Bioprocessing of hemp hurd (*Cannabis sativa)* for biofuel production

By

Reinu Elsa Abraham

B.Tech, M.S (Research)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

May, 2014

DEAKIN UNIVERSITY

ACCESS TO THESIS - A

I certify the following about the thesis entitled (10 word maximum)

Bioprocessing of hemp hurd (*Cannabis sativa*) for biofuel production

submitted for the degree of Doctor of Philosophy

This thesis may be made available for consultation, loan and limited copying in accordance with the Copyright Act 1968.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: Reinu Elsa Abraham

Signed: Signature Redacted by Library

Date: 30/10/14

DEAKIN UNIVERSITY

CANDIDATE DECLARATION

I certify the following about the thesis entitled (10 word maximum)

Bioprocessing of hemp hurd (*Cannabis sativa*) for biofuel production

submitted for the degree of Doctor of Philosophy

a. I am the creator of all or part of the whole work(s) (including content and layout) and that where reference is made to the work of others, due acknowledgment is given.

b. The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

c. That if the work(s) have been commissioned, sponsored or supported by any organisation, I have fulfilled all of the obligations required by such contract or agreement.

I also certify that any material in the thesis which has been accepted for a degree or diploma by any university or institution is identified in the text.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: Reinu Elsa Abraham

Signed: Signature Redacted by Library

Date: 30/10/14

Table of Contents

List of Figures

List of Tables

Acknowledgments

Pursuing my passion to be in Australia and completing my PhD degree was probably the most challenging dream in my life. My four years of research journey was filled with best and worst moments which I shared with many people.

I extend my gratitude to Professor Colin Barrow, my executive supervisor for helping me to pursue my PhD. I am able to complete my candidature due to his invaluable research advices and supervision which kept me ambitious and helped to achieve my goal. He was always supportive for all the decisions taken during the candidature and conducting research. His guidance served me well to outline my work and allowed to be a part of great professional people.

My sincere thanks to my co-supervisor, Associate Professor Dr. Munish Puri for providing me directions and being my moral support to head in my research and complete thesis. His focus on the work, perception of research in the area of biofuels and bioenergy are worth mentioning. He introduced me to the cutting edge area of "lignocellulosic fuels" where our contributions will be path breaking. I extend my heartfelt thanks to him for being a strong supportive advisor throughout my candidature and giving me freedom to pursue my research independently. I am thankful to him for introducing me to "nanobiotechnology" world that has its challenges but I am delighted to share that it has unique rewards. His perpetual encouragements and advices kept me determined to follow my dream.

Special thanks to Dr. Madan Verma for helping me to gain laboratory skills in the area of enzyme immobilisation. I appreciate Dr. Jitraporn Vongsvivut for providing me training in FTIR and helping me analyse the results and Dr. Jacqui Adcock for giving me guidance on gaining skills in HPLC and lab practices.

I am thankful to Dr. Andrew Sullivan for giving me training and making me acclimatised with scanning microscopy facility. My sincere thanks to Ms. Elizabeth Laidlaw for helping me settle in lab and providing me initial research guidelines. I

am thankful to Rob Pow for giving me inductions and training on various instruments in The Institute for Frontier Materials (IFM).

I extend my thanks to all my lab mates for being a great support and wonderful part of my four years of research. I would like to thank my best lab buddy Adarsha for always being kind, patient and providing honest suggestions. I am thankful to you for always being around as a moral support and a helping hand.

I thank my wonderful friends in Geelong for being supportive and dear to me. I extend my heartfelt thanks to my dear friends Janani and Sreejoyee for always being my moral support and giving me cherishing moments. Janani thanks for being an amazing company to wander around and keeping me lively. Sreejoyee, I would like to thank you for being a patient listener, coffee chats and excitements.

I would like to thank Rajesh for being a great friend and for all the fun filled trips. I extend my thanks to Mohan for being my caring brother. I am thankful to Sanjana, Pratibha, Ashwantha and Raju for being a wonderful support and good friends. I thank Mark, Angie, Henk and Ria for being kind and helpful friends.

Above all, I would like to express my sincere gratitude to Former Vice Chancellor Professor Sally Walker for encouraging me to pursue my dream and passion. I am grateful to Prof. Walker for building the confidence in me to join Deakin University. It was a great privilege to work at Deakin University.

I acknowledge Centre for Chemistry and Biotechnology for granting me Postgraduate Research scholarship. I extend my sincere thanks to School of Life and Environmental Science and Strategic Research Centre for providing academic and technical support to pursue research.

I especially thank my parents for their unconditional love and care. Their spiritual support and faith has always been my strength and confidence. I extend my sincere thanks to my wonderful friends in India and relatives for their support and prayers.

I thank almighty for bestowing his mercy and keeping me determined to achieve this goal.

List of Publications

Publications related to Thesis

Abraham RE, Barrow CJ, Puri M: Relationship to reducing sugar production and scanning electron microscope structure to pretreated hemp hurd biomass *(Cannabis sativa)*. *Biomass and Bioenergy* 2013, 58:180-87, (Impact Factor 3.9)

Puri M, **Abraham RE**, Barrow CJ: Biofuel production: Prospects, challenges and feedstock in Australia. *Renewable and Sustainable Energy Reviews* 2012, 16:6022-31, (Impact Factor 6.5)

Abraham RE, Verma ML, Barrow CJ, Puri M. Immobilising cellulase on doped ferrite nanoparticles to improve enzymatic saccharification of pretreated hemp biomass. *Biotechnology for Biofuels* 2014, 7:90, (Impact Factor-6.2)

Abraham RE, Barrow CJ, Puri M. Morphological and structural changes characterisation of pretreated and enzyme hydrolysed industrial hemp biomass for biofuel development - under preparation

Gupta A, **Abraham RE**, Barrow CJ, Puri M. Omega-3 fatty acid production from enzyme saccharified hemp hydrolysate using a novel marine microalgae thraustochytrid strain - Bioresource Technology – (Impact Factor 5.1, revised submitted 27th October 2014)

Book chapter

Abraham RE, Barrow CJ, Puri M: Developing pre-treatment technologies for biomass processing: current scenario and future prospectives in biofuel

production. Solutions to environmental challenges through innovations in research. 2013, chapter-3, pp. 41-61

Abraham RE, Barrow CJ, Puri M: Molecular characterisation of immobilised cellulase in facilitating pretreatment of lignocellulosic biomass (Elsevier book - under preparation)

Other publications

Abraham RE, Wong C, Barrow CJ, Puri M. Characterisation of cellulose microfibers extracted from industrial biomass waste (*Cannabis sativa*) - under preparation

Conference participation

- **2013:** AOCS Australasian Section Biennial Meeting Fats & Oils - The Food vs. Fuel Dilemma - Newcastle, NSW - Australia
- **2012:** CSIRO Cutting Edge 2012 Symposium - Biological and Chemical Conversion of Renewables to fuel and chemicals - Victoria, Australia
- **2011:** International Conference on Challenges in Environmental Science & Engineering (CESE -2011) - Tainan city, Taiwan
- **2010:** CSIRO Protein expression workshop - Victoria, Australia

Awards

- **2013:** AOCS Australasian Section Biennial Meeting - Student Poster Award
- **2011:** BioDeakin Postgraduate Research Scholarship
- **2010:** Best HDR presenter- ITRI Research Conference 2010 - Frontiers of Science and Technology

Abstract

Increasing fossil fuel consumption has led to concerns regarding energy security, depleting energy reserves, price increases and global climate change. Alternative renewable energy is a potential replacement for fossil fuel, but relies on the availability of large amounts of biomass. Lignocellulosic biofuel is produced from lignocellulosic feedstock, which is a carbon reserve available in abundance and can potentially serve as a sustainable biofuel. The cost and efficiency of biofuel production from lignocellulosic material needs to be improved through technology development before it will be widely adopted.

Australia has a large amount of lignocellulosic biomass, although production of second generation/lignocellulosic biofuel is in its infancy in Australia and research is required to optimise the use of local biomass. It is important not only to optimise the production of biofuel from locally obtained lignocellulose, but also to determine what valuable co-products can be produced so that the cost of biofuel is offset by the production of other valuable materials in a biorefinery. The steps involved in the conversion of biomass to biofuel include pretreatment, enzyme hydrolysis and fermentation. The compact structure of lignocellulose is expanded via pretreatment and then hydrolysed by enzyme to form hexoses and pentoses sugars that are later used in fermentation for ethanol production.

This project optimises pretreatment, enzyme hydrolysis and fermentation steps for the conversion of waste hemp (*Cannabis sativa*) into biofuel. Hemp was mechanically milled to reduce its size and then pretreated under a variety of conditions. The maximum opening of the structure was obtained with alkaline pretreatment, which gave a 74% yield of reducing sugars, the highest yield obtained among the pretreatments applied. Maximum hydrolysis was achieved through enzymatic treatment of a 5% concentration of sodium hydroxide pretreated hemp. The composition of the resulting sugar hydrolysate was analysed using reverse-phase high performance liquid chromatography (RP-HPLC) and shown to contain cellobiose, glucose, xylose and various inhibitors. An overnight washing of the pretreated biomass removed the inhibitors before hydrolysis, leading to an increased yield of reducing sugars after enzymatic treatment. Morphological and structural

characterisation of the biomass revealed that pretreatment resulted in the opening of biomass structure, removal of amorphous components and the formation of porous structures. The biomass was mainly composed of holocellulose and the crystallinity increased with pretreatment. Effective enzyme hydrolysis resulted in complete breakage of biomass into small fragments.

The cost of the enzyme used for hydrolysis of biomass is a major component of the overall cost of biofuel production. More efficient use of the enzyme can potentially be achieved through immobilisation, where the enzyme could be recovered and re-used. Immobilisation on nanoparticles may enable the enzyme to penetrate into the biomass structure to achieve hydrolysis, while also enabling recovery of the particles for reuse. We chose to immobilise the enzyme on magnetic nanoparticles, so that magnetic methods could be used to readily recover the enzyme after hydrolysis treatment. Immobilisation resulted in 94% of enzyme binding on the nanosupport, and enhanced the stability of the enzyme. This enzyme mixture provided thermostability at 80 °C, and 50% of the initial enzyme activity was retained after five repeated cycles of hydrolysis. The immobilised enzyme also showed good storage stability and resulted in 93% of hydrolysis yield from pretreated hemp hurd. The sugar hydrolysates obtained from the enzyme saccharifications were applied for biorefinery applications. These sugars were utilised in the fermentation medium to produce ethanol and grow marine microbes. The concentration of ethanol produced was detected by reversed-phase HPLC and Raman spectroscopy. The marine microbes used in the study have the potential to produce co-products such as polyunsaturated fatty acids (PUFA) and carotenoids. The growth profiles of these microbes were compared using various concentrations of sugar hydrolysate and glucose as the carbon source. Growth was similar for both glucose and sugar hydrolysate, showing that sugar hydrolysate produced from hemp was an effective carbon source for microbial production of biofuel and PUFA.

Abbreviation

~	-	approximately
°C	-	degree Celsius
µm	-	micrometre
µmol	-	micromole
µL	-	microlitre
AR	-	Analytical grade
c/L	-	cents per litre
cm	-	centimetre
emu g^{-1}		mass magnetisation
h	-	hour
g	-	gram
GL	-	Gigalitre
L	-	Litre
M	-	Molarity
mbar	-	millibar
Mha	-	Million hectare
mg	-	milligram
mg g^{-1}	-	milligram per gram
min	-	minute
ML	-	Million Litre
mL	-	millilitre
mm	-	millimetre
nm	-	nanometre
ppm	-	parts per million
rpm	-	revolution per minute

wt.	-	weight
w/v	-	weight/volume
U	-	Unit
U g^{-1}	-	Unit per gram
v/v	-	volume/volume

Chapter - 1

Introduction and literature review

1.1 Introduction

The growth of the transport, energy and industrial sectors has led to increasing pressure on the availability and cost of fossil fuels. Increasing academic and public awareness of greenhouse gas (GHG) emissions and global warming have further led to search for an alternative energy (Kopetz 2013). Growing issues of the transport sector and pressure of fossil fuel depletion brought the concept of a renewable biorefinery. This concept includes production of fuel from renewable sources like food crops, lignocellulose and waste oil; and the conversion of biological material to fuel is termed as biofuel. Biofuel is a sustainable resource as it produces energy from lignocellulosic residues. It also produces value added by-products, provides environmental benefits and can be considered as a promising substitute of fossil fuel (Fairley 2011).

Biofuel is classified into three major generations: *first generation*, *second generation* and *third generation*. First generation comprises of food crops which include sugarcane, corn, wheat, sugarbeet, barley and the research is now matured. Production of biofuel in large scale from food crops such as corn and sugarcane has developed sufficient in USA and Brazil to meet the demand (Sims et al. 2010). In the 1970's Brazil initiated the Pro-alcohol program of introducing ethanol blend fuel for vehicles which was relatively a successful program. Brazil, USA and UK are the major investors in ethanol based biofuel from food crops with new technologies (Oberling et al. 2012). The growth and development of liquid biofuel differs globally. The USA government promoted biofuel as corn derived ethanol and the European Union promoted to secure the energy supply and growing environmental issues.

However, the complete dependence of first generation biofuels on food crops made it somewhat unpopular as it competes directly with the food supply and due to sustainability issues its expansion is limited. The concept of second generation biofuel was created to overcome the issues of competition with the food supply (Graham-Rowe 2011). Second generation biofuel emphasises on lignocellulosic feedstock which includes agricultural wastes, forest residues and organic wastes. It involves the production of ethanol from lignocellulosic biomass which is a promising

prospect due to both the widespread availability and high energy density of lignocellulosic matter (Somerville et al. 2010). The basic aim in the conversion of feedstock to biofuel is to eliminate the oxygen from carbohydrates to obtain hydrocarbons. The energy content of biomass increases with the decreased oxygen content (Bond et al. 2014). Bioethanol from lignocellulose is a carbon-neutral, renewable source of energy. Besides energy security and environmental benefits it also stands to reduces waste, generates an additional income to rural sectors and eases the competition among food crops (Mohr and Raman 2013).

Different economic and technological challenges are associated with second generation biofuel. Once these are overcome, it will be preferred over first generation biofuel due to non-competition with the food supply and wide range of secondary benefits (Thompson and Meyer 2013). Second generation biofuel neither requires on a particular feedstock nor a highly fertile land for agriculture. The acceptance of biofuel is growing around the world and in international markets as it reduces energy supply issues and mitigates GHG emissions (Gabrielle et al. 2014; Venghaus and Selbmann 2014).

Third generation biofuels were developed to overcome competition and reliance on farmland for food and non-food crops. Third generation biofuel uses algal biomass having high growth yield (Wijffels and Barbosa 2010). The algal biomass targeted for fermentation gives high lipid content with good productivity. First and second generation biofuel is based on the availability of plant derived feedstock or biomass whereas third generation biofuel produces biomass and does not compete with agriculture for land (Zhu et al. 2014). Faster growth and favourable growing conditions are the advantages of third generation algal fuel over previous generations of biofuel. The feasibility of this concept requires high biomass yielding algal strains which led to the production of genetically modified strains (Adenle et al. 2013). Three generations of biofuel, its sources and respective product are represented in Figure 1.1.

Biofuel will become an important substitute for fossil fuel over time. However, more industrial scale production facilities, funding and favourable energy policies are required to increase the use of biofuel. A successful technology requires maximum

conversion with very low wastage and a good material energy balance (Florin et al. 2014). Other issues that constrain the global development of biofuel include its social acceptance and substitution of fossil fuel which is not common in many countries. For the implementation of global biofuel development, government policies, public and stakeholders opinion should be favourable which is lacking in many countries (Richard 2010).

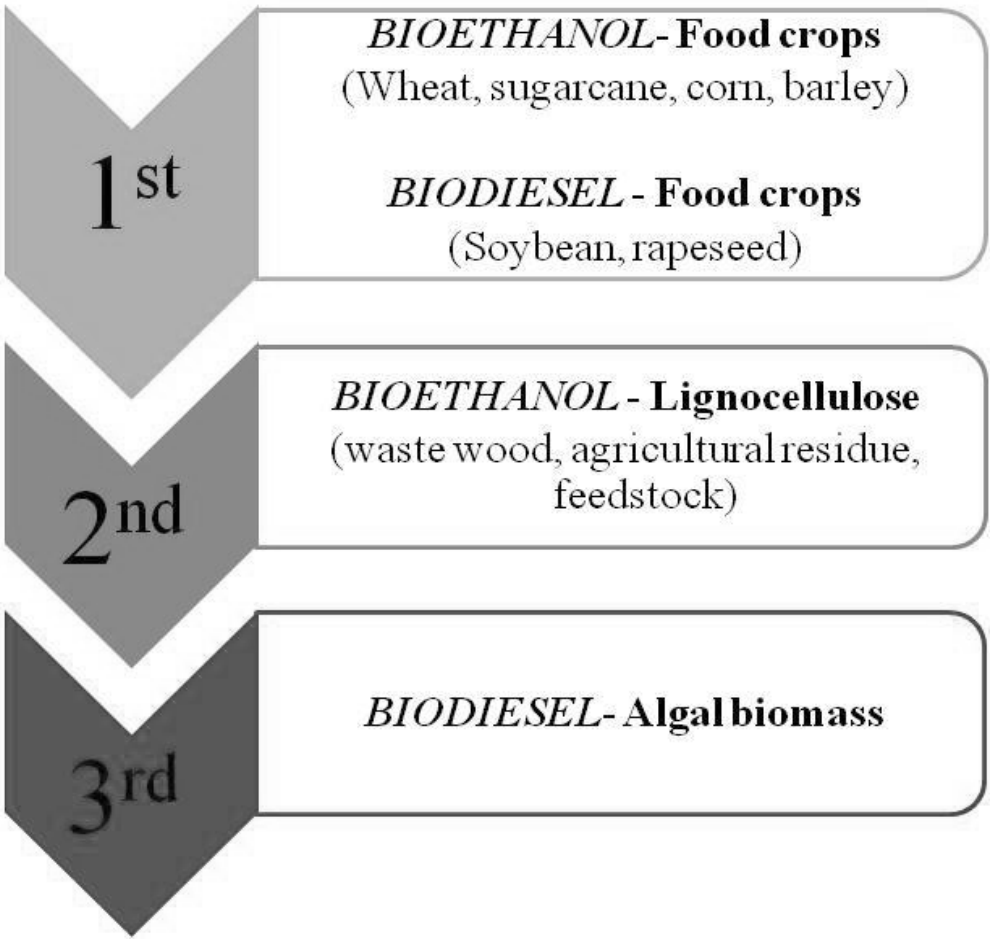


Figure 1.1 Three generations of biofuel and their sources

Countries like Germany, USA and Brazil have accepted the feasible option of biofuel production from second generation feedstock and implementing it for aviation industry. These countries are biofuel market leaders due to their interest and demand for biofuel (Kohler et al. 2014). They play a key role in setting up energy policies and

gaining investors interest to expand the global market. Government plays an important role in raising sustainability standards and providing confidence for the development of biofuel and its related industries (Berti and Levidow 2014). Increasing land usage is also a concern as biofuel production can compete with agricultural land, fertilisation and water. It is unclear how these issues can be solved other than by encouraging the production of feedstock which needs minimal land usage (Witcover et al. 2013). Studies are conducted on the basis of environmental pollution, food prices and ethical support to determine impact of biofuel on health and sustainability. Many countries have initiated marketing biofuel in various concentrations because it supports energy security and environmental benefits (Buyx and Tait 2011).

The blending concentration of biofuel in petrol and diesel varies by country. Ethanol is blended with petrol at concentrations of 10 and 85%, and marketed as E10 and E85, respectively. Biodiesel blended at a concentration of 2, 5, 20 and 100% are marketed as B2, B5, B20 and B100 blends, respectively (the latter can be used in dual-fuel automobiles) (Escobar et al. 2009).

A recent increase in the demand for ethanol has biofuel investment from investors in USA, Brazil, Germany, Sweden, and France (Subbaraman 2010). Russia, China and India are also promoting biofuel production by citing ecological viability, energy security and balance, employment, economy and rural development. The USA and Brazil together produces 78% of the world's fuel ethanol and Brazil uses 25% of ethanol blend petrol for transportation (Demirbas 2011). The biofuels market in Australia is under development where government and private entities are engaged in promoting the technology across the country. Currently E5, E10, E15 and E85 are available throughout Australia as ethanol blends with petrol (Ballinger 2008; FCAI 2006).

National concerns towards a future where first generation crops are chosen as either food or fuel can be eased by research into second-generation fuel produced from lignocellulosic biomass. Although the production of lignocellulose biomass ethanol has various technological and economic constraints it is potentially a sustainable fuel in the long term (Venghaus and Selbmann 2014). The process of converting biomass

into biofuel includes lot of technological challenges. The cost of biofuel differs with feedstock, geographical location and the industrial processes employed. The greenhouse gas (GHG) balancing depends on the feedstock used for the production of biofuel and not all feedstocks are carbon neutral (Timilsina and Shrestha 2011).

The selection of feedstock, its pretreatment, enzymatic saccharification and fermentation are key processes of ethanol production from lignocellulose. The pretreatment of lignocellulosic biomass is an essential process in the production of biofuel because the effectiveness of the pretreatment step impacts the yield of fermentable sugars and ethanol (Rubin 2008). Although the cost of lignocellulosic substrates is low but the production costs are high. Advancements in pretreatment procedures to decrease production costs are crucial to the economic viability of the technology.

1.2 Global status of biofuel and Australian perspective

Brazil and the USA have relatively matured technologies and are considered global leaders for biofuels from corn, sugarcane and lignocellulosic crops. Brazil supports its annual bioethanol production with new sugarcane varieties and agricultural technologies (Amorim et al. 2011). It's reported that the global biofuel production was up to 105 billion litres in 2010, showing a production increase of 17% from 2009. Among the European nations Germany was reported as the largest producer and consumer of biofuel (bioethanol & biodiesel) in 2010. France, Spain, Italy and the UK are the top four countries to produce biofuel after Germany and most of the countries in EU are biofuel producers (Gunnur and Nilgun 2013).

Asian countries have emerging biofuel industries. China produces a huge amount of agricultural residue suitable for biofuel production and 20% of total Chinese petroleum is comprised of ethanol blended fuel (Chen et al. 2009; Hua and Phillips 2011). In the early 2000's China invested over four large ethanol producing setup and became the world's third largest biofuel producer. Increase in the production of biofuel in China might increase commodity prices globally due to competition with agricultural food crops (Huang et al. 2012a).

In India, first generation biofuel technology is more mature than second-generation and supports its bioethanol production with sugarcane molasses and biodiesel from *Jatropha* (Findlater and Kandlikar 2011). In Southeast Asian countries such as Malaysia, Indonesia and Thailand, the production of biodiesel is primarily from palm oil and *Jatropha*. Thailand produces its ethanol from cassava and sugarcane, and is investing in the commercialisation of B5 and B10 (Zhou and Thomson 2009). According to reports on Indonesian biofuel, country is replacing 5% of total energy with biofuel from sugarcane and palm oil. They are analysing the processing and production cost, manpower, area and project plan to setup large scale biofuel production in Indonesia (Rahmadi et al. 2013).

Several Asian countries are growing *Miscanthus* for fibre and pulp production. A report on Pakistan biofuel reported promotion of national biofuel program to develop biofuel in country which might increase the prices of sugarcane, maize soybean and rapeseed (Ali et al. 2013). The USA has also planned to cultivate switchgrass for biofuel production over several million hectares and established a renewable fuel standard enforcing mandatory minimum annual production of biofuel and a mandatory fraction of second generation biofuels in its overall production volume (Gutterson and Zhang 2009).

Australia produces a large amount of second generation feedstock through forest plantations, agricultural residues and organic wastes for the production of bioenergy, mainly electricity. A considerable amount of renewable energy is produced annually, contributing significantly towards the primary energy production of Australia. However, the production of biofuel on a commercial scale using available biomass requires mature technology and experience. The production cost of ethanol depends strongly on both availability and productivity of different kinds of biomass as well as the chosen production strategy. Australia produces less than 5% of its energy from biomass (Creagh et al. 2004). The resources for the production of bioenergy (electricity and biofuels) come primarily from the sugar, wood processing and paper manufacturing industries (Syed et al. 2010).

The production of second-generation biofuel from sugar production wastes and agricultural biomass is currently in the developmental phase in Australia. In the past

five years, Australia has seen a significant increase in biofuel consumption. Currently Australia has three major bioethanol facilities in operation: The Manildra facility in Nowra - New South Wales is Australia's largest bioethanol producer (300 ML) and uses waste starch for production. Dalby bio-refineries in Queensland has capacity of 80 ML ethanol production from red sorghum and Sarina distilleries holds a capacity of 60 ML ethanol production from molasses (BAA 2013). Recently it has appeared in a report that Dalby ethanol biorefinery is under the threat of closure due to lack of government action and less demand for ethanol fuel (United 2014). The Queensland government invested AUD 6.5 million in biofuel research under "Queensland Sustainable Aviation Fuel Initiative" to produce biofuel from sugarcane bagasse, oilseed trees and algae with industry partners from aviation, biorefinery and biotechnology area (Nielsen 2014). This shows that Australia is also showing interest in introducing biofuel in aviation sector.

The Victorian government signed a memorandum to build a consortium of companies to produce an annual 200 ML of ethanol and the state is in its initial stages of producing bioethanol from cereals such as wheat and barley (Schuck 2010). Victoria has a facility to support bioenergy production and waste-water treatment through supercritical water gasification, which provides product selectivity by altering the conditions to obtain the desired products, for example, low temperature to produce natural gas and high temperature to help in developing fuel cell technology (Sanderson and Feltrin 2014). Sucrogen bioethanol (a division of CSR Limited) is another major bioethanol producer in Australia which produces 60 ML of ethanol per year from the molasses that is supplied to the food and beverage sector, industrial and fuel market (WB 2010).

Two bioethanol products, EGEN-95 and EGEN-98, were launched by the Neumann Group located in Queensland and New South Wales, comprising a 10% ethanol-enriched blend with octane ratings of 95 and 98, respectively (NP 2008). CSR spent around AUD 17.8 million to produce fuel-grade ethanol products such as E10 and E85 (Hart 2008), whereas Caltex Australia launched a high ethanol blend fuel Bio E-Flex (E85) in 2010 with 85% of ethanol being produced from first generation feedstock. It produces Bio E10 using waste starch from wheat, sugarcane molasses

and sorghum. Caltex Australia produces Bio B5 and Bio B20 biodiesel which constitutes 5% and 20% of biodiesel produced from used cooking oil and animal fats (tallow) (Caltex 2011; Hart 2008).

BP Australia is another big biofuel producer and competitor in Australia. It began marketing E10 fuel in 2001 in Queensland and is aiming to produce 400 ML of biofuel per year to exceed the target of the Federal Government. BP's new plant in Kwinana, Western Australia is using 200,000 tonnes of wheat as feedstock to produce renewable fuel (E10) and electricity (Jardine 2006). In Western Australia, Primary Energy Pty. Ltd. has established a grain based ethanol plant with the capacity to produce 500,000 L of ethanol per day (Shedden 2010). Australia as a country is on her way to understanding how its feedstock can be used for biofuel and to resolve the challenges involved in its production. To this end, the Federal and State Governments are reinforcing biofuel by providing various grants and schemes to support biofuel production with tax exemptions and excise advantages (WABTR 2007).

1.3 Technological Challenges: Production of bioethanol

Second generation lignocellulosic biomass is a renewable carbon reserve which is relatively untapped. A huge amount of biomass is available worldwide and contributes 14% of final global energy consumption (Parikka 2004). Lignocellulosic biofuel helps to mitigate agricultural residues which are burned in the field and causes environmental problems (Buyx and Tait 2011). Globally second generation lignocellulose is available abundantly in different forms that can be commercialised for biofuel. The fundamental factor is the utilisation and processing technology that determines the production cost, quantity, quality and residue after processing. The production yield may vary largely from biomass to biomass and the conversion technology applied on it. Gaps in the current research include assessing confidence, social acceptance, proper networking system to manage supply chain and analyse. Setting up of a large biofuel industry requires optimised and controlled parameters and this will include interdisciplinary areas like mathematics, statistics, networking, bioprocessing, fermentation technology, downstream processing involving quality

control, volumetric yield under one roof. This provides job opportunities and research over a wide range of disciplines.

The sources for lignocellulose biofuel include agricultural residues, wood waste materials, grasses and timber (soft and hardwood). The lignocellulosic feedstock is primarily composed of lignin, cellulose, hemicellulose with some minor components such as pectin, wax, proteins, and ash (Sedjo 2008). These carbohydrate polymers, oligomers are highly coiled with non-covalent forces and covalent cross-linkage which tends to make them rigid and robust. This structural complexity causes technological challenges and the processing difficulty differs by biomass type. The outer covering of lignocellulose is composed of lignin which protects the structure from chemical and biological threats (Sanderson 2011).

The most abundant component of lignocellulosic biomass is cellulose. It is an essential part of biomass composed of and pure polymer of glucose. Glucose is a hexose (6-carbon) sugar unit. Cellulose and its products are of both research and commercial interest (Jeoh et al. 2013). Hemicellulose is the second major component in biomass, and holds cellulose and other components in the inner core of the structure. Most hemicelluloses are of xylan and glucomannan origin, where xylan serves as a resource for the production of pentose-based sugar hydrolysate (Girio et al. 2010). Hemicellulose also finds application in the development of biofuels due to the presence of pentoses, hexoses and uronic acid.

Lignin is the binding component in the biomass structure and serves to strengthen the structure. As a phenolic polymer, lignin acts to harden the wall by providing structural rigidity and physical barrier against microbial attack (Kang et al. 2013). Lignin comprises about 15-30% of biomass composition and the percentage varies among feedstock. It is amorphous in nature and constitutes mainly phenyl propane derivatives and the monomeric composition of feedstock also brings difference in the rigidity of structure. Lignin is primarily composed of monolignols p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol monomers (Figure 1.2) (Ghaffar and Fan 2014).

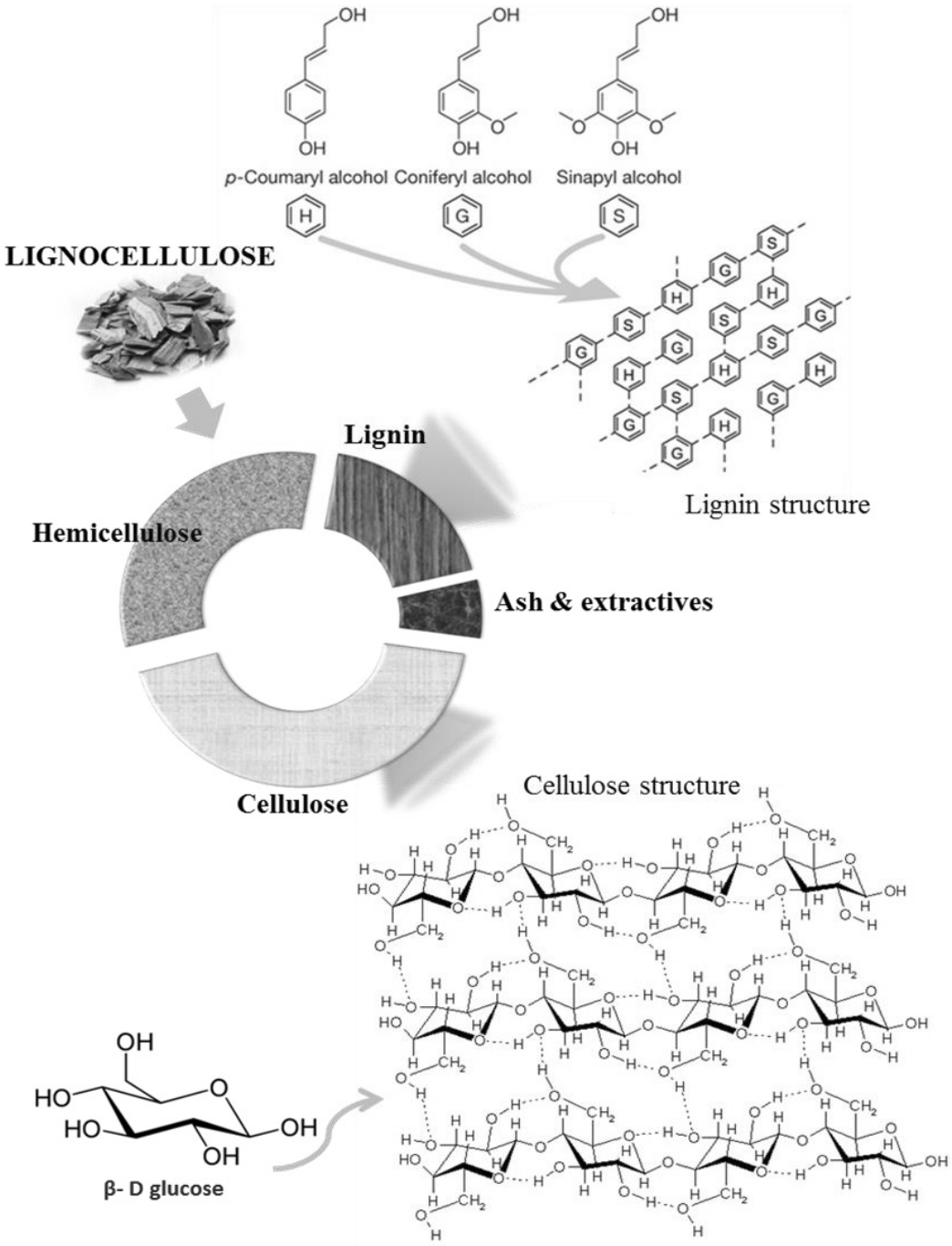


Figure 1.2 Structure of lignin and cellulose present in woody biomass. The image has been modified from Nature Publishing Group (Rubin 2008)

Research is providing genetically modified lignin that may be better for applications in bioenergy, paper and pulp industry. Transgenic plants which has modified or altered lignin content serves as improved feedstock in lignocellulose based industries

(Verma and Dwivedi 2014). Lignin chemistry and its utilisation as an industry value-add component is an emerging field of research. Lignin has potential applications in liquid fuel, syngas, char, hydrogen and aromatic-based chemicals (Azadi et al. 2013). Lignin is primarily obtained as a co-product from the pulp and paper industry and lignocellulosic based ethanol industries.

These cellulosic materials are used for enzyme hydrolysis or enzyme saccharification where cellulose gets digested to single glucose units. A successful digestion provides good yield of glucose or reducing sugars. These sugars are separated from the digested biomass and then fermented to obtain ethanol. To make this process work, the primary step is to make enzyme accessible to cellulosic material of biomass. Generally, this is achieved by pretreatment step or processing of lignocellulose which loosens the strong cross linked bonds and wide opens the structure. There are numerous advanced technological strategies practised across the world to get good hydrolysis of biomass. The bioconversion processing of lignocellulose corresponding to individual effects on the structure is shown in Figure 1.3.

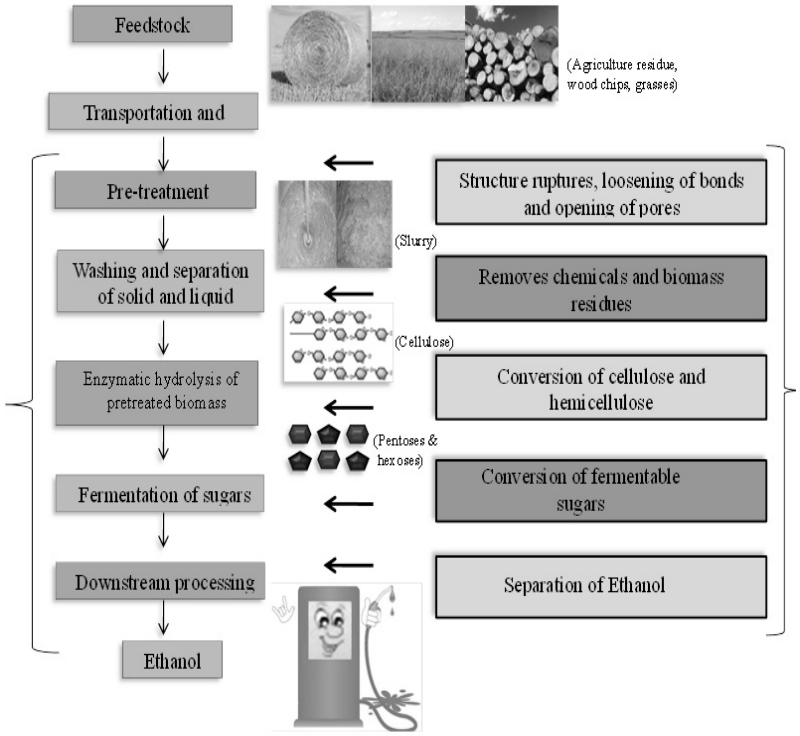


Figure 1.3 Schematic representation of lignocellulose to ethanol bioconversion

Later stages of biofuel production are fermentation of sugars into ethanol and downstream processing, which includes distillation, purification and blending of ethanol to make it compatible for transportation use. Strains are sometimes genetically modified to provide a high tolerance level acid, giving better conversion in acidic environments in the presence of inhibitors which are formed during fermentation. It has been reported that pretreatment performed under acidic conditions leads to the production of various inhibitors, such as 5-(Hydroxymethyl) furfural (HMF), a member of the furfural family. Enzymatic hydrolysis of this biomass especially in softwoods releases inhibitors, which directly influences sugar yield and ethanol production. The yield of sugar and by-product depends on the hydrolysis conditions (Kobayashi and Fukuoka 2013; Rosatella et al. 2011).

1.3.1 Pretreatment of biomass

The conversion of biomass to biofuel begins with the selection of biomass. Various computational models are employed to determine the growth of biomass, productivity, feasibility of the process, mass balance, conversion rate and, perhaps most importantly, the production costs (Festel et al. 2014). There are several more steps involved before the actual bioprocessing step begins. These include harvesting area, climatic conditions, and costs associated with harvesting and transportation per hectare (Jenkins and Sutherland 2014).

The loosening of the bonds can be accomplished by "pretreatment" of biomass which breaks the cross-linked strong ester, hydrogen and phenolic bonds (Chen et al. 2012). The major role of pretreatment is to maximise enzyme penetration by increasing the cellulose surface area. This occurs by the removal of components such as lignin and hemicellulose (Ohgren et al. 2007). The pretreatment process should be low cost, effective and provide easy residual separation and recovery (Hendriks and Zeeman 2009). In recent years, various methodologies and techniques have been adopted to increase the success of pretreatment and determine its effectiveness on biomass structure to economise biofuel production. Other factors such as enzyme consumption, occurrence of inhibitors and downstream processing also add cost to biofuel production. Minimising the cost of pretreatment can substantially reduce the overall production cost of ethanol.

Pretreatment alone poses a range of challenges that depend on the structure of biomass. It expands the structure of lignocellulose when it undergoes stress and tension. The cross linkage between lignin and the polysaccharides (cellulose and hemicellulose) with ester and ether makes the biomass structure hardwearing and robust (Hendriks and Zeeman 2009). Pretreatment normally takes place either under higher pressure, temperature or chemical influence. Generally a cost effective pretreatment and less complex lignocellulosic feedstock is preferred for biofuel production, otherwise the cost can increase drastically to delignify the biomass to obtain holocellulose. The selection of an effective pretreatment method includes treatment time and conditions, size, recovery and loss. This step is an important factor for giving quantity and quality of sugar yield during saccharification (Zhu et al. 2010).

Pretreatment methods can broadly be classified as physical (milling and grinding), physicochemical (steam explosion, oxidation), chemical (alkali, concentrated/dilute acid, oxidizing agent and organic solvents), microwave, sonication biological, or there combination (Kumar et al. 2009a; Chen and Qiu 2010) (Figure 1.4). However, the most challenging part is to identify an economic and high ethanol yielding strategy that can be used on a commercial scale. This requires extensive research and knowledge to produce a technological pathway that optimises energy consumption in biofuel production (Zhu and Pan 2010).

Physical pretreatment is the preliminary step included in almost all pretreatment techniques. It involves milling, grinding or chipping to reduce the size of the wood chip and facilitate the breaking of cellulose crystallinity (Hosseini and Shah 2009). The structure can be disrupted by extrusion, microwave or freeze pretreatment methods to break the bonds either by thermal shock, radiation or low temperature (Conde-Mejia et al. 2012). A wide range of chip sizes are used for pretreatment (300-1400 μm, 2-12 mm or higher). Usually, a small size is effective and provides good biomass digestibility but reaching such small sizes also consumes more energy (Zhang et al. 2013). In addition to the size of wood chip, the duration of the pretreatment also affects the hydrolysis of the biomass. It is a crucial step because the

recovery of holocellulose, crystallinity, amount of cellulose converted into monomers by enzymes and hemicellulose solubilisation depends on pretreatment (Tadesse and Luque 2011).

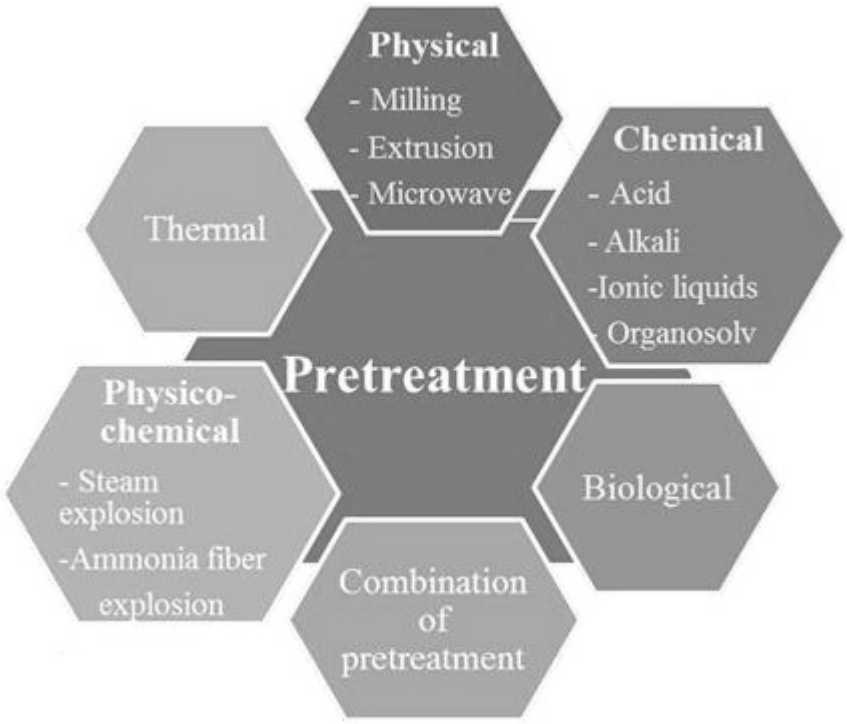


Figure 1.4 Different pretreatment methods used to disrupt the biomass structure

In some pretreatment conditions lignocellulose is pretreated at higher temperature to degrade the structure. Pyrolysis is one such process which hydrolyses polysaccharides with heat in the absence of oxygen. It forms volatiles, chars and gaseous fractions in the reaction at temperatures ranging from 250 to 500 °C. The thermal behaviour of lignin through intermediate and final products can be studied by pyrolysis (Mettler et al. 2012). Steam explosion is a pretreatment method which increases the pore volume of the biomass by degrading the hemicellulose content and provides better surface area for efficient enzymatic hydrolysis. In this temperature is the most significant factor as it affects the yield of pretreated polysaccharide (Martin-Sampedro et al. 2014). Steam explosion decreases acid soluble lignin, increases

insoluble lignin content, enhances the density and thermal stability of the biomass (Brugnagoa et al. 2011). Ammonia fibre expansion is a process that includes the exposure of lignocellulose to ammonia to increase its viability for enzymatic hydrolysis. This process is customised by four parameters that are ammonia and water loading, reaction temperature and residence time (Bals et al. 2011). It breaks the ester linkage of cell wall by ammonolytic cleavage, releases acetamides and phenolic amides which are required for downstream processing (Chundawat et al. 2010). Carbon dioxide explosion is a pretreatment method, whereby super critical carbon dioxide at varying pressures is used as an extraction solvent. It is cheap, has a low critical temperature, non-inflammable, clean and easy to recover after use (Zhang and Wu 2014).

Pretreating the lignocellulose with chemicals is the most common pretreatment method. Introducing the biomass to chemical conditions such as acid or alkaline readily opens the structure. Acid hydrolysis is generally achieved by hydrochloric acid (HCl) or sulphuric acid (H_2SO_4) and alkaline treatment occurs with lime, sodium hydroxide, alkaline peroxide, calcium carbonate (Gao et al. 2010). Earlier reports suggest that the effect of chemicals depends upon the structure of biomass and the concentration of chemical used under different pretreatment conditions (high/low temperature or pressure) (Balat 2011). The presence of extreme temperature and pressure during pretreatment adversely affect lignocellulose structure. Recent research has explored the use of ionic liquids (ILs) which can produce a greater surface area for enzymatic hydrolysis by increasing crystallinity. It reduces lignin content and can be preferred over acids due to its easy recovery and reusability (Harun and Danquah 2011). However, the cost of ionic liquid is a concern for industrial viability.

Some of the fundamental factors which are associated with pretreatment include selection of the pretreatment strategy, energy consumption required to maintain pretreatment conditions, economical large scale processing and the pretreatment technique. Preferably these should not rely on a particular feedstock so that processes are effective for a variety of biomass.

1.3.2 Enzymatic saccharification of pretreated biomass

The enzymatic saccharification of a pretreated biomass into sugars is the second step involved in the conversion process. A group of enzymes or a multi-enzyme complex is required to digest the pretreated lignocellulose into fermentable sugars (hexose and pentose) that can be fermented to ethanol. The effectiveness of pretreatment and efficiency of enzymes are critical factors which determine the substrate digestibility and release of product. Various strategies and computational models are available to determine the best biomass and enzyme loading concentration with material balance. A successful enzyme hydrolysis provides a good yield of reducing sugar ranging from 75 to 95% (Modenbach and Nokes 2013).

Cellulase is mainly required to digest the pretreated biomass. It works with the synergistic action of three other enzymes; endo-β-1, 4-glucanase (EG), cellobiohydrolase (CBH), and β-glucosidase. Endo-cellulase/ Endo-glucanase (EG) hydrolyse the internal β-(1-4) bonds of cellulose at random sites. Exo-cellulase/exo-glucanase (EXG) acts on the linear chain to form two to four units known as cellobiose which further gets hydrolysed by cellobiase/β-D-glucosidase (CB) to produce single glucose units (Sun et al. 2005). This reaction system occurs in various stages such as transfer of the enzyme from the bulk phase to a cellulose environment, reaction of the enzyme and substrate to form an enzyme-substrate complex through adsorption, hydrolysis of cellulose, transportation of hydrolysed units such as cellodextrin, glucose and cellobiose from cellulosic particles to an aqueous phase, and finally via the action of cellobiase, cellodextrin and cellobiose become hydrolysed in the aqueous phase to form glucose units (Galazka et al. 2010).

The ratio between reaction volume, enzyme and substrate loading are some critical factors which can alter the product formation. The sugars which are released from enzyme hydrolysis are glucose, xylose, mannose, arabinose, cellobiose and glucan. The yield of sugar depends on the enzyme loading and it significantly alters the overall production cost of biofuel. So the aim is to reduce the enzyme loading by making the enzyme more efficient. Advanced biotechnology and protein engineering modeling can enhance the efficiency of enzymes by constructing a synthetic enzymatic pathway. The immobilisation of enzyme on a matrix also enhances the

properties of enzyme and provides reusability with easier recovery (DiCosimo et al. 2013; Rodrigues et al. 2013). Different cellulase desorption strategies have been studied to recycle enzyme during hydrolysis and reduce the cost of enzyme loading (Tu et al. 2009).

Cell-free synthetic pathway biotransformation (SyPaB) can contribute to produce low-cost biofuel. SyPaB is constructed in an *in vitro* condition and constitutes a number of purified enzymes and coenzymes designed to catalyse a complex reaction (Barta et al. 2010). Inefficient delignification requires huge enzyme loading for biomass hydrolysis which increases the production cost. The presence of lignin residues or partial delignification can partially inhibit enzyme hydrolysis (Berlin et al. 2006). Sometimes, pretreatment methods, reaction conditions and enzyme hydrolysis results in the formation of by-products. They are inhibitors that restrict the product formation during enzyme hydrolysis. The presence of inhibitor makes recovery of product difficult (Graham-Rowe 2011).

The occurrences of different inhibitors such as phenolic compounds (4-hydroxybenzoic acid, syringaldehyde and vanillin), organic acids (acetic, succinic acid, levulinic and formic acid) cellobiose and furan derivatives (hydroxymethylfurfural, furfural) during the enzymatic saccharification reduces the sugar yield as they inhibit product formation (Kim et al. 2011; Zhang et al. 2010b). Inhibition of product formation occurs due to the solubility of the substrate, the nature of the enzyme complex, enzyme adsorption onto the substrate, the reaction steps involved, and the nature of the active sites on the enzyme (Ximenes et al. 2010).

1.3.3 Microbial Fermentation

The yield of ethanol largely depends on the efficiency of microorganism, concentration of reducing sugars, inhibitors, nutrients and growth conditions (temperature, pH, oxygen supply, and pressure). The fermentation product is greatly affected by variation in these factors (Andric et al. 2010). *S. cerevisiae*, *Z. mobilis* and recombinant *E.coli* are commonly used microorganisms for ethanol production. *S. cerevisiae* uses the Embden-Meyerhof pathway (glycolysis) to produce two

molecules of ethanol and ATP from glucose, whereas *Z. mobilis* uses the Entner-Doudoroff pathway to produce the same product with the release of one ATP molecule. There are numerous challenges associated with engineering of microbes for biofuel production (Stephanopoulos 2007). Other bacteria such as *Bacillus*, *Klebsiella* and *Clostridium* sp. use the Pentose Phosphate Pathway (PPP) for ethanol production (Geddes et al. 2011; Huang and Zhang 2011).

The fermentation of sugar hydrolysate is achieved either by simultaneous saccharification and fermentation (SSF) or separate hydrolysis and fermentation (SHF) (Wirawan et al. 2012). The bioconversion of lignocellulose using solid substrate fermentation enables the production of biofuel, synthetic enzymes, animal feeds, secondary metabolites and biofertilizers as by-products (Chandel et al. 2011). Optimal production of ethanol is achieved when sugars are completely released and utilised during fermentation. The yield of ethanol greatly depends on microbial strain, age and volume of inoculum used for fermentation (Peralta-Yahya et al. 2012). The presence of an inhibitor has different effects on the SSF (simultaneous saccharification and fermentation) and SHF (separate hydrolysis and fermentation) processes. Generally, the presence of inhibitors such as formic acid, acetic acid, furfurals and phenols restricts the production of ethanol and makes the fermentation environment acidic. These inhibitors are more likely to be produced during hydrolysis and fermentation step. Yeast cells also experience different stresses during ethanol fermentation such as high temperatures, nutrient deficiency, and contamination from yeast cell metabolism. These conditions work simultaneously and affect the growth of yeast and ethanol yield (Shields and Boopathy 2011).

Fermentation can takes place in continuous or in batch process under aerobic or anaerobic conditions. Continuous fermentation offers some advantages over a batch process such as reduced cost for conditioning inhibitory compounds by the *in situ* detoxification abilities of yeast, provides higher productivity due to higher cell density, and reduces viscosity of the substrate (Bai et al. 2008). The yield of ethanol during fermentation depends upon the capability of the microorganism to withstand the inhibitory conditions. Cost analysis at each step is essential to determine the feasibility of the entire strategy in terms of productivity and return on investment.

More focus must be given to upstream processing, as this is an important function in determining the success of ethanol production on a commercial scale (Lynd et al. 2008).

1.4 Global and Australian feedstock availability

Lignocellulose required for biofuel production is available as perennial grasses, feedstock, agricultural residues, waste wood and starch which come from agriculture, timber industry or paper and textile industry, domestic waste. Besides these, lignocellulose is available in the form of forest plantation. Furthermore a large amount of barren land is available that can be utilised for cultivating non-food crops to produce second generation feedstock. These resources are renewable and abundantly available which provides a platform for investment in biofuels. A wide range of biomass is used across the world to produce biofuel using different technologies.

The production of bioethanol in the USA is mainly from corn grains and produces around 96% of its bioethanol from corn grains (Tyndall et al. 2011). Corn stover has become a key source for bioethanol production which is increasing its market price. Excess production of corn stover is also causing problems related to agriculture and soil. It is a first generation biofuel which still exists as a major method for ethanol production in both the USA and Brazil (Blanco-Canqui and Lal 2007).

Researchers in the USA are attempting to improve soil conditions by developing databases which monitor emissions through remote data sensing. This will help to improve crop cultivation and reduce the greenhouse gases emitted from biofuel production (Kwon et al. 2013). The USA and Canada also have concerns regarding bioinvasions due to the fast growth of perennial grasses and woody species, which are introduced for the production of biofuel (Smith et al. 2013).

Asian countries like India which have strong agricultural backgrounds can support their biofuel production relying on the agricultural feedstock. Feedstocks suitable for second generation biofuel production are straw from wheat and rice which is cultivated largely in India (Ranjan and Moholkar 2013). Other Asian, European and African countries are also finding rice straw as a potential lignocellulose feedstock

suitable for bioethanol production and investing to promote and develop biofuel with mature technology (Binod et al. 2010). Ethanol has been obtained successfully from wheat and this is a growing area of interest for some countries (Talebnia et al. 2010).

Apart from bioethanol from biomass, biodiesel from biomass is also gaining attention. *Jatropha* is a plant which has been extensively used for the production of biodiesel. Many African and Asian countries are producing biodiesel from *Jatropha.* It is a pest tolerant, non-edible plant which can grow in barren and eroded land making this species of particular interest in some areas for biodiesel production (Jingura 2011). Other oilseed crops that are used largely for biodiesel production includes soybean, palm oil, canola and sunflower. Metabolic Engineering to improve the fatty acid is also useful for optimising biofuel production (Joyce and Stewart 2012).

Optimisation of various statistical, financial and networking models for biomass growth suitable for biofuel will help to make the conversion technology economical. These analyses will help to determine the biomass supply, yield, conversion rate verses growth rate that eventually result in final costs of production and determine the fuel price in the market (Kazi et al. 2010). These computational developments assist in comparing individual countries to global developments in terms of cropland usage, cultivation technique and conversion of barren land to cropland (Tran et al. 2011). Additionally, supply chain design and computational analysis are also important areas for determining biofuel production feasibility (Bai et al. 2012).

A wide range of perennial grasses can also contribute in the production of biofuel. Grass and grasslands are available in almost all the countries. These grasses are currently grown for fencing and to prevent soil erosion but generally less care is required for their growth and so can be grown in a variety of environments. Grasses such as Miscanthus and switchgrass are finding application in the production of biofuel (Chou 2009; Woli et al. 2011). Countries are endorsing the use of native grasses including pursh, wheat grass sp. and wildrye sp. for biofuel production (El-Nashaar et al. 2009). Climatic conditions of countries like Africa, Australia, Asia and European countries can support the growth of Bermuda grass which can be another potential cellulosic source for ethanol production (Xu et al. 2011b).

Some feedstocks such as wheat straw, coppice willow, miscanthus and sugar beet are popular in the UK and emerging as potential resources for biofuel expansion. The environmental benefit of biofuel is a key reason for biofuel investment in the UK (Brethauer and Wyman 2010). Ethanol is still produced primarily from molasses, cane juice and raw sugar in many countries. Zimbabwe pioneered biofuel production in African and overall biofuel production in Africa is at a very early stage of development (Taylor 2008).

