ISSN Online: 2349-7785

Isolation and Evaluation of Comparative Ethanol Production Capacity of Yeasts in Presence of Different Carbohydrate Sources from Soil

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Received 12 November 2017; accepted in revised form 21 December 2017

Abstract: Soil, is an enriched source of natural fermenting microorganisms including yeasts, which are capable of producing bioethanol. The present study was carried out to isolate and identify yeast strains from soil and screentheir ethanol tolerance capacity with a viewto select efficient yeast strains for industrial bioethanol production. Twelve yeast strains (YS1-YS12) were isolated from soil. On the basis of morpho-physiological characteristics and biochemical characterization, these yeast strains were identified as six strains of *S.cerevisiae* (YS1, YS2, YS4, YS5, YS7 and Y10), three strains of *Candidaalbicans* (YS3, YS8 and YS9) and three strains each of *Trycosporoncapitatum* (YS6, YS11 and YS12). Screening of ethanol tolerance capacity of these strains revealed that the two strains YS2 andYS3 tolerated ethanol concentration up to 12 % (v/v), while other two isolated strains YS1 and YS5 showed tolerance up to 10 % of ethanol concentration. The remaining eight strains showed tolerance of 9 % (v/v). Further these identified strains were evaluated for their 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 YS2 showed in overall the highest ethanol production and ethanol tolerance capacity in comparison to the other strains indicating as an efficient strainfor its use in ethanol production purpose.

Key Words: Yeast identification, ethanol production, ethanol tolerance.

Introduction

In recent years, due to rising prices of petroleum products has led to search for an alternate of petroleum products i.e. bioethanol from plant biomass [9, 12]. Bioethanol is ecofriendly to environment with less emission of CO_2 [17]. Bioethanol is produced by microbiological fermentation of carbohydrate rich compounds. Although many microbes have been used in ethanol production, the yeast and bacteria are primarily used in industry, to convert starch and sugars from plants biomasses to produce bioethanol [1]. Efficient ethanol production requires the use of competent microorganisms that are able to ferment a variety of sugars (pentoses and hexoses). The industrial ethanol production process, depends upon many important factors such as ethanol or sugar tolerance of microorganisms, fermentation ability at higher temperatures (thermotolerance) and enzymatic activities for transformations [15,16].

Yeast is widely distributed in nature with a variety of habitats and is commonly found onplant leaves, flowers and fruits, as well as in soil [19]. However, there is few strains which have better

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fermentative characteristic, therefore there is always a need with better features in bioethanol fermentation especially high ethanol tolerance and production on commercial scale. Yeastsbelong to the groups Ascomycetes and Basidiomycetescomprising the genera Saccharomyces, Candida, Pichia, Clavispora, Issatchenkia, Kluyveromyces, Kloeckera, Torulaspora, Geotrichum, Cryptococcus etc., have been used in ethanol production from different carbohydrate rich sources during the last three decades [20]. However,*S. cerevisiae*is the most preferred organism for industrial bioethanol production, due to its excellent fermentative capacity, high ethanol tolerance activity and good growing capacity under the anaerobic conditions [18]. The researches have been carried out to isolate and evaluate various yeast strains for enhanced bioethanol production in laboratory scale. In this regard, morphological, physiological and biochemical identification of newly isolated yeast strains is a prerequisite step for any scientific research activity.

Therefore in the present study attempt has been made to isolate, identify and screen out the ethanol production capacity of yeast species isolated from different soils with a view toenhance the for their commercial application in bioethanol industries.

Materials and methods

Isolation of yeasts

Seven types of soil samples were collected in sterile polybags from different toddy waste siteof Bhubaneswar, Khurdha, Odisha and transferred to laboratory immediately and was stored in refrigerator at 4°C. The soil samples were serially diluted and inoculated to petriplates containing tryptone glucose yeast extract (TGY) agar medium (100 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 15 g/L agar) [7]. The inoculated petriplates were incubated at 28±2°C for 48 h for proper growth of yeast. Further the indivisual isolated colonies of yeast were picked up and pure cultured TGY slants.The strains were stored at 4°C for further studies.

