Scaling-up and techno-economics of ethanol production from cassava starch via separate hydrolysis and fermentation

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**Abstract.** Nowadays bioethanol is being used extensively in fuel production because of its technical feasibility, economically competitive, and environmentally friendly. It is expected that biofuel will contribute to 30% of the global energy demand by 2050. Therefore, it is very important to investigate any cost-effective bioprocessing that can improve the overall production yield. The study aims to produce bioethanol from cassava starch by Kluyveromyces marxianus at a laboratory scale and a 5L fermentation rig. A separate hydrolysis and fermentation (SHF) process of cassava starch was introduced due to high sugar content in starch, using a thermoanaerobe able to reduce the cooling time after hydrolysis. A combination of 0.35% v/w amylase and 0.20% v/w amyloglucosidase used in the hydrolysis of cassava starch produced 19.18 g/L of sugar. A 15 g/L of K. marxianus showed to be the best yeast concentration which could produce the highest bioethanol, 42.85 g/L. When the laboratory scale was scaled up to 5 L fermentation, the result was comparable at 42.33 g/L. The same SHF parameters in laboratory scale had been proven to be effective in a larger scale fermentation based on similar results obtained. Simulation using SuperPro software indicated that 50.13% of starch can be converted into ethanol.

1. Introduction
The global rise in energy consumption has increased the energy demand and exploration of renewable as well as eco-friendly energy resources. Toxic gas emission from petroleum fuels had raised public concerns, thus bioethanol becomes one of the options for modern biofuel due to its clean combustion. Previous studies have shown that production of bioethanol is technically feasible, economically competitive, readily available throughout the year, and environmentally acceptable [1]. Besides, bioethanol has improved the vehicles’ combustion of fuel and reduced the pollutants emission into the environment [2]. It is expected that bioenergy such as bioethanol will contribute to 30% of the global energy demand by 2050 [1].Bioethanol production from cassava has received much attention because they are low in cost and is renewable.

Cassava (*Manihot esculenta* Crantz), also known as tapioca, is one of the globally important food crops because it has high starch content and high production yield [3]. Cassava is not a staple diet in many countries and the excessive cassava yield had shifted the usage of cassava to another purpose such as bioethanol production [4]. Studies had found that cassava is a potential raw material for the production...
of bioethanol. The most popular microorganisms that has been exploited for bioethanol fermentation were *Saccharomyces cerevisiae* and *Zymomonas mobilis* [5].

The use of thermophilic and thermotolerant microorganisms in bioethanol production favours the continuous removal and recovery of bioethanol from the broth which minimizes the cost for cooling, reduces the toxicity level of bioethanol for the culture and risk of contamination [6]. Fermentation at high temperature could increase the growth rate of the microbes and its productivity as the rate of diffusion and mixing of nutrients increase [7]. To date, research regarding the effects of enzyme concentration in bioconversion of cassava starch to sugar, and the concentration of *Kluyveromyces marxianus* in fermentation at elevated temperature is still insufficient for optimal bioethanol production especially in industrial application. Hence, an investigation on the hydrolysis of cassava starch to sugar before fermentation by different enzyme concentrations was performed. Then, the effect of yeast concentration after saccharification process on bioethanol production was studied on a small scale and compared with a bigger scale fermentation.

In this work, hydrolysis of cassava starch was carried out by using commercial alpha-amylase and amylglucosidase. With the ability to withstand high temperature after hydrolysis, bioconversion of glucose to bioethanol was carried out using *K. marxianus*. Besides facilitating the production of bioethanol at high temperature, thermotolerant microorganisms also offer the possibility of in-situ bioethanol recovery and reduce the enzyme requirements [1]. This was done by comparing different enzyme concentrations in the liquefaction and saccharification of gelatinized cassava starch. The selected enzymes concentration for hydrolysis and yeast concentration for fermentation are carried out in the fermentation rig to test the performance on a larger scale.

### 2. Methodology

#### 2.1. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out through three stages; gelatinization, liquefaction and saccharification.

**2.1.1. Gelatinisation**. Two hundred ml of 30% w/v slurries of cassava powder was prepared as the initial substrate concentration [5]. Gelatinisation of the cassava starch was done at 120°C on a heating plate and cooked until it becomes transparent.

