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Short Communications

Potential industrial applications of yeast capable of fermenting high gravity cane molasses despite physiological stress

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Two *Saccharomyces cerevisiae* isolates were studied for their ability to ferment cane molasses of high gravity. Supplements like soybean meal (SM), groundnut meal (GM) or castor oil meal (CM) (@2.5%) were found to be quite effective in enabling the yeast to ferment molasses of high gravity (35-40° brix). Ethanol production efficiency was increased by 45-50% with supplementation of any of these additives to concentrated molasses. The viability of yeast also improved by 24-25% in high gravity molasses. In concentrated worts with no supplement, trehalose content of yeast was increased significantly but little increase was reported in medium supplemented with SM, GM or CM, indicating the stress relieving effect of these supplements. Glycerol content did not vary with increase in the concentration of sugars in the medium. There was also no effect on invertase activity of yeast while fermenting the concentrated molasses worts.

Keywords: ethanol production efficiency, fermentation, glycerol, high gravity molasses, osmotic stress, *Saccharomyces cerevisiae*, trehalose

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Blackstrap molasses contains high level of inhibitory substances for yeast growth and fermentation. That is why it is used either in very diluted concentrations (12-17% fermentable sugars) or after various pretreatments[1-2]. Further, fermentation of diluted molasses results in the production of large quantities of effluent (*c*. 12 l/l of absolute ethanol), which is difficult to dispose off due to its high BOD (60,000-80,000). One possible solution to this problem could be the fermentation of worts of very high gravity. This technology has immense potential, like increase in the plant efficiency, increased ethanol yield per unit of fermented broth, decrease in the total energy input, labour, capital cost and amount of effluent production. However, most of the yeast strains employed at the industrial level cannot be grown in and ferment very high gravity molasses because of high osmotic stress, which reduces their growth and increases the loss of cell viability[3]. Addition of assimilable nitrogen, soy flour and yeast cell wall preparations, or osmoprotectants, like glycine, betaine and proline, to the concentrated worts have been reported to increase the growth rate, sugar utilizing ability and viability of yeast, as well as fermentation rate and final ethanol concentration in the fermented broth[3-5]. Besides, the ability of yeast isolates from nature has also been examined to ferment concentrated worts[6,7].

In the present study, two yeast isolates, SBS 13 and SBS 14, isolated from orchard soil and molasses, respectively, were examined for their ability to ferment high gravity molasses. The isolates were identified as *Saccharomyces cerevisiae* by morphological and physiological tests and have already been characterized for desirable ethanol fermentation properties[8]. The effect of various medium supplements, like soybean meal (SM), groundnut meal (GM) or castor oil seed meal (CM) on viability of cells and ethanol producing ability was also examined. Further, the physiological response of yeast cells with respect to trehalose and glycerol content under increased osmotic stress was analyzed.

Molasses was obtained from local distillery and total reducing sugar content was determined titrimetrically[8]. *S. cerevisiae* isolates, SBS 13 and SBS 14 were maintained on YEPD (yeast extract 1%, peptone 2%, dextrose 2%, agar 2%) medium. Inoculum was developed in 12^{o} brix molasses (*c*. 6% sugars) added with urea (0.3% w/v) and Na_2HPO_4 (0.15% w/v); whereas for fermentation, molasses of varying concentration, 20-40^{o} brix (12-24% sugars) was used. Fermentation media were supplemented with SM, GM or CM @ 2.5% (w/v) when required. For inoculum preparation, 24hr-old culture was inoculated into 250 ml of inoculum medium and incubated at 30^{o}C under shaking conditions (200 rpm). After 18 hr log phase, biomass was harvested and inoculated in fermentation medium @ 0.75g/ 100

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ml. Fermentation was allowed to proceed at 30°C under stationary conditions. Samples were withdrawn at different time intervals for ethanol estimation and viability determination. Ethanol content in the fermented broth was determined by the method of Caputi *et al*[9]. Efficiency of ethanol production was determined as described by Ezeogu and Okolo[10]. Viability of yeast cells was determined by methylene blue method[3].

