Biogas production from seaweed biomass: A biorefinery approach

by

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Declaration

I declare that this thesis is entirely my own work, except when otherwise stated, and that it has not been previously submitted to any other institute or university.

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CHAPTER 1

INTRODUCTION

1.1. Preface

This thesis is the result of a PhD study conducted at the Centre for Sustainability of the Institute of Technology Sligo, Republic of Ireland.

This PhD thesis follows the "manuscript model" whereby the Chapters are based on articles written by the author during the PhD study and published in peer reviewed scientific journals. Hence, each Chapter is intended to be read as single document that will address the research objectives, forming a block of knowledge for the subject of the thesis.

The work carried out within this PhD thesis was part of a joint research project between six research institutes in the Republic of Ireland, Northern Ireland and Western Scotland, called The BioMara project, which was led by the Scottish Association of Marine Sciences (SAMS). The project was funded by the European Regional Development Fund through the INTERREG IVA Programme with match funding from The Crown Estate, Highlands and Islands Enterprise. Each institute, in their individual expertise areas, implemented translational research aiming to investigate the feasibility and viability to produce biofuels from marine algae biomass.

The work is organised in 10 chapters:

Chapter 1 and 2 provides the context to the research objectives by conducting a review of literature relevant to this investigation. This includes bioenergy, seaweed as a source of energy, microbial production of methane and biorefinery. In order to integrate the anaerobic digestion of seaweed in a biorefinery model, the following Chapters assessed the potential contribution of particular methodologies (pretreatment, co-digestion and digester design) on the seaweed biofuel industry:

Chapter 3 describes the release of macromolecules from the kelp species *Laminaria digitata* and *Saccharina latissima* after exposing the biomass to different pretreatment methods (chemical, thermal and mechanical and enzymatic) (Paper 1).

Chapter 4 outlines the effect of pretreatment methods on biogas production from *L. digitata*. The selection of a suitable inoculum was also addressed (Paper 2).

Chapter 5 compares the influence of different temperatures, psychrophilic (20°C), mesophilic (35°C) and thermophilic conditions (45°C), on biogas production during the anaerobic digestion (AD) of *L. digitata* (Paper 3).

Chapter 6 evaluates the potential of five seaweed species, common in Irish waters, to produce biogas. The biogas and methane content were also compared to yields obtained from the AD of terrestrial feedstocks; grass and rice (Paper 4).

Chapter 7 focuses on a more sustainable biorefinery process to obtain biogas by co-digesting *L. digitata* and *S. latissima* with waste products from different industries (crude glycerol and bovine slurry) (Paper 5 and 6).

Chapter 8 illustrates biogas and methane differences between a stirred reactor and a two-phase AD system as a prospective alternative to a conventional one-phase AD system, overcoming the setbacks from reactor acidification during the hydrolysis phase (Paper 7).

In Chapter 9 the AD of *L. digitata* and *S. latissima* is up-scaled to a 10 L pilot plant, developing the seaweed AD concept further. The Chapter also demonstrates the suitability of the seaweed digestate as source of organic fertiliser to promote the growth of terrestrial crops (Paper 8). Finally, the main outcomes from previous chapters and the implications from a seaweed biorefinery process are outline in Chapter 10.

The contents in Chapters 3, 4, and 9 have been partially published in international peer reviewed journals. Chapters 5 and 6 are the original manuscripts, each with its own sections (introduction, materials and methods, results and discussions and conclusions). The manuscripts from Chapters 7, 8 and 10 are currently under review and preparation.

1.2. Acknowledgements

I would like to express my sincere gratitude to my supervisor Dr. John Bartlett and co-supervisor Dr. Alan Hernon.

Thanks are also due to my research colleagues from the Institute of Technology Sligo.

Finally, my great thanks to my parents, sister, wife and beto, who have supported me during these years.

1.3. Abstract

As the demand for energy is increasing worldwide, many countries are becoming increasingly dependent on fossil fuel consumption, leading to a rapid increase in carbon dioxide and reduction of petroleum reserves.

Alternative and viable options to replace fossil fuels, improve energy security and reduce greenhouse emissions have been proposed worldwide. Marine macroalgae (seaweed) has emerged as an alternative feedstock for the production of a myriad of renewable fuels, such as biogas. The implementation of the anaerobic digestion (AD) process of seaweed requires optimisation before commercialisation is feasible. This PhD study, therefore, aimed to establish a seaweed-based biorefinery approach to produce biogas as main commodity.

The study initially focused on exposing two seaweed species common in Irish waters (*Laminaria digitata* and S*accharina latissima*) to chemical, mechanical, enzymatic and physical pretreatment methods in order to enhance the release of macromolecules (lipids, protein, total carbohydrate and reducing sugars) and, ultimately, increase bio-digestibility to produce biogas.

Results showed that, among all chemical pretreatment conditions tested in this study, dilute acid hydrolysis (4% HNO_3 at 130ºC for 2 hrs) had the greatest effect in releasing macromolecules from *L. digitata* and *S. latissima.* The environmentally friendly pretreatments (freezer milling, oxalic acid and the enzymatic product *Cellulase*) improved the recovery of reducing sugars.

The two seaweed species were subjected to AD to investigate their suitability to generate biogas as source of renewable energy in 120 ml and 1.0 L size reactors. Pretreatments inhibited the anaerobic digestion (AD) process and only a 6% increase in biogas production was obtained when the biomass was subjected to a combination of 2.0% citric acid and *Cellulase*.

For an economically viable digester operation, digester temperature setting is one of the most critical factors. Reactors incubated at a mesophilic temperature were more

effective for biogas and methane production efficiency than either thermophilic or psychrophilic digesters during the AD of *L. digitata*

The AD of different seaweed species commonly found in Irish and the Northern Atlantic Ocean was compared in order to evaluate their potential to produce biogas. The lowest concentration of biogas was achieved from the AD of *Fucus serratus*. *S. latissima*, *Saccorhiza polyschides* and *L. digitata* produced the highest biogas yields, making the three species prospective candidates for the production of biogas as a renewable source of energy.

The seaweed-based biorefinery model integrates the AD of by-products from the biodiesel (glycerol) and the livestock industry (bovine slurry) to produce biogas. The anaerobic co-digestion of these waste streams with either *L. digitata* or *S. latissima* increased biogas and methane yields when compared to AD of the seaweed alone. Results show that the process could be a promising approach to integrating these by-products in order to generate biogas.

During experiments to investigate the scaling up of the process, in 10 L pilot plants, 217 and 305 ml g/VS of methane were produced from the anaerobic digestion of *L. digitata* and *S. latissima*, respectively. The low volatile solid destruction, high alkalinity and accumulation of H_2S caused a reduction in methane production. The organic residue (digestate) generated after the AD of *L. digitata* was shown to be a source of bio-fertiliser that can be used to enhance the growth rate of two biofuel crops, ryegrass and sunflower.

The results obtained from this study provided essential data to support the scale-up of anaerobic digestion of seaweed in order to generate biogas as a source of renewable energy. A seaweed-based biorefinery approach achieved the extraction of macromolecules, the co-digestion of waste products, production of biogas and digestate re-use as source of fertiliser.

1.4. Research objectives

The specific aim of this project was to establish the optimum processing conditions for biogas production from the AD of marine algae common in Irish waters as a renewable source of energy.

The working hypothesis is that marine algal biomass is an underestimated source of green energy. The enhancement of biogas production as a renewable source of energy is expected to be achievable by increasing seaweed biodigestibility, developing the most favourable condition for the AD process and integrating the process within a seaweed-based biorefinery process. To test this hypothesis, the following specific objectives were developed.

1.5. Specific objectives

i. To investigate the effect of different advanced pretreatment processes on the digestibility of the seaweed biomass by maximising macromolecule recovery.

ii. To investigate at bench level the effect of best pretreatment methods on AD of seaweed and biogas production.

iii. To develop the most efficient AD process from seaweed at 120 ml bench scale.

iv. To determine whether co-digestion technologies will enhance AD of seaweed and biogas production.

v. To upscale the best performing 120 ml conditions to 1.0 L, followed by 10 L pilot plant.

vi. To evaluate the effect of seaweed digestate as source of fertiliser.

1.6. Scientific disseminations

2013. The 23rd Irish Environmental Researcher's Colloquium, Galway, Ireland (Poster Presentation). Isolation of anaerobic bacteria capable of degrading seaweed towards biogas production. Carlos Vanegas and John Bartlett.

2013. The 23rd Irish Environmental Researcher's Colloquium, Galway, Ireland (Poster Presentation). Methanogenic community composition in an anaerobic reactor producing biogas from seaweed. Carlos Vanegas and John Bartlett.

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2012. Irish Independent. Can we produce green energy from seaweed? Carlos Vanegas and John Bartlett.
http://www.independent.ie/lifestyle/carlos-vanegas-we-could-one-day-soon-be-heating-our-houses-or-driving-our-cars-with-a-fuel-produced-from-seaweed-26848635.html

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2012. BioMara workshop, SAMS, Scotland (Oral Presentation). Anaerobic digestion of seaweed: Perspectives from IT Sligo. Carlos Vanegas and John Bartlett.

2012. 2nd International Conference on Algal Biomass, Biofuels and Bioproducts, San Diego, USA (Oral presentation). Green energy from marine algae: Biogas production from anaerobic digestion of Irish seaweed species. Carlos Vanegas and John Bartlett.

2012. 4th International Conference on Engineering for Waste and Biomass Valorisation, Porto, Portugal (Oral presentation). Anaerobic digestion *of Laminaria*

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2011. Research showcase IT Sligo, Ireland (Oral and poster presentation). Pre-treatment of seaweed for biogas production. Carlos Vanegas, Alan Hernon and John Bartlett.

2011. The 21st Irish Environmental Researcher's Colloquium, Cork, Ireland (Oral Presentation). Optimisation of seaweed biomass pre-treatment: an eco-friendly approach. Carlos Vanegas, Alan Hernon and John Bartlett.

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2011. BioMara workshop. Queen's University Belfast, Northern Ireland (Oral Presentation). Pretreatment of seaweed biomass towards biogas production. Carlos Vanegas, Alan Hernon and John Bartlett.

2011. Alg'n'Chem 2011, Montpellier, France (Oral presentation). Optimisation of algal biomass pretreatments towards biogas production. Carlos Vanegas and John Bartlett.

2011. 5th International Algae Congress, Berlin, Germany (Poster presentation). Anaerobic digestion of *Laminaria digitata*: the effect of temperature on biogas production. Carlos Vanegas and John Bartlett.

2010. 15th European Biosolids Conference, Leeds, UK (Oral presentation). A biorefinery approach to the production of biogas from algae. Alan Hernon, Carlos Vanegas and John Bartlett.

2010. BioMara Workshop, Dundalk, Ireland (Poster presentation). An Eco-friendly strategy for pre-treatment of algal biomass. Carlos Vanegas, Alan Hernon and John Bartlett.

1.7. Publications by the author

Paper 1 (Chapter 3). 2014. Vanegas C, Hernon A, Bartlett J. Influence of chemical, mechanical and thermal pretreatment on the release of macromolecules from two Irish seaweed species. *Separation Science and Technology*, 49(1), pp.30-38.

Paper 2 (Chapter 4). 2013. Vanegas C.H, Hernon A, Bartlett J. Enzymatic and organic acid pretreatment of seaweed: effect on reducing sugars production and on biogas inhibition. *Journal of Ambient Energy*. DOI 10.1080/01430750.2013.820143.

Paper 3 (Chapter 5). 2013. Vanegas C, Bartlett J. Anaerobic digestion of *Laminaria digitata*: The effect of temperature on biogas production and composition. *Waste and Biomass Valorisation*, 4(3), pp.509-515.

Paper 4 (Chapter 6). 2013. Vanegas C.H, Bartlett J. Green energy from marine algae: Biogas production and composition from the anaerobic digestion of Irish seaweed species. *Environmental Technology*, 34(15), pp.2277-2283.

Paper 5 (Chapter 7). 2012. Vanegas C.H, Bartlett J. Enhanced biogas production from macroalgae by co-digestion with crude glycerol. Bioscience and Bioengineering (Manuscript under review).

Paper 6 (Chapter 7). 2014. Vanegas C.H, Bartlett J. Co-digestion of two kelp species, *Laminaria digitata* and *Saccharina latissima*, with bovine slurry (Under preparation).

Paper 7 (Chapter 8). 2014. Vanegas C.H, Bartlett J. Comparison of a stirred, a single-phase and a two-phase reactor configuration on anaerobic digestion of *laminaria digitata* (Manuscript under review).

Paper 8 (Chapter 9). 2013. Vanegas C.H, Bartlett J. Biogas production from the anaerobic digestion of *Laminaria digitata* in a 10L pilot-plant with digestate re-use as fertiliser. *Journal of Ambient Energy*. DOI 10.1080/01430750.2013.842496.

Paper 9 (Chapter 10). 2014. Vanegas C.H, Bartlett J. Perspectives from a seaweed-based biorefinery approach (Under preparation).

1.8. Abbreviations

AD: anaerobic digestion

BS: bovine slurry

BTU: british thermal units

BSA: bovine serum albumin

DNS: dinitrosalicylic acid

GHE: greenhouse emissions

FFVs: flexible fuel vehicles

ILUC: indirect land use change

LCA: life cycle analyses

NAP: nitrates action programme

OECD: organization for economic cooperation and development

SD: seawater and sand sediments

SWW: sludge from the wastewater

SRB: sulphate reducing bacteria

SM: swine manure

RS: reducing sugars

TL: total lipids

TP: total protein

TRS: total RS

TS: total solids

VFA: volatile fatty acids

VS: volatile solids

CHAPTER 2

LITERATURE REVIEW

2.1. Bioenergy

Economic development combined with the increasing growth of the world population is contributing enormously to world energy consumption, which has been projected to expand by 41% from 2008 to 2035 (BP, 2014; EIA, 2013). Most of the world's energy consumption (88%) comes from fossil fuels with oil (35% share), coal (29%) and natural gas (24%) as the most important fuels, while nuclear energy and hydroelectricity account for 5% and 6%, respectively (BP, 2013; EIA, 2013) (Figure 2.1). However, the reserves of fossil fuels are finite, non renewable and their use produces large amounts of carbon dioxide (CO_2) and other greenhouse gases, contributing to global warming and climate change. 62% of the global warming potential of all anthropogenic greenhouse gases comes from fossil fuel combustion (BP, 2013; Marrero, 2010).

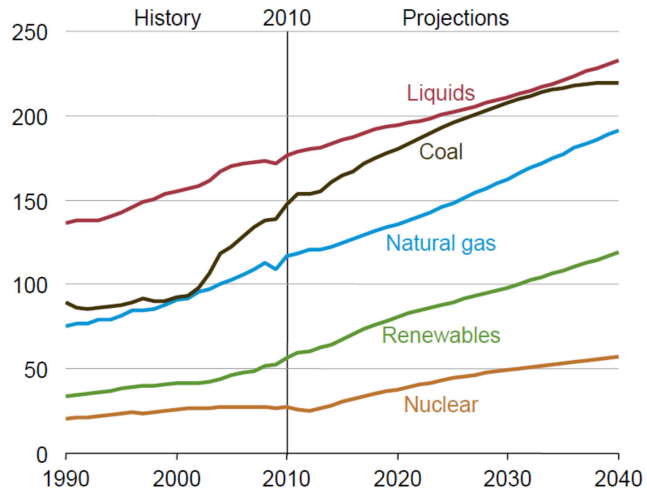


Figure 2.1. World energy consumption by fuel, 1990-2040 in quadrillion BTU (EIA, 2013).

It has been forecasted that total world energy use will increase from 505 quadrillion British thermal units (BTU) in 2008 to 630 quadrillion BTU in 2020 and 820 quadrillion BTU in 2040 (Figure 2.2). This rapid growth in energy consumption occurs in countries outside the Organization for Economic Cooperation and Development (OECD), where demand is determined by long-term economic growth. It is projected

that non-OECD nations will increase energy use by 60-85% while the increase for OECD countries will be just 18% (Chen and Chen, 2011; EIA, 2013; ExxonMobil, 2012).

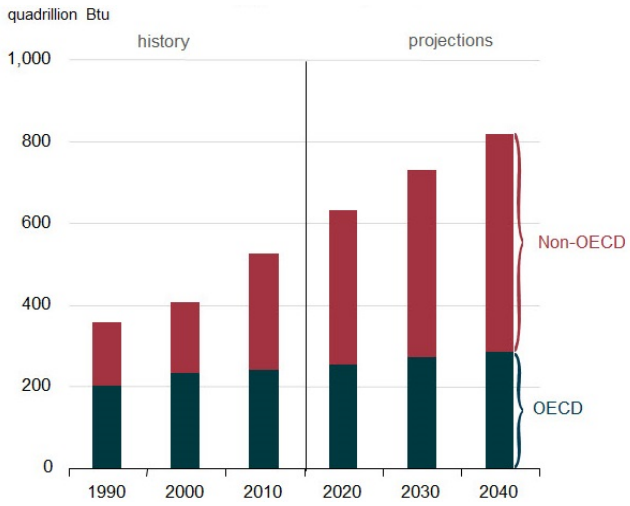


Figure 2.2. World energy consumption 1990-2040 in quadrillion BTU (EIA, 2013).

Concerns about fossil fuels, when added to increasing oil prices and worldwide economic development, are clearly incentives to promote and invest in sustainable, renewable and clean energy resources and technologies.

Renewable sources of energy, including geothermal, solar, wind, biomass, hydropower, wave and tidal action are the world's fastest-growing energy sources (11% in 2010 to 15% 2014) used in several energy fields such as electricity generation, transportation fuels, industrial processes, heating, cooling and process steam. Renewable energy has the potential to exceed current global energy demands, even with existing technologies, although renewable currently provide less than 13% of the world's energy (EIA, 2013; IEA, 2012; IPCC, 2011).

Biomass (any organic material that can be used directly as fuel or converted into other forms before combustion) can play an important role as a renewable source of energy within a domestic bio-based economy by substituting a variety of fuels and chemicals that are currently derived from petroleum (Chum and Overend, 2001; Gomez, *et al.*, 2008; Hayes, 2009; Naik, *et al.*, 2010; Shiralipour and Smith, 1984; Van Dam, *et al.*, 2005). It also represents one of the most abundant and underutilised biological resources on the planet, and is seen as a promising source of material for biofuels and raw

materials, a viable option for improving energy security, replacing fossil fuels and reducing greenhouse emissions (GHE). The carbon in biomass used as fuel, as long as the biomass was grown to an amount equal to that consumed, does not contribute to net build-up of CO_2 emissions (Demirbas, 2009; Kelly and Dworjanyn, 2008). Unlike other renewable energy sources, biomass can be stored and used when necessary.

Depending on the availability of bio-based feedstocks, many nations can potentially develop local biorefineries to produce biofuels (such as biogas, bioethanol, biodiesel, bio-oil), and biochemicals to replace their dependence on petroleum (Chum and Overend, 2001; Demirbas, 2009; Hayes, 2009; Taylor, 2008).

Developing a sustainable bio-based economy that uses eco-efficient bioprocesses and renewable bioresources is one of the key strategic challenges for the 21st century (Chum and Overend, 2001; Demirbas, 2009; Khanal, *et al*., 2010; Naik, *et al*., 2010; Sims, *et al*., 2008; Sims, *et al*., 2010; Van Dam, *et al*., 2005). A bio-based economy has the potential to generate a great number of benefits such as the one mentioned in Table 2.1.

Economic impacts	Environmental impacts	Energy security
*Sustainability	*Greenhouse gas reductions	*Supply reliability
*Fuel diversity	*Reducing air pollution	*Ready availability
*Stakeholders benefit	*Biodegradability	*Renewability
*Reducing dependency on petroleum	*Higher combustion efficiency	*Reducing use of fossil fuels
*International competitiveness	*Improved land and water	*Domestic targets and distribution
*Agricultural development	*Use Carbon sequestration	
*Increased income taxes, jobs and investments in plant and equipment		

Table 2.1. Major benefits of biofuels (Demirbas, 2009).

Biofuel/bioenergy derived from terrestrial biomass (first-generation biofuels) has received considerable attention lately and is considered a leading candidate for renewable energy generation, especially for liquid transportation fuel. It can offer some CO_2 benefits and can help to improve domestic energy security (Naik, *et al*., 2010; Nigam and Singh, 2011; Sims, *et al*., 2008; Taylor, 2008).

A ‘first-generation’ biofuel (i.e. biodiesel, bio-ethanol, and biogas) is characterised either by its ability to be blended with petroleum-based fuels, combusted in existing internal combustion engines, and distributed through existing infrastructure, or by use in existing alternative vehicle technology like FFVs (Flexible Fuel Vehicle) or natural gas vehicles. The production of first-generation biofuels is commercial today, with almost 50 billion litres produced annually (Naik, 2010) and it is expected to reach 140 billion litres a year by 2018 (IEA, 2014). However, the use of terrestrial plants as energy crops has been the subject of debate, and concerns about their utilisation have been expressed (Ajanovic 2011; Havlík, *et al*., 2011; Mueller, *et al*., 2011; Murphy, *et al*., 2011; Sims, *et al*., 2010; Nigam and Singh, 2011):

- Possible contribution to higher prices due to competition with food feedstocks
- A costly option for energy security taking in to account total production costs, excluding government grants and subsidies
- Do not meet their claimed environmental benefits, because the biomass feedstock may not always be produced sustainably
- Accelerated deforestation (with other potentially indirect land use effects also to be accounted for)
- Potentially have a negative impact on biodiversity and landscape
- Require, like any other terrestrial crops, the use of land and water which can compete for water resources in some regions
- Contribute to carbon emissions.

Second-generation biofuels produced from ‘plant biomass’ refers largely to lignocellulosic feedstock, as this makes up the majority of the cheap and abundant non food materials available from plants. But, at present, the production of such fuels faces a number of significant barriers before their potential can be realised (Carriquiry, *et al*., 2011; Havlík, *et al*., 2001; Heyne and Harvey, 2011; Melamu and von Blottnitz, 2011; Naik, *et al*., 2010; Sims, *et al*., 2010). These include the following:

- High cost of production is a fundamental barrier to deployment
- Logistic and supply chain challenges in order to effectively deliver feedstock to the gate of large plants need to be overcome
- Industry and consumer acceptance of biofuel quality

- Perceived risky investments can be significant financial barriers to commercial deployment of emerging technologies
- Agricultural/forestry sector changes needed to supply biomass feedstock imply a shift in the current business model.
- Misunderstanding of environmental/energy tradeoffs is occurring because it is still at an early stage.

Biofuels produced from microalgae and marine macroalgae (seaweed), the third-generation biofuels, has emerged as an alternative and promising feedstock for the production of a myriad of renewable fuels. Extensive research has been conducted to investigate the utilisation of macroalgae as an energy feedstock with applications being developed for the production of biodiesel, bioethanol, biogas and biohydrogen (Bruton, *et al*., 2009; Brennan and Owende, 2010; Chung, *et al*., 2011; Chynoweth, *et al*., 1987; Daroch, *et al*., 2013; Dębowski, *et al*., 2013; Demirbas, 2010; Flowers and Bird, 1984; Hansson, 1983; Jones and Mayfield, 2011; Jung, *et al*., 2013; Li, *et al*., 2008; Mata, *et al*., 2010; Roberts and Upham, 2012; Ross, *et al*., 2008; Singh, *et al*., 2011; Shi, *et al*., 2012; Tarwadi and Chauhan, 1987; Van der Wal, *et al*., 2013; Wei, *et al*., 2013).

When compared to terrestrial biomass, seaweed has a faster growth rate, lower land usage, higher CO_2 absorption and uptake rate, no need for fertilisers and no competition for food resources (Chung, *et al*., 2011; Show, 1981). Therefore, third generation biofuels derived from micro and macroalgae are considered to be a viable alternative energy resource that is devoid of the major drawbacks associated with first and second generation biofuels (Dragone, *et al*., 2010; Jones and Mayfield, 2011).

2.2. Macroalgae

The average photosynthetic efficiency of aquatic biomass is 6–8%, which is much higher than that of terrestrial biomass (1.8–2.2%) (Show, 1981; Miyamoto, 1997). Marine macroalgae can grow to considerable size (up to 80 m in length) and have growth rates ($kg/m^{-2}\ yr^{-1}$) exceeding those of terrestrial biomass (Table 2.2), mainly due to water abundance, the vigorous water movement and turbulent diffusion, which allows very high levels of nutrient uptake, photosynthesis and growth (Gellenbeck and Chapman, 1983; Ross, *et al*., 2008; Show, 1981).

Biomass	Production (Kg/ m^{-2} yr^{-1})
Trees	0.9-2.8
Grasses	1.1-6.8
Sugar cane	6-10
Algae (waste treatment ponds)	3.5-4.9
Algae (laboratory culture)	6.8-13.5
Kelp[1]	4.2-16.6
Large brown algae[1]	3.4-9.5
Palmaria palmate [2]	1-2.2
Saccharina latissima [2]	7-11
Saccorhiza polyschides [2]	12-17
Laminaria digitata [2]	6.4-8.1
Gracilaria ferox (tank cultures)	5.4

[1] Natural beds
[2] Experimental plots

Table 2.2. Productivity of marine and terrestrial biomasses (Show, 1981; Stanley, 2010; Gao and McKinley, 1994; Kelly and Dworjanyn, 2008).

Based on their pigmentation, macroalgae can be classified into three broad groups: i) brown seaweed (Phaeophyceae); ii) red seaweed (Rhodophyceae); and iii) green seaweed (Chlorophyceae) (Demirbas, 2010). Kelp species (brown seaweed) represent the largest and structurally most complex brown algae. The brown colour of these algae results from the dominance of the xanthophyll pigment fucoxanthin (Gupta and Abu-Ghannam, 2011), which masks the other pigments, Chlorophyll a and c (there is no Chlorophyll b), beta-carotene and other xanthophylls (Bartsch, 2008). The carbohydrate fractions are typically complex polysaccharides (laminarin, fucoidans and mannitol). The intercellular matrix and cell wall of brown seaweeds consists mainly of alginates, cellulose, fucoidans and a wide range of proteins (Kloareg and Quatrano, 1988; Michel, *et al*., 2010; Werner and Kraan, 2004). The chemical characteristics of some kelp species are summarised in Table 2.3.

As canopy algae, they often form dense beds, referred to as kelp forests. When compared to other algal communities the biodiversity of kelp forests is very high (Lüning, 1990). Kelp species provide additional substrata for a broad spectrum of macro and micro flora and fauna (Dayton, 1985).

Kelp inhabit the continuously submersed sublittoral and lower intertidal zones, occasionally emerging at extreme low water. Light levels determine the lower limit for algal growth in the sublittoral **(**Merzouk and Johnson, 2011; Lüning, 1990**).** In coastal

waters rich in particles, the depth limit for kelp growth is about 10 - 15 metres below mean low water, whereas in clearer waters of the open Atlantic coast kelp are found in depths down to 30 - 40 metres (Lüning, 1990).

Chemical Characteristics	*Laminaria hyperborea*	*Laminaria digitata*	*Saccorhiza polyschides*	*Saccharina latissima*	*Ulva* sp. (Green algae)
Water (%FW)[1]	77-89	73-90	90-93	78-85	65-83
Ash	16-37	13-38	26-58	22-15	11-35
Carbohydrate	-	-	-	61	33.1-52.6
Alginic acid	17-34	20-45	16-23	24-30	-
Laminarin	0-30	0-25	-	-	-
Mannitol	4-25	5.32	2-11	4	-
Fucoidan	2-4	2-4	-	-	-
Fiber	10.4	6.2	5.5-10.3	-	-
Protein	4-14	8-15	9.4-14.4	6-11	10-27
lipid	0.63	0.5-6	0.5-0.9	0.5	0.3-3.5
K	6-11	1-4	14	-	-
Na	1.6-3	0.9-2.2	4.6	-	0.9-5.9

[1]FW= Fresh weight

Table 2.3. Biochemical composition of seaweed species with biofuel potential.

Composition expressed as % dry weight except for water and ash (Garofalo, 2010).

The structure of kelp consists of a branched holdfast (root-like structure) by which the algae is anchored to the substratum (Figure 2.3a), with no known function in nutrient uptake), a cylindrical flexible stipe and a frond (blade or lamina) (Figure 2.3b). Factors such as tolerance to immersion, light penetration, tolerance to dessication, interspecies competitiveness, competition, grazing and adaptation to wave exposure affect the vertical distribution of these species on the shore (Kelly, 2005).

a b

Figure 2.3. Structure of kelp. Holdfast (a), stipe and frond (b).

2.2.1. Growth of kelp

The meristem, the major zone for longitudinal growth, is located between the stipe and the frond. New tissue is constantly formed by the basal meristem despite regular loss of the apical end of the blade. The meristem, however, is not active at the same rate over the whole year. Growth in kelp is controlled by an endogenous clock, which governs the seasonal rhythm of elongation (Lüning, 1993).

Seasons also affect the growth of kelp species. Growth rates are triggered in early spring and sustained until late summer by a combination of long days and high light levels. The lowest growth rates occur from autumn to winter resulting in a build-up of storage carbohydrates (Adams, *et al*., 2011; b; Lüning, 1979). These carbohydrates are metabolised in late winter, allowing the species to grow when light conditions and photosynthetic activity are not favourable (Lüning, 1993). Environmental factors, such as temperature, light and nutrient availability can also influence growth rates.

2.2.2. Life cycle of kelp

Laminaria plants have a two stage life cycle. Haploid spores which are produced from a large diploid thallus (comprising holdfast, stipe and frond) germinate to produce the gametophyte that, in turn, generates haploid male and female gametes. The sporophyte phase which is responsible for the primary production in the species is generated by the fusion of the gametes. The spores are found in visible dark areas on the blade, termed sori (Kelly, 2005).

The main period of sorus formation and subsequent zoospore release in the kelp species is between autumn and winter (Lüning, 1993). Table 2.4 shows general reproduction times for kelp species in European coasts.

According to recent figures, 19 million tonnes of seaweed were produced worldwide, the vast majority of which (96%) is produced by aquaculture. This makes seaweed the second largest global production after fresh water fish (by weight). The value of the seaweed harvest was estimated at €4.6 billion ($5.7 billion) in 2010 (FAO 2012).

Species	Reproduction time	European coasts
Laminaria hyperborea	October-April	General
	January	Norway
Laminaria digitata	Autum-winder	General
	June/July and October/November	Brittany/France
Laminaria saccharina	Autumn-winter	General
Saccorhiza polyschides	Autumn-winter	General
	September/October	Brittany/France
Alaria esculena	Autumn-winter	General
	February-march	Ireland

Table 2.4. Reproduction times for European Atlantic kelp species (Lüning, 1993).

Among North Atlantic kelp species *Saccharina latissima* is the fastest-growing macroalgal species. This species is similar to *S. japonica* of which 4 million tonne fresh weight is harvested annually from aquaculture in northern China, and almost 0.3 million tonne fresh weight additionally in Korea with Japan trailing at close to 50,000 tonnes (Kraan, 2010).

In Ireland and UK, commercial seaweed farms have developed over the last 2-4 years, and with tonnages of between 50-90 wet tonnes per year of the kelps *Alaria esculenta* and *Saccharina latissima*. However, cultivation of seaweed has been limited to a small number of licensed sites to date, while commercial large scale cultivation for biofuel production is still under research and current trails are mostly carried out at universities and research institutions (SAMS, Galway University, and Queen's University of Belfast) (BIM, 2013).

Preliminary studies show that seaweed production in Ireland is relatively resource-efficient and pilot scale farms will need to be scoped before realistic seaweed cultivation costs can be accurately estimated. While, only large areas of farmed seaweed could be competitive against terrestrial biomass, further research will be required to fully quantify economical aspects and life cycle footprint of the whole process chain.

Additional, the ecological effects of seaweed production to the areas of cultivation (emissions, biodiversity, nutrient bioremediation etc.), public acceptances, the costs for logistics and transportation have to be considered for future planning. By improving culture techniques and making information more available to stakeholders, further engagement can be facilitated to move the seaweed aquaculture sector forward in

Ireland (Aldridge, *et al*., 2012; Capuzzo, *et al*., 2014; Dring, *et al*., 2011; Taelman, *et al*., 2015; Watson and Dring, 2013).

Based on the energy return on investment, the limitations for developing the seaweed biomass as a source of biofuel and the areas requiring additional research can be grouped into four main groups with special focus on energy efficient process in each of these:

- cultivation techniques
- biomass harvest
- post-harvest processing technologies (preservation and storage)
- product extraction (biofuel or value-added products) and optimisation

2.3. Anaerobic digestion

Anaerobic digestion (AD) is a naturally occurring biochemical process, where organic matter is converted mainly to biogas (CO_2 and CH_4) by subsequent oxidations and reductions, in the absence of oxygen. This process occurs naturally in the environment (e.g. sediments, wetlands, swamps, paddy fields etc.), in intestinal tracts of higher animals and insects, in landfills and is applied in anoxic bioreactors. In these environments, electron acceptors, such as dioxygen, nitrate and sulphate, are depleted and replaced by carbon dioxide, resulting in the formation of methane (Brock, 1994; Gerardi, 2003; Klass, 1998).

An important feature of AD is its high degree of organic matter reduction capability, in comparison to aerobic degradation. In addition, energy conversion during the digestion process, to the form of CH_4, makes the process economically profitable (Chynoweth, 1996). Another feature is that the solid remainder from anaerobic degradation can be used as an organic fertiliser for arable land (Al Seadi and Lukehurst, 2012; Fry, 1973; Holm-Nielsen, *et al*., 2009; Lukehurst, *et al*., 2010; Vaneeckhaute, *et al*., 2013).

2.3.1. Microbiology of anaerobic digestion

The anaerobic microbiological decomposition in AD is a process in which microorganisms derive energy and grow by metabolising organic material in an oxygen-

free environment, resulting in the production of CH_4 (Gerardi, 2003; Klass, 1998; Toerien and Hattingh, 1969). Although the general microbiology of the AD process is well known, there is a lack of understanding concerning the dynamics and interactions between the different microorganisms involved in the AD process as, only a small percent of bacteria and archaea have so far been isolated (Gerardi, 2003; Nelson, *et al*., 2011). The process can be subdivided into the following four phases and each phase involves its own characteristic group of microorganisms (Figure 2.4).

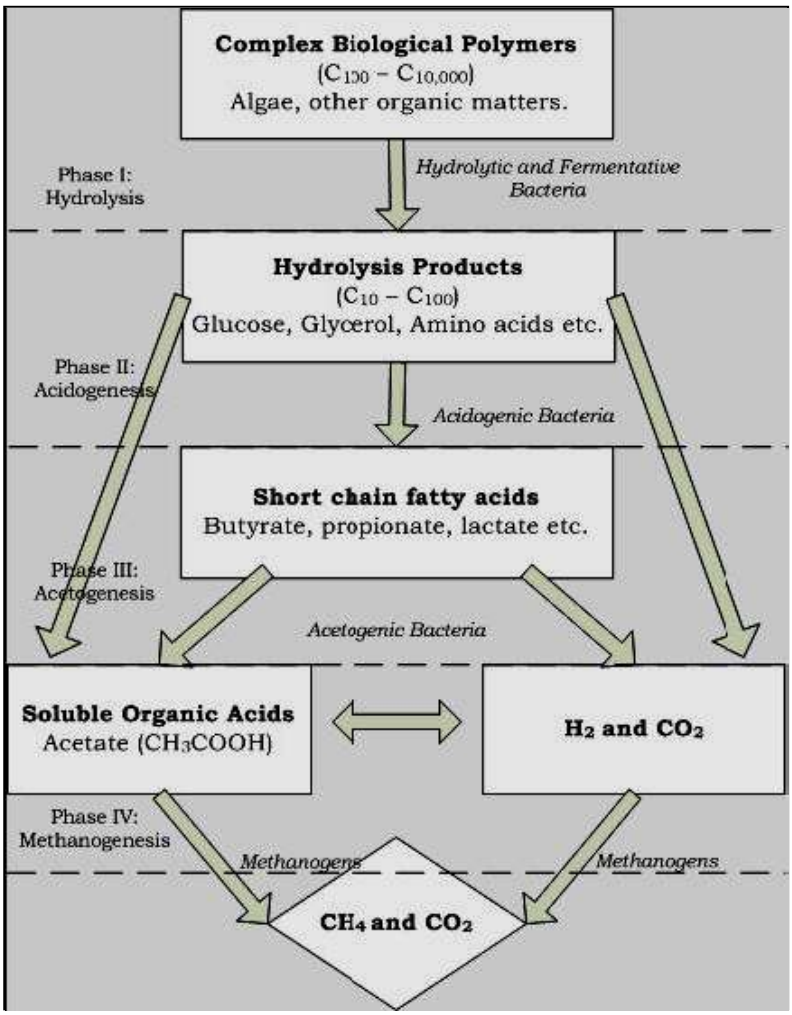


Figure 2.4. A schematic figure (modified) of AD of organic material (Chynoweth, 1996; Chen, *et al*., 2008; Nelson, *et al*., 2011).

2.3.1.1. Hydrolysis

Hydrolysis is the first, and rate limiting phase, of the AD process, where hydrolytic and fermentative microorganisms break down non-soluble biopolymers into soluble organic compounds such as monomers and oligomers. This part of the process may occur

without methanogenesis. More complex biopolymers are unavailable for intracellular metabolism, because of their size and morphology, and therefore, a pretreatment is often needed to accelerate this phase (Brock, 1994; Gerardi, 2003).

2.3.1.2. Acidogenesis

In the acid-forming stage, soluble compounds produced through hydrolysis are degraded by a large diversity of facultative anaerobes and anaerobes through many fermentative processes. The degradation of these compounds results in the production of carbon dioxide, hydrogen gas, alcohols, organic acids, and some organic-nitrogen and organic-sulphur compounds. However, hydrolytic, fermentative and acidogenic activity may be performed by the same bacterium (Brock, 1994; Gerardi, 2003).

2.3.1.3. Acetogenesis

The third phase of the AD process is acetogenesis, where fermentation products, mainly fatty acids and alcohols, are converted into acetate, CO_2 and H_2 by acetogenic bacteria. These bacteria are termed acetogens and are obligate hydrogen producers. Later, those products are used by methanogens to produce CH_4 (Brock, 1994; Klass, 1998).

Hydrogen concentration is an important factor regulating the metabolic activities in both methanogenesis and acetogenesis. Biogas formation from the fermentation products is thermodynamically possible only when the hydrogen concentration is below a threshold concentration, thus H_2 is barely detectable in the biogas formed. At the same time, the biological activity of methanogens requires a continuous supply of hydrogen to carry out the redox reaction (Klass, 1998; Volker, 2003). The relationship between the acetogens and methanogens is syntrophic, supported by a process called interspecies hydrogen transfer or interspecies electron flow (Amani, *et al*., 2011).

2.3.1.4. Methanogenesis

This is the final phase and most sensitive process in the anaerobic decomposition of biomass in AD. In this phase, microorganisms are greatly affected by the system's chemical and physical environment. The performance of any particular methane-forming species is regulated by several factors, such as accumulation of volatile fatty

acids (VFAs), hydrogen pressure, buffering capacity, bicarbonate concentration in liquid phase, CO_2 concentration in the gas phase, pH, ammonia concentration, nutrient availability, toxic substances and other environmental factors, such as temperature, light and agitation (Chen, *et al*., 2008; Chynoweth, 1996; Karakashev, *et al*., 2005; Klass 1998; Switzenbaum, *et al*., 1990).

There are three major pathways of methanogenesis, known as:

- Acetotrophic (acetate metabolised)
- Hydrogenotrophic (H_2/CO_2 metabolised) and
- Methylotrophic (methylated one-carbon compound metabolised).

Methanogens can use a limited number of substrates, of which H_2/CO_2, formate and acetate are the most common, while methanol, ethanol, isopropanol, methylated amines, methylated sulfur compounds and pyruvates are used under specific conditions (Chynoweth, 1992; Gerardi, 2003; Schink, 1997).

Methanosarcineae are the most diverse (metabolically and physiologically) methane-producing microorganisms. These species metabolise various methanogenic substrates and possess the three principal pathways for methanogenesis (hydrogenotrophic, acetotrophic and methylotrophic). In contrast, other methanogens can only utilise no more than two substrates or possess a single metabolic pathway (Zinder, 1993).

In methanogenesis, mainly acetate (Figure 2.5a) or formate (Figure 2.5b) is converted to CH_4 and CO_2 as the end products in anaerobic degradation of organic matter. CO_2 and H_2 can be used (Figure 2.5c) by methanogens in their metabolic pathway to produce CH_4 and H_2O. Carbon monoxide (Figure 2.5d) may be used by some chemolithotrophic methanogens in the production of CH_4. Methylotrophic methanogens produce CH_4 directly from substrates containing the methyl group ($–CH_3$) such as methanol and not via CO_2 (Figure 2.5e). When acetate-utilising methanogens are inhibited by ammonia, sulphides, etc., archaea will oxidise acetate to H_2 and CO_2, which is then the source of CH_4 (Brock, 1994; Deppenmeier, *et al*., 1996; Ferry, 1992; Gerardi, 2003; Toerien and Hattingh, 1969).

(a) $CH_3COOH \rightarrow CH_4 + CO_2$

(b) $2HCOOH \rightarrow CH_4 + CO_2$

(c) $CO2 + 4H_2 \rightarrow CH_4 + 2H_2O$

(d) $4CO + H_2O \rightarrow CH_4 + 3CO_2$

(e) $3CH_3OH + 3H_2 \rightarrow 3CH_4 + 3H_2O$

Figure 2.5. Methane production pathways (Gerardi, 2003).

2.4. Anaerobic digestion of seaweed

The European Commission recently published, under the EU biofuels and Indirect Land Use Change (ILUC) policy, a proposal to reduce the use of food-based biofuels from 10% to 5%. It also prioritises, in first place and among other potential biomasses, the use of algae based biofuel production (European Commission, 2012). The report identifies an enormous potential to attract development, research and investment in the European algae sector.

One contributor to the biofuel call is the use of seaweed to produce renewable bioenergy. Studies carried out in the early 70s on biogas production from macroalgae suggested the importance, advantages and technical challenges associated with the development of the technology (Bird *et al*., 1990; Bird and Benson, 1987; Chynoweth, 1987; Golueke, *et al*., 1957; Klass, *et al*., 1979; Troiano, *et al*., 1976).

This and subsequent research from a limited number of research groups have also highlighted the suitability of the process and recognised the use of seaweed as a strong driver for the development of the biofuel industry, providing the groundwork for research and increasing public and private support (Adams, *et al*., 2011; Chynoweth, 2002; Dave, *et al*., 2013; Dębowski, *et al*., 2013; Hughes, *et al*., 2012; Jard, *et al*., 2012; Kelly and Dworjanyn, 2008; Matsui and Koike, 2010; Migliore, *et al*., 2012; Morand, *et al*., 1990; Nielsen and Heiske, 2011; Østgaard, *et al*., 1993; Peu, *et al*., 2011; Roberts and Upham, 2012). These studies also concluded that the technology is within its early stages of development and process optimisation needs further research, in order to reach its full potential.

