SAIMAA UNIVERSITY OF APPLIED SCIENCES
Faculty of Technology, Imatra
Paper Technology

Shang Xueying

PREPARATION OF ETHANOL BY FERMENTATION FROM MECHANICAL GRINDING WASHING WATERS IN LABORATORY SCALE

Bachelor's Thesis 2011

ABSTRACT

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Waters in Laboratory Scale
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Instructor: Esko Lahdenperä, Senior Lecturer, SUAS

The purpose of this thesis was to produce ethanol from the black liquor of woodchips by fermentation in laboratory scale. Hemicellulose, the second most common polysaccharide in nature, represents about 20%-35% of lignocellulosic biomass. The hemicelluloses were extracted from woodchips by pressurized hot water extraction. The extract contains polysaccharides and also lignins, the polysaccharides could be hydrolyzed into monosaccharides by using sulphuric acid. The sodium hydroxide was used to neutralize the hydrolysate. The monosaccharides were fermented by *saccharomyces cerevisiae* yeasts.

Biofuel is a type of energy whose energy comes from biological carbon fixation. Biofuels are derived from biomass conversion, such as solid biomass, liquid fuels and various biogases. Cellulosic biomass comes from non-food sources such as trees and grasses. It is also being researched as a raw material for ethanol production. The choice of feedstock depends on climate, for example sugar cane is used in tropical zones, wheat is popular in Europe and corn is typical for North America. Bioethanol facilities often are linked to sugar or starch factories and use the by-products from that industry as substrates (in the form of molasses, starch hydrolysate a.s.o.).

Key words: Fermentation, Ethanol, Hemi-cellulose, Yeast, Woodchips

CONTENTS

1. BIOFUEL

Biofuel is a type of energy whose energy comes from biological carbon fixation. Biofuels are derived from biomass conversion, such as solid biomass, liquid fuels and various biogases. However fossil fuels have their source in ancient carbon fixation, they are not considered biofuels by the generally accepted definition because they contain carbon that has been "out" of the carbon cycle for a very long time. Biofuels are gaining increased public and scientific attention, driven by factors such as oil price spikes, the need for increased energy security, concern over greenhouse gas emissions from fossil fuels, and government subsidies.

Bioethanol is a high-octane, water-free alcohol produced from the fermentation of sugar or converted starch. It is a clear pellucid liquid with mild odor in its purest form . The boil point is 78°C and it freezes at -112°C. It can not be used in combustion engines either on its own nor blended with petroleum. Hydrous bioethanol (95% purity) is usually used for blending with gasoline.

Cellulosic biomass comes from non-food sources such as trees and grasses. It is also being researched as a raw material for ethanol production. The choice of feedstock depends on climate, for example sugar cane is used in tropical zones, wheat is popular in Europe and corn is typical for North America. Bioethanol facilities often are linked to sugar or starch factories and use the by-products from that industry as substrates (in the form of molasses, starch hydrolysate a.s.o.).

In its pure form, ethanol can be used as a fuel for automotive vehicles, but it is usually used as an additive of gasoline to increase octane and enhance vehicle emissions. Bioethanol is widely used in the United States and Brazil. It produces relatively lower emissions on combustion and it only releases the

same amount of carbon dioxide as plants bound while growing.

Eventually, bioethanol could considerably reduce the climate relevant greenhouse gas emissions from transport and vehicle. Biodiesel is derived from vegetable oils, animal fats or recycled greases. The pure biodiesel can be used as a fuel for vehicles, but eventually it is used to reduce levels of particulates carbon monoxide, and hydrocarbons from diesel-powered vehicles as a diesel additive. Biodiesel is produced from oils or fats using transesterification and is the most common bio-fuel in Europe.

1.1 Sustainability of bio-fuels

In the European Union, the producers and importers of bio-fuels must meet integrated criteria that insure a sustainable and ecologically responsible production. The sustainability criteria introduced in the <u>Renewable Energy Directive COM(2009)28</u> cover:

Sustainable biofuel certificates:
The whole production chain must be checked, from the farmer via manufacturing to the filling station
Protecting untouched nature:
No raw materials from (tropical) forests, wetland or nature protection areas
Substantial reductions for greenhouse gas emissions:
At least 35% compared to fossil fuels (rising to 50% in 2017, for new plants 60% in 2018)

1.2 Bio-ethanol

Nowadays, ethanol fuel becomes the most common bio-fuel worldwide, especially in Brazil. Ethanol fuels are produced by fermentation of sugars comes from wheat, corn,sugar beets, sugar cane, molasses and any sugar or starch that alcoholic beverages can be made from (like potato and fruit waste, etc.). The methods of producing ethanol used are enzyme digestion , fermentation of the sugars, distillation and drying. The distillation process requires significant energy input for heat.

Ethanol can be used in petrol engines to replace gasoline; it could be mixed with gasoline to any percentage. From now, most car petrol engines can run on mixture of up to 15% bio-ethanol with petroleum/gasoline. Ethanol has a smaller energy density than gasoline, so it takes more fuel to produce the same amount of work. An advantage of ethanol (CH_3CH_2OH) is that it has a higher rating of octane than non-ethanol gasoline available at normal gas stations which allows an increase of an engine's compression ratio for improved thermal efficiency. In high altitude region, some states mandate a mix of gasoline and ethanol as a winter oxidizer to reduce atmospheric pollution emissions.

In the current alcohol-from-corn production model in the United States, allowing for the total energy consumed by farm equipment, cultivation, planting, fertilizers, pesticides, herbicides, and fungicides made from petroleum, irrigation systems, harvesting, transport of feedstock to processing plants, fermentation, distillation, drying, transport to fuel terminals and retail pumps, and lower ethanol fuel energy content, the net energy content value added and delivered to consumers is very small. And, the net benefit (all things

considered) does little to reduce imported oil and fossil fuels required to produce the ethanol.

Even ethanol-from-corn and other food stocks have implications both on world food prices and limited, but positive energy yield (in terms of energy delivered to customer/fossil fuels used), the technology has to cause the development of cellulosic ethanol. In terms of a joint research agenda conducted through the U.S. Department of Energy, the fossil energy ratios (FER) for cellulosic ethanol, corn ethanol, and gasoline are 10.3, 1.36, and 0.81, respectively.

As dry ethanol has roughly one-third lower energy content per unit of volume compared to gasoline, so larger / heavier fuel tanks are required to travel the same distance, or more fuel stops are required. With large current unsustainable, non-scalable subsidies, ethanol fuel still costs much more per distance traveled than current high gasoline prices in the United States.

1.3 Cellulosic ethanol

Cellulosic ethanol is a type of bio-fuel produced by wood, grasses, or the non-food plants.

The cellulosic ethanol is derived from lignocellulose. Lignocellulose is a structural material that comprises much of the mass of plants. Cellulose, hemicellulose and lignin are the main three parts to compose lignocllulose. Many cellulosic materials could be used for ethanol production as feedstock, such as corn straw, switchgrass, miscanthus, woodchips and lawn and tree maintenance. The cellulosic ethanol has many advantages and diverse raw material compared to source like corn and cane sugars, but requires a complicated processing to make the sugar monomers to the microorganisms that are used to make ethanol by fermentation. Cellulose, however, is contained in nearly every natural, free-growing plant, tree, and

bush, in meadows, forests, and fields all over the world without agricultural effort or cost needed to make it grow.

According to U.S. Department of Energy studies conducted by Argonne National Laboratory of the University of Chicago, one of the benefits of cellulosic ethanol is that it reduces greenhouse gas emissions (GHG) by 85% over reformulated gasoline. By contrast, starch ethanol (e.g., from corn), which most frequently uses natural gas to provide energy for the process, may not reduce GHG emissions at all depending on how the starch-based feedstock is produced.

1.4 Fossil versus renewable energy resources

Serious geopolitical implications are caused by the fact that the human society is heavily dependent on only a few energy resources such a petroleum, mainly produced in politically unstable oil-producing countries and regions. Indeed, recording to the World Energy Council, about 82% of the world's energy needs are currently covered by fossil resources such as petroleum, natural gas and coal. Also ecological damages have come into prominence as the use of fossil energy sources suffer a number of ill consequences for the environment, including the greenhouse gas emissions, air pollution, acid rain, etc.

Moreover, the supply of these fossil resources is fixed finite. It is generally agreed that the petroleum will be exhausted with in 50 years, natural gas within 65 years and coal in about 200 years at the present tempo of consumption. With regard to the depletion of petroleum supplies, the human has to faced with the austere situation that the world is using petroleum faster than ever before, and nevertheless the "proven petroleum reserves" will still stop at the same level for 40 years, mainly as a result of new oil findings (Campbell,1998).

