Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2010, Article ID 419586, 5 pages
doi:10.1155/2010/419586

Research Article

Comparative Study on Two Commercial Strains of *Saccharomyces cerevisiae* for Optimum Ethanol Production on Industrial Scale

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Received 8 June 2009; Revised 23 December 2009; Accepted 21 January 2010

Academic Editor: Effie Tsakalidou

Two commercial strains of *Saccharomyces cerevisiae*, Saf-Instant (Baker's yeast) and Ethanol red (Mutant) were compared for ethanol production during hot summer season, using molasses diluted up to 6-7° Brix containing 4%-5% sugars. The yeasts were propagated in fermentation vessels to study the effects of yeast cell count and varying concentrations of Urea, DAP, inoculum size and Lactrol (Antibiotic). Continuous circulation of mash was maintained for 24 hours and after this fermenter was allowed to stay for a period of 16 hours to give time for maximum conversion of sugars into ethanol. *Saccharomyces cerevisiae* strain (Saf-instant) with cell concentration of 400 millions/mL at molasses sugar level of 13%–15% (pH 4.6 ± 0.2, Temp. 32°C ± 1), inoculum size of 25% (v/v), urea concentration, 150 ppm, DAP, 53.4 ppm and Lactrol,150 ppm supported maximum ethanol production (8.8%) with YP/S = 250 L ethanol per tone molasses (96.5% yield), and had significantly lower concentrations of byproducts. By selecting higher ethanol yielding yeast strain and optimizing the fermentation parameters both yield and economics of the fermentation process can be improved.

1. Introduction

Molasses contains readily utilizable carbohydrates available in the form of fermentable sugars and can be used by the alcohol producing yeasts without any pretreatment [1]. Almost 75% of the world's molasses comes from sugarcane grown in tropical climates of Asia and South America, while the remainder comes from sugar beet grown in the more temperate climates of Europe and North America [2].

In molasses-based distilleries situated in the high-temperature zones of the world, there exist problems related to ethanol production in higher yield and with full efficiency of the yeast. The optimum growth temperature for ethanol producing yeast *Saccharomyces cerevisiae* is 32°C ± 2. However, in the higher-temperature zones, the efficiency of alcohol production process drops because of temperatures of above 40°C. On the other hand, the advantages of producing ethanol at temperatures higher than those used in conventional systems include reduced running costs with respect to maintaining growth temperatures in large-scale systems, reduced risk of contamination, and increased productivity at the later stage in the batch-fed reactor systems [3].

In the distilleries the generally used yeast for ethanol production is *Saccharomyces cerevisiae*. Along with ethanol, the yeast also produces a number of byproducts and impurities including considerable amounts of acetic acid and acetaldehyde. Production of ethanol and byproduct from molasses-based media has been reported on laboratory as well as on industrial scale [4–6].

This article reports the results of a study based on the comparative analysis of ethanol production along with byproducts by two commercial yeast strains in a local distillery of Pakistan.

2. Material and Methods

2.1. Sugarcane Molasses. Sugarcane molasses procured from the Shakarganj Mills Limited, Jhang, Punjab, Pakistan was used as carbon source for ethanol production by two yeast strains without any pretreatment. The molasses containing

13% to 15% sugar content was diluted by mixing tap water in 60 m^3 tanks to reduce its viscosity.

2.2. Yeast Strains. Two commercial strains of *Saccharomyces cerevisiae*, which are already in use in the distilleries for ethanol production, were purchased from local market. An indigenous strain *S. cerevisiae* Saf-Instant (Baker's yeast) and a mutant strain *S. cerevisiae* Ethanol red (Mutant imported from France) were compared for ethanol and byproducts formation. Both of the yeast strains were in compressed dry form and were rehydrated with water and molasses along with nutrients required for yeast growth.

2.3. Inoculums Preparation. Yeast cultures were prepared in separate seed fermenters of 1.5 m^3 capacity. Molasses diluted to 6-7° Brix, and 4%-5% sugar content was supplemented with Urea (1 Kg) and Phosphoric acid (500 mL). pH of the medium was adjusted to 4.6 (Preoptimized) using M NaOH/M H_2SO_4. The medium was steam sterilized at 121°C for 30 minutes. After cooling to 32°C ± 2, compressed strains of yeast were added and the seed fermenters were aerated to facilitate the growth of yeasts. At the end of first stage of 8 hours of continuous circulation, samples withdrawn from the sample valves were subjected to analyses to get 300×10^6 cells per mL.

The cultures were transferred to second stage of propagation in individual steam-desterilized (45 minutes) vessels of 30 m^3 capacity. To each vessel molasses was added up to 25% volume of tank and essential nutrients were added and the media were adjusted to pH 4.6-4.8. Molasses (brix 12°) was gradually fed to the growing yeasts to get 300×10^6 cell/mL in about 10 hours.