The trehalose content of yeast was determined after extracting the cells with 500 m*M* trichloroacetic acid and developing the colour with anthrone reagent[11]. Glucose was used as the reference standard. Total glycerol was assayed as described by Myers *et al*[12]. Invertase was assayed according to the method of Gas'con & Lampen[13]. One unit of invertase activity corresponds to the liberation of one micromole of D-glucose per min.

In dilute molasses (20-25° brix) having no supplements, ethanol production efficiency of both the isolates of *S. cerevisiae* was found very high (87-92%). However, it decreased with further increase in the concentration. The efficiency was lowest in 40° brix molasses; isolate SBS 14 performed better (51.7%) as compared to SBS 13 (45.5%). Further, the ethanol production efficiency of isolates was considerably improved when molasses was supplemented with SM, GM or CM. Isolate SBS14 fermented 35-40° brix molasses with efficiency of *c.* 86-89% in case of SM supplement compared to 46-68% in control (molasses without supplement). Similar increase in efficiency of ethanol production was also observed in GM and CM supplements (83-87 and 82-86%, respectively).

Similarly, in diluted molasses (20-25° brix) with no supplement, viability of both the isolates was very high (90-93%) but as the concentration of molasses increased, the viability decreased and it was lowest in 40° brix (67-69%; Fig. 1). Further, the decrease was more prominent in first 24 hrs. After which, there was either no decrease at all or only slight decrease. However, when the media was added with any of the supplements, the viability of isolates was considerably improved. In molasses of 20-30° brix supplemented with SM, the viability was increased to a value of 97-98% after 24-48 hrs as compared to 83-93% in control (5-14% increase). While, in molasses of 40° brix supplemented with SM, the corresponding value was 92-93% after 24-48 hrs compared to 67-69% in control, i.e. 24-25% increase in viability. Similar

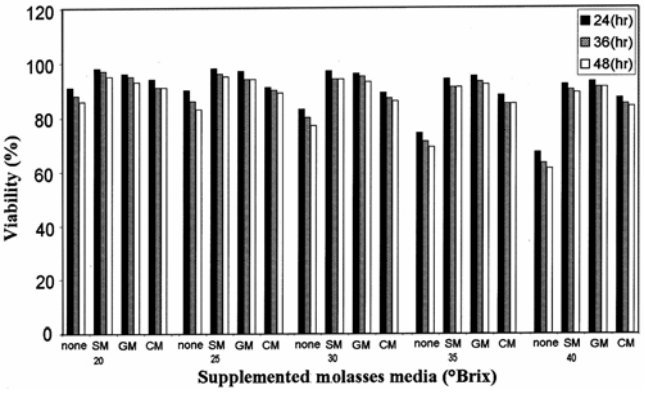

Fig. 1—Viability of yeast isolates in differentially supplemented molasses media SM-Soyabean meal, GM-Groundnut meal, CM-Castor oil seed meal

trend in improvement of viability was also seen when molasses was supplemented with GM or CM. However, CM was less effective in maintaining viability than SM or GM (Fig. 1).

Other workers have also obtained similar results[4,10]. Bafrncova *et al*[5] reported that worts of very high gravity could be fermented successfully by supplementation of media with yeast extract, glycine or soy flour and final ethanol content could be increased by 17%. They contradicted the observation that presence of adequate amount of assimilable nitrogen increases the fermentation efficiency of yeast[4]. These additives serve not only as nutrient source for yeast but also provide osmoprotection against the high osmotic pressure of concentrated molasses[10]. In the present study, additives like SM, GM or CM enabled the yeast to ferment molasses of high gravity and also increased the total sugar utilization by yeast with enhanced level of ethanol production. These supplements contained soluble components, which might have served as nutrient source for yeast, and also particulate insoluble material, which might have played role in relieving CO_2 effects and in reducing the toxic compounds from molasses. Thus, increasing the ethanol fermenting ability and viability of yeast in concentrated worts[5,14].

