De-oiled *Pongamia pinnata* seed residue:
a non-edible resource for sustainable biofuels production

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Radhakumari Muktham

June 15 2016
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STATEMENT OF AUTHORSHIP

Two papers published from this project are presented as chapters 3 and chapter 6.

Radhakumari Muktham (candidate) planed, designed and performed experiments, analyzed and interpreted experimental data, wrote manuscript draft and submitted manuscript for evaluation.

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Suresh K. Bhargava contributed to manuscript evaluation.

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Andrew S. Ball contributed in the planning of experiments, supervision of research, manuscript preparation and manuscript evaluation.

Co-authors give fully consent to Radhakumari Muktham to present these 2 above papers for examination towards the Doctor of Philosophy.

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ABSTRACT

Depleting reserves of natural resources and the deleterious effects of fossil fuel burning on the environment have led to great interest in developing renewable fuels. Biomass represents a promising renewable material for biofuels, electricity, heat, chemicals and biochar production. Consequently research has focused on aspects of biomass conversion that currently act as bottle necks to commercial development including: (i) feedstock development, (ii) selection of biomass conversion technologies, and (iii) development of large scale reactor systems. Presently, the production of biofuels from waste biomass, wood chips, forestry and agricultural residues through thermochemical and/or biochemical routes are the leading process strategies in many countries. Significant improvements in process can therefore be expected, thus making large-scale biofuels production from lignocellulosic biomass substrates possible.

Currently, non-edible bioresources including non-edible oil seeds are emerging as a potential feedstock for the biofuel industry, as both oil and seed residues can be exploited to produce biofuels. Non-edible resources have the advantage of not interfering with food supply and food pricing, or impacting on land use for feedstock supply to achieve sustainable biofuels production.

*Pongamia pinnata*, a non-edible, drought resistant tree is capable of adaptation to different climatic conditions. It belongs to the legume family and is distributed in Asia, Australia and the Pacific Islands. *P. pinnata* seeds are composed of 30-35% oil which represents a precursor for biodiesel production and has been widely studied as a potential renewable feedstock. The de-oiled seed residue is of current interest in terms of bioethanol, bio-oil and biochar production. The annual yield of the seeds reached 200,000 metric tons from India alone. *P. pinnata* seeds costs around $0.06 kg⁻¹ and the selling price of oil
extracted from the seeds is $0.06 \text{ L}^{-1}$ ($0.065 \text{ kg}^{-1}$) which makes the cost of the seed residue almost insignificant in the process, making the seed residue an economical feed-stock. In this study a range of issues in the conversion of the seed residue to biofuels were investigated. The value addition of *P. pinnata* de-oiled seed residue was carried out using chemical (acid hydrolysis), enzymatic (enzymatic hydrolysis) and thermal (pyrolysis) treatments.

In the first part of this study, chemical hydrolysis of the seed residue was carried out in the presence of sulphuric acid together with analysis of the composition of the seed residue in terms of ash (6.7%), lignin (29%), protein (22.3%) and total carbohydrates (42%). The main objective of this study was aimed at testing the seed residue for the production of glucose by acid hydrolysis in the presence of sulphuric acid and to prove the potential of the seed residue as a promising feedstock for biofuels production. The Taguchi robust design method with $L_9$ orthogonal array was applied to optimize hydrolysis reaction conditions in order to maximize the glucose yield. The effect of temperature (80, 100 and 120 °C), acid concentration (2.5, 5 and 7.5%), and liquid to seed residue weight ratio (10, 15 and 20) were considered as the main influencing factors which affects the formation of glucose. From the study it was observed that acid concentration and temperature had a principal effect on the amount of glucose formed when compared to that of the liquid to solid ratio. The maximum glucose formed was 245 g/kg seed residue. This work demonstrated the effects of different operating conditions on glucose production and the potential of the seed residue for sugar production for further application in biofuel production.

The second part of this study explores the effect of different acids including hydrochloric acid (HCl), sulphuric acid ($\text{H}_2\text{SO}_4$) and phosphoric acid ($\text{H}_3\text{PO}_4$) on sugars yield from hydrolysis of de-oiled *P. pinnata* seed residue and the application of sugars obtained for ethanol production in a three step process: acid treatment; neutralization and fermentation. The Taguchi robust design of experiments method was employed to study the effects of the
parameters for biomass pretreatment, including acid type (H$_2$SO$_4$, HCl, H$_3$PO$_4$), acid concentration (2-6%), and temperature (80-100 °C) on the formation of sugars during acid hydrolysis. Acid concentration and temperature showed a positive effect on sugar release from the biomass with hydrochloric acid the best catalyst for acid hydrolysis. The energy required for this pretreatment process using HCl was estimated, to get an insight into the process energy demand (1080-1110 KJ kg$^{-1}$). Downstream processing before fermentation for ethanol production included neutralization of the hydrolyzate to pH 4.8. Addition of alkali in the neutralization step influenced sugar concentration in terms of g sugar per litre of neutralized product together with the salts formed and the time required for complete conversion of sugars to ethanol during fermentation. The results indicated that fermentation of 2%, 4% and 6% HCl treatment followed by neutralization gave 67.52, 74.98 and 88.62 g ethanol kg$^{-1}$ dry seed cake, respectively, corresponding to ~31.45%, 34.92% and 41.28% of the theoretical ethanol formation (214 g kg$^{-1}$). The work demonstrated the selection of the best catalyst, determined the energy requirement of the acid hydrolysis process, and confirmed the potential of the seed residue as a feed-stock for ethanol production.

In the third part of this study, the enzymatic hydrolysis method was tested for sugar production from $P$. $pinnata$ seed residue for further application in ethanol production. Currently the costs associated with lignocellulases represent a key limiting factor in the development of biomass enzymatic conversion processes. The main aim of this work was to exploit the seed residue for both lignocellulase enzymes and ethanol production. $Spingomonas$ $echinoides$ (isolated from $P$. $pinnata$ seed residue) and $Iprex$ $lacteus$ (obtained from RMIT culture collection) were selected as novel sources of lignocellulases during solid state fermentation. Both organisms produced an array of lignocellulases including exoglucanase, endoglucanase, xylanase and laccase with activities of 3.9, 2.7, 0.8, 0.116 U ml$^{-1}$ min$^{-1}$ and 5.2, 8.2, 2.7, 0.129 U mL$^{-1}$ min$^{-1}$ for $S$. $echinoides$ and $I$. $lacteus$, respectively.
P. pinnata seed residue (5%, w/v) in sodium citrate buffer (pH 4.8) was pretreated at 121 °C for 15 min. Enzymatic hydrolysis of the pretreated seed residue was carried out at 50 °C with mixing at 150 rpm. Subsequent hydrolysis of the pretreated seed residue using the crude enzyme preparation from S. echinoides and I. lacteus reported appreciable sugars yields of 233 and 302 mg g⁻¹ seed residue respectively, at 10 U g⁻¹ enzyme concentrations. The yields from crude enzyme treatment were further compared with that from commercial cellulase from Aspergillus niger (330 mg g⁻¹ seed residue). The three sugar-containing liquid products from the enzymatic hydrolysis of P. pinnata seed residue using crude enzyme prepared from S. echinoides (SE), I. lacteus (IL) and cellulase from A. niger (AN) were fermented using Saccharomyces cerevisiae under anaerobic conditions at 35 °C. Ethanol yields of 81.5, 104.5 and 157.6 mg g⁻¹ and final ethanol concentrations of 4.0, 5.3 and 7.9 mg mL⁻¹ were observed from SE, IL and AN fermentations respectively, which correspond to 38%, 49% and 73% of the theoretical ethanol yield, respectively. The study demonstrated the feasibility of using the seed residue for enzyme preparation for application in hydrolysis reactions and the potential of using the hydrolysis product for ethanol production.

In the fourth part of this study thermal treatment of the seed residue was carried out under pyrolysis conditions. The aim of the work was to obtain kinetic parameters in relation to technological parameters in the conversion of non-edible seed residue biomass to bio-oil and bio-char by pyrolysis. The effects of heating rate on seed residue pyrolysis and kinetic parameters were investigated at heating rates of 5, 10, and 20 °C/min using thermogravimetric analysis. Thermogravimetric experiments showed that the onset and offset temperatures of the devolatilization step shifted towards the high-temperature range, and the activation energy values increased with increasing heating rate. In the present study iso-conversional methods (model free and model fitting methods) were applied in estimating the activation energies (118-124 KJ mol⁻¹) and the pre-exponential factors using progressive
conversion, obtained by thermogravimetric analysis. The model free methods, Kissenger-Akahira-Sunose (KAS) and Ozawa-Flynn-Wall (OFW), and the model fitting Coats and Redfern method (CRF) gave reliable activation energies and pre-exponential factors with a third order reaction model for the seed residue pyrolysis reaction. The pyrolysis reactions in the thermogravimetric analyzer at constant heating rate revealed a temperature of 450-500 °C was sufficient for carbonization of the biomass. Thermal treatment of the seed residue under pyrolysis conditions was performed in a fixed bed reactor at different temperatures including 350, 400, 450 and 500 °C. Proximate-ultimate analyses, energy value, surface structure and Fourier transformed infrared spectra of the processed biomass (biochar) were reported. Pyrolysis resulted in an increase in the heating value of the seed residue biomass from 18.1 to 24.5 MJ kg⁻¹ with the heating value of biooil determined as 33.38 MJ kg⁻¹; importantly biochar exhibited high carbon and low oxygen content. The approach represents a novel method for upgrading the seed residue resulting in a biochar which has significant commercial potential as a biosorbent for industrial effluent treatment.

The results obtained from the study have made an important contribution in value addition of the inedible and low cost bio-resource, de-oiled *Pongamia pinnata* seed residue. The scientific knowledge obtained from the present study can be useful in solving the technical barriers involved in biofuel preparation from the seed residue.
CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

********************************************************************************

1.1 INTRODUCTION

Energy sources and its utilization determine the economic status and growth of developing countries all over the world [Xu et al., 2009]. According to Navigant Researchers 2013 Smart Cities Report, the number of people living in cities will increase from 3.6 billion in 2010 to 6.3 billion in 2050. Therefore, there is an urgent need for cities across the globe to provide economic and environmentally friendly resources to satisfy the citizen’s needs.

The BP Statistical Review of World Energy estimated that in 2013 the primary sources of energy consisted of petroleum 32.88%, coal 30.00%, and natural gas 23.73%, amounting to an 86.96% share for fossil fuels in primary energy consumption in the world. In the year 2003 the world consumed 9943.8 million tonnes oil equivalent primary energy; this value increased by 7.75%, 20.23% and 28.03% in 2005, 2010 and 2013, respectively.

Today’s society is based on the use of fossil resources for transportation fuels and petrochemicals. World energy consumption by fuel type is given in Figure 1.1. It is evident that the consumption of oil, coal and natural gas greatly exceeds the consumption of renewable energy and hydroelectricity. The result of unlimited consumption of fossil energy, due to its low cost and ready availability is a severe depletion of the natural reserves. However, the use of fossil fuels also leads to environmental damage. The burning of every tonne of fossil-fuel adds 180 kg of sulphur oxides to the atmosphere, causing irritation to the respiratory system and adding to the formation of acid rain.
Figure 1.1: World energy consumption by fuel type in million tonnes oil equivalent
In addition, the burning of fossil fuels produces around 21.3 giga tonnes of carbon dioxide (CO₂) per year, but it is estimated that natural processes can only absorb about half of that amount, so there is a net increase of 10.65 billion tonnes of atmospheric carbon dioxide per year (one tonne of atmospheric carbon is equivalent to 44/12 or 3.7 tonnes of carbon dioxide).

1.2 RENEWABLE RESOURCES TO REPLACE FOSSIL RESOURCES

Depleting fossil reserves and increasing demand for energy together with environmental concerns have motivated researchers towards the development of alternative fuels which are eco-friendly, bio-degradable and economical. One such potential resource available for fuels production is lignocellulosic biomass which is renewable in nature. Lignocellulosic biomass is defined as “the biodegradable fraction of products, waste and residues from biological origin from agriculture (including vegetable and animal substances), forestry and related industry” [http://ec.europa.eu/agriculture/bioenergy/potential/index_en.htm]. Currently only 3.0% of global energy consumption is supplied from renewable sources [BP Statistical Review of World Energy. http://www.bp.com/en/global/corporate/energy-economics/statistical-review-of-world-energy.html]. In 2050, potentially around 20–80% of the world’s primary energy demand could be provided by sustainable biomass resources. Not only an energy source, biomass is also a promising raw material for the production of chemicals [Werpy et al., 2004 & Humbird et al., 2011]. As biomass represents a renewable energy source it can potentially be utilized without depleting reserves. However, the features of lignocellulosic biomass pose challenges to conversion technologies. An effective commercial technology must enable the processing of material that has a very complex and resistant structure and allow the efficient exploitation of every part of the biomass, in which the relative portions of the different parts vary greatly depending on the source. There is no
current technology available. What is now required is to develop techno-economic routes for the production of bio-based compounds to make the bio-industry competitive in the market.

Lignocellulosic biomass is composed of carbohydrate polymers (cellulose and hemicellulose), lignin and a small remaining fraction of extractive acid, salts and minerals. Figure 1.2 depicts the structural components of lignocellulosic biomass. Cellulose is a homopolymer of glucose subunits (cellobiose) with a crystalline structure; hemicellulose is a heteropolymer of pentose sugars with an amorphous structure, whereas lignin is a highly crystalline and rigid component of biomass. Cellulose and hemicellulose typically comprise two-thirds of the dry mass and varies with the type of biomass feedstock.

![Cellulose](image1.png)  ![Hemicellulose](image2.png)  ![Lignin](image3.png)

Figure 1.2: Lignocellulosic biomass structural components (cellulose, hemicellulose and lignin)

The cellulose, hemicellulose and lignin composition of different renewable feedstocks is presented in Table 1.1. These three components of biomass can be converted to
various value added products through different pathways. There are a number of recent reviews reporting the state of the art in biofuel and biochemical production and the use of different feed-stocks for this developing bioindustry [e.g. Cherubini 2010]. A comparison between the bio-industry and the fossil based industry suggests that the integration of green chemistry into the bio-industry together with the use of low environmental impact technologies will result in the sustainable production of biofuels and high value chemicals from biomass.

Table 1.1: Cellulose, hemicellulose and lignin composition of lignocellulosic biomass feedstocks. [Kumar et al., 2009; McKendry 2002; Menon & Rao 2012]

<table>
<thead>
<tr>
<th>Lignocellulosic biomass</th>
<th>% of total dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cellulose</td>
</tr>
<tr>
<td>Bamboo</td>
<td>49-50</td>
</tr>
<tr>
<td>Corn cobs</td>
<td>45</td>
</tr>
<tr>
<td>Corn stover</td>
<td>35-40</td>
</tr>
<tr>
<td>Grasses</td>
<td>25-40</td>
</tr>
<tr>
<td>Hardwood stems</td>
<td>40-50</td>
</tr>
<tr>
<td>Nut shells</td>
<td>25-30</td>
</tr>
<tr>
<td>Rice straw</td>
<td>29-35</td>
</tr>
<tr>
<td>Softwood stems</td>
<td>45-50</td>
</tr>
<tr>
<td>Sugar cane bagasse</td>
<td>25-45</td>
</tr>
<tr>
<td>Switch grass</td>
<td>30-50</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>33-40</td>
</tr>
</tbody>
</table>
1.3 NON-EDIBLE RENEWABLE RESOURCES

Currently, non-edible bioresources [Klein-Marcuschamer et al., 2013] including non-edible oil seeds are emerging as a potential feed-stock for biofuel industry, as both the oil and seed residues can be exploited to produce biofuels. Non-edible resources for biofuels production have the advantages of not interfering with food supply and food prices, nor impacting on land use for feedstock supply to achieve sustainable biofuels production.

In the present investigation de-oiled *Pongamia pinnata* seed residue was exploited as a renewable and sustainable feedstock for biofuels production. *P. pinnata* is a non-edible, drought resistant tree, adapted to different climatic conditions. *P. pinnata* belong to the legume family and is distributed throughout Asia, Australia and the Pacific Islands. It is a medium sized evergreen tree (Figure 1.3a), which has minor economic importance. The seed kernel shown in Figure 1.3b contains 27 to 39% oil. The oil contains toxic flavonoids such as karanjin and a di-ketone pongamol as major lipid associates, which make the oil non-edible. *P. pinnata* oil therefore represents an ideal feedstock for biodiesel production as it cannot be used for human consumption. *P. pinnata* seed oil has its importance as a precursor for biodiesel production [Dwivedi et al., 2014 & Aniya et al., 2015] and the residual seed is suitable for bioethanol production owing to its holocellulose composition. Following extraction, the left over seed cake (60-70%) could be used as a feed stock for the production of sugars by hydrolysis. Globally, many countries including Australia (Rural Industries Research and Development Corporation, Australian Government), India (National oil seeds and vegetable oils development board, the Ministry of Agriculture, India), Hawaii (Source: Biodiesel crop implementation in Hawaii, Hawaii Agriculture Research Centre, 2006) have initiated techno-economic models and practices for *P. pinnata* plantations to improve the economics of the biodiesel industry for sustainable energy supply. As a result of this the annual yield of the seeds reached 200,000 metric tons from India alone [Gaurav et al., 2011].
Generally, prior to biomass conversion to value added products, physical treatment such as chipping, grinding and milling are applied to reduce the size of the feedstock. It has been reported that 30 kwh energy is required for the size reduction of one ton of agricultural biomass to 3-6 mm size which is far greater than the amount of energy produced from biomass [Cadoche et al., 1989]. Therefore physical pretreatment plays a vital role in the economic viability of any bio-based industry. In the case of de-oiled *P. pinnata*, the seed cake after drying is available in a powder form, shown in Figure 1.3c, no pretreatment is required; this represents a significant economic advantage unique to the use of the *P. pinnata* seed cake as a feedstock in bio-industry.

Therefore, in the present work de-oiled *P. pinnata* seed residue was used as a renewable feedstock for bio-products production through various routes, as a part of value addition of the seed residue and to improve the economics of the *P. pinnata* biodiesel industry. An overview of integrated biorefinery system based on *P. pinnata* seeds is shown in Figure 1.4. The main concept of biorefinery systems is effective utilization of various components of lignocellullosic biomass through conversion into value added products using different processing technologies. The main components of *P. pinnata* seed bio-refinery are (i) conversion of *P. pinnata* oil to biodiesel; (ii) conversion of de-oiled seed residue to bioethanol; (iii) conversion of the solid residue from the ethanol process to biochar and biooil. Non-edible seeds represent a promising feedstock for biorefinery systems as they can be processed by a variety of treatment methods (e.g. hydrolysis and fermentation, pyrolysis and gasification) for energy and chemicals production, which will give an added economic value to the seed bio-refinery [Ramachandran et al., 2007].
Figure 1.3: (a) *Pongamia pinnata* seeds & leaves, (b) Seed kernel, (c) De-oiled and dried *P. pinnata* seed residue.
Figure 1.4: An overview of integrated biorefinery based on *Pongamia pinnata* seeds
The available techniques to separate oil from seeds are: (i) mechanical pressing [Evon et al., 2013]; (ii) solvent extraction. Mechanical pressing may not accomplish complete removal of oil from the seed. Therefore, mechanical pressing followed by solvent extraction using ethanol/hexane/toluene/isopropyl alcohol/acetone as solvents is common practice in separating oil from the seeds.

In the present work, de-oiled *P. pinnata* seed cake (oil was expelled from the seed) was obtained from a local producer in Hyderabad, India. Further, de-oiled seed cake was treated with ethanol in a Soxhlet extraction unit and used in the experiments. The oil content in ethanol treated de-oiled seed cake was <1%.

1.4 1**st** GENERATION VERSUS 2**nd** GENERATION BIOFUEL PRODUCTION

The use of renewable biomass resources to produce liquid biofuels offer attractive solutions to reducing greenhouse gas emissions, decreasing reliance on foreign oils, addressing energy security concerns, strengthening rural and agricultural economies and increasing the sustainability of the world transportation system [Demirbas 2007]. Currently bioethanol is the dominant global renewable transport biofuel and offers greenhouse gas savings of up to 80% over conventional fossil fuels depending on the feedstock. Other types of biofuels include biodiesel, biomethanol, biogas, bio-syngas, bio-oil and bio-hydrogen [Demirbas 2008] produced from a wide range of agricultural or waste sources. Apart from biofuels, many other valuable products for the chemical and pharmaceutical industry can be produced from organic byproducts through microbial fermentation [Thomsen 2005].

The starting raw materials for the production of bioethanol can broadly be classified as sucrose-containing feedstocks (sugarcane, sugar beet, etc.), starch feedstocks (wheat, corn, cassava, potatoes, etc.) and cellulosic feedstocks (straw, grasses, wood, stovers, agricultural
wastes, paper, etc.). However, bioethanol is currently produced chiefly from traditional food crops such as corn (USA), sugar cane (Brazil), wheat (France, England, Germany, and Spain), cassava (Thailand, Nigeria) and sorghum (India), the feedstock depending on location and dominant agricultural product [Mojovic et al., 2006]. Most current bioethanol production processes (1st generation) utilize more readily degradable biomass feedstock such as cereals (corn or grain) and sugar cane juice. However, the utilization of these agricultural crops exclusively for energy production conflict with food and feed production [Wheals et al., 1999]. Currently there is much focus on advancing a cellulosic bioethanol concept (2nd generation) that utilizes lignocellulosic biomass.

First generation biofuel production, mainly from food crops have several limitations as it has a direct impact on food production in terms of food price and quality and soil usage for crop growth while providing only limited greenhouse gas emission reduction benefits [Balan et al., 2013]. Second generation biofuel produced from lignocellulosic biomass, non-food crops, industrial and municipal wastes shows higher greenhouse gas reduction and does not compete for agricultural land with food crops. Bioethanol production from lignocellulosic biomass feedstock typically comprises the following steps:

- Pre-treatment: process where the structural carbohydrates that compose the biomass are made more accessible for the subsequent steps;
- Enzymatic hydrolysis: breakdown of the polymeric carbohydrates into simple sugars that can be fermented by the microorganisms into ethanol;
- Fermentation: conversion of the carbohydrates into ethanol by the selected microorganism or culture;
- Downstream processing: recovery of the ethanol from the fermentation broth (typically by distillation) and management of the remaining streams.
The economic feasibility of biofuel production from lignocellulosic feed-stocks largely depends on (i) the type of biomass and (ii) the pretreatment process before fermentation. Availability, cost, transportation to the processing facility and physical state of the biomass are major factors affecting the selection of feed-stock for bioethanol production from lignocellulosic biomass.