Morphological and biochemical characterization of yeast isolates

For morphological characterization, isolated yeast strains were inoculated on TGY agar plates and then incubated at 28±2°C for 48 h. Following incubation, colony morphology such ascolour, surface appearance, margin and elevation were studied for individual colonies. For morphological study pure culture from individual yeast isolates were stained with lactophenol cotton blue and observed under the phase contrast microscope (Olympus CHi20, India) to determine the shape and sizes etc.

For biochemical characterisation, 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 [11]. The fermentation ability the yeast isolates (BS1-BS8) were evaluated by inoculating the yeasts separately in test tubes containing different carbon sources with 10 ml of phenol red broth medium. After incubation 48 h at 30°C 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 by the yeast isolates were also studied as per the procedure of Ergul *et al.*[10].

Growth characteristics of different yeasts was also studied by growing the yeasts in the TGY medium at different temperature and pH conditions. The pH adjustment was done with 1 N NaOH and HCl. All the twelve isolated yeasts were identified based on the results of morphological and biochemical characterization according to the simplified identification system and yeast identification key proposed by Kurtzman and Fell [11].

Ethanol tolerance test

For evaluation of ethanol tolerance, the yeast strains were inoculated in 10 mL of TGY broth containing different concentration of ethanol (5, 7, 9, 10 and 12 %) and the tubes were incubated at 30°C for 48 h. After incubation, the viability of the yeast cells were checked by serially diluting the culture with sterile distilled water and plating on TGY agar medium. The survival population of the yeast was enumerated after incubation for 48 h.

Screening of yeast isolates for their ethanol production ability

For screening of ethanol production ability of isolated yeast strains, fermentation studies were undertaken in different natural substrates like molasses, sugarcane juice, mahua flower juice, grape juice and saccharified sweet potato root flour broth, as per the details given below.

Preparation of fermentation media with different substrates

Grape juice

Grape juice medium was prepared by taking 200 gm grapes, which were crushed, and the juice was extracted. Equal volume of water was added to the extracted juice and boiled to half of its volume. The broth so obtained was used for fermentation. The sugar content of the juice was adjusted to 14° brix.

Mahua juice

Dry mahua flowers (200 g) was mixed with tap water in 1:6 ratio and boiled. Then the content was cooled and grinded. Then the juice was extracted by filtering the contents using a cheese cloth. 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. The foams so generated during boilingwas removed as it contains toxic contents.

Sugarcane juice

Sugarcane juice (50 mL) was added to 150 mL distilled water. The sugar content of the juice was adjusted to14° 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 the α-amylase enzyme Palkolase- ®HT of 32 µL at pH 5.5 incubated at 90°C for 1 h following the standardized protocol of the laboratory. Then the slurry was cooled down to room temperature and a glucoenzyme, 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 carried out under anaerobic condition in Erlenmeyer flasks sealed with rubber stopper equipped with an opening for CO_2 venting. For this purpose 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 in triplicatewere incubated in an incubator-cum shaker at 30±2°C for 48 h with a constant shaking at 100 rpm. After completion of fermentation, the fermented broth was distilled to recover ethanol using alcohol distillation apparatus (Borosil Glass Works Ltd., Mumbai, India).

Results and discussion

Twelve morphologically distinct yeasts were isolated from soil samples inoculated in TGY agar plates following serial dilution. Then the strains were subjected to morphological and biochemical characterizationfor their identification. Then these strains were for their ethanol tolerance and production ability in different fermentation medium to select their potentiality for enhanced bioethanol production.

Both microscopic and macroscopic results of twelve isolated yeast strains (YS1-YS12) are presented in Table 1. The results showed a great variation with regard to their shape, colour, margin and surface. Among the strains studied, five yeast strains were white in colour, four strains were brownish white in colour and the other two strains were brown coloured. Among the colonies, some of the strains had smooth margin whereas others had rough margins.

