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A Study on Growth, Fermentation and Thermochemical Conversion of Two Microalgae Species

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ABSTRACT

Butanol is a four carbon alcohol commonly used in the chemical industry and also as a transportation fuel additive. Acetone-butanol-ethanol (ABE) fermentation is one of the processes used to produce butanol through biological conversion. The current fermentation process that uses lignocellulosic materials or food-based feedstock is not favourable because it requires high energy for the pretreatment due to the presence of lignin in lignocellulosic materials. Therefore, fermentation using an alternative feedstock such as microalgal biomass, which is a non-food based material and contains low lignin content, is considered as one of the approaches to overcome these issues. This study, therefore, was undertaken to evaluate the potential of ABE fermentation using microalgal biomass from two microalgae species, a freshwater microalgae *Chlorella* sp. and a marine water microalgae *T. suecica*. This research involves the investigation of the entire process consisting of biomass production, pretreatment, and enzymatic saccharification for reducing sugar production and ABE fermentation. A preliminary study on the thermochemical conversion of microalgal biomass was also carried out in this research.

A microalgal cultivation and carbohydrate accumulation study indicated that microalgal growth rate and carbohydrate content were significantly influenced by the cultivation conditions such as light intensity, temperature, pH, salinity and carbon dioxide concentration (CO_2). The maximum biomass production, specific growth rate (μ) and carbohydrate content for *Chlorella* sp. were 0.567 gL^{-1}, 0.252 d^{-1}, and 32.41% of dried biomass respectively, attained at 2000 lux, 30°C in a medium with initial pH of 7 without addition of NaCl. The maximum biomass production, μ and carbohydrate content for *T. suecica* of 0.54 gL^{-1}, 0.22 d^{-1}, and 20.6% of dried biomass respectively, were attained at 3000 lux, 30°C in a medium with initial pH of 7 and 30 gL^{-1} of NaCl. This study also indicated that both microalgae were able to grow in a medium supplied with 15% CO_2. Comparison of indoor and outdoor microalgal cultivation was performed at two different temperature ranges, low temperature: 10 - 20°C and high temperature range: 20 - 32°C. It was observed that higher microalgal biomass production and growth rate were obtained from the indoor cultivation compared than that of outdoor cultivation. The results suggested that the ambient temperature and natural light intensity fluctuation have a significant influence on the microalgal growth in outdoor cultivation.

The biomass obtained from the cultivation was pretreated prior to hydrolysis and ABE fermentation. Dilute alkaline pretreatment, which is a less harsh and more environmentally friendly approach compared to acid pretreatment, was applied to pretreat the microalgal biomass and the process was optimised. The pretreatment conditions (alkaline agent, alkali concentration, temperature and reaction time) were found to influence the pretreatment performance. A quadratic model that describes interaction of pretreatment conditions was developed and successfully fitted to the experimental results (r^2=0.92 for *Chlorella* sp. and r^2=0.96 for *T. suecica*). This pretreatment method is able to disrupt the microalgal cell structure and preserve the chemical compound of the microalgal

cell. The results also demonstrated that the dilute alkaline pretreatment was able to enhance the enzymatic saccharification of microalgal biomass.

The enzymatic saccharification condition for reducing sugar production was optimised by varying the temperature, pH, enzyme concentration and biomass concentration in order to obtain the maximum sugar concentration from microalgal biomass. It was found that ≈90% saccharification yield of both pretreated microalgal biomass was achieved from the saccharification at the optimum conditions (temperature: 40°C, pH: 4.5 and biomass concentration: 5-10 gL^{-1}). A high amount of glucose (50%) and xylose (45%) in both microalgal hydrolysate indicates that it can be used as chemical platform for biofuel production through the fermentation process. This study also demonstrates that a combination of dilute alkaline pretreatment followed by enzymatic saccharification can be applied to pretreat microalgal biomass prior to ABE fermentation.

Subsequently, the ABE fermentation of microalgal biomass was performed using four different forms of these two microalgal biomass; (1) untreated, (2) alkaline pretreated, (3) lipid extracted, and (4) lipid extracted followed by alkaline treated biomass. Each of the samples was subjected to enzymatic saccharification for reducing sugar production prior to the ABE fermentation. The highest ABE concentration was obtained from the fermentation of the dilute alkaline pretreated *Chlorella* sp. (0.161 gL^{-1}) and *T. suecica* (0.126 gL^{-1}) biomass. It was found that the butanol conversion yield from the fermentation of alkaline pretreated *Chlorella* sp. and *T. suecica* was 0.3% and 0.7% dried biomass respectively.

A preliminary study on thermochemical conversion of both microalgal biomass was also undertaken through pyrolysis and gasification process. The lipid extracted microalgal biomass exhibited low activation energy, which is favourable to be used in thermochemical conversion. In addition, the gasification of microalgae at 800°C and time of around 20 min were suitable conditions to complete the conversion in a thermogravimetric analyser.

The findings from this study generate significant information on the production of biofuel in an environmentally friendly manner. This has the potential to be applied not only for butanol production, but also for the production of various types of microalgal carbohydrate-based biofuel such as bioethanol, biohydrogen and biomethane.

LIST OF PUBLICATIONS

Published

1. *Mohd Asyraf Kassim*, Kawnish Kirtania, David De La Cruz, Nasser Cura, Srikanth Chakravartula, Sankar Bhattacharya. 2014. Thermogravimetric analysis and kinetic characterization of lipid-extracted *Tetraselmis suecica* and *Chlorella* sp. biomass. *Algal Research*. 6, 39-45.
2. Arash Tahmasebi, *Mohd Asyraf Kassim*, Jianglong Yu, Sankar Bhattacharya. 2013. Thermogravimetric study of the combustion of *Tetraselmis suecica* microalgae and its blend with a Victorian brown coal in O_2/N_2 and O_2/CO_2 atmospheres. *Bioresource Technology*. 150, 15-27.
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Submitted manuscripts

1. *Mohd Asyraf Kassim*, Sankar Bhattacharya. (2015). Optimization of enzymatic saccharification of dilute alkaline pretreated *Chlorella* sp. and *Tetraselmis suecica* for reducing sugar production. (Submitted to Chemical Engineering Journal)
2. *Mohd Asyraf Kassim*, Sankar Bhattacharya. (2015). Dilute alkaline pretreatment for fermentation sugar production from *Tetraselmis suecica* and *Chlorella* sp. biomass. (Resubmitted for review to Process Biochemistry PRBI-D-15-00606R1)

In preparation

1. *Mohd Asyraf Kassim*, Sankar Bhattacharya. (2015). Acetone-butanol-ethanol (ABE) fermentation of dilute alkaline pretreated *Chlorella* sp. and *T. suecica* biomass. (In preparation)

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LIST OF ABBREVIATIONS

ABE	Acetone-butanol-ethanol
ANOVA	Analysis of variance
CCM	CO2 concentrating mechanisms
Chl	*Chlorella* sp.
CO_2	Carbon dioxide
DM	Dry matter
DNS	3,5- Dinitrosalicylic acid
EIA	Energy information administration
FID	Flame ionized detector
FTIR	Fourier transform infrared
FWO	Flyn-Wall-Ozawa
GC	Gas chromatography
HPLC	High performance liquid chromatography
KAS	Kissinger-Akahira-Sunose
MLA	Microalgae cultivation medium
OD	Optical density
OVAT	One variable at a time
RCM	Reinforce Clostridium medium
rpm	Rate per minute
RSM	Response surface methodology
RuBisco	Ribulose-1,5-biphospahte carboxylase
SEM	Scanning electron microscopy
Tetra	*Tetraselmis suecica*
TGA	Thermogravimetric analyser
TYA	Tryptone-yeast extract acetate medium
UV-VIS	Ultraviolet-visible spectroscopy

LIST OF SYMBOLS

mg	Milligram
mL	millilitre
gL^{-1}	Gram per litre
h	Hour
kJ	Kilojoule
mJ	Megajoule
min	Minute
wt	Weight
t	Time
T	Temperature, K
L	Litre
g	Gram
g/g	Gram per gram
E	Activation energy
A	Pre-exponential factor
WL	Weight loss
AR	Average reaction rate
$Lmin^{-1}$	Litre per minute
w_i	Weight of the sample
µm	micrometre
R_i	Instantaneous reactivity, s^{-1}
R	Correlation coefficient
R^2	Coefficient of determination

CHAPTER 1

INTRODUCTION

1.1 Biofuel

The rapid growth of the global population and increased demand of fossil fuels have led to depletion of global energy resources reserves (BP, 2014). Moreover, over-consuming of energy for heat, electricity and transportation fuel has been identified as the primary cause of global warming and environment pollution (IEA, 2014). Therefore, substituting part of the fossil fuels with renewable energy (RE) is believed to be able to reduce greenhouse gas (GHG) emissions and promote better energy efficiency (REN21, 2014). Several RE sources such as solar, wind, geothermal and biofuel from biomass have been introduced. Of these RE sources, biofuel is considered as one with the potential energy that could partly replace fossil fuels in the near future. It is also believed to be an environmentally friendly energy source and is sustainable compared to other RE sources (Chakraborty et al., 2012).

Biofuel is a fuel derived from the conversion of biological materials into solid fuel, liquid fuel and gaseous fuels. These fuels can be produced from various types of feedstock such as woody biomass, lignocellulosic materials and other organic waste (Sims et al., 2010). Generally, this biomass is converted into biofuel through thermochemical and biological methods. Biofuels such as biodiesel, bioethanol, biobutanol, biogas, syngas and solid fuel can be produced from conversion of those biomass materials (Saxena et al., 2009; Verma et al., 2010). Production of ethanol and biodiesel are believed to have the potential to replace fossil fuels as a transportation fuel (Escobar et al., 2009). The implementation of liquid fuel such as ethanol and biodiesel as transportation fuel is being carried out in countries such as US, Brazil, Thailand and Malaysia (Balat and Balat, 2009; Silalertruksa and Gheewala, 2010; Yusoff et al., 2013). In addition, butanol, a recently introduced biofuel, is also reported to have the potential to be used as future transportation fuel due to its advantages compared to ethanol and biodiesel (Qureshi and Ezeji, 2008).

1.2 Butanol

Butanol is the second most prolific product produced after ethanol as a result of industrial fermentation (Kumar and Gayen, 2011). Typically, butanol is a four carbon alcohol, colourless liquid, less miscible with water and flammable. This chemical is widely used in the chemical industry as a solvent and chemical synthesis feedstock. In addition, it is also being used in the pharmaceutical industry. Butanol has high energy density (30 MJ/L) almost similar with gasoline (33 MJ/L), high hydrophobicity, and has high blending ability with

petroleum fuels. Butanol also has high compatibility to combustion engines and is less corrosive compared to ethanol (Qureshi and Ezeji, 2008).

Butanol can be produced through using two different approaches, chemical catalytic conversion and anaerobic acetone-butanol-ethanol (ABE) fermentation (Niemistö et al., 2013). To date, butanol is produced through a chemical reaction using propylene as a feedstock. However, the price of butanol from petrochemical feedstock is totally dependent on the petroleum price, thus making this process less favourable (Uyttebroek et al., 2015).

Another butanol production method is an anaerobic acetone-butanol-ethanol (ABE) fermentation. In this process, butanol is produced through conversion of carbohydrate-based biomass feedstock by bacteria *Clostridia* sp. as a biocatalyst (Dürre, 2007). Figure 1.1 shows the overview typical of the flow process of butanol production from biomass. Generally, in order to produce biobutanol from biomass, it has to undergo several processes consisting of pretreatment, hydrolysis and ABE fermentation to produce butanol.

Several types of feedstock can be used to produce butanol through fermentation process, for instance, starchy materials, sucrose and lignocellulosic biomass (Amiri et al., 2015; Ibrahim et al., 2012; Thang and Kobayashi, 2014). However, production of butanol through fermentation using these feedstocks displays few disadvantages. For instance, ABE fermentation using food-based feedstock has resulted in food shortages and created food versus fuel issues. In addition, the ABE fermentation using lignocellulosic materials requires high energy input during the pretreatment process due to the presence of lignin in the biomass. Therefore, the ABE fermentation using a non food-based, renewable resource with low lignin content is required to ensure the production of butanol is feasible.

Microalgal biomass is considered as an alternative butanol feedstock. By comparison to other feedstocks, this biomass does not compete with food, it has a higher growth rate than terrestrial plants, it produces high biomass production and lipid yield, it is capable of capturing carbon dioxide (CO_2), and it can be used as an agent for industrial wastewater treatment processes (Razzak et al., 2013). Moreover, microalgal cells contain 30-50% carbohydrate, depending on the species and cultivation conditions, also making this type of biomass a great candidate to be used as an ABE fermentation feedstock (Chen et al., 2013).

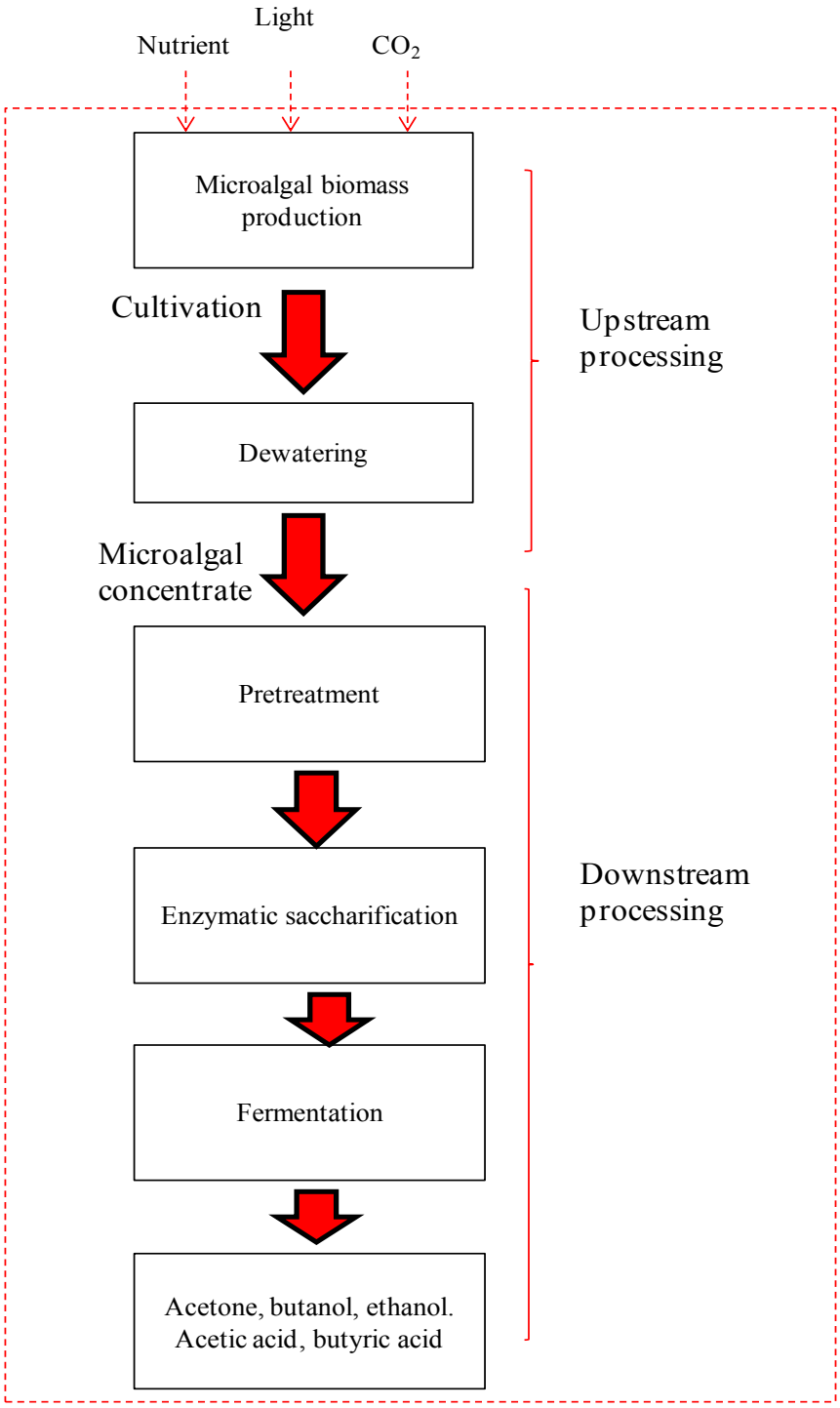

Figure 1.1 Process flow diagram for biobutanol production through biochemical conversion.

1.3 Butanol from microalgae

Microalgae is a photosynthetic microorganism that can be found either in a marine or fresh water environment. These organisms convert light and carbon dioxide (CO_2) to produce biomass and carbohydrate. There are three main chemical compositions in microalgal biomass – lipid, carbohydrate and protein. Currently, research on the conversion of microalgal-lipid into biodiesel is being carried out extensively.

Apart from microalgal lipid, microalgal carbohydrate is also considered to be one of the important components in the biofuel industry. The carbohydrate in microalgal is either accumulated as an energy storage form such as starch, or as biopolymer polysaccharide in cell walls. The cell wall composition and carbohydrate form in microalgae depends on the microalgal species and cultivation conditions (Brányiková et al., 2011; Dragone et al., 2011). The microalgal biomass with high carbohydrate content shows the potential to be used as a carbohydrate-based biofuel feedstock such as ethanol, hydrogen, methane and butanol via a fermentation process (Chen et al., 2013).

Butanol production from microalgal biomass is believed to be one of the alternative transportation fuels of the future (Yilmaz et al., 2014). Currently, the information on butanol production from microalgal biomass is scarce. There is little research on the production of butanol from carbohydrate-rich microalgal biomass. Efremenko et al. (2012) who performed ABE fermentation of seven microalgae species (*Arthrospira platensis*, *Nannochloropsis* sp, *Dunaliella tertiolecta*, *Galdieria partita*, *Chlorella vulgaris*, *Cosmarium* sp, *Nostoc* sp.) found that different thermolysis microalgae species produced different ABE concentration, and totally depended on the carbohydrate content. The study also reported that the highest butanol yield was attained by fermentation of *Arthrospira platensis*. In another study, the ABE fermentation of microalgae cultivated in wastewater using *C. saccharoperbutylacetonicum* N1-4 produced the highest butanol when the fermentation was carried out using biomass that was pretreated using acid followed by an enzymatic saccharification sample (Ellis et al., 2012). The study also indicated that the addition of 1% sugar could enhance butanol production. The ABE fermentation of acid treated *Chlorella* sp. was also reported (Wang et al., 2013). The study showed that 3.37 gL^{-1} of butanol was produced from fermentation of 111 gL^{-1} of acid pretreated *Chlorella vulgaris* biomass. The

study also suggested that pretreatment using more than 2% sulphuric acid was preferable to pretreat this microalgae species.

1.4 Scope of the thesis

Based on the discussion above, most of the ABE is being produced from starchy materials, agro-waste, lignocellulosic biomass and petroleum-based (hydrocarbon) feedstock. The increase of petroleum based feedstock and food prices have raised the prospect of the viability of butanol production using alternative and less-expensive feedstock. Feedstock such as starch, corn, sugar and other biomass has high value for food and requires large areas of land for cultivation. Thus, exploring other alternative biomass feedstock such as microalgal biomass, which is cheaper and requires less land, is one of the potential alternatives to produce cheaper butanol from biomass. The potential of producing biofuel such as ethanol and biodiesel from microalgal biomass have been reported by previous studies. However, there is limited information on the potential of butanol production from microalgal biomass. In addition, the information and understanding on the pretreatment of microalgal biomass for butanol production is also limited. As a comparison, a preliminary study on thermochemical conversion of microalgal biomass is also included in the scope of this study.

1.5 Project objectives

The main objective of this study is to explore the possibility and the potential of microalgal biomass as a butanol feedstock. This encompasses the understanding of both the bioprocess engineering from upstream processes through microalgal biomass production and carbohydrate accumulation to downstream processes that involves pretreatment, enzymatic saccharification, biochemical conversion and thermochemical conversion. This main objective is subdivided into the following specific objectives:

1. To investigate the major factors that affect microalgal growth and carbohydrate accumulation.
2. To evaluate the feasibility of the outdoor cultivation, using a centric photobiorector and CO_2 as a carbon source.

3. To explore the potential of the alkaline pretreatment method to pretreat microalgal biomass prior to subsequent process.
4. To determine the enzymatic saccharification of microalgal biomass, essential for the release of high concentrations of fermentable sugars for butanol production.
5. To evaluate the potential of butanol production from microalgal biomass through an anaerobic fermentation using *Clostridium* sp as biocatalyst.
6. To study the thermochemical conversion of microalgal biomass through pyrolysis and gasification.

1.6 Thesis outline chapter by chapter

This thesis has six distinct parts and is divided into two major sections: (1) upstream processing and (2) downstream processing. The upstream processing section involves microalgal growth characterisation and outdoor cultivation. While, the downstream processing section in this thesis involves microalgal biomass pretreatment, enzymatic saccharification, fermentation and thermochemical conversion. The detail of the thesis outline is as follows;

1.6.1 Chapter 1: General introduction

This chapter gives a general introduction and establishes the research background, research gap, project objectives and scope of the research. The organisation and outline of the thesis is also given in this chapter.

1.6.2 Chapter 2: Literature review

This chapter gives a comprehensive literature review on butanol, highlights the acetone-butanol-ethanol (ABE) fermentation and the bioprocessing of microalgal biomass. The chapter also highlights the typical process involved in biofuel production from the biomass through a biochemical pathway.

1.6.3 Chapter 3: Microalgal growth and carbohydrate accumulation

This chapter discusses the microalgal cultivation and carbohydrate accumulation under different cultivation conditions. The suitable condition to obtain maximum growth and carbohydrate accumulation of *Chlorella* sp. and *Tetraselmis suecica* is determined. The effect of light intensity, temperature, pH, salinity and carbon dioxide (CO_2) on the microalgal growth rate and carbohydrate content are investigated in this chapter. The information regarding the microalgal growth characteristic and the suitable cultivation condition that is generated from this chapter can be applied to produce high *Chlorella* sp. and *T. suecica* biomass concentration with high carbohydrate content.

1.6.4 Chapter 4: Outdoor cultivation of microalgae

This chapter presents a study on an outdoor cultivation of *Chlorella* sp. and *T. suecica*. An outdoor cultivation of both microalgae in a centric photobioreactor using natural light and carbon dioxide (CO_2) as carbon source is investigated in this study. The microalgal growth and chemical composition from indoor and outdoor cultivation of both microalgae are compared in this chapter. The outdoor cultivation of both microalgae, performed using natural light and CO_2 provides information on a basis of feasibility for mass microalgae production using ambient conditions. Furthermore, the chemical composition of the microalgal biomass produced is characterised and the results obtained gives insight on the effect of different cultivation modes towards microalgal biomass quality.

1.6.5 Chapter 5: Dilute alkaline pretreatment for reducing sugar production from microalgal biomass

This chapter discusses the pretreatment of microalgal biomass. In order to perform the fermentation, the rigid microalgal cell needs to be disrupted, thus providing better access for the enzyme to attack polysaccharide in microalgal cells. In this study, the pretreatment of *Chlorella* sp. and *T. suecica* using dilute alkaline pretreatment is evaluated for the first time. The pretreatment condition parameters that can influence pretreatment performance, such as alkali agent, alkali concentration, temperature and reaction time, are evaluated using the Response Surface Methodology (RSM) approach. The microalgal biomass residue obtained after pretreatment is also characterised using a Fourier transform infrared spectroscopy

(FTIR) and Scanning electoron microscopy (SEM). The findings of these experiments provide an alternative approach to pretreat microalgal biomass in an environmentally friendly manner that requires low energy input.

1.6.6 Chapter 6: Optimisation of enzymatic saccharification of dilute alkaline pretreated microalgal biomass

This chapter presents the details on enzymatic saccharification of pretreated microalgal biomass for reducing sugar production prior to ABE fermentation. In order to obtain high reducing sugar from microalgal biomass, an enzymatic saccharification optimisation is performed in this study. The effect of temperature, pH, enzyme concentration, and biomass concentration on reducing sugar production is examined. The sugar composition produced is also analysed using high pressure liquid chromatography (HPLC). The optimisation study performed in this study could provide a basis of an enzymatic saccharification condition that could be applied to produce the highest reducing sugar concentration from microalgal biomass.

1.6.7 Chapter 7: Acetone-butanol-ethanol (ABE) production from dilute alkaline pretreated microalgal biomass

This chapter discusses the ABE fermentation of *Chlorella* sp. and *T. suecica* biomass generated from four different pre-processing methods. The fermentation of hydrolysate generated from four pre-processing methods, namely without treatment, alkaline pretreatment, lipid extraction and a combination of lipid extraction followed with alkaline treatment is evaluated in this chapter. The carbohydrate content before and after pre-processing is determined. Also, the enzymatic saccharification and fermentation of each sample is also evaluated throughout the experiment. The findings of these experiments give information on the effect of pre-processing methods on the ABE fermentation performance.

1.6.8 Chapter 8: Pyrolysis and gasification of microalgal biomass

This chapter presents the study on the thermochemical conversion of microalgal biomass. The study covers pyrolysis and gasification of microalgal biomass. Pyrolysis of lipid

extracted microalgal biomass is evaluated using a thermogravimetric analyzer (TGA). For the gasification study, the microalgal char was prepared using two reactors: (1) entrained flow reactor (EFR); and (2) TGA. This study provides new information on the thermal characteristic of lipid extracted microalgal biomass and on the gasification of microalgal char generated from two different reactors.

1.6.9 Chapter 9: Conclusions and recommendations for future work

This chapter concludes with the findings from the current work and presents relevant outcomes toward the achievement of the main goal of the project. The chapter also proposes some future work, which could be undertaken to improve the ABE fermentation.

1.7 Organisation of the thesis

This thesis consists of nine chapters (Figure 1.2). This current chapter is the introduction. Chapter 2 represents the literature review of the study. Chapters 3 and 4 cover the upstream processing section that focuses on microalgal characterisation and outdoor cultivation. Chapter 5 discusses the pretreatment of microalgae. Chapter 6 focuses on the optimisation of enzymatic saccharification of the pretreated microalgal biomass. Chapter 7 discusses the fermentation of microalgal biomass for chemical and biofuel production, while Chapter 8 presents the thermal conversion of microalgal biomass. Finally, Chapter 9 concludes the thesis with the key findings and scope for future work.

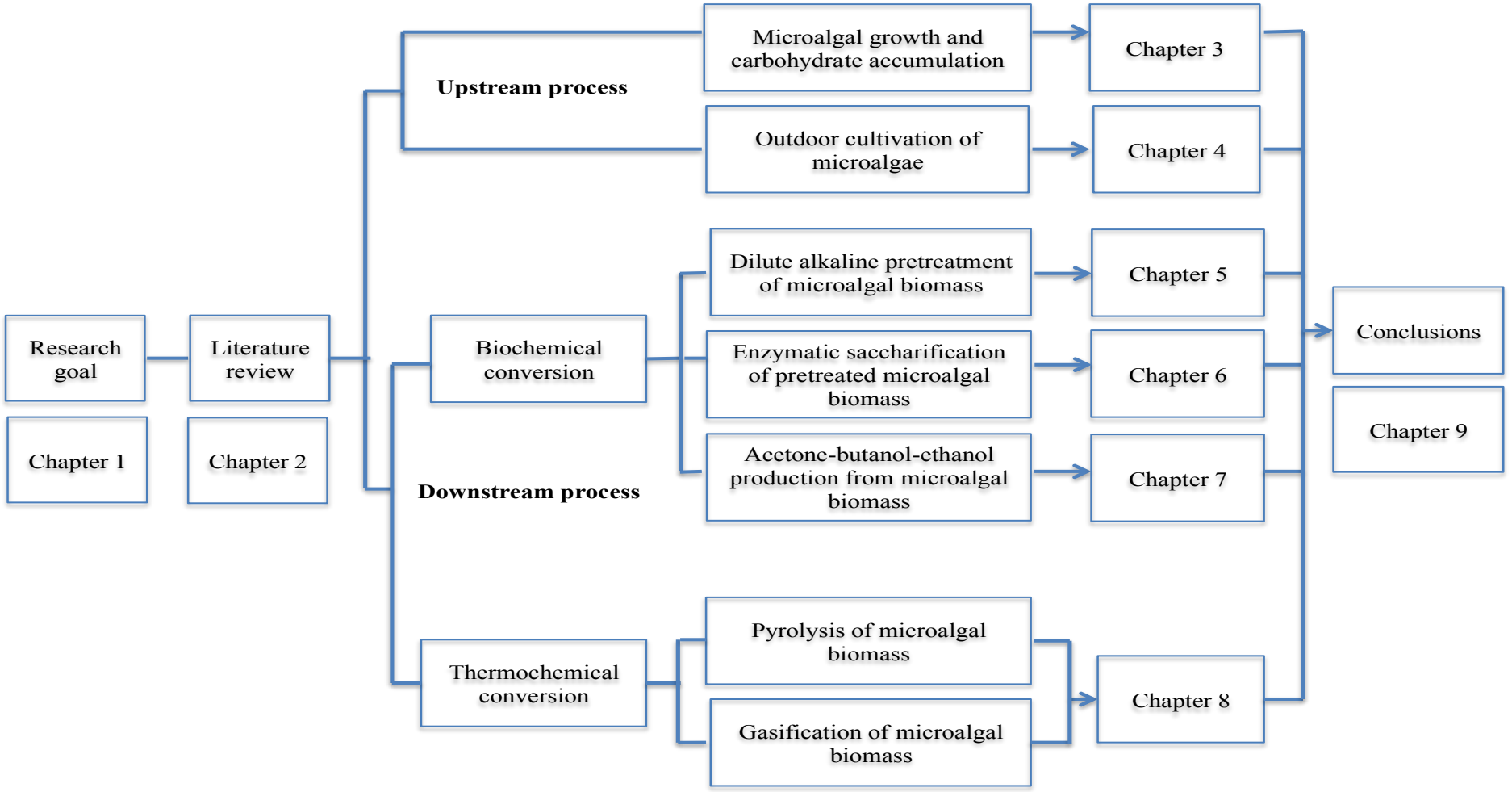

Figure 1.2 Thesis outline and their relationship.

1.8 References

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CHAPTER 2

LITERATURE REVIEW

2. 1 Biofuel

Biofuel is a fuel derived from various types of biological material such as trees, agroforestry residues, grass, plant, aquatic plant, crops and other organic waste (Figure 2.1). Various types of fuel, for instance gas, solid and liquid fuels, can be produced from the these feedstock (Naik et al., 2010).

At present, biofuel such as ethanol and biodiesel are commonly used for transportation fuel in many countries. Ethanol markets are dominated by Brazil, USA, China and to a smaller extent, the European Union, whereas, biodiesel markets are dominated by the USA, Argentina and Brazil (Shikida et al., 2014). Also, Europe and Asia produce more biodiesel from varieties of sources, including crops such as rapeseed and palm oil.

Apart from ethanol and biodiesel, butanol can also be used as transportation fuel. Butanol has more advantages compared to ethanol due to its characteristic of being miscible in petrol and it contains high-energy content compared to ethanol. It can also be blended to 85% with gasoline with or without engine modification (Hongjuan et al., 2013). At this stage, the use of butanol as an alternative fuel is very limited. Butanol production is still expensive due to the high cost of feedstock, however, considerable effort is being made to overcome this issue.

2.2 Butanol

Butanol (butyl alcohol or n-butanol) is a four-carbon alcohol with a molecular formula of C_4H_9OH. It is a colourless liquid with a distinct odor and is completely miscible with organic solvents and partly miscible with water. The butanol characteristics are summarised in Table 2.1.

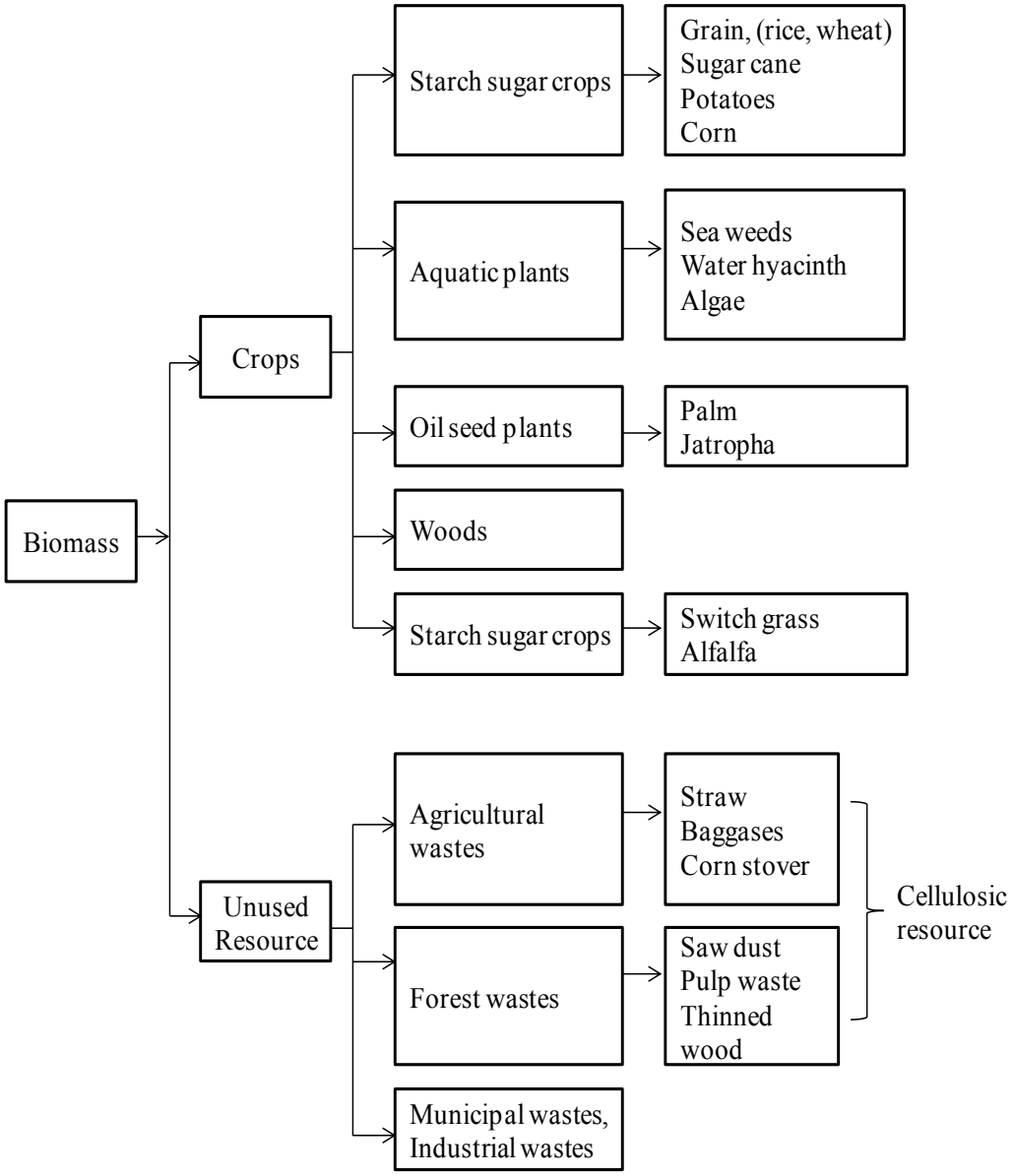

Figure 2.1 Type of biomass feedstock used for biofuel production. Modified from (Naik et al. 2010).

Table 2.1 Properties of butanol (Lee et al., 2008).

Properties	Butanol
Melting point (°C)	-89.3
Boiling point (°C)	117.7
Ignition temperature (°C)	35
Flash point (°C)	365
Density at 20 °C (g/mL)	0.8098
Critical pressure (kPa)	48.4
Critical temperature (°C)	287

Butanol has several applications, especially in the bulk chemical industries. Currently it is used as a solvent, chemical additive and as a feedstock for chemical synthesis. Butanol is commonly used as a solvent in the perfume and pharmaceutical industry, especially for the manufacturing of antibiotics, hormones and vitamins. This chemical is also used for stain removal and paint thinning. Another application of butanol is as a feedstock for glycol ether, butyl acetate and plasticiser (Durre, 2008).

Apart from being a solvent and chemical feedstock, butanol is currently being introduced as a fuel additive, it has similar characteristics to gasoline and can be used directly in the engine without any modification. Butanol has an energy density (27 MJ/L), which is close to that gasoline (32 MJ/L) (Lee et al., 2008). It also has more advantages over ethanol, such as being less miscible in water and having a higher energy content. Moreover, it can be blended with gasoline at a higher ratio compared to ethanol and has similar combustion properties to gasoline (Dürre, 2007). A comparison of fuel characteristics is shown in Table 2.2.

Table 2.2 Comparison of fuel properties (Hongjuan et al., 2013; Lee et al., 2008).

	Gasoline	Butanol	Ethanol
Energy density (MJ/L)	32	29.2	19.6
Air-fuel ration	14.6	11.3	9
Heat of vaporisation (MJ/kg)	0.36	0.43	0.92
Octane number	80-99	96	108

2.2.1 Butanol production technology

Butanol can be produced through two different routes, via a chemical reaction and through biological conversion. In a chemical reaction, there are three different chemical reactions that are typically been used to produce butanol, namely Oxo-synthesis, Reppe process and Aldol condensation. In biological conversion, the butanol is produced through acetone-butanol-ethanol (ABE) fermentation of carbohydrate. In this process, the carbohydrate is converted to a solvent including butanol by bacteria *Clostridium* sp. as a biocatalyst.

2.2.1.1 Chemical reaction route

Commercial production of butanol is derived from a chemical reaction route. In this route, three reactions, specifically Oxo-synthesis, Reppe process and Aldol Condensation, are the important processes used to produce butanol.

The Oxo-synthesis reaction involves the reaction of propylene ($CH_3CH=CH_2$) with carbon monoxide (CO) and hydrogen (H_2) in the presence of cobalt or rhodium as a catalyst. In this process, the propylene is hydroformylated to produce butyraldehyde ($CH_3CH_2CH_2CHO$), which is then hydrogenated to produce butanol ($CH_3CH_2CH_2CH_2OH$).

$$CH_3CH = CH_2 + CO + H_2 \rightarrow CH_3CH_2CH_2CHO + (CH_3)_2CHCHO \quad (2.1)$$

$$CH_3CH_2CH_2CHO + H_2 \rightarrow CH_3CH_2CH_2CH_2OH \quad (2.2)$$

The second reaction to produce butanol is the Reppe process or carbonylation of propylene. In this process, butanol is produced directly at low pressure and temperature. In this reaction, propylene ($CH_3CH=CH_2$), water and carbon monoxide reacts at pressures of 0.5-2 x 10^6 Pa and at 100°C with the presence of a catalyst. Typically, tertiary ammonium salt or polynuclear iron carbonyl hydrides catalyst is used in this reaction. This process was found to be not as commercially successfully as oxo synthesis due to the expensive technology (Uyttebroek et al., 2015).

$$CH_3CH = CH_2 \rightarrow CH_3CH_2CH_2CH_2OH + (CH_3)_2CHCH_2OH + 2CO_2 \quad (2.3)$$

The third chemical reaction process is Aldol condensation of acetaldehyde (CH_3CHO), which can be obtained by oxidation of ethanol to crotonaldehyde ($CH_3CH=CHCHO$), followed by hydrogenation to produce butanol. In this process, the dehydration is induced by acidification, using an acid such as acetic acid or phosphoric acid. The reaction is perfomed in the liquid phase in ambient temperature and pressure in the presence of an alkaline catalyst.

$$(CH_3)_2CHCHO + H_2 \rightarrow CH_3 = CHCHO \quad (2.4)$$

$$CH = CHCHO + 2H_2 \rightarrow CH_3CH_2CH_2CH_2OH \quad (2.5)$$

Production of butanol through a chemical reaction was found to be very expensive and not sustainable due to the fluctuation of the propylene price and is extremely sensitive to the price of crude oil (Dürre, 2007; Uyttebroek et al., 2015). Hence, this has led to the establishment of other alternative butanol approaches that can ensure the continuous butanol production in a cheaper manner.

2.2.1.2 Acetone-butanol-ethanol (ABE) fermentation

Another butanol production route is acetone-butanol-ethanol (ABE) fermentation. ABE fermentation is a process used to produce solvent such as acetone, butanol and ethanol using bacteria as a biocatalyst. During this process, the bacteria will convert fermentable sugar/starch into ABE under anaerobic conditions in a molar ratio of 3:6:1 with the following equation;

$$(C_6H_{10}O_5)_{10} + 9H_2O \rightarrow 3C_3H_6O + 6C_4H_{10}O + C_2C_6O + 24\,CO_2 + 16H_2 + Biomass \quad (2.6)$$

Starch Acetone Butanol Ethanol

One of the most common bacteria used in butanol production is *Clostridium* sp. (Lee et al. 2008). *Clostridium* is a rod-shape gram-positive bacteria that belongs to the Firmicutes. This bacteria is an obligate anaerobe and is capable of producing spore in its growth cycle. This bacteria has the capability to degrade a wide range of polysaccharides and produce solvents, acids and alcohols during the fermentation process (Gheshlaghi et al., 2009). The solvent-producing *Clostridium* sp. can be divided into four groups according to their genetic profile, *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutyl acetonicum*, and *C. saccharobutylicum*. The different strains share similar phenotype characteristics, for instance, the main metabolic pathway and fermentation end product. Even though these bacteria share the same similar phenotype, however, the fermentation performance and end product production totally depends on the type of feedstock and the fermentation condition.

The metabolism of ABE-fermentation by *Clostridium* sp. involves two different stages, the first stage is an acidogenesis stage, which the acetic and butyric acids are produced. Meanwhile, the second stage is a solventogenesis and usually is achieved at an early stationary stage. At this stage, the acids produced are re-assimilated into ABE solvents.

During the acidogenesis phase, organic acid is produced through an acetyl-CoA and butyryl-CoA pathway, while during solvent production, acetyl-CoA and butyryl-CoA function as the key intermediates for ethanol and butanol production. These pathways produce acetylaldehyde and butyraldehyde respectively. Ethanol can be produced independently from acetone and butanol by *Clostridium* sp. under certain culture conditions (Figure 2.2) (Kumar and Gayen, 2011). It is also understood that acetate/acetic acid, butyrate/butyric acid and ethanol are called primary metabolite, whereas acetone and butanol are called secondary metabolite.

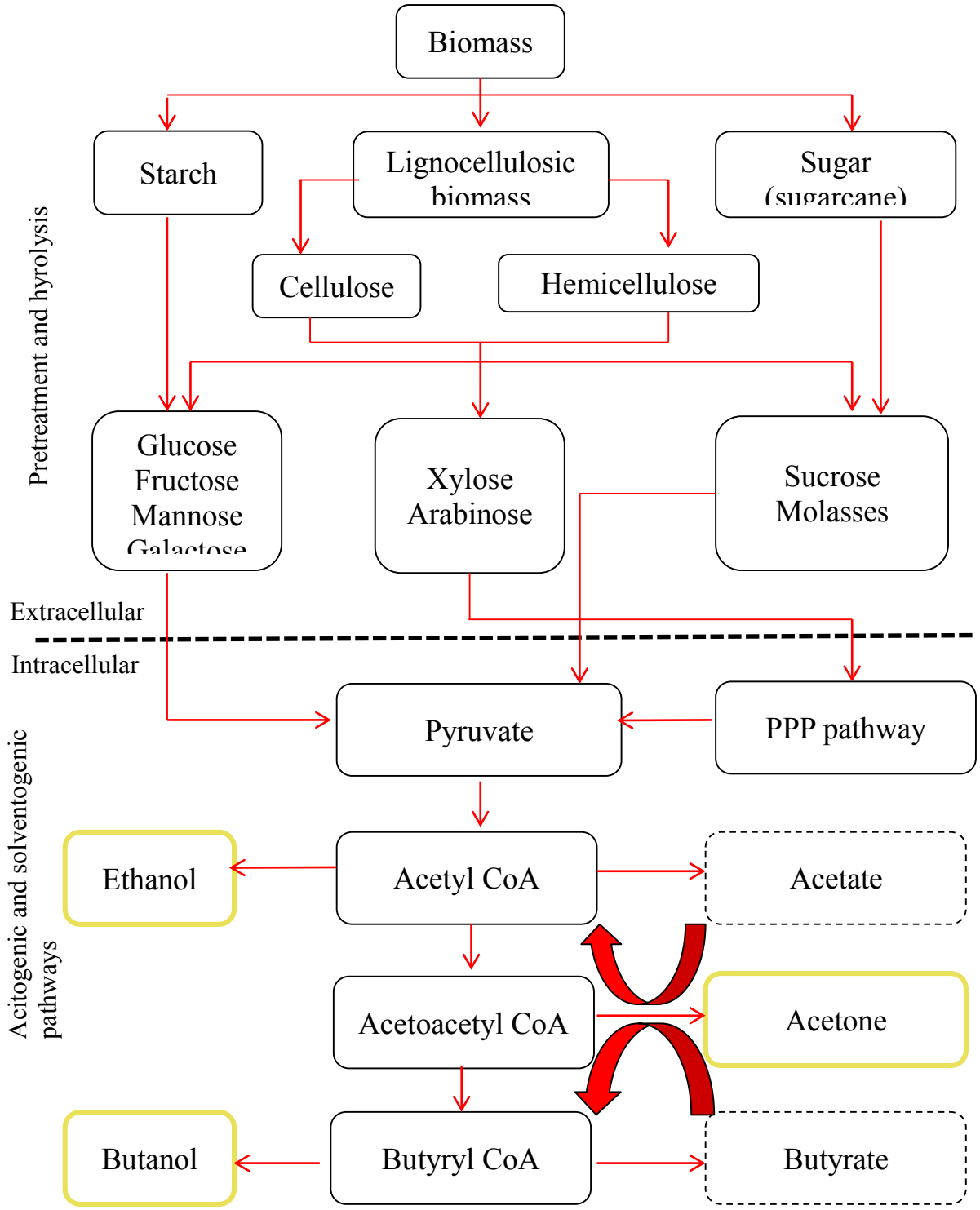

Figure 2.2 Typical Acetone-butanol-ethanol (ABE) fermentation metabolic pathway of *Clostridium* sp. (Hongjuan et al., 2013). PPP pathway: Pentose phosphate pathway

2.3 ABE fermentation feedstock

Feedstock selection is an important determinant of economic feasibility for butanol production (Pfromm et al., 2010; Qureshi and Blaschek, 2000). It was reported that feedstock costs account for approximately 60% of the butanol production cost (Demirbas, 2009). Butanol can be produced from different feedstocks such as starchy materials, agricultural residues, cheese whey, woody and lignocellulosic biomass (Table 2.3) (Cheng et al., 2012; Madihah et al., 2001; Patakova et al., 2009; Qureshi et al., 2008). Production of butanol from food-based and lignocellulosic materials has been identified to have few disadvantages (Sims et al., 2010). The application of food-based feedstock has turned out to be cost-intensive due to the increased demand of food worldwide (Antizar-Ladislao and Turrion-Gomez, 2008; Sims et al., 2010). On the other hand, several drawbacks, such as the food versus fuel issue, increase of land and water usage for biomass production and high cost of production have led to exploring new types of butanol feedstock. The use of renewable resources that can be produced continuously in a shorter period of time, are cheap with good quality, and require less complicated upstream processing to obtain the final product are the most important criteria for selecting biomass feedstock as raw materials for butanol processes (Lynd et al., 1999).

The use of a new material such as algal biomass is believed to have great potential to overcome the issues. This is due to the fact that this algal biomass has more advantages compared to terrestrial plants, such as this biomass does not compete with food, it has a high growth rate, its leads to high biomass production and lipid yield, it is able to grow using carbon dioxide (CO_2) as a carbon source, and it can be applied in wastewater treatment (Li et al., 2008). Generally, an algal cell contains 30-50% carbohydrate depending on the species and cultivation conditions (Markou et al., 2012). Algal biomass with high carbohydrate content is a great candidate to be used as an ABE feedstock. The details on the algal biomass are described in the next section.

Table 2.3 Acetone-butanol-ethanol (ABE) fermentation from different types of feedstock.

Substrate	Microoganism	Total ABE (gL^{-1})	ABE yield (g/g reducing sugar)	References
Cassava starch	*C. saccharoperbutylacetonicum* N1-4	21.0	0.33	Thang et al. 2010
Cassava chips	*C. saccharoperbutylacetonicum* N1-4	19.40	0.30	
Corn straw	*C. acetobutylicum*	6.20	nd	Lin et al. 2011
Corn stalk	*C. beijerinckii*	5.48	nd	Mu et al. 2011
Baggase	*Clostridium sp.*			Cheng et al. 2012
Rice straw	*C. acetobutylicum*	64 (alkali) 61 (acid)	0.39 0.32	Moradi et al. 2013
Empty fruit bunches (EFB)	*C. butyricum* EB-6	3.47	0.24	Ibrahim et al. 2012
Palm kernel cake (PKC)		0.34	nd	Shukor et al. 2014
Palm oil mill effluent (POME)	*C. saccharoperbutylacetonicum* N1-4	2.2	nd	Al-shorgani et al. 2012
Rice bran	*C. beijerinckii* NCIMB 8052	11.4	nd	Lee et al. 2009
De-oil rice bran	*C. saccharoperbutylacetonicum* N1-4	12.13	0.44	Al-shorgani et al. 2011

2.4 Algae

Generally, algae is a photosynthetic organism that uses sunlight and carbon dioxide (CO_2) to produce biomass through a photosynthesis reaction. According to Guiry and Guiry (2015), there are approximately 139,417 species have been registered around the world and they can be classified into two distinct categories, specifically macroalgae and microalgae. The comparison between macroalgae and microalgae is summarised in Table 2.4. Macroalgae is known as seaweed or kelp and can be divided into three different groups based on their pigmentation - brown seaweed (Phaeophyceae), red seaweed (Rhodophycease) and green seaweed (Chlorophyceae) (Ross et al. 2008). Micoralgae, on the other hand, is a microscopic organism that can be categorised into four main classes, i.e., diatom, green algae, blue algae and golden algae (Demirbas, 2010).

Table 2.4 Comparison of macroalgae and microalgae characteristic.

Macroalgae	Microalgae
• Multicellular plant growing in salt and fresh water • Able to grow up to 60 m in length • High nutrient storage capacity and has low growth rate • Utilised for food production and extraction of hydrocolloid	• Microscopy organisms growing in salt and fresh water • Small size • More nutrient uptake and has fast growth rate • Utilised to produce food, medical supplement, biofuel

Interest in producing biofuel from algal biomass has gained great attention worldwide as algal biomass shows more advantages compared to lignocellulosic biomass. The main reason of interest in algal biomass is due to its capability to grow and produce far higher biomass yield compared to any other feedstock currently being used. Algae grow 20-30 times faster than food crops and terrestrial plants (Demirbas and Fatih Demirbas, 2011; Ullah et al., 2014). Apart from having higher growth rates, studies also indicated that algae has shorter harvesting times of between 1 to 10 days with higher carbohydrate and lipid in their cell mass compared to terrestrial plants that require more than 3 months to harvest the biomass (Harun et al., 2010; John et al., 2011; Rodjareen, 2007). Both macroalgae and microalgae contain lipid, carbohydrate and protein in the cell body and the chemical components mentioned can be converted into a wide range of chemicals. Previous studies have shown that algal biomass

contains 10-40% lipid, 17-57 % carbohydrate and 20-50% protein (dry weight basis depending on the microalgae species) (Table 2.5). Some algae possess high lipid and carbohydrate content per dry biomass, which these compounds can be extracted and converted into biofuel such as diesel, ethanol and butanol. Besides, algal biomass can also be converted into other value-added products such as animal feed, bulk chemicals and other bioactive compounds, especially in the pharmaceutical industry (Milledge, 2010). Algae is also useful for bioremediation application and as a fertiliser (Kalin et al., 2004; Muñoz and Guieysse, 2006). Furthermore, algae also requires less water for its cultivation compared to other terrestrial energy crops (Dismukes et al., 2008). Moreover, the cultivation can be carried out by using either fresh water or wastewater or non-arable land, in which case the arable land can be used for food and the production of bioproducts (Parmar et al., 2011). In addition, the most important part of this organism is its capability to use light and carbon dioxide (CO_2) as energy to produce its biomass, which can be applied in carbon capture strategies (Zeng et al., 2011). Likewise, algae also potentially can be used to remove nitrogen and phosphate in wastewater streams prior to discharge to waterways (Abdel-Raouf et al., 2012).

Table 2.5 Chemical composition of algae on a dry matter basis (%) (Demirbas, 2010; Spolaore et al., 2006).

Species	Protein	Carbohydrate	Lipid
Scenedesmus oblicus	50-56	10-17	12-14
Scenedesmus dimorphus	8-18	21-52	16-40
Chlamydomonas rheihardii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Chlorella pyrenoidosa	57	26	2
Spirogyra sp.	6-20	33-64	11-21
Duneliella salina	57	32	6
Prymnesium parvum	28-45	25-33	22-38
Tetrasalmis maculata	52	15	3
Porphyridium cruentum	28-39	40-57	9-14
Spirulina plantensis	46-63	8-14	4-9
Spirulina maxima	60-71	13-16	6-7

Although both macroalgae and microalgae have the potential to be used as biofuel feedstock, microalgae is considered to have additional advantages over macroalgae. This is due to the fact that microalgae has a higher growth rate and shorter harvesting period compared to macroalgae. Theoretically, microalgae produces 158 tonnes of biomass per hectare of land, however, macroalgae produces 60-100 tonnes of biomass per hectare (Chisti, 2007). The harvesting period for microalgae is shorter than macroalgae; microalgae can be harvested in 6 to 10 days depend on cultivation conditions, while macroalgae can be harvested in 3 to 6 months. Another advantage of microalgae is having high lipid and carbohydrate content compared to macroalgae (Neveux et al., 2014). Besides, macroalgae has similar characteristics to terrestrial plants, for instance, it possesses a complex cell wall structure consisting of cellulose, hemicellulose and lignin (Michel et al., 2010). Thus, it requires higher energy input to convert macroalgae biomass to biofuel compared to microalgae. This characteristic is important in ensuring the feasibility of biofuel production from microalgal biomass.

The potential of biofuel from microalgal biomass is totally dependent on the chemical composition present in microalgal biomass. For instance, microalgal lipid can be converted into biodiesel, while microalgal carbohydrate extracted from microalgal biomass can be converted into ethanol and butanol. Besides, the microalgae that posess low lignin content exhibit great potential to be used as a liquid biofuel feedstock A number of studies have reported on the potential of microalgal biomass as a bioethanol and biodiesel feedstock. However, to the best of our knowledge, literature on the potential of butanol production from algae biomass is still limited.

In order to produce liquid fuel from microalgal carbohydrate, a few of the processes, such as upstream processing (biomass production) and downstream processing (pretreatment and conversion process), should be evaluated. The detail of the process will be described in the next section.

2.4.1 Microalgal cultivation

Microalgae can be categorised into autotrophic, heterotrophic or mixtrophic species (Figure 2.3) (Alkhamis and Qin, 2013; Frac et al., 2010; Mitra et al., 2012). The autotrophic species

is a microalgae that uses inorganic materials as a carbon source. The autotrophic microalgae can be classified into either photoautotroph or chemoautotroph. The photoautotroph species is a microalgae that uses light as an energy source, whereas a chemoautotrophic species is a microalgae that uses an inorganic compound as an energy source. For the heterotrophic species, typically this microalgae uses an organic compound as an energy and a carbon source. This heterotrophic species is also classified as a photoheterorophic and chemoheterotrophic microalgae (Chojnacka and Marquez-Rocha, 2004). For the mixtrophic species, most of the microalgae in this group use inorganic, organic and sunlight as energy sources.

Few studies on the cultivation of microalgae using heterotrophic and mixotrophic strategies have been reported (Liang et al., 2009). Cultivation using these strategies has been reported to produce higher microalgal biomass. However, cultivation using this strategy to enhance microalgal growth is not favourable due to high energy input and it being very expensive resulting from carbon and nitrogen being used as additional nutrients during the cultivation. Besides, utilisation of carbon sources for algae growth may compete with food and human consumption (Chisti, 2007). Therefore cultivation of microalgae using the autotrophic strategy is more favourable for large-scale biomass production.

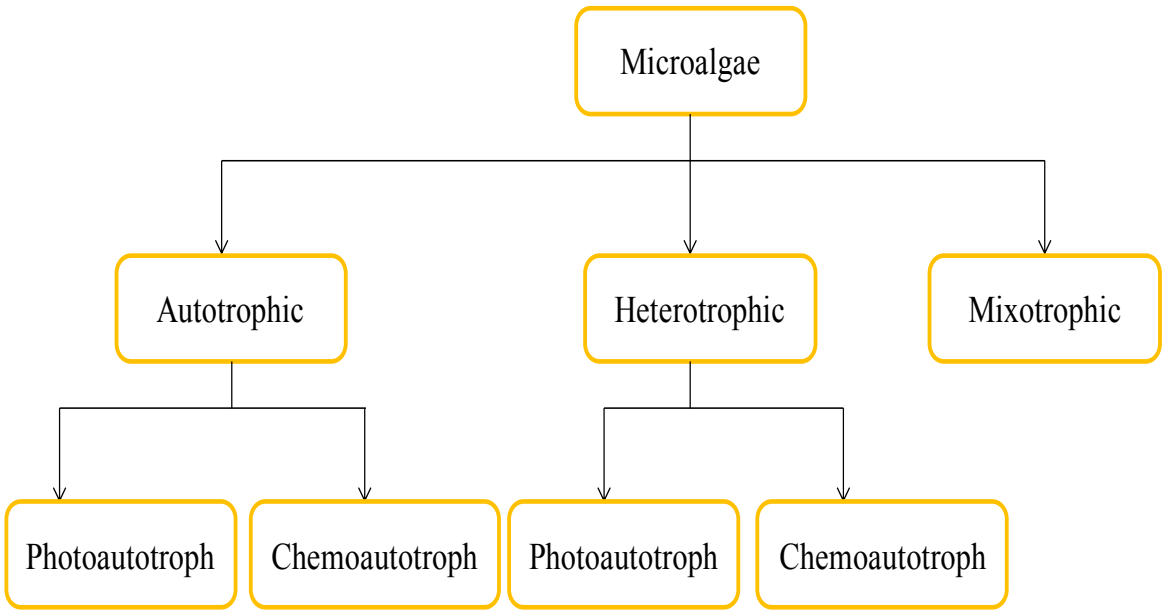

Figure 2.3 Classification of microalgal growth.

For biofuel production from microalgal biomass, mass microalgal cultivation is important in obtaining high microalgal biomass concentration. Two main cultivation systems are commonly used for mass cultivation of microalgae, namely, an open pond system and a closed system (Posten, 2009).

An open pond system is considered the oldest system used for microalgal biomass production. Generally, this system is used to produce microalgal biomass for the food and pharmaceutical industry (Tafreshi and Shariati, 2006). This system is relatively easy to operate and requires low costs due to its cheap, transparent material (Chen, 1996). Several materials can be used to build the system, for instance clay, cement, brick and plastic; or polyethylene, PVC and polyurethane (Wehr, 2007). However, a few disadvantages have been identified in this system, such as low productivity between 10-231 $mgL^{-1}d^{-1}$, contamination of other microorganisms and it requires a high water consumption rate due to its uncontrolled high evaporation rate and CO_2 diffusion compared to other cultivation systems (Brennan and Owende, 2010; Hannon et al., 2010; Moheimani and Borowitzka, 2007). In regard to these issues, much attention have been focused on using the utilisation of a closed system for microalgal cultivation and biomass production.

A closed cultivation system is a system that is carried out using a photobioreactor (PBR). Generally, the closed PBR is constructed using a transparent material such as plastic and glass with different sizes, lengths and orientations (Pulz, 2001). Microalgal cultivation using the enclosed PBR was reported to give more advantages compared to the open pond system (Roselet et al., 2013). Productivity in closed systems can be much higher than open systems, with biomass concentrations of up to 8 gL^{-1} and productivities of between 800-1300 $mg.L^{-1}d^{-1}$ (Pulz, 2001). In addition, the PBR with a higher surface area to volume of light provision is able to provide better control of gas transfer and reduction of evaporation. Moreover, the used of the enclosed PBR is able to reduce contamination from other microorganisms and is able to prevent any contamination invasion during the algae cultivation period, which facilitates higher microalgal biomass productivity (Mata et al., 2010). The comparison of microalgal cultivation using an open pond and closed system is shown in Table 2.6.

Table 2.6 Comparison of open pond and photobioreactor system in culturing algae.

Factor	Open Pond	Photobioreactor
Contamination risk	High	Low
CO_2 losses	High	Low
Evaporative losses	High	Low
Light use efficiency	Poor	Excellent
Area/volume ratio	Low	High
Area required	High	Low
Process control	Difficult	Easy
Biomass productivities	Low	High
Investment costs	Low	High
Operation costs	Low	High
Harvesting costs	High	Relatively low
Scale-up	Easy	Difficult

2.4.2 Microalgal growth and carbohydrate content

Apart from the cultivation system, the microalgal growth is also influenced by the cultivation conditions. The growth of microalgae is typically related to the photosynthesis metabolisms in the microalgal cells. During the photosynthesis process, microalgal requires carbon dioxide (CO_2), sunlight and oxygen (O_2) with the presence of water to produce carbohydrate ($C_6H_{12}O_6$) and biomass as final photosynthesis products. The overall photosynthesis reaction is described in the following equation:

$$6CO_2 + 12H_2O \longrightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O \qquad (2.7)$$

It is important to determine the environmental conditions that are optimal, favourable or merely tolerable for the growth of microalgae species. Cultivation under unfavourable conditions either could increase or decrease microalgal growth (Chen et al., 2013). Apart from microalgal growth, a suitable cultivation condition also could affect the carbohydrate accumulation in microalgal cells. Cultivation parameters, such as temperature, light intensity, salinity, nutrient, and carbon dioxide (CO_2) concentration, have been reported to influence the microalgal growth and carbohydrate accumulation in the microalgal biomass during cultivation (de Castro Araújo and Garcia, 2005). Generally, the cultivation parameters mentioned could influence microalgal carbohydrate accumulation metabolisms. The carbohydrate accumulation metabolism is presented in Figure 2.4. This subsequent section will discuss the effect of cultivation parameters on microalgal growth and carbohydrate accumulation.

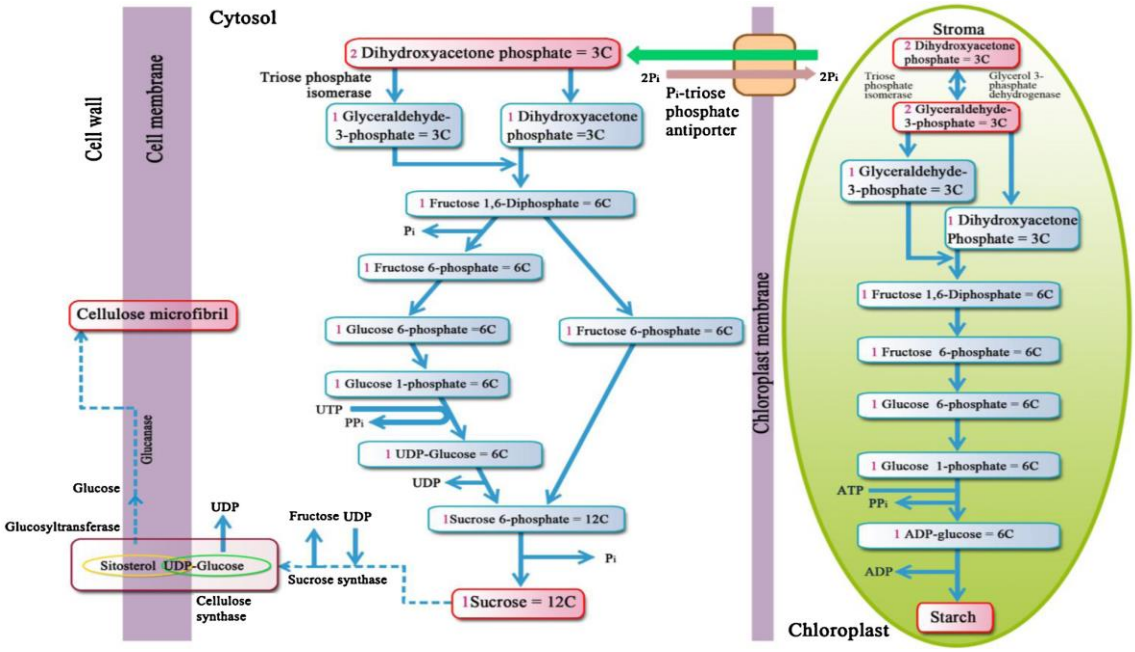

Figure 2.4 Carbohydrate accumulation metabolisms in microalgae cell (Chen et al., 2013).

2.4.2.1 *Effect of light intensity*

Light is a main energy source for microalgae for photosynthesis and biomass production. The suitable light intensity is crucial to ensure a high growth rate, and to produce high biomass concentration. Light intensity influences the photoadaptation/photoacclimation and photoinhibition process in microalgal cells. Most of microalgal growth rate increases as the light intensity increases up to a curtain limit, in which the light saturation occurs during the cultivation. Generally, the majority of microalgae are light saturated under light intensity of 200-400 umol m^3s^{-1}. Cultivation at the suitable light saturation intensity will increase biomass productivity, growth rate and biochemical composition (Amini Khoeyi et al., 2012; Ho et al., 2013). However, exposure at higher or lower light saturation intensity will cause photoinhibition (Murata et al., 2007). The photoinhibition in microalgal cells at high light intensity occurs due to the disruption of chloroplast and lamellae, resulting in enzyme inactivation that involves nutrient uptake during the photosynthesis (Huovinen et al., 2007; Neidhardt et al., 1998). Also, cultivation at low light intensity could produce low biomass concentration. Low biomass production at a low light intensity is due to insufficient energy supply to trigger photosynthesis activity. On the other hand, cultivation at low light intensity

will lead to D1 protein damage, which this protein is associated with in the photosystem PS (II) apparatus, consequently leading to the reduction of photosynthesis activities in the microalgal cell (Keren et al., 1997).

The study on the effect of light intensity on the various types of microalgal growth has been reported by previous studies (Amini Khoeyi et al., 2012; Dechatiwongse et al., 2014). For instance, *Scendesmus* sp. exhibited the maximum growth rate in cultivation at 420 umol $m^{-2}s^{-1}$ (Ho et al., 2012). Cultivation of *Scendesmus* sp. at higher than 420 umol $m^{-2}s^{-1}$ resulted in a significant drop in microalgal biomass production. The study suggested that excessive illumination led to photoinhibition, subsequently inhibiting the biomass production.