Besides these grasses, investing in woody biomass waste is also an area of interest. Second generation biofuel depends on cellulose and woody biomass is a storage hub for cellulose and hemicelluloses. Woody biomass can be obtained from various industries such as pulp and paper industry, wood processing and the timber industry. Various types of hardwood and softwood like populus, willow, pine, olive, spurce, birch are also potential sources. It is difficult to open the structure of hardwood as the bonds are very strong, making it a complex and compact structure (Cara et al. 2008; Lestander et al. 2012; Stals et al. 2010). Countries like Australia, which has large *Eucalyptus* belts, can look forward to the production of cellulosic ethanol from the huge amount of timber waste that is produced every year. The climatic condition of Australia favours the growth of *Eucalyptus* and its variety (King 1996; Meer and Dignan 2007).

In Australia, feedstock in the form of forest plantations, agricultural residues, and organic by-products from various industries are largely available. However, the adoption of mature and advanced processing technology is required to enable growth of a true biofuel industry in Australia. Biofuel adds value to biomass in different ways because biomass contains water soluble and insoluble carbohydrates and hydrocarbons which can be utilised through bioprocessing technology. Biomass plantation provides multiple benefits such as reduces soil salinity, prevents wind erosion other than providing fodder for animals, feedstock for biofuel and electricity production. Australian forest plantations are an important economic market, as they produce two-thirds of the nation's log supply. Around 1.9 Mha of forest plantations are available in Australia, and these are comprised of both hardwood and softwood species such as eucalyptus and pines. The States of Victoria and Western Australia

covers around 21% of plantation, while NSW has 19%, Tasmania 14% and Queensland 13% (Stucley et al. 2008).

Australian forests cover 19% of total Australian land area which is dominated by woodland. Reports suggest that the forest plantation has increased since 1990's and the wood supply is expected to rise to 29 million m^3 by 2020 (BRS 2009). Currently bagasses are the largest renewable resource in Australia which is used primarily in the production of electricity. Australia produces over 11 million tonnes of bagasse annually and over 9 million tonnes of cane waste which comprises leaves and tops that are traditionally burned in the field each year. This could serve as a potential source for the commercial production of biofuel. Forest residue, sugarcane and wood waste will be sufficient to meet the federal government's bioenergy target of producing 1.5 gallon litres (GL) of biofuel by 2015 (Low and Mahendrarajah 2010).

Eucalyptus is widely available in Australia, which is a fast growing hardwood as the Australian climate favours its growth. It is estimated that in 2015, Australia will be able to produce 10 million tonnes of eucalyptus wood residue from Government and "Managed Invest Schemes" (Vancov 2011). Eucalyptus is considered to be a major potential resource for the production of ethanol because it supports the current conversion technology and, in addition, the availability of a large variety can contribute to high productivity (Ravindranath et al. 2011).

Some perennial grasses such as Napier grass (*Pennisetum purpureum*), *Miscanthus* sp. and Giant Reed (*Arundo Donax*) could also be considered for the production of biofuel in the state of South Australia. A detailed study (growth curve, biomass yield, ethanol production) was carried out on Giant Reed to analyse its potential for commercial scale biofuel production (Williams and Biswas 2010). A perennial pasture (e.g. elephant grass) has good prospects for biofuel production. Currently these pastures are left for heavy grazing, burnt or left for decomposition in the field. Other agricultural biomass residues available in Australia that can serve for biofuel production are sorghum and cereal straw. In addition to agricultural residue, an urban biomass which includes timber waste can also be used for bioenergy production (CEC 2008).

1.5 Summary

Biofuel is of interest because it can be a reliable alternative to depleting fossil fuel reserve and provides environmental benefits. The resource required for second generation biofuel production is renewable, low cost and available in abundance. The rising price of fuel, increasing concern over global warming and the lack of competition from food crops also favoured the production of biofuel from the lignocellulosic biomass. With the help of advanced bioprocessing technology, agricultural wastes and feedstocks can successfully be hydrolysed and converted into useful products such as transportation fuels, nitrate and nitrogen removal from water, removal of heavy metals and dyes from aqueous solutions, and the production of electricity.

Numerous methods are practised worldwide, aiming to develop better pathways for the conversion of biomass into high yield ethanol, butanol and other improved alcohols. The development of the biofuel sector is set to raise the price and demand for feedstock because of its extensive requirement in biofuel production, along with other factors such as competition for land between non-food crops and food crops, irrigation, and nutrition (Jun et al. 2009). Currently the existing technologies are not developed enough to effectively competitiveness against fossil fuel, primarily because of higher production costs. However, in the future biofuel will become an important part of the fuel industry, since its use results in reduction of greenhouse gases while providing a politically stable, renewable and cost-effective alternative to the fuels typically employed by countries today.

Biofuel is a new market for the agriculture sector, and it provides potential opportunities for rural and regional community development, as well as supporting urban communities, improving air quality and health in cities and potentially providing a buffer against rising fuel prices. A greater use of biofuels will eventually result in a reinvigoration of some rural industries and reduce environmental problems caused by fuel usage. There is a tremendous opportunity available for transform agriculture and forestry waste biomass into biofuel if technology for its production can be improved in terms of cost and efficiency.

Research Question and Aims

Research Question:

Can effective pretreatment and enzyme saccharification of hemp hurd produce high yields of simple sugars that can be converted to biofuel?

Aims:

- Optimise the pretreatment of hemp hurd using a combination of mechanical and chemical methods.
- Develop enzyme hydrolysis methods for maximising sugar yield from hemp hurd.
- Remove inhibitors occurring during hydrolysis to optimise the yield of sugars from hemp hurd.
- Determine the impact of pretreatment and enzyme saccharification on biomass structure.
- Develop method to enable multiple re-use of cellulases through immobilisation on magnetic nanoparticles.
- Convert sugars produced enzymatically from hemp hurd into lipid and ethanol biofuels.

Chapter - 2

Pretreatment and biomass characterisation

2.1 Introduction

The availability of raw material for biofuel production is of significant concern. The dependence of first generation biofuel on food crops such as corn, sugarcane, wheat, barley, and molasses caused competition among food crops and created unstable situation. This led to the development of second generation biofuels, where the raw material comes from renewable and non-competing carbon sources such as lignocellulose and agricultural waste (Mohr and Raman 2013). Second generation biofuels are considered to be more sustainable due to greenhouse effect and food security (Hammond and Seth 2013).

The biomass used as a raw material for producing second generation biofuel is obtained from agricultural waste, feedstock, soft/hardwood and industrial waste. Generally, the lignocellulose used in the biofuel industry depends on substantial availability of raw material in the respective country (Sun and Cheng 2002). Countries with large areas of woodland prefer woody biomass to be the starting material for ethanol production, while countries with large agriculture industries prefer bio-residue as the source (Kocar and Civas 2013). Some of the commonly considered lignocellulosic biomass for biofuel production includes wheat straw, corn stover, rice straw, bagasses, miscanthus, switchgrass, napier, pine, spruce, beech, and eucalyptus. These are non-food biomass sources for ethanol production and their use does not compete with the food supply. Due to high cellulose and low lignin content these sources are useful for bioprocessing (Ziolkowska 2014). These two factors will help in producing high concentration of reducing sugars because low lignin content will permit easier access to cellulose for conversion and high cellulose will produce high quantities of hexose and pentose sugars (Figure 2.1).

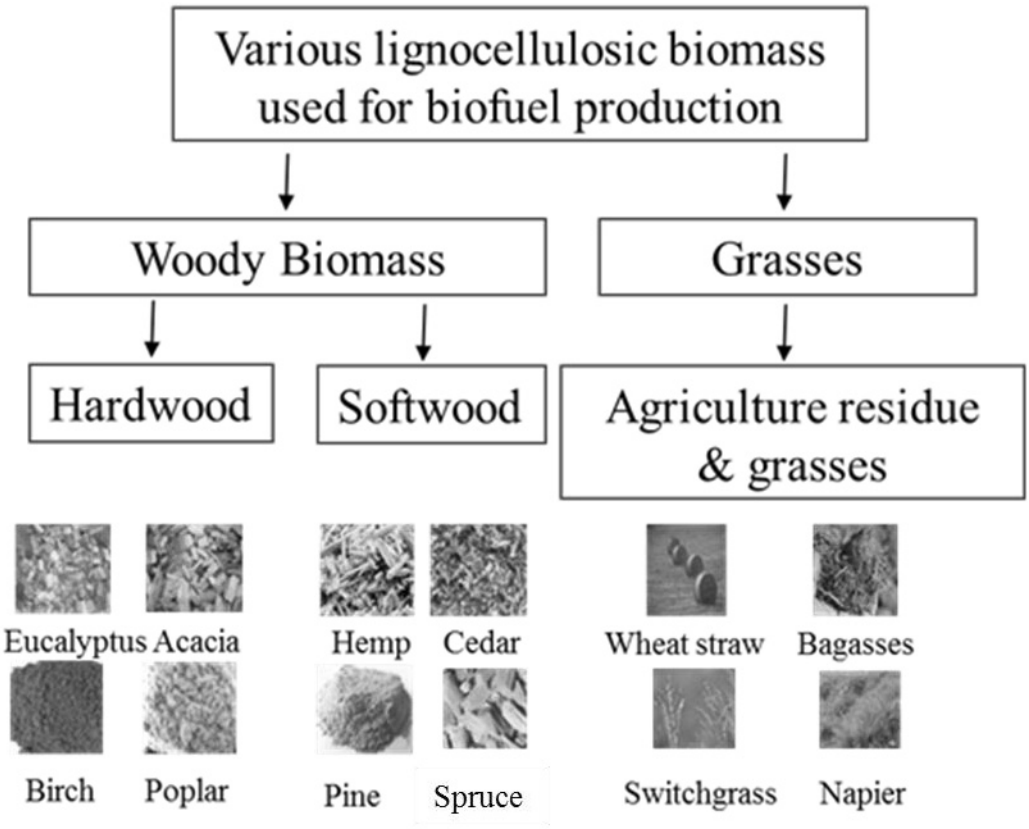


Figure 2.1 Different lignocellulosic biomass used for the production of ethanol worldwide

Hemp is an annual dicotyledonous angiosperm plant under the Cannabaceae family belonging to the Rosales order and sub order Rosidae (Chase 1998). The cross-section of hemp stalk includes bast, xylem and marrow. Xylem is used for the construction of lightweight materials due to its lower density (0.24-0.26 g/cm^3). The consumption of hemp stalk for industrial purposes is needed due to the higher volume of stalk, which is two times higher by weight than bast fibre. Utilising this waste stalk will help to promote the cultivation of industrial hemp. The fibre size, mechanical properties and degree of crystallinity are different for different parts of hemp stalk (Li et al. 2013). Hemp is a complex mixture of many volatile components, which includes monoterpenes, sesquiterpenes, cannabinoids and other terpenoid like substance. Essential oils are present in hemp and are produced in the epidermal glands (Bertoli et al. 2010). The bast fibre of hemp contains pericyclic elementary fibre in bundles and composes the lignin cell wall. Hemp comprises cellulose

(~55%), hemi-cellulose (~16%), pectin (~18%) and lignin (~4%) (Jaldon et al. 1998). Another report suggests the presence of alpha cellulose (44%), hemicellulose (25%), lignin (23%) and extraction (4%; includes oil, protein, amino acids and pectin) in hemp hurds (Gandolfi et al. 2013). The optimum sowing time in a Mediterranean environment is between February and March. It is a spring-summer crop and important for crop rotation. To maximise the biomass production a late harvest is preferred, September or October is ideal for biogas production and spring harvest for solid fuels (Amaducci et al. 2014). Studies based on hemp harvesting found significant improvement in fuel properties when harvested in the spring. The fuel properties of hemp are similar to wood and willow and superior to straw and miscanthus (Prade et al. 2012).

Hemp is mainly grown for the outer covering or bast fibre and the applications extend across many industries. Studies have reported that significant research efforts are aimed at hemp every year to improve the fibre quality and strength (Prade et al. 2012). Currently hemp has various industrial applications including the production of paper and pulp, fibre and textile, and now in bioenergy production (Rehman et al. 2013). Research has resulted in the discovery of other non-textile applications of hemp such as in essential oils, thermal insulation and the production of composite materials for the automotive industry. Hemp also exhibits various medicinal properties and is used as a food additive. The yield and quality of hemp differs with growing conditions such as soil quality, nutrition and climatic changes (Finnan and Styles 2013).

The application of hemp (*Cannabis sativa)* for biofuel production is relatively recent. Researchers are assessing the production process and feasibility of hemp as a raw material in the bioenergy industry (Gonzalez-Garcia et al. 2012). Studies have been conducted on steam pretreatment and enzyme hydrolysis for ethanol and methane production from hemp (Kreuger et al. 2011). Another study using steam pretreatment produced bioenergy including ethanol, biogas, electricity and heat from hemp (Barta et. al 2013). This indicates that steam pretreatment can be a potential way to pretreat hemp and produce bioenergy. Hemp seeds are used for oil extraction, in the diet and in cosmetic products. These seeds contain essential fatty acids such as linoleic acid

and α-linoleic acid that are good sources of omega-6 and omega-3 polyunsaturated fatty acids (PUFA) (Da Porto et al. 2012). In a recent report yeast cultures grown on hemp seed extract produced lipids which were transesterified to biodiesel (Patel et al. 2014). Due to the local availability of hemp hurd and limited studies on its use, we chose to focus on this biomass for our study (Figure 2.2).

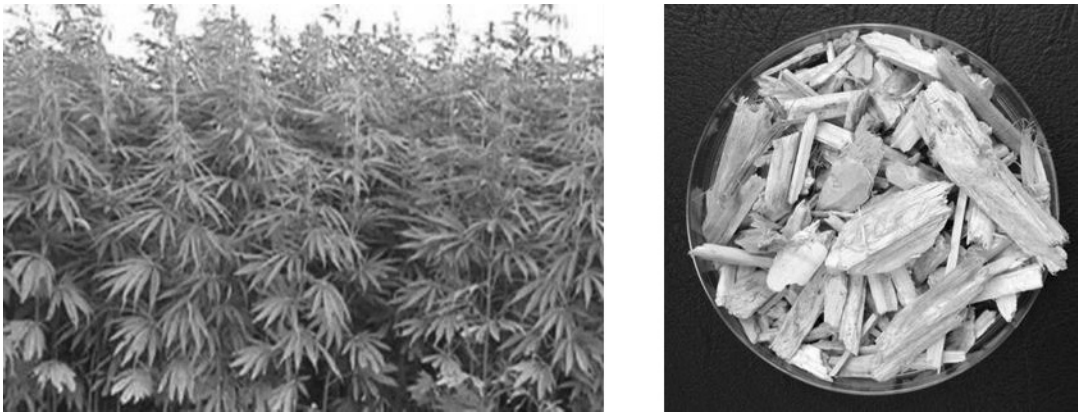


Figure 2.2 Hemp plantation and hemp chips used for the study (Hemp plantation picture is taken from - Assistance for Tasmania's industrial hemp sector by Rex.pannell. Enviroinfo, July 29, 2014).

Large amounts of biomass are available that can be utilised, after pretreatment, for bioenergy production. This study was conducted to understand the impact of existing pretreatment technologies on Australian biomass in order to determine if this biomass is useful for biofuel. Combinations of various pretreatments were used with selected biomass.

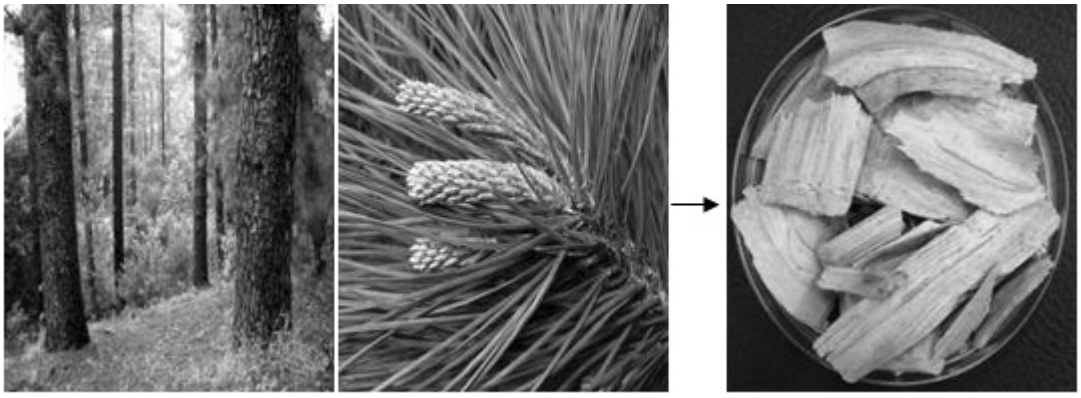
Pine is a coniferous softwood and commonly available in various parts of Australia. This softwood was investigated as the second sample for producing reducing sugars. The plantation of pine began in 1870s and by 1960s it covered an area of ~200,000 ha (Bradshaw 2012). Southeast Australian and the adjacent south west of Victoria (green triangle region) occupy 175,000 ha of plantation area. These regions are low in fertility and sandy soil. Pine is the earliest exotic planation species in these areas (O'Hehir and Nambiar 2010). Pine plantations are mainly utilised in Australia for

timber and household purposes. According to a recent report, pine can be rotated at a harvest cycle of about 30 years and is grown primarily in south-eastern Australia. *P. radiata* covers more than 1 million ha of forestry plantation in Australia (Farmilo et al. 2013). *Pinus radiata* is a native species planted prolifically and studied extensively based on various factors such as soil taxonomy, harvesting, temperature (Kasel and Bennett 2007).

The lignocellulosic biomass used in the present study was hemp hurd (*Cannabis sativa*) and pine chips (*Pinus radiata*). Hemp hurd was obtained as an industrial waste from Commins stainless manufacturing and pine chips were procured from Softwood Plantation Exporters (SPE), Geelong.

The hemp hurd used for the study was the inner core discarded as industrial waste after removal of bast fibre for industrial purposes, while the pine component consisted of waste wood chips from the timber industry. The hemp hurd used in this study first underwent physical and chemical pretreatment to reduce the size and open the fibre structure. The pretreated hemp hurd was then treated with enzyme hydrolysis for the production of sugar hydrolysate. The hemp hurd was tested with five different pretreatment conditions with the aim to determine the importance of pretreatment and the lowest concentration of certain chemicals at lowest biomass loading to expand the biomass structure.

The pine chips procured from the local industry were moist and large in size (Figure 2.3). The pine chips were dried well before considering for milling and thereafter combinations of pretreatment were applied to open the compact structure and obtain sufficient reducing sugar yields.



(a) **(b)** **(c)**

Figure 2.3 Pine tree (a), pine cones (b) and pine wood chips (c) used for this study

2.2 Materials and Methods

2.2.1 Biomass

The first biomass sample examined in this study was a Ukrainian variety of Hemp (*Cannabis sativa*) which was grown in Griffith, New South Wales (NSW), Australia (S34°34', E146 °12') and was harvested in March 2009. This study included only the hemp hurd biomass (HHB) which was the inner woody core of industrial hemp (pith and xylem) obtained as an industrial waste from Commins stainless manufacturing, Whitton, NSW. The HHB powder was achieved by mechanical decortication or milling the biomass in order to reduce the size by passing once through a Fritsch Pulverisette 19 Universal Cutting Mill which was equipped with a 1.0 mm diameter sieve. The powder was further sieved using Coles-Parmer brass test sieve-8 inch and 300 µm size. All the experiments were performed using approximately 300 µm size HHB powder dried in an oven at 70 °C until the weight was constant. Then the HHB was stored in a plastic bag at room temperature under dry conditions until further use.

The second biomass used in the study was pine (*Pinus radiata)* which was procured from a local timber industry, Geelong, Victoria, Australia. The pine chips obtained were highly moist to be considered for any study therefore it was dried at 70 °C till it attained a constant weight. The size of the wood chips was reduced with Fritsch Pulverisette 19 Universal Cutting Mill equipped with a 1.0 mm diameter sieve. This

milled biomass was further sieved using 300 μm mesh size sieve from Coles-Parmer to obtain a saw dust powder and considered for chemical pretreatment.

2.2.2 Chemicals

The chemicals used for the experiment were cellulase from *Trichoderma reesei* (EC 3.2.1.4; 700 units), carboxymethyl cellulose (CMC), 3, 5-dinitrosalicylic acid (DNS), potassium sodium tartrate, sodium citrate and sodium hydroxide procured from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals such as sulphuric acid (98%, AR grade) was obtained from Merck (Australia), hydrogen peroxide (3%) was purchased from Chem-Supply (Australia). The enzyme assay was done using Whatman filter paper no. 1.

2.2.3 Pretreatment

The pretreatment study conducted on HHB was adopted from Yamashita et al. (2010). Five pretreatments (described below) were performed on hemp hurd biomass (HHB) in which two pretreatments were done using sodium hydroxide (NaOH), another two with dilute sulphuric acid (H_2SO_4) and the last was performed with hot water. All experiments were carried out in triplicates and the mean values with standard deviation are reported. The schematic flow of the bioprocessing done on HHB including pretreatment, washing and enzyme hydrolysis is shown in Figure 2.4.

2.2.3.1 Alkaline Pretreatment

Dried raw material (HB) weighing 10 g was slurried in 1 L of aqueous NaOH (0.5%, w/v) contained in a 2 L Erlenmeyer flask. The slurry was autoclaved at 121 °C for 60 min and then cooled to room temperature. In the second alkaline pretreatment, 1 L of NaOH (1%, w/v) containing hydrogen peroxide (3%, v/v) was added to 10 g of raw material (Yamashita et al. 2010). Pretreatment was done by incubating the slurry in 2 L Erlenmeyer flask at 90 °C for 60 min in a water bath. The liquor was removed and respective slurries were washed several times to remove the alkaline traces. The alkaline treated samples were stored at 4 °C for enzymatic hydrolysis after drying at 70 °C.

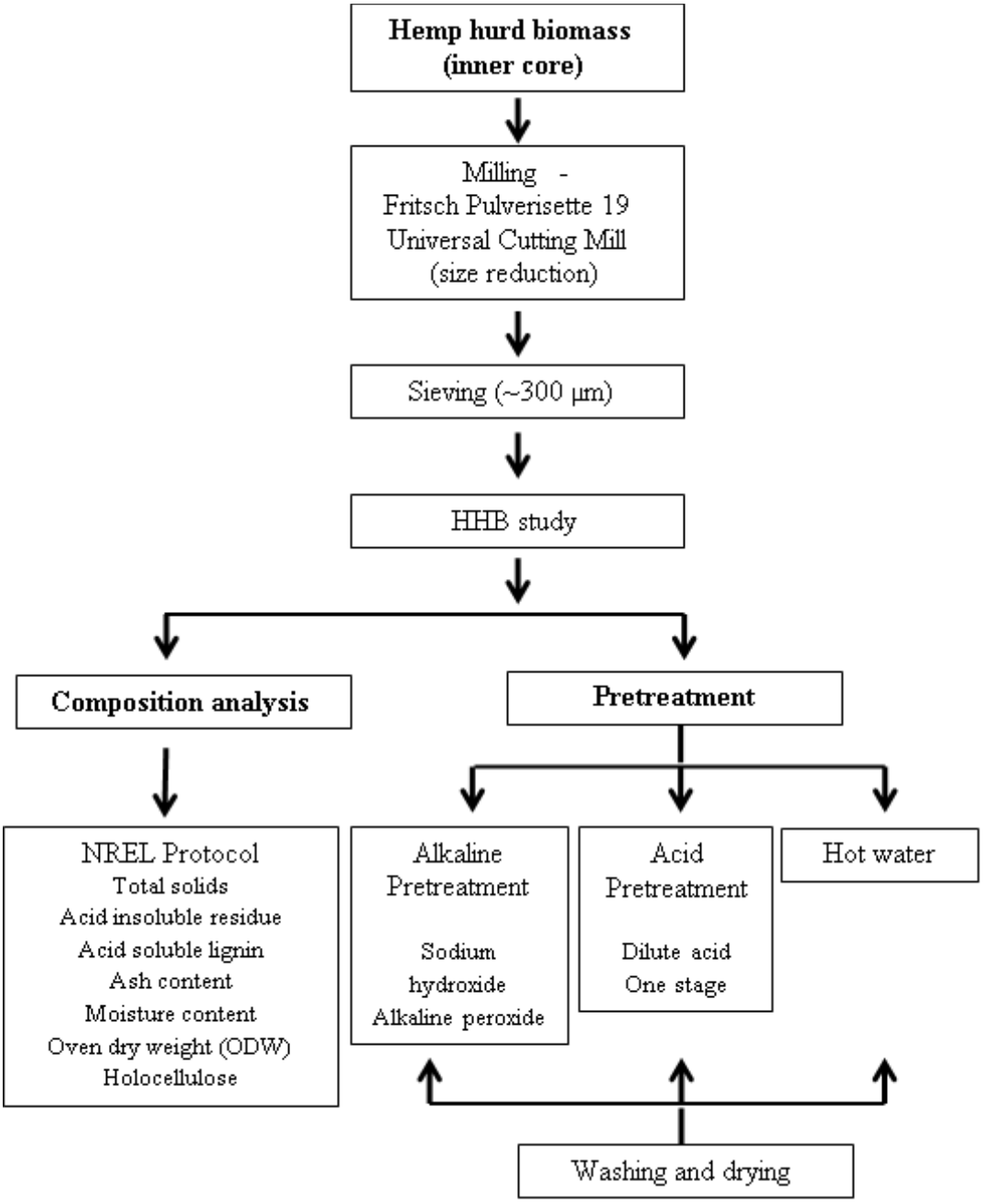


Figure 2.4 Schematic representation of biomass pretreatment and composition analysis

2.2.3.2 Dilute Acid Pretreatment

Dilute acid pretreatment on HHB was achieved by modifying acid pretreatment reference procedure. Dry HHB weighing 10 g was added to 1 L of deionised water containing 0.5% (v/v) H_2SO_4 and autoclaved at 121 °C for 60 min. Another

pretreatment was achieved by slurrying 10 g of dry sample in 0.5% H_2SO_4 concentration in 1 L of deionised water. This slurry was placed in an oil bath at 140 °C for 15 min and then it was cooled and washed once with water. The procedure was repeated again at 140 °C for 10 min in 1 L of deionised water having 0.5% H_2SO_4 concentration (Saha et al. 2005). Both the slurries were allowed to cool at room temperature and thereafter it was washed several times to remove residues and traces of acid. After drying at 70 °C it was stored at 4 °C for enzymatic hydrolysis.

2.2.3.3 Hot water treatment

Hot water treatment was performed by dissolving 10 g of HHB in 1 L of deionised water in a 2 L Erlenmeyer flask. The slurry was autoclaved at 121 °C for 60 min. After pretreatment, the slurry was allowed to cool at room temperature and then the liquor was removed. The pretreated slurry was filtered to remove the acid soluble biomass component and liquor. After the separation pretreated HHB was washed several times with distilled water and stored at 4 °C for enzymatic hydrolysis after drying at 70 °C.

2.2.4 Biomass composition analysis

The composition of biomass was investigated using standard National Renewable Energy Laboratory (NREL) protocols to determine the biomass composition (Sluiter et al. 2008a; Sluiter et al. 2008b; Davis et al. 2013). All the experiments were conducted in triplicates to determine each component and the mean was represented as the result.

2.2.4.1 Total solid content

The total solid content of HHB was determined using convection oven method and NREL protocol (Sluiter et al. 2008a). The aluminium weighing dishes were pre-dried at 105 ± 3 °C in a convection oven for a minimum of 4 h and cooled in a desiccator. The dish was weighed to the nearest 0.1 mg and recorded after cooling. The samples were mixed thoroughly and 1 g of HHB was weighed in the pre-weighed dish. The weighed sample and dish was placed into the convection oven which was set at 105 ± 3 °C for a minimum of 4 h to determine the moisture and total solid content. After removal the samples were cooled in desiccator and then weighed to the nearest 0.1

mg. The samples were placed back into the convection oven for an hour to obtain a constant weight. Constant weight is defined as ±0.1% change in the weight percentage of solids upon one hour of re-heating the sample.

Calculation:

$$\%\, Total\ solids = \frac{Weight\ (\ dry\ pan\ plus\ dry\ sample) - Weight\ (dry\ pan)}{Weight\ (sample\ as\ received)} \times 100$$

$$\%\, Moisture = 100 - (\%\, Total\ solids)$$

2.2.4.2 Acid soluble components

The acid soluble component was determined using NREL protocol (Sluiter et al. 2008b). HHB weighing 300.0 ± 10.0 mg was added to pressure tubes and 3.00 ± 0.01 mL of 72% sulphuric acid was mixed to this weighed HHB. The mixture was stirred thoroughly using a stirring rod and incubated in a water bath at 30 ± 3 °C for 60 min. The samples were stirred at every 5-10 min of interval to form a uniform hydrolysate. The concentration of acid was diluted to 4% by adding 84.00 ± 0.04 mL deionized water. The tubes were screwed tightly and the sample was mixed by inverting several times. Later these tubes were placed in the autoclave for 1 h at 121 °C. To determine the acid insoluble lignin, the crucible were placed in the muffle furnace at 575 ± 25 °C for a minimum of 4 h to attain a constant weight. Thereafter it was allowed to cool in a desiccator and the weight was recorded to the nearest 0.1 mg. The autoclaved acid hydrolysate was vacuum filtered to collect the filtrate the solid was washed with approximately 50 mL of deionised water. This residue in the pre-weighed and dried crucible was placed in the muffle furnace at 105 ± 3 °C for 4 h and then placed in the desiccator to cool and the weight was recorded to the nearest 0.1 mg. Sample were placed again in the ramp furnace at 575 ± 25 °C for 24 ± 6 h. The temperature ramp was set according the method used in NREL protocol. Firstly, it was ramped from room temperature to 105 °C and then held for 12 min at 105 °C. Secondly it was ramped to 250 °C at a temperature increasing rate 10 °C/min. The sample was held at 250 °C for 30 min and thirdly, it was ramped to 575 °C at a temperature increasing rate of 20 °C/min and the sample was held for 180 min. Thereafter it was allowed to

gradually drop down to 105 °C and held at the same temperature till the samples were removed. The ash residues were allowed to cool down in a desiccator and then weighed to the nearest of 0.1 mg. In order to obtain a constant weight the sample and crucible was placed back into the muffle furnace at 575 ± 25 °C to obtain a constant weight.

Calculation:

$$Oven\ dry\ weight\ (ODW) = \frac{Weight\ (\ air\ dry\ sample) \times \%\ Total\ solids}{100}$$

$$\%\ Acid\ insoluble\ residue\ (AIR) = \frac{Weight\ (crucible\ plus\ AIR) - Weight\ (crucible)}{ODW\ (sample)} \times 100$$

$$\%\ Acid\ insoluble\ lignin\ (AIL) = (Weight\ (AIR) - Weight\ (ash\ content)) \times 100$$

$$\%\ Acid\ soluble\ lignin\ (ASL) = \frac{UV\ (abs) \times Volume\ (filtrate) \times Dilution}{\varepsilon\ \times ODW\ (sample) \times Pathlength} \times 100$$

Where:

UV_{abs} = average UV-Vis absorbance for the sample at appropriate wavelength

$Volume_{hydrolysis\ liquor}$ = volume of filtrate, 86.73 mL

Dilution =

$$\frac{Volume\ (sample) + Volume\ (diluting\ solvent)}{Volume\ (sample)}$$

ε = Absorptivity of biomass at specific wavelength

(used 12 L/g cm which was mentioned in NREL protocol table)

ODW_{sample} = weight of sample in milligrams

Pathlength = pathlength of UV-Vis cell in cm

2.2.4.3 Ash content

The crucibles were marked and placed in the muffle furnace at 575 ±25 °C for a minimum of 4 h and then placed directly into a desiccator to avoid absorption of moisture. The crucibles were weighed to the nearest 0.1 mg and weight was recorded. Thereafter, 1 g of HHB was weighed in the crucible and was placed back into the muffle furnace at 575 ± 25 °C to attain constant weight. Constant weight is defined as less than ± 0.3 mg change in the weight upon 1 h of re-heating the crucible. The ash content of the sample was obtained using a muffle furnace equipped with a ramping program. The furnace temperature ramp program was set exactly the way mentioned in NREL protocol (Sluiter et al. 2008b) and as done above for determining acid soluble components.

Calculation:

$$\%\, Ash = \frac{Weight\ (crucible\ plus\ ash) - Weight\ (crucible)}{ODW\ (sample)} \times 100$$

2.2.5 Enzyme saccharification

The enzymatic saccharification of pretreated biomass (hemp) was performed by incubating 5 g of pretreated HHB in 250 ml citrate buffer, pH 4.8 (0.05 M) with cellulase from *Trichoderma reesei* (EC 3.2.1.4; 700 units) (Ghose 1987). The enzyme saccharification was achieved using optimised enzyme concentration in filter paper units (18 FPU g^{-1} biomass at 50 °C), 100 rpm for 72 h in an orbital shaker. The supernatant was removed at 12 and 24 h time intervals. The supernatant was centrifuged at 6,000 rpm for 10 min to remove biomass residue and stored at -20 °C for further analysis.

2.2.6 Analytical method

The concentration of reducing sugars from enzyme saccharification was determined using the 3, 5-dinitrosalicylic acid (DNS) method. The DNS reagent was prepared according to Yu et al. method (Yu et al. 1997). The reaction was done by adding 2.5 mL of reagent to 0.5 mL of supernatant. The reaction mixture was placed in vigorously boiling water bath for 10 min. The enzyme assay was performed using standard substrate and assay conditions for carboxymethyl cellulose (CMC), and filter paper assay (Ghose 1987). The CMC assay was performed using 0.5 mL of

enzyme (~20 CMC units) and 0.5 mL of 2% substrate (CMC) dissolved in 0.1 M sodium citrate buffer (pH 4.8) and incubated for 30 min. The reaction was stopped using DNS reagent and boiled in vigorously boiling water bath for 10 min. After cooling, the absorbance was measured at 540 nm. The filter paper assay was done in similar manner using different concentrations of enzymes and filter paper size of 1×6 cm size. The filter paper was rolled and placed in the test tube with citrate buffer of same concentration and cellulase for 1 h at 50 °C. The concentration of glucose was determined using the glucose standard curve and the enzyme activity was calculated accordingly. The enzyme concentration used in enzyme saccharification is determined according to FPU (filter paper units). The yield of enzymatic saccharification was calculated as (Selig et al. 2008):

$$Hydrolysis\ yield\ (\%) = \frac{Reducing\ sugar\ (g)}{Polysaccharide\ in\ substrate} \times 100$$

2.3 Results and discussion

2.3.1 Studies on Hemp

The hemp hurd biomass (HHB) used in this study was obtained as an industrial waste consisting of the inner stem core. Since hemp is softwood so the inner core required comparatively less harsh treatment to open the structure. The fibre blasting and opening of the structure enabled easy penetration of the enzyme to produce successful quantities of reducing sugars, which is discussed later in Chapter 4. This pretreatment study focussed on investigating the most suitable pretreatment condition to open the HHB structure for the production of sugar hydrolysate through enzyme hydrolysis and has been reported recently (Abraham et al. 2013).

The biomass composition study of HHB is conducted using NREL standard protocol, which determined that the HHB used in this study contained 85% mass fraction of total solids and 15% of water content. Within the total solids, approximately 8% of HHB (based on dry mass) constituted lignin, residues, ash and other extractive and the remaining 77% of biomass was composed of holocellulose (Table 2.1). A

research team studying the variation in the chemical composition of hemp at different harvesting times has reported that the cellulose content varied from 60-80%, hemicellulose ranged from 12-17%, lignin from 3-5% and ash from 1-4% in different slots of HHB harvested in different seasons (Nykter et al. 2008). Another study on sunn hemp reported 37% of cellulose, 21% hemicellulose, 13% lignin, 22% extractives and 5.2% of extractive (Kamireddy et al. 2013).This indicates that the composition of hemp in terms of cellulose content varies according to the harvesting time.

Table 2.1 Composition of hemp biomass

Components	**Mass fraction (%)**
Total solids	85.18
Acid insoluble residue	04.04
Acid soluble lignin	00.50
Ash content	03.10
Moisture content	14.81
Oven dry weight (ODW)	80.15
Holocellulose	~77.00

In comparison to other biomass, it can be seen that a major fraction of hemp (60-80%) is composed of cellulose. We have observed this in our studies and are in agreement with the existing literature (Nykter et al. 2008). In general, lignocellulosic biomass contains 30-50% cellulose, and thus hemp can be taken into consideration for producing ethanol due to its relatively high content of cellulose mass fraction and low content of lignin (Kumar et al. 2009a). The weak bonds in hemp and low content of lignin allow easy separation of biomass which helps to understand why pretreatment of hemp occurs so readily. The biomass used for this research was the

inner woody core of hemp hurd therefore the content of lignin, ash and other extractives were low. This indicates that hemp can be utilised as a raw material for industrial purpose, where outer fibre is applied in the fibre industry and the inner core or hemp hurd can serve as a substrate for the production of ethanol.

From our recent studies conducted on biomass hydrolysis it was found that HHB contains relatively a lower percentages of hemicellulose as compared to cellulose (discussed in Chapter 3). High quantity of holocellulose is useful for the production of biofuel. The weight of dry solids obtained after different pretreatments varied for all samples. An average of 73% biomass was obtained after all pretreatments, which was then further utilized for enzyme saccharification.

The remaining 27% was soluble material (Figure 2.7) which was dissolved as a result of pretreatment and biomass loss due to washing. Similar observations of significant loss of biomass (20-25%, dry wt. of biomass) under different pretreatment conditions for wheat straw was reported using a combination of steam explosion and various other solvents (Zabihi et al. 2010). The liquor collected after pretreatment and washing was acid or base depending upon the chemical used for pretreatment. Addition of this liquor to utilise the dissolved sugars during enzyme saccharification may result in higher amount of inhibitors in hydrolysis product. On the basis of biomass recovery after pretreatment, it could be seen that the maximum recovery of biomass was obtained with hot water treatment with respect to dry initial mass of HHB. The total dry initial mass obtained was 80% followed by acid pretreated samples (approximately 75%) and the lowest with alkaline pretreated HHB (approximately 65%). This infers that the amount of solid residues removed from the biomass structure correlates with the percentage conversion of biomass into product during enzyme hydrolysis (discussed later in this chapter). Studies have demonstrated that harvesting time influences the biochemical composition and glucose yield of hemp. A good conversion yield of glucose was obtained from hemp harvest in middle of August (Tutt et al. 2013).

The aim of pretreatment study was to compare lowest concentration of different chemicals at lowest possible biomass loading. The mass balance estimation and hydrolysis yield was more obvious at lower loading concentration. The experiments

were conducted at 121 °C in an autoclave which has programmed temperature, pressure and time. This temperature is set to sterile media and decontamination purpose. The boiling point of water is 100 °C so it is likely that the pretreatment slurry will get heated up at 121 °C in 20 minutes under pressure. Similar study has been reported recently on wheat straw using autoclave for pretreatment (Toquero and Bolado 2014).

We found that the colour of raw hemp changed after all the pretreatments. Different colours of dried hemp after pretreatment and washing is shown in Figure 2.5. The HHB samples treated with dilute acid in autoclave changed colour to a reddish brown whereas the samples treated at high temperature with dilute acid (one stage- without pressure) possessed a brown colour. The samples which underwent alkaline treatment in autoclave appeared as yellowish brown but the sample which was treated with alkaline and peroxide completed changed colour to off white. The HHB sample which was pretreated with hot water in autoclave showed very slight difference in colour with respect to untreated HHB. However, while comparing all the pretreatments on the basis of change in colour, we observed that the major difference was found from untreated to alkaline peroxide treatment.

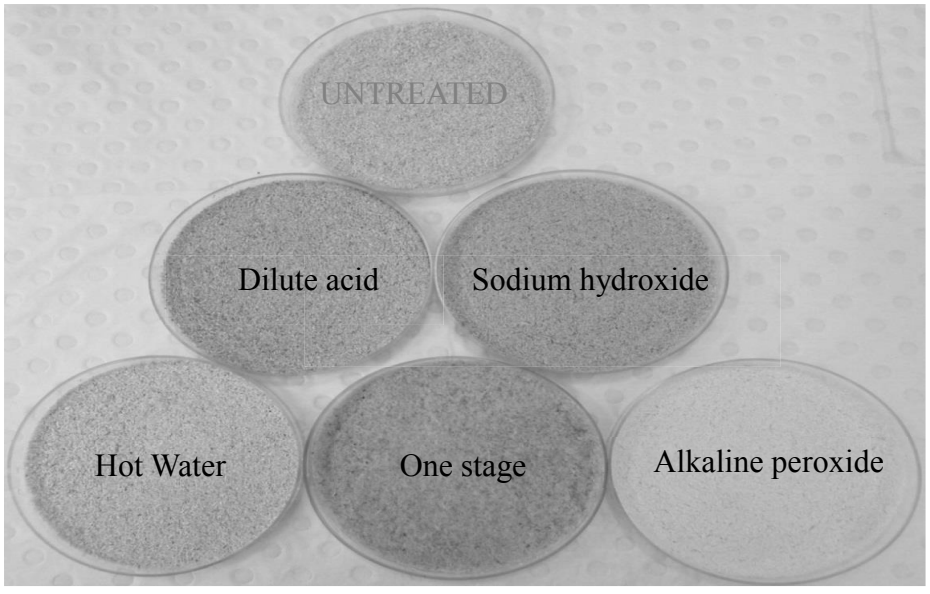


Figure 2.5 Variation in colour observed when biomass (HHB) was exposed to different pretreatments

In order to find the best treatment condition the methodology was chosen from acid to basic condition under pressure and temperature. Untreated samples or raw HHB (where no treatment was given) were considered as controls in all experiments. The pretreated HHB was washed at least 3-4 times to remove the chemicals and to attain a neutral pH. The pH of the biomass was maintained to neutral by addition of acid or alkali during washing. Due to the continuous washing of the biomass, the solid residues which were formed as a result of pretreatment were removed and resulted in loss of some initial dry mass. These residues were formed as a result of bond breakage in the biomass structure due to the opening of fibre and resulted in the separation of various components from the biomass structure. While conducting the material balance it was observed that on the basis of 1 g HHB, an average of 73% of pretreated solids were recovered after pretreatment and 23% was the solid residual mass loss.

The pretreated biomass slurry was dried at 70 °C to remove the moisture and the solid biomass powder was stored at 4 °C for enzyme saccharification. The recovery of biomass after individual pretreatment, washing and drying has been mentioned in Table 2.2. The enzymatic hydrolysis or enzyme saccharification of the pretreated biomass is performed to produce reducing sugars in biorefinery application. This perhaps evaluates the accessibility of the cellulose and indirectly determines the efficiency of pretreatment. Enzyme hydrolysis is generally conducted either with a cocktail of enzymes (cellulase, xylanase, cellobiase) or with a recombinant cellulase which is capable of producing high yields of reducing sugars. These reducing sugars mainly contain monosaccharaides with 6-C atom hexose (glucose, mannose) and/or 5-C atom pentose (arabinose, xylose) sugars or disaccharides such as cellobiose (2-glucose units).

Our study demonstrated the quantities of maximum reducing sugars which can be produced from HHB through various pretreatment conditions. Further, our interest was to develop a comparative study of different pretreatments which may lead to economical usage of single enzyme loading to better understand the efficiency of each pretreatment for delignification.

Table 2.2 Yield of reducing sugars with different pretreatments in combination with enzymatic saccharification

Pretreatment Methods [a]	Recovered biomass (g)	Recovery (%)	Reducing sugars (mg g^{-1})	Hydrolysis yield [b] (%)
Alkaline peroxide	3.20 ± 0.70	64.0	743 ± 3	74
Dilute acid	3.86 ± 0.13	77.2	349 ± 4	35
Hot water treatment	4.00 ± 0.57	80.0	299 ± 3	30
Sodium hydroxide	3.36 ± 0.34	67.2	719 ± 3	72
One stage - Temperature (140 °C)	3.75 ± 0.26	75.0	293 ± 4	29
Control	5	100	126 ± 0.01	13

[a] Hemp hurd biomass (dry matter, 10 g) used for carrying pretreatment, whereas 5 g was used for enzymatic saccharification experiments. [b] Based on the mass fraction of raw material

The effectiveness of pretreatments was determined by enzyme saccharification based on total reducing sugar yield and the conversion of cellulose. The enzyme hydrolysis profile obtained with different pretreatments for duration of 72 h is represented in Figure 2.6. After 72 h, a significant improvement was observed and the amount of reducing sugars observed at the start of the experiment was negligible. The untreated HHB also released reducing sugars though the percentage of hydrolysis was quite low. The hydrolysis profile of untreated HHB demonstrated that the conversion of holocellulose to reducing sugar reached a maximum (197 mg g^{-1}) in 24 h and negligible improvement was observed after 48 h. During 72 h of incubation of untreated HHB, it released a maximum of 126±0.01 mg g^{-1} of reducing sugar at 24 h of hydrolysis. This indicates that the recombinant cellulase was capable of penetrating into the biomass structure. However, from the hydrolysis profile of untreated HHB it could be concluded that the bonds present in the biomass structure,

from holocellulose or lower quantities of lignin, restricted the access of cellulase to cellulose due to the compressed structure of HHB.

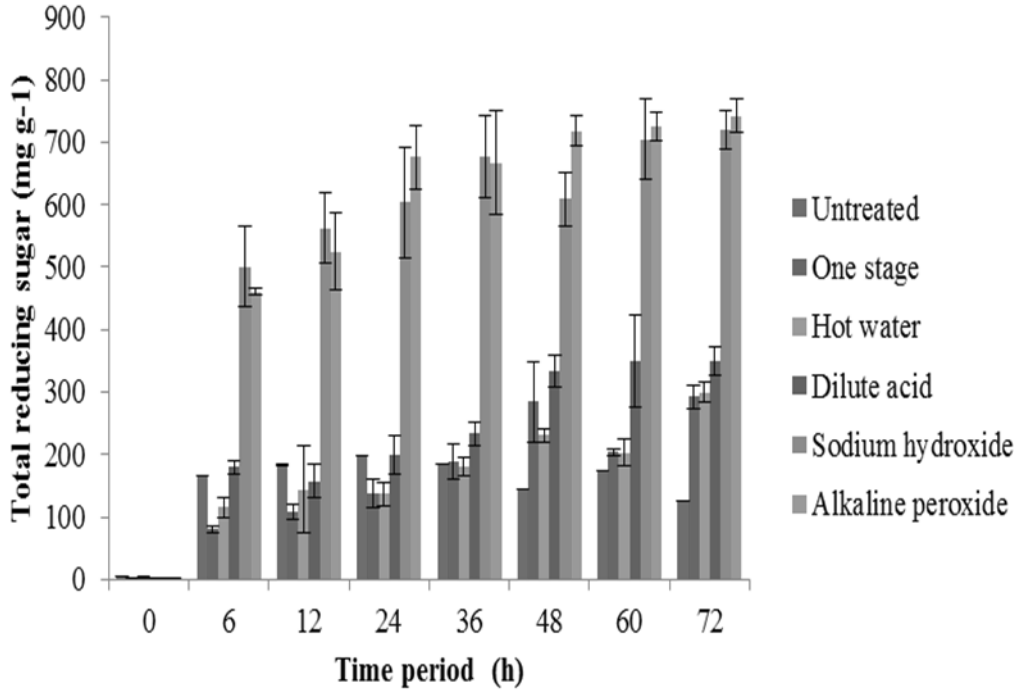


Figure 2.6 The 72 hours enzyme hydrolysis profile of different pretreated HHB samples saccharified using cellulase at 50 °C

The conversion efficiency of all pretreated samples to reducing sugars was at different levels during 72 h of enzyme hydrolysis. However, the basic hydrolysis trend was same in all pretreatments which were observed after 6 h of hydrolysis. It was seen that all pretreated samples reached up to 65-70% of their individual maximum yield in 6 h of incubation. The remaining hydrolysis occurred in following phase of hydrolysis which was extended up to 72 h. This suggest that the structure opening was efficient with less complexity, and that the activity of recombinant cellulase was quite high, which facilitated a high conversion rate during the initial 6 h of hydrolysis.

In acid treatments, dilute acid pretreated HHB provided a better yield of reducing sugars when compared to one stage and hot water pretreatments. The hydrolysis

progressed for 48 h in the one stage pretreated sample and no further improvement in yield was observed. In total it produced a maximum of 293±4 mg g^{-1}. This indicates that this pretreatment strategy was not efficient enough to separate the fibre bundle and provides access to cellulose.

Dilute acid pretreatment carried out in an autoclave under pressure also provided a similar hydrolysis pattern. The release of reducing sugars from 6 to 36 h was quite low but the release of sugar from 36 to 48 h was relatively high, and furthermore, no improvement in hydrolysis was observed. The maximum of 349 ±4 mg g^{-1} reducing sugar was produced over 72 h of incubation. The low rate of hydrolysis could be due to the lower concentration of acid. In order to prevent the formation of furfurals (known inhibitors) lower concentration of sulphuric was used during pretreatment, although this step resulted in lower production of reducing sugars. The occurrence of furfural during dilute acid pretreatment has been reported in literature. It acts as an inhibitor in later stages of processing and conversion (Jiang et al. 2013b). The occurrence of inhibitors was observed in our investigation as well which is discussed in Chapter 3.

The hot water treatment was done along with acid and alkaline condition to understand the difference in using chemicals during pretreatment and the effect of pressure under high temperature. The hydrolysis from 6 to 36 h progressed slowly but in the following 12 h the hydrolysis progressed well and reached to maximum. This indicates that only the presence of pressure under high temperature could expand the compact structure which helped the enzyme to penetrate. In 72 h of hydrolysis hot water pretreated HHB provided a maximum of 30% conversion (299±3 mg g^{-1} of reducing sugar). Hot water treatment and one stage pretreated samples provided the least amount of reducing sugars. This finding helped to understand that the presence of chemicals (absent in hot water pretreated samples) and pressure (absent in the one stage pretreated sample) are two important factors required in the loosening of the structure to allow easy penetration of enzyme for hydrolysis. The amount of reducing sugars does not significantly change after 72 h of hydrolysis in HHB. Therefore, 72 h was used for all the samples in enzymatic hydrolysis.

It was observed that the alkaline treatments (NaOH/alkaline peroxide) enhanced the digestibility of raw HHB to a greater extent when compared with acid treatments (one stage and dilute acid). Our results were in agreement with previous reports which suggested that pretreatment with sodium hydroxide degrades lignin and the acetyl group from the biomass (switchgrass) and improves enzyme accessibility (Mishima et al. 2006; Xu and Cheng 2011). A similar study has been conducted on hemp using four treatment conditions. Study reported 60% glucan and 30% xylan conversion after NaOH pretreatment (Pakarinen, 2012). Their observations were similar to those observed in our studies and also in a second study performed by them, demonstrated that addition of pectinase in enzyme cocktail enhances the hydrolysis yield (Zhang et. al. 2013). The presence of alkaline condition under pressure enabled the opening of the structure. While monitoring the hydrolysis at constant time intervals it was found that the rate of conversion in sodium hydroxide treated sample was faster in comparison to alkaline peroxide treatment up to 12 h of hydrolysis. However, the trend changed after 24 h of hydrolysis as the alkaline peroxide treatment released higher quantities of reducing sugar in later 48 h of hydrolysis.

Among all pretreatments, the alkaline peroxide treated samples provided a maximum amount of reducing sugars at 743±3 mg g^{-1}, followed by sodium hydroxide treatment which produced 719±3 mg g^{-1} of reducing sugars. This suggests that the presence of sodium hydroxide is responsible for significantly opening the biomass structure while the presence or absence of pressure did not bring large difference in hydrolysis result. This observation suggests that high temperature and the presence of sodium hydroxide are the two key factors which disrupt biomass structure. This investigation highlights that due to these factors enzymes could easily access cellulose, which could only happen when structure opens and fibre becomes porous. Thus, this finding determined the pretreatment aim of investigating the factors responsible for changing biomass structure. A similar study was reported on cotton stalk at high temperature under alkaline condition with good reducing sugar yields (Du et al. 2013).

While taking material balance into account, it was noticed that the maximum conversion of 1 g pretreated biomass to reducing sugar was 74%. The mass balance

of the HHB from dry initial mass to the yield of reducing sugar is represented in Figure 2.7. Although, the material balance indicated loss of some biomass during pretreatment and enzymatic hydrolysis, the overall reducing sugar yield was over 74%, confirming that the selected pretreatment conditions (alkaline) substantially enhanced the effectiveness of the enzymatic hydrolysis. On conducting few studies using different alkaline pretreatment conditions on various biomass researchers have reported that it is one of the successful pretreatment method to open the compact biomass structure and obtain a good yield of reducing sugar (Chen et al. 2012; Park and Kim 2012).

Furthermore, it was found that the major reducing sugar obtained during enzyme hydrolysis was mono and disaccharide 6-C atom hexose in the form of glucose and cellobiose and then 5-C atom pentose as xylose. The detailed analysis and quantification of reducing sugar using high performance liquid chromatography (HPLC) is discussed in chapter 3. Researchers have conducted similar study on elephant grass where same concentration of biomass when exposed to alkaline treatment lead to better results in comparison to dilute acid, steam explosion and ammonium soaking (Eliana et al. 2014).

There are many reports in the literature where researchers obtained a good yield of reducing sugar with dilute acid and steam explosion (Castro et al. 2014; Wood et al. 2014). Dilute acid under pressure and temperature opens the structure but simultaneously produces furfurals, HMF, acetic acid as by products. These by-products act as inhibitors during enzyme saccharification by hindering the action of enzyme and consequently product formation (Rajan and Carrier 2014). A group of researchers have succeeded in obtaining about 95% of reducing sugar from sugarcane and reported that hemicellulose was easily removed due to dilute acid pretreatment (Jiang et al. 2013b). Another group has reported about 97% of sugar yield from cattails and reported 90% of ethanol yield (Zhang et al. 2011a).

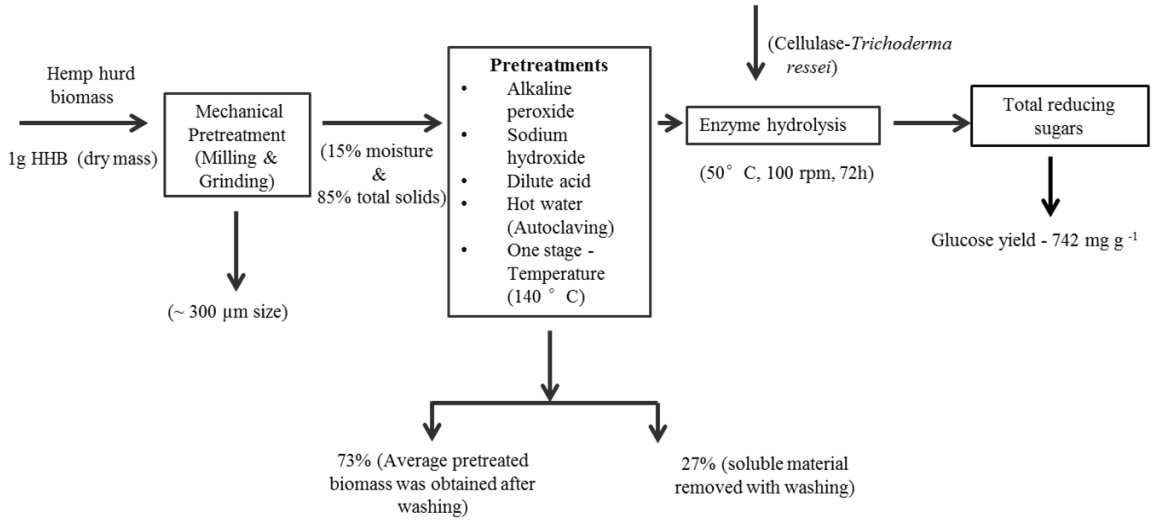


Figure 2.7 Mass balance as per g of dry weight biomass

However, in our studies, yield was not improved further under similar conditions, likely because dilute acid probably targets xylan to improve the yield while our biomass (HHB) had high cellulose content.

