Ethanol Fermentation from Rubber Cassava Starch (*Manihot glaziovii*) using Immobilized α-Amylase and Fermipan Yeast

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ABSTRACT

Rubber cassava (*Manihot glaziovii*) is one of the least attractive type of tubers for human consumption due to the high content of hydrogen cyanide (HCN) compounds, despite being rich in starch, proteins, and lipids. The variety can be used as an alternative raw material for ethanol production. Therefore, this research aimed to investigate the effects of immobilized enzymes and cells created using the entrapment method during starch hydrolysis and 84 hours of sugar fermentation, respectively. Starch, sugar, and ethanol contents were measured using the iodine, DNS, and Nicloux methods. The results showed that free enzymes in Treatment 1 (T1) hydrolyzed 100% of starch with a content of 6.50% in 30 minutes. At pH 7.07, immobilized cells in Treatment 2 (T2) produced 5.23% ethanol by converting 4.62% sugar over 84 hours. Immobilized enzymes were less productive in converting starch into sugar. Meanwhile, immobilized cells were more effective in using sugar from starch degradation to produce ethanol. These results reported the potential of rubber cassava as a viable source for ethanol production, emphasizing the efficiency of immobilized cells in the fermentation process.

Keywords: α-amylase; ethanol; Fermipan; immobilized; *Manihot glaziovii*

INTRODUCTION

In recent years, numerous global issues have raised concerns within the international community, particularly in Indonesia. The most pressing and widely discussed problems are the current and future energy crisis related to fossil fuels (Ghadge et al., 2020). In response, the Indonesian government and Pertamina Companies are collaborating to investigate the energy source transition into bioethanol as a sustainable energy source to replace fossil fuels (CNBC Indonesia, 2024).

Bioethanol is obtained from plant raw materials, often used as food sources, and is rich in carbohydrates (Sebayang et al., 2017). Rubber cassava (*Manihot glaziovii*) is considered a viable raw material for bioethanol production due to its relatively high carbohydrate content, primarily in the form of starch (75.38%) (Morgan & Choct, 2016). This variety is easy to cultivate in gardens and is less attractive to humans due to high (75-80%) and toxic cyanide acid compounds (Muiruri et al., 2021; Mellicha et al., 2021). Furthermore, the ethanol production process from rubber cassava is more economical compared to corn, sugarcane, and molasses because of lower carbon and water footprint, as well as water stress index (Machado et al., 2017; Pingmuanglek et al., 2017; Xie et al., 2017).

Starch hydrolysis by the α-amylase enzyme from bacteria (*Bacillus licheniformis*) was selected due to

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the thermostability, environmental non-toxicity, and catalytic efficiency in rapidly binding the starch substrate at its active site (Cruz-Casas et al., 2021). The next step is sugar fermentation after the enzymes hydrolyze the starch. Fermentation using commercial instant yeast Fermipan, which consists of *Saccharomyces cerevisiae* and the emulsifier sorbitan monostearate (E491), works more efficiently and reacts faster when dissolved in a medium containing essential nutrients for growth (Parapouli et al., 2020).

Immobilization is a method for trapping cells or enzymes in a gel matrix. For example, immobilized enzymes and cells can be reused and easily separated from the reaction mixture between substrate and product. Immobilization methods for enzymes and cells have been widely used during substrate hydrolysis and sugar fermentation to increase ethanol yield, reduce production costs, and control the hydrolysis and fermentation processes (Genisheva et al., 2012). Therefore, this study aims to investigate the ethanol production process using rubber cassava (*Manihot glaziovii*) as the raw material. The process includes starch hydrolysis by the α-amylase enzyme and fermentation using Fermipan as commercial yeast, comparing two different free and immobilized treatments.

METHODS

Materials

The materials used during laboratory testing included rubber cassava sourced from Kaloran Village, Kaloran District, Temanggung Regency, Central Java Province, Indonesia. The rubber cassava was processed into starch samples, which served as the raw material for ethanol production. Additional materials included Fermipan commercial yeast, commercial α-amylase enzyme Type XII-A from *Bacillus licheniformis* with an enzymatic activity of 12,143 U/mL, $CaCl_2$ solution, sodium alginate solution, and fermentation medium.

The laboratory equipment included a UV-Vis spectrophotometer (Thermo Scientific Genesys 10S Series, Madison, USA), water bath (Memmert, Southern Germany), vortex (Gemmy VM-300, Taipei, Taiwan), thermometer, hot plate (Maspion, Indonesia) with magnetic stirrer, autoclave (Hirayama Model HL 36 Ae, Japan), oven (Memmert, Southern Germany), pH meter (Ohaus ST3100-F Bench, New Jersey, USA), centrifuge (Ohaus FC5706, New Jersey, USA), and 500 mL Erlenmeyer fermentation reactors.

