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European Journal of Experimental Biology, 2013, 3(4):247-253

European Journal of Experimental Biology

Pelagia Research Library

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Bio-ethanol production from cassava effluent using *Zymomonas mobilis and Saccharomyces cerevisiae* isolated from rafia palm (*Elaesis guineesi*) SAP

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ABSTRACT

The capability of local strains of Zymomonas mobilis and Saccharomyces cerevisiae isolated from rafia palm (Elaesis guineesi) sap to produce ethanol from cassava waste water was investigated. Maximum ethanol produced after 48h period of fermentation by Zymomonas mobilis was 15.0 and16.5(% v/wt) in acid and enzyme hydrolysates respectively.In case of Saccharomyces cerevisiae 2.9 and 2.84(%v/wt) ethanol were produced in acid and enzyme hydrolysates respectively. In the course of fermentation, pH of acid hydrolysates that were seeded with Zymomonas mobilis and Saccharomyces cerevisiae dropped from 5.5 to 4.41 and 5.5 to 4.9 respectively. In enzyme hydrolysates that were fermented with Zymomonas mobilis and Saccharomyces cerevisiae pH reduced gradually from 5.5 to 4.46 and 5.5 to 5.1 respectively as fermentation duration increased from 0h to 48h. Also, sugar concentration reduced from 63.94 to 30.09 (mg/g) and 63.94 to 9.40 (mg/g) in acid hydrolysates that were fermented by Zymomonas mobilis and Saccharomyces cerevisiae respectively. The sugar concentration of enzyme hydrolystes fermented by Zymomonas mobilis and Saccharomyces cerevisiae were 20.08 and 5.28 (mg/g) respectively at the end of fermentation duration. The study demonstrates the suitability of indigenous Z.mobilis and S. cerevisiae obtained from the sap of raffia palm for the production of ethanol. Therefore, saucing and harnessing the potentials of these organisms would greatly minimize ethanol production cost in our locality – the Niger Delta region of Nigeria.

Key words: Cassava, waste water, fermentation, ethanol, *Zymomonas, Saccharomyces.*

INTRODUCTION

Ethanol or ethyl alcohol has been described as one of the most exotic chemicals because of its unique combination of properties as a solvent, a germicide, a beverage, an anti-freeze, a fuel, a depressant and especially because of its versatility as a chemical intermediate for other organic chemicals.

Ethanol is made from a variety of agricultural products such as grain, molasses, fruits, whey and sulphite waste liquor. Fermentation of any material that contain sugar can derive ethanol. Whereas, there is global emphasis in ethanol production by fermentation processes, increased yield of ethanol production by microbial fermentation, depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology [1]. The technological behavior of industrial microorganisms is the main stay of industrial secret in fermentation industry and hence most microorganisms are patented and may not be available for use outside their country of origin [1] . Consequently, there is the need to source for indigenous and suitable strains. The characteristics of an ideal organism for ethanol fermentation [2], include:

i. Tolerance to high substrate concentration
ii. High sugar uptake and ethanol yield

iii. Tolerance to high ethanol
iv. Tolerance to low pH
v. Tolerance to low/no oxygen
vi. Amenability to genetic manipulation

This paper is aimed at evaluating the potentials of two local strains for ethanol fermentation and hence determining their suitability for commercialization

MATERIALS AND METHODS

Sample Collection/Isolation of Test Organisms

Cassava liquid waste was obtained from a cassava processing mill in Abraka, Delta State. A sterile 4L container was used for the collection of the cassava liquid waste. The container was placed under the pressing machine and liquid waste was allowed to drip into the container after which, it was transported to the laboratory and analysis carried out within 30 minutes of collection.

