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RESEARCH ARTICLE

Biochemical characterization of *Bacillus paramycoides* DW1, a bacterial isolate from dairy waste water

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Bacillus paramycoides DW1 (hereafter *B. paramycoides* DW1), an isolate from dairy waste water is a gram-positive, rod-shaped, endo-spore forming bacterium. The aim of this study was to assess the ability of this bacterium to hydrolyze lactose, proteins, and lipids (major organic constituents of dairy waste water), and its potential to grow using lactose, glucose, and galactose as sources of carbon, and ability to utilize other complex carbohydrates. The results suggest that lactose is a good source of carbohydrate for *B. paramycoides* DW1 growth. Although the best growth of the bacterium was observed in the presence of 2% lactose, it was able to grow even at higher concentration of lactose (5%) usually encountered in dairy waste water, and could also utilize a variety of other carbohydrates, such as xylose, cellobiose, saccharose, trehalose, glucose, and galactose. Concomitant with its growth using lactose, *B. paramycoides* DW1 displayed lactose-hydrolyzing intracellular β-D-galactosidase activity irrespective of concentrations of lactose with the highest being at 2%. This activity was inhibited in bacterial cells grown in the presence of glucose and galactose and by a metal ion chelator ethylene diamine tetraacetic acid (EDTA) suggesting that the enzymatic reaction is catabolite repressible and requires metal ions for its activity. Further biochemical characterization revealed that a neutral pH (7.0) and mesophilic temperature (37°C) was optimum for β-D-galactosidase activity in *B. paramycoides* DW1. Additionally, the bacterium was also endowed with extracellular protease (caseinolytic) and lipase activities. Overall, the findings reveal that the potential to hydrolyze lactose, protein, and lipid probably enables *B. paramycoides* DW1 to grow and survive in nutritionally complex dairy waste water environment and implies that such a bacterium may be a significant contributor to reduction of organic pollutant load in dairy waste water .

Keywords: *Bacillus paramycoides* DW1, β-D-galactosidase, Dairy waste water, Lipase, Protease

Introduction

Bacteria are vital sources of numerous important enzymes. The gram-positive, rod-shaped, sporulating, ubiquitous firmicutes belonging to the genera *Bacillus* are worth notable in this regard.[1] The *Bacillus* spp. produce highly useful enzymes belonging to the hydrolase class, and include amylase, cellulase, protease, and lipase, among others. Such enzymes are not only of high industrial value, but are also critical to growth and survival of *Bacillus* in nutritionally diverse natural environments.[2,3] The dairy waste waters are one of many examples of nutritionally complex environment which harbor a large number of *Bacillus* spp. These include *Bacillus subtilis* VUVD001, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*,

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etc.[4,5,6,7] Their presence in dairy waste waters indicates that they can possibly utilize lactose, milk protein casein, milk fats, and mineral nutrients present in such waste waters to grow and survive.

The ability to utilize lactose, a complex disaccharide depends on the ability of bacteria to produce an intracellular hydrolytic enzyme β-D-galactosidase (E.C. 3.2.1.23), which cleaves β-(1→4) -glycosidic bond between glucose and galactose leading to release of these simple sugars for further assimilation.[8] However, β-D-galactosidase activity has been shown to vary across *Bacillus* spp., for example, 17.41 U/ml activity was detected in *Bacillus aryabhattai* and 15.10 U/ml in *Bacillus subtilis*.[5,7] The optimum pH and temperature requirements for this enzyme activity had also been reported to differ depending on the type of bacterial source.[9,10] As for biotechnological applications, some spp. with higher β-D-galactosidase activity, such as *Bacillus aryabhattai* and *Bacillus coagulans* (5978 U/mg) have been successfully used in the treatment of lactose-rich dairy waste water whey (a by-product of cheese manufacturing process and a strong environmental pollutant).[5,6] Besides β-D-galactosidase activity, many species of *Bacillus* are also able to hydrolyze proteins and lipids through production of extracellular enzymes protease and lipase, respectively.[4] A consortium of dairy waste water native *Bacillus* spp. with potential to produce such hydrolytic activities have been shown to substantially reduce the organic content of dairy waste water simulant (milk amended with minerals).[4] Such biochemical properties exhibited by *Bacillus* spp. makes them very useful candidates for exploitation in biodegradation of dairy waste waters.

