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Research Article

MOLECULAR IDENTIFICATION OF PROBIOTIC YEAST STRAINS AND THEIR CHARACTERIZATION

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ABSTRACT

Objective: The objective of this study was to identify the potential yeast isolates at themolecular level and to evaluate their probiotic characteristics.

Methods: Molecular characterization was done for five potential probiotic yeast strains. *In vitro* assays have been conducted to evaluate the probiotic properties such as NaCl tolerance, autoaggregation, and coaggregation. Hemolytic activity, urease activity, and cytotoxicity tests were carried out for safety assay during the characterization of yeast strains.

Results: In this study, the yeast strains, *viz*., LM, MR, GOI, GII2, and WI was identified at the molecular level and named as *Yarrowia lipolytica* VIT-MN01, *Kluyveromyces lactis* VIT-MN02, *Lipomyces starkeyi* VIT-MN03, *Saccharomycopsis fibuligera* VIT-MN04, *Brettanomyces custersianus* VIT-MN05, respectively. Maximum autoaggregation (92%) and coaggregation (97 %) were noted in case of *L. starkeyi* VIT-MN03. All yeast strains showed nonhemolytic activity. *In vitro* toxicity assay was performed and all the yeast strains showed nontoxic nature.

Conclusion: Five yeast strains have been studied for their probiotic characteristics and identified at molecular level. Out of five yeast strains, three strains showed maximum adhesion ability, which is a prerequisite for colonization and protection of gastrointestinal tract. All the yeast strains are validated as a safe bioresources because of their nonhemolytic activities and nonproduction of urease. It can be concluded that the identified yeast strains can serve as promising probiotics in various fields of food industry.

Keywords: Autoaggregation, Coaggregation, Cytotoxicity, Hemolytic activity, Molecular identification.

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INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate quantity confer health benefits to the host [1]. Probiotic bacteria, generally incorporated into fermented dairy products, mainly belong to the genera *Lactobacillus* and *Bifidobacterium* [2]. The use of yeasts as dietary supplements is still limited. Some yeast strains, *viz*., *Saccharomyces cerevisiae* and *Saccharomyces boulardii* have been reported as probiotics in humans for many years as they are exerting some influence on the intestinal flora [3,4]. Adhesion to the intestinal mucosa is one of the main selection criteria for probiotics. Autoaggregation is clumping of microbes which belong to the same strain, while coaggregation is the result of cell-to-cell recognition between two different microbial strains. Autoaggregation of probiotic strains has been correlated with adhesion to intestinal epithelial cells, known to be a prerequisite for colonization and enhanced persistence in the gastrointestinal system. Coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms [5]. Several studies have been conducted using human epithelial cell lines *viz.* HT-29, HT-29MTX, and Caco-2 to screen the adhesion capacities of probiotic strains [6]. The absence of hemolytic activity is considered as a safety prerequisite for the selection of a probiotic strain [7]. In this study, we aimed to identify five probiotic yeast strains at molecular level which have already been reported as potential probiotic strains in our previous study [8]. Hemolytic activity, urease activity, and cytotoxicity tests were done to evaluate and characterize the strains as potential probiotics.

METHODS

Taxonomic identification of yeast

The yeast isolates - namely, LM, MR, GOI, GII2, and WI - were subjected to molecular characterization by 18S, ITS regions, and D1/D2 domains. The genomic DNA of the isolated yeasts was amplified using the primers, UL18R:5'-TGTACACACCGCCCGTC-3' and UL28R:5'-ATCGCCAGTTCTGCTTAC-3' (universal primers; both designed by Acme progen biotech India Pvt., Ltd). The purified PCR products were characterized by partial and complete sequence analysis. The resulting consensus rRNA sequence was then compared to the nonredundant National Center for Bioinformatics database using blast similarity searches [9]. A collection of closely related sequences from this database was used to construct phylogenetic and molecular evolutionary tree by molecular evolutionary genetics analysis version 5 [10].

Autoaggregation and coaggregation

The analysis of autoaggregation was carried out following the method of Lohith and Anu [11] with minor modifications. The cell pellets were obtained after washing and resuspending the cells with phosphate buffered saline to obtain a final cell density of around 1×10^9 CFU/ml at 600 nm. 4 ml of each yeast suspension was divided into sterile test tubes. The tubes were vortexed and incubated for 3 hrs, 5 hrs and 24 hrs, respectively. Absorbance was read at 600 nm against the blank solution. The autoaggregation (%) was calculated using the following formula.

$1-(At/A0)\times100$

Where, At- Absorbance readings at different time points (t = 3, t = 5 and t = 24)

A_0 - Absorbance readings taken initially.