This fact is often used as an argument against the "prophets of doom", as there is seemingly still plenty of petroleum around for the time being. However, those" proven petroleum reserves" are increasingly found in places that are poorly accessible, inevitably resulting in an increase of extraction costs and hence, oil prices.

On the contrary, the agricultural raw materials such as wheat or corn have been continuously descending in price caused by the increasing agricultural yields. Agricultural crops such as corn, wheat and other cereals, sugar cane and beets, potatoes etc. can be treated in a bio-refinery into correlatively pure carbohydrate feedstocks, the pre-treating raw material for fermentation process. The fermentation process can transform those feedstocks into bio-fuels such as bio-ethanol.

1.5 Economic Impact

Along with technical development, from the economic perspective, the renewable resources are gradually replacing the fossil resources as a raw material. However, this is also be used as a reality for the generation of energy, considering of number of chemicals, increasingly produced from agricultural merchandises in place of petroleum.

Table1.1 *Approximate average world market prices in 2007 of renewable and fossil feedstocks and intermediates.*

Fossil		Renewable	
Petroleum	400 €/t	Corn	150 €/t
Coal	40 €/t	Straw	20 €/t
Ethylene	900 €/t	Sugar	250 €/t
Isopropanol	1000 €/t	Ethanol	500 €/t

The Table 1.1 showns the approximate average world market prices for 2007. Based on the local conditions such as distance to production site and local availability, the price of materials may vary rather widely from one place to another. All prices were converted into Euros per metric tonne (dry weight) for a number of fossil or renewable raw materials, similarly momentous feedstock intermediates like ethylene and sugar, for the only purpose of a clear indicative cost comparison of fossil versus renewable resources.

From Table 1.1, one deduction is that the renewable agricultural resources cost about half of the fossil resources based on the dry weight. At the present price of crude oil, petroleum costs about three times the price of corn. It is interesting that it notes the cost of sugar, a highly refined very pure feedstock (>99.5% purity); and very crude and unrefined mixture of chemical substances. It is cleared that agriculture feedstocks are cheaper than fossil today and are easily available in large quantities. Whereas the chemical technology based on switch fossil feedstocks into a bewildering variety of useful products very efficiently, the technology for converting agricultural raw materials into chemicals, materials and energy is still in its infancy.

It is broadly admitted that new technologies will need to be exploited and optimized to harvest the benefits of the bio-based economy. Especially industrial biotechnology is regarded a very crucial technology in this area, as it is excellently capable to utilize agricultural merchandises as a feedstock. The agricultural feedstocks processing into useful products emerge in so-called bio-refineries. However, the gradual transition from a fossil-based society to a bio-based society will take time and effort, the raw materials will win over fossil resources in the future. This is expressly true in taking into account the viewpoint of increasingly rarer, extraction problem and more expensive fossil resources.

1.6 Utilization convenience of bio-fuels

The energy content of energy carrier is only one aspect in the total contrast. The value of an energy carrier is not only defined through its energy content and yield per hectare, but also equally by its physical shape and convenience in utilization. The aspect of an energy sources is expressly important for auto applications, like transportation. In Europe, the transport part delegates 32% of all energy consumption. In practice, liquid bio-fuels are much better suited for such an application. It is authentic that almost all cars and trucks are powered by liquid fuels such as gasoline and diesel. These fuels are readily and stanchly used in classic explosion engines. They are easy to store, transport and transfer and their utilization just requires non-storage technology at all.

The liquid fuels such as bio-ethanol and biodiesel from renewable sources is based strongly on the fact that these bio-fuels show all the advantages of the classic motor fuels. They are produced by agricultural petrol and diesel, with no engine recreation required. The utilization of bio-ethanol or biodiesel hence fits perfectly within the current concept of mobility.

Table 1.2 *Energy yields of bio-energy crops in Flanders (Belgium)*

	Yield		Bio-fuel yield	Gross energy yield	
	(t dry matter/ha/yr)	Bio-fuel-type	t/ha/yr	GJ/t	GJ/ha/yr
Wheat	6.8	bio-ethanol	2.29	26.8	61
Sugar beets	14	bio-ethanol	4.84	26.8	130
Rapeseed	3.1	biodiesel	1.28	37	50
Willow/poplar	10.8	firewood	10.8	18	194

Table 1.2 compares the energy yields of the different plant resources and technologies. In accordance with comparison, fleetly growing wood species such as willow or poplar as a general renewable energy sources, are also included.

It is noted that the gross energy yield per hectare is the highest for fast growing trees like willow or poplar. Although the liquid fuel is restricted by ourselves, there remain big differences between the different bio-energy options to be interpreted.

1.7 Future of bio-fuel

The positive use of bio-ethanol coming from agricultural crops is a technically doable replacement for fossil-based gasoline. Furthermore, their use fits perfectly in the future concept and technology of our mobility. Liquid energy supporters are an expensive but very useful energy supporter for mobile applications such as transportation. It is obvious that energy sources for mobile applications can not only be compared on the basis of simple energy balances or costs, but also on the basis of their practical usefulness, quality,

environmental impact and convenience in use of the obtained energy carrier. As the discussion about the sense or nonsense of bio-fuels is under away, the transition process from a fossil-based to a bio-based society is clearly moving along, with prominent growth in the United States, Brazil, China and Europe finally gaining ground.

The large-scale introduction of bio-fuels can improve the interests of environment, mobility and agriculture and could be regard as a very important step with high symbolic value towards the sustainable society of the future.

2. Feedstock for bio-fuel

2.1 Availability of cellulosic feedstocks

Demand for chance feedstocks for fuels, chemicals and a range of commercial products has grown observably in the early years of the 21st century, motivated by the high price of crude oil, government policy to encourage chance and run-down dependence on foreign petroleum, and efforts to decay net emissions of carbon dioxide and other greenhouse gases. This is especially true for renewable feedstocks from agricultural sources.

A bio-refinery requires a large, faithful, economic and sustainable feedstock supply. Investigations at all kinds of sites manifest that economic delivery of crop residues is performable at this radius and beyond- up to 50 miles from the bio-refinery site when short line rail transport is possible. Therefore, cellulosic bio-refineries of well over 100 million gallon capacity are available.

To maintain a commercial-scale bio-refinery, cropland surrounding the site should meet the following criteria:

- Large area: minimum of 500,000 acres of available cropland;

- Sustainable: cropping practice maintains or improves long-term health of the soil;

- Faithful: consistent crop supply history with dry harvest weather;

- Economic: high-yielding cropland;

- Favorable transport: easy access from field to storage and processing facilities;

In the future, the feedstocks tend to depend on several variables, including crop acreage planted to meet competing demands; continued melioration from agricultural biotechnology; cropping practice and soil-quality maintenance considerations; and state and federal farm and energy policies. Coherence with farm and energy policies at both state and federal levels can apply to encourage production, harvest and delivery of different feedstocks to bio-refineries.

2.2 Feedstock options

- **Corn straw and cereal straw**

Figure 2.1 Baling and collection technology for wheat straw

Corn is the largest grain crop in the world. Nowadays, 50% of the corn biomass, about 250 million dry tons, is left in the field after harvest. Almost available cereal straw biomass is from wheat (see Figure 2.1). There are momentous local differences in crop characteristics to think over, also differences in harvesting mechanic for straw. The corn straw is more available than straw, but straw is more easily removed. Corn straw yields are 3-5 times greater on a per acre basis than straw from cereal crops. Only if cereal crops are irrigated, there is little straw left to collect.

- **Soybean stubble**

Soybean stubble is the surface material left after harvesting of the soy beans. Soybean stubble provides crudely the same feedstock quantity per acre as straw from dryland cereal grains. The significance of soybean stubble is on the level of rotation between soybean and corn.

● **Corn fiber**

Corn fiber is an available source of cellulose. Nowadays, the biotechnology of corn fiber processing can be applied to corn straw as well, even through big differences exist in the composition, consistency and price of the material. Corn fiber includes a small amount of lignin and a large amount of bound starch, while stover contains a much larger lignin fraction.

	Corn fiber	Stover
Cellulose	12 to 18%	32 to 38%
Hemi-cellulose	40 to 53%	28 to 32%
Lignin	0.1 to 1%	15 to 17%
Starch	11 to 22%	None

Table 2.2 Corn fiber and stover composition, dry basis

● **Process waste**

Process waste from other sources, like cotton gin trash and paper mill sludge, composes a potential source of cellulosic residues, particularly for niche conditions. Even its volume is small and as with corn fiber, there is no common view on whether these materials could provide an sufficient supply of biomass to ensure bio-refinery construction.