**2.1.2. Liquefaction**. Liquefaction was carried out after gelatinisation in 100 mL universal bottles containing 50 mL of gelatinised cassava starch at 80°C in a shaking water bath. The process was optimised using a different concentration of α-amylase (BAN 480L) in the range of 0.15-0.35% v/w. The performance of the liquefaction process was determined by using DNS reagent to measure the glucose released.

**2.1.3. Saccharification**. Amyloglucosidase (AMG 300) was added for saccharification using the selected α-amylase concentration from liquefaction in the range of 0.05-0.25% v/w. The performance of the saccharification process was measured by the determination of glucose concentration as estimated by the DNS method.

#### 2.2. Fermentation

**2.2.1. Inoculum preparation**. A 250 mL of complete medium (YPD) for inoculum preparation contained 20 g/L glucose, 10 g/L yeast extract and 20 g/L peptone was prepared and autoclaved at 121°C for 20 minutes. A 5 mL of the culture medium was transferred to a sterilised test tube and 1 mL of *K. marxianus* was added from the master bank for pre-cultured. The culture was then incubated in a flask in shaking water bath at 30°C and 250 rpm for 2 days.
2.2.2. **Lab-scale fermentation.** After cultivation, the yeast cells were harvested via centrifugation in a 50 mL centrifuge tube at 5500 g for 5 minutes at 4°C and washed with sterilised distilled water for three times. The yeast cells were re-suspended in a sterilised distilled water to a known cell mass concentration in wet weight per litre [3]. Finally, different concentration of inoculum (15, 20, 25, 30 and 35 g/L) was employed for initiating the bioethanol fermentation experiments. The carbon source for the fermentation was the optimised hydrolysed cassava starch from the previous steps. Fermentation temperature was set to 40°C by placing the bottles in a water bath equipped with a magnetic stirrer and stirring at 500 rpm. Samples were taken at 0, 4, 24, 48 and 72 h of fermentation.

2.2.3. **Fermentation rig.** The scale-up of bioethanol fermentation was carried out in a production rig with a working volume of 5L based on the selected enzyme and yeast concentration as the ones in lab scale as described in previous sections. Samples were collected through an opened nut using kerosene pump. After fermentation, bioethanol was distilled from the fermentation broth.

2.3. **Analytical methods**

2.3.1. **DNS reagent preparation.** About 2.5 g of 3,5-dinitrosalicylic acid (DNS) was mixed with 200 mL of 0.5 N sodium hydroxide and 75 g of sodium potassium tartrate (Rochelle salt), followed by distilled water up to a final volume of 250 mL in Erlenmeyer flask covered with aluminium foil [8]. Mixing was carried out at 80°C using a hot plate to ensure all the chemicals were fully dissolved. Then, the reagent was stored at 4°C for further use.

2.3.2. **Determination of reducing sugar.** One mL sample was mixed with 1 mL of DNS reagent. The mixture was placed in a boiling water bath for 10 minutes and cooled in ice water to room temperature, followed by the addition of 5 mL of distilled water. Sufficient amount of the mixture was used to determine glucose concentration using a spectrophotometer at a wavelength of 540 nm with DNS reagent as a blank. The glucose concentration was determined as a function of absorbance value by using a pre-established calibration curve.

2.3.3. **Determination of cell growth.** The samples from fermentation were centrifuged at 5500 g for 5 minutes. The residuals were added with sufficient distilled water and measured the absorbance at 600 nm. The dry cell mass was estimated as a function of absorbance value by using a pre-established calibration curve. The absorbance was measured as an indicator for yeast growth using a spectrophotometer at 600 nm [6].

2.3.4. **Analysis of ethanol and glucose on HPLC.** Samples were centrifuged in 1.5 mL Eppendorf tubes at 5500 g for 10 minutes [3]. The supernatant which consists of glucose residues and ethanol were measured by using HPLC (Shimadzu RID-10A, Japan) coupled to a refractive index detector, with an Aminex HPX-87H Ion Exclusion column (BIO-RAD) 300 mm × 7.8 mm. The mobile phase was 0.005 M H₂SO₄ at a flow rate of 0.5 ml min⁻¹. The column temperature was kept constant at 45°C, and the sample volume was 10 μL.

