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Probiotic Characterization of Cholesterol-Lowering *Saccharomyces cerevisiae* Isolated from Frass of *Pyrrharctia isabella* Caterpillars

Mayur Arjun Aswani[1], Suyash Arunrao Kathade[2], Pashmin Kaur Anand[2], Bipinraj Nirichan Kunchiraman[2], Pavan Rajendra Dhumma[2], Suresh Dnyandeo Jagtap*[1]

1- Department of Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth Deemed to be University, Pune, Maharashtra, India

2- Department of Microbiology, Rajiv Gandhi Institute for I.T. and Biotechnology, Bharati Vidyapeeth Deemed to be University, Pune, Maharashtra, India

Abstract

Background and Objective: Cholesterol hyper-accumulation is an increasing risk factor causing cardiovascular diseases. Cardiovascular diseases are leading causes of death worldwide, accounting for 16.7 million deaths annually. An increase from 25.7 to 54.7 million cases of cardiovascular diseases is reported in India. Since 1990, changes in dietary patterns have been the major causes of this condition. However, current treatments such as statins and β-blockers are inadequate and include systemic toxicity such as stent thrombosis and chronic inflammation. Probiotics with cholesterol-lowering ability are ideal safe choices for the prevention of cardiovascular diseases.

Material and Methods: The present study involved culture-dependent method for the enumeration of microorganisms from *Pyrrharctia isabella*. Microorganisms were isolated and purified and their morphology was studied microscopically. Strains that showed yeast morphology were selected for further probiotic characterization. These isolates were further characterized for probiotic potential based on guidelines from World Health Organization, Indian Council of Medical Research and World Gastroenterology Organisation. The present study assessed cholesterol-lowering potential of the best characterized isolate.

Results and Conclusion: Culture-dependent approach resulted in the isolation of four yeast cultures from the frass of *Pyrrharctia isabella* caterpillars. Cultures showed antimicrobial activity and other probiotic characteristics such as resisted pH, bile and temperature, similar to the reports by Khisti et al., 2019. However, the novel strain isolated from *Pyrrhactia isabella* could survive high concentrations of NaCl (1.02M) with an 80.32% survival rate, showing 40.23% hydrophobicity. CP-I culture showed bile salt hydrolase activity as well as susceptibility to sulphatried, penicillin-G, tetracycline and chloramphenicol. This isolate showed the potential to decrease cholesterol levels by 9.16% under *in-vitro* conditions. Molecular identification of the novel isolate demonstrated that it belonged to *Saccharomyces cerevisiae* with 97% genetic similarity. Thus, this study demonstrated for the first-time isolation of cholesterol-lowering probiotic yeast *Saccharomyces cerevisiae* from caterpillar frass under *in-vitro* conditions.

Conflict of interest: The authors declare no conflict of interest.

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***Corresponding author:**
Suresh Jagtap,
Department of Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth Deemed to be University, Pune, Maharashtra, India

Tel: 020-24366920

E-mail:
chiritatml@rediffmail.com

1. Introduction

Cardiovascular diseases (CVD) are leading causes of death worldwide, commonly includes increased blood cholesterol levels [1]. The global mortality rate of CVD accounts for 16.7 million people annually. Indian subcontinent is showing increases in the number of CVD patients from 25.7 million in 1990 to 54.7 million in 2016

due to the high-fat diet as one of the dominant factors for the highlighted condition [2]. Risk factors such as high-fat diets, obesity, blood pressure and diabetes contribute to increases in the global burden of CVD; from which, diet represents a major risk factor for CVD [3]. Cholesterol, a naturally occurring sterol in tissues that is responsible for various roles in human body such as preserving fluidity of cells, increases at high rates in the body due to sedentary lifestyle. Of the risk factors majorly, deposition of cholesterol in the human body leads to coronary heart diseases and atherosclerosis, which is a major burden in severity of CVD [4]. Conventional treatments such as use of statins and β-blockers for CVD are inadequate, including systemic toxicity such as stent thrombosis, chronic inflammation, stomach pain, constipation and muscle pain [5]. To address these problems, probiotics that possess cholesterol-lowering activity are ideal, safe alternative solutions that support human health. Probiotic therapy can regulate and decrease cholesterol levels either by bio transforming, adsorbing or sequestering cholesterol in the body and hence decreasing risks of CVD. The concept of probiotics has been revised from time to time with the recent definition stating probiotics as "live microorganisms, when administered in adequate amounts confer a health benefit on the host" by the International Scientific Association of Probiotics and Prebiotics (ISAPP) and Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) [6,7]. Probiotics include positive effects such as secretion of antibacterial agents, regulation of the body immunity, providing of anti-food antigens, lowering of cholesterol levels, anticipation of sensitivity, maintaining of good microbial balance in the gastrointestinal tract (GIT) and elimination of germs in the stomach [8,9]. Dysbiosis often results in decreases in beneficial microorganisms, the probiotic group that creates a havoc in gut microbial functionality and hence resulting in onset of diseases [10].