2.3.2 Bioprocessing of pine chips

Alkaline treatment at high temperature (based on hemp hurd pretreatment) gave best yields thus it was extended to pine chips. The milled pine saw dust weighing 10 g was slurried in 1 L of aqueous NaOH (0.5%, w/v) and pretreated at two different temperatures. The pine slurry was pretreated at 170 °C and 200 °C for 30 min in a hot air oven. The pretreated pine slurry was filtered to separate liquor and biomass. Thereafter it was washed 3-4 times to attain neutral pH. The washed biomass was allowed to dry at 70 °C until it attained a constant weight. Thereafter it was stored at 4 °C to be used for enzyme saccharification. The enzyme hydrolysis of pine chips (2%, w/v) was carried out as with HHB and under the same conditions. Studies were conducted on pretreated pine sawdust using dilute H_2SO_4. However, the maximum hydrolysis was found to be 20% within 72 h. On increasing the acid concentration, not much difference in hydrolysis percentage was observed. In comparison to acid pretreatment alkaline gave improved performance. There was no significant colour difference observed in pine pretreated samples as we observed colour variation from untreated to pretreated in HHB. The colour and size remained unchanged following pretreatment.

At 72 h, it was observed that the pine pretreated chips provided a maximum hydrolysis of 47% in the presence of alkaline at high temperature (Figure 2.8). The hydrolysis improved to 50% in the first 24 h. The raw pine chips provided a better hydrolysis profile in comparison to raw HHB. It was observed that with an increase of 30 °C in temperature the hydrolysis profile of pine demonstrated an improvement. With a difference of 30 °C, the amount of reducing sugar reached from 434 ± 0.02 mg g^{-1} to 467 ± 0.00 mg g^{-1} of pretreated pine.

The best results for pine chips were obtained from alkaline pretreatment, though some pretreatments were conducted in dilute acid. The selected conditions similar to those applied to HHB did not improve sugar yield. Increasing dilute acid concentration (0.5-15%) and temperature (140 °C) did not result improve the

hydrolysis yield. The sugar yield obtained after acid pretreatment was very low (data not shown). Improvement was not observed on increasing the concentration of alkali. Pretreatment of biomass using dilute acid at high temperature (180-200 °C) to open the structure have been reported earlier (Avci et al. 2013).

Such temperatures (200 °C) were not feasible at laboratory conditions for pretreatment of pine biomass. Since the hydrolysis percentage was found to be higher in HHB in comparison to pine chips under comparatively gentle pretreatment conditions, HHB was considered the primary source for future experimental studies.

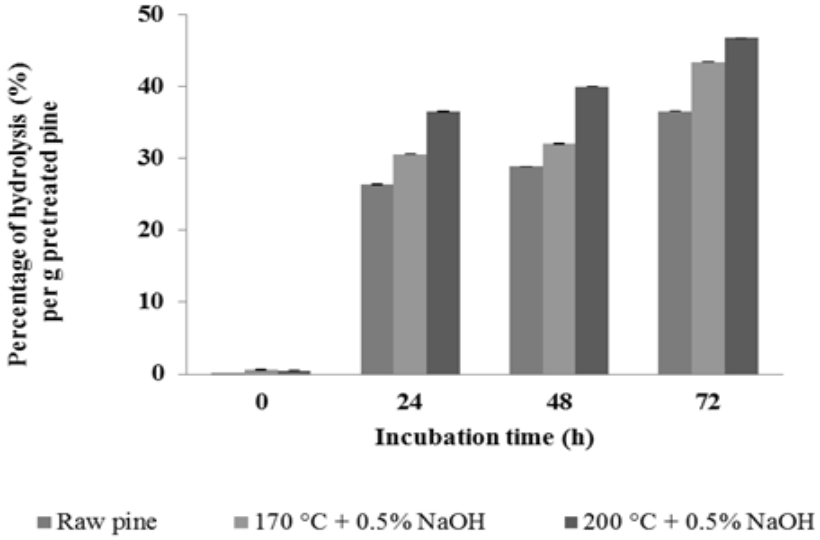


Figure 2.8 The 72 hours enzyme hydrolysis profile of pretreated pinus chips

2.4 Conclusions

Conclusions were drawn based on five different pretreatments conducted on HHB. Firstly, the presence of sodium hydroxide at high temperature expanded the biomass structure and permitted easy penetration of enzyme and thus, resulted in maximum yield of reducing sugars. The same was observed in the case of pine at higher temperature. Secondly, the presence of pressure enhances the blasting of biomass, and thirdly, recombinant cellulase used in this study retained high activity and hence converted pretreated biomass to 65-70% reducing sugar within 6 h of hydrolysis. The amount of reducing sugars released from enzyme saccharification of hot-water treated, dilute acid and one stage samples was substantially lower than for alkaline treatments. Therefore, sodium hydroxide pretreatment of HHB was used in further studies.

Chapter - 3

Enhanced enzymatic saccharification of biomass for sugar production

3.1 Introduction

The production of ethanol can be carried out by hydrolysing fermentable sugars produced during the bioprocessing of lignocellulose. These sugars are obtained by the digestion of biomass which is pretreated before hydrolysis. This pretreatment opens the structure of lignocellulose by creating pores on the surface, breaking the bonds and exploding the compact formation. The sugar yield is highly dependent on the quality of pretreatment and efficiency of enzymes. Inefficient pretreatment results in a need for higher enzyme loading, which ultimately increases the production cost of the resulting biofuel (Piccolo and Bezzo 2009).

Enzymes play a crucial role in the processing of lignocellulose, as the enzyme substrate interaction, formation of enzyme substrate complex and product release, all depend on the activity of enzymes and supportive reaction conditions. These are highly specific in nature. Bacteria and fungi are the major biomass degraders in the world. Termites contribute a small amount of endogenous cellulolytic enzymes, but mostly they harbour microbial gut symbionts that are responsible for the bulk of the degradation. The complexity in the lignocellulose structure results in poor digestibility of this substrate. The ratio of enzyme hydrolysis is estimated by the amount of cellulose or holocellulose added to the reaction mixture and the amount of product released in the total volume of reaction mixture (Chen et al. 2008). The products that are released as a result of enzyme hydrolysis are generally hexose or pentose sugars such as glucose, cellobiose, xylose, arabinose, ribose, rhaminose, glucan. The sugars generated during enzyme saccharification are predominantly monosaccharides and, to a lesser degree, disaccharides.

The main enzyme required for the hydrolysis of lignocellulose is cellulase: a complex synergistically acting mixture of endocellulase, exocellulase and β-glucosidase. According to the Carbohydrate-Active enZYmes Database (CAZy) these enzymes are classified in glycosyl hydrolase families. Endoglucanases/ Endocellulase are grouped under GH families 5-8, 12, 16, 44, 45, 48, 51, 64, 71, 74, 81, 87, 124 and 128. Exoglucanases/ Exocellulase/Cellobiohydrolases are grouped under GH families 5-7 and 48 and β-glucosidases in GH families 1,3,4,17,30 and 116 (www.cazy.org;

Juturu and Wu 2014). Endoglucanase (EC 3.2.1.4) hydrolyses the internal β-1,4-glucosidic (locates sites randomly). Subsequently a water molecule is inserted by cellodextrin/ exoglucanases (EC 3.2.1.91) at β-(1,4) bond and a new reducing and non-reducing chain end pairs are created. β-glucosidase (EC 3.2.1.21) hydrolyses cellobiose to glucose and cleaves off glucose units from cello-oligosaccharides. Cellulase works in two a dimensional environment and follows a Koshland-type mechanism. Cellulase leaves the carbon hydroxyl in the β configuration or α configuration (inversion of stereochemistry). Water molecules take the non-reducing chain end to prevent reannealing into the cellulose crystal and removal of cellodextrins occurs at this new chain end (Silveira et al. 2014; Binod et al. 2011; Himmel et al. 2007). Exoglucanase generally have longer cellulose binding site that forms tunnel to enclose catalytic residue. Endoglucanase have shorter binding sites to act directly on intact cellulose chain (Juturu and Wu 2014). The mechanism of these enzymes is shown in Figure 3.1.

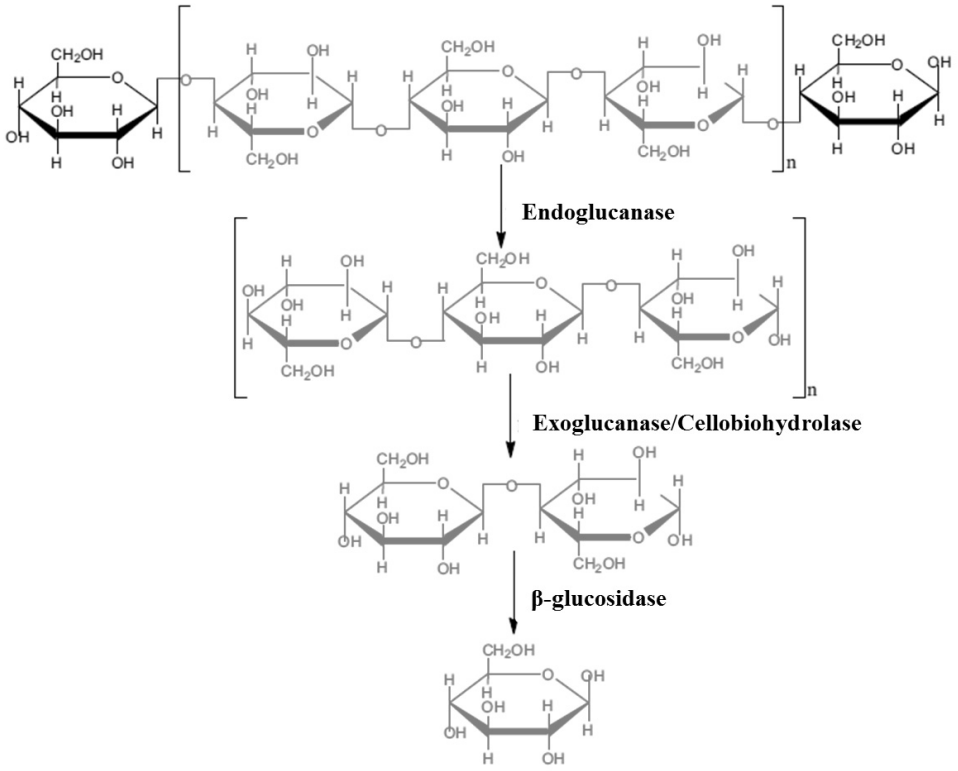


Figure 3.1 A synergistic mechanism of cellulase enzyme comprising endoglucanase, exoglucanase and β-glucosidase. This figure is reproduced from Juturu and Wu (2014).

Aside from cellulase, other enzymes such as hemicellulase, xylanase, and lignolytic enzymes are also used to hydrolyse the biomass for fermentable sugar formation. Xylan is the most common hemicellulose and found more in hardwoods. It contains a xylose backbone linked with β-1,4-glycosidic bonds and β-xylopyranose as the main chain. In softwood xylan is composed of arabino-4-0-methylglucuroxylans. The enzymatic system required to perform xylanolytic hydrolysis includes β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterase (Beg et al. 2001). Lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases are the most important lignin degrading enzymes. Lignolytic enzymes are mainly produced from white rot fungi including *Phanerochaete chrysosporium* and *Trametes* (Coriolus) *versicolour* (Call and Mucke 1997).

These enzymes can be utilised individually or in the form of a cocktail to hydrolyse the substrate. It is generally observed that product formation occurs rapidly with high concentrations of reducing sugars when an enzyme cocktail is used for hydrolysis. This occurs because various enzymes act on different components of the biomass, improving the efficiency of the process, and reducing reaction time of the hydrolysis step, thereby resulting in successful digestion and good product yield (Meyer et al. 2009). Enzyme hydrolysis is a non-corrosive process, carried under mild conditions (pH-4.8, temperature-45-50 °C) and takes several days to form product. This provides advantages over chemical hydrolysis, which is conducted under harsh conditions using inorganic acids (Binod et al. 2011).

The rate of reaction depends on the number of active sites, with more active sites yielding higher activity, as these will initiate the reaction quickly and form the complex to release the product. Cellulases are generally obtained from cellulase-producing termites and fungi. Some common sources of cellulase include *Trichoderma reesei* and *Aspergillus,* both of which are used to prepare commercial enzyme mixtures. High hydrolysis efficiency is difficult to obtain from single cellulase component or cellulase from pure culture. To reduce the production cost improved cellulases with new/more active site, multicomponent system has been proposed for commercial production of bioethanol (Kalyani et al. 2013). Some

researchers use in-house isolates obtained from soil, tree bark and termites to hydrolyse the biomass and at times allow the lignocellulose to undergo digestion for several days to yield reducing sugars. While this is a slow process, the hydrolysis of biomass using microbes to reduce sugars and produce ethanol has been frequently reported in the literature (Chen et al. 2013a; Sukumaran et al. 2009).

The activity of enzymes is calculated by enzyme assay and they are substrate specific. There are various enzymes assays and synthetic substrates available commercially, which help to determine the amount of product release, as a measure of the enzyme activity. Computational methods are available which can calculate the rate of reaction, theoretical product yield and duration of hydrolysis. Other methodologies help to design experiments which are statistically significant and can provide percentage figures for successful biomass digestibility (Garai and Kumar 2013). Quantification of the product or reducing sugars can be done using colorimetric assays or analytical methods, such as high performance liquid chromatography (HPLC). HPLC is column specific and enables the separation of carbohydrate and organic acids at different retention time. It is used to quantify different sugars and by-products formed during enzyme saccharification.

The aim of this work was to develop optimal enzyme concentrations and enzyme mixtures for hydrolysis of pretreated lignocellulosic raw materials- alkaline pretreated hemp hurd biomass. The work was carried out in two stages: first washing of the biomass to remove inhibitors, and secondly, optimal mixtures were developed using statistically designed enzyme ratio protocols. Mixtures of enzymes were also compared with commercial enzyme preparations.

3.2 Materials and methods

3.2.1 Materials

Sodium hydroxide pretreated hemp hurd biomass, cellulase from *Trichoderma reesei* (EC 3.2.1.4; 700 units) and *Acremonium cellulolyticus* (Meiji Seika, Tokyo, Japan), xylanase from *Thermomyces lanuginosus* (≥2500 units g^{-1}), cellobiase from

Aspergillus niger (≥250 units g^{-1}), carboxymethyl cellulose (CMC), Whatman No. 1 filter paper, xylan (birchwood), sodium citrate, 3,5-dinitrosalicylic acid, potassium sodium tartrate, sodium hydroxide, milliQ. The chemicals used for the study were purchased from Sigma (St. Louis, MO, USA).

3.2.2 High Performance Liquid Chromatography (HPLC)

The standards used for HPLC analysis were of high purity (HPLC grade). After running milliQ the column was thoroughly washed prior to each experiment using acetonitrile. HPLC was conducted to analyse the sugars and organic acids and concentrations were extrapolated from the standard curve obtained after running standard sugars through the column. The standard sugars used were glucose, cellobiose, arabinose, mannose, xylose and organic acids included succinic acid, oxalic acid, acetic acid, formic acid and ethanol purchased from Sigma (St. Louis, MO, USA).

3.2.3 Enzyme assays

The activity of cellulase was determined by using synthetic substrate and quantifying the product released by the DNS method. Cellulase (*Trichoderma reesei*) activity was measured in terms of CMC units or FPU (filter paper unit) (Ghose 1987). The substrate used for CMC assay was carboxymethyl cellulose and Whatman No. 1 filter paper strip (1×6 cm) was used for filter paper assay. The filter paper assay was conducted by incubating 1 mL of 0.05 M sodium citrate buffer (pH-4.8) and 0.5 mL of enzyme dilute in a test tube at 50 °C with filter paper strip roll for 60 min in shaking water bath (Ratek, Model No. SWB20D). The product release was determined using the DNS method by adding 3 mL of DNS reagent to the reaction mixture and boiling in a water bath for 5 min. After cooling, the absorbance was measured at 540 nm using UV-1800 UV-VIS spectrophotometer (Shimadzu). The concentration of glucose was obtained in accordance with the glucose standard curve. The CMC assay was conducted by adding 0.5 mL of CMC (2%, w/v in 0.05 M sodium citrate buffer, pH- 4.8) and 0.5 mL of enzyme at 50 °C for 30 min, and the product was measured by the DNS method. The enzyme activity was defined as the amount of enzyme producing 1 μmol of reducing sugar per minute. The enzyme assays were conducted in three repetitions at various enzyme concentrations, and

enzyme activity was calculated according to the standard Ghose protocol (Ghose 1987). The DNS reagent used for reducing sugar measurement was prepared according to the method of Yu et al. (Yu et al. 1997). The xylanase assay was conducted similarly to the previously described cellulase assay, using xylan as a substrate. A reaction mixture containing 1 ml of buffer (sodium citrate, pH-4.8), 1 ml of xylan and 1 ml of enzyme was incubated at 50 °C for 60 min in a shaking water bath. The reaction was stopped by adding 3 ml of DNS reagent and then boiling in a water bath for 15 min. The absorbance was measured at 640 nm and the activity of enzyme was calculated using the Ghose protocol (Ghose and Bisaria 1987).

3.2.4 Enzyme saccharification of pretreated biomass

All enzyme saccharification was conducted at 50 °C, 100 rpm in an orbital shaker (Ratek), in 0.05 M sodium citrate buffer (pH- 4.8). The duration of hydrolysis varied from 18 h to 92 h. The percentage of hydrolysis was determined by the DNS method and the concentration of sugars and organic acids were analysed by HPLC.

Enzyme hydrolysis including different concentrations of substrate (sodium hydroxide pretreated hemp hurd) and cellulase from *Trichoderma reesei* was performed using 2%, 5% and 7% (w/v) of biomass with different cellulase concentrations 10, 20, 30, 40 CMC units g^{-1} of solid biomass loading.

The enzyme saccharification using xylanase was performed using xylanase from *Thermomyces lanuginosus*. On the basis of our initial assay we could determine that the activity of xylanase is comparatively low and produces low product yields. Therefore the concentration of pretreated biomass was kept low for enzyme saccharification. The hydrolysis was conducted using 2% (w/v) of pretreated hemp hurd and five varying concentration of xylanase including 10, 15, 20, 30, 40 units g^{-1} of solid biomass loading were used. The hydrolysis was conducted for 72 h.

The enzyme hydrolysis study conducted using two different sources of cellulase were *Trichoderma reesei* and *Acremonium cellulolyticus* (Meiji Seika, Tokyo, Japan) (Fujii et al. 2009). Similar concentrations of both enzymes were taken for the study and the concentration was ~20 CMC units. The hydrolysis was conducted for 48 h at

50 °C using a concentration of 5% (w/v) of sodium hydroxide pretreated biomass and the hydrolysis yield was determined by the DNS method.

3.2.5 Modified washing and enzyme hydrolysis of biomass

The washing of pretreated biomass was modified to study its effect on hydrolysis yield. This study included two set of washes for pretreated biomass/solid fraction. After the removal of liquor from the slurry the biomass was washed four times to remove loosely bound residues and reported as PW1. This washed set of sample was stirred overnight in water. The samples were filtered the next day to separate solid and liquid fraction and reported in the study as PW2. After washing PW1 and PW2 were dried to constant weights and stored at 4 °C for later experiments. The enzyme hydrolysis was performed using PW1 and PW2 samples at biomass solid loading of 5% (w/v) using 20 CMC units of cellulase from *Trichoderma reesei*. The hydrolysis was performed for 30 h under continuous shaking condition.

3.2.6 Effect of enzyme cocktail on biomass hydrolysis

A comparative study of PW1 and PW2 was carried out using a cocktail of enzymes at various concentrations. The enzymes used for the hydrolysis were cellulase from *Trichoderma reesei*, xylanase from *Thermomyces lanuginosus* and cellobiase from *Aspergillus niger* with the combinations are outlined in Table 3.1. The solid biomass loading was conducted at 5% (w/v) concentration and the experiment was conducted for 18 h. The sugar hydrolysate obtained from hydrolysis was analysed by HPLC for sugar and organic acid quantification.

Table 3.1 Experimental design of enzyme cocktail for biomass hydrolysis

Experimental set up	Cellulase ($U\ g^{-1}$)	Xylanase ($U\ g^{-1}$)	Cellobiase ($U\ g^{-1}$)
F1	40	40	10
F2	30	60	10
F3	20	80	10
F4	30	40	12
F5	20	40	16

3.2.7 Analytical method

The quantification of sugar hydrolysate was conducted using HPLC. An Agilent 1260 Infinity series HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with a solvent degasser, quaternary pump, auto-sampler, thermostatted column compartment, refractive index detector and fitted with an Aminex HPX 87P column (300×7.8 mm), equipped with de-ashing cartridge (30×4.6 mm) and a Carbo-P micro-guard cartridge (30×4.6 mm). The column was thoroughly washed with 30% (v/v) acetonitrile before and after each set of experiments. The reaction condition was maintained at 80 °C at a flow rate of 0.6 mL/min and eluted with miliQ water. The quantification of organic acids was performed using Aminex HPX 87H columns (300×7.8 mm) equipped with Cation H microguard cartridge (30×4.6 mm). The run was performed at 65 °C at a flow rate of 0.6 mL/min under 0.01 M nitric acid as the mobile phase (Saha and Cotta 2011). The samples were filtered through a membrane filter unit (0.45 mm) purchased from Thermoscientific and the HPLC peaks were detected using a refractive index detector. All other chemicals used were of standard analytical grades and the run was performed in duplicates and represented with standard deviation (±SD). A flowchart representing use of HPLC for detection of reducing sugars and organic acids is shown in Figure 3.2.

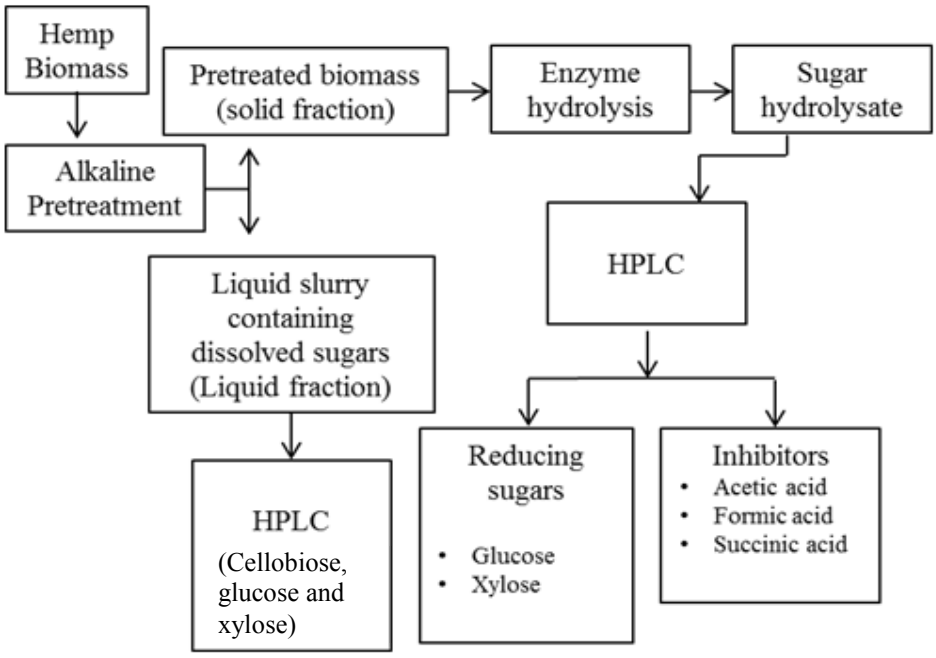


Figure 3.2 Flowchart representing the use of HPLC for detecting reducing sugars and organic acids from pretreated and enzyme saccharified hemp biomass

3.3 Results and discussion

3.3.1 Use of cellulase from different sources for saccharification

Trichoderma sp. and *Acremonium* sp. were obtained commercially and compared as a source of cellulase for saccharification. Hydrolysis was conducted for 48 h at 50 °C using sodium hydroxide pretreated hemp at a solid loading of 2% under constant shaking conditions and the results are represented in Figure 3.3. Our study demonstrated that in the initial 6 h of incubation *Trichoderma* was capable of producing about 50% hydrolysis while *Acremonium* produced only 30%. In the subsequent 6 h the hydrolysis was 54% with *Trichoderma,* with limited hydrolysis after that. Hydrolysis with *Acremonium* was 62% after 6 h and steadily increased to a maximum of 94% after 48 h.

Acremonium was better than *Trichoderma* in our study, although the yield of hydrolysis with *Trichoderma* increase to a maximum of 67% after 72 h, indicating that reactivity of this cellulase continues after our 48 h experimental period. Previous studies using *Acremonium* as a source of cellulase for biomass hydrolysis have reported high concentrations of sugar yield from biomass such as eucalyptus, douglas fir and, rice straw using *Acremonium* cellulase (Fujii et al. 2009). Another study showed about 70% sugar yield using hot-compressed water treatment and cellulase from *Acremonium* (Inoue et al. 2008).

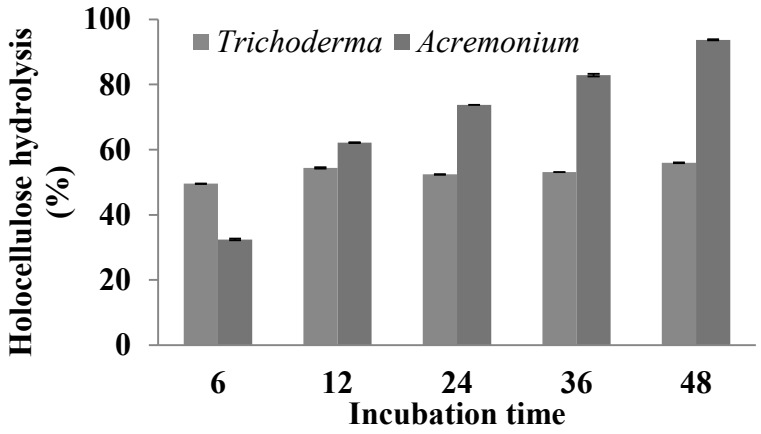


Figure 3.3 Comparative hydrolysis activity for cellulases *Trichoderma* and *Acremonium*

3.3.2 Use of xylanase with different substrate concentrations

Sodium hydroxide pretreated hemp hurds was subjected to enzyme saccharification using 2% (w/v) xylanase. Five different concentrations of xylanase were used in this study. These were 10, 15, 20, 30 and 40 unit g^{-1} of solid biomass, and hydrolysis was performed for 72 h, with samples collected and analyses at 24 h intervals (Figure 3.4). After 24 h percent hydrolysis ranged from 14-35% and increased with both enzyme concentration and time.

Yields after 48 h were 42% hydrolysis with 40 U enzyme, 32% with 30 U, 24% with 20 U, 21% with 15 U and 17% hydrolysis with 10 U of enzyme. Hydrolysis levels in our study were lower than those previously reported using xylanase, although different substrates were used (Falkoski et al. 2013). Maximum hydrolyses occurred after the longest time period (72 h) and these were 51% with 40 U enzyme, with 42% with 30 U, 29% with 20 U, 29% with 15 U and 23% hydrolysis with 10 U. One study conducted on pretreated sorghum using NaOH, dilute acid and alkaline peroxide observed 29.5-23.8 mg g^{-1} of reducing sugars from xylanase extracted from *B. altitudinis* in 48 h (Adhyaru et al. 2014). In our study we obtained higher levels of hydrolysis, with 34-84 mg g^{-1} of reducing sugars produced in 48 h.

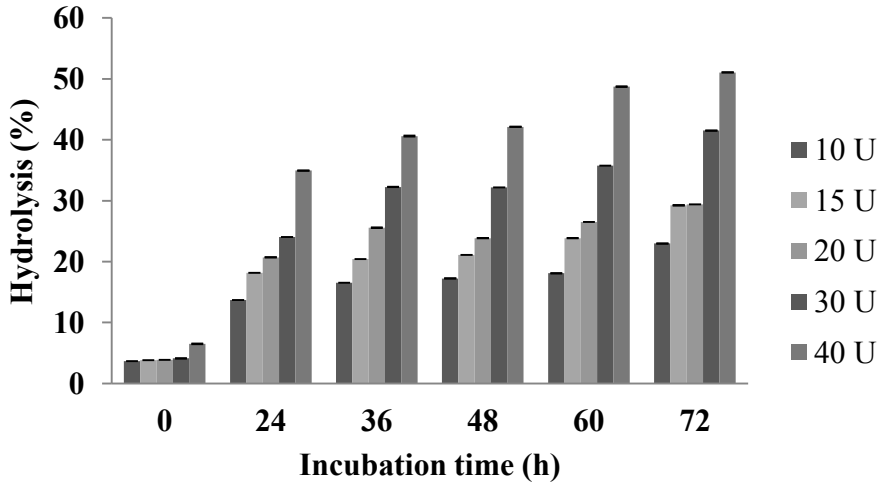


Figure 3.4 The hydrolysis of pretreated hemp at biomass loading of 2% with different concentrations of xylanase

3.3.3 Optimisation of cellulase and substrate concentrations for enhancing saccharification

This set of experiments was conducted to optimise enzyme saccharification at different cellulase and substrate concentrations. Sodium hydroxide pretreated hemp hurd gave the highest yield of reducing sugars in our previous study and so was chosen as the substrate for this study. 5% and 7% (w/v) concentration of substrate and four different concentrations of cellulase; 10, 20, 30, and 40 CMC units, were used in this study. Results obtained are shown in Figure 3.5 and 3.6.

After 72 h of enzyme hydrolysis for two different substrate concentrations the maximum holocellulose digestibility was observed with a 5% solids loading (Figure 3.4). 24 h hydrolysis of 5% substrate at different enzyme loading concentrations showed that the degree of hydrolysis increased with enzyme loading. Our hydrolysis level was slightly lower than that observed for the hydrolysis of duckweed (lower substrate loading) with an enzyme cocktail, where 85% hydrolysis was observed. We obtained 60-70% of hydrolysis in 24 h at higher substrate loading (Zhao et al. 2012).

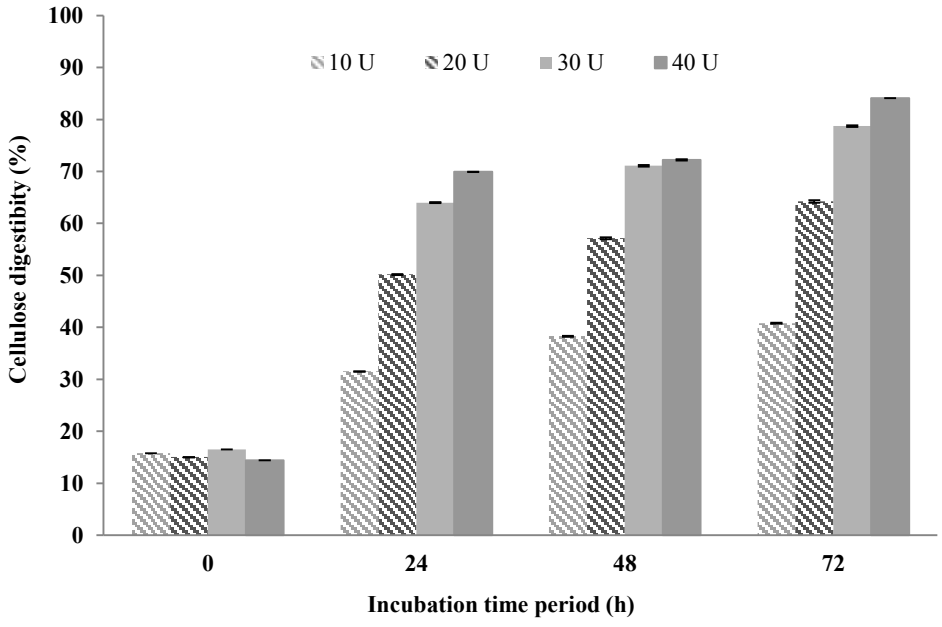


Figure 3.5 Enzyme saccharification of pretreated hemp hurds (5%, w/v) at various enzyme concentrations

A study conducted using isolated *Trichoderma* strain to hydrolyse switchgrass for about 168 h reported that maximum conversion occurred in 24 h of incubation with slight increases in the hydrolysis over the remaining time, which is consistent with our study where we observe that the majority of hydrolysis occurred in the first 24 h (Figure 3.5). The *Trichoderma* study reported a maximum hydrolysis of 9-10 mg mL^{-1} in 24 h which is lower than in our study, where we observed 15 mg mL^{-1} with 10 U and 35 mg mL^{-1} with 40 U of enzyme loading after 24 h (Cianchetta et al. 2012).

The enzyme saccharification of 7% solid loading with the same enzyme concentrations gave lower yields of reducing sugars than achieved at 5% solid loading (Figure 3.6). In 24 h, the maximum yield of reducing sugars was 40% with 30 U of enzyme loading. Another study reported that cellulase hydrolysis of pretreated pine biomass (2%) resulted in 52% hydrolysis using 30 FPU g^{-1} enzyme for, which was comparable to our *Trichoderma reesei* preparation that gave a yield of 53% (Jagtap et al. 2014). In our study we used a higher substrate loading (5%) and obtained greater levels of hydrolysis at high enzyme concentrations. A recent study reported 350 mg g^{-1} of reducing sugars production by enzymatic (20 FPU) saccharification of 0.5% alkaline pretreated Kans grass biomass (Kataria et al. 2013). In our studies we observed that the lowest concentration (10 U) produced 380 mg g^{-1} of reducing sugars in 48 h and the maximum amount obtained was 960 mg g^{-1} in 96 h with 40 U of cellulase loading. Another study which included an isolated cellulase strain and enzyme hydrolysis of sodium hydroxide pretreated *Pinus* sp. reported 507 mg g^{-1} of total reducing sugar released using a DNS method (Kalyani et al. 2013). The researchers compared their study with various commercial cellulase enzymes hydrolysing several pretreated biomass variants that provided total reducing sugars in the range of 70-488 mg g^{-1} of substrate. In our study we observed yields from 300-960 mg g^{-1} of total reducing sugars from 24-96 h of incubation at enzyme loading of 10-40 U g^{-1}.

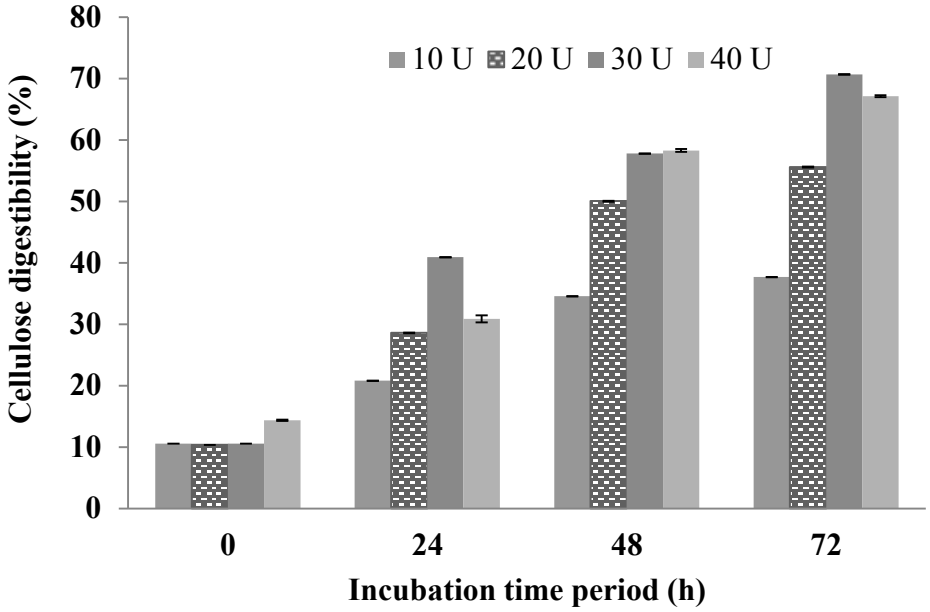


Figure 3.6 Enzyme saccharification of pretreated hemp hurds (7%, w/v) at various enzyme concentrations

3.3.4 Effect of pretreatment and washing pretreatment

Some samples were selected for a second round of washing, which included overnight washing under stirring conditions, after pretreatment and a first round of washing (PW2). This overnight washing resulted in improved digestibility as compared to samples that were washed 3-4 times after pretreatment (PW1) (Figure 3.7). After 30 h PW2 and PW1 provided maximum hydrolysis yields of 87% and 76%. The improvement in hydrolysis could be due to the removal of residuals which were inhibiting the hydrolysis in later stages. Overnight stirring helped to remove loosely bound residues and provided improved hydrolysis.

Reports show that during thermal pretreatment of biomass, D-glucose and hemicellulose degrade HMF, which further breaks into formic acid and levulinic acid. Moreover, pentose and hexose sugars also degrade to form various phenolic compounds, which act as inhibitor in later processing stages (Rasmussen et al. 2014). Severe treatment conditions are required to breakdown the complex structure of

lignocellulosic biomass and so the hydrolysis of biomass produces various hydrolytic products apart from fermentable sugars (Prado et al. 2014). A similar study where the biomass was washed prior to enzymatic hydrolysis reported a reduction in the level of inhibitors, such as acetic acid, formic acid and furfurals, after rinsing (Rajan and Carrier 2014; Ramchandran et al. 2013). The occurrence of inhibitors was from pretreatment and among them the amount of formic acid and acetic acid was more. It also suggests that xylose degrades into formic acid and furfural (Rajan and Carrier 2014). This indicates that rinsing helps in detoxification by removing some inhibitors and thereby increases hydrolysis yields. There are other detoxification methods used in the literature, such as overliming, membrane extraction, chromatography, and electrodialysis, although washing is the simplest (Grzenia et al. 2008; Laatikainen et al. 2011).

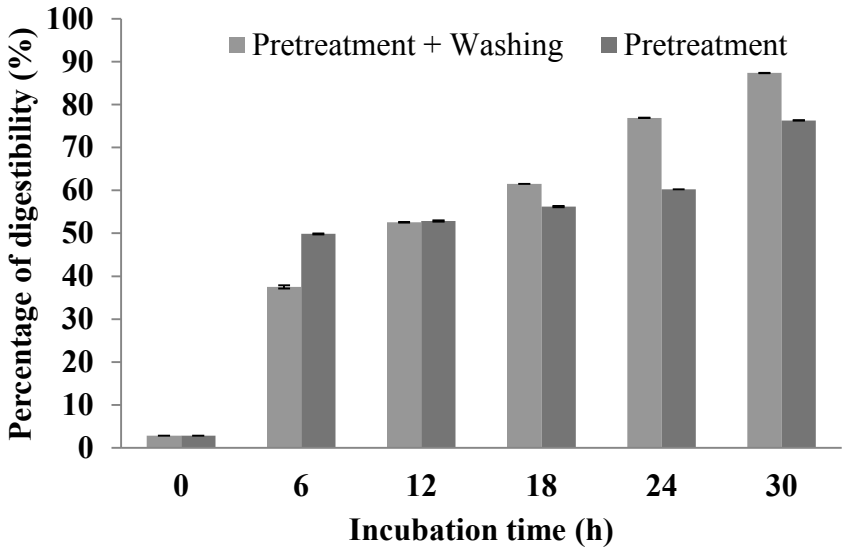


Figure 3.7 Comparison of various washings conducted on pretreated biomass and its enzyme hydrolysis

3.3.5 High Performance Liquid Chromatography analysis of saccharification

Hydrolysis of lignocellulosic biomass produces a variety of soluble, fermentable sugars that consist mainly of pentoses (xylose and arabinose) and hexoses (glucose

and mannose). Among these sugars, glucose is usually found in the highest concentration, with xylose as the second most abundant sugar from hardwoods or agriculture residues, or mannose from soft woods. Other sugars are also present, but in lower concentrations (Chu and Lee 2007). Glucose and xylose were the primary sugar products observed in our study, although other sugars including cellobiose were observed at lower levels. Mannose and arabinose was not detected in our samples.

The liquid removed from the biomass after pretreatment and before enzyme hydrolysis was analysed using HPLC to quantify the amount of reducing sugars released during pretreatment. Pretreatment led to the release of small amount of sugars into the liquid slurry, measured as 0.74% in the dilute acid pretreatment liquid slurry by HPLC. Sodium hydroxide and hot water pretreatment resulted in the loss of less sugar into the liquid slurry, with 0.24% for sodium hydroxide and 0.20% for hot water pretreatments. Alkaline pretreated biomass released the highest amount of reducing sugars, as discussed in Chapter 2. Therefore, this sugar hydrolysate was used in HPLC to detect different sugars such as pentose and hexose. HPLC of standards and sugar hydrolysate obtained from alkaline pretreated biomass (77% hydrolysis) using cellulase is shown in Figure 3.8 and 3.9. HPLC results confirmed that sugar hydrolysate contained variable quantities of glucose, xylose and cellobiose.

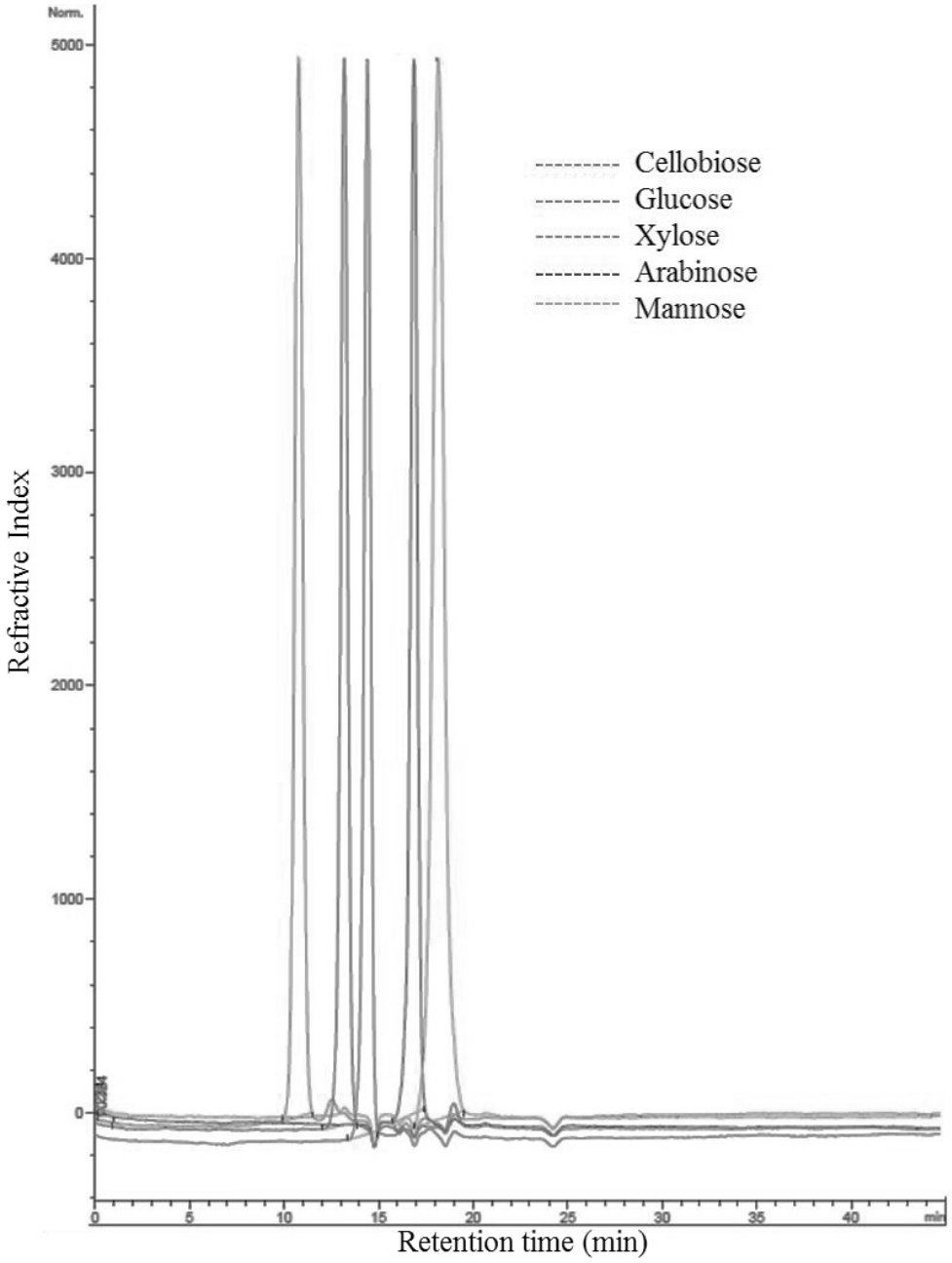


Figure 3.8 HPLC chromatogram representing standard sugars: cellobiose (RT- 10.7 min), glucose (RT- 13.1 min), xylose (RT- 14.4 min), arabinose (RT- 16.8 min), mannose (RT- 18.1 min).

* RT- Retention time

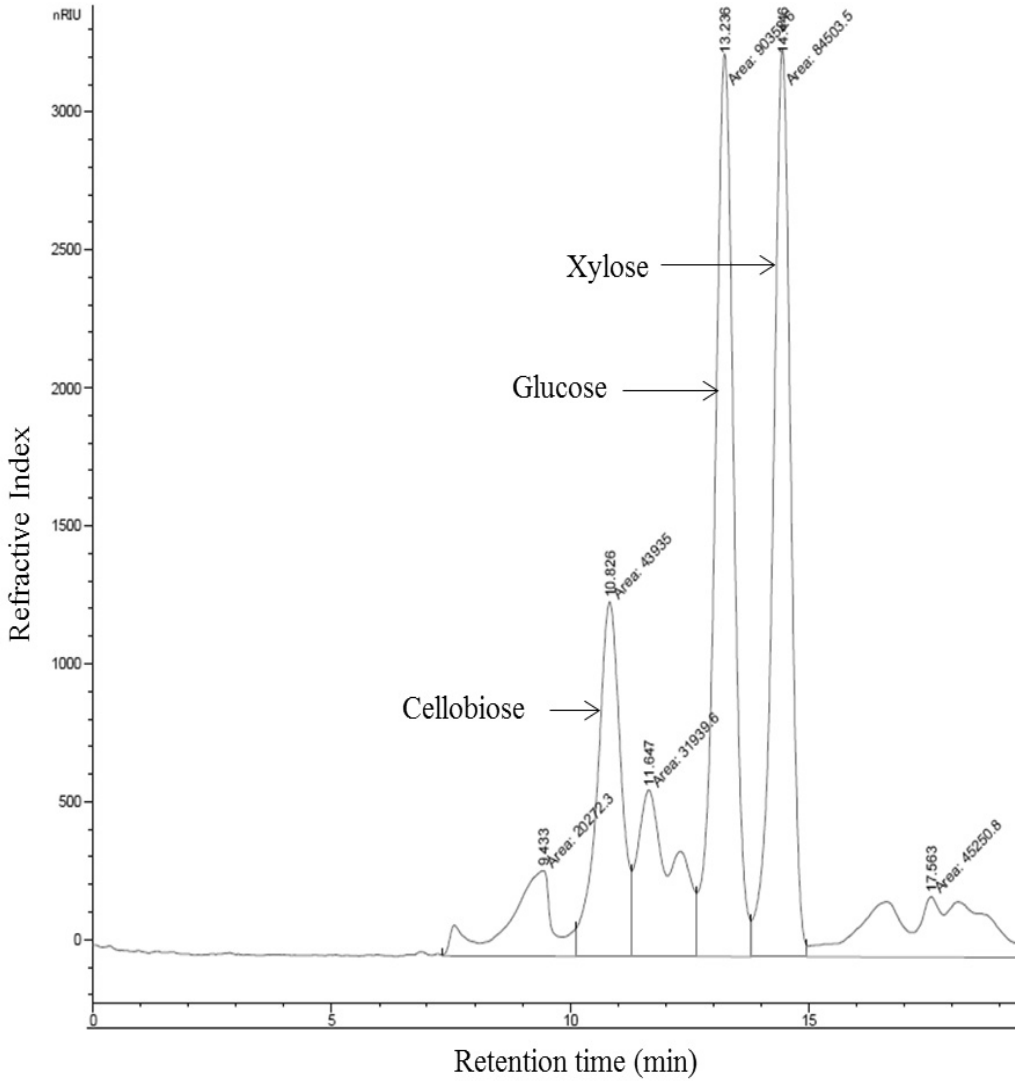


Figure 3.9 HPLC chromatogram representing reducing sugars (cellobiose, glucose and xylose) obtained from 72 hours cellulase hydrolysis of alkaline pretreated hemp hurd

3.3.6 Biomass hydrolysis using an enzyme cocktail

The cocktail experiment was conducted using a set of biomass PW1 and PW2, where PW1 constitutes pretreated biomass after four washes and PW2 consists of biomass which was washed overnight on a magnetic stirrer after an initial four sets of washing. Three sets of enzymes, namely cellulase, xylanase and cellobiase were used

in five different combinations for a quantitative study using HPLC. The combinations used for the study are shown in Table 3.1 and labelled as F1, F2, F3, F4 and F5 (refer to Section 2.6). The study was conducted for 18 h and the resulting sugar hydrolysate was analysed for reducing sugars composition and organic acids which acted as inhibitor during the hydrolysis.

The concentrations of sugars obtained from various enzyme combinations were determined for both PW1 and PW2 biomass. Organic acids (inhibitors) released during the hydrolysis of PW1 were also quantified. The total amount of reducing sugars and inhibitors obtained from PW1 and PW2 hydrolysis are shown in Figure 3.10. The F2 enzyme combination gave the highest yield of reducing sugars from PW1 after 18 h of hydrolysis, with a reducing sugars yield of 26%. F4 gave a 24% yield, F5 a 22% yield, F1 a 14% yield, and F3 a 13% yield, of reducing sugars. That is, the enzyme cocktail effectiveness was in the order F2 > F4 > F5 > F1 > F3. HPLC chromatogram of sugar hydrolysate obtained from pretreated biomass hydrolysis using cocktail of enzymes is shown in Figure 3.11.

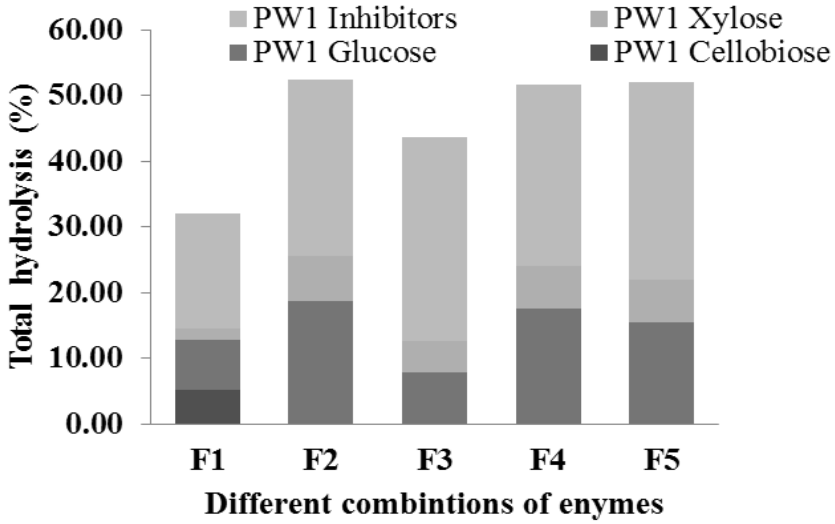


Figure 3.10 Total hydrolysis obtained from different combination of enzymes for 18 hours of hydrolysis in PW1

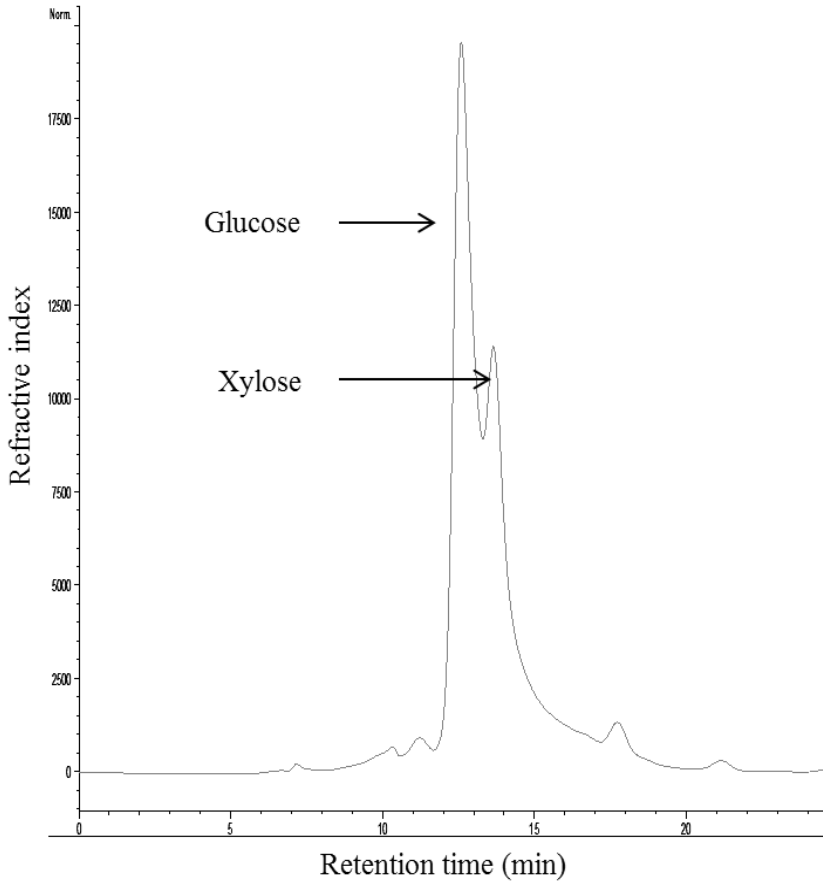


Figure 3.11 Reducing sugars detected by HPLC in sugar hydrolysate obtained using cocktail of enzymes

The concentrations of organic acid inhibitors were determined for each sample after 18 h of hydrolysis, showing that the major inhibitor was acetic acid and traces of formic and succinic acid was observed. The HPLC chromatogram of standard organic acids used to determine the inhibitor composition in the sugar hydrolysate is shown in Figure 3.12. However, the level of inhibitors did not correlate with the order of enzymatic effectiveness (Figure 3.13). Inhibitor concentrations were present from the highest to the lowest levels for F3 > F5 > F4> F2 > F1. The concentration of inhibitor increased as enzyme saccharification progressed. After 18 h hydrolysis levels were reasonably high at 50%, but inhibitor levels were still relatively low (inhibitor level was increasing with incubation), and so 18 h may represent the optimum hydrolysis time.

Researchers have described the occurrence of inhibitors such as acetic acid, formic acid, phenolics and levulinic acid during the hydrolysis of softwood such as spurce wood, with the concentration of sugar degradation to inhibitor concentration increasing after more extensive pretreatment (Hodge et al. 2009; Jung et al. 2013). That is, more extensive pretreatment results in a lower ratio of inhibitor to product. Xylooligomers are inhibitors that can also form during the enzyme hydrolysis of cellulose, although we did not determine the presence of this inhibitor in our study and so it is possible that other inhibitors are present but not detected (Qing et al. 2010). This may explain why our order of hydrolysis effectiveness did not inversely correlate with the level of inhibitors detected.