The need for a pre-treatment step is the major distinction between a first- and a second-generation process. Existing ethanol production processes have separate hydrolysis and fermentation steps (SHF) [Dahnum et al., 2015]; simultaneous saccharification and fermentation (SSF) [Liu et al., 2015] refers to saccharification and fermentation of hexose sugars taking place within the same bioreactor; simultaneous saccharification and co-fermentation (SSCF) refers to the saccharification and co-fermentation of both pentose and hexose sugars in a single step and consolidated bioprocessing step (CBP) (Figure 1.5). In CBP a single organism is used to produce the enzymes required and to perform both cellulose hydrolysis and fermentation [Horisawa et al., 2015]. CBP is considered potentially the most cost-effective process as the processes, namely enzyme production, hydrolysis and fermentation are taking place within the same bioreactor making the capital cost lower [Olson et al., 2011].

Ethanol is produced primarily by the fermentation of glucose using fermentative microorganisms, principally yeasts [Demirbas 2005]. The most common microbe used has been Saccharomyces cerevisiae (baker’s yeast) which, as Lin and Tanaka [Lin et al., 2006] reported, can produce ethanol at concentrations as high as 18% in the fermentation broth. It is a relatively easy microbe to handle as it is generally recognized as safe. Zymomonas mobilis, a Gram-negative bacterium, can also be used in fermentation of glucose into ethanol [Dien et al., 2003].
Figure 1.5: Process steps in lignocellulosic ethanol production [reproduced from Chiaramonti et al., 2012]
Biomass formed during fermentation using *S. cerevisiae* and *Z. mobilis* are recognized as safe for fodder making these organisms suitable for metabolic engineering for application in co-fermentation of both pentose and hexose sugars. Recent reports suggest that some white rot fungi [Saha et al., 2016], *Agaricus bisporus*, *Bjerkandera adusta* and *Iprex lacteus*, are able to produce ethanol from glucose under semi-aerobic conditions. Jung et al. (2015) reported the use of *Kluyveromyces marxianus* for ethanol fermentation from empty palm fruit bunches. Much research continues in this field in search of efficient fermentative microorganisms for application in the simultaneous fermentation of pentose and hexose sugars. Theoretically, 2 moles of ethanol can be obtained from the fermentation of 1 mole of hexose (Eq. 1.1).

\[
C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2
\]  \(\text{Eq. 1.1}\)

However, part of the sugars is used for growth and biomass production, and therefore experimentally obtained yields can never correspond to the theoretical ones. Furthermore, depending on the metabolic pathway that the microorganism uses, other metabolites can be produced alongside ethanol, decreasing the final yield.

Lignocellulosic biomass represents a promising but challenging substrate for ethanol production. Hydrolysis of lignocellulosic substrates results in the formation of both hexose and pentose sugars from cellulose and hemicellulose, respectively. *S. cerevisiae* can easily ferment hexose sugars but it is not able to use pentose sugars in its metabolism to produce ethanol. Therefore, the co-fermentation of hexose and pentose sugars is expected to improve ethanol yields from lignocellulosics which can be possible by applying engineered/recombinant yeast strains in the fermentation of ethanol, an area of active research at the present [Ha et al., 2011].
1.5 LIGNOCELLULOSIC BIOMASS PRETREATMENT TECHNIQUES

The main aim of lignocellulosic biomass pretreatment is to separate the biomass components i.e. cellulose, hemicellulose and lignin and eventually to remove lignin without losing hemicellulose while decreasing the crystallinity of cellulose and increasing the porosity of the biomass material. A number of techniques are available for the pretreatment of biomass; these include hot water treatment, steam explosion, ammonia fiber explosion, alkali treatment, organic solvent treatment and enzymatic hydrolysis. A brief description of the pretreatment methods is presented here.

1.5.1 Hot water treatment [Weil et al., 1997]

This type of pretreatment is also termed aqua-solve, aqueous fractionation hydrothermolysis, and uncatalyzed solvolysis. In hot water treatment, biomass is treated with liquid hot water at elevated temperature and the treatment uses pressure to maintain the water in the liquid state. Water at high temperatures acts as an acid in the fractionation of the biomass rigid structure. The main component of the operating cost for this method is the energy required to feed the water as a saturated liquid. The treatment time for this process is 15-20 minutes at temperatures in the range of 200-230 °C. Approximately 40-60% of the total biomass is dissolved in this process.

1.5.2 Steam Explosion [Sun et al., 2002]

Steam explosion is the most commonly used method for the pretreatment of biomass. In this method, biomass is treated with high-pressure saturated steam, and then the pressure is suddenly reduced, which makes the materials undergo an explosive decompression. Steam explosion is initiated at a temperature of 160-260 °C for several seconds to a few minutes before the material is exposed to lower pressure. The process causes hemicellulose
degradation and lignin transformation due to high temperature, thus improving cellulose hydrolysis. Addition of acid (≤ 3% (w/w)) in steam explosion can decrease time and temperature, effectively improve hydrolysis, and lead to the complete removal of hemicellulose.

1.5.3 Ammonia Fiber Explosion [McMillan et al., 1994]

Ammonia fiber explosion is a physicochemical pretreatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. The process is very similar to steam explosion. During pretreatment only a small amount of the material is solubilized. The structure of the material is changed, resulting in increased water holding capacity and higher digestibility in subsequent processing. Ammonia fiber explosion has been reported to be ineffective for biomass with higher lignin content (~25%).

1.5.4 Carbon dioxide explosion [Zheng et al., 1998]

In the carbon dioxide explosion method biomass is treated with supercritical carbon dioxide at comparatively lower temperatures than steam explosion. It is hypothesized that CO₂ forms carbonic acid when dissolved in water, increasing the hydrolysis rate. Increased rate of penetration of CO₂ molecules into the crystalline structure of biomass is facilitated by an increase in pressure. Carbon dioxide hydrolyzes hemicellulose as well as cellulose. Moreover, the low temperature treatment helps in preventing the decomposition of monomer sugars formed during the treatment. However, the yields are relatively low compared to those of other pretreatment methods. A comparative study on the pretreatment of sugar cane bagasse and recycled paper and its re-pulping waste using different treatment methods including CO₂ explosion, steam explosion and ammonia fiber explosion concluded that CO₂ explosion is more cost-effective than other methods.
1.5.5 Organosolvation [Pan et al., 2005]

In the organosolvation process biomass is treated with a mixture of organic/aqueous organic solvents and acid catalysts (inorganic and organic). The most commonly used solvents are methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol. The process facilitates simultaneous hydrolysis and delignification of lignocellulosic biomass. Lignin can be recovered as a fine precipitate by flash exposure of the liquor to atmospheric pressure, followed by rapid dilution with water. Other products such as sugars and sugar degradation products can be recovered from the water soluble stream. Solvents from the process can be recycled to reduce the cost.

1.5.6 Alkaline hydrolysis [Mosier et al., 2005]

Alkaline hydrolysis processes use lower temperature and pressures than other pretreatment methods. The most commonly employed alkaline pretreatment agents are sodium hydroxide, potassium hydroxide, calcium and ammonium hydroxides. Alkali pretreatments carried out under mild conditions require long pretreatment times, in the order of hours to days. However, treatment at mild temperatures (25-55 °C) selectively removes lignin and hemicellulose while cellulose is unaffected. Lignin removal increases enzyme effectiveness by increasing access to cellulose and hemicellulose and by eliminating non-productive adsorption sites. The effect of alkaline pretreatment of different biomass feed-stocks depends on the lignin content of the materials.

1.6 HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS

In biofuel production from lignocellulosic biomass the structure of the feedstock must be broken down to extract sugars from cellulose and hemicellulose fractions. Therefore, a pre-hydrolysis step is needed before the conversion of the feed stock to biofuel. The main goal of
the pre-hydrolysis is to improve the conversion of cellulose and hemicellulose to free sugars for application in further biofuel production. Hydrolysis of biomass for sugars synthesis can be carried out using either chemical or enzymatic hydrolysis.

1.6.1 Acid hydrolysis of lignocellulosic biomass for ethanol production

Acid hydrolysis is a process in which biomass is treated with water in the presence of acid to give sugars. The treatment process converts the cellulose and hemicellulose to sugars. Acid hydrolysis is the most common methodology for biomass conversion to fermentable sugars, where virtually any acid (H$_2$SO$_4$, HCl, H$_3$PO$_4$) can be used. Hydrolysis of biomass for the release of sugars takes place through either a dilute acid treatment or concentrated acid treatment. The existing acid hydrolysis processes consists of two stage acid hydrolysis [Kim et al., 2005], using double acids and heterogeneous acids. Important parameters such as reaction temperature, acid concentration, reaction time and particle size determine the conversion and yield of sugars obtained. Dilute acid hydrolysis can be carried out at lower temperature with longer reaction times and at higher temperatures with shorter reaction times. Longer reaction time results in the degradation of monomers released from hemicellulose; this observation was reported by Cruz and coworkers with barley husks [Cruz et al., 2002].

Different biomass feedstocks such as bark rich saw mill waste, rice straw, grass, silage press cakes, sugar maple wood extract, oil palm empty fruit bunch [Rahman et al., 2007], wood shavings, sweet sorghum bagasse [Heredia-Olea et al., 2012] and nitrogen rich dairy manure [Liao et al., 2004] have been processed using dilute acids for sugar release. Reports on the dilute acid hydrolysis processes, carried out in two steps with different acid concentrations at each stage [Kim et al., 2000; Kim et al., 2005 and Karimia et al., 2006], varying from 0.05 to 2.5% state that yields reached around 80-85% of the sugars available in the biomass. For example, a pre-extraction step with water at low/high temperature [Neureiter
et al., 2004] followed by acid hydrolysis of maple wood resulted in around 160 g sugar L⁻¹ concentrated wood extract [Hu et al., 2010].

A range of acids have been employed for the breakdown of the crystalline structure of biomass constituents; these include sulfuric acid, hydrochloric acid, phosphoric acid and H-USY zeolite treated with oxalic acid [Zhou et al., 2013]. The specific interest in the use of H₃PO₄ in acid hydrolysis is that after neutralization with sodium hydroxide, it will yield sodium phosphate which will remain in the hydrolyzate and subsequently be used as a nutrient by microorganisms in the fermentation for ethanol production negating the requirement for filtration [Orozco et al., 2011]. However, hydrolysis with H₃PO₄ does require higher temperatures and increased acid loading compared to hydrolysis with sulphuric acid.

The use of concentrated acid hydrolysis represents a promising process for the hydrolysis of biomass for both biofuel and bio-refinery applications, with high sugar yields, lower levels of fermentation inhibitors, good fermentability and a general robustness towards changes in raw material quality. The treatment of cellulose with concentrated sulphuric acid solution (50-60%) at room temperature [Ioelovich 2012] resulted in good solubility and the recovered cellulose had an amorphized structure characterized by high enzymatic digestibility. This regenerated cellulose had reduced crystallinity (25–30%), and a lower degree of polymerization (40–50). A two stage concentrated acid hydrolysis [Moe et al., 2012] process for the saccharification of soft wood biomass resulted in good sugar yields and a low concentration of fermentation inhibitors. However, concentrated acid hydrolysis has some major drawbacks, namely:

- Consumption of large quantities of concentrated acids.
- High costs of neutralization.
- Gypsum disposal problems.
Concentrated acid hydrolysis requires expensive materials for process equipment construction and to make the process economically feasible acid recovery is needed which itself an energy consuming step. Therefore, dilute acid hydrolysis is a more suitable option compared to concentrated acid hydrolysis.

Currently, biomass treatment technologies are energy intensive due to the large amount of water usage and the requirement for heating the process material to pretreatment temperatures of 100-200°C [Jorgensen et al., 2007]; in addition, the conversion process results in the accumulation of salts and inhibitors that are toxic to subsequent bio-refinery processes. Therefore, conversion of lignocellulosic biomass to biofuels requires efficient pretreatment technology, achieved through optimization of pre-hydrolysis in terms of both maximizing the sugars yield and minimizing the energy requirement.

**1.6.2 Enzymatic hydrolysis of lignocellulosic biomass for ethanol production**

The use of enzymes in biomass conversion processes can often eliminate the requirement for high temperatures, chemicals and extremes of pH, while at the same time offering increased reaction specificity, product purity and reduced environmental impact. Enzymatic hydrolysis of cellulose and hemicellulose components of lignocellulosic biomass is carried out by cellulase and hemicellulase enzymes which are highly specific. Cellulases are mainly a mixture of endoglucanases, exoglucanases, and β-glucosidases and catalyze the hydrolysis of cellulose to fermentable sugars. Xylanases and β-xylosidases are the enzymes that attack the backbone of hemicellulose. Pretreatment of lignocellulosic biomass is a prerequisite to achieve better conversion in the enzymatic hydrolysis of biomass. The role of pretreatment is that it usually breaks down the lignin structure, as shown in Figure 1.6, thereby facilitating the hydrolysis of cellulose and hemicellulose, resulting in the production of hexose and pentose sugars. Lignin acts as physical barrier limiting the accessibility of
enzymes to cellulose and hemicellulose substrates. The available techniques for the pretreatment of biomass have been previously discussed in Section 1.5 [Chiaramonti et al., 2012]. Biological pretreatment can represent an ecofriendly and a low cost alternative to physico-chemical and chemical pretreatments of lignocellulosic biomass. However, biological pretreatment requires an appropriate microorganism-biomass combination, as for example it is reported that fungal treatment can cause carbohydrate loss [Saha et al., 2016]. Pretreatment results in increased porosity in the biomass substrate due to the removal of the lignin, disruption of hemicellulose, size reduction of the particles and reduction in the crystallinity of cellulose depending on the specific pretreatment technology. Enzymatic delignification can also be achieved using laccase and lignin peroxidase enzymes but the technique is limited by long residence times. Improvements in enzymatic hydrolysis for the production of bioethanol from sustainable biomass are necessary in order to reduce enzyme requirements and the overall processing times.

Figure 1.6: Pretreatment for the breakdown of the rigid structure of biomass [Kumar et al., 2009 and Hsu et al., 1980].
The other major limiting factor in the enzymatic conversion of biomass to biofuels is the cost of cellulase enzymes for use in the hydrolysis of pretreated biomass [Klein-Marcuschamer et al., 2012]. Techno-economic analysis of lignocellulosic bioethanol production costs report that the enzymes cost about $132 per cubic meter of ethanol when the enzymes are supplied by commercial enzyme manufacturers, such as Novozymes [Chovau et al., 2013]. However in the case of on-site enzyme production the overall cost of enzymes was reported to be $90 per cubic meter of ethanol, significantly lower than Novozymes. Therefore, to achieve cost effective biomass conversion for biofuel production an on-site/in house enzyme production for the continuous supply of cellulases to the process appears as one of the most economically attractive options.

Much information is available on the preparation of cellulase enzymes using different substrates and a variety of cellulytic microorganisms for application in lignocellulosic bioethanol production have been reported. Both bacteria (e.g. *Bacillus*, *Bacteriodes*, *Cellulomonas*, *Clostridium*, *Streptomyces*) and fungi (e.g. *Phanerochaete chrysosporium* [Szabo et al., 1996], *Tricoderma reesei*, *Aspergillus niger* [Sukumaran et al., 2009], *Gracibacillus* species [Yu et al., 2015], *Penicillium oxalicum* [Huang et al., 2015]) can produce cellulases. A variety of substrates have been employed in cellulases production; for instance Humbird et al. reported cellulase preparation using corn syrup substrate and *T. reesei* [Humbird et al., 2011]; Jing et al. used hydrolyzed sugarcane bagasse residue as substrate for celllobiohydrolase production using *P. oxalicum* [Jing et al., 2015]; Vijayaraghavan & co-workers reported carboxymethyl cellulase production from cow dung by *Bacillus halodurans* ID18 [Vijayaraghavan et al., 2016]. The use of cheap lignocellulosic biomass substrates for enzyme production can significantly reduce the production cost of cellulases. Wheat bran has been reported to be an effective substrate for the preparation of cellulases using *T. reesei* and *A. niger* [Sukumaran et al., 2009]. Other potential woody and herbaceous substrates used in
cellulase production by white rot fungi and brown rot fungi via solid state fermentation include eucalyptus wood chips, pine wood chips, beech leaves, wheat straw, wheat bran, corn fiber, corn stover, reed grass, bean stalk and sago waste. Solid state fermentation for enzyme production is the most adopted technology as it requires less infrastructure and less skilled manpower to operate and has lower operational costs.

1.7 PYROLYSIS - A THERMAL TREATMENT FOR LIGNOCELLULOSIC BIOMASS

Asia accounts for 67% of global coal consumption, with global coal consumption increasing by 39% over the last decade. Climate change mitigation policies [Bauer et al., 2015] suggest fossil fuel markets have a profound influence on climate change caused by CO₂ emissions, especially from coal. This has led to an increase in research leading to the search for alternative sources to coal for some aspects of energy and carbon based materials production using a renewable energy resource. Pyrolysis of renewable organic material represents one of the potential methodologies leading to the generation of biochar, bio-oil and gases which have a number of key applications. Currently, research is focused on the search for alternative carbon sources that can substitute for coal in a few of its application areas, such as energy production and the production of activated carbon for use in water and air purification [Rebitanim et al., 2013]. Biochar is one such eco-friendly carbon rich product produced from the pyrolysis of renewable feed-stocks which can be used as a precursor for the synthesis of various carbon based materials.

1.7.1 Pyrolysis products and its applications

Biomass pyrolysis is the thermal decomposition of biomass in the absence of an oxidizing agent i.e. oxygen with products biochar, biooil and gases such as carbon dioxide, carbon monoxide, hydrogen, and methane. Figure 1.7 illustrates the biomass pyrolysis
reaction pathway. Pyrolysis is a promising technology for the production of bio-oil and biochar.

![Biomass pyrolysis reaction pathway](image)

Figure 1.7: Biomass pyrolysis reaction pathway

Bio-oil, also known as pyrolytic oil, can be upgraded to light hydrocarbons which contain low levels of aromatics with an almost complete absence of sulphur when compared to the sulphur content found in petroleum based fuels. Bio-oil can also be upgraded to a substitute fossil derived fuels [Mortensen et al., 2011]. Hydrodeoxygenation, catalytic cracking, emulsification and steam reforming are the main bio-oil property improvement techniques. A variety of chemicals can also be extracted from bio-oil, such as phenols, volatile organic acids, levoglucosan and hydroxyacetaldehyde for applications in the resin industry, as well as pharmaceutical, fiber synthesizing and fertilizing industries. Commercialization of chemical extraction from bio-oil requires further research focus on low cost extraction processes and refining techniques [Xiu et al., 2012].

The obtained bio-oil from fast pyrolysis of lignocellulosic biomass usually composed of water, ash, oxygen at high concentration and it is highly viscous liquid with high corrosiveness. These characteristics of bio-oil limit its application as a fuel. Intensive research has been directed towards development of efficient bio-oil upgrading techniques.

The available sustainable techniques to upgrade bio-oil are:

i. Hydrotreating
ii. Hydrocracking

iii. Process using supercritical fluids

iv. Solvent addition

v. Emulsification

vi. Steam reforming

The details of each of these technologies have been reported by Xiu et al, (2012).

Feedstock composition and pyrolysis conditions are the major factors which affect bio-oil properties. Bio-oil resulted from fast pyrolysis is composed of many compounds including, aldehydes, ketones, sugars, carboxylic acids, esters and phenolics which make upgrading of bio-oil difficult [Mortensen et al., 2011]. An elevated temperature increases the bio-oil yield and was reported by Imam and Capareda (2012). They reported that an increase in temperature from 400 to 600 °C increased both the oil and water fractions of the total bio-oil product obtained from pyrolysis of switch grass. High water content of bio-oil is one of the major limitations for its application as a fuel. The other limiting properties of bio-oil are its high viscosity, high acidity and high inorganic content. These unfavorable properties of bio-oil are due to the presence of oxygenated compounds (aldehydes, ketones and carboxylic acids) which makes it unstable and of low heating value. Therefore, bio-oil needs to be upgraded to improve its properties to make it suitable for application as a fuel.

Biochar, on the other hand, is an eco-friendly carbon rich product from the pyrolysis of renewable feed-stocks and due to its carbon sequestration ability, has already been widely used in agriculture as a soil amendment for improving soil fertility [Song et al., 2014]. Biochar is also reported to promote, nitrogen fixation thereby decreasing the emission of N\textsubscript{2}O [Suddick et al., 2013] and other greenhouse gases from agricultural soils [Wantabe et al., 2014 & Creamer et al., 2014]. In addition biochar has been used as an adsorbent in the removal of arsenic [Jin et al., 2014], cadmium [Xu et al., 2014] and chromium [Ma et al.,
2014] from aqueous solutions. Moreover, biochar derived from biomass is increasingly recognized as a multifunctional material for agricultural and industrial applications. Biochar can be used in a wide range of applications in different fields including wastewater treatment, soil conditioning, animal farming, the building sector (insulation, air decontamination, decontamination of earth foundations, humidity regulations), protection against electromagnetic radiation/electro smog, decontamination of soil, biogas slurry treatment, exhaust filters (controlling emissions, room air filters), industrial material (carbon fibers, plastics), electronics (semiconductors, batteries), metallurgy (metal reduction), energy production (pellets, substitute for lignite), thermal insulation for functional clothing, deodorant for shoe and so on [Schmidt 2012].

1.7.2 Parameters effecting pyrolysis

The main influential parameters on pyrolysis are biomass type, reaction atmosphere, temperature, heating rate and vapor residence time [Kan et al., 2016]. The composition of cellulose, hemicellulose, protein, extractives and lignin varies from biomass to biomass. Biomass rich in cellulose, hemicellulose, protein and extractives result in a high yield of bio-oil whereas lignin rich biomass contributes to high biochar formation from pyrolysis. Pyrolysis reactions are typically performed in an inert atmosphere facilitated by the continuous supply of gases such as N₂, CO₂, CO, CH₄ and H₂. The type of gas used to generate the inert atmosphere will have a significant impact on the product distribution and its characteristic properties [Zhang et al., 2011]. Zhang et al. (2011) reported that fast pyrolysis carried out in a CH₄ atmosphere contributed to high bio-oil yield, whereas the use of H₂ and CO₂ to create an inert atmosphere resulted in the formation of high surface area biochar products. The presence of excess CO in pyrolysis enhanced the conversion of oxygen present in the bio-oil to CO₂. An H₂ atmosphere promoted the conversion of oxygen in bio-oil to water. Pyrolysis under CO₂ atmosphere was reported with less bio-char formation due to
the reaction between bio-char and CO$_2$. The other parameters, temperature, heating rate and residence time also play roles in the product yield and properties. Biomass pyrolysis processes are classified into three categories based on these operating conditions (temperature (T), heating rate ($\beta$) and biomass residence time (t) in the pyrolysis reactor) [Jahirul et al., 2012]. They are:

i. Slow pyrolysis (conventional) (T: 550-950 K; $\beta$: 0.1-1 K s$^{-1}$; t: 450-550 s) [Chen et al., 2014]

ii. Fast pyrolysis (T: 850-1250 K; $\beta$: 10-200 K s$^{-1}$; t: 0.5-10 s) [Trybetskaya et al., 2016]

iii. Flash pyrolysis (T: 1050-1300 K; $\beta$>1000 K s$^{-1}$; t<0.5 s)

Slow pyrolysis leads to maximum biochar yields as a result of low operating temperatures at slow heating rates with long residence time, while fast and flash pyrolysis result in high liquid yields due to high operating temperatures, high heating rate with short residence times. Therefore, operating conditions have a strong impact on the quality and overall yield of the products.

