Bioethanol Production from Red Banana Waste *via* *Saccharomyces cerevisiae* under Optimized Conditions

Dhas Sugatha Deepa Dhas,[a,*] Pushpa Thiraviam Arokya Glory,[b] Muthumareeswaran Muthuramamoorthy,[c,*] Abdullah Alodhayb,[c] and Selvaraj Arokiyaraj [d]

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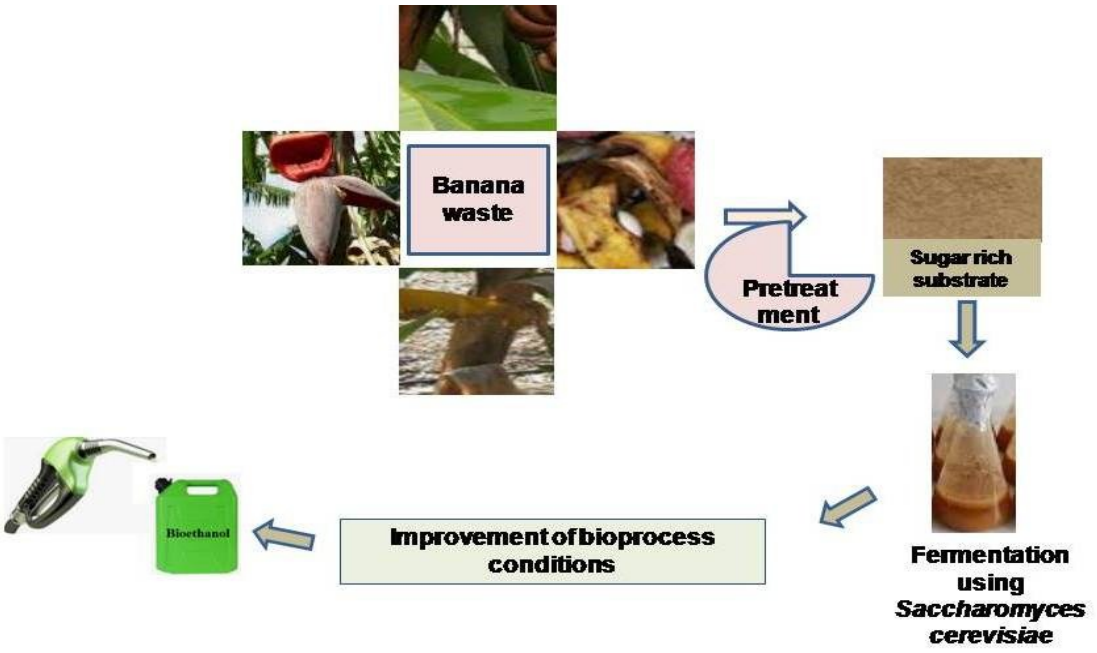
GRAPHICAL ABSTRACT



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Red banana waste (*Musa acuminata*) residue (leaves, pseudostems, banana peels, banana stalks, and flowers) was used to produce bioethanol *via Saccharomyces cerevisiae*. The banana waste was dried and pretreated individually with sodium hydroxide and sulfuric acid. Alkaline treatment increased the reducing sugar content more than acid hydrolysis. Bioethanol production from banana residue *via* enzyme saccharification and fermentation was performed *via* the filter paperase enzyme and fermentation *via S. cerevisiae*. The bioethanol production was temperature dependent. Maximum production was achieved at 36 °C (27.3 ± 0.3 g/L), after 96 h (30.4 ± 1.1 g/L), at a 7.5% substrate concentration (33.5 ± 0.91 g/L) and at pH 5.0 (36.2 ± 0.48 g/L). The maximum bioethanol production was achieved by the immobilized *S. cerevisiae* cells after 96 h of fermentation (39.8 ± 0.55 g/L). Moreover, at this stage, bioethanol production was 35.9 ± 0.51 g/L in *S. cerevisiae*-free fermentation. The bioethanol yield was 11% greater in the immobilized culture than in the free-cell fermentation after four days. Beyond its renewable energy role, bioethanol reduces fossil fuel emissions associated with neurological and developmental disabilities, a connection that will be evaluated in forthcoming research.

Keywords: Banana waste; Biomass; 2G ethanol; Saccharification; Fermentation; Neurological disabilities

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INTRODUCTION

The demand for renewable energy has increased worldwide in recent years. This trend can be attributed to a continuous decline in available resources of fossil fuel energy due to the increasing population and rapid industrialization. The relatively low price of biofuels, especially biodiesel, bioethanol, and biohydrogen, has attracted considerable attention. Biofuels are broadly classified into first- and second-generation biofuels. First-generation biofuels are obtained from agroindustrial wastes, lipids, oil, and carbohydrates. In contrast, second-generation biofuels are obtained from lignocellulosic biomass, including cellulose-rich plant biomass such as stems, stalks, and wood (Naik *et al*. 2010). Second-generation biofuels (2G), such as biomethanol, biohydrogen, and mixed alcohols, are being commercialized. 2G biofuels, also referred to as advanced biofuels, reduce the use of food and minimize the competition between food and fuels. In addition, the use of

2G biofuels reduces carbon emissions. Among the nonfood feed stocks used in bioprocesses to produce 2G biofuels are manure, food waste, spent cooking oil, sawdust, wood, leftovers from agriculture, garbage, food processing wastes and energy crops (Balasubramanian *et al*. 2021; Bello *et al*. 2022; Bhatia *et al*. 2022). Lignocellulosic materials from crops are the most promising biomass due to their widespread availability in most countries throughout the year and their low cost. The lignocellulosic material composition from the leftover layer is heterogeneous, and an additional pretreatment method is warranted during 2G-biological production (Marraiki *et al*. 2020).

