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Bioethanol Production from *Anacardium occidentale* Fruit Juiceand Preparation of Blended Fuel Using In-house Developed Bioreactor

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Abstract Ethanol has been known for a long time, being perhaps the oldest product obtained through traditional biotechnology. In the present research work, bioreactor was designed to produce bioethanol from *Anacardium occidentale* using *Zymomonas mobilis* MTCC 2427. A minimum requirement bioreactor was designed for the mass production of ethanol. *Anacardium occidentale* substrate was pretreated, total and reducing sugar concentrations were determined and found to be 3.4 mg/mL and 1.8 mg/mL, respectively. During fermentation the bacterial load, sugar content and ethanol content were analyzed for five days, where the highest concentration of ethanol (28%) was obtained on the third day of fermentation and the corresponding bacterial load was $15x10^3$CFU. Ethanol obtained from the fermentation studies were further analyzed by FT-IR. The obtained ethanol was blended with petrol in different concentrations E5%, E10%, E15% and E20% respectively and successfully tested in four stroke engine.

Keywords: *Zymomonas mobilis MTCC 2427, bioethanol, bioreactor, fermentation, Anacardium occidentale, cashew apple*

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1. Introduction

Due to the diminishing fossil fuel reserves, the demand for an alternative fuel source has increased significantly in the last three decades [1]. Ethanol (C_2H_5OH) is a clear colorless liquid; it is biodegradable, low in toxicity. Ethanol is a renewable because it is primarily the result of conversion of the sun's energy into usable energy [2]. Bioethanol is an eco-friendly alternative liquid fuel that can be used as a blended fuel in two wheelers (motorbike) without any engine modification.A bioreactor can be any device or system that sustains a biologically active environment [3]. *Zymomonas mobilis*, a gram-negative bacterium, is considered as an alternative organism in large-scale fuel ethanol production [4,5]. *Z. Mobilis* has some good characteristics for producing ethanol namely its higher sugar uptake and ethanol yield, lower biomass production, higher ethanol tolerance, require no controlled addition of oxygen during fermentation and its amenability to generic manipulations [6]. *Zymomonas mobilis* is a promising candidate for large scale production of ethanol and posses more ethanol productivity and tolerance when compared to *Saccharomyces cerevisiae* [7,8] Selection of suitable and cheap substrate is an important cost component for industrial ethanol production. The cashew apple is an agricultural based product which has a high concentration of reducing sugars [9]. As well bioethanol is economically important alternate fuel source by which it can ensure India's energy security [10]. Therefore, the studywas carried out to attain both high productivity and efficient recovery of bioethanol from broth of *Zymomonas mobilis* utilizing *Anacardium occidentale* as a substrate by an environmentally benign process. Furthermore, substrate processing and bioethanol detection was closely investigated. Emphasis was given to the design of suitable bioreactor for large scale production of ethanol from *Anacardium occidentale* using *Zymomonas mobilis* MTCC2427.

2. Materials and Methods

2.1. Bioreactor Design

The bioreactor vessel is made up of borosil glass which is autoclavable and withstands temperature up to 400°C. Thus working volume of the bioreactor was 3 L and the working volume was up to 2 LDiameter of the vessel (DT) was 60 cm and its height was measured to be 25 cm. Vessel's high to diameter ratio was 0.4 (25cm/60 cm). Vessel was equipped with one impeller made of Teflon and fixed to 20 cm long, stainless steel shaft. The shaft was made up of stainless steel and hence it was corrosion resistant. The shaft length was 20 cm and its diameter was

0.5 cm. The diameter of the impeller (Di) was 8 cm. Hence, DT /Di ratio was 7.5. The tip speed of the bioreactor was chosen in such a way that the speed is greater than 2.5 m/s. The motor has a power of 1/8 horse power. So the maximum attainable tip speed for such an agitator was 2400 rpm. To adjust the tip speed, a regulator was provided on the motor and based on the requirement, the speed was adjusted. Fish tank motor was connected to the aeration channel to aerate the fermentation medium. Thermometer was fitted through a manhole present in the reactor to monitor the temperature. Control of other parameters like pH, and substrate concentration was attained by collecting samples through a vent which was provided on the lid. Also the vent served as an outlet for excess air from the vessel.