2.5. Optimisation of anaerobic digestion

Potential ways to optimise the AD of a particular substrate range from understanding the ecology and microbial interactions within the reactor, to improving reactor performance, combining substrates, adding complementary nutrients and/or pretreating the substrates to make it more amenable for AD. Within this investigation several approaches were used to enhance biogas production from the AD of seaweed.

2.5.1. Pretreatments

Pretreatment is a process step in the biochemical conversion of biomass into biogas. During AD, the first phase-hydrolysis and acidogenesis are often regarded as rate limiting, due to the potential of substrate to hydrolysis. Subsequently, the core function of different pretreatments is to break down the complex biopolymers, disrupt cell walls and bring out the chemical substances from polymers (Kumar, *et al*., 2009). This step makes the organic matter more accessible to microorganisms and thus more easily degraded (Agbor, *et al*., 2011; Sun and Cheng, 2002).

To enhance the overall degradability of substrate, different techniques are being introduced and can be roughly categorised into (Agbor, *et al*., 2011; Alvira, *et al*., 2011; Hendriks and Zeeman, 2009; Keller, *et al*., 2003; Kumar, *et al*., 2009; Mosier, *et al*., 2005; Sun and Cheng, 2002):

- Physical, such as milling, grinding, steam, ultrasonic and thermal.
- Chemical, such as alkali, acid, solvents and oxidising agents.
- Biological or enzymatic
- A combination of some of the above.

The effects of a pretreatment on a particular substrate depend not only on the pretreatment mechanism but also on the characteristics of the substrate (Alvira, *et al*., 2011; Da Costa Sousa, *et al*., 2009; Sun and Cheng, 2002). Although pretreatments to enhance the AD of numerous substrates have been extensively studied in the scientific literature and several technologies are currently in use in commercial scale plants, only a limited number of studies have targeted the use of pretreatments on macroalgae (Tedesco, *et al*., 2014; Tedesco, *et al*., 2013; Jard, *et al*., 2013; Nielsen and Heiske,

2011; Nkemka and Murto 2010; Ross, *et al*., 2009). Therefore, the effect of chemical, physical and enzymatic pretreatment on seaweed was studied in Chapter 3 and 4.

2.5.2. AD process parameters

Temperature and digester configuration, among other operating parameters, are crucial elements affecting the successful start-up and stability of the AD process.

Generally, there are three main ranges of temperature in which the AD process can be carried out: psychrophilic (15-25°C), mesophilic (35-37°C) and thermophilic (45-60°C). The majority of applications and research effort has been concentrated on AD within the range of 30°C to 45°C. It is well known that the rate of decomposition for a determined substrate increases as temperature increases, until the optimum growth temperature is reached. During the AD, temperatures above and below the optimum growth temperature, will affect the metabolic activity of microbial consortiums resulting in a reduction in the reactor kinetics (Chen, *et al*., 2008). In Chapter 5, the optimum temperature for AD of the seaweed specie *L. digitata* was studied under psychrophilic (20±2°C), mesophilic (35±2°C) and thermophilic conditions (45±2°C).

Anaerobic digesters have different designs and configurations adapted to successfully treat a particular substrate. Reactors may be operated as batch or continuous feed, completely mixed with suspended growth or growth supporting media and/or single-phase or two-phase systems. The role of mixing, single and two-phase reactor configuration during the AD of seaweed was investigated in Chapter 8.

2.5.3. Co-digestion

Co-digestion is a method that combines the AD of a main substrate with small quantities of one or more substrates with the aim of improving biogas production. This strategy is known to balance the nutrient content of the mixture and to reduce the effect of inhibitory compounds from substrates throughout the AD process (Braun and Wellinger, 2003; Holm-Nielsen, *et al*., 2009; Mata-Alvarez, *et al*., 2014). Two co-substrates, bovine slurry and crude glycerol were used in this investigation of the AD of seaweed.

2.5.3.1. Bovine slurry

Large amounts of manure are generated by intensive animal farming. In Ireland, total slurry production is estimated as 34.89 Mt per annum and is mainly dominated by cattle slurry (29.95 Mt per annum) (Singh, *et al*., 2010). This by-product poses a waste handling problem due to the odour, its potential to increase GHE, eutrophication and the spread of pathogens (Braun and Wellinger, 2003). The most recent Ireland's Nitrates Action Programme (NAP) is designed to prevent pollution of surface waters and ground water from agricultural sources and to protect and improve water quality. The use of manure and bovine slurry for biogas production is a method widely used to mitigate the negative impacts from this by-product and at the same time generate bioenergy (Marañón, *et al*., 2012; Braun and Wellinger, 2003; Schils and Kok, 2003). In this investigation, the suitability of bovine slurry as co-substrate during the AD of seaweed was studied in Chapter 7.

2.5.3.2. Crude glycerol

Crude glycerol is a by-product from the chemical production of biodiesel. The rapid growth of the biodiesel industry has generated a surplus of glycerol that has resulted in a decrease in crude glycerol prices and environmental concerns associated with disposal (Yang, *et al*., 2012; Yazdani and Gonzalez, 2007). The use of crude glycerol as an organic substrate for biological synthesis of other materials and more recently, as a substrate for biogas production aimed at generating energy, has been the focus of great interest (Clomburg and Gonzalez, 2013; Leoneti, *et al*., 2012; Da Silva, *et al*., 2009). The use of crude glycerol as a co-substrate during the AD of *Laminaria digitata* and *Saccharina latissima* was evaluated in this investigation (Chapter 7).

2.6. Seaweed and biorefineries

The biorefinery model is analogous to that currently used within the petroleum refineries; the chemical processing and refining of petroleum or natural gas into high value products.

Similar to petroleum refineries, biorefineries seeks to convert biomass, through a combination of several technologies, into biofuels, value-added products, chemicals and

feed materials, whilst minimising waste and environmental impact, in a fully integrated, highly efficient processing plant.

Biorefineries are seen to offer an alternative solution to the increasing concern over the remaining fossil resources, climate change and energy security issues. Its implementation integrates knowledge from various subject areas; biotechnology, agriculture, environmental analysis, bio-process engineering, and socio-economic impact assessments. Extensive overviews of biorefineries and the topics surrounding this subject have been written elsewhere (Cherubini, 2010; Hayes, 2009; Jung, *et al*., 2013; Preisig and Wittgens, 2012; Taylor, 2008).

Interest in biorefineries has grown exponentially in recent years and a fully integrated model may improve the overall efficiency of biomass conversion, reduce costs and offer greater flexibility in the product mix. The current biorefinery processes are mainly focused on crops biomass to produce the desirable commodities and only a few researchers have targeted the developed of a seaweed biorefinery concept (Goh, *et al*., 2010; Jung, *et al*., 2013; Ruiz, *et al*., 2013; Subhadra, 2010; Van Hal, *et al*., 2014; Wei, *et al*., 2013).

In terms of chemical composition, physiological and morphological features, seaweeds are significantly different from terrestrial plants, especially due to their unique carbohydrates (Table 2.5) (Andrade, *et al*., 2004; Black, 1950; Gupta and Abu-Ghannam, 2011; Lobban and Wynne, 1981; Ovodov, *et al*., 1970; Percival, *et al*., 1981; Vishchuk, *et al*., 2011; Werner and Kraan, 2004; Westermeier *et al*., 2012).

Polysaccharides, oligosaccharides, pigments, lipids, proteins or amino acids, among other bioactive compounds, derived from macroalgae are widely used to produce diverse biomaterials and by-products in various industries (food, pharmaceutical and chemical). These products are extracted from the seaweed biomass using existing terrestrial biomass physiochemical treatments, where the yield and selectivity of the products are largely influenced by the reaction conditions and the chemical constituents of the biomass (El Gamal, 2010; Fleurence, 1999; Garofalo, 2010; Gupta and Abu-Ghannam, 2011; Roesijadi, *et al*., 2010; Vishchuk, *et al*., 2011; Voronova, *et al*., 1991). Therefore, new process developments to be applied to seaweed biomass are necessary.

	Macroalgae		Microalgae	Terrestrial biomass
Green algae	Red algae	Brown algae		
Mannan	Carrageenan	Laminarin	Starch	Cellulose
Ulvan	Agar	Mannitol	Arabinose	Hemicellulose
Starch	Cellulose	Alginate	Fucose	Lignin
Cellulose	Lignin	Fucoidan	Galactose	
Glucose	Glucose	Cellulose	Glucose	
Mannose	Galactose	Glucose	Mannose	
Rhamnose	Agarose	Galactose	Rhamnose	
Xylose		Fucose	Ribose	
Uronic acid		Xylose	Xylose	
Glucuronic acid		Uronic acid		
		Mannuronic acid		
		Guluronic acid		
		Glucuronic acid		

Table 2.5. Composition of macroalgae, microalgae, and terrestrial biomass (Jung, *et al*., 2012; Malihan, *et al*., 2012).

The versatility of a seaweed-based biorefinery process would be enhanced by integrating biofuel production with biomaterials and by-products in a single biorefinery model (Figure 2.6). Yet, despite the number of positive outputs and advantages from a biorefinery processes, including the energy and value-added products, the current work from academia and industry is in its infancy, and many challenges exist to prove the final value.

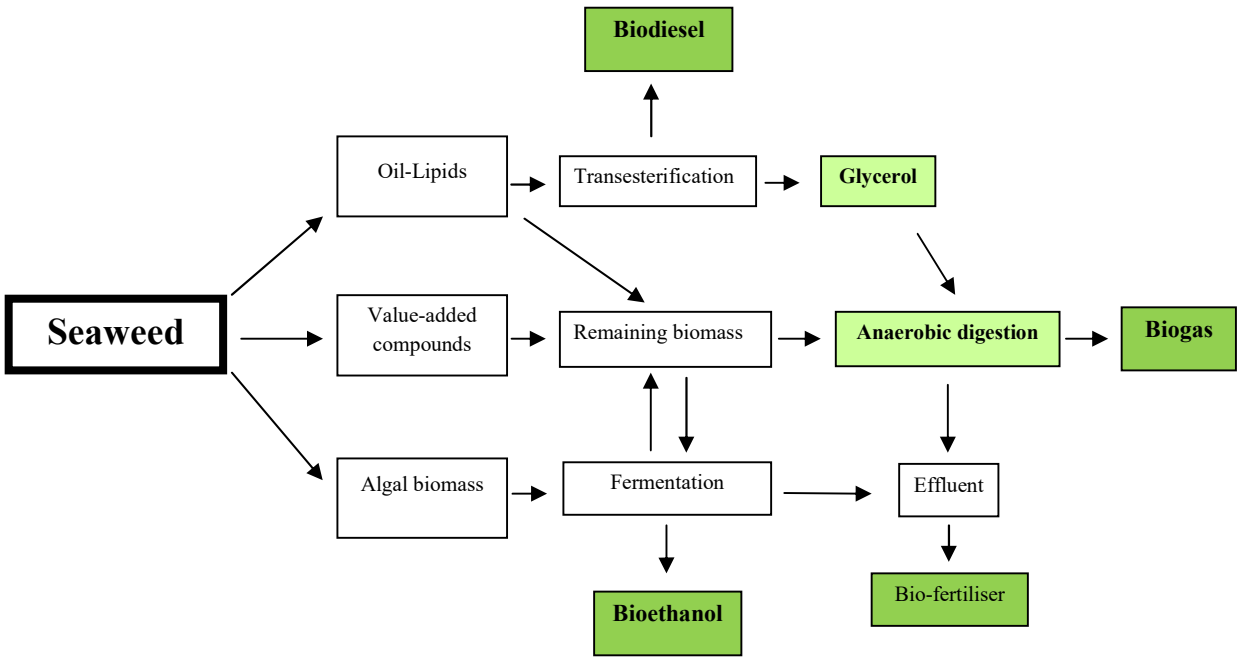


Figure 2.6. General model for a seaweed based biorefinery model (The author).

The most important challenges of this model will be addressed in further chapters, such as the resistance of the seaweed biomass to fermentation and inhibitory factors affecting the downstream microbial processes (anaerobic fermentation and biogas production),such as the C:N ratio, co-digestion, inoculum, digester system configuration and feed stock composition.

CHAPTER 3

INFLUENCE OF CHEMICAL, MECHANICAL, ENZYMATIC AND THERMAL PRETREATMENT ON THE RELEASE OF MACROMOLECULES FROM *Laminaria digitata* AND *Saccharina latissima*

Vanegas, C.H., Hernon, A., Bartlett, J. (2014) Influence of chemical, mechanical and thermal pretreatment on the release of macromolecules from two Irish seaweed species. *Separation Science and Technology*, 49(1), pp. 30-38.

3.1. Introduction

The commercialisation of the conversion of seaweed to biofuel and bioproducts requires optimisation to improve yields before scale-up is feasible. Seaweed consists of complex organic constituents wherein first phase hydrolysis and the subsequent fermentation step are often regarded as rate limiting. Therefore, pretreatment becomes an important step for the production of biomolecules with industrial interest (Behera, *et al*., 2014; Ge, *et al*., 2011; Jang, *et al*., 2013; Park, *et al*., 2012; Pham, *et al*., 2013; Rodriguez-Jasso, *et al*., 2011; Voronova, *et al*., 1991) and fermentable compounds for the biochemical conversion into biofuels (Kim, *et al*., 2013; Lee and Lee, 2011; Lee, *et al*., 2013; Ruiz, *et al*., 2013; Schultz-Jensen, *et al*., 2013; Tan, *et al*., 2013; Tan and Lee, 2014).

The core function of different pretreatments is to make the organic matter more accessible to the microorganisms by breaking down the complex biopolymers, enhancing the bio-digestibility of the algal biomass through accessibility of microbial enzymes, disruption cell walls and bringing out the chemical substances from polymers into more available compounds to ultimately improve fermentation and biofuel yield (Agbor, *et al*., 2011; Behera, *et al*., 2014; Kumar, *et al*., 2009; Mosier, *et al*., 2005).

Biomass pretreatment has been described as the second most expensive unit cost in the conversion of lignocellulose to biofuel (Agbor, *et al*., 2011; Alvira, *et al*., 2011; Behera, *et al*., 2014; Eggeman, *et al*., 2005). Hence, a careful search of pretreatment technologies is necessary to help identify conditions that have the lowest impact on the overall process.

To date, biomass pretreatment has been extensively investigated in lignocellulosic biomass. A limited number of studies in the literature have proposed the use of chemical, thermal, enzymatic and biological pretreatments to increase the hydrolysis rates of algae (Peña-Farfal, *et al*., 2005; Choi, *et al*., 2010; Kim, *et al*., 2013; Park, *et al*., 2012; Schumacher, *et al*., 2011) or to address the effect on the release of sugars from the seaweed species *L. digitata* and *S. latissima* (Adams, *et al*., 2009).

The choice of the pretreatment technology used depends on the physicochemical characteristics of each feedstock (Alvira, *et al*., 2011). Therefore, it is essential to find the most appropriate pretreatment process according to the biomass properties.

Within the implementation of the seaweed-biorefinery model outlined in Chapter 2 (section 2.6), the core objective of the present Chapter was to ascertain whether the single use or combination of different pretreatment methods (chemical, thermal, mechanical and enzymatic) could enhance biodigestibility of *L. digitata* and *S. latissima* and the recovery of macromolecules (reducing sugars, total lipids and total proteins) as value-added products.

3.2. Materials and methods

3.2.1. Biomass material

Samples of two seaweed species common in Irish waters, suitable for cultivation and biofuel production (Alvarado-Morales, *et al*., 2013; Chynoweth, 2002; Hughes, *et al*., 2012; Kelly and Dworjanyn, 2008; Roberts and Upham, 2012), *L. digitata* and *S. latissima*, were used in this study. The two species were harvested from wild stock in May 2010 during low tide from a rocky outcrop of Streedagh beach, Co. Sligo, Ireland. The collected seaweeds were brought to the laboratory for further processing. The algal blades were rinsed manually with tap water to remove sand and foreign objects. The macroalgae were dried in a drying-oven at 75°C for 24 hours (Drier Mina 50 Genlab). Dried material was milled to fine particles (<1.0 mm) using a blender (Philips HR 2000) and stored in airtight capped tubes at -20°C prior to use (Vergara-Fernández, *et al*., 2008). The rationale behind this was that in the event of scalable mariculture, drying and milling would allow preservation and more efficient handling of the seaweed

(Bruton, *et al*., 2009; Kelly and Dworjanyn, 2008; Lewis, *et al*., 2011; Roesijadi, *et al*., 2010).

3.2.2. General pretreatment procedure

1.0 g-aliquots of milled seaweed biomass were dispensed into 15 ml disposable centrifuge tubes. 5 ml (20% solids) of each solution (chemical or dH_20) was reacted under the corresponding pretreatment condition. The tubes with the pretreated biomass were centrifuged at 4500 rpm for 30 min. The solid part and supernatant were separated, and the supernatant was used to determine the concentration of macromolecules released from the seaweed biomass (Denis, *et al*., 2009). Dilutions of supernatant were made accordingly, prior to chemical analysis. For all pretreatments, two different sets of controls were included; samples treated in dH_20 at room temperature for 120 min (control) and samples treated also in dH_20 but at the corresponding temperature and reaction time of the test (thermal). Three replicate samples were run in all analytical determinations, and data are presented as the mean of replicates. The standard deviation (SD) was included in all graphs to see the variance in the data.

3.2.3. Chemical pretreatment (sodium hydroxide, inorganic and organic acids)

Chemical-based pretreatment conditions were selected from studies carried out with lignocellulosic biomass (Agbor, *et al*., 2011; Alvira, *et al*., 2011; Behera, *et al*., 2014; Hendriks and Zeeman, 2009; Kumar, *et al*., 2009; Mosier, *et al*., 2005; Sun and Cheng, 2002).

Most commonly acids/base used in laboratory and industrial practices (Hendriks and Zeeman, 2009; Kumar, *et al*., 2009; Mosier, et al., 2005) were employed during the chemical pretreatment experiments.

Sodium hydroxide (NaOH) (CP-grade), four inorganic (sulphuric, nitric, hydrochloric, phosphoric acid) and four organic acids (citric, lactic, acetic and oxalic acid) (AR-grade) were diluted with dH_20 to a range of percentages (v/v) in order to obtain the optimum chemical concentration for the release of macromolecules (0.1; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 4.0; 6.0). Due to the lack of literature concerning the use of this

pretreatment on seaweed biomass and the chemical composition of the biomass (Chapter 2, Section 2.2), mild conditions were chosen during the investigation.

Two different sets of controls were included; samples treated in dH_20 at room temperature for 120 min (control) and samples treated also in dH_20 but at 120°C (Priorclave Midas 36 autoclave) with a reaction time of 60 min at 1 atm (thermal). Samples were allowed to cool down to room temperature before analysis.

3.2.4. High temperature and reaction time pretreatment

Temperature increase and reaction time were chosen according to the most widely conditions applied toward the improvement of carbohydrate production and fermentation of land-based biomass (Kumar, *et al*., 2009; Mosier, *et al*., 2005).

Therefore, pretreatment was carried out at 80°C, 100°C, 120°C and 130°C in an autoclave with reaction times of 1, 60 and 120 min, at 1 atm. Three different acids (2% citric, 4% nitric and 3% hydrochloric) were used in order to facilitate the comparison. After the reaction, the tubes were cooled at room temperature for further analysis.

3.2.5. Physical-chemical pretreatment

Previous studies on biomass pretreatment methods (Da Silva, *et al*., 2010; Karki, *et al*., 2010; Silva, *et al*., 2010; Silva, *et al*., 2012) revealed that mechanical treatment can greatly enhance the degree of polymerisation, porosity, crystallinity and the specific surface of the biomass. Therefore, in this investigation two different particle sizes and sonication methodologies were employed:

1. The dried seaweed substrate was milled using a blender to <1.0 mm particle size (blender milling).
2. The <1.0 mm particle size seaweed samples were further milled (<0.1 mm) in a cryogenic impact grinder, SPEX 6770 cryogenic freezer, containing a liquid nitrogen bath (Freezer milling). The cylindrical grinding assembly consisted of a polycarbonate central body, a steel impactor, and two stainless steel end plugs. The pre-cooling period prior to actual grinding was 1 min and the grinding time was 1 min.

The two different particle sizes were subjected to high temperature (120°C/60 min/1 atm) and acid pretreatment (2% citric, 4% nitric and 3% hydrochloric acid).

3. The substrate resulting from the blender (particle size <1.0mm) was mixed with 4% nitric acid (20% solids), sonicated for 5, 30 and 60 minutes using a sonication bath (F5200 b ultrasound) at 38 kHz and subjected to high temperature pretreatment (120°C/60 min/1 atm).

3.2.6. Enzymatic pretreatment

Enzymatic hydrolysis has been proposed as a more economical and environmental friendly method to release fermentable sugars from biomass (Alvira, *et al*., 2010; Kovacs, *et al*., 2009). The technology does not create inhibitory compounds or byproducts; it has the lowest energy requirements among other pretreatment technologies and there is no need to use corrosive chemicals. A large number of commercial enzymes produced from a variety of microorganisms have been reported to play an important role in different industrial applications including the biofuel industry (Bhat 2000; Jegannathan and Nielsen, 2013; Klein-Marcuschamer, 2012; Lee, *et al*., 2013). Moreover, previous studies have shown a great potential of enzymatic pretreatment enhancing saccharification and conversion of lignocellulosic biomass (Choi, *et al*., 2009; Choi, *et al*., 2010; Hang and Woodams, 2001; Kovacs, *et al*., 2009). In light of the above information, the seaweed species were subjected to enzymatic hydrolysis with the following commercially available enzymes:

- Cellulase from *Trichoderma longibrachiatum* (Sigma C9748): An acid cellulase with xylanase, pectinase, mannanase, xyloglucanase, laminarase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, amylase, and protease activities. One unit will liberate 1.0 μmole of glucose from cellulose in one hour at pH 5.0 at 37°C.
- Alginate Lyase from *Flavobacterium* sp (Sigma A1603): Breaks down alginate or alginic acid to smaller molecules and reduces viscosity. One unit will produce an increase in the A_{235nm} of 1.0 per minute per mL of sodium alginate solution at pH 6.3 at 37°C.
- Celluclast® 1,5L from *Trichoderma reesei* (1500 NCU/g) (University of Reading/Novo Nordisk): The enzyme catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers. One novo cellulase unit degrades

Carboxy methyl cellulose to reducing carbohydrates with a reduction power corresponding to 1 µmol glucose per minute.

Enzymes were used in accordance with providers' recommendations. Hydrolysis of the seaweed with the range of enzymes was carried out for a period of 24 hrs in a rotator incubator at 300 rpm and the appropriate temperature. Sampling for analysis was performed after 0.5 hr, 1 hr and 24 hrs of hydrolysis. Samples were withdrawn, centrifuged at 15000 rpm for 5 min and the supernatant was used to determine the concentration of reducing sugars released from the hydrolysis process. Each test was conducted in triplicate and the mean value and standard deviation was calculated. Dilutions of supernatant were made accordingly. Controls without the addition of enzymes were included.

3.2.7. Analytical methods for pretreatments

3.2.7.1. Quantification of reducing sugars (RS)

The amount of carbohydrate in supernatants was determined by quantifying the release of RS using the dinitrosalicylic acid (DNS) method (Miller, 1959). RS, under alkaline conditions, reduces 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. A red colour is produced on boiling, which absorbs maximally at 540-560 nm. This assay was chosen to monitor the RS released from the biomass since this technique has been used widely to measure sugars and is a rapid and straightforward method (Borines, *et al*., 2013; Ge, *et al*., 2011; Kim, *et al*., 2013; Tang, *et al*., 2008; Voronova, *et al*., 1991).

1. Preparation of the DNS assay reagent
   A 150 g weight of sodium potassium tartrate was dissolved in 250 ml dH_2O and heated to 40^oC (Solution A). NaOH (8 g) was dissolved in 100 ml dH_2O and heated to 90^oC, before adding 5 g DNS (Solution B). Solution A and Solution B were mixed together, cooled and made up to a final volume of 0.5 L with dH_2O.
2. Preparation of the standards using DNS assay reagent
   A 500 µL volume of known glucose standards ranging from 0.1 to 1.0 mg/ml and a blank containing dH_2O was pipetted into individual microcentrifuge tubes. 1 ml DNS reagent was added to all glucose standards and placed on a water bath at 90ºC for a period of 10 minutes. The tubes were allowed to cool and 200 µL of each

solution was then transferred to a microwell plate. Three wells were allocated for each standard. Plates were placed in a Fluostar optima multidetection microplate reader (BMG Labtech) and read at a wavelength of 560 nm. Absorptions readings were recorded and a standard curve was plotted.

3. RS content of supernatants

   A 500 µL volume of each supernatant was pipetted into individual microcentrifuge tubes. 1 ml DNS was added to each tube and the solution was boiled for 10 min and cooled. An aliquot was transferred to a microwell plate prior to determining absorbance at 560nm using the Fluostar optima multidetection microplate reader (BMG Labtech). A glucose standard curve (Figure 3.1) was used as reference for the determination of the amount of RS in supernatants, which was expressed as mg/ml. Where absorbance readings were outside the linear region of the graph, appropriate dilutions of the supernatant were made.

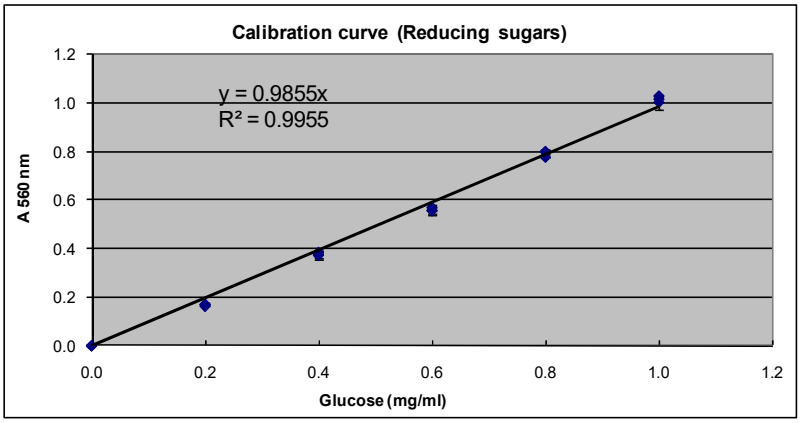


Figure 3.1. Reducing sugars standard curve (DNS method)

3.2.7.2. Estimation of total protein (TP)

While different proteins have different absorptivities at 280nm, this method was used only to give a general approximation of protein content, since it provides a rapid analysis and all protein could be recovered at the end of the procedure (Aitken and Learmonth, 2009; Stoscheck 1990). Diluted fractions from pretreated supernatants were determined using a spectrophotometer at absorbance 280nm. The concentration of protein in samples was estimated by reference to the bovine serum albumin (BSA) standard curve and results were expressed as µg/ml (Figure 3.2).

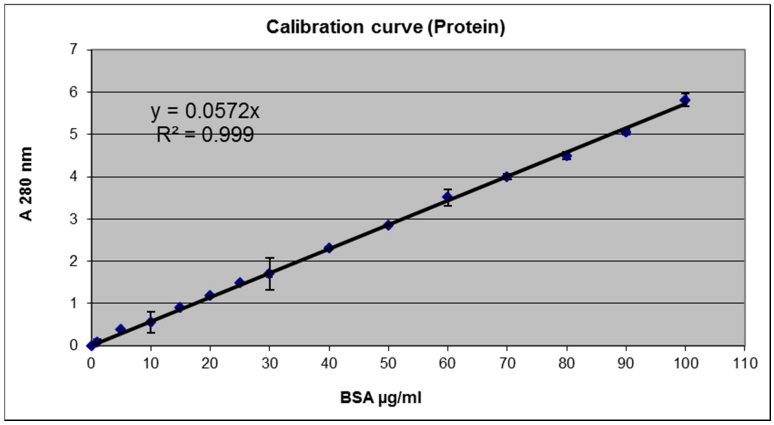


Figure 3.2: Total protein standard curve (BSA).

3.2.7.3. Estimation of total lipids (TL)

Extraction of lipids from supernatant was carried out following the method of Bligh and Dyer (Bligh and Dyer, 1959). The technique was chosen as it is a well-known method, is economic, simple and rapid (Kanda*, et al*., 2012; Smedes and Asklan, 1999). For each 1 ml of sample (supernatant) 3.75 ml 1:2 (v/v) $CHCl_3$:MeOH was added and vortexed at maximum speed for 1 min. 1.25 ml $CHCl_3$ was added and subjected to vortex as well. Finally, 1.25 ml de-ionised dH_2O was added and with a final 1 min vortex. Tubes were centrifuged at 1000 RPM for 5 min at room temperature in a table-top centrifuge to give a two-phase system (aqueous top, organic bottom). The bottom phase was recovered by inserting a pipette through the upper phase with gentle positive-pressure.

3.3. Results and discussion

In this Chapter, pretreatment methods were used to enhance the recovery of macromolecules from *L. digitata* and *S. latissima*. After pretreatment and hydrolysis, they can be transformed into biofuels or other industrially important products within a biorefinery process as has been established in the literature review (Section 2.6).

The effectiveness of these pretreatments was assessed by monitoring the amount of RS, TP and TL released from the seaweed biomass. Further, in Chapter 4, best performing pretreatments were used to improve the biodigestibility of the seaweed to enhance biogas production.

3.3.1. Inorganic acid and NaOH pretreatment

Pretreatment of *L. digitata* and *S. latissima* with inorganic acids and NaOH has not been previously reported in the literature where most of the comparisons have been carried out with different seaweed species or terrestrial biomass.

3.3.1.1. HNO_3 pretreatment

The different HNO_3 pretreatment concentrations improved hydrolysis of *L. digitata* and *S. latissima* when compared to thermal and untreated controls (Figure 3.3). It is evident from observing Figure 3.3a, and Figure 3.3b that increasing acid concentration considerably boosts RS yields for both seaweed species. 0.1% released 9.3 mg/ml and 6.0% released 36 mg/ml for *L. digitata* meanwhile for *S. latissima* 0.1% released 5.2 mg/ml and 6.0% released 39.2 mg/ml.

Maximum yields of the TL content of treated samples from both seaweed species were recovered when the samples were exposed to high acid concentrations (Figure 3.3e, f). This profile was not as evident when the release of TP in response to acid concentration was investigated (Figure 3.3c, d). Low acid concentrations were seen to enhance protein recovery in extracellular filtrates from *S. latissima* (4.0%) and to a greater extent for *L. digitata* (2.0%).

While the best nitric acid concentration for *L. digitata* and *S. latissima* hydrolysis was found in this study to be 4%, other studies (Cara, *et al*., 2008; Lenihan, *et al*., 2010) have suggested that the use of high acid concentrations during pretreatment of different biomasses will increase the production of inhibitor compounds, such as organic acids and furfural. However, the analysis of these compounds was not part of the objectives of this investigation.

3.3.1.2. HCl pretreatment

Another example of strong acid, widely used for biomass hydrolysis, is HCl (Champagne and Lie, 2009; Varga, *et al*., 2002).

The results from this acid pretreatment showed that *L. digitata* was more susceptible to hydrolysis by 6.0% HCl (28 mg/mL) than *S. latissima* (20 mg/mL). However, the sugar yield recoveries were lower (Figure 3.3a, d) when compared to the HNO_3 yields.

In a study carried out by Varga, *et al*. (2002) 5% HCl pretreatment of untreated corn stover had a better effect on the amount of total released sugars than 1% HCl. Similarly, Champagne and Lie (2009) also showed that higher loadings of HCl pretreatment had a better effect on the RS yield on lignocellulose extracted from municipal wastewater treatment process residuals. Similar results were found in this study, where increasing acid concentration showed a similar correlation with the RS yields.

As for TL and TP, HCl pretreatment did not show a clear tendency to improve the release of these macromolecules. Some of the values were comparable to thermally treated and untreated samples.

3.3.1.3. H_2SO_4 pretreatment

The most widely used and tested acid pretreatment approach is based on dilute or concentrate sulphuric acid (Agbor, *et al*., 2011; Harun and Danquah, 2011; Jang, *et al*., 2012). In this study, macromolecule recovery was higher than that of untreated *L. digitata* and *S. latissima* (Figure 3.3). Similar result was also observed by Lee, *et al*. (2013) after pretreating *S. japonica* to extract glucan. However, lower yields were obtained when compared to HNO_3 or HCl pretreatments.

Harun and Danquah (2011) found improvements in the release of fermentable sugars at high acid concentrations when pretreating microalgal biomass for bioethanol production.

Also, thermal hydrolysis of *S. japonica* (20% w/v seaweed slurry) at higher acid concentrations (from 40 mM to 94 mM) was reported to give a significant increase in the amount of RS (Jang, *et al*., 2012).

3.3.1.4. Phosphoric acid pretreatment

Diluted phosphoric acid has been used for lignocellulose pretreatment (Gámez, *et al*., 2006; Lenihan, *et al*., 2010; Um, *et al*., 2003). If neutralization of the hydrolysate after biomass pretreatment is carried out with NaOH, sodium phosphate will be formed. This would improve the economics of the process, avoiding the washing step to remove the acid, decreasing the amount of nutrient needed in the subsequent fermentation process and acting as buffer on AD (Gámez, *et al*., 2006). In addition, it is not detrimental to the environment, as the salt resulted from the reaction is not a waste end-product.

Therefore, the effect of dilute phosphoric acid pretreatment on the hydrolysis of the seaweed biomass was also investigated. Higher acid concentration (6.0%) was seen to enhance, to a greater extent, macromolecule recovery in extracellular filtrates (Figure 3.3). Nevertheless, the amount of RS recovered after exposing *L. digitata* and *S. latissima* to the highest acid concentration (6.0%) was 12 mg/ml and for 20 mg/ml, respectively. The values obtained were lower when compared to HNO_3.

For TP and TL the amount of macromolecules was slightly higher to thermal and untreated samples. The use of diluted phosphoric acid as a pretreatment of the seaweed species, *L. digitata* and *S. latissima*, has not been reported to date in the literature.

3.3.1.5. NaOH pretreatment

Pretreatment of both seaweed species with NaOH did not achieve a substantial amount of RS and TL recovery. Even under the most severe concentration condition (6.0%), the process did not contribute to the improvement of hydrolysis efficiency (Figure 3.3). Moreover, it was observed that thermal pretreatment alone yielded higher TL than with NaOH. A seaweed pretreatment process using diluted NaOH has not been reported to date in the literature.

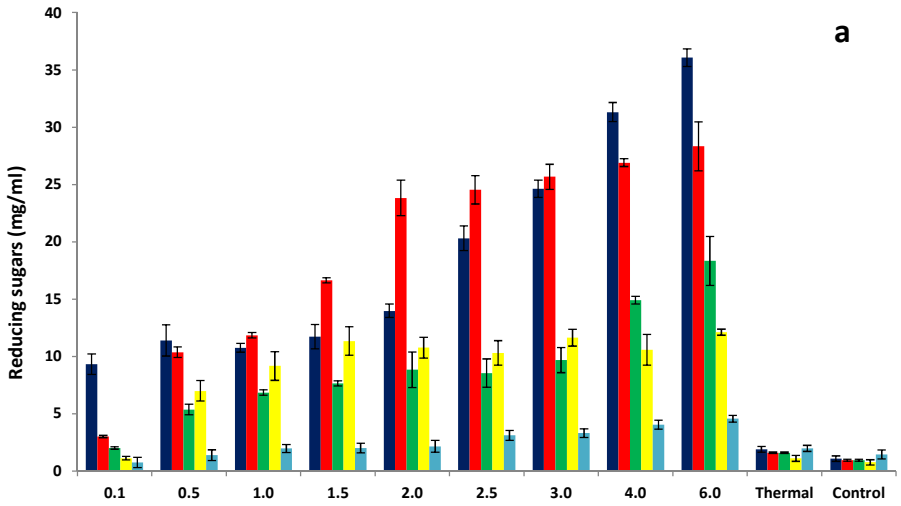
A possible explanation of the low recovery is that, depending on catalyst used, acid-thermal pretreatment of lignocellulosic biomass target the cellulose and hemicellulose content, while alkalis are more effective on lignin solubilisation (Alvira, *et al*., 2010; Mosier, *et al*., 2005). These two seaweed species lack lignin and have low cellulose content (Black 1950; Østgaard, *et al*., 1993). This explanation was also observed by

Varga, *et al*. (2002) and Chen, *et al*. (2009) where, the removal and solubilisation of lignin from corn stover was increased with NaOH.

On the other hand, the release of TP from both seaweed species was greater enhanced by 6.0% NaOH, exceeding the yields obtained from the inorganic acid pretreatments. The utilisation of alkaline solutions as method to solubilise highly water soluble proteins has been studied before. More recently, in a study carried out by Harnedy and FitzGerald (2013), the yield of alkaline soluble proteins from the seaweed *Palmaria palmata* was influenced by the NaOH concentration. Similar to the results from Figure 3.3, the study found that increasing the concentration of NaOH from 0.08 to 0.12 mol/l will enhance the quantity of protein extracted from 5.09 to 6.72 g/100 g. Therefore, it should be noted that in a seaweed biorefinery process the use of a NaOH pretreatment step will aid in development the downstream process to recover protein from seaweed.

Based on these results, it was demonstrated that the release of macromolecules was specific for each individual seaweed species and that every chemical pretreatment had a different effect on the hydrolysis reaction. For instance, pretreatment of *S. latissima* released higher concentrations of macromolecules than *L. digitata*. This observation was more evident when the species was expose to HNO_3, making this acid the most suitable catalyst tested due to its superior hydrolysis efficiency in terms of RS and TL recovery.

The release of macromolecules in these sets of experiments was attributed to the difference in the chemical constituents of the macroalgae and the susceptibility of the cell wall to hydrolysis by the different chemicals as found by other researchers (Black, 1950; El-Said and El-Sikaily, 2013; Jang, *et al*., 2012; Park, *et al*., 2012; Zvyagintseva, *et al*., 2003). This finding was also evident in a study carried out by Malihan, *et al*. (2012). In their work, the brown seaweed species *Sargassum fulvellum* was pretreated with five mineral acids (sulphuric, nitric, hydrochloric, phosphoric and citric acid) and while all five acids displayed different degrees of hydrolysis, the RS yield from the HCl pretreatment was considerably higher than the other acids.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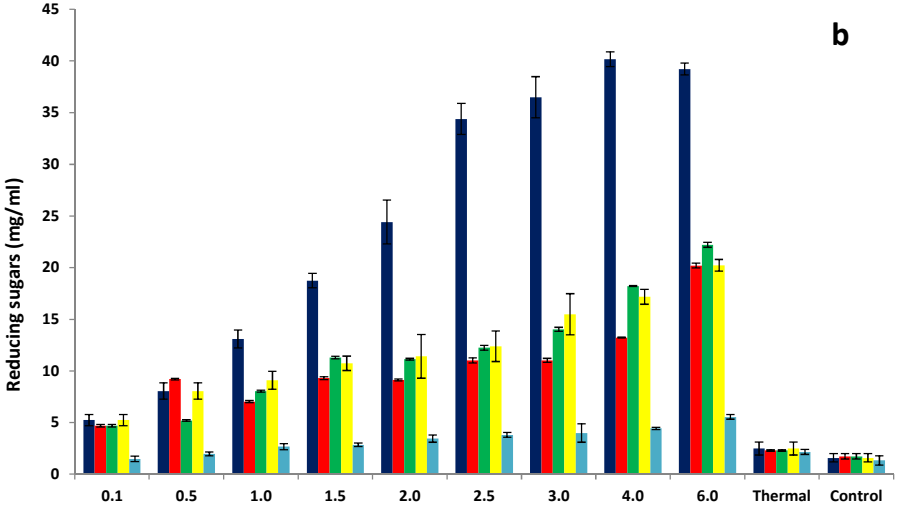
2.5
3.0
4.0
6.0
Thermal
Control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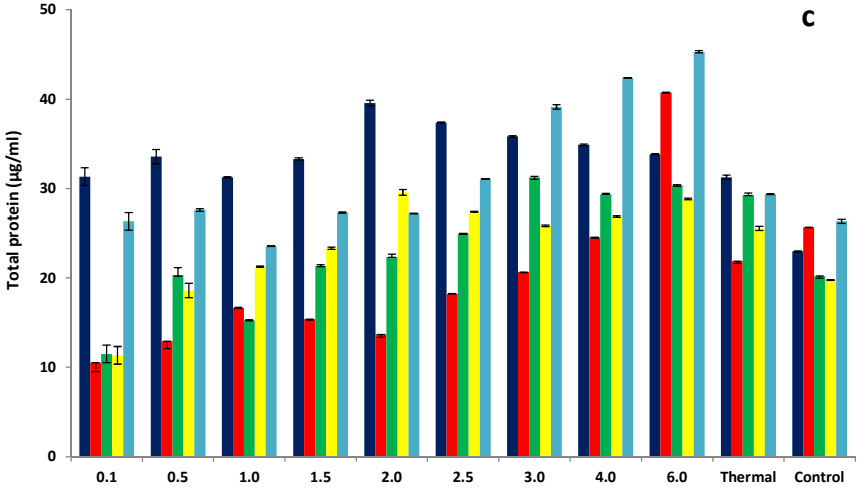
1.5
2.0
2.5
3.0
4.0
6.0
Thermal
Control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0.5
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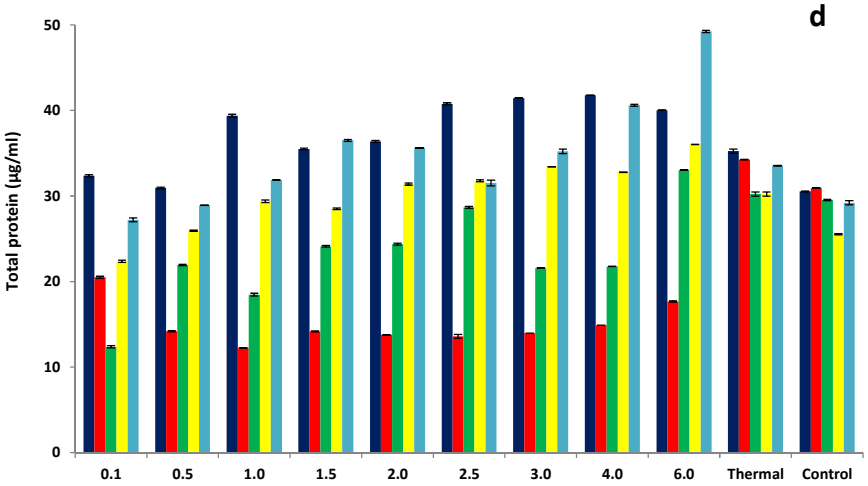
2.5
3.0
4.0
6.0
Thermal
Control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Total protein (µg/ml)
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3.0
4.0
6.0
Thermal
Control

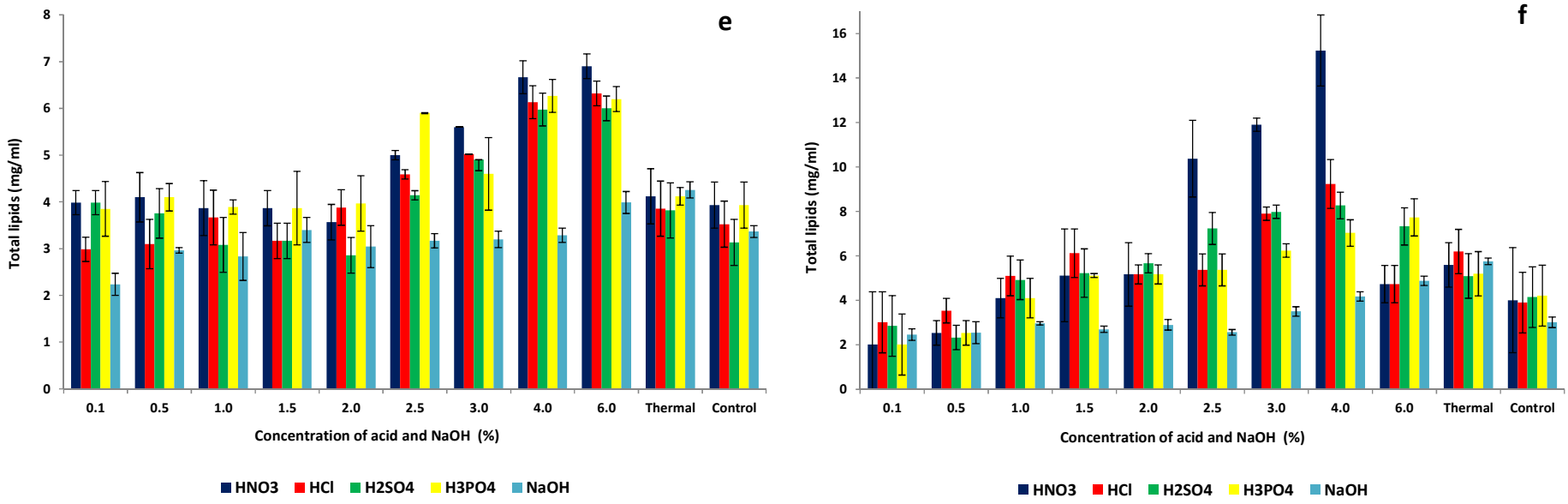


Figure 3.3. Effect of different inorganic acids and NaOH pretreatment on RS (a, b), TP (c, d) and TL (e, f) recovery from *L. digitata* (left) and *S. latissima* (right) at 120°C/60 min/1 atm (RS and TL are expressed as mg/ml, TP as µg/ml).

