A comparative study on bioethanol production from rice straw and banana pseudostem through simultaneous saccharification and fermentation using *Kluyveromyces marxianus*

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**Abstract.** To improve the efficiency of second-generation bioethanol production, simultaneous Saccharification and Fermentation (SSF) of top agricultural wastes in the Philippines such as rice straw and banana pseudostem was conducted using commercial enzymes and the thermotolerant yeast *Kluyveromyces marxianus*. Residues were initially subjected to chemical treatment prior to SSF. A constant inoculum loading of 10% v/v was introduced during SSF and mixed with a nutrient supplemented solution. Aside from the enzyme loading, the effect of reaction temperature and reaction time on ethanol concentration was assessed. Among the tested parameters, only reaction time had a significant effect on the bioethanol concentration from both biomasses. For a pre-treated rice straw with 70.93% ww-1 holocellulose, highest ethanol concentration obtained was 6.30±0.44 gL-1 at 45°C, reaction time of 48 h and with enzyme loading of 30 FPUg-1. On the other hand, 5.35±0.29 gL-1 ethanol was achieved from SSF of banana pseudostem with 67.75% ww-1 holocellulose, also at 35°C, at a lower reaction time of 24h and same enzyme loading of 30 FPUg-1. This study also proves that the thermotolerant *K. marxianus* was capable of producing bioethanol from lignocellulosics through SSF, thereby considering it as a potential alternative to *Saccharomyces cerevisiae* for bioethanol production.

1. Introduction

Bioethanol is one of the alternative renewable energy sources identified by the Philippine government and has gained much interest worldwide because of both economic and environmental relevance. In the Philippines, the Biofuels Act of 2006 or RA 9637 mandates the 10% vv-1 blending of bioethanol to gasoline. However, locally produced bioethanol can only supply at most 40% of the total demand. This is despite of the fact that at present, there are already 12 operating distilleries in the Philippines with a total rated capacity of around 380.5M liters yr-1 [1]. Hence, the lack of bioethanol supply...
resulted to importation, which is a violation of the Biofuels Act. Furthermore, locally produced bioethanol must be subsidized to be able to compete with the current global price. There is also a need to improve the local production of bioenergy feedstocks like sugarcane and sweet sorghum as U.S. corn and Brazilian sugarcane are being mass-produced at a much lower cost. Improvement in the fermentation process and meeting the challenges of ethanol distilleries can also be of help.

The use of lignocellulosic biomass such as agricultural residues like rice straw and banana pseudostem produces second-generation bioethanol. Because lignocellulosic biomass is more abundant and inedible, the pressure on food crops as a fuel will decline [2]. In addition, the common waste disposal method of excess lignocellulosic waste is by biomass burning. The smoke particles from biomass burning have adverse health and climate effects [3]. Utilizing these biomasses for bioethanol production can reduce greenhouse gas emissions.

Several studies on bioethanol production from lignocelluloses have been conducted, but developments are critical so that the large-scale process will be economically feasible. A common strategy for bioethanol production using lignocelluloses is using separate enzymatic hydrolysis and fermentation (SSF), which executes hydrolysis and fermentation at their own optimum conditions; however, the primary disadvantage is the inhibition of cellulase activity by the liberated sugars, predominantly cellobiose and glucose [4]. To improve the efficiency of bioethanol conversion by minimizing enzyme inhibition, as well as to reduce the production costs, this study employed the simultaneous saccharification and fermentation technique.

Despite the economic benefits of SSF over SHF, a critical problem could arise because of a great difference in the optimum temperature of the cellulases and the fermenting microorganism [5]. This study aimed to determine the potential of *Kluyveromyces marxianus*, a thermotolerant microorganism, to convert sugars to ethanol at different temperature values investigated. The experimental results would also provide the best possible conditions of temperature, reaction time, and enzyme loading corresponding to the highest ethanol concentration from two types of biomass, rice straw and banana pseudostem. Model equations would also be presented to easily estimate the corresponding ethanol concentration that can be obtained from each biomass at different SSF conditions.

### 2. Methodology

#### 2.1. Biomass preparation

The biomass rice straw and banana pseudostem (Figure 1) were collected from PhilRice and Institute of Plant Breeding (IPB), UPLB, Philippines respectively. Both were initially sun-dried before subjected to oven drying (70°C) for 5 days to reduce the moisture content to less than 10% w/w. The dried rice straw and pseudostem were then ground to achieve a particle size of not more than 1 mm using a Wiley mill grinder. The milled rice straw and pseudostem were then subjected to chemical pretreatment.

