High gravity fermentation of sugarcane molasses to produce ethanol: Effect of nutrients

P. Pradeep · O. V. S. Reddy

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Abstract Fermentation efficiency of more than 85% was obtained by high gravity fermentation of 33–34°Bx (spec. gravity ≈ 1.134) molasses medium with certain nutrients, instead of generally employed medium containing $\approx 16\%$ (w/v) total sugar (spec. gravity ≈ 1.090) for ethanol fermentation in distilleries to get maximum 80–85% conversion. The fermenting yeast, *Saccharomyces*, has varied capabilities, depending on the species and nutrition for fermenting the high solids medium. The fermentation period was reduced to 48 h; and the byproducts obtained were less in concentration, upon supplementation with nutrients (or osmoprotectants) like soy flour/wheat bran to the medium in the existing batch fermentation technology. This has been found partly due to improved yeast cell viability during fermentation.

Keywords High gravity fermentation · Fuel ethanol · Cane molasses · Supplementation · *Saccharomyces bayanus*

Introduction

Ethanol is produced by conventional batch fermentation of cane molasses in most of the distilleries. About 7–8% (v/v) ethanol is produced from diluted molasses (15–16%) with 80–85% efficiency [1]. In this process, huge effluent (about 12 liter effluent/liter absolute alcohol) with very high biological oxygen demand (BOD) is produced [2]. This poses a severe problem of waste disposal problem. To avoid this problem, use of very high gravity (VHG) fermentation technology could be an alternative. VHG technology is defined as “the preparation and fermentation of mashes containing 27 g or more dissolved solids per 100 g mash” [3]. Use of VHG fermentation technology saves considerable amount of water, provide more yield of alcohol and reduces labor and energy needs, capital cost and also the bacterial contamination [4]. The VHG technology may allow to approximately doubling the ethanol yield (7–10 to 15–18% v/v). However, VHG fermentations are sluggish and saccharides are rarely fermented completely. High contents of saccharides in fermentation medium cause increase of the osmotic pressure, which has a detrimental effect on yeast cells. However, *Saccharomyces cerevisiae* which is commonly used for ethanol production can ferment increased amount of sugars in the medium, when all the other required nutrients are in adequate amounts [5]. In high gravity ethanol fermentations, most of the studies have been carried out using grain starch of wheat, corn, barley, oats, etc. or pure glucose or sucrose saccharine substances. Little work has been done on fermentation with beet or sugarcane molasses using VHG technology. Hence, a study was carried out to evaluate the VHG technology for the fermentation of molasses.

P. Pradeep · O. V. S. Reddy (✉)
Department of Biochemistry,
Sri Venkateswara University,
Tirupati – 517502, India

E-mail: ovsreddy@yahoo.com

Materials and methods

Molasses

Good quality molasses having 85–88°Bx, total reducing sugars (TRS) (53% w/w) was obtained from local distillery and stored at room temperature (~30°C) until use.

Yeasts, culture media and preparation of inoculate

Flocculating, non-amyolytic laboratory yeast, *Sacchromyces bayanus* was kindly provided by Dr. Roberto Ambrosoli, University of Turin, Italy. The culture was preserved at 4°C by regular subculturing (once every 3 months) over malt extract, peptone, yeast extract, dextrose (MPYD) agar. The MPYD agar contained (g/l): malt extract, 3; peptone, 5; yeast extract, 3; dextrose, 20 and agar, 15. The inoculum was prepared by cultivating the strain in a 250 ml Erlenmeyer flask having 100 ml medium at 30°C on a rotary shaker (130 rpm) for 24 h. The medium contained (in %) molasses sugar, 5; urea, 0.2; MgSO₄, 0.05 and yeast extract, 0.1. The cells were harvested by centrifugation at 2,000 rpm for 5 min. The pellet was washed twice with 30 mM/l ethylene diamine tetra acetic acid (EDTA) to ensure floc disruption and finally washed and suspended in sterile deionized water. An amount of preculture was added to the fermenting medium which yields an initial concentration of 2×10^7 yeast cells/ml. Experiment was conducted in triplicate flasks.