Phase contrast photomicrographs of the eight isolated yeast strains are given in Figure 1. Seven yeast isolates (YS1, YS2, YS3, YS7, YS10 and YS12) were found to be oval in shape; whereas the other four strains (YS4, YS5, YS8 and YS12)

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

Yeast strains	Macroscopic characteristics	Microscopic characteristics
YS1	White, smooth and shiny surface	Oval
YS2	Brown, shiny surface	Rod
YS3	Brownish white, rough surface	Rounded and oval
YS4	Brown, shiny surface	Rounded
YS5	White, smooth and shiny surface	Oval
YS6	White, shiny surface	Rounded
YS7	White, smooth and shiny surface	Oval
YS8	White, smooth and shiny surface	Oval
YS9	Brownish white, rough surface	Oval
YS10	Brown, shiny surface	Rounded
YS11	Brownish white, rough surface	Rounded and oval
YS12	Brownish white, rough surface	Rounded and oval

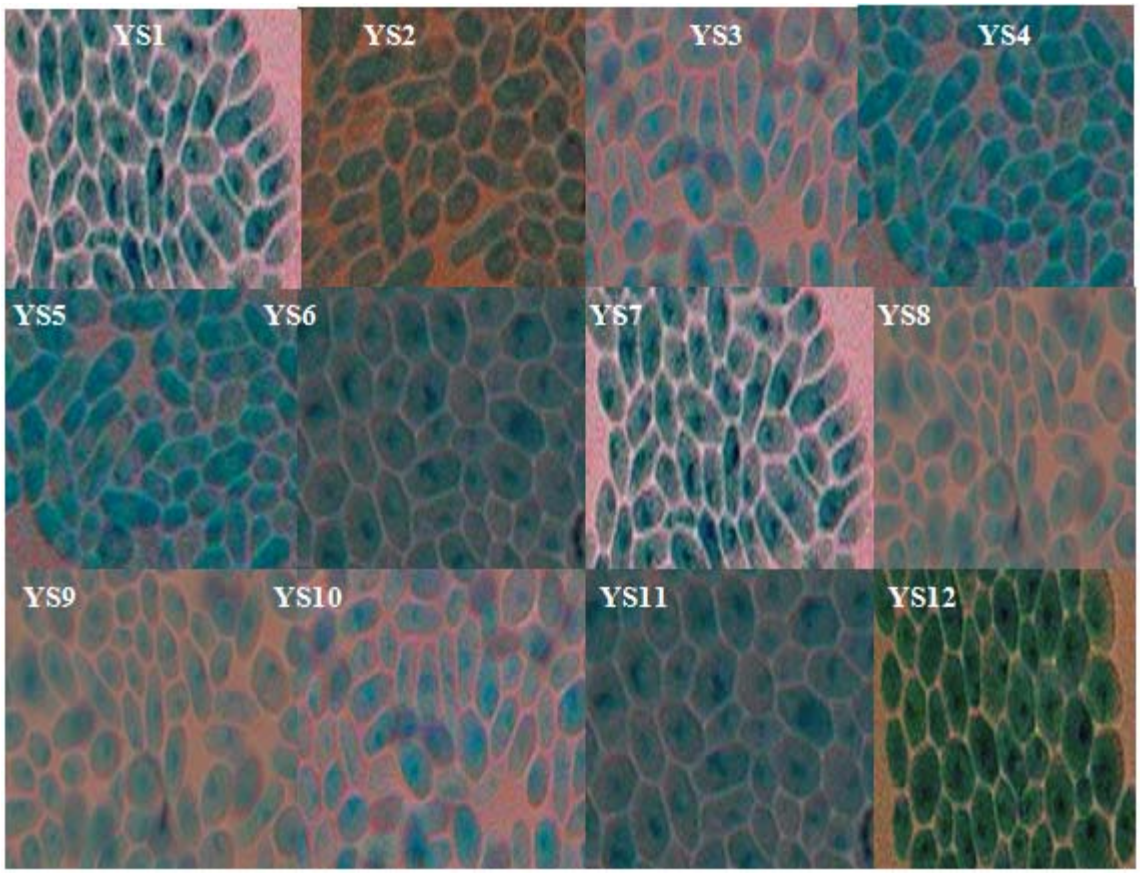

Figure 1. Photomicrographs (1000 X magnification) of yeast strains isolated from soil samples

were rod in shape. The strain YS6 and YS11 were found to be round in shape. 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. Three strains (YS1, YS3 and YS8) were found to hydrolyze urea (variable to weak), whereas all the twelve strains could hydrolyze starch. No strains were able to grow on cycloheximide. The isolated strains grew easily on etha-

