Production of bioethanol from wild cassava crude starch 
(*Manihot glaziovii* Muell. Arg) using different microbial types 
and fermentation times

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Abstract. Wild cassava (*Manihot glaziovii* Muell. Arg) is one of the varieties of cassava that contains toxic compounds cyanogenic glucosides, which cause unmarketable food products more suitable to be processed into bioethanol. This wild cassava plant produces tubers four times in weight compared to that of ordinary cassava, and the flesh contains about 40–70% starch by dry weight. This study aimed to determine the effect of the microbial types and fermentation times on bioethanol production from *M. glaziovii* Muell. Arg crude starch and to determine the type of microbes and fermentation time that can produce the highest bioethanol product from *M. glaziovii* Muell. Arg crude starch. This study consists of two factors using a factorial randomized block design (RBD). The first factor was the type of microbe which consists of 2 different microbes, namely R5I3 isolates and *Saccharomyces cerevisiae* ATCC 9763. The second factor was the fermentation time which consists of 4 levels, namely 3, 4, 5 and 6 days. The observed parameters were total dissolved solids value, pH value, reducing sugar content, and ethanol content. The data obtained were analyzed using analysis of variance (ANOVA) and continued with Tukey’s HSD post hoc tests. The results showed that fermentation using R513 isolates with a fermentation time of 5 days was the best treatment to obtain a maximum ethanol of 21.64±3.03 g/L, with a final pH value of 4.70±0.14, total dissolved solids 4.10±0.14°Brix and reducing sugar content of 0.91±0.02 g/L. Therefore, the new isolate R513 was highly potential for producing bioethanol from wild cassava crude starch.

1. Introduction

Fossil fuels that exist today are the main source of fuel but cannot be expected for a long period of time due to its non-renewable nature and causing global warming therefore other alternative sources of energy are urgently needed [1], [2]. The solution to overcome this problem is to use bioethanol as fuel because it is environmentally friendly [3]. Bioethanol is a liquid fuel derived from glucose fermentation followed by a purification process [4].

Bioethanol can be produced from plants containing starch or cellulose components. One of the tubers that have the potential to be converted into bioethanol is wild cassava (*M. glaziovii* Muell. Arg). This cassava contains a toxic compound, namely cyanide acid which is high enough so that it is not marketed for food products. In fact, the source of protein and carbohydrates is not used in food [5]. Furthermore, cassava contains about 40%–70% starch by dry weight, 20%–30% cellulose fiber and other non-starch polysaccharides, with a moisture content of 70%–80% [6].

The use of wild cassava tubers for food purposes has a few drawbacks, because they contain high toxic cyanogenic glycosides (93% linamarin and 7% lotaustralin) which makes this wild cassava tubers are more suitable to be used as a basic material for making bioethanol. Although the cyanide acid
contained in rubber cassava can be reduced by immersion in water. In the immersion process, water causes the linamarin compound to be hydrolyzed so that the cyanide acid dissolves in water [7].

After the cyanide content becomes low, pre-treatment is carried out by converting *M. glaziovii* Muell. Arg cassava tubers into starch flour [8]. Followed by the hydrolysis stage which is the main process in converting starch into bioethanol based on biological hydrolysis using enzymes. The enzymes commonly used in this process are α-amylase and glucoamylase enzymes [9], [10]. Starch hydrolysis is divided into 3 stages, namely gelatinization, liquefaction using the α-amylase enzyme and saccharification using the glucoamylase enzyme to be able to break α-1,4 glycosidic bonds and α-1,6 glycosidic bonds [11], [12], [13]. Followed by the fermentation process using microorganisms to convert glucose into ethanol. In industry, generally bioethanol production can be fermented sugar using *S. cerevisiae* [14]. *S. cerevisiae* has the advantages of being able to regenerate quickly, easily adapting to the environment and tolerant of high alcohol content up to 15% [15]. Then, continued with the last stage, namely purification which aims to increase the level of purity of bioethanol [16].