Bioethanol is obtained by physical, chemical, or enzymatic hydrolysis and fermentation of lignocellulosic biomass. Generally, a three-step procedure is utilized in bioethanol production, which includes pretreatment of biomass, saccharification, and microbial fermentation. Pretreatment is one of the important steps and is achieved *via* chemical (strong acid or alkali), physical (heat treatment or steaming), biological (enzymatic), or a combination of these three methods. The major objective of the pretreatment method is to change the structure of the lignocellulosic biomass and improve the cellulose content. Microbes utilize this pretreated biomass more easily than untreated biomass does. The yield of cellulose is affected by the selected chemical and physical treatment. The pretreatment procedure affects the lignin structure of the plant biomass, increases the available surface area, alters the solubility of the biomass, depolymerizes, and decomposes cross-links between macromolecules (Al Farraj *et al*. 2020). Alkali treatment has been found to disrupt the lignin component of the biomass. Therefore, pretreatment methods are recommended to make the biomass digestible to produce cellulase by microorganisms to release glucose (Desvaux 2005; Rajendran *et al*. 2018). The second step is microbial fermentation of cellulose and conversion of cellulose into glucose by microbial cellulases (saccharifications). The third step is the conversion of sugars into ethanol *via* the use of yeasts such as *Saccharomyces cerevisiae* (Irfan *et al*. 2016; Maceiras *et al*. 2021).

Microbial fermentation processes are widely used to produce ethanol. In the saccharification process, sugar is produced by cellulases, and the yeast *S. cerevisiae* is involved in the conversion of sugar into ethanol (Gupta and Verma 2015; Kamzon *et al*. 2016). This method is cost-effective, has improved saccharification efficacy, requires fewer enzymes, has a reduced operational time, has a high yield of ethanol, has a lower chance of inhibition and microbial contamination, and has a simple operational procedure (Sun and Cheng 2002; Chen and Fu 2016; Hakim *et al*. 2017).

Agro-industrial residues, including crop residues, are widely used to produce 2G biofuels. Approximately 130 countries cultivate banana plants worldwide, which are considered the second most common crop species, followed by citrus fruits. The share of banana fruit on the global market is 19%, and 114.08 million metric tons of banana waste is generated, which has caused environmental problems (Alzate Acevedo *et al*. 2021; Vijayaraghavan *et al*. 2021). In several countries, banana leaves are used to serve foods and act as natural packing agents. The moisture content of banana leaves protects foods and results in a fragrant aroma (Raji *et al*. 2017). Traditionally, banana waste has been used as animal feed and as organic compost for crops. Banana fruit can be used for several purposes and in biorefineries because of its lignocellulosic nature. They are used for the preparation of organic chemicals, gases, and other thermochemical compounds (Rambo *et al*. 2015). In addition, the remaining banana fruit residues can be used for heat and biogas production. Banana peels can be used for the degradation of carbohydrates, and significant amounts of xylose (9.9/100 g) and glucose (8.2/100 g) can be obtained (Pereira *et al*. 2021).

Agro-industrial wastes generated from banana processing industries are used in bioplastic production, biofuel production, and wastewater treatment (Vijayaraghavan *et al*. 2017; Alzate Acevedo *et al*. 2021). However, poor utilization of these wastes has a severe impact on the environment, contaminating water bodies, generating greenhouse gases, and causing environmental pollution (Martínez-Ruano *et al*. 2018). Biorefinery approaches are considered suitable alternatives for reducing waste volume, reducing environmental pollution, and producing high value-added products. In addition to plant-based biomass, several sectors have shown interest in the use of biorefinery methods for waste, such as sewage sludge (Banerjee *et al*. 2011), food waste (Fava *et al*. 2015), municipal solid waste (Pérez *et al*. 2020), and nonedible oils (Rojas Alfaro *et al*. 2018). Biorefinery methods are useful for the effective utilization of agroindustrial wastes and the reduction of environmental pollution (Fava *et al*. 2015). The moisture content of banana residues is high, which promotes their degradation of organic matter before production, thus affecting their transportation, handling, storage, and biorefinery technologies (Pathak *et al*. 2016). In banana rachises, the lignin content is high, whereas hemicelluloses, holocellulose, and lignin contents are high in banana leaves, which are considered reservoirs for biorefinery processing (Guerrero and Muñoz 2018). In banana, fruit peel residue composition varies according to variety, species, geographical location, seasonal variation, and maturation stage (Pathak *et al*. 2016). Banana rachises and peels are rich in several polymers, such as cellulose, pectin, lignin, proteins, fiber, water-soluble sugars, phenolic compounds, chlorophylls, and various minerals (Lavoine *et al*. 2012). Banana stalks have very low levels of lignin and high holocellulose contents; these can be regarded as favorable attributes relative to use of the resulting fibers in the pulp and paper industry. The material can also be used to produce ethanol after pretreatment (Ingale *et al*. 2014). In recent years, much attention has been given to the use of banana waste for bioenergy and to improving the circular economy (Rincón-Catalán *et al*. 2022). Among several banana varieties, red banana is rich in several essential nutrients, including vitamins, amino acids, and minerals (Tan *et al*. 2024). Bioethanol as a renewable fuel offers significant advantages in mitigating emissions of fine particulate matter, nitrogen oxides, and other toxic by-products of fossil fuel combustion. Exposure to these pollutants has been strongly associated with neurological impairments, developmental delays, and long-term disability outcomes in vulnerable populations. Thus, valorization of red banana waste for bioethanol production not only advances sustainable energy but also provides a potential pathway to reduce disability-related health risks (Landrigan *et al.* 2018). Thus, the conversion of red banana waste into bioethanol advances sustainable energy while providing a scientifically grounded basis for further investigations into its role in public health protection. In this study, red banana waste was used as a low-cost substrate to produce ethanol using *Saccharomyces cerevisiae*.

EXPERIMENTAL

Substrate

Red banana (*Musa acuminata*) waste, including leaves, pseudostems, banana peels, banana stalks, and flowers, was collected from agricultural fields. These wastes were dried in an oven at 60 ± 1 °C until a constant weight was reached, ground into powder, and sieved through a 1 mm sieve. The powdered sample (0.5 to 1.0 mm in size) was stored in an airtight container for future use. The moisture content and ash content were estimated as

described by Dziedzic *et al*. (2015). The amounts of hemicelluloses, cellulose, and lignin were analyzed *via* the standard NREL method. The amount of pectins in the banana waste was estimated as described by Oberoi *et al*. (2012). Each experiment was performed three times, and the mean value was used for data analysis (Sluiter *et al*. 2010). The total protein content of each sample was estimated *via* the AOAC method (Latimer 2012).

