THALASSIC METHANE FERMENTATION OF DIFFERENT SEAWEEDS AND RICE STRAW

GIAN POWELL B. MARQUEZ

A Dissertation Submitted to

Department of Aerospace Engineering

Graduate School of Engineering

Nagoya University

Nagoya, Japan

As Partial Fulfillment of the Requirements

for the Degree of

Doctor of Engineering

January 2016

Acknowledgments

This research was supported by the Japanese Government (Monbukagakusho) Scholarship of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan. Additional supports were received from the support of the 2013 to 2015 Research Grant of the Graduate School of Engineering, Nagoya University.

Also, I am deeply indebted to the following people whom I would like to recognize and acknowledge:

- Dr. Tatsuya Hasegawa- my supervisor, for his unwavering support, advice and guidance.
- Dr. Ichino, Dr. Kasahara, and Dr. Nagano- my thesis examiners, for their detailed reviews and valuable suggestions.
- Ms. Hisae Takeuchi - for the support, trust and encouragement.
- Ms. Nobuko Abe- for her administration, enrollment and documentation help during the first few weeks of my arrival in Japan.

Thank you to all my coauthors Dr. Wilfred John E. Santiañez, Dr. Gavino C. Trono Jr, Dr. Marco Nemesio E. Montaño, Dr. Sharon Rose B. de la Rama, Taro Noboribayashi, Tatsuo Takahashi, Yohei Fujii, and Keita Iwasaki. Thank you to my Hasegawa's laboratory Senpais. Thank you to my past and present Hasegawa's laboratory Kouhais. Thank you to the past and present Filipino students in Nagoya

University who always supported me. Thank you to my friends outside Nagoya University who kept me sane. Thank you to my parents, Arman and Lynn. Thank you to my wife, Sheena.

Thank you Almighty GOD.

Follow excellence [with honor]…. Success will chase you.

- 3 Idiots

General Abstract

Fossil fuel dependence has been linked to the unprecedented faster rate of climate change, thereby forcing us to look into other viable renewable resources such as biomass energies. However, sustaining the increasing biomass demand for biofuel production can be challenging due to the world's limited freshwater and land resources. Seaweeds are identified as a sustainable biomass that can potentially support biofuel production demands. The most suitable biomass-to-biofuel technology in rural areas is anaerobic digestion for biogas production. Hence, utilization of seaweed biomass for biogas production was explored in this study.

Biogas production of seaweed is commonly patterned to conventional digester wherein the anaerobic condition is optimized for terrestrial biomass. The high salt content of seaweed and its different structural component than the terrestrial plants may contribute to low conversion efficiency. Hence, freshwater (FW) and thalassic (TH) anaerobic digestion of *Ulva* spp. (anaaosa) were compared to determine the more suitable condition. Biological hydrolysis pretreatment (BHP) was done to improve methane yield, while NaOH pretreatment (CNP) was employed to minimize the limitation of biological hydrolysis. Higher biogasification efficiencies based on the theoretical methane yield (285.2 ml CH_4/ g Volatile Solids [VS]) were obtained using biological hydrolysis pretreatment (FW: 27.2%, TH: 63.4%). However, the biogasification time of BHP was twice as long as that of NaOH. Heating the seaweed before biological hydrolysis pretreatment increased the methane yield and shortened

the digestion time. Nonetheless, the methane yield of all pretreatments under thalassic (BHP= 180.9 ml CH_4/ g VS, CNP= 158.2 ml CH_4/ g VS, and Heat + BH= 195.7 ml CH_4/ g VS) was higher than freshwater's (BHP= 77.7 ml CH_4/ g VS, CNP= 61.7 ml CH_4/ g VS, and Heat + BH= 78.0 ml CH_4/g VS), suggesting a superior methane fermentation under thalassic condition. Therefore, thalassic (TH) biogas production using seawater as liquid substrate and marine bacteria as microbial inoculum can be used as an alternative to conventional (FW) biogas production in the utilization of seaweed feedstock in coastal communities where seaweed is an abundant feedstock for household biogas digester.

The seasonal availability of seaweed may pose problem in the continuous operation of a thalassic household biogas digester. Utilization of biomass other than the seaweed may be needed to support the continuous thalassic biogas production. Rice straw is commonly available biomass among the archipelagic Asian countries. However, the marine microorganisms used under thalassic condition may not perform well using a terrestrial biomass. Hence, we tested the methane fermentation of rice straw under thalassic condition. The performance of the co-digestion of rice straw and *Ulva* spp. (anaaosa) was also investigated. The biological hydrolysis pretreatment (BH) of rice straw under TH condition obtained the highest methane yield (75.8 ± 5.7 ml CH_4/ g VS), thereby applying the BH to the co-digestion. All co-digestion set-ups gave higher methane yield (*Ulva*:Rice straw, 25:75 = 107.6 ± 8.0 ml CH_4/ g VS, 50:50 = 130.3 ± 10.4 ml CH_4/ g VS, and 75:25 = 121.7 ± 2.8 ml CH_4/ g VS) than the expected yields of either rice straw or *Ulva* alone. The 50% *Ulva*-50% Rice straw ratio showed the highest (152.8%) methane enhancement. While the biogasification

efficiency (BE) of the biologically hydrolyzed-rice straw in terms of its theoretical methane yield (327.9 ml CH_4/ g VS) was low (23.1%), the 50:50 co-digestion of rice straw and *Ulva* increased the BE to 46%. This study successfully demonstrated the thalassic biogas production of rice straw as mono-substrate, and the improvement of its methane yield when co-digested with *Ulva*.

On the other hand, suitable pH is necessary for successful biogas start-up and stable biogas production. NaOH is commonly used pH buffer but its acquisition to isolated coastal areas proved to be difficult. Cheaper and more accessible buffer is needed to further encourage the use of biogas technology in rural communities. Hence, *Venerupis* sp. (asari) shell was tested as pH buffer on the biogas production of *Undaria pinnatifida* (wakame). Addition of 3% and 5% powdered shell (w/w) at the start of anaerobic digestion successfully started biogas production (86.4 ml CH_4/ g VS and 109.5 ml CH_4/ g VS, respectively). Biogasification efficiencies of shells (3%Shell= 24.2%, 5%Shell= 30.7%, and 'BH then 5%Shell'= 19.4%) were lower than in 'BH then NaOH' (68.3%). However, biogas start-up failed when shells were not added. Therefore, powdered shell can be a potential cheaper pH buffer to successfully start biogas production and sustain stable anaerobic digestion, targeting coastal communities.

Moreover, the potential commercial application of thalassic condition using marine microbial inoculum and seawater for biogas production was evaluated using a pilot-scale (120 L) semi-continuous fixed-bed biogas digester. The brown seaweed *Ecklonia* spp. (arame) was used as the biomass feedstock in the 10-L substrate mixture feed, with 5% total solid. The biogasification efficiency obtained for

Ecklonia in terms of its theoretical methane yield (345.6 ml CH_4/ g VS) was 68.5%. This is higher than obtained in a 1-L batch digester (5.8%). The successful thalassic biogas production of *Ecklonia* may help develop optimized condition for continuous operation. Thalassic platform for biogas production can be used as a cheaper alternative to conventional biogas platform, targeting suitable areas to maximize net energy gain.

List of Contents

List of Figures

List of Tables

Nomenclature

Roman symbols

a	Mole of elemental hydrogen
AcMY	Actual methane yield
ACD	Anaerobic co-digestion
AD	Anaerobic digestion
ANOVA	Analysis of variance
AMD	Anaerobic mono-digestion
b	Mole of elemental oxygen
BH/P	Biological hydrolysis/pretreatment
C	Carbon (element)
C	Crude carbohydrate
$Ca(OH)_2$	Calcium hydroxide
CH_4	Methane (gas)
CHO	Carbohydrates
C/N	Carbon to nitrogen ratio
CNP	Chemical NaOH pretreatment
CO_2	Carbon dioxide (gas)

Ctrl	Control
d	Days
dH_2O	Distilled water
EMY	Estimated methane yield
FW	Freshwater
H	Hydrogen (element)
H_2	Hydrogen (gas)
HB	Hydrolytic bacteria
H_2S	Hydrogen sulfide (gas)
I.D.	Inner diameter
k_C	Constant value of the crude carbohydrate
k_L	Constant value of the crude lipid
k_P	Constant value of the crude protein
L	Crude lipid
LCA	Life cycle assessment
LPG	Liquefied petroleum gas
M	Molarity
mA	Milliampere
MB	Methane bacteria
MC	Moisture content
mEq	Milliequivalent
MF	Methane fermenters
MI	Microbial inocula

n	Mole of elemental carbon
N	Replicates
N	Nitrogen (element)
N_2	Nitrogen (gas)
Na^+	Sodium ion
Na_2CO_3	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide
NAM	Non-acetate short chain fatty acids, alcohols, and methylated substrates
NaOH	Sodium hydroxide
O	Oxygen (element)
O.D.	Outer diameter
P	Crude protein
p-value	Probability value
S	Sulfur (element)
SD	Standard deviation
sp.	Species
SRB	Sulfate-reducing bacteria
TCD	Thermal conductivity detector
TH	Thalassic
TMY	Theoretical methane yield
TS	Total solids
VFA	Volatile fatty acid

VS	Volatile solids
w	weight
w/w	Weight per weight

Greek symbols

α	Significance level
β	Glycosidic bond
Ø	Diameter
μm	Micrometer (microns)

Other symbols

‰	Parts per thousand (salinity)

Chapter 1 General Introduction

1.1. Background

1.1.1. Mitigating climate change using seaweed biomass energy (bioenergy)

Oil crisis on the 1970s drove the development of biogas technology (Campbell, 2005) using several species of macroalgae (Bird et al., 1990; Chynoweth et al., 1987) as one of the renewable energy solutions. Interest on renewable energy waned when oil price stabilized due to the discovery of new oil fields. However, the worsening global warming and their dramatic and recognizable effect on the global climate and weather conditions prompted the world government to promote different forms of renewable energies, continuously and actively.

Bioenergy is one of the cheapest renewable energy. Compared to wind, hydro, geothermal, or solar powers, the utilization of biomass for energy production is readily and widely accessible in most countries due to its cheap upfront investment costs and straightforward technology (Macqueen and Korhaliller, 2011). Bioenergy can be used to generate heat, electricity, or mechanical power as solid fuel through

direct burning, liquid fuel through fermentation and hydrothermal liquefaction, and gas fuel through anaerobic digestion and gasification (Hamelinck and Faaij, 2006). A wide array of biomass resources is now being considered as feedstock for biofuel production including those that are from terrestrial (e.g. corn, sugarcane, palm, *Jatropha*), freshwater (microalgae; e.g., *Scenedesmus* spp. and *Chlorella* spp.), and marine (e.g., seaweeds) in origin. Terrestrial crops, especially corn and sugarcane, are primarily favored because of their large-scale biomass productions, and their cheap starch and sugar extraction process (Wei et al., 2013). However, the increasing demand of space, food, and freshwater from the incessantly growing world population makes food crop utilization and arable land conversion for biofuel production unsustainable in the long-term. Although theoretically advantageous compared to food crops, the use of microalgae for biofuel production is also considered unviable because of extensive energy input requirements (Lam and Lee, 2012).

Recently, interests on seaweed biomass utilization for biofuel production reemerged due to several reasons. These are (1) mitigation of climate change by lowering CO_2 emissions, and maintaining the close carbon cycle (Timilsina and Mevel, 2011), (2) energy security by decreasing the reliance on petroleum fuels, and its erratic price fluctuation (Rebhan, 2009), (3) food security by avoiding the indirect and direct competition on terrestrial food crops utilization for biofuel production, and preventing high food prices (Sexton et al., 2009), (4) better feedstock compared to terrestrial crops by having faster growth rate (Velimirov et al., 1977), and (5) environmental conservation and preservation by preventing forest conversion to biofuel crop farms (Gao et al., 2011).

Among the bioenergy technologies (e.g. bioethanol fermentation and biodiesel production), biogas production is the most efficient in terms of net energy gain (Deublein and Steinhauser, 2008), because anaerobic digestion utilizes all the degradable components (carbohydrate, protein and lipid) of the feedstock (Chandra et al., 2012a) to produce biogas (~60% CH_4, ~40% CO_2 and other trace gases). Biogas can also be upgraded to increase its methane content at the same level as found in natural gas, giving the same performance when used as fuel in internal combustion engines or as household cooking gas. The wider range of crops suitable for biogas production, as compared to biodiesel and bioethanol, made biogas technology important to the long term green energy goal, given the decreasing land resources for terrestrial biomass cultivation.

1.1.2. Biogas production

Most studies on biofuel production using marine biomass focused on microalgae for biodiesel (Brennan and Owende, 2010; Mata et al., 2010) and seaweed for bioethanol production (Borines et al., 2013; John et al., 2011). Biodiesel and bioethanol are liquid biofuels that are commercially preferred because it can readily and easily utilized by different vehicle and industrial engines for power generation. On the other hand, while biogas can be directly used in gas stoves as substitute to liquefied petroleum gas (LPG) in households, it needs further upgrading for commercial engines usage. Therefore, biogas is preferred as a renewable source of energy in rural communities (Hall, 1983; Stanley et al., 2013). Household biogas digesters can be easily managed and operated without complicated training for the

users, while wider range of biomass can be used for its feedstock. Nonetheless, anaerobic digestion process for the production of biogas involves complex interactions between diverse microorganisms and is discussed below.

1.1.2.1. Anaerobic digestion processes

There are four phases of anaerobic digestion process, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1.1) (Chynoweth et al., 2001; Nagamani and Ramasamy, 1999; Nettmann et al., 2010; Yu and Schanbacher, 2010). The hydrolysis, acidogenesis and acetogenesis phases are collectively called the acidification step, due to the drastic decrease in pH resulting from their fast occurrence, which is faster than the methanogenesis phase (Demirer and Othman, 2008). Moreover, compared to the methanogens involved in the methanation step, the faster growth rate and lower physico-chemical sensitivity of the microorganisms in the acidification step commonly cause instability to anaerobic digestion process.

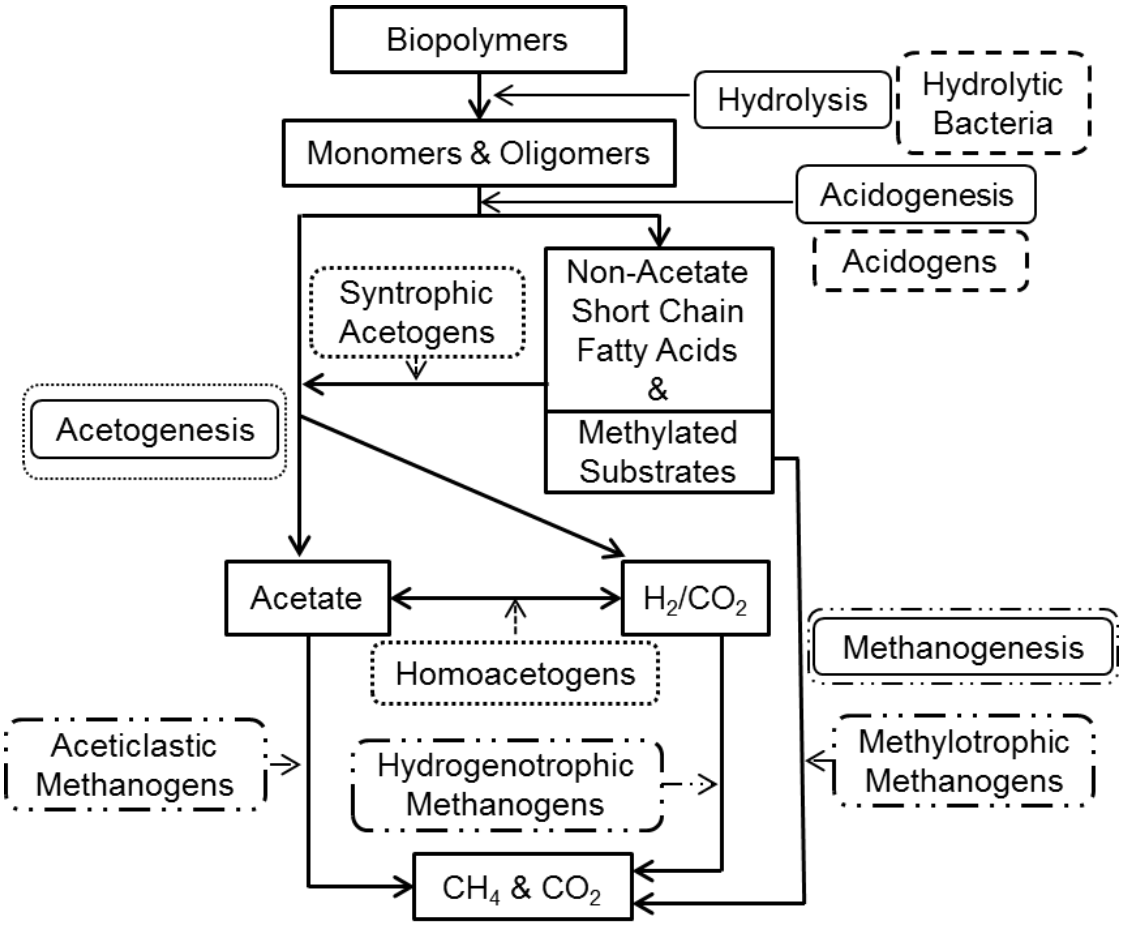

Figure 1.1 The four phases of anaerobic digestion in biogas production showing the different organisms involved (Chynoweth et al., 2001; Nagamani and Ramasamy, 1999; Nettmann et al., 2010; Stanley et al., 2013; Yu and Schanbacher, 2010).

1.1.2.2. Hydrolysis

In hydrolysis, (strictly or facultatively anaerobic) bacteria secrete extracellular enzymes that break down polymers of organic matter such as protein, polysaccharides, and lipids into their respective monomer and oligomer states. The anaerobic digestion of different biomass is completely dependent on the capability of the microorganisms, present as microflora or as microbial seed, to produce appropriate hydrolytic enzymes for specific polymers. The presence of amylolytic, proteolytic, cellulolytic, lipolytic, or phycocolloid-hydrolyzing bacteria can effectively hydrolyze the starchy crops, protein-rich waste products, cellulosic plants, fatty fruits or phycocolloid-abundant seaweeds biomass, respectively.

Lignocellulosic polymers are difficult to degrade; hence, pretreatment is needed to break down their complex structures and make cellulose accessible. Lignin cannot be degraded anaerobically since the lignin depolymerizing enzymes require oxygen. In order to proceed to acidogenesis, cellulose and hemicellulose, except lignin, are first digested by hydrolytic bacteria. Some microorganisms that can be found in anaerobic digester include *Clostridia* species which contain cellulosome — a multi-enzyme complex consisting of endo-1, 4-β-gluconase, exo-1, 4-β-gluconase, and β-galactosidase — that facilitates adhesion and complete hydrolysis of cellulose to glucose (Elshahed, 2010). On the other hand, hemicellulose such as xylan can be hydrolyzed into pentose sugar, xylose, by some microbes such as *Caldicellulosiruptor saccharolyticus* (Van De Werken et al., 2008).

1.1.2.3. Acidogenesis

Acidogenesis is the second phase in the anaerobic digestion process. The monomer and oligomer end products from the hydrolysis are instantly consumed in this phase by fermentative acidogens (strictly or facultatively anaerobic). There are three major end-product substrates produced in this phase and are grouped into (1) acetate, (2) hydrogen/carbon dioxide (H_2/CO_2), and (3) non-acetate short chain fatty acids, alcohols, and methylated substrates (NAM). Monomeric glucose from hydrolysis is converted into acetate, propionate, butyrate, ethanol (Klass, 1998), or methane with co-production of carbonic acid (Nagamani and Ramasamy, 1999). In the presence of xylose, H_2/CO_2 and acetate are produced by *Caldicellulosiruptor saccharolyticus* (Van De Werken et al., 2008) among others, which simultaneously forms a syntrophic relationship with hydrogenotrophic methanogens (Bagi et al., 2007).

1.1.2.4. Acetogenesis

The third phase, acetogenesis, is where H_2/CO_2 and NAM substrates from acidogenesis are utilized by homoacetogens and syntrophic acetogens, respectively, for acetate conversion. Homoacetogens reduce CO_2 into acetate using H_2 as electron donor. In some cases, when population of aceticlastic methanogen, which consumes acetate in methanation step, is low, backward utilization of acetate substrate to produce H_2 and CO_2 is favored by homoacetogens in the presence of hydrogenotrophic methanogens. The latter simultaneously consume the H_2/CO_2 produced (Shink, 1997; Yu and Schanbacher, 2010).

Moreover, the consumption of short chain fatty acids for acetate, CO_2 and H_2 production by syntrophic acetogens require an association with hydrogenotrophic methanogens. This syntrophic association allows immediate utilization of H_2 by hydrogenotrophic methanogen, lowering the H_2 partial pressure (<10 Pa). The simultaneous consumption of H_2 permits the endergonic conversion of acetate and H_2/CO_2 by releasing the electron from NADH as molecular hydrogen, inducing forward reaction in this pathway (Shink, 1997).

Some acetogens such as *Syntrophomonas wolfei* and *Syntrophobacter wolinii*, which consume fatty acids and propionate, respectively, are found to be in syntrophic relation with hydrogenotrophic methanogen (Boone and Bryant, 1980; McInerney et al., 1981; Nagamani and Ramasamy, 1999). On the other hand, propionate and alcohols are used by *Pelotomaculum* to produce acetate and H_2/CO_2 for methanogenesis substrate (Weiss et al., 2009). Syntrophic acetogens such as *Acetobacterium woodii* (Winter and Wolfe, 1980), *Thermotoga lettingae* strain TMO (Balk et al., 2002), *Thermacetogenium phaeum* strain PB (Kamagata and Mikami, 1989), and *Clostridium ultunense* strain B (Schnurer et al., 1996) are also found associated with hydrogenotrophic methanogens (Nettmann et al., 2010).

1.1.2.5. Methanogenesis

There are three types of methanogens — (1) methylotrophic, (2) hydrogenotrophic and (3) aceticlastic methanogens — involved in the methanogenesis phase, which are classified based on the substrate they utilize. Methylotrophic methanogens consume the methylated substrates from the NAM that is produced

during acidogenesis phase. Hydrogenotrophic methanogens utilize H_2/CO_2 substrates that are the end-products of acidogens and homoacetogens, and co-products of syntrophic acetogens. And, aceticlastic methanogens use acetate substrates that are the end products in acidogenesis and homoacetogenesis, and co-products in syntrophic acetogenesis (Chynoweth et al., 2001; Lowe et al., 1993; Nagamani and Ramasamy, 1999; Yu and Schanbacher, 2010).

Taxonomically, there are six orders of methanogens (i.e., Methanococcales, Methanomicrobiales, Methanobacteriales Methanocellales, Methanopyrales, and Methanosarcinales) (Sakai et al., 2008), four of which are commonly found in anaerobic digesters (Raskin et al., 1994). These are hydrogenotrophic Methanococcales and Methanomicrobiales — both of which mostly use CO_2, and either formate or H_2 as electron donors; hydrogenotrophic Methanobacteriales — which use mostly CO_2 and H_2 as electron donor, but some use CO_2, and both H_2 and formate; and Methanosarcinales — which use acetate (aceticlastic), methanol and methylamines (methylotrophic), and H_2/CO_2 (hydrogenotrophic).

Two families under Methanosarcinales represent aceticlastic (Methanosaetaceae) and hydrogenotrophic (Methanosarcinaceae) methanogens in anaerobic digester. Their dominance is dependent on the concentrations of short chain fatty acids and ammonia on the sludge. Methanosarcinaceae are predominant when concentrations of short chain fatty acids and ammonia in sludge are high; conversely, Methanosaetaceae predominates when these concentrations are low (Karakashev et al., 2005).

Methanogens appear to use H_2 more efficiently as shown by the low H_2 concentration, if not absent, in biogas, as compared to substantial amount of acetate substrate in the sludge after biogasification. Accordingly, enhanced CH_4 yield is observed in digesters added with hydrogen-producing bacterium *Caldicellulosyruptor saccharolyticus* (Bagi et al., 2007). Meanwhile, the slow rate of acetate utilization, as compared to H_2 utilization during methanogenesis, can be attributed to the slower growth rate of aceticlastic as compared to hydrogenotrophic methanogens, rather than their capacity to utilize the substrate itself. Hence, this suggests that more efficient utilization of H_2 as compared to acetate may not be always true (Angenent et al., 2002; Bagi et al., 2007; Lowe et al., 1993; Nagamani and Ramasamy, 1999; Valentine, 2002). Biomass which contains less protein that can be converted into ammonia during anaerobic digestion, can be predominated by homoacetogens and hydrogenotrophic methanogens under thermophilic condition (Karakashev et al., 2005; Karakashev et al., 2006; Krakat et al., 2010), while a possible shift to aceticlastic methanogen can be observed under mesophilic condition. Therefore, methanogen population dynamics can be influenced by a number of factors, including substrates availability (acetate, short chain fatty acid, and ammonia) and operational conditions such as pH and temperature, among others (Karakashev et al., 2006).

In the sulfate-rich marine environment, sulfate-reducing bacteria (SRB) can outcompete hydrogenotrophic and aceticlastic methanogens since H_2S production is thermodynamically more favorable than CH_4 using short chain fatty acids, and H_2/CO_2. Hence, only methylotrophic methanogens are commonly observed in marine environment because of the inability of SRB to utilize the methylated substrates

(Oremland et al., 1982; Zinder, 1993). Upon sulfate depletion, SRB forms a syntrophic relationship with hydrogenotrophic methanogens, making H_2 as an electron sink and CH_4 as an end product (Plugge et al., 2011).

1.1.3. Methane potential of seaweed biomass

1.1.3.1. Properties of seaweed biomass

Most seaweeds are composed of phycocolloids. *Kappaphycus alvarezii* (Doty) Doty ex Silva has high κ-carrageenan polysaccharide (Muñoz et al., 2004) while *Eucheuma denticulatum* (Burman) Collins & Hervey contains ι-carrageenan (Funami et al., 2007). *Sargassum* and *Padina* have 35% and 18.5% alginate, respectively (Omar et al., 1988). Fucose and xylose are also present in *Sargassum* (Marin et al., 2009) while agar is the main component of *Gracilaria* (Givernaud et al., 1999). In anaerobic digestion, theoretical methane yield of a certain biomass can be obtained by determining their elemental and proximate compositions. Different seaweeds contain different ratio of degradable components that can significantly affect the efficiency of biogasification.

1.1.3.2. Elemental components

The elements in organic matter are mainly carbon (C), hydrogen (H), oxygen (O), nitrogen (N) and sulfur (S). After moisture removal, the major compositions of biomass by mass are approximately 35-50% carbon and 40-45% oxygen (Chandra et al., 2012a). The suggested optimum carbon to nitrogen ratio to allow for stable anaerobic digestion is between 25 and 30 (Mital, 1996). High amount of nitrogen is

necessary for bacterial growth, especially during protein structure synthesis and nuclear matrix replication, but excessive amount may result to ammonia accumulations, which have toxic effect on methanogens (Karakashev et al., 2006; Sossa et al., 2004; Zhou and Qiu, 2006). Hence, co-digestions of different biomasses are done to adjust C/N ratio to optimum value. The presence of high amount of S as sulfate, can also affect CH_4 production by encouraging the growth of SRB. These bacteria are more efficient in utilizing acetate and H_2/CO_2 substrates as compared to aceticlastic and hydrogenotrophic methanogens, respectively (King, 1988; Lowe et al., 1993). This may cause the production of high amount of H_2S instead of CH_4 consequently decreasing biogas quality and combustibility.

Table 1.1 shows the elemental composition of the some seaweeds. Methane potential can be computed by using the elemental composition of the biomass through the stoichiometric formula of Buswell and Mueller (1952):

$$C_nH_aO_b + (n - \frac{a}{4} - \frac{b}{2})H_2O \rightarrow (\frac{n}{2} - \frac{a}{8} + \frac{b}{4})CO_2 + (\frac{n}{2} + \frac{a}{8} - \frac{b}{4})CH_4 \quad (1)$$

Where n, a, and b are the moles of the elemental carbon, hydrogen and oxygen, respectively, on a dry weight basis of the biomass.

The computation of the theoretical methane yield using the elemental composition can overestimate the methane potential of a biomass because some amount of C, such as lignin, can be a part of a non-degradable component of the biomass. Hence, the proximate compositions are preferred for the theoretical methane

yield computation of the biomass. Table 1.2 summarizes the proximate composition of some seaweed that can be used for the computation of the theoretical methane yield. The formula of the theoretical methane yield (TMY, L CH_4/ g VS) as suggested by Karpenstein-Machan (2005 in Amon et al., 2007):

$$\mathrm{TMY} = \frac{(k_P \bullet P) + (k_L \bullet L) + (k_C \bullet C)}{w} \qquad (2)$$

Where k_P, k_L, and k_C are the constant values of the crude protein (0.49 L CH_4/ g VS), crude lipid (0.85 L CH_4/ g VS), and crude carbohydrate (0.395 L CH_4/ g VS), respectively; *P*, *L*, and *C* are the actual weights (g) of crude protein, crude lipid, and crude carbohydrate, respectively; and, *w* is the actual weight of the volatile solids (g).

Table 1.1 The elemental compositions of some seaweed species.

Seaweed species	Elemental Composition (%, w/w dry)						References
	C	H	O	N	S	C/N	
Caulerpa racemosa	38.03			2.06		18.45	Kumar et al., 2011
C. scalpelliformis	27.43			1.68		16.33	
C. veravelensis	21.75			1.24		17.57	
Sargassum patens	40.18	5.22	33.85	2.00	0.98	20.09*	Li et al., 2012
S. tenerrimum	22.44	4.34		1.83	6.60	12.26*	Vijayabaskar et al., 2012
Ulva capensis				3.1			Shuuluka et al., 2013
U. fasciata				1.44		25.7	Lin et al., 2007
U. lactuca				2.9			Shuuluka et al., 2013
U. prolifera	29.7		50.1		2.97		Michalak and Chojnacka, 2009
Fucus serratus	33.5	4.78	34.44	2.39	1.31	14.02*	Ross et al., 2008
F. vesiculosus	32.88	4.77	35.63	2.53	2.44	13.00*	
Laminaria digitata	31.59	4.85	34.16	0.90	2.44	36.53*	
L. hyperborea	34.97	5.31	35.09	1.12	2.06	31.22*	
Macrocystis pyrifera	27.3	4.08	34.8	2.03	1.89	13.45*	

*Computed values by dividing N to C; C- Carbon; H- Hydrogen; O- Oxygen; N- Nitrogen; S- Sulfur

Table 1.2 The proximate compositions of some seaweed species.

Seaweed species	Proximate Values (%, w/w dry except Moisture)					References
	Lipids	CHO*	Protein	Ash	Moisture	
Caulerpa lentillifera	0.86	59.27	12.49	24.21	25.31	Ratana-arporn and Chirapart, 2006
C. racemosa	2.7	12.8	6.6	48.9		Renaud and Luong-Van, 2007
	3.8	16.6	6.8	42.2		
C. veravelensis	2.64	48.95	12.88	24.20	91.53	Kumar et al., 2011
	2.80	37.23	7.77	33.70	87.88	
Sargassum horneri	0.82	19.93	22.38	32.0	86.94	Hossain et al., 2003
Sargassum spp.	0.75	41.81	10.25	26.19	11.16	Borines et al., 2013
Ulva lactuca	4.36	35.27	8.44			Chakraborty and Santra, 2008
	1.64	14.6	7.06	21.3	10.6	Wong and Cheung, 2000
U. reticulata	0.75	55.77	21.06	17.58	22.51	Ratana-arporn and Chirapart, 2006

*CHO — Carbohydrates

1.1.3.3. Methane fermentation of seaweed biomass

Many seaweed species have been studied as feedstock for methane fermentation. Their theoretical methane yields are high but the actual methane yield of some species are low due to the presence of hydrolysis-resistant structural components and/or the absence of appropriate microorganisms that can breakdown the seaweed structures. Hence, development of suitable pretreatment for seaweed biomass is essential to maximize methane yield (Dębowski et al., 2013). In the study of Bird et al. (1990), *Sargassum fluitans* and *S. pteropleuron* yielded only 40% (450 L CH_4/ kg VS) and 35.7% (420 L CH_4/ kg VS) of their methane potential, respectively. Many approaches to enhance methane yield of different biomass such as co-digestion, physical, chemical, and biological pretreatments were discussed by Gupta et al. (2012). Physical pretreatment, specifically maceration, increased methane yield of *Ulva lactuca* from 174 L CH_4/ kg VS to 271 L CH_4/ kg VS while thermal treatment at 130°C increased to 187 L CH_4/ kg VS (Bruhn et al., 2011). Without pretreatment, *Laminaria hyperborea* and *L. saccharina* produced 280 L CH_4/ kg VS and 230 L CH_4/ kg VS, respectively (Hanssen et al., 1987). *Saccharina latissima* (*L.* saccharina) has low C:N ratio (8.8) but its methane yield (223 L CH_4/ kg VS) enhanced by 20.18% and 16.59% upon a 10-minute steam explosion pretreatment at 130°C and 160°C, respectively (Vivekanand et al., 2012). Moreover, the co-digestion of *S. latissima* with steam-exploded wheat straw (210°C, 10 minutes) to increase C:N value to 30.2 improved its methane yield to 270 L CH_4/ kg VS (Vivekanand et al., 2012).

Biomethane potential of *Ulva* sp. (196 L CH_4/ kg VS), *Gracilaria* sp. (182 L CH_4/ kg VS) and *Enteromorpha* sp. (154 L CH_4/ kg VS) decreased (167 L CH_4/ kg

VS, 170 L CH_4/ kg VS and 148 L CH_4/ kg VS, respectively) upon increasing TS input from 2.5% to 5% (Costa et al., 2012). Furthermore, seasonal variation of methane yield is also apparent in seaweeds. In *Laminaria digitata*, the highest actual methane yield (254.14 L CH_4/ kg VS) is obtained in July and the lowest (196.33 L CH_4/ kg VS) in March, corresponding to the low and high ash content, respectively (Adams et al., 2011). Also, the seasonal variations in mannitol and laminaran content of *L. hyperborea* can affect methane yield (Horn and Østgaard, 2001). On the other hand, a 2-day biological pretreatment of *L. japonica* (8.3 g Volatile Fatty Acid [VFA]/ L), *Pachymeniopsis elliptica* (6.8 g VFA/ L) and *Enteromorpha crinite* (4.4 g VFA/ L) using *Vibrio harveyi* (15.6 g VFA/ L, 12.0 g VFA/ L and 9.8 g VFA/ L, respectively) and *V. alginolyticus* (~14 g VFA/ L, ~11.9 g VFA/ L and ~7.5 g VFA/ L, respectively) boosted VFA production while chemical pretreatment using NaOH for 24 hours was less effective (~12.8 g VFA/ L, ~9 g VFA/ L and ~7.5 g VFA/ L, respectively) (Pham et al., 2013).

