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# Enhancement of bioethanol production from lignocellulosic biomass of banana by single batch fermentation using *Saccharomyces cerevisiae* and native microorganism

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**ABSTRACT**

**Bioethanol production using the lignocellulosic biomass of banana *viz*., banana raw peel, ripe peel and pseudostem were attempted. Among the three feed stocks, maximum total reducing sugar content of 21.98% was observed in the banana ripe peel. Pretreatments of the feed stocks with acid resulted in higher lignin removal and increased total reducing sugar content compared to the alkali treatment. Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF) and Single Batch Bioconversion (SBB) were carried out for the fermentation process using *Saccharomyces cerevisiae*. By SSF fermentation processs, 6.63% of ethanol was produced by *Saccharomyces cerevisiae* from the untreated samples of banana raw peel. Enhancement of bioethanol production was done using a native cellulolytic microorganism isolated from the degraded banana samples. Using the native microorganism along with *Saccharomyces cerevisiae* in SBB resulted in 6.88% of bioethanol conversion. This is the first report of using native microorganism for enhanced degradation of cellulose in banana biomass for higher bioethanol production.**

## Introduction

Global population outbursts increase the demand for biofuel since the dependence on fossil fuels is not adequate for meeting the demand of the entire population. The increase in global warming and waste generation due to the use of fossil fuel demands alternate sources of fuel for energy generation. The carbon intensity of biofuels is very low compared with that of fossil fuels, which is an added advantage (Hamzah *et al.,* 2019). Bioethanol has wide acceptance among various other biofuels. Compared with gasoline, bioethanol has a high heat of vaporization and a high amount of octane, increasing its demand (Sawarkar *et al.,* 2022). Increased blending of ethanol in petrol is needed. According to Agricultural Statistics 2023, 86 million tons of banana are produced worldwide, and after

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harvest, 60% of the banana mass is left as waste. The banana crop generates a large amount of waste in the form of fruit peels, pseudostems and leaves (www.developmentaid.org). Globally, 114.08 million metric tonnes of waste generated from bananas also results in the emission of greenhouse gases and environmental pollution (Alzate *et al.,* 2021). This massive amount of biomass waste is disposed of in agricultural fields, combusted, or dumped at plantations, thus posing environmental concerns. This has become a worrying factor for growers in many villagers. Plantain processing industries are generating large amounts of waste in the form of unripe banana peel waste. The major source of fermentable sugars in this type of plant-based material is its high cellulose and hemicellulose content. Banana waste is excellent source of cellulose. Various biological sources, such as fruit waste, can be considered potential sources for bioethanol production (Alex *et al.,* 2017; Bennurmath *et al.,* 2021; Santos *et al.,* 2020). Unripe plantain peels, especially from the chip industry, and ripe peels from the jam industry can be used as potential feedstocks for bioethanol production, and this could be an attractive alternative for the disposal of polluting residues. The effective production of bioethanol from agricultural wastes involves the characterization and pretreatment of the feedstock. A pretreatment step is necessary for the bond breaking of cellulose and lignin molecules and thus for increasing the surface area (Santiago *et al.,* 2022). Pretreatment can be performed by acid or alkali treatment. However, a major hindrance is the identification of suitable microorganisms and procedures to enhance the conversion rate during alcohol production. *Saccharomyces cerevisiae* is a promising microorganism for the production of bioethanol from various feed stocks because of its wide tolerance to different pH conditions (Derman *et al.,* 2022). Initially, fermentation of the feedstock (raw and pretreated) was carried out using *Saccharomyces cerevisiae*. Three different modes of saccharification and fermentation, namely, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and single batch bioconversion (SBB), were tested to optimize the process for enhancing the production of bioethanol from the substrate to achieve the maximum bioethanol production. Native microorganisms present in the degraded samples of biomass may have more efficiency in bioethanol production under optimum conditions. The present study is an attempt to produce cost-effective and environmentally sustainable bioethanol production using banana peels and plant residues using a mixed culture of *Saccharomyces cerevisiae* and a native microorganism isolated from decaying banana waste.

## Materials and Methods

### Preparation and pretreatment of feed stock

Three types of banana biomass, namely, the biomass of the raw peel, ripe peel and pseudostems of the variety Nendran (*Musa* AAB), were utilized for the study. Samples were collected from the Instructional Farm, College of Agriculture, Vellayani, Kerala, India. Banana peels from raw and ripe fruits and banana pseudostems were separated, washed and chopped into small pieces (~ 2-3 cm). The samples were oven-dried separately at 60°C for 48 h, finely ground and stored in airtight containers for further analysis. The feed stocks were separately blended and characterized for alcohol production, and the powdered banana samples are shown in Figure 1.

