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Simultaneous Saccharification and Fermentation of Sugarcane Bagasse by Saccharomyces Cerevisiae and Zymomonas Mobilis

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Abstract

Sugarcane bagasse represents a promising lignocellulosic feedstock for second-generation bioethanol production. This study evaluated the performance of immobilized Simultaneous Saccharification and Fermentation (SSF) systems using *Saccharomyces cerevisiae* and *Zymomonas mobilis* for ethanol production from alkali-pretreated sugarcane bagasse. Delignification using 10% NaOH enhanced cellulose accessibility for enzymatic hydrolysis by immobilized *Aspergillus niger*. SSF was conducted under anaerobic conditions at 30°C for 80 h. Reducing sugar dynamics, physicochemical properties, FTIR spectra, and GC analysis were used to evaluate ethanol formation and quality. The SSF system employing *S. cerevisiae* produced a higher ethanol concentration (2.83% v/v) and purity (99.77%) compared to *Z. mobilis* (2.20% v/v; 89.92%). Although higher residual reducing sugars were observed in the *Z. mobilis* system, ethanol conversion efficiency remained lower, indicating metabolic limitations under SSF conditions. FTIR and GC analyses confirmed ethanol formation with high water content in both distillates. These results demonstrate that microbial robustness plays a critical role in immobilized SSF performance, with *S. cerevisiae* exhibiting superior fermentative stability and ethanol yield compared to *Z. mobilis*.

Keywords: Bioethanol, Saccharomyces cerevisiae, SSF, Sugarcane bagasse, Zymomonas mobilis

INTRODUCTION

Global concerns over fossil fuel depletion and greenhouse gas–induced environmental impacts have accelerated the development of sustainable alternative energy sources. Among various alternatives, bioethanol stands out as a renewable and environmentally friendly liquid fuel. Second-generation bioethanol derived from lignocellulosic residues such as sugarcane bagasse offers several advantages, including abundance, high cellulose content, and no competition with food crops (Ayodele, Alsaffar, and Mustapa 2020; Mulyadi, Khumaisah, and Rahayu 2023).

The lignocellulosic composition of sugarcane bagasse varies depending on the source, with lignin contents ranging from 12.90% to 32.40%, hemicellulose from 22.70% to 32.00%, and cellulose from 31.88% to 45.50% (Ascencio et al. 2020; Athira, Bahurudeen, and Appari 2021; Santos et al. 2020). To improve conversion efficiency, alkaline delignification using sodium hydroxide (NaOH) has been widely employed to solubilize lignin and enhance enzymatic accessibility to the cellulose fraction (Abo et al. 2019; Bezerra, T.L., Ragauskas 2016).

Sugarcane is one of the strategic plantation commodities in Indonesia. In 2022, the total cultivated area of sugarcane reached 490,008 hectares, producing approximately 2,402,648 tons of white crystal sugar nationwide. In North Sumatra, the sugarcane cultivation area covered about 8,022 hectares, with a total sugar production of 27,645 tons. As sugar production continues to increase, the generation of sugarcane bagasse waste is also expected to rise significantly (BPS-STATISTIK INDONESIA 2023).

Microorganisms such as *Saccharomyces cerevisiae* (a yeast) and *Zymomonas mobilis* (a bacterium) have been extensively utilized in bioethanol fermentation. *S. cerevisiae* is known for its

tolerance to acidic environments and its ability to grow well under both aerobic and anaerobic conditions, which makes it widely used in industrial applications. In contrast, *Z. mobilis* has attracted increasing attention due to its high glucose uptake rate, rapid growth, and low by-product formation (Kumoro et al. 2021; Ma'As, Ghazali, and Chieng 2020).

These microorganisms utilize different metabolic pathways. *S. cerevisiae* follows the Embden-Meyerhof-Parnas (EMP) pathway, while *Z. mobilis* employs the Entner-Doudoroff (ED) pathway, resulting in distinct fermentation characteristics in terms of ATP production, stress tolerance, and final ethanol yield (Azizah, Sutamihardja, and Wijaya 2019; Febriani, Sidharta, and Pranata 2020).

Although both microorganisms have demonstrated effectiveness individually, comparative studies evaluating their fermentation efficiency on alkali-pretreated sugarcane bagasse using SSF remain limited. Therefore, this study aims to assess and compare the fermentative performance of *S. cerevisiae* and *Z. mobilis* in converting sugarcane bagasse into bioethanol, contributing to the optimization of lignocellulose-based bioethanol production.

The Simultaneous Saccharification and Fermentation (SSF) method integrates enzymatic hydrolysis and fermentation within a single bioprocess, resulting in a more efficient conversion of lignocellulosic biomass into bioethanol. This configuration reduces inhibitor formation, lowers enzyme requirements, accelerates ethanol production, and supports microbial activity on low-moisture solid substrates (50-60%) in an environmentally sustainable manner (Febriasari et al. 2021).

