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Optimizing Bioethanol Production through Separated Hydrolysis Fermentation of Rubber-Cassava (Manihot Glaziovii) Starch with K^+ and Mg^{2+} Mineral Ion Supplements

Hargono[1]
Department of Chemical Engineering, Faculty of Engineering, Diponegoro University
Semarang, Jawa Tengah

Andreas Kevin Santoso[2]
Department of Chemical Engineering, Faculty of Engineering, Diponegoro University
Semarang, Jawa Tengah

Aurellia Livia Hidayat[3]
Department of Chemical Engineering, Faculty of Engineering, Diponegoro University
Semarang, Jawa Tengah

Auxensius Rexer Fransenda[4]
Department of Biotechnology, Faculty of Sains and Mathematic, Diponegoro University
Semarang, Jawa Tengah

Abram Hasiholan Marpaung[5]
Department of Chemical Engineering, Faculty of Engineering, Diponegoro University
Semarang, Jawa Tengah

Steffanie Glory Sihombing[6]
Department of Chemical Engineering, Faculty of Engineering, Diponegoro University
Semarang, Jawa Tengah

Abstract:- This study focuses on the production of bioethanol from rubber cassava starch using hydrolysis and fermentation processes. The research includes the impact of substrate concentration on the concentration of reducing sugars in hydrolysis, the influence of mineral ion concentrations of K^+ and Mg^{2+} in the final fermentation on bioethanol yield, the effect of substrate concentration on bioethanol yield, and the influence of the final fermentation time on bioethanol yield. The results indicate that increasing substrate concentration leads to higher concentrations of reducing sugars due to more substrate molecules reacting with enzymes. Higher concentrations of mineral ions K+ and Mg^{2+} during fermentation also result in increased bioethanol yield, as these ions enhance yeast cell growth and metabolism, accelerating the fermentation process. Additionally, higher substrate concentrations positively affect bioethanol yield because more reducing sugars are available for yeast cells to convert into ethanol. Longer final fermentation times also increase bioethanol yield, as they facilitate microorganism performance in converting reducing sugars into bioethanol. However, excessively prolonged fermentation times should be avoided to prevent microbial cell death due to nutrient depletion and carbon dioxide toxicity.

Keywords:- *Bioethanol, Rubber Cassava, Mineral Ions, Alternative Energy, Starch.*

I. INTRODUCTION

The use of energy sources worldwide continues to increase every year in line with society's growing energy needs. One significant example of an energy source in high demand today is petroleum-based fuel. Petroleum fuels play a crucial role in human life, serving various purposes, including transportation, industrial and household activities, among others (Said *et al.*, 2009). However, the continued consumption of petroleum fuels has led to concerns about the world's oil reserves, which are expected to run out by 2050. Additionally, extracting energy from fossil fuels leads to pollution. The combustion of fossil fuels results in emissions of SO_2, causing acid rain, and CO_2, contributing to greenhouse gas emissions (Saxena *et al.*, 2009). The development of energy sources not aligned with consumption patterns and global environmental concerns progressively motivates the search for alternative energy sources. Similar to other countries, Indonesia faces dwindling energy availability, while natural energy production is declining. One example is crude oil. According to data from the National Energy Council Secretary General's Team (2019), crude oil production from 2009 to 2018 exhibited a consistent decline, with production falling from 949 thousand bpd in 2009 to approximately 778 thousand bpd in 2018. This decline is due to aging primary oil production wells, while new well production remains relatively limited. To meet national energy needs, especially for oil, Indonesia imports approximately 35% of its crude oil from the Middle East, despite being one of the world's oil-exporting countries (Ewaldo, 2015). The negative aspects of conventional energy sources have spurred the idea of transitioning to renewable energy sources, which can address energy needs. This transition aligns with the 17 Sustainable Development Goals established by the UN, with Goal 7 specifically focused on ensuring affordable, reliable, sustainable, and modern energy access for all (Gielen *et al.*, 2019). The Indonesian government strongly supports the development of renewable energy sources (RE) as an alternative energy, particularly biofuels. Indonesia, known for its diverse plant-based oil crops due to its long history of

