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Characterization, identification and comparative evaluation of bioethanol tolerance and production capacity of isolated yeast strains from fermented date palm sap (Toddy)

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Aims: Toddy, an alcoholic beverage produced from naturally fermented date palm sap and commonly used as traditional drink in Southern India including Odisha, is a source of natural yeasts, capable of fermenting sugar to alcohol. The present study was carried out to isolate and identify predominant yeast strains from toddy, fermented date palm sap and to evaluate their ethanol tolerance and comparative bioethanol production capacity to select potential yeast strains for efficient bioethanol production for their application in fermentation industry.
Methodology and results: Eight morphologically distinct yeast isolates (BS1-BS8) were identified on the basis of morphological and biochemical characterization. The yeasts identified include four strains of *Saccharomyces cerevisiae*, one strain each of *Pichia besseyi* and *Trycosporon capitatum* and two strains of *Candida albicans.* The strains were evaluated for their ethanol tolerance capacity in *in vitro* condition. The strains *P. besseyi* (BS2) and *S. cerevisiae* (BS7) tolerated ethanol concentration up to 10% (v/v), while two isolated *S. cerevisiae* strains (BS1 and BS5) showed tolerance up to 9% of ethanol concentration. The remaining two strains showed tolerance of 7% (v/v). The identified strains along with commercial yeast strain (*S. cerevisiae*, CTCRI) were evaluated for comparative bioethanol production capacity in different carbohydrate substrates (grape juice, mahua flower extract, molasses, sugarcane juice and saccharified sweet potato root flour broth). Taking different strains and substrate conditions into account, strain *P. besseyi* (BS2) showed in overall the highest ethanol production capacity followed by the strains of *S. cerevisiae* (BS5, CTCRI, BS2 and BS7 in an order of their performance). On the other hand strains of *C. albicans* (BS4 and BS6) and *T. capitatum* (BS3) exhibited least ethanol production capacity.
Conclusion, significance and impact study: In conclusion, the present research work characterizes eight yeast strains isolated from fermented date palm sap (toddy) by conventional morphological and biochemical methods and identified them as four species of *Saccharomyces cerevisiae*, two strains of *C. albicans* and one strain each of *P. besseyi* and *T. capitatum.* The isolate *P. besseyi* showed very high ethanol tolerance and production capacity with some promising fermentation capacity towards different substrates compared to commercial yeast strain *S. cerevisiae.*

Keywords: Date palm sap, yeast isolation and characterization, ethanol tolerance, ethanol fermentation

INTRODUCTION

A worldwide interest in the utilization of bio-ethanol as an energy source from renewable resources has stimulated studies on efficient fermentation technology for ethanol production. The economics of ethanol production by fermentation is influenced by the cost of raw materials which accounts for more than half of the production costs (Robles *et al.*, 2003). For this reason, bioethanol production from renewable agricultural resources has attracted considerable attention in recent years. For an increased yield of ethanol production by fermentation, apart from fermentation substrates, an ideal microbial strain and suitable process technology are also required. Carbohydrate-rich raw materials suitable for bio-ethanol production can be classified into three groups of agricultural products: sugar (sugarcane and molasses), starch (cassava, cereals and potatoes) and lignocelluloses (rice straw, corn cob and sugarcane waste) (Mogg, 2004).

Bioethanol production from various carbohydrate rich materials employs suitable microorganisms capable of fermenting simple and complex carbohydrates into alcohol. According to Stewart *et al.* (1984), ideal microorganism used for ethanol production must have rapid fermentative potential, improved flocculating ability, appreciable osmotolerance, enhanced ethanol tolerance and good thermotolerance. Although no microbial strain has all these desirable qualities, few strains have been found to posses appreciable characteristics for ethanol production (Hacking *et al.*, 1984). Continuous search has been made to isolate and identify efficient microorganisms for bioethanol production from various carbohydrate rich substrates.

