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INFLUENCE OF YEAST AND ENZYME VARIATION ON BIOETHANOL YIELD

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ABSTRACT

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Influence of Yeast and Enzyme Variation on Bioethanol Yield,
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This is a study concerning the procedures of bio-ethanol production from the wood based biomass hydrolysates' fermentation process. Required process conditions are analyzed and experimental data include raw material properties; bio-ethanol productivity and its impacts are evaluated to illustrate how the bio-ethanol production potential relates with the variation of the yeasts types.

Theoretical background and experimental based research are majorly applied to achieve two targets that the final thesis composition requires: first process knowledge accumulation which gives the solid foundation for principle understanding and related experimental operations; and secondly real-life process data and result analysis of the experiments that provide the scientific support to the thesis opinion establishment.

The result of this study can be concluded as follow: after the study of the bio-ethanol production from woody raw material, the related chemical and biological process as are expected to receive higher productivity and faster process period by applying the additional usage of specific and appropriate type of enzyme. According to the final results, we might understand that although the wood chips are able to be used as the raw material for bio-ethanol production, its productivity is relatively lower and process is considerably more difficult than the traditional agriculture based bio-ethanol production, for instance: bio-ethanol production from using sugar products and starch crops as raw materials.

However, due to the concerns of world`s food security and the trend of the new bio-energy developments and applications, the usage of forest based raw materials is still considered as one of the major methods of bio-ethanol production.

Key Words: Enzyme, Yeast, Bio-ethanol, Fermentation, Hydrolysis, Spruce, Lignocelluloses.

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1 INTRODUCTION

Global warming and climate change are acknowledged as the impacts from fossil fuel consumption. As the current situation that traditional fossil fuel is reaching its storage limitation quickly and global energy consumption never meets a break, it has become more necessary than ever to locate an alternative solution to reduce the fossil fuel dependence within appropriate resource and investment implantations. Nowadays, new technology and scientific research based on the alternative energy production is rapidly growing. Bio-energy, especially the bio-ethanol as energy source ranks ahead, and its importance can be noticed as approximately 30% of total biomass is transferred into bio-ethanol annually [1] and used in different fields.

Forest, or wood to be more specific is considered as one of the most essential natural resources on this planet; its valuable factors are covering not only on the aspect of traditional paper and pulp manufacturing, but a new trend of energy source such as the mentioned bio-ethanol research and production is inspired as well by the certain wood process technology. As *figure 1* indicates below, this process shows how the bio-ethanol is product from the biomass hydrolysates.

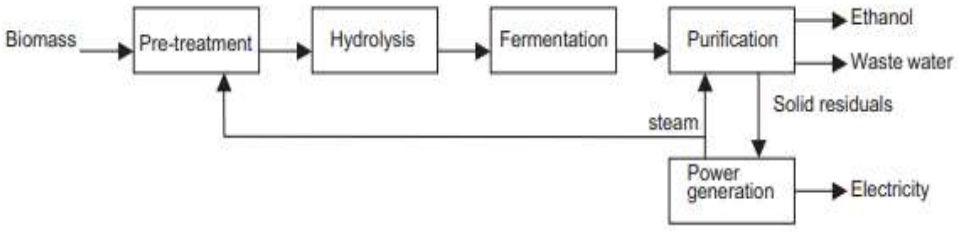

Figure1. General flowchart of bio-ethanol production from biomass [2]

This thesis project is mainly following this process with using different yeasts and specific enzymes to produce the bio-ethanol, and to clarify ethanol productivity and other related conditions according to the analytical chemistry testing results.

2 BIO-ETHANOL

Bioethanol is the principle fuel used as the petrol substitute for road transport vehicles. It is mainly produced by the sugar fermentation process. Several advantages of using ethanol as fuel should be mentioned. It is good for the environment and it can reduce dependence on oil imports

2.1 Characteristics of Bio-Ethanol

Ethanol, which is also named as ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol, is a volatile, flammable, colorless liquid. It is a psychoactive drug and one of the oldest recreational drugs. It is best known as the type of alcohol found in alcoholic beverages. Ethanol is a straight-chain alcohol, and molecular formula is C_2H_5OH, its chemical structure is given in the *figure 2* below:

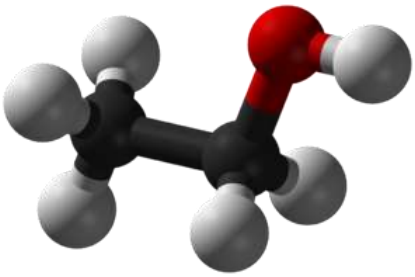

Figure2. Chemical structure of Ethanol [3]

Its empirical formula is C_2H_6O. An alternative notation is $CH_3–CH_2–OH$, which indicates that the carbon of a methyl group ($CH_3–$) is attached to the carbon of a Methylene group ($–CH_2–$), which is attached to the oxygen of a hydroxyl group (–OH). It is a constitutional isomer of Dimethyl ether. [3]

Bio-ethanol has the same chemical properties as the regular ethanol that is

actually a petroleum product; the difference only exists in their raw material of production, which regular Petroleum ethanol is made by the hydrolysis of ethylene, a major petrochemical [4]. Ethanol is used as an industrial feedstock, or solvent is often made from petrochemical feed stocks, primarily by the acid-catalyzed hydration of ethylene as the *formula 1* indicates below:

$$C_2H_4 + H_2O \rightarrow CH_3CH_2OH \qquad (1)$$

As the bio-ethanol, its production is connected with the fermentation process of sugar from living organisms, or named as biomass, which is divided into: agriculture based sources and forestry based sources.

The agriculture based biomass includes straw of cereals and pulses, stalks and seed coats of oil seeds, stalks and sticks of fiber crops, pulp and wastes of the plantation crops, peelings, pulp and stalks of fruits and vegetables and other wastes like sugarcane trash, rice husk, molasses, coconut shells etc. In another direction, harvesting and thinning residues, thinning from hazardous fuels reductions, habitat improvement, and other ecosystem restoration projects, sawdust from paper mills, trees and woody plants and their other woody parts are all included as the biomass sources from the forest [5].

The production of bio-ethanol is through the fermentation process, scientifically, the biomass fermentation is a process where microbes use sugars as food and produce alcohols (bio-ethanol) as a product of their metabolism. The fermentation process is usually anaerobic but can also be aerobic; it is depending on the microbes that are used in the fermentation process. When proceeding the biomass fermentation process, the basic stages are illustrated as the *figure 3.*

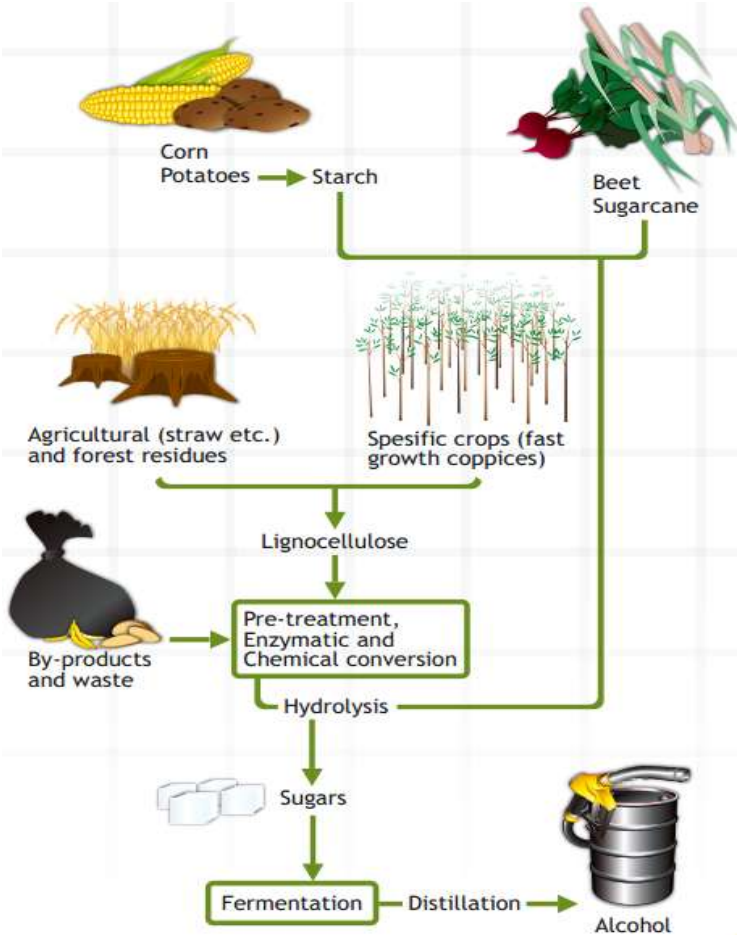

Figure3. Biomass fermentation procedures briefing

2.2 Conditions and Applications of Bio-Ethanol

Bio-ethanol, as one of the most important and valuable bio-fuels, acts an essential role in the transportation field. It is mainly used as a fuel additive for gasoline. World ethanol production for transport fuel was tripled between 2000 and 2007; it was reported from 17 billion to over 52 billion liters. In 2010 worldwide ethanol fuel production reached 86.9 billion liters, its popularity is easily being seen from this number [6].

Among the numbers of production and consumption of bio-ethanol fuel, countries of Brazil and the United States, and together both countries were responsible for nearly 90% of the world's ethanol fuel production in 2010. *Table 1*

indicates their production facts of bio-ethanol as fuel usage between 2007 and 2010. As we can notice, gloally, except for the U.S. and Brazil, many countries are interested in the bio-ethanol production and applications as well;

Table1. Annual Fuel Ethanol Productions by Country [7]

(Millions of U.S. liquid gallons per year)					
World rank	Country/Region	2010	2009	2008	2007
1	United States	13,230.00	10,600.00	9,000.00	6,498.60
2	Brazil	6,921.54	6,577.89	6,472.2	5,019.2
3	European Union	1,176.88	1,039.52	733.60	570.30
4	China	541.55	541.55	501.90	486.00
5	Thailand		435.20	89.80	79.20
6	Canada	356.63	290.59	237.70	211.30
7	India		91.67	66.00	52.80
8	Colombia		83.21	79.30	74.90
9	Australia	66.04	56.80	26.40	26.40
10	Other		247.27		
	World Total	22,946.87	19,534.993	17,335.20	13,101.7

However, today's opinions of bio-ethanol are not as positive as the trend of its production, because the bio-ethanol is produced from agricultural and forest based biomass. In developing countries there are certain concerns and worries of its production and use, related to increased food prices due to the large amount of arable land required for crops, few the energy and pollution balance of the ethanol production cycle are being argued and tested.

3 WOOD AS RAW MATERIAL

There are numbers of natural materials, for instance: agriculture residues, forestry residues, food waste, industrial waste can all be categorized as biomass, but in this specific process, wood is selected as the biomass raw material for certain mentioned process.