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agriculture, is well-suited to support biofuel development (Murtiningrum & Firdaus, 2015). Biofuels are often referred to as green energy because of their environmentally friendly origins and emissions, which do not contribute to global warming. Biodiesel and bioethanol are common biofuels in use today (Swiatek & Slawik, 2010). Currently, one dominant form of bioenergy is biofuel, specifically bioethanol, which is commonly blended with gasoline to reduce petroleum consumption. Bioethanol is a liquid produced through the fermentation of sugars and is sourced from plants rich in carbohydrates (starch). Microorganisms are used as enzymes in the fermentation process. Bioethanol is widely used as a biofuel. When compared to petroleum fuels, bioethanol offers several advantages, including a high oxygen content, high octane number, non-toxicity, and environmental friendliness due to reduced pollutant emissions such as carbon monoxide, sulfur, and nitrogen oxides (Sebayang *et al*., 2017). One of the raw materials for bioethanol production is starch-based biomass (Mahalaxmi & Williford, 2012). However, starch-based bioethanol feedstocks, such as corn, sweet potatoes, cassava, and sugarcane, have raised concerns about global food security. Therefore, it is essential to utilize non-edible starch-based biomass for bioethanol production. rubber cassava (*Manihot glaziovii*) has been identified as non-edible due to its high cyanide (HCN) content (Sebayang *et al.*, 2017). As a non-edible raw material, the potential benefits of rubber cassava have not been maximized. rubber cassava can grow in tropical and subtropical regions, typically in sandy and medium-textured soils (Moshi *et al*., 2014). Furthermore, rubber cassava can grow up to four times larger than regular cassava, making it an ideal source of biomass for bioethanol production. The utilization of rubber cassava raises no concerns about global food security as it cannot be consumed directly by humans (Hapsari & Pramashinta, 2013). The hydrolysis process breaks down carbohydrates in the raw material into glucose using enzymes. Several studies have shown that divalent mineral ions can affect enzymes by forming electrostatic interactions with charged enzyme residues (Li *et al.*, 2020). A study by Li *et al.* (2020), revealed that alkaline earth metal ions such as Ca^{2+} and Mg^{2+} can enhance enzyme activity. Transition metal ions like Cu^{2+}, Mn^{2+}, and Zn^{2+} can inhibit enzymatic hydrolysis efficiency. Additionally, various other mineral ions like K^{+}, Fe^{2+}, and Zn^{2+} can accelerate fermentation processes (Hargono *et al*., 2020). The presence of Ca^{2+} ions could hinder the fermentation process for ethanol production from molasses using Saccharomyces cerevisiae. Fermentation rates decreased as the added Ca^{2+} ion concentration increased. Therefore, it is observed that different mineral ions in different substrates can yield varying effects. Consequently, further research is needed to investigate the influence of mineral ions on the hydrolysis and fermentation processes in bioethanol production. Hence, this study aims to examine the effects of adding K^{+} and Mg^{2+} mineral ions in the hydrolysis and fermentation processes of bioethanol production from rubber cassava starch.

II. THE RESEARCH METHOD

- *Determination of the Effect of Substrate Concentration on Reducing Sugar Concentration and Bioethanol Yield*

The hydrolysis process begins by adding 1000 mL of distilled water, 10, 20, 30, 40, and 50 grams of rubber cassava starch according to the variables, and 1% (v/m) of α-amylase and glucoamylase enzymes into the Erlenmeyer flask. Insert the magnetic bar into the Erlenmeyer. Hydrolysis was carried out at a temperature of 30°C with a magnetic stirrer speed of 500 rpm for 24 hours.

