

*Optimization of the production of  
bioethanol from duckweed (Lemna  
minor)*

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Submitted for the degree of

Doctor of Philosophy

University of East Anglia

Institute of Food Research

May 2014

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**Abstract**

This project has investigated the production of bioethanol from duckweed (*Lemna minor*) biomass. The project includes four main sections: firstly, analysis of the chemical characteristics of duckweed, particularly the polysaccharides of the cell wall; secondly, exploration of suitable commercial enzymes for degrading duckweed biomass to fermentable sugars; thirdly, optimisation of pretreatments and enzymatic saccharification; finally, fermentation and optimisation of the ethanol yield.

Pond-grown *L. minor* contained 51.2 % carbohydrate (w/w dry matter) of which 77 % (including glucose, galactose and xylose) is fermentable.

A series of enzymatic hydrolyses was used to evaluate the commercial enzymes and optimise conditions for their use in the saccharification of duckweed biomass. Celluclast 1.5L (CE) and Novozyme 188 (BG) were identified as suitable for hydrolysing duckweed cell walls (prepared as alcohol insoluble residues). The additional use of thermophysical pretreatment (steam explosion) results in a dramatic decrease in the amount of enzyme required for quantitative saccharification. A more advanced commercial cellulase cocktail (Cellic<sup>®</sup> CTec 2; CTec 2) is likely to further reduce the enzyme cost.

Methods for the simultaneous saccharification, using CTec 2 and BG, and fermentation of steam exploded duckweed were developed. These resulted in an 80 % ethanol yield at a diluted substrate concentration (1 % w/v). However the ethanol yield decreased dramatically at higher substrate concentrations (to 18 % at 20 % w/v substrate concentration, which is a highly viscous suspension). Further studies involved the development of approaches to address this: (i) increasing the yeast titre in the inoculum or (ii) growing the inoculum on steam-exploded duckweed. These approaches facilitated an ethanol yield of up to 70 % (w/w) at a substrate concentration of 20 % (w/v). Maximising the final ethanol yield is of great importance in reducing the costs of production.

The optimized ethanol production process indicates the technical potential for industrial ethanol production from duckweed. Operating costs have also been estimated and are discussed in relation to the potential exploitation of protein as a co-product.

**Abbreviations**

ABS	absorbance
AIR	alcohol insoluble residue
Ara	arabinose
BG	beta-glucosidase
CDTA	cyclohexane-trans-1,2-diaminetetra-acetate
CE	Celluclast <sup>®</sup>
Conc.	concentration
CTec 2	Cellic <sup>®</sup> CTec 2
CWM	cell wall material
DCM	dichloromethane
DE	Depol <sup>™</sup> 740
DM	dry matter
DMSO	dimethyl sulphoxide
DNS	dinitrosalicylic acid
Fuc	fructose
FDM	freeze dry and freeze mill
FWM	untreated fresh material
Gal	galactose
GalA	galacturonic acid
Glc	glucose
GOPOD	glucose oxidase-peroxidase-4-aminoantipyrine
Man	mannose
OD	optical density
Rha	rhamnose
SE	steam explosion
SSF	simultaneous saccharification and fermentation
WIAIR	water-insoluble alcohol insoluble residue
WIM	water insoluble materials
WSM	water soluble materials
Vol.	volume

### ***Acknowledgements***

I am grateful for having this precious opportunity to achieve my PhD degree at Institute of Food Research and University of East Anglia. They provide fantastic facilities with everything I required in my study and an incredibly friendly environment for learning technology skills. I have had a three-year unforgettable experience in Norwich, which will impact on my future life.

I would like to particularly give my sincere appreciation to Prof Keith Waldron and Dr Mark Coleman for their inestimable effort and helpfulness throughout my PhD duration, without whom I could not have achieved my PhD.

I would also like to thank Mr Graham Moates for his invaluable help. He always enthusiastically helped me to solve any problem encountered.

I would also like to thank Dr Adam Elliston, Mr Samuel Collins and Mr Ian Wood who introduced me to many experimental techniques in this multidisciplinary project.

I would also like to thank Dr Jim Robertson, Dr Peter Ryden, Mr David Wilson, Dr Zara Merali and Dr Henri Tapp for their great help in diverse aspects: experimental techniques, data analysis, computer techniques and chemicals and consumables purchasing.

I would also like to thank Dr Mary Parker and Mrs Kathryn Cross for their great help on microscopy and their patient explanation of plant structure.

I would also like to thank Mr Rishi Ghogare, Miss Julie Burgess, Miss Susana De Val, Mr Jia Wu and Miss Nicola Cook for their great association in the laboratory.

Finally, I am also grateful for my parents' and wife's ongoing support and encouragement throughout my entire PhD life. I would like to give my most sincere appreciation to my parents, without whose sponsorship for my PhD, I could not fulfil this challenge.

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Some results of this study have been already published or submitted and some chapters of this manuscript correspond to the published papers. The published papers are listed below:

1. Zhao, X., Elliston, A., Collins, SRA., Moates, GK., Coleman, MJ., Waldron, KW., 2012. Enzymatic saccharification of duckweed (*Lemna minor*) biomass without thermophysical pretreatment. *Biomass and Bioenergy*. 47:354-361.
2. Zhao, X., Moates, GK., Collins, SRA., Wellner, N., Coleman, MJ., Waldron, KW., 2014. Chemical characterisation and analysis of the cell wall polysaccharides of duckweed (*Lemna minor*). *Carbohydrate Polymers*. 111:410-418.
3. Zhao, X., Moates, GK., Wilson, DR., Ghogare, R., Coleman, MJ., Waldron, KW., Steam explosion pre-treatment and enzymatic saccharification of duckweed (*Lemna minor*) biomass. *Biomass and Bioenergy*. Accepted.
4. Zhao, X., Elliston, A., Moates, GK., Coleman, MJ., Waldron, KW., Simultaneous saccharification and fermentation of steam exploded duckweed using commercial cellulose. Submitted to *Bioresource Technology*.
5. Zhao, X., Moates, GK., Wu, J., Tan, X., Parker, ML., Coleman, MJ., Waldron, KW., Maximising the ethanol yield by using one combined enzymatic saccharification – a modelling study on fractionated tissues of duckweed (*Lemna minor*). In preparation for Bioenergy Research.

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## ***1 Introduction***

Biofuels are derived from recently-living biological feedstocks, predominantly plants. Biofuel production has increased significantly in recent years, driven by a number of factors. The rapid development of the global economy has triggered a dramatic increase in fossil fuel demand and shortages, especially in large, rapidly developing countries, such as China, India, Brazil and Russia. These countries represent not only huge and growing commercial markets, but are also expected to have large and increasing fuel demands (see Figure 1) (U.S. Energy Information Administration., 2013). Fuel demand has increased significantly in recent years and this trend is likely to continue. The price of crude oil has increased nearly 7-fold in the last 20 years, from \$ 15 per barrel in 1994 to \$ 109.2 per barrel in 2013, and the highest price touched \$ 147.7 in July 2008 (Bloomberg, 2013; InvestmentMine, 2013). This has had a profound effect on the price of oil-derived fuels (see Figure 2). For instance, the price of unleaded petrol in UK has increased from £ 0.75 in 2000 to £ 1.30 in November 2013, a rise of 73.3 % (Deloitte, 2013). Increasing costs of oil-derived fuels is a significant driver for the adoption of biofuels that can replace their oil-derived counterparts. Biofuels is likely to be desired by those countries in which fuels are predominantly relied on the import, because the biofuel production enables these countries to become energy independent. Another major driver is the increasing concern over greenhouse gas emissions associated with fossil fuels. Global greenhouse gas (GHG, including CO<sub>2</sub>, NH<sub>4</sub>, N<sub>2</sub>O and fluorinated gases) concentrations have increased 32 % from 1990 to 2012, with CO<sub>2</sub> contributing 80 % (IPCC, 2013). The increase in GHG emissions is mainly attributed to energy supply (26 %), industry (19 %) and transport (13 %) (see Figure 3). Biofuel potentially could reduce GHG emissions through the avoidance of fossil fuels. For instance, ethanol from corn could reduce GHG emission by 20 % relative to gasoline, excluding GHG change of land use (Searchinger et al, 2008). The exhaustion of fossil fuel has been considered as the third driver since peak oil appeared at 74 million barrels per day (mb/d) in 2008. Although the peak of the production of crude oil is expected to be delayed to 2042 and it could attain to 150 mb/d, the rate of production will enter the terminal decline eventually (Kaufmann and Shiers, 2008).

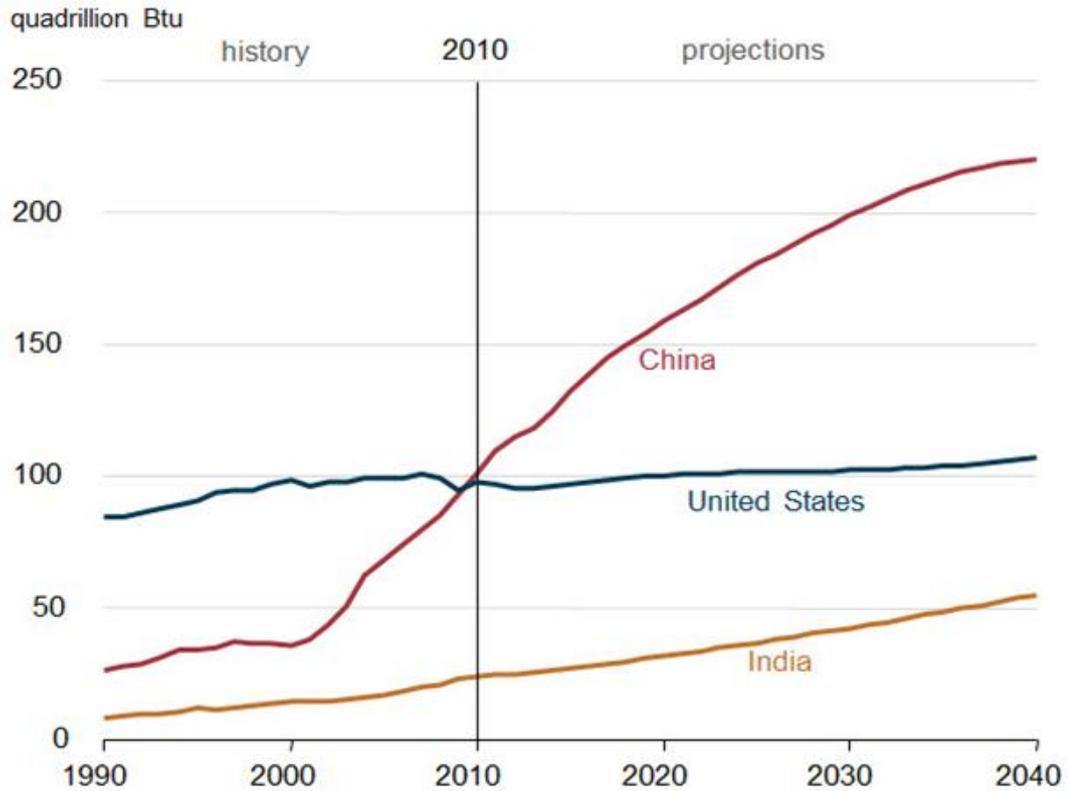


Figure 1. Fuel consumption in USA, China and India (U.S. Energy Information Administration., 2013).

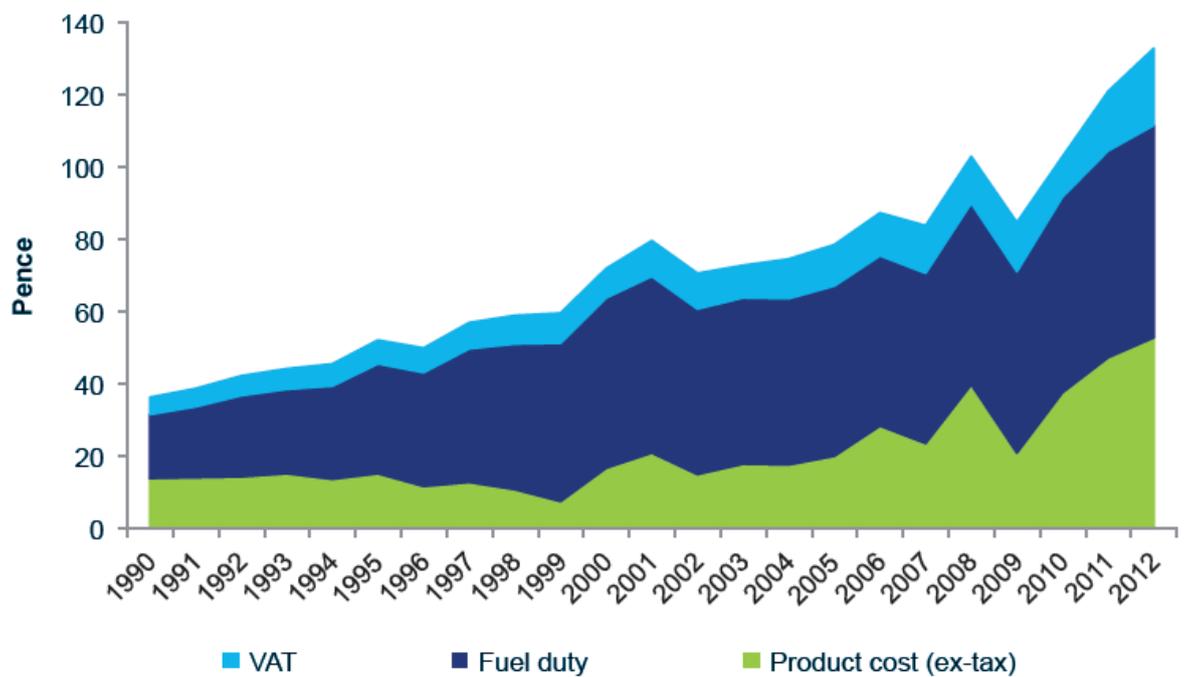


Figure 2. The petrol price over last 20 years in UK (Deloitte, 2013). The unit of money (pence) is pence/L.

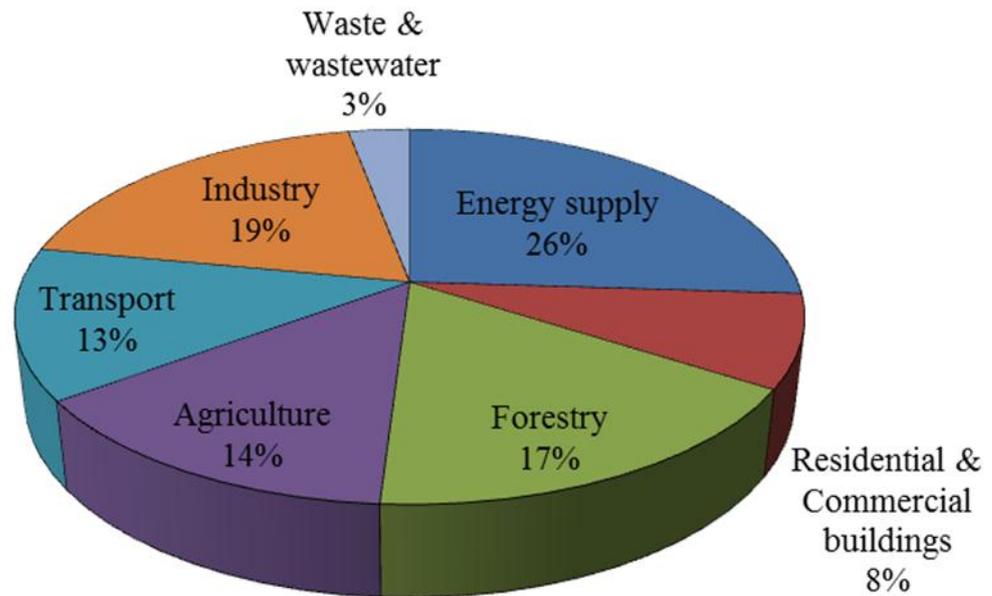


Figure 3. Global greenhouse gas emission resource (IPCC., 2007).

These factors have driven an increased adoption of biofuels to replace fossil fuels in recent years. For instance bioethanol production has increased globally from 19,000 million litres in 2001 to 85.2 billion litres in 2012 (RFA, 2013). Biofuels have been increasingly exploited as alternatives to fossil fuels for their significant advantages: sustainable production and the reduction of CO<sub>2</sub> emissions. Currently, biofuels contribute approximately 1.5 % of global transport fuels. The United States and the developing countries of Brazil, China and Thailand are the biggest biofuel producers (International Energy Agency, 2010). Much legislation has been announced worldwide to encourage biofuel development. EU Energy Policy establishes a goal of reaching a minimum 10 % of renewable biofuel in every member country by 2020 (European Commission, 2013). The legislation provides a blueprint of biofuel in the coming two decades, regulates biomass feedstock and reduces the tax on biofuel industrial profits (Megan, 2011; Global Subsidies Initiative, 2013).

### 1.1 Types of biofuels

Bioethanol, biodiesel and biogas are three major biofuels. Bioethanol as a general transport fuel has been developed for over 30 years since it was a natural extension of brewing technology. The combustion value of bioethanol has been measured by gasoline gallon

equivalency (GGE) value, in which 1.5 gallons of bioethanol generates the equivalent energy of one gallon of gasoline (U.S Department of Energy, 2013). Biodiesel is the product of the trans-esterification of plant oils (Sims et al, 2008). The particulate emissions from biodiesel are less harmful than conventional diesel (Lapuerta et al, 2008). Biogas (methane) is generated from the anaerobic digestion of organic wastes, such as animal manure and sewage. This mature technology is applied on a small domestic scale in India and China (Sims et al, 2008). It has also been systematically developed for industrial production in Germany and Denmark (Sims et al, 2008) and more recently in the UK.

The feedstocks for biofuel cover a range of biomass sources, from food crops (e.g. corn, sugarcane) to energy crops (lignocellulosic biomass), trees and grass to algae, waste paper, cardboard and municipal waste (Sims et al, 2008; Waldron, 2010). Biofuels have been classified into different generations. The 1<sup>st</sup> generation biofuels are produced from grains, sugarcane and oil seed crops and have been already extensively produced in the USA and Brazil (see Figure 4). USA is the largest producer of 1<sup>st</sup> generation ethanol, produced from corn, and Brazil is the second largest producer of bioethanol, produced from sugarcane. By 2011, US and Brazil production accounts for 87.1 % of global bioethanol production (Renewable Fuels Association., 2012). Biodiesel is extensively produced from oilseed rape in Germany and palm oil in Malaysia (Sims et al, 2008). However, biofuel from food crops has increasingly become a controversial issue since it competes with human beings and animals for food, particularly in those countries with large populations. For instance, the 1<sup>st</sup> generation biofuels provide over 1.5 % of global transport fuels but occupy 2 % of world's arable land for biomass feedstock (World Watch Institute, 2007). A series of consequent issues have been also brought to light by developing the 1<sup>st</sup> generation biofuels. Food prices will predictably rise if grains are extensively used for biofuel production, or more land, such as rainforest, peatland, savannas and grassland, will be required to create as arable land (Sims et al, 2008). GHG emissions might increase conspicuously as a consequence of the increase of agricultural waste and the decrease of the GHG emission uptake by rainforest (Fargione et al, 2008) and biodiversity will potentially decline.

More recently, 2<sup>nd</sup> generation biofuels have been considered as replacements of 1<sup>st</sup> generation biofuels. 2<sup>nd</sup> generation biofuels aim to utilise lignocellulosic biomass instead of food crops to produce biofuel (Sims et al, 2008), which enable feedstock resource to broaden to woody crops, agricultural residues (stems, leave and husks) and industrial and

municipal waste. Only 1.25 % of the entire land biomass is harvested for grains which are still shared by food and 1<sup>st</sup> generation biofuel industry, the rest of biomass is unused and recycled in global systems (Naik et al, 2010). The production of 2<sup>nd</sup> generation biofuels might be significantly increased by using the unused biomass fraction of food crops. They also potentially reduce land use change and GHG emissions (Fargione et al, 2008). However, the production of biofuel from lignocellulosic biomass requires more advanced technologies and higher energy input. The price of 2<sup>nd</sup> generation biofuels is therefore above the current market price. Using current techniques, the cost of bioethanol from lignocellulosic crops is \$ 2.65 per gallon as compared to the ethanol from corn at \$ 1.65 per gallon (Kumar et al, 2009). With the development of advanced techniques, second generation biofuels are expected to become economically viable (see Table 1). Algal biofuels are referred to as 3<sup>rd</sup> generation biofuels and 4<sup>th</sup> generation biofuels are defined as produced by using petroleum-like hydroprocessing and advanced biochemistry (Demirbas, 2009). Currently, corn, sugarcane oilseed and lignocellulosic biomass are still considered as the most feasible feedstocks for biofuel and R&D is on-going to decrease the production cost of biofuel.

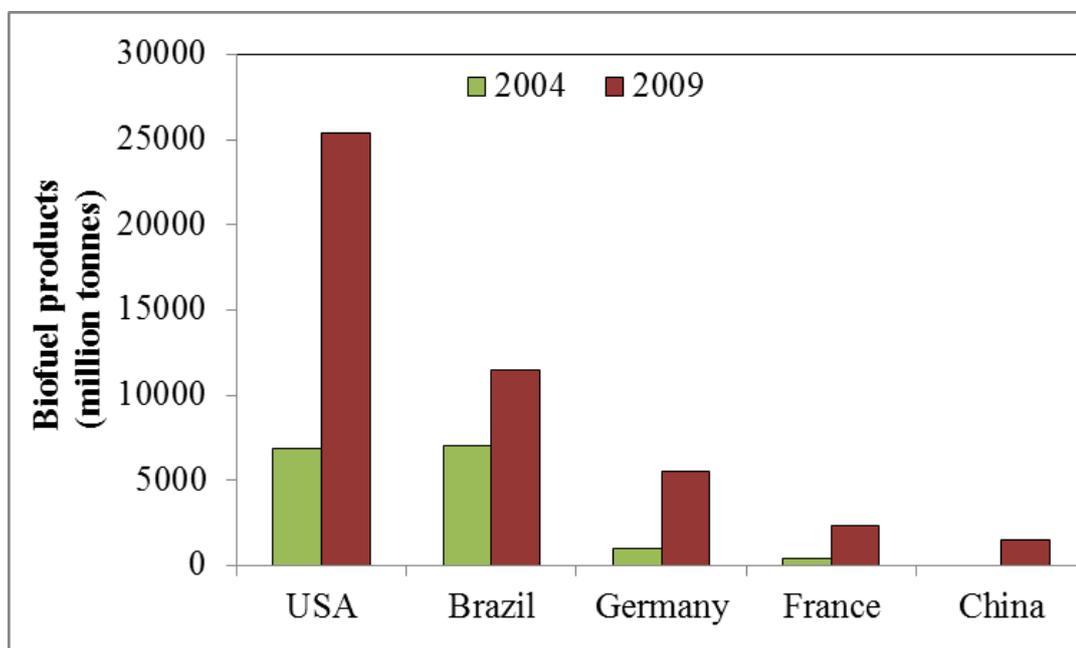


Figure 4. The 1<sup>st</sup> generation biofuel production of world main producer countries in 2004 and 2009 (data collected from International Energy Agency, 2010).

Table 1. The comparison of fossil fuel, 1<sup>st</sup> and 2<sup>nd</sup> generation biofuels (Naik et al, 2010).

	Petroleum Refinery	1 <sup>st</sup> generation biofuel	2 <sup>nd</sup> generation biofuel
Feedstock	Crude pretroleum	Vegetable oil, Corn sugars etc	None food, cheap and abundant plant waste biomass
Products	CNG <sup>a</sup> , LPG <sup>b</sup> , Diesel, Petrol, Kerosene and Jet fuel	FAME <sup>c</sup> , biodiesel, bioethanol,	bio-oil, bioethanol and mixed alcohol
Technology	Economical	Economical	Costly
Problems	Depletion/ declining of petroleum reserve	Limited feedstock (Food vs Fuel) Blended partly with conventional fuel	Large land requirement
Advantages	Mature technology	Environmentally friendly, economic and social security	Not competing with food Advance technology to reduce the cost of conversion Environmentally friendly

<sup>a</sup>CNG = compressed natural gas. <sup>b</sup>LPG = liquefied petroleum gas. <sup>c</sup>FAME = fatty acid methyl esters.

## 1.2 Cell wall structure of lignocellulosic feedstock

The plant cell wall comprises of three layers: primary wall, secondary wall and middle lamella. Primary wall forms along with the daughter cell and it is generally thin, 0.1 - 1.0  $\mu\text{m}$  (Brett & Waldron, 1996). The secondary wall forms internal to the primary wall after the daughter cell growth ceases. The secondary wall is variable in its chemical and morphological structure (Brett & Waldron, 1996). The middle lamella layer is located outside primary wall and between two adjacent cells (Brett & Waldron, 1996). The predominant composition of cell wall is carbohydrates that is potentially converted to biofuels. The cell wall carbohydrates consist of three classes of polysaccharides: cellulose, hemicellulose, pectic polysaccharides (Brett & Waldron, 1996). As Figure 5 describes, cellulose forms the main structural component of the cell wall. Cellulose microfibrils are a relatively homogenous crystalline polymer of  $\beta$ -1,4-linked glucose (Brett & Waldron, 1996); the non-crystalline phase of the cell wall is called the wall matrix and consists of a variety of polysaccharides, proteins and phenolic compounds (Brett & Waldron, 1996). The hemicellulose and pectic polysaccharides are heterogenous in nature and comprise of a number of monosaccharides including rhamnose, fucose, arabinose, apiose, xylose, mannose, galactose, glucose and galacturonic or glucuronic acids. These monosaccharides can be assigned to the groups of pectic and hemicellulosic polysaccharides because some of monosaccharides are uniquely or largely present in one specific polysaccharide group, e.g. galacturonic/glucuronic acid and apiose are unique compounds of pectin while xylose is mainly in hemicellulose (Brett & Waldron, 1996). Lignin, comprised of a number of phenolic acids, plays an important role in hornification and preventing microbial infection. As a significant component of lignocellulosic material, its quantity varies from a trace to 38 % among plants (Brett & Waldron, 1996).

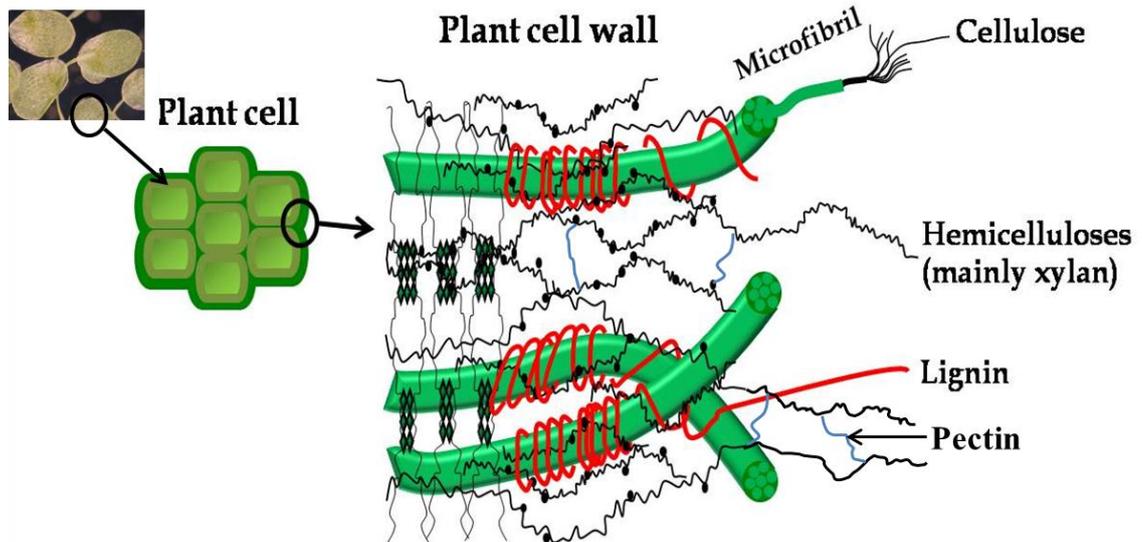


Figure 5. Micro structure of plant cell wall (Tomme et al, 1995).

### 1.3 The conversion of biomass to ethanol

The production of bioethanol from lignocellulosic biomass involves four main steps: pretreatment, enzymatic saccharification, fermentation and distillation. Figure 6 is a brief overview of how duckweed might be converted to bioethanol. In terms of lignocellulosic biomass, the ethanol production is mainly limited by enzymatic saccharification because the degradation of lignocellulose to fermentable sugars requires massive energy input and large amount of specific enzymes (Mussatto and Roberto, 2007). These problems are attributed to the nature of biomass, including: porosity of materials, cellulose fibre crystallinity and lignin and hemicellulose levels (Sun and Cheng, 2002). Diverse pretreatments are therefore applied prior to enzymatic saccharification in order to increase the ease of biomass hydrolysis. Effective pretreatment can efficiently loosen cellulose crystal structure and make biomass more accessible to enzymes (Chundawat et al, 2010). Due to the low lignin content in duckweed, the degradation of duckweed biomass might not require intensive pretreatments and therefore research has tended to concentrate on enzymatic saccharification, including investigating the synergy of enzyme mixtures, optimising enzyme dosage to an economic range or reducing enzyme costs by creating novel commercial enzymes. Fermentation and distillation have been historically used in alcohol brewing and the process in the bioethanol production closely resembles alcohol brewing. However, it is likely that the specific yeast strains and the fermentation technology both need to be enhanced.

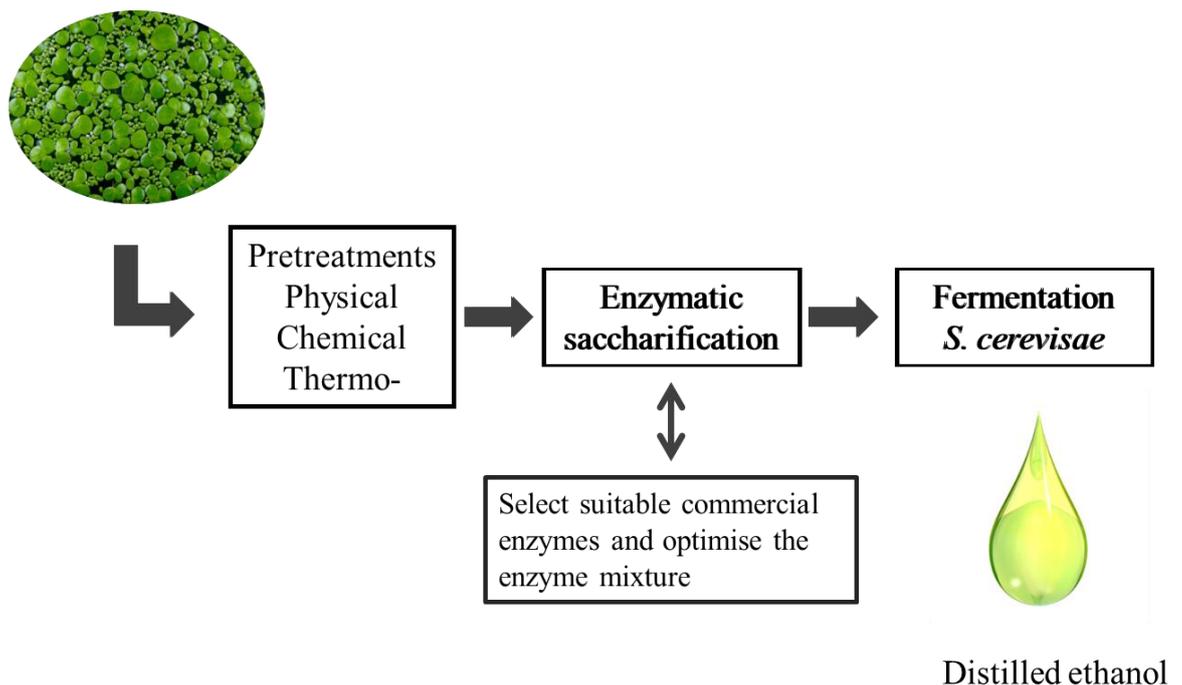


Figure 6. The conversion of duckweed biomass to ethanol.

## 1.4 Pretreatments

An effective pretreatment is characterized by several criteria: it should avoid the need for reducing the size of biomass particles; should preserve the hemicellulose fractions; should reduce formation of inhibitors that hinder growth of fermentative microorganisms, and minimize energy demands and limit cost (Mosier et al, 2005). Various pretreatments are being investigated for their effectiveness in subsequent enzymatic saccharification. The material treated by effective single or combined pretreatments is more accessible and susceptible to enzymatic saccharification and more fermentable sugars are obtained for subsequent fermentation (Chundawat et al, 2010). However, the application of pretreatment is likely to vary from material to material. Thus, utilization of an appropriate pretreatment is an important prerequisite of enzymatic saccharification and fermentation. Acid, alkaline, thermal and combination approaches may be applied and these can differ with respect to pH, temperature, retention time and material type (Pedersen and Meyer, 2010).

#### *1.4.1 Physical pretreatment*

Physical pretreatments aim to degrade the cellulose crystallinity to improve biomass digestibility by changing the physical character of materials using pyrolysis and mechanical comminution including dry, wet and vibratory ball mills (Millet et al, 1976). Mechanical comminution is a combination process involving chipping, grinding and milling which reduces the material size down to 0.2 - 2 mm (Sun and Cheng, 2002). Dry and wet mill processes are economically effective when applied in starch-to-ethanol production. Vibratory ball milling has been used to effectively generate smaller particles than other mechanical comminution methods (Millet et al, 1976). Pyrolysis is a thermal-physical pretreatment associated with higher temperature and pressure and the decomposition of polysaccharides; it results in over 80 % conversion to reducing sugars (Fan et al, 1987; Sun and Cheng, 2002). However, to achieve a conspicuous enhancement of enzymatic saccharification for lignocellulosic materials, the particle size is necessarily reduced to less than 0.05 mm (Datta, 1981). The energy demands of pure physical pretreatments are fairly uneconomic for lignocellulosic materials (Cadoche and López, 1989) and their utilization is consequently restricted or must be associated with other chemical or thermal pretreatments (Waldron, 2010).

#### *1.4.2 Chemical pretreatment*

Chemical pretreatments assist in specifically removing unwanted compounds, such as lignin or hemicellulose compounds (Fan et al, 1987, Waldron, 2010), and their application is therefore changed based on the nature of the biomass. Ozonolysis can effectively remove 60 % lignin (e.g. in wheat straw) and also avoid the generation of toxic products at room temperature conditions (Vidal and Molinier, 1988). Dilute acid hydrolysis (using H<sub>2</sub>SO<sub>4</sub>, HCl) has been successfully developed to achieve high sugar yield and avoid the toxic, corrosive issues trigger by concentrated acid hydrolysis (Kumar et al, 2009). This acid hydrolysis is also utilized to successfully convert xylan to xylose under moderate conditions (Hinman et al, 1992). Alkaline hydrolysis can also remove lignin by saponification, which causes biomass swelling, increasing the internal surface area and decreasing the extent of polymerization and crystallinity of cellulose (Fan et al, 1987; Sun and Cheng, 2002). However, the neutralization of chemical hydrolytic products is necessary prior to downstream enzymatic saccharification and fermentation. Although chemical hydrolysis significantly improves the digestibility of materials, the cost is usually higher than other pretreatments.

### 1.4.3 Physical-chemical pretreatment

Physical-chemical pretreatment is an efficient process possessing the advantages of both individual physical and chemical pretreatments, in which fine pretreated materials for downstream process are obtained and the cost and energy demands are also dramatically decreased. Steam explosion – a recognized thermal hydrolytic method – is one typical physical-chemical pretreatment and is extensively applied to enhance ethanol production for lignocellulosic biomass (Glasser and Wright, 1997). The process explodes biomass by sudden decompression following high pressure and temperature conditions. Recent studies tend to use lower temperatures combined with longer retention time (Wright, 1998). Steam exploded cellulose is more accessible to cellulase (Schwald et al, 1989). Pedersen and Meyer (2010) stated that the advantages of steam explosion are the high yield of glucose and xylose attributed to the considerable lignin transformation and hemicellulose degradation. However, disadvantages are seen as the high energy demand, a consequence of high pressure and temperature requirements, and the formation of fermentation inhibitors. Addition of H<sub>2</sub>SO<sub>4</sub> or CO<sub>2</sub> into steam explosion could improve the degradation of hemicellulose and enzymatic hydrolysis and decrease the formation of inhibitory compounds (Sun and Cheng, 2002). Ammonia fibre explosion (AFEX) is another classic physical-chemical pretreatment. It involves exposing lignocellulosic biomass to steam explosion with additional liquid ammonia. AFEX does not effectively decompose lignin and hemicellulose but generates only trace levels of inhibitors (Sun and Cheng, 2002, Waldron, 2010).

### 1.4.4 Biological pretreatment

Microorganisms have been studied and used for decomposition of plant cell wall material. Fungi are employed in the decomposition of lignocellulosic materials due to the many saccharifying enzymes they produce. Diverse fungi involving brown-, white- and soft-rot fungi have been used to target different compounds (Fan et al, 1987). For instance, brown rot fungi mainly degrade cellulose, while white and soft rots can degrade both cellulose and lignin (Fan et al, 1987). One novel approach for biological degradation of lignocellulosic biomass has been found by Malyon et al (2010): the wood-boring marine crustacean *Limnoria quadripunctata* can digest crystalline cellulose directly to fermentable glucose. King et al (2010) showed that these crustaceans potentially possess all of the enzymes for lignocellulose digestion due to the absence of the gut microbes. Although

biological pretreatment requires low energy at moderate conditions, the extent of decomposition is less (Sun and Cheng, 2002).

#### *1.4.5 Pretreatment of duckweed*

In summary, the selection of pretreatment methods depends on the characteristics of the material, particularly the proportion of lignin and hemicellulose present. Pyrolysis, ball milling, alkaline hydrolysis, steam explosion and AFEX potentially match the requirement of the decomposition of duckweed biomass. Low lignin content might mean that pretreatment can be avoided or simple pretreatment requiring less energy input could be used.

### **1.5 Enzymatic saccharification**

Enzymatic saccharification is the process that decomposes polysaccharides to monosaccharide by employing enzymes to cleave bonds. For 1<sup>st</sup> generation biofuel, amylase and amyloglucosidase required for hydrolysing starch have been already improved for the industrial ethanol production (Ghose, 1987). Enzymatic saccharification of lignocellulosic biomass is a more complex process in which diverse cell wall saccharifying enzymes are required to degrade cellulose, hemicellulose and pectin to fermentable sugars. Cellulolysis is certainly the primary pathway to obtain glucose from cellulose while the enzymatic hydrolysis of other polysaccharides, to some extent, improves cellulolysis. A number of commercial enzymes are currently available for saccharification research and these enzymes are generally cocktails of a mixture of enzymes (Forssell et al, 2009; Saha et al, 2005). For example, Depol™ 740, used in this study, is a general carbohydrase containing cellulase, xylase and ferulic acid esterase (Forssell et al, 2009; Sigma-Aldrich, 2011). The synergy of enzyme mixtures is being studied in order to maximise sugar yields.

#### *1.5.1 Essential enzymes required for the hydrolysis of cellulose*

‘Cellulase’ is a term representing enzymes involved in cellulolysis including endoglucanase, exoglucanase and  $\beta$ -glucosidase (Sulzenbacher et al, 1997). Their synergistic function in the cellulolysis is illustrated in Figure 7A. Endoglucanase is also named endocellulase, endo-1,4- $\beta$ -glucanase or carboxymethyl cellulase (CMCase); it can randomly cleave internal bonds to produce new short chains (Sulzenbacher et al, 1997). Exoglucanase (exocellulase) can cut disaccharides (cellobiose) from the ends of exposed

chains (Bommarius et al, 2008). Finally,  $\beta$ -glucosidase (cellobiase) hydrolyses disaccharides to glucose (Figure 7A). Commercial cellulase is a combination of the above three enzymes and the activity profiles of each varies among the products or suppliers depending on the source microorganism and production processes (Faulds et al, 2008; Dien et al, 2008; Forssell et al, 2009). Currently, fungi are the microorganisms generally used for industrial production of cellulase (Bommarius et al, 2008). The commercial enzymes are generally stored under mild conditions (4 °C) to maintain their activity. A summary of diverse commercial cellulase products from different suppliers reveals that the best reaction condition of saccharification for the highest cellulase activity is: temperature at 45 - 55 °C, pH at 4 - 5 (Ghose, 1987; Faulds et al, 2008).

### *1.5.2 Supplementary enzymes*

Other enzymes employed include those required for hydrolysing lignin, hemicellulose, pectin and protein; the enzymes activities are illustrated in Figure 7B. These enzymes specifically target one polysaccharide, for example, feruloyl esterase (ferulic acid esterase) attacks phenolic acid (lignin), and xylanase and xylosidase degrade xylose polymers to xylose (Aro et al, 2005). With the association of these relevant enzymes, lignin, hemicellulose and pectin are effectively decomposed and detached from cellulose that is consequently exposed to cellulase (Aro et al, 2005).

### *1.5.3 Enzyme activity and application*

Several different measures are used to express enzyme activity in literature, but it is extremely confusing to readers when comparing enzyme effectiveness in parallel. This is generally caused by the different measurement methods and description of enzyme products varying in the product profiles (solid or liquor) or different suppliers. For instance, units of IU, U, FPU, CMC, EGU are often used to measure the cellulase activity in different situations. For the convenience of studying on enzymatic saccharification, the author summarised the units of enzymes and tabulates their specific application instances in Table 2 (Ghose, 1987; Forssell et al, 2009; Saha et al, 2005; Sigma-Aldrich, 2011; Hendrickson et al, 2007).

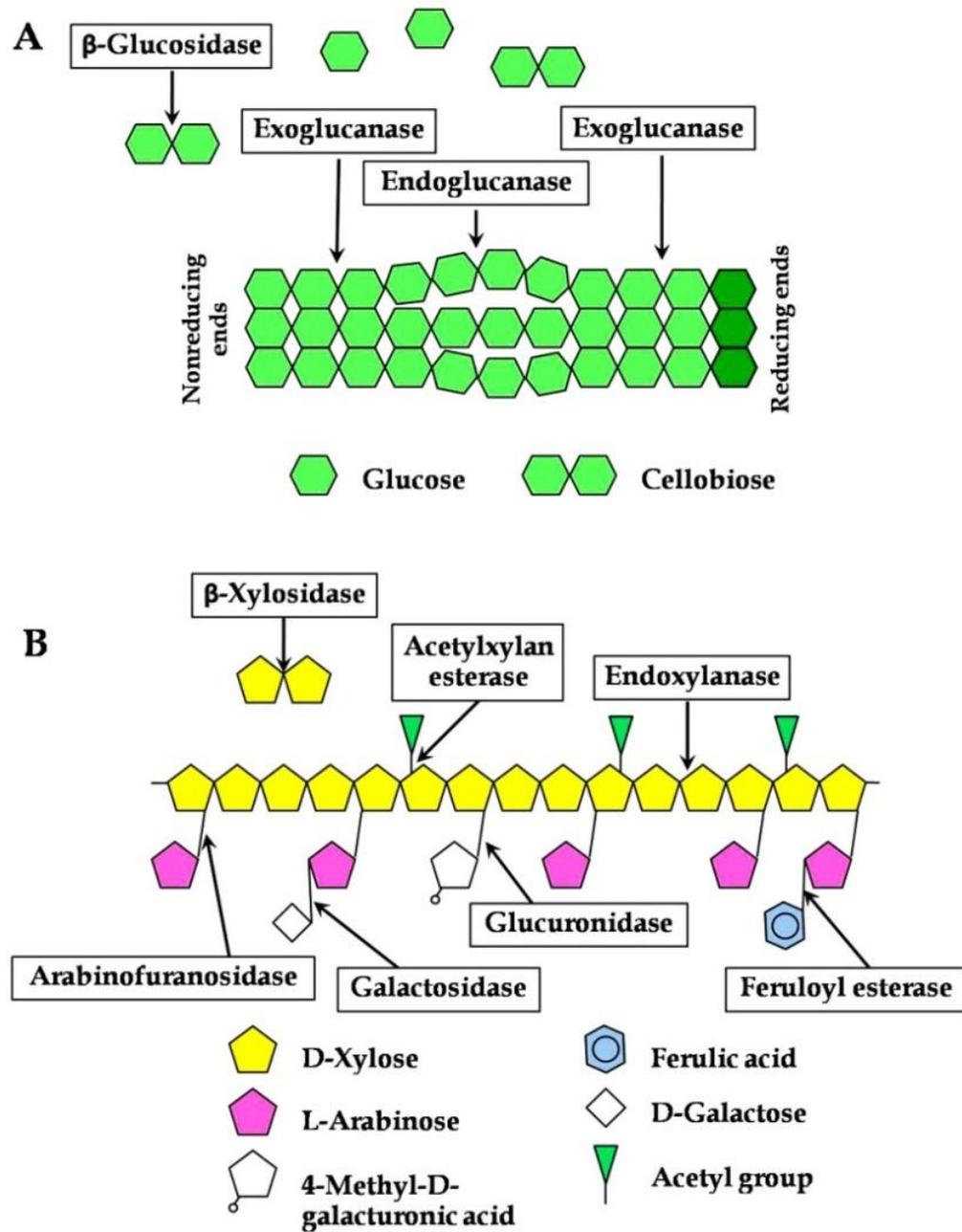


Figure 7. Enzymes involved in the decomposition of plant cell wall (Aro et al, 2005).

Figure 7A illustrates the hydrolysis of cellulose by a group of cellulase. Figure 7B shows the associated enzymes involved in the hydrolysis of cell wall and their functions.

Table 2. The definitions and application of cellulase enzymes activities. [enzyme]\* stands for the amount of enzyme required.

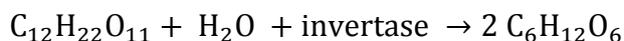
Expression	Full name	Definition	Utilization
IU	International unit	[enzyme]* <u>catalyzing</u> the conversion of 1 $\mu\text{mol}$ of <u>substrate</u> $\text{minute}^{-1}$ under standard conditions	Optimal temperature, pH value, and substrate concentration.
U	Units	[enzyme]* <u>catalyzing</u> the conversion of 1 $\mu\text{mol}$ of <u>substrate</u> $\text{minute}^{-1}$ under specific conditions	Temperature of 25°C, the pH value and substrate concentration of the maximal yield conversion rate.
FPU/CMC	Filter paper units /Carboxymethyl cellulase	[enzyme]* releasing 1 $\mu\text{mol}$ of glucose equivalents per minute from filter paper (cellulose only)	Enzyme activity is given by writer identified in Filter Paper Assay
CBU	Cellobiose unit/ $\beta$ -Glucosidase	[enzyme]* <u>catalyzing</u> the conversion of 1 $\mu\text{mol}$ of <u>cellulose</u> per minute, or releasing 2 $\mu\text{mol}$ of glucose per minute from <u>cellulose</u>	Enzyme activity is given by writer identified in Cellulbiase Assay
EGU	Endo-Glucanase unit	[enzyme]* releasing 1 $\mu\text{mol}$ of cellulose equivalents per minute from cellulose under the defined conditions	Enzyme activity is given by writer identified in their designed assay
nkat	nano katal	[enzyme]* <u>catalyzing</u> the conversion of 1 $\text{nmol}$ of <u>substrate</u> per second under defined conditions	1U = 1/60 $\mu\text{Kat}$ = 16.67 nKat
U/g (U/mL)	Unit per gram or mL	means [enzyme]* involved in solutions, which equivalents to U/mL appropriately	instructions of enzyme products, general expression in papers
U/g substrate	Unit to gram substrate	[enzyme]* reacting on 1 gram substrate under the defined experimental conditions	The activity is given to illustrate the relationship between enzyme and substrate

## 1.6 Fermentation

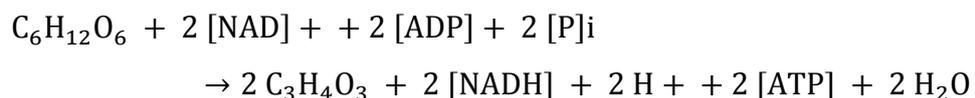
Ethanol fermentation has been used in brewing for thousands of years. Fermentation is a process in which microorganisms metabolise sugars under low oxygen conditions to produce high-energy compounds (adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) and ethanol, acids and CO<sub>2</sub> are produced as waste products (Edward, 1922). Many types of sugars (glucose, fructose, sucrose, maltose, galactose and xylose) are metabolised by various yeasts in ethanol fermentation (Donald and Judith, 1995). However, different sugars resources may require optimisation of the yeast strains employed. For example, glucose is consumed by *Saccharomyces cerevisiae* and xylose is consumed by *Pichia stipitis* to produce ethanol (Delgenes et al, 1996).

### 1.6.1 Yeasts for fermentation

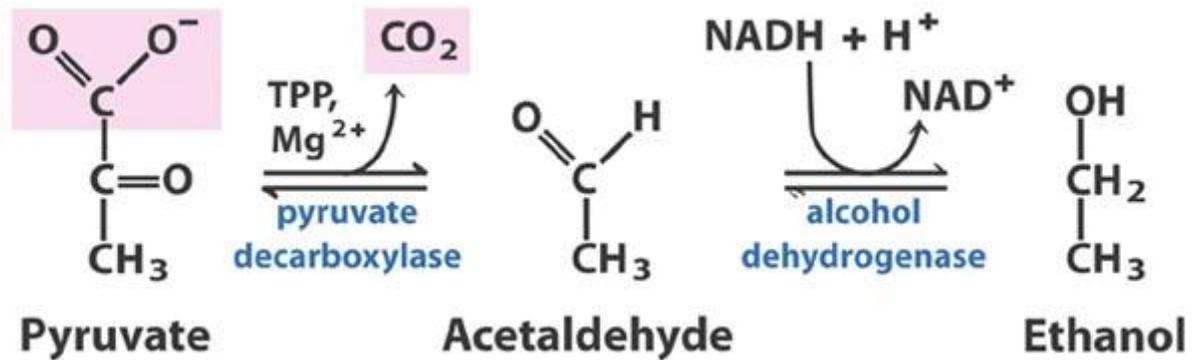
The fermentation of glucose to ethanol using *Saccharomyces cerevisiae* has the same principle and pathway as alcohol brewing and its relatively mature technology would save cost on R&D. Nevertheless, this ready-made process still demands technical enhancement of yeast strains according to biomass profiles. The traditional fermentation process mainly involves three steps: 1) disaccharides, such as sucrose, are cleaved to monosaccharide (glucose and fructose) by the enzyme invertase (Equation 1); 2) glycolysis – a crucial step in the ethanol fermentation, glucose is converted to pyruvate and high-energy compounds (ATP and NADH) under an anaerobic conditions (Equation 2) (Lubert, 1975); 3) yeast converts pyruvate to ethanol associated with enzymes of pyruvate decarboxylase and alcohol dehydrogenase (Equation 3) (Strathern et al, 1981).



Equation 1. Breakdown of di-sugar

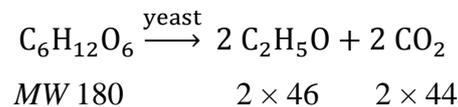


Equation 2. Glycolysis



Equation 3. The pathway of pyruvate to ethanol (Thomas, 2007)

The ethanol fermentation process was introduced and the theoretical ethanol yield could be simply calculated from a combination (Equation 4) of Equation 2 and Equation 3.



Equation 4. The mole weight relationship between glucose and ethanol.

The molecular weight of glucose and ethanol implies that the theoretical ethanol yield is 51 % (w/w) of original glucose mass, which means 1 kg glucose could theoretically convert to 0.51 kg ethanol. However, an inevitable loss of glucose mass caused by yeast growth would affect on the ethanol yield in practice. In addition, the theoretical weight of glucose and xylose released are 1.11 and 1.13 times the weight of glucan and xylan in light of the addition of water during hydrolysis (Koppram et al, 2013). Lee et al (2007) reported that the practical maximum ethanol fermentation efficiency is actually 46 % (w/w) of glucose mass.

### 1.6.2 Ethanol fermentation methodologies

Although ethanol fermentation has been applied in the brewing industry for thousands of years, novel fermentation methods are still being developed. Separate enzymatic hydrolysis and fermentation (SHF) is a process in which enzymatic hydrolysis and fermentation are carried out independently to maintain the optimal conditions for both processes (Erdei et al 2012). The significant disadvantage of SHF is that the accumulation of sugar products (cellobiose, glucose, xylose) in enzymatic saccharification process hinders the breakdown of cellulose and subsequently reduces ethanol yields (Stenberg et al, 2000). Simultaneous

saccharification and fermentation (SSF) in a combination of saccharification and fermentation; potentially it decreases inhibition by sugars accumulation and greater ethanol yield and might result (Tomás-Pejó et al, 2008). Tomás-Pejó et al also stated that SSF could improve ethanol production from SHF under the same conditions. SHF has a high initial ethanol yield in the first 3 hours, but SSF could achieve higher total ethanol yield than SHF. Additionally, SSF also requires less energy input than SHF as the process duration is considerably reduced.

SSF has been expanded to semi-simultaneous saccharification and fermentation (SSSF), simultaneous saccharification and co-fermentation (SSCF) and simultaneous saccharification and extractive fermentation (SSEF). SSSF is the process between SHF and SSF and includes a pre-hydrolysis prior to SSF to provide the optimal conditions for both enzymes and yeast in SSF (Shen and Agblevor, 2011). SSCF can convert both hexose and pentose to ethanol by using multiple microorganisms (Koppram et al, 2013). SSEF aims to reduce the influence of alcohol accumulation on yeast by periodically removing ethanol from the reaction chamber and enabling yeast to remain in a highly active state (Moritz and Duff, 1996). However, all these methods encounter the inhibition problem at higher substrate concentrations which decreases the activity of yeast. Fed-batch fermentation can reduce inhibition and achieve high ethanol yields at very high substrate concentrations (65.5 % w/v) (Elliston et al, 2013).

Direct microbial conversion (DMC) which is also known as “consolidated bioprocessing” (CBP), has been developed by Lynd et al (2002; 2005). DMC/CBP refers to an integrated process where enzymatic hydrolysis of polysaccharides and hexose/pentose fermentation are all carried out in one bioreactor using selected bacteria that secrete cellulase enzymes. The cost of this fermentation method is very low for industrial production with reasonable profit margins, but the ethanol yield of DMC is generally lower than SHF and SSF (Lynd et al, 2005).