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Toddy is a fermented product produced from date palm (Khajur) sap. It is a whitish alcoholic beverage having high nutritional value with a characteristic flavour. Date palm sap is generally very common in tropical and sub-tropical climates of India which is highly susceptible to natural fermentation at ambient temperature. Mostly date palm sap is fermented by natural yeasts after storing in the earthen pots, which is a desirable condition for yeast growth converting it into toddy (Davis and Johnson, 1987). Toddy becomes potent and its alcoholic content increase as days goes by. It contains 4 to 8% of alcohol, pH of 5.8 and has a shelf life of about 24 h (Okafor, 1972). It was found that yeasts mainly the genus of *Saccharomyces* are normal flora in toddy. Other genera including *Kloeckera*, *Pichia*, *Candida* and *Endomycopsis* were also found in Toddy (Morton, 1988). Among the different microorganisms yeasts are the most essential microorganisms used in industrial fermentation for bio-ethanol production from various substrates.

The main characteristic of the yeasts is that they ferment simple carbohydrates anaerobic ally with the release of alcohol and carbon dioxide. In most of the studies *S. cerevisiae* is the preferred organism for industrial production of ethanol, because of their good fermentative capacity, high tolerance to ethanol and other inhibitors (either formed during raw materials pretreatments or produced during fermentation) and the capacity to grow rapidly under the anaerobic conditions that are characteristically established in large scale fermentation vessels (Swain *et al.*, 2007; Watanabe *et al.*, 2007; Agbogbo and Kelly, 2008). Apart from commercial strains, various yeast strains have been isolated and evaluated for their efficient ethanol production in laboratory scale. Identification of newly isolated strains is a prerequisite for any scientific study of yeasts and is traditionally made by means of phenotypic methods by assessment of morphological, physiological and biochemical characteristics for which considerable experience and skill are required for the performance. The objective of this study was to isolate and identify predominant yeast strains from toddy, fermented date palm sap and to assess their comparative ethanol tolerance and production capacity with a view to select out potential yeast strains for efficient industrial bioethanol production.

MATERIALS AND METHODS

Isolation of yeasts

Toddy samples were collected in sterile containers in the morning (10 A.M.) from Baranga, Bhubaneswar, 12 km away from the state capital of Odisha and transferred to laboratory immediately and was stored in refrigerator at 4 °C. The samples were serially diluted and poured on to the tryptone glucose yeast extract (TGY) agar medium in petriplates (100 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 15 g/L agar) (Ergul *et al.*, 2006). The inoculated petriplates were incubated at 28±2 °C for 48 h for growth of yeast. Pure culture of the individual yeast isolates were prepared in TGY slants and stored at 4 °C for further studies.

Characterization of yeast isolates

Morphological and biochemical studies were carried out for identification of isolated yeast strains. For morphological characterization, isolated yeast strains were initially cultured on TGY agar plates and then incubated at room temperature for 48 h. Following incubation, colony descriptions, e.g. colour, surface appearance, margin and elevation were determined for individual colonies. For cell morphology study isolated colonies of individual yeast isolates were stained with lactophenol cotton blue. The stained cells were then observed under the phase contrast microscope (Olympus CHi20, India) to determine the shape and sizes for morphological identification.

In addition to the morphological investigations, 25 biochemical tests were carried out. The tests include 0.1% (w/v) urea and starch hydrolysis, growth in the presence of 0.1% and 0.01% (w/v) cycloheximide and ability to ferment glucose, sucrose, maltose, lactose (Kurtzman and Fell, 2000). For evaluation of fermentation ability the yeast isolates (BS1-BS8) were inoculated separately in test tubes containing different carbon sources with 10 mL of phenol red broth medium and incubated at 30 °C for 48 h. After incubation the tubes were observed for colour change from red to yellow due to acid production. Assimilation of 0.1% (w/v) erythritol, melliobiose, mannitol, D-raffinose, D-cellobiose, ribose and 5% (v/v) ethanol, methanol and glycerol were also studied (Ergul *et al.*, 2006). Growth of different yeasts was also detected by growing in the TGY medium in different temperature and pH conditions. The pH adjustment was done with 1N NaOH and HCl. The isolated eight yeasts were identified on the basis of morphological and biochemical tests according to the simplified identification system and yeast identification key by Kurtzman and Fell (2000).

