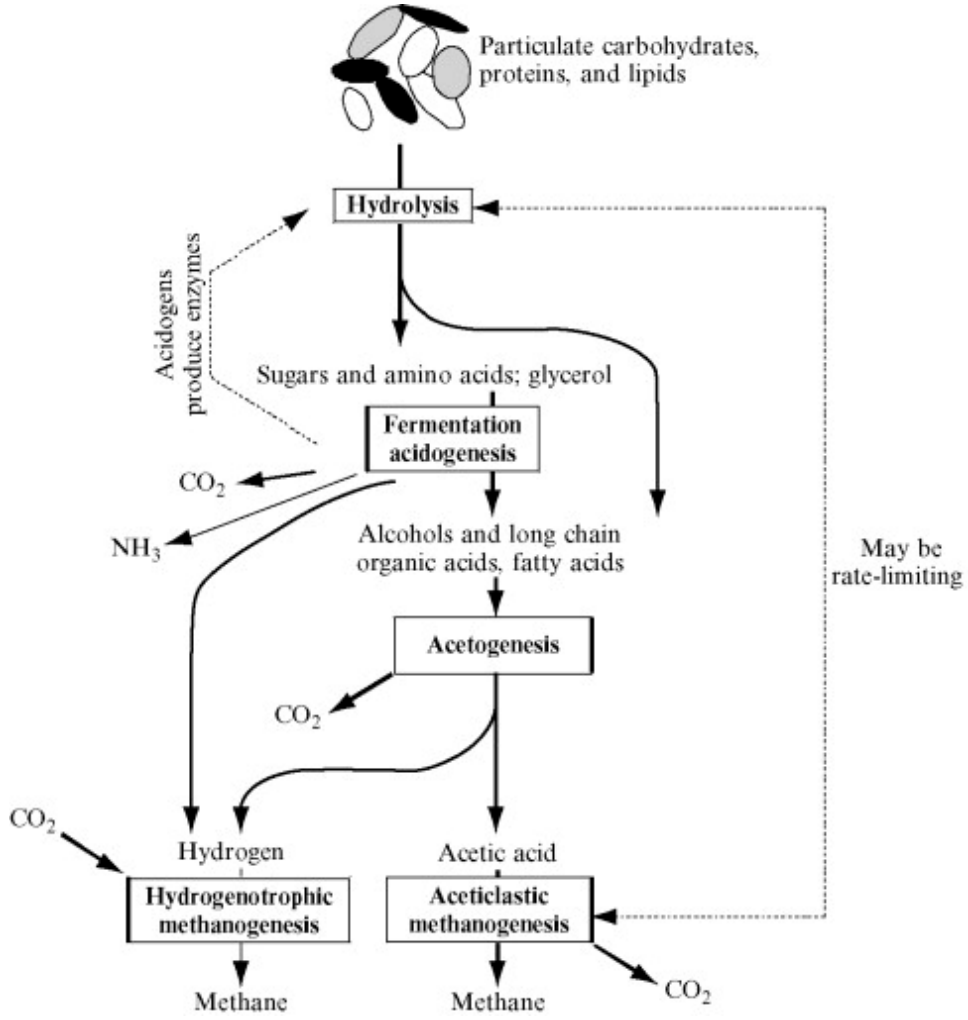

Figure 2. The main flow of the AD process in four steps [Adapted from Angelidaki et al. (2011b)]

1.1.2 Hydrolysis

The hydrolysis step is involved in the degradation of protein, carbohydrates, and lipids into amino acids, monosaccharides, and glycerol /long-chain fatty acids, respectively, through hydrolytic enzymes (Wilson 2011, Dimock and Morgenroth 2006, Chen and Chang 2017). The hydrolytic bacteria mainly belong to the phylum *Firmicutes* and *Bacteroidetes*, and they are more robust to environmental change (e.g., ammonia stress, and temperature), compared to the methanogen (Dimock and Morgenroth 2006, Chen and Chang 2017, Cazier et al. 2019).

1.1.3 Acidogenesis

During the acidogenesis step, the main products from hydrolysis, i.e., monosaccharides, amino acids, and glycerol/long-chain fatty acids (LCFA) are further degraded into smaller molecules (Myint et al. 2007).

The monosaccharides are converted into C3 products via Emben-Meyerhof-Parnas (EMP) pathway, and C2/C4/C6 products using Entner Doudoroff (ED) pathway, respectively (Zhu et al. 2019). C2/C4/C6 products (e.g., acetate, butyrate, caproate) are more commonly present in the AD process. In contrast, the C3 products (e.g., lactate) are rarely observed unless AD reactors are overloaded (Angelidaki et al. 2011a, Zhou et al. 2018a, Tian et al. 2019a).

The amino acid degradation commonly proceeds via the Stickland reaction where the paired amino acid is converted into organic acid (with one less carbon) and NH_3 in a coupled oxidation/reduction reaction (Chen et al. 2019). Most amino acids can be electron acceptors, donors, even both. In some cases, the acidogenesis of amino acid takes place via transamination, thereby pyruvic and oxaloacetic acids are formed (Tezel et al. 2011).

The LCFA is broken into shorter volatile fatty acids, acetate, and hydrogen following β-oxidation, and the shorter volatile fatty acids enter the next rounds of β-oxidation by the acetogenic-oxidizing bacteria (Tian et al. 2018a). So far, the known acetogenic β-oxidizing bacteria have been identified within the families of *Syntrophomonadaceae* and *Syntrophaceae* (Ziels et al. 2017). However, the LCFA is easily attached to the surface of microorganism, which limits the access of microorganism to the substrate (Zhou et al. 2018b). Generally, the biodegradation rate of LCFA is slower than the hydrolysis process, which causes the accumulation of LCFA quickly in the AD system. Meanwhile, the hydrogen, the by-product from the LCFA degradation, influences the distribution of the short-chain acid. Specifically, the higher hydrogen partial pressure inhibits short-chain acids production, leading to accumulation of LCFA due to the chemical equilibrium. As a consequence, it further reduces the activity of acetogenic β-oxidizing bacteria (Zhou et al. 2018b, Hao et al. 2016, Alves et al. 2001).

1.1.4 Acetogenesis

Acetogenesis refers to the process of acetate formation through two pathways, i.e., reduction of C1 units (e.g. CO_2) (Xu et al. 2011) and degradation of organic acids (Diekert and Wohlfarth 1994). The homoacetogens involved in the first pathway belong to the genera of *Acetoanaerobium*, *Acetogenium*, *Acetobacterium*, *Clostridium*, *Butyribacterium*, *Eubacterium*, and *Pelobacter* (Borja 2011). Most homoacetogens perform Wood-Ljundahl pathway for acetate generation, where H_2 and HCO_3^- are used as electron donor and acceptor, respec-

tively (Westerholm et al. 2016). Thus homoacetogens develop syntrophic co-operation with H_2-producing bacteria, while the competitive relationship with hydrogen-utilizing methanogens (Borja and Rincón 2017).

During the second pathway, the hydrogen-producing acetogens oxidize organic acids to acetate, from which the electrons produced are transferred to H^+ for H_2 and CO_2 generation (Zhu et al. 2019). However, these acetogenic bacteria can only grow in an efficient electron-removing environment. For example, the simplest co-culture involving syntrophic interaction comprises an acetogen and a hydrogen-scavenging microbe (e.g., hydrogenotrophic methanogen). Thus the presence of mutualistic co-culture is vital for the well-balanced anaerobic digestion (Angelidaki et al. 2011a, Borja 2011).

1.1.5 Methanogenesis

Methanogenesis takes place at the final stage of AD when the alternative electron acceptors (e.g., O_2, Fe^{3+}, and NO_3^-) are exhausted. Until now, all methanogens are classified into seven orders (*Methanococcales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*, *Methanopyrales*, and *Methanomassiliicoccales*) (Vanwonterghem et al. 2016). Due to their distinct metabolic traits, three different pathways are involved in methane production, i.e., the acetoclastic pathway, the hydrogenotrophic pathway, and the methylotrophic pathway (Angelidaki et al. 2011a, Lyu et al. 2018).

Firstly, acetoclastic methanogens enable the cleavage of acetate into CH_4 and CO_2. So far, only two genera of methanogens are identified for acetate utilization, namely, *Methanosarcina* and *Methanosaeta*. *Methanosaeta* is a specialist that uses acetate specifically within the range of 0-20 mM (Chen et al. 2017, Dyksma et al. 2020). Besides, *Methanosaeta* was reported as the primary methane producer on earth because it is widely present in the environment (e.g., rice paddies) and prevailed over *Methanosarcina* in nature with the low level of acetate (Smith and Ingram-Smith 2007, Rotaru et al. 2014). However, *Methanosarcina* is a metabolically versatile methanogen capable of producing methane from carbon dioxide, methanol, and methylamines as well (Smith and Ingram-Smith 2007). It is more abundant in the middle-high acetate and/or ammonia environment (Capson-Tojo et al. 2020).

Secondly, hydrogenotrophic methanogens (i.e., *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales*, *Methanocellales*, and *Methanopyrales*) are capable of converting H_2 and CO_2 to CH_4. Their presence maintains the AD

system at a low H_2 partial pressure and makes stable acetogenesis process feasible (Zabranska and Pokorna 2018, Watkins et al. 2012, Tian et al. 2019b). Compared to acetoclastic methanogens, hydrogenotrophic methanogens are more robust to ammonia stress. Thus, they are of critical importance to stable methane production at high ammonia levels (Tian et al. 2019a).

Thirdly, methylotrophic methanogens (including some *Methanosarcinales* spp. and *Methanomicrobiales* spp.) can metabolize methylated C_1 compounds to methane (Vanwonterghem et al. 2016). Besides, formic acid, methanol, methyl sulfides, methylamines, and some methylated ethanolamines can serve as carbon sources (Zabranska and Pokorna 2018). Methylotrophic methanogens are significant contributors to methane production in marine sediments, and other extreme environments, e.g. hypersaline conditions and soda lake sediments (Vanwonterghem et al. 2016, Zhang et al. 2020)

1.1.6 Syntrophic network in the anaerobic digestion process

The complete conversion of various substrates to methane depends on the obligately mutualistic metabolism performed by archaea and bacteria in anaerobic bioreactors (Morris et al. 2013). For example, during the syntrophic degradation, the H_2 derived from some bacteria can directly feed hydrogen-utilizing microbes involved in the other steps. In turn, the hydrogen-utilizing microbes are crucial to maintaining the low partial pressure of H_2 that allows the H_2-producing reaction feasible (Stams and Plugge 2009).

This mutualistic metabolism is not only limited within the transfer of reducing agents, e.g., formate or hydrogen but also involves the exchange of organic compounds or the scavenging of toxic compounds (Morris et al. 2013). Due to these unique features, syntrophic partners take advantage of each other to catabolize the organic matter, which can not be driven by the individual microbes alone. Therefore, dedicated microbial studies will shed more light on the significance of unknown metabolic interactions between microorganisms.

1.2 Anaerobic digestion of N-rich waste

During the anaerobic digestion of N-rich waste (e.g., chicken manure, slaughterhouse waste, as well as industrial waste, etc.), ammonia (TAN) consisting of free ammonia nitrogen (FAN, NH_3) and ammonia ion (NH_4^+), is a by-product of acidogenesis step. Ammonia levels within the range of 0.05-0.2 g NH_4^+-N/L are necessary for microbial growth because ammonia serves as the N-source for amino acid and nucleic acids synthesis (Jiang et al. 2019). However, TAN being above 1.5 g NH_4^+-N/L has an adverse effect on microbial activities

due to FAN inhibition (Capson-Tojo et al. 2020, Rajagopal et al. 2013). The following explanations are provided:

1) FAN can freely permeate into cells and can be converted into ammonia ion due to protonation, thus leading to intracellular proton imbalance (Jiang et al. 2019).

