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Full Length Research Paper

An industrial perspective of factors affecting molasses fermentation by *Saccharomyces cerevisiae*

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In this study, an effort has been made to address the key issue of how molasses quality and composition are key components for higher yields during ethanol fermentation. Moreover, it was also noted that the choice of a yeast strain and yeast preconditioning have a positive effect on alcohol yield during molasses fermentation. A considerably better alcohol yield (9%) was obtained with low residual sugars (< 0.3%), and an increased glycerol concentration from 0.5 to 0.9%. Microbiological analysis revealed a total aerobic count (TAC) of 1.18 × 10^6 cfu/ml and *Lactobacilli* count of 8.4 × 10^5 cfu/ml after 22 h fermentation on de Man, Rogosa and Sharpe (MRS) agar media. The wild yeast count was relatively high reaching 9 × 10^2 cfu/ml within 40 min of commencement of the fermentation but decreased to 3 × 10^4 cfu/ml after 22 h of fermentation. This study has shown some of the possible causes of poor fermentation and advantages of cell preconditioning in molasses fermentation by *Saccharomyces cerevisiae*.

Key words: Ethanol, *Saccharomyces cerevisiae*, fermentation, molasses, preconditioning.

INTRODUCTION

Fermentation is a biological process where sugars are converted to ethyl alcohol and carbon dioxide is given off as a by-product. Great strides in research together with the development of new yeast strains have led to demands to model a new yeast strain which can produce higher levels of alcohol, temperatures and pH. This requires immense knowledge of the fermentation processes to improve its efficiency which is dependent on various factors namely: process design, molasses quality, yeast strain, contamination, nutrient availability and raw material purity. Yeast alcohol is one of the most valuable products originating from the biotechnological industry with respect to both value and amount (Nissen et al., 1999). In 1996, the world ethyl-alcohol production reached an estimated 31.3 billion liters (Berg, 1998) and approximately 80% was produced by anaerobic fermentation of various sugar sources by *Saccharomyces cerevisiae.* Yeast alcohol technology has seen vast improvements to become more profitable during the last decade but profit margins have constantly been narrowed. Contamination, availability of raw materials and fermentation process design are the major limitations causing a reduction in alcohol yield and quality in the alcohol industry (Rückle, 2005). In view of the increasing importance of alcohol as an alternative source for chemicals and liquid fuel, a great deal of research interest in ethanol fermentation has been generated over the last two decades (Vega at al., 1987). The price of the sugar source is a very important process parameter in determining the overall economy of alcohol production

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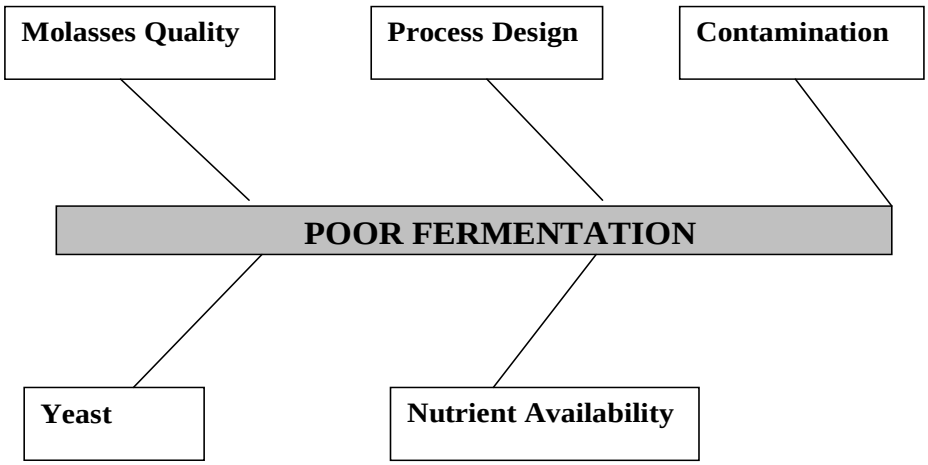

Figure 1. Fishbone diagram depicting the possible contributors to poor fermentation (Walker et al., 1996).

and it is of great interest to optimize the alcohol yields in order to ensure an efficient utilization of the carbon source (Wyman and Hinman, 1990).