Ethanol tolerance test

The yeast isolates were tested for ethanol tolerance. The yeast strains were inoculated in 10 mL of TGY broth containing different concentration of ethanol (5, 7, 9, 10 and 12%). The tubes were incubated at 30 °C for 48 h. After incubation the viability of yeast cells were checked by serially diluted with sterile distilled water and plated on TGY agar medium.

Screening of yeast isolates for their ethanol production ability

The isolated yeast strains were screened for their ethanol production ability on different natural substrates like molasses, sugarcane juice, mahua flower juice, grape juice and saccharified sweet potato root flour broth.

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Preparation of fermentation media with different substrates

Grape juice

For preparation of grape juice medium, grapes (200 g) were crushed and the juice was extracted. Equal volume of water was added to the juice and boiled to half of its volume and then used for fermentation. The sugar content of the juice was adjusted to 14° brix.

Mahua juice

Mahua flower (200 g) was mixed with tap water in 1:6 ratio and boiled. Then it was cooled and grinded. Then the juice was filtered using a cheese cloth and sugar content was adjusted to 14° brix which then used for fermentation.

Molasses

Molasses (50 g) was mixed with tap water in 1:3 ratio and the sugar content was adjusted to 14° brix. Then the mixture was boiled and the foam was removed as it contains toxic contents.

Sugarcane juice

Sugarcane juice (50 mL) was added to 150 mL distilled water and the sugar content was adjusted to 14° brix and sterilized by slight warming to remove any contamination.

Sweet potato root flour (SPRF)

SPRF (10%) slurry was prepared in flasks by adding tap water in a ratio of 1:10. The slurry was dextrinized by addition of 32 µL Palkolase- ®HT at pH 5.5 and incubated at 90 °C for 1 h and then slurry was cooled down to room temperature. Then Palkolase-®HT (329.7 µL) was added to the dextrinized slurry at pH 4.5 and incubated for 24 h at 60 °C for saccharification. The saccharified SPRF (14° brix) was used for fermentation.

Ethanol production by fermentation

Ethanol fermentation of different substrates by the isolated yeast strains were conducted under anaerobic condition in Erlenmeyer flasks sealed with rubber stopper equipped with opening for CO_2 venting. Freshly harvested starter cultures at [10% v/v (3×10^9 CFU/mL)] of different yeast cells were inoculated aseptically to different substrates in the Erlenmeyer flasks. The fermentation medium containing flasks (n = 3) were incubated in an incubator-cum shaker at 30±2 °C for 48 h with a constant shaking at 100 rpm. The fermented broth was distilled to recover ethanol using alcohol distillation apparatus (Borosil Glass Works Ltd., Mumbai, India).

RESULTS AND DISCUSSIONS

Isolation of yeast sample

Indigenous yeast strains growing in toddy were isolated following serial dilution and plating in TGY agar. Eight morphologically distinct yeast colonies grown in TGY agar plate were isolate and were subjected to morphological and biochemical characterization. Subsequently, all the strains isolated were screened for their ethanol tolerance and production capacity to select potential strains suitable for efficient ethanol production.

Morphological characterization

Data on morphological characterization (microscopic and macroscopic) of eight yeast strains (BS1-BS8) isolated from toddy samples are presented in Table 1. The isolated yeasts showed great variation with regard to their shape, colour, margin and surface. Out of 8 isolated yeast strains two colonies of strains (BS2, BS3) were brown in colour while the rest 6 strains were white coloured. Colonies of all the strains had smooth margin except one strain (BS3) which had rough margin. Similarly except strain BS3, all other isolated strains had shiny surfaces. The strain BS3 had rough surface.

