Intl. J. Food. Ferment. Technol. 6(2): 289-294, December, 2016

DOI: 10.5958/2277-9396.2016.00052.0

RESEARCH PAPER

Enhanced Ethanol Production Through Salt Pre-conditioning of *S.cerevisiae* MTCC 11815

P.S. Khanna and G.S. Kocher*

Department of Microbiology, Punjab Agricultural University, Ludhiana, India

*Corresponding author: gskocher@pau.edu

Paper No.: 138 Received: 15 June 2016 Accepted: 17 Dec. 2016

ABSTRACT

Salt preconditioning provides an alternate to very high gravity fermentation for increasing osmotolerance and thermotolerance of yeast. In the current study, a fermenting yeast strain (*Saccharomyces cerevisiae* MTCC 11815) was sequentially preconditioned at 4% (w/v) NaCl in a synthetic medium and optimized using RSM on the basis of osmotolerance and thermotolerance for maximum ethanol production. A maximum ethanol of 12.53% (v/v) was observed at a temperature of 35ºC with high residual Brix of 17 °B from initial 39 °B. Further, ethanol fermentation carried out between 20 to 30 °B revealed 24 °B as optimum with an ethanol production of 12.4 % (v/v) and residual brix of 3.5 °B. Validation studies on sugarcane juice as well as on molasses (10L) recorded the optimized fermentation parameters (Brix 24°B, Inoculum size 7.5% (v/v) and DAHP 0.3% (w/v)) with ethanol production of 12.4% (v/v) on sugar cane juice and 10.6% (v/v) on molasses having fermentation efficiencies of 86.10% and 81.20% by pre-conditioned *S.cerevisiae* MTCC 11815 cells.

Keywords: Glycerol, osmotolerance, pre-conditioning, *Saccharomyces cerevisiae*, sodium chloride, thermotolerance

Yeast is subjected to various physico-chemical stresses in the form of high initial sugar concentration ISI and low temperature, and consequently, increased ethanol concentration during industrial ethanol fermentation. Such stress factors can trigger a series of biological response which help to maintain the yeast cell viability and cell cycle progress. Hence, molasses containing 40 to 60 % of fermentable sugars are diluted with water to bring down initial sugar concentration [16-20 °B (15-16% reducing sugars)] and about 7 to 8% (v/v) ethanol with 80-85% of fermentation efficiency is produced from diluted molasses (Patil *et al.,* 1998). Due to this, huge effluent (about 12 litre effluent/litre absolute alcohol) with very high biological oxygen demand (BOD) is produced with a severe problem of disposal (Singh and Nigam 1995). As a result, very high gravity (VHG) fermentation emerged as a technology that offered great savings on process water besides providing high yield of ethanol, reduced labour and capital costs and low bacterial contamination (Thomas *et al.,* 1995). However, VHG fermentations suffer from sluggish and incomplete fermentations resulting in low ethanol production because in these fermentations the yeast cells are subjected to high osmolarity stress at initial stages when the sugar level of the medium increases above their normal tolerance limits (>30% w/v) (Panchal and Stewart 1980). Therefore, in order to reduce the negative effects caused by both the increased gravities as well as ethanol levels, research efforts are being carried out to understand the yeast mechanisms for adaptation under extreme conditions, focusing mainly on the tolerance capacities of yeast strains as the effect of osmo-protectants has been studied

during high gravity fermentation resulting in increase of ethanol yield (Thomas *et al.,* 1994). More recently, yeast cells exposed to NaCl stress prior to fermentation have been found to exhibit increased osmotolerance and thermotolerance as well as ethanol production primarily due to altered yeast cell physiology (Logothetis *et al.,* 2006). Such pre-adapted yeast cells can be used as inocula to produce high alcohol content by using VHG fermentation (Logothetis *et al.,* 2013). Earlier, in our laboratory 4% (w/v) of NaCl pre-conditioning of yeast strains *S.cereisiae* 11815 for their maximum cell viability was optimized (Khanna and Kocher 2015). In the present study, such pre-conditioned cells have been used to optimize osmotolerance, and ethanol tolerance and the results are presented in this manuscript.

