

**BACTERIAL ISOLATES FROM COMMERCIAL PROBIOTICS
AND DAIRY EFFLUENT: MOLECULAR IDENTIFICATION,
ASSESSMENT OF HYDROLYTIC ACTIVITIES AND
POTENTIAL APPLICATION IN WASTEWATER TREATMENT**

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
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Bacterial isolates from commercial probiotics and dairy effluent: molecular identification, assessment of hydrolytic activities and potential application in wastewater treatment

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CERTIFICATE

I certify that the thesis entitled “ **Bacterial isolates from commercial probiotics and dairy effluent: molecular identification, assessment of hydrolytic activities and potential application in wastewater treatment**” submitted to the Mizoram University for the award of a degree of Doctor of Philosophy in Biotechnology by Jugami Boro is a record of research work carried out by her during the period from 2018 to 2025 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles in this University or any other University or Institution of higher learning.

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I **JUGAMI BORO**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

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ABBREVIATIONS

ml	Millilitre
µl	Microlitre
L	Litre
h	Hour
min	Minute
°C	Degree celsius
nm	Nano meter
UV	Ultra-violet
Vis	Visible
w/v	Weight-to-volume ratio
%	Percent
M	Molar
mM	Millimolar

CHAPTER 1

1. Introduction

The demand and consumption of milk and milk-derived products has risen sharply over the years. Dairy industry manufactured products include processed milk, yogurt, cheese, butter, cream, ice-cream, sweets etc. According to an estimate, the per capita consumption of such products was 22.2 kg in developed countries and 10.6 kg in developing countries in 2017. This is expected to further rise by 22% by 2027 (FAO and OECD, 2018). Meeting this ever increasing demand means a proportional increase in the number of new dairy industries and/or increase in the operational capacities of existing dairy industries. This increase also means consumption of large volume of fresh water by dairy industries and wastage during milk handling and milk processing. Approximately 0.2-11 L of fresh water is wasted per L of milk processed depending on the type of operational technologies used by dairy industries (Yan and Holden, 2019). Such a huge volume of wastewater released by dairy industries is a matter of serious environmental concern (Farizoglu and Uzuner, 2011). The polluted wastewater may contaminate aquatic ecosystems, disturb the well-being of aquatic life forms by oxygen depletion (due to overgrowth of algae, bacteria, and insects), and may also cause increased spread of vector-borne diseases, such as malaria, dengue fever etc (Shete and Shinkar, 2013).

To prevent such mishaps, dairy wastewaters must be treated effectively before discharge into the environment. The dairy waste waters are generally characterized by their high content of chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS), total nitrogen (TN), total phosphorous (TP), fats, oils, and grease (FOGs), and varying pH (acidic to alkaline) (Kolev Slavov, 2017; Moodley et al., 2024). Such characteristics make efficient treatment of dairy wastewaters a highly challenging endeavour. A three stage treatment process is generally recommended to reduce the polluting strength of dairy waste waters. The three stages involve primary, secondary, and tertiary treatment (Moodley et al., 2024; de Oliveira Nunes et al., 2021). A number of primary treatment strategies are

available, including physical separation of milk solids by screening and passing through sedimentation tanks, employing oil and grease traps, and chemical separation through coagulation and flocculation (Deepa et al., 2022). In the secondary stage, the effluents released from primary treatment are exposed to biological treatment either under aerobic and/or anaerobic conditions (de Oliveira Nunes et al., 2021; Zhao et al., 2020). The effluents from the second stage is finally treated by either chlorination or exposed to ultraviolet (UV) treatment leading to pathogen removal and reusable water (Hashim et al., 2025). Although many dairy industries may be combining all the treatment approaches to treat their wastewaters, it is possible that many of them are not able to do so because of financial constraints, as physical (UV treatment) and/or chemical materials (coagulating and flocculating agents, chlorination etc.) used in the process are too costly to afford (de Oliveira Nunes et al., 2021; Hashim et al., 2025). However, biological treatment is comparatively a more cost-effective process and therefore, also commonly preferred for achieving good treatment performance (Sar et al., 2022).

1.1 Biological treatment of dairy waste water

Bacteria, fungi, yeast, and anaerobic archaea constitutes some of the major microbial groups that drive the dairy wastewater treatment process (Al-Wasify et al., 2017; Joshiba et al., 2019). Generally, a combination of several microbes (microbial consortium) with complementing metabolic activities is used as inoculum for achieving high biodegradation and mineralization of dairy wastewater (Velmurugan and Pandian 2024). When needed individual microbial strains may also be applied for degradation of specific pollutants to enhance treatment efficiency. The microbes selected for formulation of microbial consortium should preferably be native to dairy wastewaters, as they are pre-adapted to that particular environment, and therefore, may prove to be more effective for treatment of dairy wastewaters (Priyadarshini and Kumar, 2016). Additionally, the selected microbes should be the ones which are non-pathogenic, antagonize opportunistic and pathogenic microbes, exhibit resilience to fluctuating ionic strength, pH, etc. often encountered in dairy wastewaters

(Ramsuroop et al., 2024). Most importantly, such microbes must possess the ability to rapidly degrade dairy waste water-specific organics (Maghsoudi et al., 2007).

Lactose (a disaccharide made up of glucose and galactose joined by β -(1 $\rightarrow$ 4)-glycosidic bond) is a major carbohydrate present in milk (5% w/v), milk-derived products, as well as in dairy waste waters (Chollangi and Hossain, 2007). β -D-galactosidase (E.C. 3.2.1.23) is the primary enzyme responsible for catalyzing the conversion of lactose into glucose and galactose, simple sugars that can be easily assimilated further (Saqib et al., 2017). Thus, the β -D-galactosidase enzyme and/or its sources are of critical importance to dairy wastewater treatment. Presently, the major sources of this enzyme are bacteria, yeast, and some fungi. Previous studies show that introduction of the organisms or the enzyme itself can substantially reduce the content of lactose in whey, a type of dairy wastewater with high potential to pollute environment (Ramsuroop et al., 2024).

Besides lactose, dairy wastewaters are also rich in proteins (mainly casein), a complex nutrient which must be hydrolyzed by proteases before they are completely consumed (Moodley et al., 2024). Proteases represent a broad group of ubiquitous hydrolytic enzymes which are responsible for hydrolysis of peptide bonds between amino acid residues forming shorter peptides and amino acids (Kocabas et al., 2022). A large number of microbes can produce proteases, including bacteria. The bacterial proteases have high catalytic activity and are often preferred for biodegradation of dairy wastewaters (Cardoso et al, 2022; Kumar et al., 2016). The protease-producing bacteria, mainly *Bacillus* spp. have been isolated from dairy wastewaters, suggesting their ability to grow, survive, and dominate such wastewaters (Chandran et al., 2014). A previous study reported that the BOD and COD values of dairy waste water is reduced by 82% and 85%, respectively, when *Bacillus subtilis* is used as inoculant to treat dairy waste water (El-Sesy and Mustafa, 2020). Previously, various strains of *Serratia marcescens* have also been demonstrated to produce extracellular metallo-proteases when grown on whey (Ustáriz et al., 2008). In addition to proteins, dairy wastewaters also contain lipids or fats. Thus, enzymes that can hydrolyze milk fats or lipids (lipase) also play critical role in biodegradation of such wastewater (Atasoy

et al., 2020). Lipases belong to a class of serine hydrolases capable of catalyzing the hydrolysis of long-chain triacylglycerols to release glycerol and free fatty acids into lipid water interface (Garcia-Cano et al., 2020). Lipases are present in various organisms like microbes, plants and animals. *Bacillus* and *Pseudomonas* are predominantly reported lipase-producing bacteria. Other potential lipase-producing bacteria reported are *Serratia*, *Staphylococcus epidermidis*, *Thermobifida fusca* etc (Nimkande and Bafana, 2022). Many of these reported bacterial spp. have also been tested for their potential to treat dairy waste water and substantially reduce COD and BOD levels in the treated effluents (Kocabas et al., 2022; Nimkande and Bafana, 2022).

As evident from previous studies, microbes are the most important component of any biological wastewater treatment process. A better outcome in terms of effluent quality also depends on operational conditions under which treatment is carried out. In general, two broad conditions used for biological waste water treatment operations are aerobic and anaerobic with their own shares of merits and demerits.

1.2 Aerobic treatment process

The aerobic treatment process utilizes oxygen-requiring microbes to degrade dairy wastewaters. An optimal provision of oxygen is critical to its function, as oxygen is important for oxidation of organic compounds (Ramsuroop et al., 2024). One of the major drawbacks of aerobic treatment process is the cost incurred for meeting aeration requirement. The process also suffers from the inability to lower sludge production during treatment creating sludge disposal concerns. Further, during the process, inoculated microbes can be out competed by undesirable microbes leading to inhibition of the treatment process (Kolev slavov, 2017; Priyadarshini and Kumar. 2016). Over the years, several aerobic treatment technologies have been developed, including activated sludge, conventional or percolating filter, sequence batch reactor, membrane bioreactor, rotating biological contactor etc., each with its inherent advantages and disadvantages (Ramsuroop et al., 2024).

1.3 Activated sludge technology

The activated sludge technology uses microbial consortium as inoculant to treat wastewater in aeration tanks where adsorption, absorption and biodegradation of organic matter can occur simultaneously (Orhon, 2015). In this process, organic and inorganic matters are oxidized, and the end products sustain the continuous microbial growth and formation of biomass (flocs) (Orhon, 2015; Russel, 1997). The flocs formed are then maintained in the wastewater suspension by mechanical aeration or by the air blown into the bottom of the tank. The dissolved oxygen content in the aeration tank is maintained in the range of 3-5 mg/L. From the aeration tank, the mixture moves to the sedimentation tank, where the activated sludge flocs form larger compounds that settles in the sludge (Russel, 1997). A certain amount of sludge is recycled back into the aeration tank to maintain the continuity of the process. Although highly useful in reducing the organic and inorganic content of the dairy wastewater, this particular biological process leaves large amount of sludge that requires proper processing before disposal. Also, providing adequate amount of oxygen remains the main energy consuming operation and challenge during the process (Dold and Ekama, 1981).

1.4 Conventional or percolating filter

Percolating or trickling filter is one of the oldest aerobic treatment technologies that yield high quality effluents (Bali et al., 2010). It consists of a carrier medium which is 20-100 mm in diameter made up of rock, pumice, plastic pieces or gravels populated by diverse microbial community. In this process, the wastewater is allowed to trickle down the carrier medium, and the microbes on the carrier absorb and degrade the organic particles aerobically (Mottier et al., 2000). However, some of the disadvantages associated with this technology are that the deposited sludge requires periodical removal, carriers or filters may also need periodic replacement due to blockages and resulting foul smell (Makni, 2001).

1.5 Rotating biological contactors

The rotating biological contactors (RBC) involve circular discs made of high density plastic or other materials (Bella and Rao, 2023; Hassard et al., 2015). The discs designed to rotate at 1-3 revolutions per minute (rpm) are located on a horizontal shaft such that 40-60% of the disk stick out on the surface for continuous oxygen supply. Oxidation of the organic compounds in effluents are facilitated by the microbial biofilm formed on the surface of the disks, which are dislodged when the biofilm sludge becomes thicker (Hassard et al., 2015; Waqas et al., 2023). RBC is considered a better alternative to activated sludge process, as RBC requires low power input, ease of handling and low cost maintenance. Moreover, recycling of sludge and aeration are not necessary. A COD removal of 85% with organic loading rate of 500g COD/m² has been recorded previously using this technology (Bella and Rao, 2023; Rusten et al., 1992).

1.6 Sequencing batch reactor

Sequencing batch reactor require a single tank with fill and draw mode of operation, which follows a series of steps like fill, aerate, draw effluents and recycle solids (Neczaj et al., 2008). Initially, the tank is filled with wastewater without aeration, then the process of aeration follows that results in oxidation of organic matter present in the wastewater and biomass formation. The sludge is then separated from the treated wastewater in the final stage (Atasoy et al., 2020). This treatment process may demand manual oversight to regulate the cycle duration of each stage to assess the level of fluctuations in effluent composition (Gough et al., 2000; Samkutty et al., 1996).

1.7 Membrane bioreactors

In MBR system, membranes play an important role in separation of liquid and solid portions of wastewaters and they can be of two types depending on the placement of the membrane unit (Andrade et al., 2014; Bella and Rao, 2023; Sar et al., 2022). The membranes are either placed externally or are submerged in the bioreactor unit. Because of its compact system as well as low utilization of energy this treatment

process is more attractive (Andrade et al., 2014; Ueda et al., 1996). The membrane bioreactors (MBRs), particularly the submerged type membranes have received more attention due to the better quality of treated wastewater quality and lower sludge output (Iorhemen et al., 2016). However, the disadvantage of this process is the fouling of membrane in the submerged unit, as compared to externally placed membrane. The implementation of intermittent suction method and back flushing therefore becomes necessary to reutilize the membranes (Ueda et al., 1997; Visvanathan et al., 1997).

1.8 Anaerobic treatment process

Anaerobic treatment process is generally preferred over aerobic treatment because of significant drawbacks associated with the latter. The microbes involved in this process are anaerobic or facultative anaerobes. The treatment process provides several advantages, such as no requirement for aeration, lower cost of treatment equipment, low area demand, and low sludge production (Demirel and Yenigun, 2004). Furthermore, the anaerobic treatment process stabilizes organic matter in the absence of oxygen and may produce numerous useful by-products, for example, methane, biomass etc. (Mogha et al., 2017). Some previous studies have claimed anaerobic treatment process to be a very effective technology for treating dairy wastewaters with high organic load (Ahmad et al., 2019; Carvalho et al., 2013). Anaerobic treatment process, however, requires specially designed biodigesters/reactors, such as fixed-bed biodigesters, expanded bed and/or fluidized-bed digesters, completely stirred tank reactor, and upflow anaerobic sludge blanket reactors, the latter being the most preferred.

1.9 Upflow anaerobic sludge blanket reactor

In UASB, the wastewater is degraded by microbes that produce 70-80% methane, 15-25% carbon dioxide and some minor amount of hydrogen, nitrogen and other gases (Buntner, 2013). The methane gas produced can be separated and used as an alternative energy source. The design of UASB depends on the settling properties of granular sludge and the growth and development of the granules are the main

requirements for its optimum operations (Passeggi et al., 2012). The granules contribute to the separation of HRT from SRT (solid retention time) and provide more contact time to microbes to react with wastewaters (Cotta-Navarro, 2011). In this system, the wastewater to be treated is fed from the bottom and the treated effluent exits through the top with the help of internal baffle system that separates the sludge, gas, and liquid phases. UASB uses less energy for recirculation of effluent and requires no other expenses making it highly economical and also minimizes sludge disposal problem to a great extent (Passeggi et al., 2012).

1.10 Factors affecting biodegradation of dairy waste waters

Besides optimum HRT and SRT, OLR, pH, temperature etc. are also important for performance efficiency of any biological wastewater treatment system (Bella and Rao, 2023). High concentrations of ammonia, VFA (volatile fatty acids) and sub-optimal levels of alkalinity can also adversely affect the process (Bella and Rao, 2023; Demirer and Chen, 2004). Optimization of organic loading rate (quantity of volatile solids loaded into the digesters) is critical to efficiency and stability of biodegradation process. Often, low OLR is preferred. Increased OLR can lead to higher production of volatile fatty acids, decrease pH and lower the biodegradation efficiency (Bella and Rao, 2023). Similarly, high concentrations of ammonia can also have a detrimental effect on the efficiency of biodegradation process (Gil et al., 2019). Therefore, such factors should be taken into account when designing the wastewater treatment system.