In addition, a study on the effect of light intensity towards *T. suecica* displayed maximum biomass production at 133 umol $m^{-2}s^{-1}$ (Go et al., 2012). In another study, cultivation of *Nannochloropsis* sp. showed a maximum biomass production at 100 umol $m^{-2}s^{-1}$ (Wahidin et al., 2013). Those studies indicated that cultivation beyond the optimum light intensity produced low biomass production. This finding clearly suggests that the adaptability of microalgae towards light intensity is species-dependent.

The light intensity has also been reported to influence carbohydrate content in microalgal biomass (Hu, 2003). During photosynthesis, carbohydrate is produced as a final product that can be used as an energy source during respiration. The carbohydrate accumulation in microalgae during photosynthesis is greatly stimulated by three major enzymes; dihydroxyacetone, phosphate, sucrose and starch synthase. Cultivation in non-optimum conditions could influence microalgal metabolism. Previous studies on the effect of light intensity on *Pavlova lutheri* indicate that the increase of light intensity from 60 up to 150 umol $m^{-2}s^{-1}$ could slightly increase 66% of carbohydrate content (Carvalho et al., 2009). Further, a significant carbohydrate content (80%) in microalgae was increased when the light intensity was increased from 215 to 330 umol $m^{-2}s^{-1}$ (Fernandes et al., 2010). Another study demonstrates that an increase of light intensity from 30 to 400 umol $m^{-2}s^{-1}$ could increase 55% of carbohydrate content in *Scenedesmus obliqus* (Ho et al., 2012). A further increase of light intensity beyond 400 umol $m^{-2}s^{-1}$ appeared to reduce carbohydrate content in *S.obliqus*.

2.4.2.2 *Effect of temperature*

Another factor that has a significant effect on the microalgal growth and chemical composition is temperature. Cultivation temperature plays a significant role in microalgal growth, nutrient uptake, enzyme kinetic and oxygen release that is related to the photosystem II (PSII) activity (Berges et al., 2002). Cultivation at the suitable temperature will improve the microalgal growth rate, and nutrient uptake. However, cultivation at below and above the optimal temperature will reduce microalgal growth, which is attributed to the alteration of photosynthesis activity in a microalgal cell. Cultivation at a high temperature will reduce the photosynthesis enzyme, subsequently reducing specific microalgal growth (Davison, 1991). Moreover, exposing microalgal culture at an extreme temperature will limit electron transport and carbon fixation by reducing the ability of the microalgal to use light. Thus, the resulting excess light energy will indirectly damage the PS II apparatus in the microalgal cell (Murata et al., 2007). In contrast, low biomass production at a low temperature can (1) suppress the repair cycle of protein D1, thus resulting in lower photosynthetic activities, (2) slow the rate of cellular nutrient uptake. The optimum temperature range for microalgal growth is generally between 20°C to 35°C. Microalgal cultivation at beyond the suitable temperature produces low biomass due to the inactivation of the photosynthesis activity. Various microalgae have been reported to have different optimum microalgal growth temperature levels and may be species dependent (LÜRling et al., 2013).

The influence of the temperature on the various microalgal growth has been reported elsewhere (Cheng and He, 2014). For instance, the optimal temperature for the maximum specific growth rate of *Nannochloropsis oculata* and *Nitzschia laevis* was 20 and 23°C (Converti et al., 2009). On the other hand, studies on the effect of temperature on *Scendesmus* sp. indicate that different microalgal strains show a different optimal cultivation temperature. It was reported that *Scendesmus* sp. showed the highest growth rate at temperatures between 27 to 39°C (Westerhoff et al., 2010). The optimal cultivation temperature range for other *Chlorella* species was also reported between the range of 25 to 35°C (Choi et al., 2012).

Apart from microalgal growth, temperature has also been reported to significantly affect the biochemical composition in microalgal cells (Berges et al., 2002). The cultivation temperature is one of the factors that could influence the starch synthase enzyme involved in

carbohydrate metabolism in the microalgal cell. The starch synthase enzyme activity is totally dependent on the environmental temperature (González-Fernández and Ballesteros, 2012; Nakamura and Miyachi, 1982). The effect of temperature on carbohydrate accumulation varies between microalgae species. For instance, a slight increase of carbohydrate content from 14 to 20% in *Spirulina* sp. was observed as the temperature was increased from 25 to 40°C (Ogbonda et al., 2007). In another study, the maximum carbohydrate content for diatom *Chaeroceros cf. wighamii* was obtained from the cultivation at a lower temperature (de Castro Araújo and Garcia, 2005).

2.4.2.3 *Effect of pH*

The initial pH value is another factor that can influence microalgal metabolism and biochemical composition. The optimum pH value range for microalgae is generally between pH values of 6 to 9 depending on the type of microalgae species. The pH value is important in maintaining microalgal growth, especially during cultivation using CO_2 as the carbon source. The CO_2 supply in a cultivation medium will reduce the pH value to a certain level. Besides, the suitable pH value also is important to maintain the selected microalgae species and avoid contamination by unwanted organisms such as bacteria, fungi and predator (Rodolfi et al., 2009). Contamination of these unwanted organisms will provide competitive conditions during cultivation, resulting in low biomass productivity. The pH value in the cultivation medium will also affect the nutrient uptake, and enzyme kinetic involved in microalgal metabolism (Liu et al., 2007).

Generally, the pH tolerance of microalgae is species-dependent. A study on the effect of pH on *Chlorella protothecoides* growth was performed and found that the highest growth rate for this species was observed when the cultivation was carried out in mediums with an initial pH value of 5 (Guobin, 2011). However, different observations have been reported on the effect of the pH value on *C. ellipsoidea*, where its biomass production was increased with the increasing of the pH value toward an alkaline condition (Khalil et al., 2010). A study on the effect of the pH value on *Chlorella vulgaris* indicates that the highest growth rate for this species was observed in the medium with a pH value of 6.31 (Mayo 1997). The cultivation of *C. vulgaris* in a medium having lower and higher pH value than 6.5 produced low biomass concentration and displayed a slow growth rate. Furthermore, similar findings have been

observed on the marine algae *Thakassiosira pseudonana* and *Thalassiosira oceanica*, where the microalgal growth rate, nutrient uptake, and photosynthesis were reduced when these microalgae species were cultivated in a high pH value (Chenl and Durbin, 1994).

The varying of the pH in medium cultivation also could affect the metabolic process in the microalgae cell (Kosourov et al., 2003). Cultivation at lower or higher than optimal pH value will provide the an imbalanced environment between external and internal pH, thus requiring an expenditure energy to pump protons out of the cell (Lnae and Burris, 1981). Cultivation in a medium that is too alkaline will limit the availability of carbon from CO_2, which will result in the inhibition of microalgal growth (Liu et al., 2007).

Generally, the effect of pH on carbohydrate content is different between microalgal species. Certain enzymes involved in the microalgal metabolic pathway have different optimum pH, implying that the changes of pH could affect the microalgal metabolism.

Comparison of carbohydrate content in *Nannochloropsis* sp. and *Tetraselmis* sp. showed that both microalgae species produce maximum carbohydrate content at different pH values (Khatoon et al., 2014). The study indicated that the maximum carbohydrate content for *Nannochloropsis* sp. (32%) and *Tetraselmis* sp. (30%) was obtained at pH 5.5 and 8.5, respectively. Also, in another study, the maximum carbohydrate content for *C. ellipsoidea* was attained at pH 7.5 (Khalil et al., 2010).

2.4.2.4 *Effect of salinity*

Another factor that could influence microalgal growth is salinity. For microalgae that has the capability to capture CO_2 isolated from fresh water source, the ability of this microalgae to grow in a medium with a high salinity level is crucial and provides advantages for high-biomass production. It is worth mentioning that the capability of microalgal to adapt and survive in different salinity levels is also species-dependent (Kirst, 1989). Under unfavourable salinity, the growth of microalgae is inhibited and produces more lipid and carbohydrate in cytoplasm.

Studies on the effect of salinity on microalgal growth such as *D. tertiolecta*, *Botrycoccus braunii*, *Chaetoceros* cf. *Wighamii* and *Chattonella marina* have been carried out by previous studies (Chen and Jiang, 2009; Rao et al., 2007). A study on the effect of salinity on *B. braunii* indicated that the maximum biomass production was attained when the microalgae was cultivated at the lowest salinity level (Rao et al., 2007). Another study on the salinity tolerance of freshwater microalgae *Scendesmus* sp. has also been reported elsewhere. Comparison of different *Scendesmus* species indicated that different species showed a different NaCl tolerance. The maximum biomass production of *S. obliquss* and S. *almenensis* has been reported to be attained in cultivation using 0.3% and 0.58% (w/v) NaCl, respectively (Kaewkannetra et al., 2012; Sánchez et al., 2008). A study on the effect of the salinity level of three microalgal species, *Isochrysis* sp. *Nannochlorpsis oculata* and *Nitzschai flustulum* found that there was no significant effect of salinity on the growth when these microalgae were cultivated in the salinity range between 10 to 35% (w/v) NaCl (Renaud and Parry, 1994).

Apart from microalgal growth, the salinity level has also been reported to influence the carbohydrate content in microalgal cells. Microalgae will response to the osmotic stress by producing extracellular metabolite such as glycerol and carbohydrate to protect the cell from salt injury and to balance the surrounding environment (Rao et al., 2007). An increase in the salinity level in a cultivation medium has been reported to enhance intracellular carbohydrate in *Chlamydomonas reinhardtii* (Siaut et al., 2011). According to Khatoon et al. (2014), the maximum carbohydrate content for *Nannochloropsis* sp. and *T. suecica* was obtained from cultivation in a medium containing 30% (w/v) NaCl. However, different findings have been observed in *Dunaliella* sp., where the maximum carbohydrate content was attained at a low salinity level (Chen and Jiang, 2009). This clearly indicates that the inconsistent role of salinity on carbohydrate content metabolisms is also species-dependent and depends on the cultivation conditions.

2.4.2.5 *Effect of carbon dioxide (CO_2)*

Generally, microalgae use CO_2 for photosynthesis and to produce biomass. The capability of microalgae to tolerant with CO_2 concentration can be grouped into CO_2 sensitive (<2-5% v/v CO_2), and CO_2-tolerant (5-20% v/v CO_2) microalgal, and the capability of microalgal to fix

CO_2 is also species-dependent (Miyachi et al., 2003). Table 2.7 shows the microalgal capability to grow in different CO_2 concentrations. It is clearly shown that each microalgae species displayed a different level of CO_2 tolerance. The capability of microalgal to grow in a high CO_2 concentration is attributed to the CO_2 concentrating mechanisms (CCM) in the microalgal cells. Two main factors play an important role in CCM mechanism: (1) inorganic carbon transporter that assists dissolve inorganic carbon (DIC) membrane to transfer CO_2 or bicarbonate into plasmalemma and (2) chloroplast envelope and carbonic anhydrase (CAs), which are involved in stimulating the direct supply of CO_2 from outside cells to Rubisco (Baba and Shiraiwa, 2012). Based on the CCM mechanisms *Chlamydomonas reinhardtii* model, it was reported that the affinity of Rubisco for CO_2 is insufficient in the atmospheric CO_2 concentration. Cultivation at high CO_2 levels will activate CCM activity and increase their affinity on CO_2 uptake, so in turn will affect microalgal growth.

Cultivation of microalgae in different CO_2 concentrations also could influence carbohydrate content in microalgal cells (Giese et al., 2013). The CO_2 can be used to enhance starch content in the microalgal cell. Accumulation of microalgal starch and lipid at high CO_2 concentration is due to the shifting of microalgal metabolisms. Cultivation of microalgae under a stress condition will reduce the microalgal growth and increase the biosynthesis of energy rich compounds such as lipid and carbohydrate (Solovchenko, 2012; Westerhoff et al., 2010). According to Izumo et al. (2007), the cultivation of microalgae at low and high CO_2 concentrations could trigger a starch synthase enzyme and lead to the accumulation of starch content in microalgal cell. Moreover, cultivation at different CO_2 concentrations also could control the starch location. This has been proved by a previous study which found that when the cultivation was performed at 3% (v/v) CO_2, the starch accumulates in stroma, while when the CO_2 concentration decreases, the starch accumulation was shifted to the pyrenoid (Izumo et al., 2007).

The studies on the effect of CO_2 concentration on carbohydrate content in various types of microalgae species have been reported by previous researchers. Gardner et al. (2013) found that cultivation of *Chlamydomonas reinhardtii* at low 0.04% (v/v) CO_2 displayed low starch content compared to cultivation at 5% (v/v) CO_2. In the other study, cultivation of *D. salina* in a medium with 5% CO_2 slightly increased carbohydrate content, while a significant increase was observed in the protein content (Giordano and Bowes, 1997).

Table 2.7 Types of microalgae and its tolerance on CO_2 concentration (Salih, 2011).

Microalgae species	**CO_2 concentration (%) v/v**
Cyanidium caldarium	100
Scendesmus sp.	80
Chlorococcum littorale	60
Synechococcus elongatus	60
Euglena gracilis	45
Chlorella sp.	40
Eudorina sp.	20
Dunaliella tertiolecta	15
Nannochropsis sp.	15
Chlamydomonas sp.	15
Tetraselmis sp.	14

The above discussions highlight the effect of cultivation parameters on the microalgal growth and carbohydrate accumulation in microalgal biomass. It is clear that variable cultivation conditions are required for different microalgae species. Therefore, for the biofuel production from carbohydrate based liquid fuel, particularly butanol, it is important to determine the suitable cultivation condition for different microalgae species in order to obtain higher biomass concentration with high carbohydrate content. Besides, the information generated could provide a baseline for an outdoor cultivation of microalgae for biomass production.

2.5 ABE fermentation of microalgal biomass

Microalgal biomass contains three major chemical compounds in its cellular cell, namely lipid, carbohydrate and protein. Microalgal-lipid and carbohydrate can be converted into liquid fuel such as biodiesel, ethanol and butanol. Biodiesel can be produced from the transesterification process of microalgal lipid. Ethanol and butanol, on the other hand, can be produced from conversion of microalgal carbohdydrate through the fermentation process. In order to produce ethanol and butanol from microalgal biomass, several processes,

specifically, biomass production, pretreatment, enzymatic saccharification and fermentation are involved. Figure 2.5 shows the typical flow process for biofuel production from biomass through the biochemical conversion pathway. Generally, the biomass or fermentation feedstock needs to be pretreated in order to disrupt cell structure and is followed by conversion of carbohydrate to reduce sugar production prior to being subjected to the fermentation process.

2.5.1 Pretreatment of biomass

Pretreatment is one of the most important processes involved in liquid biofuel production through a biochemical conversion pathway. The main goal of pretreatment is to increase the enzyme accessibility and improve the digestibility of polysaccharides or carbohydrate available in the biomass (Potumarthi et al., 2013). In order to extract carbohydrate from intracellular cells, the rigid microalgal cell wall needs to be disrupted and pretreated before subjected to the fermentation process.

Pretreatment of biomass is totally dependent on the chemical composition of the biomass. Biomass that possesses a high recalcitrant component requires a harsh pretreatment condition to disrupt the cell structure. For the case of microalgal biomass, the composition of the microalgal cell wall varies from species to species. The microalgal cell wall primarily consists of an inner layer and an outer layer cell wall, where the outer layer can be grouped into three distinct types; (1) trilaminar outer layer, (2) thin outer monolayer, and (3) without an outer layer (Yamada and Sakaguchi, 1982). In regard to this, the microalgal cell wall disruption and pretreatment is specific for each microalgal species and totally depends on the type of cell wall. Different types of cell wall may display different resistance on mechanical and chemical stress (Miranda et al., 2012).

Various microalgal cell disruptions and pretreatment on microalgal biomass methods have been reported by previous researchers (Günerken et al., 2015; Halim et al., 2012; Prabakaran and Ravindran, 2011). The pretreatment of microalgae biomass can be categorised into four different methods, namely thermal, physical, chemical and biological (Figure 2.6). The following sections summarise the state of the art on pretreatment techniques applied for biofuel production from microalgal biomass.

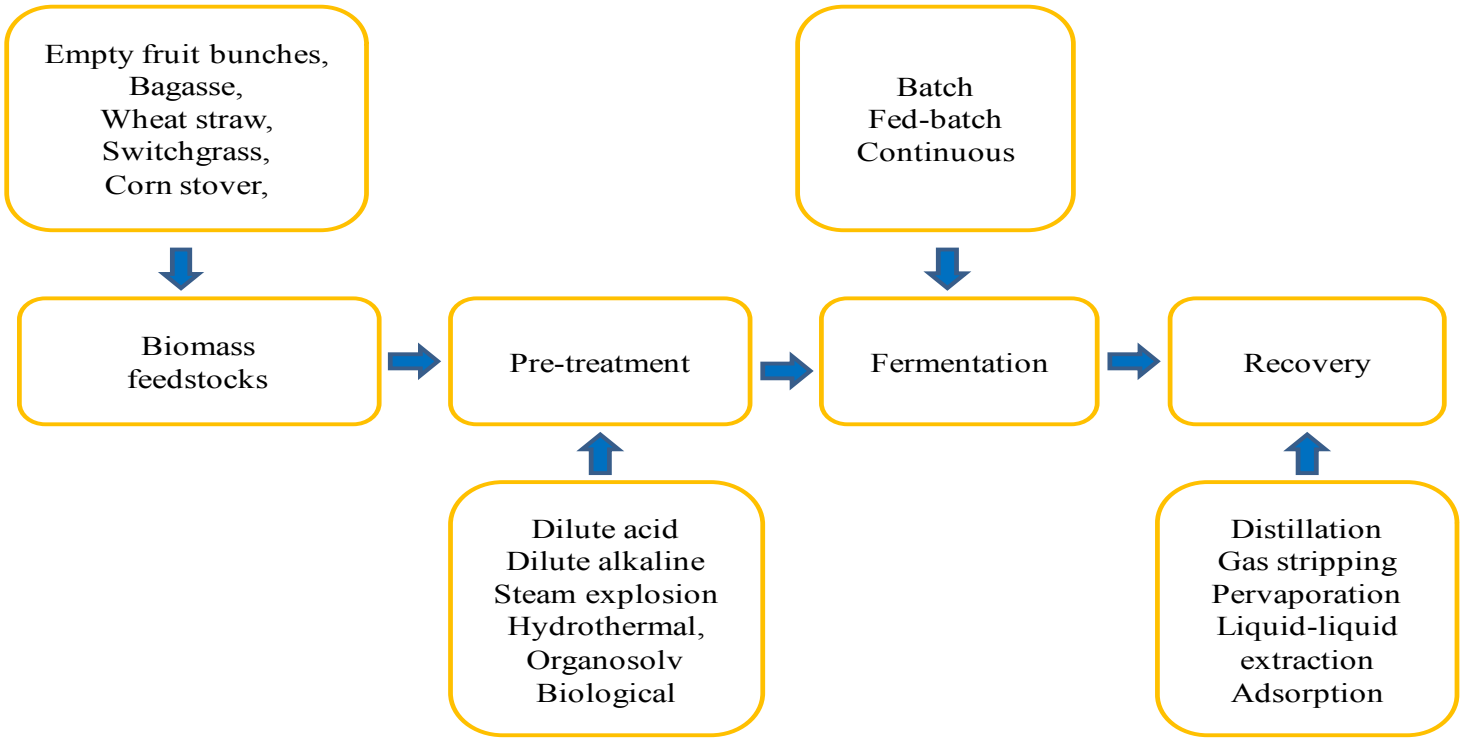

Figure 2.5 Process flow diagram for biobutanol production through biochemical conversion.

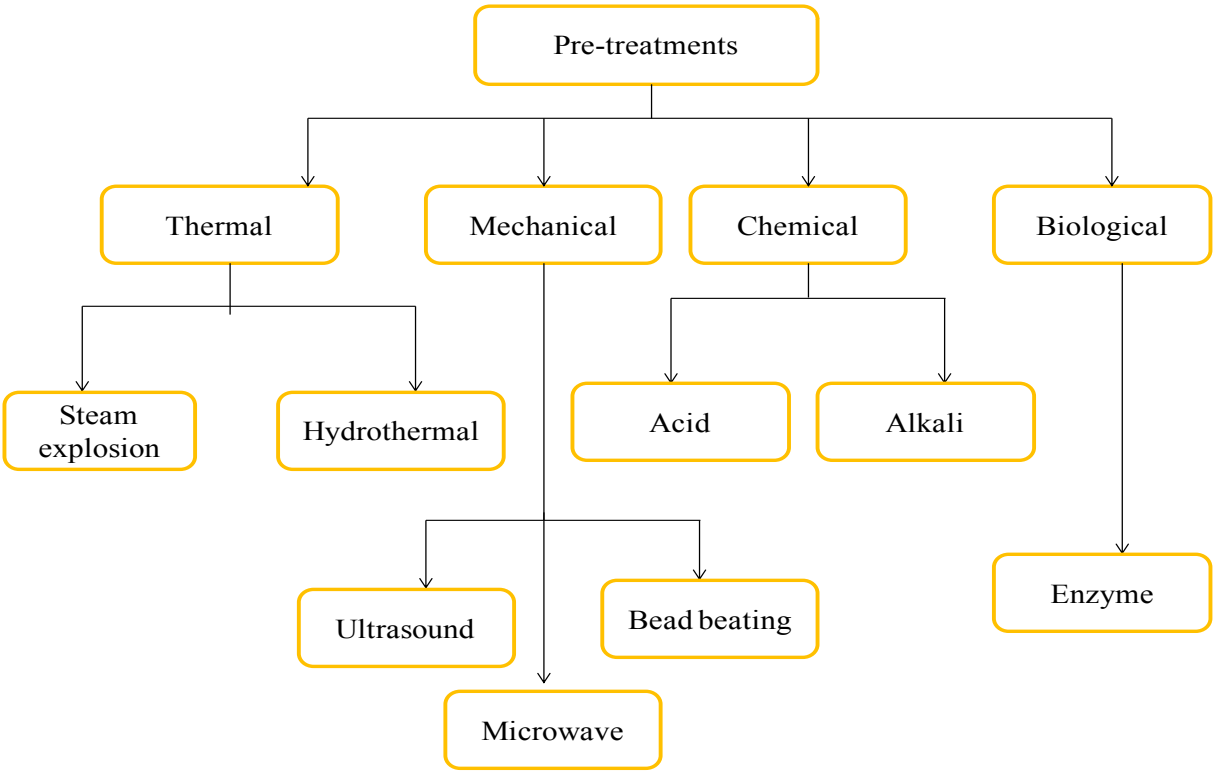

Figure 2.6 Pretreatment methods for microalgal biomass (Passos et al. 2014).

2.5.1.1 *Thermal pretreatment*

Thermal pretreatment is a treatment used to solubilise the microalgal biomass by applying heat in the pretreatment system. In this method, the biomass is heated at a temperature range of 50-170°C. Generally, the thermal pretreatment is sub-divided into three categories: (1) thermal treatment (temperature = <100°C under atmospheric pressure); (2) hydrothermal treatment (temperature = >100°C with gradual pressure release after treatment); and (3) thermal treatment with steam explosion (temperature >100°C with sudden pressure drop after pretreatment). This method is one of the most common methods used for the pretreatment of biomass. The detailed review of these pretreatments is explained elsewhere (Passos et al., 2014).

This method is proved to display a significant effect on the disruption of microalgae (González-Fernández et al., 2012b; Schwede et al., 2013). Typically, this method is applied to pretreat microalgal biomass for methane and hydrogen production. For instance, pretreatment of lipid extracted microalgal biomass for hydrogen production using a combination of thermo-alkaline treatment was explored (Yang et al., 2011). Based on their study, it was found that methane produced from lipid extracted microalgal biomass pretreated at 100°C, followed by alkaline pretreatment using NaOH, was 2 times higher compared to an untreated sample. In another study, the thermal treatment of *Scendesmus* sp. at 80°C caused the disruption of the cell wall and the methane produced from the pretreated sample was 1.6 fold higher compared to an untreated sample (González-Fernández et al., 2012b). Also, thermal pretreatment applied on methane production from microalgal biomass found that this process was able to enhance methane production from *Chlorella vulgaris*. (Mendez et al., 2014). Similarly, thermal pretreatment of *Nannochloropsis salina* biomass found that thermal pretreatment prior to anaerobic digestion could significantly enhance 64% of the methane yield (Schwede et al., 2013).

Although this method was reported to display a positive effect on biofuel production, this method is species-dependent and less effective for microalgae which possess a simple cell wall. Treatment at high temperature would degrade the polysaccharide, resulting in a reduced biofuel production yield (Ayla Sant'Ana da et al., 2013; Sun and Cheng, 2005). Moreover, this method also requires high energy and electricity to maintain the process, especially when it involves a wet feedstock (Brodeur et al., 2011).

2.5.1.2 *Physical pretreatment*

Physical pretreatment is a process that acts directly at breaking the cells through physical force. This method is not species-dependent and is widely used in lipid extraction for biodiesel production.

Various studies have been performed to investigate the potential of this method to disrupt microalgal cell structure. A few technologies on physical pretreatment, such as high-pressure homogeniser, ultrasonication and bead beating on microalgal biomass cell disruption, are the most widely used methods to disrupt microalgal cells (Halim et al., 2012).

A study on cell disruption of *Chlorococcum* sp. using a high-pressure homogeniser, ultrasonication, acid treatment and bead beating found that the most effective mechanical pretreatment method was a high-pressure homogeniser (average disruption = 73.8%), followed by bead beating (average disruption = 17.5%) and ultrasonication. The study found that after five passes of homogenisation, most of the *Chlorococcum* sp. cell had been fully disrupted.

In another study, the physical pretreatment using ultrasound on *Scendemus* sp. cell wall disruption was also evaluated (González-Fernández et al., 2012a). The study found that ultrasound treatment could disrupt *Scendesmus* sp. cell structure, and lead to an increase in methane production.

Although this procedure is quite simple, the high-energy consumption associated with it makes the process not preferable for implementation on a commercial scale (Harun and Danquah, 2011b).

2.5.1.3 *Chemical pretreatment*

Chemical pretreatment is one of the most promising methods used to pretreat biomass. Generally, this process has been proved successful, particularly when combined with heat (Mahdy et al., 2014; Mendez et al., 2014). In the chemical pretreatment method, the chemical commonly applied in this process is either an acid or alkali reagent. Acid and alkali reagents are commonly used in the chemical industry and cause a very low toxicity to the environment. The main goal of chemical pretreatment is to solubilise polymers, favouring the availability of carbohydrate in microalgal cell for enzymatic saccharification. In this chemical treatment, the concentrated acid will disrupt the hydrogen bond in the microalgal cell wall (some microalgae contains complex cellulose and hemicelluloses in the cell wall) structure and release monomer sugar and soluble oligomer into the hydrolysate. While, for the alkali treatment, the alkali reagent will attack the ester bond in the microalgal cell wall structure and release the simple monomer sugar into the hydrolysate. The potential of chemical pretreatment on microalgal has been reported by previous studies. A study by (Choi, 2010) reported that approximately 58% of sugar was released from *Chlamydomonas reinhardtii* after being pretreated with 3% sulphuric acid at 110°C for 20 min. In another study on

bieothanol production from pretreated microalgal biomass, it was indicated that the highest bioethanol of 7.2 gL^{-1} was attained from chemical pretreatment of *Chlorococcum humicola* using 1% sulfuric acid at 140°C for 20 min (Harun and Danquah, 2011b). In addition, the chemical treatment using acid has also been applied to pretreat other types of microalgal biomass such as *Chlorella* sp. and *Dunalilla tertiolecta* (Laurens et al., 2015; Lee et al., 2013).

Unlike acid pretreatment, the alkaline pretreatment method is considered very environmentally friendly as this method uses low concentration of alkali (Park and Kim, 2012). Besides, the alkaline pretreatment method has also been reported to produce less inhibitors which can affect the fermentation process and is suitable for biomass with low lignin content (Ramirez, 2013).

Mahdy et al. (2014) in their study reported that *Chlorella* sp. and *Scendesmus* sp. biomasses pretreated with NaOH followed with thermal treatment produced 17% and 20% higher methane yield compared to an untreated sample. As per to date, there is little information on the utilisation of the alkaline pretreatment to produce other biofuel such as ethanol, butanol and hydrogen. Therefore, the potential of this pretreatment needs to be explored in order to ensure the pretreatment of microalgal biomass is performed in an environmentally friendly manner.

2.5.1.4 *Biological pretreatment*

Biological treatment is a pretreatment that involves microbes and enzymes to degrade the microalgal cell wall and release fermentable sugar from microalgal biomass. In this method, microorganisms such as brown-, white- and soft-rot fungi are used to degrade the microalgal cell wall. Hydrolytic enzyme produced by the bacteria or fungi will attack microalgal cell wall to a small compound with a low molecular weight, that subsequently can be used in anaerobic fermentation. Currently, research on the direct enzymatic saccharification of microalgal biomass is still scarce.

An investigation on the enzymatic cell walls of *Chlorella vulgaris* and *Nannochloropsis* sp. using lysozyme, chitinase, peptinase, sulfatase, B-gluronidase and

laminarinase enzyme, indicates that all the enzyme has a broad effect on the microalgal cell wall (Gerken et al., 2013). Their study also indicated that a combination of the enzymes displays a significant effect on microalgal cell wall hydrolysis.

Enzymatic pretreatment of untreated *Chlorella homosphaera* using cellulase produced from *Acremonium cellulolyticus* (complete pool of cellulases plus β-glucosidase), *Trichoderma reesei* (complete pool of cellulases with low β-glucosidase) and *Aspergillus awamori* (endoglucanases and β-glucosidase) has also been evaluated and it was found that the highest hydrolysis was displayed from enzymatic saccharification using cellulase from *A. awamori* (Rodrigues et al., 2015). The saccharification yield of each enzyme produced from different microbes was not similar. Based on this study, it can be concluded that cellulase produced from different sources has different cellulase activity.

A new enzymatic saccharification method called 'whole cell' enzymatic saccharification has been newly introduced. The potential of using ''whole cell'' cellulolytic pretreatment has also been reported by Muñoz et al. (2014). Their study on pretreatment of *Nannochloropsis nagitana* and *Botryococcus braunii* using five cellulolytic bacteria (*Aeromonas*, *Pseudomonas*, *Chryseobacterium*, and *Raoultella*) found that both microalgal biomass could be pretreated by endoglucanase enzyme secreted from the bacteria. The study also exhibited that the 'whole cell' pretreatment method can be used to pretreat microalgal biomass and enhance the biogas production.

This method appears to have a few advantages, for example, it requires low energy input and this process is mildly environmentally friendly. However, the large diversity of microalgal cell walls, enzyme production, and low hydrolysis rate are among the drawbacks that need to be considered before the method is applied in large-scale biofuel production. In spite of the many pretreatment methods tested, currently available pretreatment techniques can hardly meet the requirements of commercial application due to long processing times, chemical recycle problems, or high operational costs (Agbor, 2011; Galbe et al., 2007). Therefore, more work is required to understand and generate more information on the pretreatment of microalgal biomass.

2.6 Enzymatic saccharification

In order to obtain high sugar concentration, the pretreated microalgal biomass needs to be hydrolysed into simple sugar through the enzymatic saccharification process. The enzymatic saccharification process is one of the main processes involved in biofuel production through the biochemical conversion pathway. Cellulase is a typical enzyme used in the enzymatic saccharification of microalgal biomass (Ellis et al., 2012; Harun and Danquah, 2011a). However, few combinations of the enzymes such as amylase, kinase and pectinase to saccharify microalgal biomass were also reported (Gerken et al., 2013). In this process, the carbohydrate presence in microalgal biomass is converted into fermentable sugar and subsequently can be converted into ethanol, butanol, methane and hydrogen through the anaerobic fermentation process.

The enzymatic saccharification performing at optimum condition is important in extracting high fermentable sugar concentration from microalgal biomass. The enzymatic saccharification at the suboptimum condition produces low fermentable sugar, subsequently affecting the fermentation yield. Generally, the enzymatic saccharification can be influenced by two major factors that are specifically enzyme-related (temperature and pH value) and substrate-related factors (enzyme concentration and substrate concentration) (Leu and Zhu, 2013).

An enzyme has a temperature and pH range for maximum activity (Leghilmi et al., 2013; Sharma, 2012). The optimum temperature for cellulase enzyme is generally between 40°C to 55°C. The enzymatic saccharification beyond this temperature range produces less fermentable sugar. This is because temperature changes give more energy to break the intra-molecule attraction within the enzyme structure, resulting in the alteration of the enzyme active site making it unable to accommodate the substrate, which will reduce the enzyme activity (Baker et al., 1992).

For the case of pH, the optimum pH value for maximum enzyme activity is between 4 and 6. The changes of pH value will alter the enzyme structure and inhibit the enzyme reaction. Enzymatic saccharification that is too acidic or alkali would change the electrostatic interaction in the enzyme molecule (Leu and Zhu, 2013). For the enzymatic saccharification

in an acidic environment, any basic groups such as the nitrogen groups in the enzyme molecule would be protonated. On the other hand, for the enzymatic saccharification in too alkali environment, the acid groups would be deprotonated. Hence, this would result in the inactivation of the enzyme.

Enzymatic saccharification of the different types of microalgal biomass have been reported by previous researchers (Choi, 2010; Harun and Danquah, 2011a; Markou et al., 2013). Investigation on the enzymatic saccharification of *Chlamydomonas reinhardtii* at different temperatures and pH values indicates that the pH and temperature have a significant effect on the reducing sugar production (Choi et al., 2010). It was found that the optimum condition for the sacchrification of *C. reinhardtii* was using 0.2% glucoamylase, 55°C and pH 4.5. Saccharification of this microalgal biomass beyond 54°C and pH 4.5 produced low reducing sugar concentration. In the other study, the optimum enzymatic saccharification condition of *Chlorococcum humicola* showed that 64% saccharification yield was attained from saccharification using 10 gL^{-1} biomass at 40°C and initial pH of 4.8 (Harun and Danquah, 2011a). Besides, enzymatic saccharification of a lipid-extracted *Dunaliella tertiolecta* LB999 indicated that the optimum saccharification yield (81.7%) of carbohydrate content was obtained when the enzymatic saccharification was performed at at 55°C and pH 5.5 (Lee et al., 2013).

The other important enzymatic saccharification factors are enzyme and biomass concentration. Suitable enzyme and biomass concentration are important to produce high reducing sugar concentration. Normally, enzymatic saccharification at high biomass concentration would produce higher reducing sugar concentration. However, enzymatic saccharification at lower concentration, or beyond the optimum enzyme and biomass concentration, could cause substrate inhibition during the process. Enzyme inhibition can be distinguished into three modes; competitive, uncompetitive and mixed inhibition (Kristensen et al., 2009). A competitive inhibition is a condition when the inhibitor competes with the normal substrate. Thus, reducing the enzyme affinity to substrate. For an uncompetitive inhibition, it occurs when the inhibitor binds with enzyme-substrate complexes, resulting in the distortion of the enzyme active site. In contrast, a mixed inhibition is a process, which the mixed inhibitor binds to both substrate and enzyme, consequently interfering with the substrate-enzyme binding. Hence, it would inhibit the enzyme activity.

Theoretically, for the effect of biomass concentration, the enzymatic saccharification at high biomass concentration would produce high reducing sugar concentration. However, high biomass concentration would display low saccharification yield. This could be attributed to the high viscosity of the biomass slurry, which results in insufficient dispersion of biomass and less efficient enzyme activity in the vessel (Ioelovich and Morag, 2012). A study on enzymatic saccharification of *Chlorella vulgaris* PSP-E using different biomass concentration indicated that the saccharification yield decreased when biomass concentration increased from 20 to 40 gL^{-1} (Ho et al., 2013).

Overall, a summary of the enzymatic saccharification of different types of microalgal biomasses is shown in Table 2.8. Currently, most of the microalgal enzymatic saccharifications are performed using acid pretreated microalgal biomass. Thus, it is prudent to determine the enzymatic saccharification operation condition of microalgal biomass generated from different pretreatment processes. Moreover, enzymatic saccharification of microalgal biomass can be significantly different between microalgal species.

2.7 Butanol from microalgal biomass

Butanol production from microalgal biomass is believed to be one of the alternative transportation fuels of the future (Yilmaz et al., 2014). Butanol production from microalgal could be simpler because this biomass contains low lignin content. Besides, *Clostridium* sp., which is a biocatalyst used in the conversion of carbohydrate is saccharolytic. The saccharolytic bacteria is generally a bacteria that has the capability to break the glycosidic bond in a wide range of polysaccharide.

Currently, the information on butanol production from microalgal biomass is scarce. There are only a few studies on the production of butanol from carbohydrate-rich microalgal biomass. Efremenko et al. (2012) performed an ABE fermentation of seven microalgae species (*Arthrospira platensis*, *Nannochloropsis* sp, *Dunaliella tertiolecta*, *Galdieria partita*, *Chlorella vulgaris*, *Cosmarium* sp, *Nostoc* sp.) using immobilised *Clostridium* sp. and found that different microalgae species produced a different ABE concentration. The highest butanol yield was attained by fermentation of *Arthrospira platensis*, which contains 40.8% of carbohydrate in its cellular cell.

Table 2.8 Enzymatic saccharification of different microalgal biomass.

Feedstocks	Pretreatment	Solid loading (gL^{-1})	Enzymatic saccharification conditions	Yield	References
Microalgae	5.3% H_2SO_4 at 90°C for 30 min 9.4% NaOH at 90°C for 30 min	100	Acid slurry	[a]8.92 %	(Ellis et al., 2012)
Chlorella vulgaris FSP-E	1% H_2SO_4 at 120°C for 20 min	20	Enzyme mixture pH 6 at 45°C	[b]90%	(Ho et al., 2013)
Chlorococcum humicola	Ultrasonication	10	pH 4.8 at 40°C for 72 h	[a]68.2%	(Harun and Danquah, 2011a)
Spirulina platensis	Acid treatment	13	Acid slurry	nd	(Markou et al., 2013)
Chlamydomonas reinhardtii	Acid treatment 3% H_2SO_4 at 110°C for 30 min	50	pH 5.5 at 55°C	[a]58 %	(Choi, 2010)
Microalgae *Dunaliella tertiolecta*	1.2% HCl at 121°C for 15 min	50	pH 5.5 at 55°C for 12 h	[b]80.9	(Lee et al., 2013)

(a): based on the biomass concentration; (b): based on the sugar concentration in biomass

In another study, the ABE fermentation of microalgae cultivated in wastewater using *C. saccharoperbutylacetonicum* N1-4 produced the highest butanol when the fermentation was carried out using biomass that was pretreated using acid followed by enzymatic saccharification sample (Ellis et al., 2012). Their study also indicated that the addition of 1% sugar could enhance butanol production. Further, the ABE fermentation of *Chlorella* sp. that was treated using the acid treatment produced a total of 3.37 gL^{-1} of butanol from fermentation of 111 gL^{-1} microalgal biomass (Wang et al., 2013). The study also suggested that pretreatment using more than 2% sulphuric acid was preferable to pretreat this microalgae species.

2.8 Research gap and limitation of knowledge

The interest in butanol as a future transportation fuel has gain much attention due to its advantages over ethanol. To date, most butanol is produced from starchy materials, agro-waste, lignocellulosic biomass and petroleum-based (hydrocarbon) feedstock. However, a few drawbacks have been identified from the biomass feedstock that is currently being used. In order to make the process as economically viable and sustainable as possible, the use of a new type of biomass, which is non-food based, has low lignin content and is from sustainable feedstock, is an option to ensure the possibility of butanol production.

Microalgal biomass is considered a promising butanol feedstock due to this biomass having some advantages over the biomass mentioned. A lot of research has been carried out to determine the potential of microalgal biomass as an ethanol and biodiesel feedstock (Halim, 2013; Harun and Danquah, 2011a). However, there has been little study of ABE fermentation using microalgal biomass. The microalgal biomass that contains high carbohydrate content is considered to have the most potential to be used as a butanol feedstock through the fermentation process. Generally, the carbohydrate accumulation in microalgal biomass is species-dependent and can be influenced by the cultivation condition. Most of the studies have been carried out to determine the cultivation condition on lipid and microalgal biomass production (Bartley et al., 2014; Converti et al., 2009; Toledo-Cervantes et al., 2013). In their study, the cultivation parameters' conditions such as light intensity, temperature, pH, salinity and CO_2 concentration have been reported to significantly influence

lipid content and biomass production. However, less information is available on the effect of the cultivation condition on the carbohydrate content accumulation in microalgae biomass.

In addition, although the studies on ABE production using microalgal biomass are available, most of the process used acid as a reagent to pretreat the biomass prior to the enzymatic saccharification and fermentation process, which is not environmentally friendly. Acid pretreatment is a common process applied to pretreat microalgal biomass for the fermentation process. Although this process has been proven able to disrupt the microalgal biomass, it was reported that acid pretreatment produced less sugar and produced more inhibitors that can take affect during the fermentation process (Harun and Danquah, 2011a). Besides, the harsh chemical used during the pretreatment process could give a negative effect on the environment. Thus, exploring a new approach to pretreat the microalgal biomass is needed to ensure the process is performed in an environmentally sound manner.

In order to increase sugar production, the carbohydrate or polysaccharide in the microalgal biomass needs to be converted into simple sugar through the enzymatic saccharification prior to the fermentation. Since, the enzymatic saccharification of biomass is substrate dependent, a different microalgal biomass species requires different optimum enzymatic saccharification conditions to produce maximum sugar. To date, the information on enzymatic saccharification of *Chlorella* sp. and *Tetraselmis suecica* is scarce. Therefore, further study to improve enzymatic saccharification of these microalgal biomass is indispensable.

Apart from the pretreatment and enzymatic saccharification condition, the fermentation and type of substrate also has been reported to influence the ABE fermentation performance. The use of a suitable substrate is important in obtaining the maximum ABE concentration. To date, the information on ABE fermentation of alkaline treated and lipid extracted is limited. Therefore, this study was undertaken to explore and understand the ABE fermentation of carbohydrate rich microalgal biomass using dilute chemicals as a pretreatment method.

2.9 References

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CHAPTER 3

MICROALGAL GROWTH AND CARBOHYDRATE CONTENT

3.1 Introduction

Increasing energy consumption and depleting petroleum reserves have encouraged the scientific community to explore alternative energy sources for the future. Renewable energy, such as biofuel from biomass, is one of the promising sources to at least partially replace fossil fuels. Biofuel production from biomasses, such as lignocellulose, starchy material, and microalgae, are promising feedstocks because the process is environment friendly and sustainable. Among biomasses currently being used, microalgal biomass is considered one of the potential future biofuel feedstocks to partly replace fossil fuels.

The biofuel produced from microalgal biomass has advantages over other biomass sources mentioned because microalgae has a higher growth rate than lignocellulosic material, has the capability to capture CO_2 produced from power plants, does not require arable land for biomass production, and biomass produced can be used for biofuel production. Biofuels, such as biodiesel, biohydrogen and biogas can be produced from microalgal biomass (Brennan and Owende, 2010). On the other hand, carbohydrates present in microalgal biomass can also be used to produce carbohydrate-based liquid fuels, such as bioethanol and biobutanol (Chen et al., 2013).

Microalgal carbohydrates are mainly derived from starch in chloroplasts and cellulose or polysaccharide on the microalgal cell wall. The carbohydrate content in microalgae is dependent on the microalgae species and cultivation conditions (Markou et al., 2012). Cultivation parameters, such as temperature, light intensity, salinity, nutrient content and carbon dioxide concentration, have been reported to influence the carbohydrate accumulation in microalgal biomass during the cultivation period (de Castro Araújo and Garcia, 2005). Cultivation under suboptimal conditions could either increase or decrease the carbohydrate content in microalgal cells (Chen et al., 2013).

For photoautotrophic microalgae, the effect of light intensity and temperature demonstrate an important principle for microalgal growth and chemical composition within cells (Carvalho et al., 2009). Light is an energy source for microalgae to initiate photosynthesis and to produce cell biomass. Exposure to high or low light intensity will result in phototoxicity, consequently reducing the microalgal growth rate (Takahashi and Murata,

2008). A similar effect has been observed by cultivation at a suboptimal temperature. Cultivation at a high temperature could affect enzyme metabolism kinetics and disturb cellular components involved in the photosynthetic system (Davison, 1991).

Variation in the salinity and pH of the cultivation medium could also influence microalgal growth and chemical composition. The adaptability of microalgae to varying salinity concentrations is different between microalgae species and can be classified as halophilic or halotolerant (Rao et al., 2007). Cultivation at a suboptimal salinity level provided an osmotic stress between the extracellular and intracellular space in a microalgal cell (Kirst, 1989). The osmotic stress in a microalgal cell inhibited non-cyclic electron transport, subsequently reducing photosynthetic activity (Affenzeller et al., 2009). On the other hand, cultivation at a high salinity level has been reported to produce a low concentration of microalgal biomass. This is due to the disruption of the photosynthesis apparatus located in photosystem II (PS II) by osmotic stress (Murata et al., 2007). Another important factor that could affect microalgal growth is the initial pH of the cultivation medium (Bartley et al., 2014). Changes to the pH of the cultivation medium can occur during photosynthesis and respiration of microalgae. During the daytime, photosynthesis and utilisation of CO_2 increases the pH of the medium. However, respiration activity during the night time will reduce the pH (Bartley et al., 2014). Any changes in the pH could change the chemical composition in the microalgal cell and reduce the microalgal growth rate, resulting in cell death (Khatoon et al., 2014). There is a relationship between pH and CO_2 concentration in the microalgal cultivation system. In many microalgal cultivation systems, additional CO_2 in the system will reduce the pH. Thus, the main objective of this study was to establish the suitable cultivation condition that could produce high microalgal biomass with high carbohydrate content. The effect of cultivation condition such as light intensity, temperature, pH, salinity level and carbon dioxide (CO_2) on *Chlorella* sp. and *Tetraselmis suecica* biomass production and its carbohydrate content was investigated. The suitable cultivation condition could be beneficial, especially in being able to reduce cultivation time and production cost.

3.2 Materials and Methods

3.2.1 *Microalgae and medium cultivation*

Two different microalgae species, namely fresh water microalgae *Chlorella* sp. and marine water microalgae *Tetraselmis suecica* were used in this study. Modified algae growth medium (MLA medium) with 0.49 gL^{-1} magnesium sulphate ($MgSO_4.7H_2O$), 1.7 gL^{-1} sodium nitrate ($NaNO_3$), 0.14 gL^{-1} di-potassium phosphate (K_2HPO_4) 0.03 gL^{-1} calcium chloride ($CaCl_2.2H_2O$) was used as the seed culture and biomass production medium. The medium was initially sterilised using a 0.22 μm Millipore filter. Microalgae seed cultivation was conducted in a 1 L Scott bottle containing 700 mL of modified MLA. The bottle was incubated in an illumination incubator chamber with 0.3 $Lmin^{-1}$ of compressed air or carbon dioxide (CO_2). Both microalgae cultures were cultivated under the same conditions and were harvested at late log growth. Microalgae cells from the late log phase were harvested and centrifuged at 4500 rpm for 15 min. The pellet was rinsed twice with distilled water and dried at 70°C for 24 h. The dried biomass obtained was used for further study.

3.2.2 *Preparation of microalgal inoculums*

A stock microalgal culture was cultivated in a modified Scott Bottle with 800 mL working volume at 30.0 ± 0.2°C and 3000 lux. After 10 days of cultivation, the microalgae were harvested and centrifuged at 3000 rpm for 10 min. The supernatant was removed and the microalgal pellet was washed 3 times using distilled water to produce the microalgal suspension with optical density 680 nm (OD_{680}) of 1.0. This was used for further experiments.

3.2.3 *Determination of cultivation parameters*

Study on the effect of the cultivation condition on microalgal growth and carbohydrate accumulation was carried out using a one-variable at a time design (OVAT). In this method, one factor was optimised by changing one factor at a time and keeping the other variables constant. This method is one of the simplest to be implemented, and entailed selecting the significant parameters affecting the microalgal growth and carbohydrate accumulation.

Cultivation conditions such as light intensity, temperature, pH, salinity level and CO_2 concentration were evaluated in this study. A total of 10% (v/v) standard microalgal inoculum (OD_{680}=1.0) was inoculated in each cultivation medium and the experiment was carried out in duplicate. The growth of microalgae was monitored at every 24 h interval and was harvested after 10 days of cultivation. The harvested microalgae was processed as explained in the previous section.

3.2.3.1 *Effect of light intensity*

The influence of light intensity on microalgal growth and carbohydrate accumulation was conducted at 0, 1000, 2000 and 3000 lux. The cultivation was carried out in MLA medium with initial pH of 7 at 20°C for 10 days of cultivation. The growth of microalgae at different light intensity was monitored and the biomass was collected for carbohydrate content analysis. The light intensity that promoted the highest microalgal growth was used for subsequent investigation.

3.2.3.2 *Effect of temperature*

A study on the effect of the cultivation temperature was investigated at four different temperatures; 20, 25, 30 and 40°C. The microalgae was cultivated in MLA medium having initial pH of 7 at a fixed light intensity after 10 days of incubation. The microalgal biomass and carbohydrate content was analysed as described in the previous section. The temperature that promoted highest microalgal growth and carbohydrate content was used for subsequent analysis.

3.2.3.3 *Effect of initial pH*

The effect of the initial pH medium on microalgal growth and carbohydrate content was investigated at pH 4, 7, 8, 10. The initial MLA medium was adjusted to the desired pH value using either 1M sodium hydroxide (NaOH) or hydrochloride acid (HCl). The microalgae was cultivated at a light intensity and temperature determined from previous study over 10 days. The pH value that promotes the highest microalgal growth was used for further investigation.

3.2.3.4 *Effect of salinity*

The influence of salinity on microalgal growth and carbohydrate content was investigated using five salinity levels; 0, 10, 20, 30 and 40 gL^{-1} of NaCl. The microalgae was cultivated at the light intensity, temperature and pH obtained from the previous investigation. The microalgal growth was monitored every 24 hours and the cell was harvested after 10 days of cultivation. The biomass obtained was subjected to carbohydrate content analysis, and the salinity level that promotes the highest microalgal growth and carbohydrate content was selected for subsequent investigation.

3.2.3.5 *Effect of CO_2 concentration*

The study on the effect of CO_2 concentration on microalgal growth and carbohydrate content was investigated using 5 and 15% (v/v) CO_2. In this study, microalgae cultivated using air was used as control. The experiment was carried out in duplicate and was cultivated at light intensity, temperature, initial pH value and salinity level determined from previous investigation. Microalgal cells were harvested after 10 days of cultivation, and the biomass obtained was subjected to carbohydrate content analysis.

3.2.4 *Microalgal growth kinetic parameters*

The microalgal density was determined by measuring the optical density (OD) at 680 nm. The relationship between microalgal density and OD_{680} is as shown in the following equation;

$$Chlorella \text{ sp.} = 0.549(\text{OD}_{680}) - 0.0046 \quad (3.1)$$

$$T.suecica = 0.524(\text{OD}_{680}) - 0.0129 \quad (3.2)$$

The specific growth rate (μ, d^{-1}) is calculated as:

$$\mu = \frac{l_n X_t - l_n X_0}{t - t_0} \quad (3.3)$$

The biomass productivity (P, $g^{-3}d^{-1}$) is defined as:

$$P = \frac{X_t - X_0}{t - t_0} \quad (3.4)$$

where X_0 is the initial biomass concentration (gm^{-3}) at time to (d) and X_t is the biomass concentration at any time. P_{max} ($gm^{-3}d^{-1}$) and μ_{max} (d^{-1}) refer to the maximum values obtained for each experiment. Carbohydrate productivity ($mgL^{-1}d^{-1}$) was determined by;

$$P_{carbohydrate} = \frac{XY_{carbohydrate}}{\Delta t} \quad (3.5)$$

where X is the biomass concentration (mgL^{-1}) and $Y_{carb/biomass}$ is the carbohydrate content per microalgal biomass.

3.2.5 *Chemical composition*

The lipid, carbohydrate and protein content of the microalgae biomass were determined using soxhlet extraction and the compositions determined by a gravimetric method, phenol-sulphuric acid method and the Lowry method as explained by Kassim et al. (2014).

3.2.6 *Statistical analysis*

All the samples were prepared in triplicate. A t-test was carried out to determine the significant difference between the control and the experimental parameters. The statistical analysis was performed using Minitab 14.3 software.

3.3 Results and discussion

3.3.1 *Microalgae species*

Two microalgae, *Chlorella* sp. and *Tetraselmis suecica*, were used in this study. Figure 3.1 shows the morphology of both microalgae species. *Chlorella* sp. is a single-cell freshwater green microalgae, spherical in shape. By contrast, *T. suecica* is a motile marine green microalgae and generally has an oval cell shape.

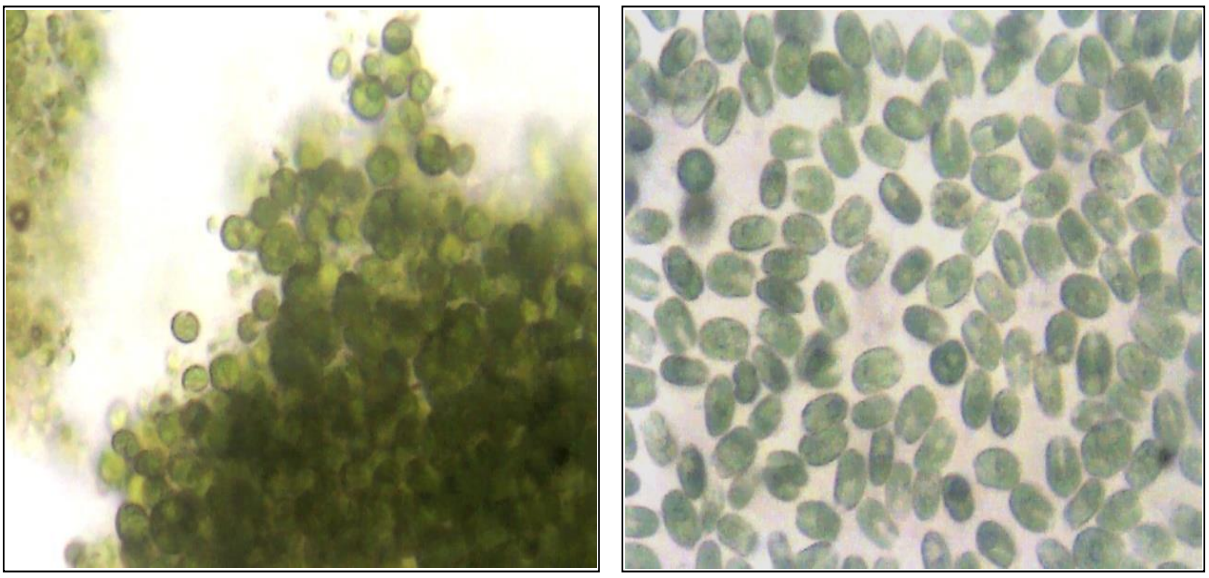

Figure 3.1 Microalgae species under the light microscope - (a) *Chlorella* sp., (b) *T. suecica*.

3.3.2 *Effect of cultivation conditions on microalgal growth and carbohydrate accumulation*

Microalgal biomass production and carbohydrate content are among the characteristics for sustainability in liquid fuel production. Carbohydrate content is important as a carbon platform for sugar production prior to the fermentation process to produce liquid fuels such as ethanol and butanol.

3.3.2.1 *Effect of light intensity on growth and carbohydrate content*

Figure 3.2 shows the growth profile and carbohydrate content of *Chlorella* sp. and *T. suecica* cultivated at four different light intensities: 0, 1000, 2000, and 3000 lux. The profiles indicated that growth of both microalgae was significantly influenced by light intensity (Figure 3.2a and 3.2b). The maximum microalgal biomass production for *Chlorella* sp. obtained by cultivation at 2000 and 3000 lux were 0.353 ± 0.015 and 0.315 ± 0.049 gL^{-1}, respectively. This result indicated that the growth potential of *Chlorella* sp. at a light intensity range between 2000 to 3000 lux is essentially the same. However, the maximum biomass production for *T. suecica* was obtained when cultivation was carried out at 3000 lux, at 0.508 ± 0.07 gL^{-1}. Increasing the light intensity increased the biomass concentration. As expected,

slow growth was displayed by both microalgae species during cultivation without a light source.

In addition to affecting microalgal biomass production, light intensity has also been reported to have a significant effect on the chemical composition of microalgal cells (Ho et al., 2013). The maximum value of carbohydrate content for *Chlorella* sp. was attained at 1000 and 2000 lux with carbohydrate content values of 30.56 ± 0.39% and 29.60 ± 0.17%, respectively. Meanwhile, the maximum carbohydrate content for *T. suecica* was attained at 3000 lux with a carbohydrate content of 37.67 ± 0.62%. The highest carbohydrate productivity for *Chlorella* sp. and *T. suecica* was obtained at 2000 and 3000 lux with values of 10.42 ± 0.00 and 19.12 ± 0.01 $mgL^{-1}d^{-1}$ respectively (Table 3.1).

A suitable light intensity is crucial to ensure a high growth rate and to produce a high biomass concentration. Based on the growth profile obtained, our results indicated that the sensitivity of both microalgae tested towards light intensity is different. It can be seen the effect of light intensity on microalgae growth are species-dependent. These findings are in agreement with other studies reported on the various types of microalgae species (Amini Khoeyi et al., 2012; Dechatiwongse et al., 2014). For instance, a study by Ho et al. (2012) reported that the specific growth rate and biomass production of *Scendesmus obliqus* increased up to 75% as light intensity increased from 50 to 3000 lux.

Light intensity generally has a significant effect on the growth and photosynthetic rate of algae (Bouterfas et al., 2002). It was reported that light intensity influences the photoadaptation and photoinhibition processes in microalgal cells. Cultivation at a suitable light saturation intensity could lead to an increase in biomass productivity, growth rate and biochemical composition (Ho et al., 2013). However, exposure to a higher or lower light saturation intensity caused photoinhibition (Murata et al., 2007). The photoinhibition in microalgal cells at a high light intensity occurred due to the disruption of chloroplasts and lamellae as well as inactivation of enzymes involved in nutrient uptake during photosynthesis (Neidhardt et al., 1998). Low biomass production at a low light intensity is due to an insufficient energy supply that fails to trigger photosynthetic activity. On the other hand, cultivation at a low light intensity will lead to the damage of protein D1 that is associated

with PS II. Consequently, this led to the reduction of photosynthetic activities in microalgal cells (Keren et al., 1997).

Light intensity not only influences microalgae growth, however, it also could alter the chemical composition of, for example, carbohydrate and lipid in microalgal cells. During photosynthesis, carbohydrate is produced as a final product that can be used as an energy source during respiration. As can be seen in Figure 3.2c, the carbohydrate content in microalgae cultivated at different light intensities was different. There was a significant increase of carbohydrate content as the light intensity increase up to 3000 lux. The maximum carbohydrate content for *T. suecica* of 37.67% of dried biomass was obtained from cultivation at 3000 lux. In contrast, there was no significant difference on carbohydrate content in *Chlorella* sp. cultivated at 1000 (31%), 2000 (32%) and 3000 lux (30% of dried biomass) respectively. This finding is consistent with other studies on the effect of light intensity on carbohydrate content in microalgal biomass.

Previous study on the effect of light intensity on *Pavlova lutheri* indicated that increasing light intensity from 2000 up to 2800 lux could slightly increase 66% of carbohydrate content (Carvalho et al., 2009). Further, a significant carbohydrate content (80%) in microalgae was increased when the light intensity was increased from 2000 to 3000 lux (Fernandes et al., 2010). Another study demonstrated that an increase of light intensity from 1200 to 3500 lux could increase 55% of carbohydrate content in *Scenedesmus obliqus* (Ho et al., 2012). A further increase of light intensity beyond 3500 lux appeared to reduce carbohydrate content in *S.obliqus*.

Shifting the light intensity from a low to high light intensity significantly increased the carbohydrate content. Generally, the carbohydrate accumulation in microalgae during photosynthesis is greatly stimulated by three major enzymes; dihydroxyacetone, phosphate, sucrose and starch synthase. Cultivation in non-optimum conditions could influence microalgal metabolism. Cultivation at unfavourable conditions could alter the carbon and nitrogen flow within the microalgal cell (Pal et al., 2011). Cultivation of microalgae at this condition will trigger carbon metabolism pathways to produce carbohydrates as energy storage and maintain growth at that condition.

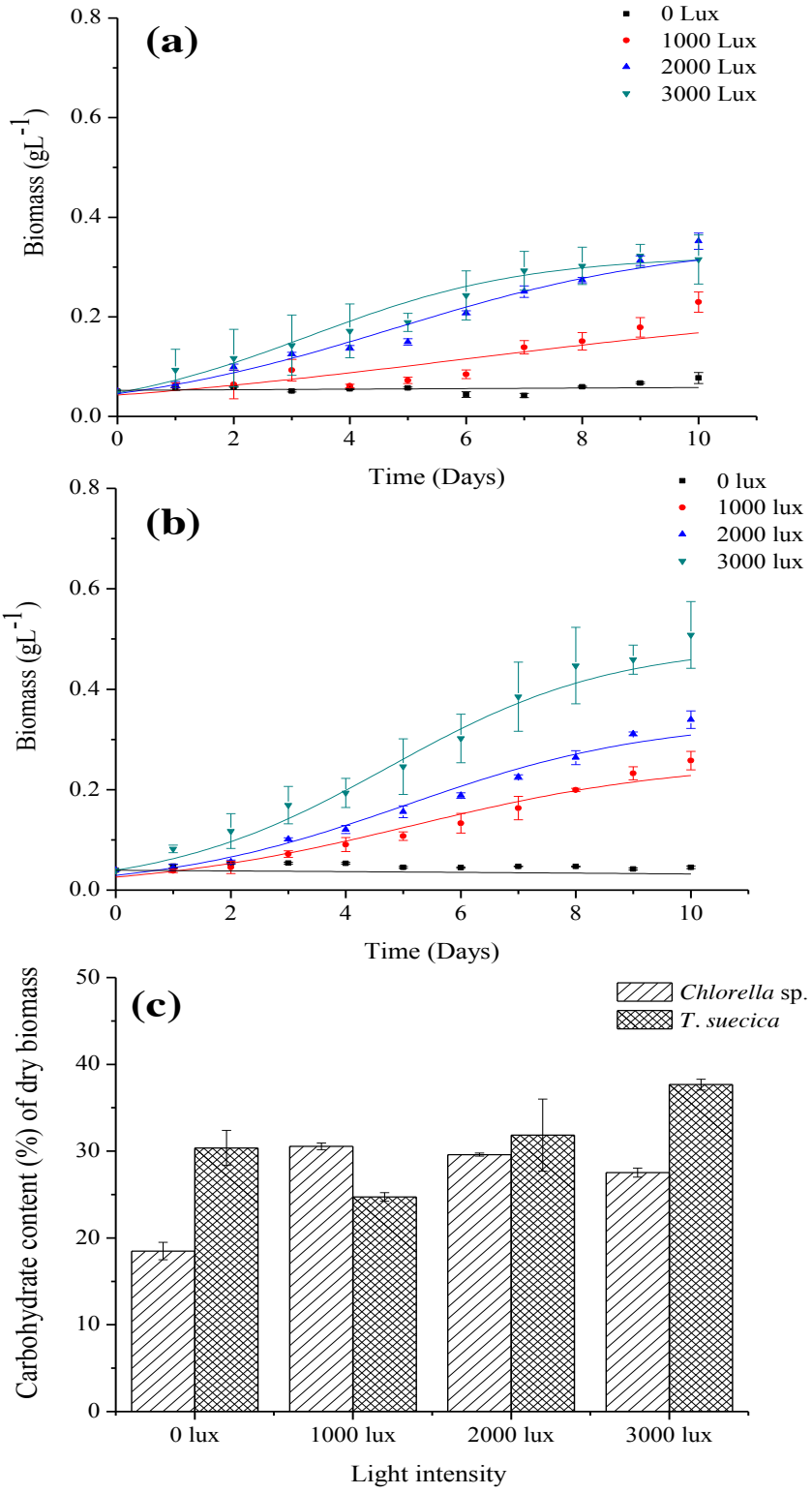

Figure 3.2 Growth profile and carbohydrate content (% of dried biomass) of microalgae at different light intensities - (a) Growth profile of *Chlorella* sp., (b) Growth profile of *T. suecica*, (c) Carbohydrate content for *Chlorella* sp. and *T. suecica*.

Table 3.1 Specific growth rate, carbohydrate content, and carbohydrate productivity at different light intensities

Species	Parameters	Light intensity (Lux)			
		0	1000	2000	3000
Chlorella sp.	Specific growth rate (μ d^{-1})	0.04 ± 0.02	0.15 ± 0.01	0.20 ± 0.01	0.18 ± 0.02
	Carbohydrate content (%)	18.49 ± 1.01	30.56±0.39	31.84 ± 4.16	37.67 ± 0.62
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	1.43±0.00	7.02±0.003	10.42 ± 0.00	8.67 ± 0.01
T. suecica	Specific growth rate (μ d^{-1})	0.01 ± 0.01	0.187 ± 0.01	0.22 ± 0.01	0.26 ± 0.01
	Carbohydrate content (%)	30.37 ± 2.03	24.74 ± 0.50	31.84 ± 4.16	37.67 ± 0.62
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	1.38 ± 0.00	6.38 ± 0.00	10.79 ± 0.00	19.15 ± 0.01

3.3.2.2 *Effect of temperature on growth and carbohydrate content*

Another factor that has a significant effect on microalgal growth and chemical composition is temperature. Figure 3.3 shows the microalgal growth profile and carbohydrate content at four different cultivation temperatures. The maximum biomass production for *Chlorella* sp. and *T. suecica* was attained at 30°C with 0.45 ± 0.113 and 0.54 ± 0.013 gL^{-1}, respectively. Cultivation at 20°C and 25°C displayed a lower biomass production and growth rate compared with cultivation at 30°C. Low biomass production was also observed when both microalgae were cultivated at 40°C. Growth of *Chlorella* sp. became inhibited after three days of cultivation. However, growth of *T. suecica* at 40°C started to decline after two days. This result clearly showed that growth of both microalgae species was inhibited at 40°C. These data also indicated that both species displayed different temperature sensitivity levels, in which *Chlorella* sp. was more temperature tolerant at 40°C compared to *T. suecica*. Overall, these findings clearly indicated that both *Chlorella* sp. and *T. suecica* could not grow well at a high temperature of 40°C.

The influence of temperature on various microalgal growth has been reported elsewhere. Various microalgae have been reported to have different optimum microalgal growth temperatures and may be species-specific (LÜRling et al., 2013). For instance, the optimal temperatures for the specific growth rates of *Nannochloropsis oculata* and *Nitzschia laevis* were 20°C and 23°C respectively (Converti et al., 2009). On the other hand, studies on

the effect of temperature on *Scendesmus* sp. indicated that different microalgal strains showed different optimal cultivation temperatures. It was reported that *Scendesmus* sp. showed the highest growth rate at temperatures between 27°C to 39°C (Westerhoff et al., 2010). The optimal cultivation temperature range for other *Chlorella* species was also reported between the range of 25°C to 35°C (Choi et al., 2012).

