

Full Length Research Paper

Comparative study of bioethanol production from sugarcane molasses by using *Zymomonas mobilis* and *Saccharomyces cerevisiae*

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The study was designed to compare the bioethanol production from *Zymomonas mobilis* and *Saccharomyces cerevisiae* using molasses as production medium. The focus was on the retention time at lab scale. Bioethanol and petroleum blend can be used in existing gasoline engines. Present study showed a more cost-effective procedure for production of ethanol from sugar-cane molasses by using bacterial strain "*Z. mobilis*". Laboratory scale unit was designed to perform the experiments through batch fermentation and to determine the impact of leading parameters, including fermentation temperature, pH, sugar concentration, and nutrients. *S. cerevisiae* produced 8.3% (v/v) bioethanol provided sugar concentration 14 g /100 ml with the fermentation efficiency of 92.5%. On the contrary, *Z. mobilis* produced 9.3% (v/v) bioethanol by utilizing 16 g/100 ml sugar with the fermentation efficiency of 90.5%. Effect of nutrients on fermentation was determined using molasses as feedstock. Thin layer chromatography was also performed to assess the possible impurities in molasses as compared to the pure sugar. The pH and fermentation temperature was optimized for the enhanced yield of bioethanol.

Key words: Bioethanol, molasses, fermentation, *Zymomonas mobilis*, *Saccharomyces cerevisiae*.

INTRODUCTION

Nowadays, petroleum products are running out of race due to unbalanced relation between supply and demand, also escalations in the oil prices for the last two decades are contributing to set trends for the use of alternative

resources. Pakistan imports million tons of oil every year to meet their energy constraint (US Department of Energy, 2014). Referable to the current scenario of energy, Pakistan needs to pay special attention to

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alternative fuels using feedstock like biomass and surplus molasses which are cheaper source of energy in some developing countries. Among the biofuels, bioethanol is very impressive and leading fuel produced in the different parts of the world (Mousdale, 2011). The literature records that the bioethanol usage cause low emissions of greenhouse gases (GHG) (Lee and Shah, 2012). Ethanol can be produced by utilizing the biomass, molasses, or any lignocellulosic material with the help of microorganisms. In this study, the sugar industry molasses was used as feedstock, which is widely available in sugar producing regions (Ayhan, 2008). Pakistan is producing 2 to 2.5 million tons of molasses every year, and 80% of molasses are being exported every year (PMSA, 2013) and if it is locally used in Pakistan for the production of bioethanol, it may be able to harvest 2 to 3% transportation fuel annually (Ali, 2013). Molasses is a byproduct of the sugar industry, has a significant quantity of sugar 40 to 50% (w/v), ash content of 5 to 15%, which is used as a substrate in the rum and bioethanol production from many years (Doelle and Doelle, 1990). The utilization of sugar cane molasses for the treatment method such as fermentation which is one of the oldest chemical processes known to human and most widely practiced by them is used to produce a variety of valuable chemicals (Mousdale, 2011).

In recent years, however, many of the products are synthesized cost effectively from petroleum feedstock, including bioethanol. The use of microorganisms is usually considered as environment friendly. The efficiency and specificity of the microorganism are an advantageous aspect to produce targeted products like bioethanol (Balat et al., 2008). From last three decades, studies were carried out to minimize the issues in the fermentation technology for efficient bioethanol production (Balat et al., 2008; Bulock et al., 1984). *Saccharomyces cerevisiae* (*S. cerevisiae*) was used widely in commercial scale, but *Zymomonas mobilis* (*Z. mobilis*) was not commercially used currently due to some constraints. Sadik et al. (2014) has reported that *Z. mobilis* has some advantages over *S. cerevisiae* with respect to time required for the completion of the fermentation process with targeted yield (Belkis et al., 1998). Diverse studies were conducted to sort out the issues in fermentation process by using yeast and bacteria (Banks and Aswad, 2013). In this study, the factors effecting the bioethanol production has been investigated for the optimum yield of bioethanol in prime conditions using molasses from local sugar mills in Pakistan.