Pyrolysis should occur with a high heat flux to the biomass and with a correspondingly high heating rate of the biomass particle. Applying heat to biomass at a high rate will result in the formation of smaller fragments (volatiles) due to the cleavage of cellulose, hemicellulose, protein and lignin, and other constituents of biomass, unstable above 400 °C due to the presence of oxygen in the fragments. Hence, very short residence times are required in the thermal treatment step and an immediate quenching is required to prevent secondary chemical changes and the formation of unstable volatiles. Pyrolysis carried out with a short residence time, optimum temperature of exposure with immediate cooling of the vapors can be achieved by taking into account the appropriate size of the reactor and heating rate in terms of heat flux. Both the heat transfer rate to biomass particles and residence time
of vapors in the reactor have strong influences on the nature and distribution of pyrolysis reaction products. Therefore, the first and foremost consideration for designing a biomass pyrolysis reactor (residence time, size and material of construction) is to develop a clear picture of thermal behavior of the feedstock biomass with the application of heat at different heating rates which correspond to the heat transfer rate to biomass particles in order to estimate the amount of energy to be supplied to pyrolysis.

Various biomass feedstocks show different thermal behavior profiles, mainly due to the variation in the composition of biomass constituents. For example, Parthasarathy and Sheeba (2014) investigated the thermal behavior of bagasse, coir-pitch, groundnut shell and casuarina leaves, all of which exhibited different degradation temperatures for the individual biomass components (hemicellulose, cellulose and lignin).

Vhathvarothai and co-workers [Vhathvarothai et al., 2014] studied the co-pyrolysis of cypress wood chips, macadamia nut shell biomass and coal using thermogravimetric techniques and Kissinger’s corrected kinetic equation for kinetic parameter determination, concluding that the activation energy of coal is greater than that of both types of biomass (wood chips and macadamia nut shells). Yan et al. (2012) carried out pyrolysis kinetics of raw and hydrothermally carbonized loblolly pine lignocellulosic biomass in a thermogravimetric analyzer. The study revealed that pyrolysis of hydrothermally carbonized biomass progressed less aggressively than that of raw biomass which also showed that after an initial significant decomposition, the pyrolysis reactions continued at much slower rates with temperature.

Thermal treatment of a variety of seed biomass such as Jatropha curcus, raspberry and safflower to produce biooil and biochar has recently been reported in the literature. Table 1.3 summarizes these results, characterizing different seed cakes for their carbon content and heating value.
Table 1.3: Biooil & biochar yield and characterization from pyrolysis of various seed biomass.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Bio oil</th>
<th>Biochar</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jatropha</td>
<td>Yield-48 wt%;</td>
<td>Yield-35.1 wt%</td>
<td>[Biradar et al., 2014]</td>
</tr>
<tr>
<td>curcas de-oiled cake</td>
<td>Density-1040 kg/m³;</td>
<td>Viscosity-1.98 cSt</td>
<td></td>
</tr>
<tr>
<td>Raspberry</td>
<td>Flash: Yield-53.6 wt%, HHV-18.6 MJ kg⁻¹</td>
<td>Flash: HHV-24.5 MJ kg⁻¹</td>
<td>[Smets et al., 2014]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slow: Yield-12.1 wt%, HHV-27 MJ kg⁻¹</td>
<td>Slow: HHV-30.9 MJ kg⁻¹</td>
<td></td>
</tr>
<tr>
<td>Safflower</td>
<td>---------------</td>
<td>Fixed carbon content:</td>
<td>[Angin et al., 2013]</td>
</tr>
<tr>
<td></td>
<td>Yield-28-36 wt%</td>
<td>80.7%, HHV-30.27 MJ kg⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yield-25-34 wt%</td>
<td></td>
<td>[Sensoz &amp; Angin 2008]</td>
</tr>
</tbody>
</table>

Biradar et al. (2014) reported pyrolysis conversion of *Jatropha curcas* de-oiled seed cake in a fluidized bed pyrolysis system. Bio-oil obtained from the process was upgraded by removing the moisture content from 31% to 3% by distillation (at 40-80 °C). Further, they reported viscosity and density of the upgraded bio-oil were 1.77 cST and 1030 kg m⁻³, respectively.
The value addition of raspberry seed residue by slow and flash pyrolysis was reported by Smets et al. (2014). Flash pyrolysis of raspberry residue resulted in the formation of bio-oil with a 18.9 MJ kg\(^{-1}\) heating value and biochar of 24.9 MJ kg\(^{-1}\) heating value. Slow pyrolysis of the same residue resulted in the formation of a bio-char product with an increased heating value of 30.1 MJ kg\(^{-1}\).

Angin et al. (2013) reported the application of activated carbon obtained from safflower seed cake biochar for adsorption of reactive dye material. Biochar has a fixed carbon content of 80.7% and the activated carbon has a surface area of 1277 m\(^2\) g\(^{-1}\).

The effects of pyrolysis operating conditions (temperature, heating rate and carrier gas flow gas) on safflower seed cake pyrolysis in a fixed-bed reactor was reported by Sensoz and Angin (2008). Pyrolysis carried out at 400 °C and 600 °C with 50 °C min\(^{-1}\) heating rate resulted into the formation of 25 and 34% bio-char, 19 and 25% gas, 28 and 36% bio-oil, respectively.

The present research is aimed at creating a platform for de-oiled *Pongamia pinnata* seed cake biomass using bio-refining by producing energy and value added products. Recent research [Rebitanim et al., 2013] has indicated the potential of biooil and biochar obtained from the pyrolysis of the seed cake biomass in areas such as energy production and the production of activated carbon.

**1.8 OBJECTIVE OF THE PROJECT**

*P. pinnata* seeds are composed of 30-40 % oil. *P. pinnata* oil has been successfully converted to biodiesel [Dwivedi et al., 2014]. As a result of biodiesel production using *P. pinnata* oil, the biodiesel industry is producing ~65-70 tonnes of de-oiled seed residue from every 100 tonnes of seeds. Moreover, by-product value addition is an important aspect in
improving the economics of the process. Like other, edible de-oiled seed residues (peanut, soybeans, sunflower and canola seed residues), *P. pinnata* seed residue is not suitable for direct consumption by humans or animals. Natanam et al. (1989) reported the toxic effects of *P. pinnata* oil and cake on growth of broiler chicks. Chicks fed on the cake diet suffered 100% mortality, whereas the consumption of autoclaved or water soaked cake showed an improvement in the weight gain and reduced mortality in the broiler chicks. Therefore, to exploit this abundant waste and toxic residue, in the present work different methodologies have been developed to convert *P. pinnata* de-oiled seed residue into bioethanol, bio-oil and biochar products.

The aim of the present work is to assess the potential of non-edible *Pongamia pinnata* seed residue for bio-refinery and to optimize the process parameters in converting the seed residue constituents to valuable products using thermo-chemical and biochemical treatment methods. The present work includes:

(i) Characterization of deoiled *P. pinnata* seed residue for carbohydrates, protein, lignin, extractives and ash composition.

(ii) Evaluation of the effects of process parameters on dilute acid hydrolysis of *P. pinnata* seed residue in terms of glucose yield.

(iii) Evaluation of the effects of different acids on sugars yield from acid hydrolysis and on the ethanol concentration from the fermentation of the hydrolysis product; estimation of energy requirement in acid hydrolysis process.

(iv) Evaluation of crude lignocellulase enzyme prepared from *Sphingomonas echinoides* and *Iprex lacteus* on hydrolysis of the seed residue and estimation of ethanol yields from enzymatic hydrolysis followed by fermentation.
Evaluation of the thermal behaviour and pyrolysis kinetics of deoiled *P. pinnata* seed residue using thermogravimetric analysis. Evaluation of bio-oil and biochar yields from the seed residue pyrolysis in a fixed bed reactor.
Chapter 2 provides a general description of the key methodologies adopted in the thesis, which are feedstock compositional characterization for moisture content, total solids, ash, protein and lignin; hydrolysis followed by fermentation and further quantification of sugars and ethanol formed, together with characterization of *P. pinnata* seed residue and biochar formed from pyrolysis of the seed cake using CHNS elemental analysis, scanning electron microscopy and Fourier transformed infrared spectroscopy.

### 2.1 FEEDSTOCK COMPOSITIONAL CHARACTERIZATION

Deoiled *P. pinnata* seed residue biomass, obtained as a waste in the oil expelling process for *P. pinnata* oil, contains significant moisture. Proper sample preparation will minimize interferences in subsequent compositional analyses. *P. pinnata* seed residue biomass sample preparation for various analyses was carried out based on the method given by Hames and coworkers (2008) [Hames et al., 2008]. Sample drying, particle size reduction and sieving are the main procedures in sample preparation for subsequent analyses. The seed residue was initially analyzed for its moisture and extractives content. The extractive free seed residue was then analyzed for protein, lignin and ash. Based on these values the composition of raw seed residue was back calculated for protein, lignin and ash contents.

#### 2.1.1 Moisture and total solids content of seed residue

Moisture and total solids content of the sample were estimated by NREL-LAP42620 [Hames et al., 2008] and NREL-LAP42621 [Sluiter et al., 2008a], respectively. The first
consideration for sample preparation is drying. Moisture and total solids content of \textit{P. pinnata} seed residue biomass sample (1 g) was determined using oven drying at 105±2 °C. The sample was oven dried overnight at 105 °C and was transferred into a desiccator for cooling before being re-weighed to determine the percentage of total solids and therefore the original moisture content. Total solids and moisture content were determined using Equation 2.1 and 2.2, respectively.

\[
\% \text{Total solids} = \frac{\text{Weight}_{\text{dried sample+cruicible}} - \text{Weight}_{\text{cruicible}}}{\text{Weight}_{\text{DOKS sample as received+cruicible}} - \text{Weight}_{\text{cruicible}}} \times 100 \quad \text{(2.1)}
\]

\[
\% \text{Moisture} = 100 - \% \text{Total solids} \quad \text{(2.2)}
\]

2.1.2 Ash

The ash content of the sample was estimated using NREL-LAP42622 [Sluiter et al., 2008b]. The amount of inorganic material in biomass, i.e. ash was determined by placing the crucible containing dried \textit{P. pinnata} seed residue biomass sample in a muffle furnace. The temperature was increased from room temperature to 200 °C and from 200 °C to 400 °C at 20 °C per min with 10 min holding at each temperature. The temperature was further increased to 575 °C at 10 °C per min and held for 10 h. Ash content was determined using Equation 2.3.

\[
\% \text{Ash} = \frac{\text{Weight}_{\text{cruicible+Ash}} - \text{Weight}_{\text{cruicible}}}{\text{Weight}_{\text{cruicible+PPSR}} - \text{Weight}_{\text{cruicible}}} \times 100 \quad \text{(2.3)}
\]

2.1.3 Extractives

A Soxhlet extraction with different solvents was employed to determine the extractives including waxes, fats, gums, resins, starches, pitch, sterols, flavonoids, tannins, terpenes, quinones and chlorophyll present in the biomass. Estimation of the percentage of extractives in \textit{P. pinnata} seed residue sample was carried out according to NREL-LAP42619 [Scarlata et
al., 2008] and was expressed on a dry weight basis. Soxhlet extractions were carried out with ethanol, acetone, toluene, isopropyl alcohol (IPA) at 5-6 siphon cycles per hour for 10 h. The experimental setup of the Soxhlet extraction unit is shown in Figure 2.1 and consists of a boiling flask, Soxhlet siphon arm, and condenser.

![Soxhlet extractor for extractives from *P. pinnata* seed residue.](image)

**Figure 2.1:** Soxhlet extractor for extractives from *P. pinnata* seed residue.

### 2.1.4 Protein

The protein content in extractive-free *P. pinnata* seed residue was estimated following the addition of 0.5 N NaOH for 3 h at room temperature with a 1:20 (v/w) dilution of cake to alkali [Madhumanchi et al., 2013]. At the end of the specified time the soluble alkali portion and the cake residue were separated by centrifugation. The alkali solution containing the
soluble matter was neutralized with 6 N HCl by adjusting the pH in the range 4-5. At this pH dissolved protein precipitated out of solution. The protein precipitate was centrifuged and dried in an oven. The amount of protein obtained was evaluated from Equation 2.4.

\[
\% \text{ Protein} = \frac{\text{Weight}_{\text{Dried protein precipitate}}}{\text{Weight}_{\text{PPSR}}} \times 100 \quad \ldots \ldots \ldots (2.4)
\]

2.1.5 Lignin

Lignin is one of the major constituents of biomass. Klason lignin is defined as the biomass component insoluble in a 72% sulphuric acid solution. Estimation was carried out by treating 1 g of extractive free *P. pinnata* seed residue biomass with 72% H₂SO₄ solution at 1:15 w/v ratio for 2 h at room temperature to hydrolyze and solubilize the carbohydrates [Jung et al., 1999]. The sample was then diluted with water (560 ml) to reduce the sulphuric acid concentration to 3 % and further boiled for 4 h. Lignin was allowed to settle before being filtered. The residue was washed using hot water until reaching a neutral pH. The dried insoluble residue represents the lignin content.

2.2 ACID HYDROLYSIS OF *P. PINNATA* SEED RESIDUE

2.2.1 Acid hydrolysis

Acid hydrolysis reactions were performed in a two necked, round bottomed glass flask (500 ml capacity) that served as a reactor in batch mode. Figure 2.2 depicts the acid hydrolysis experimental setup. A Radley's heat-on unit was used to provide the heating and stirring required for the reaction. A maximum temperature of 300°C, with heating power 600 W can be attained by the apparatus. It is manufactured from solid aluminum, and houses a carousel temperature controller and a carousel stirring hot plate (speed range 50-1200 rpm, stirred quantity max. 20 l H₂O). A magnetic bead was used for mixing and the speed was controlled
manually. The reaction vessel was equipped with a thermometer for measurement of temperature inside the reactor and a reflux condenser was provided to prevent the loss of water due to vaporization when the reactions were carried out at temperatures greater than the boiling point of water. The temperature of the cooling water was maintained with a Poly-Science automatic digital refrigerating and heating thermostat.

![Acid hydrolysis reaction experimental setup](image)

Figure 2.2: Acid hydrolysis reaction experimental setup

In a typical experiment the unit was switched on and heating was set to $T_{\text{reaction}} + 20^\circ\text{C}$. As soon as the temperature sensor/controller on the Radley's unit showed the set temperature value the reactor was filled with dried and extractive free *P. pinnata* seed residue (30 g) and acid solution and the reaction was timed. Samples were collected during the reaction at regular time intervals. At the end of the reaction the mixture was quenched immediately. Finally the cooled reaction mixture was neutralized with NaOH solution and centrifuged to
separate solid and liquid fractions. The liquid fraction was accurately weighed and analyzed to quantify the sugars formed.

2.2.2 Acid hydrolysis experimental design and analysis of results

Acid hydrolysis reactions were performed according to the Taguchi robust design of experiments method. The Taguchi method is a design of experiments method based on orthogonal arrays. It provides a set of a minimum number of experiments which will give complete information about the influence of all the factors on the performance parameter. The Taguchi method gives much reduced variance for the experiment with optimum settings to investigate the effect of different factors on the mean and variance of performance. In optimization of the performance parameter, the signal to noise ratio (S/N), which is the log transformation of mean square deviation of the desired performance is the objective function that can be optimized for the desired output. Here signal (S) is the mean (desirable value) and noise (N) is standard deviation (undesirable value). The experimental data from Taguchi arrays can be analyzed by plotting the data and performing a visual analysis, ANOVA and Fisher's exact test to assess the significance of the factor effect. Analysis of variance was performed on experimental data to estimate the sum of squares (SS), degrees of freedom (DF = number of factor levels subtracted by 1) and mean square (MS=SS/DF). The obtained data was used to estimate the F value, MS_factor/MS_residual (Fisher test), and the data was further evaluated to estimate the percentage contribution of each factorial effect.

Different criteria can be considered in defining the S/N ratio to optimize the performance parameter as detailed below.

**Criterion 1:** If the goal is to maximize the performance, the value of $\frac{S'}{N}$ should be high.

**Criterion 2:** If the goal is to minimize the performance, the value of $\frac{S''}{N}$ should be low.
**Criterion 3:** If the goal is to target the predetermined \( \frac{S}{N} \) then the value of \( \frac{S'''}{N} \) being nominal is better.

Depending on the target to be achieved as explained above the S/N ratio is defined as the three expressions 2.5, 2.6, and 2.7 for criteria 1, 2 and 3, respectively.

\[
\frac{S'}{N_i} = -10\log_{10} \left[ \frac{1}{n_j} \sum_{j=1}^{n} 1 / Y_i^2 \right] \quad \text{(2.5)}
\]

\[
\frac{S''}{N_i} = -10\log_{10} \left[ \frac{1}{n_j} \sum_{j=1}^{n} Y_i^2 \right] \quad \text{(2.6)}
\]

\[
\frac{S'''}{N_i} = -10\log_{10} \left[ \frac{1}{n_j} \sum_{j=1}^{n} [\text{Square of mean/variance}]_i \right] \quad \text{(2.7)}
\]

Where the subscript \( i \) = Experiment number

\( n \) = Number of replicates of experiment \( 'i' \)

\( j \) = Number of replicates

\( Y \) = Performance parameter

The effect of each factor was calculated by determining the range (\( \Delta \)), defined as the difference between the S/N ratio values at the maximum and minimum level of the factor.

In the present study the chosen factors that affect glucose and other sugars formation from the seed residue hydrolysis reaction were acid type, temperature, acid concentration and the acid to cake weight ratio. Three levels were considered for each factor. The performance parameters considered were: (i) % glucose in total sugars (\( Y_1 \)), (ii) g glucose per kg cake (Glucose yield, \( Y_2 \)).
The L_9 orthogonal array of the Taguchi method was selected for the experimental design. According to the L_9 orthogonal array a total of 9 experiments were required to be carried out to draw a complete picture of the effect of factors on performance (Y_1, Y_2). Here the performance parameters Y_1, Y_2 were defined as follows:

\[
Y_1 = \frac{\text{Area of glucose}}{\text{Total area of all sugars formed}} \quad \ldots \ldots (2.8)
\]

\[
Y_2 = \frac{\text{g glucose formed}}{\text{kg extractive free karanja cake}} \quad \ldots \ldots (2.9)
\]

Two sets of experiments were planned to study the effect of different factors in the acid hydrolysis reaction and to obtain the optimum factor levels. In the first set of experiments the factors considered were acid concentration (% wt in liquid), temperature and acid solution to seed cake weight ratio with Y_1 and Y_2 as performance parameters.

The next set of the experiment was planned to obtain the best acid for hydrolysis of the seed residue. Experiments were performed by fixing the acid solution to seed cake weight ratio at 1:10 with variation in the type of acid (H_2SO_4, HCl, H_3PO_4), acid concentration and temperature with Y_2 as the performance parameter.

2.2.3 Energy requirement of the acid hydrolysis process

Generally, biomass conversion technologies have been limited by the energy demand during its conversion to monomeric sugars i.e. hydrolysis of cellulose. Therefore, the present work focused mainly on estimating energy demand of hydrolysis step.

The energy requirement of the acid hydrolysis process was estimated for acid hydrolysis of the seed residue, which resulted into comparatively high quantities of sugar. Quantities of the key materials required for 1 kg sugar production were calculated based on conversions (biomass to sugars) achieved in acid hydrolysis. The energy required by the process was estimated using an energy balance equation centered around the acid hydrolysis.
process. Changes in internal energy of the substances involved in the process (P. pinnata seed cake biomass, acid, and water), heat of reaction, heat lost and the amount of energy recovered from the process streams were taken into consideration in estimating the energy demand of the process. For a closed system the energy balance equation is as follows:

\[ \Delta E = Q - W \] \hspace{1cm} (2.10)

Where \( \Delta E \) is change in internal energy of the system, \( Q \) is the heat added to the system and \( W \) is the work done by the system. The following assumptions were considered in estimating the energy requirement of the acid hydrolysis process.

1. No work was done by the pretreatment system and the energy input for agitation was not included in order to simplify the equation.
2. The heat of reaction of glucose formation (4.2 KJ/mol) [Humbird et al., 2011] was negligible compared to the energy supplied.
3. The heat losses from a well-insulated reactor system were considered negligible compared to the amount of energy supplied to the process [Mafe et al., 2015].
4. Sixty five percent of the total energy supplied was recovered from the process streams [Theodore et al., 2011]. The heat recovered in the process was of lower quality than the steam supplied to the process. However, the heat recovered can be utilized to preheat the reactants.

The heat added to the system (\( Q \)) i.e. the amount of energy required to increase the temperature of the process material from 25 to 100 °C is the sum of the heat required by the seed residue biomass, water, and HCl. Therefore,

\[ Q = Q_{p\cdot\text{pinnata}} + Q_{w} + Q_{HCl} \] \hspace{1cm} (2.11)

\[ Q = m_{p\cdot\text{pinnata}} \int_{25}^{100} c_{p\cdot\text{pinnata}} \, dT + m_{w} \int_{25}^{100} c_{w} \, dT + m_{HCl} \int_{25}^{100} c_{HCl} \, dT \] \hspace{1cm} (2.12)
Heating energies of the material streams were estimated using ASPEN plus simulation software using the HEATX model. Proximate and ultimate analysis data of *P. pinnata* seed residue was obtained experimentally according to the ASTM standard method [Cantrell et al., 2010] and the data was used to estimate the biomass properties which are not available in the ASPEN plus database.

Proximate analysis of *P. pinnata* seed residue biomass for ash, volatile matter and fixed carbon content were carried out according to ASTM D-3175-07 and ASTM D-3174-04 standard methods [Cantrell et al., 2010] in a Mettler ToledoTGA/SDTA 851e, analyser (Mettler Toledo, Switzerland). Ultimate analysis i.e., C,H,N,S,O elemental composition of *P. pinnata* seed cake was carried out using a CHNS Analyzer ELEMENTAR Vario micro cube. The detailed methodology for the estimation of biomass specific heat capacity was carried out as previously described by Doherty et al. (2013).

