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Enhanced Ethanol Production Incorporating Process Improvement Strategies in Molasses Fermentations

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ABSTRACT

Many cost effective strategies were proved to be leading to enhanced ethanol production during molasses fermentations. Strains of yeast exhibiting better tolerance to higher levels of alcohol in the broth and higher initial concentrations sugar was the most used option. Sequential batch processing resulting in nutrient balancing and incorporation of yield boosting nutrients were the choice of the methods used for enhanced ethanol yield in this study. Our experiments indicated as high as 1+/-0.5 [gg^{-1}] yield enhancements for ethanol in sequential batch process. This was run continuously for 10 cycles with appropriate nutrient supplementations. The viability and activity of the yeast cells were found to be positively influenced by the incorporation of nutrient stimulants. These yield increasing strategies when worked in tandem, it results in promising, easy to implement & economically viable strategies.

Keywords: Molasses, Sequential batch process, yield boosters for ethanol production and yeast from toddy sap.

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INTRODUCTION

As the demand for ethanol is showing a progressive rise over the years, there is an imminent requirement of high yielding production strains and economically viable process realization for the production of ethanol. The quest for cheaper rate ethanol has been pursued actively by research groups [1- 5]. There are many proven process corrections for attaining enhanced ethanol production in molasses based fermentations. Use of specially selected strains of yeast exhibiting better tolerance to ethanol and initial sucrose levels is one such reliable approach for additional yield in ethanol fermentations. Faster fermentation rates for ethanol production have been reported by following various strategies such as [1] higher cell densities per unit fermentation volume [6]. [2] enhancing ethanol tolerance [7] [3] recycling of biocatalyst population etc [8]. Yield enhancements can be thought of by employing sequential batch processes with well designed nutrient balancing. Certain micronutrients/ micronutrient preparations have been successful in contributing to better ethanol outputs [9]. Molasses is an agro-industrial by product often used in alcohol distilleries [10] due to the presence of fermentative sugars, being an optimal carbon source for the microorganism metabolism. Many studies have been done that focus on production improvement and decreasing its cost [11-13] In our investigation, regular baker's yeast [NCIM 3570] and specially isolated yeast were used for knowing the comparative significance in terms of yeast growth and activity. Yeasts were grown in both batch and sequential batch [10 cycles] formats. Appropriate nutrient supplementation was envisaged in the latter. The yield boosting was maximum when the aforesaid strategies were used in combination with indigenously made organic micronutrient stimulants.

MATERIALS AND METHODS

Microorganisms: An indigenously isolated strain of *Saccharomyces cerevisiae* [NCIM 3640] was used in this study along with a standard strain of baker's yeast [NCIM 3570]. The principal investigator's team [unpublished results] isolated this yeast culture from toddy sap employing the regular procedure of serial dilution and plating [in yeast development agar [YDA]] followed by incubation at 300C. The toddy sap isolate was subsequently deposited with NCIM [NCL, CSIR], Pune, India.

Preservation and subculture: Standard stock cultures where prepared in 50% aqueous solution of glycerol and the same was stored at -20^{0}C. Working stocks were prepared as agar slants [YDA] and stored at 40C after incubation at room temperature for 2-3 days.

Inoculum Development: Yeast cells were transferred from agar slants as two loopful of culture per 50 mL of sterilized YD broth held in 250 mL E.M. flask. The seed culture flasks were incubated in environmental shaker set to 150 rpm and 30±10C for 24 hours. After 24 hours, the cells from seed flasks were subjected to centrifugation [4,000 rpm, 10 min at 40C]. Such centrifuged cell pellets were used as inoculum. Hence, inoculums mentioned in this study comprise of the total cell pellets obtained at the end of 24 hr of cultivation from 50 mL of seed

culture developed aerobically as explained above. Cells from 50 mL were used for inoculating every 1L production medium.