As the probiotic industry is growing rapidly, there are chances of misuse of the term "probiotics" in the market [7]. Therefore, organizations such as the Joint Commission of Indian Council for Medical Research and Department of Biotechnology (ICMR-DBT), WHO and World Gastroenterology Organization (WGO) postulated the minimum essential criteria to be fulfilled by the probiotics. Probiotic strains should therefore be safe for consumption, thrive in wide ranges of pH and temperature and include the ability to withstand digestive enzymes, pH conditions of the gastrointestinal environment and inhibitory effects of bile salts and display antagonistic activity against pathogens [6,11,12]. Yeasts such as *Saccharomyces* (*S.*) *boulardii* and *S. cerevisiae* are widely used as probiotics [6,13]; however, potentials exist for investigating probiotic microorganisms with add-on activities from various environmental sources. Prokaryotes introduced as probiotic microorganisms have commercially been accepted; however, their characteristics have been responsible for various activities in eukaryotes such as ability to neutralize enterotoxin, possess synergistic effects on gut microbes and less ability to transfer genetic materials between the species [13]. Gut microflora play vital roles in maintain proper metabolic and physiological statuses for survival of the hosts. Probiotics assists the organism to act properly such as digestion of complex substrates in foods, neutralizing of the effects of alkaloids, improvement in immunity of the hosts and ability to secrete various enzymes [14]. Therefore, beneficial microorganisms must be isolated from the gut of healthy organisms. Furthermore, probiotics play important roles to improve the health status of humans. However, there is still scope to isolate probiotics from various environmental sources and characterize them for their health-promoting activities. The aim of this study was to isolate and characterize probiotic yeasts from the frass of *Pyrrharctia isabella* caterpillars collected from the barks of *Nyctanthes arbor tristis*, a commonly used medicinal plant.

2. Materials and Methods

2.1 Culture isolation

Naturally-grown *Pyrrharctia isabella* caterpillars were collected from the barks of *Nyctanthes arbor tristis* trees in Pune, India. Surface contamination decreased by washing the caterpillars with sterile distilled water three times. Caterpillars were washed on sterile Petri plates and frass samples (0.5 g) were collected from the Petri plates after 24 h. For the isolation of yeast cells, samples (0.5 g) were suspended in sterile saline (0.8%) and then streaked on yeast extract peptone dextrose (YPD) media (pH 6.5) (Himedia, India), followed by incubation at 37 ºC for 48 h under aerobic conditions to isolate the yeast cells. Morphologically distinct colonies were isolated and studied under light microscope. Purified yeast cell morphology was preserved in YPD agar slants. These isolates were inoculated into YPD broth for 24 h, before being centrifuged and re-suspended in saline to achieve a 10^7 CFU ml^{-1} cell concentration. In all experiments, this re-suspended cell suspension (1% v v^{-1}) was used as inoculum.

2.2 Hemolytic Assay

Hemolytic assay was carried out by surface spot inoculating of the cultures on sheep blood agar plates and incubating themt at 37 ºC for 24 h. To assess toxicity of the cultures, cultures were analysed for hemolytic patterns [15].

2.3 Tolerance to pH

Prior to inoculation of cultures, pH of the media was changed to 1.5-10 using 1N HCl or 1N NaOH to test their pH tolerance. Post inoculation, culture tubes were incubated at 37 ºC for 24 h and studied for turbidity in terms of growth [16].

2.4 Bile Tolerance

Bile tolerance study was carried out by introducing bile salts **(**Loba Chem, India**)** with various concentrations of 0.3, 0.6, 0.9 and 1.2% into YPD broth, inoculated with cultures. Growth was detected turbidometrically after incubation of the broth at 37 ºC for 24 h [17].

2.5 Temperature Tolerance

Yeast isolates were incubated in YPD broth at various temperatures of 28, 37 and 42 ºC. Tolerance to temperature was assessed by measuring the growth at optical density (OD) of 600 nm (Thermo Scientific, USA) after 24 h of incubation [17].

2.6 Aggregation Characteristics

Cultures were grown in YPD at 37 °C for 24 h, followed by centrifugation (8000 rpm, 10 min) and the cell pellets were suspended in sterile PBS (2 ml, pH 7.4). Auto-aggregation assay was carried out by thoroughly overtaxing 1 ml of the cell suspension in 4 ml of sterile PBS for 10 sec followed by incubation at 37 ºC for 24 h. At 600 nm, OD of the mixture was measured at 0, 2, 4 and 24 h intervals. The following formula was used to calculate auto-aggregation:

$$\text{Auto-aggregation (\%)} = A_t/A_o*100 \qquad \text{Eq(1)}$$

Where, A_t was OD at the time; t was 2, 4 or 24 h; and A_o was OD at t = 0 h. Co-aggregation was estimated as in the method described by Ogunremi et al., 2015 [18]. To achieve a final volume of 5 ml of sterile PBS, the culture was mixed with 0.2 ml of PBS, vortexed with an equivalent quantity of the pathogen suspension in PBS (0.5 OD) and co-incubated at 37 ºC for 24 h. To assess co-aggregation, absorbance of the mixture (600 nm) was measured at time intervals of 2, 4 and 24 h and then compared to that of individual pathogen suspensions. Following a 24-h incubation period, yeast cultures were pipetted, stained with methylene blue and studied under the light microscope [19]. The following formula was used to calculate co-aggregation;

$$\text{Co-aggregation (\%)} = [(Ax + Ay)/2] - A(x + y)/ Ax + Ay/2 \qquad \text{Eq(2)}$$

Where, x and y represented each of the two strains in control tubes; and (x + y) represented the mixture.

2.7 Antagonistic Activity

Antimicrobial activity of the strains was checked against gastroenteropathogens such as *Escherichia* (*E.*) *coli* NCIM 3099, *Staphylococcus aureus* NCIM 2408, *Enterococcus (E.) faecalis* NCIM 3040 and *Candida albicans* NCIM 3557. These pathogens were individually spread plated on Muller-Hinton agar (Himedia, India) and incubated at 37 ºC for 10 min. Antagonistic activity of the yeast cultures against pathogen cultures was reported by punching wells of 5-mm in diameter and loading 20 µl of cell-free supernatants collected by centrifuging (12,298 g, 20 min, RT) cultures from YPD broth (37 ºC, 24 h). After incubation for 24 h, inhibition zones were measured [20].

