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Full Length Research Paper

In vitro characterization of *Saccharomyces cerevisiae* HM535662 obtained from an indigenous fermented food "Bhaturu" of Western Himalayas

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A predominant yeast designated as SB1 was isolated from *Bhaturu,* a traditional fermented food of Western Himalayas and was identified as *Saccharomyces cerevisiae* - HM535662 on the basis of ribosomal gene (partial 18S, complete internal transcribed spacer 1 (ITS1), complete 5.8S, complete ITS2 and partial 28S ribosomal regions) analysis. It was further evaluated for probiotic traits by *in vitro* tests. The isolate was investigated for tolerance studies in simulated gastric and intestinal juices. Reduction in viability (in terms of log colony formng unit (CFU)/ml) was more in simulated gastric juice of pH 2 as compared to pH 3. The isolate was tolerant to bile conditions but without the exhibition of any bile salt deconjugation activity. The isolate also showed adhesion attributes such as cell surface hydrophobicity (58.21 ± 1.09%) and autoaggregation ability (67.42 ± 0.33%). It exhibited 38.30 ± 0.27 (intracellular cell free extract) and 42.51 ± 0.37% (whole cell extracts) antioxidative activity by scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical.

Key words: Probiotic, yeast, Western Himalayas, adhesion, bile antioxidant.

INTRODUCTION

Fermented food products are essential components of diet in a number of developing countries and are consumed either as beverage, main dish or condiment, which contribute to one third diet of people world wide (Campbell-Platt, 1994). Traditionally, fermented foods are processed through naturally occurring microorganisms; however, modern conventional methods of production generally exploit the use of defined starter cultures to ensure consistency and the quality of the final product (Ross et al., 2002).

Cultures and species involved in fermented foods do not pose any health risk, and thus are designated as 'generally recognized as safe' ('GRAS') organisms (Adams, 1999; Nout, 2001; Hansen, 2002). Therefore, some of the species of these microorganisms because of their long history of safe use in food products can be employed as protective cultures or probiotics.

Yeasts are the most common and important microorganisms associated with the fermented foods (Yarrow, 1998). Some yeast strains such as *S. cerevisiae* and *Saccharomyces boulardii* have also been used as probiotics in humans for many years because they exert some influence on the intestinal flora (Czerucka et al., 2007; Kumura et al., 2004). These probiotics that are delivered through food systems have to firstly survive during the transit through the upper gastrointestinal tract, and then persist in the gut to provide beneficial effects for the host (Chou and Weimer, 1999).

Probiotic yeasts provide an additional advantage over bacteria in a sense that there is no threat of transfer of antibiotic resistance genes between pathogenic bacteria and yeast in favorable environment of mammalian GI tract (Salyers et al., 2004), thus making probiotic yeast as more appropriate to be administered during antibiotic treatment.

Yeasts are microorganisms of great economical interest for their numerous applications in traditional and

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modern biotechnology (Orberá, 2004). They are probably the most employed biocatalyst in the biotechnological industry and a model organism in biological studies (Lachance et al., 1998).

Given that fermented food products can contain probiotics, prebiotics or both, it is not surprising that their consumption has long been associated with good health. A variety of indigenous fermented food products and beverages of Western Himalayas have been documented with respect to substrates and yeast diversity (Kanwar et al., 2007; Pathania et al., 2010).

The objective of this study was to identify and select predominant yeast from the traditional fermented food *viz., Bhaturu,* of Western Himalayas, for potential probiotic attributes.

MATERIALS AND METHODS

Isolation of yeast

Indigenous yeast were enumerated and isolated from traditional fermented food *viz., Bhaturu* (uncooked) of Western Himalayas by standard serial dilution technique on potato dextrose agar (peeled potato 250 g, dextrose 20 g, agar 20 g, distilled water 1000 ml) medium. Plates were incubated at 28°C for 48 h. All yeasts were preliminarily grouped based on their cultural morphology and physiological characteristics (Yarrow, 1998).

Colony characteristics were observed on potato dextrose agar. Ascospore shape was determined by employing modified Schaeffer-Fulton's staining (Schaeffer and Fulton, 1933) of seven days old yeast grown on glucose acetate medium. Formation of mycelium and pseudo mycelium was detected by Dalmau Plate technique (Wickerham, 1951). Fermentation pattern of different sugars was tested using fermentation basal medium.

The predominant yeast isolate based on morphological and physiological characterization was selected for further studies. All the yeast isolates were maintained on potato dextrose agar and stored at 4°C.