Net energy required by the acid hydrolysis process was defined as the difference between the heat added to the system and the heat recovered from the process streams.

\[
Net \text{ energy required, } Q_{net} = Q - Q_{recovered} \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (2.13)
\]

Where in equation (2.11), (2.12) and (2.13),

\[
Q = \text{Heat added to the system, kJ}
\]

\[
Q_{P, pinnata} = \text{Heat added to } P. \text{ pinnata stream, kJ}
\]

\[
Q_W = \text{Heat added to water stream, kJ}
\]

\[
Q_{HCl} = \text{Heat added to HCl stream, kJ}
\]

\[
Q_{recovered} = \text{Amount of heat recovered from the process streams, kJ}
\]

\[
Q_{net} = \text{Net amount of heat required by the process, kJ}
\]
\[ m_{P.\text{pinnata}} = \text{Amount of } P. \text{pinnata processed, } kg \]

\[ m_w = \text{Amount of water employed, } kg \]

\[ m_{HCl} = \text{Amount of } HCl \text{ employed, } kg \]

\[ C_{pP.\text{pinnata}} = \text{Specific heat capacity of } P. \text{pinnata seed residue, } kJ^0\text{C}^{-1}kg^{-1} \]

\[ C_{pw} = \text{Specific heat capacity of water, } kJ^0\text{C}^{-1}kg^{-1} \]

\[ C_{pHCl} = \text{Specific heat capacity of } HCl, kJ^0\text{C}^{-1}kg^{-1} \]

2.3 ENZYMATIC HYDROLYSIS

2.3.1 Isolation & Screening of cellulolytic bacteria from *P. pinnata* seed residue

Cellulolytic microorganisms were isolated from *P. pinnata* seed residue biomass at 30 °C using Bushnell-Hass salts medium (BH medium) supplemented with 0.5% w/v cellulose as the sole carbon source. An aliquot of 100 µl of serially diluted *P. pinnata* seed residue (1 g/10 ml) was spread onto BH-Cellulose agar plates and the plates were incubated at 30 °C for 7 days. Isolated colonies were subcultured repeatedly onto BH-cellulose agar plates to obtain pure isolates. Purified isolates were grown in liquid media overnight and were stored in 25% glycerol in nutrient broth at -80 °C for further work.

Screening of the isolates was carried out by measuring the diameter of clear zones [Pandey et al., 2013] on BH-cellulose agar plates. All the isolated microorganisms were grown in nutrient broth overnight and the bacterial pellet obtained by centrifugation at 4000 rpm for 5 minutes at 4 °C. Bacterial pellets were washed four times using sterile Milli-Q water to remove nutrients and the pellets re-suspended in sterile water. Nutrient free organism in water (50 µl), with an optical density adjusted to 0.9 at 600 nm, were inoculated at the center of BH-cellulose-Congo red (0.05%) agar plates. The plates were incubated at 30
⁰C for 7-14 days and the diameter of any clear zones was measured. Microbial isolates which exhibited highest clear zone diameter were selected for further investigation.

2.3.2 Cellulolytic activity of Iprex lacteus

*Iprex lacteus* is a white rot fungus and was obtained from the RMIT Culture Collection (ATCC 11245). Cellulolytic activity of *I. lacteus* was tested by inoculating a BH-cellulose agar plate with the fungus grown on potato dextrose agar medium. A 1 cm plug of fungus (nutrient free) was kept at the centre of the plate and incubated for 7-14 days. Cellulolytic activity of the fungus was assessed based on the diameter of the clear zone on the media plates. Microorganisms that showed highest clear zone were selected for quantitative screening by enzyme production.

2.3.3 Identification of screened bacterial isolates from *P. pinnata* seed residue

Genomic DNA was extracted from overnight liquid cultures of screened microbial isolates using MoBio DNA extraction kits. The extracted DNA was amplified in a polymerase chain reaction (Thermocycler, BioRad, Australia) using 2 µl DNA template, 63f forward, and 1389r reverse primers [Osborn et al., 2000]. The PCR mixture (50 µl) comprised of 2 µl forward primer, 2 µl reverse primer, 0.25 µl Taq polymerase enzyme, Taq buffer 10 µl, nuclease free water 33.75 µl, and DNA template 2 µl. The temperature program used consisted of 95 ⁰C for 5 min, 94 ⁰C for 30 s (33 cycles), 55 ⁰C for 30 s, 72 ⁰C for 1 min, and a final extension at 72 ⁰C for 10 min. Amplified DNA samples from PCR were purified using a PCR clean up kit (Promega, USA) according to the manufactures guidelines. After clean up, DNA quantification was carried out with a Nanodrop 1000 spectrophotometer. Purified DNA samples were sent to the Australian Genomic Research Facility (AGRF) for sequencing. DNA sequence data obtained from AGRF was edited in Sequencher Version 5.0 and Blast analysis was performed in National Centre for Biotechnology Information (NCBI) website to determine bacterial identities [Taha et al., 2015].
2.3.4 Crude enzyme production

One bacterial isolate, selected based on qualitative and quantitative screening and *Iprex lacteus*, a known producer of lignocellulase activity (Taha, personal communication 2015) were used in enzyme production experiments. Enzyme production was carried out in solid state fermentation mode using BH-cellulose and BH-*P. pinnata* seed residue media. Media were inoculated with 3 ml of a bacterial suspension of OD$_{600nm}$ 0.9. Fungal inoculum was prepared by growing fungi in potato dextrose broth for 3 days. Fungal biomass was harvested from the broth by centrifugation at 4000 rpm for 4 min and washed with sterile water several times to remove nutrients. Homogeneously suspended nutrient free fungal biomass (5 ml) in sterile water was used to inoculate the media for enzyme production; this corresponded to an inoculation of 0.3 g l$^{-1}$ (dry weight).

In solid state fermentation experiments cellulose and *P. pinnata* seed residue (10 g) biomass were used as substrate and were moistened with BH liquid media (60 ml) in 250 ml flasks. Fermentations were carried out with the media pH adjusted to 5 under static conditions and at 30 °C for a period of 8 days.

During enzyme preparation crude enzyme samples were collected every 2 days. Culture free enzyme samples, prepared by centrifugation followed by filtration using 0.45 μm filters, were analyzed for exoglucanase, endoglucanase, β-glucosidase, xylanase, and laccase enzyme activities.

2.3.5 Enzyme activity measurement

Secreted extracellular enzyme was examined for different lignocellulase activities based on standard assay methods. Details of the methodology of the assays are presented in Table 2.1. Activities of different enzymes were defined as:

$$\text{Exo – & Endo – glucanase activity, } U/mL \text{ min} = \frac{\text{μmole glucose formed (U)}}{\text{ml enzyme.min}} \text{ ..........(2.14)}$$
Table 2.1: Reagents and incubation conditions in crude enzyme activity estimation.

| Assay→
| Conditions↓ |
|----------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Exoglucanase                     | Endoglucanase     | β-glucosidase     | Xylanase          | Laccase           |
| Crude enzyme                     | 0.5 ml            | 0.5 ml            | 0.5 ml            | 0.5 ml            | 0.5 ml            |
| Substrate                        | 0.5 ml 2% cellulose in sodium citrate buffer (0.05 M; pH 4.8) | 0.5 ml 2% Carboxymethyl cellulose in sodium citrate buffer (0.05 M; pH 4.8) | 0.5 ml p-nitrophenyl-β-D-glucopyranoside (pNPG) (10 nM) in sodium citrate buffer (0.05 M; pH 4.8) | 1% xylan in sodium citrate buffer (0.05 M; pH 4.8) | 0.5 ml of guaiacol (0.02 M) in phosphate buffer (0.01 M; pH 6.5) |
| Temperature, °C                  | 50                | 50                | 50                | 50                | 55                |
| Time, min                        | 60                | 60                | 15                | 60                | 10                |
| Stopping reagent                 | DNS reagent (3 ml) | DNS reagent (3 ml) | Na₂CO₃ 0.2 M (2 ml) | DNS reagent (3 ml) | **                |
| Absorbance measurement at, nm    | 540               | 540               | 405               | 640               | 465               |
| Reference                        | [Ghosh 1987]      | [Ghosh 1987]      | [Jung et al., 2015] | [Ghosh & Bisaria 1987] | [Sheikhi et al., 2012] |
\[
\beta - \text{glucosidase activity, } \frac{U}{mL \text{ min}} = \frac{\mu \text{ mole } p-\text{nitrophenol formed (U)}}{\text{ml enzyme.min}} \quad \text{...........(2.15)}
\]
\[
\text{Xylanase activity, } \frac{U}{mL \text{ min}} = \frac{\mu \text{ mole xylose formed (U)}}{\text{ml enzyme.min}} \quad \text{...........(2.16)}
\]
\[
\text{Laccase activity, } \frac{U}{mL \text{ min}} = \frac{\text{Absorbance increase by 0.001 (U)}}{\text{ml enzyme.min}} \quad \text{...........(2.17)}
\]

2.3.6 Enzymatic hydrolysis of P. pinnata seed residue biomass

Enzymatic hydrolysis experiments were carried out using of P. pinnata seed residue as substrate at 50 °C, the optimum temperature for cellulase activity, with agitation at 150 rpm. Three replicates were used throughout. P. pinnata seed residue (5%, w/v) in sodium citrate buffer (pH 4.8) was pretreated at 121 °C for 15 min. Crude cellulases, obtained from solid state fermentations using the bacterial isolate and the white-rot fungus I. lacteus together with a commercial cellulase from Aspergillus niger were employed in hydrolysis of the pretreated material. Enzymes at two different loadings, 5 U and 10 U per g of P. pinnata seed residue, were used to study the effect of enzyme loading on saccharification of the material. Controls, without enzyme addition were included routinely. Samples were collected during the course of reaction and analyzed for glucose using the dinitrosalicylic acid (DNS) method [Miller 1959].

2.4 FERMENTATION OF THE HYDROLYZATE

Fermentation experiments were carried out with liquid hydrolyzates from acid hydrolysis and enzymatic hydrolysis reactions. Liquid hydrolyzate from dilute acid hydrolysis of P. pinnata seed residue contains water, dissolved acid, and sugars. The pH of the raw liquid hydrolyzate was acidic, in the range pH 1-2. The activity of yeast is pH dependent; in various experiments reporting the optimization of the fermentation of sugars by Saccharomyces cerevisiae a pH of 4-5 was reported as optimal for the growth of yeast [Buzas et al., 1989]. Therefore, prior to
fermentation, the pH of the liquid hydrolyzate was adjusted to 4.8 through the addition of NaOH solution (1N).

The hydrolyzate, adjusted to pH 4.8, was subjected to fermentation under anaerobic conditions at 35 °C using 0.5% (w/v) active dry yeast in 10 ml of hydrolysis product as the inoculum in fermentation. A round bottom flask equipped with a sampling port and an air tight rubber cork with a glass tube inserted from its center to maintain anaerobic conditions served as a fermentation reactor. Anaerobic conditions were maintained by flushing the reactor using nitrogen gas the zero time point. Fermentation flasks were completely sealed to maintain air tight conditions. The outlet of the glass tube was kept immersed in calcium hydroxide (1 M) solution. Samples were collected at regular intervals of 24 h to monitor the conversion of sugars. Theoretical ethanol yield from *P. pinnata* seed residue was estimated based on the following equation.

\[
\text{Theoretical ethanol yield from } P.\text{ pinnata seed cake} = \frac{\text{Amount of carbohydrates in } P.\text{ pinnata seed cake processed} + 0.51111 g}{\text{Amount of } P.\text{ pinnata seed cake processed} \text{ kg}} \quad \ldots (2.18)
\]

2.5 ANALYSIS OF SUGARS USING HPLC

Sugars formed during the course of the acid hydrolysis reaction were analyzed and quantified by high performance liquid chromatography (HPLC) [Karkacier et al., 2003]. HPLC analyses was carried out using a Shimadzu HPLC unit equipped with a refractive index detector (model RID-10A), column oven (model CTO-10AS VP), and an isocratic pump LC-20AT, supplied by Spinco Laboratory Pvt. Limited. In the present work, a method as developed for the analysis of sugars formed during acid hydrolysis *P. pinnata* seed residue. The conditions employed for analysis of sugars were as follows:

- Stationary phase: Phenomenex Luna 5μ NH$_2$
Mobile phase: Acetonitrile-Water in 75:25 V/V

Column temperature: 40 °C

Flow rate: 0.6 ml/min

Detector: Refractive index detector

Total analysis time: 20 min

2.6 ANALYSIS OF ETHANOL USING GC

Ethanol formed during the fermentation of the hydrolysis product mixture was analyzed using gas chromatography equipped with a thermal conductivity detector. The details of the conditions employed in the analysis are given below:

- Carrier gas: hydrogen
- Carrier gas total flow: 30 ml/min
- Injection port temperature: 200 °C
- Detector temperature: 200 °C
- Current to detector: 100 mA

Column temperature program:

<table>
<thead>
<tr>
<th>Rate, °C/min</th>
<th>Temperature, °C</th>
<th>Hold time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>200</td>
<td>4</td>
</tr>
</tbody>
</table>

2.7 PYROLYSIS

2.7.1 Pyrolysis in fixed bed reactor

Pyrolysis of P. pinnata seed residue was performed in a fixed bed reactor of 1 cm inner diameter and 35 cm length under slow pyrolysis conditions with a continuous supply of nitrogen gas at 0.1 litre per minute (LPM) flow at different temperatures including 200, 250,
300, 350, 400, 450, and 500 °C. Volatiles formed during the pyrolysis reaction were collected in a glass jacketed receiver/gas-liquid separator. The temperature of the receiver was maintained at 5±1 °C by circulating cold water using a poly-science automatic digital refrigerating and heating circulator thermostat. Condensable volatiles (bio-oil and water) were collected in the receiver and non-condensable gases from the exit of the receiver were collected in a trap. The pyrolysis transformed the *P. pinnata* seed residue to biochar (retained in the column), bio-oil (condensed liquid product collected in the receiver) and gases (collected in the trap). The solid and the liquid products obtained were weighed accurately and the yields of the same were expressed gravimetrically. Biochar obtained was characterized by ultimate (elemental) & proximate analysis, surface structure and, FTIR analyses.

### 2.7.2 Ultimate analysis

Carbon, hydrogen, nitrogen, oxygen, and sulphur elementary composition of biomass is one of the main characteristics of biomass samples. The elementary composition of dried *P. pinnata* seed residue was assessed using a CHNS Analyzer- ELEMENTAR Vario micro cube model [Osada et al., 2004].

### 2.7.3 Proximate analysis

Proximate analysis of the biomass for ash, volatile matter and fixed carbon content was carried out according to the method described by ASTM standard methods [Cantrell et al., 2010]. Volatile matter content (VM) was determined by subjecting samples to pyrolysis conditions, under a N₂ atmosphere at a flow rate of 80 ml min⁻¹, beginning at 110°C, and a 200°C min⁻¹ ramp was implemented until the temperature reached 950°C. This temperature was held for 7 min. The sample was then cooled before recording the final weight. The difference of the final weight and initial dry weight of sample served as the VM content.
The ash content was determined as the residue remaining after the following temperature program: 11°C min\(^{-1}\) increase from 110 to 750°C, 3.5°C min\(^{-1}\) increase until 950°C, isothermal hold at 950°C for 120 min, and rapid cooling to 110°C at -200°C min\(^{-1}\). The ash method used zero-grade air at a flow rate of 80 ml min\(^{-1}\).

2.7.4 Scanning Electron Microscopy (SEM)

Biomass and biochar obtained from pyrolysis of *P. pinnata* seed residue were studied by means of scanning electron microscopy [Brasquet et al., 2000]. In the present work, SEM analyses were carried out with a Hitachi S-3400N SEM microscope, Japan. Operating conditions of the SEM used to evaluate the surface of *P. pinnata* seed residue and biochar were as follows:

- **Magnification=2500**
- **Accelerating Voltage=15000 Volt**
- **Emission Current=15000 nA**
- **Working Distance=8.9 mm**
- **Sub-accelerating Voltage=15000 Volt**
- **Pixel Size=39.6875**

2.7.5 Fourier transformed infrared spectroscopy

Infrared Spectroscopy is the analysis of infrared light interacting with a molecule. This can be analyzed in three ways by measuring absorption, emission and reflection. It is used to determine functional groups in molecules. Infrared spectroscopy measures the vibrations of atoms, and based on this it is possible to determine the functional groups [Vollhardt & Schore 2007]. Generally, stronger bonds and light atoms will vibrate at a high stretching frequency.

Fourier transformed infrared spectra of *P. pinnata* seed residue biomass and biochar were collected using the KBr pelletization method on a Perkin Elmer System 100 Fourier
Transform Infrared spectrometer (Perkin Elmer Ltd., Seer Green, Beaconfield, Bucks HP9 2FX, United Kingdom) [Rutherford et al., 2012] with a caesium iodide (CsI) micro focus accessory and triglycine sulphate (TGS) detector by scanning from 4000 to 400 cm\(^{-1}\), with a resolution of 4 cm\(^{-1}\). Table 2.2 lists the nature/functionality of vibrations observed at different frequencies.

Table 2.2: FTIR stretching vibrations for different functional groups in biomass [Rutherford et al., 2012].

<table>
<thead>
<tr>
<th>Frequency, cm(^{-1})</th>
<th>Nature/functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>700-1200</td>
<td>Aliphatic nature</td>
</tr>
<tr>
<td>1200-2000</td>
<td>Aromatic nature</td>
</tr>
<tr>
<td>698</td>
<td>C-H (Aromatic ring)</td>
</tr>
<tr>
<td>1020</td>
<td>C-O stretching vibration</td>
</tr>
<tr>
<td>1420</td>
<td>C-H stretching vibration</td>
</tr>
<tr>
<td>1626</td>
<td>C=C &amp; C=O stretching vibration</td>
</tr>
</tbody>
</table>

2.7.6 Pyrolysis kinetics

2.7.6.1 Thermogravimetric analysis

Thermogravimetric analysis (TGA) of \(P. \textit{pinnata}\) seed residue was performed in a Mettler Toledo TGA/SDTA 851\(^{e}\) analyser (Switzerland) for pyrolysis kinetics evaluation. The analyses of the samples were performed at three different heating rates, 5, 10, and 20 °C/min, using nitrogen with a flow rate of 30 ml min\(^{-1}\) to a final temperature of 800 °C with a sample mass of \(\sim 11 \pm 0.1\) mg. The loss in weight of the sample was recorded as a function of temperature.

2.7.6.2 Kinetic studies
P. pinnata seed residue pyrolysis kinetics was studied using the non-isothermal data obtained from different temperature programs at a constant heating rate. The International Confederation for Thermal Analysis and Calorimetry (ICTAC) committee recommendations [Vyazovkin et al., 2011] for performing kinetic computations on thermal analysis data were followed to evaluate all the reliable kinetic parameters. Mathematical analysis to determine the kinetic triplet, activation energy (E), frequency factor (A), and reaction model f(α), was performed by the method of Coats and Redfern (model fitting approach), and model free isoconversional Kissenger-Akahira-Sunose (KAS), and Ozawa-Flynn-Wall (OFW) methods.

The conversion of biomass in the pyrolysis process is represented by the following reaction scheme:

\[ \text{Biomass} \rightarrow \text{Biochar} + \text{Volatiles} \]

and the kinetic expression with temperature dependence of the kinetic rate constant, given by Arrhenius equation as:

\[ \frac{da}{dt} = k(T)f(\alpha) \]

\[ k(T) = A \exp \left( \frac{-E}{RT} \right) \]

Where f(α) is the reaction model, dependent on the reaction mechanism, k(T) is the rate constant, A is frequency factor, E the activation energy, T the absolute temperature, t the time, α the degree of conversion, and R the universal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)). The constant heating rate is defined as:

\[ \text{Heating rate, } \beta = \frac{dT}{dt} = \text{Constant} \]

\[ dt = \frac{dT}{\beta} \]
Substituting $k(T)$ from Equation 2.20 and $dt$ from Equation 2.22 in Equation 2.19 and after rearrangement, the integration of Equation 2.19 is given as:

$$
\int_0^\alpha \frac{d\alpha}{f(\alpha)} = \int_0^T A \exp \left(\frac{-E}{RT}\right) \frac{dT}{R} = g(\alpha) \hspace{1cm} (2.23)
$$

**Model fitting approach**

The solution of Equation 2.23 based on the approximation given in Coats and Redfern [Coats and Redfern 1964] gives rise to the equation form expressed as the Coats-Redfern equation; the most frequently used expression to evaluate non-isothermal data to compute kinetic parameters is

$$
\ln \left(\frac{g(\alpha)}{T^2}\right) = \ln \left(\frac{A}{\beta E}\right) \left(1 - \frac{2RT}{E}\right) - \frac{E}{RT} \hspace{1cm} (2.24)
$$

Conversion ($\alpha$) in the pyrolysis of PPSR seed cake is calculated from TGA data using the equation given below

$$
Conversion, \alpha = \frac{m_i - m_\alpha}{m_i - m_f} \hspace{1cm} (2.25)
$$

Where $m_i$, $m_\alpha$, and $m_f$ are initial, instantaneous and final mass of the samples, respectively.