2.8 In-vitro simulated Gastrointestinal Juice Tolerance Assay

The *in-vitro* survival of the isolates under gastrointestinal environment was assessed by preparing gastric (3 $mg.ml^{-1}$ pepsin, pH 2) and intestinal (1 $mg.ml^{-1}$ pancreatin, pH 8) juices and mixing them with culture suspensions in PBS (0.2 ml) and 0.3 ml of NaCl (0.5% w v^{-1}), as described by Charteris et al., 1998 [21]. Suspensions were incubated at 37 ºC and at time intervals of 1, 90 and 180 min for gastric and 1 and 240 min for intestinal aliquots and then spread plated on YPD agar plates to calculate viable counts. Survival of the isolates under gastrointestinal environment was assessed by calculating viable count of the cultures through the following formula:

$$\text{Survival (\%)} = N_t / N_o * 100 \qquad \text{Eq(3)}$$

Where, N_t was the number of colonies at the time t (1, 90, 180 or 240 min); and N_o was the number of colonies before treatment.

2.9 NaCl Tolerance

Tolerance to NaCl was assessed by growing the cultures in YPD media with various concentration of NaCl (0.17-1.71 M) and calculating their proportional survival using OD, compared to that of the control group after incubation [22].

2.10 Hydrophobicity Assay

Hydrophobicity assay shows ability of probiotics to form adhesive interactions with human epithelial cells. For hydrophobicity, cultures (1 OD at A_{600}) were suspended in phosphate buffer (pH 6.5) and treated with xylene at a 5:1 ratio. Suspensions were thoroughly mixed for 2 min and incubated at 37 ºC for phase separation. Decreases in OD of the aqueous phase were reported as percent hydrophobicity (H %) and calculated as follows: $H\ (\%) = [(A_0 - A) / A_0] * 100$ Where, A_0 and A were absorbance of the cultures in the aqueous phase of former and latter extractions, respectively [22].

2.11 Antibiotic Susceptibility Test

The Kirby-Bauer antibiotic testing method described by Bauer et al. (1959) was used to assess antibiotic susceptibility of the yeast isolates [23]. Cultures were plated on MRS agar plates and exposed to antibiotic discs (Himedia), including ampicillin (10 mg), chloramphenicol (25 mg), penicillin-G (1 U), streptomycin (10 mg), sulphatried (300 mg) and tetracycline (25 mg). The antibiotic susceptibility was assessed as zones of growth inhibition after incubating plates at 37 ºC for 24 h.

2.12 Bile Salt Hydrolase Assay

The bile salt hydrolase (BSH) assay was carried out according to a method described by Zheng et al., 2013. Cultures were surface spot inoculated on YPD agar media added with 0.5% (w v^{-1}) sodium salt of taurodeoxycholic acid (Himedia, India) and 0.37% $CaCl_2$ (w v^{-1}). Plates were incubated aerobically at 37 ºC for 72 h and BSH activity was assessed by the presence of precipitation [24].

2.13 Cholesterol-lowering

Freshly prepared YPD broth was inoculated with yeasts that were isolated aerobically. These were incubated at 37 ºC for 48 h as the medium was supplemented with 0.3% of bile salts and filter sterilized water-soluble cholesterol (1 $mg.ml^{-1}$). Cells were concentrated after incubation and harvested by centrifugation at 12,298 g for 15 min. The cell-free supernatant (1 ml) was mixed with 3 ml of potassium hydroxide (33% w v^{-1}) and 3 ml of absolute ethanol, vortexed for 1 min and incubated at 65 ºC for 15 min. After removal of the ethanol layer, hexane (5 ml) and distilled water (2 ml) were thoroughly mixed for 1 min. The hexane layer (1 ml) was pipetted into a clean glass tube and completely dried in a 65 ºC water bath. The dried remnant was promptly dissolved in 1.5 ml of $FeCl_3$ reagent and vortexed. This was then mixed with 1 ml of concentrated sulphuric acid and vortexed for 1 min. After 30 min of incubation in dark, absorbance was measured at 550 nm [4]. The cholesterol-lowering ability ($mg.ml^{-1}$ culture broth) was calculated as follows:

$$\text{Cholesterol-lowering activity (A)} = 100 - (B / C) * 100 \quad \text{Eq (4)}$$

Where, A was proportion of the cholesterol removed; B was absorbance of the culture supernatant mixture; and C was absorbance of the control.

2.14 Molecular Identification

Yeast molecular identification was carried out as previously described by Polkade et al., 2015. Genomic DNA of CP-I isolate was extracted from growing an individual colony in YPD broth for 24 h and centrifuging to achieve cell pellets [25]. After washing with sterile DW, cell pellets were suspended in extraction buffer (100 mM Tris/HCl pH 8.0; 100 mM Na_2EDTA, pH 8.0), followed by the addition of proteinase K (Invitrogen, USA) at a concentration of 100 $mg.ml^{-1}$. The mixture was then incubated at 55 ºC for 2 h in rotary shaker. Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) reagent was used to extract DNA. Extracted DNA was washed with 70% ethanol before being dissolved in Tris-EDTA buffer (pH 8.0). ITS1 TCCGTAGGTGAA-CCTGCGG and ITS4 TCCTCC-GCTTATTGATATGC primers were used to amplify the internal transcribed spacer (ITS) region of DNA. Strain was identified by amplification and sequencing of the ribosomal ITS region. The PCR Cleanup Kit (Qiagen, Germany) was used to purify the PCR products. Using Big Dye Terminator Kit (Applied Biosystems, USA), purified amplicons were sequenced on both strands using ABI 3730xl DNA Analyser (Applied Biosystems, USA). Sequence alignment was carried out using DNA Star Pro and analysed for their closest relatives based on pairwise sequence similarity sing basic local alignment search tool (BLAST) algorithm in National Centre for Biotechnology Information (NCBI) database (minimum of 97% sequence similarity).