2) High FAN levels suppress the uptake of potassium/magnesium/sodium that are required nutrients for microbial growth (Wittmann et al. 1995)

3) High FAN levels induce intense extracellular osmotic stress which disturbs microbial metabolic activity (Kadam and Boone 1996, Chang et al. 2020)

4) High ammonia levels lead to energy-intensive metabolic process (Wittmann et al. 1995)

Methanogens are widely reported more vulnerable to ammonia than bacteria because their cell walls lack peptidoglycan and the structure is "leaky" with S-layers (Capson-Tojo et al. 2020, Engelhardt 2007). Thus the inhibited methanogenic activity at high ammonia levels leads to a reduced consumption rate of acetate/H_2 (the critical precursors of methanogenesis) (Yang et al. 2019, Fotidis et al. 2013). Once this acetate / H_2 accumulated up to certain levels, the syntrophic acetogenic and acetate oxidizing reactions may become energetically unfavourable (Morris et al. 2013, Worm et al. 2010). Thus the conversion from organic waste to methane was suppressed entirely, evidenced by the lower total solid removal rate, VFA accumulation and methane yields (Chen et al. 2018, Dai et al. 2017).

1.2.1 Multiple strategies to recover methane production from under-performing AD process

To alleviate ammonia stress in AD, many techniques including air stripping, ammonia adsorption, hollow fibre membrane contactor, conductive materials addition (e.g., magnetite) have been developed (Liu et al. 2012, Wang et al. 2018, Yabu et al. 2011, Ho and Ho 2012, Lauterböck et al. 2012). However, their commercial applications are restricted by the high-cost and/or technical challenges. Biological treatments (e.g., acclimatization and bioaugmentation) have several advantages, e.g., easy operation, high efficiency, and negligible pollution to environment. Nevertheless, more dedicated studies are required for AD process optimization.

1.3 Objectives and thesis structure

1.3.1 Objectives

This Ph.D. project focuses on 1) developing efficient and sustainable strategies to recover methane production from ammonia inhibition; 2) deciphering the microbial characteristic and their syntrophic interaction. The specific objectives are listed as follows:

1) Acclimatize ammonia tolerant inocula to achieve stable methane production in continuous stirring tank reactors (CSTR) at high ammonia loads (Paper I).

2) Investigate the metabolic interaction of ammonia tolerant microbes grown on individual carbon source (i.e., acetate, formate, methanol, H_2/CO_2) (Paper II).

3) Explore the mechanism of microbial tolerance to ammonia through genomics-centric analysis (Paper II).

4) Recover the methane yields from ammonia-inhibited reactors using bioaugmentation technology (Paper III).

5) Achieve the insights of microbial metabolic interaction triggered by the bioaugmentation with ammonia tolerant methanogen (Paper III).

6) Develop long-term microbial preservation methods to offer ready-to-use methanogenic cultures (Paper IV).

1.3.2 Structure of the thesis

In chapter 2, the stepwise acclimatization to extreme high ammonia levels is presented in mesophilic CSTR fed with organic fraction of municipal solid waste (OFMSW).

In chapter 3, the metabolic interactions among ammonia tolerant methanogenic culture fed with different single and simple carbon sources under thermophilic conditions are reconstructed using the genomics-centric analysis. The possible mechanism of microbial tolerance to ammonia is proposed.

In chapter 4, bioaugmentation with acclimatized ammonia tolerant inocula is performed to improve methane production from ammonia inhibition. Meanwhile, genomics-centric analysis shows the change in microbial composition induced by bioaugmentation.

In chapter 5, microbial preservation methods are assessed in order to provide the ready-to-use ammonia tolerant inocula. The methanogenic community composition after preservation is investigated.

In chapters 6 and 7, the main findings of the Ph.D. thesis are summarized, the future perspectives are presented.

2 Acclimatization as a method to achieve stable methane production of ammonia inhibited reactors

Acclimatization is a method where continuous exposure of microbial community to a specific environment improves its tolerance to this given environmental limitation. This bioremediation technology has been applied effectively in the AD field. For instance, microbial acclimatization successfully promoted the acidogenesis process (Saha et al. 2019), methane yield of AD from LCFAs stress (Kurade et al. 2020), and even resistance to metal toxicity (Bhakta 2016). Similar applications have been used to address ammonia inhibition which commonly occurs under AD of N-rich organic waste. The increased methane yields in batch reactors at TAN levels of 6.6 g NH_4^+-N/L (Tian et al. 2018b), and CSTR with TAN of 4.9 g NH_4^+-N /L (Dai et al. 2017) were observed. However, the ammonia threshold of acclimatization in CSTR is unclear, which may affect further technical application.

2.1 Acclimatization process

In the paper I, stepwise acclimatization to extreme ammonia levels (more than 7g NH_4^+-N /L) was performed in two mesophilic CSTRs (namely, R1 and R2) fed with OFMSW. The whole acclimatization process was divided into seven periods based on TAN levels (Table 1). Specifically, the average methane yield of P1 (no extra ammonia addition) was used as a baseline. Once anaerobic reactors reached a steady state with more than 85% of average methane yield of the P1, the TAN was increased (1-1.5 g NH_4^+-N /L each period). The mixture of urea and ammonium chloride was injected into feedstock and reactors simultaneously to achieve higher TAN levels.

Table 1. The experimental design of stepwise acclimatization in CSTR reactors. [Adapted from paper I]

Phase	Days	TAN	Extra added ammonia	
			$CO(NH_2)_2$	NH_4Cl
		(g NH_4^+-N/L)	(g NH_4^+-N/L)	(g NH_4^+-N/L)
P1	0-30	1.1	0	0
P2	31-51	4	1	1.9
P3	52-66	5	2	1.9
P4	67-74	6	2	2.9
P5	75-89	7	3	2.9
P6	90-127	8.5	4	3.4

P7	128-159	9.5	4.5	3.9

2.2 Reactor performance

The average methane yields, i.e., 345 ± 40 (R1) and 391 ± 59 (R2) mL CH_4/g VS during P1 were used as baseline references for evaluating inhibitory effect in the following phases (P2-P7) (Figure 4). Even though the two reactors experienced temporary inhibition (P2-P5) with an average reduction of 15% in methane yield compared to P1. Quick recovery with less than 6% loss was observed (Figure 3). When TAN of reactors was increased to 8.5 g NH_4^+-N/L (P7), the "inhibited steady-state" occurred with over 15% reduction in methane yields compared to P1. Meanwhile, the change in VFA from low levels (P3 to P5) to over 4000 mg /L (P6-P7) in both reactors were observed. Based on these results, 8.5 g NH_4^+-N/L was proposed as the threshold of acclimatization process in mesophilic anaerobic reactors of OFMSW. To be noticed, although the inhibitory effect of ammonia on the AD process was observed at P7, the methane yields were still higher than other acclimatization studies at the same ammonia levels (Capson-Tojo et al. 2020, Jiang et al. 2019).

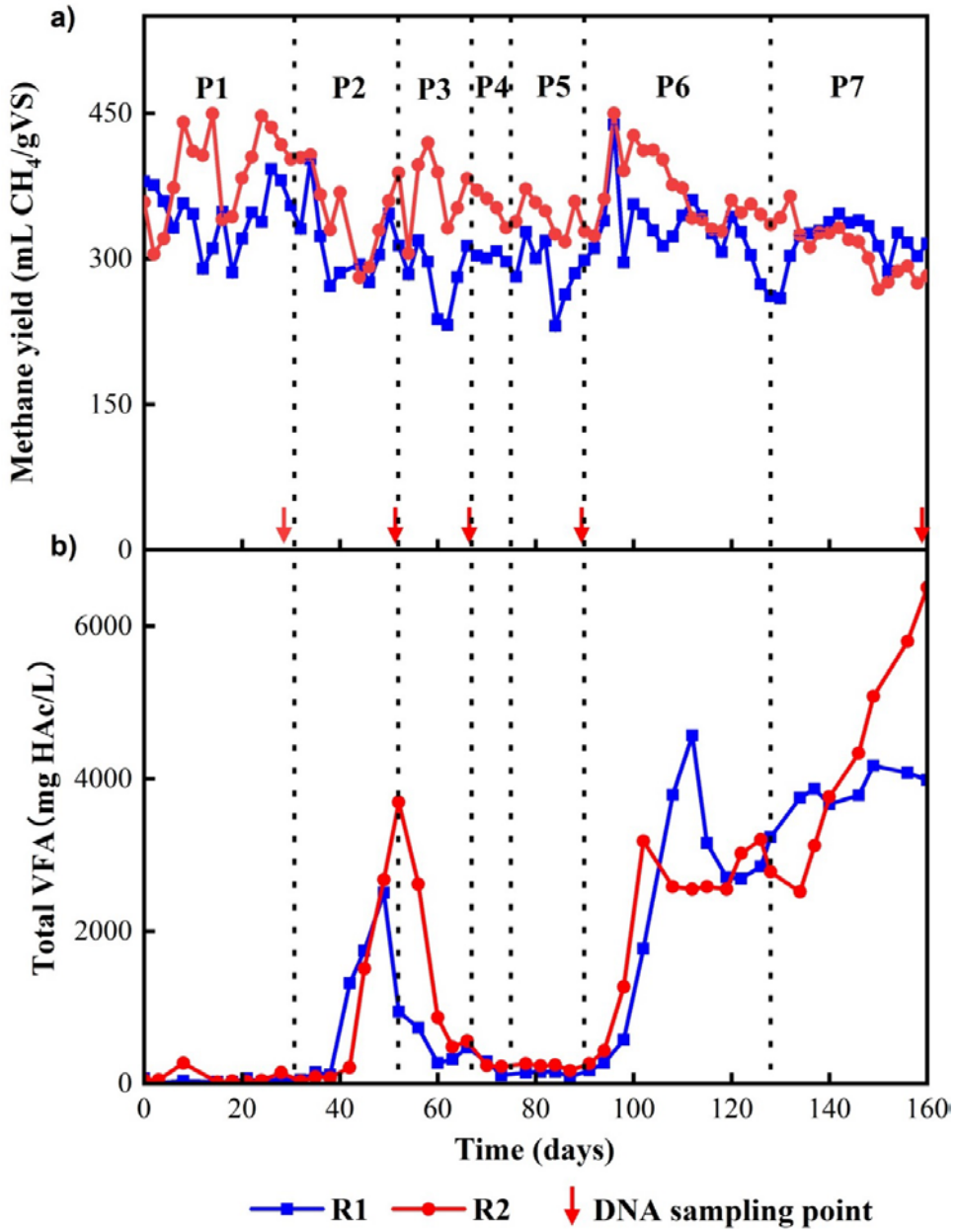

Figure 3. a) CH_4 production yields in R1 and R2, b) total VFA change from P1 to P7 [Adapted from paper I].

2.3 Microbial dynamicity

Ammonia effect on microbial shift was revealed using the 16S rRNA amplicon sequencing technology (Figure 4). Specifically, *Methanosaeta concilii* 2 was dominant methanogen with 50-70% of relative abundance of archaeal population during P1 to P2 (less than 5 g NH_4^+-N/L). As ammonia levels continuously increased, *Methanosarcina soligelidi* 1 gradually outcompeted *Methanosaeta concilii* 2 from P3 (40%) to P7 (90%). These findings agreed with other studies

that middle-high ammonia levels generally favored the growth of *Methanosarcina* spp. than *Methanosaeta* spp. in AD (Jiang et al. 2019, Tian et al. 2019c).