1.1.4. General objectives of the study

The microflora of marine biomass and marine sediments can be used as microbial seed for anaerobic digestion (Marquez et al., 2013), but operating conditions used in methane fermentation studies of seaweeds are commonly patterned after the biogas digesters running under terrestrial/conventional conditions (freshwater condition). Thus, the conventional biogas production utilizes washed seaweed biomass, freshwater liquid substrate, and conventional microbial inoculum.

The innate ability of marine bacteria to effectively digest the unique structural components of seaweeds may give better methane yield if these organisms are used as compared to the conventional inoculum. Hence, this study compared the biogas production performance of seaweed biomass in 1-L batch digester using the conventional microbial inoculum, and the developed marine microbial inoculum. Different conditions that are suitable to each inoculum — either freshwater or thalassic were used. This study is the first to compare the biogas production of seaweed using the conventional microorganisms under freshwater condition and marine microorganisms under thalassic condition. The use of marine bacteria as microbial inoculum may minimize harsh and expensive pretreatment needs for seaweed biomass because they may be more efficient in degrading seaweeds as compared to their terrestrial/conventional counterpart that has been gradually developed for thalassic condition.

The biogas production performance of rice straw as sole feedstock, and as co-feedstock of the seaweed biomass using marine microbial inoculum under thalassic condition in 1-L batch digester were also tested. The rice straw was used as a model terrestrial biomass substrate. If terrestrial biomass can be used as, substitute or supplement feedstock, to seaweed biomass whenever the supply of seaweed is lacking, then enough supply of biomass feedstock can be secured for continuous thalassic biogas operation. This study is the first to evaluate the biogas production performance of rice straw as sole substrate and co-substrate of seaweed using marine microbial inoculum under thalassic condition. Furthermore, the potential of shell of asari as substitute pH buffer to NaOH in 1-L batch digester was also examined. The

abundance of shell in the coast may lower the cost of operation of the biogas household digester in coastal communities, or may provide a temporary alternate to alkaline chemicals whenever their supply is lacking.

The potential of household digester operation in coastal communities, considering the community's ready access to seaweed as biomass feedstock and seawater as the liquid substrate, can be demonstrated under thalassic conditions. Coastal communities can greatly benefit from thalassic condition especially in terms of convenience on handling and management of digesters. However, the operation of the thalassic biogas production in 1-L batch digester may be different for continuous pilot-scale operation. Hence, in this study, the potential application of thalassic condition in a pilot-scale (120 L) fixed-bed biogas digester — semi-continuously operated using seaweed biomass — was assessed. This study is the first to test the performance of thalassic biogas production using a pilot-scale digester, which can give way to the future development of a commercial-scale operation.

1.1.5. Organization of the thesis

This thesis is divided into six chapters. Chapter 2 compared the biogas production performance of the green seaweed *Ulva* (anaaosa) in 1-L batch digester under freshwater condition using the conventional microbial inoculum, and under thalassic condition using the marine microbial inoculum. Biological hydrolysis and chemical hydrolysis pretreatments were also employed to enhance the methane yield, increase the net energy gain, and determine the limitation brought by each different condition. Chapter 3 tested the performance of marine microbial inoculum in using

the terrestrial biomass rice straw as feedstock for thalassic biogas production in 1-L batch digester. Fresh water condition using conventional microbial inoculum was also used to compare if thalassic biogas production of rice straw is better. Biological hydrolysis and chemical hydrolysis pretreatments were also tested to determine the limitation brought by each different condition. The anaerobic co-digestion of rice straw and the green seaweed *Ulva* was also done to test the possible synergistic effect of mixing different ratios of the two substrates on their hydrolysis and methane yield. Chapter 4 explored the potential of the powdered shell of the shellfish *Venerupis* (asari) as alternate pH buffer to the chemical alkaline NaOH in the biogas production of the brown seaweed *Undaria pinnatifida* (wakame) in 1-L batch digester. The performance of shell was compared to the NaOH, adding it to the digestate at the start of anaerobic digestion, and at the end of the biological hydrolysis pretreatment before the start of anaerobic digestion. Chapter 5 further evaluated the performance of the biogas production of the brown seaweed *Ecklonia* (arame) using the marine microbial inoculum under thalassic condition in a pilot-scale (120 L) fixed-bed digester that is semi-continuously operated. The performance of the semi-continuous pilot-scale fixed-bed thalassic biogas digester can be a basis for the possible commercial application of the thalassic condition in a commercial biogas plant. Chapter 6 summarizes the conclusions of these four studies and their future perspective. The contents in Chapters 1, 2, 3, 4, and 5 are extracted and organized from the three published (Marquez et al., 2014; Marquez et al., 2015a; Marquez et al., 2015b), two in press (Marquez et al., In press a; Marquez et al., In press b), and one under review papers (Marquez et al., In prep).

Chapter 2

Biogas production of biologically and chemically pretreated seaweed, *Ulva* sp., under different conditions: freshwater and thalassic

2.1. Introduction

Seaweeds are better feedstock for biogas production than terrestrial crops because of the several advantages on its cultivation, growth rate, and environmental impact mitigation (Marquez et al., 2014). The utilization of seaweed for biogas production is usually patterned in anaerobic digester that is operated using terrestrial biomass, freshwater liquid substrate, and conventional microbial seed (Nkemka and Murto, 2010; Pope et al., 2013). The high salt content of the seaweeds and the sensitivity of the methanogens to salinity (Chen et al., 2003) made washing with freshwater necessary for successful start-up of biogas production. Several studies

presented high methane yield in utilizing seaweed biomass under freshwater conditions— seeded with conventional microbial inoculum from biogas reactors and sewage treatment plants (Bird et al., 1990; Gurung et al., 2012; Vivekanand et al., 2012). However, additional operational cost and environmental footprint can be incurred upon utilization of freshwater for feedstock washing and digester maintenance. The availability of freshwater poses problem to biogas users located in island communities and areas that can experience cyclical drought like El Niño (Coelho and Goddard, 2009). Seawater is the only abundant and cheap liquid substrate that can be a substitute to freshwater. Furthermore, the carbohydrate compositions of seaweeds are mostly phycocolloids. The brown seaweeds *Saccharina* and *Sargassum* have alginate (Indergaard et al., 1990) and fucoidan (Balboa et al., 2013), respectively, while the green seaweed *Ulva* has ulvan (Lahaye and Robic, 2007). The red seaweed *Kappaphycus* contains carrageenan (Hurtado et al., 2008), while *Gracilaria* has agar (Martin et al., 2013). These phycocolloids are different from the carbohydrates found in terrestrial biomass such as starch and lignocelluloses (Marquez et al., 2014). This structural difference of seaweed may limit the hydrolysis efficiency of the conventional microorganisms. The utilization of the natural microflora from the marine environment may be a better approach when using marine biomass feedstock.

Costa et al. (2012) demonstrated the use of marine sediment (283 L CH_4/ kg VS) as microbial inoculum in anaerobic degradation of the blended *Ulva* sp. (15%) and mixed sludge (85%), but better methane yield was obtained using anaerobic

digester sludge (296 L CH_4/ kg VS). The inferior performance of marine sediment might be due to the used freshwater liquid substrate ($NaHCO_3$ [5 g/ L] and distilled water), thereby lowering the optimum salinity for marine bacteria. To fully demonstrate the performance of a marine inoculum, the digester condition must be thalassic as well. Previous study demonstrated the feasibility of biogas production under thalassic condition (Marquez et al., 2013). But the feedstock used was mainly seagrass biomass and was not further compared to the biogas production performance under optimum freshwater condition. Hence, in this study, the methane fermentation performance of the unwashed *Ulva* sp. under thalassic condition was tested. In this condition, seawater was used as substitute to freshwater liquid substrate, while marine microbial inoculum from the mixture of seaweed microflora, mud, sand, and seawater was used as the suitable microbial seed. The biogas production performance of the same but washed seaweed under optimum freshwater condition — wherein freshwater was used as liquid substrate, and the conventional microbial inoculum from activated sludge was added as microbial seed — was further compared.

Suitable microbial seed and appropriate anaerobic condition are necessary to obtain better methane fermentation performance (Migliore et al., 2012), but the biomass conversion to methane is also affected by the effectiveness of hydrolytic inoculum to hydrolyze seaweed. The limitation of seaweed hydrolysis can lower the supply of the precursor substrates for methane production (Chandra et al., 2012a). Therefore, the effect of the biological hydrolysis pretreatment (BHP) on methane fermentation under freshwater (FW) and thalassic (TH) was also compared. The

chemical NaOH pretreatment (CNP) was further employed to ensure that the difference in the methane fermentation performance between freshwater and thalassic is not only due to the difference in biological hydrolysis capability, but also to the methane fermentation ability under different conditions. Heating before BH pretreatment was also done to improve the biogas production performance of the BHP set-ups.

2.2. Materials and Methods

2.2.1. Collection, preparation, and characterization of *Ulva* spp. biomass

The *Ulva* sp. was randomly collected as “green tide” near the coast of Kirachō, Miyazaki, Nishio-shi, Aichi-ken, Japan (34°46'32.30" N, 137°05'16.90" E) on June 29, 2013 as shown in Figure 2.1. Floating *Ulva* sp. (Figure 2.2) was cleaned, removing the sands and other organic matters, before placing in resealable bags (zip lock). Seawater-submerged sands, seawater, and muds near the coast were also collected as an initial source of marine bacteria. The sands and muds with seawater were placed in a plastic container. Seawater was also obtained to be use as liquid substrate. All samples were then transported to EcoTopia Science Institute Building, Nagoya University, Nagoya-shi, Japan.

Figure 2.1 The map of the collection site (red circle) of the *Ulva* sp., seawater, sand, and muds in Nishio City, Aichi-ken, Japan.

Figure 2.2 The *Ulva* sp. bloom also known as “green tide” on the coast of Nishio City, Aichi-ken, Japan.

Chapter 2. Biogas production of biologically and chemically pretreated seaweed, Ulva sp., under different conditions: freshwater and thalassic

Freshwater and thalassic experimental set-ups were made with three replicates for each pretreatment. In freshwater set-up, the biomass was washed with freshwater, whereas in thalassic set-up, the biomass was not washed. Washing with freshwater was thoroughly done to remove salts from the biomass. The washed and unwashed biomass was allowed to drip to remove excess freshwater and seawater, respectively, before freezing (-20°C) for future use. The washed and cleaned fresh biomass of *Ulva* spp. was used to determine the moisture content (MC), total solids (TS), volatile solids (VS), and ash content following the standard procedures (AOAC, 1990) described by Marquez et al. (2013). Drying was done to obtain the moisture content and total solids using AS ONE Forced Convection Drying Oven DO-450FA at 105 °C until constant weight. The volatile solids were computed by ashing the dried seaweed at 550°C until constant weight using Burn Out Furnace KDF007EX. Seaweed samples were also sent to Chugai Technos Corporation, Yokogawa-shinmachi, Nishi-ku, Hiroshima-shi, Japan for the proximate composition analysis (lipid, protein, carbohydrate, lignin, and Carbon/Nitrogen ratio) following the standard method from the Food hygiene inspection guidelines II by Japan Food Hygiene Association, and the Compost, etc. organic matter analysis method II by Japan Soil Association. All biomass were macerated using a blender to reduce the size (~<5 mm) before pretreatment and biogas experiment.

2.2.2. Microbial seeds

2.2.2.1. Biological hydrolysis inoculum

The development of inoculum for hydrolysis was separately done for freshwater (Figure 2.3) and thalassic set-ups (Figure 2.4). The initial hydrolytic bacteria for freshwater were obtained from the slurry of the continuously-operated fixed-bed reactor that is fed with food waste. Three hundred (300) g of the initial hydrolytic bacteria was placed in a 1-L bottle (Schott Duran). Addition of 200 g of washed and macerated *Ulva* sp. as biomass feedstock and 200 ml of distilled water as liquid substrate was done as starting biomass slurry feed. The bottle was not pump with N_2 gas, but remained airtight, allowing the growth of facultative bacteria and fungi. The bottle is opened every 20 days to allow breathing and then resealed. The same experimental conditions were followed in the development of hydrolytic bacteria for thalassic set-up with the exception of using 300 g of the mixture of seawater, sands, and muds as initial source of marine hydrolytic bacteria, 200 g of unwashed and macerated *Ulva* sp. as biomass feedstock, and 200 ml of seawater as liquid substrate. Both hydrolytic bacteria of the two set-ups were incubated at 37°C in the dark, and manually shaken once a day for 30 seconds.

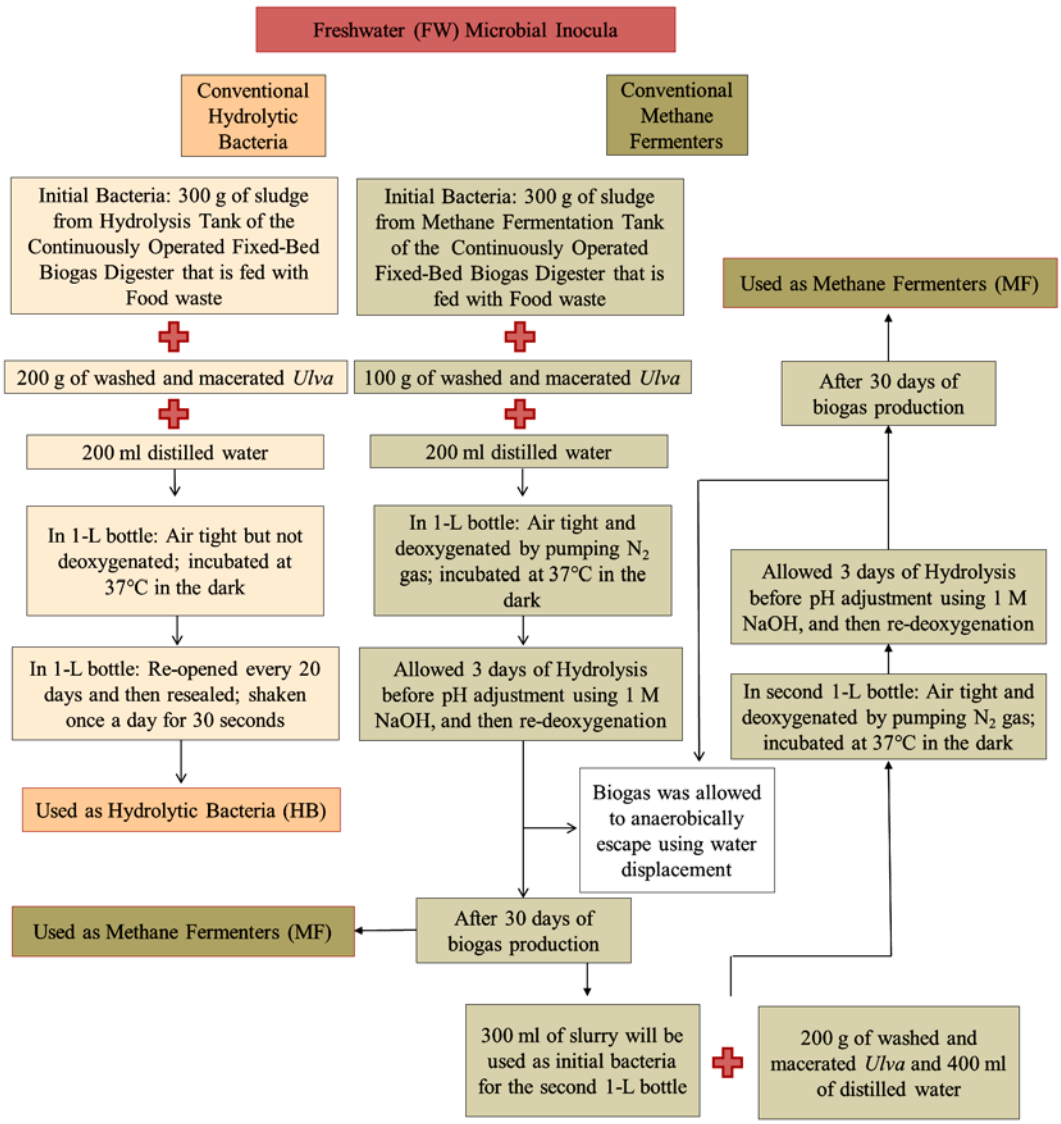

Figure 2.3 The schematic diagram of the development of the conventional hydrolytic bacteria and methane fermenters for freshwater set-up.

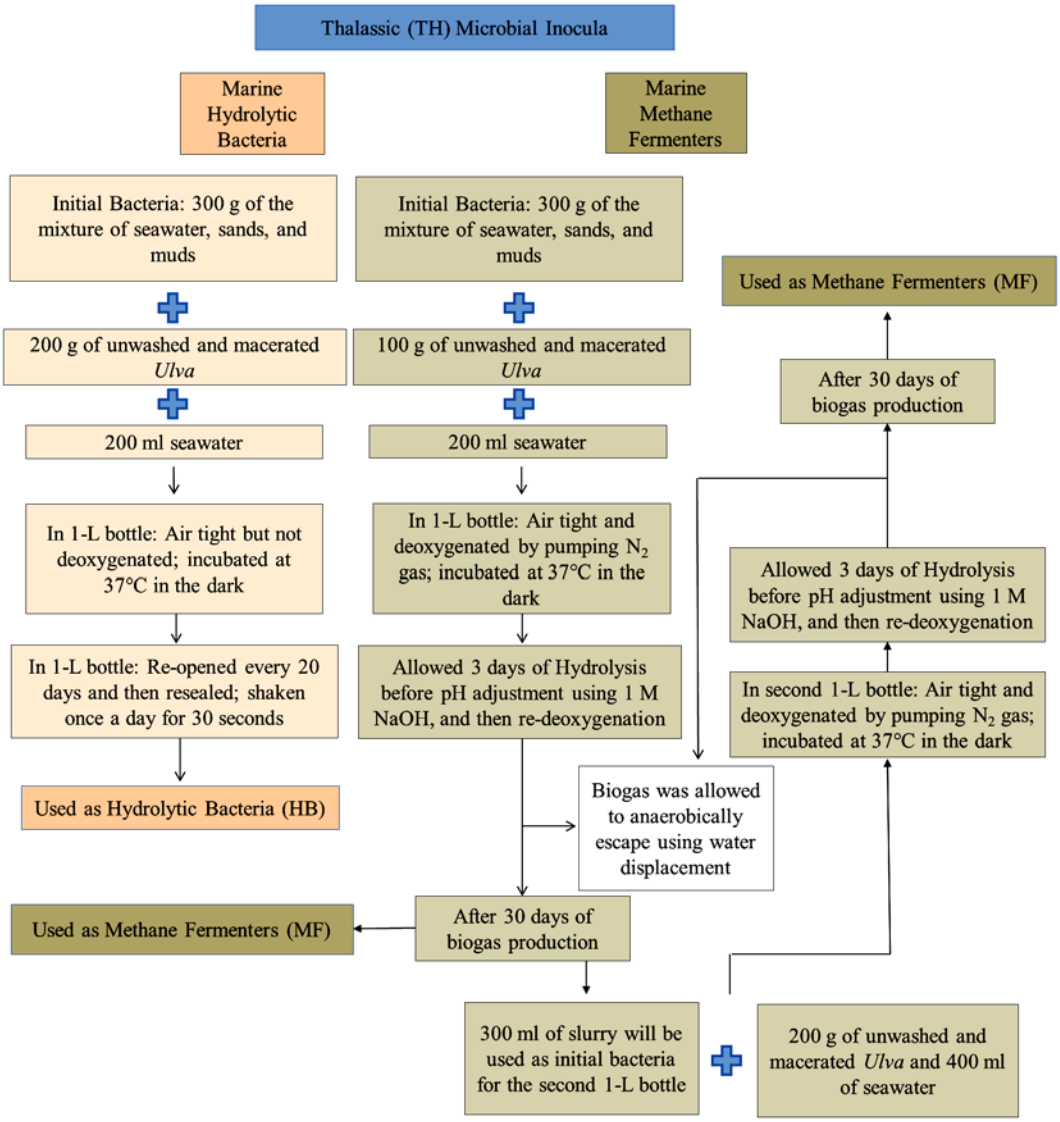

Figure 2.4 The schematic diagram of the development of the marine hydrolytic bacteria and methane fermenters for thalassic set-up.

2.2.2.2. Methane fermentation inoculum

Two separate microbial seeds for methane fermentation were developed for freshwater and thalassic set-ups. The initial microbial inoculum for freshwater was obtained from the same slurry of the continuously-operated fixed-bed reactor. Three hundred grams (300 g) of the initial microbial inoculum was mixed with 100 g of washed and macerated *Ulva* sp., and 200 ml of distilled water in an initial 1-L bottle before deoxygenation (pumping N_2 gas). After 3 days, the pH was adjusted using 1M NaOH by opening, and then deoxygenating the bottle again. The bottle was incubated at 37°C in the dark and manually shaken once a day for 30 seconds. All the biogas produced was allowed to anaerobically escape from the bottle. After a month of biogas production, 300 ml of slurry from the initial 1-L bottle was transferred to the secondary 1-L bottle. The mixture of 200 g of washed and macerated *Ulva* sp., and 400 ml of distilled water was further added to the initial and secondary 1-L bottles before starting the second biogas production using the previously described anaerobic digestion conditions. After a month of biogas production, the slurries of both initial and secondary 1-L bottles were used as microbial seed in the methane fermentation of the actual freshwater set-up experiments (Control, Biological hydrolysis pretreatment, 1% NaOH pretreatment, and Heat + BH pretreatment). The same weight ratio of hydrolytic bacteria, biomass and liquid substrate used in the development of methane fermentation seed for freshwater set-up were used in the development of the methane fermentation seed for thalassic set-up. However, a mixture of seawater, sands, and muds was used as initial marine microbial inoculum. The macerated *Ulva* sp. used as

biomass feedstock was also unwashed, while the liquid substrate used for dilution was seawater.

2.2.3. Theoretical methane yield

The constant values of crude proteins (0.49 L CH_4/ g VS), crude lipids (0.85 L CH_4/ g VS), and crude carbohydrates (0.395 L CH_4/ g VS) and the actual weights of protein (*P*), lipid (*L*), and carbohydrate (*C*) of the *Ulva* sp. biomass were used to compute for the theoretical methane yield (TMY, L CH_4/ g VS) as described in Chapter 1 (equation 2).

2.2.4. Biomass pretreatments and biogas production set-ups

Two set-ups were made to compare the biogas production performance under the freshwater conditions (Figure 2.5) and thalassic conditions (Figure 2.6). The control, biological hydrolysis pretreatment, and 1% NaOH pretreatment were made in each set-up. The Heat + BH pretreatment was further done to improve the biogas production performance of the biological hydrolysis pretreatment set-ups. Three replicates were prepared for each pretreatment of each set-up ($N = 3$). One liter-bottle (Schott Duran) was used as batch digesters. The biomass substrates, liquid substrates, and microbial seeds of the experimental set-ups are summarized in Table 2.1.

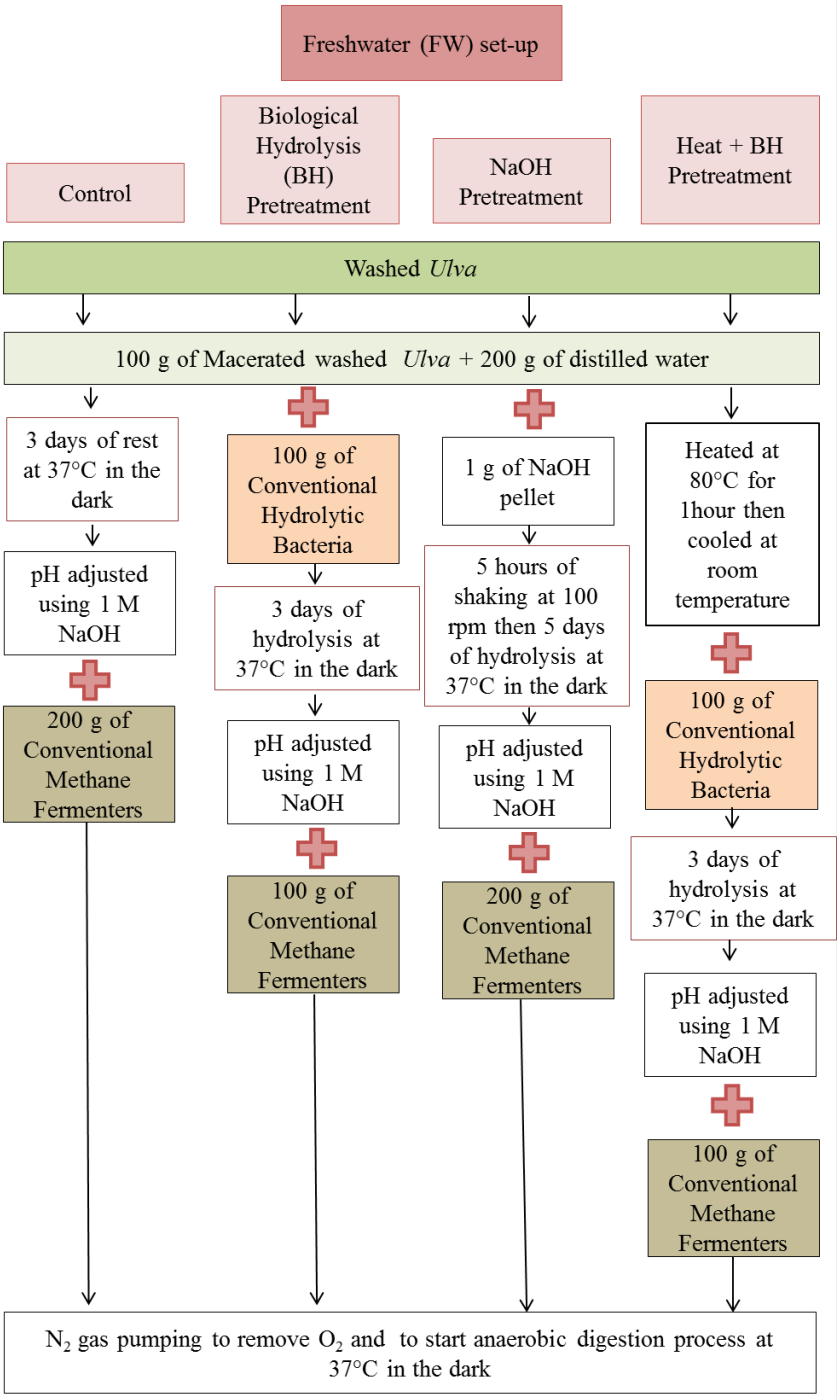

Figure 2.5 The schematic diagram of the preparation of the different pretreatment set-ups before the start of the biogas production experiment under freshwater (FW) condition.

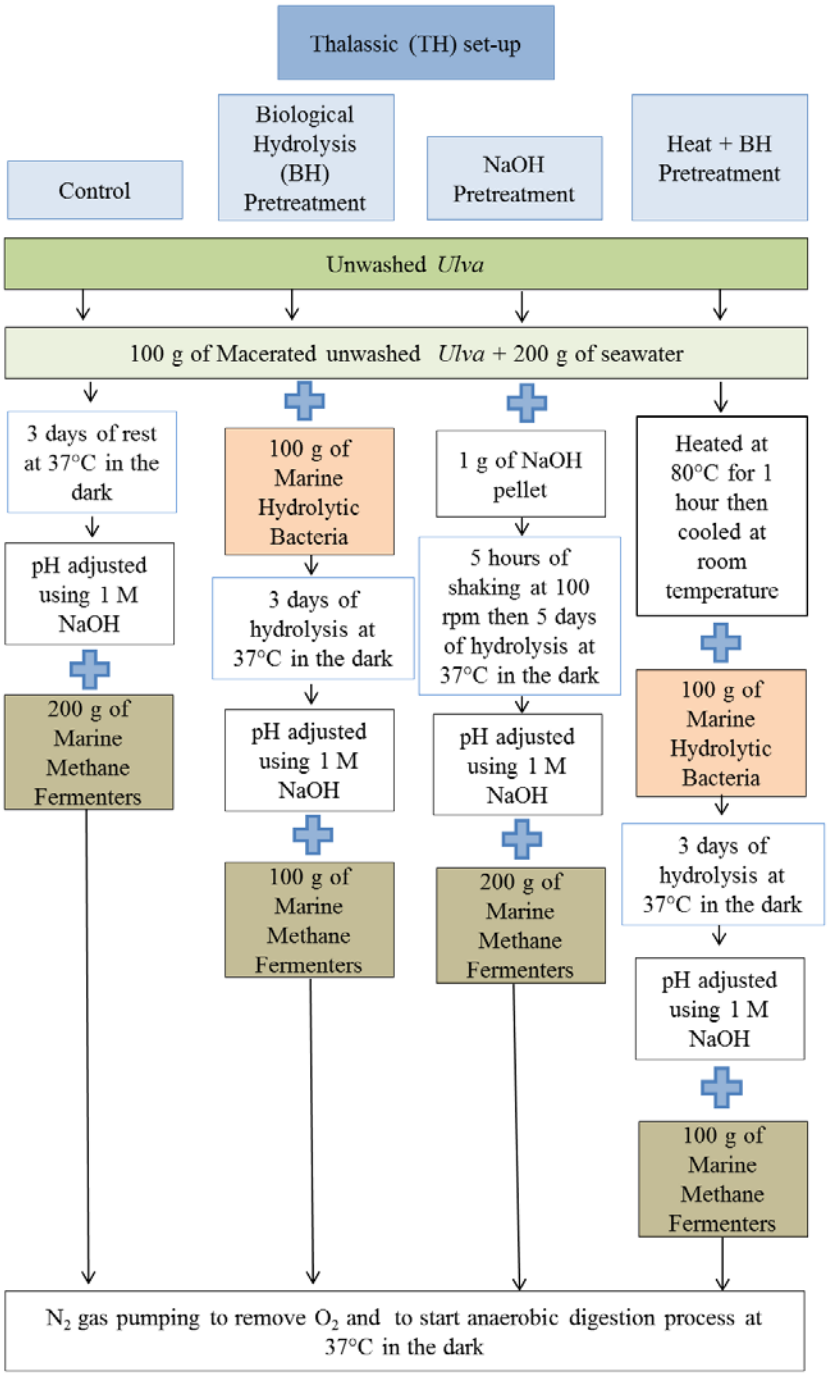

Figure 2.6 The schematic diagram of the preparation of the different pretreatment set-ups before the start of the biogas production experiment under thalassic (TH) condition.

In the control of both set-ups, the biomass substrates and liquid substrates were mixed and allowed to rest for 3 days at 37°C in the dark. The pH (Freshwater: After 3 days pH= 6.33 ± 0.06; Thalassic: After 3 days pH= 6.39 ± 0.05) was then adjusted (Freshwater: Start of Anaerobic digestion pH= 7.30 ± 0.03; Thalassic: Start of Anaerobic digestion pH= 8.06 ± 0.03) before adding the methane fermentation inoculum.

On the other hand, the biomass and liquid substrates of the biological hydrolysis pretreatment were mixed with hydrolytic bacteria inoculum before incubating at 37°C in the dark for 3 days to allow hydrolysis. The pH (Freshwater: After Biological hydrolysis pretreatment pH= 5.30 ± 0.02; Thalassic: After Biological hydrolysis pretreatment pH= 5.14 ± 0.01) was also adjusted before adding the methane fermentation inoculum (Freshwater: Start of Anaerobic digestion pH= 7.50 ± 0.03; Thalassic: Start of Anaerobic digestion pH= 8.04 ± 0.05).

Table 2.1 The summary of the substrate components and pretreatment conditions of each experimental set-ups.

Pretreatment Set-ups		Control (Ctrl)	Biological Hydrolysis (BHP)	1% NaOH (CNP)	Heat + Biological Hydrolysis (Heat + BH)
FW	Biomass Substrate	100 g washed *Ulva* sp.	100 g washed *Ulva* sp.	100 g washed *Ulva* sp.	100 g washed *Ulva* sp.
FW	Liquid Substrate	200 g dH_2O	200 g dH_2O	200 g dH_2O	200 g dH_2O
FW	Microbial Seeds	200 g FW MF	100 g FW HB, 100 g FW MF	200 g FW MF	100 g FW HB, 100 g FW MF
FW	Salinities	2.3 ‰	3.0 ‰	5.3 ‰	5.0 ‰
TH	Biomass Substrate	100 g unwashed *Ulva* sp.	100 g unwashed *Ulva* sp.	100 g unwashed *Ulva* sp.	100 g unwashed *Ulva* sp.
TH	Liquid Substrate	200 g seawater	200 g seawater	200 g seawater	200 g seawater
TH	Microbial Seeds	200 g TH MF	100 g TH HB, 100 g TH MF	200 g TH MF	100 g TH HB, 100 g TH MF
TH	Salinities	38.0 ‰	37.8 ‰	40.3 ‰	39.3 ‰

BH- biological hydrolysis; FW- freshwater; TH- thalassic; AD- anaerobic digestion; MF- methane fermenters; HB- hydrolytic bacteria; ‰- parts per thousand

For the 1% NaOH pretreatment, addition of 1 g (w/w) of NaOH pellet (97% purity, Kanto Chemicals Co.) was done after mixing the biomass substrates and liquid substrates. The 1% NaOH pretreatment of both set-ups was firstly shaken (Yamato Shaker MK161) for 5 hours with 100 rpm speed, and then incubated for 5 days at 37°C in the dark. The pH (Freshwater: After 1% NaOH pretreatment pH= 7.30 ± 0.19; Thalassic: After 1% NaOH pretreatment pH= 7.38 ± 0.15) was then adjusted (Freshwater: Start of Anaerobic digestion pH= 7.53 ± 0.04; Thalassic: Start of Anaerobic digestion pH= 7.83 ± 0.03) before addition of methane fermentation inoculum.

All pH were measured using handheld pH meter (Horiba D-52), and adjusted using 1 M NaOH. The salinities (parts per thousand, ppt) of both set-ups (Table 2.1) were measured using AS ONE refractometer (Master-AS/Millα). Deoxygenation by pumping N_2 gases (Figure 2.7) was immediately done after addition of methane fermentation inoculum to start the methane fermentation experiment.