3.1 Chemical Structure of Wood

Wood is defined as a hard, fibrous tissue type of material that exists in trees. It has been used for hundreds of thousands of years for both paper & pulp manufacturing, fuel and as a construction material. Wood is a heterogeneous, hygroscopic, cellular and anisotropic material. Cell is the basic structure unit of wood. Chemically, wood is composed principally of carbon, hydrogen, and oxygen, data is given in the *table 2* provides the percentage that each major chemical element holds inside the wood structure.

Table2. Chemical Element Distribution [8]

– Carbon is the dominant element by weight.

Element	% Dry Weight
Carbon	49.0
Hydrogen	6.0
Oxygen	44.0
Nitrogen	>0.1
Ash	0.2-0.5

There are three types of organic polymers that are responsible for the main function and structure of the wood: cellulose, hemicelluloses and lignin. In chemical terms, the difference between hardwood and softwood is reflected in the composition of the constituent lignin. Hardwood lignin is primarily derived

from sinapyl alcohol and coniferyl alcohol. Softwood lignin is mainly derived from coniferyl alcohol [9]. However, as the *table 3* indicates, due to the difference between softwood and hardwood, the distributions of mentioned organic polymers are various within certain scale of range.

Table3. Distributions of major organic polymers in wood [10]

Raw material	Lignin (%)	Cellulose (%)	Hemicellulose (%)
Hardwoods	18-25	45-55	24-40
Softwoods	25-35	45-50	25-35
Grasses	10-30	25-40	25-50

Except for the lignocellulose, wood consists of a number of low molecular weight organic compounds, such as terpenes, diterpenes, and fatty acids. For example, rosin is exuded by conifers as protection from insects.

3.2 Cellulose

Cellulose is an organic compound with the formula of $(C_6H_{10}O_5)n$. Like the *figure 4* illustrates, this compound is a polysaccharide consisting of a linear chain of hundreds to over ten thousand β (1→4) linked with D-glucose units [11].

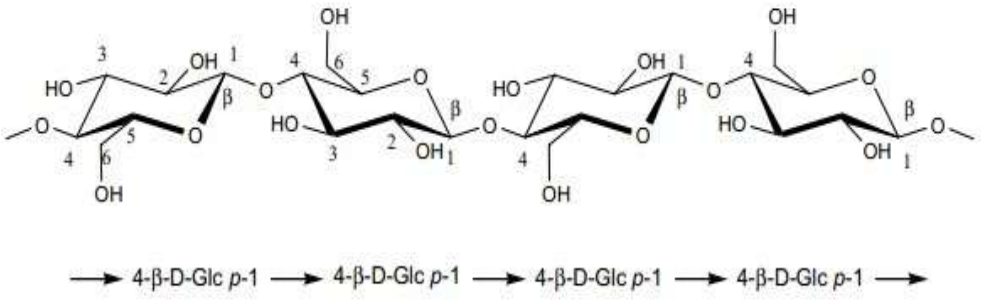

Figure4. Chemical Structure of Cellulose

It is a principal chemical constituent of the cell walls of the higher plants, and a complex carbohydrate as major structure in the form of polymer chains.

Cellulose is the key for the biological production of ethanol by proceeding two main methods: a) Cellulolysis processes which consist of hydrolysis on pretreated lignocellulosic materials, using enzymes to break complex cellulose into simple sugars such as glucose and followed by fermentation and distillation. b) Gasification process that transforms the lignocellulosic raw material into gaseous carbon monoxide and hydrogen, those gases can be converted to ethanol by fermentation or by chemical catalysis [12].

3.3 Hemicellulose

Hemicelluloses are polysaccharides in plant cell walls. These types of hemicelluloses are found in the cell walls of all terrestrial plants, the detailed structure of the hemicelluloses and their abundance vary widely between different species and cell types. The most important function of the hemicelluloses is to strengthen the cell wall by interaction with cellulose and lignin.

Glucomannans are the principal hemicelluloses in softwood. The backbone is a linear or slightly branched chain of β-(14)-linked D-mannopyranose and D-glucopyranose units like the *figure 5* illustrates [13].

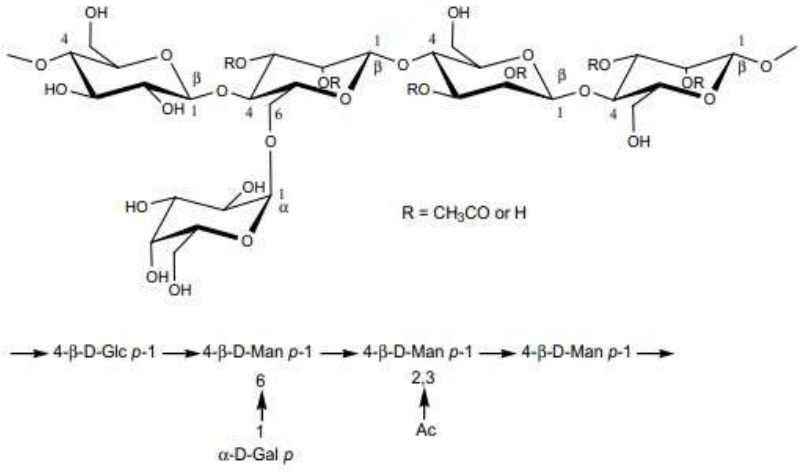

Figure5. Chemical Structure of Glucomannans

The main hemicellulose compound in the hardwood is a xylan, more specifically an O-acetyl-4-O-methylglucurono-ß-D-xylan, as the *figure 6* shows below.

The backbone consists of β-(14)-linked xylopyranose units. Most of the hydroxyl groups at C2 and/or C3 of the xylose units are substituted with acetyl groups. [13]

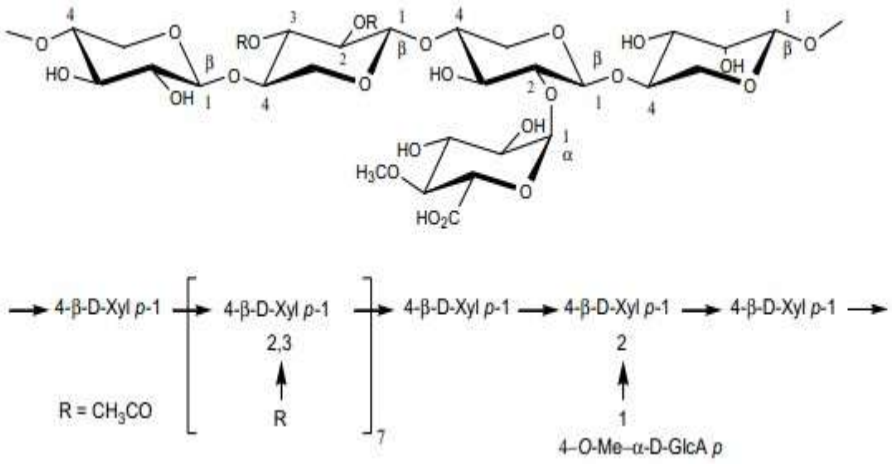

Figure6. Chemical Structure of Xylan

Hemicellulose is constructed by hexoses, pentoses and uronic acids. Comparing with cellulose, hemicellulose is easily hydrolyzed to its constituent monosaccha-rides [14].

3.4 Lignin

Lignin is defined as a chemical compound that has the cross-linked aromatic polymer property, which is complex and hydrophobic. The lignin is functioning as an integral part of the plant cell wall. Several possible monomers can be found in lignin. This molecule of phenolic character as dehydration product contains three monomeric alcohols: Trans-p-coumaryl alcohol, Trans-coniferyl alcohol and Trans sinapyl alcohol [15]. The structures of mentioned three monomers are given in the *figure 7*.

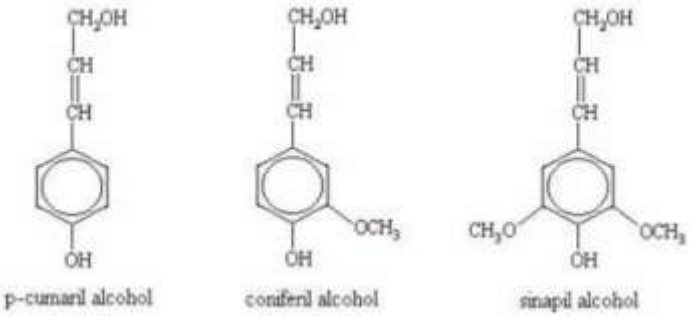

Figure7. Chemical Structure of lignin's monomers[15]

The function of the cellulosic based lignin component biomass is to provide large extent for the difficulties inherent in cellulose hydrolysis.

The main precursor of lignin in softwoods is trans-coniferyl alcohol In hardwoods, trans-sinapyl alcohol and trans -p-coumaryl alcohol are also lignin precursors [16]. The composition of lignin is different based on the source of raw material. If softwood is taken as material, it contains a higher amount of lignin of nearly 30% and the hardwood has lower lignin content of 20% approximately.

4 MATERIAL PRETREATMENT AND HYDROLYSIS

There are two fundamental stages during the lignocellulose materials' degradation in producing fermentable sugars, which are:

A) Pretreatment (chemical and mechanical)
B) Hydrolysis (chemical and enzymatic)

The pretreatment is required to increase the surface area of the feedstock, which makes the lignocellulose accessible for hydrolysis process more effectively [17].

4.1 Pretreatment

The goal of pretreatment is to destroy the lignocellulose cell structure in order to make it more approachable for further treatment. During the pretreatment, hemicellulose is chemically hydrolyzed into monomer sugars. The sugars are converted into a mixture of soluble sugars, xylose, arabinose, mannose and galactose [18]. The chemical stability of cellulose is better than that of hemicellulose. There is only a small part of cellulose that can be converted into glucose as the result of this step. The pretreatments are performed physically or chemically. In order to maximize the performance of the pretreatment stage, normally the process is done in both ways.

4.1.1 Physical Pretreatment

The most popularly used physical pretreatment method is steam explosion where the lignocellulose is heated by using high-pressure steam with the pressure between 20 and 50 bars at the temperature from 210 to 290 ^{o}C for few

minutes. During the steam explosion process, the steam with high pressure and thermal energy penetrates the structure of lignocellulose, and is released out from the closed pores of the lignocellulose [19].

The high temperature and high pressure causes the damage of hydrogen bonds of the cellulose, where new and free hydroxyl appears. As a result, the ordered structure of cellulose is changing; the adsorption capacity of cellulose is increasing. The conventional pretreatment methods only can change the solubility of hemicellulose and the enzymatic conversions rate etc. The steam explosion pretreatment is to control the temperature; manage time, and change the cellulose particle size for reaching the purpose of physical and chemical property changing of the cellulose.

The initial steam explosion was proposed and patented in 1927 by Mason. The further research which is combined with chemical treatment was done by Mason later which made the steam explosion technology more effective. The treatment effect of steam explosion is not only related with the selective chemical reagents, but also with the granularity of the raw material. By using a larger particle size (8mm - 12mm), energy can be saved, and the operating conditions can be more severe. Sugar loss in hemicellulose hydrolysis decreases, and cellulose enzyme hydrolysis rate can be raised. The advantage of steam explosion method mainly concerns the low energy consumption and performance effectiveness. However, disadvantage concerns the loss of xylose, and harmful substances produced in the fermentation. When the intensity of pre-treatment is getting greater, the easier enzymatic hydrolysis of cellulose can be done, but the less sugar is obtained from hemicellulose, and there are more harmful substances from the fermentation [19].