Despite these advantages, the performance of microorganisms in SSF can be adversely affected when cells are directly exposed to elevated ethanol concentrations, which may compromise cell wall integrity or lead to cell death. To mitigate this limitation, cell immobilization techniques have been employed, allowing controlled substrate diffusion, restricted cell proliferation and movement, and the repeated use of viable cells throughout the fermentation process (Awaltanova, E., Bahri, S., 2015).

METHODOLOGY

Materials and Instrumentals

The materials used in this study included sugarcane bagasse (locally obtained, sun-dried, and ground); *Saccharomyces cerevisiae* (baker's yeast, Fermipan®, Lesaffre, France); Nutrient Broth; Potato Dextrose Broth; *Zymomonas mobilis* strain ATCC 9029; and *Aspergillus niger* strain ATCC 2919, which was obtained from the Microbiology Laboratory, University of Sumatera Utara culture collection (ATCC, *American Type Culture Collection*). Additional chemical reagents include sodium hydroxide (NaOH, Merck, 98%; Darmstadt, Germany); sulfuric acid (H_2SO_4, Merck 98%; Darmstadt, Germany); acetic acid (CH_3COOH, Merck, 99.5%; Darmstadt, Germany); sodium acetate ($NaCH_3COO$, Merck, ≥98%; Darmstadt, Germany); sodium alginate (Sigma-Aldrich, St.Louis, MO, USA); calcium chloride ($CaCl_2$, Merck, 96%; Darmstadt, Germany); glucose, yeast extract, peptone, urea, and distilled water. All chemicals were of analytical grade.

The main equipment used in this study included a Fourier Transform Infrared (FTIR) spectrometer (Bruker Invenio-S, Karlsruhe, Germany) and a Gas Chromatograph (GC-2010 Plus, Shimadzu Corp., Kyoto, Japan) for qualitative and quantitative analysis of ethanol. FTIR spectral data were processed using Origin software (Origin Lab, Northampton, MA, USA).

Methods

Sample Preparation

The experiment was conducted at the Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan. Sugarcane bagasse was washed, dried, milled, and sieved through a 60-mesh screen. The powder was delignified using 10% NaOH solution (1:4 w/v) at 121°C for 1 hour and rinsed until reaching neutral pH. The cellulose and hemicellulose content were analyzed using the Chesson method, and lignin content was determined using the Klason method (Mulyadi et al. 2023; Narisa and Herry 2020).

Preparation of Cell Suspension

Microbial precultures were prepared as follows: *S.cerevisiae* was cultured in 100 mL of solution containing 0.5 g of peptone and 1 g of glucose; *A. niger* was cultured in 100 mL of distilled water with 2.4 g of potato dextrose broth; and *Z. mobilis* was cultured in 120 mL of distilled water containing 1.56 g of nutrient broth. The cultures were incubated at 30°C for 24 h (*Z. mobilis* and *S. cerevisiae*) and 168 h (*A. niger*). After incubation, the cultures were centrifuged at 3500 rpm for 10 min, and the pellets

were resuspended in 20 mL of distilled water (Febriani, Sidharta, and Pranata 2020; Kumoro et al. 2021; Larasati 2015).

Cell Immobilization

Cell immobilization was carried out by mixing microbial suspensions with sodium alginate solution (2% for *Z. mobilis* and *S. cerevisiae*; 4% for *A. niger*) in a 1:1 (v/v) ratio, followed by dripping the mixture into $CaCl_2$ solution (1.1% for *Z. mobilis* and *S. cerevisiae*; 7% for *A. niger*) to form beads. The beads were hardened at 4°C for 16–20 h, rinsed with 0.85% NaCl solution and distilled water. Beads were then incubated for 24 h in a nutrient solution containing 3 g/L urea, 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.45 g/L $NaPO_4$, and finally preserved in 5% glucose solution until fermentation (Awaltanova, E., Bahri, S. 2015; Widyaningrum et al. 2022).

Simultaneous Saccharification and Fermentation

For the SSF process, delignified bagasse powder was diluted with distilled water (1:10 w/v) and sterilized by autoclaving. The fermentation medium was supplemented with 1% (v/v) nutrient solution, 25 g/L yeast extract, and 40 g/L glucose (Rosanti et al. 2023). The nutrient solution had the same composition as that used for bead incubation.

Immobilized *A. niger* (3% w/v) was initially added to induce cellulase production for 72 h at 30°C (Febrianti, Syamsu, and Rahayuningsih 2017; Larasati 2015). On the fourth day, anaerobic fermentation was initiated by adding immobilized *S. cerevisiae* and *Z. mobilis* (3% w/v), and was continued for 80 h at 30°C (Rosanti et al. 2023; Ruiz-Marín et al. 2016).