2.3.5. **Determination of ethanol yield.** The ethanol yield was calculated using the equation:

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\text{Ethanol yield} = \frac{\text{Ethanol produced}}{\text{Sugar consumed}} \times 100\%
\]

2.3.6. **Determination of fermentation efficiency.** The fermentation efficiency was calculated using the equation:
Fermentation efficiency = \( \frac{\text{Sugar consumed}}{\text{Initial sugar}} \times 100\% \)

2.4. Simulation and economic analysis of ethanol production

2.4.1. Flowsheet and report generation. The process flowsheet was developed using SuperPro Designer based on material balance from the fermentation rig. The base case of cassava starch to ethanol plant has a capacity of 1000 kg/batch of cassava starch being processed. Economic analysis considering the capital and operating cost was extracted from the software.

3. Results and Discussion

3.1. Hydrolysis

3.1.1. Gelatinisation. This step was used to disrupt the starch’s molecular structure and change its characteristics by starch solubilisation, granular swelling, loss of birefringence and native crystalline melting [9]. Amylose could leak out from the radial channels of organised starch granules during gelatinisation and contribute to the solubility of the starch. It was reported that starch granules are insoluble in aqueous media below its gelatinisation temperature [10]. The temperature might cause the structure of starch to change with the expansion of the amorphous region of the channel of the starch granule, thus making it easier to be accessed by the enzymes.

3.1.2. Liquefaction. Liquefaction is important in converting starch into maltodextrins at a high temperature which aimed to reduce the viscosity of starch using \( \alpha \)-amylase [11, 12]. Observation during the experiment found that increased enzymes concentration had decreased slurry viscosity. The enzyme entered the starch granules and disrupted its structure, making it less compact [13]. In this study, liquefaction was measured using the DNS method to obtain the yield of total reducing sugars. This step generated a solution containing dextrin and little amount of glucose [14]. It is important to reduce the viscosity of fermentation medium because viscous nature will affect the mass and heat transfer, dissolved oxygen homogeneity, mixing intensity, cell growth rate and in the end, reduce the bioethanol production rate [11]. Figure 1 shows the glucose concentration obtained when using different \( \alpha \)-amylase concentrations.

![Figure 1](image.png)

**Figure 1.** Effect of alpha-amylase concentration on liquefaction of 30% w/v cassava starch.

The results show that there is no significant increase in glucose concentration between 0.15% and 0.25% of \( \alpha \)-amylase, but there is a slight increment in glucose concentration when 0.3% and 0.35% of \( \alpha \)-amylase were used in liquefaction. The highest glucose, 11.18 g/L was obtained using 0.35% of \( \alpha \)-
amylase, while the lowest α-amylase (0.15%) yielded 8.74 g/L glucose. Thus, the following process, which is saccharification was done after the addition 0.35% v/w α-amylase. It was previously reported that optimum dextrinizing activity of α-amylase was 0.35% by using CCD [15].

3.1.3. Saccharification. After liquefaction, amyloglucosidase (AMG 300) was added for saccharification. The performance of the saccharification process was measured by the glucose liberated using the DNS method. Figure 2 shows the effects of different concentrations of amyloglucosidase on the saccharification of the hydrolysed cassava starch.

![Figure 2. Effect of amyloglucosidase concentration on saccharification of cassava starch.](image)

Figure 2 shows that there were no significant increase in the total sugar contents between 0.05% to 0.15% amyloglucosidase added. But there was a slight increase in the total sugar when 0.2% amyloglucosidase was added. The final reducing sugar yield was higher than that of liquefaction. By adding 0.05% amyloglucosidase, the glucose concentration was 18.07 g/L, while 0.25% resulted in the highest yield, 19.29 g/L of glucose. Both 0.20% and 0.25% v/w of amyloglucosidase gave nearly the same performance in producing sugar. It was reported that an increase in enzyme concentration will increase enzyme activity, however the addition of more enzyme will not necessarily increase the activity of the enzyme when the peak is reached [14]. Amyloglucosidase of 0.20% v/w seemed to be the best condition for hydrolysis since the addition of higher enzymes concentration did not show significant increment. This result is in agreement with the previous research which also obtained 0.2% as the best amyloglucosidase concentration[15]. The reducing sugar concentration increased at the end of each experiment. This phenome may be due to the enzymatic activity for the conversion of liquefied starch to reducing sugar at high temperature [16]. At first, α-amylase broke the α-(1,4) glycosidic linkages of the starch to oligomers with a little amount of fermentable glucose [17]. Then, amyloglucosidase converted the oligomers to glucose which increased the glucose yield at the end of the hydrolysis.

