Development of Bioethanol Synthesis Process Based on Lignocellulosic Biomass Waste: A Sustainable Approach for Renewable Energy

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ABSTRACT

The increasing concern over climate change and the depletion of fossil fuels has encouraged greater attention toward renewable energy sources. This study explored how bioethanol can be synthesized from lignocellulosic biomass, specifically rice straw and rice husk. The process involved three main steps: pretreatment with acid-alkali to remove lignin barriers, enzyme hydrolysis to release fermentable sugars, and fermentation using *Saccharomyces cerevisiae* to produce ethanol.

The fermentation progress was observed through five days and the observable changes were performed qualitatively using multiple indicators: odor, condensation, and CO_2 bubble formation. Based on these indicators, it has been confirmed that ethanol was successfully synthesized. Additionally, the mass balance studies indicated that the biomass samples showed measurable decrease in mass during the pre-treatment and fermentation process, which further supported that sugars were broken down and converted. Due to the absence of laboratory instruments for chemical analysis, an accurate and precise ethanol concentration could not be measured.

INTRODUCTION

Renewable sources of energy have been becoming more crucial and desperately needed over the years due to the uproar of natural resources depletion, rapid climate change and environmental problems. With the average temperature in 2024 being the hottest it has ever been since 1850 and an increase of 0.06℃ in a decade, climate change has skyrocketed [1]. Furthermore, Earth's carbon emissions increased by 12.28 billion tonnes, equivalent to a near 50% increase, since 2000, which shows the severity of environmental problems in the past 20 years [2]. However, the cause of this growing destructive problem is not unfamiliar. In the rapidly evolving modern world where innovations and technology are continuously expanding at a fast pace, energy is the powerhouse that enables it to keep going, even at the expense of Earth's environment.

Currently, the most sought-after source of energy is fossil fuels due to its low cost yet large yield of energy amongst other forms of energy sources. It makes up 81.5% of the global share of energy [3]. However, its irreversible damaging impacts on our Earth and risk of the depletion of these natural resources has forced us to search for another cleaner form of energy source. One

of the commonly used clean energy sources is bioethanol. In fact, bioethanol is the most commonly used form of liquid biofuel. Bioethanol is produced through the fermentation of carbohydrates that are sugar based and starch based, with its main use as fuel mixture in light every-day vehicles (cars) and flex-fuel vehicles, as well as generating electricity in specialised plants. Second-generation bioethanol is made of lignocellulosic biomass, which mainly source from agriculture residues, such as crops, rice husks, rice straws, sugarcane, bagasse, corn cobs and sawdust. [4]

Lignocellulosic biomass' carbohydrate structures are made up of 30-50% cellulose, 20-40% hemicellulose and 20-30% lignin as shown in Figure 1.1 [5] . This carbohydrate structure is what allows it to be converted to fermented sugars: the easiest amongst other types of biomasses. However, this structure is also what prevents it from being able to be a direct source of carbohydrates for bacterial organisms to act on. Therefore, lignocellulosic biomass has to go through a pretreatment process. After, it goes through a saccharification process, followed by fermentation into bioethanol.