The results of the pretreatment also show a reduction in the solid fraction of both seaweed species, proportional to the severity of the pretreatment. Similar studies have also reported this observation, where the degree of solubilisation of the slurry was attributed to the harshness of the pretreatment; chemical concentration, high temperature, and reaction time (Cara, *et al*., 2008; McIntosh and Vanconv, 2011; Varga, *et al*., 2002; Um, *et al*., 2003).

While harsh conditions increased macromolecule recovery, chemical pretreatments with low acid concentrations have the advantage of consuming fewer amounts of neutralising agents in the following fermentation or extraction step, decreasing reactor corrosion and improving sustainability throughout the biorefinery process.

Future work comparing the effect of acid pretreatment on different feedstock (corn stover, grass, and straw among others) would give a better perspective on seaweed pretreatment process yields.

3.3.2. Organic acid pretreatment

Hydrolysis experiments were performed with four organic acids (acetic, citric, lactic and oxalic). All acids used during this study are widely used in the biochemical industry and were chosen as alternative pretreatment chemicals to inorganic acids. An added advantage is that the organic acids used during the pretreatment would be regarded as an environmental friendly catalyst, biodegradable and an extra source of organic nutrients in the medium, contributing to biomass fermentation (Mosier, *et al*., 2005; Qin, *et al*., 2012).

3.3.2.1. Acetic acid pretreatment

Although the amount of RS recoveries increased as organic acid concentration used in the pretreatment was increased, higher yields were only achieved at 6.0% with 8 mg/ml and 5 mg/ml for *L. digitata* and *S. latissima*, respectively (Figure 3.4a, b). This recovery was higher when compared to untreated controls but lower when compared to yields with inorganic acid pretreatments (Figure 3.3a, b).

Pretreatment of *L. digitata* and *S. latissima* with this organic acid has not been previously reported. On the other hand, the effect of acetic acid pretreatment of different seaweed species has been described elsewhere. Malihan, *et al*. (2012) reported that pretreatment of *Sargassum fulvellum* with acetic acid (120°C during 24h reaction) had the lowest conversion yields of total RS (20%) when compared to other inorganic acids.

In a different study, subcritical water hydrolysis of *Laminaria japonica* with 1.0% acetic acid increased the production of RS when compared to samples without catalyst (Park, *et al*., 2012). Kim, *et al*., (2013) reported that the RS yield from the acetic acid (1% to 5%, w/w) pretreatment of agarose from *Gelidium amansii* ranged from 9% to 33%.
Acetic acid was seen to effectively produce a positive response when the filtrates were assayed for TP (Figure 3.4c, d). Higher recoveries values were attained when compared to inorganic acids (Figure 3.3). The TL content (see Figure 3.4e, f) was not affected by the chemical pretreatment. Recoveries are comparable to the yields obtained from untreated and thermal controls.

3.3.2.2. Citric acid pretreatment

The amount of RS (Figure 3.4a, b) produced by citric acid hydrolysis on *S. latissima* and *L. digitata* was higher than with acetic acid pretreatment. While 6.0% acetic acid released the highest concentration of RS (8 and 5 mg/ml), citric acid at 2.5% released 12 and 11 mg/ml RS for *L. digitata* and *S. latissima*, respectively.

The amount of TL (Figure 3.4e, f) from the filtrates was comparable to thermal and control treatments and no improvement in hydrolysis could be attributed to the addition of the acid.

In contrast, the TP yields (Figure 3.4c, d) were significant higher (73 µg /ml from *L. digitata* and 63 µg/ml from *S. latissima*) at the highest acid concentration (6.0%) when compared to untreated samples or the yields obtained with the inorganic acids, demonstrating a better response from citric acid.

Seaweed pretreatment using this organic acid has not been previously reported. However, the use of citric acid during biomass pretreatment has been reported elsewhere. In a recent study, the solid fraction of corn stover was hydrolysed to certain

extend with citric acid but with a greater conversation rate with sulphuric and oxalic acid (Qin, *et al*., 2012).

Biomass with a high concentration of protein is generally considered detrimental to AD, as it will increase the ammonia content in the medium (Chen, *et al*., 2008). There is, however, great interest in examining ways to utilise proteins for other applications. As protein extractability from substrates is the rate limiting step, different methodologies such as acid-catalyzed hydrolysis, have been used to improve the yields. Therefore, the use of citric acid could add an extra value to the seaweed biorefinery model by reducing the cost of protein extraction for human and animal food production when compared to traditional processing methods (Badadani, *et al*., 2007; Fountoulakis and Lahm, 1998; Karki, *et al*., 2010; Sereewatthanawut, *et al*., 2008).

3.3.2.3. Lactic acid pretreatment

The two seaweed species were similar when the release of sugar in response to lactic acid pretreatment was compared; RS recovery increased along with increased acid concentration (Figure 3.4a, b). Pretreatment with lactic acid outperformed acetic acid pretreatment under the same conditions but was not as efficient when compared to citric acid.

There was a large increase in the TL yields released into the hydrolysate from both seaweed species when lactic acid treatment was applied, comparing to the citric, oxalic and acetic acid pretreatment (Figure 3.4 e, f). In contrast, the TP yields were the lowest among the set of organic acids used in this investigation.

Though seaweed samples treated in 6.0% lactic acid produced a comparatively large amount of macromolecules, when compared to an untreated control, yields were lower when compared to inorganic acid pretreatments. Overall, lactic acid did not show a significant increase in the release of macromolecules content.

Studies targeting the effect of lactic acid in extracting macromolecules were not found in the literature.

Successful hydrolysis and fermentability of corn stover with lactic acid was reported by Xu, *et al*. (2009), reaching high cellulose yields (74%) when compared to untreated corn stover.

In a different study, one-month pretreatment (preservative impregnation method) of sugarcane bagasse with lactic acid, resulted in similar glucose yields to the pretreatment with SO_2; about 80% of theoretical glucose yield. Unlike bagasse, pretreatment of spruce showed that the addition of lactic acid was not efficient enough and required the addition of SO_2 (Monavari, *et al*., 2011).

3.3.2.4. Oxalic acid pretreatment

The extent of hydrolysis was a function of acid concentration. When compared to citric, lactic and acetic pretreatment, 6.0% oxalic acid treatment had considerably higher RS yields (Figure 3.4a, b). 21 and 24 mg/ml of RS for *L. digitata* and *S. latissima,* respectively, were obtained when using this pretreatment.

When samples of *L. digitata* and *S. latissima* were treated with high concentrations of oxalic acid, the amount of TP recovered (Figure 3.4c, d) was higher than untreated controls but considerably lower than citric, lactic and acetic. TL yields were slightly higher when compared to controls (Figure 3.4e, f).

In a study carried out by Lee, *et al*. (2011), pretreatment of agricultural lignocellulosic biomass with oxalic acid was successfully employed and considered for further pilot scale studies and industrial scale processes. In another study, maximum xylose yield was obtained after pretreatment of corn stover with sulphuric and oxalic acid (Qin, *et al*., 2012).

The effect of steam pretreatment, reaction time, temperature and dilute oxalic acid of giant reed was investigated by Scordia, *et al*. (2011). They found that hemicelluloses can be hydrolyzed to monosaccharides in a single stage pretreatment. Additionally, after a combination of elevated severities in the pretreatment, the solid residue can be used for enzymatic hydrolysis and further fermentation to produce ethanol. Pretreatment at 190°C, 25 min and with 5% (W/W) oxalic acid was chosen for simultaneous saccharification and fermentation process.

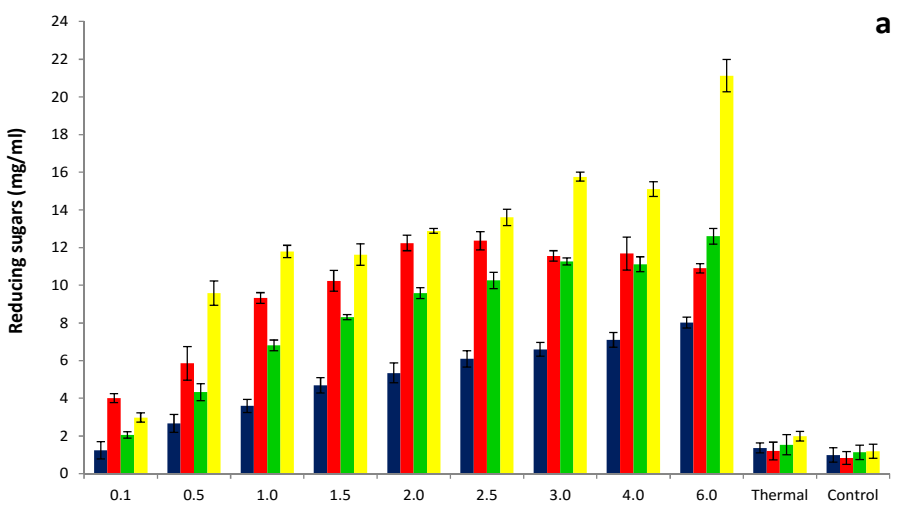
In a similar study carried out by Lee, *et al*. (2010), oxalic acid pretreatment was used to release fermentable sugar from the cob of *Zea mays* L. ssp. The process not only maximised cellulose hydrolysis but also ethanol production. Using harsh conditions, hydrolysis of the hemicellulosic fraction increased the lignin and glucan contents with only 7.5% remaining in the pretreated substrate and, at the same time, the galactan, xylan and arabinan contents were reduced considerably. Pretreatment at 168°C, 74 min and 0.027 g of oxalic acid was the optimal condition.

Although several studies have targeted the use of organic acids to enhance solubility of different biomasses, no data is available in the literature addressing the effect of organic acids on the release of macromolecules from the seaweed species *L. digitata* and *S. latissima*. However, this study has demonstrated its feasibility within a seaweed biorefinery process.

In general, it was observed that the degree of macromolecules recovery was correlated to the acid and its concentration. This similarity was also observed after both seaweed species were pretreated with inorganic acids (Figure 3.3). Terrestrial biomass pretreatment studies have also shown that the acid and the concentration employed during the process is specific for each feedstock resulting in an improvement of the hydrolysis or the extraction of value added products (Cara, *et al*., 2008; Jeong, *et al*., 2012; Lenihan, *et al*., 2010; Qin, *et al*., 2012).

All organic acid pretreatments achieved higher RS and TP yields when compared to thermal and untreated controls. In terms of RS yields, oxalic acid (4.0% and 6.0%) was the most effective pretreatment in relation to seaweed solubilisation while acetic acid was the least effective.

A noteworthy observation is that pretreatment with organic acids released higher concentrations of TP when compared to inorganic acids. In the context of this investigation, the extraction of high added value compounds, such as proteins and RS, would contribute to the implementation of the biorefinery model, adding, at the same time, commercial value to the seaweed biofuel industry.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12
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1.5
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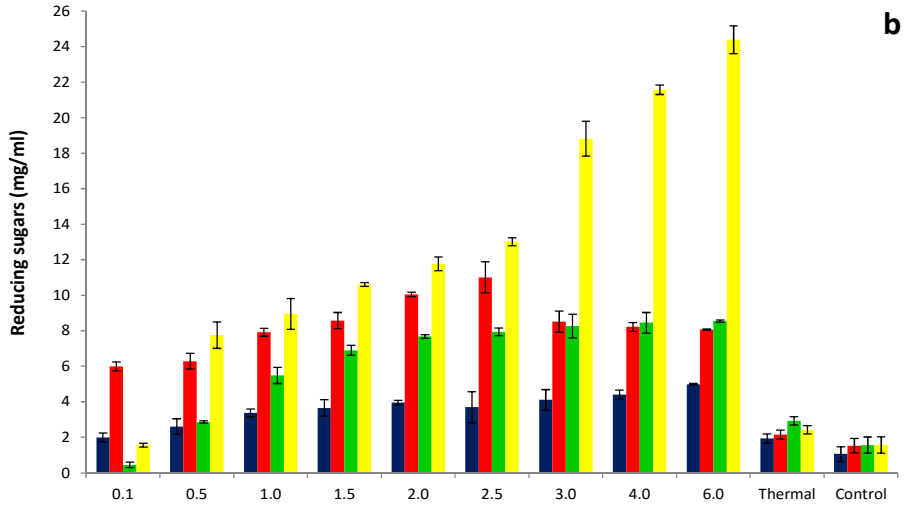
Thermal
Control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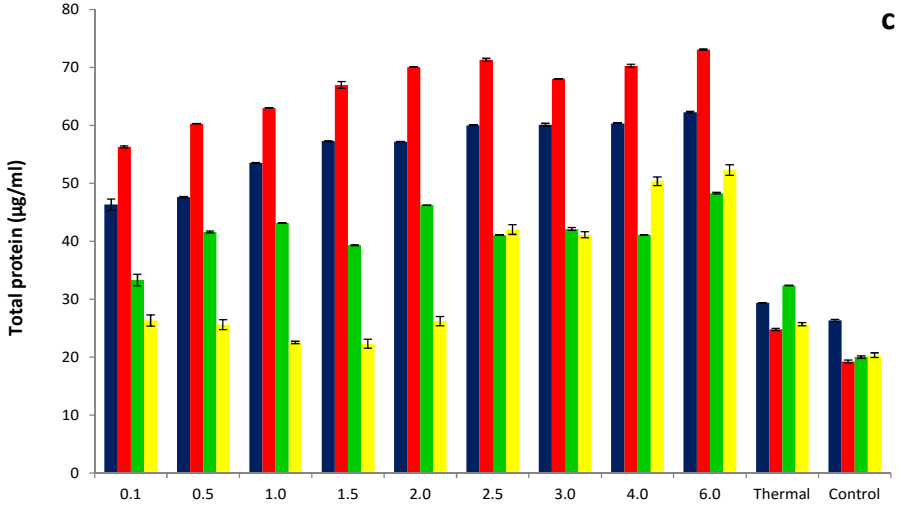
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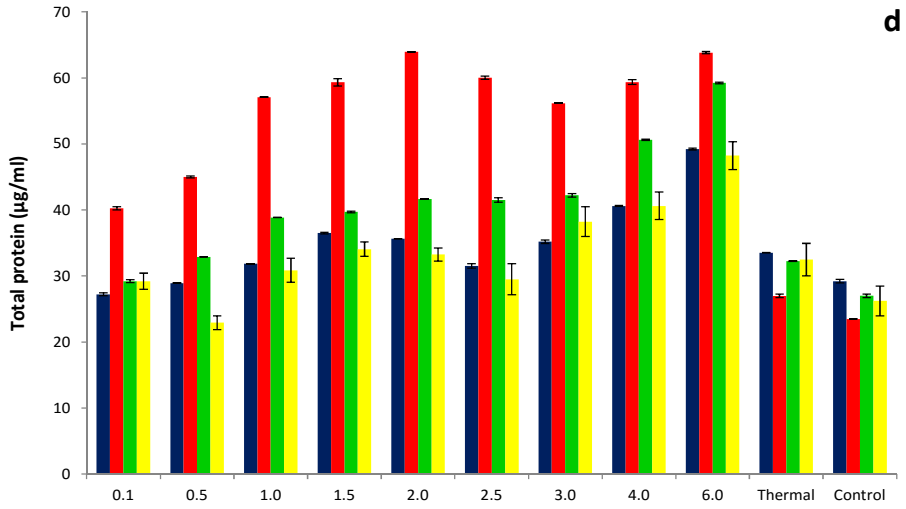
4.0
6.0
Thermal
Control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70
0.1
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1.0
1.5
2.0
2.5
3.0
4.0
6.0
Thermal
Control

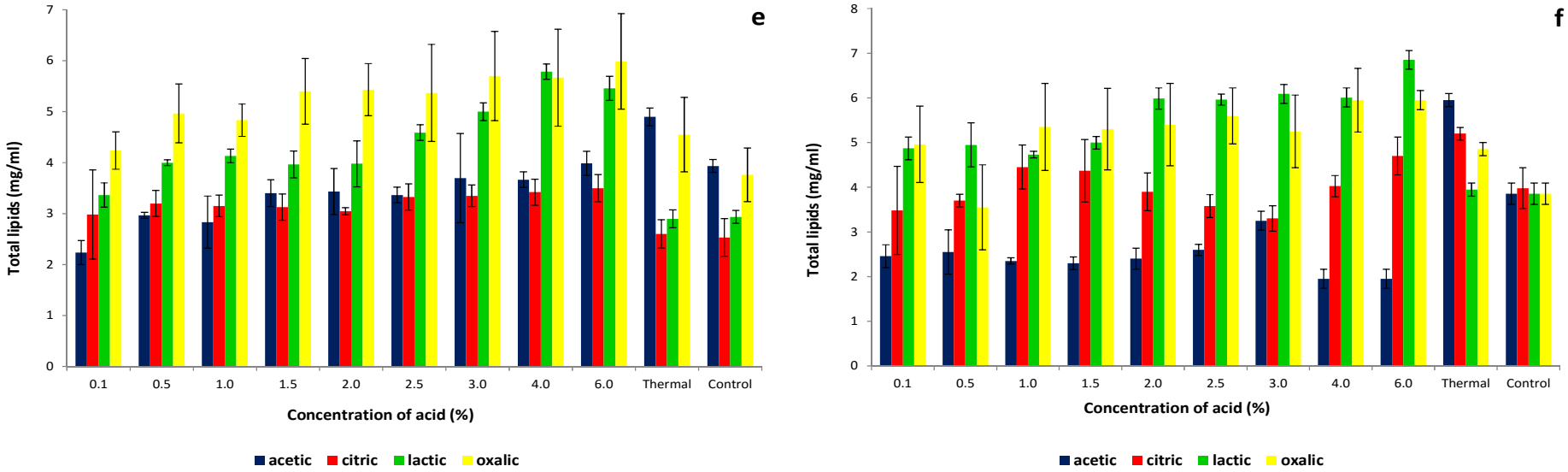

Figure 3.4. Effect of different organic acids pretreatment on RS (a, b), TP (c, d) and TL (e, f) recovery from *L. digitata* (left) and *S. latissima* (right) at 120°C/60 min/1 atm (RS and TL are expressed as mg/ml, TP as µg/ml).

3.3.3. High temperature and reaction time pretreatment

Alone, or in conjunction with chemicals or solvents, thermal pretreatment has been reported as the most commonly used method for biomass material hydrolysis (Agbor, *et al*., 2011; Cara, *et al*., 2008; Harun and Danquah, 2011; Kita, *et al*., 2010).

The goal of the process is to disrupt successfully the chemical structure of the biomass, while preserving their constituents and minimising hydrolysis of the monosaccharides. Temperature and retention time should be modulated to each particular biomass in order to avoid the formation of inhibitory compounds, such as aldehydes, so that the microorganisms may be added for effective fermentation.

From the acid pretreatment results, HNO_3 and HCl were selected as the best performing inorganic acids. Also, an organic acid widely used in food, pharmaceutical and biofuel industrial processes, citric acid, was used to investigate the effect of different temperatures and reaction times on macromolecules released from the two seaweed species. Figure 3.5 shows the macromolecule yield from the algal biomass samples pretreated at three different temperatures (80°C, 120°C and 130°C) and at four reaction times (1, 60, 120 and 240 minutes).

According to the results of these experiments, for higher temperatures and longer retention times (130°C for 120 minutes), maximum RS yields were achieved after exposing *L. digitata* to nitric and citric acid (Fig. 3.5a, b) and *S. latissima* to nitric acid. On the other hand, RS yield was reduced when the temperature and retention time was increased using HCl.

There appears to be a clear correlation between the amount of sugar released with temperature, acid nature and retention time as seen in Figure 3.5a, b. Apparently, the stability of simple sugar moieties are disrupted due to the direct solubilisation of the complex polysacharides at high temperature as suggested by Haram and Danquah, (2011).

This correlation was also reported by Nguyen, *et al*. (2009) and Borines, *et al*. (2013) where, according to acid dosage, temperature condition as well as the extension of the retention time, faster hydrolysis of *Chlamydomonas reinhardtii* and *Sargassum* spp. was

observed, releasing more sugars. In a similar study, Kim, *et al*. (2013) also reported that RS and 5-Hydroxymethylfurfural (HMF) yields from agarose hydrolysis in *Gelidium amansii* were higher after an increase in temperature (from 120°C to 130°C) and reaction time (from 20 min to 30 min).

The effect of temperature was also reported on pretreatment of corn stover. In their work, Shen and Wyman (2009) found that a combination of 180°C/10 min removed almost all the xylan available while still detected in the pretreated solids at 160°C even with an extension on reaction time.

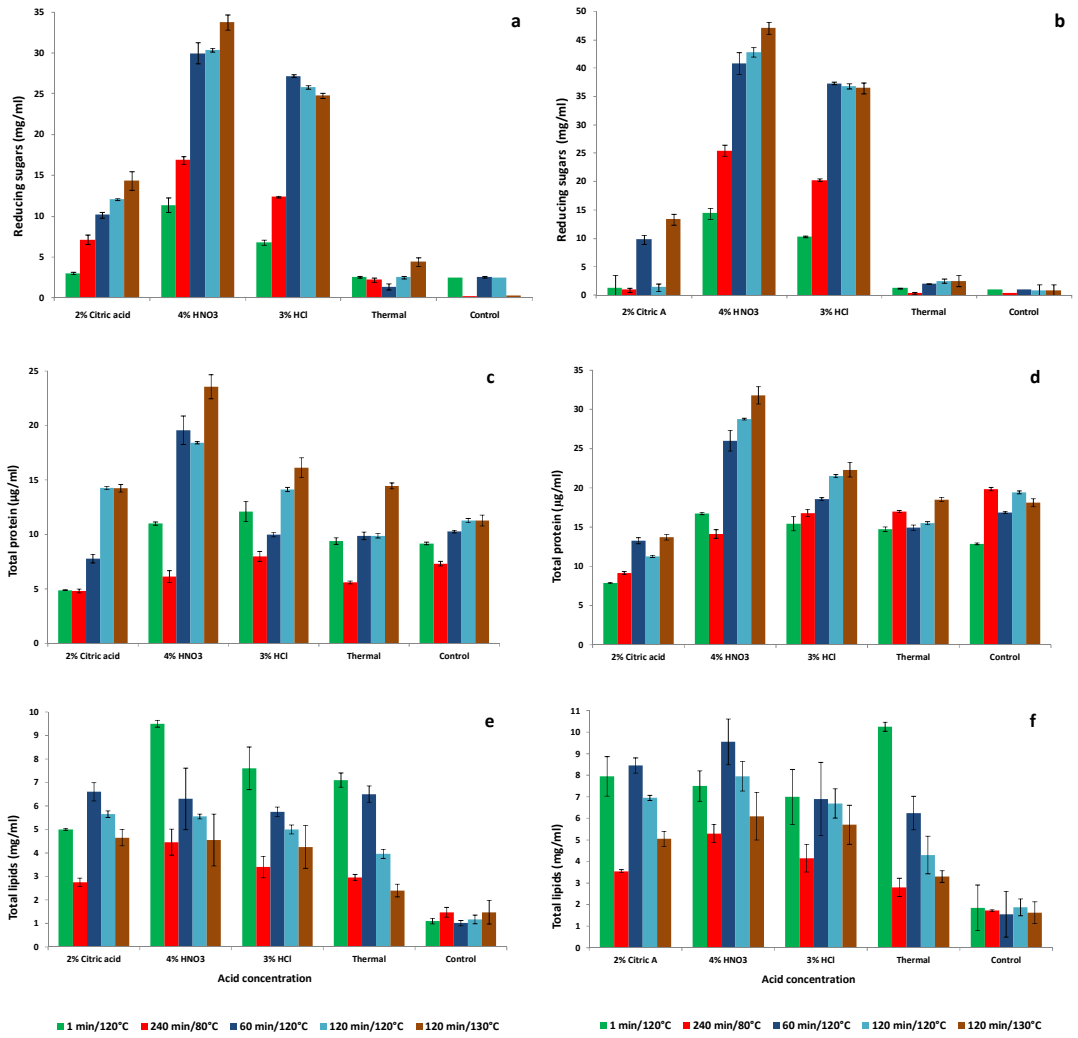

Figure 3.5. Effect of different temperatures and reaction time pretreatment on RS (a, b), TP (c, d) and TL (e, f) recovery from L. digitata (left) and S. latissima (right). (RS and TL are expressed as mg/ml, TP as µg/ml).

A noteworthy aspect found during these experiments was that the reaction time played an important role during the recovery of RS from both seaweed species. A decrease of severity of the treatment (1 and 60 min) leaded to a reduction in the overall RS yield in the prehydrolysates. This finding is attributed to the mild pretreatment condition applied to the seaweed biomass. This is in agreement with other studies where hydrolysis was enhanced by the reaction time. According to Jang, *et al*. (2012), RS content increased after 10% (w/v) slurry of the seaweed *Saccharina japonica* was pretreated with 40nM of H_2SO_4 at different retention times (from 15 to 60 min).

When samples were tested for TP (Figure 3.5c, d), the highest temperature (130°C) and longer retention time (120 minutes) was seen also to release the most protein from *S. latissima* and *L. digitata*. However, the overall yield was considerable lower when compared to NaOH (Figure 3.3) or other weak acid pretreatments (Figure 3.4).

As indicated in Figure 3.5e, f, lipid concentration was higher at 120°C for 1 minute for *L. digitata* than to the other temperatures; meanwhile for *S. latissima* no clear pattern was found.

3.3.4. Physical pretreatment

3.3.4.1. Sonication

In Figure 3.6, a sonication time of "0 min" indicates a sample exposed to acid hydrolysis without sonication. The results show that none of the sonication intervals (5, 30 and 60 min) improved the release of macromolecules.

Although sonication has been proved to be an effective pretreatment process during conversion of sugary corn to fermentable sugars (Montalbo, *et al*., 2010), to accelerate enzymatic hydrolysis of edible Atlantic red seaweed Dulse, *Palmaria* (Peña-Farfal, *et al*., 2005), to increase the sugar yield from *Enteromorpha proliferato* (Zhao and Ruan, 2011) and to extract carbohydrates and protein from *Cladophora* (Woods, *et al*., 2011), this study has suggested the opposite in these seaweed species.

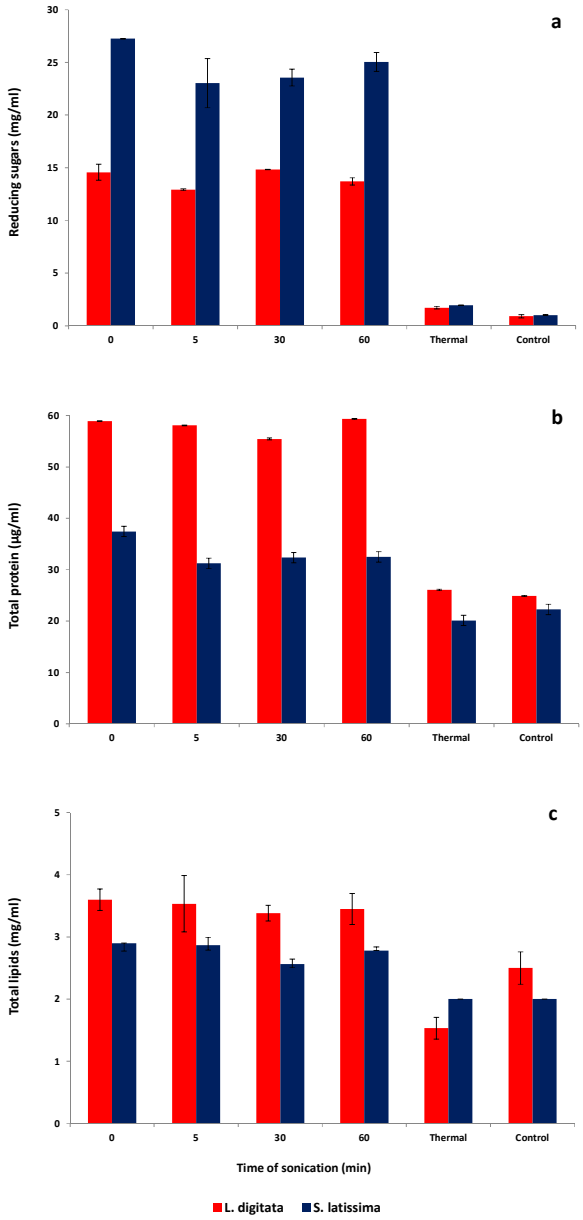

Figure 3.6. Comparison of macromolecules recovery from *L. digitata* and *S. latissima* by using a combination of acid-thermal pretreatment and sonication. RS (a), TP (b) and TL (c). (RS and TL are expressed as mg/ml, TP as µg/ml).

3.3.4.2. Particle size reduction

Particle size reduction is regarded as a necessary step to make biomass easier to handle in a full scale processing plant. Although milling pretreatments are often described as high energy requirement techniques that incur extra capital and operating costs, the suitability for scale-up this technique is very important (Da Silva, *et al*., 2010; Karki, *et al*., 2010; Silva, *et al*., 2012). However, the assessment of particle size reduction as pretreatment step for these two seaweed species has not been reported to date.

Therefore, in this set of experiments, mechanical pretreatment (two different sizes and methods) was carried out before a subsequent processing step (acid) was applied.

It was observed, as shown in Figure 3.7a, b, that *L. digitata* and *S. latissima* subjected to freezer milling with the inorganic acids (HNO_3 and HCl) showed a slight increase in the RS yield when compared to blender milling.

The effect of particle reduction on TP yields from both seaweeds was lower than blender milling, except for HCl (Figure 3.7c, d).

There were some differences between the two species for TL recovery (Figure 3.7e, f). Freezer milling pretreatment had a positive effect on *L. digitata*, releasing more than double for citric acid and HCl when compared to blender milling. This response was also higher for NHO_3 and the thermal control. On the other hand, blender milling seems to exert a better effect, releasing higher concentrations of TL from *S. latissima* than freezer milling.

While no studies have been published to date aiming to extract macromolecules from seaweed by particle size reduction, freezer milling (<0.1 mm) followed by an acid pretreatment, proved to be a potential process.

Nevertheless, and based on the pretreatment of land-based feedstocks (Agbor, *et al*., 2012; Hendriks and Zeeman, 2009), particle size reduction pretreatments are often described as high energy requirement techniques. Therefore, an economic study will help to identify the potential of this approach to extract macromolecules from seaweed.

Further, in Chapter 4, the effect of milling pretreatment on biogas production from seaweed will be evaluated. This will help to appraise the suitability and effectiveness of the pretreatment within a biorefinery process by enhancing macromolecule recovery and biogas production.

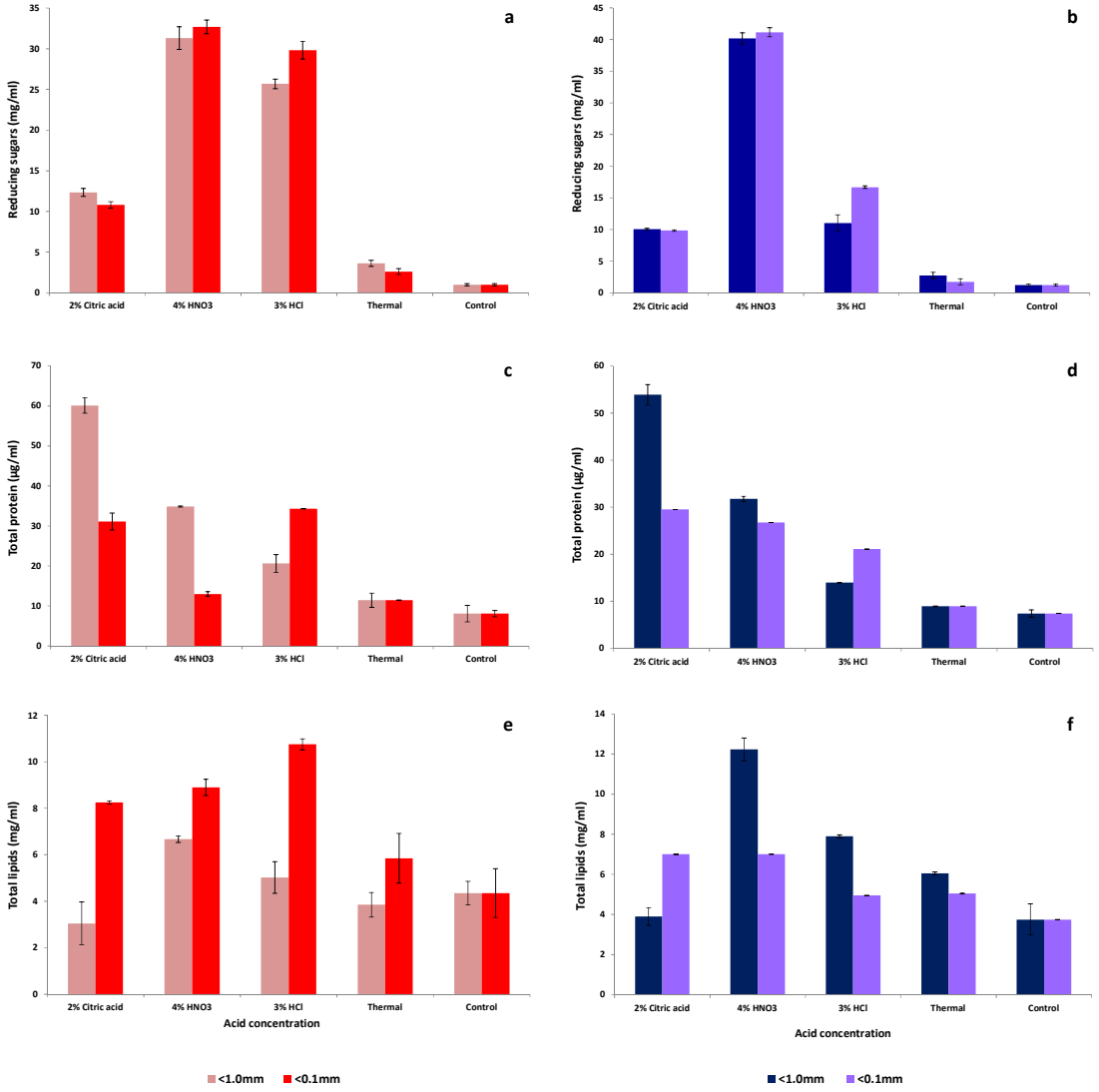

Figure 3.7. Effect of particle reduction size by freezer (<0.1 mm) and blender milling (<1.0 mm) on macromolecules released of *L. digitata* (left) and *S. latissima* (right). RS (a, b), TP (c, d) and TL (e, f). (RS and TL are expressed as mg/ml, TP as µg/ml)

3.3.5. Enzymatic pretreatment

The efficiency of enzymatic hydrolysis varies according to the biomass and should be adapted to each particular feedstock (Choi, *et al*., 2009; Choi, *et al*., 2010; Hang and Woodams, 2001; Tan and Lee, 2014). Moreover, the enzymatic pretreatment of seaweed will be influenced by the biochemical composition, physiological structure, life-cycle period and type of seaweed. Therefore, in this study three commercial available enzymatic preparations (Celluclast 1.5 L, Alginate lyase and Cellulase) were used to enhance saccharification and biodigestibility of *S. latissima* and *L. digitata*.

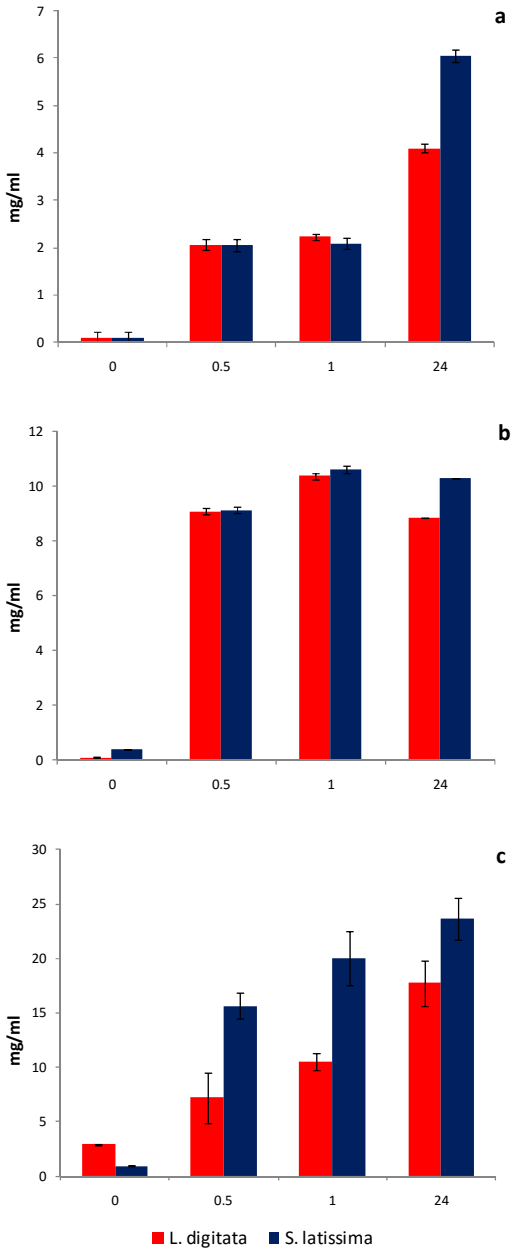

Figure 3.8. Concentration of RS (mg/ml) released after pretreatment of *L. digitata* and *S. latissima* with (a) Celluclast 1.5 L, (b) Alginate lyase and (c) Cellulase. Processing time in hours.

Saccharification with Celluclast 1.5 L released the lowest RS concentration among all enzymes; 4 and 6 mg/ml for *L. digitata* and *S. latissima*, respectively (Figure 3.8a, b).

Kovacs, *et al*. (2009) also reported lower glucose and xylose yields on lignocellulosic substrates when using the commercial Celluclast 1.5 L. In addition, the work from Hang and Woodams (2001) on corn cobs reported significantly more RS yields with other commercial available cellulase preparations than with Celluclast 1.5 L.

Conversely, both seaweed species were hydrolysed to a great extent by the Alginate lyase preparation (Figure 3.8), with an increase in the RS content of samples when compared to an untreated control (10 and 11 mg/ml for *L. digitata* and *S. latissima*, respectively).

Figure 3.8 shows the time course of RS production with Cellulase. The amount of sugars produced gradually increased through the reaction time of 24 h, reaching maximum values of 18 and 24 mg/ml for *L. digitata* and *S. latissima*, respectively.

Cellulase proved to be more efficient releasing RS than the other two enzymatic solutions. Similarly, in a study carried out by Swiatek, *et al*. (2014), the Cellulase preparation was more effective compared to the Celluclast 1,5L preparation in the saccharification of rape straw polysaccharides.

The high yield achieved with this commercial preparation may be attributed to a broad substrate spectrum composition of this product that led to a more efficient hydrolysis of the seaweed, as it was also observed by Swiatek, *et al*. (2014).

Comparing the RS yields with the inorganic acid pretreatments it is clear that, even after 24 h, there is a fraction of biomass recalcitrant to enzymatic hydrolysis. This is attributed to the mild conditions during enzymatic pretreatment. In addition, these enzymatic preparations are more active to the lignin–hemicelluloses–cellulose complex. Therefore, the enzymatic pretreatment needs to be optimised by selecting more specific enzymes capable to hydrolize the specific matrix of polysaccharides from the two seaweed species or by further improving the process conditions (enzyme dosage, temperature, pH).

Based on these results, there is a potential to optimise the use of the enzymatic preparation Cellulase during the *saccharification* step of *S. latissima* and *L. digitata*. Nonetheless, commercial enzymes may prove to be prohibitively expensive on a larger scale, thus a detail cost-benefit analysis to prove the concept will be required (Jegannathan and Nielsen, 2013; Klein-Marcuschamer, *et al*., 2012).