### *1.6.3 Inhibitors*

Fermentation is a fairly dynamic process not only because yeast growth is sensitive to physical and chemical conditions, but also because it is restricted by inhibition that limits the ethanol yield (Almeida et al, 2007). Furan, weak acids and phenolics are three major groups of inhibitors; they are generated from lignin, hemicellulose and cellulose in the

pretreatment and enzymatic saccharification phases (see Figure 8, Pedersen and Meyer, 2010). 5-hydroxymethyl-2-furaldehyde (5-HMF) and 2-furaldehyde (2-FA), which play a significantly inhibitory role in fermentation, are formed by dehydration of hexoses and pentoses respectively (Dunlop, 1948). As intermediate breakdown products, the quantities of 5-HMF and 2-FA vary with different pretreatments types and conditions. For instance, in aqueous conditions, 5-HMF and 2-FA could eventually convert to levulinic acid and formic acid (see Figure 8) (Pedersen and Meyer, 2010). Weak acids including acetic, formic and levulinic acid are irreversible inhibitors (See Figure 8). Acetic acid is a residual compound from deacetylation of hemicellulose, while levulinic and formic acids are the terminal products of cellulose decomposition. Formic acid can be additionally produced from furfural in the hydrolysis of hemicellulose under acid conditions (Almeida et al, 2007). Phenolics are another common inhibitor group; they are formed in the process of hydrolysing lignocellulosic materials and their level and types change with different biomass sources (Kumar et al, 2009). It is possible that low levels of phenolics may be generated from duckweed due to its low lignin content.

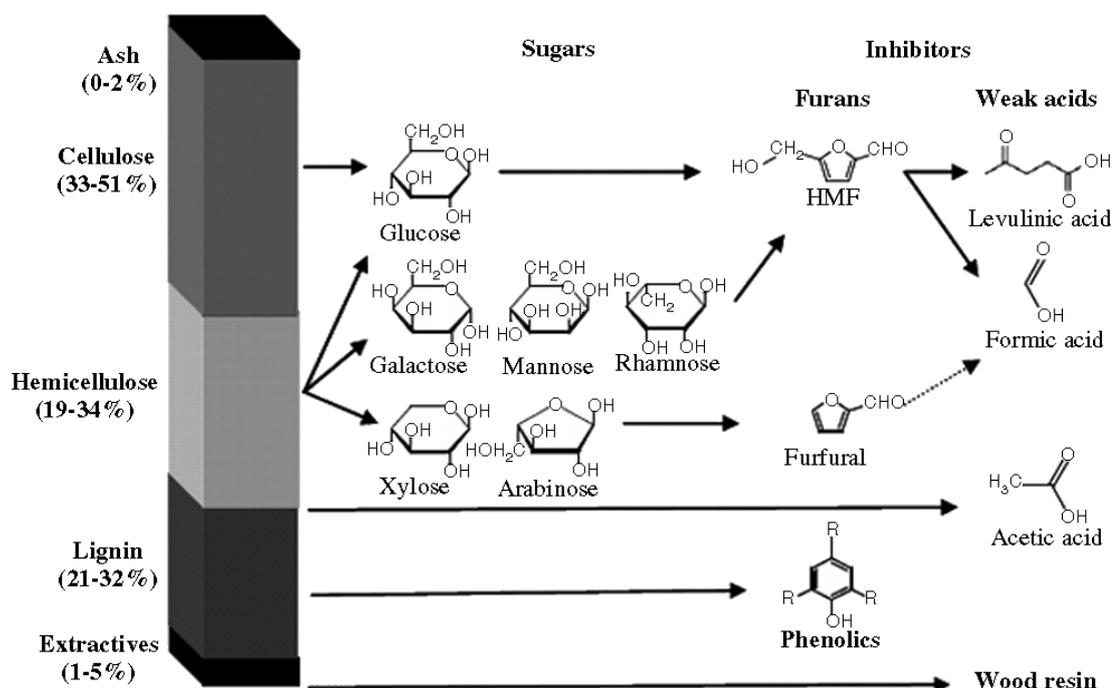


Figure 8. The hydrolysates derived from degrading lignocellulosic biomass (Almeida et al, 2007).

The inhibitory effect on ethanol fermentation using *S.cerevisiae* is tabulated in Table 3. For instance, in the fermentation of wheat straw (10 % w/w), the highest inhibitory effect was exhibited by formic acid and 2-FA when their concentrations attained to 2.7 and 4 g/L. The inhibition by 5-HMF, 2-FA, formic, levulinic and acetic acids may be considered relevant to the fermentation of duckweed.

Table 3. The inhibitory effect of common inhibitors in the fermentation of wheat straw (substrate concentration is 10 % w/w).

Inhibitors	Inhibition (%) <sup>a</sup>	Concentration	
		/volume (g/L) <sup>a</sup>	/substrate (mg/g)
HMF	50	8	80
2-FA	79	4	40
Acetic acid	74	6	60
Formic acid	80 <sup>b</sup>	2.7 <sup>b</sup>	27
Levulinic acid	50	40	400
Vanillic acid	50	1	10
Vanillin	25	1	10
4-Hydroxybenzaldehyde	72	1	10
4-Hydroxybenzoic acid	30	1	10

<sup>a</sup>from Pienkos and Zhang (2009). <sup>b</sup>from Maiorella et al (2004).

#### 1.6.4 Detoxification

Due to the potential inhibition generated from pretreatment and enzymatic hydrolysis of lignocellulosic biomass, detoxification (reduction in inhibitor concentration) needs to be considered. Taherzadeh et al (2000) stated that four approaches can be applied to minimizing the presence of inhibitors: (1) reducing the production of inhibitors in pretreatments; (2) removing or decomposing the inhibitors; (3) conversion of inhibitors to compounds that can be tolerated by yeast; (4) developing novel yeast strains to enhance their tolerability of inhibitors. A number of detoxification methods have been developed and reported including physical, chemical and biological methods. The detoxification methods and their profiles are tabulated in Table 4 (Chandel et al, 2011; Jönsson et al, 2013). The application of detoxification varies according to the nature of inhibitors but sugar loss may also need to be considered.

Table 4. The advantages and disadvantages of detoxifications.

Detoxification	Advantages (% of removal)	Disadvantages
Vacuum evaporation	Removal of volatile acids (acetic acid 54 %)	Increasing the concentrations of non-volatile inhibitors
Ion exchange	Removal of all inhibitors Furan 63.4 %, phenolics 75.8 %, acetic acid 96.3 %	High cost in commercial & industrial application
Ethyl acetate solvent	Removal of all inhibitors	Except acetic acid
Ca(OH) <sub>2</sub> / NaOH	Removal of phenolic and other compounds	A sugar loss, acetic acid remains, precipitation of toxic compounds increasing
Peroxidase, Laccase Mutant yeast	Removal of acid and phenolic compounds	Searching suitable enzymes and yeasts

### 1.6.5 Co-products

Several attractive by-products are produced during the ethanol fermentation process. In the anaerobic conditions, methane and CO<sub>2</sub> are produced from degrading organic acids (formic and acetic acids). CO<sub>2</sub> from fermentation can be extracted and reused as an industrial source for dry ice, carbonating beverages, etc (Pierantozzi, 2001). Moreover, the residual biomass contains a number of potential nutrients, such as protein, minerals and vitamins, which could be extracted for animal feed.

## 1.7 Distillation

The concentration of ethanol produced by fermentation is far too low for direct use as transportation fuel, and distillation is required to generate concentrated and purified ethanol from the mixed fermentation products. Distillation has been applied in brewing and separating fossil fuel products for many years. The principle of distillation depends upon the boiling points of components in the mixture (Hengstebeck et al, 1961) - lower boiling components preferentially vaporise at lower temperatures. In bioethanol distillation, ethanol vaporises before water as it has a lower boiling point (78.5 °C). The ethanol vapour passes to the condenser and the condensed liquid is eventually collected in a clean container.

Simple distillation requires components which have a large difference in boiling points in order to achieve high purities of the separated components (Doherty and Perkins, 1978). However, simple distillation could not purify ethanol with 100 % effectiveness because vapour is enriched in ethanol but moisture always remains. This situation is called an azeotrope. Azeotrope means the components of mixture at certain proportion have similar boiling point which leads the expected component to be no longer separated by distillation (Uragami et al, 2004). The azeotrope of ethanol is 95.6 % (v/v). To make matters worse, if the ethanol distillation proceeds over time, the purified ethanol will collect more water, which negatively affects final ethanol concentration (Uragami et al, 2004).

Fractional distillation is a technique developed from simple distillation that enables a number of components possessing similar boiling points to be separated (Kneil and Scarsdale, 1952). The presence of azeotrope in ethanol distillation limits the purity of final distilled ethanol less than 95.6 %. However, the use of fractional distillation can obtain higher pure ethanol than 95.6 %. The principle of fractional distillation is according to a temperature gradient (coolest in the top and hottest at the bottom) generated by the distance from the heat source (Kneil and Scarsdale, 1952). When the mixed vapour (ethanol and water) ascends through the temperature gradient, ethanol remains in the vapour and water condenses back to refluxing liquor. After several cycles of vaporisation and condensation, ethanol is purified and relatively water free (Kneil and Scarsdale, 1952). Industrial distillation is the process of repeated vaporisation and condensation in a huge refluxing distillation column (see Figure 9) (Packing et al, 1987).

While distillation is not part of the project described below, from a holistic point of view, it is necessary to take this into consideration for improving the overall economic efficiency of bioethanol production. A higher ethanol yield from fermentation would require less energy input for distillation. With respect to this point, the substrate concentration for fermentation will be considered in an attempt to ensure that ethanol concentration reaches an economic level.

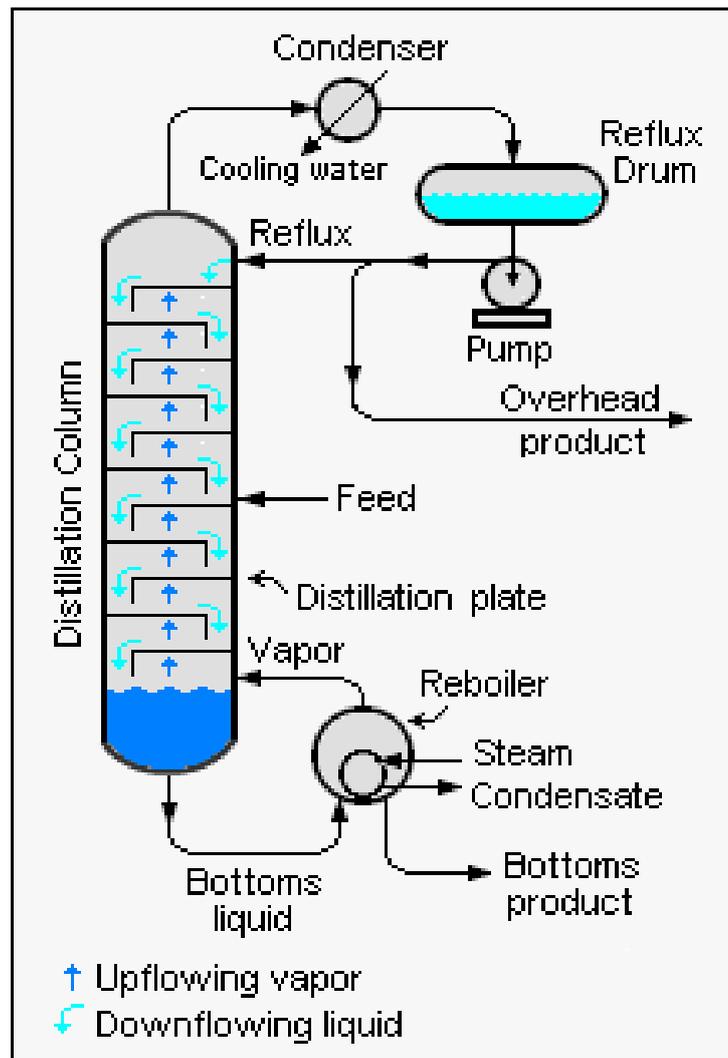


Figure 9. The schematic of industrial distillation column (Packing et al, 1987).

### 1.8 Aquatic plants – A special group of 2<sup>nd</sup> generation feedstock

Aquatic plants are considered as a potentially ideal biofuel feedstock due to their significantly common advantages: (I) aquatic plants are generally low in lignin but with a high proportion of carbohydrate; (II) aquatic plants do not compete with food crops for arable land; (III) they commonly have higher productivity because of their fast proliferation; (IV) many types of aquatic plants could be cultured in waste water system for decontamination; (V) in addition, abundant nutrients such as protein, vitamin and minerals generated from biofuel production are potentially useful as feed for animals (Wolverton and McDonald, 1979; Landolt and Kandeler, 1987; Benner et al, 1984; Cheng and Stomp, 2009). Below several aquatic plants are introduced.

### 1.8.1 Duckweed

The *Lemnaceae* family, members of which are commonly named ‘duckweed’, has four genera—*Lemna*, *Wolffia*, *Spirodela*, and *Wolffiella* (See Figure 10) (Cabrera, 2008). Duckweed is one of the world’s most abundant plants and contains the smallest higher plants, having tiny leaf-like fronds, flat growth habit (see Figure 11), and vegetative reproduction (Landolt, 1986). Two types of *Lemnaceae* can be defined: 1) the plants have one root (*Lemna*) or more roots (*Spirodela*) and two lateral reproductive pouches; 2) the plant has no root (*Wolffia* and *Wolffiella*) and a single reproductive pouch (Landolt, 1986).

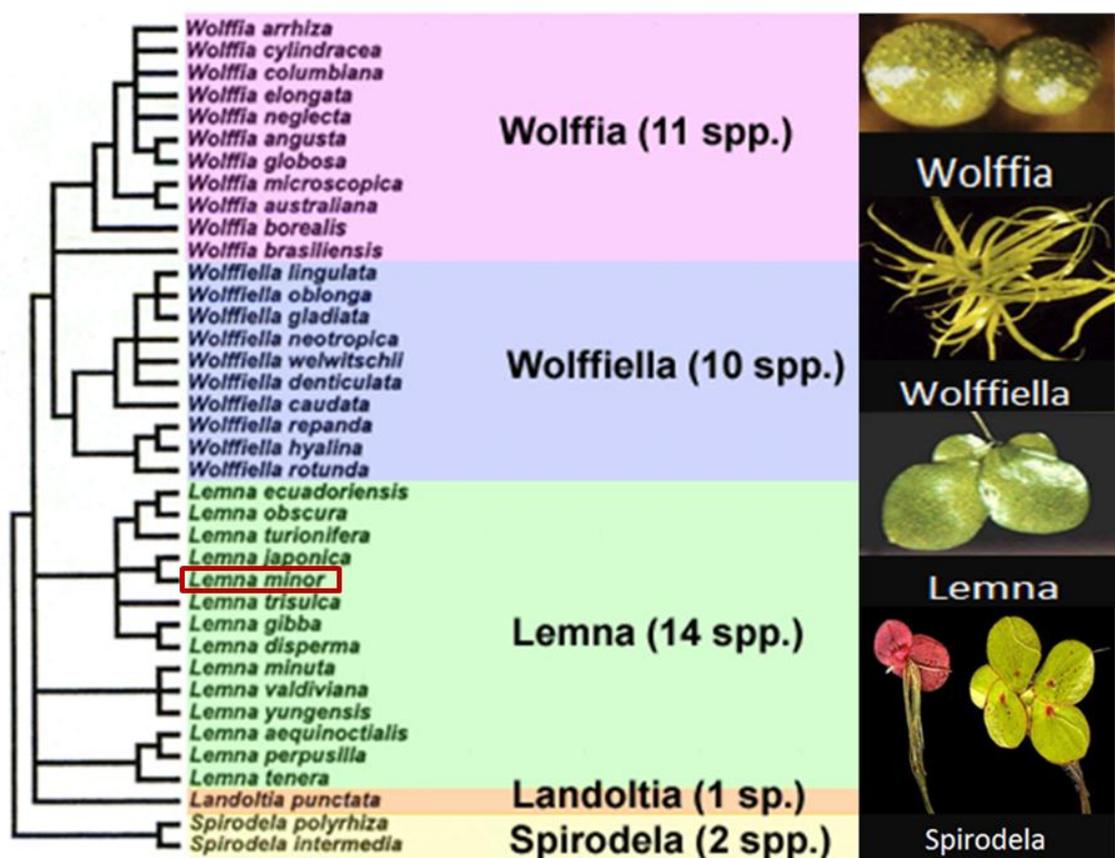


Figure 10. Duckweed genera and represented plants. The plants size: *Wolffia* < *Wolffiella* < *Lemna* < *Spirodela*.



Figure 11. Sizes of three species of duckweed (*Lemna minuta* < *Lemna minor* < *Spirodela polyrhiza*). White bar = 10 mm.

Their simple structure and morphologic characters enable duckweeds to proliferate more rapidly than most other plants. Duckweed appears to obey Kleiber's 3/4-power rule ( $G \propto M^{3/4}$ ; G means annualised rates of growth and M means body mass) (Niklas and Enquist, 2001) and they exhibit much higher specific growth rates than other, larger aquatic plants, exhibiting doubling times of between 2 to 8 days (Fujita et al, 1999). The doubling time of *Lemna minuta* can be as short as 48 hours, and in the larger *Spirodela polyrhiza* is 6 - 8 days (Fujita et al, 1999). As a result, an expected higher biomass yield, in which a large proportion of fermentable sugars are contained, is potentially achievable. Oren et al (1985) noted that the proliferation rates of duckweed are among 0.1 - 0.5 g/g per day. Cheng et al (2002) reported that a growth rate of 0.2 kg/m<sup>2</sup> per week DM was achieved by culturing *Spirodela polyrhiza* in a low nitrogen-concentration wastewater system. Also, in greenhouse conditions, production of 1 kg fresh weight/m<sup>2</sup> per week was achieved. Dry weight increase of up to 20 t/ha per year is the norm based on results obtained from 25 m<sup>2</sup> lagoons receiving dairy cattle manure (Culley et al, 1981). The latest research suggested that 105.9 t/ha per year of dry mass can be harvested in a wastewater system (Xu et al, 2012). These yields compare favourably with those of currently-considered potential energy crops, e.g. miscanthus (10 t/ha per year), willow (10 t/ha per year), poplar (9 t/ha per year), switchgrass (12 t/ha per year) (World Watch Institute, 2007).

### 1.8.2 Water hyacinth

Water hyacinth (*Eichhornia crassipes*), another aquatic plant family exhibiting rapid vegetative growth, is native of the Amazon basin (Barrett and Forno, 1982; Bolenz et al, 1990). Water hyacinth (Figure 12) is a free-floating plant and grows as much as 1 metre in height, with broad, thick, glossy, ovate leaves and violet flowers (Bhattacharya and Kumar, 2010). Water hyacinth has become a controversial plant due to its invasiveness since it was introduced to Europe, Asia, Australia, North America and Africa. The habitat of water hyacinth can range from tropical to subtropical or warm temperature desert to rainforest zones and it tolerates a range of warm temperatures (21.1 - 27.2 °C) and pH value (5.0 - 7.5) (Bolenz et al, 1990). Under appropriate conditions, it exhibits a fast growth rate. Its ability to remove nitrogen, phosphorus and iron from waste water system has been noted (Jayaweera and Kasturiarachchi, 2004; Jayaweera et al, 2008). This plant is therefore considered for the decontamination of waste water. Dry matter of 60-110 tonnes/ha per year has been harvested in wastewater system (Gumbrecht, 1993). Also, a weekly dry weight yield (71 % w/w) was harvested at a lagoon with a nutrient increase (29 % w/w DM) through May to October (Wolverton and McDonald, 1979).



Figure 12. Water hyacinth plant.

### 1.8.3 Algae

Algae are a group of simple, autotrophic eukaryotic organisms that includes unicellular and multicellular organisms (Parfrey et al, 2006). Their habitats range from small ponds to the sea and they exhibit a number of reproductive kinetic (Parfrey et al, 2006). For unicellular algae, they appear to obey Kleiber's 3/4-power rule and a very high dry matter yield is therefore potentially achieved from these very small organisms (Niklas, 2006). Green algae also parasitically live in other aquatic plants where they normally stay under cuticles or sometimes under epidermis of the host plant (Nelson, 2008). In addition, the ability of diverse biofuel (bioethanol and biodiesel) production has been demonstrated. Each algal cell has been compared to a small ethanol production plant (see **Error! Reference source not found.**) (Institute for Livestock the Environment, 2013). The essential elements including water, sunlight, CO<sub>2</sub> and nutrients can be converted to ethanol through the metabolism cycles (photosynthesis, pyruvate, and ethanol synthesis) in algae plant. Biodiesel production from algae has also been studied and reported as having some conspicuous advantages: rapid growth rate, surprising space efficiency, high yield, sulphur free fuel, non-toxicity and high biodegradability (Rout et al, 2011). However, biofuel production from algae is more costly than using other energy crops due to the requirement for artificial addition of light, CO<sub>2</sub>, water and inorganic salts (Chisti, 2007). Biofuel production from algae is commonly considered as the 3<sup>rd</sup> generation biofuel.

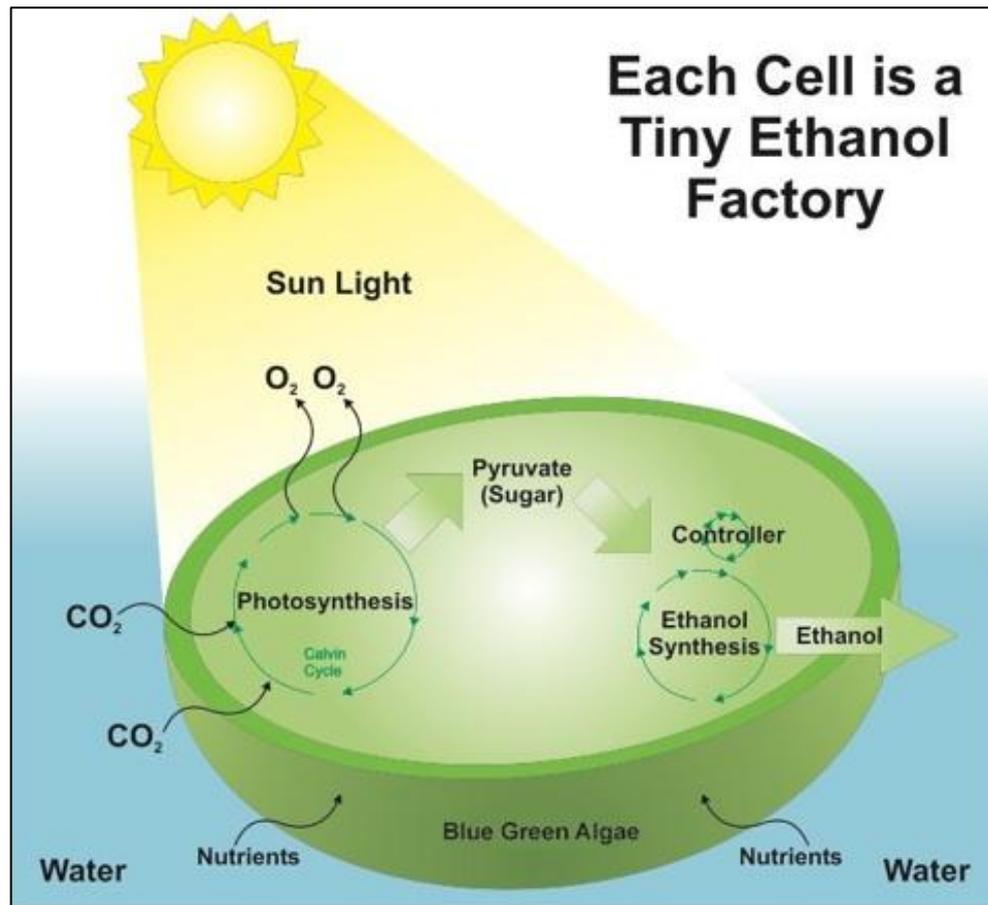


Figure 13. The pattern of alga cell as ethanol production plant (Institute for Livestock the Environment, 2013).

#### 1.8.4 The chemical characteristics of aquatic plants

The botanic and structural characteristics of aquatic plants as distinct from terrestrial plants, can be potentially advantageous for biofuel production. Some research has investigated the chemical compounds present in duckweed and water hyacinth (see Table 5). Both may have high proportions of protein and carbohydrate (Landolt & Kandeler, 1987; Gnansounou et al, 2005; Rajb and Balen, 2004). The high proportion of ash (15.9 - 19 %) in duckweed is attributed to the large storage of calcium oxalate (Landolt & Kandeler, 1987). In addition, starch can be a very significant component of duckweed; the level is variable, 3 - 75 % by DM (Cheng and Stomp, 2009), and it has been used for ethanol production (Cheng and Stomp, 2009). The relatively high proportion of protein suggests use as animal feed, and duckweed is used for this purpose (Leng et al, 1995). Water hyacinth as another classic aquatic plant which is larger than duckweed comprises of 5.2 – 10.3 % of DM. Of this dry matter, carbohydrate accounts for over 62 % (w/w), protein varies from 12.6 - 20 % (w/w) and a small of proportion of lipid (3.5 %) is reported

(Abdelhamid and Gabr, 1991; Nigam, 2002; Gunnarsson and Petersen, 2007). Only 4.1 % starch has been reported (Mishima et al, 2008). Cell walls of water hyacinth consists of cellulose (17.8 - 19.5 % by DM) and hemicelluloses (33.4 - 49.2 % by DM), plus a variable lignin content (3.5 - 26.4 % by DM) (Abdelhamid and Gabr, 1991; Nigam, 2002).

Table 5. The chemical compounds of duckweed and water hyacinth.

Parameters	Duckweed	Water hyacinth
Dry Matter (% of wet mass)	4.5 - 4.7	5.2 - 10.3
Crude fibre (% of DM)	11.0 - 18.1	18.9
Crude protein (% of DM)	29.1 - 45	12.6 - 20
Ether extract (% of DM)	3.2	3.5
Ash (% of DM)	15.9 - 19	25.9
Carbohydrate (% of DM)	14.1 - 43.6	62.3
Starch (% of DM)	3 - 75	4.1
Cellulose (% of DM)	NA	33.4 - 48.7
Hemicellulose (% of DM)	NA	17.7 - 19.5
Lignin (% of DM)	NA	3.5 - 26.4

NA means not reported (Landolt & Kandeler, 1987; Gnansounou et al, 2005; Rajb and Balen, 2004; Abdelhamid and Gabr, 1991; Nigam, 2002; Gunnarsson and Petersen, 2007; Mishima et al, 2008).

#### 1.8.5 *The decontamination of wastewater using aquatic plants*

Significant environmental pollution and diseases has arisen from wastewater generated from livestock effluents, and municipal and industrial sewage and this issue has rapidly become a global concern (Shuval, 2003). Decontamination of wastewater is therefore urgently required to prevent environmental deterioration, particularly in those countries possessing large food industries. Wastewater produced from food processing, fermentation and pharmaceuticals, includes organic and inorganic nutrients that may be toxic in the environment but an excellent nutrient resource for aquatic plants (Tripathia et al, 1991). This ability to remove toxic nutrients from wastewater, e.g. iron, mercury, magnesium, cadmium, has been extensively reported using duckweed and algae at their optimal plant growth rate (Tripathia et al, 1991; Jayaweera et al, 2008; Zimmo et al, 2004). The organic nutrients of wastewater can be reduced and Tripathi (1991) reported that nitrate (42.0 - 96.2 %) and phosphate (36.3 - 70.2 %) in wastewater could be removed by duckweed.

Another study noted that the uptake of nitrogen and phosphorus for duckweed (*Lemna gibba*) was conspicuous at a moderate concentration of nutrients and a lower nutrient condition provides a more suitable environment for aquatic plant growth (Ran et al, 2004). Oren et al (1988) reported that an optimal growth rate was achieved in a municipal wastewater containing 20 ppm nitrogen. A growth rate of 0.2 kg /m<sup>2</sup> DM per week was obtained at a low nitrogen level (10 ppm) (Cheng and Stomp, 2009). Similar results were observed in a water hyacinth study. Tripathi et al (1991) compared the capability of nitrogen and phosphorus removal of water hyacinth and duckweed, and higher uptake of nitrogen and phosphorus was observed for water hyacinth (Tripathia et al, 1991). Although iron is vital metal element for the water hyacinth, the uptake of iron is at relatively low iron concentrations (Jayaweera et al, 2008).

#### 1.8.6 Duckweed cell wall

Table 5 also tabulates the cell wall components, but few details of duckweed cell wall composition have been reported. The pectic polysaccharides of duckweed have been studied and 30 % pectin (w/w of CWM) was recorded by Kindel et al (1996). Apiogalacturonan and galacturonan were identified as the main pectic polysaccharides and galacturonic acid forms the back bone of *L. minor* galacturonan (Golovchenko et al, 2002; Cheng and Kindel, 1997). Only a low proportion of lignin is present in duckweed cell wall (Landolt & Kandeler, 1987). To the best of our knowledge, no research has been published giving a comprehensive understanding of duckweed cell wall, e.g. individual proportions of cellulose, hemicellulose and pectin and a detailed investigation of their polysaccharides groups. For bioethanol production from duckweed biomass including starch and CWM, information on duckweed cell wall is fundamental and crucial background for the subsequent pretreatments and enzymatic saccharification. Although the conversion of duckweed starch to bioethanol has been reported (Cheng and Stomp, 2009; Xu et al, 2012), the investigation of duckweed cell walls will enable further improvements in ethanol productivity and the exploitation of duckweed as a feedstock for biofuel production.

#### 1.8.7 The exploitation of duckweed as a feedstock for biofuel and other products

Duckweed as an aquatic plant with a rapid growth rate and the ability to decontaminate wastewater is being researched as a feedstock for biofuel production and other potentially high-value products. A scheme for the exploitation of duckweed is shown in Figure 14.

The use of duckweed in biofuel production has been demonstrated and in these studies involved converting starch content to ethanol (Cheng and Stomp, 2009; Xu et al, 2012). Cheng and Stomp (2009) observed that the ethanol yield from *L. minor* starch is much higher than for maize because of its higher starch productivity (28 ton/ha per year dry weight) than maize starch (5 ton/ha per year dry weight). However, the significant proportion of cellulose ought also to be considered for improving bioethanol production from duckweed. Currently, there is little information relevant to the conversion of duckweed CWM to ethanol and addressing this is the primary purpose of this project. Ethanol is not the only possible biofuel product from duckweed, other biofuel products, such as gas, oil and biochar, may be obtained through the pyrolysis (Muradov et al, 2010) and thermolysis (Campanella et al, 2011). Animal feed is another potential product. A number of research papers have reported that duckweed biomass is a good protein supplement for fish, pigs, poultry and ruminants (Hillman and Culley, 1978; Haustein et al, 1992; Leng, 1990). Furthermore, high commercial value nutrients and medicinal extracts might be considered as other potential products of a duckweed biorefinery. *Spirodela polyrhiza* has been used as a Chinese herb for hundreds of years and Chinese researchers are developing medicinal products from duckweed (Zhu, 2004). There is commercial interest in using duckweed to produce IgG antibodies (following genetic transformation) (Cox et al, 2006). Finally, the decontamination of wastewater could spread duckweed biorefinery to a broader area geographically.

There is no available pattern for the systematic biorefining of duckweed for biofuel and other products. This project will mainly concentrate on making ethanol from starch and cellulose. The studies of pretreatment, enzymatic saccharification and fermentation of duckweed biomass hopefully will provide useful information for the integrated biorefining of duckweed.

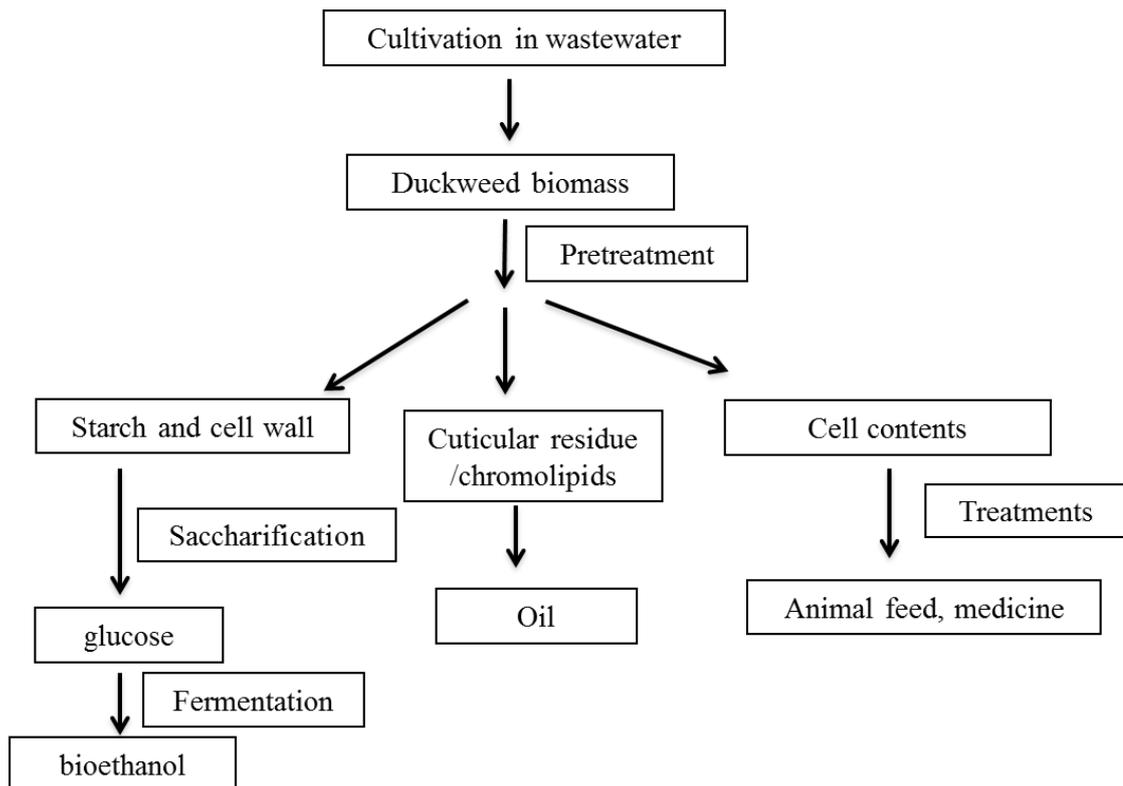


Figure 14. The exploitation of duckweed.

### 1.9 Aims of the study

The bioethanol production from duckweed is one promising research because it would play an important role as the supplementary alternative of the fuels demands and might be also a frontier research for other aquatic plants. The overall purposes of this project are summarised as: (I) to clearly understand the carbohydrate and cell wall structure; (II) to evaluate one appropriate single or combined pretreatment that will be an effective association of enzymatic saccharification; (III) to investigate the process of enzymatic saccharification on duckweed, particularly identify the enzyme preparations using commercial enzymes; (IV) to establish the ethanol yield of fermentation on pretreated duckweed. This project will mainly focus on researching the feasibility of the conversion of duckweed biomass to ethanol (see Figure 15), in light of the high productivity of duckweed and its multipurpose role in industry.

#### 1.9.1 Target A Assessment of duckweed chemical composition

The study on chemical composition is the prerequisite of how duckweed biomass might be converted to ethanol. According to a number of previous researches, duckweed is starch

rich material with low levels of lignin. However, more details of cell wall structure such as, proportions of cellulose, hemicellulose and pectic polysaccharide, were not found in the literature. This information could demonstrate the types and quantities of fermentable sugars in duckweed and the appropriate approaches to convert these sugars to ethanol.

#### *1.9.2 Target B Establishment of a suitable pretreatment approach*

The importance of pretreatment has been explained previously (see § 1.4). Fresh duckweed as a typical tiny, low lignified plant requires less energy input and specific pretreatment methods which significantly differentiate to other lignocellulosic biomass. Even if the same pretreatment is used for duckweed, it still might require different conditions which reflect on temperature, pH value (acid or alkaline) and retention time. Thus, a specific pretreatment adapted to duckweed chemical nature is a necessarily preliminary step which can dramatically increase the accessibility of duckweed biomass to enzymes.

#### *1.9.3 Target C Investigation of enzymatic saccharification on model plant*

Investigation of enzymatic saccharification on duckweed biomass is one of the main targets of this project. Enzymes are normally very specific targeted and expensive catalysts which therefore require a large amount of research to screen the suitable enzymes for duckweed. Thus, we will investigate essentially the process of enzymatic saccharification on laboratory (Hoagland's E-Medium) cultured plants. Details involve: identification of enzymes, understanding the synergy of cellulase products, establishing incubation time, the optimal conditions of enzyme, substrate concentrations.

#### *1.9.4 Target D Optimisation of enzyme preparation*

Based on the preliminary research of enzymatic saccharification, the enzyme preparation will be further optimised on pretreated materials by reducing enzyme dosages and improving the synergy between each commercial enzyme. This optimisation could reduce the cost of the process.

#### *1.9.5 Target E Optimisation of ethanol yield*

Ethanol fermentation on starch and sucrose has been applied in the brewing industry. However, ethanol fermentation on cell wall rich materials is relatively different from starch

and sucrose rich materials. Fermentation on duckweed biomass is certainly different from other energy feedstock. Thus, searching for the optimal approach for ethanol fermentation from duckweed biomass and maximising the resultant ethanol yield is another crucial target of this project.

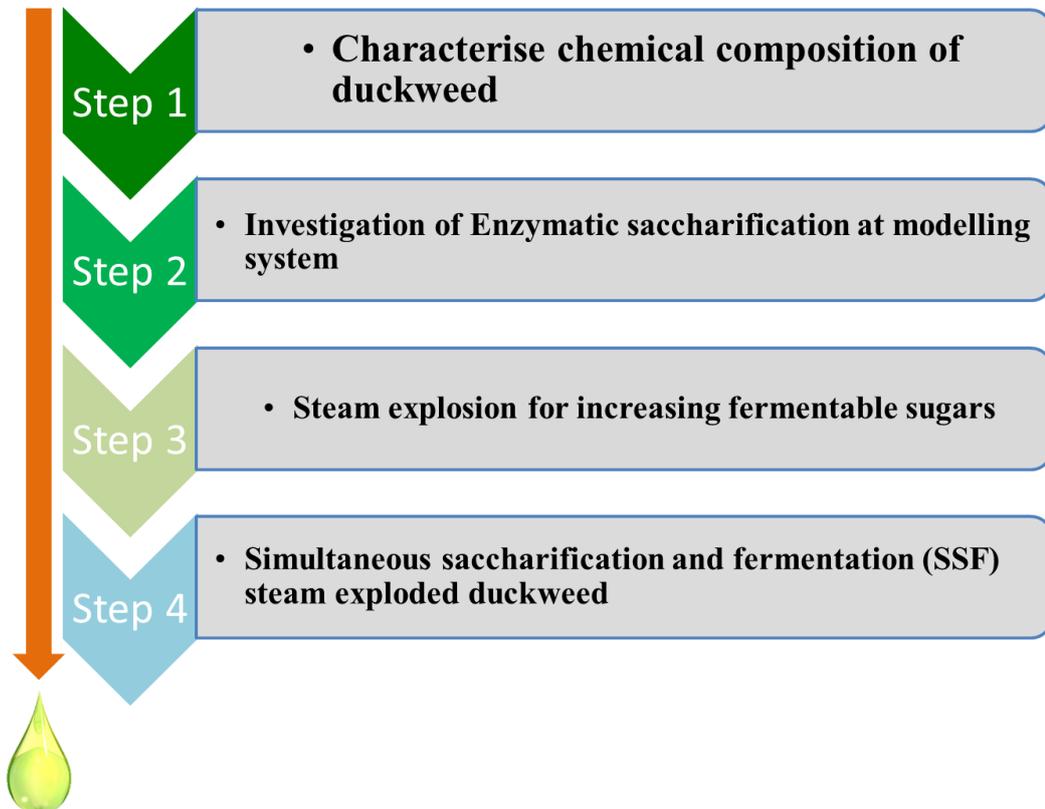


Figure 15. Demonstration of the process of the project investigation.

## 2 General materials and methods

### 2.1 General materials and chemicals

#### 2.1.1 Hoagland's E-Medium to culture *Lemna minor*

*L. minor* plants were collected from the surface of the River Yare located close to University of East Anglia, Norfolk, UK (52.61682 N, 1.243815 E) and were sterilised by immersing in diluted sodium hypochlorite (4 % v/v) for 1 min and rinsed with autoclaved distilled water to remove residual bleach. In a sterile containment cabinet, sterilised *L. minor* (10 plants) were transferred into autoclaved Hoagland's E-Medium (100 mL; pH 5.8) (Cowgill and Milazzo, 1989) in 250 mL Erlenmeyer flasks stoppered with a sponge. Hoagland's E-Medium is one of the most popular solutions for non-soil growing plant which could provide every necessary nutrient for a number of plants. The preparation of Hoagland's E-medium is given in Table 6.

Table 6. The recipe of Hoagland's E-medium.

	Stock Solution (g/100 mL)	Molecular mass	Conc. in stock (mol)	Vol in final medium mL/L	Conc. in final medium (mmol)
1. MgSO <sub>4</sub> ·7H <sub>2</sub> O	24.6	246.5	1.0	1.0	1.0
2. Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	23.6	236.2	1.0	2.3	2.3
3. KH <sub>2</sub> PO <sub>4</sub>	13.6	136.0	1.0	0.5	0.5
4. KNO <sub>3</sub>	10.1	109.1	1.0	2.5	2.5
5. Micronutrients <sup>a</sup>	Micronutrient Solution	/	/	0.5	/
6. Fe EDTA <sup>a</sup>	Fe EDTA Solution <sup>b</sup>	/	/	20.0	/

<sup>a</sup>The recipes of micronutrients and Fe EDTA are explained in Table 20 and 21 in § 8.1.

Fe EDTA Solution is added just before use.

The inoculated flasks were placed in a growth room (Figure 16) under controlled conditions: room temperature (22 °C); five fluorescent light tubes (amount of photosynthetically active radiation:  $\sim 120 \mu\text{mol}/\text{m}^2\text{s} = 25.7 \text{ W}/\text{m}^2$ ) consisting of three conventional tubes (GE F58w 33) and two Sylvania GRO-LUX tubes (F58w/GRO T8) were controlled automatically under long day conditions (16 h light and 8 h dark). The

mature plants were harvested at between 6 and 8 weeks after sub-culturing. No contamination with algae was observed. Fresh plants were used immediately after harvesting, or frozen at - 20 °C until required.



Figure 16. The plant culture room.

### 2.1.2 *Plants materials collected from John Innes Centre*

*L.minor* plants were collected from a pond located at the John Innes Centre, Norwich, UK (52.622295 N, 1.221894 E), then cleaned by tap water and rinsed by distilled water. The cleaned fresh wet biomass was packed with aluminium foil in long flat-thin packages (1 kg, 7 packages). The batches of fresh ponds sample (20 kg) were collected in different year. The packed samples were preserved in a cold room (4 °C). The original fresh material was treated by various approaches for different research purposes.



Figure 17. The location from where pond samples were collected.

### *2.1.3 Freeze dry and Freeze mill materials preparation*

The original fresh duckweed was dried by freeze drying process (Freeze Dryer 3.5, Birchover Instruments Ltd., UK) to only remove moisture content. The freeze dried materials was ground by nitrogen freeze-milling (Spex Freezer-Mill 6700, Spex Industries Inc., USA) to physically decompose plant to powders. The freeze dried and Freeze milled (FDM) duckweed was used for general chemical analysis.

### *2.1.4 Materials and chemicals*

A bulk of materials, chemicals and enzymes were used in this project, which are given in Table 7. The chemical and reagents used were of analytical grade and provided by Sigma-Aldrich Ltd (UK) unless otherwise stated.

Table 7. List of equipment, materials and suppliers.

Material	Supplier
96 deep well plate	Fisher Scientific UK Ltd, Loughborough, UK
96 well micro-titre reader plate	Fisher Scientific UK Ltd, Loughborough, UK
96 well PCR plate	Fisher Scientific UK Ltd, Loughborough, UK
Acroprep 96 filter plate 0.2 µm GHP	VWR International Ltd, Lutterworth, UK
Centrifuge tube 15, 50 mL with Centristar™ cap	Corning Incorporated, Monterrey, N.L, Mexico
Chromacol 300 µL glass vials	Essex Scientific Laboratory Supplies Ltd, Hadleigh, UK
Difco YM Media	Fisher Scientific UK Ltd, Loughborough, UK
<b>Enzymes:</b>	
Celluclast®	Novozymes Corp, Bagsvaerd, Denmark
CTec 2 Cellic®	Novozymes Corp, Bagsvaerd, Denmark
Depol™ 740L	Biocatalysts Ltd, Cardiff, UK
Novozyme 188 (BG)	Novozymes Corp, Bagsvaerd, Denmark
GF/C filter paper	Fisher Scientific UK Ltd, Loughborough, UK
Micro screw-cap tube 2 mL	SARSTEDT AG & Co. Nümbrecht, Germany
Micro safeseal tube 2 mL	SARSTEDT AG & Co. Nümbrecht, Germany
Pyrex® culture tubes	Sigma Aldrich, Gillingham, UK
Sterilin universal containers	Fisher Scientific UK Ltd, Loughborough, UK
Syringes and needle	Thermo UK Ltd, surrey, UK
Thiomersal	Sigma Aldrich, Gillingham, UK
Whatman 0.2 µm PVDF syringe filter	Fisher Scientific UK Ltd, Loughborough, UK
Whatman No.1 filter paper	Fisher Scientific UK Ltd, Loughborough, UK
Yeast Nitrogen Base	Formedium, Hunstanton, UK

## 2.2 Dry matter assessment

Duckweed contains a high and varied moisture content over 90 % (w/w) which was stated above. Thus, a precise dry matter value is required prior to all the other treatments for the different material batches. To minimise any potential degradation at high temperatures, a

small aliquot of the materials was dried under 105 °C until the moisture was completely evaporated. There are two approaches which were used in this experiment to measure the percentage of dry matter. Firstly, the percentage of dry matter of biomass (in triplicates) was determined by using a Mettler Toledo LP16 Infrared Dryer balance (Mettler Toledo Ltd, Beaumont Leys, Leicester, UK). The percentage of DM was given by the equipment. Secondly, biomass (in triplicates) was weighed and placed into aluminium plate. The aluminium plate is pre-weighed and recorded. The samples were dried in an oven at 105 °C overnight. The resulting samples were weighed. The % DM was calculated using the following formula:

$$\frac{\text{the weight of (dried biomass \& plate)} - \text{the weight of plate}}{\text{the weight of original samples}} \times 100\%$$

Equation 5. Percentage of dry matter (% w/w)

### 2.3 Alcohol Insoluble Residues (AIR) extraction

Many research studies (e.g. Yamazaki et al, 2008) have revealed that AIR is an ideal material for the investigation on cell wall of plants since the cell wall material is concentrated after AIR extraction. Duckweed biomass were extracted as AIR in order to remove water, low molecular weight moieties (including mono- and oligo-saccharides, mono- and oligo-peptides, lipid soluble components, low-to-medium chain fats and oils, chlorophyll and some salts). AIR materials therefore are cell wall rich and simply physically pretreated material which is relatively ideal materials for researching on enzymatic saccharification. The process (illustrated in Figure 18) involves: firstly, fresh (or frozen) plants were ground in ethanol (70 %) using a pestle and mortar for 10 min to physically break down the cell wall structure. The slurries were transferred to Pyrex<sup>®</sup> culture tubes after which they were heated at 80 °C for 15 min. After cooling and recovery by centrifugation (3000 x g, 10 min), the residue was re-extracted as before in ethanol (70 % v/v, 80 °C, 15 min) and then once at 80 °C in ethanol (100 % v/v). Finally the AIRs were extracted once in acetone at room temperature and dried at 30 °C overnight. The resulting AIR was continuously washed with 0.1 mol/L sodium acetic acid buffer (pH 5.0) twice then oven dried at between 30 °C and 40 °C to leave water-insoluble AIR (WIAIR). The solid and liquor were mixed at a ratio of 1: 2. Water-soluble components were further removed from the AIR.

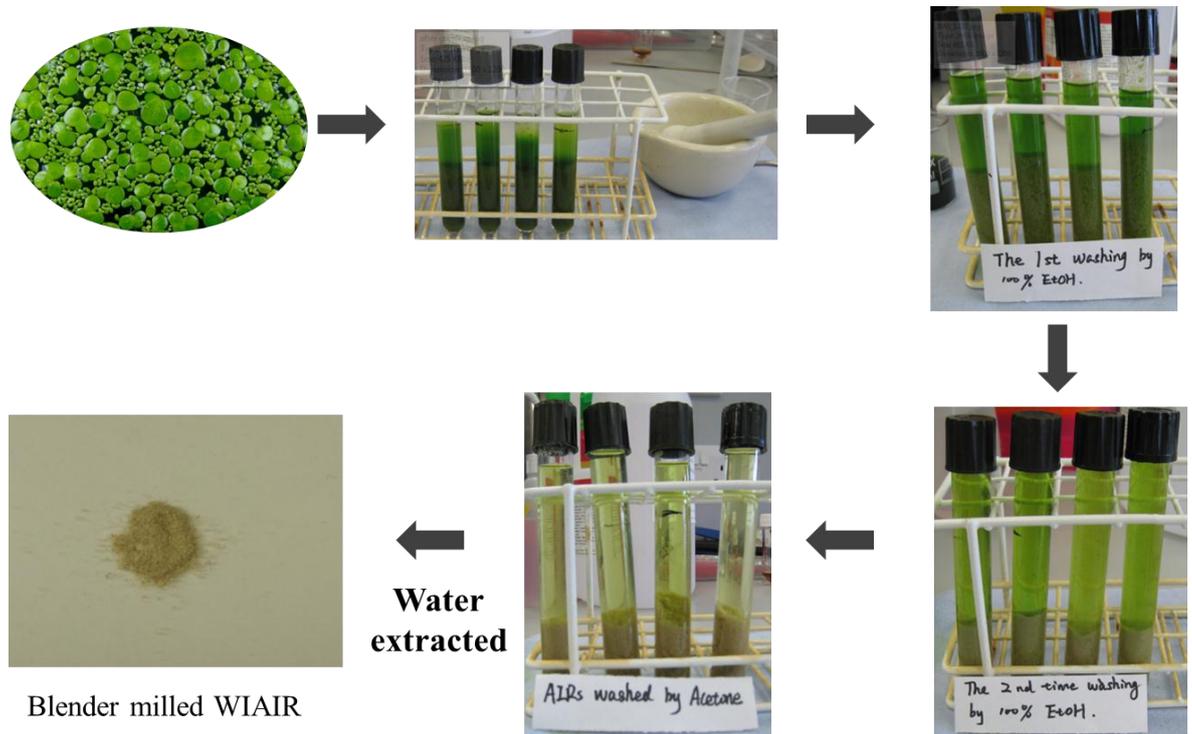


Figure 18. The process of AIR and WIAIR extraction.

#### 2.4 Steam explosion

Steam explosion is a simple thermal pretreatment which enables biomass to become more susceptible to cellulase for saccharification of cell wall materials. The steam explosion plant (1 kg) is shown in Figure 19 and its mechanism principle is illustrated in Figure 20. The maximum operating temperature of the plant is 230 °C. The fresh wet duckweed (1 kg) was introduced into the reaction chamber where biomass samples were exploded with steam at required temperature and pressure. The conditions (e.g. temperature, pressure, retention time) were maintained by computer. The steam exploded samples were depressurised into a cyclone quickly to collect the treated biomass, then steam was given off after a charcoal filtration and samples were recovered from the receptacle.



Figure 19. Steam explosion plant applied in this project.

Table 8. The parameters for the steam explosion pretreatment of duckweed.

Severity factor	Temperature (°C)	Pressure (MPa)	Retention time (min)
1.9	130	0.17	10
2.5	150	0.37	10
3.1	170	0.69	10
3.7	190	1.15	10
4.2	210	1.81	10
4.8	230	2.7	10

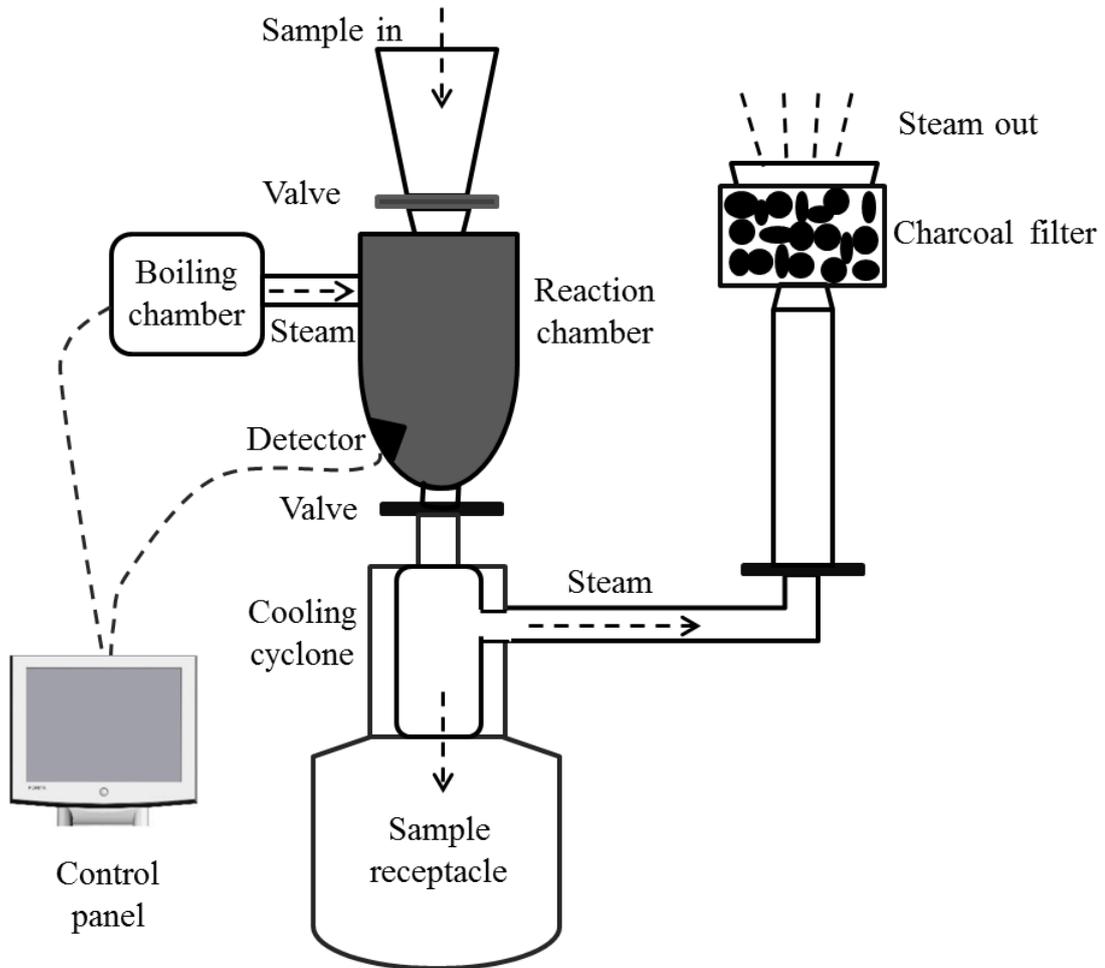


Figure 20. The construction of the steam explosion plant.