Fermentation: Fermentation experiments were carried out with commercially available cane molasses collected locally from the sugar factory [GMR Distilleries, Srikakulum]. Molasses was characterized by subjecting molasses samples to analyses such as total reducing sugars, pH, volatile acids etc. by following the standard analytical procedures. Initial studies were carried out using both the yeast cultures separately in 150 mL plastic screw cap bottles containing 100 mL each of 1.080 molasses medium with 1% each of urea & DAP [pH was adjusted to 4.5]. Static cultures were maintained at room temperature for 36 h. In a parallel set of experimentation, the same was repeated with the addition of a nutrient mix [indigenously prepared organic extract] at 0.05% [v/v] level. The samples were collected for distillation followed by estimation of ethanol using gas chromatography. Three two liter plastic reactors with provision for sending in or drawing out medium at every 200 mL levels [200 mL, 400 mL, 600 mL……] were used for this experimentation. All such extended provisions from the reactor [6mm dia] were provided with manually controlled ball valves. Two peristaltic pumps were used per reactor, one for sending in medium and other to draw out fermenting broth at regular intervals. The three tanks were entitled as A, B and C. In reactor A & B diluted molasses of specific gravity 1.080 was used at 1 liter level to initiate the fermentation on day 0. The pH of molasses medium was adjusted to 4.5 using 1 N NaOH. The fermentation was carried out under anaerobic conditions as static process with no agitation and aeration provided during the course of the experimentation. At every 24 hrs interval, 200 mL of fermented broth was drawn out of the reactors and the same quantity of specified concentration [1.020 to A and 1.060 to B] of fresh medium was sent into reactor. The table 1 indicates the feeding and medium withdrawal schedules followed for various reactors. All the molasses media used for various reactors were supplemented with 1% each of DAP and urea. If the various samples ports available in the reactors can be named as S1, S2, S3, …….to S9 [S1 here refers to sample port at 200 mL level, S2 that at 400 mL level, S3 refers to that at 600 mL and so on], at 24 hrs interval, 200mL of fermented broth was drawn out from S1; at 48 hrs the sample [200mL] was derived from S2; at 72 hrs from S3 and so on [cyclically]. The addition of fresh medium [200 mL each time] was always from S9. The reactor A was a typical batch fermentor with constant volume of medium inside the reactor at any given point of time between day 1 and day 10. Reactor B, on the other hand was showing varying levels of medium from day 1 to day 10 [the pattern of flow in to the reactor and withdrawals for reactor B is indicated in Table 1. Reactor C was essentially working as a cascade reactor for reactor B and was operational only between day 5 and day 10 i.e. reactor A, B and C were all sequential batch reactors. Reactors A & B were operated for a period of 10 days where as reactor C was operational only for last six days of the 10 day duration of the experiment. Besides this, reactor C was supplemented with the indigenously made organic extract at 0.05% [v/v] level once in every 24h. The other general nutrient substitution to molasses medium [common to all reactors] consist of commercial grade Urea and DAP [1% wt/v each] at every 40 hr interval during the entire duration of experimentation [240 hrs].

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Table 1: The pattern of medium flow into the reactors and the withdrawals indicated along with the strength of molasses media used for various reactors.

Reactor Name	A			B			C		
Days	Addititonal, ml		Withdrawal Vol, ml	Addititonal, ml		Withdrawal, Vol, ml	Addititonal, ml		Withdrawal, Vol, ml
	strength	Vol		strength	Vol,ml		Strength	Vol	
D0	1.080	1000	200	1.080	1000	-	-	-	-
D1	1.200	200	200	1.160	400	200	-	-	-
D2	1.200	200	200	1.160	400	200	-	-	-
D3	1.200	200	200	1.160	400	200	-	-	-
D4	1.200	200	200	1.120	400	200	-	-	-
D5	1.200	200	200	1.160	400	300@ +200	1.160 From B	200 300	-
D6	1.200	200	200	1.160	400	300@ 200	1.160 From B	200 300	-
D6	1.200	200	200	1.160	400	300@ 200	1.160 From B	200 300	200
D7	1.200	200	200	1.160	400	300@ 200	1.160 From B	200 300	200
D9	1.080	200	200	1.060	400	300@ 200	1.160 From B	200 300	200
D10	-	-	200	-		200	1.160 From B	200	200

Reactor C has nutrient boosters added at 500 microlitres per litre of broth for every 24 h.
Strength here refers to specific gravity of the molasses medium
@300 mL for addition to reactor C & 200 mL for sample processing

Analytical procedures: Initial molasses medium characterization for Total Reducing Sugars [TRS] and pH were carried out using standard analytical procedures [15]. The samples of fermented broth derived at various intervals during the period of experimentation were analyzed for alcohol levels using Gas Chromatography [column: Porapak P; temperatures: injector [1200C], oven, [1000C], detector [1400C], carrier gas: nitrogen]. The ethanol yield [Yps] was calculated as the actual ethanol produced and expressed as g ethanol per g total sugar utilized [gg-1]. The volumetric ethanol productivity QP [gl-1h-1] was calculated by ethanol concentration produced P [gl-1] divided by fermentation time giving the highest ethanol concentration. . Periodic samples were subjected to alcohol estimation after distilling the samples. Samples at every 48 hr interval were centrifuged [4000 rpm, 10 minutes, at 100C] and 2 mg of pellets were dispersed in 1 mL of distilled water and observed under microscope for cell viability using standard procedure of methylene blue staining. Average number of viable cells present in five microscopic fields was expressed as percentage of viability.

