PROFILING OF MALAYSIAN SEAWEEDS FOR BIOETHANOL PRODUCTION

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FACULTY OF SCIENCE
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KUALA LUMPUR

2017

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE OF DOCTOR OF PHILOSOPHY

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

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ABSTRACT

Marine macroalgae (seaweed) biomass has the potential to be an important feedstock for the production of renewable biofuel. The carbohydrate-rich seaweed shows great potential as a competitive feedstock for the production of bioethanol. Seaweeds offer a more economically feasible and environmentally-friendly bioethanol feedstock to the currently utilised corn and sugarcane. Seaweeds produce a variety of polysaccharides that require differing conditions for saccharification to produce sugars that can be fermented to alcohols. The critical step in bioethanol production is the conversion of carbohydrates to fermentable monosaccharides, which takes place *via* chemical liquefaction by acid hydrolysis or the more environmentally-friendly enzymatic saccharification, or a combination of both. In this study, 29 Malaysian seaweeds (11 green, 10 red and 8 brown seaweeds) were collected from various habitats and analysed for their potential for bioethanol production. The seaweeds' species were analysed for total carbohydrate content, while sugar production was investigated using the common method of dilute acid hydrolysis. The highest total carbohydrate content was in *Kappaphycus alvarezii* (71.22 ± 0.71 % DW), followed by *Eucheuma denticulatum* (69.91 ± 3.35 % DW). The highest reducing sugar content was found in *K. alvarezii* and *Gracilaria manilaensis*, which were 34.12 ± 1.09 % DW and 33.02 ± 1.11 % DW, respectively. Two seaweed species, *K. alvarezii* and *G. manilaensis*, were selected for further analyses based on their high sugar and carbohydrate contents. To optimise the saccharification process, factors such as temperature, incubation time, and acid concentration were applied, and based on highest reducing sugar yield and acceptable fermentation, inhibitors generated during hydrolysis the combination of 2.5 % w v^{-1} sulphuric acid, temperature of 120 °C, and 40 min incubation time were selected, which is regarded as milder, but effective parameters for hydrolysis. In the current study, this hydrolysis treatment produced total reducing sugar yields of 34% DW (*K. alvarezii*) and 33 % DW (*G. manilaensis*). Two wild-type yeasts,

plus one industrial grade yeast (*Saccharomyces cerevisiae*, Ethanol Red) were used to ferment sugar in this study. Only *S. cerevisiae* Ethanol Red, resulted in high ethanol yield and was used for further fermentation study. The hydrolysed seaweeds *via* the optimised method were converted to bioethanol, where *S. cerevisiae* resulted in bioethanol yields of 20.90 g L^{-1} (71.0 % of theoretical yield) for *K. alvarezii* and 18.16 g L^{-1} (67.9 % theoretical yield) for *G. manilaensis.* Dilute acid residues of both seaweed species were hydrolysed using enzymatic approach and assimilated to ethanol. The cumulative yield of ethanol of both dilute acid and enzymatic saccharification was 0.14 g g^{-1} biomass using *K. alvarezii*, while cumulative ethanol yield of 0.15 g g^{-1} biomass was achieved using *G. manilaensis*. In the current study, selected seaweed species were subjected to hydrolysis by dilute acid saccharification under mild condition using response surface method. Obtained results indicate that this new strategy can be effective in the saccharification of macroalgal biomass. This study simultaneously illuminated not only potential seaweed resources of Malaysia as feedstock for biofuel, but also challenges pertaining to this subject.

ABSTRAK

Biojisim makroalga marin (rumpai laut) mempunyai potensi sebagai bahan mentah yang penting untuk menghasilkan biofuel. Rumpai laut yang kaya dengan kandungan karbohidrat menunjukkan potensi besar sebagai bahan mentah kompetitif untuk sektor pengeluaran bioetanol. Rumpai Laut sebagai bahan mentah bioethanol yang lebih baik dari segi ekonomi dan mesra alam berbanding dengan jagung dan tebu yang sering digunakan. Rumpai Laut menghasilkan pelbagai polisakarida yang memerlukan keadaan yang berbeza untuk proses saccharification untuk menghasilkan gula yang boleh ditapai kepada alkohol. Langkah penting dalam pengeluaran bioetanol adalah penukaran karbohidrat kepada monosakarida penapaian melalui proses pencairan kimia dengan menggunakan hidrolisis asid atau "*saccharification*" enzim yang lebih bermesra alam, atau mengabungan kedua-dua kaedah tersebut. Dalam kajian ini, 29 rumpai laut Malaysia (11 hijau, 10 merah dan 8 perang) telah dikumpul dari pelbagai habitat dan potensi penghasilan bioethanol telah dianalisiskan. Spesies rumpai laut telah dianalisis untuk mendapatkan jumlah kandungan karbohidrat dengan menggunakan kaedah sulfurik fenol, dan penghasilan gula telah dikaji dengan menggunakan kaedah asid cair hidrolisis. Jumlah kandungan karbohidrat yang paling tinggi dihasilkan daripdada *Kappaphycus alvarezii (*71.22 ± 0.71 % dw) diikut oleh *Eucheuma denticulatum* (69.91 ± 3.35% DW). Kandungan "*reducing sugar*" yang tertinggi ditemui dalam *K. alvarezii* dan *Gracilaria manilaensis* iaitu 34.12 ± 1.09 % DW dan 33.02 ± 1.11 % DW. Dua spesies rumpai laut, *K. alvarezii and G. manilaensis,* telah dipilih untuk pengajian lanjutan berdasarkan kandungan gula dan karbohidrat yang tinggi. Untuk mengoptimumkan proses saccharification, faktor seperti suhu, masa inkubasi dan kepekatan asid telah digunakan dan berdasarkan penghasilan "*reducing sugar*" yang tertinggi serta perencat penapaian dihasilkan semasa hidrolisis yang bergabung dengan 2.5 % w v^{-1} asid sulfurik, suhu 120 °C dan 40 minit masa pengeraman telah dipilih, keadaan ini mungkin dianggap ringan

tetapi masih berkesan untuk proses hidrolisis berlaku.Dalam kajian ini, rawatan hidrolisis menghasilkan jumlah "*reducing sugar*" sebanyak 34 % DW (*K. alvarezii*) dan 33 % DW (*G. manilaensis*). Dua jenis mikroorganisma penapaian (*Saccharomyces cerevisiae*, Ethanol Red) telah digunakan untuk penapaian gula dalam kajian ini. Hanya *S. cerevisiae* , Ethanol Merah menghasilkan kandungan etanol yang tinggi dan telah digunakan dalam kajian seterusnya. Rumpai laut yang telah dihidrolisiskan melalui kaedah yang optimum ditukar kepada bioethanol, kandungan bioetanol *S. cerevisiae* adalah sebanyak 20.90 g L^{-1} bersamaan dengan 71.0 % hasil teori, untuk *K. alvareazii* dan 18.16 g L^{-1} bersamaan dengan 67.9 % hasil teori untuk *G. manilaensis*. Sisa-sisa asid cair bagi kedua-dua spesies rumpai laut telah dihidrolisis menggunakan enzim dan diasimilasikan kepada etanol. Hasil pengumpulan etanol kedua-dua asid cair dan enzim saccharification adalah 0.14 g g^{-1} biojisism dengan menggunakan *K. alvarezii* dan 0.15 g g^{-1} biojisim dengan menggunakan *G. manilaensis*. Dalam kajian ini, spesies rumpai laut yang terpilih dihidrolisis oleh asid cair saccharification di bawah keadaan sederhana menggunakan kaedah gerak balas permukaan. Keputusan yang diperolehi menunjukkan bahawa strategi baru ini boleh adalah berkesan dalam saccharification biojisim macroalgal. Kajian ini bukan sahaja menunjukan rumpai laut Malaysia sebagai sumber yang berpotensi sebagai bahan mentah untuk biofuel, tetapi juga sebagai cabaran dalam bidang ini.

ACKNOWLEDGEMENTS

I would like to begin this acknowledgement by expressing my gratitude to God, who kept me on the correct path throughout the course of my studies. I would also like to thank my first advisor, Professor Dr. Phang Siew Moi, for mentoring me, it is an honour to be your PhD student. Your guidance and attentiveness greatly encouraged my research endeavours and helped me grow as a research scientist. I would also like to thank my second advisor, Dato' Prof. Dr. Aishah Salleh. I am forever appreciative of your contributions in terms of your time and funds, both of which made my PhD more productive and intellectually stimulating. The University of Malaya and the people of Malaysia also deserve a special mention in their role of hosting me during my studies. The members of the Algae Research Lab. and IOES have indeed contributed immensely to my personal and professional development at the University of Malaya. I would like to take this opportunity to thank the past and present Algae lab and IOES members that I had the pleasure of working with, who are, but not limited to Yoon Yen, Sim, VJ, Tan Ji, Victoria, Tan, Mei Cing, Emmie, Fiona, Poh Kheng, Sze looi, Sze Wan, James, Jeannethe, Yong Hao, Wai Kuan, Kok Keong, Rydza,… and the kind officers of IOES. I would also like to mention Reza Rabiei and Jelveh Sohrabipoor, who have been instrumental during the first two years of my PhD. They make excellent friends and were more than happy to share advice(s) and collaborate when needed. I am also grateful for the collaboration we had with Martin during the course of my work. Bahram, who was also a collaborator, deserve a special mention due to his help during my studies and his subsequent friendship. I am also appreciative of Hui Yin, who was instrumental in handling official affairs. The statistical portion of my work I owe to Mahmood Danayee, in ADeC, UM, who patiently taught me statistics. Hong Sok Lai taught me HPLC-RI for some samples also deserve my gratitude. I would also like to mention friends whose enthusiasm are infectious; Vahab, Arman, Shahrooz, Vahid, Mohamad Reza, and beloved cousins, Adel and Hana.

I would like to take this opportunity to acknowledge the funds that made my PhD possible; IPPP, UM, and the Ministry of Science, Technology and Innovation Malaysia.

Last, but certainly not least, I would like to thank my family for all of their support and encouragement in the course of my work. My dear uncle, Dayee Nasser, who was always supportive, was more to me than an uncle. The love of my siblings, Nooshin, Narges, Maedeh and Sadegh, kept me going, and my loving, supportive, encouraging, and patient wife, Hoda, who remained faithfully supportive during the final stages of my PhD, deserve my special thanks. Also, my parents, who raised me with the love of science, supportive of my pursuits; my father who gave me the strength to keep going, and my mother, who remained supportive even during her battle with cancer. Love you Mom! Thanks all!

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May 2017

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percent
°C	:	Degree Celsius
µL	:	Microliter
µm	:	micrometre
ANOVA	:	Analysis of variance
AOAC	:	Association of Official Analytical Chemists
CBP	:	Consolidated Bioprocessing
CCD	:	Central Composite Design
Chl	:	Chlorophyta
Conc	:	Concentration
CV	:	Coefficient of variation
DNS	:	3, 5-dinitrosalycylic acid
DW	:	Dry Weight
EtOH	:	Ethanol
FAO	:	Food and Agriculture Organization
Fin R. Sugar	:	Final Reducing Sugar
FPU	:	Filter Paper Unit
FW	:	Fresh Weight
g	:	Gram
GC	:	Gas Chromatography
GC-FID	:	Gas Chromatography- Flame Ionization Detector
h	:	Hour
5-HMF	:	5- hydroxyl methyl furfural
HPLC-PDA	:	High Performance Liquid Chromatography- Photo Diode Array

Hyd	:	Hydrolysate
Ini R. Sugar	:	Initial Reducing Sugar
IS	:	Internal Standard
g L^{-1}	:	Gram per Litre
kg	:	Kilogram
kg m^{-2} $year^{-1}$	:	Kilogram per square meter per year
L	:	Litre
L. $ha^{-1\cdot}$ $year^{-1}$	:	Litre per hectare per year
m	:	Meter
M	:	Molar
mm	:	Millimetre
min	:	Minute
mg	:	Milligram
mg L^{-1}	:	Milligram per Litre
mg g^{-1}	:	Milligram per Gram
mL	:	Millilitre
mMol	:	Milli Mole
MTBE	:	Methyl tert-butyl ether
N.A.	:	Not Available
N/D	:	Not detected
nL	:	Nano litre
ns	:	Not Significant
NSSF	:	Non-isothermal Simultaneous Saccharification and Fermentation
pAm	:	Pico Ampere Meter
Phy	:	Phaeophyta
ppm	:	Part per million

R^2	:	Coefficient of determination
Rhd	:	Rhodophyta
rpm	:	Round per minute
RS	:	Reducing Sugars
RSM	:	Response Surface Methodology
S.A.	:	Sulphuric Acid
SD	:	Standard Deviation
SHF	:	Separate Enzymatic Hydrolysis and Fermentation
SSCF	:	Simultaneous Saccharification and Co-fermentation
SSF	:	Simultaneous Saccharification and Fermentation
temp	:	Temperature
TFA	:	Trifluoroacetic acid
TPC	:	Total phenolic compounds
UV	:	Ultra violet
Vol	:	Volume
v v^{-1}	:	Volume per volume
w v^{-1}	:	Weight per Volume
w w^{-1}	:	Weight per Weight

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

The marine macroalgae, also known as seaweeds, can be categorized generally as the green algae (Chlorophyta), brown algae (Phaeophyta) and red algae (Rhodophyta). Seaweeds are the main resource materials for phycocolloids such as agar, carrageenan (derived from Rhodophyta) and alginates (derived from Phaeophyta) (Abbott, 1982). The residues from such processing also represent a renewable source of energy (Ross *et al.*, 2008).

Seaweeds have a wide spectrum of advantages to being used as a feedstock for biofuel production. Seaweeds are capable of producing high yields of material when compared to even the most productive land-based plants. Kelp forests in shallow sub-tidal regions are amongst the most productive communities on earth, generating large amounts of organic carbon. In Nova Scotia, laminarian beds produce 1.75 kg organic carbon m^{-2} $year^{-1}$, but an average of 1.0 kg organic carbon m^{-2} $year^{-1}$ is more typical of kelp beds in general (Sze, 1993). When considering the dry weight generated, production figures between 3.3 and 11.1 kg m^{-2} $year^{-1}$ for non-cultured macroalgae are cited (Gao & McKinley, 1994). This is due to this fact that seaweeds have higher photosynthetic activity (6 – 8 %) than terrestrial biomass (1.8 – 2.2 %). This also leading to the increased CO_2 absorption by seaweeds (Aresta *et al.*, 2005).

The issues arising with increasing the proportion of land used for biofuel crops and the "food versus fuels" debate are not applicable to the seaweeds (Adams *et al.*, 2009) because the algal feedstock can be cultivated on otherwise non-productive land that is unsuitable for agriculture or in brackish, saline, and waste-water that has little-competing demands. Using algae to produce feedstock for biofuel production could have little impact on the production of food and other products derived from terrestrial crops, unlike the use of corn or sugar-cane (Searchinger *et al.*, 2008; Hughes *et al.*, 2012).

Algae have the potential to reduce the generation of greenhouse gases (GHG) and to recycle CO_2 emissions from flue gases from power plants and natural gas operations as indicated by preliminary life cycle assessments (Darzins *et al.*, 2010). Also, algae remain exempt from the negativity associated with terrestrial biomass resources, which is said to be responsible for higher food prices and which impacts water sources, biodiversity, and rainforests (Chynoweth, 2005). Another advantage of using seaweed is the low lignin content which improves the enzymatic hydrolysis of cellulose. Being immersed in water, the seaweeds do not require the support from lignified tissue and are able to absorb nutrients through the entire surface of the thallus. This saving of energy results in many seaweeds having higher biomass productivity (13.1 kg DW m^{-2} over 7 months) than land plants (0.5 – 4.4 kg DW m^{-2} $year^{-1}$) (Lewandowski *et al.*, 2003).

A diversity of useful products including food, feed, medicine and industrial materials can be produced from the seaweeds. The Phaeophyta and Rhodophyta are economically more important because they contribute 66.5 % of annual production of 4 million tones globally, of which 2.6 million tones are brown and 33 % are red seaweeds (Sahoo, 2002). The phycocolloids, comprise alginate which is produced from the brown seaweeds, and agar and carrageenan that are sourced from the red seaweeds.

The most important component of the seaweeds with regards to the production of bioethanol is the carbohydrate, which also plays an important role in the metabolism of the seaweeds, as it supplies the energy needed for respiration and other important processes (Bramarambica *et al.* 2014). Green algae accumulate cellulose as the cell wall carbohydrate, which can be used for ethanol production after enzymatic hydrolysis using cellulase (Dibenedetto, 2011). The resultant sugars are then fermented to bioethanol. The red and brown seaweeds produce different forms of carbohydrate which may or may not be easily converted to sugar through saccharification.

Presently, food crops like sugar-cane and corn are used as feedstocks for bioethanol production (Karimi & Chisti, 2015). According to Adams *et al.* (2009), by considering average world yield of different crops, sugar-cane as the most productive terrestrial crop can produce 6756 (L $ha^{-1,}$ $year^{-1}$) bioethanol, whereas this yield interestingly could reach 23,400 (L ha^{-1} $year^{-1}$) for the seaweeds. Use of seaweeds as feedstocks will not compete with their use as food, and there will be no conflicts with other land uses such as urban development or other agricultural and industrial usage.

Malaysia is rich in marine algal resources (Phang *et al.,* 2007) including species belonging to the Chlorophyta and Rhodophyta which contain biomaterial suitable for bioconversion into biofuel (Phang, 2006). While there have been reports of bioethanol production from tropical seaweeds (Khambhaty *et al.,* 2012; Kumar *et al.,* 2013, Meinita *et al.2013;* Mutripah *et al.,* 2014), the potential of using indigenous Malaysian seaweeds has not been explored.

Malaysia has a steadily expanding seaweed industry based mainly on the carrageenophytes *Eucheuma* and *Kappaphycus*. There are many other tropical seaweeds that may be commercialised if shown to be a good feedstock for bioethanol production. The search for suitable tropical seaweeds has started, and the work carried out in this thesis is to answer the question of whether local seaweed species abundantly found in Malaysia can serve as competitive feedstocks for bioethanol production.

The objective of this project was to obtain the profiles of common seaweed species in Malaysia for selection of potential species for production of bioethanol. Optimization of saccharification was conducted, followed by fermentation.

This was achieved through the following sub-objectives.

i) To collect and analyse the carbohydrate and sugar content of Malaysian seaweeds.

ii) To select two seaweeds with the potential to serve as feedstock for bioethanol production based on high carbohydrate content and type of sugar.

iii) To optimize the saccharification process for selected seaweed.

iv) To produce ethanol from selected seaweeds.

Research outputs

This research generated the following outputs.

i) List of Malaysian seaweed species and their profiles with respect to carbohydrate and sugar contents.

ii) List of Malaysian seaweeds that meet the requirements for bioethanol production.

iii) A protocol for saccharification of the seaweed carbohydrates.

iv) The potential bioethanol yield from selected seaweeds.

Figure 1.1 shows the research approach.

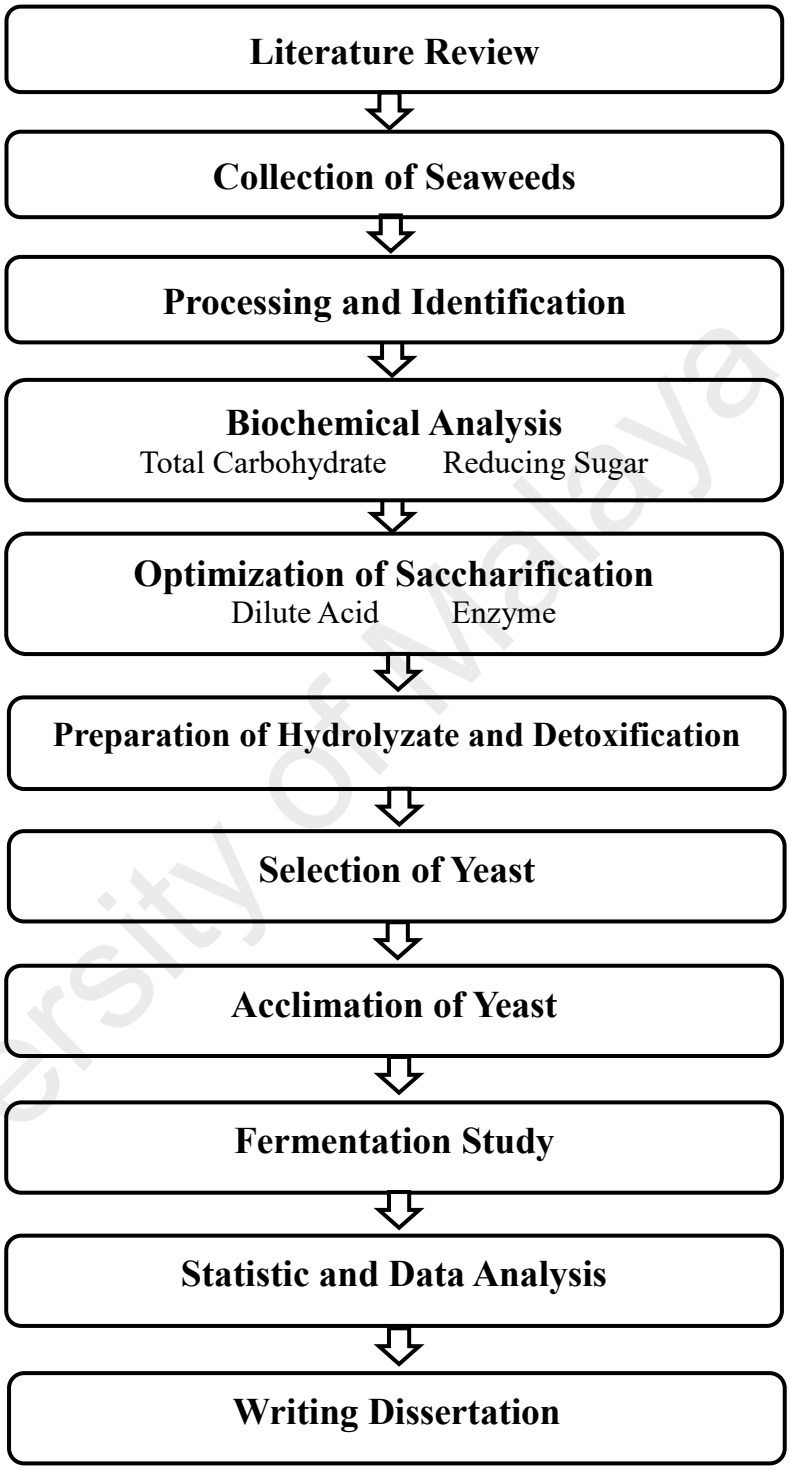

Figure 1.1: Flow-chart of research approach

CHAPTER 2: LITERATURE REVIEW

2.1 Renewable energy and biomass

Concerns over depletion of fossil fuel resources, fuel security, global warming and increasing fuel price have generated great attention towards finding alternative sources of energy to ensure the current rate of development. Renewable energy sources are essential contributors to the energy supply portfolios that contribute to world energy supply security. The advantages of renewables are well known, as far as they enhance diversity in energy supply markets; secure long-term sustainable energy supplies; reduce local and global atmospheric emissions; create new employment opportunities offering possibilities for local manufacturing and enhance security of supply since they do not require imports that characterize the supply of fossil fuels (Goldemberg & Coelho, 2004). Biomass, hydro, geothermal, wind, solar and tide are the most known types of renewable energy. Biomass, currently contributes 10 – 12 % of gross worldwide energy, due to geographical, economic, and climatic differences, the share of biomass energy in relation to total consumption differs widely among different countries, ranging from less than 1 % in some industrialized countries like the United Kingdom and The Netherlands to significantly more than 50 % in some developing countries in Africa and Asia (Kaltschmitt *et al.*, 2002). Biomass is a well-established source (80 % of total renewable energy production) of renewable energy; however, hydropower may have a higher potential than biomass (Resch *et al.*, 2008).

It is well understood that bioenergy has been used since the humans discovered how to use biomass for making fire. Biomass was the main source of energy until fossil fuels were discovered during the industrial revolution (Quaschning, 2010). Evidence of ethanol production (winemaking) gathered from residues found in the Middle East was dated back to 6,000 years ago (Berkowitz, 1996). The technology of ethanol production has progressed greatly, and it may readily be applied. Nevertheless, improvement in process

efficiency and search for cheaper and sugar-rich sources still continue (Knothe, 2010). The idea of using algae for industrial fuel production is over 60 years old (Borowitzka, 2008). At the beginning, biofuel was produced from land-crops such as corn, sugarcane, wheat or potato. The major issue with these first generation biofuel is competition with their use as food, although the process may be economic and environmentally friendly. The second generation biofuels were developed using mainly non-food feedstock such as grass, forest residues or lignocellulosic materials. The technology for industrial production of the second generation biofuel is still under development, especially with regards to reduction in the cost of production (Naik *et al.*, 2010). The third generation biofuels are derived from marine biomass, mainly from seaweeds and micro-algae (Wei *et al.*, 2013).

2.1.1 What are seaweeds?

The algae can be divided by size into two groups: macro-algae commonly known as 'seaweed' and micro-algae, microscopic single cell organisms ranging in size from a few micrometres to a few hundred micrometre (µm) (Sheehan *et al.*, 1998). The term micro-algae is often used to include the prokaryotic cyanobacteria (blue-green algae), although these are no longer classified as algae, together with the eukaryotic microalgae such as diatoms and green algae (Mata *et al.*, 2010).

Seaweeds can be classified according to their characteristics into four groups. Dissimilar to unicellular microalgae, the seaweeds are multicellular and have more plant-like structures. They generally comprise very specific structures such as holdfast, frond and the stipe (Figure 2.1).

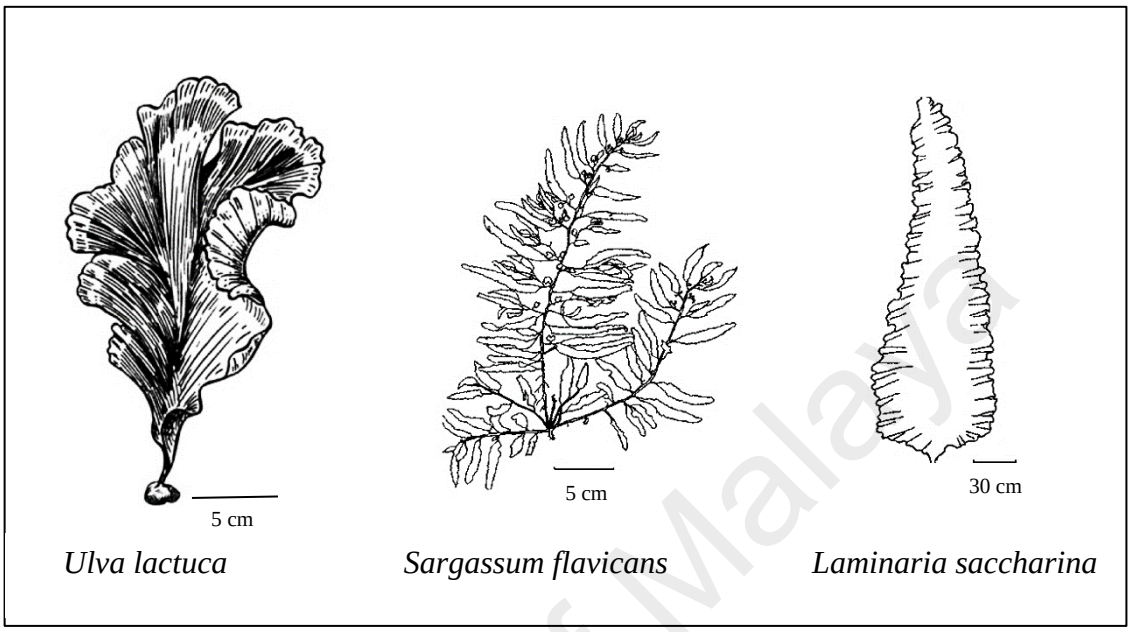

Figure 2.1: Various forms of the seaweeds.
Redrawn from:U. lactuca (Balzert, 1999); *S. flavicans* & *L. saccharina* (http://www.fao.org/docrep/006/y4765e/y4765e07.htm)

Even though seaweeds are restricted to the tidal zones and benthic photic zones, they contribute to about 10 % of the total world marine productivity (Israel *et al.,* 2010). Ecologically, they provide food, shelter and nursery grounds for marine life, and are also involved in nutrient cycling (Phang *et al.,* 2010).

2.1.2 Algae and the environment

During algal growth and photosynthesis, they remove CO_2 from the atmosphere. This gas is released again when their biomass is consumed in the various ways. However algae may provide a carbon-neutral or even a carbon reducing system if appropriate steps are taken, for example if the biomass is used to replace fossil fuel which consumes more energy in its production. In addition, algal residues after extraction of biofuel precursors, could be put to good use as mineral-rich fertilizer (Israel *et al.,* 2010). Seaweeds play

significant roles in the normal functioning of atmospheric environments. Globally changing environments on earth is more likely to severely modify the current equilibrated terrestrial and marine ecosystems (Pinto, 2013). Specifically for the marine environment, global changes will include increased carbon dioxide which will acidify the aqueous media. It has been estimated that for CO_2, the change might be from the current 350 ppm to approximately 750 ppm within 50 years, or so. Such a difference will cause higher average seawater temperatures (within 1 – 3 °C) and higher UV radiation on the water surface. These changes will affect seaweeds at different levels, namely molecular, biochemical, and population levels. While predictions of altered environments have been studied extensively for terrestrial ecosystems, comparatively much less effort has been devoted to the marine habitat. Seaweeds may also contribute significantly to pollutant reduction (heavy metals, and excessive nutrients disposed of into the marine environments) (Israel *et al.,* 2010).

2.2 Algae and biofuel

According to predictions, demand for sustainable biofuels will increase but the consumption of first generation biofuels in order to meet this goal, may result in negative environmental impacts.

Third-generation biofuels are recommended as a good solution as they can be cultivated on marginal or non-agronomical area, can use brackish water and seawater and may be more productive than former biofuel generations.

The current seaweed industry is 100 times bigger than the micro-algal industry. In 2012, 54 % of the world's seaweed produced in China which was accounted for over 12.8 million wet tonnes of the annual world production (Roesijad *et al.,* 2010; FAO, 2014).

Seaweed cultivation for bioethanol and biogas is being explored in Asia, Europe and South America, while bio-butanol from macro-algae is attracting research interest and investment in the USA.

2.2.1 Production of energy from biomass

Seaweed can be used to produce energy in various ways which can be direct combustion, pyrolysis, gasification, liquefaction, bioethanol and biomethane.

2.2.1.1 Direct combustion

Currently, direct combustion is the main method by which biomass is used to produce energy (Demirbaş, 2001). Many industries devote a considerable amount of energy to the production of steam, with the pulp and paper industry using 81 % of its total energy consumption for this purpose (Saidur *et al.,* 2011). The co-combustion of biomass with coal-fired plants is an attractive way to use biomass (Demirbaş, 2001; Saidur *et al.,* 2011). The co-generation of heat and electricity can significantly improve the economics of biomass combustion, but requires that there is a local demand for heat (Demirbaş, 2001).

It should be noted that in case of macroalgal biomass, the moisture content can reduce the heat production compared to dry biomass by 20 % (Demirbaş, 2001) and the direct combustion of biomass is feasible only for biomass with a moisture content of less than 50 % (McKinney, 2004; Varfolomeev & Wasserman, 2011). Also as seaweeds have a high amount of ash content, this also must be a considerable problem in the direct combustion of biomass due to fouling of the boilers restricting the use of high ash content biomass (Demirbaş, 2001).

2.2.1.2 Pyrolysis (bio-oil)

The using of bio-oil goes back to the time when the Egyptians discovered the way to produce tars by applying the pyrolysis of wood (Demirbaş, 2001). Fast and slow pyrolysis are two type of hydrolysis but fast pyrolysis is of the most promising thermochemical processes which produces a solid and volatile products. The products proportion is influenced by feedstock properties and operation parameters (Briens *et al.,* 2008). Fast pyrolysis is capable of achieving greater liquid product and gas yields of around 70 % – 80 %, compared to 15 % – 65 % achieved through slow pyrolysis (Varfolomeev & Wasserman, 2011). To obtain high yields of valuable liquid products or bio-oil, the biomass particles must be rapidly heated and the residence time of volatile products must be short (Briens *et al.,* 2008).

Various investigations have been conducted on producing bio-oil from lignocellulosic biomass such as sawdust, rice straw, corn cob straw and oreganum stalks, cherry and grape seeds, switch grass, etc. (Yanik *et al.,* 2013). Besides lignocellulosic biomass, some articles have been published on the feasibility of bio-oil production from macroalgal biomass (Miao & Wu, 2004; Wang *et al.,* 2013b; Bermúdez *et al.,* 2014). It is reported that, overall efficiency of the pyrolysis of seaweed is lower than that derived from lignocellulosic materials due to presence of high ash and also metal ions content in the seaweeds (Yanik *et al.,* 2013). Bio-oil has the potential to be transported and stored and generate more energy in comparison with char and syngas (Jena & Das, 2011). This makes bio-oil more interesting biofuel than char and syngas.

2.2.1.3 Gasification

During the gasification process which is carried out under high temperature (800 - 1000 °C), organic matter is converted to a combustible gas mixture which contains carbon

monoxide (20 - 30 %), hydrogen (30 - 40 %), methane (10 - 15 %), ethylene (1 %), nitrogen and water vapour. This gas mixture which is known as syngas has a calorific value of 4 - 6 M J m^{-3} (Demirbaş, 2001; McKendry, 2002; Saidur *et al.,* 2011). Syngas can be combusted to generate heat or electricity in the combined gas turbine systems that can produce an electric energy yield of 50 % of the heating value of the incoming gas. In this process, dry biomass is required to be utilized (Guan *et al.,* 2012), but for some biomass feedstock which contain high moisture, such as seaweed, supercritical water gasification (SCWG) can be employed. Moreover, the produced syngas can be converted to hydrogen or methanol that can be utilized in transportation (McKendry, 2002; Saidur *et al.,* 2011).

Increasing temperature from 302 to 652 °C, yield of the syngas increase, in agreement with a recent model of the kinetics of supercritical water gasification that indicates that higher temperatures favour generation of intermediates which are more easily gasified together with the production of gas at the expense of char (McKendry, 2002; Saidur *et al.,* 2011).

2.2.1.4 Liquefaction

Liquefaction is a low-temperature high-pressure process where biomass is converted into a stable liquid hydrocarbon fuel (bio-oil) in the presence of a catalyst and hydrogen.

In the presence of a catalyst, at the high temperature and wet environment, biomass is converted to hydrocarbons which is partially oxygenated (Demirbaş, 2001; McKendry, 2002). It is now shown that liquefaction treatment is not attractive in terms of industrial views, due to its feed system complexity and also higher costs than other processes (Demirbaş, 2001; McKendry, 2002). However this procedure has the advantage of the

conversion being carried out in an aqueous condition; therefore a prior drying process is not necessary (Minowa *et al.,* 1995; Brown *et al.,* 2010).

2.2.1.5 Biomethane

Biomethane fermentation is considered as a highly complex process which is partitioned into four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis, where in each stage, different groups of microorganisms are involved (Angelidaki *et al.*, 1993). Hydrolysing and fermenting microorganisms excrete enzymes to attack the polymers to generate simpler compounds such as hydrogen, acetate and also volatile fatty acids such as butyrate and propionate. Most of the microorganisms in this stage are strict anaerobes such as *Bifidobacteria*, *Clostridia* and *Bacteriocides.* However some facultative anaerobes also take part in this stage, including Enterobacteriaceae and *Streptococcus*. During the third stage, the obligate acetogenic bacteria convert the higher volatile fatty acids into hydrogen and acetates (Bagi *et al.,* 2007), and at the end, methanogenic bacteria produce methane from acetate or hydrogen and carbon dioxide (Schink, 1997).