Fresh palm sap tapped from raffia palm tree (*Elaesis guineesi*) was obtained in pre – sterilized 1 liter container and immediately transported to the laboratory for the isolation of test organisms. The sample was allowed to stand for 12 hours before the isolation procedure commenced. One milliliter of sample was withdrawn aseptically and diluted using the ten-fold serial dilution technique and the pour plate method was employed in inoculating 0.1ml of dilutions ranging from 10^{-3} to 10^{-7}. Standard solid media with composition: yeast extract 5.0g, glucose 20g agar agar 20g and distilled water 1000ml as prescribed by [3] was adopted for the isolation of *Zymomonas mobilis* while malt extract agar was used for the isolation of *Saccharomyces cerevisiae.* Discrete isolates from standard solid media were subjected to Gram reaction and biochemical characterization according to the criteria described in Bergey's manual of determinative bacteriology [4] while isolates obtained from malt extract agar were subjected to the criteria of [5], for the identification of *Saccharomyces cerevisiae.*

Preparation of Standard Inoculum of isolates

A loopful of cells of *Zymomonas mobilis* and *Saccharomyces cerevisiae* was respectively inoculated into 100ml of standard broth medium and malt extract broth respectively. The broth containing *Zymomonas mobilis* was incubated at ambient temperature for 2 days in anaerobic gas jar while broth that contained *Saccharomyces cerevisiae* was incubated for 4 days. At the end of appropriate incubation period, cells were harvested by centrifugation at 4000rpm for 30 minutes using 800D centrifuge. Harvested cells were re-suspended in 100ml sterile physiological saline and respective total viable counts were performed. During this process the cultures were subjected to ten-fold serial dilution up to dilution factor of 10^{-8}. An amount (0.1ml) was inoculated by pour plate technique into appropriate media and incubated appropriately. The dilution that produced 100 – 200 colonies were chosen and served as standard inoculum for preliminary screening for ethanol tolerance.

Screening for Ethanol Tolerance

The method of [6] was adopted with little modification for the determination of tolerance to ethanol by the test isolates. Ethanol concentrations of 1, 5, 10 and 20 (%v/v) were prepared using sterile distilled water. One milliliter of each standardized inoculum was aseptically introduced into nine milliliters of various ethanol concentration contained in test tubes. Incubation followed at ambient temperature and anaerobically for both *Zymomonas mobilis* and *Saccharomyces cerevisiae.* Controls contained the appropriate test organism and distilled water only. At the end of 24h incubation duration, 0.1ml were aseptically withdrawn and plated onto appropriate freshly prepared agar medium using the pour plate technique [7]. Incubation under appropriate cultural conditions as described previously for *Zymomonas mobilis* and *Saccharomyces cerevisiae*, followed immediately. At the end of which colony counts were performed and percent log survival determined by the method of [8] and Log survival greater or equal to 70% were regarded as tolerant.

$$\% \text{ log survival} = (\log C / \log c)\ 100$$

Where, C = count in each ethanol concentration
c = count in control.

Analysis of Cassava Effluent (Waste Water)

The Association of Official Analytical Chemist [9] method was used in the respective determination of cyanide, pH, starch, glucose and protein content of cassava liquid waste

Sample hydrolysis
Two methods were employed in the hydrolysis of cassava waste water sample viz: acid hydrolysis using 0.6M H_2SO_4 and enzyme hydrolysis using α- amylase. The effluent was allowed to settle for 24 hours after which, the supernatant was decanted and slurry hydrolyzed appropriately. At the end of hydrolysis, pH of hydrolysate was adjusted to 5.5 using NaOH (0.2M).

Fermentation Procedure
The destructive experimental procedure was adopted. Thus, each hydrolyzed sample was dispensed into twelve 250ml Erlenmeyer flask in 100ml amounts. Standardized inocula of *Zymomonas mobilis* was seeded into six of the twelve flasks while the remaining six flasks received standardardized *Saccharomyces cerevisiae* inocula. All flasks, were incubated anaerobically at $25^oC±2$. Fermentation was allowed to proceed for a period of 48 hours irrespective of test isolate used. At intermittent intervals of 0h, 1h, 6h, 12h, 24h and 48h, pH, concentrations of glucose and ethanol produced were determined.

Determination of Ethanol Produced
On each analysis day, a flask was selected and entire content distilled using a rotary evaporator for the recovery of ethanol produced. Ethanol which evaporated at 78^oC was condensed and collected using a round bottom flask receiver and then measured.

