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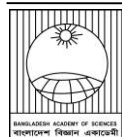

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Research Article

Protease from jute endophyte *Micrococcus luteus* MBL-Bac7 functions as a potential bating agent for the leather industry

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ABSTRACT

Ever since the discovery of jute endophytes, testing their potential for commercial uses has been a matter of interest. Considering the same, jute endophyte *Micrococcus luteus* MBL-Bac7, capable of producing extracellular proteases, was selected for *in vitro* and *in silico* analysis to assess its role as a bating agent required in rawhide processing. The presence of extracellular protease was confirmed from the plate assay. As the enzyme is tested for commercial use, the effect of various metal ions and reaction conditions (pH, temperature) have been optimized. The protease activity appears to be retained even at 85°C. It also showed significant activity in a wide range of pH (pH 3.0-8.5). Metal ion Mn^{2+} increased the protease activity significantly, but Fe^{2+}, Zn^{2+}, and Co^{2+} ions showed the opposite effect. Molecular identification of the protease was done from the whole genome sequence data. Using PSORTb v.3.0.2, SecretomeP-1.0, TMHMM-2.0, and protein molecular weight software, the physicochemical properties of the protease were predicted. The isolated protease shared a strong evolutionary link with Micrococcus species' S8 family serine peptidase. Finally, in the bating of cowhide, effects similar to that of commercial agents were obtained during finger prick, lastometer, and tensile tests. The findings of this study corroborate the possibility of using this protease as a potential bating agent. However, further studies are necessary to reduce the production cost for higher yield and commercialization.

Introduction

Endophytic bacteria are omnipresent in plants, residing either latently or actively colonising plant tissues both systematically and locally, without having any harmful effect on the plant. Reports say plants may harbour both Gram-positive and Gram-negative bacteria. However, many Gram-positive bacteria have biological control activities, such as *Bacillus*, *Brevibacteria,* and *Micrococcus* species are found inhabiting plants (Kobayashi and Palumbo, 2000). The jute (*Corchorus olitorius*) plant is home to a large number of endophytes, including both bacteria and fungi. Various biochemical and physiological tests suggest that these microbes may provide their host a wide range of benefits (Najnin et al., 2015). Among the significant benefits of endophytic bacteria, observed up to now, are growth stimulation in soya beans (Kuklinsky-Sobral et al., 2004), increasing nitrogen fixation in sugarcane (Boddey et al., 2003), assisting plants in obtaining nutrients, enhancing systemic resistance to pathogens and growth

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promotion in rice (Madhaiyan et al., 2004), regulating phytohormones resulting in stress relief and increased root growth (Hardoim, 2011). Endophytes generate extracellular enzymes such as amylase, lipase, protease, xylanase, and esterases to hydrolyze different components and initiate resistance against plant invasions (Tan and Zou, 2001). Over the years, the use of bio-based chemicals has increased. New bio-products are being developed that can reduce the dependency on mineral fuels and provide a more reliable energy source (Carole, 2004). Efficient catalytic properties of enzymes have already promoted their use in various industrial processes (Beilen and Li, 2002). Selected microorganisms, including bacteria and fungi, have been explored globally for the biosynthesis of commercially viable preparations of enzymes for industrial uses (Saxena and Singh, 2011). Protease represents one of the largest groups among commercially important enzymes (Srilakshmi et al., 2015). Proteases can hydrolyze peptide bonds. Given its many applications, it has been employed in different industries. It is used in detergent industries to remove protein stains, in food industries, in the pulp and paper industries to remove biofilm, and especially in leather industries (Kirk et al., 2002). According to the commercial standing of Bangladesh, protease's largest market is the leather industry. One of the wet blue processing steps in the leather process is bating. To prepare leather with a certain degree of softness and pliable feel and a high-quality grain, the unhaired pelts must be subjected to bating following liming and de-liming. Protease is utilised in such processes. Since bating is a crucial stage in leather processing, and there is a large market for a protease that is currently occupied by the importation of protease, this study aims to establish protease from jute endophytic bacteria *Micrococcus luteus* MBL-Bac-7 as a compatible bating agent.

Methods and materials

Bacterial strain

Bacterial strain *Micrococcus luteus* MBL-Bac7 was taken from the jute endophytic bacterial library of the Molecular Biology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka. This was previously isolated from jute in the same lab and stored in both LB agar and 30% glycerol stocks at -80°C.