2.15 Statistical analysis

Each experiment was carried out in triplicate. Data were analysed using one-way ANOVA and PRISM Software and results were expressed as mean ±SE (standard error). Differences were considered statistically significant when $p < 0.05$ ($p > 0.05$ = ns, $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***).

3. Results and Discussion

3.1 Isolation of Yeast Cultures

Pyrrharctia isabella caterpillars located on the bark of *Nyctanthes arbor tristis* were collected and surface sterilized, which were used to collect their frass for the isolation of yeasts. Yeast isolation from frass resulted in a total of 41 morphologically distinct colonies on agar plates and further microscopy of Gram-stained and negative-stained samples verified that the four cultures, CP-I, CP-II, CP-III and CP-IV, included yeast cell morphology (Fig. 1).

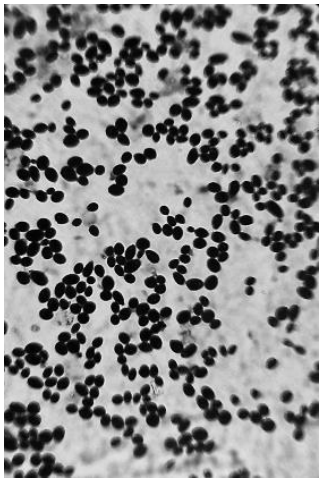

Figure 1. The CP-I culture isolated from *Pyrrharctia isabella* caterpillar frass

3.2 Hemolytic Assay

Hemolysis assay is one of the tests used for screening toxicity of the isolates. In the present study, cultures of CP-I, CP-II, CP-III and CP-IV showed γ-lysis on sheep blood agar plate (Fig. 2) and hence were non-toxic in nature, eligible for further probiotic characterization.

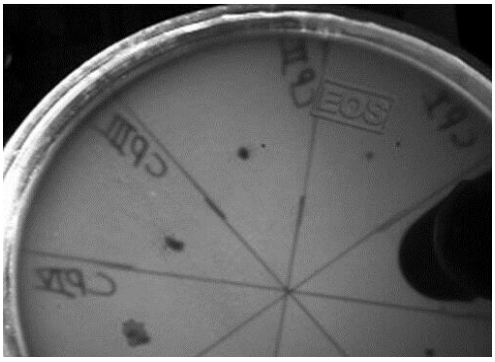

Figure 2. Non-toxic nature of the four yeast cultures on sheep blood agar plate

3.3 pH and Bile Salt Tolerance

Since probiotics are usually consumed orally, it is critical to assess culture ability to withstand harsh acidic and basic environments in the GIT [8]. By measuring turbidity of the culture media, all the four isolates were able to thrive in basic conditions up to pH 10, showing a fair growth at pH 1.5 and excellent growth at pH 7 (Table 1). After assessing growth by turbidity, CP-I and CP-II cultures showed strong growth even in a bile concentration of 1.2% (Table 1). A similar study reported that *S. boulardii* could withstand a bile concentration of 1.2% after 48 h of incubation. As the peristaltic movement progresses from the acidic stomach, microorganisms faces inhibitory and antimicrobial activity of bile in the intestine; thus, probiotic strains must include ability to survive under negative effects of bile salts in the intestine [26]. It is well accepted that functional probiotics must be safe for consumption, resist pH changes and tolerate bile toxicity [22,27,28].

3.4 Effects of Temperature

Yeast isolates of CP-I and CP-II were able to grow at three various temperatures of 28, 37 and 42 ºC. It was generally detected that CP-I and CP-II included good growth, compared to that yeast isolates of CP-III and CP-IV did (Fig. 3). Overall, CP-I isolate showed good tolerance to temperatures of 28, 37 and 42 ºC with the absorbance of 0.667, 0.756 and 0.777. For the isolate of CP-II, absorbance included 0.65, 0.71 and 0.69 at 28, 37 and 42 ºC, respectively (p = 0.023, 0.0301 and 0.0195; and p = 0.0211, 0.024 and 0.0235, respectively). Ideal probiotics must survive host environmental temperature; while tolerance to high temperatures indicates the industrial importance of the cultures that can be used in pharmaceutical industries.

Table 1. Probiotic characteristics of isolate CP-I

Parameters	Results											
Hemolysis	Negative											
pH Tolerance	1.5	2.5	3.5	7	8	9	10					
	+	+	++	+++	++	++	+					
Bile salt Tolerance	0.3 %		0.6 %		0.9 %		1.2 %					
	+++		+++		+++		++					
Temperature Tolerance (O.D. at 600 nm)	28 ºC			37 ºC			42 ºC					
	0.66			0.75			0.77					
NaCl Tolerance (% growth at) M	0	0.34		0.68		1.02		1.36		1.71		
	100	98		74		80.32		44.15		0.09		
Auto-aggregation (% aggregation at) h	2			4				24				
	91.87			100				75				
Co-aggregation (% co-aggregation at) h	*E. coli*			*E. fecalis*				*S. aureus*				
	2	4	24	2	4	24		2	4	24		
	96.71	100	76.01	94.91	100	91.52		45.37	75	100		
Bile Salt Hydrolase	Positive											
Pepsin Tolerance (% growth at)min	0		1		90			180				
	100		87.55		55.37			31.33				
Pancreatin Tolerance (% growth at)min	0			1			240					
	100			89			68					
Antimicrobial activity	*E. coli*		*E. fecalis*		*S. aureus*			*C. albicans*				
	4 mm		7 mm		4 mm			14 mm				
Antibiotic Susceptibility Test	Amp*	Strep*	S*		Tetra*		Pen*		Chl*			
	4 mm	3 mm	21 mm		23 mm		21 mm		24 mm			
Hydrophobicity	40.23%											
Cholesterol-lowering ability (% reduction at) h	0		24		48							
	0		4.21		9.16							
Molecular Characterization (ITS Sequencing)	*Saccharomyces cerevisiae* (97%) DDBJ Accession no. LC528139											