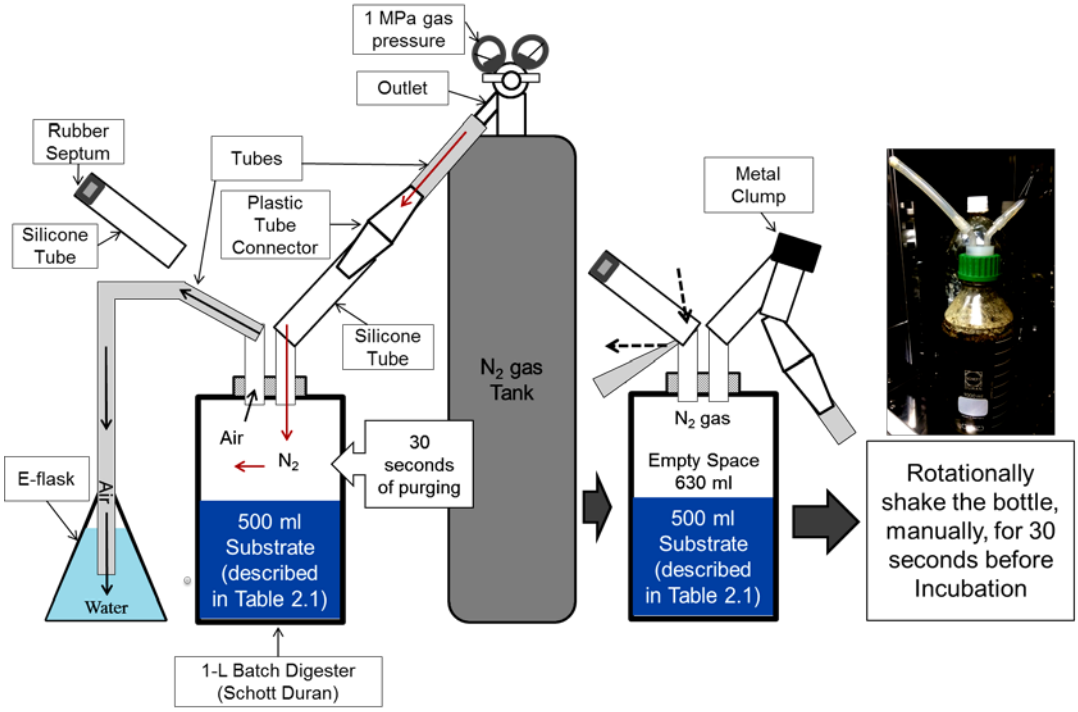

Figure 2.7 The pictorial diagram of the steps done in the pumping of N_2 gas (30 seconds) for the deoxygenation of the batch digesters.

2.2.5. Analysis of biogas compositions

Biogas components (CH_4, CO_2, and others) were analyzed using a gas chromatograph [Yanaco G1880: Injecting volume of 0.2 ml; Porapak Q Column (Length is 2 m, O.D. is 4 Ø, I.D. is 3 Ø, 80-100 mesh); Column temperature of 80°C; Injector temperature of 50°C; Helium gas flow rate of 0.098 MPa; Current 80 mA] equipped with thermal conductivity detector (TCD) (Figure A.9). The biogas sampling (Figure 2.8) was directly done using a 1-ml glass syringe. The volume of biogas was measured using a water displacement method (Figure 2.9) described by Chandra et al. (2012b). The volumetric composition of methane and carbon dioxide was obtained by multiplying the volume of biogas with the volumetric percentage obtained by gas chromatographic analysis for methane and carbon dioxide.

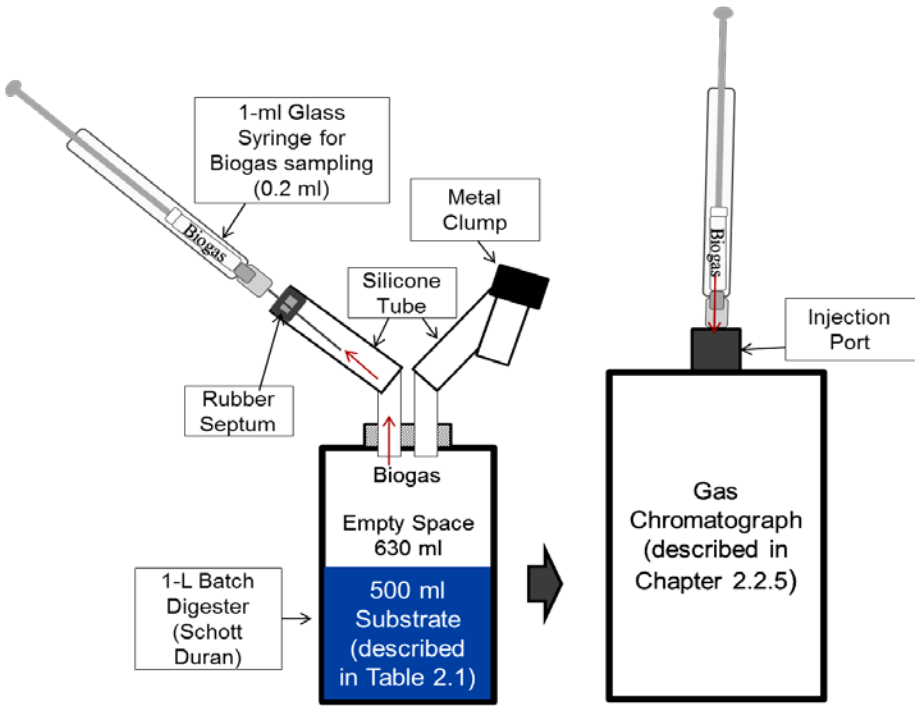

Figure 2.8 The pictorial diagram of the biogas sampling in the batch digester.

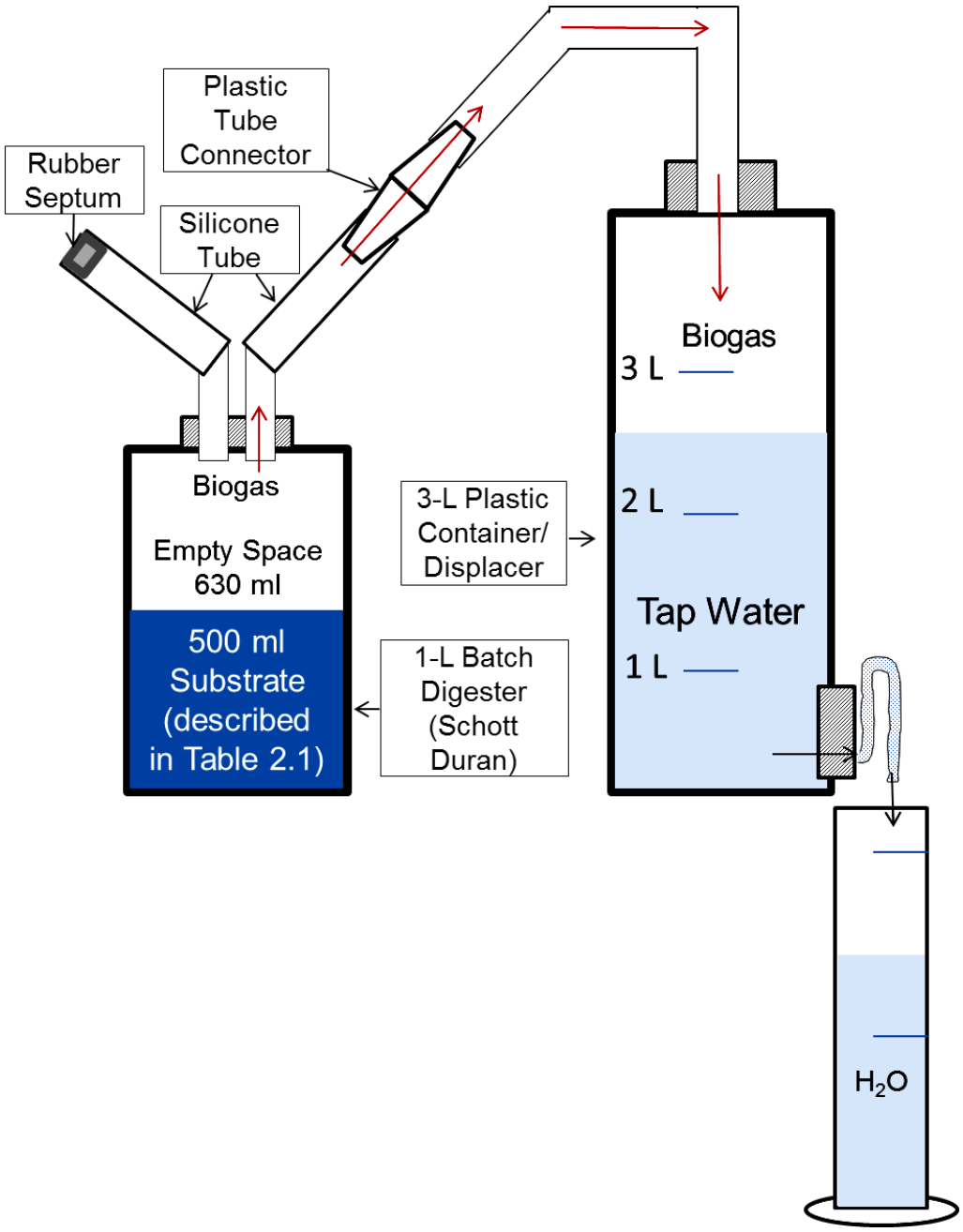

Figure 2.9 The pictorial diagram of the water displacement that is used to measure the biogas produced in the batch digester and allow the biogas to anaerobically escape from the digester.

2.2.6. Statistical data analysis

Two-way analysis of variance (ANOVA) with replication (α= 0.05) and ANOVA single factor (α= 0.05) were done to test for the significant differences of the biogas and methane yield within pretreatment in each set-up using Microsoft excel program. All data were presented as mean ± standard deviation (SD). The high standard deviation of the biogas and methane yield may have been due to the utilization of the non-homogeneous biomass substrate brought by the unthorough mixing of the blended biomass before their transfer to the batch digesters, and the addition of the microbial inocula with heterogeneous bacterial population.

2.3. Results and discussion

2.3.1. Proximate compositions and theoretical methane yield of *Ulva* sp.

The proximate compositions of *Ulva* sp. are summarized in Table 2.2. The volatile solid of *Ulva* sp. is mainly composed of carbohydrates (53.90%). Lahaye and Robic (2007) described the cell wall composition of *Ulva* as mainly cellulose and ulvan, while Wei et al. (2013) reported starch as its storage polysaccharide. The cellulose and starch are also common in terrestrial plants, hence effective hydrolysis may be observed using the conventional hydrolytic bacteria under freshwater condition. Nevertheless, the computed theoretical methane yield of *Ulva* sp. was 285.2 L CH_4/ kg VS. This is higher than the biomethane potential (196 ± 9 L CH_4/ kg

VS) obtained by Costa et al. (2012), while comparable to other seaweeds (Bruhn et al., 2011).

Table 2.2 The proximate compositions of the *Ulva* species

Proximate tests	Values (%, w/w)
Moisture	82.70
Total Solids	17.30
Volatile Solids*	69.30
Ash*	30.70
C/N	16.05
Lignin*	5.84
Crude Carbohydrate*	53.90
Crude Protein*	13.64
Crude Lipid*	0.65

Measured using fresh biomass except with * (in dry weight)

2.3.2. The pH change during the biological and chemical pretreatment of *Ulva* sp.

Biological pretreatment using the developed hydrolytic bacteria and chemical pretreatment using NaOH were done to improve the methane yield. Moreover, biological hydrolysis pretreatment further compared the effectiveness of the conventional hydrolytic bacteria and marine hydrolytic bacteria to hydrolyze *Ulva*. In case that both hydrolytic bacteria for the freshwater and thalassic set-ups are limiting, 1% NaOH pretreatments were also done to compare the performance of methane fermentation between freshwater and thalassic.

In biological hydrolysis pretreatment, the pH immediately dropped to 5.14 ± 0.01 (Thalassic) and 5.30 ± 0.02 (Freshwater) (Figure 2.9). This is most likely due to the production of organic acids after the hydrolysis of lipid, protein and carbohydrate component of *Ulva*. The higher pH difference between the start and end of biological hydrolysis under thalassic condition may signify marine hydrolytic bacteria as more effective than the conventional hydrolytic bacteria.

On the other hand, it took 5 days for 1% NaOH pretreatment to significantly lower the pH (Figure 2.9) in freshwater (7.30 ± 0.19) and thalassic (7.38 ± 0.15) to suitable level for methanogenesis. The high alkalinity of NaOH may have allowed the dissolution of some cellulose (Isogai, 1997) and ulvan (Chiellini and Morelli, 2011) for later utilization. This may also disrupt the structural integrity of the tissue, increasing its overall accessibility for microorganisms. Also, the buffering capacity of seawater could have caused the lower starting pH in 1% NaOH pretreatment of thalassic than freshwater set-up, while the seawater's slightly basic property could

have caused the higher starting pH in biological hydrolysis pretreatment of thalassic than in freshwater set-up (Figure 2.9).

The correlation of biological hydrolysis and 1% NaOH pretreatments on the methane fermentation process are further discussed in 2.3.4.

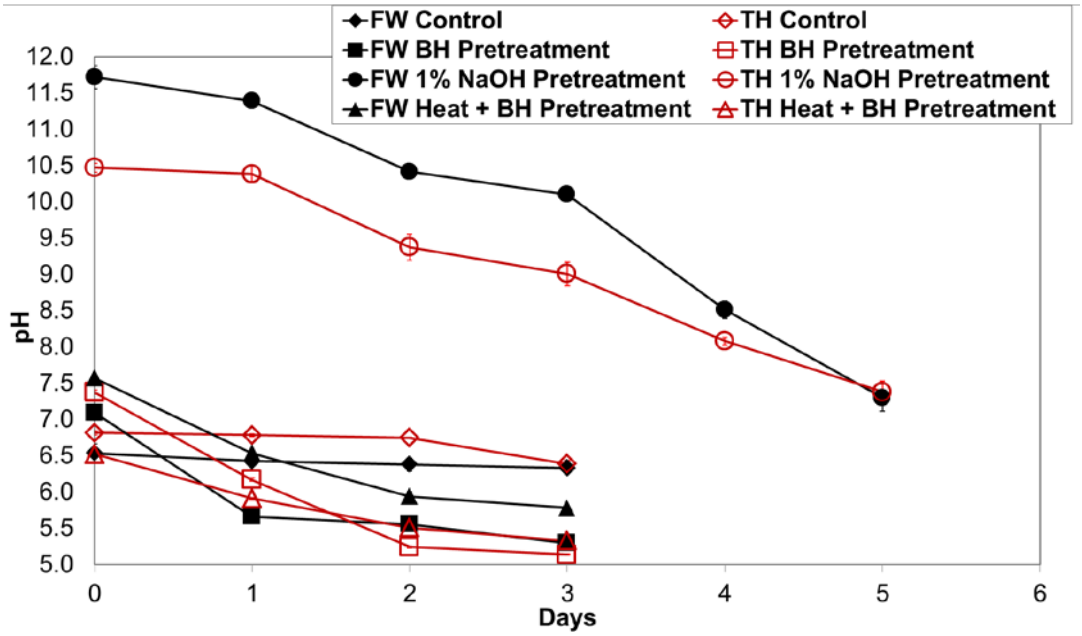

Figure 2.10 The pH change of the thalassic and freshwater set-ups under different pretreatment conditions before the start of the biogas production process. N = 3; mean ± S.D.; FW- Freshwater; TH- Thalassic; BH- Biological Hydrolysis.

2.3.3. Methane fermentation under freshwater and thalassic condition

The anaerobic digestion of *Ulva* sp., without any pretreatment (Control), showed higher cumulative methane yield under thalassic condition (Figure 2.10) by 102%. Hence, marine methane fermentation inoculum can perform better than the conventional methane fermentation seed under their corresponding suitable conditions. However, the computed biogasification efficiencies of the freshwater and thalassic controls, based on the theoretical and actual methane yield (Figure 2.11), are only 19.1% and 36.9%, respectively. These low efficiencies may be due to the difficulty of hydrolyzing the *Ulva* biomass, thereby limiting the methane fermentation. Therefore, biological hydrolysis, 1% NaOH pretreatment, and heat + biological hydrolysis pretreatment were further done to increase the anaerobic digestibility of *Ulva*.

Nonetheless, the successful development of marine methane fermentation inoculum that can be used under high salinity (38 ppt) may lower the cost of biogas technology through seawater usage.

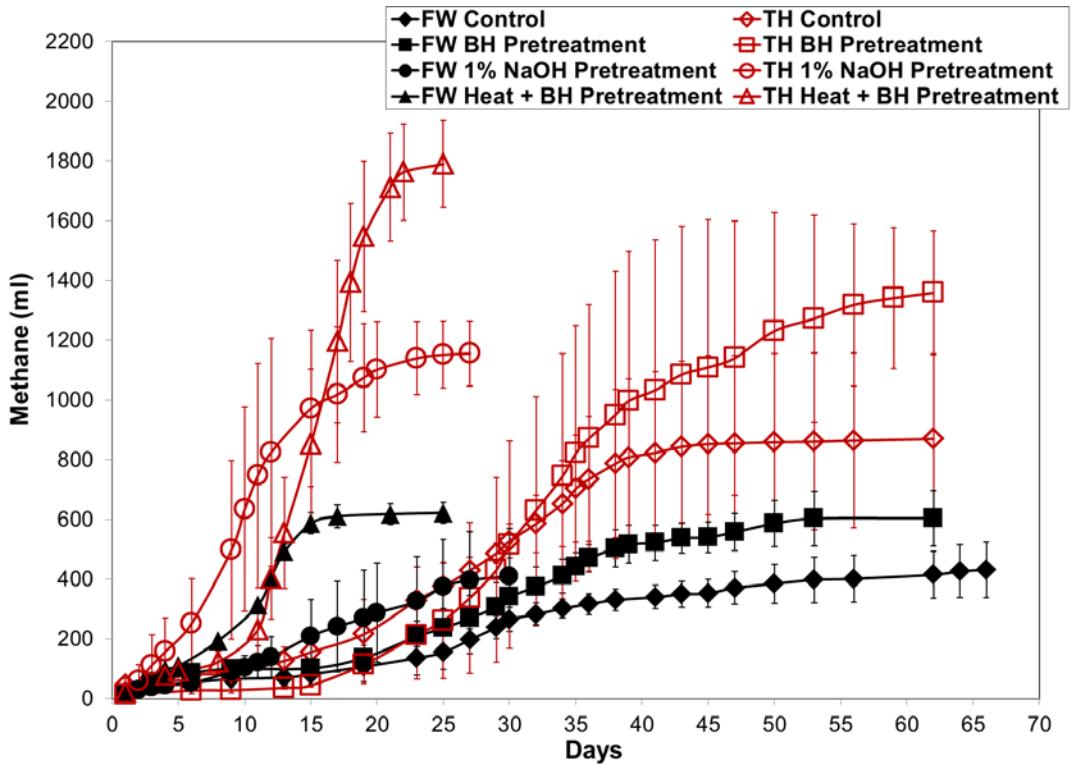

Figure 2.11 The cumulative methane production of the four pretreatments under thalassic and freshwater conditions. *N* = 3; mean ± S.D.; FW- Freshwater; TH- Thalassic; BH- Biological Hydrolysis

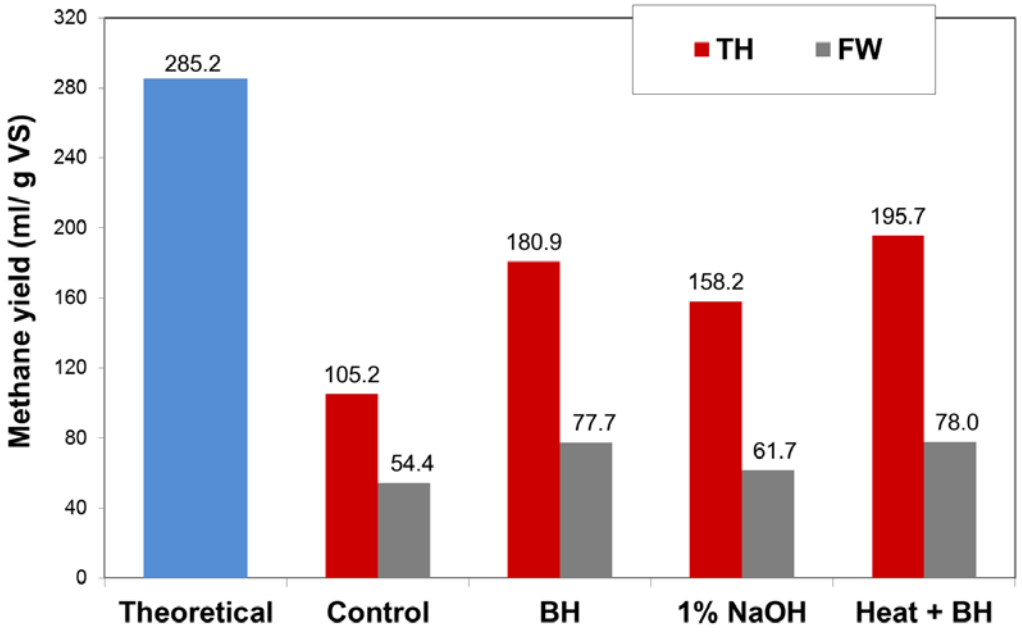

Figure 2.12 The theoretical, and average specific methane yield of the different pretreatments of freshwater (FW) and thalassic (TH) set-ups. $N = 3$

2.3.4. The effect of BHP, CNP, and heating on the methane fermentation under FW and TH conditions

The total biogas yield of the biological hydrolysis pretreatment (Freshwater: 1,112.7 ± 182.9 ml, Thalassic: 1,969.3 ± 215.0 ml) and 1% NaOH pretreatment (Freshwater: 956.0 ± 270.4 ml, Thalassic: 2,224.0 ± 449.1 ml) was higher than the control (Freshwater: 898.3 ± 152.7 ml, Thalassic: 1,496.3 ± 520.2 ml) of both set-ups (Figure 2.12). This suggests the effectiveness of both pretreatments to improve the biogas production performance. However, there is no significant difference (*p*-value = 0.12) between the cumulative biogas yield of the thalassic biological hydrolysis pretreatment and thalassic 1% NaOH pretreatment. The same relationship was observed between freshwater biological hydrolysis pretreatment and freshwater 1% NaOH pretreatment. Although the difference in biogas yield between pretreatments is not significant, the rate of biogas production of 1% NaOH pretreatment is faster than the biological hydrolysis pretreatment (Figure 2.12).

The higher cumulative methane of the thalassic biological hydrolysis pretreatment than the thalassic 1% NaOH pretreatment (Figure 2.10) is due to the difference in their methane level (Control= 58.3%, Biological hydrolysis= 68.8%, 1% NaOH= 53.2%). The same methane level trend was observed between freshwater biological hydrolysis and freshwater 1% NaOH pretreatments (Control= 47.7%, Biological hydrolysis= 54.3%, 1% NaOH= 41.7%). Comparing the specific methane yield (Figure 2.11), the improvement on the methane yield by the biological hydrolysis and 1% NaOH pretreatment under freshwater condition is not significant

(*p*-value = 0.30), suggesting the limitation on methane fermentation process. The poor fermentability of ulvan (Durand et al., 1997) by the conventional methane fermentation inoculum may have caused the poor methane fermentation performance under freshwater condition. On the other hand, the biological hydrolysis and 1% NaOH pretreatments under thalassic condition significantly improved (*p*-value = 0.03) the specific methane yield (Figure 2.11). The thalassic biological hydrolysis pretreatment improved the biogasification efficiency by 63.4%, while the thalassic 1% NaOH pretreatment by 55.5%. This suggests that thalassic biological hydrolysis pretreatment is better than the thalassic 1% NaOH pretreatment, but the faster biogasification rate of thalassic 1% NaOH pretreatment may outweighs the higher methane conversion. On the other hand, heating the seaweed before biological hydrolysis pretreatment shortened the biogasification time (25 days), while obtaining slightly higher methane yield than the other pretreatments and gaining the highest biogasification efficiency (68.6%).

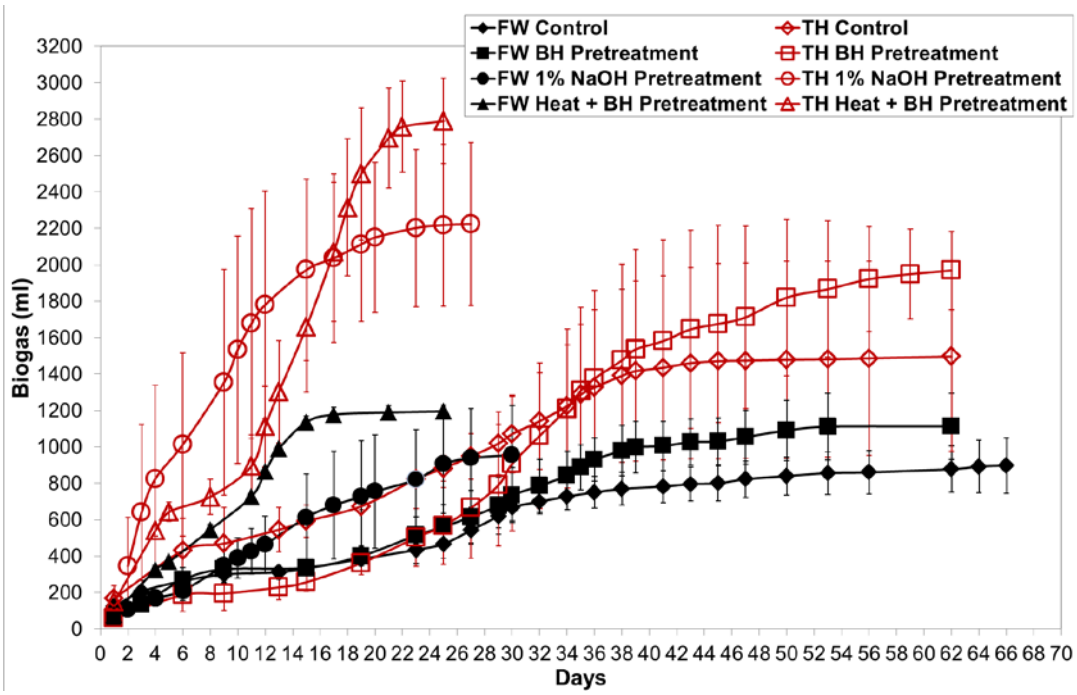

Figure 2.13 The cumulative biogas production of the four pretreatments under thalassic and freshwater conditions. *N*= 3; mean ± S.D.; FW- Freshwater; TH- Thalassic; BH- Biological Hydrolysis

The thalassic anaerobic digestion of *Percursaria percusa* and *Enteromorpha* spp. obtained higher methane yield when marine sediment was used instead of activated sludge (Schramm and Lehnberg, 1984). Higher methane yield was also reported under thalassic condition when marine sediment was added instead of cow manure (Marquez et al., 2013). The diverse population of methanogens from the marine environment might have supported the better performance under thalassic condition. Most freshwater/conventional biogas digesters (running under very low salinity condition) that are fed with either terrestrial feedstock (Ellis et al., 2012; McHugh et al., 2003; Rivière et al., 2009) or seaweed biomass (Pope et al., 2013) were reported to have aceticlastic Methanosaetaceae dominance. However, Methanosarcinaceae dominated the thalassic digester that is fed with *Saccharina japonica* (Miura et al., 2014). The increasing population of Methanosarcinaceae in different thalassic digesters was also suggested to be correlated to the better methane yield (Marquez et al., 2015c). The mixotrophic nature of Methanosarcinaceae may allow conversion of more substrate products from acido- and acetogenesis by utilizing aceticlastic, hydrogenotrophic, and methylotrophic methanogenesis. This capability may reduce the necessity of methanogen population shift that is dictated by the dynamic production of their preferred substrate. Maintaining a stable methanogen population can reduce the risk of substrate imbalance, thereby increasing the methane generation efficiency. In the marine environment, Methanosarcinaceae was found to dominate (Lowe et al., 1993). Hence, utilizing a thalassic condition like that in marine environment may further support its growth. This may be the reason of the better

performance of methane fermentation under thalassic condition. Moreover, the salt removal in the biomass is no longer needed under this condition, lowering the operational cost of the digester.

2.4. Conclusion

The low improvement of the methane yield on both pretreatments under the freshwater condition, while significantly improving that of the thalassic, suggested a superior methane fermentation under thalassic. In thalassic, the biological hydrolysis pretreatment (180.9 ml CH_4/ g VS) obtained higher methane yield than the 1% NaOH pretreatment (158.2 ml CH_4/ g VS), but the biogasification time of 1% NaOH pretreatment (27 days) is shorter than that of the biological hydrolysis pretreatment (62 days). Heating the seaweed before employing the biological hydrolysis pretreatment further improved the methane yield and shortened digestion time. The low-cost operation of the thalassic household digester can benefit from the cheaper biological hydrolysis pretreatment. Alternatively, the Heating + BH and 1% NaOH pretreatment application may be more favorable to commercial biogas plant operation.

Chapter 3

Thalassic methane fermentation of rice straw as mono-substrate, and co-substrate of the green seaweed *Ulva* sp.

3.1. Introduction

The *Ulva* sp. is widely distributed green seaweed, ranging from the tropical shores of the Philippines (Marquez et al., 2014), sub-temperate coasts of Japan, and temperate waters of the United Kingdom (Wichard et al., 2015). Their bloom is known as green tides, which is becoming a common occurrence in shallow waters that exhibit eutrophication (Leliaert et al., 2009; Teichberg et al., 2010). The most extensive green tides were reported in Brittany, France, and Qingdao, China, wherein

the biomass accumulated up to 100,000 tons (Smetacek and Zingone, 2013) and 1 million tons (Liu et al., 2013), respectively. Costly trucks and manual labors were needed to properly dispose these unwanted biomasses (Liu et al., 2013; Smetacek and Zingone, 2013). To recover some expenses for its disposal and exploit their energy potential, seaweed utilization for biofuel production has been reported (Bruhn et al., 2011; Chynoweth et al., 1993; Daroch et al., 2013; Demirbas, 2011; Marquez et al., 2015a). Among the biofuel technologies, biogas production was commonly preferred because it can be directly used for cooking or electricity generation in rural communities (Grima-Olmedo et al., 2014; Huopana et al., 2013; Marquez et al., 2015a). In the biogas production, four anaerobic digestion processes convert biomass to biogas. The first process is the hydrolysis, wherein volatile solids of organic matter (carbohydrates, proteins, and lipids) are broken down into their monomer forms (monosaccharides, amino acids, and fatty acids, respectively). These monomers are then simultaneously converted into acetate and CO_2/H_2, and non-acetate organic acids (acidogenesis process). Non-acetate organic acids are further transformed into acetates (acetogenesis process), while methane bacteria use acetate and CO_2/H_2 substrates (through aceticlastic and hydrogenotrophic methanogenesis processes, respectively) to produce approximately 50-60% methane and 40-50% carbon dioxide (Chandra et al., 2012a; Marquez et al., 2015a; Nettmann et al., 2010).

Most biogas production studies on *Ulva* focused on the different pretreatment application to enhance methane yield (Bruhn et al., 2011; Karray et al., 2015; Morand and Briand, 1999), in which conventional microorganisms and low salinity condition

were used. The different proximate and structural compositions of seaweed (ulvan, alginate, agar, or carrageenan) as compared to terrestrial biomass (starch, hemicellulose, or lignocellulose) (Grima-Olmedo et al., 2014) prompted the utilization of marine bacteria for their anaerobic digestion. In the study of Marquez et al. (2015b), utilization of marine microbial inoculum for biogas production of *Ulva* sp. gave higher methane yield than when conventional microbial inoculum was used. Under this high salinity or thalassic condition, seawater was directly utilized as liquid substrate instead of freshwater (Marquez et al., 2015b), making the operation of household digester under this condition cheaper, and their management easier. Washing of seaweed to remove salt was also not needed. However, the supply of *Ulva* sp. biomass is not constant and relies only on their seasonal bloom. Hence, utilization of other types of biomass like the agricultural wastes is desirable to supplement seaweed feedstock.

In Asia, rice straw is widely accessible because 90% of the world's rice is harvested in this region (Muthayya et al., 2014). This makes rice straw an abundant agricultural waste (Delivand et al., 2011; Shafie et al., 2014) that can be exploited as substitute or supplement feedstock in thalassic biogas digesters. Many studies reported the utilization of rice straw for anaerobic digestion (Chandra et al., 2012b; Dehghani et al., 2015; Kim et al., 2013; Sari and Budiyono, 2014). However, the biogas production performance of terrestrial biomass has not been tested yet under thalassic condition. Compared to seaweed, the rice straw is a lignocellulosic biomass that is more recalcitrant for anaerobic digestion. The activity of marine bacteria used

for seaweed may be restrained for rice straw, which may lead to low biogasification efficiency or unstable biogas production. Hence, the biogas production performance of rice straw biomass under thalassic condition using the marine microbial inoculum to freshwater condition using the conventional microbial inoculum was compared. The effectiveness of marine hydrolytic bacteria to hydrolyze the rice straw under thalassic condition was also compared to the conventional hydrolytic bacteria under freshwater condition. Moreover, the effect of alkaline NaOH pretreatment of rice straw on the biogas production performance under both conditions was examined. This alkaline pretreatment may help lessen the limitation brought by the different [conventional and marine] hydrolytic bacteria (Marquez et al., 2015b) to supply the precursor substrates for methane fermentation. The effect of the co-digestion of rice straw with *Ulva* sp. on the performance of the thalassic biogas production was further tested. If thalassic anaerobic co-digestion of rice straw and *Ulva* sp. can successfully enhance the methane yield, the continuous operation of household thalassic biogas digester may not only be improved but also ensured because of the availability of alternative feedstock. The novel approach of this study to utilize rice straw as a model terrestrial lignocellulosic biomass for thalassic biogas production may pave way for the development of an alternative biogas platform that can be exploited in the midst of worsening intensity and increasing occurrence of drought.

3.2. Materials and Methods

3.2.1. Collection and preparation of rice straw and *Ulva* sp.

The rice straw was provided by the Biomass Power Shizukuishi, Nakakurosawagawa 17-7, Shizukuishi-cho, Iwate-gun, Iwate-ken, Japan. It was manually cut into ~1cm length and then dried at 105°C for 3 hours (AS ONE Forced Convection Drying Oven DO-450FA). The dried rice straw was macerated using a force mill (TDK Y-208B) and stored in a vacuum desiccator for later use. The percentage size of the macerated rice straw was determined using a Retsch Vibratory Sieve ShakerAS200 (100 amplitudes for 20 minutes). The sizes of the mesh of the sieves were 5 mm, 2 mm, 1 mm, 500 μm, 250 μm, and 100 μm.

