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SCREENING AND CHARACTERIZATION OF STRESS TOLERANT *SACCHAROMYCES CEREVISIAE* ISOLATED FROM BREWERY EFFLUENTS FOR ANIMAL PROBIOTIC APPLICATIONS

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ABSTRACT

Based on the colony morphology and microscopic characteristics, 26 yeasts were isolated from different sources including brewery effluents. Initially they were screened for their thermotolerance at 40 ^{0}C and only 5 strains were selected. They were later grown in yeast extract peptone dextrose medium to screen their stress tolerance at five different temperatures; at different concentrations of a mixture of acetic, propionic and butyric acids; at different pH; at different concentrations of glucose and bile salts. Based on the growth at different stress conditions, yeast OBV9 was selected and characterized as Saccharomyces cerevisiae by sequencing its 5.8S rRNA gene and internal transcribed spacer (ITS) 1 and 2. The sequence obtained was most similar (99%) to S. cerevisiae, when it was blast searched in NCBI database and showed a separate branch in phylogenetic analysis.

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Keywords: ***Probiotics yeast; characterization; stresstolerance; animals***

[I] INTRODUCTION

Yeasts may be defined as microorganisms in which the unicellular form is conspicuous and which belong to fungi. No other group of microorganisms has been more intimately associated with the progress and well being of the human than yeasts [26]. The most commonly used probiotic yeast in animals is *Saccharomyces cerevisiae*. Yeasts of the genus *Saccharomyces* are usually revealed in sugar-rich natural substrates such as fruits and exudates of trees [20]. They have seldom been isolated from other habitats such as soils, plant leaves, and decaying plant debris [10]. Various yeast species found on grapes and on winery surfaces participate in spontaneous fermentations [25].

Yeast culture "*Saccharomyces cerevisiae*" is used in monogastrics and ruminants to stimulate and stabilize the processes that occur in the gastro-intestinal tract and help to increase competitive exclusion of undesired organisms in the digestive tract thereby enhancing the performance of livestock [26]. The effects of yeast culture on animal productivity are strain-dependant. So, all yeast culture preparations are not equivalent in efficiency. This aspect opens a new field of research for new strains, each being more specialized in its use [6]. Many studies did not find any difference in supplementing yeast to ruminants for increased milk production [27] and dry matter intake [15]. Some of the reasons might be high temperature i.e. 39 ^{0}C [12], bile salt concentration, osmotic pressure and organic acids in the rumen [11, 16]. Normally, yeast *Saccharomyces cerevisiae* grows at mesophilic range of temperature (30^{0}C) and pH. 4.5 - 5.5 [17]. Since the rumen environment is so diversified, some strains of this yeast may or may not tolerate and survive. Considering the above parameters, it is of importance to develop thermo, acid, bile and osmo tolerant yeast as probiotic. The present investigation was carried out to isolate, screen and characterize a new potential strain of yeast with the ability to tolerate diverse conditions of rumen.

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[II] MATERIALS AND METHODS

2.1. Isolation of yeast

Sources selected for isolation of stress tolerant yeast were toddy [18] collected in hot summer, sugarcane juice [24], sugarcane bagasse [3], bakery wastes, molasses[5,19,2], nectar[4,13,20], soil samples from thermal power plant [14], fruits such as bananas, grapes, cherries [7,23] and brewery effluents. These sources are popularly known as good supporters and have intrinsic nutritional value for yeast growth. The source samples were serially diluted to ten fold in sterile distilled water and inoculated on yeast extract peptone dextrose agar (YEPDA, Yeast extract 1%, Peptone 1%, Dextrose 2% and Agar agar 2%) medium plates by spread plate method. These plates were incubated at 32 ^{0}C in incubator for 48 hours. After incubation developed colonies were observed for their morphology and microscopic characteristic. Isolated yeasts were preserved in YEPDA slants.

