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Fermentation of Sugarcane Molasses Using Zymomonas Mobilis for Enhanced Bioethanol Production

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ABSTRACT

Bioethanol is one of the leading alternative biofuel to fossil fuels and can be use in existing gasoline engines without any major modification. Bioethanol can be produced from sugar containing biomass fermentation using different potential strains. In this study, the gram negative, facultative anaerobic, rod shaped strain '*Zymomonas mobilis*' was used as microorganism to produce bioethanol from sugar cane molasses using anaerobic fermentation. The study was conducted to investigate the optimized conditions for production of bioethanol through batch fermentation process. The fermentation unit was designed to determine the effect of process parameters such as fermentation temperature, pH, sugar concentration and supply of nutrients. The *Zymomonas mobilis* produced 9.3% (v/v) bioethanol by utilizing 16 g/100mL sugar with the fermentation efficiency 92.5%. The fertilized based nutrients were supplied to enhance the production of bioethanol yield. The bioethanol yield produced by using this strain in optimized conditions is in good compromise with previous study and also compared with commercially available yeast strain.

Keywords:
Fermentation, Sugarcane molasses, Bioethanol, Zymomonas mobilis

1. Introduction

The depletion of fossil fuels has caused a significant increase in the oil prices since last two decades [1, 2]. The energy crises are distinct threat to the sustainable development in third world countries. With reference to current energy scenario, scientific community needs to pay special attention on alternative or renewable fuels, such as biofuels as the feedstock is widely available [1, 3-5]. Among the biofuels, bioethanol is seeking attention day by day and widely produced as an important transportation fuel worldwide [6-8]. The literature records that the bioethanol can be used in substitution of gasoline in existing engines or it can be blended, producing low emissions of greenhouse gases (GHG) [9-12]. Bioethanol can be produced by utilizing the biomass, molasses, or

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any lignocellulosic material with the help of microorganisms [13, 14]. Molasses as feedstock for bioethanol production is widely available in sugar producing regions and agriculture base countries [11, 15]. Molasses is bioproducts of sugar industry, if this by-product is utilized for bioethanol production, it can contribute a huge flux in transportation sector as well as in energy sector [16-18].

Molasses is a by-product of sugar industry having significant quantity of sugar, around 40% to 50 % (w/v), and ash content around 5-15%, making it a suitable substrate for rum and bioethanol production since many years [19, 20]. The utilization of sugar cane molasses for fermentation process is one of the oldest chemical process known to human. This practice is also widely adopted for bioethanol production [21]. In recent years, however many of the products are synthesized chemically from petroleum feedstock including bioethanol often cost effectively. It depends upon the behaviour of the microorganism during fermentation and to get the useful products like bioethanol [22, 23]. From last three decades, studies were carried out to minimize the issue in the fermentation technology for efficient bioethanol production. The yeast (*Saccharomyces cerevisiae*) has been used widely in commercial scale but gram negative facultative anaerobic bacteria (*Zymomonas mobilis*) which has not been commercially practiced yet due to some constraints. *Zymomonas mobilis* has some advantages over yeast as studied by Sadik *et al* [24-26]. However, *Saccharomyces cerevisiae* has its own limitations as compared to *Zymomonas mobilis* [1, 27, 28]. Different studies were conducted to sort out the concerned issue in bacteria fermentation[27, 29]. *Zymomonas mobilis* has strength to uphold high content of sugar as well as higher temperature than yeast. Yeast cell are start degrading after 33 ° C while *Zymomonas mobilis* can tolerate up to 40 ° C. The cell growth in low pH is also adventurous property of bacteria [30, 31].

In this study, the *Zymomonas mobilis* were used for the anaerobic fermentation of sugar cane molasses in lab-scale. Furthermore the factors effecting in the bioethanol production were investigated such as pH, sugar concentration, fermentation temperature and nutrients supply. The study was developed for the optimum yield of bioethanol production by using *Zymomonas mobilis*.