Cultivation at the appropriate temperature plays a significant role in microalgal growth, nutrient uptake, enzyme kinetic and oxygen release that are related to PS II activity. However, cultivation below or above the optimal temperature will reduce microalgal growth. Low biomass production and growth rate at 20°C observed in this study could be attributed to the slow cellular nutrient uptake and alteration of photosynthetic activity in the microalgal cell (Ras et al., 2013). Low *Chlorella* sp. and *T. suecica* biomass production observed in this study could be attributed to the cultivation of microalgae at a high temperature that could damage the reaction centre of photosynthesis (D1 protein), subsequently reducing specific microalgal growth (Salleh et al., 2010) .

Apart from microalgal growth, temperature has also been reported to significantly affect the biochemical composition, especially lipid and carbohydrate content in microalgal cells (Berges et al., 2002). Figure 3.3c shows the carbohydrate content in microalgal cells at different cultivation temperatures after 10 days of cultivation. The maximum carbohydrate content for *Chlorella* sp. of 31.86 ± 1.36% of dried biomass was attained by cultivation at 30°C. Carbohydrate content for *Chlorella* sp. increased with increasing cultivation temperature. However, cultivation beyond 30°C showed a slight decrease of carbohydrate content. In contrast, the maximum carbohydrate content for *T. suecica* of 32.15 ± 0.13% of dried biomass was attained from the cultivation at 40°C. Even though the high carbohydrate content for *T. suecica* was obtained at 40°C, low biomass production at this temperature contributed to the low carbohydrate productivity (Table 3.2). Based on this result, the cultivation at 30°C was the most suitable temperature to obtain a high biomass containing high carbohydrate content. This result also indicated that temperature over 30°C is the limiting factor for both microalgal growth and carbohydrate accumulation.

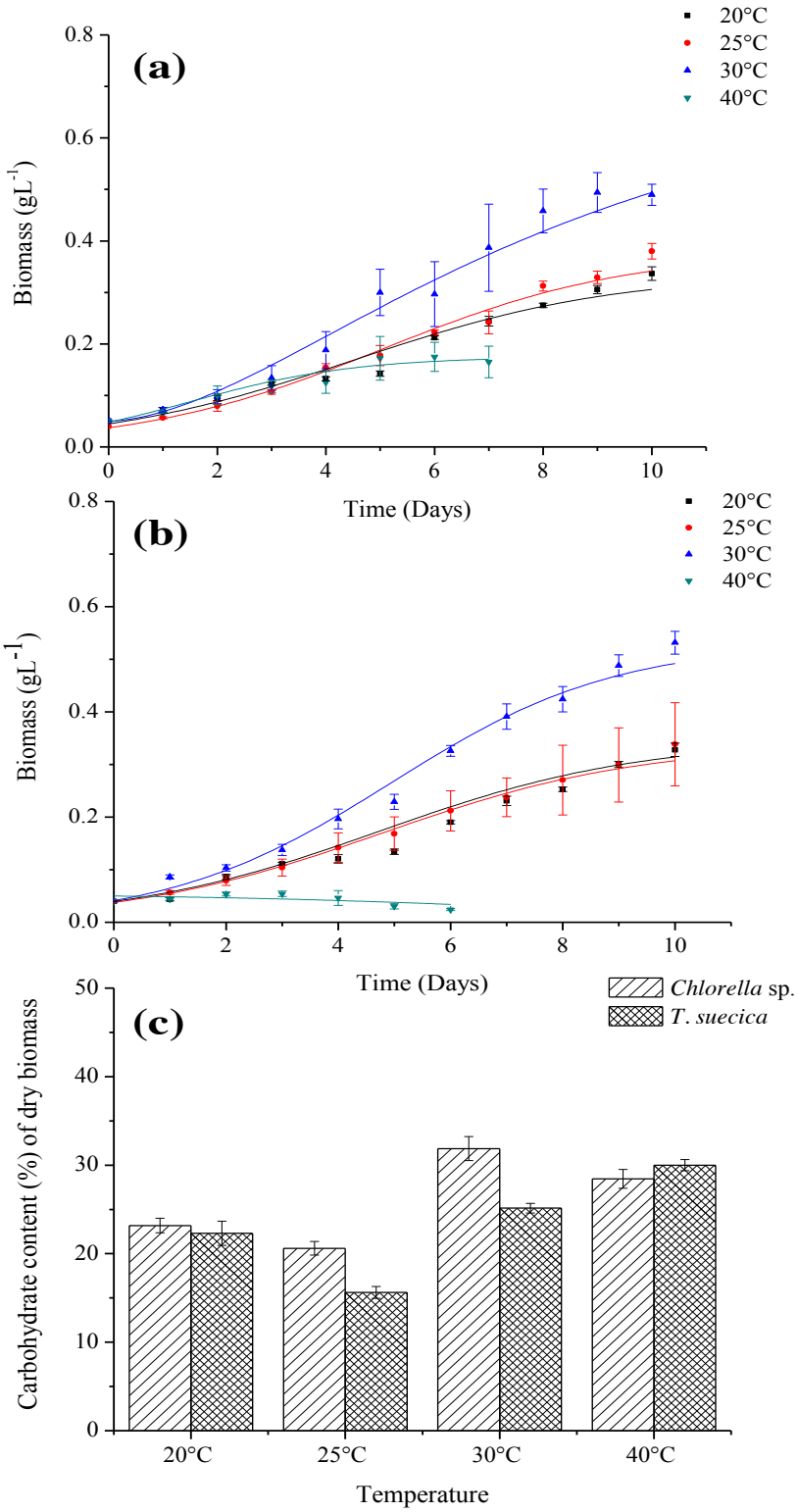

Figure 3.3 Growth profile and carbohydrate content (% of dried biomass) of microalgae at different cultivation temperatures - (a) Growth profile of *Chlorella* sp., (b) Growth profile of *T. suecica*, (c) Carbohydrate content for *Chlorella* sp. and *T. suecica*.

Table 3.2 Specific growth rate, carbohydrate content, and carbohydrate productivity at different cultivation temperatures

Species	Parameters	Temperature (°C)			
		20	25	30	40
Chlorella sp.	Specific growth rate (μ d^{-1})	0.19±0.02	0.20±0.02	0.23±0.02	0.16±0.03
	Carbohydrate content (%)	23.17±0.82	20.61±0.77	31.86±1.36	28.46±1.06
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	8.28±0.01	7.28±0.07	14.37±0.01	4.43±0.04
T. suecica	Specific growth rate (μ d^{-1})	0.21±0.03	0.22±0.02	0.26±0.04	0.07±0.04
	Carbohydrate content (%)	22.31±1.37	15.62±0.67	25.14±0.55	
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	7.19±0.01	4.82±0.07	13.59±0.02	000±0.07

3.3.2.3 *Effect of pH on growth and carbohydrate content*

The initial pH is another factor that can influence microalgal metabolism and biochemical composition. pH is important to maintain microalgal growth, especially during cultivation using CO_2 as a carbon source. The CO_2 supply in the cultivation medium will reduce the pH. Further, a suitable pH is also important to avoid contamination by unwanted organisms such as bacteria, fungi, and predators (Rodolfi et al., 2009). Contamination by these unwanted organisms will provide competition during cultivation. The pH of the cultivation medium will also affect nutrient uptake and enzyme kinetics involved in microalgae metabolism (Liu et al., 2007).

An experiment on the effect of the initial pH on microalgal growth and carbohydrate content was performed and the result is shown in Figure 3.4. The maximum biomass production for *Chlorella* sp. and *T. suecica* obtained by cultivation at an initial pH of 7 were 0.45 ± 0.113 and 0.54 ± 0.013 gL^{-1} respectively. Cultivation of *Chlorella* sp. beyond pH of 4 or 8 displayed low biomass production, while the biomass production for *T. suecica* cultivated at these two pH values was almost equivalent. Our results indicated that there was no significant difference for *T. suecica* growth cultivated at the initial pH range from 4 to 8. This clearly indicated that *T. suecica* had a relatively wide range of tolerance to pH, indicating that this species is very promising for applications of outdoor cultivation using CO_2 as a carbon source. Low biomass production for both microalgae species could be observed at a pH of 10.

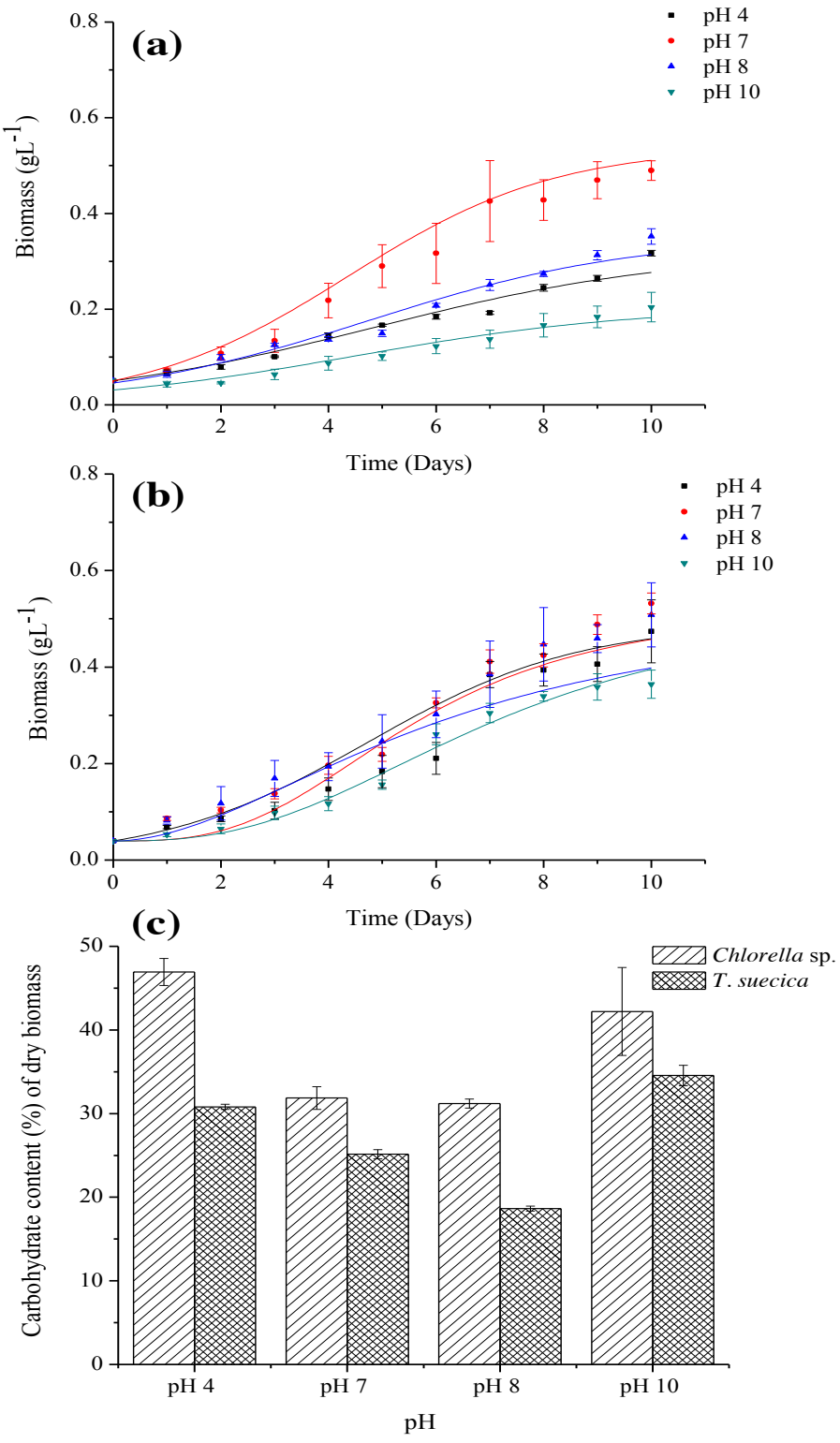

Figure 3.4 Growth profile and carbohydrate content (% of dried biomass) of microalgae at different initial pH values - (a) Growth profile of *Chlorella* sp., (b) Growth profile of *T. suecica*, (c) Carbohydrate content for *Chlorella* sp. and *T. suecica*.

The pH variation not only affected the microalgal growth rate, but also had a major effect on the carbohydrate content in the microalgal cell (Khalil et al., 2010). Figure 3.4c shows the carbohydrate composition of microalgae cultivated at different pH values. The maximum carbohydrate content for *Chlorella* sp. and *T. suecica* was obtained by cultivation at pH of 4 and 10, respectively. This clearly indicated that variation of the cultivation pH medium would change the chemical composition in the microalgal cell. Even though the maximum carbohydrate content was observed at both pH 4 and 10, the highest carbohydrate productivity for both microalgae was observed at pH 7. The carbohydrate productivity for both microalgae was almost similar to cultivation with an initial pH of 4 or 7 (Table 3.3).

Generally, the pH tolerance of microalgae is species-specific. A study by Guobin et al. (2011) reported that the highest growth rate for *Chlorella protothecoides* was observed in a culture medium with an initial pH of 5 . However, a different observation was reported on the effect of pH on *C. ellipsoidea*, where biomass production increased with an increase of pH to alkaline conditions (Khalil et al., 2010). Mayo et al. (1997), in their study on the effect of pH on *Chlorella vulgaris*, indicated that the highest growth rate for this species was observed in a culture medium with a pH of 6.31 . The low growth rate for *Chlorella* sp. at pH 4 and 10 can be explained by an alteration of nutrient uptake and a reduction in photosynthesis. A similar finding was observed for the marine algae *Thakassiosira pseudonana* and *Thalassiosira oceanica*, in which the microalgal growth rate, nutrient uptake, and photosynthetic activity were reduced when these microalgae species were cultivated at a high pH of 8.5 (Chenl and Durbin, 1994).

Varying the pH of the culture medium during cultivation also could affect metabolic processes in the microalgal cell (Kosourov et al., 2003). Cultivation at lower or higher than optimal pH will provide an imbalance between the external and internal pH, thus requiring an energy expenditure to pump protons out of the cell (Lnae and Burris, 1981). Cultivation under alkaline conditions limited the availability of carbon from CO_2, which resulted in the inhibition of microalgal growth (Liu et al., 2007).

Generally, the effect of pH on biochemical composition, especially carbohydrate and lipid, is different among microalgae species. As can be seen in Figure 3.4(c), cultivation at different pH values displayed different carbohydrate content for both microalgae species. The carbohydrate content analysis indicated that shifting the pH toward alkaline conditions significantly reduced the carbohydrate content in both microalgae species. This result also indicated that a high carbohydrate content for both microalgae could be attained by cultivation at below pH 7 and beyond pH 8. This finding indicates that cultivation at a suboptimal pH value will lead the microalgae to produce lipids and carbohydrates for energy storage. This finding is in agreement with the study on the effect of pH on carbohydrate content in *Nannochloropsis* sp. and *Tetraselmis* sp. which indicated that the maximum carbohydrate content for *Nannochloropsis* sp. and *Tetraselmis* sp. was obtained at pH 5.5 and 8.5, respectively (Khatoon et al., 2014). Also, another study on the effect of pH value on carbohydrate content in *C. ellipsoidea* indicated that the maximum carbohydrate content for this species was attained at pH 7.5 (Khalil et al., 2010). Based on this study, it clearly indicates that the effect of the pH value on the carbohydrate content in microalgae cells is species dependent. This phenomenon could be explained by the pH value influencing certain enzymes involved in the microalgal metabolic pathway and having different optimum pH, implying that changes of pH could affect the microalgal metabolism.

Table 3.3 Specific growth rate, carbohydrate content, and carbohydrate productivity at different initial pH values.

Species	Parameters	pH			
		4	7	8	10
Chlorella sp.	Specific growth rate (μ d^{-1})	0.18±0.02	0.233±0.24	0.20±0.05	0.14±0.015
	Carbohydrate content (%)	46.95±1.61	31.86±1.36	31.20±0.53	42.20±5.26
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	14.89 ±0.05	14.37±0.01	10.99±0.02	8.62±0.03
T. suecica	Specific growth rate (μ d^{-1})	0.24±0.01	0.26±0.02	0.26±0.01	0.22±0.08
	Carbohydrate content (%)	30.81±0.32	25.14±0.55	18.64±0.30	34.55±1.24
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	13.81±0.04	19.59±0.02	9.47±0.07	12.59±0.03

3.3.2.4 *Effect of salinity on growth and carbohydrate content*

Figure 3.5 shows the growth profile and carbohydrate content of microalgae cultivated in different salinity concentrations. The maximum biomass production for *Chlorella* sp. of 0.567 gL^{-1} was obtained by cultivation without NaCl. Further increases of NaCl concentration correlated with reduced microalgal biomass production. Low *Chlorella* sp. growth was displayed by cultivation in a medium with a higher NaCl concentration. In contrast, the maximum microalgal biomass for *T*. *suecica* was attained for cultivation in a medium containing 30 gL^{-1} NaCl. Cultivation of *T*. *suecica* in a medium with either lower or higher than 30 gL^{-1} NaCl showed a significant decline in the microalgal biomass production and growth rate (Table 3.5). The growth of *T*. *suecica* declined sharply by cultivation with 40 gL^{-1} NaCl. *Chlorella* sp. were very sensitive to high levels of salinity, while the *T*. *suecica* strain used in this study showed a high tolerance to high salinity levels. This result suggests that a low salinity level was appropriate to promote *Chlorella* sp. growth. By contrast, *T*. *suecica* required a high salinity level to promote its growth.

The effect of salinity on microalgal growth in *D*. *tertiolecta*, *Botrycoccus braunii*, *Chaetoceros* cf. *Wighamii*, and *Chattonella marina* was reported in previous studies (Chen and Jiang, 2009; Rao et al., 2007). However, the salinity tolerance capability for *Chlorella* sp. and *T*. *suecica* is not discussed in detail. The microalgal salinity tolerance can be influenced by several factors. Firstly, it contributed to the type of microalgae species. Generally, the capability of microalgae to adapt and survive under various salinity concentrations is species-specific (Kirst, 1989). A previous study on *B*. *braunii* indicated that the maximum biomass production was attained when the microalgae was cultivated at the lowest salinity level (Rao et al., 2007). A study on the salinity tolerance of *Scendesmus* sp. and *B*. *braunii* was reported elsewhere. The freshwater microalgae *S*. *obliquss* and S. *almenensis* had maximum biomass production at 0.05 and 0.1 M NaCl respectively (Kaewkannetra et al., 2012; Sánchez et al., 2008). Another study on the effect of salinity on three microalgae species, *Isochrysis* sp. *Nannochlorpsis oculata,* and *Nitzschai flustulum*, indicated that there was no significant effect of salinity on the growth of these microalgal species (Renaud and Parry, 1994). The study clearly indicated that these three microalgae species have a wide salinity tolerance.

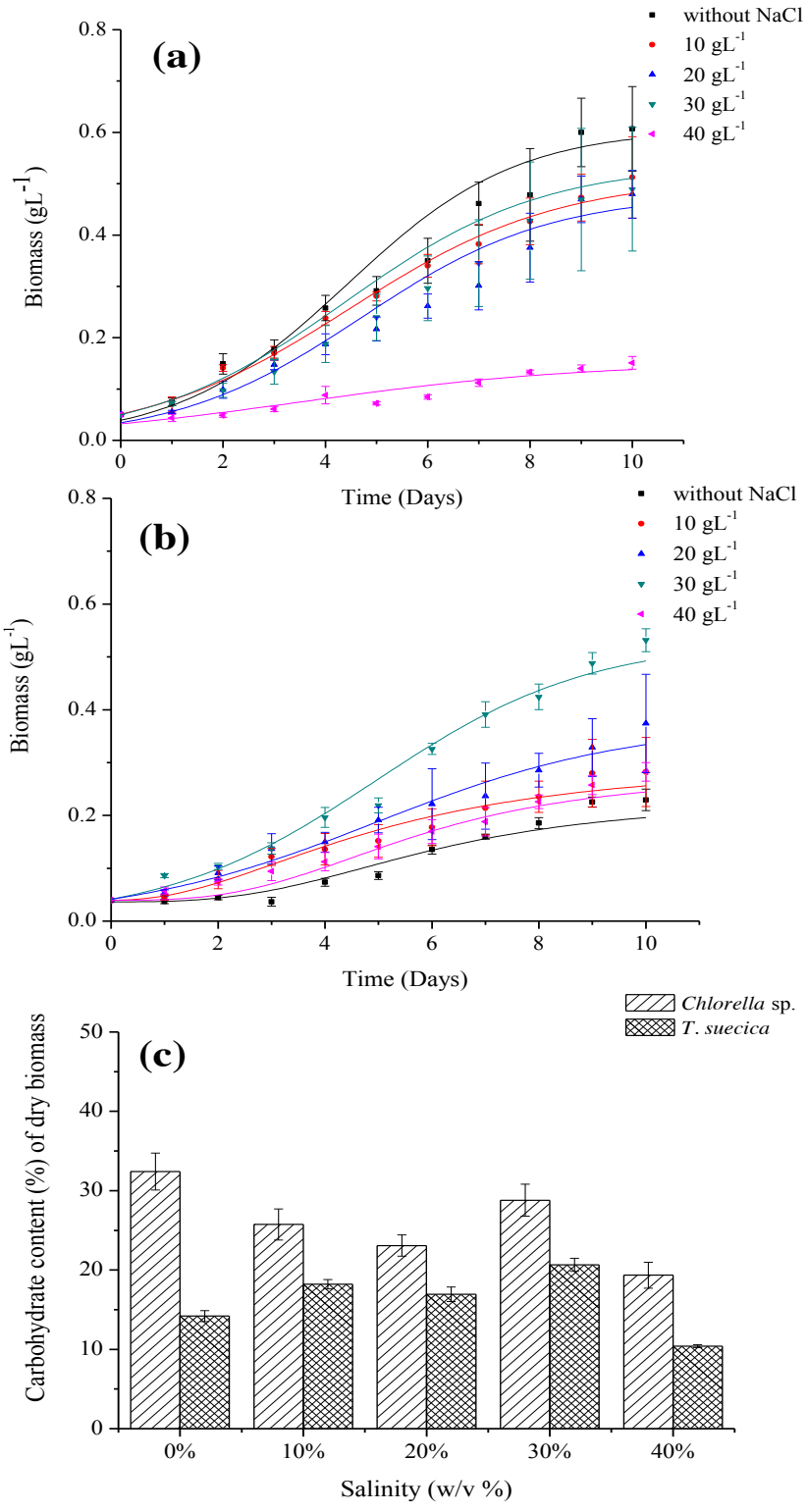

Figure 3.5 Growth profile and carbohydrate content (% of dried biomass) of microalgae at different NaCl concentrations - (a) Growth profile of *Chlorella* sp., (b) Growth profile of *T. suecica*, (c) Carbohydrate content for *Chlorella* sp. and *T. suecica*.

Secondly, the microalgal salinity tolerance contributes to the natural habitat of the microalgae. Low microalgal biomass production by freshwater microalgae (*Chlorella* sp. in this study) at a high salinity level is attributed to microalgae characteristics for which this species is incapable to adapt in a high salinity environment. This result agrees with a previous study by Gorain et al. (2013), who reported that the growth of the freshwater microalgae *S. obliqus* and *C. vulgaris* declined with increasing of NaCl concentrations. Another study on *B. baumanii* isolated from freshwater was reported to have a low biomass production at high salinity levels.

Table 3.4 shows the specific growth rates, carbohydrate content, and carbohydrate productivity for both microalgae at different salinity levels. The maximum carbohydrate content and productivity for *Chlorella* sp. of 32.41 ± 2.33% and 18.4 ± 0.003 $mgL^{-1}d^{-1}$ was attained when the microalgae was cultivated in a medium without NaCl. In contrast, the maximum carbohydrate content and productivity for *T. suecica* were attained at cultivation in a medium containing 30 gL^{-1} NaCl with 20.66 ± 1.83% and 6.37 ± 0.011 $mgL^{-1}d^{-1}$, respectively. A further increase of salinity level showed a negative trend on carbohydrate content in *Chlorella* sp. However, cultivation of *T. suecica* in a medium with less than 30 gL^{-1} NaCl produced a low carbohydrate content (Figure 3.5c). Typically, microalgae will respond to osmotic stress by producing extracellular metabolites such as glycerol as well as carbohydrate to protect the cell from salt injury and to balance the surrounding environment (Rao et al., 2007). Increased salinity level in a cultivation medium has been reported to enhance intracellular carbohydrate production in *Chlamydomonas reinhardtii* (Siaut et al., 2011). According to Khatoon et al. (2014), the maximum carbohydrate content for *Nannochloropsis* sp. and *T. suecica* was obtained by cultivation in a medium containing 30 gL^{-1} of NaCl. However, a different observation was reported for *Dunaliella* sp., where the maximum carbohydrate content was attained at a low salinity level (Chen and Jiang, 2009). This result clearly indicated that the effect of salinity on the carbohydrate content metabolism is also species-specific and depends on the cultivation conditions.

Table 3.4 Specific growth rate, carbohydrate content, and carbohydrate productivity at different NaCl concentrations.

Species	Parameters	Salinity (NaCl gL^{-1})				
		0	10	20	30	40
Chlorella sp.	Specific growth rate (μ d^{-1})	0.25±0.01	0.24±0.01	0.23±0.01	0.22±0.02	0.11±0.09
	Carbohydrate content (%)	32.41±2.33	25.74±1.96	23.08±1.34	28.80±2.01	19.35±1.63
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	18.40±0.03	13.85±0.07	11.56±0.02	12.98±0.01	2.83±0.01
T. suecica	Specific growth rate (μ d^{-1})	0.19±0.06	0.20±0.02	0.21±0.03	0.22±0.02	0.19±0.01
	Carbohydrate content (%)	14.19±0.70	18.21±1.58	16.94±1.90	20.66±1.83	10.404±0.18
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	3.52±0.02	5.49±0.06	5.55±0.09	6.37±0.06	2.87±0.002

3.3.2.5 *Effect of CO_2 concentration of growth and carbohydrate content*

Figure 3.6 shows the growth profile of *Chlorella* sp. and *T. suecica* in media supplied with different concentrations of CO_2. In this study, three different CO_2 concentrations were supplied intermittently in the cultivation medium for 10 days. Biomass produced by *Chlorella* sp. was almost similar for cultivation with air, 5%, and 15% (v/v) CO_2 with maximum biomass production values of 0.606, 0.644, and 0.613 gL^{-1} respectively. In contrast, a different growth profile was displayed by *T. suecica* cultivated in different CO_2 concentrations. The maximum biomass production for *T. suecica* was observed by cultivation using 15% (v/v) CO_2 with a biomass concentration of 0.715 gL^{-1}. The study also indicated that the growth of *T. suecica* at 5% and 15% (v/v) CO_2 were slightly similar. This result clearly indicated that both microalgae species have the capability to grow in a medium supplied with CO_2. However, *T. suecica* has a high tolerance for high CO_2 concentrations compared with *Chlorella* sp., thus it can be a good candidate to be used to fix CO_2 and at the same time produce biomass for biofuel production.

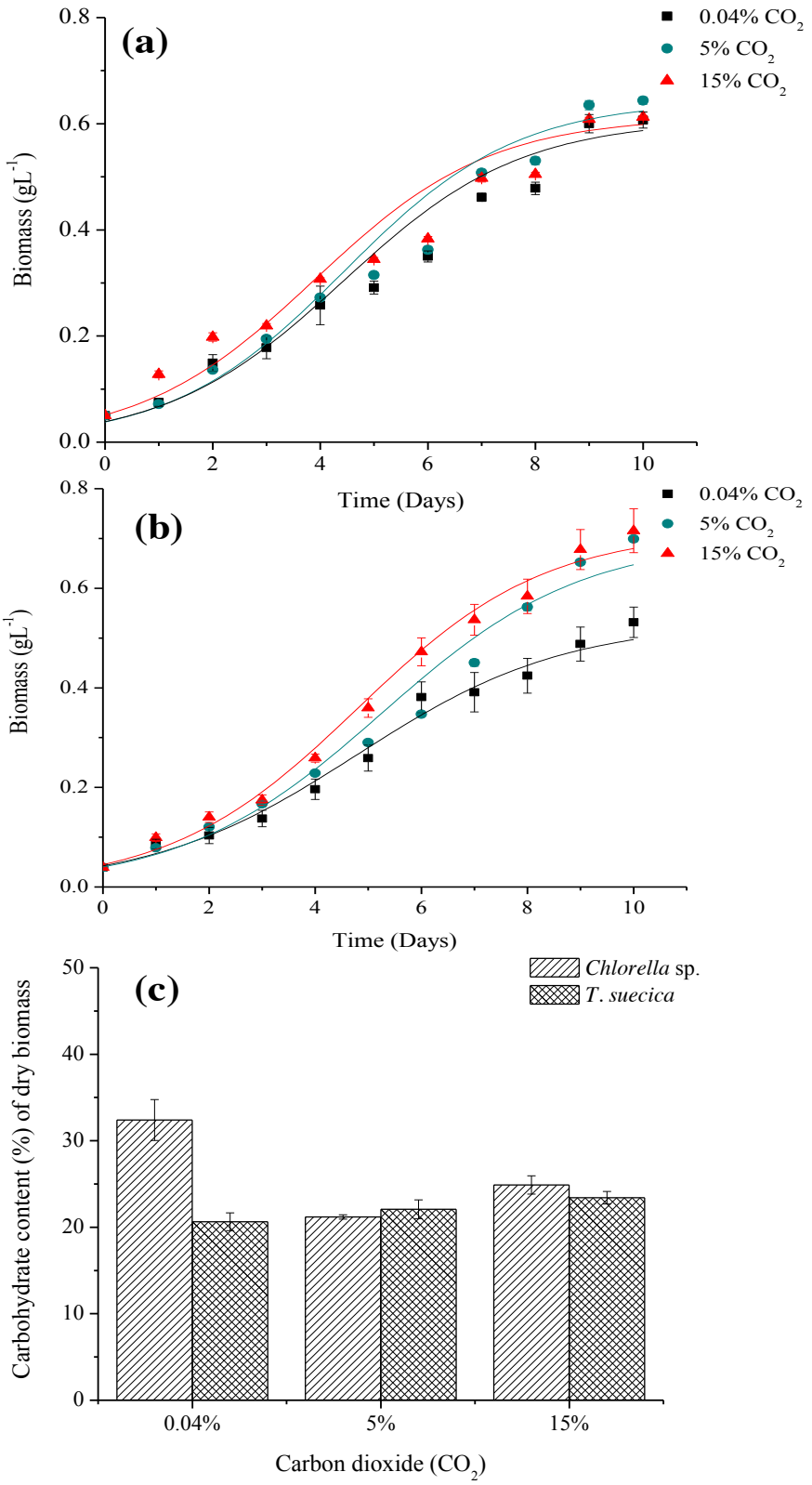

Figure 3.6 Growth profile and carbohydrate content (% of dried biomass) of microalgae at different CO_2 concentrations - (a) Growth profile of *Chlorella* sp., (b) Growth profile of *T. suecica*, (c) Carbohydrate content for *Chlorella* sp. and *T. suecica*.

Generally, microalgae utilise CO_2 for photosynthesis and production of biomass. The capability of microalgae to tolerate CO_2 concentrations can be grouped into CO_2-sensitive (<2–5% CO_2) or CO_2-tolerant (5–20% CO_2) microalgae. It is known that the capability of microalgae to fix CO_2 is species-specific (Miyachi et al., 2003). Based on this study, it is indicated that both microalgae species could be categorised as CO2-tolerant microalgae. The capability of microalgae to grow in a high CO_2 concentration is attributed to the CO_2 concentrating mechanism (CCM) in microalgal cells. The two main factors that contribute to CCM are an inorganic carbon transporter that assists in dissolved inorganic carbon (DIC) membrane transfer to CO_2 or bicarbonate into plasmalemma and chloroplast envelope and carbonic anhydrases (CAs), which are involved in stimulating the direct supply of CO_2 from outside cells to Rubisco. Based on the CCM model in *Chlamydomonas reinhardtii*, it was reported that the affinity of Rubisco for CO_2 is insufficient at an atmospheric CO_2 level. Cultivation at a high CO_2 level will activate CCM activity and increase their affinity for CO_2 uptake, in turn affecting the growth rate. Based on our results, it can be concluded that both microalgae have different tolerance levels for the CO_2 concentration.

Apart from influencing microalgae growth performance, the CO_2 concentration was also reported to affect the chemical composition, especially lipid and carbohydrate content in the microalgae cell. Figure. 3.6c shows the carbohydrate content (% of dried biomass) of *Chlorella* sp. and *T. suecica* cultivated at different CO_2 concentrations after 10 days of cultivation. The results showed that the carbohydrate content in *Chlorella* sp. was significantly influenced by the CO_2 concentration introduced during cultivation. Cultivation of *Chlorella* sp. in a medium supplied with CO_2 higher than 0.04% (v/v) reduced the carbohydrate content in the microalgae cell. The carbohydrate content in *Chlorella* sp. cultivated at 0.004%, 5% and 15% (v/v) CO_2 was 32%, 21% and 24% of dried biomass respectively. However, a different effect of CO_2 was observed on carbohydrate content in *T. suecica* biomass. It was found that the maximum carbohydrate content in *T. suecica* (23.4% of dried biomass) was obtained from the cultivation in a medium with 15% (v/v) CO_2. Even though the maximum carbohydrate content for *T. suecica* was obtained from cultivation at 15% (v/v) CO_2, statistical analysis indicated that there was no significant difference in carbohydrate content obtained from cultivation using 0.004% and 5% (v/v) CO_2. This indicated that different CO_2 concentrations supplied during the cultivation did not influence carbohydrate content in *T. suecica* cell.

Generally, the effect of the CO_2 concentration on carbohydrate content in microalgae cell is species dependent, indicating that different microalgae species displayed different metabolic metabolisms on the CO_2 concentration. The results obtained from this study are in agreement with other studies on the effect of CO_2 concentration towards carbohydrate content in different microalgae species. For instance, cultivation of *Chlamydomonas reinhardtii* at a low CO_2 condition (0.04%) displayed a lower starch content compared with cultivation at 5% (v/v) CO_2 (Gardner et al., 2013). In another study, cultivation of *D. salina* in a medium with 5% (v/v) CO_2 slightly increased carbohydrate and protein content (Giordano and Bowes, 1997).

Overall, this study indicates that the presence of CO_2 in the cultivation medium not only influences the microalgal growth, it also affects the carbohydrate accumulation in the microalgal cell specifically for *Chlorella* sp. Therefore, it can be concluded that suitable addition of CO_2 is another key approach to enhance microalgal biomass production with high carbohydrate content.

Table 3.5 Specific growth rate, carbohydrate content, and carbohydrate productivity at different CO_2 concentrations.

Species	Parameters	CO_2 (%) (v/v)		
		0.004	5	15
Chlorella sp.	Specific growth rate (μ d^{-1})	0.249±0.03	0.26±0.05	0.25±0.02
	Carbohydrate content (%)	32.40±2.33	21.19±0.23	24.88±1.05
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	19.64±0.03	13.65±0.03	15.25±0.06
T. suecica	Specific growth rate (μ d^{-1})	0.26±0.029	0.29±0.02	0.29±0.01
	Carbohydrate content (%)	20.63±1.83	22.07±4.09	23.41±0.72
	Carbohydrate productivity ($mgL^{-1}d^{-1}$)	10.96±0.06	15.43±0.01	16.75±0.03

3.4 Conclusions

The study on the effect of the cultivation condition on *Chlorella* sp. and *T suecica* was performed and the suitable cultivation condition for the maximum biomass production with high carbohydrate content was established.

This study demonstrates that cultivation conditions such as light intensity, temperature, and initial pH significantly affect microalgal growth and carbohydrate accumulation during the cultivation period. The suitable cultivation condition for both microalgae species were light intensity of 3000 lux at 30°C with initial pH of 7. Further, *Chlorella* sp. and *T*. *suecica* growth and carbohydrate content could also be affected by the presence of NaCl and CO_2 concentration in the cultivation medium. It was found that the presence of NaCl in the cultivation medium could inhibit *Chlorella* sp. growth. The study also indicated that both microalgae species had different tolerance to cultivation conditions. *T*. *suecica* was found to show high tolerance on elevated CO_2 concentration (15% (v/v) CO_2), indicating that this species has a potential to be applied in a CO_2 fixation process.

The high carbohydrate content in both *Chlorella* sp. (32% wt) and *T*. *suecica* (23% wt) biomass also shows a promising feedstock to be used for production of carbohydrate-based biofuel are chemicals such as butanol, ethanol, acetic acid, and butyric acid production through the fermentation process.

Focus on next chapter

This chapter investigated the microalgal cultivation condition and carbohydrate accumulation in different cultivation conditions. The study on the effect of cultivation conditions such as light intensity, temperature, pH, salinity level and CO_2 concentration provide information on *Chlorella* sp. and *T*. *suecica* characteristics and important information for further applications, especially for outdoor cultivation.

Further experiments on outdoor cultivation were performed using natural light and the effect of ambient conditions on microalgal growth and chemical composition were evaluated. These results will be discussed in the next chapter.

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CHAPTER 4

OUTDOOR CULTIVATION OF *CHLORELLA* SP. AND *T. SUECICA* IN CENTRIC PHOTOBIOREACTOR

4.1 Introduction

Biofuel production from biomass has gained wide attention as an alternative fuel to partially replace fossil fuels. Lignocellulosic biomass, such as terrestrial plant, agriculture crops, and algae, are currently being used for producing biofuel (Chakraborty, 2012). Algal biomass is believed to have great potential as a future biofuel feedstock. This is because algae has a higher growth rate compared with lignocellulosic biomass, and has the capability to capture CO_2 as a carbon source from industrial gas mixtures (Pittman et al., 2011).

Mass cultivation of microalgae is required to obtain high biomass. This is mainly carried out using two different methods – an open pond system and a closed system (Borowitzka, 1999). Cultivation using an open pond system is a practice to produce microalgal biomass for food and pharmaceutical products (Harun et al., 2010). However, there are a few drawbacks, such as low biomass productivity and contamination with other microorganisms that make this system not favourable for biofuel production (Mata et al., 2010). Currently, a few types of closed systems such as bubble, centric tube and bag photobioreactor have been introduced for microalgal cultivation (Chen et al., 2011). Cultivation of microalgae using a closed system is reported to show more advantages compared to an open pond system. This is due to the fact that a closed-system is easy to manage, has less contamination and shows high microalgal biomass productivity (Ugwu et al., 2008).

Production of microalgal biomass under control conditions is important to ensure the standard quality of the final product. Cultivation under fully automated mixing, with proper nutrient supply with controlled pH and temperature can keep the system in the optimum condition and maintain the biomass productivity and quality of the algae. However, the use of artificial light to provide the energy for biomass production is considered to be not sustainable. Therefore, cultivation using natural light at an ambient temperature should be used on a large scale to overcome this issue and reduce the biomass production cost.

Even though the outdoor cultivation strategy is the only approach to produce mass microalgal biomass, a few limitations have been reported that influence the microalgal growth during the cultivation period. Several factors, including: (1) abiotic factors such as

light intensity, temperature, nutrients, oxygen (O_2) and carbon dioxide (CO_2) concentrations; (2) contamination with other microorganism and competition by other algae; and (3) operational factors such as shear produced by mixing, cell density, and dilution cycle have been reported to affect the microalgal biomass production (Moheimani and Borowitzka, 2007; Raeesossadati et al., 2014). Of the factors mentioned, the effect of ambient temperature and irradiance are the most significant factors that influence microalgal biomass productivity (Feng et al., 2011). For instance, the available sunlight is variable through the day and seasons and this will affect the microalgal biomass productivity. Similar for the temperature, the fluctuation of temperature during the day could influence the microalgal metabolism. As the outdoor light and temperature condition vary, the net specific rate will be different, resulting in high variation in the biomass quality (chemical composition) and productivity.

Apart of the light intensity and temperature, the presence of CO_2 was also reported to influence the microalgal growth. The outdoor cultivation using CO_2 especially generated from the pilot plant in an integrated system could ensure the microalgal biomass is carried out in a sustainable manner. However, the synergistic effect of light intensity, temperature and CO_2 reported could affect the chemical compositions in microalgal biomass (Wahidin et al., 2013).

Thus, the main objective of this study is to determine the potential of microalgal biomass production through outdoor cultivation using a centric photobioreator. The effect of CO_2 concentration on the *Chlorella* sp. and *T. suecica* growth and chemical composition produced from cultivation using natural sunlight was also investigated.

4.2 Materials and Methods

4.2.1 *Microalgal culture and medium preparation*

Two different microalgae species, specifically fresh water microalgae *Chlorella* sp. and marine water microalgae *Tetraselmis suecica*, were used in this study. Modified algae growth (MLA) medium was used as the seed culture and biomass production medium as described in Kassim et al. (2014).

A standardised 10% (v/v) initial microalgal cell concentration of 0.03 - 0.05 gL^{-1} (OD_{680}= 1.0) was added into the medium and incubated in an illuminated incubator with 0.3 $Lmin^{-1}$ of compressed air under light with a photon intensity of 150 μmol $m^{-2}s^{-1}$. The cultivation temperature was 30 ± 0.5°C.

4.2.2 *Indoor cultivation*

Indoor cultivation of microalgae was carried out using three different CO_2 concentrations; 0.04, 5, and 15% (v/v). The microalgae was cultivated in an illuminated incubator chamber under light with a photon intensity of 150 μmol/m^2s. The cultivation temperature was set at 30 ± 0.5 °C for 10 days. The microalgal growth was monitored every 24 h and the microalgae was harvested and processed after 10 days for chemical composition analysis.

4.2.3 *Outdoor cultivation*

The outdoor cultivation was carried out in a 10 L centric tubular photobioreactor (Figure 4.1) having three transparent tubes acrylic polymer secured with an iron frame. The photobioreactor was situated at Monash University, Clayton, Melbourne (37.54E, 145.07S) and faced the northwest direction. The cultivation was carried out at two different times in two different temperature ranges; low autumn-spring (10-20 °C) ambient temperature, and summer (20-32 °C) ambient temperature. The study on the effect of CO_2 on the growth of *Chlorella* sp. and *T. suecica* was carried out using 15% (v/v) CO_2 supplied intermittently for 1 h each day over a 10-day period (Figure 2.2). The microalgae cultivated using ambient air was used as a control. Temperature and irradiance data were obtained from the Australian Bureau of Meteorology.

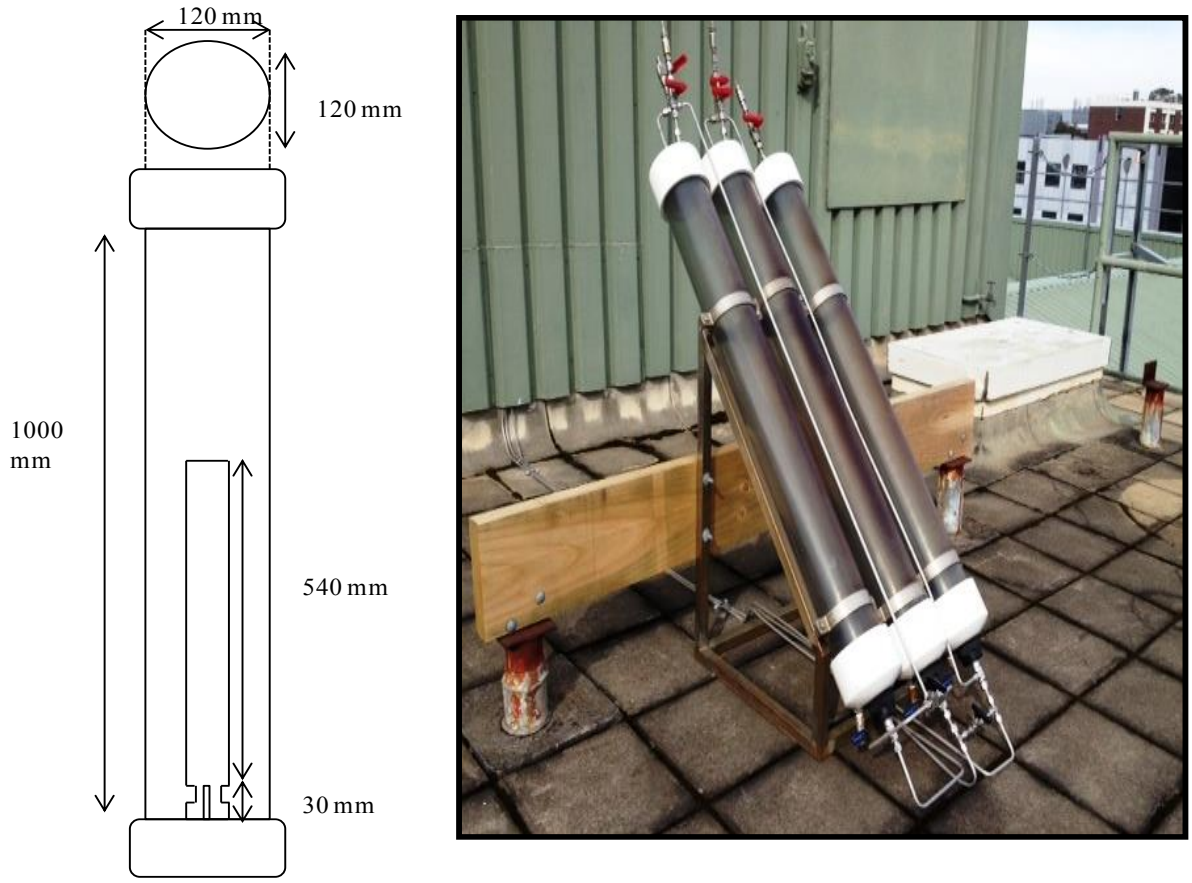

Figure 4.1 Centric tubular photobioreator for outdoor cultivation.

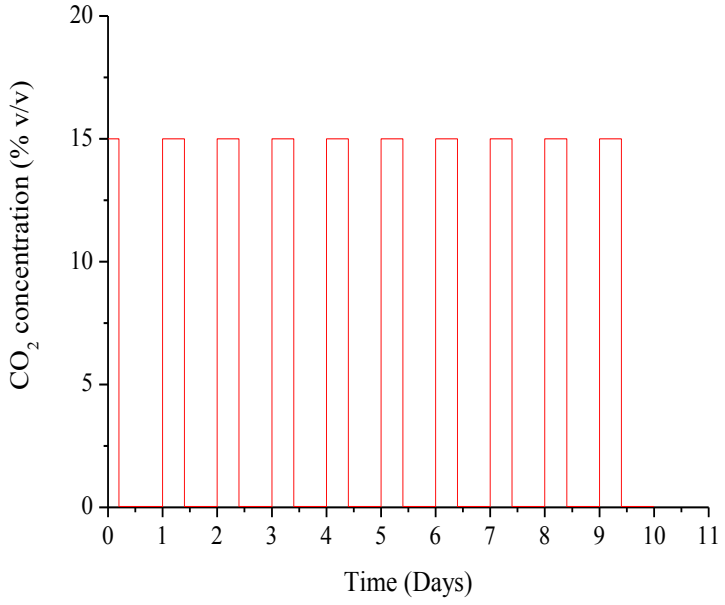

Figure 4.2 CO_2 supply profile for indoor and outdoor cultivation.

4.2.4 *Microalgal growth kinetic parameters*

The microalgal cell concentration was determined by measuring the optical density at 680 nm (denoted as OD_{680}) with a DR 5000™ UV/VIS spectrophotometer (HACH Company, US). The relationship with the microalgal cell concentration was determined by correlating the absorbance at 680 nm and dry cell weight (DCW). The microalgal DCW was calculated using the following in the following equation:

$$DCW_{Chlorella\ sp.} = 0.549(OD_{680}) - 0.0046 \quad (4.1)$$

$$DCW_{T.suecica} = 0.524(OD_{680}) - 0.0129 \quad (4.2)$$

These calibration curves were estimated by filtering 50-mL aliquots of the culture through a cellulose acetate membrane filter (0.45 um pore size, Millipore). Each loaded membrane filter was subsequently dried in an oven at 60 °C until a constant weight was achieved.

The specific growth rate (μ, d^{-1}) is calculated as:

$$\mu = \frac{l_n X_t - l_n X_0}{t - t_0} \quad (4.3)$$

The biomass productivity (P, $g^{-3}d^{-1}$) is defined as;

$$P = \frac{X_t - X_0}{t - t_0} \quad (4.4)$$

where X_0 is the initial biomass concentration (gL^{-1}) at time (day) t_0 (d) and X_t is the biomass concentration at any time. P_{max} ($gL^{-1}d^{-1}$) and μ_{max} (d^{-1}) are referred to as the maximum values obtained for each experiment.

4.2.5 *Chemical composition analysis*

The microalgae was harvested after 10 days of cultivation and was centrifuged at 4500 rpm for 15 min. The pellet generated from centrifugation was rinsed twice with distilled water and subsequently dried at 70°C for 24 h prior to further analysis. The lipid, carbohydrate, and protein contents of the lipid-extracted microalgal biomass were determined using soxhlet extraction followed by gravimetric, phenol-sulfuric acid (Nielsen, 2010), and the Lowry method (González López et al., 2010) respectively.

4.4.6 *Statistical analysis*

The samples for the chemical composition analysis were analysed in triplicates. A T-test was used to determine the significant difference between the control and the experimental parameters. The statistical analysis was performed using OriginPro software.

4.3 Results and discussion

4.3.1 *Indoor cultivation using CO_2*

The capability of microalgae to survive in CO_2 is species dependent. Some microalgae, for instance *Chlorococcum littorale* and *Chlorella vulgaris*, show good growth in high CO_2 concentration up to 40%, whereas, some microalgae such as *Chlorella* sp. WT and *Spirulina platensis* show a slow growth in high-level CO_2 (Chiu et al., 2011; Murakami and Ikenouchi, 1997; Zeng et al., 2013). In order to determine the capability of *Chlorella* sp. and *T. suecica* to grow in elevated CO_2 concentration, an indoor laboratory study was carried out using three CO_2 (0.04% (air), 5% and 15% v/v) concentrations for cultivation. For each concentration, the CO_2 was supplied intermittently and the cultivation medium incubated at 30°C for 10 days. Figure 4.3 shows the growth profiles of *Chlorella* sp. and *T. suecica* in mediums supplied with different CO_2 concentrations. Both microalgae showed a good growth in the medium supplied with CO_2. For *Chlorella* sp., the CO_2 concentration did not appear to significantly affect the biomass production and the growth rate (Figure 4.3a). The biomass concentration obtained from different concentrations of CO_2 at 0.04%, 5% and 15% (v/v) CO_2 were similar, 0.61, 0.64 and 0.61 gL^{-1} respectively.

Conversely, different growth profiles were displayed by *T. suecica* when the culture was cultivated in a medium with different CO_2 concentrations (Figure 4.3b). Within the experimental limit, CO_2 concentration was found to enhance the biomass production and growth rate. The highest biomass concentration for *T. suecica* of 0.72 g^{-1} was obtained from cultivation using 15% (v/v) CO_2. Cultivation of *T. suecica* in a medium using 0.04% (v/v) CO_2 (air) was found to produce the lowest biomass concentration.

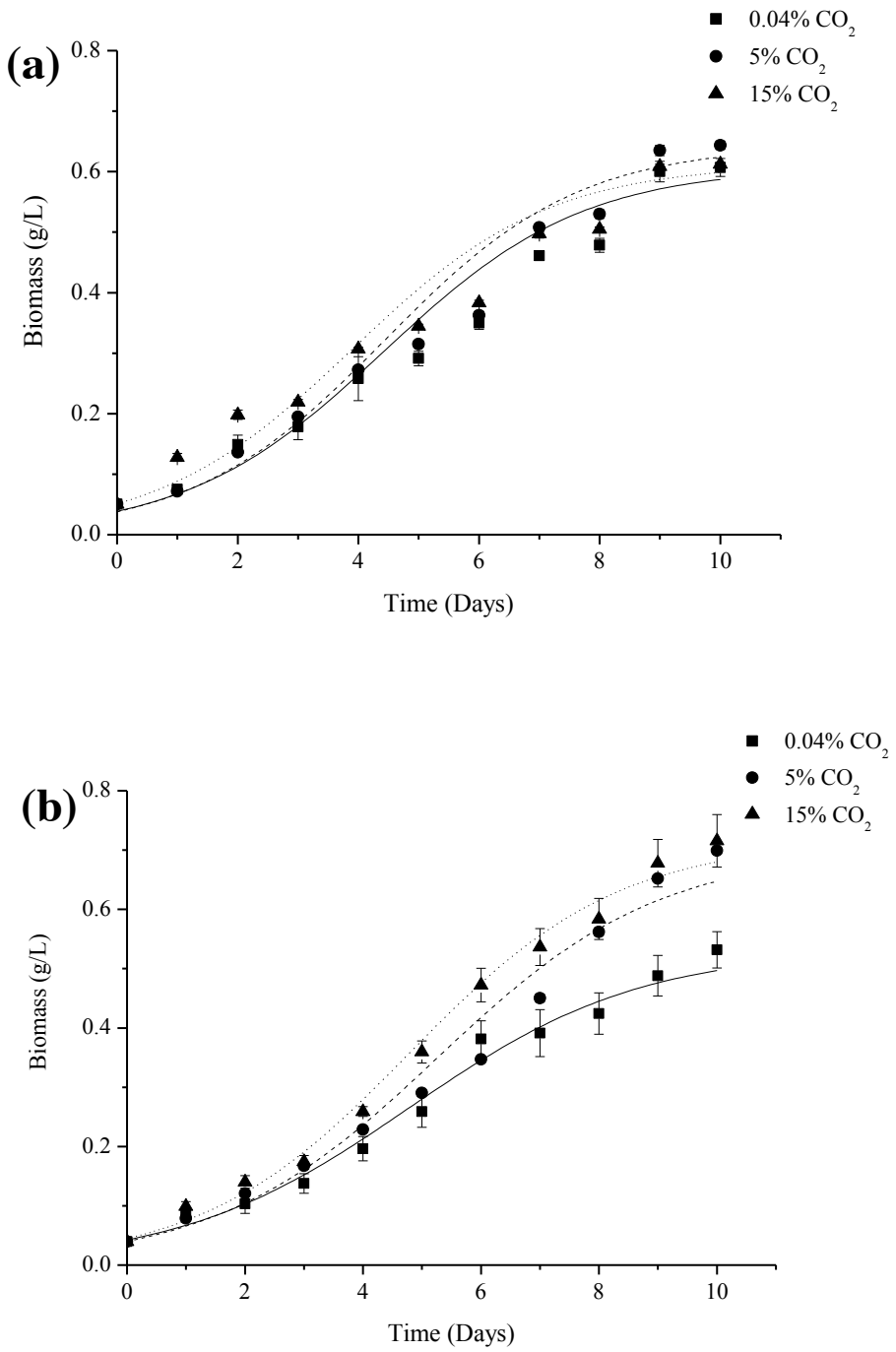

Figure 4.3 Growth profiles of microalgae cultivated at different CO_2 concentration - (a) *Chlorella* sp. (b) *T. suecica*.

Generally, microalgae utilise CO_2 for photosynthesis and production of biomass. The capability of microalgae to tolerate CO_2 concentrations can be grouped into CO_2-sensitive (<2–5% CO_2) or CO_2-tolerant (5–20% CO_2) microalgae and the capability of microalgae to grow in CO_2 is species-specific (Miyachi et al., 2003). Based on the results obtained, it is clear that both microalgae used can be categorised as CO_2-tolerant microalgae species. The ability of microalgae to grow in the high CO_2 concentration (up to 15% v/v in this study) is attributed to the CO_2 concentrating mechanism (CCM) in microalgal cells. The CCM is a mechanism that involves transfer of CO_2 or inorganic carbon available in the cultivation medium into microalgal cells. There are two main factors that contribute to CCM, specifically the (1) inorganic transfer transporter; (2) carbonic anhydrase (CA). The inorganic transfer transporter is a component that assists dissolved inorganic carbon (DIC) membranes transferring CO_2 or bicarbonate into the plasmalemma and chloroplast envelope. While carbonic anhydrases (CAs) is an enzyme that involves stimulates the direct supply of CO_2 from outside cells to ribulose-1,5-biphosphate carboxylase (RuBisco), where fixing atmospheric CO_2 during the photosynthesis process occurs (Baba and Shiraiwa, 2012).

In summary, both *Chlorella* sp. and *T. suecica* was found to be able to grow in high CO_2 concentration up to 15% v/v under indoor cultivation. In order to determine the potential of larger scale biomass production, outdoor cultivation using CO_2 as a carbon source was therefore carried out in the next part of this study.

4.3.2 *Outdoor cultivation using a centric photobioreactor*

Outdoor cultivation of *Chlorella* sp. and *T. suecica* was carried out using a centric tubular photobioreactor at two different times and ambient temperatures, specifically, a low ambient temperature range of 15 to 20°C (autumn/spring) and a high ambient temperature range of 20 to 32°C (summer) environment. The CO_2 concentration for outdoor cultivation was 15% (v/v). This concentration was based on the results obtained from indoor cultivation where 15% (v/v) CO_2 resulted in a consistently high growth rate among the three other concentrations. Besides this, the CO_2 level is typical of the CO_2 concentration released from the pilot plant.

Comparison of outdoor cultivation for *Chlorella* sp. in two different temperature ranges showed that the maximum biomass concentration and growth rate for this microalgae were obtained from cultivation in a high ambient temperature range (Figure 4.4). Increasing the CO_2 level was found to significantly enhance biomass production. When using CO_2, the maximum biomass concentration and growth rate for *Chlorella* sp. were twice that of the control. The maximum biomass concentration and growth rate for *Chlorella* sp. were 0.24 gL^{-1} and 0.16 d^{-1} respectively. It can be seen that slow growth and long lag phase was observed for 5 days and the growth of *Chlorella* sp. started to increase after 5 days of cultivation. It was found that the growth of *Chlorella* sp. started to increase as the ambient temperature started to increase from 25°C to 32°C (Figure 4.5).

Figure 4.4b represents the growth profile of *T. suecica* cultivated at different ambient temperatures using different CO_2 concentrations. Similar to *Chlorella* sp., the biomass production and growth rate of *T. suecica* was found to be significantly influenced by temperature and CO_2 concentration. The biomass production of *T. suecica* was higher for cultivation using 15% (v/v) CO_2 in a high ambient temperature compared to low ambient temperature. Cultivation of *T. suecica* in this ambient temperature range produced 0.29 gL^{-1} of microalgal biomass and growth rate of 0.2 d^{-1}. It was found that the growth of *T. suecica* started to increase after 3 days cultivation as the ambient temperature increased from 26°C to 32°C. The growth started to slow down as the ambient temperature started to decrease from 28°C to 22°C on the 5^{th} day cultivation (Figure 4.6).

Overall, this study indicates that both *Chlorella sp. and T. sueicica* displayed high biomass production and growth rate for cultivations in high ambient temperature. This can be explained by the fact that both microalgae species show good growth in a high temperature range of between 25 and 30°C. According to our indoor cultivation study on the effect of temperature towards *Chlorella* sp. and *T. suecica* growth, temperature showed a significant influence on microalgal biomass production and growth rate. Cultivation below 25°C and beyond 30°C was found to decrease the biomass production and growth rate.

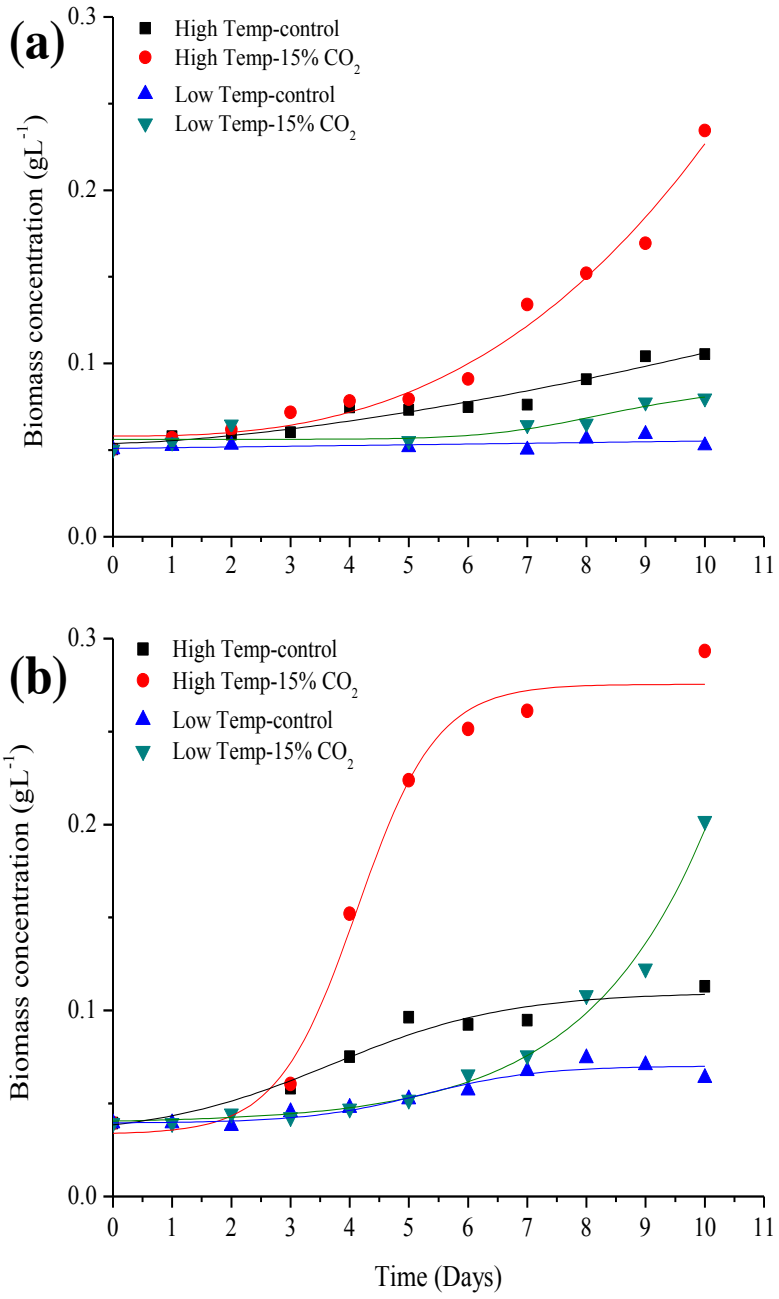

Figure 4.4 Growth profiles of microalgae cultivated in outdoor condition in two different temperature ranges - (a) *Chlorella* sp. and (b) *T. suecica*.

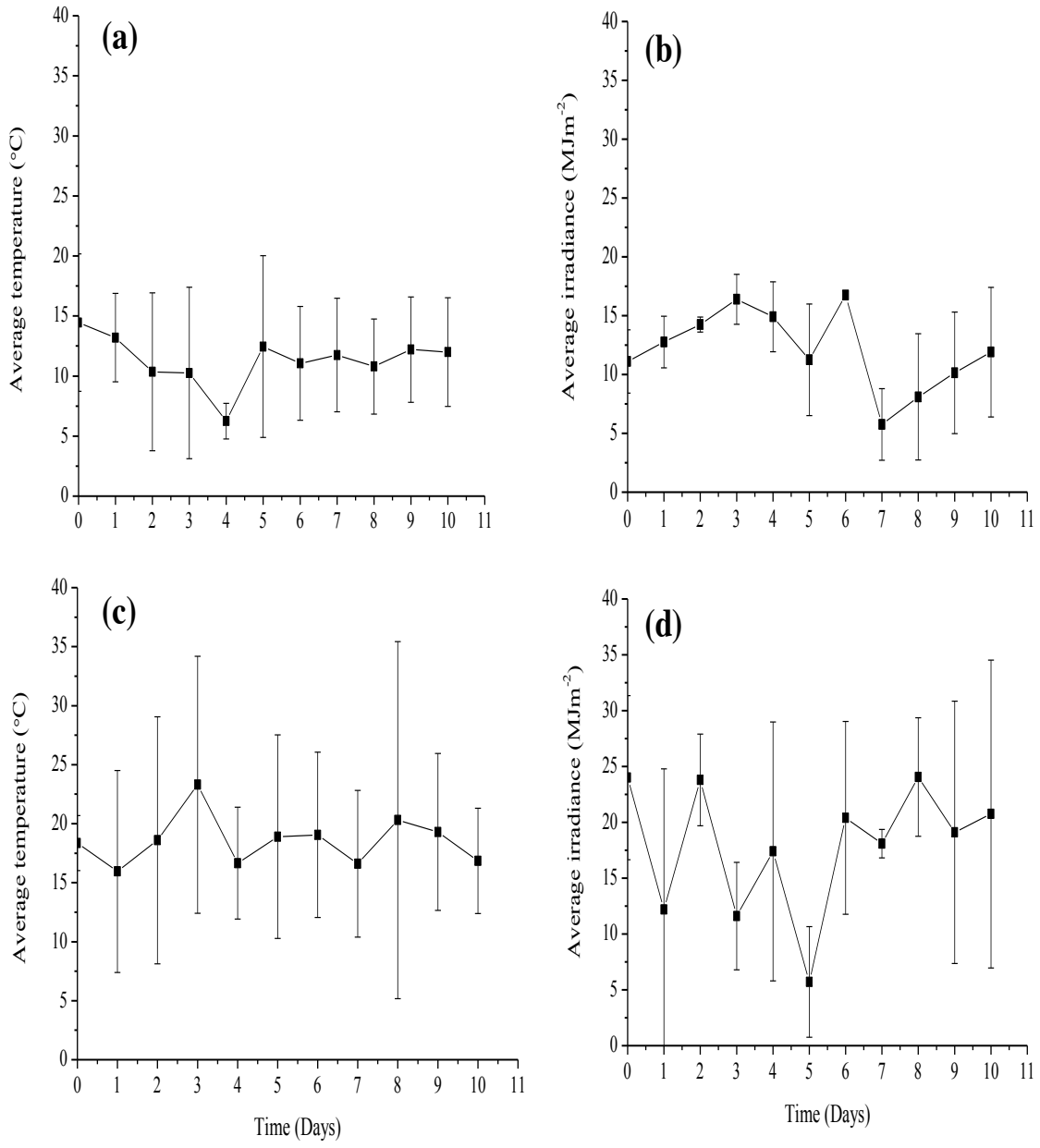

Figure 4.5 Average temperature and light intensity variation during outdoor cultivation of *Chlorella* sp. - (a-b) Low temperature range, (c-d) High temperature range.

