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Improved Ethanol Fermentation of Supplemented Sweet Sorghum Juice Using a New Thermotolerant Mutant *Saccharomyces cerevisiae* UV4 by Yeast Recycle Technique

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A new thermotolerant UV- mutant strain *Saccharomyces cerevisiae UV4* based on its invertase activity was selected and found to possess higher temperature (40°C) tolerance, higher growth rate and fermenting ability than the parent strain *Saccharomyces cerevisiae* 101. At 40°C, the final ethanol concentration was increased by more than 45% by the mutant on fermentation of sweet sorghum medium supplemented with total yeast nutrients. A 2.0 gl^{-1} addition of nitrogen sources namely urea, ammonium sulphate, yeast extract and skim milk solids has increased the ethanol yield by 29%, 27%, 37% and 33%, respectively. Batch yeast cell recycle has contributed to the formation of 17% more ethanol in the presence of skim milk supplement than the control, as it saved 5-6% of sugars required in the build up of the initial yeast inoculum.

Key words: Sweet sorghum, ethanol fermentation, mutant strain, thermotolerance, nutrients, yeast recycle.

At present there is huge demand for fuel ethanol all over the world. In India, since bio-ethanol is produced mainly by the conventional batch fermentation using cane molasses as the major feed stock, the availability of which is not only limited but also the rates are sensitive to sugar out put in the country, and therefore, there is ultimate instability in the price of alcohol as well as its availability. Hence the Indian government is likely to extend the proposed time (October 2008) for implementing E10 ethanol mandate with gasoline because of an expected shortfall (20-25%) in sugar production in 2008-09 season (Biofuels Digest, July 2008). All these circumstances necessitate the search for alternate substrates for fuel ethanol production.

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Among various alternate substrates available for bio-ethanol production, sweet sorghum tops the list at present. Because, it is not only competitive with sugarcane route, but also can act as a supplementary feedstock. Compared with sugarcane, the main advantages of sweet sorghum are as follows: a). The crop duration is short, and in areas with a long growing season it can be harvested twice a year; while the growing season of sugarcane is usually 8-12 months; b). Sugarcane is propagated from stem cuttings needing 4,500 to 6,000 kg/ha of cane, while sweet sorghum is propagated just with seed 4.5-7.5 kg/ha; and c). The quantity of water needed by sweet sorghum is only 1/3 of that needed by sugarcane.

In the present scenario of alcohol production in batch process, fermentor temperature regulation is on prime focus. Because increased inside temperatures of fermenters during summer, especially in tropical countries like India, have detrimental effect on yeast growth and viability. Hence, thermotolerant yeast strains

play important role in ethanol fermentations to counter the increased temperatures. Generally, the production of biological materials at high temperatures rather than the customary practice makes it possible to reduce the risk of contamination and the operation costs of maintaining growth temperatures in large-scale systems, and to increase the rate of productivity (Nolan *et al*., 1994).

Secondly, the yeast *Saccharomyces cerevisiae* can ferment increased amounts of sugars in the medium when all required nutrients are provided in adequate amounts. Supplementation of ergosterol, chitin, skim milk solids and fungal mycelium has improved the rate and the yield of ethanol production in cane molasses fermentation (Patil *et al.*, 1989, 1986). Increased ethanol yields were obtained by using tamarind wastes in molasses fermentation medium (Patil *et al.,* 1998). In this context, a study was needed to ascertain the role of nutrients supplementation in the sweet sorghum fermentation to ethanol.

Attempts have been made to recycle the yeast cell mass, either by centrifugation or by a natural sedimentation procedure, for ethanol production, which may lead to a saving of 6-7% sugars required for inoculum preparation (Sedha *et al.,* 1984).

The present work focuses on possible ways to overcome the problems related to industrial ethanol fermentations such as the build up of temperature in fermenters, especially during summer, the effect of inadequate nutrients in the medium, as well as to explore the yeast cell recycle advantages on the rate and final yields of ethanol.

MATERIAL AND METHODS

Microorganism

The non-amylolytic, ethanol producing yeast strain *Saccharomyces cerevisiae* 101 was obtained from CFTRI, Mysore. The culture was maintained on MPYD medium (Malt extract-0.3%, Peptone-0.5%, Yeast extract-0.3%, Dextrose-2% and Agar-1.5%) slants at 4°C.

Mutagenesis

Mutations in the parental strain were developed according to the procedure (Sridhar *et al.,* 2002) for thermotolerant yeast isolation. A loopful of the above culture was transferred to a 100ml sterilized MPYD broth medium and kept on shaking at 30°C for 12h. At log phase of growth, cell suspension was centrifuged at 4000rpm for 5 min, washed twice using sterile water, and then resuspended in 0.1M phosphate buffer of pH 7.0 and adjusted to 10^8 cells/ ml by turbidometry.