Coaggregation test

The coaggregation ability of yeast strains with bacterial pathogens was evaluated following the method of Jankovic *et al*. [12] with

modifications. Bacterial pathogens, *viz*., *Salmonella* and *Klebsiella* were obtained in log phase culture. The yeast and the pathogenic cell suspension were prepared with the final density of 1×10^9 CFU/ml at 600 nm. 2 ml each pathogen and the yeast cells were dispensed into sterile tubes. The tubes were thoroughly mixed and incubated for 60 minutes. The absorbance was read at 600 nm. Control tubes each of pathogens and the yeast cells were prepared and absorbance was read individually. The percentage of coaggregation was determined according to the formula:

$$\text{Coaggregation}(\%) = \frac{\left(\frac{Ax + Ay}{2}\right) - A(x + y)}{Ax + \frac{Ay}{2}} \times 100$$

Where x and yrepresent yeast strains in the control tubes, and (x+y) the mixture.

Hemolytic activity

The experiment was carried out to determine the hemolytic activity of the yeast cells. The strains were streaked on blood agar plates and incubated at 37°C for 24 hrs and later checked for hemolysis following the method of Manns *et al.* [13].

NaCl tolerance

For the determination of NaCl tolerance, MRS broth containing different NaCl concentration between 1% and 8% was sterilized, each test tube was inoculated with 1% (v/v) fresh overnight culture of *Lactobacillus* and incubated at 37°C for 24 hrs. After the incubation, their growth was determined by observing their turbidity at 600 nm [14].

Urease activity

The media composition for urease test are; urea (20 g/l), Na2HPO4 (9.5 g/l), KH2PO4 (9.1 g/l), and 0.01 g phenol and pH was made to 7. This enzyme converts urea to ammonia and CO_2, which convert the environment alkaline and turns pink color referred as urease positive. All yeast strains were inoculated in urea media and incubated at 37°C for 24 hrs [15].

***In vitro* cytotoxicity**

Cells were harvested at a density of 10^4 cells as 200 µl suspension. Plates were incubated for 24 hrs in saturated humid conditions at 37°C. Cells treated with medium only, served as control. At the end, sulforhodamine B (SRB) assay was conducted. In brief, old media with the formulation was discarded and 200 µl complete media was added. Then, cells were fixed by adding 50 µl of ice-cold 50% trichloroacetic acid slowly to the medium and incubated at 4°C for 1 hr. Further, plates were washed five times with deionized water and dried in air. 100 µl of 0.4% SRB dissolved in 1% acetic acid was added to the fixed cells and kept at room temperature for 20 minutes after which they were washed with 1% acetic acid to remove unbound dye. The plates were dried and 100 µl of 10 mM Tris base was added to each plate and kept for 20 minutes to solubilize the dye. Thereafter, the plates were placed on a shaker to allow mixing and the absorbance (OD) of each sample was measured at 560 nm [16]. Cell viability was measured using the formula:

% Viability = Absorbance of sample/Absorbance of control ×100

RESULTS AND DISCUSSION

Taxonomic identification of yeast

Molecular characterization of probiotic yeast isolates, *viz*., LM, MR, GOI, GII2, and WI was done. Identified yeasts showed 92-96% of confidence level with respective genus such as *Yarrowia lipolytica* (LM), *Kluyveromyces lactis* (MR), *Lipomyces starkeyi* (GOI), *Saccharomycopsis fibuligera* (GII2), and *Brettanomyces custersianus* (WI). Phylogenetic analysis was performed by the neighbor-joining algorithm as shown in Fig. 1. The partial and completed sequences of the potential probiotic yeasts (LM, MR, GOI, GII2, and WI) were submitted to the Genbank database under the accession number KY817588, KY817590, KY817589, KY852445, and KY852444, respectively (Fig. 1).

Autoaggregation ability

The autoaggregation ability of the strains is one of the proposed mechanisms to explain the protective role of *Lactobacilli* where hydrophobicity may be playing a role in the cellular interaction [17]. In this study, the autoaggregation rate was measured over a period of 6. Autoaggregation ability of the yeast strains is shown in Fig. 2.

The yeast isolates showed significant difference in their aggregation properties and most of them showed more than 50% autoaggregation. *L. starkeyi* VIT-MN03 showed the highest autoaggregation (92%), whereas lowest autoaggregation was noted in *Y. lipolytica* VIT-MN01 (52%). Therefore, the majority of the yeast isolates showed >50% autoaggregation capacity, which can prevent the invasion of various other pathogenic microorganisms through biofilm formation. This quality of adherence can also cause increased persistence in the gastrointestinal tract. Such adherence capacity is only possible through surface proteins and they are usually strain-specific [18].

