**Immobilization of *Saccharomyces Cerevisiae* and *Zymomonas Mobilis* in a Mixture of Na-Alginate and PVA for Bioethanol Production from Stale Rice Waste**

Afan Hamzah(a), Renaldi Kusuma Nurdin, Yuniar Dwi Noryanti, Baktias Fitriani, Dika Rohman Sholeh, Jihad Akbar Hadrani, Shafa Azzahra, Eva Oktavia Ningrum and Lailatul Qomariyah

Department of Industrial Chemical Engineering, Institut Teknologi Sepuluh Nopember, Surabaya, 60111, Indonesia

*Author Emails*

*a) Corresponding author:* [*afan@its.ac.id*](mailto:afan@its.ac.id)

**Abstract.** The increasing demand for transport also increases fuel consumption, leading to scarcity. Therefore, alternative energy such as bioethanol is needed. People often leave rice waste that contains carbohydrates that have the potential to be used as bioethanol. The bioethanol production process often uses the free cell fermentation method, but it is considered less effective. The weakness of free cell fermentation can be minimised by using cell immobilisation method. Cell immobilisation using *Zymomonas Mobilis* and *Saccharomyces Cerevisiae* was conducted on Na-Alginate and PVA to obtain higher yields with faster time. This study aims to utilise stale rice waste as raw material for bioethanol production using bead gel immobilised with S. cerevisiae and Z. mobilis in Na-Alginate, and to determine the best composition for S. cerevisiae and Z. mobilis in bead gel immobilised on bioethanol yield. The research method started by analysing the carbohydrate contained in the substrate, then continued with the manufacture of solid media culture, manufacture of liquid media culture, cell immobilisation, enzyme hydrolysis, fermentation and ended with analysis. The analyses conducted in this study were SEM, FTIR, sugar reduction and GC-FID analyses.The results of SEM analysis showed that the matrix was evenly distributed on the surface of the bead gel.The results of FTIR analysis showed that the presence of amine groups in the bead gel, so that the bead gel really contains cells and can be used for fermentation.The best fermentation uses the composition of S. cerevisiae and Z. mobilis in immobilised gel bead of 1:2 in sterile filtrate sample with bioethanol yield of 0.021% and fermentation time of 68 hours.

# INTRODUCTION

The usage of energy is predominantly dominated by fossil fuels, accounting for 88.69% (Setyono dan Kiono, 2021). This dependence poses a significant problem for countries that have not yet managed to reduce their reliance on fossil fuels or petroleum. Besides the depletion of fossil fuel resources, the use of fossil fuels also leads to numerous environmental issues such as air pollution, global warming, and climate change, resulting from combustion byproducts (Bian, 2020). Therefore, it is high time for countries to seek alternative renewable energy sources to address these issues, one of which is bioethanol. This is supported by the Ministry of Energy and Mineral Resources Regulation No. 12 of 2015, which mandated the use of E5 bioethanol by 2020, though this target was not met due to high production costs (Suharyati *et al.*, 2019).

Bioethanol is a biochemical liquid produced through the fermentation of sugars from carbohydrates with the help of microorganisms. Another definition of bioethanol is an environmentally friendly biofuel containing 35% oxygen, which enables a more complete combustion process, resulting in lower hydrocarbon emissions (Mobin et al., 2021). According to the Ministry of National Development Planning (2021), approximately 41.55% of carbohydrate sources are discarded daily by the community. Carbohydrate waste constitutes the largest portion of waste, followed by leftovers and vegetables. The carbohydrate waste comes from rice, potatoes, and corn. The discarded rice is no longer fit for consumption because it has gone bad and often becomes a breeding ground for microorganisms. Spoiled rice

contains starch or complex carbohydrate compounds. Spoiled rice, also known as "nasi aking," contains 83.14% carbohydrates, 3.36% protein, and 29.70% amylose (Zenel & Stewart, 2015). This meets the substrate requirements for bioethanol production, which include raw materials containing sugars or starch (Huda, 2017).