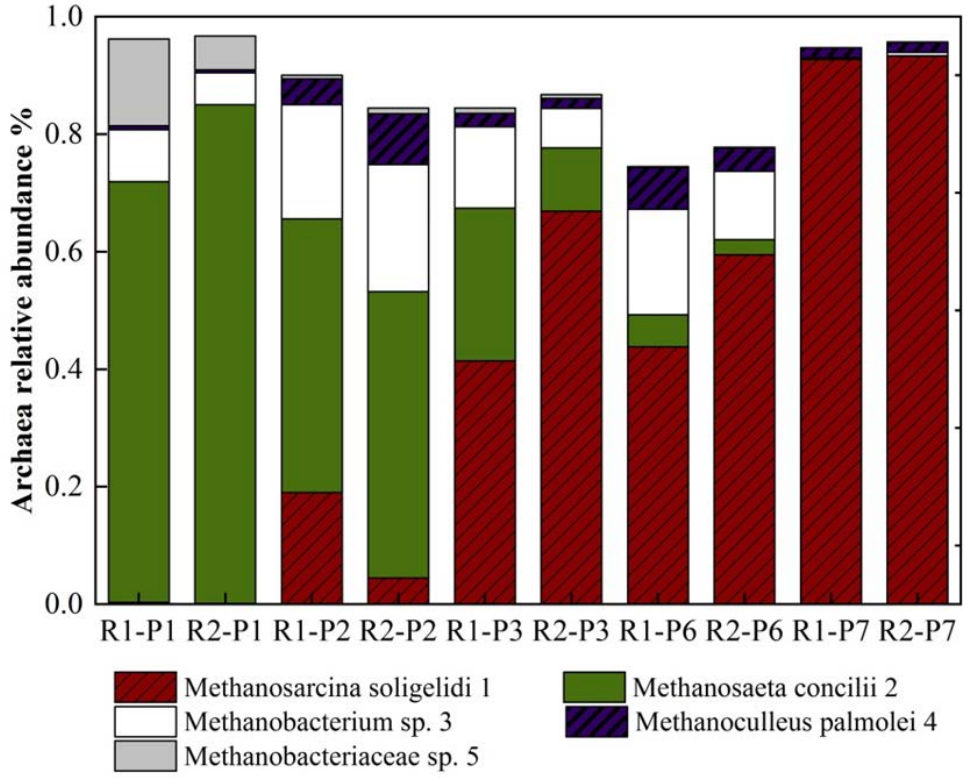

Figure 4. Archaeal relative abundance (%) in R1 and R2 at different periods. [Adapted from paper I].

In summary, successful acclimatization up to 8.5g NH_4^+-N/L was achieved to mitigate ammonia stress during AD of the OFMSW. Microbial analysis revealed that *Methanosarcina soligelidi* owned higher robustness to ammonia, which contributed to stable methane production at middle-high ammonia levels.

3 Insights into microbial tolerance to ammonia

Methanosarcina is generally more resilient to ammonia stress than *Methanosaeta* (Chen et al. 2018) and its capability to recover methane yields has been highly recognized at middle-high ammonia levels (Capson-Tojo et al. 2020, Zabranska and Pokorna 2018). Furthermore, hydrogenotrophic methanogens (e.g., *Methanothermobacter*, *Methanoculleus*, and *Methanobacterium*) can outcompete *Methanosarcina* for methanogenesis under extremely high ammonia levels in anaerobic digestors (Tian et al. 2019a, Westerholm et al. 2016, Jing et al. 2017). It seems that the distinct metabolic characteristics are linked to microbial tolerance to ammonia. Moreover, these methanogens mainly perform methanogenesis from acetate and/or H_2/CO_2, the knowledge about ammonia tolerance of methanogens grown on other methanogenic precursors (i.e., methanol and formate) is still absent. Understanding the metabolic characteristics and syntrophic interaction of ammonia tolerant microorganisms can help us to decipher the mechanisms of ammonia tolerance. In the last decades, microbial interaction and functionality were little known due to the limitation in microbial cultivation/isolation and biomolecular technology. Currently, the continuous development in metagenomics technology makes the cultivation-independent assessment feasible in terms of the most unexploited genetic reservoir of anaerobic microbial communities (Qin et al. 2010). It opens the door to the identification of unknown functional genes/pathways, and metabolic interaction. By now, most anaerobic microbial communities revealed by metagenomics were shaped by real organic waste (e.g., OFMSW, and industrial wastewater) containing complicated carbon sources (Forbes et al. 2017, Campanaro et al. 2019). A huge amount of Metagenome-Assembled Genomes (MAGs) achieved pose a challenge for the genomic metabolic analysis. Thus the ammonia tolerant microbiome grown on single carbon source (the common methanogenesis precursors: acetate, methanol, formate, and H_2-CO_2) would provide more clear insights of obligate mutualistic interactions and other functionalities.

3.1 Microbial interaction under four solely carbon sources

Four thermophilic batch reactors were inoculated with the same seed inocula that was collected from a thermophilic CSTR of cattle manure. The feedstock used in this experiment consisted of basic anaerobic medium, ammonia chloride

and single carbon source (methanol, acetate, formate, H_2/CO_2). During the acclimatization process, 5% (V/V) of fresh microbial samples were harvested to the next increased ammonia level (1g NH_4^+-N/L each step) when methane yields reached 80% of its maximal potential. The cultivation process was repeated under increased ammonia levels until the methanogenic consortia could not grow anymore (Table 2). The microbial DNA was taken from five sampling points: $G_{inocula}$, an initial inoculum (2.25 g NH_4^+-N/L); $G_{methanol}$, microbial community fed by methanol (7.25 g NH_4^+-N/L); $G_{acetate}$, microbial community fed by acetate (4.25 g NH_4^+-N/L); $G_{formate}$, microbial community fed by formate (5.25 g NH_4^+-N/L); $G_{H2/CO2}$, microbial community fed by H_2/CO_2 (7.25 g NH_4^+-N/L).

Table 2. Characteristics of the reactors. [Adapted from Paper II]

	Acetate	Formate	H_2/CO_2	Methanol	Organic loading range g.COD/L
Original ammonia level (N-NH_4^+g/L)	2.25±0.15				
pH	7.9±0.1	8.1±0.01	8.0±0.02	7.9 ±0.02	
Acclimatization process (N-NH_4^+g/L)	2.25	2.25	2.25	2.25	1
	3.25	3.25	3.25	3.25	1
	4.25	4.25	4.25	4.25	1
		5.25	5.25	5.25	1-2.5
			6.25	6.25	1-2.5
			7.25	7.25	1.7-2

* pH was maintained by NaOH solution (4 mol/L)

The microbial composition and their key functionalities in each group were unveiled using a genomics-centric approach. After the binning process, 81 MAGs were achieved. 52 out of 81 MAGs were high quality (contamination less than 5%; completeness over 90%), and the other 29 MAGs were medium quality (contamination ranging from 5% to 10%; completeness in the range of

50%-90%). The results showed that individual carbon source and ammonia shaped the common initial inocula into distinct four microbiomes (Figure 5).

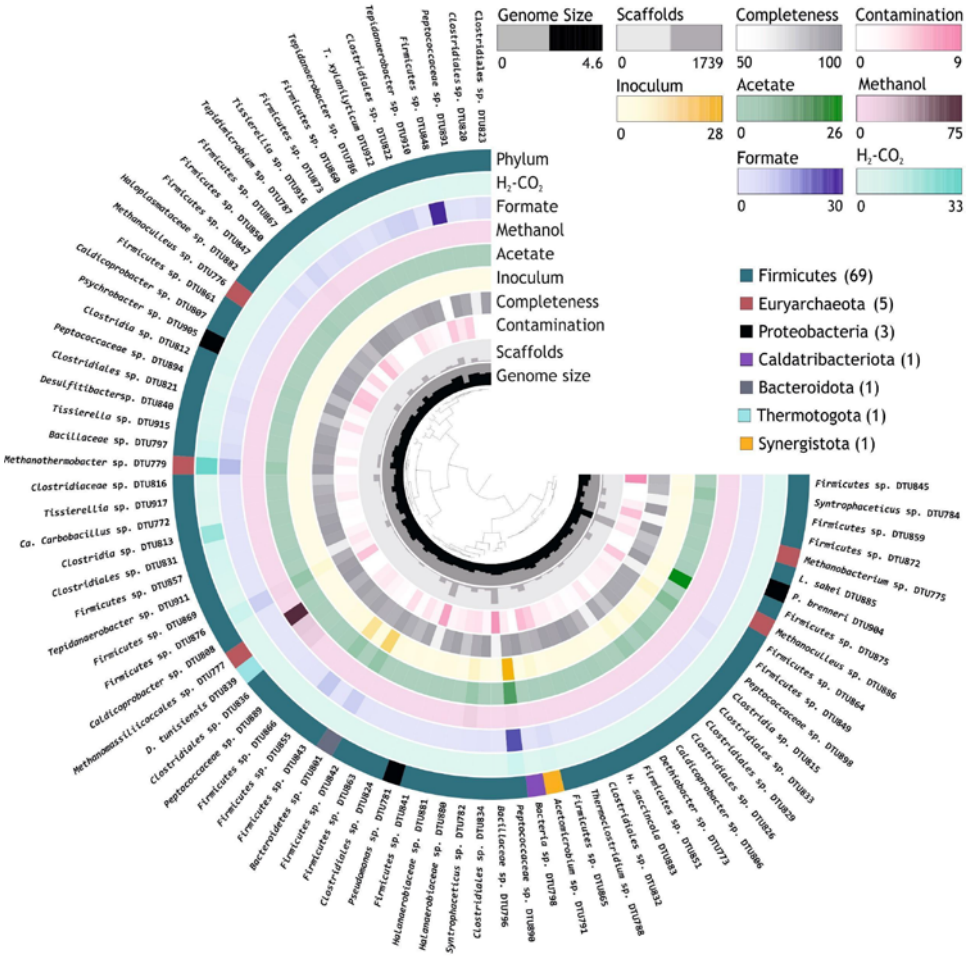

Figure 5. The characteristic (coverage, quality, and taxonomic assignment) of microbiome in $G_{inoculum}$, $G_{acetate}$, $G_{methanol}$, $G_{formate}$, and $G_{H2/CO2}$. The taxonomy at the phylum level, the relative abundance of each MAG in the different group (%), and a Pearson clustering of MAGs was listed in the outer, five middle, and inner layers. The genome size (Mbp), number of scaffolds, completeness (%), and contamination (%) are coloured in black, grey, green, and red, respectively. [Adapted from Paper II]

3.1.1 Microbiome in the $G_{inoculum}$

In $G_{inocula}$, the dominant bacterial MAGs consisted of *Peptococcaceae* sp. DTU890, *Bacteroidetes* sp. DTU801, *Firmicutes* sp. DTU855 and *Firmicutes* sp. DTU849 (Figure 5). They accounted for 58.95% of relative abundance and were assigned to sugar degraders (Table S5). The relative abundance of archaeal group was only 1.28% of the total microbial population, among them, *Methanoculleus* sp. DTU886, *Methanothermobacter* sp. DTU779, and *Methanomassiliicoccales* sp. DTU777 were 1.2%, 0.05% and 0.03% of relative

abundance, respectively. No acetoclastic methanogens were found, the possible explanation was that the TAN of 2.25 g NH_4^+-N/L in $G_{inocula}$ at 55°C suppressed their growth.

3.1.2 Ammonia tolerant microbiome in the $G_{acetate}$

$G_{acetate}$ at 4.25 g NH_4^+-N/L was mainly composed of *Methanoculleus* sp. DTU886 (26% of relative abundance), *Firmicutes* sp. DTU849 (7%) and *Peptococcaceae* sp. DTU890 (16%) (Figure 6). The syntrophic interactions in terms of the conversion of acetate to methane among these dominant MAGs were reconstructed. Specifically, the conversion of acetate to H_2/CO_2 (the precursor for methanogenesis) took place in *Firmicutes* sp. DTU849 through Wood-Ljungdahl (WL) and *Peptococcaceae* sp. DTU890 through coupling partial glycine cleavage system with the WL pathway, respectively (Zhu et al. 2019). The H_2/CO_2 was further converted to methane by *Methanoculleus* sp. DTU886, evidenced by the methane yields measurement and presence of genes (e.g. *fwd*, *mtr* and *mcr*) (Figure 6).