Total reducing and nonreducing sugar contents were estimated by the DNS method (Lindsay, 1973).

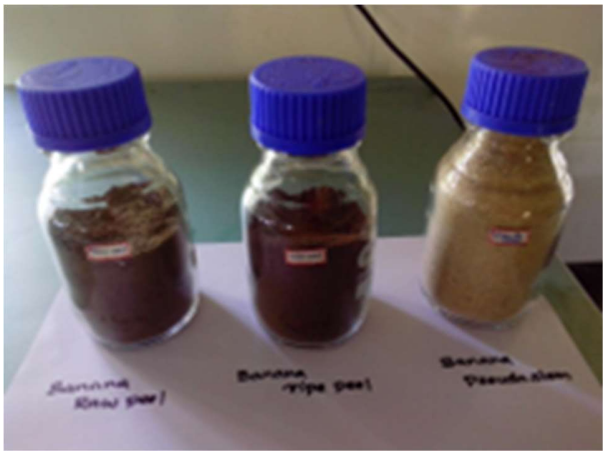

**Figure 1: Powdered banana samples**
**(a) Raw peel (b) Ripe peel (c) Pseudo stem**

The lignin content present in the sample was recorded by a gravimetric method (Lokapirnasari *et*

*al.,* 2015; Lu *et al.,* 2021). Acid or alkali treatment was performed by mixing 5 g of sample with 40 ml of 0.8 M $H_2SO_4$ or 2% $H_2O_2$, respectively, and incubating for 48 hr at room temperature (Le Tan *et al.,* 2021). The increase in the total amount of reducing and nonreducing sugars and the decrease in the amount of lignin were estimated.

**Isolation of native microorganisms and their characterization**

Cellulolytic microorganisms were isolated from degraded samples of banana peels. The morphological characteristics were determined. Cellulolytic activity was assayed using the DNS method (Dhanraj, 2014). One unit of enzyme activity was expressed as the quantity of enzyme required to reduce 1 µmol of glucose per minute under standard assay conditions (Lokapirnasari *et al.,* 2015). Molecular characterization of the microorganisms was also carried out (Das and Dash *et al.,* 2014). Genomic DNA was isolated from the culture, and PCR amplification reactions were carried out and sequenced.

**Bioethanol production using *Saccharomyces cerevisiae***

Pretreated and untreated samples were used for bioethanol production. Two grams of the oven-dried samples were placed in separate 150 ml screwed conical flasks. Then, 50 ml of distilled water was added, and the pH was adjusted to 7. Under sterile conditions, 3% (w/v) *Saccharomyces cerevisiae* was inoculated and incubated for 6 days at 37°C by continuous shaking in a shaking incubator at 60 rpm, as reported by Kulkarni *et al.* (2022). After incubation, the alcohol content was estimated. The end point of fermentation was determined by estimating the alcohol content at 48 h intervals until the reading was constant. The feedstock and fermenting microorganisms yielding more alcohol were selected for the enhancement process.

**Enhancement of bioethanol production using mixed culture of *Saccharomyces cerevisiae* and native microorganism**

Three different modes of saccharification, SHF, SSF and SBB, were tested to optimize the process for enhancing the production of bioethanol from the substrate to achieve the maximum bioethanol production. In SHF, an oven-dried banana raw peel powder was taken, 50 ml of distilled water was added, and the pH was adjusted to 7 and sterilized. Three percent (w/v) of the isolated native microorganism was inoculated into the sample for saccharification and incubated at 37°C in a shaking incubator at 60 rpm for 8 days. After incubation, 3% (w/v) *Saccharomyces cerevisiae* was inoculated into the sample, which was subsequently incubated for 6 days at 37°C in a shaking incubator at 60 rpm. After incubation, the alcohol content was estimated. In the SSF, 50 ml of distilled water was added to the oven-dried banana raw peel powder. The samples were inoculated with isolated native microorganisms and *Saccharomyces cerevisiae* under sterile conditions. The inoculated sample was incubated at 37°C at 60 rpm for 6 days. After incubation, the alcohol content was estimated. In SBB, the inoculated sample was incubated at 37°C for eight days at 60 rpm. After incubation, the sample was autoclaved to kill the native organism. Then, under sterile conditions, *Saccharomyces cerevisiae* was inoculated into the sample for fermentation. This mixture was again incubated for 6 days at 37°C and 60 rpm. After incubation, the alcohol content was estimated as reported by Mataix and De Castro (2000). There were five replications for each experiment, and the error was also calculated.