Generally, ethanol production uses free cells, which are considered inefficient due to higher cell cycle costs, significant contamination risks, limited dilution rates, and susceptibility to varying environmental conditions. Additionally, free cell fermentation leads to substrate or product inhibition from direct contact between cells and the medium (Mohd Azhar et al., 2017). The drawbacks of free cell fermentation can be minimized by using cell immobilization methods. Cell immobilization is a method of physically confining or localizing microbial cells within a certain space where they retain catalytic activity (Mehrotra et al., 2021). Some advantages of cell immobilization over free cell fermentation include easy separation after the reaction is complete or the product is formed, easy separation of immobilized cells from the reaction medium or product, higher substrate conversion, less product inhibition, shorter reaction times, and cell reuse (Saisa & Syabriana, 2018). Moreover, cells can be continuously and repeatedly used in the same reaction or process due to the maintenance of good cell activity, as microorganisms are protected by a matrix that could otherwise damage the cells if exposed directly, thereby reducing the cost and time required to replace new cells each time the process is carried out (Mehrotra et al., 2021). Immobilized yeast cells can be reused with the highest bioethanol yield of 12% achieved on the fifth cycle (Adelabu et al., 2017).

Microorganisms are crucial in the immobilization or fermentation process due to their ability to convert organic substrates into desired products, in this case, converting spoiled rice into bioethanol. Fermentation of waste into bioethanol typically uses *Saccharomyces Cerevisiae* (Faustine et al., 2021). *Saccharomyces Cerevisiae* starts consuming the substrate at pH 4 from hour 0 and shows minimal growth on the third day, entering the lag phase. Therefore, another microorganism capable of optimal growth within a similar timeframe is needed. *Zymomonas Mobilis* experiences a lag phase on the first day and a log phase on the third day (Fatimah, Ginting and Sirait, 2017).

Z. mobilis is a microorganism resistant to high ethanol concentrations and low pH (Saisa and Syabriana, 2018). *Saccharomyces Cerevisiae* produces enzymes zymase and invertase. Invertase breaks down sucrose into monosaccharides (glucose and fructose), while zymase converts glucose into bioethanol. During fermentation, these microorganisms can die if the ethanol content in the substrate is high (Mandari, Yenie and Muria, 2014). Therefore, to maximize the conversion of all raw materials into high-concentration bioethanol in a short time, a combination of both microorganisms is required.

In performing immobilization, a carrier material is needed to extend the lifespan and enhance the survival ability of the inoculant. Commonly used materials in immobilization are Na-Alginate and PVA (Polyvinyl Alcohol). These matrices have the advantage of increasing the survival rate of microorganisms by 80-95% (Cahyono et al., 2021), producing elastic gel beads similar to rubber, strong, and non-toxic (Halim et al., 2019). Therefore, the goal of combining these two matrices is to optimize the bioethanol production process. This study co-immobilized yeast cells (S. cerevisiae and Z. mobilis) in a mixture of Na-Alginate and PVA matrices for bioethanol production from spoiled rice waste to achieve higher bioethanol yields with efficient time, energy, and costs.

# MATERIAL AND METHOD

## Materials and Variables Used

The materials include spoiled rice waste, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, Potato Dextrose Agar, Na-alginate, 70% alcohol, nutrient agar, pH 5 buffer, Fehling A, Fehling B, (NH4)2SO4, KH2PO4, CaCl2.2H2O, MgSO4.7H2O, ZnSO4.7H2O, MnSO4.H2O, FeSO4.7H2O, and distilled water. The independent variables in this research are ratio of *Saccharomyces Cerevisiae* to *Zymomonas Mobilis* with ratios of 1:0, 0:1, 1:1, 1:2, and 2:1 on bioethanol yield under sterile and non-sterile filtrates .

## Preparation of Solid Media Cultures

For Sacchromyces cerevisiae and Zymomonas mobilis, the solid media consisted of 1 gram NA; 0,1 gram glucose and the rest distilled water in 50 mL. After that, stirrer by using magnetic stirrer at 200oC with 200 rpm speed. Sterilise using an autoclave at 121oC for 15 minutes. Put the media into the test tube about 1 mL only, then tilt the tube and wait for the media to solidify. After the media becomes solid, scratch the microorganisms in a zig zag manner on the solid media on the surface. Close the test tube using cotton wool that has been coated

with sterile gauze. Wait for the microorganisms to grow at room temperature for 24 hours, then put the tube in the refrigerator at 4oC.