1.11 Waste to wealth

Wastewaters are increasingly being treated not only to reduce polluted load for environmental safety, but also to recover valuable resources from it (Moodley et al., 2024). As dairy industrial wastewaters possess a plenty of organics and inorganics, they can indeed act as a precursor to a large number of biotechnologically useful products, all through the action of microbes (Kozłowski et al., 2019). These products include biofuels like biomethane (produced under anaerobic conditions, vital alternative energy source), biohydrogen (clean and highly efficient source of green

energy), important enzymes, such as β -D-galactosidase etc. (a dairy industrial enzyme used for production of low or lactose free milk, source of prebiotics such as galacto-oligosaccharides) (Kozłowski et al., 2019; Louasté and Eloutassi, 2020; Pandey et al., 2016; Wainaina et al., 2019). Additionally, bioethanol, biodiesel, and a variety of organic acids such as acetic acid, citric acid, propionic acid, lactic acid etc. can also be harvested from waste water treatment systems for applications in food, agriculture, medical, and environmental fields (Kozłowski et al., 2019; Wainaina et al., 2019).

1.12 Present study

As microbes, especially bacteria are crucial to biodegradation of organics, including lactose, proteins, and lipids present in dairy wastewaters, the purpose of this study was to screen, isolate and characterize bacterial strains competent to hydrolyze dairy wastewater organic constituents. The sources of isolated bacterial strains were a medically-used commercial probiotic (a consortium of different bacteria) with proven non-pathogenic status and dairy waste water. An attempt was also made to probe the ability of such strains to treat milk wastewater mimicking the nutritional composition of real dairy wastewaters.

CHAPTER 2

2 Materials and methods:

2.1 Sources of bacteria

Two samples were collected for isolation of β -D-galactosidase positive bacteria. One of the sample was commercially available probiotic tablet (VIBACT-DS, manufactured by Allianz Bioscience, Thiruvandar Koll, Puducherry) which was bought from a medical store in Aizawl, Mizoram, India. The probiotic tablet was containing four types of bacteria including *Streptococcus faecalis*, *Clostridium butyricum*, *Bacillus mesentericus* and Lactic acid bacillus (*Lactobacillus sporogenes*). The other sample was dairy wastewater, collected from Mizoram Milk Producers Cooperative Union Ltd (MULCO).

2.2 Screening and isolation of β -D-galactosidase positive bacterial strains

2.2.1 Screening and isolation of β -D-galactosidase positive bacterial strains from probiotic tablet

A probiotic tablet was dissolved in saline water containing sodium chloride (NaCl, 0.85%, w/v) and then diluted to 10^{-6} by serial dilution method. 50 μ l of the diluted suspension was spread on Luria-Bertani (LB)-agar plate containing 10 g tryptone, 5 g yeast extract, 10 g Sodium chloride (NaCl) and 20 g agar amended with 1 mM 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (Kalathinathan and Muthukaliannan, 2021). The plates were incubated for 20 h at 37°C. A prominent colony which appeared blue in colour was picked and streaked on a LB-agar plate. The colony was further purified by restreaking on LB-agar plate and maintained in LB-medium containing 15% glycerol at -20 °C until used for further experiments.

2.2.2 Screening and isolation of β -D-galactosidase positive bacterial strains from dairy waste water

One ml of the collected dairy wastewater was taken and diluted to 10^{-6} by serial dilution method. 50 μ l of the diluted (10^{-6}) was spread on Luria-Beratni (LB)-agar

plate amended with Xgal (Kalathinathan and Muthukaliannan, 2021). The plates were incubated for 20 h at 37°C and purified as described in the section 2.2.1.

2.3 Gram staining

For gram staining, a colony of each bacterial isolate was picked and smeared on a slide followed by standard protocol of gram staining (Bartholomew and Mittwer, 1952; Coico, 2005). To the smear, crystal violet was added and kept for 30 s and washed off the stain by distilled water. Then, iodine solution was added and kept for 30 s followed by rinsing with running water. The excess water was shaken off and few drops of decolorizer were added (Mittwer et al., 1950). Then, the slide was rinsed with running water for 5 s and counterstain (safranin) was added. After 50 s, the slide was washed and blotted. Then, a coverslip was placed and observed under microscope in 100X objective, and picture was taken using camera.

2.4 Culture methods:

2.4.1 Culture medium for determination of bacterial growth and enzyme activities

For the determination of bacterial growth, de Man, Rogosa and Sharpe (MRS)-medium was used with a little modification. The MRS medium was amended with different carbon sources of different concentrations. The carbon sources used included glucose (2%, w/v), galactose (1%, w/v), and lactose (concentration ranging from 1-5%, w/v). The final pH was made 7.0. The composition of the MRS medium is given in the Table 2.1 (de Man. C et al., 1960).

2.4.2 Sterilization

The prepared media were sterilized by autoclaving at 121°C (15 pounds per square inch) for 15 minutes (Lyne and Grange, 1995).

2.5 Carbohydrate utilization test

The isolated bacterial strains were undergone carbohydrate utilization test by using Biochemical Test Kit (Hi25™ *Enterobacteriaceae* Identification Kit, Strip-2,

KB003, HIMEDIA) (Jorgensen et al., 2015; Kumar et al., 2025). The kit strip was containing 11 wells for different carbohydrate utilization tests of along with escullin hydrolysis. The tests for different carbohydrates were arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose and lactose, respectively (Jorgensen et al., 2015).

2.5.1 Preparation of inocula for carbohydrate utilization tests

A single colony of each strain was inoculated to 5 ml LB-medium and incubated at 37°C for until the inoculums turbidity becomes 0.1 OD at 620 nm (Jorgensen et al., 2015).

2.5.2 Inoculation in the wells of the strip

The Kits were opened aseptically. Inocula (50 µl) were inoculated to each well of the strips and incubated at 37°C for 24 h. The results were interpreted by following the standards given by the manufacturer.

2.5.3 Result interpretation of the carbohydrate utilization tests

The results were interpreted as given in the Table 2.2 as per guideline given by the manufacturer (HIMEDIA).

2.6 Antibiotic susceptibility test

Antibiotic susceptibility of the isolated bacterial strains was tested by using disc diffusion method (Gajic et al., 2022). This method is based on placing of disc impregnated with several antibiotics on previously inoculated agar with bacteria.

2.6.1 Preparation of inoculums

A prominent colony of each bacterial isolate was grown for overnight in 5 ml LB medium by incubating at 37°C. The pre-cultured bacteria were re-grown in same media to 0.1 OD at 600 nm (Gajic et al., 2022).

2.6.2 Preparation of culture medium, inoculation and placement of antibiotic discs

For antimicrobial susceptibility test, LB-agar medium was used. 100 µl of the bacterial cultures with 0.1 OD at 600 nm was spread on the LB-agar plates. After inoculation, the antibiotic discs containing several antibiotics were placed on the previously inoculated LB-agar plates and incubated for 24 h at 37°C. After incubation, the inhibition zones were measured in terms of diameter (Gajic et al., 2022).

2.6.3 Interpretation of result

The results for the antimicrobial susceptibility were interpreted as per manufacturer guideline (HIMEDIA). The diameters of the inhibition zones were measured and compared with the standard diameter of inhibition zone for sensitivity and resistant following guideline of the manufacturer (HIMEDIA) as given in the Table 2.3.

2.7 Identification of bacterial cultures using 16S rDNA Sequencing

2.7.1 Genomic DNA isolation

The genomic DNA was isolated from overnight grown culture of the bacterial isolate following standard protocol (Sambrook and russel, 2001). To an eppendorf tube, 1 ml culture was pipette, centrifuged (5 min at 10,000 rpm) and the supernatant was discarded. The cell pellet was resuspended by pipetting 500 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) to the tube. 30 µl sodium dodecyl sulphate (SDS) solution (10%, w/v) was added to tube and vortexed. Then, 3 µl proteinase K solution (20 mg/ml) was added to the tube and incubated for 60 min at 37°C followed by addition of 100 µl sodium chloride (NaCl). The mixture was vortexed and 80 µl solution of 10% hexadecyltrimethylammonium bromide (CTAB) and 5 M NaCl in the ration of 1:1 was added. The mixture was mixed by inverting 3 times and incubated for 10 min at 65°C. Following the incubation, phenol/chloroform:iso-amyl alcohol (25/24:1) was added (equal volume) and emulsified by mixing. Then, the mixture was undergone centrifugation at 8,000 rpm for 5 min and the upper layer was pipetted into a fresh eppendorf tube. This step was repeated once. Ribonuclease

was added making final concentration of 20 µg/ml and kept at 37°C for 30 min. Then, phenol/chloroform:iso-amyl alcohol mixture was added (equal volume) and centrifuged. The DNA was precipitated by adding 5 M NaCl (1/10 volumes) and cold ethanol (2 volumes) and placed at -20°C for 60 min. Then, the tube was undergone centrifugation for 20 min at 12,000 rpm. The upper layer (supernatant) was discarded and the DNA pellet was washed with ethanol (70%). The tube was centrifuged again at 12,000 rpm for 20 min and the upper layer (supernatant) was discarded. The DNA pellet was air dried and dissolved in 50 µl TE buffer, and kept at -20°C until used for PCR amplification (Paramasivan et al., 2009).

2.7.2 Assessment of DNA concentration, purity and PCR amplification

The concentration and purity of DNA was assessed by using UV-Vis 1900i spectrophotometer (Shimadzu, Japan) and agarose gel (0.8%) analysis (Green and Sambrook, 2019). 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' (Lane, 1991). The thermal cycling conditions included initial denaturation for 4 min at 96°C, 30 cycles of amplification for 30 s at 94°C, annealing for 30 s at 57°C, and 1 min extension at 72°C followed by final extension for 10 min at 72°C (Green and Sambrook, 2019).

2.7.3 DNA 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), and a consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software (Mandragutti et al., 2021). The 16S rRNA gene sequence was subjected to Basic Local Alignment Search Tool (BLAST) in the __nr__ database of NCBI GenBank (Kaur et al., 2018). On the basis of maximum identity, ten sequences were selected and aligned using multiple alignment software ClustalW (Thompson et al., 1997).

The Phylogenetic tree was constructed using MEGA12 (Saitou and Nei, 1987; Tamura et. al., 2021).

2.8 β -galactosidase enzyme assay and calculation

The β -D-galactosidase activity was assayed in bacterial cells were in MRS-medium amended with different carbon sources including 2% (w/v) glucose, 1% (w/v) galactose and lactose (1-5%, w/v) for overnight at 37°C.

2.8.1 Preparation of cells

The overnight grown cultures were diluted to 10^{-2} with same media and allowed to grow for 6 h at 37°C. The cultures were incubated at in ice for 20 min to stop the bacterial growth and 2 ml of cultures were washed with chilled Z buffer containing 16 g disodium hydrogen phosphate heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), 5.6 g sodium dihydrogen phosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), 10 ml 1 M potassium chloride (KCl), 1 ml 1 M magnesium sulphate (MgSO_4), and 2.7 ml β -mercaptoethanol (BME) per litre (pH 7.0, stored at 4°C until used) by centrifugation for 10 min at 10,000 rpm at 4°C. The supernatant was discarded and resuspended with same volume of chilled Z buffer. Then, optical density (OD) was measured at 600 nm against Z buffer (blank).

2.8.2 Permeabilization of cells

Permeabilization of cell was done by addition of 100 μl chloroform and 50 μl 0.1% SDS (Chanalia et al., 2018). After addition, the cells were vortexed and equilibrated in a 28°C water bath for 5 min.

2.8.3 β -D-galactosidase enzyme activity assay

The permeabilized cells were used for β -D-galactosidase enzyme activity as per standard protocol described by Miller (1972) taking *o*-nitrophenyl- β -D-galactoside (ONPG) as substrate. 0.2 ml of ONPG (prepared by dissolving 4 g in 0.1 M phosphate buffer containing 16.1g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 5.5g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ per litre, pH 7.0) solution was added to the pearmeabilized cells and vortexed. The reaction

was carried out at 37°C for 9 min and halted by addition of 0.5 ml 1 M sodium carbonate (Na₂CO₃) (Wanarska and Kur, 2012). The reaction mixture was vortexed and 1 ml was pipetted into a new eppendorf tube followed by centrifugation at 12,000 rpm for 5 min. The debris and chloroform were removed and absorbance at 420 nm and 550 nm was measured by using UV-Vis 1900i spectrophotometer (Shimadzu, Japan). The activity was determined by using the formula given below-

$$Miller\ units = 1000 \times \left[\frac{OD_{420} - 1.75 \times OD_{550}}{t \times v \times OD_{600}} \right]$$

Where, OD₆₀₀ , *t* and *v* represent cell density, reaction time and volume of enzyme solution.

2.8.4 Determination of temperature, pH and EDTA on β-D-galactosidase enzyme activity

The impact of temperature, pH and EDTA on β-D-galactosidase activity was tested incubating the reaction mixture containing permeabilized cells (2% lactose-grown) and ONPG solution in different temperature (i.e., 25°C, 37°C), pH (6.0-9.5), and addition of EDTA (0.5-2.0 mM) for 9 min at 37°C. The assay was carried further as per standard protocol described by Miller (1972).

2.9 Determination of protease and lipase activities

2.9.1 Preparation of enzyme solution

Protease and lipase activities were assayed in the spent medium supernatant (SMS), collected from cultures grown in presence of 2% glucose, 1% galactose, and 2% lactose by centrifugation at 10,000 rpm for 10 min.

2.9.2 Protease activity assay

Protease activity was assayed as per standard protocol described by Cupp-Enyard (2008). Casein was used as substrate and its solution was prepared by dissolving 6.5 g casein in 1 L 50 mM potassium phosphate buffer (containing 11.4 g potassium phosphate dibasic trihydrate per litre, pH 7.0). The casein was solubilised by heating

with agitation at 85°C for 10 min. In a 15 ml sterilized tube 5 ml casein solution was taken and equilibrated for 5 min at 37°C. Then, 1 ml of SMS was added and incubated at 37°C for 10 min to start the reaction. The reaction was halted by adding 5 ml 110 mM trichloroacetic acid (TCA) and incubated at 37°C for 30 min. A control (1 ml enzyme solution was added after the reaction halted) was incubated along with the assay. After incubation, the test sample and the control were filtered by using 0.45 µ syringe filter. In another 15 ml tubes 2 ml filtrate of each (test sample and control) were taken and 5 ml 500 mM of sodium carbonate (NaNO₃) was added followed by rapid addition of 1 ml 0.5 mM folin-ciocalteu reagent. Then, the tubes were incubated at 37°C for 30 min.

For preparation of standard curve, 1.1 mM tyrosine solution was used as stock solution. To a series of tubes tyrosine stock was pipetted with volumes of 0, 0.05, 0.1, 0.2, 0.4, and 0.5 ml and volumes were adjusted to 2 ml with sterile Milipore Q water. To the tubes, 5 ml of Na₂CO₃ was added and followed by rapid addition of 1 ml folin-ciocalteu reagent. Then, the tubes were undergone incubation for 30 min at 37°C. The absorbance was recorded at 660 nm using UV-Vis 1900i spectrophotometer (Shimadzu, Japan) and the amount of tyrosine released was measured from the standard curve. The amount of enzyme activity was measured using following formula-

$$\frac{Units}{ml} = \frac{\mu moles\ of\ tyrosine\ released \times Total\ volume\ of\ the\ assay}{Time\ of\ assay \times Volume\ of\ test\ sample \times Colorimetric\ volume}$$

2.9.3 Lipase activity assay

Lipase activity was assayed as per standard protocol described by Kanwar et. al. (2005). p-nitrophenylpalmitate (pNPP) was used as substrate and the pNPP solution (20 mM) was prepared in isopropanol (HPLC grade). 75µl pNPP solution and 50 µl SMS were pipette in a sterilized test tube and the final volume was made to 3 ml by adding 50 mM Tris buffer (pH 8.5). The tube was incubated at 45°C for 20 min in a water bath to start the reaction and halted by addition of 1 ml chilled acetone:ethanol

solution (1:1, kept at -20°C overnight). Control containing heat inactivated (heat at boiling point for 5 min) sample was also incubated with each assay. Then, the absorbance was recorded at 410 nm against the control.