Another crucial factor in fermentation is the choice of a potent microorganism and it has been seen that microorganisms ranging from fungi, bacteria and yeasts have been harnessed for ethanol production (Vallet et al., 1996). Historically, the most commonly used microbe is the yeast, which can produce ethanol to give concentrations as high as 18% of the fermentation broth and is therefore the preferred choice for most ethanol fermentation (Balat et al., 2008). Among the yeasts, *S. cerevisiae* still remains as the prime species for ethanol production. In industry, the ethanol yield from *S. cerevisiae* is calculated based on the total sugar fed into the fermentation system and can be as high as 90 to 93%. Previous studies have shown that the ethanol tolerance and sugar utilization efficiency of yeast may be improved by altering the nitrogen source in the ferme-tation medium (Thomas and Ingledew, 1990; Thomas et al., 1993; 1996).

All factors responsible for poor fermentation (Figure 1) should be checked while trouble shooting the cause for poor fermentation (Walker et al., 1996). The quality of molasses should be traced back to the sugarcane farms from the time of soil preparation to harvesting so this will give an idea about impurities which are present in the molasses (Eggleston et al., 2008). Presence of heavy metal ions mainly potassium ions have a negative impact on the yeast and micronutrients (Zn and Mg) availability is of vital importance since it is responsible for yeast enzyme regulation (Walker et al., 1996; Ryan and Johnson, 2000). Cell preconditioning is an improved concept as yeast cells are unable to make the required microelements and pre-conditioned yeast cells have proved to have tolerance to higher alcohol levels (Walker, 1998). In addition to these, the bioprocess design should accommodate temperature controls as temperature can rise to unfavorable highs and affect yeast enzymes and also results in alcohol losses. Furthermore, an emphasis on fermentation vessel disinfection is also considered as it is susceptible to bacterial and yeast contamination (Wang et al., 1999).

Many forms of lactic acid and acetic acid bacteria can result due to poor cleaning but the main contaminant is *Leuconostoc mesenteroides* from sugar cane which are harder to detect and control. This organism causes the sucrose molecule to polymerize into long chains of dextrin that are not fermentable by *S. cerevisiae* but will appear as a reducing sugar in the TSAI (total sugars as inverts) (Eggleston et al., 2008). Therefore strong economic incentives can be realized by further improving the alcohol production processes resulting in a substantial growth in the ethanol production industry in the near future. Illovo sugar, Merebank, Durban, South Africa has taken a step forward to ferment molasses as a feed stock using *S. cerevisiae* as the yeast of choice for alcohol production (Murtagh, 1999).

The present study has undertaken to investigate some of the possible causes of poor molasses fermentation in industry and further addresses the steps followed in trouble shooting. The effects of preconditioning and microbial contamination have also been investigated in order to determine the possible impact on alcohol yield.

MATERIALS AND METHODS

Distillation and sampling

Molasses were added and the beer samples were transferred for distillation. Beer samples were sampled at every two hours intervals. Samples were analyzed for alcohol concentration, residual sugars, glycerol concentration, contamination profile and viability checks. A brief fermentation profile before and after the use of preconditioned cells was recorded.

