Ethanol Production from Alkaline-treated Rice Straw via Separate Hydrolysis and Fermentation (SHF) using *K. marxianus* UniMAP 1-1

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**Abstract.** The decreasing reserves of non-renewable energy especially fossil fuel has led to an urgent need to establish alternative fuels. Ethanol is one of the energies explored, which can be generated by fermentation method. The use of environmentally friendly material such as lignocellulosic biomass to develop a biofuel is significant. Ethanol production at high temperature was preferred as it will significantly reduce the cooling cost involved. Thus, the use of thermotolerant strain in the fermentation process was recommended. In this study, separate hydrolysis and fermentation (SHF) was employed to produce ethanol from 2% sodium hydroxide-treated rice straw using cellulase enzyme, and fermented by a thermotolerant *K. marxianus* UniMAP 1-1 strain. The fermentation process was done at two different temperatures, 37°C and 50°C, at pH 4.8. The ethanol yield from both 37°C and 50°C was 0.36 g/g and 0.38 g/g, respectively. *K. marxianus* UniMAP 1-1 showed a good production of ethanol at elevated temperature. This is the first study reporting ethanol production from rice straw using *K. marxianus* UniMAP 1-1. Thus, this study can improve our understanding of the development of thermotolerant yeast accountable to the SHF process for ethanol production.

1. Introduction

The depletion of fossil fuel supply and distress on climate change has driven the exploration of renewable and environment-friendly sources of energy. Among all of the renewable energy sources, bioethanol production from cheap and abundant feedstocks such as lignocellulosic biomass is very attractive. The United States and Brazil produced 60 and 27 million m³ of ethanol in 2017, respectively, representing 85% of the world’s production [1]. In the North American distilleries, ethanol is produced from corn starch, while in tropical countries such as Brazil, India, and Thailand, the feedstock is sugarcane juice. As for Malaysia, which is blessed with its biodiversity, wastes from the oil palms, rice straws, spent mushroom substrates and Napier grass are potential substrates for bioethanol production. In August 2005, The National Biofuel Policy has introduced bioethanol as one of the five energy sources for Malaysia [2].

Bioethanol was not only recognized as an alternative way of substituting petrol but it also helps in climate change. The development of technology in the transportation sector has made the emission of carbon dioxide increase year by year, which also affected climate change. According to Wang et al. [3], the transportation sector contributed 20-25% of the greenhouse gas emissions, and the percentage will
keep increasing as the technology expands. However, the production of biofuels from biomass could help in the reduction of the harmful gases. A study has found that greenhouse gas emissions such as hydrocarbon (HC) and sulphur dioxide (SO\textsubscript{2}) were significantly lower in bioethanol than in 100% gasoline [4]. Moreover, the replacement of bioethanol as the transport fuel can potentially reduce 3-30% of transport-related greenhouse gas emissions [5]. Thus, these statements have proved that renewable energy such as biofuel not only help the world with depletion of fossil fuel conflict but also help the earth overcome climate change and greenhouse effect issues.

Plants containing high level of starch are the main biomass sources. However, high usage of plants will affect the environment such as global warming, and increase the greenhouse effects. To reduce the risk, lignocellulosic materials from agricultural wastes were utilized. Lignocellulosic material is a plant source composed of lignin, cellulose, and hemicellulose. Examples of lignocellulosic materials are corn stovers, wheat straws, rice straws, and others. According to Thulluri [6], lignocelluloses mainly contains starch and carbohydrate that can be utilized to produce bioethanol through biological processes. Bioethanol conversion from plant materials became an interesting field of study among the researchers because the process taken is simpler than any other material. In this study, alkaline pre-treatment was evaluated for the production of ethanol from rice straw. In terms of quantity and worldwide availability, rice straw is the one of the most favourable substrates for biological products [7].