MATERIALS AND METHODS

Material and culture

Sugar cane molasses were provided by Noon Sugar mills Pvt. Limited Bhulwal Pakistan. The two strains used in the study were *Z. mobilis* and *S. cerevisiae*. *Z. mobilis* was purchased by DSMZ

Germany and a local dealer in Islamabad provided the yeast strain. The media for maintaining *Z. mobilis* culture as suggested by DSMZ Germany (2013) contain bacto-peptone (10 g/L), yeast extract, 10; 15 g/L agar for agar plates, glucose (20 g/L) with pH 7.00. Media was autoclaved at 121°C and 15 psi, for 20 min. To obtain single bacterial colonies, bacterial cells were isolated on the Petri plates by standard streak plate method under sterilized conditions and incubated at 37°C for overnight. Bacterial cells were characterized by Dichromate test and Potassium permagnate tests. The cells were inoculated in 5 ml sterilized Luria Bertani (LB) media. Number of cells were calculated by hemocytometer. *S. cerevisiae* was grown in YNPG media (Bergman, 2001) containing yeast extract (5 g/L), peptone (10 g/L), NaCl (10 g/L), glucose (10 g /L), Agar (15 g/L) for agar plates at pH 7.00. The media was sterilized at 121°C, 15 psi for 20 min. Yeast culture was streaked on YNPG agar plates by Streak Plate method and incubated at 37°C for overnight. Inoculation into test tubes for scale up and cell count was conducted. *S. cerevisiae* was maintained in glycerol stock for further use.

Analysis for total sugar concentration and impurities in molasses

Sugar concentration in molasses was determined through Fehling's test (Thorpe, 2002). Five (5) mL molasses sample was dissolved in 100 ml of distilled water and 5 ml of concentrated HCl was added to it, and heated at 70°C for 10 min. 1 M NaOH solution was taken in burette; added into the solution till neutralized; then the burette reading was recorded; its titrate value (TV). Five (5) mL of Fehling A and Fehling B solution was taken, mixed with 10 ml of distilled water in a conical flask and methylene blue indicator was added. Solution was titrated with a burette solution in boiling conditions until the disappearance of blue color and the burette reading notified as Fehling factor (FF). Fehling's "A" contains 7 g CuSO₄.5H₂O dissolved in distilled water containing 2 drops of dilute sulfuric acid. Fehling's "B" contains 35 g of potassium tartrate and 12 g of NaOH in 100 ml of distilled water. The sugar concentration was calculated by following formula:

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

TS = Total sugars, DF = Dilution factor (Dilution of molasses for sugar concentration), FF = Fehling factor, TV = Titrate value, Total sugar concentration was also determined by a second method using portable Refractometer RHB 32ATC, Japan. Total sugar found out to be 47%.

Impurities were analyzed by Thin Layer Chromatography (TLC) using water and hexane as a solvent. Two homogenous solvent chambers were maintained, one containing water as solvent and other containing hexane as solvent. Two spots of solution containing molasses were placed on two separate silica gel coated plates, about 1.5 cm from the bottom edge, allowed to dry completely and then placed in the chambers maintained with solvents. The solvent moved up the plate by capillary action, met the molasses mixture, and carries the soluble constituents up the plate. Then, the plates were removed from the chambers before the solvent front reaches the top of the stationary phase and dried.

Development of molasses inoculum for batch fermentation

One hundred (100) ml of concentrated molasses containing the sugar concentration of 7 g/100 ml were taken in a conical flask and inoculated with 5 ml of overnight grown inoculum of bacteria and yeast separately. The whole assembly was placed in incubator set