MATERIALS AND METHODS

The cultures and raw material

The fermenting yeast culture, *Saccharomyces cerevisiae* MTCC 11815 was maintained on glucose yeast extract agar slants (GYE). Cane molasses were procured from Budhewal sugar mill, Dist. Ludhiana.

Pre-conditioning of *S. cerevisiae* cells

S. cerevisiae 11815 was sequentially pre-conditioned on synthetic medium broth containing sodium chloride concentrations of upto 10% (w/v) with an increment of 1% at each treatment level. Erlenmeyer flasks (250ml) containing 150 ml synthetic medium (having 10% glucose) with 0-10% sodium chloride were prepared, inoculated @ 5.0×10^7cells/ml and incubated at 28ºC under shake flask conditions. The cultures were passaged to next higher salt concentration after 48h of incubation, when cells were still in their log phase. Total and viable counts were taken after every 12 h and were compared with initial cell counts using standard methods.

Effect of temperature and Brix on ethanol production by pre-conditioned yeast cells

The pre-conditioned cells (at 4% w/v NaCl) of yeast strain *S.cerevisiae* 11815 were optimized using Response Surface Methodology (RSM) for ethanol production using different Brix and temperature values taking respective unconditioned cells as a control. Sugarcane juice was used as a natural fermentation medium in place of synthetic medium. For RSM, two variables, Brix (20-39°B) and temperature (28-35°C) were selected that provided 13 treatment combinations. For each of the 13 combinations designed by RSM (Table 1), fermentation was carried out in 500ml capacity glucose bottles in which 350 ml of medium was taken and the response of the treatment was studied as ethanol (Caputi *et al.,* 1968), residual Brix and pH by standard methods.

Table 1: RSM design for optimization of ethanolic fermentation of *S.cerevisiae*

Run	Factor 1	Factor 2
	A : Temperature (°C)	B : Brix (°B)
1	31.5	29.5
2	31.5	29.5
3	31.5	20
4	31.5	29.5
5	31.5	29.5
6	35	29.5
7	31.5	29.5
8	28	29.5
9	31.5	39
10	28	20
11	28	39
12	35	39
13	35	20

Optimization of ethanolic fermentation

Optimization of Brix

The Brix of sugarcane juice was adjusted by using sucrose to 20, 22, 24, 26, 28 and 30 ºB, respectively and fermented after supplementing all the treatments with 0.3% DAHP. The inoculum of pre-conditioned yeast strain of *S.cerevisiae* was prepared in Erlenmeyer flasks (250ml) containing NaCl (4% w/v) in the medium with respect to control (no NaCl). The raised

inoculum was used to inoculate production medium (350 ml in 500 ml glucose bottle) and fermentation was carried out at 35ºC temperature. Samples were drawn after every 24 h and estimated for residual sugars, ethanol and glycerol by standard methods.

Validation of ethanolic fermentation

The validation of optimized ethanolic fermentation parameters was carried out at 10L scale using sugarcane juice and molasses. DAHP was supplemented @ 300ppm (Kaur and Kocher, 2014) for sugarcane juice and molasses in the production medium containing 24ºB with respect to the control (20°B). The inoculum of pre-conditioned yeast strain of *S.cerevisiae* was prepared in glucose bottles (500ml) containing NaCl in the medium with no NaCl in control. The raised inoculum was used to inoculate production medium (3.5L in 5L Erlenmeyer flasks) and fermentation was carried out at standardized temperature of 35ºC. Samples were drawn every 12 h and estimated as described earlier.

Analytical methods

The differential viable and non-viable cell counts were taken by methylene blue reagent using method of Lee *et al.* (1981). pH was measured by a digital pH meter (Hanna make). Residual sugars, ethanol and glycerol were estimated by the methods of Miller (1959), Caputi *et al.* (1968) and Lambert and Naish (1950), respectively. Specific gravity of fermentation broth was measured during fermentation with hydrometer every 12 h while initial and final counts were taken by the method of Lee *et al.* (1981). The statistical analysis of thermotolerance and sugar tolerance experiments was carried out using Response Surface Methodology (RSM), the results of fermentation at different Brix values were analyzed by STAT (Cheema and Sidhu, 2007) and fermentation of molasses and sugarcane juice was compared by one tailed t-test.