2.3 Structure of cellulose and hemi-cellulose

A variety of agricultural residues, like corn fiber, corn straw, wheat straw and sugarcane bagasse, contain about 20-40% hemi-cellulose, the second most plentiful polysaccharide in nature. It represents about 20-35% of lingo-cellulosic biomass. Xylans are the most plentiful hemi-celluloses. So far, bioconversion of hemi-cellulose has got much focus became of its practical applications in different industrial processes, such as effective conversion of hemi-cellulosic biomass to fuels and chemicals, delignification of paper pulp.
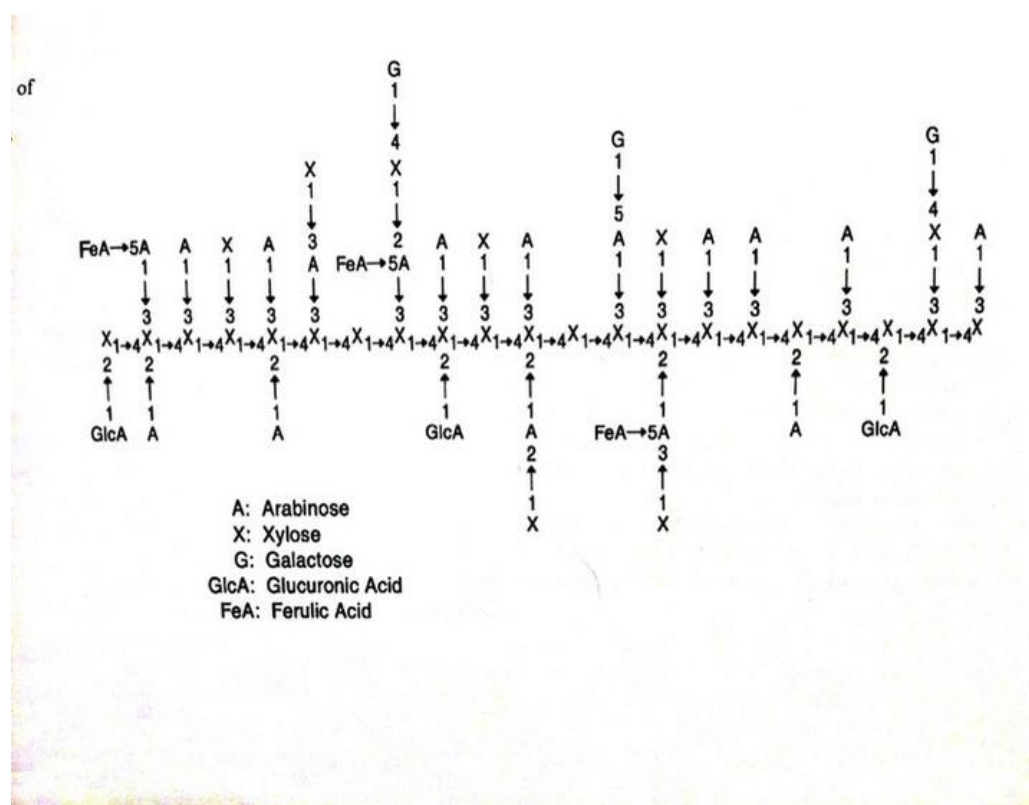
Figure 2.3 Schematic structure of corn fiber heteroxylan.

Hemi-celluloses are various polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Hemi-cellulose is different from cellulose because it is not chemically homogeneous. The hemi-cellulose in hardwood contains mostly xylans, but in softwood it contains mostly glucomannans. Different sources such as grasses, softwood and

hardwood, differ in compositions of xylans. Xylan in birch contains 89.3% xylose, 1% arabinose, 1.4% glucose, and 8.3% anhydrouronic acid. Corn fiber xylan is much more complex, it has heteroxylans containing β -(1,4)-liked cylose residues. It consists of 48-54% xylose, 33-35% arabinose, 5-11% galactose, and 3-6% glucuronic acid. About 80% of the xylan backbone is highly substituted with monomeric side-chains of arabinose or glucuronic acid liked to o-2 or o-3 of xylose residues, as well as by oligomeric side chains containing arabinose, xylose, and galactose residues (Figure 2.3). A model for the corn fiber cell wall is shown in Figure 2.4.
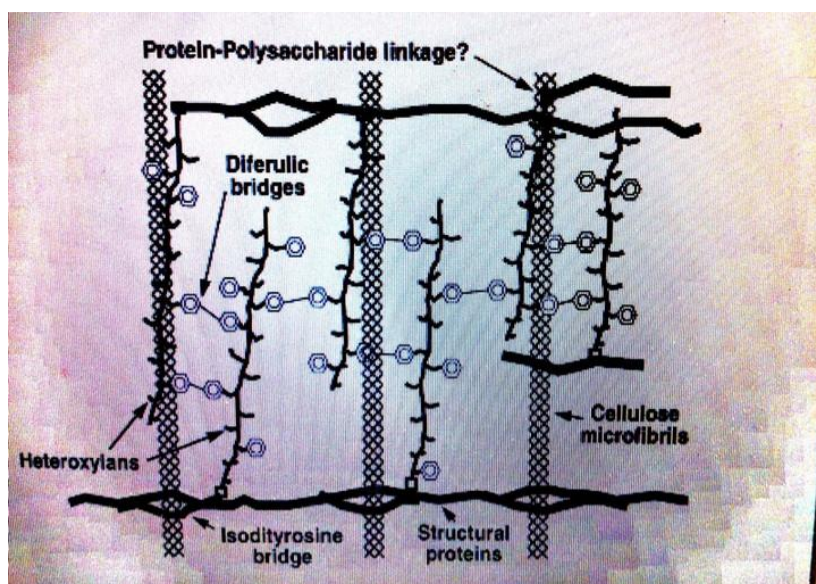
Figure 2.4 Model for corn fiber cell walls.

2.4 Raw material options in experiment

In this experiment, birch and aspen were chosen as they are common raw materials for making paper and some ready-made residual liquor after cooking was used on experiment either, the raw material is birch.

The raw materials have been divided into 8 samples, named by no.1-no.8.

Firstly, the wood chips were cooked under different conditions:

● Concentration of acid additive: to each raw material was added sulfur acid at two difference ratio: 4:1 and 3:1, the acid fractions are 1% and 1.5%.

● Solid/liquid ratio: the solid/liquid ratio of samples was set at around 6:1 and 7:1.

All samples were cooked in digester for about 4 hours at 200℃. The index of cooking process is shown in Table 2.6

Table 2.5 composition of some agricultural and wood lignocellulosic biomass

	Composition (%, dry basis)		
	Cellulose	Hemi-cellulose	Lignin
Corn fiber	15	35	8
Corn stover	45	35	15
Pine	40	28.5	27.7
Birch	41	32.4	22
Spruce	39.5	30.6	27.5

Table 2.6 Index of cooking process

	A	B	C	D	E	F	G	H	I	J	K	L	M
18	sulfur acid calculations												
19													
20	C acid	[mol/L]					2 cooking time		4 hours				
21	H2SO4 molar mass	[g/mol]					98 Tamperature		200℃				
22	acid mass concentration		[g/L]			196							
23	acid density	[g/ml]				1.13							
24	acid mass fraction	[%]				17.3 %							
25													
26						1	2	3	4	5	6	7	8
27						birch	birch	aspen	aspen	aspen	aspen	birch	birch
28	solid/liquid ratio					5.7	6.1	7.1	5.8	6.8	5.6	5.4	6.9
29	wood moisture		[%]			2.00%	2.00%	2.00%	2.00%	2.00%	2.00%	2.00%	2.00%
30	water in wood		[g]			0.54	0.42	0.40	0.48	0.42	0.56	0.58	0.42
31	amount of cooking liqour		[g]			149.72	125.44	139.08	137.05	140.59	154.75	153.69	142.75
32	H2SO4 fraction					1.00%	1.00%	1.00%	1.00%	1.50%	1.50%	1.50%	1.50%
33	amount of acid to be taken		[g]			8.69	7.28	8.08	7.95	12.31	13.54	13.44	12.50
34	dilution water required		[g]			142.0	119.0	132.0	130.0	130.0	143.0	142.0	132.0
35	water in acid		[g]			7.2	6.0	6.7	6.6	10.2	11.2	11.1	10.3
36	amount of wet solid		[g]			27	21	20	24	21	28	29	21
37	amount of abs. Dry wood		[g]			26.46	20.58	19.6	23.52	20.58	27.44	28.42	20.58
38	total amount		200g										
39	acid volume		[ml]			7.69	6.44	7.15	7.04	10.89	11.98	11.90	11.06
40													

3. EXPERIMENT PROCESS BEFORE FERMENTATION

3.1 Process modeling

1) FEEDSTOCK COLLECTION AND STORAGE

BIOTECH ENABLING TECHNOLOGY
Enzyme production coverts cellulose to sugars

2) PRETREATMENT
Making feedstock accessible to enzymatic or microbial hydrolysis.