In this study, the process of making bioethanol consist of four main stages, namely pretreatment, enzymatic hydrolysis, simultaneous saccharification and fermentation (SSF) which is the combination of enzymatic hydrolysis and fermentation carried out simultaneously in one bioreactor and this process provides advantages such as preventing inhibition by substrate concentration, saving production costs and producing more ethanol than a separate process and distillation [17],[12].

In this study, evaluation was carried out on the different types of microbial and the length of the fermentation time. The types of microbes used were *S. cerevisiae* ATCC 9763 and R5I3 isolates. Gunam et al. [1], reported that immobilized *S. cerevisiae* ATCC 9763 cells could convert simple sugars from bagasse cellulose saccharification into 63.874 ppm ethanol or equivalent to 0.006% (v/v). Afterwards, the length of the fermentation was chosen because it has a very large influence on the levels of bioethanol produced such as concentration of inoculum, activity of enzymes and composition of the substrates [18].

The objective of this study was to determine the effect of microbial types and fermentation time on bioethanol production and to determine the types of microbes and fermentation time that can produce the highest bioethanol products from wild cassava crude starch.

2. Materials and methods

2.1. Materials

The materials used in this study were *M. glaziovii* Muell. Arg cassava tubers obtained from Kintamani, Bangli, Bali, yeast *Saccharomyces cerevisiae* ATCC 9763 from the Laboratory of Microbiology IPB University, R5I3 isolates obtained from the Laboratory of Bioindustry and Environment, Faculty of Agricultural Technology, Udayana University, α-amylase enzyme and glucoamylase enzyme obtained from Novozymes, Denmark; DNS solution (Sigma-Aldrich), 70% alcohol (IKA), dextrose (Lihua Starch), buffered peptone (Merck), yeast extract (Himedia), distilled water, citric acid, sodium citrate.

The equipment used in this study include a shaker rotator (health H-MSR), centrifuge (Dragon), autoclave (Hirayama), gas chromatography (Varian 3300), chromatopac (Shimadzu C-R6A), laminar air flow (Wina Airflow), incubator (Memmert), vortex (Maxi Max II), magnetic stirrer (IKA ETS D5), measuring cylinder (Pyrex), Erlenmeyer flask (Iwaki), microliter pipette (Socorex), beaker glass (Herma), volumetric flask (Pyrex), blender (Miyako), oven (Memmert), UV-Vis spectrophotometry (Thermo scientific), water bath shaker (Shel Lab), pH meter (Senz pH), hand refractometer (Atago), bunsen, reflux distillation, distillator, 60 mesh sieve, 50 µm sieve.

2.2. Wild Cassava Substrate Preparation

Preparation began by taking wild cassava tubers weighing 4 kg, then peeled the skin and cut into smaller parts, then washed in clean water. The immersion step was carried out for approximately 72 hours with periodically water changing at every 24 hours with stirring. After that, the wild cassava tubers were dried under sunlight for 8 hours. Dried the cassava then was also dried in the oven at 85°C for 5 hours. Dried tubers were then washed using a blender then were sieved using a 60 mesh sieve [8].
2.3. Preparation of an Inoculum R5I3 Isolates and S. cerevisiae ATCC 9763

The inoculum preparation of R5I3 isolates and S. cerevisiae ATCC 9763 were carried out using a 250 mL Erlenmeyer and 1000 mL Erlenmeyer. Yeast extract peptone dextrose (YEPD) liquid media were prepared by mixing yeast extract 0.45%, peptone 0.75% and 5% dextrose as a medium for R5I3 isolates and yeast extract 0.5%, peptone 0.5% and dextrose 6% in 200 mL distilled water as a medium for S. cerevisiae. After that, the YEPD media was sterilized using an autoclave at 121°C, 1 atm, 15 minutes. One milliliter of cultured R5I3 isolates and S. cerevisiae were each inoculated on media that had been sterilized using a shaker rotator and rejuvenated for 24 hours. The rejuvenated isolates on YEPD media were inoculated back into 750 mL of YEPD liquid media for propagation for 24 hours. After that, the cell of R5I3 isolates and S. cerevisiae that had been propagated by centrifugation at 10.000 rpm for 5 minutes at 40°C. The isolates and S. cerevisiae were washed in NaCl 0.85% solution to produce cell mass, discard the resulting supernatant then adjust the cell concentration to OD660 5 [1], [19].

