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Effect of Some Fermentation Parameters on Ethanol Production from Beet Molasses by *Saccharomyces cerevisiae* CAIM13

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Abstract: Problem statement: Some component of fermentation medium showed to reduce the *Saccharomyces cerivisae* production of ethanol. **Approach:** This study was designed to evaluate the role of some fermentation parameters in affecting ethanol productivity from beet molasses BM by *Saccharomyces cerevisiae* CAIM13. **Results:** Increase in cell concentration (inoculums size) of the yeast above 3.6×105 cells/100 mL decreased the ethanol yield. The yeast could tolerate ethanol concentration up to 10% but failed to grow at concentration of 12 and 15%. Employment of a bench-scale tank fermenter enhanced the fermentation efficiency. 77% of BM sugars were assimilated after 48h giving a concentration of 5.4% ethanol. Utilization of a cell-recycling technique showed that the tested organism was capable of performing four fermentation cycles. The mud-free, H_2SO_4-treated beet molasses TBM was superior to sucrose in the repeated batch fermentation technique. A continuous-flow fermentation technique employing immobilized yeast cells yielded maximum ethanol productivity after 6 days. **Conclusion:** The present investigation has demonstrated the importance of some fermentation parameters in improving the alcoholic fermentation technology of BM. When free cells of *S. cerevisiae*. In the case of immobilized cells, the continuous-flow technique speared superior to the repeated batch-fermentation technique in production of alcohol from TBM.

Key words: H_2SO_4-Treated Beet Molasses (TBM), fermentation cycles, fermentation technology, fermentation medium, cell-recycling technique, batch fermentation, spectrophotometer model, sucrose solution, Crude Beet Molasses (CBM)

INTRODUCTION

During recent years, production of ethanol by fermentation on a large scale has been of considerable interest to meet to increased demand for new sources of energy (Akhir *et al*., 2009; Turhan *et al*., 2010). Ethanol production via yeast fermentation may provide an economically competitive source of energy (Cysewski and Wilke, 1978; Nguyen *et al*., 2009; Zhao and Bai, 2009; Csoma *et al*., 2010; Ding *et al*., 2010; Dutta *et al*., 2010; Ibrahim *et al*., 2010; Jeon and Park, 2010; Oda *et al*., 2010; Tang *et al*., 2010; Ghorbani *et al*., 2011; Razmovski and Vucurovic, 2011). Among the crucial microbial selection and adaptation are: substrate selection and preparation, microbial selection and adaptation optimization of fermentation conditions and improvement of fermentation technology.

It has long been recognized that molasses from sugar-cane or sugar provide suitable substrates for ethanol production. The present investigation aimed at evaluating the role of some fermentation parameters that might affect ethanol productivity.

MATERLALS AND METHODS

Microorganism and culture conditions: The strain of yeast used in these experiments, *Saccharomyces cerevisiae* CAIM 13 (MIRCEN). The pure isolate was maintained on slants of malt-extract-agar composed of g L^{-1}), malt extract (Difco) 20, dextrose 20, peptone 1, agar 25. The slants were incubated at 7°C for 48h and then stored at 4°C.

Beet molasses: The Crude Beet Molasses (CBM) used as a carbon source was kindly supplied by the delta sugar company, Egypt.

Preparation of yeast inoculum's: To initiate yeast growth, inocula from 3day old slants were transferred to 250 mL Erlenmeyer flasks, each containing 50 mL of a medium composed of (g L^{-1}): glucose 10, peptone 5, yeast extract3, malt extract 3. The flasks were incubated at 30±2°C for 48h on a rotary shaker (200-250 rpm). Standard inocula (2 mL each) from such liquid cultures were used to inoculate 100 mL aliquots of the fermentation medium.

Fermentation medium: The fermentation medium used was a modification of that described and composed at (g L^{-1}); mud-free, H_2SO_4-Treated Beet Molasses (TBM) 200, Urea 1.08, $MgSO_4.7H_2O$ 0.3, H_3PO_4 0.3, PH5.

Determination of the biomass yield: The fermentation beer was filtered through Whatman No. 1 study under reduced pressure and the yeast cells were dried at 90ºC to constant weight.