RESULTS AND DISCUSSION

The results presented in table 2 provide a combined effect of temperature and Brix on ethanol, Brix, and pH of *S.cerevisiae* 11815. The p-value and R^2 value of their fermentation parameters indicated the fitness of model. The yeast fermented at high Brix and temperature values but there was significant residual Brix at higher stress values along with accumulation of glycerol. The accumulated glycerol was however low at temperature and sugar concentration of 28ºC and 20ºB, respectively (Pham *et al.*, 2006).

The RSM analyses provided 11 solutions for pre-conditioned cells of *S.cerevisiae* 11815 with ethanol production (12.53%, v/v) having temperature and Brix of 35ºC and 39ºB with desirability of 90.9%. However, residual Brix content was found to be 17ºB in the same combination which was much higher and the glycerol production was also found to be maximum in this case i.e. 3g/L. Keeping in view the high amount of residual Brix, it was varied between 20 to 30ºB with the fermentation temperature kept at 35ºC to determine the optimum Brix for fermentation that doesn't produce residual Brix. The results presented in Table 3 revealed an increase in residual Brix when initial Brix was increased. Among the different initial Brix values, a significantly higher ethanol production at a Brix of 24ºB with low residual Brix of 3.5 ºB and fermentation efficiency of 92.81% was observed. Further, glycerol accumulation in the medium increased with increase in Brix from 0.65 to 2.9 g/L when brix increased from 20 to 30 ºB. This may be a critical factor in enhancing the ethanol production under stress conditions of temperature and sugar.

In earlier studies Logothetis *et al.* (2013) also observed increased in glycerol concentration while fermenting glucose (55%, w/v) by salt pre-conditioned cells of *S.cerevisiae* which they linked to the elevated stress gene expression leading to high concentration of stress tolerance metabolites like glycerol and trehalose.

The optimized fermentation parameters (Brix 24ºB, Temperature 35ºC, DAHP 300ppm, inoculum size 7.5% (v/v) for ethanol production were validated at 10L scale on sugarcane juice and molasses. Fermentation of sugarcane juice was complete in 120h and 60h, respectively with ethanol production of 12.4% (v/v) and 10.4% (v/v), respectively which validated

Table 2: Optimization of thermotolerance and ethanol tolerance using RSM in preconditioned cells of *S.cerevisiae* MTCC 11815

Run	Test parameters		Response 1	Response 2	Response 3	Final
	A:temperature (ºC)	B:Brix (ºB)	Ethanol (%v/v)	Residual Brix (ºB)	pH	Glycerol (g/L)
1	36.4497	29.5	12.25	11.85	3.2	2.0
2	31.5	16.065	8	0.15	3.3	1.0
3	28	39	12.0	20.5	3.3	2.9
4	26.5503	29.5	9.55	8.55	3.2	1.5
5	28	20	7.78	2.5	3.2	0.8
6	31.5	29.5	9.1	9.16	3.3	1.5
7	31.5	29.5	9.16	8.95	3.3	1.5
8	31.5	42.935	12.15	22.5	3.2	2.9
9	31.5	29.5	9.55	7.75	3.2	1.6
10	31.5	29.5	9.65	7.7	3.3	1.6
11	31.5	29.5	9.25	8.99	3.4	1.6
12	35	20	11.7	1.2	3.4	2.2
13	35	39	12.53	17.0	3.4	3.0

Cells	Response	Model F-value	p-value*	Predicted R^2 value	Adjusted R^2 value**
S.cerevisiae MTCC 11815	Ethanol	53.23	< 0.0001	0.8567	0.9561
	°Brix	74.48	< 0.0001	0.8864	0.9684
	pH	7.38	0.0103	0.7700	0.7266

*p-value < 0.05 and **R^2 value of > 0.75 indicates good fitness of the model

Table 3: Fermentation of sugar cane juice by *S.cerevisiae* 11815 at different initial Brix values

