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RESEARCH ARTICLE

Evaluation of growth and hydrolytic enzyme activities in *Enterococcus faecium* PB1, a probiotic bacterium with potential for application in dairy waste water treatment

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Enterococcus faecium PB1 is a gram-positive, round-shaped, commercially important (probiotic) lactic acid bacterium. The present study was aimed to evaluate the ability of this bacterium to utilize the complex disaccharide lactose as a source of carbon for growth, and its enzymatic potential to hydrolyze lactose, proteins, and lipids. The results revealed that *E. faecium* PB1 can grow in the presence of a range of lactose concentrations (1-5%) with optimum growth at 2%. As with lactose, the bacterium was able to grow well in the presence of simple sugars glucose and galactose and, utilize various other carbohydrates. *E. faecium* PB1 exhibited β-D-galactosidase activity (an intracellular lactose-hydrolyzing enzyme) at all the concentrations of lactose tested; the maximum being observed at 2%. A neutral to moderate alkaline pH range (7.0-8.5) and mesophilic temperature (37°C) was optimum for β-D-galactosidase activity in *E. faecium* PB1. Ethylene diamine tetraacetic acid (EDTA, a metal ion chelator), glucose and galactose (the breakdown products of lactose) inhibited this activity. *E. faecium* PB1 also displayed extracellular protein-degrading protease activity in cells grown with lactose and galactose. Although no extracellular lipid-degrading lipase activity could be detected in cells grown with lactose, this activity was present in cells grown with glucose and galactose. Taken together, the ability of the probiotic bacterium *E. faecium* PB1 to grow at a range of lactose concentrations and its potential to hydrolyze lactose, proteins, and lipids may be suitably exploited for biological treatment of dairy waste waters rich in similar types of organic nutrients.

Keywords: β-D-galactosidase, Dairy waste water, *Enterococcus faecium* PB1, Lipase, Probiotic, Protease

Introduction

Probiotics are gut-friendly active bacteria and yeasts.[1] The most commonly used probiotic bacteria are those belonging to the group lactic acid bacteria (LAB). Some of the well-characterized LAB include species from genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Weissella*, etc.[2] Their ability to produce a variety of hydrolytic enzymes with roles in biodegradation of complex carbohydrates, proteins, and fats makes them highly beneficial for multiple applications.[2,3,4] The metabolic versatility of LAB combined with generally-regarded-as-safe (GRAS) status has already popularized their use in health industry, for example to treat bowel-related diseases, and opens up the possibility for their potential use in bioremediation of polluted environments, which are often laden with biodegradable organics.[1,2]

Dairy industries are notorious for generating huge volume of highly polluting waste waters rich in several organics and minerals. These organics are mainly lactose (a disaccharide), casein (milk protein), and milk fats.[5] Some previous studies suggest that

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probiotic LAB, such as *Paracoccus marcusii* KGP and, some spp. of *Bacillus*, such as *Bacillus coagulans*, *Bacillus aryabhattai* GEL-09, *Bacillus subtilis* are capable of carrying out efficient biodegradation of whey, a lactose-rich waste water released by dairy industries.[6,7,8,9,10,11] The potential to biodegrade whey lies in the ability of such bacteria to hydrolyze lactose (a carbohydrate made up of D-glucose and D-galactose) through an intracellular enzyme β-D-galactosidase (E.C. 3.2.1.23), also known as lactase. This enzyme catalyzes the cleavage of β-(1→4)-glycosidic linkage in lactose and releases D-glucose and D-galactose, sugars that are easily assimilated by bacteria.[12] In addition to possession of β-D-galactosidase-mediated lactose hydrolysis ability, LAB are also expected to produce protease and lipase activities essential for hydrolysis of additional organic constituents like casein and milk fats present in dairy waste water.[13,14,15] Such strains of LAB with the potential to produce multiple hydrolytic activities are likely to be more suitable for use in the treatment of dairy waste waters.[16]

Keeping in view of the above, the aim of the present study was to screen, isolate, and identify a probiotic LAB with abilities to produce β-D-galactosidase, protease, and lipase activities. Such a bacterium was isolated from a commercial probiotic tablet containing multiple bacterial strains, and identified as *Enterococcus faecium* PB1 possessing features relevant for its potential application in treatment of dairy waste waters.