RESULTS

The result of the physico-chemical analysis of the cassava liquid waste is as presented in Table 1. The concentrations of cyanide, protein, starch, glucose as well as pH were 42.84, 4.02, 21.81, 1.37 (mg/g) and 4.98 respectively. Enzyme hydrolysis resulted in 65.04mg/g glucose and 4.74mg/g starch while values of glucose and residual starch content obtained from acid hydrolysis were 64.84 and 4.72 (mg/g) respectively

Table 1: Physico-chemical composition of Cassava Liquid waste

Component	Composition (mg/g)		
	Raw Cassava Effluent	Acid hydrolysate	Enzyme hydrolysate
Cyanide	42.84	ND	ND
Protein	4.02	ND	ND
Glucose	1.37	63.94	55.4
Starch	21.81	4.72	4.74
pH	4.98	5.5	5.5

ND = Not Determined

The result of the biochemical characterization of the bacterial test organism is as shown in Table 2. Gram negative motile rods were suggestive of *Z. mobilis* and therefore, subjected to biochemical test as highlighted in Table 2 while Gram positive, oval shaped cells obtained from malt extract agar were indicative of *S. cerevisiae.*

The isolates obtained were screened for tolerance to the toxicity of ethanol at various concentrations and results obtained are as presented in Table 3. In this preliminary screening, percent log survival that ranged from 70 to 100 (%) was taken as tolerant. Both isolates proved to be ethanol tolerant from 1% to 5% (v/v). Only *Z. mobilis* was tolerant to 10% (v/v) ethanol. Ethanol tolerance by both isolates informed further use in fermentation exercise.

Table 2: Morphological and biochemical characteristics of *Z. mobilis*

Isolate	Cellular Morphology	Biochemical Test									
		Sucrose	Catalase	Oxidase	Urease	Carbohydrate fermentation	Motility	Maltose	Arabinose	Lactose	Glucose
Z. mobilis	Gram– rod	+	+	-	-	+ (AG)	+	-	-	-	+

+ = positive
- = negative
AG = Acid and gas production

Table 3: Response of Isolates to toxicity of ethanol

Isolate	Ethanol concentration (% v/wt)			
	1	5	10	20
Z. mobilis	+++	+++	+++	++
S. cerevisiae	+++	+++	+	-

+++ = ≥ 70% log survival
++ = 50 – 69 % log survival
+ = 30 – 49 % log survival
- = < 30% log survival