Table 2. Physiological and biochemical characterization of yeast strains isolated from soil

Yeast strains Biochemical tests	YS 1	YS 2	YS 3	YS 4	YS 5	YS 6	YS 7	YS 8	YS 9	YS 10	YS 11	YS 12
Glucose fermentation (0.1 %)	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose fermentation (0.1 %)	+	+	+	+	+	+	+	+	+	+	+	+
Dextrose fermentation (0.1 %)	+	+	+	+	+	+	+	+	+	+	+	+
Maltose fermentation (0.1 %)	+	+	+	+	+	+	+	+	+	+	+	+
Erythritol assimilation (0.1 %)	+	+	+	V	+	+	+	+	+	V	+	V
Lactose fermentation (0.1 %)	+	+	+	V	W	+	-	-	W	V	V	V
Melliobiose assimilation (0.1 %)	+	+	+	-	+	+	+	+	+	-	+	+
Mannitol assimilation (0.1 %)	+	+	+	-	+	+	+	+	+	-	+	+
D-Raffinose assimilation (0.1 %)	+	+	+	V	+	+	+	+	+	V	+	V
D-Cellobiose assimilation (0.1 %)	+	+	+	+	+	+	+	+	+	V	+	V
Ribose assimilation (0.1 %)	+	+	+	+	+	+	+	+	+	+	+	+
Xylose assimilation (0.1 %)	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose assimilation (0.1 %)	-	-	-	-	-	-	-	-	-	-	-	-
Methanol assimilation (5 %)	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol assimilation (5 %)	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol assimilation (5 %)	+	+	+	+	+	+	+	+	+	+	+	+
Urea hydrolysis (5 %)	-	+	+	-	-	+	+	-	+	-	-	-
Starch hydrolysis (0.1 %)	+	+	+	+	+	+	+	+	+	+	+	+
Cycloheximide resistance (0.01 %)	-	-	-	-	-	+	+	-	-	-	-	-
Cycloheximide resistance (0.1 %)	-	-	-	-	-	+	+	-	-	-	-	-
Temperature (° C)												
20	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+
35	+	+	+	-	+	+	+	+	+	-	+	+
40	-	-	+	V	+	+	+	+	+	V	V	+
45	-	-	-	-	-	-	-	-	-	-	-	-
pH												
3	-	-	-	-	-	-	-	-	-	-	-	-
4	V	V	V	V	V	V	V	V	V	V	V	V
5	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+
7	-	-	-	-	-	+	+	-	-	-	-	-

nol and glycerol but showed no growth on methanol. In a study carried out by Dash *et al* .[6], it has been reported that the isolated yeast strains can ferment all types of sugars expect lactose. This supports to the studies by Walker *et al.*[21] 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*. The isolated strains were inoculated in TGY broth medium and incubated at different pH (3-8) and temperature (20-40°C) conditions. All the strains isolated from soils samples were well grown at temperature 35°C.

None of the yeast strain could able to tolerate temperature beyond 35°C. Tsegye.[20] studied growth of eight isolated yeast strains at different temperatures ranging from 26-45°C and the results showed that all the strains could growupto temperature 37°C.

