ETHANOL PRODUCTION FROM BIO-OIL

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF APPLIED SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Chemical and Biological Engineering)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

APRIL 2009

Abstract

Bioethanol is often viewed as one of the solutions to the tight gasoline supplies in North America. Although bioethanol is already available in the market, there are a number of problems associated with the current processes for the production of bioethanol. The current bioethanol production processes are often referred as first generation bioethanol production processes. For these first generation processes, the feedstocks for production are usually energy crops. The most common energy crops in North America are corn and wheat. The use of these energy crops has triggered debates on the problems associated with using food sources to create energy and the uptake of agricultural land to produce energy. In this project, an alternative feedstock for bioethanol is investigated. The feedstock used in the project is bio-oil, which can be derived from any biomass waste. An advantage of using bio-oil is that it is not derived from food crops but instead waste material is being converted into energy.

The objective of this study was to determine the technical viability of producing bio-ethanol using bio-oil as a substrate for fermentation. In order to maximize the ethanol yield, the extraction of levoglucosan with water was optimized and a number of detoxification techniques for inhibitor removal were evaluated. This report provides a technical overview of conditions evaluated for extracting levoglucosan from bio-oil, and methods used for improving the fermentability of bio-oil hydrolysate by detoxification. The techniques used in an attempt to improve the fermentability of bio-oil hydrolysate include: adsorption, overliming, solvent extraction, and hydrogenation. In addition, a biological approach called adaptive evolution was used to aid the yeast to adapt to the inhibitory environment of bio-oil hydrolysate in order to increase their resistance to inhibitors.

The optimal condition for aqueous extraction of levoglucosan from bio-oil was found to be 1:1 (mass water to mass bio-oil). It was found that the temperatures examined (25°C and 80°C) had minimal effect on the amount of levoglucosan extracted. Among the

detoxification techniques tested, it was found that overliming and solvent extraction were able to improve the fermentability of bio-oil hydrolysates. Overliming was able to increase the yield of ethanol from bio-oil hydrolysate by 0.19±0.01 (g ethanol/g glucose) at 50% strength hydrolysate and 0.45±0.05 (g ethanol/g glucose) at 40% strength hydrolysate. A number of extractants were examined and the three best solvents were 25% volume of tri-n-octylamine with co-solvent 1-octanol, 50% volume of alamine 336 with co-solvent 1-octanol and oleyl alcohol. These three solvents were able to selectively remove at least 84 – 93% of acetic acid, which was the targeted inhibitor in bio-oil hydrolysate. In addition, a technique called adaptive evolution of yeasts was applied, which was capable of increasing the ethanol yield by at least 6% when compared with the unadapted parental strains.

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Acknowledgements

This research project was funded by Dr. Sheldon Duff of the Department of Chemical and Biological Engineering at the University of British Columbia. I would like to acknowledge and express my gratitude to Dr. Duff for his support, guidance and contribution during the course of this research project. I would like to extend my gratitude to all of my friends and all my peers in the Biofuels Research Lab and all the staff members in the department for their support. I would also like to thank my thesis committee member Dr. Sheldon Duff, Dr. Kevin Smith and Dr. Naoko Ellis for their feedback on my thesis. A special thank to Dr. Kevin Smith and Dr. Yun Quan Yang, for their help on the catalytic reactor. Finally, I would like to thank my family and Clara Leung for their love and support.

Chapter 1 Introduction

1.1 Background

The purpose of this research is to produce ethanol in an economically and environmentally sustainable way, to relieve the dependency on petroleum fuel in North America. As an alternative source of energy, ethanol can be used as a fuel additive in gasoline and as fuel in vehicles [1]. The addition of ethanol to gasoline can reduce emissions of greenhouse gases, and reduce the consumption of gasoline. These benefits have led to an increasing demand for ethanol [2]. According to the Renewable Fuels Association (RFA) of Canada, the production of ethanol in the past 5 years has nearly tripled, and it is predicted that the production will continue to grow [3].

It is widely known that ethanol fuel can be produced by energy crops such as corn, wheat and sugar cane. While it is a cleaner alternative than petroleum, it poses many problems, such as: the negative net energy balance for the production of ethanol, the uptake of agricultural lands and the increasing demand and price of the energy crops [1]. In this project, the production of ethanol from bio-oil is being investigated. Bio-oil is a product of processing biomass in a process called fast pyrolysis. The biomass used to produce bio-oil can be any biomass wastes such as wood wastes or agricultural wastes [4]. Wood wastes like sawdust from pulp mills or beetle-killed lodgepole pine can be used to produce bio-oil in British Columbia [3]. This option has an additional benefit of reducing the forest fire hazard [3].

The feasibility of producing ethanol from bio-oil has been proven by previous researchers here at UBC and other places in the world who worked on similar projects [5, 6, 7]. The production of ethanol from bio-oil involves three stages: extraction, hydrolysis and fermentation. In the first stage, the anhydrosugars, mainly levoglucosan, are extracted using water. This process takes advantage of the hydrophilic nature of levoglucosan. In addition, water is a clean and inexpensive solvent, which reduces the risk associated with the use of organic solvents. In the second stage, the extraction product is hydrolyzed into

fermentable sugars using dilute sulfuric acid. At this stage, the yield of the hydrolysate from levoglucosan will exceed 100% due to the hydrolysis of other anhydrosugars in the bio-oil extract [6]. This will improve the fermentation by providing more free sugars to ferment into ethanol, thus increasing the production [6]. In the final stage of the process, the hydrolysate is fermented into ethanol using yeast, specifically *Saccharomyces cervisiae* strain T2. This yeast is used in the fermentation process due to its effectiveness and resistance to inhibitors.

1.2 Ethanol

Ethanol is a volatile, flammable, and a colorless liquid at room temperature. It has a strong alcohol smell and burns with a smokeless blue flame. Ethanol is also a versatile solvent, miscible with water and with many organic solvents. The discovery of ethanol can be dated back to the 15th century [1]. Ethanol has a long history as a source of energy. In the early 1800s, ethanol was the major illuminating fuel for lamps in the United States [1]. In the modern world, ethanol is used commonly as motor fuel and fuel additive.

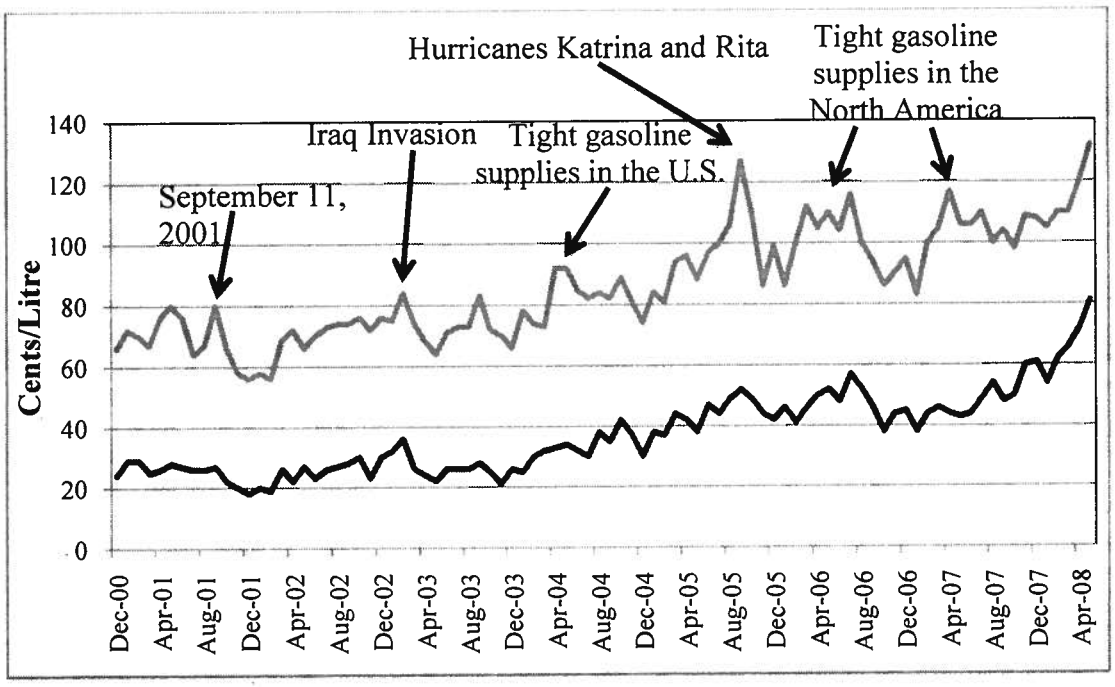

Figure 1 A timeline for gasoline price from 2000 – 2008, gasoline price (light colored line), crude oil price (dark colored line) [2]

Gasoline has dominated the fuel market for over a century. Since 2001, the retail price of gasoline has increased dramatically due to the fear of tight gasoline supply in the United States [2]. From January 2001 to May 2008, the retail price of gasoline has tripled [2]. There are a number of explanations for the increase in the price of gasoline, but the major reason is the dependency on gasoline as transportational fuel. Thus, any events that could potentially upset the supply of gasoline can drive the gasoline price up. Figure 1 has recorded the timeline of gasoline price and events in the Unites States that led to the increase in the retail price of gasoline. The jump in the gasoline price, and an increase in the environmental awareness, have sparked opportunities for alternative energy sources.

Some of the vehicles that use alternative energy sources are hydrogen vehicle, biodiesel vehicle, fuel cell vehicle, and electric/gasoline hybrid vehicle. While ethanol can also be an alternative energy source for vehicles, its major use is as a fuel additive. In the past decade, ethanol has gained importance as a fuel additive in the United States. A graph of ethanol produced in the United States from 1990 to 2008 is shown in Figure 2.

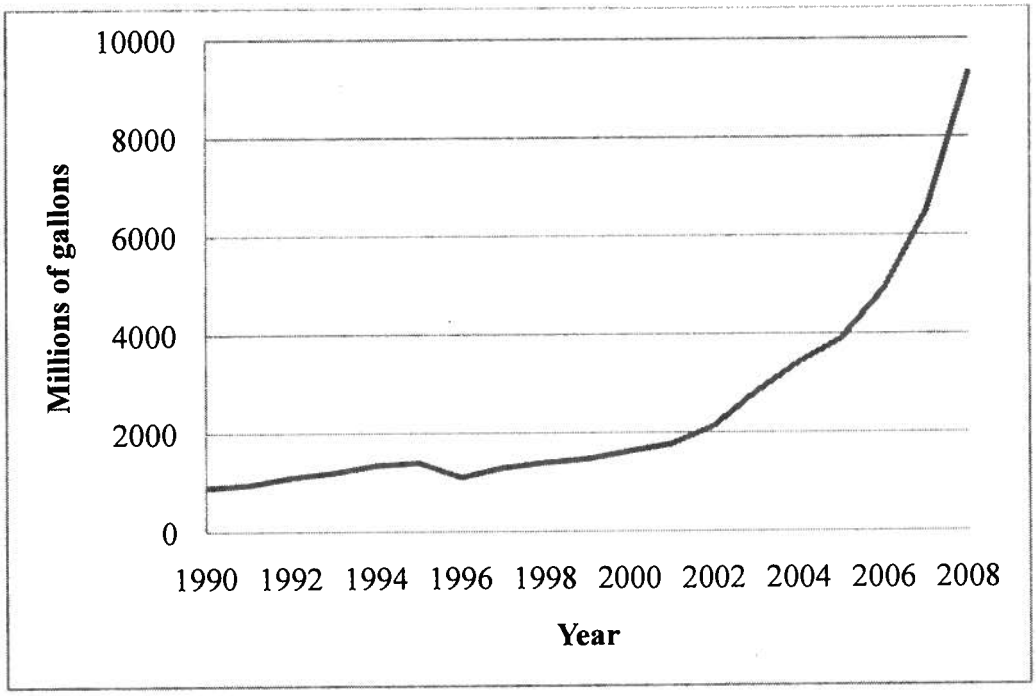

Figure 2 Historic U.S. fuel ethanol production (1990-2008) [3]

As an oxygenated fuel additive, ethanol can reduce the emission of pollutants, including particulates, and reduce the consumption of gasoline. The reduced harmful pollutants include carbon monoxide, carbon dioxide, formaldehyde and other hydrocarbons [8]. In addition, the higher octane number of ethanol (~110) increases the octane number of the mixture, thus reducing the need for toxic, octane-enhancing additives [8]. These benefits have led to the increasing demand for ethanol. The Energy Information Association (EIA) of the Unites States estimated that the gasoline market in the United State will grow from 142 billion gallons per year in 2007 to 149 billion gallons of gasoline by 2015 [1]. With this in mind, a ten percent additive in the gasoline would require 14.2 billion gallons of ethanol per year in 2007 to 14.9 billion gallons per year by 2015 [1]. The estimate was based on ten percent because most cars can be run on a mixture of 90% gasoline and 10% ethanol without adjusting their engines [9]. Newer model cars can even use mixtures of gasoline and ethanol that contain up to 20% ethanol. Furthermore, there are new engines that are designed to run on pure ethanol [8]. The new flexible fuel vehicles take advantage of the capabilities of this technology and are able to use mixtures of 0 – 85% ethanol in gasoline. This is commonly known as the E85 vehicles [9]. Ethanol can also be mixed with diesel fuel in compression ignition engines with the help of an emulsifier [9].

Canadian production of ethanol has increased dramatically in recent years as well. In 2003, ethanol production was only 212 million litres per year. By the end of 2007, production had risen to approximately a billion litres per year. Currently there are four plants under construction and are projected to be commissioned in 2009 [10]. Table 1 shows a list of all the major bioethanol producers in Canada and their production capacity [10]. When the plants are in operation, the production of ethanol in Canada is expected to reach over a billion and a half litres of ethanol (Table 1) [8, 10]. With the growing awareness of environmental issues, ethanol is attracting a lot of interest in the international and domestic market due to the environmental benefits associated with its use [11]. In addition, Canada has introduced a five percent ethanol mandate by 2010, which requires 5% ethanol to be blended in gasoline. This mandate will provide further incentive to the production of ethanol in Canada.

Table 1 Ethanol producers in Canada [10]

Ethanol producers	City	Province	Feedstock	Capacity (million litre per year)	Start Date
GreenField Ethanol	Tiverton	ON	Corn	26	1989
Pound Maker Agventures Ltd.	Laniga	SK	Wheat	12	1991
Permolex International, L.P	Red Deer	AB	Wheat	40	1996
GreenField Ethanol	Chatham	ON	Corn	150	1996
Iogen Corporation	Ottawa	ON	Straw from wheat, barley and oats	2	2004
NorAmera BioEnergy Corp.	Weybury	SK	Wheat	25	2005
Enerkem Inc.	Westbury	QC	Wood waste	5	2005
Suncor Energy	Sarnia	ON	Corn	200	2006
Husky Energy	Lloydminster	SK	Wheat	130	2006
Collingwood Ethanol LP	Collingwood	ON	Corn	50	2007
Husky Energy	Minnedosa	MB	Wheat and corn	130	2007
GreenField Ethanol	Varennes	QC	Corn	120	2007
GreenField Ethanol	Johnstown	ON	Corn	200	2008
IGPC Ethanol Inc.	Aylmer	ON	Corn	150	2008
Terra Grain Fuels Inc.	Belle Plaine	SK	Wheat	150	2008
GreenField Ethanol - Enerkem Inc.	Edmonton	AB	Municipal landfill waste	36	Under construction
North West Bio-Energy	Unity	SK	Wheat	25	Under construction
GreenField Ethanol	Hensall	ON	Corn	200	Under construction
Kawartha Ethanol	Havelock	ON	Corn	80	Under construction
Total				**1731**	

1.2.1 Overview of ethanol production

A number of different methods can be used to produce ethanol. The most common ones are hydration of ethylene and fermentation of sugars. Ethanol that is used as industrial feedstock is most often made from petrochemical feed stocks, typically by the acid-catalyzed hydration of ethylene:

$$C_2H_4 + H_2O \rightarrow CH_3CH_2OH$$

The most commonly-used catalyst is phosphoric acid, adsorbed onto a porous support such as diatomaceous earth or charcoal [12]. The reaction is carried out with high pressure steam at 300°C. In an earlier process, ethylene was hydrated indirectly by reacting with concentrated sulfuric acid to produce ethyl sulfate, which was then hydrolyzed to yield ethanol and sulfuric acid [13, 14]:

$$C_2H_4 + H_2SO_4 \rightarrow CH_3CH_2SO_4H$$
$$CH_3CH_2SO_4H + H_2O \rightarrow CH_3CH_2OH + H_2SO_4$$

Ethanol produced for alcoholic beverages and fuel, is mostly produced by fermentation. Yeast such as *Saccharomyces cerevisiae* can metabolize sugar in an oxygen limiting environment to produce ethanol and carbon dioxide [15]. The chemical equation below summarizes ethanol fermentation, in which one hexose molecule is converted into two ethanol molecules and two carbon dioxide molecules:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Yeast will perform the reactions only if oxygen is excluded or limited from the environment. Otherwise, yeast will oxidize pyruvate completely to carbon dioxide and water [15].

1.2.2 Substrates for bioethanol

In the past several decades, a number of feedstocks have been studied as potential substrates for the fermentation of ethanol [5, 16, 17]. In most bioethanol plants, the feedstock for production is an energy crop [3, 11]. The most common energy crops in North America are corn and wheat [3]. The use of energy crops to produce ethanol has received a lot of attention. This is because there are debates about whether the net energy return from these energy crops is positive. In light of this, the U.S. Department of Energy (DOE) has studied the energy return from the energy crops and reported a negative energy return regarding ethanol production using corn [17]. Several other studies have also suggested that ethanol production from corn does not provide a positive energy return and its production process contributes to global warming [18, 19]. Furthermore, the increased demand for corn to produce ethanol drives corn prices up, which can translate into higher meat, milk and egg prices for consumers [18]. Despite the studies, producing ethanol from corn is a mature technology and, with the help of government subsidies, companies are currently employing this method to produce ethanol.

However, the drawbacks of the energy crops also inspired the study of alternative feedstocks for bioethanol production. Potentially more attractive options are lignocellulosic waste materials that contain sugars [4]. Ethanol produced from biomass wastes is often referred as second generation bio-fuel, with the first generation of bio-fuels being that produced from energy crops.

1.2.3 Pyrolysis and other substrate pretreatments methods

In order to produce ethanol from lignocellulosic materials, single or multistep processes must be carried out to produce fermentable sugars. These processes are used for the removal of lignin, which allows easier access to the cellulose and hemicellulose in enzymatic or acid hydrolysis process. This helps to increase the sugar yield in the subsequent hydrolysis step. A number of pretreatment methods have been proposed for

the removal of lignin. They can be categorized into chemical, thermochemical and biological pretreatments or a combination of these pretreatment processes. Prior to the pretreatment methods, lignocellulosic materials are often pulverized by a combination of chipping, grinding, and milling [20]. This is to reduce the physical size and to reduce cellulose crystallinity of the lignocellulosic materials [20].

The chemical pretreatments include ozonolysis and the organosolv process. Ozonolysis is the addition of ozone to degrade lignin while keeping the degradation of cellulose to a minimum [20]. This process has the advantage that it does not produce additional toxic chemicals, but the process requires the use of ozone which makes the process more expensive [20]. In the organosolv process, organic solvents such as methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tertrahydrofurfurl alcohol are used to break the internal bonds of lignin and hemicellulose [21, 22]. This process can be catalyzed by the addition of organic acids such as oxalic or salicylic acid [20]. The recovery and removal of the solvents is essential to reduce the operating cost and minimize the inhibition to the organisms in the enzymatic hydrolysis and fermentation process [20].

Steam explosion is one of the most common thermochemical pretreatments of lignocellulosic materials [20]. In steam explosion, the materials are treated with high pressure saturated steam and then the pressure is swiftly reduced to cause an explosive decompression [20, 23, 24]. This process has been proven to be a good pretreatment for enzymatic hydrolysis of hardwoods. Ninety percent efficiency of enzymatic hydrolysis had been achieved in 24 hours for poplar chips that were treated by steam explosion, compared with only 15% efficiency with untreated poplar chips [25, 26].

Oxygen delignification and ammonia fibre explosion (AFEX) are examples of a combination of chemical and thermochemical processes. The AFEX process is similar to steam explosion. The lignocellulosic materials are exposed to ammonia at high temperature and the pressure is quickly reduced to cause an explosive decompression [20]. Oxygen delignification is a process that is widely used in the pulping industry. In

oxygen delignification, the materials are treated with high pressure oxygen at 100 – 150°C under alkaline conditions [27]. This triggers a series of reactions that lead to the degradation of lignin [27]. However, this process is reported to be ineffective for biomass with high lignin content (>18%) [20].

Biological pretreatments are not as commonly used as the thermochemical and chemical methods due to the long residence time and low efficiency [28]. However, it has some advantages over the other pretreatment methods, such as the low energy requirement for the process and the mild reaction conditions. These advantages attract interest from researchers trying to improve the process and overcome its weakness. The white rot fungi are used for the biological pretreatment because they are capable of selectively degrading lignin. The most common fungi used to remove lignin from plant cell walls are white rot fungi [28]. A study by Fackler et al. has reported a 1.7 - 3.2% lignin removal in 10 weeks on spruce wood veneer with various white rot fungi strains [28].

Among these pretreatment methods, pyrolysis is selected because the technology is mature and it can be applied to most lignocellulosic waste without any adjustment to the process [29]. In this project, the lignocellulosic material (pine) was pyrolyzed using a process called fast pyrolysis to produce a liquid called bio-oil. In fast pyrolysis, the biomass wastes are treated at high temperature (>300°C), resulting in a rapid decomposition of biomass into liquid, char and gas [4]. The pyrolysis liquid will contain most of the sugars (in a dehydrated or "anhydro" form) from the raw material and can be used for ethanol fermentation. The actual sugar content will depend on the raw material used in the pyrolysis process, as well as the processing conditions. These anhydrosugars can easily be hydrolyzed to produce fermentable sugars.

1.3 Characteristics of bio–oil

Bio-oil is one of the products of processing biomass using the process called pyrolysis. Hence, bio-oil is also called pyrolysis oil. Pyrolysis is a process of thermal decomposition of biomass occurring in the absence of oxygen. It is also the first step in combustion and gasification processes where it is followed by total or partial oxidation of the primary products [4, 30]. The process of pyrolysis can vary, but generally it can be categorized into three groups – fast pyrolysis, carbonization and gasification. The main variables for the pyrolysis processes are reaction temperature and residence time. Table 2 summarizes the different conditions for the pyrolysis processes and their resultant product distribution in liquid, char and gas. Fast pyrolysis for liquid bio-oil production is of particular interest because it can produce up to 75% of the original mass of wood as liquid bio-oil [4]. The remaining fractions are 12% char and 13% gas [4]. The char and gas can be recycled in the fast pyrolysis process to produce heat, thus making the process more energy efficient.

Table 2 Typical product yields (dry wood basis) for pyrolysis [4]

Process	Conditions	Liquid	Char	Gas
Fast pyrolysis	Moderate temperature, short residence time	75%	12%	13%
Carbonisation	Low temperature, very long residence time	30%	35%	35%
Gasification	High temperature, long residence time	5%	10%	85%

There are many potential applications for bio-oil, such as transportation fuel, and fuel for boilers, furnaces, and turbines [4]. In this project, one fraction of bio-oil is used to produce ethanol through a series of process such as extraction, hydrolysis, detoxification and fermentation. Several researchers have reported their work on similar project. Yu and Zhang [7] have reported that they were able to hydrolyze cotton pyrolysate, and after subsequent detoxification, obtained 14.2 g/L of ethanol from 31.6 g/L of glucose after 24 hours of fermentation [7]. Another study by Bennett [6] has reported that by diluting the bio-oil hydrolysate (2% v/v), an ethanol yield of 51% (g ethanol/g glucose) was found [6]. Figure 3 summarizes the different applications for bio-oil.