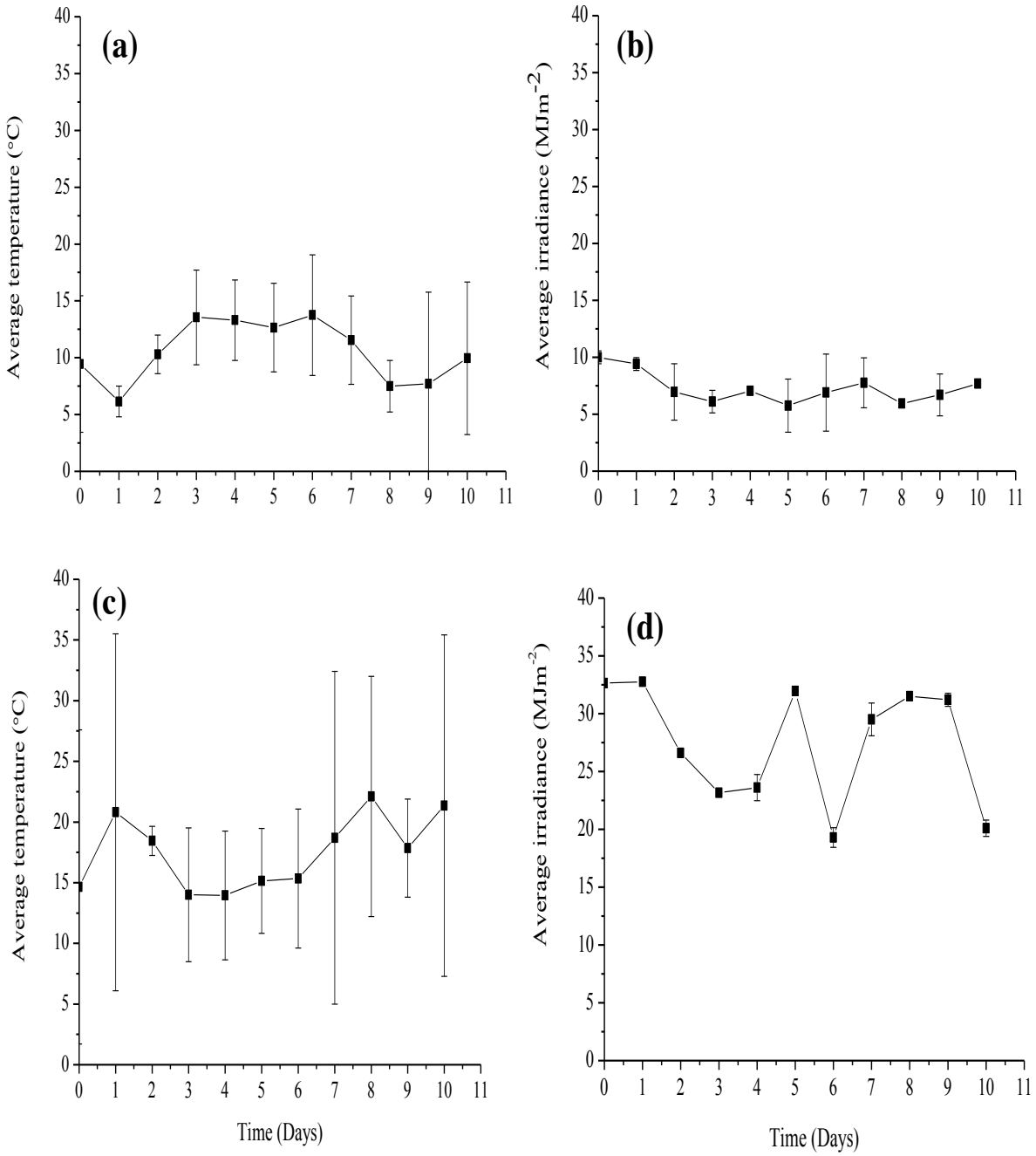

Figure 4.6 Average temperature and light intensity variation during outdoor cultivation of *T. suecica* - (a-b) Low temperature range, (c-d) High temperature range.

This study also found that biomass production and growth rate of *Chlorella* sp. and *T. suecica* were influenced by the presence of CO_2 in the cultivation medium. This finding is in agreement with our laboratory scale study, which found that CO_2 concentration showed an effect on microalgal growth. Our laboratory study indicated that CO_2 was found to increase *Chlorella* sp. and *T. suecica* growth.

4.3.3 *Comparison of indoor and outdoor cultivation*

Comparison of indoor and outdoor microalgae cultivation is discussed in this study. It was found that, the biomass production and growth rate of *Chlorella* sp. and *T. suecica* in indoor cultivation was three times higher than that of outdoor cultivation (Table 4.1). The biomass concentration for *Chlorella* sp. obtained from indoor and outdoor cultivation was 0.61 and 0.25 gL^{-1} respectively. As for *T. suecica*, the biomass concentration obtained from indoor and outdoor cultivation was 0.72 and 0.29 gL^{-1} respectively.

Similar findings have been reported on the comparison of indoor and outdoor microalgal cultivation (Khatoon et al., 2014; Shukla et al., 2013). Khatoon et al. (2014), reported that the biomass production of *T. suecica* cultivated in outdoor conditions was lower than that in indoor cultivation. Moreover, in another study, outdoor cultivation of *Chlorella* sp. was also reported to produce low biomass concentration and growth rate (Shukla et al., 2013).

Low biomass production and growth rate for *Chlorella* sp. and *T. suecica* cultivated in outdoor cultivation observed in this study is attributed to the cultivation condition. High biomass production in indoor cultivation is because both microalgae were exposed to constant temperature and light intensity. However, both microalgae cultivated in the outdoor condition were exposed to the fluctuation of temperature and light intensity (Figure 4.5 and 4.6). The large variation of temperature and light intensity influenced biomass production and growth rate of microalgae from outdoor cultivation. This is due to the fact that the fluctuation of temperature and light intensity will reduce photosynthesis activity that results from the photoinhibition mechanism, consequently decreasing microalgal biomass production and growth rate. Vonshak et al. (2001), in their study on the effect of ambient temperature on the

Table 4.1. Growth kinetic parameters and chemical compositions of *Chlorella* sp. and *T. suecica* cultivated at different cultivation conditions.

	Parameters	Indoor		Outdoor (Low-Temperature)		Outdoor (High-Temperature)	
		Control	**15% CO_2**	**Control**	**15% CO_2**	**Control**	**15% CO_2**
Chlorella sp.	P_{max} (gL^{-1})	0.61	0.61	0.06	0.18	0.11	0.24
	Biomass productivity ($gL^{-1}d^{-1}$)	0.06	0.06	0.00	0.02	0.01	0.02
	μ (d^{-1})	0.25	0.25	0.02	0.15	0.07	0.16
	Protein content (%)	15.52	68.84	60.01	51.76	32.92	24.42
	Carbohydrate content (%)	32.40	24.85	23.97	28.43	27.74	42.54
	Lipid content (%)	50.14	6.65	16.02	19.81	40.43	34.31
T. suecica	P_{max}(gL^{-1})	0.53	0.72	0.06	0.20	0.13	0.29
	Biomass productivity($gL^{-1}d^{-1}$)	0.05	0.07	0.002	0.02	0.01	0.03
	μ (d^{-1})	0.26	0.29	0.05	0.16	0.12	0.20
	Protein content (%)	64.03	63.26	60.00	70.00	56.46	57.53
	Carbohydrate content (%)	20.63	23.41	20.84	21.21	19.8	19.02
	Lipid content (%)	14.65	20.38	18.32	9.8	15.00	30.00

Low temperature range: 10-20°C; High temperature range: 20-32°C

growth of *Monodus subterraneus* reported that exposure of the microalgae to large temperature fluctuation caused photoinhibition, eventually leading to decrease of microalgal biomass production.

On the other hand, low microalgal biomass production observed in this study can also be attributed to the light intensity during cultivation. According to our microalgal growth characterisation study, a large variation of light intensity was found to influence microalgal biomass production. These results are also in agreement with Moheimani (2013) who found that outdoor cultivation of *Chlorella* sp. and *T. suecica* in a bag photobioreactor was significantly influenced by light intensity. Increased light intensity increased the microalgal biomass production.

4.3.4 *Effect of CO_2 on pH value*

The pH of the cultivation medium is one of the parameters that can affect microagal metabolisms. A decrease in pH will cause a decrease in enzyme activity related to photosynthesis, such as carbonic extracellular anhydrase, and as a result will inhibit the microalgal growth (Tang et al., 2011). The adaptability of microalgae toward pH changes during cultivation is species-dependent. Cultivation of microalgae in too acidic or alkali environment could inhibit microalgal growth. According to Zeng et al., (2012), addition of CO_2 in the cultivation medium was found to change the pH value. Hence, in order to determine the effect of CO_2 on the pH medium, the pH during the outdoor microalgal cultivation was monitored during the experiment.

Figure 4.7 shows the pH profile for both microalgae during the cultivation period. The pH of the medium decreased from pH 8 ± 0.2 to pH 6.5 ± 0.2 after 1 h of CO_2 aeration This finding is in agreement with Zhao et al., (2011) who reported that the pH value of the cultivation medium decreased as the medium was introduced with CO_2. The reduction of the pH value is due to the presence of hydrogen (H^+) in the culture medium, the formation of dissolved inorganic carbon (DIC) and carbonate species of CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} resulting from the reaction of CO_2 and water (de Morais and Costa, 2007).

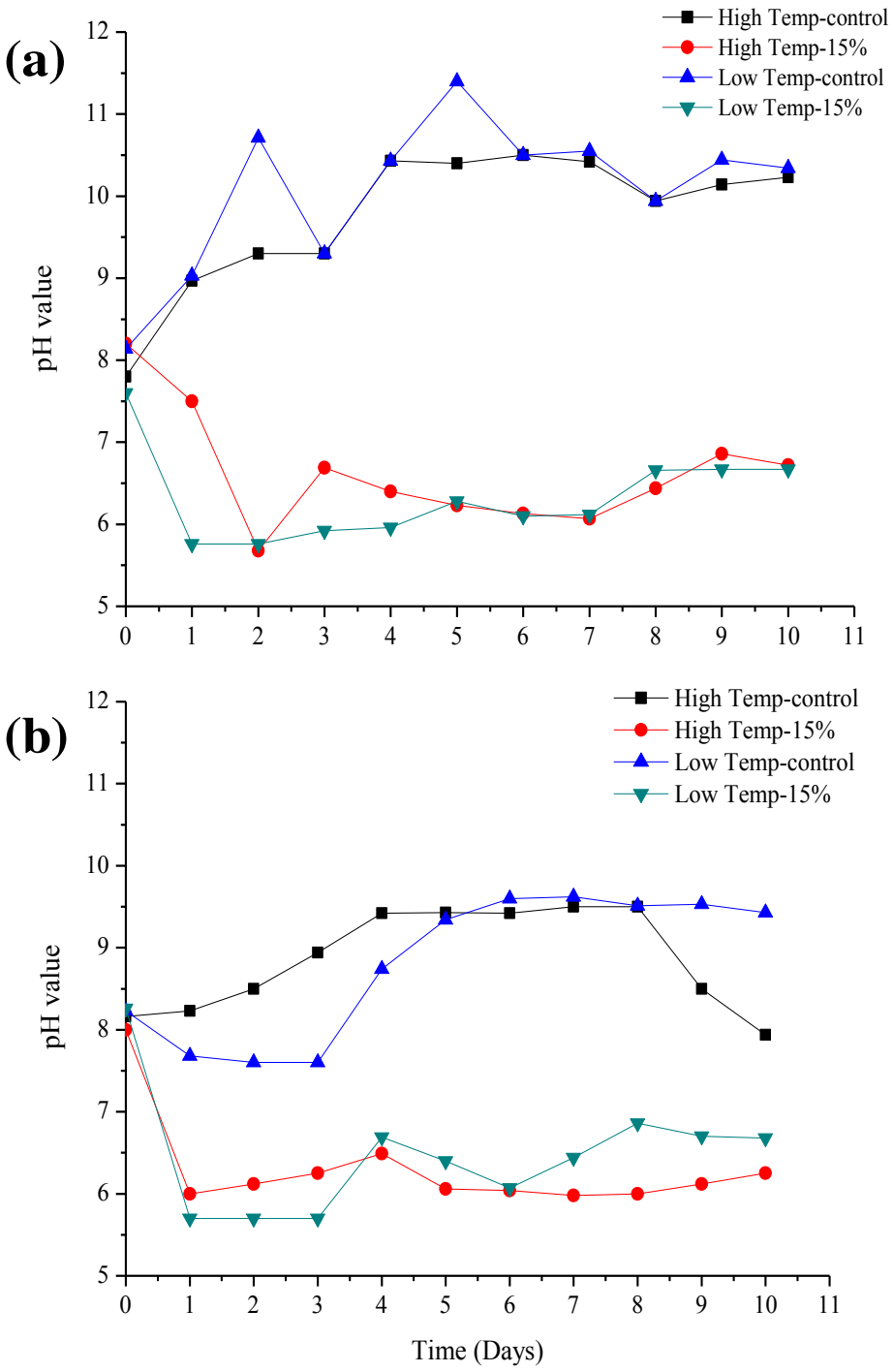

Figure 4.7 pH value variation during outdoor cultivation - (a) *Chlorella* sp. (b) *T. suecica*.

In order to determine pH changes during the cultivation period, the pH of the medium was monitored every 24 h of cultivation. It was found that the pH value started to increase from pH 6.5 ± 0.2 to pH 7.5 ± 0.2 after that period. As expected, an increased pH value during the cultivation is attributed to the photosynthesis of microalgae. This can be explained by the alkalinisation of the medium during cultivation. Alkalinisation of the cultivation medium has been widely reported by many photosynthetic microalgae as a result of the CO_2 uptake, increase of carbonate and bicarbonate, hydroxide (OH^-) and decrease of CO_2 concentration during photosynthesis (Shiraiwa et al., 1993).

Even though there were changes of pH value after addition of CO_2, this study found that the pH value does not significantly influence the growth of both microalgae. Based on the results mentioned earlier, it was found that the growth of *Chlorella* sp. and *T. suecica* in a medium with 15% (v/v) CO_2 was higher compared to that in the control experiment. This finding is in accordance with our previous study, which indicated that both microalgae species showed a better growth in slightly alkaline conditions. Moreover, the study by Moheimani (2013) also reported that higher biomass concentration of *Chlorella* sp. and *T. seucica* was obtained from cultivation in a medium with pH in the range of 6.5 to 7.5 compared to that of the control experiment.

Overall, this study reveals useful information of practical relevance. Addition of CO_2 (up to 15% (v/v) in our study) could reduce the pH value and provide a better environment for *Chlorella* sp. and *T. suecica* to grow, without requiring additional chemicals to adjust the pH during the cultivation period. Hence, this could reduce the production cost of biomass from microalgae.

4.3.5 *Chemical composition of microalgae at different cultivation modes*

The chemical composition of microalgae is dependent on the strain and cultivation conditions. Cultivation condition parameters such as temperature, light intensity, and medium composition have been reported to influence the chemical compositions in microalgal biomass (Dechatiwongse et al., 2014; Khatoon et al., 2014). In this study, the chemical composition of *Chlorella* sp. and *T. suecica* biomass obtained from different cultivation conditions was determined. The results are shown in Figure 4.8.

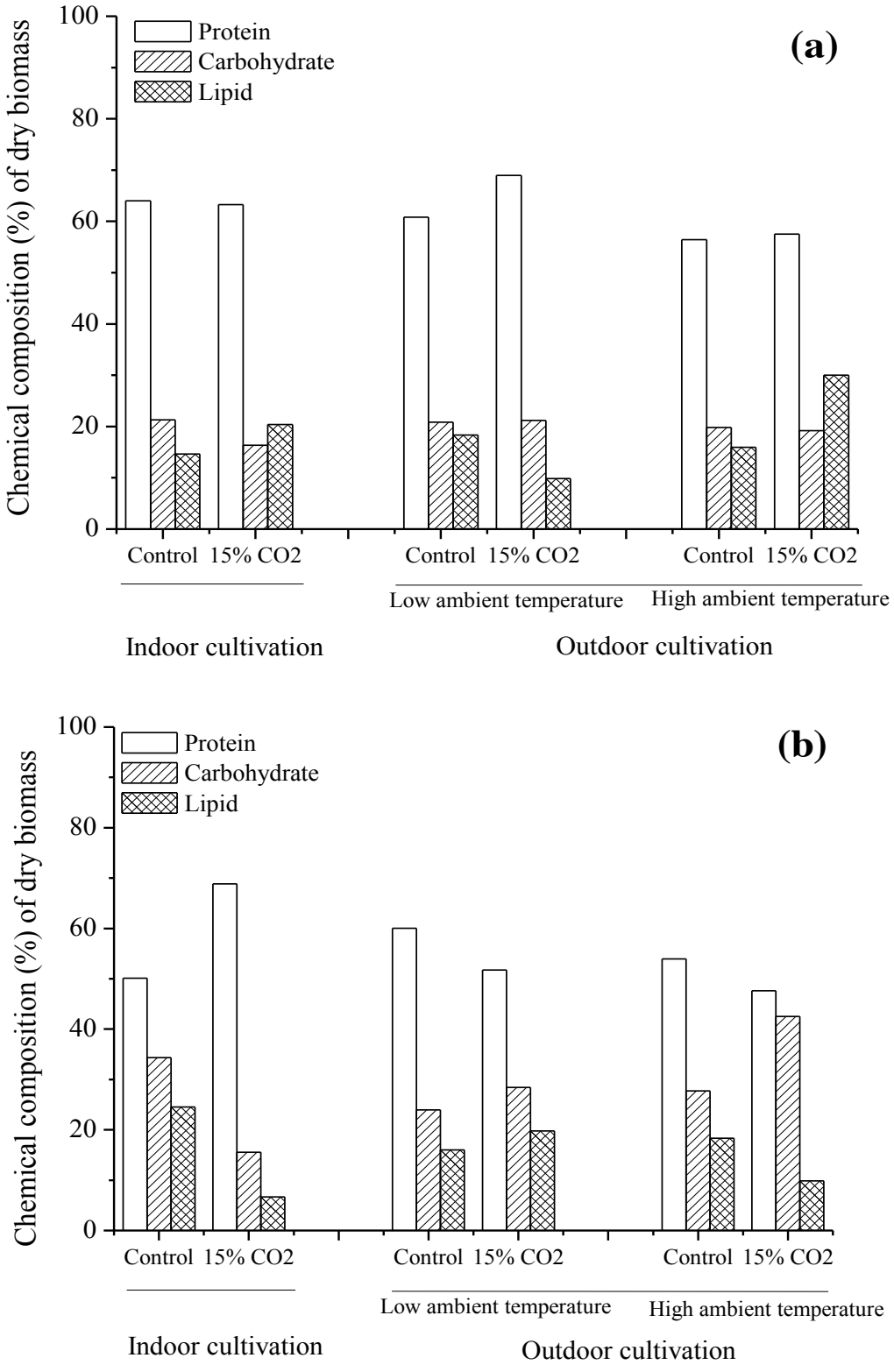

Figure 4.8 Chemical compositions of microalgae at different cultivation conditions- (a) *Chlorella* sp. (b) *T. suecic*.

Results indicate that the chemical compositions of *Chlorell*a sp. cultivated in a medium using both air and 15% (v/v) CO_2 in the low and high ambient temperature environment were different (Figure 4.8a). Protein content was the major compound for *Chlorella* sp. cultivated in all cultivation conditions (Figure 4.8a). Obvious differences can be observed in the carbohydrate and lipid content. Carbohydrate and lipid content for *Chlorella* sp. cultivated with 15% (v/v) CO_2 in high ambient temperature were 1.5 and 0.5 times higher than that in a low ambient temperature environment.The carbohydrate and lipid content for for *Chlorella* sp. cultivated in 15% (v/v) CO_2 was found to be 42.54% and 34.31% of dry biomass respectively.

The carbohydrate content for *Chlorella* sp. cultivated outdoors at 15% (v/v) CO_2 was two times higher compared to that in the control experiment. Conversely, the lipid protein content for *Chlorella* sp. was found to decrease when the cultivation was carried out using 15% (v/v) CO_2. The chemical composition for *T*. *suecica* at different ambient temperature cultivation ranges was also determined and the results are shown in Figure 4.8b. The major chemical compound in *T*. *suecica* for all cultivation conditions was protein. There was no significant change on the carbohydrate content in *T*. *suecica* biomass between the cultivation conditions. This study indicated the protein and lipid content of *T*. *suecica* was significantly affected by the cultivation condition. The lipid content for *T*. *suecica* cultivated in a low ambient temperature environment using 15% (v/v) CO_2 was two times lower than that in the control experiment. In contrast, cultivation of this microalgae species using 15% (v/v) CO_2 in indoor and outdoor cultivation in high ambient temperature was found to increase the lipid content. It was found that the lipid content of *T*. *suecica* cultivated under high ambient temperature using 15% (v/v) CO_2 was two times higher than that under the control experiment.

High carbohydrate and lipid content for both microalgae species observed during the high ambient temperature cultivation could be attributed to the environmental factors during the cultivation period. During this cultivation period, the microalgae were exposed to high irradiance and temperature. As mentioned earlier, the irradiance during high ambient temperature environment was slightly higher compared to that during low ambient temperature. A combination of light intensity and temperature at high ambient temperature

was found to increase energy storage chemicals such as lipid and carbohydrate in both microalgal cells. Irradiance and temperature have been reported to be one of the important factors for microalgae to produce biomass and energy storage (Carvalho et al., 2009; López-Rosales et al., 2014). Appropriate temperature is important to ensure the stability of microalgal metabolisms within the cell (Raeesossadati et al., 2014). The stable environment conditions could provide better growth and CO_2 uptake for biomass and chemical production (Juneja et al., 2013).

The differences in the chemical composition of *Chlorella* sp. and *T. suecica* is due to the difference in the metabolic capacity of both microalgae to adapt to changing environment conditions. High carbohydrate and lipid content for both microalgae displayed in this study also could be attributed to the large temperature fluctuation during the cultivation. Tanadul et al. (2014) reported that the cultivation at beyond favourable cultivation conditions tends to promote changes in the accumulation of lipid and starch in microalgal cells.

High carbohydrate content for *Chlorella* sp., and lipid content for *T. suecica* cultivated using 15% (v/v) CO_2 is related to the efficiency of photosynthesis and production of carbohydrate or lipid as a final product. The carbohydrate and lipid content observed in this study might be different compared to other studies due to the difference of microalgae strain, CO_2 concentration and environment condition. A study by Cheng et al. (2015) reported that high carbohydrate in *Chlorella* UTEX259 and NC64A cultivated in 2% (v/v) CO_2 compared to that in air. According to Izumo et al. (2007), high carbohydrate in microalgae obtained from cultivation using CO_2 is attributed to the activation of starch synthase, which is an enzyme involved in carbohydrate metabolisms in microalgal cell. Previous studies have reported that temperature and irradiance are among the factors that could trigger the starch synthase in microalgal cells (González-Fernández and Ballesteros, 2012). On the other hand, the high lipid content for *T. suecica* in 15% (v/v) CO_2 could be explained by the lipid synthesis mechanisms in the presence of inorganic carbon in the cultivation medium. A study by White et al. (2013) on the effect of inorganic carbon on *T. suecica* chemical composition reported that the presence of inorganic carbon in the medium may increase protein and lipid synthesis.

4.4 Conclusions

Outdoor cultivation of *Chlorella* sp. and *T. suecica* was carried out using a centric photobioreactor in two different ambient temperature ranges and two different CO_2 concentrations. Comparison of indoor and outdoor cultivation of both microalgae was also carried out and the chemical compositions in all cultivation conditions were determined.

It was apparent that the microalgal biomass production and growth rate cultivated for outdoor cultivation in a photobioreactor using natural ambient conditions were significantly influenced by the environmental factors (temperature and light intensity). The microalgal biomass production and growth rate were higher for cultivation in a high ambient temperature range compared to that in a low temperature range using 15% (v/v) CO_2. This study also indicates that the presence of CO_2 in the cultivation system could influence the pH value of the cultivation medium. However, the changes of pH value were found to be not significantly affected by the microalgal growth, indicating that no pH adjustment is required to maintain the growth of microalgae.

In comparison to indoor cultivation, the microalgal biomass production and productivity for outdoor cultivation was lower than that for indoor cultivation, indicating that cultivation in control conditions is favourable from maximum microalgal biomass production. This study also indicates that the quality of microalgal biomass (chemical compositions) is significantly affected by the cultivation mode and conditions. Overall, this finding provides information on the potential of outdoor cultivation of *Chlorella* sp. and *T. suecica*. The findings showed that the microalgal cultivation could reduce the use of artificial light, however, low biomass production and inconsistent biomass quality make this strategy indefinite.

Focus on next chapter

This chapter evaluated the feasibility of microalgal outdoor cultivation at different temperature ranges using CO_2 as the carbon source. This study provides information on the outdoor cultivation of *Chlorella* sp. and *T. suecica* under wide ranges of ambient condition fluctuation. This information is important for microalgal biomass production through an outdoor cultivation strategy. Further experiments on the conversion of microalgal biomass to biofuel, the chemical platform used and the results will be discussed in the next chapter.

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CHAPTER 5

DILUTE ALKALINE PRETREATMENT OF MICROALGAL BIOMASS

5.1 Introduction

The production of biofuel such as bioethanol and biobutanol from biomass has gained much attention as one of the alternatives to replace fossil fuel for the production of energy (Luis Caspeta et al., 2013). This is due to the fact that the production of biofuel from biomass is renewable and environmentally friendly (Naik et al., 2010). Several types of biomass have been suggested as feedstock to produce biofuel, for instance woody, lignocelluloses crops, and algae (Sims et al., 2010). Among these feedstocks, algae is considered to be a promising feedstock due to its ability to produce higher biomass compared to terrestrial crops and capturing CO_2 rapidly (Demirbas, 2011).

In order to produce bioethanol and biobutanol using biochemical methods, several processes are involved, such as feedstock production, pretreatment, hydrolysis and fermentation (Mahalaxmi and Williford, 2012). One of the most crucial parts of biofuel production through a biochemical pathway is the pretreatment process (Balat, 2011). In this process, the biomass has to be pretreated to disrupt the cell wall structure leading the way for the enzymatic saccharification prior to the fermentation process (Sun et al., 2014). Several types of pretreatment processes such as mechanical, chemical and biological methods have been applied. Among these approaches, chemical pretreatment methods, particularly using dilute alkaline pretreatment, appear promising in enhancing the accessibility of enzymatic saccharification (Gary Brodeur et al., 2011). The alkaline pretreatment method is considered very environmentally friendly as this method uses a low concentration of alkali (Park and Kim, 2012). Besides, the alkaline pretreatment method is also reported to produce less inhibitors which can affect the fermentation process (Ramirez et al., 2013).

Optimisation of pretreatment conditions should be carried out in order to extract a high concentration of reduced sugar from the microalgal biomass with a minimum of chemical use. This often means changing each variable one at a time and keeping other variables constant. To test all combinations, this involves a large number of experiments. Alternatively, response surface methodology (RSM) is an effective tool to resolve a complex parameter design problem with more than two responses. Besides, this method can be applied for a large number of data sets without compromising its ability and delivers quality results (Hill and Hunter, 1966). RSM has been widely used to determine and understand the

interaction between different process parameters especially in the biotechnology area (González-Fernández et al., 2011; Makareviciene et al., 2013). The application of RSM on alkaline treatment to pretreat biomass prior to enzymatic process has been reported by few previous studies (Rawat et al., 2013). Most of the studies have focused on lignocellulosic materials and woody biomass. However, there is currently limited information on the alkaline pretreatment on algal biomass for biofuel production.

Thus, the aim of this study is to investigate the alkaline pretreatments of two different microalgal biomass, specifically *Tetraselmis suecica* and *Chlorella* sp. using the response surface methodology (RSM) approach. The effect of several parameters of the alkaline pretreatment process such as type of alkaline reagent, alkaline concentration, temperature and reaction time on reducing sugar production from the microalgal biomass was determined. The solid residue produced after the pretreatment was also characterised.

5.2 Materials and Methods

5.2.1 *Microalgal cultivation condition*

Two different microalgae species, specifically marine water microalgae *Tetraselmis suecica* and, fresh water microalgae *Chlorella* sp. were used in this study. These species were obtained from the CSIRO Microalgae Research Centre (Hobart, Australia) and were selected based on their capability to grow in 15% carbon dioxide (CO_2), which is the typical CO_2 concentration in the flue gas from coal-fired power stations.

Modified algae growth (MLA) medium with 0.49 gL^{-1} magnesium sulfate ($MgSO_4.7H_2O$), 1.7 gL^{-1} sodium nitrate ($NaNO_3$), 0.14 gL^{-1} di-potassium phosphate (K_2HPO_4), and 0.03 gL^{-1} calcium chloride ($CaCl_2.2H_2O$) was used as the seed culture and biomass production medium. The medium was initially sterilised using a 0.22 μm Millipore filter. The microalgal seeds were cultivated in a 1 L Scott bottle containing 700 mL of the modified MLA.

A standardised 10% (v/v) initial microalgal cell concentration of 0.03-0.05 gL^{-1} (OD_{680}= 1.0) was added into the medium and incubated in an illuminated incubator with 0.3

$Lmin^{-1}$ of compressed air under light with a photon intensity of 450 $\mu molm^{-2}s^{-1}$. The cultivation temperature was 20 ± 0.5 °C. Both microalgal cultures were cultivated under the same conditions and were harvested at late log growth. The microalgal cells were centrifuged at 4500 rpm for 15 min. The pellets were rinsed twice with distilled water and subsequently dried at 70 °C for 24 h.

5.2.2 *Chemical composition*

The lipid, carbohydrate, protein content, and elemental analysis of the microalgal biomass were determined using soxhlet extraction, phenol-sulfuric acid method, and Lowry method analyses as described by Kassim et al., (2014) .

5.2.3 *Pretreatment of microalgal biomass*

A total of 50 mg dried microalgal biomass was measured and soaked in 10 mL of alkaline agent in a 50 mL Scott bottle. The mixture was placed in an oven and incubated at a specific temperature and for a specific period of time. After the incubation period, the sample was removed and cooled at room temperature. The sample was centrifuged at 3000 rpm for 10 min. The supernatant obtained was separated and subjected to a reducing sugar analysis.

5.2.4 *Reducing sugar estimation*

The total reducing sugar was determined using 3, 5 dinitrosalysilic acid method (Pradeep et al., 2013). Generally the filtered sample was added with 1 mL of DNS reagent and then boiled for 10 min in a water bath. The reaction mixture was allowed to cool and the reducing sugar concentration was estimated by using UV spectrophotometer (Hach, DR-5000) at 540 nm.

5.2.5 *Optimisation of dilute alkaline pretreatment using RSM*

Alkaline pretreatment of microalgal biomass has been carried out using two series of experiments. In the first series of experiments, the effect of type of alkaline agent on reducing sugar production from microalgal biomass was evaluated. In this first series of experiments, the pretreatment was carried out using four different alkaline agents such as potassium hydroxide (KOH), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3) and aqueous ammonia (NH_4OH).

In the second series of experiments, the optimisation of the pretreatment condition was carried out using a selected alkaline agent. The dilute alkaline pretreatment optimisation of microalgal biomass was carried out using the response surface methodology (RSM) approach. In this study, three different variables - alkaline concentration (%) (w/v), temperature and reaction time of pretreatment were chosen. Table 5.1 shows the range of values for each variable selected based on Harun et al. (2011). A total of 20 experiments as determined by the expression of 2n (2^3=8 factorial points), 2n (2×3=6 axial points) and 6 (centre points) as given in table 5.2 was carried out.

Table 5.1 Range and level of the variables in coded unit in RSM studies.

Parameters	-1	0	1
Alkaline concentration (w/v %)	0.5	1.25	2
Temperature (°C)	60	90	120
Duration (min)	30	75	120

Table 5.2 Experimental conditions.

Run	Alkaline concentration (w/v %)	Temperature (°C)	Time (min)
1	1.25	90	75
2	0.50	60	30
3	2.51	90	75
4	1.25	90	75
5	0.00	90	75
6	2.00	120	30
7	0.50	120	120
8	2.00	60	120
9	1.25	90	75
10	1.25	90	0
11	1.25	90	75
12	2.00	120	120
13	1.25	90	75
14	1.25	90	75
15	0.50	120	30
16	1.25	140	75
17	0.50	60	120
18	1.25	90	150
19	1.25	40	75
20	2.00	60	30

The response value for this study is expressed as reducing sugar concentration (Y). The quadratic model for predicting the response was expressed as described in equation (5.1):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \sum \beta_{ij} x_i x_j \quad (5.1)$$

where Y is the reducing sugar concentration (mg/g $_{\text{dry biomass}}$), β_0 is the intercept coefficient, β_i is the linear term, β_{ii} is the squared term, β_{iii} is the interaction term, and x_i and x_j are the uncoded independent variables. The model evaluated the effect of each independent variable to the response. The fit quality of the model was expressed by the correlation coefficient R^2.

5.2.6 *Enzymatic saccharification*

Enzymatic saccharification of the untreated and pretreated form of microalgal biomass was carried out using cellulase enzyme produced from *T. longibrachiatum* (Sigma Aldrich). The experiment was carried out by using 10 g/L of dried microalgal biomass in 10 mM acetate buffer (pH 5.5) and was incubated at 50 °C and 150 rpm in an orbital shaker (Thermoline Scientific) for 72 h. A total of 1.0 mL of the sample was withdrawn every 24 h and heated at 100 °C to deactivate the enzymatic reaction activity. The sample was then centrifuged at 3500 rpm for 5 min and the supernatant was used for the reducing sugar analysis. The reducing sugar estimation was carried out as described in section 5.2.4.

5.2.7 *Fourier transmission infrared (FTIR) spectroscopy*

The IR study of the untreated and pretreated microalgal biomass obtained from pretreatment at optimum conditions was carried out using an Attenuated Total Reflectance (ATR) accessory with a diamond crystal on a Perkin Elmer-Frontier-FTIR spectrometer. The single beam spectra samples were collected by 32 co-added scans at a resolution of 4 cm^{-1}. The absorbance spectrum was obtained by the equation Abs= log (1/I), in which I is the single beam spectrum of interest.

5.2.8 *Scanning electron microscopy (SEM)*

The untreated and pretreated *T. suecica* and *Chlorella* sp. biomass obtained from the optimum pretreatment conditions were dried in the oven for 24 h. The dried samples were then mounted on aluminum stub followed by a sputter coating of gold and examined under a scanning electron microscope (Phenom Pro.).

5.3 Results and Discussion

5.3.1 *Microalgal composition*

The chemical composition and ultimate analysis of *T. suecica* and *Chlorella* sp. biomass are shown in Table 5.3. Both microalgae contained high amounts of protein, followed by carbohydrate compound. Lipid content in the two samples was between 14-16%. Elemental analysis of *Chlorella* sp. and *T. suecica* biomass are also presented in table 3. The analysis revealed that there is a slight difference in the carbon (C), hydrogen (H) and nitrogen (N) composition for both algae species.

Table 5.3 Chemical composition of *T. suecica* and *Chlorella* sp.

	T. suecica	*Chlorella* sp.
Chemical composition (wt %)		
Carbohydrate	27.41	32.88
Protein	58.32	51.01
Lipid	14.25	16.11
Elemental analysis (wt %)		
Carbon	42.24	43.92
Hydrogen	7.17	6.1
Nitrogen	8.25	7.39
Sulphur	0.00	0.00
Oxygen	42.34	42.59

5.3.2 *Alkaline reagent*

In this study, alkaline treatment using four different alkaline agents, KOH, NaOH, Na_2CO_3 and NH_4OH, on *T. suecica* and *Chlorella* sp. biomass was investigated. Both dried microalgal biomass were soaked in 0.3 M of alkaline agent at 90°C for 75 min. Figure 5.1 shows the reducing sugar production from microalgal biomass after pretreatment. Results indicated that the highest reducing sugar concentration for *T. suecica* and *Chlorella* sp. can be obtained using 0.3 M of KOH and 0.3 M NaOH respectively. Pretreatment with Na_2CO_3 and NH_4OH showed an insignificant effect on reducing sugar production during the alkaline pretreatment process.

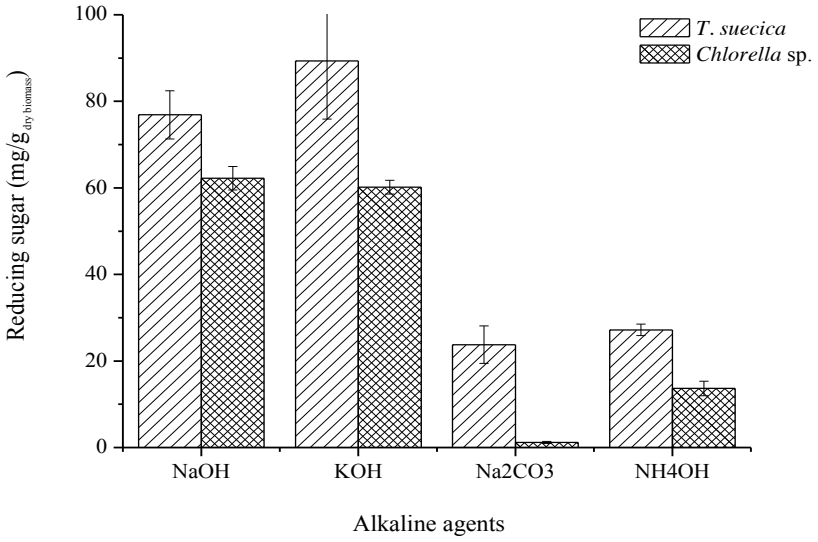

Figure 5.1 Reducing sugar concentration (mg/g $_{dry\ biomass}$) from dried microalgal biomass pretreated using different alkaline agent.

These results indicate that the extent of alkaline treatment can be influenced by the type of alkaline agent used during the process. Similar observation has been reported on the effect of KOH and NaOH on *laprix leptolepis*, *eucalyptus* and *pinus rigida* biomass (Park and Kim, 2012). A study by Hosseini et al., (2013) on the comparison between the pretreatment effectiveness of NaOH and KOH for rice straw pretreatment indicated that the use of NaOH resulted in greater solubility of the reducing sugar compared with the use of KOH.

From these results, it can be concluded that the selectivity of alkaline pretreatment is influenced by the nature of biomass feedstock. According to Ramirez et al. (2013), feedstock

composition and pretreatment condition (alkaline reagent, alkaline concentration and temperature) play a major role in the alkaline pretreatment process. Low solubility of reducing sugar in pretreatment using Na_2CO_3 and NH_4OH may be caused by the pretreatment conditions applied in the study. Generally, the pretreatment using Na_2CO_3 or NH_4OH requires an oxidative agent such as oxygen and higher temperature (over 100 °C) to obtain better performance.

5.3.3 *Model fitting and statistical analysis*

Based on Figure 5.1, a pretreatment optimisation study for *T. suecica* was carried out using KOH, while that for *Chlorella* sp. was carried out using NaOH. Identification of optimum conditions for reducing sugar extraction is very important. The three main parameters such as alkaline concentration, temperature and reaction time of the reducing sugar concentration were chosen for an optimisation study. The experiments were performed according to the central composite experimental design (CCD) to obtain the optimum condition for production of reducing sugar concentration from microalgal biomass. In this study, 20 experiments were performed and the second order polynomial equations that give the amount of reducing sugar from both biomass have been developed. Table 5.4 shows the reducing sugar concentration obtained from the dilute alkaline pretreatment of *T. suecica* and *Chlorella* sp. and the two-factor interaction (2FI) models are given in Eq (5.2) and (5.3).

$$Y_{T.suecica} = +70.42 + 14.60X_1 + 10.45X_2 + 12.30X_3 - 11.58X_1^2 - 13.66X_2^2 - 16.62X_3^2 + 0.24X_1X_2 + 8.23X_1X_3 + 5.81X_2X_3 \quad (5.2)$$

$$Y_{Chlorellas\ sp.} = +64.55 + 7.39X_1 - 12.29X_2 - 0.93X_3 - 10.03X_1^2 - 8.51X_2^2 - 4.00X_3^2 + 0.13X_1X_2 - 6.64X_1X_2 - 16.54X_2X_3 \quad (5.3)$$

where Y is the reducing sugar concentration (mg/g $_{\text{dry biomass}}$), X_1 is alkaline concentration (%), X_2 is temperature (°C) and X_3 is time (min).

The reducing sugar concentration range for *T. suecica* was 2.27 to 80.63 mg/g $_{dry\ biomass}$ and for *Chlorella* sp. was 10.40 to 87.99 mg/g $_{dry\ biomass}$ depending on the pretreatment conditions. The highest reducing sugar for *T. suecica* could be obtained when the pretreatment was carried out using 2.0% (w/v) KOH at 120°C for 120 min.

Table 5.4 Reducing sugar concentration (mg/g $_{dry\ biomass}$) obtained under different alkaline pretreatment conditions.

Run	Concentration (%w/v) (X_1)	Temperature (°C) (X_2)	Time (minutes) (X_3)	*Tetraselmis suecica*		*Chlorella* sp.	
				Predicted	Actual	Predicted	Actual
1	1.25	90	75	70.42	71.45	64.55	62.60
2	0.50	60	30	5.48	2.48	0.20	10.40
3	2.51	90	75	62.22	73.71	48.61	52.53
4	1.25	90	75	70.42	74.65	64.55	64.85
5	0.00	90	75	13.12	5.52	23.77	11.56
6	2.00	120	30	27.50	18.16	85.92	87.99
7	0.50	120	120	34.05	38.03	35.99	38.27
8	2.00	60	120	47.20	40.57	45.95	41.12
9	1.25	90	75	70.42	67.80	64.55	64.85
10	1.25	90	0	2.72	10.15	54.79	42.84
11	1.25	90	75	14.21	19.22	64.55	65.22
12	2.00	120	120	80.17	80.63	37.73	31.98
13	1.25	90	75	70.42	68.72	64.55	64.22
14	1.25	90	75	70.42	69.53	64.55	65.22
15	0.50	120	30	14.29	18.16	57.60	66.88
16	1.25	140	75	49.34	48.22	61.15	58.61
17	0.50	60	120	2.02	7.92	44.73	47.11
18	1.25	90	150	44.10	40.57	51.17	57.37
19	1.25	40	75	14.21	19.22	19.80	16.05
20	2.00	60	30	17.75	11.02	28.00	30.16

On the other hand, the highest reducing sugar concentration from *Chlorella* sp. could be obtained when the pretreatment was carried out using 2.0% (w/v) NaOH at 120°C for 30 min. The lowest reducing sugar concentration from both microalgal biomass can be observed when both biomass were pretreated at lower alkaline concentration for a shorter pretreatment period, 0.5% alkaline agent at 60 °C for 30 min.

5.3.4 *Statistical analysis*

The analysis of variance (ANOVA) for the response surface quadratic model for *T. suecica* and *Chlorella* sp. biomass showed that the p>F value for both models is less than 0.0001 (Appendix B.1). This indicated that the model shows a significant effect on reducing sugar production from both microalgal biomass. The p values ($p<0.0001$) for *T. suecica* and *Chlorella* sp. indicate that there is only a 0.01% chance that the models can occur due to the noise in the experiment. Generally, a P-value lower than 0.01 indicates that these models are statistically significant at the 99% confidence level and a P-value greater than 0.1 indicates that the model term is not significant. Therefore, based on the results obtained, parameters such as X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_3 and X_2X_3 for alkaline pretreatment of both microalgal biomass are significant model terms that affect the reducing sugar production of the biomass. The R^2 value of the model was 0.96 for *T. suecica* and 0.92 for *Chlorella* sp. (Appendix B.2). With R^2 values close to 1, the model-predicted values of reducing sugar concentration were found to be in good agreement with the actual values obtained in the experiments (Appendix B.3). The models thus appeared to be a reliable predictor of reducing sugar concentration within the design boundary of our experiments.

5.3.5 *Effect of variables on reducing sugar concentration*

The interactions of the variables involved in dilute alkaline pretreatment were visualised in 3D response surface plots. The plots indicate the interaction of two variables with one variable being kept constant at its optimal condition. The interaction of alkaline concentration, reaction temperature and reaction time for reducing sugar concentration from *T. suecica* and *Chlorella* sp. biomass during pretreatment are shown in Figure 5.2. Results show that a clear peak can be observed in the design boundary for each response surface plot, which indicates that the maximum reducing sugar can be obtained inside the design boundary.

The interaction of alkali concentration and pretreatment temperature on reducing sugar production from microalgal biomass are shown in Figure 5.2a and 5.2d. The results indicate that the reducing sugar concentration increased with an increase of alkaline concentration and temperature for both microalgal biomass. The maximum reducing sugar

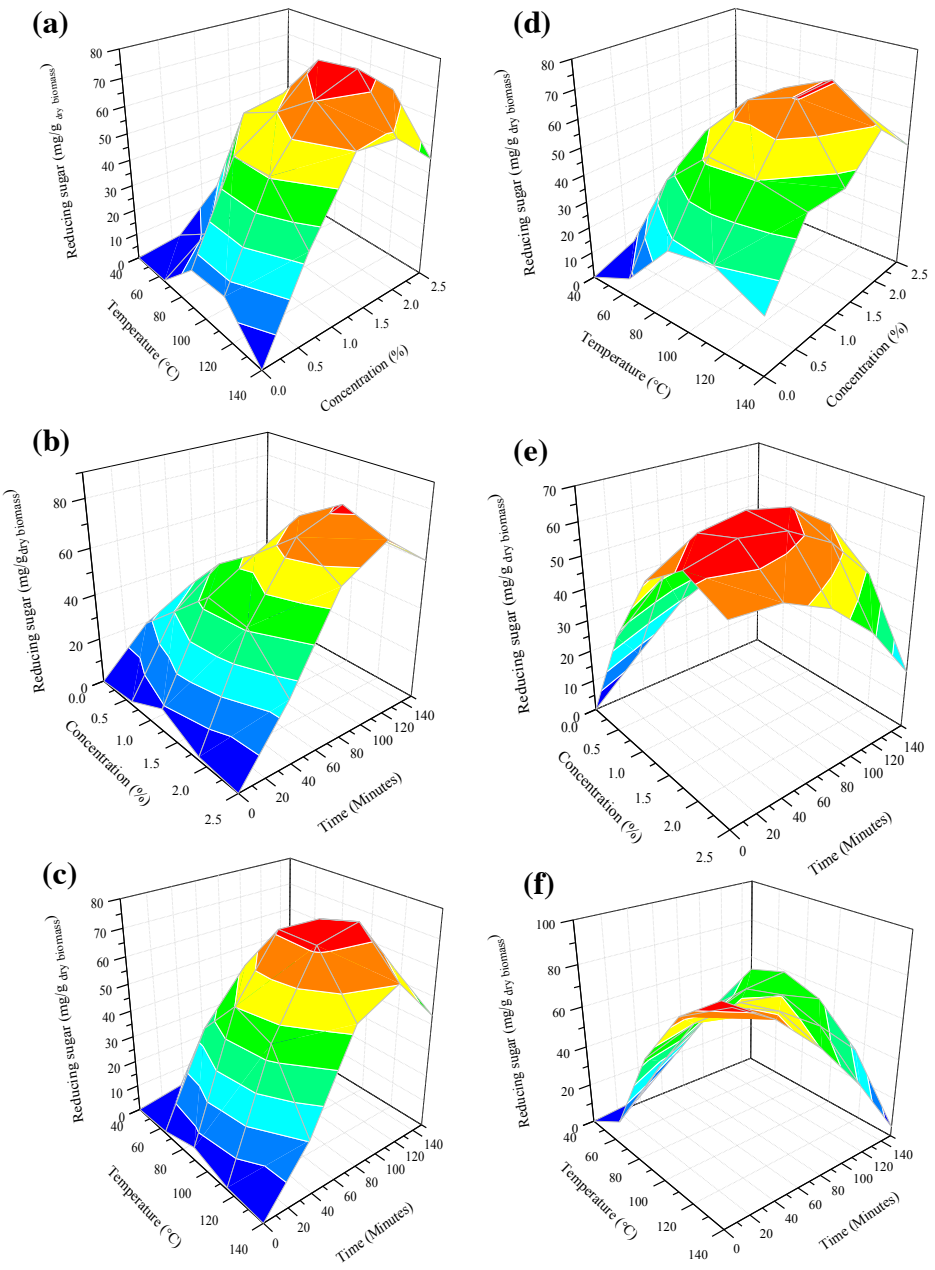

Figure 5.2 Response surface plot showing the interaction between variables during dilute alkaline pretreatment for *T. suecica* and *Chlorella* sp. biomass - (a) KOH concentration and temperature for *T. suecica*, (b) Alkaline concentration and reaction time for *T. suecica*, (c) Temperature and reaction time for *T. suecica*, (d) NaOH concentration and temperature for *Chlorella* sp., (e) Alkaline concentration and reaction time for *Chlorella* sp., (f) Temperature and reaction time for *Chlorella* sp.

from *T*. *suecica* could be obtained when the pretreatment was carried between 90°C and 120°C using 1.5 to 2.25% (w/v) of KOH (Figure 5.2a).

Likewise, the maximum reducing sugar concentration from *Chlorella* sp. could be obtained when pretreatment was carried out between 90°C to 120°C using 1.5 to 2.0% (w/v) of NaOH (Figure 5.2d). Both results indicated that pretreatment at alkaline concentration and temperature beyond the limits of our experimental design is likely to produce less reducing sugar from both microalgal biomass. Figure 5.2b and 5.2e show the interaction of the pretreatment period and alkaline concentration on reducing sugar production from *T*. *suecica* and *Chlorella* sp. biomass respectively. It was found that pretreatment of *T*. *suecica* required longer retention time and higher alkaline concentration compared to that of *Chlorella* sp. (Figure 5.2b and 5.2e). Higher reducing sugar concentration could be obtained from *T*. *suecica* biomass when the pretreatment was conducted over 100 min using 1.5 to 2.5% (w/v) of KOH. In contrast, the maximum reducing sugar concentration from *Chlorella* sp. biomass could be obtained when the pretreatment was carried out for 20 to 80 min using 1.25 to 2.0% of NaOH. Figure 5.2e indicated that the pretreatment of *Chlorella* sp. with NaOH for a period longer than 80 min will produce less reducing sugar. Less reducing sugar concentration produced from a longer reaction with high alkali concentration might be due to the undesired sugar loss through the degradation process (McIntosh and Vancov, 2011).

The interaction of the pretreatment period and temperature on reducing sugar production from microalgal biomass are shown in Figure 5.2c and 5.2f. It was found that the maximum reducing sugar concentration could be obtained from *T*. *suecica* when the pretreatment was carried out at higher temperatures between 100°C and 120°C for a longer pretreatment period of between 80 to 120 min (Figure 5.2c). Likewise, the maximum reducing sugar concentration from *Chlorella* sp. could be obtained when the pretreatment was conducted at a higher temperature of 110°C at low pretreatment duration (Figure 5.2f). Again, the pretreatment of *Chlorella* sp. using NaOH for longer (> 80 min) will produce less reducing sugar (Figure 5.2e). The study also indicated that the interaction of pretreatment temperature and time can significantly affect the reducing sugar concentration. This study showed that the combination of pretreatment at a high temperature for a longer period displayed disadvantages for pretreatment for both microalgal biomass. Low reducing sugar concentration at high temperature with longer period might be due to the reducing sugar

degradation during the process (Harun et al., 2011). This result was verified by the ANOVA test in which p-values of the X_2X_3 interaction (β_{23} terms) for both microalgal biomass were less than 0.05.

Generally, this study indicated that different types of microalgae species required different alkaline pretreatment conditions. *T. suecica* biomass required higher temperature, higher alkaline concentration, and longer retention time compared with *Chlorella* sp. to obtain optimum reducing sugar concentration. In contrast, a higher reducing sugar concentration from *Chlorella* sp. could be obtained at a higher temperature, a higher alkaline concentration, but over a shorter time. These findings are in agreement with Mahdy et al. (Mahdy et al., 2014) who reported that the pretreatment of microalgal biomass is species dependent. This difference may be attributed to the differences in their cell wall structure. Complex cell walls require more extreme conditions to be disrupted. It was reported that strain *T. suecica* have five layers of cell wall structure (Azma et al., 2010). This complex cell wall structure confims the resistance of *T. suecica* compared to *Chlorella* sp, which only have two distinct cell wall structures (Gerken et al., 2013).

5.3.6 *Enzymatic saccharification of pretreated microalgal biomass*

Enzymatic saccharification of untreated and pretreated microalgal biomass have been carried out using degradation enzyme obtained from *Trichoderma longibrachiatum*. Initially, the *T. suecica* and *Chlorella* sp. biomass were pretreated under the optimum conditions obtained from the optimisation study prior to enzymatic saccharification. Figure 5.3 shows the reducing sugar concentration from untreated and pretreated microalgal biomass. As expected, the untreated microalgal biomass showed lower reducing sugar concentration compared to the pretreated microalgal biomass. Our results indicate that reducing sugar concentration of approximately 60 mg/g $_{\text{dry biomass}}$ and 100 mg/g $_{\text{dry biomass}}$ could be obtained from untreated *T. suecica* and *Chlorella* sp. biomass. Following alkaline pretreatment, the reducing sugar concentration increased to 150 mg/g $_{\text{dry biomass}}$ and 203 mg/g $_{\text{dry biomass}}$ for pretreated *T. suecica* and *Chlorella* sp. biomass respectively.

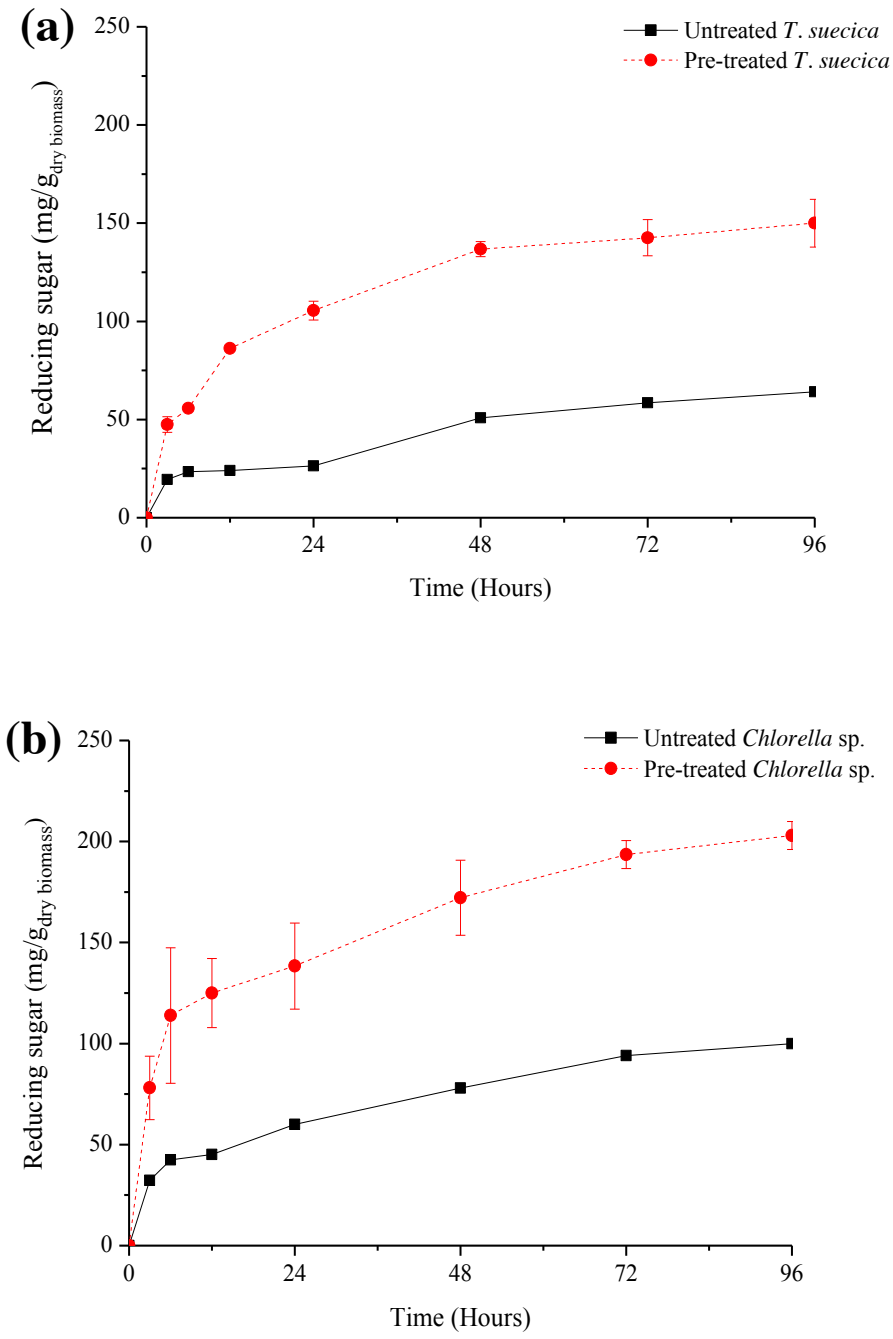

Figure 5.3 Reducing sugar produced from enzymatic saccharification of untreated and alkaline pretreated microalgal biomass - (a) *T. suecica*, (b) *Chlorella* sp.

Higher reducing sugar concentration obtained from pretreated biomass is mainly influenced by the physical-structure of biomass. Changes of biomass physical structure have been reported to improve the enzymatic digestibility of alkali pretreated biomass (Kim and Holtzapple, 2005). Thus, further FTIR and SEM analyses were carried out to determine the effect of pretreatment on the microalgal biomass cell structure.

5.3.7 *FTIR spectroscopy and SEM*

The alkaline pretreatment cause the cleavage of the hydrolysable linkage such as α- and β-aryl ether glycosidic bonds of polysaccharide and removes the acetyl group from the biomass (Chen et al., 2013). In order to determine the effect of alkaline treatment on a biomass sample, FTIR analysis has been widely used to characterise the functional group on biomass surface structure after the pretreatment process (Rawat et al., 2013). Figure 5.4 shows the comparison of FTIR spectra for both untreated and pretreated microalgal biomass. The spectra shows obvious effects peak near 3400 to 3200 cm^{-1}, 1720 to 1600 cm^{-1}, 1245 cm^{-1}, and 1098 to 900 cm^{-1}. The peak near 3400 to 3200 cm^{-1} was representative of the hydroxyl (OH) group in the samples (Siengchum et al., 2013) The peak near 1720 to 1600 cm^{-1} represents lipid and protein in the samples (Sukarni et al., 2014). A large reduction in intensity was observed in this region, which indicates that lipid and protein were removed during the pretreatment process.

A FTIR analysis also indicates that dilute alkaline pretreatment had an obvious effect on band 1245 cm^{-1}, which is associated with the acetyl group in the biomass (Sun et al., 2005). An obvious reduction of this band in both biomass after the pretreatment process strongly indicates the cleavage or alteration of the acetyl group on the biomass surface. Reduction of this band indicates that deacetylation of microalgal biomass occurred during the pretreatment process.

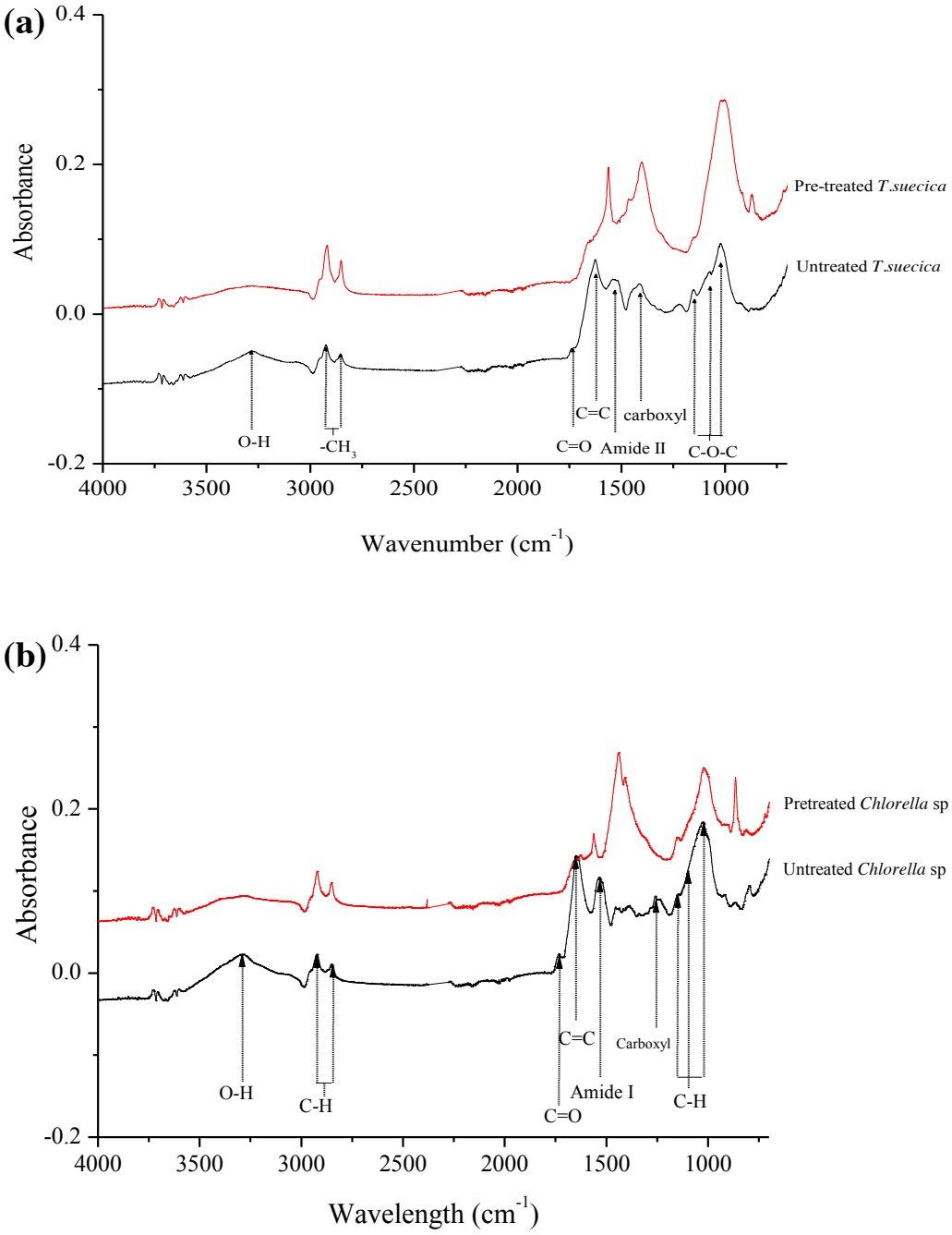

Figure 5.4 FTIR spectrum for untreated and alkaline pretreated microalgal biomass - (a) *T. suecica* (b) *Chlorella* sp.

Generally, microalgal cell wall consists of a glycoprotein and polysaccharide matrix as its main structure (Domozych et al., 2012). It was reported that *T. suecica* from class Prasinophyte consists of a thick amino sugar cell wall (Becker et al., 1994). Meanwhile, in *Chlorella* sp. that is classified from Trebouxiphycea, the cell wall contains cellulose and some coating of a chitin-like polysaccharide polymer (Kapaun and Reisser, 1995). Thus, this observation leads to the conclusion that the acetyl group in N-acetylocosamine is part of the biopolymer in the microalgal cell wall that has been cleaved off during alkali treatment.

SEM analysis was conducted to determine the structure changes and surface characteristic of the microalgal biomass. SEM images of both microalgal biomass before and after alkaline pretreatment at the optimum conditions are shown in Figure 5.5. A comparison of SEM images shows significant changes to the biomass structure after being subjected to the alkaline pretreatment. As shown in Figure 5.5(a) and 5.5(c), untreated *T. suecica* and *Chlorella* sp. biomass have a actual microalgal cell structure form. Meanwhile, uneven distribution and rough surface structure can be observed on the surface of the both treated biomass. The image clearly demonstrates that the dilute alkaline pretreatment disrupted and changed the surface structure of the microalgal biomass. The cracks and uneven structure provide rapid accessibility for the degradation enzyme to attack the inner structure of the biomass during enzymatic saccharification. Other studies have reported similar observation on the structural change of biomass after alkaline pretreatment (Chen et al., 2013; Rawat et al., 2013).

Overall, this study found that most acetyl groups and microalgal biomass structures were disrupted during the pretreatment process, thus confirming the effectiveness of dilute alkaline pretreatment for selective removal of the acetyl group and to distrupt the microalgal cell structure. The alkaline pretreatment process, therefore, provides greater accessibility for enzymatic saccharification of the microalgal biomass.

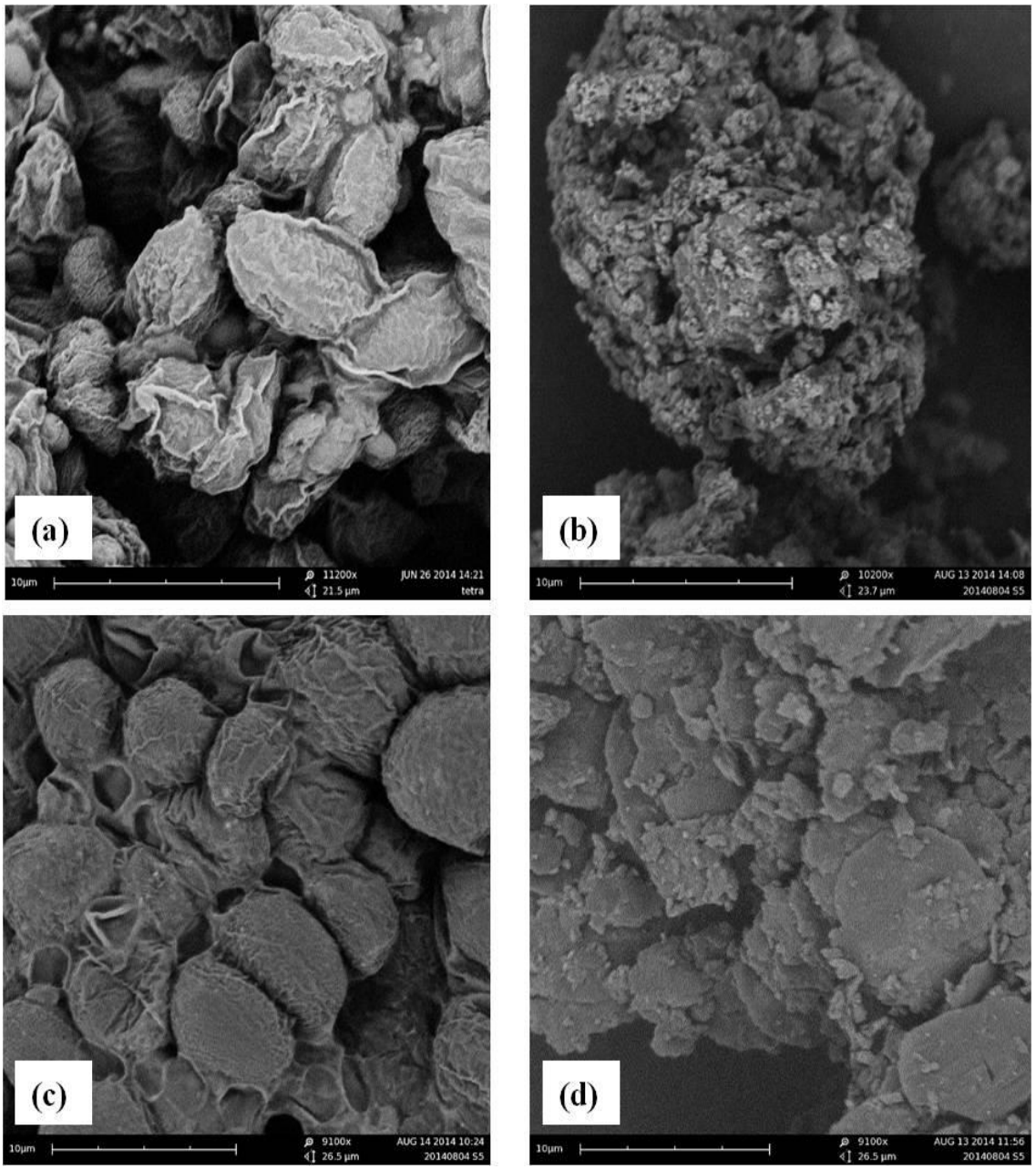

Figure 5.5 SEM of microalgal biomass before (a) *T*. *seucica*, (b) pretreated *T*. *suecica*, (c) *Chlorella* sp. (d) pretreated *Chlorella* sp.