Future work could target the effect of different commercial available enzymes on seaweed pretreatment or the development of a more specific enzymatic cocktail that

could efficiently breakdown the seaweed polysaccharides (alginate, mannitol and laminaran), achieving maximum substrate hydrolysis yields.

3.4. Conclusions

In this Chapter, the effect of different pretreatment methods (chemical, mechanical, thermal and enzymatic) on macromolecules recovery for *L. digitata* and *S. latissima* were evaluated.

The results showed that the seaweed species have the potential to be used for the development of a biorefinery process owing to the recovery of RS, TL and TP.

It was established that every pretreatment technique exerted a different effect on the amount RS, TP, and TL recovered from the two seaweed species, making essential to find the most appropriate pretreatment process according to the biomass.

All pretreatment methods enhanced the release of macromolecules to different extents when compared to samples with no pretreatment. On average, higher RS yield was observed in *S. latissima* supernatants than in *L. digitata.*

Among inorganic acids, HNO_3 was found to be the most effective inorganic acid compared to the other range of acids used during this study, while for weak acids, oxalic and citric acid released more RS and TP.

The temperature of pretreatment played an important role in the release of macromolecules as well. Increasing the temperature to 130°C and the retention time to 120 minutes released more RS and TP in *L. digitata* and *S. latissima*.

Among physical methods, sonication was not able to generate a substantial effect on macromolecule recovery, while freezer milling had a positive effect, releasing more macromolecules than blender milling.

The enzymatic preparation Cellulase proved to be the most efficient enzymatic pretreatment releasing the highest RS when compared to Celluclast 1.5L and Alginate lyase.

Results from these experiments have provided the baseline knowledge to facilitate the conversion of *L. digitata* and S. *latissima* into value-added products within a biorefinery process.

CHAPTER 4

THE EFFECT OF DIFFERENT PRETREATMENT METHODS ON BIOGAS PRODUCTION FROM *Laminaria digitata*

Vanegas, C. H., Hernon, A., Bartlett, J. (2013) Enzymatic and organic acid pretreatment of seaweed: effect on reducing sugars production and on biogas inhibition. *Journal of Ambient Energy*, DOI:10.1080/01430750.2013.820143.

4.1. Introduction

The use of seaweed as a biofuel source is primarily in the production of bioethanol and biogas, as demonstrated by several bench-top studies (Adams, *et al*., 2009; Borines, *et al*., 2013; Chynoweth, 2005; Dave, *et al*., 2013; Horn, *et al*., 2000; Hughes, *et al*., 2012; Jang, *et al*., 2012; Jard, *et al*., 2012; Jung, *et al*., 2012; Lee, *et al*., 2012; Show, 1981; Singh and Irving-Olsen, 2011; Vergara-Fernández, *et al*., 2008; Van der Wal, *et al*., 2013; Wei, *et al*., 2013).

The production yields differ considerably, depending on the type of seaweed, the biochemical composition and the treatment applied to the biomass. In the seaweed-biorefinery model outlined in Chapter 2 (section 2.6), the sugars, proteins and lipids, among other compounds, released from a pretreatment and extraction step (Chapter 3) can be recovered and used as precursors for the production of biomaterial, building blocks, industrially important chemicals or for further fermentation.

Optimising the fermentation yields from seaweed depends significantly on the solubilisation of the organic matter prior to the AD process, where, macromolecules will be released and made ready accessible for use by fermenting bacteria (Nielsen and Heiske, 2011; Østgaard, *et al*., 1993; Vivekanand, *et al*., 2012)

One of the methodologies that could be used to increase the solubilisation of the seaweed is to apply a pretreatment. An effective biomass pretreatment must be cost effective, energy efficient, easy to apply and must avoid the formation of inhibitory by-products to hydrolysis and fermentation (Agbor, *et al*., 2011; Kumar, *et al*., 2009; Mosier, *et al*., 2005).

Several pretreatment technologies have been extensively used to date during the AD of different types of feedstocks, but a limited number of studies have focused on seaweed species such as *Saccharina latissima, Saccharina japonica*, *Ulva lactuca*, *Gracillaria vermiculophylla*, *Chaetomorpha linum*, *Macrocystis pyrifera, Gelidium amansii* and *Durvillea Antarctica*, among others (Adams, *et al*., 2009; Jang, *et al*., 2012; Jard, *et al*., 2013; Jeong, *et al*., 2012; Hart and Kohler, 1986; Nielsen and Heiske, 2011; Nkemka, and Murto, 2012; Schumacher, *et al*., 2011; Vergara-Fernández, *et al*., 2008; Voronova, *et al*., 1991).

Further work needs to be carried out in this area to develop its full potential, both in terms of biomass digestibility and biogas production efficiency. In this Chapter, firstly, the effect of different inocula on reactor start-up was investigated. Secondly, the effect of different pretreatments on biogas production of *L. digitata* was evaluated.

4.2. Materials and methods

Seaweed collection, preparation and the general pretreatment procedure was as outlined in Chapter 3, section 3.2.1 and 3.2.2.

4.2.1. Selection of inocula

Several studies have emphasised the influence of different inocula during the degradation of biomass towards biogas production (Fantozzi and Buratti, 2009; Klass, 1998; Marquez, *et al*., 2013; Pandey, *et al*., 2011; Williams, *et al*., 2013). Therefore, in this Chapter, four potential inocula (Table 4.1) containing consortia of methane producing bacteria were adapted to the chemical composition of *L. digitata*. The rationale for this experiment was to examine whether the microbial consortium present in the inocula could produce enzymes to effectively hydrolyse the seaweed and, ultimately, enhancing biogas production. Selection of the inocula was according to criteria of convenience, availability and potential for use in full-scale reactors. An adaptation of Marquez, *et al*. (2103) methodology was used for this purpose:

Inocula	Chemical characteristics		
	pH	VS (%)	C:N
Bovine slurry	6.4	64	12:1
Swine manure	6.8	68	16:1
Anoxic sediments	6.8	7.5	9-11:1
Sludge wastewater	6.4	71	15-18:1

Table 4.1. Chemical characteristics of the inocula used during this study.

1. Bovine slurry (BS) and swine manure (SM) seed were collected from a local farm (Sligo, Ireland). The two inocula (BS and SM) were passed through a 0.2 mm metal sieve to remove unwanted material, thus ensuring that laboratory tubing would not be blocked.
2. Seawater and sand sediments (SD) were collected at the same location were the seaweed species were harvested. This sediment was selected for its thin oxic layer (2-6 mm depth), indicating a mixed population of methanogenics and sulphate reducing bacteria (Grossi, *et al*., 2001; Migliore, *et al*., 2012). The sediments were collected with a cylindrical corer made of PVC (10 x 40 cm). Six core samples were transported to the laboratory and placed in 1.0 L plastic containers where they were used as microbial inocula. Three bottles with the sediment and seawater were enriched with 10 g/VS of powdered *L. digitata* and maintained at 20 ± 2°C. In addition, seawater samples were taken for subsequent microbial plating. Plates were prepared with the powdered seaweed and the addition of agar base where they were incubated at 20 ± 2°C. The predominant strains growing in the plates were isolated and re-cultivated as single colonies. Approximately 10 different strains (bacteria and fungi) were frozen and preserved at -20°C.
3. The pasteurised sludge from the wastewater (SWW) treatment plant in Sligo (Ireland) was used as an inoculant.

All inocula were transferred into 1.0 L plastic containers flushed with a mix of N_2/CO_2 and stored at -20°C until further use. Frozen portions were thawed at room temperature prior to analysis. The pH, VS and C:N ratio from the four inocula were determined.

Separate bottles (1.0 L Duran glass bottle) (Figure 4.1) were inoculated with 20 g (14.6 g/VS) of *L. digitata*, 400 ml of dH_2O and 58 g/VS from each individual inoculum (ratio 1:4) (Pandey, *et al*., 2011). The headspace in the bottle was flushed with a mix of N_2/CO_2 for 5 min and placed in an incubator at 20°C, 35°C and 45°C (Angelidaki, *et*

al., 2009). This allowed the microbial communities to adapt to the chemical composition of the seaweed and develop under the corresponding temperatures. No mixing or nutrient was applied to the reactors. Where necessary, pH was controlled by the addition of 10% NaOH and $KHCO_3$.

Figure 4.1. Duran glass bottle (1.0 L).

Biogas and pH were monitored daily and after methane was detected, 3 g (2.2 g/VS) of substrate was added to the reactors (Vergara-Fernández, *et al*., 2008). The feed change was performed after the methane concentration in biogas was higher than 40-50% (every 16-22 days) for a period of 120 days. The rationality behind this was that process stability during the methanogenesis phase is regarded as the most optimal time to feed the digester. Introducing the new feed when lower concentrations of methane are present could cause unbalance in the process, especially during hydrolysis.

4.2.2. AD reactor set-up

Experiments investigating the effect of pretreatments on cumulative biogas production from *L. digitata* were carried out from December 2010 to December 2011.

The AD of *L. digitata* was based on the principles of the biomethane potential (BMP) test described by Angelidaki, *et al*. (2009) and Owen, *et al*. (1979).

After subjecting the seaweed (2.0 g/VS of *L. digitata*) to physical, acid-thermal, enzymatic or a combination of these three pretreatments (Section 4.2.3 and 4.2.4), the biomass was transferred into 120 ml Wheaton serum bottles (Figure 4.2). Thereafter, the pH of the pretreated biomass was returned to pH 7.3-7.5 using a 10% solution of NaOH followed by the addition of 4.0 g/VS of inoculum and 20 ml dH_2O. The headspace in the bottles was flushed with a mix of N_2/CO_2, sealed with rubber stoppers and capped with aluminium crimps. Experiments were carried out in triplicate at 35°C for 32 days.

For the determination of endogenous methane production, blanks containing inoculum/water and seaweed/water were run and the biogas produced was subtracted. All experiments were carried out in triplicate and the results are expressed as means. The details of individual experiments are described below.

Figure 4.2. Serum bottle reactors.

4.2.3. Chemical and enzymatic pretreatment

A limited number of studies have assessed the effect of chemical and enzymatic pretreatments to enhance the AD of seaweed (Denis, *et al*., 2009; Jard, *et al*., 2013; Nielsen and Heiske, 2011; Oliveira, *et al*., 2014 Vivekanand, *et al*., 2012). Moreover, the possibility of increasing biogas production by some form of pretreatment has never been studied in *L. digitata*.

The chemical (NaOH, inorganic and organic acids) and enzymatic pretreatment of *L. digitata* biomass was performed following the procedure in Chapter 3 (Section 3.2.2). After hydrolysis, the algal biomass was transferred into the serum bottles and the AD

was carried out as in section 4.2.2. Biogas produced from the AD of citric, lactic and oxalic acid alone was evaluated in separate reactors and the biogas generated was subtracted from the total biogas.

4.2.4. Physical pretreatment

A novel pretreatment approach addressed the effect of particle size and drying on biogas production from *L. digitata* and *S. latissima*.

4.2.4.1. Particle size

Various studies have highlighted the importance of particle size reduction on enzymatic hydrolysis and fermentation of different feedstocks to produce biofuels (Mshandetea, *et al*., 2006; Silva, *et al*., 2010; Silva, *et al*., 2012; Taherzadeh and Karimi, 2008). Hence, the impact of particle size on biogas production was also investigated.

After the biomass was dried at 75°C for 24 hours, the material was milled using a kitchen blender (Philips HR 2000) to different particles sizes by controlling the blending time; 1 min (<4.0 mm), 2 min (<2.0 mm) and 4 min (<1.0 mm) (Figure 4.3).

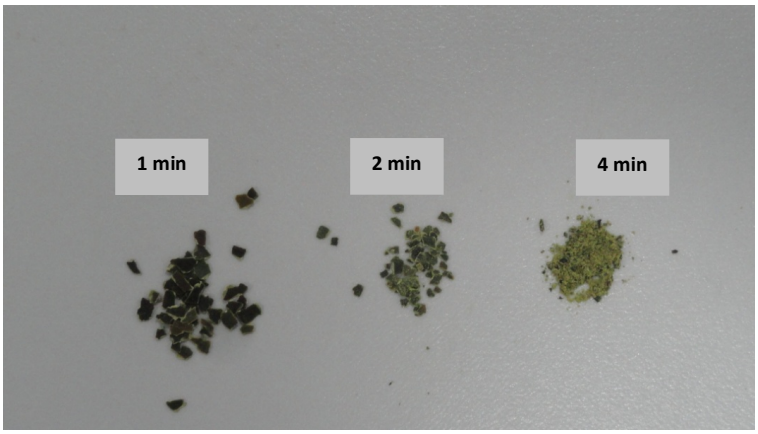

Figure 4.3. Different seaweed particles sizes after milling.

4.2.4.2. Drying

Seaweed in its fresh state (50–89% water) is perishable and subjected to microbial degradation within a few days of harvesting. They have a high water loss rate when exposed to air, and currently, drying is carried out outdoor under atmospheric conditions or by solar methods. Whether the system requires an external input of thermal heat to dry the seaweed biomass, a positive energy balance needs to be demonstrated before scaling-up or commercialisation (Bruton, *et al*., 2009; Whyte, *et al*., 1976).

Therefore, this experiment was designed to compare two different drying methods for *L. digitata* and *S. latissima* and to evaluate the extent to which drying conditions influence biogas production.

L. digitata and *S. latissima* samples were air-dried for 48 hrs at room temperature (Figure 4.4a, b) and oven-dried at 75°C for 24 hours (Figure 4.4c). The dried material was milled to fine particles (<1.0 mm) and the AD was carried out as in section 4.2.2.

a b c

Figure 4.4. *L. digitata* blades after drying (a) 4hrs RT; (b) 48hrs RT; (c) 24hrs oven-drying

4.2.5. Analytical methods AD

4.2.5.1. pH measurement

The pH value of the digested sludge was measured with pH strips (4-8 range) and with Orion 2-star bench top pH meter. A mixture of 10% NaOH and $KHCO_3$ was added to

the reactors to bring the pH to 7.0-7.5 and to boost the buffer capacity of the system, when necessary.

4.2.5.2. Biogas measurement

The biogas inside the bottles was measured using a 50 ml plastic syringe with a needle of 0.5 mm diameter, 25mm length. The needle was introduced through the rubber stopper and the piston of the syringe was released to let the gas expand to atmospheric pressure (Guwy, 2004; Owen, *et al*., 1979). Biogas measurement was performed daily during the first week of analysis and then twice a week until the end of the test.

4.2.5.3. Methane measurement by GC

A modification of the method described by Kim and Daniels, (1991) was used to measure CH_4 concentration in biogas. Analysis was carried out in a Varian 3600 (GC-FID) equipped with a capillary column (CP-PoraPlot Q, 0.53mm x 20µm x 25mm) (Bueno, 2010). The temperatures of the injector, column and detector were optimised to 150, 60 and 200°C, respectively. Helium BIP plus gas (Airproducts) was used as carrier gas. Calibration was performed with 10 ppm, 100 ppm, 1000 ppm and 10000 ppm (methane in nitrogen) gas standards (STGAS). A 250 µl syringe (Hamilton 1725SL SampleLock, point style 2) was used during collection of CH_4 from the cylinders and to inject it into the head of GC port. Three injections were performed from each methane concentration. The results (area) obtained from the GC were used for calculation of methane concentration.

4.2.5.4. Biogas measurement with a portable gas analyser

CH_4 content was also measured at regular intervals with a portable gas analyser LMS 4501, manufactured by Gas Data, configured and calibrated to measure biogas. In addition, CO_2, O_2, H_2 and H_2S profiles from the biogas produced in the reactors were also measured at room temperature once a week. H_2S and H_2 concentrations over 200 and 1100 ppm, respectively, could not be measured with this equipment, therefore, appropriate dilutions of the biogas were carried out with CO_2/N_2.

4.2.5.5. Total Solids (TS%) and Volatile Solids (VS%)

TS and VS of the seaweed were measured following EPA Method 1684 (Total, fixed, and volatile solids in water, solids, and biosolids).

4.3. Results and discussions

The seaweed *L. digitata* and *S. latissima* were subjected to different pretreatment processes as described early in the methods section. Initially, the effectiveness of these pretreatments was assessed by monitoring the amount of macromolecules released from the seaweed (Chapter 3). Subsequently, the best performing pretreatment methodologies were used to enhance the biodigestibility of the biomass and therefore, in theory, biogas production during the AD experiments.

4.3.1. AD experiments

4.3.1.1. Selection of inoculum

The study was designed to investigate the efficacy of different inocula on the degradation and consequently, biogas production from *L. digitata*. Figures 4.5 to 4.10 show the adaptation period of the microbial consortium from the inocula to algae degradation utilising *L. digitata*. Feed was added 5-6 times in a continuous AD process during the 120 days of digestion, as noted by the arrows.

The marine sediment (SD) adapted better and faster to the substrate, producing 345 ml of biogas after 24 hrs incubation compared to the 155, 196 and 110 ml of biogas produced from the BS, SWW and SM, respectively. This is in agreement with the study carried out by Ivanova, *et al*. (2002) where a highly metabolically active group of marine gamma-proteobacteria isolated from seawater (producing a wide range of glycanases and glycosidases) enhanced the degradation of *Fucus evanescens* thallus. Similarly, Zhao and Ruan, (2011) identified from sediments sample a wide group of potential bacteria degrading *Enteromorpha prolifera*. Migliore, *et al*. (2012) also reported that anoxic sediments used during the AD of *Gracilariopsis longissima* and *Chaetomorpha linum* fostered the degradation of the macroalgae and the conversion rate to methane.

In the early 16 days adaptation period, 1350, 1089, 985 and 547 ml of biogas was produced from the SD, SWW, BS and SM inoculum, respectively. These results suggest that the inoculum from SD and SWW had i) a faster degradation rate, ii) an active bacterial community, iii) microorganisms able to produce and secrete enzymes that could break down the sugars and other components from the seaweed, generating more biogas.

During the first 5 days of digestion, a reduction of biogas was observed due to a drop in the pH value (5.8-6.1), followed by a slow recovery until day 16, in particular with the BS, SWW and SM. The reduction on pH was less significant within the reactor inoculated with the SD inoculum (6.7-6.9). The buffer capacity of the SD helped to minimise changes in pH (Marquez, *et al*., 2013; Migliore, *et al*., 2012).

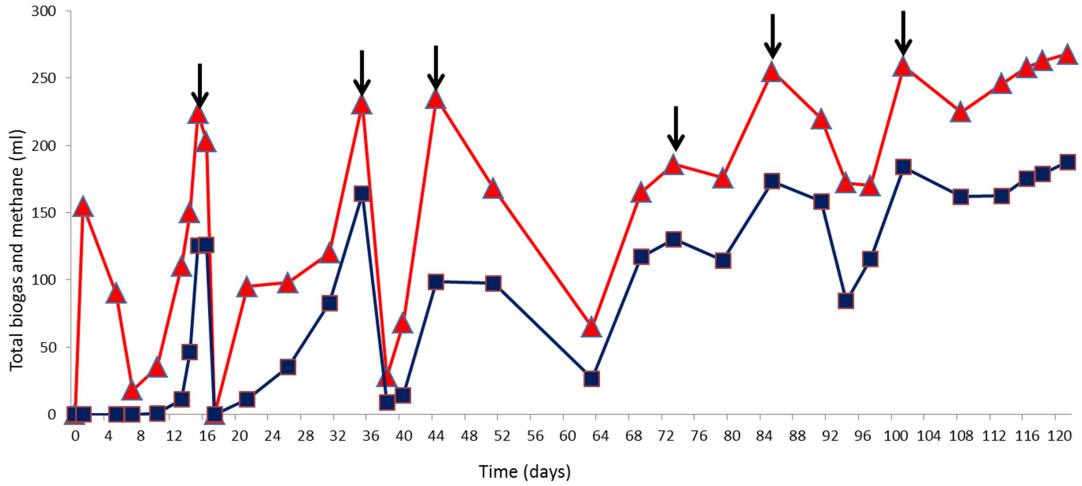

Figure 4.5. Acclimatisation of a bovine slurry (BS) inoculum to *L. digitata* at 35°C. Total biogas (red▲) and methane (blue■) production. Arrows indicate biomass addition.

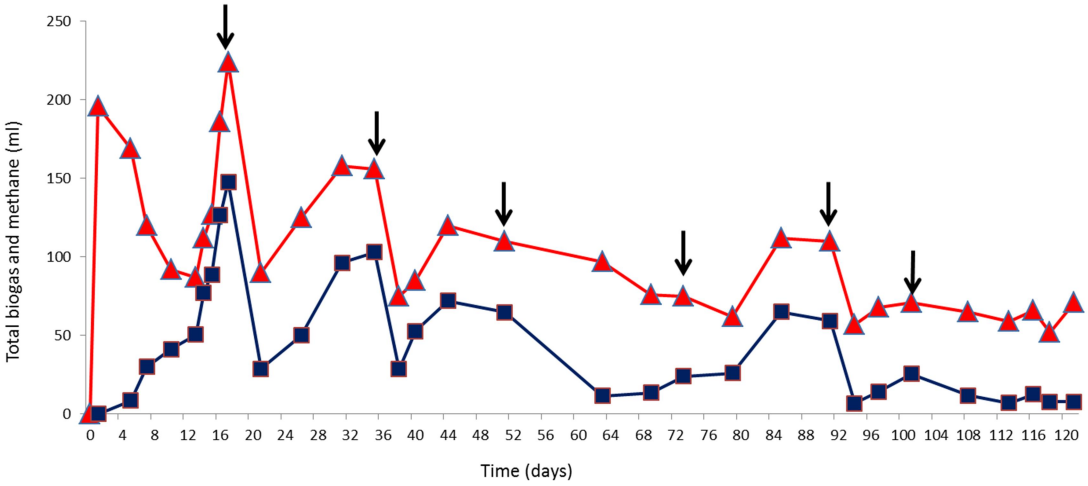

Figure 4.6. Acclimatisation of a sludge wastewater (SWW) inoculum to *L. digitata*. Production of biogas (red▲) and methane (blue■). Arrows indicate biomass addition.

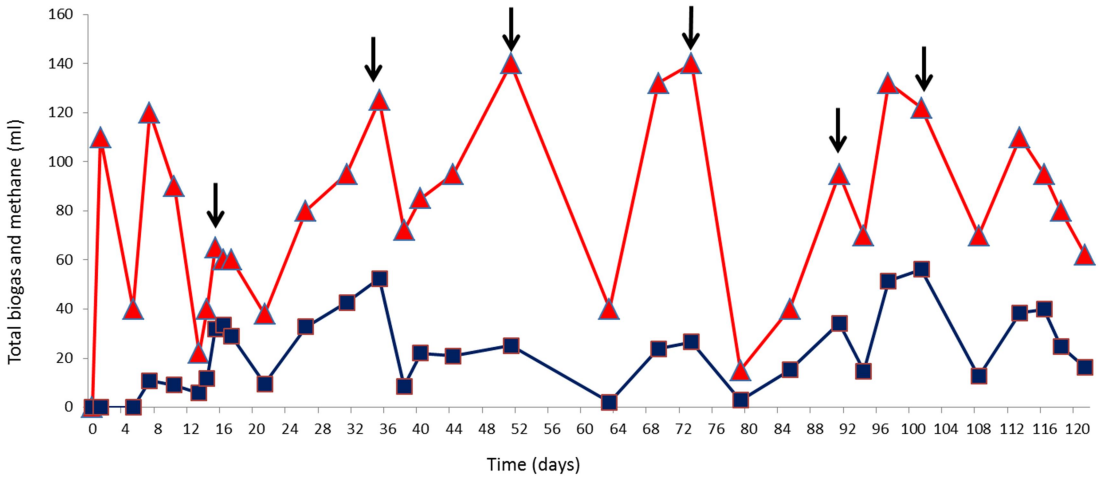

Figure 4.7. Acclimatisation of swine manure (SW) inoculum to *L. digitata*. Production of biogas (red ▲) and methane (blue ■). Arrows indicate biomass addition.

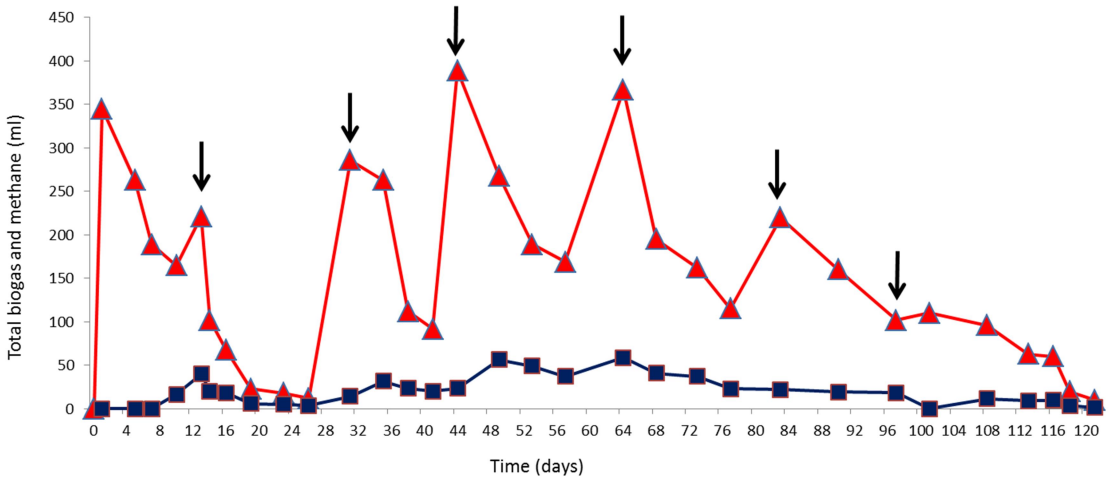

Figure 4.8. Acclimatisation of an anoxic sediment (SD) inoculum to *L. digitata*. Production of biogas (red ▲) and methane (blue ■). Arrows indicate biomass addition.

After the addition of fresh substrate to the reactors, on day 16, biogas and methane production was stopped, but, thereafter, an additional 753, 602, 544 and 338 ml of biogas for SWW, SD, BS and SW respectively, were produced until day 35. It was found for all inocula tested that whenever fresh substrate was added, a transitional change in biogas production was achieved. This suggests that inhibition due to a high input of substrate or accumulation of toxic compounds such as H_2S was present (H_2S accumulation is discussed in further Chapters). As a result, a reduction of metabolic activity or biogas/methane production rate was observed at the end of the process, especially in the reactors inoculated with the SWW and SD inocula.

From these experiments, the average methane percentage in the biogas collected was in the range of 54-58%, 48-50, 32-35% and 19-26% over the 120 days incubation period

for BS, SWW, SM and SD respectively. The results obtained show that methane concentration (%) is a function of the inoculum evaluated. Similarly, Pandey, *et al*. (2011) observed that, during the AD of dairy waste water, selection of inocula was a critical factor affecting biogas composition and methane production. Also, in a recent study carried out by Marquez, *et al*. (2013), different inocula (cow manure, marine sediment and sea wrack associated microflora) were used to produce biogas from sea wrack biomass. The study concluded that, while marine sediment was the most suitable inoculum, producing the highest methane yields, cow manure produced the lowest. This is in accordance with the results in this work, where marine sediments could have had a more diverse microbial population adapted to the seaweed biomass buried under the sediments, causing a higher metabolic activity and therefore better performance and yield.

At the end of the incubation time (120 days), biogas and methane production was reduced in reactors inoculated with SWW (Figure 4.6) and SM (Figure 4.7), whereas the reduction was greatest in reactors inoculated with SD (Figure 4.8). The microbial consortium from the BS adapted better to the chemical composition of the seaweed with a volatile solid destruction (VSD) of 59% (Table 4.2) and producing the highest methane yields among the four inocula.

Inocula	Total biogas (ml)	Biogas (ml g/VS)	Total methane (ml)	Methane (ml g/VS)	VSD[a] (%)
BS	4956	137	2792	77	59
SWW	3273	91	1360	38	38
SM	2540	70	705	19	42
SD	4854	134	620	17	39

[a] Volatile solid destruction.

Table 4.2. Cumulative biogas and methane produced from four different inocula.

Figure 4.9 and 4.10 show the biogas and methane profile of an adapted BS inoculum at 20°C and 45°C. These inocula were used during the AD experiments in Chapter 5, where the effect of temperature during the AD of *L. digitata* was further evaluated.

The selection of a temperature range for AD is dependent on the climatic conditions of the country. Therefore, the rationale behind this experiment was to develop an inoculum able to metabolise and produce biogas from seaweed at psychrophilic temperatures. The

reactor inoculated at 20°C with BS at the same loading rate as the reactor incubated at 35°C shows a different digestion profile. Low biogas was produced during the first days of digestion and, after a delay of about 4 days, biogas generation was initiated. pH adjustments using 10% NaOH and $KHCO_3$ were necessary to maintain the digestate above pH 6.9. Biogas and methane production peaked at day 17, after which time new substrate was fed.

After the addition of the second substrate, the inoculum adapted faster to the incubation temperature and was more efficient at metabolising the new feed when compared to the inoculum at 35°C. The addition of new substrate not only improved biogas production but also methane quality over the 120 day adaptation period. The last feed was added at day 108, where a small change on methane quality (from 58 to 68%) and biogas production was registered.

These results show that methanogenesis at psychrophilic temperature was a slow process that initiated later than at other temperatures, but after several substrate additions, the inoculum was able to withstand the feed change in the reactor and adapt well to the chemical composition of the seaweed at this temperature. The effect of temperature on biogas production from *L. digitata* will be further discussed in Chapter 5.

Figure 4.10 shows the value of biogas and methane produced after a 120 days inoculum adaptation at 45°C. An increase in biogas production was seen during the first days of digestion, suggesting the versatility of the microbial consortium from the BS in metabolising the seaweed at this temperature. The increase in biogas was followed by a reduction in pH (4.6-5.2), disrupting the AD process. The addition of a second feed and 10% NaOH and $KHCO_3$ helped to improve biogas production, and methane production was initiated at this period. The levels of pH in the bioreactor remained within acceptable range for the anaerobic microflora development.

The second addition of substrate affected the stability of the reactors and, only after 91 days, incubation biogas and methane production where stable. There were no differences in biogas production and methane quality after the addition of the subsequent feeds. The methane content ranged from 58-67% at the end of the experiment.

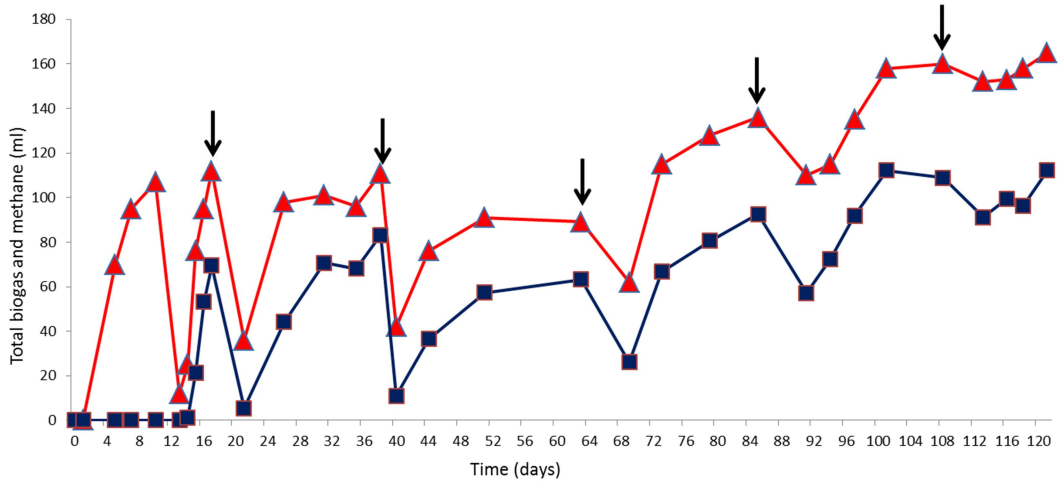

Figure 4.9. Acclimatisation of a bovine slurry (BS) inoculum incubated at 20°C to *L. digitata*. Production of biogas (red▲) and methane (blue■). Arrows indicate biomass addition.

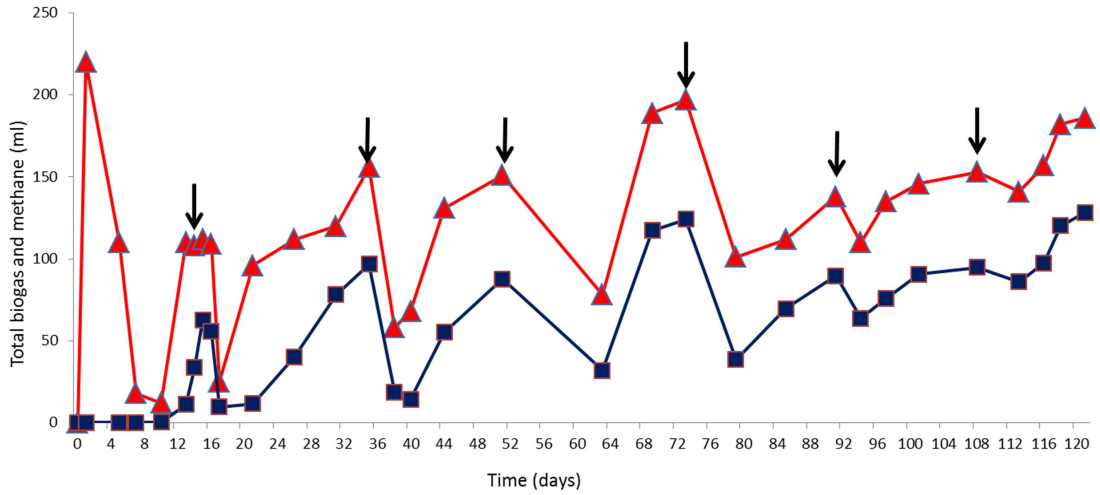

Figure 4.10. Acclimatisation of a bovine slurry (BS) inoculum incubated at 45°C to *L. digitata*. Production of biogas (red▲) and methane (blue■). Arrows indicate biomass addition.

Overall, the results suggest that selection of an inoculum capable to degrade and adapt to the chemical composition of the seaweed is a key factor in the AD of *L. digitata*. The initial days of digestion were a rate limiting phase during reactor start-up. Each inoculum exhibited different degrees of adaptation and development as it was observed in the biogas yields, demonstrating the importance of inoculum activity throughout the AD of the seaweed. This similarity has been previously reported (Angelidaki, *et al*., 2009; Karakashev, *et al*., 2005; Pandey, *et al*., 2011) and it is attributed to a wide range of enzymes produced by the microbial consortium capable of breaking down the

chemical components of the seaweed during reactor start-up (Gómez and Gareia, 1993; Moen, *et al*., 1997; Tang, *et al*., 2009; Williams, *et al*., 2013.)

Differences in VSD, biogas and methane production were observed among the four different inocula evaluated. Consequently, BS was regarded as the most optimal, and it was used in further experiments.

4.3.1.2. Effect of thermo-chemical and enzymatic pretreatment on biogas production

During biomass pretreatment, compounds are released from the biomass (Chapter 3) and made available for further industrial processing to produce bio-based materials or for microbial metabolism. The process could contribute extensively to the cost of biogas production and needs to be tailored to every biomass; therefore, investigation is required in order to make biogas production more economically attractive.

The objective of these experiments was to apply a combination of physical, chemical, thermal and enzymatic pretreatment methods to the algal biomass in order to enhance biogas production from *L. digitata*. Table 4.3 summarises the effect of the different pretreatments on total biogas production. The VSD for each pretreatment applied is also shown. The data have been organised as best pretreatment based on the total biogas produced per g/VS.

There was no correlation between the highest concentration of macromolecules (RS, TL and TP) released from *L. digitata*, after pretreatment with the most optimal pretreatment from Chapter 3, with the highest biogas yields. Moreover, a clear pattern of increasing biogas production from *L. digitata* by the different pretreatment methods was not found (Table 4.3).

The total biogas produced from untreated (Treatment 4) *L. digitata* was 228 ml g/VS. Treatment 1 (Cellulase followed by a 2.0% citric acid pretreatment) showed a 6% increase in biogas yield (243 ml biogas g/VS). The VSD percentage was greatest for Treatment 2 (2.0% citric acid) (81% VSD) followed by Treatments 1 (Cellulase + 2.0% citric acid) (79% VSD) and 3 (Cellulase) (61% VSD).

Treatment	Reagent	Biogas [a] (ml g/VS)	VSD[b] (%)
1	Cellulase + 2.0% citric acid	243 ± 3.25	79
2	2.0% citric acid	237 ± 2.48	81
3	Cellulase	232 ± 2.89	61
4	Water (control)	228 ± 0.58	52
5	Alginate lyase	225 ± 1.10	59
6	1.0% HNO_3	223 ± 2.02	56
7	Cellulase +1.0% lactic acid	219 ± 3.89	50
8	Water (thermal)	212 ± 0.45	58
9	1.0% HCl	198 ± 1.94	61
10	6.0% NaOH	186 ± 0.56	54
11	Cellulase +1.0% oxalic acid	176 ± 2.57	53
12	1.0% H_2SO_4	168 ± 1.45	61
13	1.0% lactic acid	161 ± 1.14	57
14	6.0% lactic acid	107 ± 2.45	55
15	1.0% NaOH	107 ± 1.26	56
16	Cellulase + 6.0% lactic acid	99 ± 0.98	61
17	4.0% HNO_3	94 ±1.23	67
18	6.0% oxalic acid	83 ± 0.63	57
19	3.0% HCl	81 ± 0.97	54
20	6.0% H_2SO_4	79 ± 1.42	56
21	Cellulase + 6.0% oxalic acid	76 ± 0.74	59
22	Celluclast 1.5L	72 ± 1.41	49
23	6.0% citric acid	69 ± 3.45	61
24	Inoculum	19 ± 0.12	14
25	Bovine slurry	12 ± 0.23	9
26	Seaweed (without inoculum)	8 ± 0.04	5

[a] Mean ± standard deviation.

[b] Volatile solid destruction.

Table 4.3. Biogas production from *L. digitata* after thermo-chemical and enzymatic pretreatment.

While the samples subjected to 6% acid concentration measured the highest RS and TL recoveries (Chapter 3), the biogas yields were the lowest (Treatments 10, 14, 18, 20, 23). The reduction of biogas in these treatments could be attributed to the formation of inhibitory compounds such as furfural, hydroxy-methylfurfural, phenolic/aromatic compounds, formaldehyde or some acids such as levulinic, acetic, formic and uronic acids (Taherzadeh, *et al*., 2007). These compounds would have affected the growth of the microbial consortium and therefore fermentation (Jeong, *et al*., 2012, Adams, *et al*., 2009, Scordia, *et al*., 2011). Although microbial adaptation to these type of compounds has been reported elsewhere without affecting methanogenesis (Boopathy, *et al*., 2009; Rivard and Grohmann, 1991), in these set of treatments a clear inhibition was observed.

After pretreatment, the pH of the biomass was brought to neutral range by the addition of a 10% NaOH solution. Also, during digestion, medium acidification was observed, thus, addition of NaOH was compulsory to neutralise acid formation. This would have increased the concentration of ions in the reactor, affecting microbial growth and therefore, lowering the biogas yield (Chen, *et al*., 2008; Jard, *et al*., 2012).

The results from the VSD also showed that, after 32 days incubation time, organic matter (38-51%) was still available for microbial metabolism. Similarly, Peu, *et al*. (2011), Briand and Moran, (1997), also reported a reduction on VS of 32% to 63%. This corroborates the fact that microbial inhibition was a cause of low biogas yields in the reactors and, therefore, poor VS destruction.

Numerous hydrolytic enzymes have been used extensively during pretreatment of different feedstocks for biofuel production (Adams, *et al*., 2009; Choi, *et al*., 2010; Denis, *et al*., 2009). In this study, the addition of Cellulase (Treatment 3) enhanced the biodegradability of the seaweed to some extent (61% VSD) when compared to Treatment 4 (control) (52% VSD). However, only a 1.7% increase in total biogas was observed (232 ml biogas g/VS). Therefore, no considerable improvement in biogas was attributed to the use of enzymatic preparations.

The selection of an optimum pretreatment for *L. digitata* should also consider an energy balance and life cycle analysis in order to evaluate the suitability of the pretreatment during scaling-up operations. In a seaweed-biorefinery model, high value added products such as sugars, lipids and proteins (Chapter 3) are extracted from the pretreated seaweed while the remaining biomass has the potential to be used as a source of renewable energy. Similar approaches have been proposed in the literature, simultaneous saccharification and fermentation, separate hydrolysis and fermentation and direct microbial conversion, with different bioethanol and biogas yields (Daroch, *et al*., 2013; Ge, *et al*., 2011; Jeihanipour, *et al*., 2010; Talebnia, *et al*., 2010; Tan and Lee, 2014).

4.3.1.3. Effect of particle size on biogas production

The particle size of feedstocks usually has a significant effect on digesters performance (Agbor, *et al*., 2011; Chynoweth, *et al*., 1981; Mshandetea, *et al*., 2006; Izumi, *et al*.,

2010). In addition, it is an essential pretreatment step in order to make biomass easier to handle in a commercial scale plant.

Biogas concentration was higher after reduction of the particle size when compared to control samples (Table 4.4). As particle size was reduced from <4.0 mm to <2.0 mm and further to <1.0 mm, it was found that biogas production increased from 11% (<2.0 mm) to 19% (<1.0 mm) for *L. digitata* from physical treatment through particle size manipulation. In the case of *S. latissima*, biogas production was enhanced by 13% (<2.0 mm) to 22% (<1.0 mm).

It is clear from these results and those outlined in Chapter 3 (section 3.3.4.2) that particle size reduction increased the surface area available of the substrate to the bacteria consortium, favouring the contact between the enzymes and the biomass and therefore, the biogas yield. The fact that biogas was increased means that mechanical treatment can also be used to decrease the solid retention time of the anaerobic bioreactor. Furthermore, during the operation and management of a biogas plant, the use of particle size reduction as a pretreatment step will benefit the construction of smaller storage facility and might allow the development of a smaller digester volume.

It is, however, important to highlight that grinding the seaweed requires an energy input (Da Silva, *et al*., 2010; Karki, *et al*., 2010; Silva, *et al*., 2012) and, although this energy requirement was not measured, the environmental and economic aspects of feeding the digester with smaller particle sizes should be taken into account.

Although milling as pretreatment step for these two seaweed species has not been reported to date, the study carried out by Hart and Kohler (1986) investigated the effect of particle size reduction and grinding energy on methane production from different Kelp species. They found that chopped kelp produced 228 ml g/VS at 1.9 W.h/kg and grinding the seaweed to 2.1 mm produced 247 ml g/VS at 4.3 W.h/kg, suggesting that there is no processing advantage to grinding the Kelp to smaller size except than to meet the handling needs.