Experimental Design

This research was conducted experimentally to investigate the relationship between starch hydrolysis and sugar fermentation using free and immobilized treatments, with two repetitions and four replications. Data collection during hydrolysis comprised testing starch content (%) and reducing sugar concentration (%) at three observation times (0, 15, and 30 minutes). Table 1 shows the experimental design for the two types of treatment during starch hydrolysis.

Table 1. Treatment codes during *Manihot glaziovii* starch hydrolysis and sugar fermentation in each combination of two types of treatments for the parameters of starch content and reducing sugar concentration (%), as well as residual reducing sugar and ethanol concentration (%).

Treatment codes	Variations	Description
T1	FE1	Free Enzymes 1
	FC1	Free Cells 1
T2	FE2	Free Enzymes 2
	IC1	Immobilized Cells 1
T3	IE1	Immobilized Enzymes 1
	FC2	Free Cells 2
T4	IE2	Immobilized Enzymes 2
	IC2	Immobilized Cells 2

Starch (*Manihot glaziovii*) Extraction

Approximately 10 kg of rubber cassava was peeled and washed to remove any remaining dirt. The cassava flesh was grated until smooth, forming a pulp. To extract the starch, water was added to the cassava pulp, which was squeezed to release the juice. The starch suspension was left to settle for 12 hours until a thick paste formed. This paste was sun-dried to obtain crude starch flour (Mustafa, 2015). The crude starch flour was refined by sieving through a 200-mesh screen.

Production of Immobilized Enzymes

A commercial α-amylase enzyme, diluted 10,000-fold to a concentration of 1.2143 U/mL, was mixed with a 3% sodium alginate solution as gelling agent. This mixture was dripped slowly into a 1M $CaCl_2$ solution to form beads, which were allowed to harden for one day (Talekar & Chavare, 2012; Zusfahair et al., 2020).

Production of Immobilized Cells

A solution of Fermipan (Sangra Ratu Boga, Indonesia) commercial yeast was mixed with a 3% sodium alginate solution and dripped slowly into a 1M $CaCl_2$ to form beads. These beads were also allowed

to harden for one day (Duarte et al., 2013, with modifications; Hussain et al., 2017, with modifications).

Starch Hydrolysis

Cassava starch, weighing 2.70 g and at a concentration of 1% (w/v), was mixed with a buffer solution and heated to approximately 90°C for 10 minutes with occasional stirring until fully gelatinized (Souto et al., 2016, with modifications). Subsequently, the gelatinized starch solution was treated with α-amylase enzyme at 1.2143 U/mL and heated at 80°C for 30 minutes with manual homogenization. The enzyme activity was stopped by heating the solution to 100 °C for 5 minutes (Zusfahair et al., 2020, with modifications; Sumardiono et al., 2018, with modifications). For the immobilized enzyme treatment, the hardened beads were added to the gelatinized starch solution.

Determination of Substrate Reduction Rate and Product Formation

The chemical rate equations for substrate reduction and product formation are based on the principle of reaction rate expressed as the concentration of substrate (ΔS) consumed or product (ΔP) formed per unit time. Generally, the reaction rate decreases over time as the substrate concentration decreases. The models for the equations of substrate reduction rate, product formation rate, and product yield per substrate, according to Soustelle (2011), are presented in Equations (1), (2), and (3).

$$\text{Substrate reduction rate } (\Delta S) = -\frac{\Delta[S]}{\Delta T} = \frac{S_0 - S_t}{T_t - T_0} \quad (1)$$

$$\text{Product formation rate } (\Delta P) = \frac{\Delta[P]}{\Delta T} = \frac{P_t - P_0}{T_t - T_0} \quad (2)$$

$$\text{Product yield per substrate } (Yp/s) = \frac{\Delta[P]}{\Delta[S]} = \frac{P_t - P_0}{S_0 - S_t} \quad (3)$$

Where Δ[S] (%/min or %/hrs) represents the total final starch content remaining during the time interval between St and S0, St is the starch content after a certain time interval (%), S_0 is the initial starch content before a certain time interval (%), ΔT (min or hrs) is the total final time minus the initial time after a certain time interval (min or hrs), T_t is the final time after a certain time interval (min or hrs), T_0 is the initial time after a certain time interval (min or hrs), Δ[P] (%/min or %/hrs) represents the total product yield during the time interval between P_t and P_0, P_t is the product yield after the time interval (%), and P_0 is the product yield before the time interval (%).