The fresh *Ulva* sp. and seawater were collected at the coast of Kirachō, Miyazaki, Nishio-shi, Aichi-ken, Japan (34°46'50.39" N, 137°05'43.50" E) on July 19, 2014 as shown in Figure 3.1. Foreign matters were removed from the seaweed and excess seawater was allowed to drip before placing in zip locks for immediate transport to EcoTopia Science Institute Building, Nagoya University. The seaweed biomasses were frozen (-20°C) for future use. Some *Ulva* sp. was directly dried at 105°C for 36 hours (AS ONE Forced Convection Drying Oven DO-450FA) and then powdered (force mill TDK Y-208B) for the co-digestion experiment. While, some *Ulva* sp. biomass was firstly washed with freshwater, and then dried and powdered. Figure 3.2 shows the powdered and dried rice straw and *Ulva* sp. biomass. The granular size of the powdered *Ulva* sp. was also determined, using the same sieve

shaker used for rice straw, but at 80 amplitudes for 10 minutes. The percentage sizes of the rice straw and *Ulva* sp. were shown in Figure 3.3.

Figure 3.1 The map of the collection site of the *Ulva* sp. in Nishio City, Aichi-ken, Japan.

Figure 3.2 The fresh biomass (left), and dried and powdered biomass (right) of (A) rice straw and (B) *Ulva* sp.

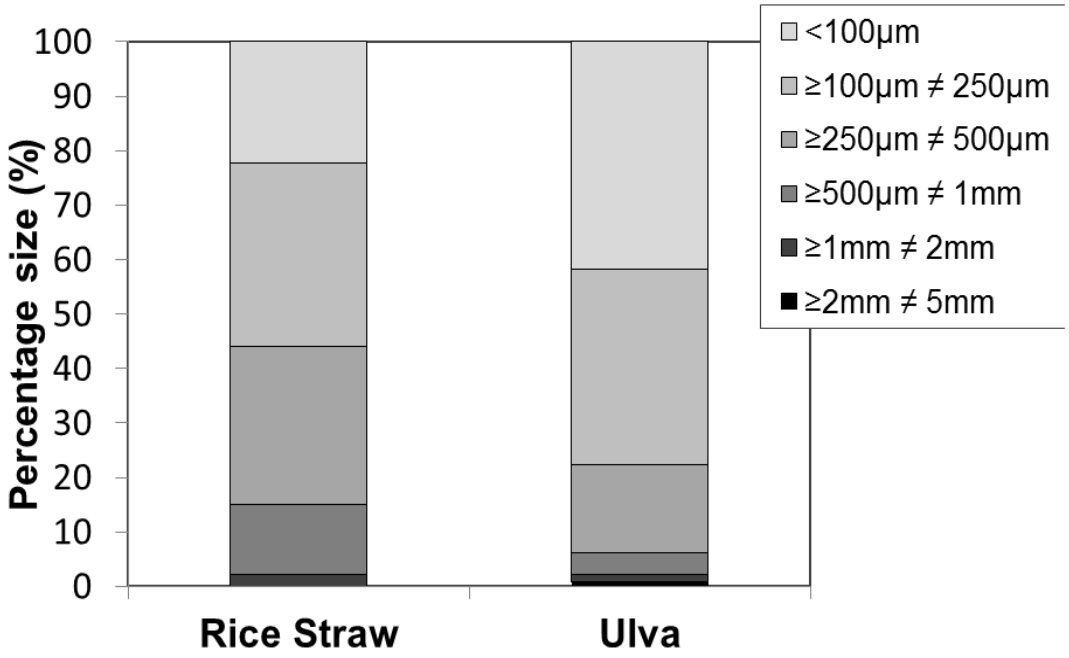

Figure 3.3 The percentage (%) size composition of the macerated rice straw and powdered *Ulva* species.

3.2.2. Characterization and theoretical methane yield of rice straw and *Ulva* species

The moisture content (MC), total solids (TS), volatile solids (VS), and ash content of both rice straw and *Ulva* sp. were determined using the standard procedure (AOAC, 1990). The forced convection drying oven (AS ONE DO-450FA) was used to measure MC and TS (105°C, until constant weight), while the burn out furnace (KDF007EX) was used for ashing (550°C, until constant weight). The proximate compositions (lipid, protein, carbohydrate, cellulose, hemicelluloses, lignin, and C/N

ratio) were measured by ChugaiTechnos Corporation (Yokogawa-shinmachi, Nishi-ku, Hiroshima-shi, Japan), using the standard procedure described by the previous study (Marquez et al., 2015b). The theoretical methane yield (TMY) was computed using the equation TMY = {[(0.49 L CH_4 / g VS) x (P)] + [(0.85 L CH_4 / g VS) x (L)] + [(0.395 L CH_4 / g VS) x (C)]} / VS (kg) of the biomass, where P, L, and C are the actual weights of the protein, lipid, and carbohydrate, respectively, of the biomass, while 0.49 L CH_4 / g VS, 0.85 L CH_4 / g VS, and 0.395 L CH_4 / g VS are their corresponding constant values as described by Marquez et al. (2013).

3.2.3. Development of microbial seeds

3.2.3.1. Biological hydrolysis inoculum

Specific biological hydrolysis inoculum was developed for each freshwater (Figure 3.4) and thalassic set-up (Figure 3.5). For freshwater set-up, 300 g of initial bacteria were acquired from the freshwater hydrolysis bacteria (HB) inoculum developed by Marquez et al. (2015b) that was originally obtained from the continuous fixed-bed reactor (Chapter 2). The substrate mixture of 20 g of macerated rice straw, 20 g of powdered washed *Ulva* sp., and 360 g of distilled water was further added in the 1-L bottle (Schott Duran). It was incubated at 37 °C (Yamato model IN602W) for 60 days in the dark before use. The HB bottle was sealed to limit oxygenation. It was manually shaken every day for 30 seconds and was quickly opened every 5 days to allow breathing. Every 20 days, 300 g of hydrolyzed substrates were removed to be later used as substrate in the freshwater methane fermentation (MF) inoculum.

Addition of the same substrate mixture, as described above, was done whenever hydrolyzed substrates are transferred to MF bottle. The same procedures and substrate ratio were used for the development of HB inoculum for the thalassic set-up, except for the utilization of initial bacteria from the developed marine hydrolytic bacteria that was cultured from the mixture of seawater, sands and sediments (Chapter 2; Marquez et al., 2015b), and the addition of powdered unwashed *Ulva* biomass and seawater. Two liters of HB inocula under freshwater and thalassic conditions were continuously maintained.

3.2.3.2. Methane fermentation inoculum

The MF inoculum was separately developed for freshwater and thalassic set-ups. The initial methane bacteria (300 g) for freshwater set-up were obtained from the previously developed freshwater MF inoculum (Chapter 2; Marquez et al., 2015b) that was also originally collected from the continuous fixed-bed reactor. It was placed in a 1-L bottle (Schott Duran), and was added with the substrate mixture of pH-adjusted (~pH 7.8, 2 M NaOH) 300 g of hydrolyzed substrates from the freshwater HB inoculum bottle, 2 g of macerated rice straw, 2 g of powdered washed *Ulva* sp., and 100 g distilled water. On the other hand, the initial methane bacteria (300 g) for thalassic set-up were from the marine MF inoculum that was also previously developed from the mixture of seawater, sands and sediments (Chapter 2; Marquez et al., 2015b). The same substrate mixture ratio was added in the marine MF inoculum, except for using the hydrolyzed substrate from the marine HB inoculum, seawater instead of distilled water, and powdered unwashed *Ulva* species. Inocula of both set-

ups were anaerobically sealed by pumping N_2 gas, and incubated at 37°C (Yamato model IN602W) in the dark for 60 days. They were manually shaken every day for 30 seconds, and allowed the produced biogas to anaerobically escape using water displacement. Methane fermentation residues (300 g) were removed every 20 days whenever 300 g of pH-adjusted hydrolyzed substrates are added, and then anaerobically resealed. Two liters of MF inocula were also continuously maintained under both conditions.

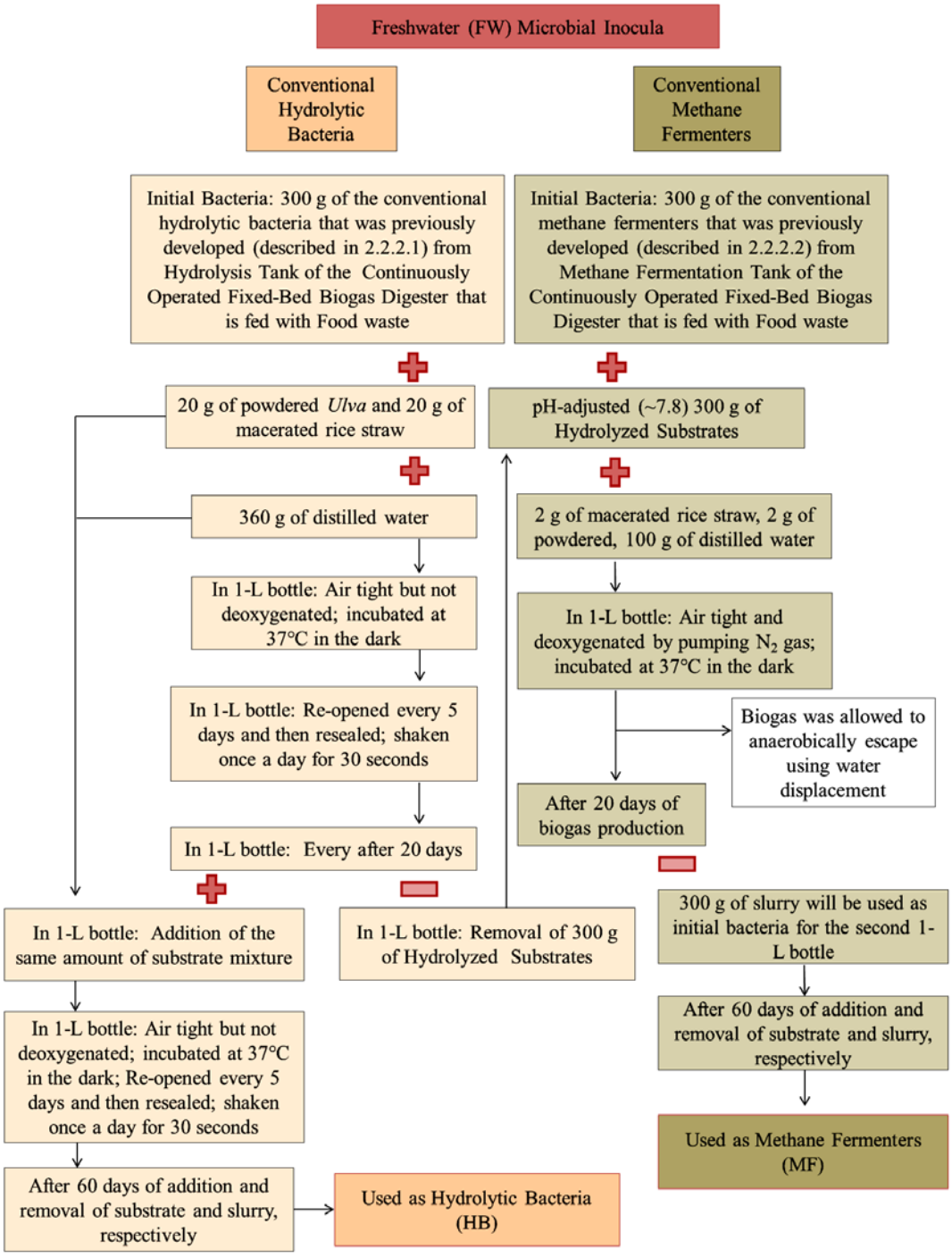

Figure 3.4 The schematic diagram of the development of the conventional hydrolytic bacteria and methane fermenters for freshwater set-up.

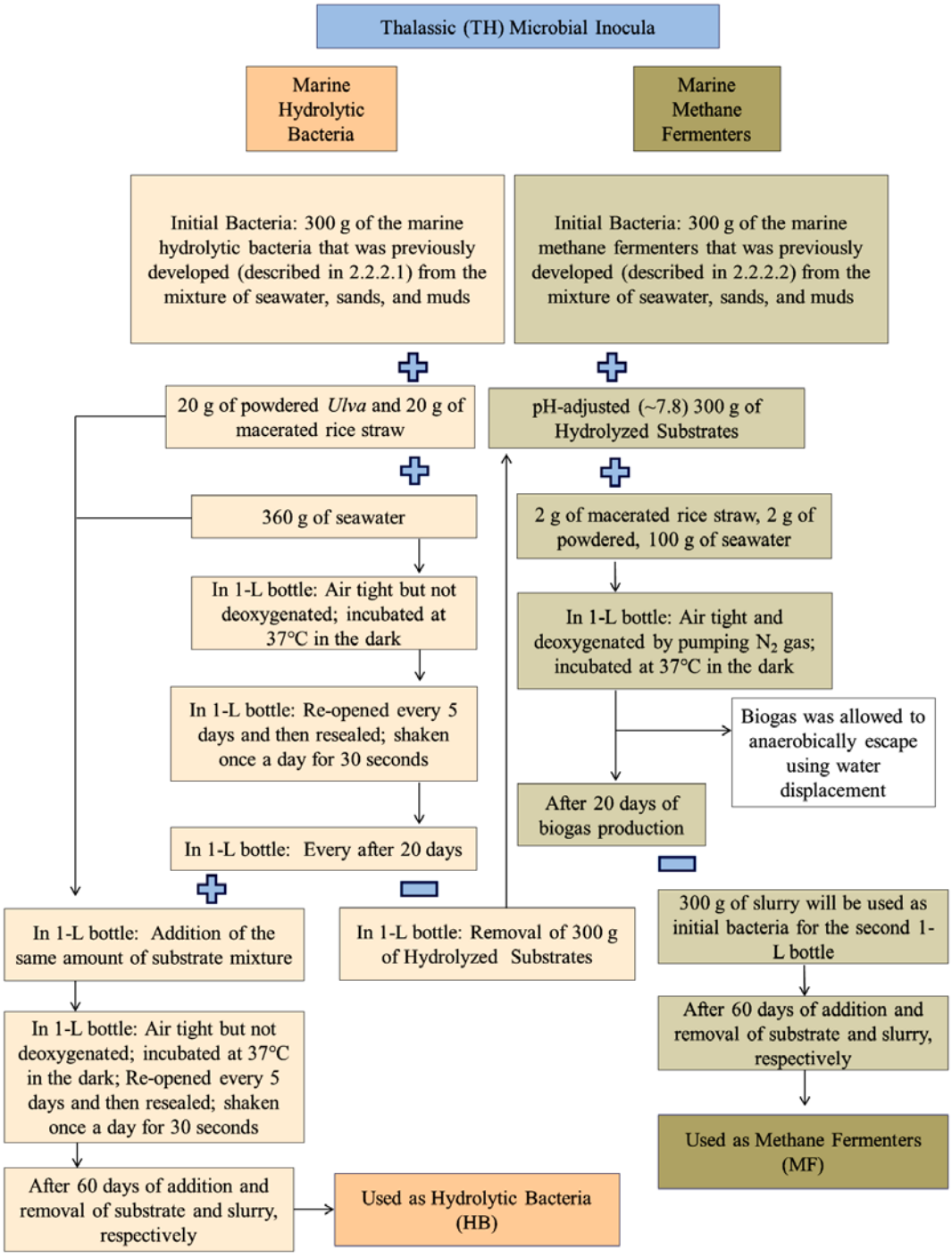

Figure 3.5 The schematic diagram of the development of the marine hydrolytic bacteria and methane fermenters for thalassic set-up.

3.2.4. Biogas production experiment of rice straw under freshwater and thalassic conditions

The biogas production performance of rice straw in freshwater condition (Figure 3.6) was compared to thalassic condition (Figure 3.7). Biological hydrolysis pretreatment (BH), before anaerobic digestion, was done to compare the hydrolytic performance of marine and freshwater bacteria. On the other hand, 3% NaOH pretreatment (NaOH) was further done to minimize the effect of hydrolysis limitation on rice straw by the different freshwater and marine hydrolytic bacteria. One-liter bottle (Schott Duran) was used as batch digester (N= 3). The microbial seeds, biomass and liquid substrates used in each pretreatment of each set-up were summarized in Table 3.1. All batch digesters during pretreatment and anaerobic digestion were incubated at 37 °C (Yamato model IN602W) in the dark. The salinities of all set-ups (FW: BH = 7.67 ± 0.58 ‰, NaOH = 7.33 ± 0.58 ‰; TH: BH = 36.0 ± 0.0 ‰, NaOH = 36.67 ± 0.58 ‰) were measured using AS ONE refractometer (Master-AS/Millα).

3.2.4.1. Biological hydrolysis pretreatment

The biological hydrolysis pretreatment on both freshwater and thalassic conditions was started by adding 125 g of FW and TH HB inocula into their corresponding biomass and liquid substrate mixtures (Table 3.1). They were then allowed to hydrolyze for 3 days. The pH of all bottles was then adjusted (pH: FW = 7.95 ± 0.01, TH = 7.91 ± 0.02) before addition of their corresponding MF inocula (pH: FW = 7.47 ± 0.03, TH = 7.62 ± 0.02). Deoxygenation was done by pumping N_2

gas to maintain anaerobic condition (in Chapter 2 Figure 2.7), and start anaerobic digestion.

3.2.4.2. NaOH pretreatment

The 3% NaOH pretreatment was done by adding 0.75 g of NaOH pellet (97% purity, Kanto Chemical Co.) that is 3% of the weight of the biomass, to the mixture of biomass and liquid substrates (Table 3.1). The substrates were then incubated for 5 days to allow alkaline dissolution and hydrolysis. The pH of both set-ups was then adjusted (pH: FW = 7.99 ± 0.09, TH = 7.90 ± 0.09) before the addition of their corresponding MF inocula (pH: FW = 7.93 ± 0.08, TH = 7.30 ± 0.16), and initiation of anaerobic digestion.

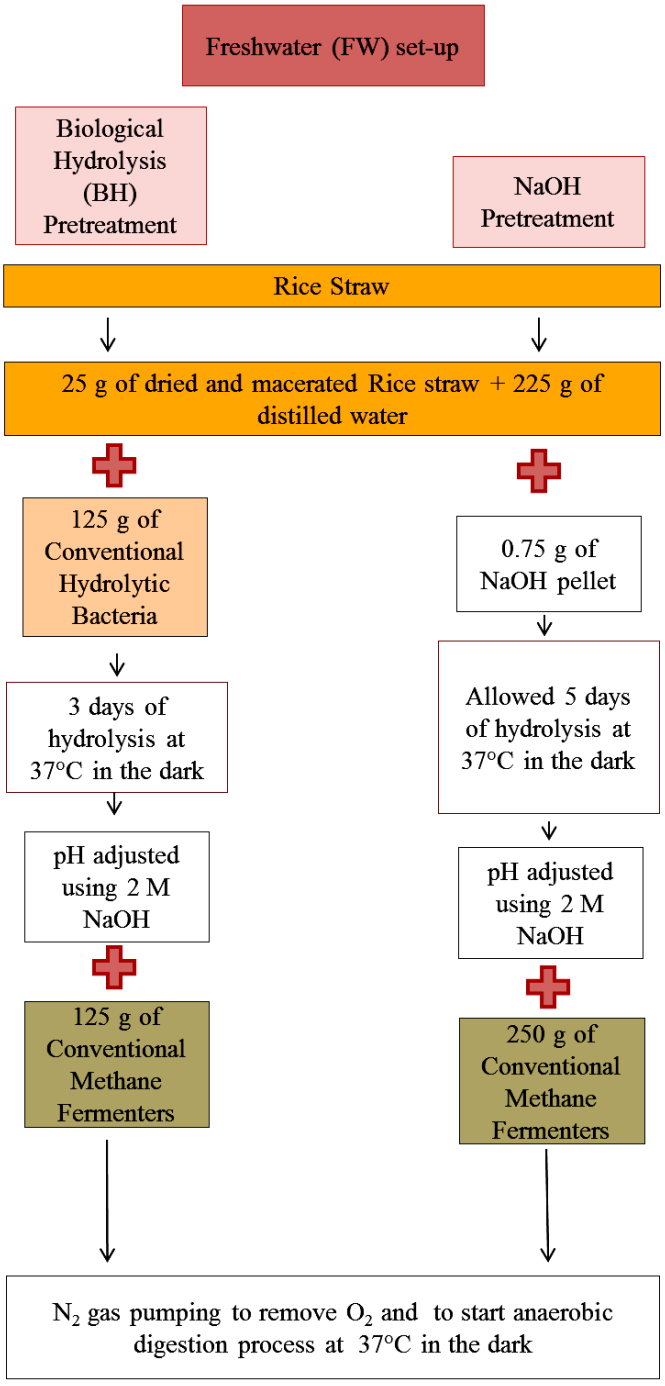

Figure 3.6 The schematic diagram of the preparation of the biological hydrolysis and NaOH pretreatment set-ups before the start of the biogas production experiment under freshwater (FW) condition.

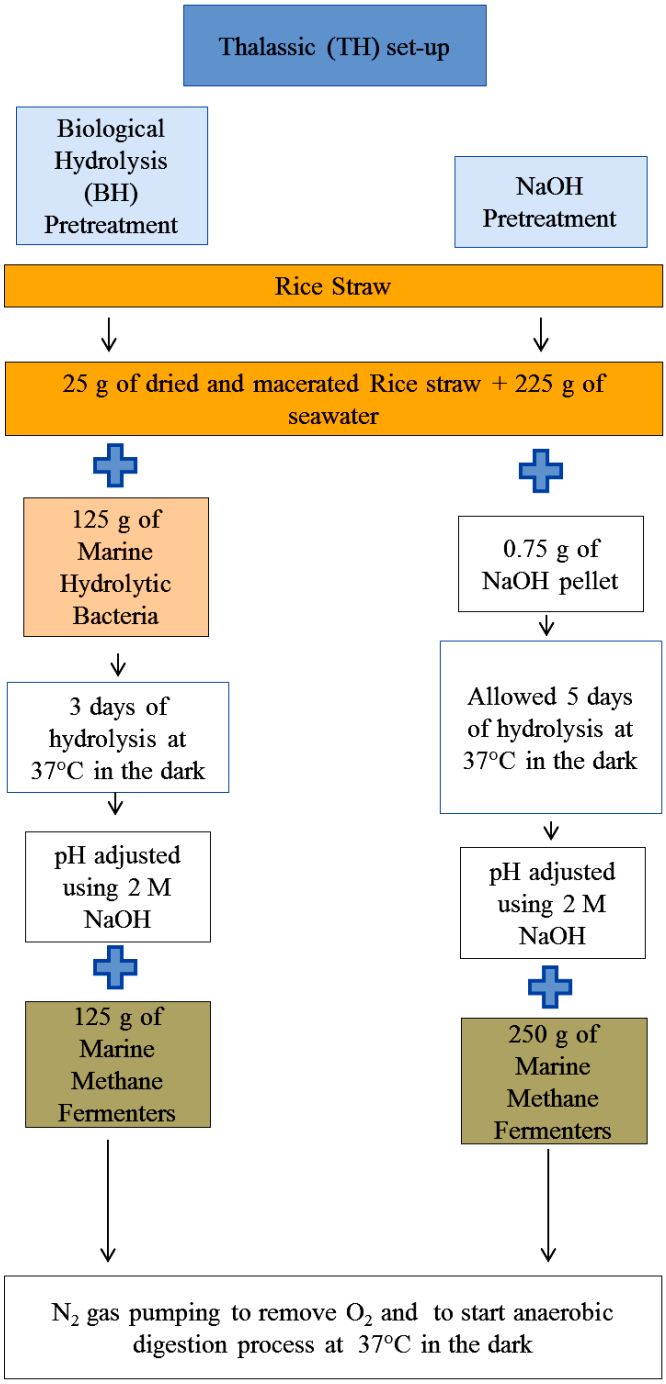

Figure 3.7 The schematic diagram of the preparation of the biological hydrolysis and NaOH pretreatment set-ups before the start of the biogas production experiment under thalassic (TH) condition.

Table 3.1 The substrate compositions of the anaerobic mono-digestion set-ups of rice straw under freshwater and thalassic conditions, and anaerobic co-digestion of rice straw and *Ulva* sp. under thalassic condition.

Set-ups			Biomass substrate	Liquid substrate	Microbial seeds
AMD	FW	BH	25 g of macerated rice straw	225 g of distilled water	125 g FW HB, 125 g FW MF
AMD	FW	NaOH	25 g of macerated rice straw	225 g of distilled water	250 g FW MF
AMD	TH	BH	25 g of macerated rice straw	225 g of seawater	125 g TH HB, 125 g TH MF
AMD	TH	NaOH	25 g of macerated rice straw	225 g of seawater	250 g TH MF
ACD	TH BH	1:0	25 g of powdered *Ulva* sp.	225 g of seawater	125 g TH HB, 125 g TH MF
ACD	TH BH	75:25	18.75 g of powdered *Ulva* sp. + 6.25 g of macerated rice straw	225 g of seawater	125 g TH HB, 125 g TH MF
ACD	TH BH	50:50	12.5 g of powdered *Ulva* sp. + 12.5 g of macerated rice straw	225 g of seawater	125 g TH HB, 125 g TH MF
ACD	TH BH	25:75	6.25 g of powdered *Ulva* sp. + 18.75 g of macerated rice straw	225 g of seawater	125 g TH HB, 125 g TH MF

AMD- anaerobic mono-digestion; ACD- anaerobic co-digestion; FW- freshwater; TH- thalassic; BH- biological hydrolysis pretreatment; NaOH- 3% NaOH (w/w) pretreatment; HB- hydrolytic bacteria; MF- methane fermenters

3.2.5. Thalassic anaerobic co-digestion experiment of rice straw and *Ulva* species

Different rice straw to *Ulva* sp. ratios were co-digested under thalassic condition (Table 3.1). All co-digestion set-ups (*N*= 3) were biologically hydrolytically pretreated for 3 days by adding 125 g of TH HB inoculum (Figure 3.8). The pH was then adjusted (*Ulva*:Rice straw; 1:0 = 7.97 ± 0.07, 75:25 = 7.98 ± 0.01, 50:50 = 7.99 ± 0.07, 25:75 = 7.91 ± 0.02) using 2 M NaOH before addition of 125 g of TH MF inoculum (*Ulva*:Rice straw; 1:0 = 7.43 ± 0.08, 75:25 = 7.50 ± 0.06, 50:50 = 7.47 ± 0.08, 25:75 = 7.52 ± 0.05). Deoxygenation was done by pumping N_2 gas. All batch digesters were incubated at 37°C (Yamato model IN602W) in the dark. The salinities of the co-digestion set-ups (*Ulva*:Rice straw; 1:0 = 45.3 ± 0.6 ‰, 75:25 = 42.7 ± 0.6 ‰, 50:50 = 41.7 ± 0.6 ‰, 25:75 = 42.3 ± 0.6 ‰) were obtained.

3.2.6. Biogas analysis

The total volume capacity of the digester bottle was 1130 ml, and the working volume was 500 ml. Water displacement (in Chapter 2 Figure 2.8) was used to measure the volume of biogas as described by Chandra et al. (2012b). The biogas composition (CH_4, CO_2, and others) was analyzed using a gas chromatograph (Yanaco G1880: Injecting volume of 0.2 ml; Column temperature of 80°C; Injector temperature of 50°C; Helium gas flow rate of 0.098 MPa; Current 80 mA) that is equipped with a Porapak Q Column (Length is 2 m, O.D. is 4 Ø, I.D. is 3 Ø, 80-100 mesh) and a thermal conductivity detector (TCD). The biogas analysis on each replicate was done twice. The volume of methane and carbon dioxide was computed

by multiplying the biogas and headspace volume to the volumetric percentage obtained from the gas chromatography analysis.

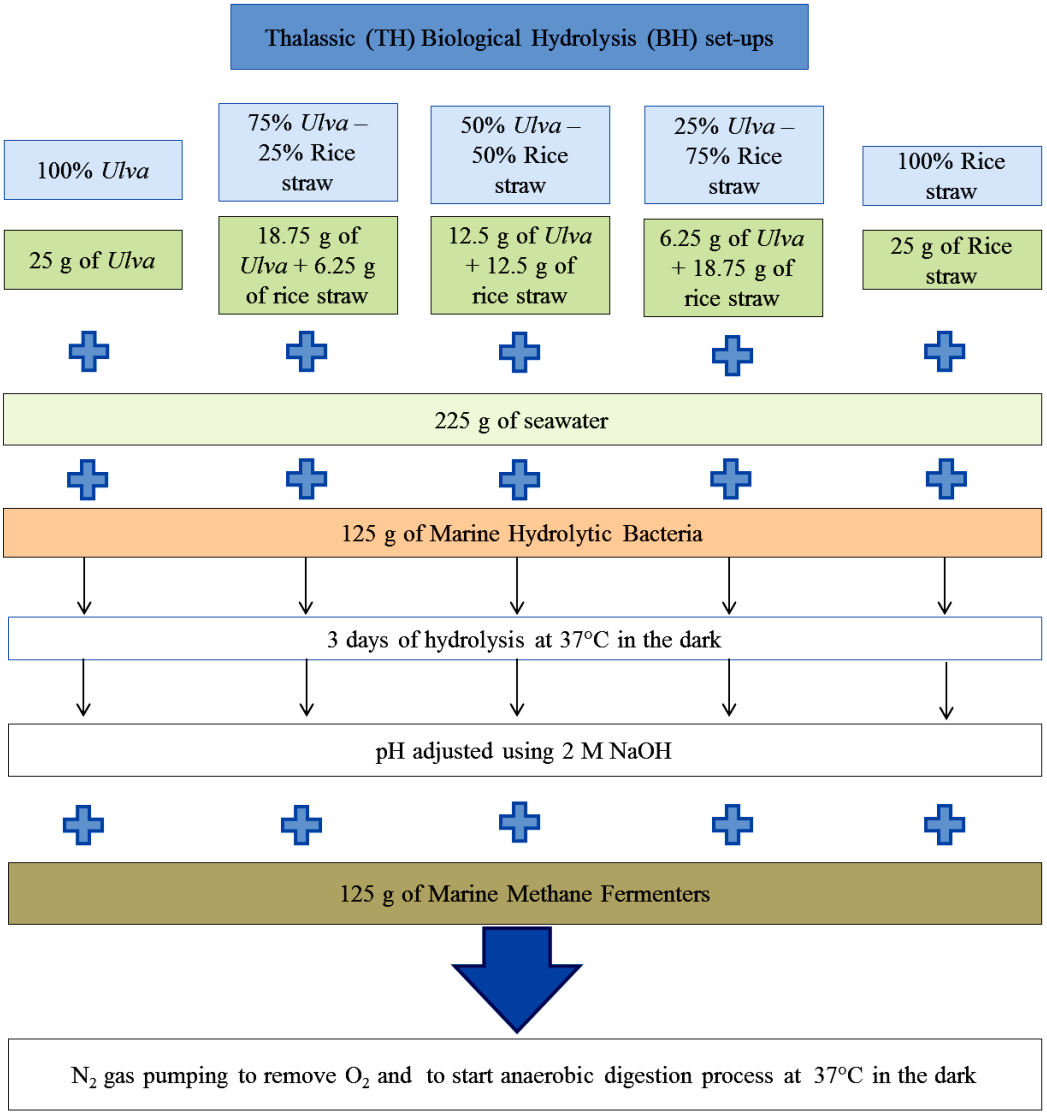

Figure 3.8 The schematic diagram of the biological hydrolysis (BH) pretreatment set-ups of the different percentage mixtures of *Ulva* and rice straw before the start of the biogas production experiment under thalassic (TH) condition.

3.2.7. Data analysis

The data were presented as mean ± standard deviation (SD). Two-way analysis of variance with replication (ANOVA, $\alpha = 0.05$) was done using Microsoft excel program to determine if the differences between set-ups in rice straw as mono-substrate are significant. One-way ANOVA ($\alpha = 0.05$) was used to compare the differences between co-digestion set-ups. The high standard deviation of the biogas and methane yield of the mono-digestion of rice straw and its co-digestion with *Ulva* may have been due to the addition of the microbial seeds with heterogeneous bacterial population, most especially the conventional microbial inocula. The utilization of non-homogeneous powdered biomass with varying granular size may have also contributed to the different fermentation efficiency within replicates, affecting standard deviation values.

3.3. Results and discussion

3.3.1. Proximate compositions and theoretical methane yield

The proximate compositions of the unprocessed rice straw and *Ulva* sp. biomasses are summarized in Table 3.2. The *Ulva* sp. biomass has high moisture and ash contents than the rice straw, thereby giving the rice straw a higher theoretical methane yield (327.9 ml CH_4/ g VS) than the *Ulva* species (238.7 ml CH_4/ g VS). Both biomasses have high carbohydrates. However, the cell wall of *Ulva* sp. is mainly composed of water-soluble ulvan and cellulose (Lahaye and Robic, 2007), making its

structure easily accessible to enzymatic actions. In contrast, the cell wall of rice straw is made up of complex lignocellulosic structure that further insulates its cellulose and hemicellulose components from bacterial attacks (Chandra et al., 2012a). Also, the lignocellulosic structure is resistant to biological degradation (Chandra et al., 2012a) under anaerobic condition because oxygen is needed in destroying the carbon-to-carbon or carbon-to-oxygen-to-carbon linkages of lignin through the production of hydrogen peroxide (Ruiz-Dueñas and Martínez, 2009). Therefore, biogas production of rice straw may obtain lower methane yield than the *Ulva* sp. To maximize degradation of rice straw, the biological hydrolysis of both FW and TH conditions were done under slightly aerobic conditions. This allows the activity of not only facultative hydrolytic bacteria but also fungi. On the other hand, the high C/N ratio of rice straw may affect its methane fermentation by limiting the available nitrogen that is required in protein synthesis of microorganisms. In the study of Vivekanand et al. (2012), co-digesting wheat straw and *Saccharina latissima* enhanced methane yield of up to 120%. Mixing of different organic wastes to obtain C/N ratio between 25 and 30, also gave better biogas production performance (Wang et al., 2014). In this study, the co-digestion of *Ulva* and rice straw was done to lower the overall C/N ratio of the substrate, but the different C/N ratios obtained from the different biomass mixtures were still higher than the suggested optimum C/N ratios (25-30) (Marquez et al., 2015b; Marquez et al., In press a; Vivekanand et al., 2012). This may limit the methane yield of the different co-digestion set-ups. On the contrary, even though the seaweed *Undaria pinnatifida* and *Ulva* sp. had low C/N ratios (10.5 (Marquez et al.,

In press a) and 16.05 (Marquez et al., 2015b), respectively), they obtained 68.3% and 63.4%, respectively, of their computed theoretical methane yield. This may suggest that the C/N may have minimum influence to the anaerobic digestion of seaweed. Furthermore, the previous C/N ratio obtained for the *Ulva* sp. that was collected at the same site and season but different year was lower (Chapter 2) (Marquez et al., 2015b) than this study. The seasonal variation of the composition of *Ulva* may be monitored to clearly demonstrate its effect on the performance of thalassic biogas production.