Pretreatment

Banana waste was pretreated with acid, alkali, or hydrogen peroxide separately. To perform acid treatment, 10 g of fine powder was mixed with 2, 4, 6, 8, and 10% sulfuric acid. The mixture was autoclaved at 121 °C for 2 h (Chiranjeevi *et al*. 2018), and the optimum acid concentration was determined. To analyze the optimum alkaline pretreatment (NaOH), various concentrations of sodium hydroxide (1, 2, 3, 4, and 5%)were mixed with the biomass and treated for 30 min at 121 °C (Fuertez *et al*. 2021). All pretreatment experiments were performed in 250 mL round bottom flasks, and the solid–liquid ratio was 1:10. The solid phase was subsequently collected, washed with distilled water three times, and dehydrated at 40 °C. The reducing sugars of the pretreated waste were determined (Brummer *et al*. 2014).

Enzyme Hydrolysis of Pretreated Banana Waste

A total of 10 g of pretreated banana waste (dry weight) was mixed with 100 mL of citrate buffer (pH 4.8, 0.05 M). Commercial β-glucosidase (>250 IU/g) and cellulases (>125 IU/g) were used for enzymatic hydrolysis. The pretreated sample was incubated at 40 °C for 42 h with shaking at 100 rpm. Enzymatic hydrolysis was initiated by loading 50 U/g cellulase and 25 U/g β-glucosidase. The samples were withdrawn every 12 h (0 h, 12 h, 24 h, 36 h, and 48 h) and centrifuged at 10,000 rpm for 10 min. The reducing sugars in the culture supernatant were determined (Brummer *et al*. 2014). The level of hydrolysis was determined *via* Eq. 1:

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugar mass after hydrolysis}}{\text{Initial mass of the pretreated substrate}} \times 100 \quad (1)$$

Inoculum

Saccharomyces cerevisiae (ATCC 7754) was used as an inoculum to produce ethanol in banana waste medium. Briefly, *S. cerevisiae* was cultivated on yeast medium agar and incubated at 28 °C. The selected yeast strain was maintained at 4 °C and subcultured every 15 days on a Petri dish before being subjected to the experiment. The yeast strain was inoculated into a 500-mL Erlenmeyer flask consisting of 400 mL of yeast extract peptone dextrose (DPY) medium containing 0.5% (w/v) yeast extract, 1% (w/v) peptone, and 2% (w/v) dextrose and incubated for 48 h at 28 °C. Yeast growth was monitored *via* a UV/visible spectrophotometer at 550 nm (Bahry *et al*. 2017).

Bioethanol Production and Analysis

The culture medium (150 mL) was composed of 3 g of yeast extract, 3 g of peptone, and 10% reducing sugars (enzymatic hydrolyzed banana waste). The culture medium (150 mL) was sterilized for 15 min at 121 °C. Then, 0.5 mL of inoculum was added, and the mixture was incubated for 72 h at 30 °C. After every 24 h, the ethanol concentration in the culture medium was determined (Mendes *et al*. 2016). The amount of ethanol produced by the yeast cells was detected *via a* Waters 2695 Alliance high-performance liquid chromatography system (Waters Inc., Milford, CT, USA).

Effect of Temperature on Bioethanol Production

The production medium comprised 10% reducing sugar from banana waste, which was sterilized and cooled, and 50 U/g cellulase and 25 U/g β-glucosidase were added. Then, 5% inoculum was introduced, and the culture was incubated at 32, 34, 36, 38, and 40 °C. The mixture was placed on a rotary shaker incubator at 150 rpm. The fermentation experiment was performed under oxygen-limited conditions. Sampling was performed, and the amount of ethanol was detected via high-performance liquid chromatography (HPLC).

Effect of pH on Bioethanol Production

The optimum pH of the culture medium was analyzed by varying the pH value of the culture medium from 3.5 to 6.0. The other variables were kept at optimum levels. A fermentation experiment was performed, and the amount of bioethanol used was determined.

Effect of Fermentation Period on Bioethanol Production

The production medium was composed of 10% reducing sugar from banana waste, which was sterilized and cooled. Then, 50 U/g cellulase and 25 U/g β-glucosidase were added. Then, 5% inoculum was added, and the culture was incubated for 24 to 144 h. The mixture was placed on a rotary shaker incubator at 150 rpm at 36 °C under oxygen-limited conditions. The amount of ethanol was detected by high-performance liquid chromatography (HPLC) up to 144 h.

Effect of Sugar Concentration on Bioethanol Production Prepared from Banana Waste

In this study, an experiment was performed to determine bioethanol production at various concentrations of sugar. Briefly, banana waste reducing sugars were added at 2.5, 5.0, 7.5, and 10% in a production medium. *S. cerevisiae* (5%) was inoculated after sterilization and cooling. The culture was subsequently maintained at 36 °C for 120 h.

Effect of Inoculum Concentration on Bioethanol Production

The enzymatically hydrolyzed reducing sugar (7.5%) was added to the production medium. The other culture conditions included shaking at 150 rpm, incubation at 36 °C, and 120 h of yeast fermentation. *S. cerevisiae* was inoculated at various concentrations (2 to 10%) after sterilization and cooling. The amount of bioethanol generated was determined after 120 h.

Immobilization of *S. cerevisiae* Cells

S. cerevisiae was cultivated in YPD broth medium, the viable cells were counted, and the cell density of the culture used for the immobilization process was 1×10^8 CFU/mL. The culture was subsequently centrifuged at 5,000 rpm for 10 min, after which the cells were harvested. The harvested cells were washed with double distilled water several times, followed by 0.85% (w/v) sodium chloride (NaCl), and was further added to 2.5% (w/v) sodium alginate solution at 28 ± 1 °C. The yeast cells were mixed with sodium alginate solution for 5 min and loaded into a 10 mL syringe. The mixture was slowly added to a 0.1 M $CaCl_2$ solution, and the immobilized beads were obtained. The diameter of the formed bead was approximately 6 mm. The Ca-alginate beads were slowly stirred *via* a magnetic stirrer for 1 h and stored in 0.1 M calcium chloride solution until further use (Waluyo *et al*. 2017).