- *Making Starter from Rubber Cassava Starch*

The process of making a starter begins by adding 15 grams of rubber cassava starch into 300 mL of distilled water. Next, 35 mL of the rubber cassava starch solution is heated to make it sterile. Then, the solution was cooled to room temperature. Add 0.15 grams of KH_2PO_4, 0.15 grams of $MgSO_4$, and 0.15 grams of urea as nutrients. Adjust the pH of the solution to 5. After the pH is adjusted, add 0.15 grams of yeast to the solution. Stir the solution until homogeneous. Next, sprinkle 0.15 grams of dry yeast on the top of the solution. The erlenmeyer is covered with aluminum foil and provided with sufficient holes. Insert the erlenmeyer flask into the incubator shaker. The starter was made for 24 hours with an incubator shaker speed of 100 rpm.

- *Determination the Effect of Adding K^{+} and Mg^{2+} Mineral Ions in Fermentation on Bioethanol Yield*

The fermentation process begins by preparing an erlenmeyer flask as a place for the fermentation process. The results of the hydrolysis process are cooled. Next, add 35 mL of starter (yeast) to the 65 mL of hydrolysis results in the erlenmeyer flask. Add the mineral ion K^{+} from the KCH_3COO compound and Mg^{2+} from the $MgCl_2$ compound with concentrations of 0, 10, 20, and 30 ppm according to the variable, 2 mL for each mineral ion. Cover the erlenmeyer using aluminum foil. Insert the erlenmeyer flask into the incubator shaker. The fermentation process was carried out for 24 and 48 hours according to variables with an incubator shaker speed of 100 rpm. The fermentation results were filtered using Whatman CAT 40 No. filter paper. 1440-125 mm.

- *Analysis of Reducing Sugar Concentration Using the Dinitrosalicylic Acid (DNS) Method*

Determination of reducing sugar concentration was carried out using the Dinitrosalicylic Acid (DNS) method. The reagent used consisted of a solution of 1% 3,5-dinitrosalicylic acid ($C_7H_4N_2O_7$), 0.05% sodium sulfite (Na_2SO_3), 20% sodium-potassium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$), and 1% sodium hydroxide (NaOH) added at a ratio of 3 : 1 into the sample in a glass beaker. Next, shaken and incubated using boiling water for 8 minutes. Next, the sample was cooled using ice water for 5 minutes. A total of 1 mL of sample solution was dissolved in distilled water to make 10 mL. Then, measure the absorbance of the sample at a wavelength of 570 nm with a UV-Vis spectrophotometer. Pure glucose 0 to 10 grams/L was used as a standard solution.

➢ *Analysis of Bioethanol Concentration using UV-Vis Spectrophotometry*

Determination of bioethanol concentration was obtained using a UV-Vis spectrophotometer. The reagent used is dichromic acid (Jones). Dichromic acid reagent is made by weighing 7.5 grams of potassium dichromate, then dissolving it with 250 mL of 5 M H_2SO_4 in cold conditions and stirring until homogeneous. Next, 5 mL of sample was added with 2 mL of dichromate reagent and heated for 5 minutes. Then, measure the absorbance of the sample at a wavelength of 580 nm with a UV-Vis spectrophotometer. Ethanol with a content of 0.1%; 0.2%; 0.3%; 0.4%; and 0.5% is used as a standard solution.

III. RESULT AND DISCUSSION

➢ *Effect of Substrate Concentration on Reducing Sugar Concentration in the Hydrolysis Process*

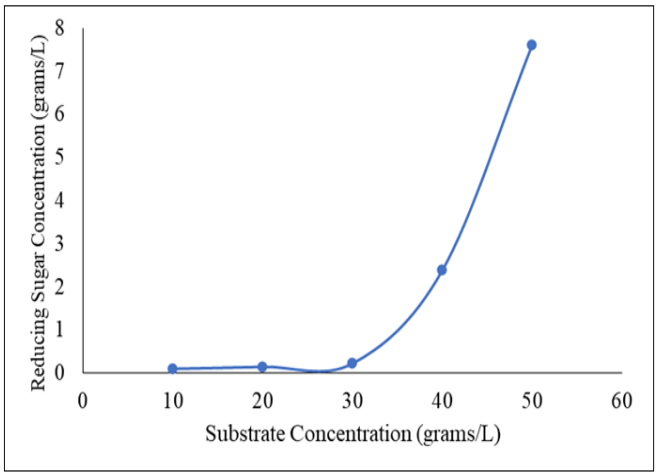

Fig 1 Effect of Substrate Concentration on Reducing Sugar Concentration in the Hydrolysis Process for 24 Hours