4.1.2 Chemical Pretreatment

Chemical method is the use of acids, alkalis, organic solvents, such as the method of lignocellulose pretreatment. The method is mainly aimed at cellulose, hemicellulose and lignin imbibed with the destruction of its crystalline structure. As similar as the physical pretreatment, there are two methods available for the chemical pretreatment:

Dilute acid hydrolysis pretreatment has been successfully used in pretreatment. The dilute sulphuric acid with concentration between 0.5 and1.5% is in use, treatment temperature should be above 160 C^{o}. It is used as the most favored pretreatment for industrial application, because it achieves reasonably high sugar yields from hemi-cellulose: minimum xylose yield is 75–90% *[20]*.

During this treatment, lignin content keeps unchanged, the average degree of polymerization of cellulose is decreased, and ability to respond is corresponding increased. As a result, the cellulose contact area is raised, additionally, dilute acid pretreatment produces fermentation inhibitors, corrosion of metal equipment, for which certain containesr and devices are required to avoid damages from happening by using the chemical pretreatment.

Alkaline pre-treatment uses chemicals of sodium hydroxide or calcium hydroxide. The function of the alkali pretreatment is to remove lignin and to increase the reactivity of the remaining polysaccharides. All lignin and part of the hemicellulose are removed, and the treated cellulose performance for later hydrolysis is sufficiently increased. The effect depends on the characteristics of raw materials with certain lignin contents and properties. If the lignin content of lignocellulose raw materials is more than 20%, the alkali treatment can hardly improve subsequent enzymatic hydrolysis rate. The mechanism of Alkali

treatment is to weaken the hydrogen bonds between hemicellulose and lignin and the saponification of the ester bond between. After the alkali treatment of wood, fiber becomes more porous, which makes wood more suitable for the growth of filamentous fungi. NaOH solution for processing of wood cellulose, which can cause swelling of wood fiber raw material profit, lowers the degree of polymerization and crystallinity. [21]

However, the use of mentioned treatments is believed to highly raise environmental concerns and may lead to prohibitive recycling, it also increases the wastewater treatment and residual handling costs.

4.2 Hydrolysis

The aim of the hydrolysis is to cleave the polymers of celluloses and hemicelluloses to monomeric sugars which are able to be fermented to ethanol by microorganisms. The hydrolysis is essential before fermentation to release the fermentable sugars. The theory difference between cellulose hydrolysis and hemicellulose hydrolysis is indicated in the *formulas 2* and *3*.

$$\text{Cellulose} \xrightarrow{\text{Hydrolysis}} \text{Glucose} \xrightarrow{\text{Fermentation}} \text{Ethanol} \quad (2)$$

$$\text{Hemicellulose} \xrightarrow{\text{Hydrolysis}} \text{Pentosed} \,\&\, \text{Hexoses} \xrightarrow{\text{Fermentation}} \text{Ethanol} \quad (3)$$

In ethanol production, the process of hydrolysis is very sophisticated, depending on several aspects, for example: properties of substrate, acidity, and decomposition rate during hydrolysis process [22]. The hydrolysis can be made either chemically or by a combined chemical and enzymatic treatment. Acids are predominantly applied in chemical hydrolysis and Sulphuric acid is the most frequently used.

4.2.1 Acid Hydrolysis

The solubility of cellulose in acid was detected already in 1815. Concentrated acid hydrolysis technology began in the 1820s, the first concentrated acid hydrolysis process was developed by the Department of Agriculture in the U.S. The required condition, the acid hydrolysis, can be performed by high acid concentration at a low temperature or that of low concentration at a high temperature in contrast [23].

The scientific explanation of concentrated acid hydrolysis is described as follows: the cellulose can be dissolved in the 72% sulfuric acid, 42% hydrochloric acid or 77% and 83% phosphoric acid solution at a lower temperature [24]. Then the cellulose is transformed into monomeric sugars. Within the concentrated hydrolysis, dimerization reaction will occur in some monosaccharose. The monomeric sugars start to rejoin and form polysaccharide. This reaction is the reverse process of cellulose hydrolysis.

The higher the hydrolyzed monomeric sugars contents and acid concentration, the greater sensitivity is obtained from the dimerization reaction. The monomeric sugars rejoin to generate the glucose disaccharide or three glycans. The hydrolytic solution must be diluted and heated in order to prevent hydrolysis forming polysaccharide. The yield of glucose will increase in the hydrolysis-operative period [24].

Dilute acid hydrolysis refers to use within 10% acid as a catalyst to hydrolysis of the cellulose and hemicellulose into monomeric sugars. The reaction condition is harder to achieve than in concentrated acid hydrolysis. The required reacting temperature is from 100 ºC to 240 ºC and the pressure is higher than 10 atmospheres in dilute acid hydrolysis process conditions.

The sugar degradation happens in high temperature and highly pressurized environment; the advantages along with some expected problems as disadvantages of the concentrated acid hydrolysis and dilute acid hydrolysis are shown in the *table 4* [25]:

Table4. Comparison between concentrated and dilute acid hydrolysis

Hydrolysis method	Advantages	Disadvantages
Concentrated acid Process	-operated at low temperature - High sugar yield	-high acid consumption -high energy consumption for acid recovery -longer reaction time (e.g. 2-6h) -equipment corrosion
Dilute acid Process	-low acid consumption -short residence time	- operated at high temperature -low sugar yield -equipment corrosion

The monosaccharose will break down into formic acid further which results in lower sugar yield and inhibition of the fermentation. But this problem can be solved by a two stage process, in which the hemicellulose is mainly hydrolysed in the initial step at temperature of 150 ºC to 190ºC and the remaining cellulose subsequently hydrolysed at more severe conditions at minimally 90 to 230ºC[26].

However, the concentrated sulfuric acid hydrolysis is still the most commonly concentrated acid hydrolysis method although obvious disadvantages exist.

4.2.2 Enzymatic Hydrolysis

The degradation of cellulose to monomer sugars in enzymatic hydrolysis is catalyzed by specific cellulolytic enzymes which are called cellulases. Cellulases are produced from both bacteria and fungi, which can decompose cellulosic material. [27]

The enzymatic hydrolysis of cellulose is a complex process. There are three different chemical reactions which take place at the same time. [28]

1. Residual (not yet solubilized) solid-phase cellulose changes chemically and physically.
2. Release of soluble intermediates from the surface of reacting cellulose molecules (primary hydrolysis).
3. Hydrolysis of soluble intermediates to lower molecular weight intermediates and finally to glucose (secondary hydrolysis).

Generally, degradation of cellulose by enzymatic hydrolysis is characterized by a rapid initial phase, and then a slow secondary phase follows. Enzymatic hydrolysis can occur under milder conditions (typically 40-50°C and pH 4.5-5), which give rise to two advantages of the process; low utility cost since there are few corrosion problems and low toxicity of the hydrolysates. In addition, it is also an environmental friendly process [29].

Enzymatic hydrolysis differs from the Acidic hydrolysis. The difference in functional environment is shown in the *table 5*.

Table5. Comparison of acid and enzymatic hydrolysis

Acid	Enzyme
1. Non-specific catalyst therefore will delignify material as well as hydrolyze cellulose.	Specific macromolecule catalyst, therefore extensive physical and chemical pretreatment is necessary to make cellulose available for degradation.
2.Decomposition of hemicellulose to inhibitory compounds	production of clear sugar syrup ready for subsequent anaerobic fermentation
3.Harsh reaction condition therefore necessary increased costs for heat and corrosion resistant equipment	Run under mild conditions (50°C,atmospheric pressure ,pH4,8)
4.Relatively low yield of glucose	High glucose yield

5 FERMENTATION

During fermentation monomeric sugars released in the hydrolysis are converted into the desired product, by a microorganism, which is required to ferment these sugars to produce bio-ethanol by different fermentation techniques [30].

The principles of the glucose fermentation can be indicated as the chemical reaction below:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (4)$$

5.1 Fermentation's Microorganisms

There are a variety of microorganisms which are able to produce ethanol in alcoholic fermentation process, including yeasts, bacteria and fungi. Among them there are several types of bacteria, yeasts and filamentous fungi. The specific organisms with their advantages and disadvantages will be discussed below.

5.1.1 Yeast

Yeast is the eukaryotic microorganism in the fungi family. There are many different strains of yeast. More than one thousand species of yeasts have been found. The most commonly used yeast is Saccharomyces cerevisiae, which can convert sugars into carbon dioxide and alcohol. Baking yeast and brewing yeast are the most important yeasts belonging to Saccharomyces cerevisiae. In brewing, the yeast is used to ferment alcoholic beverages, the ethanol is final production. While in baking, the yeast is used to leaven bread, the carbon

dioxide raises the bread and the ethanol evaporates [31]. Yeasts have recently been used to produce ethanol for the biofuel industry.

Yeast requires suitable conditions to grow. When water, nutrient, oxygen, and a proper temperature occur, the life cycle of yeast will become activated. Water is needed by the yeast in order for it to absorb nutrients. The ammonia and urea can be used as nutrient for yeast grow. Oxygen enables the yeast to metabolize nutrients and to multiply. The temperature range of yeast growing best is from 30^{o}C to 40 ^{o}C. The yeast cannot survive when the temperature is higher than 40 ^{o}C. But it can survive freezing under certain conditions.

5.1.2 Bacteria

Fermentation bacteria are anaerobic, but use organic molecules as their final electron acceptor to produce fermentation end-products. Different bacteria produce different fermentation end products. Streptococcus, Lactobacillus, and Bacillus produce lactic acid, while Escherichia and Salmonella produce ethanol, lactic acid, succinic acid, acetic acid, CO_2, and H_2. Fermenting bacteria have a characteristic in sugar fermentation that only they can decompose some specific sugars. For example, Neisseria meningitidis ferments glucose and maltose, but not sucrose and lactose, while Neisseria gonorrhoea ferments glucose, but not maltose, sucrose or lactose. This characteristic can be used to identify and classify bacteria [32].

During the 1860s, the French microbiologist Louis Pasteur studied fermenting bacteria. He demonstrated that fermenting bacteria could contaminate wine and beer during manufacturing, turning the alcohol produced by yeast into acetic acid (vinegar). Pasteur also showed that heating the beer and wine to kill the bacteria preserved the flavor of these beverages. The process of heating, now

called pasteurization in his honor, is still used to kill bacteria in some alcoholic beverages, as well as milk.

5.1.3 Fungi

Fungi are a group of organisms and microorganisms which are separate from plants, animals, and bacteria. The fungi include the fleshy fungi, the hyphae, and the yeast. Fungi are widely distributed and are found wherever moisture is present. Fungi exist primarily as filamentous hyphae. Like some bacteria, fungi digest insoluble organic matter by secreting exoenzymes, then absorbing the soluble nutrients.

