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Research Article

ISOLATION AND CHARACTERIZATION OF POTENTIAL PROBIOTIC YEASTS FROM DIFFERENT SOURCES

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ABSTRACT

Objective: The main objective of this study is to isolate yeasts from different environmental sources to investigate their potential probiotic characteristics.

Methods: Appropriate *in vitro* assays has been conducted to examine their probiotic potentiality such as acid and bile salt tolerance, temperature resistance, stimulated gastrointestinal tract tolerance, cell adhesion, and cholesterol removal. All the yeast isolates were tested under *in vitro* conditions.

Results: In this study, 20 yeast strains have been isolated from different sources, screened and their desirable probiotic properties, *viz.,* pH tolerance, bile salt tolerance, and thermotolerance have been evaluated. Screened yeast isolates treated with gastric juices showed increased survival rate above 60%. A further *in vitro* study investigates cholesterol removal and it showed increased cholesterol removal rate up to 92%. Exopolysaccharide production was estimated for selected yeast isolates and applications are under investigation.

Conclusion: Among 20 yeast isolates, 5 isolates showed increased growth under stress tolerance. It can be concluded that the screened yeast isolates lemon, millet root, goat intestine outer, goat intestine inner, and wine industry can serve as promising probiotics in various fields of food industry.

Keywords: Acid tolerance bile tolerance, Cholesterol removal, Probiotics, Yeast.

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INTRODUCTION

Probiotics are defined as living bacteria that, when administered in adequate amounts, confer a health benefit on the host [1]. The human digestive tract comprises a variety of ecological niches populated by several bacterial species that have established a symbiotic relationship with the host. This bacterial population, also called the intestinal microbiota, plays an important role in the development of the gut immune system, digestion of food, production of short-chain fatty acids and essential vitamins, and resistance to colonization from pathogenic microorganisms [2]. Criteria for selection of probiotic strains have recently been formulated by the Food and Agriculture Organization/World Health Organization. The selection of a probiotic vary, but usually includes the ability to adhere to mucus and epithelial cells [3], and survival at low pH (1.0-3.0) and bile salts of approximately 0.3% [4]. Most probiotic tests were performed *in vitro* [5]. Health benefits described for probiotics include prevention or treatment of infectious diseases, irritable bowel syndrome, allergies, lactose intolerance, colon cancer, and reduction of serum cholesterol levels [6]. In the last century, many studies have reported probiotic bacteria to play important roles in the modulation of immunological, respiratory, and gastrointestinal functions [7]. Furthermore, probiotics have been shown to play a protective role by directly competing with intestinal pathogens through the release of antibacterial substances such as bacteriocins [8] or metabolites such as acetic acid and lactic acid [9]. The most commonly used bacterial probiotics include *Lactobacillus* species, *Bifidobacterium* species, *Escherichia coli*, *Streptococcus* species, *Lactococcus lactis,* and some *Enterococcus* species. At present, the only probiotic yeast used is the non-pathogenic *Saccharomyces boulardii*. Numerous studies have investigated the use of probiotic bacteria for the delivery of gastrointestinal therapeutics; however, eukaryotic probiotics have been less well studied. A major advantage of using probiotic yeast for this application is their ability as eukaryotes to create post-translational modifications that might enable expression of a wide variety of therapeutic proteins in their proper conformation [10]. Live *Saccharomyces cerevisiae* cells are employed as biotherapeutic or probiotic agents for re-equilibration of intestinal microflora, and the latest studies have demonstrated their efficacy in treating chronic or recurrent diarrhea, especially in those cases associated with clostridium difficile [11]. Probiotic bacteria beneficially improve microbiota balance in gastrointestinal tract (GIT) to counteract local immunological dysfunction, stabilize the intestinal mucosal barrier function and prevent pathogen proliferation and function. [12]. Most of the *in vitro* studies have been studied in bacteria only not in yeast. Hence, we have studied yeast probiotics in this study.

METHODS

Sample collection and isolation

About 20 samples were collected from sources such as fruits, vegetables, plants, meats, insects, and industries. Yeast was grown to saturation in normal yeast extract peptone dextrose (YEPD) media (1% yeast extract, 2% peptone, and 2% glucose/dextrose in distilled water) followed by serial dilution of 10^4-10^6, then incubated at 37°C for 24-48 hrs. 20 yeast strains were isolated by pour plate method.