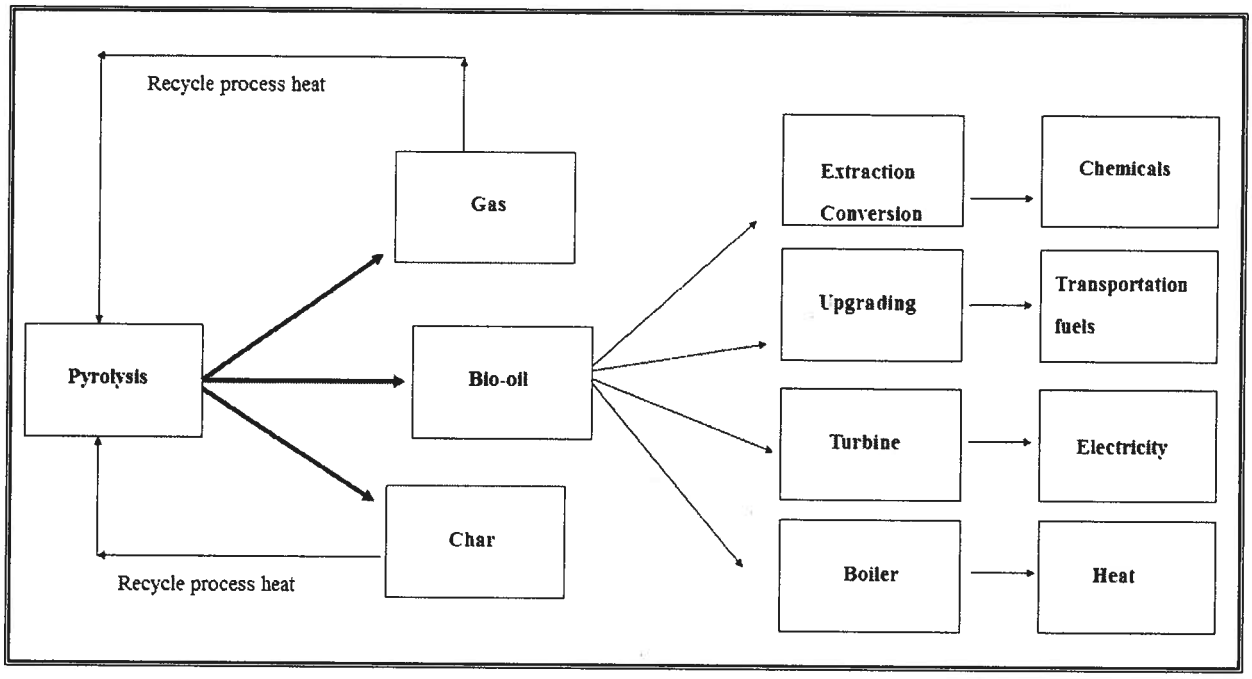

Figure 3 Summary of bio-oil applications [10]

1.3.1 Compositions of bio-oil

Bio-oil is usually dark brown with a distinctive smoke-like smell. The elemental composition of bio-oil is similar to that of the biomass before pyrolysis [30]. Bio-oil contains a complex mixture of acetic acid, formic acid, methanol, formaldehyde, aldehydes, ketones, phenols, hydroxymethyl, furans, furanones, pyranones, anhydrosugars, oligosaccharides, lignin and water (Table 19) [30, 31]. Table 3 shows the different groups of compounds that are found in bio-oil [30]. Some suspended solids may also be present in the bio-oil.

Table 3 Typical compounds in pyrolysis oil [30]

Compounds	Composition range (wt% of bio-oil)	Hydrophilicity (arbitrary scale, 4 is highest)
C1 compounds (formic acid, methanol and formaldehyde, CO_2)	5-10	4
C2 - C4 compounds (linear hydroxyl- and oxo-substituted aldehydes and ketones)	15-35	4
C5 - C6 compounds (hydroxyl-, hydroxymethyl- and/or oxo-substituted furans, furanones and pyranones)	10-20	3
C6 compounds (anhydrosugars, anhydro oligosaccharides)	5-30	4
Water-soluble carbohydrate derived oligomeric and polymeric materials	5-10	4
Monomeric methoxyl substituted phenols	6-15	2
Pyrolytic lignin	15-30	1

1.3.2 Phase behavior and stability of bio-oil

Typically bio-oil contains around 15 – 30 wt% of water depending on how it was produced and collected [4, 30, 32]. The water molecules are chemically dissolved in bio-oil. In this case, the bio-oil is in single phase due to the presence of polar carboxyl and hydroxyl compounds [30]. However, phase separation can occur with an increase in the concentration of water or lignin-derived material to a critical level [4, 7, 33]. Thus, phase separation can be induced when a critical concentration of water is added, usually 10 – 25 wt% [33]. The typical composition of bio-oil is provided in Table 4.

Table 4 Typical composition of bio-oil [30]

Component	Weight percentage (wt%)
Water	21.1
Solids	0.008
Ash	0.030
Nitrogen	0.060
Carbon	43.5
Hydrogen	7.09
Oxygen	28.2

It has been reported that bio-oil is not a product of thermodynamic equilibrium [4, 30]. Instead, it is produced with short residence time in the reactor and rapid quenching from the pyrolysis temperature. This produces liquid bio-oil that is not at thermodynamic equilibrium under normal storage conditions. Thus, the chemical composition of the bio-oil will tend to change towards thermodynamic equilibrium during storage. This results in changes in viscosity, molecular weight, chemistry and co-solubility of its many compounds [31].

The aging of the bio-oil can be monitored by its viscosity [31]. The change in viscosity is caused by the loss in volatile components in bio-oil and/or the changing of the chemical composition of bio-oil as it moves toward thermodynamic equilibrium [31]. The relationship between the change in viscosity and molecular weight of bio-oil due to aging is shown on Figure 4 [31]. According to Figure 5, the viscosity of the bio-oil used for this project had increased by 182.5 cp over the past 5 years at 4°C [30].

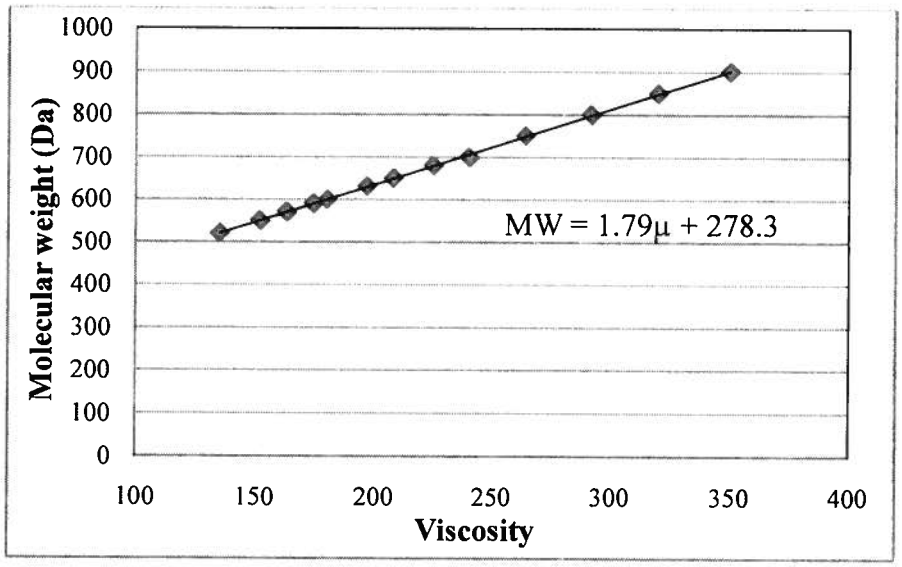

Figure 4 Change in viscosity and molecular weight of bio-oil due to aging at 40°C [31]

It is reported that the rate of viscosity change is slower at lower temperatures. This has been confirmed by a number of researchers [30, 34, 35, 36]. A graph summarizing different results from four different researchers is shown in Figure 5.

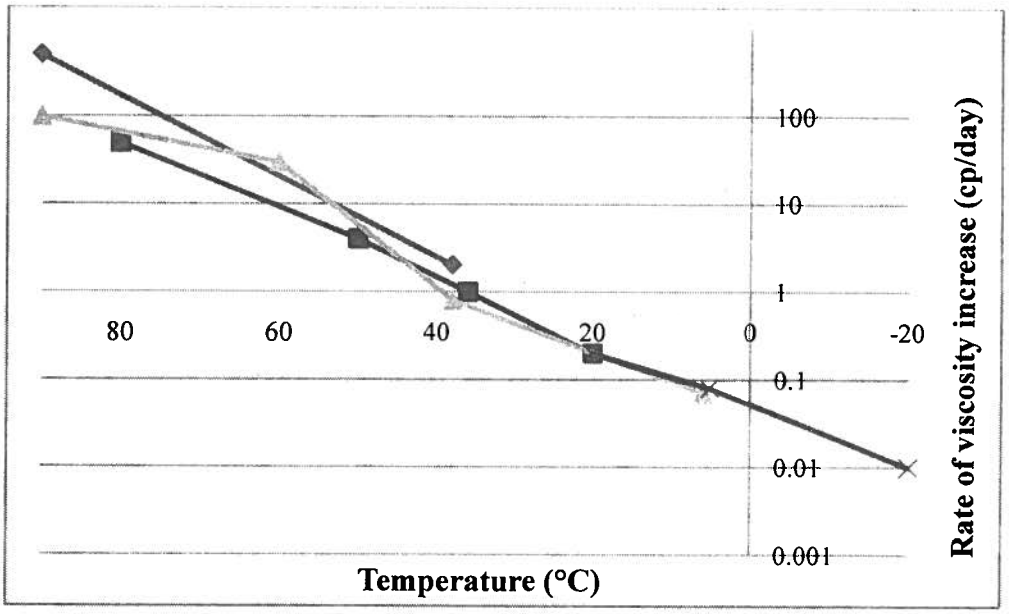

Figure 5 Rate of viscosity change at different temperatures, Czernik et al. [35] (diamond), Oasmaa and Sipila [34] (square), Diebold and Czernik [36] (triangle), Oasmaa et al. [30] (cross)

1.3.3 Anhydrosugars in bio-oil

As can be seen in Table 3, bio-oil is comprised of a significant fraction of anhydrosugars. Levoglucosan (1, 6–anhydro–β–D–glucopyranose, Figure 6) is a major component in bio-oil and the compound of interest in the project. There are a number of studies on the levoglucosan yield from pyrolysis oil. Typically, wood derived bio-oil contains about 5 – 30% of levoglucosan [4, 30]. In another study, Li and Zhang [5] has reported levoglucosan yield of 18.66% from the pyrolysis of newspaper and 33.06% from the pyrolysis of waste cotton [5]. Generally, the amount of levoglucosan depends on the types of feedstock, the pretreatment of feedstock and the pyrolysis process conditions [30]. As an anhydrosugar, levoglucosan can be hydrolyzed into glucose by acid hydrolysis, which can then be fermented into ethanol [5, 7, 37]. Thus, bio-oil provides a potentially rapid and efficient route to the supply of substrate for bioethanol production [5, 7]. Other uses for levoglucosan include the synthesis of oligosaccharides and pharmaceuticals materials [5].

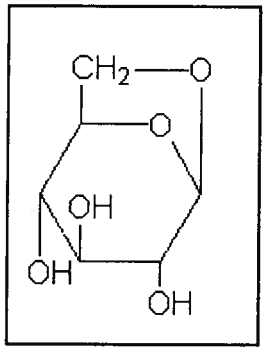

Figure 6 Structure of levoglucosan

Cellobiosan, another anhydrosugar produced during wood pyrolysis can also be hydrolyzed to glucose by acid hydrolysis [38]. However, unlike levoglucosan hydrolysis, cellobiosan is first hydrolyzed into levoglucosan and cellobiose. Then, levoglucosan and cellobiose are further hydrolyzed to glucose [38]. The exact concentration of cellobiosan in wood derived bio-oil is uncertain, but it has been reported by Helle et al. [38] that cellobiosan yield can be as high as 30% of levoglucosan yield (~1.5 – 9% cellobiosan yield) at a pyrolysis temperature of 500°C [38].

1.3.4 Extraction of levoglucosan from bio-oil

In order to utilize the levoglucosan for fermentation, solvents are required to extract it out of the bio-oil via liquid–liquid extraction. Water is selected as the solvent for the extraction because levoglucosan is hydrophilic. There are other advantages in using water as a solvent, such as availability and cost. However, other compounds with similar hydrophilicity are also extracted along with levoglucosan from the bio-oil. These compounds are listed in Table 3 [30].

A phase diagram for bio-oil and water system is given in Figure 7. The phase diagram correlates the weight percentage of water and organic compounds. In the diagram, the linear segment represents the bio-oil as a single phase liquid. Once the water content has exceeded 25 wt%, phase separation can occur for this particular bio-oil [33]. However, this phase diagram exhibits only the general trend. Other factors, such as the age and the raw materials from which the bio-oil was made have a significant effect on the water content and the organic content in the bio-oil [4]. This ultimately affects the critical amount of water required to induce phase separation.

After phase separation, the top phase is often referred as the aqueous phase because it is a homogenous solution that behaves like water. The aqueous phase contains most of the levoglucosan and other carbohydrate-derived compounds [30]. The bottom phase is often called the organic phase. In the organic phase, most of the lignin-derived compounds in the bio-oil are retained [30]. The organic phase is darker in color, behaves like a single phase and flows like a viscous fluid.

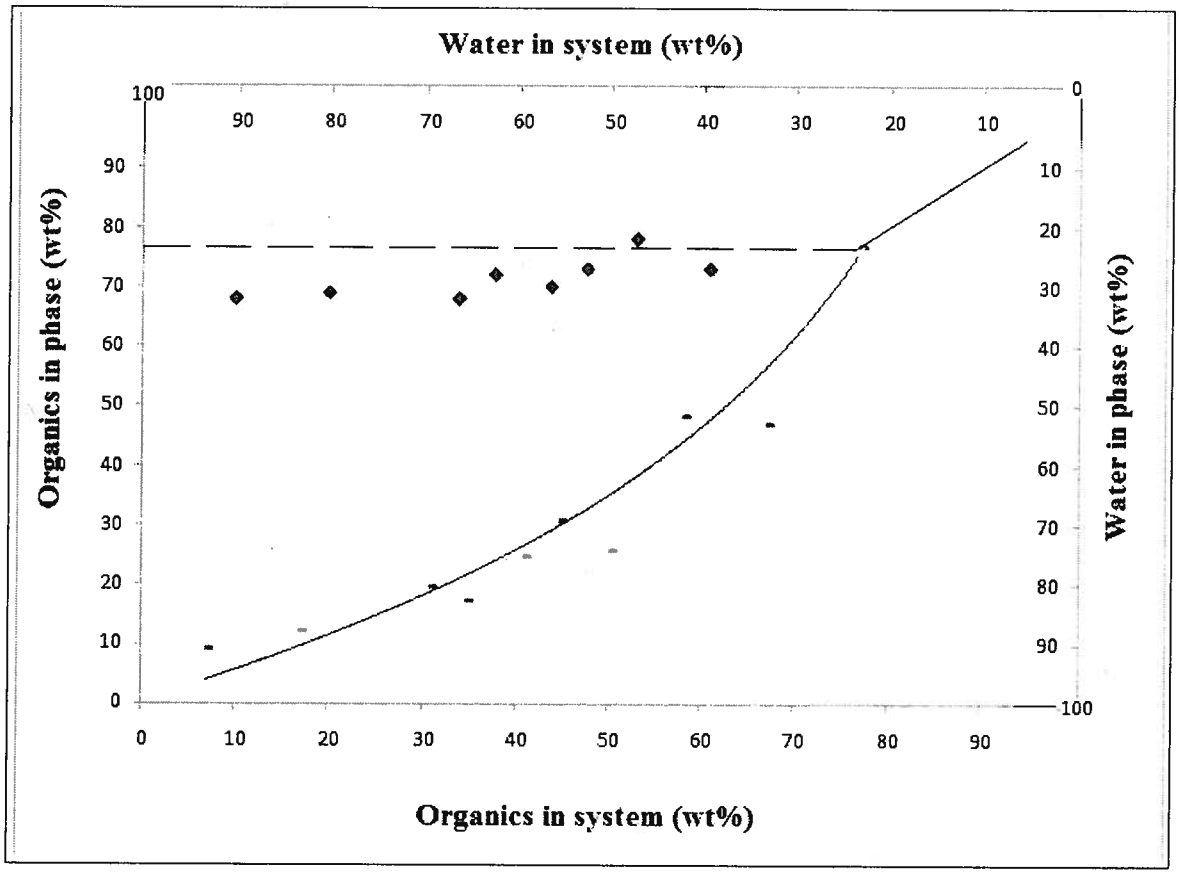

Figure 7 Phase diagram of bio-oil and water system at room temperature [33]

The extracted levoglucosan can then be hydrolyzed into fermentable sugar. The sugar solution after hydrolysis, called hydrolysate, can be used subsequently for fermentation with *Saccharomyces cervisiae* to produce ethanol. Since typical concentrations of levoglucosan range from 5 – 30% in the bio-oil, producing ethanol from bio-oil is not economically feasible [4, 30]. In order to increase the economic feasibility, producing ethanol with bio-oil in an integrated pyrolysis combined cycle (IPCC) power plant has been proposed and studied by Sandvig et al. [39]. The concept of IPCC power plant is that the bio-oil serves both as a fuel for a gas turbine cycle and as a feedstock for value-added chemicals production [39]. This increases the efficiency and economic feasibility of using pyrolysis oil due to the co-generation of products and recycling of process and pyrolysis heat [6, 39].

1.3.5 Hydrolysis of levoglucosan and other anhydrosugars

Hydrolysis is a chemical process in which a molecule is cleaved into two parts by the addition of a molecule of water. One of the molecules gains a hydrogen ion (H^+) from the water molecule. The other part of the molecules collects the remaining hydroxyl group (OH^-). In bioethanol production, the hydrolysis step is performed to break down complex sugars, hemicellulose and cellulose or in this case levoglucosan, to monomer sugars like glucose. This hydrolysis step can be performed with different methods such as using concentrated acid, dilute acid or enzymes. Each method has its advantages and disadvantages. For instance, concentrated acid hydrolysis will introduce more inhibitory compounds in the system than the other dilute acid and enzymatic hydrolysis, while enzymatic hydrolysis is more costly than acid hydrolysis [6, 40, 41, 42, 43].

In this project, sulfuric acid was used for hydrolysis because it had demonstrated to be effective and inexpensive [6]. Several researchers had reported that a glucose yield of greater than 100% was observed using dilute acid hydrolysis to hydrolyze bio-oil extract [6, 7, 38]. Helle et al. [38] had reported that the extra glucose came from the hydrolysis of cellobiosan in bio-oil [38]. The intermediate products of cellobiosan hydrolysis were cellobiose and levoglucosan [38]. These two anhydrosugars were then further reacted into glucose [38]. Thus, dilute acid hydrolysis of bio-oil extract would often result in greater than 100% theoretical glucose yield, when the calculation was based on initial concentration of levoglucosan [38].

1.4 Yeasts

Yeasts are unicellular microorganisms classified in the kingdom fungi, which are common in the natural environment. They can be found in terrestrial, aerial and aquatic environments, where the success of their colonization is closely related to their adaptability to highly variable environments [44]. Yeasts can be classified with respect to the types of energy-generating process involved in sugar metabolism, namely facultative-

fermentative, non-facultative-fermentative and obligate-fermentative yeasts [44]. The non-facultative fermentative yeasts are only capable of the respiratory metabolism and are not capable of alcoholic fermentation from glucose. An example of this type of yeast is *Rhodotorula glutinis* [44]. On the other hand, the obligate fermentative yeasts are only capable of metabolizing glucose through alcoholic fermentation. An example of this yeast is *Kazachstania slooffiae* [44]. Most of the identified yeasts are facultative fermentative. These yeasts may display either a fully respiratory or a fermentative metabolism or even both in a mixed respiratory-fermentative metabolism [44]. The type of metabolism the facultative fermentative yeasts experience depends on the growth conditions, such as the types of sugars present, the concentration of sugars and the availability of oxygen [44]. *S. cerevisiae,* the yeast used in the project, is an example of facultative fermentative yeast.

The most common microorganisms that are suitable for our application are *Z. mobilis* and *S. cerevisiae* [45]. However, many other microorganisms are also capable of fermenting sugars into ethanol [45], such as engineered *Escherichia coli* [46], *Monilia sp.* [47], *Paecilomyces sp.* [48], *Clostridium thermocellum* [49] and *Neurospora crassa* [47]. The latter four microbes are used in single stage cellulose-to-ethanol biotransformation. In this biotransformation, these microbes ferment lignocellulosic waste directly to ethanol without any pretreatment or preparation stages. However, studies on the fermentation process that utilizes these microorganisms have shown that the processes are slow and the ethanol yields are low [45]. These cellulose-to-bioethanol biotransformation processes have been found to take up to 12 days to complete [45]. Another disadvantage is that these microorganisms have lower resistance to high concentrations of ethyl alcohol than yeast. In the case of bacterial fermentation, the process produces by-products such as acetic and lactic acids, which can inhibit the fermentation reactions [45]. There is not a lot of research on the use of engineered *E. coli* due to drawbacks such as the narrow and neutral pH growth range [45]. Furthermore, *E. coli* are less robust when compared with yeast cultures [45].

1.4.1 Glucose metabolism

The two glucose metabolism pathways of interest are aerobic respiration and anaerobic metabolism. Glucose metabolism under both aerobic and anaerobic conditions begins with the process called glycolysis, which breaks down a glucose molecule into two pyruvate molecules. Under aerobic conditions, glycolysis is followed by tricarboxylic acid (TCA) cycle and oxidative phosphorylation for ATP generation and biosynthesis [15]. Under anaerobic conditions, the resulting pyruvate molecules are then converted into ethanol through a series of reactions [50].

1.4.2 Glycolysis mechanism

Glycolysis is the first step in both aerobic and anaerobic respiration and is the process by which glucose is broken down into pyruvate. The reaction sequence begins with a glucose molecule being phosphorylated by reaction with ATP and the enzyme hexokinase to yield glucose 6-phosphate (G-6P) [15, 50]. Then, G-6P is isomerized to fructose 6-phosphate with the help of phosphoglucose isomerase and subsequently converted to fructose-1, 6-bisphosphate by the phosphofrutokinase-catalyzed reaction [15, 50]. This gives us an intermediate molecule that is cleaved by aldolase [15, 50] to produce glyceraldehyde-3-phosphate (GA-3P) and dihydroxyacetone phosphate (DHAP) [15, 50].

GA-3P and DHAP are in equilibrium with each other but as GA-3P is oxidized and phosphorylated to yield 3-phospoglyceryl phosphate in the subsequent step, DHAP is converted to GA-3P due to a shift in the equilibrium [15, 50]. A phosphate in the carboxyl group of 3-phosphoglycerate (3P-GA) is transferred to ADP generating an ATP molecule and reducing 3-phosphoglyceryl phosphate to 3P-GA [15, 50]. Then, 3P-GA is converted to 2-phosphoglycerate by phosphoglyceromutase followed by a dehydration step which yields phosphoenolpyruvate (PEP) [15, 50]. In the final step of glycolysis, a phosphate group is transferred to ADP to generate ATP and yielding the final product, pyruvate [15, 50]. Pyruvate is a key metabolite; it can be converted to ethanol under

anaerobic conditions. Under aerobic conditions, pyruvate can be converted to CO_2 and NADH through the TCA cycle. A summary of the metabolic pathways of glycolysis is constructed in Figure 8.