In the industrial point of view, producing biomethane from wet biomass such as seaweeds is highly attractive. A great amount of articles have been published on the biogas production by different sources of organic materials as well as some of the recent researches on evaluating biofuel from seaweed biomass (Golueke *et al.,* 1957; Weiland, 2010; Hughes *et al.,* 2012; Vanegas & Bartlett, 2013; Marquez *et al.,* 2014; Vanegas *et al.,* 2015; Montingelli *et al.,* 2016; Tabassum *et al.,* 2016).

Seaweeds have been successfully digested to produce biogas at a low concentrations (< 1% DW), however a process that can allow for use of higher biomass concentrations

are more attractive and profitable (Oswald, 1988). Another advantage of anaerobic digestion can be the reuse of residual nutrients to enrich the seaweed farm systems (Singh & Olsen, 2011). The yield of biomethane from seaweeds have been reported between 0.09 to 0.34 cubic meters kg^{-1} of VS (Zamalloa *et al.*, 2011; González-Fernández *et al.*, 2012).

2.2.1.6 Bioethanol

Ethanol fermentation is a biological process in which reducing sugars are converted by microorganisms to ethanol and CO_2 (Lin & Tanaka, 2006). Bioethanol can be extracted from a variety of feedstocks that possess fermentable sugars generally in a mixture of polysaccharides and free sugars. Table 2.1 gives a summary of studies on ethanol production from various feedstocks.

The microorganisms used for ethanol production are divided into three categories which are mold (mycelium), bacteria (*Zymomonas* spp.) and most commonly, yeast (*Saccharomyces* spp.). These microorganisms that are isolated from the natural environment are highly selective in their substrates, metabolism and other fermentation characteristics. Some of these microorganisms can be very dependent on hexoses such as glucose and galactose or pentose such as xylose or sometimes mixtures of hexose and pentose sugars (Naik *et al.*, 2010).

Presently all vehicles, without adjusting the engine, can be run on a mixture of 10 % ethanol and 90 % gasoline. With more progress in engine technology, even consumption of higher ethanol content in fuel can become feasible. Some engines can run on 100 % ethanol whereas there are flexible-fuel cars that are capable of utilizing 85 % ethanol (E85). Diesel can also be replaced by ethanol provided that emulsifiers are used to

enhance diesel and ethanol mixing (Galbe & Zacchi, 2002). Ethanol is blended with gasoline due to its high octane number leading to increased octane number of the mixture. This would reduce the need of MTBE, the main octane enhancing additive which is considered as a carcinogenic compound. Use of ethanol can lead to reduction of carbon monoxide and other hazardous hydrocarbons as it provides oxygen for the gasoline mixture (Galbe & Zacchi, 2002). Replacement of compression-ignition and spark-ignition engines for the use of higher content of ethanol (E85), was summarized by Baily (1996). He concluded that in compression-ignition engines, ethanol possesses almost the same overall transport efficiency compared to diesel (Bailey 1996). Therefore, although ethanol possesses only about two-thirds of the energy content of gasoline, it will still be possible to run 75 – 80 % of the distance on the same amount of ethanol (Wyman 1996).

Table 2.1: Reducing sugar and bioethanol yields of some land-crops.

Biomass type (plant)	Treatment Condition	RS Yield	Yeast Spp.	EtOH% v/v	EtOH Yield	TEY %	Reference
Straw (Rice)	Enzymatic, pH 5, 45 °C	0.72 g g^{-1}	*S. cerevisiae*	N.A	0.41 g g^{-1} sugar	N.A	Abedinifar *et al.* (2009)
Bagasse (Sugarcane)	Ball milling/ Enzymatic, pH 5, 45 °C	Glucose: 89 % Xylose: 77 %	*Pichia stipitis*	0.84	0.29 g g^{-1} sugar	56.9	Buaban *et al.* (2010)
Straw (Rye)	Wet oxidation/ Enzymatic, pH 4.8, 50 °C	Glucan: 0.40 g g^{-1} Xylan: 0.22 g g^{-1}	*S. cerevisiae*	N.A	0.15 g g^{-1} DW	66	Petersson *et al.* (2007)
Straw(Oilseed rape)	Wet oxidation/ Enzymatic, pH 4.8, 50 °C	Glucan: 0.27 g g^{-1}Xylan: 0.15 g g^{-1}	*S. cerevisiae*	N.A	0.10 g g^{-1} DW	70	Petersson *et al.* (2007)
Straw (Faba bean)	Wet oxidation/ Enzymatic, pH 4.8, 50 °C	Glucan: 0.28 g g^{-1} Xylan: 0.12 g g^{-1}	*S. cerevisiae*	N.A	0.08 g g^{-1} DW	52	Petersson *et al.* (2007)
Straw (Wheat)	Dilute acid pretreatment/ Enzymatic, pH 5, 45 °C	7.83 w v^{-1}	*E. coli*	1.9	0.24 g g^{-1} DW	N.A	Saha *et al.* (2005)

RS: Reducing Sugars; TEY: Theoretical Ethanol Yield %, EtOH: Ethanol

Table 2.1: (Continued)

Biomass type (plant)	Treatment Condition	RS Yield	Yeast spp.	EtOH % v v^{-1}	EtOH Yield	TEY %	Reference
Hull (Rice)	Dilute acid pretreatment/ Enzymatic, pH4.8, 50 °C	N.A	*S. cerevisiae*	0.44	0.49 g g^{-1} sugar	84	Dagnino *et al.* (2013)
Bagasse (Sweet Sorghum)	NaOH pretreatment/ Enzymatic pH4.8, 45 °C	200 g L^{-1}	*Mucor hiemalis*	N.A	0.48 g g^{-1} glucose	81	Goshadru *et al.* (2011)
Raw Starch (Corn)	Direct hydrolysis and fermentation	N.A	*S. cerevisiae*	6.18	0.44 g g^{-1} sugar	86.5	Shigechi *et al.* (2004)
Molasses (Sugarcane)	Direct fermentation	N.A	*S. cerevisiae*	7.8	N.A	76.3	Nofemele *et al.* (2012)
Molasses (Sugarcane)	Direct fermentation	16 % w v^{-1}	*Z. mobilis*	9.3	N.A	90.5	Khoja *et al.* (2015)
Sweet potato	Enzymatic, pH5.8, 86 °C	150 g L^{-1}	*S. cerevisiae*	9	N.A	N.A	Lareo *et al.* (2013)
Potato	Dilute acid pretreatment/ Enzymatic	69 g L^{-1}	*S. cerevisiae*	2.1	N.A	60	Khawla *et al.* (2014)

RS: Reducing Sugars; TEY: Theoretical Ethanol Yield %, EtOH: Ethanol

Currently, bioethanol derived from sugarcane in Brazil is the only economically feasible biofuel that shows a significant net energy gain (Walker, 2010). By utilizing sugarcane as bioethanol feedstock, a huge amount of bagasse are produced. This can be combusted to generate heat for distillation of bioethanol, although this process has led to some environmental concerns and it is suggested that it may be more beneficial to enzymatically convert bagasse to bioethanol rather than burn it (Gressel, 2008).

Providing that the bioethanol fermentation technology can be economically feasible, with the huge amounts of feedstock available globally, it is estimated that by converting crop residues and wastes to bioethanol, about 380 million metric tonnes equal to 16 times higher than the current worldwide production of bioethanol can be produced (Balat *et al.*, 2008).

One of the technical obstacles in industrial conversion of crop waste into bioethanol is presence of lignin and hemicellulose and also crystallinity of cellulose which reduce the yield of saccharification (Gressel, 2008). Seaweeds contain very low amounts of lignin and hemicellulose, thus it is more amenable for enzymatic conversion to reducing sugars.

2.3 Use of seaweed biomass as feedstock for bioethanol production

Seaweeds are generally grouped into the green, red and brown seaweeds, which contain a diversity of carbohydrates, which exhibit different degrees of ease in saccharification, and also produce different sugars. All these influence the use of different species of seaweeds for bioethanol production, and process optimisation may have to be species-specific.

There are various methods for processing the seaweed biomass prior to fermentation. The biomass must be harvested and processed according to protocols to ensure that the

quality of the carbohydrate has not been reduced. The biomass has to undergo a series of processes including saccharification, fermentation, distillation and recovery and residue processing.

2.3.1 Saccharification of seaweed biomass

The carbohydrate polymers in the seaweed biomass need to be digested to monomers before the fermentation process through a process called saccharification. Various approaches are available for biomass saccharification but the most well-known methods are grouped into enzymatic and chemical hydrolysis (Taherzadeh & Karimi, 2007a). In addition, there are other hydrolysis methods in which no chemicals or enzymes are applied. For instance, lignocelluloses may be hydrolysed by gamma-ray or electron-beam irradiation or microwave irradiation. However, these processes are far from being commercially applied (Taherzadeh & Karimi, 2007a). Other saccharification approaches beside enzymatic or chemical treatments include electron-beam irradiation, gamma-ray microwave, that still require further development for commercial application (Taherzadeh, 1999). Seaweed carbohydrate is very different from land-crop biomass which have high carbohydrate content and ease of hydrolysis to fermentable sugars (Kim *et al.*, 2015).

Seaweeds contain unique carbohydrate compositions. Besides starch, cellulose, agar, carrageenan, alginate, they may also contain mannitol and laminarin, making them distinctively different from terrestrial biomass. Thus, it is important to apply appropriate methods to seaweed biomass and to select appropriate microorganisms that are pivotal for successful bioethanol fermentation (Tan & Lee, 2014). Table 2.3 illustrates a comparison of various chemical saccharification procedures and fermentation strategies with different microorganisms used to produce ethanol from different seaweed species.

2.3.1.1 Chemical hydrolysis

Hydrolysis includes breaking the carbohydrate polymer and randomly cleaves the constituents in the material to monomers. Cellulose breaks to glucose, hemicellulose gives some different hexoses and pentose sugars such as xylose, arabinose and glucose (Taherzadeh & Karimi, 2007a).

Acid hydrolysis of plant lignocellulosic biomass has been known since 1819. Examples are the modified Bergius process (40 % HCl) operated during World War II in Germany, and the more recently modified Scholler processes (0.4 % H_2SO_4) in the former Soviet Union, Japan and Brazil (Galbe, 2002).

however other acids such as hydrochloric acid also have been well applied (Wright & Power, 1986; Hashem & Rashad, 1993). Acid hydrolysis is mostly carried out by two methods, a) dilute-acid hydrolysis b) concentrated acid hydrolysis (Taherzadeh & Karimi, 2007a). A comparison between two methods is illustrated in Table 2.2.

a. Concentrated acid hydrolysis

This process was first discovered by Braconnot in 1819 (Sherrard & Kressman, 1945) where they found concentrated acid can convert cellulose to glucose. This process is conducted with a high concentration of acid (30 – 70 %) and at low temperature (30 - 40 °C) with a very high yield of glucose production (90 % of theoretical) therefore more ethanol yield is achievable in compare with dilute-acid treatment (Taherzadeh & Karimi, 2007a). Beside high yield of this method, use of this method might be extremely dangerous due to a corrosive attribute of concentrated acid specially once temperature increases and expensive as specialized acid resistant material must be used in reactors with high level of safety. Also acid recovery which is highly energy demanding process

is another bottleneck of this method (Taherzadeh & Karimi, 2007a) however Van Groenestijn, Hazewinkel & Bakker (2006) presented a method to use concentrated acid sulphuric and recover it by biological process and anion-selective membranes. In biological part, resulted sulphate reduced to sulphide *via* anaerobic process and sulphide is recovered as H_2S gas and then burned into sulphur dioxide and sulphur trioxide followed by conversion into sulphuric acid.

Table 2.2: Comparison between two acid hydrolysis approaches (Taherzadeh & Karimi, 2007a).

Hydrolysis type	Advantages	Disadvantages
Concentrated acid process	- Conducted at low temperature - High reducing sugar production	- High acid use - Risk of equipment corrosion - High energy use for acid recovery - Longer incubating time
Dilute-acid process	- Low acid use - Short incubating time	- High incubating temperature - Low reducing sugar production - Risk of equipment corrosion -Generation of fermentation inhibitors

b. Dilute-acid hydrolysis

Dilute-acid hydrolysis is the commonly applied chemical hydrolysis and can be used either as a pre-treatment or as the actual method of hydrolysing biomass to fermentable sugars (Qureshi & Manderson, 1995). It is reported that the first process was more likely

the Scholler process where the condition of 0.5 % sulphuric acid at 11-12 bar pressure for 45 min was applied to convert the lignocellulosic material into sugars (Faith, 1945). Single stage hydrolysis in batch reactors has been widely applied for the kinetic study of the hydrolysis of biomass to ethanol production in pilot or laboratory scales (Taherzadeh & Karimi, 2007a). The main drawback of single stage hydrolysis is degradation of parts of sugar that release from less resistant polymers into fermentation toxins such as 5-hydroxymethylfurfural, furfural, formic acid, vanillic acid, phenol, acetic acid, formaldehyde, etc. (Larsson *et al.*, 1999). It is recommended that dilute-acid hydrolysis is conducted in more than one stage (generally two stages) to avoid degradation of sugars. At the first stage, less resistant polymers convert to monosaccharides under a mild condition, while in second treatment, the residues which are more crystalline (such as cellulose) undergoes more severe condition (Nguyen *et al.*, 2000). A temperature range 140 - 170 °C can be applied in one stages hydrolysis while the temperature of 120 °C for a longer time may be used for two stages treatment (Kim *et al.*, 1993). A comparison of saccharification and fermentation yield using different seaweed species is shown in Table 2.3.

Table 2.3: Comparison of chemical saccharification and ethanol yields from different seaweed biomass.

Seaweed spp.	Type	Treatment condition Agent/ Conc/ time/ temp	Initial Conc RS g L^{-1}	RS Yield (g RS g^{-1} seaweed)	Microorganism used	EtOH Conc % v v^{-1}	EtOH Yield (g EtOH g^{-1}RS)	Fermentation yield %	Reference
Laminaria hyperborea	Phy	S.A/ pH2/ 60min/ 65 °C	20	N.A	*Pichia angophorae*	N.A	0.43	84	Horn *et al.* (2000)
Undaria pinnatifida	Phy	S.A/ 0.7%/ 60 min/ 121 °C	28.65	N.A	*Pichia angophorae*	0.942	N.A	27	Cho *et al.* (2013)
Saccharina japonica	Phy	S.A/0.4% & Saccharification with *Bacillus* sp.	45.6	N.A	*Pichia angophorae, Pichia stipites, S. cerevisiae, Pachysolen tannophilus*	0.77	0.33	NA	Jang *et al.* (2012)
Saccharina latissima	Phy	S.A/ pH=6/ 30 min /23 °C	N.A	N.A	*S. cerevisiae*	0.45	N.A	N.A	Adams *et al.* (2009)

Abbreviation: Chl: Chlorophyta, Rhd: Rhodophyta, Phy: Phaeophyta, RS: Reducing Sugar, EtOH: Ethanol, S.A: Sulphuric Acid, Conc: Concentration, Temp: Temperature, N.A: Not Available

Table 2.3: Continued

Seaweed spp.	Type	Treatment condition Agent/ Conc/ time/ temp	Initial Conc RS g L^{-1}	RS Yield (g RS g^{-1} seaweed)	Microorganism used	EtOH conc % v v^{-1}	EtOH Yield (g EtOH g^{-1} RS)	Fermentation yield %	Reference
Gelidium amansii	Rhd	S.A/ 2.5%/ 150 °C	N.A	0.42	*Brettanomyces custersii*	2.7	N.A	38	Park *et al.*, (2012)
Gelidium amansii	Rhd	S.A/ 1% / 60 min/ 121 °C	43.5	N.A	*Scheffersomyces stipitis*	2	N.A	91	Ra *et al.*, (2013)
Kappaphycus alvarezii	Rhd	S.A/ 2%/ 15 min/ 130 °C	4.4	N.A	*S. cerevisiae*	0.16	N.A	66	Meinita *et al.*, (2012)
Palmaria palmata	Rhd	S.A/ 4%/ 25 min/ 125 °C	N.A	0.16	*S. cerevisiae*	N.A	0.012	24	Mutripah *et al.*, (2014)
Kappaphycus alvarezii	Rhd	S.A/1%/ 5 min/ 140 °C	38.3	0.31	*Kluyveromyces marxianus*	1.6	0.42	N.A	Ra *et al.* (2016)
Gracilaria corticata ***(spent biomass)***	Rhd	S.A/ 1%/ 15 min / 120 °C	N.A	0.13	*S. cerevisiae*	0.3	0.10	N.A	Sudhakar *et al.*, (2016)

Abbreviation: Chl: Chlorophyta, Rhd: Rhodophyta, Phy: Phaeophyta, RS: Reducing Sugar, EtOH: Ethanol, S.A: Sulphuric Acid, Conc: Concentration, Temp: Temperature, N.A: Not Available

2.3.1.2 Enzymatic hydrolysis

Dilute acid hydrolysis is a common method applied to hydrolyse seaweed biomass but this method has its drawbacks including degradation of sugar to fermentation inhibitors. A safer method for feedstock hydrolysis is the enzymatic procedure. Enzymes are naturally found in certain plants and microorganisms that cause a chemical reaction to breakdown polymers. Cellulose as the most abundant polymer in the plant can be degraded to its monomer by the enzyme cellulase. To conduct enzymatic hydrolysis, the enzymes must obtain access to the molecules to be hydrolysed and the crystalline structure of cellulose must be reduced to increase the access of enzyme to molecules. To obtain this condition, some kind of physical or chemical pre-treatment process is applied (Badger, 2002).

Cellulase enzymes are highly specific catalysts which act under mild conditions (e.g. pH 4.5 - 5.0 and temperature 40 to 50 °C). This allows for low corrosion of equipment, low energy consumption and also the low toxicity of the hydrolysates (Taherzadeh & Karimi, 2007b). This process is performed by the synergistic action of at least three major classes of enzymes: endo-glucanases, exo-glucanases, and ß-glucosidases. These enzymes are usually called together as cellulase or cellulolytic enzymes. The endoglucanases create free chain-ends. The sugar chain is degraded by exoglucanases by removing cellobiose from the chain and ß-glucosidases cleave the cellobiose disaccharides to glucose (Wyman, 1996).

Trichoderma reesei and *T. viride* are considered the most investigated and best characterized microorganisms that produce cellulase. The enzymes extracted from these species have some advantages including their resistance to inhibitors and stability under the enzymatic hydrolysis while the disadvantage of *Trichoderma* extracted cellulase is

the low activity of ß-glucosidases. *Aspergillus* spp. have been found to be very efficient ß-glucosidase producers (Wyman, 1996; Taherzadeh & Karimi, 2007b).

Seaweeds have different polysaccharides rather than cellulose and hemicellulose that are common in terrestrial crops. Hemicellulose is only found in some green seaweeds, *Ulva* (Ventura & Castañón, 1998; Ye *et al.*, 2010), *Enteromorpha* (Ray, 2006) but unique polysaccharides such as carrageenan, alginate, agar, etc. are found in seaweeds (Sze, 1993; Barsanti & Gualtieri, 2005; Michel *et al.*, 2006). Therefore special enzymes are required for seaweed enzymatic hydrolysis. Some enzymatic treatments are reviewed in Table 2.4.

Agar is a valuable phycocolloid extracted from the cell walls of the red seaweeds, and is composed of 3,6-anhydro-L-galactoses (or L-galactose-6-sulphates) D-galactoses and L-galactoses (routinely in the forms of 3,6-anhydro-L-galactoses or L-galactose-6-sulphates) alternately linked by β-(1,4) and α-(1,3) linkages (Chi *et al.*, 2012). The main sources of agar production are from the Rhodophyceae, including *Gelidium*, *Gracilaria*, and *Porphyra* spp.

The first bacterium with an agarolytic enzyme was isolated from seawater in the early 20th century (Michel *et al.*, 2006). After that, few microorganisms were found in seawater, coastal marine sediments or water column and reported to have same attributes (Stanier, 1942). The main marine microorganisms that produce agarolytic enzymes belong to the Gammaproteobacteria class of the Proteobacteria phylum, including the genera *Pseudomonas, Alteromonas, Pseudoalteromonas, Vibrio, Alterococcus, Microbulbifer, Agarivorans, Thalassomonas*, and *Saccharophagus* (Michel *et al.*, 2006). Their enzymes are classified into α-agarase and β-agarase according to the cleavage pattern (Fu & Kim, 2010).

Carrageenan is a gel-forming and viscosifying olysaccharides which is extracted from some species of the class Rhodophyceae, mainly *Chondrus, Gigartina, Kappaphycus* and *Eucheuma* (Necas & Bartosikova, 2013). The main blocks of carrageenan are of D-galactose and 3,6-anhydro-galactose which are joined by α1→3 and β1→4 linkage. The average molecular mass of carrageenan is above 100 kDa and ester-sulphate can be detected in different content (15 - 40 %) in its structure. This sulphated polygalactan is classified into various types such as λ, κ, ι, ε, μ, which all containing 22 - 35% sulphate groups. Solubility of these carrageenan types in KCl is the base of this classification. Three factors of i) position of ester-sulphate ii) number of ester-sulphate groups and iii) content of 3.6-anhydro galactose are determining the properties of carrageenan types. For instance, higher levels of ester sulphate leads to lower solubility temperature and lower gel strength (Barbeyron *et al.* 2000). This phycocolloid has no nutritional value and it is applied due to its gelling and emulsifying characteristics in food and pharmaceutical industries (Van de Velde *et al.* 2002). In comparison to agar-degrading bacteria, much fewer microorganisms have been reported to hydrolyse carrageenan. All these bacteria were isolated in the marine environment and belong to the Gamma proteobacteria, *Flavobacteria*, or *Sphingobacteria* classes.

Alginate was first discovered by E. C. C. Stanford and patented at 12 January 1881. He believed that alginic acid contained nitrogen and contributed much to the elucidation of its chemical structure. Later by acid hydrolysis, alginate was digested into three fractions. Homopolymeric molecules of G (α-L-guluronate) and M (β-D-mannuronate) were two fractions while another fraction was a mixture (MG). Alginate was described as being composed of different blocks of G, M and MG respectively (Draget *et al.*, 2005). Alginate is an unbranched polysaccharide polymer without repeating subunit structures and can be found widely in brown seaweeds and some bacteria including *Azotobacter vinelandii* and *Pseudomonas aeruginosa* (Hansen *et al.*, 1984). Numerous bacteria are

capable of producing alginase, but unlike carrageenase, the majority of them are marine bacteria which are active in algal decomposing residues (von Riesen, 1980).

Table 2.4: Comparison of enzymatic treatments in the saccharification of selected seaweeds

Seaweed spp.	Type	Target polymer	Enzyme/ Enzyme conc	Condition pH/time/temperature	RS g L^{-1}	Yield	Reference
Ulva fasciata	Chl	Cellulose	Cellulase / 2 % (v v^{-1})	5 / 36 h / 45 °C	N.A	0.2 g g^{-1} SW	Trivedi *et al.* (2013)
Ulva rigida	Chl	Starch Cellulose	amyloglucosidase α-amylase, cellulase	5/ 48 h / 37 °C	N.A	0.19 g g^{-1} SW	Korzen *et al.* (2015)
Ulva pertusa	Chl	Cellulose, starch	Meicelase/ 5g L^{-1}	N.A / 120 h / 50 °C	43	0.82 g g^{-1} glucan	Yanagisawa *et al.* (2011)
Alaria crassifolia	Phy	Cellulose, starch	Meicelase/ 5 g L^{-1}	N.A / 120 h / 50 °C	67	0.58 g g^{-1} glucan	Yanagisawa *et al.* (2011)
Saccharina japonica	Phy	Starch	Termamyl 120 L (Amylase)	N.A	20.6	0.31 g g^{-1} CHD	Jang *et al.* (2012)
Nizimuddinia zanardini	Phy	Cellulose	Cellulase b-glucosidase	4.8 / 24 h / 45 °C	N.A	0.07 g g^{-1} SW	Yazdani *et al.* (2011)
Laminaria japonica	Phy	Cellulose	Cellobiase 55 CBU g^{-1} Cellulase 45 FPU g^{-1}	4.8 / 48 h / 50 °C	34	0.24 g g^{-1} SW	Ge *et al.* (2011)

Abbreviation: Chl: Chlorophyta, Rhd: Rhodophyta, Phy: Phaeophyta, S.A: Sulphuric Acid, Conc: Concentration, Temp: Temperature, N.A: Not Available

Table 2.4: (continued)

seaweed	Type	Target polymer	Enzyme/ Enzyme conc	Condition pH /time / temperature	RS g L^{-1}	Yield	Reference
***Laminaria japonica, Caulerpa* sp.**	Phy, Chl	Alginate	Rapidase/ Viscozyme / dextrozyme	N.A	8.3	N.A	Choi *et al.*, (2009)
Gracilaria salicornia	Rhd	Cellulose	Cellulase/ 0.5 % w v^{-1}	5/ 30 h / 50 °C	N.A	0.013 g g^{-1} wet Biomass	Wang *et al.* (2011)
Gelidium elegans	Rhd	Cellulose, starch	Meicelase/ 5g L^{-1}	N.A/ 120 h / 50 °C	49	0.67 g g^{-1} glucan	Yanagisawa *et al.* (2011)
Gracilaria verrucosa	Rhd	Cellulose	Cellulase/ 20 FPU g^{-1} SW b-glucosidase 60 U g^{-1} SW	5/ N.A / 50 °C	40	0.87 g g^{-1} cellulose	Kumar *et al.* (2013)
Kappaphycus alvarezii	Rhd	Cellulose	Cellulase 45 FPU g	5/ 24 h / 50 °C	90	0.76 g g^{-1} cellulose	Hargreaves *et al.*, (2013)

Abbreviation: Chl: Chlorophyta, Rhd: Rhodophyta, Phy: Phaeophyta, S.A: Sulphuric Acid, Conc: Concentration, Temp: Temperature, N.A: Not Available

30

2.3.2 Fermentation of algal biomass

Generally, ethanol can be produced from any material that contains sugar. Feedstock utilized in the production of ethanol by fermentation are either sugars, starches or cellulosic materials. Sugars can be converted to ethanol directly, while starches and cellulose first have to be hydrolysed to fermentable sugars by the action of enzymes (Bashir & Lee, 1994).

Ethanol is one of the most significant organic chemicals because of its unique combination of properties as a solvent, a fuel, a germicide, a beverage, an antifreeze and as an intermediate in the production of other chemicals. Thus, many processes for ethanol production have been carried out with a negative energy balance, since the ethanol was not intended for the fuel market (Horn, 2000).

In the process of ethanol production from seaweed, biomass is saccharified and then transferred to fermenters. The different sugar composition of seaweeds causes difficulty in fermentation process by using one or a few strains of microbes in fermentation. Reith *et al.,* (2005) proposed that the seaweed biomass must be grounded at the first stage to small pieces and then transferred to saccharification. The saccharified solution (hydrolyzate) can be concentrated by evaporation if low sugar content was obtained. The hydrolyzate is then transferred to the fermentation reactors to produce ethanol. The fermented product is distilled and dehydrated to achieve a concentration of 99.9 % v v^{-1} which is needed as fuel quality specifications. Also, the residues of fermentation can be utilized to produce heat and electricity (Roesijad *et al.,* 2010).

Seaweeds of Europe and East Asia have been much investigated for bioethanol production. In Europe, where brown seaweeds dominate in the cold climate and in East and South-East Asia, the red seaweeds are abundant. Among brown seaweed species, *Laminaria* spp. (Horn, 2000; Horn *et al.,* 2000; Cui *et al.,* 2002; Lee & Lee, 2010; Adams

et al., 2011; Lee & Lee, 2011; Tedesco *et al.*, 2014) *Undaria* spp. (Yoon *et al.*, 2012; Cho *et al.*, 2013; Kim *et al.*, 2013), *Saccharina* spp. (Adams *et al.*, 2009; Jang *et al.*, 2012) are the most investigated seaweeds in bioethanol production while in red seaweeds the most interest has been towards *Kappaphycus* spp. (Khambhaty *et al.*, 2012; Meinita *et al.*, 2012; Hargreaves *et al.*, 2013; Mody *et al.*, 2015;), *Gelidium* spp. (Kim, 2009; Wi *et al.*, 2009; Jeong *et al.*, 2011; Park *et al.*, 2012; Meinita *et al.*, 2013; Ra *et al.*, 2013; Cho & Kim, 2014; Kim *et al.*, 2015;), *Gracilaria* spp. (Amanullah *et al.*, 2013; Hyebeen, 2013; Kumar *et al.*, 2013; Liao *et al.*, 2013; Meinita *et al.*, 2013; Ahmad, 2014; Wu *et al.*, 2014).

2.3.3 Fermentation strategies

Accordance with biomass specification, hydrolysis techniques and also possible reducing sugar composition, different strategies must be adopted to increase the yield of bioethanol. Considering saccharification approaches to produce reducing sugar, various saccharification and fermentation procedures can be set up that can be listed as Separate Enzymatic Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), Non-isothermal Simultaneous Saccharification and Fermentation (NSSF), Simultaneous Saccharification and Co-fermentation (SSCF), and Consolidated Bioprocessing (CBP) (Taherzadeh & Karimi, 2007b). In NSSF process, saccharification and fermentation conducted in the same time but in different reactors which are adjusted to optimum temperatures for saccharification and fermentation. This strategy is used to overcome decreasing the efficiency of saccharification and fermentation process in SSF, where temperature is not suits for both process (Wu & Lee, 1998). The SSCF is another strategy to improve SSF, in which pentose and hexose sugars are fermented simultaneously (Hamelinck *et al.*, 2005).

Unlike all hydrolysis and fermentation strategies that mentioned above, in CBP, ethanol together with all of the required enzymes is produced in single reactor by applying a single microorganisms. Means, this single microorganism first hydrolyze the polysaccharides to reducing sugars then assimilate the products to ethanol itself (Taherzadeh & Karimi, 2007b). In terms of technical and economic viewpoints, each of these strategies has its pros and cons that must be studied properly before application. The first two procedures which are most well-investigated are described next.

2.3.3.1 Separate enzymatic hydrolysis and fermentation (SHF)

In this process, biomass is saccharified into reducing sugars and the hydrolyzate is transferred into a separate reactor to be converted to ethanol. Main advantage of this approach is the possibility of conducting hydrolysis and fermentation at their own optimum conditions since enzymatic hydrolysis gives optimum yield between 45 and 50 ℃ (Olsson *et al.*, 2006; Saha *et al.*, 2005; Söderström *et al.*, 2003) whereas, the optimum temperature for fermentation is 30 - 37 °C. The main disadvantage of SHF is inhibition of cellulase activity by the reducing sugars produced. For example, cellobiose concentration at 6 g L^{-1} may reduce the activity of cellulase by 60 %. On the other hand, glucose is a strong inhibitor for ß-glucosidase in which 3 g L^{-1} of glucose concentration would inhibit ß-glucosidase activity by 75 % (Philippidis et al., 1993; Philippidis & Smith, 1995). Another possible problem in SHF is that of contaminations. The hydrolysis process is rather long, e.g. one to four days, and a dilute solution of sugar always has a risk of microbial contaminations, even at rather high temperature such as 45 - 50 °C (Taherzadeh & Karimi, 2007b).

2.3.3.2 Simultaneous saccharification and fermentation (SSF)

Currently, a combination of both enzymatic hydrolysis and fermentation into one stage, is considered as the most successful method for ethanol production from biomass. In this process, the sugars generated by the enzymatic process are immediately utilized by the fermenting microorganism present in the same reactor. This is an interesting advantage for SSF compared to SHF, as no inhibition effects of enzymatic end-product may occur by keeping a low concentration of enzymatic end-product in the culture (Taherzadeh & Karimi, 2007b). It is much reported that SSF produces ethanol at higher yields than SHF and requires lower amounts of enzyme (Eklund & Zacchi, 1995; McMillan *et al.*, 1999; Sun & Cheng, 2002). Moreover, because of the presence of ethanol in media, risk of contamination in this way is lower than in the SHF process. Also, the number of vessels required for SSF is reduced in comparison to SHF resulting in a lower capital cost of the process. A key point of obtaining higher yield in SSF is to provide better conditions for the enzymatic hydrolysis and fermentation as much as possible, especially with respect to temperature (Taherzadeh & Karimi, 2007b). The optimum temperature for enzymatic hydrolysis with a most common enzyme, cellulase is between 45-50 °C, while the temperature of 30 - 35 °C is considered the optimum for fermentation process (Tengborg, 2000). Hydrolysis is usually the rate-limiting step in SSF (Philippidis & Smith, 1995). Also, the presence of ethanol may inhibit enzymatic hydrolysis in SSF. Wyman (1996) reported that 30 g L^1 ethanol reduces the enzyme activity by 25%.

2.4 Seaweeds of Malaysia

Malaysia is located in the world's richest biodiversity region, where Malaysian macroalgae biodiversity was reported as 375 specific and intraspecific taxa in 56 families of marine algae (Phang, 2006). In the region of South East Asia, mass-production of *Kappaphycus* and *Eucheuma* started around the mid-1960s in the Philippines. After

successful farming of *Kappaphycus* in the Philippines at the early 1970s, the technology was transferred to Malaysia and Indonesia in the late 1970s. At the present, Indonesia is producing more seaweeds and lower seaweed is producing in the Philippines and Malaysia (Hurtado *et al.*, 2014). Two seaweed species, *Kappaphycus alvarezii* and *Eucheuma* are growing in commercial scale especially in Sabah, East Malaysia. Several species of *Ulva*, *Gracilaria* have a wide range of distribution in Peninsular Malaysia and East Malaysia. Species of *Ulva*, *Gracilaria* and *Chaetomorpha* showed good growth in mangrove forest ecosystem which reflects their ability to grow in the high turbidity (Phang, 1994; Saifullah & Ahmed, 2007).

2.4.1 *Gracilaria manilaensis* Yamamoto & Trono

The world's first source of agar, from the middle of the seventeenth century, was *Gelidium* from Japan, but with increasing phycocolloid demand in the 20th century, *Gracilaria* was introduced in the market to meet the demands in agar production industry (Armisen, 1995) The genus *Gracilaria*, comprises more than a hundred species and is widely distributed throughout the world where the most of the species can be found in the tropical zone and warm waters (McLachlan & Bird, 1986). *Gracilaria manilaensis* productivity is reported as 8.9 to 35.7 DW g m^{-2} (Pondevida *et al.*, 1996).

2.4.2 *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva

Kappaphycus alvarezii was first described by Doty as *Eucheuma alvarezii* (Doty & Norris, 1985) from Semporna, Sabah, Malaysia, and later changed to new combination *K. alvarezii* (Silva *et al.,* 1996). *Kappaphycus alvarezii* has a tough, fleshy thallus, up to one meter in length (Phang *et al.,* 2007).

Kappaphycus has been used greatly in different applications in the world industries of food, pharmaceuticals, and nutraceuticals. Farming of this seaweed is a significant activity especially along the coastal areas between the 10° N and 10° S of the Equator (Hurtado *et al.,* 2014). In Malaysia, *K. alvarezii* has reported from Sabah on sandy habitat and this species is one of the seaweeds cultivated in commercial scale in East Malaysia using the monofilament method in the islands near Semporna for eight months a year. The average cultivation period of *K. alvarezii* is 45 days (Phang *et al.* 2007; 2014).

