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Morphological and Molecular Characterization of Isolated Probiotic Yeast

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ABSTRACT

The Experiment was conducted during 2019 in the research laboratory in the Department of Plant Pathology and Agricultural Microbiology, Post graduate Institute, Mahatma Phule Krishi Vidyapeet, Rahuri, Ahmednagar, Maharashtra, India. The objective of this study was to isolate and characterize probiotic yeast isolated using fermented jowar and bajra cereal grain flours for their efficacy as biocontrol agents. The yeast species isolated on Malt extract agar were morphologically and molecularly identified. The region obtained by PCR analysis with Internal transcribed spacer (ITS) specific primers of fungi was further sequenced and the cultures were confirmed as *Saccharomyces cerevisiae* and *Saccharomyces arboricola* by BLAST analysis. The conserved region of the isolate was amplified and the size was found to be of 758 bp for *Saccharomyces cerevisiae* and 698 bp for *Saccharomyces arboricola* in agarose gel electrophoresis and visualized through gel documentation. The nucleotide sequence obtained was subjected to Basic Local Alignment Search Tool and data was submitted to NCBI. The accession number obtained for *Saccharomyces cerevisiae* was MZ068117 and for *Saccharomyces arboricola* was MZ068118. The Phylogenetic tree was constructed individually using neighbour joining approach by using Molecular Evolutionary Genetics Analysis (MEGA X) and Motif analysis by using Multiple EM for Motif Elicitation (MEME software) bioinformatics tools of thirty closest species was performed and about five conserved sequences were obtained among the tested strains.

KEYWORDS: Biocontrol, fermented flour, identification, molecular, Probiotic yeast

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1. INTRODUCTION

Probiotic is a Latin- and Greek-derived word, meaning 'for life,' which was first used by Kollath (1953). Lilly and Stillwell (1965) were the first to propose a definition of probiotics as substances secreted by one microorganism that stimulate the growth of another. In 2002, an FAO/WHO joint panel defined probiotics as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host. Probiotics are living microorganisms that have the potential to be beneficial to host organisms when administered at the correct dosage (Hill et al., 2014). Humans have benefitted from microorganisms in food in various forms throughout history. The benefits of including certain live microbes in food were first indirectly observed in the health effects of fermented foods, though the cause would have been almost certainly unknown at the time (Gogineni et al., 2013). These benefits may exist in the form of modulating immunological homeostasis, implication in adaptive immunity or through the general maintenance of microbial homeostasis in the gut through specific interactions (Lai et al., 2019; Toma and pokrotnieks, 2006). These are also used for the health benefits of animals, birds and fishes (Abd El-Hack et al., 2020, Vieco-Saiz et al., 2019). Most probiotics are bacteria, among which bacteria such as *Bifidobacterium* and *Lactobacillus* are the most common type, but a few molds and yeasts can also be used as probiotics (Bermudez-Brito et al., 2012), The most common yeast with proposed probiotic effects is *Saccharomyces boulardii.* Also known as *Saccharomyces cerevisiae var. boulardii* or *Saccharomyces cerevisiae* Hansen CBS 5926, over-the-counter preparations of this yeast are typically recommended for the treatment of acute gastrointestinal diseases such as rotoviral and bacterial diarrhea (Kelesidis and Pothoulakis, 2012).

Probiotics occur naturally in the fermented food product such as yoghurt, kefir, sauerkraut, cabbage kimchee and soybean-based miso and natto. The range of food products conating probiotics is wide and still growing (Stanton et al., 2001) Probiotics have been used for centuries in fermented dairy products. such probiotic dairy foods beneficially affect the host by improving survival and implantation of live microbial dietary supplements in the gastrointestinal flora (Namkin et al., 2016), by selectively stimulating the growth or activating the catabolism of one or a limited number of health-promoting bacteria in the intestinal tract and by improving the gastrointestinal tract's microbial balance. (Carstensen et al., 2018) In addition to their major contribution to flavor development, their antagonistic activities toward undesirable bacteria, and fungi are now widely known (Sanders et al., 2019). These activities are associated with their competitiveness for nutrients, acidification of their growth medium, their tolerance of high concentrations of ethanol, and release of antimicrobial compounds such as antifungal killer toxins or "mycocins" and antibacterial compounds (Zhao et al., 2016). While the design of foods containing probiotics has focused primarily on *Lactobacillus* and *Bifidobacterium*, (Ran et al., 2019) the yeast *Saccharomyces cerevisiae* var. *boulardii* has long been known effective for treating gastroenteritis (Amara and Shibl, 2015). *Streptococcus thermophilicus* and *Lactococcus lactis,* two of the most commercially important lactic acid bacteria (Felis, 2007)