Acid and bile salt tolerance

Acid tolerance was evaluated by growing isolated yeasts in YEPD broth either adjusted broth to pH 1-12 using 1N HCl and 1 N NaOH [13]. For bile salt tolerance bovine salt added into broth at various concentrations from 0.2% to 1.5%, then incubated at 37°C for 24 hrs [13]. Growth was measured using spectrophotometer, reading the optical density at 600 nm against uninoculated broth.

Thermotolerance

Yeast isolates were inoculated in YEPD broth adjusted to pH-2 and incubated at different temperatures such as 25°C, 37°C, and 45°C for 24 hrs. Growth was measured at 600 nm.

Table 1: List of various sources for yeast isolation

Source	Origin	Collected from	Colony morphology
AP	Fruits	Koyambedu fruit market, Chennai, Tamil Nadu, India	White, circular, flat
GB		Koyambedu fruit market, Chennai, Tamil Nadu, India	Pale yellow, circular, raised
LM		Koyambedu fruit market, Chennai, Tamil Nadu, India	Creamy white, circular, flat
OR		Koyambedu fruit market, Chennai, Tamil Nadu, India	Creamy white, circular, flat
P		Koyambedu fruit market, Chennai, Tamil Nadu, India	Creamy white, circular, raised
BG	Vegetables	Vegetable market, Vellore, Tamil Nadu, India	Milky white, circular, convex
BS		Vegetable market, Ooty, Tamil Nadu, India	Pale yellow, circular, raised
R		Vegetable market, Ooty, Tamil Nadu, India	Milky white, circular, raised
SP		Vegetable market, Vellore, Tamil Nadu, India	Milky white, circular, raised
G		Vegetable market, Vellore, Tamil Nadu, India	Pale yellow, circular, convex
GT	Plants	Tea estate, Kashmir, India	Creamy white, circular, raised
MR		Millet field, Thuraiyur, Trichy, Tamil Nadu, India	Creamy white, circular, raised
NF		Tanjore, Tamil Nadu, India	Pale yellow, circular, convex
SF		Tanjore, Tamil Nadu, India	Creamy yellow, circular, raised
DF	Meat	Kannyakumari beach, Tamil Nadu, India	Milky white, circular, flat
GII		Madurai, Tamil Nadu, India	Creamy white, circular, convex
GOI		Madurai, Tamil Nadu, India	Pale yellow, circular, raised
GM		Madurai, Tamil Nadu, India	Pale yellow, circular, raised
SFL	Industries	Ariyalur, Tamil Nadu, India	Milky white, circular, raised
WI		Chennai, Tamil Nadu, India	Creamy white, circular, convex

SFL: Sugar factory liquid, WI: Wine industry, GM: Goat milky, GOI: Goat intestine outer, GII: Goat intestine inner, DF: Dry fish, SF: Sun flower, NF: Neem flower, MR: Millet root, GT: Green tea leaves, G: Garlic, SP: Sweet potato, R: Radish, BS: Beans, BG: Bottle guard, P: Papaya, OR: Orange, LM: Lemon, GB: Goose berry, AP: Apple

Cholesterol removal

About 1% of bile salt and 100 µg of water soluble cholesterol were added into YEPD broth adjusted to pH-2, then yeast isolates were inoculated and incubated at 37°C for different time in travels - 4th, 8th, 12th, 24th, and 48th hr. 5 ml of culture was taken in each in travel and centrifuged at 5000 rpm for 5 minutes [14,15]. Cholesterol assimilation rate was measured using supernatant optical density at 600 nm. Growth of yeast isolates was measured at 600 nm to check the rate of cholesterol removal and following formula was used to calculate the assimilation late,

$$\frac{\text{Cholesterol conc in control} - \text{Cholesterol concentration in sample}}{\text{Cholesterol concentration in control}} \times 100$$

Preparation cell suspension

Yeast isolates were inoculated in YEPD broth at 37°C for 24 hrs and centrifuged at 5000 rpm at 4°C for 5 minutes. Cell pellet was washed with PBS buffer (pH-7.0). This cell suspension was used for further analysis.