Reducing sugar concentrations were determined daily from 24 to 80 h of anaerobic fermentation, corresponding to days four through six of the SSF process, after the initial 72 h cellulase production stage and before broth filtration. The Lane–Eynon method, a titrimetric technique based on SNI 01-2892-1992, was employed to quantify reducing sugars by assessing glucose's ability to reduce Fehling's solution under alkaline conditions into brick-red Cu_2O precipitate. Fehling A contains $CuSO_4$, while Fehling B consists of potassium-sodium tartrate and NaOH. Methylene blue was added as an indicator to determine the titration endpoint during heating (Rahmat, Suhardjadinata, and Nawangsari 2022).

Distillation

The fermented broth was filtered and distilled at 78–80°C to obtain bioethanol. Ethanol was qualitatively analyzed using Fourier Transform Infrared (FTIR) spectroscopy and quantitatively determined using Gas Chromatography (GC). The results were analyzed descriptively based on ethanol concentration (% v/v), yield (g/g), and FTIR spectral characteristics (Sehwantoro, Hindarti, and Oktivina 2021). This study aimed to compare the fermentation efficiency of bioethanol production from sugarcane by two fermentative microorganisms, *S. cerevisiae* and *Z. mobilis*. As this study was exploratory and was conducted without sample replication, no inferential statistical analysis was performed.

RESULTS AND DISCUSSION

Sample Preparation

Delignification serves as a crucial pretreatment step aimed at increasing porosity and reducing cellulose crystallinity, thereby enhancing the efficiency of enzymatic hydrolysis (Sani et al. 2025). In this study, 200 g of sugarcane bagasse were treated with 10% NaOH solution at a 1:4 (w/v) ratio, resulting in 80.12 g solid residue. The treated samples were subsequently washed to neutral pH and oven-dried.

Figure 1. Reddish-brown delignified sugarcane bagasse

Under the applied alkaline pretreatment conditions (10% NaOH), phenolic hydroxyl groups in lignin underwent deprotonation by hydroxide ions, generating negatively charged phenolate species that increased lignin solubility in the alkaline medium. Simultaneously, hydroxide ions promoted the cleavage of ester linkages and partial ether bonds within the lignin–carbohydrate complex, facilitating lignin depolymerization and the release of associated hemicellulose, thereby improving cellulose accessibility. The generated phenolate ions are stabilized by sodium ions as counter-ions, forming sodium phenolate species that are soluble in the alkaline medium and impart a characteristic reddish-brown to dark coloration (Figure 1). Consequently, lignin is no longer present as an intact solid polymer but exists as soluble, fragmented species (Maharani and Rosyidin 2018; Mulyadi, Khumaisah, and Rahayu 2023).

Previous studies reported a reduction in sugarcane bagasse lignin content from 17.18% to 4.47% using 1 N NaOH at a solid-to-liquid ratio of 1:10 (w/v) at 120°C for 1 h, corresponding to a delignification efficiency of 74%. In the present study, delignification was conducted at a higher alkali concentration (10% NaOH) with a lower solvent ratio (1:4 w/v) at 121°C for 1 h, resulting in a decrease in lignin content from 15.6% to 6.4% and a delignification efficiency of 59%. These results indicate that substantial delignification can be achieved despite the reduced solvent volume, suggesting improved process efficiency. The reduction of lignin and hemicellulose fractions enhances cellulose accessibility, which is beneficial for subsequent SSF processing. The apparent increase in cellulose content after alkaline pretreatment does not indicate cellulose synthesis, but rather reflects the relative enrichment of cellulose due to the removal of lignin and hemicellulose fractions. The comparative composition of cellulose, hemicellulose, and lignin before and after delignification is presented in Table 1.

Table 1. Bagasse composition changes

	Raw material	Pretreatment NaOH	Reference
Hemicellulose	32.7%	12.13%	
Cellulose	36.8%	76.4%	Present work
Lignin	15.6%	6.4%	
Hemicellulose	22.43%	8.40%	
Cellulose	43.48%	75.91%	Rizal et al.2020
Lignin	17.18%	4.47%	

Cell Immobilization

Both the cellulase-producing fungus *Aspergillus niger* and the glucose-fermenting microorganisms *Saccharomyces cerevisiae* and *Zymo monas mobilis* were immobilized to establish a stable immobilized SSF framework by mitigating process instability associated with free-cell operation. In the absence of immobilization, fluctuations in cell density and substrate availability can lead to imbalanced hydrolysis–fermentation rates, increased microbial washout, and intensified competition for fermentable sugars. Immobilization confines microbial cells within a polymer matrix while preserving metabolic activity, thereby maintaining high local density, reducing microbial loss, and facilitating functional coordination between cellulolytic and fermentative microorganisms during SSF. Calcium alginate was selected as the immobilization matrix due to its biocompatibility, structural stability under acidic fermentation conditions, and favorable mass transfer properties, particularly for CO_2 diffusion. Through this immobilization strategy, sustained enzymatic hydrolysis and fermentation performance were achieved, enabling effective synchronization of cellulose depolymerization and ethanol production (Awaltanova, E., Bahri, S., 2015).