Analysis of Starch Content using the Iodine Method

Starch content was tested by taking samples every 15 minutes as a stock solution (0, 15, and 30 minutes). The content was calculated by adding 1, 0.5, and 8.5 mL of sample, iodine reagent, and distilled water into a test tube. The mixture was measured at a wavelength of 630 nm, and the absorbance was recorded using a UV-Vis spectrophotometer (Subroto et al., 2020, with modifications). The percentage of starch content (%) was obtained based on the starch standard curve with a linear regression equation in the form of $y = 1.0255x + 0.0218$ and $R^2 = 0.9955$, where y is the absorbance value and x is unknown, then multiplied by the dilution factor.

Analysis of Reducing Sugar Concentration using the DNS Method

Reducing sugar parameters were tested with samples taken as stock solutions every 15 minutes (0, 15, and 30 minutes), respectively two repetitions. The analysis was performed by adding 2.5 mL of the sample and 1 mL of 3,5-Dinitrosalicylic acid (DNSA) reagent, heating the mixture in boiling water for 15 minutes. The absorbance was measured at a wavelength of 540 nm using a UV-Vis spectrophotometer (Pele et al., 2018, with modifications). The percentage of reducing sugar concentration (%) was obtained using glucose as standard solution to make standard curve with a linear regression equation in the form of $y = 48.5x - 0.0617$ and $R^2 = 0.9658$, where y is the absorbance value and x is unknown, then multiplied by the dilution factor.

Yeast Culture Medium Preparation

The fermentation starter was prepared as 10% of the total volume of the medium by inoculating commercial yeast Fermipan (*Saccharomyces cerevisiae*) into 25 mL of sterile peptone, glucose, and yeast extract (PGY) medium and incubating at room temperature for 24 hours. The PGY medium was prepared by adding 0.5 g yeast extract, 1 g peptone, and 1.1 g glucose monohydrate.

Fermentation Process

Fermentation is carried out anaerobically to ensure that the reducing sugar in the fermentation medium is converted to ethanol rather than being used for yeast growth. The fermentation medium was prepared by adding 0.3375 g yeast extract, 0.5625 g NH_4Cl, 1.2376 g K_2HPO_4, 0.0562 g $MgSO_4 \cdot 7H_2O$, 0.0022 g $CaCl_2$, and 0.2250 g NaCl. The solvent used was a sugar source from starch hydrolysis. The pH

was adjusted to the optimal pH of *Saccharomyces cerevisiae*, which was 5.5, and briefly pasteurized for 30 minutes at 65 °C. The process was carried out using the Separate Hydrolysis and Fermentation (SHF) method over 84 hours (0, 12, 24, 36, 48, 60, 72, and 84 hours). Furthermore, 25 mL of Fermipan starter was aseptically added to 225 mL of sterile fermentation medium. For the immobilized cells treatment, the hardened Ca-alginate matrix was added to the solution. The Erlenmeyer flask was closed using cotton, fitted with a hose to release CO_2 gas, and incubated at room temperature. Measurements of pH and residual sugar were observed every 12 hours.

Analysis of Ethanol Concentration using the Nicloux Method

Ethanol concentration during fermentation was determined by taking samples every 12 hours (0, 12, 24, 36, 48, 60, 72, and 84 hours). The analysis was performed by taking 0.1 mL of the sample, adding 1 mL of dichromate reagent, and incubating at 60 °C for 30 minutes. The solution was allowed to cool, and 3.9 mL of distilled water was added. The absorbance value was observed at a wavelength of 578 nm using a UV-Vis spectrophotometer (Tupe et al., 2018, with modifications; Sumbhate et al., 2012, with modifications). The percentage of ethanol concentration (%) was obtained using absolute ethanol as standard solution to make standard curve with a linear regression equation in the form of $y = 0.0812x - 0.0014$ and $R^2 = 0.999$, where y is the absorbance value and x is unknown, then multiplied by the dilution factor.

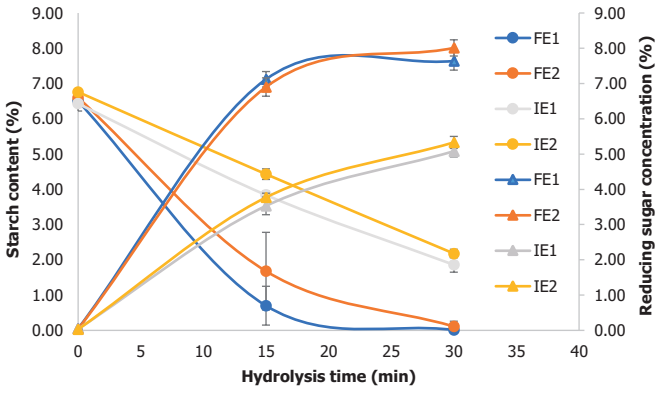

Figure 1. Starch hydrolysis by α-amylase enzymes into sugar: Free Enzyme (FE); Immobilized Enzymes (IE); starch content (rounds); reducing sugar concentration (triangles). Notes: the standard error or deviation of starch content FE2 results at 15 minutes were quite high.