At a biomass loading of 10% w/w, rice straw was treated with 5% w/w NaOH solution. The banana pseudostem, on the other hand, was treated with 0.5% v/v sulfuric acid solution. The flasks containing the mixture were placed in an incubator shaker, stored at a working temperature of 40°C, agitated at 150 rpm for 1 h. The pretreated biomass residue was collected by filtration and was subjected to subsequent washings to remove the remaining adhering chemical solution in the biomass. The pretreated biomass was brought to the Central Analytical Services Laboratory (CASL), BIOTECH, UPLB for analysis of holocellulose, hemicellulose, and alpha cellulose to determine the component of biomass that can be converted to bioethanol.
2.2. Inoculum preparation
The microorganism *Kluyveromyces marxianus* utilized as the fermenting yeast for the experiment was procured from the Philippine National Collection of Microorganisms (PNCM), UPLB. The yeast *K. marxianus* was maintained on agar slants containing 10 g L\(^{-1}\) yeast extract, 20 g L\(^{-1}\) peptone, 20 g L\(^{-1}\) glucose and 20 g L\(^{-1}\) agar. The parent stock was stored at 4°C, while the slants for acclimatization were incubated for 3 days at 30 °C. Shown in Figure 2 is the freshly acquired culture slant of *K. marxianus*.

![Figure 2](image)

**Figure 2.** Culture stock of *K. marxianus*.

For acclimatization, every 100 mL main culture medium consisting of 50 gL\(^{-1}\) pretreated straw, 3 gL\(^{-1}\) urea, 10 gL\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 3 gL\(^{-1}\) KH\(_2\)PO\(_4\), 0.5 gL\(^{-1}\) MgSO\(_4\)·7H\(_2\)O, and 0.5 gL\(^{-1}\) CaCl\(_2\) was autoclaved at 121°C and 0.18 MPa for 15 min. For each flask, yeast was added along with 5 mL of Accellerase\textsuperscript{®} 1500 and varying loadings of Novozymes\textsuperscript{®} NS-50013 (cellulase complex) corresponding to 10 FPUg\(^{-1}\), 20 FPUg\(^{-1}\) and 30 FPUg\(^{-1}\), respectively. Afterwards, the culture was incubated for 72 h at 30°C using a shaker with a speed of 120 rpm.

2.3. Simultaneous Saccharification and Fermentation experiment
Ten grams of pre-treated biomass in 250 mL flasks (with cotton stopper) were transferred in a shaker incubator with speed of 150 rpm, after sterilization using autoclave at 121°C and a pressure of 0.18 MPa for 15 min. SSF tests were performed under sterile conditions and the fermentation was started by adding 10 % v/v\(^{-1}\) inoculum with enzyme loading. At various time intervals, samples were withdrawn for subsequent determination of glucose and ethanol by DNS method and gas chromatography, respectively. Figure 3 shows the SSF equipment used.
Figure 3. Incubator shaker (IKA KS 4000i control) used for SSF.

The process was conducted at varying temperature values (35°C, 40°C and 45°C), reaction time (24h, 48h and 72h), and enzyme loading (10 FPUg⁻¹, 20 FPUg⁻¹ and 30 FPUg⁻¹). All SSF experimental runs were performed in duplicates, except for the center points that were conducted in four trials. The effects of the parameters on the responses sugar and ethanol concentrations were assessed using Analysis of Variance (ANOVA).

2.4. Analytical methods

2.4.1. Enzymatic activity measurement. The enzyme activity of Novozymes® NS-50013 (cellulase complex), in terms of filter paper units (FPU) per milliliter of original (undiluted) enzyme solution, was measured by degradation of filter paper (Whatman No.1 filter paper) in citrate buffer (pH 4.8) for 1h at 50°C, based on the procedure outlined in National Renewable Energy Laboratory [6]. The reducing sugars produced were then analyzed by 3,5- Dinitrosalicylic acid (DNS) method.

2.4.2. Reducing sugar analysis. A DNS test was used as the method to determine the reducing sugar content of each sample [7]. For each sample, 0.1 mL of the supernatant liquid was mixed with 0.1 mL of 0.05 M citrate buffer and 0.6 mL of DNS reagent in a test tube. Submerging the samples in vigorously boiling water for 5 min started the colorimetric development, and then, the absorbance of each sample was read using a spectrophotometer at 540 nm (Shimadzu UV-1800, Japan). The standard solutions were prepared using 0.05, 0.075, 0.1, 0.5, 1, 3, 10 and 15 mg mL⁻¹ glucose concentrations.