A good probiotic candidate must possess the characters such as it must be an organism that is capable of exerting a beneficial effect on the hosts, has increased growth or resistance to disease, must be nonpathogenic and nontoxic and it should be stable under storage and field conditions (Fuller, 1989). However, the potential applications of probiotics in nondairy food products and agriculture have not received formal recognition (Song et al., 2012). Among hundreds of yeast species, only a few may be useful and have been used for biocontrol against the plant pathogens (Tao et al., 2014). These Antagonistic yeasts starter cultures contribute to product safety primarily by inhibiting pathogen growth during fermentation, and to finish product sensory qualities and shelf-life by inhibiting spoilage organisms. The present study conducted to isolate the probiotic yeast and the most eminent one in biocontrol among them is identified to the species level by PCR-sequencing as the most precise molecular techniques.

2. MATERIALS AND METHODS

The Experiment was conducted in the year 2019 at the Department of Plant Pathology and Agricultural Microbiology, Mahatma Phule Krishi Vidyapeet, Rahuri, Ahmednagar, Maharashtra, India.

2.1. Isolation of probiotic yeast

Cereal grain flours of jowar and bajra are used to isolate the probiotic yeast. The flour obtained was suspended in distilled water (25 g flour in 100 ml distilled water) and incubated at 28±2°C in BOD incubator for three days to start the fermentation due to grain yeast. A loop ful of suspension from this incubated and fermented flour sample was streaked on Malt Extract Agar medium and the plates were incubated for two days at 28±2°C for appearance of yeast colonies and are further purified by streak plate method and single colonies thus obtained were maintained as pure cultures of the yeast as probiotics

2.2. Characterization of yeast isolates

The yeast isolated from jowar and bajra flour was characterized for differences among them, if any, on the basis of cultural growth, microscopic and molecular observation. The growth of the yeast with the colour of colonies was recorded for the respective yeast isolate. The shape of yeast cell isolates with budding habits and their size was recorded under microscope

2.3. Molecular characterization

The genomic DNA of isolated yeast samples was extracted from fresh culture (log phase) grown on malt extract broth. Following the method already mentioned (Aamir et al., 2015), the DNA sequences were amplified using forward (ITS1: 5´-TCCGTAGGTGAACCTGCGG-3´) and reverse (ITS4: 5´- TCCTCCGCTTATTGATATGC- 3´) universal fungal primers. Each PCR mixture contained 1 µl of each forward and reverse primers, 2 µl of extracted DNA, 2.5 µl PCR buffer, 0.25 µl Taq polymerase and sterile double distilled water up to a final reaction volume of 25 µl. the thermo cycler was programmed for intial denaturation at 94°C for 5 m and 35 cycles: of 94°C for 30 s (denaturing), 53°C for 1 m (annealing), 72°C for 1 m (extension) and final extension at 72°C for 7 m.

2.4. Product purification and submission to NCBI

Amplified PCR product was subjected to electrophoresis using Agarose gel 1% in 1X TAE buffer and stained with Ethidium bromide was observed and photographed under UV light. The PCR product was sequenced by Bioaxis DNA research Ltd., Hyderabad. The aligned sequence obtained was arranged in FASTA format using Molecular Evolutionary Genetics Analysis (MEGA) software. FASTA sequence was uploaded in National Center for Biotechnology Information (NCBI) GeneBank and final designation for species was based on the analysis of reliable sequences with basic local tool (BLAST) with the database for comparison with relevant reliable sequences. After completion of submission process in NCBI gene-bank database, accession number was obtained.