* Amp- Ampicillin (10 mg), Strep- Streptomycin (10 mg), S- Sulphatried (300 mg), Tetra- Tetracycline (25 mg), Pen- Penicillin-G (1 unit), Chl- Chloramphenicol (25 mg)

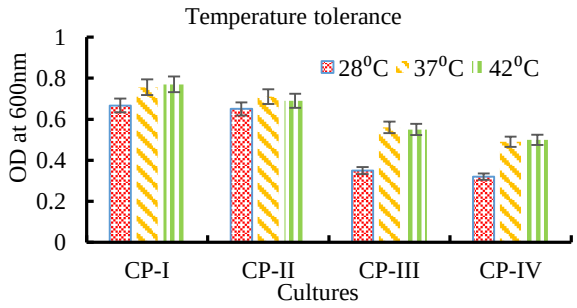

Figure 3. Temperature tolerance of the four yeast cultures at 28, 37 and 42 ºC after 24 h of incubation

3.5 Aggregation Characteristics

Capacity of the yeast isolates to aggregate was assessed by comparing the initial to final absorbance values at 600 nm. The baseline auto-aggregation proportion for successful probiotic candidates is greater than 40%. After 4 h of incubation, CP-I exhibited a better aggregation than that CP-II, CP-III and CP-IV did (Fig. 4).

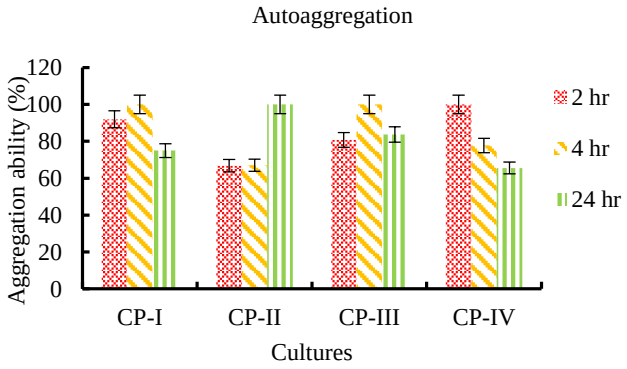

Figure 4. Auto-aggregation characteristics of the four yeast cultures

The graph clearly showed that CP-I and CP-III included 100% aggregation after 4 h, while CP-II and CP-IV included 67.01 and 77.77% aggregation after 4 h, respectively. Co-aggregation or co-culture is a process; by which, various microbial strains or species interact with each other; for example, beneficial strains eliminate harmful pathogens by either forming a cluster around pathogens or displacing pathogens from the cell surface. The proportion of co-aggregation of the yeast isolates was calculated using absorbance and microscopic studies. Co-aggregation is an indicator of the ability to suppress pathogens. When exposed to *E. coli* NCIM 3099, CP-I and CP-III demonstrated a 100% co-aggregation potential after 4 h (Fig. 5A) while CP-II (58.04%) and CP-IV (67.92%) (p = 0.0125, 0.041, 0.0168 and 0.0571) demonstrated comparatively a less ability. Similarly, isolates co-aggregated with *E. faecalis* NCIM 3040 CP-I showed 100% (Fig. 5B), CP-II showed 76.64%, CP-III showed 25.61% and CP-IV showed 26.77% aggregations after 4 h (p = 0.0215, 0.0235, 0.0351 and 0.0233, respectively). Isolates co-aggregated with *S. aureus* NCIM 2408 CP-I demonstrated 75% (Fig. 5C), CP-II demonstrated 69.69%, CP-III demonstrated 100% and CP-IV demonstrated 21.56% aggregations after 4 h (p = 0.0455, 0.0461, 0.0248 and 0.0411, respectively). This revealed that CP-I and CP-II included good abilities at controlling these pathogens.

