

In-vitro investigation of cholesterol removal, β -galactosidase synthesis, antioxidant, and antidiabetic potential of probiotic organisms

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ABSTRACT

The present study is aimed to determine some important health beneficial properties of probiotic isolates such as cholesterol removal, β -galactosidase production, antioxidant, and anti-diabetic activity. Nine probiotic isolates were screened for Bile Salt Hydrolase (BSH) activity and potential BSH producers were selected for cholesterol removal study. Isolate B11 has shown significant cholesterol removal ability ($88 \pm 0.2\%$) after 24 h and this property was also analyzed with variable concentrations, time profile, with live, and dead cells as well as from egg yolk with simulation treatment. As probiotics improve lactose intolerance, all isolates were assessed for their lactose utilizing and β -galactosidase production ability where maximum enzyme activity was observed in case of isolate D25 (226 ± 0.30 Miller units). All the isolates were also assessed for their antioxidant potential using four different methods such as reducing power assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), 2,2-diphenyl-1-picrylhydrazyl assay, and superoxide dismutase activity. Out of nine isolates, isolate D25 and B11 have shown significant antioxidant property. Further, these isolates were also subjected to anti-diabetic study, where isolate D25 ($89 \pm 0.01\%$) and B11 ($98 \pm 0.30\%$) have shown high α -glucosidase inhibition which indicate their effective anti-diabetic activity. Hence, overall probiotic isolate D25 and B11 have shown significant health beneficial properties and they were further identified as *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus*, respectively.

1. INTRODUCTION

Probiotics have gained significant research consideration in recent years because of their multiple health benefits [1]. The word "probiotic" came from the Greek word "pro-bios" which literally means "for life." Probiotics are microbial cell which when supplemented in an adequate amounts, confer beneficial effects on the health, and well-being of the consumers [2]. Health benefits of probiotic organisms are highly specific with regard to species and strains and it cannot be generalized. Therefore, bio-prospecting for novel strains of efficient probiotics has been continuously practiced [1].

There are some basic selection criteria for probiotics to be used as food supplement, that is, its safety, viability/activity in delivery carriers, acid and bile resistance, adherence and colonization in gastrointestinal tract, and production of antimicrobial substances such as bacteriocin [3,4]. Probiotics also possess some special properties such as anti-inflammatory, anti-oxidant, anti-diabetic, anti-cancer, cholesterol removal, and vitamin production [5]. Indeed, several studies have

validated that probiotics have advantageous effects on a wide range of disorders such as irritable bowel syndrome, inflammatory bowel disease, diarrhea, constipation, hypertension, lactose intolerance, and hypersensitivity [6].

In recent years, due to the food habits, people are suffering more with several cardiovascular and cerebrovascular diseases [7] associated with high cholesterol concentration. This also includes heart stroke, peripheral artery disease, diabetes, and high blood pressure [8]. Therefore, the cholesterol-lowering ability of probiotics and the associated mechanisms for cholesterol removal have received a great attention. However, the exact mechanism of the cholesterol removal by probiotics is not completely understood but several mechanisms have been proposed through *in-vivo* and *in-vitro* studies [9]. Probiotic strains such as *Lactobacillus fermentum*, *Limosilactobacillus reuteri*, *Lactocaseibacillus rhamnosus*, and *Lactobacillus acidophilus* are reported for their cholesterol removal ability. Reports have shown that consumption of these probiotics supplemented food can help in cholesterol reduction by producing bile salt hydrolase (BSH) enzyme and therefore, production of BSH has become a crucial selection criterion for probiotic organisms to determine their cholesterol removal ability. Besides producing BSH, probiotics also possess some inherent ability to scavenge cholesterol [8]. With reference to this, we have also screened out our

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isolates on the basis of BSH production and assessed their potential for cholesterol removal.

Nowadays, another problem faced by many people is lactose intolerance [10]. Lactose intolerance is not a disease but it is a physiological condition where this disaccharide can cause severe intestinal distress such as abdominal pain, bloating, and flatulence in a person with deficiency or with lower production of the intestinal enzyme β -galactosidase. Lactose intolerance severely limits the use of milk and other dairy products. Moreover, this condition becomes more severe with age and it restricts the intake of calcium-rich dairy foods when it is actually obligatory. Probiotics play a key role by producing β -galactosidase enzyme during fermentation which hydrolyses the lactose to glucose and galactose and thereby improving lactose digestion [11]. Therefore, in the present study, probiotic isolates were assessed for their ability to produce β -galactosidase enzyme.

In human body, a number of metabolic product are generated in which synthesis of superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals have been reported as reactive oxygen species (ROS) which generate oxidative stress. Moreover, nitrogen oxides, herbicides, ozonization, radiation, and some metals are also responsible for inducing oxidative stress. However, many enzymatic (glutathione reductase, glutathione peroxidase, and superoxide dismutase [SOD]) and non-enzymatic (Vitamin E, Vitamin C, thioredoxin, and glutathione) antioxidant defense mechanisms are reported in living systems that reduce oxidative stress by scavenging ROS. Other than these natural defense mechanisms, several synthetic antioxidants are also available such as butylated hydroxyl-anisole and butylated hydroxyl-toluene [12]. In this context, we have analyzed our probiotic isolates for their antioxidant property using four different methods (Reducing power assay [RPA], 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid [ABTS] assay, 2,2-diphenyl-1-picrylhydrazyl [DPPH] assay, and SOD assay).