Inhibitors in lignocellulosic biomass hydrolysate can be removed by electrodialysis. It was reported that this process removed 100% of the formic acid and about 40% of the acetic acid that were found in the initial sugar hydrolysate (Lee et al. 2014). The use of dilute acid pretreatment can increase inhibitor levels since during treatment with dilute acid, hemicellulose releases acetyl groups attached to the hemicellulose backbone simultaneously with sugars (Cheng et al. 2008; Grzenia et al. 2008).

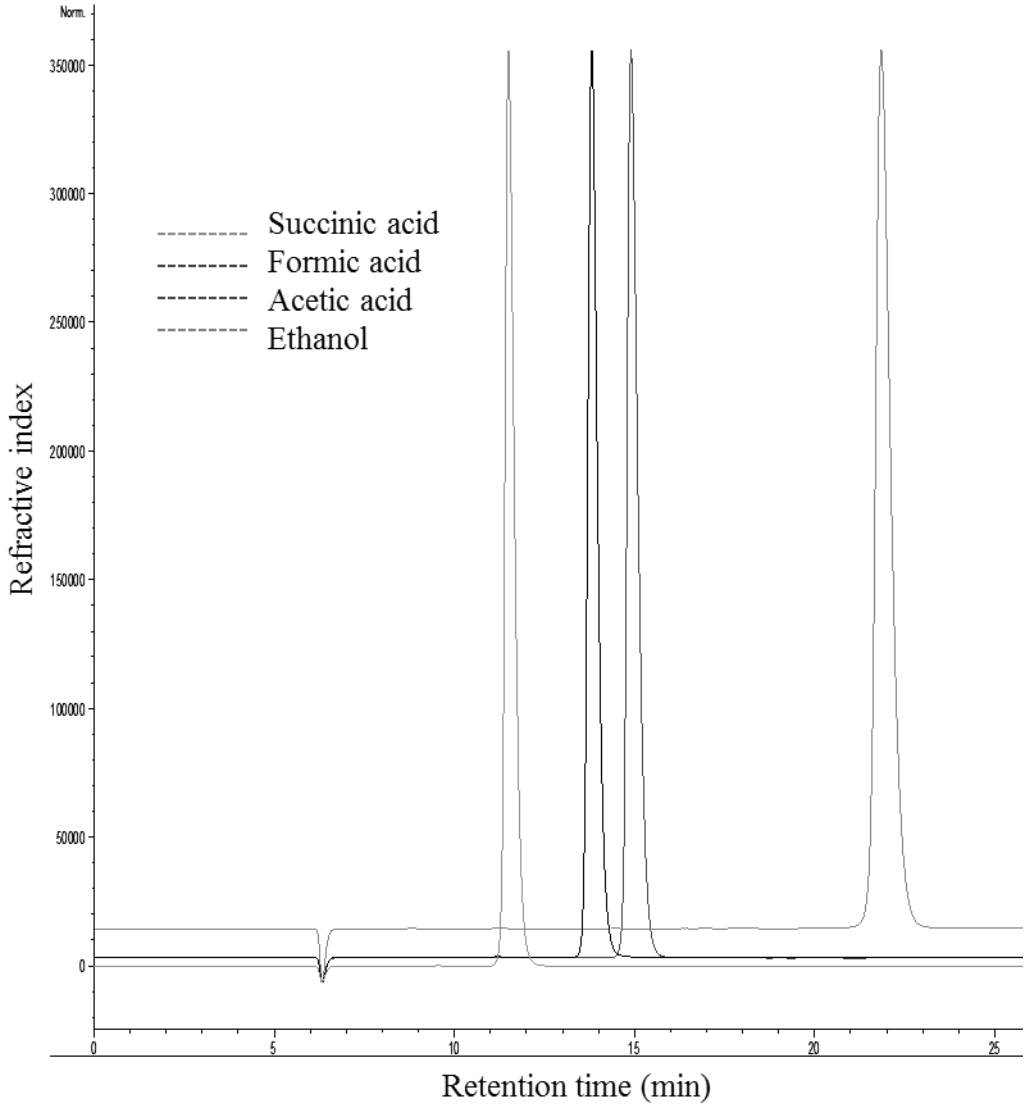


Figure 3.12 HPLC chromatogram of standard organic acids (inhibitors): succinic acid (RT- 11.5 min), formic acid (RT- 13.8 min), acetic acid (RT- 14.9 min), ethanol (RT- 21.9 min) used to determine the concentration of inhibitors in sugar hydrolysate

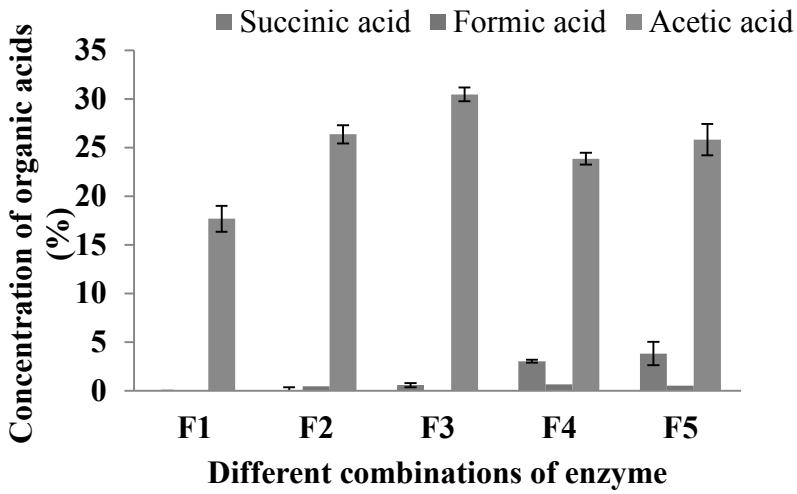


Figure 3.13 Organic acid inhibitors detected after PW1 hydrolysis

In order to remove inhibitors from the biomass we washed pretreated biomass overnight with stirring (PW2), which improved the yield of reducing sugars after 18 h of hydrolysis. The maximum concentration obtained was 35% after 18 h with enzyme combination F1 (PW2). The highest hydrolysis yield in PW1 was obtained with the F2 combination. However, overnight washing improved the concentration of reducing sugars to 34%. Compared to PW1 the concentration of reducing sugars with F3 (30%) doubled in PW2 sample. F4 and F5 combinations also showed improvement in hydrolysis after overnight washing and provided 29% and 19% of sugars, respectively. Comparison of xylose and glucose release for PW1 and PW2 is shown in Figure 3.14. PW2 essentially differs from PW1 by additional washing to remove inhibitors, shows that for each of F1 to F5 hydrolysis levels are higher for PW2, indicating that the extra washing is effective.

Overnight washing improves hydrolysis yield through improved removal of inhibitors that arise from biomass structure breakage under pretreatment conditions. It is a cheap detoxification method which allows easy separation and recovery of biomass. Earlier studies have reported that water washing and soaking helps to remove inhibitors and considerable amount of week acids (Hongqiang and

Hongzhang 2008). This observation was true in our studies also. Amorphous components of biomass can be loosely bound to the structure after pretreatment, and removed by overnight washing. The yield of reducing sugars increases substantially after washing to remove these amorphous components. The current studies were carried out using a batch system. Further improvement in inhibitor removal and efficiency could be obtained using a continuous design. Some researchers have observed the use of chelators to quench inhibitors and promote enzyme hydrolysis in a continuous system (Cavka and Jonsson 2013). The use of quenching could be used in conjunction with inhibitor removal, either batch or continuously, to further improve hydrolysis yields.

Earlier report suggested that alkaline pretreatment solubilises hemicellulose and lignin and their degradation products have inhibitory effects (Hendriks and Zeeman, 2009). Also, the presence of these inhibitors makes conditions severe for ethanol production. Another report suggested that pretreatment condition may result in degradation of hemicellulose and form acetate (acetic acid) (Vancov and Mcintosh, 2012). Furthermore, a study similar to ours using wheat straw and four pretreatment conditions in an autoclave have reported the occurrence of acetic acid, formic acid as the main by-product from the enzyme hydrolysis (Toquero and Bolado 2014). Another study has stated that a strong inhibitory effect occurs in hydrolysis by the presence of lignin-rich residue from softwood compared to lignin from other sources (Rahikainen et al. 2011).

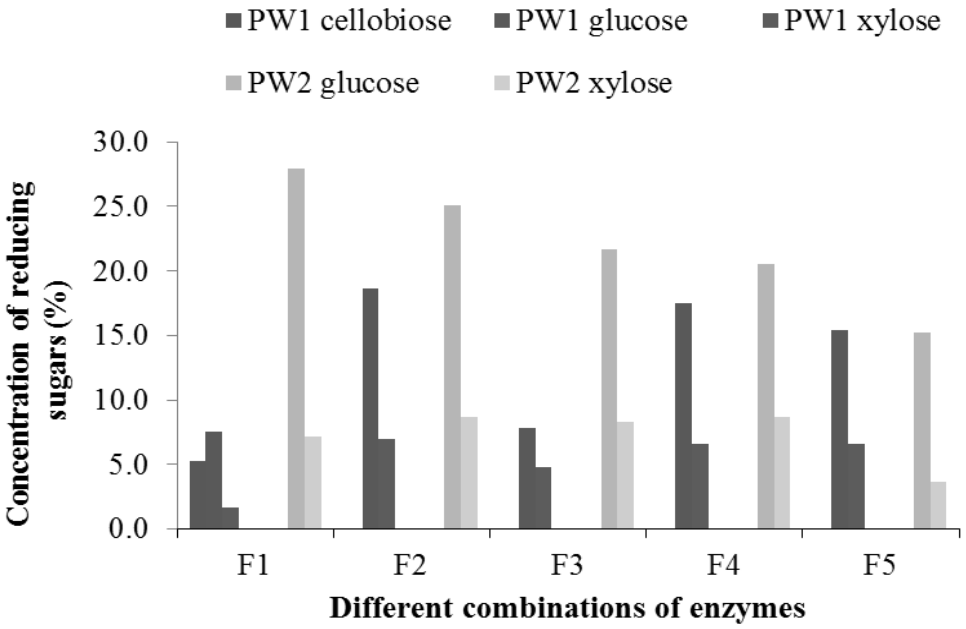


Figure 3.14 Total hydrolysis observed for different combinations of enzymes for 18 hour of hydrolysis in PW1 and PW2

3.4 Conclusion

Digestibility of alkaline pretreated hemp using cellulase from *Acremonium* sp. was greater than *Trichoderma* sp. in 72 h of hydrolysis. The maximum biomass hydrolysis exhibited by xylanase at highest enzyme loading was 51%. The highest hydrolysis (84%) of alkaline pretreated hemp at 5% (w/v) was exhibited by cellulase (*Trichoderma*). The percent hydrolysis lowered with increase in substrate concentration suggesting substrate saturation. Glucose, cellobiose and xylose peaks were detected by HPLC in the sugar hydrolysate obtained from enzyme saccharified pretreated hemp. Among all the enzyme cocktail combinations, the highest amount of reducing sugars (26%) was obtained from F2 combination. The occurrence of inhibitors during hydrolysis lowered the product release and an overnight washing of pretreated biomass increased the reducing sugars (35%) suggesting the inhibitors removal.

Chapter - 4

Characterisation of pretreated and enzyme hydrolysed biomass

4.1 Introduction

Each year extensive amounts of bio-residues in the form of holocellulose are generated from industries such as fibre and textile, paper and pulp, as well as timber processing (Brummer et al. 2014; Demirbas 2011). Cellulose based biomass is a potential feed source for the production of ethanol and value-added products which can reduce the amount of waste produced from these industrial sectors. The mass production of ethanol has been receiving increased attention due to the combination of increasing petroleum prices and global climate changes (Tirado et al. 2010). Many countries are now investing in advanced technology for ethanol production as well as improving the efficiency of existing technologies.

Pretreatment of biomass is essential to open up the structure and allow enzymatic hydrolysis (Galbe and Zacchi 2012). The degradation of the lignin component of the cell wall results in the exposure of cellulose and hemicellulose, facilitating enzymatic digestion. The digestibility of biomass increases with more favourable conditions, such as better cellulose accessibility, less inhibiting compounds and the use of efficient enzymes (Ju et al. 2013).

As a result of pretreatment various effects are observed on biomass structure, including highly porous structures, appearance of the biomass skeleton, increased crystallinity and the removal of amorphous content (lignin, pectin) (Zhang et al. 2014). Each pretreatment has a different effect on the lignocellulose structure, which varies among different biomass sources. After pretreatment the exposed cellulose structure is enzymatically digested to produce reducing sugars (Figure 4.1). This step completely disrupts the cellulose structure while amorphous biomass constituents, such as lignin, pectin and amorphous cellulose, remain undigested (Wang et al. 2013a). These compounds can subsequently be utilised by the lignin processing industry to produce value-added products.

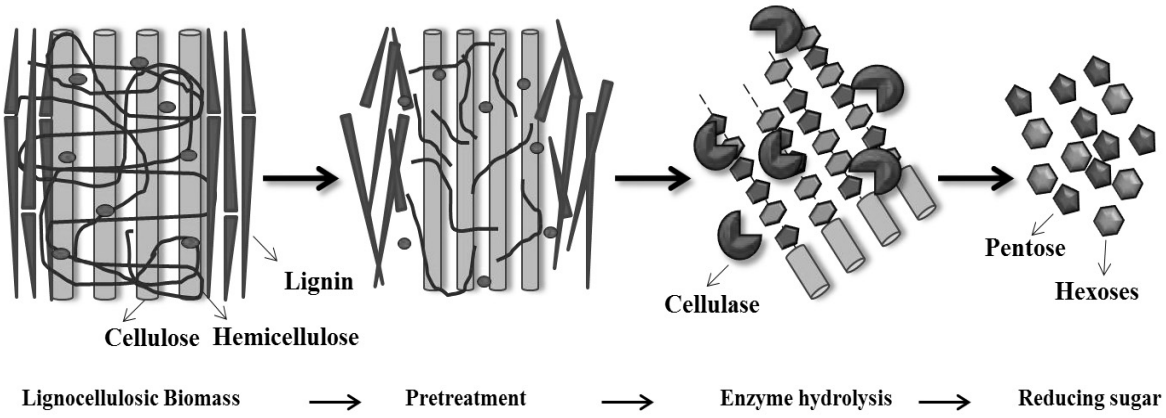


Figure 4.1 Effect of pretreatment and enzyme (cellulase) hydrolysis on biomass structure

Although hemp has been investigated as an energy source, methods of pretreatment and enzyme hydrolysis have not been well studied. Hemp is mainly grown for fibre production, but its applications have diversified in recent years, including in the field of energy production (Prade et al. 2012; Puri et al. 2012). The cultivation of hemp has intensified in many parts of world for uses including energy, despite of a limited knowledge base for the production of energy from this resource (Prade et al. 2011).

Numerous characterisation techniques are currently used to determine biomass structure, and provide information on chemical structure, appearance and composition of lignocellulose. These techniques enable the effects of pretreatment and biomass digestion to be monitored. Some techniques which are extensively used to characterise and study the structure of lignocelluloses are attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy (molecular/chemical structure), scanning electron microscopy (SEM), X-ray diffraction (XRD), thermogravimetric analysis (TGA), and nuclear magnetic resonance (NMR) (Xu et al. 2013).

SEM imaging enabled visualisation of the structure and evaluation of the changes to the raw biomass structure due pretreatment and enzyme hydrolysis. This imaging was used to evaluate particle size and facilitated in the design of further characterisation experiments. ATR-FTIR spectroscopy, TGA and NMR provided chemical composition information for biomass. After bioprocessing of biomass these analytical methods showed clear peak shifts, or reduction in peak size that provided information on structural changes due to pretreatment. NMR gave information on the carbohydrate composition of biomass and the α/β ratio of reducing sugars. XRD was used to distinguish between crystalline and amorphous material in raw and processed samples.

Lignocellulosic biomass/feedstock such as corn cob, eucalyptus, poplar, cedar, wheat and rice have been characterised to understand the morphology and effect of pretreatment on biomass structures from different sources (Cao et al. 2014; Gonzalez et al. 2012). New techniques such as near-infrared (NIR) spectroscopy have been introduced to characterise the composition of biomass used for biofuel production; specifically to determine moisture, ash and calorific content (Chadwick et al. 2014).

Researchers are also using Raman spectroscopy to analyse biomass composition and study pyrolytic behaviour (Keown et al. 2008).

There is a lack of information regarding the structural changes that hemp biomass undergoes under various pretreatment conditions and enzymatic hydrolysis. Our study assesses the effects of several pretreatment conditions and enzyme hydrolysis on biomass structure. SEM was employed to analyse biomass structure and the morphological changes that occurred in the presence of different pretreatment conditions and enzymes. Other techniques such as ATR-FTIR, ^{1}H NMR (proton nuclear magnetic resonance) and XRD were also used to investigate the structural and chemical modifications made to the biomass.

4.2 Materials and methods

4.2.1 Materials

The raw material used for the study was Ukrainian variety hemp (*Cannabis sativa)* milled and sieved. The dried biomass powder which constituted mainly xylem and pith of hemp was oven dried at 70 °C to obtain a consistent weight. This material was subsequently stored at room temperature. As described in chapter 2 the samples were pretreated with sodium hydroxide, sulphuric acid and hot water in an autoclave. Alkaline peroxide treatment was conducted in a water bath (90 °C) or through one stage treatment in an oil bath (140 °C). Enzymatic hydrolysis of these pretreated samples was carried out for 72 h using cellulase from *Trichoderma reesei* (EC 3.2.1.4; 700 units) (Sigma-Aldrich, St. Louis, MO, USA). The samples used for all the characterisation techniques were raw (control), pretreated samples and the pretreated biomass remaining after enzyme treatment in dried form. The results discussed are average of three consecutive experiments conducted at different times.

4.2.2 Scanning Electron Microscopy (SEM)

Morphology of the untreated, pretreated and enzymatically hydrolysed biomass was imaged using SEM. Sample preparation was carried out prior to the imaging step to

make the biomass electrically conductive. Samples were mounted on an aluminium stub with an adhesive double-sided carbon tape and air-dried to remove loosely attached sample particles. The stubs were hammered with specific letters for the identification of individual samples. Prior to the analysis, the samples were coated with gold using a sputter coater (BAL-TEC SCD 050, Leica Microsystems, Wetzlar, Germany) and kept in a vacuum sealed chamber to prevent the absorption of moisture. SEM was performed using a Zeiss Supra 55 VP (Carl Zeiss AG, Oberkochen, Germany) under high vacuum conditions with accelerating voltages of 7 kV and 5 kV, and a secondary electron (SE2) detector at different magnifications (10-100 μm).

4.2.3 Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy

ATR-FTIR spectroscopy was performed to analyse molecular information with respect to structural changes of the biomass after each treatment step. ATR-FTIR spectral data was acquired on an Alpha FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector and a single-reflection diamond ATR sampling module (Platinum ATR QuickSnap™) with 45° angle of incidence and a 2×2 mm^2 active sensing surface of the diamond crystal. A background spectrum was individually acquired on a clean surface of the diamond crystal prior to each sample measurement. Sample measurements were conducted by pressing hemp samples on the sensing surface using a metal clamp and high-quality spectra were collected for three individual samples in triplicate. The ATR-FTIR acquisition parameters used in OPUS 7.0 software suite (Bruker Optik GmbH, Germany) throughout the study included 4 cm^{-1} spectral resolution, 32 co-added scans, Blackman-Harris 3-Term apodization, Power-Spectrum phase correction and zero-filling factor of 2. The spectral range was observed within 4000-375 cm^{-1}. The averaged spectra of all the hemp samples were subsequently normalised for comparison purposes.

4.2.4 X-Ray Diffraction (XRD) and crystallinity analysis

The crystallinity of the samples was measured using Panalytical XRD (Panalytical XPert PRO MRD XL) at 30 kV and 40 mA. The spectrum consisted of an average of

three individual scans with intensity in the 2θ range from 5° to 30°. The crystallinity indices (CrI) of the samples were calculated using intensities of the amorphous and crystalline regions using the below formula.

$$\mathrm{CrI} = \frac{(I_{002} - I_{am})}{I_{002}} \times 100$$

I_{002} and I_{am} represent the intensities observed for the crystalline area and amorphous region at 2θ = 22° and 2θ = 15°, respectively (Segal et al. 1959).

4.2.5 Thermogravimetric Analysis (TGA)

The thermal behaviour of pretreated and enzyme hydrolysed hemp hurd biomass (HHB) samples was studied using 5 mg of biomass on a Netzsch DSC/TGA instrument (Model STA409PC) (NETZSCH- GmbH) with an elevating temperature ranging from 30-700 °C. The heating rate was set at 10 °C/min and the degradation conditions maintained under a nitrogen environment with a flow rate of 10 mL/min.

4.2.6 Nuclear Magnetic Resonance (NMR) spectroscopy

The carbohydrate composition of HHB was analysed by acid hydrolysis followed by ^{1}H-NMR spectroscopy. The acid hydrolysis of HHB was performed according to the previous study (Mittal et al. 2009). Dried HHB (0.4 g) was hydrolysed using 0.2 mL of 72% H_2SO_4 and allowed to digest at 40 °C for 1 h, with stirring at every 15 min. Thereafter, 5.4 mL of deuterium oxide (D_2O) was added and the hydrolysate was autoclaved. Once cooled 0.42 mL of 96% H_2SO_4 was added. Trimethylamine hydrochloride (TMA) was added to the acid hydrolysate as an internal standard before NMR analysis. ^{1}H NMR spectra were recorded with an AVANCE III 500 MHz NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 500 MHz in $CDCl_3$. The NMR spectrum was processed using Win NMR software (Bruker) to identify and integrate the anomeric hydrogen regions in the recorded NMR spectra.

4.3 Results and discussion

4.3.1 Effects of pretreatment and enzyme hydrolysis on biomass structure

Our investigation revealed that the HHB samples used in this study contained mainly holocellulose (77-78% of total biomass) and lignin (4-5%). The HHB has 85% total solids (including holocellulose, lignin traces, ash and other extractives) and 15% moisture (described in chapter 2). This biomass when pretreated with sodium hydroxide provided highest hydrolysis yield of reducing sugars in comparison to dilute acid and hot water treatments. The low lignin content combined with the pretreatment conditions resulted in satisfactory opening of the HHB structure and created highly porous structure.

The expansion of the structure should enable easy access to cellulose during enzyme hydrolysis. Previous studies conducted on different biomass sources have reported, that in comparison to other treatments, pretreatment with lime gives relatively high concentration of reducing sugars after enzyme hydrolysis (Chen et al. 2013b; Sasmal et al. 2012) and the reducing sugars produced can then be further utilised in fermentation for the production of ethanol (Zhou et al. 2013).

4.3.2 Scanning Electron Microscopy (SEM)

SEM was used to image untreated, pretreated and enzyme hydrolysed HHB, results are shown in Figure 4.2 and 4.3. As a result of pretreatment, the HHB samples were found to be swollen and the stacked bundles opened. The SEM of untreated/raw HHB shows a rough and compact surface (Figure 4.2). However, after different pretreatments, numerous morphological changes were observed that indicated considerable damage to the biomass. The roughness of the wood surface increased noticeably, with prominent ridges and groves demonstrating the explosion of the structure (Kapoor et al. 2001). The major changes observed in all of the pretreatments was cracking and flaking of the surface, indicating good separation of the compact structure and further facilitating enzymatic degradation. The surface flaked off readily in dilute acid, sodium hydroxide and one stage pretreated samples (Figure 4.2- b, d, and f). However, the hot water and alkaline peroxide pretreatments (Figure

4.2-c, e) resulted in the appearance of prominent holes at regular intervals (Li and Ren 2011). The size of the biomass greatly reduced in alkaline pretreated samples with extensive holes. Similar observations of sieve-like structures have been reported when corn stover was pretreated with lime, and is an indication of major lignin and hemicellulose degradation (Kumar et al. 2009b). The surface of hot water treated HHB was observed to be uneven and eroded, while sodium hydroxide pretreatment showed the degradation of lignified cell wall by showing up tracheids. These results indicate that the HHB compact structure expanded and swelled up, showing the effect of pretreatment on the structure. Similar delignification observations have been reported previously (Garcia et al. 1998). The compact tracheids bundle opened significantly with sodium hydroxide pretreatment, indicating the breakdown of cross-linked bonds, thereby increasing cellulose accessibility for enzymatic saccharification. However, on comparing the three pretreatments (i.e. sodium hydroxide, dilute acid and hot water) under the same conditions, the sodium hydroxide treatment impacted the biomass structure more than other two treatments. The loosening of the fiber bundles is evident in SEM images of alkaline treated biomass, when compared to untreated or other pretreatments. The most prominent changes that occurred with pretreatment were the expansion/opening of fibres, the appearance of flakes and consecutive holes on the surface were characterised after enzyme hydrolysed to compare the impact of each pretreatment condition on hydrolysis.

After 72 h of enzyme hydrolysis the structure of the untreated HHB became smoother and more porous. Amorphous flakes and polygonal lamellar structures in untreated HHB were similar to that found by (Bakisgan et al. 2009) with untreated wheat straw and olive bagasses. Earlier studies have reported that sodium hydroxide pretreatment increases the porosity of biomass as lignin moves away from the structure and similar observations were found in our studies (Ramsurn and Gupta 2012). By comparing SEM images of untreated and sodium hydroxide pretreated HHB, before and after enzyme hydrolysis, it is clear that opening the biomass through pretreatment has enhanced enzyme access to cellulose and facilitated biomass digestibility. Enzymatic hydrolysis of dilute acid pretreated (DAE) biomass impacted structural morphology. It resulted in the thinning of cell walls, which exposed both the transverse cross-

sections of the biomass and the tracheary cells responsible for the transport of water and minerals.

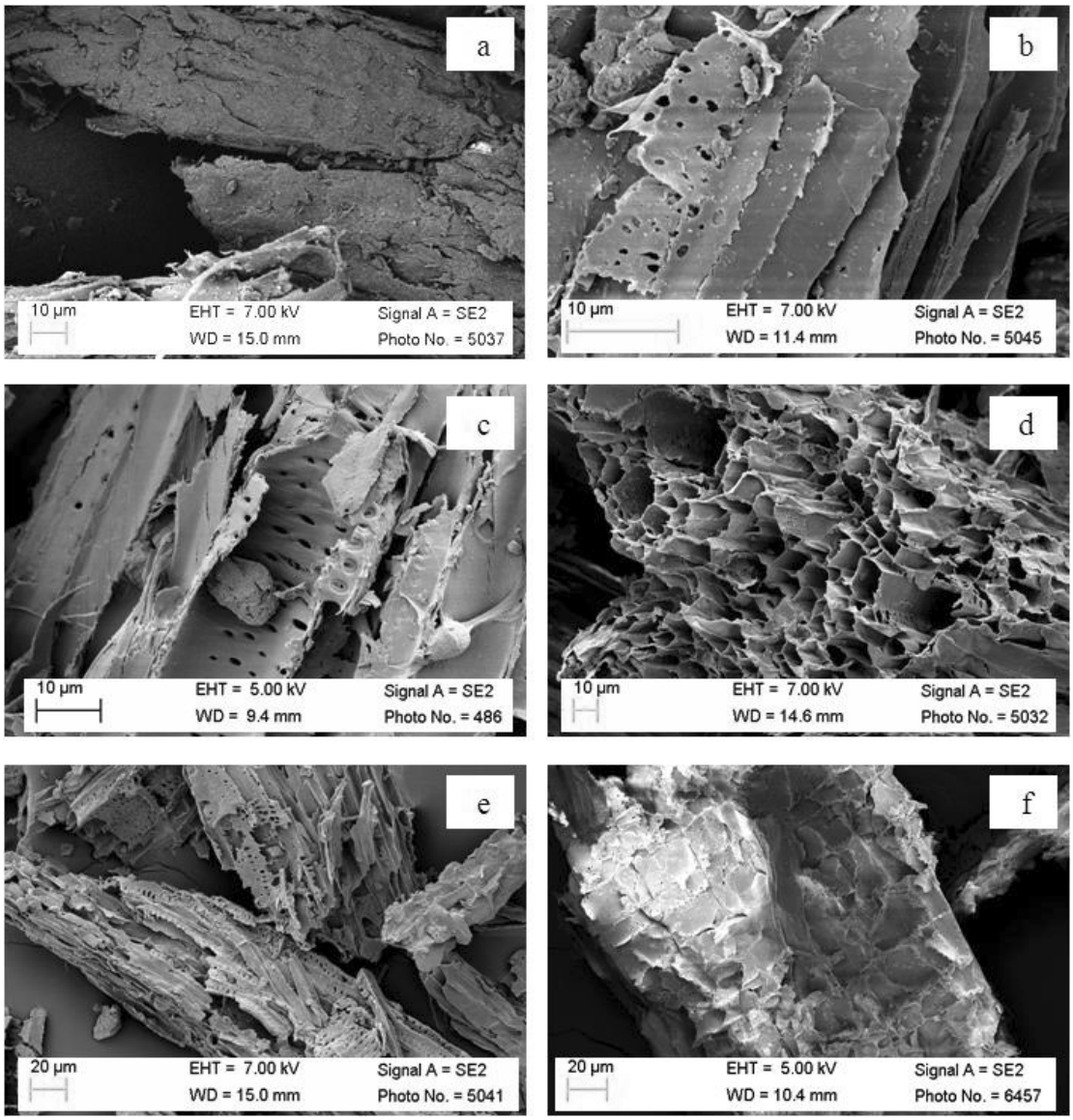


Figure 4.2 SEM images of hemp hurd biomass before and after pretreatment at accelerating voltage of 7 and 5kV and magnification ranging from 10-20 µm

Footnote: Untreated and five different pretreated HHB are presented from (a-f): (a) untreated; (b) dilute acid; (c) hot water treatment; (d) NaOH; (e) alkaline peroxide; and (f) one stage

The SEM micrographs of untreated and pretreated (sodium hydroxide, dilute acid and hot water) HHB after enzyme hydrolysis are shown in Figure 4.3. These samples
These pretreatments demonstrated that the biomass primarily contains pentagonal and hexagonal cross-sections, with four-sided cross-sections also visible; as also found by (Trtik et al. 2007). Similar cross sections were observed when hemp was treated with phosphoric acid at high concentrations (Moxley et al. 2008). Hemp biomass treated at low concentrations of sulphuric acid followed by enzymatic treatment, was able to produce similar or better morphological changes (Figure 4.3). This pretreatment caused extensive structure opening and hole formation and its enzymatic hydrolysis broke the intermolecular hydrogen bonds resulting in the release of amorphous hemicelluloses and pectin and exposed the vascular bundles and lumen. It also resulted in gaps at regular linear intervals due to the removal of lignin from the cross linked polymer matrix. Similar observations have been reported earlier (Karthika et al. 2012). Enzymatic hydrolysis of hot water treated (HWTE) hemp showed better morphological changes compared to sulphuric acid treatment (DAE) as the appearance of tracheary cells were more evident.

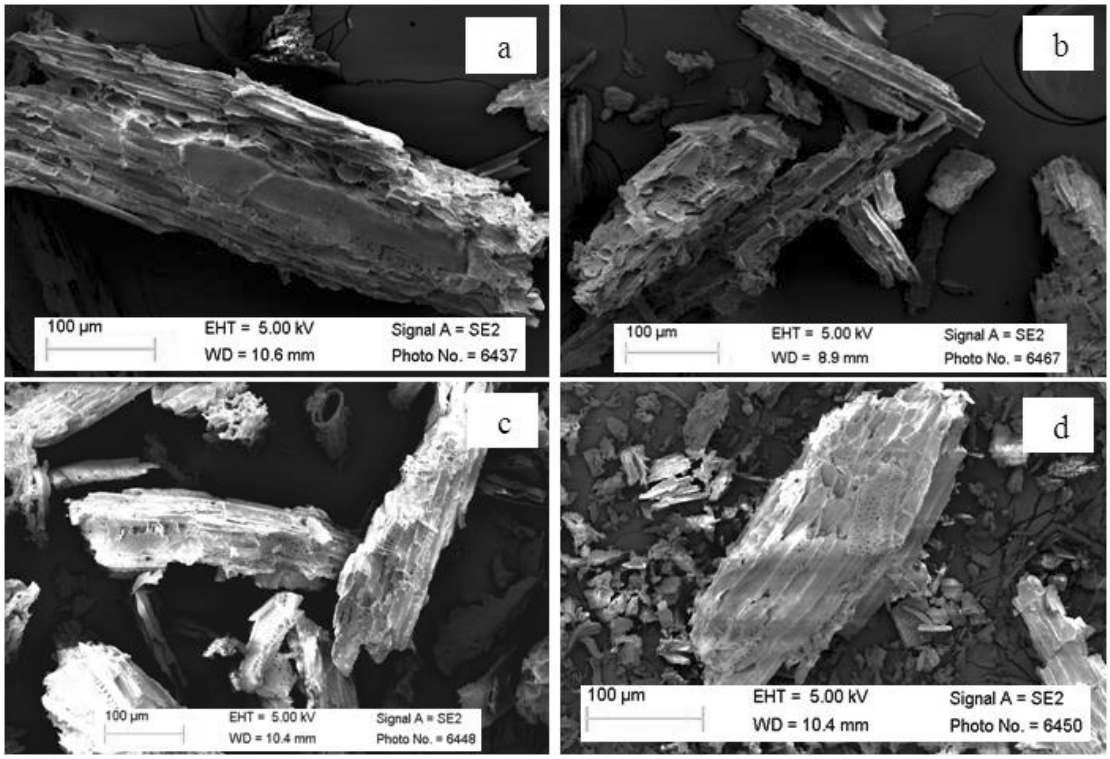


Figure 4.3 SEM images of HHB samples as a function of enzyme hydrolysis

Footnote: (a) enzyme hydrolysed untreated HHB; (b) enzyme hydrolysis of dilute acid pretreated HHB (DAE); (c) enzyme hydrolysis of hot water pretreated HHB (HWTE); and (d) enzyme hydrolysis of sodium hydroxide pretreated HHB (SHE)

4.3.3 Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy

The band assignments including specific vibrational modes of the major bands found in the spectra of HHB and their corresponding sources are given in Table 4.1. These assignments were based on those from a previous FTIR study on cellulosic fibres (Garside and Wyeth 2003). The FTIR spectra depicting the difference in peak intensity at approximately 1760 to 1030 cm^{-1} of HHB after different pretreatments are shown in Figure 4.4.

Table 4.1 FTIR band assignments for functional groups found in the spectra of HHB samples

Wavenumber values (cm^{-1})	**Band assignment[a]**	**Source**
3335	ν(O-H) of free H_2O molecules	Water/Moisture
3000-2790	ν(C-H) of methyl (CH_3) and methylene (CH_2) groups	General organic matters
1725	ν(C=O) of carbonyl and ester groups	Pectin and xylan (hemicellulose)
1590	$\nu_{in\text{-}plane}$(C=C) of aromatic ring	Lignin
1507	$\nu_{in\text{-}plane}$(C=C) of aromatic ring	Lignin
1456	δ(C-H) and δ(C-OH) of primary (1°) and secondary (2°) alcohol	Lignin, cellulose and hemicellulose
1420	$\delta_{scissor}$(CH_2)	Lignin, cellulose and hemicellulose
1370	δ(CH_2)	Lignin, cellulose and hemicellulose
1320	δ(CH_2)	Lignin, cellulose and hemicellulose
1240	γ(C-OH) of syringyl ring and ν(C-O) of acetyl group in xylan	Lignin and xylan (hemicellulose)
1160	ν_{as}(C-C) of ring breathing mode	Lignin
1050	ν(C-OH) of secondary (2°)	Cellulose and

	alcohol	hemicellulose
1032	ν(C-OH) of primary (1°) alcohol	Cellulose and hemicellulose
896	$\nu_{s,\ in\text{-}plane}$(C-O-C) of β-(1→4)-glycosidic linkages	Cellulose

[a] ν_{as} = asymmetric stretch; ν_s = symmetric stretch;
δ = in-plane deformation (bend); γ = out-of-plane deformation (bend)

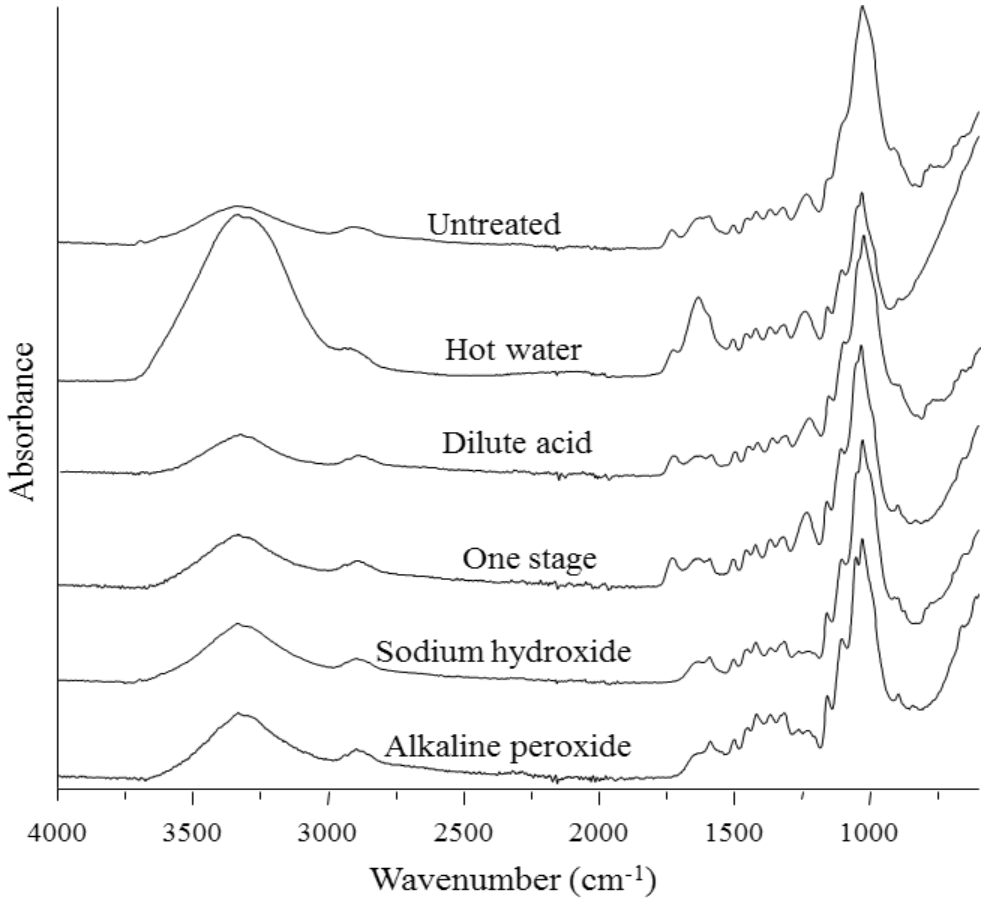


Figure 4.4 ATR-FTIR spectrum of untreated and pretreated hemp hurd biomass

A peak at approximately 1734 cm^{-1} indicates the presence of carboxylic esters from pectin and wax. This peak was observed in untreated, hot water, dilute acid and one stage pretreated biomass samples. However, it decreased in sodium hydroxide and alkaline peroxide pretreated samples, suggesting that considerable degradation of pectin and wax had occurred. In previous reports, a reduction in pectin and wax was observed at 1736 cm^{-1} when chelators and enzymes were used to separate hemp fibres (Li and Pickering 2008). The occurrence of a strong peak at 1030 cm^{-1} represents the C-C and C-O stretching at C-6. Earlier reports suggest that the region at about 1638 cm^{-1} represents water molecules and in hot water pretreatment the intensity of this peak was comparatively higher, indicating increased absorbance of water (Zhang et al. 2011b). The 1230 cm^{-1} peak in untreated, hot water treated, dilute acid and one stage pretreated biomass shows the presence of a guaiacyl ring. C-O stretches are indicative of lignin and xylan, but decrease significantly in sodium hydroxide and alkaline peroxide samples, indicating the partial removal of lignin from the HHB structure (Pandey and Pitman 2003). This indicates that delignification in was more apparent in alkaline pretreated HHB and the increased enzymatic saccharification reflects this. The alkaline pretreated HHB yielded the greatest amount of reducing sugars of all pretreatments. Another study reported that delignification of sugarcane bagasses using alkaline pretreatment resulted in hemicellulose removal by up to 90% (Rocha et al. 2012). The normalised ATR-FTIR spectra obtained for the pretreated HHB samples after enzyme hydrolysis (i.e. ET, HWTE, DAE and SHE) are shown in Figure 4.5 (a) and compared to that of the control untreated HHB sample (UT). The spectral deconvolution of SHE sample is shown in Figure 4.5 (b).

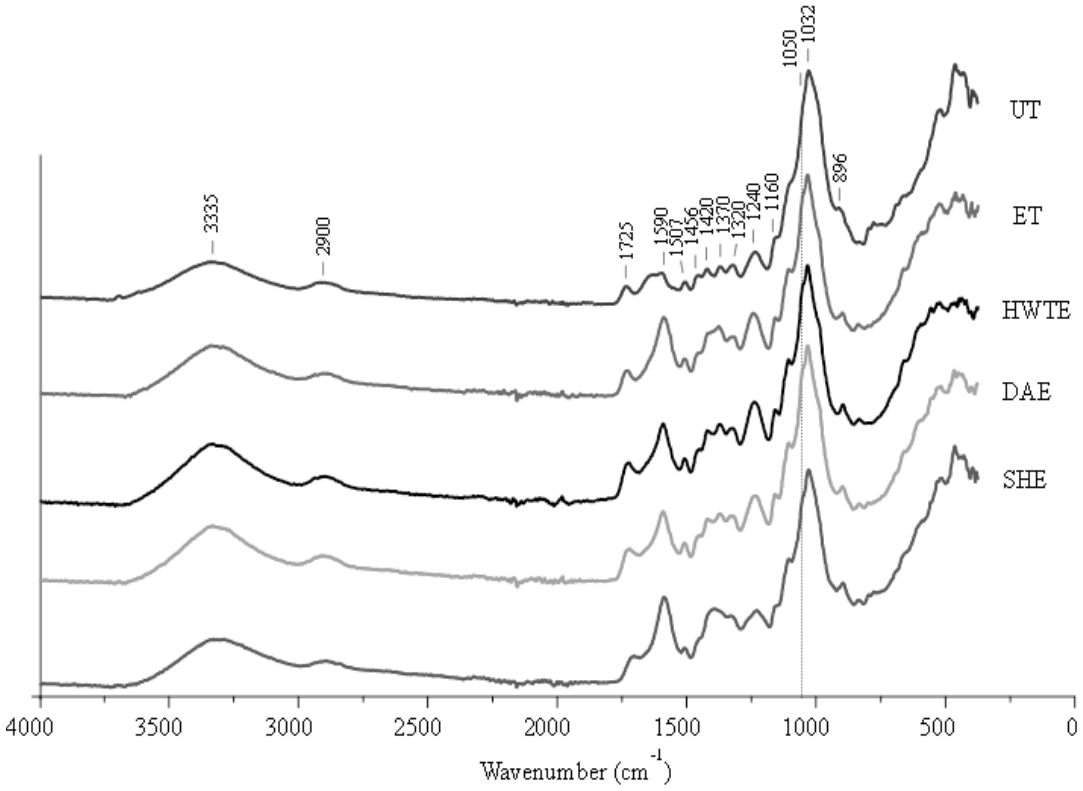


Figure 4.5 Normalised ATR-FTIR spectra of HHB samples:

(a) The spectra displayed from *top* to *bottom* represented untreated (UT - brown), untreated & enzyme hydrolysed (ET - green), hot water pretreated & enzyme hydrolysed (HWTE - black), dilute acid pretreated & enzyme hydrolysed (DAE - orange), and sodium hydroxide pretreated & enzyme hydrolysed (SHE - purple)

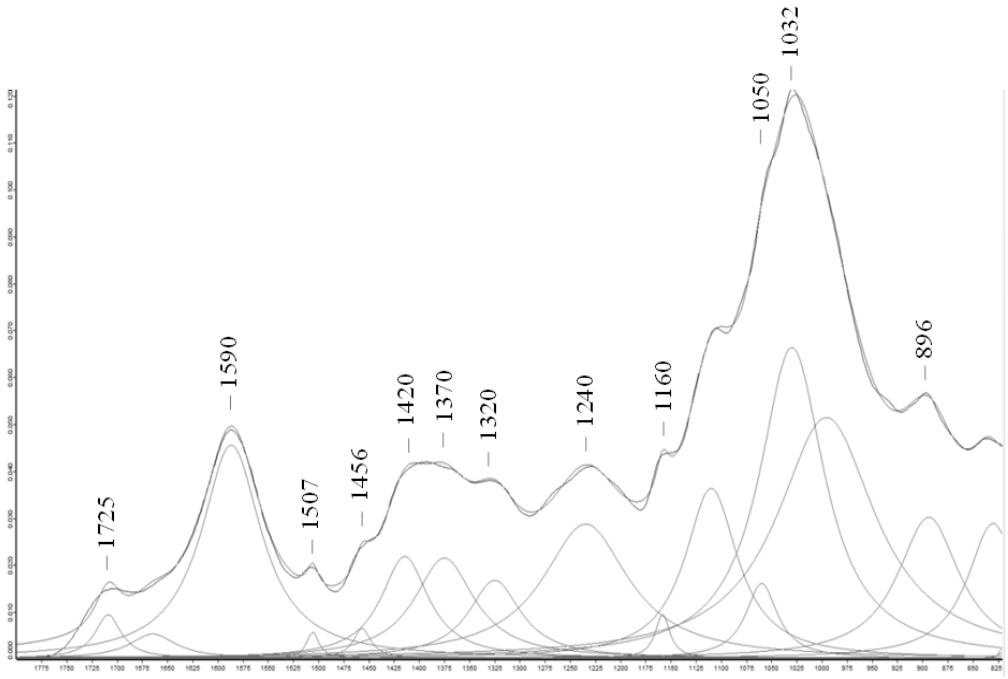


Figure 4.5 Normalised ATR-FTIR spectra of HHB samples:

(b) The example of spectral deconvolution obtained from the ATR-FTIR spectrum of the SHE sample (blue: the original experimental spectrum, red: the fitted spectrum, green: deconvoluted individual peaks)

Changes in the spectra were observed after enzyme hydrolysis of HHB samples, suggesting molecular changes in the polymer matrices of the hemp. This is in agreement with the structural changes previously observed using SEM (Section 4.3.2). In particular, the peak at 1725 cm^{-1} in all the HHB samples is attributed to ν(C=O) stretching modes of carbonyl and ester groups. As a result of sodium hydroxide pretreatment and subsequent enzyme hydrolysis, this peak appeared to shift to a lower wavenumber at 1710 cm^{-1}. This shift indicates an altered interaction of the carbonyl groups in the polymer matrices, which was previously identified as a C=O-O-C=O bonding structure (Mutje et al. 2007). The bands centred at 1590 and 1507 cm^{-1} can be assigned to the ν(C=C) in-plane stretching vibrational modes of aromatic rings in the lignin structure (Garside and Wyeth 2003). While the band intensities at 1507 cm^{-1} (which is often used as reference for lignin) are similar in all the HHB samples, the broad band at 1590 cm^{-1} is significantly stronger in the

pretreated HHB samples after the enzyme hydrolysis. The values obtained for untreated samples appear to be similar to those previously found in decayed wood (Pandey and Pitman 2003).

The overlapping features in the 1480-1290 cm^{-1} spectral region consist of a number of bands (1456, 1420, 1370 and 1320 cm^{-1}), which are primarily due to various deformation modes of C-H bonds in the methylene groups (Oliveira et al. 2014). In enzyme hydrolysed hot water (HWTE) and dilute acid (DAE) HHB samples, these peaks were similar to those in the untreated (UT) sample. The relative intensities of these bands were significantly altered in the spectra of the untreated and sodium hydroxide pretreated HHB samples after enzyme hydrolysis (i.e. ET and SHE). Bands presented in the 1500-850 cm^{-1} range were reportedly used to examine the polymorph of crystalline cellulose with the peaks at 1430 and 1420 cm^{-1}, corresponding to type I cellulose and the bonds of amorphous and type II cellulose, respectively (Nelson and O'Connor 1964). In our case, the ATR-FTIR spectra of every HHB sample revealed only the presence of the 1420 cm^{-1} band, indicating that amorphous and type II cellulose were the dominant structures in the HHB samples used in this study. In addition, the band present at 1240 cm^{-1} attributed to γ(C-OH) out-of-plane deformation of the syringyl ring in lignin and the C-O stretching modes of acetyl groups in xylan (Garside and Wyeth 2003; Sgriccia et al. 2008). The intensity of this band was found to be lower in the SHE sample compared to those in the spectra of ET and other treated samples. This indicates that the combination of sodium hydroxide pretreatment and enzyme hydrolysis was successful in removing lignin and subsequently hydrolysed cellulose into sugars.

In the low-wavenumber region, the weak shoulder band at about 897 cm^{-1} corresponds to ν(C-O-C) in-plane stretches of the β-(1-4)-glycosidic linkages that are found in both crystalline and amorphous cellulose structures (Kuo and Lee 2009). In the untreated sample, a weak shoulder band was originally observed at about 910 cm^{-1}. However, after enzyme hydrolysis this band shifted to 897 cm^{-1} and became more prominent, suggesting that hydrolysis may have led to the transformation of cellulose I networks into amorphous and cellulose II structures. This explains the reduced crystallinity of the cellulose moiety in the final products.

Spectral deconvolution was applied to the ATR-FTIR spectra after baseline correction and normalisation to better understand changes to molecular structure of HHB samples after pretreatment and enzyme hydrolysis. Figure 4.5 (b) shows the spectral deconvolution that was performed on the ATR-FTIR spectrum of the sample that was sodium hydroxide pretreated and hydrolysed (SHE) sample. This figure shows the original (experimental) spectrum compared to the combination of deconvoluted individual peaks that make up the final fitted spectrum. The band area ratios of the peaks representing the major components in the hemp samples were calculated with respect to that of the fitted peak at 1507 cm^{-1} and used as an internal standard.

The main reason for using the 1507 cm^{-1} band as an internal standard is that the band by nature is well resolved and often used as a reference for semi-quantitative purposes (Pandey and Pitman 2003). Since lignin, cellulose and hemicellulose are the major components of the hemp samples, the peaks used in the analysis of band area ratios focused on those representing these three constituents (Table 4.2). The highest band area ratios observed for the aromatic ring and methylene vibrations of lignin was at 1590/1160 cm^{-1} and 1420 cm^{-1}, respectively. These peaks were present in enzyme hydrolysed HHB samples pretreated with sodium hydroxide (SHE). This is consistent with maximum hydrolysis occurred in the sodium hydroxide treated sample resulting in a higher ratio of indigestible lignin remained in the end product. The band area ratio observed for C-OH groups at 1050 cm^{-1}, which is associated with cellulose and hemicellulose moieties, was found to be significantly higher in enzyme treated (ET) samples without any pretreatment. This indicates that high amounts of cellulose and hemicellulose still remained in the end product due to limited access of the enzyme to the hydrolysable materials. Our findings are consistent with previous studies indicating that the presence of lignin reduced biomass digestibility by binding to cellulose and thus inhibiting hydrolysis (Kumar et al. 2013).

Table 4.2 FTIR band area ratios observed for HHB samples after pretreatment and enzyme hydrolysis

Bands (cm^{-1})	Untreated (control)	Enzyme treated (ET)	Sodium Hydroxide (SHE)	Dilute acid (DAE)	Steam Explosion (SEE)
Lignin					
1590 (aromatic ring)	4.56	29.68	36.79	9.42	10.29
1420 (CH_2 and OCH_3)	2.85	8.55	9.44	4.04	4.60
1160 (aromatic ring)	1.62	2.64	5.71	3.24	1.58
Cellulose and hemicellulose					
1050	11.54	19.36	8.82	3.40	3.96
RSM error	0.00080	0.00084	0.00097	0.00081	0.00077

4.3.4 X-Ray Diffraction (XRD) studies

In the present work, XRD was used to investigate changes occurring in the crystallinity of HHB due to different pretreatment conditions and enzyme hydrolysis. The degree of hydrolysis of the pretreated HHB during enzyme saccharification can be measured using an X-ray diffractogram. This has previously been reported that this technique depends on the amount of crystalline cellulose exposed on the surface and can be used to monitor changes in cellulose crystallinity due to pretreatment (Liu et al. 2009). The XRD analyses of all pretreated and enzyme hydrolysed HHB samples are shown in Figure 4.6. The results suggest that the crystal polymorph I of cellulose was present in both treated and untreated samples. The band observed at the diffraction angle of approximately 15° corresponds to the (101) amorphous structure in lignin and hemicelluloses, whilst the crystallographic plane exhibited a broad

feature at approximately 22° corresponding to (002) cellulose polymorphs (Liao et al. 2011).

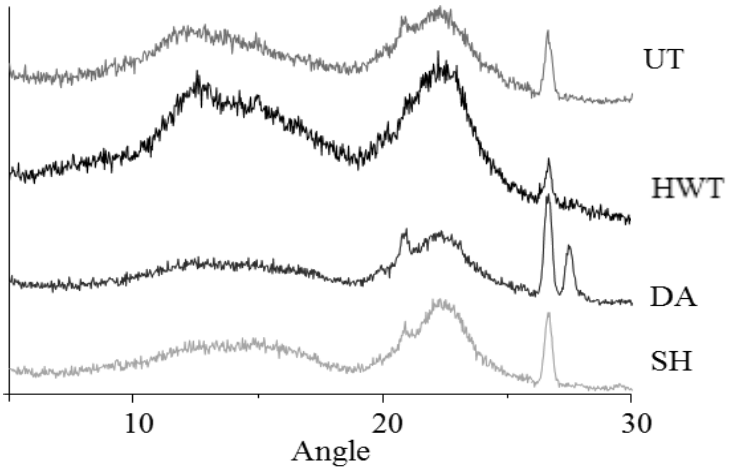


Figure 4.6 XRD spectra of HHB samples after pretreatment and enzyme hydrolysis:

(a) Comparisons of untreated HHB with pretreated samples: untreated (UT - green) and hot water pretreated (HWT - black), dilute acid (DA - blue), sodium hydroxide (SH - orange) hemp;

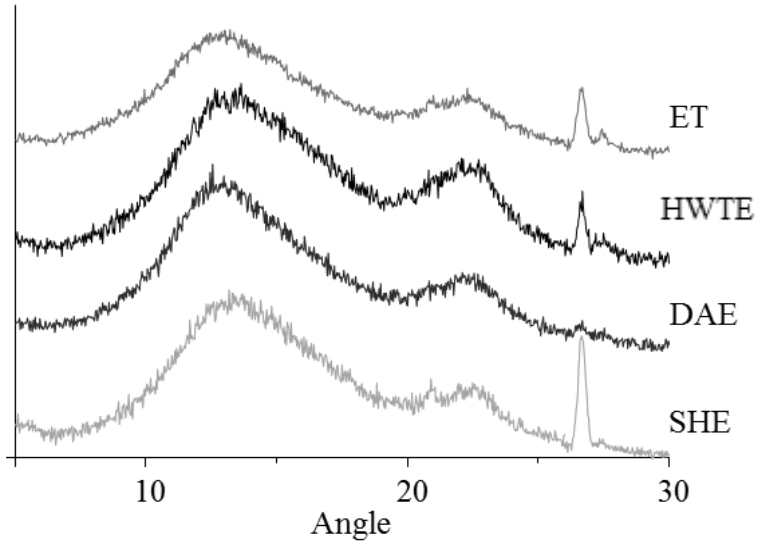


Figure 4.6 XRD spectra of HHB samples after pretreatment and enzyme hydrolysis:

(b) Comparison of pretreated samples after enzyme hydrolysis: enzyme hydrolysed HHB samples of untreated (ET - green) and hot water pretreated (HWTE - black), dilute acid (DAE - blue), sodium hydroxide (SHE - orange)

The calculated crystallinity index (CrI) values are shown in Table 4.3. When compared to untreated HHB, the degree of crystallinity (CrI) increased in all the samples after pretreatment. This indicates an improved crystalline surface area in pretreated samples resulting from the removal of lignin and hemicellulose. During pretreatment water penetrates into the para-crystalline and amorphous cellulose, and when the para-crystalline cellulose recrystallises the water molecules are released due to the high temperature conditions (Biswas et al. 2011). A previously performed crystallinity study on rice straws also showed the presence of higher amounts of cellulose as a result of the pretreatment (Yao et al. 2011).