Within the immobilized SSF framework, the cellulolytic step plays a critical role in controlling glucose generation that governs downstream subsequent fermentation performance. *Aspergillus niger* was immobilized as the primary cellulase-producing microorganism to modulate cellulose depolymerization and provide a gradual supply of fermentable sugars (Figure 2) (Herawati, Kusumawardhani, and Puspawati 2016). This controlled hydrolysis maintained a balance between enzymatic saccharification and fermentation by *S.cerevisiae* or *Z.mobilis*, thereby minimizing substrate inhibition and supporting stable SSF operation. The immobilized fungal matrix also enhanced enzyme retention, minimizing enzyme loss and contributing to sustained hydrolytic activity throughout fermentation (Figure 3).

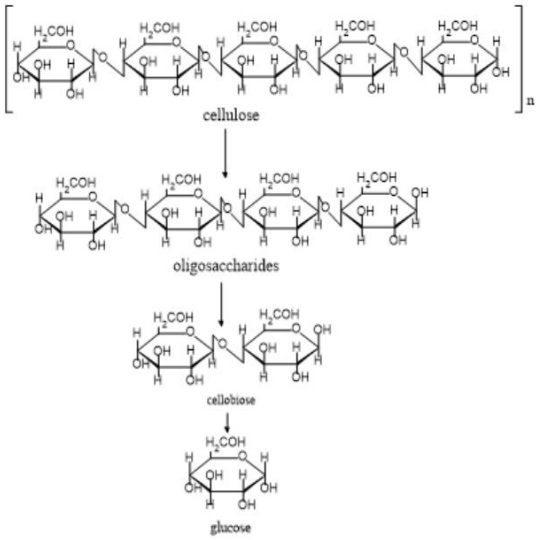


Figure 2. Mechanism of cellulose hydrolysis at β(1→4) glycosidic linkages catalyzed by cellulase enzymes from *A.niger*

Figure 3. Cell immobilization of *A.niger*

Saccharomyces cerevisiae was employed as a robust glucose-fermenting microorganism capable of maintaining consistent fermentative performance under the fluctuating substrate conditions characteristic of SSF. In the immobilized SSF system containing *A.niger*, *S.cerevisiae* primarily utilized glucose released gradually from enzymatic cellulose hydrolysis (Figure 5). Its high tolerance to acidic environments and inhibitory stresses commonly associated with lignocellulosic substrates enable*s S. cerevisiae* to maintain consistent fermentative activity despite temporal variations in glucose availability (Meyrinta, K.A., Putri, R.D., Fatoni, 2018; Mulyadi et al., 2023). Spatial confinement within the immobilization matrix buffered these fluctuations and supported sustained ethanol production driven by reducing sugars generated during hydrolysis. Within this *A.niger-S.cerevisiae* SSF configuration, immobilized *S.cerevisiae* functioned as a robust fermentative benchmark for comparison with alternative immobilized SSF systems employing *Z.mobilis* (Figure 4).