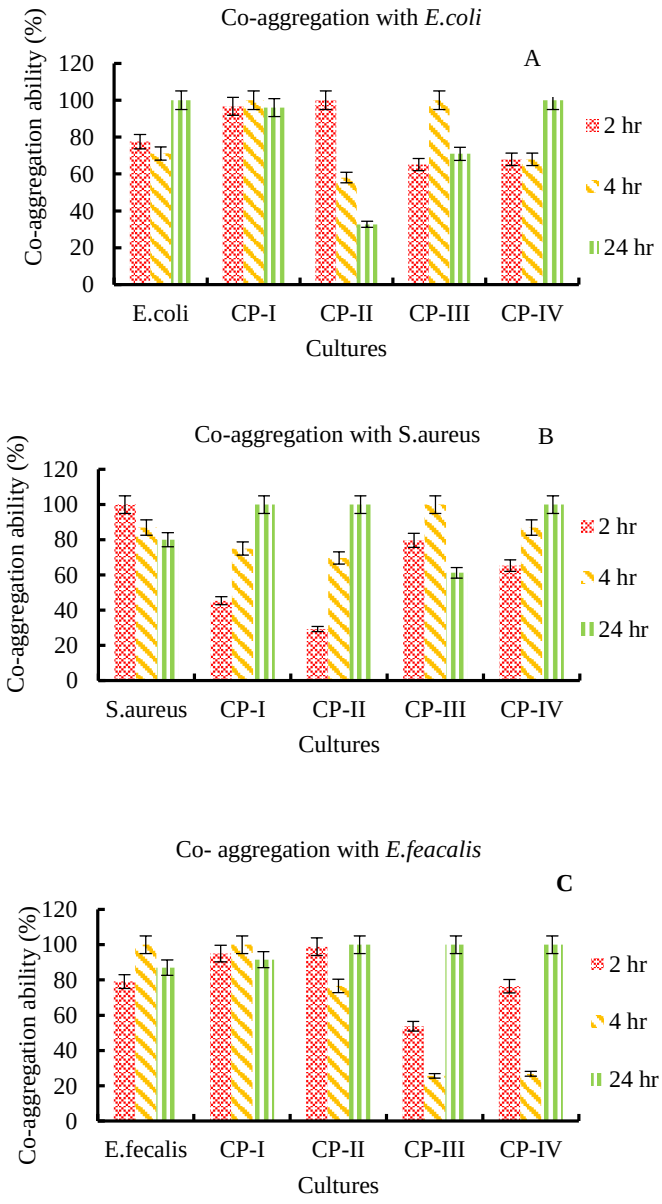

Figure 5. Co-aggregation ability of four yeast cultures with *Escherichia coli* (A), Co-aggregation ability of four yeast cultures with *Staphylococcus aureus* (*B*), Co-aggregation ability of four yeast cultures with *Enterococcus faecalis (C)*

3.6 Antagonistic Activity

Supernatant-derived bioactive compounds were assessed against Gram-positive and Gram-negative bacteria. Results

showed that isolates of CP-I, CP-II, CP-III and CP-IV could inhibit *E. coli* NCIM 3099 with an inhibition zone of 4 mm. Whereas, CP-I demonstrated antimicrobial activity against *S. aureus* NCIM 2408 with an inhibition zone of 4 mm. The inhibition zone of CP-I, CP-II isolates against *E. faecalis* NCIM 3040 included 7 and 9 mm, respectively. The inhibition zones of CP-I and CP-IV against NCIM 3557 were reported as 14 and 11 mm, respectively (Table 1). Antagonistic activity is an essential characteristics of the probiotic microorganisms in preventing or curing infections caused by pathogens. The current study detected that CP-I was effective against Gram-positive and Gram-negative pathogenic bacteria as well as *Candida. albicans*, a commensal opportunistic pathogen found in host GIT. The exact inhibition mechanisms of probiotics pathogens in *in-vivo* conditions are still unclear; however, hypotheses have suggested mechanisms; by which, probiotic microorganisms can inhibit pathogens in the GIT. Reports have stated competitive exclusion of pathogens, inhibited by secretion of antagonistic compounds from probiotics [15,17]. The present approach included all the four yeast cultures that showed antimicrobial activities against common pathogens such as *E. coli*, *E. faecalis*, *S. aureus*, inhibiting opportunistic *Candida. albicans*, the causative agent of candidiasis.

3.7 In-vitro Simulated Gastrointestinal Tolerance

The aim of this experiment was to assess resistance of cultures to effects of gastrointestinal juices. Ideal probiotic candidates should thrive after 2 h of exposure to pH-2 gastric and pH-8 intestinal juices. The present study revealed that isolates included resistance to pepsin and pancreatin at various degrees. The present study reported that isolate of CP-I survived gastric and pancreatic environments with survival rates of 55.37 and 68% after 90 and 240 min (Figs. 6A and 6B) of gastrointestinal transit times, respectively (p = 0.052 and 0.0481, respectively). However, it was seen that the cell count decreased as the gastrointestinal transit time increased. Supporting studies by Charteris et al. [23] reported that well-characterized probiotic candidates such as *Lactobacillus* and *Bifidobacterium* spp. down-regulated their growth when treated with harsh digestive enzymes and severe pH. Similar results were seen in this study as CP-I best survived in gastrointestinal transit time of up to 4 h.

3.8 NaCl Tolerance

High NaCl concentrations act as inhibitory compounds that may decrease growth of microorganisms [26]. The present study showed that the CP-I culture could significantly tolerate salt concentrations of 0.34-1.36 M with up to an 80.32% survival rate at 1.02 M concentration (p = 0.021) (Table 1).

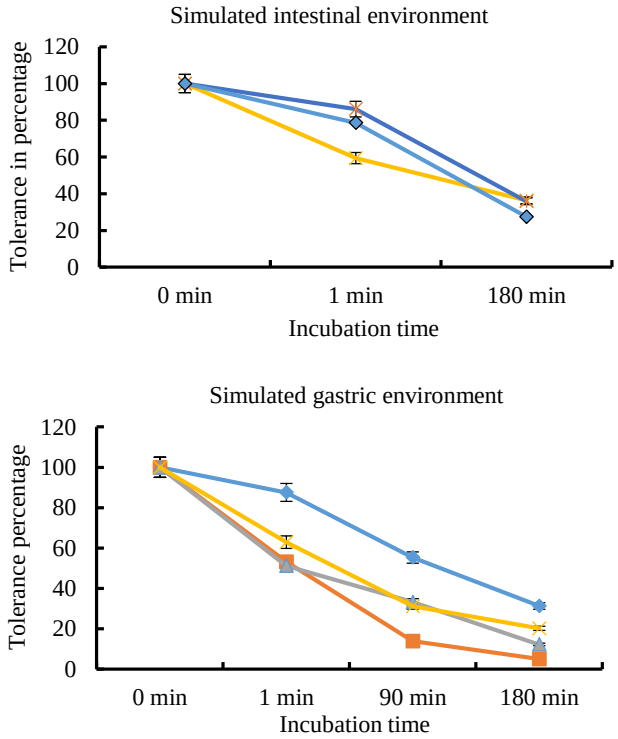

Figure 6. Effect of pancreatin on survival of four yeasts isolates (**A),** Effect of pepsin on survival of four yeasts isolates (B).