2.4.1. Phylogenetic tree

The construction of phylogenetic tree was done based on data obtained from nucleotide BLAST in NCBI. Thirty sequences showing more than 97% similarity coefficient with the native *Streptomyces* strains were used for phylogeny analysis using MEGA software.

2.4.2. Motif analysis

The nucleotide sequences of closest four from phylogenetic analysis in NCBI database which showed 100% similarity coefficient with the native strain were selected for motif analysis to find out the conserved regions among them. This analysis was performed using MEME software.

3. RESULTS AND DISCUSSION

3.1. Isolation of the probiotic yeast

After incubating for 48 h, small suppressed pale yellowish colonies were identified on MEA plates. Single colony was picked and streaked in plate and slants containing MEA. For long term storage, a loopful of culture was transferred to 20% sterile glycerol stock solution and stored at -80°C. The growth characters of Probiotic yeast isolates (Table 1) indicated that the probiotic isolate- I and II has round, Suppressed yeast colonies with pale yellowish and dull pale yellowish colony colour. It was evident that the yeast of bajra and jowar flour formed round suppressed colonies. Similar results were obtained by Disoma et al. (2014) have isolated 4 species belonging to 3 genera from probiotic kefir, Moradi (2018) isolated probiotic yeasts from 250 different fruit and dairy samples (Figure 1).

The characters of yeast cell shape, size and budding habits observed under compound microscope indicated that the shape of yeast cells of probiotic-I and II was elongate. Further the yeast isolates were variable in their cell size measuring in range of 1-1.18×0.5-0.8 µm All the yeast isolates had budding habits. Similar results were observed by Zakhartsev and Reuss (2018). Hussein et al. (2011) examined the structure and size of yeast cells with the help of scanning electron microscopy (Figure 2).

Table 1: Characterization of probiotic yeast

Probiotic isolate	Colony character	Colour of colony	Shape of yeast cell	Size (µm)	Budding habits
I	Round, suppressed	Pale yellowish	Elongated and oblong	1.09-1.18×0.6-0.7	Present
II	Round, suppressed	Dull pale yellowish	Elongated	1.15-1.40×0.81-0.86	Present

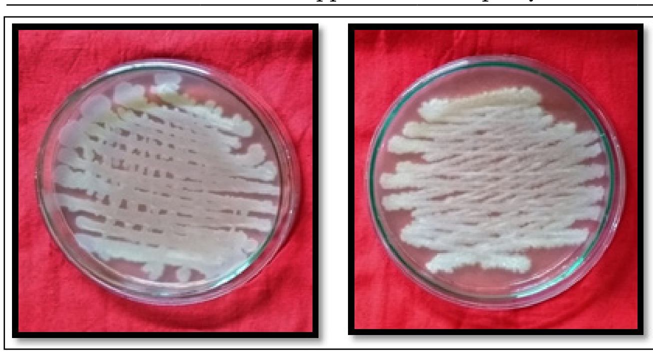

Figure 1: The growth characters of Probiotic yeast isolates

3.2. Identification of yeast probiotic isolates

With the help of specific primers, the conserved region of the isolates was amplified and the size was found to be of 758 bp and 698bp respectively in agarose gel (Figure 3). The nucleotide sequence obtained was BLAST and the results were compared with the database of NCBI. The results obtained depicted that the isolate obtained from jowar flour had 97.47% identity with \ *Saccharomyces cerevisiae*