2.2. Preparation of inoculum

Equal amount of each of the yeast cultures from slants were inoculated into 250 ml conical flasks, each containing 100 ml YEPD broth medium and incubated at 32 ^{0}C and 150 rpm agitation in the shaker incubator for overnight. After incubation, culture medium was centrifuged at 5,000 rpm for 5 minutes. Pellet was washed with sterile distilled water twice and re-suspended in equal amount of sterile distilled water. This was freshly prepared each time and 1 per cent v/v was used as inoculum. After 48 hours incubation, culture broth was diluted several times to obtain proper optical density (OD<1.0). The OD of culture medium was measured at 660 nm in UV-Visible spectrophotometer (Pathlength-1.0 cm, Systronics 117) and it is represented by multiplying it with number of dilutions.

2.3. Screening for stress (thermo, acid, osmo and bile) tolerance

To detect the thermotolerance of isolated yeasts, each of the yeasts (total 26 yeasts) were inoculated onto the yeast extract peptone dextrose (YEPD) agar medium plates and incubated at 40 ^{0}C to test their thermotolerance. Based on the growth on the medium plates, yeast isolates were selected for further studies. To detect further temperature tolerance and growth at different temperatures, 5 selected yeasts were inoculated into 100 ml sterile YEPD broth medium flasks, incubated for 48 hours at 150 rpm and at 5 different temperatures 30, 35, 40, 42 and 44 ^{0}C in shaker incubator. After incubation, optical density (OD) was measured at 660 nm.

Simultaneously, to detect the acid tolerance, the yeast strains were inoculated into sterile YEPD broth medium with different concentrations of a mixture of organic acids [1] (acetate, propionate and butyrate, 70:20:10), 0.0, 0.25, 0.5 and 1.0 per cent, respectively. Flasks were incubated for 48 hours at 32 ^{0}C and 150 rpm in the shaker incubator. This was followed by measurement of OD of the culture at 660 nm. Similarly flasks, each containing 100 ml sterile medium with different pH values 2, 3, 4, 5, 6 and 7 were also inoculated with the same strains and incubated for 48 hours at 32 ^{0}C and 150 rpm agitation in the shaker incubator and OD_{660} was measured.

The selected yeast strains were inoculated into sterile YEPD broth medium with 6 different concentrations of glucose 5, 10, 15, 20, 25 and 30 per cent, respectively. Flasks were incubated for 48 hours at 32 ^{0}C and 150 rpm agitation in the shaker incubator. After incubation, OD_{660} was measured.

Each of the selected yeast strains were inoculated into sterile YEPD broth medium with five different concentrations 0.2, 0.4, 0.6, 0.8 and 1.0 per cent of bile salts (ox bile from Qualigens) [1]. Flasks were incubated for 48 hours at 32 ^{0}C and 150 rpm agitation in the shaker incubator. After incubation, OD_{660} was measured.

2.4. Characterization of yeast by rRNA gene sequencing

Selected stress tolerant yeast OBV9 was characterized by sequencing its 5.8S rRNA gene and internal transcribed spacer (ITS) 1 and 2 [21, 23] by *Macrogen Inc. Ltd. (www.macrogen.com)* using an automated DNA sequencer (model 3730XL12-20140-014). DNA extracted from the sample was further run in the gel electrophoresis and PCR was performed to amplify the 5.8S rRNA and internal transcribed spacer (ITS) 1 and 2 with forward and reverse universal primer pairs targeted to the 5.8S rRNA gene. The primers used in this study are universal primers ITS1 and ITS4 based on the conserved regions of 5.8S rRNA gene and ITS1 and ITS2 which is designed to detect wide range of yeast strains. Sequences of forward and reverse primers are 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3', respectively.

2.5. Analysis of sequence data and Nucleotide sequence accession number

Complete Sequence of 5.8S rRNA gene and partial sequences of internal transcribed spacer (ITS) 1 and 2 were blast searched in NCBI database (www.ncbi.nlm.nih.gov) for matching. Based on the BLAST results, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [30]. Species relationships were also investigated by using unweighted pair group with mathematical average (UPGMA) cluster analysis [28]. The 659 base pair length gene sequence which we determined in this study has been deposited in the NCBI-GENEBANK data library and acquired the accession number (GU229793).

