Optimization of Upstream Processing for the Production of Bioethanol from *Leucaena leucocephala* Seeds using *Kluyveromyces marxianus* UniMAP 1-1

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Abstract. Biofuel industry is gradually growing as the market starts to shift the demand from non-renewable energy sources to renewable energy sources. The aim of this study is to produce bioethanol from biomass feedstock which is *Leucaena leucocephala*. It is a potential lignocellulosic energy source as it contains high composition of carbohydrates which can be broken down into fermentable sugar through pretreatment and enzymatic hydrolysis prior to the fermentation into bioethanol. Screening for both upstream processing were conducted by OFAT experiments to optimize several parameters tested such as pretreatment temperatures and NaOH concentrations. Fermentation process was conducted in Simultaneous Saccharification and Fermentation (SSF) conditions aided by *Kluyveromyces marxianus* UniMAP 1-1 as the fermenting microbes. This yeast is capable in converting sugar to ethanol at high temperature. The optimization of pretreatment and enzymatic hydrolysis by using OFAT gave the optimum value for both process prior to fermentation. The ethanol obtained from this SSF Fermentation is 3.15 g/L of bioethanol from 25.0 g/L of total sugars in hydrolysate. This report provides further information in the interest of producing bioethanol from *Leucaena leucocephala* seeds using *Kluyveromyces marxianus* UniMAP 1-1.

1. Introduction

Bioethanol is an alternative type of fuel based on alcohol which is produced by the fermentation of lignocellulosic biomass with high sugar contents [1]. It can be produced from any organic matter originated from the biological means with abundance amount of sugar content and materials that can be converted into sugar such as starch and cellulose. Sugarcane, sugar sorghum and sugar beetroot are examples of raw materials that consist of high sugar contents and can be used for bioethanol production [2].

*Leucaena leucocephala* is a plant which is believed to be one of the best raw material to produce bioethanol. In Malaysia, *L. leucocephala* is locally known as Petai Belalang and it can easily be found in Malaysia where tropical multipurpose tree species grow. *L. leucocephala* tree is one of the fast-growing trees and its leaves, branches and seeds contain cellulose, hemicellulose as well as lignin [3]. The lignocellulosic biomass is considered an important biomass resource to be converted into fermentable sugar for bioethanol and other chemicals production.
The process to convert lignocellulosic biomass into fermentable sugars involves two crucial steps; pretreatment and enzymatic hydrolysis. *L. leucocephala* seeds are composed of recalcitrant lignocellulosic biomasses that are cellulose, hemicellulose and lignin. Cellulose with the crystalline structure embeds in a matrix of polymers-lignin and hemicellulose are the main reasons of the recalcitrant property [4], making the hydrolysis of lignocellulosic biomass into fermentable sugar to be harder.

Pretreatment of lignocellulosic biomass is aimed to disrupt the polymer matrix in order to expose cellulose and hemicellulose and make them more accessible to the action of the enzymes. The pretreated lignocellulosic biomass then undergoes enzymatic hydrolysis to produce fermentable sugars. Enzymatic hydrolysis is an important step as it decides the yield of fermentable sugars prior to any conversion into final products such as bioethanol through fermentation. Enzymatic hydrolysis requires expensive enzymes to depolymerize cellulose and hemicellulose into sugars.

There are several types of fermentation processes which are known as Separate Hydrolysis Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). For SHF, there are two crucial steps involved which are saccharification where cellulose is degrade into fermentable sugars and the fermentation of sugar to ethanol. In contrast, SSF can hydrolyze and ferment simultaneously and produce high yield of ethanol. SSF is also believed to have lower production cost compared to SHF [5]. However, the disadvantage of using SSF is that the optimum temperature between saccharification and fermentation are not identical [6]. Optimum temperature of enzymatic hydrolysis seems to be higher compared to the fermentation itself. Hence, the use of thermo-tolerant yeast is crucial to solve this problem.

*Kluyveromyces marxianus* is a hemiascomycetous yeast capable to ferment bioethanol at higher temperature than conventional yeast. This thermostolerant yeast showed promising results to be used in the bioethanol industry as it can reduces the unit operation used, by combining the process of hydrolysis and fermentation thus reducing the operational cost. In this study, *Kluyveromyces marxianus* UniMAP 1-1 was used as the fermenting microbes.

2. Methodology

2.1. Total fermentable sugars from different plant parts

2.1.1. Plant materials. Leucaena leucocephala was obtained from Bintong, Perlis. The seeds, leaves and stalks were collected to undergo pretreatment before obtaining the fermentable sugars.