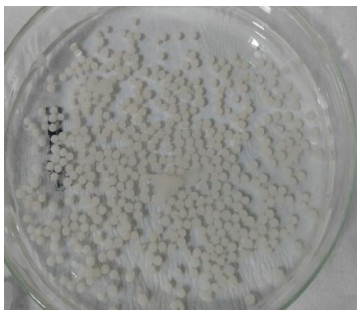


Figure 4. Cell immobilization of *S.cerevisiae*

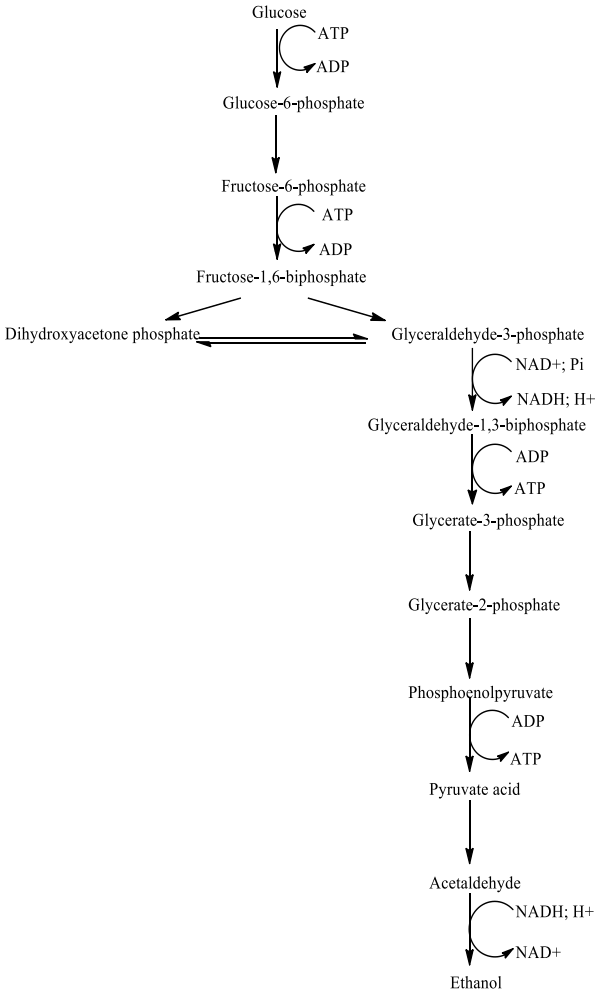


Figure 5. Embden-Meyerhof-Parnas Pathway

Zymomonas mobilis was incorporated as an alternative ethanol-producing microorganism to evaluate its performance relative to *S.cerevisiae* under identical immobilized SSF conditions (Figure 6). The selection of *Z.mobilis* was based on its rapid glucose uptake kinetics under favorable fermentation conditions and efficient ethanol biosynthesis via the Entner–Doudoroff pathway (Figure 7). However, its application in lignocellulosic systems is often constrained by its sensitivity to inhibitory compounds and limited substrate flexibility (Ramayanti and Giasmara 2020). In the present work, immobilization mitigated these limitations by reducing direct exposure to potential inhibitors and stabilizing metabolic activity during SSF. When operated in an immobilized SSF system with *A.niger*, *Z.mobilis* functioned as a fast glucose fermenter when sufficient glucose became available from enzymatic cellulose hydrolysis (Ma'As, Ghazali, and Chieng 2020). In contrast to *S.cerevisiae*, which exhibited greater

tolerance to acidic and dynamically changing conditions, the performance of *Z.mobilis* was more dependent on favorable glucose availability. These results demonstrate distinct fermentative behaviors of the two microorganisms when applied in a separated immobilized SSF system, providing a comparative basis for evaluating their respective contributions to ethanol production efficiency.

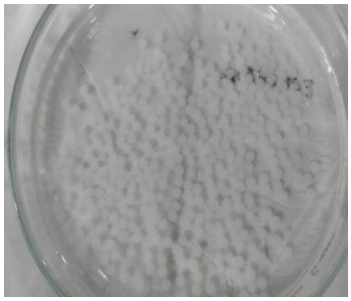


Figure 6. Cell immobilization of *Z.mobilis*

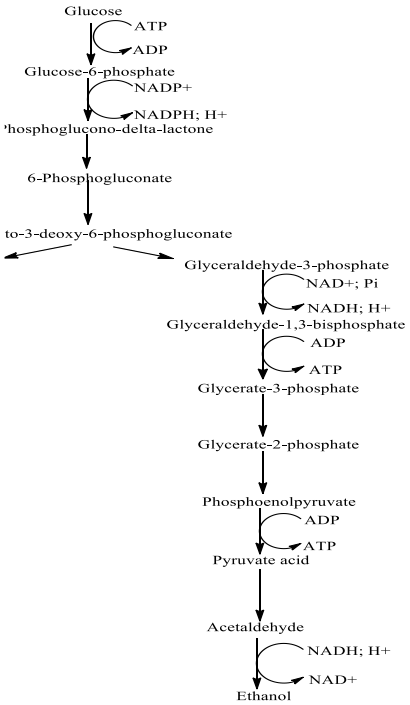


Figure 7. Entner-Doudoroff Pathway

Fermentation Process

After the cellulase production phase by *A.niger*, SSF bioethanol fermentation was conducted at 30°C for 80 h in separate immobilized systems employing either *S.cerevisiae* and *Z.mobilis* as the fermentative microorganism. Reducing sugar concentrations were determined daily from 24 to 80 h of fermentation to monitor sugar utilization during ethanol production. The reducing sugar content of the delignified bagasse fermented separately by *S.cerevisiae* and *Z.mobilis* was determined using the Lane-Eynon method, and the results are presented in Table 2.

Table 2. Comparison of reducing sugar percentages during SSFof delignified bagasse fermented by *S.cerevisiae* and *Z.mobilis*

	Time (h)	Titration Value (mL)	Reducing Sugar (%)
S.cerevisiae	24	12.167	3.12
	80	11.267	3.37
Z.mobilis	24	11.033	3.44
	80	8.667	4.38

An increase in reducing sugar concentration from 24 to 80 h in both SSF systems indicates that continuous enzymatic hydrolysis by immobilized *A. niger* proceeded faster than microbial glucose uptake under the applied SSF conditions. However, overall conversion efficiency remained limited, likely due to constraints in the initial depolymerization of cellulose to cellubiose. Although *A.niger* exhibits strong β-D-glucosidase activity that converts cellobiose to glucose, incomplete cellulose depolymerization can restrict the availability of fermentable sugars. The relatively short fermentation period may have further constrained enzyme production.

Furthermore, the glucose produced was subsequently utilized by the respective fermentative microorganism (*S.cerevisiae* or *Z.mobilis*) in each immobilized SSF system, indicating ethanol production. Differences in reducing sugar accumulation between the two SSF systems indicate that effective glucose uptake kinetics of *S.cerevisiae* and *Z.mobilis* were modulated by SSF constraints, including gradual glucose release, inhibitor presence, and immobilization effects, rather than reflecting intrinsic uptake capacity alone.