Analysis of culture beer: The ethanol was determined by the oxidation method (Benerji *et al*., 2010). Fermentation efficiency (%) was determined as (Sedha *et al*., 1984):

$$\text{Fermentationefficiency}\ (\%) = \frac{\text{Actualethanol content}}{\text{Theoreticalcontent}} \times 100$$

$$\text{Theoreticalethanol content} = \text{Totalfermentable sugar}\ \acute{}\ 0.64$$

The residual reducing-sugars content of the culture beer was photo metrically estimated at 700nm following the method described by Somogyi (1952) using a spectrophotometer model CF595, CECIL instruments, UK.

Effect of cell concentration (inoculum's size) of yeast: Different concentrations of yeast cells ranging from 1.2×10-6.0×10 mL^{-1} were each inoculated into 100 mL aliquots of fermentation medium in 250 mL Erlenmeyer flasks. Three flasks were prepared for each concentration. The flasks were incubated at 30ºC for 48h and the necessary were performed.

Ethanol tolerance of yeast: Different amounts of ethanol (4-15 m L^{-1}) were added to 100mL aliquots of an autoclaved medium composed of glucose (10%) and yeast extract (1%) in 250 m L^{-1} Erlenmeyer flasks. The fermentation process was conducted for 4days at 30ºC and daily counts of yeast cells were performed by the use of a counter model C110, New Brunswick Sci. Co., Inc., Edison, NJ, USA.

Bench-scale stirred- tank fermenter: Fermentations were performed in A 7.5liter tank (new Brunsick M 1085-1003) containing 4liters of the modified medium of Bose and Ghose (1973). The medium was autoclaved for 30min then inoculated with 3%(v V^{-1}) yeast suspension prepared as previously described. An aeration rate of 5 L min^{-1} was introduced into the medium whilst stirring at 400 rpm. Aeration and stirring rates were reduced to 0.5 L min^{-1} and 100rpm at the end of 24 h incubation at 30ºC when good yeast growth was usually observed. Samples (100 m L^{-1}) were taken daily for the assays.

Cell-recycling technique: The yeast obtained after 48h in the stirred–tank fomenter was allowed to settle, the supernatant culture broth was siphoned off and 4liters of fresh fermentation medium were added under aseptic conditions. Fermentation was continued for 24h under limited aeration (0.5 L min^{-1}) and stirring conditions (100 rpm) and the necessary analyses were carried out. The cycle was repeated suing aliquots of fresh medium and the original yeasts growth.

Immobilized- cell techniques: Batch fermentation: *S.cerevisiae* CAIM 13 was cultivated under aerobic (1.5vol min^{-1}), stirred (3000 rpm) conditions using the stirred-tank fomenter as previously described. After 48h, the cells were harvested and concentrated by centrifugation at 5000g for 15 min. A known weight of yeast cells (20g) was mixed with 2g sodium alginate and blended with 100 m L^{-1} sterile distilled water for 5min. The mixture was dribbled from a hypodermic needle into a stirred aqueous solution containing 0.1mol/liter $CaCl_2$ and 15mmol/liter KH_2PO_4. The small beads (2.3 mm in diameter) were allowed to harden in the solution.

The reactor system employed was a straight vertical column (2.5×30cm) that was filled with mud-free, H_2SO_4-treated BM (TBM) or sucrose solution (115 m L^{-1}) and autoclaved for 45min. The beads were suspended in the middle of the column in a nylon mesh bag with 1mm holes. The sugar solution (20% as total reducing sugars) was stirred continuously by a magnetic stirrer. The entire solution was removed daily for analysis and substituted by a fresh identical. Only date for days in which changes in fermentative activities were observed is presented.

Continuous fermentation: The continuous-flow reactor used consisted of a reservoir with a side arm, a peristaltic pump (Buchler instruments) and a jacketed column (2.5×30 cm). The rector was maintained at 25ºC during the course of operation using a circulating cooling bath. The column contained a multiple-disc shaft consisting of 24 glass discs (each 2.49 cm in diameter with 2mm evenly distributed holes) mounted on a glass rod and separated from each other by 1.2cm hollow glass rods. The alginate-entrapped S. cervisiae CAIM 13 beads were equally distributed on the disks. The glass column contained 1200 alginate and TBM (20% total reducing sugars), 0.1 mol L^{-1} $CaCl_2$ and 15 mmol L^{-1} KH_2PO_4 were pumped from the reservoir into the bottom of the column through the beaks at a rate of 3 m L^{-1} h with a dilution rate equal to 0.026 h^{-1}. The column containing beads had a total liquid volume of 11 5 mL and the ethanol content was periodically estimated.