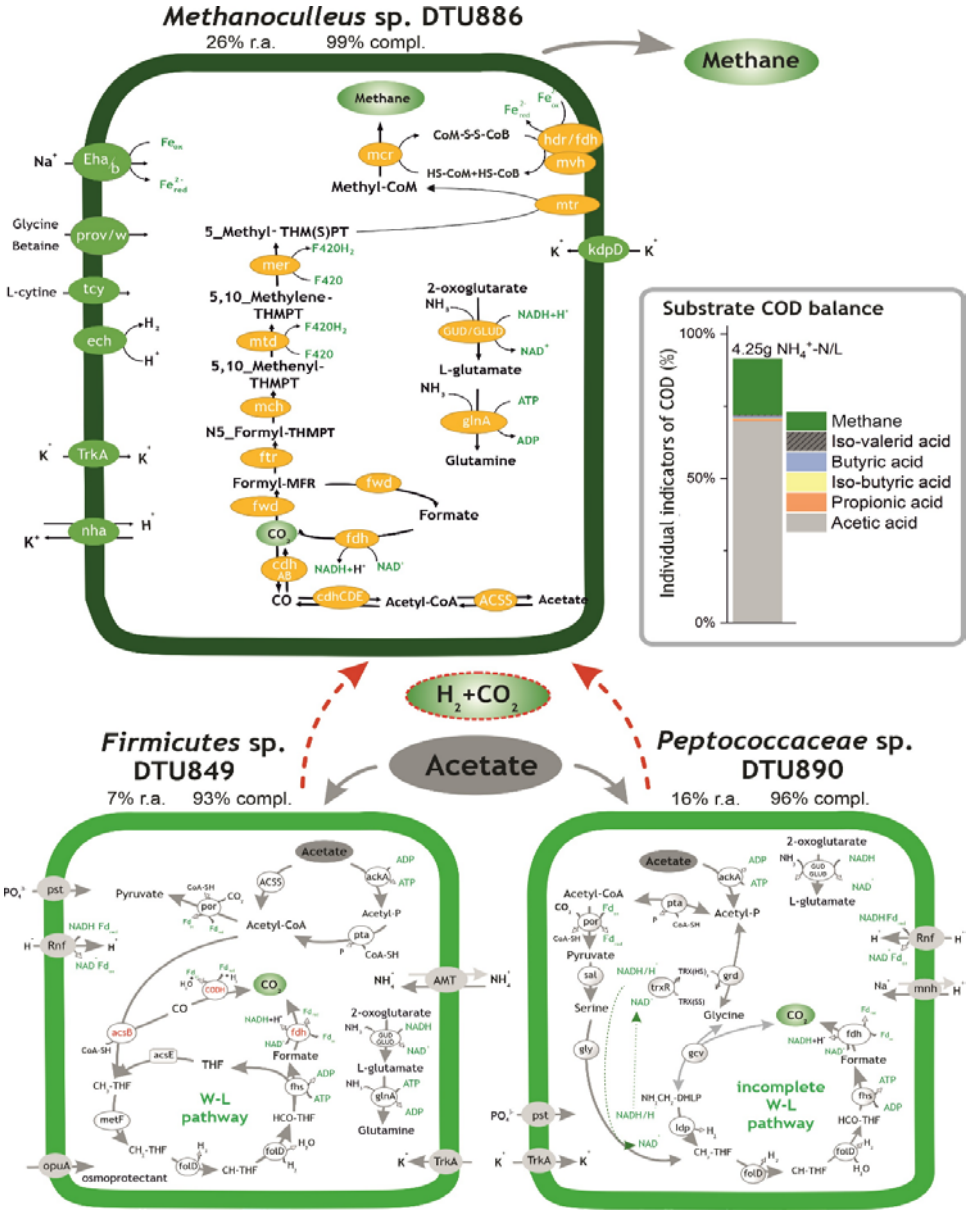

Figure 6. The figure on the right side represents the fate of acetate based on COD analysis in $G_{acetate}$. "R.a." and "compl." means "relative abundance" and "completeness", respectively. [Adapted from Paper II]

3.1.3 Ammonia tolerant microbiome in the $G_{methanol}$

$G_{methanol}$ at 7.25 g NH_4^+-N/L was dominated by *Methanomassiliicoccales* sp. DTU777, *Syntrophaceticus* sp. DTU782 and *Clostridiales* sp. DTU836 with 75%, 3%, and 5% of relative abundance, respectively (Figure 7). The complete methanogenesis pathway from methanol to methane in *Methanomassiliicoccales* sp. DTU777 suggested its methanogenic independence from other bacterial community. The co-occurrence of *acsE*, *acss*, and *metH* etc., in *Syntrophaceticus* sp. DTU782 and *Clostridiales* sp. DTU836 could be good indicators of

methanol degradation through two pathways. Briefly, the methylic group (in methanol) was firstly translocated to corrinoid Fe-S protein (CFeSP). Afterwards, CH_3-CFeSP was directly converted into acetate through acetate kinase pathway or was oxidized to acetate through partial WL pathway (Kremp et al. 2018) (Figure 7).

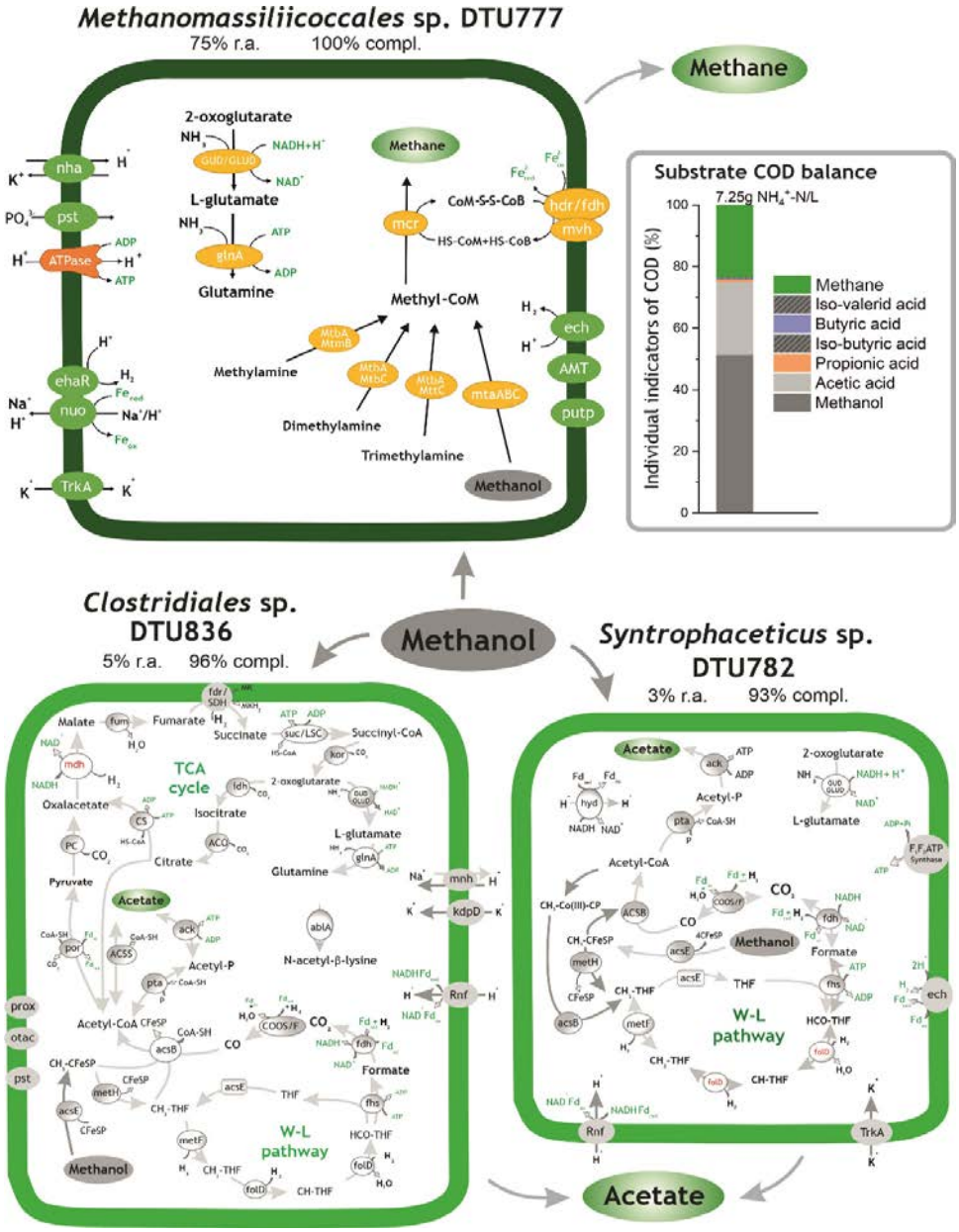

Figure 7. The figure on the right side represents the fate of methanl based on COD analysis in $G_{mehanol}$. "R.a." and "compl." means "relative abundance" and "completeness", respectively. [Adapted from Paper II].

3.1.4 Ammonia tolerant microbiome in the $G_{formate}$

The microbiome of $G_{formate}$ at 5.25 g NH_4^+-N/L was represented by *Firmicutes* sp. DTU848 (30%) and *Peptococcaceae* sp. DTU890 (22%), and *Methanothermobacter* sp. DTU779 (7%) (Figure 8). Based on the gene presence (*fdh*, *ftr*, *mch*, etc.) and methane yields, it was confirmed that *Methanothermobacter* sp. DTU779 performed hydrogenotrophic methanogenesis. Besides, incomplete reverse WL pathway and novel propionate generation pathway were detected in *Firmicutes* sp. DTU848 which explained the presence of acetate and propionate in $G_{formate}$. As mentioned before, *Peptococcaceae* sp. DTU890 possibly performed acetate oxidation using partial WL pathway coupled with the glycine cleavage system. Besides, *Peptococcaceae* sp. DTU890 and *Firmicutes* sp. DTU848 encoded formate dehydrogenase (*fdh*) and sodium ion pump (Rnf) that could be used as CO_2/H_2 sink for *Methanothermobacter* sp. DTU779 (Lins et al. 2012).

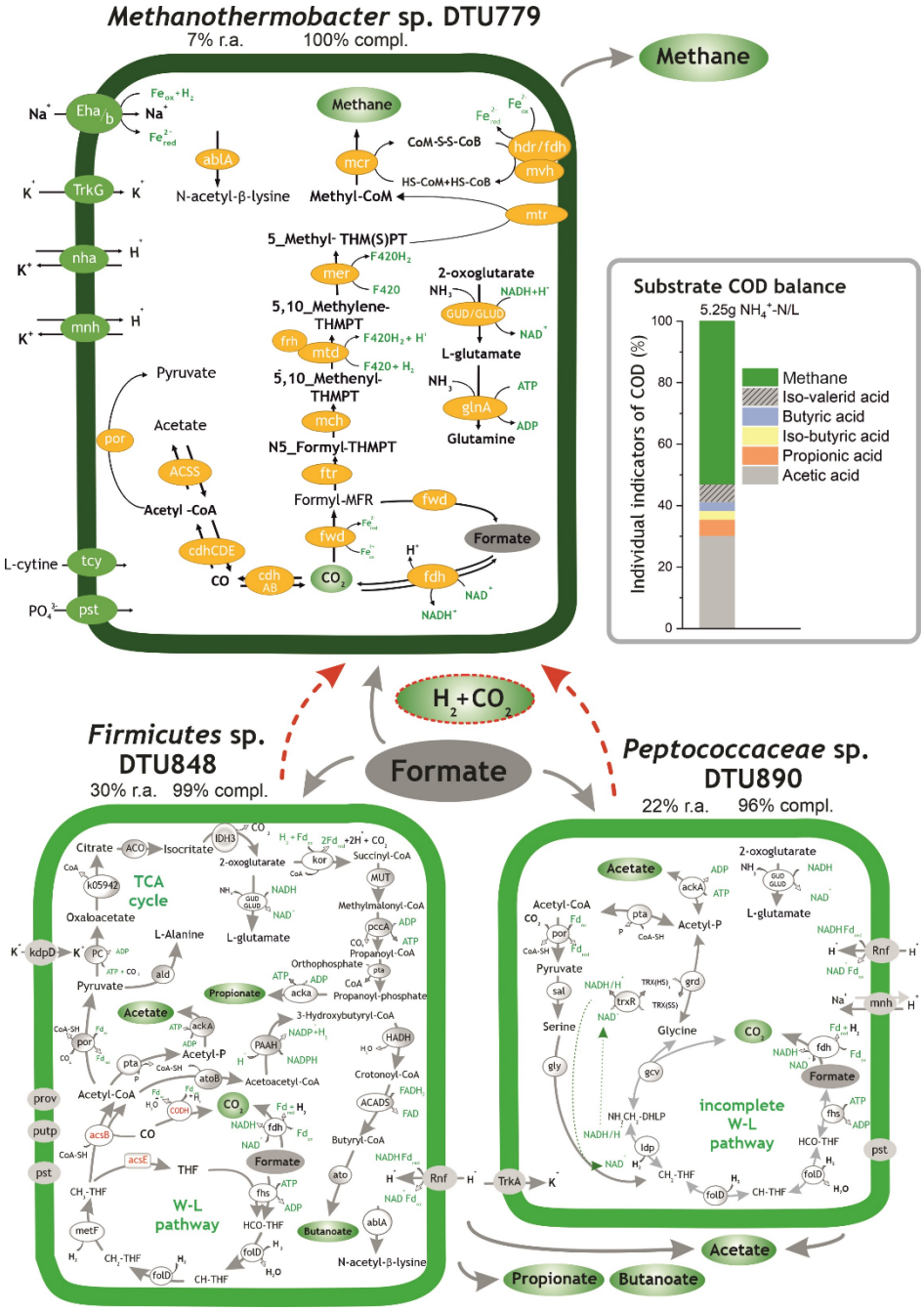

Figure 8. The figure on the right side represents the fate of formate based on COD analysis in $G_{formate}$. "R.a." and "compl." means "relative abundance" and "completeness", respectively. [Adapted from Paper II].