Materials and methods

Screening and isolation of a β-D-galactosidase-positive bacterium

A β-D-galactosidase-positive bacterium was isolated from a commercially available probiotic tablet (VIBACT, manufactured by Allianz Biosciences Pvt. Ltd, Puduchery, India). The probiotic tablet comprised of four bacterial strains, namely *Streptococcus faecalis*, *Clostridium butyricum*, *Bacillus mesentericus*, and Lactic acid bacillus (*Lactobacillus sporogenes*). To screen for β-D-galactosidase-positive bacteria, 50 µL of a 10^{-6} times diluted sterile saline water (0.85 % sodium chloride) suspension of bacterial cells contained in a probiotic tablet was spread on Luria-Bertani (LB-medium; composed of 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter) agar plates amended with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and the plates were incubated for 20 h at 37°C.[6] The β-D-galactosidase-positive bacterial colonies were identified by their blue appearance.[7] One discrete blue-coloured colony (PB1) was purified by repeated streaking on LB agar plates and used for experiments. For preliminary identification, the bacterium PB1 was Gram-stained and observed under a bright field microscope.[17]

Molecular identification of the bacterial isolate PB1

Genomic DNA isolation and PCR amplification of 16S rRNA gene

The isolated β-D-galactosidase-positive bacterium PB1 was grown in 5 ml LB medium at 37°C for overnight. Genomic DNA was prepared from an overnight grown culture of PB1, as described by Sambrook and David[18]. The concentration and purity of genomic DNA was assessed using a UV-Vis 1900i spectrophotometer (Shimadzu, Japan) and agarose gel (0.8%) electrophoresis, respectively. Then, the genomic DNA was used to PCR amplify 16S rRNA gene in 30 µl reaction volume comprising of 15 µl of PCR master mix, 0.6 µl of forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 0.6 µl of reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The thermal cycling conditions used were as follows: Initial denaturation at 96°C for 4 min followed by 30 cycles of amplification at 94°C for 30 s, annealing for 30 s at 57°C and 1 min extension at 72°C. The final extension was done at 72°C for 10 min.

16S rRNA gene sequencing and phylogenetic analysis

The PCR product was sequenced from both directions with forward and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer (AGT Biosciences, Guwahati). A consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was subjected to Basic Local Alignment Search Tool (BLAST) in the 'nr' database of National Centre for Biotechnology Information (NCBI). The closely matching 16S rRNA gene sequences with that in the bacterial isolate PB1 were retrieved and aligned using multiple alignment software ClustalW. The distance matrix and phylogenetic tree (1000 bootstraps) was constructed using MEGA12.[19]

Test for carbohydrate utilization by PB1

The ability of PB1 to utilize different

carbohydrates was analyzed using Hi25TM Enterobacteriaceae identification Kit (Strip-2, KB003-1KT, HIMEDIA). The strip comprises test wells for carbohydrates, such as esculin, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose, and lactose hydrolysis. To perform the test, a suspension of PB1 inoculum (cells equivalent to OD_{600} 0.1) was prepared and 50 µl of inoculum was introduced in each well of the strip. Then the strip was incubated for 24 h at 37°C, and the result was recorded and analyzed in terms of colour change produced in each well in the strip by following kit manufacturer's instruction.

Growth determination of PB1

The bacterium PB1 was grown in de man, Rogosa & Sharpe (MRS)-medium amended with different concentrations of lactose (1 to 5%), 1% galactose, and 2% glucose separately for 30 h at 37°C. The OD_{600} values were measured using a UV -Vis 1900i spectrophotometer at different time intervals during the entire experimental period.

Preparation of PB1 cells for determination of β-D-galactosidase activity

β-D-galactosidase activity was determined in PB1 cells grown in MRS-medium with different carbohydrates as described before. Exponentially growing PB1 cells (6 h old) were harvested by centrifugation (5000 rpm for 3 min) and ice-incubated for 20 min to stop their growth. Then the cells were washed twice with chilled Z buffer (pH 7.0) and resuspended in fresh Z buffer followed by OD_{600} measurement against a blank (Z buffer). Appropriately diluted cells of known OD_{600} were permeabilized by adding 100 µl chloroform and 50 µl 0.1% sodium dodecyl sulfate (SDS). Such cells were vortexed and equilibrated for 5 min in a 28°C water bath. Then the permeabilized cells were used for determination of intracellular β-D-galactosidase activity using ortho-nitrophenyl β-D -galactopyranoside (ONPG) as substrate by following the method described by Miller.[20]