2. Methodology

2.1 Materials

Sugar cane molasses was provided by Noon Sugar mills Pvt. Limited Bhulwal Pakistan. The strain *Zymomonas mobilis* was used in this study. *Zymomonas mobilis* (DSM 424) was procured from DSMZ Germany. *Saccharomyces cerevisiae* procured locally to compare with *Z.mobilis*. All chemicals used in this study were industrial grade and from Sigma Aldrich.

2.2 Bacteria (Zymomonas mobilis) medium

Zymomonas media suggested by DSMZ Germany, bacto-peptone 10 g/L, yeast extract 10 g/L, 15 g/L agar for agar plates, glucose 20 g/L, distilled water mark up to 1000 mL and with pH 7.00 [15][28]. Media was autoclaved at 121°C for 45 minutes , streaking was done on agar plates in Desktop Laminar Flow, and was placed in incubator (Syno Japan) for overnight. The growth cells were analysed by Hemocytometer (China) [7] and were inoculated into test tubes for scaling up and also stored in glycerol stock for preservation. The 250 mL molasses was taken in a conical flask for pre-fermentation with the sugar concentration of 7g/100 mL inoculated with 5mL growth media. The cells were counted using Hemocytometer. The desirable cell growth was achieved and used for further up scaled fermentation.

2.3 Batch fermentation and bioethanol detection

The lab scale experimental setup is presented in Fig 1. The known amount of sugar cane molasses and grown media were taken in fermenter and kept in shaker for fermentation. An anaerobic condition was maintained and kept for 48 h. The strain converted sugar into bioethanol with the evolution of CO_2. The samples were tested after 48 h, to investigate the effect of sugar concentration, pH, fermentation temperature, and supply of nutrients. 5 mL of fermented sample was taken and pinch of potassium dichromate and few drops of concentrated H_2SO_4 were added. The brownish color of sample was changed into green that indicated the presence of bioethanol. Furthermore, the identification of bioethanol was identified using GC-FID Agilent (6890).

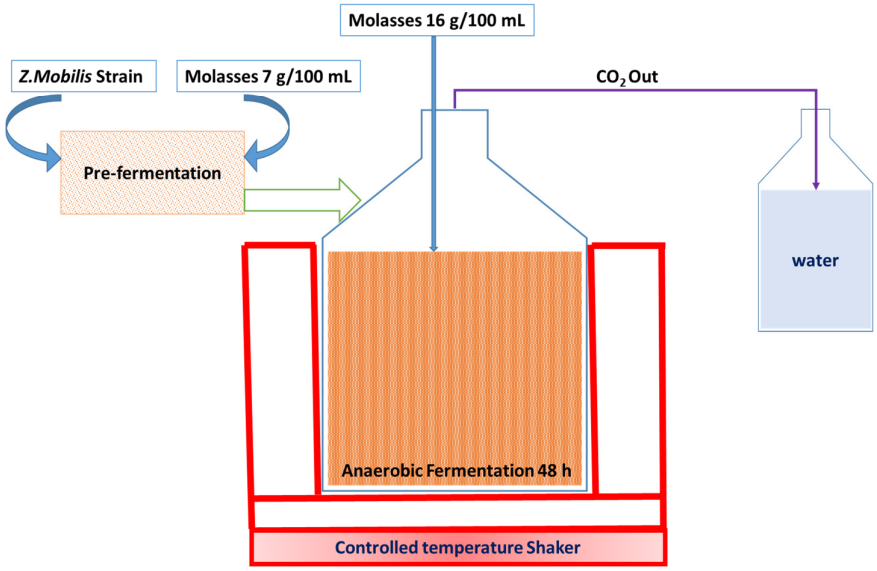

Fig. 1. Laboratory scale batch fermentation experimental setup

2.4 Analysis for total sugar concentration in molasses

The sugar concentration in molasses was determined through *Fehling's* test [16][17][28]. The 5 mL molasses sample was taken dissolved in 100 mL of distilled and 5 mL of concentrated HCl and was heated at 70 °C for 10 minutes and then it was neutralized by adding NaOH. It was prepared to 1000 mL and then taken into burette solution. The 5 mL of *Fehling A* and *Fehling B* solution were taken and mixed with 10 mL of distilled water in a conical flask and methylene blue indicator was added. The solution was titrated in boiling conditions until the blue color disappeared. The sugar concentration was calculated by Eq. (1).

$$TS = \left[\frac{DF \times FF}{TV} \times 100 \right] \quad (1)$$

where *TS* = Total sugars, *DF*= Dilution factor, *FF*=Fehling factor and *TV*=Titrate value. The second method to find the sugar concentration is the use of portable Refractometer (RHB 32ATC Japan). It

has limitations, and it can measure the maximum concentration of sugar up to 16 gm/100 mL. Total sugar found in used molasses was 47%.