Therefore, the aim of the present study was to isolate *Bacillus* spp. native to dairy industry waste waters from MULCO, the only milk-processing industry in Aizawl, Mizoram. One such isolated *Bacillus* sp. was identified by its 16S rRNA gene sequence and characterized at length with regard to its ability to utilize various carbohydrates, including lactose. Additionally, the isolated *Bacillus* sp. was assessed for its enzymatic potential to hydrolyze lactose, milk protein casein and lipids, biochemical properties relevant to its growth and survival in its natural niche.

Materials and methods

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

Waste water from the MULCO Dairy industry (Aizawl, Mizoram) was collected in 50ml sterile tubes. For screening and isolation of *Bacillus* spp. (heat-resistant endo-spore formers), an ml of dairy waste water was taken in a sterile eppendorf tube and exposed to 70°C for 20 min to kill all non-sporulating microorganisms.[11] The heat-exposed waste water was then serially diluted 10^{-6} fold with sterile saline water (0.85 % sodium chloride) and 50 µl of this diluted waste water was spread on Luria-Bertani (LB-medium; composed of 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter) agar plates added 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. The β-D-galctosidase-positive bacterial colonies were identified by their blue appearance.[12] One such blue-coloured colony (named DW1) was picked and purified by repeated streaking on LB-agar plates and used for further experiments. The bacterium DW1 was then Gram-stained and observed under a bright field microscope to determine cell shape and Gram-staining features.[13]

Molecular identification of DW1

Genomic DNA isolation and 16S rRNA gene amplification

The bacterium DW1 was grown in 5 ml LB broth at 37°C for overnight. Genomic DNA was extracted from the overnight culture by following the method described by Sambrook and David[14]. The concentration and purity of DNA was assessed using a UV-Vis 1900i spectrophotometer (Shimadzu, Japan) and agarose gel (0.8%) analysis, respectively. Then, the isolated DNA was PCR amplified for 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 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 newly isolated bacterium DW1 were retrieved and aligned using multiple alignment software ClustalW. The distance matrix and phylogenetic tree (1000 bootstraps) was constructed using MEGA12.[15]

Analysis of carbohydrate utilization by DW1

The ability of DW1 to utilize different carbohydrates was analyzed using Hi25TM Enterobacteriaceae identification Kit (Strip-2, KB003-1KT, HIMEDIA). The strip comprises test wells for esculin, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose, and lactose hydrolysis. To perform the test, a suspension of DW1 inoculum (OD_{600} equivalent to 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, as instructed by the manufacturer.

Determination of DW1 growth

The bacterium DW1 was grown in de man, Rogosa & Sharpe (MRS)-medium amended with different concentrations of lactose (1 to 5%), 1% galactose, and 2% glucose for 48 h at 37°C. At regular time intervals the optical density at 600nm (OD_{600}) values were measured using a UV-Vis 1900i spectrophotometer.

Preparation of DW1 cells for determination of intracellular β-D-galactosidase activity

To determine β-D-galactosidase activity, the DW1 was grown in MRS-medium with different carbohydrates as described for growth study. Exponentially growing cells (6 h old) were harvested by centrifugation (5000 rpm for 3 min) and ice-incubated for 20 min to stop the 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 1 ml 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 protocol described by Miller.[16]

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 DW1 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 following which the β-D-galactosidase activity was measured. 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 activity assays were performed in the spent culture supernatant (SCS). The SCS was obtained from DW1 cultures grown in MRS-medium containing different carbohydrates (1% galactose, 2% glucose, and 2% lactose) separately. After 36 h of growth, the cultures were centrifuged at 7000 rpm for 10 min and then the supernatant (spent medium) was filtered with 0.45µ syringe filter to remove any remaining bacterial cells. The extracellular protease and lipase activities were determined in such supernatants as described by Cupp-Enyard[17] for protease activity (casein as substrate) and lipase activity using p-nitrophenyl palmitate (pNPP) as substrate.[18]

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

Morphological and molecular identification of β-D-galactosidase-positive bacterium DW1

The isolation and screening procedure employed in this study led to selection of a β-D-galactosidase-positive bacterial isolate DW1 from MULCO dairy waste water (Aizawl, Mizoram). As shown in the Fig.1(a), the Gram-staining and microscopic observation of DW1 showed it to be