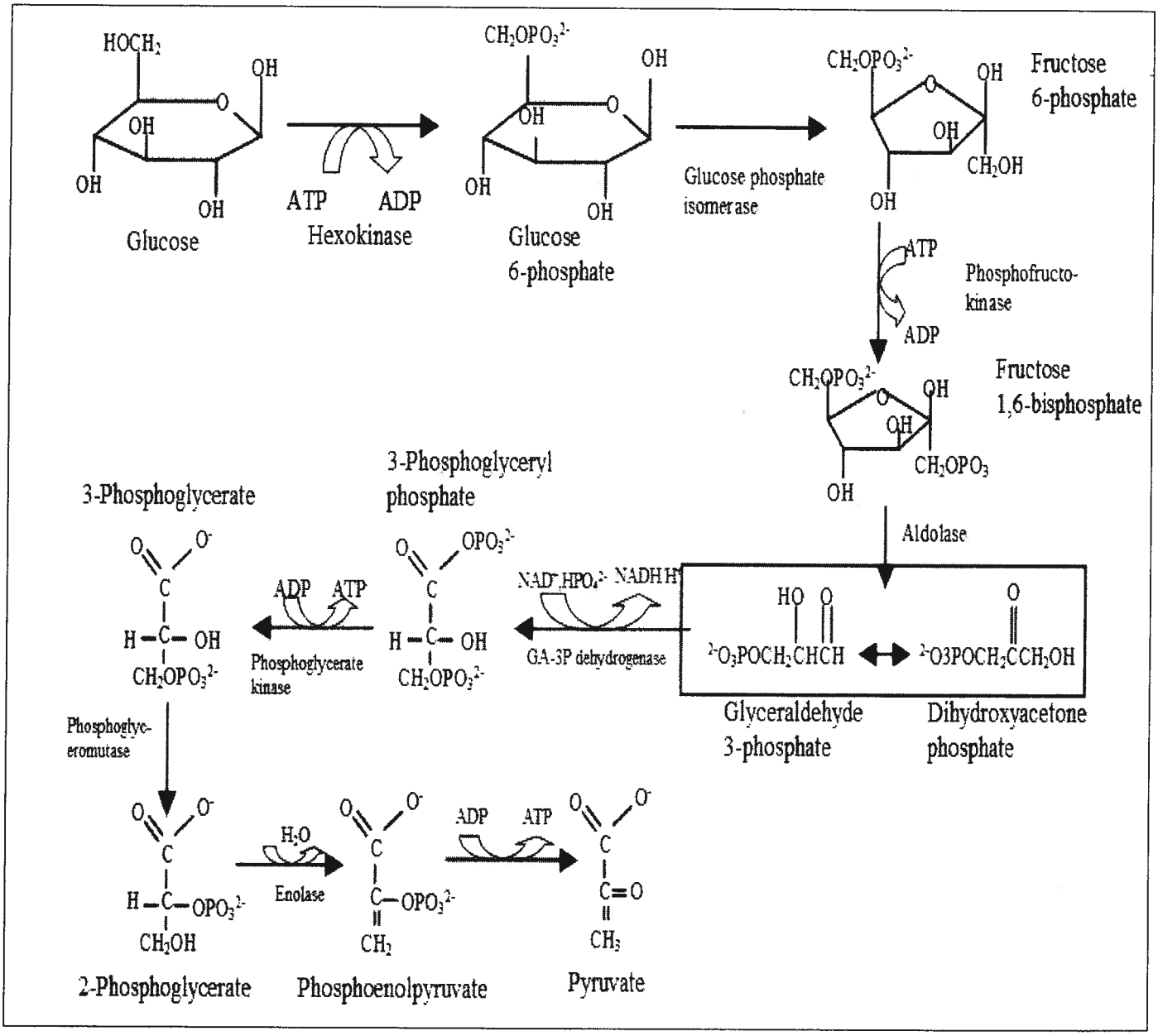

Figure 8 Glycolysis pathway [15, 50]

1.4.3 Utilization of pyruvate under aerobic conditions and the tricarboxylic acid cycle

The major roles of the tricarboxylic cycle are to supply carbon skeletons for amino acid synthesis, and to provide electrons to the electron transport chain, thereby generating energy for biosynthesis [15]. In order for pyruvate to go through the TCA cycle, it has to undergo a series of transformations to acetyl CoA. The TCA cycle is followed by oxidative phosphorylation where NAD molecules are regenerated for glycolysis and ATPs are produced for biosynthesis. A diagram of major reactions and functions of the product is shown in Figure 9.

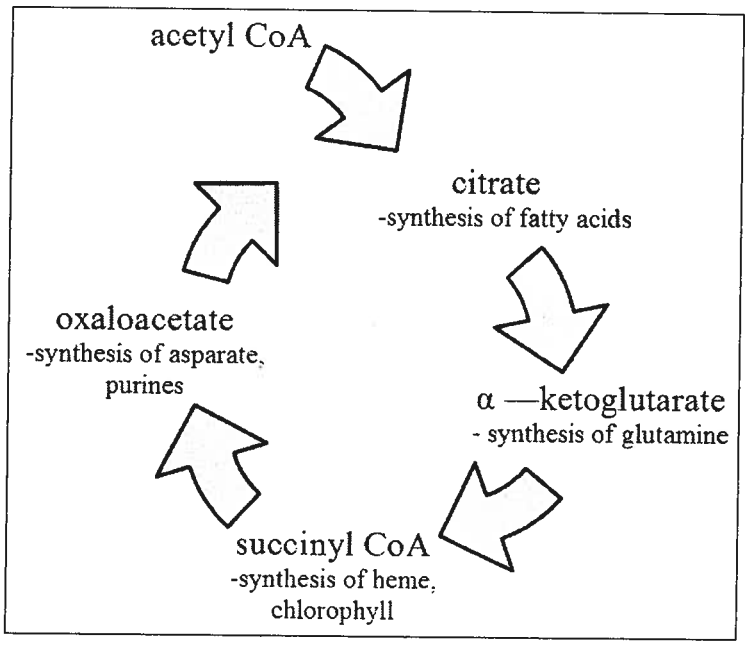

Figure 9 Tricarboxylic acid cycle

1.4.4 Utilization of pyruvate under anaerobic conditions

Fermentation is a process where yeasts derive energy from the oxidization of carbohydrates using an endogenous electron acceptor, which is an organic electron acceptor synthesized by the cell itself [51]. There are different species of yeasts that can

metabolize sugars and convert them into ethanol. In this project, sugar is being converted to ethanol by the yeast *Saccharomyces cerevisiae*.

Under anaerobic conditions, glucose metabolism begins with glycolysis, which breaks down a glucose molecule into two pyruvate molecules. Then the pyruvate molecules are converted to acetaldehyde by decarboxylation [50]. Acetaldehyde molecules are further reduced to ethanol by the enzyme called alcohol dehydrogenase [50]. The reactions require NADH as cofactor and regenerate NAD^+ to be used in glycolysis [50]. In this cycle, the organism is growing without using the electron transport chain to generate energy, which is a characteristic of fermentation [15]. A flow diagram of the major reaction pathways for fermentation is shown in Figure 10.

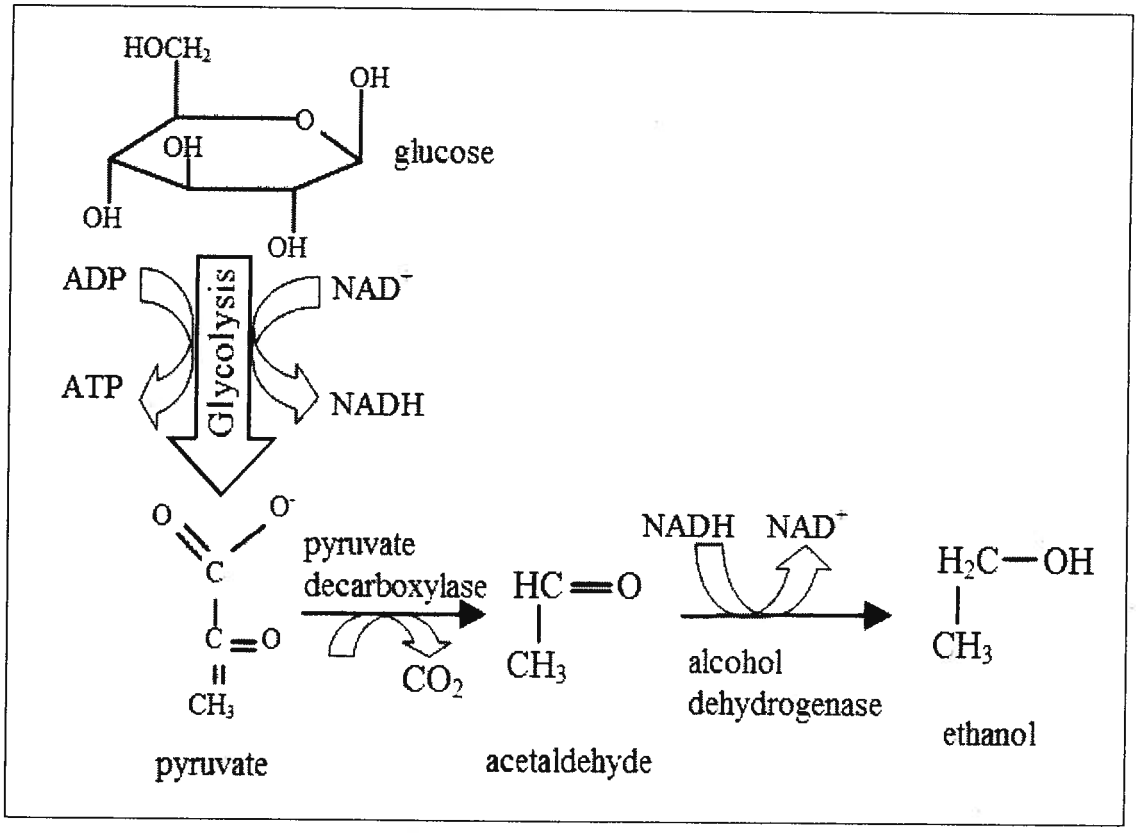

Figure 10 Major reaction pathways for fermentation [50]

1.4.5 Comparison of aerobic and anaerobic conditions

Yeasts were grown under two types of growth conditions during this project: aerobic and micro-aerophilic conditions. Since the yeasts used in the project are facultative fermentative, they are able to perform a mix of both respiratory and fermentative metabolisms. Under aerobic respiration, yeasts can generate more ATP for biosynthesis. However, the production of ethanol is not favorable under aerobic conditions [6]. Thus, the yeasts are grown under aerobic conditions for culturing (producing inoculum) while anaerobic conditions are used for ethanol production.

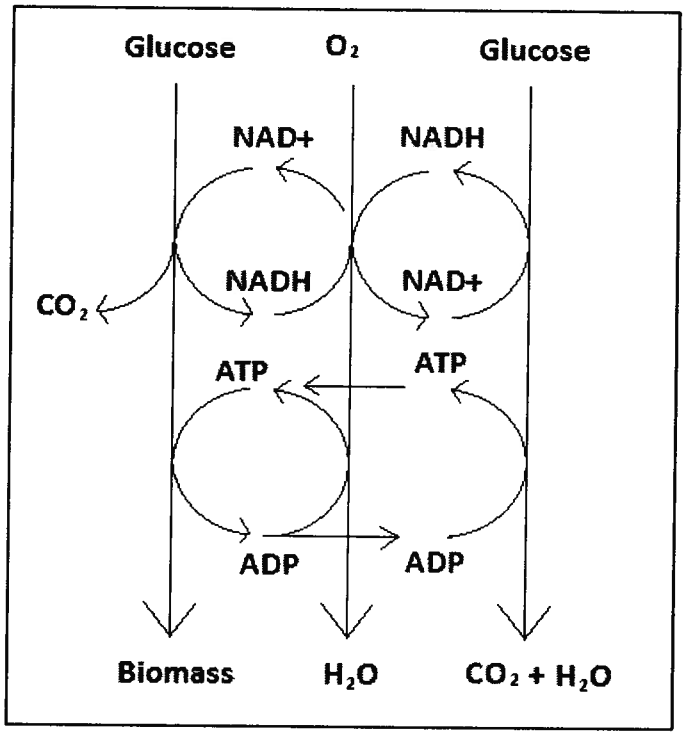

Figure 11 Aerobic energy pathway for *S. cerevisiae* [51]

Under aerobic conditions or respiratory growth, NADH produced by glycolysis can be re-oxidized to yield an additional ATP molecule [44, 51]. This is called oxidative phosphorylation, a metabolic pathway that uses energy released by the oxidation of glucose to produce ATP [51]. A flow diagram of this energy pathway is shown in Figure 11. In aerobic conditions, the reactions yield a total of sixteen ATP molecules from one glucose molecule [44].

In micro-aerophilic fermentation, substrate level phosphorylation in glycolysis is the major source of ATP in *S. cerevisiae*, because oxidative phosphorylation is limited with limited oxygen [51]. This reaction gives a net yield of two ATP molecules for each molecule of glucose [44]. A flow diagram of this energy pathway is shown in Figure 12.

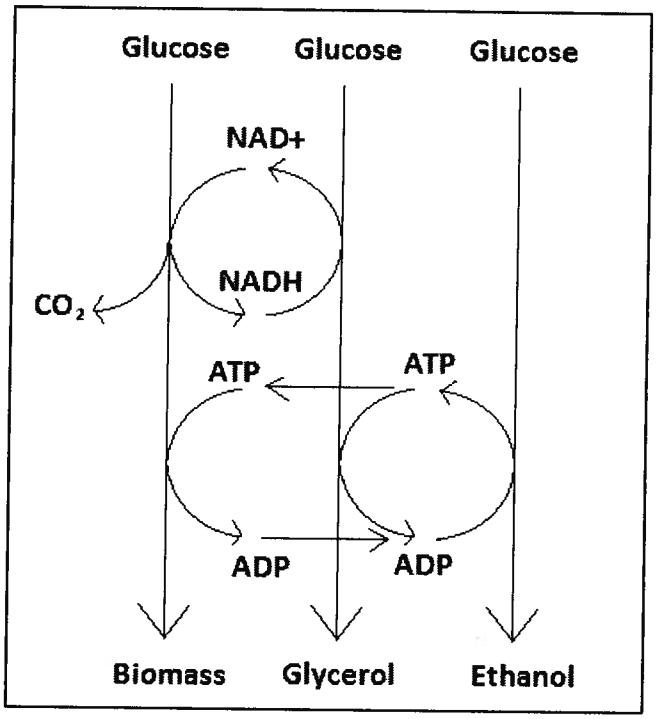

Figure 12 Alcoholic fermentation energy pathway for *S. cerevisiae* [51]

By comparing the aerobic conditions with the micro-aerophilic conditions, yeasts growing under aerobic conditions produce eight times more ATP. Thus, a much higher yeast growth rate is observed. The typical yield of biomass with aerobic fermentation is 0.5 g/g glucose, while the typical yield for micro-aerophilic fermentation is 0.1 g/g glucose [51].

1.4.6 Parameters affecting fermentation

The physical factors that can affect the production of ethanol are temperature, mixing speed and residence time. Temperature can determine the ability of the yeasts to grow and affect the growth rate of the yeasts. The optimal temperature range of the yeast *Saccharomyces cerevisiae* is reported to be 28 – 40°C [52]. Within this temperature range, the growth rate of yeasts is a linear function of temperature and is modeled using the Arrhenius equation [53]. At temperatures below the optimal growth range, yeast growth rate decreases steadily, while zero growth occurs at temperature above 40°C and below 5°C [52, 53]. The rapid decrease in growth rate is due to the decrease in the resistance to stress at temperatures outside of the optimal growth range [53].

Yeasts like *Saccharomyces cerevisiae* can tolerate a wide range of pH. They are known to be able to grow at a pH range from 2.4 – 8.6 [52]. However, the optimal pH range is reported to be 4.5 – 5.5 [53]. The actual optimal pH range for the yeasts is dependent on the type of acid dissociating in the medium. For instance, acetic acid was found to be more inhibitory than lactic and citric acids [53]. In addition, the optimal pH range is also dependent on the temperature and the presence of other inhibitory compounds [53].

1.4.7 Inhibition of fermentation

When bio-oil is extracted with water, levoglucosan as well as a number of other water soluble chemical compounds are extracted to the aqueous phase. Some of the compounds extracted are inhibitors in the fermentation process. Some of the inhibitors identified in bio-oil are dissolved solids, acetic and formic acids [52]. Acetic acid inhibition is mainly caused by the undissociated form of acetic acid [52]. Undissociated weak acids are liposoluble and can diffuse across the plasma membrane by passive diffusion [20, 52]. Upon entering the cell's cytosol, dissociation of the acids occurs due to the neutral intracellular pH, thus decreasing the internal pH [20]. At a concentration of 3 g/L acetic acid, the internal pH of *S. cerevisiae* decreases from 7.0 to 5.6, which result in a 20%

decrease in the fermentation rate [52]. A study by Larsson et al. [54] has reported that the ethanol yield is reduced from 0.45 (g ethanol/g glucose) to 0.35 (g ethanol/g glucose) at an acetic acid concentration of 29.7 g/L [54]. In addition, when other acids such as formic or levulinic acid are present, the ethanol yield is reduced to 0.33 (g ethanol/g glucose) and 0.29 (g ethanol/g glucose), respectively [54].

Other factors that can influence the toxicity of acetic acid include membrane permeability, pH gradient across the cell membrane, and buffering of the cytoplasm [52]. As dissociated acetic acids build up inside the cell, acetic acid will begin to buffer the cytoplasm thereby partially protecting the cell from additional acetic acid [52]. The increase in buffer capacity is mainly responsible for the acetic acid adaptation. In addition to the internal pH, short-chain fatty acids can also affect the membrane transport of phosphate, resulting in an increase in the maintenance energy for the cells [52]. Also, formic acid is more toxic than acetic acid, due to a lower pKa than acetic acid [52, 54].

The combined effect of these toxic compounds can result in a more severe fermentation inhibition than a single toxic compound [40]. This was observed in Nigam's [55] experiment where wheat straw-derived hydrolysate and synthetic mediums were compared. Both of the solutions contained the same concentration of acetic acid, furfural and lignin derivatives. However, the ethanol yield (g ethanol /g glucose) and biomass productivity (g biomass/L medium · hour) were 74.4% and 83% lower in the wheat straw medium than in the synthetic medium [55]. This researcher also tested the three inhibitors individually and found that the reduction in ethanol yield and biomass productivity were 30.2% and 59.6% for the medium with acetic acid, 7.0% and 12.8% for the medium with furfural and 14.0% and 44.7% for the medium with lignin derivatives [55]. From these observations, the effects of these inhibitory compounds are greater when they are combined.

1.5 Improving the fermentability of bio-oil hydrolysates

Bio-oil contains approximately 162 compounds that are detectable by gas chromatography-mass spectrometry (GC-MS) [30, 32]. Some of the compounds are identified as inhibitors in the bio-oil, and the synergistic effect of the collective compounds in the bio-oil is unknown. However, despite the unknown compounds and their synergistic effect, there are ways to approach and solve the inhibition problems in the fermentation of bio-oil hydrolysate. There are four approaches to reduce and minimize the effect of inhibition of fermentation medium. The first approach is prevention. In this case, the objective is to avoid the formation of inhibitors prior to the fermentation process [40]. The second approach is to detoxify the hydrolysate before fermentation, which is also a major focus in this project. The third approach is to develop a species of microorganism capable of resisting inhibitors [40]. This can be achieved by genetically engineering microorganisms or by a technique called adaptive evolution. The fourth approach is to convert the toxic compounds into products that do not interfere with the metabolism of the microorganism [40].

1.5.1 Air stripping

Air stripping is a common chemical engineering technique in which volatile organic compounds (VOCs) are extracted from a solution into air. In this project, air stripping is employed to remove the volatile acids such as acetic and formic acids that are in bio-oil hydrolysate. The removal of VOCs by air stripping is governed by Henry's law. Thus, a larger Henry's constant will results in greater removal of VOCs by air stripping. Consequently, temperature has an influence on air stripping since Henry's constant increases with increasing temperature. Other factors that can affect the removal efficiency of air stripping include pH and contact time. It has been reported by Helle et al. [56] that air stripping at low pH (pH not specified) could remove volatile acids while at high pH (pH 10) ammonium could also be removed [56].

1.5.2 Adsorption on activated carbon

Treatment with activated carbon is a common detoxification technique used for removing toxicants from liquids. The parameters which typically control adsorption are carbon dosage, temperature and contact time [40]. In a study using straw hemicellulose hydrolysate, it was found that temperature affects how well the activated carbon can remove lignin degradation products [57]. It was found that the removal rate of the toxic compounds increased six fold when the temperature rose from 20 to 40°C [57]. These same researchers examined effect of pH on adsorption on activated carbon and found that a low pH (pH 2) favored the adsorption of the lignin degradation products, particularly the phenolic compounds [57]. The effect of contact time was also well documented by researchers. Parajo et al. [58] found that the removal of lignin-derived compounds was at maximum at around 20 minutes and the adsorption of the compounds reached equilibrium after 20 minutes [58]. Other researchers had found that using 1% (w/w) of activated carbon to treat sugarcane bagasse resulted in the removal of 94% phenolic compounds with a sugar loss of only 0.47% [59]. Increasing the amount of activated carbon to 30% (w/w) enhanced the removal of phenolic compounds slightly but the sugar loss increased to 31.3%, which was an undesirable result [59].

1.5.3 Overliming

Overliming is regarded as a very effective method for detoxification of hydrolysates prior to fermentation [60]. Martinez et al. [60] conducted experiments on overliming of hemicellulose hydrolysate and reported removal of up to 51% of furans and 41% of phenolics, with minimal (9%) sugar loss [60]. A study by Yu et al. [61] has reported that using overliming to detoxify cotton-derived bio-oil hydrolysate, resulted in an ethanol yield of 0.39 g ethanol/ g glucose [61]. In addition, Yu et al. [61] reported that combining overliming with other adsorbent materials such as diatomite, bentonite and zeolite can increase the ethanol yield further by 5 – 13% [61]. However, another study on treating the hydrolysate with the overliming technique reported up to 20% loss of sugars [62]. The sugar loss was postulated to be due to calcium ion-catalyzed sugar degradation under

alkaline conditions [62]. In addition to the sugar loss, the formation of formic and acetic acid was observed [62]. At a temperature of 80°C and a treatment time of 3 hours, as much as a 2 to 3 fold increase in the concentration of acetic and formic acid was reported [62]. Despite the formation of formic and acetic acid, the removal of other inhibitors such as furfurals and phenols was effective [62]. Thus, the evaluation of this method and for other detoxification methods should be based upon fermentability and the yield of ethanol.

1.5.4 Coagulation and flocculation

The aim of using coagulation and flocculation to treat bio-oil is to remove the dissolved and suspended particles in the bio-oil. Coagulation and flocculation are commonly used in water treatment processes such as recovery of processed water, and treating municipal waste water. The addition of coagulants can destabilize the forces that keep the particles apart, so that formation of larger particles can occur. Then, flocculant is added to increase the cluster size of the particles, thus allowing the materials to settle.

1.5.5 Solvent extraction

Solvent extraction is a method aimed at selectively removing inhibitory compounds such as acetic acid and formic acid from the fermentation medium. It has been shown that C8–C10 saturated aliphatic tertiary amines dissolved in organic solvents are effective extractants for carboxylic acids [63]. Several aliphatic amines, for instance, alamine 336 and aliquat 336 with co-solvents such as kerosene and 1-octanol, have been used successfully to extract acetic acid [64]. It has been confirmed by Yang et al. [64] that a polar diluent can increase the extracting efficiency of a non-polar amine by providing additional solvating power that allows higher levels of polar acid-amine complexes to stay in the organic phase [64]. In the design of an amine-diluent system for acid removal, there are three factors that can influence the extraction of carboxylic acids and should be

considered. They are: the nature of the acids, the concentration of the acids and the concentration of the amines [63].

Other solvents such as oleic acid and oleyl alcohol were also evaluated in the extraction of the bio-oil hydrolysates. These two solvents were examined by researchers in the Netherlands [65]. They experimented with oleic acid and oleyl alcohol along with other solvents (hexane, octane, decane, dodecane, hexadecane, hexanol, octanol and decanol) for the feasibility of extracting inhibitory compounds from lignocellulosic hydrolysates. Zausten et al. [65] found that there was a two-fold increase in ethanol productivity by extracting the fermentation medium with oleyl alcohol [65]. In addition, oleic acid and oleyl alcohol were found to have a biocompatibility that exceeds 100% [65]. The biocompatibilities were defined by measuring the change in carbon dioxide production from the yeast after the addition of 5% solvent during the fermentation processes [65].