2.1.2. Physical Treatment. The parts of *L. leucocephala* obtained were dried in an oven (Binder, USA) at 60°C for about 48 hours to remove water content in the plants as well as to kill any microorganisms that may be present in the plants. The drying process continued until constant weight of the samples were achieved. The dried plants were then grinded into powder form by using a blender (Electrolux, Malaysia). The prepared samples were then kept in a vacuum desiccator with silica gel until further use.

2.1.3. Alkaline Pre-treatment. About 5 g of each parts of the dried *L. leucocephala* plants in powder form was pretreated with 100 mL of 1-2% (w/v) sodium hydroxide (NaOH) at a temperature of 80-100°C for about 30-90 minutes [7]. After that, the solid residues were collected and rinsed thoroughly with distilled water to completely wash out the remaining sodium hydroxide. Then, the samples were filtered to remove the excess water and dried in the oven (Binder, USA) at 100°C for about 1 hour prior to enzymatic hydrolysis.

2.1.4. Enzymatic Hydrolysis. About 2 g of dried pretreated sample was used in the enzymatic hydrolysis to produce fermentable sugar. Cellulase (Celluclast 1.5L, Novozymes, Denmark) with concentration ranging from 1-4 FPU was used. The samples and cellulase enzyme were placed in a 250 mL conical flask and mixed with 20 mL of citrate buffer (pH 4.8). Then, the conical flask was placed in an incubator shaker (Sartorius, Malaysia) at 50°C, pH 4.8 and 150 rpm for 24 hours. After that, the sample solution was centrifuged (Sigma, Germany) to separate the solids and supernatant. Then, the supernatant was collected and tested for the total reducing sugar concentration by using DNS method [7].
2.1.5. DNS Method. The reducing sugar content was estimated using the Miller method (Miller, 1959). First, 3 ml of the DNS reagent was added to 1 mL sample. The mixture was heated to 95°C in a water bath until a color change from red to brown was observed. This process took about 5 to 10 minutes. One mL of 40% potassium sodium tartrate solution was added to the mixture to stabilize the color. Then, the mixture was cooled to room temperature. The mixture was then made up to 10 mL using distilled water. The mixture was analyzed using a spectrophotometer (Genesys, Canada) at a wavelength of 540 nm. The parts of the plant which produced the highest amount of total reducing sugar after the two-step pretreatment was chosen and used throughout the experiment.

2.2. Optimization of Alkaline Pretreatment
Alkaline pretreatment was screened to obtain the optimized parameters prior to enzymatic hydrolysis. Parameters such as pretreatment temperature and NaOH concentration gave major impact on the alkaline pretreatment performance. Effect of pretreatment temperature was carried out at 80, 90 and 100 °C, at 30 minutes using 2% w/v of NaOH. The optimized temperature were then employed in the next experiment. For the effect of NaOH concentration, concentrations of NaOH chosen were 1, 1.5 and 2% w/v while temperature and residence time remained constant and set at the optimized value obtained from the previous screening. Three replications were done for each experiments.

2.3. Optimization of Enzymatic Hydrolysis
Enzymatic hydrolysis was screened to obtain the optimized parameters prior to fermentation. The effect of enzyme concentration was carried out at 1, 2, 3 and 4 FPU, at 50 °C for 72 hours. All experiments were conducted in triplicates.

2.4. SSF Assay
2.4.1. Media Preparation. Kluyveromyces marxianus UniMAP 1-1 were grown in YPD medium; 10 g/L peptone, 20 g/L peptone and 10 g/L glucose, in 125 mL Erlenmeyer flasks at 30°C with continuous shaking at 200 rpm for 24 h [8]. Glucose and YP solutions were sterilized separately at 121°C for 15 minutes by autoclave (Hirayama, Japan). Cells were washed twice and resuspended in the fermentation media.

2.4.2. Minerals and Vitamins Preparation. Minerals and vitamins were added to support the growth of the yeast. Mineral medium consists of: (NH₄)₂SO₄, 4.0 g/L; KH₂PO₄, 2.0 g/L; MgSO₄·7H₂O, 0.5 g/L; trace elements (EDTA, 15 mg/L; ZnSO₄·7H₂O, 4.5 mg/L; MnCl₂·2H₂O, 0.84 mg/L; CoCl₂·6H₂O, 0.3 mg/L; CuSO₄·5H₂O, 0.3 mg/L; Na₂MoO₄·2H₂O, 0.4 mg/L; CaCl₂·2H₂O, 4.5 mg/L; FeSO₄·7H₂O, 4.5 mg/L; H₃BO₃, 1.0 mg/L; and KI, 0.1 mg/L). Vitamin solution consists of d-biotin, 0.05 mg/L; calcium pantothenate, 1.0 mg/L; nicotinic acid, 1.0 mg/L; myo-inositol, 25 mg/L; thiamine HCL, 1.0 mg/L; pyridoxine HCL, 1.0 mg/L; and para-aminobenzoic acid, 0.2 mg/L. Both solutions were sterilized by filtration.