Based on Figure 1, the results of reducing sugar concentration for a substrate concentration of 10; 20; 30; 40; and 50 grams/L obtained respectively 0.1091; 0.1553; 0.2319; 2.3880; and 7.6042 grams/L. The relationship between substrate concentration and reducing sugar concentration obtained is that the higher the substrate concentration used, the higher or directly proportional the concentration of reducing sugar obtained. The highest concentration of reducing sugar was obtained from hydrolysis at a substrate concentration of 50 grams/L. Like research conducted by Megavitry *et al.* (2022), hydrolysis of starch by the α-amylase enzyme will produce glucose, maltose, maltotriose, and oligosaccharides which consist of 4 or more sugar residues which contain many α-1,6 glycosidic bonds. Next, the starch chains that have been hydrolyzed by the α-amylase enzyme will be further hydrolyzed into glucose by the glucoamylase enzyme. Each sugar chain that is hydrolyzed will have a simple chain of sugars called reducing sugars. Substrate concentration will affect enzyme performance and reaction rate. Increasing substrate concentration will increase the reaction rate and make enzyme performance faster. This will cause more substrate molecules to collide with enzyme molecules so that more products will be produced in the form of simple chains of glucose or reducing sugars (Megavitry *et al.*, 2022). On the other hand, a low substrate concentration will also make the enzyme work more slowly. However, when the enzyme is saturated with substrate and the substrate concentration is excessive, the enzyme's work will not increase or decrease but will remain constant (Budiyanto *et al.*, 2019). Based on Figure 1, it can be seen that the higher the substrate concentration, the higher the reducing sugar concentration obtained, where the best reducing sugar concentration is obtained with a substrate concentration of 50 grams/L. This is because the higher the substrate concentration, the more starch (polysaccharide) is available to be converted into reducing sugars (Zelvi *et al.*, 2017).

➢ *Effect of K^+ and Mg^{2+} Mineral Ion Concentrations in Fermentation on Bioethanol Yield*

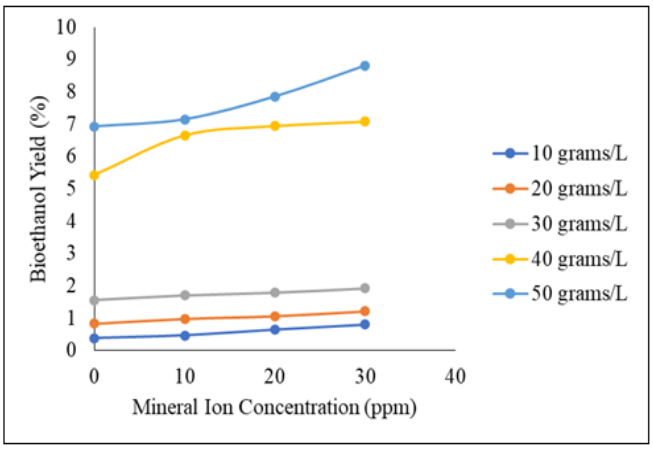

Fig 2 Effect of K^+ and Mg^{2+} Mineral Ion Concentrations in Fermentation for 48 Hours on Bioethanol Yield