By employing identical immobilized hydrolytic conditions while applying different fermentative microorganisms in the separate SSF systems, this study isolates the contribution of microbial glucose utilization kinetics to ethanol yield and downstream distillation performance. The observed differences between the *A. niger–S. cerevisiae* and *A. niger–Z. mobilis* SSF systems highlight a fundamental trade-off between fermentation robustness and conversion efficiency, providing mechanistic insight for fermenter selection in lignocellulosic bioethanol processes.

Following fermentation, the clarified broth was distilled, and the resulting bioethanol was characterized (Table 3).

Table 3. Characteristics of bioethanol after distillation

Characteristic	Bioethanol SSF	
	Saccharomyces	Zymomonas
Density (g/cm^3)	1,098	1,112
Viscosity (Cp)	0,681	0,920
Percentage of Ethanol (v/v)	2,827%	2,201%
Yield (g/g)	0,52	0,43
Form	Liquid	Liquid
Color	Colorless	Colorless

Based on the data presented, both distillates exhibited densities close to that of water and low viscosity values relative to fuel-grade ethanol, indicating a very low ethanol concentration. The viscosity of bioethanol fermented by *S.cerevisiae* was 0.681 cP, while that from *Z.mobilis* was 0.920 cP. These values deviate substantially from the standard ethanol density and viscosity of 0.7894 g/cm^3 and 1.17 cP, respectively, as specified in SNI 7390:2012 and REACH-based safety data sheets (EU No. 1907/2006), and are consistent with FTIR and GC analyses indicating high residual water content (Bakhor et al. 2022; Herawati, N., Roni, K.A., Fransiska, S. 2021). These physicochemical properties primarily reflect upstream fermentation limitation rather than distillation efficiency, as single-stage distillation was applied without additional dehydration steps.

Ethanol yield, defined as the ratio of ethanol produced to the theoretical ethanol yield from the consumed substrate, provides an integrated measure of fermentation efficiency (Zhao et al. 2015). Although previous studies have reported higher ethanol yields for immobilized *Z.mobilis* (0.48 g/g) compared to *S. cerevisiae* (0.45 g/g) (Ruiz-Marín et al. 2016; Sowatad and Todhanakasem 2020), the present study demonstrated a higher ethanol yield for the *S. cerevisiae*-based SSF system (0.52 g/g) relative to that of Z. mobilis (0.43 g/g). This discrepancy indicates that the rapid glucose uptake characteristics commonly attributed to *Z. mobilis* were not fully realized under the applied SSF conditions. Although the *Z.mobilis*-based SSF system exhibited higher residual reducing sugar levels, these sugars were not efficiently taken up and converted into ethanol, suggesting that glucose availability and metabolic stability constituted limiting factors in the immobilized SSF environment. In contrast, *S.cerevisiae* exhibited more consistent glucose-to-ethanol conversion under fluctuating substrate conditions, resulting in a higher overall ethanol yield despite lower residual sugar levels.

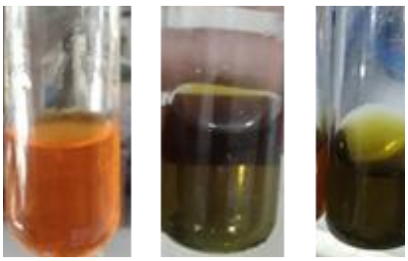
The reducing sugar dynamics observed during SSF (Table 2), therefore, directly influenced ethanol accumulation and the physicochemical characteristics of the distillates (Table 3), underscoring the importance of effective microbial metabolism rather than sugar availability alone in SSF design. By evaluating two immobilized SSF systems under identical hydrolytic conditions, these findings highlight that ethanol yield and distillate quality are governed primarily by microorganism-specific glucose utilization efficiency and fermentation robustness. Overall, the physicochemical properties of both distillates do not meet SNI specifications for fuel-grade ethanol, confirming that the produced bioethanol is unsuitable for vehicle fuel blending without further purification.

Bioethanol Qualitative Analysis

Qualitative analysis using potassium dichromate ($K_2Cr_2O_7$) was conducted to confirm the presence of ethanol in the distilled products. In this redox reaction, ethanol acts as a reducing agent, converting hexavalent chromium ions (Cr^{6+}), which impart an orange coloration, into trivalent chromium ions (Cr^{3+}), resulting in a green color in acidic conditions (Kolo 2022; Telussa et al. 2023).

The observed color change (Figure 8) qualitatively confirms ethanol formation during SSF, supporting the quantitative fermentation and distillation results discussed previously. The positive dichromate response is consistent with the ethanol yields obtained from both immobilized SSF systems, despite their low absolute ethanol concentrations. This result indicates that glucose released during enzymatic hydrolysis was at least partially converted into ethanol, in agreement with the reducing sugar dynamics (Table 2) and ethanol yield data (Table 3).