Determination of optimum temperature, pH, and the effect of EDTA on β-D-galactosidase activity

The effect of temperature, pH, and EDTA (0.5 mM) on β-D-galactosidase activity was evaluated in 2% lactose-grown PB1 cells. The permeabilized cells, as described above were added to the reaction mixtures and incubated at different temperatures i.e., 25°C, 37°C, and 60°C for 9 min for measuring β-D-galactosidase activity. To determine the effect of pH, a similar procedure was followed except that the reaction mixtures were prepared in buffers of different pH i.e., 6.0, 7.0, 8.5, and 9.5. The effect of EDTA was determined by incubating the reaction mixture (pH 7.0) with 0.5 mM EDTA at 37°C.

Determination of protease and lipase activities

The extracellular protease and lipase activities were measured using the spent culture supernatant (SCS) as source of enzymes. The SCS was obtained from PB1 cultures grown in MRS-medium containing different carbohydrates (1% galactose, 2% glucose, and 2% lactose) separately. After the cultures reached stationary-phase (36 h), they were centrifuged at 7000 rpm for 10 min and then the supernatant (spent medium) was filtered with 0.45µ syringe filter to remove residual bacterial cells. The extracellular protease and lipase activities were determined in such supernatants as described by Cupp-Enyard[21] by using casein as a substrate for protease activity, and lipase activity using p-nitrophenyl palmitate (pNPP) as substrate.[22]

Statistical analysis

All the experiments reported in this study were done in duplicate. The preparation of plots and bar diagrams, and calculation of standard deviation (SD) were done using Microsoft Excel 2010.

Results & Discussion

Screening, isolation and molecular identification of β-D-galactosidase-positive bacterium PB1

Following screening and isolation of a β-D-galactosidase-positive bacterial isolate PB1 from a commercial probiotic containing multiple bacterial strains, PB1 was subjected to Gram-staining. As shown in Fig. 1(a), PB1 appeared positive for gram-staining and coccus-shaped. This bacterium lacked catalase activity (data not shown), a feature commonly present in LAB.[2,3,6,7] For molecular identification of PB1, its genomic DNA was isolated followed by PCR amplification of 16S rRNA gene leading to a 1.4 kb DNA fragment (Fig. 1b). The

PCR amplified DNA fragment was then sequenced, subjected to BLAST, and multiple sequence alignment followed by phylogenetic tree construction. BLAST analysis revealed that the 16S rRNA gene sequence of PB1 was more than 97% similar to 16S rRNA gene sequence from other strains of *Enterococcus faecium*, indicating that the bacterial isolate PB1 is indeed *Enterococcus* faecium (Fig. 1c). The probiotic tablet from which the bacterium was isolated contained only *Streptococcus faecalis* and not *Enterococcus faecium*. However, it may be noted that the species belonging to *Streptococcus* are recently reclassified and included in the genus *Enterococci*, spp. of which are prevalent in dairy products.[23,24] The 16S rRNA gene sequence of PB1 (hereafter referred to as *E. faecium* PB1) has been submitted to NCBI with accession number PV927168.

Carbohydrate utilization potential of E. faecium PB1

LAB can utilize a variety of carbohydrates for their growth and metabolism.[25] *E. faecium* PB1 was subjected to a qualitative carbohydrate utilization