## Preparation of Liquid Media Cultures

Weigh 3 grams of yeast extract; 0.42 grams of ammonium sulfate, 0.6 grams of potassium hydrogen phosphate; 0.12 grams of calcium chloride, 0.09 grams of magnesium sulfate; 1.5 grams of zinc sulfate; 0.48 grams of manganese sulfate; 0.42 grams of ferrous sulfate then dissolve in 600 mL of distilled water. Stirrer using a magnetic stirrer at 200℃ with a speed of 200 rpm until the colour of the solution is clearer than before. Sterilise the media using an autoclave at 121℃ for 15 minutes. The equipment to be used is also sterilised first, such as erlenmeyer, spatula, watch glass, test tube, and ose. After releasing, put 50 mL of liquid media into an erlenmeyer then add 1 gram of stale rice sample and 1 tube of microorganisms. Close the erlenmeyer using cotton wool that has been wrapped in sterile gauze. Erlenmeyer was shaken for 48 hours at 150 rpm at 30℃. All preparation was done in sterile condition in laminair flow.

## Immobilization and Co-Immobilization

Invertase enzyme is immobilized by mixing it with a 2% (w/v) Na-alginate solution and then adding it drop by drop into a 3% (w/v) CaCl2 solution using a 50 mL syringe. Once added to the CaCl2 solution, Na-alginate beads with trapped enzyme form with an average diameter of 3-4 mm. For co-immobilization, S. cerevisiae and

Z. mobilis are mixed together and then immobilized. The formed gel beads are separated from the 3% CaCl2 solution using filter paper. The gel beads are stored in a refrigerator at 4°C.

## Fermentation

The fermentation process aims to convert glucose into bioethanol using immobilized yeast, namely S. cerevisiae and Z. mobilis, with the addition of invertase enzyme according to the specified variables. For ethanol fermentation, co-immobilized gel beads (2 strains of microorganisms) are incubated at pH 5.0, at a temperature of 35°C, and a shaker speed of 150 rpm for 72 hours. The immobilized yeast used in the fermentation process will be reused in subsequent fermentation processes for up to 5 cycles.

## Analytical Methods

To obtain the morphology of the cell gel beads, SEM (Scanning Electron Microscopy) analysis is used. According to Hikmah, Fadhillah and Putra (2019), FTIR (Fourier Transform Infrared) analysis is used to identify the functional groups of enzymes in the gel beads, which will determine the success of cell immobilization in the Na-Alginate and PVA matrix mixture. When cells are incorporated into the gel beads, an amine group (- NH2) will be detected in the gel beads (Homaei *et al.*, 2023). The amine group in the gel beads is obtained through the biological decarboxylation process of the substrate using microorganisms (Hanna Tiffany, Andi Marlisa Bossa Samang and Syahmidarni Al Islamiyah, 2023). Decarboxylation is a chemical process that causes the loss of a carboxyl group and the release of carbon dioxide. This aligns with the journal stating that a byproduct of bioethanol production is carbon dioxide (Fajar et al., 2019). The substrate used in this research includes polysaccharides containing carboxyl groups (Almeida *et al.*, 2016).

The concentration of reducing sugars in the raw material is analyzed using the Lane-Eynon titrimetric method to determine the success of the hydrolysis process. The Lane-Eynon method involves titrating the Soxhlet reagent (CuSO4 solution, K-Na-tartrate) with the sugar solution to be tested. This titration uses methylene blue as an indicator. The color change from blue to reddish indicates the presence of copper oxide precipitate (Afriza and Nilda, 2019). Reducing sugar analysis is conducted before and during fermentation at 12-hour intervals, collecting data twice over a 72-hour reaction time.

The bioethanol concentration is analyzed using Gas Chromatography (GC). This analysis is performed after fermentation to determine the best variable based on the highest yield. GC analysis requires an additional compound as an internal standard to achieve complete separation between sample peaks. Toluene is used as the compound because its molecular formula is similar to that of ethanol (Kolo, Obenu and Rohy, 2022). The GC

bioethanol analysis results will be compared to the standards set by the Director General of Oil and Gas Decree No: 23204.K/10/DJM.S/2008.