3) BIOTECHNOLOGY TREATMENT
Hydrolysis and fermentation of sugars

4) DOWNSTREAM
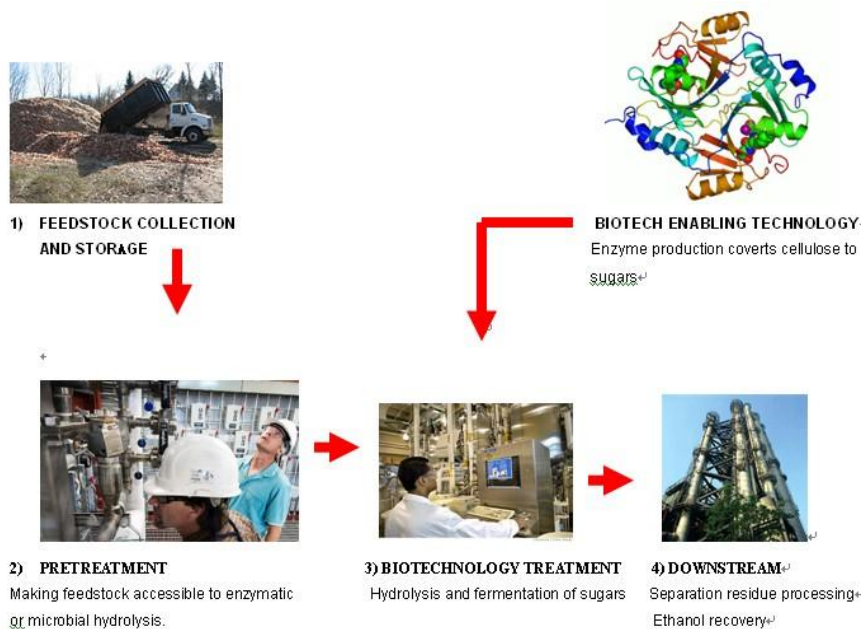Separation residue processing
Ethanol recovery

Figure 3.1 Biochemical production of cellulosic ethanol

In this experiment, the modeling was designed according to the industrial process

model. (Figure 3.2).

After cooking, the waste liquor was in the pre-treatment process (pre-filtering, pre-distillation, pre hydrolysis) to break the structure of cellulose and hemi-cellulose to make it to be a incompact model.
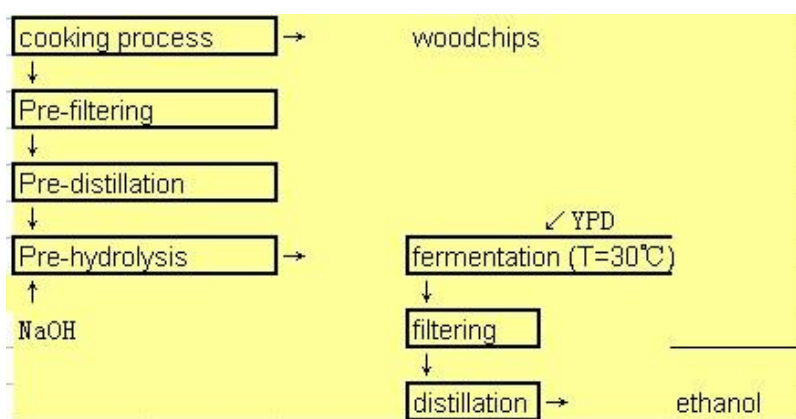
Figure 3.2 The flow sheet of processing

In the pre-distillation process, only the no.4, no.6, no.7 samples can be the available liquor, because there are too many complex compounds in the other samples resulting in the evaporation of the liquor.

3.2 Production of lignocellulosic hydrolysates

The use of hemi-cellulosic sugars is a requisite for efficient and cost-effective conversion of lignocellulosic material to fuel ethanol. A variety of waste which contains cellulose and hemi-cellulose can serve as low-cost feedstocks for production of fuel ethanol. Any hemi-cellulose including lignocellulose can form sugars upon hydrolysis process. The mixture of sugars contains any combination of xylose, arabmose, glucose, galactose, mannose, fucose and rhamnose depending on material.

The degradation of the lignocelluloseic structure usually requires two steps: first, the pre-hydrolysis in which the hemi-cellulose is broken down, and second, the hydrolysis of the cellulose fraction in which lignin will remain as a solid by product. Not only fermentable sugars are released, but a considerable

compounds, some of which could restrain the fermentation of micro-organism.

The pre-hydrolysis process was executed by physical, chemical, or biological methods such as steam pretreatment, freeze explosion, acid treatment (hydrochloric acid, sulfuric acid, sulfur dioxide), alkaline treatment (sodium hydroxide, ammonia), or organic treatment.

The sulfuric acid and sodium hydroxide solution were used in the process to hydrolyze hemi-cellulose, and the pH is 7.

4 UTILIZATION OF YEAST

4.1 Introduction of yeast (*Saccharomyces cerevisiae*)

Figure 4.1 Images of Saccharomyces cerevisiae
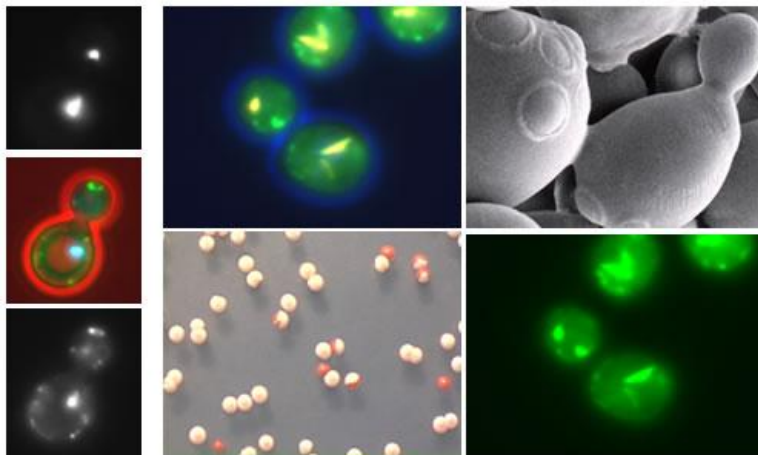
Efficient fermentation of hemi-cellulosic sugars is vital for the bioconversion of lignocellulosics to ethanol. Efficient sugar intake through the diverse expression of yeast and xylose/ glucose transporter can make fermentation better if other metabolic steps are not rate limiting. Genetic engineering and evolutionary adaptation to improve glycolytic flux coupled with transcriptomic and proteomic studies have designable targets for further rectification, as have

genomic and metabolic engineering studies in native xylose fermenting yeasts.

Bioconversion of lignocellulose to ethanol must take place in good yield, at high rate, and to concentrations that are economically recoverable. The major barrier is enzymatic saccharification for cellulose. For hemi-cellulose, it is the utilization of glucose, xylose, mannose, galactose, arabinose, and rhamnose, in the presence of acetic and ferulic acid along with a variety of degradation products from thermo-chemical pretreatment. Mostly, the yeast metabolic engineering for ethanol production from xylose was paid attention to enhancing sugar intake and the initial assimilation steps.

Saccharomyces cerevisiae is a model eukaryotic organism, often used in research because it is easy to operate and culture, and is properly similar in structure to human cells. In industry, the yeast is also widely utilize to manufacture enzymes and proteins for beer or wine because it metabolizes glucose to ethanol, and is also used to make biofuel products.

4.2 Xylose transport

Saccharomyces cerevisiae take up xylose insufficiently because of the low affinity of its native nonspecific hexose-transport system for xylose, which is approximately 130-880 mM, or about 10-100 times higher than for glucose. Furthermore, native transporters in *S. cerevisiae* are not comparatively promoted to facilitate xylose intake. Xylose transport has insufficient effect on the rate of xylose utilization when the levels of xylose reductase (XR) are limiting, but it affects utilization in cells with higher XR levels. Improved xylose transport also has a stout positive effect on *S. cerevisiae* cells engineered for assimilation through overexpression of *Piromyces* xylose isomerase (XI). Better sugar transporters could be more effective.

4.3 Baker's yeast

Baker's yeast is a strains of yeast commonly used as a leavening agent in baking bread and bakery products, where it converts the fermentable sugars present in the dough into carbon dioxide and ethanol. Baker's yeast is one of the species *Saccharomyces cerevisiae*, which is same species commonly used in alcoholic fermentation, and so is also called brewer's yeast.

Figure 4.2 A block of fresh yeast in its wrapper.
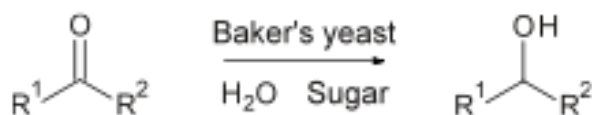
Figure 4.3 Reduction of a carbonyl to a hydroxyl with baker's yeast.