2.2. Selection of Substrate

The fruit *Anacardium occidentale* (cashew apple) was obtained from Pudhu kuppam, village near the sea shore in Puducherry, India. The fruits were transported to molecular microbiology research laboratory, VIT University, Vellore. The substrate was processed by utilizing waste cashew apples and cut into slices in order to ensure a rapid rate of juice extraction. The fruit juice was boiled in stainless steel pans at temperature 85°C in order to eliminate the wild yeast. The juice was clarified by addition of gelatin. Tannins and suspended solids are removed from juice. The clarified juice was filtered and treated with sodium Metabisulphate. The total and reducing sugars were estimated by anthrone and DNSA test [11].

2.3. Bacterial Culture

Zymomonas mobilis MTCC 2427 culture was obtained from microbial type culture collection (MTCC), IMTECH, Chandigarh – 160036, INDIA.

2.4. Inoculum Preparation

Growing cultures of *Zymomonas mobilis* MTCC 2427 wastransferred to rich medium containing 4% (w/v) glucose, 2 % (w/v) yeast extract, 0.4 % KH_2PO_4, and pH 6.0.The brothculture was monitored for an OD in the range of 0.9 –1.0 at 610 nm. When the OD value of the broth culture was in the desired range, 10 mL of the inoculum was transferred to 90 mlRM broth and incubated at 30°C for 48 h in ashaker at 150 r/min.

2.5. Ethanol Fermentation in Bioreactor

One litre of the cashew apple fruit juice was taken in fermentor and 100 mL of the inoculum was added and incubated at 30°C for 3 days. Total and reducing sugars, colony forming units and ethanol concentration were determined.

2.6. Distillation and Calcium Oxalate Treatment

The fermented broth was subjected to distillation process in a round bottom flask and the distillate was subjected to calcium oxalate treatment. The recovered ethanol was determined using potassium dichromate method [12].

2.7. Fourier Infra-Red Transform Spectroscopy

To determine the presence of functional groups in the product the control and liquid samples were analyzed.An infrared spectrum of the crude extract wasobtained using a FT-IR spectrometer (FT/IR AVATAR 330). The spectra was collected within a scanning range of 400-4000/cm and the experimental sample was scanned [13].

2.8. Blended Fuel

Various percentages of ethanol were mixed with gasoline (E5%, E10%& E15%). 5mL of ethanol was mixed with 95mL of petrol to make E5 (95% gasoline and 5% ethanol). 10mL of ethanol was mixed with 90mL of petrol to make E10 (90% gasoline and 10% ethanol), likewise E15% was prepared. Blended fuel prepared was tested as fuel to run motor cycles.

3. Results

In the present study minimum requirement bioreactor was designed and utilized throughout the study for fermentation studies. The schematic design of the bioreactor are shown (Figure 1) The pure culture of *Zymomonas mobilis* MTCC 2427 maintained on RM medium showed the morphological characteristics of gram negative, rod-shaped non motile bacterial cells. The waste cashew apples brought from Puducherry wassubjected to substrate processing. Further the total and reducing sugars were estimated. The total sugar content of the pretreated juice sample before fermentation was found to be 3.7 mg/mL. The reducing sugar content of the pretreated juice sample before fermentation was found to be 1.8 mg/mL. After five days of fermentation in bioreactor, the bacterialload was determined. It was found to be maximum ($15x10^3$CFU) on third day. 28% of ethanol was obtained on the third day of fermentation. However, prolonged incubation resulted in reduction of ethanol concentration (Table 1).

