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RESEARCH ARTICLE

Enhanced production of ethanol by high gravity glucose fermentation at temperatures above 40 °C by *Saccharomyces cerevisiae* S1 using a soya flour supplemented medium

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Abstract: A locally isolated strain of *Saccharomyces cerevisiae* S1 in peptone, yeast and mineral nutrient (PYN) medium with 100 gL^{-1} of glucose showed optimum growth at 36 °C and efficient ethanol production at 40 °C. To investigate the effect of soya flour supplementation on ethanol fermentation, PYN medium containing different concentrations of glucose (50 to 400 gL^{-1}) with and without soya flour (26.8 gL^{-1}) were used at 40 °C for 48 h. The above experiment was repeated at different temperatures (40, 43 and 45 °C) for 48 h to find the effect of soya flour supplementation on ethanol production at higher temperatures and sugar levels. The highest ethanol production efficiency (96.5 %) and the highest ethanol yield (149.3 gL^{-1}) with efficient glucose utilization (100 %) was observed in soya flour supplemented medium with a sugar concentration of 300 gL^{-1} at 40 °C. Further, it was observed that at higher temperatures (43 and 45 °C) and higher sugar concentrations, soya flour supplemented media performed well. With 300 gL^{-1} glucose at 43 and 45 °C the amounts of ethanol produced in soya flour supplemented media was 80 and 30 gL^{-1}, respectively while in unsupplemented media it was only 28 and 18 gL^{-1}, respectively. In glucose (400 gL^{-1}) – PYN medium supplemented with soya flour, 78 (48 h) and 25 gL^{-1} (48 h) ethanol was produced at 43 and 45°C, respectively. The study revealed that supplementation of media with soya flour has not only improved the glucose fermenting capacity but has also increased the efficiency of ethanol production and the ethanol production rate at higher temperatures and high sugar levels.

Keywords: Ethanol, high gravity glucose fermentation, *Saccharomyces cerevisiae,* soya flour.

INTRODUCTION

The fermentation of sugar in brewing industry is generally carried out by employing yeast *(Saccharomyces cerevisiae)* which produce 4 - 9 % (w/v) ethanol. Any increase in the ethanol concentration during fermentation would be desirable both for quality and economic considerations. The advantage of high gravity brewing includes increased plant efficiency, reduced energy, labour and capital cost (Casey *et al*., 1983). The economy of large-scale fermentation depends on the efficiency of glucose utilization and the high yield of ethanol. Low energy consumption during ethanol recovery from fermented broth is required to reduce the cost of distillation (Rose, 1976). The trend in alcohol industry is to economise wherever possible by fermenting high concentrations of sugar (Morimura *et al*., 1997). If a technology is developed to increase the concentration of ethanol while increasing the temperature to or above 40 °C, it would be a breakthrough in industrial ethanol production (Ezeogu & Emeruwa, 1993). In this context the fermentation of different concentrations of glucose (50 – 400 gL^{-1}) was carried out at 40, 43 and 45 °C in peptone, yeast extract and nutrient (PYN) medium. High substrate concentration demands additional nutrients (Casey *et al*., 1983; Viegas *et al*.,1985a) and therefore soya flour supplementation was tried. A thermotolerant *Saccharomyces cerevisiae* S1 developed using thermal adaptation and UV and EMS mutagenesis (Balakumar *et al*., 2001) was used for these studies. As it has been observed that soya flour supplementation has improved the viability of *S. cerevisiae* S1 in the presence of added ethanol and sorbitol and the fermenting ability at different sugar levels (Balakumar & Arasaratnam, 2012), further studies were carried out at high gravity fermentation at temperatures above 40 °C in the presence of soya flour.

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METHODS AND MATERIALS

Soybean purchased from the local market was powdered and dried at 80 °C. All the other materials were purchased from standard suppliers: culture media - Oxoid Limited, USA, and other chemicals - Sigma - Aldrich, USA.

***Saccharomyces cerevisiae* S1**

Saccharomyces cerevisiae S1, a locally isolated thermotolerant strain improved by treatments (Balakumar *et al*., 2001) was maintained in glucose (50 gL^{-1}), peptone, yeast extract and nutrient (PYN) agar medium slants.

Analytical methods

The determination of glucose was carried out following the dinitrosalicylic acid method described by Miller (1959); ethanol using potassium dichromate (Varley *et al*., 1980) and the viable cell count by staining (Sami *et al*., 1994).