Fermentation conditions

High gravity medium was prepared by diluting 100 g molasses with soft water (treated to remove hardness) to get medium having 30°Bx at start. Later, water to molasses ratio was decreased to get more concentrated medium having specific gravity above 1.12. TRS content was in the range of 21–26.5% (w/v), depending on the dilution. These fermenting media (100 ml each) were taken into 500 ml flasks, supplemented with various yeast assimilable nitrogen compounds like urea, peptone, yeast extract and yeast autolysate in the range of 0.1–1.0% along with magnesium sulfate. Additives such as soy flour, corn flour and wheat bran were included in the media along with nitrogen supplements. The pH of the medium was adjusted to 5 and was not sterilized. Fermentation was carried out at 30°C under stationary condition for 72 h.

Pretreatment of molasses

Diluted molasses medium was acidified to pH 4.5 by using sulphuric acid, before heating at 120°C for 10 min. It was

allowed to cool to room temperature and then centrifuged at 5,000 rpm for 15 min. The clear supernatant was used for fermentation studies. pH was adjusted to 5 using alkali and other fermenting conditions were similar to that of non-pretreated medium.

Analytical methods

Concentration of TRS in diluted molasses, after sucrose inversion using HCl and amounts of residual sugars in fermented wash was measured by Fehling-Soxhlet method [6]. Total solids content in the medium was measured by both refractometer as well as specific gravity hydrometer at 27°C. Ethanol in the fermented wash and fermentation byproducts such as aldehydes, methanol, total esters, total fusel oils and acetone contents were estimated by using gas chromatography (GC) as per standard methods [7]. Agilent systems model 6890 was used and the conditions were as follows: Graphitized packed column 5% carbowax 20 M phase, matrix 80/120 carbopack-B, Length 6 ft (1.83 m) $\times$ 2 mm ID $\times$ 1/4" OD. Nitrogen was used as carrier gas with flow rate of (20 ml/min) and eluted compounds were detected by flame ionization detector (FID). Hydrogen was used as fuel gas, with flow rate 40 ml/min, along with air at a flow rate of 400 ml/min. Secondary butyl acetate was used as internal standard.

Cell number and viability

Cell number was estimated by counting cells under the microscope in a hemocytometer, after diluting the samples in water. Viability was determined by spreading samples on Petri plates containing MPYD agar and counting colonies after 4 days of incubation at 30°C.

Results and discussion

Batch fermentations

In the present study, high gravity molasses media containing different levels of dissolved solids concentration, ranging from 30 to 40°Bx were prepared and fermentations were carried out for 72 h duration. Higher ethanol conversion efficiencies (>80%) were obtained by fermenting the media having up to 34°Bx (24.2% TRS) by supplementation. Up to this °Bx level, considerable low levels (1–2% w/v) of sugars were left-over in the wash unfermented (Table 1). Beyond 34°Bx dissolved solids concentrations, significantly high levels of residual sugars (>5% w/v) remained in the media after fermentation and further reduction of these levels has not been found even at higher levels of nutrients supplementation (Table 2).

Table 1 Approximate °Bx and corresponding specific gravities, sugar profile (TRS and RS) and ethanol concentrations of high gravity molasses fermenting media

°Bx*(approx.)	Specific gravity		TRS conc. (g/100 ml)	Residual sugar % (w/v) [#]	Alcohol % (v/v)	Fermentation efficiency %
	Initial	After 72 h fermentation				
30–31	1.120	1.037	20.5	1.8	11.4	85.9
32	1.128	1.045	22.7	1.87	12.3	83.67
33–34	1.134	1.052	24.0	1.92	12.84	82.6
35	1.142	1.059	25.2	3.2	11.2	68.7
36	1.146	1.075	26.5	5.8	10.9	63.74

*At temperature 27.5°C.

[#] Minimal levels under nutrients supplementation.**Table 2** Batch ethanolic fermentations of very high gravity molasses medium (supplemented) beyond 35°Bx

°Bx	Nutrients % (w/v)	Specific gravity		Residual sugar % (w/v)	Ethanol % (v/v)	Fermentation efficiency %
		Initial	Final			
35	Urea-0.35, MgSO ₄ -0.15	1.144	1.068	4.8	10.7	65.6
35	Urea-0.5, MgSO ₄ -0.25	1.144	1.070	5.1	9.6	58.9
35	Urea-0.35, MgSO ₄ -0.15, Wheat bran-2	1.142	1.059	3.2	11.2	68.7
35–36	Urea-0.35, MgSO ₄ -0.15 Green gram bran-2	1.146	1.066	4.0	11.3	65.8
36	Urea-0.4, MgSO ₄ -0.2	1.148	1.075	5.8	10.9	63.5