2.4.3. Inoculum Preparation. A loop of yeast was taken from the agar plate and pre-incubated in 5 mL of YPD medium at 30°C for about 24-48 hours to allow the cells to grow. Then, it was incubated in 500 mL of YPD medium at 30°C for 48 hours to grow the cells to a desired concentration. After that, the cells were washed using sterilized distilled water to remove the remaining YPD medium contains in the cells. This steps were repeated for three times.

2.4.4. SSF. Fermentation bottles were used in order to run this experiment because it can mimic the bioreactor. Fermentation bottles were added with other accessories like magnetic stirrer which act as the propeller in the bioreactor and a tube to purge CO₂ out of the bottles. SSF experiments were conducted to validate the fermentable sugar produced from the Petai Belalang seeds can be converted into bioethanol. A factor of hydrolysate concentration was conducted to verify the capability of K. marxianus UniMAP 1-1 to ferment bioethanol at high temperature using lignocellulosic hydrolysate. The working volume of this fermentation was fixed at 50 mL in 100 mL fermentation bottles.
2.5. Sugar Consumption and Bioethanol Production Analysis

Determination of sugar consumption and bioethanol produced were carried out using High-Performance Liquid Chromatography (HPLC) (Shimadzu RID-10A, Japan) with refractive index detector equipped with an Aminex HPX-87H column (300 mm × 7.8 mm). The column temperature is kept constant at 45°C. The mobile phase prepared for analysis was 0.005 M H₂SO₄. Injection volume was 10 µL with flowrate of 0.5 mL/min.

3. Results and Discussion

3.1. Sugar Contents from Different Parts of L. leucocephala

Three samples from each part of L. leucocephala which are stalks, leaves and seeds were prepared for alkaline pretreatment followed by enzymatic hydrolysis. The responses for reducing sugar content and sugar yield were obtained as shown in Figure 3.1.

![Figure 3.1. Total reducing sugar concentration in different parts of L. leucocephala.](image)

The seeds contained the highest reducing sugar when combining both treatments which was 27.65 g/L compared to stalks and leaves of L. leucocephala which were 2.82 g/L and 12.46 g/L, respectively. The results might be due to the functions of each parts in utilizing glucose. Glucose were produced by the leaves via photosynthesis, and retained very little on leaves as most of the glucose is transported elsewhere to be used and stored. Seeds on the other hand are really important to plants which function as the next generation for the plant. Large amount of glucose will be stored in the seeds to ensure survival. While stalks are a medium of transportation of water and nutrient which included glucose, therefore the reducing sugar concentration is much less than leaves and seeds.

Alkaline pretreatment promotes swelling of the lignocelluloses which can increase its porosity [9]. During alkaline pretreatment, lignin and hemicellulose can be modified or removed. For alkaline pretreatment, leaves released the highest reducing sugar concentration which is 8.76 g/L, while stalks and seeds only produced 2.78 g/L and 5.83 g/L, respectively. Differences in reducing sugar concentration yield could be a result of the different percentages of lignin, hemicellulose and cellulose content in each parts of L. leucocephala and the degree of disruption of lignin which is an undesirable component to be removed. Leaves showed the highest reducing sugar indicating that its hemicellulose percentage are much more higher than the seeds and stalks, and the lignin content were very less or nearly to zero percent which can easily be disrupted.

After alkaline pretreatment, solid residues were washed to remove excess alkaline solution and undergoes enzymatic hydrolysis. The substrate lost its amorphous region due to degradation of hemicellulose during alkaline pretreatment and made more cellulose being exposed. Therefore, during enzymatic hydrolysis, cellulose were able to be converted to reducing sugar with the help of cellulase.
which breaks down the glycosidic bond. For enzymatic hydrolysis, seeds showed the highest reducing sugar content which is 21.82 g/L, a significantly high as compared to stalks and leaves which produced 0.02 g/L and 3.71 g/L, respectively.