RESULTS AND DISCUSSION

Hydrolysis of Starch by Free and Immobilized α-amylase Enzymes

Rubber cassava starch (*Manihot glaziovii*) was hydrolyzed using free and immobilized α-amylase enzymes through gelatinization and liquefaction. According to Waterschoot et al. (2014), starch solubility shows the amount of amylose and amylopectin released from starch granules when heated at high temperatures during gelatinization. The 80°C temperature ensured full gelatinization and dispersion of starch granules. The effectiveness of the gelatinization process, influenced by the starch substrate concentration, is crucial for the success of the subsequent liquefaction stage (Li et al., 2015). The 1% (w/v) concentration of starch substrate is beneficial because enzymes cannot break down the structure at high substrate due to the re-formation of intermolecular bonds (Ratnayake & Jackson, 2007).

The starch content of each cassava variety can vary, with most types having less amylose than amylopectin, included rubber cassava, and the extraction process was performed manually, possibly leaving some impurities that can inhibit the starch hydrolysis process and cause contamination, thus becoming a limitation in this study. The starch content and reducing sugar concentration were calculated (%), as shown in Figure 1.

Figure 1 shows that free enzymes (FE) in the first treatment (T1) degraded 100% of the starch in 30 minutes, leaving the remaining content of 0.01%. However, the immobilized enzyme treatment only degraded 4.57% (T3) and 4.58% starch (T4). The hydrolysis by immobilized enzymes is slower because the effect requires the starch substrate to move from the solution in the liquid phase to the outer surface of the matrix and into the interior of the calcium chloride matrix in the solid phase (Talekar & Chavare, 2012).

The movement of immobilized enzymes includes diffusion and mass transfer between particles, affecting the rate of starch hydrolysis. However, the stability during liquefaction was showed by the consistent decrease in starch amylose and the hardness of the beads, which did not break even at 80°C. The free enzyme treatments (T1 and T2) showed good results, with maximal starch degradation within 30 minutes, leaving 0.01% (T1) and 0.11% (T2) remaining.

The next step in starch hydrolysis is reducing sugar formation. The amount formed depends on several factors, including α-amylase enzyme concentration, starch substrate concentration, and hydrolysis time (Pele et al., 2018; William et al., 2023).

Based on Figure 1, there is a clear increasing trend in the amount of sugar products produced every 15

minutes for free and immobilized enzymes. The reducing sugar concentration at 0 minutes averaged 0.04%. The free enzyme treatments (T1 and T2) produced 7.59% and 7.97% reducing sugar after 30 minutes, with a final total of 7.64% and 8.01%, respectively. The increase in sugar product at 15 minutes reports that the enzymes had started to work optimally. The rate of sugar product formation slowed by 30 minutes because the starch had been largely converted by the enzymes, causing the activity to decrease. This observation is consistent with William et al. (2023), which compares enzyme performance in producing sugar products based on factors such as pH. Liquefaction temperature also influences the amount of sugar produced. According to William et al. (2023), increasing temperature during hydrolysis can decrease reducing sugar amounts, showing that extreme acidic and alkaline conditions can slow enzyme performance. These results are contrary to the performance of the thermostable enzyme, which works optimally at temperatures up to 90°C.

The immobilized enzyme treatments (T3 and T4) produced only 5.04% and 5.30% reducing sugar after 30 minutes. The lower sugar yield in the treatments may be due to incomplete starch hydrolysis. The 1% starch substrate concentration does not significantly affect reducing sugar formation. This is because higher substrate concentrations yield more sugar (Subroto et al., 2020). The significant difference in reducing sugar concentration between treatments is due to the immobilized effect, where enzymes require 1-2 hours for complete hydrolysis. Figure 1 shows that the free enzyme treatments produced 7.59% (T1) and 7.97% (T2) reducing sugar after 30 minutes, with a final total of 7.64% (T1) and 8.01% (T2).

The ability of α-amylase enzymes to degrade starch and form sugar products can be evaluated through substrate reduction rate, product formation rate, and product yield per substrate, expressed as ΔS, ΔP, and Yp/s, respectively. Tables 2 to 5 present data analysis for these parameters in the four treatments (FE1, FE2, IE1, and IE2).