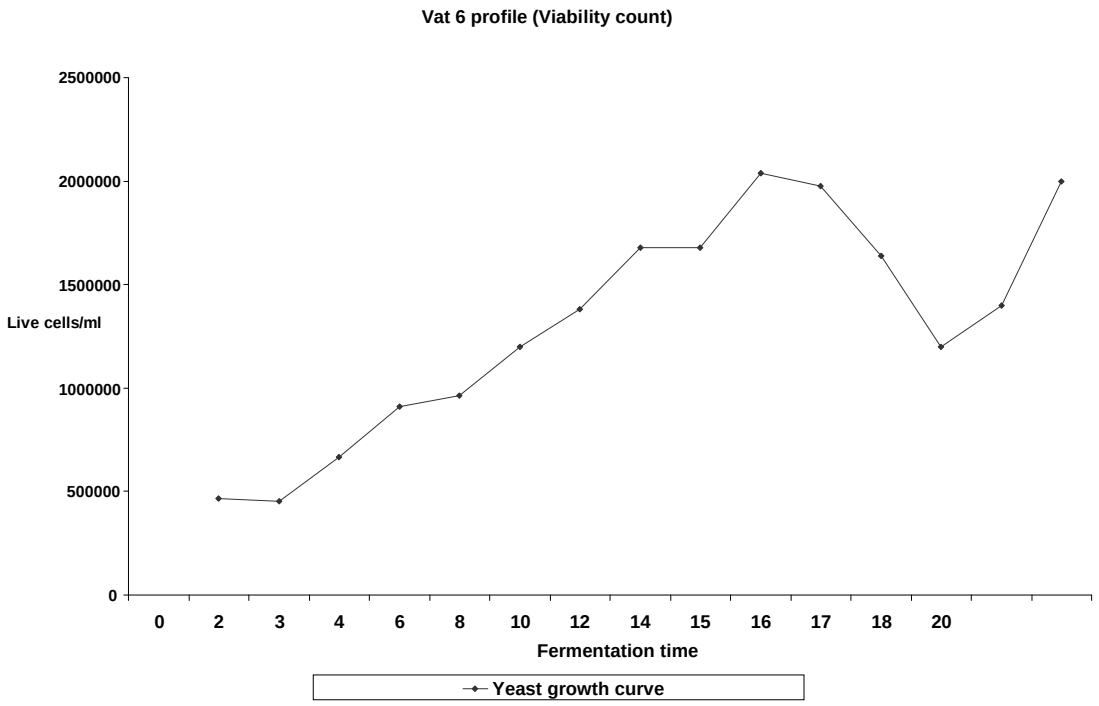

Figure 2. Profile of viability count (Vat 6).

Analytical methods

Gas chromatography (GC)

The alcohol produced after fermentation was quantified by a GC (Varian 34CX) with a FID detector at 240°C (Column type: 15QC 2.5/BP 30-0.25). Injector Temperature: 230°C; Column Temperature: 80°C; Flow rate: 10 ml min^{-1}) and 8% n-propanol, 10% ethanol (Calibration), 12% Ethanol (calibration check) as standards (Table 1).

HPLC for sugar quantification

Residual sugars were quantified by HPLC (Waters 2690) with RI detector at 40°C with a Sugarpak column (Waters) at a column oven temperature 90°C with CaEDTA (50 ppm) as the mobile phase and injection volume of 20 µl at a flow rate of 0.5 ml/min and 0.40% sucrose, 0.40% fructose, 0.20% glucose and 0.60% glycerol as standards (Table 1). The overall ethanol yield was calculated as given below. Ethanol yield (%) = Conc. of ethanol produced/ Initial conc. of sugar × 1/0.51 × 100 where, 0.51 indicates the theoretical ethanol yield (0.51 g ethanol /gram hexose)

Microbiological analysis

All the media for microbiological analysis was supplied by Oxoid Ltd, Cambridge, UK and were prepared as per Oxoid manual (Bridson, 2006). A beer sample was plated onto three different media namely: WL nutrient agar for total aerobic count, MRS agar (De Man, Rogosa, Sharpe) for *Lactobacilli* isolation and quantification, LM (Lysine media) for wild yeast isolation and quantification. Samples were plated and incubated as per manual instructions and viability counts and identification was carried out using routine microscopy.