Peptone, yeast extract and nutrient (PYN) medium

The medium contained (gL^{-1}) peptone, 3.5; yeast extract, 3.0; $MgSO_4.7H_2O$, 1.0; KH_2PO_4, 2.0; and $(NH_4)_2SO_4$, 1.0 at pH 5.0 (Balakumar & Arasaratnam, 2009). Under different experimental conditions, different amounts of glucose were added to the medium and represented as the glucose (amount in gL^{-1}) – PYN medium (Balakumar & Arasaratnam, 2009). When twice the amount of the nutrients of PYN medium was used, the medium is presented as 'glucose (amount in gL^{-1}) – 2PYN medium'.

In all the experiments conical flasks were used, and the medium : flask ratio was 1:10. The inoculum size was 10 %, (v/v). Incubation was carried out with shaking at 150 rpm. All the experiments were carried out in triplicates.

Inoculum of *Saccharomyces cerevisiae* S1

Glucose (50 gL^{-1}) – PYN medium (100 mL) was inoculated with 2 loops full of *S. cerevisiae* S1 and incubated at 36 °C for 18 h with shaking at 150 rpm.

Effect of temperature on the growth of *S. cerevisiae* S1 and fermentation of glucose

To determine the effect of temperature on the growth of *S. cerevisiae* S1 and fermentation of glucose, the inoculated glucose (100 gL^{-1}) – PYN medium was incubated at different temperatures with shaking at 150 rpm.

Effect of soya flour supplementation on ethanol production using PYN medium with different concentrations of glucose at 40 °C

To determine the effect of soya flour supplementation on ethanol fermentation using *S. cerevisiae* S1, an experiment was carried out using the PYN medium with different concentrations of glucose (100, 150, 200, 300 and 400 gL^{-1}) and PYN medium with different concentrations of glucose supplemented only with soya flour (26.8 gL^{-1}) (and not with mineral solution) at the optimum temperature (40 °C) with shaking (150 rpm) for 48 h.

Effect of soya flour on ethanol production at different temperatures (40, 43 and 45 °C) using PYN medium with different concentrations of glucose

PYN medium having different concentrations of glucose (100, 150, 200, 300 and 400 gL^{-1}) with and without soya

Table 1: Effect of temperature on ethanol production by *S. cerevisiae* S1 in glucose (100 gL^{-1}) – PYN medium at pH 5.0 and 36 h (mean values of triplicates obtained from three experiments)

Temperature (°C)	Total cell number (x 10^7)	Viable cell count (%)	Ethanol (gL^{-1})	*Ethanol yield (g / g glucose used)	**Ethanol production rate (g $L^{-1}h^{-1}$)	Residual sugar (gL^{-1})	#Sugar utilization (%)
36	8.0	98.0	40.0	0.40	1.67	0.0	85.4
40	7.7	98.0	46.5	0.47	1.93	0.0	85.0
45	4.3	85.0	29.0	0.43	1.2	33.0	66.0
50	2.0	60.0	10.0	0.33	0.42	70.0	30.0

Sugar utilization was calculated as the percentage of initial sugar content

* Ethanol yield = $\frac{\text{g ethanol produced}}{\text{g glucose used}}$

** Ethanol production rate (g ethanol / L / h) was calculated as the amount of ethanol (g) produced per hour per litre

Initial cell count in all the media was 2.0 x 10^7

flour supplementation (26.8 gL^{-1}) were inoculated with *S. cerevisiae* S1 and incubated at 40, 43 and 45 ^oC for 48 h.

RESULTS AND DISCUSSION

Effect of temperature on growth and ethanol production

When the inoculated glucose (100 gL^{-1}) - PYN medium was incubated at 36, 40, 45 and 50 ^oC, the strain showed substantial growth at 36 and 40 ^oC (24 h). The total cell counts at 36 and 40 ^oC were 8 x 10^7 and 7.7 x 10^7, respectively (Table 1; initial cell count was 2 x 10^7 cells mL^{-1}). No cell multiplication was observed at 50 ^oC; however the cell count has increased to 4.3 x 10^7 cells mL^{-1} at 45 ^oC. The percentage of viable cells was 98 % at 36 and 40 ^oC and it has dropped to 85 and 60 % at 45 and 50 ^oC. Since the percentage of viable cell count was the same at 36 and 40 ^oC, the inoculum can be prepared either at 36 or 40 ^oC.