# RESULT AND DISCUSSION

## Bacterial Growth Curve

0.50

0.45

0.40

0.35

Absorbance

0.30

0.25

0.20

0.15

0.10

0.05

0.00



0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76

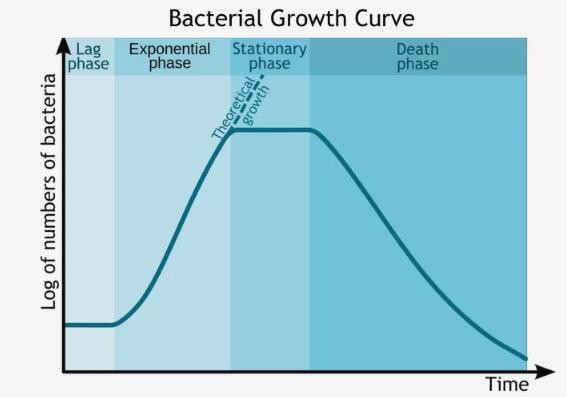
 SC  ZM

Time (h)

**FIGURE 1.** Growth Curve of S. cerevisiae and Z. mobilis

Based on **Figure 1.**, it can be seen that the bacterial growth curve obtained is very fluctuating. The bacteria did not experience optimal growth due to changes in pH during the growth process (Fajar, Yudha Perwira dan Made Ernawati, 2022). *Saccharomyces Cerevisiae* cells can grow optimally at pH 5, while *Zymomonas Mobilis* cells require a pH of

3.4 for growth.

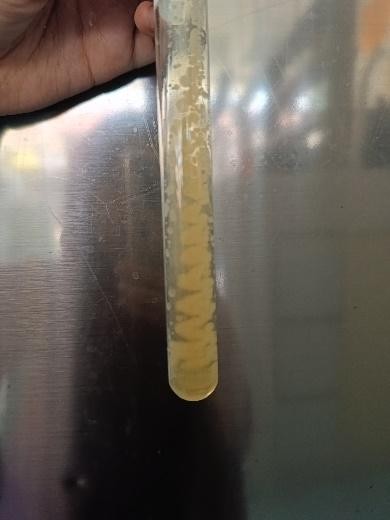
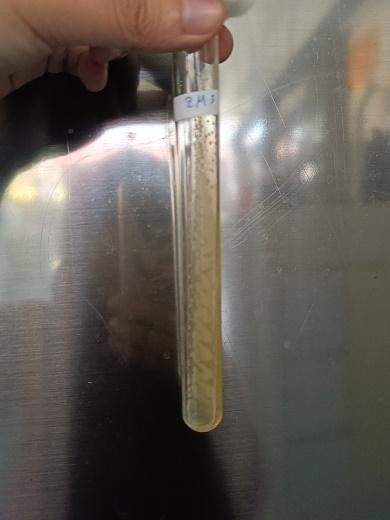


**FIGURE 2:** Theoretical Bacterial Growth Curve

The bacterial growth curve based on literature is shown in **Figure 2**. It begins with the lag phase or the adjustment phase to the environment, usually marked by no increase in cell number and a short duration, resulting in a flat curve. Next is the exponential phase, which shows an increase in the curve from the lag phase to the exponential phase as the bacteria begin to grow. The stationary phase follows, characterized by a very rapid and flat curve, which then quickly transitions to the death phase. The curve in the death phase tends to decline as the number of bacteria decreases (Respati, Yulianti dan Rahmawati, 2017)

## Bacterial Culture on Solid Media

Solid media culture immobilization is a technique used to cultivate microorganisms such as bacteria and fungi in a solid form. This medium is typically agar that contains essential elements for microorganism growth, which must meet several parameters, including an energy source, a carbon (C) source, a nitrogen source, an appropriate pH (usually neutral, but sometimes alkaline), a suitable temperature, growth factors, and the absence of contaminants in the solid culture medium (Atmanto, Asri dan Kadir, 2022).



(A) (B)

**FIGURE 3:** Solid Media Culture Containing Contaminants. (a) Solid Media Culture of Z. mobilis Cells (b) Solid Media Culture of S. cerevisiae Cells

However, based on the observation of solid media cultures shown in **Figures 3** (a) and (b), white spots are visible around the colonies, forming a zig-zag pattern. Describing bacteria and fungi begins with macroscopic morphological observation (Oratmangun, Pandiangana dan Kandou, 2017). The white spots are indicative of contamination. This contamination may occur due to non-sterile conditions of the explants or the media. It can also result from the equipment used, the environment, or insufficiently sterilized hands (Handayani *dkk.*, 2021). Subsequently, the preparation of the next solid media culture was carried out.