2.6. Scanning electron microscopy (SEM) of S. cerevisiae OBV9

For SEM analysis, *S. cerevisiae* OBV9 cells were rinsed twice with sterile double-distilled water, and distributed on a 12 mm glass cover slip coated with poly-L-lysine (Sigma Diagnostics) then fixed for 50 min by incubating in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The glass cover slip was washed twice in 0.1 M Sodium cacodylate buffer. To improve the surface architecture, fixed cells were rinsed thoroughly using 0.1 M cacodylate buffer, and treated with 6% thiocarbohydrazide. The glass cover slip was finally washed with double-distilled water and dehydrated through a graded series of ethanol solutions. It was then dried and sputter-coated (JEOL JFC-1600) with a gold layer and used for scanning.

2.7. Statistical analyses

The data obtained was analyzed statistically [29] and tested for significance by Duncan's multiple range test [8] was accepted at the level of ($P<0.01$).

[III] RESULTS

3.1. Isolation of yeast

Twenty six yeast strains were isolated from different sources (toddy, sugarcane bagasse, bakery oven wastes, molasses, soil samples from thermal power plant, nectar and samples of brewery effluents) based on their colony morphology and

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microscopic observation. They were designated as OBV1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 and 26. OBV1-6 were isolated from toddy, 7-9 from brewery effluents, 10-13 from bakery oven wastes, 14-19 from sugarcane bagasse, 20-22 from molasses, 23-24 from nectar and 25-26 from soil samples (thermal power plant). The morphological features of yeast colony grown on YEPD agar for 48 hours were off white in colour, circular in shape, convex elevated with opaque opacity and smooth texture. Yeast cells were spherical and egg shaped with budding, when observed under microscope. The scanning electron microscopic picture of *Saccharomyces cerevisiae* OBV9 is shown in **Figure–1**.

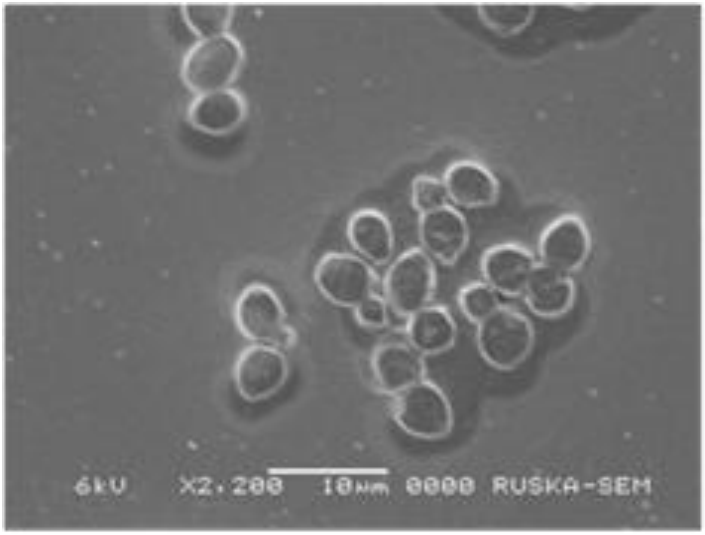

Fig: 1. Scanning electron micrograph of isolated yeast OBV9 showing budding cells under X2,200 magnification

3.2. Screening for stress tolerance and characterization

All the 26 strains were inoculated into yeast extract peptone dextrose (YEPD) agar medium plates and incubated at 40 ^{0}C to test their thermo tolerance. After 48 hours of incubation, plates were observed for growth and only 5 yeast strains were able to grow. These 5 strains (OBV4, OBV6, OBV9, OBV10 and OBV11) were later grown at five different temperatures 30, 35, 40, 42 and 44 °C.

To assess the ability of biomass production by yeasts at different temperatures, turbidity of the cultures was measured. At 42 ^{0}C significant (P<0.01) growth was observed for OBV9 and OBV11 **[Table– 1]**.