5.3.8 *Comparison of alkaline and acid treatment*

Comparison of alkaline and acid pretreatment of microalgal biomass performance was performed. In this study, the acid treatment was performed at the optimum condition obtained from the alkaline pretreatment study. Figure 5.6 indicates that different pretreatments showed different effects on the microalgal biomass and sugar production. It can be seen that higher sugar (163.9 mg/g $_{\text{dry biomass}}$) was produced from the acid treatment of *T. suecica* compared to alkaline treatment, indicating that high reducing sugar was lost and dissolved in the acid liquor during the pretreatment process. In contrast, low concentration or reducing sugar (32.48 mg/g $_{\text{dry biomass}}$) was obtained from the acid pretreatment of *Chlorella* sp. In comparison to the alkaline treatment, this could be due to the sugar degradation that occurred during the pretreatment process.

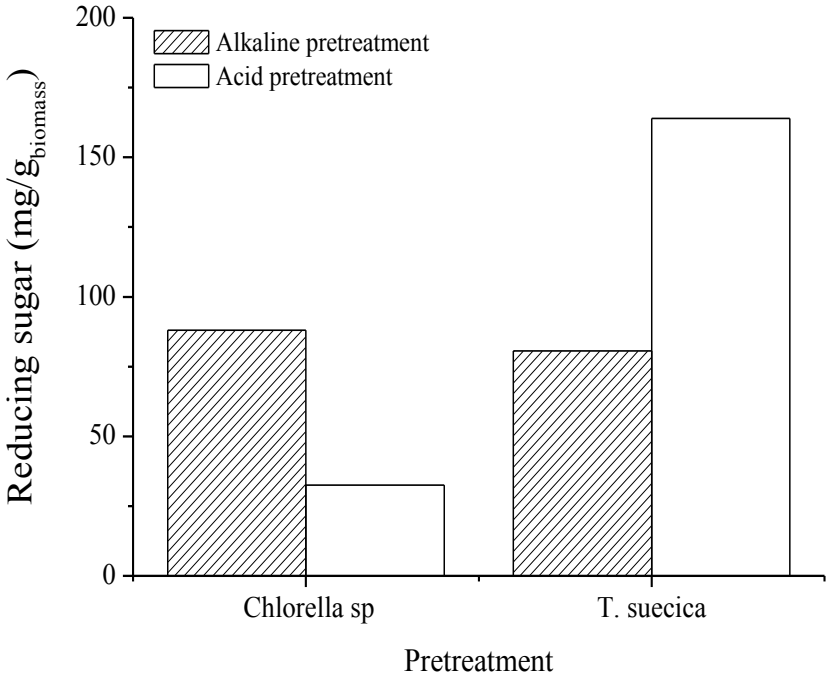

Figure 5.6 Comparison of alkaline and acid treatment for *Chlorella* sp. and *T. suecica*.

5.4 Conclusions

In this study, the effect of alkaline pretreatment on reducing sugar concentration production from *T. suecica* and *Chlorellas* sp. biomass was investigated. The highest reducing sugar concentration for *T.suecica* was obtained when the pretreatment was carried out at 120 °C using 2% KOH for 120 min, while that for *Chlorella* sp. was obtained when the pretreatment was conducted at 120°C using 2% NaOH for 30 min. Comparison of *T. suecica* and *Chlorella* sp. showed that high reducing sugar concentration from both microalgal biomass can be obtained using alkaline pretreatment. High reducing sugar concentration from pretreated microalgal biomass during enzymatic saccharification indicates that the pretreatment process successfully disrupted the microalgal cellular structure and improved the accessibility of the microalgal biomass to enzymatic reactions. The dilute alkaline pretreatment method used less hazardous chemicals thus proving to be a promising method to enhance reducing sugar production prior to fermentation. On the other hand, the high sugar concentration in the alkaline liquor produced during the alkaline pretreatment shows great potential to be used as a value-added chemical and biofuel feedstock. The results obtained from this study provide new insight into low cost and environmentally friendly solutions for the microalgal pretreatment process.

Focus on the next chapter

This chapter discussed the pretreatment of *Chlorella* sp. and *T. suecica* using a dilute alkaline pretreatment approach. These experiments provide information on the feasibility of this approach to be applied to pretreat microalgal biomass. It is very important to pretreat microalgal biomass in an environmentally friendly manner that at the same time could preserve the main chemical composition in the microalgal biomass.

Further experiments on optimisation of enzymatic saccharification, in order to extract the reducing sugar from *Chlorella* sp. and *T. suecica,* was performed and the results will be discussed in the next chapter.

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CHAPTER 6

ENZYMATIC SACCHARIFICATION OF PRETREATED MICROALGAL BIOMASS

6.1 Introduction

Renewable and sustainable energy production from biomass has gained much attention, particularly in the bioenergy and biotechnology sectors (Tustin, 2012). Liquid fuels such as bioethanol and biobutanol from biomass are believed to have the future potential to partly replace gasoline as a transportation fuel (Pfromm et al., 2010). Generally, these liquid fuels are produced through the conversion of cellulose or carbohydrate-based feedstock. Starch materials, agro-forestry biomass, energy crops and starchy materials are currently being used as a feedstock (Domozych et al., 2012; Jang et al., 2012). However, several drawbacks such as the using food-versus-fuel, an increase in land and water utilisation for biomass production, a lack of sustainability, and the high-cost of production have led to the exploration of a new type of fuel feedstock.

Renewable biomass feedstock such as algal biomass has the potential to produce liquid fuel (Posten and Schaub, 2009). Microalgae, which has a higher growth rate than lignocellulosic biomass, has the ability to use CO_2 produced from power plants as a carbon source with high carbohydrate content. Thus, it has an advantage over lignocellulosic and starchy feedstock (Suali and Sarbatly, 2012).

To produce this liquid fuel from microalgal biomass, the microalgae must go through a series of processes - biomass production, pretreatment, enzymatic saccharification and anaerobic fermentation (Balat, 2011). Pretreatment and enzymatic saccharification are crucial steps involving cell disruptions and the release of fermentable sugar from the microalgal biomass. The rigid microalgal cell wall needs to be hydrolysed through pretreatment either using acid or alkali as a catalyst prior to enzymatic saccharification (Harun and Danquah, 2011). Pretreatment using acid is widely used to pretreat microalgal biomass (Hernández et al., 2015; Lam et al., 2014), however, harsh acid treatment is believed to produce a fermentation inhibitor and is not environmentally friendly. Unlike this, the alkali treatment method is considered very environmentally friendly as this method uses a low concentration of alkali. Additionally, this method is also suitable for biomass with low lignin content. Besides, the alkaline pretreatment method also is reported to produce less inhibitor which can affect the fermentation process (Imman et al. 2014; Kataria et al., 2013; Ramirez, 2013).

Another obstacle for biofuel through the biochemical process is enzymatic saccharification. Enzymatic saccharification in optimum conditions is an important step for extracting the reducing sugar from the microalgal biomass, which can then be converted into bioethanol and biobutanol. Enzymatic saccharification parameters such as the pH, temperature, enzyme concentration and biomass concentration have been reported to have significant effects on the saccharification process (Hamzah et al., 2011). Low reducing sugar production has been reported during enzymatic saccharification at suboptimal conditions (Harun and Danquah, 2011). Although the enzymatic saccharification of microalgal biomass has been reported elsewhere, there is scarce information on the enzymatic saccharification of alkaline pretreated microalgal biomass.

Thus, the main objective of this study was to determine the suitable enzymatic saccharification conditions of alkaline pretreated *Chlorella* sp. and *T. suecica* biomass. The effect of saccharification parameters such as temperature, pH, enzyme concentration and biomass concentration that could influence reducing sugar production from the alkaline pretreated microalgal biomass were also evaluated.

6.2 Material and methods

6.2.1 *Microalgae cultivation condition*

Two microalgal species, *Chlorella sp*. and *Tetraselmis suecica* were used in this study. A modified algae growth (MLA) medium with 0.49 gL^{-1} magnesium sulfate ($MgSO_4.7H_2O$), 1.7 gL^{-1} sodium nitrate ($NaNO_3$), 0.14 gL^{-1} di-potassium phosphate (K_2HPO_4) 0.03 gL^{-1} and calcium chloride ($CaCl_2.2H_2O$) was used as the seed culture and biomass production medium. The medium was initially sterilised using a 0.22 μm Millipore filter. Microalgal seeds were cultivated in a 1 L Scott bottle containing 700 mL of the modified MLA. The bottle was incubated in an illuminated incubator chamber with 0.3 $Lmin^{-1}$ compressed air under light with a photon intensity of 450 $\mu mol/m^2s$. The cultivation temperature was 30.0 ± 0.2°C. The microalgae cultures were harvested during the late logarithmic growth phase. Each harvested sample was centrifuged at 4500 rpm for 15 min. The resulting pellet was rinsed twice with distilled water and subsequently dried at 60 °C for 24 h. The dried biomass obtained was used for further study.

6.2.2 *Chemical composition*

The lipid, carbohydrate and protein contents of microalgal biomass were determined using soxhlet extraction, phenol-sulfuric acid method, and Lowry method analyses respectively (Kassim et al., 2014).

6.2.3 *Pretreatment of microalgal biomass*

A total of 1.0 g dried microalgal biomass sample was measured and soaked in 100 mL of 2% (wt) alkaline agent in a 250 mL Scott bottle. The mixture was placed in an oven and incubated at 120°C. After the incubation period, the sample was removed and cooled at room temperature. The sample was then centrifuged at 3000 rpm for 10 min. The supernatant was separated and the solid residue was subjected to enzymatic saccharification.

6.2.4 *Enzymatic saccharification*

Preliminary enzymatic saccharification of both raw and pretreated microalgal biomass were soaked in 10 mM acetate buffer (pH 5.5) and mixed with cellulase from *Trichoderma longibrachiatum* at 45°C with a rate of agitation of 150 rpm in an orbital shaker (Thermoline Scientific) for 96 h. The cellulase enzyme (1.0 μmole of glucose from substrate) (C9748, Sigma Aldrich) used in this study is a mixture of xylanase, pectinase, mannose, β-glucosidase, amylase and potease activity. The samples were withdrawn every 24 h and heated at 100°C to deactivate the enzymatic reaction activity. The samples were then centrifuged at 3500 rpm for 5 min and the supernatant was used for reducing sugar analysis. The reducing sugar estimation will be described in next section. The saccharification yield was calculated as:

$$Saccharification\ yield\ (\%) = \frac{concentration\ of\ glucose\ at\ time\ of\ t\ x\ 0.9}{carbohydrate\ content\ after\ pre-treatment} x\ 100 \qquad (6.1)$$

6.2.4.1 *Primary screening*

Determination of suitable enzymatic saccharification conditions was carried out using two approaches. The first approach is enzymatic saccharification using a one variable at a time design (OVAT) method. In this study, a total of four series of experiments were performed. Enzymatic saccharification of both pretreated microalgal biomass were carried out by incubating the sample at a temperature ranging between 30-60°C, pH ranging between 3.5-6.5, an enzyme to biomass ratio of 0.02 to 1 and biomass concentration between 5-30 gL^{-1}. In enzyme to biomass ratio study, five different enzyme concentrations varied from 5 mg to 25 mg with an initial amount of 250 mg microalgal biomass being used. A total of 1.0 mL sample was withdrawn for every 24 h for analysis.

6.2.4.2 *Optimization of enzymatic saccharification*

The second approach is using a general full factorial matrix as shown in Table 6.1. In this study, the combination of enzymatic saccharification parameters such as temperature, pH and solid loading were evaluated. The enzymatic saccharification was carried out for 72 hours and 1 mL sample was withdrawn every 24 h for the reducing sugar production analysis. The reducing sugar estimation was carried out as described in next section.

The regression model for full factorial is expressed in the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} \beta_2 \beta_3 \qquad (6.2)$$

where Y is the saccharification yield (% of carbohydrate content), β_0 is the intercept coefficient, β_1 represents the independent effect of factor X_1 (temperature), β_2 represents the independent effect of factor X_2 (pH) and β_3 represents the independent effect of factor X_3 (biomass concentration). The model evaluated the interaction effect of each parameters and was expressed by the correlation coefficient R^2.

Table 6.1 General full factorial matrix for enzymatic saccharification of alkaline pretreated microalgal biomass.

Run	Solid loading (g/L)	Temperature (°C)	pH
1	5	40	3.5
2	5	40	4.5
3	5	40	5.5
4	5	40	6.5
5	10	40	3.5
6	10	40	4.5
7	10	40	5.5
8	10	40	6.5
9	30	40	3.5
10	30	40	4.5
11	30	40	5.5
12	30	40	6.5
13	5	50	3.5
14	5	50	4.5
15	5	50	5.5
16	5	50	6.5
17	10	50	3.5
18	10	50	4.5
19	10	50	5.5
20	10	50	6.5
21	30	50	3.5
22	30	50	4.5
23	30	50	5.5
24	30	50	6.5

6.2.5 *Reducing sugar estimation*

Total reducing sugar was determined using the 3, 5 dinitrosalysilic acid (DNS) method (Pradeep et al., 2013). Generally, the filtered sample was added with 1 mL of DNS reagent and then boiled for 10 min in a water bath. The reaction mixture was allowed to cool and the reducing sugar concentration was estimated by using a UV spectrophotometer (Hach, DR-5000) at 540 nm. The result was expressed in milligrams of reducing sugar per gram of dry microalgal (DM) biomass.

6.2.6 *High performance liquid chromatography (HPLC) analysis*

Reducing sugar composition in microalgal hydrolysate was identified by high pressure liquid chromatography (HPLC) (Waters, USA), equipped with RH sugar-pack column and a refraction index detector. The conditions for the HPLC were detector temperature: 45°C, column temperature: 75°C, flow rate: 0.6 mL/min and an injector volume of 1 µL. The

effluent consisted of a 5mM H2SO4 solution that was previously filtered using a 0.2 µm membrane filter.

6.2.7 *Statistical analysis*

All samples were analysed in triplicates. A T-test was used to determine the significant difference between the control and the experimental parameters. The statistical analysis was performed using OriginPro software.

6.3 Results and discussion

6.3.1 *Microalgal chemical composition*

Chemical compositions of *Chlorella* sp. and *T. suecica* biomass are shown in Table 6.2. The major component of both biomass was protein, followed by carbohydrate and lipids. The significant amount of carbohydrate content in both microalgal biomass shows that these species have a high potential to be used as a fermentation feedstock.

Table 6.2 Main chemical compositions in untreated *Chlorella* sp. and *T. suecica* biomass.

Percentage (wt %) on dry weigh basis	*Chlorella* sp.	*T. suecica*
Carbohydrate	38.83	26
Protein	46.24	58
Lipid	14.92	15

6.3.2 *Enzymatic saccharification of alkaline pretreated microalgal biomass*

Pretreatment of biomass using an alkaline agent has been used to alter the biomass structure to significantly increase enzymatic digestibility (Agbor, 2011). In this study, the pretreatment was applied to pretreat both biomass using an alkaline agent as a catalyst as reported in our previous study. The pretreatment of *Chlorella* sp. was carried out using 2% (w/v) NaOH at 120°C for 30 min. Meanwhile, the pretreatment of *T. suecica* biomass was carried out using 2% (w/v) KOH at 120°C for 120 min.

Enzymatic saccharification of untreated and pretreated biomass was carried out using cellulase enzyme. The results are presented in Figure 6.1. As expected, the untreated microalgal biomass produced a lower reducing sugar amount compared to the pretreated microalgal biomass. Our study for the first time shows that alkaline pretreatment was found to significantly increase the reducing sugar production from both microalgal biomass. This finding can be explained by the alkaline pretreatment having a significant effect on the biomass structure. Based on our previous SEM and FTIR analyses, the physical structure of microalgal biomass was disrupted after the alkaline pretreatment process. Thus, it provides better access for the enzyme to attack polysaccharide in the microalgae biomass.

6.3.3 *Carbohydrate content after alkaline pretreatment*

Further enzymatic saccharification was carried out to determine the suitable conditions for producing a higher reducing sugar concentration from pretreated *Chlorella* sp. and *T. suecica* biomass. It should be noted that reducing sugar from microalgal biomass can be derived from its total carbohydrate content. Table 6.3 shows the carbohydrate content before and after alkaline pretreatment. A slight reduction in the carbohydrate content for *T. suecica* was observed after pretreatment, which indicates that a significant amount of carbohydrate in the biomass was dissolved in alkaline liquor during the pretreatment process. In contrast, the carbohydrate content in *Chlorella* sp. was higher after pretreatment compared to before pretreatment. This indicated that only a slight amount of carbohydrate content was dissolved during the pretreatment process.

Table 6.3 Carbohydrate content before and after alkaline pretreatment for microalgal biomass.

Percentage (%) on dry weight basis	Before pretreatment	After pretreatment
Chlorella sp.	38.83 ± 2.39	41.44 ± 1.71
T. suecica	26.41 ± 2.08	19.26 ± 3.21

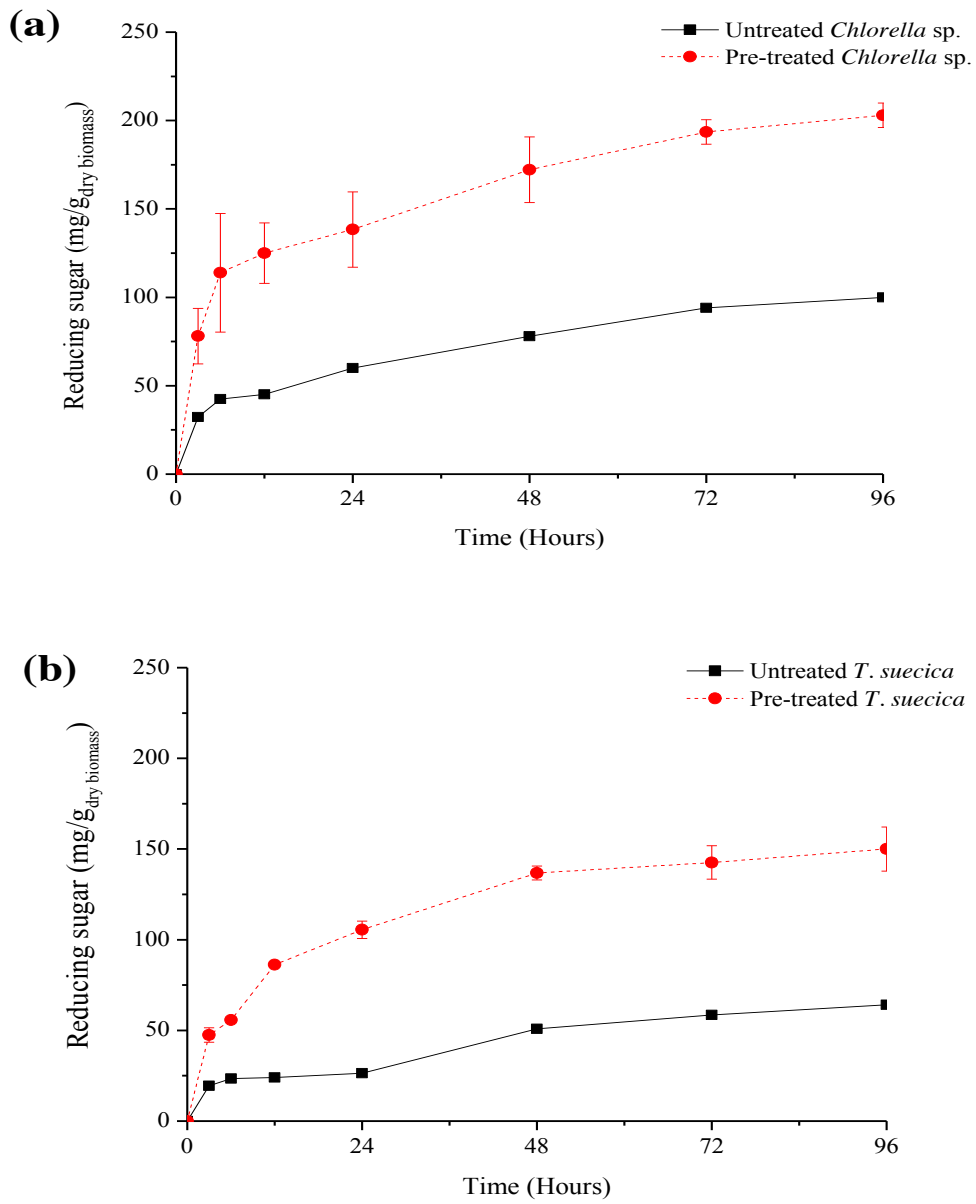

Figure 6.1 Reducing sugar produced from the enzymatic saccharification of untreated and pretreated biomass - (a) Enzymatic saccharifcation of *Chlorella* sp. biomass, (b) Enzymatic saccharifcation of *T. suecica* biomass.

6.3.4 Determination of enzymatic saccharification parameters

Experimental assessment of the suitable enzymatic saccharification conditions for achieving the maximum reducing sugar concentration and the saccharification yield were carried out in this study. Generally, the enzymatic saccharification can be influenced by two major factors that are specifically enzyme-related—temperature and pH value—and substrate-related factors—enzyme concentration and substrate concentration (Leu and Zhu, 2013). In this study, the effect of these enzymatic saccharification parameters have on reducing sugar production from pretreated microalgal biomass was evaluated.

6.3.4.1 *Effect of temperature*

The influence of temperature on reducing sugar production was evaluated using four different temperature ranges from 30 to 60°C. Figure 6.2 shows the reducing sugar concentration and enzymatic saccharification yield for both microalgal biomass after 72 hours. The maximum reducing sugar concentration for *Chlorella* sp. and *T. suecica* of 293.50 ± 2.88 and 135.66 ± 4.37 mg/g $_{\text{dry biomass}}$ were obtained at 50°C and 40°C, respectively. An approximate 66% saccharification yield was obtained from the saccharification of pretreated *Chlorella* sp. at 50°C. Similarly, a saccharification yield of 57% was observed for pretreated *T. suecica* at 40°C. Low sugar concentration was obtained from both microalgal biomass for enzymatic saccharification at 60°C. The statistical analysis showed that there was a significant difference (p=<0.05) between tested temperatures, indicating that saccharification temperature played a significant role in reducing sugar production from both microalgal biomass.

Similar observations have been reported on the saccharification of microalgae *Clorococcum* sp. (Harun and Danquah, 2011). The highest reducing sugar production obtained at this temperature could be due to cellulase enzyme activity used in this study. Cellulase enzyme produced from *T. longibrachiatum* has been reported to have an optimum activity within 30–45°C (Gautam et al., 2011). Low reducing sugar concentration produced at higher temperatures may be attributable to the inactivation of cellulase enzymes during the process (Andreaus et al., 1999). According to Gautam et al. (2011), saccharification at higher temperatures will cause modification on the enzyme active site and reduce availability for substrate binding, consequently reducing the enzymatic saccharification process.

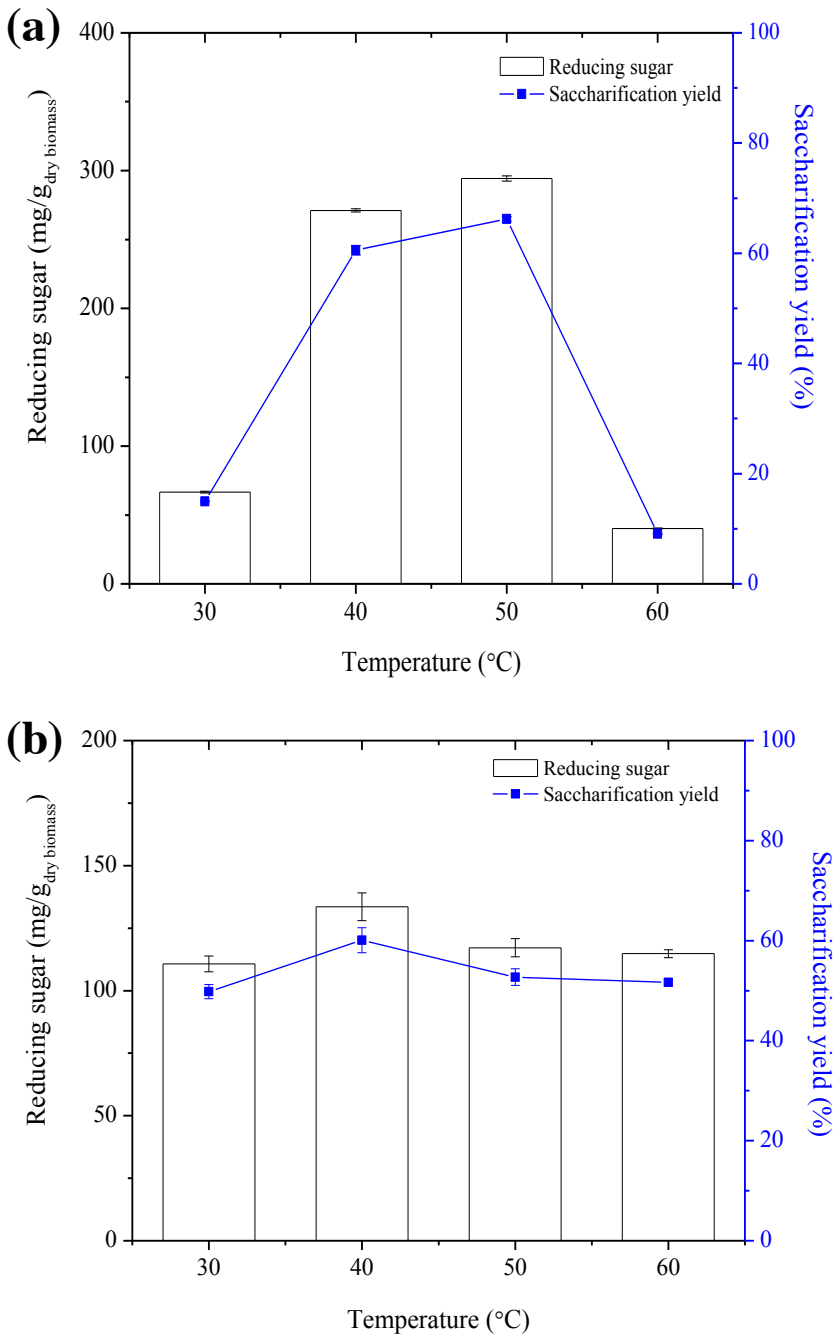

Figure 6.2 Effect of temperature on enzymatic saccharification of pretreated microalgal biomass - (a) *Chlorella* sp. (b) *T. suecica*.

6.3.4.2 *Effect of pH*

Figure 6.3 shows the reducing sugar concentration produced from saccharification at different pH buffer values. The highest reducing sugar concentration for pretreated *Chlorella* sp. biomass was obtained when saccharification was carried out at a pH value range of 4.5 to 6.5. The highest reducing sugar concentration from alkaline pretreated *Chlorella* sp. was obtained from saccharification at a pH value of 5.5 with a reducing sugar and saccharification yield of 301.61 ± 4.25 mg/g $_{\text{dry biomass}}$ and 67%, respectively. Similarly, the maximum reducing sugar from *T. suecica* of 145.55 ± 2.17 mg/g $_{\text{dry biomass}}$ was obtained at pH 4.5. Low sugar concentration was observed for enzymatic saccharification at lower and higher than optimum pH values. The one-way ANOVA analysis was also carried out and the result showed that there was a significant effect of a pH value test on reducing sugar production from both pretreated microalgal biomass (p=<0.05). This indicated that the pH value used could influence the enzymatic saccharification process and reduce the sugar production from microalgal biomass.

The results obtained are coherent with studies on other types of microalgal biomass (Choi, 2010; Harun and Danquah, 2011). Harun and Danquah (2011) investigated enzymatic saccharification of *Chlorococcum* sp. and reported that the maximum reducing sugar concentration obtained from this microalgal was observed at pH 4.8. A similar study on enzymatic saccharification of microalgae *Chlamydomonas reinhardtii* indicated that the highest reducing sugar was attained at saccharification in a buffer with a pH of 4.5 (Choi, 2010). Further, maximum reducing sugar production from *Dunaliella tertiolecta* was observed from saccharification at pH 5.5 (Lee et al., 2013). In summary, these results indicate that the suitable pH value to obtain maximum reducing sugar concentration from microalgal biomass is within a value range of 4.5 to 5.5.

In our experiment, low sugar concentration was obtained from enzymatic saccharification at pH 3.5 and 6.5. Low reducing sugar produced in strongly acidic and alkaline conditions might be due to the low enzyme activity within these conditions. Strongly

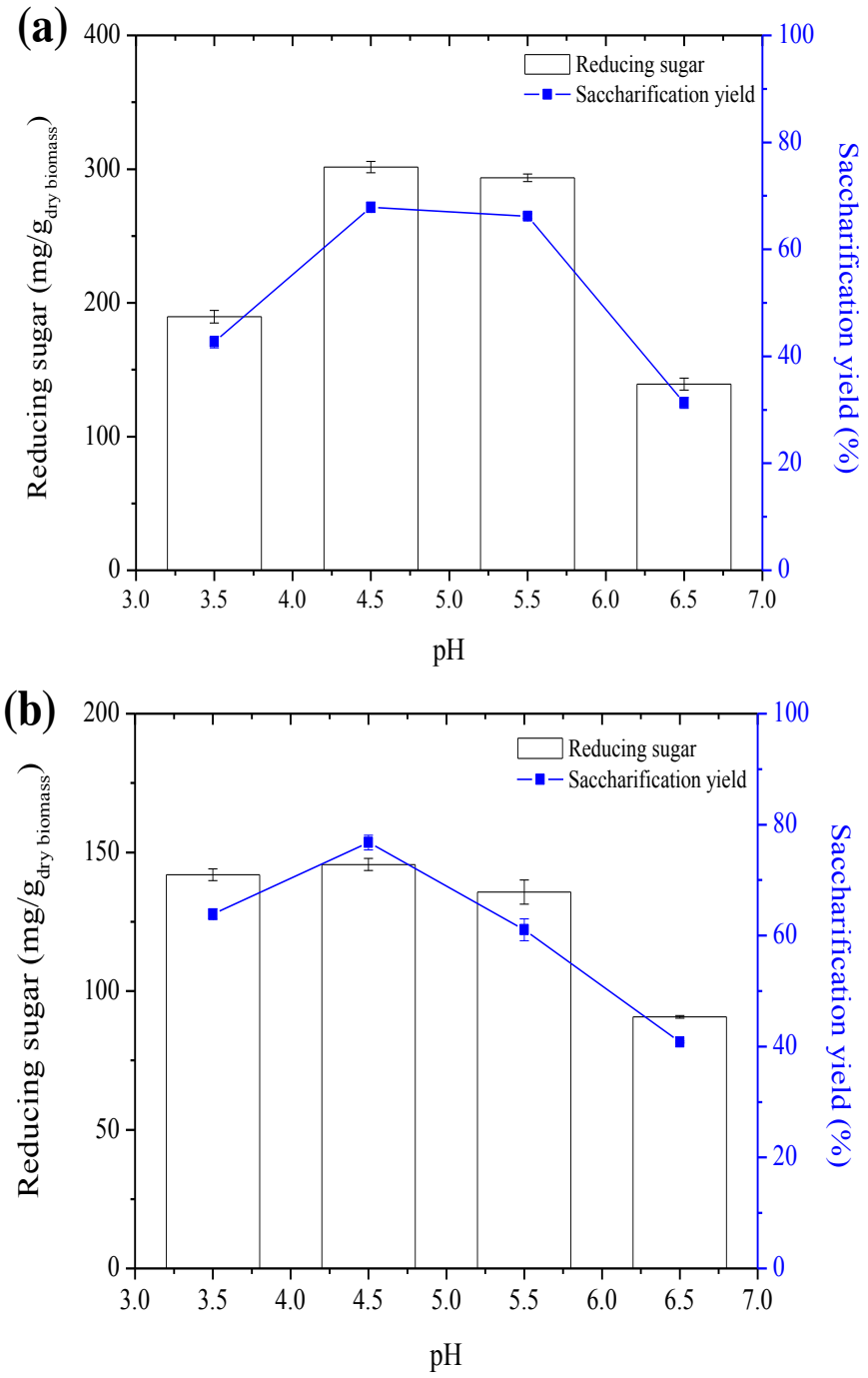

Figure 6.3 Effect of pH on enzymatic saccharification of microalgal biomass - (a) *Chlorella* sp. (b) *T. suecica*.

acidic and alkaline environments will change the electrostatic binding in the enzyme molecule, resulting in an unfolded or denatured enzyme structure (Harun and Danquah, 2011). Consequently, the cellulase enzyme becomes indirectly inactive.

6.3.4.3 *Effect of enzyme to solid ratio/ enzyme concentration*

Enzyme concentration is one of the important parameters involved in the hydrolysis process (Hamzah et al., 2011). Generally, high enzyme concentration results in better saccharification, probably through increasing the rate and saccharification yield (Kermanshahi-pour et al., 2014). A higher substrate concentration with a lesser amount of enzyme is considered the suitable approach to ensure the production of reducing sugar is done in an economic way (Kapaun and Reisser, 1995). The effect of enzyme concentrations to the substrate ratio in the range of 0.02 to 0.1 was investigated and the results are presented in Figure 6.4. The results indicated that increasing the enzyme concentration increased the reducing sugar concentration produced from both pretreated *Chlorella* sp. and *T. suecica* biomass. The maximum reducing sugar production for pretreated *Chlorella* sp. of 403 ± 7.62 mg/g $_{\text{dry biomass}}$, and *T. suecica* of 158.38 ± 9.03 mg/g $_{\text{dry biomass}}$ was obtained at E/S ratio of 0.08 and 0.1 respectively. The saccharification yield for *Chlorella* sp. and *T. suecica* at the optimum ratio was 96% and 89% respectively. The study also showed that a further increase in enzyme concentration reduced sugar concentration production from *Chlorella* sp. The maximum reducing sugar concentration for *T. suecica* was attained at a ratio of 0.04. Further, low enzyme concentration is required to produce maximum sugar concentration from pretreated *T. suecica*. This result clearly indicated that pretreated *T. suecica* biomass offers the advantage of reducing fermentation inhibition, making the process more economical.

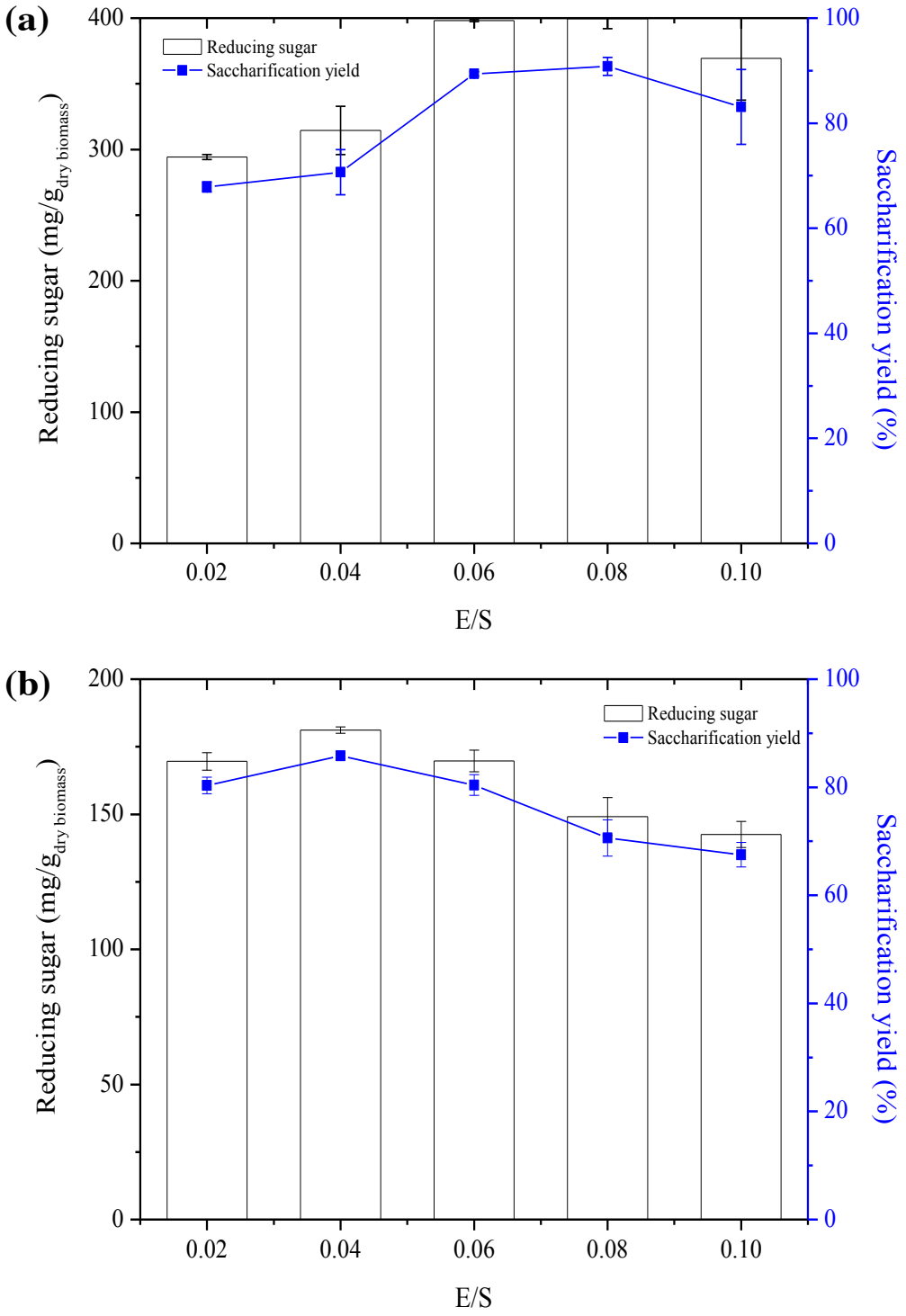

Figure 6.4 Effect of enzyme to biomass concentration ratio (E/S) on enzymatic saccharification of microalgal biomass - (a) *Chlorella* sp. (b) *T. suecica*.

Based on the results obtained, increased enzyme concentration reduced the reducing sugar production in both microalgal biomass. This finding is in accordance with a study by Lee et al. (2013), who reported that saccharification of *Dunaliella tertiolecta* beyond the optimum enzyme concentration for this microalgae species produced low concentration of reducing sugar. The main reason for low reducing sugar obtained at high enzyme concentrations is attributed to the lower absorption efficiency between enzymes and substrates (Soto et al., 1994). Low fermentable sugar produced from higher enzyme loading is from the saturation of the substrate surface with enzyme molecules, which results in a low reaction during the process (Vlasenko et al., 1997; Xu et al., 2007).

6.3.4.4 *Effect of microalgal biomass concentrations*

The effect of the pretreated microalgal biomass concentration on the reducing sugar production was evaluated using 5–30 gL^{-1} of biomass and the results are shown in Figure 6.5. The results showed that the reducing sugar concentration was increased with increments in *Chlorella* sp. biomass concentration. The highest reducing sugar concentration produced from *Chlorella* sp. of 5152 ± 72.9 mgL^{-1} and *T. suecica* of 762.11 ± 2.99 mgL^{-1} was displayed at 20 and 10 gL^{-1} respectively. Further, an increase in microalgal biomass concentration beyond 20 gL^{-1} was found to reduce sugar production. The saccharification yield of both pretreated microalgal biomass at different biomass concentrations was also evaluated in this study. Even though a high reducing sugar concentration was obtained at the high biomass concentration, a low saccharification yield was observed for both microalgal biomass. It was found that an increase of biomass concentration showed a reversal effect on the saccharification yield.

An increase in biomass concentration from 10 gL^{-1} to 30 gL^{-1} resulted in a relatively low saccharification yield (Figure 6.5). When the enzymatic saccharification of *Chlorella* sp. was carried out at higher than 10 gL^{-1}, the saccharification yield decreased drastically. A similar finding was observed for *T. suecica*, which indicated that the saccharification yield for this microalgal biomass decreased when the saccharification was carried out at higher than 20 gL^{-1}. Low rates and a saccharification yield at a higher biomass concentration is likely due to enzyme inhibition that lead to reducing enzyme activity (Zheng et al., 2009). An

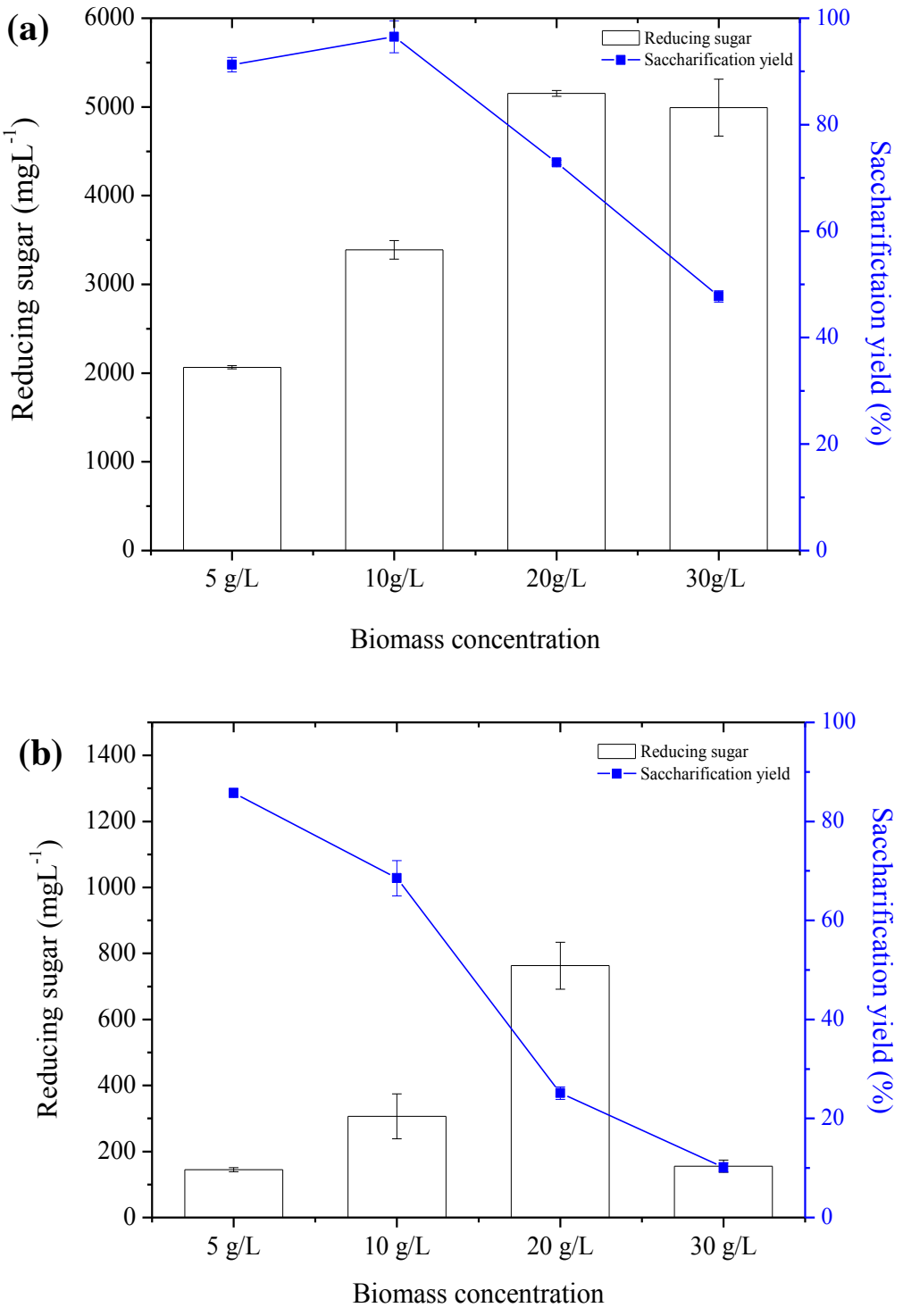

Figure 6.5 Effect of biomass concentration (in 1 L buffer) on enzymatic saccharification of microalgal biomass **-** (a) *Chlorella* sp. (b) *T. suecica***.**

increase in solid loading would reduce sugar production (Ho et al., 2013).

6.3.5 *Optimization of enzymatic saccharification*

The optimization and an interaction between the enzymatic saccharification parameters and their ability to produce reducing sugar from pretreated *Chlorella* sp. and *T. suecica* biomass was investigated in this study. The influence of temperature, pH value and biomass concentration was evaluated. Table 6.4 shows the saccharification yield obtained from enzymatic saccharification at different saccharification conditions. The results showed that the highest saccharification yield for *Chlorella* sp. (96 - 98%) was obtained saccharification using pH value range of 4.5–5.5 at 40°C and 50°C using 5 gL^{-1} of dried biomass (Figure 6.6a). In contrast, the maximum saccharification yield for *T. suecica* was obtained at 40°C with pH value range 4.5, when the saccharification was performed using 5 gL^{-1} biomass (Figure 6.6b).

This study also indicated that the maximum saccharification yield for both microalgal biomass could be obtained at biomass concentration of 5–10 gL^{-1}. A low saccharification yield was observed from saccharification at a higher than 10 gL^{-1} of biomass concentration. Increasing the biomass concentrations and pH value beyond 5.5 reduced the reducing sugar production (Figure 6.6). Saccharification of pretreated *T. suecica* at 50°C produced a low saccharification yield (less than 60%) for all conditions. The results obtained from this study are in agreement with the primary screening reported in previous section.

Analysis of ANOVA for both microalgal species was performed in order to determine the effect of saccharification parameters on the saccharification yield (Appendix C.1 and C.2). The value of the determination of the coefficient (R^2) and adjusted R^2 (Adj. R^2) for *Chlorella* sp. ($R^2 = 0.95$, Adj.$R^2 = 0.82$) and *T. suecica* ($R^2 = 0.97$, Adj.$R^2 = 0.89$) were high, indicated the high significant of the model. The most significant saccharification parameter that could influence saccharification yield of both microalgal biomass was biomass concentration followed by pH value ($P<0.5$). This indicated that these parameters had a great effect on reducing sugar production from microalgal biomass. The results also indicated that saccharification temperature tested in this study did not significantly affect saccharification yield of *Chlorella* sp. and *T. suecica*.

Table 6.4 Effect of temperature, pH value and biomass concentration on enzymatic saccharification yield of *Chlorella* sp. and *T. suecica*.

Microalgae species	Biomass concentration (gL^{-1})	Temperature (°C)							
		40°C				50°C			
		pH 3.5	pH 4.5	pH 5.5	pH 6.5	pH 3.5	pH 4.5	pH 5.5	pH 6.5
Chlorella sp.	5	83.77	98.42	96.35	4.533	60.74	65.90	92.53	27.10
	10	74.85	75.52	89.32	4.92	42.64	53.56	55.46	12.76
	30	4.19	18.11	15.83	3.79	26.15	27.84	14.45	13.24
T. suecica	5	78.62	92.43	30.15	19.98	60.07	58.32	38.72	24.20
	10	70.21	76.94	90.00	62.44	45.17	47.72	34.85	34.50
	30	3.91	25.14	2.99	2.66	44.76	49.27	18.72	15.26

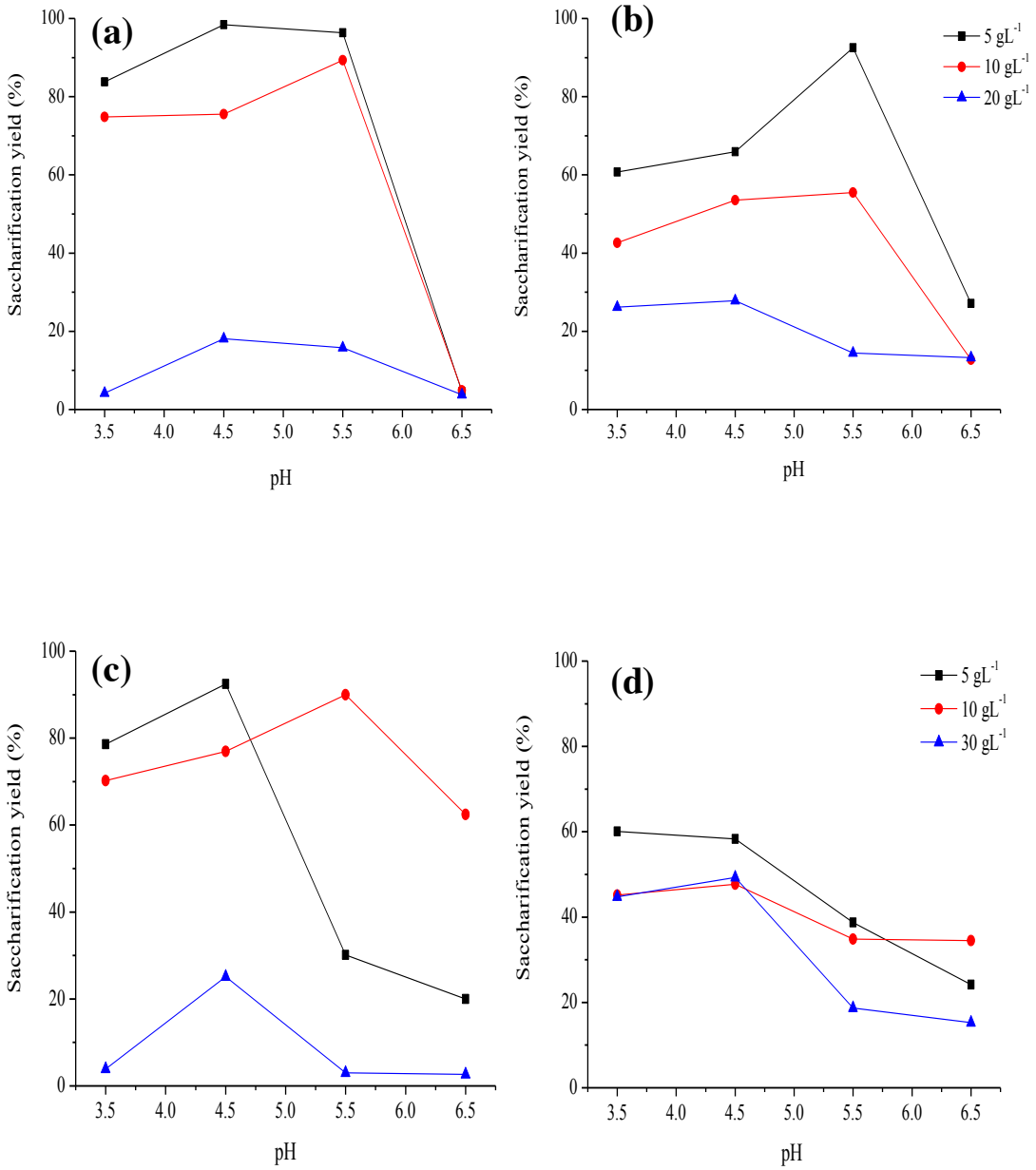

Figure 6.6 Saccharification yield at various enzymatic saccharification conditions - (a) *Chlorella* sp at 40°C; (b) *Chlorella* sp. at 50°C; (c) *T. suecica* at 40°C and (d) *T. suecica* at 50°C.

The statistical analysis on the interaction of saccharification parameters indicated that all the interactive effect did not significantly affect the reducing sugar production (P>0.5) except interaction of temperature and biomass concentration (P<0.5). The regression coefficients for the interaction model of saccharification yield of both microalgal biomass are show as following equation:

$$Y_{Chlorella\ sp.} = 43.35 - 6.09X_1 + 0.25X_2 + 13.75X_3 + 0.24X_1X_2 - 9.26X_1X_3 - 0.47X_2X_3 \quad (6.3)$$

$$Y_{T.\ suecica} = 42.21 - 2.97X_1 + 8.71X_2 + 10.19X_3 + 2.93X_1X_2 - 5.10X_1X_3 + 9.54X_2X_3 \quad (6.4)$$

The enzymatic saccharification at optimum conditions for both pretreated *Chlorella* sp. and *T. suecica* biomass was performed based on the suggested conditions Table 6.5 and the reducing sugar composition in the hydrolysate was analysed. The results indicated that the saccharification yield of *Chlorella* sp. and *T. suecica* increased 3.5 times when the saccharification was performed at the optimum conditions (Figure 6.7). A HPLC analysis indicated that the major reducing sugar present in the hydrolysate was glucose 50% (*Chlorella* sp.: 53.53%, *T. suecica*: 50.65%) followed by xylose 45% (*Chlorella* sp.: 43.43% and *T. suecica*: 48.62%). A small amount of maltose was detected in the microalgal hydrolysate (*Chlorella* sp.: 3.03% and *T. suecica*: 0.72%) (Figure 6.8). A high concentration of glucose and xylose in the hydrolysate showed the potential of the hydrolysate generated from the *Chlorella* sp. and *T, suecica* for a biofuel feedstock through the fermentation process.

Table 6.5 Model predicted value for enzymatic saccharification of pretreated microalgal biomass at optimum condition.

Microalgae	Temperature (°C)	pH	Biomass concentration (gL^{-1})	Predicted	Experimental
Chlorella sp.	40	5.5	5	99.00	98.77
	50	5.5	5	91.68	93.53
T. suecica	40	4.5	5	86.76	92.43
	40	4.5	10	78.51	87.95

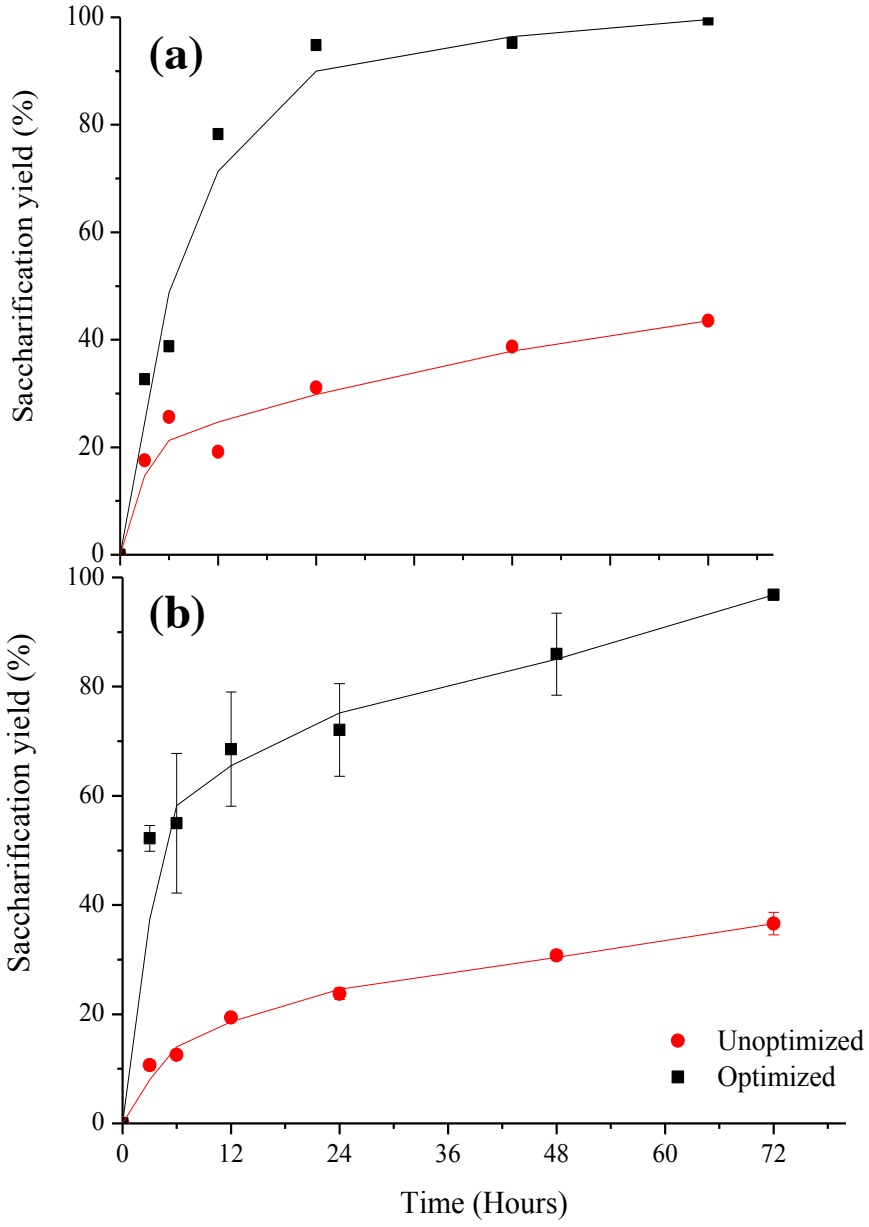

Figure 6.7 Reducing sugar production from the saccharification at unoptimum and optimum conditions - (a) *Chlorella* sp. (b) *T. suecica*.

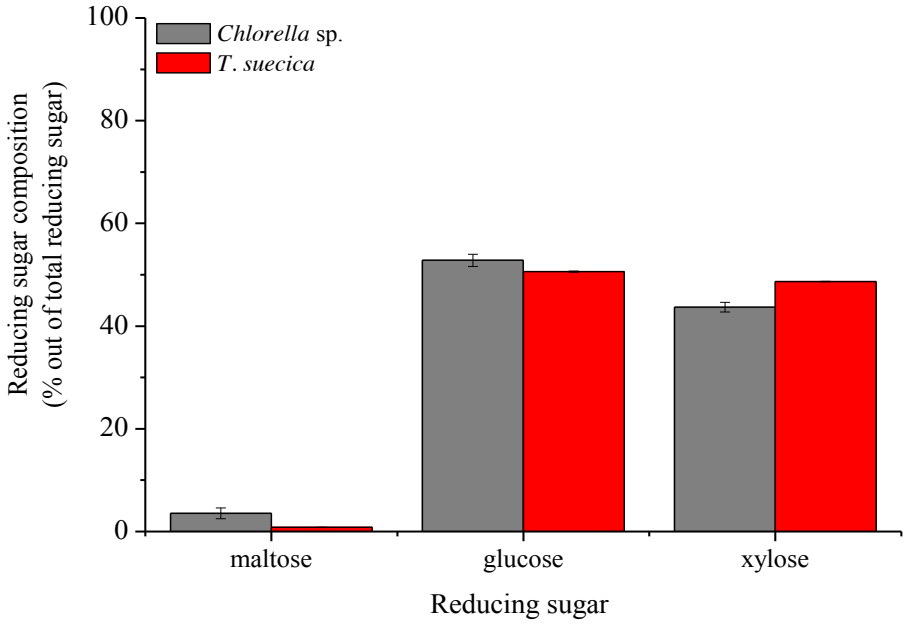

Figure 6.8 Reducing sugar composition out of total reducing sugar extracted from microalgal biomass saccharified at optimum conditions.

Overall, this study showed that *Chlorella* sp. and *T. suecica* required different enzymatic saccharification conditions to produce maximum saccharification yield. The conversion from 80–100 % for *Chlorella* sp. was obtained at 40°C using a pH buffer of 5.0–5.5 and 5 to 10 gL^{-1}. Similarly, the saccharification yield range of 80–100 % for *T. suecica* was observed at 40°C using a pH buffer of 4.5–5.5 and 10 gL^{-1}. The difference of enzymatic saccharification between *Chlorella* sp. and *T. suecica* is attributed to the microalgal species used in this study. A previous study on an enzymaticsaccharification of *Chlorella sorokina*, *Nannochloropsis gaditana* and *Scendesmus almeriensis* found that saccharification conditions are significantly influenced by the microalgae species and polysaccharide composition in the microalgal cell (Hernández et al., 2015).

This result is in agreement with other studies on saccharification on other types of microalgal biomass (Harun and Danquah, 2011). It was reported that the saccharification of *Chlorella vulgaris* FSP-E was inefficient when the biomass concentration exceed 20 gL^{-1} (Ho et al., 2013). Low reducing sugar produced at high biomass concentration may also be due to the high viscosity of the biomass slurry that results in insufficient dispersion of biomass and

less efficient enzyme activity in the vessel (Ioelovich and Morag, 2012). However, the measurement of the biomass slurry viscocity is beyond the scope of this study.

Our results can be compared with studies on enzymatic saccharification of different types of microalgal biomass (Choi, 2010; Lee et al., 2013). Table 6.6 shows the summary of the pretreatment and enzymatic saccharification studies that have been carried out elsewhere. Overall, the acid pretreatment was applied to pretreat microalgal biomass prior to the enzymatic saccharification process. However, this study suggested that alkaline pretreatment could be applied to pretreat microalgal biomass. It was found that approximately 70-90% of the saccharification yield was obtained from dilute alkaline pretreated biomass at a similar level of yield obtained from acid pretreatment. This study also revealed that the saccharification of *Chlorella* sp. and *T. suecica* using a dilute alkaline treatment along with the saccharification process occurring at a low temperature (40 and 50°C) has additional advantages, is environmentally friendly due to the absence of acids, and has low energy requirements due to a low temperature reaction.

6.4 Conclusions

Enzymatic saccharification of *Chlorella* sp. and *T. suecica* biomass was carried out in this study using *Chlorella* sp. and *T. suecica* biomass pretreated using a dilute alkaline pretreatment method. It is demonstrated that dilute alkaline was able to enhance the reducing sugar production from microalgal biomass. The study also revealed that the enzymatic saccharification parameters such as temperature, pH, enzyme concentration and biomass concentration have a remarkable effect on reducing sugar production and the saccharification yield. The maximum saccharification yield of 80–95 % for *Chlorella* sp. was obtained when the saccharification was performed using 10 gL^{-1} of biomass at a pH of 5.5 at 40°C. On the other hand, the maximum saccharification yield of 90 % for *T. suecica* was obtained when the saccharification was carried out using 10 gL^{-1} of biomass with pH 4.5, at 50°C for 72 hours.

Table 6.6 Comparison of enzymatic saccharification reported for different types of microalgae biomass with the present study.

Feedstocks	Pretreatment	Solid loading (gL^{-1})	Enzymatic saccharification conditions	Yield	References
Microalgae	5.3% H_2SO_4 at 90°C for 30 min 9.4% NaOH at 90°C for 30 min	100	Acid slurry	[a]8.92 %	(Ellis et al., 2012)
Chlorella vulgaris FSP-E	1% H_2SO_4 at 120°C for 20 min	20	Enzyme mixture pH 6 at 45°C	[b]90%	(Ho et al., 2013)
Chlorococcum humicola	Ultrasonication	10	pH 4.8 at 40°C for 72 h	[a]68.2%	(Harun and Danquah, 2011)
Spirulina platensis	Acid treatment	13	Acid slurry	nd	(Markou et al., 2013)
Chlamydomonas reinhardtii	Acid treatment 3% H_2SO_4 at 110°C for 30 min	50	pH 5.5 at 55°C	[a]58%	(Choi, 2010)
Microalgae *Dunaliella tertiolecta*	1.2% HCl at 121°C for 15 min	50	pH 5.5 at 55°C for 12 h	[b]80.9%	(Lee et al., 2013)
T. suecica	2% H_2SO_4 at 120°C for 120 min	10	pH 4.5 at 40°C for 72 h	[a]10%	This study
Chlorella sp.	2% NaOH at 120°C for 120 min	10	pH 5.5 at 50°C for 72 h	[b]90%	This study
T. suecica	2% KOH at 120°C for 120 min	10	pH 4.5 at 40°C for 72 h	[b]90%	This study

[a] based on the residual biomass, [b] based on the total amount of carbohydrates of the residual biomass

In summary, this study demonstrates that a combination of dilute alkaline pretreatment and enzymatic saccharification at a low temperature can produce a high level of reducing sugar from microalgal biomass. The main reducing sugar composition in hydrolysate was glucose followed by xylose, which are suitable for a chemical platform for biofuel feedstock such as bioethanol, biobutanol and biohydrogen. The added benefits include avoidance of acid use and a low energy requirement.

Focus on the next chapter

This chapter investigates the effect of the enzymatic saccharification condition on reducing sugar production from alkaline pretreated *Chlorella* sp. and *T. suecica* biomass. This experiment has established the suitable conditions to extract reducing sugar from both microalgal biomass. Further experiments on acetone-butanol-ethanol (ABE) production from microalgal biomass using hydrolysate through anaerobic fermentation process were performed and the results will be discussed in the next chapter.

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CHAPTER 7

ACETONE-BUTANOL-ETHANOL (ABE) FERMENTATION OF MICROALGAL BIOMASS

7.1 Introduction

Biofuel derived from a renewable resource has gained much attention, especially in the bioenergy and biotechnology sectors (Lynd et al., 2008). Liquid biofuel such as biodiesel, ethanol and butanol has the potential to partially replace gasoline in the future (Demirbas, 2008). There is renewed interest in butanol as an alternative transportation fuel. This has been renewed due to its is less miscibility in water and energy content compared to ethanol (Hongjuan et al., 2013). Additionally, butanol has similar combustion properties to gasoline and can also be blended with a gasoline at a higher ratio compared to ethanol (Yilmaz et al., 2014).

Butanol can be produced through two different methods, specifically via a petrochemical route and fermentation of carbohydrates (Niemistö et al., 2013). To date, the major production is through the petrochemical reaction route that is based on the propylene oxo-synthesis, where aldehydes from propylene are hydrogenated to produce butanol. The production of butanol through oxo-synthesis is heavily dependent on the price of crude oil (Uyttebroek et al., 2015). This has made the production of butanol through chemical reaction to be less favourable. Therefore, new and inexpensive alternative routes for butanol production need to be explored.

An alternative butanol production route is acetone-butanol-ethanol (ABE) fermentation. Generally, this technique uses carbohydrate-based feedstock that will be converted into ABE by solventogenic bacteria from *Clostridia* species. Three major chemicals such as acetone, butanol and ethanol are produced through this process (Green, 2011). The fermentation occurs in two stages: the first stage is the growth or the acidogenesis stage, and the second stage is the solventogenesis stage. During the first stage, which is after 12 to 24 h of incubation, acetic and butyric acids are produced by bacteria, while in the second stage, the acids produced are re-assimilated into ABE solvents. Usually, the second stage is achieved at an early stationary phase (after 24 h).