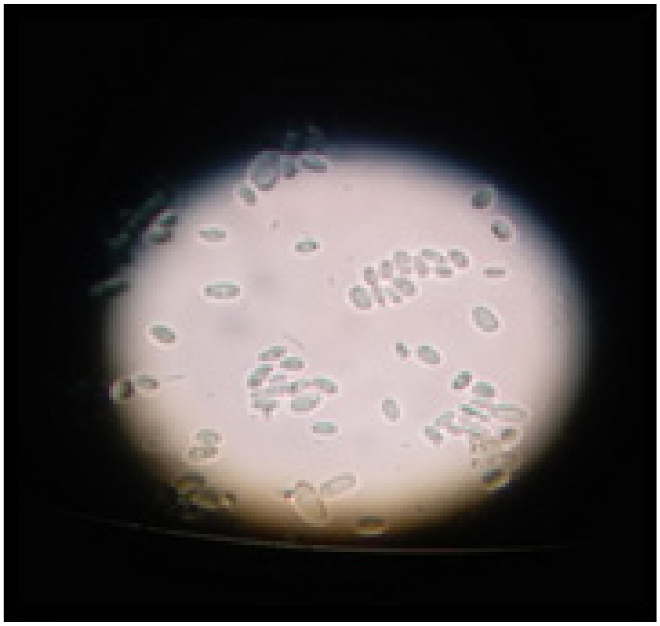

Figure 2: Micrograph of yeast cells

(CBS 1171) and that of bajra flour shows 98.57% identity to *Saccharomyces arboricola* (CBS 10644). The isolates and the sequence was submitted to NCBI The accession number obtained for *Saccharomyces cerevisiae* was MZ068117 and for *Saccharomyces arboricola* was MZ068118 (Table 2). (Zhimo et al., 2016). Isolated 29 yeasts were isolated from different sources and among the isolates, YZ 1, YZ 7 and YZ 27 showed broad spectrum of antagonistic activity (mycelial growth inhibition) against the test pathogens of banana *in vitro* which were identified by molecular methods as *Candida tropicalis* YZ 1 (CtYZ 1), *Saccharomyces cerevisiae* YZ 7 (ScYZ 7) and *C. tropicalis* YZ 27 (CtYZ 27).

3.3. *Nucleotide sequence analysis and construction of phylogenetic tree*

Thirty known sequences which showed 98% similarity with the sequences of *Saccharomyces cerevisiae* and for *Saccharomyces arboricola* submitted to NCBI database were collected and the phylogenetic tree was using neighbour joining approach in MEGA X software. (Figure 4 and 6) Wang et al. (2008) identified a novel *Saccharomyces* spp isolated from bark and developed a phylogentic tree for identification of related species. (Kurtzman and Robnett, 2003). Stated that Satisfactory resolution of the phylogenetic relationships among *Saccharomyces* species is problematic. The combined 18S–5.8S–26S rDNA sequence analysis did not resolve the species relationships of the genus

3.4. *Motif analysis*

A motif signifies repeated sequence patterns occurs among related species. MEME software discovers novel and un-gapped motifs (recurring and fixed-length patterns) in sample sequences (sample output from sequences). MEME

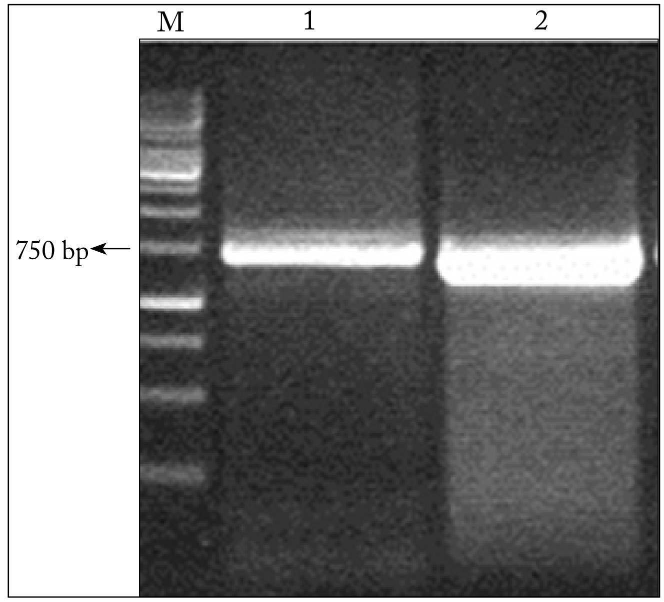

Figure 3: Gel electrophoresis of isolated Streptomyces strain (M: ladder, 1: jowarisolate, 2: bajra isolate)