## Results and Discussion

**Preparation and pretreatment of feed stock**

The total reducing and nonreducing sugar percentages and lignin content of the feed stocks before pretreatment are presented in Table 1. The variation in the sugar content after pretreatment is shown in Table 2. The values showed that acid-pretreated feed stocks had significant variations in the total reducing and nonreducing sugar contents. The alkali-treated samples did not show any significant increase in sugar content (Table 1 and Table 2). The values given in Table 3 show that the percent increase in total reducing sugars varied significantly. The greatest increase in total reducing sugars was observed in acid-treated banana raw peels (12.00%). The lowest value was observed for the alkali-treated ripe banana peel (1.86%), which was on par with that of the alkali-treated banana pseudostem (1.97%). The lignin removal percentage was highest in acid-treated banana pseudostems (25.92%), which was on par with that in acid-treated banana raw peels (25.58%). The lowest lignin removal percentage was observed in

Alkali-treated ripe banana peels (3.76%). The above results revealed that the percent increase in total sugars and percent removal of lignin were greatest in acid-treated banana samples (Table 3). These values were significantly greater than those of the alkali-treated samples. Therefore, it was inferred that acid treatment was the best pretreatment method for determining banana biomass. According to Danmaliki *et al.* (2016), bioethanol production from banana peels was performed by using three different pretreatments, and it was reported that acid treatment was more effective than alkali and water treatment.

**Table 1: Total carbohydrate content, total reducing sugar content and total nonreducing sugar content in different feed stocks of banana before treatment**

| Feedstock Banana | Total carbohydrate content (%) | Total reducing sugar content (%) | Total nonreducing sugar (%) | Lignin content (%) |
|---|---|---|---|---|
| Raw peel | 39.28±0.9 | 18.12±0.8 | 9.85±0.3 | 8.4±0.5 |
| Ripe peel | 42.63±0.9 | 21.98±1.4 | 12.05±0.4 | 8.9±0.3 |
| Pseudostem | 15.33±0.9 | 6.75±0.4 | 3.14±0.12 | 15.4±0.4 |
| CD | 1.5 | 1.2 | 0.5 | 0.5 |

**Table 2: Total reducing sugar content, total nonreducing sugar content and lignin content of the banana samples after pretreatment with acid/alkali**

| | Feedstock (Banana) | Acid treated samples | Alkali treated samples |
|---|---|---|---|
| Total reducing sugar content (%) | Raw Peel | 20.6±1.0 | 18.8±1.2 |
| | Ripe Peel | 22.9±1.6 | 22.4±1.6 |
| | Pseudostem | 7.2±0.4 | 6.9±0.4 |
| | CD | 1.4 | 1.4 |
| Total nonreducing sugar content (%) | Raw Peel | 10.2±0.4 | 9.9±0.2 |
| | Ripe Peel | 12.4±0.5 | 12.2±0.4 |
| | Pseudostem | 3.27±0.21 | 3.21±0.21 |
| | CD | 0.6 | 0.5 |
| Lignin content (%) | Raw Peel | 6.27±0.5 | 7.82±0.58 |
| | Ripe Peel | 7.22±0.46 | 8.53±0.39 |
| | Pseudostem | 11.44±0.26 | 13.45±0.56 |
| | CD | 0.6 | 0.6 |

**Table 3: Percent saccharification and percent removal of lignin after pretreatment with acid/alkali**

| | Feedstock (Banana) | Increase in total reducing sugar (%) | Increase in total nonreducing sugar (%) | Removal of lignin (%) |
|---|---|---|---|---|
| Acid treated samples | Raw peel | 12.00±1.21 | 3.51±1.93 | 25.58±2.31 |
| | Ripe peel | 3.79±0.55 | 2.83±1.03 | 18.54±3.08 |
| | Pseudostem | 6.09±0.92 | 4.11±1.68 | 25.92±0.48 |
| Alkali treated samples | Raw peel | 3.55±1.34 | 1.17±0.72 | 7.13±1.89 |
| | Ripe peel | 1.86±1.06 | 0.89±0.37 | 3.76±1.20 |
| | Pseudostem | 1.97±0.19 | 2.07±0.84 | 12.94±1.35 |
| CD | | 1.6 | 1.4 | 2.7 |