(A) (B)

**FIGURE 4:** Solid Media Culture (A) Solid Media Culture of Z. mobilis Cells (B) Solid Media Culture of S. cerevisiae Cells

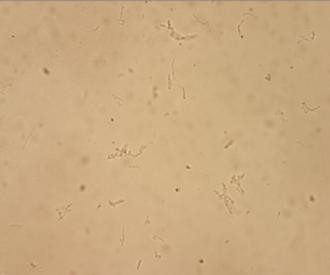
Based on the observation of the solid media cultures shown in **Figures 4** (A) and (B), white colonies in a zig-zag pattern can be seen, indicating that the solid media culture was successful. This is marked by the fact that microorganisms often show irregular growth patterns, such as zig-zags, as they move across the surface of the media during incubation (Rosmania dan Yanti, 2020).

## Bacterial Culture in Liquid Media

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**FIGURE 5:** Bacterial Culture in Liquid Media

In this liquid media culture, *Saccharomyces Cerevisiae* cells accumulate or settle at the bottom of the liquid medium, while *Zymomonas Mobilis* cells are evenly distributed throughout the liquid medium.

(A) (B)

**FIGURE 6:** (A) Observation of Z. mobilis (B) and S. cerevisiae in microscope

Based on microscopic observation of bacteria shown in **Figures 6.** (A) and (B), Z. mobilis cells are rod-shaped, whereas S. cerevisiae cells are round. From the microscopic analysis, the number of cells increased after being cultured in liquid media. Initially, Z. mobilis had 23,350,000 cfu/ml, which increased to 57,300,000 cfu/ml after growth in liquid culture media. For S. cerevisiae, the count increased from 13,100,000 cfu/ml to 32,450,000 cfu/ml. This indicates that the liquid culture media was successful, as an increase in bacterial count signifies that the culture conditions supported bacterial growth, allowing them to reproduce effectively (Nurlila dan Malik, 2024). In contrast, a failed liquid media culture is marked by the presence of mycelium and hyphae covering the media with white contaminants (Oratmangun, Pandiangana dan Kandou, 2017). These bacteria were able to grow due to the availability of sufficient energy sources, carbon sources, nitrogen sources, and appropriate pH and temperature (Atmanto, Asri dan Kadir, 2022).

## Characterization of Gel Beads

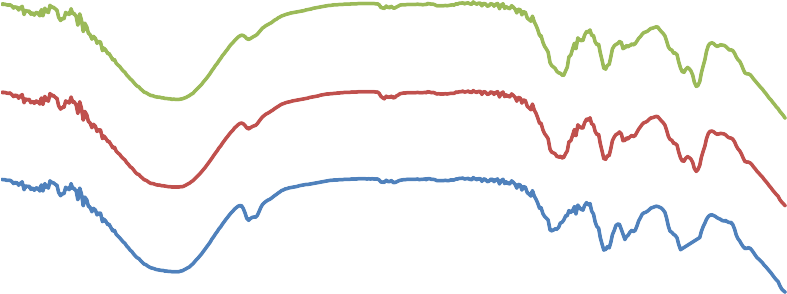
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(A)

**FIGURE 7.** SEM Micrograph (150x) of Gel Bead Interior Morphology

Figure 7 shows the results of a scan at 150x magnification. The pores in the interior of the beads are clearly visible, with round shapes indicating the presence of cells trapped within the gel bead. The pores formed on the outer surface of the beads are clearly visible. This image clearly depicts the cross-linked polymer layers formed within the beads. PVA-Na alginate demonstrates stability with evenly distributed layers. This is because higher concentrations of PVA- Na alginate result in a denser and more compact structure (Zain et al., 2021).

25000



-OH

C=O

C-O

N-H

20000

15000

10000

Tranmittance

5000

0

4000

3500

3000

2500

2000

1500

1000

500

Wavenumber (cm-1)

Empty Bead Gel Bead Gel SC Bead Gel ZM

**FIGURE 8:** FTIR Spectra of Empty Gel Bead, S. cerevisiae Gel Bead, and Z. mobilis Gel Bead

Based on the above figure, it can be seen that there is a spectral change at 1341.84 cm-1 in the gel beads containing

S. cerevisiae and Z. mobilis cells, indicating the presence of amine groups. Amine groups are functional groups of proteins (Magalhães *dkk.*, 2018). Amines are within the spectral range of 1090-1020 cm-1 (Nandiyanto, Oktiani dan Ragadhita, 2019). PVA contains hydroxyl (OH) and carbonyl (C-O) groups (Harsojuwono *dkk.*, 2022). As seen in the figure, the SC and ZM gel beads have a peak at 1602.75 cm-1, representing carbonyl (C=O) groups, as it falls within the spectral range of 1604-1411 cm-1. The peak at 2944.59 cm-1corresponds to hydroxyl (O-H) groups, as it falls within the spectral range of 3550-3200 cm-1.