Based on Figure 2, it can be seen the relationship between the concentration of K^+ and Mg^{2+} mineral ions in fermentation and bioethanol yield at various substrate concentration variables and fermentation time of 48 hours. At a substrate concentration of 10; 20; and 30 grams/L, the bioethanol yield tends to continue to increase along with the increasing concentration of K^+ and Mg^{2+} mineral ions added to the fermentation process. At a substrate concentration of 40 grams/L, the bioethanol yield will increase with the addition of K^+ and Mg^{2+} mineral ions with a concentration of 10 ppm and tends to remain constant with the addition of K^+ and Mg^{2+} mineral ions with a concentration of 20 and 30 ppm. At a substrate concentration of 50 grams/L, the bioethanol yield tends to continue to increase along with the increasing concentration of K^+ and Mg^{2+} mineral ions added during the fermentation process. The relationship between the concentration of K^+ and Mg^{2+} mineral ions in fermentation and the bioethanol yield obtained is that the higher the concentration of K^+ and Mg^{2+} mineral ions in fermentation, the higher or directly proportional the bioethanol yield obtained. The highest bioethanol yield was obtained from the addition of K^+ and Mg^{2+} mineral ions in fermentation at a concentration of 30 ppm. Several researchers have previously conducted research regarding the effect of adding mineral ions on bioethanol yield. Kounbesiou *et al.* (2010) carried out fermentation for 7 days

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using *Saccharomyces cerevisiae*by adding Mg^{2+} mineral ions at a concentration of 0; 0.1; 0.2; 0.4; 0.6; 0.8; and 1.0 gram/L. The results obtained were that the maximum bioethanol concentration was 17 grams/L when the mineral ion Mg2+ was added with a concentration of 1 gram/L. In addition, the results obtained show that bioethanol production increases by adding Mg^{2+} mineral ions. Wu *et al.* (2017) concluded that the addition of K^{+} mineral ions to the fermentation media will result in bioethanol production increasing more significantly than the addition of Mg^{2+} mineral ions to the fermentation media at the same concentration. Anggarini *et al.* (2016) conducted research by adding the mineral ions K^{+}, Mg^{2+}, and Cr^{2+} to bioethanol fermentation with *Zymomonas mobilis*. The results obtained showed that the highest bioethanol content was 2.234% in the fermentation media with the addition of K^{+} mineral ions. Fakhruddin *et al.* (2012) added K^{+}, Mg^{2+}, and Cu^{2+} mineral ions to bioethanol fermentation media with Saccharomyces cerevisiae. The results obtained were that the bioethanol fermentation media with the addition of K^{+} mineral ions produced the highest bioethanol yield of 96.38 grams/L and continued to increase over time. Magnesium and potassium ions have an important role in the growth and metabolism of yeast cells. Magnesium and potassium play a role in protecting yeast cells against environmental stress during fermentation, such as high temperatures and high osmotic pressure (Tse *et al.*, 2021). Magnesium ions have the effect of increasing the concentration of reducing sugars, but can slow down the fermentation process. Meanwhile, potassium ions have the effect of accelerating the fermentation process (Xu *et al.*, 2017). Increasing the concentration of mineral ions used will result in the growth and metabolism of yeast cells also increasing, so that the reaction rate of the bioethanol fermentation process will increase and the resulting yield will be higher. Based on Figure 2, it can be seen that the higher the concentration of K^{+} and Mg^{2+} mineral ions added, the higher the bioethanol yield obtained, where the maximum bioethanol yield is obtained with a K^{+} and Mg^{2+} mineral ion concentration of 30 ppm. This is because the higher the concentration of K^{+} and Mg^{2+} mineral ions, the growth and metabolism process of yeast cells will also increase so that they can produce high levels of bioethanol (Tse *et al.*, 2021).