Because it is quite readily available and easy to culture, baker's yeast has long been used in chemical, biological, and genetic research. Baker's yeast contains enzymes and it can reduce a carbonyl group into ahydroxyl group in properly high yield, thus making it a useful bio-reagent in chemical syntheses. It is known to reduce organometallic carbonyl compounds in very high yield. Baker's yeast is also used to manufacture ethanol via fermentation for use in chemical synthesis.

In this experiment, the baker's yeast was used to ferment sugar from waste liquor to produce ethanol. To put it into good use, the baker's yeast has been made with help of YPD media.

Figure 4.4 YPD medium bottle and YPD agar plate

Yeast Extract Peptone Dextrose (YEPD or YPD) is a complete medium for yeast growth. It contains yeast extract, peptone, water, and glucose or dextrose. It can be used as solid medium by including agar. The yeast extract includes all the amino acids necessary for growth. But now, YEPD cannot be used as a selection medium to test forauxotrophs. Instead, YEPD is used as a growth medium to grow yeast cultures.

The YEPD typically consists of 1% (mass/volume) yeast extract, 2% peptone, 2% glucose/dextrose, and is dissolved in water.

In experiment, the YPD media was made by the recipe: 10g yeast, 20g glucose, 1000ml water. Then it was rotated at $30℃$ about 24 hours.

5 FERMENTATION OF LIGNOCELLULOSE

5.1 Fermentation process in bio-ethanol manufacturing

Fermentation process used in producing wine which is made for the conversion of sugars to alcohol. When same process is followed by distillation, it can be used to obtain bio-ethanol for use as a transport biofuel.

Ethanol is manufactured by fermentation of the sugar. Fermentation only work directly with sugars. Two major components of plants, starch and cellulose, are both made up of sugars, and can be converted to sugars for fermentation in theory. Generally, only the sugar (e.g. sugar cane) and starch (e.g. corn) portions can be economically converted. There is much activity in the part of cellulosic ethanol, where the cellulose part of a plant is broken down to sugars and subsequently converted to ethanol.

One mole of glucose is converted into two moles of ethanol and two moles of carbon dioxide:

$$C_{12}H_{22}O_{11} + H_2O + \text{invertase} \rightarrow 2\ C_6H_{12}O_6 \qquad (1)$$

$$C_6H_{12}O_6 + \text{Zymase} \rightarrow 2C_2H_5OH + 2CO_2 \qquad (2)$$
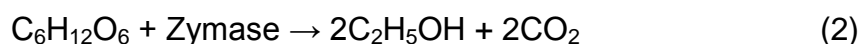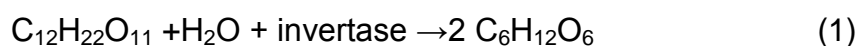
The second stage is the fermentation process to convert glucose into ethanol and CO2. Fermentation ethanol is a change of a mole of glucose into 2 moles of ethanol and 2 moles of CO2. The yeasts will mainly metabolize glucose and fructose to form pyruvic acid through the stages of the reaction pathway Embden-Meyerhof-Parnas, nevertheless pyruvic acid generated would be decarboxylated to acetaldehyde which then experiences dehydrogenation to ethanol.

Yeasts often used in alcoholic fermentation are *Saccharomyces cerevisiae,* because it can produce high tolerance to alcohol (12-18% v / v), resistance to high sugar levels and remain active in the fermentation at a temperature of 4-32℃.

After fermentation is completed, the distillation process can separate ethanol based on boiling point. The boiling point of pure ethanol is 78 ℃ while the water is 100℃ (standard conditions).

By heating the solution at a temperature range of 78 – 100℃ will result in most of the ethanol evaporated, and the condensing units will be produced with a concentration of 95% ethanol by volume.

5.2 Fermentation in experiment

In experiment, the mixture of cellulose solution and YPD solution were in a vessel and covered. The vessel was heated at 25-30℃ and stirred in the whole processing.

Table 5.1 Index of fermentation and distillation process

	Fermentation (T=30℃) →				Distillation		
Sample	pH	YPD	time	final solution	solution	boil point	sample
NO.4	7	200ml	24 hours	315ml	100ml	83℃	76ml
NO.6	7	200ml	3days	323ml	100ml	82℃	82ml
NO.7	7.5	200ml	5days	300ml	100ml	82℃	89ml

The Table 5.1 shows index in fermentation process. After fermentation, all the samples were distilled. The boiling point was around 82℃. Then, the experiment was made.

6 ANALYSIS OF D-GLUCOSE IN UV METHOD

The samples were analyzed for D-glucose in UV method. Each samples were analyzed twice.

6.1 Principle

The D-glucose concentration is determined before and after the enzymatic hydrolysis of sucrose; D-fructose is determined after the determination of D-glucose.

Determination of D-glucose before inversion:
At pH 7.6, the enzyme hexokinase (HK) catalyzes the phosphorylation of D–glucose by adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1).

(1) D-Glucose + ATP $\rightarrow$ G-6-P + ADP   (3)
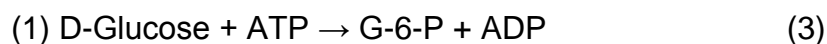
In the presence of glucose-6-phosphate dehydrogenase (G6P-DH), the D–glucose-6-phosphate (G-6-P) formed is specifically oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2).

(2) G-6-P + NADP+ <u>G6P-DH</u> D-gluconate-6-phosphate + NADPH + H+   (4)

The NADPH formed in this reaction is stoichiometric to the amount of D–glucose and is measured by means of its light absorbance at 334, 340 or 365 nm.

Determination of D-fructose:

Hexokinase also catalyzes the phosphorylation of D-fructose to D-fructose-6-phosphate (F-6-P) with the aid of ATP (3).

(3) D-Fructose + ATP <u>HK</u> F-6-P + ADP   (5)
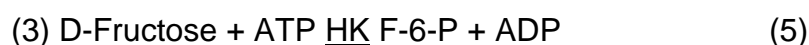
On completion of the reaction (3) F-6-P is converted by phosphoglucose somerase (PGI) to G-6-P (4).

(4) F-6-P <u>PGI</u> G-6-P   (6)

G-6-P reacts again with NADP with formation of D-gluconate-6-phosphate and NADPH (2). The amount of NADPH formed now is stoichiometric to the amount of D-fructose.

Enzymatic inversion:
At pH 4.6, sucrose is hydrolyzed by the enzyme _-fructosidase (invertase) to D-glucose and D-fructose (5).

(5) Sucrose + H2O <u>β-fructosidase</u> D-glucose + D-fructose   (7)

The determination of D-glucose after inversion (total D-glucose) is carried out according to the principle outlined above.
The sucrose content is calculated from the difference of the D-glucose concentrations before and after enzymatic inversion.

6.2 Procedure

6.2.1   Preparing of samples

The Test-Combination contains

1. Bottle 1 with approx. 0.5 g lyophilizate, consisting of:

citrate buffer, pH approx. 4.6; β-fructosidase, approx. 720 U

2. Bottle 2 with approx. 7.2 g powder mixture, consisting of:

triethanolamine buffer, pH approx. 7.6; NADP, approx. 110 mg; ATP,

approx. 260 mg; magnesium sulfate

3. Bottle 3 with approx. 1.1 ml suspension, consisting of:

hexokinase, approx. 320 U; glucose-6-phosphate dehydrogenase, approx.

160 U

The samples were adjusted to pH 8 by NaOH. The samples were measured as

blank samples (redistilled. water + sample), the photometer was adjusted to

0.000 with the blank in the beam, because the samples were colored.

6.2.2 Procedure of testing

The wavelength was 340 nm. The total volume of test solution was 3.04 ml.

The solutions were as can be seen in Table 6.1.

Table 6.1 Index of sample preparation

Pipette into cuvettes	Blank sample	NO.4 aspen	NO.6 aspen
solution 1	none	none	none

Sample solution	none	0.1 ml	0.1 ml
solution 2	1.00 ml	1.00ml	1.00ml
redist. Water	2.00 ml	1.90 ml	1.90 ml
suspension 3	0.02 ml	0.02 ml	0.02 ml

Before pipetting, the solution 1 was warmed up to 37℃ and waited to mix with sample. The no.2 solution and redistilled water were added after 5 minutes. After that, the absorbances of the solutions were read after approx. 3 minutes (A1). The solution 3 was adjusted into cuvette and waited for completion of the reaction for 15 minutes. The absorbance of the solution (A2) was read until the absorbance increased constantly over 2 minutes.