Based on its chemical structure, Na-alginate contains carboxyl (C-O) groups. The peak at 1028.74 cm-1 represents carboxyl (C-O) groups, as it falls within the spectral range of 1150-1050 cm-1. The spectra of gel beads containing cells are almost identical to those of the empty gel beads, with the exception of the additional amine peaks. This indicates the successful immobilization of cells within the PVA and Na-alginate matrix.

## The Influence of Invertase Enzyme Composition on Reducing Sugars

Before the fermentation process, hydrolysis is required to break the glycosidic bonds in starch. One method of hydrolysis involves using enzymes like alpha-amylase and glucoamylase (Olosunde, Onumadu Kelechi dan Antia, 2023). Enzymatic hydrolysis should be conducted below the gelatinization temperature because high temperatures can denature enzymes, rendering them ineffective (reference for enzyme use in hydrolysis). Conversely, lower temperatures require longer processing times (Sutikno *dkk.*, 2016)**.**

Enzymatic hydrolysis is known for its high reaction rate (Olosunde, Onumadu Kelechi dan Antia, 2023). However, it requires precise enzyme dosing. According to Ardiansyah (2018), the optimal substrate-to-water ratio for effective enzymatic hydrolysis is 1:3 (100 grams of starch / 300 ml of water) using 0.15 ml of alpha-amylase and 0.6 ml of glucoamylase. Alpha-amylase is chosen because it hydrolyzes amylose compounds in starch more rapidly than amylopectin, as glucose polymers are solely linked by glycosidic bonds. Alpha-amylase swiftly breaks these bonds in gelatinized starch (heated to 100°C). Glucoamylase, on the other hand, breaks down dextrins into glucose (Ardiansyah, Nurlansi dan Musta, 2018).

350

(0.16 :0.6

(1.5 :6)

(3:12)

300

Reducing Sugars (g/ml)

250

200

150

100

50

0

vacum (0,15 : 0,6) vacum (1,5 : 6) vacum (3 : 12)

Enzyme Ratio

**FIGURE 9.** Graph of Enzyme Composition Effect on Reducing Sugars

Based on **Figure 9**, it can be observed that enzyme variable 1 (α-amylase and glucoamylase ratio of 0.15 ml and 0.6 ml) shows a reducing sugar value of 10.990 g/ml. Enzyme variable 2 (α-amylase and glucoamylase ratio of 1.5 ml and 6 ml) exhibits a reducing sugar value of 82.760 g/ml. The highest reducing sugar value is seen in enzyme variable 3 (α-amylase and glucoamylase ratio of 3 ml and 12 ml), which shows a reducing sugar value of 331.198 g/ml. It can be concluded that increasing the volume of enzymes used results in higher levels of reducing sugars. The enzyme volume used significantly impacts enzyme concentration. Thus, increasing the concentration leads to more substrate binding with enzymes, resulting in higher product yields (Sutikno et al., 2016).

## The Influence of Yeast Composition and Fermentation Time on Reducing Sugars and Bioethanol Yield

During the fermentation process, the concentration of reducing sugars is analyzed to assess the yeast's effectiveness in converting glucose into bioethanol, using the Lane Eynon titrimetric method to determine the success of hydrolysis. Reducing sugars have the ability to reduce simple sugars into bioethanol with microbial assistance (Maryana *dkk.*, 2024). Fermentation is carried out for 72 hours, adjusted according to the growth phase of both bacteria. Observations of reducing sugars are conducted every 8 hours, specifically at 8, 16, 24, 32, 40, 48, 56, and 68 hours. Fermentation is influenced by several factors, including yeast composition. In ethanol fermentation from spoiled rice waste, S. cerevisiae and Z. mobilis bacteria are used due to the co-immobilization of more than one cell within the matrix, allowing interaction between the two cells, thereby increasing the yield due to different growth phases of the microorganisms (Ruiz-Marín *dkk.*, 2016). The yeast compositions used are S. cerevisiae: Z. mobilis in ratios of 0:1, 1:0, 1:1, 1:2, and 2:1. The data obtained is then presented to show the influence of fermentation time and yeast composition on reducing sugar concentrations.