➢ *Effect of Substrate Concentration on Bioethanol Yield*

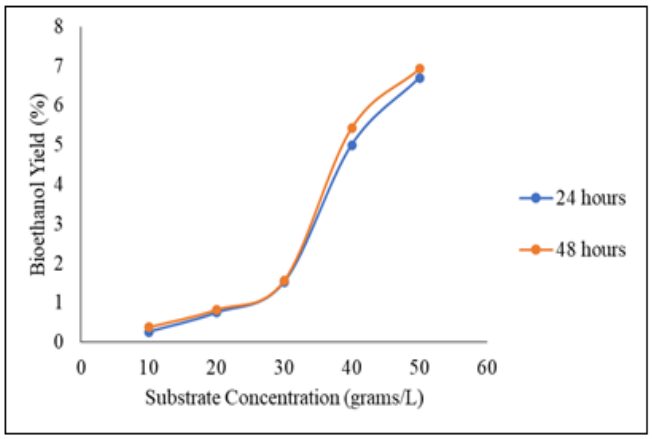

Fig 3 Effect of Substrate Concentration on Bioethanol Yield

Based on Figure 3, we can see the relationship between the concentration of toxic cassava substrate and the bioethanol yield. Substrate concentration will directly affect the results of reducing sugar into bioethanol. Each substrate concentration variable goes through a hydrolysis process for 24 hours and a stirring speed of 500 rpm, and without the addition of mineral ions in the fermentation process. Bioethanol yield obtained with a fermentation time of 24 hours and a substrate concentration of 10; 20; 30; 40; and 50 grams/L respectively are 0.2648; 0.7497; 1.5198; 5.0027; and 6.6971%. Bioethanol yield obtained with a fermentation time of 48 hours and a substrate concentration of 10; 20; 30; 40; and 50 grams/L respectively are 0.3788; 0.8258; 1.5578; 5.4351; and 6.9290%. The relationship between the substrate concentration in the hydrolysis process and the bioethanol yield obtained is that the higher the substrate concentration used in the hydrolysis process, the higher the bioethanol yield obtained or in direct proportion. The highest bioethanol yield was obtained from a substrate concentration of 50 grams/L. According to Yuda *et al.* (2018), substrate concentration affects the ethanol content and total dissolved solids because the substrate is the material that will be converted by microorganisms to become bioethanol, so the more substrate used, the higher the bioethanol content produced. By increasing the substrate concentration, the amount of cellulose that will be converted into reducing sugar also increases, so the ethanol content increases. This is in accordance with the opinion of Amalia *et al.* (2009) which states that the higher the concentration of substrate or reducing sugar that can be broken down by yeast cells into ethanol, the more ethanol will be produced. The more reducing sugars produced from a substrate that has a high concentration, the bioethanol conversion that will be obtained will have a relatively higher bioethanol yield (Wu *et al.*, 2022). Based on Figure 3, it can be seen that the higher the substrate concentration, the higher the bioethanol yield obtained, where the best bioethanol yield is obtained with a substrate concentration of 50 grams/L. This is because the higher the concentration of starch as a substrate in the hydrolysis process, the more substrate is converted into reducing sugar which is then converted into bioethanol (Hargono *et al.*, 2022).