Kappaphycus alvarezii was introduced widely for commercial purposes in the tropical warm waters. It is used for the extraction of kappa-carrageenan, as a homogenizer in milk products, chocolate milk, canned evaporated milk and medicinal purposes (Phang *et al.,* 2010). In 2010, Malaysia produced 15,000 tonnes dried carrageenan. At present, in Malaysia 12 varieties of *Kappaphycus* have been reported for cultivation. The phylogeny of Malaysian varieties of *Kappaphycus* and *Eucheuma* was recently published (Tan *et al.,* 2012).

2.5 Response surface methodology

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimization processes. Originally, Box and Wilson (1951) described the principles and fundamental aspects of this method of analysis. Reducing the number of experimental runs that are needed to provide sufficient information for statistically acceptable results is the main advantage of RSM (Ozdemir & Devres, 2000). So, it is less laborious and time-consuming than another conventional method which is required to optimize the process (Giovanni, 1983).

RSM uses quantitative data from appropriate experiments to determine and simultaneously solve multivariate equations. It is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and analysing optimum conditions of factors for desirable responses.

The most extensive applications of RSM are in the particular situations where multiple variables potentially influence some performance measure or quality characteristic of the process. The usage of RSM in the optimization stage process leads to the requirement for an experimental design, which can create a lot of samples for consumer evaluation in a short period of time, and therefore the laboratory level tests are more efficient (Lee *et al.,* 2006).

Among various design of RSM, central composite design (CCD) is a favourite type of analysis in which attention is focused on characteristics of the fit response function, in particular, where optimum response value occurs. The yield data were analysed for model fit using the RSM software (Design Expert) (Corredor *et al.,* 2006). On the other hand, CCD is an optimum design for fitting quadratic models. The number of experimental points in the CCD is sufficient to test the statistical validness of the fitted quadratic model and in addition, to test the acceptability of lack-of-fit of the model. The CCDs had its central point replicated several times to evaluate the error, resulting from experimental or

random variability. All tests were done in a randomized order to prevent the disturbing effect of environmental conditions.

A successive response surface method is an iterative method which consists of a scheme to assure the convergence of an optimization process. The scheme determines the location and size of each successive region of interest in the design space, builds a response surface in this region, conducts a design optimization and will check the tolerances on the response and design variables for termination. This RSM method has been widely used to evaluate and understand the interaction between different physiological and nutritional parameters (Hounjg *et al.,* 1989). This method has been successfully applied to optimize compositions of the fermentation medium, conditions of enzymatic hydrolysis, synthesis parameters for polymers and parameters for food processes (Li *et al.*, 2007).

CHAPTER 3: MATERIALS AND METHODS

3.1 Source of seaweeds

Twenty-nine seaweed species which are members of the Chlorophyta (green seaweeds), Phaeophyta (brown seaweeds) and Rhodophyta (red seaweeds), were collected from different habitats along the Malaysian coastline (Table 3.1). Voucher specimens were prepared as dried herbarium specimens and deposited in the University of Malaya Seaweeds and Seagrasses Herbarium. The *Eucheuma* spp. and *Kappaphycus* spp. seaweeds were obtained from a farm in Semporna, Sabah. Also, *Gracilaria manilaensis* was purchased from a farming pond in Kedah. All seaweed samples were authenticated by Prof Phang Siew Moi.

3.1.1 Seaweed storage and preparation

Collected seaweeds were washed with diluted seawater and sand, dirt and ephypites were removed from seaweed samples. Cleaned seaweeds were partially dried in oven at 55 °C for 24 h then were ground through a 2-mm screen using a grinder and the reduced sized sample were re-dried in 80 °C to reach a constant weight and preserved in a desiccator for further use.

Table 3.1: List of seaweeds used.

No		Seaweed name	Collection site
1	Chlorophyta	*Bryopsis plumosa* (Hudson) C. Agardh	Port Dickson, Negeri Sembilan
2		*Caulerpa racemosa* (Forsskål) J. Agardh	Port Dickson, Negeri Sembilan
3		*Caulerpa serrulata* (Forsskål) J. Agardh	Port Dickson, Negeri Sembilan
4		*Caulerpa sertularioides* (S.G.Gmelin) M.Howe	Port Dickson, Negeri Sembilan
5		*Chaetomorpha* sp. Kützing	Port Dickson, Negeri Sembilan
6		*Cladophora* sp. Kützing	Port Dickson, Negeri Sembilan
7		*Cladophora rugulosa* G. Martens	Port Dickson, Negeri Sembilan
8		*Halimeda* sp. J. V. Lamouroux	Perhentian Islands,Terengganu
9		*Ulva flexuosa* Wulfen	Port Dickson, Negeri Sembilan
10		*Ulva intestinalis* Linnaeus	Port Dickson, Negeri Sembilan
11		*Ulva reticulata* Forsskål	Johor
12	Rhodophyta	*Acanthophora spicifera (*M.Vahl) Børgesen	Morib, Selangor
13		*Eucheuma denticulatum* (N. L. Burman) Collins & Hervey	Semporna, Sabah
14		*Gracilaria changii* (B. M. Xia & I. A. Abbott) I. A. Abbott, J. Zhang & B. M. Xia	Morib, Selangor
15		*Gracilaria edulis* (S. G. Gmelin) P. C. Silva	Morib, Selangor
16		*Gracilaria manilaensis* Yamamoto & Trono	Kedah
17		*Gracilaria salicornia* (C.Agardh) E. Y. Dawson	Morib, Selangor
18		*Hypnea* sp. J. V. Lamouroux	Morib, Selangor
19		*Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva	Semporna, Sabah
20		*Pterocladiella caerulescens* (Kützing) Santelices & Hommersand	Port Dickson, Negeri Sembilan
21		*Solieria* sp. J. Agardh	Port Dickson, Negeri Sembilan
22	Phaeophyta	*Dictyota* sp. (Hudson) J. V. Lamouroux	Perhentian Islands,Terengganu
23		*Hormophysa* sp. (J. F. Gmelin) P. C. Silva	Port Dickson, Negeri Sembilan
24		*Lobophora variegate* (J. V. Lamouroux) Womersley ex E. C. Oliveira	Perhentian Islands,Terengganu
25		*Padina australis* Hauck	Perhentian Islands,Terengganu
26		*Sargassum baccularia* (Mertens) C. Agardh	Port Dickson, Negeri Sembilan
27		*Sargassum binderi* Sonder ex J. Agardh	Port Dickson, Negeri Sembilan
28		*Turbinaria conoides* (J. Agardh) Kützing	Perhentian Islands,Terengganu
29		*Turbinaria ornata* (Turner) J. Agardh	Perhentian Islands,Terengganu

3.2 Experiment 1. Chemical characterisation of selected seaweeds

3.2.1 Total carbohydrate

The total carbohydrate contents of the seaweed samples were estimated by the phenol-sulphuric acid method (DuBois *et al.*, 1956) with some minor changes to improve the sensitivity of analysis as follows. Dried samples (500 mg) in centrifuge test tubes were soaked in 25 mL HCl (2 M) for one hour and then incubated for one hour in a water bath (80 °C). The test tubes were shaken repeatedly to ensure complete hydrolysis. Test tubes were centrifuged for 30 min at 2500 ×g and aliquots of supernatants were diluted with distilled water to reach the concentration of 1-10 g L^{-1} sugars. Then 100 µL of diluted samples were transferred to clean glass test tubes. Then 3 mL of phenol solution (5 % w v^{-1}) were added to each tube and after shaking the glass test tubes, 5 mL of concentrated sulphuric acid were next added and the test tubes mixed thoroughly. The same procedure was applied to the standards (calibration) solutions. Test tubes were kept at room temperature to be cooled for 15 min and then readings were taken at 485 nm using the UV-visible spectrophotometer (UV-1800, Shimadzu, Japan). The standard curves were prepared based on galactose (for red seaweeds), glucose (for green seaweeds) and fucose (for brown seaweeds).

3.2.2 Moisture and ash

The moisture content was determined applying modified AOAC (2000). To conduct this, 5 g FW sample (triplicate) was placed on weighed aluminium foil, dried by an oven (Memmert, Germany) at 80 °C until constant weight was obtained. The dried sample moved to desiccator to reach room temperature, then dry weighed (DW) and amount of moisture was calculated according Eq. 3.1.

$$\text{Moisture content} = \frac{\text{FW}-\text{DW}}{\text{FW}} \times 100 \quad (3.1)$$

The ash content was measured by further combusting of the 2 g DW samples (triplicate) in a muffle furnace at 550 °C for 5 hours (AOAC, 2000). The crucibles were transferred immediately to desiccator to cool down to room temperature and reweighed. The ash content was calculated as Eq 3.2:

$$\text{Ash content} = \frac{(\text{Ash}+\text{Crucible}) - \text{Crucible}}{\text{DW}} \times 100 \quad (3.2)$$

3.2.3 Reducing sugar

The sugar contents of the hydrolysates were analysed using the modified DNS (3, 5-dinitrosalycylic acid) method (Miller, 1959). The main reagent was prepared according to basic protocol but diluted with distilled water 9 : 7, and kept in dark glass bottles. The solution of 0.1 % w v^{-1} Sodium meta-bisulphite was prepared and added to DNS reagent prior to use by a ratio of 1 : 16. To conduct the analysis, 1.5 mL of final DNS reagent, was added to 100 µL of the sample and incubated for 10 min at 90 °C. To stabilize the developed colour, 250 µL of sodium potassium tartarat (40 %) was added to reaction vials, while the vials were hot and then the vials were cooled to room temperature. The samples were read at 575 nm on a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan).

3.2.4 Soluble neutral sugar by gas chromatography

Selected seaweed species were hydrolysed with 2 M TFA (trifluoroacetic acid) for 1 h at 121 ° C. The supernatants were collected and derivatized to their alditol acetate compounds (APPENDIX A) (Melton & Smith, 2001). GC analyses of the sugar

derivatives were conducted with a 7820A gas chromatograph, Agilent, USA, equipped with a flame-ionization detector (FID), using a fused silica capillary column (30 m × 0.32 mm) wall coated with BPX70. Helium was used as the carrier gas at a column head pressure of 40 kPa and at a flow rate of 1 mL min^{-1} and a split ratio of 60 : 1 with an injection volume of 2 µL.

The initial oven temperature, 70 °C, was maintained for 5 min following injection, then increased to 170 °C at 50 °C min^{-1}, then to 230 °C at 2 °C min^{-1}, and kept at 230 °C for 20 min. The detector and inlet temperatures were held at 150 °C and 250 °C respectively. Hydrogen and zero air flow were 40 mL min^{-1} and 450 mL min^{-1} respectively and makeup flow was maintained as 50 mL min^{-1}. Glucose, galactose, mannose, fucose, rhamnose, xylose and arabinose were used as standard monosaccharides and allose as an internal standard.

3.2.5 Fermentation inhibitors

The fermentation inhibitors including furfural and 5-hydroxymethylfurfural (5-HMF) were analysed through chromatography using the HPLC-PDA machine, Varian Prostar-210, equipped with the C18 column. Isocratic elution of HPLC grade Methanol and Acetic acid 1 % in HPLC grade water was used at 20 : 80 ratio and at 27 °C and under wavelength of 254 nm. Sample of HPLC chromatogram is provided as Appendix B.

The total phenolic content (TPC) was analysed by the Folin–Ciocalteu method (Lee *et al.*, 2004; Singleton *et al.*, 1999) with some modification. Among the several assays available to quantify total polyphenols, this method is one of the most commonly used (Zhang *et al.*, 2006). 50 µL of diluted samples (range of 50 - 500 mg L^{-1}) was mixed with 3.5 mL distilled water in 5 mL self-standing base centrifuge test tubes, followed by 250

µL of Folin- Ciocalteu reagent. Between 1-8 min, 0.75 mL of Na_2CO_3 solution (20 % w w^{-1}) (APPENDIX C) was added, followed by addition of 0.45 mL distilled water and the vials were incubated in room temperature for 2 hours. The optical density was measured at 765 nm against a blank. The total phenolic contents were calculated on the basis of the calibration curve of gallic acid (APPENDIX C) and expressed as in mg L^{-1} in hydrolysate.

3.3 Experiment 2. Saccharification of *K. alvarezii* and *G. manilaensis*

The two seaweeds *Kappaphycus alvarezii* and *Gracilaria manilaensis* (Figure 3.1) were used in the following experiments.

Figure 3.1: A. *Gracilaria manilaensis;* **B.** *Kappaphycus alvarezii.*

3.3.1 Method 1: Dilute acid hydrolysis

3.3.1.1 Selection of suitable acid

To select the best acid for hydrolysis, hydrochloric, sulphuric, perchloric and acetic acid at different concentrations were used to convert the carbohydrates of *G. manilaensis* to reducing sugars. A paste of a fresh seaweed sample (1 kg) was prepared by blending the sample, using a home blender to obtain a final paste of about 10 % total solids content. Five gram of this paste which was equal to 0.5 g DW seaweed biomass, was added to a 15ml centrifuge test tube to which sulphuric acid was added to provide different acid concentrations (0.5, 1, 1.5, 2, 2.5, 3.0, 3.5, 4.0, 4.5 and 5 % w v^{-1}). This was repeated for the other three different acids (perchloric acid, hydrochloric acid and acetic acid). The test tubes were then heated at 121 °C for 60 min in an autoclave. The test tubes were cooled, centrifuged for 10 min by 2500 ×g and the supernatants were analysed for reducing sugar content by the modified DNS method (Miller, 1959) as described in section 3.2.3.

3.3.1.2 Fresh vs dry biomass

Fresh and dry biomass of *G. manilaensis* were used to investigate the effect of drying on dilute acid saccharification. Five g of paste from fresh seaweeds (as prepared in section 3.3.1.1) were added into 15 mL centrifuge test tubes followed by addition of sulphuric acid to provide acid concentration (ranging from 0.5 to 5 % w v^{-1}). This was followed by thermal treatment for 60 min at 121 °C. The test tubes were centrifuged for 10 min at 2500 ×g and the supernatant was used for reducing sugar analysis using DNS method. For dry biomass, 0.5 g of dry seaweed samples were used in place of the paste from the fresh seaweeds, and the same procedures for saccharification were applied.

3.3.1.3 Optimisation of dilute acid saccharification

Optimisation of the saccharification process was performed using sulphuric acid, on the two seaweeds *Kappaphycus alvarezii* and *Gracilaria manilaensis*. A combination of various parameters including temperature, incubation time and acid concentrations were used. To conduct this, 0.5g biomass was soaked in vials containing 10 mL dilute acid (0.5, 1.0, 2.5, 5.0 and 10.0 % w v^{-1}) giving a ratio of 1 : 20 for 2 hours at room temperature (25 ± 1 °C). The vials were then incubated in the range of different temperatures (80, 100, 120 and 140 ˚C) and for different incubation times (10, 20, 40 and 60 min). The reducing sugar contents in the resultant hydrolysate, was measured using the DNS method (Miller 1959).

3.3.1.4 Seaweed hydrolysate detoxification

Acid hydrolysis can produce fermentation inhibitors like 5-HMF. Over-liming is a process to reduce these fermentation inhibitors. The hydrolysates of the two seaweeds, from saccharification under optimised conditions (2.5 % sulphuric acid w v^{-1}, 40 min at 120 °C) were used in this study. Into beakers containing 300 mL hydrolysate, was added $Ca(OH)_2$ while stirring, until the pH of the hydrolysate reached 10, and incubated for 2 hr at 30 °C with continuous stirring using a magnetic stirrer. Sampling was conducted at regular intervals during the 2 hr, and the samples were immediately centrifuged. The supernatant was transferred to new centrifuge test tubes and the pH was adjusted to 5 using concentrated H_2SO_4 (Yadav *et al.,* 2011). The experiment was repeated to investigate the effect of the incubation pH at 11 and 12. The amount of fermentation inhibitors was determined in the samples using the HPLC-PDA machine (according the protocol described in section 3.2.5).

3.3.2 Method 2: Enzymatic saccharification

After dilute acid hydrolysis, cellulosic materials which are not converted to reducing sugars are still found in the residues. To convert these residues to reducing sugar, commercial cellulase enzyme (Cellic CTec 2) produced from Novozyme, Denmark was used. Enzyme dosage and liquid: biomass ratio were factors applied for optimization of enzymatic conversion of seaweed dilute acid treatment residues.

3.3.2.1 Optimization of the enzyme dosage

Residues from the acid hydrolysis were neutralized with 2 % w v^{-1} NaOH, washed and freeze-dried (Modulyo, Thermo, USA) for 2 days. The dried residues (2 g) were soaked in 20 mL of 0.1 M sodium citrate buffer (pH 4.8) and 0.2 % v v^{-1} Tween 80 (in blue cap bottles 50 mL), then the bottles were autoclaved for 15 min at 121 °C. Cellulase was added to each sample with different concentration from 2 %, 5 %, 10 % and 20 % g g^{-1} biomass and by adding distilled water liquid volume were adjusted to 40 mL. The samples were incubated 72 h at 50 °C on a shaker incubator with a speed of 170 rpm (Manns *et al.,* 2014). Samples were taken out periodically, centrifuged and reducing sugar was measured using the DNS method.

3.3.2.2 Optimization of liquid: biomass ratio

In order to produce glucose with high concentration, it is required to reduce the volume of liquid (sodium citrate buffer) to biomass (residue) and optimize this ratio. To conduct this, dried residue (2 g) was hydrolysed with optimum enzyme dosage of 10 % v v^{-1}, the temperature of 50 °C, pH 4.8 and in different liquid: biomass ratios ranging from

1:2.5, 1:5, 1:7.5 and 1:10. After hydrolysis treatment, reducing sugars was measured using the DNS method.

3.4 Experiment 3. Fermentation studies

3.4.1 Yeast strains and medium

Three different yeast species were used in this study:

1. *Saccharomyces cerevisiae* (NBRC 10217)
2. *Brettanomyces bruxellensis* (NBRC *0677*)
3. *Saccharomyces cerevisiae* (Ethanol Red)

Saccharomyces cerevisiae (NBRC 10217) and *Brettanomyces bruxellensis* (NBRC 0677) were purchased from the National Institute of Technology and Evaluation (NITE), Japan while *Saccharomyces cerevisiae* (Ethanol Red) was kindly provided by Fermentis, France.

The first two strains were revived and cultured on medium code 108 containing glucose 1.0 % w v^{-1}; yeast extract 0.6 % w v^{-1}; peptone 0.5 % w v^{-1}; agar 3.0 % w v^{-1}; pH 6.4–6.8 and incubated at 30 °C followed by preservation at 4 °C. The Ethanol Red was in freeze-dried condition and preserved at - 20 °C.

3.4.2 Selection of yeast strains and acclimation

The inoculum was prepared using Difco YPD broth (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) for 48 h on a rotary shaker at 30 °C. In order to conduct preliminary yeast adaptation, seaweed hydrolysate was enriched with peptone and yeast extract and the pH was adjusted to pH 5 followed by sterilization at 105 °C for 20 min; and then were inoculated (10 % v v^{-1}) with three yeast strains for three cycles of

cultivation. To conduct this, 1 L of *G. manilaensis* hydrolysate was prepared, detoxified and enriched with 0.6 % yeast extract. Reducing sugar content was adjusted to 40 g L^{-1}. The initial volume of the first batch was 50 mL of seaweed hydrolysate for all strains. The second cycle of adaptation was performed by inoculation fresh media with 5 mL of the first reaction batch medium and the same procedure was taken for the third cycle adaptation. Each cycle lasted 5 days under anaerobic condition, at 30 °C. The best yeast strain was selected based on the highest fermentation yield and used for further studies.

The selected yeast strain, Ethanol Red was used for further acclimation process using the fed-batch system in 100 mL bottles and under anaerobic condition at 30 °C. Every week 20 % of the reactor medium was withdrawn and fresh seaweed medium was added to the reactor regularly for 3 months under constant conditions.

3.4.3 Preparing seaweed hydrolysate for fermentation study

3.4.3.1 Dilute acid hydrolysis

Two seaweed species including *G. manilaensis* and *K. alvarezii* (Figure 3.1) were subjected to dilute acid treatment by the optimum condition. For that, biomass was cleaned and washed with diluted seawater and then oven dried to reach a constant weight at 80 °C, then 100 g DW of each seaweed biomass (triplicate) was soaked in 800 mL of sulphuric acid (2.5 % w v^{-1}). Samples were incubated at 120 °C for 40 min. The hydrolysate was cooled down to room temperature and residues were separated by a sieve (0.5 mm) and filtrates were detoxified by over-liming. Detoxified hydrolysates were filtered through Whatman filter paper no. 1 and immediately the hydrolysates pH were adjusted (pH 5) by hydrochloric acid. The hydrolysate was enriched with organic nitrogen source (Yeast Extract 0.2 % w v^{-1}) and was sterilized at 105 °C for 20 min.

3.4.3.2 Enzymatic hydrolysis

The residues obtained from the dilute acid hydrolysis treatment above (Section 3.4.3.1) were collected from both seaweed biomass, washed and pH adjusted to 5, followed by drying using freeze drier. Then the residues were characterized for DW and ash content.

7g of residue from each seaweed species, were transferred to 100 mL serum bottle followed by addition of 45 mL of 0.05 M citrate buffer pH 5 and 0.1 % Tween 80 as a surfactant. The samples were autoclaved for 15 min at 121 °C and after cooling down to room temperature Cellulase was added to each sample to provide a required enzyme dosage (10 % w w^{-1} biomass). Non-ionic surfactant Tween 80 (0.1 % v v^{-1}) to prevent unproductive binding of the enzyme to residues was used in all experiments (Castanon & Wilke, 1981; Alkasrawi *et al.*, 2003). Final volume of serum bottles were adjusted to 50 ml by addition of 0.05 M citrate buffer. Samples were incubation on a shaking incubator (170 rpm) for 48 h at 50 °C. Hydrolysate was filtered under the aseptic condition and transferred to clean 100 mL serum bottle for further procedures.

3.4.4 Fermentation of dilute acid-based hydrolysate

The seaweeds' hydrolysate using dilute acid treatment were fermented under anaerobic condition in 100 mL serum bottles and 1400 mL fermenter using adapted yeast (Ethanol Red). The pH of media was adjusted and maintained at 4.8 with 0.1 M citrate buffer in serum vials and using automatic adjusting in the fermenter. The volume of inoculation was 6 % v v^{-1} in all experiments at 30 °C and agitation was 150 rpm.

3.4.5 Fermentation of enzyme-based hydrolysate

Fermentation was also conducted using the enzyme hydrolysates of the seaweeds (as prepared in section 3.4.3.2). The medium was enriched with nitrogen source, yeast extract 0.6 % (Khambhaty *et al.* 2012). pH was adjusted (pH 5) using 0.05 M sodium citrate buffer followed by inoculation with adapted yeast (Ethanol Red). The samples were incubated for 3 days at 30 °C and 150 rpm in a shaking incubator.

3.4.6 Analysing bioethanol content by GC using a novel sample preparation approach

Bioethanol samples were defrosted and centrifuged for 15 min at 10625 ×g. Clear brown supernatant was collected and 100 µL of each sample (by triplicates) was added to 900 µL of solvent mixture (1% w v^{-1} iso-butanol in acetonitrile) and shaken vigorously for 15 sec, followed by centrifugation at 5976 ×g at 5 °C for 3 min. This is the first time this method is used. This is done to remove soluble compounds and to reduce the amount of water content in injection volume in GC.

Followed that sample preparation step, the concentration of bioethanol was then analysed by Gas Chromatograph (GC) (Agilent 5820-A, Agilent Inc., USA) equipped with a split/ splitless inlet, a flame ionization detector (FID) and a capillary column (HP-Innowax 30 m, 0.32 mm, 0.15 µm). The temperature programming of the GC analysis were as follow: 230 °C as injector and 230 °C as detector temperature, the column was held at 70 °C for 7 min and then the temperature was increased at a rate of 25 °C min^{-1} to 220 °C and then held for 10 min; helium at 3 mL min^{-1} was used as carrier gas, the flow rates for the FID were 40 mL min^{-1} for the makeup gas (He), 40 mL min^{-1} for hydrogen, and 450 mL min^{-1} for air with a split ratio of 1:100 and 1 µL injection sample size was used (Lin *et al.*, 2014). A standard curve of ethanol was plotted using different levels of

ethanol concentration (0.01 - 5 % w v^{-1}). A standard curve was prepared for different ethanol concentrations and the amount of ethanol was corrected by internal standard value according to Eq. 3.3:

$$\text{Response factor of EtOH} = \frac{\text{area of EtOH (pA.m)}}{\text{area of IS (pA.m)}}. \quad (3.3)$$

To evaluate the accuracy of sample preparation method and standard curve using solvents mixture, three ethanol samples with known concentrations (1.05, 0.55 & 0.30 % w v^{-1}) were prepared and the amount of ethanol was calculated based on standard curve and the variation of calculated concentration of expected value was defined as error% and extracted by Eq. 3.4:

$$\text{Error \%} = \frac{[\text{Known conc\%} - \text{experimental conc\%}]}{\text{known conc\%}} \times 100. \quad (3.4)$$

Data are presented as mean ± standard deviation (SD), and the differences in results were tested by analysis of variance.

3.4.7 Reactor systems

3.4.7.1 100 mL serum bottle

The enzymatic hydrolysis and batch experiments of fermentation were conducted in serum bottles (Wheaton, USA) with a total volume of 100 mL and 50 mL working volume (Figure 3. 2. left). The pH was adjusted to 4.8 by use of 0.1 M phosphate buffer and the serum vials were incubated in shaker incubator at 30 °C and 150 rpm.

3.4.7.2 1000 mL working volume fermenter

The final fermentation experiment was conducted in a 1.4 L fermenter, Multifors, Switzerland (Figure 3. 2. b). The pH in the fermenter was maintained at 5 by automatic addition of 2 M HCl and 2 M NaOH. The temperature was 30 °C and agitation speed was 150 rpm. Sampling was conducted through a special aseptic sampling port.

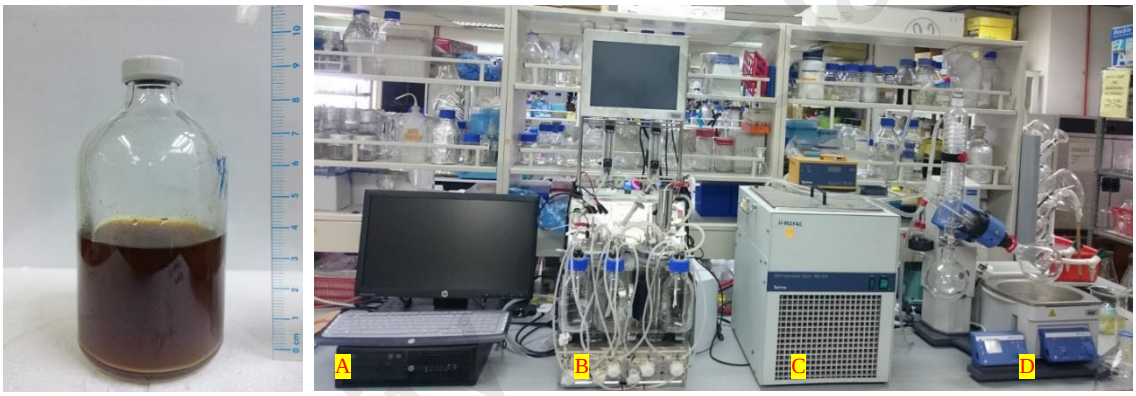

Figure 3.2: Bioreactors: Left: 100 mL serum bottle; Right: Lab scale fermentation setup (A. PC; B. 1.4 L fermenter; C. Water chiller and D. Rotary evaporator).

3.5 Experiment 4. Saccharification using dilute acid at low temperature, based on response surface methodology (RSM)

In this part, two seaweed species, were treated with dilute sulphuric acid at a lower temperature than what have been used in other studies but with longer incubation time. To optimize the reaction parameters to achieve maximum production of reducing sugars with minimum cost and faster time, the experiment was designed by Design-Expert software version 7.0.0 (Stat-Ease Inc., Minneapolis, MN, USA). Central composite design (CCD) was applied to optimize the reaction variables including temperature ranged 45-75 °C, acid concentration ranged 2.5-7.5 % w v $^{-1}$ and incubation time 2-10 h.

Dried biomass (200 mg) of *G. manilaensis* and *K. alvarezii* were transferred in 15mL centrifuge test tubes and 5 mL of 2.5, 5 and 7.5 % w v $^{-1}$ acid sulphuric added to test tubes, then test tubes were mixed thoroughly by vortexed for 30 seconds and soaked for one hour at room temperature. Test tubes later were incubated at oven (45, 60 and 75 °C) for various incubation time (2, 6 and 10 h). During incubation time, test tubes were shaken by vortex for 5s each hour to ensure effective treatment. All the experiments were done in triplicate to ensure the reproducibility of the data. The samples were immediately stored in fridge for further analysis. Amount of reducing sugars was measured using DNS method (Miller 1959) and the yield of saccharification is estimated from Eq. 3.5:

$$\text{Yield of saccharification} = \frac{\text{amount of reducing sugar}}{\text{total biomass DW}} \times 100 \qquad (3.5)$$

In present study, optimum condition for maximizing the reducing sugar generation were predicted by solving the second-order polynomial equation (Eq. 3.6) and by analysing the response surface contour plots.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (3.6)$$

Where Y represents the predicted response, B_0 is the interception coefficient B_i , B_{ii} and B_{ij} are the regression coefficients for the three variables, X_i (linear term), X_i^2 (quadratic term) and X_iX_j (interaction term) respectively.

The error of analysis for the experimental procedure is approximately ± 5 %. Regression analysis and analysis of variance (ANOVA) tested the significance of the model. The coded values of the variables are shown in Table 3.2.

Table 3.2: Coded level for variables used in the experimental design.

Factors	**Symbol**	**Coded levels**		
		-1	**0**	**+1**
Acid concentration (w v^{-1})	A	2.5	5	7.5
Incubation temperature (°C)	B	45	60	75
Incubation time (h)	C	2	6	10

3.6 Statistical analysis

In all experiments, normal distribution of data was tested using the Kolmogorov-Smirnov test. All data were analysed with the analysis of variance (ANOVA). The statistical analyses were carried out using SPSS software, version 21 (SPSS Inc., USA). Tukey's HSD comparisons were applied to determined statistically significant differences ($p < 0.05$) among time (min), acid concentration (% w v^{-1}) and temperature (°C) following ANOVA. A significance level of 95% ($p < 0.05$) was set for all the tests. Results of statistical analysis are shown in tables and figures in Appendices part.

CHAPTER 4: RESULTS

4.1 Experiment 1: Characterization of selected seaweeds

4.1.1 Total carbohydrate

The total carbohydrate content was highest in *K. alvarezii (*71.22 ± 0.71 % DW) followed by *E. denticulatum* (69.91 ± 3.35 % DW) (Table 4.1). Of the green seaweeds, *Ulva reticulata* (55.99 ± 0.98 % DW) had highest carbohydrate content followed by *Bryopsis* (43.12 ± 3.87 % DW). *L. variegata* had the highest carbohydrate content (34.83 ± 0.89 % DW). In general, the Malaysian seaweeds have comparable carbohydrate contents as similar species reported in other regions, while the Malaysian *Eucheuma denticulatum* and *Lobophora variegata* had much higher values (Table 4.1). Of the Malaysian seaweeds, *K. alvarezii* and *Gracilaria manilaensis* had highest carbohydrate content.

4.1.2 Moisture and ash

Moisture content of all seaweeds analysed in this study (Table 4.1) was high and between72.19-94.13 % FW Within green seaweeds the highest ash content were recorded in *Halimeda* (44.61 ± 1.96 % DW) and *C. racemosa* (43.12 ± 1.09 % DW) and the lowest ash contents were in *U. reticulata* (13.28± 0.32 % DW) and *U. flexuosa* (22.92 ± 1.21 % DW). Among red seaweeds, *G. salicornia* (53.11 ± 1.43 % DW) showed highest ash content followed by *Hypnea* (38.43 ± 0.67 % DW). The lowest ash content was in *E. denticulatum* (19.04 ± 0.45 % DW). Within brown seaweeds, *Padina* (41.98 ± 2.26 % DW) and *S. binderi* (37.88 ± 1.94 % DW) showed the higher ash content but *L. variegata* (24.1 ± 1.35 % DW) exhibited the lowest ash content.

Table 4.1: Total carbohydrate, reducing sugar, ash and moisture contents of selected Malaysian seaweeds.

		Amount (% DW)			
		Ash	**Total carbohydrates**	**Reducing Sugar**	**Moisture**
Chlorophyta	*Bryopsis* sp.	27.29 ± 0.98 ^{defgh}	43.12 ± 3.87 ^{d}	23.16±6.12 ^{bcde}	93.02 ± 1.09 ^{abc}
	Caulerpa racemosa	34.49 ± 1.09 ^{abc}	38.11 ± 2.09 ^{def}	15.56 ± 4.09 ^{efghi}	94.00 ± 1.44 ^{ab}
	C. lentillifera	27.31 ± 1.13 ^{defgh}	38.12 ± 2.98 ^{def}	22.65±2.77 ^{bcdef}	92.09 ± 1.91 ^{abcd}
	C. serrulata	29.34 ± 0.29 ^{cdefg}	34.12 ± 1.45 ^{efg}	20.04 ± 2.01 ^{defg}	93.19 ± 0.83 ^{abc}
	C. sertularioides	28.15 ± 2.09 ^{defgh}	32.99 ± 0.90 ^{fg}	17.14 ± 1.14 ^{efghi}	92.98 ± 1.04 ^{abc}
	Chaetomorpha sp.	30.49 ± 0.55 ^{bcde}	34.13 ± 1.11 ^{fg}	18.12 ± 1.98 ^{efghi}	87.11 ± 0.19 ^{fghi}
	Cladophora rugulosa	22.36 ± 1.41 ^{hij}	34.98 ± 2.09 ^{efg}	18.12 ± 3.18 ^{efghi}	88.12 ± 0.98 ^{efgh}
	Halimeda sp.	35.68± 1.96 ^{ab}	29.77 ± 3.48 ^{g}	10.16 ± 4.19 ^{ijk}	73.09 ± 2.09 ^{mn}
	Ulva flexuosa	18.33± 1.21 ^{jk}	34.98 ± 1.19 ^{efg}	18.12 ± 3.12 ^{efghi}	85.98 ± 0.99 ^{ghi}
	U. intestinalis	22.49 ± 0.66 ^{ghij}	33.12 ± 1.09 ^{fg}	19.15±2.74 ^{defgh}	89.56 ± 1.09 ^{cdefg}
	U. reticulata	10.62 ± 0.32 ^{l}	55.99 ± 0.98 ^{bc}	27.11±1.98 ^{abcd}	82.12 ± 0.41 ^{jk}
Rhodophyta	*Acanthophora spicifera*	27.35 ± 0.99 ^{defgh}	37.12 ± 2.01 ^{ef}	20.38 ± 1.03 ^{defg}	82.28 ± 1.09 ^{jk}
	Eucheuma denticulatum	15.23 ± 0.45 ^{kl}	69.91 ± 3.35 ^{a}	32.28 ± 3.98 ^{a}	94.13 ± 0.65 ^{kl}
	Gracilaria changii	25.68 ± 0.97 ^{efgh}	52.94 ± 0.98 ^{c}	29.66 ± 1.06 ^{ab}	89.11 ± 0.45 ^{defg}
	G. edulis	29.68 ± 1.08 ^{bcdef}	39.18 ± 0.67 ^{de}	20.12 ± 1.58 ^{defg}	91.26 ±1.11 ^{abcde}
	G. manilaensis	28.53 ± 0.68 ^{bcdef}	59.68 ± 1.83 ^{b}	33.02 ± 1.11 ^{a}	92.66 ± 0.98 ^{abcd}
	G. salicornia	38.12 ± 1.43 ^{a}	35.53 ± 1.98 ^{efg}	16.19 ± 2.05 ^{efghi}	85.12 ± 0.76 ^{hij}
	Hypnea sp.	30.43 ± 0.67 ^{bcde}	39.4 ± 1.93 ^{de}	18.19 ± 2.83 ^{efghi}	91.11 ±1.98 ^{abcde}
	Kappaphycus alvarezii	18.74 ± 1.04 ^{jk}	71.22 ± 0.71 ^{a}	34.12 ± 1.09 ^{a}	93.08 ± 0.84 ^{abc}
	Pterocladiella caerulescens	17.05 ± 0.35 ^{jk}	51.65 ± 1.48 ^{c}	28.12 ±2.09 ^{abc}	76.30 ± 1.70 ^{lm}
	Soleria sp.	30.98 ± 1.97 ^{bcdef}	36.17 ± 2.10 ^{ef}	19.13± 3.01 ^{cdefg}	91.35 ± 1.01 ^{abcde}
Phaeophyta	*Dictyota* sp.	25.98 ± 3.06 ^{cdef}	13.11 ± 1.76 ^{ij}	11.18 ± 2.12 ^{ijk}	89.11 ± 0.76 ^{defg}
	Hormophysa sp.	29.15 ± 3.18 ^{fghi}	20.14 ± 1.12 ^{h}	11.85 ± 2.11 ^{hij}	79.12 ± 0.45 ^{lm}
	Lobophora variegata	19.28 ± 1.35 ^{ijk}	34.83 ± 0.89 ^{efg}	3.62 ± 0.50 ^{k}	89.98 ± 0.65 ^{bcdef}
	Padina australis	33.98 ± 2.26 ^{abc}	29.70 ± 0.61 ^{g}	11.21 ± 1.02 ^{ijk}	91.55 ± 0.79 ^{abcde}
	Sargassum baccularia	29.30 ± 2.94 ^{cdef}	17.09 ± 0.87 ^{hij}	14.48 ± 0.49 ^{fghi}	84.59 ± 0.91 ^{hij}
	S. binderi	30.88 ± 1.94 ^{bcde}	12.16 ± 2.11 ^{j}	5.17 ± 3.70 ^{jk}	84.09 ± 0.59 ^{ij}
	Turbinaria conoides	29.30 ± 3.33 ^{abcd}	18.98 ± 2.97 ^{h}	16.63 ± 2.33 ^{efghi}	72.19 ± 0.93 ^{n}
	T. ornata	25.6 ± 1.87 ^{efgh}	17.37 ± 2.44 ^{hij}	13.49 ± 3.29 ^{ghi}	75.02 ± 1.05 ^{mn}

Values are represented as mean ± SD, replicate by independent experiments n=3. Values followed by the same letter are not significantly different at $p < 0.5$, (Tukey, HSD).