The fresh wet duckweed biomass was treated by steam explosion under different conditions. The severity factor (Chornet and Overend, 1988) representing the impact of SE condition, was determined by process temperature and the retention time by Equation 6:

$$SF = \text{Log}_{10}[t \times \exp((T - 100)/14.75)]$$

Equation 6. Severity factor of steam explosion.

where: SF = Severity factor, t = Remaining time (min), T = temperature (°C).

In this experiment, severity factor is strictly correlated to SE temperature variation (see Table 8) due to the constant time used throughout.

## 2.5 Enzymatic saccharification

Enzymatic saccharification is the most important treatment of biomass to ethanol, as described in § 1.5. The enzyme mixture was carefully prepared because enzyme activity is

extensively expressed in many different units. Enzymes concentrations in reaction solution were generally calculated based on substrate mass expressing as U/g substrate. However, a concentrated enzyme stock solution was preliminarily prepared by using unit of U/mL, which was given from enzyme companies. Thus, the total amount of enzyme (U) is the crucial parameter for adding the correct enzyme dosage (See Equation 7).

$$\begin{aligned} \text{Enzyme (U/g substrate)} \times \text{Substrate (g)} &= \text{Enzyme dosage (U)} \\ &= \text{Enzyme (U/mL)} \times \text{volume (mL)} \end{aligned}$$

Equation 7. The relationship between different enzyme activity units.

Sodium acetic acid (0.1 mol/L, pH 5.0) was used to adjust pH value of reaction solution to the required range throughout this project. Incubation was carried out with continuous agitation on a Thermoshaker at 120 rpm (Gyunter et al, 2008). Saccharification was terminated by heating to 100 °C for 5 min after which the samples were centrifuged at 16,060 x g for 5 min. The supernatants were recovered by aspiration and frozen prior to analysis. However, the quantification of the activities of commercial enzymes is a crucial step prior to enzyme preparation.

### 2.5.1 Filter paper Unit (FPU) assessment method

The activity of the cellulase enzyme products was determined by using filter paper assay (Ghose, 1987). Whatman No.1 filter paper (6 mm, 50mg) was prepared in micro tubes then mixed with diluted enzyme solution and buffered with sodium acetic acid (0.1 mol/L, pH 5.0). A range of dilutions of enzyme were measured for correcting enzyme dosage enabling the release of 2.0 mg glucose in 60 min. In the meantime, the reagent, substrate and enzyme blank were prepared. Samples and blanks were incubated for 1h at 50 °C. Hydrolysis was terminated by boiling for 5 min to inactive enzyme and tubes were centrifuged. The resultant supernatants from the samples were measured by using DNS method (described in § 2.9.1).

The sample data were plotted against the glucose standard curve with enzyme subtracted to quantify the released glucose. The logarithm of the enzyme dilution factor was plotted against the glucose from standard curve. The FPU activity was then calculated following Equation 8:

$$\text{FPU} = 0.37 \times \text{Enzyme dilution (U/mL)}$$

Equation 8. Format of FPU calculation.

The factor of 0.37 is the amount of FPU required to release 1  $\mu\text{mol}$  glucose per minute, expressed in Equation 9:

$$0.37 = \frac{2 \text{ mg (glucose)}}{0.18 \text{ (mg/mol[glucose])} \times 0.5 \text{ mL(volume)} \times 60 \text{ (min)}}$$

Equation 9. The FPU factor of 0.37.

## 2.6 Starch assessment

Starch assessment is generally grouped into acid hydrolysis and enzymatic hydrolysis procedures. Total Starch Assay (Megazyme, 2012) is the standard enzymatic hydrolysis method to assess the starch contents, and includes following treated steps: starch gelatinisation, liquefaction and dextrinisation and enzymatic hydrolysis of dextrans to glucose. Dimethyl sulphoxide (DMSO) is required to complete the starch gelatinisation (Englyst and Cummings, 1988). Today, most methods tend to employ thermal  $\alpha$ -amylase and amyloglucosidase to hydrolyse completely starch to glucose immediately following the starch gelatinisation step (Batey, 1982). The glucose released from starch was measured by using colourimetric assay – GOPOD test (this method is described in the following paragraph). Chemical reagents and enzyme are from total starch assay kit produced by Megazyme. The starch was calculated according to the following equation:

$$\frac{A \times (\text{ABS sample} - \text{ABS blank}) - B}{\text{total mass}} \times 100 \%$$

Equation 10. Percentage of Starch (% w/w)

Where 'A' stands for the gradient and 'B' represents intercept which were calculated from glucose standard curve.

The procedure was described as: the aliquot of SE products and untreated sample (triplicates) were transferred to Pyrex<sup>®</sup> culture tubes and frozen with liquid nitrogen. Then, the frozen samples were freeze dried and freeze milled. The FDM duckweed (30 mg  $\times$  triplicates) was dispersed in 80 % (v/v) ethanol (200  $\mu\text{L}$ ). After boiling for 5 min with 2 mL of dimethyl sulphoxide (DMSO), samples were hydrolysed by  $\alpha$ -amylase (3 mL, 300 U) by boiling for a further 6 min. Then, hydrolysed samples were added with sodium acetate buffer (4 mL, 200 mmol L, pH 4.5) at 50  $^{\circ}\text{C}$  following by a continuous hydrolysis with amyloglucosidase (0.1 mL, 20 U, 50  $^{\circ}\text{C}$ ) for 0.5 h. The resulting sample (100  $\mu\text{L}$ ) was

assessed by GOPOD reagent (3 mL). Absorbance was measured by using a Microplate Spectrophotometer (Benchmark Plus, BioRad, CA, USA) at 510 nm.

## 2.7 Gas chromatograph (GC) analysis for measurement sugars and fatty acids

### 2.7.1 GC principle

Cell wall polysaccharides are generally composed of aldoses in the furanose and pyranose forms and uronic acids in pyranose forms. An analysis of the monosaccharides could provide a clear understanding of the features and structure of cell wall polysaccharides. GC method allows that all neutral sugars (aldoses) are measured in a gas form. The mechanism of the GC method (Figure 21) is that the analytes are injected and immediately volatilised at high temperature (250 °C), before passing onto a capillary column (stationary phase) and eluting with a carrier gas flow, typically Nitrogen or Helium. The analytes are separated according to their relative affinity for both stationary and mobile phases and therefore the retention time of compounds differs when detected by a given method. Prior to the analytes injection, specimens were treated with a series of pretreatments, such as sugar solubilisation, sulphonation, neutralisation, reduction and acetylation, to enable analytes to be volatilised, or were transformed into a volatilisable form. Uronic acids were analysed by using a standard uronic acid analysis (see § 2.8.3). Details of setting up the facility were explained in § 8.2.

Flame Ionisation Detector (FID) is a commonly applied detection method for carbohydrate assessment by GC. The analytes were pyrolysed to carbon ions by hydrogen flame. The resulting carbon ions then were collected by electrode in opposite charge and produce a current signal which was amplified and reflected as peaks in data for analysis.

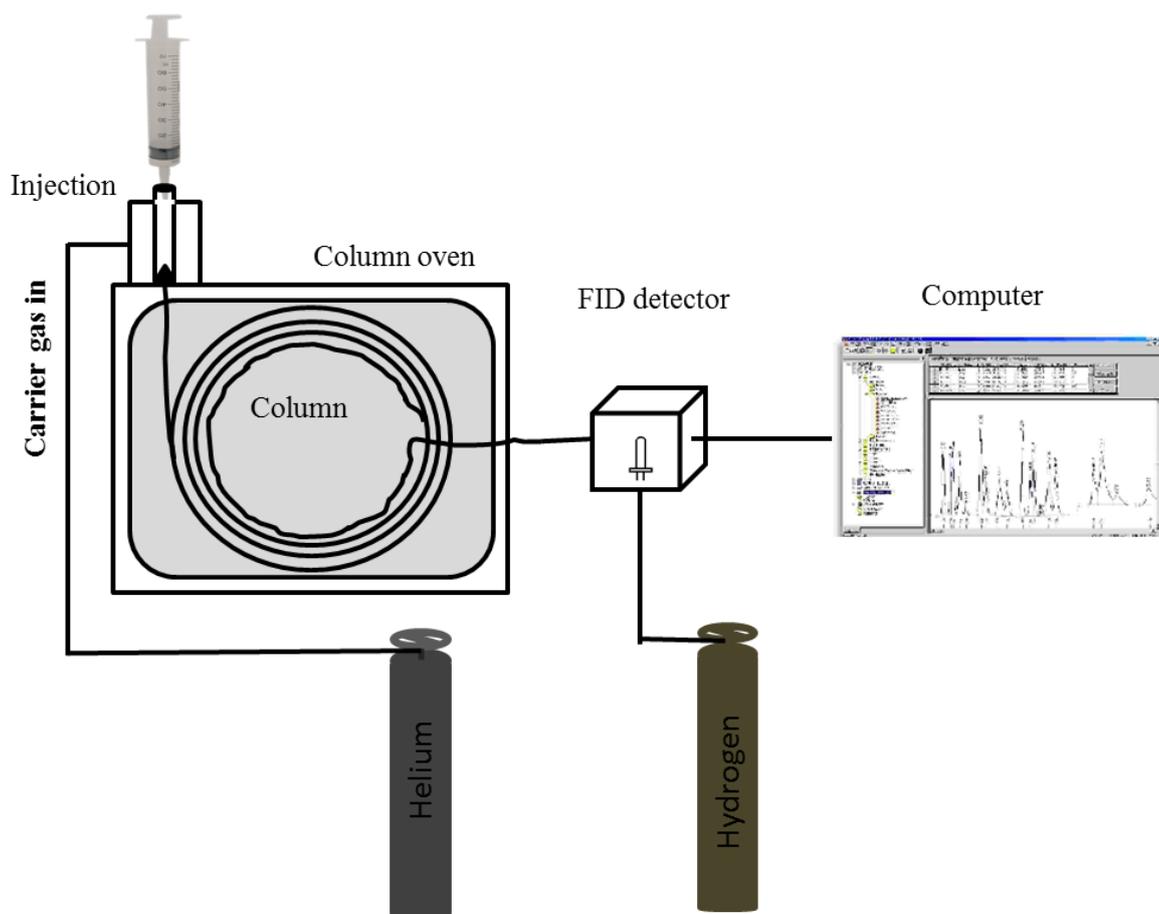


Figure 21. GC schematic

### 2.7.2 GC analysis for alditol acetates sugars

The method was according to Blakeney et al (1983). Dried solid specimens (3 mg in triplicates) were placed in Pyrex<sup>®</sup> culture tubes and were hydrolysed with 200  $\mu\text{L}$  72 %  $\text{H}_2\text{SO}_4$  (w/w) at room temperature for 3 h, following by the dilution samples to 1 mol/L  $\text{H}_2\text{SO}_4$  by adding 2.2 mL distilled water. The 1 mol/L  $\text{H}_2\text{SO}_4$  hydrolysis proceeded for 2.5 h at 100  $^\circ\text{C}$ . Polysaccharides were consequently degraded to monosaccharides (Saeman et al, 1954). Samples were taken for the colourimetric determination of galacturonic acid after 1 h of 1 mol/L hydrolysis, see §2.8.3. Samples were cooled on ice and 200  $\mu\text{L}$  2-deoxyglucose (2-DOG) was added as an internal standard. The homogenised sample (1mL) were transferred into clean Pyrex<sup>®</sup> culture tubes and neutralised with 300  $\mu\text{L}$  25 % (w/w)  $\text{NH}_3$  to pH 8 - 9. 100  $\mu\text{L}$  3mol/L  $\text{NH}_3$  containing 150 mg/mL  $\text{NaBH}_4$  was added for the reduction of sugars with 1h incubation at 30  $^\circ\text{C}$ . Acetic acid (200  $\mu\text{L}$ ) was added over ice to destroy the excess  $\text{NaBH}_4$ . 300  $\mu\text{L}$  samples were transferred to another clean Pyrex<sup>®</sup> culture tubes and directly added with 450  $\mu\text{L}$  1-methylimidazole and 3 mL acetic

anhydride over ice, then homogenised samples were incubated 30 min at 30 °C for the acetylation. The samples were cooled on ice after incubation, and meantime, 3.5 mL ultrapure water and dichloromethane (DCM) were added to extract the analytes into solvent. The homogenised samples were centrifuged to separate organic layer which was subsequently transferred to clean Pyrex<sup>®</sup> culture tubes. The remaining nonorganic layer was rewashed by 2.5 mL DCM and the organic layer was separated upon centrifugation. The nonorganic layer was aspirated and two organic layers were combined which were subsequently washed by ultrapure water twice. The nonorganic layer was aspirated each time. The resulting samples were evaporated in a concentrator (Bibby Scientific Ltd., Stone, UK) under a stream of nitrogen. Acetone (1mL) was added to dissolve analytes and transferred into a glass vial for a GC analysis by using a Perkin-Elmer Autosystem XL (Perkin Elmer Ltd, Seer Green, UK) containing a RTX-225 (Restek, Bellefonte, USA) column. This GC method also allow that non-cellulosic carbohydrates are measured by only using 1 mol/L H<sub>2</sub>SO<sub>4</sub> to hydrolyse samples (Saeman et al, 1954). In this study, the alternative sulphonation can give an approximate determination of starch content since non-cellulosic glucose predominantly comes from starch. The standard sugars (including anhydride Rha, Fuc, Ara, Xyl, Man, Gal, Glc) were prepared and internal standard 2-DOG was added.

### 2.7.3 GC analysis for Fatty acid methyl esters (FAME)

GC method is also used for the assessment of FAME. Duckweed materials were treated with Soxhlet extraction to obtain concentrated lipid samples. The lipid samples (in triplicates) were purified with 0.5 mL dry toluene containing 0.1 mg butylated hydroxytoluene (BHT) with vortex mixing. Methylation reagent (1 mol/L of methanol containing 2 % v/v H<sub>2</sub>SO<sub>4</sub>) was added and the samples were vortex mixed again. The tubes were tightly capped and heated at 50 °C overnight with occasional mixing. After heating, the samples were cooled down to room temperature and neutralised by 1 mL mixed solution of 0.25 mol/L KHCO<sub>3</sub> and 0.5 mol/L K<sub>2</sub>CO<sub>3</sub>. The resulting solutions were mixed with 1 mL hexane and centrifuged to separate FAMEs into the upper phase which were transferred to clean Pyrex<sup>®</sup> culture tubes. 200 µL of prepared solution was transferred into vial and evaluated by Hewlett Packard 5890 GC system (Hewlett-Packard Limited, Bracknell, England, UK) containing Rtx-225<sup>®</sup> column (Thames Restek UK Ltd, Saunderton, UK). Methyl heptadecanoate (Sigma Aldrich, Gillingham, UK) was added as

the internal standard and the retention time of 31 FAME components had been specifically identified for BPX 70 column (see Figure 77 in § 8.3) by using a commercial standard from SGE company (SGE Analytical Columns, New Addington, Croydon, UK).

## 2.8 High-performance liquid chromatography (HPLC) analysis

### 2.8.1 *The analysis theory*

HPLC is another commonly applied chromatographic technique, which has a very similar principle to GC, for separating mixed components in biochemical analytes with the purpose of identification, quantification and purification of individual components. In this research, HPLC was used for identifying and quantifying the phenolic acids, carbohydrates and the ethanol products of the fermentation. The details are stated in the paragraphs below (see Figure 22). Analytes are carried by pressurised liquid (mobile phase) to pass through the column made up by silica sorbents granular (size 2 - 50  $\mu\text{m}$ ). In the column, analytes were separated by their polarity and affinity to sorbents. HPLC possesses a superior resolving power than traditional liquid chromatograph due to its smaller sorbent particles (2 - 5  $\mu\text{m}$ ) in the column. This allows that smaller size analytes to be analysed and HPLC technique is therefore extensively used for compositional analysis. The separated analytes were then identified by different detectors for different purposes, such as Refractive Index (RI), Diode Array and Photo Diode Array.

Refractive Index (RI) detector (see Figure 23) is the only universal detector in HPLC. The measurement by RI detector is according to the sensitivity of RI difference between sample and mobile phase. The greater RI difference between sample and mobile phase, larger signal from analytes will be produced. In other words, a mixed sample might cover a range of refractive index, some luminousness are close to mobile phase which produce smaller RI, those possess significantly different luminousness from mobile phase can produce larger RI.

Diode Array detector (DAD, Figure 24) and Photo Diode Array detector (PDA) are both common ultraviolet (UV) detectors. This detector allows that precise absorbance can be scanned when samples pass through the flow cell. The quantification of sample by DAD and PDA is due to a new dimension of analytical capability to liquid chromatography which requires specific wavelength range.

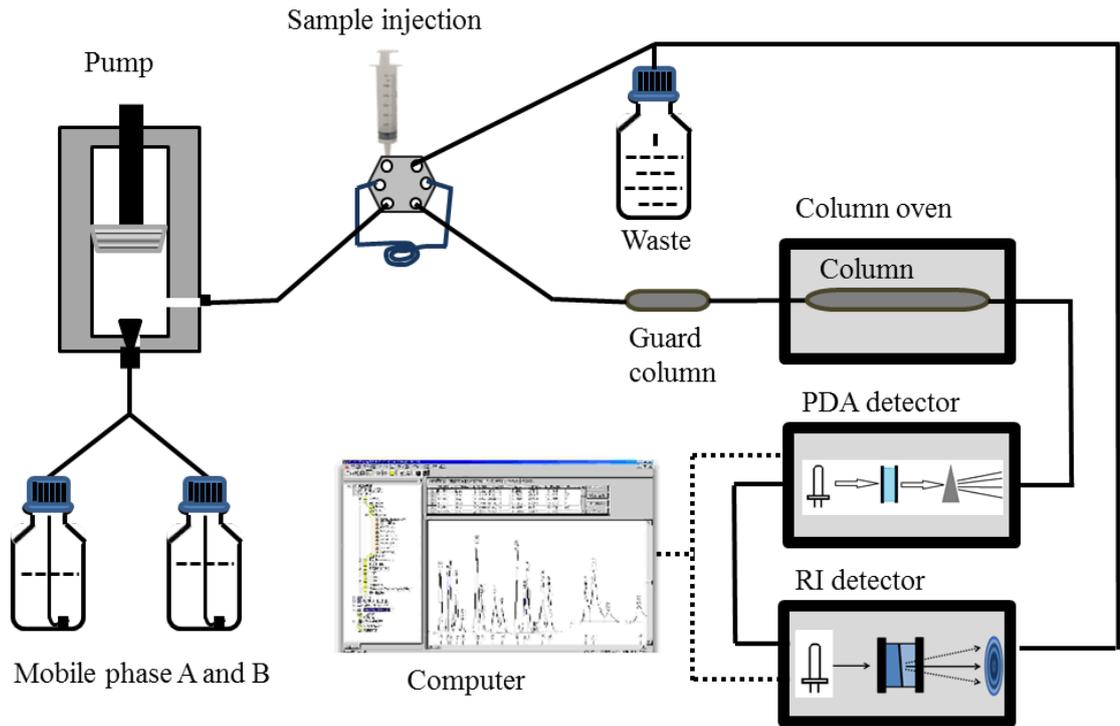


Figure 22. HPLC schematic

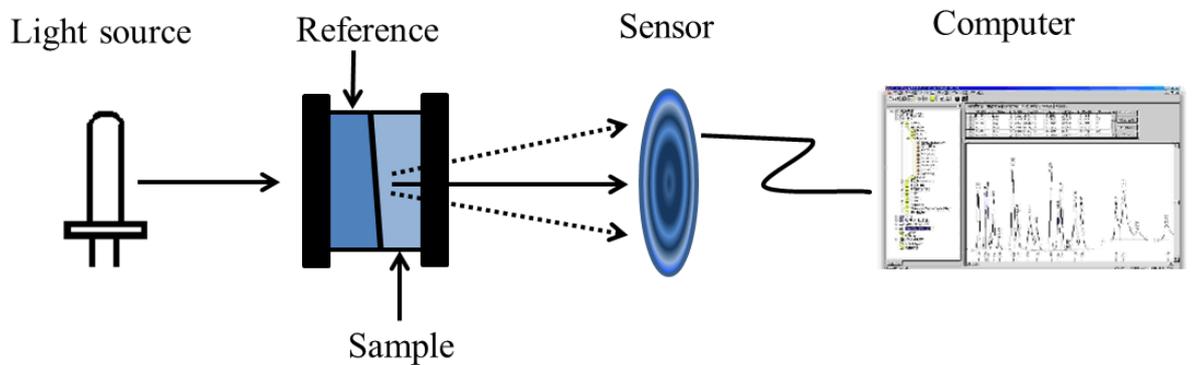


Figure 23. RI detector principle.

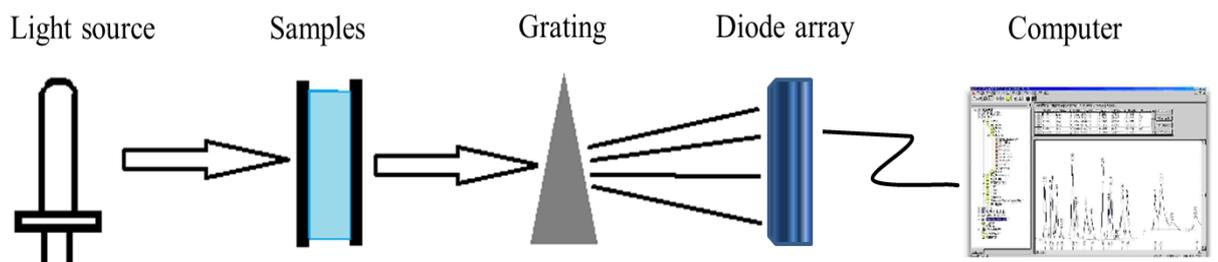


Figure 24. DAD detector principle.

### 2.8.2 *The preparation for soluble sugars and ethanol*

The aliquots (2 mL) were transferred into micro tubes with screw cap and centrifuged at 12000 x g. The supernatant were filtered with 0.2 µm GHP filter plate into 96-well reader plate. The plate was sealed with Adhesive PCR foil seal (Thermo UK Ltd, UK) and read in HPLC. Samples were assessed by using Aminex HPX-87P carbohydrate analysis column (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) matched with RI detector. A guard column was added to protect column. This method requires a mobile phase (ultrapure water) at a flow rate of 0.6 mL/min under 65 °C. The more details were listed in § 8.5. However, the superimposition of monosaccharides peaks was caused inevitably by using this method: glucose and 2-DOG are present at 12.5 minutes (retention time), rhamnose and galactose at 14.4 minutes, as well as arabinose and fucose at 15.5 minutes.

### 2.8.3 *The preparation for solid sugars*

The preparation also involves the hydrolysis by sulphuric acid. Dried solid specimens (3 mg in triplicates) were placed in Pyrex<sup>®</sup> culture tubes and were hydrolysed with 200 µL 72 % H<sub>2</sub>SO<sub>4</sub> (w/w) at room temperature for 3 h, following by the dilution samples to 1 mol/L H<sub>2</sub>SO<sub>4</sub> by adding 2.2 mL ultrapure water. The 1 mol/L H<sub>2</sub>SO<sub>4</sub> hydrolysis proceeded for 2.5 h at 100 °C. The method was according to Blakeney et al (1983) in which polysaccharides were degraded to monosaccharides. An internal standard – ribose (100 µL) was added to samples and standards. After a vortex, samples were generally neutralised with 2 mol/L CaCO<sub>3</sub> solution over ice. Aliquots (1 mL) were filtered with 0.2 µm GHP filter plate upon centrifugation at 800 rpm for 10 min into 96-well plate which was then sealed with Adhesive PCR foil seal. The method of HPLC is described in § 2.7.2.

### 2.8.4 *The preparation for acid and inhibitors*

Aliquots of sample were transferred into centrifugation tubes for centrifuging at 3500 rpm for 10 min. The supernatant (1 mL) and standards in grade of 5-HMF, 2-FA, acetic acid, formic acid were filtered with 0.2 µm GHP filter plate upon centrifugation at 800 rpm for 10 min into 96-well plate which was then sealed with Adhesive PCR foil seal. Values of organic acids which might inhibit saccharification and fermentation were measured by using HPLC containing an Aminex HPX-87H organic acid column (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and RI detector. This method requires a mobile phase (0.005 mol/L H<sub>2</sub>SO<sub>4</sub>) at a flow rate of 0.6 mL/min under 65 °C. Further details are listed in § 8.5.

### 2.8.5 *The preparation for carbohydrate size*

The preparation of samples is the same as the description in § 2.7.2. In HPLC column, samples were separated solely by molecular size instead of the interaction with sorbents. This size-exclusion chromatography (SEC) separation also enables smaller size particle penetrate quickly, thus increases their elution time. A series of columns: TSK GEL G8000, TSK GEL 3000 and TSK GEL 4000 columns (Tosoh Bioscience, Tokyo, Japan) were used to separate samples into different molecular mass profiles. RI detector was associated with a Series 200 LC equipment to identify the molecular mass profile. HPLC-SEC method requires a mobile phase (0.2 mol/L NaNO<sub>3</sub> containing 0.05 % w/v NaN<sub>3</sub>) at a flow rate of 0.5 mL/min under 35 °C. The molecular mass standard was prepared by using a polysaccharide calibration kit (Polymer Laboratories, Church Stretton, UK). § 8.6 list the details of HPLC-SEC method.

### 2.8.6 *The preparation for phenolic acids*

The preparation of phenolic acid analysis is more complicated and detailed than other HPLC analysis. Aliquots (3 mg in triplicates) were weighed and placed into foil wrapped Pyrex<sup>®</sup> culture tubes. Samples were added with 1mL 4 mol/L NaOH and immediately flushed with nitrogen flow to de-oxygenate water). The homogenised samples were incubated with an agitation at room temperature for 16 hours. Then, deoxygenated distilled water (1.5 mL), 0.5 mL HCl (37 % w/w) and 50 µL of 0.2 mg/mL trans-cinnamic acid (internal standard) were added and mixed samples after each chemical added. pH value was indicated into red colour. The solution volume was topped up to 3.5 mL by 450 µL distilled water and then extracted by ethyl acetate (3.5 mL) three times. Layers were separated by centrifugation at 3000 rpm for 3 min and the organic layer was transferred and combined into clean tubes. Evaporation with a nitrogen flow at 40 °C was carried out to remove ethyl acetate. Analytes were redissolved in 1 mL 50 % (v/v) methanol and filtered by using 0.2 µm PVDF syringe filters to 300 µL Chromacol glass vials, and capped properly to stop evaporation. Standard calibration was made up by using protococatechuic acid, chlorogenic acid, benzoic acid (p-OH-), phenyl acetic acid (p-OH-), vanillic acid, vanillin, benzaldehyde (p-OH-), CA-truxillic acid, coumaric acid (t-p-) and ferulic acid (t-p-). Samples were assessed by using a Phenomenex HPLC column (Phenomenex, Macclesfield, UK) matched with DAD detector. A guard column was added to protect column. HPLC-DAD phenolic acid method requires 4 types mobile phase: A, 50 % (v/v) methanol; B, 65 % (v/v) acetonitrile; C a mixed mobile phase of 40 % (v/v) methanol, 40

% (v/v) acetonitrile and 154  $\mu$ L Trifluoroacetic acid (TFA); D, 10 % (v/v) acetonitrile and 154  $\mu$ L TFA. The mixed mobile phase pass through column at a flow rate of 0.1 mL/min under 65 °C. The method details are illustrated in § 8.3.

## 2.9 Colourimetric assays

Colourimetric assays are simple and fast methods which can assess precise level of coloured analytes in solution by reading its absorbance in a specific wavelength of light. General colourimetric assays used in my project include Dinitrosalicylic acid (DNS) assay, Glucose oxidase/peroxidase (GOPOD) assay, Uronic Acid assay.

### 2.9.1 Dinitrosalicylic acid (DNS) assay

Dinitrosalicylic acid is an aromatic compound that can react with reducing sugars (see Equation 11) and cause a significant colour change (yellow to dark orange) (Miller, 1959). The higher the level of reducing sugars detected, the darker the colour present. Dilution of sample was sometimes necessary to bring analytes into the right range for DNS reagent. Samples were read at a wavelength at a range of 490 - 580 nm. DNS assay provides a simply fast method to assess reducing sugars in samples than using GC and HPLC method with cumbersome preparation. However, as a non-specific measurement, DNS assay is not able to measure specific glucose concentration which was necessarily measured in my research.

3,5-dinitrosalicylic acid + reducing sugars  $\rightarrow$  3-amino-5nitrosalic acid

Equation 11. DNS reaction theory.

DNS solution was prepared as: 1 % (w/v) 3,5-dinitrosalicylic acid mixed with 30 % (w/v) sodium potassium tartrate and 0.4 mol/L sodium hydroxide. The assay process was optimised by Wood and Elliston (2011) to improve operation and standardise results. Thus, there are two preparations which have been applied in this research. Traditional DNS assay was following the method stated by Bailey et al (1992): 0.025 mL of original samples were diluted 10 fold in sodium acetic acid buffer (0.1 mol/L, pH 5.0), in Eppendorf micro-centrifuge tubes to which 0.3 mL DNS reagent was added. After homogenization, the samples were heated at 100 °C for 5 min on a hotplate stirrer. The resulting solution (200  $\mu$ L) was transferred to a 96-well plate and absorbance measured in a Microplate Spectrophotometer (Benchmark Plus, Bio Rad) at 580 nm. The new DNS assay (Wood et

al, 2012) was described as: 36  $\mu\text{L}$  of original samples and 144  $\mu\text{L}$  DNS reagent (1: 4 of sample : DNS reagent) were homogenised in tall- chimney 96-well plates (Fisherbrand<sup>®</sup>, UK) stoppered with TPE PCR sealing mats (BRAND, at Fisher, UK). The solutions were heated in a Biometra<sup>®</sup> T-Gradient thermocycler (Biometra, Göttingen, Germany) at 100 °C for 3 min. A cooled aliquot (100  $\mu\text{L}$ ) was transferred to a 96-well flat-bottomed microtitre plate (Nunc, Roskilde, Denmark) and absorbance measured in a Microplate Spectrophotometer (Benchmark Plus, BioRad, CA, USA) at 580 nm. A glucose standard calibration was prepared in either process. The concentration of reducing sugars was calculated following Equation 12:

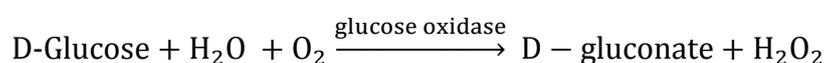
$$A \times (\text{ABS sample} - \text{ABS blank}) - B$$

Equation 12. The calculation of reducing sugars concentration.

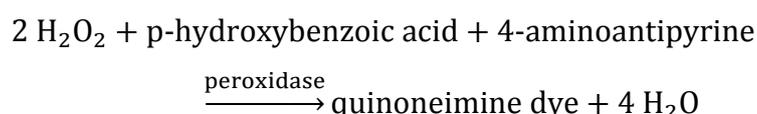
Where: A is the gradient and B is the intercept from standard calibration.

### 2.9.2 Glucose oxidase/oxidase (GOPOD) assay

Glucose was measured by using a specific method – GOPOD assay. The GOPOD kit (Megazyme, Bray, Ireland) employs high level of pure glucose oxidase and peroxidase to hydrolyse glucose to quinoimine (pink colour). The reaction formats are given below:



Equation 13. Step 1 reaction of GOPOD assay.



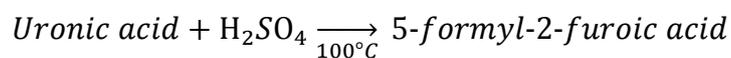
Equation 14. Step 2 reaction of GOPOD assay.

The coloured solution was measured using a plate reader at a wavelength at 490 nm. GOPOD reagent was prepared by mixing bottle 1 and 2 in GOPOD kit and stored at 4 °C. The measurable glucose range is limited by 4-aminoantipyrine. Thus, samples were necessarily diluted into the right concentration range and a glucose standard calibration (0 to 1.6 mg/mL) was prepared. The procedure is: 0.01 mL of original sample was diluted with 0.01 mL sodium acetic acid buffer (0.1 mol/L, pH 5.0) to which was added 0.3 mL GOPOD reagent. After mixing by vortexing, the samples were incubated at 50 °C for 20 min after which the absorbance was measured in a Microplate Spectrophotometer

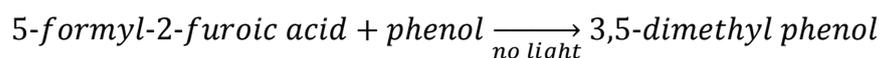
(Benchmark Plus, BioRad, CA, USA) at 510 nm. The background absorbance from blank enzyme preparations was subtracted and the concentration of sugars calculated from appropriate standard curves. The calculation of glucose concentration was similar to reducing sugars and the format referenced to Equation 12.

### 2.9.3 Uronic Acid assay

Galacturonic acid (GalA) is the predominant compound in duckweed pectic polysaccharide (Golovchenko et al, 2002) and it was measured by using uronic acid assay. GalA reacts with concentrated  $H_2SO_4$  at 100 °C to form 5-formyl-2-furoic acid which is colorimetrically determined by phenol (see Equation 15 and Equation 16).



Equation 15. Step 1 reaction of uronic acid assay.



Equation 16. Step 1 reaction of uronic acid assay.

The procedure was improved according to Blumenkrantz (1973). 3 mL sulphuric acid (98 % w/w) including 25 mmol/L sodium tetraborate was added to acid washed test tubes over ice. 0.5 mL of samples and uronic acid standard were added and tubes were vortexed strongly. Then tubes were covered with glass balls and heated in a water bath at 100 °C for 10 min. Tubes were cooled on ice. Then 50 µL of 0.15 % (w/v) 3-phenyl phenol in 0.5 % (w/v) NaOH was added into three replicates and 50 µL of 0.5 % (w/v) NaOH was added to the fourth tube as a reagent blank. After strong homogenization, the solutions were incubated in the dark at room temperature for 0.5 h. 200 µL of samples were transferred to micro-titration plate and absorbance was measured in a Microplate Spectrophotometer (Benchmark Plus, BioRad, CA, USA) at 580 nm.

## 2.10 Microscopy

Microscopy can provide visible evidence for the structural variation of cell wall and starch in micro scale. Thus, microscopy is not only a quick method for the identification of substances, but also is a complementary technique to the quantification methods for assessing duckweed compositions. For the demands of this research, a number of

compounds of starch, lignin and lipid fractions were stained by different dye solution and observed by microscopy (BX60, Olympus, Japan). Materials were stained with Lugols solution (20 g/L KI containing iodine 0.2 g/L to highlight the presence of starch. In this project, freeze dried and freeze milled duckweed powder was stained with phloroglucinol-HCl to detect the presence of lignin fractions according to Parker and Waldron (1995). The cinnamaldehyde end group of lignin reacts with phloroglucinol-HCl (saturated phloroglucinol dissolved in 20 % (v/v) HCl) for 5 minutes at room temperature to present a red-violet colour. In the meantime, wheat straw was also stained as positive control to exhibit a pinkish-red colour indicating the presence of lignin. Nile blue as a histological staining solution of biological detection is used to highlight the presence of lipid equivalents of duckweed. Lipids (e.g. fatty acids, chromolipids, phospholipids, steroids) reacts with Nile blue solution could result in a yellowish colour (Wang et al, 2012). Fresh wet duckweed, which was preliminarily immersed by cyclohexane-trans-1,2-diaminetetraacetate (CDTA) for 2 days, was stained by Nile blue solution (0.01 % w/v, Raymond A Lamb, UK) for 5 min at room temperature and was observed under blue light of fluorescence microscope.

### 3 *Compositional investigation of duckweed biomass*

The understanding of plant structure and its chemical compositions are the preconditions of researching the conversion duckweed to bioethanol. Of duckweed chemical compounds, starch and cell wall polysaccharide are the main targets of enzymatic saccharification while other compounds, such as lipids and lignin, might have significantly negative influence on the biological degradation. Thus, this chapter will individually analyse the chemical compositions of *L. minor*, particularly the types and quantities of cell wall polysaccharides. In the next research stage, all of the chemical information will be helpful to select pretreatment approaches and specific commercial enzymes for degradation of duckweed biomass.

#### 3.1 **Materials and Methods**

##### 3.1.1 *Cell wall materials preparation*

*L. minor* fresh wet plants collected from the pond in John Innes Centre (pond sample in following context) were used for the sequential extraction of polysaccharides of duckweed cell wall. The original fresh biomass was treated by a rotary ball-mill process (ball mill jar, Capco Test Equipment Ltd., UK) to disrupt plant structure and remove starch content (Tamaki et al, 1998). The blended fresh duckweed (200 g) was homogenised in 3 % (w/v) sodium dodecyl sulphate (SDS) solution (10 mmol/L  $\text{Na}_2\text{S}_2\text{O}_5$ ) by blender milling (3 min), before rinsing with 10 L of 10 mmol/L  $\text{Na}_2\text{S}_2\text{O}_5$  solution through nylon mesh (25  $\mu\text{m}$ ). The resulting material was transferred to the ball-mill jar and added with ceramic beads (750 g of 2.54 cm diameter beads, 500 g of 1.91 cm diameter beads and 500 g of 1.27 cm diameter beads). The material was dispersed in 300 mL 0.5 % (w/v) SDS (5 mmol/L  $\text{Na}_2\text{S}_2\text{O}_5$ ) solution. The ball-mill was operated at 60 rpm/min for 3.5 h. The ball milled material was homogenised in distilled water (15 min) twice and filtered through nylon mesh (25  $\mu\text{m}$ ) and stored in a freezer (- 20 °C). The residues remaining after the ball mill process consist of purified cell wall material (CWM).

##### 3.1.2 *Ash test*

The total mineral elements were established by determination using the total ash method (Sluiter et al, 2008). Freeze dry and freeze mill (FDM) materials (1g in triplicate) was placed in crucible and dried in an oven (105 °C) for 16 h and the obtained dry weight was

recorded. Then, the resulting samples were treated in a muffle furnace (Vulcan 3-550, Jencons Scientific Ltd, UK) at 200 °C to char following by increasing temperature gradually to 350 °C. Finally, the samples were treated at 500 °C for 16 h to ash.

### 3.1.3 Lignin assessment

Acid insoluble lignin was measured by the gravimetric Klason lignin method which also contains acid insoluble minerals. The FDM materials (0.1 g in triplicates) was hydrolysed in 1.5 mL 72 % (w/w) H<sub>2</sub>SO<sub>4</sub> at 30 °C for 1 h, before adding 1.5 mL water and hydrolysing at 100 °C for 2.5 h. The acid insoluble lignin was recovered on a sintered glass funnel (Porosity<sup>®</sup> 4, VWR, UK) and dried in an oven (50 °C). The resulting sample was burned in a muffle furnace to Klason lignin following the ash test method. Phenolic acids were measured by HPLC as described in § 2.8.6.

### 3.1.4 Protein assessment

The FDM pretreated pond sample (2 g) was sent to a firm of analytical chemists (Salamon & Seaber Ltd, 68 Hanbury Street, London, UK) for total protein evaluation.

### 3.1.5 The investigation of the lipid fraction

The fresh healthy plants were grown in distilled water (100 mL) in 250 mL Erlenmeyer flasks (in triplicates) stoppered with a sponge. The flasks were placed under controlled conditions described in § 2.1.1. Aliquots of representative time points during the 28 day starvation period were collected and observed with a light microscope to understand the variation in lipid content when duckweed is starved of nutrients. The total lipid fraction was measured using the Soxhlet extraction method.

#### 3.1.5.1 Soxhlet extraction

The Soxhlet extraction apparatus (Figure 25) was originally designed for extracting lipid fraction from solid samples (Laurence and Christopher, 1989). A sample (2 g) was contained in a cellulose extraction thimble capped with glass wool then the thimble was placed in the middle chamber. Hexane was used to extract lipid equivalents from sample in a solvent refluxing process. Hexane was placed in the round-bottom flask ( $\geq 2/3$  volume) before connecting the sample chamber and condenser and heated for evaporation. The hexane vapour was condensed on the top by cold water to and hexane dropped into sample to dissolve lipid equivalents. The lipids in the solvent refluxed back to flask until it

overflowed siphon top. The process was continued for 6 h and solvent containing lipids was concentrated using a rotary evaporator (Rotavapor R-114, BUCHI UK Ltd, Oldham, UK). The residual pellet was re-extracted and solvent was concentrated and combined with the first extract. The lipid contents were measured using gravimetric analysis.

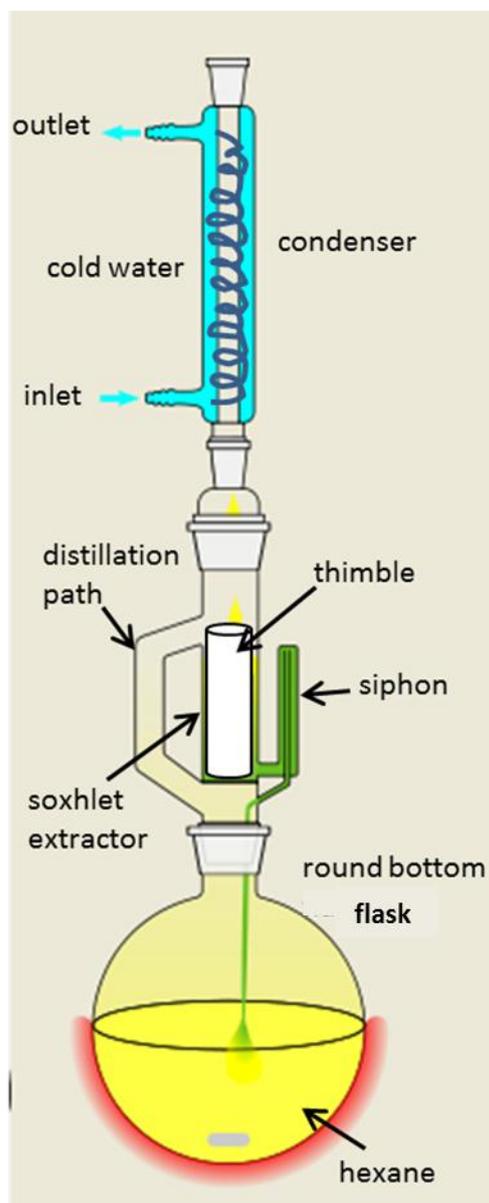


Figure 25. The illustration of the Soxhlet extractor

#### 3.1.5.2 Fatty acid methyl esters (FAME) analysis

Lipids extracted from Soxhlet extraction were prepared for FAME analysis assessed by GC method and the procedure was described in § 2.7.3.

### 3.1.6 Sequential extraction

The compositions of the duckweed cell wall including cellulose, hemicellulose and pectin could be evaluated by sequentially extracting the CWM as described by Stevens and Selvendran (1984) and Ryden and Selvendran (1990). The flowchart of Figure 26 clearly illustrates the fractionation process of duckweed CWM. CWM (2 g) was homogenised in cold water at room temperature for 2 h recovering the supernatant containing the water-soluble components. All extractants were prepared using degassed ultrapure water. Then, the residual pellet was extracted by CDTA (0.05 mol/L, pH 6.5) for 6 h following by CDTA (0.05 mol/L, pH 6.5) extraction for 2 h recovering chelator-soluble polysaccharides at room temperature. Next, the pellet was extracted with 0.05 mol/L Na<sub>2</sub>CO<sub>3</sub> including 0.02 mol/L NaBH<sub>4</sub> at 4 °C for 16 h to remove pectins with weak ester linkages followed by 0.05 mol/L Na<sub>2</sub>CO<sub>3</sub> including 0.02 mol/L NaBH<sub>4</sub> at room temperature for 2 h to remove strong ester linkage pectin. Finally, the pellet was extracted by increasing concentrations (0.32, 0.82 and 3.4 mol/L) of KOH including 0.02 mol/L NaBH<sub>4</sub> to remove hemicellulose. All the supernatant was filtered using GF/C filter paper and neutralised to pH 5. Salts in the aqueous and solid pellets were removed by using Visking dialysis tubing (Scientific Instrument Centre Ltd, UK) in 5 L flask of distilled water. The dialysis was carried out for 10 days and the water was changed twice daily. Chloroform (several drops, Fisher Scientific Ltd, UK) was added to prevent the growth of microorganisms.

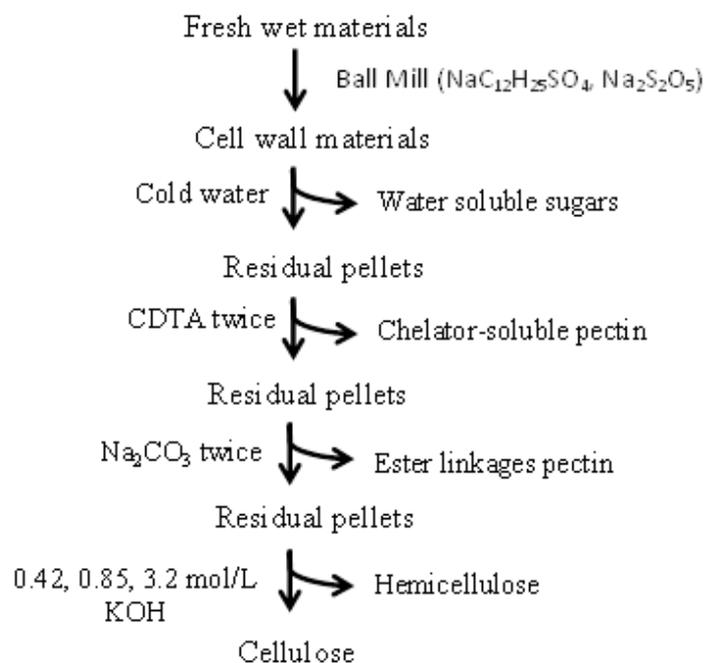


Figure 26. The process of the preparation and sequential extraction of cell wall materials.

### 3.1.7 Gas Chromatography (GC) analysis of alditol acetates

Monosaccharide of materials was analysed by GC sugar analysis method described in § 2.7.

### 3.1.8 Fourier transform infrared spectroscopy (FT-IR) analysis

FTIR-ATR method was used for identifying carbohydrate in cell wall in association with the GC sugar method. The principle is the spectrometer measures the intensity over a narrow range of wavelengths as FT-IR spectrometer simultaneously accepts a big range of spectral data. FTIR-ATR spectra were measured with a BioRad FTS175 Fourier (Bio-Rad Laboratories Inc, USA) transform infrared spectrometer equipped with a MCT detector and a GoldenGate (Specac) single reflection diamond ATR accessory. Five aliquots from each sequentially extracted sample were loaded on the ATR crystal and pressed down with the clamp. For each, 64 scans at a resolution of  $4\text{ cm}^{-1}$  in the region  $4000\text{-}800\text{ cm}^{-1}$  were averaged and referenced against a spectrum of the empty crystal.

### 3.1.9 Microscopy

Ball milled biomass was observed by microscope (BX60, Olympus, Japan) to highlight the extent of removal of starch during the ball mill process. The represented aliquots of starved fresh wet duckweed were immersed in CDTA solution (50 mmol/L  $\text{Na}_3\text{H CDTA}$  and 5 mmol/L  $\text{Na}_2\text{S}_2\text{O}_5$ , pH 7) for 2 days to split the plant (Selvendran and O'Neill, 1987), then were observed to highlight the variation of lipid fraction. The sample was observed using auto fluorescence with filter block U-MNB of the Olympus BX60 (exuter filter BP470-490, barrier filter BA 575)

### 3.2 Results and discussions

#### 3.2.1 Harvest of lab cultured duckweed

The numbers of plants and fronds of lab cultured *L. minor* in each 250 mL flask were recorded to determine growth trends and the experiments were stopped once the plants became crowded. A rapid growth rate was demonstrated and plants increased seven-fold in the beginning two weeks (Figure 27). Fresh biomass was harvested after 6 - 8 weeks of cultivation before the plants had begun to overlay each other significantly. The roots of the healthy, green plants were prolific. Under these conditions, each flask yielded between 3.5 - 4.3 g (fresh weight) of duckweed.

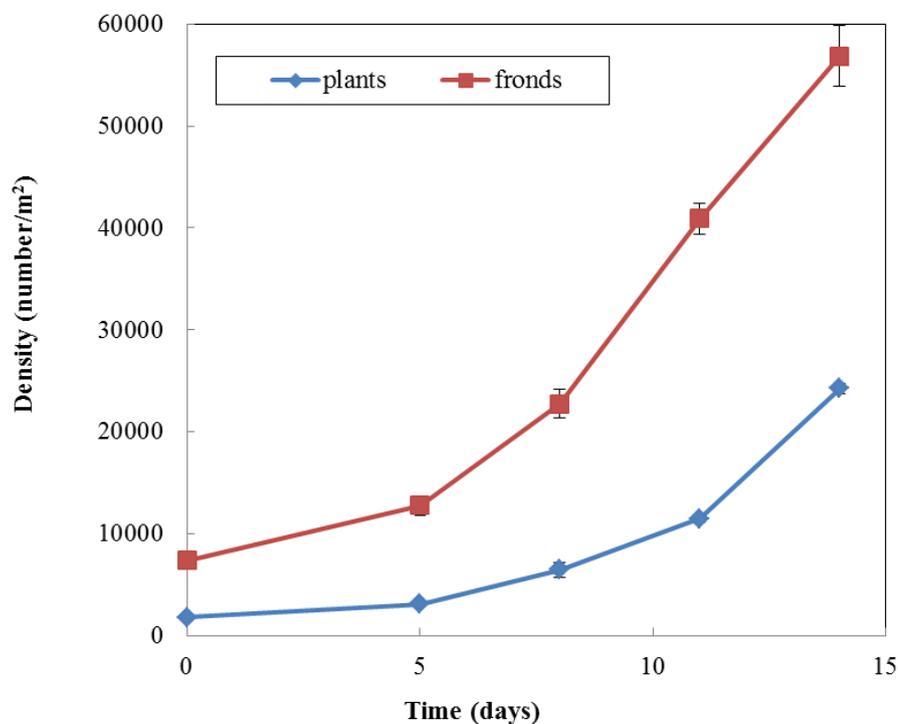


Figure 27. The growth trends of *L. minor* growing in Hoagland E-Medium for 14 days illustrated by number density of plants and fronds.

#### 3.2.2 Evaluation of the overall duckweed compounds

The chemical composition of *L. minor* evaluated by using various methods and results are tabulated in Table 9. The dry matter content of the duckweed used in this study was determined to be 8.5 % (w/w DM) - this falls within the range of 3 - 14 % reported by Landolt and Kandeler (1987). Of this dry matter, carbohydrate as the predominant compounds accounts up 51.2 % (w/w DM) while starch contents contribute to 20.0 % (w/w

DM). Ash accounts for 12.2 % – again Landolt and Kandeler (1987) reported that ash equivalents could constitute from 12- 27.6 % of dry matter. Zaher et al, (1995) give a value for ash content of 12 % of dry matter for *L. minor* cultured in a pond condition in Bangladesh. The ash value is attributed to large amounts of calcium oxalate (shown in Figure 29a) stored in a crystal form in duckweed plants (Landolt and Kandeler, 1987). However, only 12.0 % of protein is present in pond sample which is relatively lower than other published protein data, such as 14 % (w/w of dry matter) of Zaher et al (1995) and 31 % (w/w of dry matter) of Shireman et al (1977). A small amount of lipid contents (3.1 % w/w DM) were also measured in this material. The low lipid content was also stated by Landolt and Kandeler (1987) that varied from 1.8 - 9.2 %. A very low level of Klason lignin (2.4 % w/w DM) is present in *L. minor* while a trace of phenolic acids (0.03 % w/w CWM) were measured confirming that *L. minor* is a poorly-lignified plant.

Table 9. The composition of *L. minor*.

Compositions	% (w/w of dry matter)
Dry matter	8.5 ± 0.7 (of wet matter)
Carbohydrate <sup>a</sup>	51.2 ± 3.7
Starch <sup>a</sup>	20.0 ± 0.7
Protein <sup>a</sup>	12.0
Lipid <sup>a</sup>	3.1
Lignin (Klason) <sup>a</sup>	2.4 ± 0.6
Phenolic acid <sup>b</sup>	0.03 ± 0.003
Ash <sup>a</sup>	12.2 ± 0.1

<sup>a</sup> data was analysed based on FDM pond samples, <sup>b</sup> data was analysed based on CWM.

### 3.2.3 Analysis of duckweed cell wall polysaccharides

Lab cultured plants are considered as the ideal sample possessing the most reasonable compositional constitution due to its optimal medium while pond samples represent for practical samples in light of the native conditions and nutrition. Thus, they were analysed to understand the crucial carbohydrate compositions. AIR was the main substrate of enzymatic saccharification, sugars analysis was thereby carried out on AIR materials. Results of Table 10 illustrate that proportions of monosaccharide in lab cultured plants and pond samples, and differences of monosaccharide impacted by growth conditions. In the

measured seven monosaccharides and uronic acid (GalA), glucose is the predominant component accounting for 254.4 - 331.3 mg/g of AIR, following by GalA (96.2 - 104.1 mg/g of AIR), xylose (46.4 - 52.0 mg/g of AIR) and galactose (16.1 - 18.2 mg/g of AIR). Less abundant monosaccharides include rhamnose, fucose, arabinose and mannose. In comparison of lab cultured plants and pond samples, glucose relatively significantly increased in pond samples. In contrast, the proportions of the other monosaccharides decreased in pond samples. Starch was also assessed by using 1 mol/L H<sub>2</sub>SO<sub>4</sub> and the results indicate that the incremental glucose comes from the conspicuous increase of starch content (199.6 g/kg) in pond sample that is nearly 3 fold of glucose amount in lab cultured sample (72.0 g/kg). It proves that poorer growth conditions tend to stimulate duckweed plant to accumulate more starch (Xu et al, 2011). Pectic polysaccharides are rich in the monosaccharides of GalA and galactose while hemicellulose is rich in xylose, mannose and glucose. The abundant GalA and galactose and less abundant xylose and mannose highlight the profile of duckweed cell wall with a very thin secondary wall due to hemicellulose is one of main composition of secondary wall while pectin majorly contributes to primary cell wall and middle lamella (Brett and Waldron, 1996). The sugars concentration indicate that duckweed possesses 38.9 % (w/w of DM) fermentable sugars including 33.1 % (w/w of DM) glucose which theoretically suggest duckweed is likely to be one great biofuel resource. Comparing with other energy crops, such as wheat straw, rice straw and switchgrass in which fermentable sugars are 56.4 %, 46.8 % and 53.3 % (w/w of DM) respectively, including 35.5 %, 38.6 % and 33.0 % (w/w of DM) of glucose (Linde et al, 2008; Keshwani et al, 2009; Binod et al, 2010), duckweed is rich in hexose (glucose) but is low in pentose (xylose) due to its fairly thin secondary wall as discussed previously.

The individual monosaccharide compositions of the fronds and roots (pond sample) were also assessed. This work has been completed by Mr Wu and Miss Tan who were supervised by the author. The proportions of monosaccharide in frond and root (in Table 10) demonstrate the same trend as whole plant reported above. The glucose results obtained by 1 mol/L H<sub>2</sub>SO<sub>4</sub> hydrolysis (see Table 10) released approximate 50 % of glucose (153.3 mg/g of AIR) that comes from starch in the fronds while only a trace of starch (22.4 mg/g of AIR) is present in the roots.