Another serious metabolic disorder is diabetes mellitus which shows elevated blood sugar level because of insufficient insulin production (Type 1) or insulin resistance (Type 2). A number of anti-diabetic drugs have been developed to combat this disease. However, the commonly used synthetic drugs have their side effects such as flatulence, stomach pain, nausea, headache, heartburn, and diarrhea. Therefore, as an alternative to these common drugs, researchers have focused on the use of probiotics in the treatment of diabetes mellitus and probiotics have shown an incredible role in diabetes treatments due to their ability to inhibit the enzymes α -glucosidase and α -amylase [13]. Hence, in the present study, we have also assessed probiotic isolates for their anti-diabetic activity.

2. MATERIALS AND METHODS

2.1. Chemicals and Media

de Man Rogosa Sharpe (MRS) broth, Nutrient broth, Agar powder, Cholesterol, ABTS, DPPH, ONPG, pNPG, CTAB, KCl, Na_2HPO_4 , Na_2CO_3 , CaCO_3 , MgSO_4 , MnSO_4 , KOH, NaOH, HCL, H_2SO_4 , NaCl, NaHCO_3 , KI, β -mercaptoethanol, Pyrogallol, Ox-bile, Starch, Acarbose, Ascorbic acid, O-phthalaldehyde reagent, SOD, α -amylase, α -glucosidase, Trypsin and Pepsin were used in this study. DPPH was purchased from Sigma-Aldrich (U.S.A.) and all the other media, chemical and reagents were purchased from Himedia Laboratories Private Ltd. (Mumbai, India) and Loba Chemie Private Ltd. (Mumbai, India).

2.2. Determination of BSH Activity of Potential Probiotic Isolates by Plate Method

Isolation of probiotic microorganisms was carried out from various samples such as fermented idli batter, curd, human milk, lentils, and

an infant's fecal material and a total of 27 isolates were obtained. They were primarily screened for acid and bile tolerance; 12 isolates have shown resistance to low pH and bile. Further, they were proceeded for secondary screening. Among 12 isolates, nine isolates were screened out showing significant viability during gastrointestinal simulation. These nine isolates were further studied for different health beneficial properties. To determine cholesterol removal ability, BSH activity was done using plate assay method [14]. Soft MRS agar was prepared by adding 1.5% agar to MRS broth and then supplemented with bile salt (0.3% w/v, ox bile) and CaCO_3 (0.3% w/v). This mixture was autoclaved, poured on MRS plate, and was allowed to solidify. The wells of 3 mm diameter were made on agar plate and overnight grown cultures were added in wells. The plates were incubated at 37°C for 72 h and observed after each 24 h time interval for clear visible halos around the well indicated the positive BSH activity of the isolates. MRS plates without bile salts were used as the negative control.

2.3. Bacterial Growth and Cholesterol Removal

Probiotic isolates with good BSH activity were selected for cholesterol removal study. For the same, overnight grown cultures of probiotics were inoculated in medium containing cholesterol (200 $\mu\text{g}/\text{ml}$) supplemented with ox bile (0.3% w/v), MgSO_4 (0.05% w/v), and MnSO_4 (0.02% w/v). This mixture was then incubated at 37°C under shaking (100 rpm) for 24 h. After incubation, the bacterial growth was measured at 600 nm using a UV-VIS spectrophotometer and cholesterol removal was determined according to the method described by Rudel and Morris [15] with some modifications. Briefly, activated cells were centrifuged (5000 g, 4°C, 10 min) and the supernatant was saved for cholesterol estimation. In 1 ml aliquot, 1 ml of KOH (33% w/v) and 2 ml of absolute ethanol were added. The mixture was then vortexed for 1 min and incubated at 37°C for 15 min. After incubation, 2 ml of distilled water and 3 ml of n-Hexane were added. This mixture was again vortexed for 1 min and allowed to settle down. 1 ml from the hexane layer was transferred to another glass tube and evaporated. After evaporation, the residues were immediately dissolved in 2 ml of O-phthalaldehyde reagent. On complete mixing, 0.5 ml of concentrated H_2SO_4 was added and the mixture was vortexed again for 1 min. After 10 min, the absorbance of the mixture was taken at 550 nm. All experiments were performed in triplicate. The cholesterol concentration was determined from a standard curve prepared using a cholesterol stock solution (1000 $\mu\text{g}/\text{ml}$). The ability of cholesterol removal of probiotics was expressed as the percentage of cholesterol removed at each incubation interval as follows:

$$\text{Cholesterol Removal}(\%) = 100 - \frac{\text{Initial Cholesterol} - \text{Residual Cholesterol}}{\text{Initial Cholesterol}} \times 100$$

2.4. Time Profile for Cholesterol Removal Study

With the observation of bacterial growth and cholesterol utilizing ability, potential probiotic isolate was selected for time profile study. For the same, 24 h grown culture was inoculated into cholesterol containing medium. The concentration of cholesterol was estimated by withdrawing the samples at different time interval such as 0 h, 2 h, 4 h, 6 h, 12 h, and 24 h. The cholesterol concentration was evaluated in triplicate as described earlier.

2.5. Removal of Variable Concentrations of Cholesterol

Cholesterol utilizing ability of probiotic was determined by supplementing different cholesterol concentrations into the medium.

Overnight grown culture was inoculated in media having variable cholesterol concentrations ranging from 100 to 500 $\mu\text{g/ml}$ and incubated for 24 h at 37°C. After incubation, the samples were collected and estimated for residual cholesterol content as per the earlier protocol. All the experiments were performed in triplicate.

2.6. Cholesterol Removal by Live and Heat-killed (Dead) Probiotic Cells

The cholesterol removal ability of non-growing (heat-killed) probiotic cells was compared with growing cells. Heat-killed (dead) cells sample was prepared by autoclaving the overnight grown culture at 121°C for 15 min. After that, heat killed dead cells and live growing cells were processed for cholesterol removal study as described earlier. After incubation, cholesterol concentrations of the supernatants were determined. All the experiments were performed in triplicate.