RESULTS AND DISCUSSION

Studies have shown that the pH and temperature play a vital role in controlling contamination during fermentation process (Wang et al., 1998; Sugawara et al., 1994; Murtagh, 1999). *S. cerevisiae* grows better under slightly acidic conditions while *Lactobacilli* spp. grow between a pH range of 5.5 to 6.0. Results of this study indicate that waste material produced by these contaminant cells creates an unfavourable environment for other organisms including the yeast. It was evident that *S. cerevisiae* was able to outgrow all the contaminants during the early stages of fermentation but as yeast growth slowed down contaminant growth increases (Figure 2) due to competition for nutrients. In previous studies (Wang et al., 1998; Sugawara et al., 1994; Murtagh, 1999) the poor performance of the yeast was believed to be due to the shortage of trace metals in molasses and precon-ditioned yeast cells were used in this study to solve this. It was noticed that preconditioning of yeast cells boosted performance of yeast due to the availability of trace metals and there was an increase in alcohol yield (9%) as compared to non-preconditioned samples. Results also indicate a significant change in residual sugar content between the two profiles. It was noticed that prior to pre-conditioning the content of residual sugars and glycerol

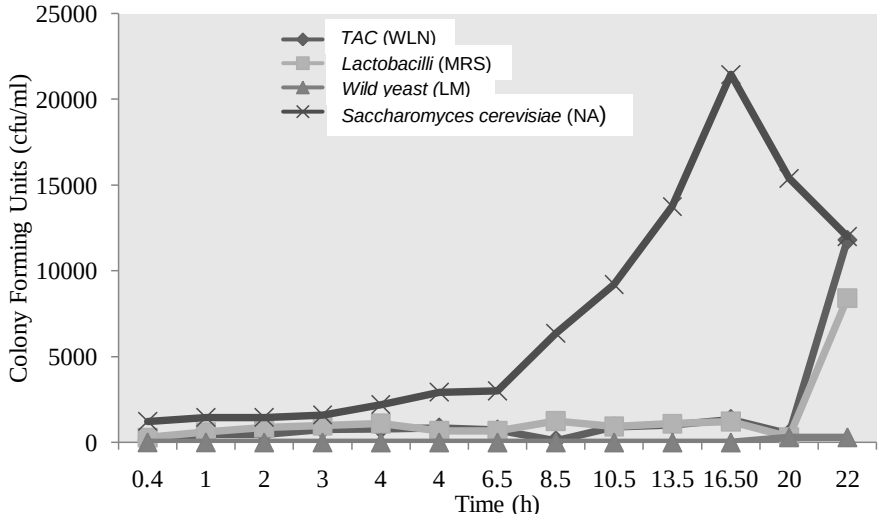

Figure 3. Contamination profile against *Saccharomyces cerevisiae* growth.