Trehalose content in yeast is a very useful indicator of stress. Exposure to high osmotic stress, high ethanol concentration, and heat and cold shocks results in increased concentration of trehalose in yeast cells. Generally, trehalose concentration is proportional to original wort gravity[15]. Also, in the present study, trehalose content in yeast increased with increase in concentration of sugar in molasses (without supplements). In dilute molasses (20-25°

Table 1—Trehalose and glycerol content in yeast cultured in differentially supplemented molasses media

Yeast cultures	Supplements[a]	Molasses (°Brix)									
		20		25		30		35		40	
		Tre	Gly	Tre	Gly	Tre	Gly	Tre	Gly	Tre	Gly
SBS 13	None	2.6	3.4	3.1	3.3	4.5	3.4	6.7	3.4	9.6	3.5
	SM	2.4	3.5	2.5	3.2	2.8	3.3	3.3	3.5	3.8	3.4
	GM	2.5	3.3	2.7	3.2	3.1	3.5	3.5	3.2	4.6	3.4
	CM	2.7	3.4	3.6	3.3	4.1	3.2	4.7	3.4	5.3	3.5
SBS 14	None	3.0	4.1	3.7	3.8	5.6	4.1	8.4	3.8	11.8	4.0
	SM	2.8	3.9	3.1	4.0	3.2	3.9	3.7	3.9	4.1	3.8
	GM	3.0	4.0	3.5	3.8	3.9	3.9	4.3	3.9	4.9	4.1
	CM	2.8	4.2	3.4	4.1	4.5	4.0	5.1	3.9	5.8	4.0

Trehalose (Tre) mg/g cells, Glycerol (Gly) % (w/v); SM-Soybean meal, GM- Ground nut meal, CM- Castor oil seed meal

Brix), trehalose content in isolates SBS 13 and SBS 14 was estimated to be 2.6-3.1 mg/g cells and 3.0-3.7 mg/g cells, respectively, which increased further with the increase in concentration of molasses (Table 1). In 40° brix molasses, SBS 13 and SBS 14 contained 9.6 and 11.8 mg/g cells of trehalose, respectively. However, when media was supplemented with SM, GM or CM, a substantial decrease in trehalose content was observed as compared to control. Trehalose content in supplemented medium (40° brix) varied from 3.8 to 5.8 mg/g cells as shown in Table 1. This clearly indicates the stress-relieving effects of these supplements.

Under conditions of high osmotic stress in concentrated media, yeast cells start the synthesis and accumulation of compatible solutes like glycerol[16]. In the present study, total glycerol content in media of different gravity varied from 3.2 to 3.5% and 3.8 to 4.1% (w/v) in SBS 13 and SBS 14, respectively (Table 1). No effect of sugar concentration or medium supplementation was observed on glycerol production; similar amounts were produced by both the isolates in molasses with different gravity whether supplemented or not. Others have also reported that glycerol production is more a function of yeast strains rather than medium[1,17,18]. In contrast, however, it has been reported that increased glycerol is produced and accumulated by yeasts in response to hyperosmotic stress of the medium[12,19]. Recently, Attfield and Kletsas[17] reported that yeast strains, inhibited by high sucrose concentration, are unable to produce significant amounts of glycerol under hyperosmotic conditions; while, those are not inhibited produce considerable amount of glycerol.

For ethanol production, yeast strains must possess invertase activity, which is required for hydrolysis sucrose and invert sugars of molasses. Myers *et al*[12] reported that high sucrose concentration in the fermentation medium results in reduced invertase activity. However, in the present study, it was observed that invertase activity of both the isolates was not affected by high sugar concentration in the medium. Further, invertase activity remained unaltered upon supplementation of medium with any of the supplements, like SM, GM or CS. Similarly, Oda & Ouchi[20] reported that invertase activity is not affected by high sugar concentration in the medium. Tauro *et al*[1] confirmed that invertase is not the limiting factor in ethanol production from diluted molasses.

It may be concluded from the present study that additives, like SM, GM or CM enable the yeast to ferment molasses of high gravity with very high ethanol production efficiency. At the same time, these supplements enhance the viability of yeast in concentrated molasses by relieving the osmotic stress. Since high concentration of ethanol is obtained in the fermented broth when concentrated worts are fermented, it will be helpful in reducing the cost of down stream processing, which in fact constitutes the most expensive component in fermentative production of ethanol, and may play a role in improving the overall economy of the process.

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