2.7. Removal of Cholesterol from Egg Yolk under Gastrointestinal Stress by Probiotics

Probiotic isolate was examined for its ability to remove cholesterol from cholesterol-rich food source (egg yolk) under gastrointestinal stress condition to mimic the *in vivo* condition. Egg yolk was added to phosphate buffer (0.2 M, pH 7.0) and then homogenized. This homogenized egg yolk was taken as a sole source of nutrition in the media and inoculated with the overnight grown culture of probiotic isolate. This system was treated with gastric (for 2 h), intestinal (for 4 h), and gastrointestinal (for 6 h) simulation. After each simulation treatment, the samples were collected and the cholesterol content was estimated in triplicate from the supernatant using the same method.

2.8. Assessment of Lactose Utilizing Ability of Probiotic Isolates

Lactose fermentation results in organic acid production [3] and thus lactose utilization ability of probiotics can be determined by observing the color change in the medium as a result of fermentation. For the same, 24 h grown probiotic cultures were inoculated in fermentation medium (10 g peptone, 15 g NaCl, 0.018 g phenol red, 5 g lactose in 1 L distilled water, and pH 7.0). These tubes were incubated at 37°C for 24 h and after incubation, the tubes were observed for the change in color from red to yellow indicating acid production on lactose fermentation.

2.8.1. Determination of β -galactosidase activity

Cultures showing good lactose utilizing ability were selected for the determination of β -galactosidase activity using the method describes by Özkan *et al.* [6] with several modifications. For this purpose, absorbance of activated cultures was taken at 600 nm and then 20 μl of 24 h grown cultures were mixed with 80 μl of permeabilization solution (100 mM Na_2HPO_4 , 20 mM KCL, 2 mM MgSO_4 , 0.8 mg/ml CTAB, 0.4 mg/ml SDS, and 5.4 $\mu\text{l/ml}$ β -mercaptoethanol) followed by incubation at 37°C for 20–30 min. Further, 600 μl substrate solution (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 1 mg/ml O-nitro-phenyl- β -D-Galactoside (ONPG), 2.7 $\mu\text{l/ml}$ β -mercaptoethanol) was added and the reaction mixture was allowed to incubate at 37°C for 15 min or until the yellow color appears and the reaction completion time period was noted down. After incubation, the reaction was stopped by adding 700 μl of stop solution (1 M Na_2CO_3). Consequently, absorbance at 420 nm was measured and the β -galactosidase activity was determined using the following formula:

$$\beta\text{-Galactoidase activity} = 1000 \times \frac{\text{Abs}_{400}}{\text{Abs}_{600} \times \text{Volume (ml)} \times \text{Reaction time (min)}}$$

2.9. Determination of Antioxidant Activity

2.9.1. RPA assay

RPA was performed as per the method used by Al-Dhabi *et al.* [12] with some modifications. 100 μl of samples (cell-free supernatant [CFS]) were mixed with 1% K_3FeCN_6 and incubated at 50°C for 20 min in boiling water bath. The mixture was allowed to cool down and 10% trichloroacetic acid was added. Further, the content was centrifuged and the upper layer was aspirated and mixed with 0.1% FeCl_3 solution. Then, the absorbance was taken at 700 nm and the reducing power potential was determined with reference to ascorbic acid standard in terms of ascorbic acid equivalent antioxidant capacity (AEAC, $\mu\text{g/ml}$).

2.9.2. ABTS assay

Antioxidant activity was also determined by ABTS assay [16]. As per the protocol, 5 ml of ABTS (7 mM) solution and 88 μl of potassium persulphate (140 mM) were mixed vigorously. This mixture was incubated at room temperature for at least 16 h in the dark. Then, this solution was diluted with distilled water until it reaches to optical density 0.7 at 734 nm and this was used as working solution. 100 μl of sample (CFS) was mixed with 3 ml of ABTS working reagent and incubated at 37°C for 10 min after which the absorbance was taken at 734 nm. A control without sample was also measured and the percent inhibition activity was calculated using the following formula:

$$\text{ABTS Inhibition (\%)} = 100 \times \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}$$

2.9.3. DPPH assay

Potential isolates were evaluated for their ability to scavenge DPPH free radicals as suggested by Sui *et al.* [17] with slight alterations. 50 μl of sample (CFS) was mixed with 3 ml DPPH (0.2 mM) solution and incubated at 37°C for 20 min. Then, the absorbance was measured at 517 nm along with the control (without sample) and the percent inhibition activity was calculated using the following formula:

$$\text{DPPH Inhibition (\%)} = 100 \times \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}$$

2.9.4. SOD activity

Superoxide scavenging activity was also determined for potential isolates [18]. Briefly, 2.35 ml of Tris-buffer (0.1 M) was mixed with 2 ml of distilled water followed by 20 μl of sample (CFS) and 150 μl of pyrogallol solution (4.5 mM in HCL). For the blank tube, same reaction mixture was prepared without adding sample and its absorbance was taken just after mixing the reagent and also after 1 min of mixing. The difference in absorbance between two aliquots indicated the rate of pyrogallol autoxidation. The superoxide radical scavenging activity was calculated as:

$$\text{SOD activity (\%)} = \frac{\frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100\%}{50\%} \times 4.5 \times \frac{1}{\text{volume of sample}} \times \text{Dilution factor}$$

2.10. Anti-diabetic Activity of Potential Probiotic Isolates

Pancreatic α -amylase and α -glucosidase are two major enzymes present in digestive system that catalyze the first step in the digestion

of starch. Inhibition of these enzymes decreases the digestion and uptake of carbohydrates, thereby curtailing postprandial blood glucose level is regularized in Type II diabetic subjects. Therefore, here in this study, anti-diabetic activity was determined by performing these two tests where acarbose was taken as standard.