The above five yeast strains were grown in YEPD medium with different concentrations (0.00, 0.25, 0.50 and 1.00 per cent) of a mixture (70:20:10) of acetic, propionic and butyric acids. After incubation, turbidity (OD_{660}) of the cultures was measured and found to be similar in 1 per cent organic acids medium for all the strains except OBV11 **[Table– 2]**. Similarly, all the 5 strains were grown in YEPD medium with different pH values 2, 3, 4, 5, 6 and 7 to screen acid tolerance. After incubation, turbidity was measured and at 2 pH, growth was found to be significant (P<0.01) for OBV9 **[Table– 3]**.

Table: 1. Effect of temperature on the growth of yeast

Strain No. (OBV)	OD_{660} at temperature				
	30 ^{0}C	35 ^{0}C	40 ^{0}C	42 ^{0}C	44 ^{0}C
4	6.34	6.41	3.99^a	0.34^a	0.33
6	5.97	6.51	4.24^a	0.29^a	0.24
9	6.38	6.49	5.15^b	4.44^c	0.34
10	6.26	6.41	3.88^a	1.28^b	0.25
11	6.42	6.48	5.73^c	4.52^c	0.31
SEM	0.11	0.07	0.20	0.52	0.02

Each value is an average of four observations

^{abc} values bearing different superscripts in a column differ significantly (P< 0.01). OD 1.0 = 2X10^6 yeast cells/ml

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Table: 2. Effect of different concentrations of organic acids i.e. mixture of acetic, propionic and butyric acids (70:20:10) on the growth of yeast at 32 ^{0}C

Strain No. (OBV)	OD_{660} at per cent organic acids 0.0	0.25	0.50	1.00
4	6.34^b	6.22	5.22	4.26^b
6	6.12^b	6.04	5.23	4.26^b
9	6.45^b	6.32	6.08	4.31^b
10	4.26^a	6.31	5.68	4.12^b
11	6.36^b	6.08	5.03	3.34^a
SEM	0.22	0.17	0.13	0.12

Each value is an average of four observations

^{ab} values bearing different superscripts in a column differ significantly (P< 0.01)

Table: 3. Effect of pH on the growth of yeast at 32 ^{0}C

Strain No. (OBV)	OD_{660} at pH 2.0	2.0	2.0	2.0	2.0	2.0
4	0.34^a	2.68^b	4.68^c	6.34^b	6.41	6.01^b
6	0.27^a	2.26^b	4.24^b	6.12^b	6.01	5.97^b
9	3.07^c	5.33^d	6.12^e	6.45^b	6.41	6.22^b
10	0.28^a	1.78^a	3.88^a	4.26^a	6.11	5.34^a
11	1.97^b	4.66^c	5.01^d	6.36^b	6.32	5.89^b
SEM	0.31	0.37	0.21	0.22	0.19	0.17

Each value is an average of four observations

^{abcde} values bearing different superscripts in a column differ significantly (P< 0.01)

To determine osmo tolerance, all the 5 strains were grown in YEPD medium with 5, 10, 15, 20, 25 and 30 per cent glucose. After incubation, tolerance at 30 per cent glucose was detected in terms of OD for two strains OBV9 and OBV11, respectively **[Table–4]**. Five sets of YEPD medium flasks with different concentrations of bile salts (0.2, 0.4, 0.6, 0.8 and 1.0 per cent) were inoculated with all the above five yeast strains to determine their bile tolerance. After incubation, turbidity (OD_{660}) of the cultures was measured. The significant (P<0.01) growth at 1 per cent level of bile was observed for yeast strains OBV9 and OBV11 **[Table–5]**. Based on the growth at different temperatures, a different concentration of organic acids, sugars, pH and bile salts, yeast isolate OBV9, isolated from brewery effluents was selected as a potential probiotic and characterized.