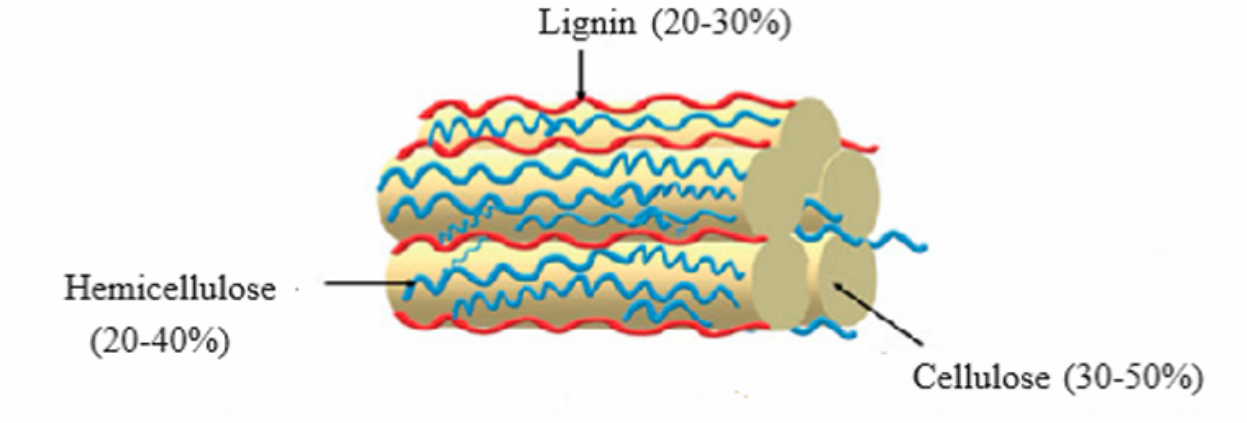


Figure 1.1: Carbohydrate Structure of Lignocellulosic Biomass

LITERATURE REVIEW

Advantages and Disadvantages of Pretreatment with Acid and Alkali

Acid pretreatment is the most efficient at hemicellulose breakdown into monosaccharides, such as xylose and arabinose, due to its hydrolysation method [11]. Reaction duration is also relatively faster than other pretreatment methods due to its chemical approach and high temperature conditions ranging from 120℃ to 200℃, increasing speed of reaction [12].

On the other hand, acid pretreatment forms inhibitory compounds, such as furfural and HMF, due to dehydration of sugars in the process [13]. This factor slows down the speed of the

reaction for the pretreatment. In addition, the strong acidity and high temperature can be dangerous, as it may significantly affect corrosive materials.

Alkali pretreatment is most efficient in breaking down lignin, as it cleaves ester and ether bonds, chemically breaking away the structural barrier the fastest [14]. The low temperature range of less than 100℃ and low pressure that alkali pretreatment works makes the conditions safer and milder. Furthermore, alkali pretreatment produces less inhibitor compounds, such as furfural and HMF, as they do not cause sugar degradation [13]. This caused the speed of reaction to be fast.

However, alkali pretreatment is least effective on hemicellulose. Alkali is unable to hydrolyse hemicellulose, leaving more pentose sugars to be unused [15]. Alkali pretreatment's total reaction time is overall longer, due to slower lignin solubilization, needing longer residence times for effective delignification [12]. A summary of the advantages and disadvantages of the different pretreatment methods are shown in Table 2.1 below.

Table 2.1: Advantages and Disadvantages of Acid Alkali Pretreatment

	Acid Pretreatment	Alkali Pretreatment
Advantages	- most efficient in breaking down hemicellulose - fast reaction rate	- most efficient in breaking down lignin - safer and milder conditions - produce less inhibitor compounds
Disadvantages	- forms inhibitory compounds - dangerous and corrosive conditions	- least effective in breaking down hemicellulose - slower reaction rate

Advantages and Disadvantages of Saccharification Enzyme Hydrolysis

Due to the specific shape of the enzyme's active site, only specific substrates can fit with specific enzymes. This shows that enzymes work highly specifically on glycosidic bonds in cellulose and hemicellulose, meaning that the rate of conversion of sugars is fastest, and yield is highest. Cellulase and pectinase's optimum condition of 50℃ temperature and pH 5.5 shows that the reaction's conditions are safer and cheaper with less energy input needed.

However, enzymes can be expensive and costly when required in large amounts. Cellulase and Pectinase have an average cost range of $3.80 to $10.10 per kilogram. Enzyme activity also requires pretreatment on substrates before being able to work, due to their inability to penetrate untreated biomass from lignin. The additional step of pretreatment is considered as increasing

cost and time required for the overall reaction to produce bioethanol. The advantages and disadvantages of saccharification enzyme hydrolysis are summarized in Table 2.2.