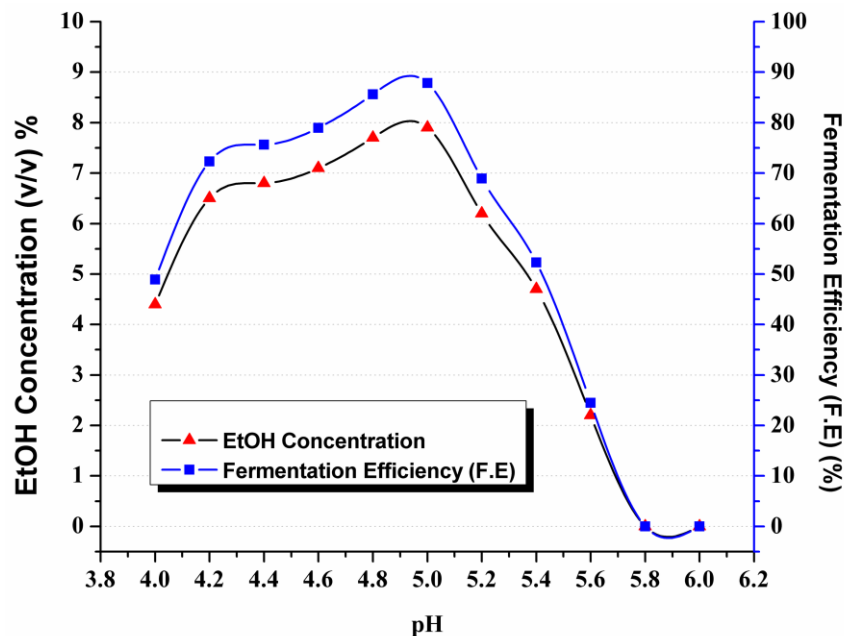


Figure 1. Effect of pH for bacterial (*Z. mobilis*) fermentation.

at 37°C overnight. Cell counts were conducted for both samples by hemocytometer; desirable growth was achieved and used for fermentation.

Batch fermentation

The known amount of sugar cane molasses and growth media was taken in fermentation flask, was inoculated and kept in shaker for fermentation. Anaerobic conditions were maintained for two days, and strains converted sugar into bioethanol with the evolution of CO₂. Samples were tested after 48 h, similar techniques were applied to *S. cerevisiae* to investigate the effect of sugar concentration, pH, fermentation temperature, supply of nutrients and effect of impurities in molasses.

Identification of bioethanol

Five (5) ml fermented sample was taken and pinch of Potassium dichromate, and few drops of concentrated H₂SO₄ were added. The brownish color of sample was changed into green which indicated the presence of bioethanol.

Determination of bioethanol concentration and pH

Ebulliometer (J. SALLERON DUJARDIN Sr. PARIS) which was approved in distilleries (US Department of Commerce, 1974) determined the bioethanol concentration, based on volatility. The reference temperature has been recorded for water, which was used in the process. The pH was determined by pH meter handy (EZDO 6011 Japan).

Acidity test

Ten (10) ml of fermented sample was taken in beaker. The beaker

was put in the stirrer and its pH was checked. The sample was taken in a burette, titrated the sample by 1 N NaOH solution until its pH reached to 7.00. The reading was noted and multiplied by 0.69, which are equivalent weight of sulphuric acid. The output obtained was acidity.

Fermentation efficiency

The fermentation efficiency was calculated by the given formula:

$$FE = (\text{actual yield} / \text{theoretical yield}) \times 100$$

FE = fermentation efficiency.

RESULTS AND DISCUSSION

Effect on pH

To obtain maximum yield of bioethanol, samples were fermented in different pH ranges from 4.0 to 6.0. The sugar concentration, cell density, and temperature were kept constant. Anaerobic conditions have been applied, and the fermented samples were analyzed after 48 h as reported (Hadiyanto et al., 2014). Figure 1 shows the result for *Z. mobilis*, maximum yield 7.9 (v/v) has been achieved in range of 5.0 pH with the fermentation efficiency of 88%. Adjustment of the pH using acid/base may cause the lower yield and production of acids that has been affirmed by acidity test however, maximum productivity was observed at 5.0 to 5.5 pH (Doelle and Doelle, 1990; Yanase et al., 2005). On the other side, the same parameters were set for *S. cerevisiae* fermentation as well and found the trends as shown in Figure 2. It