From Tables 2 to 5, the substrate reduction rate (ΔS) and product formation in the free enzyme treatment (T1) are faster, degrading starch to a maximum of 0.39%/min in 15 minutes, with a product formation rate (ΔP) of 0.47%/min since 89.30% was degraded. In contrast, the immobilized enzyme treatments (T3 and T4) had substrate reduction rates of 0.17%/min and 0.16%/min in 15 minutes, resulting in 40.28% (T3) and 34.47% (T4) of starch being converted into 3.53% (T3) and 3.77% (T4) of reducing sugar.

Although immobilized enzymes were less effective in degrading starch, the Yp/s value in T4 was 1.60 at 15 minutes, suggesting that immobilized enzymes can still be efficient in converting starch to sugars. However, since the starch consumption rate was slower, some starch remained unconverted even at 30 minutes. The slower rate of starch consumption and

Table 2. Data analysis of starch hydrolysis in the first treatment (FE1).

Time (min)	SC (%)	SD of SC (Mean)	RSC (%)	SD of RSC (Mean)	ΔS (%/min)	ΔP (%/min)	Yp/s
0	6.50	0.28	0.05	0.01	0.00	0.00	0.00
15	0.70	0.55	7.12	0.22	0.39	0.47	1.21
30	0.01	0.01	7.64	0.26	0.22	0.25	1.16

Abbreviations: SC, starch content; SD, standard deviation; RSC, reducing sugar concentration; ΔS, substrate reduction rate; ΔP, product formation rate; Yp/s, product yield per substrate.

Table 3. Data analysis of starch hydrolysis in the second treatment (FE2).

Time (min)	SC (%)	SD of SC (Mean)	RSC (%)	SD of RSC (Mean)	ΔS (%/min)	ΔP (%/min)	Yp/s
0	6.60	0.06	0.04	0.00	0.00	0.00	0.00
15	1.68	1.10	6.90	0.26	0.33	0.46	1.39
30	0.11	0.15	8.01	0.23	0.22	0.27	1.22

Abbreviations: SC, starch content; SD, standard deviation; RSC, reducing sugar concentration; ΔS, substrate reduction rate; ΔP, product formation rate; Yp/s, product yield per substrate.

Table 4. Data analysis of starch hydrolysis in the third treatment (IE1).

Time (min)	SC (%)	SD of SC (Mean)	RSC (%)	SD of RSC (Mean)	ΔS (%/ min)	ΔP (%/min)	Yp/s
0	6.43	0.04	0.04	0.00	0.00	0.00	0.00
15	3.84	0.10	3.53	0.25	0.17	0.23	1.34
30	1.86	0.21	5.08	0.16	0.15	0.17	1.10

Abbreviations: SC, starch content; SD, standard deviation; RSC, reducing sugar concentration; ΔS, substrate reduction rate; ΔP, product formation rate; Yp/s, product yield per substrate.

Table 5. Data analysis of starch hydrolysis in the fourth treatment (IE2).

Time (min)	SC (%)	SD of SC (Mean)	RSC (%)	SD of RSC (Mean)	ΔS (%/ min)	ΔP (%/ min)	Yp/s
0	6.76	0.04	0.03	0.00	0.00	0.00	0.00
15	4.43	0.15	3.77	0.12	0.16	0.25	1.60
30	2.18	0.12	5.33	0.17	0.15	0.18	1.15

Abbreviations: SC, starch content; SD, standard deviation; RSC, reducing sugar concentration; ΔS, substrate reduction rate; ΔP, product formation rate; Yp/s, product yield per substrate.

some starch remaining unconverted at 30 minutes in immobilized enzymes (T3 and T4) are thought due to diffusion limitations, steric effects, substrate affinity for the enzymes, and different ionic strengths. Santos et al. (2015) explained that the reduced activity of immobilized enzymes due to the substrate diffusion rate being lower than the substrate consumption rate by the enzymes. Moreover, the efficiency of immobilized enzymes is influenced by the affinity of the substrate to the immobilized enzymes being lower than that of the free enzymes (Bayramoğlu et al., 2002). Besides that, Cao (2005) also emphasized that changes in enzymes affinity for substrates are also caused by changes in the enzymes structure because of enzymes immobilization effect. Arica et al. (1995) also explained that the high ionic strength that causes proteins as enzyme components to only be immobilized if the protein area is simultaneously able to absorb at strong multipoint, so that it can affect the polymeric bed structure. Furthermore, if the ionic strength is low, then the protein area that has a negative charge allows for absorption and cross-linking between the enzymes and calcium alginate as a polymer.

These findings indicate that free enzymes (T1 and T2) catalyze starch degradation more rapidly, consuming most of the starch within 30 minutes, whereas immobilized enzymes work more slowly but efficiently over time. Immobilized enzymes (T3 and T4) still leaving starch after 30 minutes which is indicated by the reduction of substrate and the formation of much less product. However, it is also possible that the efficient level of immobilized enzymes will have an impact on the amount of sugar product that is greater if the hydrolysis time is more than 30 minutes.