**Isolation of native microorganisms and their characterization**

Cellulolytic microorganisms were isolated from degraded samples of raw banana peels. Yellow circular bacterial colonies were observed and are shown in Figure 2. The morphological characteristics of the isolated native microorganisms were studied by Gram's staining method. The bacterium was observed as pink-colored coccus-shaped cells after staining and was inferred to be gram-negative (Figure 2). The cellulolytic activity of the bacterial isolate was studied. It was observed that the microorganism has a slow rate of cellulolytic activity, and it was found to reach a maximum on the 8$^{th}$ day after inoculation. As cellulolytic activity was exhibited when the bacteria were grown on carboxy methyl cellulose agar plates, the organism was inferred to be *Nesterenkonia* sp. (Figure 3).

Molecular studies were carried out by bacterial DNA isolation and agarose gel electrophoresis (0.8%) of the extracted genomic DNA. The presence of good quality unsheared DNA bands on the gel is shown in Figure 4 a. Absorbance readings of the extracted genomic DNA by using a spectrophotometric method revealed good quality and quantity of DNA. The amplified products were obtained after PCR using 16S rRNA and 23S rRNA primers. Agarose gel electrophoresis (1.2%) of the amplified products revealed bands on the gel (Figure 4a and Figure 4b). Sequencing of the 16S ribosomal RNA was performed and analyzed using NBLAST. The results revealed that the bacteria showed maximum sequence similarity (99%) to the cellulolytic bacteria *Nesterenkonia* sp. EGI 80099. (Figure 5).