2.4.3. Ethanol concentration determination. The ethanol concentration was analysed by Gas Chromatography (GC) (Shimadzu GC-2014, Japan). Before the analysis, the samples were prepared by centrifugation to prevent solids from clogging the syringe in the GC auto-sampler. The supernatant liquids and an equal amount of 0.2% isopropanol v:v⁻¹ which served as the internal standard, were transferred in 1.5 mL GC vials. The ethanol concentration was obtained at a retention time of about 1.5 min. The GC condition used is as follows: Chromatopak column, isothermal at 122°C. The carrier gas N₂ was kept at 30 mLmin⁻¹ at 190°C, 155 kPa. The flame ionization detector (FID) operated at 200°C.
3. Results and Discussion

3.1. Characteristics of pre-treated biomass

Results of the compositional analysis (Table 1) show that the alpha-cellulose of pretreated rice straw is higher than that of pretreated banana pseudostem, but the hemicelluloses of the latter is higher than the first. The total holocellulose content of both biomasses are almost the same, slightly higher for pretreated rice straw, despite of different pretreatment methods employed.

| COMPOSITION     | Pretreated Rice Straw | Pretreated Banana Pseudostem |
|-----------------|-----------------------|------------------------------|
| Holocellulose   | 70.93                 | 67.75                        |
| Alpha-cellulose | 46.66                 | 16.87                        |
| Hemicellulose   | 24.27                 | 50.88                        |

The alpha-cellulose content of the alkali-treated rice straw in this study is lower than the value reported by Oberoi et al. which is 60.54% [8], under the same pretreatment conditions using 5% NaOH solution. However, the total substrate content for this study was found to be 70.93%, which is comparable with the literature value of 70.26%. With reference to the theoretical composition of untreated rice straw, the hemicellulose content slightly decreased from 28.45% to 24.27% and the alpha-cellulose from 34.12% to 46.66% after pretreatment. Dilute NaOH pretreatment causes swelling, which increases the internal surface area and porosity of the material, decreases the degree of polymerization and crystallinity, and disrupts the structure of lignin [9]. This could mean that the amount of lignin remained in the solid residue was reduced, thereby allowing the enzymes greater access for hydrolysis, which is essential for the subsequent fermentation.

A similar experiment which employed the same concentration of sulfuric acid used in this study for pretreatment of banana pseudostem was conducted by Mishra and Sheet [10]. However, the pretreatment was carried out at temperature of 125-130°C and 25 psi for one hour. The resulting cellulose content was approximately 14.07%. This was much lower than the actual cellulose content of the pretreated substrate in this study with 16.87%. The hemicellulose content of the pseudostem was 50.88%, resulting to a total holocellulose component of 67.75%. The value is lower compared to the holocellulose content of the pretreated rice straw. Low concentration of the acid (i.e. 0.5% v/v sulfuric acid solution used) coupled with short time exposure for about an hour of the substrate at an elevated temperature and pressure may also be considered as one of the factors that led to lower cellulose content. In a study by Jumarmi et al., a higher acid concentration of 2% was recommended to be employed even for a shorter reaction time of 30 minutes for pretreatment to be effective in reducing the lignin content from 19.40% to 15.92%, increasing the cellulose content from 44.60% to 52.11%, and decreasing the hemicellulose from 36% to 28.45% [11]. This accounts for a total of 80.56% holocellulose component of the banana pseudostem, which is 16% higher than that of what was obtained in this study.

3.2. Effects of enzyme loading on bioethanol concentration

As shown in Figure 4, the effect of increasing the enzyme dosage rate during SSF on the average ethanol concentrations from both banana pseudostem and rice straw was not significant. There was a recorded slight increase to around 0.4 v/v which is equivalent to approximately 3 g L⁻¹ ethanol, as the enzyme loading was increased. However, it must also be considered for SSF that too low enzyme loading was not preferred as it could generally lead to inactivation of the enzyme, decreased interaction of the enzyme with the substrate and non-specific adsorption of cellulolytic enzyme to lignin [12]. Therefore, at a higher economically acceptable enzyme loading, the larger amount of reducing sugars would be available for K. marxianus to ferment into ethanol.
Figure 4. Effect plot of enzyme loading on the final ethanol concentration from (a) banana pseudostem and (b) rice straw after SSF.