Stimulated GIT tolerance

This analysis was performed by following steps [15]; pepsin (3 mg) was added into 1 ml of prepared cell suspension, and then incubate at 37°C for 3 hrs. After incubation 1% of bile salt and 3 mg of pancreatin were added into YEPD broth adjusted to pH-8. Tubes were incubated at 37°C for 4 hrs, and then 1 ml of aliquot was plated in YEPD agar containing 0.5% $CaCO_3$. Survival rate (in %) is calculated using following formula,

$$\frac{\text{Log CFU/ml at 3 hrs}}{\text{Log CFU/ml at 4 hrs}} \times 100$$

Cell adhesion hydrophobicity

Selected isolates were measured for cell surface hydrophobicity by measuring microbial adhesion to hydrocarbons [15]. N-hexadecane was added into 1 ml of cell suspension and vortex for 3 minutes, and then tubes were incubated at room temperature for 20 minutes. Aqueous phase (lower phase) transferred into fresh tube and growth was measured at 600 nm. Hydrophobicity index was calculated using following formula

$$\frac{\text{Before OD - After OD}}{\text{Before OD}} \times 100$$

Antibiotic susceptibility test

Antibiotic sensitivity assay was carried out by disc agar diffusion method [16] in which discs of 10 antibiotics bearing different concentrations were taken: Amoxicillin (30 µg/ml), ampicillin (20 µg/ml), gentamycin (10 µg/ml), ketoconazole (10 µg/ml), levofloxacin (10 µg/ml), rifampicin (20 µg/ml), vancomycin (20 µg/ml), tetracyclin (30 µg/ml), tigecyclin (20 µg/ml), and trimethoprim (20 µg/ml). YEPD agar plates were prepared and on it pure cultures of yeast isolates from the YEPD broth were swabbed. After swabbing all the plates, antibiotics discs were placed on the agar plates using forceps. The plates were incubated at 28°C for 24-48 hrs. The drug sensitivity of the probiotic strains evaluated and zone of inhibition around probiotic growth was measured.

Antimicrobial activity

Antimicrobial activity of the screened yeast isolates was tested against *E. coli, Bacillus* sp*., Staphylococcus* sp*., Pseudomonas* sp., and *Klebsiella* sp. using agar well diffusion technique [17]. YEPD agar plates were swabbed with the pathogenic cultures and agar wells were bored using gel punctures. The freshly prepared pure culture of yeast isolates present in the YEPD broth was centrifuged at 10,000 rpm for 10 minutes at 4°C. The metabolites (supernatant) were extracted to check the antimicrobial activity of the yeast isolates. Metabolites were pipette in the wells and incubated at 28°C for 24-48 hrs. The inhibition activity of yeast isolates was evaluated measuring the zone of inhibition around the probiotic growth.

Extracellular polysaccharide (EPS) production

The cultures were streaked onto YEPD agar plates and incubated for 24 hrs at 37°C and the strains, which produced slimy colonies, were recorded as capable of producing EPS. The selected strains were inoculated to 100 ml of EPS producing media comprised sucrose (50 g/l), yeast extract (0.4 g/l), peptone (0.6 g/l), K_2HPO_4 (5 g/l), $MgSO_4.7H_2O$ (0.5 g/l), and NaCl (1 g/l). The culture was then incubated for 15 days with continuous agitation of 150 rpm at room temperature [18]. The culture was then centrifuged at 10,000 rpm for 20 minutes at 4°C and supernatant was transferred to fresh tube, and twice the volume of ice-cold isopropanol was added, and EPS was allowed to precipitate overnight. It was centrifuged at 12,000 rpm for 30 minutes. The pellet was precipitated with isopropanol for decolorization. Then, the pellet was dissolved in 1 ml of sterile distilled water. A standard graph was plotted using different concentrations of glucose from stock solution (1 mg/ml). On the basis of the standard curve, the concentrations of unknown samples which extracted from the yeast isolates were estimated [19].

RESULTS AND DISCUSSION

Isolation and identification of yeast isolates

Yeasts were isolated from different sources (Table 1) and pure culture of all yeast isolates were obtained by the pour plate method followed by the serial dilution (10^5-10^6). Isolated colonies were obtained and grown in YEPD media for analysis.

Primary screening

The pH of a human gut ranges from 1 to 2, and after food consumption, it may goes up to 5 due to the presence of digestive acid HCl. Most of the *in vitro* studies suggest extremely lower pH for selection of probiotic strains and isolated yeast growth were observed under pH-2 condition (Fig. 1a). Then, screened isolates were checked for thermotolerance at different temperatures (25°C, 35°C, and 45°C) and the growth was measured (Fig. 1b).