Photomicrograph study of yeast strains

Phase contrast photomicrographs of the eight isolated yeast strains are given in Figure 1(A-H). Four yeast strains (BS1, BS5, BS7 and BS8) were found to be oval in shape; whereas the other two strains (BS4 and BS6) were round in shape. The strain BS3 was found to be rod shaped in shape.

Table 1: Morphological characteristics of the eight strains isolated from toddy samples.

Yeast strains	Macroscopic characteristics	Microscopic characteristics
BS1	White, smooth and shiny surface	Oval
BS2	Brown, shiny surface	Rod
BS3	Brownish white, rough surface	Rounded and oval
BS4	White, shiny surface	Rounded
BS5	White, smooth and shiny surface	Oval
BS6	White, shiny surface	Rounded
BS7	White, smooth and shiny surface	Oval
BS8	White, smooth and shiny surface	Oval

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Biochemical characterizations

The isolated yeast strains were subjected to different by biochemical analysis such as urea hydrolysis, glucose fermentation, cycloheximide resistance, cellobiose assimilation, mannitol assimilation, erythritol assimilation, growth on different sugars etc. and the results are given in Table 2. All the isolated yeast strains are able to ferment glucose, sucrose, maltose but unable to ferment lactose. Three strains (BS1, BS3 and BS8) were found to hydrolyze urea (variable to weak), whereas all the eight strains could hydrolyze starch. No strain is able to grow on cycloheximide at both 0.01% and 0.1%. All the strains showed variable results with regard to assimilation of erythritol, melliobiose, mannitol, D-raffinose, D-cellobiose and ribose (Table 2). All the isolated strains showed utilization of carbon sources like glucose, sucrose, maltose and starch but had variable response (both positive and negative) on lactose utilization. The isolated strains grew easily on ethanol and glycerol but showed no growth on methanol. Similar results were reported by Walker *et al*. (2006) who reported that all the yeast isolates ferment at least one type of sugar. However, a majority of these isolates which ferment glucose, galactose, maltose, sucrose and raffinose, belonged to the genus *S. cerevisiae*.

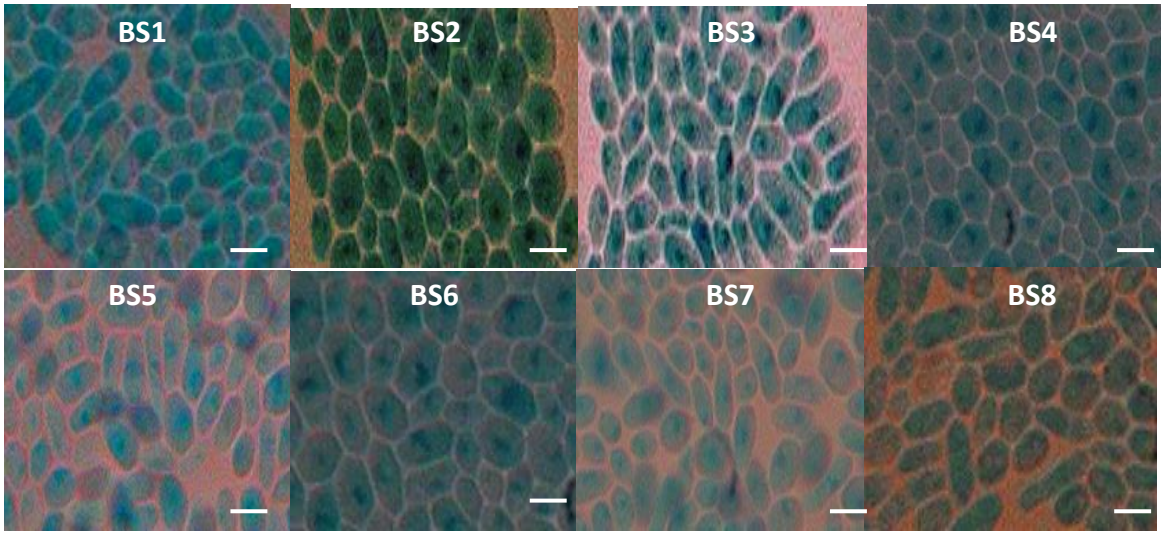

Figure 1: Photomicrographs (1000 × magnification) of isolated yeast strains. Scale bar (—) 5µm used for each photomicrograph