Ethanol Production by Immobilized and Free *Saccharomyces cerevisiae*

The optimized culture medium was prepared to produce ethanol from immobilized and free *Saccharomyces cerevisiae*. The culture medium comprised 7.5% enzymatically treated banana waste reducing sugars, and the other parameters were 96 h of fermentation, pH 5.0, and a 36 °C cultivation temperature. A total of 50 mL of 7.5% banana waste reducing sugar was prepared, and free yeast cells were inoculated after sterilization. In another experiment, 50 mL (immobilized bead volume) of immobilized cells was added to culture medium comprising 7.5% reducing sugars, and the experiment was performed for 96 h (Sirisansaneeyakul *et al*. 2013). The amount of ethanol generated was determined.

Reusability of Alginate Beads in Successive Fermentation Cycles

At the end of each reuse, to determine the amount of viable cells in the beads with 2.5% sodium alginate, the beads were liquefied *via* a chemical method adapted from Göksungur *et al*. (2005). The 10 beads were weighed and dissolved in 50 mL of a sterile 50 mM sodium citrate solution (Merck, Darmstadt, Germany), with continuous stirring for approximately 1 h at room temperature. The cell viability was determined by quantifying the number of CFUs in YPD media incubated at 25 °C for 3 to 5 days. The viability of *S. cerevisiae* cells was determined *via* the spread plate method (Waluyo *et al*. 2017). The amount of bioethanol produced in all five cycles was determined.

Statistical Analysis

Analysis of variance (ANOVA) was performed with Excel and SPSS 23 (Chicago, IL, USA). The results are expressed as the means ± standard deviations.

RESULTS AND DISCUSSION

Chemical Composition of Banana Waste

The chemical composition of banana waste before pretreatment is provided in Table 1. The ash content of the banana waste was 13.3 ± 0.15%, and the moisture content was 70.3 ± 1.5%.The amounts of cellulose, hemicelluloses, and lignin were 24.3 ± 1.1%, 19.2 ± 0.9%, and 6.45 ± 0.4%, respectively. The pectin content of the banana waste was 9.42 ± 0.1%.

Table 1. Chemical Characterization of Raw Banana Waste Utilized for the Production of Bioethanol

Chemical composition	Results
Moisture (% of fresh mass)	70.3 ± 1.5
Ash (% of dry mass)	13.3 ± 0.15
Total protein (% dry mass)	6.7 ± 0.6
Pectin (% dry mass)	9.42 ± 0.1
Cellulose (%)	24.3 ± 1.1
Hemicellulose (%)	19.2 ± 0.9
Lignin (%)	6.45 ± 0.4

Notes: Banana waste comprises leaves, pseudo stems, banana peels, banana stalks, and flowers. The means ± standard deviations are the averages of three replicate analyses.

Pretreatment of Banana Waste

Two different chemical pretreatment methods were used for the pretreatment of banana waste. The dried banana waste was pretreated with concentrated sulfuric acid (2, 4, 6, 8, and 10%), and the yield was high at 6%.The reducing sugar content obtained in the acid treatment reached a maximum at 6% sulfuric acid (13.5 ± 0.10%), which was lower than that in the alkaline treatment. After 4% NaOH treatment, 14.5 ± 0.10% reducing sugars were detected (Table 2).

Acid and alkaline treatments are considered reliable methods for the treatment of plant biomass. However, alkaline treatment is considered a most suitable method for improving product yield, and the present findings are consistent with those of previous studies (Nascimento *et al*. 2023). The present findings are consistent with previous findings on banana waste (Fiallos-Cárdenas *et al*. 2022), banana flower waste (Thokchom *et al*. 2023), banana pseudostems (Merais *et al*. 2022), and banana sheaths (Subramanian *et al*. 2022).

Table 2. Acid and Alkaline Pretreatment of Plant Biomass and Reducing Sugar Yield

Acid Treatment		Alkaline Treatment	
(Sulfuric acid, %)	Reducing sugar(%)	(NaOH, %)	Reducing sugar (%)
2	8.3 ± 0.40	1	3.5 ± 0.1
4	11.2 ± 0.22	2	8.4 ± 0.2
6	13.5 ± 0.10	3	12.1 ± 0.1
8	13.1 ± 0.10	4	14.5 ± 0.10
10	12.4 ± 0.13	5	11.3 ± 0.1
Control	6.3 ± 0.09	Control	6.3 ± 0.09

Enzymatic Hydrolysis of Pretreated Banana Waste

After the delignification step with sulfuric acid and sodium hydroxide, the pretreated banana waste samples were further treated with enzymes (β-glucosidase and cellulases). This enzymatic reaction aimed to release sugars from cellulose. This step is critical for the conversion of banana waste to the desired products. Microorganisms utilize these sugars for growth and product formation (Gonzales *et al*. 2016). The supplemented enzymes acted on cellulose and released glucose monomers. As shown in Fig. 1, after 12 h of enzymatic treatment, the reducing sugar content of the acid-treated banana waste was 14.4 ± 0.9%, and the reducing sugar content increased after 24 h (40.3 ± 1.1%).

In the alkaline-treated biomass, the reducing sugar content reached a maximum after 24 h of enzymatic hydrolysis (41.5 ± 0.9%). However, the reducing sugar content decreased after 48 h of enzymatic hydrolysis. In banana pseudostems, the glucose yield was 28.9% after acid pretreatment and enzymatic hydrolysis (Hossain *et al*. 2019). The free sugar yield was high when banana waste was pretreated with the optimum concentration of enzymes because lignocelluloses are generally heterogeneous. Hence, pretreatment methods may improve or affect product yield. The success of enzymatic saccharification is based on the type of biomass and pretreatment method. Acids generally disrupt lignin and improve cellulose susceptibility to biocatalytic hydrolysis (Łukajtis *et al*. 2018).