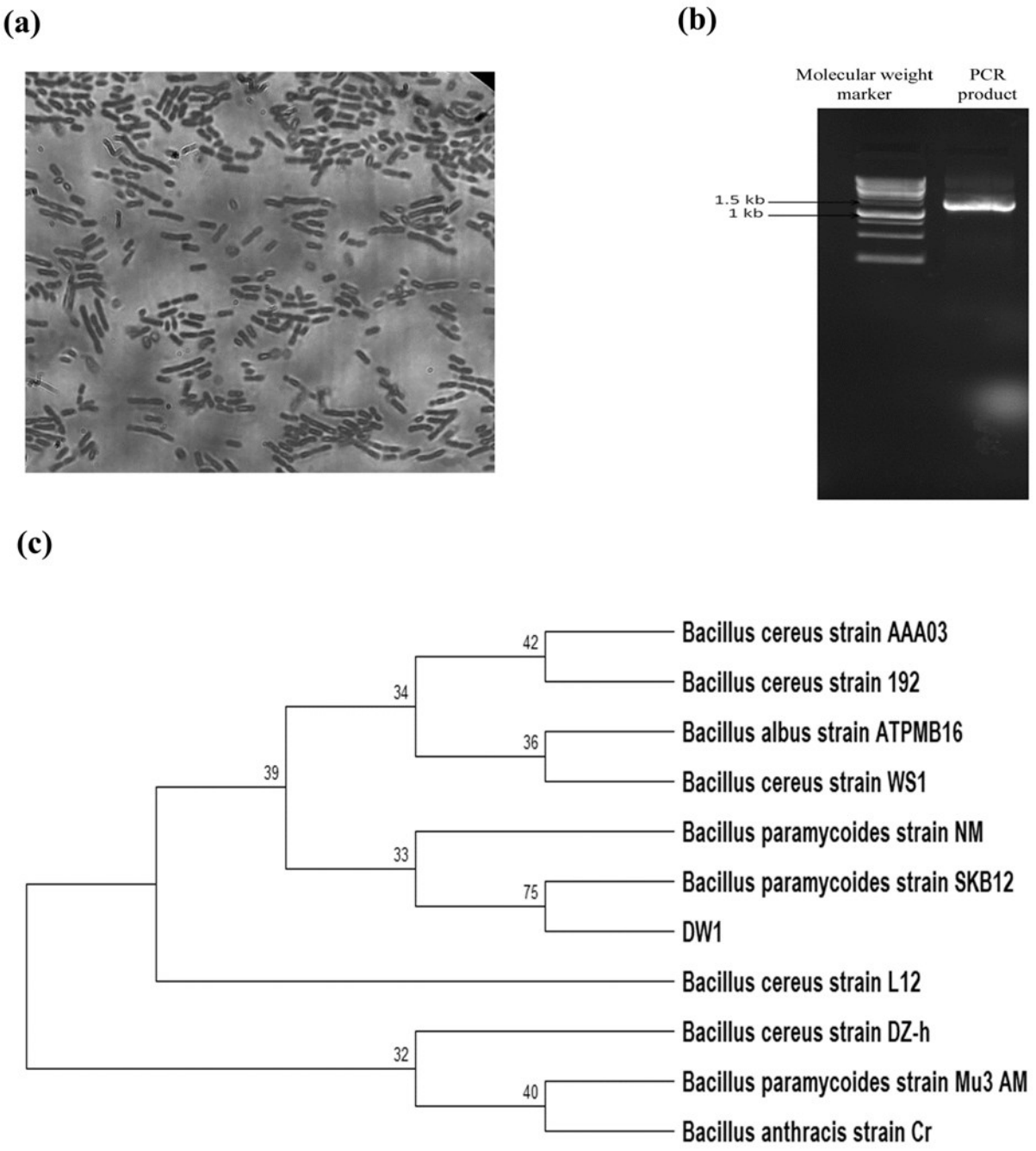

Figure 1. Morphological and molecular identification of dairy waste water bacterial isolate DW1. Gram -stained DW1 (a), PCR amplified 16S rRNA gene product of DW1 on 0.8 % agarose gel (b), and 16S rRNA gene-based phylogeny of DW1 with similar sequences from other bacterial species (c).

gram-positive, rod-shaped, and endo-spore forming bacterium, characteristics of bacteria commonly found in the genera *Bacillus*.[19] Genomic DNA isolation followed by PCR amplification of 16S rRNA gene from DW1 produced a 1.4 kb DNA fragment, as shown in Fig. 1(b). The PCR amplified DNA fragment was then sequenced, subjected to BLAST, multiple sequence alignment and phylogenetic tree construction for its further identification.[13,14,15,19] BLAST analysis revealed the 16S rRNA gene sequence of DW1 to be more than 97% similar to 16S rRNA gene sequence of *Bacillus paramycoides* SKB12 suggesting that the newly isolated bacterium DW1 is closely related to *Bacillus paramycoides* (Fig. 1c). The 16S rRNA gene sequence of the newly isolated *Bacillus paramycoides* (hereafter referred to as *B. paramycoides* DW1) has been submitted to NCBI

with accession number PV927120.