The data obtained from TGA was analyzed by different kinetic models representing chemical reaction, nucleation and nuclei growth, surface reaction between both the phases, and diffusion models [Vyazovkin and Wight 1999 & Vlaey et al., 2003]. The TGA data in the pyrolysis temperature range were analyzed by applying 14 kinetics models given in Table 2.3 using the Coats Redfern equation. The kinetic model which resulted in a straight line fit with high regression coefficient for $\ln [g(\alpha)/T^2]$ verses $1/T$ data was concluded to be the best reaction model representing the experimental data.
Table 2.3: Kinetic models used in the solid state reactions. [Vyazovkin and Wight 1999 & Vlaey et al., 2003]

<table>
<thead>
<tr>
<th>Model</th>
<th>Mechanism</th>
<th>( f(\alpha) )</th>
<th>( g(\alpha) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>First order</td>
<td>( 1-\alpha )</td>
<td>([-\ln(1-\alpha)])</td>
</tr>
<tr>
<td>2</td>
<td>Second order</td>
<td>( (1-\alpha)^2 )</td>
<td>((1-\alpha)^{-1}-1)</td>
</tr>
<tr>
<td>3</td>
<td>Third order</td>
<td>( (1-\alpha)^3 )</td>
<td>([((1-\alpha)^2-1)/2)</td>
</tr>
<tr>
<td>4</td>
<td>nth order</td>
<td>( (1-\alpha)^n )</td>
<td>([((1-\alpha)^{1-n}/n-1)</td>
</tr>
<tr>
<td></td>
<td><strong>Random nucleation and nuclei growth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Bi-dimensional</td>
<td>( 2(1-\alpha)[-\ln(1-\alpha)]^{1/2} )</td>
<td>([-\ln(1-\alpha)]^{1/2} )</td>
</tr>
<tr>
<td>6</td>
<td>Three-dimensional</td>
<td>( 3(1-\alpha)[-\ln(1-\alpha)]^{2/3} )</td>
<td>([-\ln(1-\alpha)]^{2/3} )</td>
</tr>
<tr>
<td></td>
<td><strong>Limiting surface reaction between both phases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>One Dimension</td>
<td>( 1 )</td>
<td>( \alpha )</td>
</tr>
<tr>
<td>8</td>
<td>Two dimensions</td>
<td>( 2(1-\alpha)^{1/2} )</td>
<td>( 1-(1-\alpha)^{1/2} )</td>
</tr>
<tr>
<td>9</td>
<td>Three dimensions</td>
<td>( 3(1-\alpha)^{2/3} )</td>
<td>( 1-(1-\alpha)^{2/3} )</td>
</tr>
<tr>
<td></td>
<td><strong>Diffusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>One-way transport</td>
<td>( 1/2\alpha )</td>
<td>( \alpha^2 )</td>
</tr>
<tr>
<td>11</td>
<td>Two-way transport</td>
<td>([-\ln(1-\alpha)]^{-1} )</td>
<td>( \alpha+(1-\alpha)\ln(1-\alpha) )</td>
</tr>
<tr>
<td>12</td>
<td>Three-way transport</td>
<td>( (2/3)(1-\alpha)^{2/3}/[1-(1-\alpha)^{1/3}] )</td>
<td>([1-(1-\alpha)^{1/3}]^2 )</td>
</tr>
<tr>
<td>13</td>
<td>Ginstling-Brounshtein equation</td>
<td>( (2/3)(1-\alpha)^{1/3}/[1-(1-\alpha)^{1/3}] )</td>
<td>( 1-2\alpha/3-(1-\alpha)^{2/3} )</td>
</tr>
<tr>
<td>14</td>
<td>Zhuravlev equation</td>
<td>( (2/3)(1-\alpha)^{5/3}/[1-(1-\alpha)^{1/3}] )</td>
<td>([((1-\alpha)^{1/3}-1)]^2 )</td>
</tr>
</tbody>
</table>
Model free approach (isoconversional methods)

A number of isoconversional methods are available for evaluating the kinetics of biomass pyrolysis. These methods mainly differ in the approximations of the temperature integral in Equation 2.23 and many of them give rise to linear equations. In Equation 2.23 after introducing the following terms:

\[ \text{Let } \frac{E}{RT} = p \]
\[ dp = \frac{-E}{R} T^{-2} dT \]

As \( T \to 0; p \to \infty \) and \( T \to T; p \to p \)

And by substituting \( \frac{E}{RT} = p \) on rearrangement the equation is transformed to:

\[ g(\alpha) = \int_0^\infty \frac{d\alpha}{f(\alpha)} = \frac{A E}{\beta R} \int_p^\infty p^{-2} \exp(-p) \, dp \] \[ (2.26) \]

KAS method takes the following approximation for the integral in right-side term of Equation 2.26:

\[ \int_p^\infty p^{-2} \exp(-p) \, dp = p^{-2} \exp(-p) \] \[ (2.27) \]

The solution of Equation 2.27 based on the approximation and suitable modification given as Equation 2.28 was used to determine the activation energy and frequency factor.

\[ \ln \frac{\beta}{T^2} = \ln \left( \frac{AE}{Rg(\alpha)} \right) - \frac{E}{RT} \] \[ (2.28) \]

OFW method applies Doyle's approximation [Doyle 1965] for the integral term on the right side of Equation 2.26, which becomes

\[ \log \left[ \int_p^\infty p^{-2} \exp(-p) \, dp \right] = -2.315 + 0.457p \] \[ (2.29) \]
By substituting Equation 2.29 in Equation 2.26 and on rearrangement Equation 2.30 was obtained and was used for calculating the Arrhenius parameters:

$$\log(\beta) = \log\left[\frac{AE}{Rg(\alpha)}\right] - 2.315 - 0.457 \frac{E}{RT} \ldots \ldots (2.30)$$

From the KAS method, the plot of $\ln \left(\frac{\beta}{T^2}\right)$ verses $1/T$ gives the slope $-\frac{E}{R}$. From OFW method activation energy was calculated from the slope $(-0.457E/RT)$ of the straight line obtained by plotting $\log(\beta)$ and $1/T$ data. Frequency factor from the model free isoconversional methods was determined from the intercept of the plots by making use of the best kinetic model representing the experimental data, $f(\alpha)$, evaluated from the Coats Redfern method.
Chapter 3 discusses the composition characterization of de-oiled *Pongamia pinnata* seed residue in terms of main biomass constituents. Specifically, the Chapter describes the evaluation of the seed residue as a feedstock for sugar production together with the assessment of different operating conditions including temperature, acid concentration and solid to liquid weight ratio on hydrolysis of the seed residue in the presence of sulphuric acid.

### 3.1 COMPOSITION OF DE-OILED *P. PINNATA* SEED RESIDUE

**3.1.1 Elemental analysis and ash content**

The elementary composition of *P. pinnata* seed residue together with its ash content is shown in Table 3.1. The elemental composition of the seed residue was similar to that previously reported for corn stover and other lignocellulosic biomass including cellulose pulp and poplar (*Populus nigra*). An absence of sulphur was observed in the seed residue, an added advantage of using the seed residue for biofuels production. Compared to other lignocellulosic biomass, the nitrogen content in the seed residue is higher, making it a good candidate for use as a soil organic amendment. The estimated ash content (inorganic material) in the seed residue was 4.00% which is lower than that reported for corn stover (5.45%) (Table 3.1).
3.1.2 Extractives

The amount of soluble material from *P. pinnata* seed residue using different organic solvents such as ethanol, acetone, IPA and toluene was found to vary with the solvent used: with ethanol-40 w% of deoiled *P. pinnata* seed residue cake was extracted; likewise, with acetone-29.9%; with isopropyl alcohol-29.9% and toluene 27% in a batch extraction with 5-6 syphon cycles per hour at reflux temperature for 10 h. Therefore ethanol was found to be the best solvent to obtain extractive free de-oiled and dried *P. pinnata* seed residue.

Table 3.1: Elemental analysis and ash content of *Pongamia pinnata* seed residue

<table>
<thead>
<tr>
<th>Element</th>
<th>De-oiled <em>Pongamia pinnata</em> seed residue [Present work]</th>
<th>Corn Stover [Medic et al., 2012]</th>
<th>Cellulose pulps [Carrier et al., 2011]</th>
<th>Poplar (<em>Populus nigra</em>) [Carrier et al., 2011]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>4.3</td>
<td>0.5</td>
<td>NA</td>
<td>0.9</td>
</tr>
<tr>
<td>C</td>
<td>44.2</td>
<td>44.8</td>
<td>41</td>
<td>48.2</td>
</tr>
<tr>
<td>H</td>
<td>6.5</td>
<td>6.3</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>S</td>
<td>NF</td>
<td>0.15</td>
<td>NF</td>
<td>0.4</td>
</tr>
<tr>
<td>O</td>
<td>45</td>
<td>48.25</td>
<td>52</td>
<td>42</td>
</tr>
<tr>
<td>Ash</td>
<td>4.0</td>
<td>5.45</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Extraction with organic solvent was employed to remove compounds that interfere in the hydrolysis operation. Following Soxhlet extraction, the extractive-free *P. pinnata* seed residue appeared somewhat decoloured compared to crude *P. pinnata* seed residue. The decolourization of *P. pinnata* seed residue is due to the removal of traces of oil which is
yellowish orange in color (Figure 3.1), flavonoids (e.g. karanjin, pongamol), and gums all of which were extracted in the solvent.

Figure 3.1: *Pongamia pinnata* (karanja) tree seeds, seed kernel, oil and cake

### 3.1.3 Protein and lignin content

Protein content in deoiled *P. pinnata* seed residue was estimated to be 13.4% on a dry basis and 22.3 % on a dry and extractive-free basis. A high concentration of protein was found in the seed residue confirming that oil seeds are rich in protein compared to other biomass types. For instance, de-oiled *Jatropha curcas* oil seed residue includes up to 40% of protein (dry weight) [Gofferje et al., 2015] whereas the protein content of giant brown kelp, a marine biomass source is 15.9%. Bermuda grass, a herbaceous biomass, is composed of 12.3% protein and hybrid poplar, a woody biomass, is 2.1% [Ogi 2002].

The Klason lignin content of the seed residue was determined to be 17.4% on a dry weight basis and 29% on a dry and extractive-free basis. The lignin content of the seed residue (17.4% dry weight) is comparable with that of oil palm empty fruit bunch (15.1%)
[Ying et al., 2014]. The overall composition of de-oiled and dried *P. pinnata* seed residue is summarized in Table 3.2.

Table 3.2: Compositional assay of *P. pinnata* seed residue

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Component</th>
<th>% Wt. of component in <em>P. pinnata</em> seed residue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>De-oiled &amp; dried</td>
</tr>
<tr>
<td>1</td>
<td>Ash</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Extractives</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>Protein</td>
<td>13.4</td>
</tr>
<tr>
<td>4</td>
<td>Lignin</td>
<td>17.4</td>
</tr>
<tr>
<td>5</td>
<td>Carbohydrates</td>
<td>25.2</td>
</tr>
</tbody>
</table>

From Table 3.2 it is evident that 25.2% of de-oiled and dried *P. pinnata* seed residue or 42% of the extractive free seed residue was composed of carbohydrates which potentially can be converted to sugars.

### 3.2. SULPHURIC ACID HYDROLYSIS OF *P. PINNATA* SEED RESIDUE

Batch wise hydrolysis reactions of carbohydrates in extractive-free *P. pinnata* seed residue were carried out according to the L₉ orthogonal array of Taguchi method. All the experiments were carried out for 1 h with a mixing intensity of 750 rpm. The layout of the L₉ orthogonal array along with results was shown in Table 3.3.

#### 3.2.1 Statistical analysis - effect of factors

Statistical analysis of the experimental data (Table 3.3) was carried out based on criterion 1 i.e. larger S/N ratio is best for the both performance parameters \(Y_1, Y_2\), as the
goal was to enhance the glucose yield. The mean effect plot of the three aforementioned factors on the % of glucose in total sugars and glucose yield, defined as g of glucose formed per kg extractive free *P. pinnata* seed residue is shown in Figure 3.2. For all three factors at each level the S/N ratio was tabulated (Table 3.4) and the range was calculated as the difference in the S/N values between the highest and lowest level of factor.

Table 3.3: List of experiments and results according to Taguchi - L9 array

<table>
<thead>
<tr>
<th>Real values</th>
<th>Coded values</th>
<th>% glucose in</th>
<th>g glucose/ kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1</td>
<td>X2</td>
<td>X3</td>
<td>(Y1)</td>
</tr>
<tr>
<td>80</td>
<td>2.5</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>80</td>
<td>5</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>80</td>
<td>7.5</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>2.5</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>7.5</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>120</td>
<td>2.5</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>120</td>
<td>5</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>120</td>
<td>7.5</td>
<td>15</td>
<td>3</td>
</tr>
</tbody>
</table>

From Figure 3.2 and Table 3.4 it is evident that all the factors were showing a positive effect on $Y_1$ confirming that an increase in factor level resulted in increased performance. The range of values suggests that temperature was the factor that influenced the yield of glucose most, when compared to the rest of the factors; in contrast the liquid to solid ratio was the factor that had the least effect. The same trend was also observed in the case of $Y_2$ but the liquid to solid ratio indicated a negative effect (-35.68) i.e. glucose yield decreased with increased...
liquid to solid ratio. This was also observed in mean effects plot where an increase in the liquid to solid ratio S/N ratio increased the level from 1 to 2 after which the S/N ratio decreased to a value less than the value at level 1.

Figure 3.2: Mean effects of temperature (X_1), acid concentration (X_2) and liquid to solid ratio (X_3) on % glucose in total sugars (Y_1) and g glucose/kg seed residue (Y_2).

Table 3.4: S/N ratio at each level of factors and rank of factors

<table>
<thead>
<tr>
<th>Level</th>
<th>S/N ratio corresponding to Y_1</th>
<th>S/N ratio corresponding to Y_2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X_1</td>
<td>X_2</td>
</tr>
<tr>
<td>1</td>
<td>33.50</td>
<td>40.50</td>
</tr>
<tr>
<td>2</td>
<td>71.87</td>
<td>69.07</td>
</tr>
<tr>
<td>3</td>
<td>73.09</td>
<td>68.89</td>
</tr>
<tr>
<td>Range</td>
<td>39.59</td>
<td>28.38</td>
</tr>
<tr>
<td>Rank</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
Analysis of variance (Table 3.5) confirmed that all three factors exerted a significant effect except the liquid to solid ratio ($X_3$) in the case of $Y_1$. It can be concluded that the hydrolysis reaction temperature contributed 64.4% of the sum of the total effects followed by acid concentration (34.3%); the contribution of the liquid to solid ratio was only 1.2% in the performance parameter $Y_1$. In contrast, temperature contributed 59.5%, followed in importance by acid concentration (28.5%) and liquid to solid ratio (12%) in obtaining the maximum glucose yield ($Y_2$).

Table 3.5: Analysis of variance and contribution of factors in hydrolysis reaction

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sum of square</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>$F_o$</th>
<th>$F_{a,ν_1,ν_2}$</th>
<th>Contribution of factor effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>For $Y_1$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X_1$</td>
<td>3040.9</td>
<td>2</td>
<td>1520.4</td>
<td>12.6</td>
<td>4.5</td>
<td>64.4</td>
</tr>
<tr>
<td>$X_2$</td>
<td>1621.5</td>
<td>2</td>
<td>810.8</td>
<td>6.7</td>
<td>4.7</td>
<td>34.3</td>
</tr>
<tr>
<td>$X_3$</td>
<td>58.7</td>
<td>2</td>
<td>29.3</td>
<td>0.2</td>
<td>5.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Residual</td>
<td>241.3</td>
<td>2</td>
<td>120.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>For $Y_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X_1$</td>
<td>25883.7</td>
<td>2</td>
<td>12941.9</td>
<td>465.9</td>
<td>4.5</td>
<td>59.5</td>
</tr>
<tr>
<td>$X_2$</td>
<td>12396.9</td>
<td>2</td>
<td>6198.5</td>
<td>223.1</td>
<td>4.7</td>
<td>28.5</td>
</tr>
<tr>
<td>$X_3$</td>
<td>5230.6</td>
<td>2</td>
<td>2615.3</td>
<td>94.1</td>
<td>5.1</td>
<td>12</td>
</tr>
<tr>
<td>Residual</td>
<td>55.6</td>
<td>2</td>
<td>27.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: $ν_1$ is degrees of freedom & $ν_2=(a*n)-a$ where 'n' is number of levels of factors i.e n=3; 'a' is factor number 1, 2, 3 for $X_1$, $X_2$, $X_3$ respectively.

Regression analysis was performed on the observed data of glucose yield ($Y_2$) and is given as Equation 3.1. This model equation best represented the observed data.
\[ Y_2 = 3.014X_1 + 17.108X_2 - 3.989X_3 - 206.2 \] (3.1)

\[ R^2 = 0.94 \]

The observed data is presented in a 3-dimensional surface plot in different combinations of factors \((X_1, X_2; X_2, X_3; X_3, X_1 \) vs. \(Y_1\) and \(Y_2\)) using STATISTICA software (Figure 3.3). As is evident from the surface plots it can be concluded that 50% of the surface was showing a higher percentage glucose concentration at 100-120° C temperature and 5-7.5% acid concentration and 10-15 liquid to solid ratio (Figure 3.3 (a), (b) & (c)). The yield of glucose i.e. g glucose per kg cake was also maximum at 100-120°C, 5-7.5% acid concentration and 10-15 liquid to solid ratio (Figure 3.3 (d), (e) & (f)).

From the statistical analysis of the data it was concluded that the yield of glucose increased with increased temperature and acid concentration and decreased with increased liquid to solid ratio. Therefore the optimum conditions for maximum glucose yield are 100-120 °C temperature and 5-7.5% acid concentration. Moreover, a liquid to solid ratio of 10 was found sufficient because under these conditions the glucose yield was found to be higher. The higher temperature, acid concentration, and liquid to solid ratio likely enhanced the penetration of acid to the inner matrix of biomass, breaking the crystalline structure and enhancing sugar release by breaking the glucose linkages. Moreover, at the higher temperature the energy available for the reaction was higher than the activation energy (120-130 kJ mol\(^{-1}\)) [Kunov-Kruse et al., 2013] for the reaction resulting in an enhanced rate of reaction, with the maximum sugar concentration reached in less time [Lavarack et al., 2002].

Different biomass feed-stocks have been processed for sugars using dilute acids (Table 3.6). Dilute acid hydrolysis processes are carried out in both single step and two steps with different acid concentrations in each stage and with a pre-extraction step to enhance the digestibility of the biomass. A comparison of sugar yield from various biomasses feed stocks and that from \(P. pinnata\) seed residue is presented in Table 3.6.
Figure 3.3: Three dimensional surface plot for % glucose in total sugars ($Y_1$) (a), (b), (c), and glucose yield (g glucose kg$^{-1}$ substrate) (d), (e), (f) in relation to $X_1$, $X_2$, $X_3$, $X_1$, $X_2$, $X_3$. 
Table 3.6: Dilute acid treatment of various biomass substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Process</th>
<th>Conditions</th>
<th>Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy manure</td>
<td>Dilute acid hydrolysis</td>
<td>135 °C, 1% H$_2$SO$_4$, 10% slurry concentration, 2h</td>
<td>16.5 g/L</td>
<td>Liao et al., 2004</td>
</tr>
<tr>
<td>Hemlock hog fuel/pin chips (85:15 by dry weight)</td>
<td>Two stage dilute acid hydrolysis</td>
<td>1$^{st}$ stage: 190 °C, 1.1% H$_2$SO$_4$ for 150 s 2$^{nd}$ stage: 210 °C, 2.5% H$_2$SO$_4$ for 115 s</td>
<td>269 mg sugars/g biomass</td>
<td>Kim et al., 2005</td>
</tr>
<tr>
<td>Oil palm empty fruit bunch fiber</td>
<td>Dilute acid hydrolysis</td>
<td>119 °C, 2% H$_2$SO$_4$, 60 min</td>
<td>179.9 g xylose/g biomass</td>
<td>Rahman et al., 2007</td>
</tr>
<tr>
<td>Press cakes from silage and grass</td>
<td>Dilute acid hydrolysis</td>
<td>162 °C, 0.53% H$_2$SO$_4$, dry matter concentration of 10%, 10 min</td>
<td>164.3 mg sugars/g dry matter</td>
<td>Neureiter et al., 2004</td>
</tr>
<tr>
<td>Rice straw</td>
<td>Two stage dilute acid hydrolysis</td>
<td>30 bar pressure, 0.5% H$_2$SO$_4$, for 3 min</td>
<td>343 mg sugars/g dry matter</td>
<td>Karimi et al., 2006</td>
</tr>
<tr>
<td>Sugar maple wood extract</td>
<td>Dilute acid hydrolysis</td>
<td>95 °C, 3.1% H$_2$SO$_4$, 1h</td>
<td>170 g sugars/L maple wood extract</td>
<td>Hu et al., 2010</td>
</tr>
<tr>
<td>Sweet sorghum bagasse</td>
<td>Dilute acid hydrolysis</td>
<td>15 psi(121°C), 2.5 % H$_2$SO$_4$ (%w/w), dry matter to dilue acid ratio of 6 (w/v), 150 s</td>
<td>390-415 mg sugar/g bagasse</td>
<td>Heredia-Olea et al., 2012</td>
</tr>
<tr>
<td>De-oiled <em>Pongamia pinnata</em> seed residue</td>
<td>Dilute acid hydrolysis</td>
<td>80-120°C, 2.5-7.5% H$_2$SO$_4$, 1h</td>
<td>245 g glucose/kg de-oiled &amp; extractive free <em>P. pinnata</em> seed residue</td>
<td>Present work</td>
</tr>
</tbody>
</table>
The highest yield of sugars during the hydrolysis of rice straw [Karimi et al., 2006] and sweet sorghum bagasse [Heredia-Olea et al., 2012] under high pressure conditions was reported in the range 340-415 mg g\(^{-1}\) of the sample. Kim et al. (2005) obtained 269 mg g\(^{-1}\) biomass after a two stage hydrolysis of hemlock hog fuel/pin chips (85:15 by dry weight), carried out at high temperatures in the range 190-210 °C. Hydrolysis carried out at moderate temperatures and atmospheric pressure resulted in 160-180 mg sugars g\(^{-1}\) biomass during the hydrolysis of different biomass feedstocks including oil palm empty fruit bunch [Rahman et al., 2007], silage and grass press cakes [Neureiter et al., 2004] and sugar maple wood extract [Hu et al., 2010]. In the present work the maximum glucose obtained was 245 mg from g\(^{-1}\) de-oiled *Pongamia pinnata* seed residue corresponding to 58% theoretical sugar extraction at moderate temperatures of 120 °C and 7.5% H\(_2\)SO\(_4\). Moreover, the hydrolyzate obtained was rich in glucose (Appendix B) which can easily be fermented to ethanol by *Saccharomyces cerevisiae*. Based on these results, it is feasible to obtain maximum available sugars (42 % of the seed residue) by employing either high pressure or multi step treatment during hydrolysis of the seed residue, resulting is significant improvements in the overall process yield.

### 3.3 CONCLUSIONS

Compositional analysis showed that *Pongamia pinnata* seed residue free of extractives is rich in carbohydrates (42%), protein (22%), and lignin (29%) which makes it a promising resource for bio-refinery. Dilute sulphuric acid hydrolysis of extractive free *P. pinnata* seed residue (1 kg) resulted in a maximum of 245 g glucose over a 1 h treatment i.e. 58 ±1% of theoretically available carbohydrates (420 g carbohydrates per kg de-oiled and extractive free *P. pinnata* seed residue) converted to glucose. Increases in temperature and acid concentration resulted in improved glucose yield whereas acid to seed cake weight ratio showed a negative correlation. The optimized conditions for dilute sulphuric acid hydrolysis
of extractive free *P. pinnata* seed residue biomass were 120°C temperature, 7.5 wt. % sulphuric acid, and an acid to seed cake weight ratio of 15. The present investigation revealed that hydrolysis of *P. pinnata* seed residue resulted into glucose rich hydrolyzate.
The present chapter explores the application of glucose obtained from acid hydrolysis of de-oiled *Pongamia pinnata* seed residue for ethanol production in a three step process: acid hydrolysis, neutralization and fermentation. Assessment of different acids on hydrolysis of the seed residue is presented. Finally the energy requirement for the acid hydrolysis of the seed residue is discussed.

**4.1 ACID HYDROLYSIS - EFFECT OF FACTORS**

Acid hydrolysis is the most common process to extract monomeric sugars from complex biomass structures. The most commonly employed acids used in the hydrolysis of biomass include sulphuric acid, hydrochloric acid, phosphoric acid, oxalic acid and nitric acid. Biomass acid hydrolysis has been practiced employing both dilute acids (0.1-10%) at high temperature (150-230 °C) [Noparat et al., 2015] and concentrated acids (50-60%) at low temperature (30-50 °C) [Wang et al., 2016].

In the present work, de-oiled and dried *P. pinnata* seed residue was treated in a hydrolysis reaction with different acids including H$_2$SO$_4$, HCl, and H$_3$PO$_4$, the most commonly employed acids used in biomass hydrolysis. To evaluate the best acid for glucose formation from hydrolysis of the seed residue, acid hydrolysis reactions were planned and
executed at varying concentrations of dilute acids (2-6% concentration) and at different temperatures (80-100 °C). Comparatively mild temperatures were considered in the present work to limit sugars degradation. The experimental design matrix is presented in Table 4.1 and lists the parameter values and the amount of glucose formed per kg seed residue.