Table: 4. Effect of glucose on the growth of yeast at 32 ^{0}C

Strain No. (OBV)	OD_{660} at glucose concentration (per cent) 5	10	15	20	25	30
4	6.44	6.52	6.68	6.23	3.36^b	2.04^b
6	6.48	6.54	6.58	6.21	2.27^a	1.57^a
9	6.54	6.69	6.82	6.68	6.31^c	4.12^c
10	6.34	6.41	6.68	6.28	3.25^b	1.85^{ab}
11	6.42	6.48	6.52	6.66	6.34^c	4.33^c
SEM	0.05	0.05	0.04	0.07	0.48	0.33

Each value is an average of four observations

^{abc} values bearing different superscripts in a column differ significantly (P< 0.01)

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Table: 5. Effect of bile salts (ox bile) on the growth of yeast at 32 ^{0}C

Strain No. (OBV)	OD_{660} at ox bile conc. (per cent) 0.2	0.4	0.6	0.8	1.0
4	6.12	6.22	3.34^a	1.83^a	1.56^a
6	6.07	6.11	4.02^b	2.63^b	1.38^a
9	6.44	6.49	5.85^d	4.78^c	3.74^b
10	6.22	6.04	3.91^b	2.88^b	1.51^a
11	6.30	6.38	5.13^c	4.66^c	4.17^b
SEM	0.06	0.07	0.26	0.31	0.33

Each value is an average of four observations

^{abcd} values bearing different superscripts in a column differ significantly (P< 0.01)

Characterization of stress tolerant yeast by 5.8S rRNA gene and ITS1 and ITS2 sequencing showed that it was most similar (99%) to *S.cerevisiae.* Nucleotide sequences were submitted to Gene Bank for accession number – GU229793. The optimal tree with the sum of branch length = 0.00970218 is shown in **Figure–2**. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [9]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [31] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 659 positions in the final dataset. Yeast OBV9 has exhibited an individual sub line that displayed little phylogenetic affinity with other *Saccharomyces* species. Of the 17 *Saccharomyces* species and one uncultured fungal clone, 12 species of *Saccharomyces* showed a distinct group related to each other displaying levels of 5.8S rRNA gene and ITS1 and ITS2 sequence similarity of 99% and 3 showed distinct groups with 99% similarity. Based on this analysis, stress tolerant yeast isolate OBV9 was characterized as *Saccharomyces cerevisiae* OBV9.

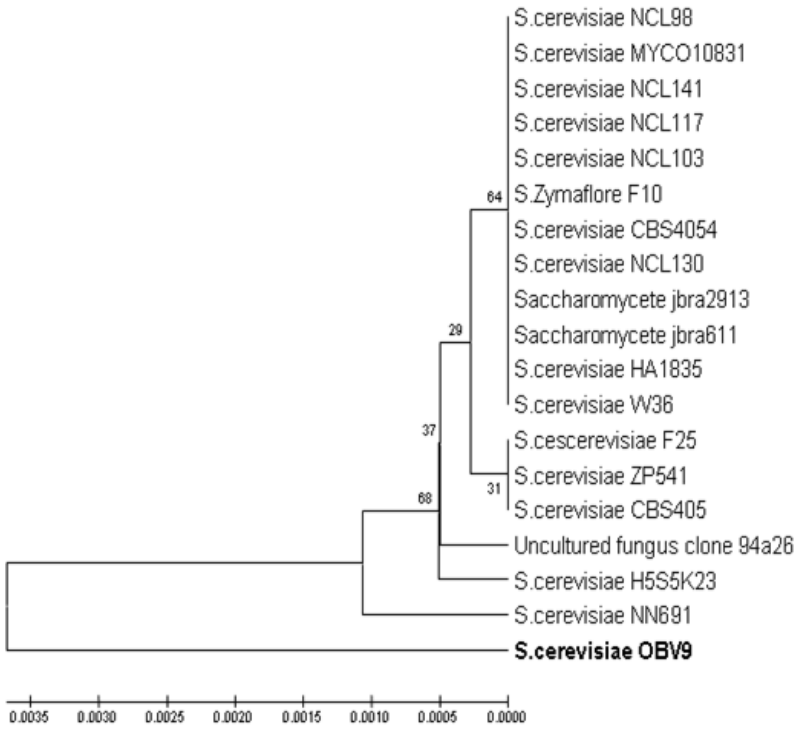

Fig: 2. Phylogenetic tree of *Saccharomyces cerevisiae* OBV-9 associated with other members of *Saccharomyces* genus. Distance matrix was calculated using UPGMA and topology was inferred by Neighbor-joining method based on the bootstrap analysis of 500 trees