Figure 1. Bioreactor

Table 1. Count of colony forming units (CFU/mL) of *Zymomonasmobilis* MTCC 2427 on different days of fermentation

Incubation period (day)	Colony forming units (CFU/mL)
1st	$3.6 \times 10^3 \pm 0.52$
2nd	$7.0 \times 10^3 \pm 0.54$
3rd	$15 \times 10^3 \pm 0.23$
4th	$6.0 \times 10^3 \pm 0.47$
5th	$4.3 \times 10^3 \pm 0.51$

The total and reducing sugar content after fermentation was found to be 3.4 mg/mL and 1.8 mg/mL respectively. From day two it was gradually decreased to 2.6 mg/mL and 1.6 mg/mL. A complete decrease in total and reducing sugar was observed on day three. It was well evident that on day 3 sugar content was highly utilized by the organism (Table 2, Figure 2).

Table 2. Ethanol estimation during fermentation

Sampling date (day)	Concentration of ethanol (%)
1st	12.3±0.54
2nd	17±0.52
3rd	28±0.43
4th	26±0.87
5th	25±0.62

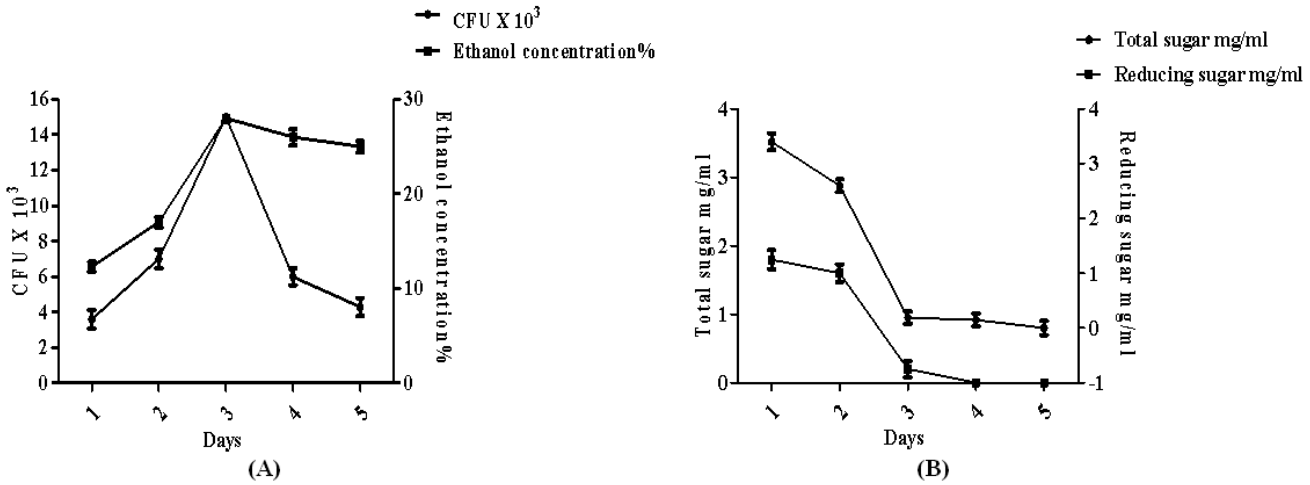

Figure 2. (a) Bacterial count and ethanol concentration after fermentation. (b) Comparison of total and reducing sugar after fermentation

3.1. FTIR Analysis

The FTIR spectrum for the ethanol (CH_3CH_2OH)was more complicated. It has a CH stretch, an OH stretch, a CO stretch and various bending vibrations. The OH stretch appears as a broad band at approximately 3300-3500 cm^{-1}.By comparing the band position and the intensities observed for standard ethanol. A broad OH stretch was found at 3464 cm^{-1} wave length. This indicates the presence of OH group(Figure 3).