For preparation of standard curve, p-nitrophenol solution was used as standard solution. A series of tubes with p-nitrophenol, concentrations ranging from 2-20 µg/ml was incubated at 45°C for 20 min and then halted by adding acetone : ethanol mixture. The p-nitrophenol produced was measured from the standard curve and the lipase activity was measured using the following formula-

$$\frac{\text{Units}}{\text{ml}} = \frac{\mu\text{moles of } p - \text{nitrophenol produced} \times \text{Total volume of the assay}}{\text{Time of assay} \times \text{Volume of test sample} \times \text{Colorimetric volume}}$$

2.10 Waste milk biodegradation potential

Milk biodegradation potential was measured in batch studies. The experiment was run for 10 days and the physicochemical characteristics of the waste milk before and after treatment with bacterial isolates were measured. The physicochemical characteristics included tCOD, sCOD, turbidity, TS, VS, concentrations of carbohydrate and protein, pH, and alkalinity.

2.10.1 Experimental set up

The reactors with dairy waste waster were inoculated with the bacterial strains separately and in a form of combination of the both strains. Experiments were run in duplicate. The reactors were placed at 37°C and for 10 days. After 10 days, the physicochemical and its organic contents were measured.

2.10.2 Determination of total and soluble chemical oxygen demand (COD)

The total COD (tCOD) was measured in a COD vial containing 2 ml of the sample, 0.25 of ml COD solution 1 (Hach, Cat. 29509830), and 2.8 ml of COD solution 2 (Hach, Cat. 2950915) (Bhushan et al., 2021). The vial was incubated for 2 h at 150°C in a COD reactor (Hach, DRB 200) and allowed to cool to room temperature. The

absorbance was recorded by using a DR6000 UV-Vis spectrophotometer and the concentration of tCOD calculated. The soluble COD (sCOD) concentrations was measured in the filtrate of effluents after centrifugation (10 min at 10,000 rpm) and filtration through 0.45 µm filters by following a procedure similar to tCOD (APHA, 2005).

2.10.3 Determination of total solid (TS), volatile solid (VS), turbidity, pH and alkalinity

The TS and VS were measured gravimetrically according to the standard methods prescribed for the examination of water and wastewater (APHA, 2005). Turbidity and pH were measured using a HACH HQ series multi-meter (HQ4200, U.S.A.), Alkalinity was determined by using total alkalinity kit (TNT 870, HACH) and the absorbance values were recorded using DR6000 UV-Vis Spectrophotometer (HACH, U.S.A).

2.10.4 Determination of organics content

Preparation of test sample: In a sterilized tube, 5 ml of the test sample was taken and subjected to centrifugation at 10,000 rpm for 8 min. The supernatant was taken and filtered by using 45 µ syringe filter and collected in another sterilized test tube. The filtered sample was used for determination of soluble carbohydrate and protein concentrations.

Determination of carbohydrate concentration: The concentration of carbohydrates was determined using anthrone method (Layne, 1975; Plummer, 1990). A series of sterilized tubes containing volume ranging from 50-500 µl of the standard solution (200µg glucose per ml water) was taken. The volume was made 1 ml by addition of distilled water. Along with the standard series, 1 ml of the test sample was pipette into a sterilized tube. To each tube 5 ml anthrone reagent (prepared by dissolving 2g of anthrone in 1 L concentrated sulphuric acid, H₂SO₄) was added and vortexed. The tubes were tightened with cap and allowed to cool to room temperature. Then, the tubes were incubated for 10 min in a boiling water bath. A blank containing 1 ml of

distilled water was also incubated along with the tubes. After incubation, the tubes were cooled down to room temperature and read absorbance at 620 nm. The absorbance values of the standard series were plotted and the calibration curve was prepared. The carbohydrate concentration was determined from the calibration curve putting the value of the absorbance of the test sample in the equation.

Determination of protein concentration: The protein concentration was determined using Lowry method (Lowry et. al., 1951). A series of tubes containing standard solution (1 mg/ml Bovine Serum Albumin, BSA) of volumes ranging from 50-500 μ l was taken along with a tube containing 1 ml of test sample and a tube containing 1 ml distilled water was taken as blank. To each tube, 4.5 ml of reagent A (a mixture of 2% sodium carbonate in 0.1 N sodium hydroxide, 1% sodium-potassium tartarate in water, and 0.5% copper sulphate pentahydrate in water in a ration of 48:1:1) was added, vortexed and the tubes were incubated at room temperature for 10 min. Then, 1 ml folin-ciocalteu reagent (1N) was added and incubated at room temperature. After 30 min of incubation, absorbance was read at 660 nm by using UV-Vis 1900i spectrophotometer. The absorbance values of the standard series were plotted in a graph to prepare calibration curve. Using the equation from the calibration curve, the concentration of protein was determined by putting the absorbance value of the test sample.

2.11 Statistical analysis

All the experiments were done in duplicate and the values were reported as mean $\pm$ standard deviation (SD). The plots and bar diagrams were prepared by using Microsoft Excel 2010.

Table 2.1 Compositions of MRS-medium.

Sr. No	Compositions	Concentrations (g/L)
1	Peptone	10.00
2	Beef extract	10.00
3	Yeast extract	5.00
4	Dextrose (Glucose)	20.00
5	Tween 80 (Polysorbate 80)	1.00
6	Ammonium citrate	2.00
7	Sodium acetate	5.00
8	Magnesium sulphate	0.10
9	Manganese sulphate	0.05
10	Dipotassium hydrogen phosphate	2.00

Table 2.2 Result interpretation chart for carbohydrate utilization test kit (Strip-II).

Strip-2		Result interpretation chart		
Sr. No.	Test	Original colour of the medium	Positive reaction	Negative reaction
1	Esculin hydrolysis	Cream	Black	Cream
2	Arabinose	Pinkish red/Red	Yellow	Red/Pink
3	Xylose	Pinkish red/Red	Yellow	Red/Pink
4	Adonitol	Pinkish red/Red	Yellow	Red/Pink
5	Rhamnose	Pinkish red/Red	Yellow	Red/Pink
6	Cellobiose	Pinkish red/Red	Yellow	Red/Pink
7	Melibiose	Pinkish red/Red	Yellow	Red/Pink
8	Saccharose	Pinkish red/Red	Yellow	Red/Pink
9	Raffinose	Pinkish red/Red	Yellow	Red/Pink
10	Trehalose	Pinkish red/Red	Yellow	Red/Pink
11	Glucose	Pinkish red/Red	Yellow	Red/Pink
12	Lactose	Pinkish red/Red	Yellow	Red/Pink

Table 2.3 Result interpretation chart for antibiotic susceptibility test.

Antibiotic	Symbol	Disc contents	Standard zone diameter (mm)
Neomycin	N	30 µg	12-17
Gentamicin	GEN	10 µg	12-15
Vancomycin	VA	30 µg	14-17
Bacitracin	B	10 units	8-13
Erythromycin	E	10 µg	13-23
Penicillin G	P	10 units	14-15
Chloramphenicol	C	30 µg	12-18
Cefotaxime	CTX	30 µg	18-35
Augmentin	AMC	30 µg	18-32
Ofloxacin	OF	5 µg	17-33
Co-Trimoxazole	COT	25 µg	24-32
Polymyxin-B	PB	300 units	13-19
Ciprofloxacin	CIP	5 µg	22-40
Sparfloxacin	SPX	5 µg	21-38
Gatifloxacin	GAT	5 µg	20-35
Teicoplanin	TEI	30 mcg	15-21
Azithromycin	AZM	15 µg	21-26
Doxycycline HCl	DO	30 µg	18-29
Cefoperazone	CPZ	75 µg	23-34
Cefpodoxime	CPD	30 µg	19-28
Ceftazidime	CAZ	30 µg	16-32
Cefepime	CPM	30 µg	23-35
Imipenem	IPM	10 µg	20-32
Amikacin	AK	30 µg	18-26
Moxifloxacin	MO	5 µg	17-35

CHAPTER 3

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

3.1 Introduction

Probiotics are gut-friendly active bacteria and yeasts (Butel, 2014). The most commonly used probiotic bacteria are those belonging to the group lactic acid bacteria (LAB). Some of the well-characterized LAB include spp. from genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Weissella*, etc. (Wang et al., 2021). 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 (Kocabaş et al., 2022; Maske et al., 2021; Wang et al., 2021). 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. (Butel, 2014; Wang et al., 2021).

Dairy industries are notorious for generating huge volume of highly polluting wastewaters rich in several organics and minerals. These organics are mainly lactose (a disaccharide), casein (milk protein), and milk fats (Kolhe et al., 2011). Some previous studies suggest that 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 (Kalathinathan and Muthukaliannan, 2021; Kolev et al., 2022; Liu et al., 2019; Luan and Duan, 2022; Vasiljevic and Jelen, 2001; Venkateswarulu et al., 2020). 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 (Kalathinathan et al., 2023). 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 (Camarota and Freire, 2006; Kieliszek et al., 2021; Mp and Manjunath, 2011). 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 wastewaters (Loperena et al., 2009).

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 wastewaters.

3.2 Materials and methods

3.2.1 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*). The screening of β -D-galactosidase-positive bacteria was done as describe in chapter 2 (section 2.2.1) and the blue colour colony was picked and named as PB1. The bacterial strain was further purified and Gram-stained for preliminary identification as described in chapter 2 (section 2.3).

3.2.2 Molecular identification of the bacterial isolate PB1

3.2.2.1 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 in chapter 2 (section 2.7.1). 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 as stated in the section chapter 2 section 2.7.3).

3.2.2.2 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 (Janda and Sharon, 2007).

3.2.3 Test for susceptibility to antibiotics and carbohydrate utilization by PB1

The antibiotics susceptibility of PB1 was tested against 25 antibiotics using disc based method. The utilization of different carbohydrates by PB1 was analyzed using Hi25™ *Enterobacteriaceae* identification Kit (Strip-2, KB003-1KT, HIMEDIA) as described in chapter 2 (section 2.5.1, 2.5.2 and 2.5.3).

3.2.4 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 OD₆₀₀ values were measured using a UV-Vis 1900i spectrophotometer at different time intervals during the entire experimental period.

3.2.5 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. The preparation of cells and enzyme assay were done as described in chapter 2 (sections 2.8.1, 2.8.2 and 2.8.3).

3.2.6 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 as described in chapter 2 (section 2.8.4).

3.2.7 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 0.45 μ syringe filter to remove residual bacterial cells. The extracellular protease and lipase activities were determined in such supernatants as described in chapter 2 (sections 2.9.2 and 2.9.3).

3.2.8 Statistical analysis

Statistical analysis was done as described in chapter 2 (section 2.11).

3.3 Results and discussion

3.3.1 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.3.1(a), PB1 appeared positive for gram-staining and coccus-shaped. This bacterium lacked catalase activity (data not shown),

a feature commonly present in LAB (Kalathinathan and Muthukaliannan, 2021; Kolev et al., 2022; Maske et al., 2021; Wang et al., 2021). 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.3.1 b). 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.3.1 c). The probiotic tablet from which the bacterium was isolated contained only *Streptococcus faecalis* and not *Enterococcus faecium*. However, it may be noted that the spp. belonging to *Streptococcus* are recently reclassified and included in the genus *Enterococci*, spp. prevalent in dairy products (Giraffa, 2003; Schleifer and Kilpper-Bälz, 1984). The 16S rRNA gene sequence of PB1 (hereafter referred to as *E. faecium* PB1), a LAB has been submitted to NCBI with accession number PV927168.

3.3.2 Antibiotics susceptibility and carbohydrate utilization potential of *E. faecium* PB1

LAB exhibits differential susceptibility towards clinically relevant antibiotics (Floris et al., 2025). The LAB *E. faecium* PB1 was exposed to 25 different antibiotics to determine its susceptibility pattern (Fig. 3.2 a-d). As shown in Table 3.1, *E. faecium* PB1 was sensitive to all the antibiotics, except ceftazidime, imipenem, co-trimoxazole, and azithromycin. This suggest that susceptibility of *E. faecium* PB1 to a large number of commonly used antibiotics may qualify it as safe in terms of transmission of antibiotic resistance to other microbes in the environment

LAB can utilize a variety of carbohydrates for their growth and metabolism (Petrut et al., 2019). *E. faecium* PB1 was subjected to a qualitative carbohydrate utilization test to determine its potential to use different types of carbohydrates. As shown in Table 3.2, *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. Nevertheless, its ability to utilize some disaccharides and pentose sugar may be a useful trait that can be exploited for various biotechnological applications.

3.3.3 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.3.3, *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 (Zang et al., 2020). 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 wastewaters.

3.3.4 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 to assimilate sugars glucose and galactose (Kalathinathan et al., 2023). The exponentially growing cultures of *E. faecium* PB1 were used for the determination of β -D-galactosidase activity. As shown in Fig.3.4, 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 (Kalathinathan and Muthukaliannan, 2021; Kolev et al., 2022; Liu et al., 2019; Luan and Duan, 2022; Vasiljevic and Jelen, 2001). 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} (Badarinath and Halami, 2011). 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 (Badarinath and Halami, 2011; Liu et al., 2019).

3.3.5 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.3.5 (a), the β -D-galactosidase activity was only marginally lower at 25°C (615.57 $\pm$ 18.5 Miller Unit) and 60°C (635.37 $\pm$ 18.1 Miller Unit) than that at 37°C (702.8 $\pm$ 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 (Badarinath and Halami, 2011; Yao et al., 2019). Further, as shown in Fig.3.5 (b), the highest β -D-galactosidase activity was observed in reaction mixture having pH 8.5 (765.86 $\pm$ 26.8 Miller Unit) followed by that in pH 7.0 (702.8 $\pm$ 15.81 Miller Unit) and pH 9.5 (175.93 $\pm$ 8.004 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 to alkaline pH range consistent with β -D-galactosidases characterized from other bacteria (Badarinath and Halami, 2011; Yao et al., 2019).

3.3.6 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 oligo-peptides and amino acids and these simpler forms can serve as nutrients for bacterial growth (Biscola et al., 2018; Kieliszek et al., 2021; Savijoki et al., 2006). Milk fats or lipids are broken down by lipases to smaller organic acids and can serve as sources of carbon (Cammarota and Freire, 2006; Katz et al., 2001; Mp and Manjunath, 2011). 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.3.6(a), the highest extracellular protease activity (0.04 ± 0.001 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 (Biscola et al., 2018; Savijoli et al., 2006). Furthermore, as shown in Fig.3.6(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 (Loperena et al., 2009).

Taken together, the findings presented in this study suggest that the isolated probiotic bacterium *E. faecium* PB1 is an useful LAB, as it is sensitive to most antibiotics and metabolically versatile with the ability 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.