3.1.5 Ammonia tolerant microbiome in the $G_{H2/CO2}$

In the $G_{H2/CO2}$ at 7.25 g NH_4^+-N/L, *Methanothermobacter* sp. DTU779 showed higher ammonia tolerance to 7.25 g NH_4^+-N/L and accounted for an even higher relative abundance of 33% (5 times higher than in $G_{formate}$) (Figure 9). This finding confirmed *Methanothermobacter* sp. DTU779 preferred H_2 over formate as electron donor for methanogenesis (Lins et al. 2012). Additionally, the

bacterial community was dominated by *Pelotomaculum* sp. DTU813 (20% of relative abundance) and *Peptococcaceae* sp. DTU890 (6% of relative abundance). According to metabolic reconstruction, the parasitic relationship might develop between *Methanothermobacter* sp. DTU779 and *Pelotomaculum* sp. DTU813 where *Methanothermobacter* sp. DTU779 provided pyruvate as the substrate for *Pelotomaculum* sp. DTU813. This hypothesis was in agreement with other literature where the conversion of pyruvate to acetate and propionate occurred in *Pelotomaculum thermopropionicum* (Imachi et al. 2002). Since *Peptococcaceae* sp. DTU890 had versatile metabolic capabilities, it could produce or consume CO_2/H_2 and acetate depending upon metabolites' concentrations in the reactors. Thus the role of *Peptococcaceae* sp. DTU890 needed further investigation.

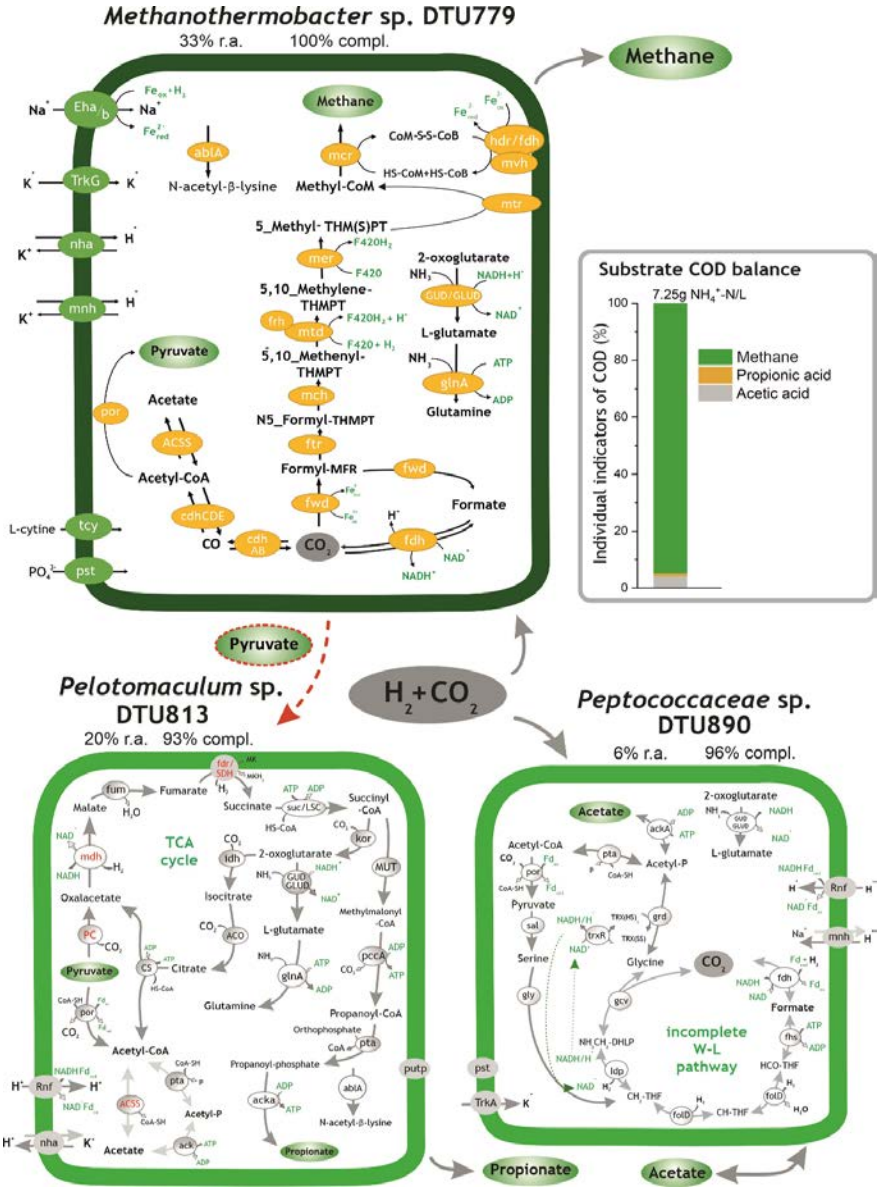

Figure 9. The figure on the right side represents the fate of H_2/CO_2 based on COD analysis in $G_{H2/CO2}$. "R.a." and "compl." means "relative abundance" and "completeness", respectively. [Adapted from Paper II].

3.2 Mechanism of microbial tolerance to ammonia

The single carbon source and stepwise increased ammonia concentrations drove the original inocula into four highly divergent communities with varied ammonia tolerance. The genes related to osmotic regulators, such as the K^+ uptake system (TrKA), sodium/proton antiporter (*nha*) system, osmoprotectant (glutamate, glycine betaine, and N^{ε}-acetyl-L-lysine, etc.), and energy converting complexes (e.g. Eha/b and Ech), might contribute microbes to counteract ammonia stress (Figure 10) (Kadam and Boone 1996, Sudmalis et al. 2018, Kraegeloh et al. 2005, Müller et al. 2005). Furthermore, the presence of distinct bacteria was linked to their role in supporting methanogen adaptation to ammonia through metabolic complementarity (Capson-Tojo et al. 2020, Zhu et al. 2020). This study expended the knowledge about the intricate syntrophic-supported food web among ammonia tolerant microbiomes grown on acetate, formate, H_2-CO_2, and methanol.

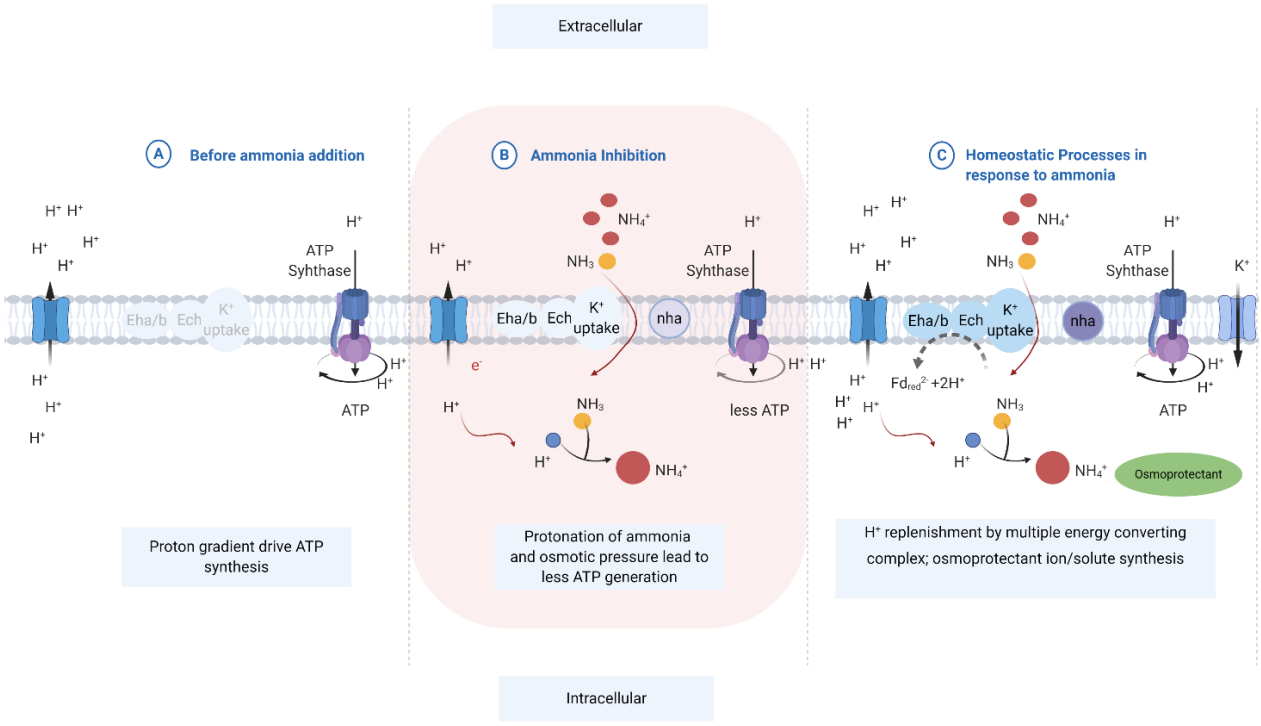

Figure 10. The response of methanogen to ammonia in different situations was proposed: a) before ammonia inhibition. b) under ammonia inhibition. c) homeostatic regulation to counteract ammonia stress. [Adapted from Paper II].

4 Bioaugmentation as an effective strategy to recover AD from ammonia inhibition

Bioaugmentation is the introduction of specialized microbial consortia into an under-performing system to speed up the degradation rate of organic matters. Bioaugmentation has been applied to increase methane yields from the N-rich substrate, reduce the recovery period of the AD process (Fotidis et al. 2014, Schauer-Gimenez et al. 2010), and decrease the total solid (TS) content (Tambone et al. 2010). Besides, discharged digestate containing bioaugmentation inocula can be used as a soil amendment or fertilizer, which is exactly following the circular economy (Favaro et al. 2019, Tambone et al. 2010). Conversely, the other technical attempts like magnetite and zeolite addition need extra separation steps before discharge, which poses technical challenges. Many researchers have proved that the bioaugmentation with ammonia tolerant methanogen promoted the conversion of organic matter to methane at middle-high ammonia levels (3-6 g NH_4^+-N /L) (Tian et al. 2019b, Yang et al. 2019, Town and Dumonceaux 2016, Li et al. 2017). Even though these findings provide good guidance on AD operation, the biological mechanisms behind bioaugmentation performance remains absent. It raises the doubt that why the bioaugmentation inocula own remarkble tolerance to ammonia and how they develop specific syntrophic networks, which awaits further discovery.

4.1 Bioaugmentation with pure *M. bourgensis* MS2 on two mesophilic CSTR reactors

To recover methane production from ammonia inhibition, bioaugmentation was applied in two identical mesophilic CSTRs (namely, R_{con} and R_{bio}) at an organic loading rate of 3.4 g VS/L/day. The whole process was divided into five periods, during which urea ($CO(NH_2)_2$) and ammonium chloride (NH_4Cl) were mixed into OFMSW as simulated N-rich feedstock. Ammonia levels in the CSTRs were increased stepwise from 9.5 g NH_4^+-N /L (P1) to 13.5 g NH_4^+-N /L (P3). During P4, ammonia acclimatized *M. bourgensis* MS2 strain with volatile suspended solids (VSS) of 67 mg/L was injected into the R_{bio} and the same volume of cultivation medium into the R_{con}. The experimental design was presented in Table 3.