Effect of supplementation of 34°Bx medium

Fermentation efficiency was very low even at 34°Bx molasses concentration without supplementation. The role of nitrogen rich nutrients viz. urea, peptone, yeast extract and skim milk solids on ethanol production was examined by supplementation of these to 34°Bx medium. The final ethanol concentrations were almost similar, 95.4 g/l, 99.8 g/l, 98.9 g/l and 100.2 g/l with skim milk solids,

peptone, urea and yeast extract compared to control 72.9 g/l. Among these, urea was found to be the most suitable nitrogen source for molasses fermentation, since it is cheaper in cost, available in plenty and does not increase the viscosity of medium, as compared to other organic nitrogen sources. Ethanol productivity was increased significantly from 1.21 g/l/h to 2.08 g/l/h (maximum) upon supplementation using the above nitrogen sources (Fig. 1).

Fig. 1 Effect of nutrients supplementation on sugar utilization by the *S. bayanus* in 34°Bx high gravity molasses medium.

Table 3 High gravity ethanol fermentations of initial 33–34°Bx (24% TRS) containing molasses medium (without pretreatment) using supplements at different concentrations by *S. bayanus*

Additive/nutrient added	Concentration % (w/v)	Specific gravity (final)	Residual sugar %	Alcohol % (v/v)	Fermentation efficiency %
Unsupplemented (control)	—	1.062	2.4	9.8	63.0
Urea + MgSO ₄	0.4+0.2	1.050	2.3	11.5	74.0
Urea + MgSO ₄	0.6+0.25	1.052	2.42	12.1	77.8
Urea+MgSO ₄ + Wheat bran	0.35+0.15+1.0	1.047	2.1	12.68	81.5
Urea+MgSO ₄ + Wheat bran	0.35+0.15+2.0	1.053	2.6	12.11	77.9
Wheat bran + Soy flour	1.0+1.0	1.052	2.5	11.8	75.9
Wheat bran + Corn flour	1.0+1.0	1.049	2.37	11.5	74.0
Urea + MgSO ₄ + Soy flour	0.4+0.15+1.0	1.052	1.92	12.84	82.6

Table 3 summarizes the importance of yeast assimilable nitrogen (free amino nitrogen [FAN]) source in the medium. The addition of FAN leads to higher final ethanol concentrations in the fermented media and amounts of the cell mass accumulation. In media containing 120 g saccharides per liter, adequate usable nitrogen must be in the 140–150 mg FAN per liter range [8]. In the media of higher gravity, the recommended concentration increases [5]. Although molasses contain total FAN (267 mg/l) in ~22% dissolved solids mash, usable FAN (141 mg/l) is low [9]. For high gravity molasses fermentations still more amount of FAN is required to accelerate fermentation rate. But, surprisingly the amount of nitrogen required to attain similar yield level is halved when the medium was supplemented with additives, as shown in the Table 3. These additives are rich sources of protein, lipids, minerals, vitamins (Table 4) and antioxidants. The nutrients may be due acts as osmoprotectants and/or yeast nutrients. And the bran may act as natural immobilization support for the yeast to adsorb on.

Therefore, supplementation of 34°Bx medium with yeast nutrients has increased the final ethanol concentration by 31% and decreased the fermentation duration from 72 to 48 h, thereby ethanol productivity was improved

(>2.0 g/l/h). All the added supplements has shown significant ($P < 0.05$) increase of ethanol concentrations when compared with unsupplemented medium. Among the various additives tested, soy flour and wheat bran at 1% concentration have shown superior performance along with urea, in terms of productivity and final ethanol concentration. Soy flour and wheat bran are relatively inexpensive and abundant source of protein with lipid which can be easily assimilated into cellular materials.

Effect of pretreatment

In order to reduce the osmotic pressure on yeast, suspended solids were removed from the 34°Bx medium by pretreatment process and then the fermentation was carried out using *S. bayanus*. Pretreatment of molasses medium has shown significant influence on ethanol production. Final concentration of ethanol was improved by 21% in the fermentation of pretreated medium even without any nutrient supplementation when compared to non-pretreated control medium. Further, nutrients supplementation has raised the ethanol concentration of pretreated molasses to 13.6% (v/v) (Table 5). Thus, improved fermentation efficiency (above 87%) was observed in pretreated

Table 4 Nutrients composition of supplemented additives in high gravity molasses medium

	Soy flour	Nutrients composition/100g		Skim milk solids
		Wheat bran	Corn flour	
Protein	34.5 g	13.3 g	6.93 g	35%
Total lipid	20.6 g	4.5 g	3.86 g	1% (Max.)
Carbohydrate	35.2 g	15.4 g	76.87 g	51%
Minerals	K, P, Ca, Mg etc.	K, P, Mg, Ca, Fe, Zn etc.	K, P, Mg, Ca etc.	K, Ca, P, Na etc.
Vitamins	Thiamine, niacin, riboflavin, folate etc.	Niacin, riboflavin, vit.E, folate etc.	Niacin, riboflavin, thiamin etc.	Niacin, vit. C, riboflavin, pyridoxine etc.