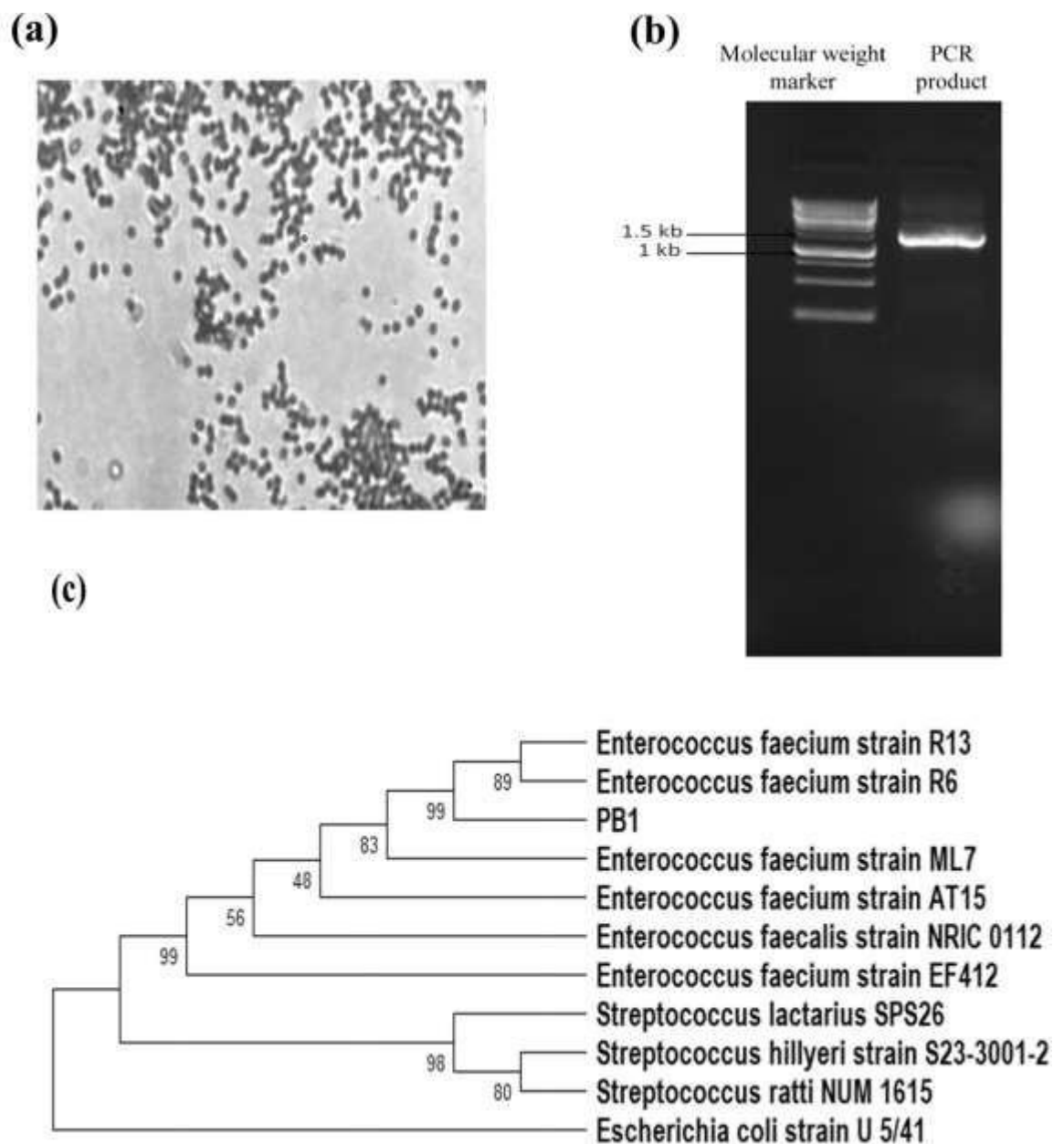


Fig. 3.1 Morphological and molecular identification of a probiotic bacterium PB1. Gram-stained PB1 (a), PCR amplified 16S rRNA gene product of PB1 on 0.8 % agarose gel (b), and 16S rRNA gene-based phylogeny of PB1 with similar sequences from other bacterial species (c). Different species of *Streptococcus* and an *Escherichia coli* strain were used as out-groups

(a)



(b)



(c)



(d)



Fig.3.2 Effect of different antibiotics on survival of *E. faecium* PB1. An exponentially growing culture of *E. faecium* PB1 equivalent to OD₆₀₀ of 0.1 was used to spread plates (a-d) followed by placing the antibiotic-containing discs on top of the cultures to assess susceptibility pattern.

Table 3.1 Antibiotics susceptibility pattern of *E. faecium* PB1 scored as resistant (R) or sensitive (S).

Antibiotics	Result
Penicillin G (P 10)	S
Polymixin B (PB 300)	S
Neomycin (N 30)	S
Bacitracin (B 10)	S
Chloramphenicol (C 30)	S
Gentamicin (GEN 10)	S
Amikacin (AK 30)	S
Moxifloxacin (MO 5)	S
Cefoparazone (CPZ 75)	S
Cefpodoxime (CPD 30)	S
Ceftazidime (CAZ 30)	R
Cefepime (CPM 30)	S
Imipenem (IPM 10)	R
Ofloxacin (OF 5)	S
Co-trimoxazole (COT 25)	R
Cefotaxime (CTX 30)	S
Augmentin (AMC 30)	S
Erythromycin (E 10)	S
Gatifloxacin (GAT 5)	S
Teicoplanin (TEI 30)	S
Azithromycin (AZM 15)	R
Vancomycin (VA 30)	S
Doxicyclin (DO 30)	S
Ciprofloxacin (CIP 5)	S
Sparfloxacin (SPX 5)	S

Table 3.2 Carbohydrate utilization potential of *E. faecium* PB1.

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

An exponentially growing culture of *E. faecium* PB1 equivalent to OD₆₀₀ of 0.1 was used to estimate carbohydrate utilization potential according to kit manufacturer's instructions and result interpretation. —+|| sign indicate positive utilization.

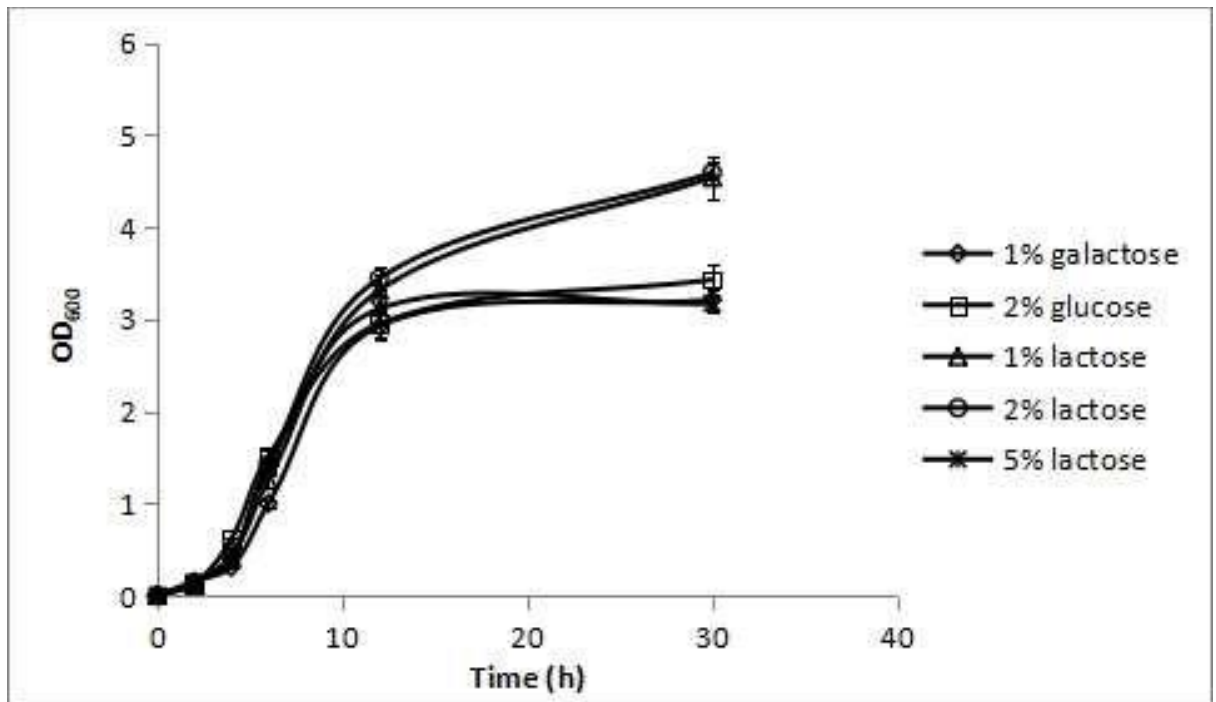


Fig.3.3 Effect of different carbon sources on the growth of *E. faecium* PB1. Exponentially grown culture of *E. faecium* PB1 was inoculated in MRS medium amended with different carbon sources, as indicated, and growth was measured periodically by determining OD₆₀₀ of cultures. The experiments were done in duplicate and results are presented as mean±SD.

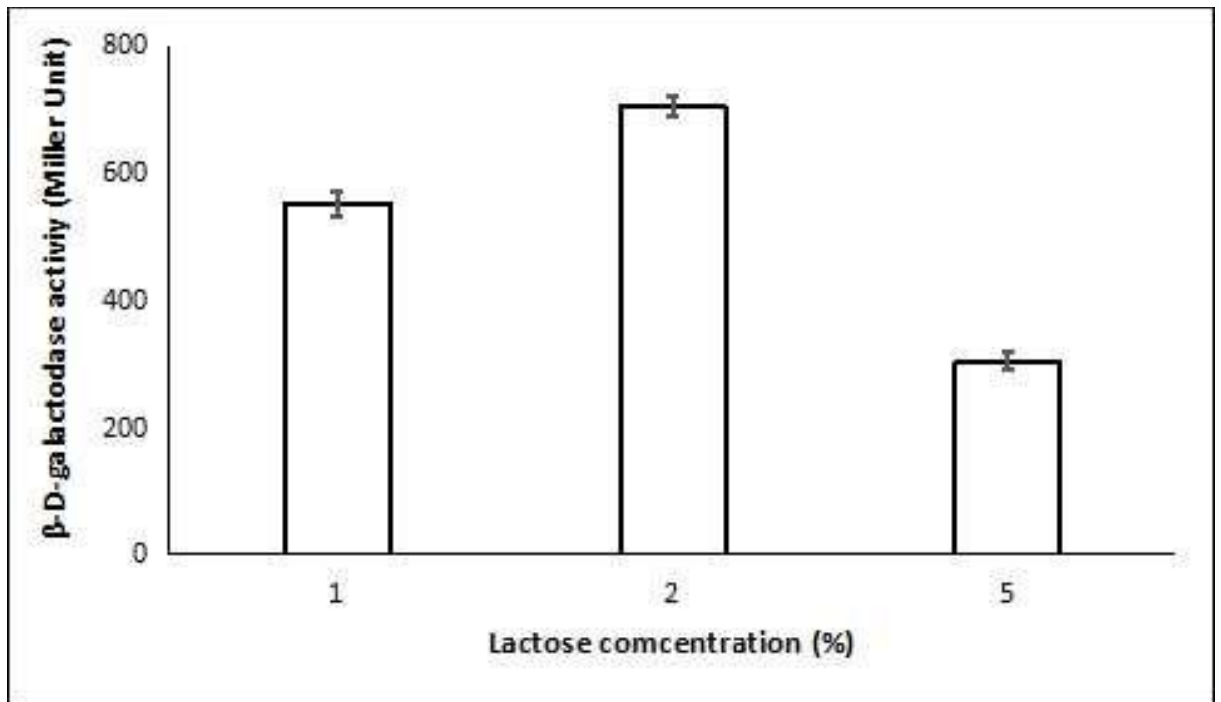
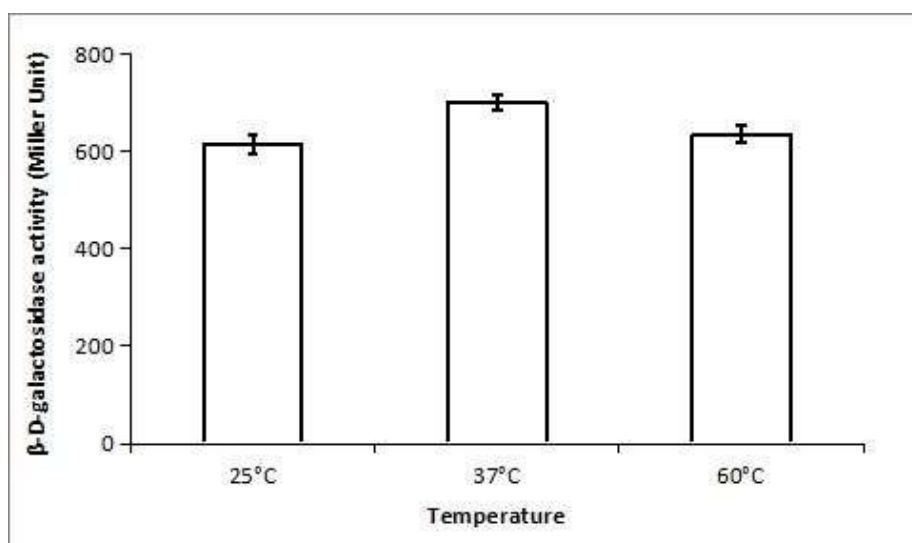


Fig.3.4 Effect of different lactose concentrations on β -D-galactosidase activity in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with different concentrations of lactose, as indicated, and grown for 6 h. The cultures were then harvested and permeabilized cells were used in the activity assay. The experiments were done in duplicate and the results are presented as mean $\pm$ SD.

(a)



(b)

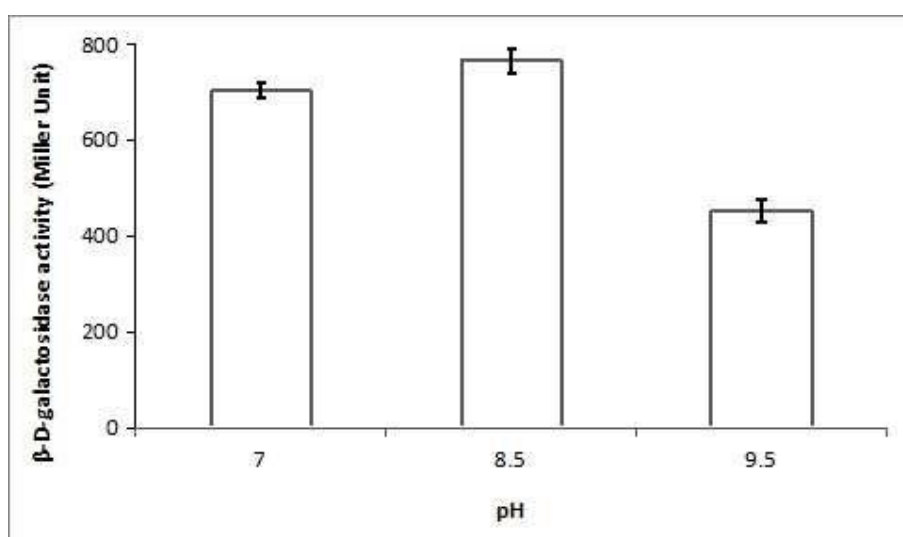
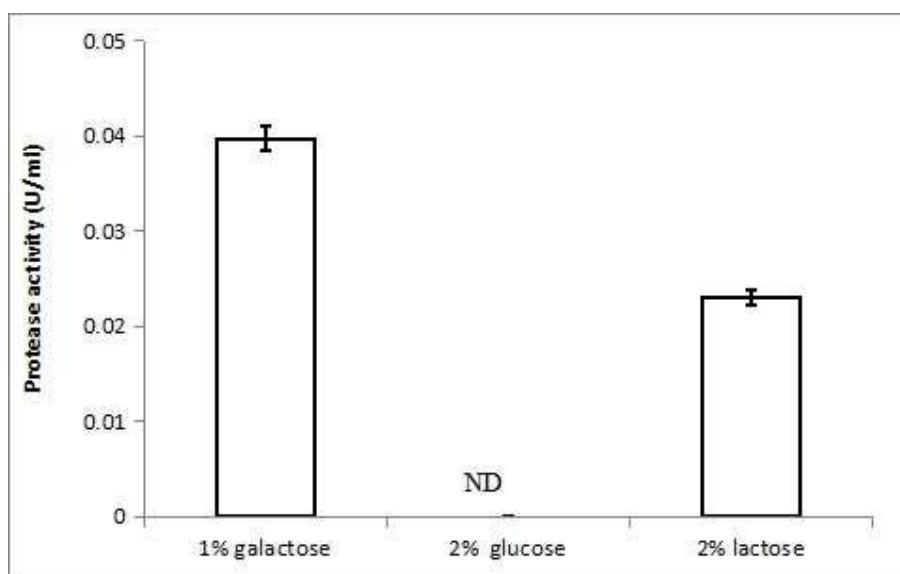


Fig.3.5 Effect of temperature (a) and pH (b) on β -D-galactosidase activity in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with 2% lactose and grown for 6 h. The cultures were then harvested and the bacterial cells permeabilized. Such permeabilized 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 $\pm$ SD.