The XRD results of pretreated HHB samples after enzyme hydrolysis support the ATR-FTIR results. The strong ATR-FTIR peak at 897 cm^{-1} in enzyme hydrolysed samples indicated the presence of amorphous cellulose, resulting from the transformation of crystalline cellulose I. Similarly, the XRD investigation showed the conversion of crystalline cellulose into simple sugars after enzyme hydrolysis by the increased presence of amorphous cellulose, lignin and hemicellulose in the sample. After hydrolysis the crystalline peak intensity at approximately at 22° was lower and the amorphous peak intensity increased.

Table 4.3 Crystallinity index of untreated & pretreated hemp samples

Samples	**CrI (%)**
Untreated	10.35
Steam Exploded	13.48
Diluted acid	29.79
Sodium hydroxide	36.71

4.3.5 Thermogravimetric Analysis (TGA)

During the thermal degradation process wood undergoes various stages of pyrolysis in which the major components of biomass (cellulose, hemicelluloses and lignin) undergo a complex decomposition and form ash (Jiang et al. 2013a). The three-stage oxidative pyrolysis of the HHB samples (untreated, pretreated and enzyme hydrolysed) in the presence of nitrogen at a heating rate of 10 °C min^{-1} is shown in Figure 4.7. The pyrolysis of all HHB samples produced a TG and DTG curves with three degradation phases. The first stage of degradation corresponds to devolatilisation where components such as inorganic compounds, carbon dioxide and water are removed in the temperature range of 50-150 °C (Popescu et al. 2010). With the increase of the temperature, a well distinguished peak was observed in this region at around 80 °C, as shown in Figure 4.7 (a-h). Weight loss for all HHB samples was low indicating that the samples have low moisture content.

Degradation progressed with the gradual increase in temperature. The active pyrolysis or second stage of degradation occurs between 150 and 400 °C. Due to the pyrolytic degradation of hemicelluloses and pectin a small peak/shoulder has appeared between 150 and 300 °C in all samples except the sodium hydroxide pretreated samples, and the occurrence of similar hemicellulose and pectin degradation peaks have been reported in literature (Carrier et al. 2011; Damartzis et al. 2011). The absence of hemicellulose/pectin peaks in the sodium hydroxide pretreated sample indicates the removal of these components during pretreatment. Furthermore, appearance of a slight shouldering in the untreated samples at around 300 °C indicates the presence of hemicellulose/pectin. As the structure of untreated HHB was compact, the hemicellulose and cellulose remained as holocellulose resulting in the merge of peak. The holocellulose degraded at higher temperature, unlike other samples. The curve indicating the mass loss of the biomass during the pyrolysis is shown in Figure 4.8.

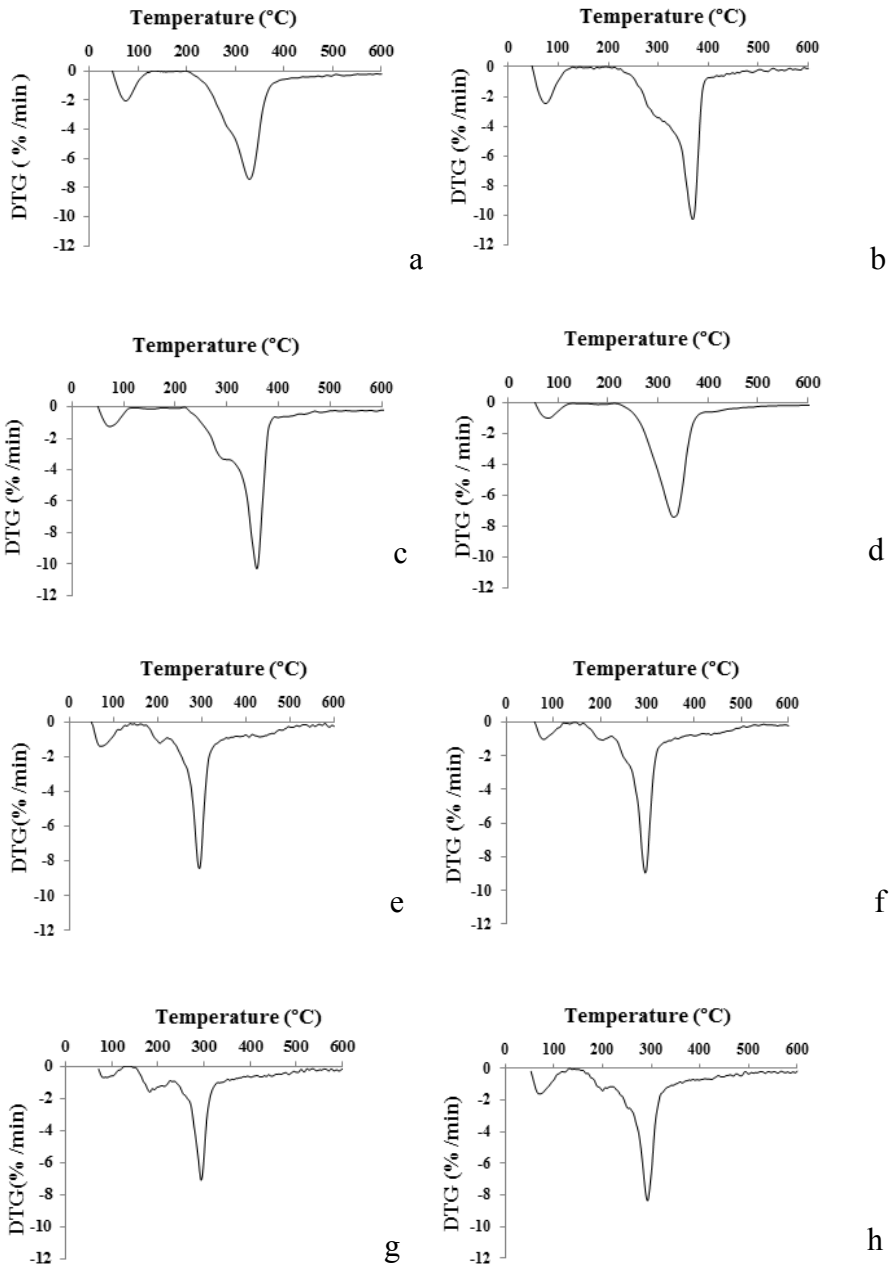
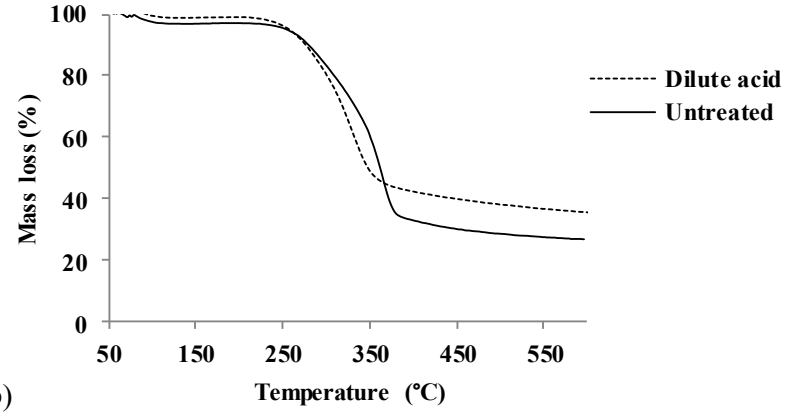


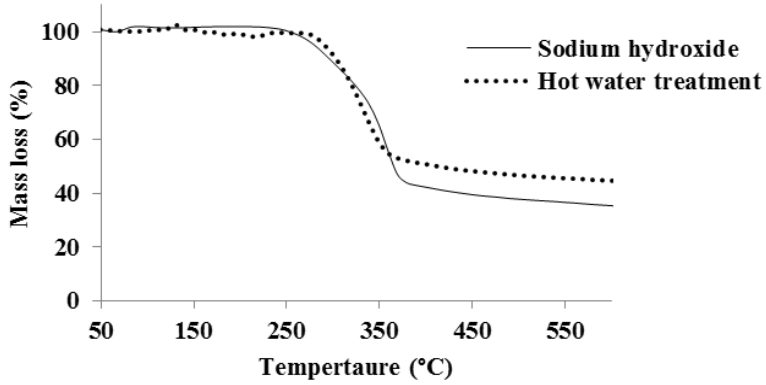
Figure 4.7 DTG pyrolysis profiles of HHB samples after pretreatment and enzyme hydrolysis

(Footnote: (a) untreated, (b) hot water pretreated, (c) dilute acid pretreated, (d) sodium hydroxide pretreated, (e) untreated and enzyme hydrolysed, (f) hot water pretreated and enzyme hydrolysed, (g) sodium hydroxide pretreated and enzyme hydrolysed, and (h) dilute acid pretreated and enzyme hydrolysed)

a



b)



c)

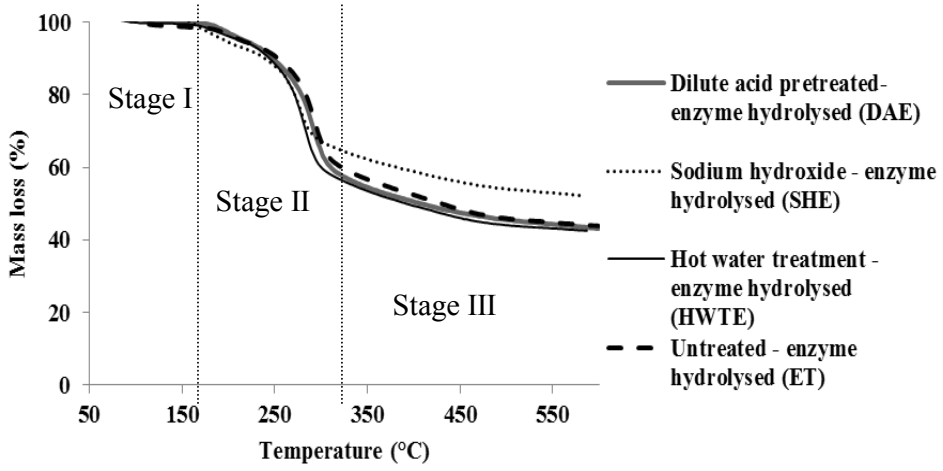


Figure 4.8 TGA curves indicating the mass loss with increasing temperature

Footnote: (a) untreated and dilute acid pretreated samples, (b) sodium hydroxide and hot water pretreated samples, and (c) all enzyme hydrolysed samples

The thermal degradation of biomass was maximal from 300 to 400 °C. The peak in this region corresponds to the decomposition of primary hemicelluloses and cellulose (Damartzis et al. 2011). All the pretreated samples showed a major loss of mass between 350 and 400 °C, indicating that cellulose was the primary biomass constituent.

The thermal degradation of the pretreated samples after enzyme hydrolysis showed peak shifts in this region. In particular, the pyrolytic degradation temperatures assigned for hemicelluloses, pectin and cellulose shifted from 300-400 °C to 250-300 °C. The loosening of cellulose and hemicellulose created higher porosity in the structure, resulting in ready penetration of enzyme for cellulose hydrolysis, and subsequently left amorphous material as hydrolysis residue. Above 400 °C the third stage of pyrolysis began where reaction achieved passive pyrolysis. It led the degradation of lignin traces, volatile components and the formation of ashes. No substantial mass loss was observed in this temperature range as the majority of the hemp biomass had already been degraded. Table 4.4 represents the onset and peak degradation temperature of the HHB after pretreatment and enzyme hydrolysis.

Table 4.4 The onset and peak degradation temperature of pretreated and enzyme hydrolysed samples

Samples	Onset degradation temperature (T_o)	Peak degradation temperature (T_{max})
Pretreated		
Untreated	220	326
Dilute acid	220	359
Hot water treatment	235	367
Sodium hydroxide	220	329
Enzyme hydrolysed (Pretreated samples)		
Untreated	168	295
Dilute acid	141	293
Hot water treatment	160	292
Sodium hydroxide	140	290

4.3.6 Nuclear Magnetic Resonance (NMR)

^{1}H NMR was used to determine carbohydrate composition of acid hydrolysed HHB. The ^{1}H NMR spectrum obtained for the hydrolysate is shown in Figure 4.9. The carbohydrate composition was determined from this spectrum and is presented in Table 4.5. The composition of HHB was partially determined by integration of anomeric hydrogen peaks in the ^{1}H NMR spectrum. The TMA peak (2.8 ppm) was used as an internal standard. The peak observed at 2.0 ppm represents free and bound acetyl groups. The monomeric sugars such as glucose and xylose resulted in a α- and β-anomeric C_1 proton. The doublet peaks at 4.5 and 4.6 ppm are attributed to β-xylose and the anomeric proton of β-glucose, respectively. The doublet peaks at 5.15 and 5.19 ppm, represent α-xylose and α-glucose, respectively (Mittal et al. 2009). The amount of furfural in the hydrolysate was subsequently determined by integrating the anomeric hydrogen peaks between 6.7 to 9.5 ppm. Xylose is known to be converted to furfural during acid hydrolysis (Shin and Cho 2008).

The carbohydrate composition determined from ^{1}H NMR analysis indicated that about 50% of the HHB biomass was sugars, of which 36.4% was glucose (calculated by adding α and β-anomeric peaks) and 13.8% was xylose. By summation of all the integrated peaks, approximately 12.1% of xylose was converted into furfural. The remaining component of the hydrolysate was acetate (37.6%), with TMA (internal standard). This suggests that furfurals and acetates appeared in the bioprocessing of biomass acted as inhibitors during hydrolysis and inhibited product formation.

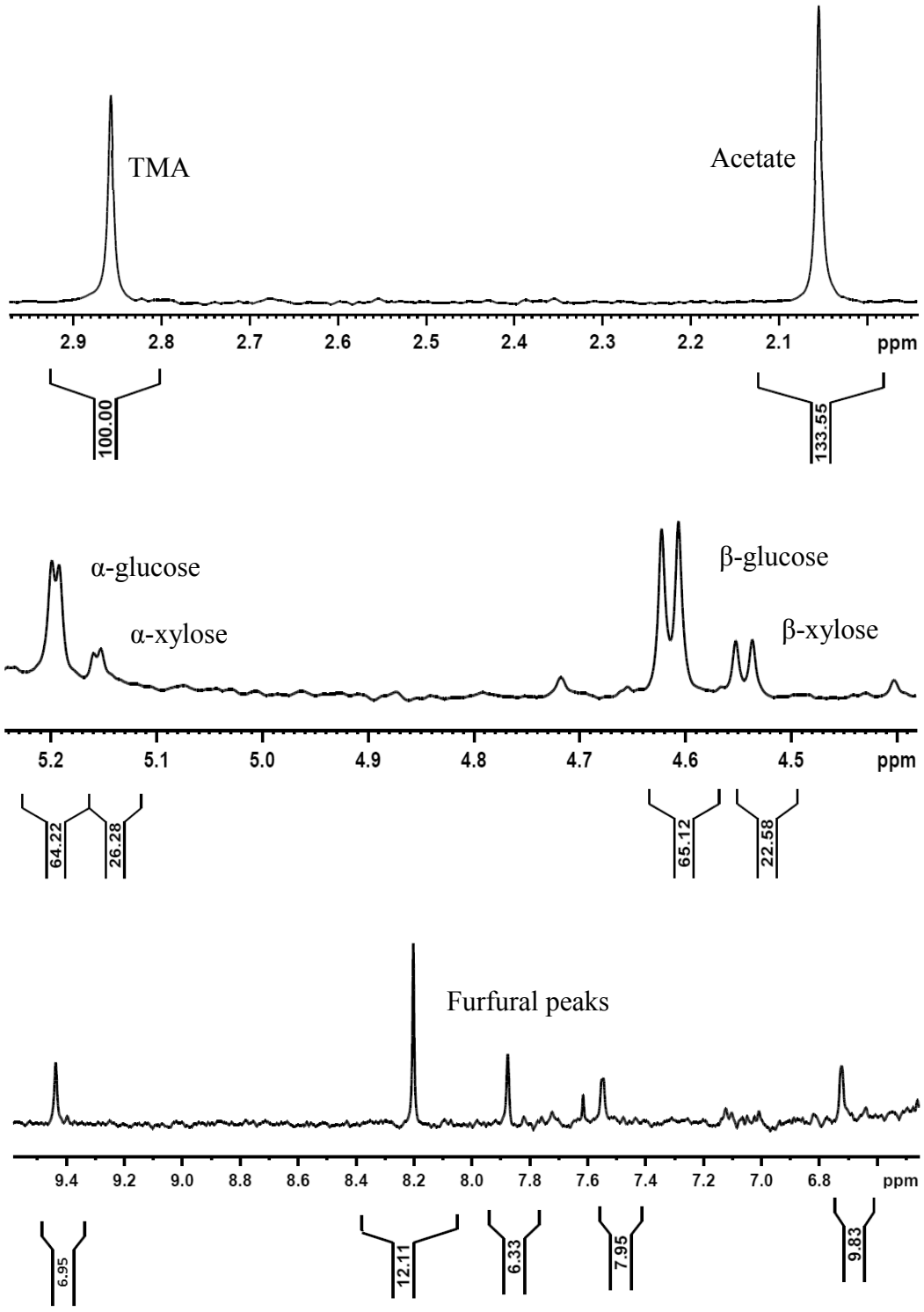


Figure 4.9 [1]HNMR spectra of HHB showing the composition peaks after acid hydrolysis

Table 4.5 Composition and location of sugar and non-sugar peak in the ^{1}HMR spectra of wood hydrolysate

Composition	Mass fraction (%)	Peak location (ppm)
Glucose	36.4	4.6/5.19
Xylose	13.8	4.5/5.15
Furfural	12.1	6.7/7.5/7.8/8.2/9.4
Acetate	37.6	2.0

4.4 Conclusion

The structural and morphological characterisation of HHB biomass was performed using SEM, XRD and ATR-FTIR. Modifications made to the HHB structure as a result of pretreatment and enzyme hydrolysis were monitored. It was found that the pretreatments decreased the amount of covalent cross-linking and expanded the pore volume in the biomass. This enabled the enzyme to penetrate into the rigid lignocellulose structure further leading to: (1) Characteristic structural and morphological changes to the surface of the biomass; and (2) reducing sugars produced through partial hydrolysis. Images of enzyme hydrolysed HHB captured by SEM, indicated that the enzyme could hydrolyse the biomass into small pieces. Sodium hydroxide pretreatment resulted in the highest production of reducing sugars after hydrolysis, indicating that this pretreatment method was best at opening the structure to enable enzyme access. This demonstrates the potential application of sodium hydroxide for hemp pretreatment. ATR-FTIR and XRD analyses demonstrated that sodium hydroxide pretreatment and enzyme hydrolysis converted crystalline cellulose into sugars, with hemicellulose, lignin and amorphous cellulose remaining intact. The thermal behaviour study of biomass determined that this biomass (HHB) contains mainly hemicellulose and cellulose with traces of lignin. Furthermore, ^{1}H NMR data supported the presence of holocellulose, through the presence of xylose and glucose peaks.

Chapter - 5

Immobilisation of cellulase on nanoparticles

5.1 Introduction

The highly complex lignocellulosic structure does not open completely under pretreatment processes and so requires a large amount of enzyme to hydrolyse biomass to produce fermentable sugars. The high enzyme cost is a major limitation for commercial biofuel production. To overcome this challenge either highly effective pretreatment or highly efficient and low cost enzyme systems are required (Liu et al. 2013). Enzyme immobilisation can enable multiple re-use of enzymes for industrial processes, thereby significantly lowering production costs.

Our previous work as discussed in chapter 2 focused on devising effective pretreatment of biomass and its structural characterisation. Another objective is to make the process more economical by using less enzyme, or multiply reusing enzymes for sugar production. This chapter describes methods tested for enzyme immobilisation on nanomaterials to provide a high loading of enzyme and enable enzyme reusability. Cellulase was immobilised on magnetic nanoparticles enabling easy removal and reusability of the immobilised enzyme.

Immobilisation is used to enhance the biocatalytic properties of an enzyme, in particular stability and reusability. Immobilised enzyme can be more readily separation from the reaction mixture and multiple reuses can lower the costs of enzyme processes dramatically (Kennedy et al. 1984; Puri et al. 2013; Verma et al. 2012; Verma et al. 2013a). Immobilised enzyme has been used for various industrial biotechnology applications such as the production of organic acids (Abdel-Rahman et al. 2013), antibiotics (Trabelsi et al. 2014) secondary metabolites (Dornenburg 2004), oligosaccharides (Aragon et al. 2013; Urrutia et al. 2013) and sweeteners (DiCosimo et al. 2013).The immobilised enzyme reaction system includes a biocatalyst binding to a carrier for use in hydrolysing substrate to form a product under optimised parameters of reaction time, temperature, pH, concentration of enzyme and substrate (Hanefeld et al. 2009). Immobilisation is achieved through methods including entrapment, cross-linking, encapsulation, ionic interaction, covalent attachment and non-covalent adsorption (Brady and Jordaan 2009). Some of the commonly used immobilisation techniques are shown in Figure 5.1.

Immobilisation is carried out to improve some of the characteristics of enzymes to make them more suitable as large scale industrial catalysts. Immobilisation can enhance enzyme biochemical properties such as catalytic activity, and stability, in addition to recovery and reusability (Garcia-Galan et al. 2011).

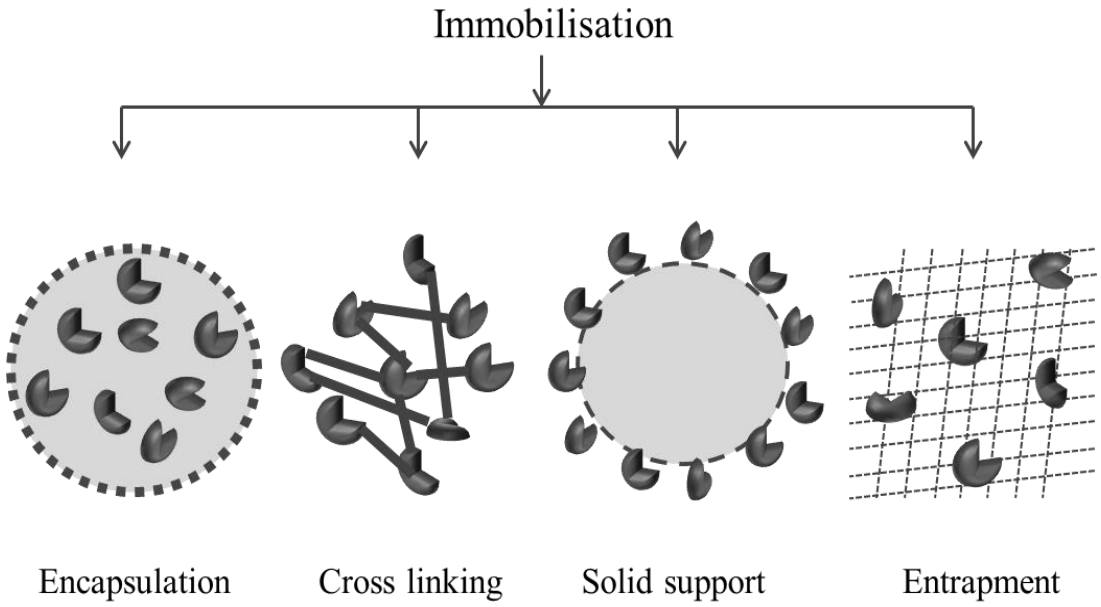


Figure 5.1 Different immobilisation techniques used to bind carrier and enzyme

(This figure has been modified from (Asgher et al. 2014; Sassolas et al. 2012).

Enzyme - (as shown in the figure)

The properties of immobilised enzyme preparations are governed by the properties of both the enzyme and the carrier material. The specific interactions provide an immobilized enzyme with distinct chemical, biochemical, mechanical and kinetic properties (Asgher et al. 2014). For successful immobilisation the reactive groups on the support should be able to effectively bind the enzyme without destroying activity (Mateo et al. 2007). There are various organic and in-organic supports such as functionalised silica, carbon, gold nanoparticle, and zinc oxide that are commercially available and used for enzyme immobilization. Some researchers prefer synthesising

their own matrix or using supports which are optimally compatible with the enzyme structure and properties (Bellino and Regazzoni 2011; Laurent et al. 2008; Sureshkumar and Lee 2011).

The binding of enzymes onto a nanosize magnetic particle provides ready separation of the enzyme from the reaction mixture. The system developed using these nanocarriers provides long term thermal and storage. A major advantage of the use of nanomaterials, as opposed to larger materials, is that they provide a larger surface area which can support high enzyme loading. Nanomaterials also have other potential advantages such as improved flow properties and robustness (Verma et al. 2013a). Previous reports indicate that magnetic nanoparticle supports have low toxicity and may be useful for applications in medical, textile and waste recycling processes (Miletic et al. 2012). In bioenergy production, the immobilisation of enzymes onto nanomaterials has the potential to improve the economic viability of the process (Puri et al. 2013). Activated nanosized supports such as iron oxide, chitosan, polystyrene, and activated gold nanoparticle provides high surface area and strong cross-linking through covalent bonds (Ansari and Husain 2012).

Enzymes such as β-glucosidase, which can be used for the hydrolysis of lignocellulosic biomass, have been immobilised on various supports such as porous solid silica and magnetic chitosan microspheres to improve their biochemical properties and stability. Studies have also been conducted to immobilise cellobiase on magnetic chitosan microspheres for hydrolysing pretreated straw (Das et al. 2011; Zheng et al. 2013). Three cysteine-tagged cellulases have been co-immobilised on AuNP for the hydrolytic degradation of cellulose (Cho et al. 2012). Cellulase immobilised on silica through the assistance of L-cysteine functionalised gold nano-particle was applied for the continuous hydrolysis of waste bamboo chopsticks powder to produce glucose (Cheng and Chang 2013). Recently, in our laboratory a model was developed where β-glucosidase immobilised on nanoparticle led to hydrolysis of a synthetic CM-Cellulose that has potential application in biofuel production (Verma et al. 2013b).

Occasionally, experimental investigations have produced unexpected results such as significant reduction or increase in activity compared with soluble enzymes (Cao

2005; Mateo et al. 2007; Nwagu et al. 2012). Utilising immobilised enzymes in establishing a biorefinery (a facility that integrates biomass conversion process into the production of fuel, power, co-products, chemicals) to hydrolyse pretreated lignocellulosic biomass is an important industry objective. Researchers have successfully hydrolysed synthetic substrate using immobilised enzyme and exhibited improved enzyme properties in short hydrolysis times (Bayramoglu et al. 2004). However, the use of immobilised enzyme to hydrolyse pretreated real biomass for the cost effective production of ethanol or sugars has not been fully investigated. (Maitan-Alfenas et al. 2014; Singh et al. 2013).

The present work focuses on the hydrolysis of microcrystalline cellulose (CMC) and natural cellulosic biomass obtained from hemp hurd (HHB) using an immobilised enzyme. Immobilisation of cellulase onto an activated magnetic nanoparticle was achieved using glutaraldehyde as a cross-linker. Being magnetic in nature a magnetic nanoparticle provides an advantage of easy separation of the immobilised enzyme from the reaction mixture. After thorough washing this separated immobilised mixture can be reused. The biochemical characterisation of the free and immobilised enzyme at different temperatures and substrate concentrations was investigated. The catalytic efficiency of immobilised cellulase was assessed based on its thermostability, reusability and storage.

5.2 Materials and Methods

5.2.1 Materials

5.2.1.1 Chemicals

The present study utilised recombinant cellulase (EC 3.2.1.4; 700 units) from *Trichoderma reesei* for immobilisation as discussed in chapters 2 and 3. Other chemicals used for the study were ferric chloride, zinc chloride, potassium hydrogen phthalate, sodium acetate, sodium citrate, potassium phosphate, trizma hydrochloride, and carboxymethyl cellulose (CMC) and these were procured from Sigma-Aldrich (St. Louis, MO, USA). Glutaraldehyde was procured from SAFC and was used as a

cross linker during nanomaterial immobilisation. The protein assay kit used for estimating protein concentrations (Bio-Rad protein dye reagent concentrate) was sourced from Bio-Rad.

5.2.1.2 Cellulosic biomass

The biomass used for the study was hemp hurd biomass (*Cannabis sativa*) - HHB, procured as an industrial residue as described in previous chapters. The same biomass which was milled using a Fritsch Pulverisette 19 Universal Cutting Mill was used for the study. The milled biomass was sieved using a mesh of pore size circa 300 μm.

5.2.2 Nanomaterial

The strong magnetic properties of magnetic nanoparticles assist in the efficient recovery of the immobilised enzyme. To increase the saturation magnetisation of nanoparticles, zinc was doped into magnetite for the present study. Magnetic nanoparticles were synthesised using a hydrothermal method. To achieve this, aqueous solutions of iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), iron(II) chloride tetrahydrate ($FeCl_2.4H_2O$) and zinc chloride ($ZnCl_2$) were mixed in a molar ratio of Fe^{3+} :Fe^{2+} : Zn^{2+}=2.0 : 0.6 : 0.4. An aqueous sodium hydroxide (NaOH) solution was subsequently added to neutralise the pH. The precipitates were subjected to hydrothermal treatment at 150 °C for 12 h, followed by repeated rinsing with deionised water and freeze-drying at -80 °C and 0.014 mbar for 24 h (Verma et al. 2013b).

The crystalline structure of the nanopowder was characterised using an X'Pert pro X-ray diffractometer (Pan-Analytical, Netherlands) with Cu K-alpha radiation (40 KV, 30 mA). The morphology of the synthesised particles was characterised by transmission electron microscopy (TEM) using a JEOL2100M microscope (JEOL, Japan) with electron beam energy of 200 kV. Magnetic hysteresis of the particles was measured using a semiconductor quantum interference device magnetometer (Quantum Design Inc., USA) at room temperature.

5.2.3 Immobilisation of cellulase on the activated magnetic nanoparticle

The magnetic nanoparticles were suspended in deionised water at a concentration of 5 mg mL^{-1} and sonicated for 1 h (Verma et al. 2013b). The sonicated nanoparticle was thoroughly washed and allowed to dry at room temperature.

Due to their magnetic property, washing, separation and recovery is quick and easy with minimal loss of nanoparticle. To attain good binding efficiency the activation of these magnetic nanoparticles was achieved in glutaraldehyde solution having a concentration of 1 M. This solution was incubated for 1 h with glutaraldehyde solution at 25 °C and 250 rpm in a shaker. After activation the magnetic support with enzyme was separated from the solution using a magnet. The immobilised mixture was washed twice with deionized water and once with sodium acetate buffer after separation to remove unreacted glutaraldehyde.

The activated nanoparticle was for immobilisation immediately after washing. A pictorial representation of nanoparticle activation and enzyme immobilisation is shown in Figure 5.2. The covalent binding of the enzyme to the nanoparticle was achieved by incubating the activated nanoparticle support with enzyme at a concentration of 5 mg mL^{-1} at 25 °C for 2 h in a shaker at 250 rpm. The supernatant obtained after separating the immobilised mixture from solution was used for protein estimation. The immobilised cellulase mixture on the nanoparticle support was thoroughly washed with deionised water and buffer to remove any loosely bound protein.

The binding efficiency of the enzyme was determined by calculating the ratio of total protein bound, as determined by the Bradford assay, to the total protein available for immobilization.

$$Binding\ efficiency\ (\%) = \frac{Total\ amount\ of\ protein\ binded}{Total\ amount\ of\ protein\ added} \times 100$$

$$Immobilisation\ efficiency = \frac{\alpha i}{\alpha f} \times 100$$

Where, αi is the total activity of the immobilized enzyme and αf is the total activity of the free enzyme (Verma et al. 2012).

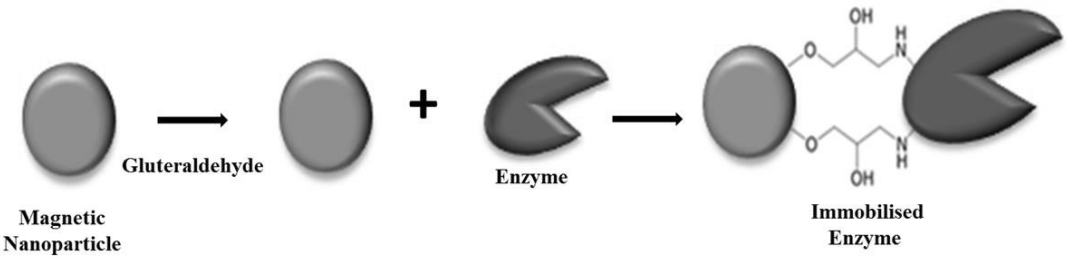


Figure 5.2 The activation of nanoparticle and binding of an enzyme

5.2.4 Enzyme assay

To determine the enzyme activity, enzyme assays for free and immobilised enzyme was carried out using CMC assays (Ghose 1987). The assay for free enzyme was conducted at 50 °C with a reaction mixture containing 0.5 mL enzyme (~20 CMC units) and 0.5 mL of 2% substrate (CMC) dissolved in 0.1 M sodium acetate buffer (pH 4.0) and incubated for 30 min. The reaction was stopped by adding 3 mL of DNS reagent and heating for 10 min in a vigorously boiling water bath. The reagent was prepared in the same manner as mentioned in earlier chapters. The concentrations of glucose released were measured at 540 nm. The estimation of reducing sugars produced during enzyme hydrolysis was carried out using the DNS method. The assay for immobilised enzyme was performed under the same conditions at 60 °C. One unit of enzyme activity is defined as 1 μmol of glucose liberated per minute of enzyme assay. All experiments were conducted in triplicates and reported as mean values ± standard deviation.

Protein estimation from the supernatant after immobilisation was performed using the Bradford method (Bradford 1976). The Bradford assay was done by adding 5 mL of Bradford commercial reagent in 10 μL of protein supernatant obtained from immobilisation mixture diluted in 90 μL of deionised water. The commercial protein dye reagent concentrate (1 mL) was diluted in 4 mL of deionised water before adding to the supernatant mixture. The mixture was thoroughly vortexed and incubated for 5 mins at room temperature. The optical density was measured at 600 nm to determine the concentration of eluted protein.

5.2.5 Characterisation of immobilised enzyme and biomass using Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) and Scanning Electron Microscopy (SEM)

The binding of cellulase onto magnetic nanoparticle supports was determined using ATR-FTIR (Bruker Optik GmbH- Germany). The detector was deuterated triglycinesulfate (DTGS) with a single-reflection diamond ATR sampling module (Platinum ATR QuickSnap™). The specifications of the instrument and software description have been described in the previous chapters. The scanning range was

from 2200 to 400 cm^{-1} with scanning resolution of 4 cm^{-1} and 64 scans per sample and the results were analysed using the OPUS 6.0 suite (Bruker) software.

The untreated and pretreated HHB were characterised by SEM (Zeiss Supra 55 VP-Germany). Sample preparation for SEM was conducted by mounting the sample over an aluminium stub and then sputtered with gold and allowing the sample to set under vacuum overnight. The imaging was done at an accelerating voltage of 7 kV using a secondary electron (SE2) detector.

5.2.6 Determination of enzyme kinetics

The kinetics study of the free and immobilised enzyme was conducted using different concentrations of CMC substrate (0.5% to 2.5%, w/v). The enzyme assays for the free and immobilised enzymes were performed at 50 °C and 60 °C, respectively, using 0.1 M sodium acetate buffer at pH 4.0 for 30 min in a shaking water bath. The glucose concentration was estimated by the DNS method as described earlier. Data analysis was done with graph pad prism 6 software using Michaelis-Menten kinetic derivation.

5.2.7 Thermostability and storage study

The thermal stability of free and immobilised enzymes was determined at selected temperatures (60 °C and 80 °C) in the absence of substrate. The enzyme assays for immobilised and free enzymes were performed at intervals of 2 h and 30 min, respectively. The immobilised enzyme was stored at 4 °C and its activity measured after an interval of 1, 5, 7 and 45 days. The activity was measured via the CMC assay.

5.2.8 Reusability of immobilised enzyme

The reusability of the immobilised enzyme was determined by enzyme assay conducted at 60 °C. The immobilised preparation was washed with deionised water followed by enzyme assay buffer. After performing each cycle of the assay, the immobilised mixture was separated using a magnet (Figure 5.3). The immobilised nanoparticles were resuspended in buffer and CMC substrate solution. The activity

obtained in the 1st cycle for the immobilised enzyme was taken as the control and represents 100% activity.

5.2.9 Hemp hurd pretreatment

The hemp hurds were pretreated at high temperature and pressure to remove lignin, ash and other residual components, and also to open the hurd structure to improve enzyme accessibility. Pretreatment was conducted as per the optimised study discussed in chapter 2. HHB was pretreated with sodium hydroxide at 0.5% w/v concentration with 2% of milled and dried HHB in deionised water at autoclave conditions. The pretreated biomass (10 g) was washed five times to remove alkaline traces and stored at 4 °C after drying for use in enzyme saccharification experiment (Abraham et al. 2013).

5.2.10 Enzyme saccharification of biomass

The pretreated biomass (HHB) and CMC were used for hydrolysis with free and immobilised enzymes. Hydrolysis was carried out for 48 h using 0.1 M sodium acetate buffer and a substrate blank at pH 4.0 for both cases. The optimised temperatures for carrying out enzyme hydrolysis for free and immobilized enzyme were found to be 50 °C and 60 °C, respectively. The samples were removed at 12 h interval and tested for reducing sugars by the DNS method. The schematic flow in Figure 5.3 represents the steps involved during cellulase immobilisation and substrate hydrolysis. The hydrolysis percentage of cellulose was calculated using the formula below (Correia et al. 2013).

$$\begin{aligned} &Cellulose\ digested\ (g) \\ &\quad = glucose\ concentration \times \upsilon\ (total\ reaction\ volume) \\ &\quad \times 0.9\ (correction\ factor) \end{aligned}$$

$$Cellulose\ hydrolysis\ (\%) = \frac{Amount\ of\ cellulose\ digested}{Amount\ of\ cellulose\ added} \times 100$$

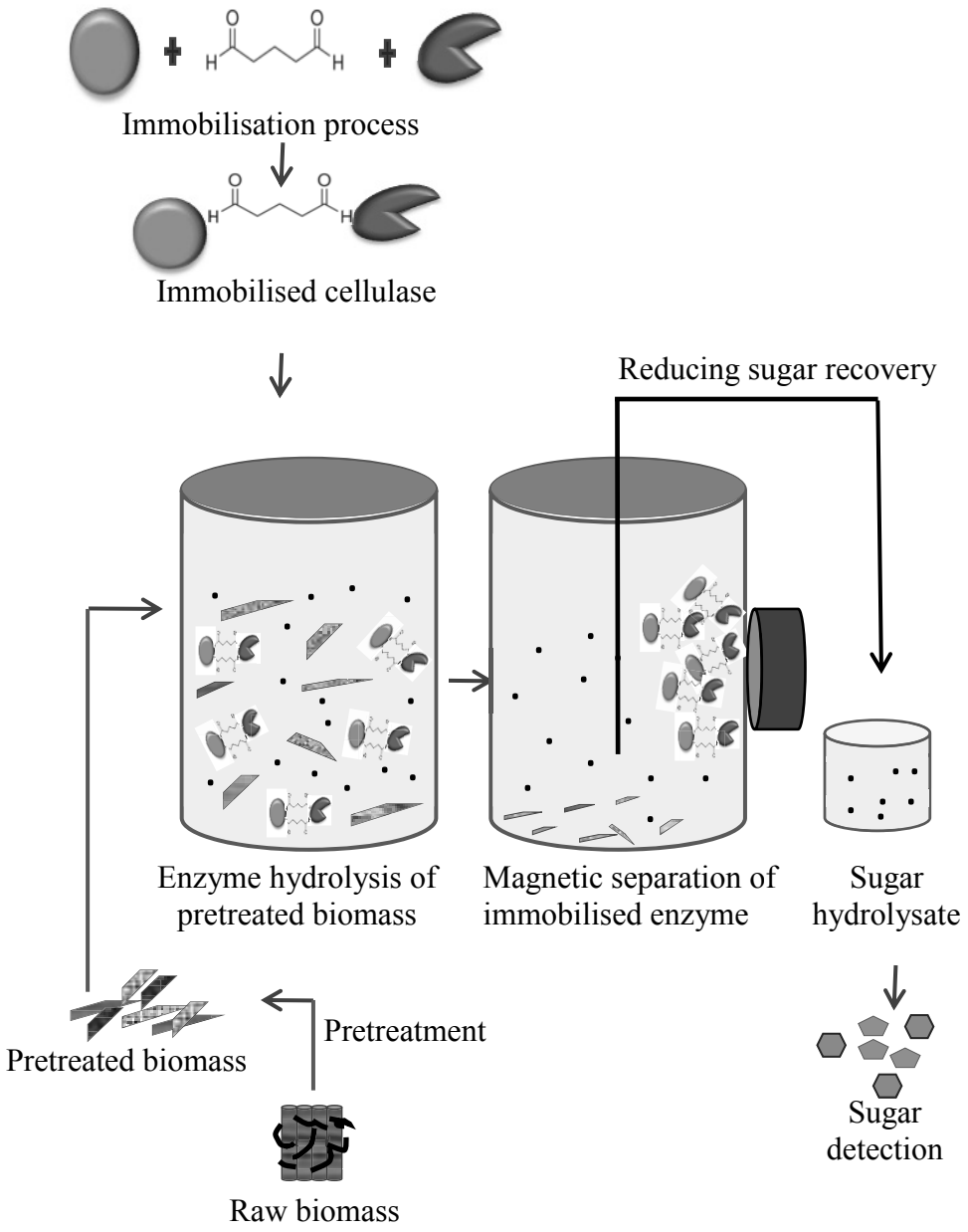


Figure 5.3 Schematic flow of biorefinery to produce reducing sugars using immobilised enzyme

5.3 Results and discussion

5.3.1 Characterisation of magnetic nanoparticles

XRD results showed that the magnetic nanoparticles consisted of a mixture of hematite (Fe_2O_3) and ferrite ($Zn_{0.4}Fe_{2.6}O_4$). Hematite is a very weak magnetic material. Nevertheless, the saturation magnetisation value was 109 emu/g at 50 kOe, which is considerably higher than undoped Fe_3O_4 (typically <60 emu/g) despite the mixed crystal phases. The magnetic hysteresis loop of nanoparticles showed that the coercivity is zero, indicative of their superparamagnetic nature. This ensured a stable dispersion of nanoparticles in the absence of an external magnetic field so as to sustain a high surface area for efficient enzymatic reactions to occur. TEM studies (Figure 5.4) indicated that the nanoparticles were near-spherical in shape with diameter of about 40 nm (Verma et al. 2013b). As a result of immobilisation the overall size of the nanoparticle was found to be bigger and spherical. Moreover, a dense agglomeration of nanoparticle was observed after immobilisation suggesting a successful binding of enzyme on an activated nanoparticle.

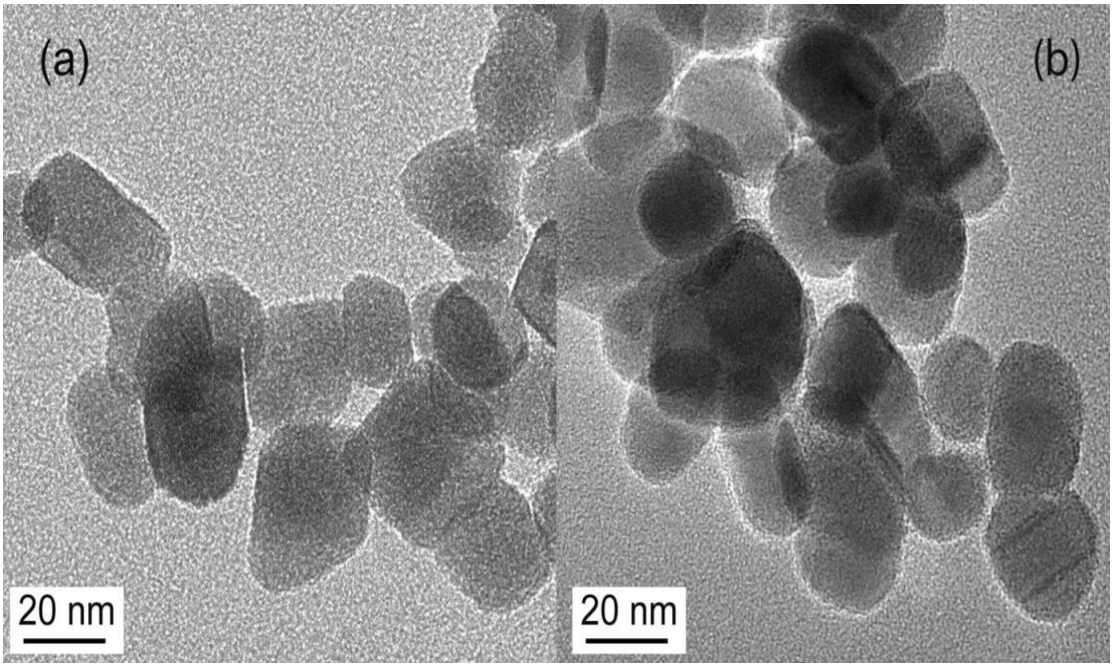


Figure 5.4 TEM images of: (a) free; and (b) immobilised nanoparticle (The images are included with permission (Verma et al. 2013b))

5.3.2 Binding efficiency of immobilisation

The binding efficiency and protein loading of cellulase onto magnetic nanoparticles was confirmed using the Biorad protein assay kit. The immobilisation was done in different protein: nanoparticle ratios, as shown in Figure 5.5. The activation of nanoparticle supports was tested for 1 h as optimised earlier (Verma et al. 2013b). The quantity of protein loading and the binding time were studied over 3.5 h (data not shown). The binding rate of protein onto the nanoparticle supports increased for 1.5-2 h, thereafter elution of protein from the immobilised mixture slowed indicating the onset of equilibrium. Therefore, 2 h was the optimum time for protein-nanoparticle immobilisation at 25 °C. The binding of protein onto nanoparticles showed a broad range of binding efficiency, varying with the protein: nanoparticle ratio of with maximum binding efficiency at a ratio of about 1:1 (Figure 5.5).

The maximum percentage of binding was obtained at a protein: nanoparticle weight ratio of 1:1. A binding of 94% was achieved at this ratio and the immobilisation efficiency was 39.7%. The percentage of immobilisation efficiency is lower compared to other reports (Verma et al. 2013b). Previous research conducted on a magnetic nanomaterial cellulase immobilisation found 95% binding after a long incubation of 7 h (Khoshnevisan et al. 2011). The efficiency obtained after immobilisation was found to be 86.3%. The immobilisation studies conducted on another nanomaterial found the optimal cellulase immobilisation time to be 30 min on a 7.5 min activated support at a temperature of 40 °C (Hung et al. 2011). In the present study we started detecting unbound proteins from the reaction mixture after 30 min of immobilisation. The concentration of unbound proteins gradually lowered from 30 min to 2 h of incubation. Statistically significant elution was not observed after 2 h of incubation. A similar study observed 90% binding in 2 h with a weight ratio of 0.02. Compared with this study we observed a higher percentage and weight ratio (Jordan et al. 2011).

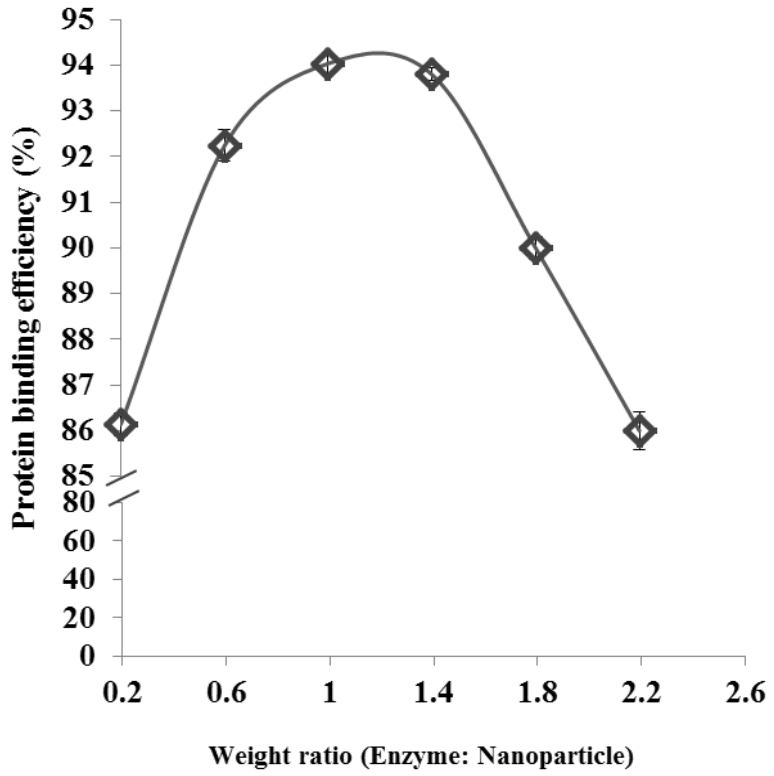


Figure 5.5 Binding efficiency of cellulase onto nanoparticles with varying concentration of protein

5.3.3 Characterisation studies

The binding of cellulase onto activated nanoparticles was confirmed by FTIR spectroscopy analysis. The FTIR spectra in Figure 5.6 shows the spectra of activated nanoparticles, cellulase bound nanoparticles, and cellulase. Stretches at 1636 cm^{-1} and 1036 cm^{-1} and a shift from 1541 cm^{-1} to 1226 cm^{-1} for the cellulase bound nanoparticle resembles the peaks in the cellulase enzyme FTIR spectra. This characteristic shift in the frequency of cellulase bound nanoparticles from the activated nanoparticles is indicative of covalent binding of cellulase onto the nanoparticle. Peaks at 1636 cm^{-1} and 1541 cm^{-1} indicate the stretching of C=O and C-

O groups. The stretching near 1541 cm^{-1} also indicates binding of the carboxyl group of the enzyme and the amine group of the nanoparticles (Jordan et al. 2011). The peaks from cellulase were observed on the activated nanomaterial after immobilisation indicating that immobilisation was successful. Immobilisation of cellulase onto glutaraldehyde activated nanomaterial support provided a maximum of 94% enzyme binding efficiency in 2 h of incubation at 25 °C. In this system, glutaraldehyde acted as a good cross linker which covalently linked cellulase to the nanoparticle. The immobilization of cellulase on to the glutaraldehyde activated nanomaterial support resulted in a maximum of 94% enzyme binding efficiency in 2 h of incubation at 25 °C. Under optimum reaction conditions we obtained a good amount of binding with minimal loss of protein in the supernatant.

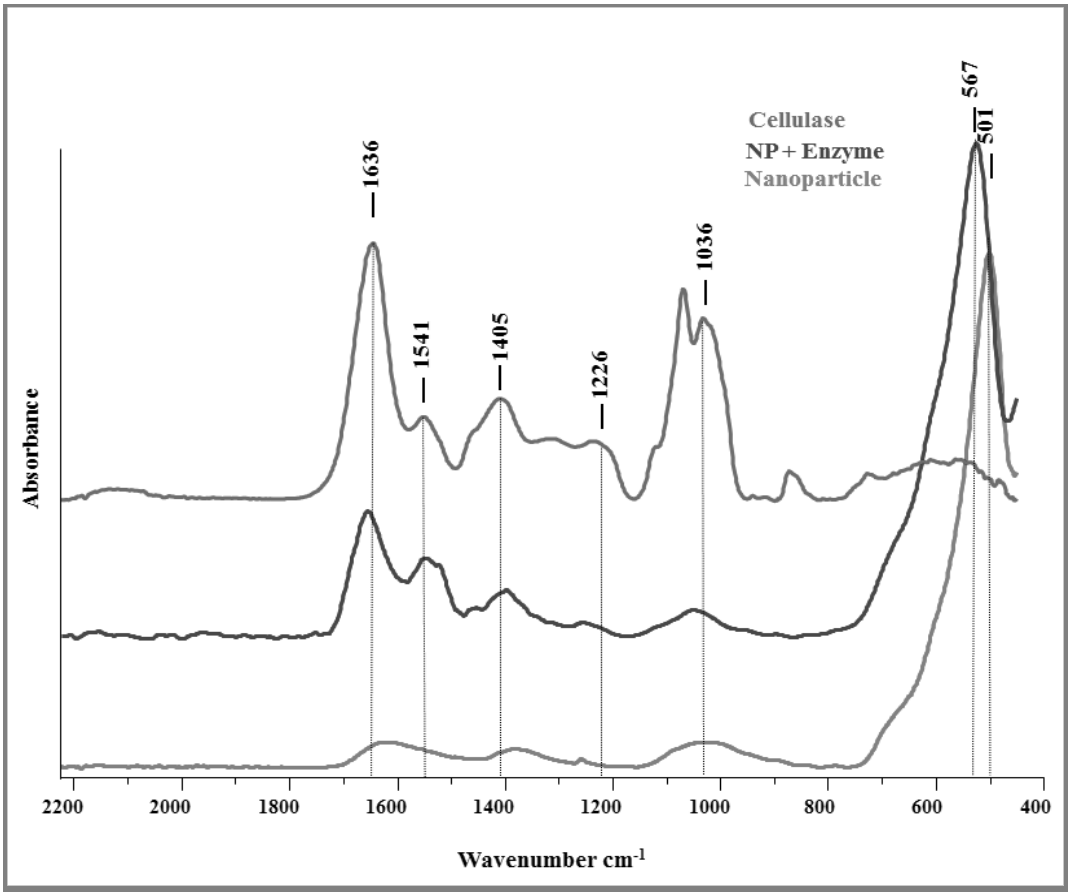


Figure 5.6 FTIR spectra of free (shown in green colour), immobilised cellulase (blue colour) and activated nanoparticle (red colour)

This chapter describes the use of immobilised enzyme preparations to hydrolyse alkaline pretreated biomass (natural substrate) and characterise their effect on biomass structure by SEM. Our optimised pretreatment conditions showed that this treatment gave the best result among the pretreatments tested for HHB, resulting in increased hydrolysis of biomass during enzyme saccharification to produce reducing sugars (Chapter 2). The SEM images of untreated, alkaline pretreated and enzyme hydrolysed HHB are shown in Figure 5.7. The structure of the untreated biomass was found to be compact and rigid (Figure 5.7 (i)), as discussed in chapter 4, and after pretreatment this structure was broken down under alkaline conditions and made the structure easily accessible to enzymes (Figure 5.7 (ii)). After enzyme hydrolysis, the biomass structure was broken into small fragments (Figure 5.7 (iii)).

