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Full Length Research Paper

Improving bio-ethanol yield: Using virginiamycin and sodium flouride at a Pakistani distillery

Muhammad Arshad*, Muhammad Anjum Zia, Muhammad Asghar and HaqNawaz Bhatti

Department of chemistry & Biochemistry, University of Agriculture Faisalabad.

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Bacterial contamination is a major problem in bio-fuel production industry; whether from molasses or corn. To improve ethanol yield at industrial scale fermentation, virginiamycin and sodium fluoride was tested. Virginiamycin gave maximum efficiency at 2 ppm and increased the alcohol level to about 1% in fermenters with the reduction of sulfuric acid consumption. By doing cost benefit analysis, it has been shown that use of this antibiotic saved a lot of money for the distillery industry.

Key words: Virginiamycin, sodium fluoride, distillery, ethanol.

INTRODUCTION

Bacterial contamination is a major cause of reduced yields in ethanol production (Connolly, 1997). Contaminants constantly utilize carbon available for conversion to ethanol and compete for growth factors needed by yeast (Kelly et al., 2004). The contaminating agents produce deleterious end products such as lactic and acetic acids that inhibit the growth of *Saccharomyces cerevisiae* (Makanjuola et al., 1992; Narendranath et al., 1997). Fermentation tanks and yeast propagation systems can act as reservoirs of bacteria that can continually reintroduce contaminants (Day et al., 1954).

A number of antimicrobial agents to control bacterial contamination in ethanol fermentations under laboratory conditions have been described. Urea hydrogen peroxide reduced the numbers of Lactobacillus while providing nutrients to aid performance of the yeast (Narendranath et al., 2000). Various agents have been tested for control of bacterial contaminants under laboratory conditions, such as hydrogen peroxide, potassium metabisulfite and 3,4,4-trichlorocarbanilide (Chang et al., 1997; Gibbons and Westby 1986; Narendranath et al., 2000; Oliva-Neto and Yokoya, 1998). Hop acids are reported as replacing agents of antibiotics (Rückle and Senn, 2006). Penicillin and antibiotic monensin have shown effective results against the strains of *Bacillus* and *Lactobacillus* isolated from Brazilian alcoholic fermentation units (Stroppa et al., 2000). Different antibiotics including penicillin, virginiamycin and tetracycline have been reported to control contamination by lactic acid bacteria in experimentally infected alcoholic fermentations (Aquarone, 1960; Bayrock et al., 2003; Hynes et al., 1997).

Although, there are different designs and operating practices in distilleries, numerous chances exist for contaminants to persist or thrive in the system. To clean and sterilize the propagation and fermentation tanks is common in distilleries and as there are batch process so much time is available. Yeast propagation is a potential point of contamination which increases during fermentation. The contamination load comes continuously with molasses and this causes a great problem in fermentation. Sugar cane molasses produced by sugar mills in Pakistan are stored in very poor conditions and so when they reach the distilleries, they are found to be loaded with contaminating microorganisms.

The profitability of ethanol production is dependent on favorable sugar cane molasses price and the quality of molasses (sugar% and contamination level). After the year 2000, alcohol business in Pakistan expanded rapidly. Contamination was controlled by reducing pH with sulfuric acid.

We have used virginiamycin and sodium fluoride to treat the bacterial infections in our fuel ethanol fermentation facility at Distillery Shakarganj Mills Ltd Jhang and very successful results were got. This was done by raising the alcohol level in fermenters and reducing the

*Corresponding author. E-mail: arshad_877@yahoo.com.

use of sulfuric acid. Results have been obtained in very hard scientific conditions and it is the first work being reported on the use of antibiotic at industrial scale in distillery.

MATERIALS AND METHODS

Substrate and nutrients

Sugarcane molasses was used by diluting it with tap water in mild steel tank of 60 m^3. Sugar level was maintained by measuring indirectly by ^{0}brix (Arshad et al., 2008).