3.9 Hydrophobicity Test

Hydrophobicity activity provides clear ideas of the cell surface and cell adherence ability to surfaces of the intestinal walls. Presence of glycolipids, glycoproteins and complex carbohydrates on the microbial cell surface is reported to play vital roles in adherence interactions [22,29]. Surface biomolecules present on microbial cells directly correlate with their capability to adhere to epithelial cells [30,31]. The CP-I culture showed a good hydrophobicity with an initial value of 0.863 and a final value of 0.516. In this experiment, proportion of hydrophobicity was calculated using formula and showed 40.23% with p = 0.0047 (Table 1). To report health beneficiary effects on hosts, probiotics must be able to colonize the intestinal epithelial cells. Interacting with the human cells, probiotics are reported to use hydrophobic interactions that can be assessed by assessing its hydrophobicity [22,30].

3.10 Antibiotic Susceptibility Test

The CP-I culture showed resistant to ampicillin (10 mg), streptomycin (10 mg) (Table 1) (Fig. 7) and susceptibility to suphatried (300 mg), tetracycline (25 mg), penicillin-G (1 U) and chloramphenicol (25 mg), based on the interpretation of inhibition zones (in mm) by Reynolds et al., 2009 [32]**.** Resistance of the strains was reported based on the breakpoint proposed by European food safety authorities. Usually, probiotic supplements are prescribed with antibiotics to restore the intestinal microflora as antibiotics

lead to disturbance in the normal flora of the gut, the beneficial microorganisms are removed that result in symptoms such as abdominal pain and antibiotic-associated diarrhea, which commonly lead to dysbiosis. Therefore, probiotic microorganisms should survive in presence of antibiotics [33,34].

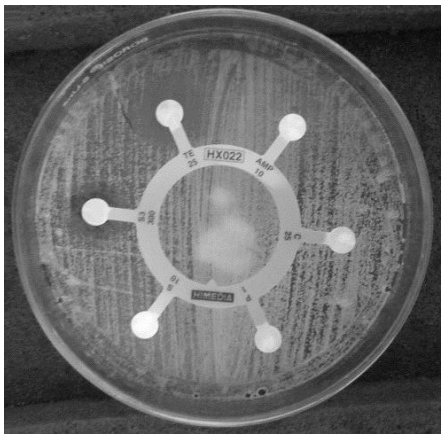

Figure 7. Antibiotic resistance and susceptibility pattern of the CP-I culture. AMP 10, ampicillin (10 mg); TE, tetracycline (25 mg); S3, sulphatried (300 mg); C, chloramphenicol (25 mg); S, streptomycin (10 mg); and P, penicillin G (1 U)

3.11 Bile Salt Hydrolysis

The BSH activity was assessed based on the white precipitation around colonies after 72 h of growth at 37 ºC in presence of sodium salt of taurodeoxycholic acid. In the present study, precipitation was observed around the CP-I colonies, showing BSH activity of the culture [24,35]. Bile salt hydrolysis is one of the important activities where probiotic strain detoxifies bile salts by producing BSH enzymes. This assay is one of the most important methods for screening probiotics. The BSH enzyme catalysis deconjugation of bile into free primary bile acids. Nowadays, probiotics with BSH activity are important as the activity helps lower blood cholesterol concentration of the host. It not only includes ability to decrease cholesterol levels, it is also reported that the presence of BSH can improve survival rates in the host GIT and thereby increase the adhesion ability, which was significantly established in *L. plantarum* [36].

3.12 Cholesterol-Lowering Activity

High blood cholesterol levels are suggested as the most important risk factors for the occurrence of CVD such as coronary heart diseases. Therefore, lowering serum cholesterol levels is important for disease prevention [4,37]. The CP-I showed ability to decrease cholesterol levels from the media, with 9.16% decrease in cholesterol levels (Table 1). Ability of probiotic strains to lower cholesterol levels depends on various mechanisms such as BSH activity, assimilation of cholesterol, binding of cholesterol to the probiotic cell surfaces or their physiological actions such as production of short-chain fatty acids (SCFA) [38]. In molecular investigation, it has been reported that certain enzymes secreted by the probiotic microorganisms played vital roles in assimilation and reduction of cholesterol levels. Enzymes such as bile salt hydrolase are responsible for the deconjugation of bile acids. To maintain homeostatic conditions, cholesterol is utilized for conjugation reactions. Studies have reported presence of cholesterol dehydrogenase/isomerase, which is responsible for the conversion of cholesterol to coprostanol compounds that is directly excreted in feces. The chemical assimilation in probiotics occurs by the microbial ability to adsorb cholesterol on their cell membranes [39]. By introducing probiotics in lifestyle, they establish healthy balanced gut microbiomes that perform the functions of probiotics as well as prevent risks of CVD.

3.13 Molecular Identification

The ITS region of CP-I yeast isolate was genotypically sequenced and analyzed. After comparing ITS gene regions in NCBI database, the CP-I isolate was identified as *S. cerevisiae* with 97% similarity (Table 1). Sequences were deposited in DNA Data Bank of Japan (DDBJ) (accession no. LC528139).