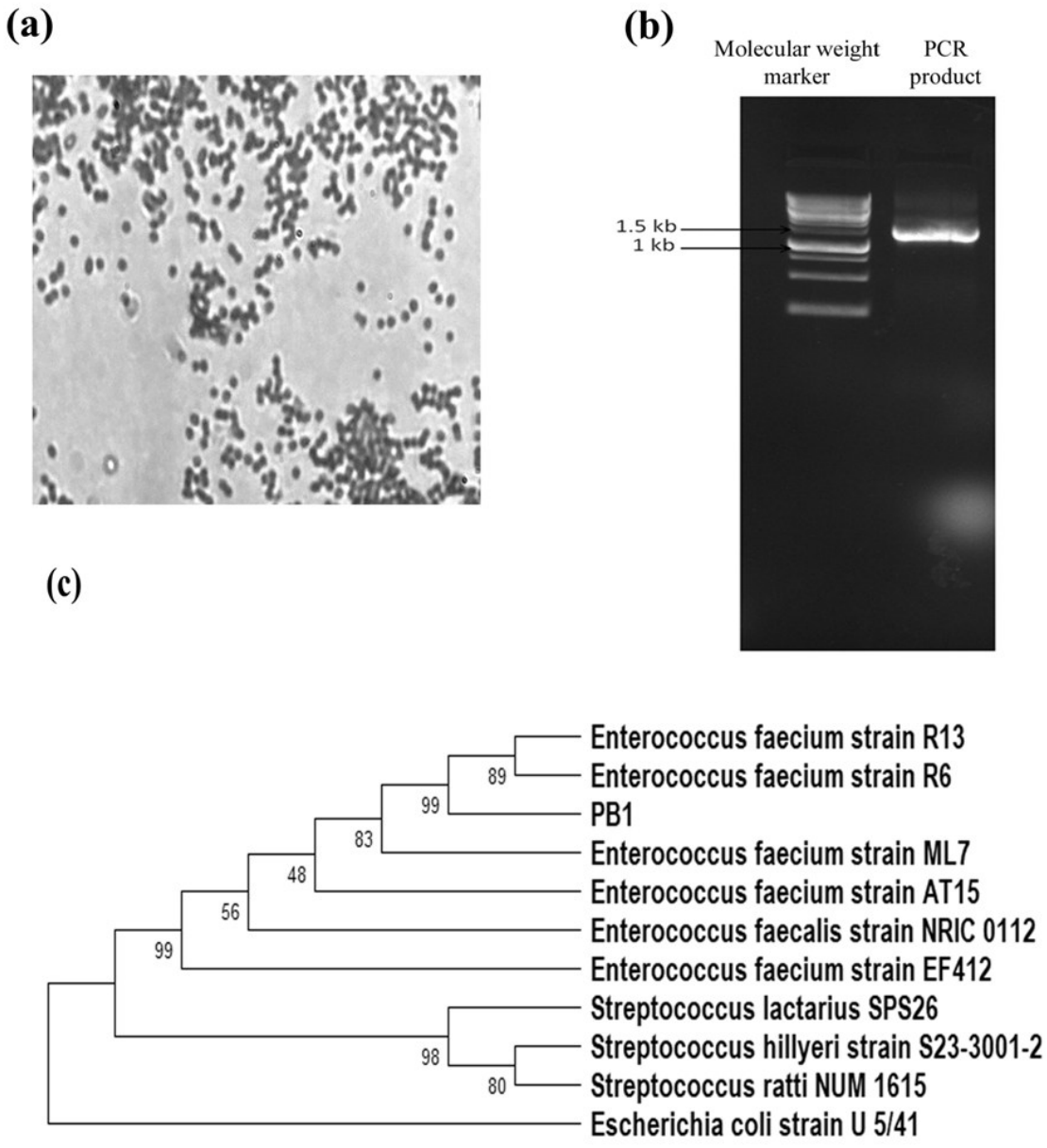

Figure 1. Morphological and molecular identification of a probiotic bacterium PB1. Gram-stained PB1 (a), PCR amplified 16S rRNA gene product of PB1 on 0.8 % agarose gel (b), and 16S rRNA gene-based phylogeny of PB1 with similar sequences from other bacterial species (c). Different species of *Streptococcus* and an *Escherichia coli* strain were used as out-groups

test to determine its potential to use different types of carbohydrates. As shown in Table 1, *E. faecium* PB1 could utilize esculin, arabinose, cellobiose, melibiose, glucose, and lactose, the latter being a major carbohydrate in dairy waste water. However, *E. faecium* PB1 could not utilize xylose, saccharose, trehalose, adonitol, rhamnose, and raffinose.