In the third stage the yeast cultures from these vessels were transferred to the propagation tanks of 60 m^3 capacities. The yeast cultures having 300×10^6 cells/mL, reducing sugars contents below 1% and ethanol content in the range of 4.0-4.5% v/v, were prepared for use in fermentation of molasses to ethanol.

2.4. Fermentation Process. Fed-batch culture system was employed for optimization of fermentation parameters for both strains. The yeast cultures were transferred to fermenters having working volume of 300 m^3. Initially a bed of 20% volume was made by yeast culture at the bottom of fermenter, but afterwards a continuous feeding of diluted molasses of brix 25 to 27° (15% to 17% sugars) was fed to the fermenters to enable yeast cells to utilize sugars in the molasses for conversion into ethanol. Feeding of molasses was adjusted so that fermenter vessels were filled to 100% working capacity with a level rise of 5% h^{-1} in a time period of 16 hours.

During fermentation, no nutrient or aeration was provided. However, circulation of mash was continued to control the temperature of mash up to 32°C ± 2. Continuous circulation of mash was maintained for 24 hours and after this fermenter was allowed to stay for 16 hours to allow the maximum conversion of sugars into ethanol. After 16 hours, the samples collected through sample valves were analyzed for ethanol content, residual sugars, viable cell count, brix, acetic acid, and potassium permanganate test time (PTT).

2.5. Process Optimization. During third propagation stage, all the parameters to be optimized were varied. During optimization, temperature and pH were maintained at previously optimized levels (temperature 32°C ± 2; pH 4.6-4.8). The process parameters were optimized by applying Classical Method of medium optimization, varying one parameter at a time in fed-batch culture. During optimization, temperature and pH were maintained at 32°C ± 2 and 4.6-4.8, respectively.

Cell count optimization was performed by using varying yeast cell counts like 300×10^6, 350×10^6, 400×10^6, and 450×10^6 cells/mL for each strain.

Varying concentrations of urea (100, 150, 200, 250 ppm) and DAP (35.49, 47.32, 59.15, 70.98 ppm) were added to the fermentation media inoculated with optimum yeast cell counts.

Varying volumes of inoculum (% v/v) of both strains were used to inoculate the respective fermentation vessels under optimized parameters of cell count, Urea, and DAP to investigate the effect of inoculum size on ethanol production and side products formation.

Acetic acid bacteria contaminations have a major impact on ethanol production in industrial fermentations. The effect of varying concentrations of antibiotic Lactrol (Virginiamycin + dextrose) was studied on ethanol and bacterial acid production under optimum fermentation conditions.

2.6. Analytical Procedures. Ethanol content of the fermented samples was measured with ebulliometer and confirmed on high-performance liquid chromatography (HPLC) [7, 8]. Molasses Brix was measured with the help of ATAGO densitometer (model 2313; ATAGO Co. Ltd., Tokyo, Japan) to maintain the sugar percentage [7].

Concentration of aldehydes was measured as potassium permanganate test time (PTT), as described earlier (ASTM-D-1363). Ethanol sample of 50 mL was taken in test tube and 2 mL of $KMnO_4$ (0.02%) was added and made up to 50 mL volume with distilled water. The time of change in color (as compared with control) was noted at the end. Acidity was measured titrimetrically using phenol red as indicator with light pink color endpoint [9].

Cell count was determined using electron microscope with the help of haemocytometer. Cell viability was checked by using methylene blue indicator. The dead cells were stained with blue indicator while viable cells remained uncolored [7].

3. Results and Discussion

During process optimization, the preoptimized temperature and pH were maintained. In a previous study fermentation of medium at 32°C ± 2 temperature and pH 4.6–4.8 gave maximum yield of ethanol with lower concentrations of acids [7].

Table 1: Effect of yeast cell count of inoculum on ethanol and acetic acid production by two commercial strains* using sugarcane molasses in fed-batch cultures.

Yeast cell count (10^6/mL)	Analysis											
	Ethanol yield (%v/v)		Residual sugar (%)		Final brix (°)		Final cell count (10^6/mL)		Acetic acid (mg/100 mL)		PTT (sec.)	
	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER
300	7.7	7.5	0.99	0.90	10.99	10.46	280	325	96.84	48.95	5	7
350	7.9	7.7	1.01	0.87	10.70	9.86	310	365	81.16	55.80	9	8
400	8.2	8.0	0.97	0.97	10.08	10.75	316	375	66.31	45.10	11	9
450	7.4	7.2	1.11	1.00	11.13	11.00	395	405	110.0	49.50	5	5

* SI: Saf-Instant and ER: Ethanol Red.