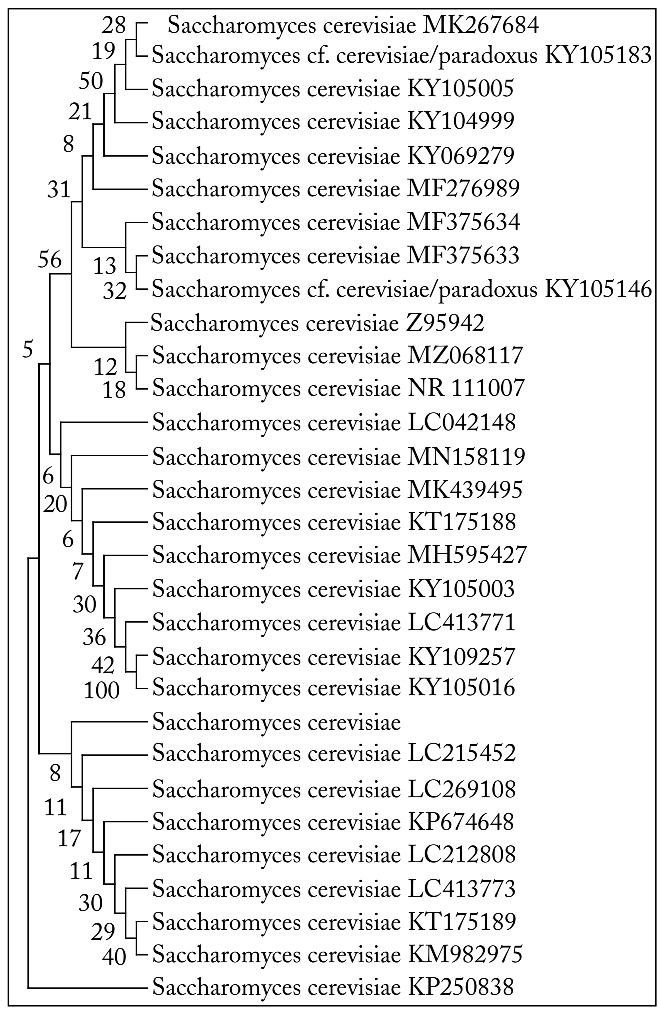

Figure 4: Phylogenetic analysis of 30 related isolates of *Saccharomyces cerevisiae*

Table 2: Details of culture submitted to NCBI

Organism	Classification	Definition	NCBI Submitted	Sequence
Saccharomyces cerevisiae	Eukaryota; Fungi; Dikarya; Ascomycota Saccharomycotina Saccharomycete Saccharomycetales Saccharomycetaceae Saccharomyces.	Saccharomyces cerevisiae isolate KGR9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	MZ068117	1tgaacttaagcatatcataaaaagaaatttaataattttgaaaatg- gattttttgtttt 61ggcaagagcatgagagcttttactgggcaagaagacaagaga- gagtccagccgggcctgc 121gcttaagtgcgcggtcttgctaggcttgtaagtttctttcttgc- tattccaaacggtgag 181agatttctgtgcttttgttataggacaattaaaaccccatcaata- caacacactgtggag 241ttttcatatctttgcaactttttctttgggcattcgagcaatc- ggggcccagaggtaaca 301aacacaaacaatcattaaatttttgtcaaaaacaagaattttcg- taactggaaattttaa 361aaatattaaaaactttcaacaacggatctcttggttctcgcatc- gatgaagaacgcagcg 421aaatgcgatacgtaatgtgaattgcagaattccgtgaatcatc- gaatctttgaacgcaca 481ttgccccccttggtattccagggggcatgcctgtttgagcgt- catttccttctcaaacatt 541ctgtttggtagtgagtgatactctttggagttaacttgaaatt- gctggccttttcattgg 601atgtttttttccaaagagaggtttctctgcgtgcttgaggtata- atgcaagtacggtcg 661ttttaggtataaccaactgcggctaatctttttatactgagcg- tattggaacgttatcg 721ataagaagagagcgtctaggcaacaatgttcttaaagt
Saccharomyces arboricola	Eukaryota; Fungi; Dikarya; Ascomycota Saccharomycotina Saccharomycete Saccharomycetales Saccharomycetaceae Saccharomyces.	Saccharomyces arboricola isolate KGR92 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	MZ068118	1ctgggcaagagtataagagatggagagttccaggggcctgcgct- taagtgcgcggtcttt 61 ctagacttgtaagtttctttcttgctattccaaacagtga gaga- tttctctgtttttgtt 121ataggacaaataaaaccgttttgtatacaacacactgtggagttt- tatatctttgcaact 181ttttctttgggttttcgagcaatcgttagcccagaggaacaaa- cacaaacaattttatt 241tattcattatcaaatttttgtcaaaaacaagaattttcgtaactg- gaaattttaaaaata 301ttaaaaactttcaacaacggatctcttggttctcgcatcgat- gaagaacgcagcgaaatg 361cgatacgtaatgtgaattgcagaattccgtgaatcatcgaatcttt- gaacgcacattgcg 421ccccttggtattccagggggcatgcctgtttgagcgt- catttccttctcaaacattctgt 481ttggtagtgagtgatactctctggagttaacttgaaattgctg- gccttttcattggatgt 541tttttttccaaagagaggtttctctacgtgcttgaggttaatg- caagtacggtcgtttt 601aggttttaccaactgcggctaatctttttgtactgagcgtattg- gaacgttatcgataa 661gaagagagcgtctaggcgaacaatgttcttaaagttga