3.2. Fermentation

3.2.1. Lab-scale fermentation. By implementing the optimum enzyme concentrations during hydrolysis (0.35% α-amylase and 0.20% amyloglucosidase), the fermentation performance by different yeast concentration was studied. This is to determine the suitable yeast concentration by SHF of cassava starch. Figure 3 shows the effects of yeast concentrations on glucose utilisation and bioethanol production.
Figure 3. Glucose utilisation and ethanol production using 30% cassava starch.

According to figure 3, 30% of the cassava starch had an initial glucose concentration ranging from 126.36 to 135.22 g/L. Yeast concentrations of 30 and 35 g/L produced almost the same amount of bioethanol, 46.85 and 47.30 g/L, respectively. The initial yeast concentration of 20 and 25 g/L also gave nearly the same final bioethanol concentration, which was 44.53 and 43.57 g/L, respectively. The lowest yeast concentration, 15 g/L implemented in this experiment produced 42.85 g/L of ethanol. However, reducing sugar remained in the fermentation broth about 6.20 to 23.73 g/L after 72 hours under agitated condition. The factors of this phenomena might be due to the inability to tolerate the bioethanol in the fermentation broth as it exerts a toxicity effect on yeast [6]. Bioethanol produced by microorganisms has been claimed to be more inhibitory as compared to ethanol added during a fermentation period, thus reduces the cell membrane permeability to some nutrients [18].

The high concentration of glucose in the fermentation media also could toxicate the yeast and reduced the fermentation rate [5, 18, 19]. Besides that, high sugar concentration will increase the viscosity of fermentation broth which gives an inhibitory effect on yeast growth, decrease the sugar utilization and thus reduces the capability to produce bioethanol [6]. Yeast may be confronted with a variety of environmental stresses that can cause the loss of yeast viability, reduced yeast growth and increased fermentation time, which reduced yeast fermentation rates [6]. The bioethanol yield and fermentation efficiency is shown in Figure 4.
**Figure 4.** Effect of yeast concentration on ethanol yield and fermentation efficiency.

It shows that there were no significant difference on bioethanol yield based on the yeast concentrations tested (15 - 35 g/L). The fermentation efficiency increased slightly with the increase in yeast concentration. The excessive yeast in the fermentation medium might affect the fermentation efficiency by reducing the fermentation time when most of the substrate was immediately converted to bioethanol [20].

The SHF process with yeast concentration of 35 g/L produced 47.30 g/L of bioethanol and the yield was the highest (39.71%) among the five experiments carried out. This is comparable with the bioethanol produced from cassava starch using co-immobilised cells of *Zymomonas mobilis* and *Saccharomyces diastaticus* at 30°C, 46.7 g/L [21]. However, the bioethanol produced from all the five yeasts concentration showed almost similar productivity. As compared to Zhang et al (2011) [22], bioethanol production from raw sweet potato using *S. cerevisiae* produced 93.56 g/L of ethanol in SHF process at 30°C. From literature, most of the bioethanol production was carried out at a lower temperature and it could affect the cost of cooling before fermentation. The utilization of high-temperature fermentation by using thermoanaerobe, *K. marxianus* could potentially be performed directly after the hydrolysis process. Hence, the maximum bioethanol produced (42.85 g/L) and bioethanol yield (39.87%) which were obtained with an initial *K. marxianus* concentration of 15 g/L, was selected to be implemented in the rig fermentation as it seems to be economically feasible in a large-scale fermentation.

### 3.2.2. Rig fermentation

The main objective of scaling-up was to identify problems that were not significantly noticed at lab-scale and to verify the critical fermentation parameters. According to the results at lab scale, liquefaction by using 0.35% α-amylase, saccharification by using 0.20% amyloglucosidase, and 15 g/L of inoculum concentration was chosen to be used in the fermentation rig with a working volume of 5 L. Figure 5 shows the bioethanol concentration and residual sugar in the 5 L fermentation.
Figure 5. Glucose consumption and ethanol production in a 5L fermentation.

An initial glucose concentration of 103.81 g/L after hydrolysis produced 42.33 g/L of bioethanol after 72 hours under agitation at room temperature. However, the residual sugar concentration, 45.26 g/L in the rig fermentation was higher than that of the lab-scale, 23.73 g/L. This might be due to the configuration of the fermentation rig where mixing was not as efficient as the ones in fermentation bottles. Figure 6 shows the comparison between shake flask and scaling-up fermentation.

Figure 6. Comparison between lab-scale and rig fermentation.