3.2. Effect of Temperature on Alkaline Pretreatment
Alkaline pretreatment refers to application of alkaline solution to modify the structure and composition of the lignocelluloses. Alkaline solution used for this experiment is sodium hydroxide (NaOH) which can supply sodium ions diffused into lignocellulose and promote swelling especially in cellulose [9]. Temperature during the alkaline pretreatment is a considerable parameter to be examined. At elevated temperature, the heat energy will weaken the intermolecular force and the intermolecular hydrogen bond will become unstable and break easily [10]. Figure 3.2 shows the effect of temperature on alkaline pretreatment in producing total reducing sugar, followed by enzymatic hydrolysis.

![Figure 3.2: Effect of Temperature for Alkaline Pretreatment on Total Reducing Sugar Concentration followed by Enzymatic Hydrolysis (72 hours, 50℃)](image)

Based on the data shown in Figure 3.2, 100℃ pretreatment temperature for alkaline pretreatment produced 18.56 g/L reducing sugars, and continue to increase in production at a total of 45.77 g/L when combining sugars production in both treatments. At 80℃ and 90℃, it only produced a total of 28.22 g/L and 40.94 g/L of reducing sugar, respectively. Degree of polymerization of hemicellulose will directly show on the molecular weight of hemicellulose. As the molecular weight of a polymer getting smaller, it indicated that the degree of polymerization also higher and depolymerization of hemicellulose become harder compare to shorter chain of polymer (hemicellulose). The higher the temperature applied, it will increase the molecular weight of hemicellulose. Therefore, the degree of polymerization of hemicellulose become smaller and degradation becomes easier. As the degradation of hemicellulose were complete, hemicellulose will be dissolved and cellulose is exposed for subsequence enzymatic hydrolysis. For alkaline pretreatment at 80 ℃, hemicellulose might not be fully degraded. Some cellulose might still bond with hemicellulose and limit the accessibility of enzyme which resulted in lower conversion reducing sugar. Hence, it can be concluded that higher temperature is preferable for alkaline pretreatment in achieving high fermentable sugar.

3.3. Effect of NaOH Concentration on Alkaline Pretreatment
Alkaline concentration gives a significant effect to the rate of reaction [11]. During alkaline pretreatment, sodium hydroxide ions need to penetrate through the lignocellulosic material in order to
disrupt the recalcitrance structure of hemicellulose and lignin. Hence, size of ions play an important factor to be considered as it affected the alkalinity of the solution.

As the concentration of alkaline are diluted, ions will be surround by water molecule and hydrodynamic diameter are too large to diffuse into the cellulose region. On the other hand, when too concentrated alkaline solution are used, hydrogen bonding of cellulose are too difficult break by hydration of alkali ions [8]. Figure 3.3 shows the effect of alkaline concentration on the total reducing sugar obtained.

![Figure 3.3](image-url)

**Figure 3.3.** Effect of NaOH concentration for Alkaline Pretreatment on Total Reducing Sugar followed by Enzymatic Hydrolysis (72 hours, 50°C).

Temperature was fixed at 100 °C for alkaline pretreatment as optimized from the previous experiment. From the data obtained, 2% w/v of NaOH produced the highest reducing sugar which was 23.04 g/L. This showed that the concentration of alkaline used were successful, due to the suitable size of ions that were able to penetrate into the cellulose layer, increasing the solubility of cellulose and restructure the hemicellulose [10]. When 1% w/v of NaOH used, it only produced 18.93 g/L of reducing sugar during alkaline pretreatment. This may due to inhibitor appear from the biomass which treated with 2% w/v of NaOH. Suitable alkaline concentration should be considered to achieve maximum conversion of reducing sugar since too diluted or too concentrated also affect the size of ion molecule that will diffused into amorphous area of cellulose.

### 3.4. Effect of cellulase concentration on enzymatic hydrolysis

Enzyme activity are normally calculated in terms of filter paper unit (FPU), by calculating the rate of total amount of enzyme used to produce 1 g of reducing sugar [12]. For lignocellulose material, ideal usage for enzyme loading is 5 FPU until 35 FPU per one gram of substrate. Figure 3.4 shows the effect of enzyme concentration on the reducing sugar concentration after the substrates were pretreated using 2% w/v of NaOH at 100 °C for 30 minutes.
Figure 3.4. Effect of enzyme concentration on Reducing Sugar Concentration (2 w/v% of NaOH, 100 °C).