splits variable-length patterns into two or more separate motifs. With the help of TOMTOM programme, DNA sequences of the *Saccharomyces* strains under the study were subjected to similar motifs analysis. Analysis showed 5 motifs which were found to be conserved in the DNA sequences of S. *cerevisiae* (Figure 5 and Table 3) and *Saccharomyces arboricola* (Figure 6 and Table 4). Conserved sequences with the help of motif analysis helps us to find homology among different organisms and species during computational analysis (Wong et al., 2015). In relation to biological significance, the conserved sequences found between species are the coding sequences which may retain the structural and functional integrity of any particular protein present in the organism (Janda and Abbott, 2007)

Probiotic bacteria are increasingly used in food and pharmaceutical applications to balance disturbed intestinal microflora and related dysfunction of the human gastrointestinal tract (Kailasapathy and Chin, 1999). The pharmaceutical applications of probiotics have been reported by several other workers also (Molin, 2001). As most of the commercial preparation of probiotics contain the edible yeast and bacteria these were isolated from natural sources under laboratory condition (Oliveria et al., 2002).

The antagonistic properties of yeasts have been used in numerous promising agricultural applications as natural bio-control agents, both as soil treatments and for preventing diseases in pre- and post-harvest crops. In 1995, the USA environmental protection agency registered *Candida oleophila* as bio-control post-harvest yeast (El-neshawy and Wilson, 1997). probiotic yeast was effective in controlling post-harvest diseases in grapes when the inoculum load was less and also reported that it improved the quality of fruits (Greeshma et al., 2020).