Table 4.1: Experimental design matrix for acid hydrolysis of *P. pinnata* seed cake together with yield of sugars formed (g/kg seed cake).

<table>
<thead>
<tr>
<th>Run</th>
<th>Acid type</th>
<th>Acid concentration, %wt</th>
<th>Temperature, °C</th>
<th>Sugars formed, g/kg seed cake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2</td>
<td>80</td>
<td>26.06</td>
</tr>
<tr>
<td>2</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>4</td>
<td>90</td>
<td>53.25</td>
</tr>
<tr>
<td>3</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>6</td>
<td>100</td>
<td>156.53</td>
</tr>
<tr>
<td>4</td>
<td>HCl</td>
<td>2</td>
<td>90</td>
<td>117.47</td>
</tr>
<tr>
<td>5</td>
<td>HCl</td>
<td>4</td>
<td>100</td>
<td>146.71</td>
</tr>
<tr>
<td>6</td>
<td>HCl</td>
<td>6</td>
<td>80</td>
<td>129.50</td>
</tr>
<tr>
<td>7</td>
<td>H&lt;sub&gt;3&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2</td>
<td>100</td>
<td>43.49</td>
</tr>
<tr>
<td>8</td>
<td>H&lt;sub&gt;3&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>4</td>
<td>80</td>
<td>55.88</td>
</tr>
<tr>
<td>9</td>
<td>H&lt;sub&gt;3&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>6</td>
<td>90</td>
<td>61.78</td>
</tr>
</tbody>
</table>

As the aim of the work was to obtain the maximum amount of glucose available per kg biomass the results were analyzed based on the *Criteria I*: “If the goal is to maximize the performance or response, the value of $\frac{t}{N}$ should be high”. Where $\frac{t}{N}$ is defined as follows.
The effect of the three aforementioned factors on sugars formed per kg seed residue is shown in Figure 4.1. For all three factors at each level the S/N ratios were calculated and the range values were calculated as the difference of the S/N values at the highest and lowest level of the factors. From Figure 4.1 it is evident that acid concentration (Var2) and temperature (Var3) showed positive effects on the amount of sugars produced from the de-oiled \( P. \text{pinnata} \) seed residue, confirming that an increase in factor level resulted in increased performance. The range values suggest that type of acid was the factor that influenced the reaction most, with the highest average S/N ratio (ETA) being observed with HCl and the lowest ratio observed when H\(_3\)PO\(_4\) was used. In Figure 4.1 pink and red dotted lines represent the mean of S/N (ETA) for all runs in the experiment and standard error of the mean S/N, respectively. The data was further analyzed for variance and the contribution of parametric effect on amount of glucose formed (Table 4.2). Assessment of the impact of each of the variables on glucose formation from hydrolysis and analysis of variance confirmed that Var1 (type of acid used) represented the most significant variable, accounting for 54.4% of the total variation (Table III). Var2 (acid concentration) showed the next largest contribution (25.1%) while Var3 (temperature) contributed 20.5%. The results obtained from the mean effects measurement and ANOVA were in agreement. Higher temperature and acid concentration may have enhanced the penetration of acid to the inner matrix of the biomass, resulting in increased degradation of the crystalline structure and enhanced sugar release through breaking of the glucose linkages. Moreover, at the higher temperature the energy available for the reaction was higher than the activation energy (120-130 kJ mol\(^{-1}\)) [Andreas et al., 2013] resulting in increased rate of reaction with the maximum sugar concentration obtained in less time [Lavarack et al., 2002].
Figure 4.1: Plot of mean effects of variables on sugar release from the biomass. Var1-Acid type (level 1-H$_2$SO$_4$, level 2-HCl, level 3-H$_3$PO$_4$; Var2-Acid concentration (level 1-2%, level 2-4%, level 3-6%); Var3-Temperature (level 1-80°C, level 2-90°C, level 3-100°C)
In the acid hydrolysis reaction, acid catalyzes the cleavage of hydrogen bonds in the cellulose molecule, which result in the formation of simple sugars. The mechanism of the reaction was previously reported by Xiang et al. (2003). Based on the amount of glucose obtained from HCl, H$_2$SO$_4$ and H$_3$PO$_4$ treatments it can be observed that the amount of glucose obtained from the HCl treatment>H$_2$SO$_4$ treatment>H$_3$PO$_4$ treatment. The $K_a$ values, a measure of acid strength, of HCl, H$_2$SO$_4$ and H$_3$PO$_4$ are 1.3*10$^6$, 1.0*10$^3$ and 7.1*10$^{-3}$, respectively [http://depts.washington.edu/eooptic/links/acidity.html]. From this data it is evident that the acid with maximum strength employed in P. pinnata seed residue hydrolysis process resulted into maximum glucose formation.

From the study of effect of factors, HCl was selected as the best acid for hydrolysis of the seed cake biomass as it showed better performance at all three concentrations. Further experiments were performed at 100 °C using HCl at different concentrations to study the energy requirement for 1 kg sugars production and energy demand to process 1 kg seed cake biomass. The effect of chemical addition on the biomass conversion process consisting of acid hydrolysis, neutralization of acid followed by fermentation to obtain ethanol was further

Table 4.2: ANOVA for the S/N value of each variable

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>Contribution, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAR1</td>
<td>9395.8</td>
<td>2</td>
<td>4697.9</td>
<td>4.2</td>
<td>54.4</td>
</tr>
<tr>
<td>VAR2</td>
<td>4338.7</td>
<td>2</td>
<td>2169.3</td>
<td>1.9</td>
<td>25.1</td>
</tr>
<tr>
<td>VAR3</td>
<td>3532.8</td>
<td>2</td>
<td>1766.4</td>
<td>1.6</td>
<td>20.5</td>
</tr>
<tr>
<td>Residual</td>
<td>2210.9</td>
<td>2</td>
<td>1105.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
examined. The amount of glucose formed during hydrochloric acid hydrolysis at 100 °C employing 2%, 4% and 6% HCl concentrations were 132.1, 146.7 and 173.4 g glucose kg\(^{-1}\) seed residue, respectively.

Hu et al. (2010) studied dilute acid hydrolysis of maple wood extract at atmospheric pressure and reported 170 g sugar per litre of maple wood extract at 95 °C in the presence of 3.1% sulphuric acid concentration. Ge et al. (2011) studied the transformation of seaweed wastes to sugars and reported a maximum glucose yield of 277.5 g kg\(^{-1}\) seaweed residue from a two-step hydrolysis process: sulphuric acid hydrolysis followed by enzymatic hydrolysis. Comparison of the results obtained in the present work with that already reported work using different biomass feed stocks reveal that the *Pongamia pinnata* seed residue is a promising feed stock for sugars production at mild temperatures and at atmospheric pressure. The process adopted in the present work resulted in glucose formation without the requirement of high pressures and a second stage enzyme treatment.

### 4.2 ENERGY REQUIREMENT FOR ACID HYDROLYSIS PROCESS

From the optimization studies on the acid hydrolysis of *P. pinnata* seed residue to obtain sugars, HCl showed comparatively better sugar formation. Therefore, the energy requirement of HCl catalyzed acid hydrolysis of the seed residue was estimated at different acid concentrations. The material and energy inputs required for 1 kg sugars production is presented in Table 4.3.

An increase in acid loading resulted into an increased conversion of the seed residue to sugars which in turn decreased the quantities of the biomass and process water required to obtain 1 kg sugars.
Table 4.3: Material and energy inputs for acid hydrolysis of *P. pinnata* seed cake biomass

<table>
<thead>
<tr>
<th>Acid hydrolysis process→</th>
<th>2% HCl</th>
<th>4% HCl</th>
<th>6% HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and energy input↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Materials input</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. pinnata</em> seed residue, kg</td>
<td>7.57</td>
<td>6.82</td>
<td>5.77</td>
</tr>
<tr>
<td>Water, kg</td>
<td>71.61</td>
<td>60.80</td>
<td>48.32</td>
</tr>
<tr>
<td>HCl (37%), kg</td>
<td>4.09</td>
<td>7.37</td>
<td>9.35</td>
</tr>
<tr>
<td>Sugars obtained, kg/kg seed cake</td>
<td>0.1321</td>
<td>0.1467</td>
<td>0.1734</td>
</tr>
<tr>
<td>Energy supplied</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. pinnata</em> seed residue Q_{P.pinnata}, kJ</td>
<td>596</td>
<td>537</td>
<td>454</td>
</tr>
<tr>
<td>Water Q_{W}, kJ</td>
<td>22449</td>
<td>19060</td>
<td>15148</td>
</tr>
<tr>
<td>HCl (37%) Q_{HCl}, kJ</td>
<td>964</td>
<td>1735</td>
<td>2202</td>
</tr>
<tr>
<td>Total energy supplied Q, kJ</td>
<td>24010</td>
<td>21333</td>
<td>17805</td>
</tr>
<tr>
<td>Energy recovered Q_{recovered}, kJ</td>
<td>15606</td>
<td>13866</td>
<td>11573</td>
</tr>
<tr>
<td>Net Energy for 1 kg sugars production, kJ</td>
<td>8403</td>
<td>7466</td>
<td>6232</td>
</tr>
<tr>
<td>Net energy to process 1 kg seed cake, kJ</td>
<td>1110</td>
<td>1095</td>
<td>1081</td>
</tr>
</tbody>
</table>

Among the three different concentrations of HCl treatments, 2% HCl treatment (8403 kJ) was estimated as the most net energy demanding process, followed by 4% HCl treatment
(7466 kJ), with the 6% HCl treatment the least energy intensive process (6232 kJ) to produce 1 kg sugars. However, when considering the energy required to process (1 kg seed cake) rather than to produce 1 kg sugar no significant variation in net energy requirement was observed, with the three processes requiring between 1080-1110 kJ/kg seed residue. It can be concluded that the impact of different HCl treatment methods was not very significant when 1 kg seed cake is processed.

4.3 NEUTRALIZATION

Neutralization of the acid hydrolysis product is one of the detoxification techniques (others include ion exchange resin, activated charcoal and over liming treatments) used to improve ethanol formation during fermentation [Chandel et al., 2007]. Neutralization of the acid hydrolysis reaction mixture was carried out using NaOH (1N). In the present work neutralization of the acid hydrolysis product was carried out to study the effect of the salt formed during neutralization on the rate of fermentation and final concentration of glucose and ethanol from the process. In neutralized hydrolyzates, sugar concentration and the amount of salt formed in hydrolysis treatments using HCl at different concentrations is presented in Table 4.4.

In the present work, 2%, 4%, and 6% HCl treatments resulted 21.41 g, 31.93 g and 38.19 g salt per l of neutralized hydrolyzate, respectively, from 1 kg seed cake processing (Table 4.4). The amount of glucose formed per kg seed cake increased with increasing concentration of HCl in the acid treatment step. However, the concentration of sugars (g l⁻¹) decreased and salts concentration increased after the neutralization step with increase in acid concentration due to the addition of extra alkali in the neutralization step; an increase in acid concentration resulted in increased quantities of alkali required for neutralization of the acid employed. Among the three different concentrations of HCl treatments, comparatively higher
sugar concentrations and lower salt concentration per litre were observed in 2% HCl treatment after the neutralization step. However, maximum glucose formation per kg of the seed residue was observed in the 6% HCl treatment.

Table 4.4: Sugars and salt concentration in neutralized hydrolyzates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acid concentration, g l⁻¹</th>
<th>Temperature, °C</th>
<th>Sugars Yield, g kg⁻¹ seed cake</th>
<th>NaOH (1N) added, l</th>
<th>Sugars, g l⁻¹</th>
<th>Salt, g l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% HCl</td>
<td>20</td>
<td>100</td>
<td>132.1</td>
<td>5.48</td>
<td>8.71</td>
<td>21.41</td>
</tr>
<tr>
<td>4% HCl</td>
<td>40</td>
<td>100</td>
<td>146.7</td>
<td>10.97</td>
<td>7.21</td>
<td>31.93</td>
</tr>
<tr>
<td>6% HCl</td>
<td>60</td>
<td>100</td>
<td>173.4</td>
<td>16.46</td>
<td>6.80</td>
<td>38.19</td>
</tr>
</tbody>
</table>

**4.4 FERMENTATION**

Fermentation of neutralized liquid hydrolyzates from 2%, 4% and 6% HCl acid hydrolysis were performed at 35 °C using dry yeast as the fermentation agent. Quantitative and qualitative assessment of the sugars in the fermentation mixture was determined using HPLC with quantification assessed using calibration curves prepared from standard glucose, fructose and sucrose concentrations. The ethanol formed during fermentation was quantified by Gas Chromatography using a calibration curve prepared from analytical grade ethanol in water at known concentrations; the calibration constant obtained was 1.086.

The glucose conversion profile in terms of glucose concentration (g l⁻¹) in the fermentation media with respect to time during fermentation is shown in Figure 4.2 (a).
Figure 4.2: (a) Glucose concentration during fermentation; (b) Ethanol formed from HCl hydrolysis followed by neutralization and fermentation.
Glucose conversion was estimated based on the following expression:

\[ Glucose\ conversion,\% = \frac{C_{G0} - C_{Gi}}{C_{G0}} \times 100 \]

Where,

- \( C_{G0} \): Initial glucose concentration before fermentation at time ‘0’
- \( C_{Gi} \): Glucose concentration during fermentation at time ‘i’

A rapid consumption of glucose within 24 h was observed in fermentation of hydrolysis product obtained from 2% HCl treatment when compared with glucose consumption in fermentation of hydrolysis product obtained from 4% and 6% HCl treatments (Figure 4.2a). The amount of glucose converted steadily increased over 72 h and then levelled out, except for the hydrolyzate obtained at 6% HCl treatment. The fermentation of 6% acid hydrolysis reaction products showed a lag in total sugar conversion in the initial period (24 h) followed by a linear increase until 96 h and a slow rate of fermentation after that. Maximum glucose conversion values of 99.5%, 96.8%, and 91.02% were observed in the fermentation runs of 2% HCl, 4% HCl, and 6% HCl treatments, respectively, at the end of 144 h (6 days) fermentation.

The acid hydrolysis reactions in the present work were carried out at mild temperatures in the range 80-100 °C. It was therefore assumed that no significant amounts of fermentation inhibitors were formed during the reaction. Moreover, the acid hydrolysis reaction product was subjected to detoxification by neutralization. It has been reported that salt has a significant inhibitory effect on the fermentation capacity of yeast and results in a significantly slower fermentation rate [Casey et al., 2013]. Therefore, the lag phase observed during the fermentation may be attributed to the presence of salt. The slower fermentation rates at increased acid concentration were likely due to the salts originating from the addition of chemicals during biomass processing and the subsequent pH adjustment. The presence of
salts in fermentation media can lead to osmotic stress. Yeasts have several different mechanisms to combat osmotic stress, many of which require energy or carbon [Olz et al., 1993]. This need for additional energy and carbon could explain the enhanced glucose consumption rates at low salt concentrations. However, at higher salt concentrations, sugar consumption rates were decreased (Figure 4.2 (a)).

Ethanol yield in terms of g ethanol formed per liter of fermentation media is presented in Figure 4.2 (b). From the present investigation it was observed that neutralization of excess acid after acid hydrolysis had a profound influence on the ethanol concentration. Comparatively higher ethanol concentrations (4.43 g/l) were achieved from the fermentation of neutralized hydrolyzates from 2% HCl treatment compared to those obtained at 4% and 6% HCl treatments (3.56 g/l and 3.37 g/l ethanol after 144 h of fermentation, respectively). Moreover ~41% of theoretical ethanol (g ethanol formed*100/g carbohydrates present in the seed residue*0.511111) was achieved from the 6% HCl treatment process per kg seed cake, showing the potential of the seed cake for application in ethanol production, as has previously been suggested for other biomass feedstocks [Kim et al., 2004]. As the ethanol yield depends on the amount of fermentable sugars present in the media, and from the analysis of results presented in Table 4.4, Fig. 4.2 (a) and (b), it was concluded that the conditions employed in the acid hydrolysis step had a strong influence not only on the fermentation rate but also on the final ethanol concentration. Chandel et al. (2007) reported an ethanol concentration of 3.46 g l⁻¹ from a 2.5% HCl treatment followed by neutralization and subsequent fermentation of bagasse.

Based on the glucose concentration (g/L) from acid hydrolysis followed by neutralization experiments and simulated data of energy requirement (1080-1110 kJ/kg seed cake) of the acid hydrolysis process the optimum acid hydrolysis conditions for conversion
of de-oiled *P. pinnata* seed residue to obtain maximum ethanol concentration were HCl treatment at 100 °C.

### 4.5 CONCLUSIONS

The present work proposed the practical use of de-oiled *Pongamia pinnata* seed residue via acid hydrolysis, neutralization followed by fermentation. The first step of the work identified the effect of acid hydrolysis treatment conditions on sugar formation. The optimum conditions based on the Taguchi robust method in the considered range of variables were HCl treatment at 100 °C. The net energy demand of acid hydrolysis using 2%, 4%, and 6% HCl to process 1 kg seed residue was estimated and found to be in the range 1080-1110 kJ. The amount of sugars liberated increased with increasing HCl concentration but the concentration of sugars (g l⁻¹) decreased per litre of neutralized mixture. Fermentation of neutralized liquid hydrolyzates obtained from different concentrations of HCl revealed the effect of acid concentration employed in the hydrolysis step in terms of fermentation time and ethanol yield. Fermentation of neutralized hydrolyzate from 2%, 4%, and 6% HCl treatments resulted in the production of 4.43 g/l, 3.56 g/l and 3.37 g/l ethanol yield after 144 h of fermentation, respectively. A 2%, 4% and 6% HCl treatment followed by neutralization and fermentation gave ~31.45%, 34.92% and 41.28% of theoretical ethanol formation. The results confirm that the de-oiled *P. pinnata* seed cake represents a promising feedstock for 2nd generation ethanol production.
Chapter 5 discusses the application of de-oiled *Pongamia pinnata* seed residue as a substrate for lignocellulose enzymes and ethanol production. *Sphingomonas echinoides* and *Iprex lacteus* were selected as sources of lignocellulases during solid state fermentation. The study demonstrated the feasibility of using the seed residue for enzyme preparation for application in hydrolysis of the same seed residue and the potential of using the hydrolysis product for ethanol production.

5.1 MICROORGANISMS

*Sphingomonas echinoides* and *Iprex lacteus* were selected as sources of lignocellulases during the fermentation for enzyme production. *Sphingomonas echinoides* is a bacterium isolated from de-oiled *P. pinnata* seed residue, not previously used as a source of lignocellulases. *Iprex lacteus* is a white rot fungus and was obtained from the RMIT Culture Collection (ATCC 11245). Lignocellulolytic microorganisms represent promising sources of new enzymes for lignocellulosic biofuel production. *P. pinnata* seed residue was targeted to isolate cellulolytic microorganisms because the resident microbes decompose the seed residue quickly and effectively, suggesting the natural microflora may contain microorganisms producing high concentrations of lignocellulolytic enzymes. Isolates were initially screened qualitatively based on growth on cellulose media. Twenty seven bacterial
isolates were purified from *P. pinnata* seed residue using BH-cellulose agar plates. From the clear zone diameter method 8 bacterial isolates were selected for further screening, quantitatively, and were identified as *Bacillus firmucutes* (5 isolates), *Leclercia enterobacteria* (1 isolate), *Methylobacterium a-proteobacteria* (1 isolate), and *Sphingomonas a-proteobacteria* (1 isolate). The selected isolates were further tested for cellulolytic activities (exoglucanase) by quantitatively measuring the enzyme activities in cellulose liquid media. Quantitative screening was performed by estimating the crude enzyme activity from submerged fermentation carried out at 30 °C with mixing at 150 rpm using BH-cellulose liquid media. *Sphingomonas echinoides a-proteobacteria* exhibited highest cellulolytic activities among the isolates.

### 5.2 ENZYME PRODUCTION IN SOLID STATE FERMENTATION

Solid state fermentation for enzyme production was carried out employing *S. echinoides* and *I. lacteus* on both cellulose and *P. pinnata* seed residue substrates separately. The fermentations were carried out for a period of 8 days at 30 °C, pH 5 and the enzyme activities detected at the end of the 8 day incubation are presented in Figure 5.1. In Figure 5.1 PIL, PSE, CIL and CSE represent solid state fermentations using *P. pinnata-I. lacteus*, *P. pinnata-S. echinoides*, cellulose-*I. lacteus* and cellulose-*S. echinoides*, respectively.

The crude enzyme preparation exhibited appreciable levels of exoglucanase, endoglucanase, and xylanase activities in *P. pinnata* seed residue media inoculated with *S. echinoides* (PSE) and *I. lacteus* (PIL) but with very low laccase activity. With pure cellulose media (CSE and CIL in Figure 5.1) enzyme activities were significantly lower compared to that with *P. pinnata* seed residue media. Of the two organisms assessed, *I. lacteus* produced higher enzyme activities than *S. echinoides*. *S. echinoides* produced higher exoglucanase activity compared to endoglucanase while *I. lacteus* exhibited more endoglucanase than
exoglucanase activity (Figure 5.1). The highest exoglucanase, endoglucanase, xylanase activities obtained from *S. echinoides* and *I. lacteus* were 3.9, 2.7, 0.8 U ml\(^{-1}\) min\(^{-1}\) and 5.2, 8.2, 2.7 U ml\(^{-1}\) min\(^{-1}\), respectively, in the presence of *P. pinnata* seed residue media.

![Graph showing enzyme activities](image)

**Figure 5.1:** Crude enzyme activities U ml\(^{-1}\) min\(^{-1}\) from solid state fermentations using *P. pinnata-I. lacteus* (PIL), *P. pinnata-S. echinoides* (PSE), cellulose-*I. lacteus* (CIL), cellulose-*S. echinoides* (CSE).

Enzyme production by solid state fermentation is a well-adapted process and has significant advantages over submerged fermentation [Raimbault, 1998]. Solid state fermentation processes are unaffected by the purity/impurity level of the substrate resulting in highly concentrated enzyme production. The present investigation revealed similar results, with the production of concentrated (exhibiting high activity) lignocellulases from solid state
fermentation from both the strains. These activities were some 10-50 times greater than those observed in liquid cultures (data shown in Appendix C).