Table 1. Carbohydrate utilization potential of *E. faecium* PB1. An exponentially growing culture of *E. faecium* PB1 equivalent to OD_{600} of 0.1 was used to estimate carbohydrate utilization potential according to kit manufacturer's instructions and result interpretation. "+" sign indicate positive utilization ability and "-" indicate negative/non utilization ability

Test	Result
Esculin	+
Arabinose	+
Xylose	-
Adonitol	-
Rhamnose	-
Cellobiose	+
Melibiose	+
Saccharose	-
Raffinose	-
Trehalose	-
Glucose	+
Lactose	+

Growth of E. faecium PB1 with different carbohydrates

The growth of *E. faecium* PB1 was evaluated in MRS medium amended with 1-5% lactose, 1% galactose, and 2% glucose. As shown in Fig. 2, *E. faecium* PB1 grew in the presence of all the different carbohydrates. The exponential growth occurred between 4 to 12 h after inoculation in lactose-, glucose-, and galactose-containing medium. However, the maximum cell density of the cultures grown with different carbohydrates varied by the end of the experimental period; the highest cell density was observed in the presence of 1 and 2% lactose and lower cell densities in 5% lactose, glucose and galactose. These results suggest that 2% lactose was optimum for the growth of *E. faecium* PB1, but the bacterium has the potential to grow even at higher concentrations of lactose, as has also been reported in other LAB.[26] Such a property of *E. faecium* PB1 may be useful for its growth and survival in environments with high concentrations of lactose, including in dairy waste waters.

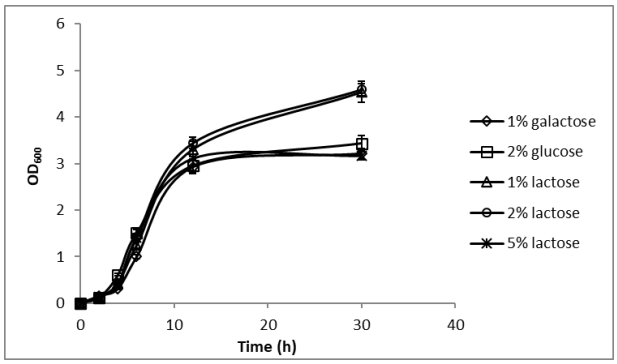

Figure 2. Effect of different carbon sources on the growth of *E. faecium PB1*. Exponentially grown culture of *E. faecium* PB1 was inoculated in MRS medium amended with different carbon sources, as indicated, and growth was measured periodically by determining OD_{600} of cultures. The experiments were done in duplicate and results are presented as mean±SD.

β-D-galactosidase activity in E. faecium PB1

The ability of bacteria to grow in the presence of lactose is dependent on their ability to produce β-D-galactosidase, an intracellular enzyme that catalyzes the conversion of lactose into simple easy

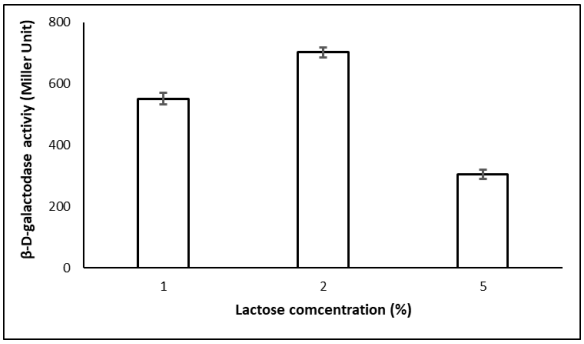

Figure 3. Effect of different lactose concentrations on β-D-galactosidase activity in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with different concentrations of lactose, as indicated, and grown for 6 h. The cultures were then harvested and permeabilized cells were used in the activity assay. The experiments were done in duplicate and the results are presented as mean±SD.

to assimilate sugars glucose and galactose.[12] The exponentially growing cultures of *E. faecium* PB1 were used for the determination of β-D-galactosidase activity. As shown in Fig. 3, the highest β-D-galactosidase activity of 702.8±15 Miller Unit was produced in the cultures grown with 2% lactose. This activity was 551.6±19.3 and 304.44±15.22 Miller Units in the cultures grown with 1 and 5% lactose, respectively. In galactose-grown cells, this activity was considerably low (83.12±3.24 Miller Units) and no activity could be detected in glucose-grown cells. These results suggest that, as in other β-D-galactosidase producing bacteria, lactose is an inducer of β-D-galactosidase activity in *E. faecium* PB1 and the products of the reaction i.e., glucose and galactose are inhibitors of this activity.[6,7,8,9,11] Furthermore, the lactose concentration, which was optimum for its growth is also optimum for β-D-galactosidase activity. It is a well established fact that the β-D-galactosidase activity is influenced by metal ions in bacteria. A previous study has demonstrated that the β-D-galactosidase activity in *E. faecium* MTCC 5153 is stimulated by metal ions, such as Mg+2, Mn+2, Ca+2, K+ and Na+, and inhibited by Zn+2, Co+2 and Cu+2.27 It is likely that such stimulatory metal ions may also be required for β-D-galactosidase activity in *E. faecium* PB1, as addition of 0.5 mM EDTA (a metal chelating agent) leads to complete activity inhibition.[9,27]