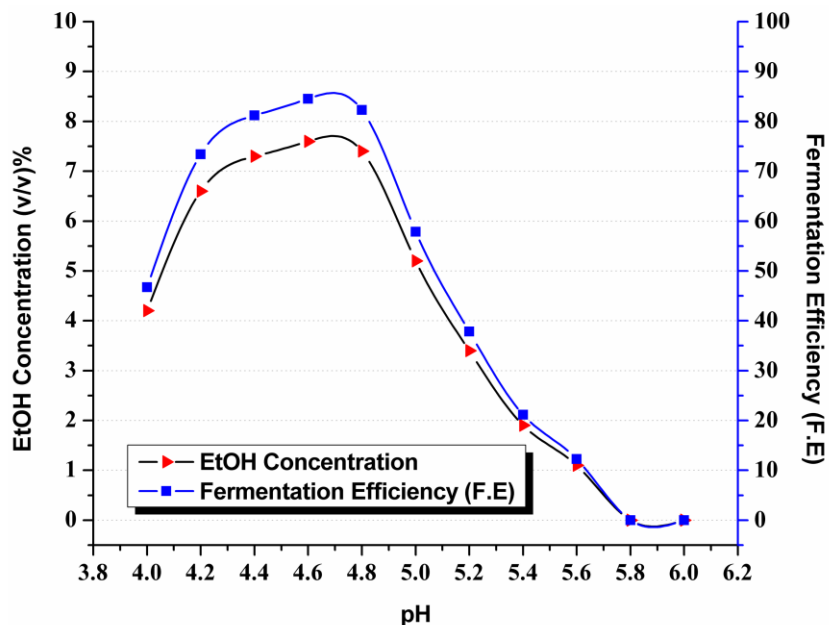


Figure 2. Effect of pH for yeast (*S. cerevisiae*) fermentation.

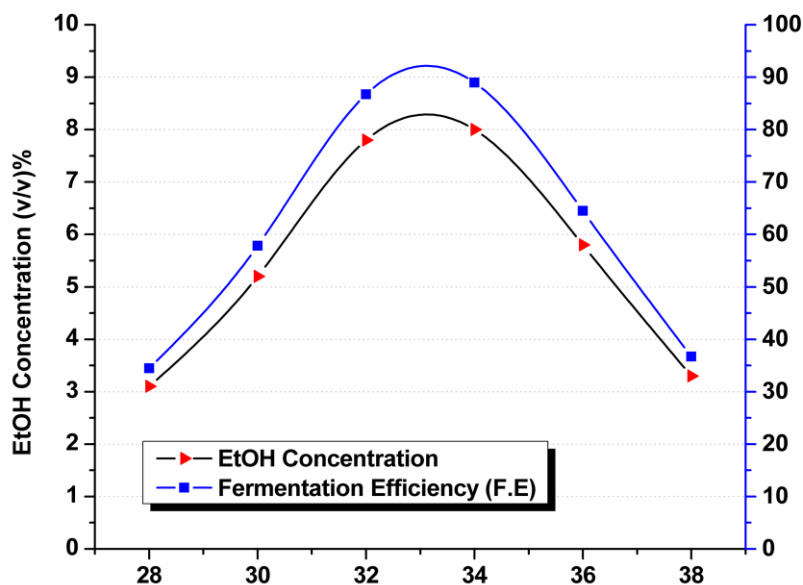


Figure 3. Effect of fermentation temperature (*Z. mobilis*).

shows that increasing pH causes increase in bioethanol yield until pH 4.6, further increase in pH cause decreases in bioethanol yield. A yield of 7.6(v/v) was achieved in 4.6 pH with the fermentation efficiency of 88%. It is clear from above assessment that the *Z. mobilis* can produce optimal yield in the high pH as compared to *S. cerevisiae*. The current studies also reveal that the optimum pH for yeast is in the range of 4.0 to 4.6 (Hemamalini et al., 2012) (Nigam, 1999). The fermentation time was also investigated and it was recorded that the optimum yield of bioethanol was achieved at 30 to 36 h of incubation for