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[IV] DISCUSSION

Strains isolated from a particular region are usually more adapted to their own climatic conditions and therefore the isolation of such organism was obvious because these strains were isolated from high temperature environment (Bakery oven wastes and soil samples from thermal power plants) and high sugar concentration as observed in fruits, molasses, nectar etc. In the present study, Yeast Extract Peptone Dextrose (YEPD) medium [32] was used for the screening of yeast strains.

Though significant growth was obtained at 40 ^{0}C by OBV11, among all the strains OBV9 and OBV11 were able to produce significant ($P<0.01$) biomass at 42 ^{0}C in terms of OD_{660} and therefore described as thermotolerant yeasts. This thermotolerance is in agreement with Preeyaporn *et al*. [22] where the authors isolated thermotolerant yeast which can tolerate 41 ^{0}C. At 44 ^{0}C there was no significant ($P>0.01$) growth obtained for any of the yeast strains. Significant ($P<0.01$) biomass in terms of OD was obtained by the strain OBV9 in the medium with pH 2. This might be due to its high acid tolerance. Significant lower biomass was observed for OBV11 compared to all other strains in the medium containing 1 per cent organic acids. The yeast strains OBV9 and OBV11 were able to produce significant ($P<0.01$) biomass in the medium containing 25 per cent glucose. Significant ($P<0.01$) biomass in 30 per cent glucose medium was also obtained by OBV9 and OBV11 compared to other strains. This might be due to their high osmo tolerance. The growth obtained by OBV6 and OBV10 was not significantly ($P>0.01$) different from each other in 0.6 per cent bile salts medium. However, the significant ($P>0.01$) growth in 0.6 per cent bile salts medium was obtained by the strain OBV-9. In the medium containing 0.8 per cent bile salts, significant ($P<0.01$) growth was obtained by OBV9 and OBV11. Similarly, in 1 per cent bile salts medium also, significant ($P<0.01$) growth was observed for OBV9 and OBV11. But it was not significantly ($P>0.01$) different between both of the strains. Based on growth performance in all the above conditions OBV9, isolated from brewery effluents was selected and characterized. The gene sequence showed similarity of 99% with *Saccharomyces cerevisiae*, while the similarity was less with other organisms.

Alignment with corresponding sequences of the 17 other strains of *Saccharomyces* and one uncultured fungal clone was performed and a phylogenetic tree was constructed using the neighbor-joining method. The 5.8S rRNA gene and ITS1 and ITS2 based phylogenetic analysis revealed the presence of two branches. A first branch comprising the 9 strains of *Saccharomyces cerevisiae* 2 *Saccharomycetes* and one *Saccharomyces zymaflore* were appeared as a sister group while based on the sequence data and other molecular characters, yeast strain OBV9 appeared as a separate branch within the *Saccharomyces cerevisiae* strains. It is concluded that the stress tolerant *Saccharomyces cerevisiae* OBV9 is a promising candidate for ruminant probiotic applications.

[V] CONCLUSION

Since the yeast *Saccharomyces cerevisiae* OBV9 tolerates temperature up to 44 ^{0}C, pH 2, sugar concentration up to 30 per cent and 1 per cent bile salt, it is described as a stress tolerant yeast. All these stress conditions prevail in gastro intestinal tract (rumen) of animals. Since it tolerates all the stress conditions present in gastro intestinal tract of animals, OBV9 is a promising candidate for ruminant probiotic applications.

FINANCIAL DISCLOSURE

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