Journal of Scientific & Industrial Research
Vol. 68, May 2009, pp. 401-405

High efficiency alcohol tolerant *Saccharomyces* isolates of *Phoenix dactylifera* for bioconversion of sugarcane juice into bioethanol

Nidhi Gupta[1]*, Ashutosh Dubey[2] and Lakshmi Tewari[1]

[1]Department of Microbiology, [2]Department of Biochemistry, G B Pant University of Agriculture and Technology (GBPUAT), Pantnagar 263 145, India

Received 27 October 2008; revised 04 February 2009; accepted 03 March 2009

Various indigenous strains of *Saccharomyces* sp. (SCP-1, SCP-3, SCP-4, SCP-5, SCP-7) isolated from datepalm (*Phoenix dactylifera*) sap were evaluated for alcohol dehydrogenase (ADH) enzyme activity, ethanol production and alcohol tolerance limits and compared with standard culture of *S. cerevisiae* (S.C.Std). Alcoholic contents in juice samples fermented with different yeast strains varied considerably (8.9-12.5%, $^{v}/_{v}$) as determined by GLC. Yeast cultures showed varied *in vitro* ethanol tolerance (3-12%). Isolate SCP-1 was found superior showing 12.5% ethanol production, high ADH enzyme activity (4.38 units/ml) and higher alcohol tolerance maintaining cell viability at 12% ethanol in YPD medium up to 48 h.

Keywords: Bioconversion, Bioethanol, *Phoenix dactylifera*, *Saccharomyces* sp. Sugarcane juice

Introduction

Alternative energy sources based on sustainable, regenerative and ecologically friendly processes are urgently needed[1]. Bioethanol is generally produced from fermentable raw materials[2] containing sugar or starch (sugarcane, wheat, cellulose as well as industrial waste). Brazil is biggest exporter of ethanol, delivering 70% of worldwide supply and US is country's biggest client as it imported 1.74 billion l in 2006, which represents 58% of Brazil's exports[3]. Ethanol can be blended with gasoline as a fuel extender, an oxygenating agent for spark ignition (SI) engines without significant changes in vehicle performance. Ethanol produced from sugarcane is used as transport fuel-as a blend (24%) in petrol (gasohol) and in neat form (100% alcohol) in Brazil[4].

Organisms generally employed for bioethanol production are strains of yeast *Saccharomyces cerevisiae* and bacteria *Zymomonas mobilis.* But *S. cerevisiae* is commonly employed for bioconversion of substrate to the higher yield of bioethanol under controlled optimization parameters[5]. Date palm (*Phoenix dactylifera*) sap is also a very good source of fermentation microorganisms[6]. Several factors influencing overall rate of brewing wort fermentation, including osmotic pressure and ethanol concentration of media and ethanol tolerance of yeast strain employed[7]. Alcohol dehydrogenase (ADH), responsible for alcoholic fermentation from sugary substrates, plays an important role in fermentating efficiency of yeast catalyzing reaction as follows[8]:

$$RCHO + NADH + H^{+} \underset{}{\overset{ADH}{\rightleftharpoons}} RCH_2OH + NAD^{+}$$

This study presents selection of a potential microbial strain of *S .cerevisiae* having high ethanol tolerance for production of bioethanol from sugarcane juice.

Materials and Methods

Strains and Culture Media

Five isolates of *Saccharomyces* sp. (SCP-1, SCP-3, SCP-4, SCP-5, SCP-7) were isolated from sap samples by serial dilution pour plate and streak plate method on YPD medium. Sap samples were collected from different palm trees growing in Kashipur, Uttarakhand, India. Standard culture of *S. cerevisiae*, ATCC-9763 (Sc. Std) was procured from MTCC, Chandigarh, India. All yeast cultures were routinely sub-cultured at an interval of 15 d and maintained on YPD (w/v) [yeast extract (1%), peptone (2%), glucose (2%), agar (2%)] medium at 4ºC.

*Author for correspondence
Tel: 05944-233410; Fax: 05944-233341
E-mail: nidhigupta_2411@yahoo.co.in

Fermentation Media

Fresh juice of sugarcane (var. CoPant -90223) collected from Crop Research Centre (CRC) of GBPUAT, Pantnagar, Uttarakhand, India, was pasteurized at 85°C for 1 h and centrifuged at 5000 rpm for 10 min to remove solid particles. Cleared juice was sterilized at 10 lb psi for 20 min and stored at 0°C for further use.