To arrive with higher ethanol yields, high substrate concentration was ensured than increasing the enzyme dosage rate to improve the rate of reaction. This is despite of result of the study that revealed that doubling the enzyme loading from 7.0 FPU g\(^{-1}\) of dry matter (DM) to 15.0 FPU g\(^{-1}\) DM greatly increased the ethanol concentration from 52.1 % to 75.9 % or 40.6 to 59.3 g L\(^{-1}\), for instance, on SSF of steam-exploded corn stover [13]. A higher loading of enzyme will be able to liberate simple sugars at a faster rate, however, low enzyme loadings is preferred for economic reasons.

3.3. Effects of reaction time on bioethanol concentration

As shown in Figure 5, the ethanol concentration decreases with increasing reaction time. The ethanol concentrations peaked at 0.6 v v\(^{-1}\) or around 5 g L\(^{-1}\) from banana pseudostem and at 0.4 v v\(^{-1}\) or around 3 g L\(^{-1}\) from rice straw with 24 hours of reaction time.

Figure 5. Effect plot of reaction time on the final ethanol concentration from (a) banana pseudostem and (b) rice straw after SSF.
As saccharification and fermentation proceeds, there are other metabolic compounds and by-products formed aside from the desired ethanol, such as weak acids. There may be a deficiency in nutrients or the condition of the sample that favored the production of other metabolites. Furfural and 5-HMF have a direct inhibition effect on either the glycolytic or the fermentative enzymes of the yeast. These inhibitors could affect the growth of the yeast more than the ethanol formation [14]. It was further explained that weak acids, including acetic acid and formic acid, upset the intracellular pH by accumulating in the yeast cells, thereby lowering the biomass formation and ethanol yield. For the case of this study, the pH of the mixtures was found to remain constant at around pH 5, which reduces the toxicity of the acids. In a study by Moreno et al. for SSF of steam-exploded biomass with laccase treatment, the viability of \textit{K. marxianus} declined at the 12\textsuperscript{th} hour of SSF when it was completely inhibited, despite its ability to assimilate most of the inhibitory compounds mentioned [14].

Another point to consider is the viability of \textit{K. marxianus} during the fermentation period. In a study conducted by Lopez, the viability of \textit{K. marxianus} cultivated in YEPD agar was observed. It was found out that after 23 h of fermentation, the viability of the yeast decreased from 100\% to 59.6\%. After the fermentation period of 72 h, only 47.5\% was viable. When cell viability loss, the ethanol concentration will decrease [15].

### 3.4. Effects of reaction temperature on bioethanol concentration

The temperature had insignificant effect on ethanol production as shown in Figure 6. The insignificant effect of temperature to ethanol production could be due to the thermotolerance of \textit{K. marxianus}. The thermotolerance of yeast was the ability to withstand relatively hot or cold condition. In a study published by Arora et al., it was mentioned that \textit{K. marxianus} was capable of thriving and fermenting ethanol at a temperature range of 30 to 65\°C [16]. This could imply that possibly at the temperature range (35 to 45\°C) employed in this study, the growth rate and kinetics of \textit{K. marxianus} were the same. Thus, no significant effect was seen in terms of ethanol production even if the temperatures were varied. A similar result was acquired by Bajpai and Margaritis [17], they found out that ethanol yield approached a constant value when immobilized cells of \textit{K. marxianus} was exposed to a temperature ranging from 25 to 45\°C, while the free cells of the same yeast had constant ethanol yield at temperature range of 25 to 35\°C only. The ethanol yield dropped drastically at a temperature beyond 35\°C. In another experiment, the findings showed that ethanol yield of two strains of \textit{K. marxianus} used in the study was good even at temperature reaching up to 45\°C [18].

![Figure 6](image-url)

**Figure 6.** Effect plot of temperature on the final ethanol concentration from both banana pseudostem and rice straw after SSF.
In the study of Abdel-Banat et al., they reported that the optimal fermentation performance of the *K. marxianus* DMKU3-1042 strain was observed at 40°C [19]. In an experiment conducted by Du Le et al., it was found that increasing the temperature from 30°C to 40°C did not change the production of ethanol by *K. marxianus* after fermentation for 48 h. However, enzyme activity was inhibited at 45°C [20]. In this study, the major trend observed was the decrease in ethanol concentration after 24 h, regardless of the temperature value. The decline in ethanol formation could be attributed to the evaporation of ethanol that, however, efficiently occurs at 50 °C.