Treatment	Condition	Biogas [a] (ml g/VS)	
		L. digitata	*S. latissima*
1	< 1.0 mm	273 ± 1.34	366 ± 2.21
2	< 2.0 mm	255 ± 0.96	339 ± 1.76
3	< 4.0 mm (control)	228 ± 0.58	298 ± 2.32

[a] Mean ± standard deviation.

Table 4.4. Biogas production from *L. digitata* and *S. latissima* after milling pretreatment, by particle size reduction, on biogas production.

4.3.1.4. Effect of drying on biogas production

Drying is a conventional process that aims to reduce the water content or moisture from a product to a level at which microbial spoilage is reduced. Although drying biomass provides an important benefit in terms of transportation and storage volume, this must be balanced against the energy required to evaporate the water, avoiding an increase in capital and operating costs. Consequently, drying the seaweed biomass is a crucial step before it can be used in industrial-scale processes (Bruton, *et al*., 2009; Gupta, *et al*., 2011; Schlarb-Ridley and Parker, 2013).

The results from this experiment show that cumulative biogas production was affected by the physical state of the seaweed. Raw seaweed generated 41-43% less biogas when compared to oven dried (Table 4.5). On the other hand, drying *S. latissima* and *L. digitata* at room temperature resulted in a 3% and 4% reduction of biogas, respectively, when compared to the seaweed subjected to oven drying. While a small difference in biogas production was observed, an important reduction in the energy consumption from the overall process is expected.

Treatment	Condition	Biogas [a] (ml g/VS)	
		L. digitata	*S. latissima*
1	Oven (Control)	228 ± 0.58	298 ± 2.32
2	Room temperature	218 ± 1.15	289 ± 1.07
3	Raw	135 ± 2.87	170 ± 0.69

[a] Mean ± standard deviation.

Table 4.5. Biogas production from *L. digitata* and *S. latissima* reactors after different drying regimes.

Several studies have suggested that the extraction and yields of phytochemicals with high-added value are affected by the drying temperature. Also, changes in the physicochemical composition of the seaweed affect fermentation and therefore, biofuel yields (Chan, *et al*., 1997; Tello, *et al*., 2011; Wong and Cheung, 2001).

For instance, in a study carried out by Gupta, *et al*. (2011), the total phenolic and flavonoid content from the brown seaweed, *Himanthalia elongate*, was reduced by a 49-51% after the seaweed was dried at 25°C. However, when the temperature was increased to 30-40°C the phytochemical content was higher.

Mussgnug, *et al*. (2011) demonstrated that drying microalgae, as a pretreatment step, is detrimental in terms of biogas production with a decrease of approximately 20% of the biogas production potential. The reduction was attributed to loss or decrease of essential fermentable volatile organic compounds for bacterial metabolism.

On the other hand, Bruhn, *et al*. (2011) and Nielsen and Heiske, (2011), reported that drying *Ulva lactuca* did not affect the methane yield (ml g/VS) but, instead, enhanced the weight specific methane yield (ml g/algae), reduced the volume of the seaweed and increased the TS/VS content.

There are several factors which must be taken into account before bringing this process to full scale, such as the heat source to dry the biomass, a process to remove the evaporated water and an effective method to expose the full blade to dryness. Therefore, from this study, some considerations should be taken into account when drying the seaweed at room temperature.

1. The size of the drying rack had to be designed in a way that air should easily circulate through the seaweed to assure good ventilation and quick drying of the blades.
2. It was found that if water was not removed from the surface of the blades during the first 4-6 hrs, microbial colonisation of the surface was seen, therefore, upsetting the biochemical composition of the seaweed. This is in agreement with a study carried out by Ivanova, *et al*. 2002, where a metabolically active group of marine bacteria was associated with the brown seaweed *Fucus evanescen*. Suggesting that the seaweed hosts microbial communities that play an important role during the

degradation of the seaweed in the microbial food web (Ivanova, *et al*., 2002; Tang, *et al*., 2009.

3. To avoid this, the blades were exposed to a stream of air generated by a pedestal floor fan.
4. The average room temperature was 17-21°C

4.4. Conclusions

In this chapter, the use of chemical, enzymatic and physical pretreatment methods to enhance biogas production from seaweed was investigated.

Throughout the AD experiments, bovine slurry was selected as most optimal inoculum for seaweed biogas production.

A combination of enzymatic (Cellulase) and acid-thermal (2.0% citric acid) pretreatment improved biodigestibility and biogas yield of *L. digitata* by 6.0% (243 ml g/VS), whereas other chemical and enzymatic treatments had little effect or inhibited biogas production.

Among physical pretreatments, milling the seaweed to fine particles (<1.0 mm) was the most suitable pretreatment method, enhancing biogas yields by 11% to 22%.

While drying the seaweed biomass at room temperature for 48 hrs reduced biogas yields by 3% to 4% when compared to oven drying, the energy input to dry the seaweed would be expected to be lower.

Information gathered from these experiments contributes to the development of a more efficient seaweed-based biogas production process. While most of the chemical pretreatment methods successfully released macromolecules from the two seaweed species (Chapter 3), the results presented in this Chapter have shown that the same pretreatment methods inhibited biogas production. Therefore, within the seaweed biorefinery model outlined in Chapter 2, chemical pretreatments should be employed to extract macromolecules from the seaweed rather than to increase biogas yields.

Research to achieve higher biogas and methane conversion yields by means of co-digestion will be investigated (Chapter 6 and 7) in order to make the seaweed-biofuel process a more feasible process.

The conclusions reached in this study apply to small-scale (120 ml) batch operations, and further investigation will be carried out to relate these findings to larger laboratory scale reactors (Chapter 5 to 9).

CHAPTER 5

ANAEROBIC DIGESTION OF *Laminaria digitata:* THE EFFECT OF TEMPERATURE ON BIOGAS PRODUCTION AND COMPOSITION

Vanegas, C., Bartlett, J. (2013) Anaerobic digestion of *Laminaria digitata*: The effect of temperature on biogas production and composition. *Waste and Biomass Valorisation*, 4 (3), pp.509-515.

5.1. Introduction

Biofuels produced from biomass are considered to be an important option for renewable energy generation (Singh, *et al*., 2011; Gunaseelan, 1997). In Ireland, the cost of imported fuels (oil, gas and coal) was over €6 billion per annum in 2010, accounting for 95% of all energy (Howley, *et al*., 2011). As a result, the Irish government is developing instruments to change the fossil-based energy economy towards to one based on sustainable energy.

The utilisation of seaweed as an energy feedstock, in particular biogas, has an important potential impact in replacing fossil fuels, because it can be used as an alternative for natural gas, fuel in the transport sector, and for power and heat generation in Ireland (Bruton, *et al*., 2009; Gunaseelan, 1997; Hughes, *et al*., 2012; Singh, *et al*., 2011).

The microbial activity and, therefore, the fermentation process during the production of biogas by AD, are strongly dependent on several factors such as temperature (Holm-Nielsen, 2009; Ahring, 2001) and pH (Switzenbaum, *et al*., 1990; Chen, *et al*., 2008). AD can be carried out at psychrophilic (15–25°C), mesophilic (30–37°C) and thermophilic (45–60°C) temperatures. Commonly, the selection of a temperature range for AD is dependent on the climatic conditions of the country, the feedstock, and process performance.

In Ireland, for example, inland air temperatures normally reach 8°C during winter and about 15 to 20°C during summer. Several studies have highlighted the advantages and disadvantages of AD at different temperature ranges. The majority of applications and

research effort has been concentrated on AD within the mesophilic (30–40°C) temperature range. This is largely due to the view that mesophilic conditions are optimal for microbial activity and biogas production rates.

Digestion under thermophilic conditions has many advantages over mesophilic or psychrophilic, such as greater metabolic and conversion rates, a faster solid-liquid separation and minimisation of pathogens (Golueke, *et al*., 1957; Holm-Nielsen, *et al*., 2009; Zamalloa, *et al*., 2012). On the other hand, thermophilic processes have some limitations, such as reduced stability to environmental changes and the cost of heat energy requirements (Ahring, *et al*., 2001).

Researchers have also targeted biogas production of different wastes and feedstocks under psychrophilic ranges (Kashyap, *et al*., 2003). Results from some studies on the effect of temperature on biogas and methane production have proposed that biogas production is linearly correlated with temperature (Bouallagui, *et al*., 2004). Other authors have suggested that temperature has no effect on the ultimate methane yield (Chae, *et al*., 2008), and that an increase in temperature could result in a reduction of the biogas yield, due to low solubilisation rate (Komemoto, *et al*., 2009) and increased inhibition by ammonia (Samson and LeDuy, 1986).

Studies carried out in the life cycle analyses (LCA) of biofuel production from seaweed highlighted that the energy associated for digester heating comprises a large portion of the overall operating cost and investigation to minimise this element is crucial (Alvarado-Morales, *et al*., 2012; Dave, *et al*., 2013; Langlois, *et al*., 2012).

Production of biomethane from *L. digitata* has been reported at temperatures of 37°C (Adams, *et al*., 2011). In order to further enhance the understanding of biogas production from this seaweed species, the optimum temperature for the AD of *L. digitata* was studied under psychrophilic (20°C), mesophilic (35°C) and thermophilic conditions (45°C), maximising hydrolysis rate and consequently biogas and methane production efficiency.

5.2. Materials and methods

5.2.1. Biomass material

Samples of *L. digitata* were harvested and processed as in Chapter 3, section 3.2.1.

5.2.2. Selection of inoculum

Bovine slurry and inoculum adaptation was processed as outlined in Chapter 4, section 4.2.1. Briefly, the selection of the inocula was according to criteria of convenience, availability and potential for use in full-scale reactors. The BS (58 g/VS) was passed through a 0.2 mm metal sieve and mixed with 20 g (14.6 g/VS) of *L. digitata* and 400 ml of dH_2O in 1.0 L Duran glass. The headspace in the bottle was flushed with a mix of N_2/CO_2 for 5 min and placed in an incubator at 20°C, 35°C and 45°C. After methane was detected 3 g (2.2 g/VS) were added to the reactors. The chemical parameters of the individual substrates used in this Chapter are represented in Table 5.1.

Characteristics	Inoculum	Bovine slurry	*L. digitata*
C/N	16/1	12/1	25/1
pH	7.6	6.4	6.6
VS (%)	58	64	74

Table 5.1. Characteristics of substrates used during the experiments.

5.2.3. Batch experiments

Batch laboratory AD experiments were carried out in 120 ml serum bottles. 1.4 g/VS of powdered *L. digitata* were transferred into each bottle. 2.84 g/VS of inoculum adapted to the seaweed was taken from the 1.0 L laboratory scale anaerobic digester (Chapter 4, section 4.2.1), and mixed with 30 ml of distilled water. pH was adjusted to 7.2-7.4 in all cases. After the set-up of each reactor, the headspace in the bottles was flushed with a mix of N_2/CO_2 for 1 min. The bottles were sealed with rubber stoppers, capped with aluminium crimps and incubated at 20°C, 35°C and 45°C for 54 days.

For the determination of endogenous biogas and methane production, blanks containing only the anaerobic inoculum and seaweed were run. All experiments were carried out in triplicate and the results are expressed as means. The biogas inside the bottles was measured as outlined in Chapter 4, section 4.2.5.2.

5.2.4. Analytical methods

Methane (CH_4) content and CO_2, O_2, H_2 and H_2S profiles were measured as previously described in Chapter 4, section 4.2.5.3 and 4.2.5.4.

The analytical methods for TS, VS (EPA Method 1684), and pH of the samples are described in Chapter 4, section 4.2.5.

5.3. Results and discussion

A critical factor for a viable seaweed digestion process is an optimum operating temperature, and although the effect of temperature on biogas production from different feedstocks is well investigated, no studies have been carried out in this seaweed species which is the novelty of the present work.

5.3.1. Effect of temperature on cumulative biogas

Cumulative biogas from the degradation of *L. digitata* as a renewable source of energy is presented in Figure 5.1. The data shown are the biogas generation from reactors incubated at thermophilic (45°C), mesophilic (35°C) and psychrophilic (20°C) conditions over 54 days. Maximum biogas production occurred at 35°C (333 ml biogas/gVS) when compared to the other two temperatures. Second best was at 45°C (236 ml biogas/gVS), followed by 20°C (198 ml biogas/gVS) with 30% and 41% less biogas produced respectively, when compared to mesophilic digesters.

The rapid conversion of the chemical components of *L. digitata* to biogas during the first days of digestion was evident. The duration of the start-up phase was also depended on the inocula, playing an important role in the biogas production rate. Results show that throughout the first 4 days of operation, the thermophilic temperature had a better effect on biogas generation. A rapid start-up was more distinct at the

thermophilic temperature (96 ml biogas/gVS) followed by mesophilic (76 ml biogas/gVS) and psychrophilic (6 ml biogas/gVS) temperatures. This accounts for approximately 40%, 23% and 3% of the total volume of biogas generated over the 54 days digestion. This rapid start-up is explained by the process of adaptation of the inoculum to the thermophilic temperatures and the chemical composition of the seaweed, producing a number of specific enzymes with the ability to hydrolyze the main polysaccharides of *L. digitata* (alginate, laminaran and mannitol) to biogas. The use of a thermophilic temperature during the first days of digestion could shorten the retention time of the biomass therefore, reducing costs. Alternatively, the hydrolysis process can be carried out at this particular temperature while the methanogenesis step at mesophilic in a two-phase AD system.

Biogas production at both thermophilic and mesophilic temperatures proceeded parallel from day 8 to 37, at which point an increase at the mesophilic temperature was seen. Bacterial communities in AD reactors exhibit diverse levels of dynamism and complexity, especially under different temperatures (Karakashev, *et al*., 2005). The results suggest that a different and more active consortium of microorganisms was developed, or may be dominant in the mesophilic and thermophilic reactors, as found in other studies (Karakashev, *et al*., 2005; Mladenovska, *et al*., 2000).

The results are in agreement with previous studies of other seaweeds that showed a better biogas production rate with reactors incubated at mesophilic temperatures than those at thermophilic ranges. Varel, *et al*. (1988), for instance, found that the methane production rate from anaerobic degradation of *Spirulina maxima* was higher at 35°C than at 55°C. The study suggested that this was due to a lack of nutrients, an imbalance in the C/N ratio, or toxic compounds that may have been generated during the digestion at 55°C. Hansson (1983) also reported lower gas yields and high levels of VFA during the thermophilic AD of marine green algae *Ulva*, *Chaetomorpha* and *Cladophora*, when compared to mesophilic fermentation. In a continuous hybrid flow-through reactor digesting the microalgae *Scenedesmus obliquus* and *Phaeodactylum tricornutum*, the thermophilic (54°C) reactor had a higher biogas production rate than one at mesophilic (33°C) conditions for *S. obliquus*. However, for *P. tricornutum*, the difference between operational temperatures was not significant (Zamalloa, *et al*., 2012). It is concluded that when considering scaling up the AD of *L. digitata*, mesophilic temperature should operate better.

While AD at mesophilic and thermophilic ranges is well understood and documented, very little work has been done on biomethanation at psychrophilic temperatures. In Figure 5.1, it can be seen that biogas production in reactors incubated at 20°C was lower than the reactors incubated at 35°C and 45°C. Despite the fact that the inoculum was adapted to the seaweed chemical composition at this particular temperature (20°C), metabolism occurred at a very low rate until day 4, when biogas was detected. These findings indicate that the bacterial consortium in the psychrophilic digestor had a different metabolism to those in the other two reactors.

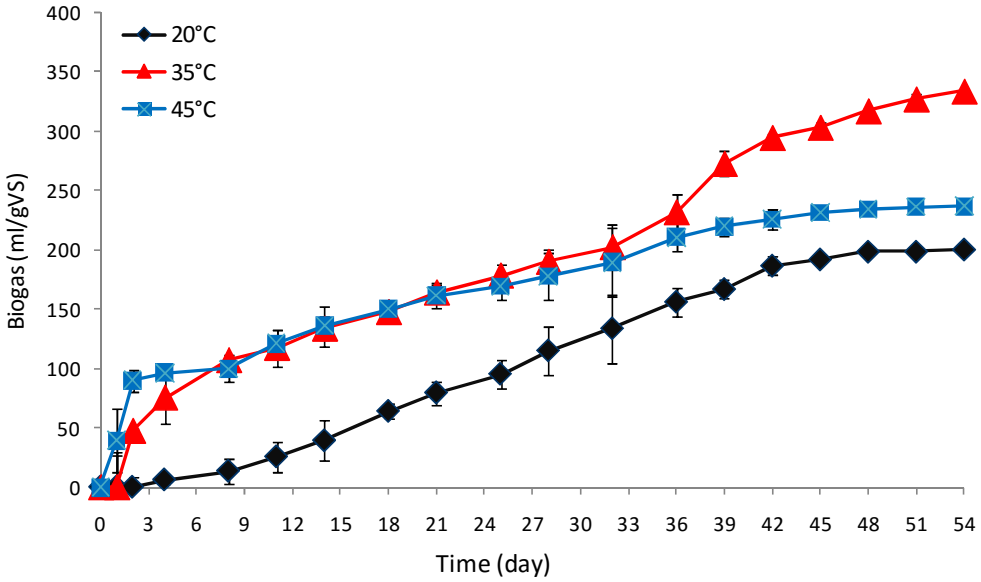

Figure 5.1. Cumulative biogas production (ml) per g volatile solids of *L. digitata* over 54 days digestion

5.3.2. Effect of temperature on cumulative methane

It can be seen from Figure 5.2 that the trend in methane production was similar to that for biogas. According to the results, the cumulative methane production was greatest at 35°C with 184 ml CH_4/gVS. The reactors incubated at 45°C produced 23.3% (141 ml CH_4/gVS) less methane when compared to reactors incubated at 35°C. Previous work on AD at different temperatures has suggested that the reduction of methane yields is related to the sensitivity of the methanogenic community to accumulation of inhibitory compounds produced during the AD, such as ammonia and H_2S (Samson and LeDuy, 1986) or volatile fatty acids (VFAs) (Komemoto, *et al.*, 2009), rather than the temperature. Golueke, *et al.* (1957), for instance, indicated that during the AD of green algae, a moderate increase of the reactor's temperature (from 35°C to 50°C) facilitated the adaptation of the inocula to the new condition and, therefore, higher biogas yields

and the effective destruction of volatile matter was achieved. During the start-up period, methane was only detected after day 3 and day 1 in reactors incubated at mesophilic and thermophilic conditions, respectively.

High levels of acids accumulated temporarily in the reactors incubated at mesophilic conditions, reducing the pH, and consequently the activity of methanogens. The reactors incubated at psychrophilic temperature produced the lowest methane yield, as was found with biogas production. Cumulative methane production was 111 ml CH_4 g/VS which was a 39.7% lower than digestion at the mesophilic temperature. In a study where the anaerobic degradation of lipids from the marine microalgae *Nannochloropsis salina* was performed at 20°, methane production was observed up to day 210, followed by a slow reduction in concentration until the end of the experiment (Grossi, *et al.*, 2001). Longer digestion time would be necessary to verify whether higher biogas/methane rates could be achieved for AD of *L. digitata* at psychrophilic conditions. From the data presented here, psychrophilic temperature had a negative impact on biogas/methane production and will not be recommended during the scaling up of the process.

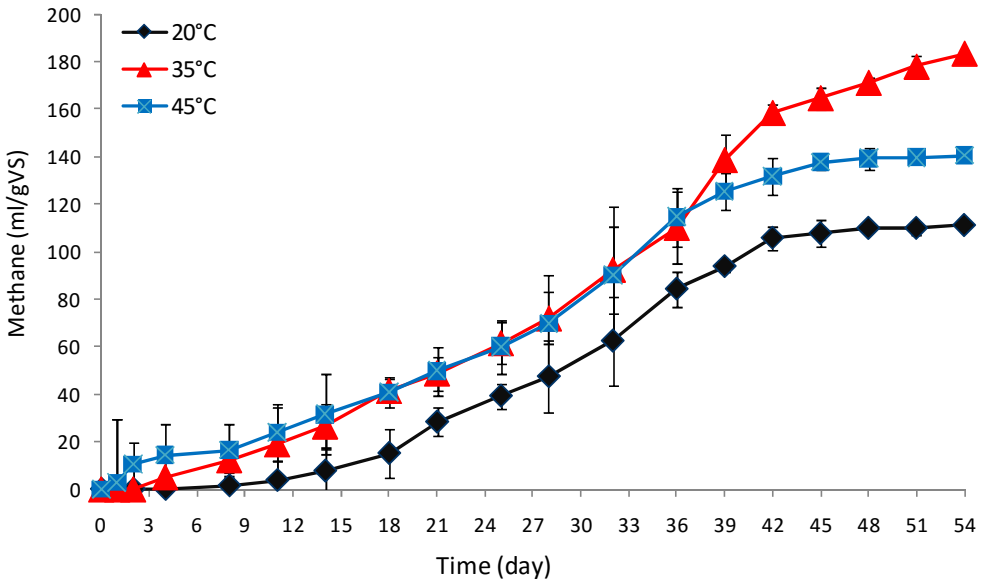

Figure 5.2. Cumulative methane production (ml) per g volatile solids of *L. digitata* over 54 days digestion.

Although no studies on AD of *L. digitata* at 20°C and 45°C have been carried out to date, production of methane at 37°C has been reported elsewhere. While seaweed samples harvested in May produced 210 cm^3 g/VS of methane, higher yields were

reached from samples harvested in July (254 cm^3 g/VS) (Adams, *et al*., 2011). In a full scale digestor fed with *Laminaria*, Morand, *et al*. (1990) shows the average daily methane production of 0.5 m^3 Kg/VS during the last 21 days of the experiment, of which 61.2% was CH_4 and 38.3% CO_2. The methane concentrations found in this study compare favourably with the values from Morand, underlining the potential for bioenergy harvesting from AD of *L. digitata* (Table 5.2).

5.3.3. Effect of temperature on biogas composition

The methane concentration of the biogas from the reactors incubated at the mesophilic temperature ranged from 8-34% during the first 10 days of digestion, followed by a constant increase to 62-75% until day 48, with lower concentrations (62%) at the end of the experiment. For reactors at thermophilic temperature, the methane gas concentration was lower than at mesophilic, ranging from 2-15% over the first 10 days, with an increase to 51-60% towards the end of the digestion process. On the other hand, reactors at 20°C had a higher methane composition of 12-38% over the first weeks of digestion, followed by 45-58% during the remaining digestion period (Table 5.2). This higher methane concentration, when compared to the other temperatures, is attributed to a neutral pH over the first days of digestion, contributing to the stability of the system and the methanogenic community.

	CH_4 (%)			CO_2 (%)			H_2 (ppm)		H_2S (ppm)
Days	0-10	11-48	49-54	0-10	11-48	49-54	0-25	26-54	0-54
Temperature									
20°C	12-38	45-58	45	50-80	28-49	10-27	>1200	1190-15	>200
35°C	8-34	62-75	62	52-76	51-33	9-32	>1200	1150-10	>200
45°C	2-15	42-60	51	51-79	50-36	15-35	>1200	1180-13	>200

Table 5.2. Biogas composition during the AD of *L. digitata* with varying temperatures.

Carbon dioxide, hydrogen and hydrogen sulphide generation during AD was also measured. The gas produced throughout the first 3 days of digestion reached 50-80% CO_2 with a rapid reduction after methane was produced. Lowest values ranged between 7% and 20%. For all temperature conditions, maximum values of hydrogen were observed during the first days of digestion ranging from 1200 ppm to 10 ppm at the end of the experiment. This indicates that the fermentative and acetogenic bacteria were more metabolically active at the beginning of the process than methanogens.

Klass, *et al*. (1979) found higher concentrations of carbon dioxide (89 mol% after 103h) and hydrogen (28 mol% after 13h) during the early stage of a study of the AD of *Macrocystis pyrifera*. As methane started increasing, carbon dioxide and hydrogen concentrations rapidly decreased, reaching 10-15 mol% and zero, respectively.

H_2S was also detected in higher quantities (more than 200 ppm) over the 54 days digestion. The H_2S content of *L. digitata* is relatively high when compared to other biomass sources. This is due to the unique chemical composition of the seaweed and the amount of sulphated polysaccharides (fucoidan) present (Black, 1950). H_2S production from the AD of other marine algae species has also been reported (Nkemka and Murto, 2010; Grossi, *et al*., 2001; Peu, *et al*., 2011; Vergara-Fernández, *et al*., 2008). Hydrogen sulphide production may reduce the methane yield of the AD by competition between methanogens and sulphate reducing bacteria, or even inhibiting methanogenesis. For conventional co-generation gas engines, the hydrogen sulphide content should be kept below approximately 700 ppm in order to avoid unnecessary corrosion, production of sulphur dioxide and rapid deterioration of lubricating oil (Aoki, 2006). Biological desulphurisation process could be used as an environmental friendly solution if problems are encountered during scaling up of the process.

5.3.4. Effect of temperature on pH

During the hydrolysis/acidogenesis phase, significant amounts of VFA are produced, disturbing the pH balance. Accumulation of VFAs causes the drop in the pH, affecting the activity and equilibrium between acetogens and methanogens, and ultimately the collapse of the system (Komemoto, *et al*., 2009). Therefore, the change of the pH values during the AD of *L. digitata* was monitored continuously (Figure 5.3). The initial value was 7.2-7.4 for all reactors. After day 1, pH decreased rapidly to 5.5 and the conversion of substrate to biogas was reduced in reactors incubated at mesophilic and thermophilic temperatures.

This reduction in pH was also detected during the AD of brown algae, where Hanssen, *et al*. (1987) observed a drop in pH to 5.6-5.9 after 1-2 days digestion, with an associated decline in gas production. NaOH was added to the reactor to bring the pH to 7.5 resulting in continued biogas production with more than 50% methane concentration after the 10 day digestion. A lack of buffering capacity is associated with high

hydrolysis rates causing an increase of VFAs, lowering the pH value and consequently reducing the performance of the system (Switzenbaum, *et al*., 1990; Karakashev, *et al*., 2005). After day 1, pH was brought progressively to neutral ranges by the addition of NaOH and 10% $KHCO_3$, boosting the buffer capacity of the system. This observation is corroborated by the low but constant biogas production throughout day 2 to day 9 (Figure 5.1). On day 9, the pH increased to 6.8 and 7.2, indicating process stability and the optimal activity of methanogenic bacteria. Under psychrophilic conditions, the pH did not decrease rapidly, and, after day 2, only $KHCO_3$ was added to boost the buffer capacity of the system. Throughout the first days of digestion at mesophilic and thermophilic temperatures, pH was a key parameter contributing to reactor failure during the AD of *L. digitata*. Therefore, careful monitoring must be carried out in future experiments.

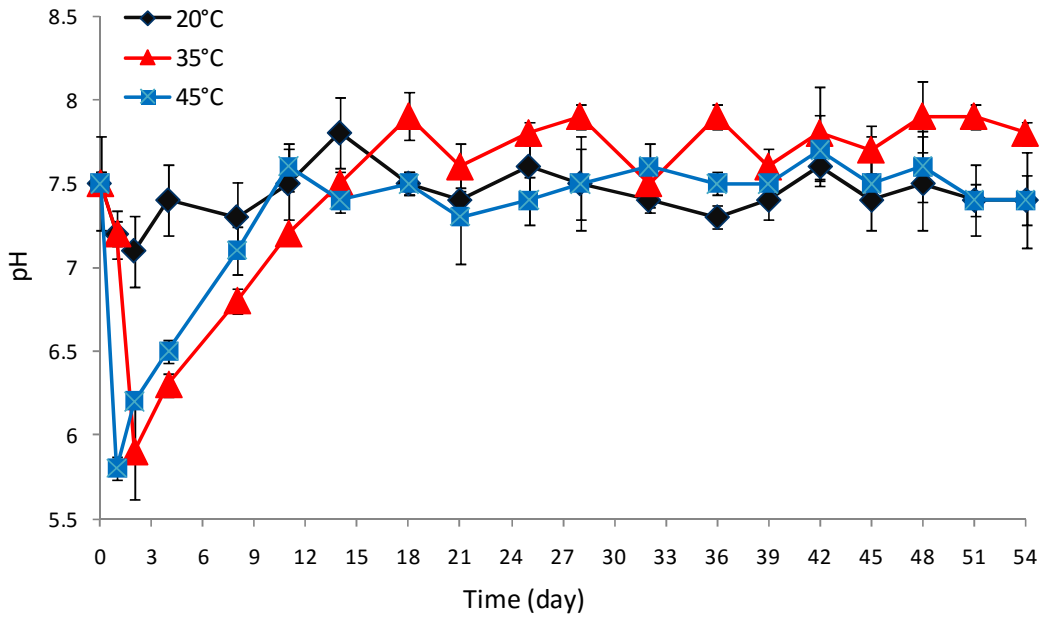

Figure 5.3. Change of pH during batch AD of *L. digitata* at different temperatures.

Further studies should be carried out to examine differences in microbial communities' dynamics within reactors when assessing the response of temperature on biogas production from AD of *L. digitata*. Additionally, the use of mesophilic and thermophilic temperatures to produce VFAs from the AD of seaweed could be an interesting concept to explore within a seaweed-biorefinery model.

It would also be important to take into account the net energy balance between bioreactor heating energy demand and biogas/methane production, as this will also decide the economic viability of the process.

5.4. Conclusions

Thermophilic, mesophilic and psychrophilic temperatures were used during the AD of the seaweed *L. digitata*. The results of the laboratory-scale experiment demonstrated the feasibility of producing biogas and proved that digestion temperature had an influence on cumulative and methane concentration.

Among the 3 temperature ranges, the reactors at a mesophilic temperature were more effective for biogas and methane production than either thermophilic or psychrophilic digesters. Consequently, mesophilic condition (35°C) was selected as the most optimal temperature, thus providing the context to implement it in further Chapters.

The results from this Chapter also demonstrated the effect of temperature on hydrolysis rate of *L. digitata* where higher rates were observed at a thermophilic (45°C) range. Inhibition at this range caused a reduction in biogas and therefore methane yields. Similarly, results from Chapter 4 (Section 4.3.1.1) also established that, the successful start-up and the stability of the reactor were influenced by the temperature.

In future studies, potential obstacles such as increased proportions of H_2S in the biogas composition and pH regulation during the initial hydrolysis step should be evaluated further, if the results are to be extrapolated to large scale.

CHAPTER 6

BIOGAS PRODUCTION FROM ANAEROBIC DIGESTION OF IRISH SEAWEED SPECIES

Vanegas, C.H., and Bartlett, J. (2013). Green energy from marine algae: biogas production and composition from the anaerobic digestion of Irish seaweed species. *Environmental Technology*, 34 (15), pp.2277-2283.

6.1. Introduction

The development of the seaweed-based biorefinery process, such as the one outlined in Chapter 2, Section 2.6, should also include the use of several seaweed species to extract value-added products (reducing sugars, lipids and proteins in Chapter 3) or to transform the biomass into biofuel by the AD process (Chapter 4 and 5). The European seas are particularly suitable to cultivate these seaweed species due to the temperature, high nutrient and light conditions (Bruton, *et al*., 2009; Dave, *et al*., 2013; Jung, *et al*., 2013; Lüning, 1990; Merzouk and Johnson, 2011).

Seaweed species have the potential to be an aquatic energy crop for the production of biofuels, due to their low concentration of cellulose, lack of lignin, and easily biodegradable sugars (60% carbohydrates), such as mannitol, alginate and laminarin (Chynoweth, *et al*., 2001; Horn, *et al*., 2000; Kelly and Dworjanyn, 2008).

Technical feasibility data on AD of algal biomass have been reported for a number of marine species such as *Laminaria* spp*, Sargasum* spp*, Macrocystis* spp*, Gracilaria* spp*, Ulva* spp and, more recently, microalgal strains (Chynoweth, *et al*., 2001; Kelly and Dworjanyn, 2008; Peu, *et al*., 2001; Vivekanand, *et al*., 2012; Zamalloa, *et al*., 2012). These studies have concluded that macroalgae are suitable feedstocks for the AD process and the production of renewable energy, where methane yields could range between 0.15 to 0.41L g/VS. Therefore, it is of great importance to investigate the suitability of other seaweed species to produce biogas.

In this context, the goal of the present Chapter was to compare the potential of five seaweed species, commonly found in Irish and the Northern Atlantic Ocean, to produce

biogas. The cumulative biogas produced was also compared to yields obtained from the AD of terrestrial feedstocks; grass and rice. Their biogas potential was initially estimated in 120 ml batch digesters and the scale-up was evaluated in 1000 ml reactors at a later stage.

6.2. Materials and methods

6.2.1. Substrates

The seaweed species *Laminaria digitata, Saccharina latissima*, *Saccorhiza polyschides*, *Fucus serratus* and *Ulva* sp. were harvested from wild stock in September 2011, during low tide, from a rocky outcrop of Streedagh beach, Co. Sligo, Ireland. The collected seaweeds were rinsed manually with tap water to remove sand and dried at room temperature for 48 hours. Rice and grass substrates were used to compare the AD of terrestrial biomass against that of seaweed. Commercially available rice was used for the test. Grass was harvested at the same month for seaweed harvesting, in September 2011, and dried at room temperature for 96 hours. All feedstock samples were milled to fine particles (<1.0 mm) and stored in airtight capped tubes at -20°C prior to use.

6.2.2. Batch experiments in 120 ml reactors

Equal amounts of each substrate (seaweed, grass and rice), 2.0 g/VS, were transferred individually into separate bottles (120 ml serum bottles) and mixed with 30 ml of distilled water. 4.0 g/VS of bovine slurry was used as bacterial seed. pH was adjusted to 7.2-7.4 in all cases with a 10% solution of NaOH. The headspace in the bottles was flushed with a mix of N_2/CO_2 for 50-60 seconds, sealed with rubber stoppers and capped with aluminium crimps.

For the determination of endogenous biogas and methane production, reactors containing bovine slurry/water and seaweed/water were used and the biogas/methane produced was subtracted. A set of experiments (Table 6.1) were carried out at 20°C and at 35°C for 32 days. Measurements were carried out 2-3 times a week during the first weeks of incubation and approximately once a week afterwards until the end of the experiment. All the experiments were carried out in triplicate and the results are expressed as means.

Experiments	Conditions tested		
	Feedstocks	Temperature	g/VS[a]
1	*L. digitata*	35°C and 20°C	0.73
2	*S. latissima*	35°C and 20°C	0.81
3	*Ulva* sp.	35°C and 20°C	0.54
4	*Fucus serratus*	35°C and 20°C	0.74
5	*Saccorhiza polyschides*	35°C and 20°C	0.59
6	Rice	35°C	0.98
7	Grass	35°C	0.69

[a] Grams per volatile solid (VS)

Table 6.1. Feedstocks used for the 120 ml experiments: 5 seaweed species, rice and grass.

6.2.3. Batch experiments in 1000 ml reactors

The total volume of the bench-top reactor was 1000 ml, with a working volume of 600 ml. A Duran glass bottle with a GL 45 threads screw cap and two connection ports was used. The feed was introduced into the reactor via the inlet feeding port. A second port (outlet) with a GL 14 tube adapter was used for gas collection in a 1.0 L Altef gas bag. Gas was measured by water displacement at room temperature.

In order to re-suspend the sediments and avoid scum layers, the reactors were stirred manually (5-10 seconds) every 3-4 days and incubated at 35°C in a water bath for 109 days. Biogas production from the slurry and the feed alone was also recorded. Experiments were carried out in duplicate and the results expressed as means.

6.2.4. Analytical methods

Total solids, volatile solids, pH and the biogas composition (CH_4, CO_2, O_2, H_2 and H_2S content) were determined as previously described (Chapter 4, section 4.2.6). Ammonia was measured following the 4500-NH_3 Nitrogen standard method.

6.3. Results and discussions

Early studies have demonstrated that the biochemical composition of seaweed species and consequently their biodegradation and conversion rates are affected by the growth cycle, harvested season and location of the seaweed (Adams, *et al*., 2011a; Moen, *et al*.,

1997). Hence, all species were harvested on a single month to avoid inconsistency due to the season.

6.3.1. Biogas production in 120 ml batch reactors

At the start of the experiment, the pH in reactors digesting rice and grass had to be adjusted several times. Over the first days of digestion the pH dropped rapidly to 4.5-5.0, and, as a result, gas production was reduced. By adding 10% $KHCO_3$ and 10% NaOH, pH was increased to 7.5, resulting in continued gas production. For all seaweeds species, low amount of biogas was produced after the end of the experiment (day 32), hence, longer digestion time would be necessary.

It can be seen from Figure 6.1 that the highest biogas values were obtained from the AD of rice with 264 ml biogas/gVS. *S. latissima* produced 244 ml biogas/gVS, followed by *S. polyschides* with 177 ml biogas/gVS, grass with 168 ml biogas/gVS, *L. digitata* with 161 ml biogas/gVS and *Ulva* sp. with 97 ml biogas/gVS. The lowest value among all substrates evaluated was obtained from the digestion of *F. serratus* with 65 ml biogas/gVS. The difference in biogas yields can be explained by the chemical composition of the different feedstocks (Table 2.3) and the bacteria capable to reduce easy fermentable compounds. Other studies have discussed in more detail how these compounds and the harvesting season influence AD and consequently biogas and methane yields (Adams, *et al*., 2011a; Adams, *et al*., 2011b; Bird, *et al*., 1990; Jard, *et al*., 2013).

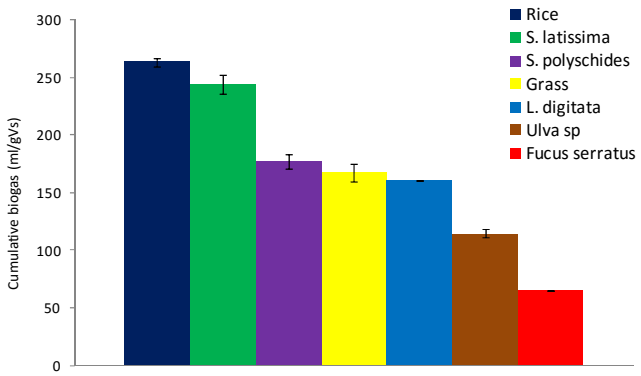

Figure 6.1. Total biogas production from different substrates in 120 ml reactors incubated at 35°C.

To date, there is no information available in the literature on biogas or biofuel production from *F. serratus*. Low biogas values in this species could be the result of inhibitory or recalcitrant compounds, and the inaccessibility of microbial enzymes to the cellular components of the seaweed.

The AD of rice gave the greatest biogas yields among all biomasses. This is attributed to the high content of fermentable sugars and high degree of biodegradability. However the use of this substrate for biofuel production is not considered a sustainable option, as it will compete directly with food, water and land resources.

During the AD of *S. latissima* the total biogas yield obtained was approximately 7.5% lower than the yield obtained from rice. The lack of lignin, low content of cellulose and the biochemical constituents (Table 2.3) make this seaweed species a relative easy substrate for fermentation (Jard, *et al*., 2012; Moen, *et al*., 1997; Nielsen and Heiske, 2011; Østgaard, *et al*., 1993; Vivekanand, *et al*., 2012). This finding corroborates the hypothesis that seaweed is a suitable and alternative biomass for the production of biofuels, such as biogas.

S. polyschides produced more biogas than grass and *L. digitata*, making it a good candidate for future AD trials. There is no evidence currently available in the literature where *S. polyschides* has been used on AD to produce biogas.

L. digitata and grass produced similar biogas yields. While fermentation conditions, digestion temperature and the nature of the inoculum were different, the yield of biogas was lower to the values reported previously (Adams, *et al*., 2011b; Hanssen, *et al*., 1987).

Low biogas yields were obtained from *Ulva* sp. due to the low VS content and a late methanogenic phase when compared to the other biomasses used. A reduced buffering capacity in the system and high hydrolysis, causing abrupt changes in pH values, also affected the production of biogas. As reported by other authors, Briand and Morand, (1997) and Bruhn, *et al*. (2011), there was a low degradation rate during the AD of this seaweed species.

When digesters were incubated at 20°C, as shown in Figure 6.2, the biomass that produced the highest cumulative biogas was *S. latissima* with 198 ml biogas/gVS, followed by *S. polyschides* producing 185 ml biogas/gVS, *L. digitata* with 93 ml biogas/gVS and *Ulva* sp. with 40 ml biogas/gVS. Lowest biogas yield was obtained from the digestion of *F. serratus* with 7 ml biogas/gVS. This low yield was also reported with the reactors incubated at 35°C, corroborating the unsuitability of this seaweed species for biogas production in these experimental conditions.

Total biogas was lower for all seaweed species when compared to biogas yields in digesters operated at 35°C. This finding is in agreement with the results from previous Chapters and studies on AD of different algae where incubation temperature has an effect on biogas production (Zamalloa, *et al*., 2012; Bruhn, *et al*., 2011).

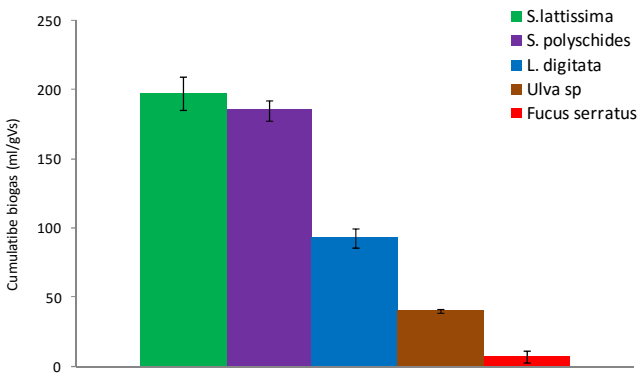

Figure 6.2. Total biogas production from different substrates in 120 ml reactors incubated at 20°C.

6.3.2. Biogas production and composition in 1000 ml batch reactors

To establish the suitability of the collected seaweed species for the scaling up of the AD process, 1000 ml batch reactors were set up. While all reactors were fed with a similar substrate: bovine slurry ratio, the results show that the biogas yield from the 1000 ml reactors was different than the 120 ml reactors after 32 days digestion. Biogas production from *S. latissima*, *S. polyschides*, *L. digitata* and *Ulva* sp. was 21 %, 23%, 11% and 39% higher, respectively, than the 120 ml reactors. This difference is attributed to the periodic mixing applied to the 1000 ml reactors and the reactor configuration that allowed an efficient control of the pH changes.

Figure 6.3 shows the cumulative biogas production from the AD of seaweed species over 109 days. In these batch experiments, *S. latissima* was considered to be the most suitable species for AD, followed by *S. polyschides*, *L. digitata* and finally *Ulva* sp. *F. Serratus* was excluded from these trials due to the low biogas and methane recoveries from the 120 ml batch reactors experiment.