Identification of yeast isolate

Predominant yeast isolate was identified by using 18S ribosomal sequence analysis. Total genomic DNA of yeast isolate was extracted following the procedure of Sharma et al. (2005) with minor modifications. The DNA extracted was stored at -20°C for further use. Universal primers viz., ITS 5 (GGAAGTAAAAGTCGTAACAAGG) and ITS-4 (TCCTCCGCTTATTGATATGC) synthesized from Imperial Life Sciences (P) Ltd, CA, USA, targeting ribosomal gene region [Intergeneric spacer region (ITS1 and ITS-2), 5.8S and partial regions for 18S -28S] were used to amplify the genomic DNA of yeast isolate (Sumari et al., 2010).

Amplification was carried out in the thermal cycler (Gene-Amp PCR system 9700, Applied Biosystems, USA) with an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 55°C for 1 min , 72°C for 1 min and a final elongation step at 72°C for 10 min. The PCR products were stored at -20°C until analyzed. The amplified PCR products were resolved/checked by electrophoresis using 1.2 to 1.6% agarose gel in 0.5 x tris acetate EDTA buffer (2 M Tris base, 57.10 ml acetic acid and 0.5 M EDTA, pH 8.0, 50 x) containing ethidium bromide (0.5 µg/ ml).

The PCR products obtained through amplification were freeze dried (CHRIST *ALPHA I-2LD*) and then custom sequenced (ABI 3730xl automated sequencer) by using same upstream and downstream primers (Life Technologies India, Pvt. Ltd), by a commercial sequencing facility (Ocimum biosolutions, Pvt. Ltd. Hyderabad, India). The corrected sequence of yeast obtained after sequencing was blasted using on-line NCBI BLASTN program http://www.ncbi.nih.gov/blast (Altschul et al., 1997). All the phylogenetic analyses were conducted in MEGA 4.1 software programme (Tamura et al., 2007). After identification the selected yeast isolate was subjected to various *in vitro* probiotic attributes.

Screening for *in vitro* probiotic attributes

Preparation of simulated gastric and small intestinal juices

Simulated gastric and intestinal juices were prepared as per the method described earlier (Sourabh et al., 2010). Simulated gastric juice was prepared by dissolving pepsin (Merck Specialities Pvt. Ltd, Mumbai, India) in sterile saline (0.85 % w/v) to a final concentration of 3 g/L. The pH was adjusted to 2.0 and 3.0, separately. Similarly, simulated intestinal juice was prepared by dissolving pancreatin from porcine pancrease, USP specifications (Sigma-Aldrich Inc. USA) to a final concentration of 1 g/L and its pH was adjusted to 8. These juices were then filter sterilized separately, through Millipore filter assembly using 0.22 µm Durapore membrane filter.

Preparation of washed cell suspension

The isolate was incubated in potato dextrose broth (PDB) at 37°C for 18 h and then centrifuged at 2500 x g at 4°C for 10 min. The cell pellet obtained was washed three times in phosphate buffer saline (PBS) buffer solution (pH 7.0). The collected cells were resuspended in sterile saline and viable count was determined by serial dilution method on potato dextrose agar (PDA) plates prior to the assay of transit tolerance.

Upper gastrointestinal transit tolerance assay

The tolerance of washed cell suspensions of yeast isolate to simulated gastric and small intestinal transit was determined by following the method of Charteris et al. (1998) as described in Sourabh et al. (2010). For screening gastric transit tolerance, 0.1 ml aliquot was removed after every 30 min of intervals (upto 4 h) for determining the viable count. The small intestinal transit tolerance was evaluated by determining the viable count after 1, 4, and 8 h intervals.

Effect of ox bile on the growth rate

The effect of ox bile (HiMedia Laboratories, Pvt. Ltd., Mumbai, India) on growth of the yeast isolate was studied by adapting the method of Walker and Gilliland (1993). This effect was measured on the basis of time required to increase the absorbance at 620 nm by 0.3 units in PDB-thio broth with and without 0.3 % ox bile. The difference in time (h) for attaining desirable absorbance between both culture media was considered as the lag time (LT) (Usman and Hosono, 1999).

Bile salt deconjugation activity

The ability of the yeast isolate to deconjugate bile salts *viz.,* sodium salts (Calbiochem) of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC) and glycodeoxycholic acid (GDC) was

determined according to the method of Taranto et al. (1995) and Vinderola and Reinheimer (2003) as employed by Sourabh et al. (2010).