Cellulase (Cellulast 1.5L Novozymes) with 1.22 g/mL of density was used. Based on the data obtained from Figure 3.4, there was no difference between the three volumes of enzyme used in the total reducing sugar. For 0.05 mL of enzyme used, 50.98 g/L of total reducing sugar from both treatments were produced, while for 0.1 mL and 0.15 mL of enzyme, it produced 50.92 g/L and 50.95 g/L of total reducing sugar, respectively. This phenomena occurred due to the enzyme loading are over the substrate loading which made substrate binding sites fully occupied [12]. Enzyme loading is a parameter which affect the rate of conversion of reducing sugar since substrate inhibition occurred and slow down the process. Since the results did not shower significant difference, the lowest enzyme loading was chosen to avoid substrate inhibition, speed up conversion rate and consequent detection of structure. Also, minimum use of enzymes are preferable by the industries to lower the cost of production.

3.5. SSF Fermentation
According to [13], increase in substrate loading in SSF Fermentation is one of the most important challenges to make bioethanol production more economical. Hence, different concentrations of hydrolysate have been introduced in this study. The results was shown in Figure 3.5.
From Figure 3.5, the overall trend of glucose consumed by *K. marxianus* for all substrate loading were almost the same. For the first six hours, all substrates were slightly decreasing, hence it can be said that *K. marxianus* were consuming the hydrolysate to get the carbon source and nutrient required for them to grow. Moreover, if we look at the trend of bioethanol production, it also slightly increasing from zero gram per liter for all hydrolysate loading. In short, for the first six hours, it can be said that when glucose were consumed, bioethanol were produced.

The trend for both glucose concentration and bioethanol concentration graph were continued to decrease and increase, respectively for the next hours until 24 hours. All substrate loading were decreasing linearly from nine hours to 24 hours. Additionally, it was found that at this point, the substrate were in their lowest concentration which means that *K. marxianus* were consuming all of the sugar in the hydrolysate. However, the ethanol concentration produced was still low, 3.68 g/L at highest. This might be due to the presence of inhibitors such as furfural, acetic acid and HMF that can hinder the production of ethanol.

As the trend of glucose consumed and bioethanol produced by the yeast at all hydrolysate loading of 30, 50 and 70% were not significantly different, hence, 50% of hydrolysate was chosen as the percentage of substrate loading due to consistency of production. According to [13], increasing substrate loading can increase in ethanol production, but over-increase it can lead the formation of unwanted inhibitors that can inhibit enzyme and fermenting microbes.

4. Conclusion

This study was designed to optimize the upstream processing of bioethanol production and produce bioethanol from *Leucaena leucocephala* using a thermo-tolerant yeast *Kluyveromyces marxianus* UniMAP 1-1. The biomass of *L. leucocephala* undergoes alkaline pretreatment followed by enzymatic hydrolysis to achieve maximum of reducing sugar yield. Seeds have been found to produce the highest yield of total reducing sugar compared to stalk and leaves after alkaline pretreatment and enzymatic hydrolysis process.

Pretreatment and enzymatic hydrolysis are the key processes in the production of fermentable sugar for further bioconversion into biofuel, therefore this project was done to optimize the alkaline pretreatment and enzymatic hydrolysis of *L. leucocephala* seeds by employing OFAT experiments. The optimized conditions of alkaline pretreatment were observed at 100 °C of pretreatment temperature and 2% w/v of sodium hydroxide. The results show that increasing the severity of the alkaline pretreatment could improve the degradation of the recalcitrant structure of *L. leucocephala* seeds.

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**Figure 3.5:** Glucose consumption and bioethanol production during fermentation using different substrate loading.
For OFAT experiments in the screening of the enzymatic hydrolysis parameters, the optimized range for cellulase concentration is 3 to 4 FPU. Low hydrolysis temperature, long hydrolysis time and high cellulase concentration have resulted in a higher total sugar yield during enzymatic hydrolysis of *L. leucocephala* seeds.

The fermentation of this sugar source is using Simultaneous Saccharification and Fermentation (SSF). The optimize parameters using OFAT have been studied and therefore can be used to produce a high bioethanol production. The results of research conducted therefore will be collected from the designs parameters and the glucose consumption and bioethanol produced will be measured using HPLC.

The best condition for fermentation of bioethanol from *L. leucocephala* using *K. marxianus* UniMAP 1-1 were at 50% of hydrolysate and 4 FPU of enzyme loading. From the experiment conducted, the highest ethanol produced was 3.68 g/L.

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Acknowledgement
This work was supported by the Fundamental Research Grant Scheme awarded by the Ministry of Higher Education, Malaysia (FRGS/1/2018/STG05/UNIMAP/02/4).