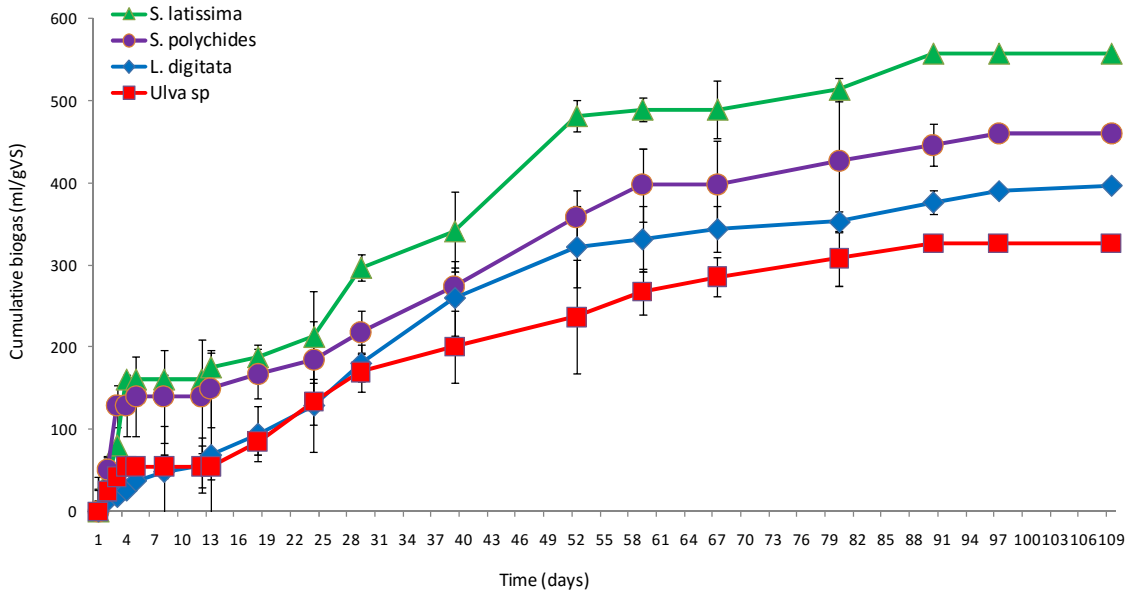

Figure 6.3. Cumulative biogas production of different seaweed species in 1000 ml reactors incubated at 35°C over 109 days digestion.

The mixture of *S. latissima* and bovine slurry showed a high hydrolysis rate when compared to the other seaweed species. 35% of the final biogas yield was produced during the first 3 days, along with a depletion of the pH (4.7-5.2). This pH reduction was also detected during the AD of brown algae with a drop to 5.6-5.9 after 1-2 days digestion (Hanssen, *et al*., 1987). A mixture of 10% of NaOH and $KHCO_3$ was added to the reactors to bring the pH to 7.7-7.9, and to boost the buffer capacity of the system, respectively, resulting in continued biogas production rate after the 12^{th} day. However, the use of these reagents may increase the likelihood of Na^+ / K^+ ion inhibition. A lack of buffering capacity is associated with high hydrolysis rates, causing an increase of VFAs, lowering of the pH value and consequently reducing the performance of the system.

The cumulative biogas curve gives a value of 565 ml biogas/gVS. From day 80 until the end of the experiment, marginal amounts of biogas were measured. The cumulative

methane production reached by the 109 digestion day was 335 ml/gVS (Figure 6.4). The AD of *S. latissima* and the effect of pretreatment and co-digestion on methane yields has been reported elsewhere. For instances, Nielsen and Heiske, (2011), shows that chopped and macerated *S. latissima* produced 340 and 333 ml CH_4 g/VS, respectively, when incubated at 53°C for a period of 34 days. Vivekanand, *et al*. (2012), reported that the AD of *S. latissima* at 37°C over 119 days produced 223 ml methane yield /gVS^{-1} from untreated samples, whereas the seaweed exposed to steam explosion at 130°C for 10 min recorded 268 ml CH_4 g/VS, with a methane content of 57-59%. Østgaard and co-workers (1993), working in a semi-continuous experiment, reported methane yields of 0.22 L CH_4 per g/VS from raw *S. latissima* harvested in spring. In our set of experiments, low methane percentages were detected over the first 3 digestion days, (5-12%) with a simultaneous production of high concentrations of CO_2 (50%-72%). Between day 4 and 8, no methane was registered, due to a reduction in pH levels affecting methanogenics. On day 15, methane percentage reached 60% with a constant increase to 68-72% until day 80, where lower concentrations were measured (50%-35%). The H_2S values registered were above 200 ppm during the 109 days of digestion.

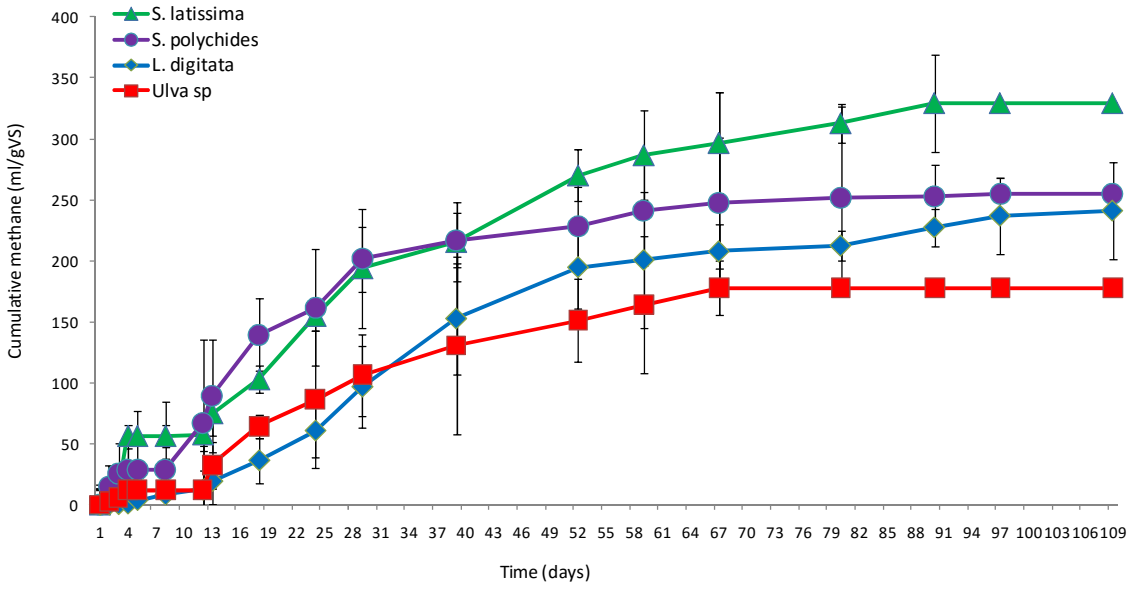

Figure 6.4. Cumulative methane production of different seaweed species in 1000 ml digesters incubated at 35°C over 109 days digestion.

All reactors emitted a very strong H_2S odour and a black sulphide precipitate was observed in the digestate surface. H_2 concentration during AD is an important factor regulating the metabolic activities in both methanogenesis and acetogenesis. When acetate-utilising methanogens are inhibited, bacteria will oxidise acetate to H_2 and CO_2

which is then the source of methane. Thus, there is a syntrophic relationship between acetogens and methanogens, with an interspecies electron flow taking place (Schink, 1997). H_2 concentration during the first 3 days of the acid-forming step was above 1100 ppm, followed by a progressive reduction until day 24 (0-100 ppm). As the methanogenic activity was low at the beginning of the process, due to low pH, acetate and H_2 utilisation by methanogens was hampered, hence the high concentration of H_2. As seaweeds contain a high percentage of protein, prevention of ammonia build up is a concern. The ammonia concentration in the reactors started at 94 ppm and rose as high as 350 ppm. Methane production was not likely reduced during the AD process as ammonia concentrations over 1700 ppm have been reported to inhibit methane fermentation (Chen, *et al*., 2008).

The mixture of *L. polyschides* showed that the maximum value of biogas production was between the 13^{th} and 58^{th} day with a cumulative biogas production of 468 ml/gVS (Figure 6.3). The hydrolysis rate during the first days of digestion, as well as the decrease in pH followed a similar pattern as with *S. latissima*. The high hydrolysis is attributed to the level of biodegradable carbohydrates from both kelp species. Moreover, the large number of microbial species in the bovine slurry facilitates the digestion of the seaweed cell wall (Orpin, *et al*., 1985; Williams, *et al*., 2013). The cumulative methane increased from 19% to 71% at day 24, followed by a slow reduction to about 40% at day 90. In these batch reactors, cumulative methane reached 255 ml/gVS (Figure 6.4). A 0.8-11% concentration of O_2 was found. The H_2S values were above 200 ppm over the digestion time. Despite the concentrations found in this study, no substantial inhibition of methane production was observed. No studies targeting the AD of *L. polyschides* have been reported to date. The average ammonia concentration remained at a lower level (69-110 ppm) until the end of the experiments, hence no bacterial inhibition was observed with the substrate concentration ratio used.

For *L. digitata*, a slow but constant biogas rate was observed during the first weeks of digestion. This is explained by the difference in the biochemical composition of the seaweed when compared to the other kelp species. Furthermore, the slight reduction on pH levels (6.0-6.5) demonstrated a slow adaptation process of the bacteria present in the bovine slurry to the substrate, producing a reduced amount of specific enzymes able to hydrolyse the recalcitrant fraction of this seaweed and therefore, effectively producing biogas. A 10% $KHCO_3$ solution was added to boost the buffer capacity of the system. A

steady biogas rate was seen from day 32, reaching 407 ml biogas/gVS until the end of the experiment. While methane generation was constrained over the first weeks of digestion, due to the reduced metabolic activity of the bacteria consortium implicated in the hydrolysis and methanogenesis steps, the cumulative methane production at the end of the experiment was 246 ml CH_4/gVS. Whilst Adams, *et al*.(2011b), reported 210 cm^3 of methane yield /gVS^{-1} after 36 days of digestion at 35°C, in this study, 152 ml CH_4/gVS was generated at the 39 day. Looking at the ammonia content, low concentrations (110-215 ppm) were measured over the first 30 days of digestion. The highest values were registered between day 56 (450 ppm) and day 97 (910 ppm), followed by a slow decrease to 560 ppm at the end of the experiment. This suggested that, despite the ammonia content, the initial and final concentrations could not inhibit the AD process. The H_2S concentrations in the biogas remained above 200 ppm until the end of the experiment. H_2 measurements were higher (more than 1100 ppm) until day 15, where a progressive reduction was observed until day 112.

The AD test result indicates that *Ulva* sp. yielded the lowest biogas production in relation to *L. polyschides*, *S. latissima* and *L. digitata*. The yield obtained within this experiment was 327 ml biogas/gVS. A slow hydrolysis was seen along the first days of digestion when compared to the other seaweed species. While biogas production was low, methane yields were very similar to the other seaweed species during the first digestion days. The metabolic activity of the bacteria was not a cause of medium acidification and, therefore, reduction of pH (6.0-6.5) was not as evident during the AD of this seaweed species, hence a more steady digestion was present. pH was controlled by the addition of a mixture of 10% of NaOH and $KHCO_3$.

The cumulative methane production of *Ulva* sp. over the 109 days was lower than the other feedstocks. Methane yield reached 191 ml/ gVS after the digestion time and the concentration in the biogas collected was in the range of 54-70%. This result suggests that methane concentration is a function of the algae species evaluated. In a study where co-digestion of cattle manure with dry *Ulva lactuca* showed that methane can be significantly improved by the addition of the seaweed (707 to 1049 ml l^{-1} d^{-1} at 60% manure/20% seaweed) (Nielsen and Heiske, 2011). A more modest increase in the amount of ammonia was observed (from 220 ppm to 390 ppm) during the AD of *Ulva* sp. This concentration was lower than those reported by other researchers (Costa, *et al*.,

2012; Peu, *et al*., 2011), thus no reduction or inhibition on biogas and methane yields could be attributed to these levels.

Further studies should target the effect of high H_2S concentrations on methane production as this parameter will strongly inhibit the process. Harvesting of hydrogen and CO_2 as by-product during the AD of seaweed should be considered as part of an integrated strategy for biofuel production.

6.4. Conclusions

The present study supported the hypothesis that four of the seaweed species used in this Chapter are potential feedstocks to produce biogas. Based on the results, *S. latissima* and *S. polyschides* offered the highest biogas yields, of all feedstocks.

Digestion temperature was found to influence biogas production where reactors operated at 35°C produce higher quantities than at 20°C, corroborating the conclusions from Chapter 5.

Careful monitoring of pH should be carried out during the first days of hydrolysis as medium acidification was a limiting parameter, inhibiting biogas and methane rate.

Ammonia concentrations did not significantly affect the AD process. Biogas production showed that increasing the digester volume size from 120 ml to 1000 ml was a feasible process with a difference in the biogas production per g/VS.

The results presented here will be of valuable assistance providing initial data for the design of a larger scale digester and enabling the valorisation of a seaweed-based biorefinery process.

CHAPTER 7

ANAEROBIC CO-DIGESTION OF SEAWEED WITH CRUDE GLYCEROL AND BOVINE SLURRY

Vanegas, C.H., and Bartlett, J. (2014). Enhanced biogas production from macroalgae by co-digestion with crude glycerol. Bioscience and Bioengineering (Manuscript under review).

Vanegas, C.H., and Bartlett, J. (2014).Co-digestion of two kelp species, *Laminaria digitata* and *Saccharina latissima*, with bovine slurry (Manuscript under preparation).

7.1. Introduction

Biorefineries have been proposed as platforms, similar to a petroleum refinery, to co-produce high value products along with the biofuel from biomass (Cherubini, 2010; Hayes, 2009; Jung, *et al*., 2013; Preisig and Wittgens, 2012; Taylor, 2008). A more efficient biorefinery model will combine their material flows in order to reach a complete utilisation of all biomass components; the residue from one bio-industry becomes an input for other industries, giving rise to a more sustainable, economic and competitive biorefinery (Chapter 2, Section 2.6).

The production of biodiesel generates crude glycerol as an end by-product. 100 kg of biodiesel could produce 10 kg of crude glycerol, with a glycerol content of 38-96% (Yang, *et al*., 2012; Yazdani, *et al*., 2007). However, as biodiesel production capacity is expanding, glycerol remains a relatively low commercial value by-product, representing a cost in the process of biodiesel production. Hence, utilisation of glycerol in another, new process would increase demand and therefore, value, making the biodiesel process more viable.

Although glycerol has been used in the food, medicine and cosmetic industries, crude glycerol from biodiesel production has to undergo a costly refining and purification process to comply with standards, adding an extra cost to the process (Yang, *et al*., 2012; Viana, *et al*., 2012; Yazdani, *et al*., 2007). Therefore, economically and ecologically viable alternatives for this substrate have to be found.

Crude glycerol can be transformed to high value-added products by gasification, oxidation, pyrolysis, reduction and microbial conversion (Da Silva, *et al*., 2009; Leoneti, *et al*., 2012; Varrone, *et al*., 2013; Viana, *et al*., 2012).

A microbial conversion strategy would be an ideal option as chemical catalysis has a number of disadvantages (e.g. high levels of contaminants within the crude glycerol, low product specificity, use of high pressure and/or temperatures) (Da Silva, *et al*., 2009; Leoneti, *et al*., 2012; Yang, *et al*., 2012).

Crude glycerol is a suitable carbon source for the production of biogas by microbial anaerobic digestion (AD) processes and successful studies have demonstrated the advantages of its valorisation as a co-substrate (Fountoulakis, *et al*., 2010; Holm-Nielsen, *et al*., 2008; Robra, *et al*., 2010; Viana, *et al*., 2012). As glycerol lacks fundamental compounds for bacterial biomass formation, digestion with feedstocks having a sufficient nutrient content may represent a promising approach.

Interest has also been increasing in the AD of organic wastes from farm origin (Amon, *et al*., 2006; Astals, *et al*., 2012; Holm-Nielsen, *et al*., 2009; Nuchdang and Phalakornkule, 2012; Robra, *et al*., 2010). The simultaneous digestion of an organic residue together with a main substrate, can aid in generating additional revenue from waste products (Braun and Wellinger, 2003; Mata-Alvarez, *et al*., 2014). In addition, the digested substrate could be used as fertiliser in agriculture fields.

Bovine slurry (BS) is a valuable organic waste widely used in AD. Co-digestion of this product with different substrates has been proved to increase methane yields, reduce the amount of waste and therefore, to be an economically and sustainable waste management system (Cecchi, *et al*., 1996; Costa, *et al*., 2012; Holm-Nielsen, *et al*., 2009).

Furthermore, the use of manures as co-digestion substrate has overcome several problems in AD (Esposito, *et al*., 2012; Mata-Alvarez, *et al*., 2014), such as ammonia inhibition resulting from poor substrate digestion, insufficient nutrients and C/N ratio, stable pH as a consequence of accumulation of VFAs and the reduced buffering capacity. Moreover, co-digestion will provide additional benefits to overcome economic

aspects in the livestock industry (Esposito, *et al*., 2012; Holm-Nielsen, *et al*., 2009; Mata-Alvarez, *et al*., 2014).

In Ireland, total slurry production is estimated as 34.89 Mt per annum and is dominated by cattle slurry (Smyth, *et al*., 2009; Smyth, *et al*., 2011). Hence the potential to use this waste product as co-digested for biogas is significant.

Few studies have targeted the co-digestion of algae biomass with different waste products (Cecchi, *et al*., 1996; Costa, *et al*., 2012; Peu, *et al*., 2011). In a recent study, Nielsen and Heiske, (2011) illustrated that co-digestion of cattle manure with *Ulva lactuca* can significantly improve the performance of the AD process, with a 48% increase in methane production rate.

Despite the number of studies on anaerobic co-digestion of glycerol and BS, there has not been a report to date on the use of these by-products as a co-substrate source in the AD of marine macroalgae.

In Chapter 6, the diversification of the seaweed-based biorefinery process was discussed with the production of biogas from different seaweed species. In this Chapter, the process is extended by establishing a novel integrated system that combines the AD of *L. digitata* and *S. latissima* with crude glycerol or bovine slurry to enhance biogas production during the AD, potentially enhancing the economic viability of the seaweed biorefinery.

7.2 Materials and methods

7.2.1. Substrates preparation

Samples of *L. digitata* and *S. latissima* were harvested and processed as described in Chapter 3, section 3.2.1, while the BS samples were processed as described in Chapter 4, section 4.2.1.

The material used as co-substrate was the crude glycerol, obtained after a laboratory scale manufacturing process for biodiesel. The transesterification process was carried out with sunflower oil-waste (0.9 L) at 55-60°C. After 1 hr, sodium hydroxide (3.0 g),

as catalyst, and methanol (150 ml) were added and mixed continuously. The mixture was transferred to a separation funnel and allowed to settle by gravity overnight. The bottom fraction was collected and used as the crude glycerol (94% VS), without any purification before use.

7.2.2. AD of seaweed and crude glycerol in 120 ml batch reactors

The inoculum (31% VS) was obtained from an active methane producing lab-scale anaerobic digester treating seaweed and bovine slurry at a temperature of 35°C (Chapter 4, section 4.2.1).

Biogas produced from the inoculum was measured in parallel reactors (without the substrates) and subtracted from the data. The test materials, *L. digitata* (74% VS) and *S. latissima* (81% VS), were weighted individually to approximately 2.0 g/VS and transferred into 120 ml serum bottles (reactors). The bottles were fed with 4.0 g/VS of inoculum, 30 ml dH_2O and the corresponding concentration of crude glycerol as per Table 7.1. The headspace in the bottles was flushed with a mix of N_2/CO_2 for 1 minute, sealed with rubber stoppers and capped with aluminium crimps. Experiments were carried out in triplicate and the biogas produced was measured as previously described (Chapter 4, section 4.2.5.2). Digestion experiments were carried out at 35°C for 32 days.

Treatment 7 corresponds to a mix of crude glycerol and inoculum without the addition of the seaweed. Treatment 8 (control) is a reactor with seaweed and inoculum without crude glycerol addition.

7.2.3. AD of seaweed and crude glycerol in 1.0 L batch reactors

Co-digestion of seaweed with the most productive glycerol loading from previous experiment (Treatment 2) was selected for scaling up in 1.0 L batch reactors. The reactors set-up is described in Chapter 6, section 6.2.3. 20 g/VS of *L. digitata* and *S. latissima* were loaded individually, mixed with 60 g/VS of inoculum and 2 ml/VS of glycerol. 400 ml of dH_2O was added to the mix for a final working volume of 600 ml.

Control reactors were incubated in parallel without the addition of the crude glycerol. In addition, the AD of the crude glycerol was also monitored in order to determine synergist effects. The vessels were incubated at 35°C for 109 days in duplicate and the results were expressed as means. The biogas produced was collected and measured as in Chapter 6, section 6.2.3.

7.2.4. Co-digestion of bovine slurry and seaweed in 120 ml batch reactors

In this set of experiments different concentrations of BS (Figure 7.3) were mixed with each individual seaweed (2.0 g/VS) and 30 ml of H_2O to find the most optimal substrate:BS ratio for biogas production. No inoculum was added and the hydrolysis depended on the natural consortium of microorganisms of the BS. The headspace in the bottles was flushed with a mix of N_2/CO_2 for 1 minute, sealed with rubber stoppers and capped with aluminium crimps. Digestion experiments were carried out at 20°C and at 35°C for 32 days and the biogas measurement were performed as previously described in Chapter 4, section 4.2.6.

Treatment 6 (Figure 7.3) was designed as control reactor, inoculated with an inoculum adapted to the chemical composition of the seaweed (Chapter 4, section 4.2.1), water and the corresponding seaweed (2.0 g/VS). Reactors containing only the BS/water and seaweed/water were incubated in parallel, and the biogas generated was subtracted from the total biogas produced in the assay bottles. All sets of experiments were carried out in triplicate and the results are expressed as means.

7.2.5. Analytical methods

Total solids, volatile solids pH, ammonia and the biogas composition (CH_4, CO_2, O_2, H_2 and H_2S content) were determined as in Chapter 4, section 4.2.5. The elemental content of the seaweed species was analysed in triplicate using a CHNOS elemental analyser Vario El Cuber. 5 mg of sulfanilamide was used as standard.

7.2.6. Statistical analysis

The statistical significance of differences between the addition of the different concentrations of the crude glycerol on biogas from both seaweed species was assessed

by analysis of variance (ANOVA: two-factor without replication). Differences of $p<0.05$ were considered significantly different. Values are expressed as mean ± standard deviation.

7.3. Results and discussion

7.3.1. Influence of crude glycerol addition on AD of the seaweed species (120 ml reactor)

Higher biogas yields were obtained when reactors were loaded with lower concentrations of crude glycerol (Table 7.1). Digesters containing 0.1 ml of crude glycerol, 0.31% VS (v/v), showed the highest biogas recoveries, producing 361 and 538 ml biogas/gVS for *L. digitata* and *S. latissima*, respectively, compared to 211 and 286 ml biogas/gVS in reactors without the addition of glycerol (Treatment 8). A small biogas yield (62 ± 0.97 ml biogas/gVS) was observed from digesters with only the addition of glycerol as the sole substrate (Treatment 7). This was possible because the nutrients for microbial metabolism and biomass formation were provided by the inoculum itself. Furthermore, the crude glycerol represented the most readily available carbon source for the microbial consortium present in the reactors.

Treatment	Condition tested		*L. digitata*		*S. latissima*	
	Crude glycerol		Biogas	C:N ratio	Biogas	C:N ratio
	ml	%VS [a]	ml g/VS [b]		ml g/VS [b]	
1	0.1	0.31	361 ± 0.32	26:1	538 ± 0.75	30:1
2	0.2	0.62	355 ± 0.19	28:1	532 ± 0.43	31:1
3	0.5	1.56	207 ± 1.63	29:1	276 ± 1.15	35:1
4	1.0	3.13	148 ± 7.14	34:1	207 ± 3.07	38:1
5	2.0	6.26	146 ± 2.75	36:1	201 ± 4.12	42:1
6	3.0	9.39	130 ± 2.56	49:1	186 ± 3.12	53:1
7 (glycerol only)	1.0	3.13	62 ± 0.97	64:1	62 ± 0.97	64:1
8 (control)	0.0	0.0	211 ± 0.58	25:1	286 ± 1.04	27:1

[a] (v/v)
[b] Mean of replicate tests ± standard deviation

Table 7.1. Cumulative biogas produced from the AD of *L. digitata* and *S. latissima* with varying concentrations of crude glycerol (ml) in 120 ml batch reactors. Reactors were incubated at 35°C for 32 days.

A decrease of biogas from the digesters supplemented with concentrations above 0.2 ml of crude glycerol, 0.62% VS (v/v), was observed. Digestion failure may have been caused by inhibitory compounds from the crude glycerol such as the high pH and Na^+ content, originating from the catalyst used during the production of the biodiesel (Viana, *et al*., 2012) or the organic overloading. Lower glycerol concentrations (Treatment 1 and 2) led to the dilution of these compounds, reducing them under their toxic thresholds, with no effect in the hydrolysis.

Another possible explanation is the high C:N ratio found in the treatments. The results in Table 7.1 show a correlation between the C:N ratio and the reduction in cumulative biogas. The increase in the carbon content of the reactors (Treatments 3 to 6) intensified the metabolic activity of the microbial consortium causing the build-up of volatile fatty acids (VFA) followed by the decline on the pH value in the slurry (from 7.4 to 6.6) and therefore, the reduction of biogas. This inhibition was also observed by Holm-Nielsen, *et al*. (2008), who found that low concentrations (less than 5-7 g L^{-1}) of glycerol showed no sign of disruption during the AD of manure and organic food industrial waste. Fountoulakis, *et al*. (2010), also reported that co-digestion of sewage sludge with glycerol can boost biogas yields only if a limiting 1% (v/v) concentration in the feed is not exceeded. In another study, the addition of glycerine to the AD of pig manure and maize silage resulted in a significant increase in methane (Amon, *et al*., 2006). Although a 3-6% glycerine was used, the feedstock had a lower C:N ratio than that used in our experiments, which may explain a higher tolerance. In addition, this could also be explained by a lack of toxic compounds from the crude glycerol (e.g. methanol).

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	263938	7	37705.43	19.61781	0.000425	3.787044
Columns	27889	1	27889	14.51041	0.006634	5.591448
Error	13454	7	1922			
Total	305281	15				

Table 7.2. ANOVA for crude glycerol addition on AD.

The significance level employed in this analysis was 0.05. Table 7.2 present variance analyses for *L. digitata* and *S. latissima*. It shows that the value of F (19.61 and 14.51) is greater in comparison to the value of F-critical (3.78 and 5.59) and also, the P-values

are smaller than 0.05. Therefore, considering the two above premises, it can be concluded that the effect of glycerol addition is significant. It can be similarly concluded that the impact on both seaweed species is also significant.

7.3.2. Influence of crude glycerol addition on biogas production (1.0 L reactors)

The crude glycerol loading from Treatment 2 (Table 7.1) was scaled up in to 1.0 L reactors over a period of 109 days. While a higher biogas yield was reached with Treatment 1, the threshold for inhibition was established at loadings below 0.62% VS (v/v). Higher biogas yield (480 ml/gVS) (Figure 7.1) was observed during the hydrolysis phase of the mixture of *S. latissima*-crude glycerol (SL+Gly). Maximum biogas yield was reached at day 71 with 813 ml biogas/gVS for the SL+Gly reactors, where no biogas was detected subsequently (to day 109). The highest biogas yield obtained for the *S. latissima* (SL) control reactors was 548 ml biogas/gVS at day 109. For the *L. digitata* (LD) control reactors, 381 ml of biogas/gVS where produced, whereas the *L. digitata*-crude glycerol (LD+Gly) reactors produced 552 ml of biogas/gVS (44% increase).

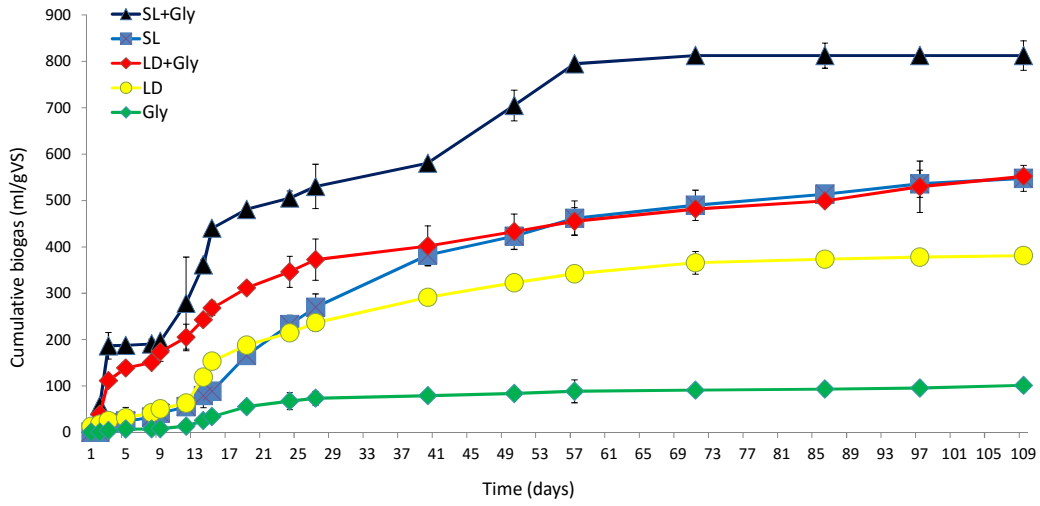

Figure 7.1. Cumulative biogas production from 1.0 L digesters co-digesting crude glycerol with seaweed. Gly: crude glycerol, SL: *S. latissima*, SL+Gly: *S. latissima* and crude glycerol, LD: *L. digitata*, LD+Gly: *L. digitata* and crude glycerol.

The addition of crude glycerol represented an important supply of readily available organic carbon that stimulated the production of specific enzymes, leading to an

increase in the metabolic rate of microorganisms and therefore, the digestion of the blend.

This was also found in a study carried out by Yen and Brune, (2007), where a significant increase of methane production (1170 ml/L day vs. 570 ml/L day) was observed when algal sludge was co-digested with paper waste. The specific nature of waste paper helped to increase the cellulase activity. In this study, the higher biogas production yield using glycerol waste from the biodiesel production process led to an improvement in the energy balance of the biodiesel process, improving its viability.

7.3.3. Influence of crude glycerol addition on methane and biogas composition (1.0 L reactors)

The results obtained from the methane monitoring are presented in Figure 7.2 Methane production in the SL+Gly reactor started at a later point (8 day) than the others, followed by a constant increase between day 9 and 50. Methane production from the LD+Gly reactor followed a different pattern than the SL+Gly mixture. Methane was measured at day two, from where it continued at a constant rate and lasted longer than the SL+Gly reactors.

This variability in methane production profile is due to optimal process stability within the first days of digestion in the LD+Gly reactor when compared to the SL+Gly reactor. High hydrolysis rates were observed during the first days of digestion (Figure 7.1) in the SL+Gly reactor, reducing the pH (from 7.3 to 6.4) and therefore, delaying the methanogenesis phase.

In Figure 7.2, the digestion of the SL+Gly mixture achieved 42% more methane overall (406 ml g/VS) than the SL alone (286 ml g/VS). The LD+Gly mixture produced 26% more methane (294 ml g/VS) than the LD reactor (232 ml g/VS). Mixing both substrates clearly increased the methane yield when compared to reactors incubated with the seaweed alone. However, the synergist effect was more evident in the reactors with SL+Gly than LD+Gly.

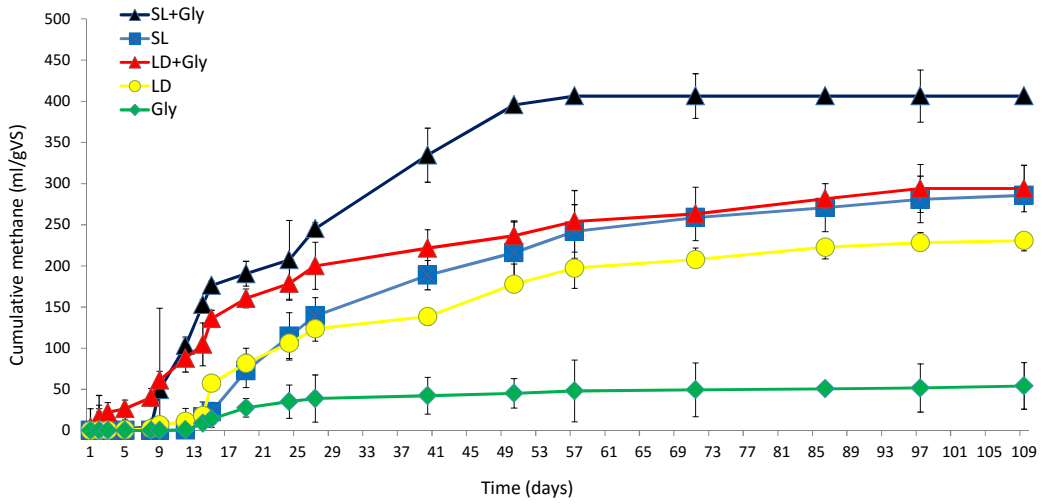

Figure 7.2. Cumulative methane production from 1.0 L digesters co-digesting crude glycerol with seaweed. Gly: crude glycerol, SL: *S. latissima*, SL+Gly: *S. latissima* and crude glycerol, LD: *L. digitata*, LD+Gly: *L. digitata* and crude glycerol.

In this study, the yields obtained from the AD of *S. latissima*-crude glycerol mix were higher than those reported by Jard, *et al*. (2012) (209 ml g/VS), Vivekanand *et al*. (2012) (223-268 ml g/VS), Nielsen and Heiske, (2011) (340 ml g/VS) and Østgaard *et al*. (1993) (220 ml g/VS). In the case of *L. digitata* crude glycerol mix, the yields were comparable to the study carried out by Chynoweth, *et al*. (1993) (260-280 ml g/VS) and higher than those reported by Adams, *et al*. (2011b) (219 ml g/VS) and Alvarado-Morales, *et al*. (2013) (200 ml g/VS).

Based on the same amount of algae biomass (g/VS), more bioenergy was generated with the addition of the crude glycerol, as was found in this investigation. The conversion of the seaweed constituents not only supplied nitrogen in the process, but also the different micronutrients necessary for optimal microbial metabolism.

The highest methane concentration measured in the SL+Gly reactors was at day 27 with 75%, 11% CO_2 and 430 ppm of H_2. The average methane content over the 109 days was in the range of 61-65%, where the lowest percentages were registered throughout the first days of digestion (2.5% - 55%). For the LD+Gly reactor, the highest methane concentration was produced at an earlier stage. At day 15, the measured methane was 72%, 17% CO_2 and 290 ppm H_2, followed by a constant reduction until the end of the

experiment. The average methane content over the 109 days was in the range of 58-62% and the lowest methane concentrations were achieved between day 12 and 38.

Looking at the H_2 profile, higher concentrations were observed at the beginning of the process, with more than 1100 ppm, followed by a constant reduction until day 49-57 where no H_2 was detected in all four reactors.

Generation of H_2S was constant throughout the process and concentrations as high as 200 ppm was observed during the first 30 days of digestion.

Ammonia was also measured in all reactors since lower C:N ratios will release higher concentrations, which eventually will accumulate in the digester, decreasing the methanogens activity (Chen, *et al*., 2008; Matsui, *et al*., 2010) . Throughout the first days of digestion, ammonia concentration remained at a low level (120-170 ppm), followed by a constant increase to 450-490 ppm until the end of the experiment. The level of ammonia may not have been high enough to prevent methane fermentation.

The pH was stable during the methanogenic phase (7.5-7.8) and the final pH was similar for all digested fractions. The high hydrolysis rate accompanied by the low pH values during the first days of digestion must be monitored closely in future experiments.
Further studies should also aim to investigate the feasibility of scaling up the process to pilot plant scale.

7.3.4. Influence of bovine slurry on AD of *L. digitata* and *S. latissima*

Different concentrations of BS were used to find the most optimal ratio for biogas production. The digesters were run for 32 days and results were compared to biogas production from an inoculum (Treatment 6, Figure 7.3) which was already adapted to the chemical composition of the seaweed and generating constant biogas (Chapter 4, section 4.2.1).

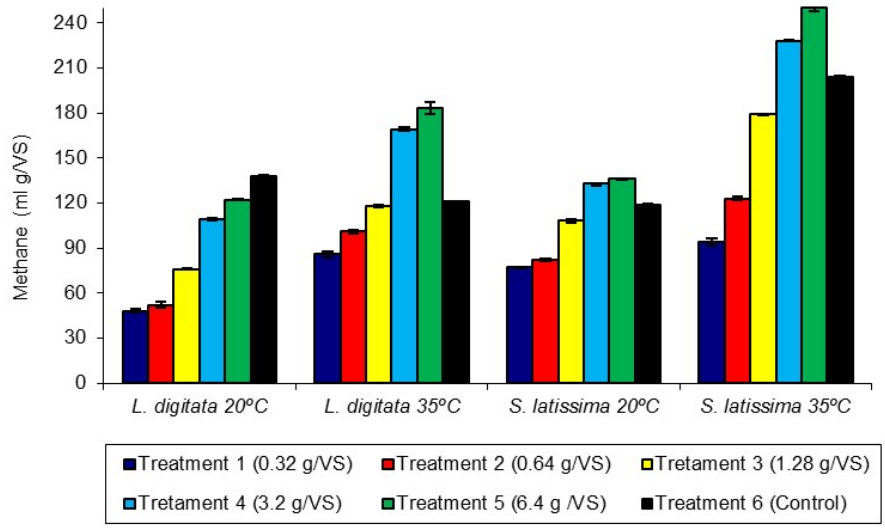

Figure 7.3. Total biogas produced from co-digestion of *L. digitata* and *S. latissima* with bovine slurry at different concentrations in 120 ml reactors incubated at 20°C and 35°C.

In treatment 1, 2 and 3 (0.32, 0.64, 1.28 g/VS of BS, respectively), a reduction in methane was observed (Figure 7.3). This was attributed to a low seaweed:BS ratio that triggered the accumulation of volatile fatty acids (VFA), lowering the buffer capacity of the reactor (from pH 7.3 to 6.1) and therefore, reduction of the metabolic activity of methanogens. This has been described previously by Migliore, *et al*. (2012), who found that the accumulation of total VFAs during the AD of marine macroalgae led to a strong decrease of the pH and a definitive inhibition of methanogenic activities.

Although the build-up of VFAs during the AD of these seaweed species was detrimental to reactor performance, VFAs (acetic, propionic and butyric acids) could be also used as value-added products or converted to alcohol fuels (Pham, *et al*., 2012; Pham, *et al*., 2013). Alternatively, in a two-phase AD system, the mild acid condition produced during the first phase would act as catalyst for a more efficient biomass hydrolysis. The effect of reactor configuration during the AD of seaweed will be assessed in Chapter 8.

In treatment 4 and 5, where the seaweed:BS ratios were higher, the methane yield was enhanced. The higher ratios contributed to the buffer capacity and a smaller effect on pH value. Moreover, it is seen from Figure 7.3 that the total methane produced was considerable higher than the adapted inoculum. Consequently, in this condition, there is no actual benefit in using a more adapted/active inoculum to the seaweed biomass. The BS harboured a potential consortium of microorganisms, capable of metabolising the

seaweed components and thus improving the efficiency of the AD process. This agrees with the findings of Costa, *et al*. (2012), Williams, *et al*. (2012) and Migliore, *et al*. (2012) who found that, the microbial seed had an adequate source of natural bacteria for the efficient AD of the main substrate in renewable biofuel production.

Similar experimental conditions were applied at 20°C. Methane yield from *L. digitata* followed the same trend as *S. latissima* (Figure 7.3). In treatments, 4 and 5, higher yields were observed when compared to an adapted inoculum. It is evident that the addition of high concentrations of BS (3.2 and 6.4 g/VS), recorded maximum methane production yields than reactors with an adapted inoculum, suggesting the possibility to use high substrate concentration on future AD experiments.

Results show that for the same amount of seaweed (2.0 g/VS) added to the reactors, 18 to 33% more methane can be obtained by co-digestion with BS when compared to the single-digestion (seaweed/inoculum). As a result more bioenergy would be generated when the co-digestion method is integrated within the seaweed-based biorefinery process.

An explanation for the higher yields obtained is attributed to the synergistic effect from the BS. Not only the BS supplied the adequate microorganisms to metabolise the seaweed biomass, but also it supplied additional nutrients to the digester microbes. Moreover, the synergistic effect from the BS greatly enhanced the buffering capacity of the reactor helping to maintain the stability of the AD system and the efficient digestion (Cecchi, *et al*., 1996).
This synergic effect was also reported during the anaerobic co-digestion of Taihu blue algae (Zhong, *et al*., 2012). In their study, corn straw was used as an external source of carbon enhancing the biogas yield from the algae sludge when compared to the mono-digestion system. Similarly, Costa, *et al*. (2012) also found that the co-digestion of macroalgae with waste activated sludge (WAS) was a promising choice, with a synergetic effect, increasing the methane yield by 26% when compared to the WAS digestion alone.

Co-digestion of seaweed and BS could efficiently balance feedstock carbon and nitrogen. Although an optimum C:N range for AD differs and depends on the biomass used, a 20:1 to 30:1 range is the most widely acceptable range (Li, *et al*., 2011). The

fact that *L. digitata* had a C:N of 26:1, *S. latissima* 27-29:1 and BS of 14-15:1, could be the reason why no methane inhibition due to a C:N imbalance was observed. Consequently, an optimal C:N ratio was beneficial to methanogen activity, resulting in a reduction of VFA levels due to higher methane conversion. Results from Vivekanand, *et al*. (2012) also shown that blending wheat straw with *S. latissima* enhanced the C:N ratio and therefore, biogas production.

To date, no published data is available on biogas production from the co-digestion of BS with these two seaweed species at different ratios.

7.4. Conclusions

The results showed that co-digestion of both substrates with the seaweed were a feasible process improving the overall AD.

Biogas and methane production from the AD of *L. digitata* and *S. latissima* was enhanced by addition of the crude glycerol. Supplementing the 120 ml reactors with concentrations below 0.2 ml (0.62% v/v) of crude glycerol increased biogas production.

Experiments in 1.0 L digesters produced 44% and 48% more biogas, respectively, than reactors with the seaweed alone. Methane yield was improved by 26% and 42% with the addition of glycerol. Reuse of a biodiesel by-product in production of renewable energy from AD extends the biorefinery model, improving its overall viability and sustainability.

BS, a waste product from intensive farming in Ireland enhanced methane production from seaweed. Co-digestion of both seaweed species with BS at higher ratios (over 1:2) were optimal for biogas production at mesophilic conditions. The bacterial consortium and buffer capacity of the slurry promote the efficient AD of the seaweed when compared to reactors incubated with the inoculum.