Separate bile salt plates were prepared by adding 0.5% (w/v) of sodium salts (Calbiochem) of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC) and glycodeoxycholic acid (GDC) to PDA, autoclaved (121°C, 15 min) and immediately used. The isolates were spot inoculated on the media and the plates were anaerobically (GasPak System-Hi Media) incubated at 37°C for 72 h. The presence of precipitated bile acid around growth was considered a positive result.

Microbial adhesion to hydrocarbon (MATH)

The test of adhesion to hydrocarbon n-hexadecane was adopted to screen yeast isolate for its cell surface hydrophobicity property. Microbial adhesion to hydrocarbon (MATH) in terms of the cell surface hydrophobicity (H in percent), was determined according to the method of Rosenberg et al. (1980) with slight modification as described by Vinderola and Reinheimer (2003).

Autoaggregation ability

Yeast isolate was grown at 37°C for 24 h in PDB. The cells were harvested by centrifugation and suspended in phosphate buffered saline (PBS) to 0.5 optical density (O.D.) units at 600 nm. Autoaggregation assay was performed as described by Collado et al. (2008) with minor modifications. Yeast isolate was grown at 37°C for 24 h in PDB. The cells were harvested by centrifugation and suspended in phosphate buffered saline (PBS) to 0.5 optical density (O.D.) units at 600 nm.

Five millliliter of this yeast suspension was incubated at 37°C for 20 h and then 3 ml of the upper suspension was carefully transferred to another tube and the O.D. was measured at 600 nm. Percent autoaggregation ability was calculated as 1 - (O.D. upper suspension/O.D. total bacterial suspension) × 100.

Total antioxidative activity (TAA)

Preparation of whole cell extracts and intracellular cell free extracts

The yeast isolate was grown in PDB at 37°C for 24 h and harvested by centrifugation at 10000 × g at 5°C for 15 min. For the preparation of intact cells, cells were washed three times with phosphate buffered saline (PBS)/water and then were resuspended in the same. Total cell number was adjusted to 10^9 cfu/ml for subsequent preparation of whole cell and intracellular cell-free extracts. Ultrasonic disruption (B. Braun Biotech International, Germany) was performed for five times at 1-min intervals in an ice bath.

The sonicated cell lysate was divided into two parts. In one part, cell debris was removed by centrifugation at 7800 × g for 10 min, and the resulting supernatant was the intracellular cell-free extract. In second part, the cell debris was not removed and the resulting lysate was used as whole cell extract.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay

1**,** 1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay (Sharma and Bhat, 2009) in methanolic and buffered methanol systems employing 200 µM of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma-Aldrich Inc. USA) solution was used for screening yeast isolate for free radical scavenging (antioxidant) activity.

In one set of assay (methanol), to 100 µL of whole cell extract or intracellular cell free extract, methanol was added to make the volume to 3 mL. To this reaction mixture, 1 ml of DPPH solution (200 µM) was added, vortexed and kept at 30°C for 30 min in darkness. Absorbance was measured at 517 nm against control.

In another set of assay (buffered methanol), to 100 µl volume of whole cell or intracellular cell free extract, methanol was added to make the volume to 1 ml. One millliter (1 ml) of DPPH solution (200 µM) was added to each tube wrapped with aluminium foil, followed by 2 ml of buffered methanol. Buffered methanol was prepared by mixing 40.0 ml of 0.1 M acetate buffer (pH 5.5) with 60 ml of methanol. The contents were vortexed and incubated at 30°C for 30 min under darkness. Absorbance was measured at 517 nm against control. Butylated hydroxytoluene (BHT) a standard antioxidant was used as positive control in all antioxidant assays.

RESULTS AND DISCUSSION

Isolation of yeast

The total viable yeast count was 8.5 log CFU/ml in Bhaturu and on the basis of morphological and biochemical characteristics four yeast were isolated (Table 1). The predominant yeast was designated as SB1 and its viable count was 7.6 log CFU/mL. On the basis of standard traditional characterization techniques as described by Lodder (1970), Barnett et al. (1983), Kreger-van-Rij (1984) and Yarrow (1998), four isolated yeast *viz.,* SB1, SB2, SB3 and SB4 were found to be *S. cerevisiae, Issatchenkia orientalis, Saccharomyces fragilis* and *Kluveromyces marxianus*. Earlier workers also encountered similar type of yeasts in various traditional fermented foods and beverages (Jespersen et al., 2005; Zhang et al., 2011).