Nutrients

Although, molasses generally contain most of the nutrients required for yeast fermentation, ammonium salts and phosphates are added to supply nitrogen and phosphorus (Prescott and Dunn, 2002). Commercially available urea (2 kg) and phosphoric acid (500 ml), previously optimized (Borzani, 1996), were used as the main nitrogen and phosphorus sources only at inoculum preparation stage.

pH

Sulfuric acid was used to adjust the pH and as sulfur source (Arshad, 2005).

Propagation (inoculum preparation)

Before full-scale fermentation was performed, yeast was propagated up to 60 m^3 in three stages. Air is supplied during the propagation by the air blower.

Stage-I

By transferring the 10-liter mutant strain active-yeast culture (prepared in flask) into a 1 m^3 working capacity vessel, further propagation was carried out in three stages.

Stage-II

In this stage, the vessel with 20 m^3 working volume was used and 1 m^3 yeast was transferred to it. Aeration was carried out at this stage and temperature varied between 30 – 32°C. This stage continued for 8 h.

Stage-III

During this stage, the propagation vessel was of 60m^3 working volume. Aeration continued. When cell count reached 350 10^6 it was transferred to fermenters.

Fermentation

This stage lasted approximately 30 h during which no aeration took place and temperature was kept at 30 – 32°C. During this stage, the culture fermented statically until near consumption of all available sugars. Foaming was controlled by adding castor oil as an antifoaming agent.

Antibiotic

Different concentrations of commercially available antibiotic virginiamycin and sodium fluoride were used to control contaminating bacteria during fermentation process for optimum ethanol recovery. Virginiamycin (lactrol) was obtained from Phibro Chemicals USA and sodium fluoride from M/s shah traders (Origin China). Virginiamycin was dissolved in 96% ethanol (rectified Spirit) and added to give concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 ppm in the fermentation tank. Sodium fluoride was dissolved in fresh water and added in the dose ranges of 5, 10, 15, 20 and 25 ppm at propagation stage.

Analytical methods

Cell count and viability

A hematocytometer was used to determine bacterial and yeast cell counts in each fermenter. A 1 ml fermenter broth sample was serially diluted with a sterile saline solution (0.89% w/v NaCl) to a point where a reasonable number of cells could be counted (Alfenore et al., 2007).

Sugar and ethanol analysis

Total sugar was estimated using a Brix hydrometer (Abdel-Fattah, 2000). Reducing sugars were determined using the method of Arshad (2005). Ethanol in fermented samples was determined with ebulliometer and confirmed on GC as described previously by Arshad et al. (2008).

RESULTS

Contamination was controlled by reducing the pH with sulfuric acid. Commonly used antibiotic was the sodium fluoride; which is not much effective against the continuous contamination load (Arshad et al., 2005).

Virginiamycin and sodium fluoride were used to improve the ethanol by controlling the contamination in fermentation. Both antibiotics were used at five levels and the results are shown in Tables 1 and 2, respectively. For each experiment, five fermenters were used and the mean value was taken. Control was separately used for each experiment and the mean value of all the controls was reported.

Pure culture is always required for fermentation. Our aim was to get inoculum free of any kind of bacteria. As molasses has continuous load of contamination in distil-leries, substrate was not sterilized. Therefore, to combat this contamination, antibiotics were used at different levels and maximum achievable results were obtained keeping all other factors constant. When virginiamycin was used at only 0.5 ppm concentration, their effect was clear and it increased the ethanol l% in fermenters than control. The level of ethanol percentage increased with the increasing level of virginiamycin and it achieved maximum at 2 ppm, when there was no

Table 1. Effect of varying concentrations of lactrol on fermentation.