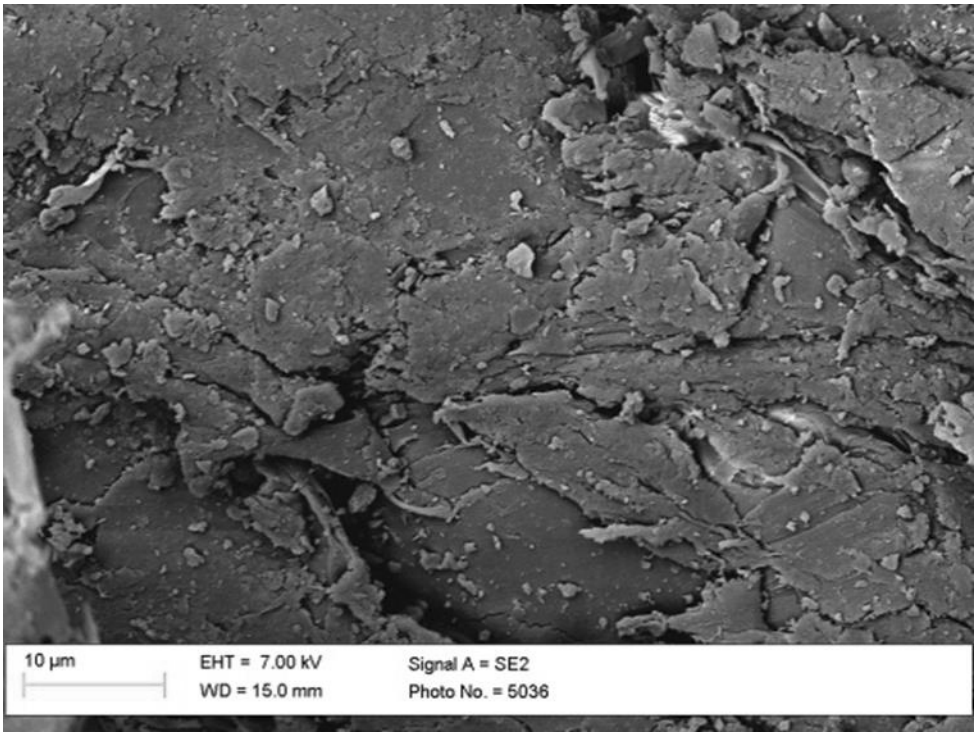
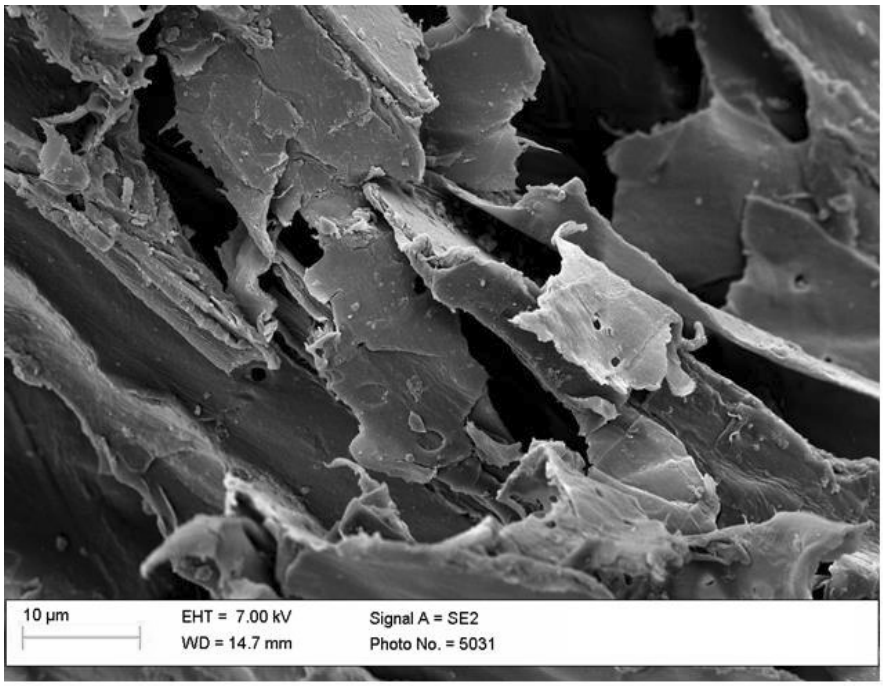


Figure 5.7 SEM images of untreated hemp hurd (upper)

(ii) middle



(iii) lower

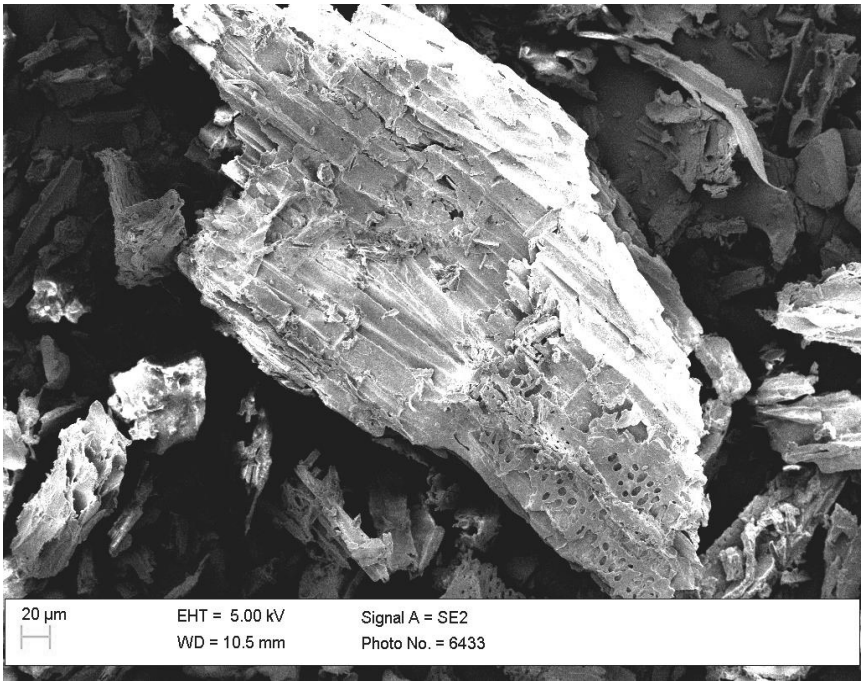


Figure 5.7 SEM images of alkaline pretreated hemp hurd (middle) enzyme treated (lower) hemp hurd after alkaline pretreatment

5.3.4 Enzyme activity variation with pH and temperature

The pH profile demonstrating the relationship between pH buffer and catalytic activity of free and immobilised enzymes is shown in Figure 5.8. The enzyme activity of free and immobilised enzymes was maximal at pH 4.0 in sodium acetate buffer (0.1 M). Activity for both free and immobilised enzyme decreased with increasing pH. The immobilised enzyme was most stable in the pH range of 4.0-5.0. Similar pH stability observation has been reported for cellulase immobilised onto acrylonitrile copolymer nanofibre, where immobilised cellulase activity was found to increase from pH 3.0, attain stability between pH 4.0-5.0, and subsequently decrease again with higher pH values (Yuan et al. 1999). Another study conducted using immobilised cellulase showed that the enzyme preparation was quite stable at a wide pH range (pH-1.5-12.0) (Hegedus et al. 2012). A study conducted on cellulase immobilised on cation-exchange membranes demonstrated enzyme stability in a broad pH range, with maximal activity being observed at pH 3.8 (Cheng et al. 2010).

Enzyme activity of free and immobilised enzymes was investigated at various temperatures ranging from 30 to 60 °C, with an aim of understanding the effects of temperature on the activity of cellulase following immobilisation. Figure 5.9 shows the increase in enzyme activity with temperature for both the free and immobilised forms. The relative enzyme activity increased up to a temperature of 50 °C for the free enzyme, while it increased up to 60 °C for the immobilised enzyme, thereafter declining with further increases in temperature. The temperature profile study demonstrated that the optimum temperatures for the free and immobilised enzymes were 50 °C and 60 °C, respectively. Enzyme activity for the immobilised enzyme retained about 60% activity in the 50-70 °C range, with activity peaking at 60 °C. Immobilisation increased the thermal stability of the enzyme, which was also observed in previously studies for immobilised cellulase (Hung et al. 2011).

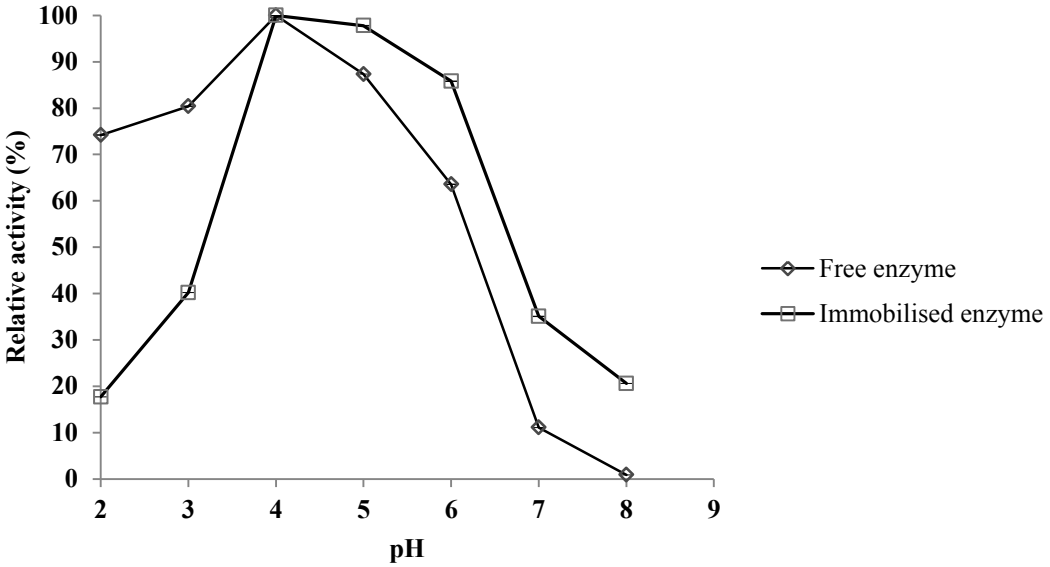


Figure 5.8 Effect of pH on the activity of free and immobilised enzyme

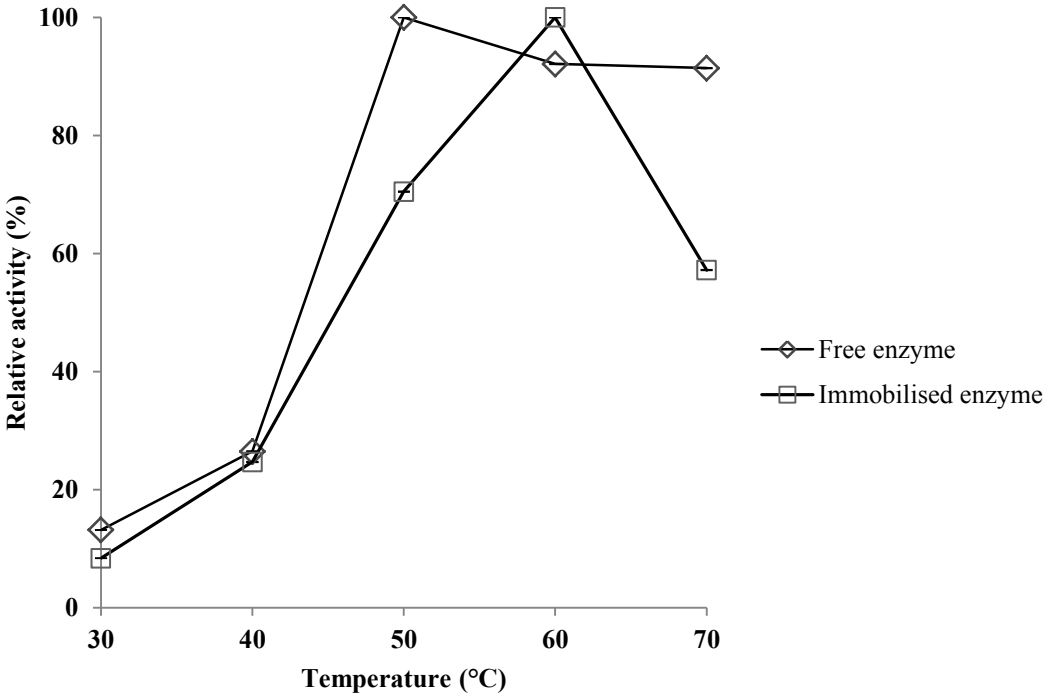


Figure 5.9 Effect of temperature on the activity of free and immobilised enzyme

5.3.5 Kinetic study of immobilised and free enzyme

The kinetic study of free and immobilised enzymes was done using varying concentrations of CMC. The rate of reaction with respect to substrate concentration using Michaelis-Menten kinetic derivation is shown in Figure 5.10. The K_M (half-maximal velocity) and V_{max} (maximal velocity) values obtained using graph pad prism 6 software (software has enzyme kinetic study as a built in feature) at 95% confidence is given in Table 5.1. Michaelis-Menten kinetic derivation is a non-linear regression and a good model to study enzyme kinetics at varying substrate concentration where the rate of reaction is plotted against concentration (Golicnik 2011). The K_M values of the free and immobilised enzymes are 0.87 and 2.6, respectively. A 3-fold increase in the K_M value of immobilised versus free enzyme was observed. Similar results were observed in a previous study where K_M values increased about 2.7 times following immobilisation of cellulase onto a nanomaterial support (Liang and Cao 2012). Other reports on immobilising cellulase on various supports reversibly soluble matrix Eudragit L-100 have suggested that the K_M value of the enzyme changes as a result of immobilisation (Zhang et al. 2010a).

Increases in the K_M value following BGL immobilisations on gamma-Fe_2O_3@SiO_2 core-shell magnetic nanoparticles have also been reported (Georgelin et al. 2010). The minor change in apparent K_M values suggested that the substrate binding affinity of the enzyme active site was altered by immobilisation. The K_M values for the free and immobilised BGL *Agaricus arvensis* were 2.5 mM and 3.8 mM, respectively (Singh et al. 2011). The product release was found to increase with the increase of concentration from 0.5-2.5% of CMC. The activity of free cellulase reached saturation from 2-2.5% of CMC concentration, whereas the activity and the product release were high for immobilised enzyme, suggesting improvement in cellulase performance. This experiment provided preliminary data on cellulase efficiency variation with substrate concentration and the impact of immobilisation, and aided in the design of enzyme hydrolysis experiment with natural and synthetic substrate.

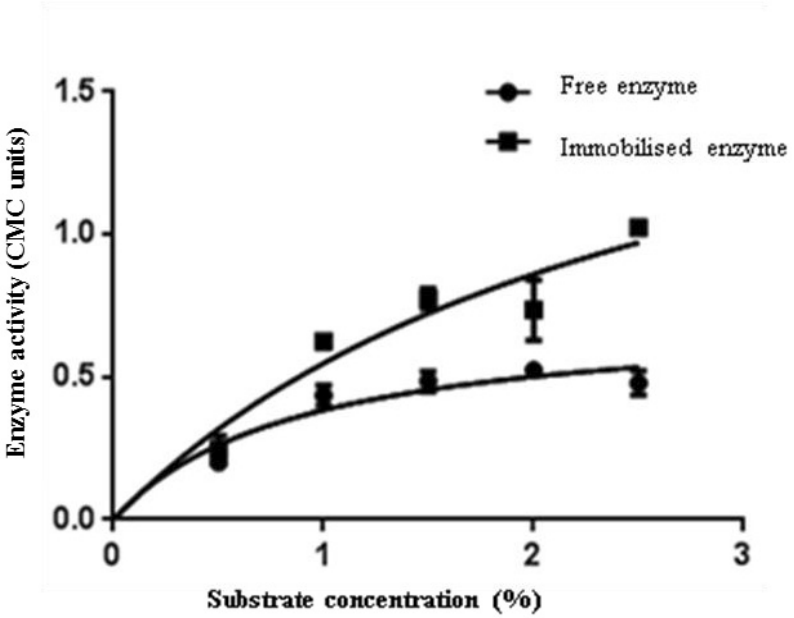


Figure 5.10 Kinetic study of cellulase using CMC substrate

Table 5.1 Rate of reaction for free and immobilised enzyme at different substrate concentration using Michaelis-Menten kinetics

	Free Enzyme	**Immobilised Enzyme**
V_{max} $(mg\ mL^{-1}.min)$	0.72 ± 0.1	2.0 ± 0.6
K_m $(mg\ mL^{-1})$	0.87 ± 0.3	2.6 ± 1.3
95% confidence level (Graph pad Prism 6)		

5.3.6 Thermal stability of free and immobilised cellulase

Improvement in the stability of the immobilised cellulase at higher temperatures is shown in Figure 5.11. The results obtained from the thermal stability studies show that the immobilised enzyme was stable for approximately 4 h at 80 °C. It retained about 66% of its initial activity in the first 2 h of incubation and gradually decreased in the next 4 h of incubation, providing a maximum of 26% activity after 4 h. After incubation for 4 h the enzyme had lost about 73% of its activity. The immobilised preparation completely lost activity by 6 h of incubation at 80 °C. The loss of activity for the immobilised cellulase preparation was slower than that for the free enzyme. The activity of free cellulase was found to decrease in the first half hour of incubation, which suggests that the free enzyme denatured at 80 °C, as shown in Figure 5.11. The loss in activity of the free cellulase after 2 h of incubation at 80 °C is shown in Figure 5.12. The immobilised enzyme retained 72% of its initial activity for up to 6 h of incubation at 60 °C. At 60 °C the activity was 90% after the first 2 h of incubation and gradually reduced to 75% after 4 h of incubation, thereafter reducing to a negligible rate up to 6 h. Free enzyme activity reduced to 61% after 2 h of incubation at 60 °C, and thereafter no significant decrease in the activity from 4 to 6 h was observed. This indicated that the free enzyme retained about 60% of its initial activity for up to 6 h of incubation at 60 °C. These results indicate that the properties of the enzyme were not impacted by immobilisation, with the exception of higher stability at elevated temperatures.

In addition to better thermal stability, immobilisation provides storage stability by binding the enzyme onto a support and inhibiting denaturation over time. The immobilised cellulase preparation was stored at 4 °C in acetate buffer (0.1 M, pH 4.0) for a period of 7 weeks and activity was measured at intervals of 1, 5, 7 and 45 days (Figure 5.13). The immobilised enzyme retained almost full activity over the 45 day period.

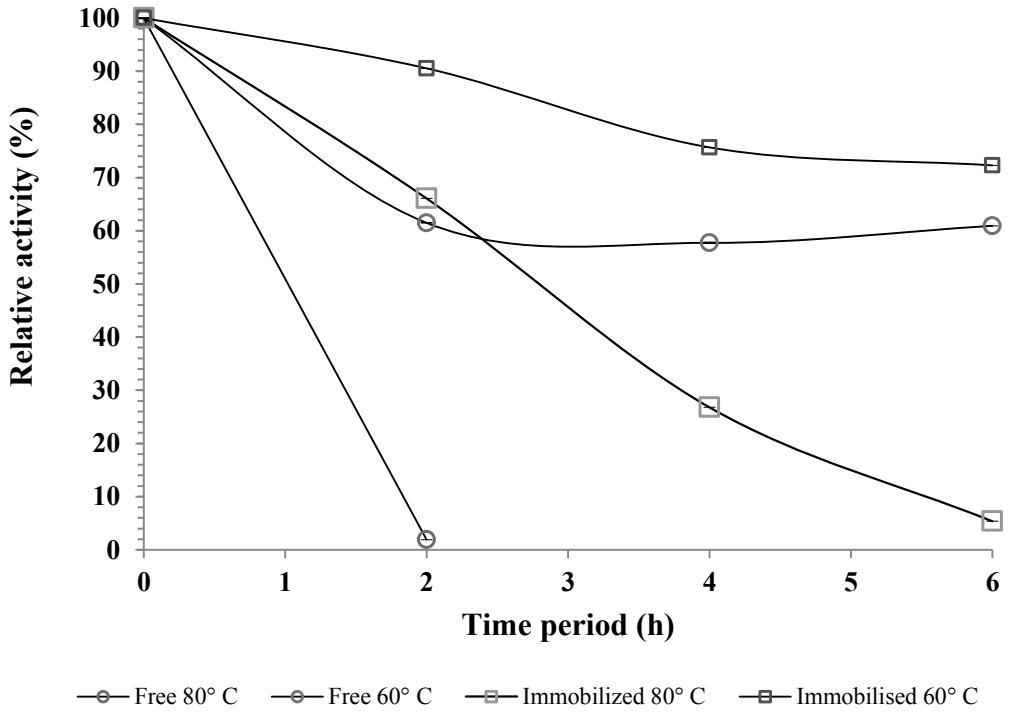


Figure 5.11 Thermostability study of free and immobilised enzyme conducted at 60 and 80 °C for 6 hours

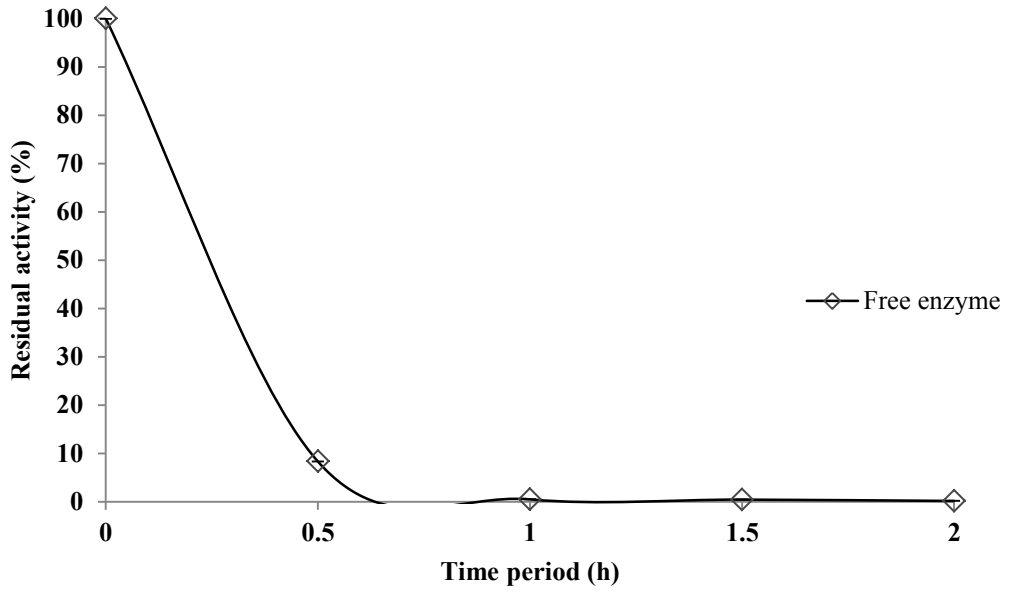


Figure 5.12 Thermostability study of free enzyme at an interval of 30 min at 80 °C

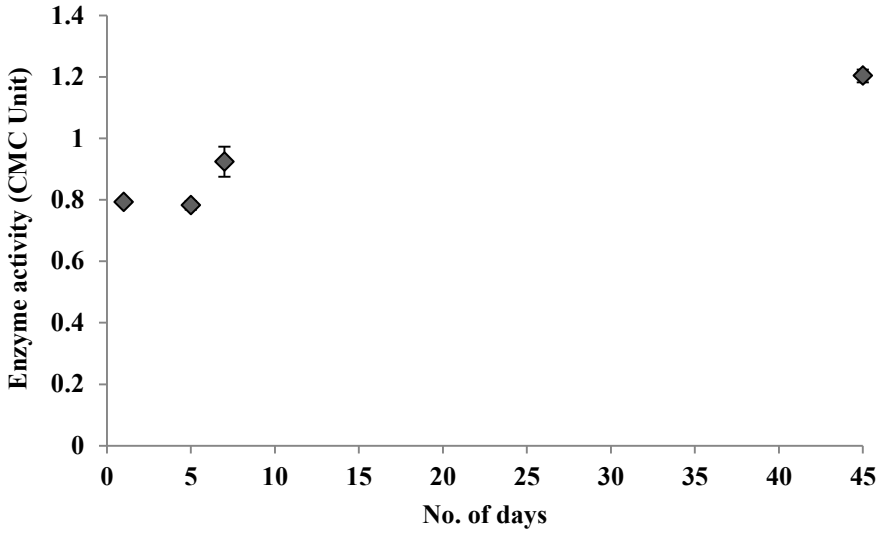


Figure 5.13 Storage stability of immobilised enzyme preparation

5.3.7 Reusability of immobilised enzyme

Immobilisation of cellulase can facilitate enzyme recycling in a sequential batch-wise process, thereby lowering enzyme cost per cycle. The immobilised enzyme was stable for up to 7 consecutive cycles at 60 °C of CMC hydrolysis for 30 min, and thereafter the activity reduced significantly (Figure 5.14). The immobilised enzymes maintained their about 70% of their activity up until the 3rd cycle. Earlier studies of cellulase immobilisation on PAMAM-grafted silica resulted in 75% of activity being retained after 3 cycles. In another study, 41% of activity was retained after 6 cycles when the enzyme was attached using adsorption and 67% when the enzyme was covalently crosslinking (Wang et al. 2013b). Some reports have suggested that the gradual loss of enzyme activity after only a few cycles occurs due to factors such as product inhibition, structural modification of the enzyme, protein denaturation and/or inactivation of the enzyme (Jordan et al. 2011). We observed the CMC hydrolytic activity of immobilised cellulase dropped gradually after each cycle. On average the loss of activity was 10-12% after each cycle. The immobilised cellulase mixture was readily recovered using the magnetic properties to separate from the reaction mixture, although sequential washing appeared to decrease enzyme recovery.

Enzyme activity decreased from cycle two to cycle 6, but didn't decrease much further from cycle 6 to 9, but again reduced at cycle 10. Although not quite linear, stability essentially decreased progressively with each cycle. The immobilised cellulase activity was retained for 5-6 h at 60 °C even after sequential washing, indicating strong binding of enzyme to the nanoparticle.

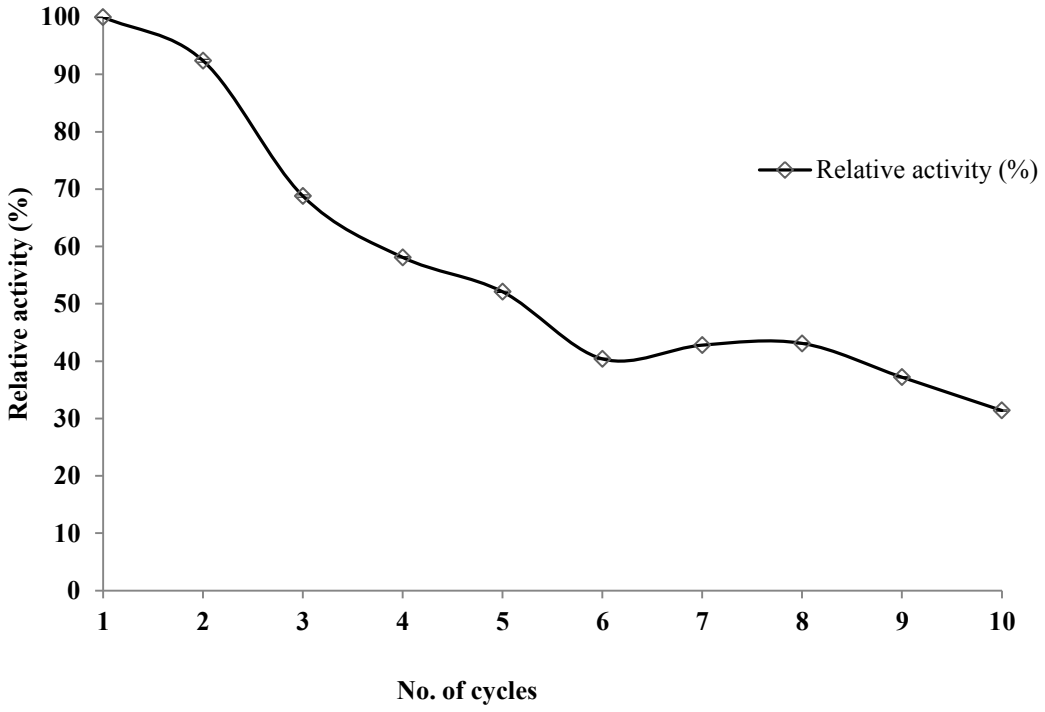


Figure 5.14 Reusability study of immobilised enzyme using CMC substrate

(Footnote: The enzyme assay was conducted using the same batch of immobilised enzyme washed with deionised water followed by buffer after performing each run of experiment with 30 min incubation)

5.3.8 Hydrolysis of CMC using free and immobilised enzymes

Free and immobilised cellulase were used for up to 48 h to hydrolyse varying concentrations of untreated synthetic substrate (CMC), ranging in enzyme: substrate ratio from 1:1 to 1:4 (Figure 5.15). Hydrolysis increased with enzyme: substrate ratios from 1:1 to 1:3 for both free and immobilised enzymes, with no significant

increase observed at a 1:4 ratio of enzyme: substrate. The immobilised and free enzyme showed optima at 1:3 enzyme:substrate ratio, with immobilised enzyme hydrolysing 83% of substrate and free enzyme hydrolysing 88% of substrate over 48 h. The level of hydrolysis at a 1:1 ratio was 26% for the immobilised enzyme and 17% for the free enzyme. When the level of CMC was increased to a 2:1 ratio of substrate to enzyme, hydrolysis increased to 81%. During a previous study, researchers obtained 85% glucose yields using free enzymes, and a maximum of 83% glucose yield with immobilised cellulase when hydrolysing CMC pretreated with ionic liquid (Chang et al. 2011). Another study hydrolysing CMC resulted in a lower yield with immobilised compared with free enzyme and concluded that dilution of enzyme on the support accounted for the lower yield after immobilisation (Ungurean et al. 2012). A further study conducted using immobilised cellulase to hydrolyse ionic liquid treated cellulose found 0.95 g glucose/g cellulose in 8 h of hydrolysis with addition of 4% of EMIM-DEP (1-ethyl-3-methylimidazoliumdiethyl phosphate) compared to our 24 h hydrolysis with untreated cellulose (Jones and Vasudevan 2010).

5.3.9 Enzymatic saccharification of hemp hurd biomass (HHB)

Varying concentrations of pretreated HHB (0.5-4 mg) were incubated with free and immobilised enzymes (5 mg protein), with hydrolysis percentages shown in Figure 5.15. Enzyme saccharification at 48 h resulted in a maximum of 89% hydrolysis using free cellulase. However, 93% hydrolysis was achieved with immobilised cellulase at a 1:1 enzyme: substrate ratio. Enzyme saccharification using the immobilised enzyme was found to reduce with an increasing biomass concentration. Hydrolysis reduced when the enzyme: substrate ratio was increased from 1:1 to 1:2 (Figure 5.15). Partial hydrolysis resulted either from reduced accessibility of cellulase with increasing substrate concentration, or from interference of lignin during the hydrolysis. The immobilised mixture was forming aggregates at higher biomass loading, which restricted the movement of cellulase to penetrate and break the structure. However, free enzyme had no restricted movement and had ready access to biomass, resulting in higher yield at higher biomass concentrations. With increased shaking enzyme biomass interaction did not improve. The rate of

hydrolysis initially increased over a 24 h period, thereafter stabilising for both the free and immobilised enzymes. The free enzyme showed optimum hydrolysis of 85% at 1:3 enzyme:substrate ratio. Similar observations were reported for a study on enzyme saccharification with ionic liquid pretreated yellow poplar using immobilised enzyme. This study gave a maximum of 45.3% hydrolysis in 24 h, with no improvement observed even after a second addition of cellulase preparation (Wang et al. 2011). A study with immobilised enzymes using glutaraldehyde as a cross linker, applied to the hydrolysis of steam exploded corn stalk and bagasse substrate, demonstrated that 24 h of hydrolysis was optimum and that further hydrolysis time did not improve the yield (Xu et al. 2011a). Our study conducted in similar conditions provided higher levels of hydrolysis.

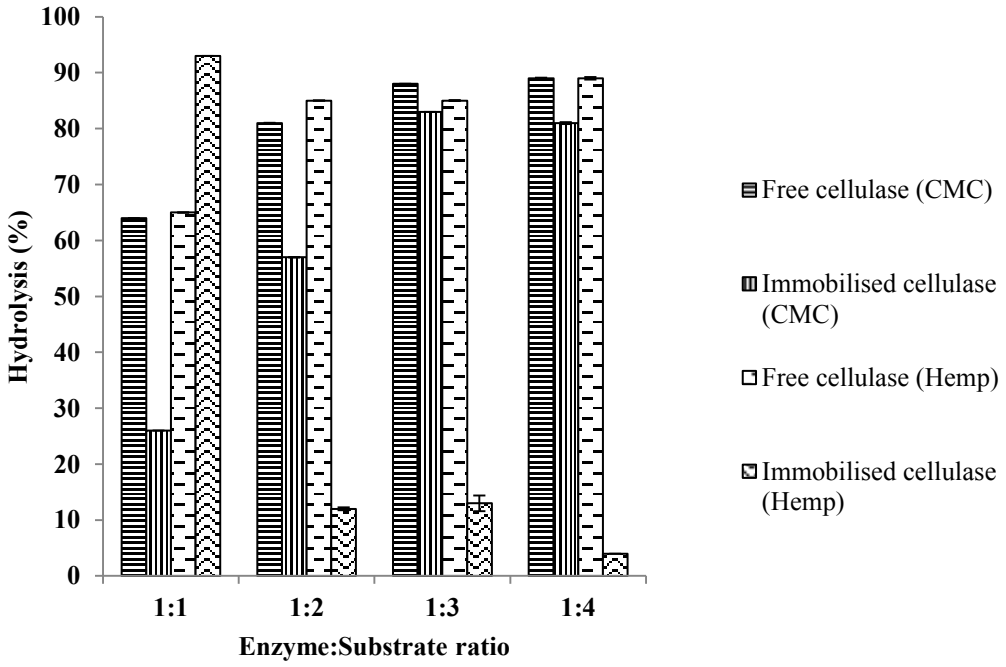


Figure 5.15 Hydrolysis of CMC and pretreated hemp hurd using free and immobilised enzyme for 48 hours (This experiment was conducted using different concentrations of CMC and pretreated hemp hurd biomass. The hydrolysis was conducted for 48 hours at 50 °C (free enzyme) and 60 °C (immobilised enzyme).)

Better enzyme/substrate ratio is required for translating evolved model in real biofuel industry. However, the activity of immobilised cellulase used in this study (procured from Sigma) is comparatively lower than commercial series of cellulase available from other manufacturers. A commercial preparation (Cellucast, Meiji Japan) was used and it resulted in significant percentage of biomass hydrolysis however, same preparation couldn't be immobilised to economise its usage. Different conditions during the binding of commercial enzyme to a support such as protein concentration, protein ratios were tried to achieve immobilization but didn't lead to positive results. A more powerful enzyme will allow higher hydrolysis, less enzyme loading and bring cost-affectivity to the process.

5.4 Conclusions

The immobilisation of cellulase onto a functionalised nanoparticle was achieved and used to investigate the hydrolysis of synthetic (CMC) and natural pretreated substrate (HHB). The confirmation of cellulase and nanoparticle binding (maximum 94%) was done using FTIR spectroscopy. The comparative assessment of the effects of pH and temperature on free and immobilised enzymes demonstrated superior stability for the immobilised enzyme over the free enzyme at elevated temperature. The thermostability of the immobilised enzyme increased to 80 °C, and it retained 50% of initial activity for up to five runs with superior storage stability (45 days). An optimum of 88% CMC hydrolysis and maximum of 89% hydrolysis with pretreated hemp hurd biomass was obtained using the free enzyme. The immobilised enzyme provided successful hydrolysis of 83% with CMC and 93% with hemp hurd biomass. There is scope to further improve the hydrolysis percentage of biomass during enzyme saccharification using immobilised enzymes at higher substrate ratios. The reusability and stability of immobilised mixture could be further improved by using cellulases that have higher activity and stability.

Chapter - 6

Biorefinery: Utilisation of sugars for biofuel and co-product generation

6.1 Introduction

Various forms of waste wood from plantations are underutilised and can be used as feedstocks in biorefining applications to produce energy. The bioprocessing of these untapped carbon resources could yield a large amount of fermentable sugars which can be used for biofuel production. Generally, these sugar hydrolysates are a combination of five and six carbon sugars which are usually xylose and glucose, respectively. Other sugars that may be present in sugar hydrolysate include arabinose, mannose and cellobiose (Goh and Lee 2011). The composition of the sugar hydrolysate varies depending on the biomass source and type of enzyme (lignolytic enzyme, cellulase, xylanase or cellobiase) added to degrade the biomass (Skoulou et al. 2011). The concentration of sugar is highly dependent on the specific activity of the enzyme for the different carbohydrates and the amount of inhibitors produced during the process, which either inhibit the production release or subsequently degrades the product (Kim et al. 2011). All biomass do not end up with high yields of fermentable sugar after processing. This is due to the structure of some biomass being inherently resistant to enzymatic hydrolysis.

Biorefining is a process which converts biomass into multiple products that can be used for fuel, power, co-products or chemical production (Liu et al. 2012). Investment in biorefinery technologies encourages the production of various co-products, fuel and power that stimulates socio-economic development, while reducing carbon emissions (Contestabile 2014). According to the origin and composition of the lignocellulosic biomass, it can be classified for different applications. The delignification ratio and carbohydrate conversion yield helps decide where the feedstock is placed in the biorefining application flowchart (Savage 2011). The major biorefining pathways and applications for lignocellulosic biomass and co-products are presented in Figure 6.1.

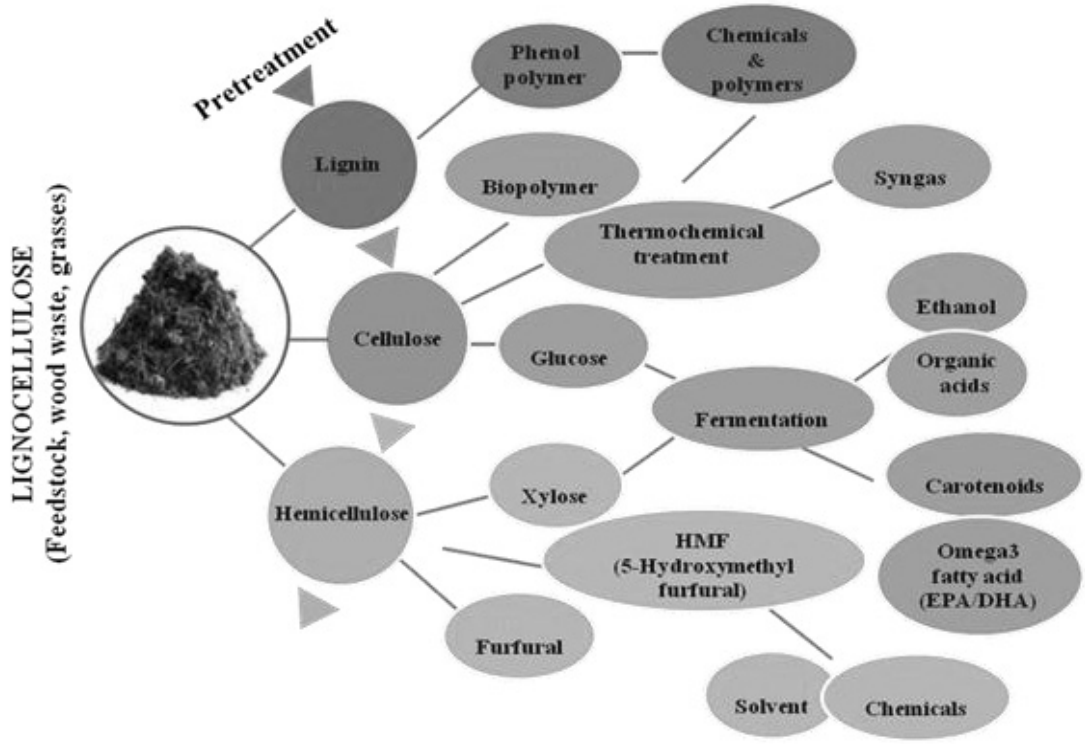


Figure 6.1 Potential biorefinery products obtained from lignocellulosic biomass

Sugars sourced from low-cost lignocellulosic biomass provide an economically viable feed stock that can be used for growing microbes. Microbes may be grown for the production of commercial valuable compounds, such as ethanol and polyunsaturated fatty acids (PUFA) (Figure 6.2) (Skoulou et al. 2011). Some eukaryotic algae are capable of synthesising high value lipids such as eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) (Guschina and Harwood 2006). These PUFA are widely used nutritional products (Gupta et al. 2012) and numerous studies have demonstrated health benefits associated with these compounds (Dewapriya and Kim 2014). In addition to PUFA, some of these marine microbes can produce other useful metabolites such as carotenoids (Gupta et al. 2013). With the appropriate low cost feed stock (biorefined sugars) the production of these valuable compounds could potentially provide a cost-competitive source for the growing nutraceutical industry (Dewapriya and Kim 2014; Zhu et al. 2007). The

utilisation of sugar hydrolysate (produced from low-cost feedstock) for different biorefinery applications is described in this chapter.

During the production of ethanol from these sugar hydrolysates various parameters must be considered, such as sugar concentration, inhibitor concentration, microorganism species used, growth conditions and the ethanol yield (Lin et al. 2012). Microorganisms such as *Saccharomyces* sp*., Zymomonas* sp., *E. coli, P. stipitis* and microalgae are widely utilised for the fermentation of sugar hydrolysates under controlled conditions (Gupta and Verma 2015). Genetically modified strains gives good productivity and have higher tolerance at varied pH, temperature and inhibitors (solvents) (Guo et al. 2011; Ndimba et al. 2013). These organisms are used commercially for industrial scale ethanol and bioactive production in the biofuel industry (Gronenberg et al. 2013). The production of ethanol can be carried out in two different ways. These are separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) which can be done under aerobic and anaerobic conditions. SHF involves the saccharification of biomass followed by fermentation, whereas SSF involves the simultaneous saccharification of biomass and fermentation for production of ethanol (Ask et al. 2012; Wirawan et al. 2012). During the fermentation of sugar hydrolysates additional products such as acetic acid and formic acid may form as co-products. These have been frequently reported to inhibit the growth of the organism (Tian et al. 2011) and have reported strains which can tolerate presence of inhibitors during enzyme hydrolysis and fermentation.

In the present study, a novel isolate *Schizochytrium* sp. was employed to utilise the sugar hydrolysate obtained after the enzymatic saccharification of pre-treated hemp biomass (*Cannabis sativa*), for the production of PUFAs. The production of PUFAs was investigated using sugar hydrolysate as the sole carbon source, with comparison to glucose.

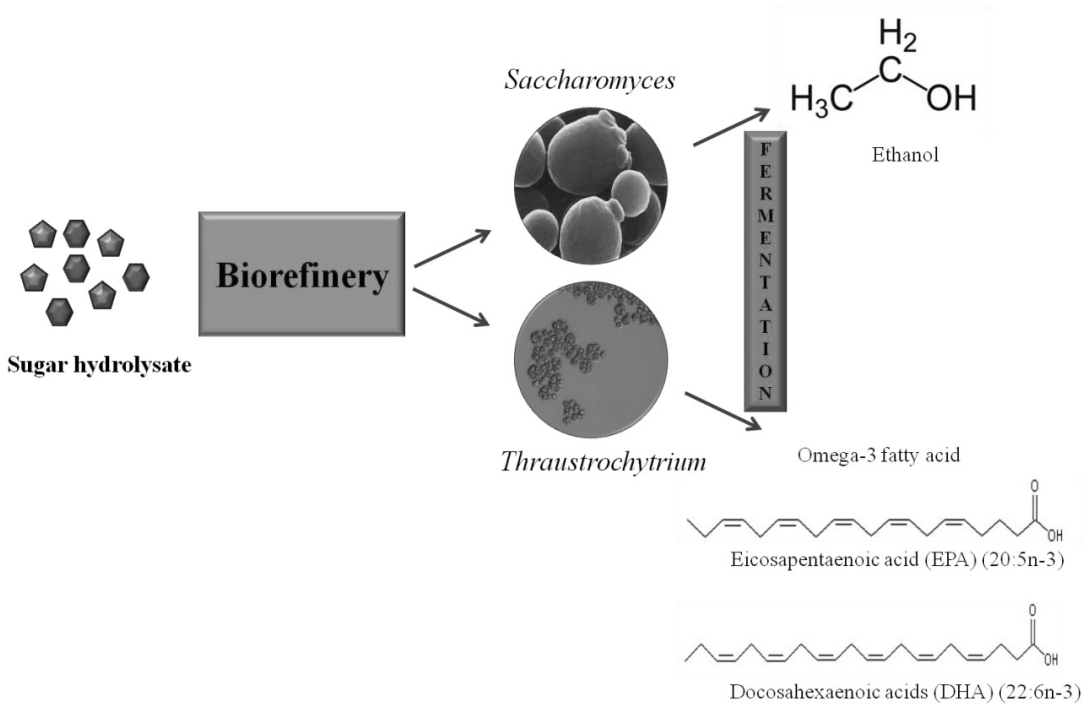


Figure 6.2 The applications of sugar hydrolysate for the production of valuable co-products using various microbes

6.2 Materials and methods

6.2.1 Chemicals

All chemicals used in this study were of analytical grade and were obtained from Sigma-Aldrich, (St. Louis, MO, USA) and Merck Chemicals (Darmstadt, Germany). Instant ocean sea salts, were obtained from Aquarium Systems Inc. (Blacksburg, VA, USA). The hemp biomass for the study was obtained from Commins Stainless manufacturing (Whitton, NSW, Australia).

6.2.2 Sugar hydrolysate

The sugar hydrolysate used for the study was obtained from hemp hurd that had been pretreated with sodium hydroxide and enzymatically hydrolysed (Chapter 2). The sugar hydrolysate studied in this chapter was from two different solid loading concentrations (2 and 5%) and was detoxified using NaOH. This was done by over-titrating hydrolysate with NaOH to pH 8.5 and kept for 1 h at room temperature. The suspension was subsequently filtered and boiled at 100 °C for 15 min. The hydrolysate was cooled to room temperature and readjusted to pH 5.5.

6.2.3 Ethanol fermentation media

The fermentation was carried using *Saccharomyces cerevisiae* culture obtained from American Type Culture Collection (ATCC) (7B 7000). The fermentation medium was prepared using 50.0 g L^{-1} glucose, 1.0 g L^{-1} yeast extract, 5.0 g L^{-1} monopotassium phosphate (KH_2PO_4), 2.0 g L^{-1} ammonium sulphate ($(NH_4)_2SO_4$) and 0.4 g L^{-1} magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) and adjusted to pH 5.0 (Cheng et al. 2009). The medium was sterilised by autoclaving. The culture was maintained at 4 °C on a fermentation medium agar plate and replated every month. The fermentation was carried out in 50 mL flasks containing 10 mL medium at 30 °C with shaking (200 rpm). The fermentation was carried out for 24-48 h in duplicate. The volume and age of the inoculum was optimised and discussed in section 3.1. The experiment was conducted in duplicates for 24-48 h. The optical density (OD) of biomass was measured at 600 nm and the initial concentration of ethanol was estimated using a QuantiChrom^{TM} Ethanol Assay Kit (DIET-500) (BioAssay Systems, USA). The assay was carried by adding 400 µL of reagent A to 400 µL of sample and incubated for 30 min in a water bath (30 °C). Reagent B was quickly added to stop the reaction and mixed briefly. The optical density of reaction mixture was measured at 580 nm and the concentration of ethanol was determined using standard curve of ethanol prepared using the kit. This assay is based on dichromate method; a bluish colour is produced when dichromate is reduced by ethanol.

6.2.4 Culture maintenance

A new Thraustochytrid strain (DT3) was isolated in our laboratory and used for this study (Genbank accession number KF682125). It was maintained on sterile (autoclaved) Glucose yeast and peptone (GYP) agar medium (glucose 5 g L^{-1}, yeast extract 2 g L^{-1}, peptone 2 g L^{-1}, agar 12 g L^{-1} in 50% artificial seawater [ASW]).

6.2.5 Thraustochytrid growth at different sugar hydrolysate and glucose concentrations

The fermentation medium (GYP) was prepared using different glucose and sugar hydrolysate concentrations (2, 4, 6, 8 and 10%) to evaluate the effect on biomass, lipid production and fatty acid composition. The medium contained 1 g L^{-1} yeast extract and 1 g L^{-1} mycological peptone in 50% (v/v) artificial seawater and had an initial pH of 7. Fermentation was initiated with a 5% inoculum and was performed in 100 ml flasks at 25 °C with shaking at 150 rpm. The fermentation was conducted for 5 days and biomass growth was monitored by measuring the optical density (OD) at 600 nm at an interval of 24 h (Gupta et al. 2013).

6.2.6 Microscopic studies

The isolate used for the study was observed using differential interference contrast (DIC) and Nomarski microscopy using an Axio-imager (Zeiss, Germany). Thraustochytrid cells were fixed on a glass slide and air-dried before observation

6.2.7 Raman Spectroscopic Measurements

Raman microspectroscopic measurements were conducted using a InVia Raman microspectrometer (Renishaw plc, Gloucestershire, UK), which was equipped with a 63x water immersion objective, a 785-nm Near-IR laser (HPNIR) (Renishaw plc, Gloucestershire, UK) and a thermo-electrical cooled CCD detector. To prevent heating and photochemical damage to the samples the original laser power was reduced by 10%, which was ~30 mW at the sample droplet. Acquisition parameters used for spectral data collection were 10 s exposure time and 10 accumulations at 4-cm^{-1} spectral resolution. Spectral post-processing (baseline correction and smoothing) was performed using WiRE 3.4 (Renishaw plc, Gloucestershire, UK) software.

6.2.8 High Performance Liquid Chromatography (HPLC)

Ethanol detection was also carried out using reverse-phase HPLC. An Agilent 1260 Infinity series HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with a solvent degasser, quaternary pump, auto-sampler, thermostatted column compartment, diode array detector and fitted with an Aminex HPX 87H column (Bio-Rad, Hercules, CA, USA) with Cation H micro-guard cartridge. The column was maintained at 65 °C and eluted with 0.01 M nitric acid (HNO_3) in milliQ at a flow rate of 0.6 mL/min. Ethanol was detected by refractive index or UV absorption (277 nm) (Saha and Cotta 2011). The concentration of ethanol was determined using a standard curve obtained with HPLC-grade ethanol.

6.2.9 Gas Chromatography (GC) analysis of FAMEs

Fatty acid methyl esters (FAMEs) were concentrated under a nitrogen stream and analysed by gas chromatography-flame ionization detection (GC-FID) system-Model No. 6890N (Agilent Technologies, Santa Clara, USA). A modified protocol of Gupta et al. was used for FAME analysis (Gupta et al. 2013). The GC was equipped with a capillary column (SGE, BPX70, 30 m × 0.25 mm, 0.25 μm thickness). The injector was maintained at 250 °C with a sample volume of 1 μL injected with a 50:1 split ratio. Helium was used as the carrier gas and its flow rate maintained at 1.5 $mL.min^{-1}$. The oven temperature was held at 140 °C for 5 min, and then ramped to 240 °C at a rate of 4 $°C/min^{-1}$. This final temperature was held for a further 10 min. Fatty acid peaks were identified with Chemstation chromatography software (Agilent Technologies, Santa Clara, CA, USA) by comparison with the retention times of internal standards (Sigma-Aldrich, St. Louis, MO USA) and corrected using theoretical relative FID response factors. Methyl nonadecanoate (C19:0) (mg mL^{-1}) was used as an internal standard. All samples were analysed in duplicate and represented with mean ± SD.

6.3 Results and discussion

6.3.1 Optimisation of inoculum (Saccharomyces cerevisiae)

Experiments were carried out to investigate the production of ethanol using *Saccharomyces cerevisiae* grown in a medium containing different concentrations of sugar hydrolysate and glucose. Initial work focussed on optimising the inoculum size and age for hydrolysate fermentation. This was done to determine the stage of inoculum which gave the greatest yield of ethanol via fermentation. Cultures were grown in glucose fermentation medium for 24 h, with microbial growth monitored at 6 h intervals (Figure 6.3). The cultures grew exponentially from 0 to 6 h and reached stationary growth after 12 h. The maximum cell density was observed between 6-12 h and thereafter steady state was attained. Thus an optimum time period of 12 h was selected for further studies.

A similar study used both 6 and 12 h old inoculums to optimise age of culture for ethanol fermentation from sugarcane leaves and obtained highest yield with 6 h inoculum (Jutakanoke et al. 2012). Another study, where fermentation was conducted by simultaneous saccharification and fermentation (SSF) from pretreated rice straw, used a 24 h old mixed culture (Suriyachai et al. 2013). These studies suggest that a starter inoculum of 6-24 h of *S. cerevisiae* can be used for the fermentation of ethanol. After optimisation of the inoculum age the inoculums/starter volume was investigated. This experiment was conducted using culture volume of 2.5, 5, 7.5 and 10% in glucose fermentation medium (Figure 6.4). The different inoculation volumes were added to the medium and the culture growth measured after (12 h). The marginal difference was observed in the cell density from 2.5-10%, though it was decreasing at higher concentration. The highest cell density was observed at 5% inoculum volume which used in later studies.

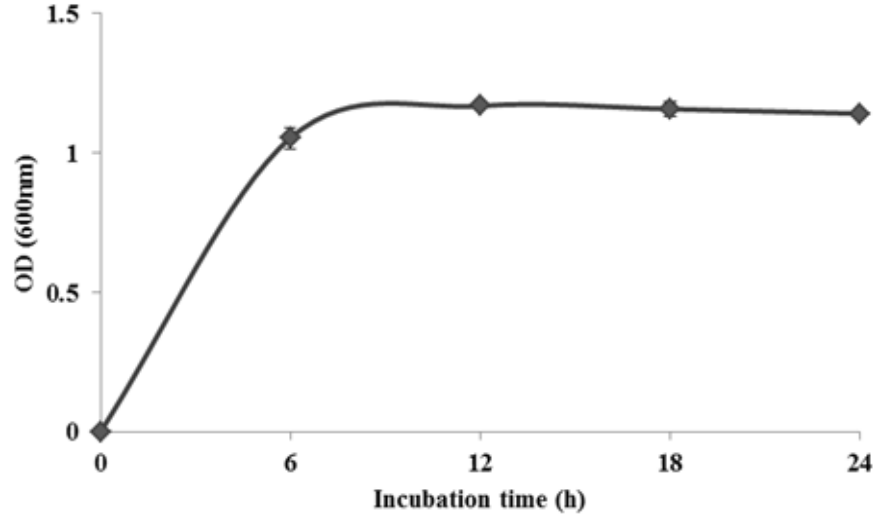


Figure 6.3 Growth of *Saccharomyces cerevisiae* inoculum over time

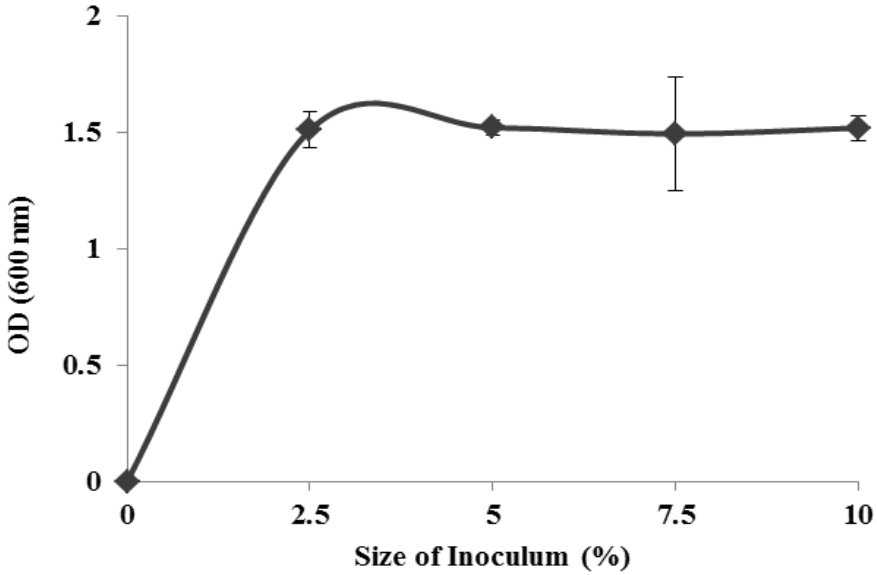


Figure 6.4 Effect of *Saccharomyces cerevisiae* inoculum volume on cell density

6.3.2 Growth of Saccharomyces cerevisiae in glucose and sugar hydrolysate fermentation media

The fermentation of sugars such as glucose was conducted using optimised inoculum of *Saccharomyces cerevisiae* for 48 h. When the culture was grown on the glucose-containing medium it yielded 1.3 and 1.4% of ethanol after 24 h and 48 h, respectively. A maximum of 1.6% ethanol was detected over the 48 h when medium containing used.