The fungi contain a large-scale diversification of classification with varied ecologies, life cycle strategies. Its biological conformation ranges from single-celled aquatic chytrids to large mushrooms. Fungi present a significant role in the decay of organic matter and they have elementary role in nutrient cycling and exchange. They can be used as a direct source of food, such as mushrooms and truffles; they can also be used as a leavening agent for bread, and in fermentation of various food products. *[33]*

5.2 Fermentation Techniques

The fermentation process can be performed in majorly three types of operations, depending on different conditions such as properties of microorganisms and types of lignocellulosic hydrolysates. They are batch process, fed batch process and continuous process *[34]*.

5.2.1 Batch Process

The batch process is a closed fermentation process. In the batch process, nutrients and the inoculums are added to the reactor only once at the start of the process. When the maximum amount of product is present in the reactor, the product is extracted from the solution. Then the reactor is cleaned and used for other batch processes.

During the batch fermentation process, the microorganism works in high substrate concentration initially and a high product concentration in the end [35]. The batch process is a multi-vessel process, allows flexible operation and easy control over the process. Generally batch fermentation is defined as low productivity with an intensive labor. For batch fermentation, elaborate preparatory procedures are needed; and because of the discontinuous start up and shut down operations, high labor costs are incurred. This inherent disadvantage and the low productivity offered by the batch process have led many commercial operators to consider the other fermentation methods [35].

5.2.2 Fed batch Process

The fed batch process is based on feeding the reacting solution into the reactor. This is called controlled feeding process. During the process, feed solution contains substrate yeast culture, important minerals and vitamins. They are added at regular intervals after the start [36].The concentration of substrate in the reacting solution must be kept constant in the reactor while the feeding is made.

Fed batch process is a very popular fermentation process which is mostly used in ethanol industry. It is a production technique in between batch and continuous fermentation process. It combines the advantages from them both. No more

equipment is needed compared to the batch process requirement. And it provides better yield than batch process for the production of ethanol under controlled conditions in the fed-batch process.

5.2.3 Continuous Process

During continuous process, nutrients are continuously supplied to the bioreactor and metabolites and other wastes are continually removed at the same rate as the supply, resulting in a constant volume. This method prolongs the exponential growth phase of microbial growth and promotes continual growth of the microorganisms [37].

Two control methods are used in continuous culture fermentation, namely, chemostat and turbidostat. Continuous fermentation can be completed in different kind of reactors – stirred tank reactors (single or series) or plug flow reactors [37].

Continuous fermentation often gives a higher productivity than batch fermentation. Continuous operation offers ease of control and is less labor intensive than batch operation. The continuous process eliminates much of the unproductive time associated with cleaning, recharging, adjustment of media and sterilization.

5.3 Distillation

Distillation is a separation process for a mixture of liquids by taking advantage of their difference in boiling point temperatures. A distillation step is required after fermentation to separate the ethanol from the mixed solution. The boiling point of ethanol is 80^{o}C, and water is 100 ^{o}C. Ethanol will preferentially vaporize first

during heat the mixed solution to be boiling. The ethanol concentration in the condensate of the vapor is high. [38]

6 EXPERIMENTAL SECTION

Experimental operation is the most essential and direct method to allocate the performance, effects and appearance of scientific research and study. The way how experimental devices, tested raw materials and other related factors are prepared and processed is the key to the accuracy of the mentioned chemical/biological process.

6.1 Materials

In this process, only spruce wood chips are used lignocellulosic raw material. Besides, two types of yeast and two types of enzyme are used for the biological treatment during the bioethanol production process.

6.1.1 Raw Lignocellulosic Material

Wood chips from spruce were used as lignocellulosic raw material in my thesis work. Spruce is one kind of softwood. Table 6 shows representative values which are taken from the literature for the composition of spruce. However, the values can differ quite much due to species and environmental variations for each material [39].

Table6. Composition of the lignocellulosic material in spruce (Percentage of dry material)[39]

Glucan	Galactan	Mannan	Xylan	Arabinan	Lignin
44.0	2.3	13.0	6.0	2.0	27.5
43.4-45.2	1.8-2	12-12.6	4.9-5.4	0.7-1.1	27.9-28.1
49.9	2.3	12.3	5.3	1.7	28.7
44.8-45.0	2.2	12.0	5.2	2.0	29.9-32.3

6.1.2 Categories of Yeast

Three different kinds of yeast are used in my thesis work. They are baking yeast and brewing yeast. The characteristics of dry baking yeast are given in the *table 7* below:

Table7. Characteristics of Dry Baking yeast

Name	Dry Baking yeast (Active dry yeast)
Ingredients	Yeast(Saccharomyces cerevisiae), Rehydrating agent
Properties	Very long standing natural product. Commonly used as a leavening agent in baking bread and bakery products.
Dry matter and density	Dry matter range is 92 – 96 %. Density is about 0.75 – 0.95
Pitching instructions	The yeast is rehydrated to reactivate it in axenic water at around 38 ^{o}C before use.
Fermentation T and pH	T is about 30^{o}C. pH is 6-7.
Packaging	1 X 11 g packed in paper bag
Storage	Store at room temperature (24^{o}C) for a year. Frozen for more than a decade. Once opened, the yeast is best stored dry and refrigerated and used as quickly as possible.

For the dry brewing yeast, characteristics are given in the *table 8*:

Table8. Characteristics of Dry Brewing yeast

Name	Dry Brewing yeast
Ingredients	Yeast(Saccharomyces cerevisiae), Rehydrating agent
Properties	Very popular general purpose yeast. Used for the production of a varied range of top fermented special beers. Excellent performance in beers with alcohol contents of up to 7.5% v/v but can ferment up to 11.5% v/v.
Dosage	50 g/hl to 80 g/hl in primary fermentation 2.5 g/hl to 5.0 g/hl in bottle-conditioning
Pitching instructions	Re-hydrate the dry yeast into water before utilization. Sprinkle the dry yeast in 10 times its own weight of sterile water at 27±3^{o}C.
Fermentation T	T is about 135^{o}C. PH is 6-7.
Packaging	1 X 6 g vacuum-packed in paper bag
Storage	Store in cool (< 10^{o}C), dry conditions. Opened sachets must be sealed and stored at 4^{o}C and used within 7 days of opening.

6.1.3 Categories of Enzymes

Two types of enzymes were used in the hydrolysis process. They are cellulase and xylanase.

Cellulase is a digestive system enzyme that helps to break down the cellulose into glucose. Xylanase is a class of enzymes which degrade the hemicellulose into xylose.

The Biobake TR and Biobake Optum 815 are the commercial products that contain mentioned enzymes, and thus they were both in use for the experiments. Biobake TR holds the Cellulase, and the Biobake Optum 815 contains the Xylanase. The concentration of each enzyme in the product is 10% to 25%. The working conditions of the enzymes are almost the same:

Active temperature range: 45^{o}C – 60 ^{o}C
Active pH range: 4.3 – 6.3

6.2 Experimental Equipment and Procedures

The experimental operation was done under the laboratory scale by using the laboratory facility at the Saimaa University of Applied Sciences, Imatra, Finland. All equipment operations and safety precautions are following certain supervisions from the university regulations.

6.2.1 Wood Selection

The yeast fermentation requires certain experimental equipment to provide reaction environment and other required conditions for further treatment with quality. Wood chips are used as the raw material in this experiment, but due to

its production process, the difference of wood chip sizes is very significant, which is not ideal for the further treatment. Under this condition, the selection process of wood chips becomes is necessarily required. In order to select the wood chips within proper sizes, Gyratory Screening device is in use as the *figure 8* indicates:

Figure8. Gyratory Screening for wood chips selection

In this process, only select-sized wood chips, which are in the range from 5 x 10 x 3 mm to 15 x 20 x 3 mm (length x width x thickness) are able to be gathered. Approximately 1 kg of the wood chips was collected and stored in an air tight bag under the room temperature.

6.2.2 Hydrolyzation

As the illustration in the *figure 9* shows, the equipment of Ethylene Glycol Bath is in use to produce hydrolyzed and treated lignocellulosic materials from the wood chips. The control panel is shown on the left of the figure and working part of rotating reactor is on the right.

Figure9. Ethylene Glycol Bath

During this stage, sulfuric acid with 1.5% concentration is needed; however only 98% concentrated sulfuric acid was available in the laboratory, where a manual dilution of the concentrated sulfuric acid was applied. Safety operations had to be taken into account when proceeding: Adding concentrated sulfuric acid into water.

This step can be divided into two parts. In the first part, 22g of wood chips and 165ml of 1.5% sulfuric acid were added into the small reaction unit inside Ethylene Glycol Bath for a reaction, which temperature requirement is 130 C^o and duration is 2 hours. After the reaction, through the filtration process, the solvent is left for further treatment, which contains the pH of 1.9. Then the hydrolysate can be treated in the next fermentation stage.

In the second part, the mentioned process is considered as the pretreatment. The enzymatic hydrolyzation is executed right after completion of the pretreatment. The pH of the solution should be modified through the sodium hydroxide solution in order to achieve the suitable condition for enzymes working. 100ml of solution is taken into the flask and the enzymes are added directly into the solution. Flux is sealed by cotton. The solution is kept in the water base for 48 hours at 55 ^oC. Then the solution is ready to be fermented in the next phase.

6.2.3 Yeast Cultivation

Yeast cultivation is critical to ensure the success and quality of the final results of the experiment; there are five major stages to properly control the cultivation, which are:

1. Water boiling: this is to provide disinfected water as reaction media and bacterial-free experimental environment. Water is cooked inside flux for 10 minutes.

2. Water cooling: it is impossible to perform the experiment when water is at 100 ^{o}C, so the water has to be cooled. The water is removed from flame and cooled to be 40 ^{o}C, which is the same as the best temperature of the yeast's cultivation.

3. Yeast adding: when required temperature of 40 ^{o}C is reached, yeast is added into the flux. The mixture is well mixed.

4. Nutrition adding: 10 minutes after adding the yeasts, necessary nutrition is added to provide the cultivation fundamentals. To this stage, 2g of $(NH_4)_2PO_4$ is added.

5. After building the cultivation fundamentals, the flux is sealed by cotton, in order to maintain the air exchange (O_2 is required during the cultivation) and keep any external contamination outside.

6. Keeping the yeasts cultivation in the water base for 24 hours. The temperature is 35 ^{o}C.

As the figure 10 illustrates below, the preparation stage is ready for yeast cultivation.

Figure10. Preparation and Environment for Yeasts Cultivation

6.2.4 Fermentation

The batch process is run in this fermentation stage. The pH of the hydrolysate should be modified through the sodium hydroxide solution in order to achieve the suitable condition for yeast working. 100ml of hydrolysate is mixed with the active yeast solution in flask. The flask is closed with a rubber balloon. The function of the curving conduit is to eliminate the carbon dioxide which is produced by the fermentation process. One side of the curving conduit is on the top of the mixed solution in the flask. The other side is immerged to the NaOH solution in the beaker. The oil layer floats on the solution. The oil is used to prevent the air from entering into the NaOH solution, and then further come into the ferment mixed solution. Fermentation is an anaerobic process which does not need oxygen. The connective structure of the laboratory equipment for the fermentation process is shown in the next figure.