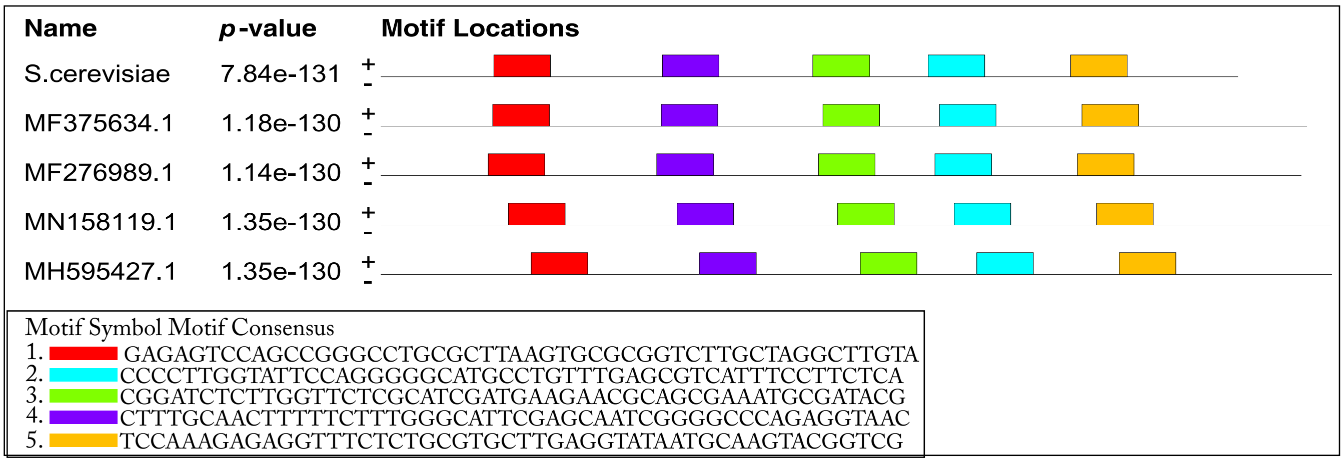

Figure 5: Motif locations and consensus among the tested *Saccharomyces* isolates

Table 3: Motif locations of *Saccharomyces cervisiae* with closest related other *Saccharomyces* isolates

Isolates	e-values	Start	Probability value	Site count	width	Isolates	e-values	Start	Probability value	Site count	width
S. cerevisiae	5.6e-067	101	1.89e-32	5	50	MN158119.1		405			
MF375634.1		100				MH595427.1		425			
MF276989.1		96				*S. cerevisiae*	5.9e-062	485	1.06e-31	5	50
MN158119.1		114				MF375634.1		495			
MH595427.1		134				MF276989.1		491			
S. cerevisiae	1.3e-063	250	2.50e-31	5	50	MN158119.1		508			
MF375634.1		249				MH595427.1		528			
MF276989.1		245				*S. cerevisiae*	4.1e-061	611	3.84e-31	5	50
MN158119.1		263				MF375634.1		621			
MH595427.1		283				MF276989.1		617			
S. cerevisiae	8.7e-063	383	1.62e-31	5	50	MN158119.1		634			
MF375634.1		392				MH595427.1		654			
MF276989.1		388									

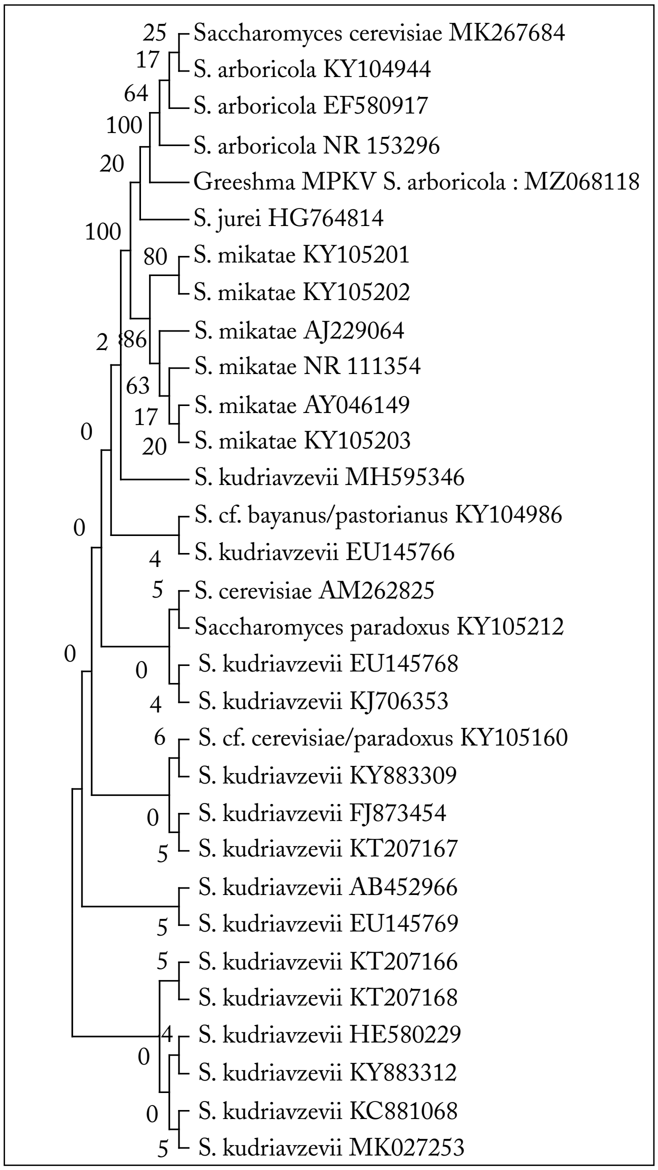

Figure 6: Phylogenetic analysis of 30 related isolates of *Saccharomyces arboricola*