(a)



(b)

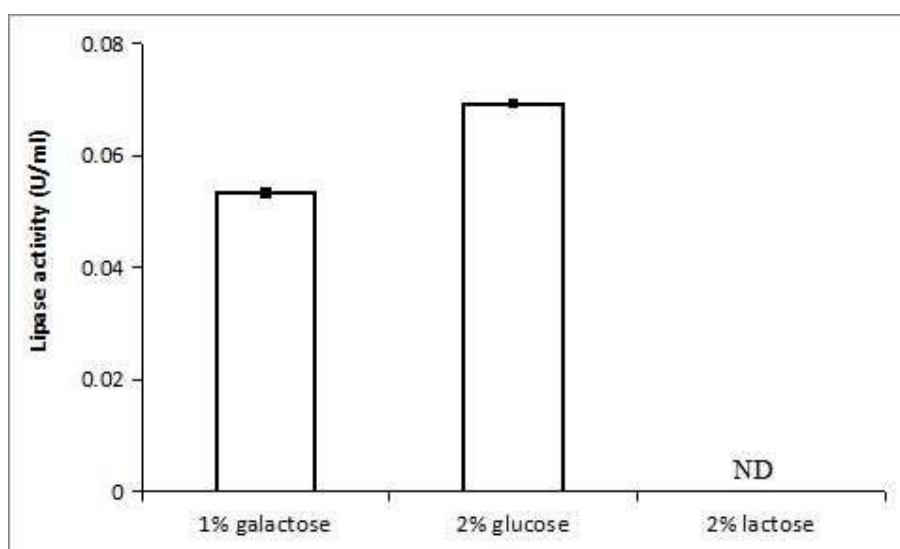


Fig.3.6 Effect of different carbon sources on protease (a) and lipase (b) activities in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with different carbon sources, as indicated, and grown for 36 h (stationary phase). Such cultures were then centrifuged 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 $\pm$ SD. The abbreviation —ND refers to non-detectable.

CHAPTER 4

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

4.1 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 (Su et al., 2020). 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 (Earl et al., 2008; Elshaghabee et al., 2017). The dairy wastewaters 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*, etc. (Liu et al., 2019; Loperena et al., 2009; Luan and Duan, 2022; Venkateswarulu et al., 2020). Their presence in dairy wastewaters 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 $\rightarrow$ 4)-glycosidic bond between glucose and galactose leading to release of these simple sugars for further assimilation (Lu et al., 2020). 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* (Luan and Duan, 2022; Venkateswarulu et al., 2020). The optimum pH and temperature requirements for this enzyme activity had also been reported to differ depending on the type of bacterial source (Luan and Duan, 2023; Yao et al., 2019). 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 wastewater whey (a by-

product of cheese manufacturing process and a strong environmental pollutant) (Liu et al., 2022; Luan and Duan, 2022). 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 (Loperena et al., 2009). 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) (Loperena et al., 2009). Such biochemical properties exhibited by *Bacillus* spp. makes them very useful candidates for exploitation in biodegradation of dairy wastewaters.

Therefore, the aim of the present study was to isolate *Bacillus* native to dairy industry wastewaters 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.

4.2 Materials and Methods

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

Wastewater from the MULCO Dairy industry (Aizawl, Mizoram) was collected in 50ml sterile tubes. For screening and isolation of *Bacillus* spp. (heat-resistant endospore 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 (Wakisaka and Koizumi, 1982). The heat-exposed waste water was then serially diluted 10^{-6} fold with sterile saline water (0.85 % sodium chloride) and 50 μ l of this diluted waste water was spread on Luria-Bertani (LB-medium amended with 1 mM X-gal as described in chapter 2 (section 2.2.2). A colony, which appeared blue in colour was picked and named as DW1. It was subjected to further purification by streaking on LB-plate. For pliminary identification, the bacterium was Gram-stained as described in chapter 2 (section 2.3).

4.2.2 Molecular identification of DW1

4.2.2.1 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 Russell (2001) as described in chapter 2 (section 2.7.1). 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 as described in chapter 2 (section 2.7.3).

4.2.2.2 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 (Janda and Sharon, 2007).

4.2.3 Analysis of antibiotics susceptibility pattern and carbohydrate utilization by DW1

The antibiotic susceptibility of DW1 was tested against 25 antibiotics using antibiotic containing discs and its ability to utilize different carbohydrates was analyzed using Hi25TM *Enterobacteriaceae* identification Kit (Strip-2, KB003-1KT, HIMEDIA) as described in chapter 2 (section 2.5.1, 2.5.2 and 2.5.3).

4.2.4 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 (OD₆₀₀) values were measured using a UV-Vis 1900i spectrophotometer.

4.2.5 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. The preparation of cells and enzyme assay were done as described in chapter 2 (sections 2.8.1, 2.8.2 and 2.8.3).

4.2.6 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 as described in chapter 2 (section 2.8.4).

4.2.7 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 0.45 μ syringe filter to remove any remaining bacterial cells. The extracellular protease and lipase activities were determined in such supernatants as described in chapter 2 (sections 2.9.2 and 2.9.3).

4.2.8 Statistical analysis

Statistical analysis was done as described in chapter 2 (section 2.11).

4.3 Results and discussion

4.3.1 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 wastewater (Aizawl, Mizoram). As shown in the Fig.4.1(a), the Gram-staining and microscopic observation of DW1 showed it to be gram-positive, rod-shaped, and endo-spore forming bacterium, characteristics of bacteria commonly found in the genera *Bacillus* (Harinisri et al., 2024). Genomic DNA isolation followed by PCR amplification of 16S rRNA gene from DW1 produced a 1.4 kb DNA fragment, as shown in Fig.4.1(b). The PCR amplified DNA fragment was then sequenced, subjected to BLAST, multiple sequence alignment and phylogenetic tree construction for its further identification (Coico, 2005; Sambrook and David, 2001; Janda and Sharon, 2007; Harinisri et al., 2024). 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.4.1c). 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.

4.3.2 Antibiotics susceptibility pattern and carbohydrate utilization potential of *B. paramycoides* DW1

Resistance to antibiotics is common in bacteria and poses serious health and environmental risks (Aslam et al., 2021). *B. paramycoides* DW1 was exposed to 25 different antibiotics in order to reveal its susceptibility pattern (Fig. 4.2 a-d). As shown in Table 4.1, *B. paramycoides* DW1 was sensitive to all the antibiotics tested in this study, proving its worth as a safe bacterium, as it may not be able to contribute to spread of antibiotic resistance to other bacteria in the environment

A previous study has suggested that *B. paramycoides* is a potent degrader of various carbohydrates (Rashid et al., 2021). 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 4.2, *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. Such differential pattern of carbohydrate utilization in bacteria has also been revealed earlier (Rashid et al., 2021).

4.3.3 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.4.3, *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 wastewater (Lu et al., 2020).

4.3.4 β -D-galactosidase activity in *B. paramycoides* DW1

β -D-galactosidase is an intracellular enzyme produced by certain microorganisms, including *Bacillus* spp. (Duan and Luan, 2023; Liu et al., 2019; Lu et al., 2020; Luan and Duan, 2022; Venkateswarulu et al., 2020;). 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.4.4, 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-galactosidase producing bacteria, lactose induces β -D-galactosidase activity in *B. paramycoides* DW1 and the enzyme is inhibited by reaction products glucose and galactose (Duan and Luan, 2023; Kolev et al., 2022; Liu et al., 2019; Luan and Duan, 2022; Rashid et al., 2021; Venkateswarulu et al., 2020). 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 (Liu et al., 2019).

4.3.5 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. 4.5(a), the β -D-galactosidase activity in *B. paramycoides* DW1 varied 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 (Duan and Luan, 2023; Liu et al., 2019; Luan and Duan, 2022; Yao et al., 2019). 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. 4.5(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* DW1 is optimum at neutral pH, and is sensitive to alkaline and acidic pH (Luan and Duan, 2022; Liu et al., 2019).

4.3.6 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 (Mp and Manjunath, 2011; Rajashekhar et al., 2017, Sangeetha et al., 2010). 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.4.6, 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 (Mp and Manjunath, 2011; Sangeetha et al., 2010). 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 type of carbohydrates, as observed for protease activity. Nevertheless, the potential to produce diverse hydrolytic enzymes possibly enables the sustenance of *B. paramycoides* DW1 in dairy wastewater.

Overall, the results presented in this study described the isolation of a bacterial strain DW1 from dairy wastewater and its identification as *B. paramycoides* based on 16S rRNA gene sequence. Importantly, *B. paramycoides* DW1 was sensitive to all the antibiotics tested. Further 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. Furthermore, 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 may imply the utility of *B. paramycoides* DW1 in treatment of dairy waste water.

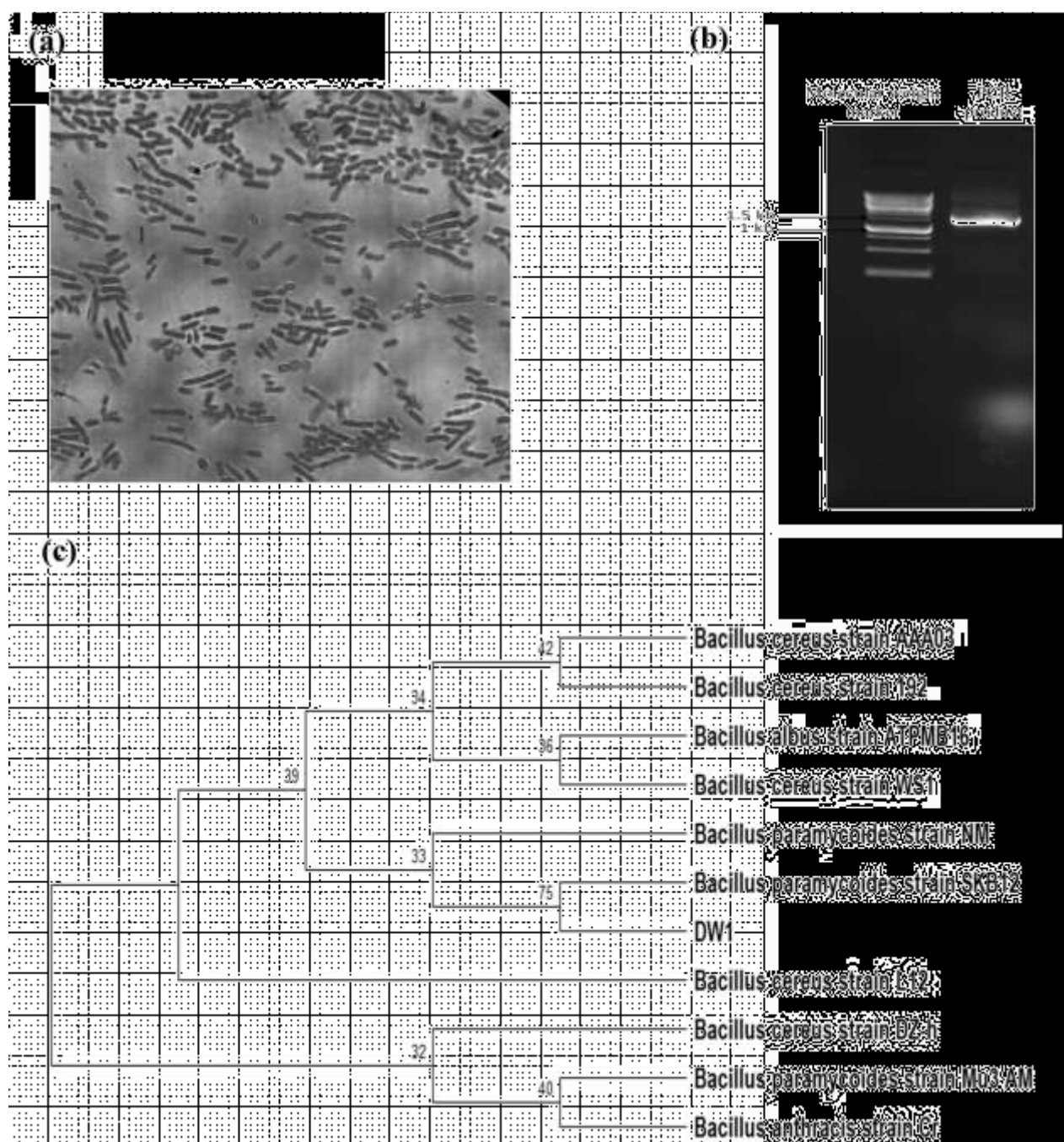


Fig.4.1 Morphological and molecular identification of dairy waste water bacterial isolate DW1. Gram-stained DW1 (a), PCR amplified 16S rRNA gene product of DW1 on 0.8 % agarose gel (b), and 16S rRNA gene-based phylogeny of DW1 with similar sequences from other bacterial species (c).

(a)



(b)



(c)



(d)



Fig.4.2 Effect of different antibiotics on survival of *B. paramycoides* DW1. An exponentially growing culture of *B. paramycoides* DW1 equivalent to OD₆₀₀ of 0.1 was used to spread plates (a-d) followed by placing the antibiotic-containing discs over the cultures to assess susceptibility pattern.

Table 4.1 Antibiotics susceptibility pattern of *B. paramycoides* DW1 scored as resistant (R) or sensitive (S).

Antibiotics	Result
Penicillin G (P 10)	S
Polymixin B (PB 300)	S
Neomycin (N 30)	S
Bacitracin (B 10)	S
Chloramphenicol (C 30)	S
Gentamicin (GEN 10)	S
Amikacin (AK 30)	S
Moxifloxin (MO 5)	S
Cefoparazone (CPZ 75)	S
Cefpodoxime (CPD 30)	S
Ceftazidime (CAZ 30)	S
Cefepime (CPM 30)	S
Imipenem (IPM 10)	S
Ofloxacin (OF 5)	S
Co-trimoxazole (COT 25)	S
Cefotaxime (CTX 30)	S
Augmentin (AMC 30)	S
Erythromycin (E 10)	S
Gatifloxin (GAT 5)	S
Teicoplanin (TEI 30)	S
Azithromycin (AZM 15)	S
Vancomycin (VA 30)	S
Doxicyclin (DO 30)	S
Ciprofloxacin (CIP 5)	S
Sparfloxacin (SPX 5)	S

Table 4.2 Carbohydrate utilization potential of *B. paramycoides* DW1.

Sr. 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	+

An exponentially growing culture of *B. paramyoides* DW1 equivalent to OD₆₀₀ 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.

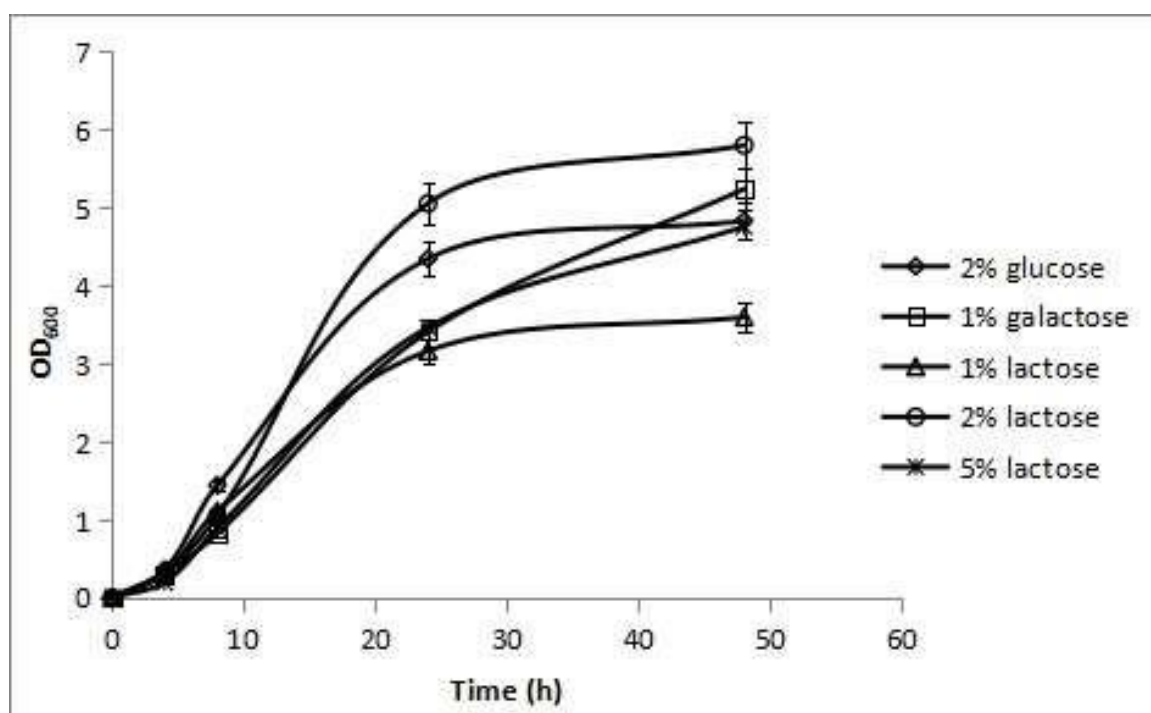


Fig.4.3 Effect of different carbon sources on the growth of *B.paramyoides* DW1. Exponentially grown culture of *B. paramyoides* DW1 was inoculated in MRS medium amended with different carbon sources, as indicated, and growth was measured periodically by determining OD₆₀₀ of cultures. The experiments were done in duplicate and results are presented as mean±SD.