While this qualitative test does not provide concentration information, it serves as complementary evidence validating ethanol production under the applied SSF conditions and reinforces the interpretation that limitations in ethanol content arise from upstream hydrolysis and fermentation constraints rather than the absence of ethanol formation.



a b c

Figure 8. Qualitative ethanol test. a) control (reagent only), b) *S.cerevisiae* bioethanol, c) *Z.mobilis* bioethanol

Bioethanol Characterization by FTIR

The FTIR spectra of bioethanol produced via SSF using Saccharomyces cerevisiae and Zymomonas mobilis are presented in Figure 9, with corresponding peak assignment summarized in Table 4.

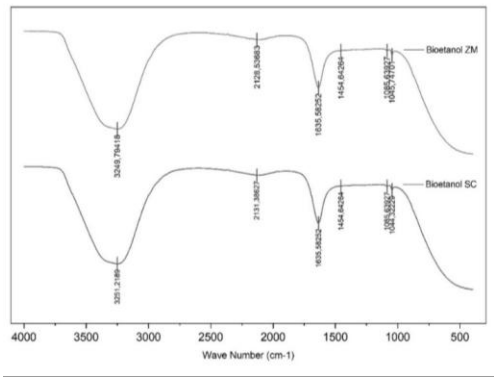


Figure 9. FTIR spectra of bioethanol derived from sugarcane bagasse via SSF using *S.cerevisiae* (SC) and *Z.mobilis* (ZM)

Both spectra exhibited broad absorption bands at 3251.22 cm^{-1} (SSF-SC) and 3249.79 cm^{-1} (SSF-ZM), corresponding to -OH stretching vibrations associated with alcohol and residual water, confirming the presence of ethanol in low-concentration aqueous distillates. Characteristic C-O stretching bands were observed at 1044.32 cm^{-1} and 1045.75 cm^{-1} and O-H bending at 1085.64 cm^{-1}, which are consistent with reported ethanol reference spectra. However, typical ethanol C-H stretching vibrations in the range of 2976, 2931–2875 cm^{-1} and the C-C vibration near 881 cm^{-1} were not clearly resolved in either sample, indicating low ethanol purity and substantial water content. This observation is consistent with the physicochemical properties reported in Table 3, where both distillates exhibited densities close to that of water and low viscosity values, reflecting limited ethanol accumulation during fermentation. The weaker spectral definition in the *Z.mobilis*- derived bioethanol further aligns with its lower ethanol yield despite higher residual reducing sugar levels (Table 2), suggesting incomplete conversion of available glucose into ethanol under the applied SSF conditions.

Minor absorption features near 2139.39 cm^{-1}–2128.54 cm^{-1} observed in the *Z.mobilis* sample may indicate trace impurities or spectral interference, potentially arising from metabolic by-products or matrix effects associated with lignocellulosic fermentation. Overall, the FTIR analysis corroborates the quantitative fermentation results by confirming ethanol formation at low concentrations and demonstrating that differences in microbial glucose utilization efficiency, rather than sugar availability alone, govern ethanol yield and distillate quality in immobilized SSF systems.

Table 4. FTIR spectral interpretation of bioethanol derived from sugarcane bagasse via SSF using *S.cerevisiae* (SC) and *Z.mobilis* (ZM)

Bioethanol SSF-SC (cm^{-1})	Bioethanol SSF-ZM (cm^{-1})	Reference (cm^{-1})	Functional Group Interpretation
3251.22	3249.79	3355	O-H stretching
1635.58	1635.58	1641	H-O-H bending
1454.64	1454.64	1453	C-H bending
1085.64	1085.64	1088	O-H bending
1044.32	1045.75	1045	C-O stretching

Bioethanol Quantitative Analysis by Gas Chromatograph

The ethanol concentration produced from the immobilized SSF of sugarcane bagasse using *S.cerevisiae* and *Z.mobilis* was quantified by gas chromatography. Ethanol content was determined by comparing the sample peak areas with those of a 96% ethanol standard (Kolo 2022). The chromatograms of bioethanol produced by *S.cerevisiae* and *Z.mobilis* are shown in Figures 10 and 11, respectively, with detailed chromatographic parameters summarized in Table 5.