Determination of Growth of Yeast Cultures

Growth and population dynamics of various yeast isolates of date palm sap were studied by measuring cell number (cfu counts) in YPD broth up to 48 h at 30°C. An aliquot (1 ml) was withdrawn from appropriate dilution of each sample at 0 and 48 h of incubation and plated on YPD medium. Colonies were counted after incubation at 30±2°C for 48 h. Total viable counts (cfu/ml) were determined and specific growth rate constant for each culture was calculated as

$$\log_{10} N - \log_{10} N_0 = K/2.303(t-t_0) \quad \ldots(1)$$

where, N =cfu ml^{-1} at 48 h, N_0 = cfu ml^{-1} at 0 h, t_0 = time (0 h), t = time (48 h)

Determination of Alcohol Dehydrogenase (ADH) Activity of Yeast Cultures

A) Preparation of Cell Extract (Crude Enzyme)

Broth cultures (48 h old) were harvested by centrifugation at 8000 rpm for 8 min and cell pellets washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA and stored at -20°C. Before cell breakage, samples were thawed at room temperature, washed and resuspended in 10 mM potassium phosphate buffer containing 2 mM $MgCl_2$ and 2 mM dithiothreitol. Cell extracts were prepared by sonicating cell preparations with glass beads (0.7 mm diam) at 0°C for 2 min at an interval of 30 s with LABSONIC U sonicator (133 V, 0.5 repeating cycles per s). Unbroken cells and cell debris were removed by centrifugation at 4°C for 20 min at 12000 g. Purified cell extracts were used as crude enzymes and for protein estimation[9].

B) Enzyme Assay

Crude enzyme preparations of various yeast cultures were analyzed for ADH enzyme activities. Reaction velocity was determined using the method described by Brady *et al*[10] with slight modifications. Standard assay mixture contained 0.1 M sodium pyrophosphate buffer, pH-9.6 (1.5 ml), 2.0 M ethanol (0.5 ml), 0.025 M NAD (1.0 ml) and crude enzyme (0.8 ml). Increase in absorbance at 340 nm for 3-4 min at room temperature (25°C) was recorded. Absorbance (340 nm/min) was calculated from initial linear portion of the curve. One enzyme unit is the amount of enzyme that reduces one micromole of NAD^+ per min at 25°C under specified conditions. ADH enzyme activity was calculated as

$$\text{ADH units/mg protein} = \frac{\text{A340/min}}{\text{6.22 x mg protein/ml reaction mixture}}$$

Determination of Ethanol Tolerance

*In v*able concentrations of ethanol (3-15%,(v/v)and inoculated with active yeast cultures @ 10 % (v/v) individually and incubated for 48 h at 30°C. Cultures were then evaluated for cell viability (cfu/ml). Ethanol concentration that completely suppressed growth was determined for each culture.

Fermentation of Sugarcane Juice and Estimation of Alcoholic Content

Erlenmeyer fRRlasks containing 40 ml of sugarcane juice were sterilized at 15 lb psi for 20 min and inoculated individually with active cultures @ 10% (v/v) Inoculated juice samples were incubated for first 16 h in an incubator shaker (120 rpm) and then incubated in static conditions up to 48 h. Fermented juice samples were centrifuged at 8000 rpm for 10 min and analyzed for alcohol yield. Alcoholic contents from fermented samples were recovered by distillation of each sample collected in a separate graduated dry tube immersed in ice-cooled water. Distillates were analysed by a Nucon Gas Chromatograph model 5700 for separation and quantification of ethanol. Ethanol concentrations (%) in different fermented samples were determined by computing peak area of samples against standard curve drawn for ethanol.

Results and Discussion

In order to select date palm sap as a rich source of indigenous rapid ethanol producing *Saccharomyces* sp., growth rate and population dynamics of various isolates (SCP-1, SCP-3, SCP-4, SCP-5, SCP-7) were studied by determining total viable cell counts in shake cultures (YPD broth) incubated for 48 h at 120 rpm and compared with standard culture of *S. cerevisiae* (Table 1). Yeast isolates (SCP-4 and SCP-5) showed significantly higher cfu counts (142 $x10^7$ and 145.5 $x10^7$ ml^{-1} respectively) as compared to other isolates and standard culture of

S. cerevisiae (56.20 x10^7 ml^{-1}) in broth cultures at 48 h. Growth rate constants (k) were also higher of SCP-4 (0.10 h^{-1}) and SCP-5 (0.08 h^{-1}) as compared to standard culture (0.070 h^{-1}). Higher growth rate of yeast isolates indicated that cultures were in physiologically active phase with rapid multiplication. Thus, date palm sap is a rich source of fast growing yeast. A specific growth rate (0.10 h^{-1}) in a chemostat at steady state is also reported[11].