On the basis of morpho-physiological characteristics and biochemical characterization, the yeast strains isolated from soil samples were identified as six strains of *S. cerevisiae* (YS1, YS2, YS4, YS5, YS7 and Y10), three strains of *Candida albicans* (YS3, YS8 and YS9) and three strains each of *Trycosporon capitatum* (YS6, YS11 and YS12).In another study Dash *et al.*[6] has reported isolation of eight distinct yeast strains from fermented beverage samples and identified as four strains of *Saccharomyces cerevisiae*, one strain each of *Pichia besseyi* and *Trycosporon capitatum* and two strains of *Candida albicans* on the basis of microscopic and macroscopic observation. Bullock.[2] stated that *S. cerevisiae* is the most widely used microorganism for ethanol fermentation due to its ability to hydrolyse sucrose into fermentable sugars.

Ethanol tolerance is the most important criteria for selection of yeast strains for their use in industrial ethanol production. Hence, for the purpose of evaluation of ethanol tolerance capacity, all theisolated 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. The results (Table 3) showed that, all the strains were able to grow at 5 and 7 % of ethanol concentrations. The strains YS2 and YS11 showed growth at 10 % ethanol concentrations. Haggran and Nivien.[8] isolated two yeast isolates from soil samples and concluded that two strains were able to grow and tolerate up to 12 % ethanol. Miguel *et al.*[14] screened several yeast strains for ethanol tolerance, among them the *Candida parapsilosis* could only grow at 4 % ethanol. Different researchers reported that most of the ethanol producing yeast strains isolated were able to tolerate ethanol concentration between 10 to 12 %[3, 13]. Naturally growing yeast strains showed higher ethanol tolerance up to 13 % has been reported by Kumar *et al.*[10]. Dash *et al.*[6] studied on ethanol tolerance capacity of isolated yeast strains from toddy and found that the yeast strains could tolerate upto 10 % of ethanol. 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 [4].

Ethanol production capacity of isolated yeast strains wasscreened in different sugary and starchy substrates (grape juice, mahua flower extract, molasses broth, sugar cane juice and saccharified SPRF broth). The results showed a great variation in ethanol production with different substrates taken after fermentation (Table 4 and Figure 2). The isolate strain YS2 showed maximum ethanol production from in all substrates except molasses and sugarcane juice followed by YS7, which shared maximum ethanol production in molasses broth. The YS6 strain also good ethanol production in all the substrates after the strains YS2 and YS7. Choi *et al.*[5] isolated two ethanol producing yeast strains from soil sampls and studied their ethanol production capacity on cassava starch. Dash *et al.* [6] also isolated and screened fermentation ability of eight yeast strains on different sugary and starchy substrates for further use in industrial scale.

Table 3. Ethanol tolerance of yeast strains isolated from soil

Ethanol concentration (%)	Ethanol tolerance											
	YS1	**YS2**	**YS3**	**YS4**	**YS5**	**YS6**	**YS7**	**YS8**	**YS9**	**YS10**	**YS11**	**YS12**
5	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	V	W	+	W	+	W	+	V	+	+
10	W	+	-	-	V	-	+	-	-	-	+	-
12	-	+	+	-	+	-	-	-	-	-	-	-

Table 4. Ethanol production of twelve yeast strains isolated from soil samples on different substrates

Yeast strains	Ethanol production (g/kg substrate)				
	Grape juice	Mahua flower extract	Molasses broth	Sugarcane juice	Saccharified SPRF broth
YS1	248.9±0.03^{cd}	298±0.03^{bc}	235.5 ±0.03^{cd}	127.7±0.01^{ef}	21.9±1.30^{fg}
YS2	317.7±0.01^{bc}	378.8±0.08^{a}	244.9±0.02^{cd}	204.4±0.05^{cd}	55.5±1.30^{f}
YS3	212.2±0.07^{cd}	280.7±0.04^{bc}	222.7±0.06^{cd}	98.9±0.02^{ef}	27.6±0.51^{fg}
YS4	204.4±0.05^{cd}	262.9±0.03^{cd}	198.9±0.02^{de}	104.4±0.10^{ef}	44.4±0.94^{fg}
YS5	277.7±0.04^{bc}	274.9±0.04^{c}	279.2± 0.01^{bc}	157.2±0.07^{de}	42.7±0.29^{fg}
YS6	299.9±0.13^{bc}	370±0.08^{ab}	289.9±0.02^{bc}	192.5±0.02^{de}	33.9±0.05^{fg}
YS7	307.7±0.08^{bc}	362.8±0.10^{ab}	322.9±0.03^{b}	217.4±0.03^{cd}	45.7±0.51^{fg}
YS8	211.2±0.015^{cd}	280.4±0.05^{bc}	235.7±0.03^{cd}	111.2±0.10^{ef}	29.4±1.30^{fg}
YS9	235.5±0.01^{cd}	274.9±0.08^{c}	244.7±0.02^{cd}	135.5±0.02^{e}	19.7±0.02^{g}
YS10	212.2±0.03^{cd}	204.5±0.03^{cd}	202.2±0.02^{d}	112.2±0.02^{ef}	34.4±0.03^{fg}
YS11	228.9±0.05^{cd}	268.8±0.08^{cd}	227.7±0.01^{cd}	128.9±0.03^{ef}	22.7±0.02^{fg}
YS12	194.9± 0.01^{de}	174.3±0.03^{de}	204.6±0.01^{cd}	94.9±0.03^{ef}	24.9±0.03^{fg}