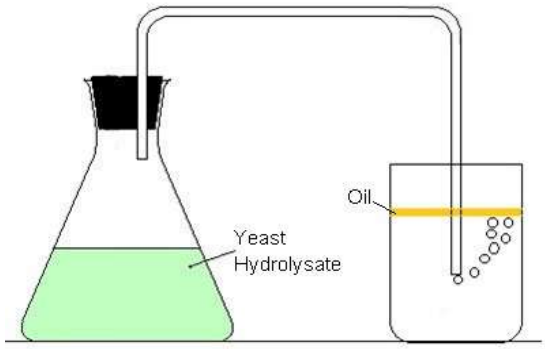

Figure11. Connective structure of the equipments

After building the fermentation fundamentals, it is then put into the oven which can keep the temperature at 35^{o}C well for 24 hours.

6.2.5 Distillation

The distillation apparatus is set up as *figure 12* indicates below.

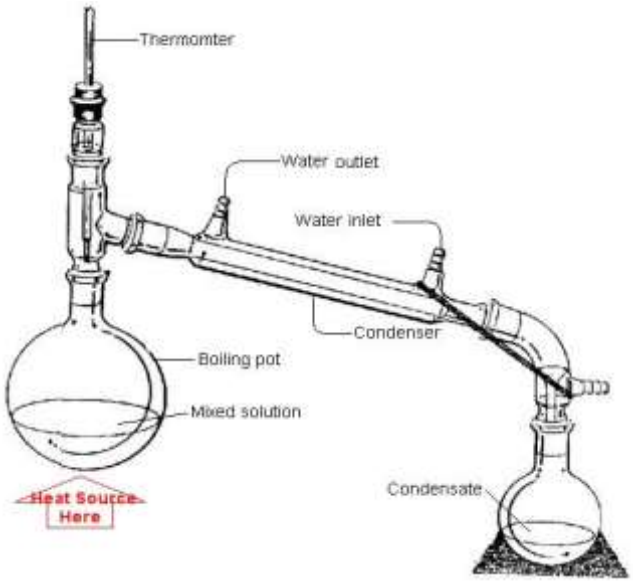

Figure12. Connective structure of the distillation apparatus

The side of water inlet and outlet must be carefully identified while installing the equipment. The mixed solution is heated by the heat source. When temperature achieves a certain degree, the ethanol vapor passes into the condenser. The

vapor is cooled and liquefied through the condenser. Then the resulting liquid is collected in a flask. The heat source should be closed, when the temperature achieves 100^{o}C.

6.2.6 Distillate Analyzed by Gas Chromatograph

Each distillate was analyzed twice through the Gas Chromatograph in order to identify the ethanol concentration based on the areas; the reason of the double analysis is to increase the accuracy of the results. During the GC analysis, the “Ethanol - 1” method was applied. The device of the GC analysis is presented in the *figure 13* below:

Figure13. Gas Chromatograph analysis device

The figure 14 describes the principle of how the ethanol concentration can be calculated from the area, based on the “Ethanol-1” analysis method.

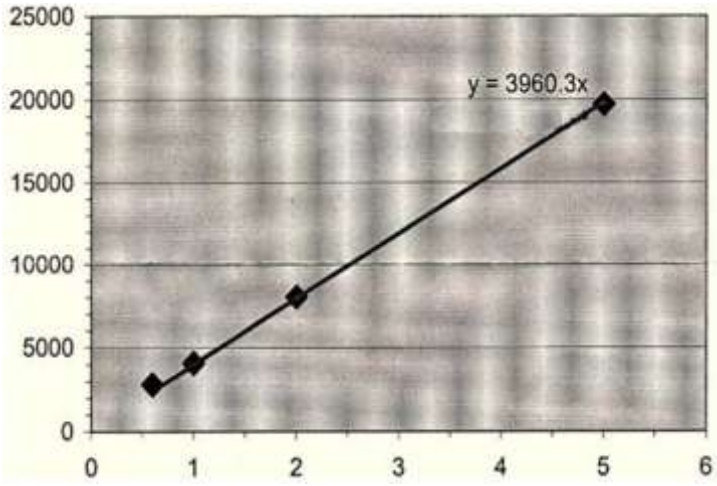

Figure14. Ethanol concentration calculation principle function

In the *figure 14*, the calculation principle formula is presented as*:*

$$y = 3960.3 * x \quad (5)$$

Where “y” represents the area which is obtained from the analysis;

“3960.3” is a constant number of this function

“x” is the ethanol concentration that indicates the percentage.

7 RESULTS AND DISCUSSION

7.1 Yield Comparison between Two Yeasts

After the fermentation process by using two different types of yeast without enzymatic hydrolysis, totally 4 kinds of distillates samples were received from each mixture which is indicated below:

A) 40ml of Baking Yeast solution + 100ml of hydrolysate
B) 60ml of Baking Yeast solution + 100ml of hydrolysate
C) 40ml of Brewing Yeast solution + 100ml of hydrolysate
D) 60ml of Brewing Yeast solution + 100ml of hydrolysate

Based on the calculation principle *formula 5*, the 8 analysis results are given in the *table 9*. (The results from the GC analyses are given as the appendix.)

Table9. Analysis results and ethanol concentration (A, B, C, D)

Sample Type	Area (pA*s)	Ethanol Concentration (%)	Average C. (%)
A1	8638.2	2.18	2.205
A2	8852.8	2.23	
B1	8770.4	2.21	2.220
B2	8829.9	2.23	
C1	8788.3	2.22	2.145
C2	8215.9	2.07	
D1	10142.1	2.56	2.540
D2	10023.2	2.53	

The volumes of all the distillates were 10ml. The yield of the ethanol can be calculated based on the concentration. So the concentration of the distillate is in direct proportion to the yield of ethanol. In the other words, the raise of the ethanol concentration in the distillates reflex the increase of the ethanol yield.

The differences of average concentration values in two groups of A-B samples and C-D samples were compared. In each group, all conditions in the process were the same, expect for the amount of the yeast which was used in the fermentation process. It shows that when the volume of hydrolysate solution is constant, the demand for the yeast has not reached saturation point in the fermentation process; with more yeast used, the more ethanol is fermented. On the other hand, if the yeast had reached saturation point in the fermentation process, more yeast will be an inhibitor which can restrict the process.

The comparison was also made between the A-C samples and B-D samples groups; however the regular change did not appear. But if another method was applied on the comparison, if A and B samples are considered as one unified object which is named AB sample and the same method was applied to C and D samples, then under this condition, both AB and CD samples contain 100ml of yeast solution and 200ml of hydrolysate solution. The total ethanol concentration from AB samples (Baking yeast) is smaller than the CD sample (Brewing yeast). This fact proves that the ethanol productivity when using the Brewing yeast is slightly higher than that of the Baking yeast. However, considering they belong to the same yeast family, the differences of functions and working conditions are very similar.

7.2 Yield Comparison between Two Enzymes

After the fermentation process by using Baking yeast with enzymatic hydrolysis, totally 2 kinds of distillates were received from each mixture, which is indicated below:

E) 40ml of Baking Yeast solution + 100ml of hydrolysate with xylanase
F) 40ml of Baking Yeast solution + 100ml of hydrolysate with cellulase

Based on the same calculation principle of *formula 5*, the 4 analysis results are given in the *table 10*. (The results from the GC analyses are given as the appendix.)

Table10. Analysis results and ethanol concentration (E, F)

	E1	E2	F1	F2
Area (pA*s)	11605.6	13691.2	13717.2	11584.3
Ethanol Concentration (%)	2.93	3.45	3.72	3.14
Average C. (%)	3.19		3.43	

The average ethanol concentration values in E and F samples are obviously higher than in samples A, B, C, and D. This data describes that adding specific enzyme can enlarge the ethanol yield.

By comparing the average ethanol concentration values between samples E and F, the results prove that the ethanol yield from the enzymatic hydrolyzation with xylanase is lower than the ethanol yield from the enzymatic hydrolyzation with

cellulose which concludes that the working efficiency of cellulase is better than that if the xylanase in this process.

7.3 Discussion

By analyzing the results from the experiments, no solid evidence was found to prove that large difference in ethanol yield exists between the usage of baking yeast and the usage of brewing yeast in the fermentation process. Biologically, the baking yeast and brewing yeast are both categorized as fungus, formally known as Saccharomyces cerevisiae. Both of them can be used in alcoholic fermentation. The functions of these two yeasts are almost the same. This is why no significant difference in the ethanol yield between these two yeasts was appeared.

The amount of the cellulose is much greater than hemicellulose in wood structure. The cellulase is active on the cellulose which holds the largest share of the wood structure. The xylanase is a type of hemicellulase which is active on the hemicellulose. Based on the biological characteristics of enzymes, specific type of enzyme must be properly applied to certain raw materials in order to release its maximum efficiency. An efficient enzymatic hydrolyzation acts an import role in the ethanol production by using the lignocellulosic materials as source, since it largely increases the ethanol productivity.

However, this work is done within the laboratory scale; there are still several factors which can influence the ethanol yield, for example: experimental device restrictions and the controls of the experimental conditions.

8 CONCLUSION

The theoretical study and experimental operations of the particular wood process have offered the understanding on how wood as raw material is used in the bio-ethanol production.

During this study, two types of yeasts were in use for the fermentation process, the variation of the yeast influenced the ethanol production, but the difference is not significantly large since the biological characteristics of mentioned yeast are similar. But compared with the yeast, the difference between using and not using the specific enzyme is obvious; the same appearance can also be found in the comparison between acidic hydrolyzation and the enzymatic hydrolyzation, which both comparisons indicate that the usage of enzyme is able to highly enlarge the productivity. This fact tells that if the condition is available, it is best to apply a certain enzyme to the chemical/biological process to reach higher productivity and faster production period.

After analyzing the final results, we might understand that although the wood chips are able to be used as the raw material for bio-ethanol production, its productivity is relatively lower and process is considerably more difficult than in the agricultural bio-ethanol production. For instance: producing bio-ethanol by using sugar products and starch crops. However, due to the concerns of world`s food security and the trend of the new bio-energy developments and applications , the usage of forest based raw materials is still considered as one of the major methods of bio-ethanol production.