2.10.1. Anti- α -amylase activity

Anti- α -amylase activity was determined by the method suggested by Xia *et al.* [19]. For the same, 100 μ l aliquot (CFS) was mixed with 300 μ l of sodium phosphate buffer (0.1 M, pH 6.9), then 200 μ l of α -amylase (2 U/mg) enzyme was added and the mixture was incubated at 37°C for 10 min. On incubation, 200 μ l of starch (1% w/v) was added and enzymatic reaction was allowed for 30 min at 37°C. After completion, the reaction was stopped by adding 200 μ l of HCl followed by 200 μ l of KI for color development and then the absorbance was taken at 620 nm. A solution mixture without substrate and enzyme was used as blank and without enzyme was used as a control and the inhibition activity was calculated using the following formula:

$$\alpha\text{-amylase Inhibition}(\%) = 100 \times \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}$$

2.10.2. Anti- α -glucosidase activity

Anti- α -glucosidase activity of selected probiotic cultures was also determined [20]. Briefly, 100 μ l of sample (CFS) was taken and mixed with 400 μ l sodium phosphate buffer (0.1 M, pH 6.9). 100 μ l of α -glucosidase enzyme (1 U/ml) was added and tubes were incubated at 37°C for 10 min. After this pre-incubation, 200 μ l of p-nitro-phenyl α -glucopyranoside (pNPG, 5 mM) was added as a substrate solution and enzymatic reaction was allowed for 30 min at 37°C. After incubation, the reaction was stopped by adding 100 μ l of 0.1 M sodium carbonate and then the absorbance was measured at 405 nm. Anti- α -glucosidase activity was determined by measuring the release of p-nitro-phenol from pNPG at 405 nm. A solution mixture without sample was used as a control and without sample and enzyme was used as blank and the inhibition activity was calculated as follows:

$$\alpha\text{-glucosidase Inhibition}(\%) = 100 \times \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}$$

2.11. Statistical Analysis

All experimental measurements were repeated independently in triplicate and results are expressed as mean \pm standard deviation and they were evaluated by performing the analysis of variance (ANOVA) considering the significance level of $P < 0.05$. The analysis was performed using MS-Excel.

3. RESULTS AND DISCUSSION

3.1. Determination of BSH Activity of Potential Probiotic Isolates by Plate Method

The BSH enzyme produced by probiotics perhaps deconjugates the bile salts which leads to less cholesterol uptake from the gut [17]. This can be used as a vital marker for selection of unique probiotics having hypocholesterolemic activity [1]. Total nine different probiotic isolates were tested for the production of BSH enzyme on MRS agar plate supplemented with bile salt and calcium carbonate by agar well diffusion method. Among them, isolate D25, B11, B32, M2, and M3 have shown positive BSH activity by showing a clear zone around the well [Figure 1] and the zone diameters are shown in

Figure 2. Similarly, Foley *et al.* [21] have determined BSH activity of *L. acidophilus* and *Lactobacillus gasseri* and suggested that BSH enzyme cleave the conjugated glycine or taurine from primary bile acids which may help further in cholesterol removal. In addition, Liang *et al.* [22] have reported BSH activity of 54 highly hydrophobic probiotic isolates by the plate assay against taurodeoxycholic acid (TDCA) or glycinodeoxycholic acid (GDCA). They found that 15 isolates have shown positive activity for both the substrates, 13 had ability to hydrolyzed TDCA, and only five strains tended to hydrolyze GDCA. Hence, they concluded that different strains might have different substrate specificity of BSH. Moreover, Zhang *et al.* [9] have determined BSH activity of 10 different *Streptococcus thermophilus* strains and compared it with standard reference strain where they found that three isolates have higher, three isolates have lower, and four isolates have similar BSH activity to the reference strain.

3.2. Bacterial Growth and Cholesterol Removal

Total three potential BSH producing probiotic isolates (isolate B11, D25, and B32) were selected for cholesterol removal study. Activated cultures were inoculated into the medium with cholesterol and bile and isolate B11 has shown the highest cholesterol removal efficiency ($88 \pm 0.2\%$) with significant growth [Figure 3]. On similar notes, Liu *et al.* [23] have studied cholesterol removal ability of *Lactiplantibacillus plantarum* Y15 through both *in-vitro* and *in-vivo* studies. They have observed high BSH activity *in-vitro*. In *in-vivo* study, they have observed a decrease in total cholesterol and triglyceride level in serum and liver while level of cholesterol and bile salts was increased in feces. Likewise, Singhal *et al.* [24] have isolated *Enterobacter faecium* which was able to assimilate $>50\%$ of cholesterol from the medium. This percent assimilation could be improved with addition of bile salts in the medium as *E. faecium* LR13 has shown 98% and 76% assimilation with and without bile salts, respectively. In addition, El-Zahar *et al.* [25] have supplemented camel and cow's milk with *Bifidobacterium longum* and observed that it significantly improved the body weight, blood lipid profile, serum proteins, and liver and kidney markers in mice fed with high fat diet. Moreover, Pereira *et al.* [26] have studied cholesterol removal from MRS medium containing cholesterol along with ox-bile. They concluded that the amount of bile salt present in the medium has affected both the growth rate of bacteria as well as its cholesterol removal ability. As the amount of ox-bile was increased, a significant decrease was observed in the growth rate and so as in cholesterol removal. At lower bile concentration ($<0.5\%$), cholesterol reduction remained constant, and it increased till 2% cholesterol concentration and then with further rise in concentration, no significant removal was observed.