4.1.3 Reducing sugars

The use of dilute acid treatment gave the highest yield of reducing sugar in *K. alvarezii* (34.12 ± 1.09 % DW) and *G. manilaensis* (33.02 ± 1.11 % DW) (Table 4.1). Among the green seaweeds, the reducing sugar contents from *U. reticulata* (27.11 ± 1.98 % DW) and *Bryopsis* (23.16 ± 6.12 % DW) were the highest whereas the lowest value was detected in *Halimeda* (10.16 ± 4.19 % DW). Within red seaweeds, *K. alvarezii and G. manilaensis* showed higher reducing sugar contents, 34.12 ± 1.09 % and 33.02 ± 1.11% respectively. The lowest value of reducing sugar was recorded in *G. salicornia* (16.19 ± 2.05 % DW). The lowest amount of reducing sugars were detected in brown seaweeds, where the highest value was detected in *S. baccularia* (14.48 ± 0.49 % DW) and *Turbinaria (*13.63 ± 2.33 % DW) but the lowest was detected in *S. binderi (*5.17 ± 3.71% DW).

4.1.4 Neutral sugars

The monosaccharide composition of selected seaweed species is shown in Table 4.2. Glucose and galactose were found in all seaweeds and ranged from 8.93 ± 1.49 to 159.60 ± 9.83 mg g^{-1} DW and 7.77 ± 0.04 to 262.28 ± 25.09 mg g^{-1} DW respectively. In this study, fucose was only detected in brown seaweeds. Of the green seaweeds, *U. reticulata* (159.60 ± 9.83 mg g^{-1} glucose), *U. flexuosa* (97.25 ± 3.66 mg g^{-1} glucose) and *Cladophora* sp. (68.62 ± 2.48 mg g^{-1} galactose) are suitable seaweeds; while of the red seaweeds, *G. changii* (288.51 ± 29.36 mg g^{-1} galactose), *G. manilaensis* (262.28 ± 2.48 mg g^{-1} galactose) and *K. alvarezii* (253.96 ± 19.41 mg g^{-1} galactose) are best. The brown seaweeds showed low glucose and galactose contents.

Table 4.2: Monosaccharide composition of some selected seaweed species conducted with gas chromatography.

	Monosaccharide composition (mg g $^{-1}$ DW)						
Seaweed species	Glucose	Galactose	Mannose	Arabinose	Xylose	Fucose	Rhamnose
C. racemosa	47.21 ± 2.31^{d}	19.13 ± 0.88^{e}	14.17 ± 0.12^{ef}	3.34 ± 0.91^{c}	101.76 ± 4.18^{a}	N/D	N/D
Cladophora sp.	23.15 ± 5.36^{e}	68.62 ± 2.48^{d}	20.31 ± 0.21^{de}	60.66 ± 3.19^{a}	14.19 ± 2.05^{cd}	N/D	6.47 ± 0.52^{e}
U. flexuosa	97.25 ± 3.66^{b}	22.71 ± 1.74^{e}	N/D	N/D	21.15 ± 0.08^{b}	N/D	70.09 ± 4.47^{b}
U. reticulata	159.60 ± 9.83^{a}	24.26 ± 3.31^{e}	14.84 ± 2.30^{ef}	N/D	22.71 ± 1.04^{b}	N/D	80.65 ± 3.91^{a}
A. spicifera	31.24 ± 5.09^{e}	147.20 ± 14.84^{c}	19.84 ± 0.59^{de}	N/D	15.91 ± 0.57^{c}	N/D	26.80 ± 2.75^{c}
G. changii	21.91 ± 2.28^{e}	288.51 ± 29.36^{a}	7.21 ± 1.19^{fg}	N/D	8.73 ± 0.98^{e}	N/D	10.24 ± 1.65^{d}
G. manilaensis	61.10 ± 5.92^{c}	262.28 ± 25.09^{ab}	23.60 ± 0.35^{d}	10.40 ± 2.11^{b}	2.41 ± 0.47^{gh}	N/D	N/D
G. salicornia	23.22 ± 4.08^{e}	126.00 ± 5.46^{c}	22.68 ± 1.18^{de}	4.50 ± 0.07^{c}	N/D	N/D	N/D
K. alvarezii	91.20 ± 10.73^{b}	253.96 ±19.41^{b}	N/D	N/D	5.08 ± 0.48^{fg}	N/D	N/D
Hormophysa sp.	11.73 ± 1.67^{f}	15.64 ± 0.55^{e}	78.01 ± 2.49^{b}	2.48 ± 0.00^{c}	8.58 ± 0.11^{e}	25.80 ± 3.27^{c}	3.99 ± 0.65^{a}
Padina sp.	8.93 ± 1.49^{f}	7.77 ± 0.04^{e}	93.90 ± 12.13^{a}	N/D	7.40 ± 2.01^{ef}	20.40 ± 0.97^{d}	N/D
S. baccularia	13.13 ± 2.14^{f}	20.32 ± 1.68^{e}	93.03 ± 5.98^{a}	N/D	12.16 ± 0.08^{d}	36.16 ± 5.32^{b}	N/D
Turbinaria sp.	9.41 ± 0.77^{f}	20.56 ± 1.06^{e}	66.87 ± 8.93^{c}	N/D	8.43 ± 1.05^{e}	41.10 ± 5.28^{a}	5.34 ± 0.62^{e}

Values are represented as Mean ± SD, replicate by independent experiments n=3. Values followed by the same letter are not significantly different at $p < 0.5$, (Tukey, HSD). N/D= Not detected

4.1.5 Fermentation inhibitors

Fermentation inhibitors found in seaweed hydrolysates are illustrated in Table 4.3. The highest TPC observed in the present study was from the red seaweeds, ranging from 634.06 ± 59.35 mg L^{-1} (*Solieria*) to 1221.55 ± 65.90 mg L^{-1} (*G. changii*). The lowest TPC was obtained in the green seaweeds, *Halimeda* sp. (219.08 ± 39.56 mg L^{-1}) and *U. flexuosa* (275.03 ± 13.19 mg L^{-1}), as well as the brown seaweeds, *Dictyota* sp. (284.36 ± 21.76 mg L^{-1}) and *P. australis* (298.35 ± 6.59 mg L^{-1}). Highest 5-HMF was produced by the red seaweeds, *G. changii* (638.17 ± 18.39 mg L^{-1}) and *Hypnea* (628.97 ± 63.78 mg L^{-1}). The lowest level of 5-HMF was found in *P. caerulescens* (276.50 ± 15.02 mg L^{-1}). Among the green seaweeds, *U. reticulata* (128.15 ± 7.33 mg L^{-1}) showed highest level 5-HMF followed by *C. rugulosa* (96.28 ± 7.82 mg L^{-1}) and the lowest amount was detected in *Halimeda* (27.88 ± 1.20 mg L^{-1}) and *U. flexuosa* (27.44 ± 3.89 mg L^{-1}), respectively. The brown seaweed, *S. baccularia* (30.87 ± 6.47 mg L^{-1}) showed the highest amount of 5-HMF, while the lowest amount was in *Dictyota* (1.89 ± 0.97 mg L^{-1}). Furfural content was higher in the green algae (ranged from 21.44 ± 0.36 in *C. rugolosa* to 43.64 ± 0.07 mg L^{-1} in *C. racemosa*) compared to the rest. In the red seaweeds, *K. alvarezii* and *E. denticulatum* showed the highest furfural levels of 21.37 ± 1.71 and 24.99 ± 3.45 mg L^{-1}, respectively. The lowest amount was in *Solieria* (17.39 ± 0.23 mg L^{-1}). In the brown seaweeds, *Hormophysa* (23.26 ± 0.99 mg L^{-1}) exhibited the highest amount and *S. baccularia* (16.26 ± 0.02 mg L^{-1}) the lowest.

Table 4.3: Composition of some fermentation inhibitors including 5-hydroxymethylfurfural, (5-HMF); furfural and total phenolic compounds (TPC) in hydrolysates obtained from saccharification of selected tropical seaweeds.

		Amount (mg L^{-1} in hydrolysate)		
		5-HMF	**Furfural**	**TPC**
Chlorophyta	***Bryopsis* sp.**	81.08 ± 25.43 ^{fg}	30.14 ± 2.48 ^{b}	764.61 ± 98.91 ^{bcdefgh}
	Caulerpa racemosa	64.97 ± 28.22 ^{fg}	43.64 ± 0.07 ^{a}	447.54 ± 72.52 ^{ijklm}
	C. lentillifera	53.04 ± 3.63 ^{fg}	46.30 ± 2.16 ^{a}	508.17 ± 158.26 ^{hijklm}
	C. serrulata	48.39 ± 9.57 ^{fg}	46.83 ± 1.42 ^{a}	671.36 ±217.60 ^{efghijk}
	C. sertularioides	67.15 ± 10.02 ^{fg}	34.11 ± 1.37 ^{b}	680.68 ± 19.78 ^{efghij}
	***Chaetomorpha* sp.**	66.26 ± 14.08 ^{fg}	24.37 ± 0.05 ^{cd}	904.49 ± 72.53 ^{bcdef}
	Cladophora rugulosa	96.28 ± 7.82 ^{fg}	21.44±0.36 ^{cdefgh}	937.13 ± 39.56 ^{abcde}
	***Halimeda* sp.**	27.88 ± 1.20 ^{fg}	21.37 ± 1.35 ^{cdefg}	219.08 ± 39.56 ^{m}
	Ulva flexuosa	27.44 ± 3.89 ^{fg}	20.56 ±0.65 ^{defghi}	275.03 ± 13.19 ^{m}
	U. intestinalis	46.69 ± 18.91 ^{fg}	33.31 ± 2.50 ^{b}	330.99 ± 29.10 ^{lm}
	U. reticulata	128.15 ± 7.33 ^{f}	22.92 ± 3.67 ^{cde}	927.80 ± 118.69 ^{abcd}
Rhodophyta	***Acanthophora spicifera***	482.06 ± 115.56 ^{bc}	21.63±0.99 ^{cdefgh}	955.78 ± 52.75 ^{abcde}
	Eucheuma denticulatum	561.85 ± 88.89 ^{ab}	24.99 ± 3.45 ^{c}	1044.37 ± 164.85 ^{ab}
	Gracilaria changii	638.17 ± 18.39 ^{a}	18.82 ± 0.45 ^{ghij}	1221.55 ± 65.90 ^{a}
	G. edulis	505.21 ± 18.52 ^{bc}	19.33 ± 1.13 ^{fghij}	1053.70 ± 32.97 ^{ab}
	G. manilaensis	411.67 ± 90.51 ^{cd}	20.04 ± 0.32 ^{efghij}	913.82 ± 72.53 ^{bcdef}
	G. salicornia	435.82 ± 59.24 ^{cd}	19.40 ± 0.14 ^{fghij}	904.49 ± 138.47 ^{bcdef}
	***Hypnea* sp.**	628.97 ± 63.78 ^{a}	19.61 ± 0.19 ^{efghij}	988.42± 19.78 ^{abc}
	Kappaphycus alvarezii	586.23 ± 61.74 ^{ab}	21.37±1.71 ^{cdefgh}	685.35 ± 131.88 ^{defghij}
	Pterocladiella caerulescens	276.50 ± 15.02 ^{e}	22.00 ± 2.90 ^{cdefg}	1024.25 ± 134.25 ^{ab}
	***Soleria* sp.**	329.35 ± 29.36 ^{de}	17.39 ± 0.23 ^{hij}	634.06 ± 59.35 ^{fghijk}
Phaophyta	***Dictyota* sp.**	1.89 ± 0.97 ^{g}	16.35 ± 0.02 ^{j}	284.36 ± 21.76 ^{lm}
	***Hormophysa* sp.**	7.35 ± 1.30 ^{g}	23.26 ± 0.99 ^{cdef}	340.31 ± 39.56 ^{klm}
	Lobophora variegata	2.78 ± 1.27 ^{g}	21.93 ± 0.56 ^{cdefg}	424.31 ± 88.25 ^{ijklm}
	Padina australis	3.72 ± 2.92 ^{g}	16.39 ± 0.07 ^{ij}	298.35 ± 6.59 ^{lm}
	Sargassum baccularia	30.87 ± 6.47 ^{fg}	16.26 ± 0.02 ^{j}	708.66 ± 19.78 ^{cdefghi}
	S. binderi	4.75 ± 1.07 ^{g}	21.84±0.29 ^{cdefgh}	821.70 ± 76.40 ^{bcdefg}
	Turbinaria conoides	4.89 ± 0.16 ^{g}	21.74±0.11 ^{cdefgh}	400.93 ± 59.35 ^{jklm}
	T. ornata	7.99 ± 3.11 ^{g}	21.53 ± 1.67 ^{cdefg}	573.44 ± 79.13 ^{ghijkl}

Thermal-acidic treatment using1%w v-1 sulphuric acid; incubation time 1 h, at 121 °C; ratio of solid: liquid= 1:20. Values are represented as Mean ± SD, replicate by independent experiments n=3. Values followed by the same letter are not significantly different at $p < 0.5$, (Tukey, HSD).

4.2 Experiment 2. Saccharification of *K. alvarezii* and *G. manilaensis* biomass

4.2.1 Dilute acid saccharification

4.2.1.1 Selection of suitable acid

Kappaphycus alvarezii and *G. manilaensis* were selected for further studies based on their total carbohydrate and high reducing sugar contents and also these seaweeds are currently the main seaweeds cultivated in Malaysia and the surrounding region. The effect of different acids on *G. manilaensis* is shown in Figure 4.1. Acid acetic produced the lowest reducing sugar content, at all concentrations used ($p < 0.05$). No significant difference was seen between the rest of the acids at most concentrations, however at 0.5 % perchloric acid gave highest sugar yield ($p < 0.05$). Also at 1% and 1.5 % levels, sulphuric acid and perchloric acids gave highest reducing sugar yield ($p < 0.05$) compared with hydrochloric acid.

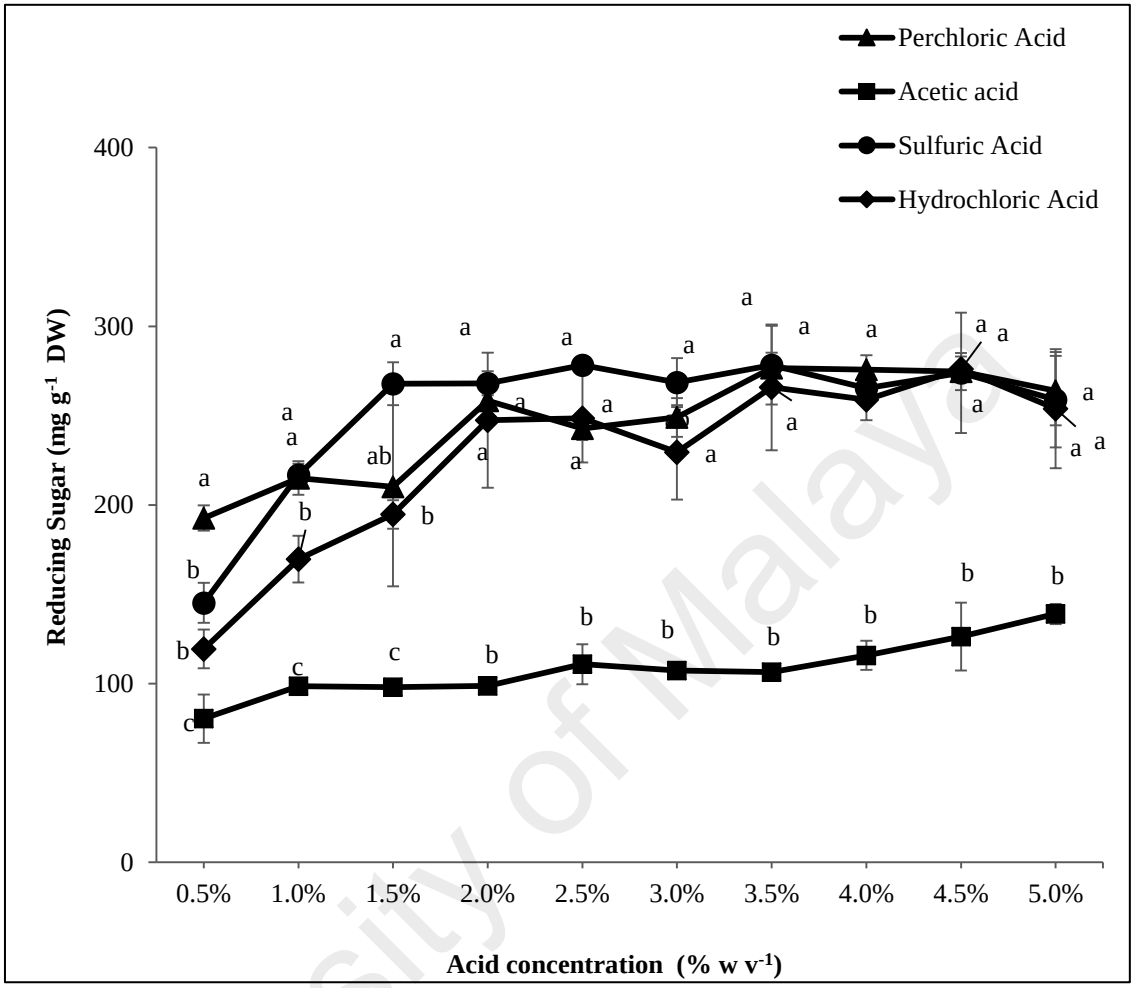

Figure 4.1: Effect of four different acids on saccharification of *G. manilaensis* samples under different concentrations (0.5 – 5 % w v^{-1}) and incubation time of 60 min, at 121 °C. (Hydrochloric acid ♦; Sulphuric acid ●; Perchloric acid ▲, Acetic acid ■). Mean ± SD: n=3. Means with different letter are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD).

4.2.1.2 Fresh vs dry biomass

Figure 4.2 shows the effect of biomass physical condition on hydrolysis yield at different acid concentrations. At 1 %, 2.5 % and 3% sulphuric acid concentrations, dry biomass yielded higher than fresh biomass ($p < 0.5$) and the bigger difference was seen at acid concentrations of 1.5 % and 2 % sulphuric acid ($p < 0.01$). No significant difference was seen in other acid concentrations between dry and fresh biomass ($p > 0.05$).

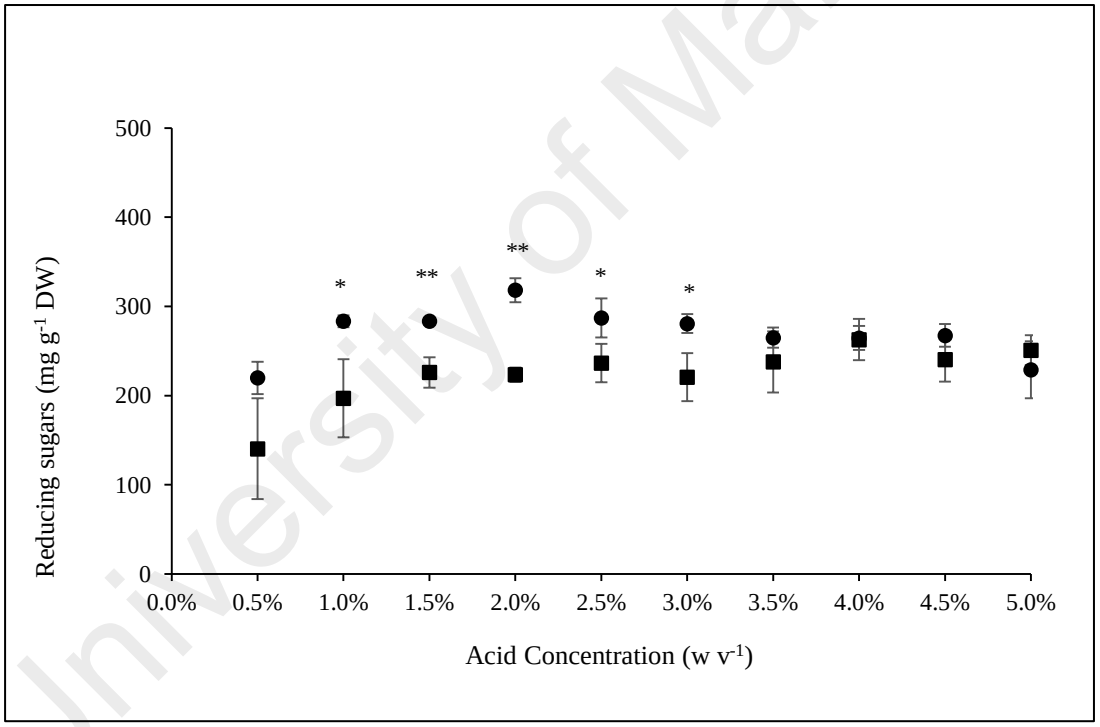

Figure 4.2: Evaluation of the effect of biomass (*G. manilaensis*) condition (Dry ● Fresh ■) on the yield of saccharification. Mean ± SD, n=3, Independent t-Test df 4, $p < 0.05$ *, $p < 0.01$ ** **.**

4.2.1.3 Dilute acid treatment

Figure 4.3 to Figure 4.10 show the reducing sugar content obtained by dilute acid treatment of biomass of the two seaweeds. Figure 4.3 shows *K. alvarezii* hydrolysed at 80 °C, where reducing sugar produced increased with increasing acid concentration. At 20 min, 40 min and 60 min incubation the best acid concentrations were 5 % w v^{-1} (18.24 ± 1.70 % DW), 2.5 % (17.89 ± 1.93 % DW) and 10 % (21.67 ± 1.53 % DW) respectively. Figure 4.4 shows *K. alvarezii* hydrolysed at 100 °C. The highest (30.40 ± 3.07 % DW) reducing sugar content was produced in 5 % acid concentration and 40 min incubation. However, at 10 min (22.93 ± 0.94 %DW) and 20 min (26.47 ± 2.19 % DW) incubation, the highest sugar yields were obtained in 10 % acid concentration. Figure 4.5 Figure 4.5 shows *K. alvarezii* at 120 °C where highest (35.98 ± 3.33 % DW) sugar yield was found after 40 min and in 5 % sulphuric acid. However, high sugar yields were also obtained at 20 min incubation in 5 (33.96 ± 1.19 % DW) and 10 % (34.12 ± 1.24 % DW) acid respectively. Incubation at 140 °C did not further increase the sugar yield (Figure 4.6). At 10 min and 20 min incubation, high sugar yields were obtained at both 2.5 (30.99 ± 1.28 % DW for 10 min; 31.60 ± 2.19 % DW for 20 min) and 5 % (31.89 ± 1.92 % DW) at 10 min and 32.69 ± 2.56 % DW at 20 min) respectively.

Figure 4.7 to Figure 4.10 show dilute acid treatment effect on *G. manilaensis* biomass. In general, acid concentration higher than 2.5 % did not increase the sugar yield significantly except at 100 °C (Figure 4.8). Saccharification at 120 °C gave highest sugar yield ($P < 0.05$). The acid concentration of 2.5% was best and gave sugar yields of 30.26 ± 1.69 % DW at 20 min; 37.10 ± 0.72 % DW at 40 min and 33.96 ± 3.56 % DW at 60 min incubation respectively. Incubation at 140 °C did not increase sugar yields with increasing acid concentration except at 10 and 20 min respectively (Figure 4.10). Statistical analysis for seaweeds tested in this study were shown in APPENDIX D-APPENDIX M.

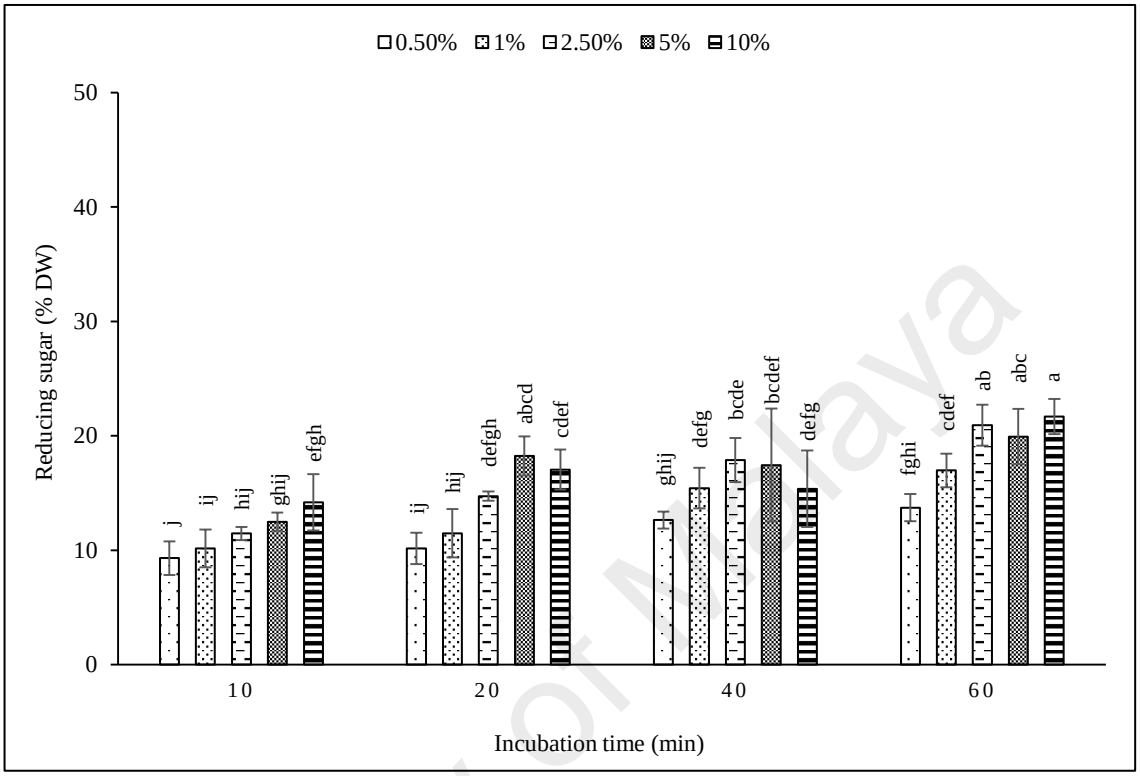

Figure 4.3: Reducing sugar content obtained under different conditions during thermal-acidic treatment of *K. alvarezii* (80 °C). Means with different letter are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD), n=3.

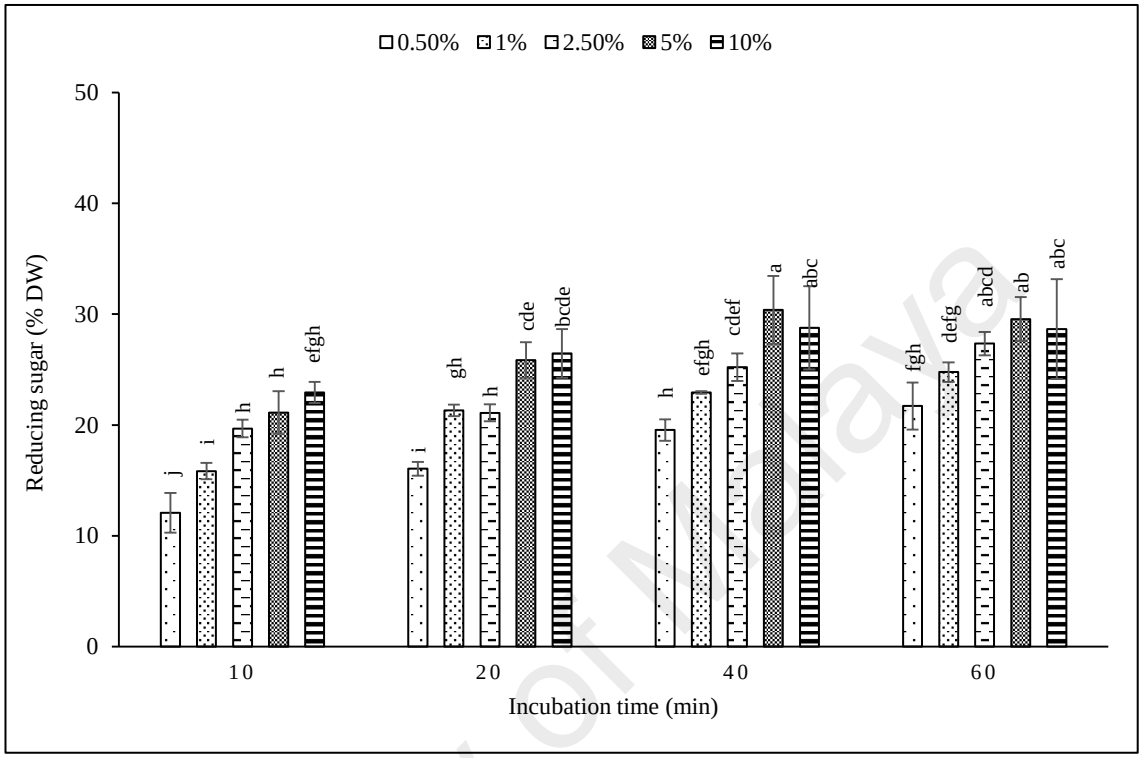

Figure 4.4: Reducing sugar content obtained under different conditions during thermal-acidic treatment of *K. alvarezii* (100 °C). Means with different letters are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD), n=3.

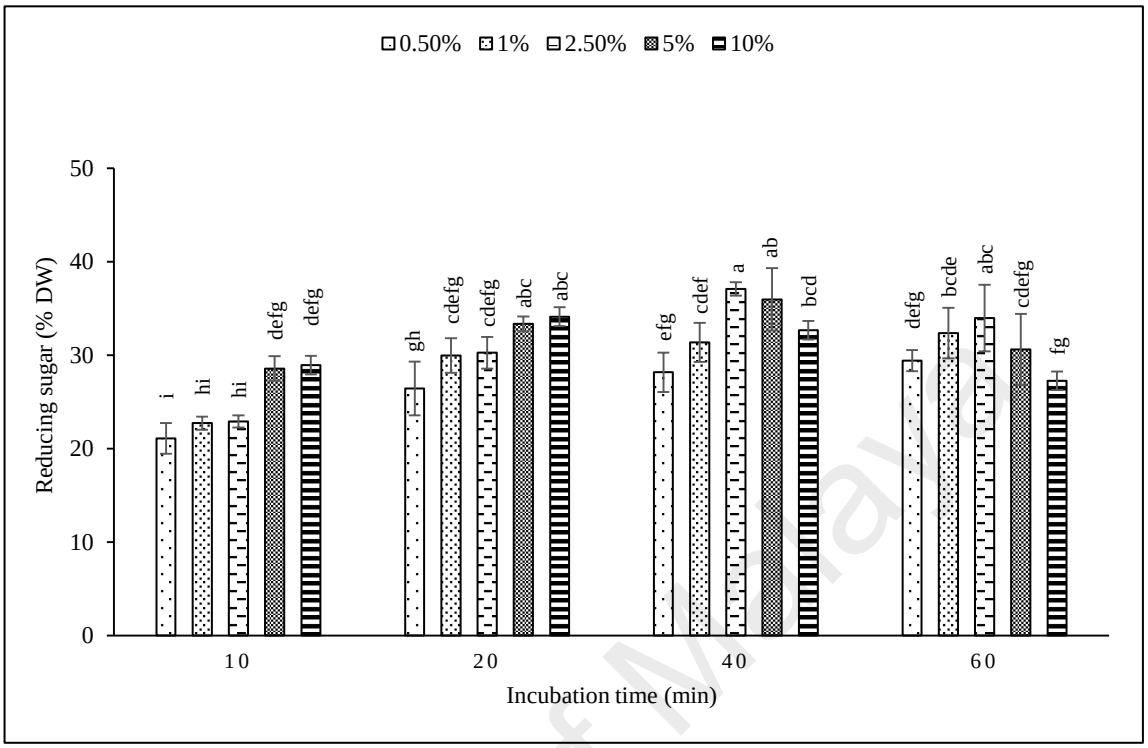

Figure 4.5: Reducing sugar content obtained under different conditions during thermal-acidic treatment of *K. alvarezii* (120 °C). Means with different letter are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD), n=3.