Table 10. The monosaccharide assessment of *L.minor* carbohydrate in different materials and fractions (mg/g). Rha = rhamnose, Fuc = fucose, Ara = arabinose, Xyl = xylose, Man = mannose, Gal = galactose, Glc = glucose, GalA = galacturonic acid and Totals = the total carbohydrate. 72 % of H<sub>2</sub>SO<sub>4</sub> is equivalent to 16.6 mol/L H<sub>2</sub>SO<sub>4</sub>.

Sample	Hydrolysis (H <sub>2</sub> SO <sub>4</sub> )	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Totals
Lab Whole plants	72% 1 mol/L	5.9±0.4 3.0±0.9	4.8±0.8 2.4±0.5	15.8±0.8 8.8±2.9	52.0±4.0 30.3±9.5	4.8±0.3 1.5±0.6	18.2±1.0 10.2±3.4	254.4±18.5 72.0±28.8	104.1±5.9 26.3±7.2	459.0±21.0 154.6±49.4
Whole plant	72%	4.6±0.7	2.3±0.8	8.0±0.8	46.4±0.2	6.1±1.6	16.1±1.1	331.3±13.7	96.2±1.7	512.0±8.6
Pond Frond plants	72% 1 mol/L	4.3±0.3 4.9±0.1	2.1±0.2 3.5±0.1	6.5±0.4 11.1±0.7	38.9±2.1 78.3±4.6	3.3±0.1 5.4±0.1	15.4±0.7 19.2±1.5	199.6±7.9 304.7±10.0	44.3±3.5 158.5±6.9	314.4±11.6 585.5±23.6
Root	72% 1 mol/L	4.2±0.3 4.7±0.5	3.2±0.1 1.7±0.1	10.5±0.8 5.9±8.0	62.5±5.2 105.5±1.8	2.2±0.1 8.7±0.3	17.2±1.7 16.3±0.2	153.3±17.2 244.7±28.9	58.8±2.9 168.1±7.5	312.0±22.4 555.7±30.9
		4.6±0.1	2.1±0.6	10.2±1.3	87.3±5.0	3.6±0.1	14.7±0.2	22.4±0.8	49.5±2.4	194.4±2.9

Table 11. The monosaccharide compositions of freeze dry and freeze mill (FDM) material and cell wall materials (CWM) materials (Units: mg/g). 72 % of H<sub>2</sub>SO<sub>4</sub> is equivalent to 16.6 mol/L H<sub>2</sub>SO<sub>4</sub>.

Sample	Hydrolysis	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Totals
FDM	72% H <sub>2</sub> SO <sub>4</sub> 1 mol/L H <sub>2</sub> SO <sub>4</sub>	4.4±0.5 4.3±0.2	2.3±0.4 2.1±0.2	8.0±0.6 6.5±1.0	46.4±4.3 38.8±1.8	7.1±0.9 3.3±0.3	16.3±1.5 15.42±0.5	331.3±33.9 199.6±6.3	96.1±4.9 44.2 ±3.5	512.0±37.4 314.3±11.1
CWM	72 % H <sub>2</sub> SO <sub>4</sub> 1 mol/L H <sub>2</sub> SO <sub>4</sub>	7.0±0.4 6.1±0.3	2.2±0.3 2.1±0.2	6.9±0.3 6.8±0.2	83.8±0.9 75.1±3.6	8.5±0.5 3.1±0.2	14.2±0.3 13.2±0.5	308.0±5.3 31.6±0.8	202.2±22.6 86.5±10.6	632.7±27.8 224.5±6.1

### 3.2.4 Assessment of starch content

Starch as one of the important carbohydrate constituents has been assessed by using 1 mol/L H<sub>2</sub>SO<sub>4</sub> hydrolysis. Although these results include a trace of cellulosic glucose, the data clearly reflects the changes of starch content between different materials and plant fractions (see Figure 28). The amount of starch (dry untreated materials) in lab cultured plants (2.4 % w/w DM) and pond plants (19.9 % w/w DM) imply that starch accumulation in duckweed varies according to the nutrient condition but is in the range of 3 - 75 % (w/w DM) stated by many studies (e.g. Landolt & Kandeler, 1987; Cheng and Stomp, 2009; Xu et al, 2011). Thus, starch is one of the important fermentable sugar resources for the ethanol production from duckweed. Moreover, starch is predominantly located in the fronds whilst only a little starch is present in the roots (see Figure 29b & c).

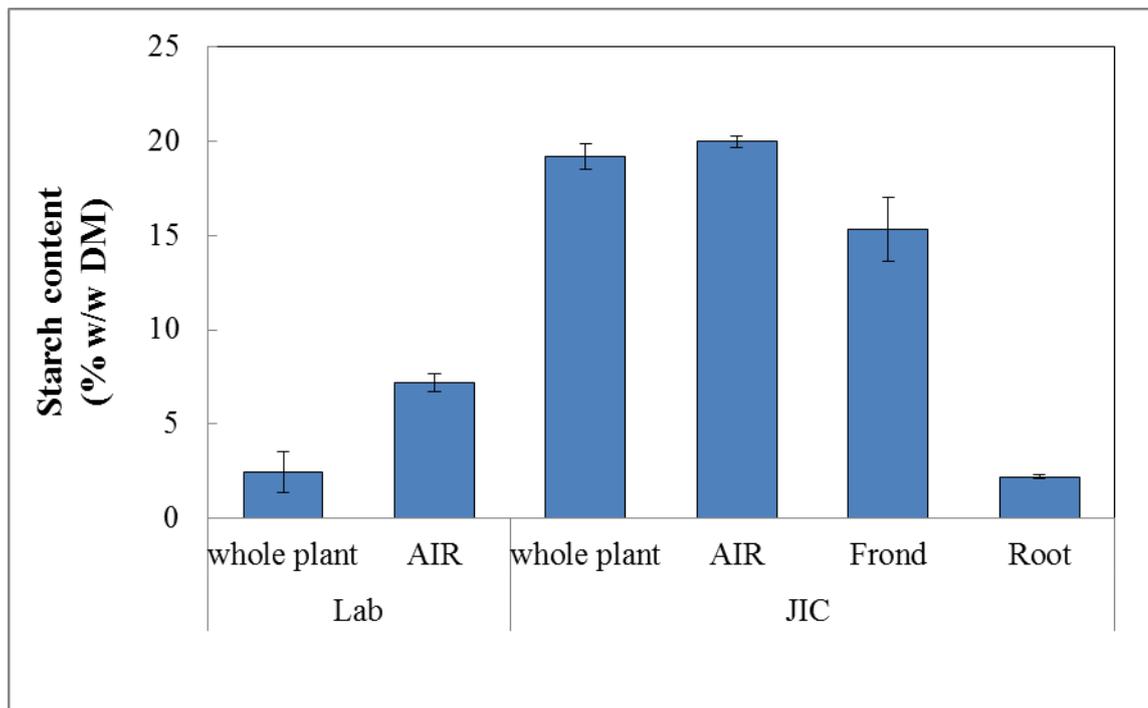
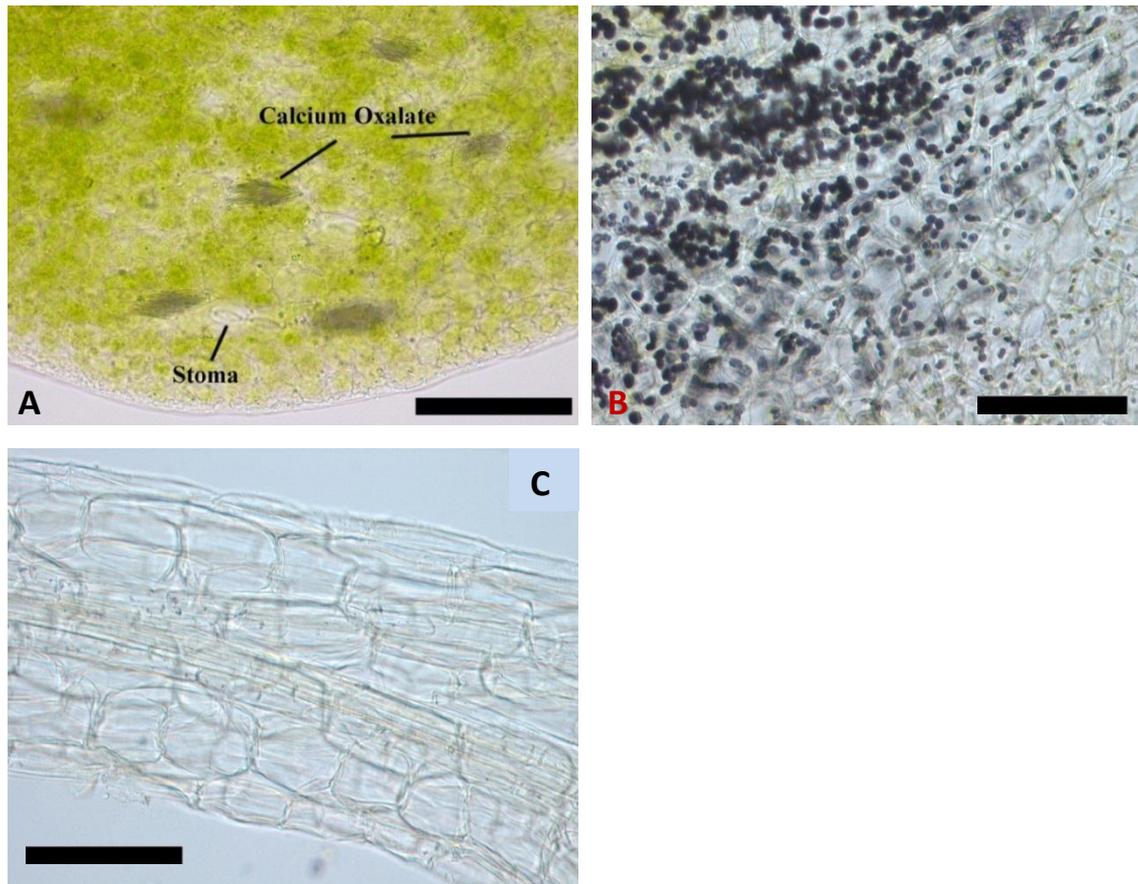


Figure 28. The summary of starch results in different materials. Whole plant represents the dry untreated materials.



a Leaf of healthy plant

b Leaf tissue stained with Lugols solution

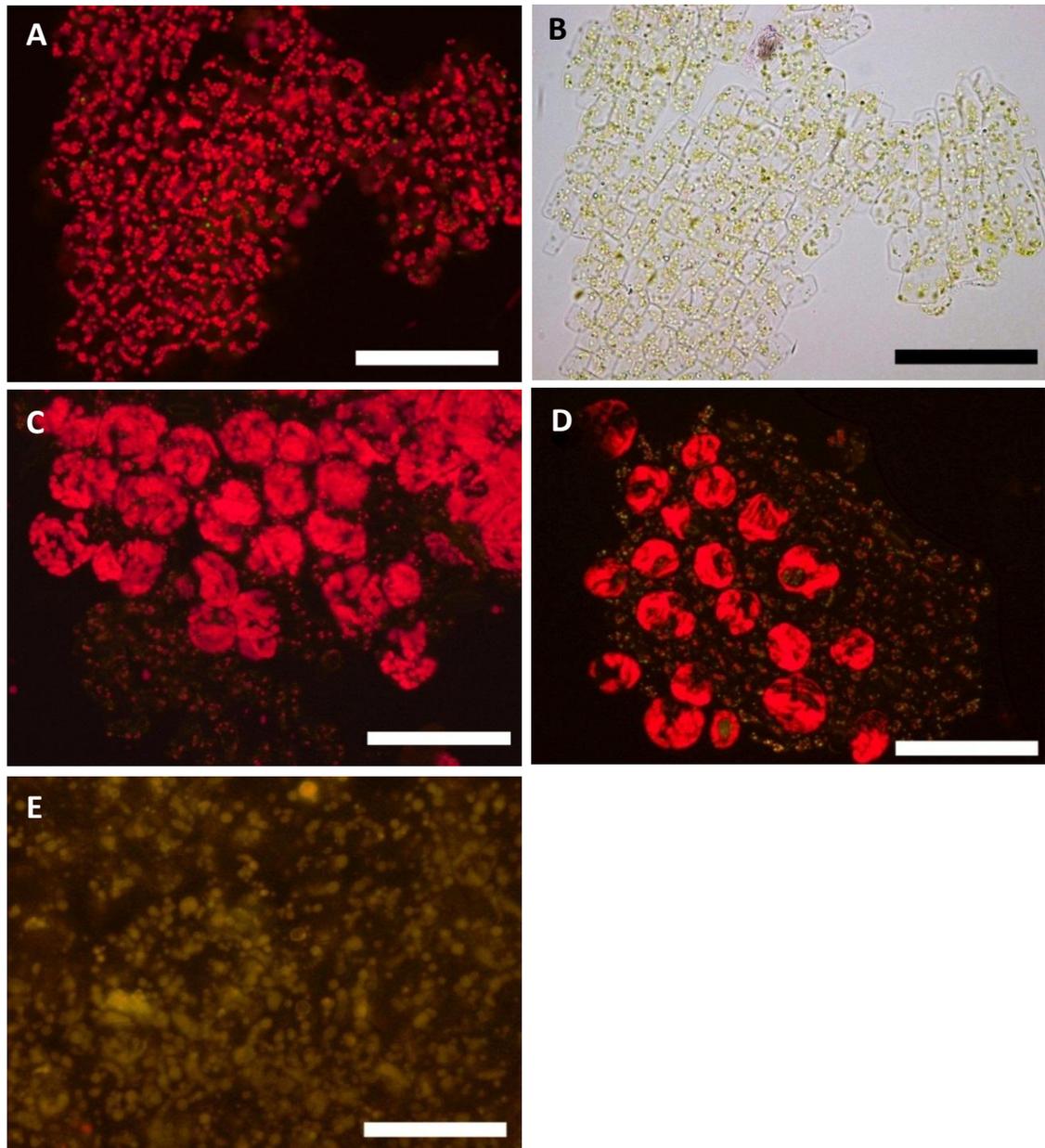
c Root tissue stained with Lugols solution

Figure 29. Images of duckweed plant and tissues stained with Lugols solution and unstained tissues.

### 3.2.5 Evaluation of lipid contents

The total fatty acids (3.1 % w/w DM) were assessed by using Soxhlet extraction and visual evidence was also obtained by using fluorescent microscopy (see Figure 30). Under the blue light, lipid equivalents are present in yellow colour, whereas chlorophyll is present in red colour. In fresh healthy duckweed, lipid contents are more likely stored in epidermal cells than palisade cells (Figure 30a and c). Figure 30b shows that the same palisade tissue of Figure 30a observed under bright field as a reference. After duckweed had undergone 28 days of nutrient-starved conditions, more lipid equivalents were formed and stored in epidermal tissues (Figure 30d). It is notable that abundant lipid equivalents are stored in epidermal tissues of dead plants (Figure 30e). These images of the long term nutrient-starved duckweed imply that more lipids could be released from cytoplasmic membrane or

chloroplasts and starch is consumed when the plant is dying due to lack of nutrition. Duckweed growing in nutrient deficient conditions might be a good method for biorefining lipid products from duckweed but it is not beneficial for converting duckweed sugars to ethanol due to the reduced starch levels.



- a Palisade tissue of healthy plant under blue light
- b Palisade tissue of healthy plant under bright field
- c Epidermal tissue of healthy plant under blue light
- d Epidermal tissue of healthy plant day 28 under blue light
- e Epidermal tissue of dead plant day 28 under blue light

Figure 30. The indication of lipid fraction and the variation of lipid under nutrient-starved conditions (bars = 100  $\mu\text{m}$ ). Blue light means using fluorescence filter block (U-MNB).

FAME components were assessed by GC method and the fatty acid profile is tabulated in Table 12. Fatty acids only account for 0.8 % (w/w) of DM, but contain a high proportion of the essential fatty acids (EFA), e.g. linoleic and  $\alpha$ -linolenic acid. Of the total fatty acid, saturated FA contributes of 27.4 % (equivalent to 6.9 % of total lipid) while unsaturated FA contributes of 72.6 % (equivalent to 18.3 % of total lipid). Due to low proportion of fatty acids, both the proportions of saturated and unsaturated FA are less than most vegetables (Holland, Buss & Unwin, 1991). In the saturated FAs, palmitic acid accounts for 22.4 % (w/w of total FA), whereas the other detectable saturated FAs of myristic, stearic, eicosanoic and lauric acids only account for 1.9, 1.7, 1.0 and 0.5 % (w/w of total FA) respectively. Of the unsaturated FAs,  $\alpha$ -linolenic acid was detected as the primary compound (33.4 %), followed by linoleic/linoelaidic acid (25.2 %). These unsaturated FAs are well known types of EFA and are potentially high-value byproducts of biofuel production. Other unsaturated FAs with nutritional and medicinal value are relatively low, e.g. stearidonic acid (3.6 %), oleic acid (3.0 %),  $\gamma$ -linolenic acid (1.8 %) and eicosatrienoic acid (1.8 %).

Table 12. The assessment of fatty acids in *L.minor*.

Fatty acids	C: D	n-x	% (w/w of FA)	% (w/w of lipid)
<b>Total FA</b>			0.8 ± 0.12 (of DM)	25.2
<b>Saturated FA</b>			<b>27.4</b>	<b>6.9</b>
Lauric acid	12: 0		0.5 ± 0.03	0.1
Myristic acid	14: 0		1.9 ± 0.02	0.5
Palmitic acid	16: 0		22.4 ± 0.13	5.6
Stearic acid	18: 0		1.7 ± 0.1	0.4
Eicosanoic acid	20: 0		1.0 ± 0.13	0.3
<b>Unsaturated FA</b>			<b>72.6</b>	<b>18.3</b>
Palmitoleic acid	16: 1	n-7	3.0 ± 0.03	0.8
Vaccenic acid	18: 1	n-7	1.0 ± 0.12	0.3
Oleic acid ( $\omega$ -9)	18: 1	n-9	3.0 ± 0.12	0.7
Linoleic/Linoelaidic acid ( $\omega$ - 6)	18: 2	n-6	25.2 ± 0.04	6.4
$\alpha$ -linolenic acid ( $\omega$ - 3)	18: 3	n-3	33.4 ± 0.21	8.4
$\gamma$ -Linolenic acid ( $\omega$ - 6)	18: 3	n-6	1.8 ± 0.07	0.4
Stearidonic acid ( $\omega$ - 3)	18: 4	n-3	3.6 ± 0.09	0.9
Eicosadienoic acid ( $\omega$ - 6)	20: 2	n-6	0.5 ± 0.04	0.1
Eicosatrienoic acid ( $\omega$ - 3)	20: 3	n-3	1.0 ± 0.12	0.3

C: D represents the ratio of carbon and double bonds while n-x represents the position of the first double counted from methyl end.

### 3.2.6 Compositional analysis of phenolics

Phenolic acids are the main low molecular weight compounds of lignin and also play a crucial role in the linkages between hemicellulosic polysaccharides (Brett and Waldron, 1996). Thus, phenolic acids are worthy of examination even if only low levels of lignin (2.4 % w/w DM) are present in *L. minor*. Total phenolics account for 0.25 mg/g DM which mainly involve 5 types of identified phenolic acids (see Figure 31). The proportions of lignin and phenolic acids are much lower than well-known energy crops, such as lignin in switchgrass and wheat straw is 15-29 % and 15-20 %, respectively (Harris and DeBolt, 2010; Theander and Aman, 1982) and phenolics in wheat straw is less than 1 % (Waldron, 2010). *p*-coumaric acid is the most predominant composition (0.15 mg/g), which accounts for 60 % of phenolics, following by truxillic acid (0.04 mg/g), protocatechuic aldehyde (0.02 mg/g) and ferulic acid (0.02 mg/g). A trace of *p*-OH-benzaldehyde (0.01 mg/g) was also detected. Coumaric acid and ferulic acid are common phenolic components– indeed, they are the most abundant phenolics present in wheat straw (Merali et al, 2013).

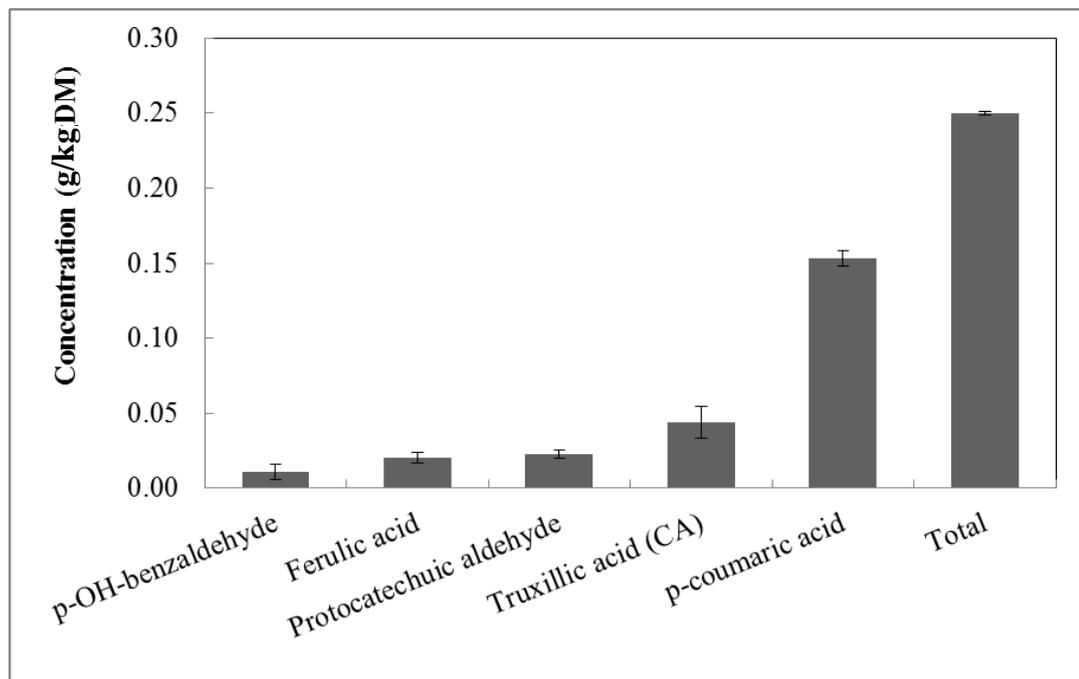
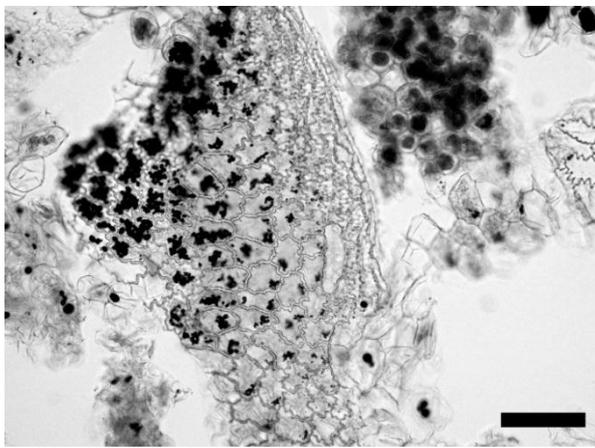


Figure 31. Phenolic acids of *L. minor* in ball mill prepared CWM.

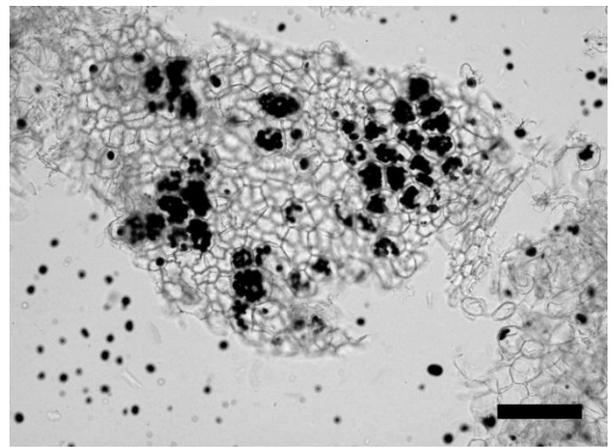
### 3.2.7 Analysis of sequential-extracted polysaccharides

The materials used for the sequential extraction were prepared with the ball mill method to remove non-cell wall carbohydrate, such as starch. The extent of starch removal was evaluated by microscope (shown as Figure 32). Starch granules are conspicuously present

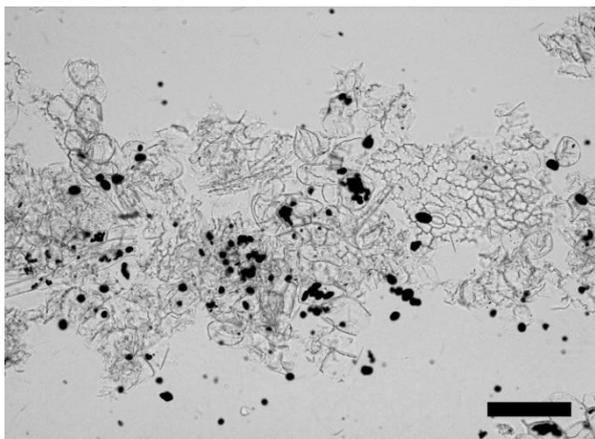
in duckweed cells (Figure 32a). In the ball milling process, starch granules had gradually been released from disrupted tissues (Figure 32b - d) and dissolved into liquor phase during the distilled water wash (Figure 32e). Thus, the majority of the starch contents were washed off after 3.5 h of ball milling and double distilled water washing. Monosaccharide compositions of cell wall materials (CWM) (Table 11) also implied the extent of starch removal. Concentrations of all the monosaccharide and the overall carbohydrate increase in CWM against the concentrations of FWM, besides of a drop of glucose concentration. In addition, only 31.2 mg/g DM starch was detected in CWM. In summary, any glucose detected in the fractions from the sequential extraction is therefore likely to be of cell wall origin.



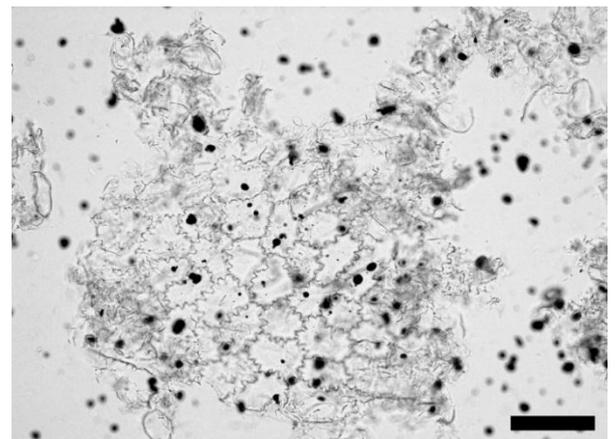
a Blender milled material



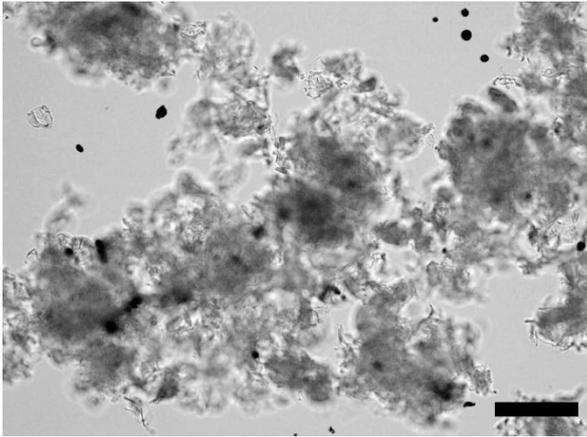
b 1 h ball milling



c 2 h ball milling



d 3.5 h ball milling



e Distilled water washing residues

Figure 32. The extent of removal of starch during the ball-milling process observed by microscopy (Bars = 100  $\mu\text{m}$ ).

Starch-free CWM (12.8 % w/w of fresh wet material) was yielded through the ball mill process and 2 g of this material was used for sequential extraction. In the fractionation process, 18.8 % (w/w) of dry mass was extracted by CDTA solution and only 1.7 % (w/w) was extracted by  $\text{Na}_2\text{CO}_3$  solution (see Table 13). It is notable that KOH only extracted a small amount (3.5 % w/w) of dry mass. Over 62 % (w/w) of dry mass remained in the residual pellets. As Brett and Waldron (1996) described, CDTA and  $\text{Na}_2\text{CO}_3$  extraction remove pectic polysaccharides and KOH extraction removes hemicellulose, the residual matter is cellulose. In this fractionation, over 20.3 % (w/w) of pectic polysaccharides and only 3.5 % (w/w) of hemicellulose were removed. In the combination of GC sugar results and weights of recovered fractions, cellulose is the most component of CWM and accounts for 43.7 % of cell wall polysaccharide (deducting by starch). Approximately 20 % of pectin contributes to duckweed cell wall, which is lower than the 30.1 % of pectin in *L. minor* CWM reported by Kindel, Cheng, & Ade (1996). The major pectic polysaccharides were extracted by CDTA and 1st  $\text{Na}_2\text{CO}_3$  extraction, which suggests pectic polysaccharides are predominantly bond with weak ester linkage (Brett & Waldron, 1996). Only small amount of hemicellulose (approximately 3.5 %) exits *L. minor* CWM. We found no literature reporting hemicellulose in duckweed cell wall.

Monosaccharide data in Figure 33 further visually reflects the proportions of cell wall sugars extracted into different solutions. Glucose mainly remained in the residual pellets and a small amount of glucose was extracted by KOH. For cell wall material, glucose predominantly constitutes to cellulose and a small portion might be the component of

glucuronoxyylan, glucomannan and glucan. Another possibility that starch were intensively extracted by KOH should be considered. GalA as the predominant component of pectic polysaccharide was largely extracted by CDTA & Na<sub>2</sub>CO<sub>3</sub>. Fucose, arabinose and galatose were uniformly extracted by CDTA & Na<sub>2</sub>CO<sub>3</sub> and KOH. Thus, fucose, arabinose and galatose are the main components of pectin and hemicellulose. Rhamnose, xylose and mannose were only intensively extracted by KOH. However, the proportion of each monosaccharide does not aggregate to 100 % (w/w) because the materials were lost in the fractionation process.

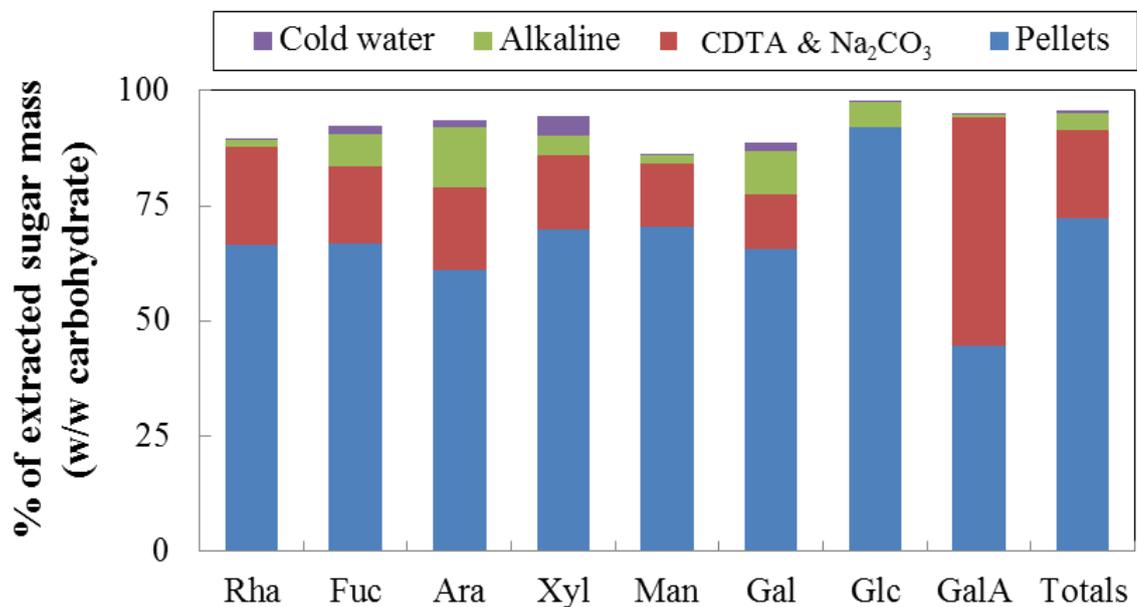


Figure 33. The proportions of cell wall polysaccharides in the fractionation reflected by the proportions of their monosaccharides compositions. Alkaline represents KOH solution only.

Table 13. The concentration of polysaccharides and dry mass recovery of fractions in sequential extraction.

Fraction	Rha (mg/g)	Fuc (mg/g)	Ara (mg/g)	Xyl (mg/g)	Man (mg/g)	Gal (mg/g)	Glc (mg/g)	Gala (mg/g)	Total sugars (mg/g)	Recovered mass (mg)	% of extracted mass
CWM 72%	7.0±0.4	2.2±0.3	6.9±0.3	83.8±0.9	8.5±0.5	14.2±0.3	308.0±5.3	202.2±22.6	632.7±27.8	2000	100
CWM 1 mol/L	6.1±0.3	2.1±0.2	6.8±0.2	75.1±3.6	3.1±0.2	13.2±0.5	31.6±0.8	86.5±10.6	224.5±6.1	/	/
Cold water	2.7±0.3	3.4±0.4	7.8±1.2	315.9±26.9	1.6±0.3	22.3±2.6	13.6±1.6	44.5±8.4	411.7±26.1	23.9 ±13	1.2 ± 0.7
CDTA 1st	7.9±0.3	1.7±0.1	5.5±0.2	77.5±10.5	6.0±0.9	7.8±0.8	1.1±0.1	562.1±28.8	669.5±32.9	337.6 ±38	16.9 ± 1.9
CDTA 2 nd	6.7±1.3	2.0±0.4	6.1±2.2	63.8±16.9	3.5±0.6	7.2±3.1	2.9±1.5	282.0±60.4	374.3±81.7	37.4 ±18	1.9 ±0.9
Na <sub>2</sub> CO <sub>3</sub> 1st	7.6±0.2	4.2±0.3	15.1±0.3	27.7±0.1	9.2±1.5	20.0±0.1	5.5±0.4	516.2±1.06	605.7±1.24	27.2 ±8	1.4 ± 0.4
Na <sub>2</sub> CO <sub>3</sub> 2 nd	7.2±0.4	5.1±0.5	20.1±1.2	14.5±3.2	12.6±0.4	21.3±1.1	21.9±1.3	274.8±3.4	377.5±0.2	6.3 ±1	0.3 ± 0.1
0.42 mol/L KOH	3.3±0.1	1.9±0.3	15.3±1.5	15.0±2.1	3.0±0.2	10.8±2.0	559.2±14.5	32.1±2.9	640.7±9.1	39.7 ±6	2.0 ± 0.3
0.85 mol/L KOH	2.4±0.1	8.6±0.5	35.8±0.9	253.6±12.2	4.4±0.2	84.5±4.2	398.9±20.7	24.4±1.3	812.6±38.2	19.3 ±3	1.0 ± 0.1
3.4 mol/L KOH	2.5±0.3	5.6±0.4	48.1±2.4	115.9±8.9	11.3±0.6	56.4±0.9	213.2±5.9	32.6±2.1	485.5±7.2	10.6 ±7	0.5 ± 0.3
Pellet	7.5±0.4	2.4±0.1	6.8±0.2	94.4±5.3	9.6±0.3	15.0±0.8	457.2±25.3	145.4±7.1	738.2±30.6	1241.0 ±3	62.1 ± 0.2
Aggregation	/	/	/	/	/	/	/	/	/	1742.8 ±4	87.1 ± 0.2

The technique of FT-IR can be used to measure the unique bond positions of polysaccharides in order to identify polysaccharide types combined with GC sugars results (Table 13). Even after the extensive washing to remove starch during the milling, further small amounts of material are solubilised with distilled water. The cold water extracted fraction contained 411.7 mg/g of carbohydrate which is high in xylose (315.9 mg/g) suggesting the presence of xylan polysaccharides, possibly complexed with pectic polymers as found in asparagus (Waldron and Selvendran, 1991). The presence of fucose implies the presence of water soluble xyloglucans (Jacobs et al, 2003). FT-IR (Figure 34 Spectra 3) also shows corresponding spectra of glucuronoxylan described by Kacuráková et al, (2000). The majority of extracted sugars of CDTA and Na<sub>2</sub>CO<sub>3</sub> extraction are galacturonic acid that is the hydrolysed residues of galacturonan that is the back bone of pectin. In the meantime, a trace of xylose, galactose, arabinose and mannose were extracted, which are the main compositions of side chains of xylogalacturonan, rhamnogalacturonan. Ovodova et al, (2000) stated a similar proportion of monosaccharides of pectic polysaccharides of *L minor*. FT-IR Spectra 4-7 (Figure 34) have a characteristic shape which contains many specific bonds peaks corresponding to pectic polysaccharides. Homogalacturonan is present in terms of the strongest specific bonds at 1010 and 1090 cm<sup>-1</sup> (Kacuráková et al, 2000). The specific bonds at 1045 and 1074 cm<sup>-1</sup> indicate that pectic polysaccharides mixtures including rhamnogalacturonan, arabinan and arabinogalactans (Kacuráková et al, 2000). Glucose and xylose were the major monosaccharide constituents of polymers extracted in KOH. A trace of arabinose, galactose and galacturonic acid were present in alkaline fractions. The presence of large amounts of glucose in both the 0.42 and 0.85 mol/L KOH extracts were probably due to the gelatinisation and solubilisation of small amounts of residual starch in KOH. Han and Lim (2003) reported that corn starch was dissolved extensively in 1 mol/L NaOH with vigorous agitation at room temperature. The FT-IR spectra (Figure 34, Spectra 8-9) of these fractions were dominated by the solubilised starch. The overall band pattern is very similar to that of the initial starch (Figure 34, Spectrum 1), but the increased peak at 1022 cm<sup>-1</sup> and the smaller shoulder at 1045 cm<sup>-1</sup> clearly indicate a much more amorphous structure (vanSoest et al, 1995). The 3.4 mol/L KOH extract mainly contained xyloglucan and xylan and might include glucan, as indicated by the FT-IR spectrum (Figure 34, Spectrum 10). The bonds at 1130 - 60 cm<sup>-1</sup> were dominated by the glycosidic linkage (C-O-C) from xylan, xyloglucan and glucan (Kacuráková et al, 2000). Moreover, the small bonds at 930 - 40 cm<sup>-1</sup> are likely to be from glucomannan and galactoglucomannan (Kacuráková et al, 2000).

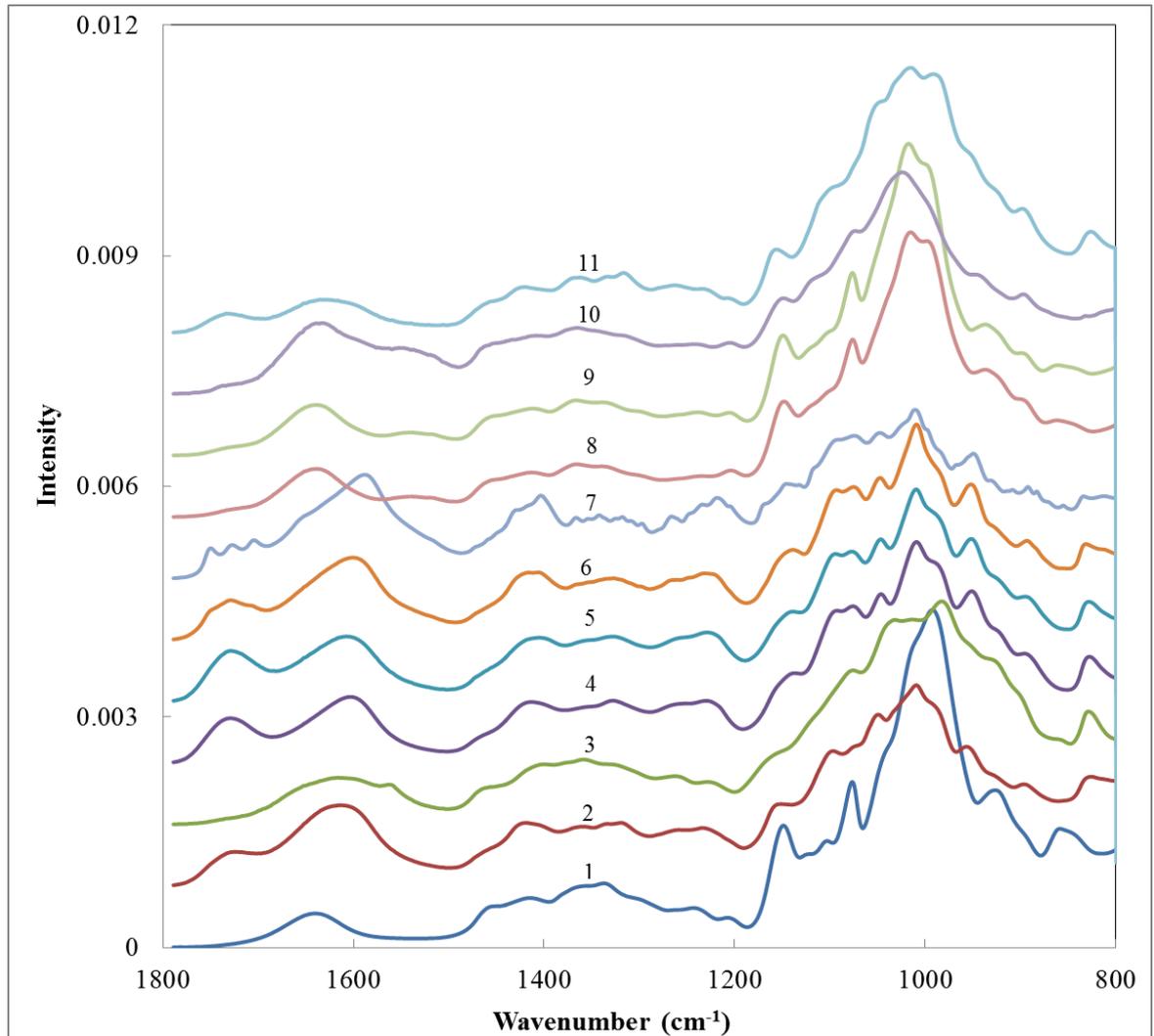


Figure 34. FT-IR spectrum of cell wall polysaccharides of sequential extraction – extracting in cold water (3), 1 st CDTA (4), 2 nd CDTA (5), 1 st  $\text{Na}_2\text{CO}_3$  (6), 2 nd  $\text{Na}_2\text{CO}_3$  (7), 0.42 mol/L KOH (8), 0.85 mol/L KOH (9), 3.4 mol/L KOH (10) and cellulose pellets(11). (1) and (2) represent starch blank and CWM.

### 3.2.8 Assessment of sequential-extracted phenolics

As we introduced before, phenolics play a major role of the linkage in hemicellulosic and pectic polysaccharide, phenolic acids were therefore extracted following polysaccharides in sequential extraction was mainly extracted by CDTA and low concentration alkali extraction while truxilic acid and ferulic acid were only extensively extracted by CDTA solution (Table 14). In the contrast, Protocatechuic aldehyde was only solubilised in  $\text{Na}_2\text{CO}_3$  and low concentration alkali solution. However, p-OH-benzaldehyde was averagely removed from each step. All compounds were conspicuously detected in residual pellets. These data indicate that protocatechuic aldehyde and p-coumaric acid are the

important compounds of linkage in hemicelluloses while truxillic acid, ferulic acid and p-coumaric acid were proven their linkage role of pectic polysaccharides. Merali et al (2013) also detected large amounts of several phenolic acids retained in residual pellet of sequential extracted wheat straw.

Table 14. Phenolic compositions of *L.minor* cell wall ( $\mu\text{g/g}$  CWM) following sequential extraction.

Fractionation	Protocatechuic aldehyde	p-OH-benzaldehyde	Truxillic acid (CA)	Ferulic acid	p-coumaric acid	Total
CWM	22.5 $\pm$ 10.6	10.7 $\pm$ 7.1	43.8 $\pm$ 5.4	20.1 $\pm$ 10.4	153.0 $\pm$ 21.2	250.1 $\pm$ 20.6
Cold water	0	13.7 $\pm$ 0.2	51.3 $\pm$ 0.1	26.4 $\pm$ 0.3	33.4 $\pm$ 0.2	125.2 $\pm$ 0.7
CDTA 1st	0	9.5 $\pm$ 1.9	44.5 $\pm$ 1.5	39.2 $\pm$ 3.5	23.8 $\pm$ 0.9	117.0 $\pm$ 6.7
CDTA 2nd	0	6.6 $\pm$ 0.1	44.7 $\pm$ 0.2	29.6 $\pm$ 0.2	8.6 $\pm$ 0.1	89.5 $\pm$ 0.4
Na <sub>2</sub> CO <sub>3</sub> 1st	10.0 $\pm$ 0.3	23.2 $\pm$ 0.1	44.6 $\pm$ 0.5	113.4 $\pm$ 0.6	79.5 $\pm$ 0.2	284.1 $\pm$ 3.7
Na <sub>2</sub> CO <sub>3</sub> 2nd	45.8 $\pm$ 0.5	32.1 $\pm$ 0.1	36.6 $\pm$ 0.1	34.4 $\pm$ 0.1	123.0 $\pm$ 0.1	271.8 $\pm$ 0.9
0.42 mol/L KOH	12.8 $\pm$ 0.2	21.0 $\pm$ 0.1	27.3 $\pm$ 0.2	55.1 $\pm$ 0.6	334.6 $\pm$ 1	450.8 $\pm$ 3.8
0.85 mol/L KOH	11.1 $\pm$ 0.2	23.1 $\pm$ 0.1	27.6 $\pm$ 0.2	36.7 $\pm$ 0.1	361.2 $\pm$ 0.4	460.0 $\pm$ 0.6
3.4 mol/L KOH	0	17.0 $\pm$ 0.1	47.6 $\pm$ 0.1	86.4 $\pm$ 0.2	97.2 $\pm$ 0.1	248.2 $\pm$ 0.3
Pellets	17.0 $\pm$ 0.5	4.6 $\pm$ 5.0	21.2 $\pm$ 6.1	0	17.0 $\pm$ 6.0	42.8 $\pm$ 12.7

### 3.3 Conclusions

This study provides a detailed examination of the chemical constituents of duckweed (*L. minor*). Duckweed biomass contains a high proportion of fermentable sugars (including glucose, 33.1 % w/w DM) and a low amount of lignin (3 % w/w DM). The results of the fractionation shows that 20 % pectin is extracted by CDTA and Na<sub>2</sub>CO<sub>3</sub> solutions consisting of galacturonan with small amounts of xylogalacturonan, rhamnogalacturonan, only 3.5 % hemicellulose is extracted by KOH solution predominantly consisting of xyloglucan and xylan, and the insoluble residue is rich in cellulose. EFA ( $\alpha$ -linolenic and linoleic/linoelaidic acid) and *p*-coumaric acid are the most abundant fatty acids and phenolics of *L.minor* respectively. The profiles of cell wall structure will play an important role in the enzymatic saccharification and fermentation of duckweed biomass to ethanol, such as, the selection of enzymes (cellulase) and yeast (*S. cerevisiae*).

## ***4 The preliminary investigation -- enzymatic saccharification on duckweed without thermophysical pretreatment***

The previous chapter characterised the compositions of duckweed as a potential bioethanol feedstock and its high level of fermentable sugars was also identified. However, the conversion of cellulose/starch to ethanol requires significant improvement for bioethanol production processing. Seeking the efficient enzyme preparations for duckweed biomass and optimizing the enzymatic saccharification conditions are the fundamental and effective approaches to maximize the yields of glucose and other cell-wall-derived sugars. This chapter will mainly identify the enzyme preparations by selecting the most efficient enzymes from a range of commercial cellulase and other relevant cell-wall degrading enzymes. The enzymatic digestibility has been studied on alcohol-extracted, water-insoluble preparations of lab cultured *L.minor* materials. The optimization of the enzyme preparations enables the cost of saccharification to fall into an acceptable range. Eventually, saccharification can be achieved within about 8 h using commercial cellulase at 100 U or 4.35 FPU/g substrate in conjunction with additional beta-glucosidase at 100 U/g substrate.

### **4.1 Materials and methods**

#### *4.1.1 Plant resource*

The materials used in this section were cultured as described in § 2.1.1.

#### *4.1.2 Alcohol insoluble residue (AIR) extraction*

Fresh duckweed biomass was ground by mortar & pestle and extracted to AIR based on AIR preparation which was described in § 2.3.

#### *4.1.3 Enzymatic hydrolysis*

Enzymatic saccharification of the duckweed WIAIR employed three commercial enzyme preparations: Celluclast<sup>®</sup> (CE; cellulase, Sigma Chemical Co., St. Louis, MO), Novozyme<sup>®</sup> 188 (BG;  $\beta$ -glucosidase, Sigma Chemical Co., St. Louis, MO), and Depol<sup>™</sup> 740 (DE; cell wall degrading enzyme cocktail, Biocatalysts Limited, Wales, UK). Depol<sup>™</sup> 740 contains mainly ferulic acid esterase along with cellulase and significant xylanase activities. The enzyme activities are defined by the manufacturer for CE and BG as 700

U/mL (Sigma-Aldrich, 2011) and 250 U/mL (Sigma-Aldrich, 2011) respectively, and by Hendrickson et al (2007) for DE as 170 U/mL. The FPU activity of cellulase (Celluclast<sup>®</sup> and Depol<sup>™</sup> 740) was also assessed following the standard measurement of cellulase (Ghose, 1987). The enzymes were separately loaded in the designated cocktails, i.e. Depol + Celluclast (DE+CE), Depol + BG (DE+BG), Celluclast + BG (CE+BG) and Depol + Celluclast + BG (DE+CE+BG), in triplicates of each cocktail. Prior to enzyme addition, the CE and DE were desalted using a PD-10 Column (GE Healthcare Life Sciences, Little Chalfont, Bucks., UK) (Rosengren et al, 1996) and BG was centrifuged at 12000 x g to remove insoluble particulates. Digestions were carried out in triplicate and contained 10 mg of WIAIR and enzyme in 0.1 mol/L sodium acetate (pH 5.0) containing thimerosal (Sigma Chemical Co., St. Louis, MO; 0.1 g/L) in a total volume of 2 mL. The enzymatic hydrolysis process was described earlier § 2.5.

#### 4.1.4 Analytical methods

##### 4.1.4.1 DNS & GOPOD test

The reducing sugars released by enzymolysis were measured by using DNS method described in § 2.9.1 while the liberated glucose was detected by specific GOPOD method described in § 2.9.2.

##### 4.1.4.2 Gas Chromatography (GC) analysis of alditol acetates

Monosaccharide of AIR, WIAIR and dry recalcitrant pellets remaining after enzymatic saccharification was analysed by GC sugar method described in § 2.7.

##### 4.1.4.3 Microscopy

AIR, WIAIR and dry recalcitrant pellets remaining after enzymatic saccharification were observed by microscopy (BX60, Olympus, Japan). More details were described in § 2.10.

## 4.2 Results and discussion

### 4.2.1 WIAIR preparation and yields

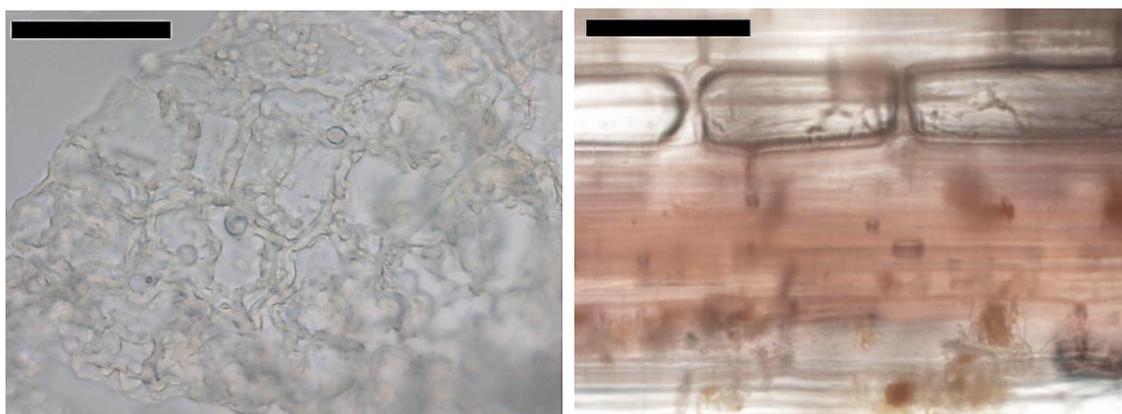
Batches of lab cultured duckweed were harvested and prepared as WIAIR. AIR yields were between 3.1 to 3.4 % (w/w) of fresh weight and after extraction of soluble components, the final WIAIR accounted for approximately 2.3 - 2.4 % of the initial fresh weight. Microscopy revealed small quantities of starch stained by KI/I<sub>2</sub> solution present within the cells.

### 4.2.2 Chemical composition of WIAIR

The component cell wall sugars (rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, galacturonic acid) and any starch-derived glucose of hydrolysates (H<sub>2</sub>SO<sub>4</sub>; 72% and 1 mol/L) are shown in Table 15. Carbohydrate accounted for 45.9 % of duckweed WIAIR. Of this, glucose accounted for 25.4 % and would have arisen from cellulose and non-cellulosic glucan including starch. GalA accounted for 10.4 % and would have arisen predominantly from pectic polysaccharides whilst xylose accounted for 5.2 % and would have been derived from arabinoxylan and xyloglucan (Albersheim et al, 1996; Brett and Waldron, 1996; Weightman et al, 1994). Furthermore, a theoretical yield of ethanol from the glucose component of duckweed WIAIR would equate to approximately 164.2 L/metric ton (and therefore about 4 litres/metric ton fresh weight). Conversion of all sugars would increase the theoretical yield further by approximately 30 %. Hydrolysis in 1 mol/L H<sub>2</sub>SO<sub>4</sub> (Table 15) resulted in release of one third of the carbohydrate (17.1 %). Of this, 8 % of Glc was obtained providing an estimation of non-cellulose glucans including starch. Also, one third of GalA (2.9 %) and half of the xylose (Xyl; 3.4 %) were produced by 1 mol/L H<sub>2</sub>SO<sub>4</sub>. The predominance of glucose implies that *S. cerevisiae* will be appropriate for subsequent fermentation and it will be inappropriate to specifically seek to ferment the relatively low levels of xylose. The remaining uncharacterised material will be due to intracellular protein and cuticular material present on the upper leaf surfaces (Albersheim et al, 1996; Wersal and Madsen, 2009). The low levels/absence of lignin was confirmed by lack of colour upon staining with phloroglucinol-HCl (Figure 35a), in contrast with the pinkish-red colour observed upon staining wheat straw (Figure 35b).