Growth in different temperature and pH condition

The isolated strains were inoculated in TGY broth medium and incubated at different pH (3-8) and temperature (20-40 °C) conditions**.** The all eight isolated strains in the present study were growing well at 30 °C while BS2 and BS8 strains were able to grow at a higher temperature of 35 °C. None of the yeast strain could able to tolerate temperature beyond 35 °C. This result agrees with previous reports that the yeast cell viability decreases with increase in temperature (Cassey 1996). It has been reported by D'Amore *et al.* (1989) that, at temperature 30-37 °C yeasts will grow and multiply faster but the growth of the yeasts were decreased as the temperature increased further. Moreover, it is observed that, at higher temperature (>50 °C) the yeast cells become stressed and die (Sathees *et al.*, 2011). All the strains showed positive growth at pH 4, 5 and 6, while no growth was seen at pH lower than 4 and higher than 6. It has been reported by Narendranath and Power (2005) that yeasts generally have an optimum pH between 4.0 and 6.0 although they can grow in a wide range of pH 2.5 to 8.5.

Identification of strains

From the morphological, microscopic and biochemical characterization, the isolated yeast strains are identified as four strains of *S. cerevisiae* (BS1, BS5, BS7 and BS8), two strains of *Candida albicans* (BS4 and BS6) and one strain each of *Pichia besseyi* (BS2) and *Trycosporon capitatum* (BS3). *Saccharomyces cerevisiae* is found to be the main fermenting organism in toddy, which has also been supported by the observations of by Sathees *et al.* (2011) from toddy. Martini (1996) reported that yeast *S. cerevisiae* with very high ethanol producing capabilities in the natural environment used for the conversion of hexoses such as glucose and mannose. *Pichia besseyi* (BS2) was found to ferment ribose sugar (pentose). There have been reports that the strains of *P. besseyi* could show utilization capacity of pentoses such as xylose, ribose and arabinose (Olsson and Hahn, 1993). Besides, the species of *Saccharomyces* and *Pichia* species, *Candida* and *Trycosporon* species were also isolated in the present study. Sheperd and Sullival (1975) also showed that *Candida* species were able to utilize various carbon

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sources for their growth in fermentation medium. Bullock (2002) stated that *S. cerevisiae* is the most widely used microorganism for ethanol fermentation due to its ability to hydrolyse sucrose into fermentable sugars. *P. stipilis*, *Candida shehatae* and *C. parapsilosis* are the natural xylose fermenting yeasts, can convert xylose to xylitol and xylitol to xylulose from lignocellulosic biomass (Bullock, 2002). *Saccharomyces cerevisiae* isolated from toddy showed maximum yield of ethanol (40 g/L) compared with baker's yeast *S. cerevisiae* in the optimum pH 3.0, temperature 30 °C and initial sugar concentration 20% (Fukuda *et al.*, 2009).

Table 2: Physiological and biochemical characterisation of yeast strains isolated from toddy.