Ethanol is well known as an inhibitor of microbial growth. It damages mitochondrial DNA in yeast cells and causes inactivation of some enzymes, such as hexokinase and dehydrogenase[12]. Ethanol is toxic to living cells, even in ethanol producing species, limiting their growth, metabolic activity and ethanol yield[13]. Some strains of *S. cerevisiae* show tolerance and can adapt to high concentrations of ethanol[14]. Since higher concentration of ethanol in culture medium becomes inhibitory for microbial growth, therefore, various ethanol producing isolates were also evaluated for *in vitro* ethanol tolerance limits in YPD broth containing varying concentrations (0-15%) of ethanol. With increasing alcohol concentration (0-12%, $^{v}/_{v}$), cfu counts of all the isolates decreased gradually (Table 2) and only SCP-1 (0.64 x10^5 cfu/ml) and SCP-5 (0.29 x10^5 cfu/ml) depicted growth at 12% ethanol concentration at 48 h. Standard culture of *S. cerevisiae* had lower alcohol tolerance (17.2 x10^5 cfu/ml) at 9% alcohol concentration but no viable counts at higher (12%) alcohol concentrations at 48 h. SCP-3 showed minimum alcohol tolerance with 3.81 x10^5 cfu/ml at 3% alcohol while no growth at higher (>3%) alcohol concentrations in the medium. Thus SCP-1 and SCP-5 were found superior showing higher alcohol tolerance (up to 12% ethanol conc.) as compared to standard culture of *S. cerevisiae* (tolerant to only 9% ethanol). Higher alcohol tolerance limits of yeast observed in present study have also been reported[15]. High ethanol tolerance of *S. cerevisiae* is due to unique lipid composition of its plasma membrane as it synthesizes ergosterol rather than cholesterol and phospholipids, containing very high proportion of unsaturated fatty acyl residues[16].

A comparative evaluation of various strains of *Saccharomyces* sp. isolated from date palm sap for ethanol producing capacity and ethanol tolerance (Fig. 1) indicates that isolates were having differential potential for ethanol production and ethanol tolerance. However, SCP-1, with maximum ethanol producing potential and tolerant to maximum ethanol concentration in culture medium, was found superior among all cultures as well as standard culture of *S .cerevisiae*.

Batch fermentation of sugarcane juice for ethanol production using yeast isolates (Table 3) indicates that isolates differed in ethanol producing capacity. Maximum ethanol concentration (12.5%, $^{v}/_{v}$) was recorded in juice fermented with SCP-1 followed by SCP-4 (12.16%), Sc Std (12.10%) and SCP-3 (12.06%). However, no significant difference in alcoholic content in juice fermented with three isolates (SCP-1, SCP-3, SCP-4) and standard culture (Sc Std) could be recorded.

Table 1—Viable cell counts and specific growth rate of various *Saccharomyces* isolates of date palm sap in YPD broth

Isolates	Viable cell counts cfu (x10^7) ml^{-1}		Specific growth rate constant, h^{-1}
	0 h	48 h	
SCP-1	1.25	2.96	0.0739
SCP-3	2.88	35.0	0.0029
SCP-4	1.00	142.0	0.1008
SCP-5	0.38	145.5	0.0825
SCP-7	2.74	3.11	0.0017
Sc. Std	0.45	56.20	0.0670

Table 2—Evaluation of yeast isolates for *in vitro* ethanol tolerance efficacy as determined by cell viability in YPD broth cultures at 48 h

Isolates	cfu (x10^5)ml^{-1} at different concentrations of ethanol, %				
	0	3	6	9	12
SCP-1	2960	2850	276	0.72	0.64
SCP-3	350	3.81	-	-	-
SCP-4	14200	12700	30.0	0.03	-
SCP-5	14500	13600	34.6	0.21	0.29
SCP-7	311	2.17	0.03	-	-
Sc. Std	5600	370	25.6	17.2	-