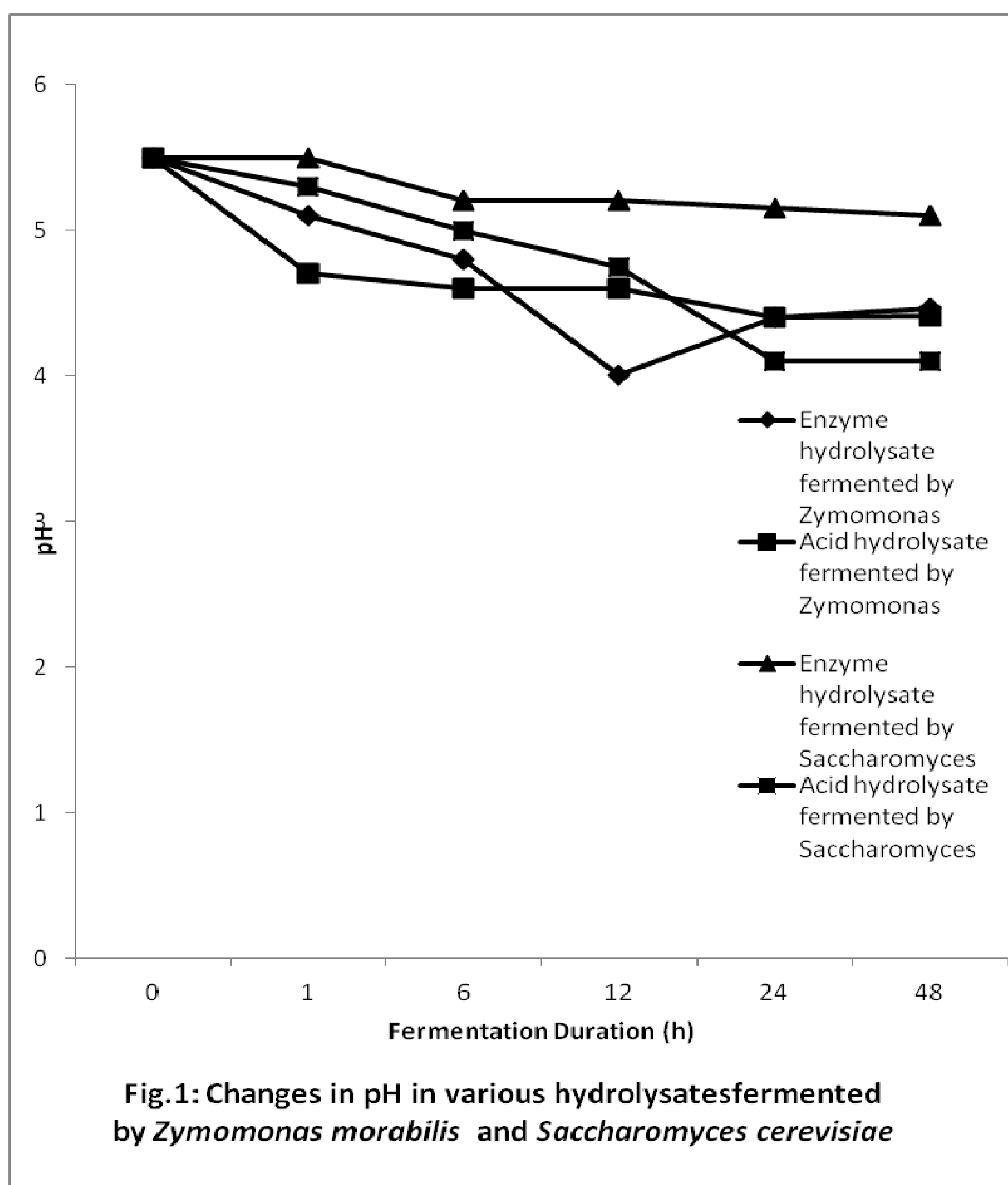

Fig.1: Changes in pH in various hydrolysatesfermented by *Zymomonas morabilis* and *Saccharomyces cerevisiae*

During fermentation, gradual reductions in pH of fermentative medium were recorded as duration of fermentation increased (fig. 1). In enzyme hydrolysates fermented by *Z. mobilis*, values dropped from 5.5 to 4.46. Similarly, pH values of acid hydrolysates containing *Z. mobilis* dropped from 5.5 to 4.41. In fermentors that received *S. cerevisiae* reduction in pH were 5.5 to 5.1 (enzyme hydrolysate) and 5.5 to 4.9 (acid hydrolysate).

Residual glucose concentrations obtained in enzyme hydrolysate fermented with Z. mobilis were 55.41, 41.23, 37.16 and 31.34, 25.02 and 20.08 (mg/g) at 0h,1h, 6h, 12h, 24h and 48h respectively. Corresponding values obtained in acid hydrolysate were 63.94, 48.06, 41.64 and 35.74, 31.00 and 30.09 (mg/g) respectively as depicted in fig.2. Also, residual glucose concentration in various fermentors containing *S. cerevisiae* reduced with increase in fermentation

duration. At the end of fermentation, values recorded were 5.28 mg/g in enzyme hydrolysate and 9.40mg/g in acid hydrolysate.

However, the amount of ethanol produced in both hydrolysates by *Z. mobilis* increased progressively as fermentation duration increased from 0h to 48h. Afterwards a gradual reduction was observed. Peak ethanol produced by *Z. mobilis* in enzyme and acid hydrolysates were 16.5 and 15 (%v/wt) respectively. Similar trends in ethanol production were observed in hydrolysates that contained *S. cerevisiae* but beyond 24h of fermentation, there were no noticeable increase in ethanol concentration rather a plateau was formed as shown in fig.3 indicating inability for further generation of ethanol. Maximum ethanol concentration obtained in these fermentors 2.84% v/wt (enzyme hydrolysate) and 2.9% v/wt (acid hydrolysate). There were significant difference between ethanol yield by *Z. mobilis* and *S. cerevisiae* at P> 0.05 confidence limit. Sugar conversion efficiency by *Z. mobilis* and *S. cerevisiae* were 46.7% and 5.67% respectively in enzyme hydrolyzed set – ups while in acid hydrolyzed set – up, the respective sugar conversion efficiencies were 44.31 and 3.89 (%).