Table 15. The quantities of the monosaccharide in the substrate (WIAIR) and the undigested residual pellets (Units: mg/g).

Enzyme preparation	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Total	% pellets recovered
WIAIR (72 % H <sub>2</sub> SO <sub>4</sub> )	5.9±0.4	4.8±0.8	15.8±0.8	52.0±4.0	4.8±0.3	18.2±1.0	254.4±18.5	104.1±5.9	459.0±21.0	100
WIAIR (1 mol/L H <sub>2</sub> SO <sub>4</sub> )	3.0±0.9	2.4±0.5	8.8±2.9	30.3±9.5	1.5±0.6	10.2±3.4	72.0±28.8	26.3±7.2	154.6±49.4	100
Buffer+AIR	3.8±0.1	3.1±0.1	10.3±0.1	44.8±0.1	3.3±0.1	11.4±0.1	188.5±0.4	91.3±0.4	356.4±0.2	89
DE+CE	4.8±0.1	5.8±0.1	6.0±0.1	29.4±0.1	13.2±0.1	7.8±0.1	108.9±0.3	75.3±0.2	251.0±0.1	68
DE+BG	2.2±0.1	4.6±0.1	4.2±0.1	31.3±0.1	23.0±0.1	6.9±0.1	61.7±0.2	30.3±0.2	164.0±0.5	69
CE+BG	1.9±0.1	3.5±0.1	4.3±0.1	27.6±0.1	30.8±0.1	6.4±0.1	35.6±0.1	26.6±0.2	136.7±0.1	61
DE+CE+BG	1.6±0.1	3.3±0.1	3.2±0.1	26.2±0.1	22.2±0.2	5.7±0.1	28.3±0.1	25.9±0.1	116.4±0.2	52



a duckweed sample

b wheat straw (control blank)

Figure 35. The presence of lignin in duckweed WIAIR stained with phloroglucinol-HCl.

#### 4.2.3 Enzymolysis of duckweed cell walls with excess enzyme

In a preliminary evaluation, a 24 h time course of enzymolysis was performed using excess Depol 740L (xylanase 1700 U/g WIAIR; 50 °C; pH 5.0). The extent of digestion was evaluated by recovering the insoluble residue by centrifugation, drying and quantifying gravimetrically. It was found that digestion had tailed off by 8 h, so this time was chosen for initial studies. After 8 h, 36 % of the initial weight of material had been digested. The ability of commercial enzymes to degrade duckweed cell walls was investigated using cocktails of (initially excess) Celluclast (cellulase 4000 U/g WIAIR equivalent to 174 FPU/g WIAIR), Depol 740 (xylanase 1700 U/g WIAIR and cellulase 6 FPU/g WIAIR) and Novozyme 188 (BG; 4000 U/g WIAIR). Four enzyme cocktails were evaluated consisting of: DE+CE, DE+BG, CE+BG and DE+CE+BG and were incubated with WIAIR for 30 min and 8 h periods. The yields of total reducing sugars and released glucose (% of theoretical maxima sugars or glucose respectively) are shown in Figure 36 & 37. The highest yield of released sugars was achieved in 8 h by CE+BG (85.2 %) followed by DE+CE+BG (74.4 %), then DE+BG (73.4 %) (Figure 36). The yields of released glucose showed a similar trend (Figure 37) in that the CE+BG cocktail released the largest quantities of glucose (90.7 %) following by DE+CE+BG (78.1 %) and DE+BG (70.7 %). The DE+CE cocktail is much less effective, releasing sugar and glucose at 33.1 % and 57.7 % respectively. The blank incubations released only minor quantities of sugars (5.4 %) and glucose (3.2 %). The results demonstrate that in excess enzyme, the bulk of duckweed WIAIR sugars can be hydrolyzed and released over an 8 h period.

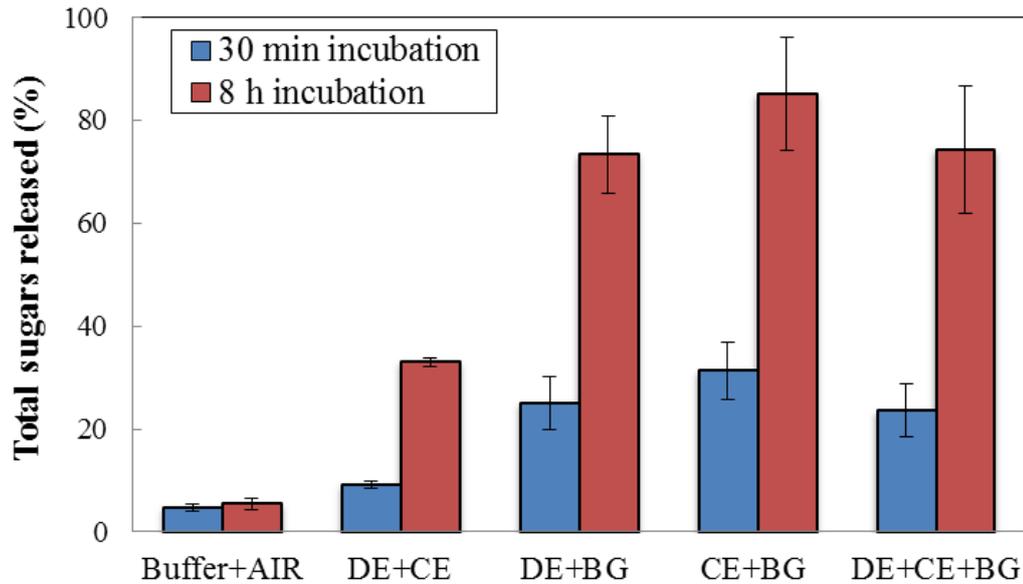


Figure 36. The % (w/w of total sugars) yield of reducing sugars released hydrolysed by the enzyme cocktail (CE + BG) over 30 min and 8 h. Buffer + AIR represents the enzyme absent control. DE = Depol 740, CE = Celluclast and BG = Novzyme 188.

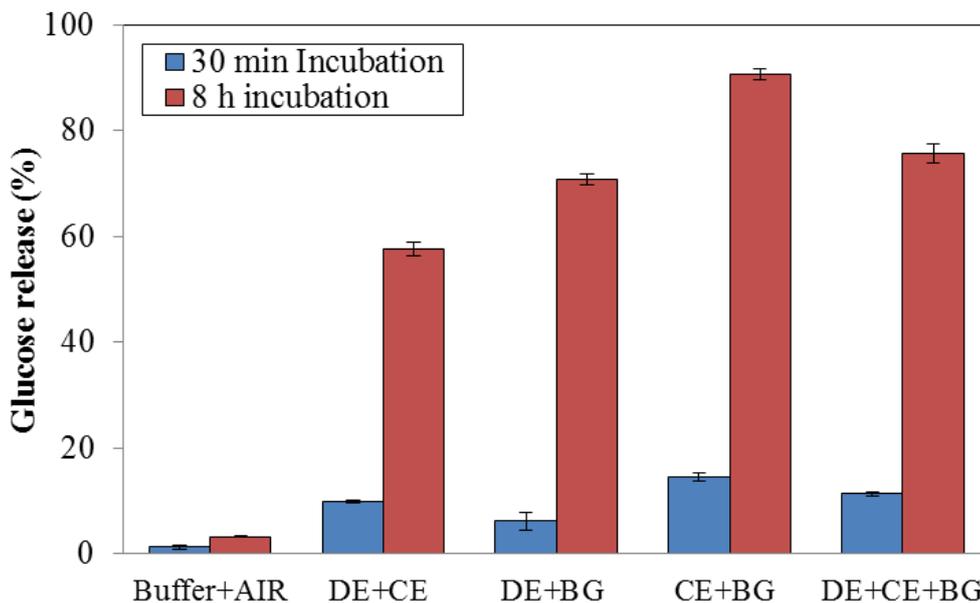


Figure 37. The % (w/w of total glucose) yield of glucose released hydrolysed by the enzyme cocktail (CE + BG) over 30 min and 8 h. Buffer + AIR represents the enzyme absent control. DE = Depol 740, CE = Celluclast and BG = Novzyme 188.

The compositions of the WIAIR and the recalcitrant residues are shown in Table . The extent of WIAIR solubilisation is shown in Figure 38. From the compositions of the recalcitrant residues, the percentages of Glc, GalA and Xyl released by enzymolysis have

been calculated and are shown in Figure 39. Nearly 40 % of WIAIR was digested by CE+BG, whereas, 48 % of WIAIR could be digested by DE+CE+BG (Figure 38). Saccharification was most effective for Glc (less than 10 % remaining in the recalcitrant residue from DE+CE+BG) and GalA (reduced to about 23 %). However, only about half of the xylose was liberated at best. Interestingly, the calculated “release” of glucose (Figure 39) was similar for both the DE+CE+BG and CE+BG cocktails, differing slightly from the results in Figure 37. It is possible that this difference reflects the presence of undegraded dextrans in the DE+CE+BG hydrolysate.

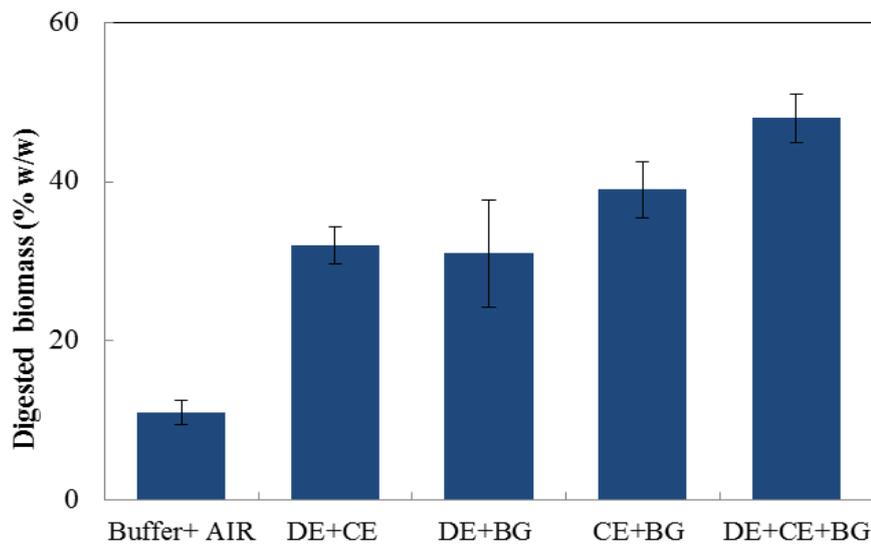


Figure 38. The extent of saccharification of WIAIR indicated by the weight of solubilised biomass. Buffer AIR represents the enzyme absent control, DE = Depol 740, CE = Celluclast, BG = Nobvozyme 188.

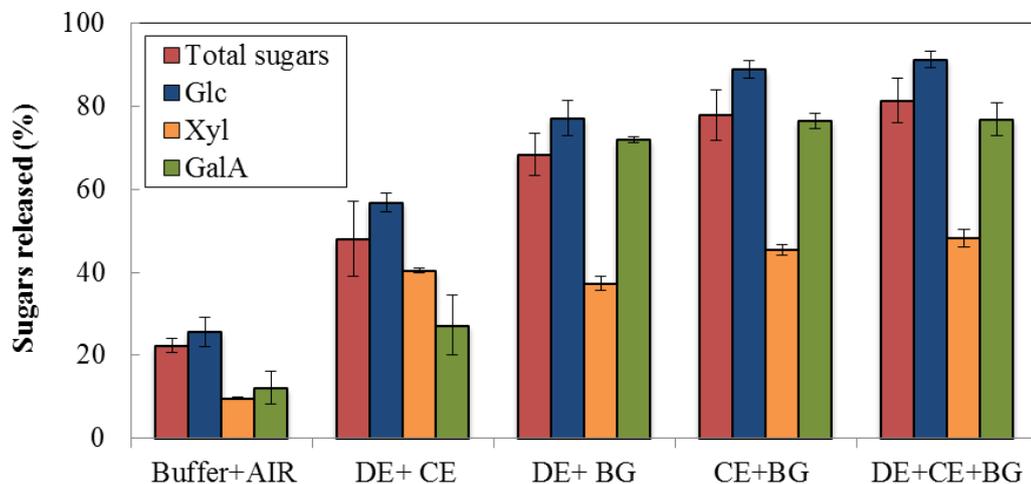
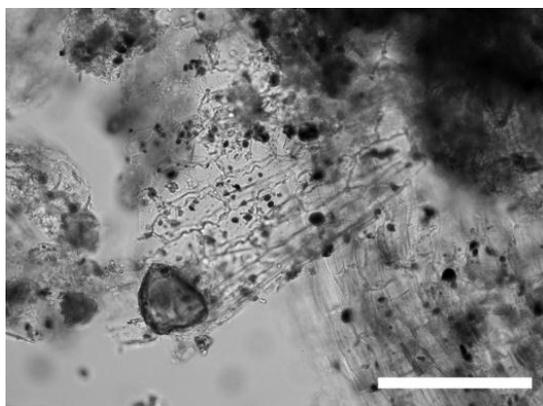


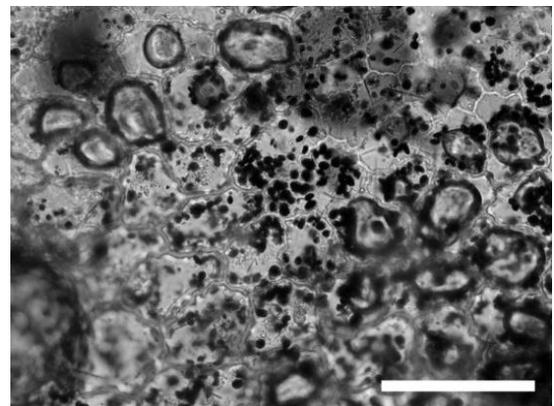
Figure 39. The extent of dissolution of the key component sugars as indicated by the % of each sugar remaining in the recalcitrant residues. Total sugars include Rha = rhamnose, Fuc = fucose, Ara = arabinose, Xyl = xylose, Man = mannose, Gal = galactose, Glc = glucose, GalA = galacturonic acid.

#### 4.2.4 Visible measurement of enzymatic saccharification

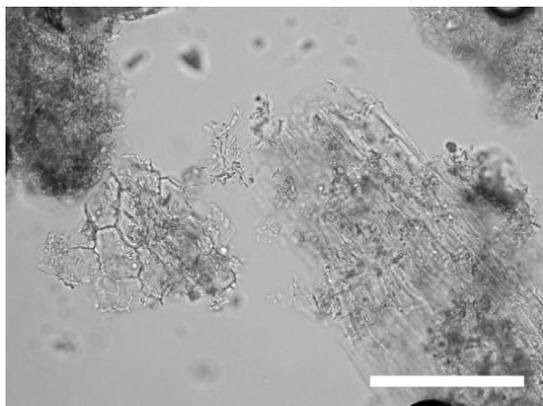
Microscopic evaluations of the WIAIR and selected recalcitrant residues are shown in Figure 40. Image (a) shows that significant plant structure remained after AIR extraction and a significant amount of liberated and intracellular starch were observed in the AIR. In WIAIR (Figure 40b), the structure of root and large tissues of leaves including stoma and epidermal cells were still observed along with significant intracellular starch (iodine staining). However, enzymolysis resulted in the loss of clear structures (Figure 40c-f) along with the loss of starch which indicates the presence of amylase activity in the enzyme cocktails suggesting that no additional amylase is needed for the starch degradation. It is likely that the residual and unclear structures are composed of the epidermal cuticular layers which present an imprint of the degraded cellular structure.



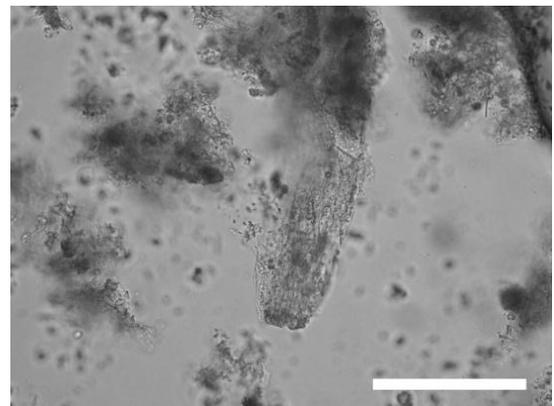
a AIR



b WIAIR



c DE+CE



d DE+BG

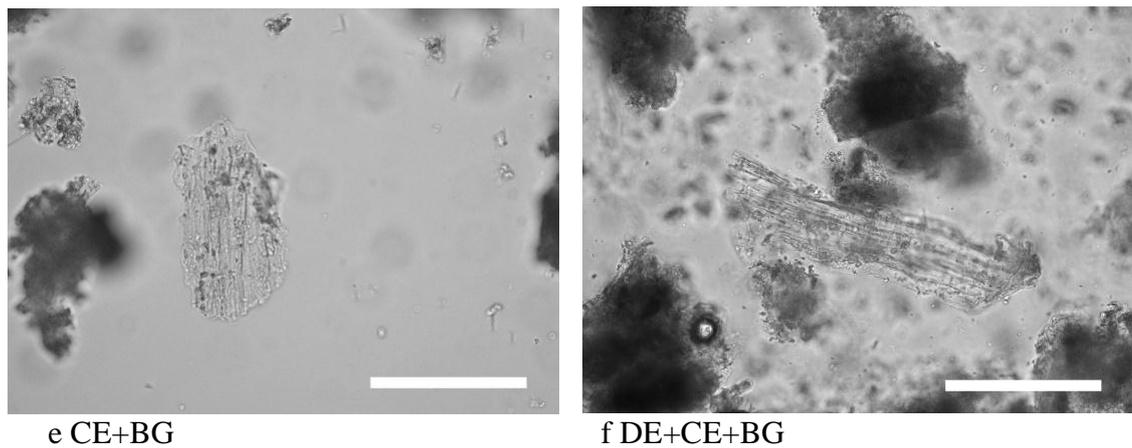


Figure 40. The microscope observations of AIRs and the residual pellets after enzymatic hydrolysis. The images were observed under 20 x magnification and dyed using iodine (Bars = 100  $\mu\text{m}$ ).

#### 4.2.5 Optimisation of enzyme concentrations

Having established that in excess concentration, CE+BG was most effective at saccharification of duckweed WIAIR polysaccharides, the effect of reducing enzyme concentration on the theoretical maximum yields of reducing sugars (Figure 41) and glucose (Figure 42) were evaluated over an 8 h and 24 h period. The longer (24 h) incubation time provided an indication as to how close the digestion was to completion at 8 h. The results (Figure 42) shows that reducing the enzyme concentrations from 4000 to 200 U/g WIAIR had relatively little impact on glucose release, and the 8 h and 24 h results were relatively similar (although a little lower). However, at 100 U/g WIAIR and below, the extent of glucose release was very much reduced and at 10 U/g WIAIR the saccharification was about a third of the maximum level. Similar trends were shown for the release of total reducing sugars by DNS (Figure 41). The results indicate that 100 U/g WIAIR would be a minimum requirement for a 24 h treatment. The cellulase activity of the optimal concentration can be also expressed as 4.35 FPU/g WIAIR to facilitate comparison with other studies (Ghose, 1987; Adney and Baker, 2008).

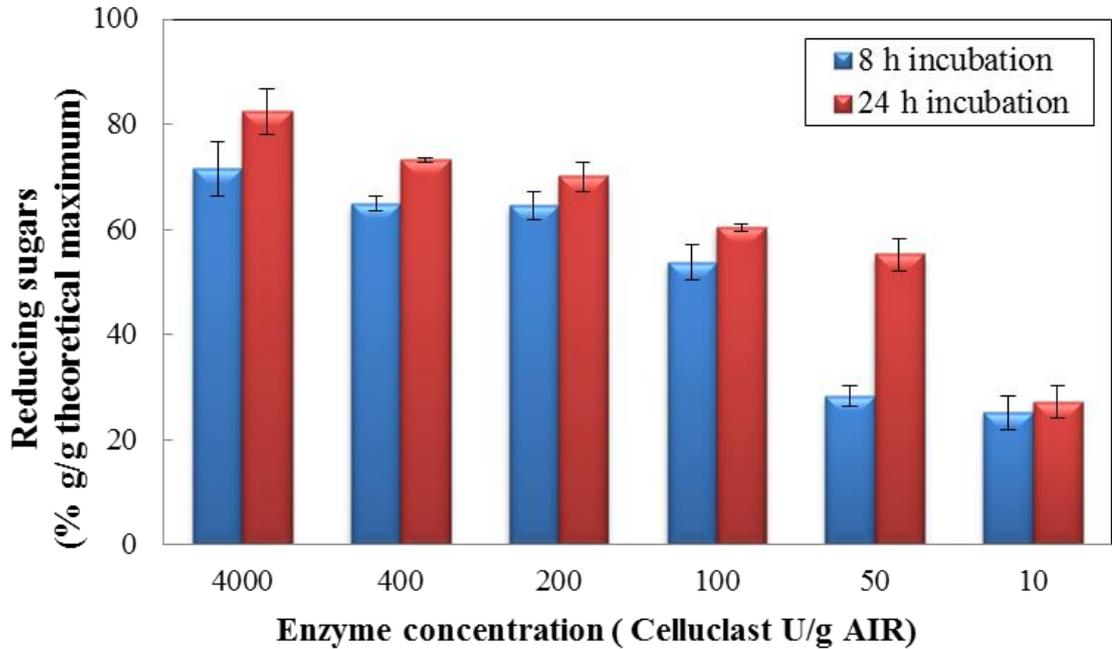


Figure 41. The effects of reducing levels of CE+ BG as a function of yields in the % release of reducing sugars. BG was proportionally reduced.

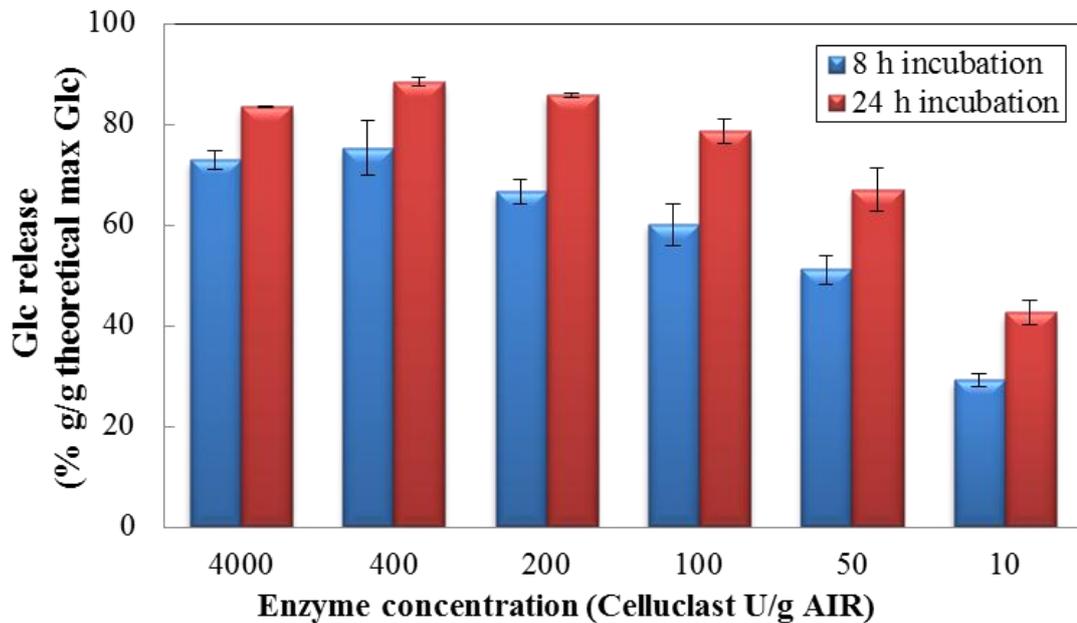


Figure 42. The effects of reducing levels of CE+ BG as a function of yields in the % release of glucose. BG was proportionally reduced.

In the initial optimisation study, the ratio of CE to BG had been 1:1. The importance of this ratio was investigated by investigating the ratio down to 20:1, at a CE concentration of 100 U/g WIAIR. The results (Figure 44) show that for duckweed cell walls, the optimal release of reducing sugars and glucose over both 8 h and 24 h requires a ratio of 1:1. At the lower

CE concentrations, the lower yields of reducing sugars, when compared with yields of glucose may reflect a reduction in the concentration of accessory (non cellulase) enzymes to well below optimal levels.

Depol 740 was initially chosen for evaluation in this study because previous work on grass digestion (Anderson and Akin, 2008) indicated that it was synergistic with cellulase. However, for duckweed saccharification, DE+CE+BG did not enhance saccharification as compared with CE+BG alone. Interestingly, a relatively high ratio of BG to CE (1:1) was found to be optimal, indicating the required synergy between the two enzyme systems. This also indicates that cellobiose may be reaching concentrations necessary to inhibit the cellulases (Klinke et al, 2004; Palmqvist et al, 1999; Tengborg et al,2001). The range of high ratios of CE to BG, e.g. CE:BG at 5:1, 1:2, 1:3 were employed to optimize commercial enzyme preparations (Dien et al, 2008; Gáspár et al, 2007; Xiao et al, 2004).

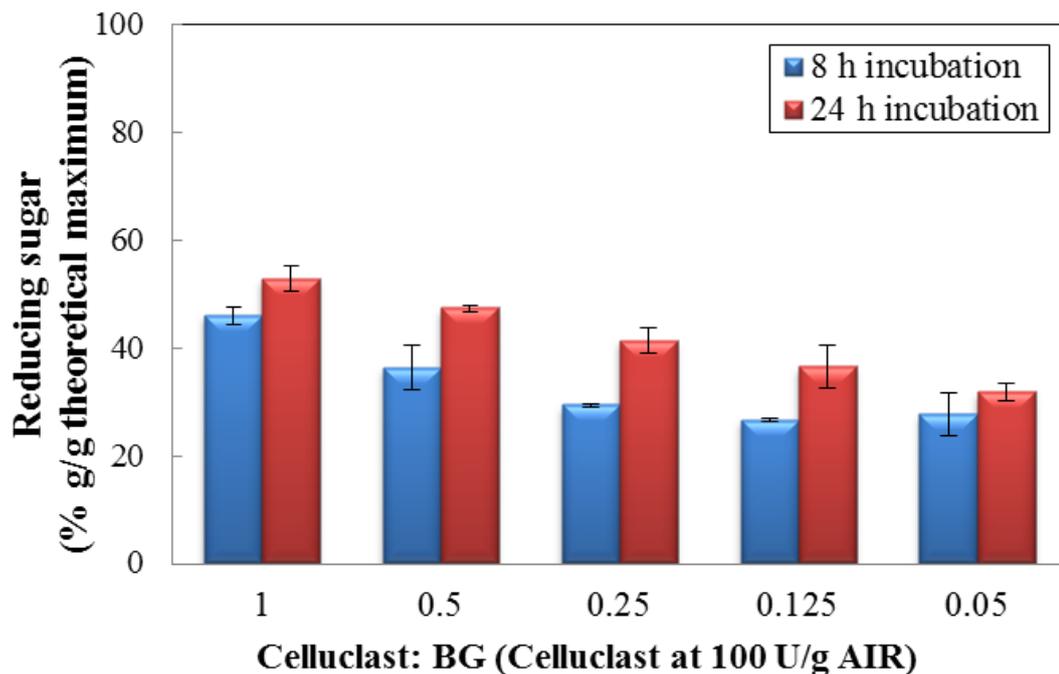


Figure 43. The effects of reducing the ratio of Novozyme 188 to Celluclast as a function of yield of reducing sugars. (where initial ratio is 100 U/g substrate of Celluclast and 100 U/g substrate of BG).

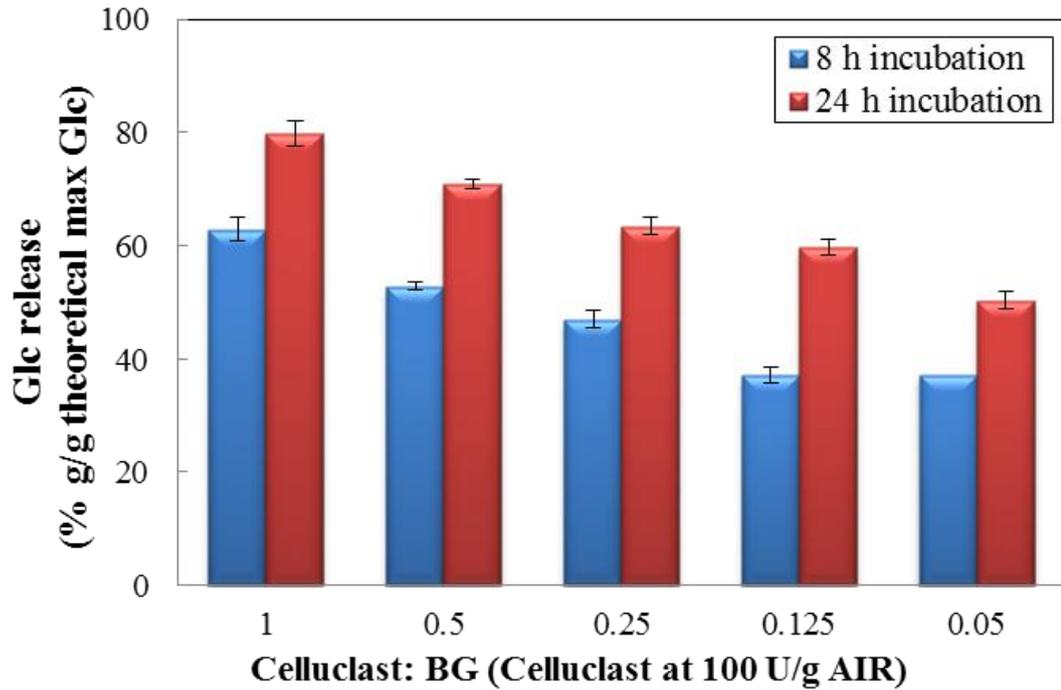


Figure 44. The effects of reducing the ratio of Novozyme 188 to Celluclast as a function of yield of reducing sugars. (where initial ratio is 100 U/g substrate of Celluclast and 100 U/g substrate of BG).

The current study has used WIAIR from duckweed as a model material. Further research will be required to evaluate the potential for saccharification of fresh duckweed biomass, and conversion of the released sugars to ethanol. Recent research (Xu et al, 2011) has demonstrated the use of high-starch duckweed for production of ethanol whereas this study has tried to exploit a novel enzyme cocktail to saccharify effectively both the cell-wall material and the small quantity of starch.

### 4.3 Conclusions

The enzymatic saccharification study on lab cultured duckweed has demonstrated that duckweed biomass (AIR) has the potential to be effectively saccharified to produce glucose (25 % of WIAIR, which is equivalent to 0.6 % of fresh weight) and other cell-wall-derived sugars which might be converted to ethanol by fermentation. Without thermal pretreatment, the saccharification was optimized within 8 h using commercial CE (Celluclast) at 100 U or 4.35 FPU/g WIAIR and added  $\beta$ -glucosidase (BG) at 100 U/g WIAIR. Depol and BG can also be used to saccharify the cell walls, but not to quite the same extent. Hence, duckweed has the potential to provide a means to decontaminate effluent streams and at the same time provide a useful source of biomass for ethanol or sugar production.

## ***5 Enzymatic saccharification on a thermophysical pretreatment (steam explosion) of duckweed.***

Our previous study has shown that duckweed is potentially an ideal feedstock for the production of biofuels because it can be effectively saccharified enzymatically. This chapter will set about reducing the cost of enzymatic saccharification by using an additional pretreatment – steam explosion that enable biomass to be more susceptible to cellulase and enzyme preparations to be consequently optimised. Steam explosion (SE) of duckweed prior to saccharification is thought to be one effective approach to reduce the cost. A range of temperatures, from 130 - 230 °C with a fixed retention time of 10 minutes, are employed. The degradation of fresh duckweed at a range of SE temperature (130 – 230 °C) is measured and the best SE temperature is therefore identified. The enzymatic preparation achieved from last chapter is continuously optimised based on steam exploded slurry at the best conditions.

### **5.1 Material and methods**

#### *5.1.1 Plant material*

*L.minor* biomass materials were collected from the pond of John Innes Centre and were prepared for this sectional research as described in § 2.1.2. Various duckweed pretreated materials including fresh wet, freeze dry, FDM and WIAIR materials were used to identify the effectiveness of the optimised enzyme cocktails.

#### *5.1.2 Steam explosion pretreatment (SE)*

The raw wet *L. minor* biomass (1 kg) was treated by steam explosion apparatus (Cambi™ A/S, Asker, Norway) under different conditions. The process was introduced in § 2.4. Steam exploded products were obtained as slurries and the volume of the slurries was measured before freezing in a coldroom (- 80 °C) until required. An aliquot (200 mL) of each SE product was stored in individual bottles with added thimerosal (0.1 g/L) in the fridge (4 °C) for subsequent analysis.

#### *5.1.3 The subsequent treatment of steam exploded materials*

Representative aliquots (7 mL, triplicates) of steam exploded slurries were transferred to Pyrex® culture tubes and centrifuged to separate the supernatant including water soluble

materials (WSM) from the solid residue (water insoluble material; WIM). The pellets were then washed twice using distilled water. Supernatants were filtered using GF/C filter paper and frozen (- 20 °C). The residual pellets were prepared as alcohol insoluble residues (AIR) using the following procedure. The wet residual pellets (3 mL) were homogenised twice with ethanol (100 %), and the steam-exploded samples prepared at 130 °C and 150 °C were ground with a pestle and mortar for 10 min to break down plant tissues. The resulting slurry was transferred to Pyrex® culture tubes. The pestle and mortar was rinsed out, and the volume was made up to 10mL with additional pure ethanol giving a final ethanol concentration of 70 % (v/v). The slurries were heated at 80 °C for 15 min. After cooling and recovery by centrifugation (3000 x g, 10 min), the residue was re-extracted as before in ethanol (70 % v/v, 80 °C, 15 min) and then once at 80 °C in pure ethanol. Finally the AIRs were extracted once in acetone at room temperature and dried overnight. The frozen residual pellets and half amounts of WSM samples were dried using a freeze dryer (Birchover Instruments Ltd, Hitchin, UK) to recover fully dry mass. Aliquots (40 mg, duplicates) of the resulting dry mass were dried at 105 °C to test the moisture content.

#### 5.1.4 *Enzymatic saccharification of steam exploded slurries*

The enzyme cocktail of CE (cellulase: 100 U or 4.35 FPU/g substrate) with additional BG (100 U/g substrate) optimised from the last chapter was used as the initial enzyme dosage and was further optimised based on steam exploded materials. Consequently, the final optimised enzyme cocktail was designed to hydrolyse various pretreated materials. A novel cellulase product – Cellic® CTec 2 (CTec 2; Novozymes A/S Ltd, Bagsvaerd, Denmark) was identified based on steam exploded material for replacing Celluclast, which is not economically used in a large scale production, in the fermentation study. The FPU activity of CTec 2 was assessed as 189 FPU/mL, using the standard measurement for cellulase (Ghose, 1987). The details of digestion process were already introduced in § 2.5.

#### 5.1.5 *Analytical methods*

##### 5.1.5.1 Moisture Assessment

Because of the different growing conditions of pond sample and lab cultured sample, the percentage of dry matter was preliminarily measured as described in § 2.2.

##### 5.1.5.2 Starch Assessment of SE slurry

The starch content is a crucial parameter to assess the effectiveness of steam explosion on duckweed. The proportions of starch in SE slurry, WSM, WIM were measured following the methods described in § 2.6.

### 5.1.5.3 DNS & GOPOD test

Reducing sugars and glucose released from steam explosion process and subsequent enzymatic saccharification were assessed by using DNS and GOPOD assay methods (see § 2.9.1 and § 2.9.2).

### 5.1.5.4 Microscopy of SE slurry

The microscopy observations were carried out as a complementary technique for understanding changes in cell wall structure and starch variation during steam explosion. Details of operation were described in § 2.10.

### 5.1.5.5 Carbohydrate analysis

The assessment of monosaccharide compositions were analysed by using GC sugar method (see § 2.7) and the identification of carbohydrate size was analysed by using HPLC carbohydrate size method (see § 2.8.5).

### 5.1.5.6 Inhibitors assessment

2-furfuraldehyde (2-FA), 5-Hydroxymethylfurfural (5-HMF) and weak acids (e.g. formic and acetic acid produced from the breakdown of hemicellulose and phenolics) have been assessed since they are thought to be significant fermentation inhibitors. The concentration of organic inhibitors was analysed by HPLC using a Flexar LC instrument (Perkin Elmer, Seer Green, Bucks., UK) equipped with refractive index and photo diode array detectors (reading at 210 nm wavelength) in series. Details of analysis method was described in § 2.8.4.

## 5.2 Results and Discussions

### 5.2.1 Recovery of material following steam explosion

The recovery of dry matter following SE employing different conditions was assessed. Table 16 shows that increasing the severity of steam explosion resulted in a reduction in total dry matter recovery. Up to 35.4 % (w/w) of biomass was lost at 230 °C. This is likely to have been due to the carriage of some of the solubilised and hydrolysed material and possibly small particles through the machine during depressurisation. Larger, insoluble particles will have been more readily recovered in the cyclone recovery system. Jacquet et al (2011) reported that mass loss as a consequence of the SE of cellulose fibre starts at from 70 °C and increased temperature results in increased loss. Mass loss (4 - 27 %) has also been observed following SE of birch wood (Shimizu et al, 1998). Shimizu et al (1998) found that the weight loss of birch wood is positively correlated with SE retention time. At the same pressure (1.47 MPa), dry mass loss of 15, 25 and 26.3 % (w/w) was observed at, respectively, 5, 10 and 15 min retention times.

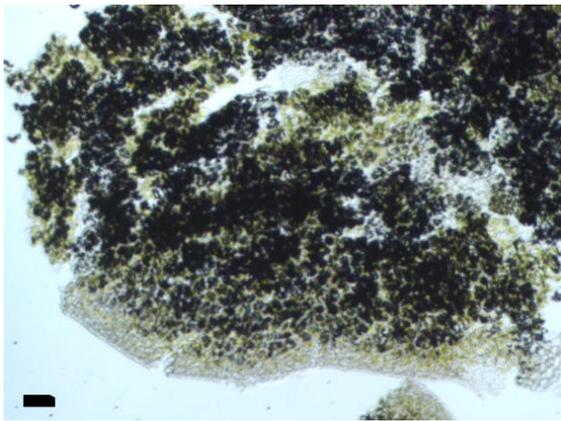
Table 16. Dry mass recovery of SE products. The SE retention time was 10 min for all treatments. SF= severity factor.

Parameter	SF	Vol (mL)	Density (g/mL)	Weight (g)	% DM (w/w)	DM (g)	Recovery (%)
Untreated	/	1000	1.00	1000.0	8.5±0.69	84.7	100
130 °C	1.9	1450	0.97±0.01	1405.2	4.9±0.05	69.3	81.8
150 °C	2.5	1860	0.95±0.05	1762.9	3.6±0.34	62.6	73.9
170 °C	3.1	2293	0.92±0.08	2116.0	2.8±0.25	59.0	69.7
190 °C	3.7	2080	0.96±0.05	2003.4	3.4±0.07	66.9	79.0
210 °C	4.2	2070	0.97±0.05	2002.0	3.1±0.05	60.9	71.9
230 °C	4.8	2310	0.94±0.01	2170.8	2.5±0.01	54.1	63.8

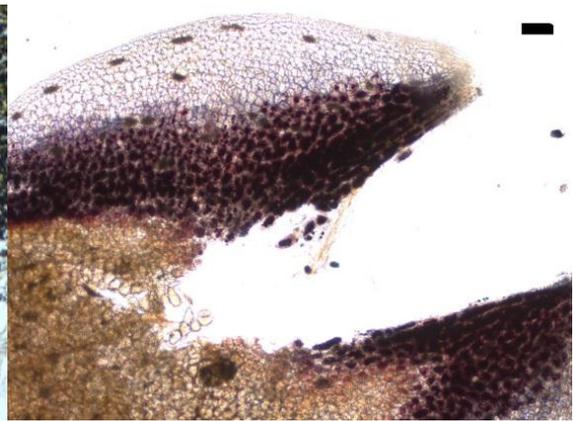
### 5.2.2 Visual impact of steam explosion on duckweed tissues

Steam explosion has a significant impact on duckweed tissue disruption (Figure 45). At a constant retention time of 10 min, increasing temperature resulted in increased tissue disruption observed by microscopy. Following staining with Lugol's solution, a large amount of starch granules were observed in the untreated fresh materials (FWM; Figure 45a; starch granules are stained black). At the lower pretreatment temperatures (130 - 170

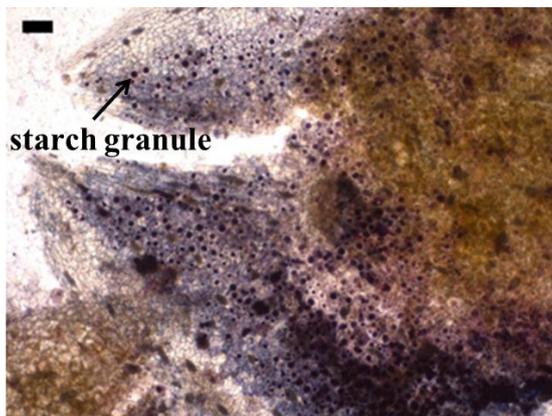
°C) the cellular structure remained visually intact. At 130 °C, fronds were slightly disrupted, but starch granules remained embedded in the cells (Figure 45b). At 150 °C, the plant structure was further disrupted and a significant amount of starch and sodium oxalate (needle shaped) crystals were released from the cell (Figure 45c). At 170 °C, the tissue was further decomposed and the starch had started to gelatinise (Figure 45d). At 190 °C, the tissue began to become less well defined as cells started to separate. The starch gelatinisation and the decomposition was very pronounced (Figure 45e). At 210 °C, only a small amount of residual, structured tissue was observed and intracellular contents had apparently been liberated into the aqueous phase - starch granules were extensively released from plant cells (Figure 45f). At 230 °C, plant tissue integrity was completely disrupted but the liberated and gelatinised starch appeared to be reduced (Figure 45g). This is probably due to polysaccharides degradation (see below).



a. Fresh plant tissue



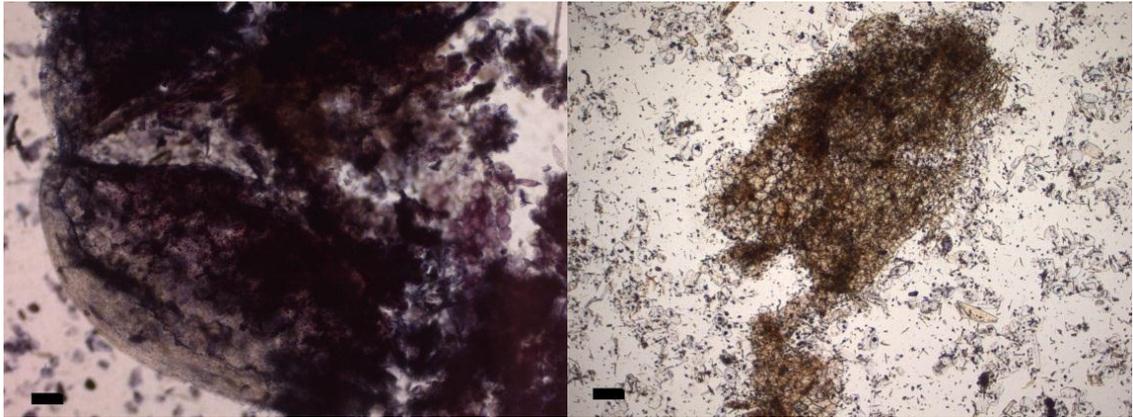
b. 130 °C pre-treated leaf tissue



c. 150 °C pre-treated leaf tissue

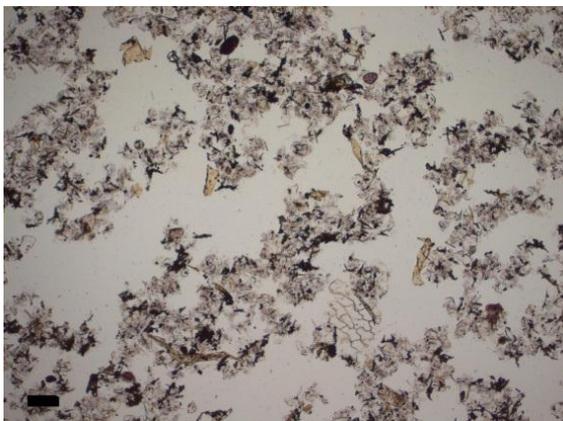


d. 170 °C pre-treated leaf tissue



e. 190 °C pre-treated leaf tissue

f. 210 °C pre-treated leaf tissue



g. 230 °C pre-treated leaf tissue

Figure 45. Microscopy of steam exploded duckweed biomass. Tissue has been stained with iodine. Bar = 100  $\mu\text{m}$ . Black spots are starch granules labelled in the picture.

### 5.2.3 Assessment of the chemical composition of steam exploded materials.

Figure 46 shows the relative levels of soluble (WSM) and insoluble (WIM) dry matter recovered, and shows that the severity-related increase in tissue disruption is associated with an increase in soluble material in the aqueous phase. The highest proportion of WSM (70 %) appears after pretreatment at 210 °C; SE using this temperature and these conditions would thus be an effective means of solubilising duckweed biomass. In comparison, Sun et al (2005) observed that the highest WSM conversion (40 % of dry mass) by SE of wheat straw (lignocellulose) occurred at a severity factor around 4.44 (200 °C, 33 min and 220 °C, 8 min). SE with a severity factor 4.2 (230 °C, 2 min) was found to be the pretreatment that was most effective in solubilising rice straw (generating 30% WSM; Nakamura et al, 2001). Jacquet et al (2011) noted that depolymerisation of cellulose fibres occurs at a severity factors in the range of 4.0 - 5.2. On this basis, for our work with

a constant retention time of 10 min, depolymerisation would be expected to occur in a range from 200 - 245 °C. However, *L. minor* is poorly lignified (Jarvis et al, 1988), and this is likely to account for the tissue disruption at low severities which is probably initiated by the depolymerisation of pectic polymers involved in cell adhesion at 170 °C (Figure 45).

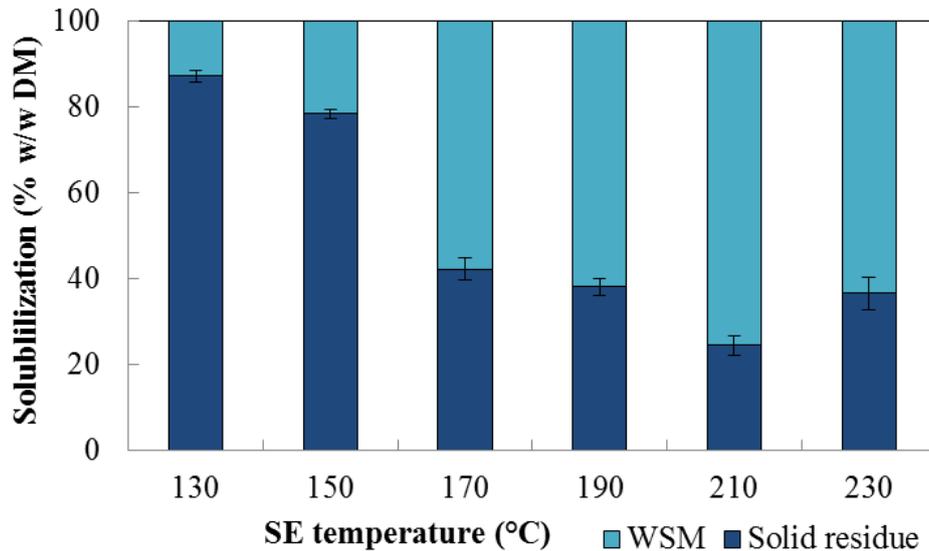


Figure 46. Solubilisation of biomass pretreated with steam explosion at different temperatures. WSM = water soluble materials.

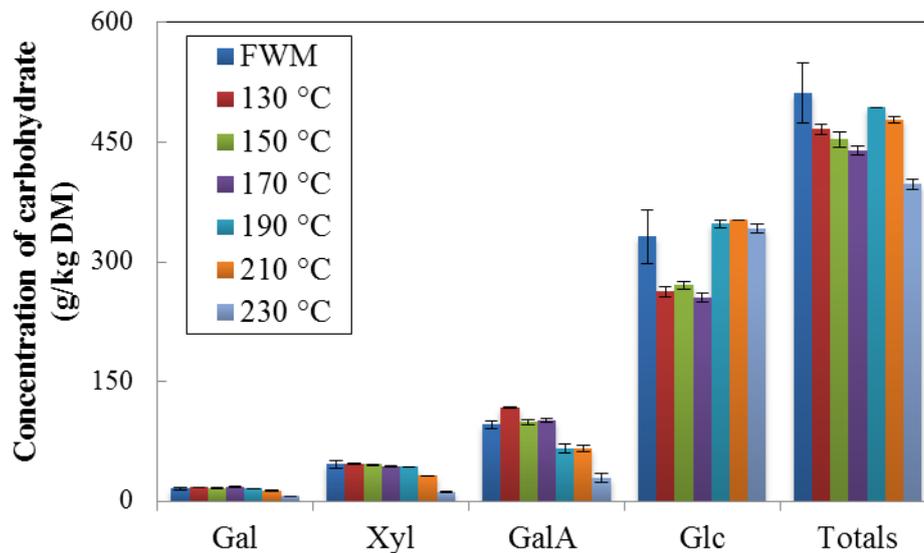


Figure 47. Carbohydrate concentration of dry SE total slurry analysed by GC method of 72 % (16.6 mol/L) H<sub>2</sub>SO<sub>4</sub> hydrolysis. Xyl = xylose, Gal = galactose, Glc = glucose, GalA = galacturonic acid and Totals = the total carbohydrate including Rha, Fuc, Ara, Xly, Man, Gal, Glc and GalA.

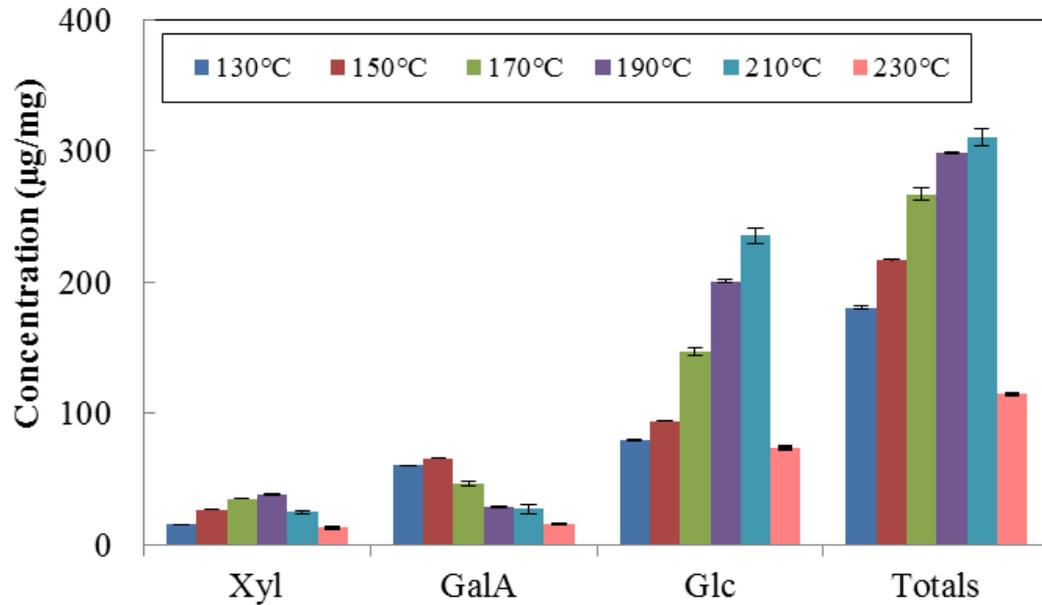


Figure 48. Carbohydrate concentration of steam exploded WSM analysed by GC method of 72 % (16.6 mol/L)  $H_2SO_4$  hydrolysis. Xyl = xylose, Gal = galactose, Glc = glucose, GalA = galacturonic acid and Totals = the total carbohydrate including Rha, Fuc, Ara, Xly, Man, Gal, Glc and GalA.

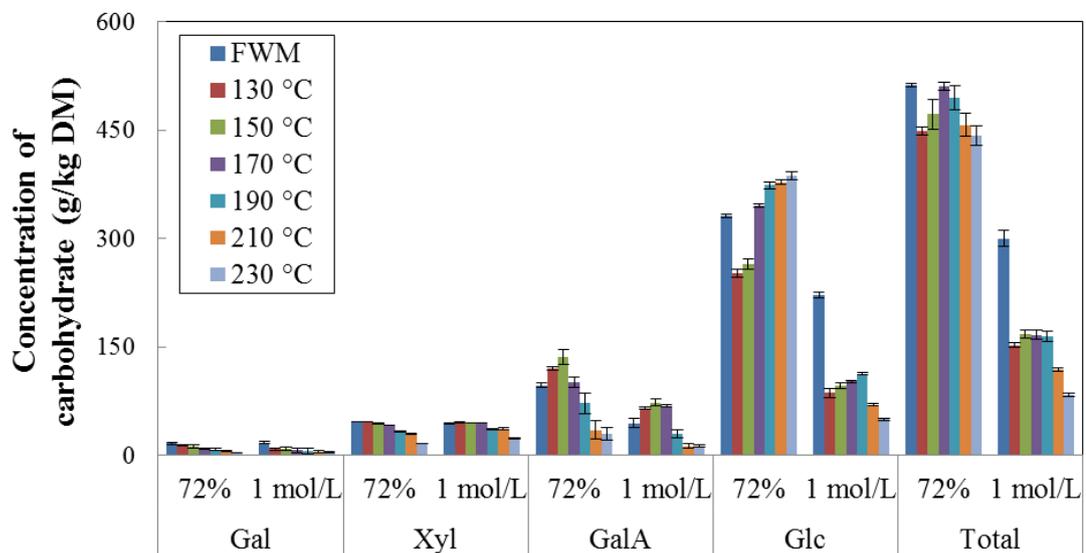


Figure 49. Carbohydrate concentration of SE WIM (AIR) by GC method of 72 % (16.6 mol/L) and 1 mol/L  $H_2SO_4$ . Xyl = xylose, Gal = galactose, Glc = glucose, GalA = galacturonic acid and Totals = the total carbohydrate including Rha, Fuc, Ara, Xly, Man, Gal, Glc and GalA.