1.5.6 Hydrogenation

Hydrogenation is a reaction that results in the addition of a pair of hydrogen atoms to the reactants. The process has been investigated by a number of researchers to upgrade and stabilize bio-oil for transportation and storage [66, 67, 68]. The hydrogenation process requires metal catalysis as well as high temperature and pressure. One study found that two catalysts, Re_2S_7 and Re_2O_7, were able to hydrogenate dilute acetic acid solution into ethanol at 230°C and 12.2 MPa of hydrogen pressure [69]. The reaction took about six hours, the ethanol yield was found to be 68.6% and the acetic acid reduction was found to be 76.9% for hydrogenation with the Re_2S_7 catalysis [69]. Under the same conditions, the ethanol yield is reported to be 41.1% and the acetic acid reduction was found to be 62.6% for experiments with Re_2SO_7 catalysis [69]. Other studies that are related to hydrogenation reactions and reaction kinetics are focused on phenolic and furanic groups in the bio-oil [67]. Moreover, the hydrogenation experiments on bio-oil were focused on stabilizing and upgrading of bio-oil. Thus, very little information can be found regarding the removal of inhibitors from bio-oil hydrolysate with hydrogenation.

1.5.7 Adaptive evolution

Adaptive evolution is, by definition, a set of mutations that occur in response to a specific challenges or changes in the environment and is advantageous to the cells under these conditions [70]. This technique can potentially be used to develop an adapted yeast strain that can resist the inhibitory compounds in the fermentation broth. Thus, the adapted yeast strain can show an increase in the ethanol yield and biomass productivity without the need of any additional detoxification stage [70].

The stresses that can cause yeasts to function poorly include: temperature, pH, inhibitory compounds and limiting substrate [52, 70]. The mechanism of adaptive evolution is still not fully known, but there are three models that can potentially explain the process. The three models are: the directed mutation model, the hypermuation model and the cryptic growth model [70, 71]. In the directed mutation model, it is hypothesized that mutations are targeted toward genes in which a mutation directly relieves the stresses caused by the environment [70, 71]. In the hypermutation model, it is postulated that genome-wide mutation rates increase such that both adaptive and non-adaptive mutations are stimulated [70, 71]. In this situation, most of the mutated genes are unhelpful in the adaptation of the stress but some mutations are useful, thus able to survive and out-compete other mutants. In the cryptic growth model, the mutation rate is hypothesized to be unaffected by the environmental conditions, but mutations that occurred randomly appear to be adaptive due to the survival of some mutants [70, 71].

The adaptive evolution models can be applied to yeast cultures. When a yeast population is propagated asexually under continuous long term stress, it undergoes a series of adaptive shifts. The clones that are well adapted to the environment successively replace one another and eventually replace the entire population of yeast [71]. Such changes have been observed to occur in every 50 generations [71]. The evolution can be identified by the appearance of mutants in the population, as each new and fitter mutant outgrows the population of the progenitor genotype [71].

Chapter 2 Research Objectives

The objective of this project is to determine the technical viability of producing bio-ethanol using bio-oil as a substrate for fermentation. The major tasks involve in the project include:

1. Optimize the extraction of levoglucosan from bio-oil

2. Screen and evaluate techniques that could remove inhibitors and/or enhance the yield of ethanol
    a. Air stripping
    b. Adsorption on activated carbon
    c. Overliming
    d. Coagulation and flocculation
    e. Solvent extraction
    f. Hydrogenation
    g. Adaptive evolution of yeast

Chapter 3 Materials and Methods

3.1 Process overview

Production of ethanol from bio-oil began with the extraction of levoglucosan with water. Adding a sufficient amount of water to bio-oil causes the bio-oil to separate into an aqueous phase and an organic phase. The aqueous phase would contain most of the levoglucosan. It can then be hydrolyzed with dilute sulfuric acid. The product of hydrolysis, the hydrolysate, is used as fermentation medium to produce ethanol [6, 7]. However, it was found that bio-oil hydrolysate contained fermentation-inhibiting compounds, thus the hydrolysates must first be detoxified [6]. After the detoxification stage, the detoxified hydrolysates were then used as fermentation medium for *Saccharomyces cerevisiae*, strain T2 to produce ethanol.

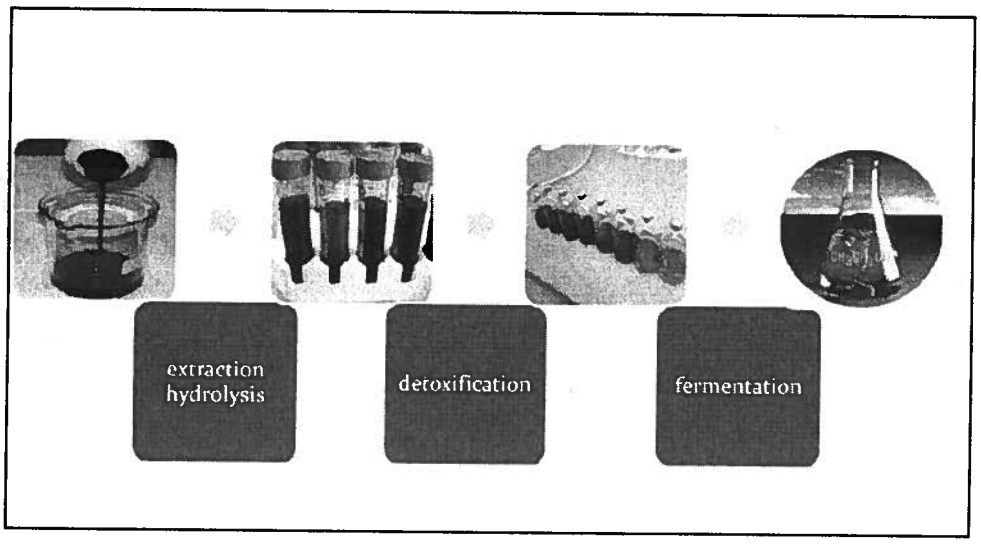

Figure 13 Process overview for ethanol production from bio-oil

3.2 Procedure for extraction of levoglucosan

The objective of this part of the experiment was to optimize the extraction of levoglucosan and other anhydrosugars from bio-oil. The experiments were designed to

find the critical concentration of water required to induce phase separation and to determine the optimal mass ratio of water-to-bio-oil for levoglucosan extraction.

3.2.1 Storage and preparation of bio-oil

The bio-oil used in the project came from the Technical Institute of Finland. It was stored in the refrigerator at about 3°C, to slow the aging of the bio-oil. The bio-oil used in this project has been stored for approximately 5 years. Before any experiment with the bio-oil, the oil was placed in an environmental shaker at 20°C and 120 rpm for approximately 20 minutes. This decreased the viscosity of the bio-oil and allowed the bio-oil to be poured out of the container easily.

3.2.2 Titration of bio-oil to determine the critical amount of water required to induce phase separation

Water is soluble in bio-oil and the bio-oil will behave like a single phase until a critical amount of water is reached. As soon as the critical amount of water is added, phase separation will occur. In order to determine the critical point, a titration experiment with water and bio-oil was conducted. In the experiment, water was added to the bio-oil slowly until phase separation could be observed. When phase separation was observed, the volume of the water added to bio-oil was recorded. A photograph of the experimental apparatus is shown in Figure 14.

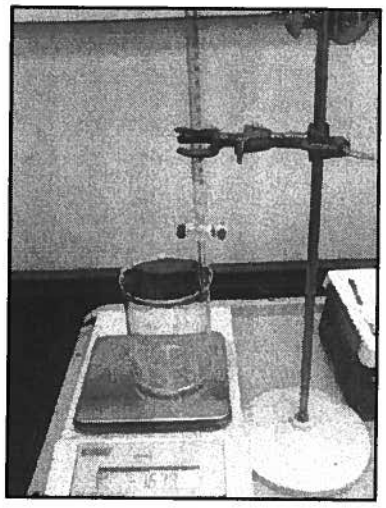

Figure 14 Bio-oil titration equipment

It is important to note that addition of a few drops of water might give the illusion of phase separation if there is inadequate mixing. Therefore, it was vital to provide sufficient mixing during the titration experiment. A photograph of the bio-oil at the point of phase separation is shown in Figure 15.

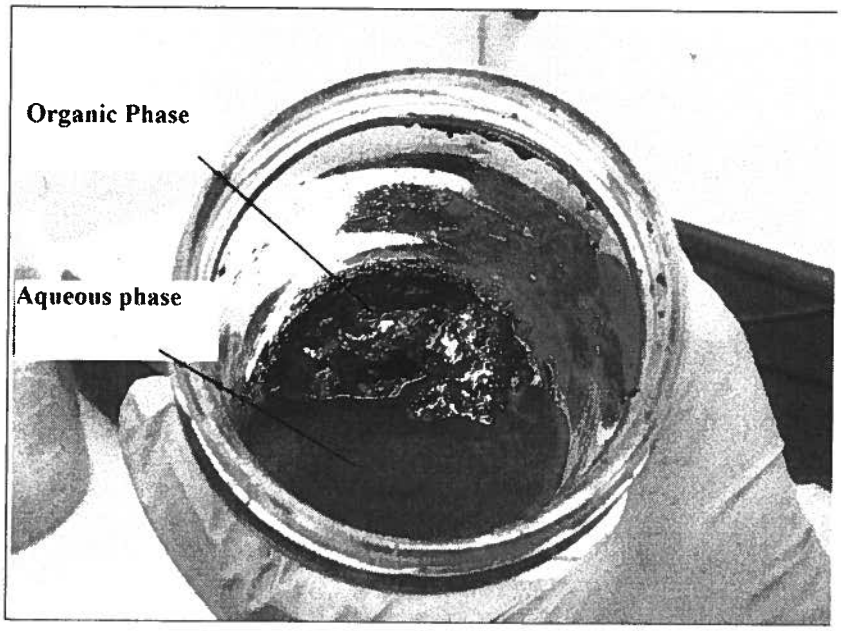

Figure 15 Phase separation of bio-oil

3.2.3 Levoglucosan extraction from bio-oil

A preliminary experiment was conducted to test whether it was beneficial to add water to bio-oil or bio-oil to water. The ratios tested were 50 and 100 wt% of water added to bio-oil. Before the experiment, both the bio-oil and water were weighed and measured according to the predetermined mass ratios as shown in Table 5. Then, the water was slowly added to the bio-oil (or vice versa) in a 50 mL beaker with a magnetic stirrer. During the extraction experiment, it was necessary to provide sufficient mixing time for the two liquids, which was approximately 20 minutes [6]. After 20 minutes, the aqueous phase samples were decanted into falcon tubes for storage and analysis.

Table 5 Parameters for levoglucosan extraction with water

Wt% of water added to bio-oil	Water add to bio-oil		Bio-oil add to water	
	Weight of Bio-oil (g)	Weight of water (g)	Weight of Bio-oil (g)	Weight of water (g)
50%	10	5	10	5
100%	10	10	10	10

Two methods of mixing were examined to determine which method would provide optimal extraction: the mixer method and the vortex method. Schematics of both mixing methods are shown in Figure 16. In the mixer method, bio-oil was poured into the water and a handheld kitchen mixer (Braun, Multiquick MR405) with steel blades was used to mix the liquids and grind the tar like organic phase formed in the initial separation of water and bio-oil. In the vortex method, bio-oil droplets were dripped into a falcon tube, which was secured on a vortex mixer. The flow of the bio-oil droplet was controlled by a valve at the end of a buret. The bio-oil was slowly dropped into the swirling liquid to enhance contact time and mixing between the liquids.

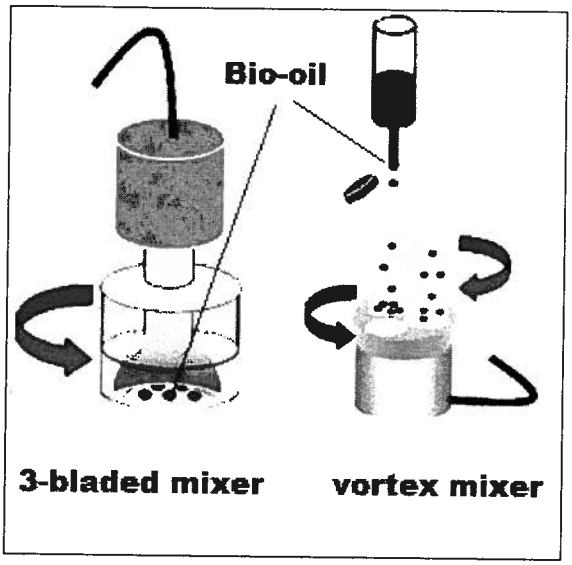

Figure 16 Schematic for extraction of levoglucosan

In finding the optimal extraction ratios, the bio-oil and water were weighed and measured according to the predetermined ratios shown in Table 6. The extraction ratios examined ranged from 10 – 2000 wt% of water added into bio-oil. In this set of experiments, the mixer method was used and bio-oil was added to water. These were chosen based on the results from previous set of experiments on levoglucosan extraction.

Table 6 Extraction ratios examined to determine the optimal ratios

Wt% of water added to bio-oil	Weight of Bio-oil (g)	Weight of water (g)
10%	10	1
20%	10	2
40%	10	4
50%	10	5
100%	10	10
200%	10	20
400%	10	40
600%	10	60
800%	10	80
1000%	10	100
2000%	10	200

Small aliquots (1 mL) from the aqueous phase from the extraction experiments were taken with a pipette and analyzed by gas chromatography (GC).

Since previous works had shown that temperature played a major role in the properties of bio-oil, it was beneficial to determine if temperature had any effect in the extraction of levoglucosan [31]. The two temperatures tested in levoglucosan extraction were 25°C and 80°C. The extraction ratio tested for this experiment was 100 wt% of water added into bio-oil. Similar to other extraction experiments, when the bio-oil extracts were cooled to room temperature, small aliquots from the aqueous phase were taken and analyzed by GC.

3.3 Dilute acid hydrolysis of the aqueous fraction of bio-oil

The purpose of this part of the experiment was to hydrolyze anhydrosugars, such as levoglucosan and cellobiosan into glucose for fermentation in later stages of the experiments. In the project, sulfuric acid was used for hydrolysis because it had demonstrated to be effective and inexpensive [6]. The procedure for hydrolysis of levoglucosan was based on published methods [6, 61]. Prior to hydrolysis, samples from the aqueous phase were analyzed with gas chromatography to determine the molar concentration of levoglucosan. Then, an equal molar ratio of sulfuric acid and levoglucosan was used in the hydrolysis [6]. The hydrolysis was carried out with an autoclave (Steris Amsco Century, SV-1262 prevac steam sterilizer) at 121°C for 20 minutes (excluding heating and cooling time). After hydrolysis, the hydrolysate was cooled in a cold water bath until room temperature was reached. Then, the hydrolysate was sampled and filtered with a syringe filter with a pore size of 20 μm. Finally, the filtered sample was injected to a high pressure liquid chromatography system for analysis. A picture of the hydrolysate in 50 mL falcon tubes is shown in Figure 17.

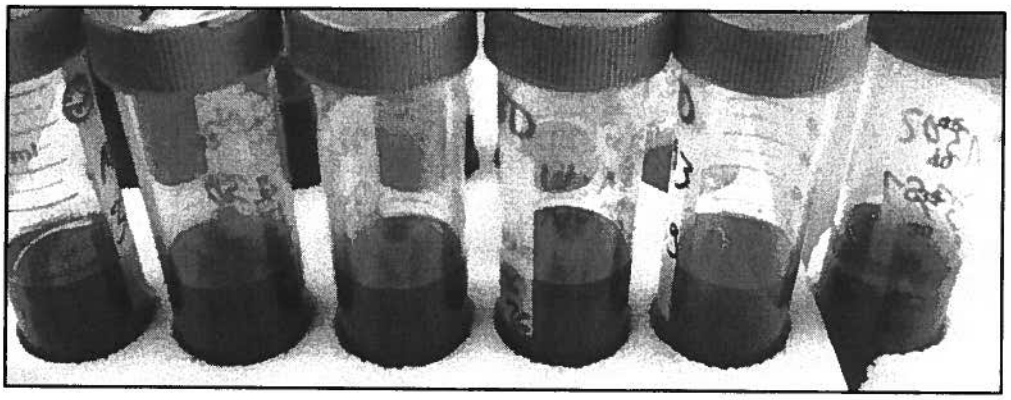

Figure 17 Hydrolysate in 50mL falcon tubes

3.4 Procedure for fermentation of bio-oil hydrolysate

Fermentation was one of the most important stages of the experiment. The optimal conditions for ethanol production were governed by a number of different factors such as temperature, nutrients concentration, oxygen concentration, pH, residence time, mixing speed, and the presence of bacteria and antimicrobial compounds [53, 73]. Some of these factors could interact and act synergistically to affect the well-being of yeasts, which made the realization of true optimal conditions more complex [53].

3.4.1 Preparation of yeast inoculum for fermentation

The yeasts were stored in refrigerator at 4°C on agar plates. When preparing the yeast for fermentation, a loop full of yeast from the agar plate was transferred aseptically to YPG medium containing 1% (w/v) of yeast extract, 2% (w/v) of peptone and 2% (w/v) of glucose. YPG medium contains nutrients that provide for starter yeast cultures. Yeast extract and peptone are complex substances that contain vitamins, minerals and proteins needed for cell growth. Glucose is needed to provide carbon and energy for the yeasts.

The YPG medium was sterilized by autoclaving for 20 minutes at 121°C. The yeast was grown in the YPG medium for 1 full day in an environmental shaker at 30°C and 150

rpm. Then, 2 mL of the starter culture broth was transferred to fresh YPG medium to grow for another day. After two days of culturing, the yeast cultures were ready to be used as fermentation inoculum. Yeast broths from second stage growth flasks were transferred aseptically to 50 mL falcon tubes and centrifuged for 10 minutes at 2285g (Damon/IEC division CU-5000). This caused the yeast broth to be separated into a yeast pellet and supernatant (YPG medium). The supernatant was then discarded and the yeast pellet was resuspended with 10 mL of sterile water. This process was repeated once to wash the yeast. The purpose of washing the yeast was to remove any trace amount of YPG medium that might still be present during the first separation of yeast and YPG medium. After the yeast pellet was washed, it was ready to be inoculated into the fermentation medium. The volume of the yeast inoculated to fermentation medium was 2 mL, which yielded a concentration of approximately 2.5 g/L (in 50 mL fermentation medium). A flow diagram of the yeast preparation procedure is shown in Figure 18.

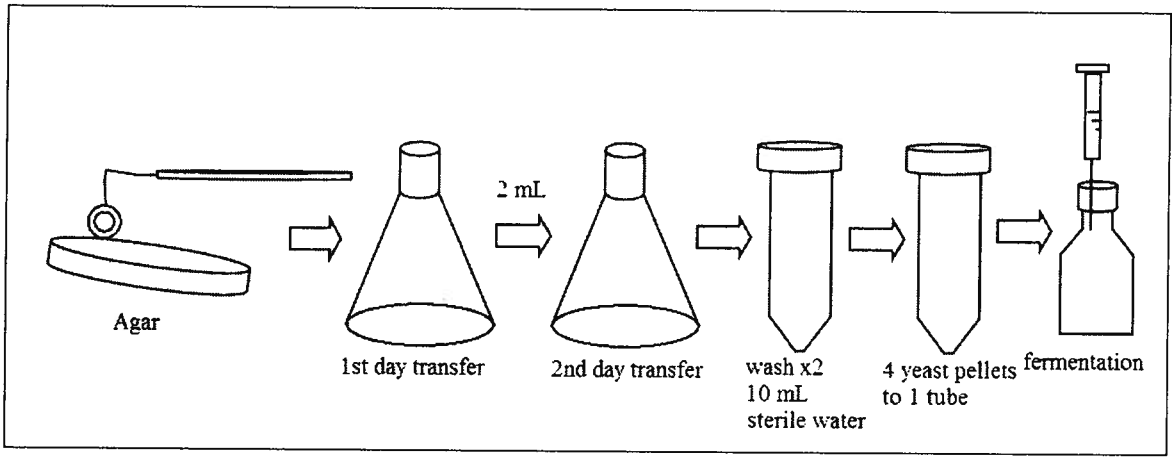

Figure 18 Yeast preparation for use as inoculum

It was important that sterile water was used for resuspending the yeast. In addition, yeasts were transferred in an aseptic environment; otherwise contamination by other microorganisms might generate inaccurate results.

3.4.2 Fermentation of bio-oil hydrolysate

In this work, the fermentations were carried out in 15 and 150 mL serum vials. The 15 mL serum vials were used for determining the ethanol yield after 48 hours of fermentation, whereas the 150 mL serum vials allowed more samples to be taken during the 48 hour fermentation period. Depending on the goal of the experiments, the volume of the fermentation medium such as YPG medium and bio-oil hydrolysate ranged from 10-100 mL.

As previously mentioned, yeasts under micro-aerophilic conditions favour the production of ethanol. In order to achieve this condition, the serum vials were capped with a rubber stopper and sealed tightly with aluminum caps so that the vials were hermetically sealed (Figure 19). An 18-gauge, 1.5 inch needle and 5 mL syringe filled with foam was inserted into the vial through the rubber stopper to allow a small amount of air to circulate in the vials, thus simulating the micro-aerophilic conditions.

The vials were incubated in an environmental shaker for maintaining the mixing speed and temperature at optimal conditions. In this work, the rotating speed of the shaker was set at 150 rpm and the temperature was set at 30°C. Short term modifications to the rotating speed of the shaker were made from time to time because the yeast would adhere to the wall of the serum vial as the cultures grew. The residence time was usually 48 hours, and samples were usually withdrawn from the serum vials at 1, 2, 4, 8, 12, 24, and 48 hours via syringes. In all of the fermentation experiments for this project, the pH of the hydrolysate was adjusted to 5.5 prior to fermentation but, overtime, the pH typically decreased to 4.5 – 4.7.

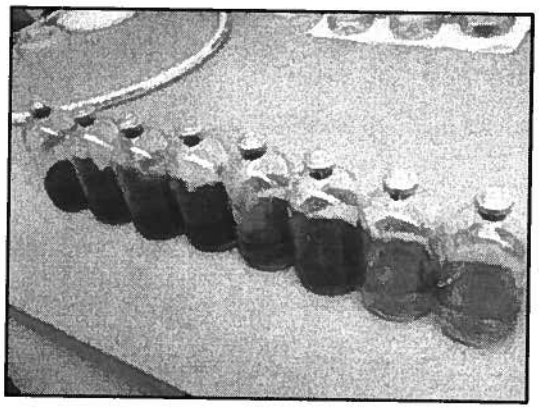

Figure 19 Fermentation medium in serum vials

3.5 Procedure for detoxification of bio-oil hydrolysates

A detoxification stage was necessary in order to increase the fermentability of the bio-oil hydrolysates and improve the production of ethanol. The detoxification techniques examined include air stripping, overliming, hydrogenation, adsorption and solvent extraction. The selected methods were targeted at different inhibitory compounds in the bio-oil. The targeted inhibitors in the bio-oil were furfural, phenolics, acetic acid, formic acid, and dissolved solids. The evaluation of these detoxification methods was based upon the fermentability of the bio-oil hydrolysates. In addition, a technique called adaptive evolution was used to help the yeast to adapt to the bio-oil hydrolysate, thereby increasing the yield of ethanol.

3.5.1 Air stripping

An experiment was conducted to test if air stripping could increase the fermentability of bio-oil hydrolysate. The purpose of air stripping was to remove the volatile organic components in the hydrolysate. The parameters tested for this experiment were temperature and time. In this experiment, air was bubbled through a nozzle into flasks that contain bio-oil hydrolysate. Therefore, factors, such as packing materials, size of the packing materials and size of the stripper tower, were not studied. Prior to air stripping,

the pH of the bio-oil hydrolysate was adjusted to 10 by adding NaOH (50% w/w). The experiments were conducted at 25°C, 50°C and 80°C for 30 minutes and 1 hour.