3.3. Time Profile for Cholesterol Removal Study

Time profile of cholesterol removal was carried out with isolate B11. The significant finding of this study is that this culture was able to utilize more than 60% of supplemented cholesterol within 6 h and the highest cholesterol removal was observed $88 \pm 0.17\%$ after 24 h as shown in Figure 4. Similar kind of results has been reported by Kimoto *et al.* [27] where maximum cholesterol removal by isolate N7 was achieved within 12–18 h of incubation which corresponds to the exponential growth of the culture. Comparable statement was also given by Shivangi *et al.* [18] that cholesterol removal depends on bacterial cellular state and growth phase (log, stationary, and decline phase). Their isolate *Bacillus tequilensis* showed maximum cholesterol removal (64.41%) in log phase of growth.

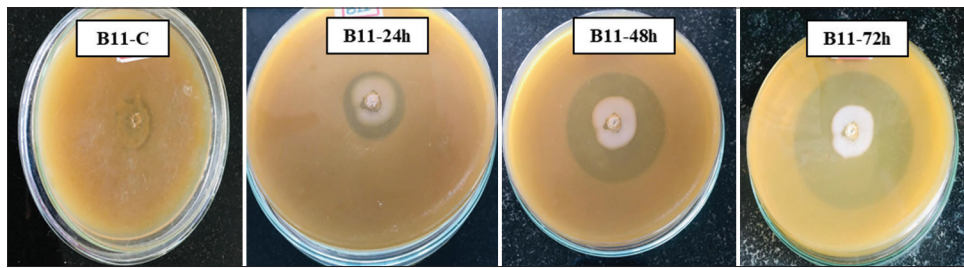


Figure 1: Bile salt hydrolase activity zone on bile salt supplemented MRS agar plates.

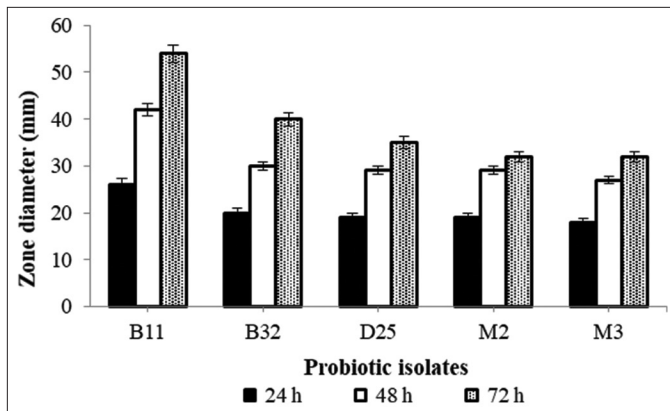


Figure 2: Bile salt hydrolase activity of probiotic isolates by plate method.

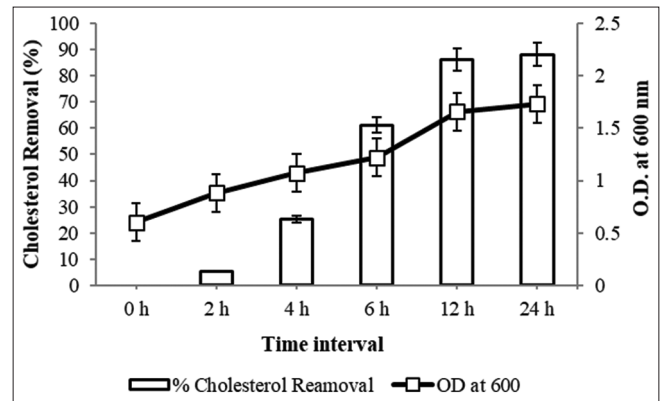


Figure 4: Time profile study for cholesterol removal.

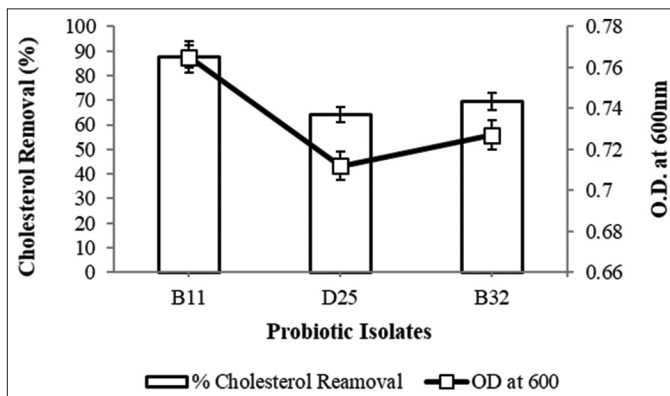


Figure 3: Bacterial growth and cholesterol removal efficiency.

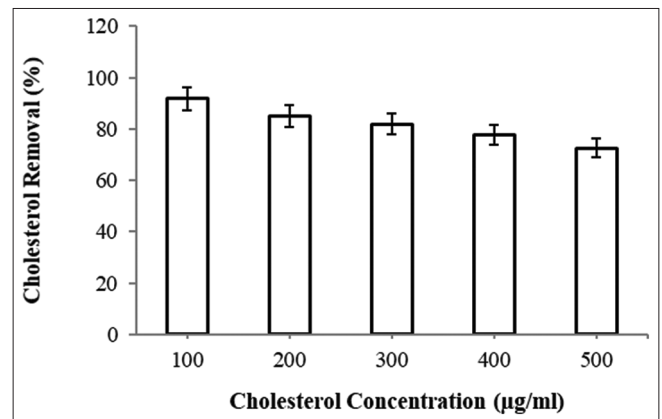


Figure 5: Cholesterol removal at variable cholesterol concentration.