6.3 Calculations

The absorbance differences (A2-A1) for both blanks and samples were determined. The absorbance difference of the blank was subtracted from the absorbance difference of the corresponding sample.

$$\triangle A = (A2-A1)sample-(A2-A1)blank \qquad (8)$$

The difference between $\triangle A_{total}$ D-glucose (from the sucrose sample) and $\triangle$ AD-glucose (from the D-glucose sample) yields $\triangle A_{sucrose}$.

It follows for the determination of D-fructose:

The absorbance differences (A3-A2) for both blank and sample were determined (D-glucose/D-fructose sample). The absorbance difference of the blank was subtracted from the absorbance difference of the sample. This results in $\triangle A$ D-fructose.

The measured absorbance differences should, as a rule, be at least 0.100

absorbance units to achieve sufficiently precise results.

According to the general equation for calculating the concentrations:

$c = (V \times MW) / (\varepsilon \times d \times v \times 1000) \times \triangle A$ [g/l]   (9)

V = final volume [ml]

v = sample volume [ml]

MW= molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of NADPH at

340 nm = 6.3 [l × mmol-1 × cm-1]

Hg 365 nm = 3.5 [l × mmol-1 × cm-1]

Hg 334 nm = 6.18 [l × mmol-1 × cm-1]

for D-glucose:

$$c = \frac{3.020 \times 180.16}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D\text{-glucose}} = \frac{5.441}{\varepsilon} \times \Delta A_{glucose}$$

[g D-glucose/l sample solution]   (10)

The calculations are shown in the Table 6.2

Table 6.2 UV Analysis

UV Analysis				
ε=6.3		Aspen	Aspen	Birch
	Blank sample	NO.4 Aspen	NO.6 Aspen	NO.7 Birch
A1	0.184	0.285	0.614	0.319
A2	-0.154	0.415	0.986	0.961
△A		0.468	0.71	0.98

| C (D-gulcose) | 0.404189 | 0.613192 | 0.846378 g/L |

6.4 Conclusion

The concentration of D-glucose in samples is presented in Table 6.3

Table 6.3 Concentration of D-glucose
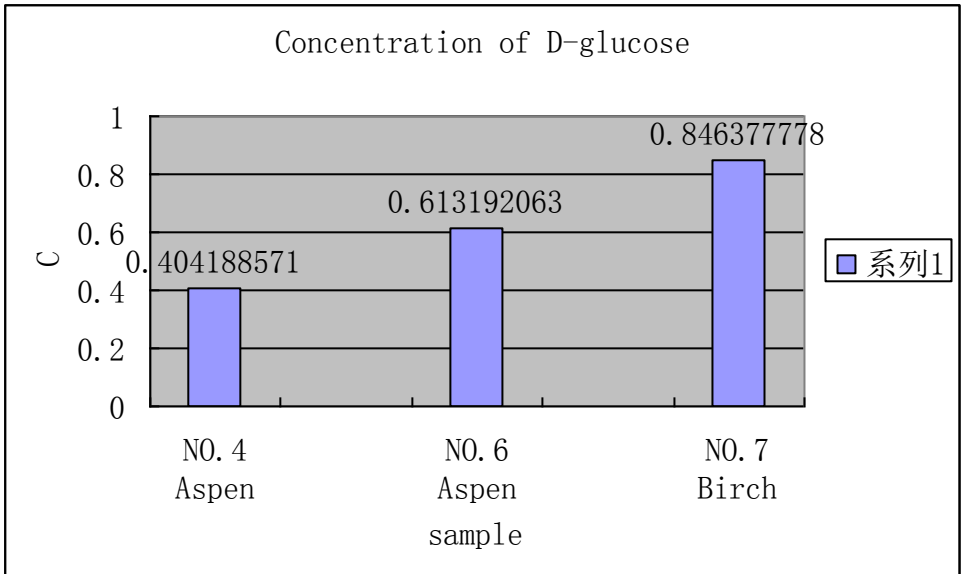
As the table 6.3 showed, the no.7 sample has the highest concentration of the D-glucose.

Table 6.4 Concentrations Versus Absorbance

Concentration VS Absorbance
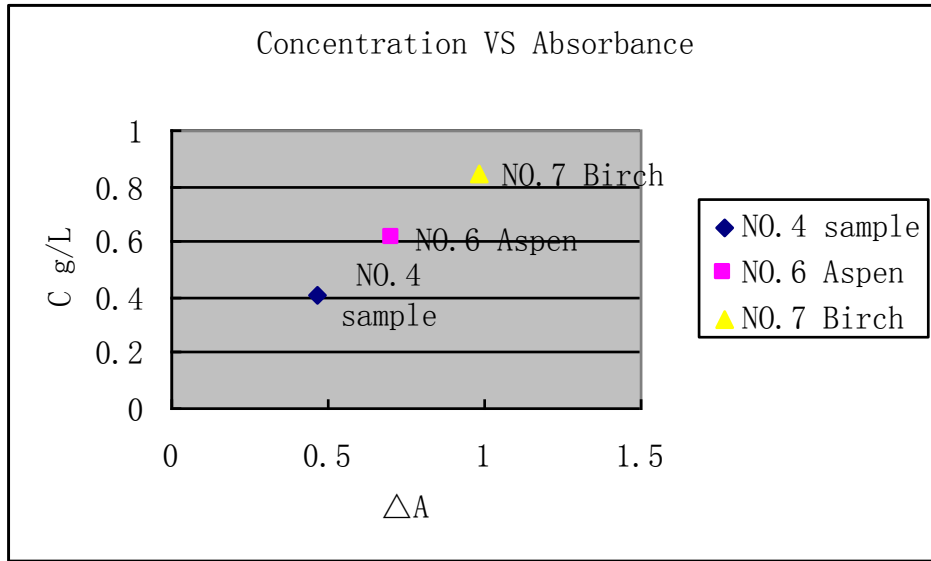
The Table 6.4 shows the relationship between the concentration of D-glucose and △A. If the △A increased , the concentration of D-glucose would be increased. In other words, the differences of absorbance defined the concentration of D-glucose.

Table 6.5 Concentration of D-glucose VS Concentration of Ethanol in Sample

C of D-glucose VS C of ethanol in sample
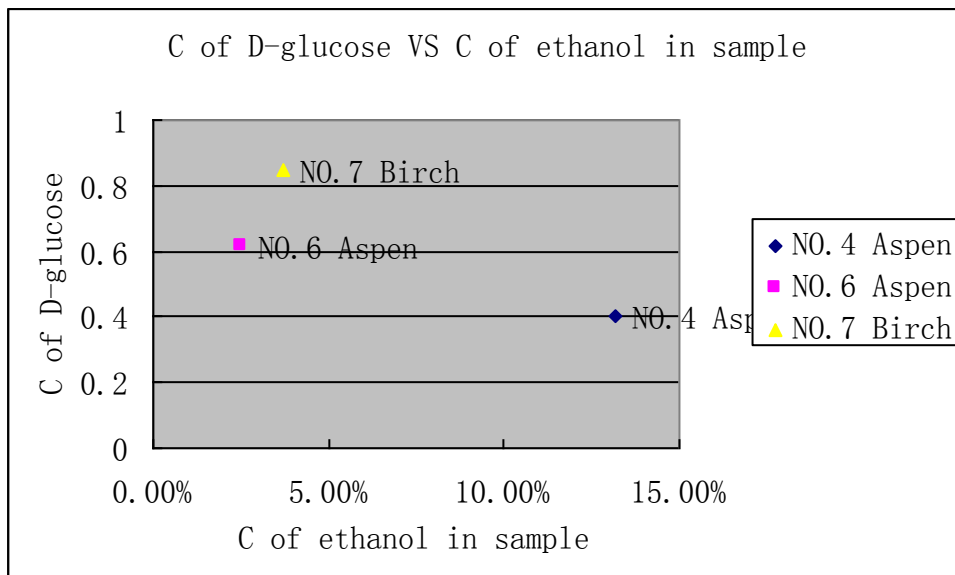
The Table 6.5 presented the influence of concentration of D-glucose in the cooking water influence on the concentration of the final product.

7. CONCLUSION

Table 7.1 the concentration of ethanol in experiment

Sample	Concentration of ethanol	C ethanol in sample
NO.4	55.19%	13.20%
NO.6	10.22%	2.50%
NO.7	12.54%	3.70%

The target of experiment was to produce ethanol from waste waters of industrial pulping. Compared with birch, aspen was more available to produce ethanol by bio-conversion because it has a lower content of pentose. Pentose is hard to be hydrolyzed by the fermentation process.

Fermentation time is another factor effecting the processing. According to the experiment results, the activity of yeast generally descended after 24 hours and ethanol could volatilize into air, if the vessel was not in a vacuum condition.