The data in the table represents the mean±SE of replicates (n=6) values in the table carrying different letters `are significantly different at Pd ≤ 0.05

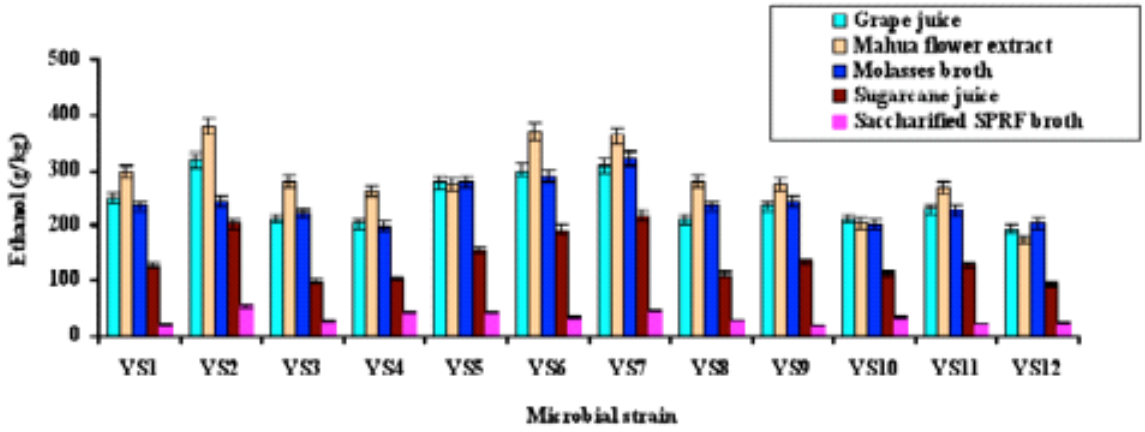

Figure 2. Ethanol production of twelve yeast strains isolated from soil samples on different substrates

It may be noted that the strain YS2 and YS7 has shown high ethanol production and tolerance capacity among the others. Thus these two strains can be considered as promising ethanol producing strains isolated from soil samples.

Conclusion

The results from the present research work concluded that the twelve isolated yeast strains isolated from soil samples are identified as six strains of *S. cerevisiae*, three strains of *Candida albicans* and three strains each of *Trycosporon capitatum*. Among the isolated yeast strains, YS2 showedgood ethanol tolerance and production capacity in most of the substrates studied. Thus the strainscould 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 in industrial application. In conclusion the YS2 strain isolated from soil sample

showed best fermentation ability in different sugary and starchy substrates and can be further utilized as an efficient microbial strain in industrial application.

Acknowledgement

We thank the Principal, College of Engineering and Technology for providing necessary facilities for this research work. We are thankful to Dr. P.N. Chowdhary, National Centre of Fungal Taxonomy, New Delhi for identifying the yeast strains. The authors are grateful to the Dr. C. K. Pradhan, Head, P. G. Department of Botany, Utkal University, VaniVihar for kind help and consideration.

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