2.4. Enzymatic Hydrolysis

Gelatinization was done by mixing sifted wild cassava with water in a ratio of 1:5. After that, the mixture was stirred and heated to a temperature of 80°C until a thick slurry was formed. Gelatinized cassava was liquefied by adding 1.2 mL α-amylase enzymes (212.25 U/mL) at 90°C within 2 hours with continuous agitation at 100 rpm to form a dextrin. Then the slurry was cooled to 32°C. After that, sterilized at 121°C for 15 minutes [11], [12], [20].

2.5. Simultaneous saccharification and fermentation (SSF)

The sterilized substrate was adjusted to pH 5 by adding 0.05 M citric acid. Then 0.3 mL of a solution glucoamylase enzyme (109.05 U/mL) and the suspension with 5% (v/v) concentration of each microbe were also added. The mixture containing enzyme and microbe was mixed in a 250 mL bioreactor which then fermented according to a predetermined time in a water bath shaker at a temperature of 40°C [12], [21]. The SSF process was carried out with various times of 3, 4, 5, and 6 days at 40°C. Analysis of pH value [22], reducing sugar content [23], and total dissolved solids [24] were carried out at each time.

2.6. Distillation

After the fermentation process was done, the residue was filtered while the filtrate (bioethanol) was distilled by reflux distillation at 80°C and then stopped after the ethanol was separated [25]. Analysis of ethanol content was carried out at each time interval [26]. Ethanol content determination was carried out using gas chromatography (GC).

2.7. Data analysis

The data obtained were analyzed for its diversity using analysis of variants (ANOVA) and continued with the HSD Tukey. Observed parameters measured were total dissolved solids value, pH value, reducing sugar content, and ethanol content

3. Results and Discussions

3.1. pH analysis

The results of the ANOVA showed that the type of microbial treatment and fermentation time had a very significant effect (p<0.01), while the interaction had no significant effect (p≥0.05) on the pH of crude starch fermentation of M. glaziovii Muell. Arg cassava. The average pH value of fermented wild cassava starch can be seen in Figure 1.
Figure 1. The average pH value of the fermented wild cassava starch on the treatment of microbial types and fermentation time.

The results in Figure 1 show that there are differences in pH values for different types of microbes. The highest average pH value based on microbial type was obtained from isolate R5I3 which was 4.71±0.12 and the lowest was obtained from *S. cerevisiae* which was 4.56±0.17. Based on the data obtained from Figure 1, there is a decrease in pH at each increase in fermentation time and differences in pH values for different types of microbes. According to Dompeipen and Dewa [22], this happens because during fermentation, CO₂ and other organic acids are formed. Organic acids formed include acetic acid, pyruvic acid and lactic acid can reduce the pH value, while other acids such as butyric acid and other fatty acids have only a slight effect in reducing the pH of the substrate [21].

3.2 Total dissolved solids analysis

The results of the ANOVA showed that the type of microbial treatment and fermentation time had a very significant effect (p≤0.01), while the interaction had no significant effect (p≥0.05) on the total dissolved solids of crude starch fermentation of *M. glaziovii* Muell. Arg cassava. The average total dissolved solids value of fermented wild cassava starch can be seen in Figure 2.

The results in Figure 2 show that there are differences in the total value of dissolved solids for different types of microbes. The highest average total dissolved solids based on the type of microbes was obtained from *S. cerevisiae*, namely 4.6±0.16°Brix and the lowest was obtained from R5I3 isolates which was 4.3±0.28°Brix. The high total dissolved solids in *S. cerevisiae* was caused by the less optimum of these microbes converting glucose into ethanol. R5I3 isolates converted more glucose into ethanol than *S. cerevisiae*.