4. Conclusion

The present study documented isolation of four non-toxic yeast cultures that could grow at a wide range of pH, bile and temperatures as well as in presence of the intestinal environment. These cultures inhibited pathogenic species such as *E. coli, E. fecalis, S. aureus and Candida albicans*. Furthermore, the probiotic strains demonstrated good auto-aggregation characteristics. However, CP-I and CP-II included good co-aggregation capabilities with *E. coli, S. aureus* and *E. fecalis*. The CP-I isolate showed the best survival in the gastrointestinal transit environment within the four isolates. It could interact with organic solvents such as xylene; thus, including probiotic characteristics *in vitro*. In addition to being a good probiotic candidate, the isolate included BSH enzymes. To include beneficial functions for the host, the strain showed decreases in cholesterol by 9.16% *in vitro*. Based on these results, the CP-I isolate seems an excellent candidate for further probiotic characterization, identified as *S. cerevisiae* using molecular methods. These results provide an initial screening of the probiotic cultures for their efficacy as cholesterol-lowering therapeutics, reported for the first time from caterpillar frass isolates.

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6. Conflict of Interest

The authors report no conflicts of interest.

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ویژگی‌های زیست‌یاری کاهش‌دهندگی کلسترول *ساکارومیسس سرویزیه* جداشده از پودر کرم ابریشم *پیرهارکتیا ایزابلا*

مایورآرجون آسوانی[1]، سویاش آرونارو کاتاد[2]، پاشمین کوآر آناند[2]، بیپین‌راج نیریچان کانچیرامان[2]، پاوان راجندرا دوما[2]، سورش دی جاکتاپ[1*]

۱- گروه داروهای گیاهی، مرکز تحقیقات مرتبط با امور بهداشتی (IRSHA)، شبه دانشگاه باهاراتی ویدیاپیت، پونا ، ماهاراشترا ، هند.
۲- گروه میکروبیولوژی، انستیتو زیست‌فناوری و فناوری اطلاعات راجیو گاندی، شبه دانشگاه باهاراتی ویدیاپیت، پونا ، ماهاراشترا ، هند.

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***نویسنده مسئول**

سورش دی جاکتاپ

گروه میکروبیولوژی، انستیتو زیست‌فناوری و فناوری اطلاعات راجیو گاندی، شبه دانشگاه باهاراتی ویدیاپیت، پونا ، ماهاراشترا ، هند

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پست الکترونیک:
chiritatml@rediffmail.com

چکیده

سابقه و هدف: تجمع زیاد کلسترول عامل خطر فزاینده‌ای است که موجب بیماری قلبی عروقی می‌شود. بیماری‌های قلبی عروقی عامل مرگ و میر سالانه حدود ۱۶/۷ میلیون نفر در جهان می باشد. افزایش ۲۵/۷ میلیون به ۵۴/۷ میلیون مورد ابتلا به بیماری قلبی عروقی در هند گزارش شده است. از سال ۱۹۹۰، تغییرات الگوی غذایی عامل اصلی این وضعیت بوده است. با این‌حال، درمان‌های فعلی، مانند استاتین‌ها و مسدود کننده‌های بتا[1] ناکافی می‌باشند و سمیت‌هایی سیستمیک مانند ترمبوز فنر قلب[2] و التهاب مزمن را موجب می‌شوند. زیست‌یارها[3] با توانایی کاهش کلسترول انتخاب‌هایی ایده‌آل سالمی برای پیشگیری از بیماری‌های قلبی عروقی می‌باشند.

مواد و روش ها: مطالعه حاضر شامل روشی وابسته به کشت برای شمارش میکروارگانیسم‌های حاصل از *پیرهارکتیا ایزابلا* می‌باشد. میکروارگانیسم‌ها جداسازی، خالص‌سازی شدند و ریخت‌شناسی آنها با میکروسکوپ مورد مطالعه قرار گرفت. سویه‌هایی که ریخت‌شناسی مخمر داشتند، انتخاب شدند تا ویژگی‌های زیست‌یاری آنها تعیین شود. این جدایه‌ها براساس راهنمای سازمان جهانی بهداشت، شورای تحقیقات پزشکی هند و سازمان جهانی گوارشی از نظر توانایی زیست‌یاری بررسی شدند. مطالعه حاضر توانایی کاهش‌دهندگی کلسترول بهترین جدایه را مورد ارزیابی قرار داد.

یافته‌ها و نتیجه‌گیری: نتیجه روش وابسته به کشت جداسازی چهار کشت مخمر از پودر کرم ابریشم *پیرهارکتیا ایزابلا* بود. کشت‌ها فعالیت ضدمیکروبی و سایر ویژگی‌های زیست‌یارها مانند مقاومت در برابر pH، صفرا و درجه حرارت را از خود نشان دادند، مشابه گزارش Khisti و همکاران در سال ۲۰۱۹. با این‌حال، سویه جدید جداشده از *پیرهارکتیا ایزابلا* قادر به زنده‌ماندن در غلظت‌های بالای سدیم کلرید (۱/۰۲M) با درصد زنده‌مانی ٪۸۰/۳۲ بود و ٪۴۰/۲۳ آبگریزی نشان داد. کشت CP-I فعالیت آبکافتی نمک صفراوی و حساسیت به سولفاترید، پنی‌سیلین-G ، تتراسیکلین و کلرامفنیکل را نشان داد. در شرایط برون تنی[4]، این جدایه توانایی کاهش سطح کلسترول تا ٪ ۹/۱۶ را داشت. شناسایی مولکولی جدایه جدید تعلق آن به *ساکارومیسس سرویزیه*، با ٪۹۷ تشابه ژنتیکی، را نشان داد. از این‌رو، این مطالعه برای اولین بار جداسازی مخمر زیست‌یار کاهش‌دهنده کلسترول *ساکارومیسس سرویزیه* از پودر کرم ابریشم در شرایط برون‌تنی را نشان داد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] β-blockers
[2] stent thrombosis
[3] Probiotics
[4] *in-vitro*