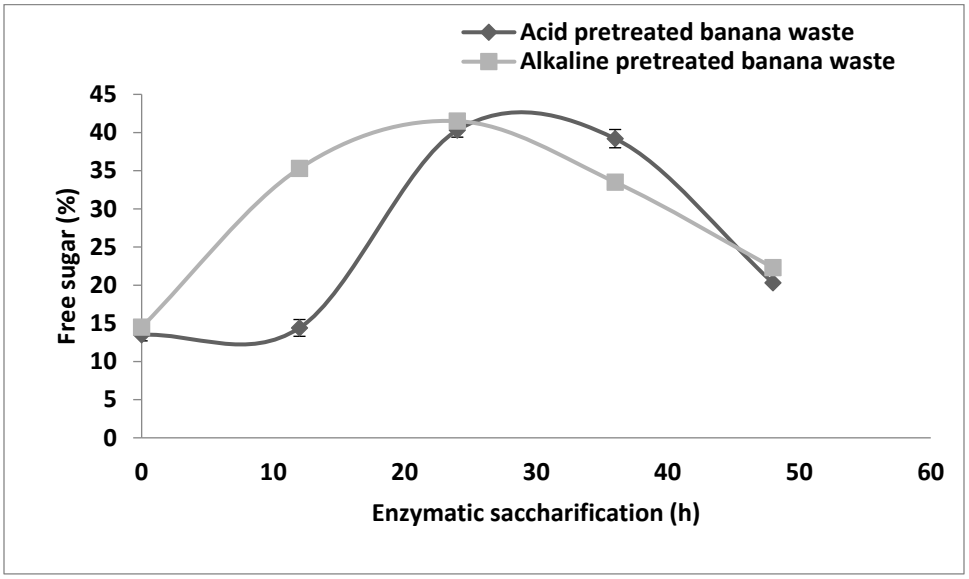

Fig. 1. Enzymatic hydrolysis of pretreated red banana waste for the production of bioethanol by yeast fermentation. The error bar represents the standard deviation.

Effect of Temperature on Bioethanol Production by *S. cerevisiae*

The optimum temperature for improving bioethanol production was determined. Bioethanol production was temperature dependent, and maximum production was achieved at 36 °C (27.3 ± 0.3 g/L) and was lower at higher incubation temperatures (Fig. 2).

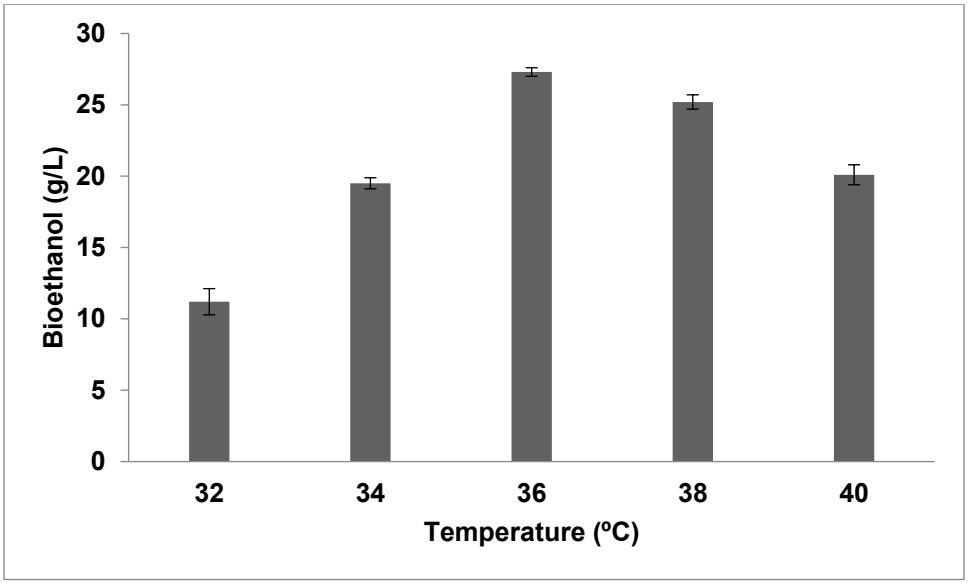

Fig. 2. Effect of temperature on bioethanol production. The error bars represent the standard deviation.

Temperature is one of the critical factors affecting membrane turgidity of yeast cells, and enzymatic activity and thermostable yeast cells are preferred for industrial processing. The amount of bioethanol produced by *S. cerevisiae* in this study was compared

with that in previous studies. The yeast *S. cerevisiae* ITV-01 has been isolated from sugarcane molasses and produces the maximum amount of bioethanol at 30 °C, and the yield was 58.4 g/L (Ortiz-Muñiz *et al*. 2010), which was higher than that in this study. The optimum incubation temperature increased yeast growth and improved product formation. Lin *et al*. (2012) optimized ethanol production *via S. cerevisiae* BY4742 and reported maximum ethanol production between 30 °C and 40 °C. Moreover, ethanol production was reduced significantly at 50 °C, which was consistent with the results of this study. The variation in ethanol production at higher temperatures might be due to changes in the transport system, which might increase the accumulation of ethanol in yeast cells. The optimum temperature for ethanol production by *Saccharomyces cerevisiae* was 30 °C when yeast was cultivated in sugarcane molasses substrate (Rasmey *et al*. 2018), 41 °C in 10% glucose medium (Benjaphokee *et al*. 2012), and 45 °C in pretreated wheat straw substrate (Ruiz *et al*. 2012). The present investigation and previous studies revealed that the optimum incubation conditions for the growth of *S. cerevisiae* may vary on the basis of the strain type, culture medium, fermentation type, and ethanol concentration in the medium.

Effect of pH on Bioethanol Production

Bioethanol production reached a maximum at pH 5.0 (36.2 ± 0.48 g/L), and a slight reduction in ethanol yield was observed at higher pH values (Fig. 3). The pH is an important factor for yeast growth and metabolism, and the amount of H^+ determines the pH of the culture medium, which affects the permeability of nutrients into yeast cells through the membrane (Lee *et al*. 2011; Zabed *et al*. 2014). Alterations in the pH of the culture medium affect the vacuolar and cytosolic pH of *S. cerevisiae*, and a moderate bioethanol yield was observed above pH 5.5. Changes in the pH of the culture medium affect the ATPase activity of the plasma membrane (Peña *et al*. 2015); hence, an optimum pH value is required for yeast growth and ethanol fermentation. At acidic pH values, the intracellular pH reduces the permeability of the plasma membrane to protons and the consumption of adenosine triphosphate, which significantly reduces the glucose uptake of microorganisms and glycolytic activity (Woo *et al*. 2014).