Initial Brix	Final Brix(ºB)	11815				
		Residual sugars (%)	Ethanol (%v/v)	Fermentation efficiency (%)	Glycerol (g/L)	pH
20 (control)	0.0	1.0	10.4± 0.20	81.2	0.65	3.5
22	1.0	0.76	12.00±0.23	85.23	1.50	3.6
24	3.5	3.5	12.40±0.70	92.81	1.95	3.4
26	5.5	4.5	12.50±0.45	75.12	2.66	3.4
28	7.5	6.4	12.55±040	70.03	2.72	3.5
30	9.0	7.2	12.60±040	65.62	2.90	3.2
CD (5%)	Ethanol-0.555					

Fermentation conditions: No. of fermentation days: 3, Temperature: 35ºC, Scale: 500ml, DAHP concentration: 300ppm, Inoculum size: 7.5% (v/v).

Table 4: Scaled up comparative fermentation of sugarcane juice and molasses by pre-conditioned cells *S.cerevisiae* 11815

Hours	Molasses*						Sugarcane juice**					
	Specific gravity	Brix (ºB)	Residual sugars (%)	Ethanol (%v/v)	Glycerol (g/L)	pH	Hours	Brix (ºB)	Residual sugars (%)	Ethanol (%v/v)	Glycerol (g/L)	pH
0	1.190	47.50	23.5±0.50	0.0±.0.10	0.00	5.5	**0**	24.0±0.25	22.6±.050	0.0±0.00	0.0	3.5
12	1.150	35.00	19.4±0.25	1.5±.0.20	0.25	4.9	**24**	12.50±0.25	11.5±0.20	5.90±0.25	0.25	3.6
24	1.110	25.00	12.3±0.20	3.9±.0.20	0.43	4.1	**48**	8.50±0.20	7.10±0.25	7.80±0.20	0.89	3.4
36	1.074	17.50	9.74±0.35	6.7±.0.25	0.60	3.5	**72**	5.50±0.25	5.12±0.15	9.24±0.35	1.40	3.4
42	1.062	15.25	7.44±0.30	8.1±.0.20	0.79	3.5	**96**	2.50±0.20	2.20±0.15	10.92±0.30	1.85	3.4
60	1.055	12.5	6.14±0.25	10.4±.0.15	1.10	3.4	**120**	0.0±0.10	0.22±0.20	12.40±0.25	2.00	3.2
			Fermentation efficiency (Residual sugar)-81.82%						Fermentation efficiency (Residual sugar)-86.10%			
			Fermentation efficiency (Total Brix)-61.2%						Fermentation efficiency (Total Brix)-80.72%			
p (5%)$_{ethanol}$				0.00047								

***Fermentation conditions:**
No. of fermentation hrs: 60h
Temperature: 35ºC
Scale: 10L
DAHP concentration: 300ppm
Inoculum size: 7.5% (v/v)

**** Fermentation conditions:**
Fermentation hrs: 60h
Temperature: 35ºC
Scale: 10L
DAHP concentration: 300ppm
Inoculum size: 7.5% (v/v)

earlier results of table 3 particularly with sugarcane juice though molasses produced significantly lower ethanol which can be due to higher initial Brix (47ºB) though it had reducing sugars of 23.6 ºB (Table 4).

During molasses fermentation, more than 12ºB was left unfermented which corresponded to residual reducing sugars of 6.14%, hence the fermentation efficiencies w.r.t. total and residual reducing sugars differed significantly which was not so different in fermentation of sugarcane juice. This might be the reason that the current industrial practices use 15-17 of reducing sugars in molasses for producing 7-8% (v/v) ethanol in a single lot of 48h (Patil *et al*., 1998).

In the current study, the pre-conditioned cells of *S.cerevisiae* 11815 produced higher ethanol in 60h of molasses fermentation that may further help in saving the cost on process water (for diluting molasses). Earlier, Logothetis *et al.* (2006 and 2013) reported that salt pre-conditioning of cells imparted yeast an ability to tolerate fermentation stresses due to high ethanol, high sugar concentrations, low pH and high temperature. Further, Logothetis *et al.* (2013) also reported as high as 23.5% (v/v) ethanol production by pre-conditioning yeast cells (*Syrah*) in fermentation time of 12 days. Moreover, as yeast strain becomes thermotolerant, lesser amount of energy is consumed to maintain the fermentation temperature and there are low chances of contamination.