Table 3.2 The proximate compositions of the unprocessed biomass of the rice straw and *Ulva* species.

Proximate tests	Values (%, w/w)	
	Rice Straw	*Ulva* species
Total Solids	90.1	19.7
Moisture	9.9	80.3
Volatile Solids	90.1	65.6
Ash	9.9	34.4
Crude Carbohydrate	78.9	53.0
Crude Protein	2.1	5.3
Crude Lipid	0.7	0.4
Lignin	11.7	3.3
Cellulose	18.4	1.2
Hemicellulose	28.5	4.9
C/N	125	29

3.3.2. pH behavior during pretreatments

3.3.2.1. Rice straw as mono-substrate

The pH of BH pretreatment set-up under both TH and FW conditions exponentially dropped within a day of hydrolysis (Figure 3.9), indicating that some easily degradable substrates such as amorphous cellulose and hemicellulose may be readily accessible. While, the slow decreased of pH from the 1st day onwards may have been possibly due to the abated hydrolysis of the more recalcitrant residual substrates, thereby leading to the slower production of organic acids. The unproductive binding of the microbial enzymes on the substrate activation sites may have also lowered the hydrolysis rate (Eriksson et al., 2002). Nonetheless, the behaviours of pH in the BH pretreatment under the TH and FW conditions were the same. However, the pH values under FW were lower than the TH, suggesting either the better hydrolytic activity of conventional HB than the marine HB in hydrolyzing rice straw, or the seawater may have helped buffered (0.3 mEq/L) the drastic change of the pH under TH (Marquez et al., 2013). The comparison of the methane yield of the BH pretreatment set-ups between both conditions supported the latter premise. On the other hand, the exponential decline of pH in the NaOH pretreatment under both FW and TH conditions was immediately observed until the 2nd day, indicating the alkaline hydrolysis of lignocellulosic complexes. Without applying high temperature to alkaline pretreatment, the early pH change in NaOH pretreatment was mainly caused by the lignin removal (Zhang et al., 2015), exposing more cellulose and hemicellulose for later anaerobic degradation. The porosity of amorphous and

crystalline cellulose fiber may have also been increased (He et al., 2008). If cellulose and hemicellulose are more accessible, the anaerobic microorganisms can easily convert these substrates to organic acids during biogas production.

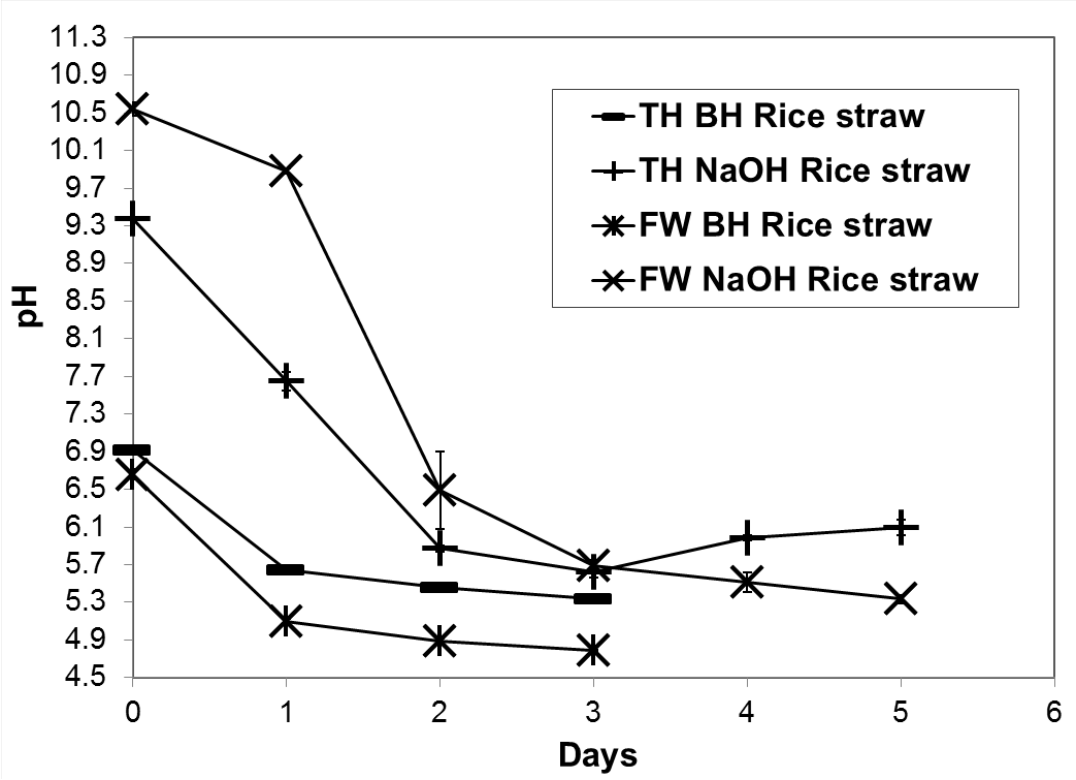

Figure 3.9 The pH change during the biological hydrolysis (BH) and chemical (NaOH) pretreatment of rice straw under freshwater (FW) and thalassic (TH) conditions.

3.3.2.2. Co-digestion of rice straw and *Ulva* sp.

The same pH behavior in the BH of 100% rice straw under TH condition (described in 3.3.2.1) was observed on the BH of 100% *Ulva* sp. for the first 24 hours (Figure 3.10). This drastic decreased of pH may be due to the availability of easily digestible components of *Ulva* sp. such as starch and hemicellulose (Yanagisawa et al., 2013). Ulvan in *Ulva* may have also been simultaneously hydrolyzed during the first day (Lahaye et al., 1997). This is because some microorganism like *Persicivirga ulvanivorans*, needs other carbon source whenever ulvan is digested (Collénet al., 2011). It was also reported that while ulvan is present in the substrate, partial inhibition on the cellulase activity for hydrolysis of α-cellulose was observed (Bobin-Dubigeon et al., 1997). The decline of the pH from the 1st to the 2nd day, although slower than within the first 24 hours, was significantly lower than the 100% rice straw's. This change may be due to the later hydrolysis of cellulose, which may have started after the hydrolysis of ulvan (Bobin-Dubigeon et al., 1997).

The pH change of the 75% *Ulva*-25% Rice straw and 50% *Ulva*-50% Rice straw was comparable to the pH change of 100% *Ulva*. This suggested that *Ulva* mainly influenced the pH behavior of the co-digestion set-ups. Also, the pH of the 25% *Ulva*-75% Rice straw decreased more than the 100% rice straw during the BH, but less than the remaining co-digestion set-ups. This may have been due to the presence of more recalcitrant structures from the rice straw. Overall, under thalassic condition, better pH change was observed when portion of *Ulva* was increased in the

mixture, showing the more effective hydrolytic activity of marine HB to the seaweed *Ulva* than the rice straw.

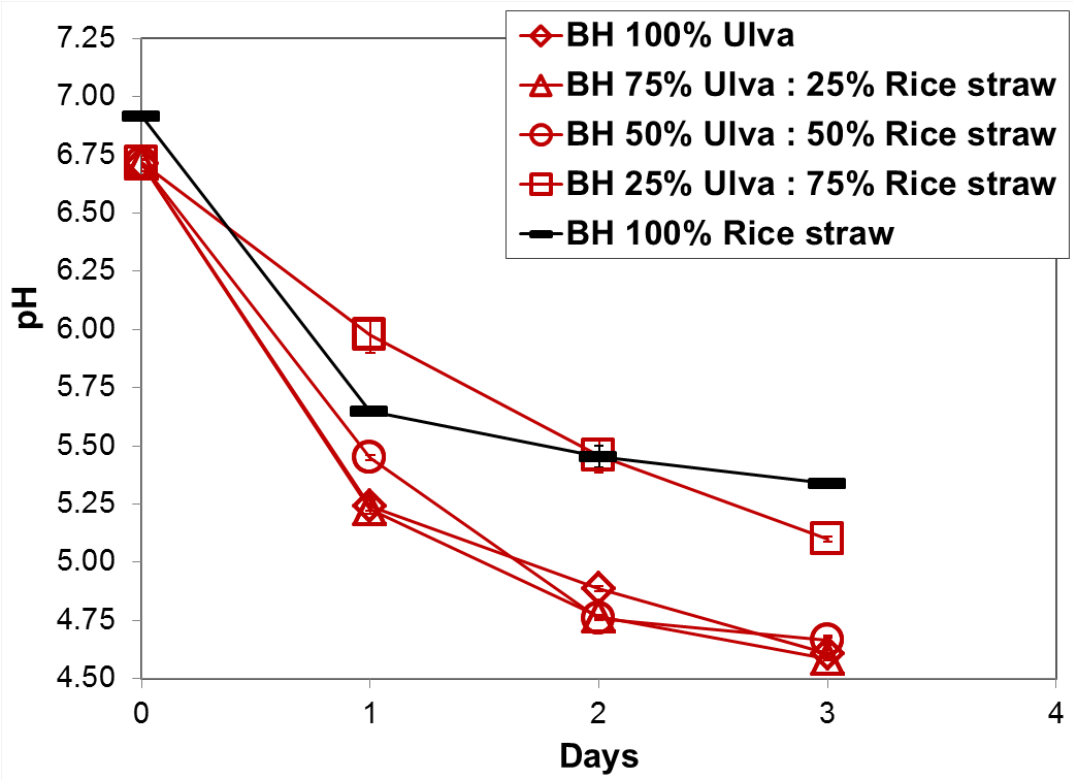

Figure 3.10 The pH change during the biological hydrolysis (BH) of the different percentage mixtures of rice straw and *Ulva* sp. biomasses under thalassic (TH) condition.

3.3.3. Anaerobic mono-digestion of rice straw under freshwater and thalassic conditions

The alkaline pretreatment was previously used by many authors to improve the methane yield of rice straw. Dehghani et al. (2015) obtained 0.292 L CH_4/ kg VS (125% improvement) upon pretreatment of rice straw with 0.5 M Na_2CO_3 at 110°C for 2 hours. To yield 0.225 L CH_4/ kg VS from rice straw, the pretreatment condition of 9.81% $Ca(OH)_2$ for 5.89 days was suggested by Song et al. (2013). Zhang et al. (2015) also obtained 0.288 L CH_4/ kg VS using 3% NaOH pretreatment at 35°C for 48 hours. In this study, 3% NaOH (w/w) was employed for 5 days before biogas production under both FW and TH conditions. The NaOH can disrupt ester bonds between lignin and cellulose or hemicellulose, and cleave β-O-4, β-5, and β-β bonds within lignin, thereby allowing the release of cellulose and hemicellulose, and the dissolution of lignin, respectively (He et al., 2008). It can also increase the porosity of the crystalline cellulose, and completely hydrolyze the hemicellulose (He et al., 2008). This makes the rice straw components easily accessible and digestible to anaerobic bacteria, enhancing the methane yield. However, the BH pretreatment of rice straw under both FW and TH conditions gave higher cumulative biogas (Figure 3.11A) than the NaOH set-ups (*p*-value= 0.008). Under the FW condition, the low biogas yield and unstable methane fermentation process of the NaOH set-up may have been due to the Na^+ inhibition of methane bacteria (Chen et al., 2008). While the hydrolysis and acidogenesis of rice straw successfully proceeded as indicated by the low unconsumed VS (33.1 ± 11.0%), the low pH (6.78 ± 1.00) at the end of the

experiment suggested the restricted consumption of organic acids for methane production. The slightly elevated salinity (described in 3.2.4.2) that may have resulted to this methane bacteria inhibition may have been due to the added NaOH during pretreatment. On the other hand, although the TH condition produced higher methane yield than the FW condition, the methane yield of TH NaOH set-up was still low when compared to the BH set-ups of both conditions. This low methane fermentation performance may have been mainly caused by the limitation on hydrolysis and acidogenesis of rice straw as implied by the high unconsumed VS (76.9 ± 3.0%). Under TH condition, the Na^+ inhibition on methanogens may have not occurred because marine bacteria were used. The slightly basic end pH (8.23 ± 0.03) of the TH NaOH set-up further suggested the successful consumption of organic acids for methane fermentation.

Table 3.3 The different performance of methane fermentation of rice straw as mono-substrate.

Set-ups			(ml CH_4/ g VS)			Methane content (%)	C/N ratio	H_2S (%)
			TMY	EMY	AcMY			
AMD	FW	BH Rice straw	327.9	—	62.2 ± 30.9	66.4 ± 3.8	125	—
AMD	FW	NaOH Rice straw	327.9	—	15.0 ± 22.8	31.1 ± 20.6	125	—
AMD	TH	BH Rice straw	327.9	75.8	75.8 ± 5.7	59.5 ± 0.8	125	0.06 ± 0.03
AMD	TH	NaOH Rice straw	327.9	—	21.4 ± 4.2	45.9 ± 1.6	125	—

AMD- anaerobic mono-digestion; FW- freshwater; TH- thalassic; TMY- theoretical methane yield; EMY- estimated methane yield; AcMY- actual methane yield; BH- biological hydrolysis pretreatment; NaOH- 3% NaOH pretreatment

Table 3.4 The different performance of methane fermentation of rice straw as co-substrate of *Ulva* sp.

Set-ups			(ml CH_4/ g VS)			Methane content (%)	C/N ratio	H_2S (%)
			TMY	EMY	AcMY			
ACD	TH BH	100% *Ulva*	238.7	94.8	94.8 ± 6.8	51.6 ± 1.3	29	3.82 ± 0.08
ACD	TH BH	75% *Ulva* : 25% Rice straw	261.0	90.0	121.7 ± 2.7	59.2 ± 1.5	53	1.15 ± 0.18
ACD	TH BH	50% *Ulva* : 50% Rice straw	283.3	85.3	130.3 ± 10.3	60.2 ± 2.4	77	0.417 ± 0.10
ACD	TH BH	25% *Ulva* : 75% Rice straw	305.6	80.5	107.6 ± 7.9	60.7 ± 1.6	101	0.375 ± 0.11

ACD- anaerobic co-digestion; TH- thalassic; TMY- theoretical methane yield; EMY- estimated methane yield; AcMY- actual methane yield; BH- biological hydrolysis pretreatment

On the other hand, lower pH under the FW condition was obtained during the BH pretreatment (Figure 3.9) than under the TH. This may be due to the higher hydrolytic activity of the conventional HB than the marine HB, which translated to the earlier methane production peak (20^{th} day) under FW condition (Figure 3.11B). However, the TH BH set-up of rice straw still gave higher cumulative biogas yield than the FW condition (Table 3.3). While the methane production of the TH condition peaked at a later time (35^{th} day) (Figure 3.11B), the higher specific methane yield obtained under this condition (Table 3.3) indicated that the difference between FW and TH is not their hydrolytic potency, but their rate of activity. Moreover, although the biogasification efficiency of BH in terms of the theoretical methane yield of rice straw was higher under the TH (23.1%) than the FW (19.0%), the methane yields of BH set-up under both conditions were not significantly different (*p*-value= 0.182). This suggests that the ability of marine bacteria to utilize rice straw is the same as that of the conventional bacteria. Still, higher specific methane yield in the TH BH set-up was obtained than the FW BH set-up (Table 3.3) of this study and the 3% NaOH-pretreated rice straw (74.1 ml CH_4/ g VS) that was previously reported by Chandra et al. (2012b). The marine bacteria under TH demonstrated their ability to utilize terrestrial biomass, as good as, if not better than the conventional bacteria. The successful degradation of terrestrial lignocellulosic biomass by marine microorganisms may have been due to their ability to produce lignocellulase enzyme complex, as individual species (Sethi et al., 2013) or consortium (Wongwilaiwalin et al., 2010). Lignocellulolytic and saccharifying activities on terrestrial plants were

reported for the marine bacterium *Isoptericola* sp. JS-C42 (Santhi et al., 2014). A marine bivalve symbiont *Teredinibacta turnerae* can produce cellulase with cellobiohydrolase and β-1,4(3) endoglucanase activities (Ekborg et al., 2005). The β-1,4 endoglucanase can cut the amorphous cellulose, creating a reactive end for the attack and release of cellobiose by cellobiohydrolase (Suvorov et al., 2011). The *Saccharophagus degradans* gen. nov., sp. nov. 2-40^{T} was also described to be a versatile marine bacterium that can degrade agar, alginate, fucoidan, laminarin, chitin, pectin, starch, xylan, and pullulan (Ekborg et al., 2005). Hence, the successful biogas production of terrestrial biomass, like rice straw, under thalassic condition can be expected. This may help the continuous operation of thalassic biogas digester, without heavily relying to the seaweed feedstocks.

Moreover, 87.8% of the cumulative biogas and 86.4% of the total methane were already produced on the 51st day of anaerobic digestion in the TH BH set-up. This was faster than the FW BH set-up where only 86.9% of the cumulative biogas and 85.6% of the total methane were obtained on the 55th day (Figure 3.11). Lowering the retention time to 45 days can give the same percentage of total methane yield in both conditions (TH: 82.2%, FW: 82.9%). Therefore, either condition may be used in the biogas production of rice straw without significantly affecting the fermentation performance. But, the co-digestion of the seaweed *Ulva* with the rice straw may affect the microbial activities under the FW condition. Different enzymes are used for the hydrolysis of *Ulva* and rice straw, which may change the effectiveness of the

conventional bacteria. Since marine bacteria can effectively utilize both rice straw and *Ulva*, then the co-digestion under thalassic condition was preferentially done.

3.3.4. Anaerobic co-digestion of rice straw and *Ulva* sp. under thalassic condition

The thalassic biogas production of fresh *Ulva* gave higher specific methane yield (180.9 ml CH_4/ g VS) (Chapter 2; Marquez et al., 2015b) than the obtained from the dried *Ulva* (Table 3.4). The biogas production of dried *Ulva* sp. by Bruhn et al. (2011) gave 176 ml CH_4/ g VS, which is comparable to the reported fresh *Ulva* (Chapter 2; Marquez et al., 2015b). The difference in the result between the dried *Ulva* of Bruhn et al. (2011) (45°C) and this study (105°C) may be due to the employed high drying temperature. Ulvan with higher molecular weight was favored when extracted at higher temperature range (80°C to 90°C) (Lahaye and Robic, 2007), which may have resulted to a more difficult fermentation process. Some volatile fatty acids that may have been produced from the auto-hydrolysis of *Ulva* could have also been evaporated, lowering the substrates for methane conversion. Nonetheless, the specific methane yield of the dried *Ulva* was higher than the rice straw. This difference is expected because of the more complex structure of the rice straw than the *Ulva*.

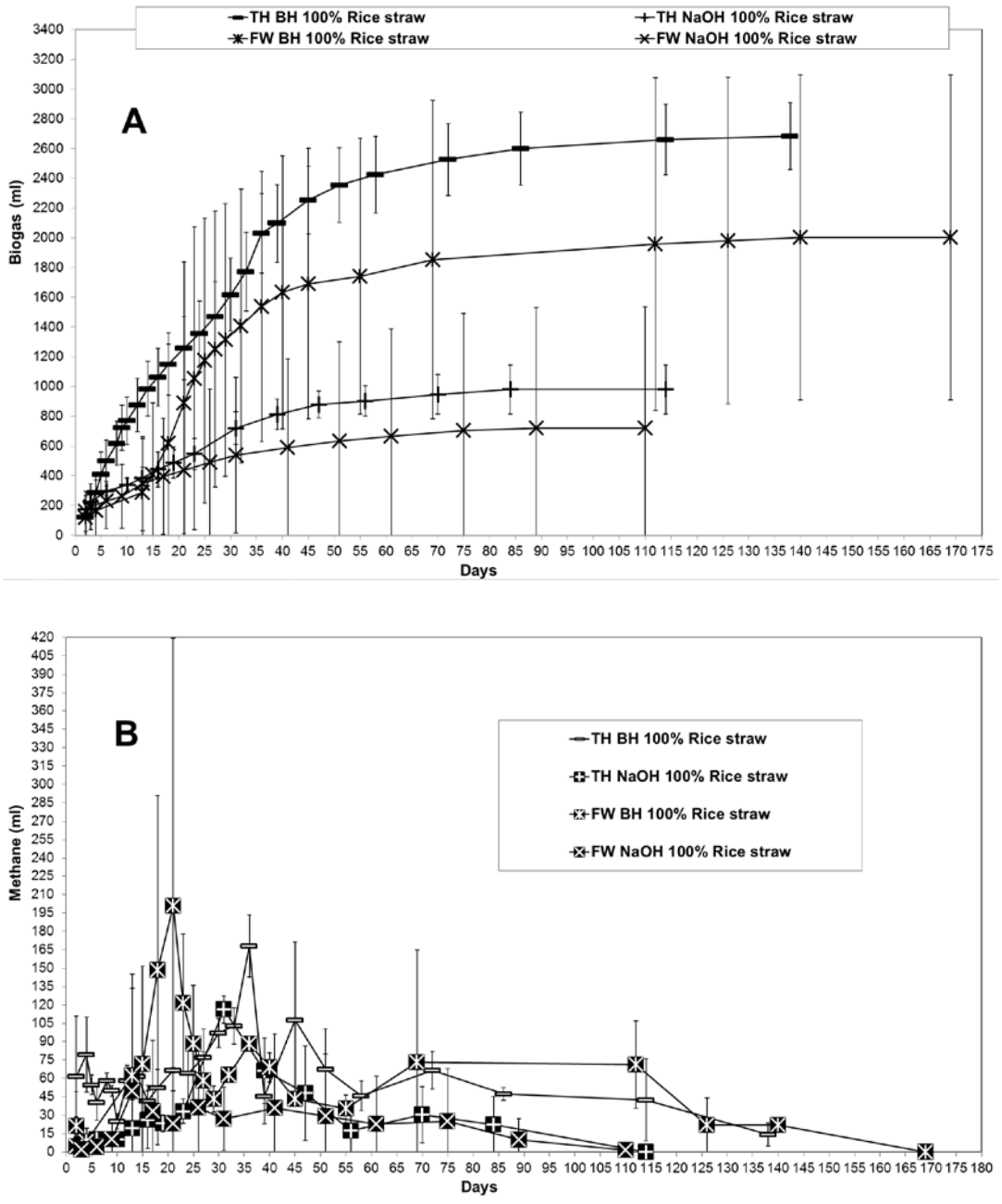

Figure 3.11 The (A) cumulative biogas production and (B) methane yield per day of the biologically hydrolyzed (BH) and chemically (NaOH) pretreated rice straw under freshwater (FW) and thalassic (TH) conditions.

To further increase the methane yield of the rice straw, different weight percentage of *Ulva* was added with the rice straw as co-substrate. Higher cumulative biogas yield was observed on all co-digestion set-ups than the mono-digestion of both *Ulva* and rice straw (Figure 3.12A). All co-digestion set-ups obtained higher actual methane yield than their corresponding estimated methane yield —computed from the actual methane yield of *Ulva* and rice straw (Table 3.4). This suggests a synergistic effect on the methane fermentation of rice straw and *Ulva*. The same methane yield improvement (from 46 ml CH_4/ g VS to 340 ml CH_4/ g VS) was observed in the co-digestion of rice straw and piggery wastewater (Mussoline et al., 2013). The lake water blue algae from Taihu (201 ml CH_4/ g VS) also obtained higher methane yield (325 ml CH_4/ g VS) when corn straw was mixed (Zhong et al., 2012). While methane improvement was previously reported in the batch co-digestion of the seaweed *Saccharina latissima* and wheat straw (Vivekanand et al., 2012), and the pilot-scale co-digestion of *Laminaria*, *Ulva*, and milk (Matsui and Koike, 2010), this study is the first to use seawater as liquid substrate and marine bacteria as microbial seed in the co-digestion of rice straw and *Ulva*.

On the other hand, the methane production peaked earlier in the co-digestion set-ups, of which the ratio of *Ulva* was lower than the rice straw (Figure 3.12B). As the ratio of rice straw increases, the earlier the methane production peak was observed. But with rice straw as mono-substrate, the peak of methane production was the slowest. *Ulva* has β-1,4-D-xyloglucan (Lahaye and Robic, 2007) that can be degraded by xylanase. Increasing the amount of *Ulva* in the co-digestion may have

increased the xylan in the co-digestion substrates. Soluble xylan was found to decrease the hydrolytic activity of cellulase (Zhang et al., 2012), which may have restricted the hydrolysis of cellulose in the rice straw. Consequently, this may have caused the lower biogas yield in the co-digestion set-up with 75% *Ulva* than with 25% *Ulva* (Figure 3.12A).

The cumulative methane yields of 25% *Ulva*-75% Rice straw and 50% *Ulva*-50% Rice straw were significantly higher than the 100% *Ulva* (*p*-value= 0.002 and 0.011, respectively) and 100% Rice straw (*p*-value = 0.003 and 0.004, respectively). However, the 75% *Ulva*-25% Rice straw attained the highest biogasification efficiency (46.6%) in terms of their theoretical methane yield (Table 3.4), followed by the 50% *Ulva*-50% Rice straw (46.0%) and then the 25% *Ulva*-75% Rice straw (35.2%). Almost the same biogasification efficiency in terms of their estimated methane yield was obtained by the 25% *Ulva*-75% Rice straw (133.6%) and the 75% *Ulva*-25% Rice straw (135.2%) (Table 3.4). The highest was given by the 50% *Ulva*-50% Rice straw set-up (152.8%). This indicates that among the thalassic co-digestion set-ups, the 50:50 ratios enhanced the methane production the most in terms of their estimated methane yield (Table 3.4).

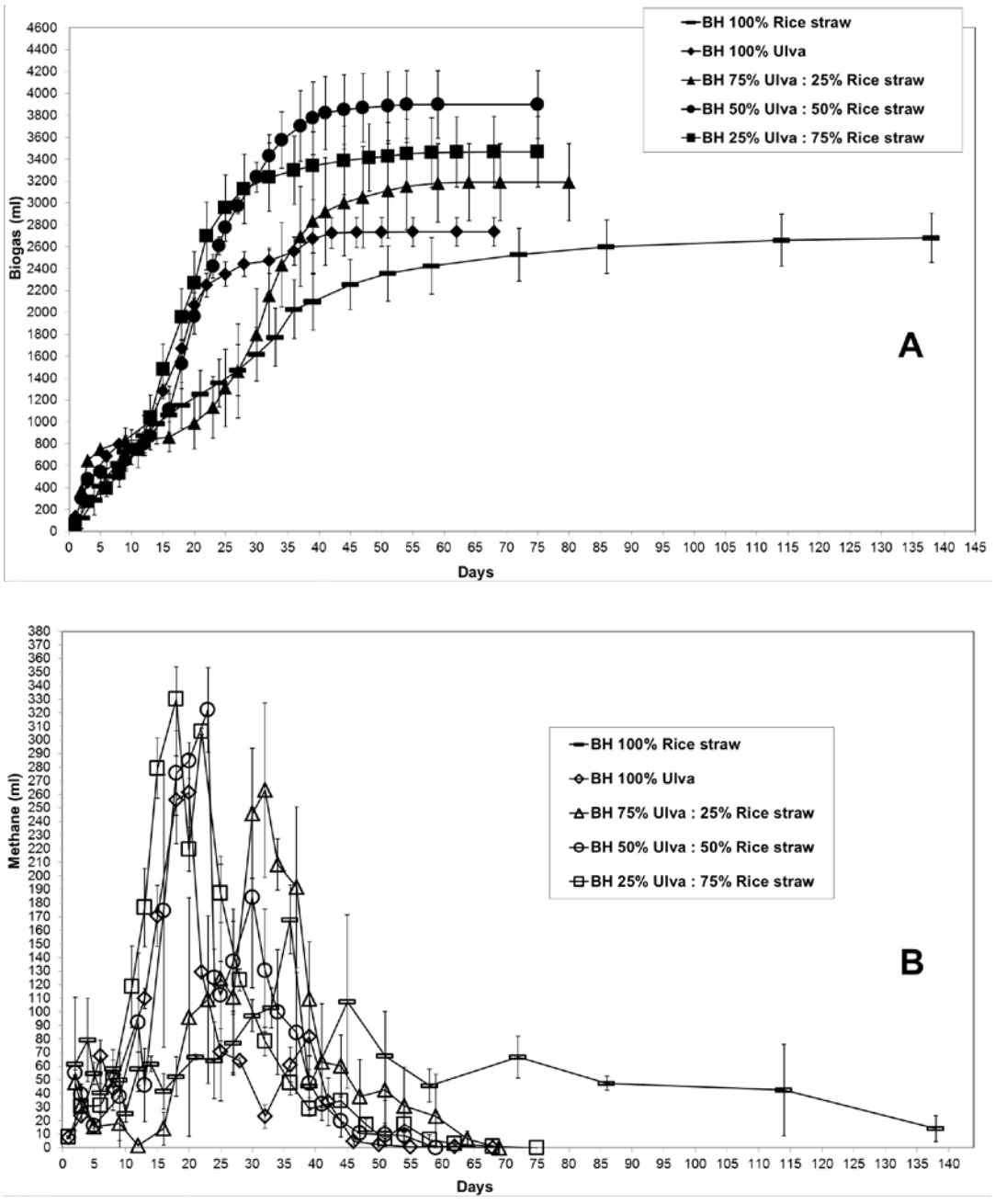

Figure 3.12 The (A) cumulative biogas production and (B) methane yield per day of the biologically hydrolyzed (BH) mixtures of rice straw and *Ulva* sp. biomasses (w/w) under thalassic (TH) condition.

Different pretreatments were done on rice straw to improve its methane yield. It was reported that the 3% NaOH (w/w) pretreatment, and the combination of hydrothermal (200°C, 10 minutes) and 5% NaOH (w/w) pretreatment, only increased the methane yield of rice straw from 59.8 ml CH_4/ g VS to 74.1 ml CH_4/ g VS and 132.7 ml CH_4/ g VS, respectively (Chandra et al., 2012b). By just mixing 25% *Ulva* with 75% rice straw, the methane yield was already higher (107.6 ml CH_4/ g VS) than the alkaline-pretreated rice straw (Chandra et al., 2012b). Higher methane yield than the hydrothermal pretreated-rice straw (Chandra et al., 2012b) was further obtained by increasing the ratio of *Ulva* by 50% (Table 3.4). Also, the biogas production rate of the co-digestion set-ups was faster than the 100% rice straw. At the 39th day, 88.9%, 96.8%, and 96.3% of the cumulative biogas, and 85.7%, 96.5%, and 95.9% of the total methane were already obtained by the 75:25, 50:50, and 25:75 (*Ulva*:Rice straw) set-ups, respectively. Hence, the co-digestion of rice straw and *Ulva* can be a better and cheaper approach for enhancing methane yield and fermentation rate without the utilization of a more energy expensive pretreatment. Furthermore, as the portion of *Ulva* was reduced in the co-digestion set-ups, the lower the H_2S was obtained (Table 3.4). This may be due to the decreased production of sulfated polysaccharide substrates from *Ulva* (Yanagisawa et al., 2013), which can be utilized by sulfur-reducing bacteria for H_2S production. Hence, the co-digestion of rice straw with *Ulva,* not only improved the methane yield but also decreased the H_2S level in the biogas. This can help minimize the health risk that is posed by the H_2S to the biogas users.

In the coastal and isolated communities, the utilization of thalassic condition for biogas production can help lower the operational cost of household digesters by using seawater instead of freshwater and removing the need to wash the salt in the seaweed. The successful thalassic biogas production of rice straw demonstrated the potential of terrestrial biomass as a substitute feedstock if not supplement to seaweed. Also, co-digestion of rice straw and *Ulva* is an excellent and suitable approach to improve methane yield of thalassic biogas digester among poor coastal households.

3.4. Conclusion

The rice straw was successfully used as mono-substrate feedstock for thalassic biogas production, showing a more stable biogas production under thalassic than under freshwater conditions. Although thalassic BH pretreatment of rice straw as mono-substrate gave the highest methane yield (75.8 ml CH_4/ g VS), the biogasification efficiency was only 23.1% of the theoretical methane yield. Co-digestion of rice straw with *Ulva* further improved the methane yield and shortened the retention time, with the 50:50 as the most suitable ratio in terms of specific methane yield (130.3 ml CH_4/ g VS). Nevertheless, methane fermentation of rice straw as mono-substrate and as co-substrate of *Ulva* was successfully observed under thalassic condition.

Chapter 4
Biogas production performance of *Undaria pinnatifida* using a bio-based pH buffer — shell of *Venerupis* species (Asari)

4.1. Introduction

Climate change drives the development of biofuel technologies. However, utilization of terrestrial crops for biofuel production resulted to higher food prices (HLPE, 2013) because of its demand in the fuel industry. Also, conversion of grasslands and forest areas to grow biofuel crops released carbon that is previously stored in these ecosystems (Searchinger et al., 2008). Hence, interests on marine biomass utilization for biofuel production were resumed as solution to the drawbacks brought by terrestrial feedstock.

Chapter 4. Biogas production performance of Undaria pinnatifida using a bio-based pH buffer — shell of Venerupis species (Asari)

Undaria pinnatifida (Harvey) Suringar is a brown seaweed. Its harvest from the wild and mariculture have been widely improved that its biomass production increased from 147,000 t (1976, in Japan alone) to around 500,000 t wet weight in both Japan and Korea (FAO, 2013). Although it is consumed as food in East Asia, *U. pinnatifida* is considered as pest in Australia and New Zealand due to their invasive characteristics (TNS, 2008). Disturbance on natural seaweed bed by biological grazing or bad weather conditions resulted to its opportunistic colonization (Stuart, 2004). Physical removal in Tasmania, Australia has been done to further combat its proliferation (TNS, 2008). The invasiveness of *U. pinnatifida* and its well-established aquaculture technique may assure its biomass supply. Hence, *U. pinnatifida* was tested as feedstock for biogas production.

Biogas production is the commonly preferred rural energy (Hall, 1983) among the biomass conversion technologies because of its cheap initial investment cost and easy digester operation (Marquez et al., 2014). However, understanding the biogas production process is necessary to properly manage a biogas digester. The biogas production is an anaerobic digestion (AD) process that can be divided into acidification phase (hydrolysis, acidogenesis and acetogenesis steps) and methanation phase (methanogenesis steps). In the acidification phase, the biomass is hydrolyzed into its simple structures (fatty acid, amino acid, monosaccharide, and oligosaccharide). These simple structures are quickly converted into organic acids. The organic acids are then simultaneously used for the production of biogas (CH_4, CO_2, and other trace gases) in the methanation phase. The rate of acidification is

faster than the methanation phase mainly because of the faster growth rate of the microorganisms involved in acidification phase. Consequently, the faster production of organic acids in acidification phase compared to its consumption in methanation can dramatically decrease pH, which can further inhibit the growth of methane bacteria (Nettmann et al., 2010). Hence, the adjustment to suitable pH range (6.4 to 7.9) is necessary for methanation to occur (Chen et al., 2008; Marquez et al., 2014).