The carbohydrate compositions of SE slurries, WIM and WSM were quantified in order to assess variation and the impact of severity on the conversion of WIM to WSM (Figure 47 -

49 respectively). In total slurry (Figure 47), galactose (Gal), xylose (Xyl) and galacturonic acid (GalA) content generally decreases as temperature increases, and glucose (Glc) content as a proportion of the total generally increases with temperature although it is not a clear trend (Figure 47). Further clarity could be obtained by evaluating the WSM and WIM. In WSM (Figure 48), Gal, Xyl and GalA increased from 130 - 170 °C, after which they all decreased to low levels also. Increase in severity up to 210 °C resulted in an increase in soluble glucose, after which it decreased dramatically to low levels. In WIM (Figure 49), Gal, Xyl and GalA increased from 130 - 170 °C, after which they all decreased to low levels. Glc present in the WIM increased concomitantly, then decreased at high severities. Evaluation of Glc using 1 mol/L hydrolysis alone decreased dramatically at high severities. More information on the origin of changes in Glc was also obtained by measuring the levels of starch present in SE slurry, WSM and WIM (Figure 50) using total starch assay.

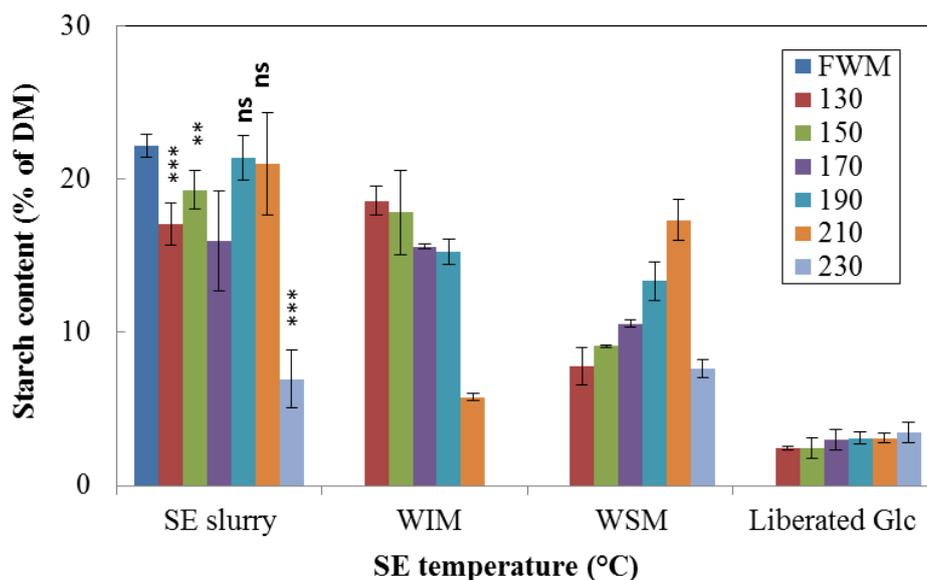


Figure 50. The % (w/w) starch content present in FWM, SE slurry, SE WIM and SE WSM, and % (w/w) liberated glucose present in SE slurry. The percentages of starch were calculated based on the dry matter of the original SE slurry. \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , ns = not significant ( $p > 0.05$ ) compared with FWM sample.

The results showed that in total slurry, starch levels were relatively stable as a proportion of the dry matter until 210 °C, but dropped markedly after pretreatment at 230 °C. In WIM, the level of starch decreased by about 25% up to 190 °C, then dropped considerably after treatment at 210 °C and was undetectable by 230 °C. This was reflected in the increase in

soluble starch in WSM from 130 °C up to 210 °C after which the starch content dropped to low levels. GOPOD analysis of the WSM directly revealed a proportion of liberated glucose which comprised about 2.5 % of the WSM DM at 130 °C, but rose to about 3.5 % after pretreatment at 230 °C. The fact a small amount of free glucose was produced under all SE conditions suggests that pretreatment solubilisation of starch may have also involved the production of di-, tri- or other oligosaccharides (not evaluated).

Details of sugar products (mainly including oligosaccharides of glucose) of steam explosion pretreatment might be unveiled by carbohydrate size (Figure 51) evaluated by HPLC methods. Several oligosaccharides of glucose were used as the standard: glucose (Glc), cellobiose (CBO), cellotetraose (DP4) and celloheptaose (DP7). This group of sugars is also referred to water soluble sugars products since carbohydrate size smaller than cellooctaose (DP8) are water soluble (Huebner et al, 1978). To some extent, this figure also indicates the solubilisation trend of duckweed biomass in steam explosion pretreatment. Increase in severity up to 210 °C results in large amount of low carbohydrate size products, which might continuously convert to organic acids at 230 °C. Molecular weights of common organic acids are smaller than glucose because their peaks are present after glucose peak. The first peak from left might be gelatinised starch due to changes of this peak over different temperature is corresponding to the starch results of WSM.

The above results clearly demonstrate the solubilisation, possibly hydrolysis and breakdown of cell wall and starch polysaccharides during SE pretreatment. The movement of sugars from solid residue to water phase increases as temperature rises from 130 - 210 °C. Starch results (Figure 50 - Figure 51) show clearly that starch is gelatinised and eventually completely solubilised. However, the loss of measurable starch at 230 °C suggests that the soluble starch is destroyed. Other non-cellulosic sugars follow a similar trend. The galactose and glucose decrease in WIM, and their increase in the WSM up to 170 - 190 °C suggests solubilisation. However, above 190 °C, they decrease, again suggesting that they are degraded. The degradation of non-cellulosic sugars is consistent with studies on pretreatment of lignocellulose (Kabel et al; Merali et al; Waldron, 2010) and accounts for the increase in breakdown products shown in Figure 52 (see below).

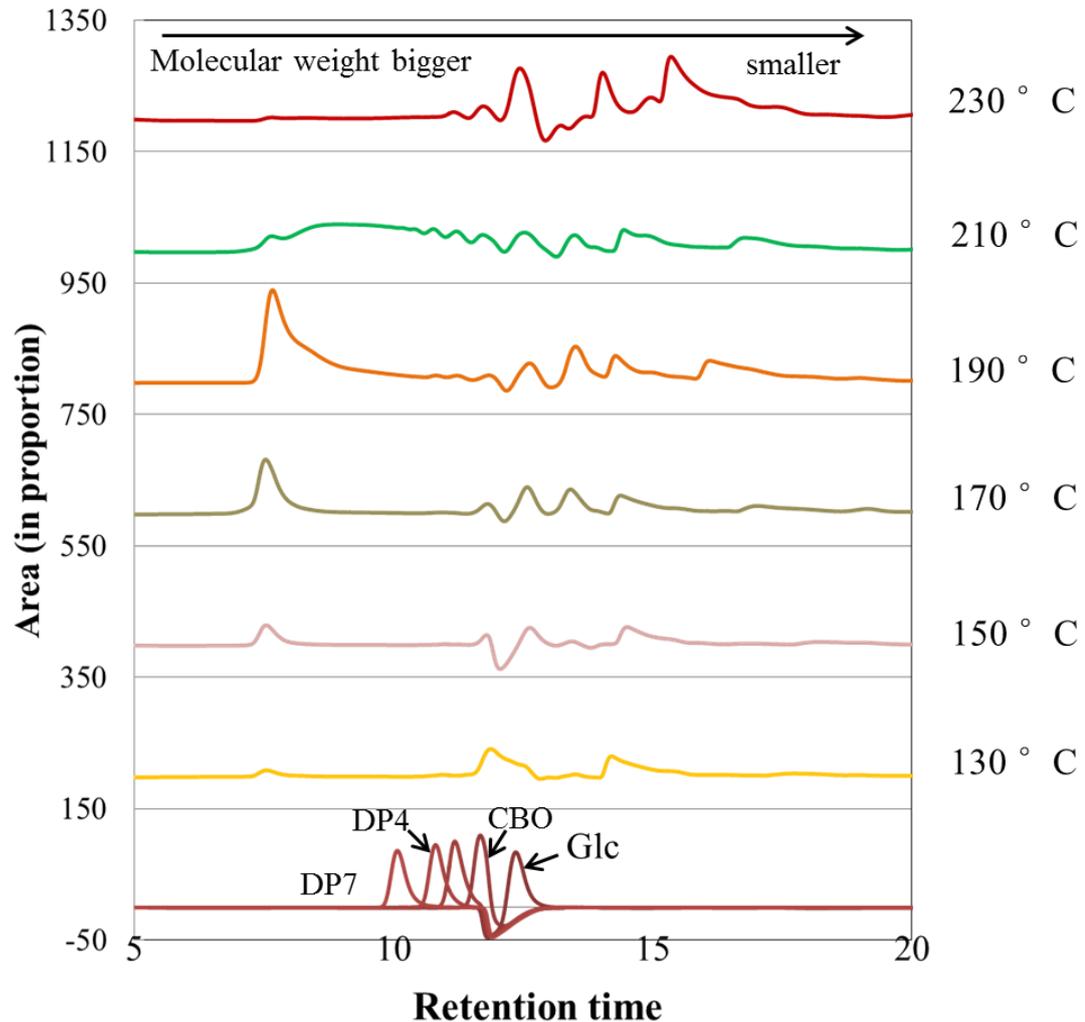


Figure 51. The HPLC analysis of carbohydrate size of SE slurries at different temperature. CBO = cellobiose; DP4 (degree of polymerization) = cellotetraose; DP7 = celloheptaose.

#### 5.2.4 Quantification of fermentation inhibitors in steam exploded materials

Significant quantities of fermentation inhibitors were detected in the WSM and these were most prominent after the higher severity treatments of 210 °C and 230 °C coincidental with the greatest loss of carbohydrate material. 2-furfuraldehyde (2-FA) and 5-hydroxymethylfurfural (5-HMF) are known to inhibit glycolytic enzymes and thus hinder sugar fermentation by yeast (Almeida et al., 2007). Fermentation inhibition by these acid products has been reported by Pienkos and Zhang (2009); the degree of inhibition by 5-HMF, 2-FA, acetic acid and levulinic acid were reported as 50, 79, 74 and 50 % respectively when their concentrations reached 80, 40, 60 and 400 mg/g. A formic acid concentration of 27 mg/g has been reported to cause 80 % fermentation inhibition (Maiorella et al, 2004). 2-furfuraldehyde is derived from xylose and 5-HMF is produced from glucose under acidic conditions (Pedersen and Meyer, 2010). Furthermore, 5-HMF

and 2-FA will continue to transform to levulinic acid and formic acid if sufficient water is present (Pedersen and Meyer, 2010).

The low pH conditions of steam explosion on fresh wet duckweed are thus likely to result in the formation of 2-FA and 5-HMF and high level of organic acids. The generation of 2-FA, 5-HMF and organic acids was assayed (Figure 52). 2-FA and 5-HMF were detectable following SE at 190 °C and the levels increased up to 5.6 and 7.3 mg/g respectively at 230 °C. These are not high levels of 2-FA and 5-HMF and it is not expected that at these concentrations there would be a significant inhibitory effect on subsequent fermentation. However, significantly higher levels of formic acid (23.8 and 38 mg/g at 210 and 230 °C respectively) and acetic acid/ levulinic acid (53.8 and 67.7 mg/g) were produced at 210 and 230 °C respectively. Almeida et al (2007) showed that monosaccharide products of hydrolysis of cellulose and hemicellulose could convert to 5-HMF and 2-FA. Acetic acid is a hydrolysis product of hemicellulose and lignin (Almeida et al, 2007), but since duckweed has a low proportion of lignin (Jarvis et al, 1988), it is likely that the observed acetic acid, as well as the levulinic acid and formic acids, are derived from reducing sugars. In keeping with the production of acidic breakdown moieties, SE led to a reduction in pH. This was comparatively slight from 130 °C to 190 °C (pH 6.5 to 6.2) but more pronounced at higher temperatures: pH 5.6 at 210 °C and pH 4.6 at 230 °C.

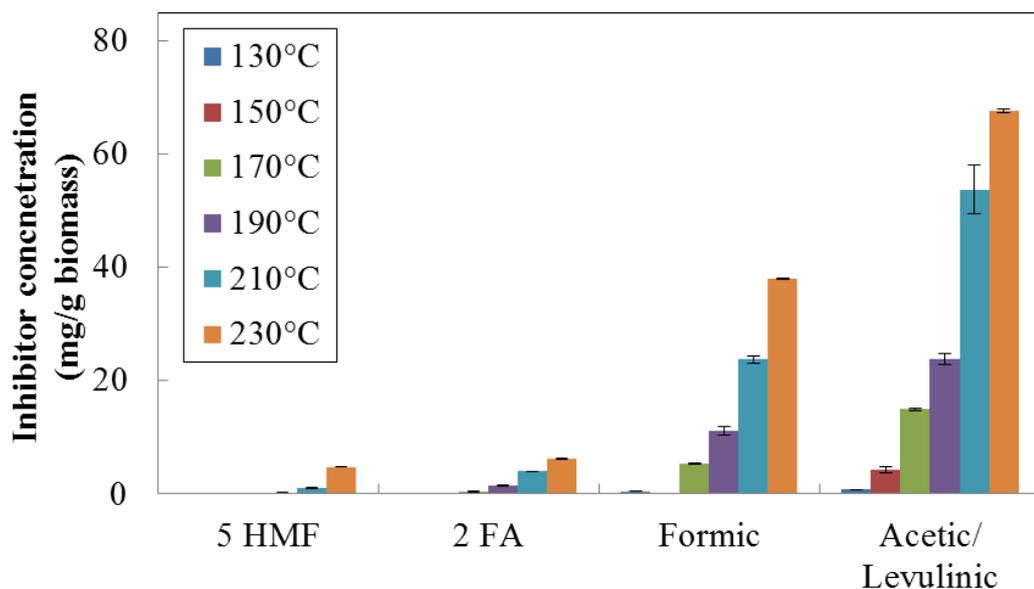


Figure 52. Variation in levels of fermentation inhibitors at different SE severities.

### 5.2.5 Investigation of enzymatic saccharification on steam exploded raw slurry

To test the hypothesis that steam explosion increases the ease of hydrolysis by cellulolytic enzymes, enzymatic saccharification was carried out to hydrolyse the carbohydrate components (cellulose, hemicellulose and pectin) of the total slurry. In keeping with the analysis of WSM (Figure 48), the total slurry was found to contain both solid residual CWM and soluble glucose liberated by SE (Figure 53). In addition, using DNS analysis, it was found that significant quantities of additional reducing sugars were present (Figure 53). The levels of SE-solubilised reducing sugars and Glc were taken into account when evaluating the potential for enzymatic saccharification of the slurry.

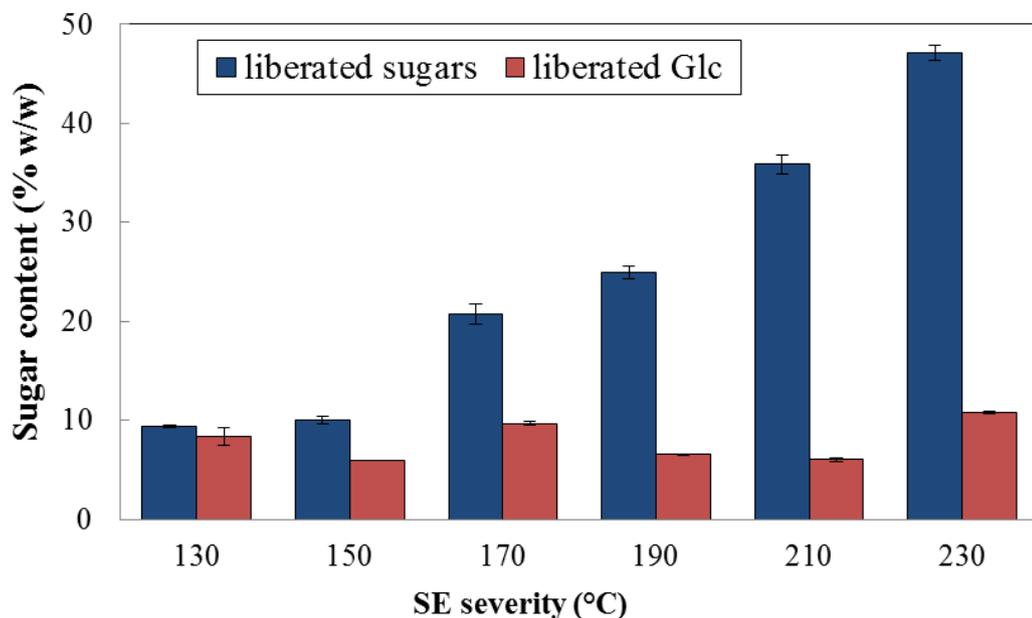


Figure 53. The concentration of the reducing sugars and glucose solubilised in the SE process.

Initial studies screened for the total yield of reducing sugar released from SE slurry by enzyme treatment using Celluclast (CE, 100 U or 4.35 FPU/g substrate) and Novozyme 188 (BG, 100 U/g substrate), identified previously as optimal for digesting purified duckweed cell wall material. Saccharification was found to be positively correlated with the severity of SE (Figure 54). Slurry treated by SE at 210 °C and 230 °C was digested completely (~100 % reducing sugar yield) within 8 h. Over the same period the SE 190 °C material exhibited a reducing sugar yield of 86.5 %. The increasing initial hydrolysis rates following the increase in SE severity imply that more carbohydrate was depolymerised at the higher temperatures. For all SE samples the bulk of the saccharification occurred in the

first 2 h of incubation and was stable after 8 h. This indicated that it might be possible to improve the efficiency of the saccharification by identifying the minimum severity of pretreatment required, and the minimum levels of enzymes. Since SE at both 210 and 230 °C gave 100 % saccharification, then material SE at 210 °C was chosen for further optimisation. Not only would this involve less energy in the process, but it would also significantly reduce the quantities of fermentation inhibitors produced (Figure 52; Jacquet et al, 2011).

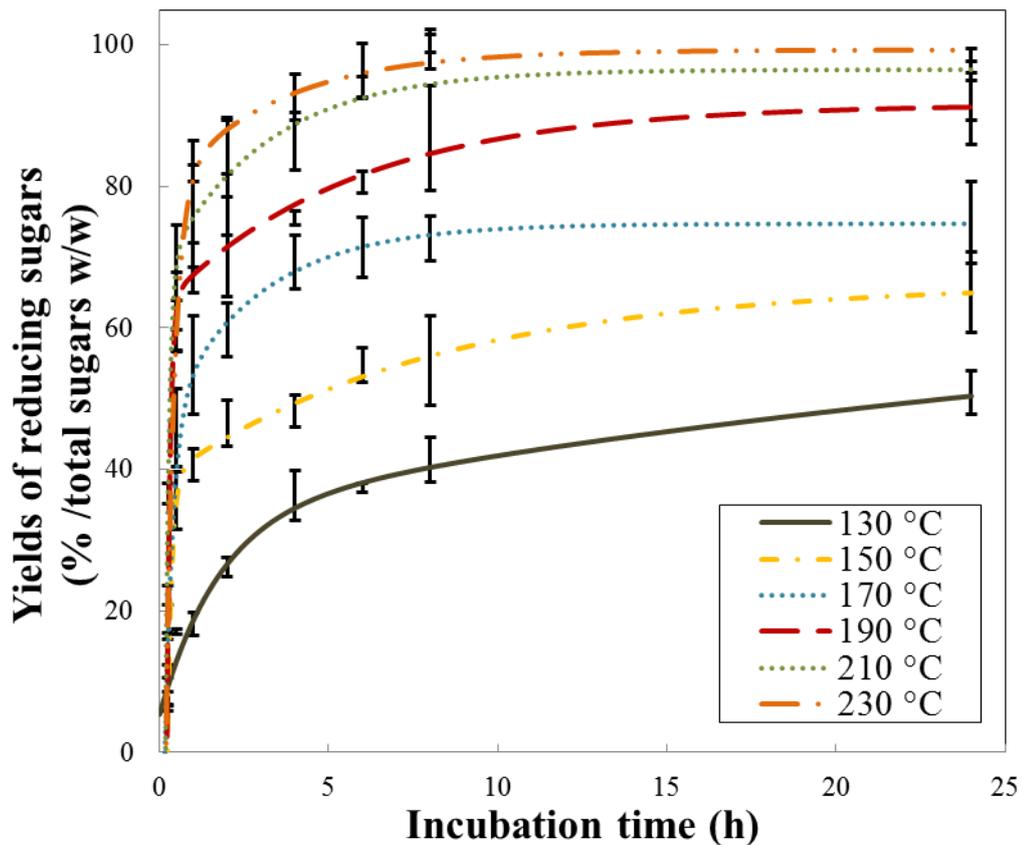


Figure 54. The reducing sugar yield following hydrolysis of residual CWM using 100 U (4.35 FPU)/g substrate of CE and 100 U/g substrate of BG.

To investigate the efficacy of lower enzyme doses, slurry from SE at 210 °C was treated with enzyme cocktails of CE at 100, 50, 20 and 10 U/g substrate (equivalent to 4.35 to 2.18, 0.87 and 0.44 FPU/g substrate) and BG (concentration at CE: BG of 1:1). Digestion with 100, 50 and 20 U/g substrate resulted in similar reducing sugar yields: 97.9, 93.7 and 87.3% respectively after 8 h, at which point the digestion stabilised (Figure 55). Digestion using CE at 10 U/g was less effective, with a maximum yield of 70.9 %. Initial rates were

similar at all concentrations studied. The optimum enzyme dose for slurry from SE at 210 °C is thus of the order of 20 to 50 U/g substrate (0.87 to 2.18 FPU/g substrate).

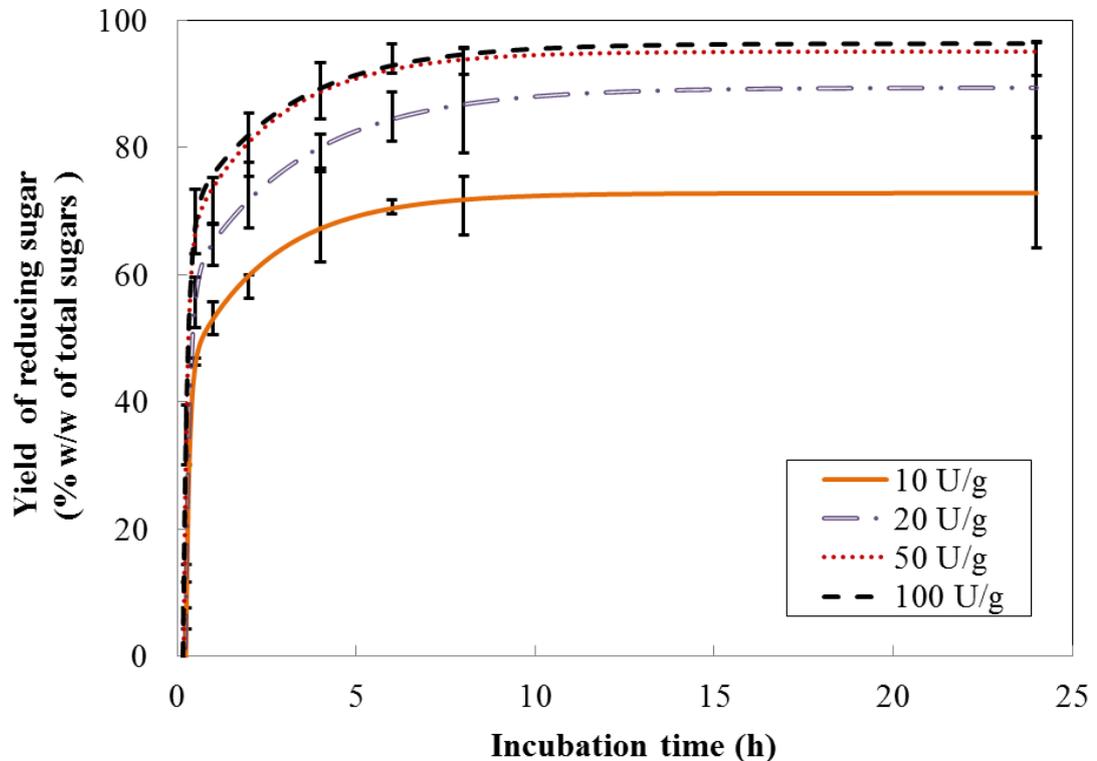


Figure 55. Optimization of enzyme saccharification using reduced CE concentrations (100, 50, 20 and 10 U/g substrate) with additional BG (CE: BG at 1:1).

To further optimize enzyme use, various CE: BG ratios were investigated. Optimal CE concentrations of 50 and 20 U/g substrate were chosen and CE: BG ratios of 1: 1 down to 1: 0.1 were employed to digest SE 210°C slurry. After a 24 h digestion, CWM was completely hydrolysed at the CE: BG ratios of 1: 1 and 1: 0.5 with a CE concentration of 50 U/g and the digestions exhibited high initial rates (Figure 56). The next most effective conditions were CE: BG ratios of 1:1 and 1: 0.5 at CE 20 U/g, which exhibited 95.6 and 94.1 % reducing sugar yields respectively. Notably, however, the lower CE: BG ratios also achieved reducing sugar yields approaching 90 %, with the exception of CE: BG ratio 1: 0.1 at CE 50 U/g, which hydrolysed only 84.9 % of the carbohydrate. This clearly shows the synergy between CE and BG. In realistic enzyme concentration ranges, the CE: BG ratios of 1: 0.1 to 1: 0.5 achieve better digestion than the lower ratios (1: 0.25 and 1: 0.1) and the addition of BG facilitates the use of CE at lower concentrations. When enzyme costs are also considered, the data suggest that CE at 20 U or 0.87 FPU/g substrate and BG at 2 U/g substrate is an appropriate enzyme cocktail for the digestion of SE treated

duckweed material, offering both efficient digestion and being relatively more cost effective.

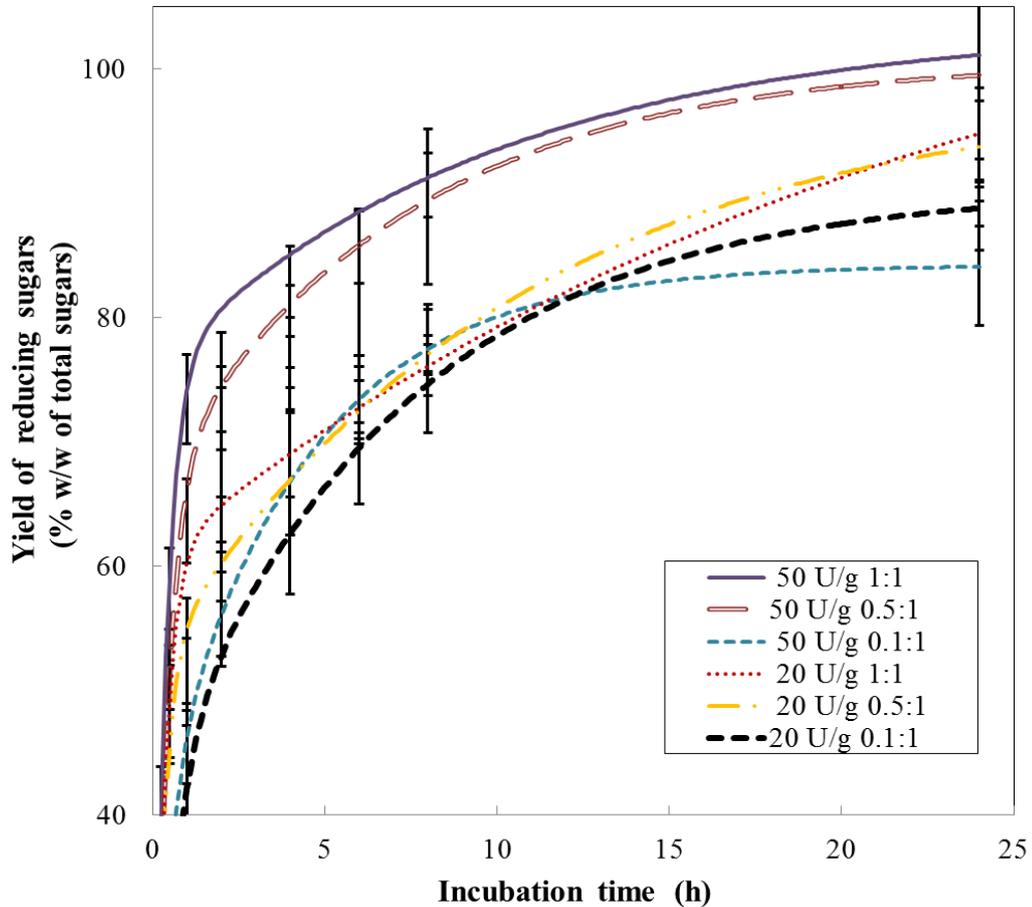
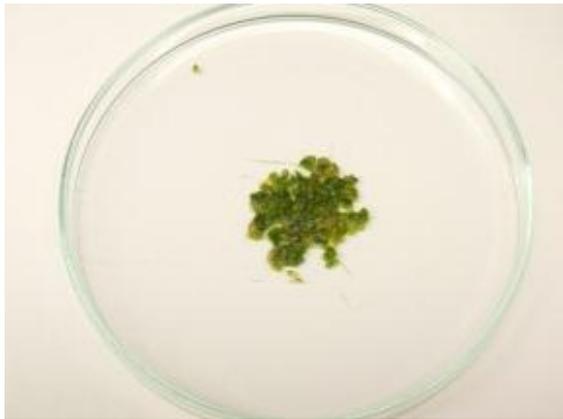


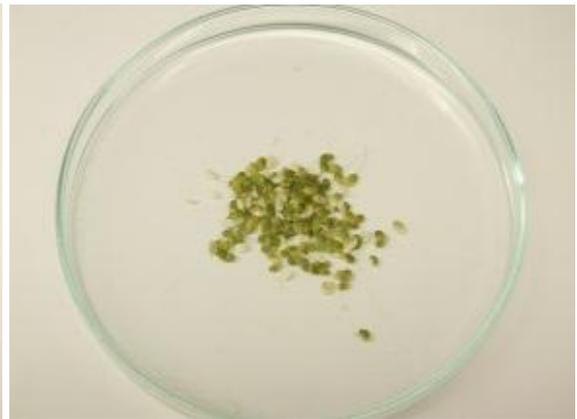
Figure 56. Optimization of enzyme saccharification using reduced CE: BG ratios (1: 1, 1: 0.5, and 1: 0.1), at CE concentrations of 50 and 20 U/g substrate.

Having established optimal CE and BG concentrations the comparative effects of a number of pretreatments on the saccharification of duckweed were investigated. These involved steam explosion, freeze drying, freeze drying and freeze milling, preparation of a water-insoluble alcohol insoluble residue and untreated (fresh) material as a control (see Figure 57). The same mass of material prepared by the different pretreatment methods was hydrolysed using the optimised enzyme cocktail, CE 20 U (0.87 FPU)/g substrate, BG 2 U/g substrate. The results (Figure 58) show that freeze drying alone is a poor pretreatment, producing little if any increase in glucose yield when compared to untreated material. Saccharification of blender-milled, water-insoluble alcohol-insoluble residue (WIAIR) resulted in a glucose yield of only 40 %. Freeze milling increased glucose yield to 55 %, 1.4 fold higher than the glucose yield of WIAIR but steam explosion was by far the most

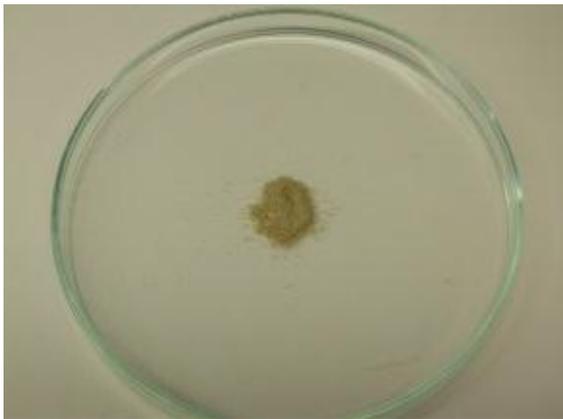
effective pretreatment tested, resulting in a glucose yield to 80 %. Previously, we have obtained similar glucose yields from enzymatic saccharification of WIAIR but only by using much higher enzyme concentrations (CE at 100 U (4.35 FPU)/g substrate plus additional BG at 100 U/g substrate). Steam explosion pretreatment thus greatly enhances the digestibility of duckweed material and enables effective saccharification at reduced enzyme concentrations.



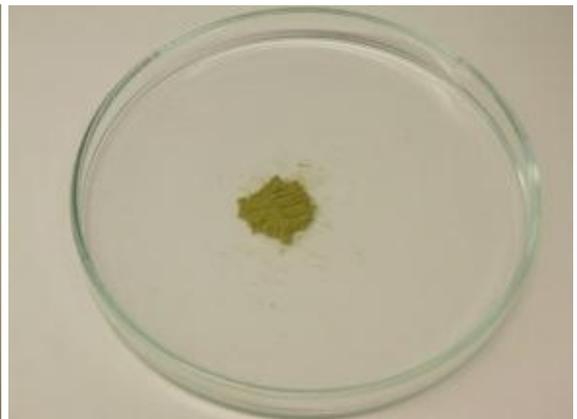
a. Fresh wet duckweed



b. Freeze dry materials



c. Freeze dry and freeze mill



d. WIAIR



e. steam exploded slurry (210 °C)

Figure 57. The different pretreated materials were used for enzymatic saccharification.

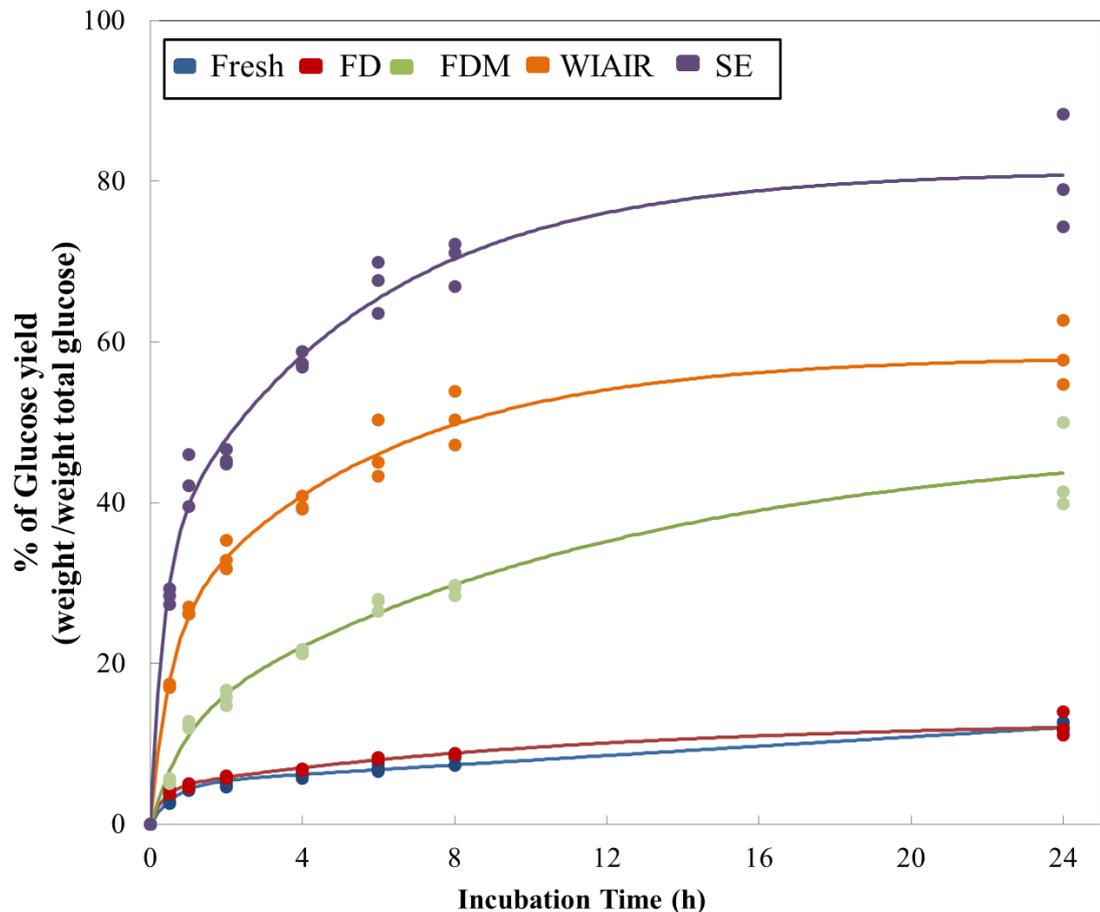


Figure 58. Glucose yield produced by hydrolysing different pretreated duckweed samples with the optimised enzyme cocktail (CE 20 U/g and BG 2 U/g). ‘Fresh’ - untreated duckweed material; ‘FD’ - fresh material that has been freeze dried; ‘FDM’ – freeze-dried and freeze-milled material; ‘WIAIR’ - blender-milled, water-insoluble alcohol-insoluble residue; ‘SE’ – 210 °C steam exploded material.

An effective pretreatment is determined by following criteria: it avoids the cost imposed by reducing the size of biomass particles, avoids the loss of fermentable sugars, resists the formation of fermentation inhibitors, and minimises input energy and cost (National Research Council, 1999). SE requires energy input but, especially at higher severities, results in tissue disruption and renders duckweed biomass much more susceptible to enzymatic saccharification without further treatment. SE pretreatment removes the requirement for physical treatments such as grinding and drying. It also greatly reduces the enzyme dosages required in the saccharification process. Enzymes are a major economic cost in conversion of biomass to bioethanol. Fermentation inhibitors were detected, but only at relatively low concentrations. The low levels of these inhibitors and the pH of the

slurry provide environment that is suitable for subsequent enzymatic saccharification and should be suitable for fermentation. It is concluded that steam explosion alone appears to be an effective pretreatment of duckweed biomass.

#### 5.2.6 CTec 2 identification

Our previous research has illustrated a good digestibility with enzyme cocktail CE+BG on steam exploded *L.minor* at a very low concentration of 20 U or 0.87 FPU/g substrate of CE and 2 U/g substrate of BG. Unfortunately, CE is relatively expensive and not economic for industrial production. CTec 2, as a new generation of cellulase product, contains higher cellulase activity reported to vary from 119 to 132 FPU/mL (Reye et al., 2011; McIntosh et al., 2012). To replace CE by using CTec 2 in fermentation study, the appropriate CTec 2 dosage was identified by a comparative saccharification with Celluclast. Steam exploded (210 °C) materials were hydrolysed by using CTec 2+BG with different ratios of CTec 2: BG, 10 fold of CTec 2 only and CE+BG as a control. The enzyme cocktails were prepared following cellulase (0.87 FPU/g substrate) + BG (2 U/g substrate). The amount of glucose (6 %, Figure 59) released by the steam explosion pretreatment was measured using GOPOD method and deducted from the glucose yield calculations. Figure 59 shows that the enzyme cocktail of CTec 2+BG could result in a very similar glucose yield (76.6 %) over a 24 h incubation when compared with CE+BG. However, the higher dosages of CTec2+BG enhanced little glucose yields. Enzymatic hydrolysis using only CTec 2 produced a much lower glucose yield (59.4 %) even when the cellulase dosage was increased to 10 times. These data reflect the facts that CTec 2+BG has very similar digestibility to CE+BG, the necessity for additional  $\beta$ -glucosidase and the important synergy between CTec 2 and BG for the saccharification of steam exploded duckweed. Eventually, CTec 2+BG containing CTec 2 (20 U or 0.87 FPU/g substrate) and BG (2 U/g substrate) was selected as the optimal enzyme preparation for the simultaneous saccharification and fermentation work.

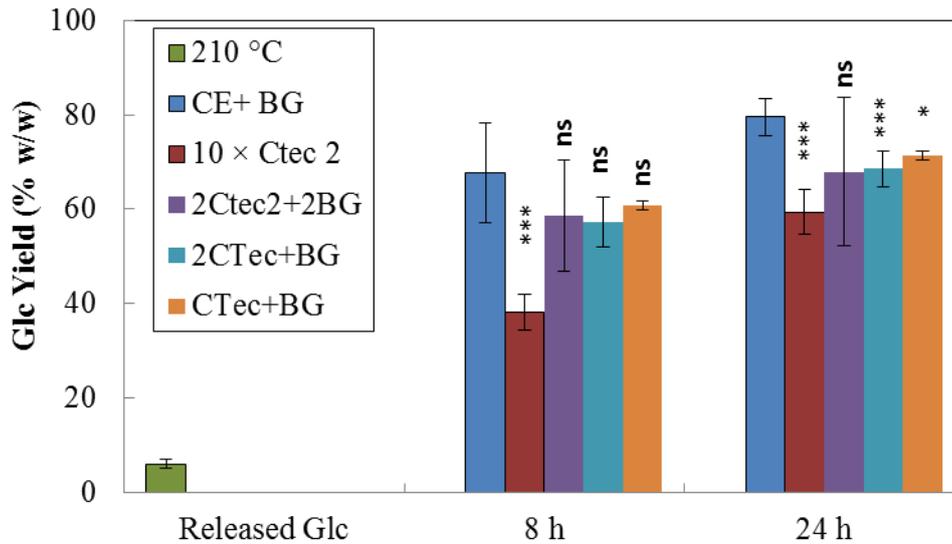


Figure 59. The identification of CTec 2 dosage by using the same amount enzyme as Celluclast to hydrolyse steam exploded (210 °C) materials. The enzyme dosage is cellulose at 0.87 FPU/g substrate, BG at 2 U/g substrate. Released Glc represents the amount of glucose produced in steam explosion. \*\*\*  $p < 0.001$ , \*  $p < 0.05$ , ns = not significant ( $p > 0.05$ ) compared with the sample of CE + BG.

The measurement of  $\alpha$ -amylase in the enzyme cocktail of “CTec 2+BG” is one supplementary experiment to understand the activity of this cocktail. The existence of  $\alpha$ -amylase in cellulase commercial enzymes has been unveiled by high glucose yields (> 80 % of total glucose) of enzymatic saccharification in previous research § 4.2.3 and § 5.2.5. In this experiment, the standard starch from wheat was hydrolysed by the enzyme cocktails of CE+BG and CTec 2+BG and the three individual commercial enzymes. Two methods were used for hydrolysing starch: one is following the enzymatic saccharification method (Method A) and the other is following the total starch assay (Method B) as the method control. Results of Figure 60 illustrate that the overall commercial cellulase enzymes contain certain levels of  $\alpha$ -amylase. The highest digestibility (10.4 % of total starch) of starch was obtained using BG in Method A whereas only 2.4 % starch was hydrolysed by BG in Method B. However, antipodal results of starch hydrolysis are gained using CTec 2 between two methods as 7.5 % of starch hydrolysed by Method B and 3.8 % of starch hydrolysed by Method A. Only approximately 2.5 % of starch was degraded by CE in both methods. The detail of  $\alpha$ -amylase in CE, CTec 2 and BG has not been further researched, but literature suggested that  $\alpha$ -amylase is secreted by fungi strains of *Trichoderma spp* – the strain used for producing cellulase industrially (El-awamry et al, 2012). The starch-rich

duckweed biomass might result in the enhancement of amylase secreting of *Trichoderma spp* and starch is therefore hydrolysed dramatically. It should be noted that similar amounts of starch was hydrolysed by enzyme cocktails of CE+BG (22.7 %) and CTec 2+BG (20.6 %), but these are significantly greater than the aggregations of the individual enzymes. This implies that enzymatic synergy of CE and CTec 2 with BG contributes to the increase of starch degradation. As we investigated previously, the supplement of additional  $\beta$ -glucosidase intensifies the degradation of cell wall. This important improvement is likely to enable the intracellular starch to be exposed to  $\alpha$ -amylase. As the best of our knowledge, no research has reported the synergy of cellulase and starch. However, duckweed as one starch- and cellulose-rich biofuel feedstock requires specific commercial enzyme containing cellulase and amylase supplementing with  $\beta$ -glucosidase. Figure 59 & Figure 60 demonstrate that the optimised enzyme cocktail of CTec 2+BG possesses relatively ideal digestibility of duckweed biomass and potentially economically utilized for fermentation research which generally requires concentrated enzymes.

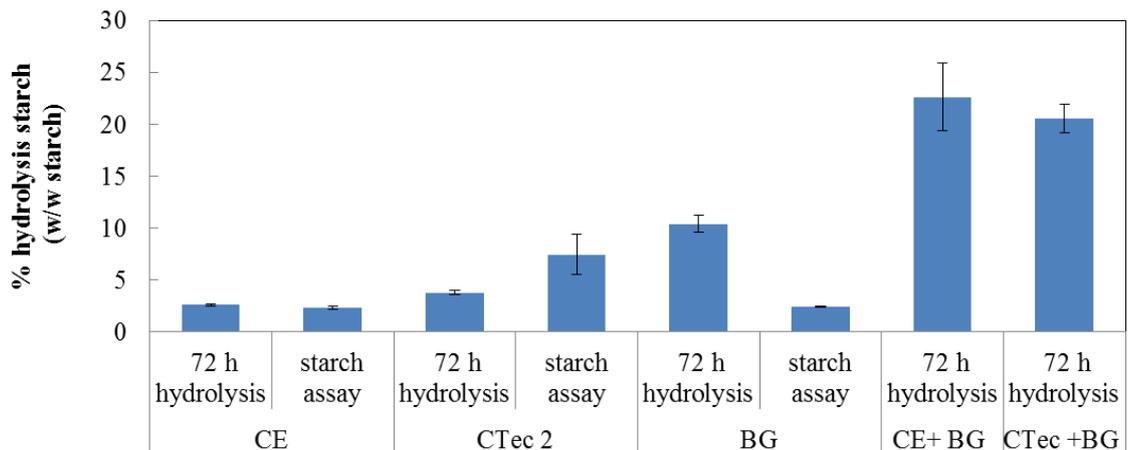


Figure 60. The identification of  $\alpha$ -amylase activity of cellulase commercial products. Substrate is wheat starch (96 % w/w)

### 5.3 Conclusion

Steam explosion as a thermochemical pretreatment effectively damages the plant structure and produces easily digestible CWM in tiny particle pellets at SE temperatures above 210 °C. However, a significant mass loss is observed at 230 °C which reduces the final ethanol yield. The high conversion (70 %) of WSM has been obtained from SE slurry at 210 °C since a large proportion of starch (20 % DM) is solubilised and hemicellulose (60 % by carbohydrate) and pectin (60 % by carbohydrate) are degraded by SE pretreatment at severity 4.2. The most economic and efficient enzymatic saccharification is optimised by reducing Celluclast from 100 U or 4.35 FPU/g substrate down to 20 U or 0.87 FPU/g substrate, with additional beta-glucosidase (from 100 to 2 U/g substrate, CE: BG at 1: 0.1) and yields of reducing sugars (88.3 %) and glucose (70.9 %) are achieved for the subsequent fermentation. Moreover, low concentrations of fermentation inhibitors and a favourable pH (5.6) are produced in the SE process at 210 °C which is beneficial for the subsequent fermentation. The severity factor 4.2 (210 °C, 10 min) is the optimal steam explosion pretreatment for *L. minor* biomass to achieve maximum yield of reducing sugars.

## 6 *Simultaneous saccharification and fermentation of steam exploded duckweed*

After a series of pretreatments, enzymatic saccharification, stubborn polysaccharides have been hydrolysed to fermentable monosaccharide which will be continuously converted to ethanol in a further study. Although ethanol fermentation has been developed for hundreds of years, the insufficiency of ethanol fermentation for bioethanol production from aquatic plants still requires extensive improvements, e.g. the innovation of specific fermentation processing, genetic transformation of yeast and identification of substrate concentrations. With current fermentation techniques, the energy conversion rate of 2<sup>nd</sup> generation biofuel production is inefficient (International Energy Agency, 2010) which demands the huge consumption of enzymes and feedstock. The energy efficiency of biochemical conversion mean of biomass to ethanol is only 28.6 % (IATA, 2009). Simultaneous saccharification and fermentation (SSF) will be considered as the prime option based on stacks of literature research and the advantages of SSF has been already stated in § 1.6.2. Another precondition of SSF of duckweed has been already provided by steam explosion pretreatment as high level of fermentable sugars were produced. SSF of SE slurry allows reducing the fermentation duration and energy input of the entire production. In this chapter, SSF of SE duckweed slurry will be intensively researched as the prime means for producing ethanol from duckweed.

### 6.1 **Material and methods**

#### 6.1.1 *Plant material*

*L. minor* biomass materials are collected from the pond of John Innes Centre and the preparation process was as described in § 2.1.2. The prepared frozen materials were used for steam explosion pretreatment. Another two types of pretreated duckweed materials -- fresh wet and FDM materials were also fermented in a comparative experiment.

#### 6.1.2 *Steam explosion pretreatment (SE)*

The raw wet *L. minor* biomass was treated by steam explosion at 210 °C and the products (SE slurry) were prepared for subsequent saccharification and fermentation. The steam explosion process was introduced in § 2.4. Steam exploded slurry was frozen in a coldroom (-40 °C) until required.

### 6.1.3 *Enzymatic saccharification of steam exploded slurries*

The new enzyme cocktail of CTec 2 (cellulase: 20 U or 0.87 FPU/g substrate) with additional BG (2 U/g substrate) identified in previous section § 5.2.6 was onwards involved in the fermentation research.

### 6.1.4 *The preparation of substrate for fermentation*

SE slurry and pellets were fermented by using a range of dry matter levels. The original % DM of SE slurry is from 2.3 - 2.8 % (w/w). The dry matter varied among different batches of fresh duckweed and their % DM was measured individually. Different means were used for preparing the fermentation substrates required in the concentrations.

#### 6.1.4.1 The preparation of SE slurry (% DM $\leq$ 2 %)

For low levels of dry matter substrate (% DM  $\leq$  2 %), the slurry was straight used as fermentation substrate.

#### 6.1.4.2 The preparation of SE slurry (% DM $\geq$ 2 %)

For high levels of dry matter substrate (% DM  $\geq$  2 %), the slurry was condensed by using rotated vacuum- evaporation method. 200 mL of original SE slurry was transferred into a pre-weighed round-bottom flask. The flask was mounted on the Rotavapor R-114 (BUCHI UK Ltd, Oldham, UK) with adaptors and was fasten by clips. The evaporation was progressed under vacuum condition at 50 °C. The percentage of dry matter was monitored by measuring gravimetric difference of samples and finally identified by using a Mettler Toledo LP16 Infrared Dryer balance (Mettler Toledo Ltd, Beaumont Leys, Leicester, UK). The concentrated SE slurry was eventually recovered to the percentage of dry matter required in the fermentation process.

#### 6.1.4.3 The preparation of pellets

The original SE slurry (50 mL) was transferred into a centrifuge tube and centrifuged at 3500 rpm for 10 min. The supernatant was removed and pellets were washed using 10 mL distilled water twice. The washed pellets were used as fermentation substrate and recovered to the percentage of dry matter required in the fermentation process.

### 6.1.5 Yeast preparation

#### 6.1.5.1 Yeast culture in YM medium

*Saccharomyces cerevisiae* strain (NCYC 2826) obtained from the National Collection of Yeast Cultures (NCYC, Norwich, UK) was used throughout this project due to its high ethanol tolerance of 15 - 20 % (v/v). The strain was sub-cultured from a slope culture by inoculation into 1 L of Difco™ Yeast and Mould (YM) broth (Fisher Scientific UK Ltd., Loughborough, UK) which contains 0.3 % (w/v) yeast extract, 0.3 % (w/v) malt extract, 0.5 % (w/v) peptone and 1 % (w/v) dextrose (Elliston et al., 2013). Yeast was grown in this medium over 2 days at 25 °C. Then, the yeast suspension was stored in a fridge at 4 °C for up to 1 month before use (Elliston et al., 2013). Before yeast was inoculated to fermentation reaction solution, the cultured yeast medium was centrifuged (3000 rpm, 5 min, Centrifuge 5810 R, Eppendorf UK Ltd., Stevenage, UK) and the supernatant (YM media) was disposed and the yeast cells were reconstituted in nitrogen base (ForMedium™, Formedium Ltd, Hunstanton, UK). The total viable yeast cells were measured by using a cell count reader (NucleoCounter® YC-100™, ChemoMetec, Allerød, Denmark).

#### 6.1.5.2 Subculture of yeast in SE medium

Yeast was also raised in the steam exploded liquor (containing water soluble sugars) to investigate the adaptability of yeast to SE medium. The SE medium was prepared as follows: a range of SE slurries (150, 170, 190, 210 and 230 °C) were centrifuged and supernatants were transferred into sealed bottles which were subsequently autoclaved before yeast suspension was added. With 10 % (v/v) yeast inoculation, yeast was grown in the SE medium for 2 days. The total viable yeast cells were measured using a cell count reader. On the other hand, the kinetics of yeast growing in the YM and SE media were established using a Microplate Spectrophotometer (Benchmark Plus, BioRad, CA, USA). A small scale yeast culture (200 µL) in SE medium was carried out on a 96-well flat-bottomed microtitre plate with lid (Nunc, Roskilde, Denmark). Yeast was also cultured in YM medium (200 µL) as a positive control and yeast free media were prepared as blank controls simultaneously. The turbidity of culture solution was measured in a Microplate Spectrophotometer (Benchmark Plus, BioRad, CA, USA) at  $\lambda = 590$  nm at 30 min intervals, which can be referred to the quantity of yeast cells (Blomberg, 2011).

### 6.1.6 A small scale SSF process

SSF of steam exploded duckweed (1, 5, 10, 20 % w/v) was performed in glass universal bottles (Figure 61). The reaction solution (10 mL) contained: substrate (1, 5, 10, 20 % w/v), 10 % (v/v or  $8.0 \times 10^7$  cells/mL) of *S. cerevisiae* NCYC 2826, CTec2 (20 U or 0.87 FPU/g substrate) + BG (2 U/g substrate) and Nitrogen base (6.9 g/L) (Elliston et al., 2013). The concentration of enzymes and nitrogen base were increased in proportion to the substrate concentration. Upon the yeast inoculum 10 % (v/v) of final fermentation solution, yeast was further concentrated to 4, 10, 20 and 50 times in the investigation of the effect of yeast inoculum. The SE slurries (20 % w/w DM) were separately inoculated with YM cultured yeast in 4 times ( $3.2 \times 10^8$  cells/mL), 10 times ( $8.0 \times 10^8$  cells/mL), 50 times ( $4.0 \times 10^9$  cells/mL) of the norm and SE medium cultured yeast in 1 time ( $1.8 \times 10^7$  cells/mL), 2 times ( $3.6 \times 10^7$  cells/mL), 4 times ( $7.2 \times 10^7$  cells/mL) and 20 times ( $3.6 \times 10^8$  cells/mL) of the norm and the enzyme cocktail and nitrogen base were proportionally added to top the volume up to 10 mL. Substrate blanks were prepared as a control to detect fermentable sugars and ethanol from YM solution or enzymes and subtracted from the sample readings. SSF samples were incubated over 3 days at 25 °C with moderate agitation (120 rpm) in general although strong agitation (300 rpm) was used to assess the effect of agitation on ethanol yield of higher substrate concentration (20 %). Aliquots (2 mL) of fermented samples were transferred to screw-cap tubes and were boiled for 5 minutes to terminate the SSF. The resulting samples were centrifuged and the supernatants were assessed for ethanol and fermentation inhibitors using HPLC methods.



Figure 61. The fermentation vessels for a small scale SSF.