Among the 20 yeast isolates, 12 isolates showed good growth under acidic condition (pH-2) and temperature resistance (35°C) which can be potentially used as probiotic strains. Similar results were found in case of other probiotic yeast species [13].

Secondary screening

Optimum bile concentration of our human gut environment ranges from 0.30% to 0.60%. Thus to check salt tolerance, the isolates were inoculated in 0.20-1.5% concentrations of bile salt mixed YEPD broth. Among the 12 isolates, 9 yeast isolates were selected from bile salt tolerance assay. Few strains showed the best tolerance against bile salt at 0.6% (Fig. 2a). Then, selected yeast isolates were treated with gastric juices (pepsin and pancreatin), and survival rate was measured using pour plate method (Fig. 2b). The ability to adhere to mucus and epithelial cells is proposed as an important selection criterion for potential probiotic strains. The adhesion ability of probiotic strains has been studied *in vitro* model systems, which are commonly used to select and assess probiotic strains for *in vivo* studies [15].

To detect their hydrophobicity index n-hexadecane was used in selected yeast strains. Most of the isolates showed better adhesion rate above 60% (Fig. 2c). Selected yeast isolates were treated with known amount (100 µg) of cholesterol and samples were tested for their cholesterol removal at a different time in travels (4^{th}, 8^{th}, 12^{th}, and 24^{th} hr). The removal rate was increased on 24^{th} hr compared with 48^{th} hr. Cholesterol assimilation rate was calculated and shown in Fig. 2d.

Final screening

To access their safety assays, antibiotic sensitivity was carried out with 10 antibiotics with known concentrations. After incubation zone formation was measured to determine their sensitivity against antibiotics (Fig. 3a).

Table 2: Antibiotic sensitivity test for screened isolated yeasts

Antibiotics/yeast isolates	LM	P	MR	SF	GOI	GII	GM	SFL	WI
Amoxicillin	-	-	-	-	-	+	+	-	-
Ampicillin	+	-	+	+	+	+	-	-	+
Gentamycin	+	+	+	+	+	+	+	-	+
Ketoconazole	+	+	-	+	+	+	+	+	-
Levofloxacin	-	-	+	+	-	-	-	-	+
Rifampicin	-	-	-	-	-	-	-	-	-
Vancomycin	-	-	+	-	-	-	-	-	-
Tetracyclin	+	-	+	-	+	-	+	-	
Tigecyclin	+	+	-	+	+	-	+	-	+
Trimethoprim	+	-	+	+	-	-	-	-	+

Zone formation (-) negative, (+) positive, SFL: Sugar factory liquid, WI: Wine industry, GM: Goat milky, GOI: Goat intestine outer, GII: Goat intestine inner, SF: Sun flower, MR: Millet root, P: Papaya, LM: Lemon