There are several types of feedstock that can be used to produce butanol by fermentation, such as starchy materials, sucrose, lignocellulosic and algal biomass (Amiri et al., 2015; Ellis et al., 2012; Ibrahim et al., 2012; Thang and Kobayashi, 2014). ABE

fermentation using these feedstocks has been reported to have disadvantages because it is non-renewable and food-based feedstock, which could lead to food shortages and it also requires more arable land for feedstock production (Alam et al., 2012).

Microalgal biomass is believed to have the potential to be used as an ABE fermentation feedstock due to this organism being a non food-based feedstock, it has a high growth rate compared to terrestrial plants, it has high lipid yield, it is able to capture carbon dioxide (CO_2) as a carbon source, and it can be used for the industrial wastewater treatment process (Ellis et al., 2012; Razzak et al., 2013; Wang et al., 2013). As microalgal cells contain 30-50% carbohydrate, it is a great candidate to be used as an ABE fermentation feedstock (Chen et al., 2013). There are a limited number of studies on ABE production from microalgal biomass. Thus, the objective of this study was to evaluate the potential of microalgal biomass as an ABE fermentation feedstock. Two different microalgae species, freshwater microalgae, *Chlorella* sp. and marine water microalgae *T. suecica,* were investigated in this study. Four types of microalgal biomass, (1) untreated, (2) alkaline pretreated biomass, (3) lipid extracted biomass, and (4) lipid extracted followed by alkaline pretreatment biomass, were evaluated in this study.

7.2 Materials and Methods

7.2.1 *Microalgae and cultivation conditions*

The two species were obtained from the CSIRO Microalgae Research Centre (Hobart, Australia) and were selected based on their capability to grow in 15% carbon dioxide (CO_2), which is the typical CO_2 concentration in the flue gas from coal-fired power stations. Chapters 3 and 4 discussed the growth profile of these two species in indoor and outdoor conditions.

Modified algae growth (MLA) medium with 0.49 gL^{-1} magnesium sulfate ($MgSO_4.7H_2O$), 1.7 gL^{-1} sodium nitrate ($NaNO_3$), 0.14 gL^{-1} di-potassium phosphate (K_2HPO_4), and 0.03 gL^{-1} calcium chloride ($CaCl_2.2H_2O$) was used as the seed culture and biomass production medium. The medium was initially sterilised using a 0.22 μm Millipore

filter. The microalgal seeds were cultivated in a 1 L Scott bottle containing 700 mL of the modified MLA.

A standardised 10% (v/v) initial microalgal cell concentration of 0.03 - 0.05 gL^{-1} (OD_{680}= 1.0) was added into the medium and incubated in an illuminated incubator with 0.3 $Lmin^{-1}$ of compressed air under light with a photon intensity of 450 μmol/m^2s. The cultivation temperature was set at 30 ± 0.5°C. Both microalgal cultures were cultivated under the same conditions and were harvested at late log growth. The microalgal cells were centrifuged at 4500 rpm for 15 min. The pellet produced from the centrifugation process was rinsed twice with distilled water and subsequently dried at 70°C for 24 h.

7.2.2 *Chemical analysis*

The lipid, carbohydrate, and protein content of the microalgal biomass were determined using soxhlet extraction, the phenol-sulfuric acid method, and Lowry method analyses (Kassim et al., 2014).

7.2.3 *Enzymatic saccharification*

Enzymatic saccharification of microalgal biomass was carried out using cellulase enzyme produced from *T*. *longibrachiatum* (Sigma Aldrich). The experiment was performed using 10 g/L of dried microalgal biomass in 10 mM acetate buffer (pH 5.5) and was incubated at 50°C and 150 rpm in orbital shaker (Thermoline Scientific) for 72 h. The sample was withdrawn every 24 h and heated at 100°C to deactivate the enzymatic reaction activity. The sample was then centrifuged at 3500 rpm for 5 min and the supernatant was used for the reducing sugar analysis. The reducing sugar estimation was carried out as described in the subsequent section. The saccharification yield was expressed as showed in the following equation:

$$Saccharification\ yield\ (\%) = \frac{concentration\ of\ glucose\ at\ time\ of\ t\ x\ 0.9}{carbohydrate\ content\ after\ pre-treatment} x\ 100 \qquad (7.1)$$

7.2.4 *ABE fermentation by Separate Hydrolysis and Fermentation (SHF)*

Throughout this study, a fermentation of microalgal biomass was performed in a separate hydrolysis and fermentation (SHF) approach. The SHF method involves the enzymatic saccharification that was performed separately from the fermentation process. The enzymatic saccharification was performed at the optimum conditions obtained from Chapter 6. The details of the process are described below.

7.2.4.1 *Microorganisms and media preparation*

The solvent-producing bacteria, *Clostridium saccharoperbutyliticum* N-14 was used throughout this study. The bacteria was obtained from the American Type Culture Collection (ATTC, USA). A Reinforce Clostridia Medium (RCM) medium was used to prepare the bacteria inoculum and consisted of 150 gL^{-1} potato dextrose, 0.5 gL^{-1} ammonium sulfate ($(NH_4)_2SO_4$) and 3 gL^{-1} of calcium carbonate ($CaCO_3$).

Two types of mediums were used for active inoculum preparation and fermentation, TYA and P2 as described by Al-Shorgani et al. (2011). The TYA medium was used to prepare active culture and consisted of 20 gL^{-1} glucose, 6 gL^{-1} tryptone, 2 gL^{-1} yeast extract, 3 gL^{-1} ammonium acetate, 0.5 gL^{-1} monopotassium phosphate (KH_2PO_4), 0.3 gL^{-1} of magnesium sulphate ($MgSO_4.7H_2O$), and 0.01 gL^{-1} iron sulphate ($FeSO_4.7H_2O$).

In this study, P2 medium was used as a fermentation medium, based and consisting of 0.5 gL^{-1} monopotassium phosphate (KH_2PO_4), 0.5 gL^{-1} dipotassium phosphate (K_2HPO_4), 0.4 gL^{-1} magnesium suphate ($Mg_2SO_4.7H_2O$), 0.01 gL^{-1} manganese sulphate ($MnSO_4.4H_2O$), 0.01 gL^{-1} iron sulphate ($FeSO_4.5H_2O$), 1.0 gL^{-1} yeast extract and 0.5 gL^{-1} cysteine. A final concentration of 80 ugL^{-1} biotin and 1 mL of a solution containing 1 mgL^{-1} of aminobenzoic acid were added into the 1 L P2 medium.

7.2.4.2 *ABE batch fermentation*

Batch fermentation was conducted using a 100 mL serum bottle with a working volume of 70 mL medium. The hydrolysate obtained from the enzymatic saccharification process was used

as a fermentation medium. The pH value of the medium was adjusted to 6.5 ± 0.2 using 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH) followed by sterilisation by autoclaving at 121°C for 10 min. After sterilisation, an anaerobic condition was attained by passing nitrogen gas through the medium for about 2 to 5 min prior to inoculation with 10% active *Clostridium saccharoperbutylicticum* N-14. The sample was then incubated in an incubator oven at 35°C for 96 hours. The fermentation sample was withdrawn at regular intervals for analytical monitoring. All experiments were conducted in duplicate and the average value was reported.

The microalgal biomass derived from each process is denoted as untreated *Chlorella* sp. (Chl), alkaline pretreated *Chlorella* sp. (AkChl), lipid extracted *Chlorella* sp. (ExChl), combined lipid extracted and alkaline pretreated *Chlorella* sp. (ExAkChl), untreated *Tetraselmis suecica* (Tetra), alkaline pretreated *T. suecica* (AkTetra), lipid extracted T. suecica (ExTetra) and combined lipid extracted and alkaline pretreated *T. suecica* (ExAkTetra) throughout this study (Figure 7.1).

7.2.5 *Sample analysis*

In this study, the fermentation products such as solvent (acetone-butanol-ethanol) and organic acid (acetic acid and butyric acid) produced during the fermentation are analysed as described below.

7.2.5.1 *Fermentation products estimation*

A total of 1 mL fermentation sample was withdrawn periodically every 24 h for analysis. The withdrawn sample was centrifuged at 3000 rpm for 10 min and the supernatant was filtered using a 0.45 um cellulose acetate filter. The solvent (ABE) concentrations were determined using gas chromatography equipped with a flame ionization detector (Shimadzu GC-FID 2010). A 50-m capillary HP-FFAP column was used in this study. The oven temperature was programmed to increase from 50°C to 200°C at a rate of 20°C/min. The injector and detector temperature were set at 260°C. Helium gas was a carrier gas and was set at a flow rate of 29 mL/min.

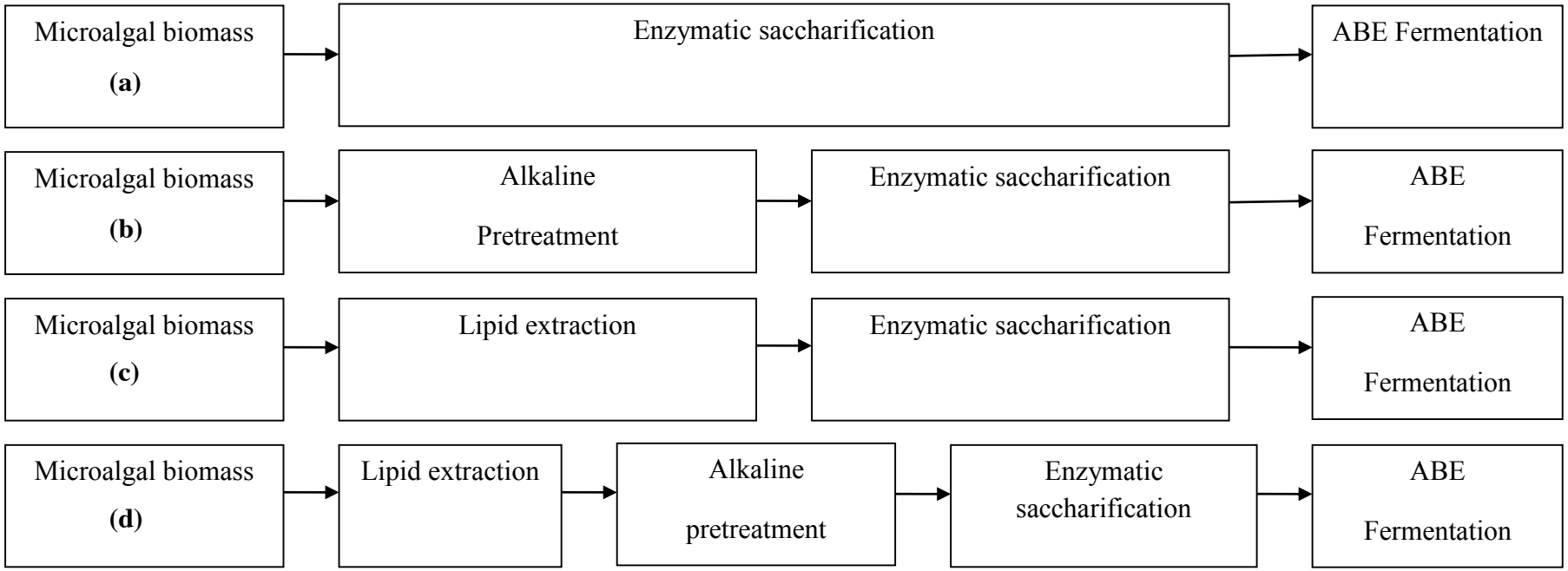

Figure 7.1 Experimental designs for ABE production from *Chlorella* sp. and *T*. *suecica* biomass.

7.2.5.2 *Reducing sugar estimation*

Total reducing sugar was determined using the 3, 5 dinitrosalysilic acid (DNS) method (Pradeep et al., 2013). Generally the filtered samples were added with 1 mL of DNS reagent and then boiled for 10 min in a water bath. The reaction mixture was allowed to coole the reducing sugar concentration was estimated using a UV spectrophotometer (Hach, DR-5000) at 540 nm.

7.2.5.3 *Statistical analysis*

All samples were analysed in triplicate. A T-test was used to determine the statistical difference between the control and the experimental parameters. The statistical analysis was performed using OriginPro software.

7.3 Results and Discussion

In the following subsection, the final carbohydrate content in the microalgal biomass after pre-processing is presented followed by enzymatic saccharification of microalgal biomass. Finally, the ABE fermentation of microalgal biomass hydrolysate obtained from the enzymatic saccharification process is discussed in the subsequent section.

7.3.1 *Carbohydrate content*

Microalgal carbohydrate is the main chemical component involved in ABE production through the fermentation process. This carbohydrate has to be converted into sugar prior to the fermentation process. In this study, four different microalgal biomass were prepared using different pre-processing strategies as explained in section 7.2. The carbohydrate content of each microalgal biomass was determined prior to enzymatic sacchrification and the results are shown in Figure 7.2. It can be seen that the carbohydrate content in the alkali treated (AkChl and AkTetra), lipid extracted (ExChl and ExTetra) and combination of lipid extracted followed by alkaline treated (ExAkChl and ExAkTetra) biomass was lower compared to the untreated sample. A direct comparison of carbohydrate content loss indicated that the highest carbohydrate content loss for *Chlorella* sp. (~23%) and *T. suecica* (~45%) was observed for

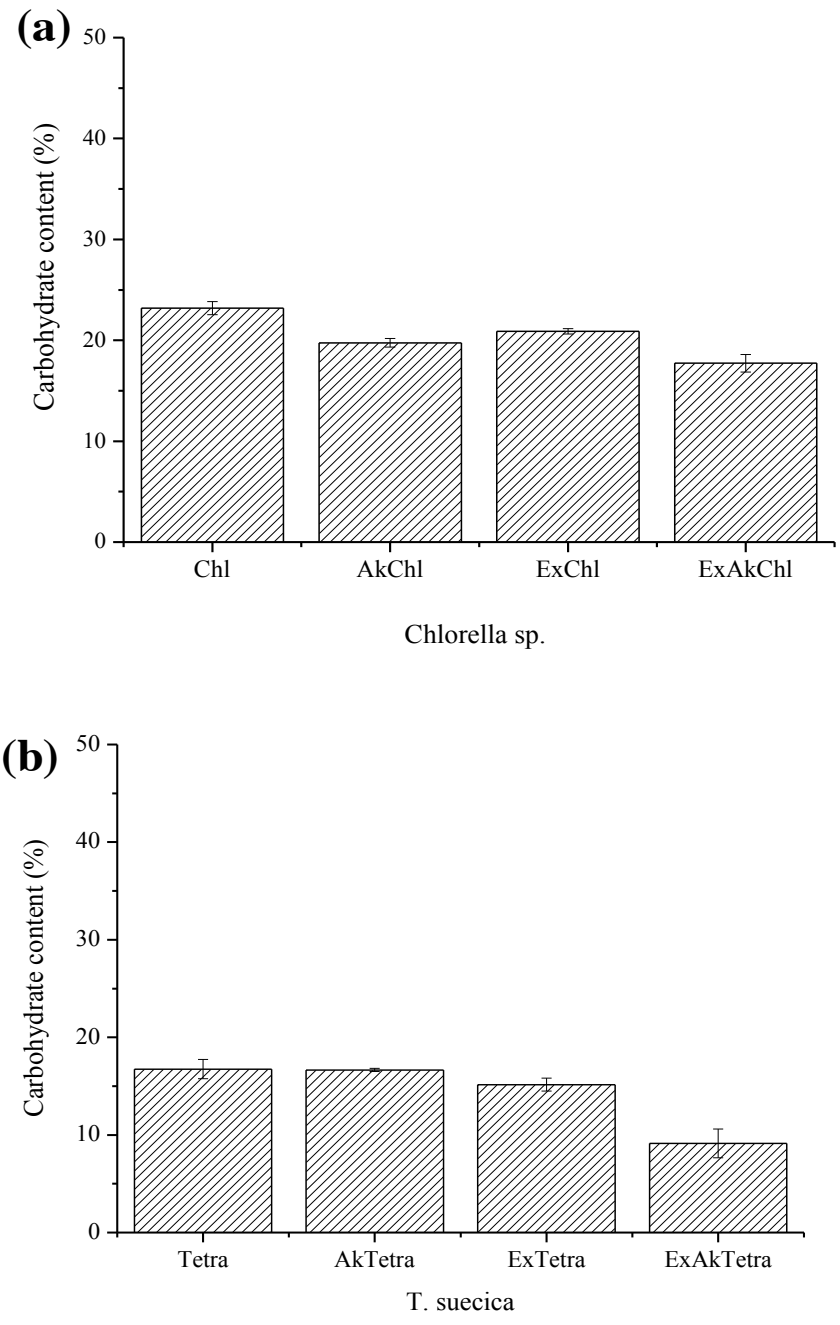

Figure 7.2 Carbohydrate content of different types of microalgal biomass - (a) *Chlorella* sp. (b) *T. suecica*. Chl: untreated *Chlorella* sp.,Tetra: untreated *T. suecica*, AkChl: alkaline pretreated *Chlorella* sp., AkTetra: alkaline pretreated *T. suecica*, ExChl: lipid extracted *Chlorella* sp., ExTetra: lipid extracted *T. suecica*, ExAkChl: lipid extracted followed by alkaline pretreatment *Chlorella* sp., ExAkTetra: lipid extracted followed by alkaline pretreatment *T. suecica*.

the sample treated with a combination of lipid extracted followed by alkaline treatment (ExAkChl and ExAkTetra). In contrast, approximately ~9% of carbohydrate loss was observed for the lipid extraction of *Chlorella* sp. and *T. suecica* biomass (ExChl and ExTetra). For the case of alkaline treatment, it was found that approximately ~14% and ~1% of carbohydrate content loss was observed for the alkaline pretreatment of *Chlorella* sp. and *T. suecica* biomass (AkChl and AkTetra).

This study clearly showed that the treatment methods used to prepare *Chlorella* sp. and *T. suecica* biomass had a significant effect on the carbohydrate content in the biomass. A similar observation was also reported on the carbohydrate content changes in *Scendesmus* sp., *Spirulina* sp, and *Chlorella vulgaris* after the lipid extraction (Lam et al., 2014; Vardon et al., 2012). This is because the solvent diffuses into the cell and dissolves the lipid during the extraction process.

The results also showed that small amounts of carbohydrate content loss were observed from *Chlorella* sp. and *T. suecica* biomass after alkaline pretreatment. The reduction of carbohydrate content observed after alkaline pretreatment can be explained by the chemical reaction occurring during the process. Based on our previous study in Chapter 5, a small portion of carbohydrate in the biomass was also extracted during the pretreatment process. The extraction of this carbohydrate content occurred due to the deacetylation of microalgal biomass that occurred during the pretreatment process. The acetyl group in N-acetylocosamine that is part of the biopolymer in microalgal cell wall is cleaved off during alkali treatment resulting in the dissolution of biopolymer into the treatment solution.

Interestingly, the results also indicated that reduction in carbohydrate content was observed from the lipid extracted followed by alkali treated (ExAkChl and ExAkTetra) samples. This finding indicates that a combination of the lipid extraction followed by the alkaline pretreatment has a negative effect on the microalgal carbohydrate content. This observation is likely due to the weak cell wall during the extraction and further cell disruption through the pretreatment process, leading to an increase in carbohydrate loss from the microalgal biomass. During the lipid extraction process, the solvent penetrates through the cell membrane into the cytoplasm and interacts with the lipid complex. The solvent-lipid complex then diffuses out of the cell (Halim et al., 2012). There is scant information on the

effect of solvent on the cell walls of *Chlorella* sp. and *T. suecica*. However, according to Zhang et al., (2011) the cell wall of *Botryococcus braunii* FACHB 357 became loose and collapsed when this microalgae was exposed to solvent during extraction. This study demonstrates that the alkaline pretreatment, lipid extraction and combination of lipid extraction followed by alkaline treatment has a significant effect on the carbohydrate content in microalgal biomass.

7.3.2 *Enzymatic saccharification of microalgal biomass*

In order to produce reducing sugar, which is a platform for ABE fermentation, all the microalgal biomass samples were subjected to the enzymatic saccharification. The enzymatic saccharification of each *Chlorella* sp. and *T. suecica* biomass samples were carried out at optimum conditions obtained in Chapter 6. Enzymatic saccharification of *Chlorella* sp. samples were performed at 40°C, initial pH of 5.5 for 72 h. On the other hand, the saccharifications of *T. suecica* were carried out at 50°C, with initial pH of 4.5 for 72 h.

Figure 7.3 shows the saccharification profile of the different types of microalgal biomass. It was found that microalgal biomass treated using different treatment strategies displayed different enzymatic saccharification profiles. The study indicated that the treatment methods used have a significant effect on the enzymatic saccharification of microalgal biomass. The highest saccharification yield was obtained from the saccharification of alkaline pretreated biomass (AkChl: ≈72% and AkTetra: ≈95%), followed by untreated (Chl: ≈52% and Tetra: ≈59%) and a combination of lipid extracted followed by alkaline treatment (ExAkChl: ≈47% and ExAkTetra: ≈74%) biomass. The lowest enzymatic saccharification yield was obtained from the saccharification of lipid extracted biomass (ExChl: ≈33% and ExTetra: ≈57%).

As expected, the saccharification of pretreated microalgal biomass was higher compared to that of untreated microalgal biomass. This finding can be explained by the fact that microalgal biomass structure after alkaline pretreatment is easily hydrolysed by enzymes compared to the untreated sample. As discussed in chapter 6, the alkaline pretreatment

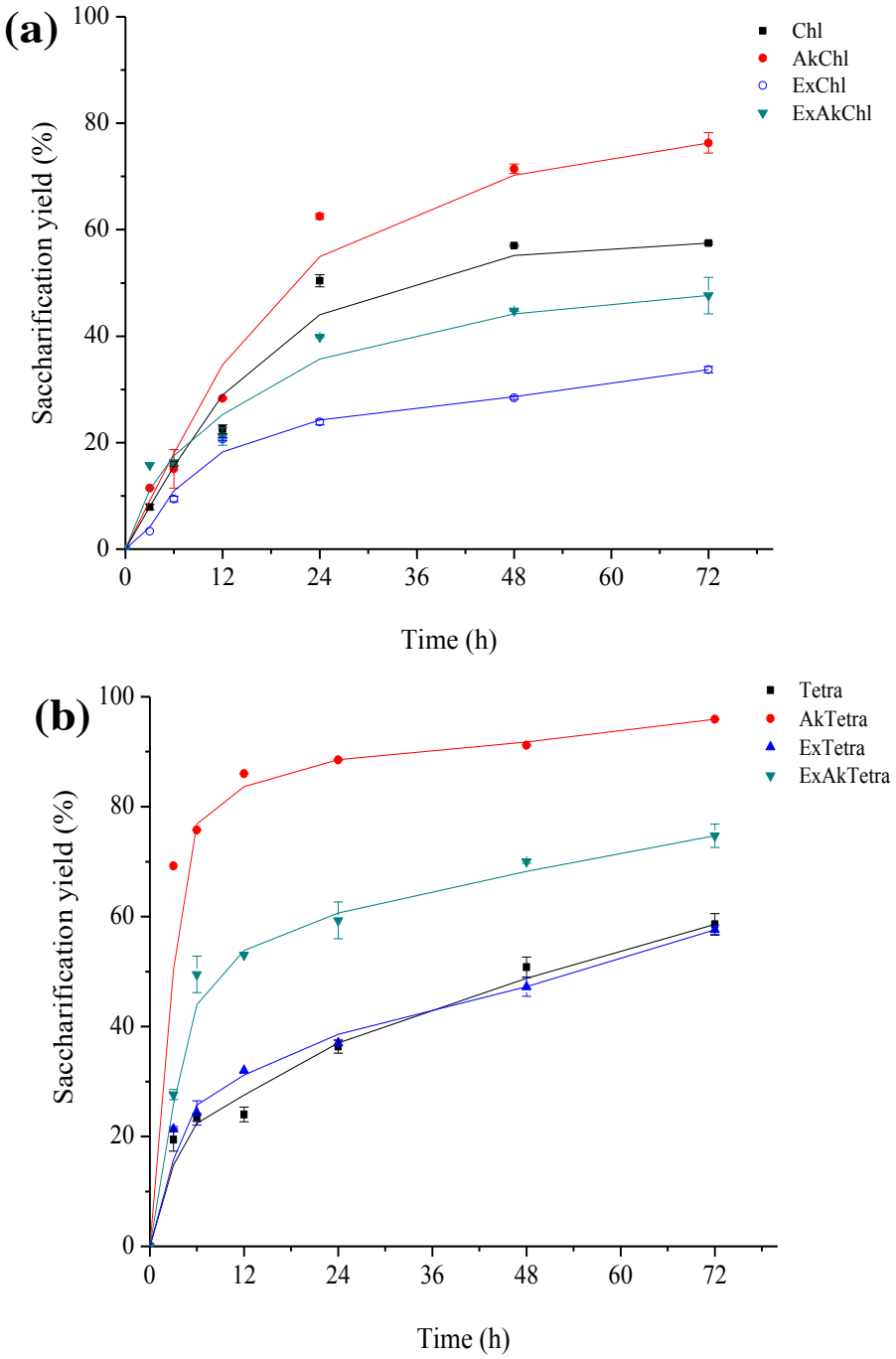

Figure 7.3 Enzymatic saccharification profile of different types of microalgal biomass -(a) *Chlorella* sp. (b) *T. suecica*. Chl: untreated *Chlorella* sp.,Tetra: untreated *T. suecica*, AkChl: alkaline pretreated *Chlorella* sp., AkTetra: alkaline pretreated *T. suecica*, ExChl: lipid extracted *Chlorella* sp., ExTetra: lipid extracted *T. suecica*, ExAkChl: lipid extracted followed by alkaline pretreatment *Chlorella* sp., ExAkTetra: lipid extracted followed by alkaline pretreatment *T. suecica*.

process was found to break down the microalgal cell wall and provide better access for enzymes to attack polysaccharide in the microalgal cell. A similar finding has also been reported by enzymatic saccharification of alkaline pretreated *Chlorella vulgaris* ESP6 (Liu et al., 2012).

Interestingly, although the carbohydrate content for untreated and lipid-extracted samples was almost similar, however, the enzymatic saccharification of lipid extracted samples showed the lowest saccharification yield. It was found that only 33 and 57% of carbohydrate in lipid extracted *Chlorella* sp. (ExChl) and *T. suecica* (ExTetra) was converted into sugar. Low saccharification yield for lipid-extracted samples observed in this study is attributed to the presence of organic solvent in biomass after lipid extraction. The presence of organic solvent in microalgal biomass inhibits enzymatic saccharification activity (Kilbanov, 1997). Overall, this study demonstrates that the enzymatic saccharification of microalgal biomass is significantly influenced by the pre-processing strategies performed prior to the saccharification process.

7.3.3 *ABE fermentation*

7.3.3.1 *ABE production using sugar-based medium*

In order to evaluate the ABE fermentation of microalgal biomass, a positive control (baseline) fermentation was carried out using glucose as a substrate. Two glucose concentrations, 2 gL^{-1} and 4 gL^{-1}, were used in this study. These represent the carbohydrate content in 10 gL^{-1} of dried *Chlorella* sp. and *T. suecica* biomass.

Figure 7.4 shows the ABE fermentation profile from two different glucose concentrations. The study showed that different ABE fermentation profiles were observed from fermentation of 2 gL^{-1} and 4 gL^{-1} glucose. Results showed that the fermentation of glucose-based medium occurred rapidly after 24 h of incubation. Total ABE concentration for 2 gL^{-1} and 4 gL^{-1} glucose was almost similar with 0.112 gL^{-1} and 0.096 gL^{-1} respectively. The ABE yield for fermentation of 2 gL^{-1} and 4 gL^{-1} glucose was 0.484, and 0.282 $g/g_{reducing}$

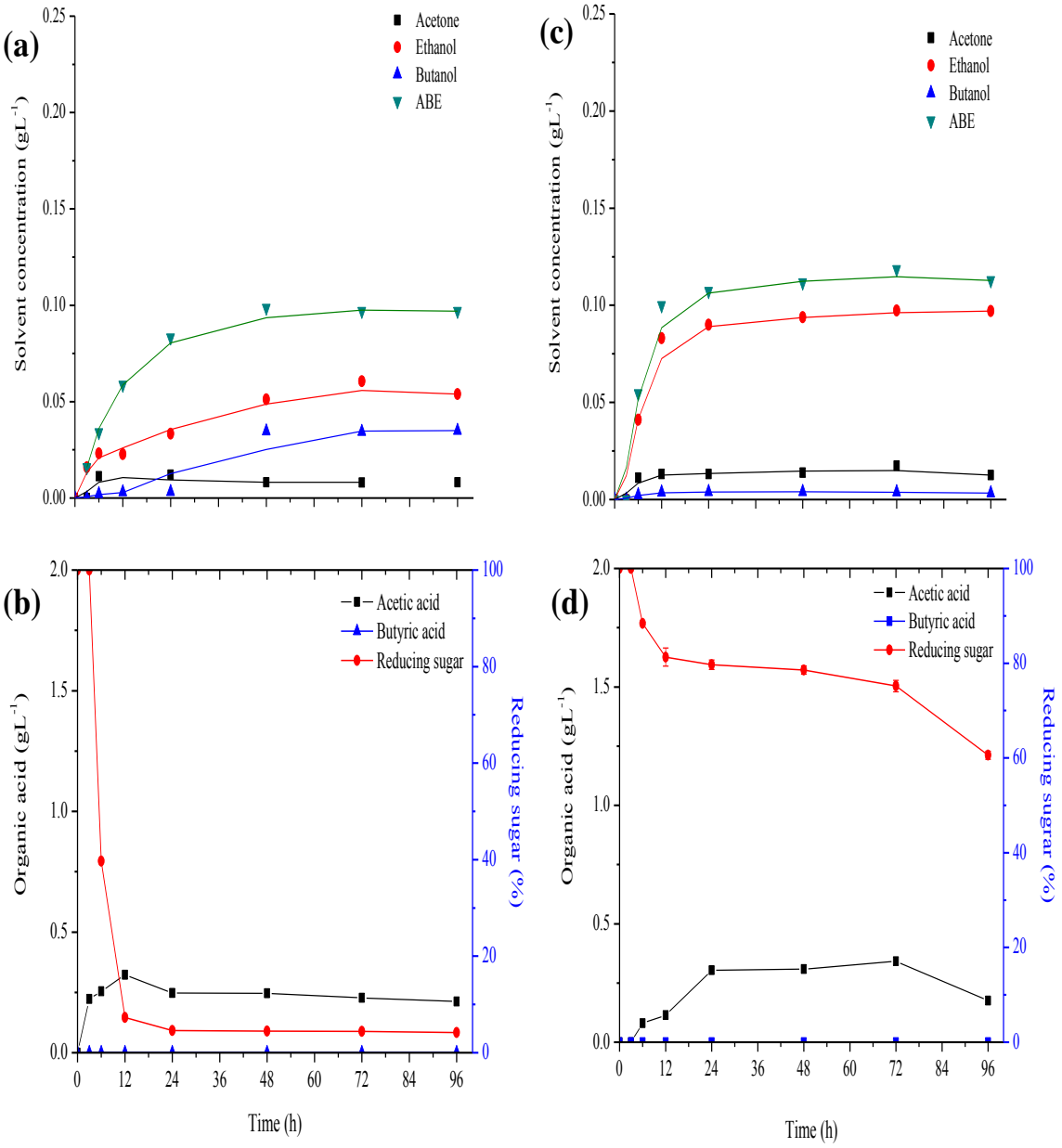

Figure 7.4 ABE fermentation using different glucose concentration (a-b) 2 gL^{-1} of glucose, (c-d) 4 gL^{-1} of glucose.

$_{sugar}$. Ethanol was found to be the major solvent produced from the fermentation of both glucose concentrations. Comparison of butanol production between fermentation using 2 and 4 gL^{-1} of glucose indicated that higher butanol production was obtained from fermentation with 2 gL^{-1} glucose. It was found that the butanol concentration started to increase after 24 h of incubation.

The fermentation of glucose-based medium also indicated that organic acid (acetic acid and butyric acid) was produced during the fermentation. The acetic acid was found to be the major organic acid produced from the fermentation of 2 gL^{-1} glucose. It can be seen that, butyric acid produced from ABE fermentation of 2 gL^{-1} glucose started to decrease after 12 h of incubation, and butanol started to accumulate after that period. A clear transition from acidogenesis to solventogenesis in fermentation of 2 gL^{-1} glucose was observed after 24 h for fermentation. In contrast, a different fermentation profile pattern was observed for ABE fermentation of 4 gL^{-1} glucose, in which the butyric acid was the major organic acid produced from the fermentation process. The butyric acid concentration was found to be stagnant after 24 h of fermentation. No changes were observed in ABE concentration after 24 h. The relationship between the ABE and organic acid production in this fermentation could be explained by the metabolic pathway of the *Clostridium* sp. Generally, ABE fermentation involves two phases, specifically, the acidogenesis and the solventogenesis. The acidogenesis phase normally occurs at an early stage of the fermentation and a significant amount of acid (acetic acid and butyric acid) is produced during this phase. While, the second phase is solventogenesis, which occurs when the bacteria growth reaches a stationary phase, and acids produced are converted to solvent. This study indicates that high reducing sugar concentration reduces the fermentation yield. Fermentation using higher than 4 gL^{-1} glucose was found to inhibit the fermentation process. This finding is in accordance with study by Ibrahim et al., (2012) who reported that substrate concentration or reducing sugar showed a significant effect on the ABE production yield from the fermentation of empty fruit bunches as substrate. This could be explained by the fact that reducing sugar is considered as one of the inhibitors for *Clostridium* sp metabolism and resulted in reducing the fermentation performance.

7.3.3.2 *ABE fermentation of Chlorella sp*.

The ABE fermentation of each *Chlorella* sp. biomass was carried out using hydrolysate obtained from the enzymatic saccharification. Bacteria *C*. *saccharoperbutyliticum* N1-4 was used as biocatalyst. Figure 7.5 shows the ABE and organic acid production profiles from different types of *Chlorella* sp. biomass. It can be seen that each of the *Chlorella* sp. biomass displayed a different ABE production profile. Comparison of ABE fermentation from different types of *Chlorella* sp. biomass indicated that the highest ABE concentration was produced from the fermentation of AkChl with 0.161 gL^{-1}. In the case of the fermentation of Chl and ExAkChl, the ABE concentration was 0.144 and 0.153 gL^{-1} respectively. The fermentation of the ExChl sample was poor and produced the lowest ABE concentration with 0.133 gL^{-1}. Conversely, the highest ABE yield was obtained from the fermentation of ExChl, followed by ExAkCl, AkCl and Chl with 0.202, 0.181, 0.111 and 0.110 $g/g_{\text{reducing sugar}}$ respectively. The ABE concentration obtained from this study was significantly lower compared to the ABE concentration obtained from other biomass. However, the ABE yield obtained from fermentation of *Chlorella* sp. biomass is comparable with ABE yield reported from fermentation of other types of microalgal biomass (Efremenko et al., 2012; Ellis et al., 2012). For instance, ABE fermentation of microalgae cultivated in wastewater using the same bacteria displayed ABE yield of 0.2 $g/g_{\text{reducing sugar}}$ (Ellis et al., 2012).

The results also indicated that ethanol was the major solvent produced from the fermentation of all the *Chlorella* sp. biomass. Butanol was only produced from the fermentation of alkaline pretreated biomass (AkChl), whereas no butanol was produced from the fermentation of untreated (Chl), lipid exterated (ExChl) and the combination of lipid extracted followed by alkaline treatment (ExAkChl) biomass (Table 7.1). Butanol from AkChl started to accumulate after 6 h and achieved the maximum concentration at 24 h of incubation. The fermentation ceased after 72 h and there was no discernible change of the ABE thereafter. The order preference of ABE production from *Chlorella* sp. biomass is AkChl>Chl>ExAkChl>ExChl.

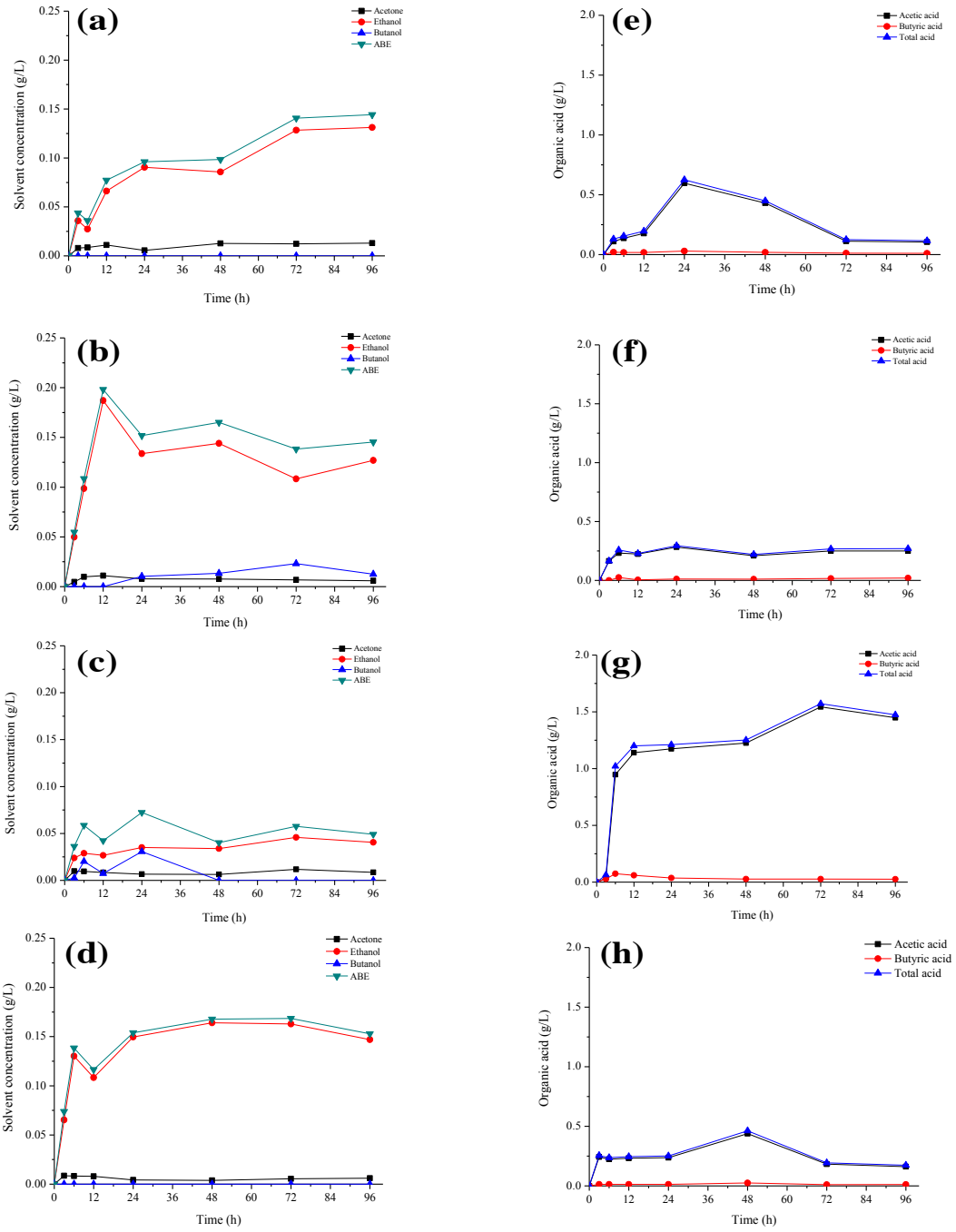

Figure 7.5 Production of ABE and total organic acid profile from 10 % (w/v) *Chlorella* sp. biomass- (a) ABE for Chl (b) ABE for AkChl (c) ABE for ExChl (c) ABE for ExAkChl (d) ABE ExAkChl (e) Acid for Chl (f) Acid for AkChl (g) Acid for ExChl and (h) Acid for ExAkChl.

Table 7.1 ABE and organic acid production from *Chlorella* sp. by *C. saccharoperbutyliticanum* N1-4 after 96 h of fermentation.

	Chl	**AkChl**	**ExChl**	**ExAkChl**
Initial sugar (gL^{-1})	1.311	1.444	0.660	0.846
Initial pH	6.630	6.650	6.52	6.49
Final pH	5.730	5.600	5.44	5.42
ABE (gL^{-1})	0.144	0.161	0.133	0.153
Acetone (gL^{-1})	0.013	0.003	0.006	0.006
Butanol (gL^{-1})	nd	0.027	nd	nd
Ethanol (gL^{-1})	0.131	0.131	0.127	0.147
ABE yield ($g/g_{\text{reducing sugar}}$)	0.109	0.111	0.202	0.180
Acetic acid (gL^{-1})	0.096	0.249	1.448	0.162
Butyric acid (gL^{-1})	0.029	0.020	0.026	0.011
Total organic acid (gL^{-1})	0.113	0.270	1.474	0.174

nd: not detected

The ABE fermentation of *Chlorella* sp. biomass resulted in a significant amount of organic acid. The organic acid concentration was found to be higher than the solvent concentration in this fermentation. The highest organic acid concentration was found from the fermentation of the ExChl sample with 1.474 gL^{-1} consisting of 1.448 gL^{-1} acetic acid and 0.026 gL^{-1} butyric acid. The fermentation of the Chl (0.113 gL^{-1}) produced the lowest organic acid concentration consisting of 0.096 gL^{-1} acetic acid and 0.029 gL^{-1} butyric acid.

7.3.3.3 *ABE fermentation of T. suecica*

Figure 7.6 presents the ABE fermentation profiles of four different *T. suecica* biomass. Similar to the ABE fermentation of *Chlorella* sp., a different ABE fermentation profile was observed from fermentation of different treated *T. suecica* biomass. The ABE fermentation of untreated (Tetra) and alkaline pretreated (AkTetra) biomass were found to produce almost similar ABE concentrations of 0.124 and 0.126 g/L respectively. The study showed that the lowest ABE concentration was obtained from the fermentation of lipid extracted (ExTetra) and combination of lipid extracted followed by alkaline treatment (ExAkTetra) biomass with 0.08 and 0.026 gL^{-1} respectively (Table 7.2). Similar to the ABE fermentation of Chlorella sp., it was found that ethanol concentration was higher than butanol for all the fermentation samples. No butanol was produced from the fermentation of ExTetra and ExAkTetra.

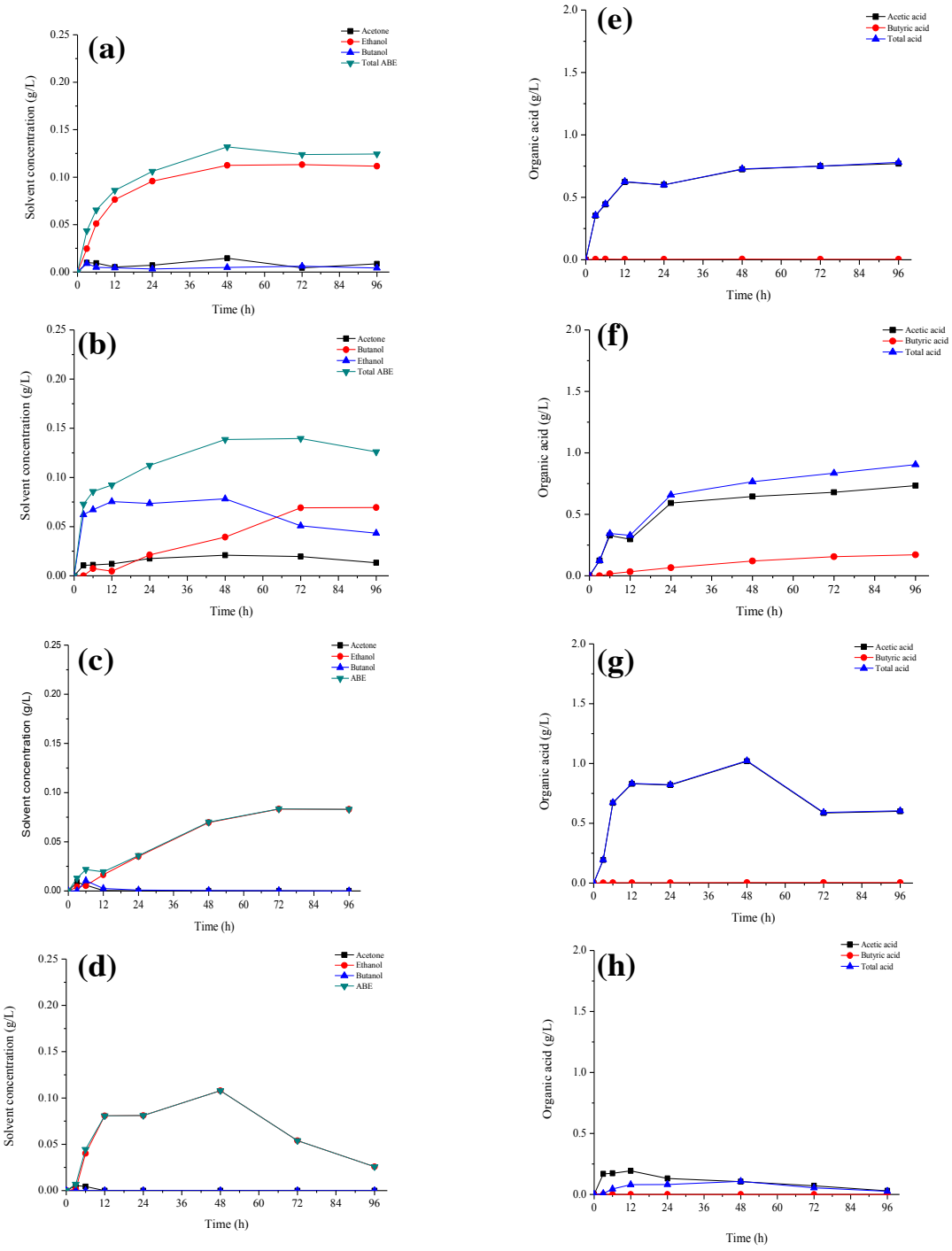

Figure 7.6 Production of ABE and total organic acid profile from 10% (w/v) *T. suecica* biomass - (a) ABE for Tetra (b) ABE for AkTetra (c) ABE for ExTetra (c) ABE for ExAkTetra (d) ABE ExAkTetra (e) Acid for Tetra (f) Acid for AkTetra (g) Acid for ExTetra and (h) Acid for ExAkTetra.

It can be seen that butanol was produced from the fermentation of AkTetra after 12 h of incubation. In contrast, ethanol started to accumulate after 3 h of incubation. This is due to the presence of the acetic acid in the hydrolysate buffer that could trigger the ethanol production during the process. For the fermentation of *T. suecica* biomass, the order for the ABE fermentation was AkTetra>Tetra>ExTetra>ExAkTetra. It can also be noticed that the ABE fermentation of all *T. suecica* biomass produced a higher concentration of the organic acid compared to the solvent concentration. It was found that the acetic acid was the major acid produced from all the fermentations. The total of organic acid produced from the fermentation of Tetra, AkTetra, ExTetra and ExAkTetra was 0.881, 0.904, 0.605 and 0.533 gL^{-1} respectively. This study clearly indicated that the organic acid production was preferred in ABE fermentation of *T. suecica* biomass.

Table 7.2 ABE and organic acid production from *T. suecica* by *C. saccharoperbutyliticanum* N1-4 after 96 h of fermentation.

	Tetra	**AkTetra**	**ExTetra**	**ExAkTetra**
Initial sugar (gL^{-1})	0.9	1.52	0.905	0.666
Initial pH	6.58	6.5	6.5	6.6
Final pH	5.58	5.69	5.58	5.67
ABE (gL^{-1})	0.124	0.126	0.026	0.083
Acetone (gL^{-1})	0.009	0.013	nd	nd
Butanol (gL^{-1})	0.004	0.069	nd	nd
Ethanol (gL^{-1})	0.112	0.043	0.026	0.083
ABE yield ($g/g_{\text{reducing sugar}}$)	0.08	0.083	0.088	0.040
Acetic acid (gL^{-1})	0.877	0.733	0.600	0.533
Butyric acid (gL^{-1})	0.004	0.170	0.005	nd
Total organic acid (gL^{-1})	0.881	0.904	0.610	0.533

nd: not detected

7.3.4 *Comparison of ABE fermentation from microalgal biomass*

Figure 7.7 represents the ABE concentrations and yield obtained from the fermentation of different types of *Chlorella* sp. and *T. suecica* biomass. A direct comparison of ABE concentration from the different types of microalgal biomass revealed that the highest ABE concentration was obtained from the alkaline pretreated microalgal (AkChl and AkTetra) biomass. While, the lowest ABE concentration for *Chlorella* sp. and *T. suecica* biomass was obtained from the fermentation of lipid extracted (ExChl: 0.133 gL^{-1} and ExTetra: 0.026 gL^{-1})

biomass respectively. Even though the ABE concentration obtained from this study was low, this concentration was comparable with the ABE concentration produced from the fermentation of palm kernel cake (PKC) (0.169 gL^{-1}) using the same fermentation condition (10 gL^{-1} of biomass) and biocatalyst (Shukor et al., 2014).

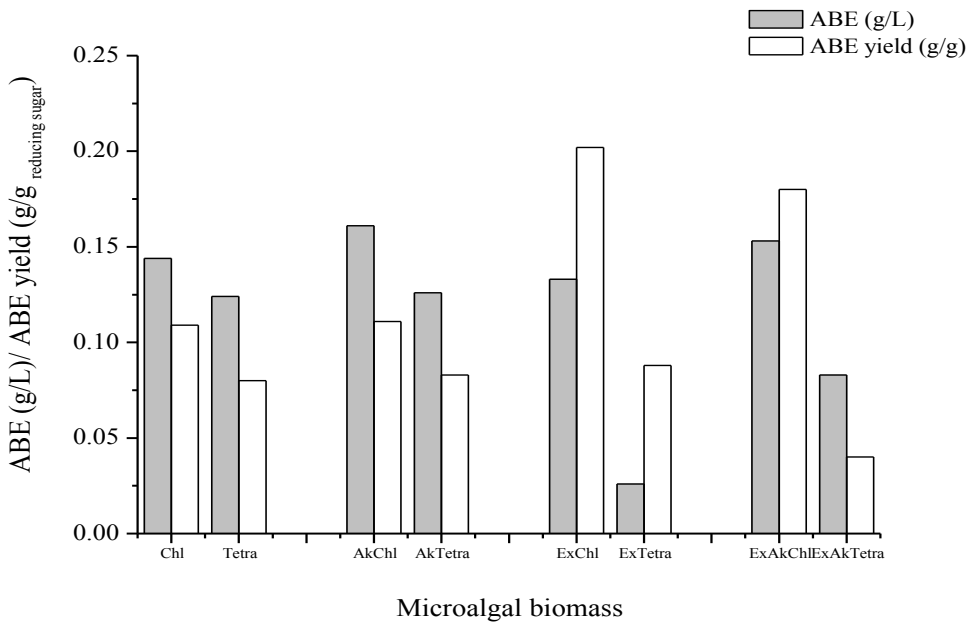

Figure 7.7 ABE concentration and yield from different microalgal biomass (*Chlorella* sp. and *T. suecica*) - Chl: untreated *Chlorella* sp.,Tetra: untreated *T. suecica*, AkChl: alkaline pretreated *Chlorella* sp., AkTetra: alkaline pretreated *T. suecica*, ExChl: lipid extracted *Chlorella* sp., ExTetra: lipid extracted *T. suecica*, ExAkChl: lipid extracted followed by alkaline pretreatment *Chlorella* sp., ExAkTetra: lipid extracted followed by alkaline pretreatment *T. suecica*.

This study also showed that the fermentation of *Chlorella* sp. produced higher ABE concentration compared to *T.suecica*. This observation is most likely due to the microalgal biomass used in this study. Microalgal biomass with high carbohydrate content tends to produce more ABE concentration compared to that having low carbohydrate content. This is due to the fact that carbohydrate or sugar is the main carbon source for bacteria used during the fermentation process. This finding is in accordance with a study on the ABE fermentation of seven microalgae species (*Arthrospira platensis*, *Nannochloropsis* sp, *Dunaliella*

tertiolecta, *Galdieria partita*, *Chlorella vulgaris*, *Cosmarium* sp, *Nostoc* sp.) which found that different microalgae species with different carbohydrate content produced different ABE concentration (Efremenko et al., 2012). This study clearly indicates that the chemical composition or the carbohydrate content present in the microalgal biomass has a significant effect on ABE fermentation.

Another reason for higher ABE concentration obtained from the pretreated microalgal biomass is due to the reducing sugar concentration present in the microalgal biomass hydrolysate. Notably, high reducing sugar or substrate is important to produce higher ABE concentration. The presence of high reducing sugar concentration is important to change the shifting of the acidogenesis to the solventogenesis phase. According to the enzymatic saccharification study reported in the previous section, it was found that the highest saccharification yield was obtained from the alkaline pretreated (AkCl and AkTetra) biomass with 75% and 95% respectively. The high saccharification yield produced high reducing sugar in the hydrolysate, resulting in producing high ABE concentration from this biomass. This finding also suggests that pretreatment of microalgal biomass is necessary to extract reducing sugar from microalgal biomass and enhance the ABE production.

In this study, the fermentation of *Chlorella* sp. and *T. suecica* biomass using *C. saccharoperbuyliticum* N1-4 was found to produce higher concentration of the organic acid than solvent. It is worthwhile to mention that ABE fermentation by *Clostridium* sp. is a biphasic process. Typically, the *Clostrodium* sp. metabolism during ABE fermentation can be divided into two stages. The first phase is the initial growth phase called acidogenesis, in which acetic acid and butyric is produced. The second stage is the solventogensis stage, normally achieved at an early stationary phase of bacterial growth. In this stage acids produced in acidogenesis stage will be re-assimilate and converted into acetone-butanol-ethanol (Jang et al., 2012).

High organic acids produced from ABE fermentation of *Chlorella* sp. and *T. suecica* biomass could be attributed to several reasons. Firstly, the reducing sugar present in the hydrolysate is not sufficient to change the acidogenesis to solventogenesis phase in the bacterial cell. The reducing sugar present in microalgal biomass hydrolysate may only be sufficient to support the production of acid during the fermentation. This finding is in

accordance with the study on ABE production from the oil-extracted rice bran and empty fruit bunches (EFB) biomass (Al-Shorgani et al., 2012; Lee, 2009). A study by Al-shorgani et al. (2011) reported that high organic acid concentration from rice bran and oil-extracted rice bran was due to the lack of reducing sugar concentration in the hydrolysate. It was reported that the presence of less than 10 gL^{-1} of reducing sugar or feedstock assisted the bacteria to maintain the acidogenesis phase without shifting to solventogenesis phase (Fond et al., 1985).

Another possible reason for the high organic acid produced from this study is the fermentation condition. The reason behind the low ABE concentration obtained from the ABE fermentation of *Chlorella* sp. and *T. suecica* is because the fermentation was performed under non-optimum conditions. The ABE fermentation by *Clostridium* sp. under non-optimum conditions prefers to produce high organic acid compared to solvent concentration (Lin et al., 2011; Ranjan et al., 2013). Fermentation at this condition assists the *Clostridium* sp. to maintain the 'ideal' environment without any further metabolism and does not lower the acid concentration produced by *Clostridium* sp., resulting in the accumulation of organic acid in the medium (Kumar et al. 2013). Several parameters that can influence ABE fermentation performance such as initial pH, temperature and biomass concentration have been reported on fermentation of other types of biomass (Al-Shorgani et al. 2015).

7.3.5 *Overall ABE fermentation of microalgal biomass*

Figure 7.8 shows the entire chain ABE and organic acid production from *Chlorella* sp. and *T. suecica* biomass through the ABE fermentation process. It can be seen that approximately 37.4% of solvent (consisting of 0.7% acetone, 6.2% butanol and 30.51% ethanol) was produced from the fermentation of alkaline pretreated *Chlorella* sp. While, approximately 16.32% of solvent (consisting of 1.15% acetone, 9.34% butanol and 5.82% ethanol) was produced from the fermentation of alkaline pretreated *T. suecica*. Overall, butanol conversion yield for the fermentation of alkaline pretreated *Chlorella* sp. and *T. suecica* was 0.3% and 0.7% of dry biomass respectively.

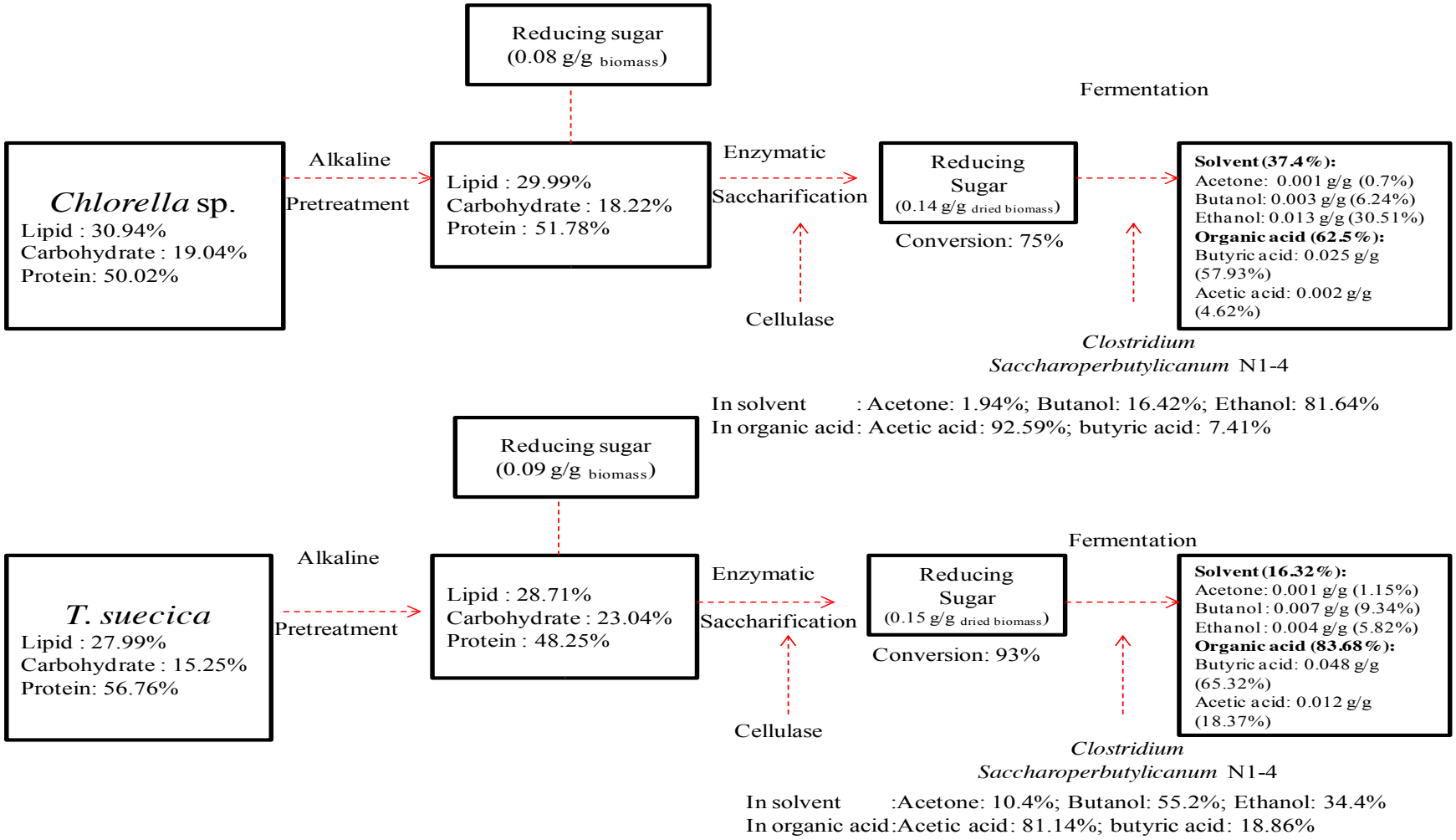

Figure 7.8 Process flow diagram of the entire chain ABE fermentation from microalgal biomass.

7.4 Conclusions

The ABE fermentation of microalgal hydrolysate obtained from enzymatic saccharification of (Chl and Tetra), dilute alkaline pretreated (AkChl and AkTetra), lipid extracted (ExChl and ExTetra), and combination of lipid extracted and alkaline pretreated (ExAkChl and ExAkTetra) microalgal biomass were evaluated. The carbohydrate content, enzymatic saccharification, and ABE concentration of each biomass was determined.

Based on this study, the treatment applied to each biomass had a significant effect on the final carbohydrate content. The highest carbohydrate loss (ExAkChl: 22% and ExAkTetra: 45%) was observed from the biomass that was treated with lipid extraction followed by alkaline treatment. This study also indicated that biomass treatment such as lipid extraction had a negative effect on enzymatic saccharification and ABE fermentation due to the presence of organic solvent, which reduces enzyme activity.

The ABE fermentation of different treated *Chlorella* sp. and *T. suecica* biomass resulted in different ABE fermentation profiles. The highest ABE concentration was obtained from the fermentation of alkaline pretreated microalgal (AkChl: 0.161 gL^{-1}, ABE yield: 0.111 $g/g_{\text{reducing sugar}}$ and AkTetra: 0.126 gL^{-1}, ABE yield: 0.083 $g/g_{\text{reducing sugar}}$) biomass. The study also showed that the ABE concentration obtained from *Chlorella sp*. was higher compared to the *T. suecica* biomass due to the high carbohydrate content present in the biomass. It should be noted that the fermentation condition was not optimised in this study. Overall, the production of ABE from microalgal biomass is possible, however, an optimisation study should be carried out to improve the ABE production yield and the fermentation performance.

7.5 References

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CHAPTER 8

PYROLYSIS AND GASIFICATION OF MICROALGAL BIOMASS

As part of this thesis, preliminary work was also undertaken on pyrolysis and gasification of the microalgal biomass. This chapter comprises two sections:

Part one: Thermogravimetric analysis and kinetic characterization of lipid-extracted *Tetraselmis suecica* and *Chlorella* sp.
(Published in Algae Research)

This section of the chapter determines the thermal characteristic of lipid extracted microalgal biomass. The thermal characteristic of microalgal biomass is determined using thermogravimetric analyser and the devolatilazation and activation energy are determined using the Flyn-Wall-Ozawa and Kissinger-Akahira-Sunose methods.

Part two: Comparison of CO_2 and steam gasification reactivity of algal and woody biomass chars
(Published in Fuel Processing Technology)

This section investigates the gasification of microalgal char produced from two different reactor with two gasifying agents (CO_2 and steam). The gasification reactivity of char produced is perfomed and compared with woody biomass. Further analysis of char by Scanning Electron Microscope (SEM) is performed for morphological information.

Chapter 8 – Part One

Thermogravimetric analysis and kinetic characterization of lipid-extracted *Tetraselmis suecica* and *Chlorella* sp.

8.1 Thermogravimetric analysis and kinetic characterization of lipid-extracted *Tetraselmis suecica* and *Chlorella* sp.

8.1.1 Abstract

In this study, the pyrolysis behavior of two lipid-extracted microalgal biomass, specifically freshwater microalgae *Chlorella* sp. and marine microalgae *Tetraselmis suecica*, was examined using a thermogravimetric analyzer. These studies assessed the effects of different heating rates (5, 10, and 15°C/min) on the devolatilization stage and determined the kinetics using the Flyn-Wall-Ozawa and Kissinger-Akahira-Sunose methods. The activation energy and pre-exponential factor values for *T. suecica* were slightly lower compared with *Chlorella* sp. and other types of microalgal and lignocellulose biomass. The results obtained from this study provide useful information for designing a pyrolytic processing system using lipid-extracted microalgal biomass as a feedstock.

Keywords: lipid-extracted biomass, microalgal biomass, TGA

8.1.2 Introduction

Biofuel derived from biomass is one of the promising future energy sources with low carbon dioxide (CO_2) emissions. Several types of biomass, such as agricultural and forestry waste, energy crops, and algal biomass, can be used to produce biofuel (Tustin et al., 2012). The biofuel produced from microalgal biomass is a promising feedstock due to its advantages over lignocellulosic biomass: algal biomass has higher growth rates than lignocellulosic biomass, does not compete for arable land, can to grow in concentrated CO_2 produced from power plant flue gas, and can produce higher biomass and lipid yields (Brennan and Owende, 2010; Verma et al., 2010).

Biofuel can be produced from biomass through chemical, biochemical, and thermochemical routes. Biomass can be converted into biofuel through thermochemical processes (Sims et al., 2010) that are more favorable than biochemical and chemical processing because thermochemical processing can produce different types of fuels using a single step. Among the different thermochemical pathways, pyrolysis can be used to produce biofuel by cracking the polymeric structure of the biomass and converting it into bio-oil, gas, and solid residue (Jahirul et al., 2012).

Several studies probing the potential of algal biomass (macroalgae and microalgae) conversion via pyrolysis process have been published previously (Grierson et al., 2011; Miao, 2004; Sanchez-Silva et al., 2013). In contrast, few studies have proposed using lipid-extracted algal biomass as a feedstock for biofuel production through this process. Generally, an algal cell contains approximately 30 to 50% lipid depending on the species and cultivation conditions (Singh et al., 2010; Talebi et al., 2013). The biomass remaining after lipid extraction contains higher carbohydrate and protein contents. The algal biomass with higher carbohydrate and protein contents is a potential feedstock for producing bio-oil that contains complex components with various molecular weights through pyrolysis (Li et al., 2011).

Thermogravimetric analysis (TGA) has been used to investigate the characteristics of thermochemical reactions. TGA study is useful for understanding pyrolysis degradation processes and the mechanisms involved. The pyrolysis characteristics of terrestrial biomass, such as palm biomass, cassava pulp residues, coconut shell, rice husk, and paddy straw, have been extensively investigated (Slopiecka et al., 2012; Yang et al., 2006). However, few studies have assessed the pyrolysis behavior of microalgal biomass (Shuping et al., 2010;

Tahmasebi et al. 2013), and the pyrolysis behavior and kinetics of lipid extracted microalgal biomass during the thermochemical conversion process have received limited attention.

Consequently the aim of this study is to examine the pyrolysis behavior and determine pyrolysis kinetics of two different lipid-extracted microalgal biomass, specifically *Chlorella* sp. and *Tetraselmis suecica*, through thermogravimetric analysis. We also report the activation energy of pyrolysis determined through the Flynn–Wall–Ozawa (FWO) and the Kissinger–Akahira–Sunose (KAS) methods.