Z. mobilis and 48 to 60 h for *S. cerevisiae*.

Effect of fermentation temperature

Samples were maintained in the optimum pH 5.0 for *Z. mobilis* and pH 4.6 for *S. cerevisiae* with the temperature range from 28, 30, 32, 34, 36 and 38°C as shown in Figures 3 and 4. The samples were fermented for 48 h; after analyzing the samples it found that the optimum yield was achieved in 34°C for *Z. mobilis* where the

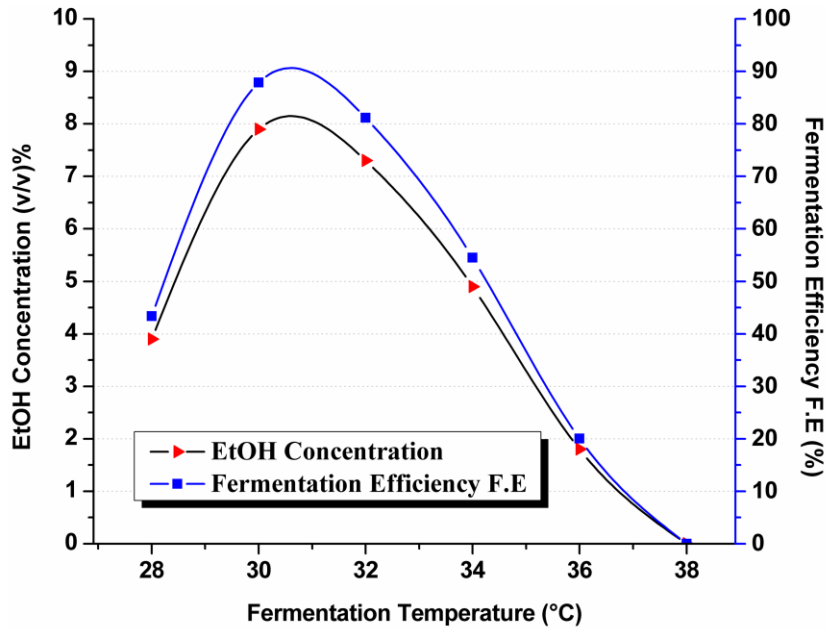


Figure 4. Effect of fermentation temperature (*S. cerevisiae*).

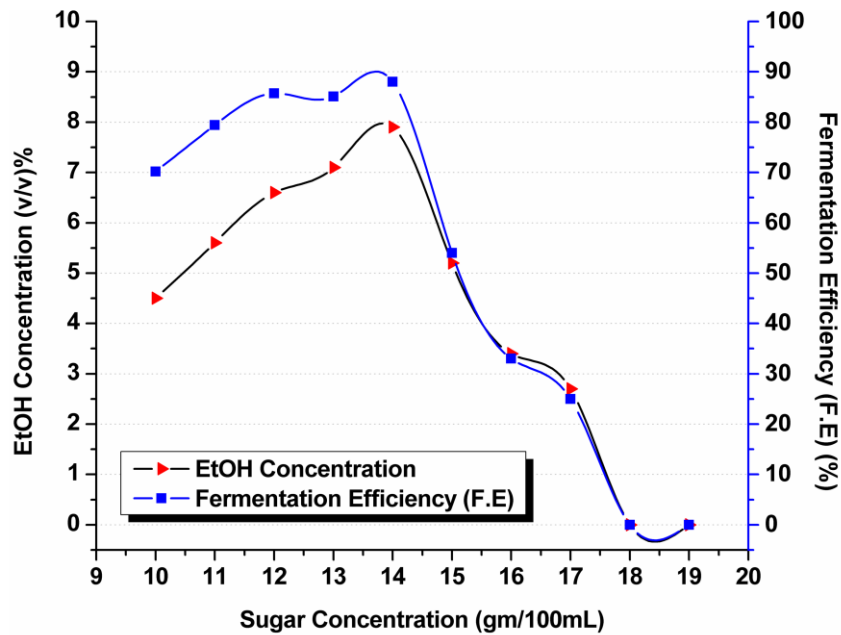


Figure 5. Effect of sugar concentration on yeast (*S. cerevisiae*) fermentation.