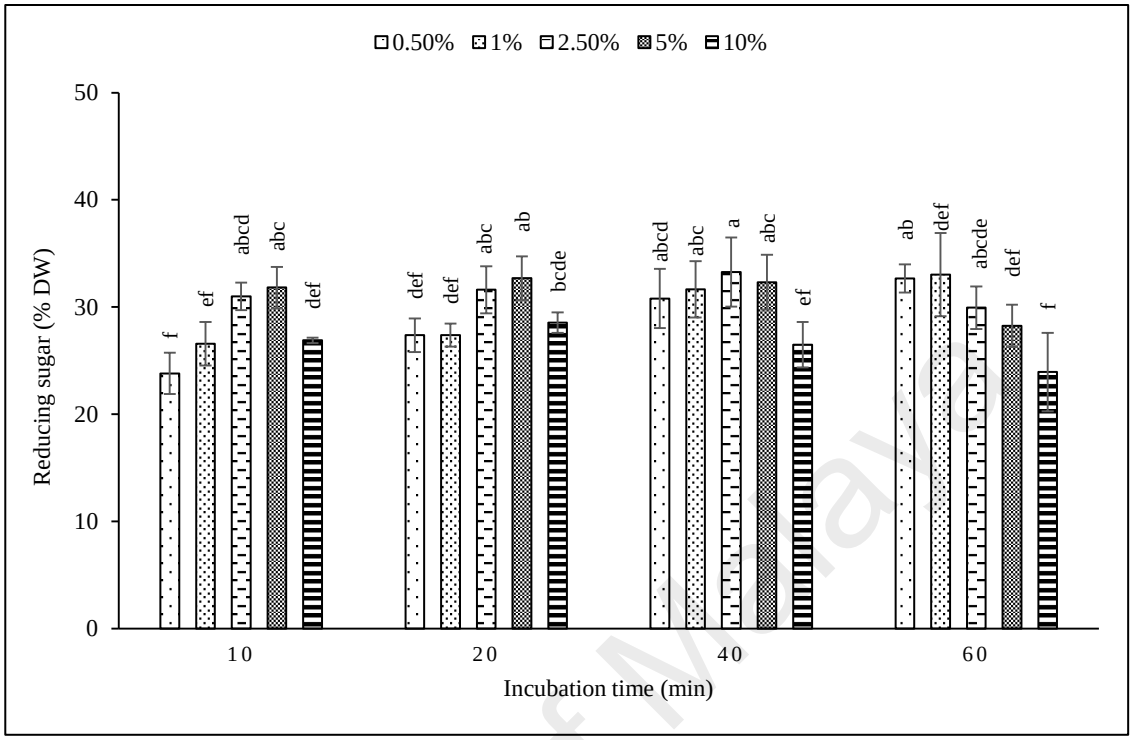

Figure 4.6: Reducing sugar content obtained under different conditions during thermal-acidic treatment of *K. alvarezii* (140 °C). Means with different letter are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD), n=3.

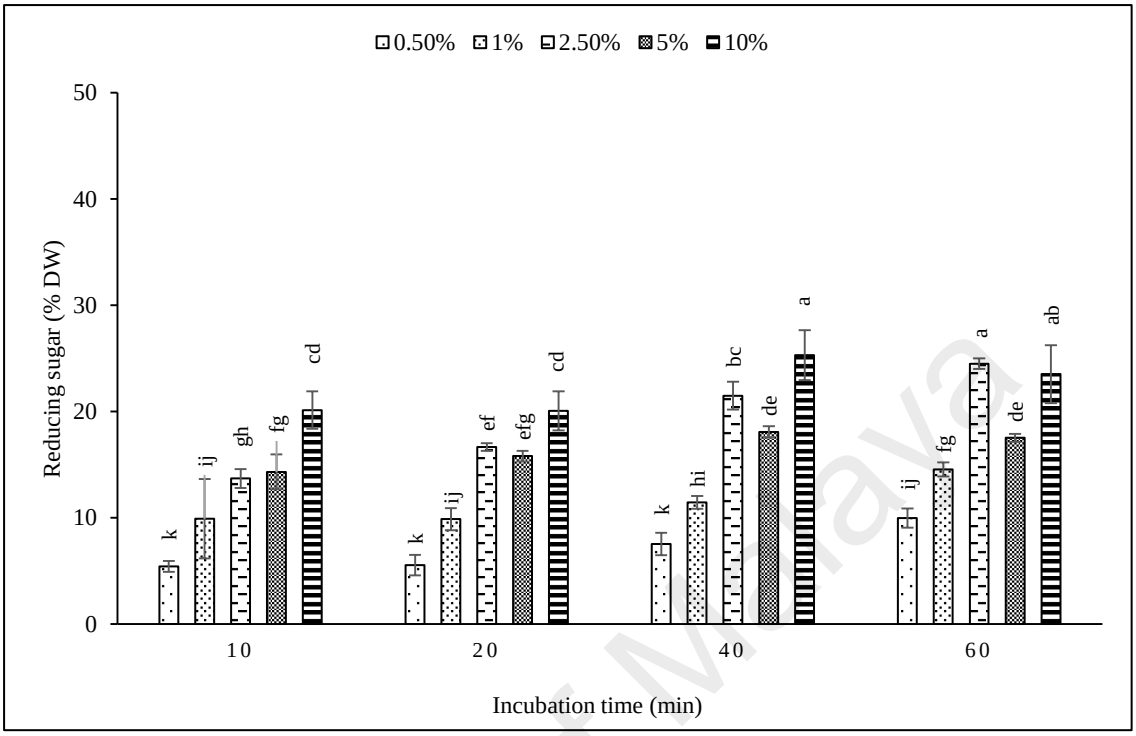

Figure 4.7: Reducing sugar content obtained under different conditions during thermal-acidic treatment of *G. manilaensis* (80 °C). Means with different letter are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD), n=3.

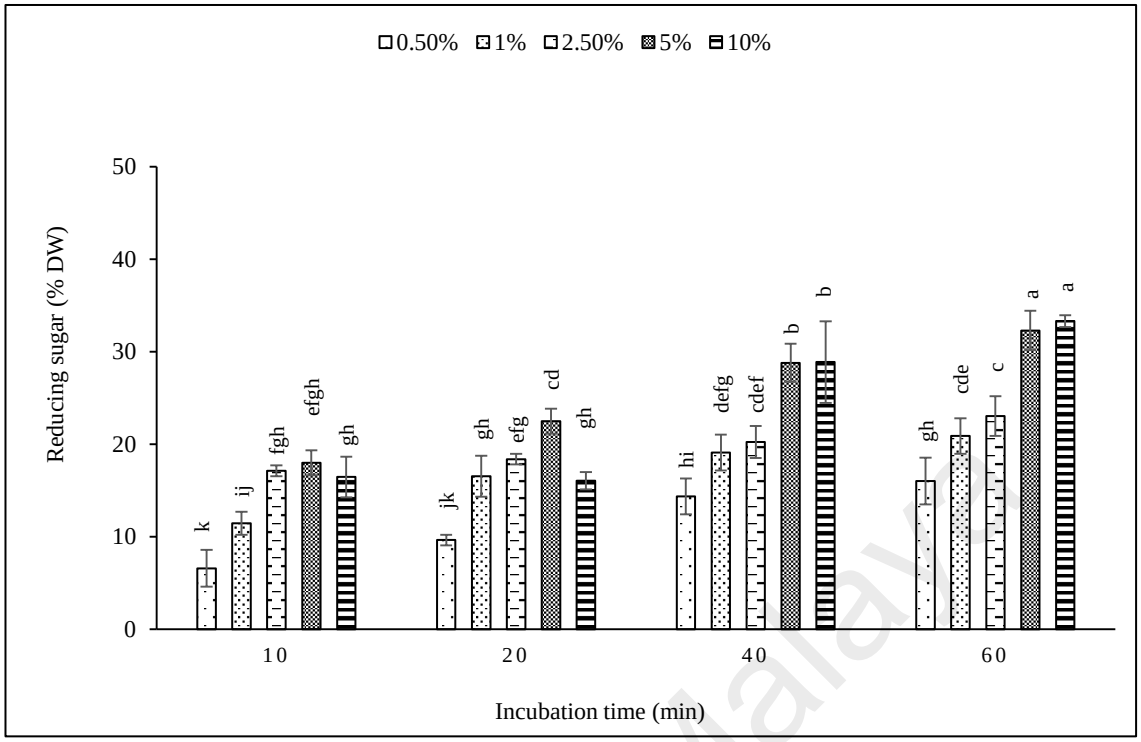

Figure 4.8: Reducing sugar content obtained under different conditions during thermal-acidic treatment of *G. manilaensis* (100 °C), Means with different letter are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD), n=3.

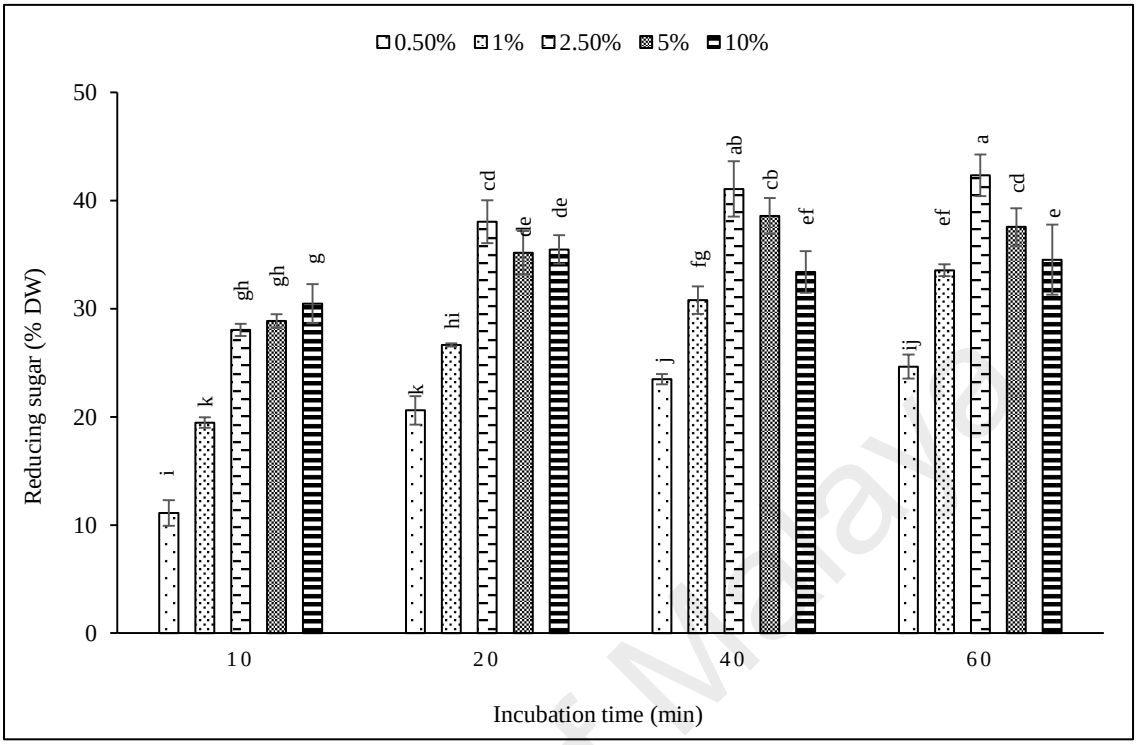

Figure 4.9: Reducing sugar content obtained under different conditions during thermal-acidic treatment of *G. manilaensis* (120 °C). Means with different letter are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD), n=3.

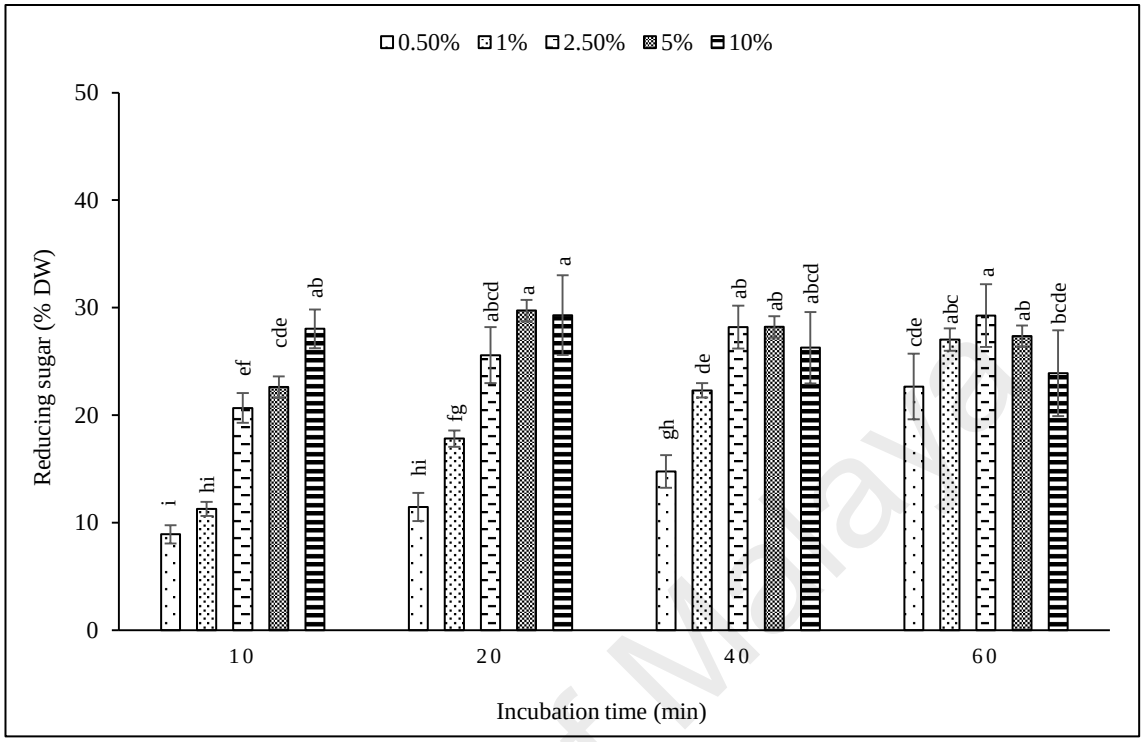

Figure 4.10: Reducing sugar content obtained under different conditions during thermal-acidic treatment of *G. manilaensis* (140 °C). Means with different letter are significantly different at each acid concentration level ($p < 0.5$, Tukey, HSD), n=3.

All data variables prior to data analysis were subjected to normality test based on Skewness and Kurtosis. The results (APPENDIX D-APPENDIX M) showed that all values for Skewness and Kurtosis ranged between an acceptable range of - 0.8 to + 0.8 for Skewness and - 2 to + 2 for Kurtosis, thus data were distributed normally and full factorial analysis was conducted for results of both seaweed species.

4.2.2 Seaweed hydrolysate detoxification

Figure 4.11 and Figure 4.12 shows the trends of 5-HMF removal in *K. alvarezii* and *G. manilaensis* respectively. Difference between removal effect of pH 11 and pH 12 was not significant ($p < 5\%$) therefore pH 11 for 60 min was selected for main treatment procedure. Sugars are sensitive to alkaline condition and by increasing over-liming more sugar degradation would occur (Martinez *et al.*, 2000).

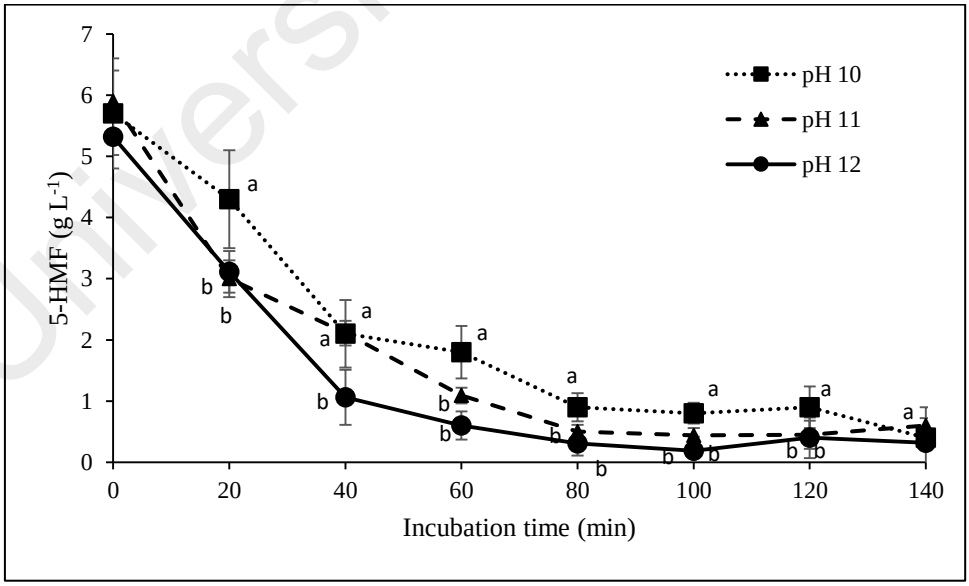

Figure 4.11: Reduction of 5-HMF during over liming process in *K. alvarezii* hydrolysate. Different letters are representing significant difference at $p < 0.05$ by Tukey, HSD between yeast species, (n=3).

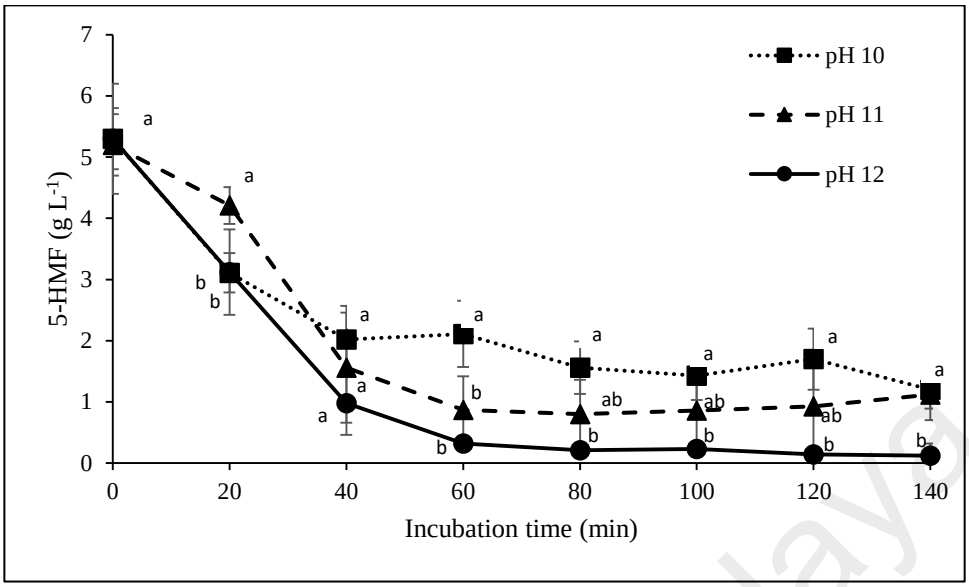

Figure 4.12: Reduction of 5-HMF during over liming process in *G. manilaensis* hydrolysate**.** Different letters are representing significant difference at $p < 0.05$ by Tukey, HSD (n = 3).

4.2.3 Enzyme-based saccharification

4.2.3.1 Optimization of the enzyme dosage

Figure 4.13 illustrates the hydrolysis of *G. manilaensis* dilute acid treatment residues by different ratio of cellulase enzyme (CTech 2). In general, amount of 2 % w w^{-1} and 5 % w w^{-1} showed same hydrolytic effectiveness and similarly 10 and 20 % w w^{-1} did not show significant differences ($p > 0.05$). Highest yield (87.5 % conversion) was achieved after 48 h in the sample with 20 % w w^{-1} enzyme where the yield of the sample with 10 % w w^{-1} enzyme was 85.5 % and no significant difference was observed ($p > 0.05$). Highest yield in 2 % enzyme was 82.5 % and 87.5 % for 5 % enzyme loading after 72 h incubation.

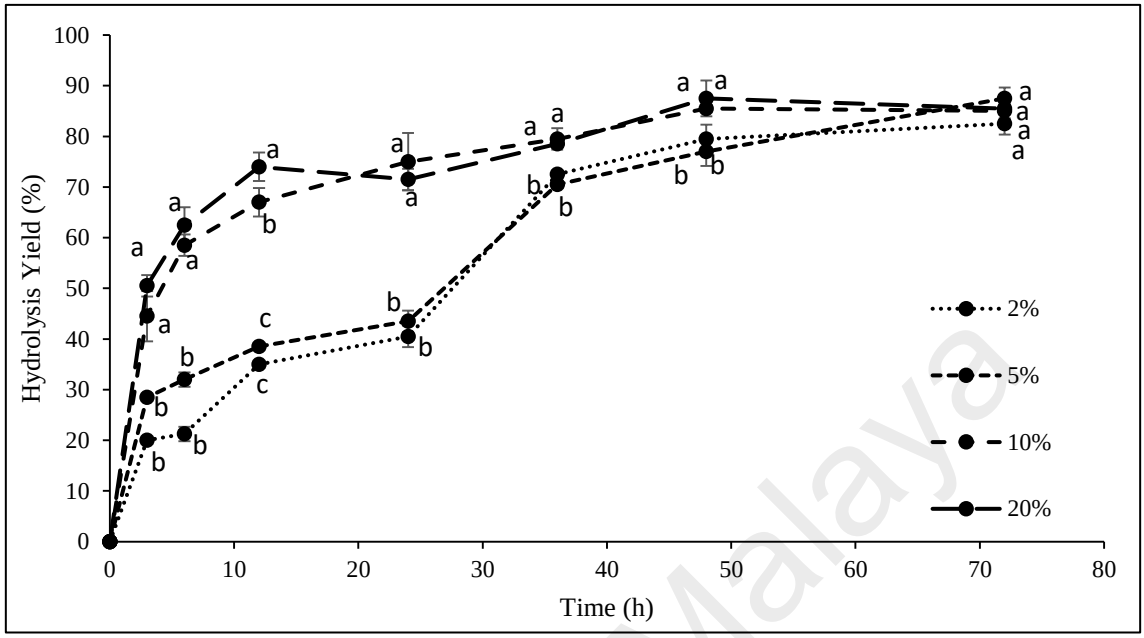

Figure 4.13: Enzymatic hydrolysis of *G. manilaensis* residues by different cellulase concentration loading. Different letters are representing significant difference at $p < 0.05$ by Tukey, HSD (n = 3).

4.2.3.2 Optimization of liquid: biomass ratio

Figure 4.14 illustrates the effect of the ratio of liquid to seaweed biomass on enzymatic hydrolysis yield and glucose concentration in the hydrolysate. In ration of 2.5 : 1 no reducing sugar was produced also 5.31 % w w^{-1} glucose was produced after 3 days incubation in sample with liquid: biomass ratio 5 :1. Highest hydrolysis yield was achieved in liquid: biomass ratio 10 : 1 where 85.12 % of biomass was converted to glucose, whereas highest glucose concentration 20.89 % w v^{-1} was achieved in sample with liquid: biomass ratio 7.5 : 1.

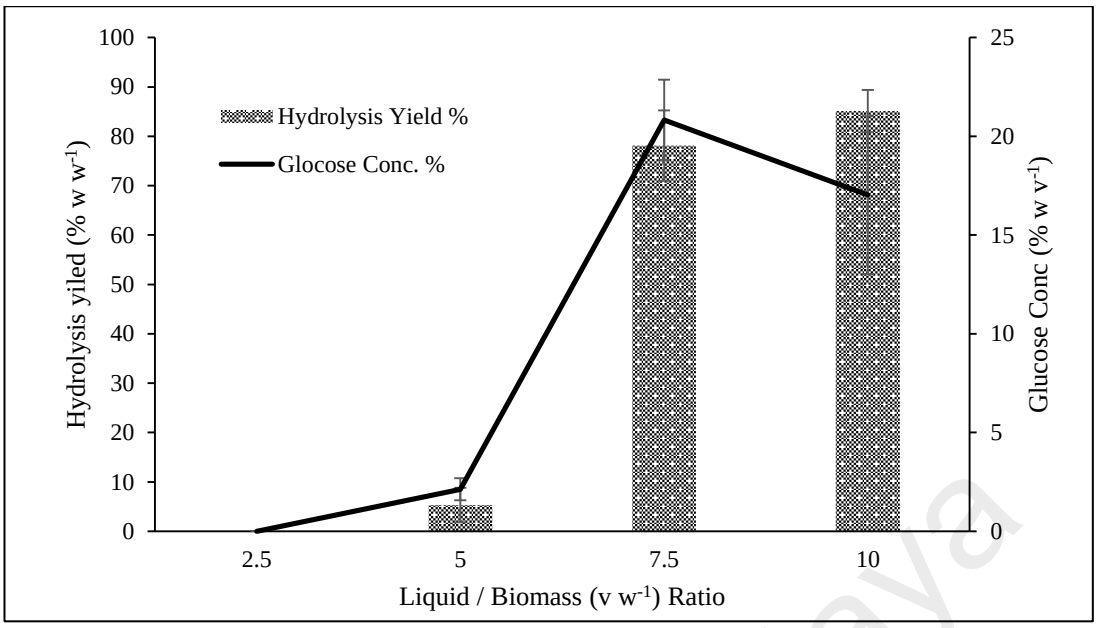

Figure 4.14: Effect of ratio of liquid to biomass (*G. manilaensis* cellulosic residues) in hydrolysis yield and final glucose concentration.

4.2.4 Preparation of seaweed hydrolysate for fermentation study

4.2.4.1 Dilute acid-based hydrolysis

The process of dilute acid treatment for two seaweed species was conducted by optimized condition, includes, using 2.5 % w v^{-1}, biomass: acid ratio 1:8, incubation time and temperature of 40 min and 120 °C respectively. Hydrolysates were filtered and detoxified by over-liming treatment using Ca (OH) $_2$. Material balance during the process of hydrolysis and detoxification is illustrated in Table 4.4.

Table 4.5 presents the effect of over-liming on sugar and main fermentation inhibitor in two seaweed hydrolysates. 5-HMF content in *G. manilaensis* and *K. alvarezii* hydrolyzate were reduced 62.15 % and 76.98 %, respectively. Over-liming also reduced the amount of sugar in both *G. manilaensis* and *K. alvarezii* hydrolyzate by 11.43 % and 10.88 %, respectively. The volume of hydrolyzate in both specimens is also decreased because of absorption of liquid to dry $Ca(OH)_2$.

Table 4.4: Material balance obtained during dilute acid hydrolysis treatment for fermentation study.

	Seaweed spp.	*K. alvarezii*	*G. manilaensis*
Before Hydrolysis	**Initial Biomass (g)**	100 ± 0.00	100 ± 0.00
	Sulphuric acid added (mL)	800 ± 0.00	800 ± 0.00
Hydrolysis Process	**Recovered Hyd (mL)**	580.33 ± 3.51	680.67 ± 9.02
	Hyd Sugar Conc (g L^{-1})	58.81 ± 1.33	56.29 ± 2.75
	Produced Sugar (g)	34.13 ± 0.60	38.30 ± 1.43
After Detoxification Process	**Detoxified Hyd Vol (mL)**	489.67 ± 8.50	615.00 ± 6.24
	Detoxified Hyd Sugar Conc (g L^{-1})	61.28 ± 2.98	55.77 ± 3.28
	Vol Hyd Loss (mL)	72.00 ± 3.46	72.00 ± 3.46
	Vol Hyd Loss (%)	12.41 ± 0.60	10.58 ± 0.40
	Sugar Recovered (g)	29.99 ± 0.94	34.28 ± 1.71
	Sugar Loss (g)	4.14 ± 0.54	4.02 ± 0.32
	Sugar Loss (%)	12.14 ± 1.68	10.51 ± 1.19

Hyd: Hydrolysate, Vol: Volume

Table 4.5: Effect of over-liming treatment to remove fermentation inhibitors on two seaweed hydrolysates.

		Before treatment g L^{-1}	After treatment g L^{-1}	Decrease %
K. alvarezii	**Reducing sugar**	58.18 ± 1.33	61.28 ± 2.98	12.14 ± 1.68
	5- HMF	5.68 ± 0.41	1.42 ± 0.42	79.23 ± 4.35
	Furfural	0.46 ± 0.07	0.31± 0.05	42.63 ± 6.39
	Phenolic compounds	3.88 ± 0.67	1.96 ± 0.73	54.59 ± 23.79
G. manilaensis	**Reducing sugar**	56.29 ± 2.75	55.77 ± 3.28	10.51 ± 1.19
	5- HMF	5.24 ± 0.79	1.30 ± 0.17	77.16 ± 5.04
	Furfural	0.76 ± 0.14	0.52 ± 0.17	36.99 ± 22.57
	Phenolic compounds	3.17 ± 1.02	1.05 ± 0.09	68.26 ± 8.08

The hydrolysates were adjusted to pH 11 by adding $Ca(OH)_2$ and were shaken for 30 min at 30 °C, Mean ± SD, n = 3.

4.2.4.2 Enzyme-based hydrolysis

Table 4.6 illustrates the enzymatic hydrolysis of residues of the two seaweeds, after dilute acid treatment. Seven g of each residue were used in this experiment and results were used to calculate the potential amount of sugar that can be generated (Table 4.7).

Table 4.6: Results of enzymatic hydrolysis of two seaweeds by dilute acid treatment residues from 7 g DW residue.

	K. alvarezii	*G. manilaensis*
Initial Residue used (g)	7.00 ± 0	7.00 ± 0
Ash %	8.75 ± 0.54 ns	7.03 ± 0.19 ns
Ash Free DW (g)	6.38 ± 0.43 ns	6.90 ± 0.73 ns
Total Buffer (mL)	50 ± 0	50 ± 0
Recovered Hyd (mL)	44.17 ± 2.25 ns	45.77 ± 1.66 ns
Hyd Sugar Conc (g L^{-1})	120.33 ± 10.97 ns	105.67 ± 4.16 ns
Produced Sugar (g)	5.30 ± 0.24 ns	4.83 ± 0.08 ns
Saccharification Yield %	82.97 ± 4.23 ns	74.15 ± 1.73 ns

ns: Not Significant at t-Test analysis $p > 0.05$

Table 4.7: Calculated values of enzymatic hydrolysis of two seaweed dilute acid treatment residues obtained from 100 g DW biomass.

	K. alvarezii	*G. manilaensis*
Residues (g)	13.07 ± 2.18 ns	13.81 ± 0.54 ns
Ash %	8.75 ± 0.54 ns	7.03 ± 0.19 ns
Total Ash Free DW (g) *	11.93 ± 2.03	12.86 ± 0.60
Hyd Sugar Conc (g L^{-1}) *	120.33 ± 10.97 ns	105.67 ± 4.16 ns
Produced Sugar (g) *	9.94 ± 1.38 ns	9.54 ± 0.32 ns
Sugar Yield (g g $biomass^{-1}$)*	0.82 ± 0.01 ns	0.74 ± 0.01 ns

ns: Not Significant with t-Test analysis at $p > 0.05$
*The values are calculated based on saccharification yield of 7.00 g DW residue in Table 4.6

4.3 Experiment 3. Fermentation studies

4.3.1 Selection of microorganism and acclimation to seaweed hydrolyzate

Figure 4.15, Figure 4.16 and Figure 4.17 show the trend of sugar consumption and ethanol production during 3 cycles of acclimation by three yeast species. Figure 4.15 shows ethanol production was increased from 2.13 ± 0.25 g L^{-1} to 3.90 ± 0.56 g L^{-1} in *B. bruxellensis* while ethanol was increased from 3.55± 0.48 g L^{-1} to 5.3 ± 0.90 g L^{-1} in *S. cerevisiae* NBRC 10217 (Figure 4.16), where no significant difference on ethanol

production by the third phase was observed ($p > 0.05$) between these two strains (Figure 4.17). In Ethanol Red strain, ethanol concentration in the third phase was 7.20 ± 0.70 g L^{-1} and statistically significant difference ($p < 0.05$) was seen between Ethanol Red and other strains (Figure 4.18).

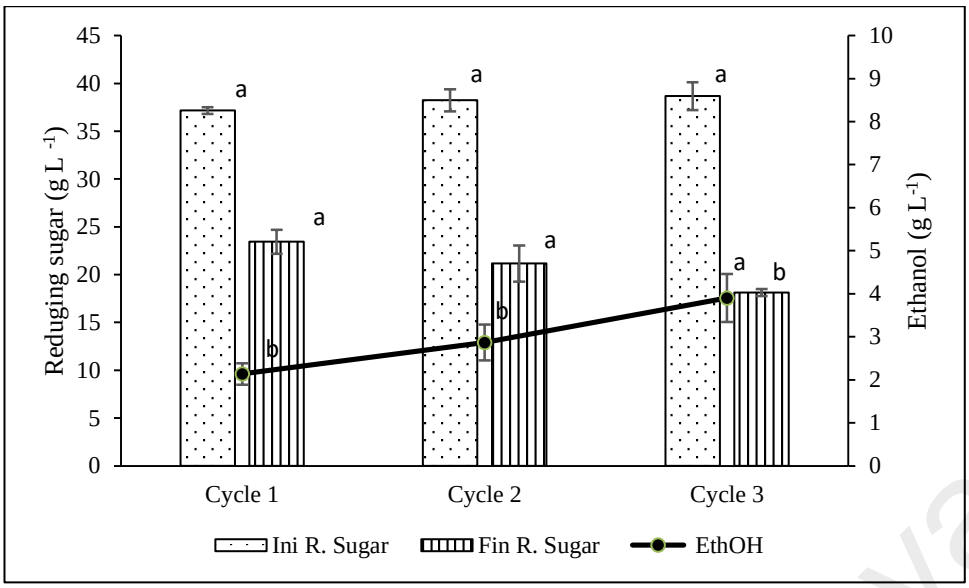

Figure 4.15: Fermentation of hydrolysate of *G. manilaensis* by *B. bruxellensis*- NBRC 0677, during cyclic adaption. Different letters are representing significant difference at $p < 0.05$ by Tukey, HSD (n = 3).

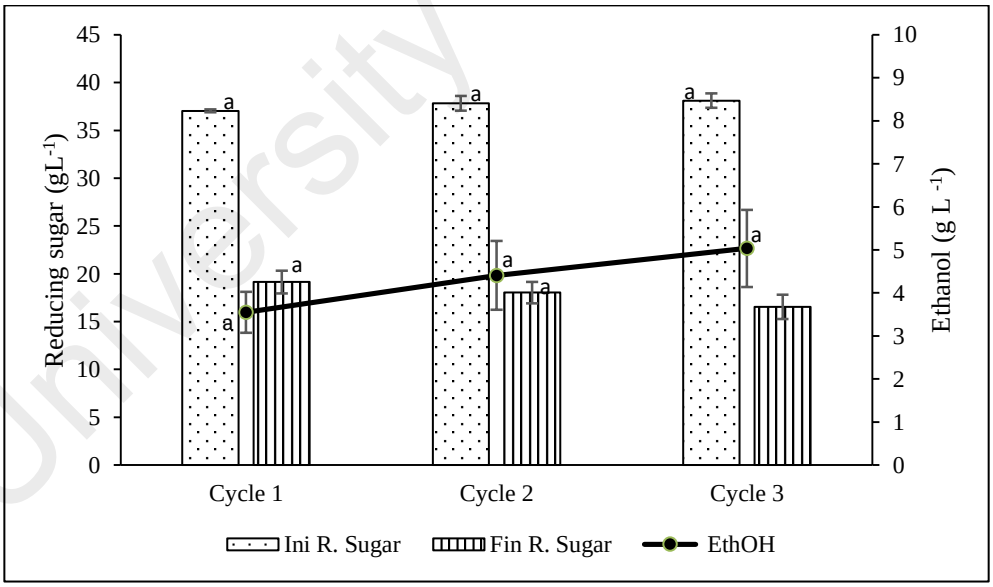

Figure 4.16: Fermentation of hydrolysate of *G. manilaensis* by *S. cerevisiae*-NBRC 10217, during cyclic adaption. Different letters are representing significant difference at $p < 0.05$ by Tukey, HSD (n = 3).