3.5.2 Adsorption on activated carbon

The procedure for adsorption on activated carbon was to first determine the weight of the bio-oil hydrolysate. Then, activated carbon powder (Fisher Scientific, Fisherbrand AC 50-200 mesh, Diameter ~0.074-0.297 mm) was measured according to the predetermined concentration (Table 7) with an analytical balance (Ohaus, AP110S analytical plus). Afterward, the bio-oil hydrolysate and the activated carbon were placed into an environmental shaker to allow the adsorption to take place in controlled conditions (Table 7). Finally, the bio-oil hydrolysates were transferred to 50 mL falcon tubes and centrifuged at 2285g for 5 minutes (Damon/IEC division CU-5000). The bio-oil hydrolysates were decanted and used for fermentation in subsequent experiments. Table 7 summarizes the parameters for the adsorption on activated carbon in this study. The parameters were chosen based on journal articles that had similar experiments [40, 57, 58, 59].

Table 7 Parameters for activated carbon adsorption

Parameter	Condition A	Condition B
Temperature	25°C	40°C
pH	2	2
Concentration	1% (w/v)	10% (w/v)
Contact time	30 min	1 h.

3.5.3 Overliming

In overliming, $Ca(OH)_2$ was added to the bio-oil hydrolysate (with adequate mixing) until a pH of 10 was reached. The pH of the bio-oil hydrolysate was measured with a pH meter (Thermo Orion, model 710 pH meter). To ensure the accuracy of the equipment, the pH

electrode was calibrated with buffer solutions (pH 4, 7, 10). The hydrolysate was then mixed with a magnetic stirrer for 30 minutes. Afterward, the hydrolysate was centrifuged for 15 minutes at 2285g (Damon/IEC division CU-5000). The supernatant was decanted to be used in subsequent fermentation experiments.

3.5.4 Coagulation and flocculation

The coagulant and flocculant used in the project is called Callaway C-4907 and Profloc 170RR. These two chemicals can be used in low pH (pH 2) environments similar to that of bio-oil hydrolysate. Both substances are called cationic polyacrylamide copolymers in water-in-oil emulsions. The chemistries and compositions of these two chemicals are listed as petroleum distillate and surfactant, whereas the concentrations of the substances are confidential.

The procedure for treating bio-oil hydrolysate with coagulation and flocculation was to pipette the predetermined concentration of the coagulant and flocculant (Table 8) into the bio-oil hydrolysate. Then, the liquids were mixed with magnetic stirrer for 10 minutes and allowed 20 minutes for the materials to congregate. Finally, the congregated material was removed by filtration (Whatman filter paper, Grade 1, 11 μm).

Table 8 Summary of the volume of coagulant and flocculant added

Chemicals	Volume of chemicals (μL)	Volume of hydrolysate (ml)
Callaway C-4907	10	10
Callaway C-4907	20	10
Callaway C-4907	50	10
Profloc 170RR	10	10
Profloc 170RR	20	10
Profloc 170RR	50	10
Callaway C-4907&Profloc 170RR	10	10
Callaway C-4907&Profloc 170RR	20	10
Callaway C-4907&Profloc 170RR	50	10

3.5.5 Solvent extraction for detoxification of bio-oil hydrolysate

The solvents used in the project were alamine 336, aliquat 336, tri-n-octylamine, tributyl phosphate, primene-JMT, oleic acid and oleyl alcohol. Alamine 336 and aliquat 336 are the trade name of chemicals, which are tri-octyl/decyl amine and had a structure of R_3N and R_3NCH_3, respectively [74, 75]. Primene JMT is also a trade name of a primary aliphatic amine which had a structure of R_3-C-NH_2 [76]. Each of the five amines was used in conjunction with two different co-solvents. The co-solvents were 1-octanol and kerosene.

In order to study the effect of the co-solvent, a set of four different co-solvent concentrations was tested on the amines. Solvent extractions were carried out in 50 mL falcon tubes at room temperature. Equal volume of the solvents and hydrolysates were used in the extraction experiment. They were mixed for approximately two hours in an environmental shaker at room temperature (~20°C). After two hours, the solvents and the hydrolysates were separated by centrifugation at 2285g for 10 minutes (Damon/IEC division CU-5000). The hydrolysates were then transferred by pipette to serum vials for fermentation. Table 9 shows the experimental design for the different sets of chemicals used for solvent extraction.

The solvent extraction experiments were initially conducted using a model solution containing 10 g/L glucose and 60 g/L acetic acid. Performances of the solvents were evaluated based on the amount of acetic acid removed and the amount of sugar retained after the extractions. Then, experiments were conducted to test if the solvents were inhibitory to fermentation. This was tested by extracting 15 mL water with 15 mL of the proposed solvents. The water was then added to 35 mL of YP medium and supplemented with 10 g/L of glucose for fermentation.

Table 9 Experimental design for solvent extraction using model hydrolysate

Solvents	Co-solvents (1 – octanol or kerosene)
Alamine 336	0 vol%
Alamine 336	25 vol%
Alamine 336	50 vol%
Alamine 336	75 vol%
Aliquat 336	0 vol%
Aliquat 336	25 vol%
Aliquat 336	50 vol%
Aliquat 336	75 vol%
Primene-JMT	0 vol%
Primene-JMT	25 vol%
Primene-JMT	50 vol%
Primene-JMT	75 vol%
Tri-n-octylamine	0 vol%
Tri-n-octylamine	25 vol%
Tri-n-octylamine	50 vol%
Tri-n-octylamine	75 vol%
Tributyl phosphate	0 vol%
Tributyl phosphate	25 vol%
Tributyl phosphate	50 vol%
Tributyl phosphate	75 vol%
Oleic acid	0 vol%
Oleyl alcohol	0 vol%

3.5.6 Hydrogenation for detoxification of bio-oil hydrolysate

The reactors used in hydrogenation treatments are cylindrical reactors from PARR and Autoclave Engineers. The two reactors are similar and can be used interchangeably when the other reactor is not available. Both reactors are capable of handling a pressure of at

least 2000 kPa and are equipped with a rupture disk as a safety mechanism. The temperature and the stirring speed of the two reactors are controlled by controllers with digital output. Figure 22 – 24 shows the two reactors and the controllers used in this project.

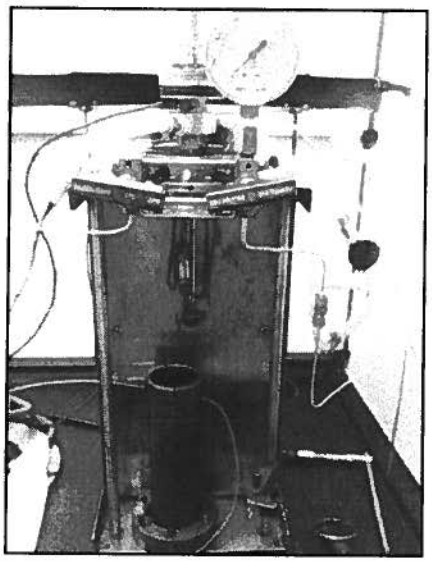

Figure 20 Reactor from Autoclave Engineers used for hydrogenation of artificial hydrolysates

Figure 21 Temperature control (bottom unit) and stirrer control (top unit) for Autoclave Engineers reactor

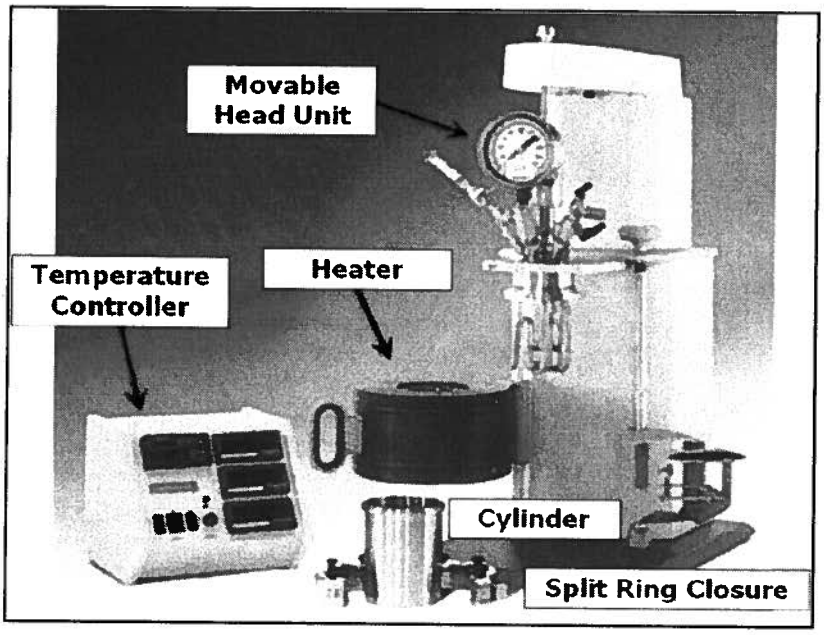

Figure 22 PARR reactor used for hydrogenation of artificial hydrolysate

The catalyst used in the reaction was molybdenum disulphide (MoS_2), which was directly prepared in the reactor. The preparation of the catalyst involved the addition of 0.1 mL of carbon disulphide and 2 mL of ammonium heptamolybdate (AHM) solution. Reactions of hydrogen gas with carbon disulphide and AHM solution would generate the desired catalyst.

$$CS_2 + H_2 \rightarrow CH_4 + H_2S$$

$$H_2S + AHM \rightarrow MoS_2 + NH_3 + H_2O$$

==========

$$CS_2 + H_2 + AHM \rightarrow CH_4 + NH_3 + H_2O + \mathbf{MoS_2}$$

The concentration of the catalyst MoS_2 prepared in the reactor was approximately 600 ppmw. The experiments were conducted with 100 mL of artificial solutions that simulate the bio-oil hydrolysate and the aqueous phase. The temperature was set at 250°C. The stirrer was set at approximately 400 rpm. During the startup, the reactor was purged with nitrogen for 5 minutes and then pressurized with hydrogen to 150 psi for the low pressure condition and 400 psi for the high pressure condition. The compositions of the artificial solutions are shown in Table 10.

Table 10 Concentration of levoglucosan, glucose and acetic acid in the artificial solutions

Compounds	Solution A (g/L)	Solution B (g/L)
Levoglucosan	7	0
Glucose	0	10
Acetic acid	50	50

3.5.7 Adaptive evolution

Adaptive evolution is not a detoxification method, but rather a technique that is aimed at alleviating the effect of the inhibitory compounds through the adaptation of the yeast to inhibitory environments. A known percentage of bio-oil hydrolysates were diluted in YP medium (Table 11). Yeast inoculums were cultured and prepared in YPG medium. Then, the yeasts were inoculated to the YP medium that contained bio-oil hydrolysate, which allowed the yeast to adapt at a non-lethal level. The yeasts were cultured in shake flasks and transferred to new fermentation medium under the same stress conditions every day. It had been reported that the mutants will replace their progenitor genotype every 50 generations [72]. A growth curve was prepared for the yeasts and by solving the equation of the curve, each generation took approximately 7 hours (Figure 23). In this work, the yeasts were adapted for a total of 36 days. In this period of time, the yeasts were adapted for approximately 121 generations. The parameters investigated were concentration of hydrolysate and the growth conditions of the yeasts (aerobic and micro-aerophilic). Table 11 shows the experimental design for this experiment.

Table 11 Growth conditions tested for adaptive evolution of yeast

Sample	Culturing conditions	Hydrolysate volume in medium (vol %)
1	aerobic	35%
2	aerobic	35%
3	aerobic	10%
4	aerobic	10%
5	Micro-aerophilic	35%
6	Micro-aerophilic	35%
7	Micro-aerophilic	10%
8	Micro-aerophilic	10%

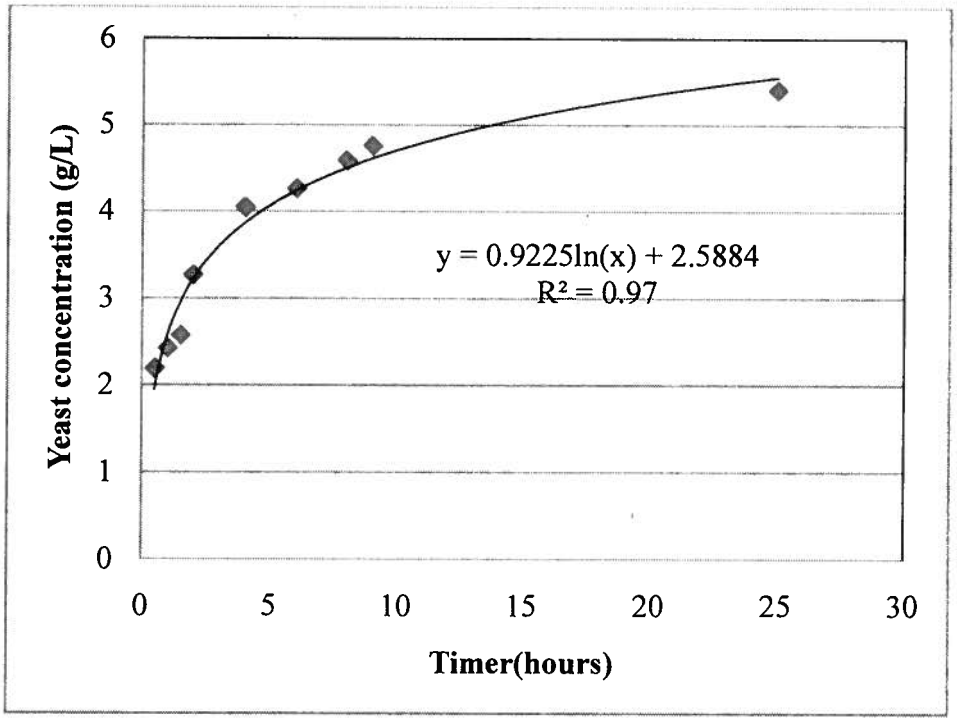

Figure 23 Typical yeast growth curve for *S. cerevisiae* strain T2

3.6 Analytical methods

The samples taken from hydrolysates and fermentation broths were analyzed with gas chromatography (Varian Inc., CP-3800) and high pressure liquid chromatography (Dionex Corp., ED-50) for ethanol and levoglucosan analysis, and sugar analysis, respectively. The concentration of yeast was determined by generating a standard curve formulating the linear relation with optical density and concentration. The optical density was measured with a spectrophotometer (Mandel Scientific Inc., Pharmaspec UV-1700) at 600 nm.

3.6.1 Determining yeast concentration

In order to determine the yeast concentration during fermentation, 1 mL of sample was taken from the fermentation medium at the predetermined sampling time. Each sample was then spun down by centrifugation at 10000g for 10 minutes (Sanyo, MSE micro centaur). The supernatants were used for ethanol analysis, while the yeast pellets were resuspended with 1 mL of deionized water. This process was repeated twice to wash the yeast and ensure the complete removal of any fermentation medium. The re-suspended yeast pellets were then stored in a freezer and used for optical density (OD) analysis.

In order to analyze the yeast samples, they were thawed, diluted 20 – 40 times and were transferred to a cuvette for absorbance analysis in a spectrophotometer. The wavelength used for yeast analysis was 600 nm. The OD was compared with the standard curve prepared for the yeast to determine the yeast concentration. The standard curve was prepared by diluting a known concentration of the yeast. The yeast sample was diluted to an OD reading between 0.2–0.9 and the OD reading was recorded at each dilution (Figure 24).

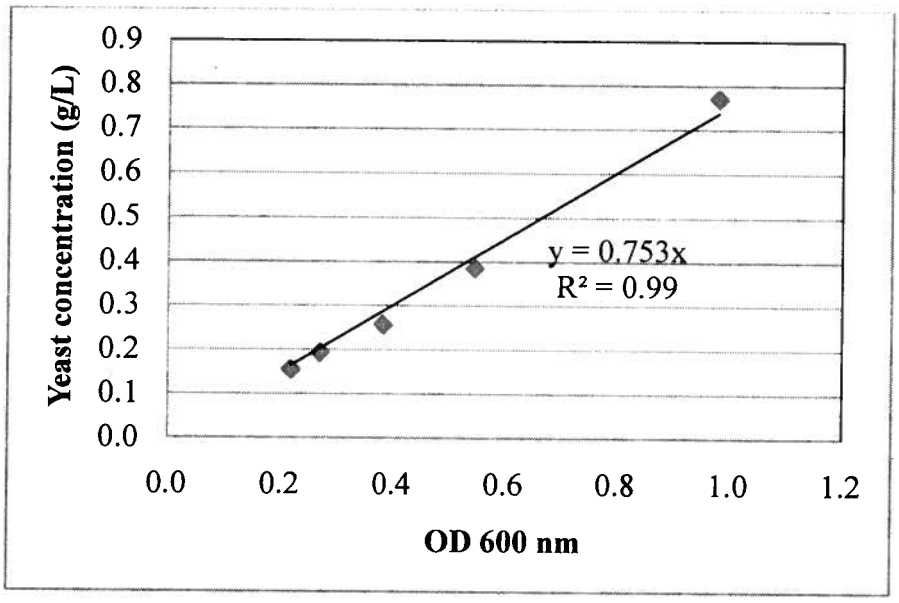

Figure 24 Optical density calibration curve for *S. cerevisiae* strain T2

3.6.2 Analysis of ethanol and levoglucosan by gas chromatography

A gas chromatography (GC) system (CP-3800, Varian Inc.) was used in the project to analyze the concentration of levoglucosan and ethanol. Samples used for ethanol analysis were stored in a freezer. Prior to analysis, the samples were thawed and diluted ten times. The solvent used for dilution was either deionized water for ethanol analysis or acetone for levoglucosan analysis. Then, an internal standard was added to determine the accuracy of the signal output by the GC. The internal standard used was 1-butanol for ethanol analysis or 1-octanol for levoglucosan analysis. The diluted samples and internal standards were added to the sample vials manufactured specifically for the auto-sampler (CP-8400, Varian Inc.) and mixed for 2 minutes by placing them on a vortex.

A software called Star was used to communicate between the GC and the computer. There were five methods programmed to operate the GC. The methods were called standby, ethanol analysis, sugar analysis, condition-ethanol and condition-sugar. When the GC was not in use, the standby method was selected to keep the column at 40°C and maintain the flames for the flame ionization detectors (FIDs). Prior to ethanol analysis

and levoglucosan analysis, the condition-ethanol or condition-sugar method was selected to prepare the GC column for analysis. The condition methods would run for about an hour to purge any impurities out of the system. When one of the analysis methods was selected, the auto-sampler would inject, and analyze the samples automatically. A summary of the methods is tabulated in Table 12.

Table 12 Summary of gas chromatography methods used for ethanol and levoglucosan analysis

	GC methods				
Specification	**Ethanol analysis**	**Levoglucosan analysis**	**Condition ethanol**	**Condition sugar**	**Standby**
Volume of injection	1 μL	1 μL	0 μL	0 μL	0 μL
Temperature of column-oven	50 °C	250 °C	45 °C	250 °C	40 °C
Injector temperature	200 °C	270 °C	200 °C	270 °C	200/270 °C
Detector temperature	200 °C	285 °C	200 °C	285 °C	200/285 °C
Auto-sampler cleaning solvent	Deionized water	Methylene chloride	N/A	N/A	N/A
Internal standard	1-butanol (10 g/L)	1-octanol (10 g/L)	N/A	N/A	N/A
Analysis time/duration	~16 minutes	~72 minutes	~ 1 hour	~ 1 hour	N/A

3.6.3 Analysis of sugars by high pressure liquid chromatography

The high pressure liquid chromatography (HPLC) system (ED-50, Dionex Corp.) is connected to a computer and controlled by a program called Chromeleon Chromatography Management System. This program can control the temperature, pressure, injection volume, as well as the output of the results. Table 13 summarizes the operating conditions for carbohydrate analysis with the HPLC.

Table 13 Operating conditions for high pressure liquid chromatography analysis of carbohydrate

Specification	Carbohydrate method
Temperature	30 °C
Pressure	200 - 2000 psi
pH	10 - 13
Injector volume	25 µL
Flush volume	250 µL
Internal standard	Fucose (5g/L)
Analysis time	60 minutes

The procedures for sample preparation for HPLC analysis are similar to those for the GC. Samples in storage were thawed and diluted 50 times in nanopure water. Due to the sensitivity and vulnerability of the HPLC column, nanopure water must be used throughout the entire system. The specification of the nanopure water is shown in Table 14.

Table 14 Specifications of nanopure water used in high pressure liquid chromatography

Specification	
Resistivity at 25 °C	18.2 MΩ cm2/cm
Total organic carbon (TOC)	< 1 ppb
Bacteria	< 1 CFU/ml
pH at 25 °C	7
Particles	<0.2 µm

In order to analyze the sugar content of the samples with the HPLC system, a number of preparation procedures are required. Before every set of analysis samples, it was necessary to prime the GP-50 gradient pump and the TTL pump in the HPLC system to make sure that there were no leaks throughout the piping network. Then, a ready check was performed using the Chromeleon program. When all the preparations were complete, data acquisition was activated and ran for 10 – 15 minutes until signals from the HPLC stabilized. After the signal from the HPLC system was stabilized, the analysis could then begin manually.

Chapter 4 Levoglucosan extraction from bio-oil

4.1 Bio-oil titration to determine phase behavior

The first goal in levoglucosan extraction was to determine the critical weight ratio of water and bio-oil that would induce phase separation. This was determined through several titration experiments. In the experiments, 20.14 mL of bio-oil was used as the solvent and water was slowly added to bio-oil until phase separation occurred. Phase separation was observed at about 2.38 mL of water. In mass ratio, this was approximately 9.86 wt% of water added to bio-oil. Comparing this with the results from Peacocke et al. [33], who indicated that phase separation occurred at 20 – 23 wt%, shows a significant difference [33]. Other researchers have reported that phase separation occurs at 27 wt% for birch, 23 – 25 wt% for pine and 31 wt% for poplar [6, 30]. However, these results were reported as the weight percentage of total water within the system [6, 33]. The water content of the bio-oil for this project (produced by VTT) was reported to be in the range of 17.4 – 21.1 wt% [6, 30, 77]. Thus, the total water content that induced phase separation was found to be in the range of 27.3 – 31.0 wt%.

4.2 Suspended solids in aqueous phase

Upon separation, the aqueous phase was poured into a falcon tube for centrifugation at 4650g (Damon/IEC division, CU-5000). This was to remove any suspended solids that might be present in the aqueous phase. Initially, the aqueous phase was clear after centrifugation and solid particles were removed. However, suspended solids were observed at the bottom of the container when aqueous phase samples were stored in a refrigerator at 4°C or at room temperature for more than one day. Since bio-oils were known to be chemically unstable, it was hypothesized that suspended solids were forming in the aqueous phase.

4.3 Effect of temperature on levoglucosan extraction

An experiment was conducted to determine if temperature has any effect on the extraction of levoglucosan. Levoglucosan was extracted with 100 wt% of water at two different temperatures (25°C and 80°C). It was found that altering the temperature had no significant effect on the amount of levoglucosan being extracted from bio-oil. The average concentration for levoglucosan extracted at 25°C was found to be 51.4±5.2 g/L and 50.3±1.5 g/L at 80°C. At high temperature, bio-oil was observed to be completely dissolved into water in equal mass ratio (1:1). This resulted in a single phase solution. However, phase separation was observed once the solution cooled down and the amounts of levoglucosan extracted were similar to cold water extraction.

4.4 Effect of the order of solvent addition on levoglucosan extraction

Initial experiments were conducted to determine whether the addition of water to bio-oil and the addition of bio-oil to water would generate the same results. The results obtained from this experiment are shown in Figure 25. It was found that at water to bio-oil extraction ratios of 50 wt% and 100 wt%, the differences were 1.96% and 1.89%, respectively. The standard deviation of the levoglucosan method was evaluated with five runs of standard aqueous phase extracted from the same batch of bio-oil and was found to be 4.9%. Comparatively, the differences in levoglucosan concentrations between adding bio-oil to water and adding water to bio-oil were small. Thus, it was safe to conclude that the amount of levoglucosan extracted was independent of whether water was added to bio-oil or vice versa. From this result, the subsequent levoglucosan extraction experiments were conducted by the addition of bio-oil to water.