Carbohydrate utilization potential of B. paramycoides DW1

A previous study has suggested that B. paramycoides is a potent degrader of various carbohydrates.[20] It is likely that *B. paramycoides* DW1 may also biodegrade several carbohydrates. Thus, *B. paramycoides* DW1 was subjected to a qualitative carbohydrate utilization test to determine its potential to use different types of carbohydrates. As shown in Table 1, *B. paramycoides* DW1 indeed has the potential to utilize a variety of carbohydrates, including esculin, xylose, cellobiose, saccharose, trehalose, glucose, galactose, and lactose, the latter being a major carbohydrate present in dairy waste water. However, it lacks the ability to utilize arabinose, adonitol, rhamnose, melibiose, and raffinose.[20]

Table 1. Carbohydrate utilization potential of *B. paramycoides* DW1. An exponentially growing culture of *B. paramycoides* DW1 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.

Sl. No	Carbohydrates	Utilization potential
1	Esculin Hydrolysis	+
2	Arabinose	-
3	Xylose	+
4	Adonitol	-
5	Rhamnose	-
6	Cellobiose	+
7	Melibiose	-
8	Saccharose	+
9	Raffinose	-
10	Trehalose	+
11	Glucose	+
12	Lactose	+

Growth of B. paramycoides DW1 in the presence of different carbohydrates

The effects of a range of lactose concentrations (1-5%), glucose (2%), and galactose (1%) were evaluated on the growth of *B. paramycoides* DW1. As shown in Fig. 2, *B. paramycoides* DW1 exhibited growth (measured as OD600) in the presence of all the different carbohydrates. The lag-phase lasted for initial 4 h followed by exponential growth for approximately 16 h at all concentrations of lactose, glucose, and galactose. However, the maximum cell density of the cultures differed depending on the type and concentrations of carbohydrates; the highest cell density was observed in the presence of 2% lactose and lower cell densities in other concentrations of lactose, glucose and galactose by the end of the experimental period. These results suggest that *B. paramycoides* DW1 can grow optimally using 2% lactose. Although lower, its ability to grow even at higher concentrations of lactose may be important for its survival in lactose-rich dairy waste water.[8]

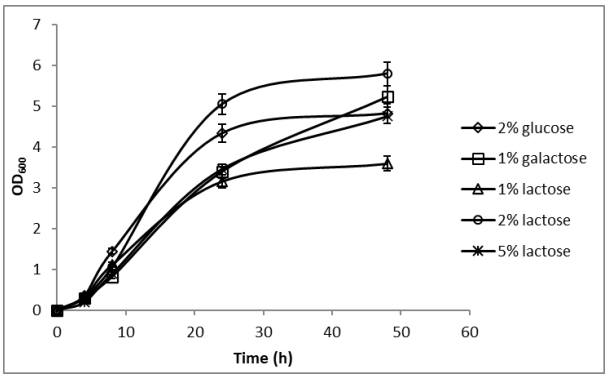

Figure 2. Effect of different carbon sources on the growth of *B.paramycoides* DW1. Exponentially grown culture of *B. paramycoides* DW1 was inoculated in MRS medium amended with different carbon sources, as indicated, and growth was measured periodically by determining OD_{600} of cultures. The experiments were done in duplicate and results are presented as mean±SD.

β-D-galactosidase activity in B. paramycoides DW1

β-D-galactosidase is an intracellular enzyme produced by certain microorganisms, including *Bacillus* spp.[5,6,7,8,10] This enzyme is produced by bacteria to hydrolyze lactose to glucose and galactose, sugars that can be easily assimilated. The mid log-phase cultures of *B. paramycoides* DW1 grown with 1-5% lactose, 2% glucose, and 1% galactose were used to determine β-D-galactosidase activity. As shown in the Fig. 3, the highest β-D-galactosidase activity (275.7 ± 9.6 Miller Unit) was found in the culture grown with 2% lactose. In the cultures grown with 1 and 5% lactose, this activity was reduced to 137 ± 6.7 and 176.1 ± 7.9 Miller Units, respectively. Comparatively, much lower β-D-galactosidase activity was detected in the cells grown with

glucose (15.86±0.58 Miller unit) and galactose (18.78±0.69 Miller unit). This result suggests that, as in other β-D-galctosidase producing bacteria, lactose induces β-D-galctosidase activity in *B. paramycoides* DW1 and the enzyme is inhibited by reaction products glucose and galactose.[5,6,7,10,12,20]