Characterization of predominant yeast isolate

It has been shown that the unambiguous taxonomic identification of *Saccharomyces* at species level requires combinations of several methods (Dellaglio et al., 2003; Antunovics et al., 2005). Therefore ribosomal gene analysis was employed for identification of selected yeast isolate. Analysis of ribosomal gene sequence of yeast containing 18S, complete Intergenic spacer (ITS)-1, complete 5.8S, complete ITS2 and 28S ribosomal regions has been employed by various workers for rapidly and accurately identifying and discriminating yeasts (Masneuf et al., 1998; De Barros lopes et al., 1998; Yamagishi et al., 1999; Foschino et al., 2004; Arroyo-Lo´ pez et al., 2006). This technique was employed in the present study for the identification of predominant yeast isolate-SB 1 and it was identified as *S. cerevisiae* after BLASTN analysis. The sequence was submitted at GenBank National Center for Biotechnology Information (NCBI), USA and GenBank accession number HM535662 was allotted. Phylogenetic tree (Figure 1) of indigenous yeast isolate-SB 1 was constructed by using rRNA gene sequences of selected

Table 1. Morphological and physiological characteristics of yeasts isolated from Bhaturu.

Isolate	SB1	SB2	SB3	SB4
Colony morphology	White-cream, raised, smooth, round, small, shiny	White, irregular margins, furrowed , wrinkled, rough	Cream, convex, smooth, dull	Cream, convex, smooth, dull
Cell shape	Spherical oval	Ellipsoidal oval	Oval, ellipsoidal	Oval, ellipsoidal
Ascospore shape	Spherical	Spherical ellipsoidal	Oval	Spherical
Pseudomycellium	-	+	+	+
Growth at 30°C	+	+	+	+
Growth at 37°C	+	+	+	+
Growth at 40°C	+	+	+	+
Growth in ethanol (10%)	+	+	+	+
Growth in potassium nitrate media	-	-	-	-
Growth in cycloheximide (0.01%)	-	-	-	-
Growth in cycloheximide (0.1%)	-	-	-	-
Characteristics in broth	Smooth sediments	Pellicle formation	Smooth sediments	Smooth sediments
Fermentation of sugars:				
Arabinose	-	-	-	-
Cellobiose	-	-	A	-
Lactose	-	-	A	A
Maltose	A	-	-	-
Melibiose	A	-	-	-
Galactose	AG	AG	A	AG
Glucose	AG	-	AG	A
Raffinose	AG	-	A	A
Starch	A	-	A	A
Sucrose	A	-	AG	A
Trehalose	AG	-	-	-
Inulin	-	-	A	A

A: Acid production; G: gas production; +: Presence of particular characteristic; -: Absence of particular characteristic.

yeast isolates from NCBI.

Tolerance to simulated gastric and small intestinal juices

In vitro screening based on the capacity of microorganisms to survive in the simulated conditions of the digestive tract is used for selection of probiotic microorganisms, as it is indispensable property of probiotic. The tolerance study on indigenous yeast isolate *S. cerevisiae* (HM535662) was conducted in simulated gastric juice of pH 2 and 3 (Table 2) and this isolate was found to tolerate these low pH conditions**.** At pH 2 of simulated gastric juice, the reduction in viability was more (2.71 to 4.12 log CFU/ml) as compared to reduction in pH 3 (0.88 to 3.06 log CFU/ml) after 240 min exposure indicating inherent tolerance of this isolate.

The tolerance to acidic pH has already been reported for *S. cerevisiae* strains (Psomas et al., 2001; Hernández et al., 2011). Survival in low pH may be due to various factors like cell size, composition of cell wall etc. as reviewed by Czerucka et al. (2007). The viable count of yeast isolate in simulated intestinal juice of pH 8 was found to be 6.63 $\pm$ 0.02 CFU/ml after 8 h of incubation from the initial value of 6.82 $\pm$ 0.02 CFU/ml.

The indigenous food borne yeast isolate demonstrated high tolerance to simulated human upper gastrointestinal tract juices and thus, it fulfills the preliminary *in vitro* selection criteria for being designated as potential probiotic. Tolerance to gastrointestinal juices is an important trait of good probiotic because probiotics entering the GI tract must be resistant to local stresses such as the presence of GI enzymes and pH.