	Yeast strains							
	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8
Biochemical tests								
D-Glucose fermentation (0.1 %)	+	+	+	+	+	+	+	+
Sucrose fermentation (0.1 %)	+	+	+	+	+	+	+	+
Maltose fermentation (0.1 %)	+	+	+	+	+	+	+	+
Lactose fermentation (0.1 %)	-	-	-	-	-	-	-	-
Erythritol assimilation (0.1 %)	V	V	V	V	W	W	W	V
Melliobiose assimilation (0.1 %)	+	+	-	-	-	-	W	W
Mannitol assimilation (0.1 %)	+	W	+	+	+	V	V	V
D-Raffinose assimilation (0.1 %)	+	+	V	V	V	-	W	W
D-Cellobiose assimilation (0.1 %)	+	+	V	-	-	-	-	W
Ribose assimilation (0.1 %)	-	+	-	-	-	W	-	W
Methanol assimilation (5 %)	-	-	-	-	-	-	-	-
Ethanol assimilation (5 %)	+	+	+	+	+	+	+	+
Glycerol assimilation (5 %)	+	+	+	+	+	+	+	+
Urea hydrolysis (0.1 %)	V	+	V	-	-	-	-	W
Starch hydrolysis (0.1 %)	+	+	+	+	+	+	+	+
Cycloheximide resistance (0.01 %)	-	-	-	-	-	-	-	-
Cycloheximide resistance (0.1 %)	-	-	-	-	-	-	-	-
Temperature (°C)								
20	W	W	W	-	-	-	-	-
25	W	W	W	-	V	-	-	W
30	+	+	+	+	+	+	+	+
35	-	+	-	-	-	-	-	V
pH								
3	-	-	-	-	-	-	-	-
4	V	V	V	V	V	V	V	V
5	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
7	-	-	-	-	-	-	-	-

V, Variable; W, Weakly Positive; +, Positive; - , Negative.

Ethanol tolerance of yeasts

The yeast strains were inoculated in TGY broth containing different concentrations of ethanol (5, 7, 9, 10 and 12%) and incubated at 30 °C for 48 h for evaluation of ethanol tolerance capacity. Ethanol tolerance capacity of yeast varies with strains. The results (Table 3) showed that, all the strains showed their growth at 5 and 7% of ethanol concentrations. The strains BS2 and BS7 showed growth at 10% ethanol concentrations. No isolated yeast strains were able to tolerate ethanol concentrations above 10%. Ethanol tolerance is one of the criteria for selection of strains for industrial ethanol production. From different findings it is reported that most of the ethanol producing yeast strains isolated were able to tolerate ethanol concentration from 10 to 12% (Cassey, 1996; Manikandan *et al.*, 2010). Yeast naturally growing in toddy showed higher ethanol tolerance up to 13% has been reported by Kumar *et al.* (2011). Cassey (1996) reported that the yeast strains survive in palm wines must have some degree of ethanol tolerance, which is important in choosing a yeast strain for industrial ethanol fermentation process. Use of efficient yeast strains with higher ethanol tolerance to improve ethanol yields in the fermentation product (cane molasses) would reduce distillation costs and hence the profitability of the overall process (Chandrasena *et al.*, 2006).

Screening of yeast isolates on their fermentation ability

Fermenting capacity of different yeast strains varies greatly with different substrates. The fermentation capacity of the eight isolated yeast strains along with a commercial

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Table 3: Ethanol tolerance of yeast strains isolated from toddy.

Ethanol concentration (%)	Ethanol tolerance							
	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8
5	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+
9	+	+	V	W	+	W	W	W
10	W	+	-	-	V	-	+	-
12	-	-	-	-	-	-	-	-

V, Variable; W, Weakly Positive; +, Positive; - , Negative.

yeast strain (*S. cerevisiae* strain, CTCRI) were evaluated inoculating the strains on different sugary and starchy substrates (grape juice, mahua flower extract, molasses broth, sugar cane juice and saccharified SPRF broth) and distilling ethanol as fermented product after 48 h of incubation. The results are given in Table 4 and Figure 2. The isolate BS2 (*P. besseyi*) showed maximum ethanol production from substrate SPRF in comparison to other strains. BS2 strain produced 208 mL/kg of ethanol which is 6.5% more than that of the commercial *S. cerevisiae* strain (CTCRI) in the same incubation period. BS5 and BS7 strains produced maximum ethanol of 66 mL/kg from sugar cane juice. BS1 and BS7 strains produced maximum ethanol from grape juice and sugarcane juice. BS1, BS2 and BS8 strains showed maximum ethanol production from mahua flower extract. For evaluation of ethanol production capacity the strains were scored on order of their ethanol production capacity (1-9) in a particular substrate. Based on score ranking taking in account all the substrate conditions and strains (Table 4), the strain BS2 (*P. besseyi*) was found to be the efficient strain in terms of ethanol production followed by BS5, BS7 and commercial *S. cerevisiae* strains.