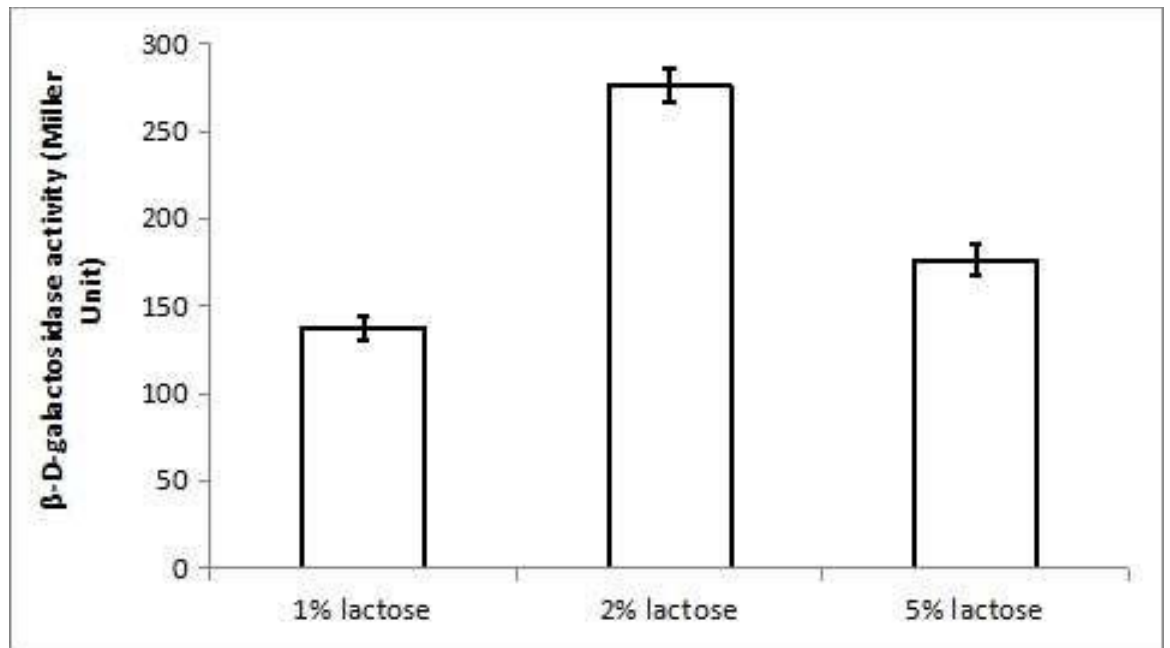
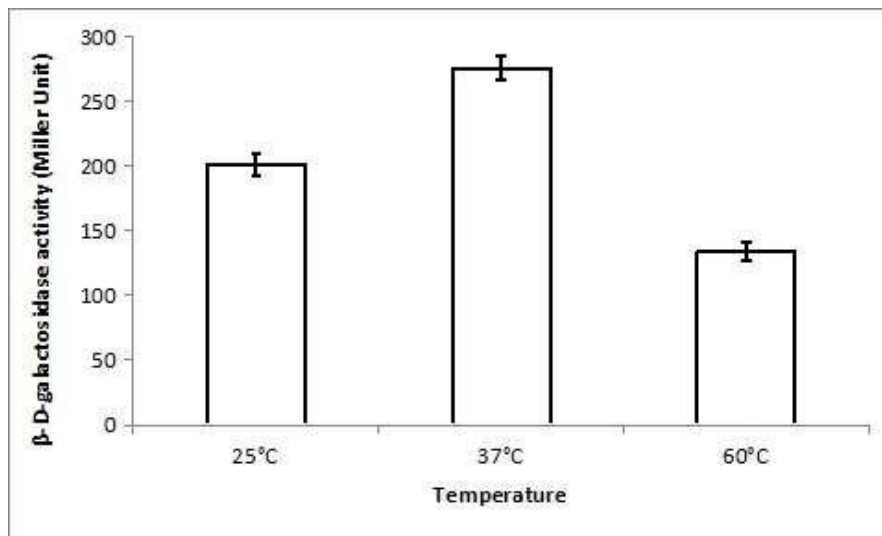


Fig.4.4 Effect of different lactose concentrations on β -D-galactosidase activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with different concentrations of lactose, as indicated, and grown for 6 h. The cultures were then harvested and permeabilized cells were used in the activity assay. The experiments were done in duplicate and the results are presented as mean $\pm$ SD.

(a)



(b)

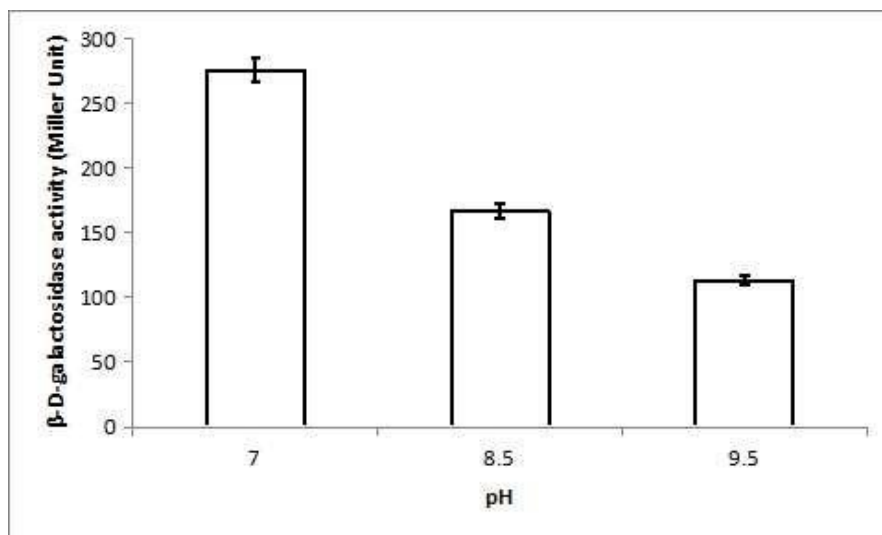


Fig.4.5 Effect of temperature (a) and pH (b) on β -D-galactosidase activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with 2% lactose and grown for 6 h. The cultures were then harvested and the bacterial cells permeabilized. Such 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 $\pm$ SD.

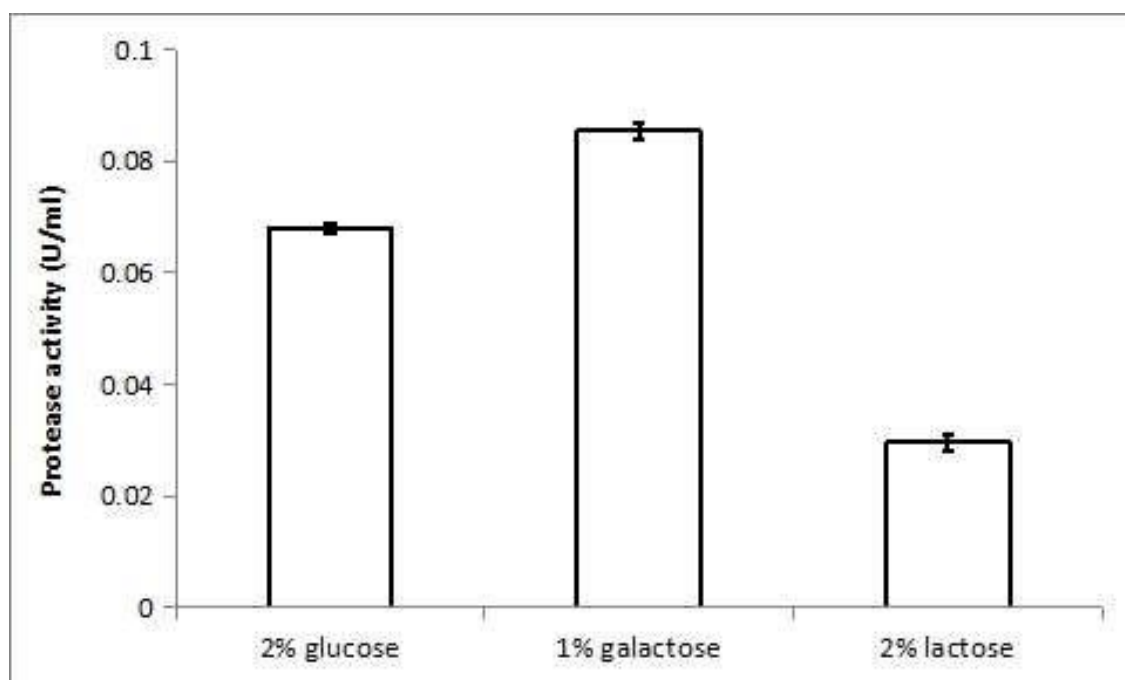


Fig.4.6 Effect of different carbon sources on protease activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with different carbon sources, as indicated, and grown for 36 h (stationary phase). Such cultures were then centrifuged 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.

CHAPTER 5

Biodegradation of waste milk by *Bacillus paramycoides* DW1, *Enterococcus faecium* PB1, and their mixed cultures

5.1 Introduction

Milk-processing dairy industries are one among the most polluting food industries. The volume of wastewater generated by such industries depends on their milk-processing capacity, and can vary from 0.2-10 L with each L of milk processed (Yan and Holden, 2019). Owing to their high content of biodegradable sugars (mainly lactose), proteins (casein), milk fats, and inorganics, the dairy industry waste water is considered a serious and high strength pollutant and should be treated efficiently before disposal (Farizoglu and Uzuner, 2011) . Various physico-chemical and biological treatment methods and/or their combination are generally applied to treat dairy wastewaters. The physico-chemical methods include coagulation-flocculation, membrane adsorption, electrochemical treatment etc., which are costly and when applied alone and not in conjunction with other methods achieve low treatment efficiency (de Oliveira Nunes et al., 2021; Hashim et al., 2025). In comparison, biological treatment method is regarded more cost-effective and efficient, and is in wide practice (Sar et al., 2022).

Microbes are at the core of biological treatment of dairy wastewater due to their ability to metabolize a wide range of substrates, fast growth, and tolerance to harsh environmental conditions. However, the ability to rapidly biodegrade different types of organic waste varies considerably across microbial species and strains (Al-Wasify et al., 2017; Joshiba et al., 2019). Therefore, many previous studies emphasize the need for proper selection of microbes with high efficiency to biodegrade organics present in dairy wastewaters (Loperna et al., 2009; Velmurugan and Pandian, 2024). It has been demonstrated that microbes, particularly bacteria with potential to produce lactose-hydrolyzing intracellular enzyme β -D-galactosidase, protein- and lipid-hydrolyzing extracellular protease and lipase, respectively, may be more competent to biodegrade dairy wastewaters (Maghsoudi et al., 2007). Additional

emphasis has also been placed on the habitats from which such bacteria are isolated, their pathogenic potential, and resistance to clinical antibiotics. Indeed, the bacterial isolates from dairy wastewater have been shown to be more efficient in treatment of milk, compositionally similar precursor to dairy wastewater (Loperna et al., 2009). Furthermore, a number of probiotic bacteria have also been evaluated and recommended for use in dairy wastewater treatment, not only due to their efficiency, but also due to their safety status of not being pathogenic, sensitivity to clinical antibiotics, which reduces the scope of antibiotic resistance spread to other co-inhabiting microbes.

The aim of the present study was to evaluate the potential of the bacterial strains *Bacillus paramycoides* DW1 (a dairy wastewater isolate), *Enterococcus faecium* PB1 (a probiotic bacterium), and their mixed cultures to treat waste milk, organic and inorganic composition of which is similar to dairy wastewaters. Such bacterial strains have earlier been shown to exhibit lactose, protein, and lipid biodegradation activities in synthetic-medium (chapters 3 and 4). Thus, they are also expected to biodegrade waste milk. The biodegradation potential were measured in terms of their ability to reduce total and soluble chemical oxygen demand (tCOD and sCOD), total solids (TS), volatile solids (VS), carbohydrate and protein levels, pH, and alkalinity, standard water quality parameters in waste milk before and after treatment with such strains.

5.2 Materials and methods

5.2.1 Collection of waste milk

The milk samples or waste milk (milk or its residues remaining in milk supply bottles) were collected in sterile bottles from households in Mizoram University campus. After collection, such milk samples were brought to the laboratory and stored at 4 °C till enough volume of milk was obtained for experiments. The total chemical oxygen demand (tCOD) of the accumulated waste milk was determined immediately after thawing to room temperature. Such samples approximately diluted to 1500 mg/L tCOD concentrations were used as substrate in biodegradation studies.

5.2.2 Experimental set-up for waste milk biodegradation

The waste milk biodegradation studies were conducted in batch mode using six sterile serum bottles. The total and working volume of such serum bottles was 0.5 L and 0.4 L, respectively. To start with, separate bottles in duplicate were inoculated with *Bacillus paramycoides* DW1, *Enterococcus faecium* PB1, and their mixed cultures (10% of working volume), except in the control. Then the remaining working volume was filled with waste milk (same tCOD as described above) in the test bottles. The control bottles were filled only with 0.4 L of waste milk. All the bottles were incubated for 10 days at 35 ± 1 °C.

5.2.3 Preparation of microbial inocula

Bacillus paramycoides DW1 and *Enterococcus faecium* PB1 were grown to mid-logarithmic phase in LB-broth, washed with sterile Milli-Q water, and individual bacterial cultures equivalent to 0.1 OD₆₀₀ were used to seed serum bottles. For mixed-culture biodegradation test, both the bacterial cultures each of 0.1 OD₆₀₀ were mixed in 1:1 proportion for use as microbial inoculum.

5.2.4 Analytical methods

The waste milk was evaluated for standard water quality parameters, such as tCOD, sCOD, TS, VS, turbidity, pH, alkalinity, and concentrations of carbohydrates and proteins before and after treatment, as described in chapter 2.

5.2.5 Statistical analysis

The experiments were performed in duplicate and the results are presented as mean $\pm$ standard deviation.

5.3 Results and discussion

5.3.1 Changes in tCOD and sCOD of waste milk after microbial treatment

The tCOD concentration of wastewaters is a reflection of their quality; higher the tCOD concentration, poor is the effluent quality (Porwal et al., 2015). As shown in

Table 1, incubation of waste milk (collected from households) with *Bacillus paramycoides* DW1, *Enterococcus faecium* PB1, and their mixed cultures reduced the tCOD concentrations substantially from the initial value. The highest reduction in tCOD concentration of waste milk was achieved with mixed cultures (63%) followed by that with *Enterococcus faecium* PB1 (60%), and the lowest reduction was achieved with *Bacillus paramycoides* DW1 (57%). These results suggest that individually, as well as when mixed, isolated bacterial strains have the potential to reduce the load of pollutants in waste milk. The tCOD reduction efficiency of the bacterial cultures is consistent with dairy wastewater biodegradation studies using mono- or mixed-bacterial cultures (Porwal et al., 2015; Al-Wasify et al., 2017). Like tCOD, sCOD also showed a similar pattern, except that the sCOD reduction efficiencies were higher. The highest reduction in sCOD concentration of waste milk was achieved with mixed culture (77%) followed by that with *Enterococcus faecium* PB1 (75%), and the lowest reduction was achieved with *Bacillus paramycoides* DW1 (71%). These results suggest that the bacterial cultures were able to solubilize or hydrolyze organic matter present in waste milk for thie further consumption.