Table 3. Details of the experimental process. [Adapted from Paper III]

Phase	Days	TAN	Extra added ammonia	
		(g NH_4^+-N/L)	$CO(NH_2)_2$ (g NH_4^+-N/L	NH_4Cl (g NH_4^+-N/L)
P1	0-5	9.5	4.5	3.9
P2	6-22	11.5	5.5	4.9
P3	23-50	13.5	6.5	5.9
P4 (Bioaugmentation)	51-54	13.5	-	-
P5	55-120	13.5	6.5	5.9

* "-": No extra ammonia addition

4.1.1 Reactor performance

Compared to the period without extra ammonia addition (described in section 2.1), the 25% reduction of methane yields in two reactors (P1 to P3) indicated severe inhibition occurring. While after bioaugmentation, a 21% increase in methane production yields (P5) was achieved in comparison to P3 (Figure 11). Correspondingly, VFA levels in R_{bio} reduced from 15,000 to 5,398 mg/L, and the VFA levels in R_{con} had been exceeding 7,500 mg/L revealing a severe ammonia inhibition occurring (Figure 11). The results were consistent with the previous research that bioaugmentation could alleviate the inhibitory effect of ammonia on AD performance (Capson-Tojo et al. 2020, Fotidis et al. 2017)

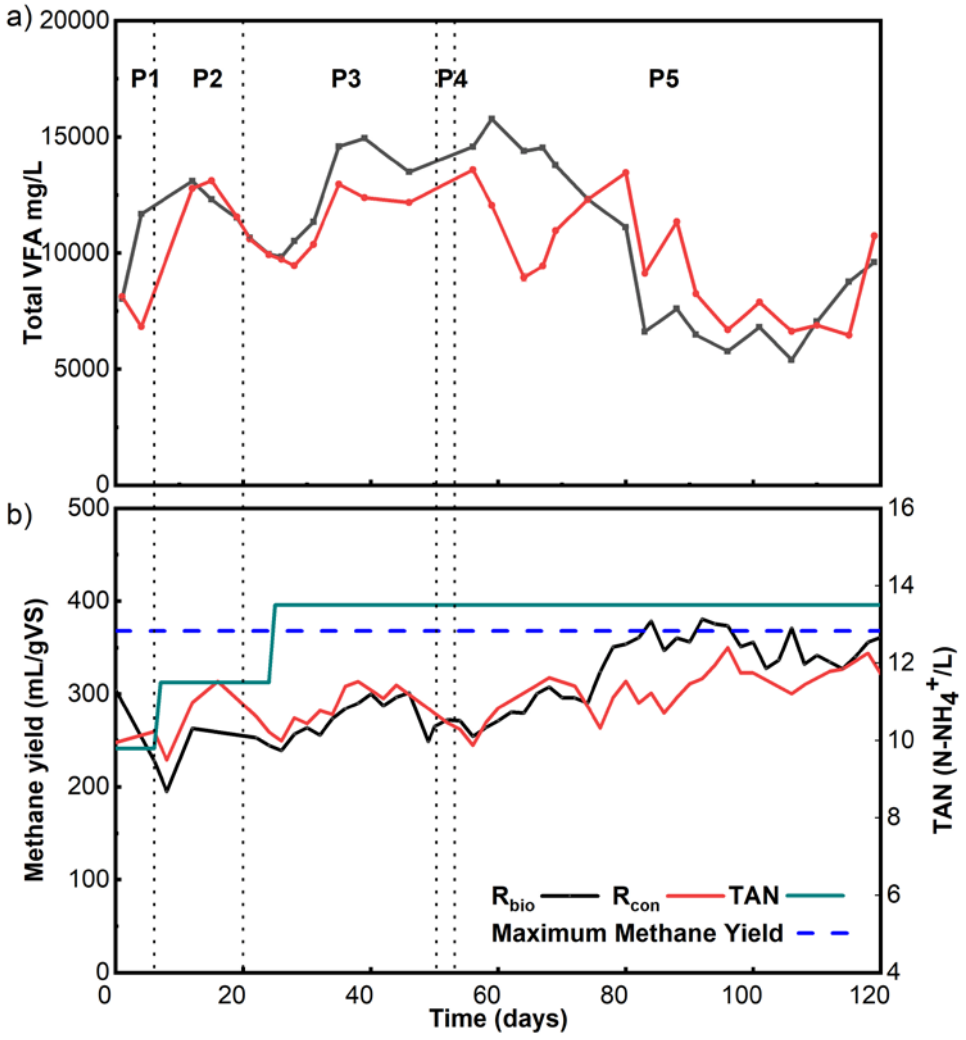

Figure 11. a) the VFA and b) methane production change through the experimental process.

4.1.2 Representative microbial metabolic interaction

The bioaugmentation with *M. bourgensis* triggered a 4.7, 2.4, and 1.5-fold increase in the relative abundance of *Peptococcaceae* spp. (DTU895, DTU900, and DTU 903), *Syntrophaceticus* sp. DTU 783 and *Tissierellales* sp. DTU879, respectively, between P3 to P5 (Figure 12).

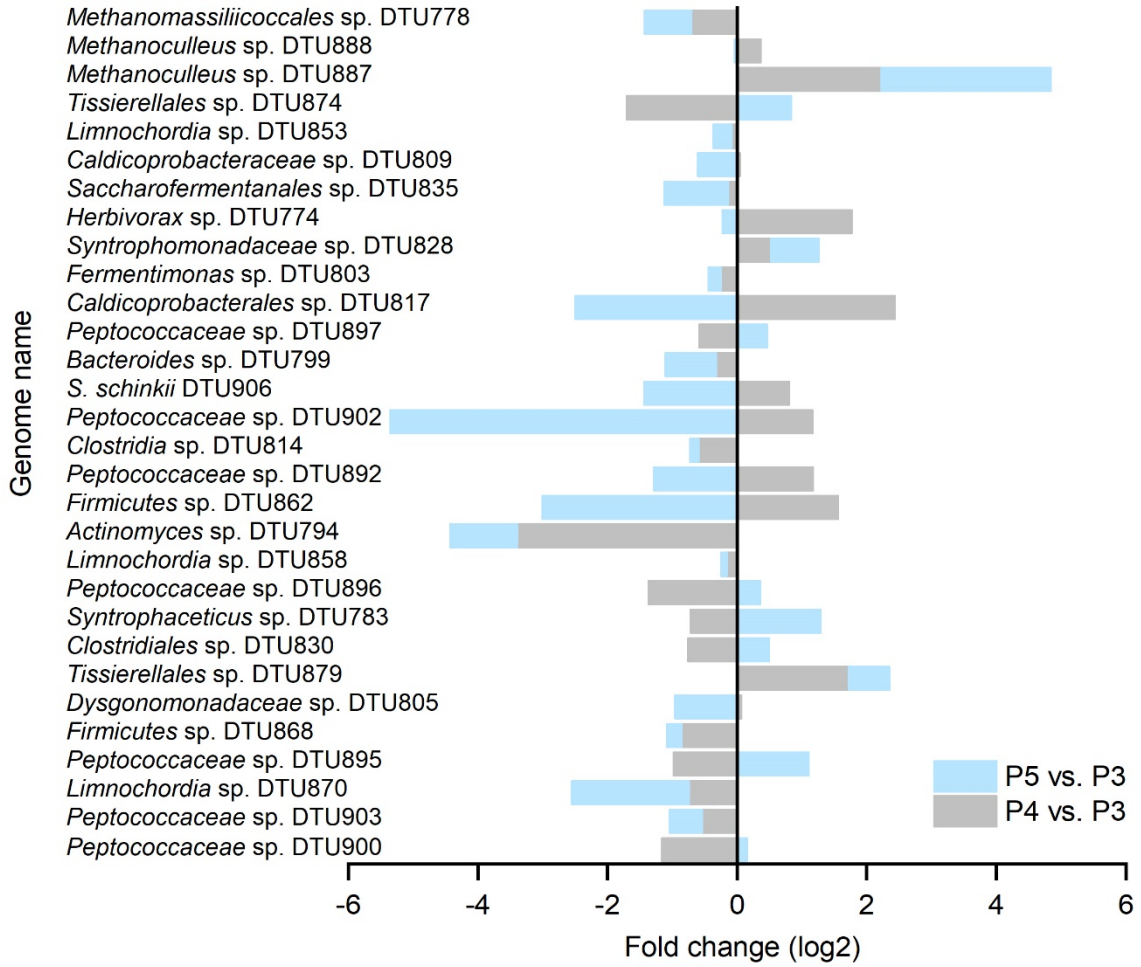

Figure 12. The shift of dominant MAGs was based on the fold change (log2) between the different periods. [Adapted from Paper III]

Correspondingly, the metabolic interactions among these dominant MAGS were reconstructed using metagenomics analysis (Figure 13). Two glucose catabolic pathways were detected in *Peptococcaceae* spp., and *Tissierellales* sp. DTU879, which might explain their dominant role in degrading carbohydrates-rich OFMSW. Specifically, pyruvate as intermediate, was derived from Embden-Meyerhof-Parnas (EMP) process. It was further converted to H_2/CO_2 through the first pathway coupling glycine cleavage system with tetrahydrofolate pathway. During the second pathway, the pyruvate was directly converted to acetate and H_2/CO_2, as evidenced by the presence of functional genes i.e., *kor*, *pta*, and *ackA* (Zhu et al. 2020).

Furthermore, the addition of *M. bourgensis* stimulated the 1.5-fold increase of *Syntrophaceticus* sp. DTU783 (Figures 12 and 13). Two acetate degradation pathways (i.e. WL pathway and glycine cleavage system coupling with the tetrahydrofolate pathway) were reconstructed in *Syntrophaceticus* sp. DTU783.

These multiple acetate degradation pathways possibly contributed its flexibility to varying environmental conditions or the absence of a syntrophic partner. The negative correlation between *Syntrophaceticus* sp. DTU783 and acetate suggested *Syntrophaceticus* sp. DTU783 played an essential role in scavenging acetate at elevated ammonia levels. However, the bioaugmentation with SAOB alone hardly affected the AD performance under ammonia inhibition (Yang et al. 2019, Westerholm et al. 2012).

Methanoculleus sp. DTU887 (the bioaugmentation inocula) contained the genes i.e., *putp*, *proW/V*, *kdp* and *mnhBCDE*, that were responsible for cells osmoregulation (Engelhardt 2007, Meury and Kohiyama 1992) (Figure 13). Besides, the multiple energy regulating complexes (Mtr, Eha, Ech and V/A type ATP synthase) could be the winning strategy to optimize its energy metabolism under severe environment (Vanwonterghem et al. 2016, Sapra et al. 2003). These genetic characteristics enabled its superior tolerance to ammonia stress and capability to perform stable methanogenesis process in ammonia-inhibited reactors. The establishment of *Methanoculleus* sp. DTU887 consumed the H_2 promptly, which stimulated the growth of *Syntrophaceticus* sp. DTU783, thus enhanced the acetate oxidizing rate. Like a domino effect, the complete chain of electron transfer between bacteria and methanogen was resumed. Eventually, the complete syntrophic substrate degradation was accelerated with a 21% increase in methane yield (Figure 11).

In summary, this study confirmed the feasibility of bioaugmentation as an efficient strategy to counteract high ammonia stress up to 13.5 g NH_4^+-N /L. Genome-centric analysis revealed that bioaugmentation inocula stimulated the growth of glucose degraders and acetate oxidizers in CSTR systems. The presence of parallel degradation pathways of both glucose and acetate in these increased bacteria might be their survival strategy under stressed conditions. Meanwhile, the exceptional ammonia tolerance of *Methanoculleus* sp. DTU887 might owe to its various complexes that were involved in energy conversion and osmoregulation.