Coaggregation assay

The coaggregation assay is a reliable method to evaluate the close interaction between probiotic microbes and pathogenic bacteria. Coaggregation ability of the yeast strains associated with *Salmonela* sp. is shown in Fig. 3a. All the strains showed aggregation ability more than 67% after 6 hrs. The yeast strains *L. starkeyi* VIT-MN03 (96%) showed highest coaggregation ability followed by *S. fibuligera* VIT-MN04 (91%), *B. custersianus* VIT-MN-05 (86%), *K. lactis* VIT-MN02 (81%), and *Y. lipolytica* VIT-MN01 (67%). Fig. 3b shows the percentage of coaggregation ability of yeast strains with *Klebsiella* sp. Three strains have proved to have binding capacity >70%. The yeast strains *L. starkeyi* VIT-MN03 (97%) showed highest coaggregation ability followed by *S. fibuligera* VIT-MN04 (93%), *B. custersianus* VIT-MN-05 (85%), *Y. lipolytica* VIT-MN01 (78%) and *K. lactis* VIT-MN02 (72%). This study showed the ability of yeast cells to coaggregate with other bacteria with a capability of >70%. Hence, these yeast strains can serve as potential probiotic candidates who can help in preventing bacterial colonization and secreting antimicrobial substances [19].

Hemolytic activity

The yeast strains were tested for hemolytic activity. None of the isolates exhibited hemolysis (clear blood lysis zones) in blood agar plates which proved that the yeast strains under study are non-pathogenic (Fig. 4).

NaCl tolerance

Fig. 5 shows survival and good growth of yeast isolates at different NaCl concentrations which shows that these organisms can combat the pathogen and can stop their establishment in the presence of high salt concentrations [20]. Among the five yeast strains, 3 strains, *viz*., *L. starkeyi* (GOI), *S. fibuligera* (GII2), and *B. custersianus* (WI) showed increased growth at 8% NaCl. Similar result was reported in probiotic bacteria by Modi *et al.,* (2014).

Urease activity

All the yeast strains exhibited negative result without producing pink color which indicated the nonproduction of urease (Fig. 6). This confirmed the nontoxic nature of the yeast isolates [21].

***In vitro* cytotoxicity**

In vitro cytotoxicity assay was performed using SRB, the chemical which is used to predict human toxicity and for the general screening of toxic chemicals [22]. No cytotoxic nature of the yeast isolates was noted as there was no loss of viability (Fig. 7).