As shown in figure 6, these values of bioethanol yield, bioethanol produced and fermentation efficiency in rig fermentation demonstrated no significant difference compared with those obtained by shake flask fermentation. The ethanol produced in the rig fermentation (42.33 g/L) were nearly similar to those in the lab scale (42.85 g/L). The results were comparable in both experiments from the fermentation efficiency and bioethanol yield. A pilot scale of ethanol production from oil palm empty fruit bunch fibre using S. cerevisiae in the 350L fermenter showed a 6.36% v/v of ethanol produced [23] from the available glucose in the broth. This result shows that the experiment conducted in fermentation rig could be useful in addressing the need of the pilot-scale study for further bioethanol production.

3.3. Simulation and economic analysis of ethanol production

3.3.1. Flowsheet preparation. The simulation of ethanol production from cassava starch was developed using SuperPro Designer. The purpose of conducting the simulation is to find out the differences that
would arise on scale-up of the lab scale ethanol production with the industrial scale. The process simulated for 1 tonne of cassava starch showed that 139.61 hours per batch was required, equivalent to 56 batches per year with a total operating hours of 7818.32 per year. Aspen Plus V10 software was used to simulate a bioethanol plant from microalgae cellulosic residue which was found to be able to operate for 330 days, 7920 hours annually [24]. Our findings from the simulation showed similar operating hours of bioethanol production as the considering of plant shutdown and maintenance activities. From the simulation of material balance per batch, 99% starch conversion to glucose could be achieved and more than 98% of substrate is utilised by yeast to produce about 50.12% of ethanol. The glucose was consumed by yeast and released 478.56 kg/ batch of carbon dioxide. The pre-treatment process; liquefaction and saccharification were able to convert cassava starch into glucose by hydrolysis. conducted simulation of saccharification from brown algae yield 75% of total carbohydrates.

![SuperPro flowsheet of ethanol production from cassava starch.](image)

**Figure 7.** SuperPro flowsheet of ethanol production from cassava starch.

3.3.2. Economic analysis. The economic evaluation report was generated from the software. Simulation of ethanol production indicated that operating cost would be $5,832,076/ year. This estimation is based on an ethanol plant processing about 56,000 kg cassava starch per annum, and producing 26,667.76 kg of ethanol. The flowsheet was prepared based on the rig fermentation process. The ethanol production resulted in a 50.13% w/w (ethanol/ cassava feed). This is higher compared to the ones reported [25], only 25% w/w of ethanol produced from brown algae using S. cerevisiae. A similar process had reported that the annual operating cost for bioethanol production from Sri Kanji 1 cassava was $2,120,719.80 [26], and the production cost for 1 L bioethanol from Sri Kanji 1 cassava was $0.10. As compared to another researcher, they reported that the cost of ethanol produced was $0.67 per kg [24]. The operating cost in this research is higher than [26] may be due to SuperPro software does not take into account for the by-products. A simulation using SuperPro Designer software to produce 1000L/ day of bioethanol using a genetically modified microalgae, Synechocystis sp showed a loss as the revenue from ethanol was unable to cover the operating costs [27]. They concluded that their simulation was non-feasible with the innovative approach of microalgae for direct ethanol production. Although this study showing that ethanol could be produced on a larger scale, it would be better to optimise the process flowsheet to generate more ethanol from the same amount of cassava starch.
4. Conclusion
Cassava starch is a potential source for bioethanol production in Malaysia as it does not compete with food supply, and it has a higher yield. Alpha-amylase and amyloglucosidase are important hydrolytic enzyme to degrade starchy material into reducing sugar. Since it provided the necessary glucose for fermentation, there was no need for additional sugar supplement. *K. marxianus* is one of the microorganisms that has the capability of producing bioethanol at high temperature. The same SHF parameters in the lab-scale had proven to be effective in a larger scale fermentation as it showed similar results. Hydrolysis of 30% w/v cassava starch slurries was optimized by using 0.35% v/v amylase and 0.20% v/v amyloglucosidase. An initial yeast concentration of 15 g/L was selected to be implemented into the rig fermentation with its maximum bioethanol produced, 42.85 g/L with a yield of 38.97%. When scaled up to a working volume of 5 L, 42.33 g/L of bioethanol was produced. This study had proven that fermentation in the laboratory scale and rig fermentation showed similar results. Optimization of $\alpha$-amylase concentration in liquefaction was important to optimize the amyloglucosidase and yeast concentration to obtain higher glucose and bioethanol production. Simulation using SuperPro software indicated that 50.13% of starch could be converted into ethanol.

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