In archipelagic nations, many poor rural communities are located near the coast. Poor fisher folks can be targeted as users of household biogas digesters, using seaweed as feedstock. The common problem in household operation is maintaining the optimum pH. Some commonly used chemical pH buffers are NaOH, KOH, Na_2CO_3 and CaOH (Manyi-Loh et al., 2013). But these chemicals are difficult and expensive to acquire, especially if the digesters are operated in isolated areas. If chemical alkaline buffers are absent, complete failure of biogas production, if not low biogas yield can occur in household digester. Utilization of local materials that can easily be gathered on the coast and can be used as pH buffer is necessary. Therefore, the shell of the widely distributed shellfish *Venerupis* species (Asari) was tested as potential bio-based pH buffer on the biogas production of *U. pinnatifida*. Effective buffering potential of the shell can lower the maintenance cost and can increase the safeness of digester operation in terms of chemical handling.

4.2. Materials and Methods

4.2.1. Characterization of *Undaria pinnatifida* and preparation of *Venerupis* shell

Fresh *U. pinnatifida* biomass (Figure 4.1) and *Venerupis* spp. (Figure 4.2) were acquired from Seiyu (Shiotsuketori, Showa-ku, Nagoya-shi, Aichi-ken, Japan) on April, 2013. The seaweed was washed before freezing (-20°C) for future use. The blade of seaweed was macerated (< ~5mm) using a force mill (TDK Y-208B). Proximate compositions (protein, lipid, carbohydrate, C/N) were measured by Chugai Technos Corporation, Yokogawa-shinmachi, Nishi-ku, Hiroshima, Japan, using the standard method described by Marquez et al. (2015b; in Chapter 2). Total solids (TS), volatile solids (VS) and ash content (Burn Out Furnace KDF007EX) were determined using the standard procedure (AOAC, 1990; in Chapter 2). Theoretical methane yield of *U. pinnatifida* was computed as described in Chapter 1 (equation 2). The *Venerupis* spp. were boiled (100°C, 10 minutes) to remove the shell from the meat, washed, and then dried (ASONE Forced Convection DO-450FA) before crushing into powder using the force mill. Size composition of the shell (Retsch Vibratory Sieve Shaker AS200, 80 amplitude, 10 minutes) is composed of 30% <5 mm to 500 μm and 70% <500 μm granules.

Figure 4.1 The fresh blade (left), and macerated biomass (right) of *Undaria pinnatifida*.

Figure 4.2 The whole shell (left), and dried and powdered shell (right) of the *Venerupis* sp. or Asari.

4.2.2. Development of microbial inocula for hydrolysis and methane fermentation

The microbial inocula (MI) (Figure 4.3) for hydrolysis and methane fermentation were developed by obtaining initial bacteria (300 g) from the slurry of the hydrolysis tank and fermentation tank, respectively, of the continuous fixed-bed biogas reactor that is fed with food waste. Both inocula were mixed with 200 g of macerated *U. pinnatifida* and 200 ml of distilled water (dH_2O) in separate 1-L bottles (Schott Duran), and then incubated at 37°C [60 days, in the dark] before use. The hydrolysis bacteria (HB) inoculum was not deoxygenated, but tightly sealed. The HB was shaken every day for 30 seconds and opened every 5 days. The hydrolyzed substrates (300 g) were collected every 20 days and pH-adjusted (~pH 7.6, 1 M NaOH), before transferring to the methane bacteria (MB) bottle. Addition of 100 g of macerated *U. pinnatifida* and 200 ml of distilled water to the HB bottle was also done whenever 300 g of hydrolyzed substrate is removed. On the other hand, the bottle of MB inoculum was deoxygenated (N_2 pumping) before incubation, and shaken every day (30 seconds). The biogas was allowed to anaerobically escape. Three hundred grams of residues were removed from the MB bottle every 20 days, while simultaneously adding 300 g of pH-adjusted hydrolyzed substrate. The MB was maintained under anaerobic condition.

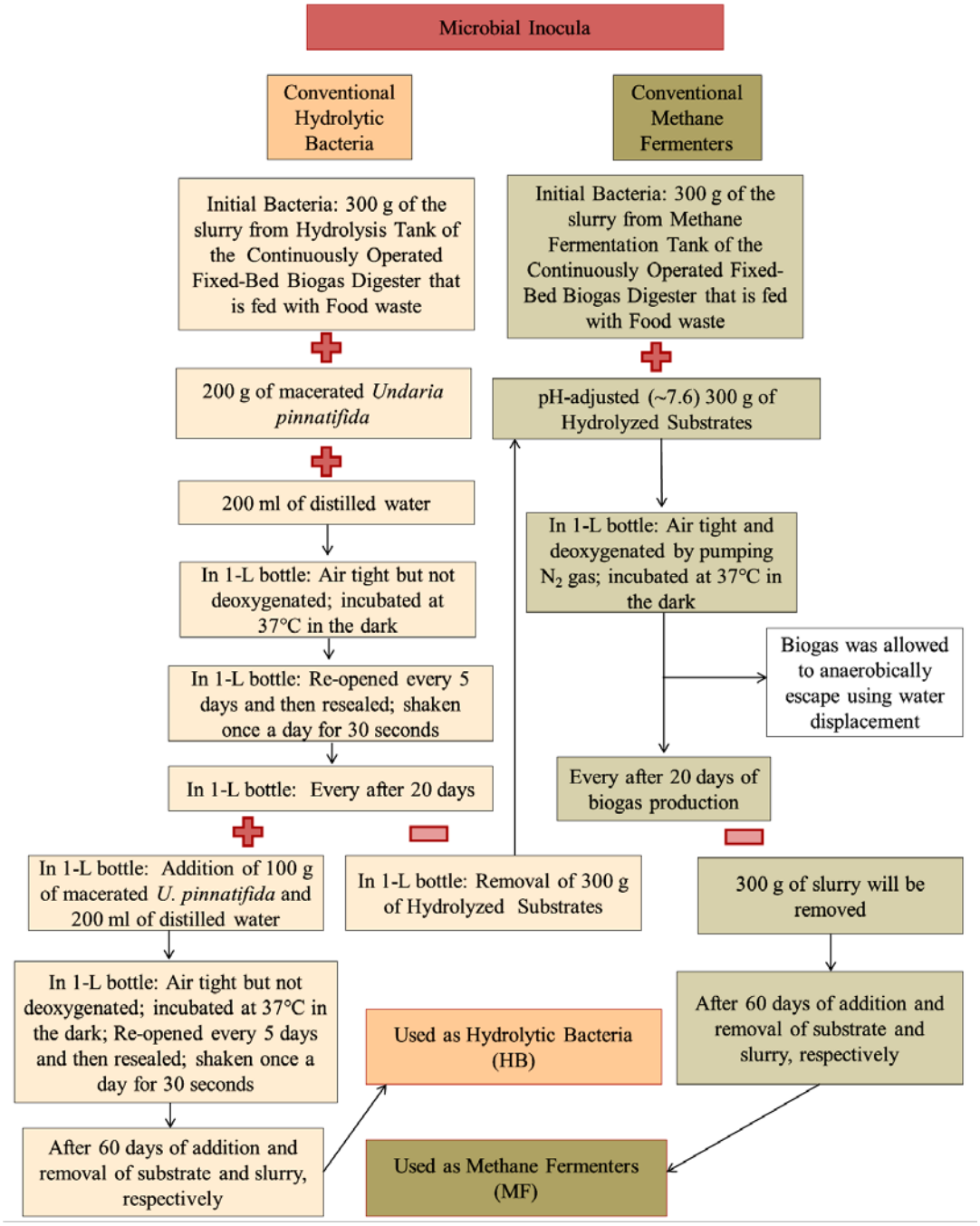

Figure 4.3 The schematic diagram of the development of the hydrolytic bacteria and methane fermenters for shell experimental set-ups.

4.2.3. Effect of different amount of shells on the pH behavior during acidification phase

pH adjustment is essential to the successful start-up and stabilization of the biogas production process. To determine the effect of shell on pH behavior during acidification phase of *U. pinnatifida*, 8 set-ups (*N*= 3) were made using different amount of powdered shell (Figure 4.4). In the negative control set-up, 30 g of biomass were added with 15 g of HB and 15 g of dH_2O (biomass to HB and dH_2O ratio [2:1:1]) in a 100-ml bottle (Schott Duran).The same substrate ratio was used in the other set-ups (Table 4.1). The changes of pH were measured using a handheld pH meter (Horiba D-52). All bottles were not deoxygenated, however airtight cap was maintained. All set-ups were incubated at 37°C in the dark (YAMATO model IN602W) and manually shaken for 30 seconds every day.

4.2.4. Biogas production experiment

Five set-ups (*N*=3) were made for the biogas production experiment (Figure 4.5). The AD condition, feedstock, microbial inoculum, and liquid substrate used in the experiment are summarized in Table 4.1.The biogas production experiment was done using 1-L bottle (Schott Duran) as batch digesters. All digesters were deoxygenated (pumping N_2 gas, 1minute) before the start of biogas production. All digesters were incubated at 37°C in the dark.

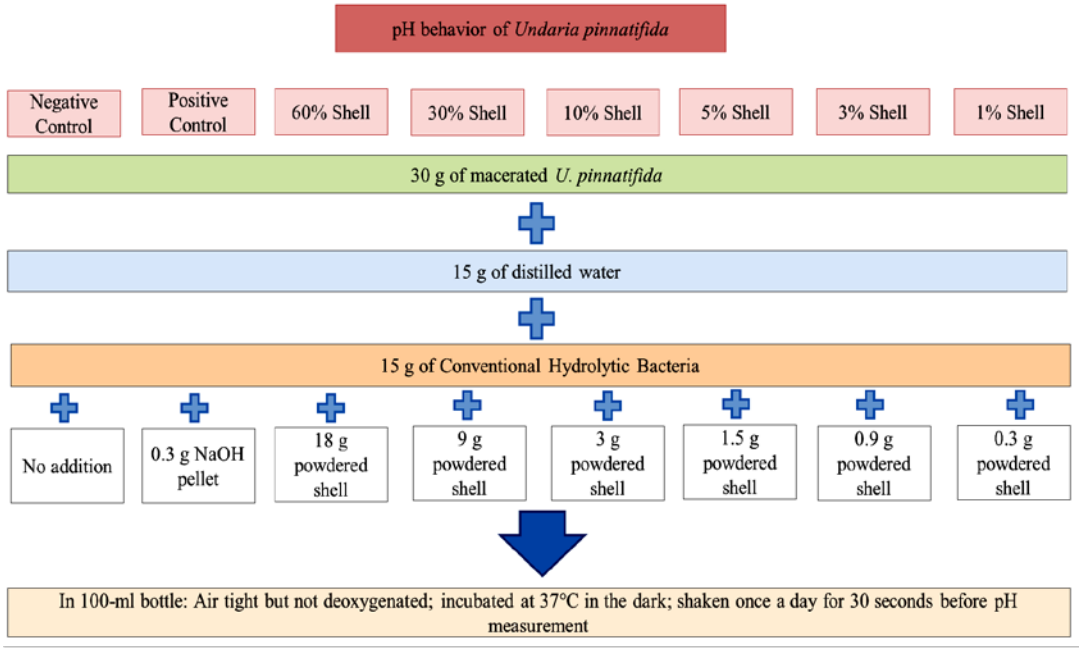

Figure 4.4 The schematic diagram of the experimental condition during the optimization of the amount of shell to be used in the shell experimental set-ups.

Table 4.1 The summary of the substrate composition used in the experiment.

Set-ups	AD conditions	pH buffer	Biomass substrate	Liquid substrate	Microbial inocula
Control	AD started immediately	None	200 g macerated *Undaria pinnatifida*	100 ml distilled water	Mixed 100 g HB and 100 g MB
3% Shell	AD started immediately	6 g Shell	200 g macerated *Undaria pinnatifida*	100 ml distilled water	Mixed 100 g HB and 100 g MB
5% Shell	AD started immediately	10 g Shell	200 g macerated *Undaria pinnatifida*	100 ml distilled water	Mixed 100 g HB and 100 g MB
BH then 5% Shell	3 days BH then add pH buffer before AD	10 g Shell	200 g macerated *Undaria pinnatifida*	100 ml distilled water	100 g HB, then 100 g MB
BH then NaOH	3 days BH then add pH buffer before AD	1 M NaOH	200 g macerated *Undaria pinnatifida*	100 ml distilled water	100 g HB, then 100 g MB

AD- anaerobic digestion; BH- biological hydrolysis; HB- hydrolytic bacteria inoculum; MB- methane bacteria inoculum

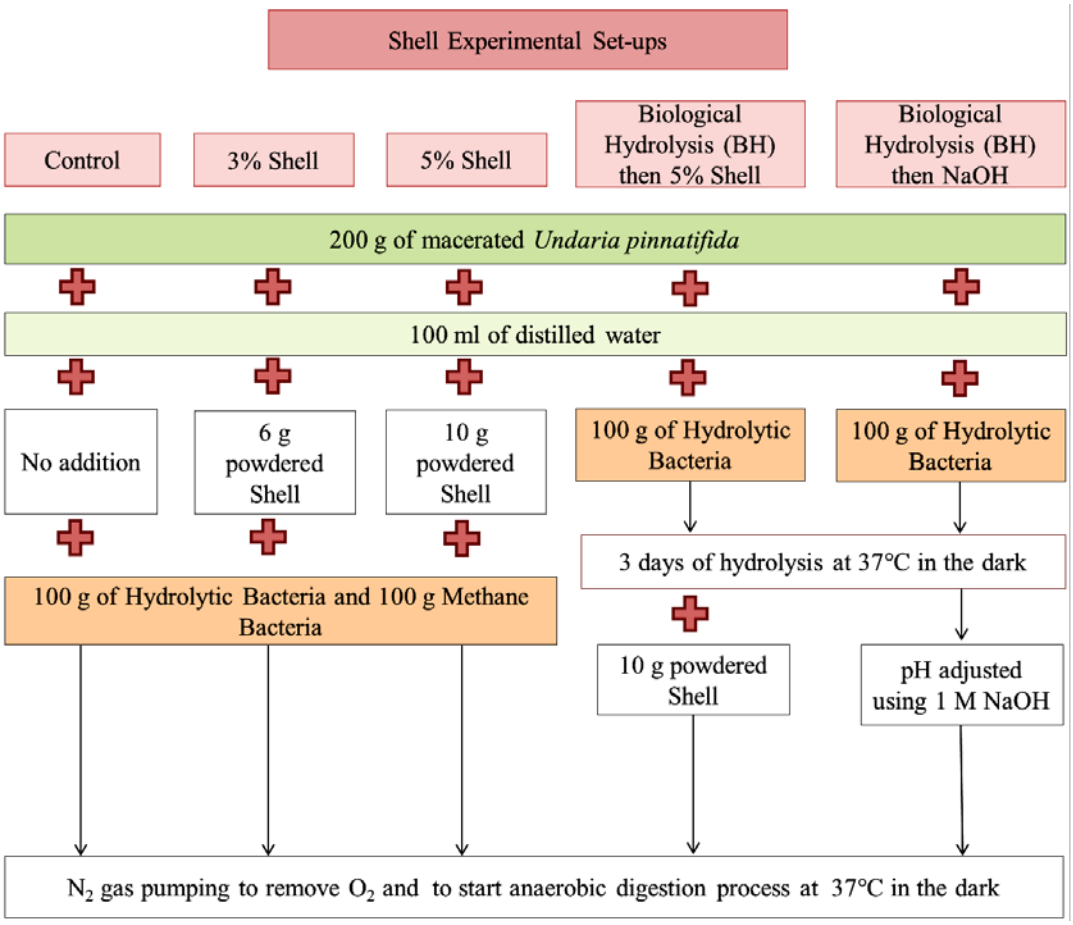

Figure 4.5 The schematic diagram of the preparation of the different shell set-ups before the start of the biogas production experiment.

4.2.5. Biogas composition and data analysis

The total volume capacity of the digester bottles was 1130 ml. Working volume of the digesters was 500 ml. Water displacement method (described in Chapter 2) was used to determine the volume of biogas produced as described by Chandra et al. (2012b). The biogas components and their volumes were analyzed using a gas chromatograph as described by Marquez et al. (2015b; in Chapter 2). All data were presented as mean (N= 3) with standard deviation (SD) as error bar. The high standard deviation of the methane yield may have been due to the utilization of the non-homogeneous biomass substrate before their transfer to the batch digesters, the addition of the microbial inocula with heterogeneous bacterial population, and the varying granular size of the powdered shell that may have affected its dissolution.

4.3. Results and discussion

4.3.1. Proximate composition and theoretical methane yield of *Undaria pinnatifida*

U. pinnatifida is mainly composed of moisture (94.7 %), with carbohydrate (64.32%) as the main component of VS (83.07%). The protein (17.87%) is higher than lipid (1.79%). Theoretical methane yield was computed to be 356.8 ml CH_4/ g VS. On the other hand, C/N value (10.5) was low, indicating high N content. Although it has lower C/N value as compared to the suggested values (20 to 30) by Mital (1996), the biogas and CH_4 production were stable during the experiment.

Therefore, low C/N could not necessarily have a negative effect on anaerobic digestion of *U. pinnatifida*. Also, the same study on *Laminaria saccharina*, gave better methane yield even though its C/N value was low (Chynoweth et al., 1987).

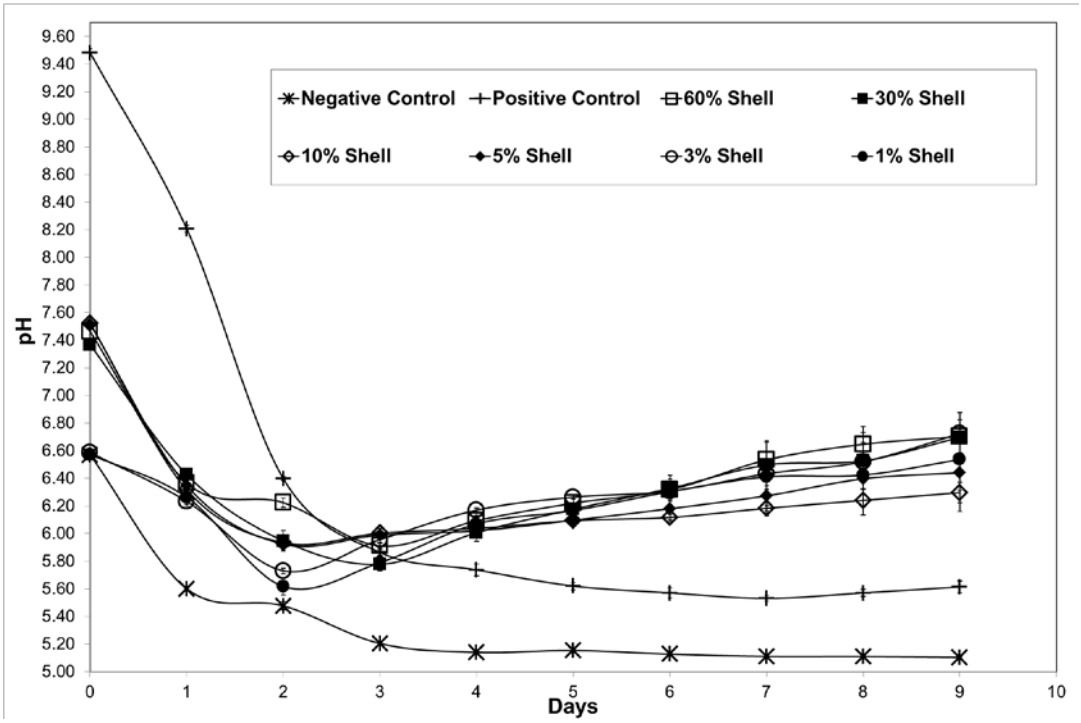

Figure 4.6 Effect of pH buffer on the change of pH during the biological hydrolysis of *Undaria pinnatifida* with different amount of shells (*N*= 3; ± S.D.). Positive control was added with NaOH pellet [1% (w/w) weight (g) of biomass; 97% purity (Kanto Chemical Co.)], while shell set-ups were added with different amount of shells [60%, 30%, 10%, 5%, 3%, and 1% (w/w) weight (g) of biomass, respectively].

4.3.2. Effect of shell on the pH behavior during acidification phase

The change of pH of the different set-ups during the acidification of *U. pinnatifida* was shown in Figure 4.6. Exponential decreased of the pH was observed immediately until the 2nd day. All set-ups with shell showed an increase of pH after the 2nd day, suggesting a buffering effect of the shell. Highest increased of pH was obtained from 60% and 30% Shell set-ups, but this amount of shell in proportion to the biomass may cause problem on the operation of the household digester and may lower the conversion efficiency. Among the remaining shell set-ups, the buffering effect exhibited by 5%, 3%, and 1% Shell was enough to allow methane fermentation, while not having too much amount of shell. On the other hand, the pH value of the negative control significantly decreased at the 3rd day of hydrolysis. Hence, the biological hydrolysis (BH) pretreatment before AD was done for 3 days in the 'BH then NaOH' and 'BH then 5% Shell' set-ups.

4.3.3. Effect of biological hydrolysis pretreatment on biogas production

Control set-up exhibited biogas production failure after the 4th day due to the low pH. This failure can be due to the high biodegradability of *U. pinnatifida* which can affect the anaerobic digestion system to self-regulate (pH), suggesting the importance of pH adjustment. The failure of biogas start-up was avoided by separating the acidification and methanation phase as shown by the 'BH then NaOH' set-up. The total biogas yield of the 'BH then NaOH' set-up was 2,616.3 ± 117.7 ml (Figure 4.7A) in 35 days. Different CH_4 and CO_2 contents were observed throughout

the digestion period, ranging from 35.9% to 82.9% and 64.1% to 17.1%, respectively. On the other hand, CH_4 production rapidly increased from the 8th day until the 25th day (Figure 4.7B), producing 70% of the total CH_4 yields within that period. Although digestion time continued until the 35th day (Figure 4.7B), 92.1% and 97.3% of the total CH_4 yield were already measured at 25th and 30th day, respectively, hence, retention time can be selected from these days. The biogasification efficiency of 'BH then NaOH' set-up was 68.3% (Figure 4.7C). This is higher than the biological methane potential test (41.6%, 34 days) from Kim et al. (2010). Therefore, application of BH pretreatment before biogas production is an effective and cheap way to increase CH_4 yield and degradation rate.

4.3.4. Powdered shell as pH buffer during anaerobic digestion

The better CH_4 yield in 'BH then NaOH' than in 'BH then 5% Shell' set-up (Figure 4.7) can be mainly due to the capacity of the NaOH to immediately change the pH before the start of AD. After BH pretreatment, both pH of the set-ups were low ('BH then NaOH' set-up= 5.47 ± 0.007; 'BH then 5% Shell' set-up= 5.67 ± 0.007). During the start of AD, the pH changed to only 6.44 ± 0.028 upon addition of 5% of shell (w/w) as compared to using NaOH (pH= 8.00 ± 0.028). Although successful biogas production start-up was observed, the acidic condition of the 'BH then 5% Shell' set-up could have affected the growth of methanogens from the start. Successful adaptation and proliferation of the different methanogen populations at the start of AD can dictate the stability of the digestion process. Therefore, shell is less effective than NaOH when used after BH pretreatment.

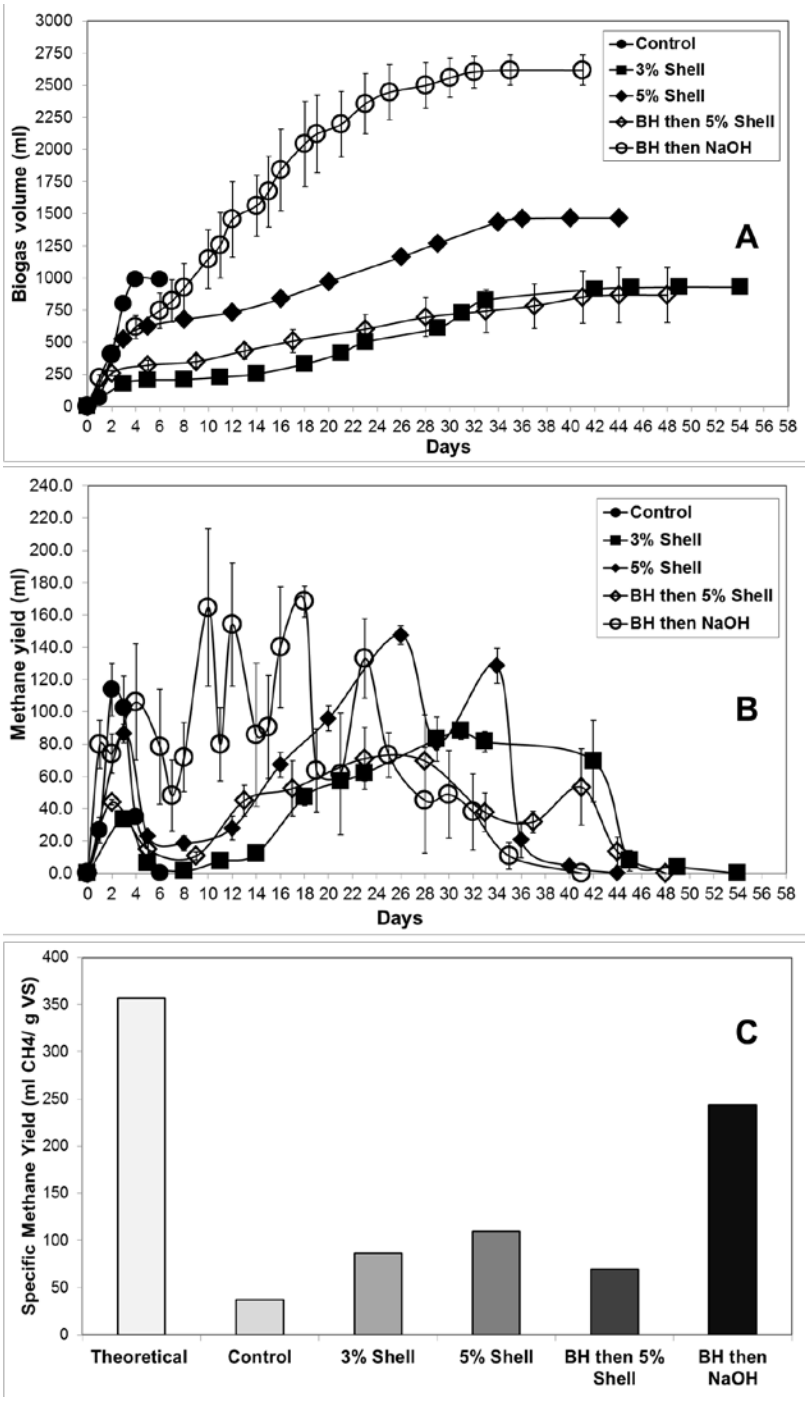

Figure 4.7 The (A) cumulative biogas production, (B) volume of methane yield per day, and (C) specific methane yield of the different set-ups using *Undaria pinnatifida* (*N*= 3; ± S.D.).

Both 3% and 5% Shell set-ups obtained higher total biogas (Figure 4.7A) and specific methane yield (Figure 4.7C) than 'BH then 5% Shell' set-up, suggesting that the shell was more effective when mixed with the biomass at the start of anaerobic digestion. The application of shell at the start of anaerobic digestion may have given ample time for the shell to dissolve. The composition of shell is mainly $CaCO_3$ (Jacob et al., 2008), which is a natural pH buffer in the marine environment. Acetate is the major organic acid formed; hence, the $CaCO_3$ in the shell may react to acetic acid. This reaction may permit the formation of acetate ion and H_2O instead of H^+, buffering the pH and lessening the impact of pH shock on methane bacteria. Moreover, some species of methane bacteria, specifically in the Order Methanomicrobiales (Gerardi, 2003) can tolerate lower pH range. Their presence could have helped the early consumption of organic acids, thereby slowly increasing the pH.

The 5% Shell set-up had the highest CH_4 yield among the shell set-ups, but its specific methane yield is still lower than the 'BH then NaOH' set-up (Figure 4.7C). Nonetheless, the specific CH_4 yield of 5% Shell set-up is 67.3% of the BMP that was previously reported (Kim et al., 2010), and 44.9% of the 'BH then NaOH' set-up. While this specific CH_4 yield (109.5 ml CH_4/ g VS) is only 30.7% of the theoretical methane yield of *U. pinnatifida*, without adding shell, biogas production completely failed. Hence, in the absence of NaOH, shell can be solely used as pH buffer to allow successful and stable biogas production.

4.4. Conclusion

The application of biological hydrolysis pretreatment, together with NaOH as pH buffer, gave stable biogas production and the highest specific methane yield (243.6 ml CH_4/ g VS). Also, successful biogas production was observed upon the utilization of shell as bio-based pH buffer. Among shell set-ups, higher CH_4 yield can be obtained when shell is mixed at the start of anaerobic digestion using 5% shell (109.5 ml CH_4/ g VS). Although lower CH_4 yield was obtained with shell, the biogas production can successfully start without using chemicals. The cheaper and easier acquisition of shell can encourage isolated coastal communities to utilize household biogas digesters.

Chapter 5
Performance of semi-continuous fixed-bed digester for thalassic biogas production of the brown seaweed, *Ecklonia* sp.

5.1. Introduction

Seaweed biomass has been suggested to have a high potential for harvest expansion to support a future bio-based economy (Marquez et al., 2015a). Also, compared to terrestrial crops, seaweed has faster growth rate, environment-friendly cultivation (Marquez et al., 2014), and excellent carbon sink (Radulovich et al., 2015). In the utilization of seaweed for biofuel conversion, anaerobic digestion for biogas production was the most energy efficient in terms of biomass conversion (Marquez et al., 2015a). Various seaweeds have been used by many studies for biogas production

(Bruhn et al., 2011; Hanssen et al., 1987; Vivekanand et al., 2012). Different pretreatments such as thermal (Vivekanand et al., 2012), biological, and chemical (Marquez et al., 2015b) were also done to further increase their methane yield. However, the sufficient supply of biomass is important to allow continuous operation of a biogas digester. The rotation of several seaweed species as biomass feedstock is needed because each species is only available in certain period of the year. The brown seaweed *Ecklonia* species can be harvested during summer (Hwang et al., 2009) when the production of other brown seaweeds *Laminaria* and *Undaria* is not in season. The successful utilization of *Ecklonia* for biogas production can help secure the steady supply of seaweed biomass feedstock. Therefore, in this study, the seaweed *Ecklonia* sp. was tested as feedstock for biogas production.

Previous study of Marquez et al. (2015b) suggested the better performance of marine bacteria in biogas production of the green seaweed *Ulva* sp. under high salinity (thalassic) condition. But, the methane fermentation under thalassic condition has been only done in a 1-L batch digester. This simple digester may have different or limited conversion performance. Hence, the operation of a pilot-scale semi-continuous fixed-bed digester has been tested under thalassic condition. This study is the first to report the performance of a semi-continuous fixed-bed digester under thalassic condition using *Ecklonia* sp. as feedstock.

5.2. Materials and Methods

5.2.1. Characterization of *Ecklonia*

The fresh *Ecklonia* sp. biomass (100 kg) was purchased from Nakiri Port, Mie Prefecture, Japan (Figure 5.1) on August, 2014. The thalli and blades of the seaweed were both used as feedstock. The seaweeds were stored in the freezer (-20°C) for future use. Some were immediately dried at 105°C (ASONE Forced Convection DO-450FA), and then powdered using a blender (≤500 μm size) (Figure 5.2). Seawater was delivered from Ise bay, Ise, Mie Prefecture, Japan several times (September, 2014, March, 2015, May, 2015, August, 2015). The Chugai Technos Corporation, Yokogawa-shinmachi, Nishi-ku, Hiroshima, Japan measured the proximate compositions (protein, lipid, carbohydrate, C/N) using the standard method described by Marquez et al. (2015b; in Chapter 2). Standard procedures (AOAC, 1990) were used to determine the total solids (TS), volatile solids (VS) and ash content (Burn Out Furnace KDF007EX) of the seaweed. The computation of the theoretical methane yield of *Ecklonia* sp. was described in Chapter 1 (equation 2).

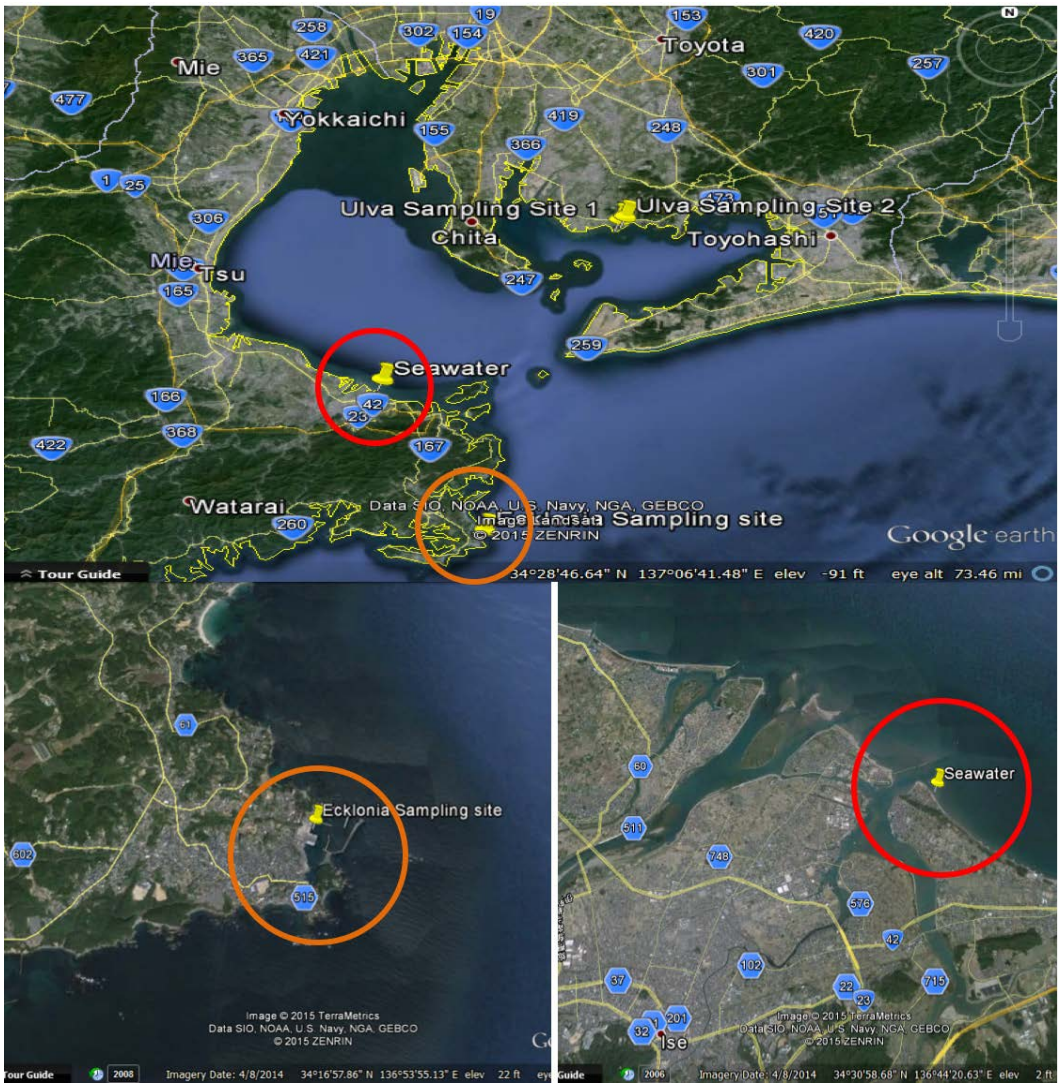

Figure 5.1 The map of the collection site of the *Ecklonia* sp. seaweed (orange circle) and seawater (red circle).