Optimum temperature and pH for β-D-galactosidase activity in E. faecium PB1

Besides metal ion inhibitors, temperature and pH are critical to enzyme activities in bacteria. The optimum temperature and pH for β-D-galactosidase activity was determined in 6 h old mid-log phase cells of *E. faecium* PB1 grown with 2% lactose. As shown in Fig. 4(a), the β-D-galactosidase activity was only marginally lower at 25°C (615.57±18.5 Miller Unit) and 60°C (635.37±18.1 Miller Unit) than that at 37°C (702.8±15.81 Miller Unit). Thus, the optimum temperature for β-D-galactosidase activity in E. faecium PB1 was 37°C, but the bacterium can retain β-D-galactosidase activity even at temperatures lower or higher than 37°C.[27,28] Further, as shown in Fig. 4(b), the highest β-D-galactosidase activity was observed in reaction mixture having pH 8.5 (765.86±26.8 Miller Unit) followed by that in pH 7.0 (702.8±15.81 Miller Unit) and pH 9.5 (175.93±8.0 Miller Unit). However, no β-D-galactosidase activity was detected in reaction mixtures with pH lower than 7.0, i.e., pH 6.0. These results suggest that the β-D-galactosidase in *E. faecium* PB1 is active in neutral

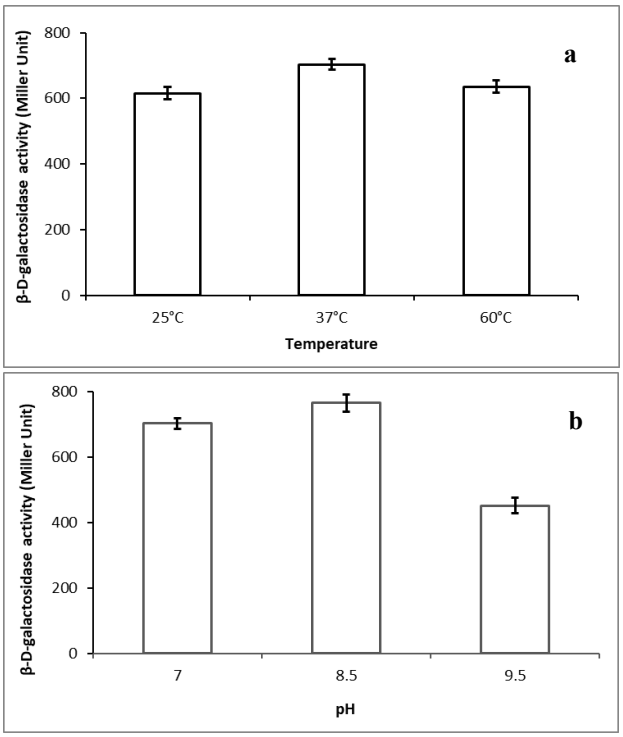

Figure 4. Effect of temperature **(a)** and pH **(b)** on β-D-galactosidase activity in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with 2% lactose and grown for 6 h. The cultures were then harvested and the bacterial cells permeabilized. Such permeabilized cells were used as the source of the enzyme in the reaction mixtures (pH 7.0) incubated for 9 min at different temperatures or with reaction mixtures of different pH incubated at 37°C, as indicated, for the activity assay. The experiments were done in duplicate and the results are presented as mean±SD.

to alkaline pH range consistent with β-D-galactosidases characterized from other bacteria.[27,28]