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Formula

Formula1 $C_2H_{4(g)} + H_2O_{(g)} \rightarrow CH_3CH_2OH_{(l)}$

Formula2 $\text{Cellulose} \xrightarrow{\text{Hydrolysis}} \text{Glucose} \xrightarrow{\text{Fermentation}} \text{Ethanol}$

Formula3 $\text{Hemicellulose} \xrightarrow{\text{Hydrolysis}} \text{Pentosed} \ \& \ \text{Hexoses} \xrightarrow{\text{Fermentation}} \text{Ethanol}$

Formula4 $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

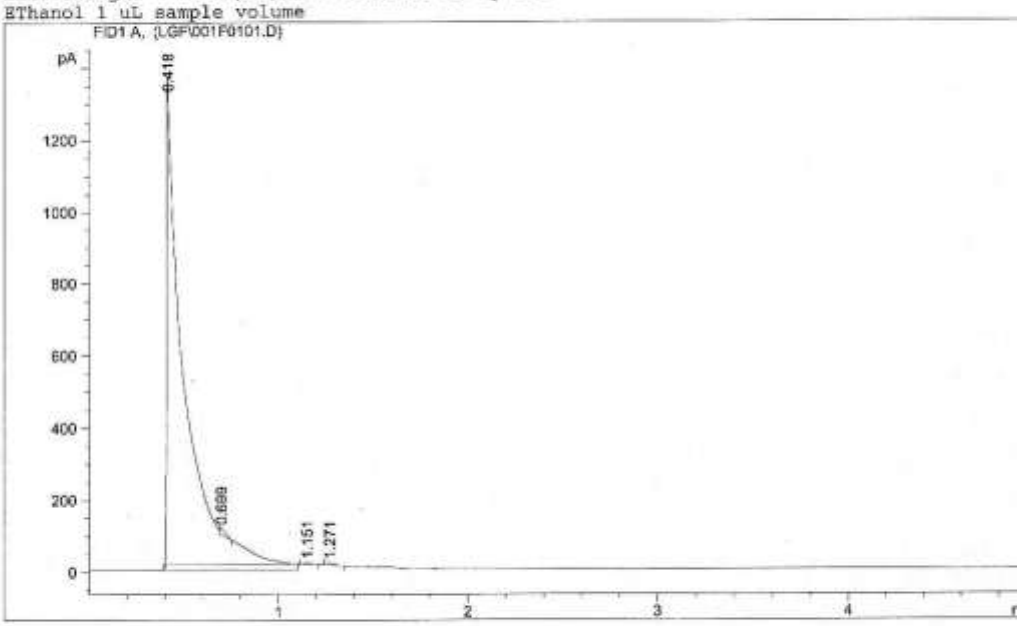
Formula5 $y = 3960.3 * x$

APPENDIX 1

GC analysis report: Sample A1

Data File D:\HPCHEM\1\DATA\LGF\001F0101.D Sample Name: exp 1,

```
Injection Date  : 11/25/2011 12:22:07 PM        Seq. Line :   1
Sample Name     : exp 1/1                       Location : Vial 1
Acq. Operator   : Liu Guifang                       Inj :   1
                                               Inj Volume : 1 µl
Sequence File   : D:\HPCHEM\1\SEQUENCE\SKTYO12.S
Method          : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed    : 11/23/2011 11:12:42 AM by Liu
EThanol 1 uL sample volume
```



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,
```

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	0.418	PB S	0.0791	8582.48828	1315.11829	99.35505
2	0.699	BB X	0.0360	38.07068	17.61040	0.44072
3	1.151	BP	0.0295	7.80823	3.43779	0.09039
4	1.271	BP	0.0345	9.83328	4.02547	0.11383
Totals :				8638.20047	1340.19194	

```
 Results obtained with enhanced integrator!
                        *** End of Report ***
```

Agilent 6850 GC 11/25/2011 12:27:07 PM Liu Guifang Page 1 of 1

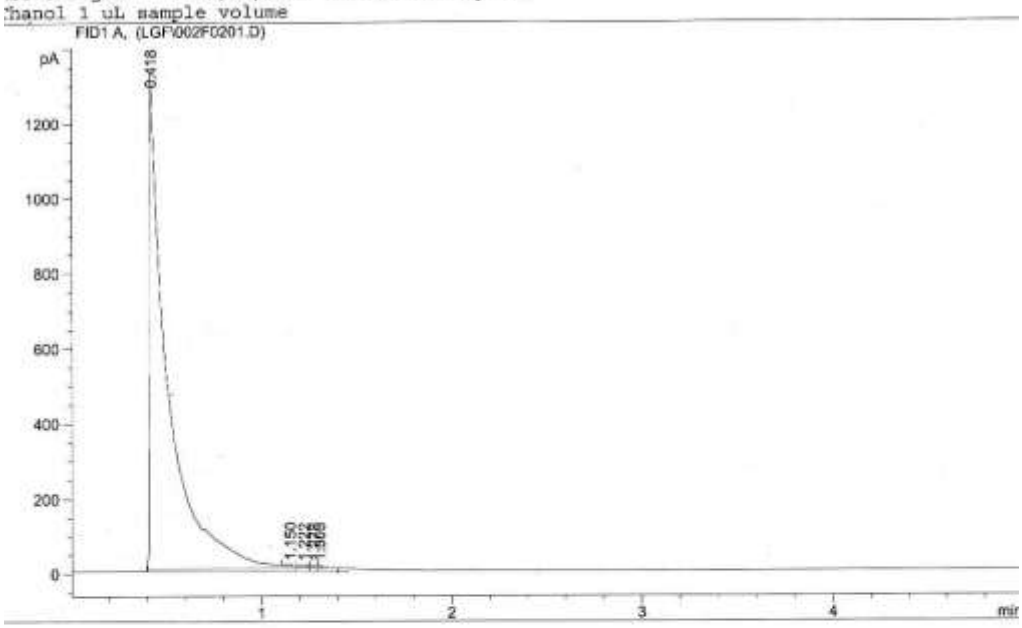
APPENDIX 2

GC analysis report: Sample A2

ile D:\HPCHEM\1\DATA\LGF\002F0201.D Sample Name: exp 1/2

jection Date : 11/25/2011 12:28:14 PM Seq. Line : 2
mple Name : exp 1/2 Location : Vial 2
q. Operator : Liu Guifang Inj : 1
Inj Volume : 1 µl

thod : D:\HPCHEM\1\METHODS\ETHANOL1.M
st changed : 11/23/2011 11:12:42 AM by Liu
hanol 1 uL sample volume



Area Percent Report

rted By : Signal
ltiplier : 1.0000
lution : 1.0000

gnal 1: FID1 A,

ak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	0.418	PB S	0.0834	8813.19043	1278.48828	99.55253
2	1.150	VV X	0.0272	6.79330	3.57230	0.07674
3	1.222	VV X	0.0339	10.35088	4.04257	0.11692
4	1.278	VV X	0.0325	14.87811	5.88966	0.16806
5	1.305	VB X	0.0190	7.59180	6.66997	0.08576

tals : 8852.80451 1298.66279

esults obtained with enhanced integrator!

*** End of Report ***

t 6850 GC 11/25/2011 1:12:08 PM Liu Guifang Page 1 of 1

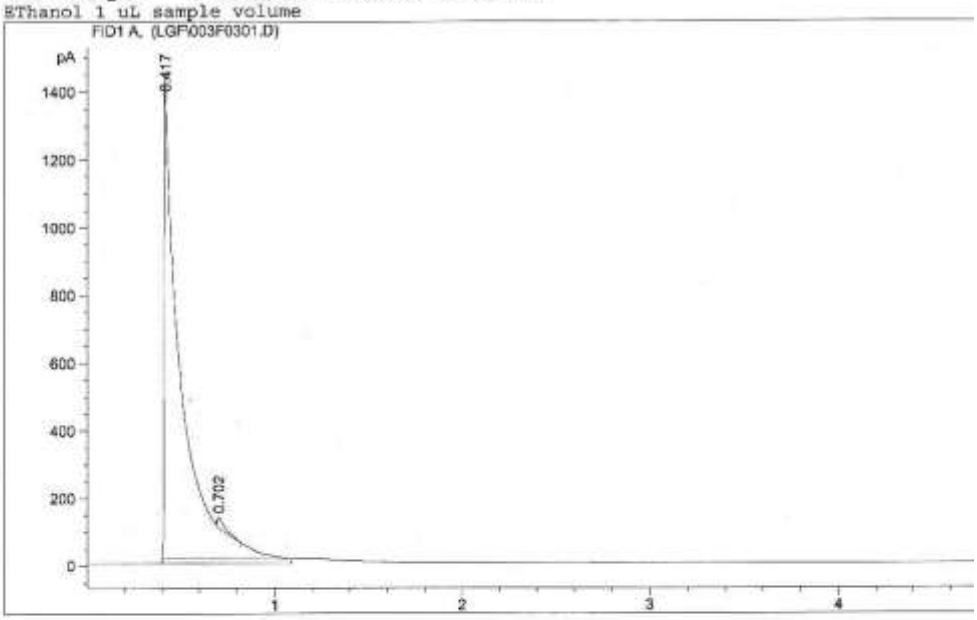
APPENDIX 3

GC analysis report: Sample B1

Data File D:\HPCHEM\1\DATA\LGF\003F0301.D Sample Name: exp

Injection Date : 11/25/2011 12:34:17 PM Seq. Line : 3
Sample Name : exp 2/1 Location : Vial 3
Acq. Operator : Liu Guifang Inj : 1
Inj Volume : 1 µl

Method : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed : 11/23/2011 11:12:42 AM by Liu
EThanol 1 uL sample volume



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	0.417	PB S	0.0772	8664.16016	1380.35791	98.78920
2	0.702	BB X	0.0460	106.19143	33.48462	1.21080

Totals : 8770.35159 1413.84253

Results obtained with enhanced integrator!

*** End of Report ***

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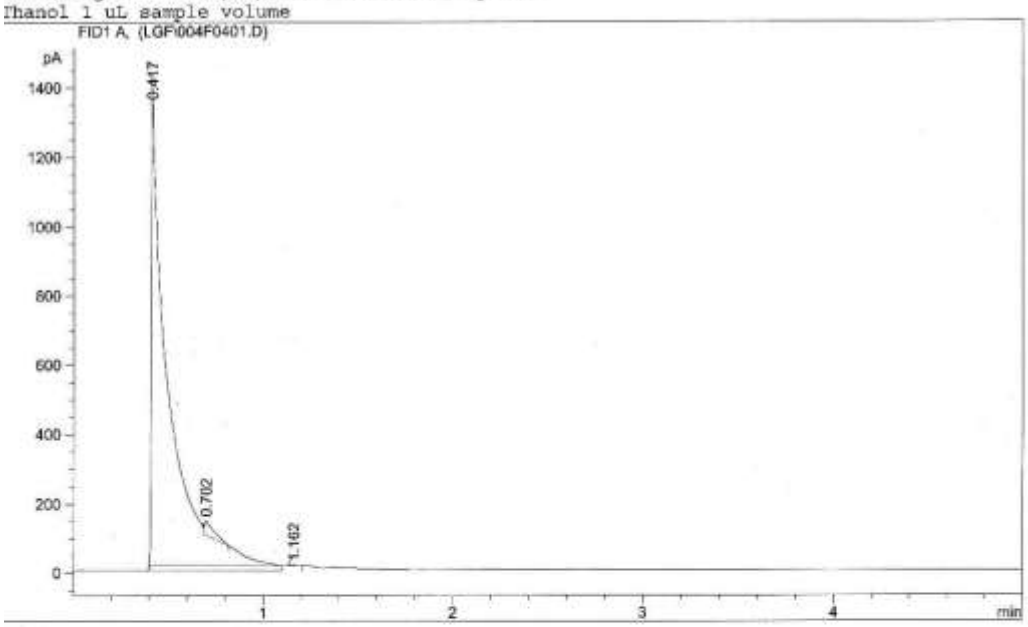
APPENDIX 4

GC analysis report: Sample B2

File D:\HPCHEM\1\DATA\LGP\004F0401.D Sample Name: exp 2/2

njection Date	: 11/25/2011 12:40:19 PM	Seq. Line :	4
ample Name	: exp 2/2	Location :	Vial 4
eq. Operator	: Liu Guifang	Inj :	1
		Inj Volume :	1 µl
ethod	: D:\HPCHEM\1\METHODS\ETHANOL1.M		
ast changed	: 11/23/2011 11:12:42 AM by Liu		

Ethanol 1 uL sample volume



Area Percent Report

orted By	:	Signal
ultiplier	:	1.0000
ilution	:	1.0000

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	0.417	PB S	0.0781	8666.59180	1346.29700	98.15028
2	0.702	BB X	0.0635	161.85495	42.50209	1.83303
3	1.162	PP	0.0176	1.47397	1.47697	0.01669
Totals :				8829.92072	1390.27606	

Results obtained with enhanced integrator!

*** End of Report ***

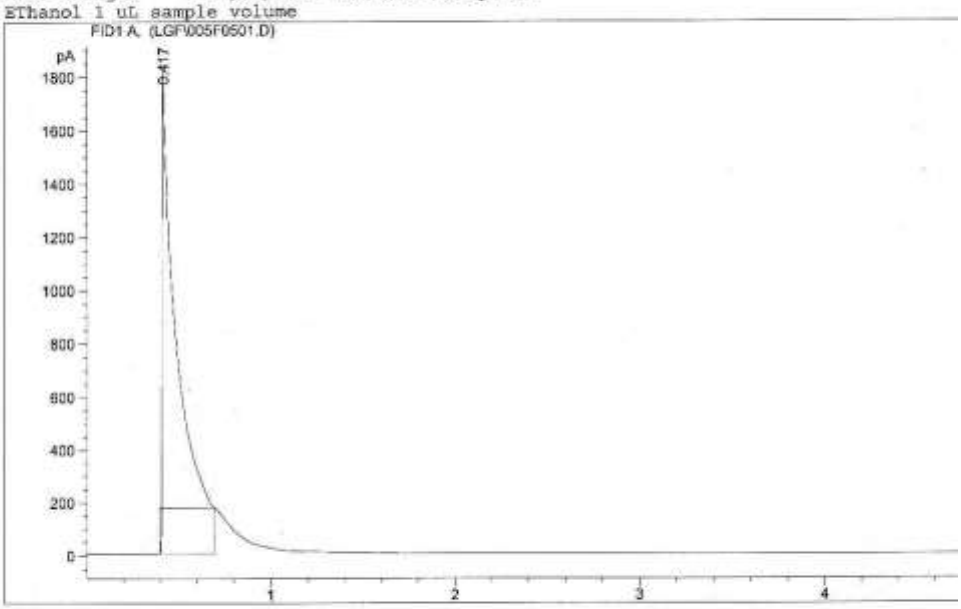
APPENDIX 5

GC analysis report: Sample C1

Data File D:\HPCHEM\1\DATA\LGF\005F0501.D Sample Name: exp

Injection Date : 11/25/2011 12:46:20 PM Seq. Line : 5
Sample Name : exp 3/1 Location : Vial 5
Acq. Operator : Liu Guifang Inj : 1
Inj Volume : 1 µl

Method : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed : 11/23/2011 11:12:42 AM by Liu
EThanol 1 uL sample volume



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	0.417	PB S	0.0590	8788.25879	1829.94128	1.000e2

Totals : 8788.25879 1829.94128

Results obtained with enhanced integrator!

*** End of Report ***

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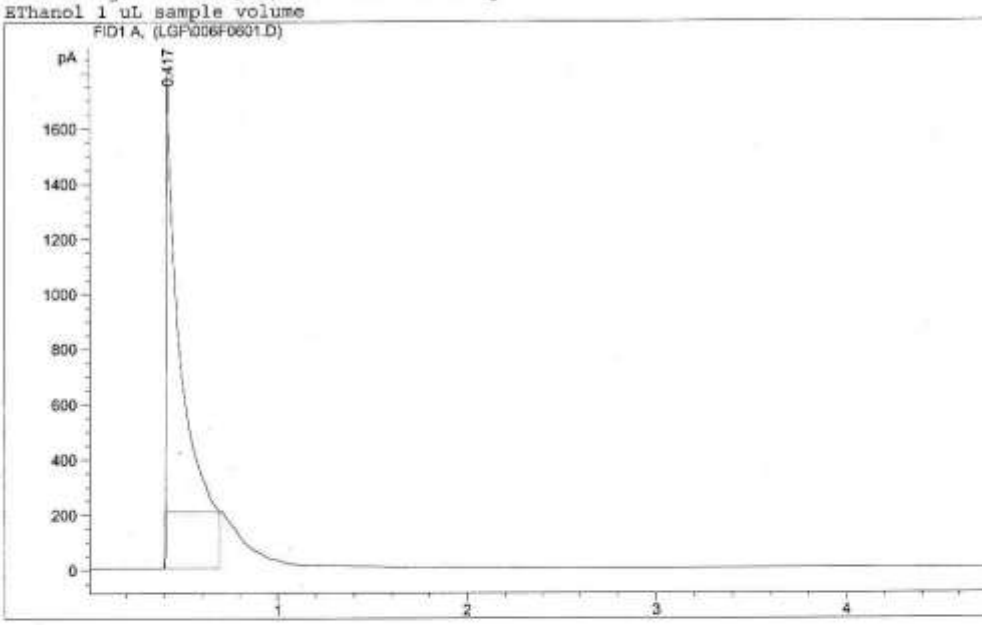
APPENDIX 6

GC analysis report: Sample C2

ata File D:\HPCHEM\1\DATA\LGF\006F0601.D Sample Name: ex