About 10ml of the culture was transferred to sterile Petri plates and were irradiated with 254 nm wavelength emitting by a 15 watt UV lamp at a distance of 75 cm. Samples were taken at every 5 min intervals for a period of 30 min. Then they were plated onto YPD medium, after serial dilution in buffer. The plates were incubated at 30°C for 3-4 days and observed for viable colonies/ cells.

Inoculum preparation

Inoculum was prepared in step-wise manner to a required volume by transferring a loopful of fresh culture of selected UV4 mutant to 5 ml, and then to 100 ml sterile sorghum juice medium in a flask, having added total sugars-5.0%, urea-0.25%, $MgSO_4.7H_2O$-0.05%, yeast extract-0.2%. The flask was kept on rotary shaker (100 rpm) for 24h at 40ºC temperature. A cell suspension having $1x10^8$ cells/ml viable counts was used as inoculum in all the experiments.

Substrate

Stalk juice of sweet sorghum variety ICSV 700 was procured from S.V. Agricultural College, Tirupati. Refractometer brix of the juice was 16-17°Bx. Total reducing sugar concentration was found to be 16% (w/v), after inversion using HCl. Sterilization of the juice was done at 121°C for 15min., after cooling to room temperature, it was centrifuged at 5000 rpm and clear supernatant was used in the experiments.

Nutrients and fermenting conditions

A set of experiments were performed in order to evaluate the role of various nutrients on ethanol fermentation. Batch fermentations were performed in 250 ml Erlenmeyer flasks containing each 100 ml of juice and plugged with rubber bung. Each flask was provided with a tube connected to 0.2µ PTFE (Polytetrafluoroethylene) filter for CO_2 removal. Nutrients such as urea, ammonium sulphate, yeast extract, peptone and skim milk solids (along with magnesium sulphate) were used as nitrogen sources, to study their role

in the process against control (without supplementation). The pH was adjusted to 5.2, and the temperature was maintained at 40ºC through out the fermentation period of 72h at stationary conditions. Samples were drawn at different intervals and assayed for alcohol, residual sugar and biomass.

Recycling of yeast cells

Fermentation media of three sets were prepared as follows for yeast cell recycling studies: (A). Total juice sugars-16.0%+ urea-0.25%+ $MgSO_4$ $7H_2O$; (B). Same as A+ Yeast extract 0.2%; (C). Same as A+ Skim milk solids 0.2% (90 ml in 150ml conical flask) . The required sets of fermentation media and inoculating media were autoclaved at 15 psi (121°C) for 20min and fermentation at stationary conditions was carried out at 40°C for a specified period.

After inoculating the first set with 10% inoculum, the flasks were incubated at 40°C for 48 h and then 90 ml fermented media was removed by decanting without disturbing the cell mass. The fresh medium of 90 ml each of similar composition was then transferred to the respective flasks, under aseptic conditions. On total seven cycles were carried out, the remaining six cycles of decantation and fresh transfer of media were completed by incubating only for 24 h, and by keeping other conditions constant. Samples (5 ml) were collected periodically from all these sets for ethanol estimation.

Analytical methods

Total reducing sugars concentration was measured by using the DNS method (Miller, 1959) after inversion using dilute HCl. Residual sugar level was determined by the Lane Eynon analytical method (Egan *et al.*, 1981). Biomass was determined by gravimetric method on dry weight basis. Ethanol concentration at the end of fermentation was measured by Sikes Hydrometer, after distilling the wash, using Sikes table with temperature correction. Cell growth was measured by direct counting of cells using microscope (X400 magnification) in a Haemocytometer.

RESULTS

Isolation of thermotolerant mutant UV4

About 100% killing of cells was observed at 25min exposure to UV light. And LD_{50} was found to be 12.5 min exposure. Sharp reduction in the % survivors was observed between the 10-20 minutes of exposure time (Fig. 1). About 50 colonies from different plates exposed to different time intervals were selected based on colony morphology and size. 5% inoculum of these selected 50 colonies grown in YPD broth at 30°C was transferred to YPD broth flasks containing 10% sucrose. These tubes were incubated at 40°C for selecting strains capable of showing good growth at this temperature. Five best performing mutant isolates of *S. cerevisiae* 101 strain were screened out based on their invertase activity and cell density, and numbered as UV1, UV2, UV3, UV4 and UV5.