The pre-treatment process is particularly crucial, as the enzymatic hydrolysis of lignocellulosic biomass is impeded by the substantial presence of lignin and crystalline nature of cellulose. The lignin component in lignocellulosic biomass acts as a physical barrier and pre-treatment step is needed to structurally alter the barrier to make carbohydrates accessible for transformation processes. According to Oberoi et al. [8], to modify the macroscopic and microscopic biomass size and structure, as well as its sub-microscopic chemical composition and structure, pre-treatment is required so that hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more efficiently with higher yields. Alkaline pre-treatment method was used as it utilizes low temperature and resulted in less sugar degradation compared to other methods such as acid pre-treatment.

In this study, Separate Hydrolysis and Fermentation (SHF) were used as the main method to produce the ethanol. The key advantage of this approach is the two processes; hydrolysis and fermentation will be performed at different optimal conditions. The optimal temperature for cellulase is 45 - 50°C and the temperature for the fermenting organism is 30 - 37°C [9]. Therefore, this method was recommended as the temperature used can be more flexible and contamination during the fermentation can be reduced. Moreover, SHF method was simpler and cheaper compared to other methods.

Kluyveromyces marxianus is widely known for its capability of assimilating a variety of sugars, such as lactose, xylose, and arabinose, which are abundantly found in lignocellulosic biomass. This species was likely to be used compared to Saccharomyces cerevisiae due to its capability in consuming both sugars (pentose and hexose). It also has been widely used for the development of economic and biotechnological biomolecules such as production of ethanol, aromatic compounds, enzymes (β-galactosidase, inulinase, and pectinase), and recombinant proteins [10, 11]. Moreover, it also has the highest specific growth rate among eukaryotic microorganisms at temperatures up to 50°C [12]. In a nutshell, this yeast was a fulfillable yeast to be used in the lignocellulosic ethanol production. Thus, the present work aims to produce ethanol from rice straw using a newly isolated K. marxianus UniMAP 1-1 via SHF process.

2. Methodology
2.1. Rice straw pre-treatment
The rice straw from a rice field in Perlis, Malaysia was milled to obtain particles size between 0.25 and 0.84 mm. Then, the rice straw was pretreated with 2% NaOH (v/w) with a ratio of 1:10 (solid:liquid). The pre-treatment was carried out at 80°C. After 24 hours, the rice straw was washed with distilled water until the colour of the water residue became clear.
2.2. Enzymatic hydrolysis

Cellulase (Celluclast 1.5L, Novozymes, Denmark) with concentrations ranging from 5 - 30 FPU was used. Three grams of dried pre-treated samples and cellulase enzyme was inserted into a 100 mL conical flask, and 25 mL of citrate buffer (pH 4.8) was added to the mixture. The sample was then incubated at 50°C for 24 hours.

2.3. Inoculum preparation

2.3.1. Growth media. K. marxianus UniMAP 1-1 strain was cultured in Yeast extract-Peptone-Dextrose (YPD) media. The media was prepared by dissolving 10 g/L of yeast extract, 20 g/L of dextrose and 20 g/L of peptone in a 1L schott bottle and autoclaved at 121°C for 15 minutes.

2.3.2. Minerals and vitamins. Fermentation medium contained YPD medium, minerals and vitamins to support the growth of the microorganisms. Mineral medium consists of: (NH₄)₂SO₄, 4.0g/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) [13]. The medium was sterilized at 121°C for 15 minutes by autoclave. In vitamin solution, it consists of d-biotin, 0.05 mg/L; calcium pantothenate, 1.0 mg/L; nicotinic acid, 1.0 mg/L; myo-inositol, 25mg/L; thiamine HCL, 1.0 mg/L; pyridoxine HCL, 1.0 mg/L; and para-aminobenzoic acid, 0.2 mg/L [13]. This solution was sterilized using filter.

2.3.3. Inoculum cultivation. Inoculum cultivation was done in the laminar flow to prevent contamination. All of the apparatus was sterilized before the cultivation activity. One colony of K. marxianus UniMAP 1-1 strain from YPD agar was streaked using an inoculating loop and was inserted into 5 mL of YPD media for pre-incubated process. The cells was pre-incubated at 37°C for 24 – 48 hours with continuous shaking at 150 rpm to ensure good growth. Then, the pre-incubated cells was transferred into 250 mL of YPD media and further incubated for 48 hours to allow the cells to double. After that, the cells were centrifuged and washed using sterilized distilled water for several times to wash out the remaining YPD media.