5.3.2 Changes in turbidity, TS and VS content in waste milk after microbial treatment

The turbidity, TS and VS content of wastewaters is often considered as indicators of its polluting strength (Porwal et al., 2015). As shown in Table 1, *Bacillus paramycoides* DW1, *Enterococcus faecium* PB1, and their mixed cultures reduced the turbidity in waste milk and the percentage reduction values ranged from 6-42% after treatment; *Bacillus paramycoides* DW1 produced the lowest reduction in turbidity in treated waste milk. Furthermore, gravimetric studies revealed that *Bacillus paramycoides* DW1, *Enterococcus faecium* PB1, and their mixed cultures reduced the TS content in waste milk and the reduction percentage values ranged from 67-75 after treatment. The VS content was also reduced by the bacterial cultures in waste milk by 73-86%. Thus, except turbidity, which showed lower reduction percentage particularly in the case of *Bacillus paramycoides* DW1, TS and

VS reduction was pretty efficient, in consonance with the observed reduction in tCOD and sCOD following waste milk treatment.

5.3.3 Changes in the concentrations of carbohydrate and protein in waste milk after microbial treatment

The carbohydrates, proteins, and lipids are the major constituents of dairy wastewater or milk from which the waste water gets generated after processing (Kolhe and Pawar, 2011). Lactose (carbohydrate) and casein (protein) are the major organics present in milk or its wastewater. The intracellular enzyme β -D-galactosidase hydrolyzes lactose and extracellular protease hydrolyzes casein. Both the enzyme activities were detected in *Enterococcus faecium* PB1 and *Bacillus paramycoides* DW1. Notably, the β -D-galactosidase activity was approximately 3-fold higher in the former bacterium than that in the later in standard MRS growth medium (compare chapter 3 and 4). As shown in Table 1, *Bacillus paramycoides* DW1, *Enterococcus faecium* PB1, and their mixed cultures reduced the carbohydrate concentration considerably in waste milk and the percentage reduction values ranged from 80-92% after treatment. Similarly, the bacterial cultures also reduced the concentration of proteins in waste milk by 44-63%. The lower reduction observed in protein content compared to carbohydrate content after treatment may suggest that the bacterial cultures prefer to utilize carbohydrate first and then protein. It is likely that given more contact time with waste milk, such cultures may hydrolyze and consume remaining proteins. These results clearly suggest that lactose and casein hydrolytic bacteria isolated during this study are indeed very effective in reducing the organic load in waste milk.

5.3.4 Changes in pH and alkalinity in waste milk after microbial treatment

pH is a critical factor that determines the growth and metabolic activities of bacteria. As waste milk degradation may result in decrease in pH due to production of a variety of acids, such as lactic acid, the pH values were determined in the wasted milk samples before and after treatment. As shown in Table 1, *Enterococcus faecium* PB1 reduced pH in waste milk to 6.08 from its initial value of 6.71. On the other

hand, the pH value was further reduced to 5.85 and 5.26 after treatment of waste milk by *Bacillus paramycoides* DW1 and the mixed cultures, respectively. These results suggest that the bacterial cultures reduce the pH while biodegrading waste milk possibly by producing metabolic acids and the resulting acidic pH may interfere with both growth and biodegradation efficiency. The increase in acidity can be countered by bases, such as bicarbonate and/or ammonium/ammonia, product of microbial metabolic activities. Alkalinity levels in waste milk increased after treatment with *Enterococcus faecium* PB1 from 24 mg/L (before treatment) to 52 mg/L. Similarly, after treatment of waste milk by *Bacillus paramycoides* DW1 and the mixed cultures, the alkalinity levels increased to 66 and 68 mg/L, respectively. The results show that the bacterial cultures possess the potential to counter acidity by increasing the alkalinity levels during milk waste biodegradation. However, such an increase in alkalinity levels may not be sufficient to raise the pH to neutral (pH 7.0) or near neutral level sufficient to prevent the adverse effects of acid. These findings are consistent with acidic pH being one of the constraining factors for achieving higher biodegradation efficiency during microbial treatment of wastewaters, and can possibly be tackled by addition of common bases like sodium/potassium or calcium hydroxides.

Overall, the findings suggest that the probiotic bacterium *Enterococcus faecium* PB1 and dairy waste isolate *Bacillus paramycoides* DW1 are both suitable for waste milk biodegradation and pollutant load reduction implying that such bacteria may also be highly useful for treating real dairy waste waters when applied either in the form of a consortium or even individually as bioaugmentative agents.

Table 5.1 Characteristics of waste milk before and after treatment with different bacterial isolates.

Parameters	Before treatment	After treatment		
		<i>E. faecium</i> PB1	<i>B. paramycoides</i> DW1	Mixed culture
tCOD (mg/L)	1564 ± 30	631.5±9.19	673.5±2.12	584±2.83
sCOD (mg/L)	1512 ± 21.17	378±12.85	430±14.62	344±11.67
Turbidity (NTU)	641 ± 30.5	394±18.5	602±30	370±17.5
TS (g/L)	1.8 ± 0.063	0.45±0.016	0.6±0.021	0.45±.015
VS (g/L)	1.45 ± 0.05	0.2±0.007	0.4±0.014	0.2±0.007
Carbohydrate (mg/L)	273.25 ± 0.61	47.57 ± 0.32	21.86 ± 0.32	54.238±0.16
Protein (mg/L)	174.42 ± 0.57	96.52 ± 0.34	63.79 ± 0.32	88.91 ± 0.28
pH	6.7 ± 0.34	6.08 ± 0.3	5.85 ± 0.29	5.26 ± 0.26
Alkalinity (mg/L)	24 ± 0.08	51.6 ± 1.29	66.2 ± 1.62	67.7 ± 1.7

The treatments were conducted at 37°C for 10 days and the reported values are mean±SD from duplicate experiments.

CHAPTER 6

Summary

The dairy wastewaters are high strength pollutants with severe implications for human and environmental health when released without proper treatment. Currently, biological treatment is considered one of the most effective approaches to reduce the polluting potential of dairy wastewaters. The milk-originated lactose (carbohydrate), casein (protein), and fats are the major organic pollutants present in dairy wastewaters. A variety of microbes, including bacteria have the potential to break down such organics into simpler compounds which can be readily assimilated by them. It is an established fact that such a potential to utilize organics can vary across different bacterial species. Therefore, effective treatment of dairy wastewaters must involve efficient organics biodegrading bacteria. The purpose of this study was to isolate such bacteria from commercial probiotic (safe for environmental applications) and dairy wastewater and characterize them with regard to their ability to utilize dairy wastewater relevant organics and waste milk for their prospective application in real dairy waste water treatment system.

The bacterial isolate from a commercial probiotic was identified as *Enterococcus faecium* PB1 (hereafter *E. faecium* PB1), a gram-positive, round-shaped lactic acid bacterium. *E. faecium* PB1 was evaluated for its potential to utilize the complex disaccharide lactose as source of carbon for growth, and its ability 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% in MRS-medium. 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 (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.

Another bacterium isolated during this study from dairy wastewater was *Bacillus paramycoides* DW1 (hereafter *B. paramycoides* DW1), a gram-positive, rod-shaped, endo-spore forming bacterium. As in *E. faecium* PB1, this bacterium was also assessed for its ability to hydrolyze lactose, proteins, and lipids (major organic constituents of dairy wastewater), and its potential to grow using lactose, glucose, and galactose as sources of carbon, and 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.

Given such abilities of the isolated bacterium *E. faecium* PB1 and *B. paramycoides* DW1, an attempt was made to evaluate their potential to biodegrade waste milk and reduce its pollution strength when applied individually or as mixed cultures. The results suggest that the bacterial isolates are indeed capable of substantially reducing tCOD, sCOD, turbidity, TS, and VS (standard parameters used to assess the quality

of waste water) content in waste milk. Consistent with this ability, the bacterial isolates also reduced the concentrations of carbohydrate and protein in waste milk though with slightly different efficiencies. However, increase in acidity and insufficient alkalinity observed during the waste milk treatment may be some of the factors of concern in achieving better treatment efficiency warranting further investigations into remedial measures.

Overall, the findings reveal that the potential to hydrolyze lactose, protein, and lipid probably enables the probiotic bacterium *E. faecium* PB1 and dairy waste water isolate *B. paramycoides* DW1 to grow and survive in waste milk and these highly useful bacterial isolates may be exploited in future for treatment of real waste waters discharged from dairy industries to reduce their organic pollutant load.

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Mizoram Science congress	Biochemical characterization of β - galactosidase activity from <i>Streptococcus faecalis</i>	MISTIC and MSS, MAS, STAM, GSM, MMS, BIOCONC and MITS	November 24 th -25 th 2022

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1. **Jugami Boro.**, Mintu Medhi., Asahan Ali., Nchebui Zeliang., Tovikali Zhimo., Jyotirmoy Bhattacharya*. (2025). Biochemical characterization of *Bacillus paramycoides* DW1, a bacterial isolate from dairy waste water. *Science Vision*, Iss 1-3, p. 26-33
2. **Jugami Boro.**, Mintu Medhi., Asahan Ali., Tovikali Zhimo., Jyotirmoy Bhattacharya*. (2025). Evaluation of growth and hydrolytic enzyme activities in *Enterococcus faecium* PB1, a probiotic bacterium with potential for application in dairy waste water treatment. *Science Vision*, Iss 1-3, p. 10-18

Declaration: I hereby declare that all the above information is true to the best of my knowledge and belief.

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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.

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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

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 $\rightarrow$ 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 μ L 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 David¹⁸. 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 μ L reaction volume comprising of 15 μ L of PCR master mix, 0.6 μ L of forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 0.6 μ L 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 OD₆₀₀ 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 OD₆₀₀ 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 OD₆₀₀ measurement against a blank (Z buffer). Appropriately diluted cells of known OD₆₀₀ 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-Enyard²¹ 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

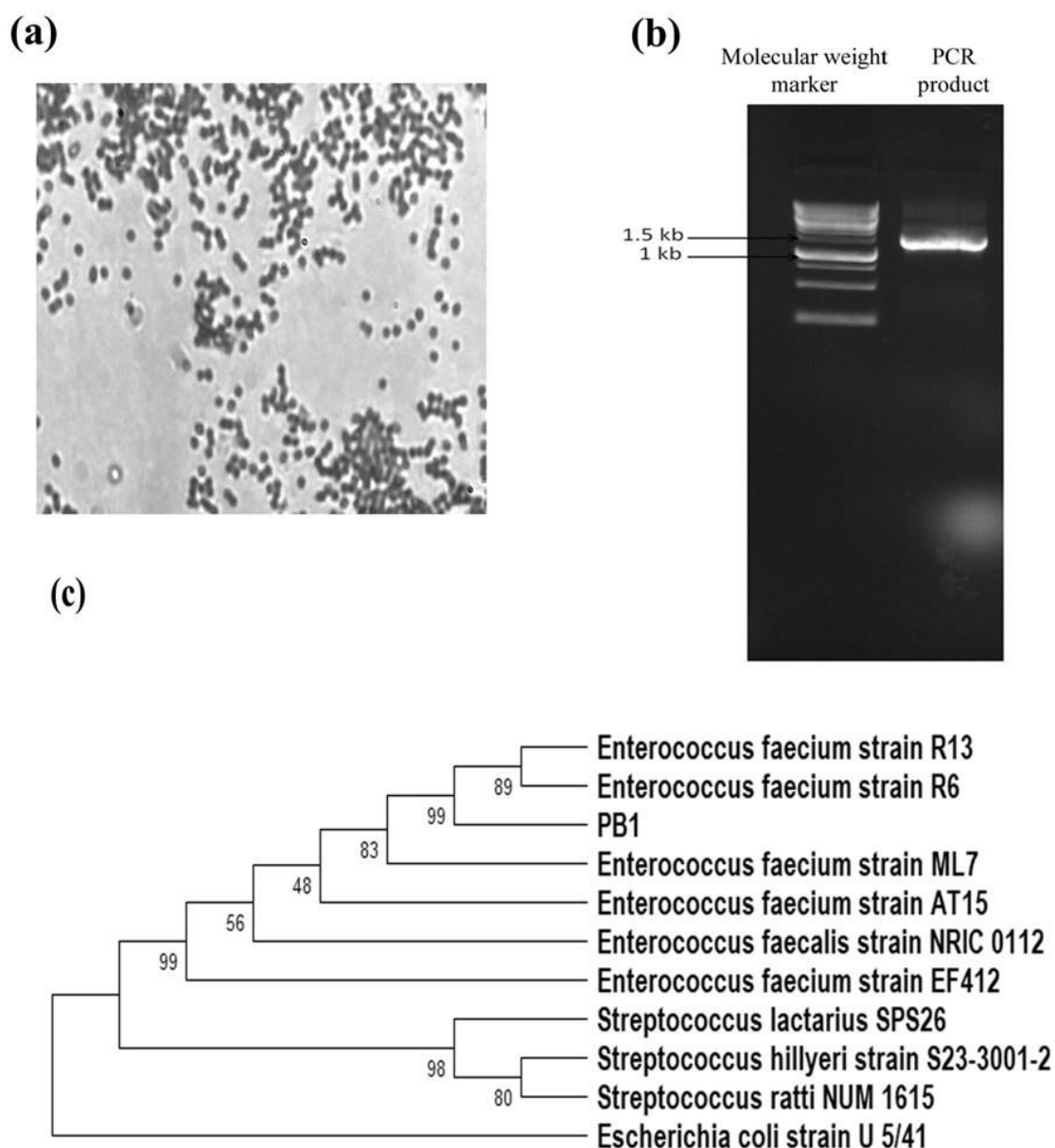


Figure 1. Morphological and molecular identification of a probiotic bacterium PB1. Gram-stained PB1 (a), PCR amplified 16S rRNA gene product of PB1 on 0.8 % agarose gel (b), and 16S rRNA gene-based phylogeny of PB1 with similar sequences from other bacterial species (c). Different species of *Streptococcus* 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 OD₆₀₀ 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.