```
Injection Date  : 11/25/2011 12:52:20 PM        Seq. Line :   6
Sample Name     : exp 3/2                       Location : Vial 6
Acq. Operator   : Liu Guifang                       Inj :   1
                                               Inj Volume : 1 µl
Method          : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed    : 11/23/2011 11:12:42 AM by Liu
EThanol 1 uL sample volume
```




```
                        Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [pA*s]      [pA]       %
----|-------|----|-------|----------|----------|--------|
   1   0.417 PB S  0.0566 8215.88574 1789.78662 1.000e2

Totals :                  8215.88574 1789.78662

 Results obtained with enhanced integrator!

                        *** End of Report ***
```

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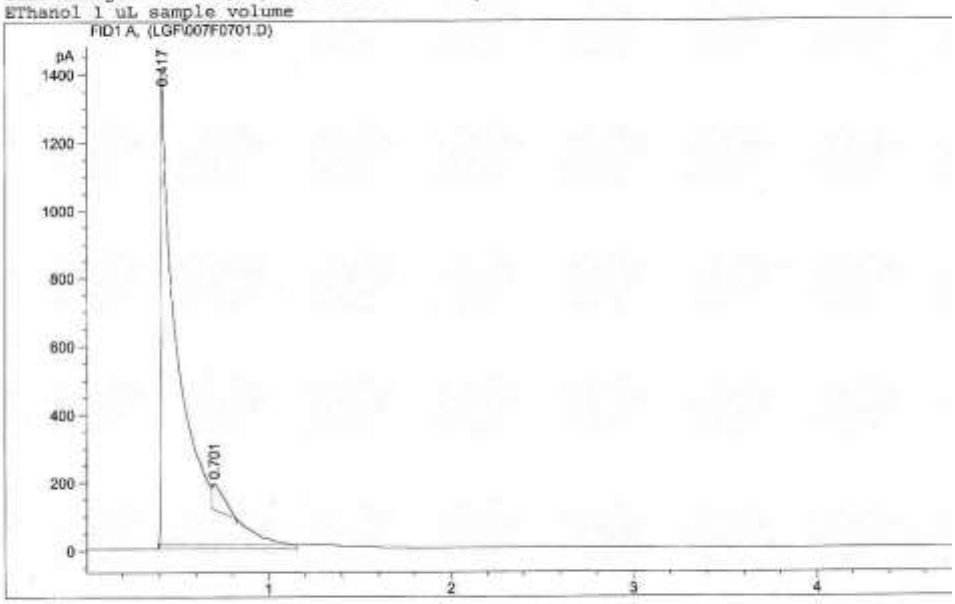
APPENDIX 7

GC analysis report: Sample D1

Data File D:\HPCHEM\1\DATA\LGF\007F0701.D Sample Name: exp

```
Injection Date  : 11/25/2011 12:58:19 PM        Seq. Line :   7
Sample Name     : exp 4/1                      Location : Vial 7
Acq. Operator   : Liu Guifang                      Inj :   1
                                              Inj Volume : 1 µl

Sequence File   : D:\HPCHEM\1\SEQUENCE\SKTYO12.S
Method          : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed    : 11/23/2011 11:12:42 AM by Liu
EThanol 1 uL sample volume
```



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,
```

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	0.417	PB S	0.0879	9793.07422	1346.30273	96.55849
2	0.701	BB X	0.0748	349.04190	77.81986	3.44151
Totals :				1.01421e4	1424.12260	

```
 Results obtained with enhanced integrator!
                        *** End of Report ***
```

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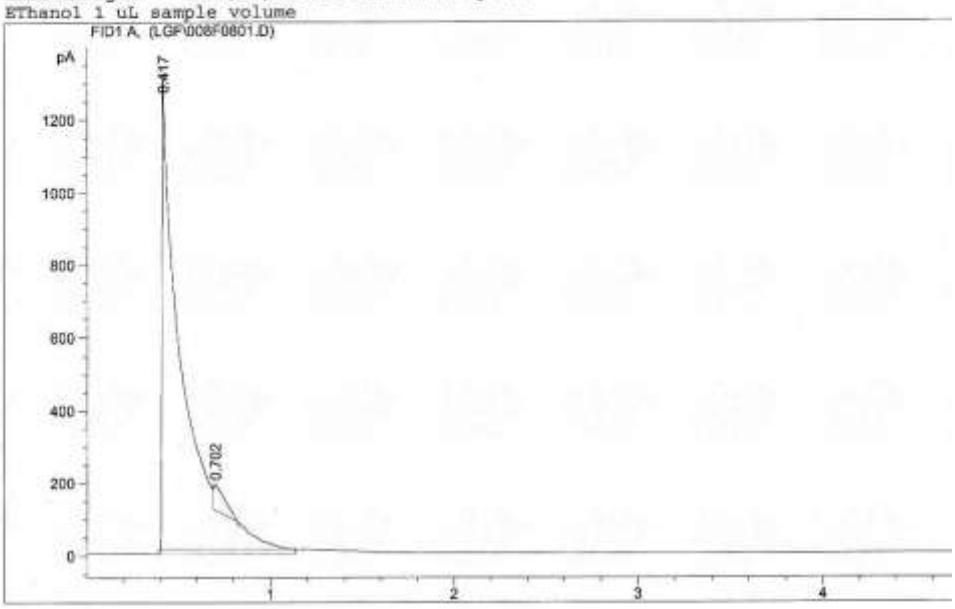
APPENDIX 8

GC analysis report: Sample D2

Data File D:\HPCHEM\1\DATA\LGF\008F0801.D Sample Name: ex

Injection Date : 11/25/2011 1:04:17 PM Seq. Line : 8
Sample Name : exp 4/2 Location : Vial 8
Acq. Operator : Liu Guifang Inj : 1
Inj Volume : 1 µl

Sequence File : D:\HPCHEM\1\SEQUENCE\SKTYO12.S
Method : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed : 11/23/2011 11:12:42 AM by Liu
EThanol 1 uL sample volume



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	0.417	PB S	0.0932	9705.23340	1255.11633	96.82790
2	0.702	BB X	0.0736	317.94525	72.04700	3.17210
Totals :				1.00232e4	1327.16334	

Results obtained with enhanced integrator!