Table 2.2: Advantages and Disadvantages of Saccharification

Advantages	- Enzyme works specifically on specific substrates - Faster rate of conversion - Optimum conditions are safer and cheaper
Disadvantages	- Enzymes can be expensive - Enzyme requires pretreatment before - Longer total time required

Advantages and Disadvantages of fermentation with Saccharomyces cerevisiae

Fermentation is the most efficient method to obtain the highest yield of bioethanol from biomass. Fungi Saccharomyces cerevisiae ferments glucose, fructose and mannose into ethanol at the highest conversion rate of 0.51 grams of ethanol per gram of sugar [16]. Furthermore, Saccharomyces cerevisiae has the highest tolerance to high ethanol concentration of 12-15%, meaning contamination risk is reduced and downstream processing is simplified [17]. Saccharomyces cerevisiae is also the safest fungi and bacterial microorganisms for several industrial processes, in comparison to other organisms such as Pre-cultured M. circinelloides.

On the other hand, fermentation has a limited range of types of sugars that can be converted to bioethanol. Many sugars, such as xylose and arabinose, are not able to be naturally fermented, oftentimes leaving them unused [18]. Furthermore, oxygen levels have to be constantly maintained, as a small amount of oxygen is needed by these microorganisms for anaerobic respiration, the method fermenting bioethanol, which is around 1.5 to 3.5 mg of oxygen per gram of dry mass of yeast [18]. A summary of *S. cerevisiae* fermentation is outlined in Table 2.3.

Table 2.3: Advantages and Disadvantages of *S. cerevisiae* fermentation

Advantages	- Obtain highest yield of bioethanol - Highest tolerance to high ethanol concentration - Safest microorganism to be used
Disadvantages	- Limited range of types of sugars to be fermented - Oxygen levels have to be maintained

METHODOLOGY

The overall experimental process for this study is outlined in the Figure 2.1 below.

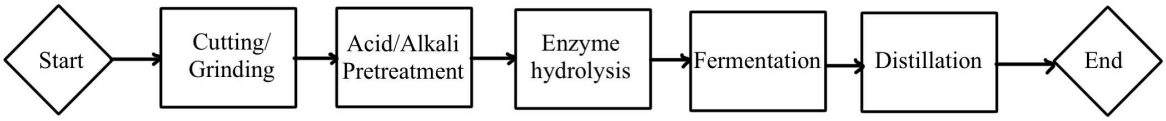


Figure 2.1: Process Mapping of Bioethanol Production

The steps of the experiment involves cutting or grinding the biomass into smaller pieces, pretreated with either acid or alkali, hydolized with enzyme mixtures, fermented, and then distilled in order to collect the bioethanol.

Pretreatment with Acid and Alkali

Pretreatment is necessary in order to break down the “closed up” structure of lignocellulosic biomass that is preventing bacterial organisms from being able to work on it. It does this by breaking down the surface structure and reducing the particle size by compression forces [7], which then allows bacterial organisms to work and reactions to take place on these lignocellulosic biomass structures.

There are a few types of pretreatments that can be done: Physical Pretreatment, Microwave Pretreatment, Ultrasound Pretreatment, Mechanical Pretreatment and Chemical Pretreatment [8]. The most effective out of these 5 pretreatment methods is Chemical Pretreatment using Acid and Alkali. Chemical Pretreatment immediately and efficiently breaks down the structure of the lignocellulosic biomass by breaking the protective barrier made of hemicellulose and lignin that blocks enzymes from reaching the glycosidic bonds [9]. Strong acids, such as sulfuric acid (H2SO4), hydrochloric acid (HCl), and strong alkalis, such as sodium hydroxide (NaOH), potassium hydroxide (KOH), are most effectively used.

Sources of lignocellulosic biomass were taken from rice straws and rice husks. Rice straws and husks were cut into 2 cm pieces, then pulverised until thin. In 1 Mole concentration (1 Mole per litre solution) of sodium hydroxide (NaOH) or potassium hydroxide (KOH), rice straws are soaked in with a concentration of 80 grams per litre solution. The solution is then heated to a temperature of 100℃ for 60 minutes in an autoclave, a pressure cooker or a boiling water bath with aluminium foil seal work as an alternative.

Solid residue is collected through filtration, where it is then neutralised by 1 M concentration (1 Mole per litre solution) of hydrochloric acid (HCl) or sulfuric acid (H2SO4). After, the residue is washed 2-3 times with distilled water until pH is neutral (pH 7). Solid residue is then dried to 50℃ in an oven, then pulverised until powdered by a pulverizer, a blender or mortal and pestle work as an alternative.