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RESULTS AND DISCUSSION

Initial fermentative studies indicated the superiority of the toddy sap isolate of yeast for higher ethanol production in 48 h. In the usual static fermentation of molasses medium with urea and DAP, NCIM 3640 yielded 0.43 [gg-1] and 0.44for 1.080 and 1.120 molasses media respectively [table2]. This was confirming superiority of the local isolate. The indigenously prepared organic booster mix when supplemented in the production medium [1.120], both strains of yeast gave better production with NCIM 3640 producing superior yield in this trial too [Table2]. All the three reactors produced better ethanol levels than the static flasks used in the preliminary studies with the same local isolate of yeast [NCIM 3640]. The typical batch fermentations yielded just 0.43 gg-1 for 1.120 medium and 0.44 gg-1 for1.120 medium. Even in the presence of yield booster the ethanol concentration was only 0.46 gg-1. While in sequential batch process, the yield was as high as 0.48 gg-1for reactor A and 0.49 gg-1for reactor B. Beyond a level if the medium sugar levels are increased, the rate of production of ethanol is not proportionately increasing for this isolate. The ethanol concentrations depicted in table 3 clearly depicts the superiority of the sequential batch process for ethanol production. The findings from the reactor C when read in comparison with the corresponding findings for reactor B, it is clear the inductive role of the yield booster used in this study [Table 3]. 1.080 medium is better suited for the sequential batch process with NCIM 3640 than 1.120 medium.

The viability of cells observed under microscope also indicated more viable cells in the slides made with 1.080 medium than 1.120 medium especially after 5th day of process. Ernandes et al. [1990] reported the diversified response of various isolates of yeasts for medium sugar levels, rate of ethanol production and cell viability etc. [16]. From the aforementioned studies, it is possible to conclude that the sequential batch process is a desirable practice for enhanced ethanol production. For molasses based fermentations, this will be a better option compared to fed-batch culturing in terms of volume of alcohol produced per reactor per unit time. If we can carry out such production process with yeasts capable of withstanding higher initial levels of sugar, it is more desirable. In our studies we could easily lift the initial concentration of sugars in molasses to 1.12 [gravity of molasses] from the regular 1.08 being normally followed for industrial production of ethanol. Further rise to 1.16 did produce better results but the net rate of production of ethanol indicated a drop as a result we did not register a proportional rise in yield. Viability of yeast cells is largely preserved through this mode of fermentation is another noteworthy aspect [fresh yeast cells were given only after every 96h that too in very negligible amounts]. Nutrient balancing by maintaining carbon, nitrogen, phosphorous and micronutrients possible through sequential medium addition coupled with periodical removal of excess alcohol produced would have helped the system to give better results.

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Table 2: Ethanol production by yeast cultures in molasses media and the influence of an Indigenously made nutrient mix on the biosynthesis of ethanol

Isolate type	Regular Yeast			Toddysap isolate of yeast (NCIM 3640)			
Molasses mix	P (gl^{-1})	Qp($gl^{-1}h^{-1}$)	Yps(gg^{-1})	P (gl^{-1})	Qp($gl^{-1}h^{-1}$)	Yps(gg^{-1})	t(h)
1.080	46.6	1.29	0.38	51.8	1.43	0.43	48
1.120	59.2	1.64	0.39	62.6	1.73	0.44	48
1.120 with nutrient mix (500 ul/L)	62.8	1.74	0.41	69.6	1.93	0.46	48

P, ethanol concentration; Qp, volumetric ethanol productivity; Yps, ethanol yield and t, fermentation time.

Table 3: Ethanol concentrations obtained from various samples from reactors (A, B&C) operated as sequential batch reactors

Day	P (gl^{-1})	Yps(gg^{-1})	P (gl^{-1})	Yps(gg^{-1})	P (gl^{-1})	Yps(gg^{-1})
	Reactor A		Reactor B		Reactor C	
1	44.0	0.36	43.7	0.36	-	-
2	82.0	0.48	73.0	0.40	-	-
3	79.4	0.46	83.4	0.49	-	-
4	78.9	0.46	83.0	0.46	-	-
5	79.4	0.46	77.6	0.43	-	-
6	74.4	0.43	76.0	0.42	-	-
7	72.0	0.42	73.3	0.40	75.9	0.42
8	71.2	0.41	72.5	0.40	73.3	0.40
9	61.0	0.35	69.2	0.38	72.6	0.40
10	55.0	0.45	63.0	0.35	66.2	0.36

P, ethanol concentration and Yps, ethanol yield.
All the experiments were performed in Triplicate.

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