**400**

**Reducing Sugar (g/ml)**

**350**

**300**

**250**

**200**

**150**

**100**

**50**

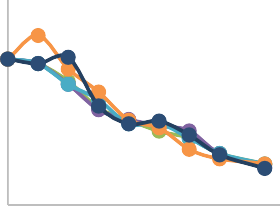
 **0:1**

 **1:0**

 **1:1**

 **1:2**

**200**

**150**

**Reducing Sugar (g/ml)**

**100**

**50**

 **0:1**

 **1:0**

 **1:1**

 **1:2**

**0**



**0 8 16 24 32 40 48 56 64 72**

**Time (hours)**

 **2:1 0**

**0 8 16 24 32 40 48 56 64 72**

**Time (hours)**

 **2:1**

(A) (B)

600.00

Reducing Sugar (g/ml)

500.00

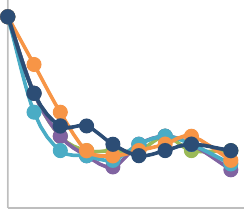
400.00

300.00

200.00

100.00

0.00



0 8 16 24 32 40 48 56 64 72

Time (hours)

 0:1

 1:0

 1:1

 1:2

 2:1

500.00

400.00

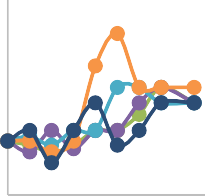
Reducing Sugar (g/ml)

300.00

200.00

100.00

0.00



0 8 1624324048566472

Time (hours)

 0:1

 1:0

 1:1

 1:2

 2:1

(C) (D)

**FIGURE 10.** Influence of Fermentation Time on Reducing Sugars in (A) Sterile Filtrate, (B) Non-Sterile Filtrate, (C) Sterile Substrate Slurry using SSF Method, (D) Non-Sterile Substrate Slurry using SSF Method

Based on Figure 10, it is evident that as fermentation time increases, the concentration of reducing sugars produced decreases. This is because the sugars present in the substrate (sterile filtrate) are converted into bioethanol, with some used as a carbon source for microbial growth (Mohd Azhar et al., 2017**)**. There is a significant reduction in reducing sugars during the 8-24 hour fermentation period, attributed to the short lag phase of both S. cerevisiae and Z. mobilis, which accelerates the growth phase. The growth phase occurs between 8-24 hours for S. cerevisiae and 2-22 hours for

Z. mobilis, resulting in a significant decrease in sugars during this range and a stationary phase thereafter (Ma’As, Ghazali dan Chieng, 2020). Figure 10 (A) shows that the average yeast composition in sterile filtrate at 48 hours experiences a decrease in reducing sugar concentration, followed by an increase at 56 hours and another decrease at 68 hours. This fluctuation is due to environmental changes such as pH and temperature during fermentation, which affect the activity of microorganisms in consuming sugars (Fajar, Yudha Perwira dan Made Ernawati, 2022). Figure 10 (B) indicates that at 68 hours, the lowest reducing sugar concentration is observed across all yeast compositions, with details for yeast compositions 0:1, 1:0, 1:1, 1:2, 2:1, and reducing sugar concentrations sequentially at 40.095 g/ml, 36.793 g/ml, 35.072 g/ml, 35.703 g/ml, and 40.169 g/ml, compared to the initial non-sterile filtrate reducing sugar concentration of 142.4 g/ml. The SSF (Simultaneous Saccharification and Fermentation) method is a method that combines hydrolysis and fermentation processes simultaneously or simultaneously so that it effectively minimises the presence of inhibitors during fermentation (Prasetyo *dkk.*, 2024). Figure 10 (C) shows that at hour 68 is the smallest reducing sugar concentration for the entire yeast composition, the following details for yeast composition 0: 1, 1: 0, 1: 1, 1: 2, 2: 1 and reducing sugar concentrations are sequentially 165.59 g/ml, 110.365 g/ml, 127.352 g/ml, 137.969 g/ml, and 165.573 g/ml with a comparison of the reducing sugar concentration of the SSF method sterile slurry sample at hour 0 of 552.03 g/ml. Figure 10(A), Figure 10 (B) and Figure 10 (C) are in accordance with the theory that the longer the fermentation time, the smaller the concentration of reducing sugar produced (Fatimah, Ginting dan Sirait, 2017). Figure 10 (D) is not in accordance with the literature, it should be that the longer the fermentation time there is a constant decrease for reducing sugar in non-sterile slurry substrates with the SSF method. The results of reducing sugar fluctuate in SSF fermentation because cell activity to ferment glucose is inhibited so that it has an impact on the increase in sugar.