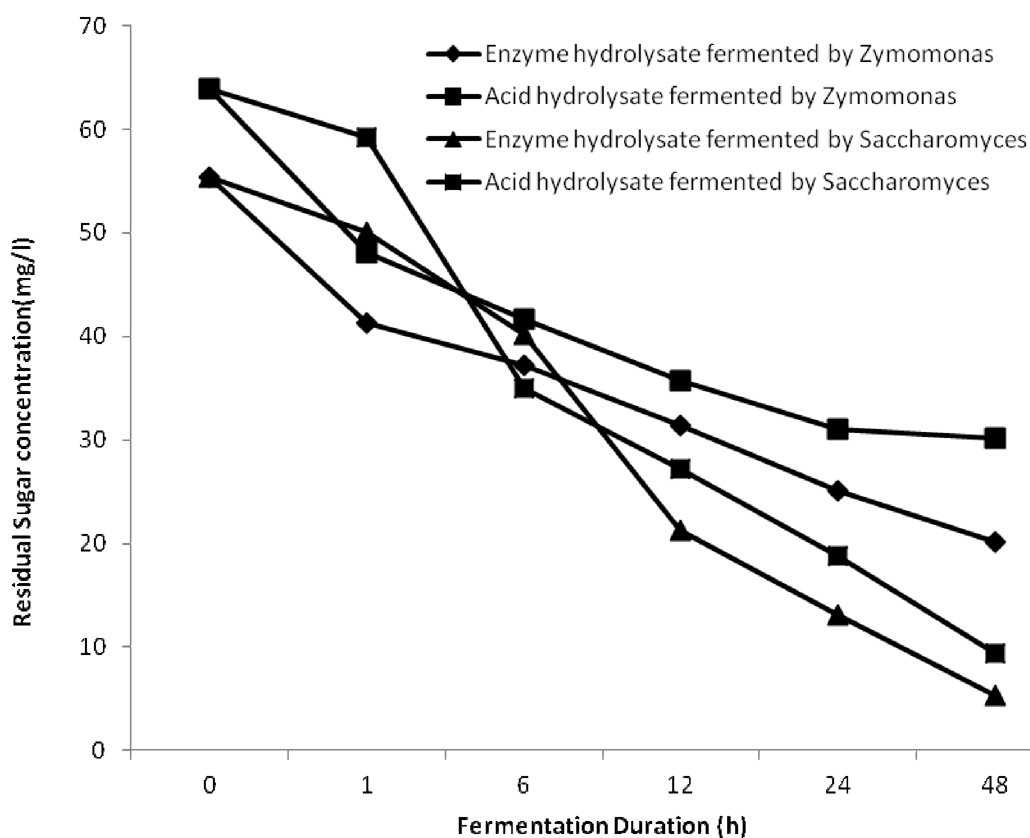

Fig. 2: Changes in sugar concentration in various hydrolysates fermentated by *Zymomonas morabilis* and *Saccharomyces cerevisiae*