Figure 5.2 The fresh (top), dried (center), and powdered (bottom) *Ecklonia* sp.

5.2.2. Development of marine inocula for hydrolysis and methane fermentation tanks

The marine microbial inocula (MI) for the hydrolysis and methane fermentation tanks were initially obtained from the mixture of sands, seawater, and unprocessed seaweed (in Chapter 2 Figure 2.4) as described by Marquez et al. (2015b). A sealable10-L plastic container was used to scale up the volume of the marine microbial inoculum. Fresh macerated *Ecklonia* was used as feedstock during the inoculum development. Untreated seawater was used as liquid substrate. The inocula were incubated at 37°C for 60 days in the dark before transferring to their corresponding hydrolysis and fermentation tanks.

Substrate feedstock (1 kg of powdered *Ecklonia* and 19 L of seawater) was immediately added to the hydrolysis tank after putting the inoculum (10 L), while 30 L of seawater was added in the fermentation tank after also adding the 10-L inoculum. The substrates in the hydrolysis and methane fermentation tank were recirculated for 20 days. Additional feeding of substrate (1 kg of powdered *Ecklonia* and 18 L of seawater) to hydrolysis tank was done after transferring 10 L of hydrolyzed substrates (all hydrolyzed substrates were pH adjusted to 7.8 using 10% NaOH solution) to the methane fermentation tank. After 20 days, the 10-L hydrolyzed substrates from hydrolysis tank were transferred to the methane fermentation tank before adding 1 kg of powdered *Ecklonia* and 18 L of seawater substrate feedstock. In this feeding, the retention time was lowered down to 10 days. Removal of 10 L waste sludge was done from the methane fermentation tank before transferring 10 L of hydrolyzed substrates

from the hydrolysis tank to the methane fermentation tank. Substrate feedstock (500 g of powdered *Ecklonia* and 9.5 L seawater) was then added to the hydrolysis tank after transferring the hydrolyzed substrates to the methane fermentation tank. Removing of 10 L of waste sludge from the methane fermentation tank, transferring of 10 L of hydrolyzed substrates to the methane fermentation tank, and feeding of 10 L of substrates (500 g of powdered *Ecklonia* and 9.5 L seawater) to the hydrolysis tank were done every 10 days (2 times), then every 5 days (3 times), and finally every 3 days (5 times) before the start of the semi-continuous experiment (Figure 5.3).

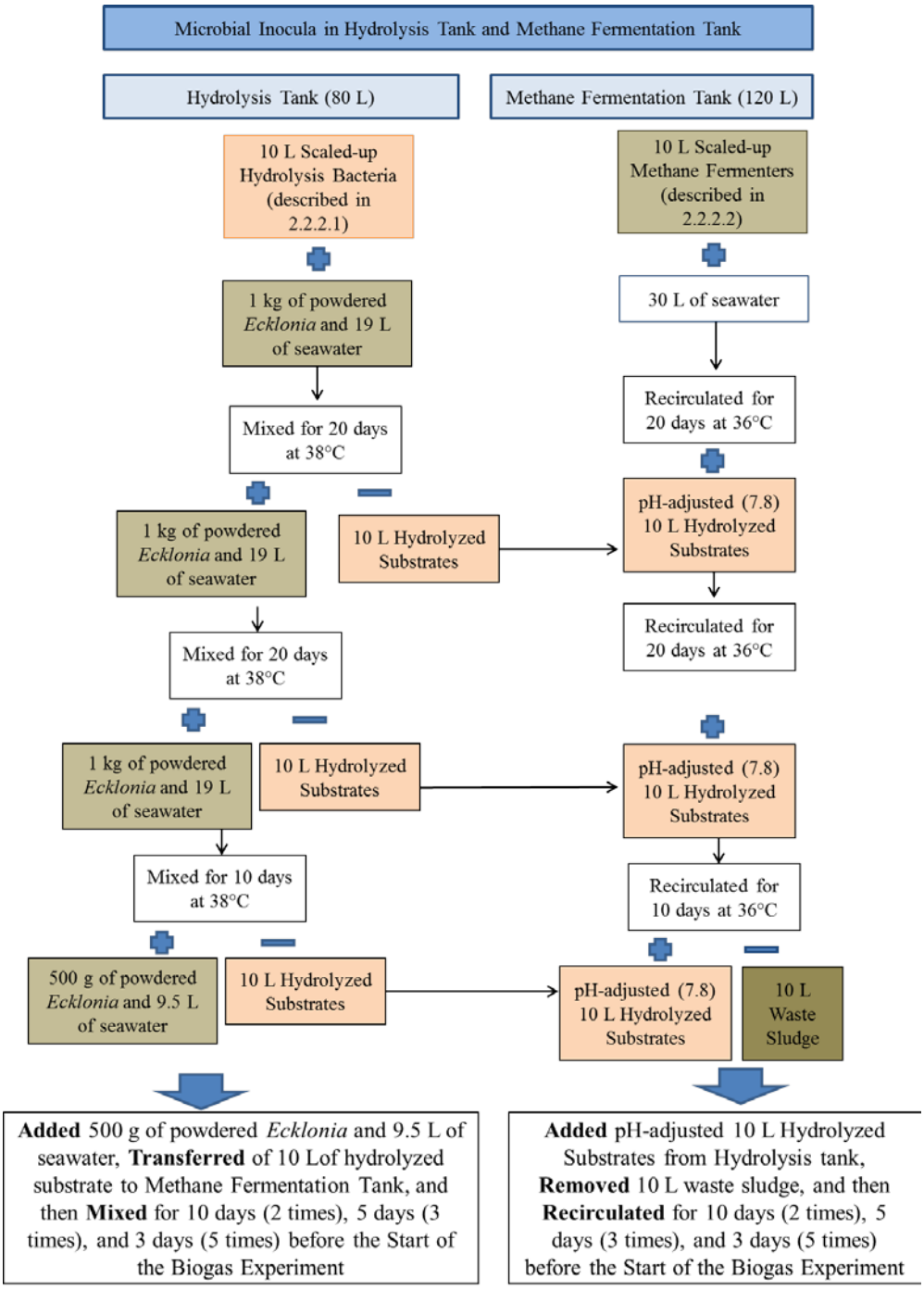

Figure 5.3 The schematic diagram of the development and scaling up of the marine hydrolytic bacteria and marine methane fermenters for hydrolysis tank and methane fermentation tank, respectively.

5.2.3. Description and operation of the semi-continuous fixed bed digester

The semi-continuous fixed bed digester (Figure 5.4) was composed of hydrolysis (80 L), pH adjustment (50 L), methane fermentation (120 L), and waste sludge tanks (50 L) which are made up of stainless steel. The polyvinylidene chloride sponges with a density of 0.04 g/ cm^3 was used in the methane fermentation tank (occupying 50 L volume). These are used as fixed-bed to retain and increase the density of the methane bacteria in the tank. The hydrolysis tank was continuously mixed with a CEMCO chemical mixer (TCM-45515-PSS), while the methane fermentation tank was continuously recirculated using a magnet pump (SANSO PMD-581B2E). The working volumes of the hydrolysis tank, pH adjustment tank, and methane fermentation tank were 50 L, 30 L, and 50 L, respectively. Substrates were transferred from each tank using a reversible motor pump (YOKOGAWA CRM-H8A25Z). The temperature of hydrolysis tank (38°C) and methane fermentation tank (36°C) were maintained using a recirculating (SANSO PMD-331BK) hot water bath. The pH of all tanks was measured using the YOKOGAWA pH meter (PH10HLD). Addition of 100 ml of 10% NaOH solution in the pH adjustment tank was done on the 12^{th} day or the start of the 5^{th} feeding to adjust the pH of the methane fermentation tank. The feeding of 10 L of substrates (500 g of powdered *Ecklonia* and 9.5 L seawater) to the hydrolysis tank, transferring of 10 L of hydrolyzed substrates to the methane fermentation, and removing of 10 L of waste sludge from the methane fermentation tank were done every 3 days (Figure 5.5). The

salinity of the methane fermentation tank was measured using ASONE refractometer (Master-AS/Millα).

5.2.4. Biogas and data analysis

The volume of biogas was continuously measured using a dry test gas meter (Shinigawa DC-1C-M). Biogas sampling was done every day for the compositional analysis. Biogas components (CH_4, CO_2, and others) were analyzed using a gas chromatograph [Shimadzu model 14B: Injecting volume of 0.2 ml; Porapak Q Column (80-100 mesh; F-3619); Column temperature of 80°C; Injector temperature of 50°C; Helium gas flow rate of 0.098 MPa; Current 100 mA] equipped with thermal conductivity detector (TCD) as described by Chandra et al. (2012b). Gastec detection tubes were used to measure the H_2S (no. 4HH) and NH_3 (no. 3M). All data were presented as mean with standard deviation (SD) as error bar.

Figure 5.4 The overview of the (A) pilot-scale fixed-bed biogas digester, the (B) hydrolysis tank (80 L capacity), the (C) pH adjustment tank (50 L capacity), the (D) methane fermentation tank (120 L capacity), the (E) top part of the methane fermentation tank where biogas is collected using the aluminum bag, and the (F) polyvinylidene chloride sponge (occupying 50 L volume of methane fermentation tank).

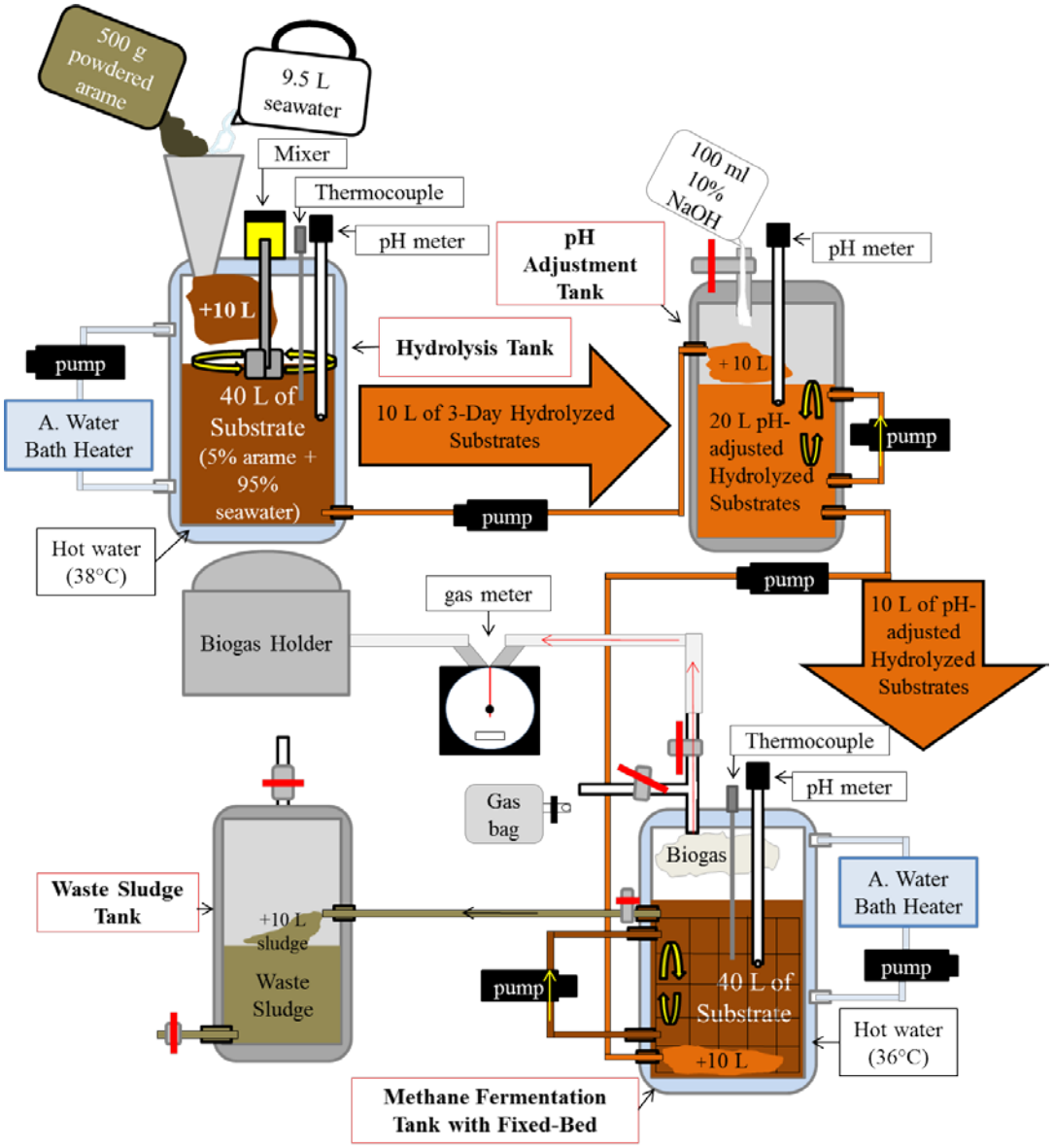

Figure 5.5 The semi-continuous operation of the biogas digester system, which shows the operating condition, working volume capacity, and retention time of the hydrolysis tank and methane fermentation tank under thalassic condition.

5.3. Results and discussion

5.3.1. Proximate composition and theoretical methane yield of *Ecklonia*

The moisture content of *Ecklonia* (65.3%) is relatively lower than the other seaweed. The VS is high (Table 5.1), making its computed theoretical methane yield (345.6 ml CH_4/ g VS) comparable to terrestrial crops (Chandra et al., 2012a). The summary of the proximate compositions of *Ecklonia* is shown in Table 5.1.

Table 5.1 Proximate compositions of *Ecklonia* species.

Proximate values	Percentage (%, w/w)
Protein	3.5
Lipid	0.1
Carbohydrate	25.8
Cellulose	1.4
Hemicellulose	1.6
Lignin	2.3
Moisture content[a]	65.3
Total Solid[a]	34.7
Volatile Solid[b]	75.57
Ash[b]	24.43
C/N	22.17

[a]Measured in fresh weight
[b]Measured in dry weight

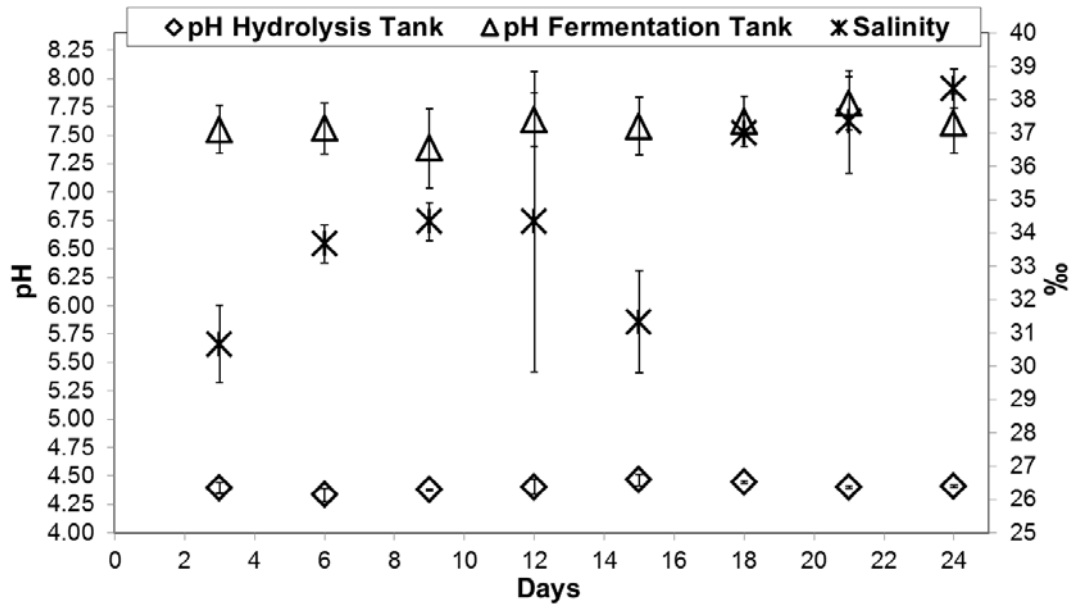

Figure 5.6 The pH of the hydrolysis and fermentation tanks, and salinity of the methane fermentation tank of the thalassic semi-continuous fixed-bed digester using *Ecklonia* as feedstock (*N*= 3; ± S.D.).

5.3.2. pH and salinity

The pH of the hydrolysis under thalassic condition was stable (Figure 5.6). However, lower pH (3.5-3.8) was obtained when food waste was fed under freshwater condition (unpublished). This was expected due to the easier degradability of food waste — mostly composed of cooked rice —, than *Ecklonia* species. The pH of methane fermentation tank fluctuated between 7.3 and 7.8 (Figure 5.6), which was still within the optimum pH range for the methane fermentation. Transferring of hydrolyzed substrates to the methane fermentation tank caused the pH to abruptly decrease. The pH slowly increased up to the 3rd day of retention time for each feeding, which coincided with the consumption of the organic acids during the biogas production. However, the overall pH change was declining; hence, the pH was adjusted at the start of the 5th feeding which resulted to the increased of pH at the 12th day (Figure 5.6). On the other hand, the salinity of the methane fermentation tank increased to 37 ppt at the end of retention time upon pH adjustment (Figure 5.6). This may be due to the pH buffer used, which could have increased the Na^{+}.

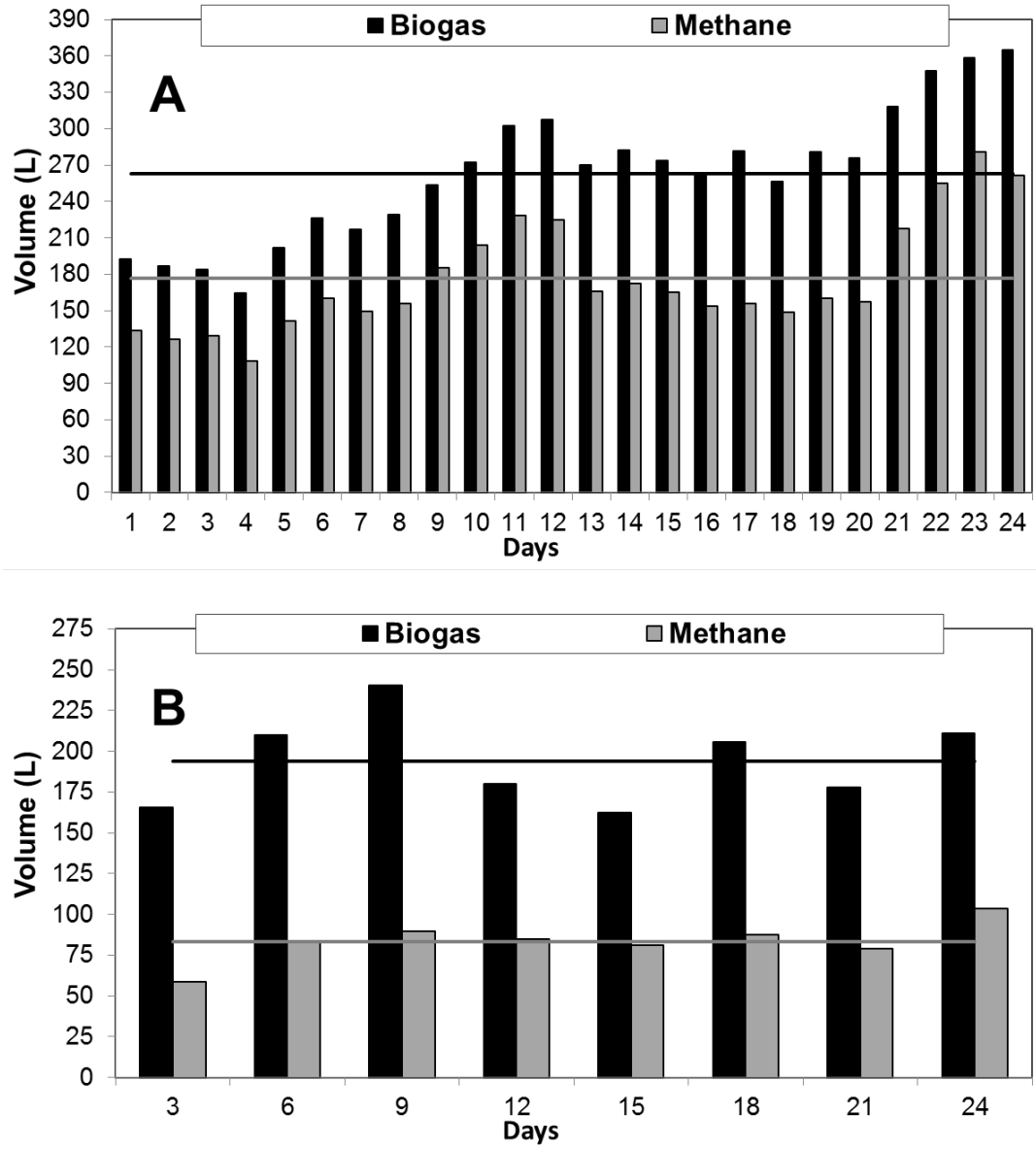

Figure 5.7 The cumulative biogas and methane yield of the (A) food waste, and (B) *Ecklonia* sp. in the fixed-bed digester under freshwater and thalassic conditions, respectively. The fixed-bed digester was continuously operated using food waste, while semi-continuously operated using *Ecklonia* at different times.

5.3.3. Biogas production

The biogas production rate and methane yield of the thalassic condition using *Ecklonia* (Figure 5.7B) were lower than the food waste under freshwater condition (Figure 5.7A, previously operated with the same feeding ratio but 1-day retention time). This was expected because of the more difficult degradation characteristics of *Ecklonia*. Generally, the methane yield per day was high at the start of methane fermentation for each feeding (Figure 5.8). This is due to the high organic acid concentration at the start of fermentation.

The sudden change of salinity coincided with the lower m ethane yield (Figure 5.6 and 5.7B), but the subsequent increase of biogas and methane yield showed the ability of marine bacteria to adjust to the salinity fluctuation. Furthermore, the biofilm formation within the fixed-bed may have helped the fast recovery of the fermentation system. This may have also countered the possible inhibition on methane bacteria brought by phenolic compounds from brown seaweeds (Horn, 2000). Inhibition on methane fermentation was observed during the thalassic biogas production of *Ecklonia* in 1-L batch digester. Only 5.8% of the theoretical methane yield was obtained in this digester. This is very low when compared to 63.8% obtained in the semi-continuous digester (Figure 5.8). Nonetheless, improvement on the biogasification efficiency and degradation rate through pretreatment may further increase the conversion efficiency of the thalassic semi-continuous fixed-bed digester.

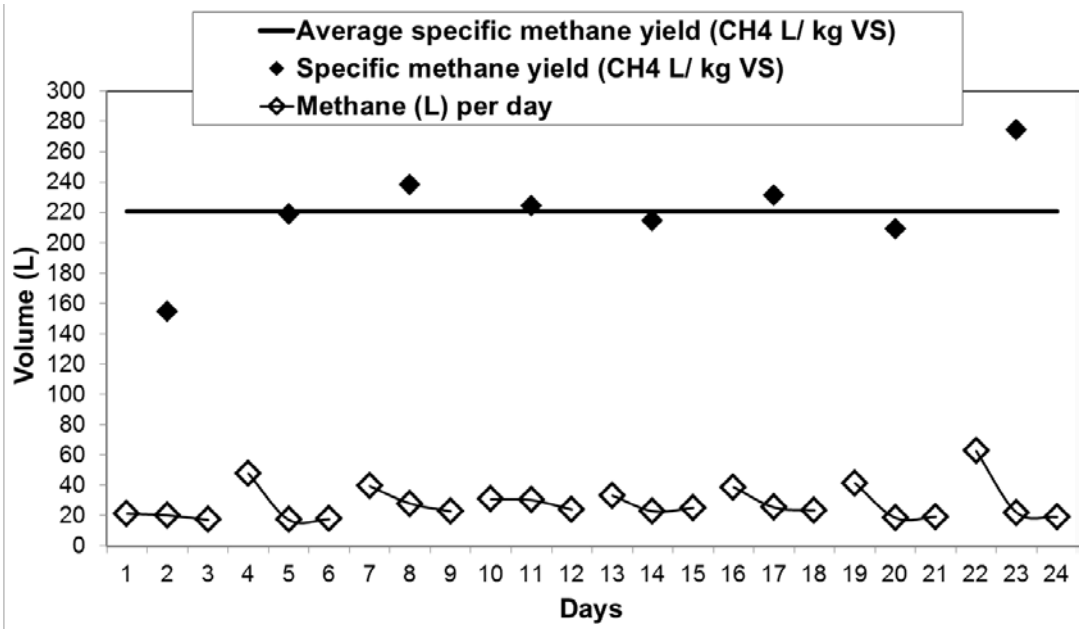

Figure 5.8 The methane yield per day and specific methane yield of *Ecklonia* in a pilot-scale semi-continuous fixed-bed digester as compared to the specific methane yield in a 1-L batch digester.

5.4. Conclusion

The utilization of *Ecklonia* sp. as biomass feedstock on thalassic biogas production obtained higher specific methane yield (220.5 ml CH_4/ g VS) in a semi-continuous fixed-bed digester than in batch digester (19.9 ml CH_4/ g VS). This may have been due to the possible presence of phenolic compounds, which can inhibit methane bacteria. The biofilm formation in fixed-bed could have helped lessen this inhibition. Still, 63.8% of the theoretical methane yield of *Ecklonia* was obtained in the thalassic semi-continuous fixed-bed digester.

Chapter 6
General Conclusion and Future Work

6.1. General Conclusion

The higher methane yield obtained under thalassic condition than under freshwater condition suggested a better anaerobic digestion performance of the marine microbial inoculum than their conventional counterpart. Utilization of thalassic condition can lower the cost of digester operation by using seawater in washing inorganic materials that is mixed in the biomass, and in digester as liquid substrate. Under thalassic, the biological hydrolysis pretreatment (180.9 ml CH_4/ g VS) obtained higher methane yield than the 1% NaOH pretreatment (158.2 ml CH_4/ g VS), but the biogasification time of 1% NaOH pretreatment (27 days) was shorter than that of the biological hydrolysis pretreatment (62 days). Further heating the seaweed before employing the biological hydrolysis pretreatment shortened the biogasification time by half while slightly enhancing the methane yield. The biological hydrolysis can be used as a cheap pretreatment in household digesters.

Alternatively, the Heating + BH and 1% NaOH pretreatment application may be more favorable to commercial biogas plant operation.

The rice straw was successfully used as mono-substrate feedstock for thalassic biogas production, showing a more stable biogas production under thalassic than under freshwater conditions. Although thalassic BH pretreatment of rice straw as mono-substrate gave the highest methane yield (75.8 ml CH_4/ g VS), the biogasification efficiency was only 23.1% of the theoretical methane yield. Co-digestion of rice straw with *Ulva* further improved the methane yield and shortened the retention time, with the 50:50 as the most suitable ratio in terms of specific methane yield (130.3 ml CH_4/ g VS). Therefore, it is better to use rice straw as a supplement feedstock to *Ulva*. Furthermore, the successful biogas production start-up upon the utilization of powdered shell as a bio-based pH buffer was observed. Among the shell set-ups, higher CH_4 yield was obtained when the shell is mixed at the start of anaerobic digestion using 5% shell (109.5 ml CH_4/ g VS). Although low CH_4 yield was obtained in using shell than NaOH, the cheaper and easier acquisition of shell can encourage its utilization as temporary substitute pH buffer in household biogas digesters. Moreover, the successful application of thalassic condition in a pilot-scale fixed-bed semi-continuous biogas digester using *Ecklonia* sp. as biomass feedstock can help the development and optimization of a commercial-scale thalassic biogas digester.

The finite land resources limit the expansion of the production of the conventional biomass resources. The increasing population and the burgeoning middle class in the society further increase the demand on this biomass resource

through the food industry. Only through the expansion of farming in the ocean may sufficiently support this expanding biomass demand, especially in the biofuel industry. Hence, the future of biomass resources may only be through massive seaweed cultivations. This study provides a cheaper biogas production platform through the utilization of seawater as liquid substrates, marine bacteria as microbial inocula, and various types of seaweeds as biomass feedstock that can suitably tackle the challenges that may be brought by the future problems on biomass supply.

6.2. Recommendations for Future Work

Although this study presented the feasibility of thalassic fermentation of seaweed for biogas production, more research can be done to further understand and improve the operation of the thalassic biogas production.

6.2.1. Identification of the most suitable seaweed feedstock

The biogas production varies among the species of seaweeds, the geography where the seaweed was collected, the time when the seaweed was cultivated, and the sufficient supply of seaweed biomass. Hence, parallel studies on the species of seaweed that can be farmed in massive amount can be done to support the demand of future biogas plant that will use seaweed feedstock. Evaluation on the impact of massive seaweed farm on the marine ecosystems should also be done to develop proper management for future ocean expansion.

6.2.2. Improvement of methane yield

Different pretreatments were tested to further improve the methane yield in this study; however, other pretreatments such as hydrothermal, microwave, enzymatic, thermoacidic, or thermoalkaline can be further done to enhance the conversion efficiency. Evaluation of the effect of specific operational parameters such as organic loading rate, temperature, pH, H_2S level, salinity or organic acids on the biogas production can also be done to better understand the optimum operational parameters of thalassic digester for better control and easier management.

6.2.3. Continuous operation of the pilot-scale digester

Extension of the period of operation of the pilot-scale digester can be done to further support the feasibility of thalassic biogas production for commercial application, only concentrating on the utilization of a specific species of seaweed like *Ulva*. Further optimization of the operational condition is needed to establish suitable operational condition for commercial application and maximize profitability of thalassic biogas plant.

6.2.4. Evaluation of bacterial species and their population dynamics

Determine the important bacterial species that are present in the thalassic biogas digester and their population dynamics as dictated by the changing physico-chemical parameters of the digester during operation. This microbial knowledge can help increase the biogas conversion efficiency and allows the comparison of thalassic digester and conventional digester.

6.2.5. Analysis of life cycle assessment (LCA)

To further elaborate the feasibility of thalassic biogas production, the LCA can be done to determine the economic feasibility of the thalassic biogas platform and the impact of this platform on the environment.

References

Adams JMM, Toop TA, Donnison IS, Gallagher JA. (2011). Seasonal variation in *Laminaria digitata* and its impact on biochemical conversion routes to biofuels. Bioresour Technol 102, 9976-84.

Amon T, Amon B, Kryvoruchko V, Machmuller A, Hopfner-Sixt K, Bodiroza, V, Hrbek R, Friedel J, Potsch E, Wagentristl H, Schreiner M, Zollitsch W. (2007). Methane production through anaerobic digestion of various energy crops grown in sustainable crop rotations. Bioresour Technol 98, 3204-12.

Angenent LT, Sung S, Raskin L. (2002). Methanogenic population dynamics during startup of a full-scale anaerobic sequencing batch reactor treating swine waste. Water Res 36, 4648-54.

Association of Official Analytical Chemists (AOAC). (1990). Official Methods of Analysis. 13th ed. USA: AOAC.

Bagi Z, Acs N, Bálint B, Horváth L, Dobó K, Perei KR, Rákhely G, Kovács KL. (2007). Biotechnological intensification of biogas production. Appl Microbiol Biotechnol 76, 473-82.

References

Balboa EM, Rivas S, Moure A, Dominguez H, Parajó JC. (2013). Simultaneous extraction and depolymerization of fucoidan from Sargassum muticum in aqueous media. Mar Drugs 11, 4612-27.

Balk M, Weijma J, Stams AJM. (2002). *Thermotoga lettingae* sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol 52, 1361–8.

Bird KT, Chynoweth DP, Jerger DE. (1990). Effects of marine algal proximate composition on methane yields. J Appl Ecol 2, 207-313.

Bobin-Dubigeon C, Lahaye M, Guillon F, Barry J-L, Gallant DJ. (1997). Factors limiting the biodegradation of *Ulva* sp cell-wall polysaccharides. J Sci Food Agric 75, 341-51.

Boone DR, Bryant MP. (1980). Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl Environ Microbiol 40, 626-32.

Borines MG, de Leon RL, Cuello JL. (2013). Bioethanol production from the macroalgae *Sargassum* spp. Bioresour Technol 138, 22-9.

Brennan L, Owende P. (2010). Biofuels from microalgae- a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 14, 557-77.

Bruhn A, Dahl J, Nielsen HB, Nikolaisen L, Rasmussen MB, Markager S, Olesen B, Arias C, Jensen PD. (2011). Bioenergy potential of *Ulva lactuca*: biomass yield, methane production and combustion. Bioresour Technol 102, 2595-604.

Buswell AM, Mueller HF. (1952). Mechanism of methane fermentation. Ind Eng Chem 44, 550–2.

Campbell CJ. (2005). Oil crisis. United Kingdom: Multi-Science Publishing Co. Ltd.

Chakraborty S, Santra SC. (2008). Biochemical composition of eight benthic algae collected from Sunderban. Indian J Mar Sci 37, 329-32.

Chandra R, Takeuchi H, Hasegawa T. (2012a). Methane production from lignocellulosic agricultural crop wastes: a review in context to second generation of biofuel production. Renew Sustain Energ Rev 16, 1462-76.

Chandra R, Takeuchi H, Hasegawa T. (2012b). Hydrothermal pretreatment of rice straw biomass: a potential and promising method for enhanced methane production. Appl Energy 94, 129-40.