They were initially identified based on colony size and morphology. Size of colony was smaller compared to native and also rough colonies were formed, compared to smooth in native strain. Among the selected mutants, UV4 was taken up for further studies based on its high invertase activity and high cell density at this temperature (Table.1).

Table 1. Cell density and invertase activity of selected mutants grown at 40°C for 12 h in a 2% sucrose medium

Yeast strain	Cell density	Invertase activity*
UV1	2.1×10^8cells/ml	38
UV2	1.91×10^8cells/ml	52
UV3	2.32×10^8cells/ml	23
UV4	3.1×10^8cells/ml	78
UV5	2.39×10^8cells/ml	36

*Units shown are micromoles of glucose released per minute per 100 mg of dry weight cells

Effect of nutrient supplementation

Supplementation with yeast nutrients such as urea, ammonium sulphate, yeast extract and milk powder at concentrations 0.1%, 0.2% and 0.3% (w/v) has increased the ethanol productivity and the final yield significantly (Table. 2). A 0.2% concentration of these nutrients along with 0.05% (w/v) $MgSo_4$ was optimal for maximum ethanol productivity and final yield. Final ethanol concentration as high as 6.8% (w/v), theoretical yield of 92% was reached with

in 48 h, when the medium was supplied with 0.2% skim milk powder together with nitrogen source, whereas in unsupplemented medium, the fermentation was incomplete as 4% residual sugars were left over in the medium.

Yeast cell recycling studies

Addition of skim milk solids and yeast extract yields maximum productivity and final ethanol concentration. In the present study, about 17% and 10% increment in ethanol production was observed after seven yeast cycles using skim milk solids and yeast extract as supplements, respectively (Table.3). Addition of skim milk solids was found to be superior to yeast extract; and the former is a cheaper product than yeast extract for use in sweet sorghum fermentation. In the present study, nearly 90% fermentation efficiency was maintained and was consistent in all seven cell recycles in the skim milk supplemented medium. In supplemented medium, cell viability was maintained to the level of

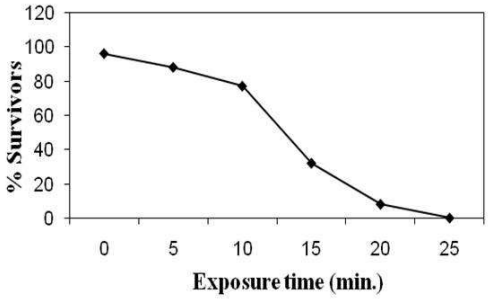

Fig. 1. Rate of reduction in the viability of yeast during UV irradiation (254nm)

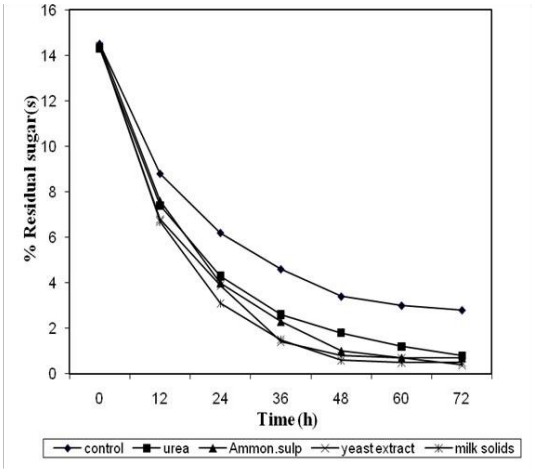

Fig. 2: Effect of various nutrient supplements (0.2%w/v) on sugar depletion rate by mutant *S. cerevisiae* UV4 strain in the medium at 40°C

Table 2. Effect of supplementation on ethanol concentration % (w/v) by UV4 mutant strain of *S. cerevisiae,* at 48 h of fermentation

Supplement	Concentration	Final ethanol concentration % (w/v) at		
	% (w/v)	48 h	% IMP*	%EFF**
None	-	4.58	-	62
Urea	0.1	5.4	18	72
	0.2	5.9	29	79
	0.3	6.2	35	83
Ammonium	0.1	5.3	15.7	71
sulphate	0.2	5.85	27.7	79
	0.3	5.8	26.6	78
Yeast extract	0.1	5.7	24.4	77
	0.2	6.28	37	84
	0.3	6.1	33	82
Skim milk	0.1	5.5	19.5	74
solids	0.2	6.1	33	82
	0.3	6.5	37.5	85
Urea + yeast extract	0.2 + 0.2	6.7	46	90.5
Urea + skim milk solids	0.2 + 0.2	6.8	48.5	92

IMP*- Improvement; %EFF**-Fermentation efficiency.