According to Simatupang et al. [27], the decrease in total dissolved solids occurs because the process of breaking down glucose into alcohol is carried out by yeast so that the glucose content decreases which results in the value of total dissolved solids also decreased. Yeasts need substrates and nutrients for the necessities of life. Substrates and nutrients in the media decrease, causing the total dissolved solids in the media to also decrease [28].
3.3. Reducing sugar analysis
The results of the ANOVA showed that the type of microbial and fermentation time had a very significant effect (p≤0.01), while the interaction had a significant effect (p≤0.05) on the reducing sugar of crude cassava starch fermentation. The average pH value of fermented wild cassava starch can be seen in Figure 3.

Figure 2. The average total dissolved solids value of the fermented wild cassava starch on the treatment of microbial types and fermentation time

![Figure 2](image1.png)

Figure 3. The average reducing sugar value of the fermented wild cassava starch on the treatment of microbial types and fermentation time

![Figure 3](image2.png)

The results in Figure 3 show that there are differences in reducing sugar levels for different types of microbes. The level of reducing sugar produced by the R5I3 isolates was lower than that of the reducing sugar produced by *S. cerevisiae*. This happened because the temperature used at the time of fermentation was 40°C, where the optimum temperature of *S. cerevisiae* was in the range of 30–35°C [29], while R5I3 isolates was able to work optimally at a temperature of 40°C.
The value of reducing sugar content in the fermentation process decreased from the 3rd day to the 6th day. This happened because both R5I3 and *S. cerevisiae* consumed glucose which was characterized by a decrease in reducing sugar levels at an increase in fermentation time. According to Wignyanto *et al.* [30], the more sugar consumed by microbes, the higher the ethanol produced. Conversely, the less sugar consumed by microbes, the lower the amount of ethanol produced.

3.4. Bioethanol Analysis

The results of the ANOVA showed that the type of microbial treatment and fermentation time had a very significant effect (p≤0.01), while the interaction had no significant effect (p≥0.05) on the ethanol levels of crude cassava starch fermentation. The average ethanol content of fermented wild cassava starch can be seen in Figure 4.

![Figure 4](image.png)

**Figure 4.** The average ethanol value of the fermented wild cassava starch on the treatment of microbial types and fermentation time

The results in Figure 4 show that there are differences in the ethanol content for different types of microbes. The highest average value of ethanol content based on microbial type was obtained from R5I3 isolates which was 21.64±3.03 g/L and the lowest was obtained from *S. cerevisiae*, namely 10.99±0.11 g/L. This happened because the temperature used during fermentation was 40°C, where the optimum temperature of *S. cerevisiae* was in the range of 30–35°C [29], while isolate R5I3 was able to work optimally at 40°C.

As the fermentation time goes on, the production of CO₂ gas also increases. The increase in CO₂ gas production along with the increase in alcoholic fermentation time, inhibits the activity of microbes which results in decreased alcohol formation while the acid formed can reduce the pH of the substrate which causes yeast to not grow optimally [20], [31]. The increase in fermentation time also causes more sugar to be converted into ethanol. In fact, the length of the fermentation process also has an optimum time so that when the optimum time has been reached, the ethanol content also decreases. Because, the longer the time the number of microbes also decreases and will go to the death phase caused by the nutrients that become microbial food decreases [32].

4. Conclusion

The type of microbes and the fermentation time affect the pH value, total dissolved solids, and ethanol content of the fermented wild (*M. glaziovii* Muell. Arg) cassava starch while the interaction between treatments affected the reducing sugar produced by the wild cassava starch fermentation. Fermentation
using R513 isolates with a length of the time fermentation of 5 days was the best treatment to obtained a maximum ethanol content of 21.64±3.03 g/L, with a final pH value of 4.7±0.14, total dissolved solids 4.1±0.14°Brix and reducing sugar content of 0.91±0.02 g/L. The new isolate is potential yeast in the production of bioethanol from crude wild cassava starch and based on the ethanol content produced. Wild cassava substrate has potential as a material for bioethanol production.

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