Table 2: Table 1 Effect of varying concentrations of urea (as nitrogen source) on ethanol and acetic acid production by two commercial strains* of yeast on sugarcane molasses in fed-batch cultures.

Urea concentration (ppm)	Analysis											
	Ethanol yield (%v/v)		Residual sugar (%)		Final brix (°)		Final cell count (10^6/mL)		Acetic acid (mg/100 mL)		PTT (sec.)	
	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER
100	8.0	7.3	0.98	0.95	10.31	9.91	290	304	74.14	38.65	10	10
150	8.3	7.9	0.91	0.89	9.53	9.91	306	340	38.04	35.31	13	11
200	7.9	7.5	1.05	1.08	10.53	10.80	280	365	89.06	39.56	9	8
250	7.5	7.0	1.15	1.20	11.13	11.05	265	289	98.75	48.08	7	6

* SI: Saf-Instant and ER: Ethanol Red.

Varying yeast cell counts were used for inoculation of fermentation vessels. Results indicated that for both of the strains the maximum ethanol content with minimum sugar loss and minimum undesirable products formation was with inocula having cell counts of 400×10^6 cells/mL. For *S. cerevisiae* Saf-Instant ethanol content was 8.2% v/v, remaining sugars (R.S) (0.97%), final brix 10.0^0, final viable cell count 316×10^6/mL, acetic acid 66.31 mg/100 mL, and PTT 11 seconds. For *S. cereviseae* Ethanol Red, ethanol content was 8.0% v/v, R.S 0.97%, final brix 10.75^0, final viable cell count 375 mg/100 mL, acetic acid 45.10 mg/100 mL, and PTT 09 seconds (Table 1). The results revealed that varying yeast cell counts had significant effect ($P \leq .05$) on ethanol yield. However the difference between the two strains regarding ethanol production and all other parameters was nonsignificant ($P \leq .05$). However, acetic acid production by Ethanol Red strain was significantly ($P \leq .05$) lower as compared to Saf-Instant.

Varying concentrations of urea were added as nitrogen supplement for yeast growth. Results showed that cell growth and ethanol yield increased with urea addition and 150 ppm urea concentration gave maximum ethanol content of 8.3% v/v. Optimum ethanol yield of 7.9% was obtained for Ethanol Red strain at same concentrations of Urea (Table 2). The two strains showed significant difference ($P \leq .05$) in ethanol yield, acetic acid content, and sugar loss.

Varying concentrations of DAP were used as phosphorus and supplementary nitrogen source to promote yeast growth and increase ethanol production. At DAP concentration of 59.15 ppm, *S. cerevisiae* Saf-Instant produced 8.5% (v/v) ethanol with remaining sugars, 0.8%, final brix 9.95^0, final cell count 355×10^6/mL, acetic acid 75.32 mg/100 mL, and PTT 13 seconds (Table 3). Ethanol Red also gave optimum results at the same concentration (59.15 ppm) of DAP but ethanol (8.1%) was nonsignificantly lower ($P \leq .05$) and acetic acid content (80.31 mg/100 mL) was significantly ($P \leq .05$) higher as compared to Saf-Instant.

Nitrogen and phosphorus are the main nutritional requirements for the yeast growth and maximum ethanol production efficiency. Although molasses contains most of the nutrients required for yeast growth, generally nitrogen and phosphate are added to enhance yeast growth and ethanol production [10]. For optimum yeast efficiency in molasses medium, urea was used as nitrogen source and DAP (Diammonium phosphate) was used as phosphate as well as nitrogen source. Phosphorus has the major role in the glycolysis cycle in the yeast cell. Extensive studies were previously performed to optimize the nitrogen and phosphorous sources and other supplements [11]. Higher ethanol production has also previously been reported with urea, phosphoric acid, and sulfuric acid making the process very economical [9].

Ethanol yield and production of coproducts has a major relationship during ethanol fermentation. Extensive studies have been carried out to investigate the effect of yeast inoculation rate to help out the yeast cells overcome the bacterial cells on the basis of size and number. Effect of varying inoculum sizes on ethanol yield and side products formation was studied under optimized parameters of cell count (400×10^6), Urea (150 ppm), and DAP (53.42 ppm). For both yeast strains maximum ethanol content was found at an inoculation rate of 20%. Results have shown that at 20% inoculation rate ethanol content was 8.4% and 8.7% for SI and ER strains, respectively (Table 4). Statistical analysis of data showed a significant ($P \leq .05$) effect of inoculum size on ethanol production. However, the difference between

Table 3: Effect of varying concentrations of DAP on ethanol and acetic acid production by two commercial yeast strains* using sugarcane molasses in fed-batch cultures.