The results outlined in this Chapter offers a feasible and more effective process to obtain renewable energy from waste streams, stimulating the exploration of the value of very diverse ranges of feedstocks and the integration in a seaweed-based biorefinery.

CHAPTER 8

LABORATORY SCALE STUDY ON THE ANAEROBIC DIGESTION OF *Laminaria digitata* WITH DIFFERENT DIGESTER CONFIGURATIONS

Vanegas, C.H., Bartlett, J. (2014) Laboratory scale study on the anaerobic digestion of *Laminaria digitata* in different digesters configuration. (Manuscript under review)

8.1. Introduction

The anaerobic digester configuration is an important operating parameter that needs to be optimised for each specific feedstock (Chapter 2, section 2.5.3). In previous Chapters, the AD of seaweed was carried out in the traditional single-phase AD system, where hydrolysis, acidogenesis, acetogenesis and methanogenesis phase, all take place in the same reactor. During the hydrolyse phase of AD, complex macromolecules are hydrolysed into smaller units by a diverse group of enzymes produced from the microbial community present in the inoculum (Chapter 2, section 2.3.1). This phase, which is often the fastest step in AD, gives a high-energy yield for the microorganisms (Gerardi, 2003; Klass, 1998; Yu, *et al*., 2002), frequently created by an imbalance between the substrate and the inoculum. As a result, this may cause VFAs to accumulate, affecting the stability of the process and therefore, reduction of biogas yields (Chen, *et al*., 2008). Consequently, pH regulation is needed for a balanced biogas production level.

To avoid such problems, various anaerobic reactor design configurations have been developed in order to prevent the imbalance between the process of acidogenesis and methanogenesis (Goblos, *et al*., 2008; Sarada and Joseph, 1996).

In the two-phase system, this imbalance is eliminated by physically isolating the acidogenic and methanogenic microbial groups in two different reactors. Hydrolysis takes place in a fermentation reactor, whereas the methanogenesis is carried out by an active consortium of methane-producing in a separate reactor (Klass, 1998). Because the growth and the nutrient requirements of the acidogenic and methanogenic consortiums are different; the two-phase system can be operated simultaneously to

provide optimal conditions for the microorganisms in each phase for greater efficiency in digestion (Demirel and *Yenigün*, 2002).

Numerous studies have proposed the different advantages of a two-phase AD system in comparison to the conventional single-phase during the conversion of different substrates to biogas (Chynoweth, *et al*., 1987; Costello, *et al*., 1991; Demirel and *Yenigün*, 2002; Goblos, *et al*., 2008; Jung, *et al*., 2012; Nasr, *et al*., 2012; Sarada and Joseph, 1996; Vergara-Fernández, *et al*., 2008; Yu, *et al*., 2002). However, factors such as the complexity of the system and the cost of building and operating a commercial full scale AD system could add an extra expense for its commercialisation (Costello, *et al*., 1991; Demirel and *Yenigün*, 2002). Moreover, the theoretical higher biogas yields have also been questioned, since the acidogenic phase separation prevents the hydrogen to methane pathway (Conrad, 1999). Nevertheless, the potential of the system to enhance performance has encouraged research, and commercial plants have been successful as well (Costello, *et al*., 1991).

Mixing is another essential parameter to consider in the configuration of an anaerobic digestor. Continuous mixing helps to develop a consistent blend by preventing stratification and formation of floating layers of solids, ensures an optimal retention time, enables heat and gas transfer, particle size reduction from active bacteria and a progressive release of gas from the mixture (Kaparaju, *et al*., 2008; Karim, *et al*., 2005a; b; Kowalczyk, *et al*., 2013; Lindmark, *et al*., 2014).

An intermediate level of mixing intensity and duration appears to be optimal for each substrate conversion, whereas inadequate mixing conditions could result in digester collapse (Kowalczyk, *et al*., 2013; Lindmark, *et al*., 2014). Despite the implications of mixing for higher biogas yields and substrate degradation, there are no studies targeting the effects of mixing on AD of seaweed, neither are there data in the literature on the most optimal digester configuration using seaweed as a feedstock. Consequently, in order to achieve a successful start-up and stability of the seaweed AD process there is a need for additional research in this area.

The purpose of this Chapter, therefore, was to compare the performance of single-phase and two-phase AD batch reactors for the production of biogas from *L. digitata*. In

addition, the effect of mixing on the performance of a two-phase AD reactor was also evaluated.

8.2. Materials and methods

For all experiments, bench-top reactors (1.0 L) were assembled and the biogas produced was collected and measured as previously described (Chapter 6, section 6.2.3). The chemical characteristic of the substrate and inoculum are described in Chapter 5, section 5.2.2. Experiments were carried out in duplicate and the results expressed as means.

8.2.1. Experimental set-up in the one-phase AD system

20 g/VS of *L. digitata* were mixed with 400 ml of dH_2O and 60 g/VS of inoculum (Chapter 7, section 7.2.4). The reactors were operated at mostly inactive conditions and only stirred manually for 2-3 seconds prior to gas analysis. In this system the acid-forming and the methane-forming microorganisms were kept together in the same single reactor (Figure 8.1).

8.2.2. Experimental set-up in the two-phase AD system

During the hydrolysis phase, the reactors were loaded with 20 g/VS of *L. digitata*, 10 g/VS of inoculum and 400 ml of dH_2O for a 2:1 ratio. The starting pH of the mix was 7.3 and no further pH adjustment was carried out. The rationale behind this approach was that a high substrate:inoculum ratio will allow a fast hydrolysis rate of the seaweed, producing more soluble substrates, higher levels of VFAs, and therefore promoting the acidogenesis phase and inhibition of methanogens (Demirel and *Yenigün*, 2002).

Hydrolysis conditions were maintained throughout the initials days of the experiment until no biogas production was observed. The methanisation stage was started in the same reactor. The pH of the pre-acidification reaction was adjusted to pH 7.7 ± 0.2 by addition of a 10% $NaOH/KHCO_3$ mix to ensure a neutral medium for methanogenics present in the inoculum to develop in the reactor. Afterwards, the methane production stage and in order to ensure a high concentration of methanogens, the reactors were fed with 40 g/VS of inoculum and flushed with a mix of N_2/CO_2 for 4-5 minutes. Digesters

were operated at mostly inactive conditions and only mixed for 2-3 seconds prior to gas analysis (Figure 8.1).

8.2.3. Experimental set-up in the stirred reactor system

In this experiment, the reactors were started and operated following the same procedure as for the two-phase system. The mixing scheme was accomplished by constant magnetic stirring at 10 rpm to provide a continuous mild stirring until the end of the experiment. The rationale behind this approach was to apply constant stirring during the hydrolysis and the methanisation stage enhancing biomass conversation and consequently biogas yields.

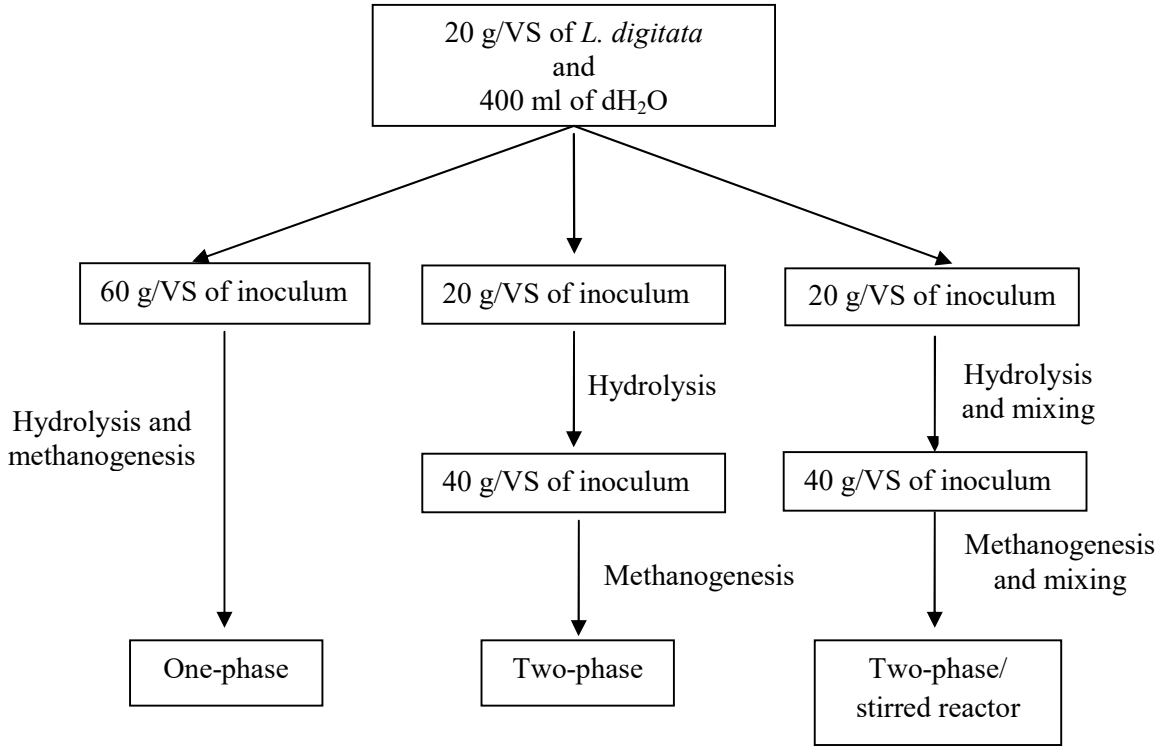

Figure 8.1. Description of the AD processes and stages to each system.

8.2.4. Analytical methods

Cumulative biogas content (CH_4, CO_2, O_2, H_2 and H_2S), TS, VS and pH were determined as previously described (Chapter 4, section 4.2.5).

8.3. Results and discussion

The results from previous Chapters showed that the hydrolysis rate proceeded at a much faster rate than the rate of conversion to methane, thus causing a drop in pH and, consequently, the reduction and further inhibition of methanogenesis.

Thus, in this Chapter, laboratory scale anaerobic digesters (1.0 L) were operated to evaluate the effect of a single-phase, two-phase and stirred AD system on biogas production from *L. digitata*. In Figure 8.1 and 8.2, the performance characteristics of the digesters over 109 days are shown.

8.3.1. Effect of digester configuration on cumulative biogas production

Total cumulative biogas production varied in the three systems (Figure 8.1). The single-phase and two-phase AD systems generated 409 and 462 ml biogas g/VS, respectively. The cumulative biogas in the stirring system was higher among all treatments, generating 516 ml biogas g/VS after 109 days incubation. Mixing regime is regarded to be an important parameter during AD and its effects on biogas production have been discussed extensively elsewhere (Karim, *et al*., 2005a; b; Kowalczyk, *et al*., 2013; Lindmark, *et al*., 2014; Pandey, *et al*., 2011). However, no information is available on the efficiency of stirred and unstirred systems during the start-up of AD of *L. digitata*.

Through the first 5 days digestion, the lowest concentrations were obtained in the single-phase system accounting for 22 ml g/VS. In contrast, biogas production in the two-phase reactor was fairly high (82 ml g/VS) when compared to the stirred system (33 ml g/VS).

The last two AD systems were designed to provide faster hydrolysis rates, leading to a rapid biodegradation of the seaweed biomass and therefore higher concentration of VFAs in the slurry. After the biomass is hydrolysed and fermented, a methanogenic inoculum is added reducing the possibility of methane inhibition by pH changes. From day 6 to 29 differences in the biogas concentration were clearly distinguished in the three reactors (Figure 8.1). While the single-phase system followed a constant but slow biogas production, the other two systems experienced a reduction in biogas. At day 16,

cumulative biogas reached 80, 92 and 106 ml g/VS for the single, two-phase and the stirred reactors, respectively.

These results suggest that the digester configuration was a rate-limiting step that caused detrimental conditions in the medium affecting the development of hydrolytic and methanogenic consortia. Moreover, reactor acidification due to VFA accumulation led to a reduction in pH as in the case of the stirred and the two-phase system.

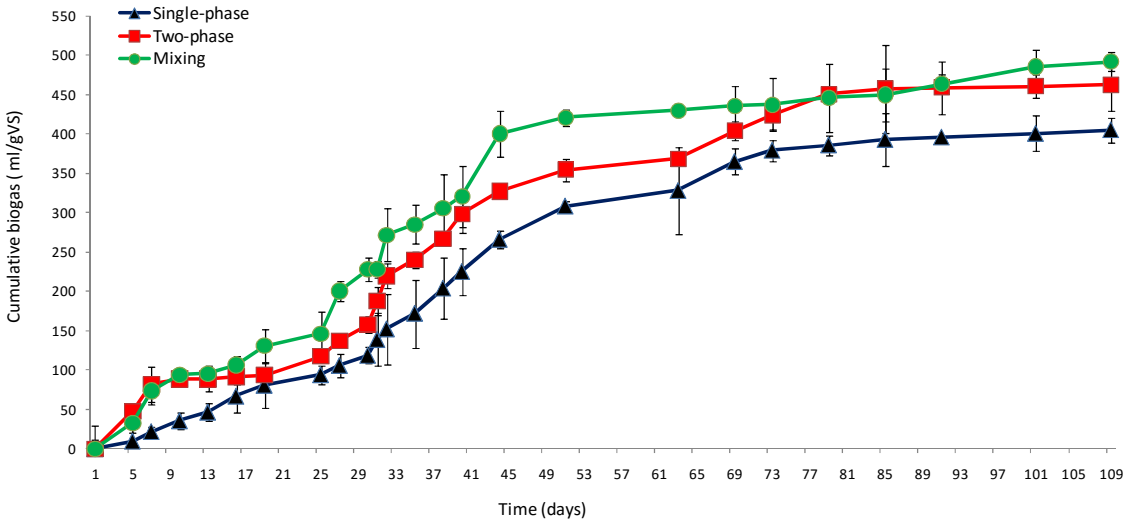

Figure 8.1. Cumulative biogas production from the AD of *L. digitata* in a single-phase, two-phase and stirred system.

The role of pH in the stability of the process was a key parameter for biogas reduction in these experiments as well as in previous Chapters. During the start-up of the two-phase and stirred system, the pH values declined quickly to as low as 4.9-5.6 leading to a reduction in biogas concentrations. Although a small drop in pH (from 7.3 to 6.5-6.8) in the single-phase system was observed, biogas production was not drastically reduced and gradually increased afterwards where it remained within the optimum range (pH 7.1–7.5) for methanogenenics to develop (Gerardi, 2003).

8.3.2. Effect of digester configuration on cumulative methane

Gas composition, quality and quantity are the most important parameters during biogas upgrading and injection in to the grid system. They are directly influenced by the nature of feedstock and the process conditions (Börjesson and Ahlgren, 2012; Rasi, *et al.*, 2011). In this study, all three parameters were influenced by the digester configuration.

An example of the evolution of the methane production obtained from the systems is presented in Figure 8.2.

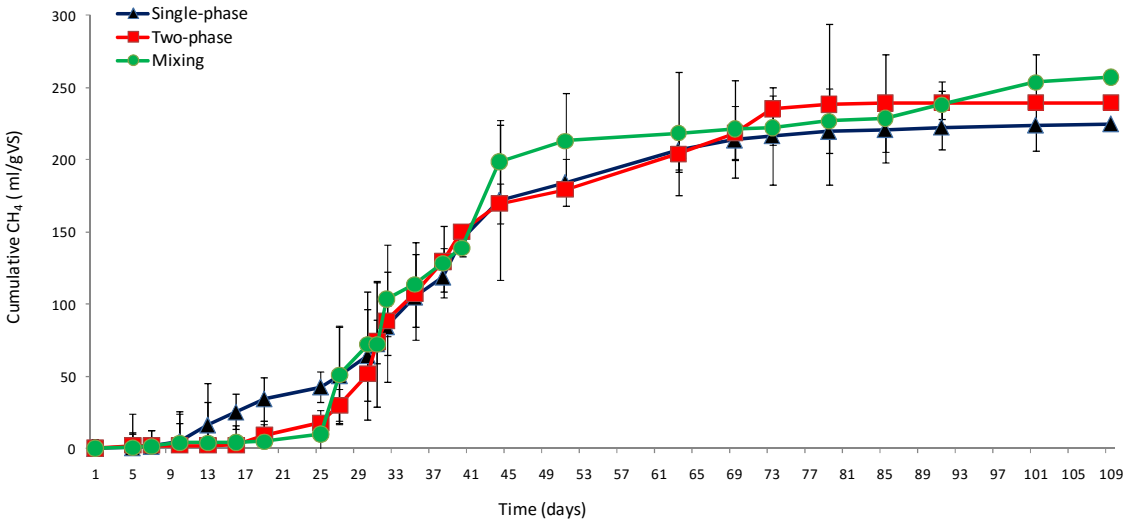

Figure 8.2. Cumulative methane production from the AD of *L. digitata* in a single-phase, two-phase and stirred system.

The time required for the methanogenic community present in the slurry to start producing methane was between 6 to 7, 16 to 19 and 7 to 16 days in the single-phase, two-phase and stirred system, respectively. In these experiments, the start-up period (initial 1-3 weeks) was crucial for the reactors in which the inoculum acclimatised to the seaweed chemical composition. The innoculum or seed used in these experiments did not contain sufficient levels of metabolically active methanogens therefore, a delay in the methanogenic phase was observed. Furthermore, the slow growth rate of methanogens increased the time required for the consortium to establish in the reactor (Gerardi, 2003). Therefore, a balanced consortium of microorganism would be required during the reactor start-up.

Methane was highest after day 26 when it decreased with time, reaching the lower levels after day 73 for all reactors. Cumulative methane after 109 days of incubation was 224 and 240 ml g/VS for the single and two-phase system, respectively. The highest methane concentration was obtained in the mixing system with 267 ml g/VS. The establishment of a prompt methanogenic phase, therefore, would be a key aspect in order to prevent digester failure, making the AD process more economical profitable by maximising the energy recovery.

Maximum substrate utilisation occurred throughout days 30-44, 25-44, 27-44 for the single-phase, two-phase and stirred reactors, respectively. The digestibility of *L. digitata* biomass was about 11% (single-phase) and 14% (two-phase) lower than that of stirred system, indicating that the stirred system offered a higher biodegradation rate than the other two. After the easily biodegradable constituents from the seaweed were consumed, the availability of the feed for the methanogens would have been considerably reduced. Consequently, the effluent released from the single and two-phase process would be composed of undigested material which was not quickly hydrolysed and thus decreasing methane production. These results confirm that the hydrolysis phase during the AD of *L. digitata*, particularly depends on the reactor configuration used and will affect the establishment of active methanogens.

8.3.3. Effect of digester configuration on biogas quality and composition

The two-phase and stirred systems significantly enhance methane quality when compared to single-phase system. Methane concentration ranged from 68-73%, 65-69% and 60-66, respectively. The differences in percentage can be attributed to the fact that at the start of the process there was a different hydrolysis rate in each system, converting the seaweed biomass at different rates. This would have provided higher or lower concentration of substrates for the methanogens and thus allowing divergent growth rates (Abu-Dahrieh, *et al*., 2011). The methane content generated in the present study is comparable to the percentage obtained from a two-phase AD system digesting *M. pyrifera* and *D. antartica* , where both seaweed species produced 180.4 (±1.5) ml g^{-1} dry algae d^{-1}, with a 65% in CH_4 concentration (Vergara-Fernández, *et al*., 2008).

Overall, high H_2 concentrations corresponded to high biogas production rates. H_2 was generated in the single-phase system until day 35, meanwhile for the two-phase and mixed system, production stopped at day 91 and 96, respectively. On average, higher concentrations (more than 1100 ppm) were registered along the first 10 days of digestion followed by a slow reduction, demonstrating that acetogenic bacteria were producing hydrogen which was readily usable by methanogenics. Thus, the hydrogen generation could have improved the methane production and overall digestion efficiency.

It would be also important to mention that, although hydrogen is a more ideal energy source with higher heating values (122 kJ/g for hydrogen and 55.6 kJ/g for methane), production was not as high as methane. Nevertheless, from an energy point of view, the recovery of hydrogen as by-product during the AD of seaweed would be an added advantage within a seaweed-based biorefinery process and should be evaluated further as an alternative source of energy. Feasibility studies suggested that, the production of biogas rich in hydrogen and methane from seaweed is a suitable process, with great potential to increase the energetic value of the seaweed biomass (Jung, *et al*., 2011; Jung, *et al*., 2012; Lee, *et al*., 2012; Liu and Wang, 2014; Shi, *et al*., 2011).

In the single-phase system, CO_2 concentration (first 10 days) was between 40-58%, followed by a slow reduction to 10-15% until day 109. In the two-phase system, higher percentages were observed (60-71%), followed by a fast reduction to 13-15% until the end of the experiment. The stirred system also produced higher concentrations of CO_2 during the first 10 days, accounting for 68-72%, followed by a progressive reduction to 7-10% until day 109.

The differences in CO_2 are correlated with the high hydrolysis rates during the first days of digestion where hydrolytic/acidogenic bacteria were mostly consuming the biomass and no methanogenic communities were actively producing CH_4. As CO_2 is inert in terms of combustion and was present in large quantities, a promising technology for its re-use is the biological capture using microalgae to produce biomass available for biodiesel (lipids) or as high added value materials (Collet, *et al*., 2011; Converti, *et al*., 2009; Harun, *et al*., 2011; Kao, *et al*., 2012; Jones and Mayfield, 2012; Pires, *et al*., 2012). In addition, by removing the CO_2 from the biogas the CH_4 content from the biogas can be increased. A number of technologies have been developed to date and few will include absorption of CO_2 by physical and chemical solvents, by cryogenic and membrane separation or a more environmental friendly process such as biological fixation (Abatzoglou and Boivin, 2009; Converti, *et al*., 2009; Rasi, *et al*., 2011).

The integration of this process, therefore, within a seaweed-based biorefinery process will be regarded as an added venture, expanding the versatility and viability of the biorefinery.

The H_2S generated in the three systems was above 200 ppm throughout the experiment. This profile has been also observed in Chapter 5 and 6 and is in agreement with the high S content of some seaweed species when compared to terrestrial biomass. However, elevated concentration of H_2S in the reactor might reduce methane production caused by its toxicity effect as well as direct competition for nutrients between sulphate reducing bacteria and methanogens (Gerardi, 2003). This gas is detrimental and corrosive to combustion systems and its conversion eventually will generate environmental hazardous compounds. Therefore, its removal is essential before any eventual utilisation of biogas (Rasi, *et al*., 2011).

From the results obtained in this Chapter, it is recommend that further studies should target the optimisation of the acid-phase reaction process with carbon rich co-substrates such as crude glycerol (Chapter 7). Increasing temperature rate during the first days of digestion could also enhance the hydrolysis as it was demonstrated in Chapter 5, after reactors were incubated at 45°C.

8.4. Conclusions

Results from the lab-scale experiments showed that reactor configuration influenced process performance. The quality and concentration of the methane produced during the AD of *L. digitata* was also influenced.

Among the three configurations evaluated, the stirred system produced higher concentrations of biogas and methane when compared to single and two-phase system.

The increase in biogas accounted for 12% and 23% more in the two-phase and stirred system, respectively, compared to single-phase system.

Reactor configuration had a great influence on hydrolysis, decreasing the pH values and therefore biogas production.

These 1.0 L lab-scale reactors were a preliminary step toward the configuration of a 10 L pilot plant AD process (Chapter 9) to generate biogas from seaweed.

CHAPTER 9

A 10 L ANAEROBIC DIGESTER SYSTEM FOR BIOENERGY RECOVERY FROM *Laminaria digitata* AND *Saccharina latissima* WITH DIGESTATE RE-USE AS FERTILISER

Vanegas, C.H, Bartlett, J. Biogas production from the anaerobic digestion of *Laminaria digitata* in a 10 L pilot-plant with digestate re-use as fertiliser. *Journal of Ambient Energy*. DOI:10.1080/01430750.2013.842496.

9.1. Introduction

Before the design and construction of an anaerobic digester, essential parameters such as, temperature, organic loading, hydraulic retention time, nutrient dosage, mixing, co-substrates and biogas yields, among others, require optimisation in laboratory scale reactors (Chapters 4 to 8).

The AD of different seaweed species have been studied by several researchers with different co-substrates (Hanssen, *et al*., 1987; Hughes, *et al*., 2012; Jard, *et al*., 2012; Kelly and Dworjanyn, 2008; Matsui and Koike, 2010). However, the majority of published data was collected from bench-scale reactors (100-5000 ml) and only two studies have investigated the suitability of the process at a pilot or larger scale (Morand, *et al*., 1990; Matsui and Koike, 2010).

Whereas the AD potential of seaweed can be better understood through bench-scale studies, reducing time and costs, it is very difficult to design and operate a farm-scale plant based on the data generated from these reactors. Furthermore, the estimated biogas potential can be greater than the biogas produced in full-scale digesters where operational constrains exists (Malmqvist, *et al*., 1998). This could lead to a large uncertainty in estimating the biogas production from a commercial-scale operation. Hence, investigating biogas production in a pilot-scale process can offer a more practical assessment of the energy values of seaweed.

Depending on the stream in the digester, the organic residue produced from the AD (digestate) is considered a valuable agricultural product that could offset nutrient loses

during cultivation of terrestrial crops (Alburquerque, *et al*., 2012a; Alburquerque, *et al*., 2012b; Lukehurst, *et al*., 2010; Möller and *Müller, 2012;* Vaneeckhaute, *et al*., 2013). Normally, the digestate is distributed on fields displacing 30% to 48% of mineral fertiliser, closing the nutrient and carbon cycle. The use of the digestate as a new form of organic fertiliser is therefore crucial in a seaweed-based biorefinery.

Different marine macroalgae have had a long tradition as soil enhancer around the world (González, *et al*., 2012; Sivasankari, *et al*., 2006). However, only a small number of studies have highlighted the benefits of digestate produced from the AD of seaweed (Hanssen, *et al*., 1987, Kelly and Dworjanyn, 2008; Nkemka and Murto, 2010).

The objective of this Chapter was to describe the basic operational condition for the design and implementation of a larger-scale two-phase AD process using seaweed in combination with bovine slurry (BS) as source of inoculum and co-substrate. For this purpose the seaweed species *L. digitata* and *S. latissima* were co-digested individually in a 10 L pilot plant reactor. The overall aim was to assess the feasibility to scale up the AD of seaweed and produce biogas. Thereafter, the digestate was used as a source of fertiliser during a 60-80 day laboratory experiment to enhance seed germination and the growth rate of two crops extensively used as biofuel feedstocks, sunflower (*Helianthus annuus*) and perennial ryegrass (*Lolium perenne*).

9.2. Materials and methods

9.2.1. Substrate

Although the initial experimental design was to evaluate the 3 best performed seaweed species from Chapter 6, however, only sufficient quantities of *L. digitata* and *S. latissima* were available at the time of the experiment.

L. digitata was collected and processed as previously described (Chapter 3, section 3.2.1). Samples of *S. latissima* were provided by SAMS. The dried material was further milled to small particle sizes (< 1.0 mm) and the TS (91%) and VS (63%) were measured as previously described Chapter 4, section 4.2.5. BS was collected and processed as previously described Chapter 4, section 4.2.1.

The reactors were inoculated following a similar loading pattern as that described in Chapter 8 for a two-phase AD process with constant mixing. The rationale behind this approach was based on the results obtained from Chapter 8, where a more stable process was observed after subjecting the seaweed to a two-phase AD system, particularly due to the abrupt changes of pH during the hydrolysis phase. For this reason, the reactors were initially fed with 260 g/VS of BS, 222 g/VS (300 g of dried seaweed weight) and 6.0 L of dH_2O. The headspace in the reactors was flushed with a mix of N_2/CO_2 for 10 min, applying constant mixing.

The starting pH of the blend was 6.8 and no further adjustment was carried out. Hydrolysis conditions were maintained throughout the initial days of the experiment and the methanisation stage was started when biogas production from the hydrolysis phase stopped. The pH of the pre-acidification reaction was adjusted to 7.0 ± 0.1 with the addition of a 10% $NaOH/KHCO_3$. To initiate the two-phase, 320 g/VS of BS was added subsequently to the reactors giving a total of 580 g/VS (900 g of sieved slurry) and a ultimate seaweed:BS ratio of 1:3.

Endogenous biogas generated from the slurry alone was calculated based on the 1.0 L reactors incubated in Chapter 6 and 7, and subtracted from the cumulative biogas/methane.

All analytical determinations were carried out as described in previous Chapters (cumulative biogas and biogas content, TS, VS, ammonia, and pH). The total alkalinity, expressed as mg $CaCO_3$/L digestate, was measured by titration to pH 4.0 with 1N HCl following APHA method 2320 (Alkalinity). The determinations were carried out in triplicate and all the results are expressed as means.

9.2.2. Digester design

The AD reactors used in this chapter were two stainless steel pilot plants with a 10 L volume running in parallel. The blend inside the reactor was stirred using a Lenze gear motor with a torque of 38 Nm (Figure 9.1).

Figure 9.1. Pilot plant digester unit (10 L).

A stainless steel top plate lid was originally built with four outlets. A 50 cm long hose was connected to a gas nipple outlet. A single polypropylene fitting was connected to the end of house where gas was collected in a 5.0 L Tedlar bag. A filter was attached on to the line connecting the digester gas outlet to the polypropylene fitting to absorb water from the biogas, as the water could damage the sample cell in the gas analyser (LMS 4501, Gas Data).

A wider outlet fitted with a mechanical valve was used for manual feeding. The bottom of the digester was fitted with another mechanical valve for digestate collection. There is also an inverted well at the bottom of the digesters to allow for a PT 100 temperature probe (Figure 9.2).

Figure 9.2. Bottom view of digester with wide port opening for digestate sampling.

A manually controlled circulation pump with a thermostat allowed hot water to circulate through the digester wall and back to the heating system in a loop system. Hot water in the circuit was kept at 35 ± 2°C (Figure 9.3).

Figure 9.3. Water heating pump.

The control panel, located in a protective and closed plastic box, contains all the electric system controls required for the functioning of the digester.

9.2.3. Fertiliser potential

The digestate used during this experiment originated from the AD of *L. digitata*.

9.2.3.1. Digestate

Samples were taken after the end of the AD processes and stored at -20°C without further post-treatments or processing. The chemical characteristics of the digestate used during the cultivation experiments are shown in Table 9.1. The digestate was prepared with dH_2O at concentrations of 100% (pure), 50%, 20%, 10%, 5% and 1% (volume fraction).

Parameters	Digestate
VS	32%
RS	1.3 mg/ml
Ammonia	432 mg/L
Alkalinity	12500 mg/L $CaCO_3$
pH	7.9
BOD	490.08 mg/L

Table 9.1. Characteristics of the digestate used during the fertilisation experiment.

9.2.3.2. Pot experiment setup

For the plant growth bioassays a novel methodology was developed. Plant seeds were purchased from a local greenhouse provider (Connacht Gold Garden Centres, Sligo, Ireland). 20 ryegrass seeds were grown in 100 x 7 cm square plastic pots. For the sunflower experiments, 10 plants were grown from seed inside a 5"Stewart Bio Flower pots. All pots (in duplicate for each treatment tested) were filled with Jonn Innes multi-purpose compost and used for the bioassay. For all plants, pots were amended initially with 100 ml of the corresponding concentration of digestate with further additions every 20 days. Irrigation with 100 ml of dH_2O was carried out every 2-3 days to prevent dehydration.

The cultivation was done inside a Mylar hobby tent GT2010, set at 21 ± 3 ºC air temperature, 16 ± 1 soil temperature and a relative humidity of 70% ± 5 (Figure 9.4). Inside, a HPS bulb 400W (Mission lighting SCC Super Cool Compact Ballast Kit) was used, giving a photon flux density of 650 µmol m^{-2} s^{-1} (approximate) for a 24 hr light constant cycle.

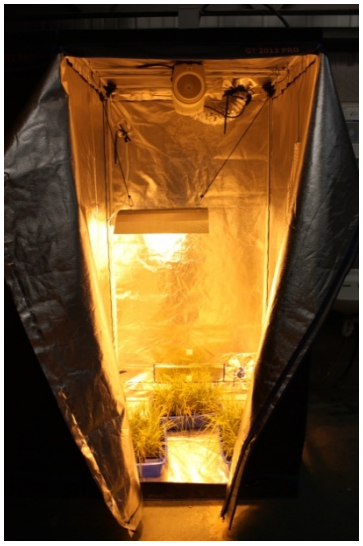

Figure 9.4. Set up of the mylar hobby tent used to incubate the grass and sunflower.

The percentages of germinated seeds were determined and the ryegrass material was harvested every 20 days (three cuts in total) and stored immediately in airtight capped tubes at -20°C prior to testing in order to avoid microbial degradation. Samples (20% solids in dH_2O) were subjected to thermal hydrolysis (121°C/1 atm/1 min) and analysed

for RS, using the DNS method (Miller, 1959) (Chapter 3). In the case of sunflowers, harvesting was carried out after approximately 80 days of growth, by which time the plants had started flowering.

9.3. Results and discussions

Previous Chapters demonstrated that the AD of seaweed was a suitable and feasible process where production rates can be enhanced by different conditions; pretreatment (Chapter 4), co-digestion (Chapter 6 and 7), and digester configuration (Chapter 8).

Results established the feasibility of biogas production in 120 ml and 1.0 L bench scale digesters. Here, the main findings from a 10 month pilot-scale experiment that investigated the performance of two 10 L pilot plants to produce biogas from the AD of the seaweed species *L. digitata* and *S. latissima* in co-digestion with BS are reported.

9.3.1. Optimisation of digester performance

Once all parts from the digester were assembled, the vessel was filled with water and pressurised air. Temperature, mixing and gas leaks were monitored continuously. Operating optimisation from each digester was carried out over a three week's period.

9.3.1.1. Temperature

Initially, the temperature was monitored by inserting one of the thermocouple probes into the vessel via one of the outlet ports from the lid and the other thermocouple was slotted into the thermo wells at the bottom of the digesters. Heating pups were initiated and temperature readings were recorder in a Pico Tech Enviromon EL008 data logger every 5 minutes. The same procedure was carried out for digester 2, once the water temperature inside of the reactor was stabilised at 35ºC ± 2.

It was found that there was a slight difference in temperature between the reading given by the probe immersed inside the vessel and the reading obtained from the probe slotted in the inverted well. Since the difference in temperature was constant, the readings obtained by the probe were considered consistent as long as this difference was taken into account.

9.3.1.2. Stirring

After the temperature in the reactor was verified, the stirring operation was optimised. AD of cattle slurry is often associated with floating layers and crusts thus, in order to avoid this, the stirrer was set at 50 rpm (maximum speed) to allow for adequate mixing. Moreover, Chapter 8 also highlighted the benefits of mixing in biogas and methane production, thus the importance of optimising this parameter.

After initiating the stirrer, a gas leak from the rubber o-ring around the shaft of the stirrer was found and a new replacement had to be designed and ordered.

9.3.2. Reactors start-up

Digesters were heated up to 35ºC ± 2 prior to feed additions. Once the feed was added (seaweed, BS and water), mixing was set at 50 rpm. It was observed that this speed affected the microbial community resulting in a reduction on biogas production and inhibition of the AD process after 2 weeks. Therefore, mixing rate was reduced to 10 rpm (as previously reported in Chapter 8) resulting in a mild agitation regime until the end of the experiment.

The biogas and methane produced from the BS was determined to be negligible (less than 2%) based on the preliminary assays in Chapter 6 and 7.

9.3.2.1. Biogas production from *L. digitata* in 10 L digesters

A drop in pH was observed after 6 hrs digestion (Figure 9.5), as the easily digestible fraction of the seaweed was hydrolysed and converted to biogas. After 72 hrs, the pH dropped to 5.4-5.5 where it was maintained until biogas production stopped completely. The second-phase was initiated on the 7th day with the addition of a 10% NaOH/$KHCO_3$ mix. After pH was successfully increased to 7.0 ± 0.1 (7th day), BS was added at day 10 and biogas production began to rise gradually as the substrates were mainly consumed by methanogens. Fluctuations of pH were observed over the experiment, but overall there was a progressive increase from day 13 to 80 (Figure 9.5).

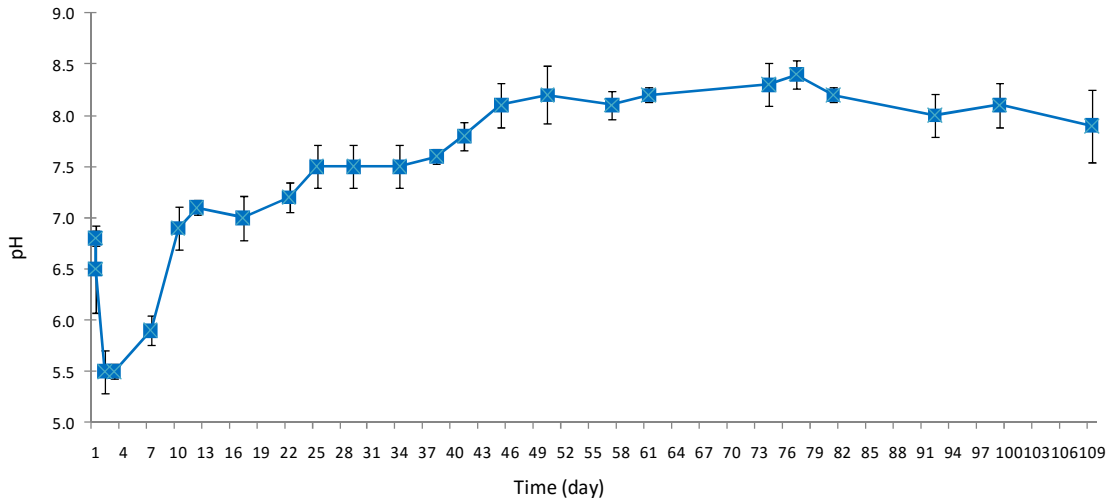

Figure 9.5. Change of pH during AD of *L. digitata* in the 10 L reactors.

The trend of biogas production is presented as an average of the two reactors in Figure 9.6. Total cumulative biogas and methane yields of 375 and 217 ml g/VS, respectively, were obtained. The cumulative biogas curve represents the two phases of the experiment. The first-phase (day 1 to 7) is characterised by a rapid hydrolysis stage with a cumulative biogas production of 172 ml g/VS. Low concentrations of methane were detected (0-15 ml g/VS) since the phase was designed to break down the main constituents of the seaweed, facilitating the growth of methanogenics at a later stage.

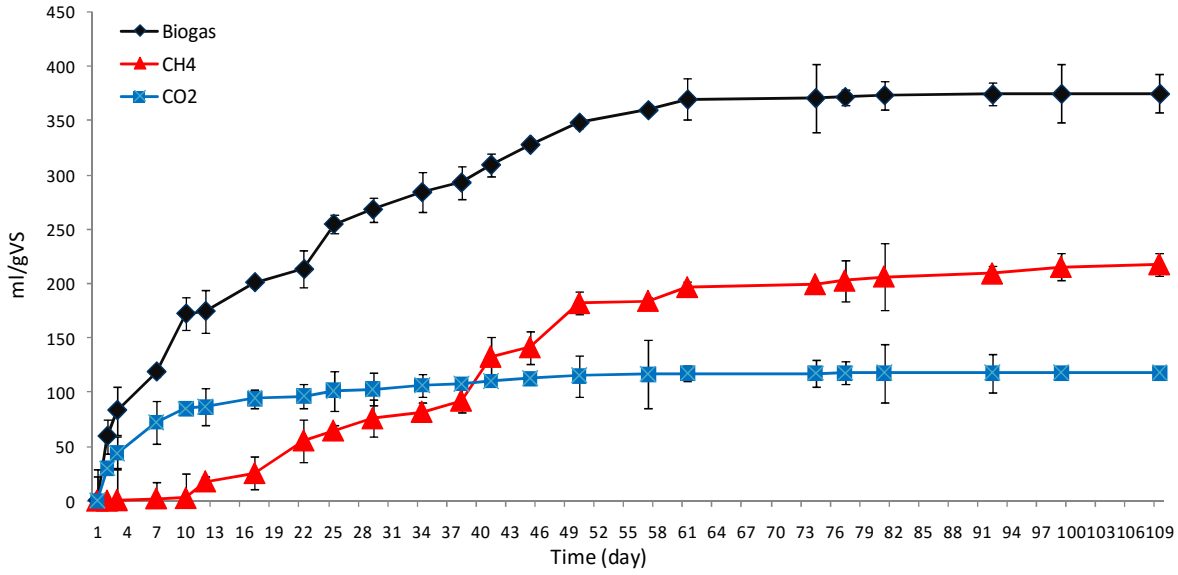

Figure 9.6. Cumulative biogas, CH_4 and CO_2 production from 10 L reactors digesting *L. digitata.*

Greater quantities of CO_2 were produced, accounting for 70% to 92% of the total biogas. Hydrogen was also generated in high concentrations (more than 1200 ppm). In a biorefinery process, as already discussed in Chapter 8, CO_2 would be used to cultivate microalgae, whereas H_2 can be stored as fuel energy source. While 1.1 ± 0.4% of H_2S was measured throughout the initial phase of the AD, inhibition on biogas production was not observed.

During the second-phase (after day 10), 202 ml g/VS of biogas was produced together with a progressive accumulation of methane. 93% of the methane was generated within this period with an average concentration of 61%. While the production of methane was found to be higher, a decline on CO_2 (from 69% to 0.6%) and H_2S (from 1.0% to 0.1 %) was observed. The last part of the curve, day 61 to 109, becomes almost horizontal with a reduction of biogas (5 ml g/VS). Comparable biogas production yields from the AD of *Laminaria* sp. were achieved in previous Chapters and reported elsewhere (Adams *et al*. 2011b, Hanssen *et al*. 1987).

At the beginning of the experiment, ammonia concentration was 96 ± 4 ppm, followed by a progressive increase, reaching 470 ± 9 ppm after the 109 days incubation time.

The increase in alkalinity may have played a role in reducing cumulative biogas and methane concentration. While the initial alkalinity of the feed was 68-72 mg/L $CaCO_3$, at the end of the process it averaged 12750-13750 mg/L $CaCO_3$. This increase in alkalinity is attributed to the addition of NaOH and $KHPO_3$ to control the low pH levels reached during the hydrolysis phase. Moreover, the high levels of ammonia reacted with CO_2 and water, producing ammonium bicarbonate increasing the alkalinity of the digestate (Gerardi, 2003).

The VSD was monitored in order to examine the bio-digestibility of the process. A 60% to 67% of VSD was observed at the end of the experiment. This relatively low VSD could be attributed to the recalcitrant organic fraction (Cellulose) from the BS that affected the AD process. This similarity was also observed during the AD of *S. latissima* and *Palmaria palmata* (Jard, *et al*., 2012). In their study, refractory compounds such as fibre, soluble xylans, alginates and sulphated fucans slowed down degradation and biogas production. In a different study, Bird, *et al*. (1990) suggested

that there exist several types of recalcitrant material in *Gracilaria* spp. and *Sargassum* spp. which reduce biodegradability.