8.1.3 Materials and methods

8.1.3.1 *Microalgae*

Two different microalgae species, specifically fresh water microalgae *Chlorella* sp. and marine water microalgae *Tetraselmis suecica*, were used in this study. These species were obtained from the CSIRO Microalgae Research Centre (Hobart, Australia) and were selected based on their capability to grow in 15% carbon dioxide (CO_2), which is the typical CO_2 concentration in the flue gas from coal-fired power station. The lipids extracted from this microalgae can be converted into biodiesel.

8.1.3.2 *Medium and cultivation conditions*

Modified algae growth medium (MLA medium) with 0.49 gL^{-1} magnesium sulfate ($MgSO_4.7H_2O$), 1.7 gL^{-1} sodium nitrate ($NaNO_3$), 0.14 gL^{-1} di-potassium phosphate (K_2HPO_4), and 0.03 gL^{-1} calcium chloride ($CaCl_2.2H_2O$) was used as the seed culture and biomass production medium. The medium was initially sterilized using a 0.22 μm Millipore filter. The microalgal seeds were cultivated in a 1 L Scott bottles containing 700 mL of the modified MLA.

A standardized 10% (v/v) initial microalgal cell concentration of 0.03-0.05 gL^{-1} (OD_{680}= 1.0) was added into the medium and incubated in an illuminated incubator with 0.3 $Lmin^{-1}$ of compressed air under light with a photon intensity of 150 $\mu mol/m^2s$. The cultivation temperature was 20±0.5°C. Both microalgal cultures were cultivated under the same conditions and were harvested at late log growth. The microalgal cells were centrifuged

at 4500 rpm for 15 min. The pellets were rinsed twice with distilled water and subsequently dried at 70°C for 24 h. The cultivation was conducted using 6 replicates, and the dried biomass was used for further study.

8.1.3.3 *Determination of the microalgal cell concentration*

The microalgal cell concentration was determined by measuring the optical density at 680 nm (denoted as OD_{680}) with a DR 5000^{TM} UV/VIS spectrophotometer (HACH Company, US). The relationship with the microalgal cell concentration was determined by correlating the absorbance at 680 nm and dry cell weight (DCW). The microalgal DCW was calculated using the following equation:

$$DCW_{Chlorella\ sp.} = 0.549(OD_{680}) - 0.0046 \quad (8.1)$$

$$DCW_{T.suecica} = 0.524(OD_{680}) - 0.0129 \quad (8.2)$$

This calibration curve was estimated by filtering 50-mL aliquots of the culture through a cellulose acetate membrane filter (0.45 um pore size, Millipore). Each loaded membrane filter was subsequently dried in an oven at 60°C until a constant weight was achieved.

8.1.3.4 *Chemical composition*

The lipid, carbohydrate, and protein contents of the lipid-extracted microalgal biomass were determined using soxhlet extraction followed by gravimetric, phenol-sulfuric acid (Nielsen, 2010), and the Lowry method analyses (González López et al., 2010) respectively.

8.1.3.5 *Solvent Extraction of lipid from microalgal biomass*

The lipids were extracted from the microalgal biomass using a Soxhlet apparatus. Briefly, 1.0 g of oven dried microalgal biomass was packed in a cellulose thimble inside the Soxhlet extraction apparatus unit. During this process, 120 mL of isopropanol and 180 mL of hexane were used to extract the lipids for 6 h at 10 refluxes per hour. After the extraction, the solvents were evaporated, and the extract, which mainly contained lipids, was weighed; the

total lipid content was determined gravimetrically. The analysis was conducted in triplicate for each sample.

8.1.3.6 *Determination of carbohydrate content*

The carbohydrate content of the microalgal biomass was determined through the phenol–sulfuric acid method (Hempel et al., 2012). Approximately, 50 mg of biomass was suspended in 2.5 mL of 2.5 N hydrochloride acid (HCl), and the mixture was incubated in water bath at 90°C for 3 h. Afterwards, the mixture was neutralized by adding sodium carbonate (Na_2CO_3) until the effervescence ceased, and the mixture was diluted to 50 mL with distilled water. The mixture was subsequently centrifuged at 3000 rpm for 15 min to separate the solid residues and dissolved sugar.

After centrifugation, 0.2 mL of the supernatant was transferred to a Falcon tube. The supernatant solution then was diluted to 1.0 mL with distilled water. Subsequently, 1.0 mL 5% phenol solution and 5 mL 96% sulfuric acid were then added to the solution. The mixture was kept in a 30-°C water bath for 30 min, and the UV absorbance of the sample was read at 485 nm. Glucose was used as the standard for this analysis. The carbohydrate content was calculated based on the following equation;

$$\text{Carbohydrate content (\%)} = \frac{C}{V} \times M \qquad (8.3)$$

where C is the carbohydrate content (mg mL^{-1}) obtained from the calibration curve, V is the volume (mL) of the supernatant used for the analysis and M is the total volume (mL) of the microalgal sample solution. The analysis was conducted in triplicate for each sample.

8.1.3.7 *Determination of protein content*

The protein content of the microalgal biomass was determined through the Lowry method (Derrien et al., 1998). The chemicals used during this analysis were purchased from Sigma-Aldrich. The following solution was prepared in distilled water: (1) Lysis buffer (5 mL L^{-1} of Triton X-100, 0.372 gL^{-1} of ethylenediaminetetraacetic acid disodium salt, and 0.0348 g L^{-1} of phenyl methyl sulfonyl fluoride); (2) SDS solution (0.05 g L^{-1} of sodium dodecyl sulfate

salt); (3) bovine serum albumin (BSA) solution; (4) reagent A (4.0 g L^{-1} of sodium hydroxide and 20.0 g L^{-1} of sodium carbonate); (5) reagent B1 (0.001 g L^{-1} of copper II sulfate pentahydrate); (6) reagent B2 (0.002 g L^{-1} of potassium sodium tartrate tetrahydrate); (7) reagent C (20 mL of reagent A, 0.2 mL of reagent B1 and 0.2 mL of reagent B2 prepared immediately before use); (8) Folin–Ciocalteu reagent (1:1 v/v Folin reagent/distilled water), which was also prepared immediately before use.

During this analysis, 20 mg of the dried microalgal biomass were suspended in 10 mL of lysis buffer in a Falcon tube for 20 min. An aliquot of this well-mixed suspension was diluted with the lysis buffer such that the protein concentration ranged from 0 to 1000 mgL^{-1}. A 0.1 mL sample of each dilution was transferred to an Eppendorf tube before 0.1 mL sodium dodecyl sulfate salt solution was added in the tube. The mixture was vortexed and 1.0 mL of reagent C was added to the Eppendorf tube. After 10 min of incubation, 0.1 mL of Folin reagent was added in the mixture; the mixture was incubated in the dark for 30 min. Afterwards, the UV absorbance of the sample was measured at 750 nm with a spectrophotometer (HACH, US). The absorbance was converted into the protein concentration by using a calibration curve established using bovine serum albumin (BSA) as a standard for this analysis. The protein content of the microalgal biomass was calculated as follows:

$$\text{Protein content } (\%) = \frac{\text{CVD}}{\text{m}} \times 100 \qquad (8.4)$$

where C is the protein concentration (mg L^{-1}) obtained from the calibration curve, V is the volume (L) of the lysis buffer used to resuspend the biomass, D is the dilution factor and m is the amount of biomass (mg). The analysis was conducted in triplicate for each sample.

8.1.3.8 *Final analysis*

The final analysis was performed to determine the carbon, hydrogen, nitrogen and sulfur content in microalgal biomass while oxygen content was determined by difference. A Perkin Elmer Analyzer (Model 2400, Series II USA) was used for this analysis. Two mg of microalgal biomass was used in each sample and the analysis was conducted in triplicate for each sample.

8.1.3.9 *Pyrolysis*

TGA was carried out by using thermal analyzer (Model STA 449 F3 Jupiter, NETZSCH-Geratebau GmbH, Germany). The ground biomass was sieved, and the particles smaller than 20 μm were used. In each experiment, 10 mg of dried microalgal biomass was spread in an alumina crucible. Small particles and sample sizes were used to minimize the mass and heat transfer effects during the process. The pyrolysis process was performed from ambient temperatures to 600°C at three different heating rates: 5, 10, and 15°C/min. High purity nitrogen (99.99%) was at 100 ml/min as an inert purge gas to displace the air during pyrolysis, avoiding unwanted oxidation in the sample. The weight loss was recorded as a function of temperature. The rate of weight loss (dx/dt) in the biomass during pyrolysis was recorded as a derivative thermogravimetry (DTG) curve. At least two replicates were performed for each experiment to estimate the kinetic parameters.

8.1.3.10 *Kinetic parameters*

TGA measures the overall weight loss due to pyrolysis and provides general information about the overall reaction kinetics rather than the individual reactions. For the solid biomass, the reaction was assumed to occur as follows:

$$Biomass \rightarrow Solid\ residue + volatile$$

Generally, the reaction rate (% min^{-1}) for the decomposition reaction is described as;

$$\frac{d\alpha}{dt} = k\, f(\alpha) \tag{8.5}$$

where

$$\alpha = \frac{(w_o - w)}{(w_o - w_f)} \tag{8.6}$$

where w_o is the initial weight, w is the sample weight at the corresponding time (min) or temperature T(K), and w_f is the final weight after the reaction.

The reaction rate constant k (min^{-1}) is defined by the Arrhenius equation:

$$k = A \exp(-\frac{E}{RT}) \tag{8.7}$$

where A (min^{-1}) is the pre-exponential factor of the Arrhenius equation, E ($Jmol^{-1}$) is the activation energy, and R (8.314 $Jmol^{-1}$ K^{-1}) is the universal gas constant..

If the temperature of the sample is changed by a controlled and constant heating rate (β = dT/dt) Eqs. (8.7) and (8.5) result in:

$$\frac{d\alpha}{dt} = A \exp(-\frac{E}{RT})\ f(\alpha) \tag{8.8}$$

The integrated form of Eq. (8.8) is generally expressed as

$$G(\alpha) = \int_0^{\alpha} \frac{d\alpha}{dt} = \frac{A}{\beta} \int_{T_0}^{T} \exp(-\frac{E}{RT})\ dT \tag{8.9}$$

where $G(\alpha)$ is the integrated form of the conversion dependence function $f(\alpha)$. Based on this equation, different kinetic methods were used in this study.

8.1.3.11 *Estimation of the activation energy (E) and pre-exponential factor (A)*

The activation energy and pre-exponential factor were determined by using two isothermal methods:

the Flynn-Wall-Ozawa (FWO) equation

$$ln\beta = \ln\left[\frac{AE}{Rg(\alpha)}\right] - 5.331 - 1.052\frac{E}{RT} \tag{8.10}$$

and the Kissinger (KAS) equation;

$$\ln\left[\frac{\beta}{T^2}\right] = \ln\left[\frac{AR}{EG(\alpha)}\right] - \frac{E}{RT} \tag{8.11}$$

With a given conversion rate, plotting ln β, ln (β/T^2) versus 1/T gave straight lines with slopes of -1.052E/RT and –E/R, respectively. The activation energy was determined from the slope of the line. All of the plots were generated and the lines fitted using the Origin 8.0 software (OriginLab Corporation).

8.1.4 Results and discussion

8.1.4.1 *Microalgae chemical composition*

The chemical composition and ultimate analysis of the lipid-extracted *T. suecica* and *Chlorella* sp. are shown in Table 8.1. Both species showed a significant difference in chemical composition. Both microalgal biomass samples contained high amounts of protein, followed by carbohydrates. The total organic carbon for both microalgal biomass after the lipid extraction was still ≥ 80%. The higher protein and carbohydrate contents in the solid residues after the lipid extractions suggested that biomass might be used to produce fuels through pyrolysis.

Table 8.1.1 Major chemical compositions of the two lipid-extracted microalgal biomass (dry basis).

Component (%)	*Chlorella* sp.	*T. suecica*
Chemical composition		
Protein	55.28±1.61	63.04±2.80
Lipid	<1	<1
Carbohydrate	24.77±2.08	19.81±1.32
Ultimate analysis		
Carbon	39.34±4.71	24.09±2.92
Hydrogen	6.60±0.43	3.64±0.36
Nitrogen	7.91±1.40	4.12±0.39
Sulphur	0.65±0.58	0.61±0.54
Oxygen	45.50±6.24	67.54±3.08
Proximate analysis		
Volatile matter (%)	56±1.02	54±0.71
Moisture	6±1.14	6±1.41
Fixed carbon	18±1.12	20±1.14
Ash	20±1.10	20±1.14

8.1.4.2 *Characterisation of pyrolytic degradation process*

The weight loss curves from the TGA analyses and the curves showing the rates of weight loss from the DTG of the lipid-extracted *Chlorella* sp. and *T. suecica* biomass at 10°C/min up to 600^{o}C under a nitrogen atmosphere are shown in Figure 8.1.1. The TGA curves for both of the biomass samples indicated that there were three different stages of pyrolysis (Figure 8.1.1a). The first stage (Stage I) occurs from the ambient temperature to the initial

devolatilization temperature (T_1), where the cellular and externally bound water are removed. The second stage is devolatilization (200-550ºC), which is where the volatiles present in the biomass are released. The final stage is the decomposition of, the carbonaceous matter in the solid residues (>550ºC), where the carbonaceous matter is continuously decomposed to leave behind bio-char. The pyrolysis behaviors of the *Chlorella* sp. and *T. suecica* were the same, and the profiles of the lipid-extracted biomass were similar to those of other types of microalgal biomass, such as *Dunaliella tertiolecta* and *Spirulina platensi* (Shuping, et al., 2010).

The first stage of pyrolysis occurred from room temperature to 187°C for the *Chlorella* sp. and at 155°C for the *T. suecica*. Only a negligible weight loss occurred during this stage due to the dehydration process during which the cellular and external water bound by surface tension were eliminated (Li et al., 2010: Li et al., 2011). The second stage (stage II) of pyrolysis corresponded to the major pyrolysis process (devolatization stage). Most of the volatiles in the biomass samples were released during this stage. This study indicated that stage II for *Chlorella* sp. and *T. suecica* occurred from 188 to 511°C and 156°C to 475°C, respectively. Approximately 56.48% and 54.85% weight losses were observed for *Chlorella* sp. and *T. suecica*, respectively. The DTG curves for both samples showed only one major peak as the temperature increased from 150 to 350°C (Figure 8.1.1b). A similar observation had been reported for *Dunaliella tertiolecta*, which showed only one strong peak during pyrolysis (Shuping, 2010). However, different observations have been reported for the pyrolysis of microalgae *Chlorella pyrenoidosa, Chlorella protothecoides*, and *Nannochloropsis gaditana*: two different peaks were observed at this stage (Gai et al., 2013; Sanchez-Silva et al. , 2013). Generally, the second stage could be divided into two zones based on the chemical components present in the microalgal biomass. The first zone included the decomposition of proteins and soluble polysaccharides, while the second zone included the decomposition of insoluble polysaccharides and crude lipids. The single major weight loss peak in this study

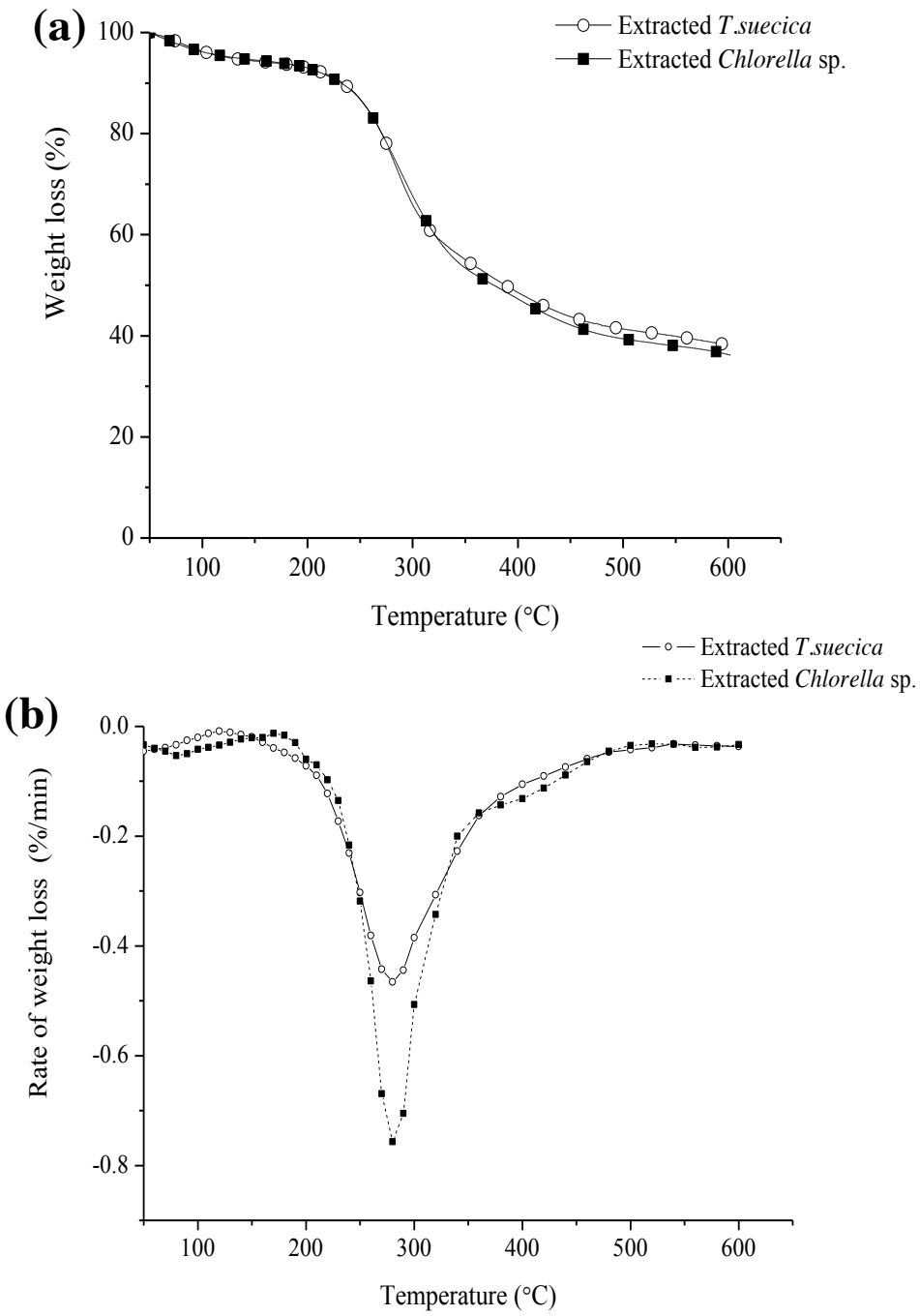

Figure 8.1.1 Thermogravimetric curves for the oil-extracted *Chlorella* sp. and *T. suecica* at 10°C /min under nitrogen - (a) Weight loss curve and (b) Derivative thermogravimetry curve (DTG).

indicated that the proteins and carbohydrates degraded simultaneously between 150 to 350°C. However, two different peaks were observed in *C. protothecoides* and *Nannochloropsis gaditana* due to the presence of lipids in the biomass during decomposition at higher temperatures (Peng et al., 2001; Sanchez-Silva et al., 2013).

This study also showed that the pyrolysis of both *Chlorella* sp. and *T. suecica* was different from the pyrolysis of macroalgae and lignocellulosic materials (Li et al., 2010; Rao, 1998). A pyrolysis study on red macroalgae (*Plocamium telfairiae*) by Li et al., (2011) showed that two different major peaks could be observed when the biomass was pyrolyzed at 10°C/min under nitrogen. Similar observations have been reported for two brown macroalgae: *Laminaria japonica* and *Sargassum pallidum* (Li et al., 2010).

The differences in the pyrolysis behavior of the microalgae relative to that of macroalgae and woody biomass might be due to their different chemical compositions. According to Shuping et al., (2010), the thermal degradation of biomass is directly influenced by its composition. Generally, the micro- and macroalgal biomass consist of lipids, carbohydrates, and proteins, while lignocellulose materials consist mainly of cellulose, hemicelluloses, and lignin. Although macroalgal biomass has a chemical composition similar to that of microalgal biomass, macroalgae are composed of many low polymeric polysaccharides compared with microalgae. The carbohydrate fraction in macroalgae can be divided into free sugars, such as alginic acid, laminarin, mannitol, and other sugars (Ross et al., 2008). However, microalgae carbohydrates are complex and include a mixture of neutral sugars, amino sugars, and uranic acid (Templeton et al., 2012).

Sanchez-silva et al. (2013) showed that the devolatilization of protein and carbohydrates occurred between 180 to 450°C. However, the devolatilization of the chemical components including cellulose, hemicellulose, and lignin in woody biomass occurred at 200-430°C, 250-350°C, and 250-550°C, respectively (Abdullah et al., 2010). During our study, the devolatilization temperature range for both microalgal biomass was lower compared than that of the woody biomass. Therefore, less energy was required to convert the microalgal biomass compared with the woody and lignocellulosic materials.

The DTG curve also indicated that the maximum decomposition temperatures for *Chlorella* sp. and *T. suecica* were only marginally different: 290°C and 287°C, respectively

(Figure 8.1.1b). The rate of weight loss for the lipid-extracted *Chlorella* sp. was also marginally different from that of the lipid-extracted *T. suecica* biomass. An analysis of the molecular structure of the polysaccharides and proteins in the two species might clarify the reasons for these differences, but this analysis has not yet been performed. This difference may also be attributed to the differences in their protein and carbohydrate concentrations (Bothara and Singh, 2012). Moreover, both microalgae species contained high amounts of protein, which is composed of different types of amino acids. *Chlorella* sp. contains high amounts of alanine, glutamic acid, and leucine (Hempel et al., 2012). However, aspartic acid, arginine, and glutamic acid are the major amino acids present in *T. suecica* (Derrien et al., 1998). According to Maddi et al., (2011), different microalgae species exhibit varied pyrolysis behaviors based on the composition of the constituent proteins. Gai et al., (2013) investigated the pyrolysis behavior of *Chlorella pyrenoidosa* and *Spirulina platensis* and concluded that the types of amino acid present in the protein affect the rate of weight loss during pyrolysis. Olafsson and Bryan (1970) reported that the maximum decomposition temperature and rate of weight loss for different amino acids are different. A study of 14 different amino acids indicated that the pyrolysis behavior of amino acids was influenced by the presence of amine group at the ends of the aliphatic chains (Wesolowski and Erecińska, 2005).

The last stage of thermal degradation was observed at 511°C and 475°C for *Chlorella* sp. and *T. suecica,* respectively. The pyrolysis characteristics of *Chlorella* sp. and *T. suecica* were compared, and the final residue for both microalgal biomass after the pyrolysis process accounted for 38% and 40%, respectively (Figure 8.1.1a).

8.1.4.3 *Kinetic analysis of the pyrolysis process*

The pyrolysis of lipid-extracted microalgal biomass was carried out using three different heating rates: 5, 10, and 15°C/min. Generally, heating rates affect the weight loss, maximum decomposition, and maximum temperature (T_{max}) during pyrolysis. Within the range studied, however, the heating rate had a minor effect on the pyrolysis behavior of the lipid-extracted *Chlorella* sp. and *T. suecica* biomass (Appendices E.1 and E.2).

Table 8.1.2 shows the effects of the heating rate on the weight loss of the microalgal biomass at different stages during pyrolysis. Increasing the heating rate increased the weight loss percentage for both microalgal biomass. The weight loss percentage of *Chlorella* sp. was

slightly higher than that of *T. suecica*. The total weight losses for *Chlorella* sp. were 55.48%, 56.78%, and 58.82% when pyrolyzed at 5, 10, and 15°C/min, respectively. Under the same experimental conditions, the total weight losses for *T. suecica* were 50.88%, 54.85%, 58.24, respectively. Therefore, increasing the heating rate increased the total release of volatile matter.

Table 8.1.2 Weight losses of the microalgal biomass at different stages.

Algae species	Heating rate (°C/min)	Stage						Final residue at 600°C (%) original weight
		I		II		III		
		WL (%)	AR (%/ min)	WL (%)	AR (%/ min)	WL (%)	AR (%/ min)	
Chlorella sp.	5	4.75	0.28	55.48	0.89	9.57	0.45	38.27
	10	5.36	0.29	56.48	1.65	7.06	0.80	38.02
	15	6.39	0.33	58.82	2.98	5.79	0.63	36.26
T. suecica	5	3.84	0.31	50.88	0.89	8.71	0.27	43.26
	10	4.15	0.32	4.85	1.54	7.30	0.59	40.18
	15	5.73	0.35	58.24	2.31	7.51	1.20	36.43

WL: weight loss AR: Average rate of weight loss

The heating rate also influenced the amount of final residue produced during pyrolysis (Appendices F.1 and F.2). Increasing the heating rate decreased the residual char formation for both microalgal biomass at the end of the process (Table 8.1.2). This result agrees with other studies of various microalgal biomass (Kirtania and Sankar, 2012; Shuping et al., 2010). The higher weight losses at higher heating rates are attributed to the higher rates of thermal energy transfer between the medium and the particle interiors (Shuping et al., 2010). However, enacting pyrolysis at lower heating rates leads to longer retention time in the reactor and favors the secondary reactions, such as cracking, re-polymerization, and recondensation, to form solid char (Li et al., 2010).

This study also showed that increasing the heating rate increased the initial and maximum devolatilization temperatures (T_{max}) of both biomass samples. The T_{max} for both samples shifted toward higher temperatures as the heating rate increased. The results agree with other studies of the effects of the heating rate on T_{max} for other types of microalgal

biomass, such as *D. tertiolecta* (Shuping et al., 2010). According to Maiti et al., (2007), the major reason for this shift is that biomass conducts heat poorly, producing a temperature gradient throughout the biomass particle. At lower heating rates, the temperature is usually the same at both the outer face and inner core of the biomass particle, depending on the particle size. At higher heating rates, the temperature at the core of the biomass particle is usually lower than the temperature on the surface, resulting in different devolatilization rates during pyrolysis.

8.1.4.4 *Activation energy (E) and pre-exponential factor (A)*

The results obtained from the TGA were elaborated using model-free methods to calculate the kinetic parameters for the pyrolysis of the microalgal biomass. During this study, the activation energy (E) and pre-exponential factor (A) values were obtained using the KAS and FWO methods.

According to Eqs. (8.10) and (8.11), plotting ln (β/T^2), lnβ against 1/T generates a straight line. The activation energy of the dynamic degradation at various conversions could be estimated from the lines in Figure 8.1.2 and 8.1.3. The activation energy and pre-exponential factor values (ln A) obtained from the slope of both samples are shown in Tables 8.1.3 and 8.1.4. The correlation coefficients (R^2) for both methods while determining the activation energy of both microalgal biomass ranged from 0.94 to 0.999, validating the data.

The activation energy for both microalgal biomass showed fluctuations based on the conversion. This study showed that the activation energy for the lipid-extracted *Chlorella* sp. was higher than that of *T. suecica*. The activation energy and pre-exponential factor for the lipid-extracted *Chlorella* sp. and *T. suecica* based on the FWO method were as follows: E: 298.42 kJ/mol, A: 64.33 min^{-1}, and E: 99.69 kJ/mol, A: 21.66 min^{-1}, respectively. In addition, the activation energy and pre-exponential factor values for the lipid-extracted *Chlorella* sp. and *T. suecica* using the KAS method were as follows: E: 301.70 kJ/mol, A: 47.19 min^{-1} and E: 94.77 kJ/mol, A: 9.39 min^{-1}, respectively.

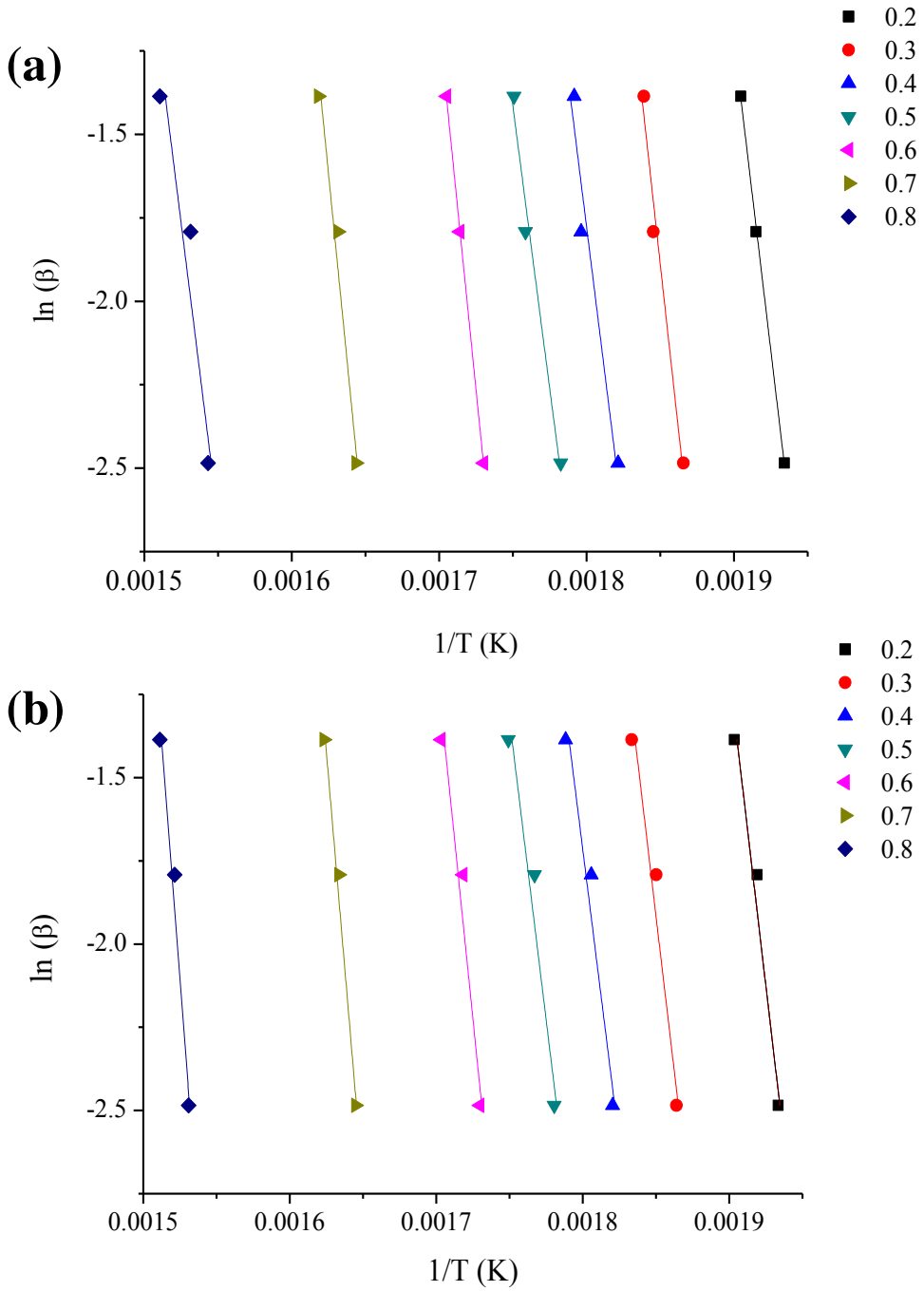

Figure 8.1.2 FWO plot of the lipid-extracted microalgal biomass at different conversions for (a) *Chlorella* sp. and (b) *T. suecica*.

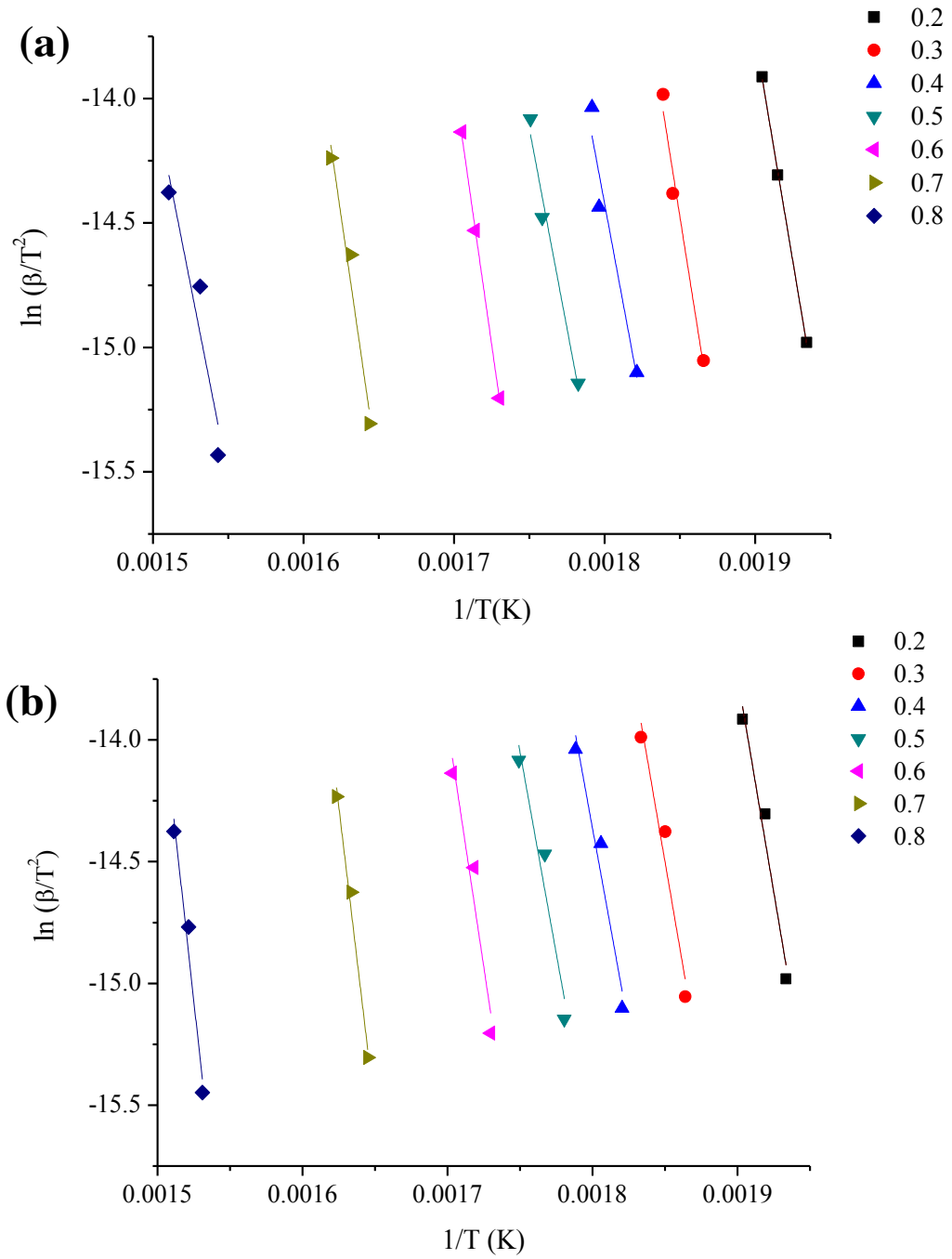

Figure 8.1.3 KAS plot of the lipid-extracted microalgal biomass at different conversions for (a) *Chlorella* sp. and (b) *T. suecica*.

Table 8.1.3 The activation energy of the extracted *Chlorella* sp. biomass obtained from the TGA data at different conversions.

Conversion	FWO			KAS		
	E (kJ/mol)	lnA (min^{-1})	R^2	E (kJ/mol)	lnA (min^{-1})	R^2
0.2	293.26	69.50	0.99	299.84	49.64	0.99
0.3	310.71	71.40	0.98	317.89	51.08	0.98
0.4	265.82	62.96	0.95	270.44	39.73	0.94
0.5	262.66	57.98	0.98	266.90	37.91	0.98
0.6	344.77	69.82	0.99	353.02	54.00	0.99
0.7	358.55	69.71	0.98	348.36	49.52	0.97
0.8	253.21	48.95	0.91	255.48	48.47	0.90
Average	298.42			301.70		

Table 8.1.4 The activation energy of the extracted *T. suecica* biomass obtained from the TGA data at different conversions.

Conversion	FWO			Kissinger		
	E (kJ/mol)	lnA (min^{-1})	R^2	E (kJ/mol)	lnA (min^{-1})	R^2
0.2	29.78	8.23	0.99	23.22	11.32	0.99
0.3	51.09	12.82	0.99	45.10	6.95	0.99
0.4	71.95	16.36	0.99	62.97	4.78	0.99
0.5	141.80	31.91	0.97	139.83	18.89	0.96
0.6	178.47	32.65	0.98	146.59	11.62	0.98
0.7	131.36	24.93	0.94	128.14	7.72	0.94
0.8	123.40	24.73	0.94	117.55	4.46	0.92
Average	99.69			94.77		

Table 8.1.5 compares activation energy values obtained from this study those reported in the literature for various types of biomass. The activation energy values for the lipid-extracted *Chlorella* sp. and *T. suecica* were higher than for the other microalgae: *C. platensis*, *C. protothecoides*, and *Spirulina platensis*. The activation energy for the lipid-extracted *Chlorella* sp. biomass was higher compared with corncob, sawdust, wheat straw, *Chlorococcum humicola, D. tertiolecta*, *Spirulina platensis*, and a macroalgae (*Ulva pertusa*) (Cai and Bi, 2009; Kirtania and Sankar, 2012; Kumar et al., 2008; Sonobe and Worasuwannarak, 2008; Ye et al., 2010). However, the activation energy for the lipid-extracted *T. suecica* was slightly lower than that of the lignocellulosic biomass mentioned above, as well as macroalgae species such as *Laminaria japonica* and *Sargassum pallidum* (Cai, 2009; Li et al., 2010). This study also suggested that the pyrolysis behavior and kinetic parameters (E and A) of microalgal biomass were influenced by the chemical composition of the biomass feedstock. Based on this study, lipid-extracted *T. suecica* biomass with a lower

activation energy is a suitable feedstock for biofuel production through thermochemical processing. These results provide information that is useful for designing a pyrolytic processing system using lipid-extracted microalgal biomass as a feedstock.

Table 8.1.5 Comparison of the various kinetic parameters for the different types of biomass.

Biomass	Activation energy (kJ/mol)	References
Chlorella sp.	298.42 (FWO) 301.70 (KAS)	Present study
T. suecica	99.69 (FWO) 94.77 (KAS)	Present study
Poplar wood	158.58 (FWO) 157.27 (KAS)	(Slopiecka et al., 2012) (Slopiecka et al, 2012)
Spirulina platensis	76.20	(Peng et al, 2001)
Chlorella protothecoides	42.20	(Peng et al, 2001)
Dunaliella tertiolecta	145.71 (KAS) 146.42 (FWO)	(Shuping et al, 2010)
Chlorococcum humicula	189.59	(Kirtania and Bhattacharya, 2012)
Ulva pertusa	148.7	(Hui et al., 2010)
Maize straw	153.0	(Hui et al., 2010)
Rice straw	170	(Sonobe and Worasuwannarak, 2008)
Rice husk	174	(Sonobe and Worasuwannarak, 2008)
Corncorb	183	(Sonobe and Worasuwannarak, 2008)

8.1.5 Conclusions

The pyrolysis behavior of lipid-extracted *Chlorella* sp. and *T. suecica* was investigated using thermogravimetric analysis. Based on this study, the pyrolysis curves for both microalgae species could be divided into three stages: dehydration, devolatilization, and decomposition of the carbonaceous matter. The activation energy for the devolatilization stage of both microalgal biomass was obtained using the Flyn-Wall-Ozawa and Kissinger-Akahira-Sunose methods. The activation energy for the pyrolysis of lipid-extracted *Chlorella* sp. was higher than that of *T. suecica* due to the differences in their chemical composition. However, the mineral composition of the two species may also exert an effect that requires further investigation.

8.1.6 References

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Chapter 8 – Part Two

Comparison of CO_2 and steam gasification reactivity of algal and woody biomass chars

8.2 Comparison of CO_2 and steam gasification reactivity of algal and woody biomass chars

8.2.1 Abstract

This study undertook gasification reactivity measurement of an algal biomass (*Chlorella* sp.) char prepared in two different reactors with two gasifying agents (CO_2 and steam) and compared that with similar measurements on woody biomass (commercial wood mix) char in a thermo-gravimetric analyser at three different temperatures. In general, the woody char from entrained flow reactor showed higher reactivity during gasification. At 800 °C and 950 °C, similar reactivity was exhibited by algal char from thermo-gravimetric analyser whereas at 1100 °C, the woody char became more reactive than the algal char. For algae, the char prepared in entrained flow reactor showed lower reactivity than the char from thermo-gravimetric analyser. The scanning electron microscope images of the char samples showed significant difference in morphology with respect to the char preparation condition and species. For chars of both the species, a temperature of 800 °C and time of around 20 min are found to be sufficient to accomplish most conversion; this information is of practical relevance.

8.2.2 Introduction

Biomass is an important source of fuel for alternative energy. Along with the conventional woody biomass, algae have become one of the most promising sources of biomass for its high growth rate and capability for capturing carbon dioxide. The algal biomass can be used for extracting oil for which several methods are available (Demirbas, 2011). This oil can be used for production of biodiesel by trans-esterification (Kusdiana & Saka, 2001). However, the separation process of oil from algae is difficult and energy intensive (Pimentel, 2008). A potentially attractive method to produce fuel from algae, fuel gas, is through thermochemical processing. Most of the thermochemical processing studies on algae are based on thermo-gravimetric experiments on pyrolysis of algae (Li et al., 2012; Li et al., 2011; Li et al., 2010b; Li et al., 2010) and (Ross et al., 2009) and its carbohydrates (Li et al., 2010a) and (Anastasakis et al., 2011). To take a step further, gasification of algae was done by Demirbas (2009) to observe the gas evolution rate. However, in general the research on thermochemical conversion of algae is in preliminary stage. Also life cycle assessment

studies indicate that it is too early to decide whether it is better to convert algae biochemically or thermochemically (Singh & Olsen, 2011). Therefore, research is required to explore the potential of thermochemical conversion of algal biomass.

Among different kinds of biomass, woody biomass has been the most commonly studied in literature for thermochemical conversion. Several studies can be found on the gasification reactivity of woody biomass, varying operating parameters as well as char preparation environment. For woody char, high heating rates during pyrolysis produce a more reactive char for gasification (Cetin et al., 2005; Cetin et al., 2004; Chen et al., 1997; Fushimi et al., 2003; Kumar & Gupta, 1994; Mermoud et al., 2006; Moilanen & Mühlen, 1996). To understand gasification characteristics, treatment condition of raw biomass during pyrolysis is of utmost importance. However, studies assessing the reactivity of algal char prepared under various conditions are rare.

This study was undertaken to evaluate and compare the reactivity of woody and algal chars prepared under different pyrolysis conditions. Analysis of the gasification reactivity considers isothermal data unlike the oxidation of biomass char where dynamic thermo-gravimetric analyses are performed. The reactivity of all types of char was evaluated at 800°, 950° and 1100 °C under both CO_2 and steam environment with the structure of the char particles studied by scanning electron microscope imaging. The following section discusses the materials and methods for the experiments, followed by the presentation and discussion of the results.

8.2.3 Experimental

8.2.3.1 *Sample preparation*

Modified MLA medium with 0.494 gL^{-1} $MgSO_4 \cdot 7H_2O$, 1.7 gL^{-1} $NaNO_3$, 0.14 gL^{-1} K_2HPO_4, and 0.029 gL^{-1} $CaCl_2 \cdot 2H_2O$ was used as the seed culture and alga production medium. The medium was initially sterilised using a 0.22 μm Millipore filter. Microalga seed cultivation was conducted in a 1 L Scott bottle containing 500 mL of modified MLA. The bottle was incubated in illumination incubator chamber with 0.1 L/min of compressed air sparging, a photon intensity of 150 $\mu mol/m^2s$. The cultivation temperature was 20.0 ± 0.5 °C. Alga cells from late log phase were harvested and centrifuged at 4500 rpm for 15 min. The pellets were

rinsed twice with distilled water to remove salt and then dried at 80 °C for 12 h. Two desired particle sizes were produced by grinding with hand mortar.

Commercial mix of woodchips were collected from sawmill and then dried at 80 °C for 12 h. The woodchip samples were ground and sieved to obtain two different particle size ranges — less than 38 μm and 150–250 μm by grinder. It is known (Zanzi et al., 1996) that particles below 0.20 mm showed kinetically controlled behaviour whereas the larger particles exhibit heat transfer control during fast pyrolysis. Less than 38 μm particles were only used for char preparation in TGA to perform gasification in a kinetically controlled regime whereas 150–250 μm particles were used for char preparation in the entrained flow reactor.

8.2.3.2 *Ultimate and proximate analyses*

The ultimate analysis was performed with an analyzer (Model 2400, Perkin-Elmer). Ash content was determined by combusting the samples at 800 °C. The oxygen concentration was calculated by difference as the concentrations of other materials are in ppm range. Three samples were analysed to have an average elemental composition. Proximate analysis was carried out in a Thermo-Gravimetric Analyser (Model STA 449F3 Jupiter®).

8.2.3.3 *Pyrolysis and gasification*

The pyrolysis experiments were performed in an entrained flow reactor (EFR) enclosed in a vertical furnace which can operate up to 1000 °C. An EFR was selected because it can provide very high heating rate and mimic the industrial reactors. The description of the experimental set up can be found elsewhere (Zhang et al., 2010). The reactor is made of quartz having 50 mm diameter and an effective reaction length of 1.8 m. First the furnace was heated up to 1000 °C. Then particulate sample was fed using a piezo-feeder under 5 L/min constant flow of nitrogen. The feeding rate was in the range of 20–25 g/h. 150–250 μm particles of both algae and commercial mix of sawdust were used as feed. The char was collected in a conical flask and thimble filter at the bottom of the reactor. Char was also produced under slow pyrolysis condition in thermo-gravimetric analyser at the same temperature as EFR with less than 38 μm particles of both samples. The pyrolysis experiments were carried out in the thermo-gravimetric analyser. For all the pyrolysis, two steps of heating were used at two heating rates. At first, the temperature was raised to 200 °C

at a heating rate of 5 K/min. Then the temperature was raised up to 1000 °C at a constant heating rate of 10 K/min. N_2 flow was kept constant at 100 mL/min in all pyrolysis experiments. The smallest particle size (< 38 μm) is pyrolysed in thermo-gravimetric analyser to see the difference in behaviour with the larger particles as it is assumed to exhibit intrinsic reactivity.

Isothermal gasification was performed at 800, 950 and 1100 °C respectively in the thermo-gravimetric analyser. The amount of sample loaded was 4–5 mg for each run to eliminate diffusion effects. All the gasification runs were performed for 2 h. Both steam and CO_2 gasification were performed by introducing 20 mL/min of steam and CO_2 respectively with 80 mL/min of N_2 in the system. The detailed experimental flow diagram is presented in Figure 8.2.1. The instantaneous reactivity was calculated using the following equation:

$$R_i = \frac{-1}{w_i} \left(\frac{dw}{dt}\right)_i$$

where w_i is the weight of the sample at time t_i and $(dw/dt)_i$ is the instantaneous rate of decomposition at the time.

8.2.3.4 *Scanning electron microscopy*

The particles of the samples were dispersed on a carbon tape attached over a metal stab. The raw and pyrolyzed samples were platinum coated to avoid charging by electron interaction. Images taken at similar scale were compared for the surface structure of particles before and after pyrolysis.

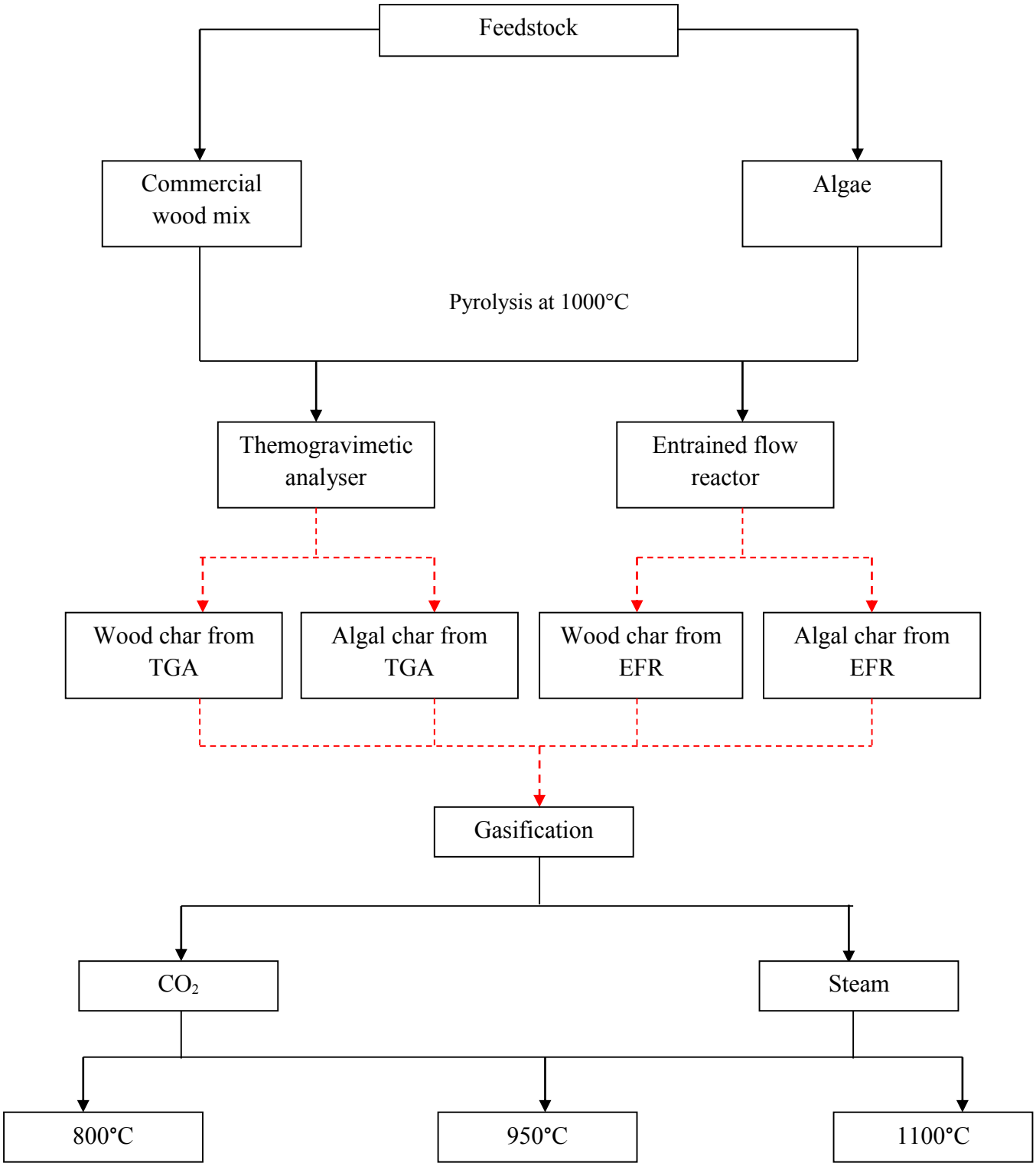


Figure 8.2.1 Experimental flow diagram.

8.2.4 Results and discussion

8.2.4.1 *Ultimate and proximate analysis*

Proximate analysis of commercial wood mix and *Chlorella* sp. showed the basic difference in ash content represented in Table 8.2.1. Even though both the biomass have same amount of fixed carbon, lower weight loss during gasification is expected due to higher ash content. Ultimate analysis showed that commercial wood mix has higher carbon, nitrogen and oxygen contents with respect to *Chlorella*. Only nitrogen content of *Chlorella* sp. is higher than that of commercial wood mix.

Table 8.2.1 Proximate and ultimate analyses of commercial wood mix and *Chlorella* sp. as a fed to the reactor (wt.%).

	Commercial wood mix	*Chlorella* sp.
Proximate analysis		
Moisture	3.30	5.5
Volatile matter	66.70	56.75
Fixed carbon	24.80	24.45
Ash	5.2	13.3
Ultimate analysis		
C	49.02	43.92
H	4.95	6.1
N	0.8	7.39
S	-	-
O	40.03	29.29
Ash	5.2	13.3

8.2.4.2 *Comparison of morphology*

The structure of biomass changes significantly with the heating rate. For slow heating rates, volatile pyrolysis products are released through the natural porosity and no major change takes place in the particle morphology (Rocca et al., 1999) whereas for fast heating rates the original cellular structure is lost (Septien et al., 2012) as a consequence of melting (Cetin et al., 2005; Cetin et al., 2004; Fushimi et al., 2003). To observe the surface structure of the particles under study, scanning electron microscopy was carried out on both raw samples and char produced at different reactors. Figure 8.2.2 shows less than 38 μm sized particles of

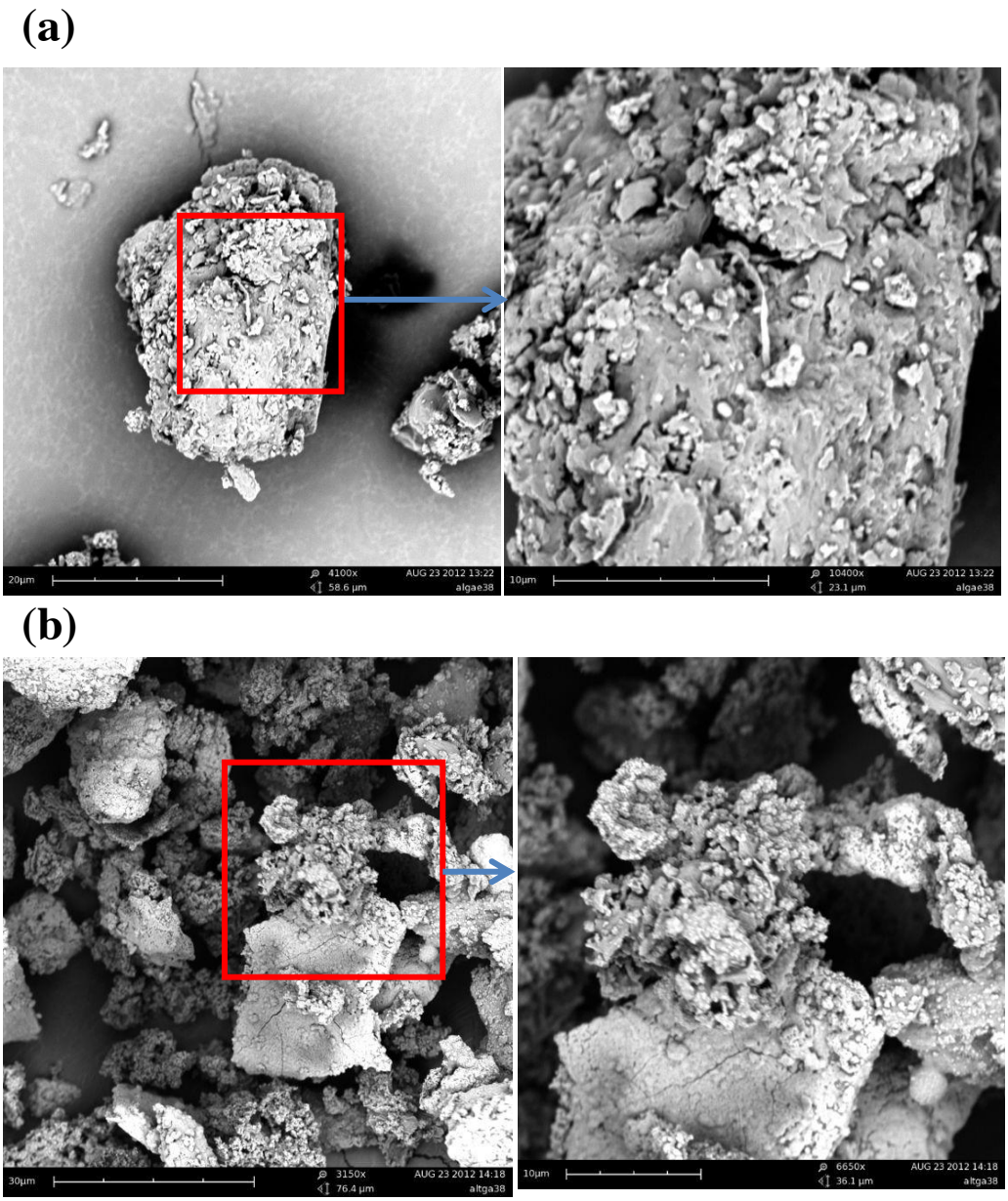


Figure 8.2.2 Structure of *Chlorella* sp. (less than 38 µm)- (a) raw (b) char obtained from TGA.

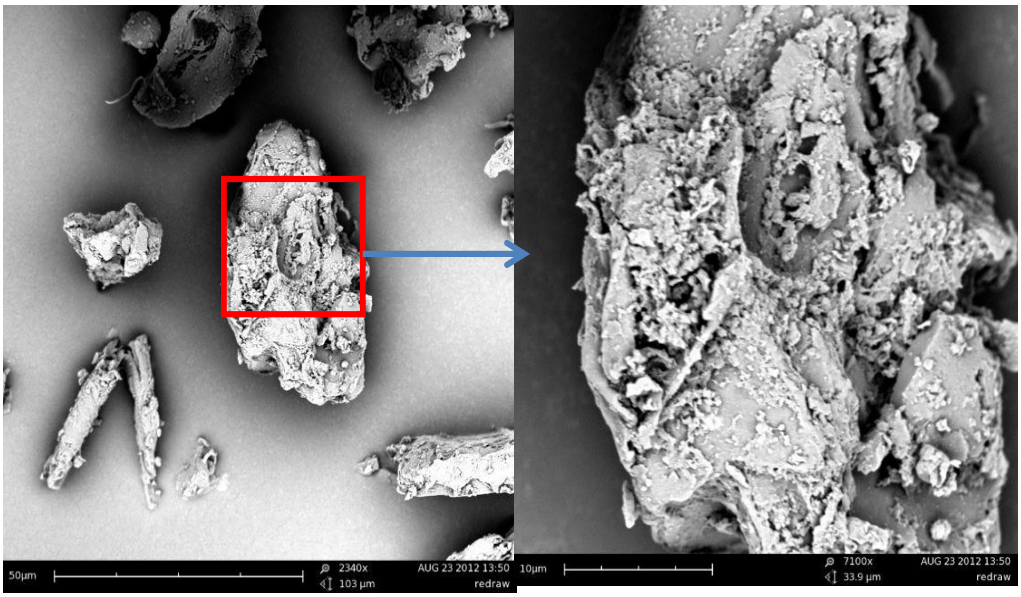
Chlorella sp. before and after pyrolysis in TGA. Raw particles of the alga showed non-porous or quite rigid structure. Few tiny particles were observed on the rough surface of the raw particles. After pyrolysis, the surface of the particles became either smooth or partly formed into clinker like structure. The smooth surface retained the original structure of raw particles with only melting of the fine particles on the surface, whereas clinker shaped particles were with porous surface expected to be more reactive.

SEM images of commercial wood mix particles less than 38 μm size are shown in Figure 8.2.3. The surface of the raw particles was rough because of attrition during grinding and has some loose extrusions or finer particles on the surface. The char particles produced in the TGA from that particle size showed two major types of structure — one type showed solid shape with no visible pores whereas the other type showed breakage and opening of what can be classified as macropores. The woody structure was also evident from the char prepared at a slower heating rate.

Figure 8.2.4 represents the structure of *Chlorella* before and after the pyrolysis in entrained flow reactor. In this case, the particle size was 150–250 μm. The raw alga particles are like bricks with some smaller particles on the surface. After treatment at a faster heating rate at 1000 °C in an EFR, the particles went through a heat shock. The char particles resulting from this process were observed to be completely converted into a clinker like structure. Numerous macro and mesopores were visible from the image. The porous structure resembles that the surface passed through the melting phase with volatiles possibly released through the pores. This type of structure is very common in secondary char or soot (Septien et al., 2012). While forming soot, part of the product gas condenses to solid with completely porous structure like clinkers. The soots are generally known to be less reactive than primary char (Zhang et al., 2006).

In the case of commercial wood mix particles of 150–250 μm, woody structure is visible in Figure 8.2.5. The ligaments of the woody structure were visible on the surface of the particle. The surface shows the presence of tiny particles and extrusions. Figure 8.2.5b shows a particle after pyrolysis in the EFR. This structure is quite different from the structure observed for the alga at the same condition in that this one was only partially molten.

(a)



(b)

Figure 8.2.3 Structure of commercial wood mix (less than 38 µm) - (a) raw (b) char obtained from TGA.

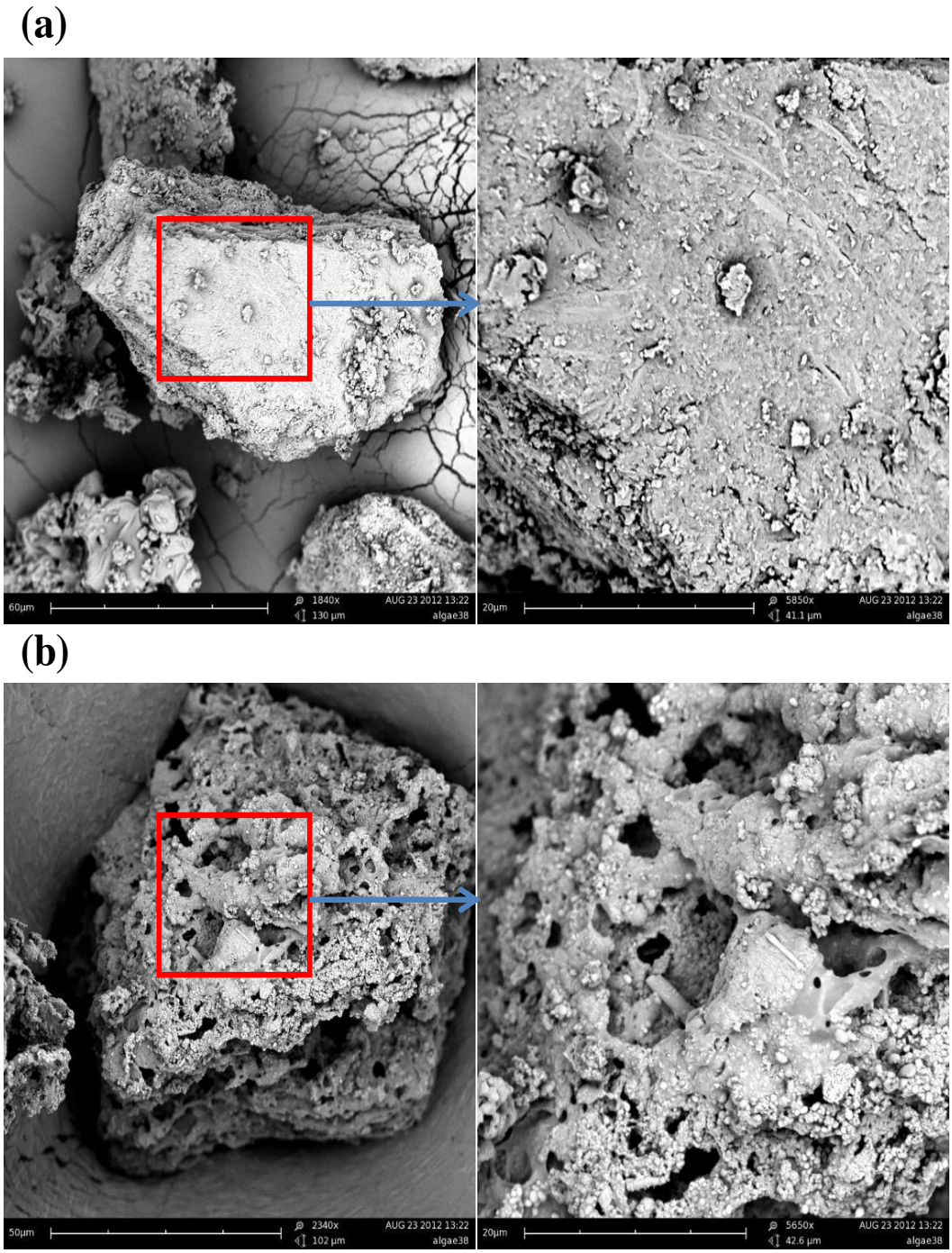


Figure 8.2.4 Structure of *Chlorella* sp. (150-250 µm) – (a) raw (b) char obtained from EFR.

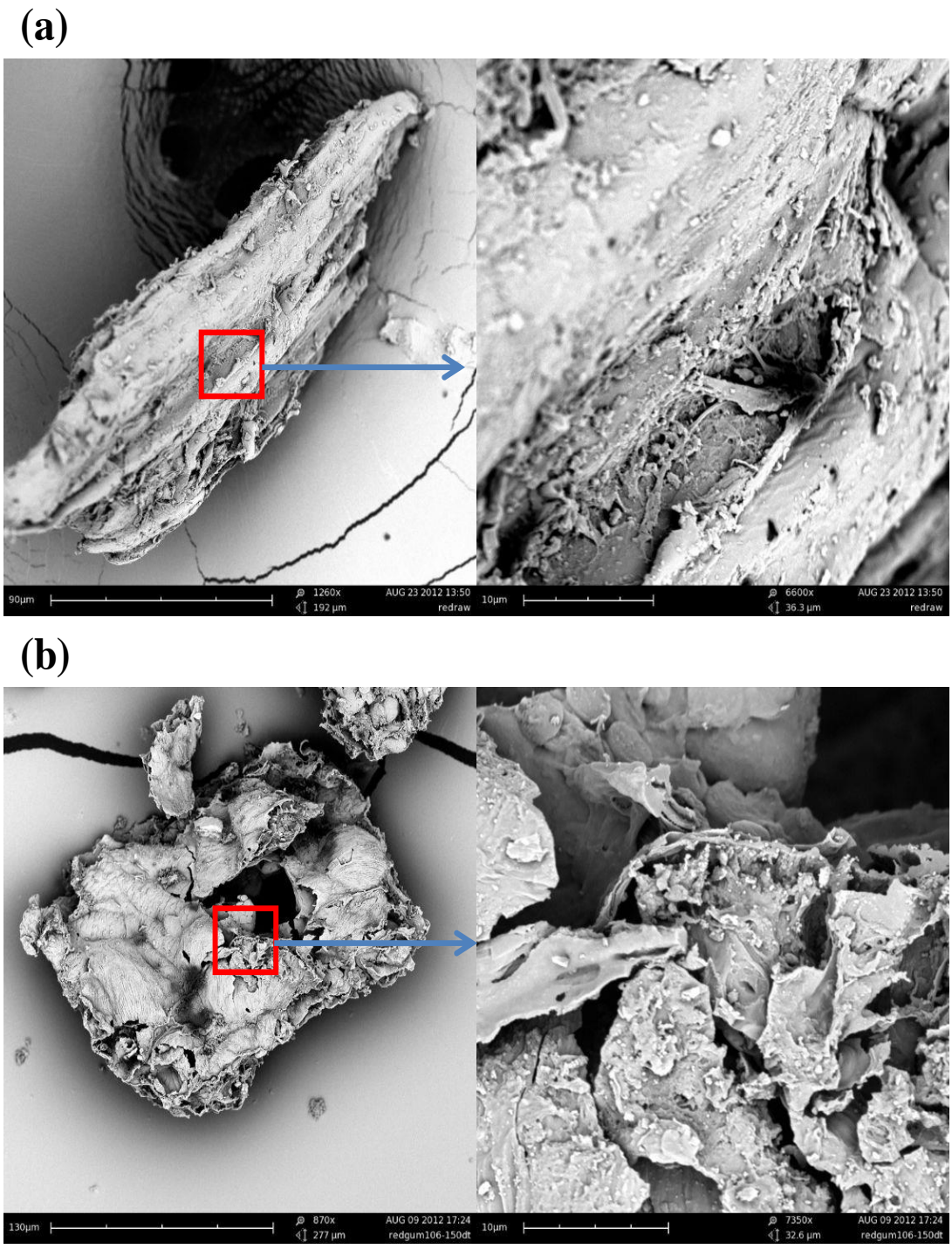


Figure 8.2.5 Structure of commercial wood mix (150-250 µm) – (a) raw (b) char obtained from EFR.