Further investigation was carried out with media containing different concentrations of sugar hydrolysate with glucose-only medium used as a control. *Saccharomyces cerevisiae* grown on sugar hydrolysate obtained from 5% HHB substrate concentration (enzymatically hydrolysed) produced more ethanol than that grown on 2% HHB substrate (Figure 6.5). In 48 h the fermentation of sugar hydrolysate from 5% HHB provided 1.6% of ethanol, while 2% HHB produced 0.72%. There was no significant difference observed in the concentration of ethanol between samples taken at 36 and 48 h. This confirmed that the culture was capable of consuming sugar hydrolysate and converting it into ethanol. Additionally, the yield of ethanol increased as the concentration of sugar hydrolysate increased.

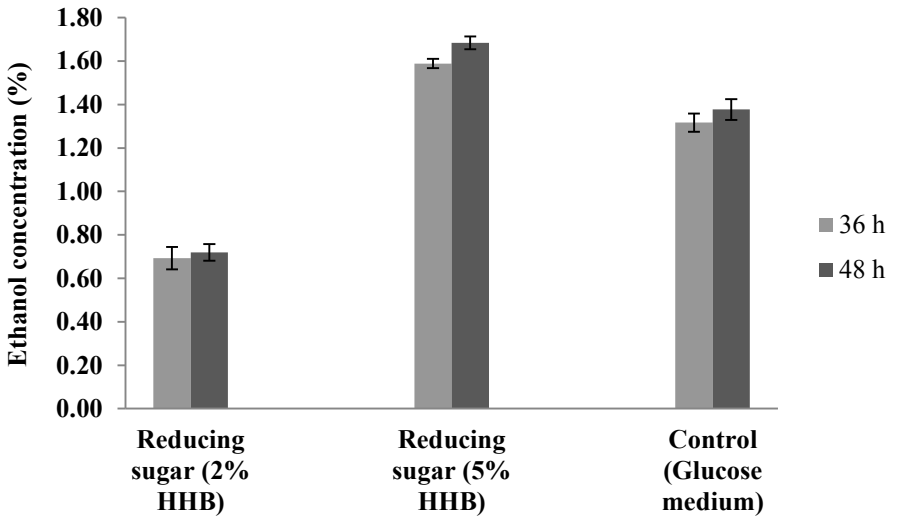


Figure 6.5 *Saccharomyces cerevisiae* grown on different concentration of sugar hydrolysate

6.3.3 Raman spectroscopy detection of ethanol produced under aerobic and anaerobic conditions

The fermentation of sugar hydrolysates to produce ethanol by *Saccharomyces cerevisiae* was investigated under aerobic and anaerobic conditions. Cultures were grown under optimised conditions for 48 h and the concentration of ethanol determined by Raman spectroscopy. Prior to Raman spectroscopy the ethanol was first detected and the concentration estimated using an ethanol estimating kit (BioAssay Systems, USA). As a considerable improvement was not observed from the 24 h to 48 h fermentation samples (Section 3.2 and 3.3), the 24 h sample was used for Raman analysis. Raman spectroscopy detected an ethanol peak in both aerobic and anaerobic fermentation samples (Figure 6.6). The peak representing ethanol can be seen an 877 cm^{-1} and indicates the production of ethanol from the sugar hydrolysate under both aerobic and anaerobic conditions. Another study using an isolated strain (*Caulobacter* sp.) capable of hydrolysing synthetic substrates also produced ethanol under aerobic and anaerobic fermentation. However, the concentration of ethanol was comparatively better than our studies (Song et al. 2013).

Although the concentration of ethanol was found to be relatively low in both samples, the peak was greater in the aerobic sample. Using Raman spectroscopy for measuring ethanol has been explored in recent times. This study demonstrated that Raman spectroscopy is capable of detecting minute concentrations of ethanol in fermentation media. A similar study using Raman spectroscopy for the analysis of fermentation product from pretreated corn stover reported relatively better concentrations of ethanol (Shih and Smith 2009). The same group has also used Raman spectroscopy for quantification of enzyme hydrolysis products, such as glucose and xylose after dilute acid pretreatment (Shih et al. 2011). Others have compared this technique to HPLC analysis of fermentation products such as ethanol (Ewanick et al. 2013). This study was conducted on sugar hydrolysate obtained from switchgrass. They used sugar hydrolysate obtained from switchgrass for ethanol fermentation. The concentrations of ethanol obtained in our study are low as compared to above mentioned studies. However, their studies have used simple high glucose yielding substrates, while we have used more complex softwood residue. Our

substrate is more complex which comprise of lignocellulosic waste material which is relatively difficult to hydrolyse and get converted to ethanol.

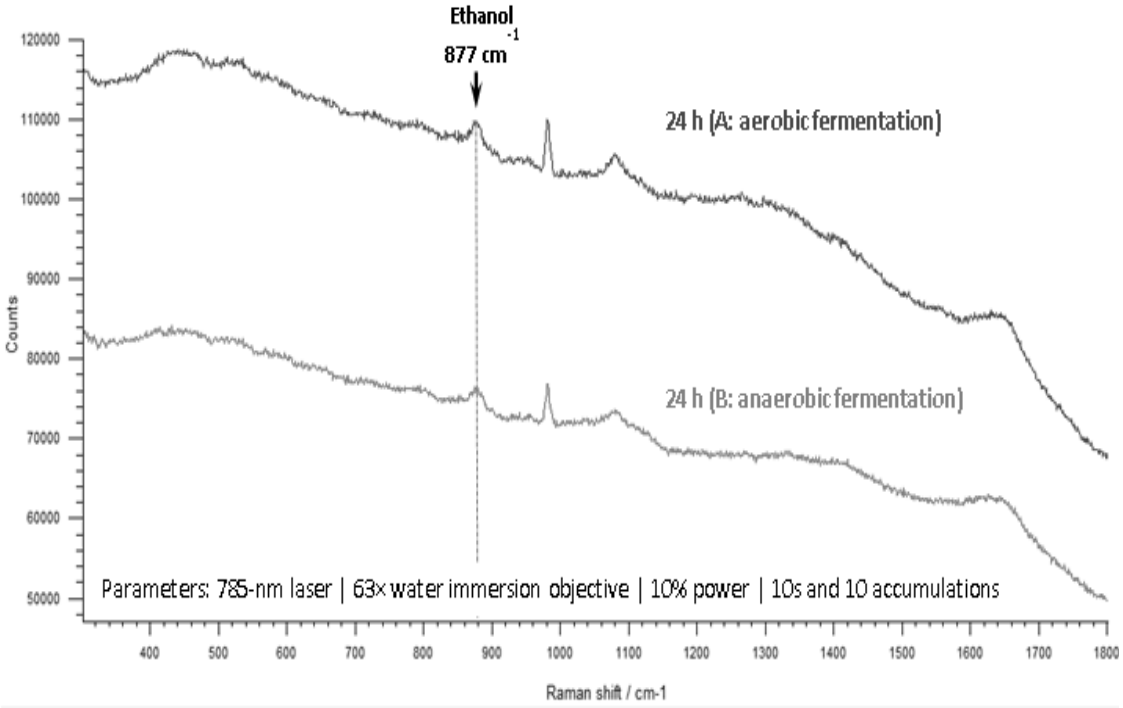


Figure 6.6 Detection of ethanol by Raman spectroscopy in cultures grown under aerobic and anaerobic conditions

6.3.4 High Performance Liquid Chromatography (HPLC) analysis of ethanol production

The quantification of ethanol from fermentation media was carried out using reverse-phase HPLC and the chromatogram is represented in (Figure 6.7). The sugar hydrolysate was taken from 5% of HHB digested with an enzyme cocktail (cellulase, xylanase and cellobiase) for 72 h and detoxified by over-liming using NaOH. The experiment was conducted under aerobic conditions using *Saccharomyces cerevisiae* with optimised conditions. Ethanol peak of concentration 0.04 ± 0.0 g L^{-1} was detected by HPLC analysis in the fermentation medium. Peaks representing sugars were also observed, indicating that the yeast did not convert all of the reducing sugars

into ethanol. The low concentration of ethanol detected by HPLC was in agreement with those found using Raman spectroscopy.

Previous studies using 6% of pretreated sugar cane leaves as substrate have reported ethanol yields of 4.7 g L^{-1} in 24 h (Jutakanoke et al. 2012). However, the substrate used was relatively simple compared to the softwood used in our study. Another group used enzyme-treated waste paper as a feed stock for fermentation to produce ethanol (Elliston et al. 2013). They obtained a maximum of 11% ethanol after 408 h of enzyme saccharification and fermentation, though the concentration of ethanol produced after 24 h was comparable to our results. Also similar to our results is the observation that unsuccessful ethanol fermentation can result from inhibitor interference originating from the sugar hydrolysate. Some have reported that high concentration of inhibitors such as hydroxymethylfurfural (HMF) and furfural repress the production of ethanol by *P.stipiti* (Lee et al. 2013). Another study demonstrated that the presence of synthetic medium helps to increase the concentration of ethanol in the fermentation (Arslan and Eken-Saracoglu 2010). They observed that microbial growth and fermentability was comparatively low in hydrolysate-containing media than synthetic media and the presence of inhibitor restricted ethanol production.

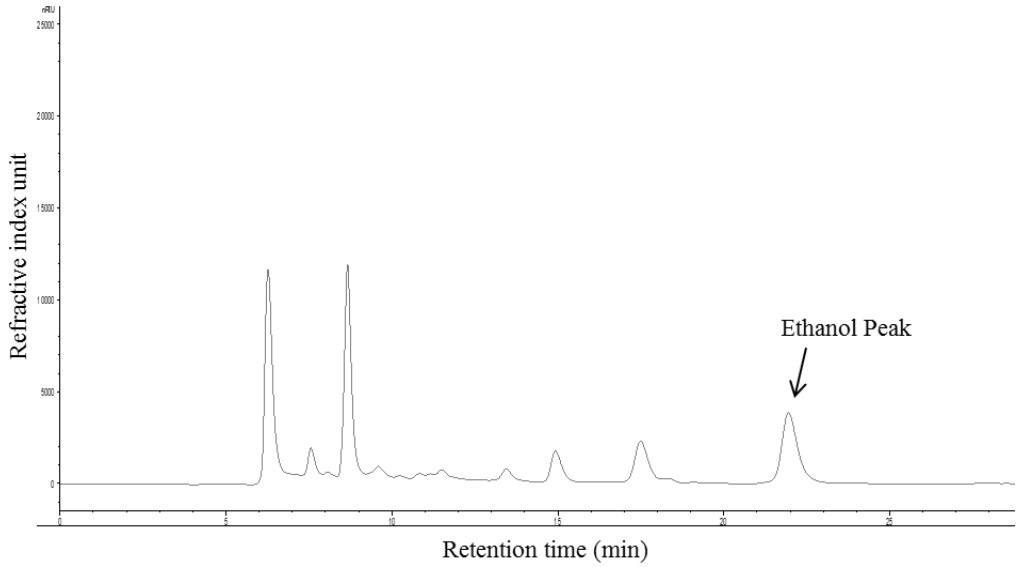


Figure 6.7 Reverse-phase HPLC chromatogram representing ethanol peak in sugar rich media

The higher concentration of sugar in the fermentation medium produced lower ethanol concentration. Even after few repeated trials employing fresh yeast culture, ethanol concentration didn't improve which in fact was perplexing. Upon further quantification of SH by HPLC analysis, various inhibitors such as acetic acid, succinic acid and formic acid were found which probably have restricted the growth of microbes (Table 1). Similar study carried on wheat straw reported the occurrence of acetic acid, formic acid as the main by-product from the enzyme hydrolysis (Toquero and Bolado, 2014).

Investigations were done to modify the pretreatment and enzyme hydrolysis process to reduce the concentration of inhibitors. Overnight washing of pretreatment helped to reduce the concentration of inhibitors to a larger extent as observed later in Chapter 3 (section 3.3.4 and 3.3.6) and this difference may have helped to improve the ethanol concentration.

Table 6.1 Reducing sugars detected in HPLC analysis of sugar hydrolysate from hemp

Enzyme concentration (FPU)	Glucose (g/l)	Xylose (g/l)	Total sugars (g/l)	Total inhibitors* (g/l)
15	547.5 ± 0.5	42.5 ± 1.1	590.0 ± 0.9	29.5 ± 0.8
30	829.0 ± 1.7	69.3 ± 0.7	898.3 ± 1.0	44.9 ± 1.2
60	815.6 ± 1.0	75.7 ± 0.9	891.4 ± 1.3	40.0 ± 0.7
90	782.0 ± 1.9	58.4 ± 1.2	782.0 ± 1.7	39.1 ± 0.9

*acetic acid, formic acid and succinic acid were the major inhibitors obtained in the hemp hydrolysate

6.3.5 Biomass growth observed in glucose and sugar hydrolysate

Thraustochytrids (marine microalgae) have been found to grow in different industrial waste residues such as distillery waste water, liquid residues from the food industry, beer and potato processing waste, cellulosic biomass and spent yeast from breweries (Hong et al. 2012). Utilisation of these alternative carbon sources helps to minimise the cost of fermentation process and simultaneously reduces the biological waste

residues. This study was conducted using *Schizochytrium* strain DT3 (an in house isolate), which is capable of producing omega-3 fatty acids. *Schizochytrium* spores were found in a size range of 10-50 μm and were found to form lumps after budding (Gupta et al. 2013). The cells were found to be highly refractive, which suggests the presence of oil, as shown in Figure 6.8.

Growth curves were prepared for cultures grown for 120 h on a media containing either reducing sugar hydrolysate or glucose (available commercially), using above mentioned strains (Figure 6.9). Comparison of the growth curves shows that the biomass growth in both glucose and sugar hydrolysate media was relatively similar up to 24 h. From this point onwards biomass growth was more prolific in the glucose medium and it was exponential till 72 h. The lag phase of the strain was till 48 h and reached early stationary phase in sugar hydrolysate medium which is relatively shorter than glucose medium.

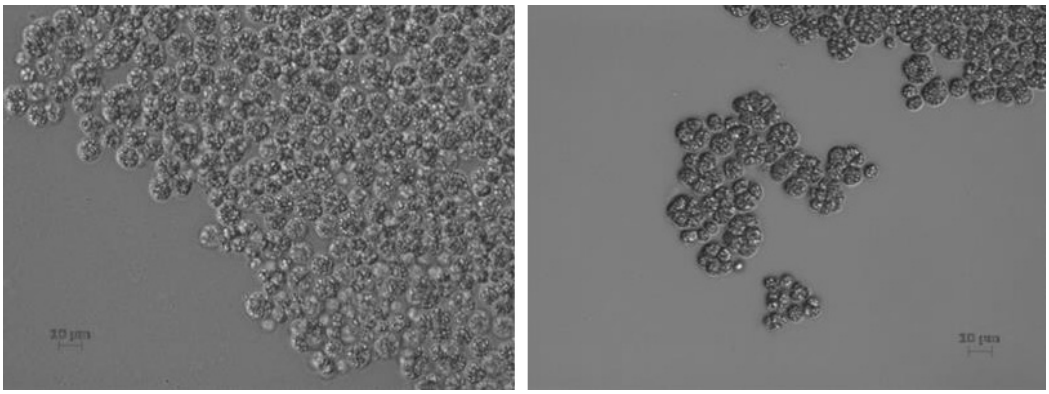


Figure 6.8 Microscopic images of Schizochytrium strain (DT3) grown in an in-house optimised medium [cell size (8-15 μm) and magnification (10 μm)]

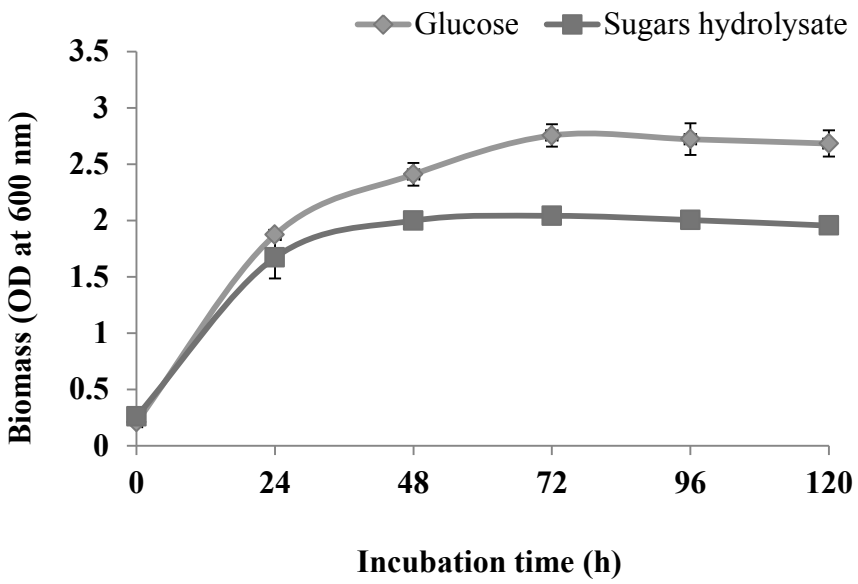


Figure 6.9 Growth profiles of *Schizochytrium* strain - DT3 grown in media containing glucose or sugar hydrolysate

6.3.6 Cell dry weight yields at different initial glucose and sugar hydrolysate concentrations

The effect of different concentrations of sugar hydrolysate and glucose on cell dry weight was investigated using our in-house strain DT3 (*Schizochytrium* sp.). Cell growth was monitored in all the concentrations (Figure 6.10). Cultures grown on 2% concentration of either carbohydrate substrate were found to yield similar cell dry weight. As the glucose concentration was increased the cell dry weight values also increased. However, when thraustochytrids were fed with sugar hydrolysate the dry cell mass decreased as substrate concentration increased. Comparing the biomass concentrations from 2 to 10% with glucose fed cultures, the cell dry weight was found to be high in 6% (4.1 g L^{-1}) and 8% (4.3 g L^{-1}), though the cell weight reduced slightly in 10% (4.0 g L^{-1}). While taking lower concentrations of sugars in the medium, 2% (1.9 g L^{-1}) and 4% (2.9 g L^{-1}) produced relatively good amount of cell dry weight. Biomass growth lowered at higher sugar hydrolysate concentration indicating substrate saturation and the excess sugar was not utilised by the microbes.

A similar study using different concentration of glucose has reported lowering of biomass growth at higher glucose concentration (Burja et al. 2006).

Various studies have been conducted using different carbon sources for growing thraustochytrids. A recent study on *Thraustochytrium* sp. using glycerol as carbon source observed good biomass growth until 4% and the growth decreased when glycerol concentration was increased to 10% (Gupta et al. 2013). Other studies employing *Schizochytrium* sp. SR21 with different carbon sources also reported low biomass at higher sugar concentration (Ethier et al. 2011; Huang et al. 2012b). In this study, the maximum biomass was attained with 8% glucose concentration and 2% sugar hydrolysate concentration. The lowest cell mass (0.9 g L^{-1}) was obtained in cultures grown at 8% sugar hydrolysate. Raw glycerol from biodiesel waste has also been used for thraustochytrid fermentation (Pyle et al. 2008), where impurities in the biodiesel waste (such as soap) were found to inhibit the biomass growth. During the production of sugar hydrolysates via enzyme saccharification, numerous inhibitors such as furfural, hydroxyl methyl furfural (HMF), phenols and acetate compounds can be produced.

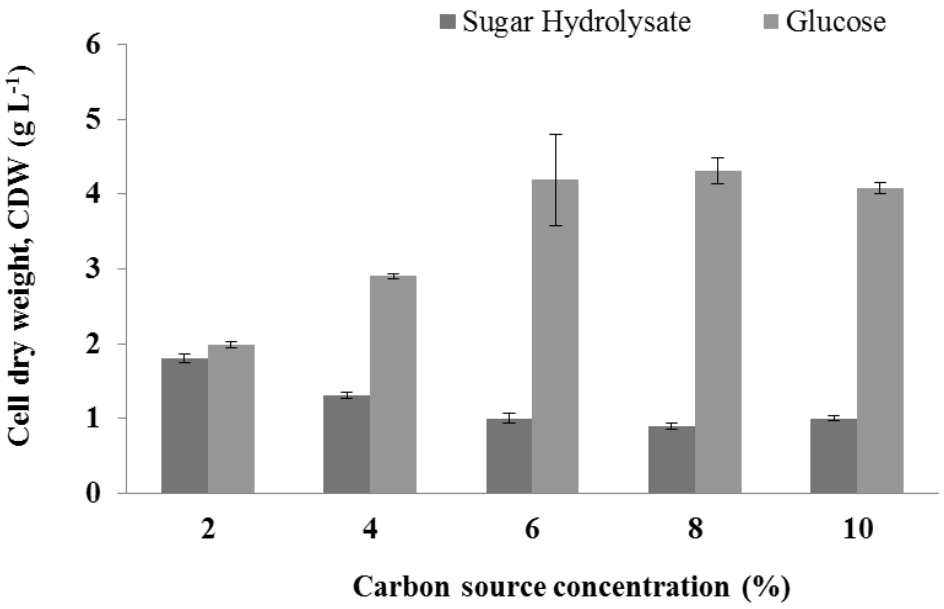


Figure 6.10 Cell dry weight (g L^{-1}) of *Schizochytrium* DT3 in different concentrations of glucose and sugar hydrolysate

These compounds have been reported to inhibit the product formation (Cao et al. 2013). In our studies we have also observed the presence of these inhibitors (chapter 3). It is likely that the presence of these inhibitors in the hydrolysate solution would have limited the growth of the thraustochytrid cells.

GC analysis was conducted to study the fatty acid profile produced in *Schizochytrium* DT3 grown in different concentration of sugar hydrolysate. The total fatty acid profile of 2, 4 and 6% sugar hydrolysate with major fatty acids is shown in Figure 6.11. The major fatty acids found in all concentrations were palmitic acid (C16:0), stearic acid (C18:0), EPA (C20:5n3), docosapentaenoic acid (C22:5n6) and DHA (C22:6n3). The accumulation of DHA decreased with the increase of hydrolysate concentration (Figure 6.11- 2, 4 and 6% has 38, 31 and 30% of DHA [C22:6n3]). The total fatty acids (TFAs) were higher in 2% concentration with more polyunsaturated fatty acids (PUFAs) in it. The saturated fatty acids (SFAs) were observed higher in 4% sugar hydrolysate concentration. A similar study has been done using *Aurantiochytrium mangrovei*, where biomass was allowed to grow in different concentrations of glucose. The biomass growth and DHA content increased as substrate concentration increased and obtained 20 g L^{-1} of biomass yielding 30-35 (mg mL^{-1}) of DHA in a glucose medium (Wong et al. 2008).

The time course experiment was carried by employing sugar hydrolysate (2%) as the carbon source in fermentation medium for growing *Schizochytrium* DT3 for 7 days (Figure 6.12). The fermentation profile was conducted to evaluate the potential of sugar hydrolysate to be used as a low cost carbon source for growing marine microbes. The cultures grew exponentially in 2 days with rapid consumption of sugar hydrolysate. The growth slowed down after 3 days followed by minimal sugar consumption. Difference in cell density or sugar consumption was not observed after 7 days of fermentation suggesting the attainment of stationary phase. The cell dry weight 1.82 g L^{-1} produced 38% DHA after 7 days of fermentation (Figure 6.11). Similar biomass growth profile of microalgae (*Chlorella vulgaris*) was observed while using reducing sugars from low cost substrate for biodiesel production (Wang et al. 2013c).

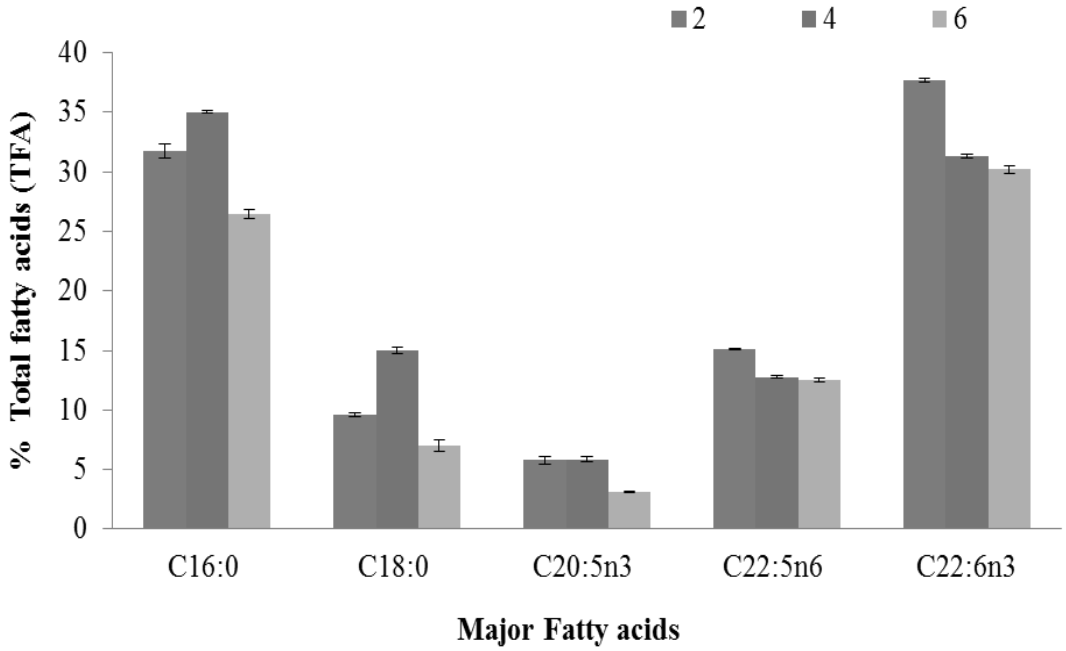


Figure 6.11 Fatty acid profile of DT3 at various sugar hydrolysate concentrations

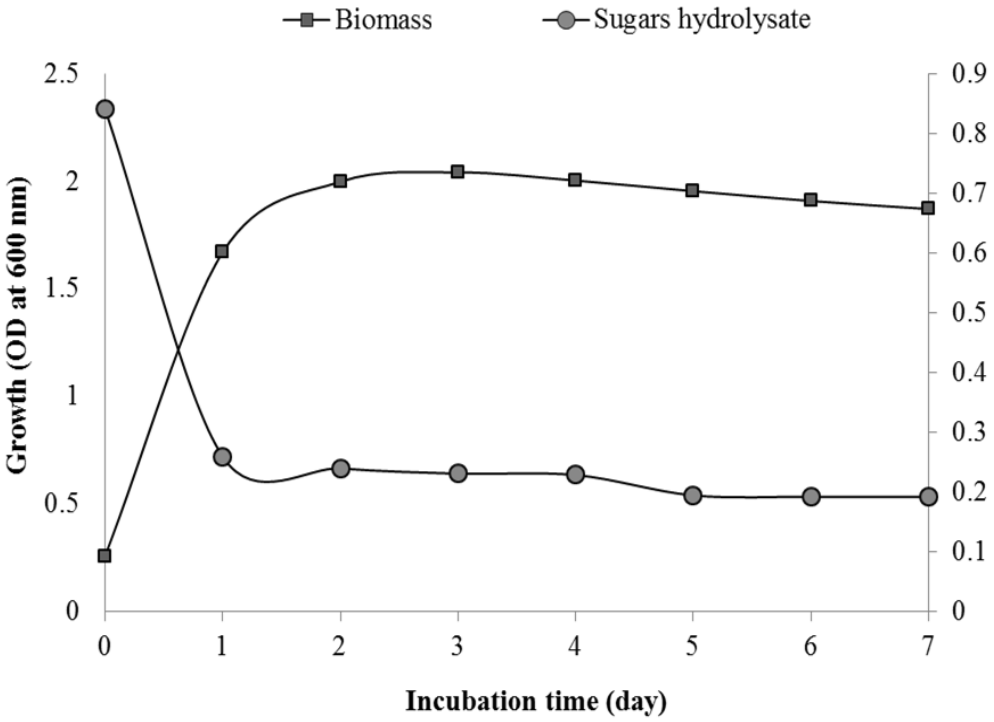


Figure 6.12 The time course of cell growth and reducing sugar concentration of DT3

6.4 Conclusion

The biorefinery aspect was applied by utilising sugar hydrolysate for ethanol production using *Saccharomyces cerevisiae* and PUFA production by thraustochytrids. *Saccharomyces cerevisiae* was able to ferment sugar hydrolysate into ethanol. Ethanol was detected and the concentration was determined by Raman spectroscopy and reverse-phase HPLC. The maximum amount of ethanol detected was relatively low at 1.6% after 48 h of fermentation. The culture was capable of producing ethanol under aerobic conditions.

The thraustochytrids utilised the sugar hydrolysate and demonstrated good DHA accumulation at different concentrations. The growth of microbes was greatest at low concentrations (2%) of sugar hydrolysate, as higher concentrations repressed microbial growth. Cultures grown on glucose were not found to exhibit this inhibition and yielded maximum cell growth at 8% concentration. The maximum concentration of total fatty acid was observed in media containing 2% of either carbon source

Summary and future directions

The production of second generation lignocellulosic biofuel using locally availability Victorian feedstock was the foundation to this project. Australia has a large amount of grassland, woodlands and feedstocks potentially available for biofuel production. In this project we explored the bioprocessing of locally produced waste biomass for the production of biofuel and commercially valuable co-products. The bioprocessing of lignocellulose is required to simplify the complex structure and enable its hydrolysis to produce a high yield of simple sugars.

Hemp (*Cannabis sativa*) which was used for this study was decorticated and the inner core (xylem and phloem) was milled to reduce the size and rigidity. The obtained hemp powder was dried and used for later bioprocessing steps. Although the particle size was reduced, pretreatment was still required to open the compact structure and make it porous. This was accomplished by using different pretreatment combinations such as chemicals (acid and alkaline) under autoclave conditions, higher temperature to obtain better porosity and bond breakage. The most successful pretreatment among these combinations was alkaline pretreatment because it provided the highest hydrolysis yield (74%) after enzyme saccharification. Alkaline pretreatment resulted in an improved open structure that was more accessible to enzyme hydrolysis. About 60-70% of the hydrolysis occurred in the initial 6 hours of incubation, irrespective of the pretreatment method, indicating high activity of the recombinant cellulase.

The different pentose and hexose sugars in the sugar hydrolysate detected by HPLC included cellobiose, glucose and xylose. This softwood comprises mainly holocellulose and other sugars such as mannose, arabinose, and glucan were not detected in the sugar hydrolysate. The maximum conversion of holocellulose to reducing sugars was obtained at a substrate concentration of 5% with different enzyme loadings and the activity of cellulase from *Acremonium* was higher than *Trichoderma*. The overnight washing of pretreated hemp under stirring conditions increased the yield of reducing sugars, indicating the removal of inhibitors which were previously detected in the sugars hydrolysate. This observation indicates that detoxification before enzyme hydrolysis of biomass is essential to obtain higher yields, since inhibitors are known to inhibit the product formation.

The morphological and structural characterisation of the hemp biomass were characterised before and after pretreatment. The hemp biomass was shown to have a compact and rigid structure with traces of lignin and extractives embedded in it. As a result of pretreatment the fibre bundles opened, crystallinity increased, and the amorphous biomass component were removed, enabled more ready access of the enzymes and improved biomass digestibility.

The enzyme loading is an important factor during the bioprocessing of biomass as it alters the production cost. To enable multiple reuse of the enzyme and lower the cost per hydrolysis cycle, cellulase was immobilised on magnetic nanoparticles. The activation of nanoparticle with a cross-linker helped to achieve the maximum observed immobilisation binding (94%). Immobilisation increased the stability properties of enzyme, making it stable at higher temperature (80 °C). The magnetic nanoparticle provided the advantage of easy separation from the reaction mixture, with the enzyme retaining 50% of its initial activity after five consecutive hydrolysis cycles. Immobilisation also resulted in improved storage stability. This preparation was successful for the hydrolysis of synthetic (CMC) and natural (pretreated hemp) substrate with good hydrolysis yield.

The utilisation of reducing sugars, obtained from different enzyme hydrolysis methods, was applied to the fermentation of microbes to realise the creation of a biorefinery for biofuel and co-product production from hemp biomass. The sugar hydrolysate was used as a carbon source in the fermentation medium to grow microbes capable of producing industrial important products such as ethanol and polyunsaturated fatty acids (PUFA). Techniques including reversed phase HPLC and Raman spectroscopy were used to quantify ethanol produced. Marine microbes were grown using different concentrations of sugar hydrolysate and their growth and lipid profiles were compared with those obtained using glucose as the carbon source (control). Both carbon sources produced similar amounts of cell dry weight and PUFA. This indicates that the project was successful in utilising and converting low cost, locally available feedstock into industrially important value added products via bioprocessing steps.

Future work could include a comparative study on the conversion of different Australian biomass into biofuel and co-products. Computational and statistical analysis can help to understand local availability of feedstock, land usage, conversion yield, statistical significance, feasibility with experimental design with the biomass available in Australia. The feasibility of the experimental design can be compared with theoretical and actual/experimental data.

Pretreatment methodologies have been development, including microwave pretreatment and the use of ionic liquid for hydrolysing synthetic cellulose however, same may be explored with respect to real biomass. Pretreatment chambers are also available commercially which allows control over pretreatment conditions, including pH, temperature, pressure and residence time. This will allow higher solid biomass loading under controlled pretreatment conditions and make the process more economical. These chambers can facilitate continuous or batch pretreatment processes, with controlled supervision of different parameters. Pretreated biomass should be dried at optimum or room temperate, as drying at higher temperature may damage the biomass structure. Another future area of investigation is enzyme loading, which is an important parameter for cost of biofuel production. Some of the commercially available cellulases (such as the accellerase series- Accellerase® 1500, DuPont) are very powerful. These products can be used for further enhancing the yield of reducing sugar from pretreated lignocellulosic biomass. A statistical approach can be used to understand the hydrolysis of biomass using enzyme cocktails at different experimental conditions and enzyme loading. Measures can be taken to improve the yield of ethanol by using monitoring systems/fermenters, recombinant strains, higher concentration of reducing sugars, or more efficiently pretreated biomass. It would also enhance process viability if simultaneous saccharification and fermentation (SSF) were applied to pretreated lignocellulosic biomass. The cost of processing can be lowered by using immobilised enzymes, low cost biomass, highly efficient recombinant enzymes, and backed by efficient pretreatment. Improving process efficiency is critical to future uptake of biofuel and cost competitiveness with fossil fuel production.

References

Abdel-Rahman, M. A., Tashiro, Y., Sonomoto, K., 2013. Recent advances in lactic acid production by microbial fermentation processes. Biotechnology Advances, 31 (6), 877-902.

Abraham, R. E., Barrow, C. J., Puri, M., 2013. Relationship to reducing sugar production and scanning electron microscope structure to pretreated hemp hurd biomass (*Cannabis sativa*). Biomass and Bioenergy, 58, 180-7.

Adenle, A. A., Haslam, G. E., Lee, L., 2013. Global assessment of research and development for algae biofuel production and its potential role for sustainable development in developing countries. Energy Policy, 61, 182-95.

Adhyaru, D. N., Bhatt, N. S., Modi, H. A., 2014. Enhanced production of cellulase-free, thermo-alkali-solvent-stable xylanase from *Bacillus altitudinis* DHN8, its characterization and application in sorghum straw saccharification. Biocatalysis and Agricultural Biotechnology, 3 (2), 182-90.

Ali, T., Huang, J., Yang, J., 2013. Impact assessment of global and national biofuels developments on agriculture in Pakistan. Applied Energy, 104, 466-74.

Amaducci, S., Scordia, D., Liu, F. H., Zhang, Q., Guo, H., Testa, G., Cosentino, S. L., 2014. Key cultivation techniques for hemp in Europe and China. Industrial Crops and Products, Article in Press.

Amorim, H. V., Lopes, M. L., Oliveira, J. V. C., Buckeridge, M. S., Goldman, G. H., 2011. Scientific challenges of bioethanol production in Brazil. Applied Microbiology and Biotechnology, 91, 1267-75.

Andric, P., Meyer, A. S., Jensen, P. A., Dam-Johansen, K., 2010. Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of cellobiose and glucose inhibition on cellulolytic enzymes. Biotechnology Advances, 28, 308-24.

Ansari, S. A., Husain, Q., 2012. Potential applications of enzymes immobilized on/in nano materials: A review. Biotechnology Advances, 30 (3), 512-23.

Aragon, C. C., Mateo, C., Ruiz-Matute, A. I., Corzo, N., Fernandez-Lorente, G., Sevillano, L., Diaz, M., Monti, R., Santamaria, R. I., Guisan, J. M., 2013. Production of xylo-oligosaccharides by immobilized-stabilized derivatives of endo-xylanase from *Streptomyces halstedii*. Process Biochemistry, 48 (3), 478-83.

Arslan, Y., Eken-Saracoglu, N., 2010. Effects of pretreatment methods for hazelnut shell hydrolysate fermentation with *Pichia Stipitis* to ethanol. Bioresource Technology, 101 (22), 8664-70.

Asgher, M., Shahid, M., Kamal, S., Iqbal, H. M. N., 2014. Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology. Journal of Molecular Catalysis B: Enzymatic, 101, 56-66.

Ask, M., Olofsson, K., Di, F. T., Ruohonen, L., Penttila, M., Liden, G., Olsson, L., 2012. Challenges in enzymatic hydrolysis and fermentation of pretreated *Arundo donax* revealed by a comparison between SHF and SSF. Process Biochemistry, 47 (10), 1452-9.

Avci, A., Saha, B. C., Kennedy, G. J., Cotta, M. A., 2013. High temperature dilute phosphoric acid pretreatment of corn stover for furfural and ethanol production. Industrial Crops and Products, 50, 478-84.

Azadi, P., Inderwildi, O. R., Farnood, R., King, D. A., 2013. Liquid fuels, hydrogen and chemicals from lignin: A critical review. Renewable and Sustainable Energy Reviews, 21, 506-23.

BAA. 2013. Biofuels Association of Australia. Ethanol Production facilities in Australia. (available online: http://www.biofuelsassociation.com.au/index.php?option=com_content&view=article&id=74&Itemid=91 latest visited 15 May 2014).

Bai, F. W., Anderson, W. A., Moo-Young, M., 2008. Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnology Advances, 26, 89-105.

Bai, Y., Ouyang, Y., Pang, J. S., 2012. Biofuel supply chain design under competitive agricultural land use and feedstock market equilibrium. Energy Economics, 34 (5), 1623-33.

Bakisgan, C., Dumanli, A. G., Yurum, Y., 2009. Trace elements in Turkish biomass fuels: Ashes of wheat straw, olive bagasse and hazelnut shell. Fuel, 88 (10), 1842-51.

Balat, M., 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Conversion and Management, 52 (2), 858-75.

Ballinger, R., 2008. Flexfuel-E85 Ethanol. Can my car use ethanol blended fuel E10? (available online: http://www.flexfuelkit.com.au/articles/8/1/Can-my-car-use-ethanol-blended-fuel-E10/Page1.html last visited 15 May 2014).

Bals, B., Wedding, C., Balan, V., Sendich, E., Dale, B., 2011. Evaluating the impact of ammonia fiber expansion (AFEX) pretreatment conditions on the cost of ethanol production. Bioresource Technology, 102 (2), 1277-83.

Barta, Z., Kovacs, K., Reczey, K., Zacchi, G., 2010. Process design and economics of on-site cellulase production on various carbon sources in a softwood-based ethanol plant. Enzyme Research, 2010, 1-8.

Barta, Z., Kreuger, E., Bjornsson, L., 2013. Effects of steam pretreatment and co-production with ethanol on the energy efficiency and process economics of combined biogas, heat and electricity production from industrial hemp. Biotechnology for Biofuels, 6 (1), 56.

Bayramoglu, G., Yilmaz, M., Arica, M. Y., 2004. Immobilization of a thermostable α-amylase onto reactive membranes: Kinetics characterization and application to continuous starch hydrolysis. Food Chemistry, 84 (4), 591-9.

Beg, Q., Kapoor, M., Mahajan, L., Hoondal, G., 2001. Microbial xylanases and their industrial applications: a review. Applied Microbiology and Biotechnology, 56 (3-4), 326-38.

Bellino, M. G., Regazzoni, A. E., 2011. Immobilization of enzymes into self-assembled iron(III) hydrous oxide nano-scaffolds: A bio-inspired one-pot approach to hybrid catalysts. Applied Catalysis A: General, 408 (1-2), 73-7.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., Saddler, J., 2006. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. Journal of Biotechnology, 125 (2), 198-209.

Berti, P., Levidow, L., 2014. Fuelling expectations: A policy-promise lock-in of UK biofuel policy. Energy Policy, 66, 135-43

Bertoli, A., Tozzi, S., Pistelli, L., Angelini, L. G., 2010. Fibre hemp inflorescences: From crop-residues to essential oil production. Industrial Crops and Products, 32 (3), 329-37.

Binod, P., Janu, K. U., Sindhu, R., Pandey, A. 2011. Hydrolysis of lignocellulosic biomass for bioethanol production. Biofuels: alternative feedstocks and conversion processes, chapter-10, 229-50.

Binod, P., Sindhu, R., Singhania, R. R., Vikram, S., Devi, L., Nagalakshmi, S., Kurien, N., Sukumaran, R. K., Pandey, A., 2010. Bioethanol production from rice straw: An overview. Bioresource Technology, 101 (13), 4767-74.

Biswas, A. K., Umeki, K., Yang, W., Blasiak, W., 2011. Change of pyrolysis characteristics and structure of woody biomass due to steam explosion pretreatment. Fuel Processing Technology, 92 (10), 1849-54.

Blanco-Canqui, H., Lal, R., 2007. Soil and crop response to harvesting corn residues for biofuel production. Geoderma, 141 (3-4), 355-62.

Bond, J. Q., Upadhye, A. A., Olcay, H., Tompsett, G. A., Jae, J., Xing, R., Alonso, D. M., Wang, D., Zhang, T., Kumar, R., Foster, A., Sen, S. M., Maravelias, C. T., Malina, R., Barrett, S. R. H., Lobo, R., Wyman, C. E., Dumesic, J. A., Huber, G. W., 2014. Production of renewable jet fuel range alkanes and commodity chemicals from integrated catalytic processing of biomass. Energy and Environmental Science, 7 (4), 1500-23.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72 (1-2), 248-54.

Brady, D., Jordaan, J., 2009. Advances in enzyme immobilisation. Biotechnology Letters, 31 (11), 1639-50.

Bradshaw, C. J. A., 2012. Little left to lose: deforestation and forest degradation in Australia since European colonization. Journal of Plant Ecology, 5 (1), 109-20.

Brethauer, S., Wyman, C. E., 2010. Review: Continuous hydrolysis and fermentation for cellulosic ethanol production. Bioresource Technology, 101, 4862-74.

BRS. 2009. Australian forest profiles: Eucalypts (Bureau of Rural Science). 1-8.

Brugnagoa, R. J., Satyanarayanab, K. G., Wypycha, F., Ramos, L. P., 2011. The effect of steam explosion on the production of sugarcane bagasse/polyester composites. Composites Part A: Applied Science and Manufacturing, 42 (4), 364-70.

Brummer, V., Jurena, T., Hlavacek, V., Omelkova, J., Bebar, L., Gabriel, P., Stehlik, P., 2014. Enzymatic hydrolysis of pretreated waste paper - Source of raw material for production of liquid biofuels. Bioresource Technology, 152, 543-7.

Burja, A. M., Radianingtyas, H., Windust, A., Barrow, C. J., 2006. Isolation and characterization of polyunsaturated fatty acid producing *Thraustochytrium* species: screening of strains and optimization of omega-3 production. Applied Microbiology and Biotechnology, 72 (6), 1161-9.

Buyx, A., Tait, J., 2011. Ethical Framework for Biofuels. Science, 332 (6029), 540-1.

Call, H. P., Mücke, I., 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). Journal of Biotechnology, 53 (2-3), 163-202.

Caltex. 2011. Bio Fuels. (available online: http://www.caltex.com.au/ProductsAndServices/Pages/BioFuels.aspx last visited 16 May 2014).

Cao, G., Ximenes, E., Nichols, N. N., Zhang, L., Ladisch, M., 2013. Biological abatement of cellulase inhibitors. Bioresource Technology, 146, 604-10.

Cao, L., 2005. Immobilised enzymes: science or art? Current Opinion in Chemical Biology, 9 (2), 217-26.

Cao, X., Zhong, L., Peng, X., Sun, S., Li, S., Liu, S., Sun, R., 2014. Comparative study of the pyrolysis of lignocellulose and its major components: Characterization and overall distribution of their biochars and volatiles. Bioresource Technology, 155, 21-7.

Cara, C., Ruiz, E., Oliva, J. M., Saez, F., Castro, E., 2008. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. Bioresource Technology, 99 (6), 1869-76.

Carrier, M., Loppinet, S. A., Denux, D., Lasnier, J. M., Ham, P. F., Cansell, F., Aymonier, C., 2011. Thermogravimetric analysis as a new method to determine the lignocellulosic composition of biomass. Biomass and Bioenergy, 35 (1), 298-307.

Castro, E., Nieves, I. U., Mullinnix, M. T., Sagues, W. J., Hoffman, R. W., Fernandez-Sandoval, M. T., Tian, Z., Rockwood, D. L., Tamang, B., Ingram, L. O., 2014. Optimization of dilute-phosphoric-acid steam pretreatment of *Eucalyptus benthamii* for biofuel production. Applied Energy, 125, 76-83.

Cavka, A., Jonsson, L. J., 2013. Detoxification of lignocellulosic hydrolysates using sodium borohydride. Bioresource Technology, 136, 368-76.

CEC. 2008. Australian Bioenergy Roadmap (available online: http://www.energyfarmers.com.au/bioenergy-roadmap-a-good-plan-for-the-future/ last visited 16 May 2014).

Chadwick, D. T., McDonnell, K. P., Brennan, L. P., Fagan, C. C., Everard, C. D., 2014. Evaluation of infrared techniques for the assessment of biomass and biofuel quality parameters and conversion technology processes: A review. Renewable and Sustainable Energy Reviews, 30, 672-81.

Chandel, A. K., Chandrasekhar, G., Radhika, K., Ravinder, R., Ravindra, P., 2011. Bioconversion of pentose sugars into ethanol: A reveiw and future directions. Biotechnology and Molecular Biology Reveiw, 6 (1), 008-20.

Chang, R. H. Y., Jang, J., Wu, K. C. W., 2011. Cellulase immobilized mesoporous silica nanocatalysts for efficient cellulose-to-glucose conversion. Green Chemistry, 13 (10), 2844-50.

Chase, M., 1998. The Angiosperm phylogeny group: an ordinal classification for the families of flowering plants. Annals of the Missouri Botanical Garden, 85, 531-553.

Chen, B. Y., Chen, S. W., Wang, H. T., 2012. Use of different alkaline pretreatments and enzyme models to improve low-cost cellulosic biomass conversion. Biomass and Bioenergy, 39, 182-91.

Chen, C. L., Qi, W., Wang, J. Y., 2013a. Microbial cocktail for bioconversion of green waste to reducing sugars. Journal of Bioscience and Bioengineering, 115 (1), 82-5.

Chen, H., Qiu, W., 2010. Key technologies for bioethanol production from lignocellulose. Biotechnology Advances, 28 (5), 556-62.

Chen, L., Xing, L., Han, L., 2009. Renewable energy from agro-residues in China: Solid biofuels and biomass briquetting technology. Renewable and Sustainable Energy Reviews, 13 (9), 2689-95.

Chen, M., Zhao, J., Xia, L., 2008. Enzymatic hydrolysis of maize straw polysaccharides for the production of reducing sugars. Carbohydrate Polymers, 71 (3), 411-5.

Chen, Y., Stevens, M., Zhu, Y., Holmes, J., Xu, H., 2013b. Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification. Biotechnology for Biofuels, 6 (1), 8.

Cheng, C., Chang, K. C., 2013. Development of immobilized cellulase through functionalized gold nano-particles for glucose production by continuous hydrolysis of waste bamboo chopsticks. Enzyme and Microbial Technology, 53 (6-7), 444-51.

Cheng, K. K., Cai, B. Y., Zhang, J. A., Ling, H. Z., Zhou, Y. J., Ge, J. P., Xu, J. M., 2008. Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochemical Engineering Journal, 38 (1), 105-9.

Cheng, N. G., Hasan, M., Kumoro, A. C., Ling, C. F., Tham, M., 2009. Production of ethanol by fed-batch fermentation. Journal Science and Technology Pertanika, 17 (2), 399-408.

Cheng, Z., Wu, C., Yang, W., Xu, T., 2010. Preparation of bromomethylated poly(2,6-dimethyl-1,4-phenylene oxide) hollow fiber cation-exchange membranes and immobilization of cellulase thereon. Journal of Membrane Science, 358 (1-2), 93-100.

Cho, E. J., Jung, S., Kim, H. J., Lee, Y. G., Nam, K. C., Lee, H. J., Bae, H. J., 2012. Co-immobilization of three cellulases on Au-doped magnetic silica nanoparticles for the degradation of cellulose. Chemical Communications, 48 (6), 886-8.

Chou, C. H., 2009. *Miscanthus* plants used as an alternative biofuel material: The basic studies on ecology and molecular evolution. Renewable Energy, 34 (8), 1908-12.

Chu, B. C. H., Lee, H., 2007. Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation. Biotechnology Advances, 25 (5), 425-41.

Chundawat, S. P. S., Vismeh, R., Sharma, L. N., Humpula, J. F., Sousa, L., Chambliss, C. K., Jones, A. D., Balan, V., Dale, B. E., 2010. Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. Bioresource Technology, 101 (21), 8429-38.

Cianchetta, S., Galletti, S., Burzi, P. L., Cerato, C., 2012. Hydrolytic potential of *Trichoderma* sp. strains evaluated by microplate-based screening followed by switchgrass saccharification. Enzyme and Microbial Technology, 50 (6-7), 304-10.

Conde-Mejia, C., Jimenez-Gutierrez, A., El-Halwagi, M., 2012. A comparison of pretreatment methods for bioethanol production from lignocellulosic materials. Process Safety and Environmental Protection, 90 (3), 189-202.

Contestabile, M., 2014. Energy: European biorefineries. Nature Climate Change, 4 (2), 87.

Correia, J. A. C., Junior, J. E. M., Goncalves, L. R. B., Rocha, M. V. P., 2013. Alkaline hydrogen peroxide pretreatment of cashew apple bagasse for ethanol production: Study of parameters. Bioresource Technology, 139, 249-56.

Creagh, C., Jennings, P., Dale, M., 2004. Fact Sheet 8 Biomass. Facts and Figures. The Australian Institute of Energy, 1-7.

Damartzis, T., Vamvuka, D., Sfakiotakis, S., Zabaniotou, A., 2011. Thermal degradation studies and kinetic modeling of cardoon (*Cynara cardunculus*) pyrolysis using thermogravimetric analysis (TGA). Bioresource Technology, 102 (10), 6230-8.

Da Porto, C., Decorti, D., Tubaro, F., 2012. Fatty acid composition and oxidation stability of hemp (*Cannabis sativa L.*) seed oil extracted by supercritical carbon dioxide. Industrial Crops and Products, 36 (1), 401-4.

Das, S., Berke-Schlessel, D., Ji, H. F., McDonough, J., Wei, Y., 2011. Enzymatic hydrolysis of biomass with recyclable use of cellobiase enzyme immobilized in sol–gel routed mesoporous silica. Journal of Molecular Catalysis B: Enzymatic, 70 (1-2), 49-54.

Demirbas, A., 2011. Competitive liquid biofuels from biomass. Applied Energy, 88 (1), 17-28.

Davis, R., Tao, L., Tan, E.C.D., Biddy, M.J., Beckham, G.T., Scarlata, C., Jacobson, J., Cafferty, K., Ross, J., Lukas, J., Knorr, D., and Schoen, P., 2013. Process Design and Economics for the Conversion of Lignocellulosic Biomass to Hydrocarbons: Dilute-Acid and Enzymatic Deconstruction of Biomass to Sugars and Biological Conversion of Sugars to Hydrocarbons; Technical Report (NREL/TP-5100-60223); National Renewable Energy Laboratory: Golden, Colorado, pp 1-128.

Dewapriya, P., Kim, S., 2014. Marine microorganisms: An emerging avenue in modern nutraceuticals and functional foods. Food Research International, 56, 115-25.

DiCosimo, R., McAuliffe, J., Poulose, A. J., Bohlmann, G., 2013. Industrial use of immobilized enzymes. Chemical Society Reviews, 42 (15), 6437-74.

Dornenburg, H., 2004. Evaluation of immobilisation effects on metabolic activities and productivity in plant cell processes. Process Biochemistry, 39 (11), 1369-75.

Du, S., Zhu, X., Wang, H., Zhou, D., Yang, W., Xu, H., 2013. High pressure assist-alkali pretreatment of cotton stalk and physiochemical characterization of biomass. Bioresource Technology, 148, 494-500.

El-Nashaar, H. M., Griffith, S. M., Steiner, J. J., Banowetz, G. M., 2009. Mineral concentration in selected native temperate grasses with potential use as biofuel feedstock. Bioresource Technology, 100 (14), 3526-31.

Eliana, C., Jorge, R., Juan, P., Luis, R., 2014. Effects of the pretreatment method on enzymatic hydrolysis and ethanol fermentability of the cellulosic fraction from elephant grass. Fuel, 118, 41-7.

Elliston, A., Collins, S. R. A., Wilson, D. R., Roberts, I. N., Waldron, K. W., 2013. High concentrations of cellulosic ethanol achieved by fed batch semi simultaneous saccharification and fermentation of waste-paper. Bioresource Technology, 134, 117-26.

Escobar, J. C., Lora, E. S., Venturini, O. J., Yanez, E. E., Castillo, E. F., Almazan, O., 2009. Biofuels: Environment, technology and food security. Renewable and Sustainable Energy Reviews, 13, 1275-87.

Ethier, S., Woisard, K., Vaughan, D., Wen, Z., 2011. Continuous culture of the microalgae *Schizochytrium limacinum* on biodiesel-derived crude glycerol for producing docosahexaenoic acid. Bioresource Technology, 102 (1), 88-93.

Ewanick, S., Thompson, W., Marquardt, B., Bura, R., 2013. Real-time understanding of lignocellulosic bioethanol fermentation by Raman spectroscopy. Biotechnology for Biofuels, 6 (1), 28.