2.4. Separate Hydrolysis and Fermentation (SHF)

Enzymatic hydrolysis was done before the fermentation. The dried pre-treated rice straw was soaked in citrate buffer (pH 4.8) with ratio of 1:10 w/v. Cellulase enzyme was inserted into the mixture with concentration of 30 FPU/g and the mixture was heated at 50°C for 24 hours at 500 rpm. The 50 mL SHF reaction mix was composed of 25 mL of hydrolysate, 50 g/L of yeast, 5 mL mineral and 0.05 mL of vitamin. The fermentation was done at two different temperatures, 37°C and 50°C. Then, the samples was withdrawn every 3 hours for the first 24 hours and was withdrawn every 24 hours afterwards until 72 hours of fermentation. The samples were centrifuged and kept at -20°C for further use in the next analysis. All experiments were done in triplicates.

2.5. Products analysis

The substrates and products of fermentation (glucose, ethanol, xylose, xylitol, glycerol and acetic acid) were analysed using the HPLC (Perkin Elmer, USA) with a refractive index detector. The column HPX-87H (Bio-rad Lab, USA) with size of 300 x 7.8 mm was used. A 5mM sulphuric acid (H₂SO₄) was used as the mobile phase. The flow rate was set at 0.6 ml/min with 45°C of oven temperature. The concentrations of each component was determined by their peak area using standard calibration curves.
3. Results and discussion

3.1. Effect of enzyme concentrations on fermentable sugar production

The degradation of sugars by acid hydrolysis can be reduced by performing enzymatic hydrolysis using specific enzymes. In enzymatic hydrolysis, cellulase and hemicellulase react specifically on cellulose to produce glucose, and hemicellulose to produce both pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose) [14]. In this study, cellulase enzyme was used and the optimum temperature for this enzyme is 50℃. Three different concentrations of cellulase were tested; 5, 10, and 30 FPU. Figure 1 shows the glucose and xylose produced after enzymatic hydrolysis. The glucose and xylose concentration increased as the enzyme concentration increased. The highest glucose and xylose produced were 16.6 g/L and 8.2 g/L, respectively using 30 FPU of enzyme. Enzyme concentrations are proportional with the glucose production, however for the xylose, the production did not improve after 10 FPU. This might be due to the need of an additional enzyme; xylanase, to breakdown the hemicellulose composition, thus releasing more xylose.

![Figure 1](image-url)

**Figure 1.** Effect of enzyme concentrations on the production of glucose and xylose.

3.2. Fermentation performance of K. marxianus UniMAP 1-1 at 37℃ via SHF.

The current work shows the possibility of successful production of ethanol from rice straw, by enzymatic hydrolysis followed with fermentation by *K. marxianus* UniMAP 1-1. Most of the common yeasts, including *Saccharomyces cerevisiae* were only capable of fermenting hexose sugars to ethanol, but not xylose [15] [16]. However, *K. marxianus* UniMAP 1-1 had been proven to be capable of assimilating both hexose and pentose sugars. Figure 2 shows the fermentation profile of *K. marxianus* UniMAP 1-1 at 37℃.

3.2.1. Fermentation profiles. The maximum ethanol concentration produced by *K. marxianus* UniMAP 1-1 at 37℃ was 6.4 g/L with ethanol yield of 0.36 g/g, equivalent to 72% of theoretical yield. Compared to the ethanol production using *Clostridium acetobutylicum* bacterium with alkaline pretreated rice straw, the maximum ethanol produced was about 0.1 g/L with initial sugars about 7 g/L [17]. Thus, this research study has shown that *K. marxianus* UniMAP 1-1 has great potential in producing ethanol from rice straw hydrolysate.

Besides, xylitol production also has been observed. As illustrated in Figure 2, xylose were consumed simultaneously with glucose, but the consumption rate of xylose were a bit slower than glucose.
Figure 2. Fermentation profiles of *K. marxianus* UniMAP 1-1 at 37°C.