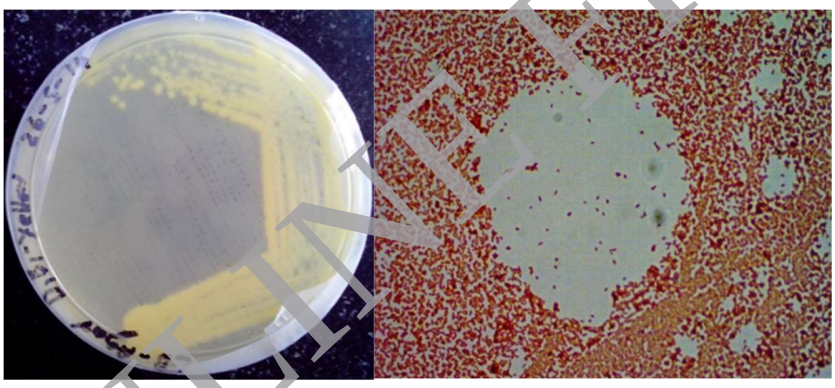

**Figure 2: Gram staining of native microorganisms isolated from banana waste**

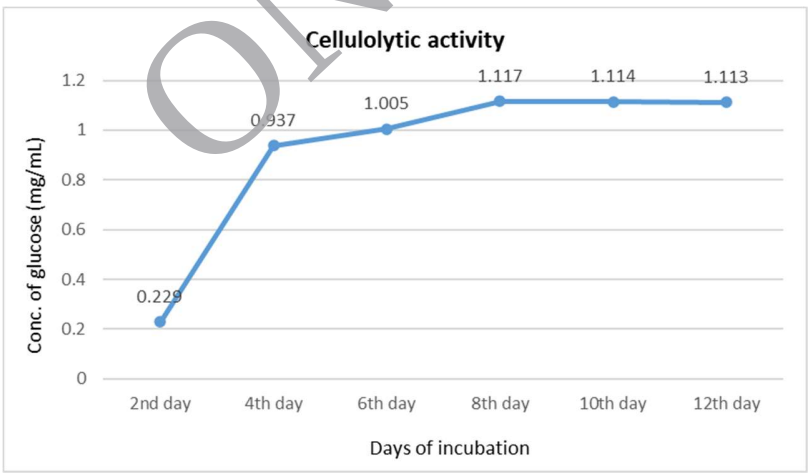


**Figure 3: The cellulolytic activity of native microorganisms isolated from banana biomass**

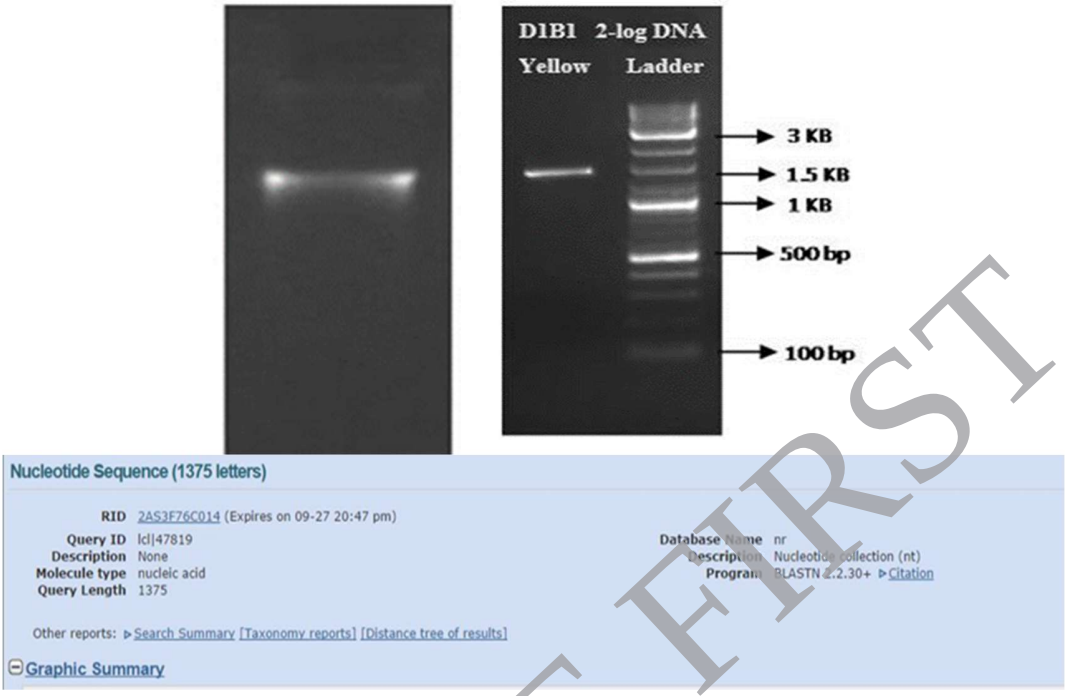


**Figure 4 (a) Bacterial genome (b) Amplification profile of isolated bacterial DNA using 16S rRNA RNA primer DNA**

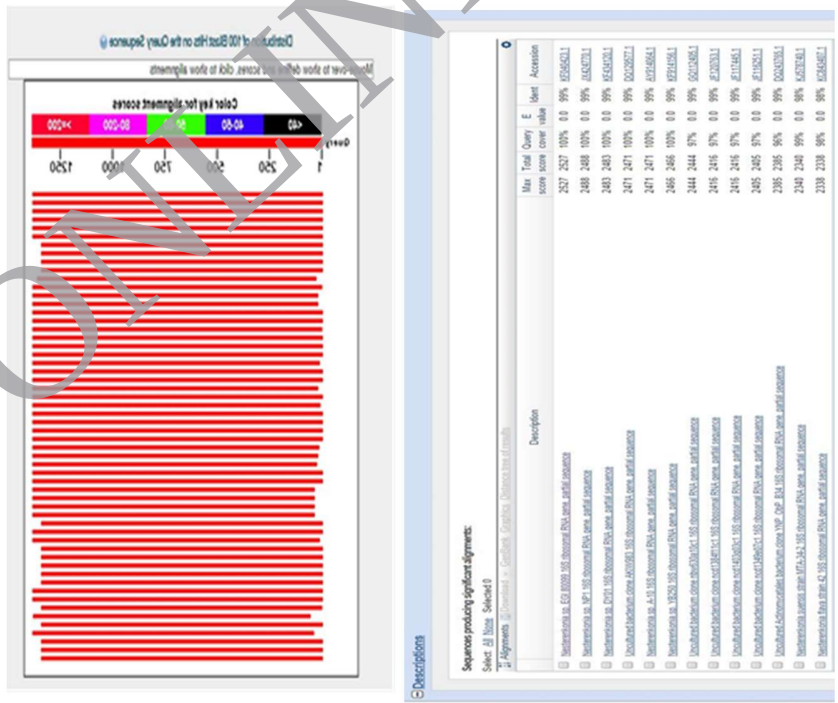

**Figure 5: Screenshot showing the result of Nblast**

**Bioethanol production using *Saccharomyces cerevisiae***

The percentage of ethanol produced after the banana samples were fermented with *Saccharomyces cerevisiae* are shown in Table 4. The percentage of ethanol obtained after fermentation showed that the samples without pretreatment differed significantly from the acid-treated and alkali-treated samples. The maximum percentage of ethanol was obtained for the untreated raw banana peel sample (6.63%), and the lowest percentage was observed for the alkali-treated banana pseudostem sample (0.82%). The percentage of total reducing sugars and total nonreducing sugars converted to ethanol were determined, and the values are given in Table 4. The highest percentage of conversion to alcohol was observed in the raw banana peel, which was 87.87% of the total reducing sugars. The lowest value of 47.12% was detected in the banana pseudostem. The maximum percentage conversion of total nonreducing sugars to alcohol was observed in the raw banana peels (45.90%), which was on par with that in the ripe banana peels (44.12%). The lowest value was observed for the banana pseudostem (16.88%) (Table 4). The initial sugar content and nonfermentable sugar content of the untreated banana feed stocks after fermentation using *Saccharomyces cerevisiae* were determined. The results shown in Table 5 revealed that the values showed significant variation. The production of bioethanol from banana peels using *Saccharomyces cerevisiae* was reported by Hamzah *et al.