### 6.1.7 Analytical methods

#### 6.1.7.1 The assessment of ethanol and residual fermentable sugars

The ethanol product and the residual fermentable sugars were measured by using HPLC method (see § 2.8.2). Briefly, SSF samples were centrifuged, filtered, measured using HPLC fitted with carbohydrate analysis column with RI detector (Elliston et al., 2013). The ethanol yield is expressed as Equation 17 to demonstrate the effectiveness of SSF on steam exploded duckweed.

$$\text{Ethanol yield} = \frac{\text{ethanol product (g)}}{\text{theoretical ethanol products(g)}} \times 100\%$$

Equation 17. The calculation of ethanol yield (% w/w).

The amount (g) of theoretic ethanol product was obtained from molecular weight of glucose (180.2 g/mol) and ethanol (46.1 g/mol) which was explained in Equation 4. Thus, the theoretical ethanol product is 51.2 % (w/w) of glucose matter.

#### 6.1.7.2 GC sugar analysis

The assessment of monosaccharide compositions of fermented residue were analysed by using GC sugar method (see § 2.7).

#### 6.1.7.3 GOPOD test

The hydrolysed glucose and unfermented glucose were detected by the specific GOPOD test method (McCleary et al, 1994). The preparation and measurement were introduced in § 2.9.2.

#### 6.1.7.4 Inhibitors assessment

Fermentation inhibitors of 2-FA, 5-HMF, formic and acetic acids produced in steam explosion process were measured to estimate their inhibitions of SSF. The concentration of organic inhibitors was analysed by HPLC using a Flexar LC instrument (Perkin Elmer, Seer Green, Bucks, UK) equipped with refractive index and photo diode array detectors (reading at 210 nm wavelength) in series. Details of analysis method was described in § 2.8.4.

## 6.2 Results and discussion

### 6.2.1 The adaptability of *S.cerevisiae* to steam exploded slurry

The adaptability of the chosen yeast strain to YM and media containing liquor from steam exploded duckweed (SE media) was assessed by a 96 h yeast culture in a small scale (200  $\mu$ L) in a 96-well plate. Figure 62 shows the growth kinetics of yeast in YM medium and a range of SE media. Over a 96 h incubation, yeast growing in YM medium exhibits three growth phases: a very short lag phase, a short and fast exponential growing phase (less 24 h) and a long stationary phase (72 h). Ciani and Picciotti (1995) observed similar growth kinetics for various yeasts used in wine production. In our study, yeast growing in the SE media generally exhibited a variable lag phase then underwent a linear growth phase longer than that observed in YM medium. The exponential growing phase of SE media continued throughout the entire 96 h incubation (after the lag phase) except SE medium with liquor for SE at 230 °C, for which growth phase terminated about 80 h with the turbidity of 0.65 OD<sub>590</sub> units (Figure 62). The turbidity of 150, 170 and 190 °C SE media is all close to 0.5 OD<sub>590</sub> units. Unexpectedly, yeast cultured in SE medium made with liquor from SE at 210 °C only showed in a small increase of turbidity, 0.3 OD<sub>590</sub> units, over the 96 h incubation. The turbidity in SE media from SE at 150, 170, 190, 210 and 230 °C are respectively 58, 58, 58, 35 and 76 % that of yeast in YM medium. Considering the fermentable sugars concentrations of YM (10 mg/mL of glucose) and SE medium (1 mg/mL of glucose), yeast displays a great adaptability to SE medium. However, 210 °C was already established as the optimal temperature condition for steam explosion of duckweed (unpublished data) and a larger quantity of SE slurry at 210 °C was produced for subsequent fermentation. In practice, yeast pre-cultured by SE medium of 210 °C is thereby considered for the further ethanol fermentation.

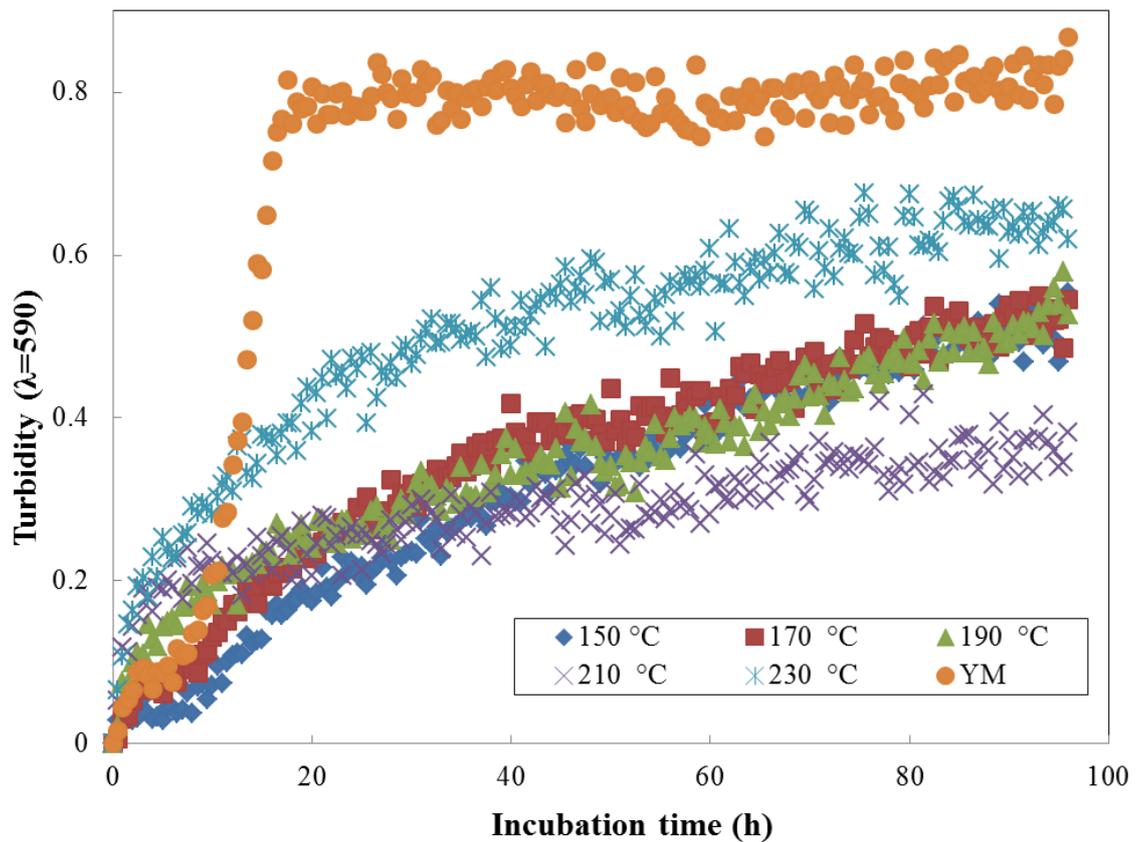


Figure 62. A 96 h yeast culture in YM medium and various SE liquors pretreated at different temperature. Sugars concentrations of SE liquors are 1 mg/mL while the concentration of YM is 10 mg/mL. Turbidity represents the concentration of yeast cells.

Yeast strain adaptability to YM and SE (210 °C) medium was continuously established by viable yeast cells. The yeast growth in the standard YM medium proliferated to  $8 \times 10^7$  of viable yeast cells which was 4 times of SE medium ( $1.8 \times 10^7$  cells/mL) (see Table 17). However, a high number of dead cells ( $2.9 \times 10^6$  cells/mL) were present in YM medium compared to dead cells ( $\leq 6 \times 10^3$  cells/mL) in SE medium (210 °C). The ratio of viable yeast cells between YM medium and SE medium (210 °C) closely matches the ratio of yeast densities in Figure 62. These results from a combination of previous data of Figure 62 illustrate that in 2 days incubation, yeast growth in SE medium is likely to remain in the linear growth phase but with less increase of yeast cells whereas yeast growth already enters the stationary phase or the dead phase in light of the large amount of dead yeast cells (Table 17) (Lo et al, 1997). Thus, the incubation time of yeast subculture in YM media can be condensed to 24 hr (see Figure 62) and the relatively high quantity of viable yeast cells is potentially obtained using SE media within 2 days.

Table 17. Viable yeast cultured by using YM and SE liquor. Sugars concentrations of SE liquors are 1 mg/mL while the concentration of YM is 10 mg/mL.

Medium	Total cells (numbers/mL)	Dead cells (numbers/mL)	Viable cells (number/mL)
SE medium	$1.8 \times 10^7$	$\leq 6 \times 10^3$	$1.8 \times 10^7$
YM	$8.3 \times 10^7$	$2.9 \times 10^6$	$8 \times 10^7$

### 6.2.2 Ethanol productivity of SSF different pretreated duckweed

The effect of pretreatments on ethanol yield was initially investigated by fermenting various pretreated materials including fresh raw (untreated), FDM and SE (210 °C) materials. Ethanol yields were assessed using the HPLC method and the results are shown in Figure 63. Ethanol yield of SSF on untreated materials only obtained 31.4 % (w/w of theoretical ethanol) whereas FDM pretreatment dramatically enhanced the ethanol yield to 61.3 % (w/w of theoretical ethanol) which is almost 2 times ethanol yield of untreated materials. However, steam explosion resulted in a further increase in ethanol yield up to 79.9 % (w/w of theoretical ethanol) at a low substrate concentration (1 % w/w DM). The conspicuous leap of ethanol yields on different pretreated materials indicates that physical breakdown of botanic structure in FDM pretreatment effectively intensifies ethanol yield and SE pretreatment exerts a stronger effect on the degradation of botanic structure than FDM pretreatment for its higher ethanol yield. Extra pretreatments prior to enzymatic saccharification enable lignocellulosic biomass to be more susceptible to enzyme accessibility (Waldron, 2010). The great effect of thermal-pretreatment including steam explosion on removal of lignin and hemicellulose fractions has been clearly stated by Chundawat et al (2010). Ethanol yield (85.3 % of theoretical ethanol) was also obtained by SSF on SE wheat straw at 16.7 % (w/w of DM), but requires extra alkaline peroxide pretreatment and high concentration of cellulase (40 FPU/g substrate) (Chen et al, 2008). Steam explosion on duckweed biomass investigated previously by authors (publication in progress) demonstrated that SE considerably exposed cellulose to enzyme without extra pretreatment and enzyme dosage was thereby reduced to a very low concentration (0.87 FPU/g substrate).

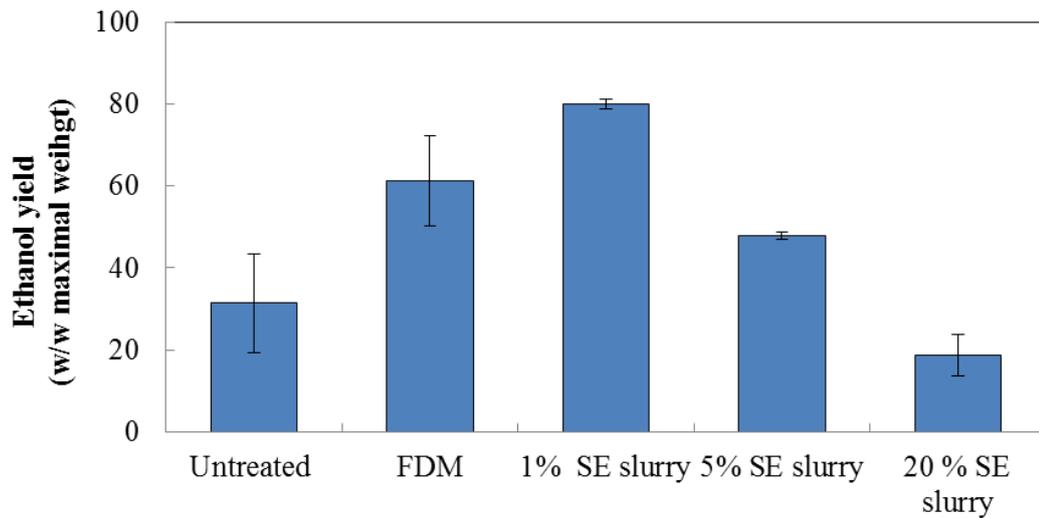


Figure 63. Ethanol yields of SSF from different pretreated materials and SE materials at different substrate concentrations.

The effectiveness of SSF on SE (210 °C) duckweed is reflected in sugars concentrations of the dilute SE slurry (1 % w/w substrate concentration) and remaining in SSF pellets analysed. The concentrations variation of total sugars and monosaccharides (see Figure 64) potentially illustrates fermentable sugars consumption during the SSF process. In the substrate, carbohydrate accounts for 477.4 mg/g, while glucose as the predominant monosaccharide accounts for 352.5 mg/g followed by GalA (66.1 mg/g) and xylose (31.7 mg/g), with traces of galactose (13.3 mg/g), mannose (6.6 mg/g), arabinose (3.7 mg/g), rhamnose (2.4 mg/g) and fucose (1.1 mg/g). In the pellet, total sugars decrease to 162.3 mg/g which is attributed to the dramatical decrease in glucose (from 352.5 mg/g to 98.2 mg/g) and GalA (from 66.1 mg/g to 10.8 mg/g). The other monosaccharides were usually unchanged in their concentrations. During the SSF process, 66 % of carbohydrate was degraded, of which 72.2 % of the glucose was converted to ethanol and 85 % of the GalA was degraded. A trace of xylose and galactose were metabolised to ethanol by *S. cerevisiae* during SSF. The amount of glucose consumption is closely matched to the ethanol yield (Figure 63). In terms of the remarkable disappearance of GalA, it might be caused by the uptake of GalA by *S. cerevisiae* at pH 3 - 6.5 since GalA is a hydrophilic compound (Souffriau et al., 2012). However, GalA cannot be metabolised by *S. cerevisiae* and also inhibit fermentation (Huisjes et al., 2012). The authors reported that GalA inhibited the metabolism of galactose, xylose and arabinose at pH 3.5 in *S. cerevisiae* but not including

glucose. Since duckweed is rich in pectinaeous materials, the inhibition of GalA might be considerable for the fermentation of xylose and galactose compounds.

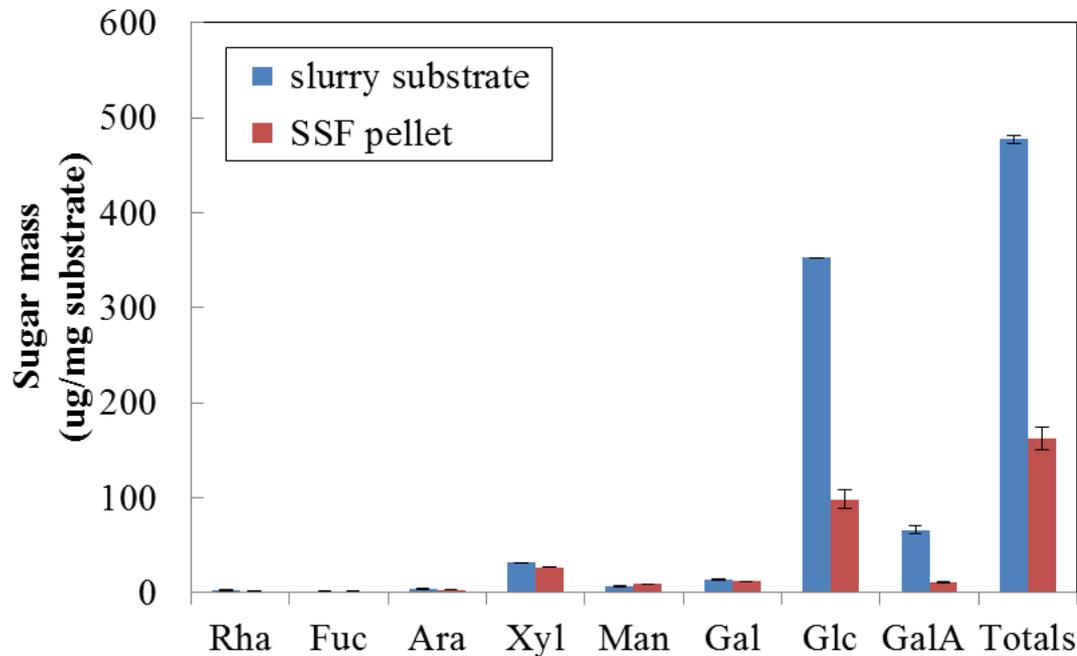


Figure 64. Sugar analysis of SSF substrate and pellet. Substrate is SE slurry at 210 °C. the small scale SSF.

### 6.2.3 Investigation of digestibility of concentrated SE materials

The low substrate concentration (1 % w/v) provides a good insight to determine the maximum ethanol yield of SSF SE duckweed (Figure 63). However, ethanol yield decreased markedly when substrate concentration increased. In Figure 63, only 47.7 % and 18.8 % (w/w of theoretical ethanol) ethanol yields were achieved by fermenting 5 % and 20 % (w/v) samples respectively. The dramatic decrease in ethanol yield suggests that substrate concentration would strongly limit the ethanol yield under the original experimental conditions. It should be noted that fermentation at lower substrate concentration will increase considerably the cost of downstream distillation. Since the starting ethanol concentration required for distillation is generally considered to be in the order of 5 - 7 % (Monash Scientific Glass Blowing Services, 2013), SSF of duckweed should contain 20 % (w/v) of biomass due to its 35.2 % of glucose. Thus, concentrating the substrate prior to fermentation is the prerequisite for SE duckweed materials. The vacuum evaporation process was aimed to condense the original SE slurry (around 3 % w/w DM) to 20 % (w/v). The moisture content was approximately controlled by gravimetric

difference at certain time points. The relationship of percentage of dry matter and evaporation interval (Figure 65) shows that the aimed dry matter could be complete in a 6.5 hour. In general, the evaporation tends to display in an exponential curve that might shift based on the vacuum and temperature conditions.

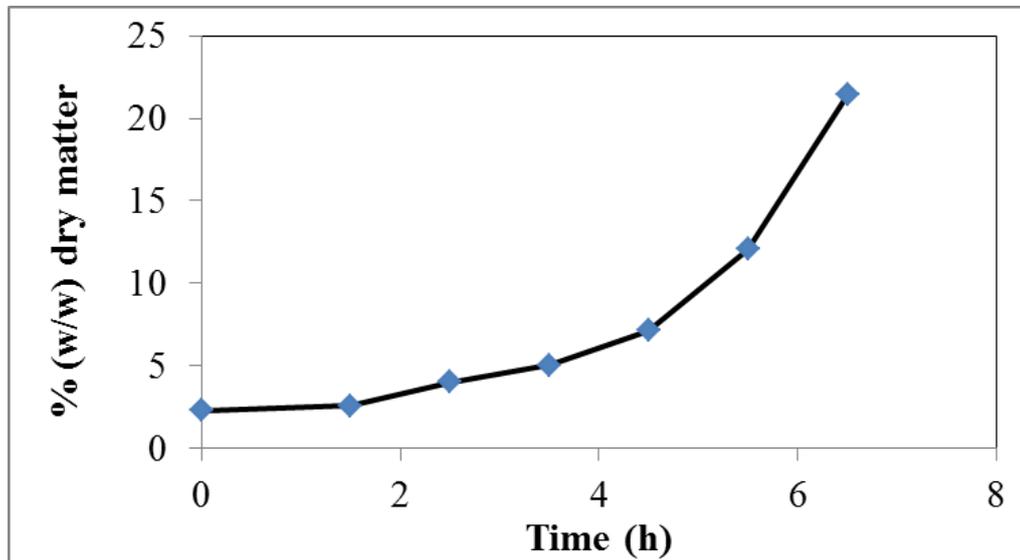


Figure 65. The relationship of the dry matter and interval of vacuum evaporation.

The vacuum evaporation method was used to concentrate steam exploded duckweed and to potentially remove volatile inhibitors (formic and acetic acids) in duckweed. However, vacuum evaporation might cause the hornification of concentrated SE materials and consequently affect the ethanol yield (Luo and Zhu, 2011). The impact of evaporation on the digestibility of concentrated SE slurry was assessed by enzymatic saccharification (see Figure 66 and Figure 67). In Figure 66, although the enzyme cocktail of CTec 2 (0.87 FPU/g) + BG (2 U/g) was added into a range of concentrated materials in proportion, the trend shows that the glucose yield still declined in contrast to substrate concentration. Only averagely 47.7 % of glucose was released in 20 % slurry, in which released glucose was reduced by 39 % compared to that of the original SE slurry (2.3 % DM) (Figure 66). To understand the decrease in glucose yield shown in Figure 66, an additional enzymatic saccharification was carried out on the same materials which were reconstituted to the same dry matter content using a consistent enzyme cocktail. In comparison with Figure 66, similar glucose yields (2.5 mg/mL) were obtained by the samples in overall concentrations (Figure 67), which suggests vacuum evaporation (up to 20 % w/w DM) does not lower the

digestibility of SE materials. Thus, the drop of glucose yield is probably due to the high viscosity of the material and low fluidity of the enzyme.

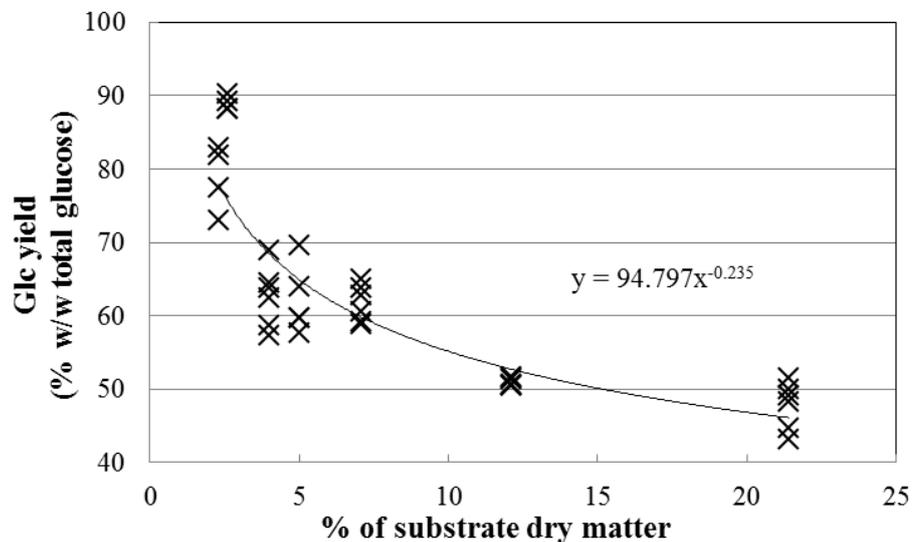


Figure 66. Enzymatic saccharification on concentrated materials.

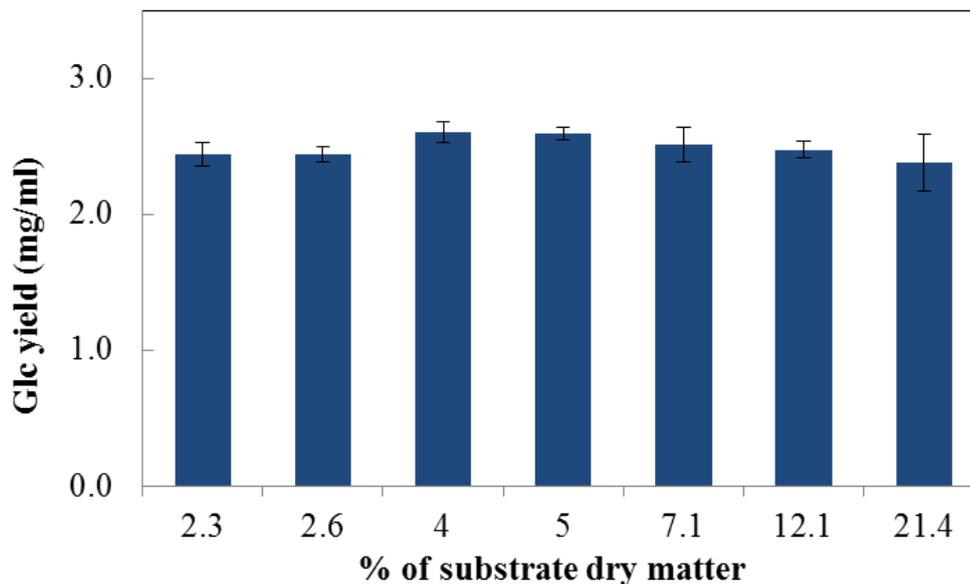


Figure 67. Enzymatic saccharification on concentrated materials with diluting to the same amount of dry matter. The whole samples in this experiment do not display a significant difference ( $F(6, 35) = 1.77, p = 0.133$ ).

Fermentation inhibitors produced from SE duckweed biomass including breakdown products, such as organic acids (formic and acetic acid), 5-hydroxymethylfural (5-HMF) and 2-furfuraldehyde (2-FA) were studied previously (§ 5.2.4) and measured here to

establish the fermentation inhibition of SE slurry at higher substrate concentrations (Figure 68). The concentrations of 2-FA and 5-HMF in the original SE slurry (2.3 % DM) are only 3.0 and 6.2 mg/g of DM, which are not expected to cause a significant inhibitory effect on subsequent fermentations (Almeida et al, 2007). However, the concentrations of acetic and formic acids are 44.0 mg/g and 89.0 mg/g which are significantly higher than the inhibitory levels reported by Almeida et al (2007). The inhibition degree of formic acid (80 %) and acetic acid (74 %) were expressed when their concentrations reached 27 and 60 mg/g respectively (Pienkos and Zhang, 2009; Maiorella et al, 2004). Figure 68 illustrates the concentration variations of inhibitors in the evaporated residual pellets. In the vacuum evaporation process, the volatile acids reduced considerably in the overall evaporated residues although independently of substrate concentrations which released similar levels for each inhibitor. The reduction of formic and acetic acids is attributed to the effectiveness of vacuum evaporation on volatile acids (Chandel et al, 2011). 2-FA and 5-HMF stabilised in the process due to they did not volatilize in the process but the quantities of 2-FA and 5-HMF perform unclear trends (Figure 68). However, this effect was not reflected in the concentrations of volatile acids in the aqueous phase (Figure 68) which was removed from SE slurry by the vacuum evaporation. In summary, at higher substrate concentrations (10, 20 %), 2-FA and 5-HMF are not likely to give any inhibitory effects but the concentrations of acetic and formic acid must be considered in subsequent fermentations.

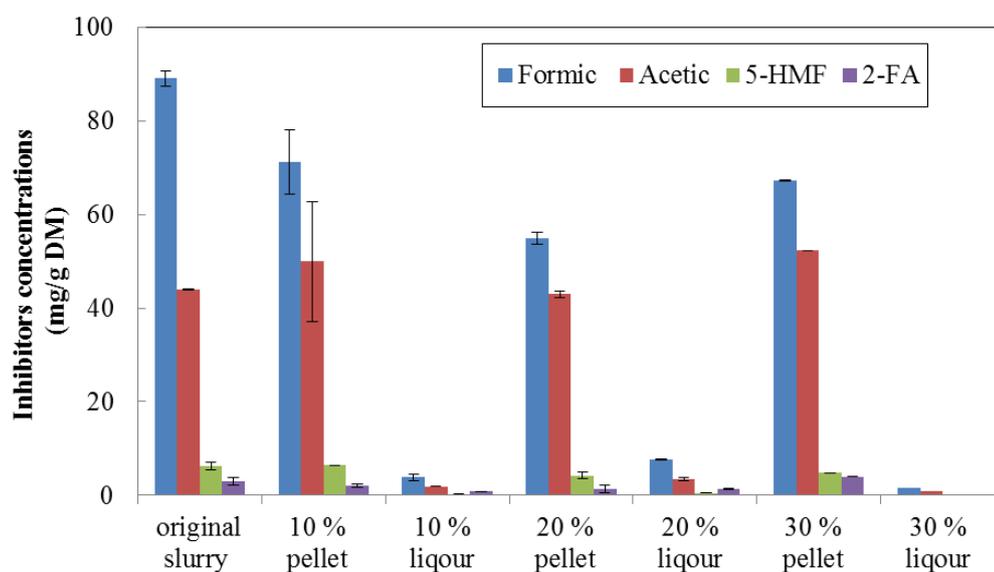


Figure 68. Inhibitors measurement of various concentrated SE slurries. Original slurry contains 2.3 % DM. Liquor represents the sample containing the redissolved inhibitors from the concentrated materials.

#### 6.2.4 Improvement of ethanol yield of SSF on 20 % substrate concentration

Our previous study on enzymatic saccharification indicated that increased agitation may be beneficial for enhancing the ethanol yield of SSF concentrated materials. Given the reduced ethanol yield observed under mild agitation (120 rpm) (Figure 63), strong agitation was investigated. This experiment was designed to ferment SE slurries at a range of substrate concentrations using 4 times concentration (yeast cells) of YM yeast inoculum together with strong agitation (300 rpm). Figure 69 illustrates the agitation effect on enhancing the ethanol yield (% w/w of theoretical ethanol). The ethanol yield across the samples were increased by certain amounts compared with Figure 63, but were still lower (40 - 60 %) than expected at higher substrate concentration. Agitation does not impact on the ethanol yield for 10 % sample. However, it resulted in an increase of ethanol yield and considerable consumption of glucose for 20 % sample which reveals the necessity of agitation at higher substrate concentration. With the combined effect of the agitation and yeast inoculum, the decrease in ethanol yields among the concentrated samples was mitigated to some extent, particularly in the sample of 20 % substrate concentration. These results together suggest that the increase of yeast inoculum (the concentration of yeast cells) might promote the conversion of sugars to ethanol under strong agitation (300 rpm).

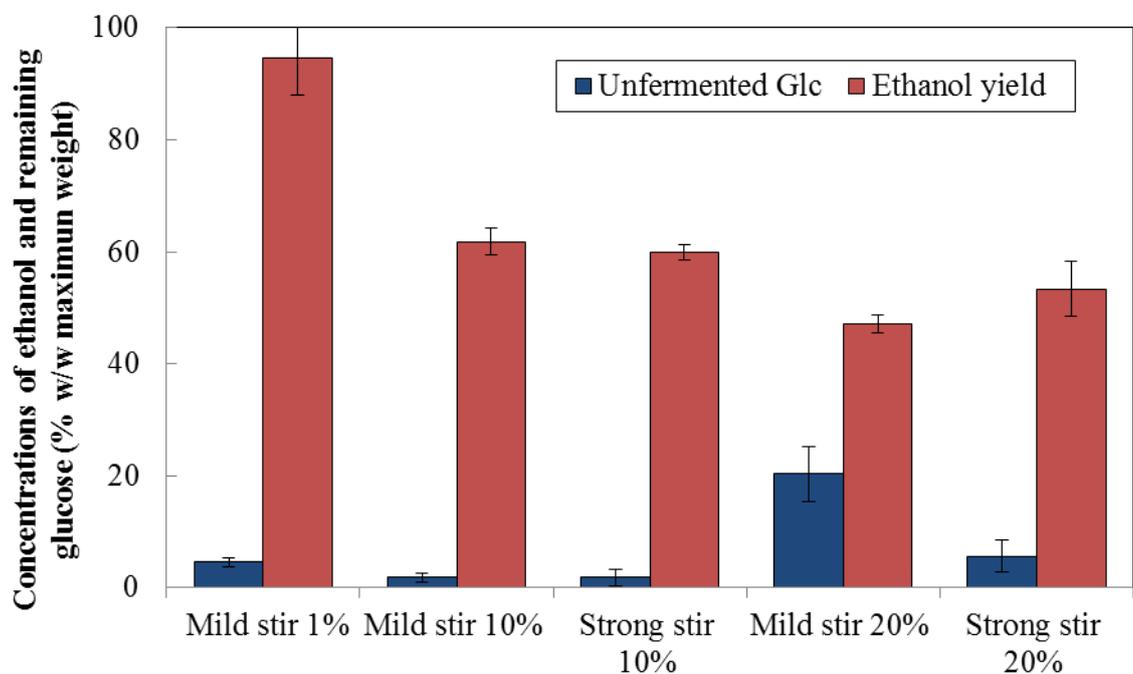


Figure 69. Ethanol yield and the unfermented glucose of SSF treated with the different level of agitation (mild: 120 rpm and strong: 300 rpm).

To further improve ethanol yield at 20 % substrate concentration, there are two approaches taken in this study: (I) the enhancement of YM cultured yeast titre (YM yeast below, the concentration of yeast cells) and (II) the adoption of SE medium cultured yeast (SE yeast below) and increase of yeast titre. The relationship between ethanol yield and yeast inoculum is demonstrated in Figure 70. The ethanol yields of YM yeast samples were increased along with the strength of the yeast inoculum dosage, but the ethanol yields of SE yeast samples do not show this clear trend. The low level SE yeast dosage (1 and 2 times) only achieved 45.2 and 48.6 % ethanol yield (equivalent to 2.2 % v/v of total solution) and a big increase occurs at both 4 and 20 times with 68.4 and 67.4 % ethanol yields (equivalent to 3.5 % v/v of total solution) respectively. This is reflected in the decrease of unfermented glucose in those samples with higher ethanol yields. Both ethanol yields of YM and SE yeast inoculated samples were limited at around 70 % which is likely to be caused by two reasons: firstly, the inhibitory effect of significant amount of formic and acetic acids (discussed previously); secondly, starch could not be fully hydrolysed in the concentrated materials. Although previous data has constantly illustrated the effect of  $\alpha$ -amylase in these commercial enzyme products, the lower temperature condition of SSF (25 °C) might largely reduce the activity of  $\alpha$ -amylase in contrast with the optimum temperature condition of enzymatic saccharification (50 °C).

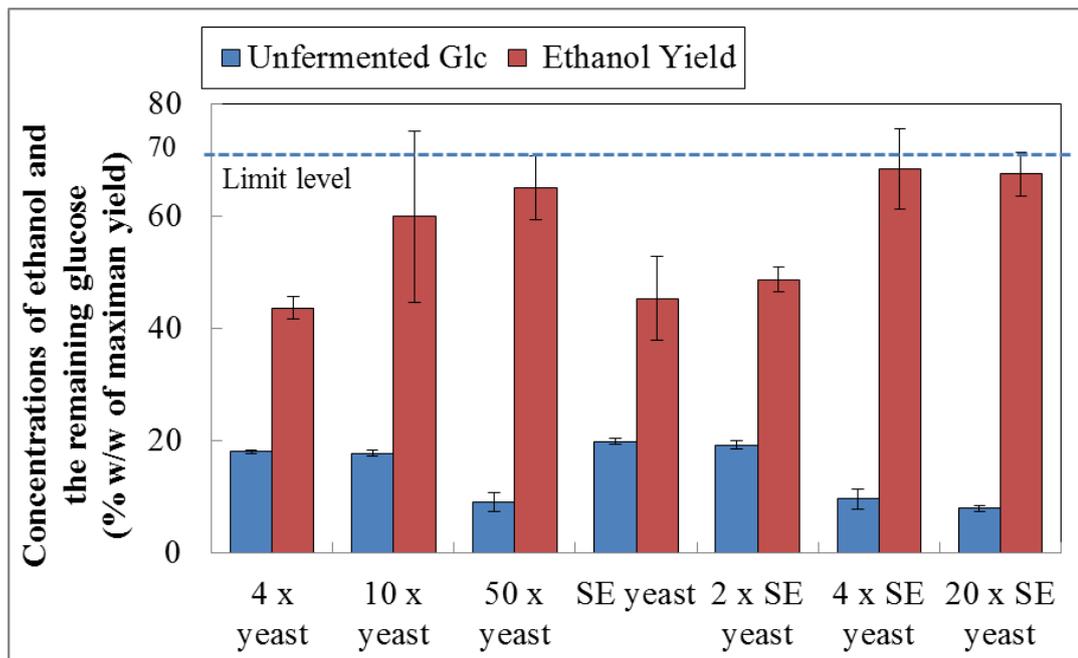


Figure 70. SSF using different levels of yeast inoculum at 20 % substrate concentration.

To clearly understand the limitation of ethanol yield and further improvement, the inhibitors (formic acid, acetic acid, 5-HMF and 2-FA) of SSF residual pellets were assessed and results are given in Figure 71. The inhibitors concentrations were increased in SSF residue samples compared to the SE slurry (substrate). The concentrations of formic acid markedly increased from 55 to 120 mg/g while acetic acid were slightly increased from 42.3 to over 50 mg/g in the overall SSF samples. Even though the quantities of 2-FA are very low in the materials, it dramatically increased from 1.4 mg/mL to around 5 mg/mL. The concentration of 5-HMF has not changed during the SSF process. According to Pienkos and Zhang (2009) and Maiorella et al (2004), formic acid, acetic acid, 5-HMF and 2-FA could produce 80, 74, 50 and 79 % of inhibition when their concentrations reached 27, 60, 80 and 40 mg/g respectively. Although the concentrations of 2-FA had increased by several folds, it is not expected that at these concentration there would be any inhibitory effect of 5-HMF and 2-FA on fermentation. However, for each of the four inhibitors, the concentration did not vary significantly during the SSF process. The concentration of formic acid in the SSF residual pellets is much higher than the inhibition level and acetic acid is also fairly close to the significant inhibition level. However, the ethanol yields of those samples with higher yeast dosage levels were not affected by the higher organic acid levels but were increased to an expected level (nearly 70 % in Figure 70). This situation indicates that the adaptability of *S. cerevisiae* strain to the material is potentially intensified along with large yeast inoculum. In addition, similar ethanol yields achieved by lower SE yeast inoculum dosage (4 times) suggest that SE medium pre-cultured possesses greater adaptability than YM yeast. Nevertheless, it is unavoidable that the presence of high levels of formic and acetic acid is likely to limit the conversion of the remaining glucose (10 %) to ethanol.

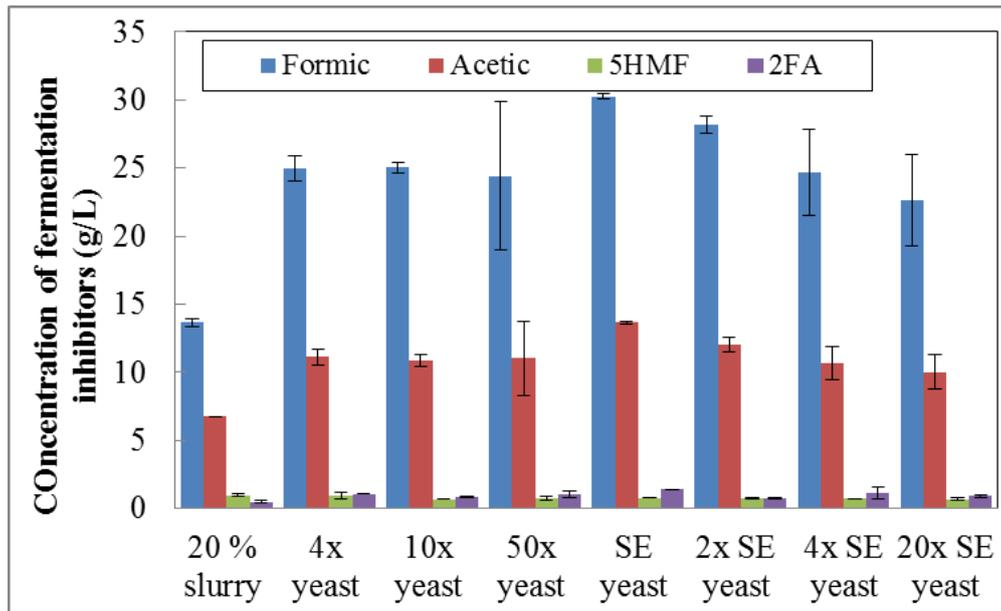


Figure 71. Inhibitors assessment of SSF pellets using different levels of yeast inoculations at 20 % substrate concentration.

At a substrate concentration similar to that used in brewing (20 %), ethanol yield could be improved to nearly 70 % (equivalent to 3.5 % v/v of total solution) using YM pre-cultured *S. cerevisiae* inoculum dosage of 50 times or using SE medium pre-cultured *S. cerevisiae* at a much lower level (4 times). Although yeast inoculated on SE liquor produces fewer viable cells ( $1.8 \times 10^7$  cells/mL) and takes longer to culture (2 days) compared to that inoculated on YM ( $8.3 \times 10^7$  cells/mL; 1 day) it is still more economical to use. Firstly, SE liquor is a by-product of the process whereas YM would have to be purchased. Secondly, due to the dosage required for optimum SSF being 12.5 (50/4) times smaller and accounting for the fact that two batches of YM inoculum can be cultivated at the same time as one SE inoculum, based on an equivalent volumes, SE inoculated yeast can dose 6.25 times more than YM. Compared with using other associated means or increasing enzyme cocktail dosage to improve the ethanol yield, the adoption of SE inoculum could considerably reduce the production cost.

Other ethanol yields from duckweed starch have been researched. Xu et al (2011) unveiled that 97.8 % (w/w of theoretical ethanol) of ethanol yield was achieved by fermenting *Spirodela polyrrhiza* containing 31 % (w/w DM) of starch hydrolysed by  $\alpha$ -amylase, pullulanase, and amyloglucosidase. Chen et al (2012) reported that over 90 % ethanol yield (3.9 % v/v) could be obtained by fermenting pentinase (26.5 PECTU/g mash) pretreated

duckweed (*Landoltia punctata*) which contains 75 % (w/w DM) of starch. In the studies reported in this thesis, 70 % ethanol yield was obtained using a very low enzyme cocktail of CTec 2 (0.87 FPU/g substrate) and BG (2 U/g substrate) and high yeast inoculum which demonstrates the effectiveness of steam explosion on ethanol yield of SSF duckweed biomass and the potential of cost reduction in the ethanol production of duckweed feedstock. This process is also utilised on ethanol production from wheat straw reported by Luo et al (2008): 65.8 % (theoretical ethanol) ethanol yield was achieved using a SSF approach on steam exploded wheat straw (substrate: 10 % w/v) loading with an enzyme cocktail of Celluclast 1.5 (30 FPU/g substrate) and additional BG under similar fermentation conditions. The total of the recovered glucose from the ethanol yield (68.4 %) and unfermented glucose (9.6 %) in the SSF process is similar to the maximum glucose yields (80 % w/w) obtained by hydrolysing steam exploded duckweed, but not match the glucose concentration in the substrate. This indicates that 20 % of glucose was not saccharified in the SSF process or some of the glucose might be oxidised (Cannella et al, 2012). Only 10 % of the unfermented glucose is probably attributed to inhibitory effects. A tiny amount of SSF materials were subsequently inoculated onto a PCA plate and cultured for 2 days to establish the bacteria contamination in the SSF solution. Figure 72 clearly illustrates that only small amounts of yeast colonies has grown on PCA plates and no significant bacteria is present, which suggests that SSF solution has not been contaminated by bacteria throughout the SSF process. This information excludes the potential of bacteria consumption of the glucose resource.



Figure 72. The yeast culture in PCA plate – pure yeast is evident from the formation of orange colonies (NCYC 2826 strain). Black spots are inoculated SSF material.

### 6.3 Conclusions

Simultaneous saccharification and fermentation of steam exploded duckweed by using the finally optimised enzyme cocktail CTec 2 + BG can produce 80 % ethanol yield at 1 % (w/v) substrate concentration, but the yield dramatically decreased to 18 % at 20 % (w/v) substrate concentration (a highly viscous suspension). Further optimization of SSF is addressed by using these two approaches: (i) increasing the yeast titre in the inoculum or (ii) growing the inoculum on steam-exploded duckweed. Association of the increase of yeast titre in both YM cultured and steam-exploded duckweed cultured yeast, 94.7 % ethanol (w/w of theoretical ethanol) was obtained from the diluted substrate (1 % w/v) and nearly 70 % (w/w of theoretical ethanol) or 3.5 % (v/v of total solution) was achieved from the concentrated substrate (20 % w/v). The enhanced SE yeast inoculum (4 times) is further considered as more economical than YM yeast inoculum (50 times).

## 7 *General conclusions, discussions and further research*

### 7.1 **Conclusions and discussions**

The bioethanol production from aquatic plants has been researched by using the model plant - duckweed and the process has been systematically improved and developed with regard to the following important aspects: (I) duckweed was identified as a potential biofuel resource due to its high content of fermentable sugars; (II) the thermal pretreatment (steam explosion) can effectively pretreat duckweed biomass to improve degradability and enzyme accessibility; (III) the enzymatic saccharification has become feasible because a high glucose yield was effectively achieved by using the enzyme preparation at a fairly low concentration and the cost of enzyme falls into a affordable range; (IV) the ethanol yield at a distillable ethanol concentration is achieved by using simultaneous saccharification and fermentation method. The detailed outcomes are stated below:

#### 7.1.1 *Characterisation of L. minor*

Results of chapter 3 demonstrate that duckweed could be developed as a useful industrial feedstock in many aspects, e.g. bioethanol, animal feed, nutrients & traditional Chinese medicine. The bioethanol production from duckweed is significantly attributed to its high proportion of carbohydrate that accounts for half of dry matter and predominantly contains a significant amount of fermentable sugars (glucan) 33.2 % (w/w DM). The glucan of duckweed (pond samples) predominantly comes from starch (20 % of DM) and cellulose (12 % of DM) and their ratio here is nearly 2:1. Compared with other energy resources, duckweed can provide a reasonable glucan content, comparable with hardwood (40 -55%), wheat straw (37 %), grasses (25 - 40 %), leaves (15 - 20 %) and waste paper from chemical pulps (60 - 70 %). Duckweed also has conspicuously lower levels of lignin (3 %), compared to hardwood (18-25 %), wheat straw (16 %), grasses (10-30 %) and waste paper from chemical pulps (5 - 10 %) (Kumar et al, 2009; Merali et al, 2012). This compositional profile in combination with its fast growth rate could eventually provide a useful ethanol resource from duckweed. In addition, the variable starch content (3 - 75 %) (Cheng and Stomp, 2009) might further increase the ethanol yield to a much higher level. The results of fractionation demonstrate a cell wall structure comprising middle lamella and primary wall with a very thin secondary wall. In addition, the establishment of other duckweed compounds further improves the potentially commercial value of the exploitation of duckweed. The discovery of EFA and phenolics provides the possibility of the exploitation

of duckweed for high value products accompanying the ethanol production. It is not to be neglected that the residual materials from ethanol production may potentially be used for animal feed because of its protein content (12 %) of dry matter. Indeed the protein content may often be in a range of 29.1 - 45 % (Landolt & Kandeler, 1987). This enhances the duckweed utilization in a holistic view of sustainable production and reduces the cost of the waste disposal.

### 7.1.2 *The improvement of pretreatment*

Steam explosion as a common effective thermal physical pretreatment for lignocellulosic biomass was evaluated for use on duckweed biomass in chapter 5. The effectiveness of the pretreatment was expressed in two ways: (I) the glucose yield of saccharification or ethanol yield of fermentation; (II) the reduction of enzyme dosages or yeast inoculum. In the combination of glucose yields (Figure 58) and ethanol yield (Figure 63), it is found that steam explosion could largely reduce the required dosage of enzyme preparation and the titre of yeast inoculum without creating a significant drop of sugar and ethanol yields. The results further indicate that steam explosion of duckweed does not require any further chemical addition, e.g. acids/alkalis. Thus, steam explosion was identified as an effectively and potentially economically helpful pretreatment for bioethanol production from duckweed.

In this project, a few other pretreatments have been used to break down duckweed biomass and their utilization and effectiveness are summarised here: (I) FDM is a purely physical pretreatment that could maximally retain the matter and components of materials. FDM materials are finely ground particles and were therefore generally prepared as a control blank. (II) AIR production is a physical-chemical pretreatment and AIRs are made up of mainly CWM and starch which were solely used for the investigation of enzymatic saccharification in a lab scale. (III) Ball milling is another physical-chemical pretreatment in which the fairly pure CWMs were obtained and starch was removed. However, the complex and costly pretreatment process could not afford enough materials for the investigation of enzymatic saccharification. Thus, this pretreated duckweed biomass was specifically prepared for the fractionation analysis of duckweed cell wall polysaccharides only. In terms of glucose and ethanol yields, FDM resulted in a significant ethanol yield and greater glucose yield than alcohol extraction. This suggests that physical milling is one

more efficient means to increase ethanol yield from duckweed biomass. This might be attributed to the thin secondary cell structure and low lignin content in duckweed.

### 7.1.3 Enzymatic saccharification

A more cost effective enzyme preparation was identified by screening a number of commercial enzymes for the enzymatic saccharification of duckweed individually or in combination. By using WIAIRs materials, the enzyme cocktail of CE + BG including 100 U (4.35 FPU)/g substrate of CE and 100/g substrate of BG was identified as the most efficient enzyme preparation. Commercial cellulase with additional  $\beta$ -glucosidase is a common enzyme cocktail used in the saccharification of lignocellulosic biomass (Eklund et al, 1990; Mosier et al, 2005; Gáspár et al, 2007; Dien et al, 2008). This enzyme cocktail was further optimised on the steam exploded material and the dosage was reduced to 20 U (0.87 FPU)/g substrate of CE plus 2 U/g substrate of BG. This dramatic drop would reduce the cost of ethanol production from duckweed but without conspicuous reduction of the ethanol yield. A novel and cheaper commercial cellulase – CTec 2 was identified to replace CE by using the same level of dosage as CE. By using the final optimised enzyme cocktail of CTec 2 + BG (CTec 2: 20 U (0.87 FPU)/g substrate and BG: 2 U/g substrate), 80 % of glucose yield was obtained. The high glucose yield achieved by such a low enzyme dosage of CTec 2 + BG is comparable to other glucose yields achieved by using much greater dosage of CTec 2, e.g. 11.5 FPU/g glucan (Lan et al, 2012) and 8.7 FPU/g glucan (Eckard et al, 2012). In addition, a certain amount of  $\alpha$ -amylase was also proven to present in each commercial enzyme and the activity in the combination is greater than the aggregation of individual enzymes.

### 7.1.4 Fermentation

Chapter 6 particularly describes how the fermentation was developed from a dilute substrate (1 % w/v) to a highly viscous substrate (20 % w/v) which is required to achieve a distillable ethanol concentration. The ethanol yield was also considerably increased from 18.8 % to nearly 70 % (w/w of theoretical ethanol or 3.5 % v/v) by using a concentrated yeast titre (50 times ( $4.0 \times 10^9$  cells/mL) of YM *S. cerevisiae*). In addition, the more effective yeast strain can be cultured in the liquid from steam exploded slurry and the same ethanol yield was achieved by using much less yeast titre (4 times or  $7.2 \times 10^7$  cells/mL) of SE yeast inoculum than YM yeast inoculum. This method allows reducing the production cost instead of using concentrated enzyme or additional pretreatment. The ethanol yield

(70 %) is lower than some reported fermentation efficiency (90 %) of bioethanol production from duckweed (Xu et al, 2011; Chen et al, 2012), but they were focusing on high-starch duckweed. The utilization of the low enzyme dose and self-cultured yeast are likely to make this process more economical than those requiring high concentrations of enzymes and complex enzymatic saccharification and fermentation processes.

## 7.2 Estimation of the production cost

Combining the entire optimizations in this project together, an integrated ethanol production pattern is established and by that the production cost might be evaluated. This rough estimation might provide a chance to estimate how far the cost of the biofuel production from duckweed is away from a realistic cost. Some fundamental figures need to be addressed prior to the estimation. Firstly, an optimized production pattern achieved in this project is outlined (see Figure 73). Secondly, the ethanol yield per kg dry matter is figured out using following relationships: (I) 350 g glucose can be obtained from one kilogram dry matter; (II) the conversion of glucose to ethanol (Equation 4); (III) the ethanol yield is 70 % of theoretical yield. Thus, 0.13 kg (0.16 L) ethanol is produced from 1 kg duckweed dry matter via this pattern. Thirdly, the industrial electricity price in UK is 8.5 penny/KWh in 2012 (Department of Energy & Climate Change, 2013). Finally, the currency exchange rates of Euro – GBP, Dollar – GBP etc. are based on the currency exchange rate on 6<sup>th</sup> Jan 2014 (FXTOP, 2014).

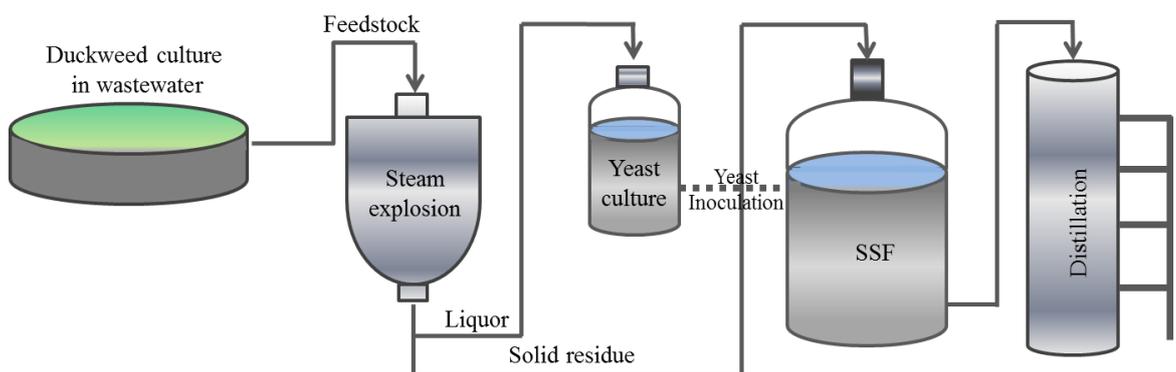


Figure 73. The flowchart of the optimized production pattern.

### 7.2.1 The estimation of the capital cost

Capital costs refer to the capital spent on establishing the bioethanol production plant, which include purchasing cost of facilities, place and roads, buildings, instalment of

facilities etc. Capital costs are generally fixed in the ethanol production, but are variable in different countries and locations. The referable capital costs are tabulated in Table 18 according to Anbarasan et al (2012) (see Table 28 in § 8.9) and Zimbardi et al (2002) (see Table 29 in § 8.9) and their depreciation values are independently calculated excluded from the operating cost of each treatment.

Table 18. The capital cost of the facilities

Equipment	£ million	Depreciation*** (£/m <sup>3</sup> ethanol)
Steam explosion plant*	6.75	131
Fermenter**	3.3	0.6
Distiller**	4.01	0.8
Centrifuge**	1.69	0.3
Holding tanks**	8.6	1.7
Building, roads and place*	1.93	40
Total cost	26.28	174.5

\* According to the productivity of steam explosion plant (25000 t/y) (Zimbardi et al, 2002).

\*\* According to the productivity of ethanol production plant (10000 bbl per day with assumed 250 working days per year) (Anbarasan et al, 2012).

\*\*\* Depreciation is calculated based on an expected working life of 15 years.

## 7.2.2 The estimation of the operating cost

### 7.2.2.1 Biomass harvest

In this estimation, the biomass is presumably obtained from wastewater system so that the cost spent on the cultural of duckweed is reckoned as zero. The harvest cost only takes account of collection, transportation and labours required. A parallel cost of harvesting water hyacinth could be referenced that the harvesting cost per hectare ranges from \$ 1235 – 1976 (Washington State Department of Ecology, 2001). According to 105.9 t/ha per year of dry mass could be harvested (Xu et al, 2012), the harvesting cost of duckweed dry biomass is \$ 0.014/kg DM and subsequently \$ 0.11/kg or \$ 0.09/L for ethanol. The cost is further updated to \$ 0.14/kg or \$ 0.12/L adjusted by inflation of USA (30 % of consumer price index from 31<sup>st</sup> June 2001 to 6<sup>th</sup> Jan 2014) which is equivalent to £ 0.09/kg or £ 0.08/L (FXTOP, 2014). This cost does not obviously count the additional cost for mobilization and equipment used (\$ 35000 - 110000) and potential disposal of plant

materials (Washington State Department of Ecology, 2001) which would shift dramatically when it is apportioned to different acreage.