Ethanol production rate and the yield gave the highest values, 1.93 g ethanol $L^{-1}h^{-1}$ and 46.7 gL^{-1}, respectively at 40 ^oC and these values decreased with the increase in temperature (Table 1). These results suggest that 40 ^oC is the optimum temperature for higher ethanol production rate and optimum ethanol yield for *S. cerevisiae* S1. Jeyaseelan and Seevaratnam (1986) have reported an optimal growth temperature of 30 ^oC for a locally isolated *Saccharomyces* Y7 (from palmyrah palm sap). This strain significantly produced ethanol (95 – 85 % yield) at temperatures 28 – 35^oC. Therefore *S. cerevisiae* S1 is superior in terms of ethanol production at high temperatures. The ethanol yield obtained for *S. cerevisiae* S1 is comparable to *Kleuveromyces marxianus* (Hughes *et al*., 1984), which recorded 0.41, 0.47, 0.43 and 0.37 (g ethanol / g glucose) ethanol yields at 30, 40, 43 and 45 ^oC, respectively. Therefore *S. cerevisiae* S1 is a better strain (Table 1) than *K. marxianus* giving higher yields of ethanol at the respective temperatures reported.

Effect of soya flour supplementation on ethanol fermentation using PYN media with different concentrations of glucose

In this experiment the inoculum was developed at 36 ^oC and the ethanol fermentation was carried out at 40 ^oC for 36 h. Different concentrations of glucose in PYN medium with or without soya flour were used in this study (Table 2). The amount of soya flour (26.8 gL^{-1}) added was equivalent to the total organic nitrogen present in the PYN medium. The total nitrogen content of soya flour was 38 % (w/w) (Wikramanayake, 1996) and the total nitrogen in peptone and yeast extract was 14 and 9.8 % (w/w), respectively (Budavari *et al*., 1996). When glucose only was supplemented with soya flour (medium D_2 and E_2), the ethanol production efficiency and sugar utilization were less than those of glucose - PYN media (D_1 and E_1). However the ethanol production efficiency and sugar utilization were not

Table 2: Effect of soya flour supplimentation on ethanol production by *S. cerevisiae* S1 in different concentrations of glucose-PYN medium at 40 ^oC (mean values of triplicates obtained from three experiments)

Medium	Glucose (gL^{-1})	Nutrient composition		Ethanol (gL^{-1})	*Ethanol production efficiency (%)	Residual sugar (gL^{-1})	#Sugar utilization (%)
		PYN	Soy flour (gL^{-1})				
A	50	PYN	-	24.5	98.0	0.0	100.0
B	100	PYN	-	46.5	93.0	0.0	100.0
C	150	PYN	-	66.6	88.8	15.8	89.5
D_1	200	PYN	-	73.8	73.8	46.4	76.8
E_1	300	PYN	-	80.1	53.4	128.8	57.1
F_1	400	PYN	-	67.4	33.7	253.2	36.7
D_2	200	-	26.8	68.4	68.4	58.2	70.9
E_2	300	-	26.8	74.3	49.5	143.4	52.2
D_3	200	PYN	26.8	96.5	96.5	0.0	100.0
E_3	300	PYN	26.8	149.3	99.5	0.0	100.0

Sugar utilization was calculated as the percentage of initial sugar added

* Ethanol production efficiency = $\frac{\text{The amount of ethanol produced}}{\text{Theoretical amount of ethanol that could be produced}} \times 100$

Table 3: The amount of ethanol produced, ethanol production rate (EPR), residual glucose (RG), efficiency of ethanol production (EPE) and glucose utilization (GU) at different temperatures by *S. cerevisiae* S1 in PYN medium and supplemented with soya flour containing different concentrations of glucose. Time taken for maximum ethanol production is given in parenthesis (mean values of triplicates obtained from three experiments)