2.5 Determination of bioethanol concentration and pH

The different bioethanol concentrations were determined by Ebulliometer (J. SALLERON DUJARDIN Sr. PARIS) which is approved in commercial distilleries and it determined on the basis of volatility [32]. The boiling point of water, used in the process was set as reference temperature. The pH has been determined by pH meter handy (EZDO 6011 Japan). For acidity test, 10 mL fermented sample was taken in beaker and put in the stirrer and pH meter dipped in this sample to check the pH. The sample was taken in burette, titrated the sample by 1M NaOH solution till its pH reached to 7.00. The reading was noted and multiplied by 0.69, which is equivalent weight of sulphuric acid. The acidity values were calculated by this method. The fermentation efficiency (FE) was calculated by the given Eq. (2).

$$FE = \left[\frac{Actual\ yield}{Theoretical\ yield} \times 100 \right] \quad (2)$$

3. Results and Discussion

3.1 Effect of pH

The samples were fermented using *Zymomonas mobilis* in different pH ranges from 4.0 to 6.0 to obtain the maximum yield of bioethanol by adding diluted sulphuric acid. The sugar concentration and temperature were kept constant. Anaerobic conditions were applied and the fermented samples were analysed after 48 hours. Table (1) show the bioethanol yields and fermentation efficiency. The maximum yield 7.9% (v/v) was achieved on pH 5.0 and fermentation efficiency was recorded 88% demonstrated in Table (1). While lowering the pH depicts less yield and production of acids, which was confirmed by acidity test. The pH higher than 5.4 revealed a smaller amount of yield were recorded. The observation in the study was also supported by literature and maximum yield can achieved at 4.8 to 5.4 pH [30, 33]. *Zymomonas mobilis* can produce optimum yield in the high pH as compared to commercial available yeast [31]. The elevated tolerance towards pH depicts that *Zymomonas mobilis* will consume fewer sulphuric acid to maintain the pH in fermentation. As we know that the molasses pH is range in between 5.0 to 6.0. It is a major challenge to maintain pH in the fermentation. This study also reveals that the optimum pH for *Zymomonas mobilis* is in the range of 4.0 to 4.6 [6, 24].

3.2 Effect of fermentation temperature

The samples were maintained in the optimum pH 5.0 for *Zymomonas mobilis* with the fermentation temperature ranging from 28 °C, 30 °C, 32 °C, 34 °C, 36 °C and 38 °C demonstrated in Table (2) The samples were kept for fermentation for 48 h, after analysing the samples it was found that the optimum yield for bioethanol was achieved at 34 °C. At that temperature bioethanol yield was 8 % (v/v) along with the fermentation efficiency of 88.96 % presented in Table (2). The increase in temperature increased bioethanol yield till 34°C, furthermore, the yield decreased with increasing fermentation temperature. The effect of temperature for bacterial fermentation was studied by [30] and [34], both recorded 35 °C as optimum temperature. *Zymomonas mobilis*

produced the maximum yield at high temperature, it was observed that it can be used in a high temperature fermentation process for the conversion of non-reducible sugars. The effect of fermentation temperature were studied by [18] and found 32 °C as optimum for *Zymomonas mobilis* [6, 30]. The values may be varying because during fermentation the temperature increases due to exothermic reaction [31, 35].