### 3.5. Ethanol concentration equations considering multiple factor interaction

The relationship of ethanol concentration with the factors varied in this study and their interactions could be presented using mathematical equations. In Equations 1 and 2, the exact value of the response ethanol concentration from banana pseudostem and rice straw respectively, as a function of varying parameters, could be estimated. Note that based on the ANOVA, only the reaction time had a significant effect on ethanol concentration.

\[
E = 0.4259 + 0.0537A + 0.0218B - 0.1976C - 0.0437AB + 0.0065AC + 0.0413BC - 0.0403ABC
\]

where:
- \( E \) is the ethanol concentration from banana pseudostem (in % v v\(^{-1}\))
- \( A \) is the enzyme loading (in mL)
- \( B \) is the SSF temperature (in °C)
- \( C \) is the reaction time (in h)

\[
\frac{1}{E} = 2.25 - 0.1685A + 0.1882C - 0.1460AB + 0.3342AC + 0.4034BC - 0.6956ABC
\]

where:
- \( E \) is the ethanol concentration (in % v v\(^{-1}\))
- \( A \) is the enzyme loading (in FPU g\(^{-1}\))
- \( B \) is the temperature (in °C)
- \( C \) is the reaction time (in h)

Based on Equation 1 and considering interaction of parameters, the highest concentration of ethanol was 0.7145 % mL mL\(^{-1}\) or equivalent to 5.35 ± 0.29 g L\(^{-1}\), recorded at highest enzyme loading of 30 FPU g\(^{-1}\), temperature of 35°C, and reaction time of 24 hours. For the pretreated rice straw, the highest ethanol concentration acquired in the study was 6.23 ± 0.69 gL\(^{-1}\), obtained at the SSF conditions of 35°C, 10 FPU g\(^{-1}\) enzyme loading and reaction time of 72 h. However, a comparable concentration of 6.30 ± 0.44 gL\(^{-1}\) was observed at the 48th hour under the conditions of 45°C and enzyme loading of 30 FPUg\(^{-1}\) using the same Equation 2. This peak indicates that the production of ethanol at a faster rate, signifying a similarly high concentration achieved at a shorter reaction time but at a higher enzyme loading and reaction temperature. Therefore, the latter combination of parameters appears to be a more suitable combination of conditions than the former if considering shorter SSF processing time.

This study also proves that *K. marxianus* was capable of producing bioethanol from lignocellulosic biomass rice straw and banana pseudostem through SSF. This can be attributed to the characteristics of the thermotolerant *K. marxianus*, having higher growth rate and resistance to toxic compounds than the typically used industrial yeast *S. cerevisiae* [21]. With a doubling time of approximately equal to 70 minutes, the organism is considered to have one of the fastest growth rates compared to other eukaryotic microbes [22]. A study conducted also by Abdel-Banat et al. concluded that a derivative strain of *K. marxianus*, *K. marxianus* DMKU3-1042, was able to ferment cellubiose, xylose, xylitol,
arabinose, glycerol, and lactose [23]. This therefore means that *Klyveromyces marxianus* can efficiently metabolize several industrial substrates of economical relevance, thereby showing its potential to replace *S. cerevisiae* for bioethanol production [24].

4. Conclusion

The viability of banana pseudostem and rice straw as feedstocks for bioethanol production using *K. marxianus*, a thermostolerant yeast, was studied. Three factors were varied namely: enzyme loading, SSF temperature, and reaction time. According to the experimental data, the highest concentration of ethanol from pseudostem was 5.35 ± 0.29 L⁻¹ recorded at highest enzyme loading of 30 FPU g⁻¹, temperature of 35°C, and reaction time of 24 h. At the same enzyme loading, 6.30 ± 0.44 g L⁻¹ bioethanol concentration was observed at the 48th h at SSF working temperature of 45°C.

Also, it was concluded that among the tested parameters, only the reaction time had a significant effect on the bioethanol concentration for both utilized feedstocks. The ethanol concentration had an inverse relationship with the reaction time. The ethanol concentration for this experiment reached its peak value after fermentation period of 24h for the case of banana pseudostem, while 48h for the pretreated rice straw. Although, the tested temperature ranging 35 to 45°C had an insignificant effect on the ethanol concentration, the ability of *K. marxianus* to produce ethanol at the working temperature conditions was still proven. Moreover, it was found out that both biomasses could be used as a source of bioethanol.

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