Furthermore, the concentration of lactose that supports the optimum growth also leads to optimum β-D-galactosidase activity in *B. paramycoides* DW1. Furthermore, the β-D-galactosidase activity in *B. paramycoides* DW1 is metal-dependent is proved by the fact that the metal chelating agent EDTA at a concentration of 0.5 mM completely inhibited this activity.[6]

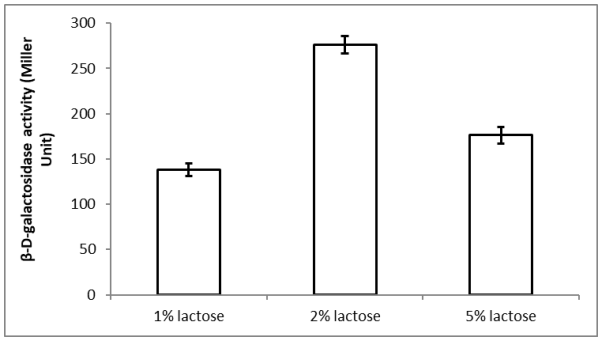

Figure 3. Effect of different lactose concentrations on β-D-galactosidase activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with different concentrations of lactose, as indicated, and grown for 6 h. The cultures were then harvested and permeabilized cells were used in the activity assay. The experiments were done in duplicate and the results are presented as mean±SD.

Optimum temperature and pH for β-D-galactosidase activity in B. paramycoides DW1

It is a well established fact that temperature and pH play a critical role in modulating enzyme activities in bacteria. The optimum temperature and pH for β-D-galactosidase activity was determined in mid-log phase cells of *B. paramycoides* DW1 grown with 2% lactose. As shown in Fig. 4(a), the β-D-galactosidase activity in *B. paramycoides* DW1varied with changes in temperature at which the reaction mixtures were incubated with the time remaining constant (9 min). In comparison to 37°C (275.7 ± 9.6 Miller Unit), the β-D-galactosidase activity was lower at 25°C (201.25±9.05 Miller unit) and reduced further at 60°C (133.84±6.6 Miller unit). Thus, 37°C is the optimum for β-D-galactosidase activity in B. *paramycoides* DW1. The results suggest that this bacterium can retain β-D-galactosidase activity from 25-60°C, a feature conducive to its lactose utilization ability under low to high temperature conditions.[5,6,9,10] To determine the optimum pH for β-D-galactosidase activity, the reaction mixtures as before were incubated for 9 min at 37°C in buffers of different pH (6.0, 7.0, 8.5 and 9.5). As shown in Fig. 4(b), the highest β-D-galactosidase activity was observed in pH 7.0 reaction mixture (275.7 ± 9.6 Miller Unit) followed by that in pH 8.5 (187.8 ± 6.5 Miller Unit) and pH 9.5 (175.93±8.0 Miller Unit). However, no activity was detected in reaction mixtures with pH lower than 7.0, suggesting that the β-D-galactosidase activity in *B. paramycoides*