Concentration	Yeast cell count	Bacterial cell count	Ethanol %	Brix^0	Reducing sugar
0.5 ppm	301x10^6±6	271x10^6±5	7.8±0.15	11.01±0.16	1.15±0.02
1.0 ppm	311x10^6±3	27x10^4±3	7.9±0.10	10.81±0.10	1.12±0.02
1.5 ppm	354x10^6±5	32±2	8.3±0.18	10.01±0.12	1.01±0.01
2.0 ppm	375x10^6±4	None	8.5±0.11	9.49±0.11	0.90±0.01
2.5 ppm	346x10^6±4	None	8.5±0.09	9.41±0.16	0.95±0.05
Control	302x10^6±3	320x10^6±6	7.5±0.17	11.51±0.13	1.25±0.01

Table 2. Effect of varying concentrations of sodium fluoride on ethanol fermentation.

Concentration	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm	Control
Yeast cell count (M)	277.67±1.46	304.67±3.20	315.33±2.04	352.33±3.31	343.00±2.66	301.67±2.62
Bacterial cell count (M)	338.33±2.04	218.33±4.44	128.33±4.94	0.00	0.00	346.33±1.21
Ethanol	6.02±0.01	6.06±0.04	6.10±0.01	6.21±0.01	6.04±0.02	5.86±0.02
Brix	11.890±0.05	11.373±0.03	11.133±0.07	10.560±0.03	10.920±0.10	12.183±0.14
RS (%)	19.300±5.79	15.500±3.57	14.033±3.23	13.300±4.32	11.633±3.93	25.867±8.32

contaminant in the culture. At 1 ppm, the remaining reducing sugars were 1.12% (w/v) and the trend was decreasing with the increase of antibiotic level. At the 1.5 ppm concentration, bacterial count remained at 32 only. At 2 ppm, maximum alcohol level that was matching to the potential of molasses used was obtained.

Alcohol level in the case of sodium fluoride also increased but does not equal that of sodium fluoride. It was 0.3% higher than the control.

Cost benefit analysis

Difference in ethanol % (optimum - control) = 8.5 - 7.5 = 1.0
More ethanol in one fermenter (300 M^3) = 3000 L
Difference in alcohol per day per 100,000 L production = 12000 L per day
Cost of extra ethanol produced per day = 12000 × 30 = 360000
Cost of virginiamycin used = 10,000 per day
Net profit = 350,000 Rs ($4375)

DISCUSSION

Comparing the control with the antibiotic treated at 0.5 ppm, there was difference of 0.3% ethanol v/v. When bacteria population decreased to the range of 10^4, more 0.4% ethanol was produced. When the antibiotic dose increased to the 1.5 ppm, ethanol increased by 0.8% v/v and optimally 8.5% ethanol was obtained at 2 ppm concentration of antibiotic used. These results are in agreement with the finding of Narendranath et al. (1997). Occurrence of 10 x 10^6 lactobacilli/ml mash resulted in approximately 1% v/v reduction in the final ethanol produced by the yeast; this depended on the strain of the contaminant bacteria. The over loss in ethanol yield is 1% in our study and which is a significant and huge financial loss. Our results are in agreement with Makanjuola et al. (1992) who reported 1% reduction in ethanol yield which is quite significant to distillers of fuel alcohol since their profit margins are very narrow.

There is need to optimize the concentration of antibiotic use. If antibiotics are not administered correctly, the development of antibiotic resistant strains cannot become a reality (Neelakantam and Narendranath, 2004). High level of contamination will not make any impact to yeast growth nor have affect on the recovery of ethanol. In the terms of viability, there is heavy lost in yeast viability when culture is contaminated (Thomas et al., 2001).

Virginiamycin has certain advantages over other antibiotics such as temperature and pH stability and high resistance level. Loss was up to 13-14% when lactrol was not used in our study and available literature supports this. Hynes et al. (1997) stated that 6 - 12% loss of total produced alcohol (0.8-1.5% v/v ethanol concentration in fermentation) were seen when particularly aggressive contamination were present in high number.

CONCLUSION

Use of Virginamycin at industrial scale ethanol fermentation is a reliable source to avoid losses due to contamination. Its use should be carefully done as per requirement. It can save more $4000 per day for the distillery producing 100, 000 L/day; which is a big amount

for distilleries.

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