* (2019) to be more efficient at acidic pH values than at alkaline pH values (Table 5). The production rate was dependent on the type of yeast cells used. The percentage of bioethanol produced from banana peels using *Saccharomyces cerevisiae* was approximately 6.5% (Table 5). There are reports indicating that biofuel production from rotten banana using *Saccharomyces cerevisiae* at different agitation speeds produces variation in the bioethanol content, with a maximum of 6.35% (Cardona *et al.*, 2010; Dhanraj, 2014). Even though the acid pretreatment of the banana feed stocks improved the sugar content of the samples, the ethanol production was less than that of the untreated samples. This may be due to the production of inhibitors such as acetic acid, furfural and 5 hydroxy methyl furfural, which may hinder the growth of microorganisms, as reported by Benjamin *et al.* (2014). The results of the present study revealed that among the three feed stocks utilized in this study, the untreated banana raw peel sample exhibited the greatest conversion. Banana raw peel yielded 6.63% ethanol after fermentation, which was significantly greater than that obtained for ripe banana peel and pseudostem.



**Table 4: Ethanol production using *Saccharomyces cerevisiae* from untreated/pretreated feed stocks of banana biomass**

| Fermentation | Feedstock (Banana) | Ethanol % (Untreated samples) | Ethanol % (Acid pretreated samples) | Ethanol % (Alkali pretreated samples) |
|---|---|---|---|---|
| *Saccharomyces cerevisiae* | Raw peel | 6.63±0.18 | 5.22±0.39 | 4.60±0.81 |
| | Ripe peel | 4.26±0.19 | 3.50±0.23 | 3.16±0.24 |
| | Pseudostem | 1.65±0.21 | 1.29±0.44 | 0.82±0.18 |

**Table 5: Percentage of conversion of sugar to alcohol in banana biomass**

| | Feedstock Banana | Initial sugar content (%) | Nonfermentable sugar content (%) | Conversion to alcohol (%) |
|---|---|---|---|---|
| Total reducing sugar | Raw Peel | 18.12±0.8 | 2.19±0.2 | 87.87±1.4 |
| | Ripe Peel | 21.98±1.4 | 8.78±0.7 | 59.85±4.9 |
| | Pseudostem | 6.75±0.4 | 3.57±0.4 | 47.12±3.7 |
| | CD | 1.2 | 0.7 | 5.8 |
| Total nonreducing sugar | Raw Peel | 9.85±0.3 | 5.33±0.3 | 45.90±2.9 |
| | Ripe Peel | 12.05±0.4 | 6.73±0.5 | 44.12±4.1 |
| | Pseudostem | 3.14±0.1 | 2.61±0.2 | 16.88±0.9 |

**Enhancement of alcohol production from raw banana peels using mixed culture media (*Nesterenkonia* sp. and *Saccharomyces cerevisiae*)**