Four samples were taken with an average mass of 20 grams of rice straws each, with samples 1-4 having a mass of 20.04 grams, 19.95 grams, 20.06 grams, 19.97 grams respectively. In a beaker, each sample of the rice straw is soaked into 250 mL solution of 1M sodium hydroxide (NaOH). Using a boiling water bath and aluminium foil seal, each sample is heated up to an average temperature of 97.3℃. With 250 mL solution of 1M hydrochloric acid (HCl), the samples are neutralised. After rinsed with distilled water and being pulverised to powdered, the final samples mass recordings are recorded to be lower than its initial mass. Samples 1-4 masses post pre-treatment are 17.57 grams, 16.98 grams, 15.32 grams, 15.24 grams respectively.

Saccharification (Enzyme Hydrolysis)

Enzyme hydrolysis is a crucial step prior to fermenting bioethanol, which can be done by using enzymes, such as cellulase and pectinase. Enzymes break down the β-1,4-glycosidic bonds in the cellulose of these lignocellulosic biomass by hydrolysis with water, and target the bonds cleavage to release glucose units [9]. Glycosidic bonds are covalent bonds connecting monosaccharides that form cellulose and hemicellulose.

An acid-base catalysis occurs where an acid catalyst donates a proton to the glycosidic oxygen and a base catalyst accepts a proton which turns a water molecule into hydroxide ion (OH-). The hydroxide ion attacks the anomeric carbon (C1) of the carbohydrate, acting like a nucleophile. The breakdown of the glycosidic bond is complete [10].

Pretreated rice straws are mixed into an enzyme solution with concentration of 10 grams per litre solution, where the enzymes used are a mixture of Cellulase Onozuka 3S, Cellulase T Amano 4 and Pectinase G Amano at a ratio of 1:1:1 by mass. The optimized enzyme solution used is at a concentration of 1 gram per litre solution per enzyme, with the solvent being 50 mM (milimole) concentration of acetate buffer of pH 5.5. At 30℃, each sample of solution is stirred with an agitation of 120 rpm for 48 hours.

7.5 grams of pretreated rice straws are taken from each sample, then mixed into 75 mL (mililitres) of acetic acid with pH of 5.5, acting as acetate buffer solvent. 0.075 grams of Cellulase Onozuka 3S, Cellulase T Amano 4 and Pectinase G Amano each are added to the mixture. Using an electronic stirrer, each solution sample is stirred at an average rate of 120 rpm for 48 hours.

Fermentation

Fermentation, the last step of producing bioethanol from lignocellulosic biomass, is where bacterial or fungus microorganisms convert sugars into ethanol through the process of anaerobic respiration. Microorganisms break down 1 molecule of glucose into 2 molecules of ethanol and 2 molecules of carbon dioxide, generating 2 molecules of ATP energy in the process.

Chemical equation: $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + 2ATP$

Bacterial and/or fungus microorganisms that are most commonly used in fermentation are Saccharomyces cerevisiae (brewer's yeast). With an optimal temperature of 35℃ and pH of 5.5, its total fermentation duration is within 72 to 120 hours.

Saccharomyces cerevisiae is added to the solution in a concentration of 3 grams per litre, with the solvent of the solution being the same 50 mM (milimole) of acetate acid (buffer) with pH of 5.5. The substrate of treated rice straws are mixed in at a concentration of 100 grams per litre. At a temperature of 28℃, the solution is stirred using a stirrer at an agitation of 120 rpm for 96 hours.

Each of the samples containing 75 mL of solution is mixed with 0.225 grams of Saccharomyces cerevisiae (brewer's yeast), with the same 75 mL of 50 mM (milimole) of acetate buffer used as the solvent. At room temperature of 25℃, the solution is stirred at 120 rpm for 72-120 hours continuously.