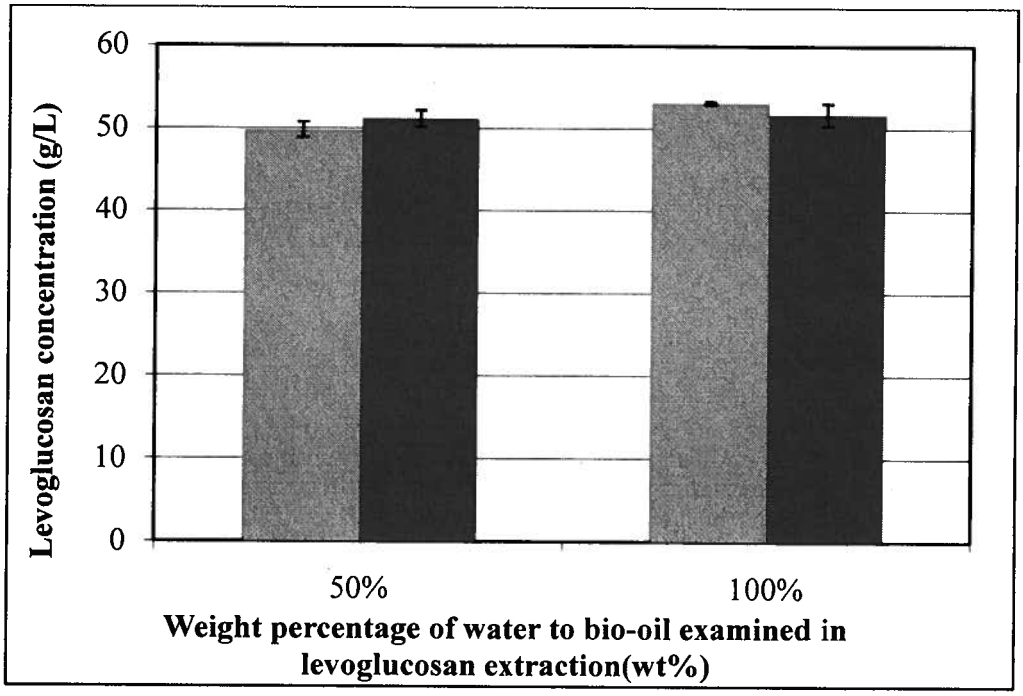

Figure 25 Comparison of levoglucosan extracted between the addition of water to bio-oil (light colored bars) and bio-oil to water (dark colored bars)

4.5 Effect of mixing on levoglucosan extraction

In addition to the order of adding bio-oil and water, two different mixing methods were evaluated. They were referred as the handheld mixer and the vortex mixer method (Figure 16). The extractions ratios examined were 50 wt% and 100 wt% of water in bio-oil. These two methods were to examine if the tar like organic phase formed during the initial contact of bio-oil and water was interfering with the levoglucosan extraction. Thus, the handheld mixer was used to break and mash the congealed organic phase, while the vortex mixer provided only gentle mixing. It was found that the difference between the two method of mixing were small (Figure 26). At 50 wt% and 100 wt% the difference between the two mixing methods were 1.5% and 4.6%, respectively. This indicated that the organic phase did not act as a barrier to the extraction of levoglucosan.

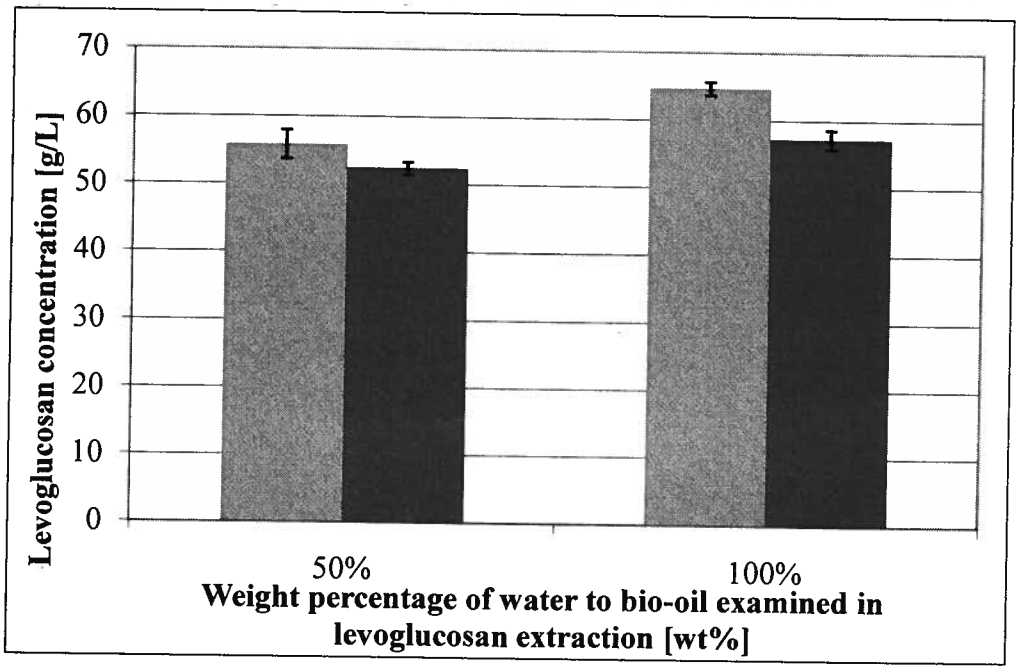

Figure 26 Comparison of levoglucosan extraction between handheld mixer (light colored bars) and vortex mixer (dark colored bars)

4.6 Optimal water-to-bio-oil ratio for levoglucosan extraction

It was seen in section 4.3 – 4.5 that variation of the parameters (temperature, mixing, adding water to bio-oil or vice versa) had minimal effect on levoglucosan extraction. However, a common trend was observed for both of the above experiments, which exhibited a decrease in the concentration of levoglucosan extracted when the extraction ratio decreased from 100 wt% to 50 wt%. The average concentration of levoglucosan at 100 wt% extraction ratio was 4% and 7% higher than extraction at 50 wt% in the first experiment (addition of water to bio-oil or vice versa) and the second experiment (different mixing method), respectively. This suggested that the amount of water used in levoglucosan extraction could have more influence than the other parameters. Thus, a set of experiments with extraction ratios from 100 wt% to 2000 wt% was conducted. The results were shown in Figure 27. A decreasing trend was observed for the ratios examined. The data were fitted with a second degree polynomial with coefficient of determination of 0.97.

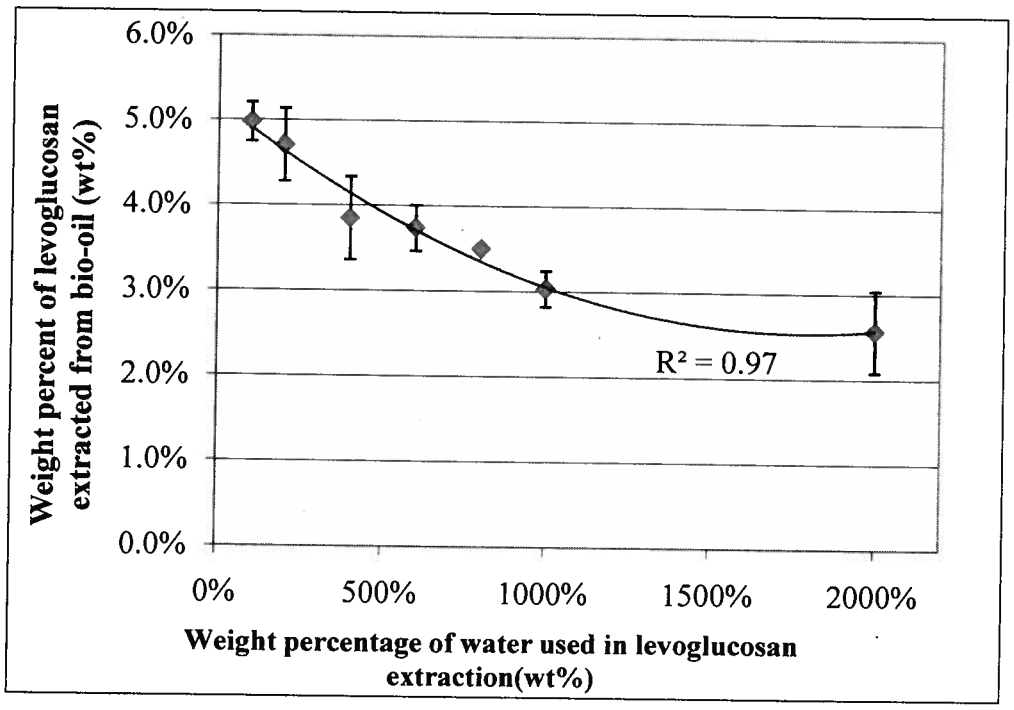

Figure 27 Effect of water addition on levoglucosan extraction (100 – 2000 wt% water-to-bio-oil)

Further extraction experiments with less than 100 wt% of water are needed in order to determine the optimal extraction ratio of water to bio-oil. From the titration results, 9.86 wt% of water in bio-oil was needed to induce phase separation. Thus, the lowest ratio chosen for examination was 10 wt%. Figure 28 shows the results for experiments with low extraction ratios (10 wt% - 50 wt%).

It can be seen that there was an increasing trend in the extraction of levoglucosan with an increase in the mass ratio of water used in the extractions (Figure 28). A logarithmic trend was able to fit the set of results with a coefficient of determination value of 0.86. Combining Figure 28 with previous results for higher water to bio-oil extraction ratios (Figure 27) it was found that the optimal ratio for levoglucosan extraction with water was 100 wt%.

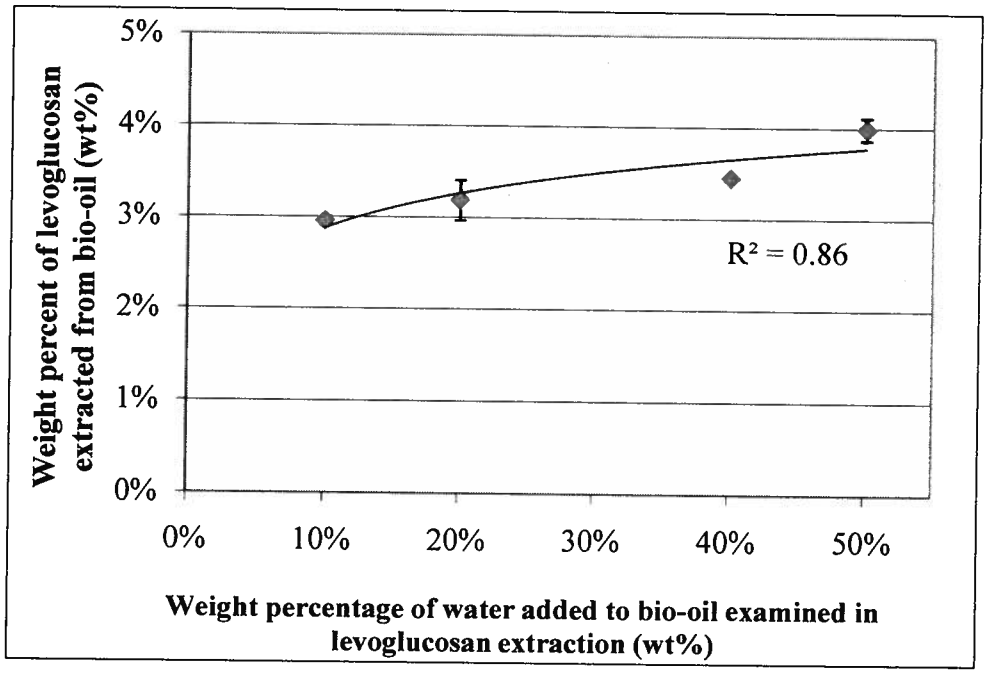

Figure 28 Effect of water addition on levoglucosan extraction (10 – 50 wt% water-to-bio-oil)

The maximum levoglucosan concentration obtained was approximately 4.98 wt% (g levoglucosan/ g bio-oil). In Bennett's work, the maximum value of levoglucosan extracted was reported to be 5.1 wt% (g levoglucosan/ g bio-oil) at an extraction ratio of 60 wt% (water to bio-oil) [6]. Although the maximum value of levoglucosan extracted coincided with Bennett's work, the optimal extraction ratio was significantly different. In Bennett's work, at 100 wt% extraction ratio of water to bio-oil, the weight of levoglucosan extracted was 2.2 wt% (g levoglucosan/ g bio-oil) [6]. A possible explanation to the differences was that the aging of the bio-oil caused a change in the composition of compounds and properties of bio-oil.

4.7 Acetic acid in aqueous phase

Acetic acid was one of the inhibitory compounds that got extracted along with levoglucosan. The amount of acetic acid found in the aqueous phase varies depending on

the extraction conditions. Figure 29 shows the amount of acetic acid extracted for the tested extraction ratios of water to bio-oil.

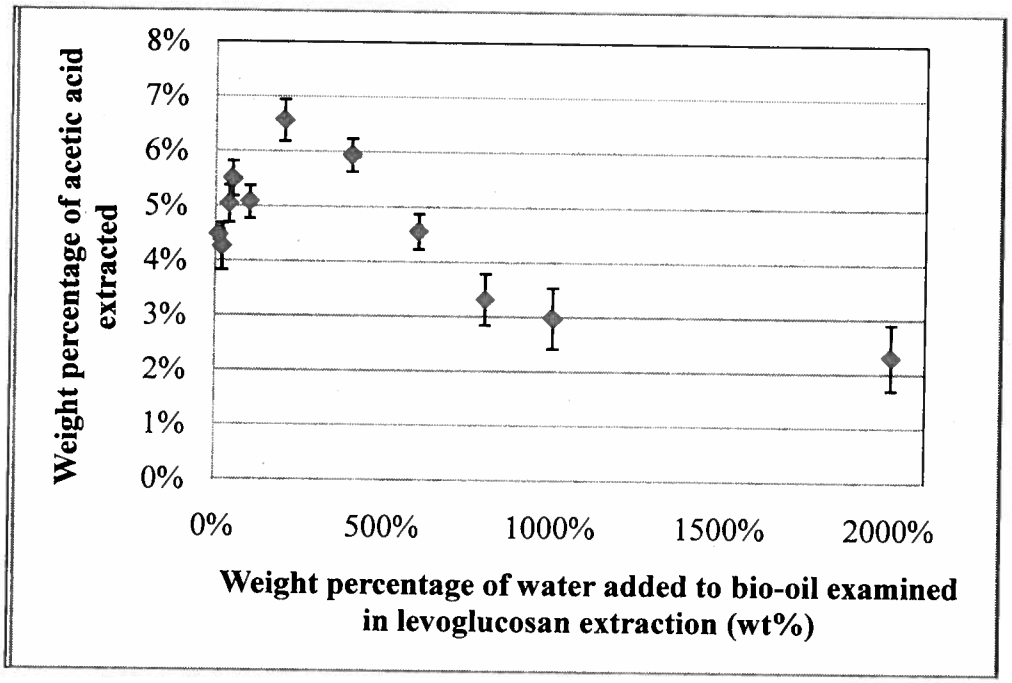

Figure 29 Weight percentage of acetic acid extracted from bio-oil (10-2000 wt% water-to-bio-oil)

An increase in the concentration of acetic acid was observed as the extraction ratio increased from 10 wt% to 50 wt%. The maximum amount of acetic acid extracted from bio-oil was 6.56 wt% (at extraction ratio of 100 wt% water to bio-oil). Similar to the levoglucosan samples, the consistencies of the GC results were tested with five aliquots from the same sample of bio-oil extract. The standard deviation was found to be 8.2%.

4.8 Other compounds in aqueous phase

A number of compounds were extracted to the aqueous phase. An average of 63 peaks (excluding internal standard) were detected in the aqueous phase for the proposed extraction ratios. In a similar experiment, Siplar et al. [78] induced phase separation by adding water to bio-oil at a mass ratio of 1:10 and identified 45 compounds in pine wood

derived bio-oil with gas chromatography and mass spectrometry [78]. The name of the compounds can be found in Table 18 in the Appendix. In this project, the identities and concentrations for most of the compounds were unknown. However, they were quantified indirectly according to the mass transferred from the bio-oil fraction to the aqueous fraction. Figure 30 shows the results of mass transferred from bio-oil to the aqueous fraction after extracting bio-oil with water in various extraction ratios.

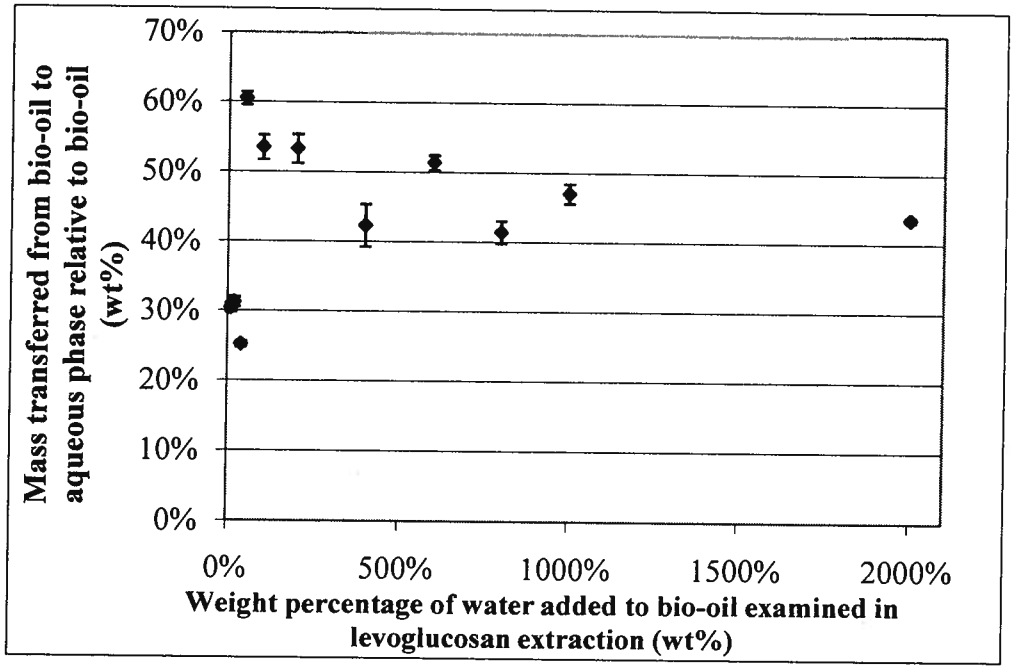

Figure 30 Mass transferred from bio-oil to aqueous phase (10-2000 wt% water-to-bio-oil)

From the results obtained, it was found that the mass transferred from the bio-oil to the aqueous phase decreases by 7 – 30% as the water-to-bio-oil extraction ratios deviated from the 1:1 mass ratio of water to bio-oil. This finding reinforced previous finding that at 100 wt% water to bio-oil or 1:1 mass ratio, extraction was most efficient. However, the efficiency was not limited to levoglucosan but also includes some of the compounds that are extractable by water.

Chapter 5 Methods for improving the fermentability of bio-oil hydrolysate

5.1 Effect of hydrolysate concentration on fermentation by *S. cerevisiae*

Due to the presence of inhibitors in bio-oil, it was necessary to dilute the bio-oil hydrolysate with sterile water to lower the concentration of the inhibitors so that the yeasts were able to survive in the medium. An experiment was conducted to determine the highest concentration of the bio-oil hydrolysate that the yeasts were able to survive. The bio-oil hydrolysates were diluted based on the volume in milliliters and were referred as the strength of the hydrolysate. For example, 25% strength hydrolysate would mean 25 vol% of hydrolysate and 75 vol% of sterile water. The strength of the hydrolysate tested ranged from 2 – 45%. Figure 31 shows the concentration of ethanol produced in the fermentation medium (52 mL), and Table 15 shows the ethanol yield based on available glucose for each set of the experiments.

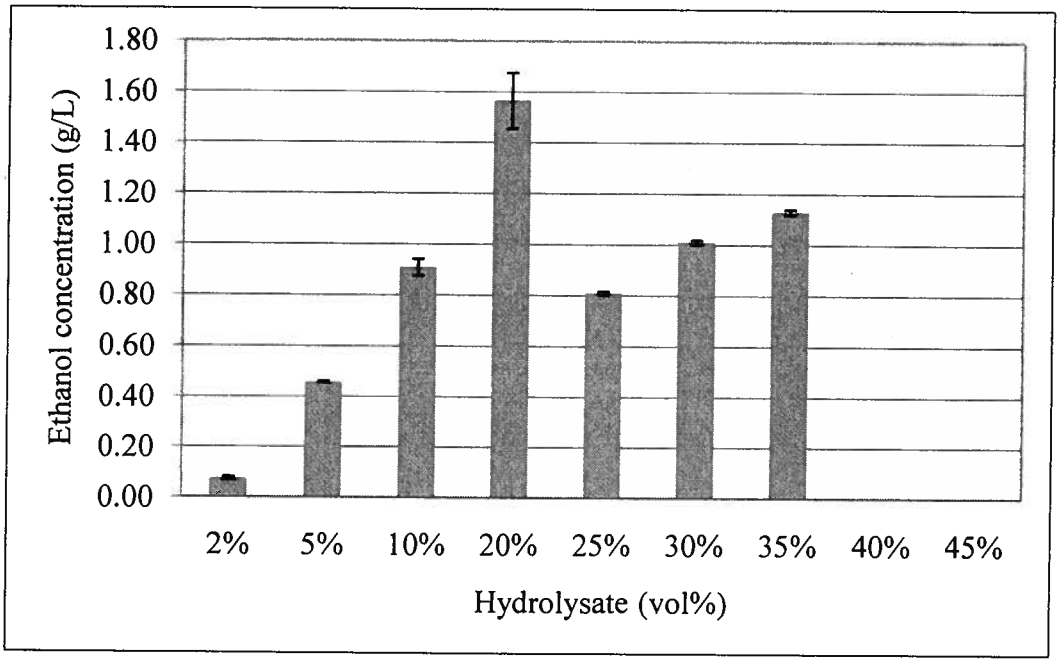

Figure 31 Ethanol produced in various strengths of hydrolysate

Table 15 Effect of hydrolysate concentration on ethanol yield

Fermentation medium	Yield (g ethanol / g glucose)
2% hydrolysate	0.20±0.08
5% hydrolysate	0.49±0.01
10% hydrolysate	0.49±0.04
20% hydrolysate	0.42±0.09
25% hydrolysate	0.18±0.01
30% hydrolysate	0.18±0.01
35% hydrolysate	0.17±0.01
40% hydrolysate	0.0
45% hydrolysate	0.0

It was found that the ethanol yield was highest for 5% hydrolysate. At 2% hydrolysate, the ethanol yield was only 0.20±0.08 (g ethanol/g glucose). This was probably due to the scarce amount of the sugar available to the yeast. In terms of toxicity, hydrolysates at 5% and 10% strength were able to achieve a relatively high yield of 0.49±0.01 and 0.49±0.04 (g ethanol/g glucose). These two values were close to the theoretical ethanol yield of 0.57 (g ethanol/g levoglucosan), which had an equivalent concentration of 0.51 (g ethanol/ g glucose) reported by Bennett [6]. However, it was found that the hydrolysate became more inhibitory at 20% strength. The yield of ethanol was decreased by 14% when the strength of hydrolysate increased from 10 to 20%. As the strength of hydrolysates increased from 20% to 25%, the ethanol yield was decreased by 58.4%. At 40% v/v hydrolysate, the yeasts were not able to produce any ethanol because the concentration of bio-oil hydrolysate was too high in the fermentation medium. This set of experiments provided insights for evaluating the detoxification methods proposed.