5.3 ENZYMATIC HYDROLYSIS OF P. PINNATA SEED RESIDUE

Hydrolysis reactions were carried out using crude enzyme from S. echinoides and I. lacteus from solid state fermentation and for comparison a commercial cellulase preparation (from A. niger) at two different loadings, 5 U, and 10 U per g of P. pinnata seed residue at 50 °C. Pretreatment of the seed residue prior to enzymatic hydrolysis resulted in the formation of ~50 mg glucose per g of P. pinnata seed residue. Pretreatment enhances the digestibility of lignocellulosic biomass by improving access of the enzymes to the substrate [Hendriks and Zeeman, 2009]. Enhanced hydrolysis rates can be expected with a pretreatment that facilitates the breakdown of the rigid structure of lignocellulosic biomass allowing better access for cellulases. Hydrolysis of P. pinnata seed residue in terms of the amount of reducing sugars formed per g of seed residue i.e. sugars yield (mg/g) in the presence of different enzymes at two different enzyme concentrations is presented in Figure 5.2.

In the presence of crude enzyme from S. echinoides sugar yields from P. pinnata seed residue reached 171 mg g⁻¹ and 233 mg g⁻¹, at enzyme concentration of 5 and 10 U g⁻¹ respectively. Crude enzyme from I. lacteus resulted in higher values, 280 mg g⁻¹ and 306 mg g⁻¹ sugar formation at 5, 10 U g⁻¹ enzyme concentration, respectively. These values were comparable to those obtained with the commercial cellulase preparation, with a sugar yield of 331 mg g⁻¹ at 10 U g⁻¹ and 302 mg g⁻¹ sugar yield with the enzyme at 5 U g⁻¹.

The results suggest that the presence of exoglucanase, endoglucanase and xylanase in the crude enzyme samples can effectively breakdown the cellulose and hemicellulose constituents present in P. pinnata seed residue for application in bioethanol production. In our earlier work [Chapter 4, Radhakumari et al., 2014], sulphuric acid hydrolysis of the same
seed residue resulted in sugar yield of 245 mg g\(^{-1}\) whereas in the present work enzymatic hydrolysis showed a yield of \(\sim 300-330\) mg/g. Improved sugar yields were achieved from enzymatic hydrolysis compared to chemical hydrolysis of the same seed residue. The results were further compared with the sugars yield (343 mg sugars g\(^{-1}\) rice straw) from rice straw in a two stage dilute sulphuric acid hydrolysis under high pressure (30 bar) [Karimi et al., 2006]. Therefore, enzymatic hydrolysis using crude enzyme preparation is a promising option for the conversion of \textit{P. pinnata} seed residue to sugars and the seed residue has the potential to compete with the lignocellulosic feed-stocks for ethanol production.

Figure 5.2: Sugar yield (mg g\(^{-1}\)) from hydrolysis of \textit{P. pinnata} seed residue using crude enzyme from \textit{S. echinoides} (ESE), \textit{I. lacteus} (EIL), and commercial cellulase from \textit{A. niger} (CAN) at 5 and 10 U g\(^{-1}\) concentration.
5.4 FERMENTATION OF ENZYMATIC HYDROLYSIS PRODUCT

The three liquid products from the enzymatic hydrolysis of *P. pinnata* seed residue using crude enzyme prepared from *S. echinoides*, *I. lacteus* and cellulase from *A. niger* were fermented using *S. cerevisiae* for 4 days under anaerobic conditions. The conversions of sugars and ethanol formed during the course of fermentations are presented in Figure 5.3 (a) and (b), respectively. In Figure 5.3 SE, IL and AN represent fermentation of liquid product from the enzymatic hydrolysis of *P. pinnata* seed residue employing crude enzyme from *S. echinoides*, *I. lacteus* and cellulase-*A. niger*, respectively.

A rapid conversion of sugars, ~80%, was observed in AN fermentation within 1 day followed by a slow increase in the conversion. During AN fermentation the conversion plateaued out after 2 days and a maximum conversion of 93% was observed at the end of day 4. Fermentation of SE and IL resulted into a sugars conversion of ~50% after 1 day and a slow increase in the conversion was observed thereafter. An almost similar sugar conversion profile was observed during the fermentation of SE and IL and a maximum conversion of ~68% was observed at the end of day 4. Ethanol yield in terms of mg ethanol formed from g of the seed residue is presented in Figure 5.3 (b). Highest ethanol yield was observed from AN fermentation, 157.6 mg/g seed residue. In the case of fermentation of hydrolyzates from crude enzyme treatment ethanol yields of 81.5 and 104.5 mg/g were observed from SE and IL, respectively. Final ethanol concentrations of 4.0, 5.3 and 7.9 mg/ml were observed from SE, IL and AN fermentation, respectively, Figure 5.3 (b).

The highest sugar conversion and ethanol yield observed from the fermentation of AN is attributed to the presence of hexose sugars alone in AN. It is well reported that cellulases are highly specific and are able to hydrolyse cellulose alone resulting in the release of hexose sugars from the cellulose matrix.
Figure 5.3: (a) Sugar conversion % w/w); (b) Ethanol concentration (mg/ ml) and yield (mg/g) during fermentation using *Saccharomyces cerevisiae* for 4 d.
The sugar conversion rate is high during the initial period followed by a very slow conversion rate (Figure 5.3a). The availability of reactant (glucose) at high concentration during the initial period (1st day) led to ~80% conversion of the glucose at the end of 1st day. The reduced amount of the reactant (from day 2 onwards) may lead to a decrease in the rate of conversion in AN.

IL and SE followed a similar sugar conversion profile but the initial concentration of sugars in IL and SE were different. The initial glucose concentration of IL was 6.7 mg mL$^{-1}$ (306 mg g$^{-1}$ biomass) and that of SE was 5.2 mg mL$^{-1}$ (233 mg g$^{-1}$ biomass). This makes the difference in ethanol yield from the two species.

When cellulase from A. niger was employed in the hydrolysis of P. pinnata seed residue the enzyme will ultimately act on the cellulose portion of the seed residue resulting in the formation of hexoses which can easily be fermented by S. cerevisiae. In contrast in the case of SE and IL, the presence of hexoses and pentoses can be expected in the products as the crude enzyme preparation from S. echinoides and I. lacteus contained both cellulase and xylanase activities acting on both the cellulose and hemicellulose portion of the seed residue resulting in the formation of hexose and pentose sugars. S. cerevisiae is highly efficient in hexose sugars fermentation, but it is unable to ferment pentose sugars. This likely explains the lower sugar conversion and ethanol yield from the fermentation of SE and IL [Stambuk et al., 2008]. These results suggest that the onsite enzyme production strategy will also require a co-fermentation technology for ethanol production from both pentose and hexose sugars to improve ethanol yields. The co-fermentation of hexose and pentose sugars can be possible by applying engineered/recombinant yeast strains in bioethanol production from lignocelluloses, an area of active research at the present [Ha et al., 2011].

Techno-economic analysis of lignocellulosic bioethanol production costs reports that the enzymes cost about $ 132 per cubic meter of ethanol when the enzymes were supplied by
the commercial enzyme manufacturers, Novozymes [Chovau et al., 2013]. In the case of on-site enzyme production the overall cost of enzymes were reported to be $ 90 per cubic meter of ethanol, significantly lower than that of Novozymes. Humbird et al. (2011) estimated the cost of enzymes prepared from cultures grown using pure glucose as the carbon and energy source and reported that the carbon source is the most significant expense in enzyme production. In the present work, *P. pinnata* seed residue, after extracting oil from the seeds, was utilised as the carbon source during the enzyme preparation. *P. pinnata* seeds costs around $0.06 kg\(^{-1}\) and the selling price of oil extracted from the seeds is $0.06 L\(^{-1}\) ($0.065 kg\(^{-1}\) [Murphy et al., 2012] which makes the cost of the seed residue almost insignificant in the process. Therefore, the work suggests that on-site/in-house enzyme preparation using *P. pinnata* seed residue represents a promising option in the enzymatic conversion of the same seed residue for further application in bioethanol production.

Overall, *P. pinnata* seed residue has significant potential to be exploited as a feedstock for the production of sugars. The on-site/in house enzyme production using *S. echinoides* and *I. lacteus* also represents a promising strategy to make the enzymatic hydrolysis process economically feasible. Importantly, the process adopted in the present work is environmentally friendly as no chemicals or organic solvents were employed in pretreatment and enzymatic hydrolysis of the seed residue. However the fermentation of the total sugars formed during hydrolysis using crude enzyme preparations needs improvement to achieve a better ethanol yield, which may be possible using engineered *S. cerevisiae* strains.

**5.5 CONCLUSIONS**

The high cost of cellulases, a major limiting factor in the seed residue conversion process can be addressed by on-site/in-house enzyme preparation. *Spingomonas echinoides* and *Iprex lacteus* have the potential of producing highly active lignocellulases for application in the
hydrolysis of the seed residue. Crude enzyme from *S. echinoides*, *I. lacteus* and commercially available cellulase from *A. niger* resulted in appreciable sugar yields at a minimum enzyme concentration (10 U g\(^{-1}\)) and ethanol yields of 82, 104 and 158 mg g\(^{-1}\) seed residue were observed, respectively. *P. pinnata* seed residue is a potential substrate for both lignocellulases and sugars production for further application in bioethanol preparation.
Chapter 6 discusses the pyrolysis of *Pongamia pinnata* seed residue in a fixed bed reactor and also in a thermogravimetric analyzer. Assessment of the effects of heating rate on the pyrolysis behavior of the seed residue and evaluation of pyrolysis kinetics using isoconversional methods is presented. The characteristic properties of pyrolysis products are also reported.

### 6.1 THERMOGRAVIMETRIC ANALYSIS OF *P. PINNATA* SEED RESIDUE

The thermal behavior of *P. pinnata* seed residue in the temperature range 26±2 °C to 800 °C was studied at three different heating rates. The reduction in the mass of the sample (11±0.1 mg) with temperature at a heating rate 20°C min⁻¹ is shown in Figure 6.1. The complete pyrolysis reaction proceeds with an initial dehydration step followed by decomposition of protein, hemicellulose, cellulose and lignin [Kok et al., 2013]. In the initial dehydration step free and bound moisture and volatile extractives of biomass are carried away by the inert gas flow. The dehydration step occurs to a temperature of 200°C. Most of cellulose and hemicellulose decomposes above 200°C [Carrier et al., 2011] and a vigorous mass loss was observed in the temperature range 250-450°C. Lignin decomposition takes place over a wide temperature range [Burhenne et al., 2013], 160°C to 900°C because of its recalcitrant structure and in the present study a very slow mass loss after a temperature of 450°C was attributed to the lignin mass loss.
In the initial dehydration step, a 1.35% weight loss was observed as moisture was removed. In the decomposition of biomass constituents, considered as an active pyrolysis zone, a 73.8% weight loss was observed with the remaining 24.85% representing residual matter. During the pyrolysis, the intermolecular associations and weaker chemical bonds are destroyed. The side aliphatic chains may be broken and some small gaseous molecules are produced at lower temperature. At higher temperatures chemical bonds in lignin are broken and the parent molecular skeletons are destroyed [Raman et al., 1981 & Singh and Shadangi 2011].

Figure 6.1: Thermogram of *Pongamia pinnata* seed residue at 20 °C min⁻¹ heating rate

### 6.2 PYROLYSIS KINETICS

Biomass pyrolysis includes complex heat and mass transfer phenomena; therefore, only about 11 mg of *P. pinnata* seed residue biomass samples were used for thermogravimetric analysis
to minimize/overcome the endothermic and exothermic effects on the sample due to the furnace temperature. The thermogravimetric data was evaluated using 14 different reaction models, previously given in Table 2.3. The best correlation was obtained with the third order reaction model at three different heating rates and the regression plots according to Coats Redfern method are shown in Figure 6.2.

![Figure 6.2: Coats Redfern method, third order reaction model fitted to TG data at different heating rates.](image)

The subsequent calculations determining the values for activation energy (E) and frequency factor (A) using the Coats Redfern method are listed in Table 6.1. The activation energy data obtained ranged from 82.95 to 98.21 kJ mol⁻¹. An increase of reaction kinetics parameters with the heating rate employed was observed in the present study. The obtained
kinetic triplet was used to simulate the seed residue pyrolysis process. A good agreement was observed between the model and experimental data (Appendix D).

Table 6.1: Kinetic triplet for karanja seed cake pyrolysis reaction from the Coats Redfern method

<table>
<thead>
<tr>
<th>Heating rate, °C min⁻¹</th>
<th>Reaction model, f(α)</th>
<th>Activation energy (E), kJ mol⁻¹</th>
<th>Frequency factor (A), min⁻¹</th>
<th>Correlation coefficient, R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>(1-α)³</td>
<td>82.95</td>
<td>3.43*10⁶</td>
<td>0.9949</td>
</tr>
<tr>
<td>10</td>
<td>(1-α)³</td>
<td>96.31</td>
<td>9.73*10⁷</td>
<td>0.9974</td>
</tr>
<tr>
<td>20</td>
<td>(1-α)³</td>
<td>98.21</td>
<td>2.25*10⁸</td>
<td>0.9967</td>
</tr>
</tbody>
</table>

The data obtained from thermogravimetric analysis was also analyzed for isoconversional kinetics according to the Kissinger-Akahira-Sunose method (KAS) and the Ozawa-Flynn-Wall method (OFW), standard methods for isoconversional kinetics evaluation. The regression lines obtained using KAS and OFW methods are depicted in Figure 6.3 (a) and (b), respectively.

Activation energies, calculated from the KAS and OFW methods are presented in Table 6.2. It is evident from the table that the values obtained from both methods are similar. Regression coefficients of the plots increased with progressive conversions. Activation energies from KAS method ranged from 122.8 kJ mol⁻¹ to 106 kJ mol⁻¹ for conversions ranging from α=0.2 to α=0.9. The activation energies from the OFW method were in the range 126-110 kJ mol⁻¹ for the same conversions. Activation energies decreased with an increase in conversion, as high conversions were observed at high temperatures, due to the requirement of comparatively lower activation energies at elevated temperatures. More reactions were triggered at higher temperatures, leading to a sharp rise in reaction rates with more unstable intermediates and lower activation energies.
Figure 6.3: Regression lines for determination of activation energy by (a) the KAS method; (b) the OFW method.
The reported values of pyrolysis reaction activation energies for hazelnut husk are in the range 128-131 kJ mol\(^{-1}\) [Ceylan and Topcu 2014], 129 kJ mol\(^{-1}\) for corn cob [Gai et al., 2013], 62-206 kJ mol\(^{-1}\) for pine wood waste [Amutio et al., 2012]; the activation energy of pyrolysis of \(P.\ pinnata\) seed residue are in the range of the hazelnut husk, corn cob but there was considerable deviation from the reported activation energy of pine wood biomass. The activation energy of pyrolysis is highly dependent on the extent of conversion and the type and composition of biomass feedstock as seen in the activation energy for pine wood waste which is a woody biomass, rich in lignin and very different from that of seed cake biomass and nut husk and other biomass types. The KAS and OFW methods gave reliable activation energies that were used for the calculation of the frequency factor in order to get a complete picture of the kinetic parameters with progressive conversions; the frequency factors were determined by making use of the reaction model obtained from the CRF method. The calculated frequency factor values from the OFW method are shown in Table 6.2. Therefore, the kinetic expression obtained for pyrolysis of \(P.\ pinnata\) seed residue was:

\[
\frac{da}{dt} = Ae^{\frac{-E}{RT}}(1 - \alpha)^3 \quad \text{(6.1)}
\]

Where the Arrhenius parameters obtained from the model-free isoconversional methods were in good agreement with the values determined using the model fitting method.

Determination of the pyrolysis kinetic parameters, based on thermogravimetric experimental data is extremely useful in the design and control of the pyrolysis process, especially in large scale operations with desired composition and yield of products. Moreover, pyrolysis kinetic data links technological parameters, including biomass retention times at preset temperature program that determines pyrolysis product profiles.
Table 6.2: Arrhenius parameters and regression factors of pyrolysis reaction from KAS and OFW methods.

<table>
<thead>
<tr>
<th>Conversion, α</th>
<th>KAS method</th>
<th>OFW method</th>
<th>OFW method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ea, KJ mol⁻¹</td>
<td>R²</td>
<td>Ea, KJ mol⁻¹</td>
</tr>
<tr>
<td>0.2</td>
<td>122.81</td>
<td>0.907</td>
<td>126.03</td>
</tr>
<tr>
<td>0.3</td>
<td>120.41</td>
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<td>123.87</td>
</tr>
<tr>
<td>0.4</td>
<td>117.92</td>
<td>0.943</td>
<td>121.61</td>
</tr>
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<td>0.5</td>
<td>114.26</td>
<td>0.958</td>
<td>117.95</td>
</tr>
<tr>
<td>0.6</td>
<td>112.97</td>
<td>0.967</td>
<td>117.10</td>
</tr>
<tr>
<td>0.7</td>
<td>108.24</td>
<td>0.975</td>
<td>114.92</td>
</tr>
<tr>
<td>0.8</td>
<td>108.24</td>
<td>0.982</td>
<td>112.83</td>
</tr>
<tr>
<td>0.9</td>
<td>106.00</td>
<td>0.987</td>
<td>110.81</td>
</tr>
</tbody>
</table>

6.3 PYROLYSIS OF P. PINNATA SEED RESIDUE IN A FIXED BED REACTOR

Thermal treatment of P. pinnata seed residue was performed in a fixed bed reactor of 1 cm ID and 35 cm length under slow pyrolysis conditions with a continuous supply of nitrogen gas at 0.1 LPM flow at different temperatures including 200, 250, 300, 350, 400, 450, and 500 °C. The pyrolysis transformed the seed residue to biochar (thermally treated biomass retained in the reactor), bio-oil (condensed liquid product) and gases. The solid and the liquid obtained were weighed and the yields expressed gravimetrically. Biochar was characterized by proximate analysis carried out according to ASTM standard methods [Cantrell et al., 2010] using a Mettler Toledo TGA/SDTA 851e analyser (Switzerland), ultimate/elemental analysis using a CHNS Analyzer- ELEMENTAR Vario
micro cube model; surface structure was obtained by scanning electron microscopy using a Hitachi S-3000N Scanning electron microscope and FTIR was performed using a Perkin Elmer System 100 Fourier Transform Infrared spectrometer [Rutherford et al., 2012].

Pyrolysis temperature showed a profound influence on the product distribution in the reaction. Figure 6.4 shows the pyrolysis products, biochar and total volatiles (sum of condensable and non-condensable) and yields obtained in relation to temperature. Pyrolysis at higher temperature (≥500°C) resulted in increased carbon conversion to volatiles [Antony et al., 2011 & Chutia et al., 2014] and reduced biochar.

![Figure 6.4: Effect of temperature on pyrolysis of de-oiled P. pinnata seed residue to biochar.](image)

The *P. pinnata* seed residue, processed in the present work was composed of 25.2% carbohydrates, 17.4% lignin, 13.4% protein, and 4.0 % ash with 40% water and ethanol soluble matter [see Chapter 3, Radhakumari et al., 2014]. The biochar obtained from the
thermal treatment of *P. pinnata* seed residue was analyzed for its composition in terms of proximate and ultimate analysis and the data is presented in Table 6.3 along with evaluated higher heating value (HHV), carbon carry over, H/C and O/C ratios.

Table 6.3: Composition and energy value of biochar obtained from the pyrolysis of *P. pinnata* seed cake at different temperatures.

<table>
<thead>
<tr>
<th>Element, %wt.</th>
<th>P. pinnata seed residue</th>
<th>Biochar from Pyrolysis at 350 °C</th>
<th>Biochar from Pyrolysis at 400 °C</th>
<th>Biochar from Pyrolysis at 450 °C</th>
<th>Biochar from Pyrolysis at 500 °C</th>
<th>Biooil from pyrolysis at 500 ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximate analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>03.20</td>
</tr>
<tr>
<td>Ash</td>
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<td>15.42</td>
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<tr>
<td>N</td>
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<td>05.84</td>
<td>05.91</td>
<td>06.07</td>
<td>05.95</td>
<td>3.97</td>
</tr>
<tr>
<td>C</td>
<td>44.20</td>
<td>58.39</td>
<td>60.80</td>
<td>63.64</td>
<td>65.06</td>
<td>67.24</td>
</tr>
<tr>
<td>H</td>
<td>06.50</td>
<td>05.21</td>
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<td>O</td>
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<td>28.84</td>
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<td>HHV, MJ/kg</td>
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<td>22.9</td>
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<td>H/C</td>
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<td>O/C</td>
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<tr>
<td><em>Carbon carry over, %</em></td>
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<td>56.82</td>
<td>53.29</td>
<td>52.55</td>
<td>51.52</td>
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</table>

*{(C content in biochar X Biochar yield)/(C content in karanja seed cake)}; *not found
From the elemental analysis of the feed *P. pinnata* seed residue and biochar at different temperatures it was observed that the carbon content increased while the hydrogen and oxygen content decreased with increasing temperature. H/C and O/C values give a picture of the degree of carbonization, aromaticity and maturity of organic materials. The observed decrease in oxygen (O/C) with increasing temperature was probably due to the increased decomposition of oxygen containing functional groups. The percent biochar obtained from the seed cake by experimentation was 56.82%, 53.29%, 52.55%, and 51.52% at 350, 400, 450, and 500 °C respectively indicating a decrease in the carbon carryover from the seed cake to biochar with temperature. Therefore, the value addition of *P. pinnata* seed residue by thermal treatment represents a promising pre-treatment method to obtain biochar for multiple applications.

### 6.3.1 Scanning electron microscopy

As porosity play a vital role in determining the efficiency and suitability of bio-sorbents in the adsorption of contaminants from effluent treatment, with maximum adsorption occurring when pores are large enough to admit contaminant molecules, scanning electron microscopy of *P. pinnata* seed residue and biochar was carried out to assess the surface structure of the samples. The SEM pictures obtained are presented in Fig. 6.5 a and b. As is evident from the pictures the char formed (Fig. 6.5b) has a highly porous structure compared to the original seed residue biomass (Figure 6.5 (a)). This can be attributed to the fact that thermal treatment improves the porous structure of biochar due to the loss of hydrogen, oxygen (major quantity) and carbon (minor quantity) atoms in the form of volatiles from the parent biomass material leaving the skeletal structure.
Figure 6.5: Surface structure (from SEM) of (a) de-oiled *P. pinnata* seed residue; (b) biochar; (c) Fourier transformed infrared (FTIR) spectra of biochar obtained at 500 °C.
6.3.2 Fourier transformed infrared spectroscopy

Fourier transformed infrared spectra of biochar was collected using the KBr pelletization method on a Perkin Elmer System 100 Fourier Transform Infrared spectrometer (FTIR) with a CSI TGS detector by scanning from 4000 to 400 cm\(^{-1}\), with a resolution of 4 cm\(^{-1}\). The biochar spectra as shown in Figure 6.5 (c) depicts vibrations in the frequency range 700-1200 cm\(^{-1}\) and 1200-2000 cm\(^{-1}\) confirming the aliphatic and aromatic nature of the sample. The peaks at frequencies of 698 (aromatic ring C-H), 1020 (C-O stretching vibration), 1420 (C-H stretching vibration) and, 1626 cm\(^{-1}\) (C=C and C=O stretching vibrations) show the carbon structure of biochar. This spectrum is comparable to reference spectra obtained by Rutherford et al. (2012) who studied changes in composition and porosity occurring during the thermal degradation of wood and wood components. The result further emphasize the fact that thermal treatment of biomass converts aliphatic carbon to aromatic on prolonged treatment [Rutherford et al., 2012].