RESULTS

Qualitative Observations

In the Fermentation Phase, the presence of condensation of a colourless liquid was observed on the inner surface of the plastic wrap covering the beakers of the bioethanol being fermented. The condensation indicates that a volatile liquid substance was produced, which indicates the formation of a volatile compound. Considering the nature of bioethanol that evaporates and condenses at low temperatures, it suggests that the naturally warm and humid conditions of the experimental setup caused the bioethanol to evaporate. The condensation also suggests that some of the bioethanol may have escaped to the surroundings, resulting in a partial loss of bioethanol to the resulting mixture.

In addition, during the fermentation, a strong yeast smell was given off, which is consistent with the metabolic activity of yeasts that occur as it breaks down sugars by enzyme saccharification.

As the Fermentation Phase progresses from Day 1 to Day 5, the odor transitions from yeasty to a pungent alcoholic scent. The change in odour indicates that alcohol (bioethanol) has been produced by the reaction, and accumulates as the reaction progresses and yeast continues to produce more bioethanol. However, the odour also indicates that some bioethanol has evaporated and diffused into the air, causing a slight pungency in scent.

Gas bubbling was also observed in all samples, serving as a clear indicator of active yeast metabolism. The release of CO_2 was due to yeast converting organic substrates like sugars in simpler compounds, such as ethanol and simple sugars. The intensity of the bubbling in each sample indicated the rate of fermentation. Samples with more vigorous and visible bubbling showed a faster metabolism rate, a higher substrate breakdown, giving faster fermentation rate. The production of CO_2 also explains the decrease in mass of the samples as CO_2 gas escapes to the surroundings. Therefore, this bubbling confirms the process of fermentation and that pretreatment enhances the availability and potential of fermentable materials for microbial utilization. Table 2.4 summarizes the qualitative observation of the fermentation of bioethanol over the five days period.

Table 2.4: Qualitative observations of fermentation progress across five days, showing odor evolution, condensation, and CO_2 bubbling intensity as indicators of yeast metabolic activity and bioethanol production.

Day	Odor	Condensation	Bubbling Intensity
1	Strong yeast smell	None	Minimal
2	Mild sweet odor	Light	Low/Moderate
3	Slight alcohol scent	Moderate	High
4	Strong, pungent alcohol smell	Heavy	Moderate
5	Strong alcoholic	Heavy	Low

Overall, the qualitative results have demonstrated that the fermentation process of ethanol was successful.

Mass Balance

The recorded data shows a noticeable decrease in the mass of all samples after the pretreatment process and fermentation process. The data was recorded and summarized in Table 2.5.

Table 2.5: Mass balance data of lignocellulosic biomass samples before pretreatment, after pretreatment, and after fermentation.

Sample #	Initial Mass (g)	Post-Pretreatment Mass (g)	Post-Fermentaion Mass (g)	Total Mass Loss (%)
1	20.04	17.87	16.52	17.6 %
2	19.95	16.98	15.23	23.7 %
3	20.06	14.46	12.98	35.3 %
4	19.97	12.27	10.95	45.2 %

As shown in Table 2.5, the higher the mass reduction means that there are higher ethanol yields, showing effective cellulose breakdown facilitated by acid-alkali pretreatment and enzyme hydrolysis with Sample #4 yielding the highest ethanol.

DISCUSSION

The results of this study demonstrated that bioethanol production from lignocellulosic biomass, particularly rice straws and rice husks, was achieved through acid-alkali pretreatment, enzyme saccharification, and yeast fermentation, based on multiple qualitative indicators, such as odor, condensation, and gas bubble formation. The results were only validated based on the qualitative observation and mass balance due to the inaccessibility of chromatographic analytical equipment during the study.

The condensation that took place on the plastic wrap covering the beakers suggested the formation of a volatile product, which is consistent with ethanol's known volatility and low boiling point. The gradual transition in odor from a strong yeast smell to a strong, alcoholic scent further supports the formation of ethanol, aligning with the expected biochemical activity of *Saccharomyces cerevisiae* converting sugars into alcohol and carbon dioxide.

The formation of gas bubbles that was observed during fermentation also served as an indicator of active yeast metabolism and CO_2 production, confirming that anaerobic respiration was taking place. The decline in bubbling intensity toward the later stages of fermentation was due to the depletion of the available sugars in the solution.This pattern of activity corresponds with previous studies reporting that *S. cerevisiae* fermentation slows as ethanol accumulates and nutrient sources diminish [16,17].