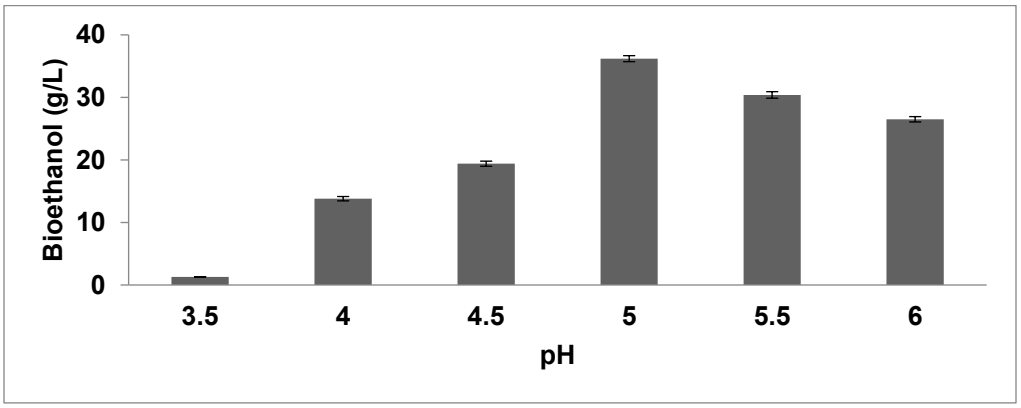

Fig. 3. Effect of pH on bioethanol production. The error bars represent the Standard Deviation.

Effect of Fermentation Period on Bioethanol Production by *S. cerevisiae*

The bioethanol production was 3.1 ± 0.92 g/L after 24 h at 36 °C. The bioethanol content was highest (30.4 ± 1.1 g/L) after 96 h when initial inoculum level was 5%. The bioethanol production decreased to 23.2 ± 0.75 g/L after 144 h of fermentation (Fig. 4). In an earlier study, ethanol production reached a maximum at the 38 h incubation period, and

the initial inoculum concentration was 2% (Nikolić *et al*. 2009). The type of culture medium, amount of glucose, incubation temperature, and initial inoculum level may affect the fermentation process. Sudhakar *et al*. (2021) recently reported increased ethanol yield after four days of incubation, and the final yield was 1.57 g/L when *Ganoderma lucidum* was used as the test organism to meet industrial demand. The yield obtained in this study was greater than that reported previously. The bioethanol conversion efficacy of *S. cerevisiae* was greater than that of *G. lucidum*. Banana peels were used as the culture medium to produce ethanol, and the yield was 13 g/L by *S. cerevisiae* after 10 to 12 h, whereas it was lower (13 g/L) in the culture media containing *K. marxianus* at 10% inoculum and at 41 °C. Conversely, presaccharification, simultaneous saccharification and fermentation increased ethanol production (32.6 g/L) within 64 h of incubation in banana peel medium (Palacios *et al.* 2021).

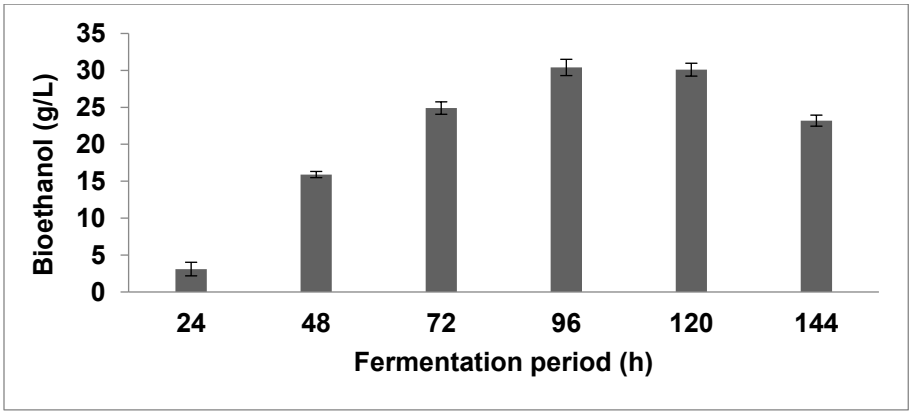

Fig. 4. Effect of fermentation period on bioethanol production. The error bar represents the standard deviation.

Effect of Sugar Concentration on Bioethanol Production Prepared from Banana Waste

The effect of sugar concentration on bioethanol production was determined, and the results are shown in Fig. 5. The bioethanol yield was 14.9 ± 0.53 g/L at a 2.5% substrate concentration after 96 h of incubation. During the same incubation period, bioethanol production reached a maximum at a 7.5% substrate concentration (75 g L^{-1}) (33.5 ± 0.91 g/L). Moreover, bioethanol production decreased at a 10% substrate concentration (30.1 ± 0.88 g/L). The initial sugar concentration in the culture medium increased the specific growth rate of the yeast cells, and the average ethanol productivity increased. In this study, ethanol production was 2.24-fold greater with the 7.5% substrate than with the 2.5% substrate, which was consistent with a previous report. Le Man *et al*. (2011) used reducing sugars from food waste and achieved a 2.3-fold ethanol yield. The reported optimum culture conditions were pH − 4.0, temperature − 40 °C, and a reducing sugar concentration of 7.5% (75 g L^{-1}). In the present study, bioethanol production was greater than that reported by Le Man *et al*. (2011), and the ethanol conversion ratio was greater in this study. The variation in bioethanol in the medium may be due to substrate inhibition and the tolerance of the yeast to relatively high ethanol concentrations. Yeasts utilize sugars anaerobically and ferment into ethanol and carbon dioxide. Yeast can convert 1 kilogram of glucose into 500 g of ethanol on the basis of theoretical estimation. However, it may vary on the basis of the organism and culture conditions (Righelato 1980; Borges Filho and Dettmer 2025).