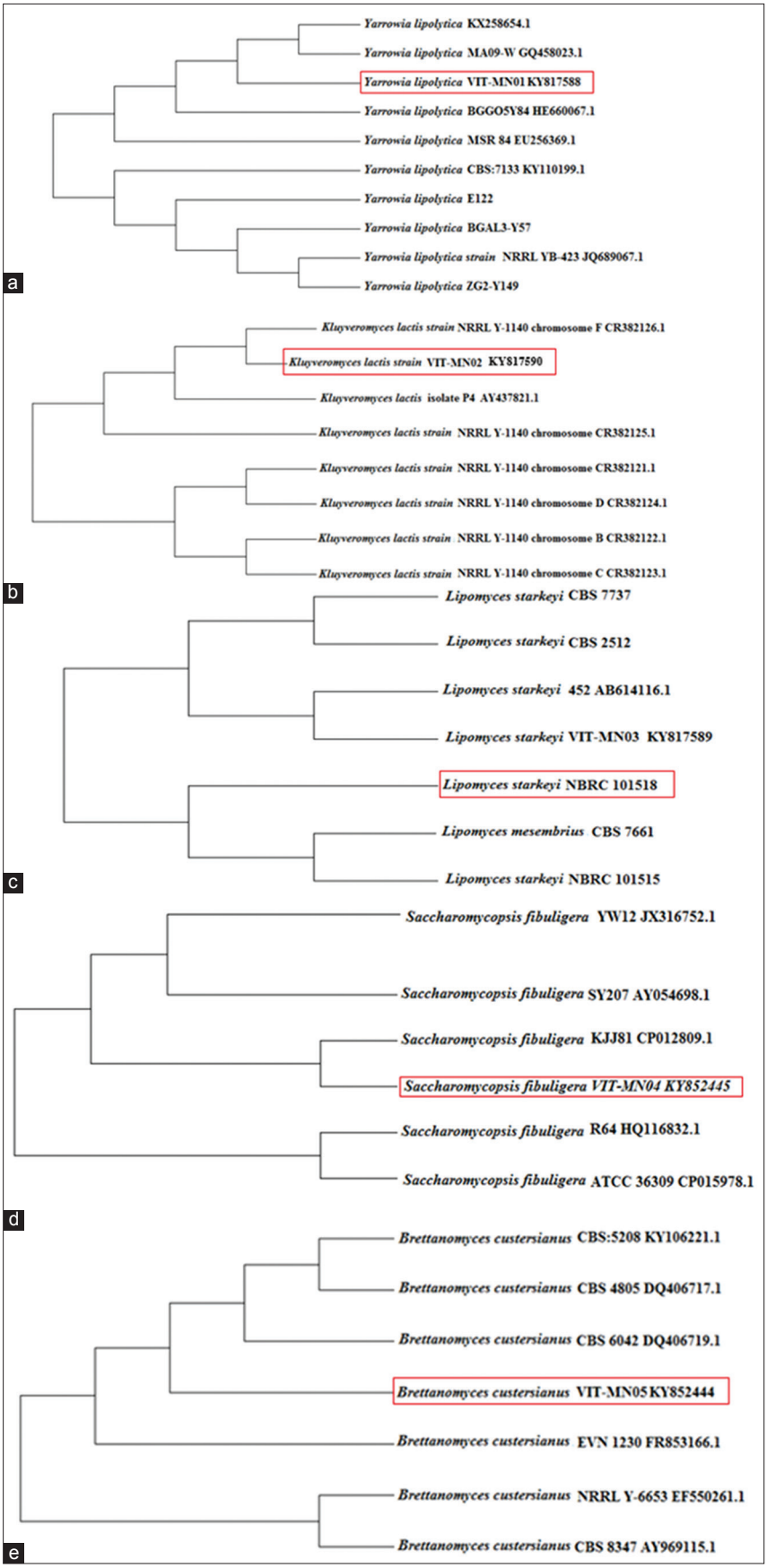

Fig. 1: Molecular identification of probiotic yeast strains, (a) LM, (b) MR, (c) GOI, (d) GOI, and (e) GII2

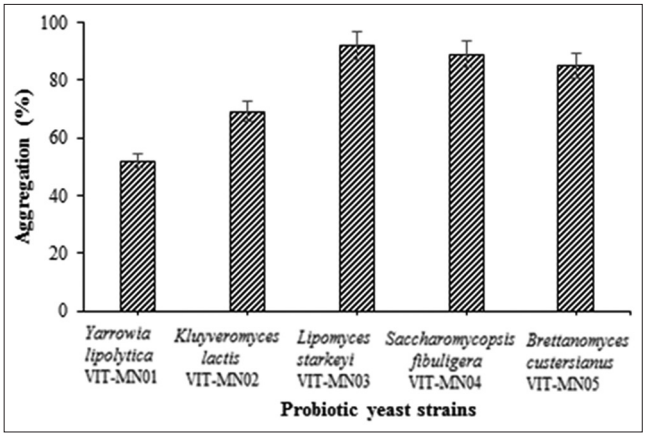

Fig. 2: Autoaggregation of probiotic yeast strains

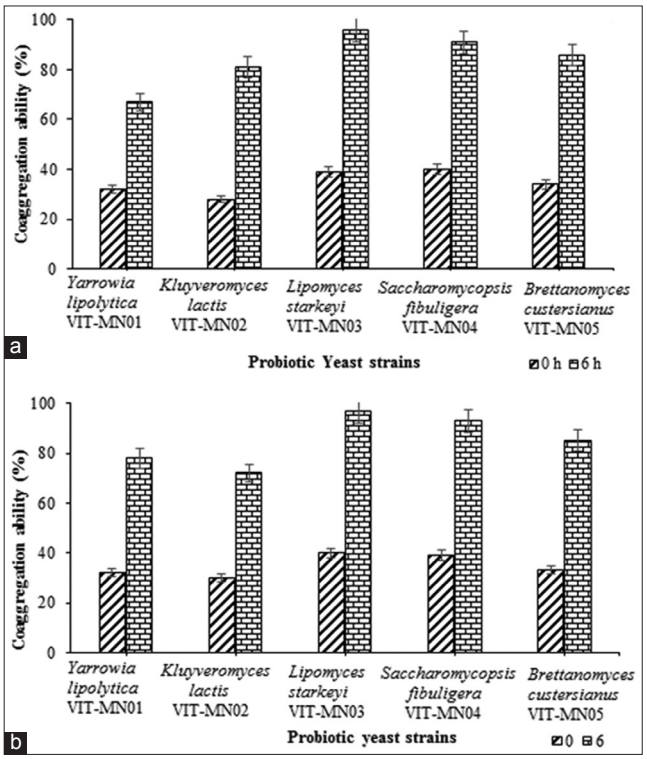

Fig. 3: Coaggregation of probiotic yeast with pathogenic bacteria, (a) *Salmonella* sp. and (b) *Klebsiella* sp.