All the data presented in this study were the mean values of 3 replicate results.

RESULTS

Effect of cell concentration (inoculum's size) of yeast: The amounts of sugars consumed and ethanol outputs increased linearly with increase in initial cell concentration from 1.2×10^3-3.6×10^5 cells/100 mL with the latter producing the maximum effect (5.4% ethanol; fermentation efficiency 94.7%).

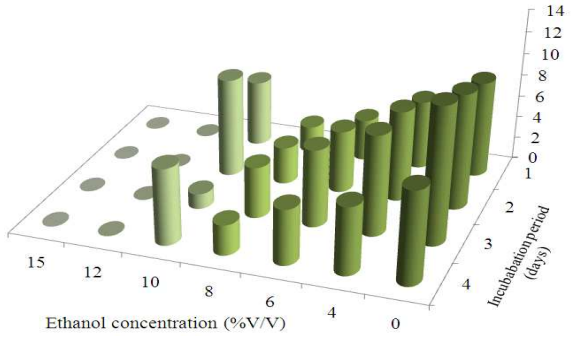

Fig. 1: Number of cells of *S. cerecisiae* CAIM13 per mL medium recorded in different ethanol concentrations (% V/V) after various incubation periods (days)

Table 1: Number of cells of *S.cerevisiae* CAIM13 per mL medium[a] recorded in different ethanol concentrations (%V/V) after various incubation periods(days)at 30°C

Ethanol concentrations	Incubation period (days)			
	1	2	3	4
0	8.5	12.5	10.6	8.8
4	6.2	9.2	8.3	6.3
6	5.1	7.1	5.7	3.8
8	2.8	4.7	3.4	2.4
10	7.0	1.4	9.0	6.0
12	0.0	0.0	0.0	0.0
15	0.0	0.0	0.0	0.0

[a]: Medium Composition (g/litre):glucose,100; yeast extract,10; pH5

Table 2: Dry weight yields of *S.cervisiae* CAIM13(g) and amounts of ethanol produced per 100mL medium[a] after various fermentation periods(h) at 30ºC using a laboratory tank fermenter (7.5 liters capacity)

Fermentation Period (h)	Consumed sugar (g)	Cell dry weight (g)	Ethanol content (%v/v)	Fermentation efficiency (%)
24	7.365	4.592	2.2	49.7
36	8.850	4.621	4.6	86.6
48	9.693	4.891	5.4	93.1
60	9.804	4.502	5.1	86.7
72	9.971	4.029	4.3	71.9

[a]: Fermentation medium (g/L): TBM, 200; urea, 1.08; $MgSO_4.7H_2O$,0.3; H_3PO_4,0.3,pH5

Ethanol tolerance: The results obtained showed that the tested yeast could tolerate ethanol concentration up to 10% but failed to grow at concentrations of 12 and 15% with a progressive decrease in yeast cell numbers in the concentrations ranging from 4-10% Table 1 and Fig. 1. In all cases, maximum yeast growth was attained after 2 days.

Utilization of bench- scale tank fermenter: About 77% of the initial BM sugars were consumed at the end of the first 24 h incubation Table 2. Extension of the incubation period to 48h represented the phase of active sugar assimilation which favored ethanol production. At the end of this period, 89.2% of BM sugars were converted, giving ethanol concentration of 5.4% and fermentation efficiency of 93.1%. Ethanol concentration gradually decreased in the later phases of the fermentation process (60-72 h).

The utilization of a cell-recycling technique showed that test organism was capable of performing 4 fermentation cycles and that relatively high fermentative activity was attained in the first reuse of the cells Table 3. Under these conditions, the added sugars were almost totally assimilated with the production of the highest ethanol yield as well as the maximum fermentation efficiency. However, repeated reuse led to lower fermentative activities.

Utilization of immobilized-cell techniques: Repeated-batch fermentations using TBM and sucrose feed-solutions were conducted with immobilized cells. TBM was found to be superior to sucrose; maximum ethanol concentration (11.2%) was achieved between 20-26 days using TBM Table 4. Moreover, the yeast remained its ability to produce ethanol over a longer period in the case of TBM. The continuous-flow fermentation technique using immobilized cells yielded maximal ethanol output after 6days Table 4.