#### 7.2.2.2 Steam explosion

The operating cost of steam explosion mainly comprises of a number of factors which is hardly evaluated from our trial steam explosion plant. A referable steam explosion plant with a large capacity of 25000 t/y DM is provided by Zimbardi et al (2002) (Table 29 in § 8.9) in Italy and the operating cost was estimated as € 0.19/kg DM including the spending on feedstock and depreciation. Depreciation of steam explosion will be calculated separately in the depreciation section. Due to duckweed is self-cultured in wastewater system, the cost is revived to € 0.076/kg DM and the updated cost on is € 0.098/kg DM equivalent to £ 0.081/kg DM adjusted by inflation of Italy (29 % of consumer price from 31<sup>st</sup> June 2002 to index 6<sup>th</sup> Jan 2014) (FXTOP, 2014). To be noticed, the dry matter of fresh duckweed (8 %) is much lower and demands more steam than the loading material in Zimbardi's pattern (50 %), but the steam explosion duckweed is easily operated and might require less manpower. This cost is therefore only considered as a referable value for duckweed. In the combination of ethanol yield from duckweed biomass (0.16 L/kg DM), the cost is further revived to £ 0.51/L for ethanol product.

#### 7.2.2.3 Simultaneous saccharification and fermentation

The cost of SSF is predominantly evaluated from three aspects: (i) the energy and water input; (ii) the spending on commercial enzymes and yeast inoculum; (iii) the manpower required for the process. The costs of energy and water input are calculated according to the study stated by Akiyama et al (2003) in which the polyhydroxyalkanoates were produced by using a fed-batch bacterial fermentation with the productivity of 824 kg/h operating at 34 °C over 50 h. Akiyama et al (2003) evaluated the cost of the energy and including electricity, steam, processing water and cooling water and the aggregation is 12.78 MJ/kg or expresses in the total electricity demand (3.55 kwh/kg product). Based on the industrial electricity price in UK 2012, the energy cost of fermentation is £ 0.28/kg or £ 0.22/L ethanol. Due to self-cultured yeast inoculum in this study, the operating cost of yeast inoculum only comes from energy input. The process and conditions of yeast culture resemble the fermentation process. Because the loading yeast inoculum is only 10 % (v/v) of fermentation solution, the operating cost of yeast culture is consequently estimated as 1/10 of the cost of fermentation which is £ 0.03/kg or £ 0.02/L ethanol. To be noticed, the

ethanol fermentation of duckweed and yeast culture are carried out at 25 °C that requires significant lower energy than Akiyama's fermentation. Thus, the energy cost is likely to be much lower than the estimated cost. The loading proportions of CTec2 and BG in the final SSF solution are 0.09 % (v/v) and 0.16 % (v/v) and their dosage required for 1 L ethanol production are calculated as 0.028 L/L ethanol product and 0.05 L/L ethanol product respectively. The industrial price of CTec 2 and Novozyme 188 are reported as £ 150/m<sup>3</sup> (Novozymes, 2012) and £ 45/t (Shi et al, 2009). The expense on CTec 2 and Novozyme 188 for 1 L ethanol is £ 0.0042 and £ 0.0018, together, the enzyme cocktail of "CTec 2+BG" costs £ 0.006/L ethanol.

#### 7.2.2.4 Distillation

By using an ethanol distillation facility with capacity of 1738 m<sup>3</sup>/h water, the distillation of ethanol products including original ethanol concentration of 7 g/L or 0.9 % (v/v) generates 12166 kg/h distilling ethanol that requires energy of  $1.02 \times 10^7$  KJ/h with 100 % efficiency (Anbarasan et al, 2012; Sacia et al, 2012). With respect of the original ethanol concentration of 27 g/L or 3.5 % (v/v) in this study, 46926 kg/h distilling ethanol is potentially produced and requires  $3.93 \times 10^7$  KJ/h. Combining with the industrial electricity price in UK, the distillation cost for 46926 kg distilling ethanol from duckweed per hour is estimated as £ 923. The distillation cost also is expressed as £ 0.02/kg ethanol or £ 0.015/L ethanol with 100 % distillation efficiency. With a realistic efficiency of 50 %, the distillation cost would increase to £ 0.04/kg ethanol or £ 0.03/L ethanol.

#### 7.2.2.5 Other operating costs

Some additional costs are necessarily taken account into the total operating cost, including depreciation, maintenance, insurance, plant management (Zimbardi et al, 2002). These additional costs are dominated by depreciation that is estimated as £ 174.5/m<sup>3</sup> ethanol or £ 0.18/L ethanol (see Table 18). The rest additional cost is not established here because they are very variable based on the plant distribution.

#### 7.2.3 *The profits of using duckweed in the industrial wastewater plant*

It is not be neglected that there are remarkable profits generated from using duckweed in the industrial wastewater plant which is necessarily take account into the cost estimation of ethanol production. The profits are mainly attributed to the purchasing fees of the conventional chemical and microbes saved by growing duckweed in the discharge ponds.

The annual chemical cost in one industrial wastewater plant with capacity of 100 000 m<sup>3</sup>/d is £ 153 720 (COSTWater, 2014) which is potentially saved by using duckweed. In terms of this effluent of wastewater, it requires one massive reservoir to contain the wastewater for duckweed growing. Otherwise, wastewater can not be completely cleaned without enough retaining time. Thus, the detailed establishment of growing duckweed in wastewater system demands more precise investigation further.

#### 7.2.4 *The potential overall cost*

The total cost (£ 1.05/L ethanol) of the optimised pattern associated with steam explosion is summarised as Scenario II in Table 19. The major contribution of the cost is the steam explosion (£ 0.51/L ethanol) and SSF energy requirement (£ 0.22/L ethanol). To highlight the effectiveness of the steam explosion pretreatment for reducing the production cost, another pattern using sufficient enzyme dosage was estimated and its cost is illustrated as Scenario I in Figure 74. The enzyme preparation of Scenario I is CTec 2 + BG and their dosages are according to a common range of cellulase (20 – 40 FPU/g substrate) and the ratio of CE:BG at 1:1 reported by a number of literature (e.g. Xiao et al, 2004; Gáspár et al, 2007; Anderson et al, 2008; Chen et al, 2012). Thus, 30 FPU (700 U) /g substrate of CTec 2 and 700 U/g substrate of BG are used for the cost estimation of Scenario I and their cost are £ 0.15/L and £ 0.62/L respectively. The cost of enzyme preparation is the predominant contribution of the total cost of Scenario I. To be noticed that the materials in those studies (Xiao et al, 2004; Gáspár et al, 2007; Anderson et al, 2008; Chen et al, 2012) were also treated by basic pretreatment, e.g. physical milling, which generates certain amount of cost and has not been estimated here. According to Littlewood et al (2013), a selling price of Scenario I is estimated as £ 2.08/L ethanol (in Figure 74) while the selling price of Scenario II is estimated as £ 1.83/L ethanol (in Figure 74), topped with fuel duty (£ 0.58/L ethanol, Dec, 2013), VAT (17.5 % of cost) and distribution cost (£ 0.021/L ethanol). This parallel ethanol production from wheat straw pretreated by SE was estimated as: the operating cost (£ 0.453/L ethanol) and the bioethanol selling price (£ 1.18/L ethanol) (Littlewood et al, 2013). It also stated that the major contributions of his estimation came from enzyme cost and feedstock harvest while steam explosion exhibited an economic cost. Kumar and Murthy (2011) also illustrated a relatively low ethanol production cost of using SE pretreatment as \$ 0.86/L ethanol (equivalent to £ 0.53/L ethanol). However, none of these literature revealed a detailed evaluation of the steam explosion cost that can be applied to estimate the operating cost of SE in our study, despite they suggest that the

operating cost of SE could be potentially largely trimmed. In addition, the depreciation would be also reduced according to Littlewood's research. We are also necessarily aware of the less energy content in bioethanol, the energy generated by 1 L bioethanol is equivalent to 0.68 L of petrol (Littlewood et al, 2013). This study provides a rough cost estimation based on a number of literature after all, it only demonstrates that the commercialisation of this technology is likely to be within reach.

Table 19. The estimation of the ethanol production from duckweed by using our optimised pattern. The cost is estimated based on per litre of ethanol.

Parameter	Cost value (£/L)
Biomass harvest	0.08
Steam explosion	0.51
SSF (energy input)	0.22
Enzyme preparation	0.006
Yeast inoculum	0.02
Distillation	0.03
Depreciation	0.18
Total cost	1.05

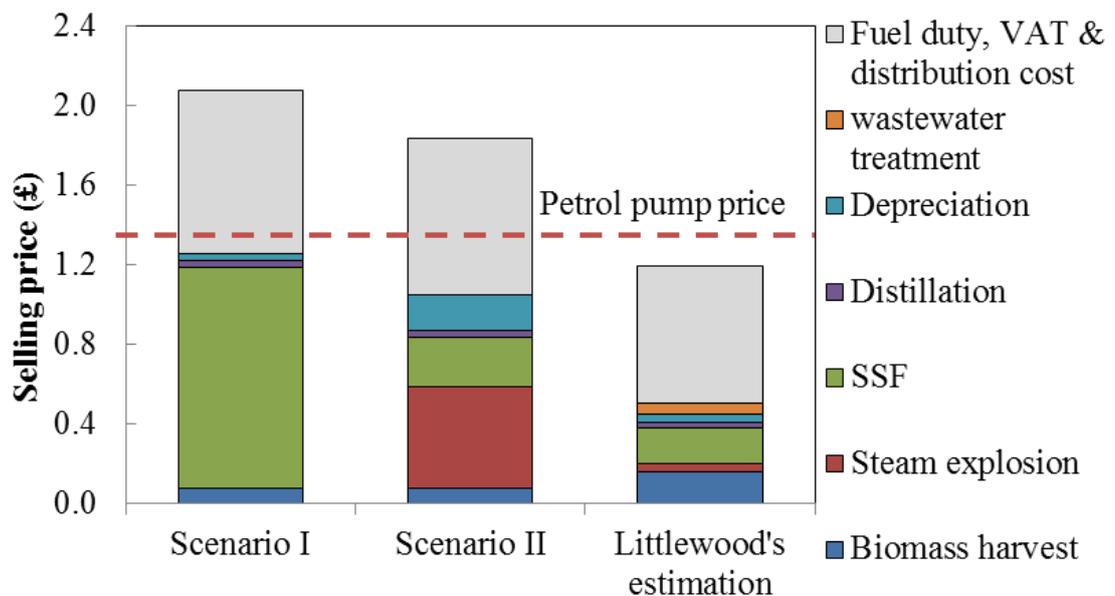


Figure 74. The comparison of the estimation in the cost and the selling price with other bioethanol product (Littlewood et al, 2013). Petrol pump price is £ 1.31/L in December, 2013.

### 7.3 Limitations and future research

This study provides a technical potential for bioethanol production from duckweed. The outcomes achieved in this project demonstrate how duckweed biomass could be converted to bioethanol technologically. However, this study is still restricted by many factors that lead the possibility of bioethanol production to remain at a trial stage. It is questionable if the object – *L. minor* could represent the whole duckweed family. Will the same ethanol yield be achieved in the industrial scale by using this optimized pattern? Nevertheless, these limitations or disadvantages will be perceivably overcome if the bioethanol production is enhanced by following further approaches:

#### 7.3.1 *The geographical distribution of this technology*

The ethanol production from duckweed appears to distribute geographically in certain area affected by two reasons: one is the suitable habit for duckweed growth to ensure the throughout of feedstock, the other is feedstock harvest cost. Bonnomo et al (1997) found that the application of duckweed in the wastewater system seemed difficult in Italy because of the requirement of large area for growing land and the cessation of growth in winter period. However, the ceasing of growth seems not happened in the tropical, subtropical zone. Iqbal (1999) reported the duckweed growing wastewater system can supply sufficient food for carp cooperatively raised in the wastewater system throughout year. Thus, this technology should be primarily considered for applying in the perennial mild-wettish countries. Harvesting duckweed biomass is labour and equipment intensive and the harvesting cost varies depending on the plants density, the nutrient condition of water system, equipment used, transportation and labours. Hence, the estimated harvesting cost (£ 0.06/L of ethanol) will shift in different countries and years. The application in those countries with cheaper labour is probably more feasible than in the developed countries. As a conclusion, this ethanol production is potentially adopted in those countries or area possessing mild-wettish weather and cheaper labour, such as, southern China, South and Southeast Asian countries, Middle Africa and South American countries.

#### 7.3.2 *Improvement of the processing technique*

The cost estimation has illustrated that this optimised technology is still beyond the petrol pump price and the biofuel from wheat straw. Even though this high evaluation was calculated based the cited information, promoting the processing technique is bound to

optimize the ethanol production and consequently reduce the operating cost. The optimized ethanol production pattern might be improved from the following aspects:

Firstly, the preservation of duckweed biomass appears knotty due to the high moisture content. Direct storage of the fresh biomass in the frozen condition is unfeasibly enforced for the massive freezer required and the expensive cost. The biomass therefore demands drying treatment prior to the preservation. Drying in the sun is most likely low technology solution, but may cause compounds denaturation (Sridhar, 2008). Other dry methods with association of heating would obviously increase the cost. Furthermore, a loss of dry matter is found in diverse preservation methods and it significantly varies for the different duration and conditions including temperature, light and volume (Leuven et al, 1985). Thus it can be seen that selection of the preservation methods will be possibly considered as the equilibrium of denaturation and the cost as well as the requirement of subsequent pretreatment.

Secondly, steam explosion as the major contribution of the operating cost, requires greater technical innovation to lower the cost. From our disciplinary perspective, this could be optimized on duckweed material. In this project, the fresh wet duckweed biomass was directly treated by steam explosion for the purpose of avoiding the cost and compounds denaturation in the drying process. However, it appears to demand large energy input to achieve the required steam explosion conditions. Kahr et al (2012) stated that the water content of the total introduced biomass of steam explosion would be a key factor on the steam consumption. In another word, steam demand would increase when more moisture is present in the materials. It seems that the low moisture content of materials is not only beneficial for feedstock preservation, but it also caters to the reduction of energy input in the steam explosion pretreatment. Besides, the reduction of steam demand is likely to be improved by preheating materials and modifying the density of loading samples (Kahr et al, 2012).

Thirdly, the bottleneck of ethanol yield and the energy efficiency need to be further improved by technically promoting the fermentation process. A reasonable ethanol yield (70 % of theoretical ethanol) was obtained in this optimized SSF, which appears a bottleneck of the ethanol yield. On the other hand, the energy efficiency of biomass to ethanol using biochemical conversion mean is only 28.6 % (IATA, 2009). These figures

indicate that there is a huge room to technically optimize the fermentation as one more efficient process. This ethanol yield ought to be technologically increased by reducing inhibitors, innovating yeast strain and fermentation method. In addition, a variety of fermentation methods have been reported which might enable to advance the fermentation process. Elliston et al (2013) stated that a novel method – fed batch semi simultaneous saccharification and fermentation of waste paper achieved high ethanol concentrations (12 % v/v) at a reasonable ethanol yield (65.5 %) using a very viscous substrate (65 % w/v). With the respect of the profile of steam exploded duckweed slurry, the fermentation method could be improved as: (I) simultaneous subculture yeast and fermentation on SE duckweed liquids and (II) fed batch simultaneous saccharification and fermentation by constantly loading with concentrated solid SE slurry.

### 7.3.3 Innovation of enzyme preparation and yeast

Enzyme and yeast as the most important biochemical catalyst play a crucial role for the bioethanol production from duckweed. This study unveils that duckweed is one starch- and cellulose-rich feedstock that requires specific commercial enzyme in the combination of highly active cellulase and amylase supplementing with  $\beta$ -glucosidase. As the best of our knowledge, no commercial enzymes containing both the highly active cellulase and amylase is specifically produced for the starch- and cellulose-rich feedstock. However, the equal effectiveness of this specific enzyme combination could be alternatively achieved by optimizing saccharification pattern. One associated study completed by Mr Wu and Miss Tan and supervised by author investigated one alternative hydrolysis pattern to maximise the glucose yield up to 100 % (w/w) (see Figure 78 in § 8.8). This pattern is one combined enzymatic saccharification in which duckweed biomass is initially hydrolysed by using the enzyme cocktail of CTec 2 + BG then followed by loading of additional  $\alpha$ -amylase. The increase of 20 % glucose yield would provide a theoretical potential of ethanol yield increase (20 %). Demand of specific *S. cerevisiae* strain for the duckweed biomass is possibly stronger in the industrial ethanol production. As our results illustrated previously, SE medium pre-cultured *S. cerevisiae* has better adaptability in the fermentation solution than YM pre-cultured and the more efficient ethanol productivity was also performed by SE yeast.

### 7.3.4 Promoting the production in a sustainable perspective

The perspective of sustainable developing and environmental protection are bound to be

one important motivation of the optimization of the bioethanol production industry. In this optimized production pattern, the waste steam from steam explosion and wastewater effluent are likely reused in the production system. As we discuss previously, several steps of the production require to be improved with additional heating treatments that are possibly replaceable by using waste steam from steam explosion. Firstly, preheating of materials prior to steam explosion might be completed by refluxing the emitted waste steam with great heat capacity (see Figure 75). Secondly, the starch saccharification is suggested to be immediately carried out after SE pretreatment (discussed in §7.3.3). The steam exploded materials remain at a very high temperature condition and the gelatinised starch is possibly still in the loosened formation just after SE pretreatment. Thus, the amylase hydrolysis of starch applied immediately after SE pretreatment is likely to be more efficient and energy saved. Furthermore, the extra waste steam is also potentially used in the distillation or other steps that potentially require heating treatment. The yeast cultivation in steam-exploded duckweed slurry might be reckoned as another aspect of sustainable bioethanol production from duckweed. This method will not only provide specifically targeted and efficient yeast for fermentation, but also sufficiently reuse extra liquid generated from SE pretreatment that is essentially removed for the subsequent fermentation. However, the high level of liberated glucose generated from steam explosion might be largely wasted in the yeast culture. In addition, the waste water generated in the production process might be considered for growing duckweed biomass prior to discharge.

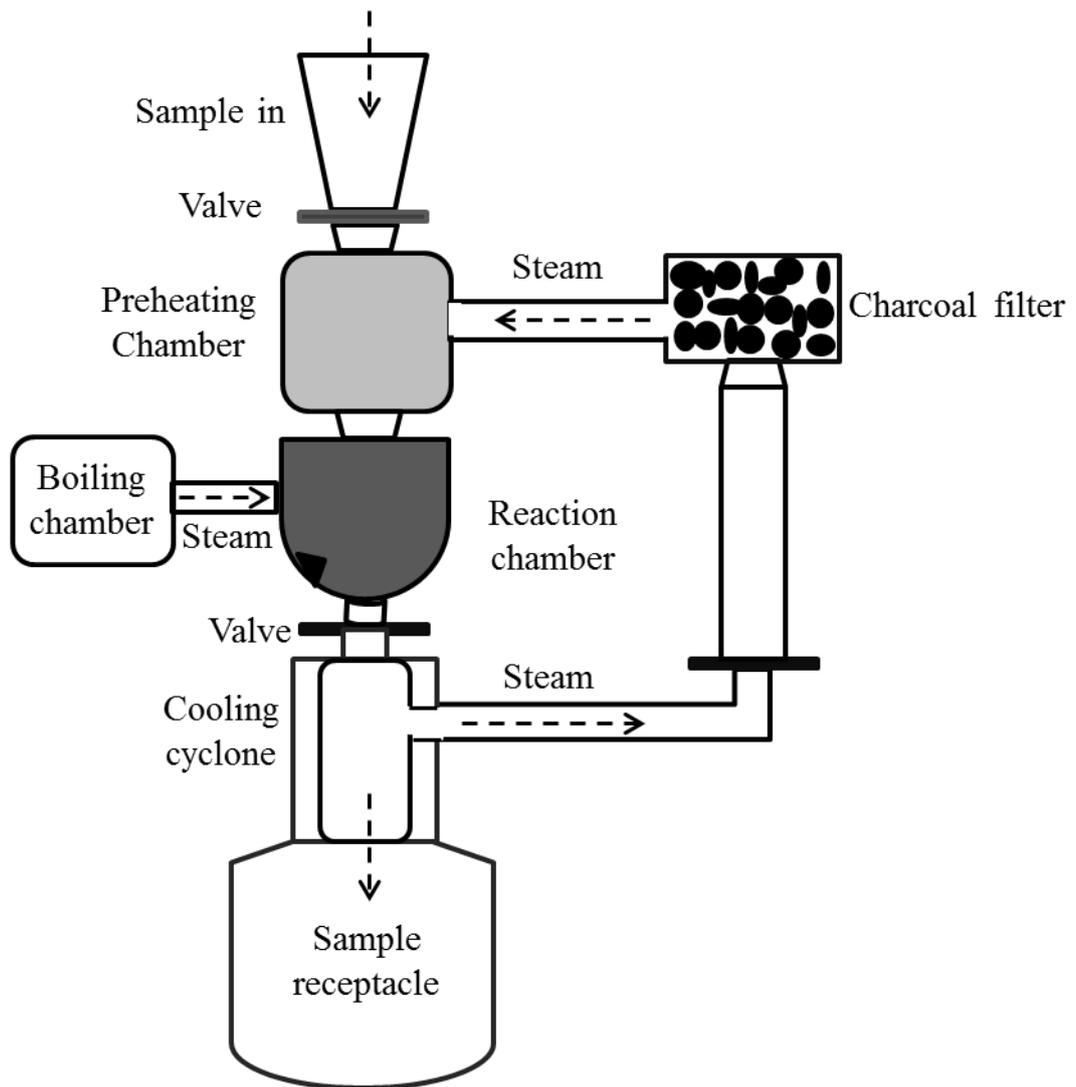


Figure 75. The steam explosion plant with preheating treatment.

### 7.3.5 One potentially industrial pattern of bioethanol production from duckweed

The overall statements discussed above are likely to draw one constructive pattern of the bioethanol production from duckweed that might be adapted to other aquatic plants. This new pattern (Figure 76) appears more efficient and energy saving expressed in following aspects: firstly, starch hydrolysis that required a thermal treatment can be implemented immediately downstream after steam explosion for possibly exerting the heat generated in the SE process; secondly, self-culturing yeast by using SE medium will not only save capital for buying yeast medium, but also promote the yeast adaptability for subsequent fermentation and reduce the dosage of yeast inoculum; finally, the reuse of the emitted steam from steam explosion pretreatment into either material drying or distillation would significantly save energy input. Certainly, this pattern only provides the possibility in theory and it needs to be evaluated in a real industrial plant.

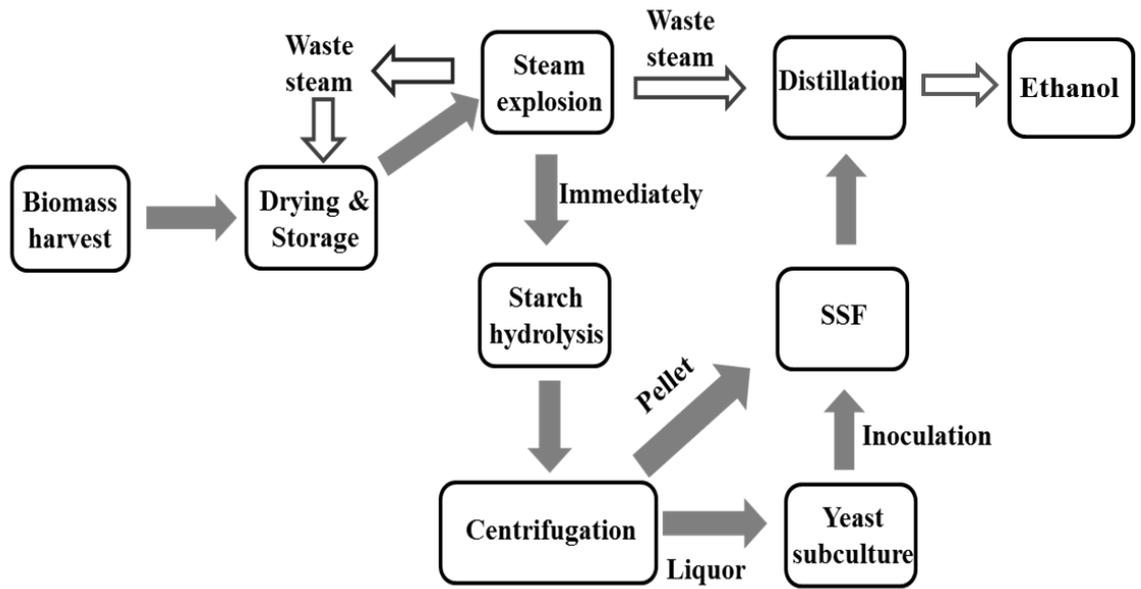


Figure 76. The new bioethanol production pattern.

## 8 Appendices

### 8.1 Hoagland E-Medium preparations

Table 20. Micronutrient solution recipe

	Molecular mass	g/L	Conc. in stock (mmol)	Conc. in final medium (mmol)
H <sub>3</sub> BO <sub>3</sub>	61.83	2.86	46.0	23
MnCl <sub>2</sub> .4H <sub>2</sub> O	197.90	1.82	9.20	4.6
ZnSO <sub>4</sub> .7H <sub>2</sub> O	287.60	0.22	0.765	0.382
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	241.90	0.09	0.372	0.186
CuSO <sub>4</sub> .5H <sub>2</sub> O	249.68	0.09	0.360	0.180

Table 21. Fe EDTA recipe

	g/250 mL	Conc. in stock (mmol)	Conc. in final medium (mmol)
FeCl <sub>3</sub> .6H <sub>2</sub> O	0.121	1.79	35.8
EDTA	0.375	4.0	80

## 8.2 The setting parameters for GC analysis of sugars

### Instrument Control Method

Instrument Name : AutosystemXL

Instrument Type : PE AutoSystem GC with built-in Autosampler

### Channel Parameters

Data will be collected from channel A

Delay Time : 0.00 min

Run Time : 50.00 min

Sampling Rate : 12.5000 pts/s

Channel A Channel B

Signal Source: Detector A

Analogue Output: NT

Attenuation -6

Offset 5.0 mV

### Autosampler Method

Syringe Capacity: 5.0 µL

Injection Speed: Normal

Viscosity Delay: 0

Injection Volume: 1.0 µL

Sample Pumps: 6

Wash/Waste Vial Set: 1

Pre-injection Sample Washes: 2

Pre-injection Solvent Washes: 0

Post-injection Solvent Washes (A): 8

### Carriers Parameters

Carrier A control: PFlow – He

Column A length: 15.00 m

Vacuum Compensation: OFF

Split Flow: 0.0 mL/min

Initial Set point: 2.0 PSIG

Diameter: 320 µm

Initial Hold: 999.00 min

### Valve configuration and settings

Valve 1: SPLIT On

Valve 2-6: NONE

### Detector Parameters

Detector FID

Range 1

Time Constant 200

Autozero ON

## Heated Zones

Injector A: PSSI	Initial Set point : 250°C
Initial Hold: 999.00 min	Injector B: NONE
Set point: OFF	Detector A: 250°C
Detector B: 0°C	Auxiliary (NONE): 0°C

## Oven Program

Cryogenics: Off	Initial Temp: 140°C
Initial Hold: 5.00 min	Total Run Time: 50.00 min
Maximum Temp: 240°C	Equilibration Time: 2.0 min
Ramp 1: 2.5 0/min to 210°, hold for 17.00 min	

## Timed Events

SPL1 set to 60 at 4.00 min	SPL1 set to 10 at 10.00 min
----------------------------	-----------------------------

## Real Time Plot Parameters

Offset (mV): -2.000	Scale (mV): 32.000
---------------------	--------------------

## Processing Parameters

Bunch Factor: 12 points	Noise Threshold: 20 µV
Area Threshold: 100.00 µV	

## Peak Separation Criteria

Width Ratio : 0.200	Valley-to-Peak Ratio : 0.010
---------------------	------------------------------

## Exponential Skim Criteria

Peak Height Ratio : 5.000	Adjusted Height Ratio : 4.000
Valley Height Ratio : 3.000	

## Baseline Timed Events

Event #1 - Disable Peak Detection at 0.010  
Event #2 - Enable Peak Detection at 10.000

Table 22. Component information of GC monosaccharides assesment.

Component	Retention time	Search window
Rha	19.900 min	0.00 s, 0.50 %
Fuc	20.270 min	0.00 s, 1.00 %
Ara	22.730 min	0.00 s, 1.00 %
Xyl	25.170 min	0.00 s, 1.00 %
Man	30.720 min	0.00 s, 1.00 %
Gal	31.520 min	0.00 s, 1.00 %
Glc	32.500 min	0.00 s, 1.00 %
2DOG	26.840 min	0.00 s, 1.00 %

Reference Component : Find peak closest to expected RT in window

### 8.3 The setting parameters for GC analysis of FAME

#### Parameters of Hewlett Packard 5890 GC system

- Packed columns
  - corrected length, 250
- ECD flows, 153
  - flows for NPD, 140
- flows for TCD, 123
  - flows with FPD, 161
- gas flows for FID, 113
- flow ranges, 73
- installing glass columns, 25
- septum purge, 74
- Pressure control
  - capillary columns with FPD, 164
  - detectors, 231
  - EPC, zeroing, 222
  - for FID makeup gas, 120
  - inlet programming, 226
  - NPD with capillary columns, 143
- Programming
  - detector pressure, 232
  - inlet pressure, 226
  - start/stop, 184
- Safety shut down, 220
- septum purge, 74
  
- SETPOINTS
  - loading, 206,207
  - Shutdown, instrument, 11
  - Signal sand INET, 181
  - assigning, 171
  - attenuation on/off, 179
  - on/off control, 176
  - Single column compensation, 129,190
  - Split mode, flows in capillary inlet, 79
  - storing, 206
  - signal output, 170
    - as timed events, 200
  - attenuation, 176
  - display or monitor, 173
  - zeroing, 175
- Packed inlet
  - installing capillary inlets, 27
  - installing metal columns, 23
- Polarity inversion, TCD, 128, 180
  - auxiliary EPC, 230
  - detector programming, 232
  - ECD, 156
  - for FID, 117
  - for TCD, 125
  - inlets with EPC, 222
  - restrictors, 234
- checking inlet pressures, 229
- inlet flow, 246
- oven temperature, 58
- start/stop using INET, 185
- Sensitivity, TCD, 128,201

**Parameters of BPX 70 column**

Phase: BPX70 0.25 µm film	Constant Flow: On
Column: 25 m ×0.22 mm ID	Ave. Linear Velocity: 35 cm/sec at 155 °C
Sample: 200ppm in dichloromethane	Injection Mode: Split
Initial Temperature: 155 °C	Split Ratio: 80:1
Rate 1: 2 °C/min to 180 °C	Injection volume: 0.5 µL
Rate 2: 4 °C/min to 220 °C	Injection temperature: 250 °C
Final Temperature: 220 °C, 5 min	Liner Type: 4mm ID
Detector type: MSD	Double Taper Liner
Carrier Gas: He, 35.3psi	Liner Part Number: 092018
Carrier Gas Flow: 1.6mL/min	Full Scan / SIM: Full scan 45-450

**The standard components of FAME**

1. Methyl butyrate (C4:0)
2. Methyl hexanoate (C6:0)
3. Methyl octanoate (C8:0)
4. Methyl decanoate (C10:0)
5. Methyl undecanoate (C11:0)
6. Methyl laurate (C12:0)
7. Methyl tridecanoate (C13:0)
8. Methyl myristate (C14:0)
9. Methyl myristoleate (C14:1)
10. Methyl pentadecanoate (C15:0)
11. Methyl 10-pentadecenoate (C15:1)
12. Methyl palmitate (C16:0)
13. Methyl palmitoleate (C16:1)
14. Methyl heptadecanoate (C17:0)
15. Methyl stearate (C18:0)
16. Methyl oleate (C18:1)
17. Methyl elaidate (C18:1T)

18. Methyl linoleate (C18:2)
19. Methyl linolenate (C18:3)
20. Methyl gamma linolenate (C18:3)
21. Methyl arachidate (C20:0)
22. Methyl 11-eicosenoate (C20:1)
23. Methyl 11-14 eicosenoate (C20:2)
24. Methyl behenate (C22:0)
25. Methyl erucate (C22:1)
26. Methyl 11-14-17 eicosatrienoate(C20:3)
27. Methyl homogamma linolenate (C20:3)
28. Methyl arachidonate (C20:4)
29. Methyl nervonate (C24:1)
30. Methyl docosadienoate (C22:2)
31. Methyl docosahexaenoate (C22:6)

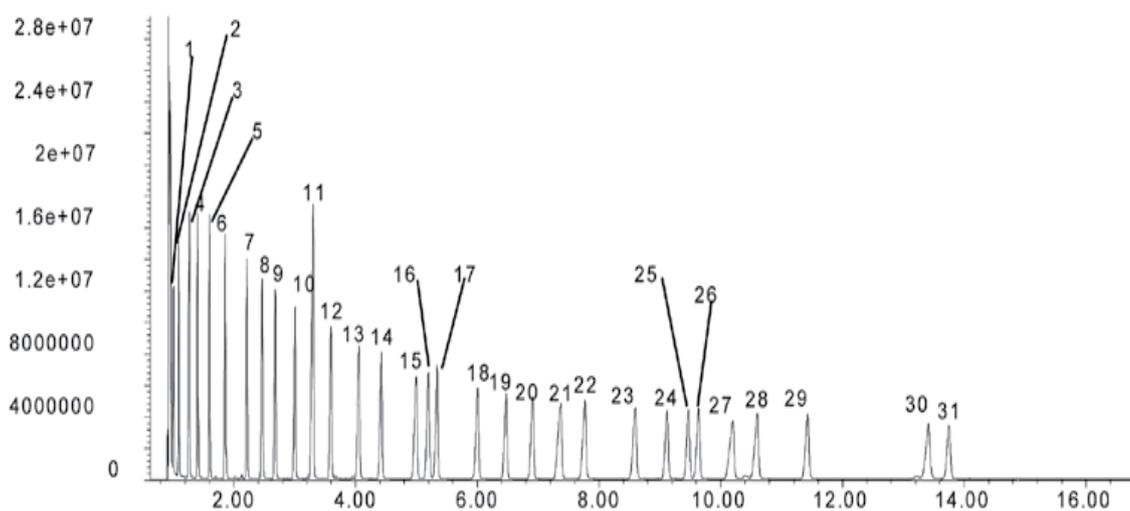


Figure 77. The retention time of 31 components of FAME in BPX 70 column.

#### 8.4 HPLC setting parameters for phenolic acids

Instrument Control Method

Instrument Name : HPLC-DAD

Instrument Type : Quaternary LC Pump Model 200Q/410 with ISS-200 Autosampler

##### Channel Parameters

Delay Time: 0.00 min

Run Time: 42.00 min

Sampling Rate: 1.1364 pts/s

##### Autosampler Method

Injection Source: Autosampler

Injection volume: 40  $\mu$ L

Loop size: 150  $\mu$ L

Fixed mode: Off

Excess volume: 5  $\mu$ L

Sample syringe size: 250  $\mu$ L

Needle level: 5%

Inject delay time: 0.00 min

Flush volume: 1000  $\mu$ L

Flush speed: Fast

Flush cycles: 2

Air cushion: 5  $\mu$ L

Sample speed: Medium

##### Detector Parameters

A (nm): 280 nm

BWA (nm): 5 nm

RWA (nm): 360 nm

B (nm): 325 nm

BWB (nm): 5 nm

RWB (nm): 360 nm

Spectral Acquisition Mode: Time

Lamp off at end of run: No

Sampling Period: 1.76 s

##### Real Time Plot Parameters

Offset (mV): -30.000

Scale (mV): 200.000

##### Processing Parameters

Bunch Factor : 5 points

Noise Threshold : 24  $\mu$ V

Area Threshold : 122.00  $\mu$ V

##### Peak Separation Criteria

Width Ratio : 0.200

Valley-to-Peak Ratio : 0.010

## Exponential Skim Criteria

Peak Height Ratio : 5.000

Adjusted Height Ratio : 4.000

Valley Height Ratio : 3.000

## Baseline Timed Events

Event #1 - Disable Peak Detection at 2.400

Event #2 - Enable Peak Detection at 4.070

Event #3 - Disable Peak Detection at 30.000

Table 23. Component information of HPLC phenolic acids assessment.

Component	Retention Time	Search Window
Protocatechuic Acid	6.42	1.00 s, 2.00 %
Chlorogenic Acid	7.45	1.00 s, 2.00 %
(Aldehyde) Protocatechuic	8.94	1.00 s, 2.00 %
Benzoic Acid (p-OH-)	9.76	1.00 s, 2.00 %
Phenyl Acetic Acid (p-OH-)	10.1	1.00 s, 2.00 %
Vanillic Acid	10.26	1.00 s, 2.00 %
Caffeic Acid	12.58	1.00 s, 2.00 %
(CA)Truxillic Acid	12.99	1.00 s, 2.00 %
Vanillin	13.35	1.00 s, 2.00 %
t-p-Coumaric Acid	14.28	1.00 s, 2.00 %
t-Ferulic Acid	14.88	1.00 s, 2.00 %
c-p-Coumaric Acid	15.2	1.00 s, 2.00 %
c-Ferulic Acid	15.96	1.00 s, 2.00 %

Find peak closest to expected RT in window

Component standard purity percentage: 100.0000%

**8.5 HPLC setting parameters for carbohydrate**

## AutoSampler Section

Device Name: FX10ASCO-3

Model: Flexar FX-10 UHPLC Autosampler Cool Only

Injection Volume ( $\mu\text{L}$ ): 20Syringe size ( $\mu\text{L}$ ): 250

Flushes: 2

Flush volume ( $\mu\text{L}$ ): 250

Sample speed: Medium

Pre-inject flush cycles: 0

Air cushion ( $\mu\text{L}$ ): 5

Post-inject flush cycles: 1

Flush speed: Medium

Tray Temperature ( $^{\circ}\text{C}$ ): 4Loop size ( $\mu\text{L}$ ): 50

Needle level (mm): 4

Tolerance ( $\pm$   $^{\circ}\text{C}$ ): 2Mode: Partial loop (45  $\mu\text{L}$ )

Injection Delay Time (min): 0.000

## Pump Section

Device Name: FX10Pump-2

Transition type: Isocratic

Pressure units: psi

Initial equil time (min): 0.100

Upper pressure limit: 10000

Stop time after equil (min): 999 Lower pressure

limit: 0

Standby time (min): 120.000

Standby flow (mL/min): 0.2

Program				Solvent Reservoir				
Step	Type	Time (min)	Flow (mL/min)	A	B	C	D	Curve
0	Equil	0.1	0.6	0	100			0
1	Run	42.000	0.6	0	100			0

## Oven Section

Device Name: FXPOven-4

Model: Flexar Peltier Column Oven

Temperature ( $^{\circ}\text{C}$ ): 65Tolerance ( $\pm$   $^{\circ}\text{C}$ ): 1

Equil Time (min): 0

## Detector Section

Device Name: FXRIDet-1

Model: Flexar Refractive Index Detector

Temperature ( $^{\circ}\text{C}$ ): 35

Autozero: True

Sampling rate (pts/s): 2                      End Time (min): 42.000  
 Time(min): 0.000                              Polarity: +

Channel Name: RI  
 Model Name: Flexar Refractive Index Detector  
 Time Adjustment (min): 0.000  
 Unretained peak time (min): None

#### Peaks and Calibration

Bunching Factor: 1                              Area Threshold: 0.01  
 Noise Threshold: 0.00                        Matching: Use tallest  
 Outlier Limit (%): 15                        Internal Standard: Ribose  
 RRT Reference Component: Unidentified Peak Quantitation

Table 24. Components information of carborhydrate size measurement.

Component	Retention time (min)	Peak search start (min)	Peak search end (min)
Cellobiose	10.025	9.641	10.409
Maltose	10.514	10.115	10.913
Glucose	12.255	11.693	12.588
Xylose	13.369	12.759	13.721
Galactose	13.979	13.979	13.979
Ethanol	17	16.5	17.5
Ribose	34.926	33.422	36.095

**8.6 HPLC setting parameters for inhibitors / organic acids**

## AutoSampler Section

Device Name: FX10ASCO-3

Model: Flexar FX-10 UHPLC Autosampler Cool Only

Injection Volume ( $\mu\text{L}$ ): 25Syringe size ( $\mu\text{L}$ ): 250

Flushes: 2

Flush volume ( $\mu\text{L}$ ): 1000

Sample speed: Medium

Pre-inject flush cycles: 1

Air cushion ( $\mu\text{L}$ ): 5

Post-inject flush cycles: 1

Flush speed: Fast

Tray Temperature ( $^{\circ}\text{C}$ ): 4Loop size ( $\mu\text{L}$ ): 50

Needle level (mm): 4

Tolerance ( $\pm$   $^{\circ}\text{C}$ ): 1Mode: Partial loop (45  $\mu\text{L}$ )

Injection Delay Time (min): 1.000

## Pump Section

Model: Flexar FX-10 UHPLC pump

Device Name: FX10Pump-2

Transition type: Isocratic

Pressure units: psi

Initial equil time (min): 0.100

Upper pressure limit: 1600

Stop time after equil (min): 999.9

Lower pressure limit: 0

Standby time (min): 120.000

Standby flow (mL/min): 0.2

Program				Solvent Reservoir				Curve
Step	Type	Time (min)	Flow (mL/min)	A	B	C	D	
0	Equil	0.000	0.6	0	100			0
1	Run	60.000	0.6	0	100			0

## Oven Section

Device Name: FXPOven-5

Model: Flexar Peltier Column Oven

Temperature ( $^{\circ}\text{C}$ ): 65Tolerance ( $\pm$   $^{\circ}\text{C}$ ): 1

Equil Time (min): 0

## Detector Section

Device Name: FXRIDet-4

Model: Flexar Refractive Index Detector

Temperature ( $^{\circ}\text{C}$ ): 35

Autozero: True

Sampling rate (pts/s): 2

End Time (min): 60.000

Time(min): 0.000

Polarity: +

Device Name: PDADet-1

Model: Photo Diode Array Detector

Sampling rate (pts/s): 2

End Time (min): 60.000

Unretained peak time (min): 0

Channel Name: RI

Model Name: Flexar Refractive Index Detector

Time Adjustment (min): 0.000

Unretained peak time (min): None

Plot Title: RI

Scaling type: Autoscale

Y min: -200.000

Y max: 500

Start (min): 0.000

End (min): 999.990

Annotations:

Sample ID: Yes

Baselines: Yes

RTs: Yes

Wavelengths: Yes

X axis label: Yes

Y axis label: Yes

Peak Names: Yes

Timed Events: Yes

Overlay: none

Channel Name:

Model Name: Photo Diode Array Detector

Plot Title: 210

Scaling type: Autoscale

Y min: -1000.000

Y max: 4000

Start (min): 0.000

End (min): 999.990

Annotations:

Sample ID: Yes

Baselines: Yes

RTs: Yes

Wavelengths: Yes

X axis label: Yes

Y axis label: Yes

Peak Names: Yes

Timed Events: Yes

Overlay: none

Peaks and Calibration

Bunching Factor: 1

Area Threshold: 0.01

Noise Threshold: 0.00

Matching: Use closest

Outlier Limit (%): 15

RRT Reference Component: Unidentified Peak Quantitation

Table 25. Peak Identification by using channel RI

Component	Retention time (min)	Peak search start (min)	Peak search end (min)
TFA	6.360	6.310	6.420
Citric	7.643	7.330	7.955
Malic	9.067	8.950	9.200
Succinic	9.067	8.950	9.200
Formic	14.385	12.747	15.506
Acetic	15.554	14.960	16.987
5-HMF	28.459	26.360	32.749
2-FA	41.796	39.100	45.101

Table 26. Peak Identification by using channel 210

Component	Retention time (min)	Peak search start (min)	Peak search end (min)
TFA	6.162	5.894	6.431
Citric	7.460	7.153	7.767
Malic	8.879	8.529	9.229
Succinic	10.756	10.350	11.162
Formic	14.209	13.699	14.719
Acetic	15.378	15.361	17.854
5-HMF	30.072	29.000	31.557
2-FA	43.000	40.766	45.671

**8.7 HPLC setting parameters for carbohydrate size**

## AutoSampler Section

Device Name: AS-3

Model: LC 200 Series Autosampler

Injection Volume ( $\mu\text{L}$ ): 40Syringe size ( $\mu\text{L}$ ): 250

Flushes: 2

Flush volume ( $\mu\text{L}$ ): 1000

Sample speed: Medium

Pre-inject flush cycles: 3

Air cushion ( $\mu\text{L}$ ): 10

Post-inject flush cycles: 3

Flush speed: Fast

Tray Temperature ( $^{\circ}\text{C}$ ): 20Loop size ( $\mu\text{L}$ ): 100

Needle level (%): 10

Tolerance ( $\pm$   $^{\circ}\text{C}$ ): 1Excess volume ( $\mu\text{L}$ ): 10Flush syringe size ( $\mu\text{L}$ ): 250

Fixed Mode: Off

Injection Delay Time (min): 0.000

## Pump Section

Model: LC 200 Series Quaternary Pump

Device Name: QPump-2

Transition type: Isocratic

Pressure units: psi

Initial equil time (min): 5.000

Upper pressure limit: 725

Stop time after equil (min): 60.00

Lower pressure limit: 40

Standby time (min): 120.000

Standby flow (mL/min): 0.2

Program				Solvent Reservoir				
Step	Type	Time (min)	Flow (mL/min)	A	B	C	D	Curve
0	Equil	1.000	0.5	0	100	0	0	0
1	Run	60.000	0.5	0	100	0	0	0

## Oven Section

Device Name: Oven-5

Model: LC 200 Column Oven

Temperature ( $^{\circ}\text{C}$ ): 25Tolerance ( $\pm$   $^{\circ}\text{C}$ ): 1

Equil Time (min): 0.5

Channel name: RIDet-4 1

## Detector Section

Device Name: RIDet-4

Model: LC 200a Series Refractive Index Detector

Channel name: RIDet-4 1

Temperature (°C): -1

Autozero: True

Sampling rate (pts/s): 1

End Time (min): 60.000

Time(min): 0.000

Polarity: +

Device Name: PDADet-1

Model: Photo Diode Array Detector

Sampling rate (pts/s): 1

End Time (min): 60.000

Unretained peak time (min): 0

#### Channels

Channel Name:

Model Name: Photo Diode Array Detector

Time Adjustment (min): 0.000

Unretained peak time (min): None

Channel Name: RIDet-4 1

Model Name: LC 200a Series Refractive Index Detector

Time Adjustment (min): 0.000

Unretained peak time (min): None

Plot Title: RIDet-4 1

Scaling type: Autoscale

Y min: -200.000

Y max: 500

Start (min): 0.000

End (min): 999.990

Annotations:

Sample ID: Yes

Baselines: Yes

RTs: Yes

Wavelengths: Yes

X axis label: Yes

Y axis label: Yes

Peak Names: Yes

Timed Events: Yes

Overlay: none

#### Peaks and Calibration

Bunching Factor: 4

Area Threshold: 0.03

Noise Threshold: 0.01

Matching: Use tallest

Outlier Limit (%): 15

Table 27. RRT Reference Component: Unidentified Peak Quantitation

Component	Retention time (min)	Peak search start (min)	Peak search end (min)
Mw $\geq$ 23000	3.923	3.719	4.121
Mw 12200	4.000	3.797	4.203
Mw 5800	4.195	3.986	4.404
Mw 738	5.311	5.068	5.554
Cellobiose	5.895	5.635	6.155
Glucose	6.297	6.025	6.569

### 8.8 The combined enzymatic hydrolysis using cellulase and amylase

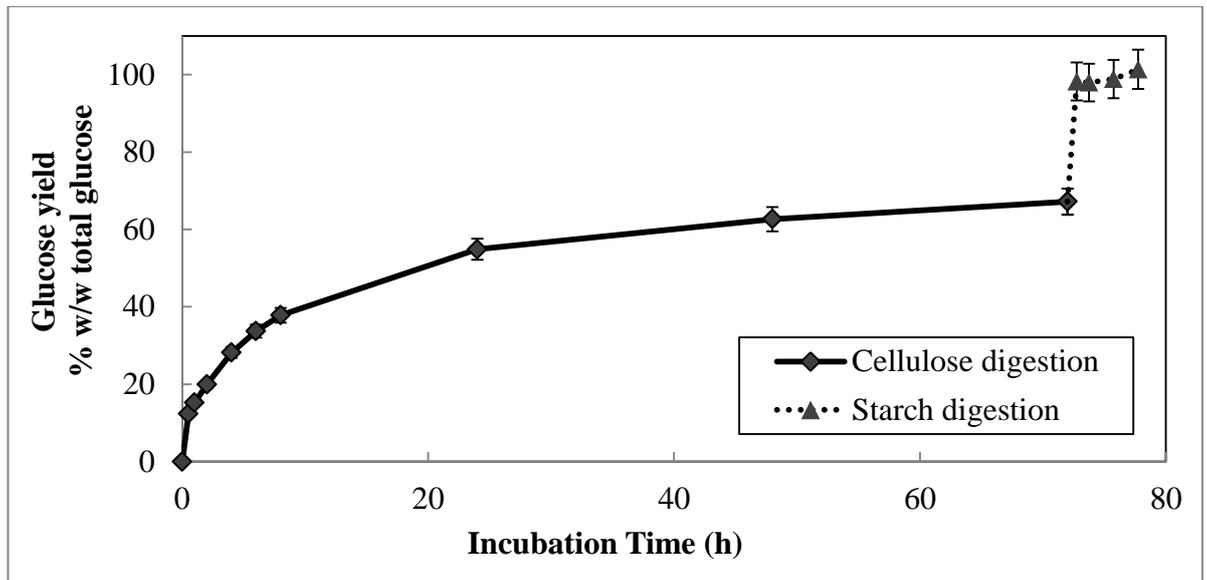


Figure 78. The combined enzymatic hydrolysis of starch and lignocellulosic carbohydrate.

## 8.9 The cost estimation original data

Table 28. The capital cost of the ethanol production plant with the productivity at 10000 barrel per day (Anbarasan et al, 2012).

Unit	Size	Cost (\$Million)	Cost (%of total)
Fermenter	42000 m <sup>3</sup>	5.41	1.93
Ethanol Distiller	18 stages	0.45	0.16
Tributylin Distiller	4 stages	0.06	0.02
2× Toluene Distiller	20 stages	0.96	0.34
Condensation PFR	3440 m <sup>3</sup>	6.13	2.18
Centrifuge	50 kg solid/s	2.77	0.99
3× Holding Tanks	20000 m <sup>3</sup>	14.1	5.03
Pretreatment	14500 m <sup>3</sup>	4.02	1.43
Hydrolysis	2400 m <sup>3</sup>	0.75	0.27
Dryer	6.4 Mkg/day	2.21	0.79
Other <sup>a</sup>	/	36.9	13.1
<b>Total Units</b>	/	<b>73.8</b>	<b>26.3</b>
Solvents <sup>b</sup>			
Tributylin <sup>c</sup>	42000 m <sup>3</sup>	68.1	24.3
Toluene	4800 m <sup>3</sup>	3.5	1.3
<b>Total solvent</b>	/	<b>71.7</b>	<b>25.5</b>
Catalysts			
Palladium	3.57 tons	132	47
K <sub>3</sub> PO <sub>4</sub>	3400 tons	3.4	1.2
<b>Total Catalyst</b>		<b>135.4</b>	<b>48.2</b>

<sup>a</sup>Other accounts for all neglected costs. <sup>b</sup>Industrial Chemical Prices from ICIS. <sup>c</sup>Tributylin estimated using glucerol, butanal.

Table 29. Breakdown cost for a biomass pretreatment plant based on the steam explosion having a capacity of 25.000 t/y (DM) expressed in milion Euros (Zimbardi et al, 2002).

Direct Fixed Cost	Expression	Cost
<b>Total plant direct cost, TPDC</b>		
Purchasing cost, PC	/	4.65
Installation	0.4PC	1.86
Piping	0.35PC	1.63
Instruments	0.3PC	1.4
Electricity supplying	0.15PC	0.7
Building	0.35PC	1.63
place and Adaptation	0.15PC	0.7
<b>Total</b>	<b>2.7 PC</b>	<b>12.56</b>
<b>Total plant Indirect cost, TPDC</b>		

Plant engineering	0.15TPDC	1.88
Building engineering	0.10TPDC	1.26
Total	0.25TPDC	3.14
Total plant cost, TPC (TPCD +TPIC)	3.375PC	15.69
Income	0.17PC	0.79
Total Direct Fixed Cost	3.545PC	16.48
<b>Operational Cost</b>	<b>Expression</b>	<b>Cost</b>
<b>Cost related to DFC</b>		
Devaluation <sup>a</sup>	DFC(1-f)/n	0.99
Maintenance	0.02DFC	0.33
Insurance	0.005DFC	0.08
Plant Management	0.03DFC	0.49
Total	0.122DFC	1.9
<b>Cost related to manpower</b>		
Direct manpower <sup>b</sup>	MP	0.66
Benefits	0.4MP	0.26
Supervision	0.4MP	0.26
Labour equipment	0.1MP	0.07
Chemical Analyses	0.15MP	0.1
Administration	0.6MP	0.4
Total	2.65MP	1.75
Raw materials (straw) <sup>c</sup>	/	1.32
Fuel (Methane) <sup>d</sup>	/	0.63
<b>Chemical</b>		
NaOH <sup>e</sup>	/	0
H <sub>2</sub> SO <sub>4</sub> (98 %) <sup>f</sup>	/	0
Total		0
<b>Utilities</b>		
Electricity supplying <sup>g</sup>	/	0.52
Water <sup>h</sup>	/	0.03
Steam (thermoregulation) <sup>i</sup>	/	
Total	/	0.55
Waste management <sup>l</sup>	/	
Total Operational cost	/	4.82
Specific cost (Euros/kg DM)	/	0.193

<sup>a</sup>n= years of plant activity; set as 15; f= plant value at dismissal set as 10 %. <sup>b</sup>Twelve workers on shift, with individual gross wage of 20 Euros /h. <sup>c</sup>0.043 Euros/kg. <sup>d</sup>0.284 Euros/m<sup>3</sup>. <sup>e</sup>0.36 Euros/kg. <sup>f</sup>0.10 Euros/kg. <sup>g</sup>0.12 Euros/kWh. <sup>h</sup>0.67 Euros/m<sup>3</sup>. <sup>i</sup>0.013 Euros/kg. <sup>l</sup>6 Euros/m<sup>3</sup>.

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