The recorded mass reduction across all samples was analyzed and showed substantial degradation of cellulose and hemicellulose into simpler sugars and gases. The highest mass

loss observed in Sample 4 suggests a more effective breakdown of lignocellulosic structure, likely due to better pretreatment efficiency. These results are consistent with findings from Robak and Balcerek (2020) [5], who observed that strong alkaline pretreatment enhances enzyme accessibility to cellulose fibers, improving overall conversion efficiency.

However, despite these successful qualitative indicators, it was not possible to determine an accurate and precise ethanol concentration being produced due to the absence of chromatographic equipment. As a result, the confirmation of the ethanol production was based primarily on qualitative observations. Note that this limitation does not undermine the validity of this study's objective. The aim was to demonstrate the feasibility of bioethanol production using accessible laboratory methods. Nevertheless, future studies will include quantitative measurement in order to establish the conversion efficiency and the effective yield of the process.

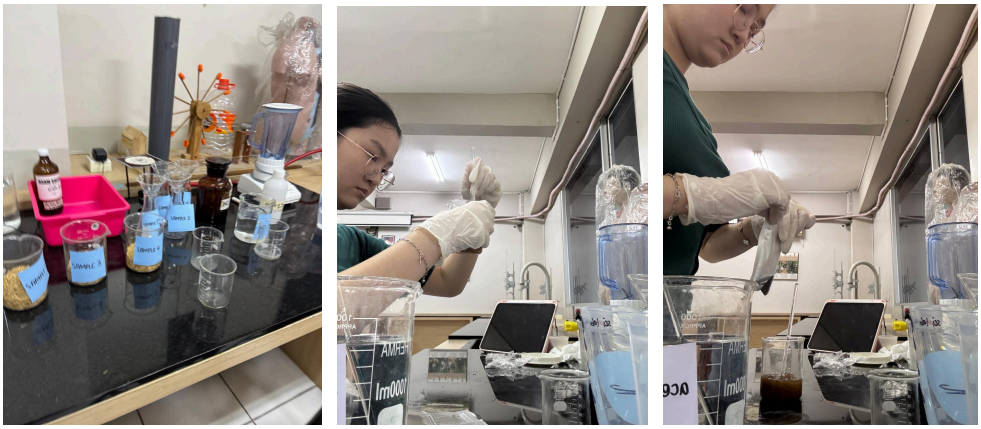
CONCLUSION

The method of producing bioethanol from lignocellulosic biomass through pretreatment with acid and alkali, saccharification by enzyme hydrolysis and fermentation with yeast proved to be a reliable method for small-scale laboratory fermentation of bioethanol, as the observations of the bioethanol product can be directly seen. However, it may not be suitable for large-scale factory production of bioethanol, this is due to the lack of ability to directly and instantaneously measure the amount of bioethanol produced without needing to get it lab-tested, as it would be more costly and time-consuming.

Furthermore, the result also showed a detrimental limitation. Due to the naturally warm temperature and high pH conditions of the reactions taking place, it makes deriving fermented liquid bioethanol a lot more difficult, as a significant amount of the ethanol produced has evaporated into gas due to its volatile and low boiling point nature. This is due to the volatile and low boiling point nature of bioethanol that is difficult to retain under uncontrolled experiment conditions. As a result, the yield of liquid bioethanol measured was smaller than the actual amount produced.

In future further studies, several modifications may be made to increase the bioethanol derived. Firstly, optimizing pH levels by having an electronic pH indicator with precise readings may ensure that the reaction occurs at its optimal pH. In addition, creating a closed system with an electronically-controlled temperature would be ideal in order to obtain the liquid yield of bioethanol from the experiment. For example, when temperature drops to below the enzyme's suited temperature, the electronic system increases the temperature using a heater. Lastly, other collection methods may be used, such as distillation apparatus with a condenser, to capture the evaporated bioethanol.

PHOTOGRAPHS AND EVIDENCES



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