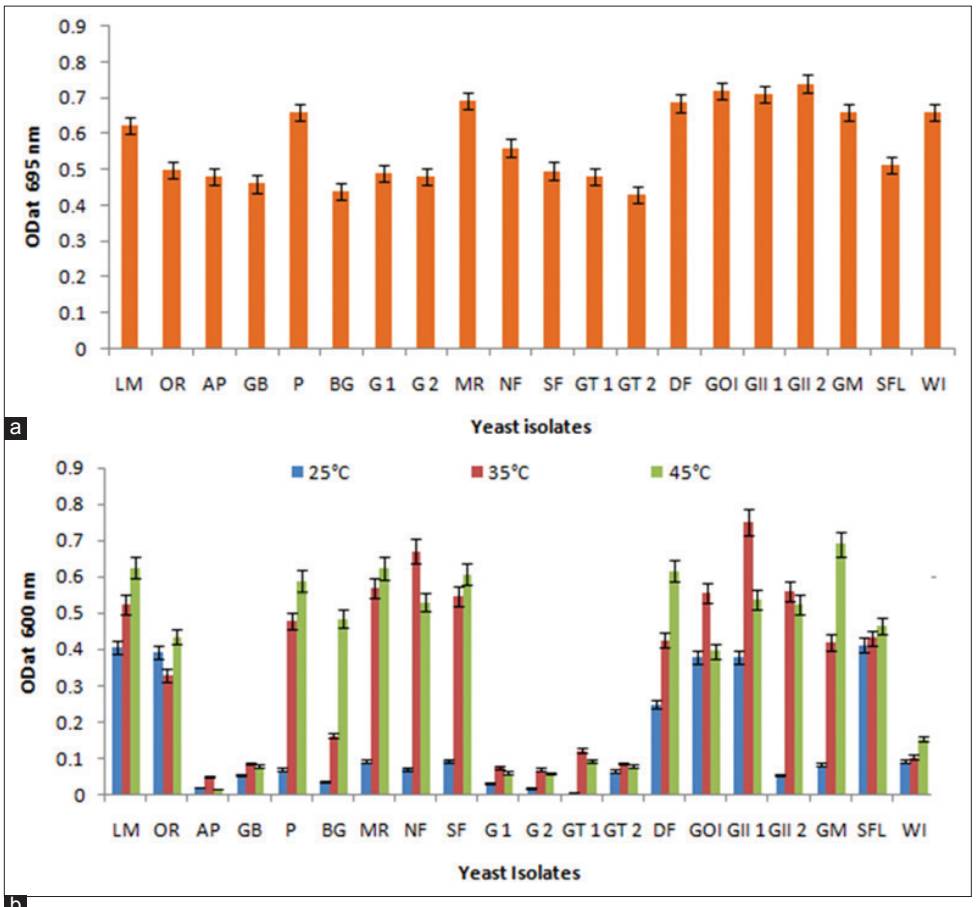

Fig. 1: (a) Effect of pH-2, (b) Effect of temperature in isolated yeast strains

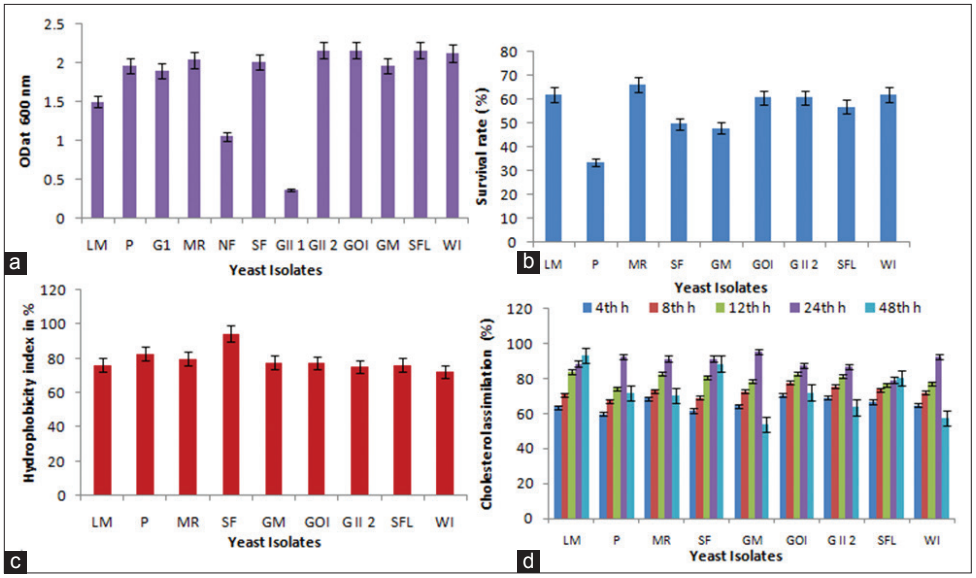

Fig. 2: (a) Bile salt tolerance, (b) Survival rate under gastrointestinal tract, (c) Cell surface adhesion and (d) Cholesterol assimilation by yeast isolates (d) percentage of cholesterol assimilation by yeast isolates

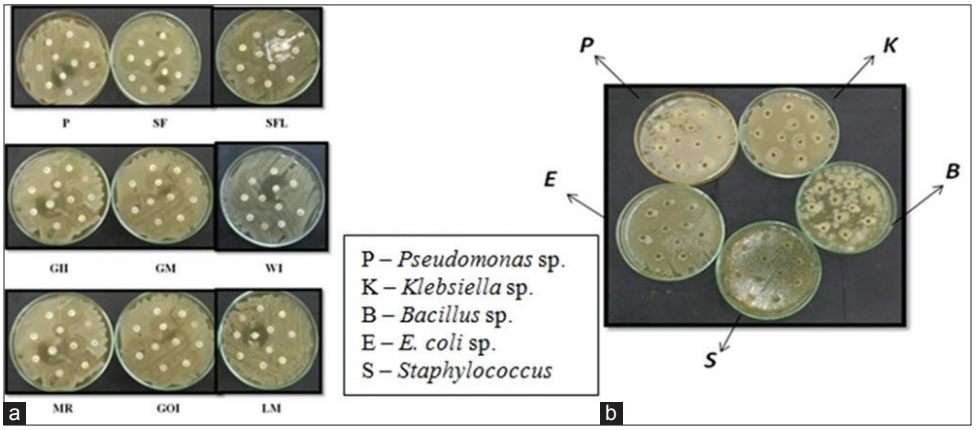

Fig. 3: Zone of diffusion, (a) Caused by antibiotic sensitivity test, (b) Caused by metabolites of screened yeast isolates against pathogenic culture