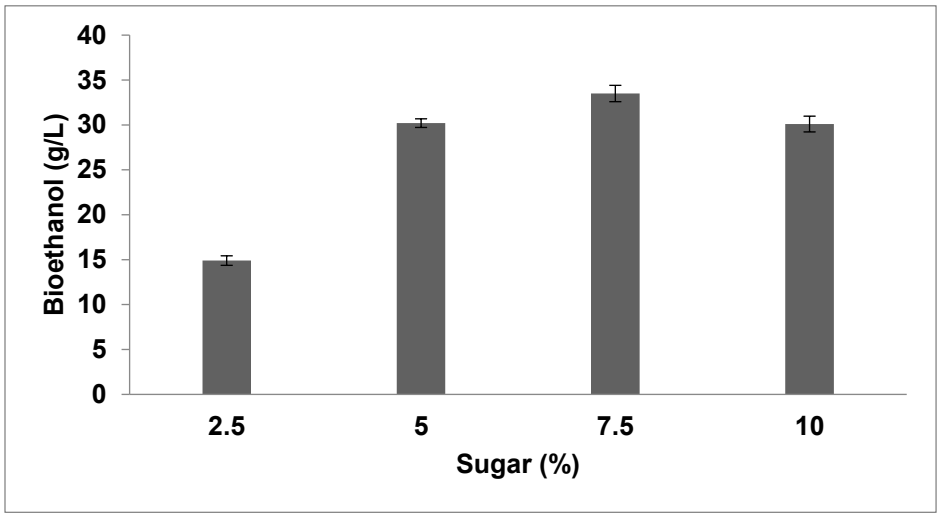

Fig. 5. Effect of sugar concentration (2.5 to 10%) on bioethanol production. The error bar represents the standard deviation.

Effect of Inoculum Concentration on Bioethanol Production

Five concentrations of inoculums (2, 4, 6, 8, and 10%, v/v)were used to determine the effect of the inoculums on bioethanol production. At the 6% inoculum concentration, glucose was rapidly consumed by the yeast and improved the production of bioethanol (35.9 ± 0.55 g/L) (Fig. 6). Moreover, the bioethanol amount detected declined at increased inoculum levels.

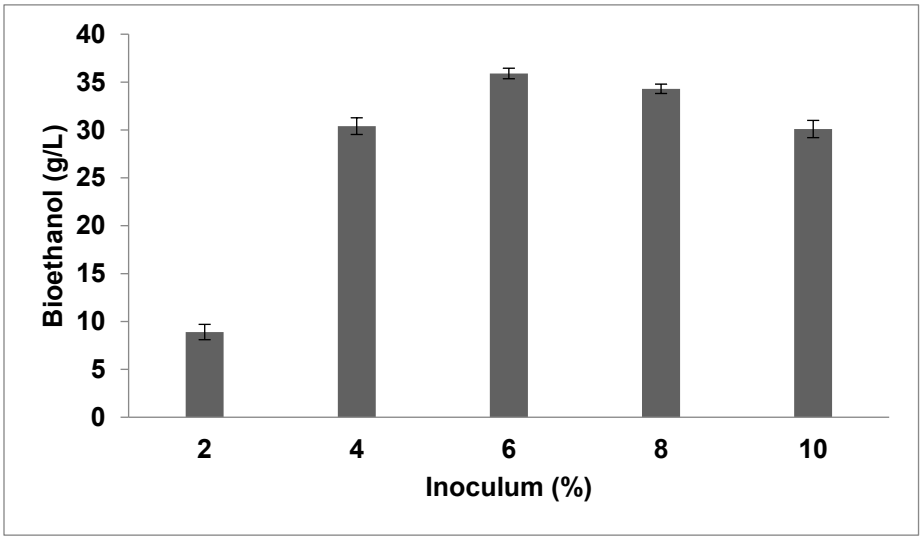

Fig. 6. Effect of inoculum concentration on bioethanol production. The error bar represents the standard deviation.

A smaller inoculum size is preferable for fermentation to reduce the production cost of ethanol. However, increasing the concentration of inoculum affected ethanol production. The inoculum dosage requirement varies on the basis of the substrate and composition of the medium. In coffee husk medium (5 g/L), 3 to 4% was considered the optimum ethanol production (Gouvea *et al*. 2009), and 10% was suggested to improve the ethanol yield in cassava starch medium without any nitrogen source (Akaracharanya *et al*. 2011). The

optimum inoculum size for ethanol production was 40 g/L yeast dry cell mass. The ethanol production rate was increased from 1.29 to 2.35 g/L/h when the yeast concentration increased from 0.5 to 5 g/L (Laluce *et al*. 2009). In fermentation technology, inoculum concentration is an important factor that improves bioethanol production potential (Altınışık *et al*. 2025).

Production of Bioethanol *via* Immobilized *S. cerevisiae* and Free Cells

The bioethanol production of free and immobilized *S. cerevisiae* cells was analyzed, and the results are depicted in Fig. 7. The maximum bioethanol production was achieved by the immobilized *S. cerevisiae* cells after 96 h of fermentation (39.8 ± 0.55 g/L). Moreover, at this stage, bioethanol production was 35.9 ± 0.51 g/L in *S. cerevisiae*-free fermentation. After four days, bioethanol production was 11% greater in the immobilized culture than in the free-cell fermentation. This bioethanol yield was similar to bioethanol production by the thermotolerant *Kluyveromyces marxianus* (Khumsupan *et al*. 2025). After six days of fermentation, bioethanol production decreased in both the immobilized and the free *S. cerevisiae* cultures when 7.5% sugar was used. Ethanol productivity is greater in immobilized cultures than in *S. cerevisiae*-free cell cultures because of its potential to tolerate diverse environmental conditions, such as acidic environments, high temperatures, and high sugar concentrations (Krisch and Szajani 1997; Najafpour *et al*. 2004; Zhu 2007).