3.4. Cholesterol Removal with Different Cholesterol Concentration

Isolate B11 has shown significant removal within 24 h and therefore to determine cholesterol removal efficacy of this culture, variable concentrations of cholesterol (100, 200, 300, 400, and 500 µg/ml) were taken. After 24 h of incubation, cholesterol removal was determined and linear decrease was observed with low to high concentrations. The observations indicated lower removal efficiency with increase in cholesterol concentration into the media [Figure 5]. Isolate B11 showed 92 ± 0.11% removal with the lowest concentration (100 µg/ml) which was decreased to 72 ± 0.55% with 500 µg/ml cholesterol. In contrast, Majeed *et al.* [28] observed regardless of minimum (25 µg/ml) or maximum (200 µg/ml) concentrations of cholesterol, *Bacillus coagulans* was able to remove nearly 50% of total cholesterol from the medium.

3.5. Cholesterol Removal by Live and Heat-killed (Dead) Probiotic Cells

The actual mechanism of cholesterol removal is still unknown; and therefore, this study was also carried out with live and dead biomass to determine whether the dead cells can assimilate the cholesterol or not. Two different concentrations 200 and 500 µg/ml were taken under study and cholesterol removal was observed 86 ± 0.04% and 73 ± 0.13% with live cells and 8 ± 0.2% and 9 ± 0.04% with dead biomass, respectively. These observations indicated that dead biomass has no significant effect on cholesterol removal. The capability of dead cells to confer health benefits has attracted many food manufactures due to certain advantages such as longer shelf-life, better handling, easy transportation and storage, and possibility of addition of non-bacterial biologically active metabolite. However, simultaneously, inactivated or killed cells possess negligible functional properties and

therefore do not extend any health benefits in diseased conditions [29]. In this reference, Miremadi *et al.* [30] reported significant difference in cholesterol removal by growing (34–65%), resting (29–56%), and dead (9–37%) cells of *L. acidophilus* and *B. longum*. Likewise, Anila *et al.* [31] have described higher cholesterol removal (47.70 µg/ml) by live cells of *Lactocaseibacillus casei* PLA5 which was only 4.74 µg/ml by dead cells indicating the attachment of cholesterol on the cell membrane of live bacterial cells [32].

3.6. Removal of Cholesterol from Egg Yolk under Gastro-intestinal Stress by Probiotics

Cholesterol removal in a synthetic medium was studied with many different parameters so the isolate B11 was assessed for its removal efficiency from a cholesterol rich food (egg yolk). To mimic *in vivo* condition, simulation treatment was also given and the cholesterol removal was observed 15 ± 0.12% after 2 h of gastric (GJ), 15 ± 0.24 % after 4 h of intestinal (IJ), and 45 ± 0.09 % after 6 h of gastrointestinal (G + I) simulation [Figure 6]. When the treatment was continued for 24 h, it resulted in 52 ± 0.02% removal. In a similar way, Mikulski *et al.* [33] have observed 12.33% cholesterol removal from egg yolk by *Pediococcus acidilactici*. With a step ahead, Lokapinasari *et al.* [34] aimed to study the combinatorial effect of *Bifidobacterium* spp. and *L. acidophilus* on egg yolk cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) content. With supplementation of equal amount (0.5% each) of both the probiotics, they observed lower cholesterol content (155.86 mg/dl), lower LDL content (48.46 mg/dl), and higher HDL (50.00 mg/dl) which were earlier observed 224.02 mg/dl, 58.63 mg/dl, and 41.59 mg/dl, respectively, suggesting probiotics as a significant diet supplement. Researchers have also studied cholesterol removal potential of probiotics with other cholesterol rich foods than egg yolk. With reference to this, Liu *et al.* [35] have determined cholesterol removal ability of *L. plantarum* and *Enterococcus faecalis* from egg yolk as well as skimmed milk. They observed 58.15% and 38.45% of cholesterol removal from egg yolk and 22.35% and 11.22% removal from skimmed milk by *L. plantarum* and *E. faecalis*, respectively. Their removal pattern is also similar to that of observed in our study.

3.7. Determination of Lactose Utilizing Ability

Probiotic organisms have been proved very useful for lactose intolerant people as they can easily utilize lactose. Total nine potential isolates were screened for their lactose utilizing ability by tube method where phenol red was used as an indicator. Organism with lactose utilizing ability produces organic acids on fermentation of lactose that leads to

yellow color production in the medium. In this study, total six isolates (Isolate B32, D25, F, M1, M2, and M3) have shown positive lactose utilizing ability. This lactose utilizing ability is considered as screening criteria for the selection of potential probiotic [36] and Zeng *et al.* [37] have studied fermentation patterns of three different *L. plantarum* strains (GS083, GS086, and GS090) and observed all of them were able to utilize lactose as a carbon source.

3.8. Determination of β-galactosidase Activity

Many children and adults are unable to digest lactose due to insufficient production of β-galactosidase enzyme and suffer from lactose intolerance which is a common digestive problem when person consume milk or any other dairy products [38]. Probiotic organisms have been found beneficial in improving these symptoms through the production of β-galactosidase [6]. In this study, all lactose utilizing probiotic isolates were screened for β-galactosidase activity by ONPG method. From the six potential lactose utilizing isolates, isolate D25 has shown maximum activity of 226 ± 0.30 Miller units followed by isolate B32 (190 ± 0.13) and M2 (110 ± 0.15) as shown in Figure 7. Similarly, Son *et al.* [39] have studied β-galactosidase activity of *L. plantarum* Ln4 isolated from Kimchi that significantly showed 3320.99 Miller units activity. In addition, Chanalía *et al.* [40] have reported β-galactosidase activity of *P. acidilactici* to be 65.08 ± 0.9 Miller units which was actually 22 times higher than earlier reports. In addition, Zhao *et al.* [41] have determined β-galactosidase activity of *Lactobacillus bulgaricus* with the use of whey-based medium and observed two-fold higher (2034 U/L) activity than the activity observed with the traditional MRS medium. However, probiotic strain *Lactobacillus lactis* Gh1 was not able to produce β-galactosidase enzyme but they suggested that absence of this enzyme would not be a disadvantage since permeability of cells is necessary for efficient lactose hydrolysis in the small intestine [42].