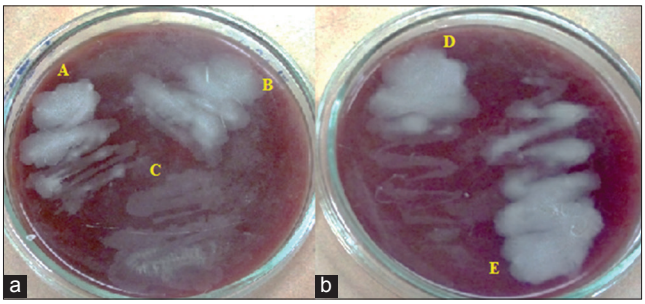

Fig. 4: (a and b) Hemolytic activity of yeast strains (A) *Yarrowia lipolytica* VIT-MN01, (B) *Kluyveromyces lactis* VIT-MN02, (C) *Lipomyces starkeyi* VIT-MN03, (D) *Saccharomycopsis fibuligera* VIT-MN04, and (E) *Brettanomyces custersianus* VIT-MN05

CONCLUSION

In this study, five yeast strains were identified at the molecular level and found to possess the desirable *in vitro* probiotic properties based on their autoaggregation, coaggregation, and nonhemolytic capacities. Being non producer of urease, all the yeast strains were confirmed as safe bioresources. *In vitro* studies on cytotoxicity with SRB demonstrated that no toxic substance was produced by the probiotic yeast strains. Therefore, the identified yeast strains *viz. Y. lipolytica* (LM), *K. lactis* (MR), *L. starkeyi* (GOI), *S. fibuligera* (GII2), and *B. custersianus* (WI) can be used as potential probioticsin functional foods and health-related products.

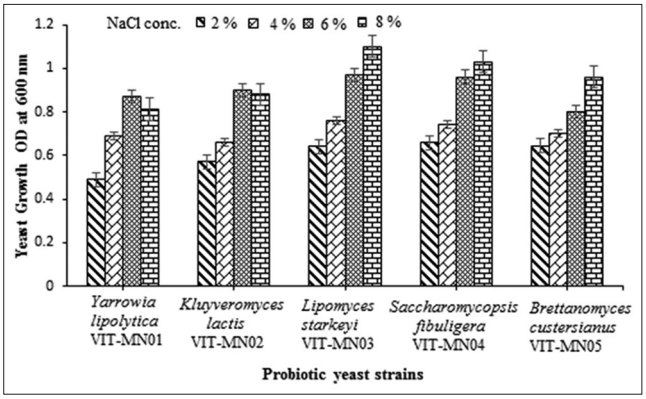

Fig. 5: NaCl tolerance of probiotic yeast strains

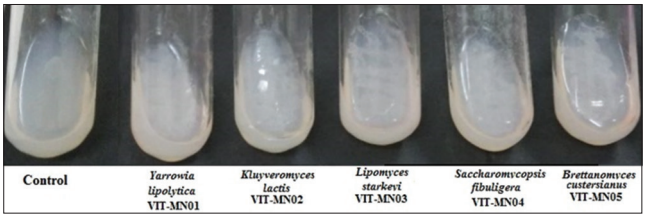

Fig. 6: Urease activity of probiotic yeast strains

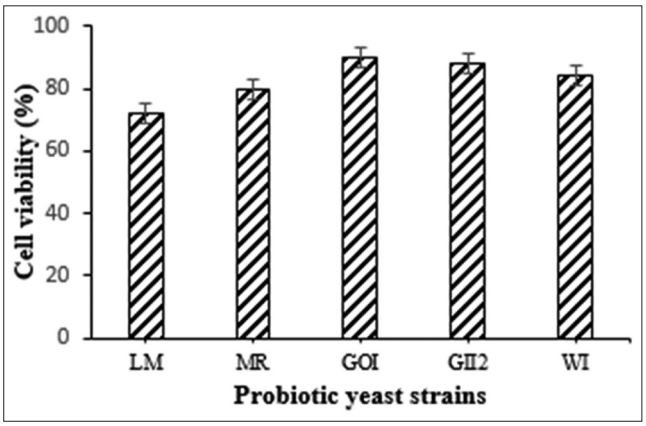

Fig. 7: Cytotoxicity of probiotic yeast strains

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