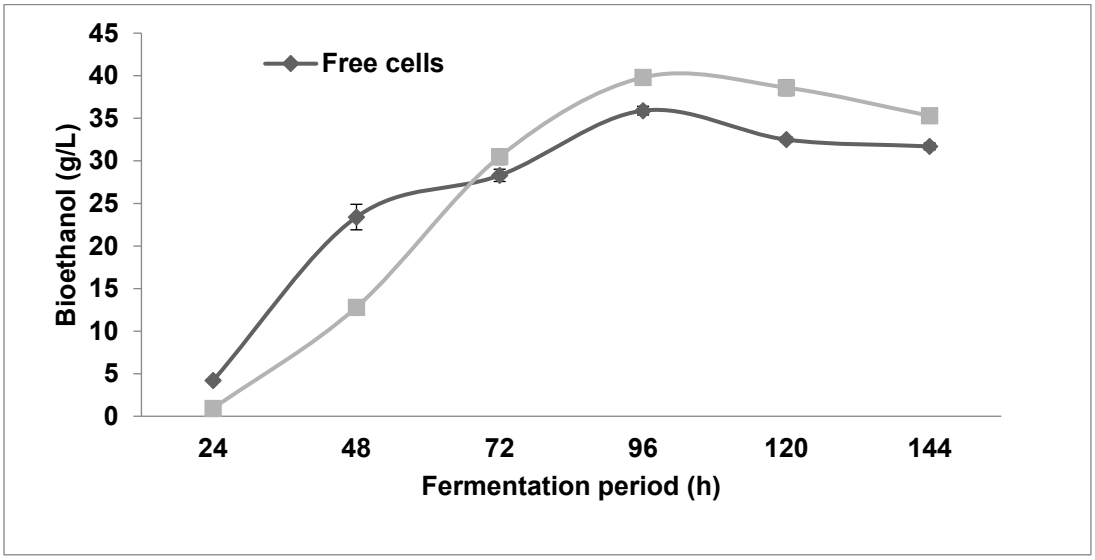

Fig. 7. Bioethanol production by immobilized *Saccharomyces cerevisiae* and free yeast cells. The error bar represents the Standard Deviation.

The small size of the immobilized beads affected the ability of *Saccharomyces cerevisiae* SC90, which was previously reported by Najafpour *et al*. (2004). The immobilization method is considered a suitable method for ethanol production by *S. cerevisiae via* pretreated banana waste sugars under optimum culture conditions. The continuous search for potent *Saccharomyces cerevisiae* strains to improve bioethanol yield is a challenge that involves several factors, such as temperature, initial pH, osmotic stress, and viable yeast cell concentration (Techaparin *et al*. 2017). The thermotolerant yeast *Saccharomyces cerevisiae* SC90 has been used to produce ethanol, and the maximum product yield was achieved at 40 °C (Tareen *et al*. 2021). *Saccharomyces cerevisiae* was

found to be suitable for producing ethanol *via* the immobilization technique by entrapment in a porous matrix, mechanical containment behind a barrier, and attachment on a support surface (Moreno-Garcia *et al*. 2018). In the present study, entrapment was used for the preparation of immobilized cells. The immobilization method has several advantages over free-cell cultivation, including minimal risk of contamination, high product yield, and improved overall fermentation process and reuse of immobilized beads. In this sense, the calcium alginate entrapment method used in this study effectively served as an important protective barrier against ethanol toxicity, similar to previous studies (Milessi *et al*. 2020). The immobilization technique is useful because it significantly reduces the lag phase of microbial growth and effectively improves stress tolerance, thus improving product yield (Khumsupan *et al*. 2025).

Reusability of Yeast Immobilized with Alginate Beads

The mortality of yeast cells and ethanol yield were estimated for five successive fermentation cycles. At the end of the first fermentation cycle, the yeast cell count was 2.3 ± 0.14 $\times 10^8$/bead, and the yeast population and ethanol production improved during the second cycle. This can be attributed mainly to the adaptation of yeast cells to the alginate beads and the ethanol content. After the fourth cycle, rupture was observed on the surface of the immobilized cells during fermentation. During this cycle, the yeast cell population and subsequent bioethanol production decreased. Table 3 shows the profile of yeast growth and ethanol concentration during the first and fifth fermentation cycles. The present findings are similar to previous results (Clementz *et al*. 2015). In addition, ethanol production was greater during the second cycle than during the first fermentation cycle. The increased production in the second and third cycles was due mainly to the increased population of yeast cells within the sodium alginate beads. This finding is in line with previous reports that microbial cells can grow within alginate beads, and increased production has been reported previously in sodium alginate beads entrapped with *S. cerevisiae* **(**Clementz *et al*. 2015). In the present study, a significant amount of bioethanol was detected up to the third cycle, and this result was consistent with that of a previous study. The immobilization method has been used for the production of bioethanol by *Saccharomyces cerevisiae* using cellulose from wood waste. Bioethanol production was consistent up to the 3rd cycle and declined after the fourth and fifth cycles (Pratama *et al*. 2023).

Table 3. *Saccharomyces cerevisiae* Population and Bioethanol Production in the Immobilized Sodium Alginate Beads

Number of Cycle	Yeast cells ($\times 10^8$)/bead	Bioethanol (g/L)
1	2.3 ± 0.14	39.4 ± 3.5
2	3.1 ± 0.52	45.2 ± 1.1
3	5.3 ± 0.33	48.3 ± 0.9
4	4.2 ± 0.71	41.2 ± 1.1
5	1.9 ± 0.55	15.2 ± 0.8

CONCLUSIONS

1. Banana waste was pretreated with either sulfuric acid or sodium hydroxide. The reducing sugar yield in the acid treatment was greatest at 6% (13.5 ± 0.10%), and it was 14.5 ± 0.10% in the 4% sodium hydroxide treatment. The reducing sugar content reached a maximum after 24 h of enzymatic hydrolysis (41.5 ± 0.9%).
2. The reducing sugar was used as the substrate for ethanol production by *Saccharomyces cerevisiae* in anaerobic fermentation. Bioethanol production was optimal at 36 °C, pH 5.0, and after 96 h, the inoculum concentration was 6%, and the substrate concentration was 7.5%.
3. The immobilization method was used to reuse yeast cells in fermentation cycles. In this work, a significant amount of bioethanol was detected up to the third cycle and declined after the fourth and fifth cycles. The present findings revealed that immobilized *S. cerevisiae* may be useful for achieving ecofriendly ethanol production, while contributing to ongoing research on reducing pollutant emissions and associated disability-related health risks.

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