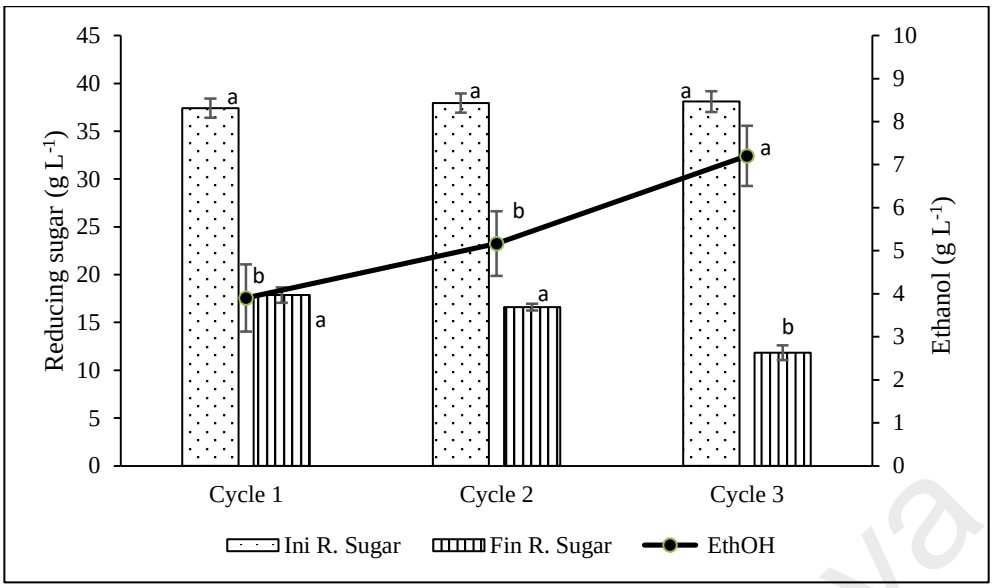

Figure 4.17: Fermentation of hydrolysate of *G. manilaensis* by *S. cerevisiae*- Ethanol Red, during cyclic adaption. Different letters are representing significant difference at $p < 0.05$ by Tukey, HSD (n = 3).

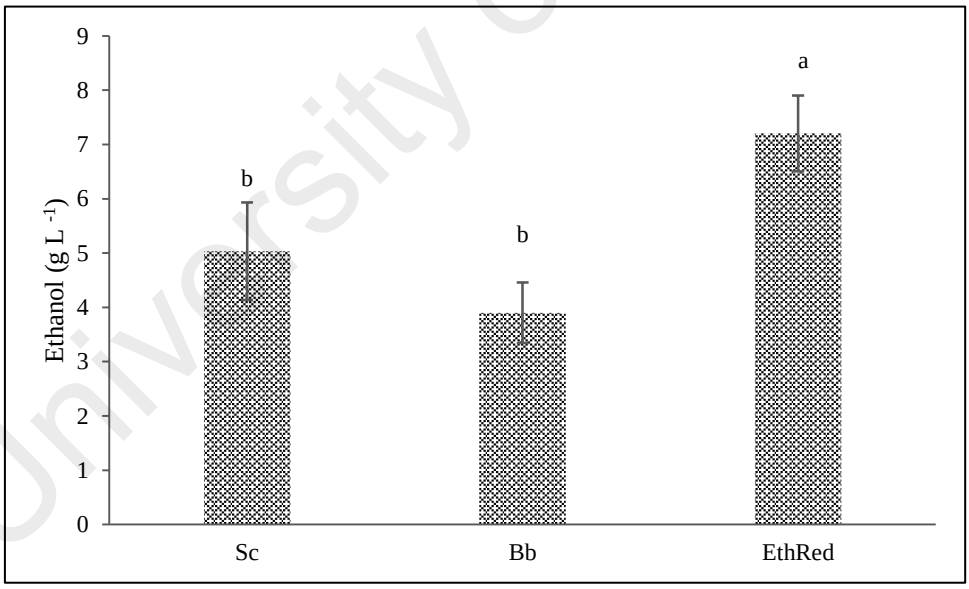

Figure 4.18: Ethanol production from hydrolysate of *G. manilaensis* by three yeast strains after 3 cyclic acclimations. Sc: *S. cervisies* NBRC 10217; Bb: *B. bruxellensis*- NBRC 0677; Ethanol Red: *S. cerevisiae*- Ethanol Red. Different letters are representing significant difference at $p < 0.05$ by Tukey, HSD between yeast species, (n=3).

4.3.1.1 Acclimation of selected strain

Ethanol production, initial sugar concentration and remaining sugar concentration of acclimation process is illustrated in Figure 4.19. Significant differences t ($p < 0.05$) n=3, were observed between ethanol production and remaining sugar concentration in P0 and P1.

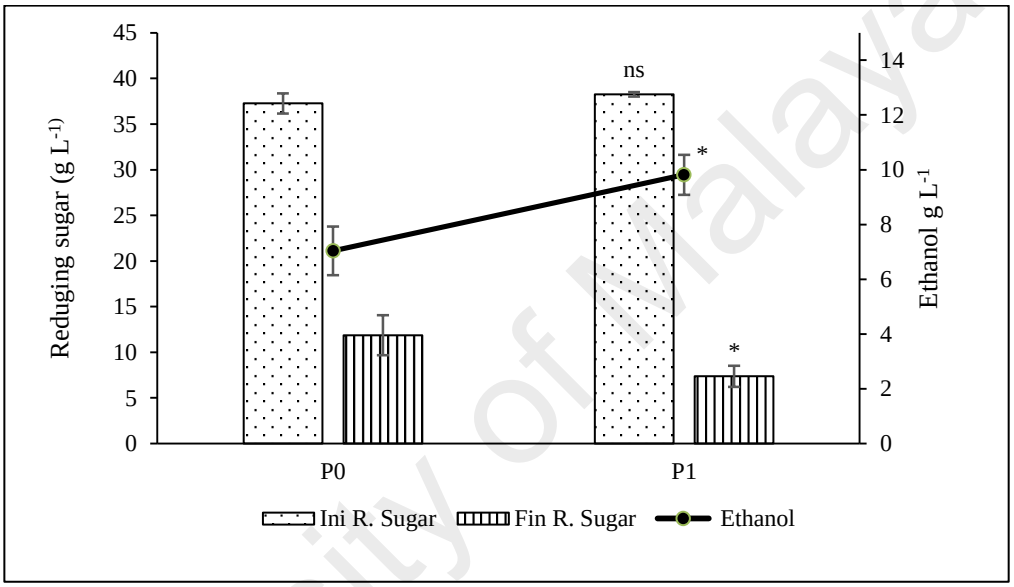

Figure 4.19: Ethanol production from *G. manilaensis* hydrolysate, initial reducing sugar concentration and remaining reducing sugar concentration of acclimation process in *S. cerevisiae*- Ethanol Red, n=3. (* Significant difference $p < 0.05$, ns: Not Significant).

4.3.2 Fermentation of dilute acid-based hydrolysate

S. cerevisiae is a well-established microorganism used in anaerobic fermentation for ethanol production. Figure 4.20 and Figure 4.21 illustrate the production of ethanol in relation to consumption of reducing sugar at different incubation times during the fermentation of hydrolysates of *K. alvarezii* and *G. manilaensis* by *S. cerevisiae.* Initial reducing sugar concentration in *K. alvarezii* hydrolysate was 61.28 ± 2.98 g L^{-1} and

maximum ethanol production (20.90 ± 1.81 g L^{-1}) was achieved at 36 h corresponding to 71.06 % of theoretical yield conversion of reducing sugar on the basis of glucose yield (0.48 g g^{-1}), while maximum ethanol production in *G. manilaensis* was 20.62 ± 1.68 g L^{-1} at the same time which was corresponding to 72.50 % of theoretical yield. In the present study, all sugar were not fully consumed in both hydrolysates after 72 h, were 3.47 ± 1.11 g L^{-1} and 6.24 ± 1.15g L^{-1} were seen in *K. alvarezii* and *G. manilaensis,* respectively.

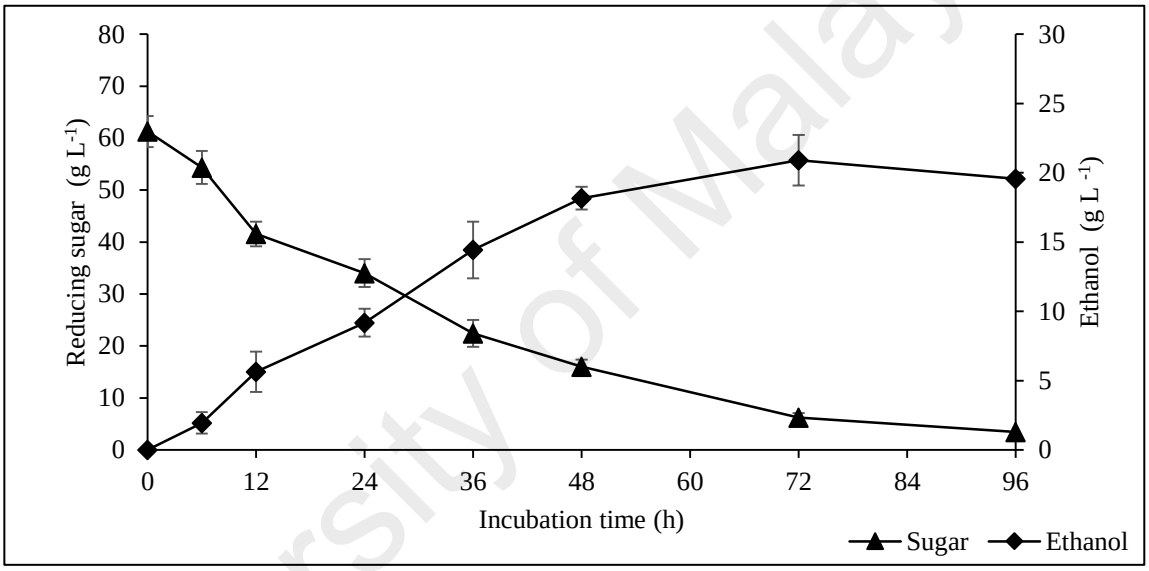

Figure 4.20: Fermentation with dilute acid hydrolysate of *K. alvarezii* hydrolysate using Ethanol Red, *S. cerevisiae.*

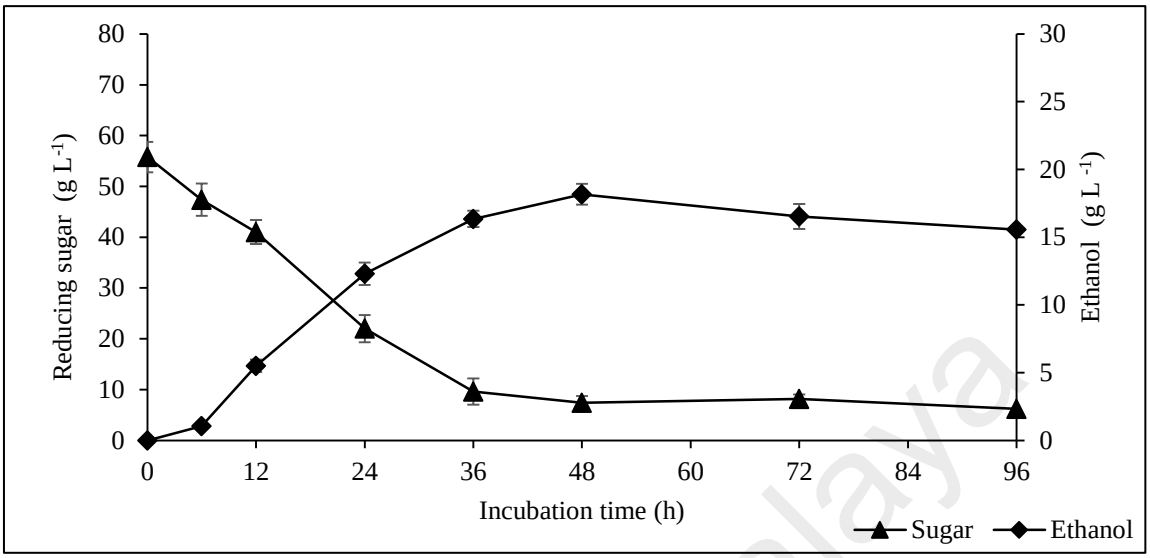

Figure 4.21: Fermentation with dilute acid hydrolysate of *G. manilaensis* hydrolysate using Ethanol Red, *S. cerevisiae.*

4.3.3 Fermentation of enzyme-based hydrolysate

Figure 4.22 illustrates the fermentation of hydrolysate of *K. alvarezii.* Maximum ethanol (56.26 g L^{-1}) was achieved after 48 h in *K. alvarezii* corresponding to 91 % of theoretical yield while highest ethanol production was after 36 h (51.10 g L^{-1}) in *G. manilaensis* hydrolysate (Figure 4.23) and 95 % of the theoretical yield of fermentation was achieved.

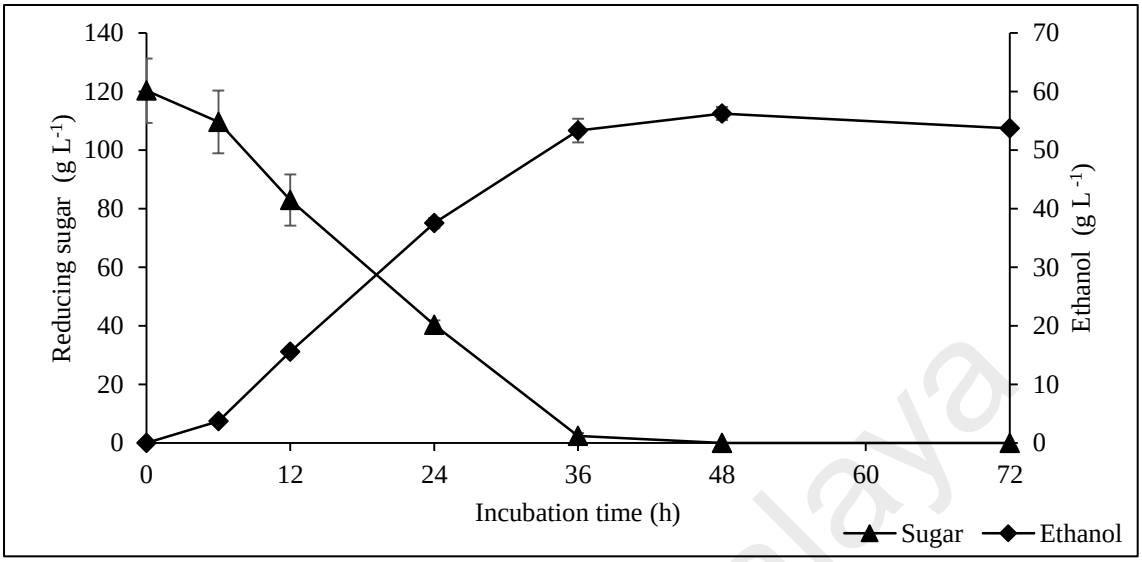

Figure 4.22: Fermentation with enzymatic hydrolysate of *K. alvarezii*.

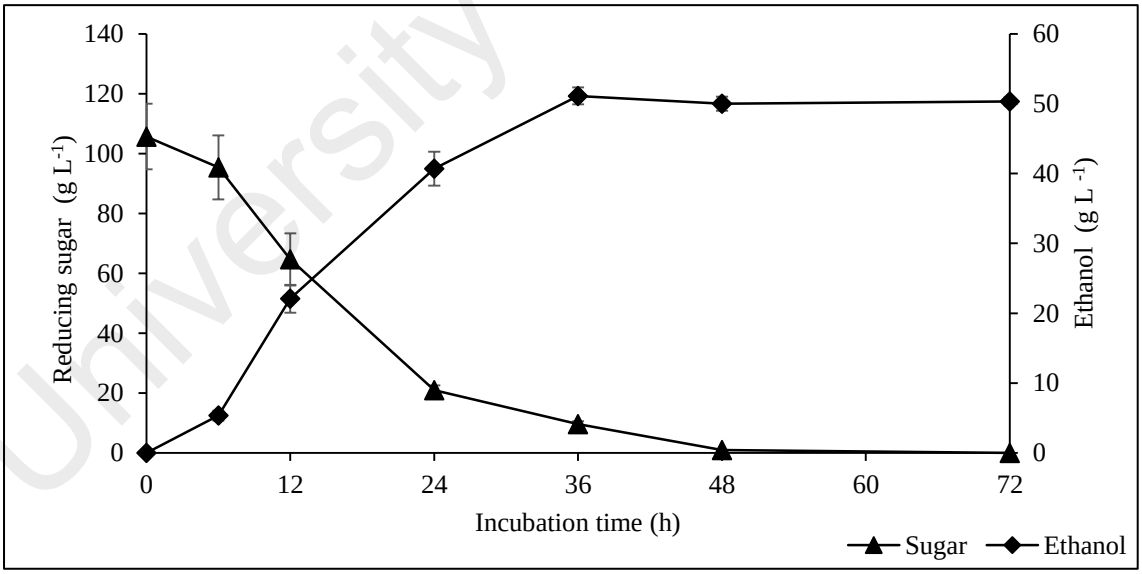

Figure 4.23: Fermentation with enzymatic hydrolysate of *G. manilaensis.*

4.3.4 Calculating the bioethanol production potential in *K. alvarezii* and *G. manilaensis*

The potential of bioethanol production from *K. alvarezii* and *G. manilaensis* in this study was calculated based on 100 g DW of each seaweed biomass and data are plotted in Figure 4.24 and Figure 4.25 respectively. Applying optimum condition for dilute acid treatment, soluble polysaccharide were converted to reduced sugars and residues were characterized for ash and DW content. In *K. alvarezii*, 34.13 ± 0.60 g of reducing sugars was produced and after over liming treatment 29.99 ± 0.94 g of reducing sugars remained in detoxified *K. alvarezii* hydrolysate. These values were 38.30 ± 1.43 g and 34.28 ± 1.71 g for *G. manilaensis* biomass. Obtained residues for *K. alvarezii* and *G. manilaensis* were 13.07 ± 2.18 g and 13.81 ± 0.54 g respectively, and the amount of ash in both seaweed residues were not high. Enzymatic conversion generated 9.94 ± 1.38 g glucose in *K. alvarezii* corresponding to 82.97 ± 4.23 % enzymatic conversion while 9.54 ± 0.32 g glucose was produced in *G. manilaensis* with a value of 74.15 ± 1.73 % for enzymatic conversion.

Taking into account the reducing sugar yields and losses in each step, it can be estimated that the process herein studied resulted in a ratio 14.88 g corresponding[1] to 18.83 mL ethanol in *K. alvarezii* (Figure 4. 24**)** and 15.79 g corresponding to 19.98 mL of ethanol in *G. manilaensis* (Figure 4.25) per 100 g DW seaweed, respectively.

[1] The density of ethanol is equal to 0.789 g/cm^3

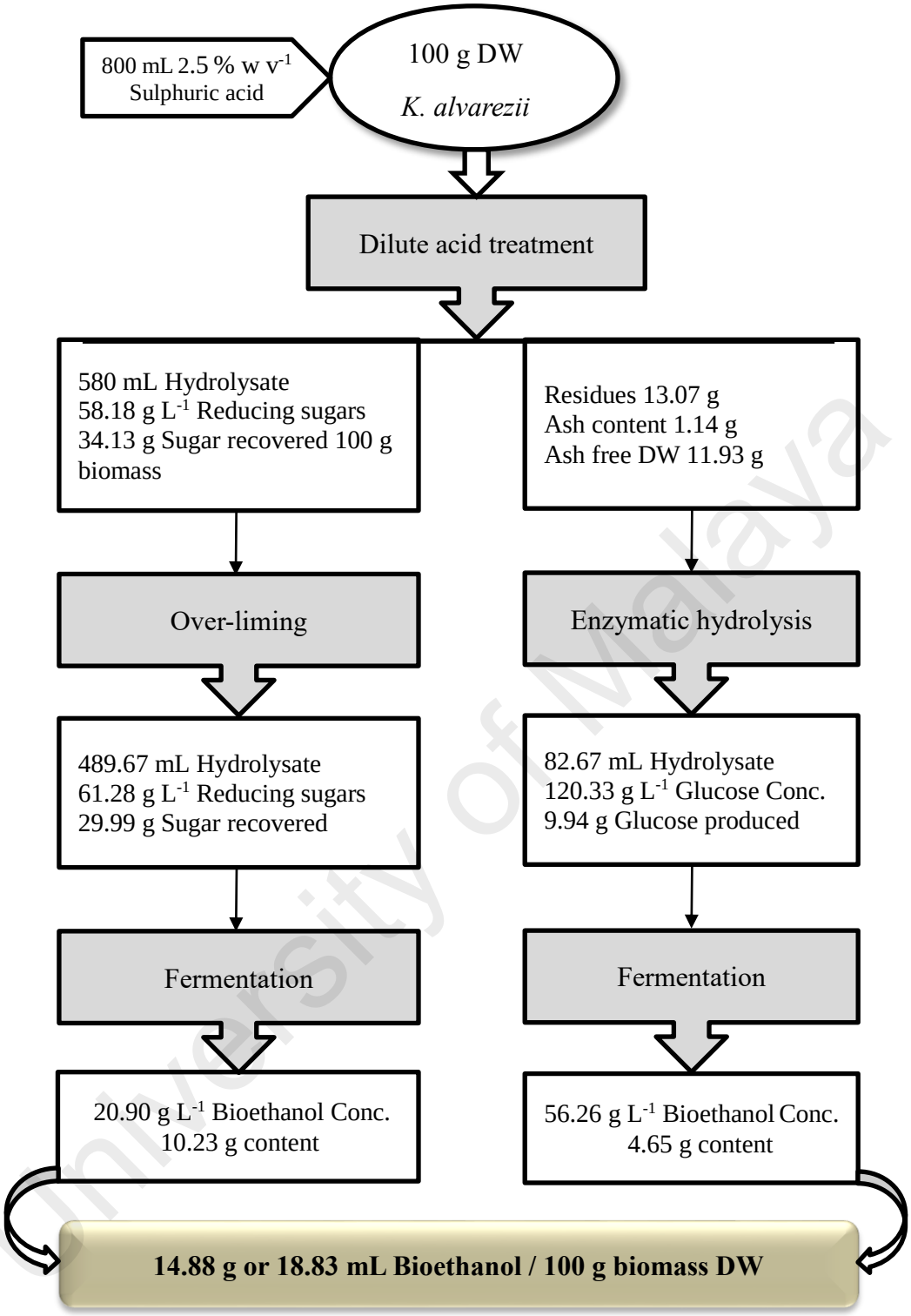

Figure 4.24: Material balance chart for the conversion of *K. alvarezii* biomass to bioethanol

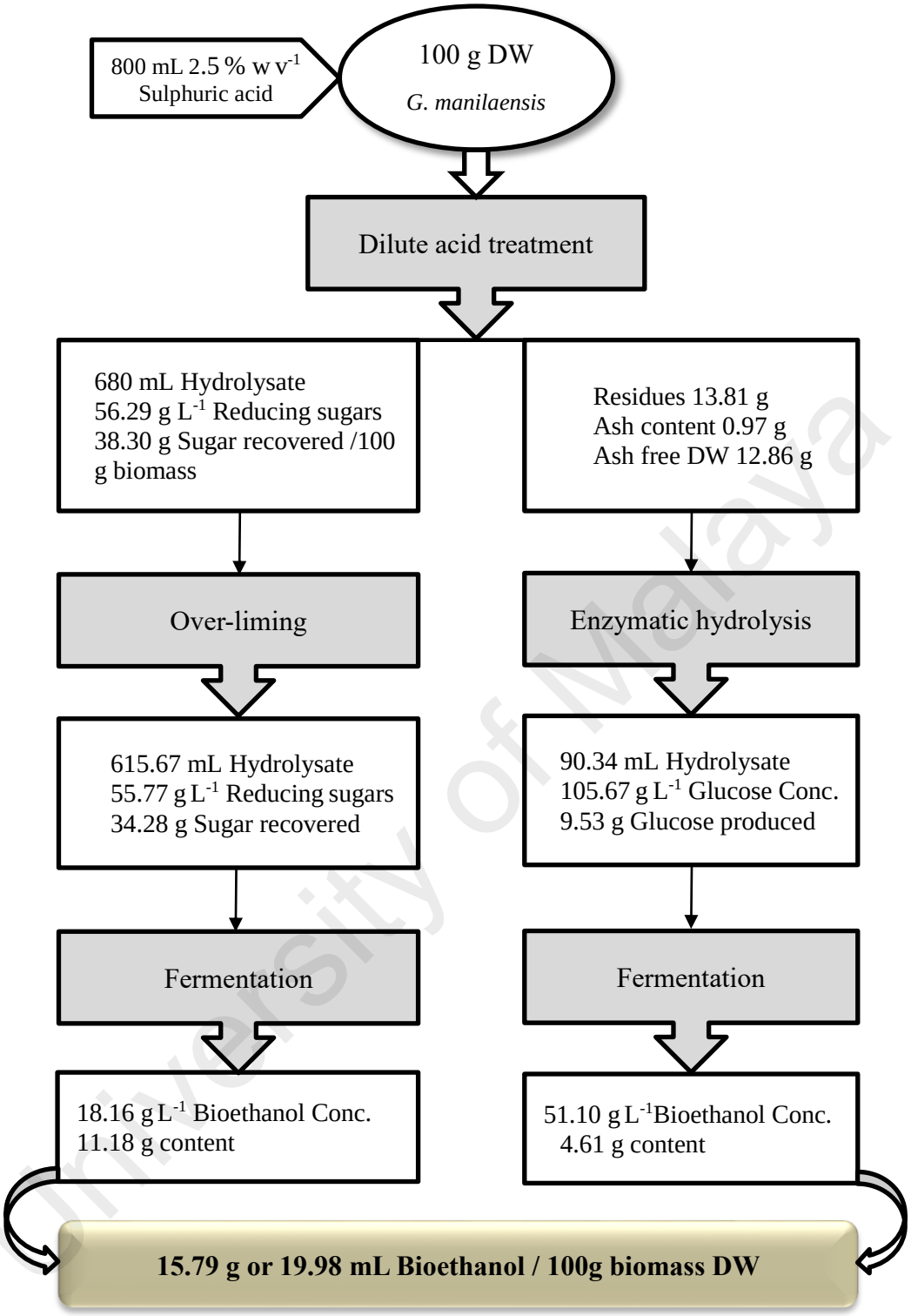

Figure 4.25: Material balance chart for the conversion of *G. manilaensis* biomass to bioethanol

4.3.5 Analysing bioethanol content by GC using a novel sample preparation approach

In this study mixture of two solvents, acetonitrile and isobutanol was used. These two solvents can be separated from ethanol efficiently (Canfield *et al.,* 1998) (APPENDIX N). Figure 4.26 shows the effect of solvent mixture (Acetonitrile / Iso-butanol) on a sample by which adding the solvent mixture to samples caused a precipitation of the water-soluble complex organic compounds (Figure 4.26, Vial C) and followed by centrifugation. Water-soluble compounds become precipitated at the bottom of the vial and a clear yellowish supernatant was achieved (Figure 4.26, Vial D) which would be injected into GC machine.

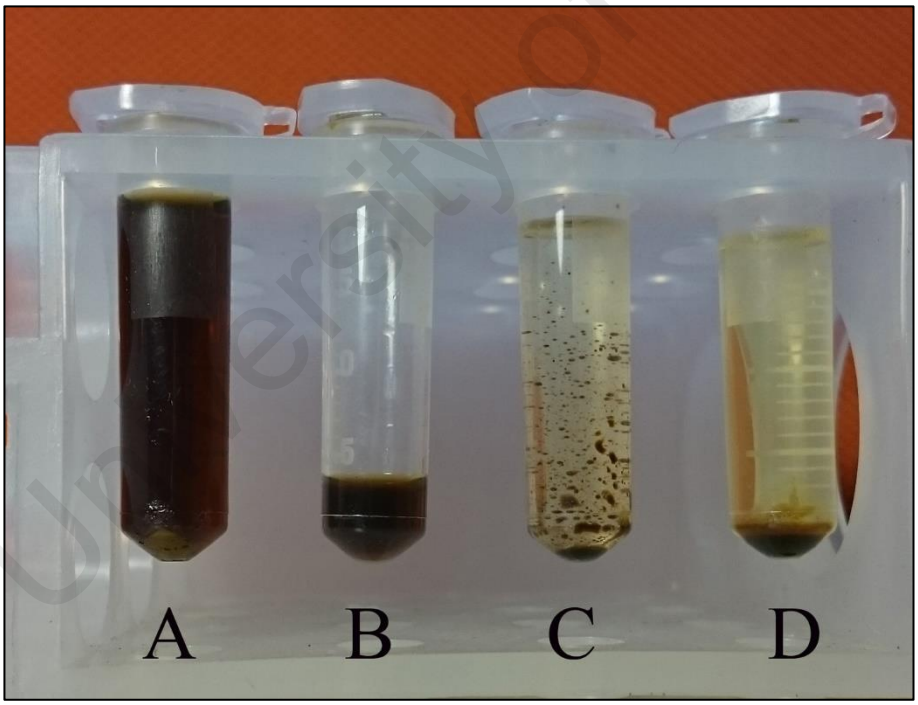

Figure 4.26: Effect of solvent mixture on fermented sample, A. Centrifuged fermented sample, B. supernatant of centrifuged sample from vial A, C. Solvent mixture is added to sample, D. Centrifuged precipitated sample

Figure 4.27 shows the chromatogram of ethanol analysis as described before. Ethanol is the first eluent detected in GC after 2.30 min, followed by the main matrix, acetonitrile (2.66 min) and internal standard, iso-butanol (3.06 min). Using this method a sufficient and fast separation of the compounds were achieved.

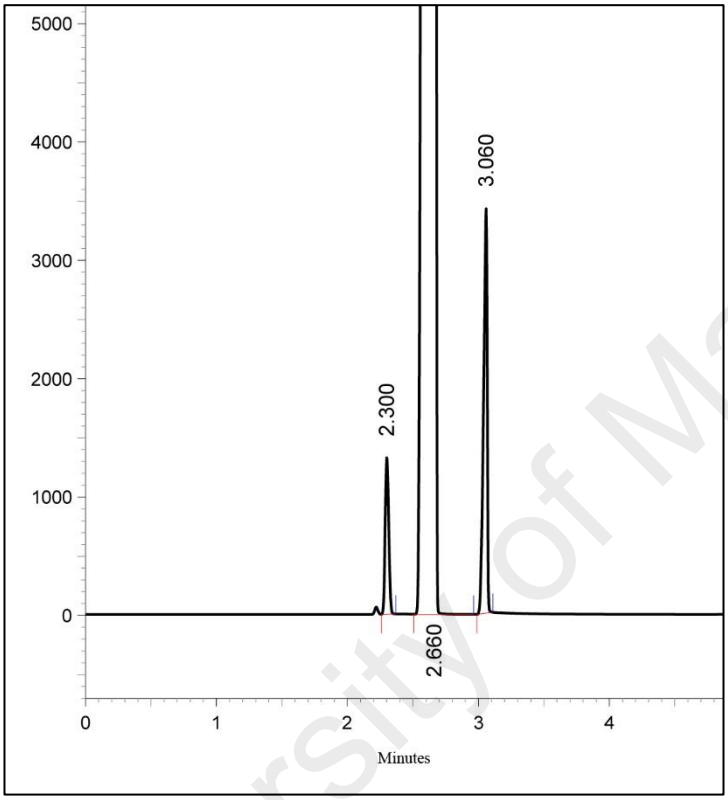

Figure 4.27: Chromatogram of three compounds (retention time, min) including; Ethanol (2.30), Acetonitrile (2.660) and Iso-Butanol (3.060).

To evaluate the accuracy of sample preparation methodology, a triplicate of known ethanol concentrations was prepared and the error was calculated as shown in Table 4.8. High accuracy was achieved using this method where, the lowest error was observed in ethanol test sample 0.3 % w v^{-1} with 0.460 %. Maximum error was observed in 1.765 % in ethanol test sample 1.050 % w v^{-1}. Error % was calculated as the following Eq:

$$\text{Error } \% = \frac{[\text{Known conc.} \% - \text{calculated conc.} \%]}{known\ conc. \%} \times 100$$

The standard curve plotted with and without applying the method is shown in APPENDIX O.

Table 4.8: Evaluating the solvents mixture method by known ethanol concentration samples.

	Known sample Conc. (% w v^{-1})	Calculated Conc. (% w v^{-1})	Error %
A	**1.050**	1.068 ± 0.043	1.745
B	**0.550**	0.546± 0.019	0.739
C	**0.300**	0.299± 0.012	0.460

4.4 Experiment 4. Saccharification at low temperature and dilute acid

4.4.1 RSM modelling for reducing sugar production

Experimental design matrix for optimization using a new approach of dilute acid treatment of *K. alvarezii* and *G. manilaensis* are illustrated in Table 4.9 and Table 4.10.

Table 4.9: Experimental design matrix for the optimization of the dilute acid pretreatment of *K. alvarezii*.

Run	Acid Conc. (%)	Temp (°C)	Time (h)	Reducing sugar (%)
1	5.0	60	6	23.0
2	5.0	60	6	23.0
3	7.5	75	10	21.9
4	2.5	75	10	25.3
5	5	60	6	21.7
6	5	60	6	21.0
7	7.5	45	10	11.9
8	2.5	45	2	1.3
9	2.5	75	2	22.7
10	2.5	45	10	6.9
11	7.5	45	2	2.3
12	7.5	75	2	24.4
13	5	60	6	22.0
14	5	60	2	10.0
15	5	75	6	25.0
16	5	60	10	21.3
17	5	45	6	5.3
18	7.5	60	6	24.1
19	5	60	6	22.2
20	2.5	60	6	20.9

Table 4.10: Experimental design matrix for the optimization of the dilute acid pretreatment of *G. manilaensis.*

Run	Acid Conc. (%)	Temp (°C)	Time (h)	Reducing sugar (%)
1	2.5	45	10	6.5
2	2.5	75	2	10.9
3	5.0	60	6	14.0
4	5.0	60	6	15.0
5	7.5	45	10	13.0
6	2.5	45	2	1.7
7	7.5	45	2	5.6
8	7.5	75	2	16.4
9	5.0	60	6	14.0
10	2.5	75	10	18.0
11	7.5	75	10	22.0
12	5.0	60	6	15.0
13	2.5	60	6	12.4
14	5.0	45	6	7.8
15	7.5	60	6	17.0
16	5.0	60	6	13.0
17	5.0	60	6	15.0
18	5.0	60	10	16.0
19	5.0	75	6	19.3
20	5.0	60	2	8.5

Highest reducing sugar yield in *K. alvarezii* was achieved in Run 4 (25.30 % DW) while the lowest was observed in Run 8 (1.30 % DW). In *G. manilaensis* highest and lowest yield of reducing sugar were in Run 11 (22.00 % DW) and Run 6 (1.70 % DW), respectively.

Reducing sugar yield in both seaweed species were not met by the RSM assumption so Natural log was applied for both seaweed species. The Quadratic model for reducing sugar yield showed the highest order model with significant terms (Prob > F is less than 0.05), therefore, it was selected as a final model for this data (Table 4.11 and Table 4.12).