Protease and lipase activities in E. faecium

The protease and lipase, unlike β-D-galactosidase, are hydrolytic enzymes that are secreted by bacterial cells to enable utilization of proteins and lipids. The proteins, such as casein present in milk are cleaved by proteases to oligopeptides and amino acids and these simpler forms can serve as nutrients for bacterial growth.[13,29,30] Milk fats or lipids are broken down by lipases to smaller organic acids and can serve as sources of carbon.[14,15,31] The protease and lipase activities were determined in the SCS of stationary-phase (36 h) cells of *E. faecium* PB1 grown with 2%

lactose, 2% glucose, and 1% galactose. As shown in Fig. 5(a), the highest extracellular protease activity (0.04±0.0 U/ml) was detected in the SCS of cells grown with 1% galactose. This activity was 0.023±0.0 U/ml when cells were grown with 2% lactose and no activity could be detected in the SCS of cells grown with 2% glucose. These results suggest that *E. faecium* PB1 has the ability to produce extracellular protease activity, but this activity can vary depending on the type of carbohydrates available for its growth.[29,30] Furthermore, as shown in Fig. 5(b), the extracellular lipase activity (0.07±0.0 U/ml) was highest in the SCS of cells grown with 2% glucose followed by that in 1% galactose (0.053±0.0 U/ml), and no lipase activity was detected in the SCS of cells grown with 2% lactose. Thus, as with protease, lipase activity in *E. faecium* PB1 is also influenced by the type of carbohydrates. The possession of multiple hydrolytic enzyme activities, including β-D-galactosidase, protease, and lipase suggests that the probiotic bacterium *E. faecium* PB1 may have the potential to efficiently utilize lactose, casein, and milk fats, the major organics present in dairy waste waters.[16]

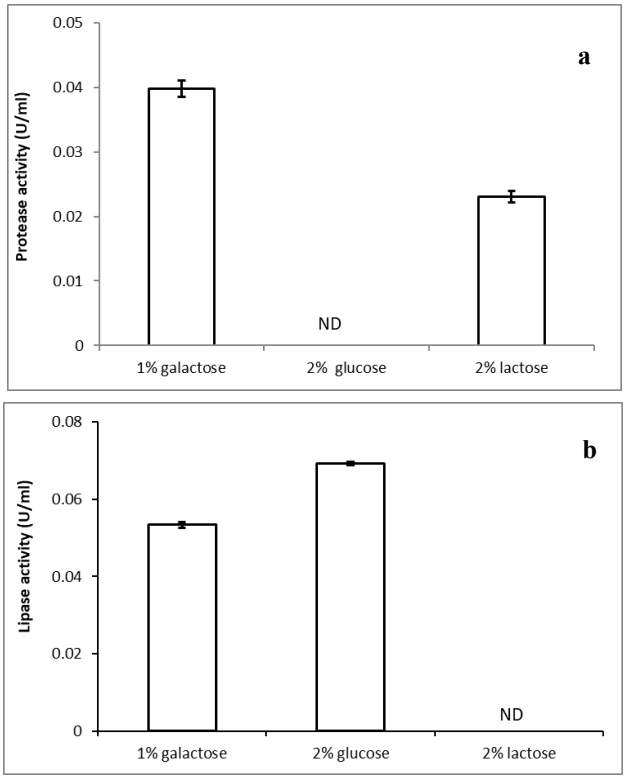

Figure 5. Effect of different carbon sources on protease (a) and lipase (b) activities in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with different carbon sources, as indicated, and grown for 36 h (stationary phase). Such cultures were then centrifuged and the spent culture media were used as the source of enzyme for activity assay. The experiments were done in duplicate and the results are presented as mean±SD. The abbreviation "ND" refers to non-detectable.

Conclusion

The findings presented in this study suggest that the isolated probiotic bacterium *E. faecium* PB1 is a metabolically versatile LAB able to utilize a wide variety of carbohydrates, such as lactose, glucose, and galactose for its growth. Its growth in various concentrations of lactose was accompanied by production of β-D-galactosidase activity with optimal activity detected at mesophilic temperature (37°C) and neutral to alkaline pH range 7 to 8.5. This lactose-hydrolyzing enzymatic activity was inhibited by glucose, galactose (reaction products) and metal ion chelating agent EDTA. Additionally, *E. faecium* PB1 also demonstrated the potential to produce protein- and lipid-hydrolyzing extracellular protease and lipase activities. Given its probiotic, GRAS status, and diverse metabolic capacities, *E. faecium* PB1 could be a suitable candidate for environmental applications, such as in the biological treatment of dairy waste waters, a pollutant rich in biodegradable organics.

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Conflict of interests

The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

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