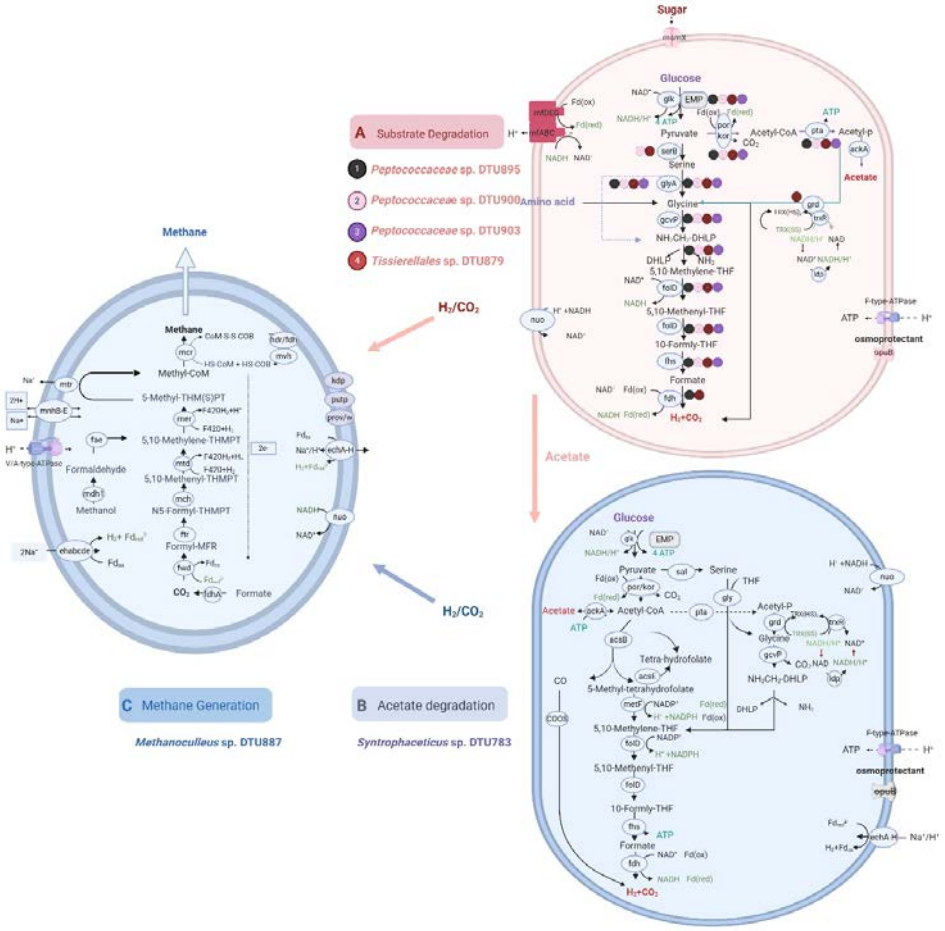

Figure 13. Metabolic reconstruction of the OFMSW degrading syntrophic community. Metagenomic data revealed pathways involved in substrate degradation, in the utilization of intermediates compounds (H_2/CO_2, acetate), and methanogenesis. [Adapted from Paper III]

5 Long-term preservation and fast recovery of ammonia tolerant methanogenic culture

Bioaugmentation effect to increase methane production in the AD system has been recognized (Fotidis et al. 2014, Tian et al. 2019d). However, methanogens are slow-growing, which means the period of cultivation is long (Imachi et al. 2002, Rincón et al. 2010, Cheng et al. 2008, Xue et al. 2006). It poses a technical challenge to perform timely bioaugmentation. The delay in recovering full-scale anaerobic digester from ammonia stress results in economic loss. Therefore, bioaugmentation with ready-to-use inocula is required for stable AD operation of N-rich organic waste. Until now, ready-to-use inocula have been rarely investigated to speed up bioaugmentation process (Massalha et al. 2015, Yarberry et al. 2019). Thus, it is crucial to develop a fast and easy method where ammonia tolerant consortia could be preserved. Several microbial preservation strategies have been explored, such as freeze-drying (Yarberry et al. 2019), heat-drying (Bhattad et al. 2017), liquid nitrogen storage (Rothrock et al. 2011), living cells entrapped in a gel or liquid medium (Banu et al. 2018, Iacobellis and DeVay 1986). In practice, there are many challenges in preserving anaerobic microorganism, due to their vulnerability to oxygen, operation cost, and transportation cost. However, agar gel and liquid medium seem to show tremendous potential as preservation carrier and their effectiveness for maintaining the metabolic activity of ammonia tolerant inoculum needs to be investigated.

5.1 Evaluation of microbial preservation method

In paper IV, two carriers (i.e. agar gel and liquid BA media) and storage temperatures of 4°C and 24°C were used, and ammonia tolerant enrichments were preserved with different time frames (i.e., 1, 7, 14, 28, 84, and 168 days). Specifically, the inocula collected from the previous research (Tian et al. 2019c) were cultivated with BA medium containing 4 g/L acetate and 9 g NH_4^+-N/L. The microbial enrichments were harvested for the preservation step when over 80% of the theoretical methane potential of acetate (373.33 mL CH_4/g VS) was achieved. Four different preservation strategies were performed as follows: 1) agar gel at 24°C ($PG_{(24°C)}$), 2) liquid BA medium at 24°C ($PL_{(24°C)}$), 3) agar gel at 4°C ($PG_{(4°C)}$), and 4) liquid BA medium at 4°C ($PL_{(4°C)}$). After 1, 7, 14, 28, 84, and 168 days of preservation, their efficiencies were evaluated based on

the methane production yields and lag phases. The detail of the whole process was presented in Table 4.

Table 4. Experimental setup. [Adapted from paper IV]

Strategy	Preservation medium	Preservation temperature °C	Reactivation
$PSG_{(24°C)}$	5 mL Inoculum + 2 mL Agar solution	24	33 mL BA medium+ 4g HAc/L+ 9.00 g NH_4^+-N/L
$PSG_{(4°C)}$	5 mL Inoculum + 2 mL Agar solution	4	33 mL BA medium+ 4g HAc/L+ 9.00 g NH_4^+-N/L
$PSL_{(24°C)}$	5 mL Inoculum	24	35 mL BA medium+ 3.77g HAc/L + 9.0 g NH_4^+-N/L
$PSL_{(4°C)}$	5 mL Inoculum	4	35 mL BA medium+ 3.77 g HAc/L+ 9.00 g NH_4^+-N/L

5.2 Methanogenic activity test after preservation

Among the four strategies, $PG_{(24°C)}$ showed advantages in maintaining microbial methanogenic activity for up to 168 days, and the lag-phase was less than 25 days during the revival test (Figure 14). The maximum time that microbes required to retain microbial metabolism in $PL_{(24°C)}$ were 84 days with a lag-phase of 41 days. Furthermore, when the preservation frame was within 14 days, $PL_{(24°C)}$ showed a shorter lag phase (less than 18 days) compared to $PG_{(24°C)}$. Conversely, both $PG_{(4°C)}$ and $PL_{(4°C)}$ were slow recovery or inactive after seven days of preservation, suggesting storage under 4°C was inefficient.

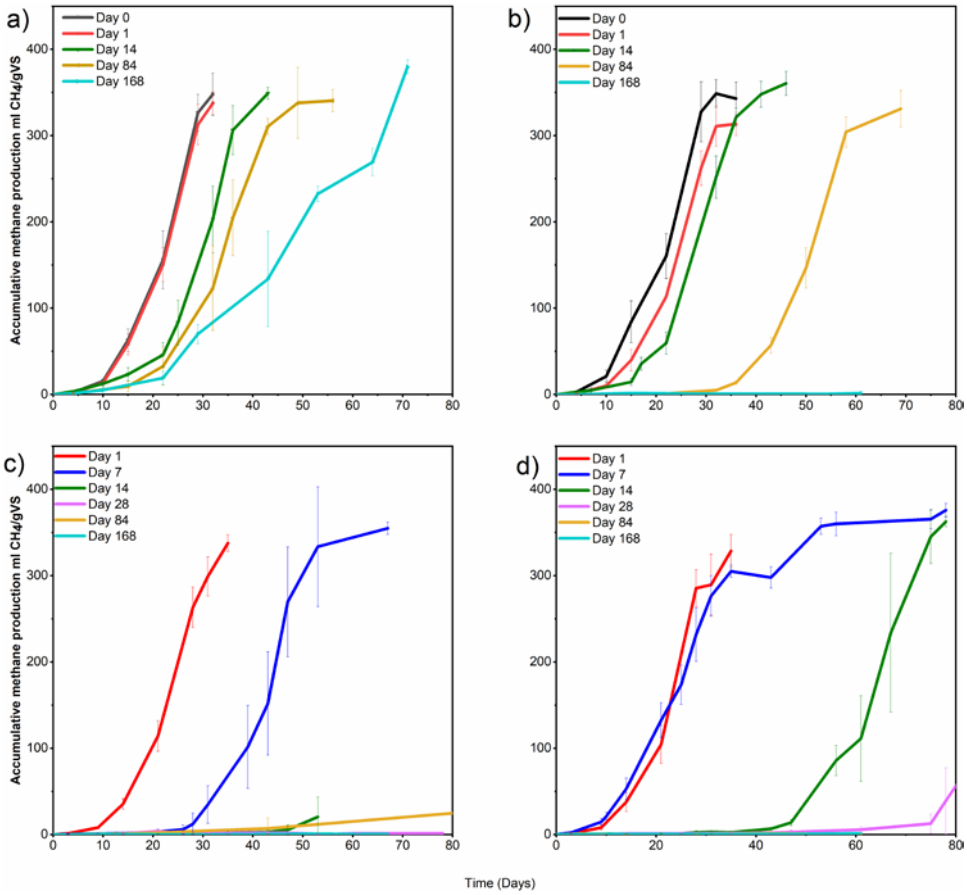

Figure 14. The accumulative methane production yield of the preserved ammonia-tolerant methanogenic enrichments during reactivation process: a) $PG_{(24°C)}$; b) $PL_{(24°C)}$; c) $PG_{(4°C)}$; d) $PL_{(4°C)}$. [Adapted from paper IV]

5.3 Microbial response to different preservation approaches

The microscopy using live/dead differential staining showed that alive cells were present in $PG_{(24°C)}$ and $PL_{(24°C)}$ after 84 days of preservation (Figures 15.a and b). When it was up to 168 days of preservation, the alive cells were only observed in $PG_{(24°C)}$ (Figure 16.a), which confirmed the cells entrapped in $PL_{(24°C)}$ were dead and not just under inactive state (Oliver 2010).

However, few alive cells were detected in $PG_{(4°C)}$ and $PL_{(4°C)}$ after 84 and 168 days of preservation which was consistent with their meagre methane yields (Figures 15.c, d, and Figures 16.c, d). Thus, 4°C was not appropriate temperature to maintain microbial viability in this study. These results agreed with other literature that carbon source addition could not stimulate microbial activity after 28 days of storage at 4±1°C (Scherer et al. 1981). Based on the results above, $PG_{(24°C)}$ was proposed as the most efficient long-term preservation method compared to others.

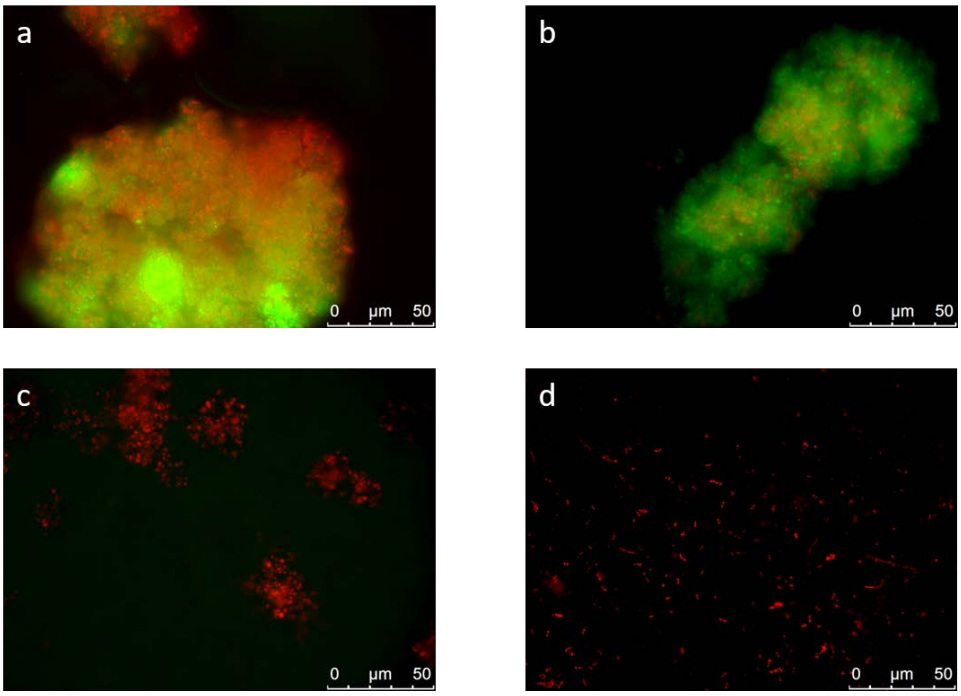

Figure 15. Identification of live or dead microorganisms in different preservation conditions: a) $PG_{(24°C)}$; b) $PL_{(24°C)}$; c) $PG_{(4°C)}$; d) $PL_{(4°C)}$ after 84 days preservation. DNA in dead or damaged microbial cell walls/membranes were stained with red fluorescence; DNA in alive microbial cell walls/membranes were stained with green fluorescence. [Adapted from paper IV].