Fermentation of Sugar to Ethanol by Free and Immobilized Cells

After forming reducing sugar, the next stage includes converting the sugar to ethanol using Fermipan yeast. The relationship between reducing sugar conversion to ethanol is based on three test parameters, residual reducing sugar, ethanol concentration (Figure 2), and fermentation pH (Figure 3).

Based on Figure 2, the free cell treatment consumed 6.23% (T1) and 3.78% (T3) of sugar over 84 hours. In contrast, the immobilized cells consumed 4.62% (T2) and 3.03% (T4) of sugar. This difference is because the optimal pH for yeast is 5.5, and the fermentation temperature supports the Fermipan yeast environment. Additionally, instant yeast does not require a long time to grow, and the available nutrients are used to maintain survival. This is supported by research conducted by Da Silva Fernandes et al. (2022), where nutrients in the fermentation medium are stored as food reserves for yeast.

Ethanol concentration is a key parameter for assessing the success of the fermentation process. The amount produced can be determined from the residual reducing sugar concentration and the performance of

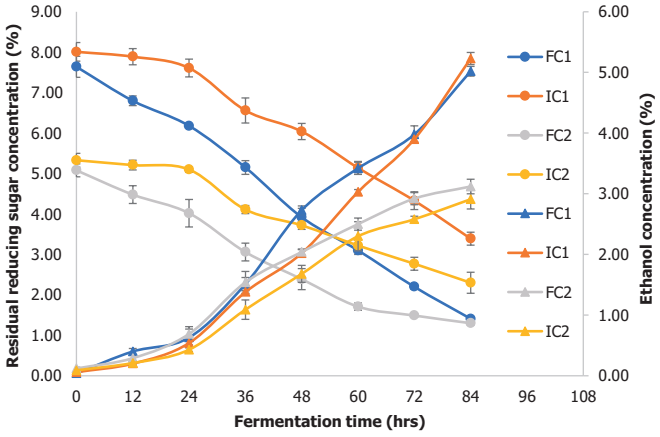

Figure 2. Sugar fermentation by yeast cells into ethanol over 84 hours: Free Cells (FC); Immobilized Cells (IC); residual sugar content (rounds); ethanol content (triangles).

immobilized or non-immobilized yeast cells. However, high concentrations can be toxic to yeast cells. The yeast cells will experience death and stress when the ethanol level increases (Da Silva Fernandes et al., 2022). Based on Figure 2, the concentration at 0 hours ranges from 0.04% to 0.12%. The immobilized cell treatment (T2) produced 5.17% ethanol after 84 hours, with a total concentration of 5.23%.

The immobilized cell treatment (T4) produced 2.82% ethanol after 84 hours, with a final concentration of 2.91%. Even though both treatments include immobilized cells, the difference is in the starch hydrolysis stage, where T4 has less sugar than T2. The immobilization effect is the main cause of the difference in ethanol concentration between the free and immobilized cell treatments, as well as the impact of the sugar conditions formed during hydrolysis. Other influencing factors include alginate concentration and the storage duration of Ca-alginate beads. In this research, a 3% (w/v) alginate concentration was used to increase immobilization yield. Talekar and Chavare (2012) found that a 3% (w/v) alginate concentration reduced matrix porosity and leakage of immobilized cells. Similarly, storing Ca-alginate beads for one day helps reduce cell leakage since shorter storage times lead to softer, easily broken beads.

The positive impact of cell immobilization in treatment T2 on increasing ethanol concentration is due to the better ability to convert reducing sugar into ethanol. The extended fermentation time also enhances the capacity, but the immobilized cells in T2 may experience limited substrate diffusion. In contrast, treatment T4 produced the least ethanol compared to the others using immobilized cells. This is consistent with research by Nikolić et al. (2010), where lower ethanol production by immobilized cells is caused by low diffusion rates of substrate and fermentation medium, as well as product inhibition.

The rate of sugar substrate reduction and ethanol product formation, with product yield per substrate during fermentation, shows that Fermipan yeast cells can effectively use sugar to produce ethanol. Tables 6 to 9 present data analysis of the rate of sugar substrate reduction (ΔS), ethanol product formation (ΔP), and product yield per substrate (Yp/s) for each treatment during 84 hours of sugar fermentation.

From Tables 6 to 9, after 84 hours of fermentation, the immobilized cells treatment (T2) had a sugar substrate reduction rate (ΔS) and product formation rate

Table 6. Data analysis of sugar fermentation in the first treatment (FC1).