➢ *Effect of Fermentation Time on Bioethanol Yield*

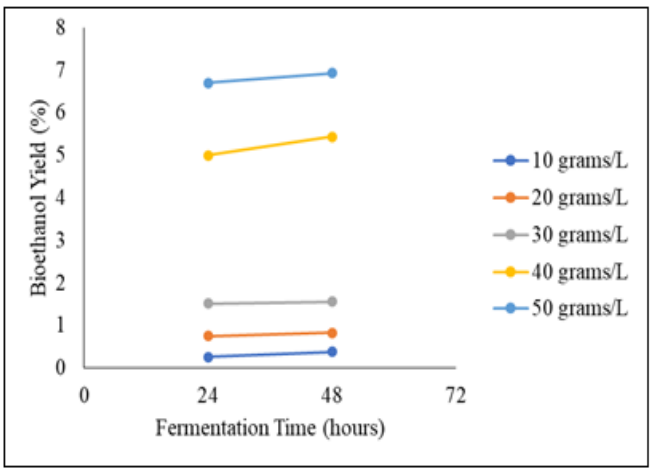

Fig 4 Effect of fermentation time on bioethanol yield

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Based on Figure 4, you can see the relationship between fermentation time and bioethanol yield. The bioethanol yield obtained with a substrate concentration of 10 grams/L and fermentation time of 24 and 48 hours respectively was 0.2648 and 0.3788%. The bioethanol yield obtained with a substrate concentration of 20 grams/L and fermentation time of 24 and 48 hours respectively was 0.7498 and 0.8258%. The bioethanol yield obtained with a substrate concentration of 30 grams/L and fermentation time of 24 and 48 hours respectively was 1.5198 and 1.5578%. The bioethanol yield obtained with a substrate concentration of 40 grams/L and fermentation time of 24 and 48 hours respectively was 5.0027 and 5.4351%. The bioethanol yield obtained with a substrate concentration of 50 grams/L and fermentation time of 24 and 48 hours respectively was 6.6972 and 6.9290%. The relationship between fermentation time and the bioethanol yield obtained is that the longer the fermentation time, the higher or directly proportional the bioethanol yield obtained. The highest bioethanol yield was obtained with a fermentation time of 48 hours. However, the efficient fermentation time is 24 hours. According to Alfonsín *et al.* (2019), the longer the fermentation time given will facilitate the performance of microorganisms and increase the success of the conversion of reducing sugar into bioethanol. However, fermentation time has an optimum time. When the optimum time has been reached, no significant addition to the bioethanol yield is obtained. Ethanol production is determined by fermentation time involving the use of a logarithmic clock, where a large number of microorganisms must be present to convert reducing sugars into ethanol. Microorganisms that increase in large numbers during the logarithmic period will increase the ability of microorganisms to use nutrients which will affect product yield. If the fermentation period is too long, ethanol production can be reduced due to microbial cell death caused by lack of nutrition and carbon dioxide poisoning which is a by-product of the anaerobic fermentation process (Hidayati *et al.*, 2022). The same thing has also been stated by Prasasti & Herdyastuti (2022), that the time in the fermentation process greatly influences the volume of bioethanol obtained and the bioethanol content. The longer the fermentation time, the more bioethanol is obtained and the higher the ethanol content obtained. However, fermentation that is too long can cause the nutrients in the substrate to run out and the *Saccharomyces cerevisiae*yeast can no longer work properly. Based on Figure 4, it can be seen that the longer the fermentation time, the higher the bioethanol yield obtained, where the best bioethanol yield was obtained with a fermentation time of 48 hours. This is because more and more reducing sugars can be converted into bioethanol over time, thereby increasing the yield and volume of bioethanol. However, if fermentation is carried out for too long it can cause the nutrients to run out so that there is no significant change in the bioethanol yield obtained.

IV. CONCLUSION

Increasing substrate concentration in the hydrolysis process can enhance the concentration of reducing sugars obtained. The higher the concentration of substrate used, the higher the concentration of reducing sugars produced. The addition of 50 grams/L of substrate resulted in the highest concentration of reducing sugars compared to other substrate concentrations. The addition of potassium (K^+) and magnesium (Mg^{2+}) mineral ions in the final fermentation process can lead to an increase in bioethanol yield. The use of a combination of potassium (K^+) and magnesium (Mg^{2+}) ions results in a higher bioethanol yield. The highest yield was obtained with a combination of potassium (K^+) and magnesium (Mg^{2+}) ions at a concentration of 30 ppm compared to other variables. Increasing substrate concentration can also boost bioethanol yield. The higher the concentration of substrate used, the higher the bioethanol yield. The addition of 50 grams/L of substrate resulted in the highest bioethanol yield compared to other substrate concentrations. A longer fermentation time in the final fermentation process can increase bioethanol yield. The longer the final fermentation time, the higher the bioethanol yield. A fermentation time of 48 hours resulted in the highest bioethanol yield compared to other fermentation times. However, a 24-hours final fermentation time is more efficient than a 48-hours final fermentation time.

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