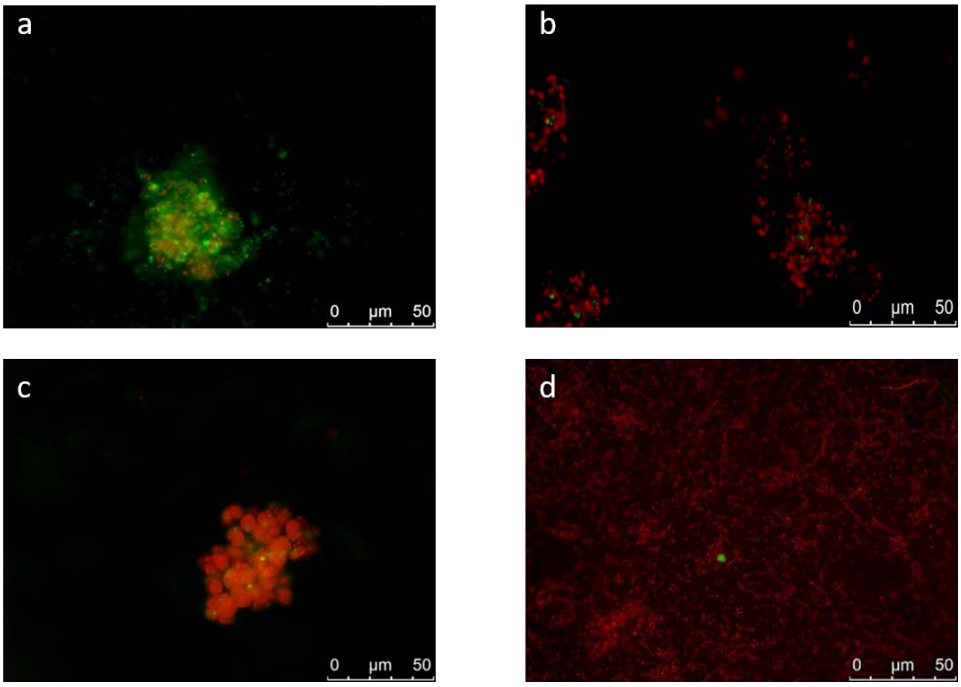

Figure 16. Identification of live or dead microorganisms in different preservation conditions: a) $PG_{(24°C)}$; b) $PL_{(24°C)}$; c) $PG_{(4°C)}$; d) $PL_{(4°C)}$ after 168 days preservation. DNA in dead or

damaged microbial cell walls/membranes were stained with red fluorescence; DNA in alive microbial cell walls/membranes were stained with green fluorescence. [Adapted from paper IV].

The initial inoculum was dominated by *Methanomassiliicoccus luminyensis* OTU18 (17.4% of relative abundance), *Methanosarcina soligelidi* OTU01 (12%), and *Methanoculleus palmolei* OTU12 (0.6%), respectively (Tian et al. 2019c). However, the preservation process changed the microbial community composition. In detail, the relative abundance of *M. luminyensis* OTU18 in all groups decreased significantly to less than 3% ($p < 0.05$) of the total population, revealing the sensitivity of *M. luminyensis* OTU18 to the preservation environment (Figure 17). Meanwhile, the increase in relative abundances of *M. soligelidi* OTU01, ranging from 21.6% to 33.7% suggested its high potential as long-term preserved methanogen. The other dominant methanogen was *M. palmolei* OTU12 with the 4.5-fold increase in $PL_{(24°C)}$-84 and $PG_{(24°C)}$-168 compared to the initial inocula, indicating its high robustness to long-term preservation.

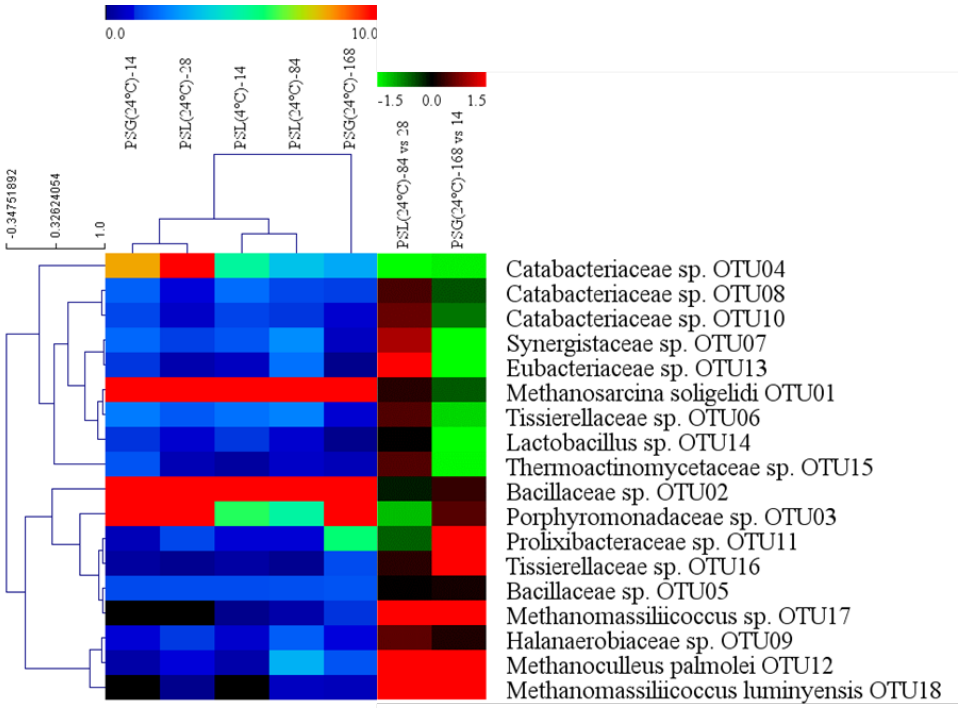

Figure 17. Hierarchical cluster analysis of the abundant microbes after preservation. Two right columns represent the fold change (log2) of each OTU between the two periods. [Adapted from paper IV].

Overall, paper IV assessed different approaches for the long-term preservation of ammonia tolerant methanogenic cultures and found that the $PG_{(24°C)}$ was the best preservation method, followed by the $PL_{(24°C)}$. The microbial analysis showed that *M. soligelidi* OTU01 and *M. palmolei* OTU12 were robust enough

to long-term preservation environments, including temperature and starvation shock. Thus this study developed successful and easy preservation methods and made the ready-to-use inocula possible for the timely bioaugmentation in full-scale biogas reactors.

6 Conclusions

This Ph.D. project mainly focused on developing methane recovery strategies to overcome ammonia inhibition and unveiling the microbial response to ammonia using high-throughput sequencing technology. Firstly, acclimatization and bioaugmentation approaches were applied to improve methane production during AD of N-rich substrate. Secondly, 16S rRNA amplicon sequencing technology and genome-centric analysis were used to decipher the effect of microbial composition on AD performance. Thirdly, the methods to preserve ammonia tolerant inocula were achieved. Specifically, the key findings of this Ph.D. project are listed:

1) Acclimatization up to 8.5 g NH_4^+-N /L was proved as an efficient approach to achieve stable methane yields with fluctuation less than 10% during AD of OFMSW.

2) During the stepwise increase of TAN, the dominant methanogen gradually shifted from *Methanosaeta concilii* to *Methanosarcina soligelidi*.

3) The different microbial tolerances to ammonia stress were observed: the consortia grown on methanol or H_2/CO_2 could adapt to ammonia levels up to 7.25 g NH_4^+-N/L, followed by formate group to 5.25 g NH_4^+-N/L and acetate group to 4.25 g NH_4^+-N/L.

4) The osmoprotectant synthesis/uptake (K^+, glutamate, glutamine, N^{ε}-acetyl-L-lysine, and glycine betaine, etc.) and energy regulating complexes (Ech and Eha/b), were proposed to improve microbial tolerance to ammonia stress.

5) The dominant bacteria in each microbiome was linked to their role in supporting the methanogens through catabolic complementarity.

6) Bioaugmentation with *Methanoculleus* sp. DTU887 remarkably improved the methane production yield by 21% and reduced VFA by 10% in CSTR at 13.5 g NH_4^+-N/L.

7) Genome-centric analysis revealed that the dominant bacteria (i.e., *Peptococcaceae* spp. and *Tissierellales* sp. DTU879) were important glucose degraders. The derived acetate was further degraded by *Tissierellales* sp. DTU879 and *Syntrophaceticus* sp. DTU783 at 13.5 g NH_4^+-N/L.

8) *Methanoculleus* sp. *DTU887* as an H_2 scavenger might play an essential role in supporting the growth of *Peptococcaceae* spp., *Tissierellales* sp.

DTU879 and *Syntrophaceticus* sp. DTU783 by complementary metabolic reactions at 13.5 g NH_4^+-N/L.

9) The presence of two parallel degradation pathways for both glucose and acetate possibly provided the dominant species (e.g., *Peptococcaceae* spp., *Tissierellales* sp. DTU879, and *Syntrophaceticus* sp. DTU783) enough flexibility against extremely high ammonia stress.

10) Ammonia tolerant methanogens could be well preserved in agar gel for168 days and liquid media for 84 days at 24°C, respectively.

11) *Methanosarcina soligelidi* and *Methanoculleus palmolei* showed robustness to long-term preservation, as evidenced by a high methanogenic activity after 168 days of preservation.

Overall, this PhD project proved the feasibility of stable methane production in the full-scale AD of N-rich organic waste. The knowledge about microbial tolerance to ammonia could be used to optimize AD process by controlling microbial composition. Based on these findings, the customized bioaugmentation method was provided according to the different conditions in AD, e.g, feedstock composition, ammonia levels.

7 Future perspective

This Ph.D. project provides sustainable approaches to improve biomethane production yields from N-rich organic waste. More insights into microbial tolerance to ammonia are revealed using high-throughput sequencing technology. To further optimize the biomethane process, the following suggestions are listed:

- Mathematical modelling can be applied to predict the timing point of bioaugmentation during AD of N-rich substrate to avoid the loss of methane.

- Metatranscriptomic analysis is vital to identify the real-time metabolic pathway that occurred under different ammonia levels when parallel pathways are present in critical microbes.

- The flux balance analysis is necessary to identify the microbial roles in the food web of AD, which can provide guidance for optimizing the microbial composition in the AD system.

- The possibility of gene transfer in terms of ammonia tolerance among anaerobic microorganisms could be tested to understand ammonia acclimatization further.

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9 Papers

I **Yan, M.,** Fotidis, I.A., Tian, H., Khoshnevisan, B., Treu, L., Tsapekos, P. and Angelidaki, I. (2019) Acclimatization contributes to stable anaerobic digestion of organic fraction of municipal solid waste under extreme ammonia levels: focusing on microbial community dynamics. Bioresource technology 286, 121376. **(IF=7.5)**

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III **Yan, M.,** Treu, L., Campanaro, S., Tian, H., Zhu, X., Khoshnevisan, B., Tsapekos, P., Angelidaki, I. and Fotidis, I.A. (2020) Effect of ammonia on anaerobic digestion of municipal solid waste: inhibitory performance, bioaugmentation and microbiome functional reconstruction. Chemical Engineering Journal, 126159. **(IF=10.7)**

IV **Yan, M**., Fotidis, I.A., Jéglot, A., Treu, L., Tian, H., Palomo, A., Zhu, X. and Angelidaki, I. (2020b) Long-term preserved and rapidly revived methanogenic cultures: Microbial dynamics and preservation mechanisms. Journal of Cleaner Production, 121577. **(IF=7.24)**

In this online version of the thesis, **papers I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from:

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