Time (hrs)	RRSC (%)	SD of RRSC (Mean)	EC (%)	SD of EC (Mean)	ΔS (%/hrs)	ΔP (%/hrs)	Yp/s	pH
0	7.64	0.26	0.04	0.02	0.00	0.00	0.00	5.53
12	6.80	0.12	0.40	0.05	0.07	0.03	0.43	5.47
24	6.18	0.03	0.63	0.14	0.06	0.02	0.40	5.44
36	5.15	0.17	1.51	0.11	0.07	0.04	0.59	5.39
48	3.92	0.22	2.73	0.07	0.08	0.06	0.72	5.31
60	3.10	0.10	3.42	0.10	0.08	0.06	0.74	5.26
72	2.20	0.06	3.98	0.14	0.08	0.05	0.72	5.21
84	1.41	0.04	5.02	0.08	0.07	0.06	0.80	5.12

Abbreviations: RRSC, residual reducing sugar concentration; SD, standard deviation; EC, ethanol concentration; ΔS, substrate reduction rate; ΔP, product formation rate; Yp/s, product yield per substrate.

Table 7. Data analysis of sugar fermentation in the second treatment (IC1).

Time (hrs)	RRSC (%)	SD of RRSC (Mean)	EC (%)	SD of EC (Mean)	ΔS (%/hrs)	ΔP (%/hrs)	Yp/s	pH
0	8.01	0.23	0.06	0.03	0.00	0.00	0.00	5.53
12	7.89	0.20	0.20	0.04	0.01	0.01	1.16	7.47
24	7.61	0.22	0.54	0.03	0.02	0.02	1.20	7.37
36	6.56	0.31	1.38	0.03	0.04	0.04	0.91	7.34
48	6.04	0.20	2.02	0.06	0.04	0.04	0.99	7.30
60	5.14	0.16	3.03	0.04	0.05	0.05	1.03	7.15
72	4.33	0.22	3.90	0.04	0.05	0.05	1.04	7.13
84	3.39	0.16	5.23	0.10	0.06	0.06	1.12	7.07

Abbreviations: RRSC, residual reducing sugar concentration; SD, standard deviation; EC, ethanol concentration; ΔS, substrate reduction rate; ΔP, product formation rate; Yp/s, product yield per substrate.

Table 8. Data analysis of sugar fermentation in the third treatment (FC2).

Time (hrs)	RRSC (%)	SD of RRSC (Mean)	EC (%)	SD of EC (Mean)	ΔS (%/hrs)	ΔP (%/hrs)	Yp/s	pH
0	5.08	0.16	0.12	0.02	0.00	0.00	0.00	5.53
12	4.48	0.22	0.29	0.05	0.05	0.01	0.28	7.31
24	4.02	0.34	0.69	0.12	0.04	0.02	0.54	7.32
36	3.06	0.22	1.54	0.18	0.06	0.04	0.70	7.31
48	2.39	0.26	2.04	0.05	0.06	0.04	0.71	7.27
60	1.71	0.09	2.50	0.10	0.06	0.04	0.70	7.18
72	1.49	0.04	2.92	0.10	0.05	0.04	0.78	7.09
84	1.30	0.04	3.12	0.12	0.05	0.04	0.79	6.93

Abbreviations: RRSC, residual reducing sugar concentration; SD, standard deviation; EC, ethanol concentration; ΔS, substrate reduction rate; ΔP, product formation rate; Yp/s, product yield per substrate.

(ΔP) of 0.06%/hr. This treatment converted 57.68% of reducing sugar into 5.23% ethanol, with a product yield per substrate (Yp/s) of 1.12. The results showed that the biomass of Fermipan yeast cells grew and entered the exponential phase, leading to increased cell division and activity, as well as producing more ethanol. The immobilized cells in the second treatment (T2) gave the best results, proving productive in producing higher ethanol levels.

Figure 2 shows that the ethanol concentration in all treatments increased slowly until reaching a maximum at 84 hours. However, the concentration could continue to increase when fermentation time was extended. Free and immobilized cell treatments reported a trend of decreasing residual reducing sugar concentration every 12 hours. T2 and T4 had residual reducing sugar concentrations that did not decrease with free cell treatments. The highest concentration was in the immobilized cell treatment (T2) at 84 hours, with a total of 3.39%.

This situation is due to the immobilization effect, which limits the diffusion of sugar substrates and nutrients into the immobilized cell particle layer. Therefore, ethanol concentration is influenced by the structure of the Ca-alginate matrix and the hydrodynamics of the fermentation medium, which affect sugar consumption and transfer (Hussain et al., 2017; Galaction et al., 2012). The immobilized treatment (T2) at 84 hours had

Table 9. Data analysis of sugar fermentation in the fourth treatment (IC2).