5.2 Detoxification of artificial aqueous phase and hydrolysate by hydrogenation

The first detoxification method experimented was hydrogenation of artificial solutions. As mentioned in the materials and methods (section 3.5.6), the solutions contained 7 g/L of levoglucosan and 50 g/L of acetic acid in solution A and 10 g/L of glucose and 50 g/L of acetic acid in solution B. The use of levoglucosan and glucose could provide some

insight as to whether this detoxification method was best applied before, or after the hydrolysis of the aqueous phase. The results for the two sets of experiment are tabulated in Table 16.

Table 16 Concentration of levoglucosan, glucose and acetic acid in artificial solution A and B after hydrogenation

Rxn time (hr)	Artificial solution A		Artificial solution B	
	LG conc. (g/L)	AA conc. (g/L)	Glucose conc. (g/L)	Acetic acid conc. (g/L)
0	7.0	50.0	10.0	50.0
1	4.4	43.6	3.4	30.4
2	0.8	25.8	0.9	26.3
3	0	17.3	0	19.4
4	0	23.0	0	20.2

It was found that the degradation of levoglucosan was very rapid. This was especially true at higher temperature. This observation was made because the temperature was steadily increasing from 0 hour to 1 hour. After about 25 minutes of reaction time, the temperature reached 250°C, which was the proposed hydrogenation temperature. Thus, the slower degradation rate of levoglucosan during the first hour was very likely due to the relatively milder overall reaction temperature. This was also true for acetic acid degradation. However, the degradation of acetic acid was much slower than levoglucosan. It took about 2 hours to reduce the amount of acetic acid by 50% while levoglucosan degraded by 88.6% in 2 hours. It was found that the degradation of levoglucosan, glucose and acetic acid followed first order reaction kinetics (Figure 32). The first order reaction constant was found to be 1.08 hr^{-1}, 1.20 hr^{-1} and 0.34 hr^{-1} for levoglucosan, glucose and acetic acid, respectively (Figure 32).

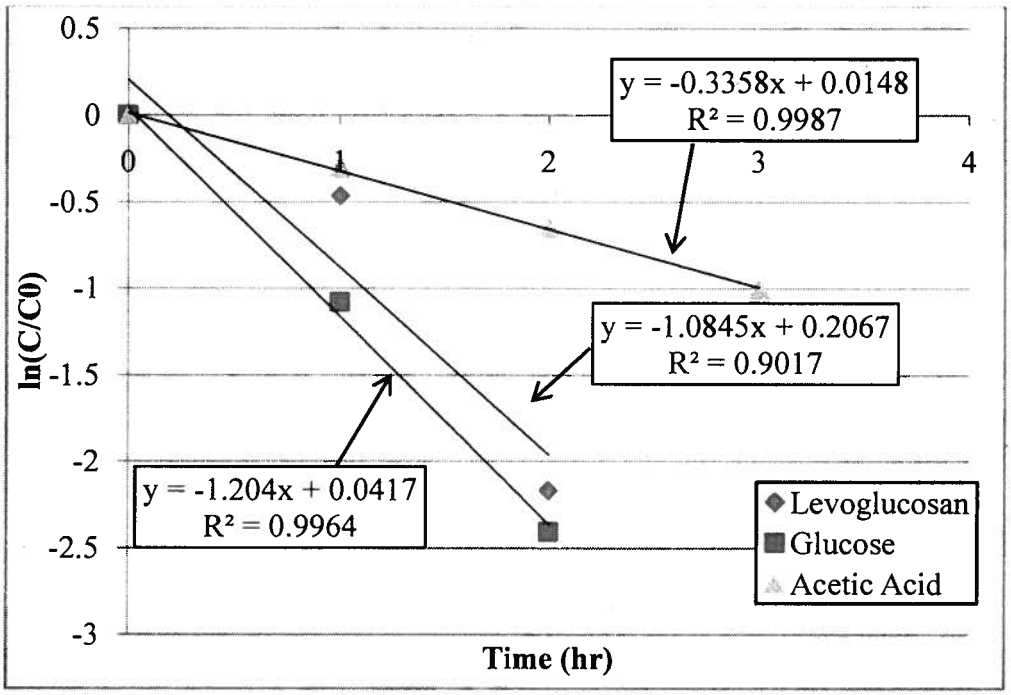

Figure 32 First order reaction kinetics for hydrogenation of levoglucosan, glucose and acetic acid

In order to determine the feasibility of this technique the reaction time of sugar was also considered. This was to determine if the bio-oil hydrolysate could generate better results. Table 16 shows the results of hydrogenation of the artificial hydrolysate. It can be seen that dextrose had been reacted completely in 3 hours while a substantial amount of acetic acid remained unreacted. This was similar to the results reported by van Gorp et al [79]. In van Gorp's work [79], glucose was hydrogenated at 100°C and 120°C with a hydrogen pressure of 40 bar [79]. All of the glucose was reacted in approximately 80 minutes at 100°C and 140 minutes at 120°C [79]. Comparing the rate of degradation of dextrose and levoglucosan, it was found that dextrose degraded 30% more than levoglucosan during the first hour of hydrogenation.

There was a possibility that the difference in concentration between the sugars and acetic acid could generate biased results. Nevertheless, the initial experiments suggested that hydrogenation would not be very beneficial if the degradation was not selective. In addition to the non-selective nature, the degradation rate of acetic acid was relatively

slow compared to the sugars. All in all, hydrogenation might not be beneficial for the project under the proposed operating conditions and catalyst.

5.3 Detoxification using activated carbon, air stripping and overliming

Commonly used detoxification techniques were applied to treat bio-oil hydrolysate. The techniques used were air stripping, adsorption on activated charcoal and overliming. These techniques were targeted toward compounds such as phenolics, furans and volatile organic compounds in bio-oil hydrolysates, which were inhibitory to fermentation. As mentioned in the materials and methods section, instead of identifying and quantifying the compounds adsorbed, the evaluation of the detoxification techniques were based on the ethanol yield from the fermentation of the treated bio-oil hydrolysate. All of the treated bio-oil hydrolysates were diluted with sterilized water to 40% and 50% strength before initiating fermentation. Air stripping at the proposed temperature, time and pH (25°C, 50°C and 80°C for 30 minutes and 1 hour, pH 10) failed to detoxify the bio-oil hydrolysates to a sufficient level for ethanol production. A possible explanation was that the volatile components in the aqueous phase were not the major inhibitors in the fermentation process. Thus, removing the volatile components via air stripping did not improve the fermentability of the hydrolysates or none of the inhibitory compounds was removed from the hydrolysate.

For adsorption with activated charcoal, the same results were observed. In the experiment, 1 wt% and 10 wt% of activated charcoal were added to the hydrolysate at two different temperatures (25°C and 40°C). Hydrolysates treated with activated charcoal under these conditions showed no improvement in the fermentability. In addition, no ethanol was produced at 40% and 50% strength of bio-oil hydrolysate. However, the treated hydrolysates did become lighter in color which suggested some degree of chemical removal by the activated charcoal.

The overliming technique showed more promising results. At 50% strength of bio-oil hydrolysates, the yield of ethanol was 0.19±0.01 (g ethanol/g glucose). This was a significant improvement in the fermentability of the hydrolysates. The improvement in the fermentability and the yield of ethanol was most significant on the 40% strength bio-oil hydrolysate. As one might recall from previous results, the hydrolysate was found to be too toxic for the yeast to produce any ethanol at 40% strength. However, the ethanol yield at 40% strength was 0.45±0.05 (g ethanol/g glucose) when treated with the overliming techniques. This ethanol yield was not far from the ethanol yield from the controlled fermentations with YPG mediums (0.47 g ethanol/g glucose) and the theoretical ethanol yield reported (0.51 g ethanol/g glucose) [6].

5.4 Detoxification by coagulation and flocculation

Treatments using coagulant and flocculant removed 3.4 – 6.5 wt% of materials from bio-oil hydrolysate by settling. Table 17 shows the weight of the materials removed, as well as the weight percentage of the settled material. However, the removal of these materials did not improve the fermentability of bio-oil hydrolysate at 40% and 50% strength. Thus, a higher dosage of coagulant and flocculant (100 μL/10 ml) was attempted but this resulted in the hydrolysate forming a thick paste which behaved like single phase. This was undesirable as no material could be removed by settling or filtration.

Table 17 Materials removed by treatment with coagulation and flocculation

Chemicals added	Settled weight (g)	Settled weight (wt%)
Callaway C-4907 (10μL/10ml hydrolysate)	0.5751	3.60%
Callaway C-4907 (20μL/10ml hydrolysate)	0.5983	3.82%
Callaway C-4907 (50μL/10ml hydrolysate)	0.7018	4.91%
Profloc 170RR (10μL/10ml hydrolysate)	0.5488	3.38%
Profloc 170RR (20μL/10ml hydrolysate)	0.6846	4.76%
Profloc 170RR (50μL/10ml hydrolysate)	0.8501	6.30%
Both (10μL/10ml hydrolysate)	0.6136	4.03%
Both (20μL/10ml hydrolysate)	0.6000	3.96%
Both (50μL/10ml hydrolysate)	0.8663	6.52%

5.5 Detoxification by extraction with organic solvents

In the solvent extraction experiments conducted, the targeted inhibitor in bio-oil hydrolysate was acetic acid. Initial experiments with solvent extractions involved artificial hydrolysates, which consisted of only sugar and acetic acid. Further experiments were then conducted to determine if the selected solvents were inhibitory to fermentation. This was achieved by comparing the amount of ethanol produced with fermentation medium extracted with the proposed solvents and the control medium as described in materials and methods (Chapter 3, section 3.5.5). Based on these results, the solvents were screened and selected to apply to bio-oil hydrolysates. In addition, the results of the best three solvents were compared.

5.5.1 Solvent extraction for acetic acid removal from artificial bio-oil hydrolysate

The artificial hydrolysates used in the experiments contain approximately 10 g/L of glucose and 60 g/L of acetic acid. Figure 33 shows the percentage removal of acetic acid with 1-octanol and kerosene as the co-solvent. The percentage removals were calculated based on the concentration of acetic acid after the extractions. From Figure 33, it can be seen that the removal of acetic acid increases as the volume of 1-octanol in the system increases for alamine 336, primene-JMT and tri-n-octylamine. The two highest amounts of acetic acid removal were by the solvents alamine 336 and tri-n-octylamine. At 25 vol% alamine 336 in 1-octanol and 25 vol% tri-n-octylamine in 1-octanol removal of up to 86±5.2% and 90.4±6.8% of acetic acid were achieved.

Kerosene, another co-solvent examined in the extraction experiments, was compared against 1-octanol. The comparison is shown in Figure 33. It was found that the amount of acetic acid removed by the solvents with kerosene was lower than with 1-octanol. There was a decreasing trend in the amount of acetic acid being removed with an increasing volume of kerosene in the solvent system. This indicated that as a co-solvent, kerosene was impairing the extraction of acetic acid by other solvents. However, contrary to this general trend, an increase in acetic acid removal was observed with increasing volume of kerosene for primene-JMT.

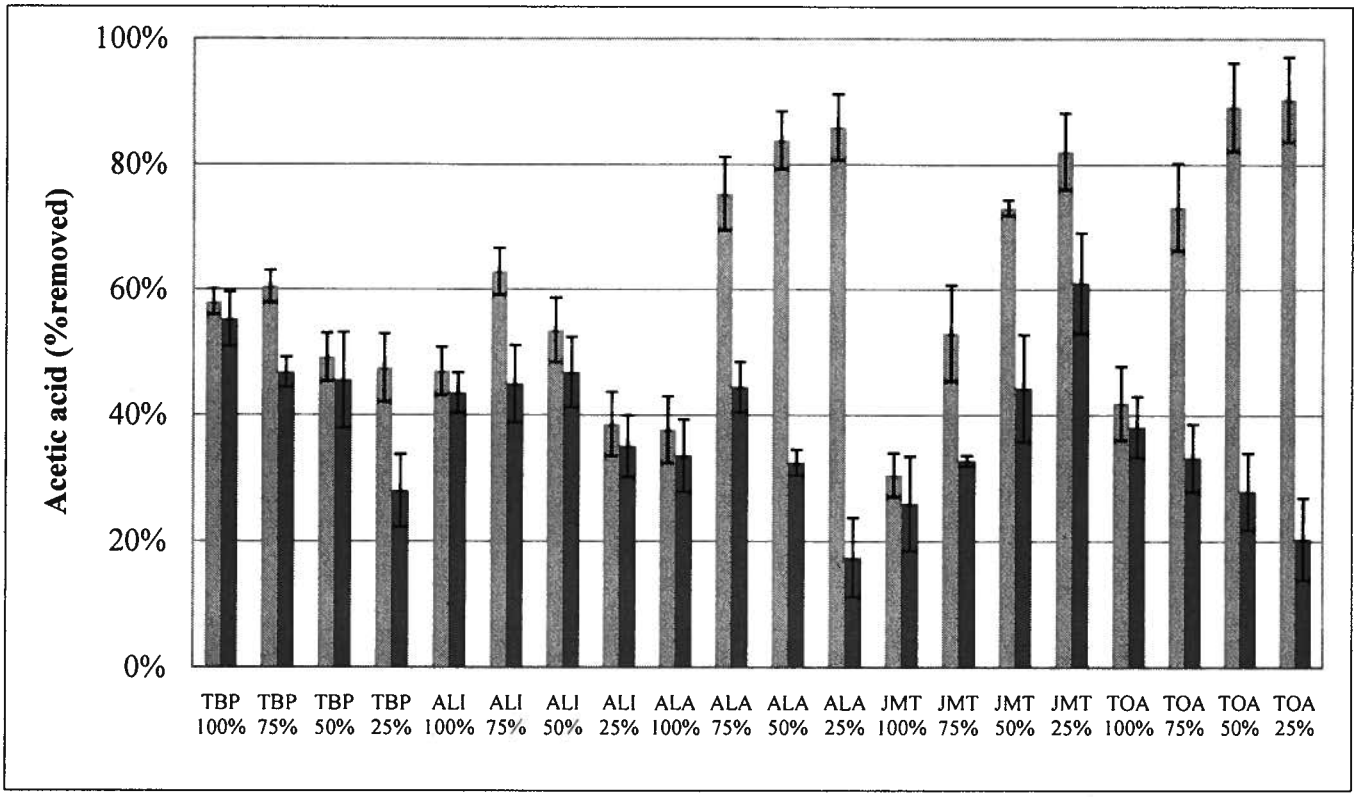

Figure 33 Acetic acid removals, co-solvents: 1-octanol (light colored bars) and kerosene (dark colored bars), solvents: TBP = tributyl phosphate, ALI = aliquot 336, ALA = alamine 336, JMT = primene JMT, TOA = tri-n-octylamine

The amount of glucose retained was also an important aspect to the evaluation of the solvent extraction methods. The results of this experiment are shown in Figure 34. Some of the tested solvents were able to selectively remove acetic acid while completely retaining glucose. This was true for aliquot 336 in 1-octanol, alamine 336 in 1-octanol, tributyl phosphate in 1-octanol and tri-n-octylamine in 1-octanol. The rest of the solvents also showed very good selectivity towards acetic acid removal. The retention of glucose ranged from 76.3% to 100%. The two best solvents for acetic acid removal, 25 vol% alamine 336 with 1-octanol and 25 vol% tri-n-octylamine with 1-octanol retained 97.3±1.9% and 100±1.4% glucose, respectively. The selective removal of acetic acid further supported the feasibility of detoxifying the bio-oil hydrolysates with these two solvents.

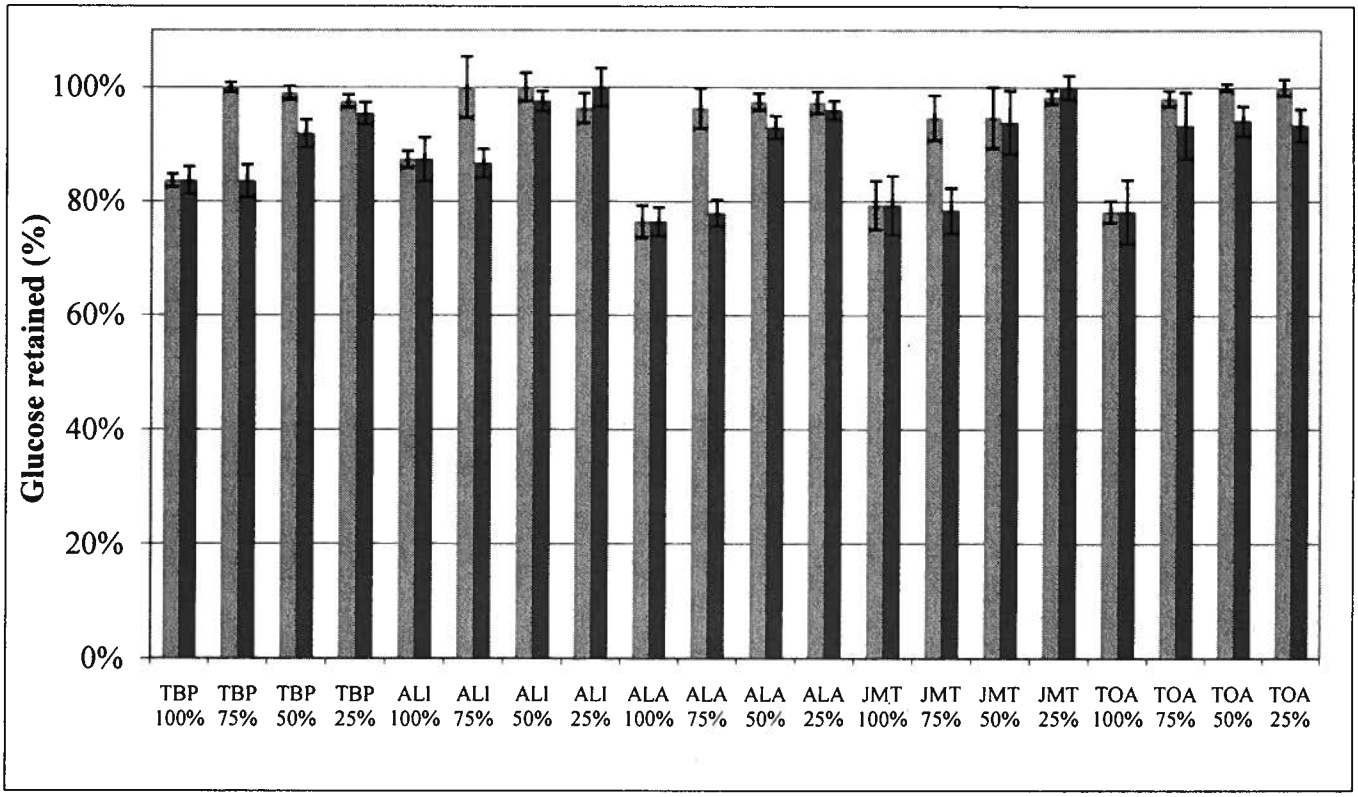

Figure 34 Percentage of glucose retained after extraction, co-solvents: 1-octanol (light colored bars) and kerosene (dark colored bars), solvents: TBP = tributyl phosphate, ALI = aliquot 336, ALA = alamine 336, JMT = primene JMT, TOA = tri-n-octylamine

5.5.2 Evaluation of the toxicity of the solvents towards fermentation

During the extraction process, trace amounts of the solvents could be dissolved in the bio-oil hydrolysate. This could introduce new inhibitors to the hydrolysate. Thus, it was necessary to evaluate the toxicity of the solvents. This was done by comparing the yeast growth and ethanol yield of extracted artificial hydrolysates with control samples. The control samples were YP medium (1% yeast extract, 2% peptone) with a supplemented sugar concentration of 10 g/L. The proposed solvents were used to extract distilled water as described in the materials and methods section (Chapter 3, section 3.5.5). Thus, the extractions were only meant to contaminate the model hydrolysate with the proposed solvents from the detoxification experiment. In this experiment, acetic acid was not

added to the artificial hydrolysate to reduce the number of factors and make the results easily comparable.

It was found that three of the solvents, aliquot 336, primene-JMT and tributyl phosphate severely inhibited yeast activity, with no growth observed in the artificial hydrolysates (Figure 35). Furthermore, no ethanol was detected in the hydrolysates after 48 hours of fermentation which agreed with the result that there were no yeast activity. The other two solvents tri-n-octylamine in diluted 1-octanol and alamine 336 diluted in 1-octanol exhibited less severe inhibition of yeast growth (Figure 35). Nevertheless, these two solvents did exhibited 22 – 30% reduction in yeast growth for alamine 336 in 1-octanol and 12 – 20% reduction for tri-n-octylamine in 1-octanol. From this results, the only suitable solvents for the project were alamine 336 in 1-octanol and tri-n-octylamine in 1-octanol.

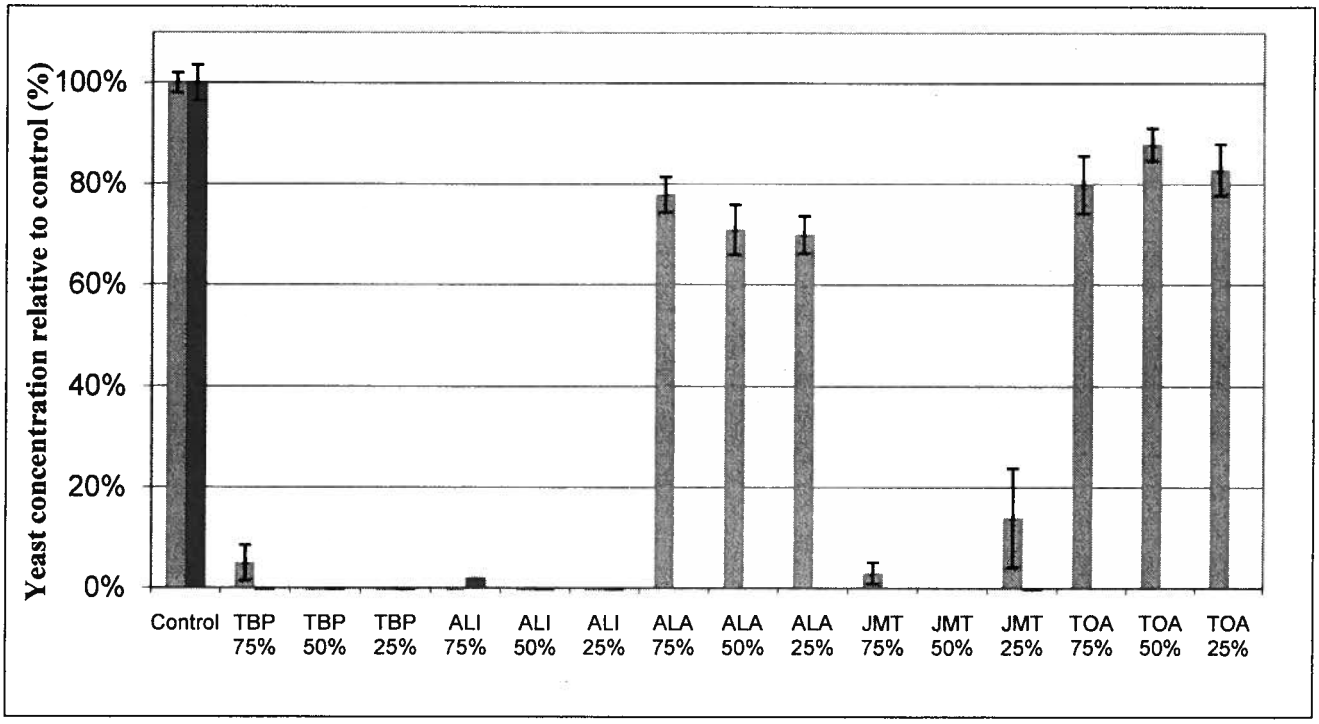

Figure 35 Comparison of yeast concentration after 48 hours fermentation, co-solvents: 1-octanol (light colored bars) and kerosene (dark colored bars), solvents: TBP = tributyl phosphate, ALI = aliquot 336, ALA = alamine 336, JMT = primene JMT, TOA = tri-n-octylamine

The inhibition of ethanol production was also evaluated by comparing with the control (Figure 36). Surprisingly, for both of the solvents the inhibition of biomass production was more severe than the ethanol production. This could be due to the difference in mechanism between biomass production and ethanol production for the yeast. Once the ethanol production was compared, it was clear that the best solvent for detoxifying the bio-oil hydrolysates for the project was tri-n-octylamine in 1-octanol.