Chen W-H, Han S-K, Sung S. (2003). Sodium inhibition of thermophilic methanogens. J Environ Eng 129, 506–12.

Chen Y, Cheng JJ, Creamer KS. (2008). Inhibition of anaerobic digestion process: a review. Bioresour Technol 99, 4044-64.

Chiellini F, Morelli A. (2011). Ulvan: A versatile platform of biomaterials from renewable resources. In: Pignatello R, editor. Biomaterials- Physics and Chemistry, Croatia: InTech; pp. 75-98.

Chynoweth DP, Fannin KF, Srivastava VJ. (1987). Biological gasification of marine algae. In: Bird KT, Benson PH, editors. Seaweed cultivation for renewable resources, Amsterdam: Elsevier; pp. 285-95.

Chynoweth DP, Owens JM, Legrand R. (2001). Renewable methane from anaerobic digestion of biomass. Renew Energ 22, 1-8.

References

Chynoweth DP, Turick CE, Owens JM, Jerger DE, Peck MW. (1993). Biochemical methane potential of biomass and waste feedstocks. Biomass Bioenergy 5, 95-111.

Coelho CAS, Goddard L. (2009). El Niño-induced tropical droughts in climate change projections. J Clim 22, 6456-76.

Collén PN, Sassi J-F, Rogniaux H, Marfaing H, Helbert W. (2011). Ulvan lyases isolated from the Flavobacteria *Persicivirga ulvanivorans* are the first members of a new polysaccharide lyase family. J Biol Chem 286, 42063-71.

Costa JC, Gonçalves PR, Nobre A, Alves MM. (2012). Biomethanation potential of macroalgae *Ulva* spp. and *Gracilaria* spp. and in co-digestion with wasted activated sludge. Bioresour Technol 114, 320-6.

Daroch M, Geng S, Wang G. (2013). Recent advances in liquid biofuel production from algal feedstocks. Appl Energy 102, 1371-81.

Dębowski M, Zieliński M, Grala A, Dudek M. (2013). Algae biomass as an alternative substrate in biogas production technologies — review. Renew Sust Energ Rev 27, 596-604.

Dehghani M, Karimi K, Sadeghi M. (2015). Pretreatment of rice straw for improvement of biogas production. Energy Fuels 29, 3770-5.

Delivand MK, Barz M, Gheewala SH, Sajjakulnukit B. (2011). Economic feasibility assessment of rice straw utilization for electricity generating through combustion in Thailand. Appl Energy 88, 3651-8.

Demirbas MF. (2011). Biofuels from algae for sustainable development. Appl Energy 88, 3473-80.

References

Demirer GN, Othman M. (2008). Two-phase thermophilic acidification and mesophilic methanogenesis anaerobic digestion of waste-activated sludge. Environ Eng Sci 25, 1291-300.

Deublein D, Steinhauser A. (2008). Biogas from waste and renewable sources: an introduction. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA.

Durand M, Beaumatin P, Bulman B, Bernalier A, Grivet JP, Serezat M, Gramet G, Lahaye M. (1997). Fermentation of green alga sea-lettuce (*Ulva* sp) and metabolism of its sulphate by human colonic microbiota in a semi-continuous culture system. Reprod Nutr Dev 37, 267-83.

Ekborg NA, Gonzalez JM, Howard MB, Taylor LE, Hutcheson SW, Weiner RM. (2005). *Saccharophagus degradans* gen. nov., sp. nov., a versatile marine degrader of complex polysaccharides. Int J Syst Evol Microbiol 55, 1545-9.

Ellis JT, Tramp C, Sims RC, Miller CD. (2012). Characterization of a methanogenic community within an algal fed anaerobic digester. ISRN Microbiol, DOI: 10.5402/2012/753892.

Elshahed MS. (2010). Microbiological aspects of biofuel production: current status and future directions. J Advanced Research 1, 103-11.

Eriksson T, Karlsson J, Tjerneld F. (2002). A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of *Trichoderma reesei*. Appl Biochem Biotechnol 101, 41-60.

References

FAO. (2013). Fisheries and Aquaculture Department, species fact sheets: *Undaria pinnatifida* (Harvey) Suringar, 187). [cited 25 July 2013]. Available from: http://www.fao.org/fishery/species/2777/en. Accessed 25 July 2013.

Funami T, Hiroe M, Noda S, Asai I, Ikeda S, Nishinari K. (2007). Influence of molecular structure imaged with atomic force microscopy on the rheological behavior of carrageenan aqueous systems in the presence or absence of cations. Food Hydrocolloids 21, 617-29.

Gao Y, Skutsch M, Masera O, Pacheco P. (2011). A global analysis of deforestation due to biofuel development: a working paper 68. CIFOR, Bogor, Indonesia.

Gerardi MH. (2003). The Microbiology of Anaerobic Digesters. Waste Water Microbiology Series. USA: John Wiley and Sons Inc.

Givernaud T, El Gourji A, Mouradi-Givernaud A, Lemoine Y, Chiadmi N. (1999). Seasonal variations of growth and agar composition of *Gracilaria multipartite* harvested along the Atlantic coast of Morocco. Hydrobiologia 398/399, 167-72.

Grima-Olmedo C, Ramírez-Gómez Á, Alcalde-Cartagena R. (2014). Energetic performance of landfill and digester biogas in a domestic cooker. Appl Energy 134, 301-8.

Gupta P, Singh RS, Sachan A, Vidyarthi AS, Gupta A. (2012). A re-appraisal on intensification of biogas production. Renew Sust Energ Rev 16, 4908-16.

Gurung A, Van Ginkel SW, Kang W-C, Qambrani NA, Oh S-E. (2012). Evaluation of marine biomass as a source of methane in batch tests: A lab-scale study. Energy 43, 396-401.

Hall DO. (1983). Biomass for energy in developing countries. Geojournal 7, 5-14.

References

Hamelinck CN, Faaij APC. (2006). Outlook for advanced biofuels. Energy Policy 34, 3268–83.

Hanssen JF, Indergaard M, Østgaard K, Bævre OA, Pedersen TA, Jensen A. (1987). Anaerobic digestion of *Laminaria* spp. and *Ascophyllum nodosum* and application of end products. Biomass 14, 1-13.

He Y, Pang Y, Liu Y, Li X, Wang K. (2008). Physicochemical characterization of rice straw pretreated with sodium hydroxide in the solid state for enhancing biogas production. Energy Fuels 22, 2775-81.

HLPE. (2013). Biofuels and food security. Rome: A report by the High Level Panel of Experts on Food Security and Nutrition of the Committee on World Food Security.

Horn SJ. (2000). Bioenergy from brown seaweed. PhD Dissertation. Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway.

Horn SJ, Østgaard K. (2001). Alginate lyase activity and acidogenesis during fermentation of *Laminaria hyperborea*. J Appl Phycol 13, 143-52.

Hossain Z, Kurihara H, Takahashi K. (2003). Biochemical composition and lipid compositional properties of the brown alga *Sargassum horneri*. Pak J Biol Sci 6, 1497-500.

Huopana T, Song H, Kolehmainen M, Niska H. (2013). A regional model for sustainable biogas electricity production: a case study from a Finish province. Appl Energy 102, 676-86.

References

Hurtado AQ, Critchley AT, Trespoey A, Bleicher-Lhonneur G. (2008). Growth and carrageenan quality of *Kappaphycus striatum* var. sacol grown at different stocking densities, duration of culture and depth. J Appl Phycol 20, 551-5.

Hwang EK, Baek JM, Park CS. (2009). The mass cultivation of *Ecklonia stolonifera* Okamura as a summer feed for the abalone industry in Korea. J Appl Phycol 21, 585-90.

Indergaard M, Skják-Bræk G, Jensen A. (1990). Studies on the influence of nutrients on the composition and structure of alginate in *Laminaria saccharina* (L.) Lamour. (Laminariales, Phaeophyceae). Botanica Marina 33, 277-88.

Isogai A. (1997). NMR analysis of cellulose dissolved in aqueous NaOH solutions. Cellulose 4, 99-107.

Jacob DE, Soldati AL, Wirth R, Huth J, Wehrmeister U, Hofmeister W. (2008). Nanostructure, composition and mechanisms of bivalve shell growth. Geochim Cosmochim Ac 72, 5401-15.

John RP, Anisha GS, Madhavan Nampoothiri K, Pandey A. (2011). Micro and macroalgal biomass: a renewable source for bioethanol. Bioresour Technol 102, 186-93.

Kamagata Y, Mikami E. (1989). Diversity of acetotrophic methanogens in anaerobic digestion. In: Hattori T, Ishida Y, Maruyana Y, Morita RY, Uccida A. editors. Recent advances in microbial ecology. Tokyo, Japan: Japan Scientific Societies Press; Pp. 459–64.

References

Karakashev D, Batstone DJ, Angelidaki I. (2005). Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Appl Environ Microbiol 71, 331-8.

Karakashev D, Batstone DJ, Trably E, Angelidaki I. (2006). Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of *Methanosaetaceae.* Appl Environ Microbiol 72, 5138-41.

Karpenstein-Machan M. (2005). Energiepflanzenbau für Biogasanlagenbetreiber (Energy crop for biogas plant operators). DLG-Verlags-GmbH, Frankfurt am Main, Germany.

Karray R, Hamza M, Sayadi S. (2015). Evaluation of ultrasonic, acid, thermos-alkaline and enzymatic pretreatments on anaerobic digestion of *Ulva rigida* for biogas production. Bioresour Technol 187, 205-13.

Kim M, Liu C, Noh J-W, Yang Y, Oh S, Shimizu K, Lee D-Y, Zhang Z. (2013). Hydrogen and methane production from untreated rice straw and raw sewage sludge under thermophilic anaerobic conditions. Int J Hydrogen Energy 38, 8648-56.

Kim J, Lee Y, Jung S, Lee J, Cho MH. (2010). Production of methane from anaerobic fermentation of marine macro-algae. Clean Technol 16, 51-8.

King GM. (1988). Methanogenesis from methylated amines in a hypersaline algal mat. Appl Environ Microbiol 54, 130-6.

Klass DL. (1998). Biomass for renewable energy, fuels, and chemicals. San Diego: Academic Press.

References

Krakat N, Westphal A, Schmidt S, Scherer P. (2010). Anaerobic digestion of renewable biomass: thermophilic temperature governs methanogen population dynamics. Appl Environ Microbiol 76, 1842-50.

Kumar M, Gupta V, Kumari P, Reddy CRK, Jha B. (2011). Assessment of nutrient composition and antioxidant potential of Caulerpaceae seaweeds. J Food Compost Anal 24, 270-8.

Lahaye M, Brunel M, Bonnin E. (1997). Fine chemical structure analysis of oligosaccharides produced by an ulvan-lyase degradation of the water-soluble cell-wall polysaccharides from *Ulva* sp. (Ulvales, Chlorophyta). Carbohydr Res 304, 325-33.

Lahaye M, Robic A. (2007). Structure and functional properties of ulvan, a polysaccharide from green seaweeds. Biomacromolecules 8, 1765-74.

Lam MK, Lee KT. (2012). Microalgae biofuels: a critical review of issues, problems and the way forward. Biotechnol. Adv 30, 673–90.

Leliaert F, Zhang X, Ye N, Malta E, Engelen AH, Mineur F, Verbruggen H, De Clerck O. (2009). Identity of the Qingdao algal bloom. Phycol Res 57, 147-51.

Li D, Chen L, Xu D, Zhang X, Ye N, Chen F, Chen S. (2012). Preparation and characteristics of bio-oil from the marine brown alga *Sargassum patens* C. Agardh. Bioresour Technol 104, 737-42.

Lin H-J, Wu C-Y, Kao S-J, Kao W-Y, Meng P-J. (2007). Mapping anthropogenic nitrogen through point sources in coral reefs using $\delta^{15}N$ in macroalgae. Mar Ecol Prog Ser 335, 95-109.

References

Liu D, Keesing JK, He P, Wang Z, Shi Y, Wang Y. (2013). The world's largest macroalgal bloom in the Yellow Sea, China: formation and implications. Estuarine, Coastal Shelf Sci 129, 2-10.

Lowe SE, Jain MK, Zeikus JG. (1993). Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. Microbiol Rev 57, 451-509.

Macqueen D, Korhaliller S. (2011). Bundles of energy: the case for renewable biomass energy. Natural Resource Issues No. 24. IIED, London.

Manyi-Loh CE, Mamphweli SN, Meyer EL, Okoh AI, Makaka G, Simon M. (2013). Microbial anaerobic digestion (bio-digesters) as an approach to the decontamination of animal wastes in pollution control and the generation of renewable energy. Int J Environ Res Public Health 10, 4390-417.

Marín A, Casas-Valdez M, Carrillo S, Hernández H, Monroy A, Sanginés L, Pérez-Gil F. (2009). The marine algae *Sargassum* spp. (Sargassaceae) as feed for sheep in tropical and subtropical regions. Rev Biol Trop 57, 1271-81.

Marquez GPB, Reichardt WT, Azanza RV, Klocke M, Montaño MNE. (2013). Thalassic biogas production from sea wrack biomass using different microbial seeds: cow manure, marine sediment and sea wrack-associated microflora. Bioresour Technol 133, 612-17.

Marquez GPB, Santiañez WJE, Trono Jr GC, Montaño MNE, Araki H, Takeuchi H, Hasegawa T. (2014). Seaweed biomass of the Philippines: sustainable feedstock for biogas production. Renew Sustain Energ Rev 38, 1056-68.

References

Marquez GPB, Santiañez WJE, Trono Jr GC, de la Rama SRB, Takeuchi H, Hasegawa T. (2015a). Seaweeds: a sustainable fuel source. In: Tiwari BK, Troy DJ, editors. Seaweed Sustainability: Food and Non-food Applications, USA: Academic Press; p. 421-58.

Marquez GPB, Takeuchi H, Hasegawa T. (2015b). Biogas production of biologically and chemically-pretreated seaweed, *Ulva* spp., under different conditions: freshwater and thalassic. J Jpn Inst Energy 94, 1066-73.

Marquez GPB, Reichardt WT, Azanza RV, Onda DFL, Lluisma AO, Montaño MNE. (2015c). Dominance of hydrogenotrophic methanogens at the peak of biogas production in thalassic digesters. Waste Biomass Valorization 6, 201-7.

Marquez GPB, Takeuchi H, Hasegawa T. (In press a). Biogas production performance of *Undaria pinnatifida* using a bio-based pH buffer- shell of *Venerupis* species (Asari). Energy Sources, Part A.

Marquez GPB, Noboribayashi T, Takahashi T, Fujii Y, Takeuchi H, Hasegawa T. (In press b). Performance of semi-continuous fixed-bed digester for thalassic biogas production of the brown seaweed, *Ecklonia* spp. Energy Sources, Part A.

Marquez GPB, Iwasaki K, Takeuchi H, Montaño MNE, Hasegawa T. (In prep) Thalassic biogas production of rice straw as mono-substrate and co-substrate of *Ulva* spp. Appl Energy.

Martin LA, Rodriguez MC, Matulewicz MC, Fissore EN, Gerschenson LN, Leonardi PI. (2013). Seasonal variation in agar composition and properties from *Gracilaria gracilis* (Gracilariales, Rhodophyta) of the Patagonian coast of Argentina. Phycol Res 61, 163-71.

Mata TM, Martins AA, Caetano NS. (2010). Microalgae for biodiesel production and other applications: a review. Renew Sust Energ Rev 14, 217-32.

Matsui T, Koike Y. (2010). Methane fermentation of a mixture of seaweed and milk at a pilot-scale plant. J Biosci Bioeng 110, 558-63.

McHugh S, Carton M, Mahony T, O'Flaherty V. (2003). Methanogenic population structure in a variety of anaerobic bioreactors. FEMS Microbiol Lett 219, 297-304.

McInerney MJ, Bryant MP, Hespell RB, Costerton JW. (1981). *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl Environ Microbiol 41, 1029-39.

Michalak I, Chojnacka K. (2009). Edible macroalga *Ulva prolifera* as microelemental feed supplement for livestock: the fundamental assumptions of the production method. World J Microbiol Biotechnol 25, 997-1005.

Migliore G, Alisi C, Sprocati AR, Massi E, Ciccoli R, Lenzi M, Wang A, Cremisini C. (2012). Anaerobic digestion of macroalgal biomass and sediments sourced from the Orbetello lagoon, Italy. Biomass Bioenerg 42, 69-77.

Mital KM. (1996). Biogas systems: principles and applications. New Delhi: New Age International (P) Limited Publishers; p. 412.

Miura T, Kita A, Okamura Y, Aki T, Matsumura Y, Tajima T, Kato J, Nakashimada Y. (2014). Evaluation of marine sediments as microbial sources for methane production from brown algae under high salinity. Bioresour Technol 169, 362-6.

Morand P, Briand X. (1999). Anaerobic digestion of *Ulva* sp. 2. Study of *Ulva* degradation and methanisation of liquefaction juices. J Appl Phycol 11, 165-77.

Muñoz J, Freile-Pelegrin Y, Robledo D. (2004). Mariculture of *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae) color strains in tropical waters of Yucatan, Mexico. Aquaculture 239, 161-77.

Mussoline W, Esposito G, Lens P, Spagni A, Giordano A. (2013). Enhanced methane production from rice straw co-digested with anaerobic sludge from pulp and paper mill treatment process. Bioresour Technol 148, 135-43.

Muthayya S, Sugimoto JD, Montgomery S, Maberly GF. (2014). An overview of global rice production, supply, trade, and consumption. Ann NY Acad Sci 1324, 7-14.

Nagamani B, Ramasamy K. (1999). Biogas production technology: an Indian perspective. Current Science 77, 44-55.

Nettmann E, Bergmann I, Pramschüfer S, Mundt K, Plogsties V, Herrmann C, Klocke M. (2010). Polyphasic analyses of methanogenic archaeal communities in agricultural biogas plants. Appl Environ Microbiol 76, 2540-8.

Nkemka VN, Murto MJ. (2010). Evaluation of biogas production from seaweed in batch tests and in UASB reactors combined with the removal of heavy metals. Environ Manage 91, 1573-9.

Omar S, Ahmad N, Ahmad F. (1988). Composition of alginates from brown seaweeds, *Sargassum* and *Padina* spp. Pertanika 11, 79-85.

Oremland RS, Marsh LM, Polcin S. (1982). Methane production and simultaneous sulphate reduction in anoxic, salt marsh sediments. Nature 296, 143-5.

Pham TN, Um Y, Yoon HH. (2013). Pretreatment of macroalgae for volatile fatty acid production. Bioresour Technol 146, 754-7.

Plugge CM, Zhang W, Scholten JCM, Stams AJM. (2011). Metabolic flexibility of sulfate-reducing bacteria. Front Microbiol 2, 1-8.

Pope PB, Vivekanand V, Eijsink VGH, Horn SJ. (2013). Microbial community structure in a biogas digester utilizing the marine energy crop *Saccharina latissima*. 3 Biotech 3, 407-14.

Radulovich R, Neori A, Valderrama D, Reddy CRK, Cronin H, Forster J. (2015). Farming of seaweeds. In: Tiwari BK, Troy DJ, editors. pp. 27-59. Seaweed Sustainability: Food and Non-Food Applications. UK: Academic Press.

Raskin L, Stromley JM, Rittmann BE, Stahl DA. (1994). Group- specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl Environ Microbiol 60, 1232–40.

Ratana-arporn P, Chirapart A. (2006). Nutritional evaluation of tropical green seaweeds *Caulerpa lentillifera* and *Ulva reticulate*. Kasetsart J (Nat Sci) 40, 75-83.

Rebhan E. (2009). Challenges for future energy usage. Eur Phys J Special Topics 176, 53-80.

Renaud SM, Luong-Van JT. (2007). Seasonal variation in the chemical composition of tropical Australian marine macroalgae. In: Anderson R, Brodie J, Onsøyen E, Critchley AT, editors. Eighteenth International Seaweed Symposium. Netherlands: Springer; pp. 155-61.

Rivière D, Desvignes V, Pelletier E, Chaussonnerie S, Guermazi S, Weissenbach J, Liu T, Camacho P, Sghir A. (2009). Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J 3, 700-14.

Ross AB, Jones JM, Kubacki ML, Bridgeman T. (2008). Classification of macroalgae as fuel and its thermochemical behavior. Bioresour Technol 99, 6494-504.

Ruiz-Dueñas FJ, Martínez Á.T. (2009). Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microb Biotechnol 2, 164-77.

Sakai S, Imachi H, Hanada S, Ohashi A, Harada H, Kamagata Y. (2008). *Methanocella paludicola* gen. nov., a methane-producing archaeon, the first isolate of the lineage 'Rice Cluster I', and proposal of the new archaeal order *Methanocellales* ord. nov. Int J Syst Evol Microbiol 58, 929-36.

Santhi VS, Gupta A, Saranya S, Jebakumar SRD. (2014). A novel marine bacterium *Isoptericola* sp. JS-C42 with the ability to saccharifying the plant biomasses for the aid in cellulosic ethanol production. Biotechnol Rep 1-2, 8-14.

Sari FP, Budiyono. (2014). Enhanced biogas production from rice straw with various pretreatment: a review. Waste Tech 2, 17-25.

Schnurer A, Schink B, Svensson BH. (1996). *Clostridium ultunense* sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. Int J Syst Bacteriol 46, 1145-52.

Schramm W, Lehnberg W. (1984). Mass culture of brackish-water-adapted seaweeds in sewage-enriched seawater. II: Fermentation for biogas production. Hydrobiologia 116, 282-7.

Searchinger T, Heimlich R, Houghton RA, Dong F, Elobeid A, Fabiosa J, Tokgoz S, Hayes D, Yu T-H. (2008). Use of U.S. croplands for biofuels increases greenhouse gases through emissions from land-use change. Science 319, 1238-40.

Sethi R, Padmavathi T, Sullia SB. (2013). Lignocellulose biomass degradation by marine microorganisms. Eur. J Exp Biol 3, 129-38.

Sexton S, Rajagopal D, Zilberman D, Hochman G. (2009). Food versus fuel: How biofuels make food more costly and gasoline cheaper. Agricultural and Resource Economics Update 12, 1-6.

Shafie SM, Masjuki HH, Mahlia TMI. (2014). Rice straw supply chain for electricity generation in Malaysia: economical and environmental assessment. Appl Energy 135, 299-308.

Shink B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61, 262-80.

Shuuluka D, Bolton JJ, Anderson RJ. (2013). Protein content, amino acid composition and nitrogen-to-protein conversion factors of *Ulva rigida* and *Ulva capensis* from natural populations and *Ulva lactuca* from an aquaculture system, in South Africa. J Appl Phycol 25, 677-85.

Smetacek V, Zingone A. (2013). Green and golden seaweed tides on the rise. Nature 504, 84-8.

Song Z, Yang G, Han X, Feng Y, Ren G. (2013). Optimization of the alkaline pretreatment of rice straw for enhanced methane yield. BioMed Res Int 968692, DOI: 10.1155/2013/968692.

Sossa K, Alarcon M, Aspe E, Urrutia H. (2004). Effect of ammonia on the methanogenic activity of methylaminotrophic methane producing Archaea enriched biofilm. Anaerobe 10, 13-8.

References

Stanley AM, Stanley DM, Dadu DW, Abah AM. (2013). Appraising the combustion of biogas for sustainable rural energy needs. African J Environ Sci Technol 7, 350-7.

Stuart MD. (2004). Review of research on *Undaria pinnatifida* in New Zealand and its potential impacts on the eastern coast of the South Island. DOC Science Internal Series 166, 1-40.

Suvorov M, Kumar R, Zhang H, Hutcheson S. (2011). Novelties of the cellulolytic system of a marine bacterium applicable to cellulosic sugar production. Biofuels 2, 59-70.

Teichberg M, Fox SE, Olsen YS, Valiela I, Martinettos P, Iribarnes O, Muto EY, Petti MAV, Corbisier TN, Soto-Jiménez M, Páez-Osuna F, Castro P, Freitas H, Zitelli A, Cardinaletti M, Tagliapietra D. (2010). Eutrophication and macroalgal blooms in temperate and tropical coastal waters: nutrient enrichment experiments with *Ulva* spp. GCB 2010;16:2624-37.

The National System (TNS). (2008). National control plan for the Japanese seaweed or wakame *Undaria pinnatifida*, The National System for the Prevention and Management of Marine Pest Incursions. Tasmania: Aquenal Pty Ltd.

Timilsina GR, Mevel S. (2011). Biofuels and climate change mitigation: a CGE analysis incorporating land-use change, World bank policy research working paper 5672. Washington, DC: The World Bank.

Valentine DL. (2002). Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: a review. Antonie van Leeuwenhoek 81, 271-82.

Velimirov B, Field JG, Griffiths CL, Zoutendyk P. (1977). The ecology of kelp bed communities in the Benguela upwelling system, Analysis of biomass and spatial distribution. Helgol Wiss Meeresunter 30, 495-518.

Vijayabaskar P, Vaseela N. (2012). *In vitro* antioxidant properties of sulfated polysaccharide from brown marine algae *Sargassum tenerrimum*. Asian Pac J Trop Dis S890-6.

Vivekanand V, Eijsink VGH, Horn SJ. (2012). Biogas production from the brown seaweed *Saccharina latissima*: thermal pretreatment and codigestion with wheat straw. J Appl Phycol 24, 1295-301.

Wang X, Lu X, Li F, Yang G. (2014). Effect of temperature and carbon-nitrogen (C/N) ratio on the performance of anaerobic co-digestion of dairy manure, chicken manure and rice straw: focusing on ammonia inhibition. PLoS One 9, DOI:10.1371/journal.pone.0097265.

Wei N, Quarterman J, Jin Y-S. (2013). Marine macroalgae: an untapped resource for producing fuels and chemicals. Trends Biotechnol 31, 70–7.

Weiss A, Jérôme V, Burghardt D, Likke L, Peiffer S, Hofstetter EM, Gabler R, Freitag R. (2009). Investigation of factors influencing biogas production in a large-scale thermophilic municipal biogas plant. Appl Microbiol Biotechnol 84, 987-1001.

Wichard T, Charrier B, Mineur F, Bothwell JH, De Clerck O, Coates JC. (2015). The green seaweed *Ulva*: a model system to study morphogenesis. Front. Plant Sci 6, DOI: 10.3389/fpls.2015.00072.

Winter JU, Wolfe RS. (1980). Methane formation from fructose by syntrophic associations of *Acetobacterium woodii* and different strains of methanogens. Arch Microbiol 124, 73–9.

Wong KH, Cheung PCK. (2000). Nutritional evaluation of some subtropical red and green seaweeds Part I — proximate composition, amino acid profiles and some physico-chemical properties. Food Chem 71, 475-82.

Wongwilaiwalin S, Rattanachomsri U, Laothanachareon T, Eurwilaichitr L, Igarashi Y, Champreda V. (2010). Analysis of a thermophilic lignocellulosic degrading microbial consortium and multi-species lignocellulolytic enzyme system. Enzyme Microb Technol 47, 283-90.

Yanagisawa M, Kawai S, Murata K. (2013). Strategies for the production of high concentrations of bioethanol from seaweeds. Bioengineered 4, 224-35.

Yu Z, Schanbacher FL. (2010). Production of methane biogas as fuel through anaerobic digestion. In: Singh OV, Harvey SP, editors. Sustainable Biotechnology, Netherlands: Springer; pp. 105-27.

Zhang J, Tang M, Viikari L. (2012). Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. Bioresour Technol 121, 8-12.

Zhang Y, Chen X, Gu Y, Zhou X. (2015). A physicochemical method for increasing methane production from rice straw: extrusion combined with alkali pretreatment. Appl Energy 160, 39-48.

Zhong W, Zhang Z, Luo Y, Qiao W, Xiao M, Zhang M. (2012). Biogas productivity by co-digesting Taihu blue algae with corn straw as an external carbon source. Bioresour Technol 114, 281-6.

Zhou H, Qiu G. (2006). Inhibitory effect of ammonia nitrogen on specific methanogenic activity of anaerobic granular sludge. J Cent South Univ Technol 13, 63-7.

Zinder SH. (1993). Ecology, physiology, biochemistry and genetics. In: Ferry JG, editor. Methanogens, USA: Springer; pp. 128-206.

Appendix

A. Equipment used in the experiment

Figure A.1 The (left) pH meter (Horiba D-52) used to measure the pH, and the (right) refractometer (AS ONE Master-AS/Millα)) used to measure the salinity in the experiment

Figure A.2 The AS ONE Forced Convection Drying Oven DO-450FA used to dry the biomass (105°C, until constant weight).

Figure A.3 The (left) muffle furnace (Burn Out Furnace KDF007EX) that is used to measure the ash and volatile solid content of the biomass (550°C, until constant weight), containing (right) crucible (dried and powdered biomass).

Figure A.4 The sieves and shaker (Retsch Vibratory Sieve ShakerAS200) used to determine the size of the powdered biomass.

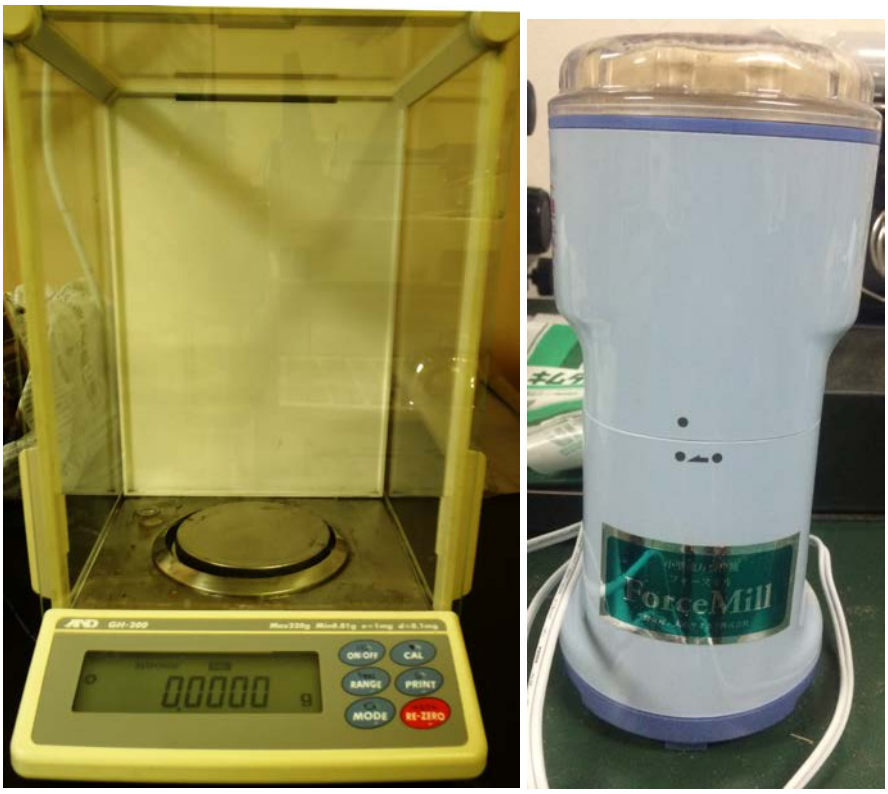

Figure A.5 The (left) weighing scale (AND GH-200) used to measure the weight of the biomass, and the (right) force mill (TDK Y-208B) used to macerate the biomass.

Figure A.6 The shaker (Yamato Shaker MK161) used to automatically mix the digestate.

Figure A.7 The (left) incubator (Yamato model IN602W) used to maintain the temperature and dark condition, and the (right) inside of the incubator showing the 1-L batch digesters (Schott Duran).

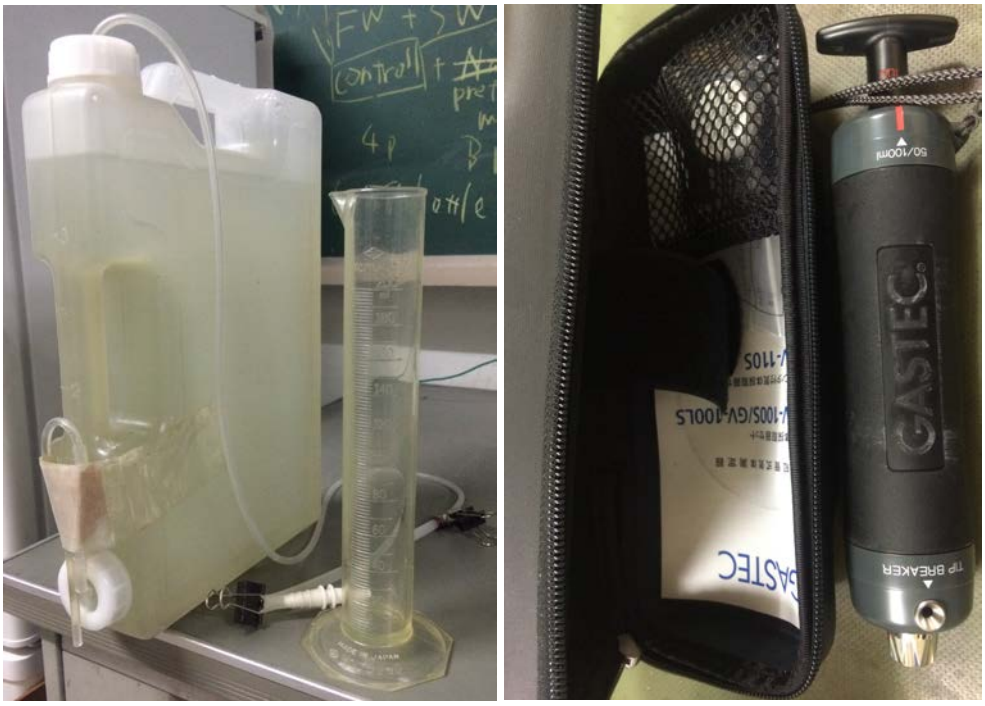

Figure A.8 The (left) water displacement 3-L container that is used to measure the biogas volume from the batch digester, and the (right) Gastec detection tubes used to measure the H_2S (no. 4HH) and NH_3 (no. 3M).

Figure A.9 The gas chromatograph (Yanaco G1880: Injecting volume of 0.2 ml; Column temperature of 80°C; Injector temperature of 50°C; Helium gas flow rate of 0.098 MPa; Current 80 mA) that is equipped with a Porapak Q Column (Length is 2 m, O.D. is 4 Ø, I.D. is 3 Ø, 80-100 mesh) and a thermal conductivity detector (TCD) and used to measure the biogas composition in batch digesters.

Figure A.10 The gas chromatograph (Shimadzu model 14B: Injecting volume of 0.2 ml; Porapak Q Column (80-100 mesh; F-3619); Column temperature of 80°C; Injector temperature of 50°C; Helium gas flow rate of 0.098 MPa; Current 100 mA) that is equipped with thermal conductivity detector (TCD) and used to measure the biogas composition from the pilot-scale methane fermentation tank.