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APPENDIX 1

Data File D:\HPCHEM\1\DATA\ANALYZE4\003F0301.D **Sample NO.4 a**   Sample Name: Ethanol4a

```
**************************************************************
   Injection Date  : 4/5/2011 2:39:18 PM          Seq. Line :   3
   Sample Name     : Ethanol4a                     Location : Vial 3
   Acq. Operator   : Esko                               Inj :   1
                                              Inj Volume : 5 µl

   Method          : D:\HPCHEM\1\METHODS\ETHANOL2.M
   Last changed    : 2/10/2011 11:57:45 AM by Esko
   Ethanol analysis. Volume 5 uL
```
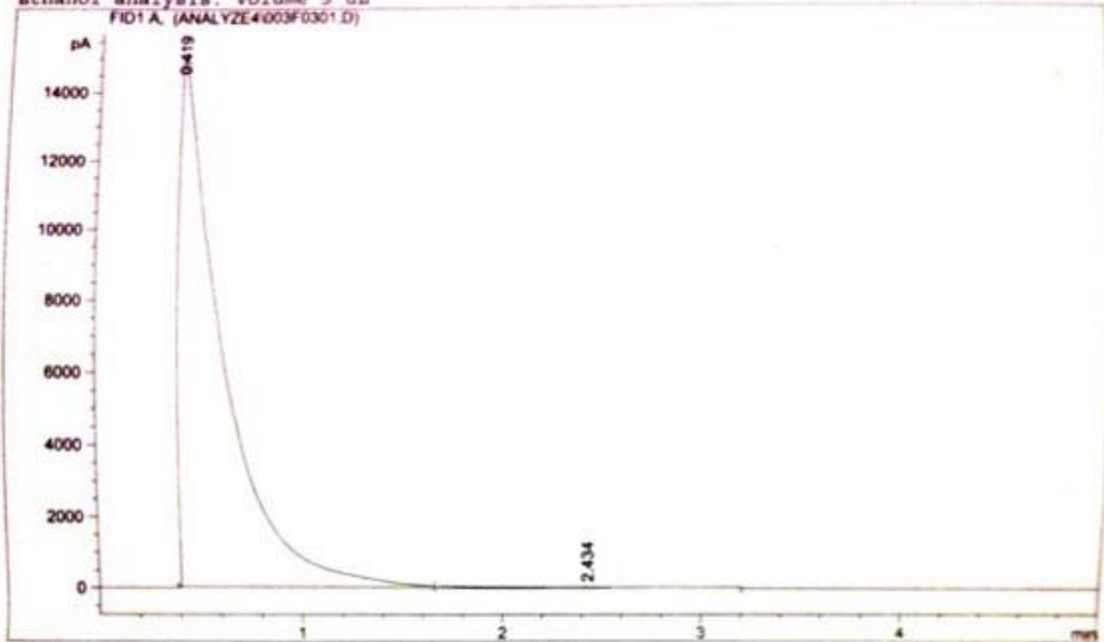


```
**************************************************************
                       Area Percent Report
**************************************************************

   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   0.419 PV S  0.1812 2.20996e5   1.49714e4   99.64593
     2   2.434 VBA+  0.2278  785.27081   57.44399    0.35407

   Totals :                 2.21781e5   1.50289e4
```

Results obtained with enhanced integrator!

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

APPENDIX 2

Data File D:\HPCHEM\1\DATA\ANALYZE4\004F0401.D Sample No.4 b   Sample Name: Ethanol4b

```
===============================================================
  Injection Date  : 4/5/2011 2:45:20 PM        Seq. Line :    4
  Sample Name     : Ethanol4b                   Location  : Vial 4
  Acq. Operator   : Esko                        Inj    :    1
                                                Inj Volume : 5 µl
  Sequence File   : D:\HPCHEM\1\SEQUENCE\ETHANOL.S
  Method          : D:\HPCHEM\1\METHODS\ETHANOL2.M
  Last changed    : 2/10/2011 11:57:45 AM by Esko
  Ethanol analysis. Volume 5 uL
```

FID1 A, (ANALYZE4\004F0401.D)
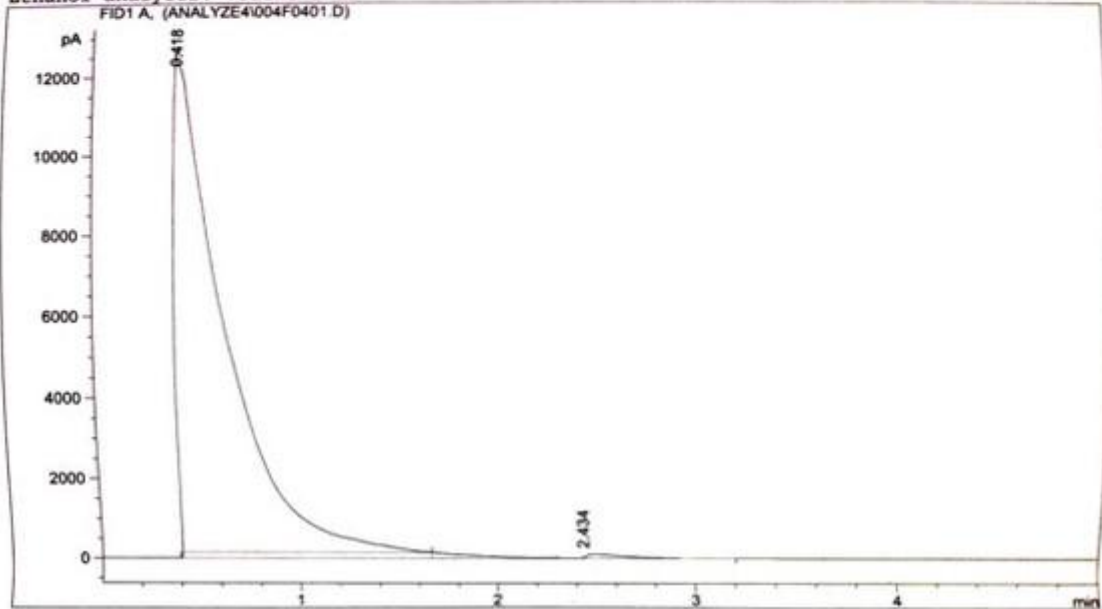
```
===============================================================
                    Area Percent Report
===============================================================

  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   0.418 PV S  0.2054 2.16142e5  1.25192e4  98.47594
   2   2.434 VBA+  0.3838 3345.12744  145.25531  1.52406

  Totals :                 2.19487e5  1.26644e4

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

APPENDIX 3

40

Data File D:\HPCHEM\1\DATA\ANALYZE3.2\001F0101.D **Sample NO. 6 a**   Sample Name: Ethanols1

```
========================================================================
Injection Date  : 3/28/2011 2:41:36 PM          Seq. Line :   1
Sample Name     : Ethanols1                      Location : Vial 1
Acq. Operator   : Esko                                Inj :   1
                                              Inj Volume : 5 µl
Sequence File   : D:\HPCHEM\1\SEQUENCE\ETHANOL.S
Method          : D:\HPCHEM\1\METHODS\ETHANOL2.M
Last changed    : 2/10/2011 11:57:45 AM by Esko
Ethanol analysis. Volume 5 uL
```

FID1 A, (ANALYZE3.2\001F0101.D)

```
========================================================================
                          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 PV S  0.1690 4.17726e4  2935.16479 90.53969
  2   1.465 BV X  0.0422   36.40666   12.80270  0.07891
  3   1.516 VB X  0.0222   35.46255   24.05582  0.07686
  4   2.434 VBA+  0.1207 4292.86279  592.84601  9.30454