Effect of ox bile on the growth rate of yeast isolate

The indigenous yeast isolate *S. cerevisiae* (HM535662) showed a lag time of 0.15 h in growth in the presence of 0.3% ox bile indicating that it is fairly resistant to tested concentration. Van der Aa Kqhle et al. (2005)

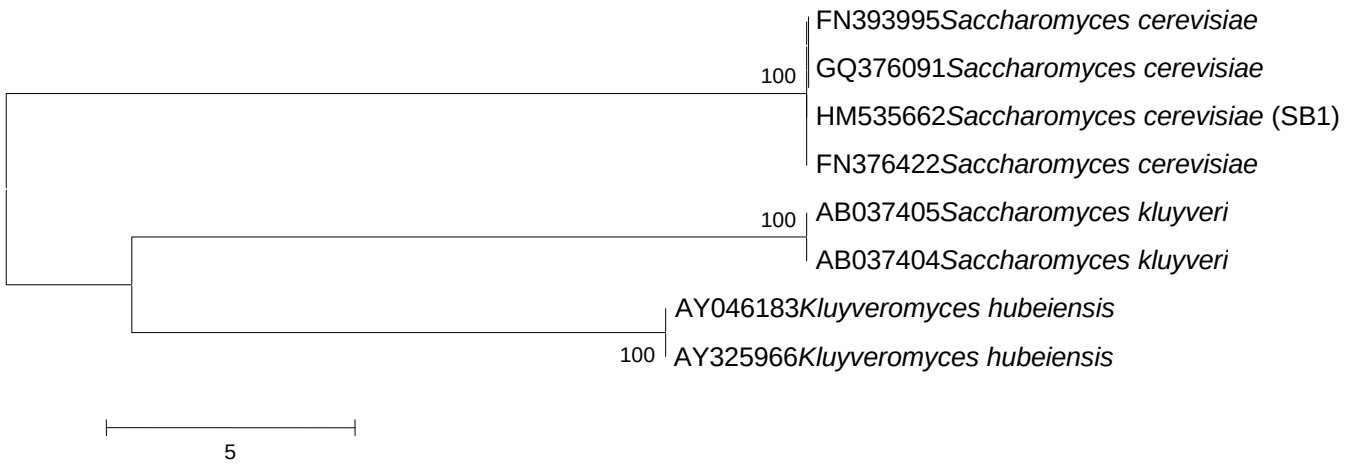

Figure 1. Phylogenetic tree of yeast isolates based on rRNA gene sequences. NCBI GenBank accession numbers are followed by organism's name.

Table 2. Survival of indigenous yeast isolate in simulated gastric juice of pH 2 and 3.

S/N	pH	Viable count (log CFU/ml)						
		0 min	30 min	60 min	90 min	120 min	180 min	240 min
1	2	10.86 ± 0.01	10.75 ± 0.03	9.93 ± 0.01**	9.45 ± 0.03**	9.04 ± 0.03**	8.65 ± 0.02**	8.15 ± 0.04**
2	3	10.79 ± 0.02	10.66 ± 0.04*	10.46 ± 0.03**	10.35 ± 0.03**	10.26 ± 0.03**	10.04 ± 0.03**	9.91 ± 0.04**

Results are shown as mean (± standard deviation), Number of replications=3; Independent sample, testing of significance of variances by F -test, significance testing of two means by using Fishers t-test and Cochran and Cox t-test,*P < 0.05 but > 0.01; **P< 0.01.

investigated 18 *S. cerevisiae* strains isolated from various foods or beverages, and found that all yeast strains were able to withstand 0.3% oxgall which is a important probiotic trait. In earlier study also 23 indigenous yeast isolates of *S. cerevisiae* of same region were found to exhibit tolerance to gastrointestinal juices and ox bile (Sourabh et al., 2011). Several other workers have also reported the survival of yeast strains in presence of bile (Psomas et al., 2001; Kumura et al., 2004; Van der Aa Kqhle et al., 2005). The indigenous isolate was also able to grow in the presence of 3% concentration of ox bile. The yeast strains such as *S. cerevisiae* have already been reported to tolerate bile concentrations as high as 0.9% (Agarwal et al., 2000).

Bile salt deconjugation activity

World Health Organization (WHO) has included deconjugation as one of the main activities of intestinal microorganisms (FAO/WHO, 2002). This indigenous yeast isolate *S. cerevisiae* (HM535662) did not show precipitation/deconjugation activity on PDA plates supplemented with 0.5% (w/v) of various glycine and taurine conjugated bile salts; however, it could grow in presence of these bile salts, indicating thereby that these two traits are independent of each other.