From the characterization of the seed residue biomass and biochar, based on elemental composition, surface structure and FTIR it is evident that the biochar is rich in carbon, has a porous structure and can be effectively used for energy generation and as a biosorbent for industrial effluent treatment and electrode material in activated carbon electrodes manufacture.

6.4 CONCLUSIONS

The present study is the first report on non-edible seed residue biomass pyrolysis kinetics (in particular \textit{P. pinnata} seed residue biomass), with the work presented helpful in assessing the potential for the value addition to \textit{P. pinnata} seed residue through thermal treatment. Thermogravimetric analysis at constant heating rate revealed that a temperature of 450-500 °C was sufficient for carbonization of the biomass. The KAS, OFW, and CRF
methods gave reliable activation energies and frequency factors with a third order reaction model for the seed residue pyrolysis reaction. Characterization results showed that the thermally treated biomass has a highly porous structure, rich in carbon, suitable for various applications as biosorbent and precursor for carbon materials. The kinetic data thus obtained is extremely useful in assisting in the appropriate design of pyrolysis systems for seed residue biomass.
CHAPTER 7
GENERAL DISCUSSION

7.1 DISCUSSION

Second generation biofuel production using lignocellulosic biomass is a promising alternative to fossil fuels and first generation biofuels. Our dependence on fossil fuels has resulted in severe environmental damage. However first generation biofuel production is hampered by usage of food grade raw material for biofuel preparation as it has a direct impact on the food price [Balan et al., 2013]. For every 2.5 million tonnes used for fuel ethanol production by the US fuel ethanol industry the price of corn was estimated to increase by $1.2-2.0 ton\(^{-1}\) [Elander and Putsche 1996]. As a result, second generation non-edible lignocellulosic biomass has emerged as the most suitable precursor for biofuels production. The advantage of second generation biofuels production is that residues from agriculture, forest as well as other lignocellulosic wastes can be exploited as raw materials which facilitates not only the utilization of low cost feed stock in the bio-industry but also utilizes a renewable, carbon neutral substrate.

The aim of this research study was to assess the potential of a non-edible seed residue, de-oiled and dried *Pongamia pinnata* seed residue, as a precursor for biofuel production. The seed residue is a by-product from the biodiesel industry which utilizes the seed oil as feedstock. A major advantage of the seed residue as feedstock is that it is available in powder form after de-oiling and drying which eliminates the need for size reduction. Size reduction is a primary unit operation in the case of woody biomass and the conversion of other agricultural biomass into biofuels. Energetically this is an expensive procedure; size
reduction of one ton of biomass to 3-6 mm size requires 30 kwh energy [Cadoche and Lopez 1989]. Therefore, from the point of process energy demand, *P. pinnata* seed residue represents a promising feed stock for biofuels production.

The present thesis focused on studying the application of a non-edible seed residue as a sustainable feedstock for bioethanol production. The seed residue was considered as a sustainable feedstock based on its annual production in India and Australia. The annual productivity of the seeds in India is 2,00,000 metric tonnes annum\(^{-1}\) and Australia has already started a commercial trial site of 300 ha for *Pongamia* tree plantation in Roma, Central Queensland. Based on this data the seed residue was considered as a sustainable feedstock for biofuels production as biomass feedstock availability is also a key factor for sustainable biofuel production. The present thesis discussed the sustainability issue by considering feedstock availability. Moreover, the de-oiled *P. pinnata* seed residue is available in a flowing powder form. The physical state of the seed residue eliminates the size reduction step from the process. In the case of woody biomass, size reduction is an essential step that requires around 30 kwh energy per tonne of biomass.

In the first part of this study, *P. pinnata* seed residue was characterized for composition in terms of ash, extractives, lignin and protein together with CHNS elemental composition using well established methods [Sluiter et al., 2008a; Sluiter et al., 2008b; Scarlata et al., 2008; Jung et al., 1999; Madhumanchi et al., 2013]. Compositional characterization showed the seed residue to be rich in lignocellulosics and protein (42 % carbohydrates, 29 % lignin and 22 % protein after removing water and ethanol soluble components from the seed residue) and therefore a potential candidate for conversion into sugars for application in bioethanol production. CHNSO elemental composition of the seed residue found this biomass to be similar to that of other lignocellulosic biomass which have
been reported in biofuels production, for instance corn stover [Medic et al., 2012], cellulose pulps and poplar *Populus nigra* [Carrier et al., 2011].

Assessment of *P. pinnata* seed residue as a feedstock for biofuel production was carried out by acid hydrolysis in the presence of sulphuric acid. Sulphuric acid in water at different concentrations was employed to convert the biomass into soluble sugars. During sulphuric acid hydrolysis of the seed residue the effects of temperature and solid to liquid mass ratio on selective glucose formation were studied, based on the Taguchi robust design of experiments method. Identification and quantification of sugars formed (glucose, fructose, xylose and sucrose) were carried out using high performance liquid chromatography. Among the hydrolysis conditions tested 7.5 % sulphuric acid concentration at 120 °C with a solid to liquid weight ratio of 1:15 resulted in the highest recovery of glucose of 245 mg glucose g\(^{-1}\) seed residue over a treatment time of 1 h with mixing (750 rpm). Selective formation of glucose was also measured in order to study the effect of reaction factors on the hydrolysis of the seed residue. The results confirmed that temperature and acid concentration were the key factors in *P. pinnata* seed residue hydrolysis. An increase in the solid to liquid weight ratio from 1:10 to 1:15 and 1:20 had no significant effect on glucose yield. The study also revealed that hydrolysis of the seed residue resulted in glucose rich product formation. Rehman et al. (2007) reported that dilute acid hydrolysis of oil palm empty fruit bunches at 119 °C using 2% H\(_2\)SO\(_4\) for 60 min resulted in a xylose rich hydrolyzate whereas hydrolysis of dairy manure reported by Liao et al. (2004) gave a mixture of sugars comprising arabinose, xylose and galactose from 1% H\(_2\)SO\(_4\) treatment at 135 °C for 2 h. Therefore, from this part of the work it was concluded that *P. pinnata* seed residue represented an ideal substrate for glucose production, a readily fermentable sugar for bioethanol production.
Acidity is dependent on the type of acid and its concentration [Taherzadeh and Karimi 2007]. Different acids have been reported as catalysts for the acid hydrolysis of lignocellulosic biomass. The most commonly employed acids include sulphuric acid, hydrochloric acid, phosphoric acid, oxalic acid and nitric acid. In order to evaluate the best acid for hydrolysis of the seed residue for glucose extraction different acids including sulphuric acid, hydrochloric acid and phosphoric acid were tested, employing 2, 4 and 6% concentrations at very mild temperatures of 80-100 °C to limit the further degradation of glucose [Noparat et al., 2015]. The experimental investigation showed that sulphuric acid showed better hydrolysis performance only at high temperature (100 °C) and at high concentration (6%) whereas hydrochloric acid showed better hydrolysis performance at both low and high levels of temperature and acid concentration, confirming that hydrochloric acid was the best agent compared to other acids for the hydrolysis of the seed residue. The hydrolysis product obtained from the HCl assisted hydrolysis of the seed residue was subjected to further processing which includeds neutralization followed by fermentation. Neutralization has been reported as a detoxification techniques (along with ion exchange resin, activated charcoal and over liming treatments) used to improve ethanol formation during fermentation [Chandel et al., 2007] of the acid hydrolysis product. In the present work neutralization of acid hydrolysis reaction mixture was carried out using NaOH (1N). Fermentation of the neutralised acid hydrolysis product was carried out to study the effect of acid concentration in hydrolysis on the rate of fermentation and final concentration of ethanol from the process. Fermentation of 2%, 4% and 6% HCl treatments followed by neutralization gave 67.5, 75.0 and 88.6 g ethanol kg⁻¹ dry seed cake, respectively, corresponding to ~31.5%, 34.9% and 41.3% of theoretical ethanol (214 g kg⁻¹) formation. Chandel et al. (2007) reported 3.46 g l⁻¹ ethanol concentration from 2.5% HCl treatment followed by neutralization and fermentation of bagasse. The levels of ethanol concentration observed in the present work
confirmed the potential of the seed residue as a feedstock for ethanol production to compete with other lignocellulosic biomass resources.

The unconverted seed residue in acid hydrolysis of *P. pinnata* seed residue was the byproduct obtained from the process which can be utilized as a boiler feed to supply heat to the process. The carbon conversion efficiency was estimated based on the amount of glucose formed from acid hydrolysis treatment using HCl (6%) carried out at 100 °C. It was observed that 0.1734 kg glucose was obtained from HCl treatment of 1 kg of seed residue (data given in Table 4.3). The percent weight of carbon in glucose is 40% (=72*100/180) and that in *P. pinnata* seed residue is 44.2% [data presented in Table 3.1]. Therefore, carbon conversion efficiency in acid hydrolysis of *P. pinnata* seed residue is 15.7% [=0.1734*0.40*100/(1*0.442)]. This signifies that 15.7% of the total carbon of *P. pinnata* seed residue was converted to glucose in the acid hydrolysis process.

The other important factor involved in acid hydrolysis of lignocellulosic biomass is the energy demand of the process in heating the process material to the desired temperature [Mafe et al., 2015]. The energy requirement of the seed residue hydrolysis was estimated in order to get an insight into the process. The net energy demand of hydrochloric acid (2-6%) assisted hydrolysis of the seed residue was 1080-1110 KJ kg⁻¹ seed residue processing. Water was the highest energy demanding stream when compared to the seed residue and acid streams.

The other well-known strategy for hydrolysis of lignocellulosic biomass is enzymatic hydrolysis using a lignocellulosic enzyme mixture. The major limiting factor in the enzymatic hydrolysis process for ethanol production is the cost of cellulase enzymes for use in the hydrolysis of the pretreated biomass [Klein-Marcuschamer et al., 2012]. To achieve cost effective biomass conversion for bioethanol production, on-site/in-house enzyme
production for the continuous supply of cellulases to the process appears to be one of the most cost effective options. Currently, a large number of lignocellulose degrading microbes are available and capable of producing an array of lignocellulases. A great deal of published material exists regarding cellulase production using different substrates by submerged or solid state fermentation using cellulolytic microorganisms (especially *Trichoderma reesei*, *Aspergillus niger* [Sukumaran et al., 2009] and *Gracibacillus* species [Yu et al., 2015]) for application in lignocellulosic bioethanol production. Despite the availability of a large number of lignocellulosic biomass degrading microorganisms, to date there is no report of lignocellulolytic microorganisms capable of efficiently converting the biomass components into its monomers [Wang et al., 2012]. Therefore, in the present investigation, the ability of two selected microorganisms, one a novel isolate, *Sphingomonas echinoides*, a bacterial strain isolated from *P. pinnata* seed residue, and *Iprex lacteus* (ATCC® 11245™) a white rot fungi, were investigated to produce crude lignocellulase enzymes during their growth on *P. pinnata* seed residue biomass, a cheap substrate as carbon and energy source. In addition the crude enzymes were employed in the hydrolysis of the seed residue. Both microorganisms showed appreciably more enzyme activities on *P. pinnata* seed residue substrate than on pure cellulose substrate. The results confirm reports in the literature which concluded that lignocellulosic biomass represents a promising substrate for enzyme preparation in solid state fermentation mode [Pandey et al., 2000 and Raimbault 1998]. The presence of cellulose, hemicellulose and lignin in the seed residue and the ability of *Sphingomonas* species [Hu et al., 2014 & Masai et al., 1999] and *I. lacteus* [Kawai et al., 1978] in utilizing these biomass components resulted in the formation of an array of enzymes including exoglucanase, endoglucanase, xylanase and laccase. It was observed that enzyme activities obtained from *I. lacteus* were greater than that from *S. echinoides*. The results confirmed that the white rot fungus has the capability of producing significantly more cellulase activity than the bacterial
isolate [Kalyani et al., 2013; Sharma et al., 2013]. Prior to the application of these crude enzyme mixtures on the seed residue, a simple pretreatment of the seed residue (5% w/v) at 121 °C for 15 min was carried out to make the biomass structure accessible to enzymes [Hendrinks and Zeeman, 2009]. Subsequent hydrolysis of the pretreated seed residue using this crude enzyme mixture from *S. echinoides* and *I. lacteus* resulted in sugar yields of 233 and 302 mg g⁻¹, respectively, at an enzyme concentration of 10 U g⁻¹. The amount of sugars formed from one gram of the seed residue from crude enzymes from *S. echinoides* and *I. lacteus* were compared with those produced using commercial cellulase from *Aspergillus niger* (10 U g⁻¹). Commercial cellulase from *A. niger* at 10 U g⁻¹ enzyme concentration resulted in 330 mg sugars g⁻¹. Overall, crude enzyme from *I. lacteus* resulted in higher values of sugar formation and these values were comparable to those obtained with the commercial cellulase (*A. niger*) preparation. Fermentation of the three hydrolysis products was carried out using *Saccharomyces cerevisiae*. During the fermentation of hydrolyzates obtained from *S. echinoides* and *I. lacteus* enzyme treatments a sugar conversion of 68% was achieved, whereas 93% conversion was observed during the fermentation of hydrolyzate from commercial cellulase (*A. niger*) treatment. This can be explained as when cellulase from *A. niger* was employed in the hydrolysis of *P. pinnata* seed residue the enzyme acts only on the cellulose portion of the seed residue resulting in the formation of hexoses which can easily be fermented by *S. cerevisiae*. In contrast in the case of hydrolyzate from *S. echinoides* and *I. lacteus* enzyme treatment the presence of both hexoses and pentoses can be expected in the products as crude enzyme preparation from *S. echinoides* and *I. lacteus* has both cellulase and xylanase activities resulting in the formation of hexose and pentose sugars. *S. cerevisiae* is highly efficient in hexose sugar fermentation, but it is unable to ferment pentose sugars. This likely explains the lower sugar conversion and ethanol yield from the fermentation of hydrolyzates from *S. echinoides* and *I. lacteus* enzyme treatments. These results suggest that
the on-site enzyme production strategy will also require a co-fermentation technology for ethanol production from both pentose and hexose sugars to improve ethanol yields [Stambuk et al., 2008]. The co-fermentation of hexose and pentose sugars is possible by applying engineered/recombinant yeast strains in bioethanol production from lignocelluloses, an area of active research at the present [Ha et al., 2011]. Ethanol yields of 81.5, 104.5 and 157.6 mg g\(^{-1}\) and ethanol concentrations of 4.0, 5.3 and 7.9 mg ml\(^{-1}\) were achieved from the fermentation of hydrolyzates from \textit{S. echinoides}, \textit{I. lacteus} and commercial cellulase (\textit{A. niger}) enzyme treatments, respectively. The study demonstrated the feasibility of using the seed residue for enzyme preparation and for application in hydrolysis of the same seed residue, and the potential of using the hydrolysis product for ethanol production.

Amount of ethanol formed (mg g\(^{-1}\) seed residue) from enzymatic hydrolysis followed by fermentation was further compared with that from acid hydrolysis followed by fermentation and it was found that the amount of ethanol obtained from enzymatic hydrolysis was 2.7 times greater than that from acid hydrolysis. Moreover, enzymatic hydrolysis employing crude lignocellulase enzymes acted on the cellulose and hemicellulose compounds of the seed residue resulting in to the formation of hexose and pentose sugars. The application of co-fermentation technology [Novy et al., 2013; Kim et al., 2012] together with the enzymatic hydrolysis can improve ethanol formation, leading to enhanced process economics.

Biomass pyrolysis is another treatment methodology employed in the present work for conversion of biomass to produce biochar, bio-oil, and gases such as carbon dioxide, carbon monoxide, hydrogen, and methane. Pyrolysis is defined as the thermal decomposition of biomass in the absence of an oxidizing agent and is a promising technology for the production of bio-oil (which can be upgraded to light hydrocarbons) and biochar (an eco-
friendly carbon rich product) from renewable feedstocks like non-edible seed cakes after the extraction of oil.

In the present work pyrolysis of the seed residue was carried out to assess the potential of the seed residue in bio-oil and biochar production. The first and important consideration for the design and development of biomass pyrolysis protocols is evaluation of thermal behavior of the feedstock biomass with the application of heat at different rates. This corresponds to the heat transfer rates to biomass particles and is used to estimate the amount of energy required for pyrolysis. Therefore, one of the objectives of this part of the work was to study the thermal behavior and pyrolysis kinetics of the deoiled *Pongamia pinnata* seed residue with different temperature programs using thermogravimetric techniques. The thermal behavior of the seed residue was studied in a thermogravimetric analyzer at different heating rates (5, 10 and 20 °C/min). The thermal degradation of deoiled *P. pinnata* seed residue under pyrolysis conditions proceeded with an initial dehydration step followed by decomposition of protein, hemicellulose, cellulose and lignin [Kok et al., 2013]. The thermogravimetric data was further evaluated using 14 different reaction models according to the Coats Redfern method and the best correlation was obtained with the third order reaction model \( f(\alpha) = (1 - \alpha)^3 \) at all the three different heating rates. The activation energy data from the Coats-Redfern method ranged from 82.95 to 98.21 kJ mol\(^{-1}\) while the activation energy calculated from isoconversional methods including the KAS and OFW methods were in the range 122.8-106 kJ mol\(^{-1}\) and 126-110 kJ mol\(^{-1}\), respectively, for conversions ranging from \( \alpha=0.2 \) to \( \alpha=0.9 \). The present work is the first report on the use of the non-isothermal methods including model fitting and model free methods to calculate the kinetic parameters for the pyrolysis of the seed residue. Ceylan et al (2014) reported activation energies for hazelnut husk pyrolysis reaction which are in the range 128-131 kJ mol\(^{-1}\) whereas Gai et al (2013) reported activation energy of 129 kJ mol\(^{-1}\) for corn cob pyrolysis reaction and 62-206
kJ mol\(^{-1}\) for pine wood waste was reported by Amutio et al (2012). The activation energy of pyrolysis of the seed residue is in the range of the hazelnut husk, corn cob but there was considerable deviation from the reported activation energy of pine wood biomass.

Further investigation on the pyrolysis of the seed residue was carried out in a fixed bed reactor of 1 cm ID and 35 cm length under slow pyrolysis conditions with a continuous supply of nitrogen gas at 0.1 LPM flow at different temperatures in the range 200-500 °C. Evaluation carried out on characteristic properties (proximate-ultimate analyses [Cantrell et al., 2010], surface structure [Rutherford et al., 2012], surface active functional groups [Rutherford et al., 2012] and higher heating value (HHV) [Channiwala et al., 2002]) of the seed residue, biochar and biooil revealed that the pyrolysis treatment improved the surface porosity of the seed residue by converting it into biochar which may have an application as an adsorbent. Moreover, improved energy density (MJ kg\(^{-1}\)) was achieved by transforming the seed residue (18.11 MJ kg\(^{-1}\)) into bio-oil (33.38 MJ kg\(^{-1}\)) and biochar (24.5 MJ kg\(^{-1}\)). The approach used in this investigation has potential application in the design and development of an efficient thermal treatment system for conversion of *Pongamia pinnata* seed residue to biochar and biooil components.

Overall, this study has shown:

- Composition estimation and evaluation of the surface properties (using SEM and FTIR) of de-oiled *Pongamia pinnata* seed residue.
- The suitability of enzymatic hydrolysis of the seed residue for increased ethanol production compared with that obtained during acid hydrolysis.
- The potential of using the seed residue as a low cost substrate for lignocellulosic enzymes preparation.
Pyrolytic conversion of the seed residue into high density energy bio-oil and biochar compounds. Evaluation of the pyrolysis reaction kinetics was found to be useful in process development.

The potential of *Pongamia pinnta* seed residue as a feedstock for biofuels (bioethanol, biochar and biooil) production based on different treatment methodologies.

### 7.2 FUTURE RESEARCH

Although the work presented in the present investigation addresses some features of deoiled *Pongamia pinnata* seed residue conversion to ethanol (hydrolysis followed by fermentation), bio-oil and biochar (pyrolysis), there is still a need for further investigation of integrated biofuels production which will significantly improve the economics of *Pongamia pinnata* seed biorefinery for biofuels and biomaterials production.

Further studies should be performed:

- To integrate pyrolysis with the seed residue conversion to ethanol process by utilising the solid residue from the hydrolysis process as feedstock [Girisuta et al., 2012]. The solid residue from hydrolysis represents a lignin rich material [Huang et al., 2012] and the effective utilization of this residue could help in minimizing the cost of ethanol production.

- To understand the mechanism of pretreatment and enzymatic hydrolysis of the seed residue at high solids loading [Modenbach et al., 2013]. For example, the effect of high solid loading (10-30 % w/w) on pretreatment efficiency and enzymatic hydrolysis of the pretreated seed residue could be carried out to obtain improved sugar and ethanol concentrations [Ramos et al., 2015].
studies will also address the limitations of the hydrolysis process at high solids loading such as insufficient mixing and mass and heat transfer in the hydrolysis reactor.

To search for a suitable fermentative microorganism and its subsequent assessment for application in the co-fermentation of mixed sugars [Kim et al., 2012] obtained from the enzymatic hydrolysis of the seed residue.

To assess the application of biochar generated from the pyrolysis of the seed residue as a precursor for the development of carbon based materials such as activated carbon for application in color and odour removal from industrial effluents and also in wastewater treatment [Huggins et al, 2016 & Rajapaksha et al., 2015]. The surface properties (surface area and pore volume) of the biochar can be improved by steam activation or chemical activation [Nielsen et al., 2014 & Zhu et al., 2016]. Further studies may be required to design and optimize activated biochar preparation by optimizing the main process parameters such as temperature, time, activation agent and its concentration.
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Appendix A

HPLC analysis of a synthetic water-glucose mixture
Appendix B

HPLC analysis of the product of acid hydrolysis of *P. pinnata* seed residue
Appendix C

Enzyme activity (U ml\(^{-1}\) min\(^{-1}\)) data from submerged and solid state fermentations of \textit{I. lacteus} and \textit{S. echinoides} grown in the presence of cellulose and \textit{P. pinnata}

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SBF: Submerged fermentation using \textit{P. pinnata-I. lacteus}
SBB: Submerged fermentation using \textit{P. pinnata-S. echinoides}
SCF: Submerged fermentation using cellulose-\textit{I. lacteus}
SCB: Submerged fermentation using cellulose-\textit{S. echinoides}
PIL: Solid state fermentation using \textit{P. pinnata-I. lacteus}
PSE: Solid state fermentation using \textit{P. pinnata-S. echinoides}
CIL: Solid state fermentation using cellulose-\textit{I. lacteus}
CSE: Solid state fermentation using cellulose-\textit{S. echinoides}
Appendix D

Validation of the pyrolysis reaction kinetic model using the Coats-Redfern method