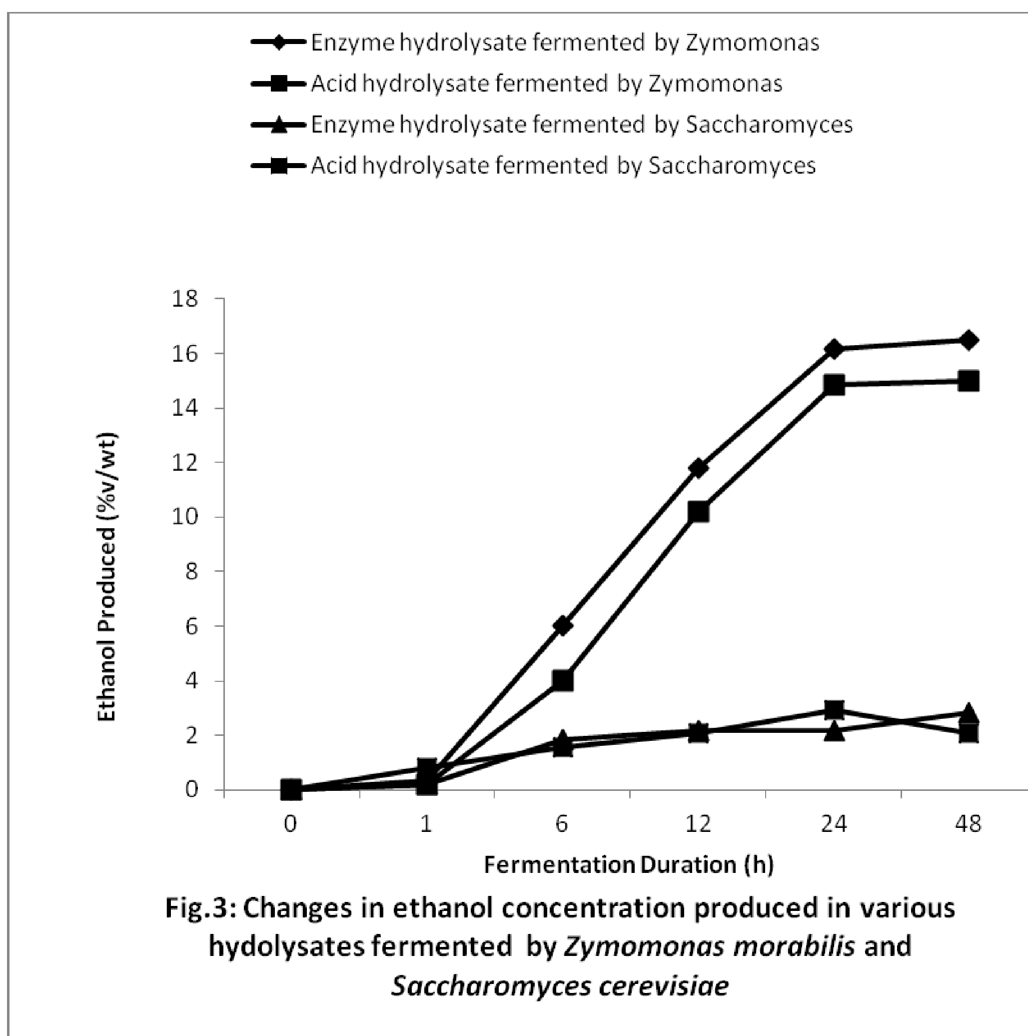

Fig.3: Changes in ethanol concentration produced in various hydolysates fermented by *Zymomonas morabilis* and *Saccharomyces cerevisiae*

DISCUSSION

Z. mobilis and *S. cerevisiae* isolated from raffia palm (*Elaesis guineesi*) sap were found to be successful tools in the production of ethanol in the order *Z. mobilis* > *S. cerevisiae*. The ability of the test isolate *Z. mobilis* to produce ethanol may be due to the fact that it has the ability to degrade sugar using the Entner – doudoroff pathway as well as high tolerance to ethanol [10]. The high tolerance to ethanol observed might be due to the characteristic occurrence of lopanoids in the cell membrane.

The results obtained on the fermentation activities of *Z.mobilis* and *S. cerevisiae* revealed that the amount of ethanol generated by *Z.mobilis* was about 500% more than that generated by *S. cerevisiae*. This difference in ethanol produced may be attributable to the ethanol tolerance of *Z.mobilis* in addition to its higher sugar uptake ability as well as lower biomass production [10]. The unique and dual presence of pyruvate decarboxylase and alcohol dehydrogenase in *Z.mobilis* might have facilitated the rapid conversion of glucose to ethanol. Simultaneous saccharification and fermentation of cassava starch using *Z.mobilis* and *S. cerevisiae* ATCC 26602 was investigated by [11]. They reported that fermentation by *Z.mobilis* was considerably faster than *S.uvaran* completing fermentation in 20h resulting in a yield 95% of the theoretical yield while *S.uvaran* required a period of 33h to complete fermentation resulting in a yield of 90% of the theoretical value.

In this study both *Z.mobilis* and *S. cerevisiae,* exhibited potential for ethanol production. However, they could be manipulated genetically for higher ethanol tolerance/production but more importantly, the study demonstrates the ethanol production suitability of indigenous *Z.mobilis* and *S. cerevisiae* obtained from the sap of raffia palm which is abundant in the Niger Delta region of Nigeria. Therefore, saucing and harnessing the potentials of these organisms would greatly minimize ethanol production cost in our locality.

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