Microbial adherence to hydrocarbon

After surviving the upper gastrointestinal transit, next challenge for an effective probiotic is to adhere to small intestinal cells. Cell surface hydrophobicity is considered to be an important factor in the adhesion and proliferation of microorganisms on the intestinal epithelial cells (Del Re et al., 1998). Therefore, hydrophobicity was used to ascertain the adhesive potential of the indigenous isolate. Hydrophobicity was determined with n-hexadecane because it has been reported to give more reliable results without any cell lysis as compared to other hydrocarbons for evaluation of adhesion ability of probiotics (Richard et al., 1999).

Hydrophobicity value of 58.21 $\pm$ 1.09% was observed for indigenous yeast isolate *S. cerevisiae* (HM535662), which was close to the highest value of hydrophobicity (59.65 $\pm$ 0.58%) reported earlier for indigenous strains of *S. cerevisiae* (Sourabh et al., 2011) isolated from fermented foods of Western Himalayas.

Strains possessing high hydrophobicity exhibits good adhesion property to intestinal cell lines (Pan et al., 2006; Marin et al., 1997). In yet another study, yeast

Wickerhamomyces anomalus (LV-6) isolated from broilers excreta showed 25% hydrophobicity (Hernández et al., 2011) which came out relatively low when compared with other probiotic microorganisms (Rondón et al., 2008) and indigenous yeast isolate *S. cerevisiae* (HM535662). In some of the studies, this characteristic is not considered important for adhesion to the intestinal mucosa and colonization (Tovar-Ramírez et al., 2003). However in comparison to bacteria, the yeast strains generally show variable adherence property and that is why these microorganisms are required to be administered repeatedly to achieve steady-state concentrations in the colon (Kumura et al., 2004).

Autoaggregation ability

Autoaggregation ability of probiotics is another trait which is associated with the adhesion ability of microorganisms (Del Re et al., 1998; Perez et al., 1998). An autoaggregation (%) ability of 67.42 ± 0.33% was observed for indigenous yeast isolate *S. cerevisiae* (HM535662) and this was almost close to the autoaggregation ability of 67.59 ± 0.27% reported earlier for *S. cerevisiae* (Sourabh et al., 2011) associated with fermented foods of Western Himalayas.

Autoaggregation ability has been more strongly associated to adhesion as compared to hydrophobicity (Del Re et al., 2000) therefore, good amount of autoaggregation ability may account for adherence property of this indigenous isolate. Isolates having good autoaggregation ability in conjunction with the good hydrophobicity values can strongly be related to the adhesion ability of microorganisms. Though both of these traits are independent of each other; they are still related to adhesion property of a particular microbe (Rahman et al., 2008).

Antioxidant activity

Potential probiotic yeast isolate showed scavenging of DPPH free radical in both methanol and buffered methanol reaction systems. The intracellular cell free and whole cell extract of isolate showed antioxidant activity of 19.77 ± 0.25 and 23.70 ± 0.10%, respectively with DPPH assay employing methanolic system.

In case of DPPH assay employing buffered methanol, isolate SB1 showed antioxidant activity of 38.30 ± 0.27 and 42.51 ± 0.37% with intracellular cell free and whole cell extracts, respectively. These results indicate that the radical scavenging ability of both intracellular cell-free extracts and whole cell extract of native isolate contribute towards the antioxidative effect.

Antioxidative activities ranging from 11.43 ± 0.40 to 40.40 ± 0.20% have been previously reported for yeast (Sourabh et al., 2011). In yet another study Chen et al. (2010) reported 12 to 41% ability to scavenge 1, 1-diphenyl-2-picrylhydrazyl free radicals for yeast.

Various workers have reported the exhibition of antioxidative activity by bacteria like *Lactobacillus* species (Kullisaar et al., 2002, Saide and Gilliland, 2005, Jarvenpaa et al., 2007) whereas in yeasts it is less reported (Sourabh et al., 2011).

The results of the present study confirm that indigenous yeast *S. cerevisiae* (HM535662) isolated from traditional fermented food *viz., Bhaturu* (uncooked) possess tolerance to simulated gastric and intestinal juices. It can also grow in the presence of ox bile but without exhibition of any bile deconjugation ability and exhibits good hydrophobicity and autoaggregation abilities which might help in surviving gastrointestinal environment. Along with other traits the exhibition of strong antioxidative ability makes indigenous yeast an appropriate candidate for the development of functional foods or natural antioxidant supplements in food industry.

Since, indigenous *S. cerevisiae* has been isolated from traditional fermented food being consumed regularly by people of Western Himalayas; therefore, its role as potential probiotic seems to be of paramount importance. Further studies are required to explore the effects of the potential probiotic yeast under *in vivo* conditions.

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