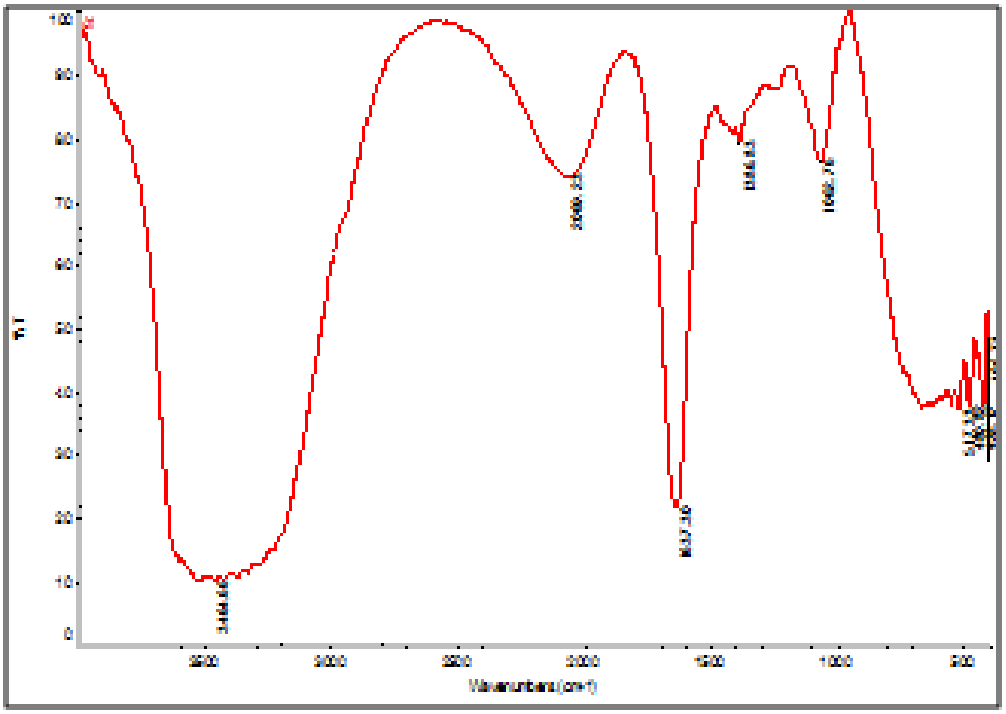

Figure 3. FT-IR spectra obtained for ethanol produced by fermentation

3.2. Blended Fuel

The blended fuel prepared was tested in running Hero Honda splendor plus motor cycle. The findings revealed that there was no difficulty in starting the engine or running the vehicle.

4. Discussion

Current research approaches have been directed towards the development or the improvement of continuous bioreactor designs due to improvements in the

volumetric productivity and the ability to maintain stable environments for maximum microbial rates. *Z. mobilis* is an obligate fermentative bacterium of industrial interest for ethanol production [14]. This organism has been reported to be capable of converting sugar with greater ratesby several fold increase [15] and has a higher ethanol tolerance than *S. cerevisiae* [16].The organism ferments glucose to ethanol and carbon dioxide at a level of up to 98% of the theoretical yield (two ethanol molecules per glucose molecule), achieving high concentrations of ethanol that are higher than those achieved by *Saccharomyces cerevisiae* [17] (98% in *Z. mobilis* and 86% in *S. cerevisiae* from 25% glucose. Similarly the present study showed highest concentration of ethanol which was obtained at the third day of fermentation with bacterial load of$15x10^3$. This level of metabolic activity is made possible by high-level expression of the glycolytic and ethanologenic enzymes, which account for approximately 50% of the soluble proteins [18].

In view of the backdrop of rising crude oil prices, depletion of resources, political instability in producing countries and environmental challenges in recent years, biomass fuels, like ethanol produced by microorganisms, have a promising future [19,20,21,22]. *Zymomonas mobilis* presents some advantages when compared to yeasts: 98% efficiency in ethanol production and a specific rate twice bigger [23]. Researches indicate that *Zymomonas mobilis* ferment sugar from cashew fruit extract into ethanol [24,25]. The observations of the cashew apple are also supported by other reports showing highest ethanol in 24 h with 33.02 g/L of ethanol with 90.19% of its yield. Hence ethanol is a liquid biofuel produced from sugar rich biomass and ethanol can be blended up to 20% with diesel or petrol [26].

5. Conclusion

Hence the research works from agricultural based substrates enhanced production and serveto be more economical and alternative substrate for the production of bio ethanol and could prevent the environment to stop the negative impact.

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Conflict of Interest

Mohanasrinivasan, Anand, SubathraDevi, JemimahNaine, Karthikeyandeclare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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