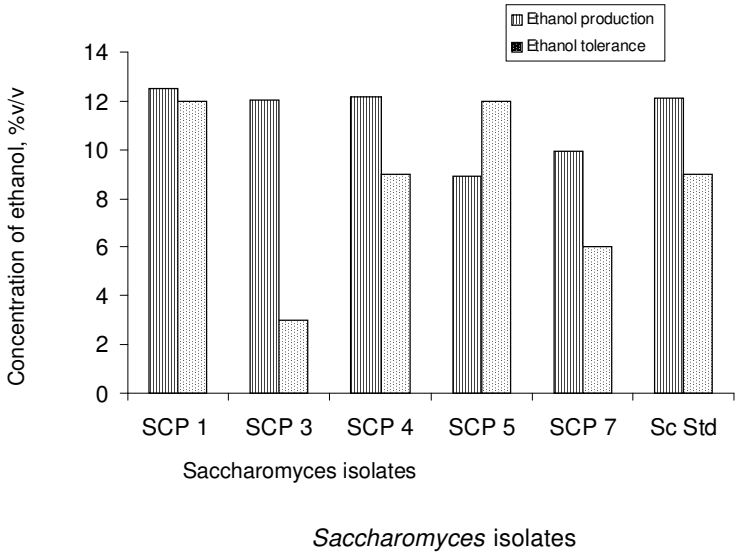

Fig. 1—Comparative evaluation of various *Saccharomyces* isolates for ethanol production and ethanol tolerance

Table 3—Comparative evaluation of *Sacccharomyces* isolates for specific ADH activity and ethanol production in sugarcane juice at 48 h of fermentation

Isolates	Ethanol concentration in fermented juice, % (v/v)	Specific activity of ADH, U mg^{-1} protein
SCP-1	12.50	43
SCP-3	12.06	0.8
SCP-4	12.16	18.6
SCP-5	8.90	9.0
SCP-7	9.90	0.2
Sc. Std	12.10	55

Least ethanol production was shown by SCP-5 (8.9%) and SCP-7 (9.9%). Variability of ethanol production (5-20%) by *S. cerevisiae* and *Z. mobilis* has been reported[17].

Crude enzyme preparations of various yeast isolates of date palm sap grown in sugarcane juice for 48 h showed significant differences in ADH enzyme activity (0.03-4.38 Uml^{-1}) (Fig. 2). Maximum ADH activity (4.38 Uml^{-1}), recorded for SCP-1, was significantly higher than ADH activity of all other isolates but was at par from ADH activity (4.135Uml^{-1}) shown by standard culture of *S. cerevisiae*. Among various isolates tested, maximum specific activity for ADH (43 Umg^{-1} protein) was recorded for SCP-1, which was significantly higher than all other isolates tested (Table 3). Thus results in present study are in agreement with earlier studies, wherein specific ADH activity of 4.5 Umg^{-1} protein for *Clostridium beijerinkii* have been reported[18].

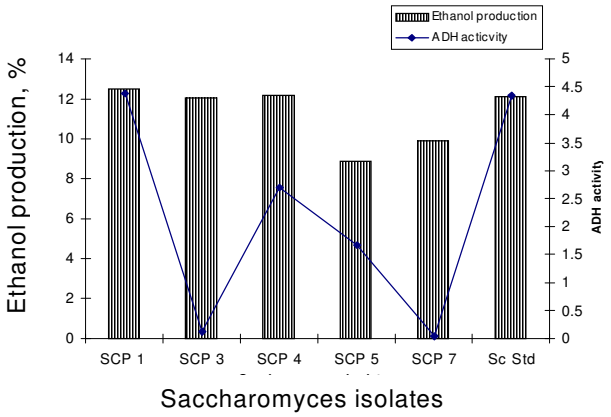

Fig. 2—Correlation between alcohol dehydrogenase activity of yeast isolates and ethanol production in sugarcane juice at 48 h of fermentation

Isolate SCP-1 (Fig. 2) with higher ADH activity (4.38 Uml^{-1}) also has higher ethanol producing capacity (12.5%) but isolates SCP-3 and SCP-4 showed lower ADH activity (0.12Uml^{-1} and 2.69Uml^{-1} respectively) but higher ethanol production (12.06% and 12.16% ethanol conc. in fermented juice, respectively). This may be because enzymes from different isolates have their specific turn over number, which is equivalent to the number of substrate molecules, converted to product in a given unit of time by a single enzyme molecule when enzyme is saturated with substrate[19]. Therefore, SCP-3

with lower ADH activity but higher ethanol production might have high turn over number than isolate SCP-1. Moreover, high intracellular ethanol concentrations are accompanied by inactivation of ADH indicating that ADH is under regulation by end product inhibition and loss of cell viability in SCP-3 that might occur due to rapid fermentation as also reported by previous workers[20]. Also, there may be some effector (activator) molecules required for ADH activity, which might have been removed away during crude enzyme preparation.