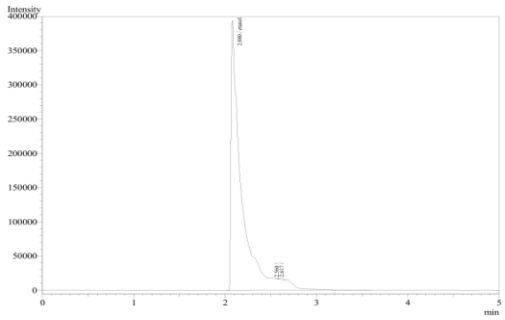


Figure 10. Chromatogram of *S.cerevisiae* (SC) bioethanol

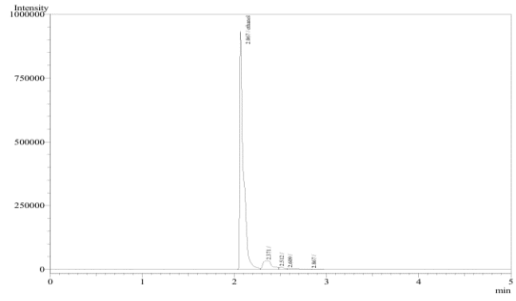


Figure 11. Chromatogram of *Z.mobilis* (ZM) bioethanol

Table 5. Chromatogram data showing peak number, retention time(RT), area, area percentage (purity), and sample identity

Peak	RT (min)	Area	Area%	Sample name
1	1.987	112457811	99.9857	Ethanol pa
1	2.080	3311429	99.7676	Bioethanol SC
1	2.067	2577934	89.9175	Bioethanol ZM

Both SSF systems exhibited ethanol peaks at similar retention times (2.080 min for *S. cerevisiae* and 2.067 min for *Z. mobilis*), confirming ethanol as the dominant volatile component in both samples. The close retention times indicate comparable physicochemical properties of the ethanol produced, while differences in peak area and area percentage reflect variations in ethanol concentration and purity. These results are consistent with the reducing sugar dynamics observed during SSF (Table 2) and the ethanol yield data obtained after distillation (Table 3). Although the *Z. mobilis*-based system exhibited higher residual reducing sugar levels, this did not translate into higher ethanol concentration or purity, indicating that glucose uptake and conversion were constrained under the applied SSF conditions. In contrast, *S. cerevisiae* demonstrated more stable glucose-to-ethanol conversion under fluctuating substrate availability, resulting in higher ethanol accumulation despite lower residual sugar levels.

The GC results further corroborate FTIR analysis (Figure 9), where strong O–H and H–O–H absorption bands indicated high water content in both distillates, consistent with their low ethanol concentrations and single-stage distillation without dehydration. Overall, by applying identical immobilized hydrolytic conditions while varying the fermentative microorganism, this study demonstrates that ethanol yield and purity in SSF are governed primarily by microorganism-specific metabolic stability rather than intrinsic glucose uptake capacity alone.

The *S. cerevisiae*-based SSF system produced a higher ethanol concentration (2.827% v/v) and purity (99.77%) than the *Z. mobilis* system (2.201% v/v; 89.92%). Figures 10-11 show chromatograms of standard, *S.cerevisiae*, and *Z.mobilis*, summarized in Table 5. *S.cerevisiae* produced a higher ethanol yield (2.827% v/v) than *Z.mobilis* (2.201% v/v), with respective purities of 99.77% and 89.92%. Retention times were similar: 2.080 min (*S.cerevisiae*) and 2.067 min (*Z.mobilis*), which may be due to environmental stress such as low pH or toxic lignin compounds, which may compromise fermentation stability. These GC results align with FTIR data, particularly the 1635.58 cm-1 band, indicating a strong H-O-H bending vibration.

CONCLUSION

Sugarcane bagasse, a lignocellulosic by-product rich in cellulose, represents a promising substrate for glucose and subsequent ethanol production. In the Simultaneous Saccharification and Fermentation (SSF) process, optimizing cellulase activity plays a crucial role in maximizing ethanol yield. Quantitative GC analysis revealed that fermentation with *S.cerevisiae* produced a higher ethanol concentration (2.83% v/v) and purity (99.77%) compared to *Z.mobilis* (2.20% v/v) ethanol and purity (89.92%). Both distillates exhibited densities close to that of water and low viscosity values, indicating high residual water content and low ethanol concentration. The viscosity and density of bioethanol fermented by *S. cerevisiae* were 0.681 cP and 1,098 g/cm^3, while those from *Z. mobilis* were

0.920 cP and 1,112 g/cm^3 (Table 3). These values deviate from the standard density and viscosity of pure ethanol (0.7894 g/cm^3 and 1.17 cP) as specified in SNI 7390:2012 and REACH-based safety data sheet (EU No. 1907/2006), which is consistent with FTIR and GC analyses. The relatively low ethanol quality is attributed to the use of single-stage distillation without further purification; therefore, advanced separation methods such as fractional distillation or adsorption are recommended.

Under immobilized SSF conditions, ethanol yield and purity were governed primarily by microorganism-specific fermentative robustness rather than glucose availability alone. Although the *Z.mobilis* system exhibited higher residual reducing sugar levels, its ethanol conversion efficiency remained lower, indicating metabolic sensitivity under fluctuating SSF conditions. In contrast, *S. cerevisiae* demonstrated greater adaptability and stable glucose-to-ethanol conversion, resulting in higher yield and distillate quality. These findings highlight the importance of microbial robustness in immobilized lignocellulosic SSF systems and support the preferential use of *S.cerevisiae* for stable bioethanol production from sugarcane bagasse.

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