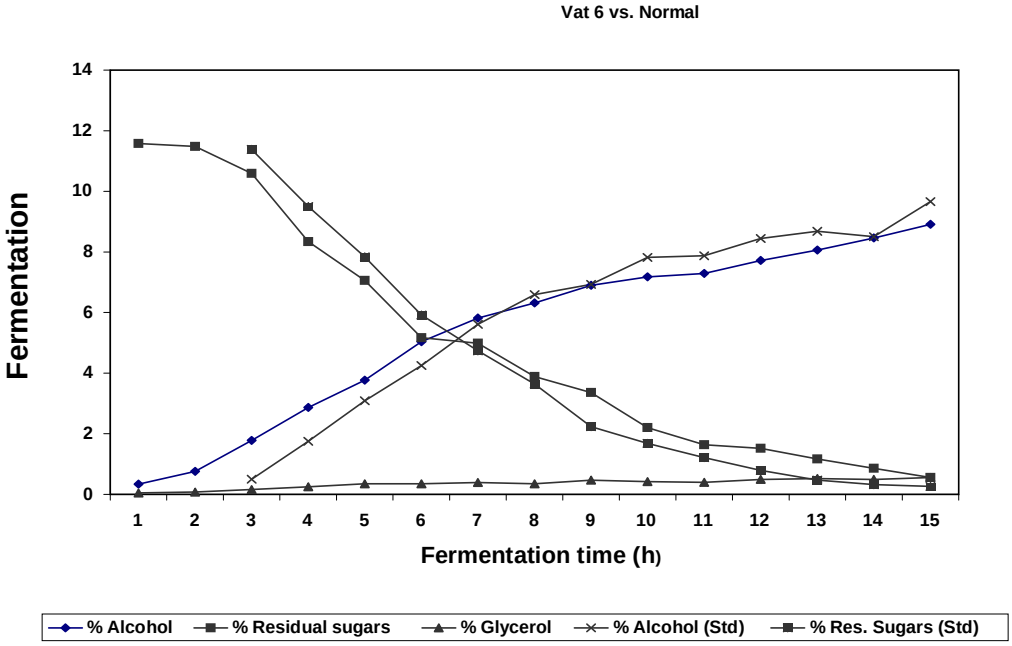

Figure 4. Vat profile prior to use of preconditioned cells against a Standard vat sample profile.

concentration were 0.56 and 0.50%, respectively (Figure 4) while after preconditioning the residual sugars and alcohol content remained 0.22 and 9.6% (Figure 5).

Based on results of this study and with the above advantages in mind, it is suggested that though there was no significant difference in glycerol levels amongst the two profiles but residual sugars were reduced (< 0.3%), and alcohol content was increased to 9.6% compared to the beer sample without the use of preconditioned cells. A contamination profile (Figure 3) and microscopic studies revealed that yeast cells were stressed out even though they were actively growing. A total aerobic count (TAC) of 1.18×10^6 cfu/ml was recorded from yeast cells in WLN plate after 22 h of fermentation whereas the maximum count of 1.352×10^5 cfu/ml was observed after 16.5 h of fermentation. On the other hand the maximum *Lactobacilli* count in MRS plate was 1.240×10^5 cfu/ml in 8.5 h followed by 8.4×10^5 cfu/ml after 22 h. The wild yeast count was high (9×10^2 cfu/ml) at the start of fermentation but it decreased to 3×10^4 cfu/ml after 22 h. The minimum contamination was observed at 20 h for both TAC and *Lactobacilli* (5×10^4 and 3×10^4 cfu/ml),