Table 1
Effect of pH on fermentation process using *Zymomonas mobilis* (ZM 424)

pH	Sugar Conc. (g/100 mL)	Fermentation temperature (°C)	Acidity	EtoH yield (v/v)%	FE %
4	14	33	8.1	4.4	49
4.2	14	33	6.3	6.5	72
4.4	14	33	5.2	6.8	75
4.6	14	33	4.9	7.1	79
4.8	14	33	4.2	7.7	85
5	14	33	4	7.9	88
5.2	14	33	6.5	6.2	69
5.4	14	33	7.5	4.7	52
5.6	14	33	6.3	2.2	24
5.8	14	33	13.8	0.0	0

Table 2
Effect of the fermentation temperature on bacterial fermentation using *Zymomonas mobilis* (ZSM 424)

Fermentation Temperature (°C)	pH	Sugar Conc. (g/100 mL)	Acidity	EtoH yield % (v/v)	FE %
28	5	14	7.4	3.1	34.5
30	5	14	5.2	5.2	58
32	5	14	4.4	7.6	84.5
34	5	14	4.1	7.9	87.6
36	5	14	6.7	5.8	64.5
38	5	14	8.1	2.2	25

3.3 Effect of sugar concentration

Sugar molasses concentration was set from 10g/100 mL to 18g/100 mL by keeping the pH and temperature constant (pre-optimized). Increasing sugar concentration the bioethanol productivity enhanced and optimum at 16g/100 mL with 8.9 % (v/v) with the fermentation efficiency (FE) of 87 %, after this increase in concentration yielded lesser quantity of the product Table (3). Above analysis indicated that the *Zymomonas mobilis* has capability to tolerate the higher sugar content with better efficiency. [30] studied the sugar concentration effect 20 g/100 mL but the enzymes were used for optimum yield. Due to enzymes production quality, the *Zymomonas mobilis* tolerated higher sugar concentrations [31].

Table 3
Effect of the sugar concentration on bacterial fermentation of *Zymomonas mobilis*

Sugar Conc. (g/100 mL)	Density of molasses	pH	Fermentation Temperature °C	Acidity	EtoH yield % (v/v)	FE %
10	1.079	5	33	6.2	4.70	73
12	1.097	5	33	5.9	6.2	80.4
14	1.116	5	33	5.2	7.8	86.7
16	1.135	5	33	4.4	8.9	87
18	1.155	5	33	9.4	5.5	50

3.4 Effect of nutrients

Fadel *et al.,* [20] studied the effect of different nutrients in an anaerobic fermentation. Nutrients are quite effective in the production of bioethanol from sugar cane molasses. Di-Ammonium phosphate (DAP) was supplied to the fermentation with pre-optimum parameters. The contribution of the DAP and urea in the process were 1:1. Different nutrients can be supplied for the fermentation process, the most effective nutrients found are DAP and Urea reported by Fadel *et al*., [20]. But their quantity has been adjusted according to our set parameters. The yield increases with the addition of nutrients in the fermentation process with a notable efficiency. Table (4) shows that the 2g/L dose was found as best quantity to get the optimum yield of 9.3 % (v/v) with the fermentation efficiency 92.5%. The addition of nutrients was effective for higher sugar concentrations, no more effective in low concentration of sugar.

Table 4
Effect of the nutrients (DAP) in the fermentation using *Zymomonas mobilis*

Nutrients (DAP) (g/L)	pH	Sugar Conc. (g/100 mL)	Fermentation Temperature °C	Acidity	EtoH yield % (v/v)	FE %
1	5	16	33	4.4	8.9	86.6
2	5	16	33	4.1	9.3	92.5
3	5	16	33	4.2	9.1	88.6

4. Conclusions

The sugarcane molasses batch fermentation were carried out using *Zymomonas mobilis* in an anaerobic conditions. The effect of different parameters such as pH, sugar concentration, fermentation temperature and nutrients supply were investigated for bioethanol production using *Zymomonas mobilis*. The optimal condition achieved for *Zymomonas mobilis* based anaerobic fermentation as 9.3 % (v/v) bioethanol has been produced with efficiency of 92.5%, supplied sugar concentration of 16g/100 mL, pH 5.0 and fermentation temperature is found 34°C. Furthermore 2 g nutrients were supplied for this for this process to get optimum yield (9.3% v/v). The effect of nutrients were significant and enhance the fermentation efficiency in the anaerobic process. The *Zymomonas mobilis* has capability to use as commercial strain, as it tolerate high temperature and pH as compared to conventional yeast.

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