Table 4: Scoring* of ethanol production capacity of isolated yeast isolates.

Yeast strains	Grape juice	Mahua flower extract	Molasses broth	Sugarcane juice	Saccharified SPRF broth	Total score	Rank
S. cerevisiae CTCRI strain	8	4	4	1	8	33	3
S. cerevisiae BS1	6	6	8	5	5	30	4
P. besseyi BS2	9	7	9	9	9	43	1
T. capitatum BS3	3	3	3	2	1	12	8
C. albicans BS4	2	1	1	7	2	13	7
S. cerevisiae BS5	7	8	5	8	3	34	2
C. albicans BS6	1	2	2	3	4	12	9
S. cerevisiae BS7	5	5	7	6	7	30	5
S. cerevisiae BS8	4	9	6	4	6	29	6

*Scoring 1-9 for 9 strains with 9 as maximum scoring for highest ethanol production which decreases in order of decreasing ethanol production within a particular substrate condition.

As reported by Kularatnam *et al.* (1971) the members of the genera *Saccharomyces*, *Kloeckera*, *Pichia*, *Candida*, *Trycosporon* and *Endomycopsis* have a great role in industrial bioethanol production. Wilkins et al., (2007) reported that the two ethanologenic yeast strain *S. cerevisiae* and *Kluyveromyces marxianus,* were used to ferment hydrolyzed sugars extracted from orange peel waste and *S. cerevisiae* produced more ethanol than *K. marxianus* at 72 h of incubation period. Ferrai *et al*. (1992) reported that production of maximum ethanol concentration 12.6 g/L at a fermentation time of 72 h from eucalyptus wood hemicellulose by *Pichia stipitis.* In the present study, maximum ethanol production (208 mL/kg) shown by BS2 is higher than other strains reported above and also the commercial strain used. This yeast strain is a pentose utilizing strain which is also found to efficiently utilize the hexose sugar. Therefore, this strain could have great scope for fermentation of agro-based substrates including both hexose and pentose sugars. However, a detail study in this regard is required for its industrial applications. Apart from the *P. besseyi,* high ethanol tolerant and ethanol producing *S. cerevisiae* isolated in the present study could also be exploited for commercial ethanol production with proper biotechnological evaluation.

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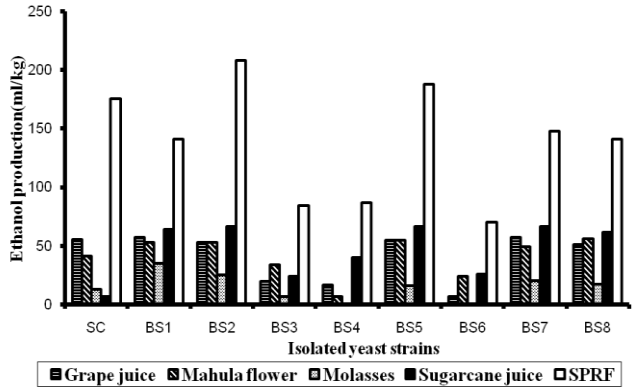

Figure 2: Ethanol production of yeast isolates on different substrate.

CONCLUSION

In conclusion, the present research work characterizes eight yeast strains isolated from fermented date palm sap (toddy) by conventional morphological and biochemical methods and identified as four species of *Saccharomyces cerevisiae*, two strains of *Candida albicans* and one strain each of *Pichia besseyi* and *Trycosporon capitatum.* The isolate *P. besseyi* showed very high ethanol tolerance and production capacity along with promising fermentation ability towards different substrates compared to commercial yeast strain *S. cerevisiae.* Thus *P. besseyi* could serve as a potential strain for ethanol fermentation from different substrates. Among the other strains, the isolated *S. cerevisiae* is found to be a suitable where as *Trycosporon capitatum* and *Candida albicans* are less suitable for ethanol production compared to the commercial *S. cerevisiae* strain.

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