Time (hrs)	RRSC (%)	SD of RRSC (Mean)	EC (%)	SD of EC (Mean)	ΔS (%/hrs)	ΔP (%/hrs)	Yp/s	pH
0	5.33	0.17	0.09	0.03	0.00	0.00	0.00	5.57
12	5.21	0.12	0.21	0.03	0.01	0.01	1.00	7.94
24	5.10	0.07	0.43	0.03	0.01	0.01	1.48	7.93
36	4.11	0.10	1.09	0.16	0.03	0.03	0.82	7.91
48	3.72	0.10	1.68	0.14	0.03	0.03	0.99	7.94
60	3.22	0.04	2.30	0.24	0.04	0.04	1.05	7.89
72	2.77	0.16	2.58	0.05	0.04	0.03	0.97	7.85
84	2.30	0.26	2.91	0.16	0.04	0.03	0.93	7.79

Abbreviations: RRSC, residual reducing sugar concentration; SD, standard deviation; EC, ethanol concentration; ΔS, substrate reduction rate; ΔP, product formation rate; Yp/s, product yield per substrate.

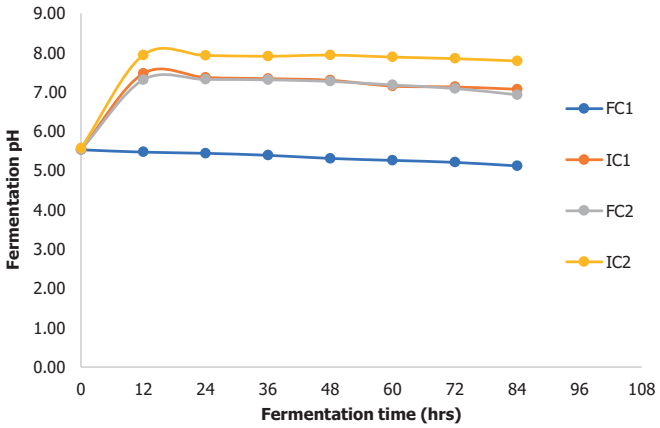

Figure 3. Fermentation pH value in four different treatments: Free Cells (FC) and Immobilized Cells (IC).

a total remaining sugar of 3.39%, with a consumption rate of 4.62% to produce 5.23% ethanol. The free cells in T1 consumed 6.23% more sugar than T2 to produce 5.02% ethanol.

The pH condition suitable for the yeast environment allows long survival and increases the capacity to convert sugar into ethanol. The pH value of fermentation depends on the conditions in the bioreactor (Hussain et al., 2017), as shown in Figure 3.

Based on Figure 3, the immobilized treatments (T2 and T4) experienced pH increases of 1.94 and 2.37, respectively. Similarly, the free cell treatment (T3) had a pH increase of 1.78. However, the free cell treatment (T1) showed a decrease in pH from 5.47 at 12 hours to 5.12 at 84 hours, showing that the Fermipan instant yeast was at the optimal point and productive in producing ethanol. This observation is consistent with Wu et al. (2022), where the optimum pH for *Saccharomyces cerevisiae* yeast is in the range of 4-6.

The free (T2) and immobilized cell treatments (T3 and T4) were more resistant to pH changes when observed from 12 to 84 hours. The most extreme pH trend was in the fourth treatment, which initially increased by 2.37 at 12 hours, then decreased by 0.15 at 84 hours. The main cause of the pH increase is the immobilization effect. Even though T2 and T3 had only one immobilized condition, the fermentation pH was affected. This situation was explained by Duarte et al. (2013), where immobilized cells trapped in the Ca-alginate matrix were subjected to an adaptation phase and may experience stress due to immobilization. The pH value of fermentation in Figure 3 shows that all treatments had a stagnant range of 5.53 starting from 0 hours. However, T2, T3, and T4 increased the pH value by 1.94, 1.78, and 2.37 at 12 hours, respectively.

CONCLUSION

Free enzymes (FE1) in the first treatment, with a concentration of 1.2143 U/mL, were able to degrade 100% of rubber cassava (*Manihot glaziovii*) starch with a content of 6.50% in 30 minutes. In contrast, the immobilized enzymes (IE1 and IE2) in the third and fourth treatments, with the same concentration, were only able to degrade 4.57-4.58% of starch in 30 minutes. The use of immobilized Fermipan yeast (IC1) with 3% alginate and free cells (FC1) used 4.62% and 6.23% sugar to produce 5.23% and 5.02% ethanol, respectively. Immobilized enzymes and cells showed good potential for reuse in starch hydrolysis

and fermentation processes. The results reported the efficiency of free enzymes in starch degradation and the potential of immobilized cells for sustainable and reusable applications in industrial processes. This research also provided valuable insights for optimizing enzyme and yeast cell use in biotechnological applications.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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