Based on **Figure 10**, there are various analysis results on the concentration of reducing sugar influenced by yeast composition. Yeast composition affects the concentration of reducing sugar, because bacteria need sugar for growth, both reproducing and maintaining cell life (Rahmadani, Muria dan Utami, 2017). The longer the fermentation time, the more initial reducing sugar is utilised by microbes so that the concentration of reducing sugar will decrease. Figure 11 (A) obtained the lowest residual reduction sugar, namely the use of yeast composition 1: 2 of 110.365 g/ml at 48 hours and 106.914 g/ml at 68 hours with a comparison of the reduction sugar concentration of sterile filtrate samples at 0 hours of 331.19 g/ml. Figure 11 (B) obtained the lowest reducing sugar concentration at yeast composition 0:1, 1:0, 1:1, 1:2, 2:1 at hour 68, respectively 40.095 g/ml, 36.793 g/ml, 35.072 g/ml, 35.703 g/ml, and 40.169 g/ml with the ratio of reducing sugar concentration of non-sterile filtrate sample at hour 0 of 142.4 g/ml. Figure 11 (A) and Figure 11 (B) are in accordance with the literature that *Zymomonas Mobilis* is more effective than Sacchromyces cerevisiae in decomposing reducing sugar. Figure 11 (C) obtained the lowest concentration of reducing sugar in the composition of yeast 0: 1, 1: 0, 1: 1, 1: 2, 2: 1 at 68 hours, sequentially 165.59 g/ml, 110.36 g/ml, 127.35 g/ml, 137.96 g/ml, and 165.57 g/ml with a comparison of the reducing sugar concentration of the SSF method sterile slurry sample at 0 hours of 142.4 g/ml. Figure 11 (D) is less appropriate because, supposedly, the longer the fermentation time there

is a constant decrease for reducing sugar in non-sterile slurry substrates with the SSF method. The graph with the highest reduction sugar results indicates the composition of ineffective cell use in SSF method fermentation due to bioethanol hoarding which inhibits cell growth and can cause cell death (Wignyanto et al., 2021).

0.025

Yield Bioethanol (%)

0.02

0.015

0.01

0.005

0

0:1 1:0 1:1 1:2 2:1

S cerevisiae :Z mobilis

**Figure 11.** Effect of Yeast Composition on Bioethanol Yield on Sterile Filtrate Substrate

Based on Figure 11 shows the relationship of yeast composition with bioethanol yield against fermentation time. From the graph, it can be seen that the highest bioethanol yield is obtained from yeast composition 1:2 at fermentation time for 68 hours with a yield value of 0.021%. This is in accordance with the research (Fatimah, Ginting dan Sirait, 2017).This is in accordance with the research (Fatimah et al 2017), for bioethanol yield, the best ratio of microbial composition is the ratio of S. cerevisiae : Z. mobilis (1:2) at fermentation time of 3 days. Due to the growth phase of Z.mobilis faster than S. cerevisiae so that the addition of Z. mobilis ratio is more effective in producing yield. At the fermentation time of 68 hours there is a decrease in bioethanol yield, this is likely due to the sugar contained in the solution has largely been broken down into ethanol, so that microbial activity will be reduced due to lack of sugar as food to be broken down into bioethanol (Fitriani et al., 2021).

# CONCLUSION

Based on the research conducted, it can be concluded that stale rice waste can be used as raw material for bioethanol production. The co-immobilised bead gel of S. cerevisiae and Z. mobilis in a mixed matrix of Na-Alginate and PVA was successfully prepared as evidenced by the results of FTIR analysis which indicated the presence of N-H groups in the bead gel. The best composition of S. cerevisiae and Z. mobilis in the immobilised gel bead was 1:2 on sterile filtrate samples with a bioethanol yield of 0.021%. with a fermentation time of 68 hours**.**

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