Fairley, P., 2011. Introduction: Next generation biofuels. Nature, 474 (7352), S2-S5.

Falkoski, D. L., Guimaraes, V. M., de Almeida, M. N., Alfenas, A. C., Colodette, J. L., de Rezende, S. T., 2013. *Chrysoporthe cubensis*: A new source of cellulases and hemicellulases to application in biomass saccharification processes. Bioresource Technology, 130, 296-305.

Farmilo, B. J., Nimmo, D. G., Morgan, J. W., 2013. Pine plantations modify local conditions in forest fragments in southeastern Australia: Insights from a fragmentation experiment. Forest Ecology and Management, 305, 264-72.

FCAI. 2006. Can my vehicle operate on Ethanol blend petrol? (Federal chamber of Automotive Industries) (available online: http://www.fcai.com.au/environment/can-my-vehicle-operate-on-ethanol-blend-petrol last visited 20 May 2014).

Festel, G., Wurmseher, M., Rammer, C., Boles, E., Bellof, M., 2014. Modelling production cost scenarios for biofuels and fossil fuels in Europe. Journal of Cleaner Production, 66, 242-53.

Findlater, K. M., Kandlikar, M., 2011. Land use and second-generation biofuel feedstocks:The unconsidered impacts of *Jatropha* biodiesel in Rajasthan, India. Energy Policy, 39, 3404-13.

Finnan, J., Styles, D., 2013. Hemp: A more sustainable annual energy crop for climate and energy policy. Energy Policy, 58, 152-62.

Florin, M. J., van de Ven, G. W. J., van Ittersum, M. K., 2014. What drives sustainable biofuels? A review of indicator assessments of biofuel production systems involving smallholder farmers. Environmental Science and Policy, 37, 142-57.

Fujii, T., Fang, X., Inoue, H., Murakami, K., Sawayama, S., 2009. Enzymatic hydrolyzing performance of *Acremonium cellulolyticus* and *Trichoderma reesei* against three lignocellulosic materials. Biotechnology for Biofuels, 2 (24), 1-8.

Gandolfi, S., Ottolina, G., Riva, S., Fantoni, G. P., Patel, I., 2013. Complete chemical analysis of carmagnola hemp hurds and structural features of its components. BioResources, 8(2), 2641-56.

Gabrielle, B., Gagnaire, N., Massad, R. S., Dufosse, K., Bessou, C., 2014. Environmental assessment of biofuel pathways in Ile de France based on ecosystem modeling. Bioresource Technology, 152, 511-8.

Galazka, J. M., Tian, C., Beeson, W. T., Martinez, B., Glass, N. L., Cate, J. H. D., 2010. Cellodextrin transport in yeast for improved biofuel production. Science, 330 (6000), 84-6.

Galbe, M., Zacchi, G., 2012. Pretreatment: The key to efficient utilization of lignocellulosic materials. Biomass and Bioenergy, 46, 70-8.

Gao, M., Xu, F., Li, S., Ji, X., Chen, S., Zhang, D., 2010. Effect of SC-CO_2 pretreatment in increasing rice straw biomass conversion. Biosystems Engineering, 106 (4), 470-5.

Garai, D., Kumar, V., 2013. A Box-Behnken design approach for the production of xylanase by *Aspergillus candidus* under solid state fermentation and its application in saccharification of agro residues and *Parthenium hysterophorus L*. Industrial Crops and Products, 44, 352-63.

Garcia-Galan, C., Berenguer-Murcia, A., Fernandez-Lafuente, R., Rodrigues, R. C., 2011. Potential of different enzyme immobilization strategies to improve enzyme performance. Advanced Synthesis and Catalysis, 353 (16), 2885-904.

Garcia, C., Jaldon, Dupeyre, D., Vignon, M. R., 1998. Fibres from semi-retted hemp bundles by steam explosion treatment. Biomass and Bioenergy, 14 (3), 251-60.

Garside, P., Wyeth, P., 2003. Identification of cellulosic fibres by FTIR spectroscopy: thread and single fibre analysis by attenuated total reflectance. Studies in Conservation, 48 (4), 269-75.

Geddes, C. C., Nieves, I. U., Ingram, L. O., 2011. Advances in ethanol production. Current Opinion in Biotechnology, 22, 312-9.

Georgelin, T., Maurice, V., Malezieux, B., Siaugue, J. M., Cabuil, V., 2010. Design of multifunctionalized γ-Fe2O3@SiO2 core–shell nanoparticles for enzymes immobilization. Journal of Nanoparticle Research, 12 (2), 675-80.

Ghaffar, S. H., Fan, M., 2014. Lignin in straw and its applications as an adhesive. International Journal of Adhesion and Adhesives, 48, 92-101.

Ghose, T. K., 1987. Measurement of cellulase activities. Pure and Applied Chemistry, 59 (2), 257-68.

Ghose, T. K., Bisaria, V. S., 1987. Measurement of hemicellulase activities part 1: xylanase. Pure and Applied Chemistry, 59 (12), 1739-52.

Girio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., Bogel-Lukasik, R., 2010. Hemicelluloses for fuel ethanol: A review. Bioresource Technology, 101 (13), 4775-800.

Goh, C. S., Lee, K. T., 2011. Second-generation biofuel (SGB) in Southeast Asia via lignocellulosic biorefinery: Penny-foolish but pound-wise. Renewable and Sustainable Energy Reviews, 15 (6), 2714-8.

Golicnik, M., 2011. Evaluation of enzyme kinetic parameters using explicit analytic approximations to the solution of the Michaelis–Menten equation. Biochemical Engineering Journal, 53 (2), 234-8.

Gonzalez-Garcia, S., Luo, L., Moreira, M. T., Feijoo, G., Huppes, G., 2012. Life cycle assessment of hemp hurds use in second generation ethanol production. Biomass and Bioenergy, 36, 268-79.

Gonzalez, G. S., Moreira, M. T., Feijoo, G., Murphy, R. J., 2012. Comparative life cycle assessment of ethanol production from fast-growing wood crops (black locust, eucalyptus and poplar). Biomass and Bioenergy, 39, 378-88.

Graham-Rowe, D., 2011. Agriculture: Beyond food versus fuel. Nature, 474 (7352), S6-S8.

Gronenberg, L. S., Marcheschi, R. J., Liao, J. C., 2013. Next generation biofuel engineering in prokaryotes. Current Opinion in Chemical Biology, 17 (3), 462-71.

Grzenia, D., Schell, D., Wickramasinghe, S., 2008. Membrane extraction for removal of acetic acid from biomass hydrolysates. Journal of Membrane Science, 322 (1), 189-95.

Gunnur, K., Nilgun, C., 2013. An overview of biofuels from energy crops: Current status and future prospects. Renewable and Sustainable Energy Reviews, 28, 900-16.

Guo, Z., Qiu, C., Zhang, L., Ding, Z., Wang, Z. X., Shi, G. Y., 2011. Expression of aspartic protease from *Neurospora crassa* in industrial ethanol-producing yeast and its application in ethanol production. Enzyme and Microbial Technology, 48 (2), 148-54.

Gupta, A., Singh, D., Barrow, C. J., Puri, M., 2013. Exploring potential use of Australian thraustochytrids for the bioconversion of glycerol to omega-3 and carotenoids production. Biochemical Engineering Journal, 78, 11-7.

Gupta, A., Verma, J. P., 2015. Sustainable bio-ethanol production from agro-residues: A review. Renewable and Sustainable Energy Reviews, 41, 550-67.

Gupta, A., Vongsvivut, J., Barrow, C. J., Puri, M., 2012. Molecular identification of marine yeast and its spectroscopic analysis establishes unsaturated fatty acid accumulation. Journal of Bioscience and Bioengineering, 114 (4), 411-7.

Guschina, I. A., Harwood, J. L., 2006. Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research, 45 (2), 160-86.

Gutterson, N., Zhang, J., 2009. Important issues and current status of bioenergy crop policy for advanced biofuels. Biofuels, Bioproducts and Biorefining, 3 (4), 441-7.

Hammond, G. P., Seth, S. M., 2013. Carbon and environmental footprinting of global biofuel production. Applied Energy, 112, 547-59.

Hanefeld, U., Gardossi, L., Magner, E., 2009. Understanding enzyme immobilisation. Chemical Society Reviews, 38 (2), 453-68.

Hart, C., 2008. Biofuel demand fuels CSR's move to more ethanol production. Business with The Wall Street Journal. (available online: http://www.theaustralian.com.au/business/biofuel-demand-fuels-csrs-ethanol/story-e6frg8zx-1111117084732 last visited 15 May 2014).

Harun, R., Danquah, M. K., 2011. Influence of acid pre-treatment on microalgal biomass for bioethanol production. Process Biochemistry, 46 (1), 304-9.

Hegedus, I., Hancsok, J., Nagy, E., 2012. Stabilization of the Cellulase Enzyme Complex as Enzyme Nanoparticle. Applied Biochemistry and Biotechnology, 168 (6), 1372-83.

Hendriks, A. T. W. M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology, 100, 10-8.

Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., Foust, T. D., 2007. Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production. Science, 315 (5813), 804-7.

Hodge, D. B., Andersson, C., Berglund, K. A., Rova, U., 2009. Detoxification requirements for bioconversion of softwood dilute acid hydrolyzates to succinic acid. Enzyme and Microbial Technology, 44 (5), 309-16.

Hong, W. K., Kim, C. H., Rairakhwada, D., Kim, S., Hur, B. K., Kondo, A., Seo, J. W., 2012. Growth of the oleaginous microalga *Aurantiochytrium* sp. KRS101 on cellulosic biomass and the production of lipids containing high levels of docosahexaenoic acid. Bioprocess and Biosystems Engineering, 35 (1-2), 129-33.

Hongqiang, L., Hongzhang, C., 2008. Detoxification of steam-exploded corn straw produced by an industrial-scale reactor. Process Biochemistry 43, 1447-51.

Hosseini, S. A., Shah, N., 2009. Multiscale modelling of hydrothermal biomass pretreatment for chip size optimization. Bioresource Technology, 100, 2621-8.

Hua, M., Phillips, F., 2011. Technological evolution and interdependence in China's emerging biofuel industry. Technological Forecasting & Social Change, 78 (7), 1130-46.

Huang, J., Yang, J., Msangi, S., Rozelle, S., Weersink, A., 2012a. Global biofuel production and poverty in China. Applied Energy, 98, 246-55.

Huang, T. Y., Lu, W. C., Chu, I. M., 2012b. A fermentation strategy for producing docosahexaenoic acid in *Aurantiochytrium limacinum* SR21 and increasing C22:6 proportions in total fatty acid. Bioresource Technology, 123, 8-14.

Huang, W., Zhang, Y. H. P., 2011. Analysis of biofuels production from sugar based on three criteria:Thermodynamics, bioenergetics, and product separation. Energy and Environmental Science, 4, 784-92.

Hung, T. C., Fu, C. C., Su, C. H., Chen, J. Y., Wu, W. T., Lin, Y. S., 2011. Immobilization of cellulase onto electrospun polyacrylonitrile (PAN) nanofibrous membranes and its application to the reducing sugar production from microalgae. Enzyme and Microbiology Technology, 49 (1), 30-7.

Inoue, H., Yano, S., Endo, T., Sakaki, T., Sawayama, S., 2008. Combining hot-compressed water and ball milling pretreatments to improve the efficiency of the enzymatic hydrolysis of eucalyptus. Biotechnology for Biofuels, 1 (2), 1-9.

Jagtap, S. S., Woo, S. M., Kim, T. S., Dhiman, S. S., Kim, D., Lee, J. K., 2014. Phytoremediation of diesel-contaminated soil and saccharification of the resulting biomass. Fuel, 116, 292-8.

Jaldon, C. G., Dupeyre, D., Vignon, M.R., 1998. Fibers from semi-retted hemp bundles by steam explosion treatment. Biomass Bioenergy 14, 251-260.

Jardine, J., 2006. BP Brings Biofuels into the Mainstream. (available online: http://www.bp.com/en_au/australia/media/media-releases/bp-brings-biofuels-into-the-mainstream.html last visited 16 May 2014).

Jenkins, T. L., Sutherland, J. W., 2014. A cost model for forest-based biofuel production and its application to optimal facility size determination. Forest Policy and Economics, 38, 32-9.

Jeoh, T., Santa-Maria, M. C., O'Dell, P. J., 2013. Assessing cellulose microfibrillar structure changes due to cellulase action. Carbohydrate Polymers, 97 (2), 581-6.

Jiang, L., Hu, S., Sun, L., Su, S., Xu, K., He, L., Xiang, J., 2013a. Influence of different demineralization treatments on physicochemical structure and thermal degradation of biomass. Bioresource Technology, 146, 254-60.

Jiang, L. Q., Fang, Z., Li, X. K., Luo, J., Fan, S. P., 2013b. Combination of dilute acid and ionic liquid pretreatments of sugarcane bagasse for glucose by enzymatic hydrolysis. Process Biochemistry, 48 (12), 1942-6.

Jingura, R. M., 2011. Technical options for optimization of production of *Jatropha* as a biofuel feedstock in arid and semi-arid areas of Zimbabwe. Biomass and Bioenergy, 35 (5), 2127-32.

Jones, P., Vasudevan, P., 2010. Cellulose hydrolysis by immobilized *Trichoderma reesei* cellulase. Biotechnology Letters, 32 (1), 103-6.

Jordan, J., Kumar, C. S. S. R., Theegala, C., 2011. Preparation and characterization of cellulase-bound magnetite nanoparticles. Journal of Molecular Catalysis B: Enzymatic, 68 (2), 139-46.

Joyce, B. L., Stewart, J. C. N., 2012. Designing the perfect plant feedstock for biofuel production: Using the whole buffalo to diversify fuels and products. Biotechnology Advances, 30 (5), 1011-22.

Ju, X., Engelhard, M., Zhang, X., 2013. An advanced understanding of the specific effects of xylan and surface lignin contents on enzymatic hydrolysis of lignocellulosic biomass. Bioresource Technology, 132, 137-45.

Jun, Y., Jikun, H., Huanguang, Q., Scott, R., Sombilla, M. A., 2009. Biofuels and the Greater Mekong Subregion: Assessing the impact on prices, production and trade. Applied Energy, 86 (Supplement 1), S37-S46.

Jung, C. D., Yu, J. H., Eom, I. Y., Hong, K. S., 2013. Sugar yields from sunflower stalks treated by hydrothermolysis and subsequent enzymatic hydrolysis. Bioresource Technology, 138, 1-7.

Jutakanoke, R., Leepipatpiboon, N., Tolieng, V., Kitpreechavanich, V., Srinorakutara, T., Akaracharanya, A., 2012. Sugarcane leaves: Pretreatment and ethanol fermentation by *Saccharomyces cerevisiae*. Biomass and Bioenergy, 39, 283-9.

Juturu, V., Wu, J. C., 2014. Microbial cellulases: Engineering, production and applications. Renewable and Sustainable Energy Reviews, 33, 188-203.

Kalyani, D., Lee, K. M., Kim, T. S., Li, J., Dhiman, S. S., Kang, Y. C., Lee, J. K., 2013. Microbial consortia for saccharification of woody biomass and ethanol fermentation. Fuel, 107, 815-22.

Kamireddy, S. R., Li, J., Abbina, S., Berti, M., Tucker, M., Ji, Y., 2013. Converting forage sorghum and sunn hemp into biofuels through dilute acid pretreatment. Industrial Crops and Products, 49, 598-609.

Kang, S., Li, X., Fan, J., Chang, J., 2013. Hydrothermal conversion of lignin: A review. Renewable and Sustainable Energy Reviews, 27, 546-58.

Kapoor, M., Beg, Q. K., Bhushan, B., Singh, K., Dadhich, K. S., Hoondal, G. S., 2001. Application of an alkaline and thermostable polygalacturonase from *Bacillus* sp. MG-cp-2 in degumming of ramie (*Boehmeria nivea*) and sunn hemp (*Crotalaria juncea*) bast fibres. Process Biochemistry, 36 (8-9), 803-7.

Karthika, K., Arun, A. B., Rekha, P. D., 2012. Enzymatic hydrolysis and characterization of lignocellulosic biomass exposed to electron beam irradiation. Carbohydrate Polymers, 90 (2), 1038-45.

Kasel, S., Bennett, L. T., 2007. Land-use history, forest conversion, and soil organic carbon in pine plantations and native forests of south eastern Australia. Geoderma, 137 (3-4), 401-13.

Kataria, R., Ruhal, R., Babu, R., Ghosh, S., 2013. Saccharification of alkali treated biomass of Kans grass contributes higher sugar in contrast to acid treated biomass. Chemical Engineering Journal, 230, 36-47.

Kazi, F. K., Fortman, J., Anex, R., Kothandaraman, G., Hsu, D., Aden, A., Dutta, A., 2010. Techno-Economic Analysis of Biochemical Scenarios for Production of Cellulosic Ethanol Technical Report (NREL/TP-6A2-46588); National Renewable Energy Laboratory: Golden, Colorado, pp 1-92.

Kennedy, J. F., Kalogerakis, B., Cabral, J. M. S., 1984. Surface immobilization and entrapping of enzymes on glutaraldehyde crosslinked gelatin particles. Enzyme and Microbial Technology, 6 (3), 127-31.

Keown, D. M., Li, X., Hayashi, J., Li, C. Z., 2008. Evolution of biomass char structure during oxidation in O_2 as revealed with FT-Raman spectroscopy. Fuel Processing Technology, 89 (12), 1429-35.

Khoshnevisan, K., Bordbar, A. K., Zare, D., Davoodi, D., Noruzi, M., Barkhi, M., Tabatabaei, M., 2011. Immobilization of cellulase enzyme on superparamagnetic nanoparticles and determination of its activity and stability. Chemical Engineering Journal, 171 (2), 669-73.

Kim, Y., Ximenes, E., Mosier, N. S., Ladisch, M. R., 2011. Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. Enzyme and Microbial Technology, 48, 408-15.

King, D. A., 1996. A model to evaluate factors controlling growth in Eucalyptus plantations of southeastern Australia. Ecological Modelling, 87 (1-3), 181-203.

Kobayashi, H., Fukuoka, A., 2013. Synthesis and utilisation of sugar compounds derived from lignocellulosic biomass. Green Chemistry, 15 (7), 1740-63.

Kocar, G., Civas, N., 2013. An overview of biofuels from energy crops: Current status and future prospects. Renewable and Sustainable Energy Reviews, 28, 900-16.

Kohler, J., Walz, R., Marscheder-Weidemann, F., Thedieck, B., 2014. Lead markets in 2nd generation biofuels for aviation: A comparison of Germany, Brazil and the USA. Environmental Innovation and Societal Transitions, 10, 59-76.

Kopetz, H., 2013. Renewable resources: Build a biomass energy market. Nature, 494 (7435), 29-31.

Kreuger, E., Sipos, B., Zacchi, G., Svensson, S.-E., Bjornsson, L., 2011. Bioconversion of industrial hemp to ethanol and methane: The benefits of steam pretreatment and co-production. Bioresource Technology, 102 (3), 3457-65.

Kumar, P., Barrett, D. M., Delwiche, M. J., Stroeve, P., 2009a. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial and Engineering Chemistry Research, 48 (8), 3713-29.

Kumar, R., Hu, F., Sannigrahi, P., Jung, S., Ragauskas, A. J., Wyman, C. E., 2013. Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion. Biotechnology and Bioengineering, 110 (3), 737-53.

Kumar, R., Mago, G., Balan, V., Wyman, C. E., 2009b. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresource Technology, 100 (17), 3948-62.

Kuo, C. H., Lee, C. K., 2009. Enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments. Carbohydrate Polymers, 77 (1), 41-6.

Kwon, H. Y., Mueller, S., Dunn, J. B., Wander, M. M., 2013. Modeling state-level soil carbon emission factors under various scenarios for direct land use change associated with United States biofuel feedstock production. Biomass and Bioenergy, 55, 299-310.

Laatikainen, M., Heinonen, J., Sainio, T., 2011. Modeling of chromatographic separation of concentrated-acid hydrolysates. Separation and Purification Technology, 80 (3), 610-9.

Laurent, N., Haddoub, R., Flitsch, S. L., 2008. Enzyme catalysis on solid surfaces. Trends in Biotechnology, 26 (6), 328-37.

Lee, H. J., Lim, W. S., Lee, J. W., 2013. Improvement of ethanol fermentation from lignocellulosic hydrolysates by the removal of inhibitors. Journal of Industrial and Engineering Chemistry, 19 (6), 2010-5.

Lee, J. W., Trinh, L. T. P., Lee, H. J., 2014. Removal of inhibitors from a hydrolysate of lignocellulosic biomass using electrodialysis. Separation and Purification Technology, 122, 242-7.

Lestander, T. A., Finell, M., Samuelsson, R., Arshadi, M., Thyrel, M., 2012. Industrial scale biofuel pellet production from blends of unbarked softwood and hardwood stems-the effects of raw material composition and moisture content on pellet quality. Fuel Processing Technology, 95, 73-7.

Li, H., Ren, Y., 2011. The effect of SEM preparation on pit membrane remnants in vessel element end-walls of primitive angiosperms. Flora, 206 (4), 310-5.

Li, X., Wang, S., Du, G., Wu, Z., Meng, Y., 2013. Variation in physical and mechanical properties of hemp stalk fibers along height of stem. Industrial Crops and Products, 42, 344-8.

Li, Y., Pickering, K. L., 2008. Hemp fibre reinforced composites using chelator and enzyme treatments. Composites Science and Technology, 68 (15-16), 3293-8.

Liang, W., Cao, X., 2012. Preparation of a pH-sensitive polyacrylate amphiphilic copolymer and its application in cellulase immobilization. Bioresource Technology, 116, 140-6.

Liao, Z., Huang, Z., Hu, H., Zhang, Y., Tan, Y., 2011. Microscopic structure and properties changes of cassava stillage residue pretreated by mechanical activation. Bioresource Technology, 102 (17), 7953-8.

Lin, Y., Zhang, W., Li, C., Sakakibara, K., Tanaka, S., Kong, H., 2012. Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. Biomass and Bioenergy, 47, 395-401.

Liu, G., Qin, Y., Li, Z., Qu, Y., 2013. Development of highly efficient, low-cost lignocellulolytic enzyme systems in the post-genomic era. Biotechnology Advances, 31 (6), 962-75.

Liu, L., Sun, J., Li, M., Wang, S., Pei, H., Zhang, J., 2009. Enhanced enzymatic hydrolysis and structural features of corn stover by $FeCl_3$ pretreatment. Bioresource Technology, 100 (23), 5853-8.

Liu, S., Lu, H., Hu, R., Shupe, A., Lin, L., Liang, B., 2012. A sustainable woody biomass biorefinery. Biotechnology Advances, 30 (4), 785-810.

Low, K., Mahendrarajah, S., 2010. Future directions for the Australian forestry industry. Australian Bureau of Agricultural and Resource Economics, 1-23.

Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J. D., Sheehan, J., Wyman, C. E., 2008. How biotech can transform biofuels. Nature Biotechnology 26 (2), 169-72.

Maitan-Alfenas, G. P., Lage, L. G. d. A., Almeida, M. N. d., Visser, E. M., Rezende, S. T. d., Guimaraes, V. M., 2014. Hydrolysis of soybean isoflavones by *Debaryomyces hansenii* UFV-1 immobilised cells and free β-glucosidase. Food Chemistry, 146, 429-36.

Martin-Sampedro, R., Eugenio, M. E., Moreno, J. A., Revilla, E., Villar, J. C., 2014. Integration of a kraft pulping mill into a forest biorefinery: Pre-extraction of hemicellulose by steam explosion versus steam treatment. Bioresource Technology, 153, 236-44.

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., Fernandez-Lafuente, R., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme and Microbial Technology, 40 (6), 1451-63.

Meer, P. J. v. d., Dignan, P., 2007. Regeneration after 8 years in artificial canopy gaps in Mountain Ash (*Eucalyptus regnans* F. Muell.) forest in south-eastern Australia. Forest Ecology and Management, 244 (1-3), 102-11.

Mettler, M. S., Vlachos, D. G., Dauenhauer, P. J., 2012. Top ten fundamental challenges of biomass pyrolysis for biofuels. Energy and Environmental Science, 5 (7), 7797-809.

Meyer, A. S., Rosgaard, L., Sorensen, H. R., 2009. The minimal enzyme cocktail concept for biomass processing. Journal of Cereal Science, 50 (3), 337-44.

Miletic, N., Nastasovic, A., Loos, K., 2012. Immobilization of biocatalysts for enzymatic polymerizations: Possibilities, advantages, applications. Bioresource Technology, 115, 126-35.

Mishima, D., Tateda, M., Ike, M., Fujita, M., 2006. Comparative study on chemical pretreatments to accelerate enzymatic hydrolysis of aquatic macrophyte biomass used in water purification processes. Bioresource Technology, 97 (16), 2166-72.

Mittal, A., Scott, G. M., Amidon, T. E., Kiemle, D. J., Stipanovic, A. J., 2009. Quantitative analysis of sugars in wood hydrolyzates with ^{1}H NMR during the autohydrolysis of hardwoods. Bioresource Technology, 100 (24), 6398-406.

Modenbach, A. A., Nokes, S. E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings – A review. Biomass and Bioenergy, 56, 526-44.

Mohr, A., Raman, S., 2013. Lessons from first generation biofuels and implications for the sustainability appraisal of second generation biofuels. Energy Policy, 63, 114-22.

Moxley, G., Zhu, Z., Zhang, Y. H. P., 2008. Efficient sugar release by the cellulose solvent-based lignocellulose fractionation technology and enzymatic cellulose hydrolysis. Journal of Agricultural and Food Chemistry, 56 (17), 7885-90.

Mutje, P., Lopez, A., Vallejos, M. E., Lopez, J. P., Vilaseca, F., 2007. Full exploitation of *Cannabis sativa* as reinforcement/filler of thermoplastic composite materials. Composites, Part A, 38 (2), 369-77.

Ndimba, B. K., Ndimba, R. J., Johnson, T. S., Waditee-Sirisattha, R., Baba, M., Sirisattha, S., Shiraiwa, Y., Agrawal, G. K., Rakwal, R., 2013. Biofuels as a sustainable energy source: An update of the applications of proteomics in bioenergy crops and algae. Journal of Proteomics, 93, 234-44.

Nelson, M. L., O'Connor, R. T., 1964. Relation of certain infrared bands to cellulose crystallinity and crystal lattice type. Part II. A new infrared ratio for estimation of crystallinity in celluloses I and II. Journal of Applied Polymer Science, 8, 1325-41.

Nielsen, L., 2014. Queensland Sustainable Aviation Fuel Initiative. (available online: http://www.aibn.uq.edu.au/qsafi last visited 15 May 14).

NP. 2008. Fuels (Neumann Petroleum). (available online: http://www.neumannpetroleum.com.au/?page=4 last visited 13 May 2014).

Nwagu, T. N., Aoyagi, H., Okolo, B. N., Yoshida, S., 2012. Immobilization of a saccharifying raw starch hydrolyzing enzyme on functionalized and non-functionalized sepa beads. Journal of Molecular Catalysis B: Enzymatic, 78, 1-8.

Nykter, M., Kymalainen, H. R., Thomsen, A. B., Lilholt, H., Koponen, H., Sjoberg, A. M., Thygesen, A., 2008. Effects of thermal and enzymatic treatments and harvesting time on the microbial quality and chemical composition of fibre hemp (*Cannabis sativa L.*). Biomass and Bioenergy, 32 (5), 392-9.

Oberling, D. F., Obermaier, M., Szklo, A., La Rovere, E. L., 2012. Investments of oil majors in liquid biofuels: The role of diversification, integration and technological lock-ins. Biomass and Bioenergy, 46, 270-81.

O'Hehir, J. F., Nambiar, E. K. S., 2010. Productivity of three successive rotations of P. radiata plantations in South Australia over a century. Forest Ecology and Management, 259 (10), 1857-69.

Ohgren, K., Bura, R., Saddler, J., Zacchi, G., 2007. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresource Technology, 98 (13), 2503-10.

Oliveira, R. C., Hammer, P., Guibal, E., Taulemesse, J. M., Garcia, J. O., 2014. Characterization of metal–biomass interactions in the lanthanum(III) biosorption on *Sargassum* sp. using SEM/EDX, FTIR, and XPS: Preliminary studies. Chemical Engineering Journal, 239, 381-91.

Pakarinen, A., Zhang, J., Brock, T., Maijala, P., Viikari, L., 2012. Enzymatic accessibility of fiber hemp is enhanced by enzymatic or chemical removal of pectin. Bioresource Technology, 107, 275-81.

Pandey, K. K., Pitman, A. J., 2003. FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. International Biodeterioration and Biodegradation, 52 (3), 151-60.

Parikka, M., 2004. Global biomass fuel resources. Biomass and Bioenergy, 27, 613-20.

Park, Y. C., Kim, J. S., 2012. Comparison of various alkaline pretreatment methods of lignocellulosic biomass. Energy, 47 (1), 31-5.

Patel, A., Pravez, M., Deeba, F., Pruthi, V., Singh, R. P., Pruthi, P. A., 2014. Boosting accumulation of neutral lipids in *Rhodosporidium kratochvilovae* HIMPA1 grown on hemp (*Cannabis sativa Linn*) seed aqueous extract as feedstock for biodiesel production. Bioresource Technology, 165, 214-22.

Peralta-Yahya, P. P., Zhang, F., del Cardayre, S. B., Keasling, J. D., 2012. Microbial engineering for the production of advanced biofuels. Nature, 488 (7411), 320-8.

Piccolo, C., Bezzo, F., 2009. A techno-economic comparison between two technologies for bioethanol production from lignocellulose. Biomass and Bioenergy, 33 (3), 478-91.

Popescu, C. M., Lisa, G., Manoliu, A., Gradinariu, P., Vasile, C., 2010. Thermogravimetric analysis of fungus-degraded lime wood. Carbohydrate Polymers, 80 (1), 78-83.

Prade, T., Finell, M., Svensson, S. E., Mattsson, J. E., 2012. Effect of harvest date on combustion related fuel properties of industrial hemp (Cannabis sativa L.). Fuel, 102, 592-604.

Prade, T., Svensson, S. E., Andersson, A., Mattsson, J. E., 2011. Biomass and energy yield of industrial hemp grown for biogas and solid fuel. Biomass Bioenergy, 35 (7), 3040-9.

Prade, T., Svensson, S. E., Mattsson, J. E., 2012. Energy balances for biogas and solid biofuel production from industrial hemp. Biomass Bioenergy, 40, 36-52.

Prado, J. M., Forster-Carneiro, T., Rostagno, M. A., Follegatti-Romero, L. A., Maugeri Filho, F., Meireles, M. A. A., 2014. Obtaining sugars from coconut husk, defatted grape seed, and pressed palm fiber by hydrolysis with subcritical water. The Journal of Supercritical Fluids, 89, 89-98.

Puri, M., Abraham, R. E., Barrow, C. J., 2012. Biofuel production: Prospects, challenges and feedstock in Australia. Renewable and Sustainable Energy Reviews, 16 (8), 6022-31.

Puri, M., Barrow, C. J., Verma, M. L., 2013. Enzyme immobilization on nanomaterials for biofuel production. Trends in Biotechnology, 31 (4), 215-6.

Pyle, D. J., Garcia, R. A., Wen, Z., 2008. Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. Journal of Agricultural and Food Chemistry, 56 (11), 3933-9.

Qing, Q., Yang, B., Wyman, C. E., 2010. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresource Technology, 101 (24), 9624-30.

Rahikainen, J., Mikander, S., Marjamaa, K., Tamminen, T., Lappas, A., Viikari, L., Kruus, K., 2011. Inhibition of enzymatic hydrolysis by residual lignins from softwood-study of enzyme binding and inactivation on lignin-rich surface. Biotechnology and Bioengineering, 108 (12), 2823-34.

Rahmadi, A., Aye, L., Moore, G., 2013. The feasibility and implications for conventional liquid fossil fuel of the Indonesian biofuel target in 2025. Energy Policy, 61, 12-21.

Rajan, K., Carrier, D. J., 2014. Effect of dilute acid pretreatment conditions and washing on the production of inhibitors and on recovery of sugars during wheat straw enzymatic hydrolysis. Biomass and Bioenergy, 62, 222-7.

Ramchandran, D., Rajagopalan, N., Strathmann, T. J., Singh, V., 2013. Use of treated effluent water in ethanol production from cellulose. Biomass and Bioenergy, 56, 22-8.

Ramsurn, H., Gupta, R. B., 2012. Production of biocrude from biomass by acidic subcritical water followed by alkaline supercritical water two-step liquefaction. Energy Fuels, 26 (4), 2365-75.

Ranjan, A., Moholkar, V. S., 2013. Comparative study of various pretreatment techniques for rice straw saccharification for the production of alcoholic biofuels. Fuel, 112, 567-71.

Rasmussen, H., Sorensen, H. R., Meyer, A. S., 2014. Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms. Carbohydrate Research, 385, 45-57.

Ravindranath, N. H., Lakshmi, C. S., Ritumbra, M., Balachandra, P., 2011. Biofuel production and implications for land use, food production and environment in India. Energy Policy, 39 (10), 5737-45.

Rehman, M. S. U., Rashid, N., Saif, A., Mahmood, T., Han, J. I., 2013. Potential of bioenergy production from industrial hemp (*Cannabis sativa*): Pakistan perspective. Renewable and Sustainable Energy Reviews, 18, 154-64.

Rex.pannell, 2014. Assistance for Tasmania's industrial hemp sector. Enviroinfo, July 29. (available online: http://www.enviroinfo.com.au/assistance-for-tasmanias-industrial-hemp-sector/#.VCoo5Z1--72).

Richard, T. L., 2010. Challenges in scaling up biofuels infrastructure. Science, 329 (5993), 793-6.

Rocha, G. J. M., Goncalves, A. R., Oliveira, B. R., Olivares, E. G., Rossell, C. E. V., 2012. Steam explosion pretreatment reproduction and alkaline delignification reactions performed on a pilot scale with sugarcane bagasse for bioethanol production. Industrial Crops and Products, 35 (1), 274-9.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, A., Torres, R., Fernandez-Lafuente, R., 2013. Modifying enzyme activity and selectivity by immobilization. Chemical Society Reviews, 42 (15), 6290-307.

Rosatella, A. A., Simeonov, S. P., Frade, R. F. M., Afonso, C. A. M., 2011. 5-Hydroxymethylfurfural (HMF) as a building block platform: Biological properties, synthesis and synthetic applications. Green Chemistry, 13 (4), 754-93.

Rubin, E. M., 2008. Genomics of cellulosic biofuels. Nature, 454 (7206), 841-5.

Saha, B. C., Cotta, M. A., 2011. Continuous ethanol production from wheat straw hydrolysate by recombinant ethanologenic *Escherichia coli* strain FBR5. Applied Microbiology and Biotechnology, 90 (2), 477-87.

Saha, B. C., Iten, L. B., Cotta, M. A., Wu, Y. V., 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry, 40 (12), 3693-700.

Sanderson, J., Feltrin, M., 2014. Gasification Australia Pty. Ltd. (available online: http://www.gasificationaustralia.com/ last visited 14 May 2014).

Sanderson, K., 2011. Lignocellulose: A chewy problem. Nature, 474 (7352), S12-S4.

Sasmal, S., Goud, V. V., Mohanty, K., 2012. Ultrasound assisted lime pretreatment of lignocellulosic biomass toward bioethanol production. Energy Fuels, 26 (6), 3777-84.

Sassolas, A., Blum, L. J., Leca-Bouvier, B. D., 2012. Immobilization strategies to develop enzymatic biosensors. Biotechnology Advances, 30 (3), 489-511.

Savage, N., 2011. Fuel options: The ideal biofuel. Nature, 474 (7352), S9-S11.

Schuck, S., 2010. Bioenergy Australia (Newsletter). (available online: http://www.bioenergyaustralia.org/newsletters/news38/Newsletter38_June2010.pdf last visited 16 May 2014), 38.

Sedjo, R. A., 2008. Biofuels: Think Outside the Cornfield. Science, 320 (5882), 1420-1.

Segal, L., Creely, J. J., Martin, A. E., Conrad, C. M., 1959. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. Textile Research Journal, 29 (10), 786-94.

Selig, M., Weiss, Y., Ji, Y., 2008. Enzyme saccharification of lignocellulosic biomass. Laboratory analytical procedures (TP-510-42629); National Renewable Energy Laboratory: Golden, Colorado, pp 1-8.

Sgriccia, N., Hawley, M. C., Misra, M., 2008. Characterization of natural fiber surfaces and natural fiber composites. Composites, Part A, 39 (10), 1632-7.

Shedden, U., 2010. Primary Energy Grain Fuel Ethanol Plant. (available online: http://www.docstoc.com/docs/39287277/Primary-Energy-Grain-Fuel-Ethanol-Plant-Kwinana-Western-Australia last visited 16 May 14).

Shields, S., Boopathy, R., 2011. Ethanol production from lignocellulosic biomass of energy cane. International Biodeterioration and Biodegradation, 65 (1), 142-6.

Shih, C. J., Lupoi, J. S., Smith, E. A., 2011. Raman spectroscopy measurements of glucose and xylose in hydrolysate: Role of corn stover pretreatment and enzyme composition. Bioresource Technology, 102 (8), 5169-76.

Shih, C. J., Smith, E. A., 2009. Determination of glucose and ethanol after enzymatic hydrolysis and fermentation of biomass using Raman spectroscopy. Analytica Chimica Acta, 653 (2), 200-6.

Shin, S. J., Cho, N. S., 2008. Conversion factors for carbohydrate analysis by hydrolysis and ^{1}H-NMR spectroscopy. Cellulose, 15 (2), 255-60.

Silveira, M. H. L., Aguiar, R. S., Siikaaho, M., Ramos, L. P., 2014. Assessment of the enzymatic hydrolysis profile of cellulosic substrates based on reducing sugar release. Bioresource Technology, 151, 392-6.

Sims, R. E. H., Mabee, W., Saddler, J. N., Taylor, M., 2010. An overview of second generation biofuel technologies. Bioresource Technology, 101 (6), 1570-80.

Singh, A., Sharma, P., Saran, A. K., Singh, N., Bishnoi, N. R., 2013. Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized *Saccharomyces cerevisiae* on various matrices. Renewable Energy, 50, 488-93.

Singh, R. K., Zhang, Y. W., Nguyen, N. P. T., Jeya, M., Lee, J. K., 2011. Covalent immobilization of β-1,4-glucosidase from *Agaricus arvensis* onto functionalized silicon oxide nanoparticles. Applied Microbiology and Biotechnology, 89 (2), 337-44.

Skoulou, V., Mariolis, N., Zanakis, G., Zabaniotou, A., 2011. Sustainable management of energy crops for integrated biofuels and green energy production in Greece. Renewable and Sustainable Energy Reviews, 15 (4), 1928-36.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Wolfe, J., 2008a. Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples Laboratory analytical procedures (TP-510-42621); National Renewable Energy Laboratory: Golden, Colorado, pp 1-6.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008b. Determination of structural carbohydrates and lignin. Laboratory analytical procedures (TP-510-42618); National Renewable Energy Laboratory: Golden, Colorado, pp 1-15.

Smith, A. L., Klenk, N., Wood, S., Hewitt, N., Henriques, I., Yan, N., Bazely, D. R., 2013. Second generation biofuels and bioinvasions: An evaluation of invasive risks and policy responses in the United States and Canada. Renewable and Sustainable Energy Reviews, 27, 30-42.

Somerville, C., Youngs, H., Taylor, C., Davis, S. C., Long, S. P., 2010. Feedstocks for lignocellulosic biofuels. Science, 329 (5993), 790-2.

Song, N., Cai, H. Y., Yan, Z. S., Jiang, H. L., 2013. Cellulose degradation by one mesophilic strain *Caulobacter* sp. FMC1 under both aerobic and anaerobic conditions. Bioresource Technology, 131, 281-7.

Stals, M., Carleer, R., Reggers, G., Schreurs, S., Yperman, J., 2010. Flash pyrolysis of heavy metal contaminated hardwoods from phytoremediation: Characterisation of biomass, pyrolysis oil and char/ash fraction. Journal of Analytical and Applied Pyrolysis, 89 (1), 22-9.

Stephanopoulos, G., 2007. Challenges in engineering microbes for biofuels production. Science, 315 (5813), 801-4.

Stucley, C. R., Schuck, S. M., Sims, R. E. H., Larsen, P. L., Turvey, N. D., Marino, B. E., 2008. Biomass energy production in Australia: Status, costs and opportunities for major technologies. A report for the RIRDC/ FWPRDC L & W Australia/ MDBC Joint Venture Agroforestry Program (in conjunction with the Australian Greenhouse Office).

Subbaraman, N., 2010. Ethanol blend hike to jump start cellulosic investment. Nature Biotechnology, 28 (12), 1229-30.

Sukumaran, R. K., Singhania, R. R., Mathew, G. M., Pandey, A., 2009. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. Renewable Energy, 34 (2), 421-4.

Sun, X. F., Xu, F., Sun, R. C., Geng, Z. C., Fowler, P., Baird, M. S., 2005. Characteristics of degraded hemicellulosic polymers obtained from steam exploded wheat straw. Carbohydrate Polymers, 60 (1), 15-26.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology, 83 (1), 1-11.

Sureshkumar, M., Lee, C. K., 2011. Polydopamine coated magnetic-chitin (MCT) particles as a new matrix for enzyme immobilization. Carbohydrate Polymers, 84 (2), 775-80.

Suriyachai, N., Weerasaia, K., Laosiripojana, N., Champreda, V., Unrean, P., 2013. Optimized simultaneous saccharification and co-fermentation of rice straw for ethanol production by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture using design of experiments. Bioresource Technology, 142, 171-8.

Syed, A., Melanie, J., Thorpe, S., Penney, K., 2010. Australian energy projections to 2029-30. Australian Bureau of Agricultural and Resource Economics, research report, 1-51.

Tadesse, H., Luque, R., 2011. Advances on biomass pretreatment using ionic liquids: An overview. Energy & Environmental Science, 4 (10), 3913-29.

Talebnia, F., Karakashev, D., Angelidaki, I., 2010. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresource Technology, 101 (13), 4744-53.

Taylor, G., 2008. Biofuels and the biorefinery concept. Energy Policy, 36, 4406-9.
Thompson, W., Meyer, S., 2013. Second generation biofuels and food crops: Co-products or competitors? Global Food Security, 2 (2), 89-96.

Tian, S., Zhu, J., Yang, X., 2011. Evaluation of an adapted inhibitor-tolerant yeast strain for ethanol production from combined hydrolysate of softwood. Applied Energy, 88 (5), 1792-6.

Timilsina, G. R., Shrestha, A., 2011. How much hope should we have for biofuels? Energy, 36 (4), 2055-69.

Tirado, M. C., Cohen, M. J., Aberman, N., Meerman, J., Thompson, B., 2010. Addressing the challenges of climate change and biofuel production for food and nutrition security. Food Research International, 43 (7), 1729-44.

Toquero, C., Bolado, S., 2014. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresource Technology, 157, 68-76.

Trabelsi, I., Ayadi, D., Bejar, W., Bejar, S., Chouayekh, H., Salah, R. B., 2014. Effects of *Lactobacillus plantarum* immobilization in alginate coated with chitosan

and gelatin on antibacterial activity. International Journal of Biological Macromolecules, 64, 84-9.

Tran, N., Illukpitiya, P., Yanagida, J. F., Ogoshi, R., 2011. Optimizing biofuel production: An economic analysis for selected biofuel feedstock production in Hawaii. Biomass and Bioenergy, 35 (5), 1756-64.

Trtik, P., Dual, J., Keunecke, D., Mannes, D., Niemz, P., Stahli, P., Kaestner, A., Groso, A., Stampanoni, M., 2007. 3D imaging of microstructure of spruce wood. Journal of Structural Biology, 159 (1), 46-55.

Tu, M., Zhang, X., Paice, M., MacFarlane, P., Saddler, J. N., 2009. The potential of enzyme recycling during the hydrolysis of a mixed softwood feedstock. Bioresource Technology, 100 (24), 6407-15.

Tutt, M., Kikas, T., Olt, J., 2013. Influence of harvesting time on biochemical composition and glucose yield from hemp. Agronomy Research, 11 (1), 215-20.

Tyndall, J. C., Berg, E. J., Colletti, J. P., 2011. Corn stover as a biofuel feedstock in Iowa's bio-economy: An Iowa farmer survey. Biomass and Bioenergy, 35 (4), 1485-95.

Ungurean, M., Paul, C., Peter, F., 2012. Cellulase immobilized by sol-gel entrapment for efficient hydrolysis of cellulose. Bioprocess Biosystem and Engineering, 1-12.

United. 2014. Dalby Ethanol Bio-Refinery under threat of closure. (available online: http://www.unitedpetroleum.com.au/united/news/news-item/news/2014/02/17/dalby-ethanol-bio-refinery-under-threat-of-closure last visited 15 May 2014).

Urrutia, P., Mateo, C., Guisan, J. M., Wilson, L., Illanes, A., 2013. Immobilization of *Bacillus circulans* β-galactosidase and its application in the synthesis of galacto-oligosaccharides under repeated-batch operation. Biochemical Engineering Journal, 77, 41-8.

Vancov, T., 2011. Forest biomass available, biofuels investment urged (Agriculture Today). (available online: http://www.dpi.nsw.gov.au/archive/agriculture-today-stories/ag-today-archives/march-2011/forest-biomass-ready-for-biofuels last visited 16 May 2014).

Vancov, T., McIntosh, S., 2012. Mild acid pretreatment and enzyme saccharification of *sorghum bicolor* straw. Applied Energy, 92, 421-28.

Venghaus, S., Selbmann, K., 2014. Biofuel as social fuel: Introducing socio-environmental services as a means to reduce global inequity? Ecological Economics, 97, 84-92.

Verma, M. L., Barrow, C. J., Kennedy, J. F., Puri, M., 2012. Immobilization of β-d-galactosidase from *Kluyveromyces lactis* on functionalized silicon dioxide

nanoparticles: Characterization and lactose hydrolysis. International Journal of Biological Macromolecules, 50 (2), 432-7.

Verma, M. L., Barrow, C. J., Puri, M., 2013a. Nanobiotechnology as a novel paradigm for enzyme immobilisation and stabilisation with potential applications in biodiesel production. Applied Microbiology and Biotechnology, 97 (1), 23-39.

Verma, M. L., Chaudhary, R., Tsuzuki, T., Barrow, C. J., Puri, M., 2013b. Immobilization of β-glucosidase on a magnetic nanoparticle improves thermostability: Application in cellobiose hydrolysis. Bioresource Technology, 135, 2-6.

Verma, S. R., Dwivedi, U. N., 2014. Lignin genetic engineering for improvement of wood quality: Applications in paper and textile industries, fodder and bioenergy production. South African Journal of Botany, 91, 107-25.

WABTR. 2007. Western Australia Biofuels Taskforce Report. The economics of biofuels for Western Australia. (available online: http://www.thecie.com.au/content/publications/CIEfinalbiofuelsreportApr07.pdf last visited 16 May 14).

Wang, F. Q., Xie, H., Chen, W., Wang, E. T., Du, F. G., Song, A. D., 2013a. Biological pretreatment of corn stover with ligninolytic enzyme for high efficient enzymatic hydrolysis. Bioresource Technology, 144, 572-8.

Wang, S., Su, P., Ding, F., Yang, Y., 2013b. Immobilization of cellulase on polyamidoamine dendrimer-grafted silica. Journal of Molecular Catalysis B: Enzymatic, 89, 35-40.

Wang, W., Zhou, W., Liu, J., Li, Y., Zhang, Y., 2013c. Biodiesel production from hydrolysate of *Cyperus esculentus* waste by *Chlorella vulgaris*. Bioresource Technology, 136, 24-9.

Wang, Y., Radosevich, M., Hayes, D., Labbe, N., 2011. Compatible ionic liquid-cellulases system for hydrolysis of lignocellulosic biomass. Biotechnology and Bioengineering, 108 (5), 1042-8.

WB. 2010. About Sucrogen BioEthanol. (available online: http://wilmarbioethanol.com/about-csr-ethanol last visited 13 May 2014).

Wijffels, R. H., Barbosa, M. J., 2010. An Outlook on Microalgal Biofuels. Science, 329 (5993), 796-9.

Williams, C., Biswas, T., 2010. Commercial Potential of Giant Reed for Pulp, Paper and Biofuel Production. Rural Industries Research and Development Corporation Publication 10/215, 1-152.

Wirawan, F., Cheng, C. L., Kao, W. C., Lee, D. J., Chang, J. S., 2012. Cellulosic ethanol production performance with SSF and SHF processes using immobilized *Zymomonas mobilis*. Applied Energy, 100, 19-26.

Witcover, J., Yeh, S., Sperling, D., 2013. Policy options to address global land use change from biofuels. Energy Policy, 56, 63-74.

Woli, K. P., David, M. B., Tsai, J., Voigt, T. B., Darmody, R. G., Mitchell, C. A., 2011. Evaluating silicon concentrations in biofuel feedstock crops *Miscanthus* and switchgrass. Biomass and Bioenergy, 35 (7), 2807-13.

Wong, M. K. M., Tsui, C. K. M., Au, D. W. T., Vrijmoed, L. L. P., 2008. Docosahexaenoic acid production and ultrastructure of the thraustochytrid *Aurantiochytrium mangrovei* MP2 under high glucose concentrations. Mycoscience, 49 (4), 266-70.

Wood, I. P., Elliston, A., Collins, S. R. A., Wilson, D., Bancroft, I., Waldron, K. W., 2014. Steam explosion of oilseed rape straw: Establishing key determinants of saccharification efficiency. Bioresource Technology, 162 (0), 175-83.

Ximenes, E., Kim, Y., Mosier, N., Diend, B., Ladischa, M., 2010. Inhibition of cellulases by phenols. Enzyme and Microbial Technology, 46 170-6.

Xu, F., Yu, J., Tesso, T., Dowell, F., Wang, D., 2013. Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: A mini-review. Applied Energy, 104, 801-9.

Xu, J., Cheng, J. J., 2011. Pretreatment of switchgrass for sugar production with the combination of sodium hydroxide and lime. Bioresource Technology, 102 (4), 3861-8.

Xu, J., Huo, S., Yuan, Z., Zhang, Y., Xu, H., Guo, Y., Liang, C., Zhuang, X., 2011a. Characterization of direct cellulase immobilization with superparamagnetic nanoparticles. Biocatalysis and Biotransformation, 29 (2-3), 71-6.

Xu, J., Wang, Z., Cheng, J. J., 2011b. Bermuda grass as feedstock for biofuel production: A review. Bioresource Technology, 102 (17), 7613-20.

Yamashita, Y., Shono, M., Sasaki, C., Nakamura, Y., 2010. Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo. Carbohydrate Polymers, 79 (4), 914-20.

Yao, R., Hu, H., Deng, S., Wang, H., Zhu, H., 2011. Structure and saccharification of rice straw pretreated with sulfur trioxide micro-thermal explosion collaborative dilutes alkali. Bioresource Technology, 102 (10), 6340-3.

Yu, S., Olsen, C. E., Marcussen, J., 1997. Methods for the assay of 1,5-anhydro-d-fructose and α-1,4-glucan lyase. Carbohydrate Research, 305 (1), 73-82.

Yuan, X., Shen, N., Sheng, J., Wei, X., 1999. Immobilization of cellulase using acrylamide grafted acrylonitrile copolymer membranes. Journal of Membrane Science, 155 (1), 101-6.

Zabihi, S., Alinia, R., Esmaeilzadeh, F., Kalajahi, J. F., 2010. Pretreatment of wheat straw using steam, steam/acetic acid and steam/ethanol and its enzymatic hydrolysis for sugar production. Biosystems Engineering, 105 (3), 288-97.

Zhang, B., Wang, L., Shahbazi, A., Diallo, O., Whitmore, A., 2011a. Dilute-sulfuric acid pretreatment of cattails for cellulose conversion. Bioresource Technology, 102 (19), 9308-12.

Zhang, H., Wu, S., 2014. Enhanced enzymatic cellulose hydrolysis by subcritical carbon dioxide pretreatment of sugarcane bagasse. Bioresource Technology, 158, 161-5.

Zhang, J., Ma, X., Yu, J., Zhang, X., Tan, T., 2011b. The effects of four different pretreatments on enzymatic hydrolysis of sweet sorghum bagasse. Bioresource Technology, 102 (6), 4585-9.

Zhang, J., Pakarinen, A., Viikari, L., 2013. Synergy between cellulases and pectinases in the hydrolysis of hemp. Bioresource Technology, 129, 302-7.

Zhang, J., Wang, Y., Zhang, L., Zhang, R., Liu, G., Cheng, G., 2014. Understanding changes in cellulose crystalline structure of lignocellulosic biomass during ionic liquid pretreatment by XRD. Bioresource Technology, 151, 402-5.

Zhang, Q., Zhang, P., Pei, Z. J., Wang, D., 2013. Relationships between cellulosic biomass particle size and enzymatic hydrolysis sugar yield: Analysis of inconsistent reports in the literature. Renewable Energy, 60, 127-36.

Zhang, Y., Xu, J. L., Li, D., Yuan, Z. H., 2010a. Preparation and properties of an immobilized cellulase on the reversibly soluble matrix Eudragit L-100. Biocatalysis and Biotransformation, 28 (5-6), 313-9.

Zhang, Y. H. P., Sun, J., Zhong, J., 2010b. Biofuel production by in vitro synthetic enzymatic pathway biotransformation. Current Opinion in Biotechnology, 21, 663-9.

Zhao, X., Elliston, A., Collins, S. R. A., Moates, G. K., Coleman, M. J., Waldron, K. W., 2012. Enzymatic saccharification of duckweed (*Lemna minor*) biomass without thermophysical pretreatment. Biomass and Bioenergy, 47, 354-61.

Zheng, P., Wang, J., Lu, C., Xu, Y., Sun, Z., 2013. Immobilized β-glucosidase on magnetic chitosan microspheres for hydrolysis of straw cellulose. Process Biochemistry, 48 (4), 683-7.

Zhou, A., Thomson, E., 2009. The development of biofuels in Asia. Applied Energy, 86, S11-S20.

Zhou, W., Yu, Y., Liu, D., Wu, H., 2013. Rapid recovery of fermentable sugars for biofuel production from enzymatic hydrolysis of microcrystalline cellulose by hot-compressed water pretreatment. Energy Fuels, 27 (8), 4777-84.

Zhu, J. Y., Pan, X. J., 2010. Woody biomass pretreatment for cellulosic ethanol production: Technology and energy consumption evaluation. Bioresource Technology, 101, 4992-5002.

Zhu, L., Zhang, X., Ji, L., Song, X., Kuang, C., 2007. Changes of lipid content and fatty acid composition of *Schizochytrium limacinum* in response to different temperatures and salinities. Process Biochemistry, 42 (2), 210-4.

Zhu, L. D., Hiltunen, E., Antila, E., Zhong, J. J., Yuan, Z. H., Wang, Z. M., 2014. Microalgal biofuels: Flexible bioenergies for sustainable development. Renewable and Sustainable Energy Reviews, 30, 1035-46.

Zhu, W., Zhu, J. Y., Gleisner, R., Pan, X. J., 2010. On energy consumption for size-reduction and yields from subsequent enzymatic saccharification of pretreated lodgepole pine. Bioresource Technology, 101, 2782–92.

Ziolkowska, J. R., 2014. Optimizing biofuels production in an uncertain decision environment: Conventional vs. advanced technologies. Applied Energy, 114, 366-76.