Furthermore, the high alkalinity (13750 mg/L $CaCO_3$) and concentration of H_2S (competition of substrate with SRB) during the second-phase, would have affected the microbial community in the digester, decreasing process performance and, therefore, the degradation of the organic material.

9.3.2.2. Biogas production from *S. latissima* in 10 L digesters

Figure 9.7 shows the cumulative biogas production from *S. latissima* throughout the 109 days of digestion. The cumulative biogas and methane produced was 514 ml g/VS and 305 ml g/VS, respectively.

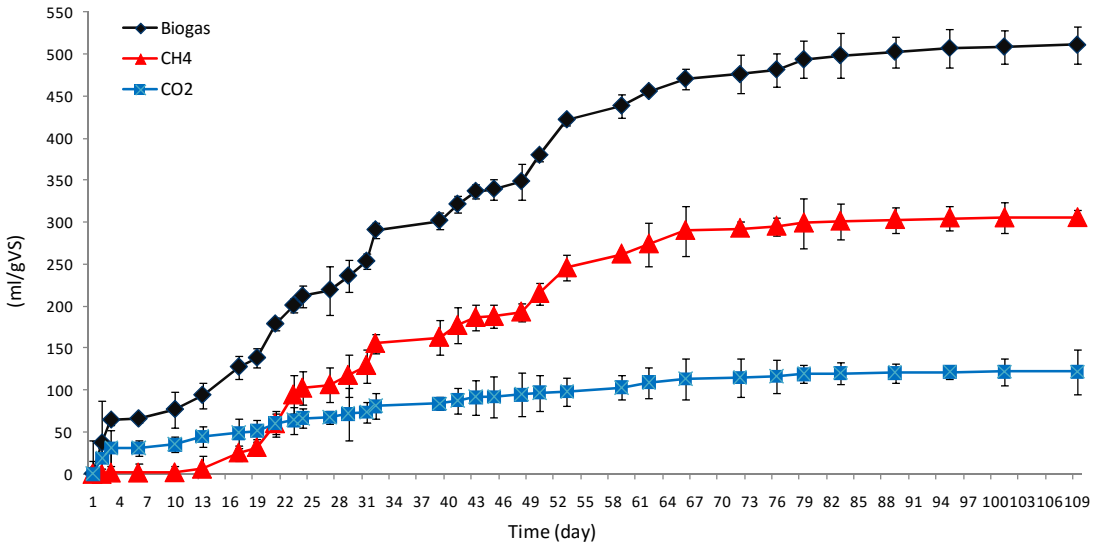

Figure 9.7. Cumulative biogas, CH_4 and CO_2 production from 10 L reactors digesting *S. latissima.*

Unlike the AD of *L. digitata*, low concentrations of biogas and methane (94 and 7 ml g/VS, respectively) were produced during the hydrolysis phase (day 1 to 10). Biogas production was more efficient in the second phase (day 11 to 62) accounting for (69%) of the total cumulative biogas (420 ml g/VS). A higher methane concentration was reached during this period (263 ml g/VS) with an average in composition of 64% in the biogas. In the last stage of the AD process (day 63 to day 109), a reduction on methane

concentration (from 68% to 7%) was observed with a cumulative production of approximately 35 ml g/VS.

In previous Chapters and during the AD of *L. digitata*, H_2S (in gas phase) was produced in high concentrations. Depending on the pH of the medium, H_2S is present in two forms. At higher pH levels, H_2S is found in the alkaline sulphide form and at lower pH levels it is found in the gaseous form (Gerardi, 2003). During the hydrolysis phase, at pH 5.2-5.9, (Figure 9.8) about 90% could be found as gaseous H_2S (2.1-2.6%) and 10% as the alkaline sulphide. After the start of second phase, an increase of pH (7.8-8.3) along with a reduction on H_2S (0.8-1.5%) was observed, possible caused by the dissociation of H_2S in to the alkaline form or a reduction in the number of SRB (Gerardi, 2003). Although this assumption was not corroborated in this study, further work should investigate the H_2S pathway during AD of these seaweed species.

High concentrations of H_2S are problematic for the development of AD technology to produce electricity and heat (CHP units) or during upgrading to natural gas. However, several technologies have been developed to treat the biogas from farm plants origin: aeration, biological desulphurisation, iron oxide (iron sponge), alkaline-based scrubbers, molecular sieves (zeolites, activated carbon), and liquid-based processes among others (Abatzoglou and Boivin, 2009; Kohl and Neilsen, 1997; Wellinger and Linberg, 2000).

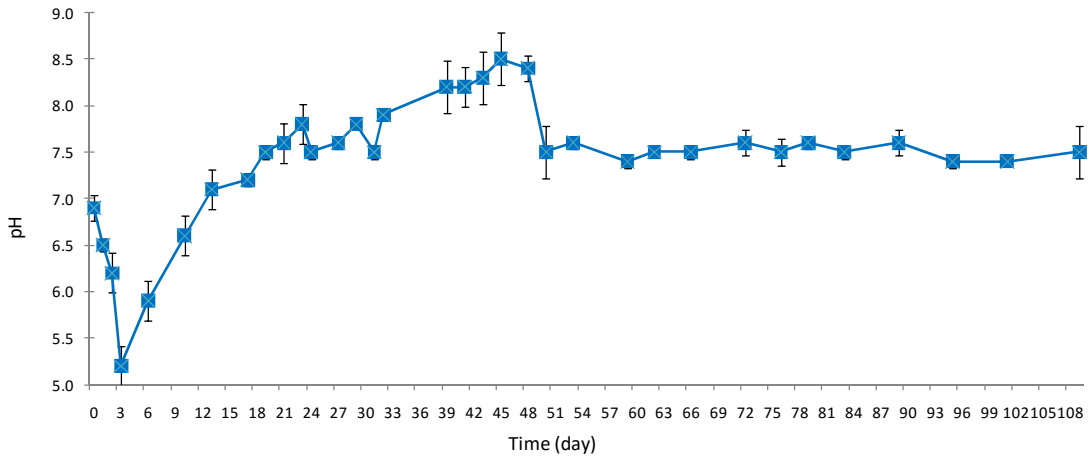

Figure 9.8. Change of pH during AD of *S. latissima* in the 10 L reactors.

Large quantities of CO_2 were generated (122 ml g/VS) during the AD of *S. latissima*, particularly during the first days of digestion. The benefits and potentials from this by-product within a seaweed-based biorefinery process have been discussed above and in previous Chapters.

A noteworthy result was the production of 15-23 ml g/VS of hydrogen during the 109 days of digestion. As previously discussed in Chapter 8, a process generating biogas rich in methane and hydrogen is an extra advantage from the AD of seaweed (Jung, *et al*., 2011, Jung, *et al*., 2012; Kaparaju, *et al*., 2009; Lee, *et al*., 2012; Liu and Wang, 2014). Although purification of hydrogen is a costly process, particularly when H_2S is produced in large quantities (Klass, 1998), further investigation is required to include this by-product in a seaweed biorefinery process.

Alkalinity at the end of the process was in the range of 8500- 14000 mg/L $CaCO_3$. The inadequate levels of alkalinity seen in the reactors digesting both seaweed species, together with high production of H_2S, contributed to the alkalinity in the medium. This high concentration appears to have been a cause of biogas reduction.

The concentration of ammonia (180-470 ppm) which was very similar to the 1.0 L reactors from Chapter 7 (120-490) may have not been high enough to prevent methane production.

VSD is associated to the extent of hydrolysis and solubilisation of biodegradable material carried out during AD. Although the VSD concentration decreased over time in the digester effluent, the two digesters had similar VSD profiles (59-61%), therefore, a considerable amount of organic material remained available for degradation. The reduction in VSD is in agreement with the low biogas yield obtained in the 10 L reactors. Although a similar VSD profile was obtained during the AD of *Sargassum tenerrimum* (57-62.9%) (Anjaneyulu, *et al*., 1986), the remaining organic material can be used and integrated as a form of organic fertiliser within the seaweed-based biorefinery process.

Different factors may have reduced the degradation of the substrate and therefore, the biogas yield when compared to the 1.0 L reactors:

- A temperature increase of 3-4^{o}C on day 24 accounted for a 21% reduction on methane production whereby, on day 43 a 31% reduction was measured. Once the temperature was returned to 35^{o}C ± 1, the methane quality and quantity was improved. The changes in temperature would have disturbed the activity of the microbial consortia in the digesters, thus resulting in a reduction in the methane yield. Negative effects of temperatures shifts have also been reported for different biomasses (Batstone, *et al*., 2002; Kaparaju, *et al*., 2009). This has led to a reduction in methane (in terms of quantity and quality), as methanogens are usually more sensitive than hydrolytic bacteria (Chapter 5).
- Low hydrolysis rates were observed during the first 10 days of digestion (94 ml/ gVS) when compared to the biogas yields obtained in Chapter 6 (180 ml/ gVS). This difference would be explained by a poor microbial activity and quality from the BS inoculum. Because the biogas produced through the hydrolysis phase did not differ between the two 10 L reactors running in parallel (181 and 179 ml/ gVS for reactor 1 and 2, respectively), it is assumed that the inoculum (BS) had a significant influence over reactor start-up and consequently, the biogas variation between the 1.0 L and the 10 L reactors.
- Differences were observed during the hydrolysis stage in the two-phase AD system when compared to the hydrolysis from previous Chapter, affecting process performance and, therefore, inhibiting biogas yield. In Chapter 5 and 6, after 1-2 days digestion, the drop in pH was in the range of 5.6-5.9 and during the AD of *S. latissima* the lowest pH reached after 4 days digestion was 5.2. Thus, the differences in pH, when compared to previous Chapters, are correlated with the low biogas yields, suggesting that the degradation of the seaweed took longer as the reactors progress towards an acidic state, consequently, affecting the activity of the microbial community (Elefsiniotis and Oldham, 1994; Zhang, *et al*., 2005). During the acidogenesis of glucose, Zoetemeyer, *et al*. (1982) recommended a pH of 5.7 - 6.0 for the acid reactor, providing a more stable and favourable substrate for the methane reactor.
- Variations in the chemical composition of the substrate (Adams, *et al*., 2011a; Rioux, *et al*., 2009; Shi, *et al*., 2012; Shiralipour and Smith, 1984) might have also influenced the metabolic activity of the inoculum and therefore, hydrolysis rates and biogas yields (Bird, *et al*., 1990). For instance, a difference in terms of VS content when compared to the biomass used in Chapter 6 was observed (28% lower). This is in agreement with the recalcitrant fraction of the seaweed that remained (57-61% VSD) after the

utilisation of the most easily hydrolysable material. A similar finding was observed in a study carried out by Shi, *et al*. (2012) where the low methane yields and VSD of macroalgae are likely to be reduced due to differences in ash, lipid or mineral content among the species investigated (*Gelidium corneum* and *Cladophora glomerata*).

- The degradation of the seaweed also released, in great quantities, other inhibitory compounds which accumulated in the digester, including specific VFAs (not determined in this investigation) and by-products (H_2S) from the metabolic reduction of sulphated compounds (amino acids and polysaccharides) by SRB with the expense of organic matter. Hence, not all the available organic matter in the seaweed was converted into methane but into other gasses.

9.3.3. Assessment of digestate on plant growth

While the use of cow manure and different varieties of seaweed species as source of soil enhancer has been reported elsewhere, experimental trials assessing the effect of digestate generated from the AD of *L. digitata* or *S. latissima* to enhance crop cultivation have not been studied.

The implementation of the seaweed-based biorefinery process would integrate the production of biogas together with the recycling of the digestate generated, converting it into a new form of fertiliser or soil conditioner. Oleskowicz-Popiel, *et al*. (2012) investigated the operation and economics of a biorefinery process within an organic farm. While ethanol was produced from rye grains and whey, the fermentation effluent was further used to produce biogas. The solid fraction was regarded as a protein fodder for animal feeding and the effluent from the AD to serve as natural fertiliser. In a most recent study, Nkemka, *et al*. (2014) suggested that the use of seaweed digestate as a source of fertiliser needs further research and practical applicability prior to farmland application. Therefore, the effect of the digestate generated from the AD of *L. digitata* on seed germination and plant growth was investigated.

9.3.3.1. Sunflower

Sunflower is mainly cultivated for commercial oil production and more recently as an alternative source to produce biodiesel. Seed development and plant growth are affected by nutrient availability in the soil (Mohammadia, *et al*., 2013; Russo and Fish, 2012). In

this study, the different digestate concentrations had a minor effect in the percentage (85-95%) of germinated seeds (Figure 9.9a) (Table 2) and the shoot height (Figure 9.9b).

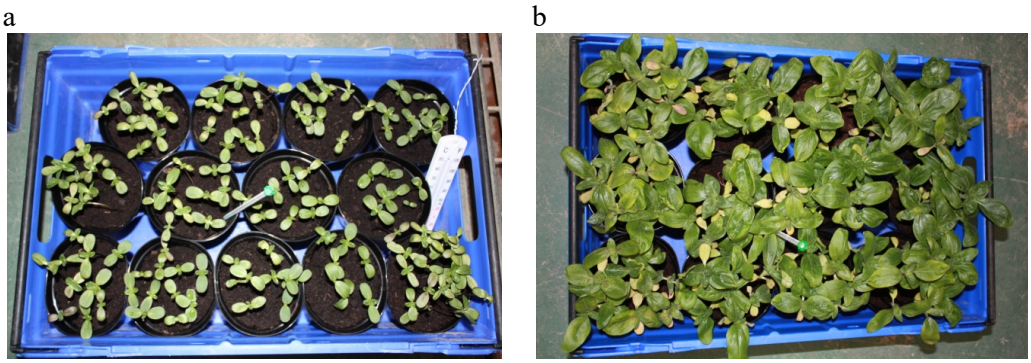

Figure 9.9. Germinated sunflower seeds after 2 days (a) and 12 days (b).

Treatment	Germinated seeds (%)	
	Grass	Sunflower
Control	71	90
1%	73	90
5%	75	90
10%	74	95
20%	78	95
50%	88	85
100%	89	85

Table 9.2. Percentage of germinated seeds after 4 days growth.

By day 22 (Figure 9.10), higher concentrations of the digestate (20%, 50% and 100%) improved the growth rate of the plants. This increase is explained by the enhancing effect of macro and micro-nutrients present in the digestate that stimulated plant development.

On the other hand, by day 32, a reduction (Figure 9.11) was observed in pots amended with the highest concentrations of the digestate; 50% and 100%. This reduction in growth is attributed to accumulation of inhibitory compounds from the digestate and nutrient overloading.

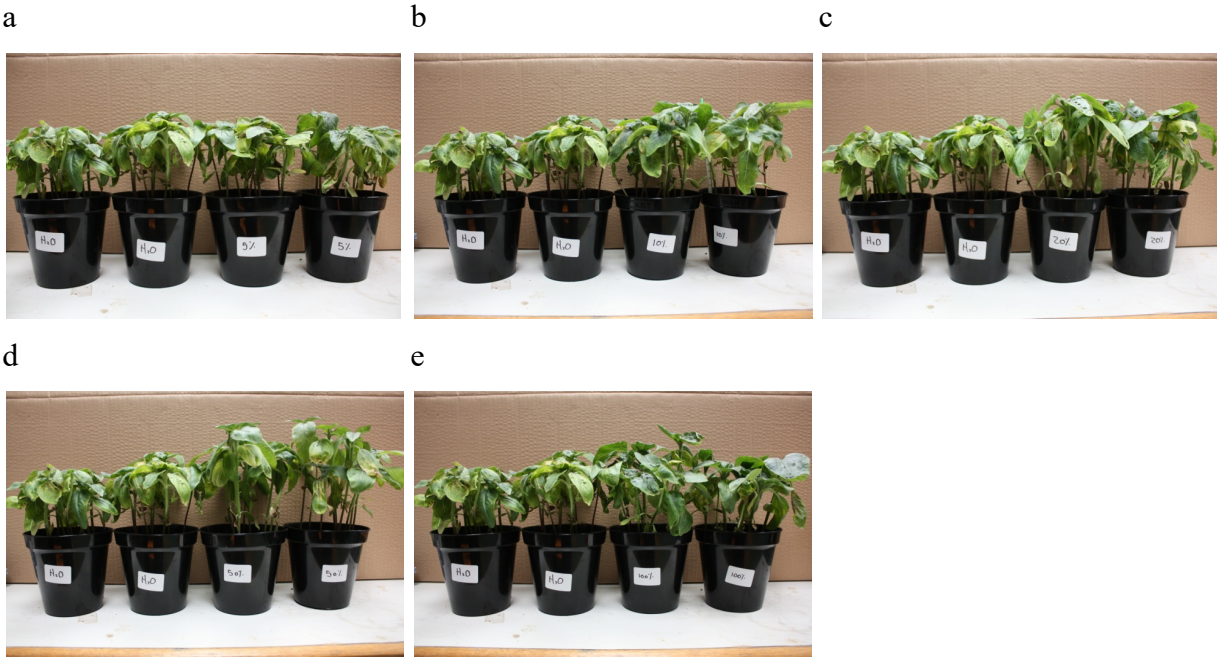

Figure 9.10. Germinated sunflower seeds after 22 days. (a) 5%, (b) 10%, (c) 20%, (d) 50%, (e) 100% digestate addition.

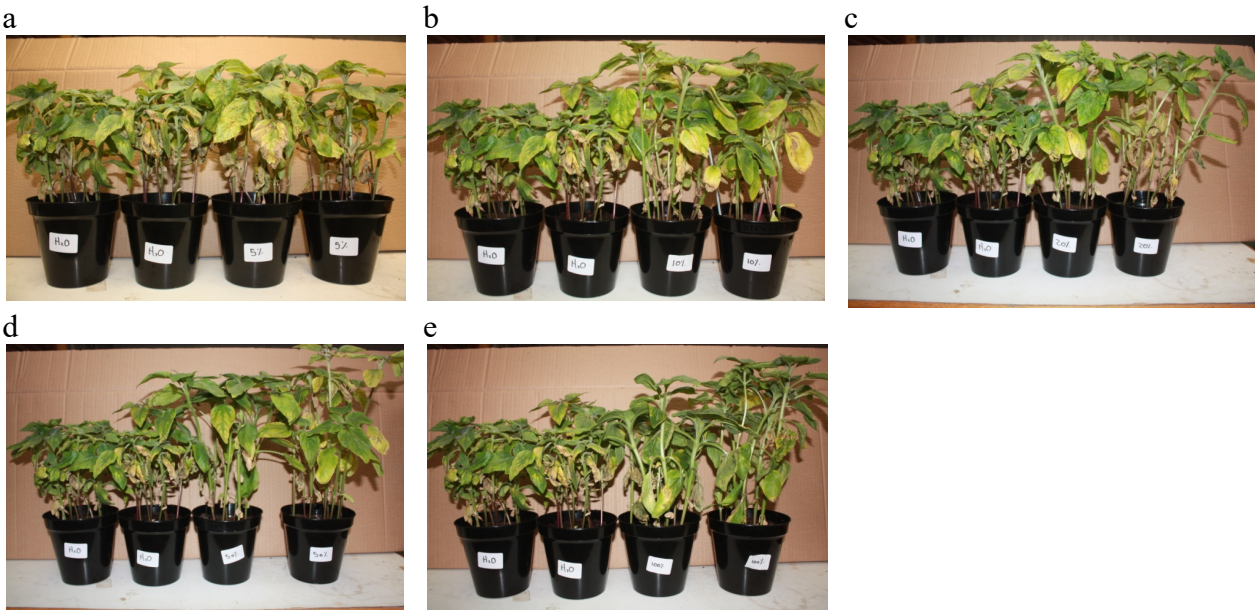

Figure 9.11. Germinated sunflower seeds after 32 days. (a) 5%, (b) 10%, (c) 20%, (d) 50%, (e) 100% digestate addition.

Between day 50 and 65 the first terminal buds appeared, forming a small floral head in pots amended with the 50% and 100% digestate. By the end of the experiment (day 75-80), the pots showed the highest number of flowers (Figure 9.12).

Based on these conditions, digestate dosage is not recommended for seed development and maturation. However, it is useful throughout the vegetative development of the plant where the higher concentrations (20%, 50% and 100%) stimulated flower production. These results contribute to an efficient digestate management by reducing dosages without affecting crop yields (Russo and Fish, 2012).

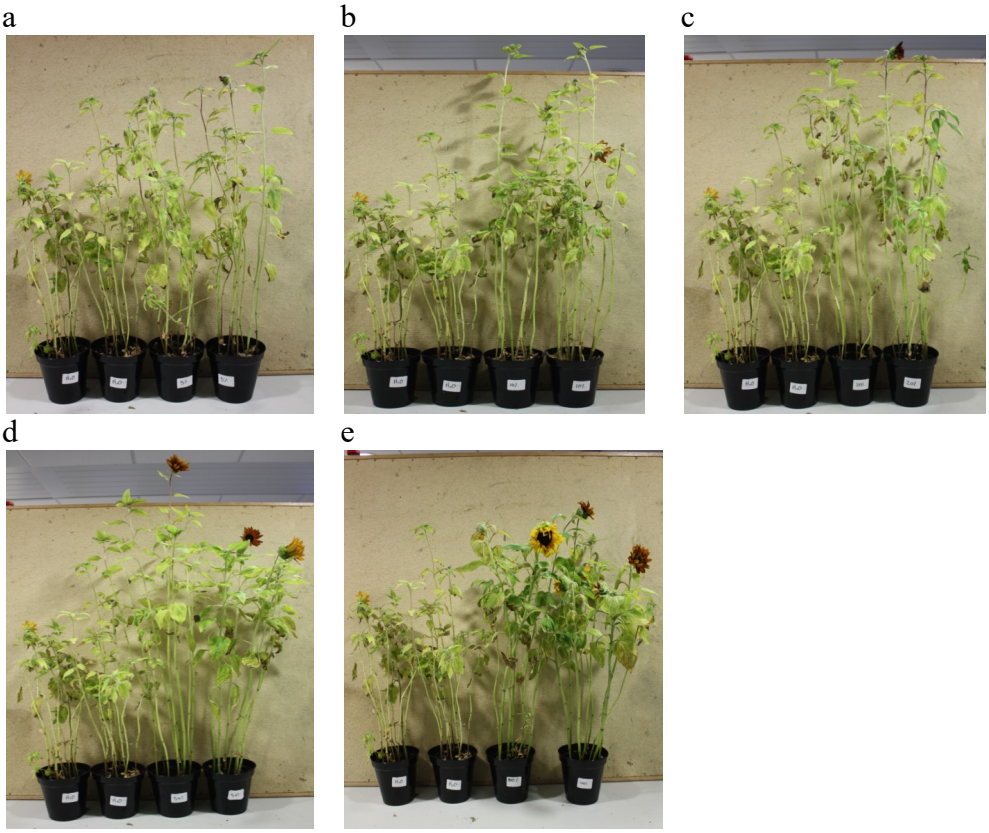

Figure 9.12. Germinated sunflower seeds after 80 days. (a) 5%, (b) 10%, (c) 20%, (d) 50%, (e) 100% digestate addition.

9.3.3.2. Grass

Ireland possesses the highest grass yields among western EU countries, hence the opportunity to use this feedstock as a source of biomethane (Abu-Dahrieh, *et al*., 2011; Smyth, *et al*., 2009; Smyth, *et al*., 2011). The grass growth rate could be increased and improved by manure spreading at different rates and the addition of fertilisers (Kaffka

and Kanneganti, 1996). In this experiment, the effect of digestate addition on ryegrass seed germination, plant growth and RS content was evaluated.

After 4 days growth, the higher concentrations of the digestate clearly enhanced seed germination (Table 9.2). Before the first cut (20 days) similar leaf colour and length was observed for all treatments (Figure 9.13a). The response to digestate addition was different just after the second cut (40 days). Although the grass length was improved by the increase in digestate concentration, the leaf colour intensity was reduced (Figure 9.13b).

At the end of the experiment (60 days), only the treatments with the highest dosage of digestate (20% 50% and 100%) enhanced grass growth (Figure 9.13c) (two rows at the back). This observation is in agreement with the higher concentration of nutrients available in the digestate that were consumed at different rates by the plant, hence influencing grass growth.

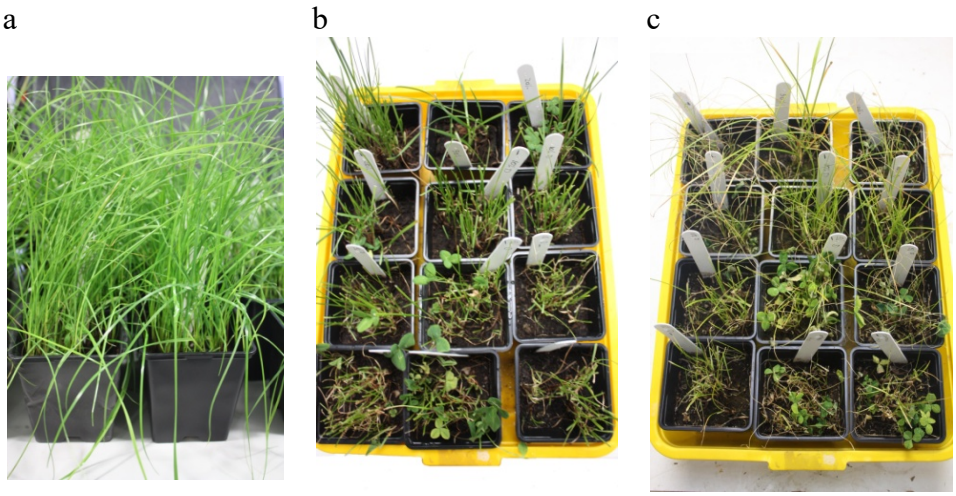

Figure 9.13. Germinated ryegrass seeds after 20 days (a), 40 days (b) and 60 days (c). In Figure b and c: First two front rows correspond to 1%, 5% and 10%. Two rows at the back correspond to 20%, 50% and 100%.

Grass quality is assessed not only by seed germination and plant growth but also by crude protein and carbohydrate content (Wilson, 1982). The effect of digestate addition on the RS content of ryegrass leaves was investigated.

Figure 9.14 shows that digestate concentration had an influence on the amount of RS (mg/ml) released from ryegrass hydrolysis after each cut. During the first cut, pots amended with 10% and 20% of digestate released more RS. Maximum concentrations of RS were recovered after the second cut (40 days), particularly in pots amended with 50% and 100% of the digestate. After the third cut (60 days), a decrease in the RS concentration was observed in pots amended with 1%, 5%, 50% and 100% of digestate.

The gradual reduction of RS may be attributed to the use of the RS for plant development or the translocation of sugars to other parts of the plant (cell wall polysaccharides) to support re-growth (Turner, *et al*., 2001). In a study carried out by McGrath, (1988), water-soluble carbohydrate content from perennial and Italian ryegrass was affected by the cuts in each year. Temperature, nitrogen uptake, an extended period of sunshine and plant survival parameters affected the concentration of WSC in the plants.

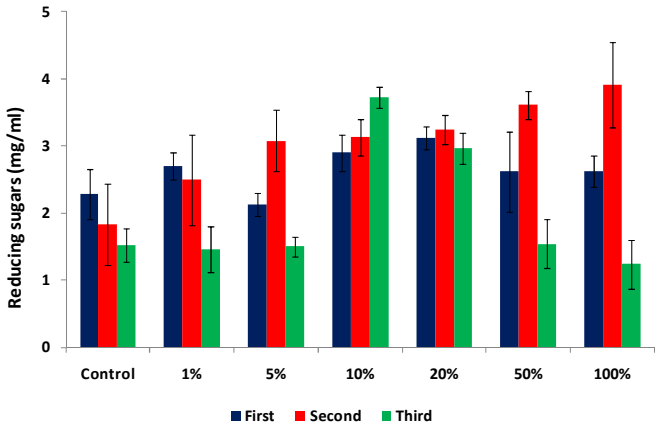

Figure 9.14. Concentration of reducing sugars (mg/ml) released from ryegrass after three cuttings in response to fertiliser dosage (percentage).

A clear variability in the total RS (TRS) released from the grass cuts was observed (Table 9.3). Compared to control and the other dosages, TRS concentration was higher in pots supplemented with 10% and 20% digestate. While 20% digestate concentration increased the TRS content in ryegrass, it also maintained the RS constant (Figure 9.14) after each cutting period improving at the same time plant growth. From a bioenergy perspective, the carbohydrates stored in the plant (among other nutrients) would be fermented by microorganisms to generate higher biofuel yields.

Concentration (%)	RS (mg/ml)
0 (control)	5.995
1	6.65
5	6.71
10	9.75
20	9.97
50	7.77
100	8.07

Table 9.3. Total RS recovered from grass cuts after the 60 days experiment.

Before the digestate can be integrated into a fertilisation programme, further considerations need to be taken into account:

- Biological and chemical analyses are recommended in order to avoid any risk of contamination, environmental pollution or phytotoxic effects on plant growth from excessive application of the digestate (Al Seadi and Lukehurst, 2012; Greger, *et al*., 2007; Lukehurst, *et al*., 2010; Möller and *Müller, 2012*).

- Soil microbial ecosystem functioning is subject to alteration by digestate addition (Abubaker, *et al*., 2012; Galvez, *et al*., 2012). Therefore, analysis of microbial biomass activity should be further investigated in order to profile changes in soil conditioning under different fertilising regimens. This will directly contribute to crop development and agricultural sustainability.

9.4. Conclusions

The results presented in this Chapter not only provided valuable data to scale-up the AD of seaweed in co-digestion with BS, but also demonstrated the benefits of digestate re-use as organic fertiliser in a seaweed-based biorefinery model.

The cumulative biogas and methane yield reached from two 10 L pilot scale experiments over a period of 109 days was 375 and 217 ml for *L. digitata* whereas for *S. latissima* the yields accounted for 514 and 305 ml g/VS.

Sunflower growth and flowering was enhanced by the addition of higher concentrations of digestate concentration (20%, 50% and 100%) but no distinctive effect was observed

on seed germination. While 20% digestate enhanced TRS content in ryegrass, higher digestate concentrations (50% and 100%) improved grass growth.

CHAPTER 10

GENERAL CONCLUSIONS AND OUTLOOK

Vanegas, C.H., Bartlett, J. (2015) Perspectives from a seaweed-based biorefinery approach (Manuscript under preparation).

Research on alternative energy is vital to attain objectives set by the EU to replace fossil fuels use for renewable sources (European Commission, 2012; European Commission, 2013; Ecofys, *et al*., 2013; Kampman, *et al*., 2013) such as the production of biogas from seaweed.

In order to make the technology commercially available and competitive against other biofuel lines, research is required to find the most optimal and viable process. This PhD thesis investigated the different processes influencing the AD of seaweed, by looking at the practicalities of a seaweed-based biorefinery process (Chapter 2, Figure 2.6).

The starting point of this study considered the use of existing data and the current understanding of the biorefinery concept (Chapter 2) so that the seaweed would be transformed to valuable biomaterials and bioenergy, maximising the economic value of the biomass, while reducing and recycling waste streams produced.

This final chapter summarises the core findings that have led to the development of a seaweed biorefinery and its significance in the biofuel industry, starting by the optimisation of pretreatment methods to enhance seaweed biodigestibility and macromolecules recovery, followed by the AD of different seaweed species, the effect of co-substrates and digester configuration on biogas yields, and scaling up the process to a 10 L pilot plant with digestate re-use as source of fertiliser.

Pretreatment is a key step in the biorefinery process, since it has a great impact on the efficiency and yield on the subsequent processes (Agbor, *et al*., 2011; Alvira, *et al*., 2010; Da Costa Sousa, *et al*., 2009; Menon and Rao, 2012; Ruiz, *et al*., 2013). As shown in Chapter 2, the seaweed biomass exhibit pronounced differences in the chemical composition and growing dates when compared to typical lignocellulosic feedstocks (Table 2.5). In this respect, for every biomass a particular pretreatment

application is required. The results in Chapter 3 established that the the most widely applied land-based biomass pretreatment methods (chemical, mechanical, thermal and enzymatic) enhanced, to different extents, the release of macromolecules. The recovery of RS, TL and TP from the two seaweed species, *Laminaria digitata* and *Saccharina latissima*, was influenced by the pretreatment method, the intensity and the species. It was concluded that, among all pretreatment methods, a combination of a freezing milling procedure (<0.1 mm particle size of the biomass), followed by a dilute-acid thermal step (4% HNO_3/130°C/1atm/2hr) released the highest concentration of RS and TL from the two seaweed species. The implication of these results is that the existing pretreatment methods can be used within a seaweed-based biorefinery to extract value-added products. Furthermore, the recovery of macromolecules was greater for *S. latissima* which suggests that this species is a better feedstock for the production of chemical building blocks with industrial interest when compared to *L. digitata*.

Variations on macromolecule extraction yields would also be affected by the type of seaweed, the changing biochemical composition of the seaweed through the year (Table 2.3) and harvesting season (Table 2.4) (Adams, *et al*., 2011a; b; Rioux, *et al*., 2009). Therefore, special considerations should be taken into account when finding the most appropriate month of the year to extract and process the seaweed biomass.

In recent years, there has been a great interest on seaweed biogas production and the methods associated to increase the yields have been investigated. Many of these studies documented a pronounced increase in the biogas yield (Nkemka and Murto, 2012; Nkemka and Murto, 2013; Tedesco, *et al*., 2013; Tedesco, *et al*., 2014), while others found a reduction or no additional effect (Jard, *et al*., 2013; Nielsen and Heiske, 2011; Nkemka, *et al*., 2014; Oliveira, *et al*., 2014; Vivekanand, *et al*., 2012). Based on the highest yields of macromolecules recovered (RS, TL and TP) (Chapter 3), the most favourable pretreatment methods were used as starting point to evaluate the effect on the AD of *L. digitata* (Chapter 4). A clear correlation between the amount of macromolecules released from the seaweed and the biogas yield was not observed, whereas the majority of the acid-based and chemical-thermal pretreatments inhibited biogas production. A combination of enzymatic treatment (*Cellulase*) followed by acid hydrolysis with 2.0% citric acid at 120ºC/1atm/1hr reaction time was selected as the most optimal condition from a range of chemical, thermal and enzymatic pretreatment methods. While biogas production was higher (6% increase) when compared to control

reactors, further research is required to determine whether the use of this pretreatment strategy has a significant contribution to the biogas yield during the AD of seaweed.

A pre-processing step, however, is still required so that after the seaweed is harvested the biomass will be more amenable for further processing. From a commercial standpoint, this would reduce transportation costs, storage, increase the shelf life of the seaweed and more importantly, it will decrease natural degradation (Bruton, *et al*., 2009; Gupta, *et al*., 2011; Schlarb-Ridley and Parker, 2013). Among mechanical pretreatments, milling (particle size reduction to <1.0 mm) was the most suitable method, increasing the biogas yield by 11-22%. Although drying the seaweed at room temperature for 48 hrs reduced the biogas yield by 3-4%, it is expected that the energy input to dry the biomass would be lower than oven drying. Drying and milling are straightforward techniques widely used in agricultural businesses which can be employed within a biorefinery process to pretreat the seaweed. These processes would be crucial for a commercially viable macroalgae biofuel enterprise thus; it is recommended including a detailed life cycle assessment of the techniques.

It is important to highlight that most of the pretreatment strategies discussed above will incur in high costs which would influence the overall yields gained from the AD. While, this investigation focused on the effect of macromolecule recovery and the biogas yield of the seaweed, it is recommended to consider the energy, economic and environmental aspects in order to determine, whether the use of these technologies are favourable within a seaweed biorefinery.

The results in Chapter 4 also established that the choice of an effective inoculum was a limiting and critical step during the hydrolysis process of *L. digitata*. Among the different inocula evaluated, BS contained the most suitable bacterial consortium capable of degrading the seaweed and generating biogas. Whereas anoxic sediments offered an optimum buffer capacity and the highest biogas rates during the first days of digestion, the microbial consortium from the BS inoculum adapted better to the chemical composition of the seaweed, producing a more stable biogas in the long term. The selection of a suitable inoculum or consortium of microorganisms may hold the key to efficiently transforming seaweed biomass in to the desirable biofuel (Tang, *et al*., 2009; Wargacki, *et al*., 2012; Williams, *et al*., 2013).

Chapter 5 highlighted that the incubation temperature was a rate-limiting step during the AD of *L. digitata*. Throughout the first days of digestion, the reactors incubated at 45ºC produced the highest biogas yields, leading to a reduction on pH and consequently, an unbalance between acidogenics and methanogenics. While this unbalance constrained methane production, the temperature enhanced the hydrolysis rate of the seaweed. It is recognised that higher temperatures have an enhancing effect on bacteria metabolism (Brock, *et al*., 1994), thus from a reactor design point of view, separating the acid producing microorganisms from the methane producers in a two-phase AD system will be of great convenience (Chapter 8). Ideally, the process will integrate a high-rate hydrolysis reactor running at 45ºC, reducing the retention times by 2 to 4 days when compared to 35 ºC, and a second reactor with a methanogenic consortium running at 35ºC.

In the context of this investigation, it is clear that the comparable performance at 35ºC to that at 45ºC or 20ºC strongly suggests that mesophilic treatment was the most optimal and effective for biogas and methane production. The Chapter also emphasised the importance to establish a balance between the bioreactor heating energy demand and methane yield which should be considered when bringing the technology to commercial scale. The increased proportions of H_2S in the biogas composition and low pH throughout the initial hydrolysis phase were regarded as potential inhibitors during the AD of seaweed. These limitations were further analysed in Chapter 9.

In Chapter 6, five seaweed species commonly found in Irish coasts were subjected to AD in order to compare their suitability to produce biogas. The results from the 1.0 L digesters clearly demonstrated that the cumulative biogas and methane yield were very wide and species dependent. *S. latissima* and *S. polyschides* offered the highest potentials, while *Fucus serratus* proved to be a poor substrate for biogas production highlighting the importance of selecting the most suitable species for biofuel production. Potential inhibitory compounds from the seaweed (mainly polyphenols in *F. serratus*) could have affected the microbial consortium and consequently biogas yields. It is recommended carrying out chemical screenings of potential inhibitory compounds that could affect the AD process. Wild harvesting of *S. polyschides* was not possible in the sampling location and, therefore, further trials with this species were not carried out. Based on the results from this Chapter, it was foreseen the diversification of the seaweed biorefinery by integrating different native species with high biogas potential

into the process. This could lead to a more sustainable use of natural stocks or in any commercial seaweed mariculture initiative (Bruton, *et al*., 2009; Hughes, *et al*., 2013; Roberts and Upham, 2012).

In Chapter 7, *S. latissima* and *L. digitata* were co-digestated with waste products from the livestock industry (BS) and the biodiesel industry (crude glycerol) in 1.0 L batch reactors. The feasibility of the process was demonstrated, as both co-substrates increased biogas and methane yields when compared to control reactors without the addition of the co-substrate. The increase was in proportion to the amount of crude glycerol added (up to 0.62% VS) and the substrate:BS ratio (higher than 1:2). The rationale behind this approach was to integrate various industries in to the seaweed biorefinery thus, enhancing the sustainability of the process.

In Chapter 8, the influence of reactor configuration on process performance and consequently, the quantity and quality of the biogas produced during the AD of *L. digitata* was investigated enhancing the prospects for a seaweed biogas production system. Since the reactors were fed, in terms of VS, with equal quantities of substrate, a clear comparison of the biological activity in the 3 systems (single-phase, two-phase and stirred reactors) can be made for practical reactor design purposes. Reactor configuration exerted a great influence on hydrolysis, decreasing the pH value and therefore, biogas production. A stirred two- phase anaerobic reactor system produced more biogas and methane when compared to the other systems. Since hydrogen was detected along the AD process, it is recommend (1) quantifying hydrogen production during the hydrolysis phase and its integration in a biorefinery process, (2) investigating synergic effects on methanogenic communities, or (3) recycling hydrogen surplus into the methanogenic reactor to increase methane concentration. The results from the 1.0 L lab-scale reactors were a preliminary step toward the configuration of a 10 L pilot plant AD process to generate biogas from seaweed. Commercial scale digester systems are operated in day-to-day feeding process therefore; further research should examine feeding the digester on a continuous base.

Information describing a suitable approach to scale-up the AD process from bench work to full-scale operation is limited, whereas on seaweed is not available in the literature. For this purpose, the feasibility to scale up the AD of *L. digitata* and *S. latissima* in two 10 L pilot plant digesters was investigated in Chapter 9, providing a more

comprehensive understanding of the process that could lead to the development of a larger digester unit. Variations in the methane yield (8-12%), in terms of ml/gVS, were observed when compared to the yields obtained from the 1.0 L digesters (Chapter 6-7). The results from this Chapter are valuable indicators of the importance of scaling-up the AD process. Before commercialisation, the effect of inhibitory compounds on methane yields, particularly H_2S, need to be studied further.

The slurry produced after the AD (digestate) is considered by many researchers and businesses enterprises an end product with valuable qualities that can be used as a soil biofertiliser rather than as a waste stream (Alburquerque, *et al*., 2012a; Alburquerque, *et al*., 2012b; Lukehurst, *et al*., 2010; Mata-Alvarez, *et al*., 2014; Moller and Muller, 2012; Smith, *et al*., 2007). The digestate generated during the AD of *L. digitata* enhanced the growth rate of terrestrial crops with biofuel potential; sunflower and ryegrass. The results from Chapter 9 established that beside biogas production, an additional source of revenue from the AD of seaweed biomass is represented by the nutrients embedded in the digestate, which can be applied in agriculture lands as source of fertiliser or processed further into soil conditioner.

The results and conclusions reached in this PhD demonstrated the viability to establishing a seaweed-based biorefinery process. The recovery of macromolecules, the use of byproducts from other bioindustries to enhance biogas yields and the plant-growth promoting properties of seaweed-based digestate were the main pillar from the biorefinery. While the different scenarios investigated in these Chapters developed the sustainability of the seaweed biofuel industry further, there is still much research necessary to elucidate the economic potential of such scenarios. Some of the key results raised during this PhD can be part of further research that is worth considering; they may include purification of macromolecules, development of an efficient inoculum capable to degrade the seaweed and the compositional analysis of microbial communities. The future direction of the research presented in this PhD is the development of the AD of seaweed in a large scale bioenergy recovery system.

The work in this thesis was completed within the framework of the BioMara project where all the deliverables at the Institute of Technology Sligo were accomplished beyond the objectives of the working package. The key contributions of this PhD to the

body of knowledge of the BioMara project lay within the development of the seaweed biofuel industry in Ireland and the interactions with local SMEs.

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