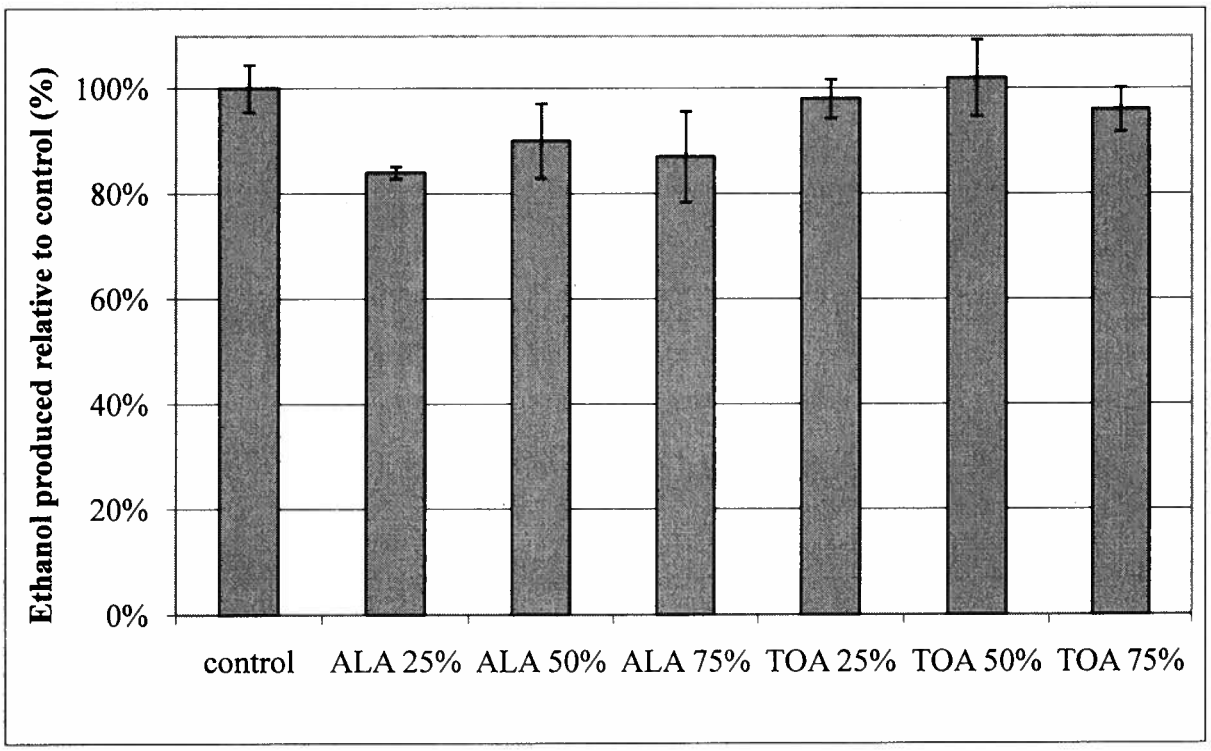

Figure 36 Comparison of ethanol concentration after 48 hr fermentation (alamine 336 and tri-n-octylamine in 1-octanol)

5.5.3 Fermentation of bio-oil hydrolysate treated with solvent extraction

Oleic acid and oleyl alcohol were used in this project to compare with the best solvent found from the extraction results. Their abilities to selectively extract acetic acid while retaining glucose were compared (Figure 37). As well, the practicability of these solvents was compared based on ethanol production.

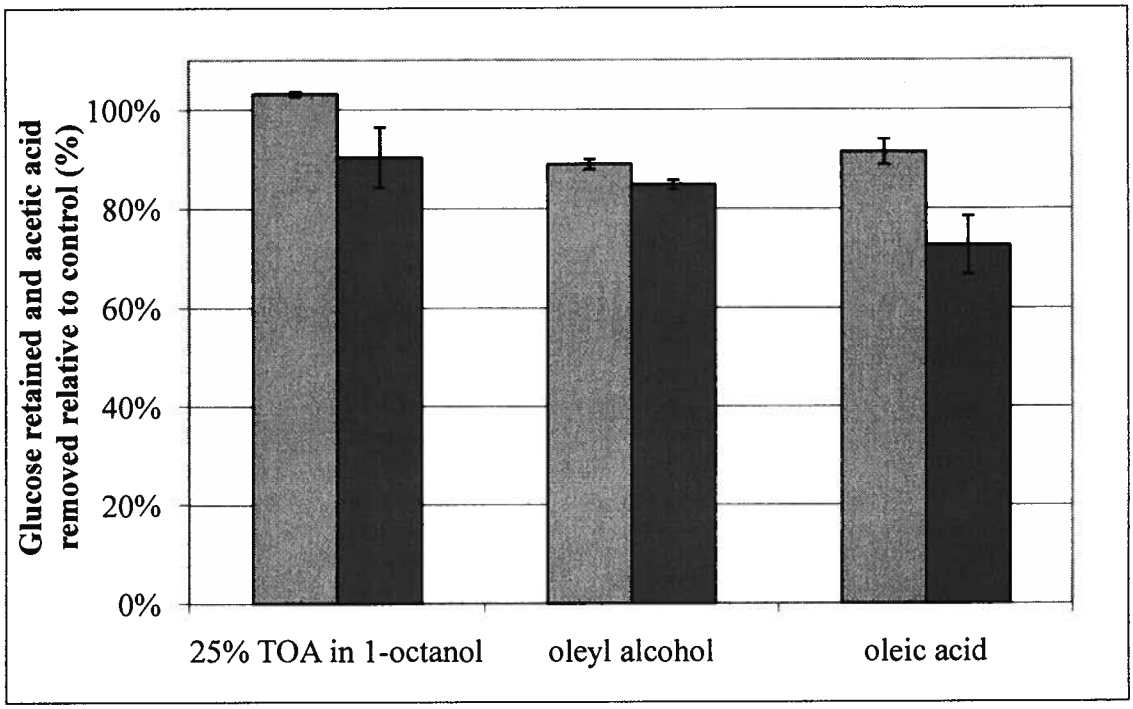

Figure 37 Comparison of acetic acid removed (dark colored bars) and glucose retained (light colored bars) between 25% TOA with 1-octanol, oleyl alcohol and oleic acid

A comparison on the amount of glucose retained after the extractions indicated that tri-n-octylamine was able to achieve 11% higher glucose retention than oleyl alcohol and 10% higher than oleic acid. For the removal of acetic acid, tri-n-octylamine was able to remove 6% more than oleyl alcohol and 18% more than oleic acid. Similar to other solvent extraction experiments, the ethanol yields from the fermentation of the extracted artificial hydrolysates were compared with the control fermentation medium. The comparison between the solvents and the control were based on the ethanol concentration after 48 hours of fermentation.

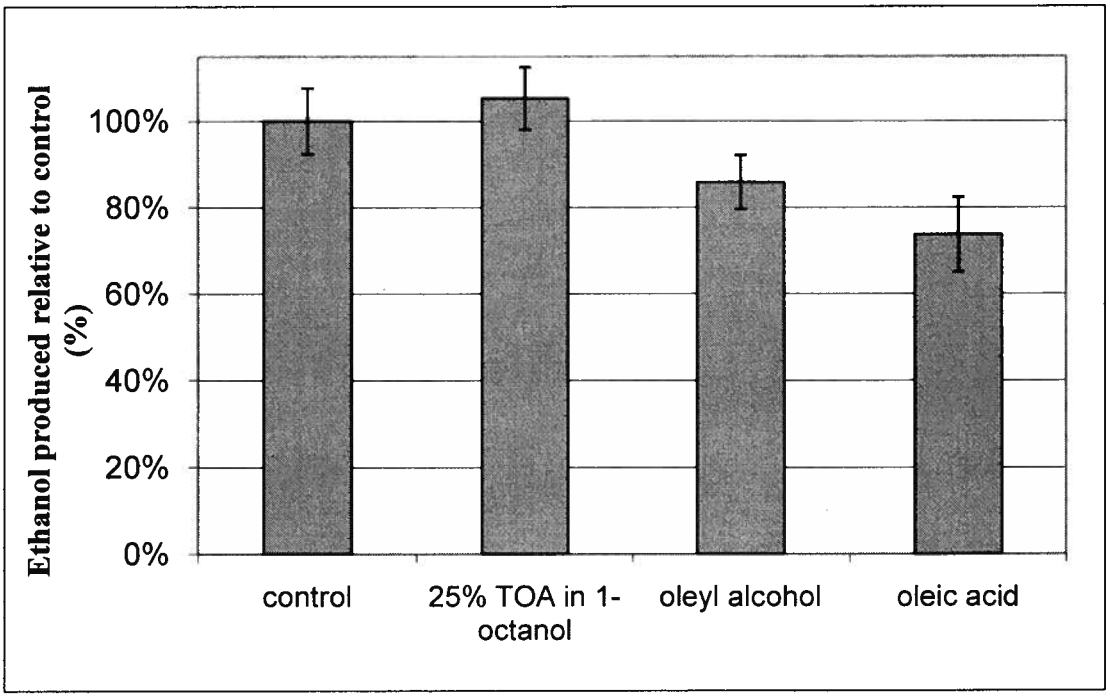

Figure 38 Comparison of ethanol concentration after 48 hr fermentation (tri-n-octylamine, oleyl alcohol and oleic acid)

It was found that oleic acid showed a 26% inhibition towards ethanol production, and oleyl alcohol showed 14% inhibition (Figure 38). The key objective of the project was to increase the productivity of ethanol. Thus, while oleic acid showed adequate extraction results, the inhibition towards ethanol production exhibited by the solvent would limit its usefulness.

The best three solvents were selected to extract actual bio-oil hydrolysate. The bio-oil hydrolysates were then diluted to 40% strength for fermentation. The ethanol yields from these detoxified bio-oil hydrolysates were compared. It was found that the three solvents had similar ethanol yields. The ethanol yield was found to be 0.24±0.01 (g ethanol/g glucose), 0.24±0.02 (g ethanol/g glucose) and 0.23±0.02 (g ethanol/g glucose) for 25 vol% tri-n-octylamine with 1-octanol, 50 vol% alamine 336 with 1-octanol and oleyl alcohol, respectively. This was not foreseeable for 25 vol% tri-n-octylamine and oleyl alcohol as their results for the retention of glucose differed by 15% and ethanol production in YPG medium differed by 14%. The reason for the slight difference in ethanol yield was unknown.

Although an increase in ethanol yield from 0 to 0.24 (g ethanol/g glucose) was quite significant, the improvement was only half of the overliming technique. The overliming technique generated an ethanol yield of 0.45±0.050 (g ethanol/g glucose) under the same fermentation conditions. However, the cost of the two detoxification methods should also be considered in order to determine the practicability of the two methods.

5.6 Adaptive evolution of yeast

Adaptive evolution is a more passive approach than the other detoxification methods that increase the fermentability of bio-oil hydrolysates. Adaptive evolution occurs when bacteria and yeast experience a growth limiting or stressful environment. Under these conditions, they have the potential to survive and to adapt to these stresses. An example of this phenomenon is the antibiotic resistance of bacteria.

The two parameters studied in this experiment were to adapt the yeast in aerobic or micro-aerophilic conditions and to adapt the yeast in either high or low concentration of bio-oil hydrolysates. The high and low concentrations were chosen based on the results of fermenting bio-oil hydrolysate obtained from section 5.1. The high concentration medium contained 35 vol% bio-oil hydrolysate, which was the highest concentration of bio-oil hydrolysate that had shown ethanol production. The low concentration medium contained 10 vol% bio-oil hydrolysate, which was the highest concentration of bio-oil hydrolysate that exhibited an ethanol yield close to the theoretical yield.

It was observed that under high concentration of bio-oil hydrolysates, each successive transfer would make the yeast darker in color. In addition to the color change, when the yeasts were spun down, the pellets became less compact with each successive transfer. On the seventh day, all of the yeasts had perished under high concentration of bio-oil hydrolysate and micro-aerophilic conditions. This was determined when there was no observable yeast pellet when the fermentation medium was centrifuged at 10000g for 10 minutes (Sanyo, MSE micro centaur). Two days later, the same result was found for the

yeasts that were under high concentration of bio-oil hydrolysate and aerobic conditions. This revealed that the yeasts were able to survive longer under aerobic conditions. Nevertheless, it was a surprising result because previous experiments confirmed that the yeasts were able to ferment and produce an ethanol yield of 0.17±0.01 (g ethanol/g glucose) at 35% volume of bio-oil hydrolysates. The failure of the adaptations was probably due to the prolonged exposure to the high toxic level in the bio-oil hydrolysate.

All cultures adapted using the lower (10% v/v) concentration of bio-oil hydrolysate under micro-aerophilic conditions (samples ML 1 and 2) and aerobic (samples AL 1 and 2) conditions survived. They were transferred to agar plates and were used in the fermentation of bio-oil hydrolysates of 30% strength. The results were compared with an unadapted strain of T2 yeast and are shown in Figure 39.

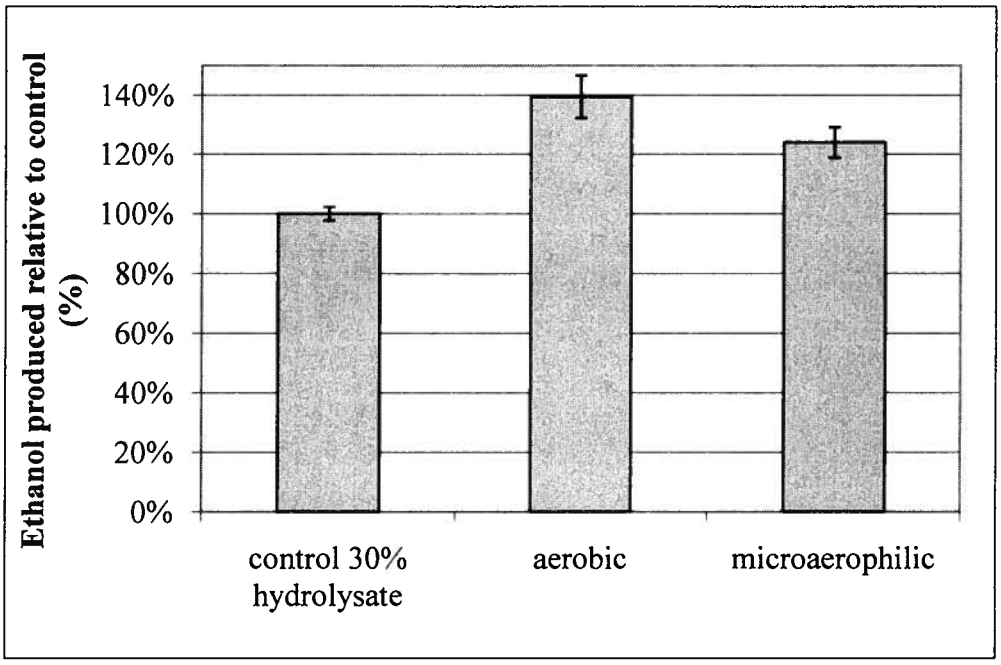

Figure 39 Comparison of ethanol produced after 49 hours of fermentation (yeast adapted in aerobic and low hydrolysate concentration, yeast adapted in micro-aerophilic and low hydrolysate concentration)

Yeast adapted under aerobic conditions was able to produce 39.4% more ethanol than a normal strain and 15.4% more ethanol than yeasts adapted under micro-aerophilic

conditions. It was hypothesized that aerobic culturing of the yeast produced more biomass under the same stress conditions, which could result in higher mutation rate. In addition, yeast under micro-aerophilic condition would have slower growth rate, which would mean that these yeast adapted for less than 121 generations. A comparison of the yield of ethanol was also prepared in Figure 40.

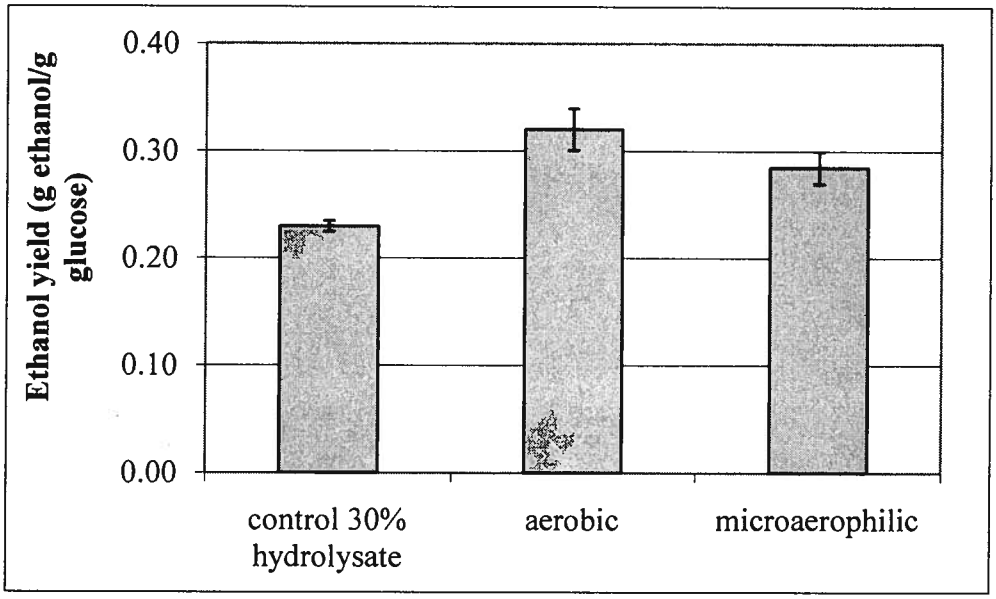

Figure 40 Effect of hydrolysate concentration and culture conditions on ethanol yield after 49 hours fermentation

In previous results with 30% v/v hydrolysate, the ethanol yield was 0.18±0.01 (g ethanol/g glucose). In this set of experiments, the control showed an increase in the ethanol yield when compared with previous results. Perhaps the increase in the ethanol yield could be a result of the additional hour of fermentation and random errors during the fermentation process. The improvement with the adapted yeasts was much more significant. Yeasts from sample AL had 9% higher ethanol yield than the control, while the yeast adapted at ML conditions had 6% higher ethanol yield than the control. The adapted yeast strain had the potential to increase the ethanol yield and it was likely that further adaptation could increase the ethanol yield further. In addition, this technique could be used in combination of other detoxification methods without incurring extra costs once the yeasts were adapted.

Chapter 6 Conclusions

One of the objectives in this project was to find the optimal conditions to extract levoglucosan from bio-oil. The dominating parameter in levoglucosan extraction was the amount of water used for the extraction. It was found that the maximum amount of levoglucosan was extracted when 100 wt% of water-to-bio-oil was used for the extraction. Under the optimal conditions, 4.98 wt% of levoglucosan was extracted from the bio-oil. Other parameters such as sufficient mixing and extracting with hot water at 80°C would also be beneficial to the extraction process. Extraction with hot water had the benefit of lowering the viscosity of the organic phase of bio-oil. This could reduce the maintenance cost of the equipment.

The other objective was to reduce the toxicity of the bio-oil hydrolysate, thereby increasing the yield of ethanol. It was found that at 10% and 20% strength of bio-oil hydrolysate, the ethanol yield was comparable to the control (ethanol yield in YPG medium). However, the ethanol yields decreased as the concentration of the hydrolysates were increased. At 40% strength, the medium was too inhibitory for any ethanol production. The detoxification methods used in an attempt to remove the inhibitors included: air stripping, adsorption with activated carbon, overliming, hydrogenation, solvent extraction and coagulation and flocculation. Among these detoxification methods, overliming and solvent extraction were found be the most efficient. Bio-oil hydrolysates treated with overliming and fermented at 40% strength produced an ethanol yield of 0.45±0.05 (g ethanol/g glucose). Detoxification by solvent extractions (40% hydrolysate) produced ethanol yields of 0.24±0.01 (g ethanol/g glucose), 0.24±0.02 (g ethanol/g glucose) and 0.23±0.02 (g ethanol/g glucose) for the solvents 25 vol% tri-n-octylamine in 1-octanol, 50 vol% alamine 336 in 1-octanol and oleyl alcohol, respectively. A technique called adaptive evolution of yeast was applied to increase the resistance of yeast to inhibitors in bio-oil hydrolysates. Using high concentration of hydrolysate (35% strength), the yeast failed to adapt under both aerobic and micro-aerophilic cultivation. At low concentration of hydrolysates (10% strength), the yeast were able to adapt to the

hydrolysate. When subsequently used to ferment 30% v/v hydrolysates the adapted yeasts achieve an ethanol yield of 0.32±0.09 (g ethanol/g glucose) and 0.29±0.02 (g ethanol/g glucose) for yeast adapted under aerobic conditions and micro-aerophilic conditions, respectively. This was an increase of 9% and 6% compared with the controls (unadapted yeast) for yeast adapted under aerobic conditions and micro-aerophilic conditions, respectively.

Chapter 7 Recommendations for future work

The optimal extraction ratio (water to bio-oil) of levoglucosan has been found. However, it would be beneficial to test if other solvents can be used to extract more levoglucosan from bio-oil. In addition, using dilute sulfuric acid as a solvent for extraction has the potential to combine extraction and hydrolysis in one step.

This project has showed that overliming, solvent extraction and adaptation of yeasts were able to increase the ethanol yield from the fermentation of bio-oil hydrolysate. However, a more detail analysis with additional parameters could be added to the investigation. For example, in solvent extraction, a more detailed study of the ratio of the solvents and co-solvent system could be conducted to find an optimal ratio for extraction. In addition, investigation of other organic solvents such as 2-ethylhexyl alcohol, hexanol and decanol to remove fermentation inhibitors would contribute positively to this project. For adaptive evolution, it would be beneficial to see if the yeast adapt to other concentrations of hydrolysate and/or adapt better after more mutation cycles.

Modifications to the examined experimental parameters and further research can potentially improve the detoxification methods that failed to provide promising results, such as air stripping, hydrogenation, and adsorption on activated charcoal,. For example, a screening of a number of different catalysts can be performed for hydrogenation of bio-oil hydrolysates to find catalysts that are able to degrade acetic acid much faster than the sugars. Finally, the monetary costs and benefits of these methods have yet to be studied and quantified. Therefore, an economic analysis on the project is highly recommended.

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Appendix

Table 18 List of identified compounds in the aqueous phase for pine wood derived bio-oil [46]

Compounds		Compounds	
1	acetic acid	24	5-(hydroxymethyl)-2-fural-dehyde
2	1-hydroxy-2-propanone	25	3-methylcatechol
3	1-hydroxy-2-butanone	26	3-methoxycatechol
4	2-cyclopenten-1-one	27	4-methylcatechol
5	2-furaldehyde	28	4-vinylquaiacol
6	2-methyl-2,3-dihydrofuran-3-one	29	dimethylpyranone
7	1-acetyloxypropan-2-one	30	dimethylcatechol
8	2-methyl-2,3-dihydrofuran-3-one	31	eugenol
9	acetylfuran	32	4-ethylcatechol
10	2-methyl-2-cyclopenten-1-one	33	vanillin
11	isomer of a-angelica lactone	34	isoeugenol
12	4-(hydroxy-methyl)tetrahydropyran-3-one	35	4-propylquaiacol
13	5-methyl-2-furaldehyde	36	acetoquaiacone
14	phenol	37	coniferyl alcohol (cis)
15	2-hydroxy-3-methyl-2-cyclopenten-1-one	38	vanillic acid
16	2-methylphenol	39	con-iferyl alcohol (trans)
17	3- or 4-methylphenol	40	a-oxypropioquaiacone
18	quaiacol	41	dihydroconiferyl alcohol
19	3-hydroxy-2-methyl-(4H)-pyran-4-one	42	acetosyringone
20	2,4- or 2,5-dimethylphenol	43	coniferaldehyde
21	ethylphenol	44	syringyl acetone
22	catechol	45	sinapaldehyde
23	4-methylquaiacol		