*** End of Report ***

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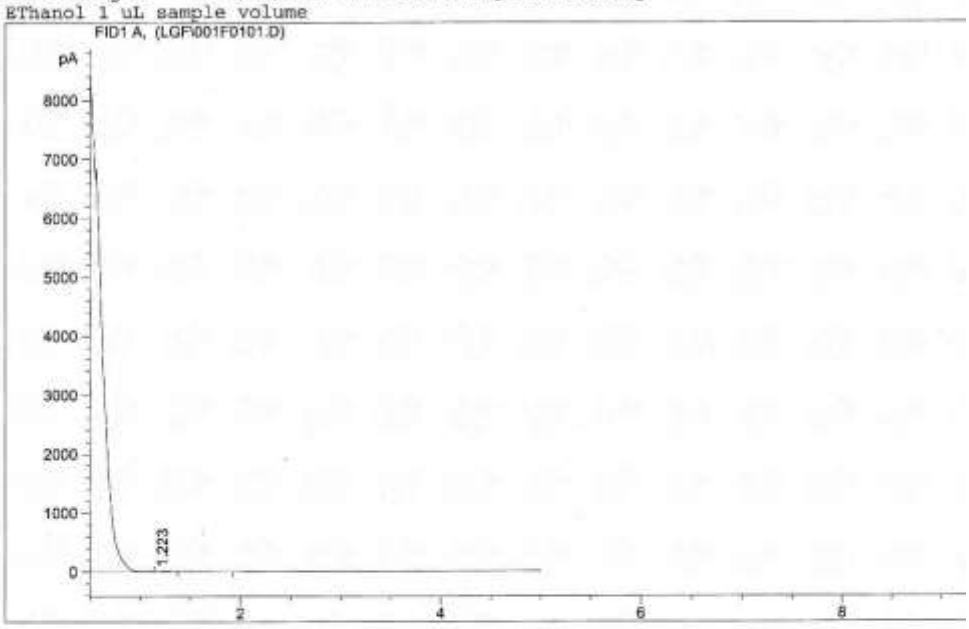
APPENDIX 9

GC analysis report: Sample E1

Data File D:\HPCHEM\1\DATA\LGF\001F0101.D Sample Name: lop

Injection Date : 12/16/2011 4:55:15 PM Seq. Line : 1
Sample Name : loptim815 Location : Vial 1
Acq. Operator : Liu Guifang Inj : 1
Inj Volume : 1 µl

Sequence File : D:\HPCHEM\1\SEQUENCE\SKTYO1.S
Method : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed : 11/28/2011 10:39:25 AM by Liu Guifang
EThanol 1 uL sample volume



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	0.416	HH S	0.1312	1.16029e5	1.05581e4	99.97654
2	1.223	B X	0.1113	27.22224	3.19679	0.02346
Totals :				1.16056e5	1.05613e4	

Results obtained with enhanced integrator!

*** End of Report ***

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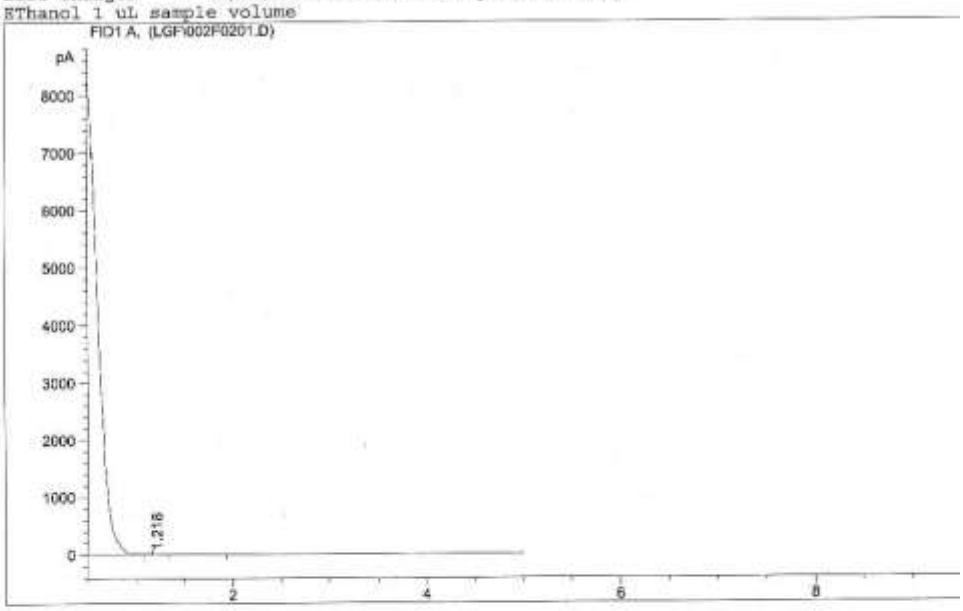
APPENDIX 10

GC analysis report: Sample E2

Data File D:\HPCHEM\1\DATA\LGF\002F0201.D Sample Name: 2opti

```
Injection Date  : 12/16/2011 5:01:17 PM          Seq. Line :   2
Sample Name     : 2optim815                     Location : Vial 2
Acq. Operator   : Liu Guifang                       Inj :   1
                                                Inj Volume : 1 µl
Sequence File   : D:\HPCHEM\1\SEQUENCE\SKTYO1.S
Method          : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed    : 11/28/2011 10:39:25 AM by Liu Guifang
EThanol 1 uL sample volume
```



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [pA*s]      [pA]       %
----|-------|----|-------|----------|----------|--------|
   1   0.416 HH S  0.1225 1.15830e5  1.12181e4  99.98894
   2   1.218 BB X  0.0924   12.80691    1.83521  0.01106

Totals :                  1.15843e5  1.12199e4

 Results obtained with enhanced integrator!
```

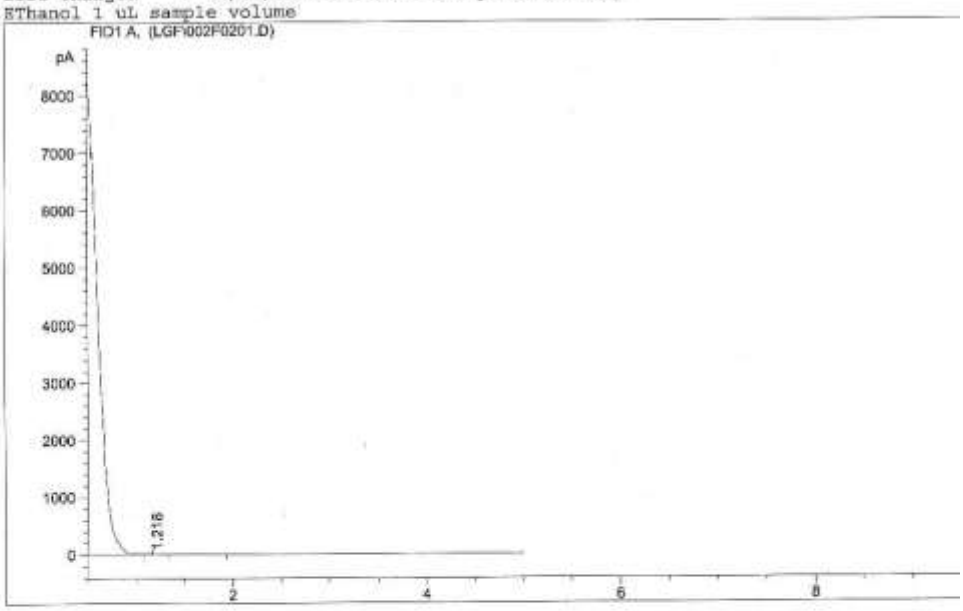
*** End of Report ***

APPENDIX 11

GC analysis report: Sample F1

Data File D:\HPCHEM\1\DATA\LGF\002F0201.D Sample Name: 2opti

```
Injection Date  : 12/16/2011 5:01:17 PM          Seq. Line :   2
Sample Name     : 2optim815                     Location : Vial 2
Acq. Operator   : Liu Guifang                       Inj :   1
                                                Inj Volume : 1 µl
Sequence File   : D:\HPCHEM\1\SEQUENCE\SKTYO1.S
Method          : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed    : 11/28/2011 10:39:25 AM by Liu Guifang
EThanol 1 uL sample volume
```



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [pA*s]      [pA]       %
----|-------|----|-------|----------|----------|--------|
   1   0.416 HH S  0.1225 1.15830e5  1.12181e4  99.98894
   2   1.218 BB X  0.0924   12.80691    1.83521  0.01106

Totals :                  1.15843e5  1.12199e4

 Results obtained with enhanced integrator!
```

*** End of Report ***

APPENDIX 12

GC analysis report: Sample F2

Data File D:\HPCHEM\1\DATA\LGF\004F0401.D Sample Name

```
Injection Date  : 12/16/2011 5:13:19 PM          Seq. Line :   4
Sample Name     : ITR                           Location : Vial 4
Acq. Operator   : Liu Guifang                       Inj :   1
                                                Inj Volume : 1 µl
Sequence File   : D:\HPCHEM\1\SEQUENCE\SKTYO1.S
Method          : D:\HPCHEM\1\METHODS\ETHANOL1.M
Last changed    : 11/28/2011 10:39:25 AM by Liu Guifang
EThanol 1 uL sample volume
```


```
                        Area Percent Report

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: FID1 A,

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [pA*s]      [pA]       %
----|-------|----|-------|----------|----------|--------|
   1   0.416 HH S  0.1346 1.36884e5  1.20404e4  99.97961
   2   1.102  H X  0.0482    6.00241    2.07661  0.00438
   3   1.171  B X  0.0858   21.91874    3.37258  0.01601

Totals :                  1.36912e5  1.20459e4

 Results obtained with enhanced integrator!

                        *** End of Report ***
```

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