bioethanol yield was 8.0% (v/v) with the fermentation efficiency of 88.96%. By increasing temperature, increased bioethanol yield was observed until 34°C. Furthermore, the yield decreases by further increasing fermentation temperature. The effect of temperature for bacterial fermentation was studied before by Doelle and Doelle (1990) and Panesar et al. (2007); they recorded

35°C as optimum temperature. Similarly, the *S. cerevisiae* showed, the maximum yield of 7.9%(v/v) at 30°C with fermentation efficiency of 87.85% as shown in Figure 5. The Figure reflects that the temperature is a very sensitive parameter for *S. cerevisiae* because it produced maximum yield at 30°C but bioethanol production declined at higher temperature due to the

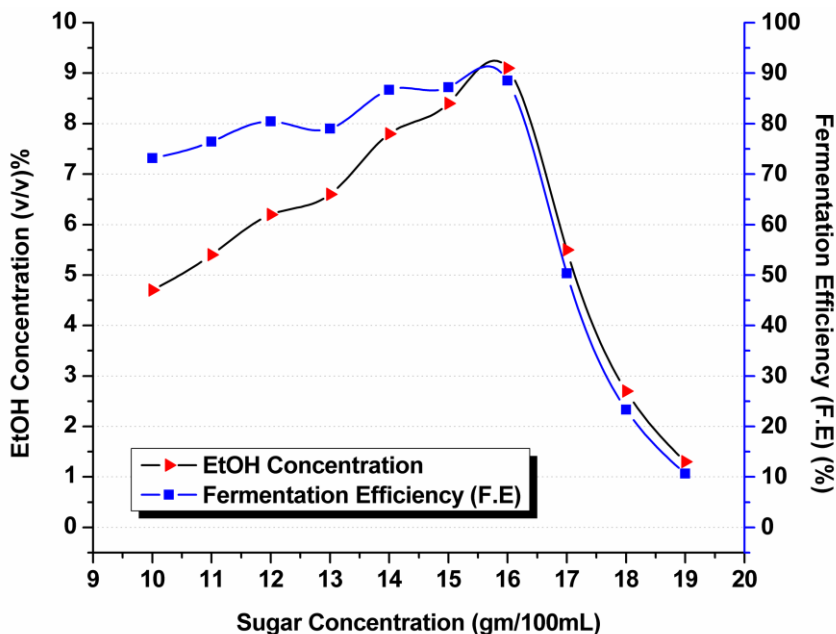


Figure 6. Effect of sugar concentration on bacterial (*Z. mobilis*) fermentation.

denaturation of *S. cerevisiae* cells. *S. cerevisiae* was unable to tolerate the elevated temperature, due to this factor in summer; the yield of bioethanol production is quite low in distilleries. On the contrary, the *Z. mobilis* produced the maximum yield in high temperature. From these results, it was observed that the *S. cerevisiae* was suitable for low temperature process while *Z. mobilis* can be used in regions having an elevated-temperature process. Kopsahelis et al. (2007) studied the effect of fermentation temperature and found 30°C as optimum in case of yeast fermentation. Kirk and Aswad (2013) followed the same method and found that the optimum yield can be obtained at 32°C for yeast fermentation though exothermic reaction values may differ from process to process because of rise in temperature during fermentation (Table 2).

Effect of sugarcane molasses concentration

Sugar molasses concentrations were varied from 10 g/ to 18 g/100 ml for both *S. cerevisiae* and *Z. mobilis*, keeping the pH and temperature constant (pre-optimized). In the case of bacteria (Figure 6) increasing sugar concentration caused the enhanced bioethanol productivity and reached optimum at 16 g/100 mL with 8.5% (w/v) with the fermentation efficiency of 81%, after the yield goes down by increasing concentration.