Table 3. Effect of skim milk powder supplementation on ethanol production in a yeast cells recycle medium

Supplements used (0.2%)	Ethanol % (w/v) at periodic cycles and at specific hours							Total ethanol	Ethanol (%) increment
	48h	72h	96h	120h	144h	168h	192h		
A. Control	5.6	5.3	5.5	5.9	5.8	5.6	5.9	39.6	-
B. Yeast extract	6.6	6.3	6.4	5.9	6.0	6.2	6.4	43.8	10.6
C. Skim milk	6.5	6.6	6.6	6.8	6.7	6.7	6.6	46.5	17.4

Control: Total juice reducing sugars-16.0%+urea-0.25%+$MgSO_4 7H_2O$-0.05%.
(Fermentable reducing sugars-14.5%); Temperature-40ÚC, pH-5.2; Values are average of duplicate experiments.

$70x10^6$cells/ml. Fermentation completion time was reduced significantly from initial 48 h to 24 h by recycling of yeast to achieve similar final ethanol concentration.

DISCUSSION

Several potential benefits are associated with operating ethanol fermentation at temperatures exceeding 40°C. The optimum temperature for fermentation has been reported to be higher than that for growth in strains of *S. cerevisiae* (Hacking *et al.*, 1984). Osmotolerant, thermotolerant and ethanol tolerant mutant microbes were isolated by several researchers. Earlier, a thermotolerant mutant strain *S. cerevisiae VS3* was isolated by UV exposure technique (Sridhar *et al.,* 2002); and a high level thermotolerant mutant strain *Saccharomyces cerevisiae HU-TY-1*, which has been widely applied in ethanol fermentation industry in china, was obtained by UV mutagenesis as well (Chengtao *et al.,* 2005).

Although UV4 mutant showing better growth at 40ºC temperature, the nutrients supplementation influenced complete utilization of sugars in the fermentation process. Incomplete fermentations were due to lack of essential yeast nutrients in the medium. Yeast requires sufficient nitrogen for optimal growth and fermentation. As most of the substrates are low in assimilable nitrogen in their composition (Ingledew, 2005), external addition is required in the form of free amino nitrogen (FAN). Nutritive supplements provide necessary nitrogen in yeast assimilable form, and enhance ethanol tolerance and the rate of ethanol production (O'Connor-Cox, *et al.*, 1991). In media containing 120 g saccharides /L, adequate usable nitrogen must be in the range of 140-150mg FAN/L. Besides nitrogen, minerals and vitamins supplementation is vital for yeast growth. Complex nutrient supplements provide all necessary yeast nutrients for ethanol fermentation, since better ethanol yield was observed in cases of yeast extract and skim milk supplemented media, instead of only nitrogen in excess.

According to earlier reports, apparently there was no beneficiary role of external addition of nutrients and mineral salts in ethanol fermentation of sweet sorghum (Kargi and Curme, 1984), which is contrary to the present findings. However, it was reported that varieties of sweet sorghum and their cultivation practices differ, which may contribute to variations in accumulation of sugars and other yeast nutrients in the stem juice. This could be the reason for the positive effect of the supplementary nutrients in the present study. On the other hand, in control medium, ethanol formation rate was slow and was unable to reach its maximum level of ethanol production even up to completion of seven cell recycles. Earlier Patil *et al* (1986) reported that skim milk supplement improved the rate of ethanol production by 29% after 48 h at 30°C.

The present study showed a 50% reduction in the total fermentation time, which could be achieved by increased cell density through cell recycling technique and supplementation with required nutrients. Considerable reduction in the fermentation duration upon yeast cell recycling would improve the plant productivity. Increased cell density and reduced lag phase of cells before fermentation initiation were responsible for this effect. Verma

et al (1983) observed a net saving of 30.5% in fermentation time using cell recycle technique with a simultaneous increase in alcohol production. Patil *et al.* (1989) reported significantly more (20-30%) ethanol production and 87% process efficiency, after completion of 16 yeast cycles, in presence of supplements using cane molasses as substrate.

CONCLUSIONS

It can be concluded that the development of thermotolerant yeasts would be of an immense use to the alcohol industry and represent an advancement in the countries where the ambient temperature remains over 25-35°C for many months and exceeds 40°C during summer months. Nutrients supplementation has shown to be increase the fermentation efficiency. The significant advantage of the cell recycle technique is that it does not require any change in the existing batch fermentation technology and it saves 6-7% sugars, required for inoculum build-up, which in turn contributes to more ethanol yield. All these features contribute to rapid fermentation and final high ethanol concentration, which are desirable features in alcohol fermentation industry.

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