Table 4.11: Sequential model sum of squares for reducing sugars yield in *K. alvarezii*

Source	Sum of sq	df	Mean sq	F Value	p-value Prob>F	
Block vs Mean	0.3992	1	0.3992			
Linear vs Block	9.3351	3	3.1117	11.4448	0.0004	
2FI vs Linear	1.5598	3	0.5199	2.4773	0.1112	
Quadratic vs 2FI	2.4705	3	0.8235	154.3815	< 0.0001	Suggested
Cubic vs Quadratic	0.0343	4	0.0086	3.1212	0.1218	Aliased
Residual	0.0137	5	0.0027			
Total	155.3182	20	7.7659			

Table 4.12: Sequential model sum of squares for reducing sugars yield in *G. manilaensis*

Source	Sum of sq	df	Mean sq	F Value	p-Value Prob>F	
Block vs Mean	0.1764	1	0.1764			
Linear vs Block	5.0546	3	1.6849	20.8817	< 0.0001	
2FI vs Linear	0.5266	3	0.1755	3.0813	0.0683	
Quadratic vs 2FI	0.5872	3	0.1957	18.2631	0.0004	Suggested
Cubic vs Quadratic	0.0767	4	0.0192	4.8555	0.0567	Aliased
Residual	0.0197	5	0.0039			
Total	126.8670	20	6.3433			

The test for lack of fit breaks up the sum of squares of error into a sum of squares for lack of fit and an experimental error sum of squares (Lazic, 2006). Thus, to finalize the model lack of fit test should be considered. In this study, based on Table 4.13 and Table 4.14, showed that quadratic model is a better model to meet our results.

Table 4.13: Lack of fit tests for reducing sugars yield in *K. alvarezii*

Source	Sum of Sq	df	Mean Sq	F Value	p-value Prob > F	
Linear	4.0722	11	0.3702	241.1626	< 0.0001	
2FI	2.5124	8	0.3141	204.5850	< 0.0001	
Quadratic	0.0419	5	0.0084	5.4549	0.0626	Suggested
Cubic	0.0076	1	0.0076	4.9434	0.0903	Aliased
Pure Error	0.0061	4	0.0015			

Table 4.14: Lack of fit tests for reducing sugars yield in *G. manilaensis*

Source	Sum of Sq	df	Mean Sq	F Value	p-value Prob > F	
Linear	1.1953	11	0.108664	28.97901	0.0026	
2FI	0.668662	8	0.083583	22.2903	0.0046	
Quadratic	0.081458	5	0.016292	4.344746	0.0898	Suggested
Cubic	0.004749	1	0.004749	1.266546	0.3234	Aliased
Pure Error	0.014999	4	0.00375			

Also, considering R-square and Press factors that should be highest and lowest respectively, applying the quadratic model for a yield of reducing sugars in both seaweed species was suggested (Table 4.15 and Table 4.16), where R-square was 0.9964 for *K. alvarezii* and 0.9846 for *G. manilaensis.* R-square is ranged between 0-1 and closer value to 1 indicates better effectiveness in the prediction of responses.

Table 4.15: Model Summary Statistics for reducing sugar in *K. alvarezii*

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.5214	0.6960	0.6351	0.3841	8.2614	
2FI	0.4581	0.8122	0.7184	0.0167	13.1892	
Quadratic	0.0730	0.9964	0.9928	0.9700	0.4020	Suggested
Cubic	0.0524	0.9990	0.9963	-0.9345	25.9482	Aliased

Table 4.16: Model Summary Statistics for reducing sugar in *G. manilaensis*

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.2841	0.8068	0.7682	0.5945	2.5405	
2FI	0.2387	0.8909	0.8363	0.3516	4.0619	
Quadratic	0.1035	0.9846	0.9692	0.8548	0.9099	Suggested
Cubic	0.0628	0.9968	0.9887	-1.5947	16.2553	Aliased

The analysis of models showed a high coefficient of determination (R^2) for reducing sugar production in *K. alvarezii*, which was 0.9964 implying that 99.64 % variance can be explained by the model. R^2 for reducing sugar production in G. manilaensis was 0.9846, which showed 98.46 % of total variance explained by the model. The R^2 value ranged between 0 and 1. The closer the R^2 is to 1, the stronger the model and the better it predicts the response (Nelofer *et al.*, 2011).

For reducing sugar yield, generated in both seaweed species, the model included an intercept, three main terms, three interaction and three-second order effect (Table 4.17 and Table 4.18). According to these tables, that showing coefficient estimates, resulted coefficient of variation were 3.0753 and 2.6721 in *K. alavrezii* and *G. manilaensis* respectively, which are less than 10 % and this indicates that the model cannot be

considered as a reproducible model. The coefficient of variation (CV) is the ratio of the standard error to the average of response (as a percentage) which measure the reproducibility of the model and in the reproducible model must be greater than 10 % (Rustom *et al.*, 1991).

Table 4.17: Model coefficient estimated by regression for reducing sugar yield in *K. alvarezii.*

	Coefficient Estimate	95 % CI Low	95 % CI High	VIF
Intercept	3.0753	3.0166	3.1340	
A-Acid Conc	0.1209	0.0687	0.1732	1.0000
B-Temp	0.8677	0.8155	0.9200	1.0000
C-Time	0.4074	0.3551	0.4596	1.0000
AB	-0.1510	-0.2094	-0.0926	1.0000
AC	-0.0316	-0.0900	0.0268	1.0000
BC	-0.4137	-0.4722	-0.3553	1.0000
A^2	0.0839	-0.0169	0.1848	1.8627
B^2	-0.5814	-0.6822	-0.4805	1.8627
C^2	-0.3459	-0.4468	-0.2451	1.8627

Table 4.18: Model coefficient estimated by regression for reducing sugar yield in *G. manilaensis.*

	Coefficient Estimate	95 % CI Low	95 % CI High	VIF
Intercept	2.6721	2.5889	2.7553	
A-Acid Cons	0.2841	0.2100	0.3581	1.0000
B-Temp	0.5414	0.4674	0.6155	1.0000
C-Time	0.3628	0.2887	0.4369	1.0000
AB	-0.1628	-0.2456	-0.0800	1.0000
AC	-0.0911	-0.1739	-0.0083	1.0000
BC	-0.1762	-0.2590	-0.0934	1.0000
A^2	-0.0156	-0.1586	0.1273	1.8627
B^2	-0.1840	-0.3269	-0.0410	1.8627
C^2	-0.2348	-0.3777	-0.0918	1.8627

The 3D response surface plot is a graphical representation of the regression equation. It is plotted to understand the interaction of the variables and locate the optimal level of each variable for maximal response. Each response surface plotted for reducing sugar

production represented the different combinations of two test variables at one time while maintaining the other variable at the zero level. This graphic representation helps to visualize the effects of the combination of factors. Figure 4.28, Figure 4.30 and Figure 4.32 show the 3D plot generated from Design expert software by fitting the data to a predictive model. The predictive models generated for reducing sugar in in *K. alvarezii* based on actual factors is as follows:

$$\text{Ln (Sugar)} = 3.08 + 0.12 \times A + 0.87 \times B + 0.41 \times C - 0.15 \times A \times B - 0.032 \times A \times C - 0.41 B \times C + 0.084 B \times A^2 - 0.58 \times B^2 - 0.35 \times C^2$$

Similarly, 3D plots for fitting the data to a predictive model are illustrated in Figure 4.29, Figure 4.31 and Figure 4.33. The predictive models generated for reducing sugar in *G. manilaensis* based on actual factors is as follows:

$$\text{Ln(Sugar)} = +2.67 + 0.28 \times A + 0.54 \times B + 0.36 \times C - 0.16 \times A \times B - 0.091 \times A \times C - 0.18 \times B \times C - 0.016 \times A^2 - 0.18 \times B^2 - 0.23 \times C^2$$

Figure 4.28 shows the effect of acid concentration and temperature in *K. alvarezii*, where the maximum reducing sugars observed in high temperature and during all acid concentrations and once temperature reduce the amount of reducing sugar falls in all acid concentrations. Also, in the area of maximum temperature fall of reducing sugar is obvious.

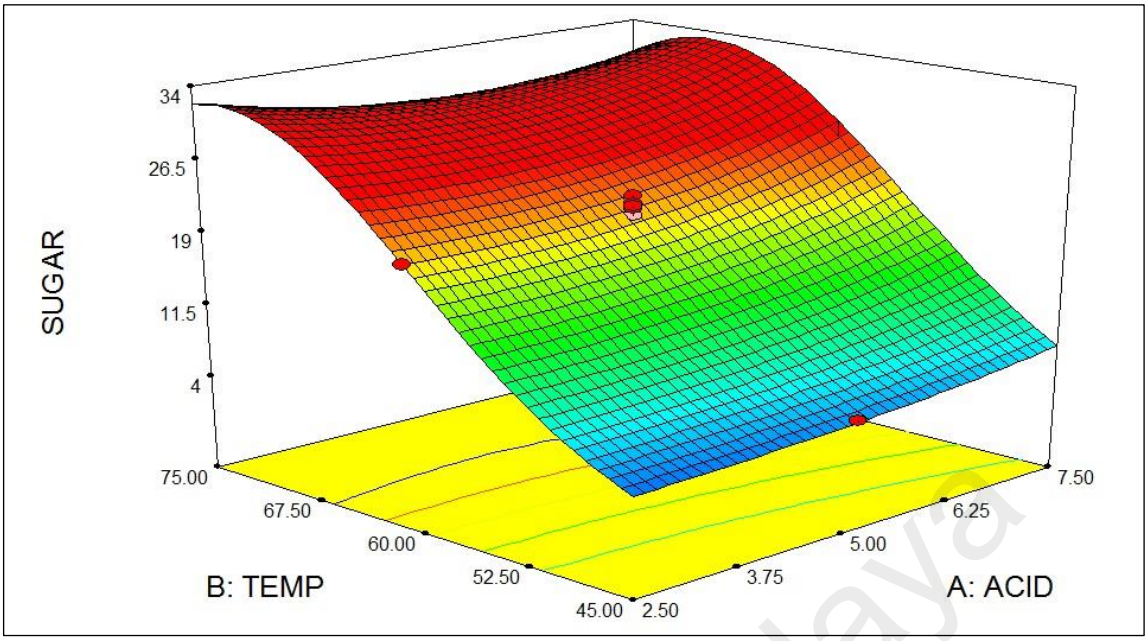

Figure 4.28: Effect of "A" Acid concentration (% w v^{-1}) and "B" Temperature (°C) on reducing sugar yield in dilute acid treatment of *K. alvarezii*.

Figure 4.29 illustrates the interaction of acid concentration with temperature in *G. manilaensis.* Unlike what is observed in same interaction in *K. alvarezii* less fall of reducing sugar is seen in high temperature and with increasing of temperature in all acid concentration levels, elevation of reducing sugar occurs.

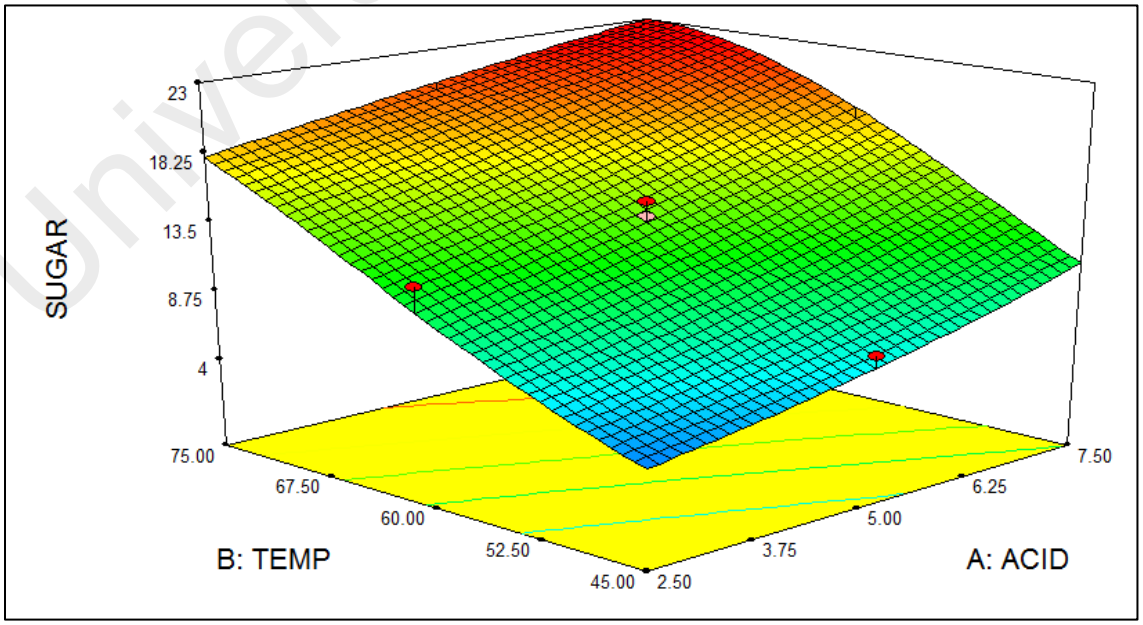

Figure 4.29: Effect of "A" Acid concentration (% w v^{-1}) and "B" Temperature (°C) on reducing sugar yield in dilute acid treatment of *G. manilaensis.*

Interaction of acid concentration and time for *K. alvarezii* and *G. manilaensis* is illustrated in Figure 4.30 and Figure 4.31 respectively. Difference among these two species is distinct where reducing sugar generations in *K. alvarezii* is higher in all levels of acid concentration from short to long incubating time in compare with *G. manilaensis*.

The interaction of time and temperature in both seaweed species show similar distribution (Figure 4.32 and Figure 4.33) however slight decrease of reducing sugar is seen in high temperature and long incubation time in *K. alvarezii* in compare with another seaweed species.

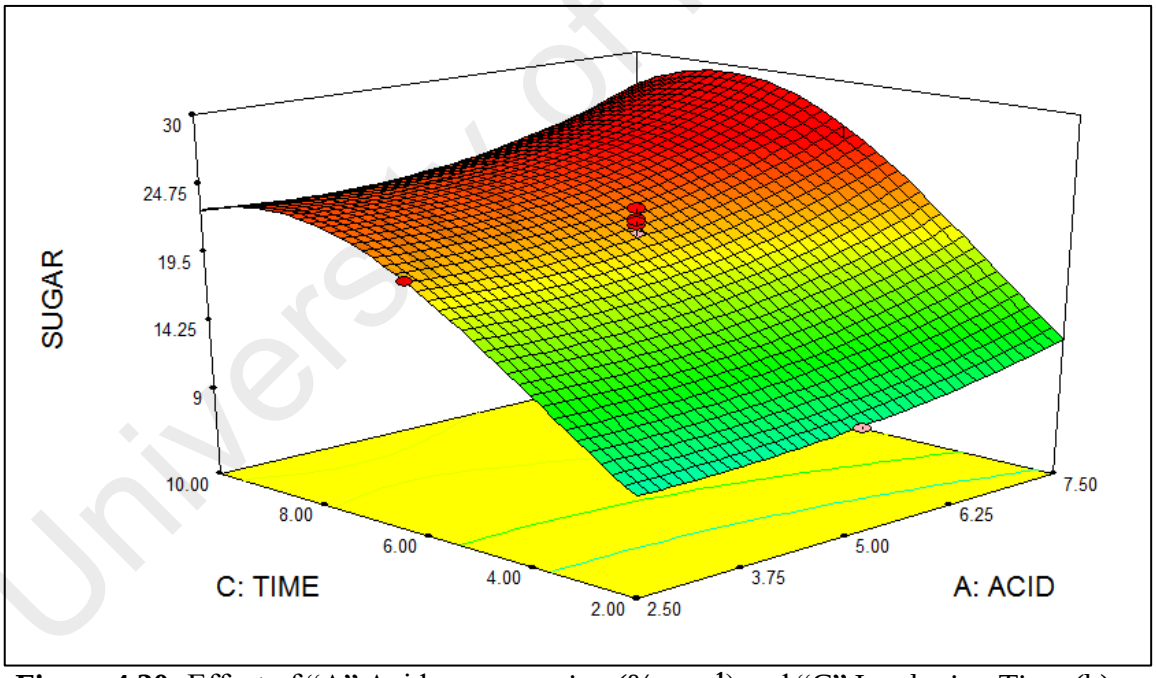

Figure 4.30: Effect of "A" Acid concentration (% w v^{-1}) and "C" Incubation Time (h) on reducing sugar yield in dilute acid treatment of *K. alvarezii*.

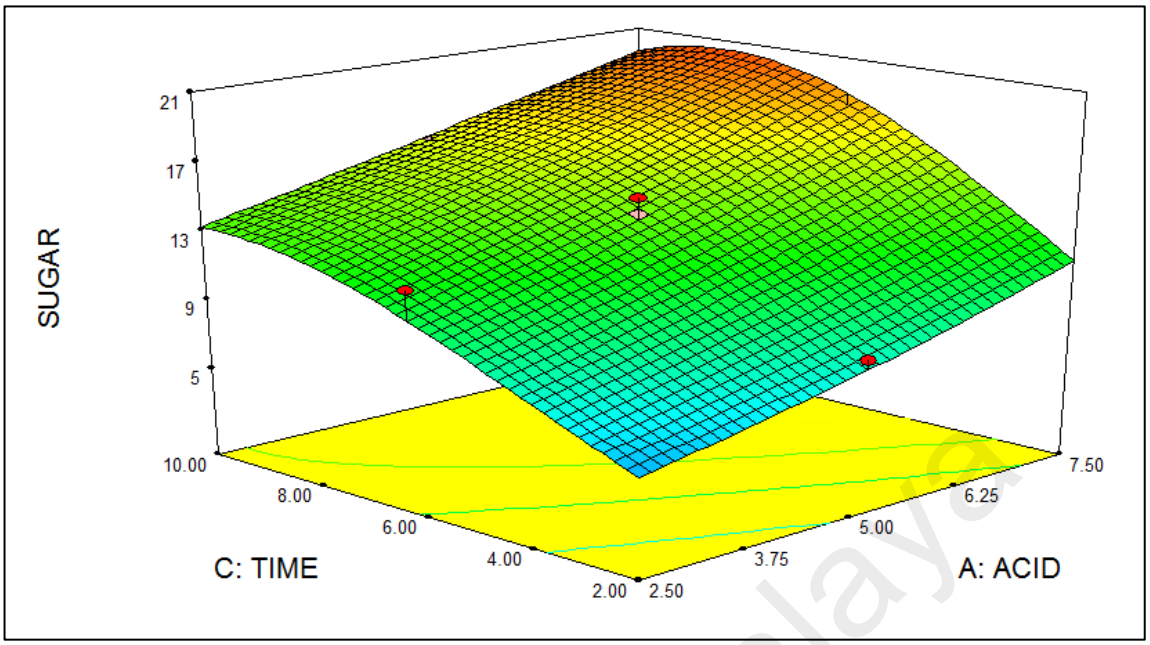

Figure 4.31: Effect of "A" Acid concentration (% w v^{-1}) and "C" Incubation Time (h) on reducing sugar yield in dilute acid treatment of *G. manilaensis.*

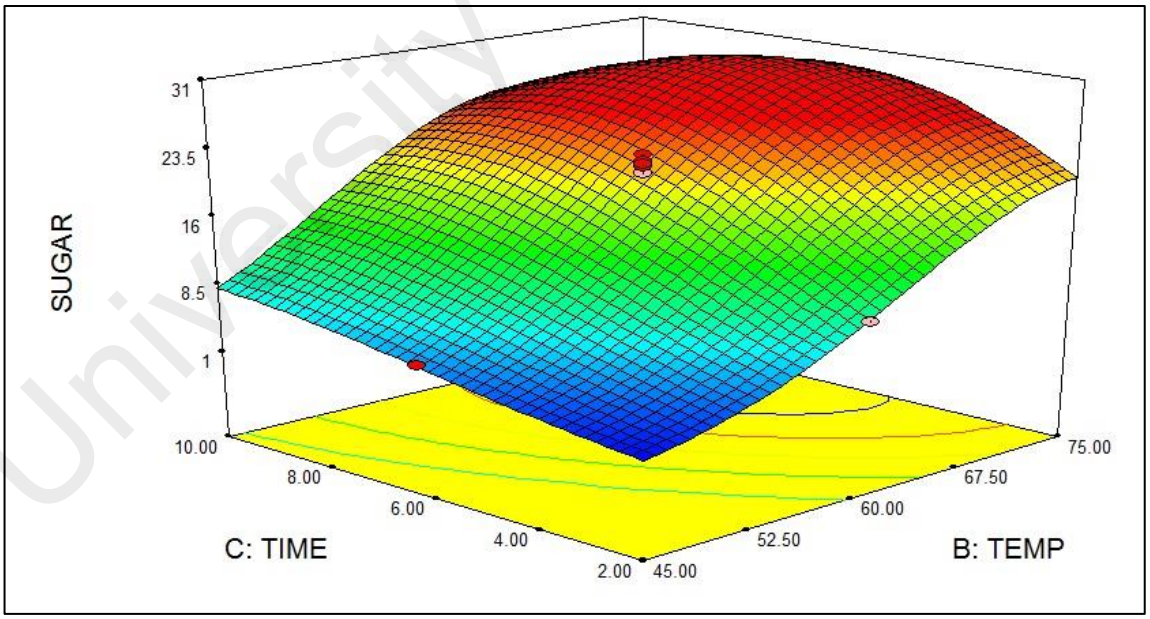

Figure 4.32: Effect of "C" Time (h) and "B" Temperature (°C) on reducing sugar yield in dilute acid treatment of *K. alvarezii.*

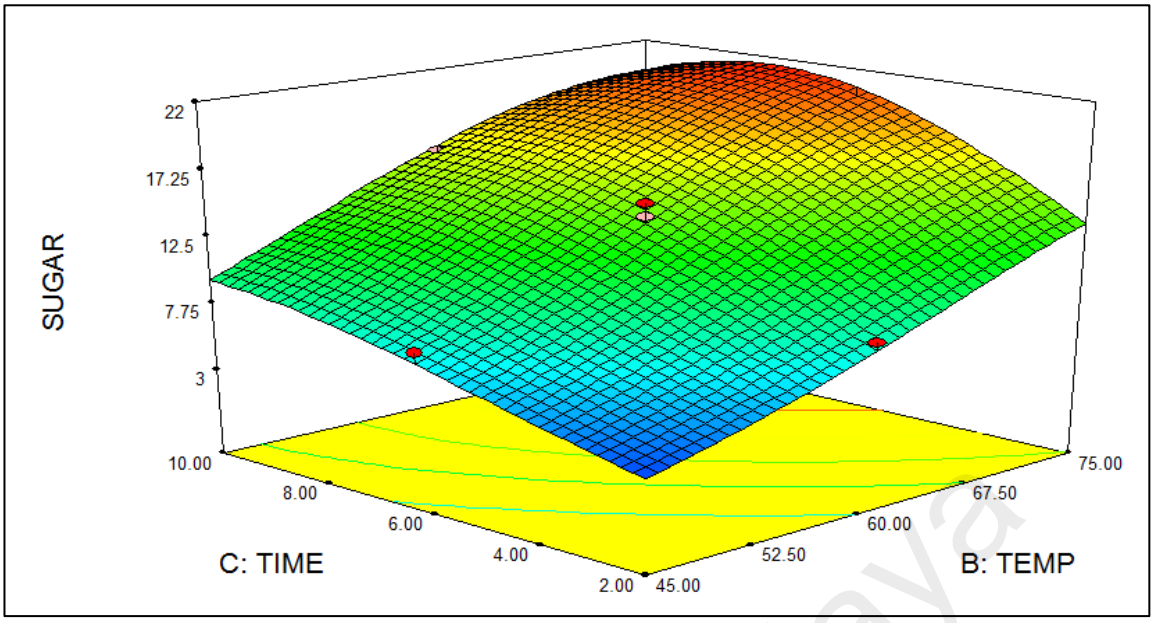

Figure 4.33: Effect of "C" Time (h) and "B" Temperature (°C) on reducing sugar yield in dilute acid treatment of *G. manilaensis.*

4.4.1.1 Validation of optimum conditions using RSM

Table 4.19 and Table 4.20 present the optimum conditions of reducing sugar in *K. alvarezii* and *G. manilaensis* respectively. To find such optimum condition for both seaweed species, the acid concentration was adjusted to a minimum while the rest of factors including temperature and time were in the range of actual experiment values (time: between 2 – 12 h, temperature: 40 - 80 °C). Highest desirability (1) was achieved in *K. alavarezii* optimization model (Table 4.19) while the acceptable value of this factor (0.989) seen in *G. manilaensis* (Table 4. 20).

Also, it can be observed that lower temperature (63.65 °C) in former seaweed species can be applied to achieve highest desirability in compare with *G. manilaensis* (79.98 °C) .

Table 4.19: Predicted and experimental sugar yield % DW at optimum condition in *K. alvarezii*.

No.	Condition			Predicted Sugar	Experimental Sugar
	Acid Conc % -	Temp -	Time	% DW	% DW
1	2.5	69.42	6.77	32.14	25.73 ± 0.76 *
2	2.5	64.87	8.42	28.94	22.87 ± 0.32 *

*Significant at $p < 0.05$, ns: Not Significant

Table 4.20: Predicted and experimental sugar yield % DW at optimum condition in *G. manilaensis.*

No.	Condition			Predicted Sugar	Experimental Sugar
	Acid Conc % -	Temp -	Time	% DW	% DW
1	2.5	75	8.43	19.58	19.94 ± 0.22 ns
2	2.5	75	8.17	19.57	19.67 ± 0.49 ns

*Significant at $p < 0.05$, ns: Not Significant

CHAPTER 5: DISCUSSION

5.1 Characterization of selected tropical seaweeds with reference to their use as feedstock for bioethanol production

In the context of bioethanol production, carbohydrate content is the most important biochemical property in seaweeds. The method of measuring carbohydrate content is a challenging issue, as some methods are detecting soluble carbohydrate only but there are other methods refer to total carbohydrate content. Also in some studies, carbohydrate content is determined by calculation and using subtraction of ash, moisture, lipids, and proteins from the total dry weight. (McDermid & Stuercke, 2003).

The aim of this study was to select two seaweed species with the highest total carbohydrate contents that also possessed the potential to be easily converted to fermentable sugars, within the framework of this criteria, more focus was placed on the collection of red and green seaweeds, due to the fact converting reducing sugars of brown seaweed to bioethanol is not promising (Takeda et al. 2011, Wang et al. 2013a).

Kappaphycus alvarezii and *E. denticulatum* showed the highest carbohydrate content in this study. *Kappaphycus* and *Eucheuma* are two closely related genera with high economic importance (Tan *et al.*, 2012), and their cultivation in Southeast Asia is common. Compared to our findings, a lower value of 56.8 % total carbohydrate in *K. alvarezii* was reported by (Fayaz *et al.*, 2005), while a higher amount (78.3 ±11.5 % DW) was reported in Papua, Indonesia (Meinita *et al.*, 2012). *K. alvarezii* contains 74 % k-carrageenan and 3 % μ-carrageenan (Estevez, Ciancia & Cerezo, 2004). MacArtain and Stuercke (2008) reported the value of soluble carbohydrate content of Hawaiian *E. denticulatum* as 28.0 ± 0.7 % DW, where crude fibre is not included. *U. reticulata* was collected from India (Shanmugam & Palpandi, 2010) and Indonesia (Mutripah *et al.*, 2014), and had carbohydrate contents of 50.24 % DW and 46.81 % DW, respectively.

Among the brown seaweeds, *L. variegata* (34.83 ± 0.89 % DW) was lower than that of (50.60 ± 1.62 % DW) reported by Castro *et al.* (2013). *Padina australis* (29.70 ± 0.61 % DW) in the present study was higher than those reported as 18.4 % DW and 19.3 % DW in winter and summer, respectively (Renaud & Luong-Van, 2006).

In short, carbohydrate content was high in both green seaweeds (29.77 - 55.99 % DW) and red seaweeds (35.53 - 73.22), but the lower content of carbohydrate was detected in brown seaweeds (12.16- 34.83 % DW). The carbohydrate content of *U. reticulate* was previously reported to be 50.24 % DW, which is lower than the present study (Shanmugam & Palpandi, 2010). Higher content for another species of *Ulva* has been reported for *U. lactuca* (61.5 ± 2.3 % DW) by Ortiz *et al.,* (2006). Unlike our results demonstrating sharp differences of carbohydrate content between studied seaweed species, studies of several genera of brown, green, and red seaweed conducted by Manivannan *et. al*. (2009) showed similar amounts of carbohydrate for all types seaweeds, ranging from 14.73 ± 0.07 to 17.49 ± 1.18 % DW. The highest total carbohydrate contents generated by other studies are compared with highest content in the Malaysian seaweed in Table 5.1.

Ash content in seaweeds is another factor that should be considered for in the production of bioethanol. Seaweeds generally contain a high amount of ash. Among seaweeds, there are types of calcified algae which contain very high ash content. According to Renaud & Luong-Van (2006), very high amount of ash (64.4 - 74.4 %) was recorded in calcified-seaweed, *H. macroloba,* which is higher than that determined in this study. Obviously, high amounts of ash in seaweed biomass may interfere in bioethanol production process in two ways; first, higher amount of ash might result in lower amounts of biomass to be converted to sugar. Moreover, ash content might result in problems in the down-stream process, such as increasing of salt in hydrolysate and the need to remove

it. In this study, all seaweeds showed high amounts of ash, and this character was not useful for selection of suitable seaweeds to produce bioethanol.

Table 5.1: Comparison of reported total carbohydrate content in seaweed species with the present study.

Type of seaweed	Name	TCHD % DW	Method used	Location	Reference
Rhodophyte	*Kappaphycus alvarezii*	78.3	Ph-sulphuric	Papua, Indonesia	Meinita *et al.* (2012)
	Kappaphycus alvarezii	71.22	Ph-sulphuric	Sabah, Malaysia	Present study
	Euchema denticulatum	69.91	Ph-sulphuric	Sabah, Malaysia	Present study
	Gracilaria manilaensis	59.68	Ph-sulphuric	Kedah, Malaysia	Present study
Chlorophyte	*Ulva reticulata*	50.24	Ph-sulphuric	Vellar Estuary, India	Shanmugam & Palpandi, (2010)
	Bryposis plumosa	56.9	Ph-sulphuric	Argentina	Ciancia *et al.* (2012)
	Ulva reticulata	55.99	Ph-sulphuric	Johor, Malaysia	Present study
	Bryopsis plumosa	43.12	Ph-sulphuric	Port Dickson, Malaysia	Present study
Phaeophyte	*Lobophora variegata*	19.34	Ph-sulphuric	India	Thennarasan (2015)
	Padina fernandeziana	44.07	Calc.	Chile	Goecke *et al.* (2012)
	Lobophora variegata	34.83	Ph-sulphuric	Perhentian Island	Present study
	Padina australis	29.7	Ph-sulphuric	Perhentian Island	Present study

Ph.sulpuric: Phenol-sulphuric based on DuBois et al., (1956), Calc.: calculation based on McDermid & Stuercke (2003).

Song *et al.* (2010) reported that the monosaccharide composition of *Bryopsis* spp. are mainly galactose, arabinose and glucose, but the ratio might vary in different samples (galactose 2.38 - 43 %, arabinose 4.36- 31 %, and glucose 4.62- 90.30 % of total sugars). In a previous study on tropical Australian seaweeds, *Halimeda macroloba* was reported as a seaweed with very low soluble carbohydrate (4.7 % DW in summer and 2.7 % DW in winter), which may be the result of its remarkable ash content (74.4 % DW in summer and 64.4 % DW in winter) (Renaud & Luong-Van, 2006).

Thermo-chemical hydrolysis conducted as a dilute acid treatment at high temperature is an inexpensive process compared with the enzymatic process from an economic perspective, but the disadvantage of this treatment is the possible occurrence of

fermentation inhibitors. On the other hand, the enzymatic hydrolysis is more effective, and fermentation inhibitor does not occur in the process (Taherzadeh *et al.* 2007; Larsson *et al.* 1999; Klinke *et al.* 2004). Currently, enzymatic hydrolysis is considered an expensive process in the bioethanol industry. Hence, in this study, we used diluted sulphuric acid and high temperature to aid carbohydrate hydrolysis. According to our results (Figure 4.1) for both seaweeds, *K. alvarezii* and *G. manilaensis*, hydrolysis yields in both 80 °C and 100 °C were low compared to 120 °C and 140 °C, where significant differences of $p > 0.05$ was observed.

On one hand, using sulphuric acid at high concentrations and temperature require better equipment and more energy and chemicals. However, harsh treatments might result in increased fermentation inhibitors. Therefore, in this study, we selected the optimal condition of 2.5 % w v^{-1} sulphuric acid, temperature of 120 °C, and 40 min incubation time, which might be considered a milder but more effective condition. Khambhaty *et al.* (2013) applied the same treatment with minor modifications, where they used sulphuric acid (2.5 % w v^{-1}) and treated *K. alvarezii* biomass for 1 hour in 100 °C. Meinita *et al.* (2012) used sulphuric acid (2 % w v^{-1}) and 15 min treatment in 130 °C for the same seaweed species.

The mild condition is utilised to prevent the over-decomposition of carbohydrate (Yang *et al.,* 2009). Khambhaty *et al.* (2012), using dilute acid (0.9 N H_2SO_4) hydrolysis, obtained up to 30.6 % DW reducing sugar from *K. alvarezii*, while the value was increased to 62.35 % DW with a combined acid-enzyme (Celluclast) method (Abd-Rahim *et al.*, 2014).

Agar contains D-and L-galactose, whereas carrageenan consists entirely of the D-galactose (Percival 1979). Glucose is also another dominant reducing sugar in red seaweeds. Wi *et al.* (2009) reported galactose and glucose as main reducing sugars in

Gelidium amansii, with values of 23.4 and 22.3 % DW, respectively. Other monosaccharides such as rhamnose, arabinose, xylose, and mannose were detected at very low amounts. The content of galactose may reach 87.3 % of total carbohydrate in red seaweeds, such as *Gracilaria cornea* (Melo *et al.*, 2002).

Hemicellulose encompasses xylans, xyloglucans, glucomannans, mannans, and beta-glucans (Scheller & Ulvskov, 2010). Main sources of xylose that can be converted to furfural is absent in seaweeds (Kraan, 2012). Another pentose sugar, arabinose, are also scarce in seaweeds (Percival, 1979), resulting in low furfural levels. Generally, dilute acid saccharification of seaweeds in the current study resulted in lower amounts of furfural and TPC compared to 5-HMF. Galactose is the main component of galactan, which is the major polysaccharide, namely agar and carrageenan, of red seaweeds, and consists of galactose or modified galactose units (Percival, 1979).

Monosaccharide profiling confirmed that fucose was only detected in brown seaweeds. Fucose is a deoxyhexose (6-deoxy-galactose) that is present in a wide variety of organisms (Becker & Lowe, 2003). Fucoidans are polysaccharides consisting of L-fucose and sulphate ester groups, found in brown seaweeds and some marine invertebrates (Li *et al.*, 2008). The presence of high glucose and galactose content after saccharification confirms the viability of the seaweed to be used as feedstock for fermentation.

Acid hydrolysis can result in the degradation of carbohydrates to fermentation inhibitors, including furfural, 5-HMF, acetic acid, levulinic acid, formic acid, uronic acid, and formaldehyde and phenolic compounds, such as 4-hydroxybenzoic acid, vanillic acid, vanillin, phenol, and cinnamaldehyde (Taherzadeh, 1999; Larsson *et al.*, 2000). The TPC such as 5-HMF and furfural were determined early on in the seaweed hydrolysates in the present study. TPCs are derived from the degradation of lignin, while furfural is derived from the degradation of pentose monosaccharides such as xylose and arabinose. Other

hexose sugars tend to decompose to 5-HMF and levulinic acid (Palmqvist & Hahn-Hägerdal, 2000). Phenolic compounds are naturally present in some brown seaweeds; 29.01 ± 0.50 mg g^{-1} of phenolic compounds was reported in *Turbinaria conoides* (Chandini *et al.,* 2008), but less phenolic compounds are expected to be present in the hydrolysates of *Kappaphycus* and *Gracilaria*, since they contain very little lignin (Wi *et al.* 2009; Ge *et al.* 2011).