DAP concentration (ppm)	Analysis											
	Ethanol yield (%v/v)		Residual sugars (%)		Final Brix (°)		Final cell count (10^6/mL)		Acetic acid (mg/100 mL)		PTT (sec.)	
	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER
32.05	7.5	7.1	0.99	0.95	10.25	10.50	305	315	47.69	43.91	7	5
42.74	7.9	7.5	0.95	1.01	10.10	10.25	318	340	39.87	37.25	8	7
53.42	8.4	8.0	0.87	0.89	9.89	9.90	345	360	35.59	30.12	12	11
64.11	8.0	7.8	1.10	1.15	10.95	11.10	365	385	47.33	45.75	6	5

* SI: Saf-Instant and ER: Ethanol Red.

Table 4: Effect of varying inoculum sizes on ethanol and acetic acid production by two commercial yeast strains* using sugarcane molasses in fed-batch cultures.

Inoculum size (%v/v)	Analysis											
	Ethanol (%v/v)		R.S (%)		Final Brix (°)		Final cell count (10^6/mL)		Acetic acid (mg/HL)		PTT (sec.)	
	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER
15	8.1	8.1	1.3	1.21	11.73	12.15	289	297	114.48	107.20	6	5
20	8.5	8.7	1.0	1.11	10.61	10.95	305	317	90.61	87.57	9	8
25	8.4	8.5	0.97	0.99	9.71	9.85	335	348	75.81	74.36	13	11
30	7.9	8.0	1.15	1.25	9.89	10.13	341	365	83.69	79.29	7	6

* SI: Saf-Instant and ER: Ethanol Red.

Table 5: Effect of varying concentrations of lactrol on ethanol and acetic acid production by two commercial yeast strains* using sugarcane molasses in fed-batch cultures.

Lactrol concentration (ppm)	Analysis											
	Ethanol (%v/v)		R.S (%)		Final Brix (°)		Final cell count (10^6/mL)		Acetic acid (mg/HL)		PTT (sec.)	
	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER	SI	ER
0.5	7.9	7.7	1.05	0.99	11.23	11.57	285	311	62.93	65.34	5	3
1.0	8.2	8.0	0.95	0.95	11.01	11.23	319	328	54.39	57.10	7	5
1.5	8.8	8.7	0.85	0.89	10.50	10.65	345	360	35.37	38.65	12	10
2.0	8.0	8.1	0.95	1.01	10.85	11.25	260	285	37.83	40.27	11	8

* SI: Saf-Instant and ER: Ethanol Red.

the two strains was nonsignificant ($P \leq .05$). In brewing, higher yeast inoculation rates cause attenuation to initiate the process more rapidly, and reduce viability losses that occur immediately after pitching [12]. In a previous study, the ethanol yield increased with increasing inoculum size and yield of methanol, acetic acid, fusel alcohols, or aldehydes was the lowest at inoculum size above 30% [7].

The basic requirements for *Saccharomyces cerevisiae* are fermentable sugars and micronutrients. However, during fermentation, contaminating bacteria compete with yeast cells for sugar and nutrients causing significant decrease in ethanol production. An antibacterial Lactrol (Virginiamycin + dextrose) was added at varying concentrations to control the growth of contaminating bacteria. Optimum ethanol content (8.8%) for Saf-Instant was found in the medium receiving 1.5 ppm Lactrol (Table 5). Remaining sugars were 0.85%, final brix 10.50°, cell count 345×10^6, acetic acid 35.37 mg/100 mL, and PTT 14 seconds for the Saf-Instant. Ethanol Red strain also gave optimum ethanol content (8.7%) at similar concentration of Lactrol. The remaining sugars were 0.89%, final brix 10.65°, final viable cell count 360x10^6/mL, acetic acid 38.65 mg/100 mL, and PTT 13 seconds. Addition of Lactrol caused significant bacterial growth inhibition that is reflected by lower acetic acid yields of both yeast strains

In our distilleries the major problem was to control the temperature during hot season (from June to August) that lowers the ethanol yield and efficiency of *Saccharomyces cerevisiae* (optimum activity at temperature 32°C ± 2). To overcome this problem, the mutant strain of yeast with trade mark Ethanol Red was imported from France.

The results of our study showed that under optimum conditions there were nonsignificant ($P \leq .05$) differences between the two strains regarding ethanol yield. However, acetic acid production of ER mutant was significantly ($P \leq .05$) lower than our indigenous strain Saf-Instant. On the average ethanol production by *S. cerevisiae* Saf-Instant was better as compared to imported strain Ethanol Red. Side products production efficiency (other than acetic acid) differed nonsignificantly for both strains. However,

optimization of process parameters improved ethanol production and decreased side products formation by the local yeast strains of *S. cerevisiae* Saf-Instant.

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