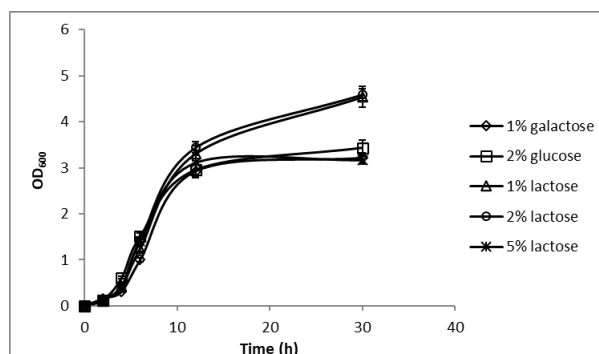


Figure 2. Effect of different carbon sources on the growth of *E. faecium* PB1. Exponentially grown culture of *E. faecium* PB1 was inoculated in MRS medium amended with different carbon sources, as indicated, and growth was measured periodically by determining OD₆₀₀ of cultures. The experiments were done in duplicate and results are presented as mean $\pm$ 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

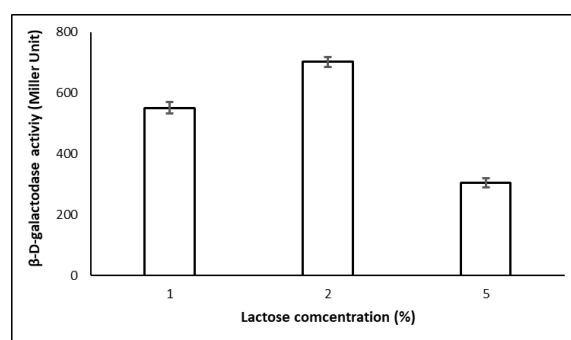


Figure 3. Effect of different lactose concentrations on β -D-galactosidase activity in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with different concentrations of lactose, as indicated, and grown for 6 h. The cultures were then harvested and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

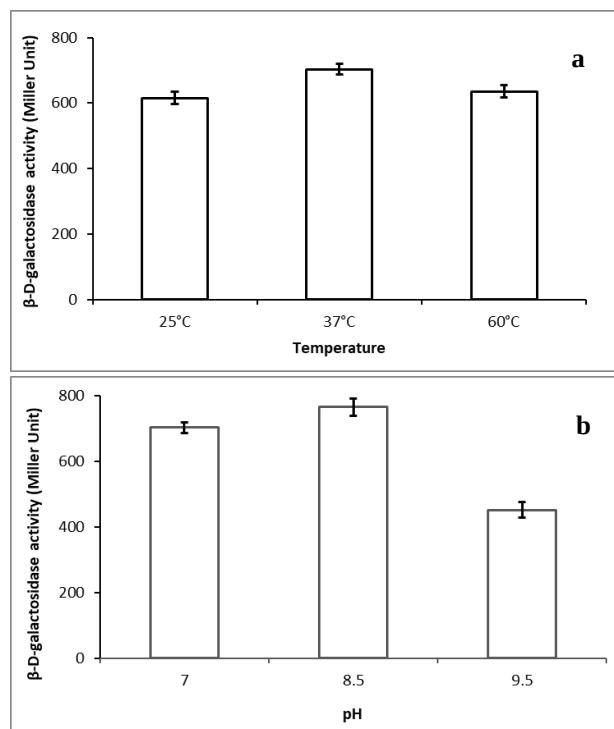


Figure 4. Effect of temperature (a) and pH (b) on β -D-galactosidase activity in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with 2% lactose and grown for 6 h. The cultures were then harvested and the bacterial cells permeabilized. Such permeabilized 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 $\pm$ 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

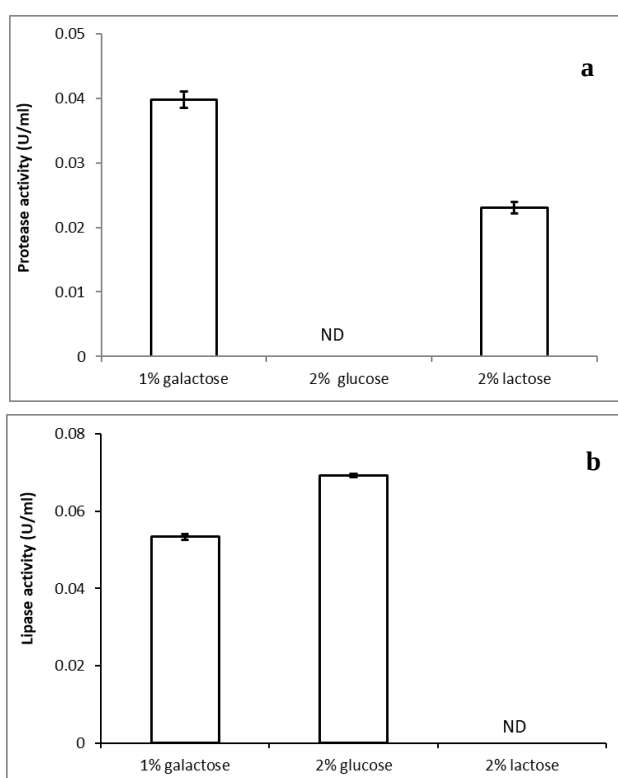


Figure 5. Effect of different carbon sources on protease (a) and lipase (b) activities in *E. faecium* PB1. Exponentially grown cultures of *E. faecium* PB1 was inoculated in MRS medium amended with different carbon sources, as indicated, and grown for 36 h (stationary phase). Such cultures were then 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 $\pm$ SD. The abbreviation “ND” refers to non-detectable.

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

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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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.

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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*,

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 $\rightarrow$ 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 μ l 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-galactosidase-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 David¹⁴. 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 μ l reaction volume comprising of 15 μ l of PCR master mix, 0.6 μ l of forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 0.6 μ l 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 (OD₆₀₀ 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 (OD₆₀₀) 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 OD₆₀₀ measurement against a blank (Z buffer). Appropriately diluted 1 ml cells of known OD₆₀₀ 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-Enyard¹⁷ 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

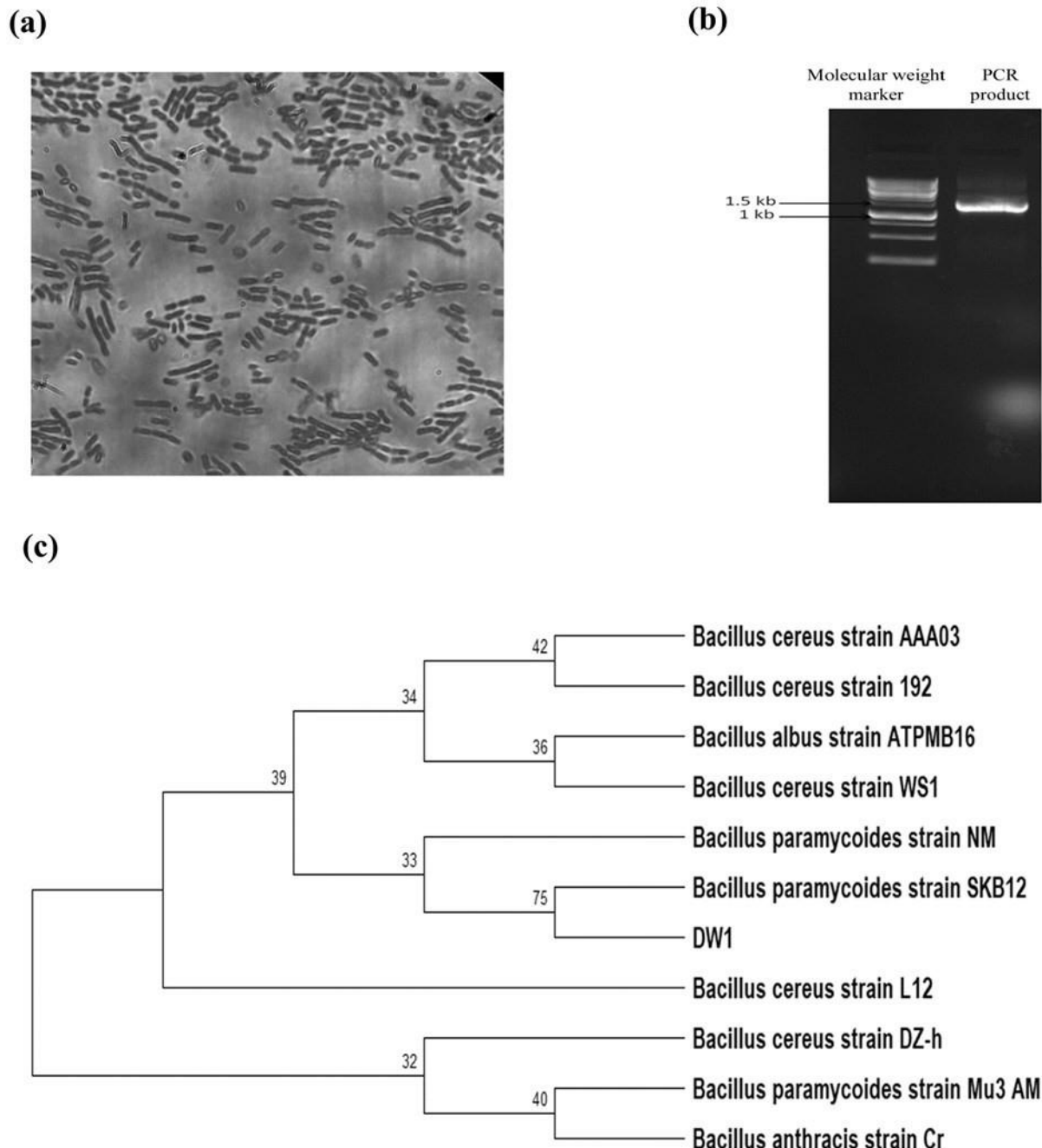


Figure 1. Morphological and molecular identification of dairy waste water bacterial isolate DW1. Gram-stained DW1 (a), PCR amplified 16S rRNA gene product of DW1 on 0.8 % agarose gel (b), and 16S rRNA gene-based phylogeny of DW1 with similar sequences from other bacterial species (c).

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 OD₆₀₀ 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

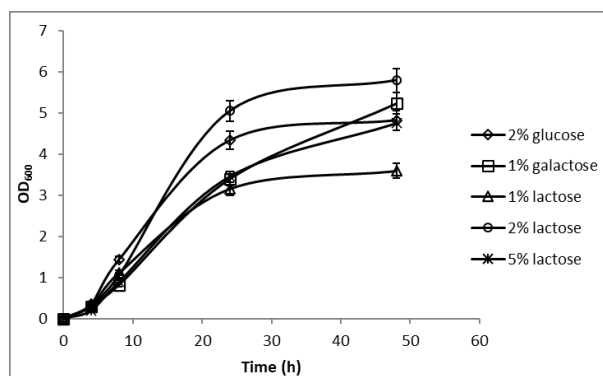


Figure 2. Effect of different carbon sources on the growth of *B. paramycoides* DW1. Exponentially grown culture of *B. paramycoides* DW1 was inoculated in MRS medium amended with different carbon sources, as indicated, and growth was measured periodically by determining OD₆₀₀ of cultures. The experiments were done in duplicate and results are presented as mean $\pm$ SD.

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

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-galactosidase producing bacteria, lactose induces β -D-galactosidase 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

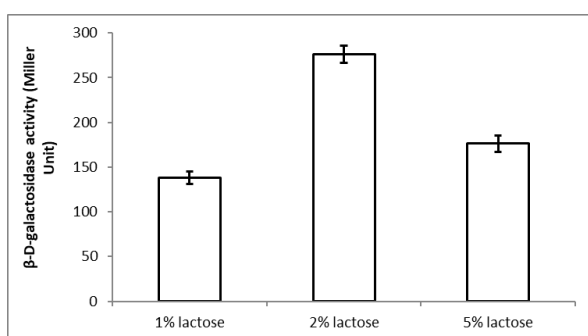


Figure 3. Effect of different lactose concentrations on β -D-galactosidase activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with different concentrations of lactose, as indicated, and grown for 6 h. The cultures were then harvested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

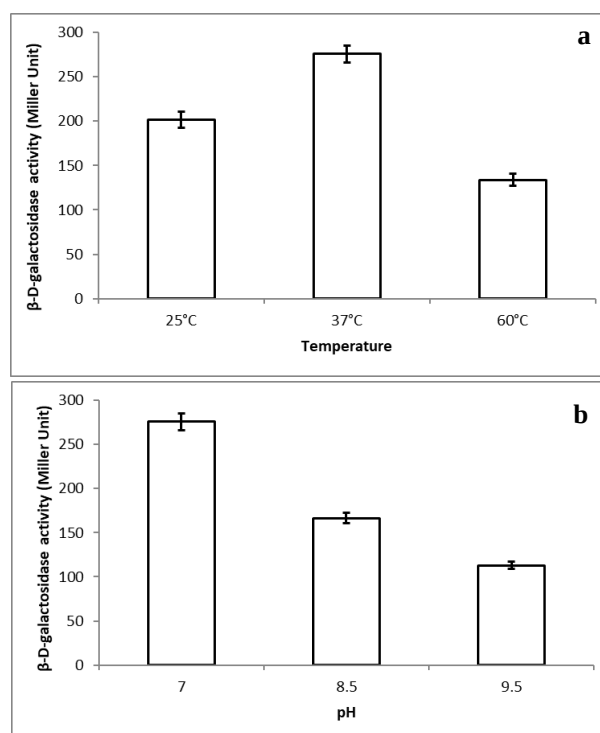


Figure 4. Effect of temperature (a) and pH (b) on β -D-galactosidase activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with 2% lactose and grown for 6 h. The cultures were then harvested and the bacterial cells permeabilized.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

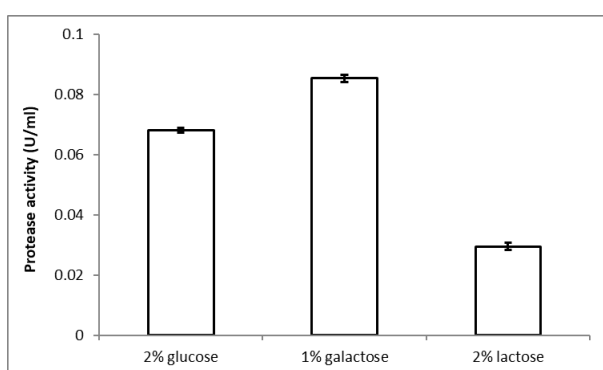


Figure 5. Effect of different carbon sources on protease activity in *B. paramycoides* DW1. Exponentially grown cultures of *B. paramycoides* DW1 was inoculated in MRS medium amended with different carbon sources, as indicated, and grown for 36 h (stationary phase). Such cultures were then centrifuged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

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.

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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DEPARTMENT	: Biotechnology
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ABSTRACT

BACTERIAL ISOLATES FROM COMMERCIAL PROBIOTICS AND DAIRY EFFLUENT: MOLECULAR IDENTIFICATION, ASSESSMENT OF HYDROLYTIC ACTIVITIES AND POTENTIAL APPLICATION IN WASTEWATER TREATMENT

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Bacterial isolates from commercial probiotics and dairy effluent: molecular identification, assessment of hydrolytic activities and potential application in wastewater treatment

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ABSTRACT

The dairy waste waters are high strength pollutants with severe implications for human and environmental health when released without proper treatment. Currently, biological treatment is considered one of the most effective approaches to reduce the polluting potential of dairy waste waters. The milk-originated lactose (carbohydrate), casein (protein), and fats are the major organic pollutants present in dairy waste waters. A variety of microbes, including bacteria have the potential to break down such organics into simpler compounds which can be readily assimilated by them. It is an established fact that such a potential to utilize organics can vary across different bacterial species. Therefore, effective treatment of dairy waste waters must involve efficient organics biodegrading bacteria. The purpose of this study was to isolate such bacteria from commercial probiotic (safe for environmental applications) and dairy waste water and characterize them with regard to their ability to utilize dairy waste water relevant organics and waste milk for their prospective application in real dairy waste water treatment system.

The bacterial isolate from a commercial probiotic was identified as *Enterococcus faecium* PB1 (hereafter *E. faecium* PB1), a gram-positive, round-shaped lactic acid bacterium. *E. faecium* PB1 was evaluated for its potential to utilize the complex disaccharide lactose as source of carbon for growth, and its ability 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% in MRS-medium. 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 (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.

Another bacterium isolated during this study from dairy waste water was *Bacillus paramycoides* DW1 (hereafter *B. paramycoides* DW1), a gram-positive, rod-shaped, endo-spore forming bacterium. As in *E. faecium* PB1, this bacterium was also assessed for its ability 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 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.

Given such abilities of the isolated bacterium *E. faecium* PB1 and *B. paramycoides* DW1, an attempt was made to evaluate their potential to biodegrade waste milk and reduce its pollution strength when applied individually or as mixed cultures. The results suggest that the bacterial isolates are indeed capable of substantially reducing tCOD, sCOD, turbidity, TS, and VS (standard parameters used to assess the quality of waste water) content in waste milk. Consistent with this ability, the bacterial

isolates also reduced the concentrations of carbohydrate and protein in waste milk though with slightly different efficiencies. However, increase in acidity and insufficient alkalinity observed during the waste milk treatment may be some of the factors of concern in achieving better treatment efficiency warranting further investigations into remedial measures.

Overall, the findings reveal that the potential to hydrolyze lactose, protein, and lipid probably enables the probiotic bacterium *E. faecium* PB1 and dairy waste water isolate *B. paramycoides* DW1 to grow and survive in waste milk and these highly useful bacterial isolates may be exploited in future for treatment of real waste waters discharged from dairy industries to reduce their organic pollutant load.