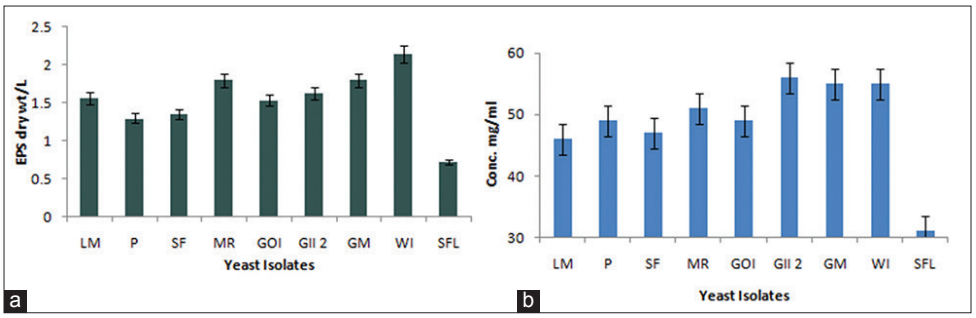

Fig. 4: (a) Exopolysaccharides production, (b) Total carbohydrate content of exopolysaccharides in screened yeast isolates

The antimicrobial potentiality was checked against five different pathogens *Pseudomonas* sp., *Klebsiella* sp., *Bacillus* sp., *E. coli* sp., and *Staphylo Coccus* (Fig. 3b). Among the 9 isolates, 5 yeast isolates were showed the highest sensitivity against antibiotics as well as antimicrobial activity.

Based on the zone diffusion selected yeast isolates results were tabulated below (Table 2 for antibiotic sensitivity test and Table 3 for antimicrobial activity).

EPS production

A standard graph was plotted using different concentrations of glucose. On the basis of standard curve, the concentrations of unknown samples which were extracted from the yeast isolates were estimated (Fig. 4b) by phenol-sulfuric acid method [20]. Extracted EPS was calculated in dry weight (Fig. 4a) and isolates lemon (LM), millet root (MR), goat intestine outer (GOI), goat intestine inner (GII 2), and wine industry (WI) showed increased EPS production.

Table 3: Antimicrobial activity of isolated yeast

Yeast isolates	*Pseudomonas* sp.	*Klebsiella* sp.	*Bacillus* sp.	*E. coli* sp.	*Staphylococcus* sp.
LM	+	+	+	+	+
P	+	+	+	+	-
MR	+	+	+	+	+
SF	+	+	+	-	-
GII	+	+	+	+	+
GOI	+	+	+	+	+
GM	+	+	+	-	-
SFL	+	-	+	-	-
WI	-	+	+	+	+

Zone formation (-) negative; (+) positive, SFL: Sugar factory liquid, WI: Wine industry, GM: Goat milky, GOI: Goat intestine outer, GII: Goat intestine inner, SF: Sun flower, MR: Millet root, P: Papaya, LM: Lemon, *E. coli: Escherichia coli*

CONCLUSION

Morphology of 20 yeast isolates was studied by simple staining and their growth was determined on YEPD media. Desirable probiotic characteristics have been investigated in all the yeast isolates. The probiotic characteristics of all the yeast isolates were checked based on tolerance to pH, temperature, and bile salt. The screened 9 isolates out of 20 were subjected to optimize their susceptibility on gastric juice stimulation, cell adhesion and cholesterol removal. The best 5 isolates were chosen based on their antimicrobial activity and antibiotic sensitivity. Exopolysaccharide produced by yeast isolates was estimated for finally selected yeast strains. In conclusion, the results of this study showed that yeast isolates, namely, LM, MR, GOI, GII 2, and WI were found to possess desirable *in vitro* probiotic properties which may serve as promising probiotics. Selected yeast isolates have been sent for molecular identification.

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