3.9. Determination of Antioxidant Activity

In the present study, antioxidant activity of potential isolates was determined using four different methods such as RPA, ABTS, DPPH, and SOD. RPA was determined in terms of ascorbic (AEAC, µg/ml) and isolates have shown significant reduction ability except isolate F [Figure 8a]. Isolate M3 (1074 ± 0.32 µg/ml) showed the highest AEAC followed by isolate B32 (953 ± 0.37 µg/ml) and D25 (905 ± 0.26 µg/ml). When ABTS assay was performed [Figure 8b], isolate D25 (72 ± 0.04%) showed maximum ABTS scavenging ability followed by isolate B11 (69 ± 0.17%) and B32 (66 ± 0.46%). DPPH assay was also performed where isolate D25 (71 ± 0.22%) has again shown maximum DPPH scavenging activity followed by isolate B11 (64 ±

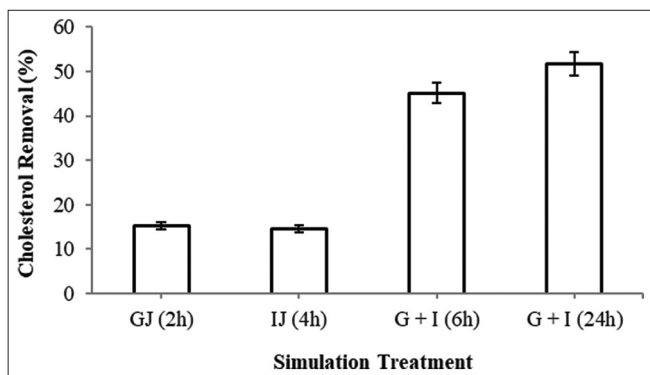


Figure 6: Cholesterol removal from egg yolk with simulation treatment. (GJ - Gastric juice, IJ - Intestinal juice, G + I - Gastrointestinal simulation).

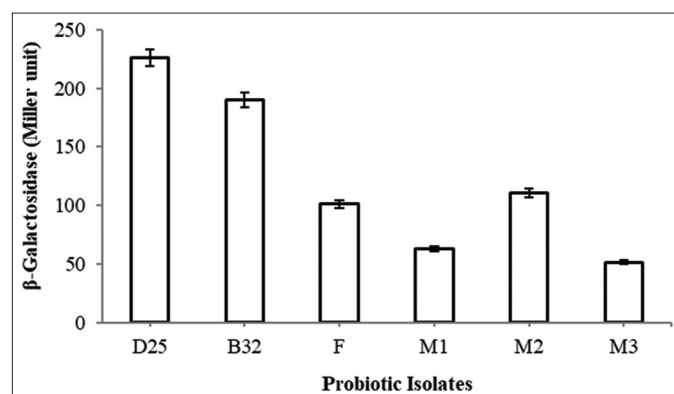


Figure 7: β-Galactosidase activity of probiotic isolates.

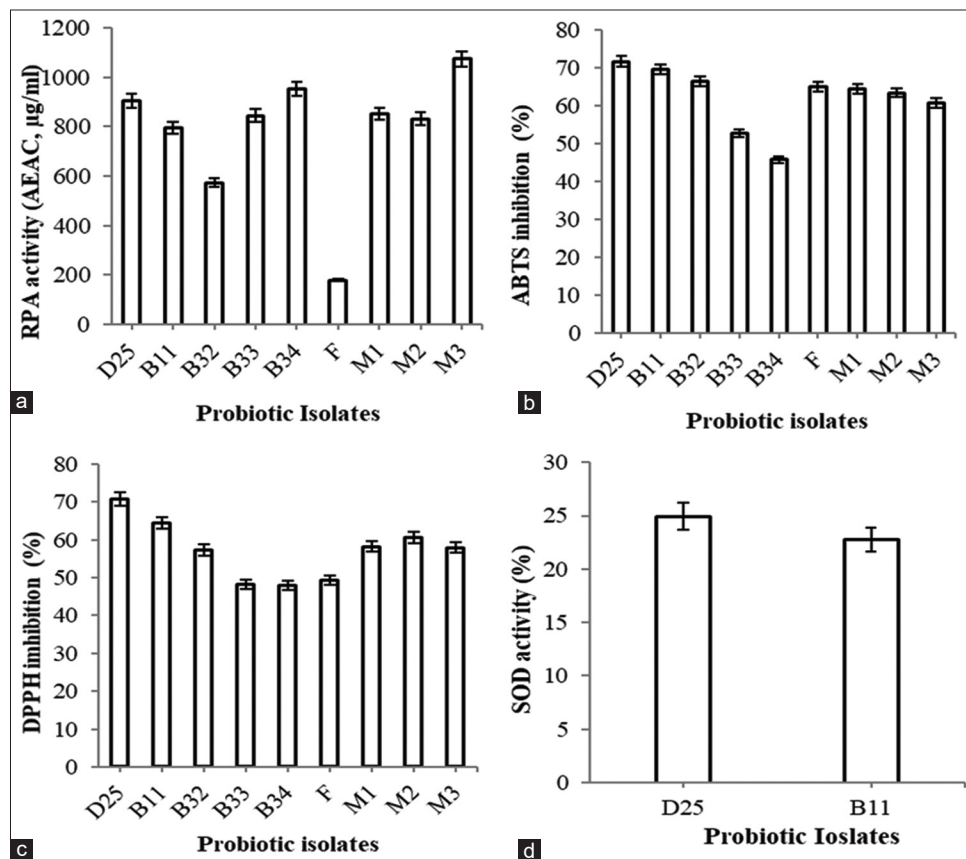


Figure 8: Antioxidant activity of probiotic isolates (a) reducing power activity, (b) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid inhibition activity, (c) 2,2-diphenyl-1-picrylhydrazyl inhibition activity, and (d) superoxide dismutase activity.