CONCLUSION

To sum up, the present study successfully employed salt pre-conditioned cells of *S.cerevisiae* to ferment molasses diluted to 47.5 ºB (23.5% reducing sugars) at 39 ºC to produce 10.4% ethanol thus, reducing the amount of process water generated post-fermentative distillation.

REFERENCES

Caputi, A.J., Ueda, M. and Brown, T. 1968. Spectrophotometric determination of ethanol in wine. *Am. J. Enol. Vitic*., **19**: 160-165.

Cheema, H. and Sidhu, S.S. 2007. GSTAT: *A software package of STAT* Punjab Agricultural University, Ludhiana, India.

Kaur, M. and Kocher, G.S. 2014. Effect of recycled yeast inoculum on fermentation and quality of red wine produced from Punjab purple (Syn H516) grapes., *Indian J of Nat Prod and Res*., **5**(3).

Khanna, P.S. 2016. Pre-conditioning of fermenting yeast with sodium chloride for ethanol production. M.Sc. Thesis, Punjab Agricultural University, Ludhiana, Punjab. India.

Khanna, P.S. and Kocher, G.S. 2015. Acquisition of osmotolerance and thermotolerance in *Saccharomyces cerevisiae* MTCC 11815 through NaCl preconditioning. 52nd Annual Conference of Association of Microbiologists of India, Dec 8-12, 2015,New Delhi,Abstract number. EMP 82.

Lambert, M. and Naish, A.C. 1950. Rapid method for estimation of glycerol in fermentation solutions. *Can. J. Reds.,* **28**: 83-89.

Lee, S.S., Robinson, F.M. and Wang, H.Y. 1981. Rapid determination of yeast viability *Biotechnol Bioeng Symp* (United States): Journal Volume **11**: Conference: 3. sympo-sium on biotechnology in energy production and conservation, Gatlinburg, TN, USA, 1981 Research Org. University of Michigan, Ann Arbor.

Logothetis, S. Walker, G. and Neratzis, E.T. 2006. Osmotic stress and yeast cell Viability. 2nd International Congress on Bioprocesses in Food Industries, Patras Greece, pp. 82-83.

Logothetis, S., Tataridis, P., Kanellis, A. and Neranizits, E. 2013. The effect of preconditioning cells under osmotic stress on high alcohol production. *J Nat Sci.,* **124**: 405-414.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem*., **31**: 426-428.

Panchal, C.J. and Stewart, G.G. 1980. The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast strain. *J Inst Brew.,* **86**: 207–210.

Patil, B.G., Gokhale, D.V., Bastawde, K.B., Puntambekar, U.S. and Patil, S.G. 1998. The use of tamarind wastes to improve ethanol production from cane molasses. *J Ind Microbiol Biotechnol*., **21**: 307–331.

Pham, T.K., Chong, P.K., Gan, C.S. and Wright, P.C. 2006. Proteomic analysis of *Saccharomyces cerevisiae* under high gravity fermentation conditions. *J. Proteome. Res*., **5**: 3411–3419.

Singh, D. and Nigam, P. 1995. Treatment and disposal of distillery effluents in India. In: Environmental Biotechnology: Principles and Applications. Moo-young M, *et al.* (Eds.), Kluwer Academic Publishers, pp. 735–750.

Thomas, K.C., Dhas, A. Rossnagel, B.G. and Ingledew, W.M. 1995. Production of Fuel Alcohol from Hull-less Barley by very High Gravity Technology. *Cereal Chem*., **72**(4): 360–364.

Thomas, K.C., Hynes, S.H. and Ingledew, W.M. 1994. Effects of particulare materials and osmoprotectants on very-high-gravity ethanolic fermentation by *Saccharomyces cerevisiae*. *Appl Environ Microbiol*., **60**: 1519–1524.