Totals :                 4.61373e4  3564.86932

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

APPENDIX 4

41

Data File D:\HPCHEM\1\DATA\ANALYZE3.2\002F0201.D **Sample No.6b**   Sample Name: Ethanols2

```
========================================================================
 Injection Date   : 3/28/2011 2:47:40 PM         Seq. Line :   2
 Sample Name      : Ethanols2                     Location : Vial 2
 Acq. Operator    : Esko                              Inj :   1
                                              Inj Volume : 5 µl
 Sequence File    : D:\HPCHEM\1\SEQUENCE\ETHANOL.S
 Method           : D:\HPCHEM\1\METHODS\ETHANOL2.M
 Last changed     : 2/10/2011 11:57:45 AM by Esko
 Ethanol analysis. Volume 5 uL
```
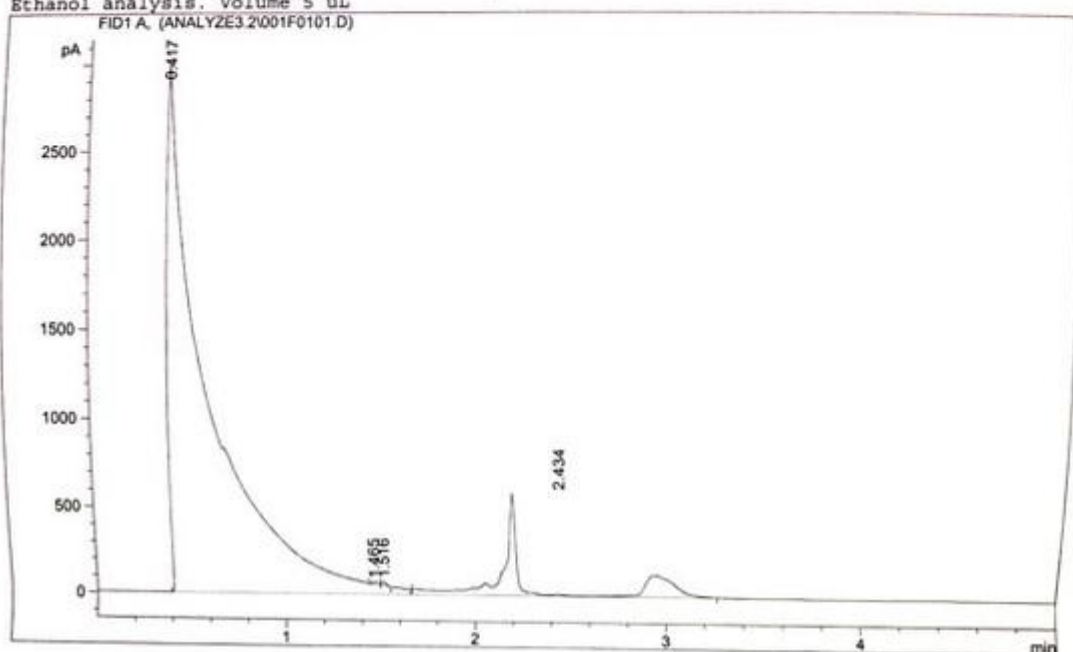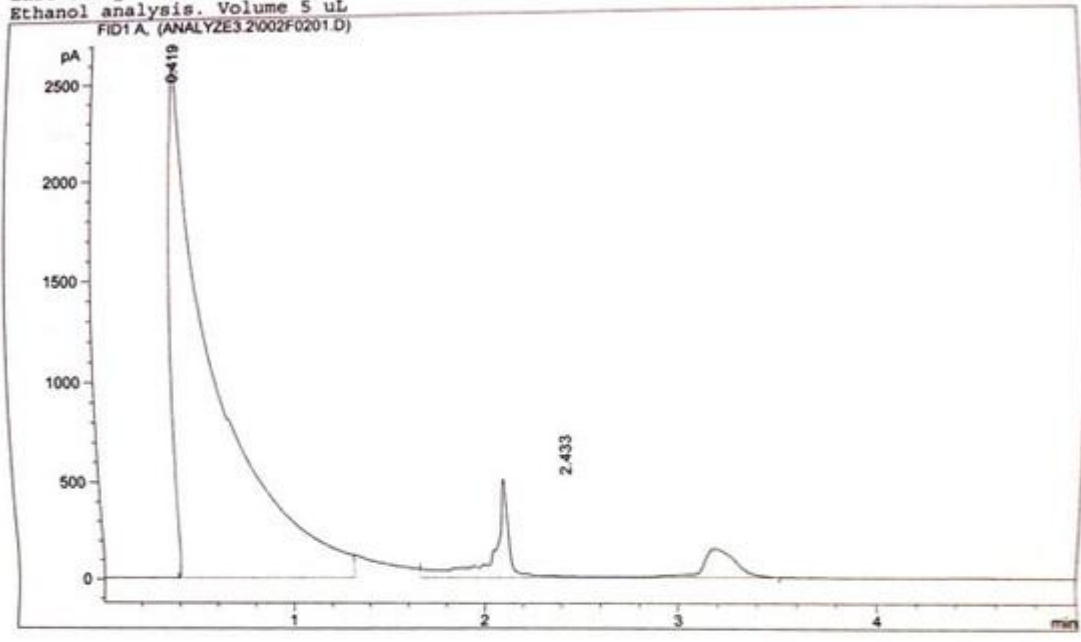


```
========================================================================
                        Area Percent Report
========================================================================

 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   0.419 PV S  0.1860 3.91839e4   2568.00342 89.51391
  2   2.433 VBA+  0.1524 4590.18945  502.06079  10.48609

 Totals :                  4.37741e4  3070.06421

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

APPENDIX 5

Data File D:\HPCHEM\1\DATA\ANALYZE4\001F0101.D *Sample NO.7 a*   Sample Name: Ethanol7a

```
============================================================================
Injection Date  : 4/5/2011 2:27:12 PM            Seq. Line :    1
Sample Name     : Ethanol7a                      Location  : Vial 1
Acq. Operator   : Esko                                Inj  :    1
                                                Inj Volume :  5 µl

Method          : D:\HPCHEM\1\METHODS\ETHANOL2.M
Last changed    : 2/10/2011 11:57:45 AM by Esko
Ethanol analysis. Volume 5 uL
```

FID1 A, (ANALYZE4\001F0101.D)
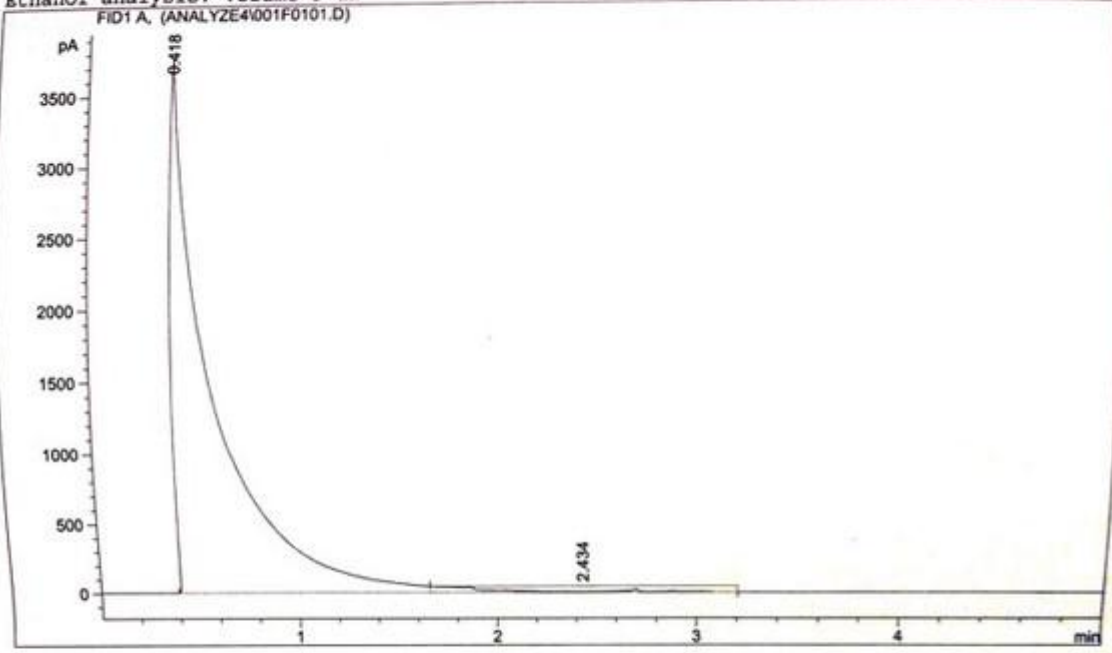

```
============================================================================
                         Area Percent Report
============================================================================

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   0.418 PV S  0.1621  5.02814e4   3735.09570  97.83530
  2   2.434 VBA+  0.4095  1112.52368    45.27650   2.16470

Totals :                  5.13939e4   3780.37220

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

APPENDIX 6

```
===============================================================
Injection Date  : 4/5/2011 2:33:15 PM          Seq. Line :    2
Sample Name     : Ethanol7b                     Location : Vial 2
Acq. Operator   : Esko                               Inj :    1
                                              Inj Volume : 5 µl

Sequence File   : D:\HPCHEM\1\SEQUENCE\ETHANOL.S
Method          : D:\HPCHEM\1\METHODS\ETHANOL2.M
Last changed    : 2/10/2011 11:57:45 AM by Esko
Ethanol analysis. Volume 5 uL
```

FID1 A, (ANALYZE4\002F0201.D)


```
===============================================================
                    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 BV S  0.1595 4.90990e4  3683.69067 97.69866
  2   2.434 VBA+  0.3926 1156.54846   49.09621  2.30134

Totals :                  5.02555e4 3732.78689

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

44