Glucose was fully consumed within 3 hours however xylose was fully consumed within 24 hours. According to Nitiyon et al. [18], after exhaustion of glucose concentration, xylose was sequentially assimilated and resulted in higher ethanol yield. The maximum concentration of xylitol produced was 0.95 g/L.

During pretreatment process, several inhibitors were also released. The formation of inhibitors such as acetic acid, phenol, furfural and HMF could affect the inhibition of the growth and fermentation of the microorganism [19]. Formation of acetic acid was evaluated in this study. As shown in Figure 2, formation of acetic acid increased as the fermentation occurred. The highest concentration of acetic acid formed during the fermentation was 1.7 g/L. However, acetic acid formation does not affect the formation of ethanol. Ethanol started to deplete after glucose was fully consumed by the yeast after 9 hours of fermentation.

3.2.2. Fermentation profiles of *K. marxianus* UniMAP 1-1 at 50°C via SHF. The main conflict in handling ethanol fermentation is the different temperatures needed by the enzyme used and fermenting microorganism. High temperature-tolerant strains need to be explored in order to produce high ethanol as required in a SSF process. Based on this study, *K. marxianus* UniMAP 1-1 demonstrated a good production of ethanol at high temperature. The maximum ethanol produced was 6.9 g/L with 0.38 g/g ethanol yield, equivalent to 76% of theoretical yield. Surprisingly, the performance of *K. marxianus* UniMAP 1-1 during fermentation at condition 50°C was better than the ones in 37°C. In 2016, a fermentation using *K. marxianus* strain (K21) was done at 40°C and 50°C which resulted in ethanol concentrations of 43.46 g/L and 19.15 g/L, respectively [20]. In another research done by Ryabova et al. [21], they used *Saccharomyces stipitis* as the fermenting organism and they also reported the sensitivity of the strain to higher temperatures. At 30°C, the strain was able to produce approximately 2.5 g/L of ethanol, but as the temperature increased to 37°C, ethanol was barely produced by the strain which confirmed the sensitivity of the strain to high temperatures [21]. Thus, this research has shown that *K. marxianus* UniMAP 1-1 was a good candidature of fermenting organism in producing ethanol at high temperature. Figure 3 shows the fermentation profiles of *K. marxianus* UniMAP 1-1 at 50°C via SHF.
Both glucose and xylose consumption were involved in the ethanol production. The exhaustion of glucose sources would affect the xylose due to the need for carbon sources as a food supply for the yeasts to produce ethanol [22]. After 24 hours, glucose and xylose concentration was increased. This can be explained by the activation of enzyme used since cellulase’s optimum temperature is 50°C. Both 37°C and 50°C conditions showed a decreased of ethanol concentration after 24 hours.

Xylitol production during 50°C also higher than 37°C. The maximum production of xylitol during 50°C was 3.3 g/L while during 37°C, the highest xylitol production was 3 g/L. Compared to the research done by Zhang et al. [23], the fermentation using un-engineered strain of *K. marxianus* (YZB001) resulted the highest concentration of xylitol, 0.23 g/L. Therefore, this strain not only proved to be a good consumer for both hexose and pentose sugars, but it also proved to be a good ethanol and xylitol producer at high temperatures. Based on the result, acetic acid production during the fermentation process at 50°C condition did not significantly affected the production of ethanol. The concentration of acetic acid increased as the time taken for the fermentation to occur increased.

### 4. Conclusion

This study is the first to examine the production of ethanol using *K. marxianus* UniMAP 1-1 via SHF. This strain could ferment ethanol from rice straw hydrolysate, even when the temperature was elevated to 50°C. The ethanol yield resulting from the hydrolysate fermentation using *K. marxianus* UniMAP 1-1 were 0.38 g/g and 0.36 g/g at the temperatures 50°C and 37°C, respectively. This study is an effort to improve the understanding of thermotolerant yeast accountable for the SHF process for lignocellulosic ethanol production.

### References

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