Source: <http://www.fineli.fi/food> and USDA.

Table 5 Effect of supplementation in pretreated molasses fermentation (24% w/v TRS) on ethanol production using *S. bayanus*

Supplement	Final ethanol concentration % (v/v)	Residual sugars % (w/v)	Fermentation efficiency %	Ethanol productivity g/l/h
Unsupplemented	11.9	2.2	76.5	1.56
Urea (0.35%) + MgSO ₄ (0.15%)	13.1	1.1	84.3	2.15
Urea (0.35%) + MgSO ₄ (0.15%) + Soy flour (1.0%)	13.6	0.7	87.5	2.2
Urea (0.35%) + MgSO ₄ (0.15%) + Wheat bran (1.0%)	13.5	0.5	86.8	2.18

molasses than in medium without pretreatment (82%), upon supplementation. During pretreatment process, contaminant microorganisms could also be eliminated from the medium along with suspended inorganic and organic fermentation inhibitory compounds, eventually leading to greater conversion efficiency. Pretreatment of cane molasses with tricalcium phosphate, hydrochloric acid, and Sephadex fractionation was shown to increased production of citric acid by *Aspergillus niger* [10]. About 18% increase in ethanol production and 42% reduction in the residual sugars was reported in a batch ethanolic fermentation of pretreated molasses by ceramic microfiltration membrane [11].

Effect on growth and viability

The yeast cell number was increased to $\approx 4.9 \times 10^8$ cells/ml from initial 2×10^7 cells/ml in 1% soy flour supplemented with in 48 h period. In unsupplemented medium $\approx 3.1 \times 10^8$ cells/ml was observed. As high as 72% viability of cells were maintained even at 48 h of fermentation time compared to

control 40% (Fig. 2). This clearly indicates that the yeast cells are provided with some nutrients in order to overcome the osmotic stress at initial and ethanol induced oxidative stress at the end of fermentation. Similar type of results were obtained by adding 4% soy flour (w/v) to a simple medium with 300 g/l glucose [12].

Fermentation byproducts

Aldehyde concentration in the form of acetaldehyde and total fusel oil concentrations were found to be less in the supplemented media than the control. Acetaldehyde concentrations of 28.07 and 30.5 mg/100 ml absolute alcohol were found in the soy flour and wheat bran (at 1%) supplemented media, respectively, in comparison with control medium that showed 59.24 mg/100 ml of absolute alcohol. Total fusel oils content in the unsupplemented medium was found to be higher 219.54 than the supplemented ones with soy flour and wheat bran 159.73 and 180.61 mg/100 ml absolute alcohol, respectively. Significant reduction in the byproducts was observed upon supplementation of nutrients and osmoprotectants such as horse gram flour, finger millet [13, 14] to the high gravity fermenting medium. In general, the formation of higher levels of fusels by yeasts takes place under certain unfavorable fermenting conditions such as higher temperatures, low pH and also when yeast activity is limited by low nitrogen content.

Fig. 2 Viability percentage of yeast *S. bayanus* in supplemented medium during 72 h fermentation period. (▲) 1% soy flour, (■) 1% wheat bran against control (◆).

Conclusion

The nutritional supplements have increased the ethanol yield in the present study, which are inexpensive and abundantly available. Supplements addition has shown increased the final ethanol concentrations to nearly 14.0% (v/v) in high gravity medium compared to 7–9% (v/v), the concentration presently obtaining in the industry. These nutrients were helped in achieving greater productivity, as rapid and higher ethanol concentrations are the major objectives of any ethanol industry. Higher ethanol concentrations in the

medium would certainly lead to reduced outflow effluents and lower distillation costs, thereby improving the economics of ethanol fermentation. Generally, flocculent yeast strains have more tolerance to ethanol induced growth inhibition and viability loss. Further studies are needed to confirm the suitability of the high gravity technology for molasses fermentation, although it has established well in cases of wheat, oats, and other starchy materials fermentation.

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