Effect of nutrients

Nutrients are quite effective in the production of bioethanol

from sugar cane molasses (Fadel et al., 2013; Cazetta et al., 2007). Di-Ammonium phosphate (DAP) and urea was supplied to the fermentation with pre-optimum parameters. The contribution of the DAP and urea in the process was 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. (2013). However, their quantity was adjusted according to set parameters in experiment. Yield increased with the addition of nutrients in the fermentation process with a notable efficiency in case of *S. cerevisiae*. Table 1 show that the 2 gm /L was found as best quantity to get the optimum yield of 9.30%(v/v) with the fermentation efficiency 90.5% for *Z. mobilis* while, yeast produces 8.3%(v/v) with the fermentation efficiency of 92.3%, which is considered as more yield than bacteria. Using nutrients as supplement, the *S. cerevisiae* showed more increments in the yield as compared to *Z. mobilis*.

Effect of impurities in fermentation

To check the effect of impurities in fermentation, an experiment was conducted, in pre-optimized conditions, which were already conducted and results were observed. Using pure sugar as a substrate instead of molasses, (Table 3) results were affirmative. Thin Layer Chromatography (TLC) analyzed the impurities and find whether there are any impurities that inhibited the bioethanol production, and suppress the activity of enzymes (Cazetta et al., 2007). While, utilizing pure sugar the bioethanol yield for *S. cerevisiae* is 8.6% (v/v) with fermentation efficiency of 95.5%. *Z. mobilis*

Table 1. Effect of nutrients (DAP+Urea) in bacterial fermentation.

DAP+Urea (g/L)	p.H	Sugar conc. (g/100 ml)	F. temp°C	Acidity	EtOH Yield (v/v)	F.E %
1	5	16	33	4.4	8.9	86.6
2	5	16	33	4.1	9.3	90.5
3	5	16	33	4.2	0.1	88.5

Table 2. Effect of nutrients (DAP+Urea) in yeast fermentation.

DAP+Urea (g/L)	p.H	Sugar conc. (g/100 ml)	F. temp°C	Acidity	EtOH Yield (v/v)	F.E %
1	4.6	14	30	4	8.3	92.3
2	4.6	14	30	5.1	7.9	87.9
3	4.6	14	30	4.7	8	88.9

Table 3. Effect of impurities in fermentation.

Microorganism	Nutrients (g/L)	p.H	Sugar conc. (g/100 ml)	F. temp °C	Acidity	EtOH Yield (v/v)	F.E %
<i>S. cerevisiae</i>	1	4.6	14	30	4.1	8.6	95.5
<i>Z. mobilis</i>	2	5	16	33	4.2	9.6	93

Table 4. Effect of fermentation time.

Microorganisms	Concentration of EtOH (v/v)	Fermentation time (h)	F.E (%)
<i>S. cerevisiae</i>	8.67	52	95.6
<i>Z. mobilis</i>	9.59	33	93

produced 9.6%(v/v) with the efficiency of 93%. It can be concluded that impurities in molasses may influence on enzymatic activity and yield can be enhanced using some enzyme stabilizers or some agents/additives, which may nullify the effects of impurities. Thin layer chromatography of molasses also reveals that the impurities are water-soluble only and cannot be dissolved in hexane.

Effect of fermentation time

For optimum yield, the fermentation time found was given in Table 4 while applying the pre-optimized parameters.

Conclusion

The optimized conditions were found by analyzing different parameters for *Z. mobilis* and *S. cerevisiae*. The optimum condition for bacteria was recorded as 9.3%(v/v); bioethanol can be produced with efficiency of 90.5%, at sugar concentration of 16 g/100 mL, pH 5.0 and fermentation temperature of 34°C. Two (2) g/L

nutrients (DAP, urea) were supplied to get the optimum yield. For yeast, it was found that optimum bioethanol yield like 8.3%(v/v) can be obtained at pH 4.6, sugar concentration of 14 g/100 ml, fermentation efficiency of 92.3% and fermentation temperature of 30°C. One (1) g/L nutrients (DAP, urea) were supplied in the same ratio. The impurities in the molasses are also responsible for the lesser bioethanol yield in fermentation process. The fermentation time was investigated while keeping other parameters in optimized condition.

Conflict of interests

The authors did not declare any conflict of interest.

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