Polyphenolic compounds are derived from the degradation of lignin, while furfural is derived from pentose monosaccharides degradation products, mainly xylose and arabinose. Other hexoses sugars tend to form 5-HMF and levulinic acids (Palmqvist *et al.,* 2000). However, phenolic compounds are naturally present in some brown seaweeds, (29.01 ± 0.50 mg g^{-1} of phenolic compound was reported in *Turbinaria conoides*) (Chandini *et al.,* 2008), while low phenolic compounds were expected to be present in the hydrolysate, since seaweed carbohydrate contain low amounts of lignin (Wi et. al 2009; Ge *et al.*, 2011). Furthermore, pentose sugars (xylose and arabinose) are detected in low amount in seaweeds (Percival, 1979; Ly *et al.* 2005), therefore, low furfural such as pentose sugar degradation product is expected. 5-HMF and levulinic acid are detected in higher amounts in seaweed hydrolysate. Meinita *et al.* (2012) reported a value of 4.67 ± 0.96 g L^{-1} 5-HMF in *K. alvarezii*. They used activated charcoal to remove this compound, and reduced it to 1.14 ± 0.02 g L^{-1}. In another study on the same red seaweed, a total of 4.23 ± 1.50 g L^{-1} of 5-HMF was detected in seaweed hydrolysate. Over-liming and activated charcoal were applied to remove the fermentation inhibitor (Jeong *et al.*, 2013). The presence of 5-HMF is also reported in *G. amansii* in hydrolysate at 4.8 g L^{-1}, but no detoxification treatment was applied prior to the fermentation study (Cho & Kim, 2014).

In summary, the data gathered in this study proved that two red seaweed species, *K. alvarezii* and *G. manilaensis,* are suitable for further studies and can be utilized as feedstock for generating reducing sugars to produce bioethanol.

5.2 Optimization of saccharification of *K. alvarezii* and *G. manilaensis*

In this study, we used dilute acid treatment to screen seaweeds. Dilute acid conversion is the most feasible technology for generating reduced sugars. This technology represent the best commercialisation option (Kaylen *et al.,* 2000).

Based on high total carbohydrate contents, high reduced sugar contents and the ease of mass cultivation, *K. alvarezii* and *G. manilaensis* were selected for evaluating their potential as a feedstock for bioethanol production. The advantage of using *K. alvarezii* is that carrageenan, as the main component of this seaweed, contains the basic unit of D-typed galactose, which is easily fermented by yeast and bacteria (Meinita *et al.,* 2012), but agar hydrolysate from *Gracilaria* consist of galactose and 3,6-anhydro-L-galactose, where the latter cannot be metabolised by common microorganisms, thus precluding it from producing ethanol (Yun *et al.,* 2014).

To select the best acid for dilute acid treatment, *G. manilaensis* was used as seaweed sample (Figure 4.1). Sulphuric acid showed better performance in this study, along with perchloric acid. Currently, most studies have been conducted using sulphuric acid, not only due to its high acidity, but also its reasonable associated costs (Harris *et al.,* 1945; Wright & Power 1986; Hashem & Rashad 1993). However, Abd Rahim *et al.* (2014) found no significant difference between the use of H_2SO_4 and HCl, and obtained sugar yields of 42.8 and 44.8 % DW, respectively, under conditions of 110 °C and incubation time of 90 min.

The physical condition of the biomass was also studied, where higher yield achieved from the dry biomass that showed that the drying makes the seaweed easier to break (Moore *et al.* 2008) thus facilitating easier saccharification.

Dilute-acid hydrolysis is less expensive compared to the use of enzymes for saccharification. The former allows the direct transfer of the treated sample to a secondary enzymatic hydrolysis process to further increase the sugar yield (Ge *et al.* 2011). However, a disadvantage of acid treatment is the production of fermentation inhibitors (Taherzadeh & Karimi, 2007).

Kinetic studies on the dilute acid treatment of various biomass indicated that the hydrolysis kinetic factors are strongly dependent on the biomass and acid concentrations, incubation time, and temperature (Malester *et al.*, 1988; Lenihan *et al.*, 2010).

Kappaphycus alvarezii and *G. manilaensis* were hydrolysed at various acid concentrations, temperature, and incubation times. In this study, we selected 2.5 % w v^{-1} sulphuric acid, a temperature of 120 °C, and 40 min incubation time, which is regarded as milder, but still effective, as the most suitable conditions for hydrolysis. In the present study, the hydrolysis treatment reduced sugar yields to 34 % DW (*K. alvarezii*) and 33 % DW (*G. manilaensis*). Meinita *et al.* (2012) used a range of acid hydrolysis conditions on *K. alvarezii*, and reported that the best conditions were 0.2 M sulphuric acid and 15 min incubation time at 130 °C. The sugar yield was 30.5 g L^{-1} with 25.6 g L^{-1} galactose and the ethanol yield reached 1.7 g L^{-1} hydrolysate.

Increasing hydrolysis time decreased production due to the sugar degrading into fermentation inhibitors such as 5-HMF and levulinic acid. Khambhaty *et al.* (2012) used sulphuric acid (2.5 % w v^{-1}) at 100 °C and an incubation time of 60 min, obtaining up to 30.6 % DW sugar yield from *K. alvarezii*. In another study, the best condition for

hydrolysing *G. salicornai* was a temperature of 120 °C , 2 % w v^{-1} sulphuric acid, and 30 min incubation time (Wang *et al.*, 2011).

Park *et al.* (2012) used continuous acid hydrolysis in a reactor to obtain higher sugar yields and lower levels of fermentation inhibitors from *Gelidium amansii*. Park *et al.* (2012) used 4 % sulphuric acid at 190 °C, and obtained 38.09 % glucose from *G. amansii*.

In another strategy, lower temperatures and longer incubation times compared to our pre-set optimal conditions were used in other studies. In one study, *Laminaria hyperborea* was treated for 60 min at 65 °C (Horn *et al.*, 2000), while Khambhaty *et al.* (2013) set a temperature of 100 °C and 60 min incubation time to treat *K. alvarezii*.

However, dilute-acid hydrolysis is an inexpensive process compared to the enzymatic process. The generation of fermentation inhibitors is regarded as one of its main drawbacks (Taherzadeh *et al.*, 2007). Generally, increasing the hydrolysis time decreased production, due to sugar degradation into fermentation inhibitors such as 5-HMF and levulinic acid (Ra *et al.*, 2013).

In order to detoxify dilute acid hydrolysate, several approaches has been applied prior to fermentation process, such as over-liming, neutralisation, activated charcoal, extraction with ethyl acetate, membrane-mediated detoxification, evaporation, and certain biological procedures (Chandeli *et al.*, 2011). Among these approaches, over-liming is an effective way of reducing the toxicity of hydrolysates generated from dilute acid treatment of biomass (Mohagheghi *et al.*, 2006) using over-liming and activated charcoal to remove fermentation inhibitors (Jeong *et al.*, 2013). 5-HMF (4.8 g L^{-1}) was reported in *G. amansii* hydrolysate (Cho & Kim, 2014). Generally, the use of over-liming eliminates fermentation inhibitors to a safe(r) level. Activated charcoal (26 %) was used by Hargreaves et al (2013) in *K. alvarezii* to detoxify 20 g L^{-1} 5-HMF, reaching <1 g L^{-1}.

Dissimilar to dilute acid hydrolysis, mild conditions are applied in enzymatic treatment, resulting in the absence of fermentation inhibitors and high yields of hydrolysis. Nonetheless, the bottleneck of the enzymatic approach in biomass treatment is high, which hinders its commercial application in the biofuel industry (Taherzadeh & Karimi, 2007b). There has been great interest in lower enzyme consumption or recycling enzyme in order to render it feasible for industrial applications (Jordan & Theegala, 2014; Tu *et al.,* 2009; Weiss *et al.,* 2013).

In the current study, results indicated that 10 % w v^{-1} of cellullytic enzyme is effective for the saccharification of biomass. Other studies reported lower dosage of the enzyme, where Baghel *et al.* (2015) used 2 % dosage to convert seaweed biomass, while in another study, 5 % cellulolytic enzyme (CTec 2) was used (Manns *et al.,* 2015).

Distillation is one of the most energy-intensive steps in ethanol production (Hoyer *et al.,* 2009), and several studies have concluded that the ethanol concentration being generated should reach ~ 4 – 5 % to render the process economically feasible (Fan *et al.,* 2003; Lu *et al.,* 2010). The trend of energy demand for distillation of fermented syrup at dilute ethanol concentration is shown in Figure 5.1. Some attempts have been made to increase the concentration of ethanol obtained from lignocellulose. Lu *et al.* (2010) obtained up to 49.5 g L^{-1} ethanol by applying high solid concentration to the fermentation of steam-exploded corn stover. (Yamashita *et al.,* 2010) succeeded in producing an approximate concentration of 73 g L^{-1} ethanol by using organosolv pre-treated Japanese cedar. Therefore, we attempted to determine the optimum condition in order to realise the highest enzymatic conversion efficiency and most concentrated glucose. So, the effect of the ratio of liquid: solid on enzymatic hydrolysis yield was evaluated, and the ratio of 7.5 : 1 was confirmed to be the best condition, where 78.11 % of biomass was converted to glucose, and a 20.82 % glucose concentration was achieved. Hargreaves *et al.* (2013)

used a 5.5 : 1 ratio, which is more concentrated than our result. Mechanical agitation during enzymatic provided better contact (Radhakrishnaiah *et al.*, 1999). The addition of more surfactants, such as Tween 80 or Tween 20 (Börjesson *et al.*, 2007), may also increase efficiency while substrate loading is high (Taherzadeh & Karimi, 2007b).

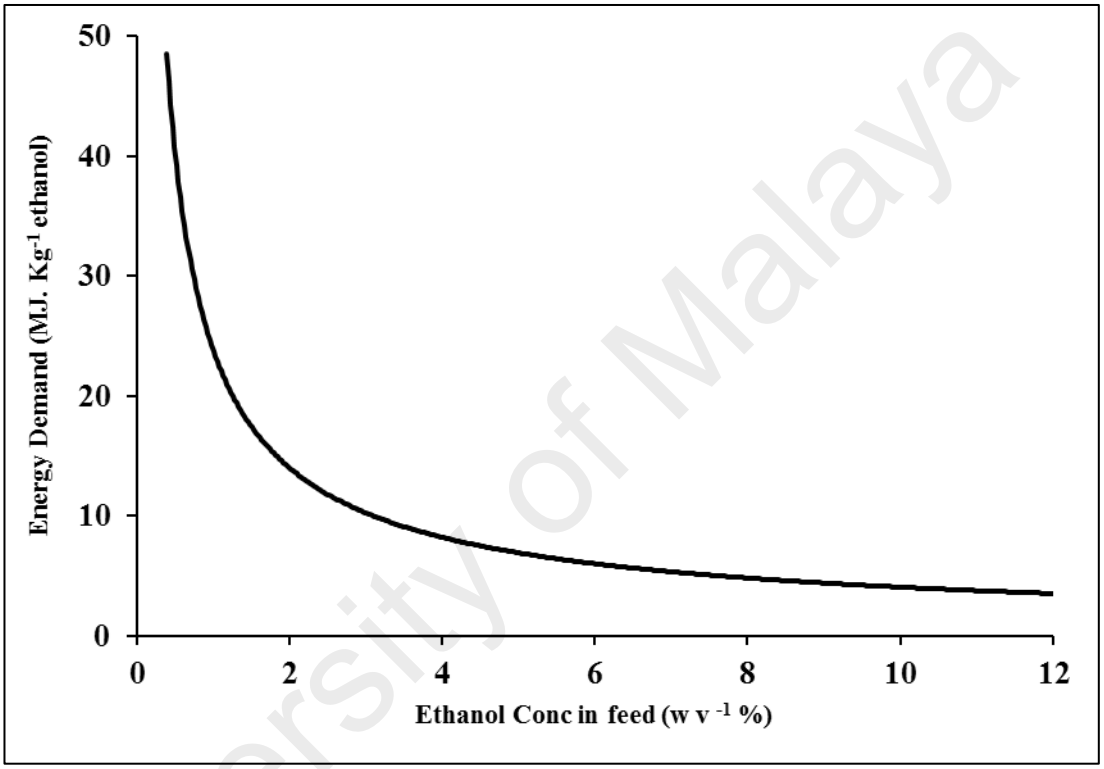

Figure 5.1: Energy demand in a single distillation unit for concentration of the dilute ethanol stream to 94.5 % (w w^{-1}) (Galbe, 2002).

Two red seaweed species were examined by enzymatic treatment, and a 48-h hydrolysis yield (%) of 82.97 ± 4.23 and 74.15 ± 1.73 was achieved for *K. alavrezii* and *G. manilaensis*, respectively. The glucose concentration was 120.33 ± 10.97 g L^{-1} and 105.67 ± 4.16 g L^{-1} for *K. alavrezii* and *G. manilaensis*, respectively. Our results for *K. alavrezii* agree with other studies. Hargreaves *et al.* (2013) investigated both 12-h

enzymatic efficiency and glucose concentrations, which are critical factors in enzymatic hydrolysis. They achieved the highest glucose concentration under 18 % cellulosic residue loading (92.3 g L^{-1} glucose), with 77.3 % enzymatic efficiency, which is lower than our values. Moreover, they reported 84.1 % as their highest enzymatic yield. To ensure complete hydrolysis of biomass, a 48 - 72 h treatment is recommended by enzyme provider. In the current study, overall, after 48 h of incubation, further enzymatic hydrolysis did not significantly increase the concentration of glucose.

As far as we are aware, there have been no reports pertaining to the enzymatic hydrolysis of *G. manilaensis.* Instead, other species of *Gracilaria* have attracted more attention. *G. dura* was investigated for enzyme treatment at the optimum condition of 2 % enzyme and a hydrolysis period of 36-h at a temperature of 45 °C (Baghel *et al.,* 2015). They reported 910 mg glucose g^{-1} cellulosic. The dry weight of this species was 12.24 ± 0.09 %, while the cellulosic matter had a fresh weight of 3.70 ± 0.13 %. In another study, *G. salicornia* was hydrolysed by cellulose, and 15.1 mM glucose was achieved after 4 h incubation (Wang *et al.,* 2011). Kawaroe *et al.* (2013), with similar seaweed species, reported 0.80 g glucose g^{-1} biomass.

Gracilaria verrucosa was studied in another research, and 0.87 g sugars g^{-1} cellulose was generated under enzymatic treatment. Another study on *G. verrucosa* was conducted by adding 16 U mL^{-1} of single and mixed enzymes using Spirizyme Fuel, Viscozyme L, and Celluclast 1.5 L (Ra *et al.,* 2015). In a slurry with 60 g DW total carbohydrate L^{-1}, 21.7 g L^{-1} glucose was generated after 24 h of treatment with mixed enzymes (Viscozyme L and Celluclast 1.5 L).

Low saccharification yield was reported in acid treatment residues, as notable amount of residues that are not cellulosic matter exist in the form of ash or residual

phycocolloids. It might be more effective to use an enzyme cocktail rather than a single enzymatic treatment.

All in all, the data obtained from this study seemed to suggest that both seaweed species carbohydrate can be converted to reduce sugar by applying dilute sulphuric acid at 120 °C for 40 min, which results in the highest amount of reduced sugar. Enzymatic hydrolysis is another effective approach to convert seaweed cellulosic residues by applying 10 % w w^{-1} commercial cellulase for 48 h incubation time at 50 °C. The data obtained from this study seems to imply that higher hydrolysis efficiency and concentrated reduced sugar (glucose) can be generated by enzymatic approach, while faster hydrolysis can be done with dilute acid treatment. It is also believed that the higher enzymatic cost of enzymatic hydrolysis is a limiting factor, and efforts are ongoing to reduce cost *via* recycling.

5.3 Fermentation of seaweed hydrolysate to bioethanol

In fermenting seaweed hydrolysates, fermentative microorganism (yeast or bacteria) consume reduced sugar to produce bioethanol in an anaerobic condition. *Saccharomyces cerevisiae* is the most common microorganism used in anaerobic fermentation, and it has proven itself to be highly vigorous and well fitted for conversion of cellulosic hydrolysates into bioethanol. *Zymomonas mobilis* can ferment glucose to ethanol with higher yields due to the reduced production of biomass, but is less robust (Galbe, 2002). It is assumed that the selection of proper fermentative microorganism and the acclimation to seaweed hydrolyzate are key factors in the successful seaweed usage in biofuel production, which resulted in many studies pertaining to this area (Kawai & Murata, 2016).

In the present study, two starins of *S. cerevisiae* and *B. bruxellensis* were evaluated, and from these, one strain of *S. cerevisiae,* Ethanol Red, Fermentis, France, showed significantly higher ethanol production efficiency (Figure 4.18). It was reported that *B. bruxellensis* performed well on co-fermentability using reagent grade of mixed sugar, comprising of galactose and glucose as substrates to produce ethanol (Park *et al.,* 2012). They used *B. bruxellensis* to co-ferment *G. amansii* hydrolysate containing galactose and glucose, and a 91 % fermentation efficiency was reported.

Compared with *B. bruxellensis,* many studies have been conducted using Ethanol Red, which is the industrial strain of *S. cerevisiae* (Yan *et al.,* 2011, Gill *et al.,* 2012; Bischoff *et al.* 2016; Pedersen, 2016;). Furthermore, this strain is reported to perform well in mixed sugar media (Klaassen *et al.,* 2015), and few studies have been conducted using this strain to ferment seaweed hydrolysate (Adams *et al.,* 2009; Adams *et al.,* 2011).

In the present study, seaweed hydrolysates were fermented by Ethanol Red. The initial reduced sugar content in acid hydrolysate of *K. alvarezii* and *G. manilaensis* were 61.28 ± 2.98 g L^{-1} and 55.76 ± 3.28 g L^{-1}, respectively. The highest ethanol concentration in *K. alvarezii* was achieved after 72 h (Figure 4.20), while in *G. manilaensis*, the maximum ethanol concentration was generated after 48 h (Figure 4.21). Adams *et al.* (2009) reported that when using Ethanol Red, the highest ethanol concentration from fresh and defrosted seaweeds (*S. latissima*) was achieved at 55 and 48 h, respectively. The ethanol yield was 20.90 ± 1.81 g L^{-1}, corresponding to 76.75 % of theoretical yield, while these values for *G. manilaensis* were 20.62 ±1.68 g L^{-1} and 72.50 %, respectively.

The low ethanol production rate and fermentation efficiency in both seaweeds are due to the presence of galactose in dilute acid hydrolysate as the main reducing sugar content as yield and productivity from galactose are notably lower than yields from glucose (Hong *et al.,* 2011; Lee *et al.,* 2011), since D-galactose undergoes conversion *via* the Leloir

pathway (Frey, 1996). Basically, in this pathway, a five-step enzymatic pathway converts D-galactose to glucose-6-phosphate, resulting in higher energy consumption (Timson, 2007). For starters, galactose is transported into the cell by galactose permease (Gal2) (Tschopp *et al.*, 1986). The conversion of D-galactose to glucose-1-phosphate is achieved by the four reactions, catalysed by Gal10, Gal1, and Gal7, which constitutes the Leloir pathway (Holden *et al.*, 2003). All these processes lead to higher energy consumption in galactose metabolism, thus lower fermentation yield compared with glucose.

Some studies have been carried out to improve the efficiency of ethanol from galactose using the transformed *S. cerevisiae* (Ostergaard *et al.*, 2000; Bro *et al.*, 2005; Hong *et al.*, 2011; Lee *et al.*, 2011). Bro *et al.* (2005) reported a fermentation yield of 0.29 g g^{-1} in a genetically transformed strain of *S. cerevisiae*, demonstrating better yield compared to the control (0.18 g g^{-1} galactose), while in another study, Meinita *et al.* (2012) reported higher fermentation yields from pure galactose, at 0.32 g g^{-1}.

According to our results, the fermentation of enzymatic hydrolysate from both seaweeds dilute acid residues is less difficult, and higher fermentation yield was realised, where 91 % and 95 % theoretical fermentation efficiency was produced in *K. alvarezii* and *G. manilaensis.* Unlike ethanol concentration from dilute acid hydrolysates, the ethanol concentration generated by both seaweed residue *via* the enzymatic approach falls within an acceptable range of > 5% v v^{-1} (Fan *et al.*, 2003; Lu *et al.*, 2010).

Based on the results of the current study, 33.4 g and 33.2 g ethanol can be extracted from 1 kg DW of *K. alvarzeii* and *G. manilaensis* through enzymatic treatment of dilute acid residues, respectively, which is comparable to the yield of ethanol in *G. verrucosa, at* 38 g ethanol per kg DW (Kumar *et al.*, 2013). These findings are indicative of the fact that more opportunities can be expected in enzymatic hydrolysis of seaweeds rather than dilute acid treatment. However, it is not cost effective.

Overall, the cumulative ethanol production by both dilute acid and enzymatic treatment shows that 105.9 g ethanol, which is equivalent to 134 mL of ethanol, can be extracted from 1 kg DW of *K. alvarezii*, while 112.5 g ethanol, or 142.4 mL, can be obtained from *G. manilaensis*. This yield is higher compared to a similar study conducted on *G. Salicornia*, which was 79.1 g per kg DW (Wang *et al.,* 2011).

In the same study, *Gracilaria* sp. was converted to reduced sugar using sequential acid and enzymatic hydrolysis (Wu *et al.,* 2014) processes. They reported 0.48 g g^{-1} ethanol per reduced sugar corresponding to a 94 % fermentation efficiency, where 236 g of ethanol was extracted from 1 kg DW, and 38 g of ethanol was extracted per kg DW *G. from verrucosa* (Kumar *et al.,* 2013).

Previous studies have introduced *K. alvarezii* as a promising feedstock for the production of bioethanol (Khambhaty *et al.,* 2012; Hargreaves *et al.,* 2013; Mansa *et al.,* 2013), and our study indicates that *G. manilaensis* can also be cultivated to serve as a bioethanol feedstock.

In summary and based on the gathered results, it can be concluded that although the efficiency of fermentation from seaweed reducing sugars was lower than land-based crops, the advantages of seaweed cultivation over land-based crop render these seaweeds as viable feedstock for the production of bioethanol in Malaysia.

Regardless of the developments in GC techniques, injecting aqueous samples for gas chromatography analyses is a topic of great interest, due to the fact that it is hazardous to not only gas chromatograph machine and capillary column, but it is also capable of interrupting the results of the analyses. There is a need to minimise the negative effects of sample preparation, which is assumed to be the most time-consuming and labour-intensive task involved in the analytical scheme (Santos & Galceran, 2002). Due to the

need for an effective, robust, and reliable sample preparation, many procedures have been developed with the aim of achieving fast, simple, and, if possible, solvent-free or solvent-minimised operations.

The main issue with water in GC-samples is its large expansion volume. It starts in the injector, where the samples are vaporised so that the analytes can be swept into the column by the carrier gas; a problem known as back-flash (Kuhn, 2002). Some common solvents and their corresponding vapour expansion volumes are tabulated in Table 5.2. Another concern regarding the presence of water in the samples are the degradation of the stationary phase, since water is capable of interacting with the stationary phase of the polymer (de Zeeuw & Luong, 2002).

The presence of water-soluble compounds in fermented samples, such as plant pigments, proteins, lipids (Miyazawa *et al.*, 1991; Palmqvist & Hahn-Hägerdal, 2000; Wu *et al.*, 2007; Hou *et al.*, 2015) and fermentation additives (yeast extract, meat peptone, vitamins, enzymes, *etc.*) are matters of concern in the context of GC analysis. Generally, these compounds are non-volatile and are retained in GC-units, mostly the injection chamber, column, and even detectors.

Table 5.2: Solvents and their corresponding vapour volume in injector temperature 250 °C; pressure 20 psi.

Solvent sample 1uL	Approximate Vapour Volume (µL)
Isooctane	110
n-Hexane	140
Toluene	170
Ethyl acetate	185
Acetone	245
Methylene chloride	285
Carbon disulphide	300
Acetonitrile	350
Methanol	450
Water	1010

The values are calculated using flow calculator application can be downloaded from Agilent Technologies' web site (http://www.chem.agilent.com).

Overall, the addition of this solvent mixture result in the reduction of water by almost 10 % (added solvent to the ratio of 9:1). Considering 1 µL sample injection and a split ratio of 100 : 1 in split/split less injector, a maximum of 1 nL of water could enter the injection chamber and capillary column, respectively, which is 10 times lower compared to direct sample injection.

Also, applying this simple approach, fewer unwanted compounds would pass through the GC machine, including the path of injection part, column, and detector. Thus, it would lead to the increased life-span of capillary column and maintenance of the system's cleaner over injections.

Moreover, the accuracy of the method did not fall within an acceptable R^2 in a standard plot (APPENDIX O).

5.4 Dilute acid hydrolysis at low temperature, a novel approach

Currently, two main approaches of acid based hydrolysis were introduced, which are dilute and concentrated acid methods (Taherzadeh & Karimi, 2007a). Each of these approaches encompasses various advantages and disadvantages that have been reviewed previously (Table 2.2). In this study, two red seaweed species, including *K. alvarezii* and *G. manilaensis* were examined using response surface methodology (RSM) for a new approach of acid based hydrolysis, which is the application of dilute acid at lower than 80 °C incubating temperature range at longer incubation times.

In this experiment, the lack of fit was not statistically significant in both seaweed species (Tables 4.10 and 4.11), as the P values exceeded 0.05, indicating that the RSM can be applied for predicting the optimum. However, the validation of optimum condition resulted in different values for *K. alvarezii* and *G. manilaensis*, where a significant difference ($p < 0.05$) was observed in the predicted and experimental reduced sugar yields in *K. alvarezii* (Table 4.9), while in *G. manilaensis*, the method was validated as the difference between the predicted and experimental yield as not being significantly different ($P > 0.05$). Failure in validating the optimisation method for *K. alvarezii* might be due to the occurrence of sugar decomposition in this species.

Up till this point, dilute acid treatment under mild temperature (below 80 °C) of any biomass has not been reported in literature. Hereby, the acceptable yield of reducing sugars in both seaweed species by this approach indicates that the application of low temperature and low acid concentration at longer incubation time can be assumed to be an effective method for saccharification of the macrolagal biomass, although this claim needs to be evaluated by other seaweed species.

The possible advantages of this approach are:

1. The usage of high temperatures would reduce the cost of facility and heat generation in the reactors (Taherzadeh & Karimi, 2007a).

2. The generation of fermentation inhibitor is expected to decrease due to the smaller amount of carbohydrate decomposition in mild temperatures (Larsson *et al.,* 1999). Therefore, we expect to be able to optimise this approach, not only in the context of the efficiency of saccharification, but also decreasing fermentation inhibitors in hydrolysate, resulting in immediate fermentation post-pH adjustment. The loss of reduced sugars during the detoxification process have been reported.

3. Providing optimum temperatures (65-80 °C) will provide a feasible approach of biomass treatment once this method is coupled with other sustainable heat production systems, such as solar thermal heating, which is suitable for tropical climates (Mekhilef *et al.,* 2012).

CHAPTER 6: CONCLUSION

6.1 Conclusion

Based on the medium variant projection, the global population of 7.2 billion in mid-2013 is expected to reach 8.1 billion in 2025, and 9.6 billion in 2050 (DeSA, 2013). The need to develop alternatives to fossil fuels is therefore inevitable. This study evaluated seaweed resources of Malaysia in the event they could be utilised as feedstock to produce bioethanol.

Among all of the seaweeds that were examined, the red seaweeds showed the highest carbohydrate content, particularly two red seaweed species, *K. alvarezii*, which is well-studied in terms of bioethanol production, and *G. manilaensis*. Both were hydrolysed by applying dilute acid treatment and enzymatic approach, followed by fermentation using an acclimated yeast. Calculated ethanol yield per kg DW with *K. alvarezii* and *G. manilaensis* were 105.9 g and 112.5 g, respectively.

Therefore, these seaweed species can be utilised as feedstock for bioethanol production in Malaysia, however, this process is not without its problems. The main difficulty with ethanol production using seaweeds is the nature of carbohydrate, which is mostly made up of galactose, and also the presence of sulphated bonds. Galactose yield was determined to be low, and its metabolism is even slower compared to glucose. Proper yeast or bacteria that was acclimated with galactose was suggested for use in the process of removing the sulphate bond, however, the overall process needs to be improved (Cho & Kim, 2014; Kim *et al.*, 2014; Kim*et al.*, 2013). More investigation is also needed to develop a more effective thermo-chemical treatment using different acid types and concentrations to optimise the hydrolysis process. Effective and cheap enzymes must be applied to increase the efficiency of hydrolysis and optimise fermentation in order increase bioethanol yield from seaweeds.

One can challenge the cultivation of red seaweed for bioenergy production, as phycocolloids have a higher value of 10.5- 18 USD kg^{-1} (Bixler & Porse, 2011) compared to biofuel, which is much cheaper. However, taking into account the market size for global phycocolloids production and demand, which was 86,000 tonnes in 2009 (Bixler & Porse, 2011), it illustrates the huge distinction between these two industries, revealing the fact that in the context of economics, seaweed cultivation can be logical for the generation of biofuels.

Although most prior reports showed that substituting biofuels for gasoline will reduce greenhouse gasses because biofuels sequester carbon *via* growth of the feedstock, using a worldwide agricultural model to estimate emissions from land use change, Searchinger *et al.* (2008) reported that final result of using land crop-based bioethanol instead of producing a 20% savings, nearly doubles greenhouse emissions over 30 years and increases greenhouse gasses for the next 167 years (Searchinger *et al.*, 2008). They also pointed out that biofuels from switchgrass, if grown on U.S. corn lands, increase emissions by 50%. This raises concerns about large corn-based biofuel, and highlights the value of utilising other sources to produce biofuels. Therefore, producing bioethanol from marine algae has recently attracted more attention.

Moreover, in this study, a novel approach to sample preparation for analysing ethanol in the fermented sample was introduced, which enhances the accuracy of measurement and increase the life-span of capillary column and gas chromatograph parts.

We also introduced a new cost effective procedure for seaweed biomass hydrolysis using dilute acid treatment. This method can examine other polysaccharides that can be digested in a mild condition, such as starch in marine and land-based crops.

All in all, despite the fact that ethanol yield range obtained in this investigation is lower than land-based crops, taking into account the unique advantages of cultivating seaweeds over land-based crop, it can be concluded that red seaweeds are a suitable alternative feedstock for the production of ethanol in Malaysia. It should also be pointed out that the cultivation of seaweed for biofuel production is not economically feasible, and we strongly suggest that the industry produce bioethanol as petroleum additive to replace methyl tert-butyl ether (MTBE), which is a highly carcinogenic compound, currently added to petroleum in developing countries such as Malaysia. Many investigations have confirmed its carcinogenic attribute (Mehlman, 1998; Mehlman, 1996). MTBE is an oxygenate compound that is added to petroleum to raise its octane number. Its production and consumption have been banned in the USA from 2004 (Metcalf *et al*., 2016), and has now been replaced by ethanol.

This could present an additional incentive for the replacing MTBE with bioethanol from renewable resources such as seaweeds. Seaweed cultivation can also remove nutrients from wastewaters (Rabiei *et al.*, 2015; Rabiei *et al.*, 2016), as well as reduce the content of carbon dioxide from the atmosphere (Hughes *et al.* 2012; Kader *et al.*, 2013; Liu 2013). Seaweed cultivation for biofuel can be a sustainable and environment friendly process, rendering the cultivation of *K. alvarezii* and *G. manilaensis* in Malaysia an important activity for the production of bioethanol on an economically feasible basis.

6.2 Appraisal of this study

This study illustrated a real image of potential seaweed resources of tropical region in order to be used as bioethanol feedstock, and simultaneously, issues with using seaweed biomass as well. We found that two red seaweeds, *K. alvarezii* and *G. manilaensis*, are well-established in Malaysia in the context of mass production (Phang *et al*. 1996; Phang 2010). They have the highest potential for use as feedstock to produce bioethanol.

The fermentation yield in this work was in line with other crops, so in the event that the production of bioethanol becomes vital in the near future, seaweeds might serve to be an alternative that warrants further investigations, and the results of this study would be good start.

Moreover, a new approach of biomass hydrolysis was tested in this study, where dilute acid treatment in mild condition showed promising results. Provided that optimisation can be conducted in order to increase the yield of reduced sugars, our proposed approach can be efficiently applied for the production of biofuels.

6.3 Areas for future research

Due to unique nature of seaweed carbohydrate, up till this point, the complete utilisation of reduced sugar obtained by seaweeds is unfeasible, therefore, more investigations is warranted in order to increase the efficiency of fermentation in order to increase the yield of bioethanol. Part of the problem is the fact that industrial yeast is generally isolated or engendered, acclimated, and used to assimilate land-crop based sugars to bioethanol, which are obviously inefficient for marine-based sugars. Thus, isolating or engineering fermentative microorganisms (yeasts or bacteria) that are capable

of tolerating higher salinity or the presence of sulphate in media, and more importantly in different reduced sugars rather than glucose or xylose in seaweed biomass.

Moreover, as seaweeds are aquatic plants, they tend to absorb and retain a great amount of water, thus obtaining concentrated slurry of seaweed biomass is not a simple affair, and consequently, reduced sugar and ethanol content can hardly touch industrial requirements. Therefore, creative methods in order to solve this issue to increase the reduced sugar contents in the seaweed hydrolysate is needed.

One of the bottlenecks of bioethanol production from seaweeds towards industrial scale is that researches have not focused heavily upon the production of bioethanol from macrolagal biomass, although some researchers and institutes are claiming to pursue this technology. More organised research with powerful governmental support is needed to develop applicable approaches for bioethanol production from seaweeds.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publication

Hessami M. J., Aishah Salleh, & Phang S. M. (2016) Bioethanol as a by-product of agar and carrageenan production industry from the tropical red seaweeds, *Gracilaria manilaensis* and *Kappaphycus alvarezii*, *Iranian Journal of Fisheries Sciences* (Accepted).

Hessami M. J., Aishah Salleh, Rabiei R., & Phang S.M (2016) Evaluation of tropical seaweeds as feedstock for bioethanol production, *International Journal of Environmental Science and Technology* (Submitted).

Hessami M. J., Phang S. M., Aishah Salleha, & Cheng S. F. (2017) Evaluation of a simple gas chromatography sample preparation for bioethanol analysis using the red seaweed, *Gelidium elegans* - Short comunication; (In preparation).

Hessamia M. J., Aslanzadeh S., Rabieie R., Aishah Saliha, & Phang S. M. (2017) A comparative study on biogas production from residues of agar industry, (In preparation).

Conference and seminar presentation

Hessami M. J., Wong M. M., Teoh T. C., Aishah Binti Salleh, Zainudin Bin Arifin & Phang S. M. (2012) Evaluating perchloric acid for the saccharification of selected seaweeds, Poster presentation in *South China Sea 2012 (SCS2012): Sharing Knowledge, Resources and Technologies for Sustainable South China Sea*. Second Conference for Regional Cooperation in the South China Sea conference, 22-26 October 2012 at University of Malaya, Malayisa.

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