0.26%) and isolate M2 with $61 \pm 0.14\%$ activity [Figure 8c]. Moreover, in case of SOD activity [Figure 8d], isolate D25 and B11 have shown $25 \pm 0.06\%$ and $23 \pm 0.33\%$ activity, respectively. Similarly, Cao *et al.* [16] studied *L. plantarum* ST with significant ABTS (47%) and DPPH (59%) scavenging activity. Antioxidant activity with CFS, cell free extract and intact cells of *L. plantarum* and *L. rhamnosus* was determined by DPPH method and highest antioxidant activity with CFS followed by cell free extract and intact cells was observed [43]. In addition, Romero-Luna *et al.* [44] have reported 1336.72 ± 345.05 Trolox_{eq} ABTS scavenging activity from the CFS of *L. paracasei* CT12. Furthermore, tremendous DPPH scavenging activity was observed from the CFS of *Weissella confusa* MD1 ($69.15 \pm 2.73\%$) and *Weissella cibaria* MD2 ($74.34 \pm 1.4\%$) [45]. In addition, 50% reducing power potential of *L. plantarum* RJF4 by RPA was also reported [46]. In addition, Kostelac *et al.* [47] determined SOD activity of their isolate *L. plantarum* M2 (0.02 ± 0.002 U/ml) and *L. plantarum* KO9 (0.05 ± 0.009 U/ml).

3.10. Anti- α -amylase and Anti- α -glucosidase Activity

Amylase is a first enzyme to start digestion and it will convert starch to glucose molecule. Similarly, α -glucosidase enzyme also helps in digestion by breaking down starch and other disaccharides into glucose molecules. Inhibition of these two enzymes by probiotic cells leads to low serum glucose level and proves them as potent anti-diabetic agents. This activity was performed with two potential isolates, that is, isolate D25 and B11 [Figure 9]. None of them have shown significant inhibitory effect on α -amylase as the inhibition observed was $11 \pm 0.24\%$ and $10 \pm 0.20\%$, respectively. This might have happened as

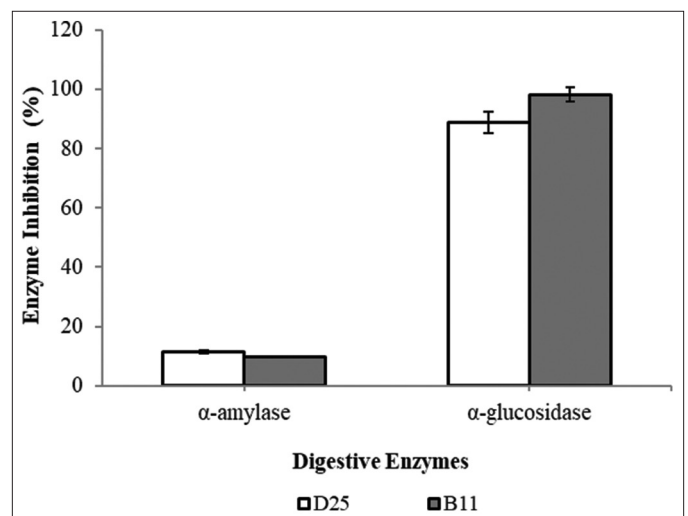


Figure 9: Anti-diabetic activity of potential probiotic isolates.

these cultures are potential amylase producers and therefore they do not possess any inhibitory effect on this enzyme. However, $89 \pm 0.01\%$ and $98 \pm 0.30\%$ α -glucosidase inhibition was observed with isolate D25 and B11, respectively. Similarly, potent inhibitory activity of α -glucosidase (91.7%) by *L. plantarum* C70 [48]. Likewise, Zeng *et al.* [13] have assessed α -glucosidase inhibition activity of seven different strains of *L. plantarum* and *L. brevis* and observed inhibitory activities ranged from 18.4% to 34.9% with the highest value for *L. plantarum* ZF06-3. Our result with α -glucosidase enzyme was

considerably significant than the earlier reported observations but our α -amylase inhibition is not significant. Similar kind of observation was also reported by Shin *et al.* [49]. They observed no amylase inhibition activity with their 17 probiotic isolates and have suggested that high amylase activity is a fundamental property of probiotics for starch hydrolysis and this might be a reason behind low inhibitory activity.

As isolate D25 and B11 have shown very significant results in terms of various health beneficial properties, they were further identified by 16S rRNA sequencing method. Isolate D25 and B11 were identified as *Lactiplantibacillus plantarum* with accession number MW362778 and *Pediococcus pentosaceus* with accession number MW362744, respectively.

4. CONCLUSION

The results of the present study indicate that all the nine isolates taken under study have shown significant results in terms of cholesterol removal, β -galactosidase production, antioxidant property, and anti-diabetic activity. Among them, isolate B11 and D25 have shown highly potent results for all the properties. Now a day, many synthetic drugs are available in market to solve such health issues but they all have some side effects on human and animal health. Hence, as an alternative to these synthetic drugs, probiotic organisms can be used which can give multifarious health benefits. Moreover, these probiotics can be consumed separately as well as they can be added in the preparation of functional foods, can be utilized as an animal feed supplement, and can be utilized in cosmetic industry and also for some clinical purposes.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All data generated or analysed during this study are included in this article.

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