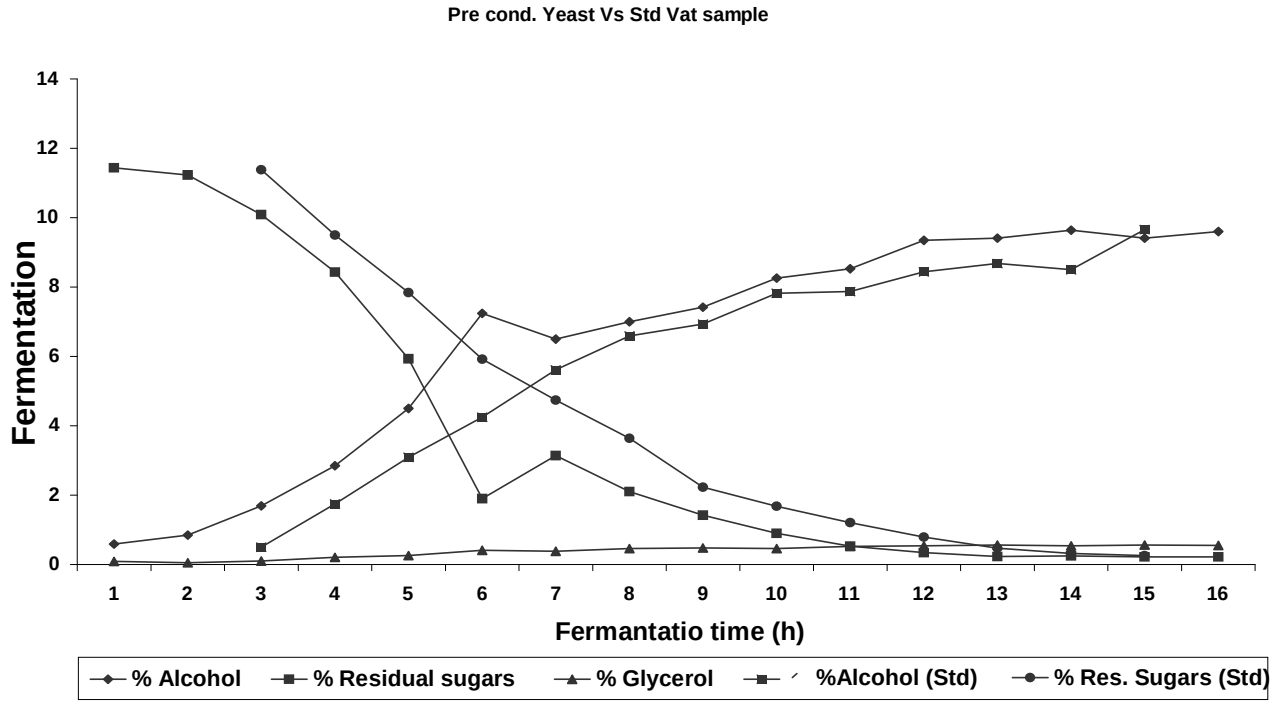

Figure 5. Vat Profile after the use of preconditioned yeast cells against a Standard vat sample profile.

Table 1. Standards used for calculation.

GC	HPLC
8% Internal Standard (n-Propanol)	0.40% Sucrose
10% Ethanol standard – calibration	0.40% Fructose
12% Ethanol standard-calibration check	0.20% Glucose
	0.60% Glycerol

Table 2. An outline of typical stress factors affecting yeast fermentation.

Stress factor	Limit (Optimum)
Lactic acid	> 0.8% w/v
pH	3.0 – 4.0
Sulphite	>100 mg/L (varies with strain)
Sodium ion	> 500 mg/L
Temperature	32^0C – 37°C
Acetic acid	>0.05 %w/v
Ethanol	23 %
Sugar content	38 % w/v

respectively followed by wild yeast count of 1×10^2 cfu/ml at 6.5 h and 16.5 h of fermentation. The availability of fermentable sugars in molasses makes it attractive to different microorganisms and it is the most common source of contamination. An outline of different stress factors affecting yeast fermentation is outlined in Table 2. Our studies are well supported by some recent reports (Tang et al., 2010; Thrune et al., 2009) which indicated that sugarcane that has been left to stand after harvesting in a humid environment is susceptible to

Leuconostoc messenteriodes contamination (Eggleston et al., 2008).

Microscopic studies identified three classes of contaminants namely: wild yeast, *Lactobacillus* spp. and other microbes recorded as TAC (total aerobic count). *S. cerevisiae* produces an average of 9.5% v/v alcohol per vat in approximately 25 h. This alcohol is further transferred to distillation plant for concentration to 95% and further purification. Sugawara et al. (1994) found that the yeast was unable to ferment the molasses to completion, thus higher residual sugars were recovered at the end of fermentation and less alcohol content. To rectify this problem more yeast was added into the fermentation vessel but it failed and more molasses with urea were added as a source of nitrogen for scale up fermentation. It was assumed that cooling jackets, connected to heat exchangers in vessels may be the possible cause of contamination leading to poor yield of alcohol (Wang et al., 1999).

Nevertheless, it is assumed that further studies on use of preconditioned cells in wide variety of molasses samples are needed for increased yields and it is necessary to achieve an economical process.

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