Glucose (gL^{-1})		40 ºC		43 ºC		45 ºC	
		PYN	PYN-Soy	PYN	PYN-Soy	PYN	PYN-Soy
100	Ethanol (gL^{-1})	46.5 (36h)	47.0 (36h)	38.0 (48h)	44.0 (48h)	26.0 (48h)	31.0 (48h)
	EPR ($gL^{-1}h^{-1}$)	1.29	1.30	0.78	0.92	0.54	0.64
	EPE (%)	88.8	94.0	74.4	86.0	59.4	85.0
	RG (gL^{-1})	0.0 (36h)	0.0 (36h)	17.5 (48h)	6.3 (48h)	30.6 (48h)	63.0 (48h)
	GU (%)	100.0	100.0	80.5	93.3	64.4	92.5
150	Ethanol (gL^{-1})	66.6 (36h)	70.0 (36h)	55.0 (48h)	62.0 (48h)	35.0 (48h)	42.0 (48h)
	EPR ($gL^{-1}h^{-1}$)	1.85	1.94	1.14	1.3	0.73	0.87
	EPE (%)	88.8	93.3	71.4	75.0	47.5	54.8
	RG (gL^{-1})	0.0 (36h)	0.0 (36h)	30.6 (48h)	18.8 (48h)	56.9 (48h)	18.8 (48h)
	GU (%)	100.0	100.0	77.5	86.3	61.3	86.3
200	Ethanol (gL^{-1})	75.0 (48h)	95.0 (48h)	38.0 (48h)	73.0 (48h)	19.0 (48h)	32.0 (48h)
	EPR ($gL^{-1}h^{-1}$)	1.56	1.97	0.8	1.52	0.4	0.62
	EPE (%)	72.5	93.0	37.2	70.0	30.0	70.6
	RG (gL^{-1})	42.0 (48h)	0.0 (48h)	118.1 (48h)	43.8 (48h)	126.9 (48h)	43.3 (48h)
	GU (%)	79.1	100	59.7	77.3	21.0	35.0
300	Ethanol (gL^{-1})	82.0 (48h)	149.0 (48h)	28.0 (48h)	80.0 (48h)	18.0 (48h)	30.0 (48h)
	EPR ($gL^{-1}h^{-1}$)	1.7	3.1	0.58	1.67	0.37	0.62
	EPE (%)	53.4	97.0	17.5	51.3	17.5	51.3
	RG (gL^{-1})	120.0 (48h)	0.0 (48h)	236.3 (48h)	125.0 (48h)	231.9 (48h)	121.9 (48h)
	GU (%)	60.0	100	20.0	56.9	21.3	57.5
400	Ethanol (gL^{-1})	68.0 (36h)	150.0 (48h)	23.0 (48h)	78.0 (48h)	6.0 (48h)	25.0 (48h)
	EPR ($gL^{-1}h^{-1}$)	1.9	3.1	0.47	1.62	0.12	0.52
	EPE (%)	33.7	73.4	10.2	37.5	11.3	37.5
	RG (gL^{-1})	250.0 (36h)	81.3 (48h)	347.8 (48h)	228.9 (48h)	332.5 (48h)	228.1 (48h)
	GU (%)	30.6	79.5	11.3	38.0	15.0	41.3

significantly different between media, which had either PYN or soya flour as the nitrogen source. However, soya flour supplemented media with 200 and 300 g glucose L^{-1} (medium D_3 and E_3) gave higher ethanol production efficiency and increased sugar utilization. In media A, B, D_3 and E_3 *S. cerevisiae* S1 completely utilized the glucose in 48 h (Table 2). Incomplete glucose utilization was observed in glucose (200 gL^{-1}) – PYN medium (Table 2), however complete fermentation was observed when the medium was supplemented with soya flour. The efficiency of fermentation achieved in this media was extremely high when compared with the results reported previously (Morimura *et al*., 1997; Barfroonva *et al*., 1999). Supplementation of horse gram flour (Reddy & Reddy, 2005) and finger millet (Reddy & Reddy, 2006) reduced the fermentation time and enhanced the ethanol concentration. Soya flour supplementation has been used in many studies (Damiano & Wang, 1985; Dombeck & Ingram, 1986; Ernardes *et al*., 1990) because it is relatively inexpensive and has abundant amounts of protein (38 %) with lipid (20 %), which can be assimilated into intracellular materials (Damiano & Wang, 1985). It has been reported that soya flour supplementation has improved the growth

and fermentation of yeast (Ernardes *et al*., 1990). This could be due to the abundant protein-lipid complex present in soya (Damiano & Wang, 1985). Soya flour supplementation could yield extremely high amounts of ethanol (21.5 %, v/v) (Dombeck & Ingram, 1986) and the addition of 4 % (w/v) soya flour to 300 gL^{-1} glucose improved the ethanol production to 12.8 % at 64 h by *S. bayanus* (Viegas *et al*., 1985b, D'Amore *et al*., 1989). At all glucose concentrations considered in this study, the ethanol production and glucose utilization were increased with the addition of soya flour. Soya flour was reported to be a good yeast nutrient supplement for the production of high concentrations of ethanol (D'Amore *et al*., 1989 ; Barfroonva *et al*., 1999).

Effect of soya flour on the fermentation of different concentrations of glucose at 40, 43 and 45 ºC