Table 3: Dry weight yields of *S.cerevisiae* CAIM13 and concentrations of ethanol produced per 100 mL sample at the end of each cycle (i.e., after 24h from adding fresh medium to the original growth)

Cell recycling number[a]	Consumed sugar (g)	Cell dry weight (g)	Ethanol content (%v/v)	Fermentation efficiency (%)
0[b]	0.693	4.891	5.4	93.1
1	10.695	4.772	6.3	98.2
2	9.752	3.502	5.7	98.2
3	6.506	3.175	3.2	82.0
4	2.759	1.152	0.8	50.0

[a]: Number of recycles of yeast growth obtained after 48 h in the stirred-tank fermenter (original growth). Data for each cycle were recorded after 24 h from siphoning the supernatant and adding fresh medium to the original growth. [b]: Data for the stirred-tank fermenter

Table 4: Concentrations[a] of ethanol produced per 100 mL sample using immobilized cells of *S.cerevisiae* CAIM13 and either sucrose or TBM in a batch fermentation method and TBM only in a continuous fermentation method, after various incubation periods at 30°C

	Batch fermentation		Continuous fermentation	
Incubation period (days)	Sucrose ethanol (%)	TBM ethanol (%)	Time (days)	Ethanol (%)
1	7.0	7.9	1	4.0
2- 7	7.9	9.5	2	6.7
8-19	9.3	10.4	3	7.0
20-26	5.7	11.2	4	7.2
27-29	1.7	9.6	5	7.2
30-34	0.0	8.5	6	7.5
35-38	0.0	6.3	7	7.5
-	-	-	8	7.5
-	-	-	9	6.7
-	-	-	10	6.0

[a]: Average concentration of ethanol produced every 24 h after changing the medium within the different ranges of incubation periods

DISCUSSION

Since the relationship between the fermentative ability and viability of yeast is intimate (Singh *et al*., 2009; Yamada *et al*., 2009; Ghorbani *et al*., 2011and Razmovski and Vucurovic, 2011) the ethanol tolerance of the experimental yeast was studied by Cortes *et al*. (2010). These observations are consistent with the findings of Stanley *et al*. (2010) and Razmovski and Vucurovic (2011).

At present, ethanol fermentation technology generally employs batch bioreactor systems and only occasionally continues reactors where the cells are freely suspended in the liquid phase (Choi *et al*., 2009; Hong *et al*., 2009; Zhang *et al*., 2009; Turhan *et al*., 2010; Ghorbani *et al*., 2011; Li *et al*., 2010; Tang *et al*., 2010) in recent years, many workers have used immobilized-cell systems to ferment a wide variety of carbohydrates to ethanol (Liu *et al*., 2009; Singh *et al*., 2009; Pacheco *et al*., 2010; Ghorbani *et al*., 2011; Pruksathorn and Vitidsant, 2009) reported that certain strains of *S. cerveisiae* can undergo up to 10 fermentation cycles using molasses-containing medium. Alshiyab *et al*. (2009) reported that by using bigger reactor size, the effect of gaseous products in fermentation medium was reduced and enhanced both bacterial productivity and biomass concentration, where Brethauer and Wyman (2010) reported that some continuous fermentations are now employed for commercial ethanol production from cane sugar and corn to take advantage of higher volumetric productivity, reduced labor costs, and reduced vessel down time for cleaning and filling.

This study and others have demonstrated that free cells of *S.cerevisiae* maintain about 10% viability for 2 weeks whilst less than 1% of entrapped cells remain viable after one month (Ghose and Bandyopadhyay, 1982; Laopaiboon *et al*., 2009; Yamada *et al*., 2009; Basso *et al*., 2010; Orlic *et al*., 2010; Turhan *et al*., 2010; Yu *et al*., 2010; Ghorbani *et al*., 2011).

CONCLUSION

In conclusion, the present investigation has demonstrated the importance of some fermentation parameters in improving the alcoholic fermentation technology of BM. When free cells of *S. cerevisiae* were utilized, an inoculum's size of 3.6×10^5 cells/100 mL TBM and incubation period of 48h at 30°C gave optimal fermentation efficiency in the first use of yeast in the tank fermenter. In the case of immobilized cells, the continuous-flow technique speared superior to the repeated batch-fermentation technique in production of alcohol from TBM.

Selection of the proper technique depends on the type of carbohydrate substrate and nature of the yeast-strain utilized.

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