Conclusions

This study presents occurrence of high ethanol producing microorganisms (*Saccharomyces* sp.) with faster growth rate in date palm sap. Isolate SCP-1 showed higher ADH enzyme activity, higher alcohol tolerance and higher alcohol production, and was found superior to standard strain of *S. cerevisiae*. Thus, SCP-1 can be exploited for production of bioethanol to be used as biofuel in transportation sector replacing either partially or fully the fossil fuel.

References

1 Kalscheuer R, Stolting T & Steinbuchel A, Microdiesel: *Escherichia coli* engineered for fuel production, *Microbiology*, **152** (2006) 2529-2536.

2 Reimelt S, Winkler F, Mogel K & Kirchhof M, Bioethanol technology of lurgi life science, *Zckerindustrie,* **127** (2002) 770-781.

3 Wüst, C, Thanksgiving in the car tank. Fuel from biomass can replace the oil — if better technology is used, *Der Spiegel*, **8** (2007) 104-111.

4 Ghosh P & Ghose T, Bioethanol in India: Recent, past and emerging future, *Adv Biochem Engg/Biotechno,* **84 (**2003) 1-7.

5 Noor A A, Hameed A, Bhatti K P & Tunio S A, Bioethanol fermentation by the bioconversion of sugar from dates by *Saccharomyces cerevisiae*, *Biotechnol (Pakistan)*, **2** (2003) 8-17.

6 Manay N A & Shadaksharaswamy M, In *Foods, Facts and Principles* [New Age International (P) Ltd. Publishers, India] 1998, 192.

7 Stewart G C, D'Amore T, Pauchol C J & Russell I, Factors that influence the ethanol tolerance of brewer's strains during high gravity wort fermentations, *Masters Brewers Assoc America,* **25** (1988) 47-53.

8 Sarcar S, Jain T K & Maitra A, Activity and stability of yeast alcohol dehydrogenase (ADH) entrapped in aerosol OT reverse micelles, *Biotechnol Bioengg,* **39** (1992) 474-478.

9 Vurlhan Z, Morais M A, Jai S L, Riper M D W & Prank J T, Identification and characterization of phenyl pyrunate decarboxylase genus in *S. cerevisiae*, *Appl Environ Microbiol,* **69** (2003) 4534-4541.

10 Brady C J, Conjhurt T J & Tung H F, Developmental regulation of the expression of alcohol dehydrogenase in ripening tomato fruits, *J Food Biochem,* **14** (1990) 421-433.

11 Gombert A K, Moreira M S, Christensen B & Nielsen J, Network identification and fluse quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression, *J Bacteriol,* **183** (2001) 1441-1451.

12 Ibeas J I & Jimenez J, Mitochodrial DNA loss caused by ethanol in *Saccharomyces* flour yeasts, *Appl Environ Microbiol,* **63 (**1997) 7-12.

13 Hallsworth J E, Ethanol induced water stress in yeast, *J Ferment Bioengg,* **85** (1998) 125-137.

14 Alexandre H, Rousseaux I & Charpentier C, Ethanol adaptation mechanisms in *Saccharomyces cerevisiae*, *Biotechnol Appl Biochem,* **20 (**1994) 173-183.

15 Rios E M, Salazans G M T, Antvnes R V, Accidy L G A, Otamar J & Morais F, Glucose fermentation by *Zymomonas mobilis*- effect of addition of ethanol and of cell free fermented must on the microbial growth and ethanol production, *Arg Biol Technol,* **34** (1991) 45-52.

16 Ingram L O & Buttke T M, Effects of alcohol on microorganisms, *Adv Physiol,* **25 (**1984) 253-300.

17 Gorzynska A, Krgzwacka J & Przem T, Teleranceja drozdy na alcohol, *Ferment Rolny,* **17 (**1973) 8-9.

18 Hiu S F, Zhu C X, Yan R T & Chen J S, Butanol ethanol dehydrogenase and Butanol ethanol isopropanol dehydrogenase : Different Alcohol Dehdrogenases in two strains of *Clostridium beijernikii* (*Clostridium butylicum*), *Appl Environ Microbiol,* **53** (1987) 697-703.

19 Nelson D L & Cox M M, Enzymes, In Lehninger Principle of Biochemistry, **4th edn** (W H Freeman & Company, New York) 2005, 206-207.

20 Nagodawithana T W & Steinkraus K H, Influence of the rate of ethanol production and accumulation on the viability of *Saccharomyces cerevisiae* in rapid fermentation, *Appl Environ Microbiol,* **31** (1976) 158-162.