Table 4: Motif locations of *Saccharomyces arboricola* with closest related other *Saccharomyces* isolates

Isolates	e-values	Start	Prob-ability value	Site count	width
S. arboricola	9.0e-067	34	3.44e-31	5	50
NR_153296.1		138			
EF580917.1		117			
KY104944.1		70			
KY105203.1		133			
S.arboricola	3.0e-063	318	1.38e-31	5	50
NR_153296.1		417			
EF580917.1		396			
KY104944.1		349			
KY105203.1		413			
S. arboricola	1.3e-060	407	2.23e- 32	5	50
NR_153296.1		506			
EF580917.1		485			
KY104944.1		438			
KY105203.1		502			
S. arboricola	1.5e-059	570	5.42e-31	5	50
NR_153296.1		669			
EF580917.1		648			
KY104944.1		601			
KY105203.1		713			
S. arboricola	2.3e-059	629	5.42e-31	5	50
NR_153296.1		728			
EF580917.1		707			
KY104944.1		709			
KY105203.1		772			

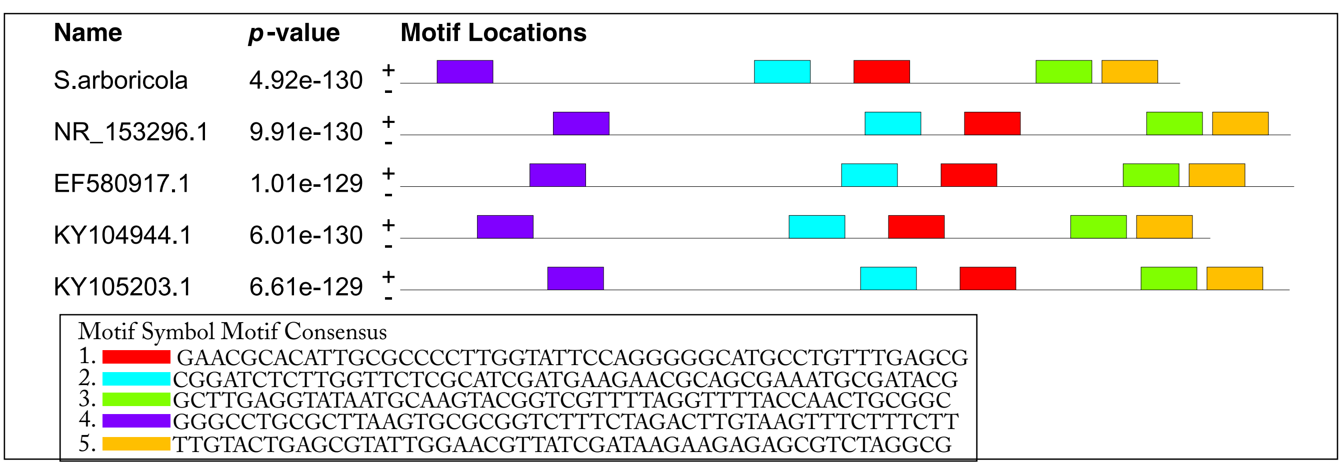

Figure 7: Motif locations and consensus among the tested *Saccharomyces arboricola* isolates

4. CONCLUSION

Cereal grain flours of jowar and bajra were used to isolate the probiotic yeast and these isolates were characterized for differences among them on the basis of cultural growth, microscopic and molecular observation. The results depicted that the isolate obtained from jowar flour had 97.47 per cent identity with *Saccharomyces cerevisiae* and that of bajra flour shows 98.57 per cent identity to *Saccharomyces arboricola*. Motif Analysis with the aid of TOMTOM programme showed 5 motifs which were found to be conserved in the DNA sequences of *S. cerevisiae* and *Saccharomyces arboricola*.

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