S. cerevisiae S1 showed an ethanol production efficiency of 90 % at 40 ºC in glucose (100 gL^{-1}) – PYN medium (Table 3) but in 400 gL^{-1} glucose at the same temperature it was reduced. The sugar utilization beyond 150 gL^{-1} was incomplete. Even though a higher ethanol production was observed in 300 gL^{-1} (82 gL^{-1} ethanol), the sugar utilization and ethanol production efficiency were 60 and 53.4 %, respectively. Inadequate levels of nitrogen could be the cause for the reduction of fermentation at high glucose concentrations as nitrogen is a major element involved in cell metabolism. Free α-amino nitrogen is the limiting nutrient in high gravity fermentation (Ingledew, 1993). The addition of substances containing available nitrogen to yeast growing media could alleviate the problem of poor fermentative capacity at high glucose levels. Therefore soya flour was used as the nutrient supplement and the ethanol production efficiency was increased significantly (Table 3). The addition of soya flour to glucose (300 gL^{-1}) – PYN medium has significantly increased the ethanol production efficiency from 53.4 to 97 %. Also soya flour addition has increased the sugar utilization to 100 % as against 79 and 60 % in 200 and 300 gL^{-1} glucose, respectively. An ethanol production efficiency of 73.4 and 33.7 % and a sugar utilization of 79.5 and 30.6 % were observed with and without soya flour in the fermentation of 400 gL^{-1} glucose, respectively (Table 3). The experiments revealed that at an initial sugar level of 300 gL^{-1}, *S. cerevisiae* S1 is able to produce 149 gL^{-1} ethanol within 48 h with 97 % production efficiency and with complete sugar utilization at 40 ºC with soya flour supplementation.

At 43 ºC the ethanol production efficiency and sugar utilization were 74.4 and 80.5 %, respectively. When glucose (100 gL^{-1}) – PYN was supplemented with soya flour, the ethanol production efficiency and sugar utilization were increased to 86.0 and 93.3 % (Table 3). However beyond 100 gL^{-1} glucose level the ethanol production efficiency was severely affected and dropped to 38 % in glucose (400 gL^{-1}) – PYN medium even after supplemented with soya flour.

The ethanol production at 45 ºC was studied at different glucose contents (100 – 400 gL^{-1}) – PYN medium with and without soya flour supplementation. In glucose (150 gL^{-1}) – PYN medium with and without soya flour, 42 and 35 gL^{-1} ethanol was produced with production efficiencies of 54.8 and 47.5 %, respectively (Table 3). Further increase in the glucose concentration at 45 ºC decreased the performance of *S. cerevisiae* S1 whereas at 40 ºC the ethanol production efficiency was maximum (97 %) up to 300 gL^{-1} glucose concentration and dropped to 73.4 at 400 gL^{-1} glucose. Even though this strain is capable of performing the fermentation at 43 or 45 ºC, it is not cost effective to ferment at 45 ºC, (Balakumar & Arasaratnam, 2012) due to underutilization of added sugar.

In industrial-scale fermentation the temperature of the culture increases, particularly in hot regions like Jaffna (Balakumar & Arasaratnam, 2009). During large scale fermentation at 5000 L capacity, a rise in temperature (heat shock) has been observed with the concomitant decrease in the viability of the baker's yeast cells (Balakumar *et al*., 2001). Therefore the use of high temperature tolerant yeast in fermentation would be a good option to overcome the heat shock.

The ethanol production was higher with soya flour supplementation at all the temperatures studied with concomitant utilization of glucose (Table 3). A maximum ethanol production efficiency of 97 % was achieved at 40 ºC in glucose (300 gL^{-1}) – PYN – soya flour medium at 48 h. *S. cerevisiae* fermented 200 gL^{-1} sugar in 90 h (Converti *et al*., 1985). The time taken for the complete fermentation of 200 gL^{-1} dissolved solids was 90 h (Thomas & Ingledew, 1992) while *S. cerevisiae* S1 only took 48 h. Therefore considering the temperature and the duration of fermentation, *S. cerevisiae* S1 seems to be better than the strains reported earlier.

The highest ethanol production rate was 3.1 $gL^{-1}h^{-1}$ in glucose (300 and 400 gL^{-1}) – PYN media supplemented with soya flour (26.8 gL^{-1}) at 40 ºC. Even though the rates were the same at 300 and 400 gL^{-1} glucose levels, the fermentation was incomplete in the latter. The ethanol production rate in PYN- soya flour was increased significantly with the increase in glucose concentration at

40 °C. At 43 and 45 °C, soya flour supplement improved the ethanol production efficiency. The addition of soya flour to the medium greatly influenced the ethanol production rate when the levels of glucose in the medium were 200, 300 and 400 gL^{-1} at 43 and 45 °C. This study indicated the usefulness of soya flour nutrients at high gravity glucose fermentation at higher temperatures. The organism was able to grow and ferment high concentrations of sugar without the addition of mineral solution.

CONCLUSION

S. cerevisiae S1 can perform well at glucose concentrations up to 300 gL^{-1} in PYN medium with soya flour supplementation. Further studies are needed to find the correct amount of soya flour that should be added to the medium to improve the fermentation at higher glucose concentrations and at higher temperatures.

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