The cellulolytic bacterium *Nesterenkonia* sp. isolated from banana waste was utilized in mixed culture with *Saccharomyces cerevisiae* to enhance bioethanol production. SHF, SSF and SBB were the fermentation systems used. In this process, the stages were virtually the same, and both analyses were performed in the same bioreactor. In the present study, among the three fermentation methods, SBB produced the highest percentage of alcohol (6.88%), and the lowest percentage of alcohol was observed in SSF (5.88%). The probable reason might be that *Nesterenkonia* sp. inhibited the growth of *Saccharomyces cerevisiae* when cultured together in the SSF. Another possible problem in SHF is contamination. The hydrolysis process is rather long, and a dilute solution of sugar always has a risk of microbial contamination, even at rather high temperatures, such as 45-50°C. The highest percentage of ethanol from raw banana peels was observed when it was degraded using the cellulolytic bacterium *Nesterenkonia* sp. and fermented using *Saccharomyces cerevisiae* (6.88%). The higher yield in the raw peel when *Nesterenkonia* sp. was used might be due to the increased cellulose content in the raw peel (15.14%) compared to that in the ripe peel (9.97%). The percentages of ethanol produced by mixed cultures of *Saccharomyces cerevisiae* and *Nesternkonia* sp. are presented in Table 6. The results showed significant variation. The highest value was observed for single batch bioconversion (6.88%), and the lowest value was obtained for simultaneous saccharification and fermentation (5.88%) (Table 6 and Figure 6). The production of bioethanol from rotten banana fruit increased when the enzymes pectinase and cellulase acted along with the enzyme produced by *Saccharomyces cerevisiae*. Previous reports by Itelima *et al.* (2013) showed that bioethanol production from banana, plantain and pineapple peels by the SSF process resulted in a 3.98% yield of bioethanol from banana peels. According to Benjamin *et al.* (2014), a symbiotic coculture of the amylolytic fungus *Aspergillus niger* and the nonamylolytic fungus *Saccharomyces cerevisiae* produced 6.54% bioethanol, and the production rate increased with increasing fermentation time.

**Table 6: Ethanol production percentage using *Nesternkonia* sp. and *Saccharomyces cerevisiae* for the bioconversion of raw banana peels**

| Fermentation method | Ethanol percentage |
|---|---|
| Separate Hydrolysis and Fermentation | 6.43±0.14 |
| Simultaneous Saccharification and Fermentation | 5.88±0.19 |
| Single Batch Bioconversion | 6.88±0.06 |
| **CD** | 0.2 |

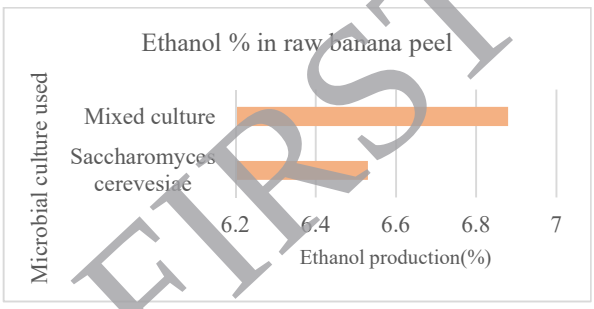


**Figure 6: Percentage of ethanol produced by the microbial culture**

## Conclusion

The present study is an attempt to produce cost-effective and environmentally sustainable ethanol using banana fruit and plant residues, which are major agro-wastes worldwide. Different pretreatments were applied, and acid treatment resulted in the release of more sugars, with values of 22.85% reducing sugars and 12.41% nonreducing sugars, from ripe banana peels. The values of the percentage of ethanol obtained after fermentation showed that the maximum percentage of ethanol was obtained for the untreated raw banana peel sample (6.63%), and the lowest percentage was observed for the alkali-treated banana pseudostem sample (0.82%) when *Saccharomyces cerevisiae* was used for fermentation. An attempt was made to increase the production of bioethanol from banana feedstock using a mixed culture of cellulolytic microorganisms isolated from the degraded samples of raw banana peels identified as *Nesterenkonia* sp. EGI 80099 and *Saccharomyces cerevisiae.* Among the fermentations, the highest value was observed for single batch bioconversion (6.88%), and the lowest value was obtained for simultaneous saccharification and fermentation (5.88%). This is the first report regarding the production of bioethanol using native microorganism along with *Saccharomyces cerevisiae.*

By optimizing the reaction parameters, it is possible to increase ethanol production from banana peel waste.

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## Conflict of interest
The authors declare that they have no conflicts of interest.

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