8.2.4.3 *Gasification characteristics*

The gasification behaviour of the two types of char from the two types of biomass is compared at 800 °C in Figure 8.2.6. Figure 8.2.6a and 8.2.6b shows the weight loss in CO_2 and steam atmosphere respectively. *Chlorella* char from EFR displayed higher weight loss in CO_2 than steam whereas the reverse behaviour was observed for char prepared in TGA. In general, commercial wood mix char prepared from both EFR and TGA showed consistent behaviour irrespective of the gasifying agent and lost more weight than algae. Figure 8.2.6c and 8.2.6d describes the reactivity at 800 °C under CO_2 and steam. It is interesting to note that among all the chars, the highest reactivity at 800 °C was observed for *Chlorella* char prepared in TGA during steam gasification. The reactivity of the *Chlorella* char from EFR was the lowest. It might be due to the structure observed under a scanning electron microscope.

The CO_2 and steam gasification behaviour of the four chars at 950 °C is presented in Figure 8.2.7. The highest weight loss is observed for commercial wood mix char prepared in EFR. *Chlorella* char from TGA is found to be highly reactive in CO_2 whereas the commercial wood mix char from EFR was the most reactive in steam. Alga char from the EFR is found to have the lowest reactivity among the chars. At 1100 °C, commercial wood mix char from EFR showed maximum reactivity as well as weight loss in both the gasifying agents. The second highest reactivity was observed for the alga char prepared in TGA. These characteristics are demonstrated in Figure 8.2.8. Again the lowest reactivity was observed for the EFR char from *Chlorella*, consistent with the structural analysis in the previous section. The structure of the commercial wood mix char from EFR was found to be the most reactive one.

Therefore, the general idea of higher reactivity with a faster heating rate does hold for woody biomass but not for algal biomass. The reactivity of algal biomass was higher for the char prepared in TGA. At 800 °C and 950 °C, gasification reactivity of the algal char from TGA was similar to the commercial wood mix char derived from EFR in relation to gasification reactivity irrespective of gasifying agent. At 1100 °C, however, reactivity of algal chars was lower than the reactivity of commercial wood mix char in both CO_2 and steam.

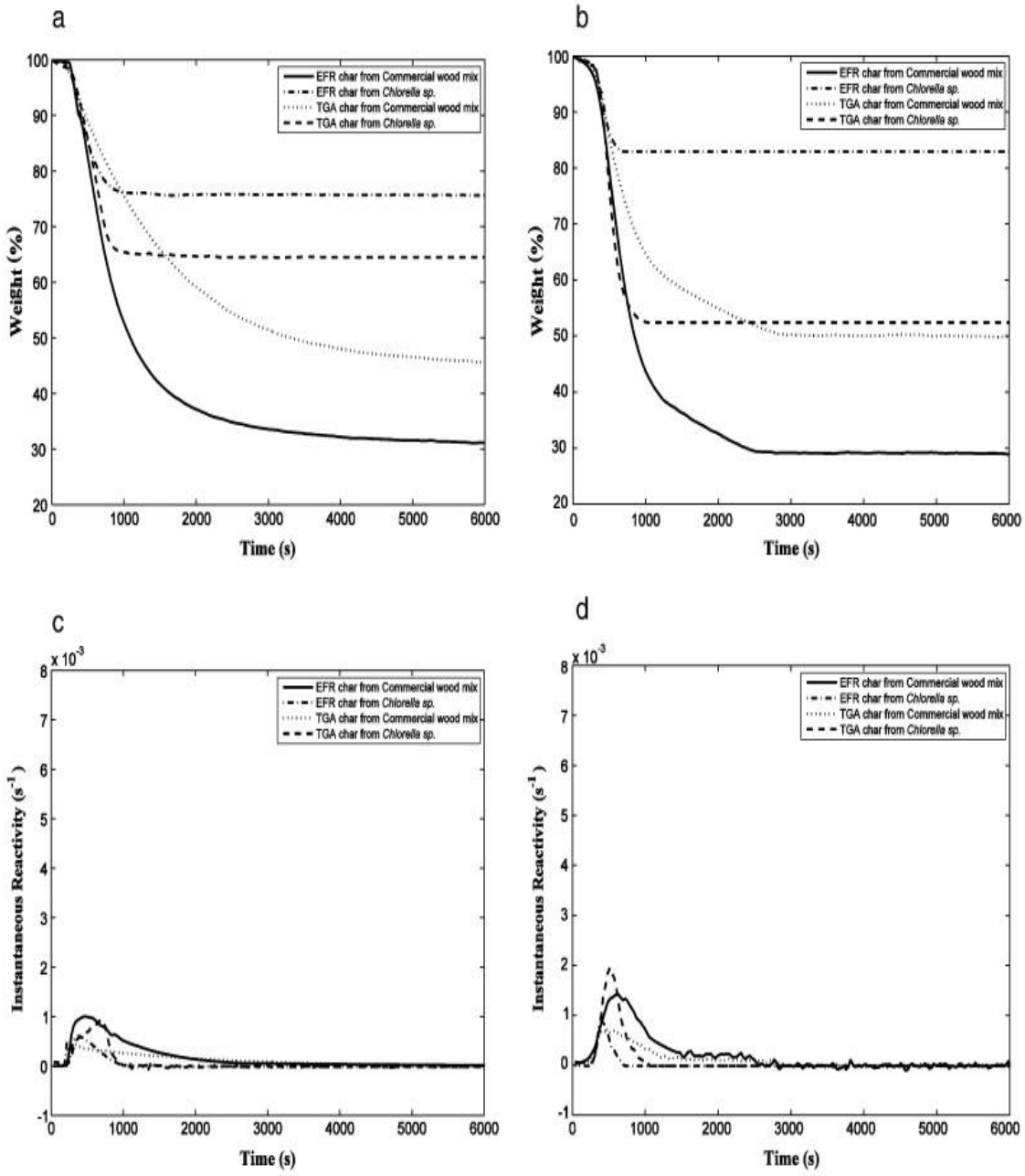


Figure 8.2.6 Gasification characteristics of different types of char at 800°C – (a) weight loss in 20% CO_2 (b) weight loss in 20% steam (c) reactivity in 20% CO_2 and (d) reactivity in 20% steam.

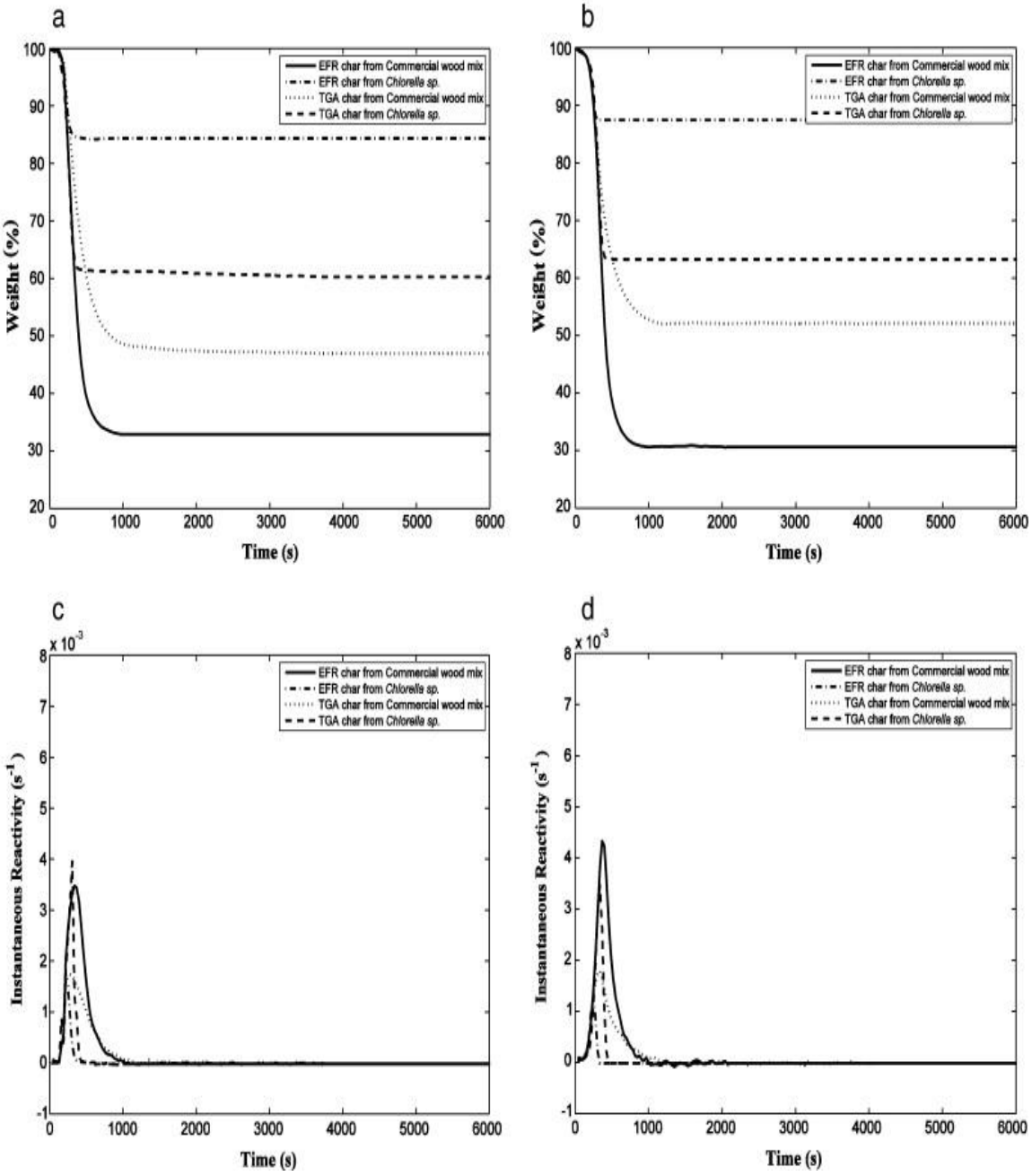


Figure 8.2.7 Gasification characteristics of different types of char 950°C – (a) weight loss in 20% CO_2 (b) weight loss in 20% steam (c) reactivity in 20% CO_2 and (d) reactivity in 20% steam.

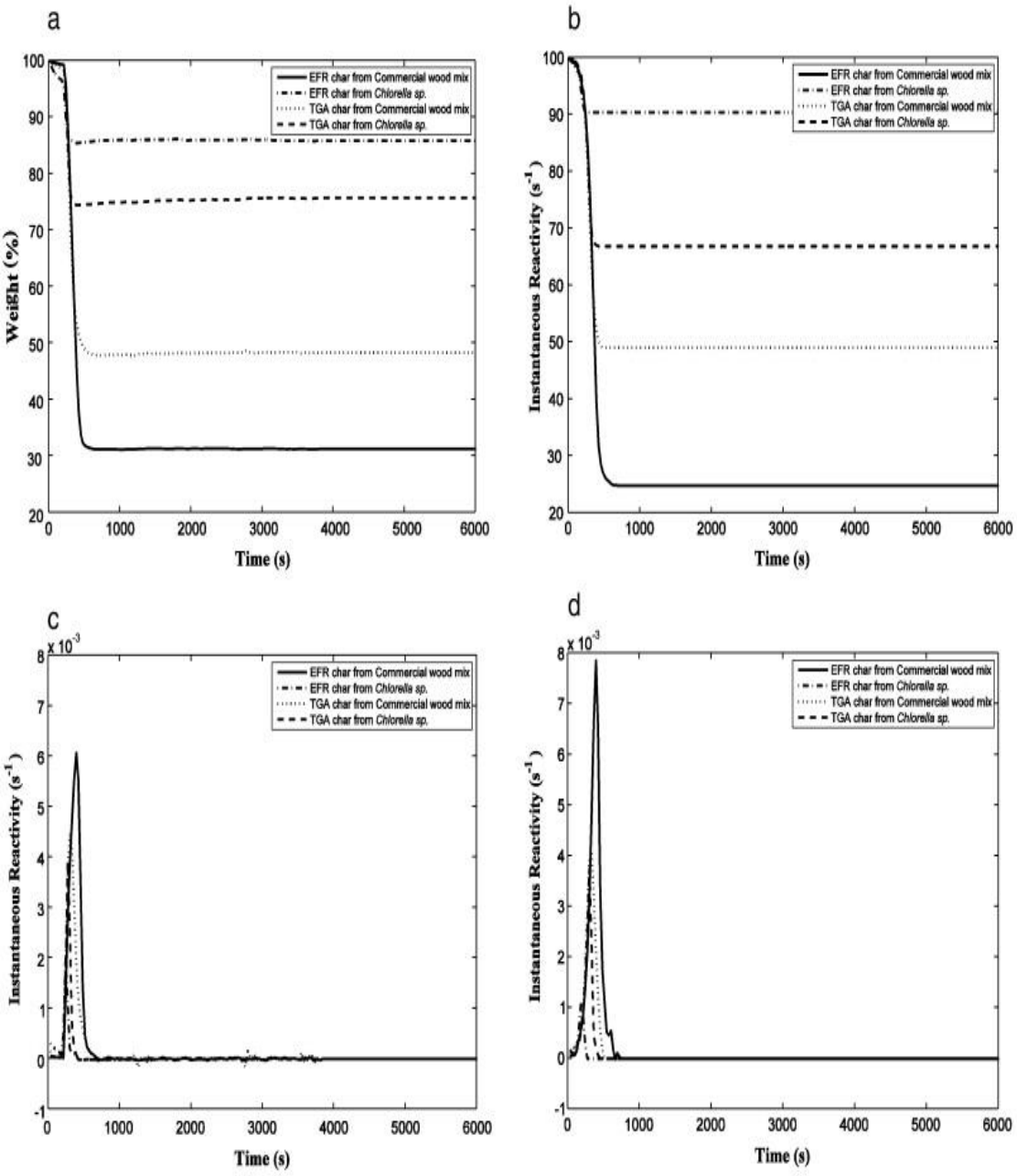


Figure 8.2.8 Gasification characteristics of different types of char 1100°C – (a) weight loss in 20% CO_2 (b) weight loss in 20% steam (c) reactivity in 20% CO_2 and (d) reactivity in 20% steam.

Comparison of weight loss and estimated reactivities, as shown in Figure 8.2.6, Figure 8.2.7 and Figure 8.2.8, also reveals two important pieces of information of practical relevance. A temperature up to 800 °C and about 1000 s (eg. roughly 20 min) is sufficient to accomplish major conversion of char from both the species in a fixed bed arrangement. If thermochemical processing of these chars is to occur in a fluidized bed or entrained flow reactor, the time requirement for a certain conversion is likely to be less.

Also FT-IR characterisation was carried out on raw woody and algal biomass, and their char produced in the EFR and the TGA. Although the results have not been presented here, it was clear that the major functional groups were lost in the char produced after pyrolysis. Therefore, the char reactivity of both these species was not affected by the functional groups present in the raw algae or woody biomass.

8.2.5 Conclusion

Algal and woody biomass chars prepared in similar conditions showed significant difference in structure and gasification reactivity. Clinker like structure was observed for algal char prepared in entrained flow reactor and it showed the lowest reactivity in all cases studied. The algal char obtained at a lower heating rate from TGA showed rigid structure despite its smaller particle size in comparison to the EFR char. At temperatures below 950 °C, the reactivity of algal char from TGA was similar to that of the commercial wood mix char derived from EFR in both gasifying agents. In the case of woody biomass, high reactivity was observed for commercial wood mix char from EFR. Woody chars from both EFR and TGA showed higher reactivity than the algal char at 1100 °C under both CO_2 and steam. It is likely that pyrolysis of algae at a lower heating rate would result in highly reactive char during low temperature gasification regardless of the gasifying agent. For chars of both the species, a temperature of 800 °C and time of around 20 min are found to be sufficient to accomplish most conversion.

8.2.6 References

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CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

9. Conclusions and Recommendations for Future Work

The aim of this research is to explore the potential for the production of acetone-butanol-ethanol (ABE) by fermentation of two different microalgal biomass, freshwater microalgae *Chlorella* sp. and marine water microalgae *Tetraselmis suecica*. This research included investigation of the entire bioprocess chain for biofuel (butanol) production including cultivation, pretreatment, enzymatic saccharification and fermentation. A preliminary investigation on the pyrolysis and gasification of microalgal biomass was also performed in this study. The results establish that dilute alkaline pretreated *Chlorella* sp. and *T. suecica* biomass have the potential to produce ABE under specific process conditions. The key conclusions drawn from this study are summarised in section 9.1. Recommendations for future work are also presented in the 9.2.

9.1 Conclusions

Conclusions from different chapters are summarised in the following sub-sections.

9.1.1 Microalgal growth and carbohydrate accumulation

Cultivation parameters significantly affected microalgal growth and carbohydrate accumulation during the cultivation period. Overall, the microalgae species displayed a different growth profile at different cultivation conditions. The suitable cultivation of *Chlorella* sp. and *T. suecica* are at a light intensity range between 2000 lux and 3000 lux, temperature range between 25°C and 30°C and pH value between 6 and 7. The presence of NaCl in the cultivation medium was found to inhibit *Chlorella* sp. growth, while a different finding was observed for *T. suecica*. Maximum biomass production, specific growth rate (μ) and carbohydrate content for *Chlorella* sp. of 0.567 gL^{-1}, 0.252 d^{-1} and 32.41% of dried biomass was attained at 2000 lux, 30°C in a medium having an initial pH of 7 without the addition of NaCl. In the case of *T. suecica*, the maximum biomass production, specific growth rate (μ) and carbohydrate content of 0.54 gL^{-1}, 0.22 d^{-1} 20.6% of dried biomass was attained at 3000 lux, 30°C in a medium having an initial pH of 7 and 30 gL^{-1} of NaCl. Cultivation beyond these conditions could produce low microalgal biomass concentration. Additionally, the results also indicated that both microalgae have the capability to grow in a medium containing 15% (v/v) CO_2.

9.1.2 Outdoor cultivation of *Chlorella* sp. and *T. suecica* in centric photobioreactor

Outdoor cultivation of *Chlorella* sp. and *T. suecica* displayed a good growth rate during cultivation at a high temperature range (Temperature: 20-32°C). The ambient temperature and light intensity during cultivation were found to have a significant effect on the growth of both microalgal species. Outdoor cultivation (*Chlorella* sp.: 0.24 gL^{-1} and *T. suecica*: 0.29 gL^{-1}) produced lower biomass concentration compared to indoor cultivation (*Chlorella* sp.: 0.61 gL^{-1} and *T. suecica*: 0.61 gL^{-1}). Large temperature and light intensity fluctuations during the cultivation were found to influence the growth of microalgae in outdoor cultivation. Chemical composition analysis indicated that the chemical composition of microalgal cultivated in different cultivation modes was different. Protein content was the major component in all microalgal biomass cultivated in different conditions. However, storage energy compound such as carbohydrate and lipid in microalgal biomass from outdoor cultivation using 15% (v/v) CO_2 was 2 times higher compared to that in indoor cultivation. Overall, this study concludes that environmental factors such as ambient temperature and light intensity are amongst the challenges for outdoor cultivation. For the case in this study, the most suitable period for outdoor cultivation of both microalgae species is during a high temperature range (Temperature: 20-32°C). Therefore, the ambient cultivation parameters such as temperature and light intensity should be considered for mass microalgal biomass production through outdoor cultivation.

9.1.3 Dilute alkaline pretreatment of microalgal biomass

The dilute alkaline pretreatment conditions for of *Chlorella* sp. and *T. suecica* conditions were optimised. With r^2 close to 1.0, a quadratic surface model was found to describe the relationship of the three pretreatment variables (temperature, alkaline concentration and reaction time) that were involved in the process. The suitable pretreatment condition for *T. suecica* was at 120°C using 2% KOH for 120 min, while that for *Chlorella* sp. was at 120°C using 2% NaOH for 30 min. Pretreatment at higher temperature (>120°C) using higher alkaline concentration (>2% w/v) leads to the reducing sugar degradation.

Preliminary enzymatic saccharification of pretreated microalgal biomass was performed, and it was found that 2 fold higher reducing sugar was produced compared to untreated samples for both *Chlorella* sp. and *T. suecia*. Higher reducing sugar production

from pretreated microalgal biomass is attributed to the disruption of biomass structure after pretreatment process. It was confirmed by the SEM and FTIR analysis, that the microalgal biomass structure and functional group after pretreatment were changed significantly, which resulted in high reducing sugar production during enzymatic saccharification. This clearly indicates that dilute alkaline pretreatment has potential as an alternative pretreatment method to be used to enhance enzymatic saccharification of microalgal biomass.

9.1.4 Enzymatic saccharification of microalgal biomass

This study demonstrated that dilute alkaline pretreatment was able to enhance reducing sugar production from microalgal biomass. Temperature, pH, enzyme concentration and biomass concentration have a significant effect on the reducing sugar production and the saccharification yield. The maximum saccharification yield of 80–95% for *Chlorella* sp. was obtained when the saccharification was performed using 10 gL^{-1} of biomass at a pH of 5.5 and at 40°C 72 h. On the other hand, the maximum saccharification yield of 90% for *T. suecica* was obtained when the saccharification was carried out using 10 g/L of biomass with pH 4.5, at 50°C for 72 h. This study also indicates that glucose and xylose are the major sugars present in the enzymatic hydrolysate which can be used as a chemical platform for biofuel production especially through the fermentation process. In summary, this study demonstrates that a combination of dilute alkaline pretreatment and enzymatic saccharification at low temperature could produce a high level of reducing sugar from the microalgal biomass. The added benefits include avoidance of acid use and a low energy input requirement.

9.1.5 Acetone-butanol-ethanol (ABE) fermentation of microalgal biomass

The enzymatic saccharification and ABE fermentation of the samples were evaluated. The final carbohydrate content of the biomass after treatment, the saccharification yield and the ABE concentration were determined in this study. The study indicates that the final carbohydrate content in both *Chlorella* sp. and *T.suecica* samples were reduced after the alkaline treatment, lipid extraction and combination of lipid extraction followed by alkaline treatment.

For enzymatic saccharification study, the highest enzymatic saccharification yield for both *Chlorella* sp. and *T. suecica* was obtained from the alkaline pretreated sample with 75% of carbohydrate content for AkChl and 95% of carbohydrate content for AkTetra respectively. Interestingly, the results also indicated that the enzymatic saccharification of the lipid-extracted biomass displayed a negative effect on the saccharification yield. Although the carbohydrate content for lipid-extracted biomass was similar with alkaline pretreatment biomass, however, enzymatic saccharification of lipid-extracted biomass (ExChl and ExTetra) exhibited the lowest saccharification yield.

With respect to the fermentation of microalgal biomass, the ABE fermentation of AkChl and AkTetra produced the highest ABE concentration. The ABE production yield for *Chlorella* sp. and *T. suecica* was 0.016 g/g and 0.013 g/g $_{\text{dried biomass}}$ respectively. The butanol conversion yield for the fermentation of alkaline pretreated *Chlorella* sp. and *T. suecica* was 0.3% and 0.7% of dried biomass respectively. The ABE fermentation of all microalgal biomass found that a higher organic acid was produced compared to a solvent concentration. The reason behind this finding is due to the low reducing sugar concentration in hydrolysate, which is insufficient to assist the shifting from the acidogenesis to the solventogenesis phase. Furthermore, these results also concluded that the ABE fermentation of microalgal biomass performance appeared to be influenced by the type of biomass feedstock used (sugar concentration and pre-processing strategy). Overall, this finding suggests that alkaline pretreated microalgal is the suitable option to be used as ABE fermentation feedstock due to high enzymatic saccharification yield and the produced high ABE concentration compared to other treated biomass.

9.1.6 Pyrolysis and gasification of microalgal biomass

Determination of the pyrolysis characteristic of the lipid extracted microalgal biomass was carried out using a thermogravimetric analyser (TGA) and the activation energy was determined using the Flyn-Wall-Ozawa (FWO) and Kissinger-Akahira-Sunose (KAS) methods. Based on this study, the pyrolysis curve for both microalgal biomass are similar and could be divided into three stages: dehydration, devolatilisation, and decomposition of the carbonaceous matter. The activation energy of lipid-extracted *Chlorella* sp. and *T. suecica* biomass was compared with the activation energy of other lignocellulosic biomass. The results indicated that low activation energy was observed for lipid-extracted *T. suecica*

biomass (FWO: 99.69 kJ/mol and KAS: 94.77 kJ/mol) compared than that of lipid-extracted *Chlorella* sp. (FWO: 298.42 kJ/mol and KAS: 301.70 kJ/mol) and other types of lignocellulosic biomass. The differences of activation energy displayed by lipid-extracted microalgal biomass are attributed to the difference on the chemical composition presence in the biomass. The lipid extracted microalgal biomass exhibited low activation energy, which was favourable to be used in thermochemical conversion. In addition, the gasification of microalgae at 800°C and the time of around 20 min were the suitable condition to complete the conversion in a thermogravimetric analyser.

9.2 Recommendations for future work

The findings from this study provide information on the consolidating processing steps involved in biofuel and chemical production from microalgal biomass through the ABE fermentation pathway. Although the results suggested that butanol and other value-added chemicals (acetone, ethanol, acetic acid and butyric acid) could be produced from microalgal biomass, much work needs to be done to realise full-scale application of the process. The following recommendations for future research are made based on the outcomes of the work presented in this study:

1) **Biomass production improvement**: Microalgal cultivation using heterotrophic and mixotrophic strategies for *Chlorella* sp. and *T. suecica* biomass production should be explored using an additional cheap carbon source. The presence of additional cheap carbon such as waste glycerol produced from the biodiesel conversion (transesterification) process in the cultivation medium could be beneficial for microalgal biomass and biofuel production. In order to understand the mechanisme of microalgal growth, the effect of cultivation condition at molecular level also should be carried out in future study.

2) **Microalgal biomass production through biorefinery strategy**: The results obtained in chapters 3 and 4 indicate that both microalgae species have the capability to grow in high CO_2 concentration. Thus future work on the potential of microalgal to capture the real flue gas emitted from the pilot plant could significantly make the biofuel and chemical production from microalgal biomass sustainable. Moreover, the microalgae cultivation in wastewater pretreatment plant using flue gas would be useful in

upstream processing.

3) **Enzymatic saccharification using high biomass loading**: It is crucial to produce high reducing sugar before the fermentation process. Another technique to obtain high reducing sugar concentration is using a high biomass concentration. Enzymatic saccharification at high biomass concentration could lower capital costs due to low liquid volume and lower operating costs due to less energy being required for heating and cooling during the process. However, based on the results obtained from this study, the enzymatic saccharification using a high solid biomass concentration (>20 gL^{-1}) produced low reducing sugar and saccharification yield. Thus, future work on enzymatic saccharification using high biomass concentration through the fed-batch enzymatic saccharification approach should be explored in order to ensure the process is carried out in a cheap and sustainable manner.

4) **Fermentation optimisation and product improvement**: This study indicates that biofuel such as ethanol and butanol can possibly be produced from microalgal biomass. However, low concentration and yield produced from this study requires further investigation. Hence, the further fermentation optimisation of microalgal biomass should be carried out in order to improve and increase the ABE concentration and yield. An optimisation study of the ABE fermentation parameters such as initial pH, initial biomass concentration and temperature should be part of the future work. On the other hand, in order to understand the relationship between enzymatic saccharification and fermentation, Simultaneous Saccharification and Fermentation (SSF) should be explored in the future study.

5) **Biofuel production using a combination of alkaline pretreatment followed by enzymatic saccharification hydrolysate**: This study showed that combination of alkaline pretreatment followed by enzymatic saccharification is a promising approach to produce biofuel from microalgal biomass. This approach can also be applied to produce other microalgal carbohydrate-based fuels, for instance bioethanol, methane and biohydrogen.

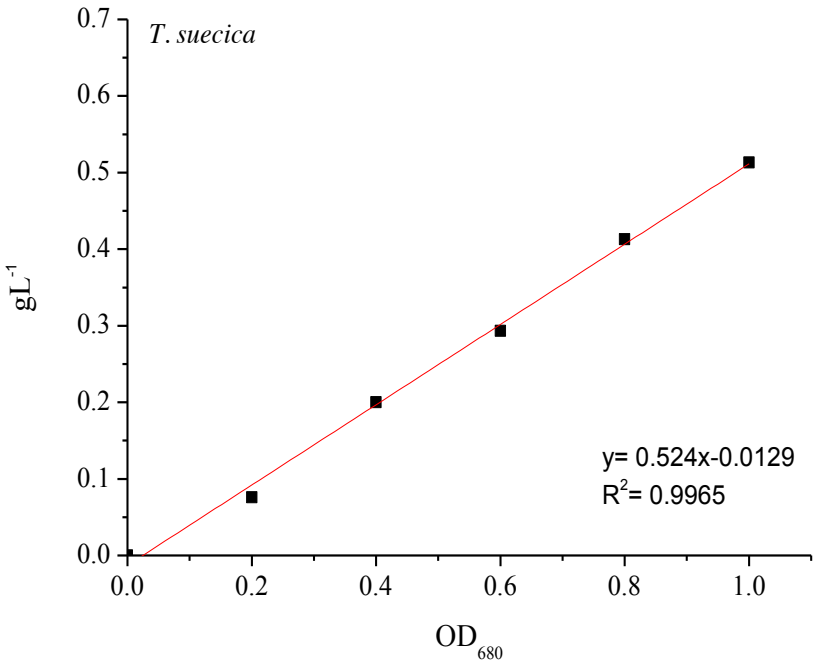
6) **Pyrolysis and gasification of lipid extracted biomass:** Another approach for butanol production is through chemical reaction using syngas. The potential of syngas

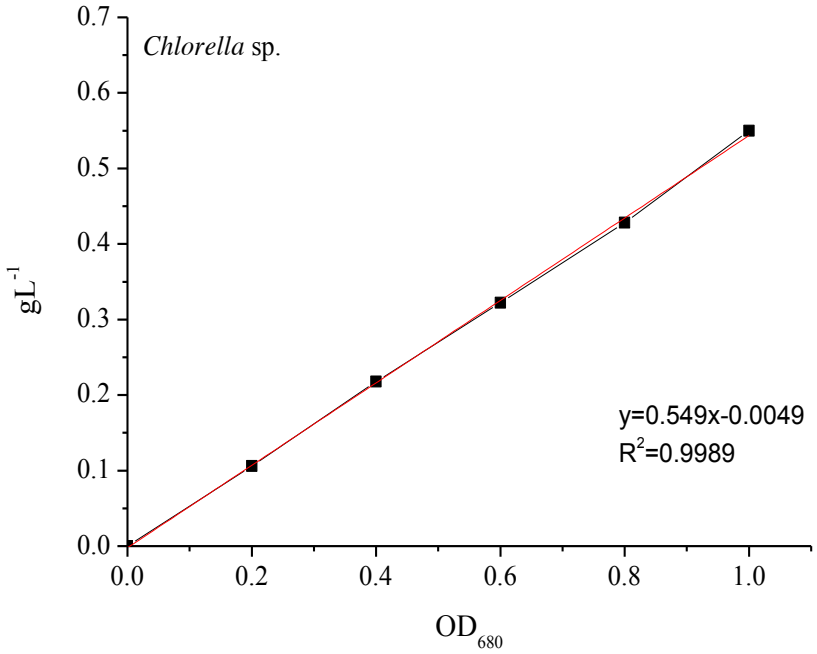
production from the lipid extracted biomass should be explored and studied in detail. This study should include the determination of the gasification operation condition and syngas composition analysis. On the other hand, a study on the effect of the mineral composition of the microalgal biomass on the pyrolysis performance should also be part of the future work. Study on the butanol production using gas produced from gasification of microalgal biomass should be explored in order to ensure the possibility and potential of this biomass as a potential alternative butanol feedstock.

APPENDICES

Appendix A

A.1 Standard curve for microalgal biomass





A.2 Statistical analysis for determination of the effect of different light intensities on biomass production from *Chlorella* sp.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	1221.41	407.13	1102.70	6.55E-15
Error	12	4.43	0.369		
Total	15	1225.84			

Null Hypothesis: The means of all level are equal
Alternatve Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significant different

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1000	0	24.27	0.43	79.87	0	0.05	1	22.99	25.54
2000	0	8.3	0.43	27.31	0	0.05	1	7.02	9.57
2000	1000	-16.00	0.43	52.56	0	0.05	1	-17.24	-14.69
3000	0	12.09	0.43	39.79	0	0.05	1	10.81	13.37
3000	1000	-12.18	0.43	40.08	0	0.05	1	-13.45	-10.9
3000	2000	3.79	0.43	12.48348	7.03E-06	0.05	1	2.51707	5.07

Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

Statistical analysis for determination of the effect of different light intensities on biomass production from *T. suecica* sp.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	134.03325	44.67775	25.13097	1.85E-05
Error	12	21.33355	1.7778		
Total	15	155.3668			

Null Hypothesis: The means of all level are equal
Alternatve Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significant different

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
1000	0	-2.54593	0.94281	3.81888	0.07911	0.05	0	-5.34504	
2000	0	1.2336	0.94281	1.85038	0.57516	0.05	0	-1.56551	
2000	1000	3.77953	0.94281	5.66926	0.00814	0.05	1	0.98042	
3000	0	5.45932	0.94281	8.18893	4.32E-04	0.05	1	2.66021	
3000	1000	8.00525	0.94281	12.00781	1.10E-05	0.05	1	5.20615	
3000	2000	4.22572	0.94281	6.33855	0.00361	0.05	1	1.42662	

Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

A.3 Statistical analysis for determination of the effect of different temperatures on biomass production from *Chlorella* sp.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	446.36783	148.78928	110.57201	5.23E-09
Error	12	16.14759	1.34563		
Total	15	462.51541			

Null Hypothesis: The means of all level are equal
Alternatve Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significant different

Turkey's Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
25	20	-2.3622	0.82025	4.07272	0.05828	0.05	0	-4.79744	0.07303
30	20	11.07612	0.82025	19.09652	0	0.05	1	8.64088	13.51135
30	25	13.43832	0.82025	23.16924	0	0.05	1	11.00308	15.87356
40	20	6.29921	0.82025	10.86058	2.94E-05	0.05	1	3.86398	8.73445
40	25	8.66142	0.82025	14.9333	9.17E-07	0.05	1	6.22618	11.09665
40	30	-4.7769	0.82025	8.23594	4.10E-04	0.05	1	-7.21214	-2.34167

Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

Statistical analysis for determination of the effect of different temperatures on biomass production from *T. suecica*.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	292.5414	97.5138	84.7661	2.41E-08
Error	12	13.80464	1.15039		
Total	15	306.34604			

Null Hypothesis: The means of all level are equal
Alternatve Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significant different

Turkey's Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
30	20	2.82983	0.75842	5.27677	0.0132	0.05	1	0.57818	5.08147
30	25	9.52178	0.75842	17.75523	2.76E-08	0.05	1	7.27014	11.77343
40	20	4.52263	0.75842	8.43334	3.31E-04	0.05	1	2.27099	6.77428
40	25	11.21459	0.75842	20.9118	0	0.05	1	8.96295	13.46624
40	30	1.69281	0.75842	3.15657	0.16974	0.05	0	-0.55884	3.94445

Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

A.4 Statistical analysis for determination of the effect of different pH on biomass production from *Chlorella* sp.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	726.52358	242.17453	29.88295	7.53E-06
Error	12	97.24923	8.1041		
Total	15	823.77282			

Null Hypothesis: The means of all level are equal
Alternatve Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significant different

Turkey's Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
7	4	-15.08385	2.01297	10.59717	3.76E-05	0.05	1	-21.06013	-9.10758
8	4	-15.74567	2.01297	11.06213	2.45E-05	0.05	1	-21.72195	-9.7694
8	7	-0.66182	2.01297	0.46496	0.98712	0.05	0	-6.63809	5.31445
10	4	-4.73853	2.01297	3.32906	0.13997	0.05	0	-10.71481	1.23774
10	7	10.34532	2.01297	7.26811	0.00121	0.05	1	4.36905	16.3216
10	8	11.00714	2.01297	7.73307	7.14E-04	0.05	1	5.03087	16.98342

Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

Statistical analysis for determination of the effect of different pH on biomass production from *T. suecica*

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	578.42637	192.80879	386.28271	3.38E-12
Error	12	5.98967	0.49914		
Total	15	584.41604			

Null Hypothesis: The means of all level are equal
Alternatve Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significant different

Turkey's Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
7	4	-5.66821	0.49957	16.04593	3.45E-07	0.05	1	-7.15137	-4.18505
8	4	-12.16738	0.49957	34.4442	0	0.05	1	-13.65054	-10.68421
8	7	-6.49917	0.49957	18.39827	0	0.05	1	-7.98233	-5.016
10	4	3.74803	0.49957	10.61018	3.72E-05	0.05	1	2.26487	5.2312
10	7	9.41624	0.49957	26.65611	0	0.05	1	7.93308	10.8994
10	8	15.91541	0.49957	45.05438	0	0.05	1	14.43225	17.39857

Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

A.5 Statistical analysis for determination of the effect of different NaCl concentrations on biomass production from *Chlorella* sp.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	4	406.63006	101.65752	28.61412	7.28E-07
Error	15	53.29057	3.5527		
Total	19	459.92063			

Null Hypothesis: The means of all level are equal
Alternatve Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significant different

Turkeys's Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
10	0	-6.66472	1.3328	7.07184	0.00127	0.05	1	-10.78026	-2.54917
20	0	-9.32859	1.3328	9.89843	3.65E-05	0.05	1	-13.44414	-5.21305
20	10	-2.66387	1.3328	2.8266	0.31274	0.05	0	-6.77942	1.45167
30	0	-3.60964	1.3328	3.83013	0.09967	0.05	0	-7.72518	0.50591
30	10	3.05508	1.3328	3.2417	0.20085	0.05	0	-1.06046	7.17063
30	20	5.71895	1.3328	6.0683	0.00495	0.05	1	1.60341	9.8345
40	0	-13.05977	1.3328	13.85753	5.16E-07	0.05	1	-17.17531	-8.94422
40	10	-6.39505	1.3328	6.7857	0.00187	0.05	1	-10.5106	-2.27951
40	20	-3.73118	1.3328	3.9591	0.08475	0.05	0	-7.84672	0.38437
40	30	-9.45013	1.3328	10.0274	3.14E-05	0.05	1	-13.56568	-5.33459

Sig equals 1 indicates that the means difference is significant at the 0.05 level

Sig equals 0 indicates that the means difference is not significant at the 0.05 level

Statistical analysis for determination of the effect of different NaCl concentrations on biomass production from *T. suecica*.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	4	246.97893	61.74473	30.90105	4.40E-07
Error	15	29.97215	1.99814		
Total	19	276.95108			

Null Hypothesis: The means of all level are equal
Alternatve Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significant different

Turkey's Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
10	0	4.02127	0.99954	5.68958	0.00832	0.05	1	0.93481	7.10774
20	0	2.75288	0.99954	3.89497	0.0919	0.05	0	-0.33358	5.83934
20	10	-1.26839	0.99954	1.79461	0.71298	0.05	0	-4.35486	1.81807
30	0	6.43411	0.99954	9.10343	9.44E-05	0.05	1	3.34764	9.52057
30	10	2.41284	0.99954	3.41385	0.16488	0.05	0	-0.67363	5.4993
30	20	3.68123	0.99954	5.20846	0.0161	0.05	1	0.59477	6.76769
40	0	-3.78776	0.99954	5.3592	0.01309	0.05	1	-6.87423	-0.7013
40	10	-7.80904	0.99954	11.04877	9.87E-06	0.05	1	-10.8955	-4.72257
40	20	-6.54064	0.99954	9.25416	7.86E-05	0.05	1	-9.62711	-3.45418
40	30	-10.22187	0.99954	14.46263	2.78E-07	0.05	1	-13.30834	-7.13541

Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

Appendix B

B.1.1 Sequential Model Sum of Squares for *T. suecica*

Model	Sum of Square	DF	Mean of Square	F value	Prof > F	
Mean	35000.83	1	35000.83			
Linear	6467.73	3	2155.91	4.01	0.03	
2FI	811.52	3	270.51	0.45	0.72	
Quadratic	7227.55	3	2409.18	43.48	0.00	Suggested
Cubic	486.15	4	121.54	10.73	0.01	Aliased
Residual	67.94	6	11.32			
Total	50061.72	20	2503.09			

B.1.2 Sequential Model Sum of Squares for *Chlorella* sp.

Model	Sum of Square	DF	Mean of Square	F value	Prof > F	
Mean	0.32	1	0.32			
Linear	0.012	3	0.00	1.66	0.22	
2FI	0.02	3	0.01	4.256	0.03	
Quadratic	0.02	3	0.01	25.77	0.03	Suggested
Cubic	0.00	4	0.00	4.937	0.04	Aliased
Residual	0.00	6	0.00			
Total	0.36	20	0.02			

B.2.1 Analysis of variance (ANOVA) for *T. suecica*

Regression statistic	
R square	0.96
Adjust R-square	0.93
Standard error	7.44

ANOVA

	Df	SS	MS	F	Significant F
Regression	9	14506.79	1611.87	29.09	0.00
Residual	10	554.08	55.41		
Total	19	15060.88			

Variables	Coeffecient	Std error	P-value
Intercept	70.42	3.04	0.00
Alkaline concentration	14.60	2.01	0.00
Temperature	10.45	2.01	0.00
Time	12.30	2.01	0.00
Concentration × Concentration	-11.58	1.96	0.00
Temp × Temp	-13.66	1.96	0.00
Time × Time	-16.62	1.96	0.00
Concentration × Temp	0.24	2.63	0.93
Concentration × Time	8.23	2.63	0.01
Temp × Time	5.81	2.63	0.05

SS: Sum of squares,, MS: Mean of square

B.2.2 Analysis of variance (ANOVA) for *Chlorella* sp.

Regression statistic	
R square	0.92
Adjust R-square	0.85
Standard error	8.10

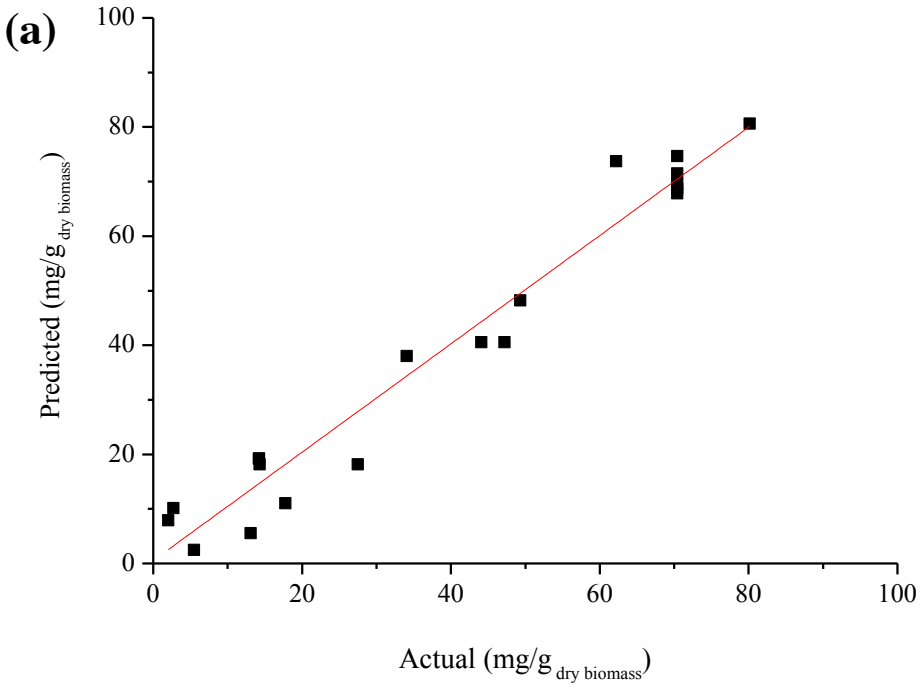
ANOVA

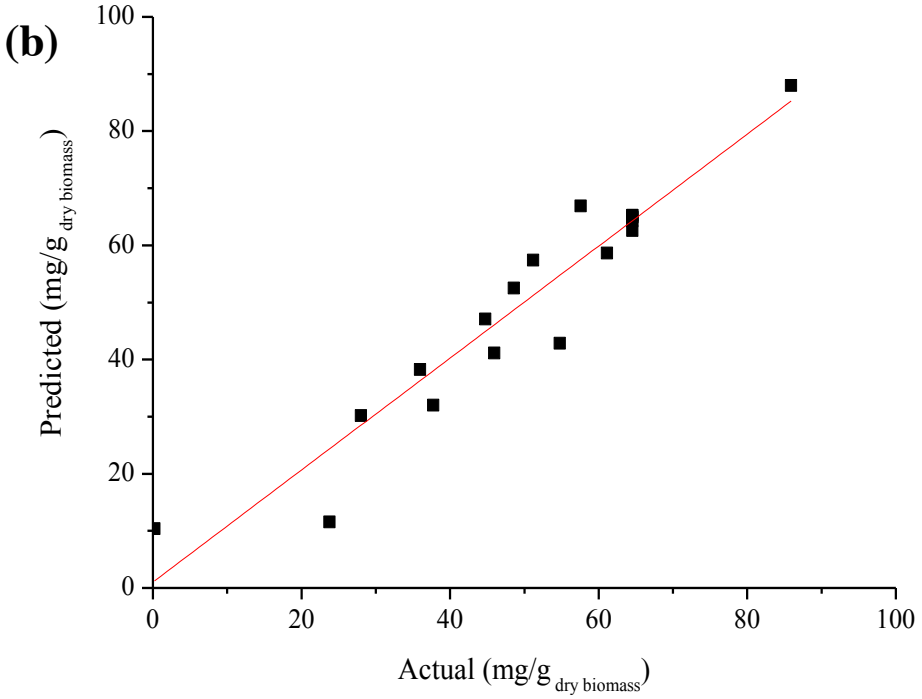
	Df	SS	MS	F	Significant F
Regression	9	7711.81	856.87	13.07	0.00
Residual	10	655.63	65.56		
Total	19	8367.45			

Variables	Coeffecient	Std error	P-value
Intercept	64.55	3.30	0.00
Alkaline concentration	7.39	2.19	0.00
Temperature	12.29	2.19	0.00
Time	-0.92	2.19	0.68
Concentration × Concentration	-10.03	2.13	0.00
Temp × Temp	-8.51	2.13	0.00
Time × Time	-3.99	2.13	0.09
Concentration × Temp	0.13	2.86	0.10
Concentration × Time	-6.64	2.86	0.04
Temp × Time	-16.53	2.86	0.00

SS: Sum of squares,, MS: Mean of square

B.3 Reducing sugar concentration predicted *vs* actual value (a) *T. suecica* (b) *Chlorella* sp.





Appendix C

C.1 Analysis of variance (ANOVA) for *Chlorella* sp.

Regression statistics					
R square	0.9531				
Adjust R-square	0.8202				
Standard error	13.42				
ANOVA					
	Df	SS	MS	F	Significant F
Regression	17	21953.86	1291.40	7.17	0.0112
Residual	1080.56				
Total	23034.42				
Variable	Coeff	Std error			P-value
Intercept	47.35	2.74			
Temperature	-6.09	2.74			0.0681
pH	13.39	4.74			0.0065
Biomass concentration	13.75	3.87			0.0027
Temperature X pH	-0.63	4.74			0.9815
Temperature X biomass concentration	-9.26	3.87			0.0072
pH X biomass concentration	1.22	6.71			0.0883

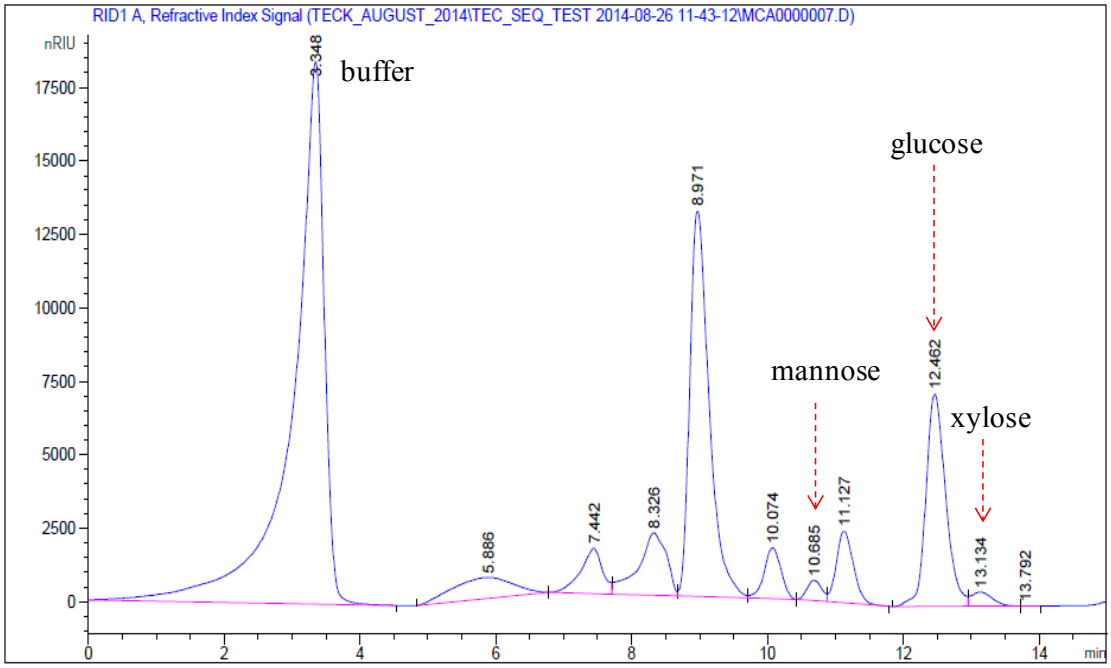
Df: Degree of freedom SS: Sum of squares MS: Mean of squares

C.2 Analysis of variance (ANOVA) for *T. suecica*

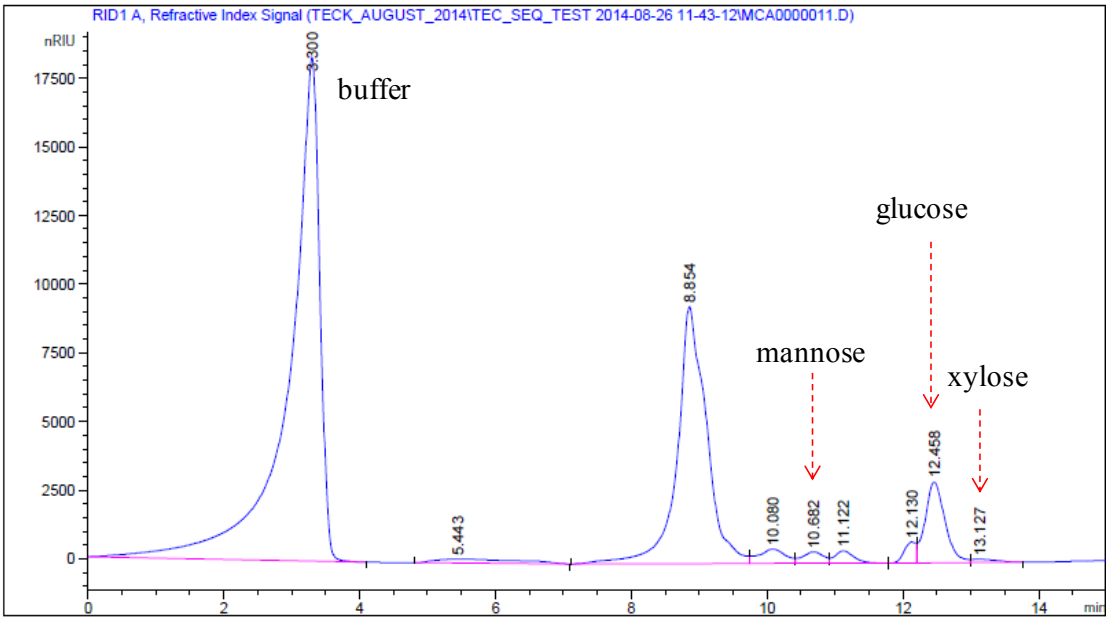
Regression statistics					
R square	0.9683				
Adjust R-square	0.8784				
Standard error	8.62				
ANOVA					
	Df	SS	MS	F	Significant F
Regression	17	13628.03	801.65	10.78	0.0038
Residual	446.34				
Total	14074.37				
Variable	Coeff	Std error			P-value
Intercept	42.21	1.76			0.0
Temperature	-2.97	7.76			0.149
pH	15.87	3.05			0.0025
Biomass concentration	10.19	2.49			0.0004
Temperature X pH	2.93	3.05			0.4904
Temperature X biomass concentration	-5.10	2.49			0.0030
pH X biomass concentration	-7.24	4.31			0.1921

Df: Degree of freedom SS: Sum of squares MS: Mean of squares

C.3 HPLC chromatogram of alkaline pretreated *Chlorella* sp. hydrolysate



C.4 HPLC chromatogram of alkaline pretreated *T. suecica* hydrolysate



Appendix D

D.1 ANOVA analysis of carbohydrate content for *Chlorella* sp. biomass

	DF	**Sum of square**	**Mean Square**	**F Value**	**Prob>F**
Model	3	81.598	27.200	75.927	0
Error	12	4.299	0.358		
Total	15	85.898			

R-Square	Coeff Var	Root MSE	Data Mean
0.94995	0.0275	0.599	21.749

		Mean Diff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Alk-Chl	Chl	-4.778	0.423	15.966	0	0.05	1	-6.035	-3.522
ExChl	Chl	-2.482	0.423	8.292	0	0.05	1	-3.738	-1.225
ExChl	Alk-Chl	2.297	0.423	7.674	0	0.05	1	1.040	3.553
ExAk-Chl	Chl	-5.880	0.423	19.648	0	0.05	1	-7.136	-4.623
ExAk-Chl	Alk-Chl	-1.102	0.423	3.681	0.093	0.05	0	-2.358	0.155
ExAk-Chl	ExChl	-3.398	0.423	11.355	0	0.05	1	-4.655	-2.142

Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

D.2 ANOVA analysis of carbohydrate for *T. suecica* biomass

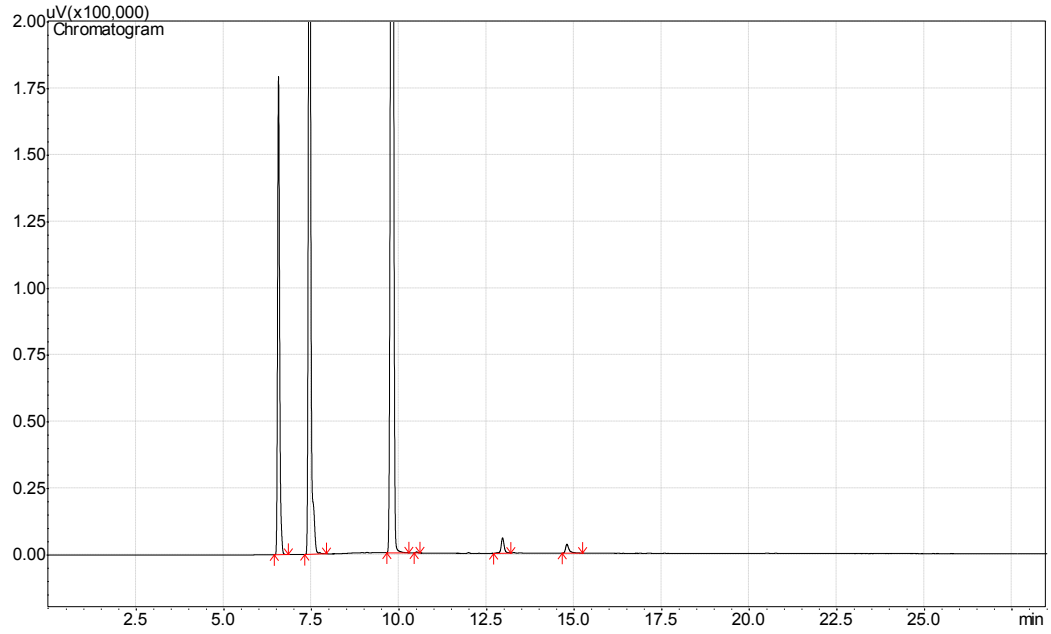
	DF	Sum of square	Mean Square	F Value	Prob>F
Model	3	112.292	37.431	49.215	0
Error	12	9.127	0.761		
Total	15	121.418			

R-Square	Coeff Var	Root MSE	Data Mean
0.99639	0.021	0.608	29.583

		MeanDiff	**SEM**	**q Value**	**Prob**	**Alpha**	**Sig**	**LCL**	**UCL**
AkTetra	Tetra	2.549	0.617	5.847	0.007	0.05	1	0.719	4.380
ExTetra	Tetra	1.685	0.617	3.863	0.075	0.05	0	-0.146	3.515
ExTetra	AkTetra	-0.865	0.617	1.984	0.521	0.05	0	-2.696	0.966
ExAkTetra	Tetra	-4.329	0.617	9.927	0	0.05	1	-6.160	-2.497
ExAkTetra	AkTetra	-6.878	0.617	15.774	0	0.05	1	-8.709	-5.047
ExAkTetra	ExTetra	-6.013	0.617	13.790	0	0.05	1	-7.844	-4.182

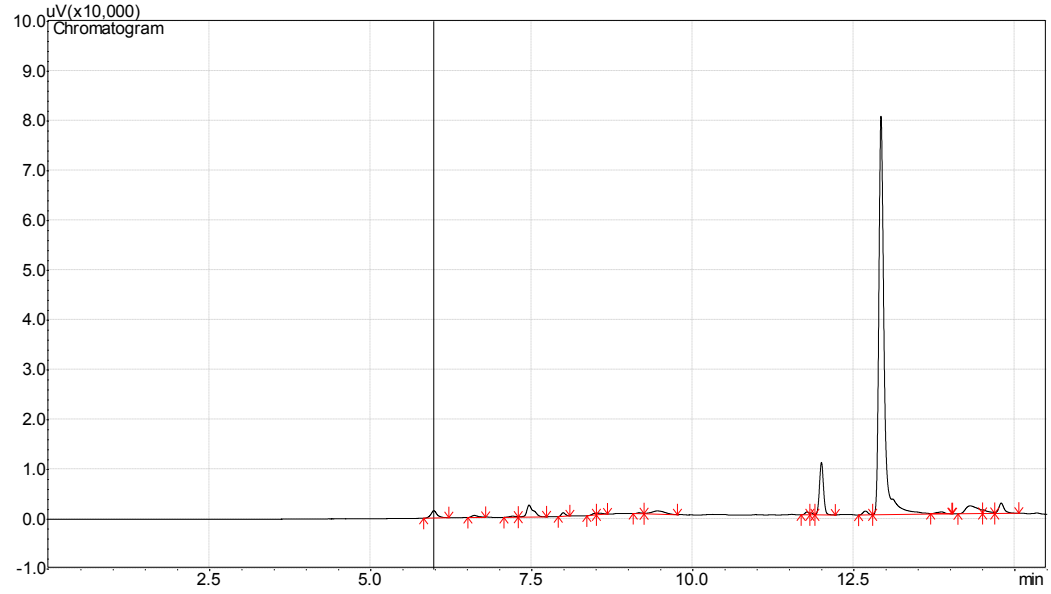
Sig equals 1 indicates that the means difference is significant at the 0.05 level
Sig equals 0 indicates that the means difference is not significant at the 0.05 level

D.3 Standard GC-FID

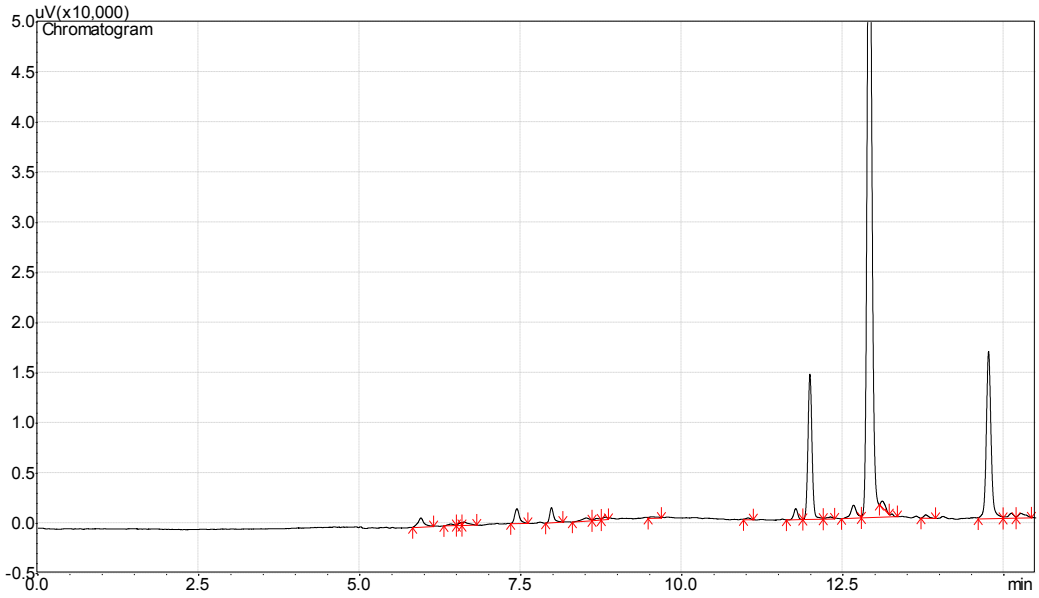


Peak#	Ret.Time	Area	Height	Units
1	6.559	721816.6	178905.4	Acetone
2	7.443	1565540.3	347990.6	Ethanol
3	9.802	2645808.1	495142.0	Butanol
4	10.503	1499.9 400.7	0.00000	
5	12.955	35063.8 5774.3	0.00000	Acetic acid
6	14.793	24392.5 3426.8	0.00000	Butyric acid

D.4 GC chromatogram from Alkaline pre-treated *Chlorella* sp. (AkChl)

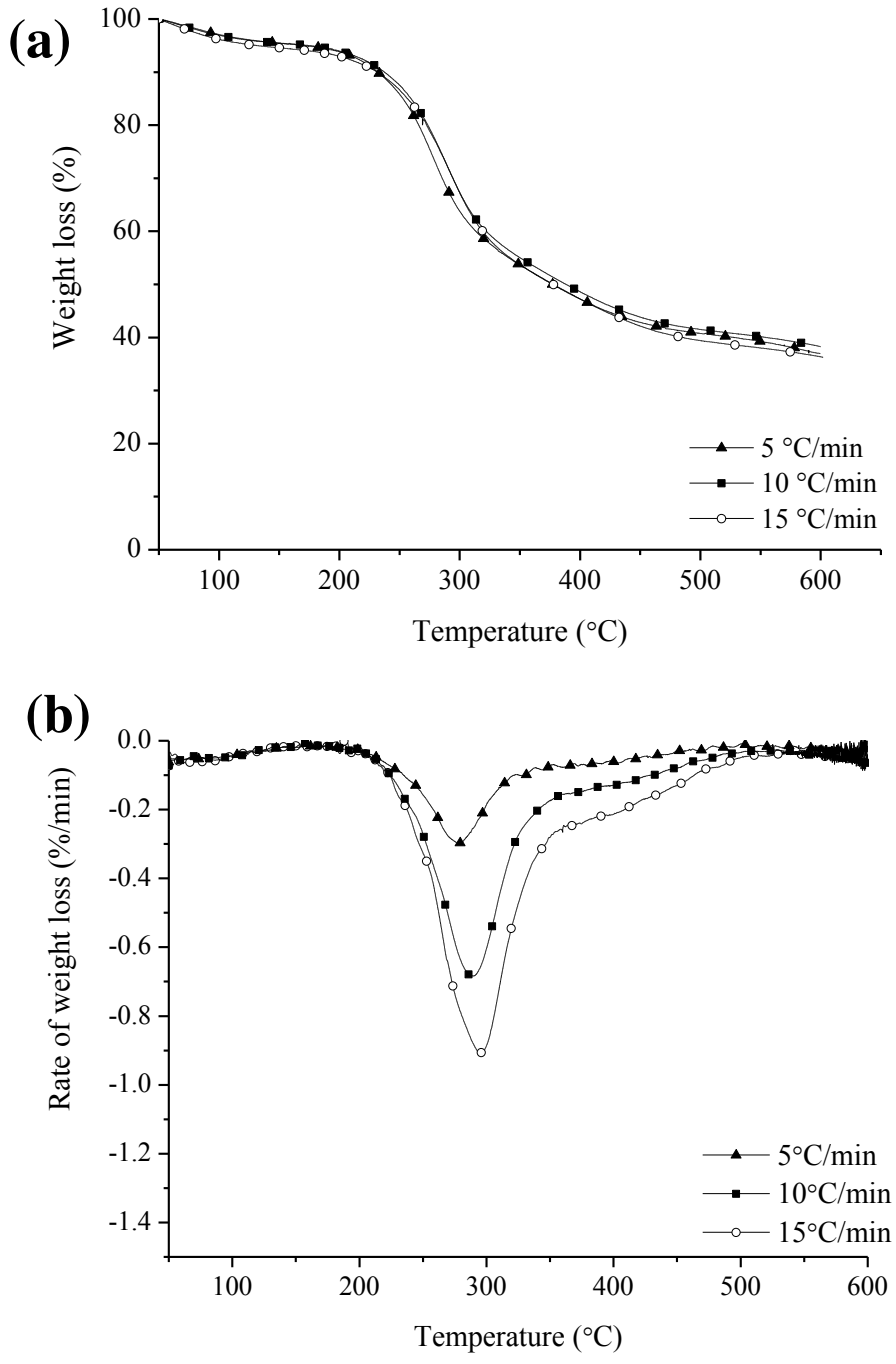


D.5 GC chromatogram from Alkaline pre-treated *T. suecica* (AkTetra)



Appendix E

E.1 Pyrolysis behaviour of *Chlorella* sp.



E.2 Pyrolysis behaviour of *T. suecica*