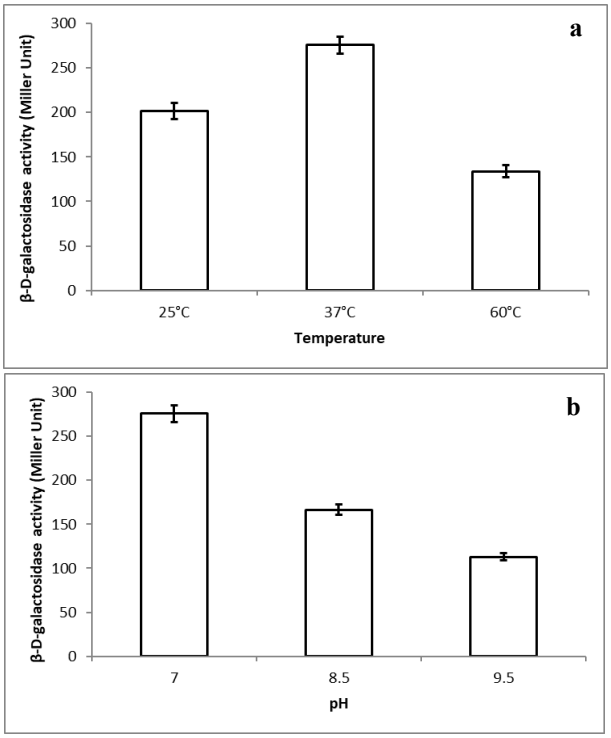

Figure 4. Effect of temperature (a) and pH (b) on β-D-galactosidase activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with 2% lactose and grown for 6 h. The cultures were then harvested and the bacterial cells permeabilized. 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.

DW1 is optimum at neutral pH, and is sensitive to alkaline and acidic pH.[5,6]

Protease and lipase activities in B. paramycoides DW1

The protease and lipase activities are crucial for bacteria to utilize proteins and lipids for their growth. Unlike β-D-galactosidase, these hydrolytic enzymes are secreted by bacterial cells to chop off proteins and lipids into their respective monomers for easy utilization.[21,22,23] The protease and lipase activities were determined in SCS of stationary-phase (36 h) cells of *B. paramycoides* DW1 grown with 2% lactose, 2% glucose, and 1% galactose. As shown in Fig. 5, the highest protease activity of 0.086±0.0 U/ml was detected in SCS of 1 % galactose-grown cells. This activity was lower (0.07±0.0 U/ml) in SCS of 2% glucose-grown cells, and lowest (0.0209±0.0 U/ml) in the SCS of 2% lactose-grown cells. These results suggest that though *B. paramycoides* DW1 is endowed with the ability to produce extracellular protease, its activity can vary depending on the carbohydrates used for growth.

The lipase activity was performed with similarly treated SCS samples as protease activity.21,23 Except in SCS of 2% glucose-grown cells (21.1 ± 0.07 U/ml lipase activity), no lipase activity was detected in SCS of 1% galactose- and 2% lactose-grown cells, suggesting that the lipase activity in *B. paramycoides* DW1 can vary significantly depending on the carbohydrates, as observed for protease activity. Nevertheless, the potential to produce diverse hydrolytic enzymes possibly enables the sustenance of *B. paramycoides* DW1 in dairy waste water.

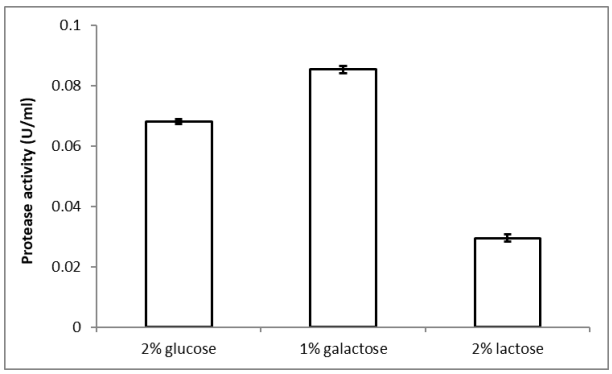

Figure 5. Effect of different carbon sources on protease activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with different carbon sources, as indicated, and grown for 36 h (stationary phase). Such cultures were then centrifuged 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.

Conclusion

A bacterial strain DW1 was isolated from dairy waste water and identified as *B. paramycoides* based on 16S rRNA gene sequencing. The characterization of this bacterium revealed that it can utilize a range of lactose concentrations for its growth. Besides lactose, *B. paramycoides* DW1 was also able to utilize a large number of other carbohydrates, such as xylose, cellobiose, saccharose, trehalose, glucose, and galactose, indicating its diverse metabolic potential. The ability of this bacterium to utilize lactose was conferred by β-D-galactosidase activity, an intracellular lactose-hydrolyzing enzyme. Further characterization showed that a neutral pH (7.0) and mesophilic temperature (37°C) was optimum for this activity, and the activity was inhibited in cells grown in the presence of glucose and galactose, possibly due to catabolite repression effect, and by metal ion chelator EDTA suggesting metal ion requirement for function. Additionally, *B. paramycoides* DW1 also exhibited the ability to produce extracellular protease (caseinolytic) and lipase, properties relevant to dairy waste water degradation. Such multiple abilities to hydrolyze lactose, proteins, and lipids attest the importance of *B. paramycoides* DW1 in dairy waste water biodegradation process.

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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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