Morphological analysis

MBL-Bac7 was cultured on a plate containing tryptic soy agar (TSA). A drop of water along with a loopful of bacteria was placed on a clear slide and spread very thinly. After the thin film dried, crystal violet was applied to the stain for 30 s and rinsed with water. Then it was covered with gram's iodine for another 30 s and again washed with water. Decolourisation was done with 90% ethanol for 30 s, which was later washed with water followed by staining with safranin. Further, it was stained for the 30s, washed with water, and allowed to dry. A coverslip was fixed on the stained bacteria with 60% glycerol. Finally, the slide was observed under a microscope.

Production of enzyme

The enzyme was produced by fermentation with bacteria in the presence of substrate, which is specific to the enzyme. For submerged fermentation media was prepared with glucose (1.0%), peptone (0.5%), yeast extract (0.5%), salt solution (5.0%). Salt solution was prepared with $MgSO_4.7H_2O$ (0.5%), KH_2PO_4 (0.5%), $FeSO_4.7H_2O$ (0.01%), and NaCl (0.01%). The components were mixed in Erlenmeyer flasks with distilled water, and pH was fixed around 7.0-7.2 and autoclaved to sterilise at 121ºC for 20 min. Then a loop of the bacterial colony was inoculated in liquid media and incubated at 37ºC with 2×g relative centrifugal force (RCF) for 24 h.

Isolation of enzyme

After incubation, the culture medium was centrifuged at 3920×g RCF for 10 min, and the supernatant was collected. The collected supernatant was measured in a measuring cylinder, and a required amount of ammonium sulphate for 80% saturation was

calculated. Ammonium sulphate was mixed with the collected supernatant and was aliquoted in different falcon tubes. After overnight incubation at 4ºC, they were centrifuged at 10000×g for 30 min, the supernatant was discarded, and the precipitated protein was dissolved in phosphate buffer.

Qualitative screening of the enzyme

The qualitative screening of the enzyme was carried out on the medium containing gelatin (1%), peptone (0.5%), yeast extract (0.5%), salt solution (5%) $MgSO_4.7H_2O$, (0.5%), KH_2PO_4, (0.5%), $FeSO_4.7H_2O$ (0.01%) NaCl) (0.01%) and agar (1.5%). Inoculation was done using 10.0 µL of an overnight liquid bacterial culture and incubated for one day. After one day of incubation, the plate was flooded with a saturated solution of ammonium sulphate [$(NH_4)_2SO_4$]. The gelatin unutilised by the bacteria was precipitated by $(NH_4)_2SO_4$, which created a clear zone around the bacterial colony.

Protease activity assay

Protease activity was measured by a slightly modified method of Cupp-Enyard. The reaction mixture was prepared by mixing 100.0 µL enzyme with 5 mL of 0.65% casein (Nacalai Tesque Inc., Japan) solution dissolved in phosphate buffer and incubated at 37ºC in a water bath for 10 min. The reaction was stopped with 5.0 mL of 110 mM trichloroacetic acid (TCA), which precipitated the proteins interrupting their activities, and incubated again under the same condition for 30 min. After incubation, 2 mL of supernatant was collected by centrifuging the mixture at 10000×g for 10 min. Meanwhile, standard samples were prepared with different amounts of L-tyrosine, making the volume up to 2.0 mL with distilled water where the concentration of a stock solution L-tyrosine (for use as a standard) was 1.1 mM. 5.0 mL 500 mM sodium carbonate and 1.0 mL Folin and Ciocalteu's phenol reagent (Sigma-Aldrich, USA) were added. Again, all the tubes were incubated at 37ºC for 30 min. Readings were taken in a spectrophotometer at 660 nm. A standard curve was prepared using the absorbance against different concentrations of tyrosine. Each µmole of tyrosine released by protease per min per mL was defined as one unit of protease.

Quantitation of protein content

The Bradford method was used to calculate protein content in the culture supernatant using bovine serum albumin (BSA) as a reference. The absorbance was taken at 595 nm, and the specific activity was measured in units per mg of protein.

Characterization of the protease

Effect of temperature on protease activity

To study the effect of various temperatures, crude protein extracts were taken in different falcon tubes and incubated at 37ºC, 45ºC, 60ºC, and 85ºC. The activity of protease was measured according to the procedure described above.

Effect of pH on protease activity

To study the effect of different pHs on protease, gelatin was used as a substrate instead of casein, as casein does not dissolve at a pH lower than 6.0. Gelatin was dissolved in pH 3.0, 4.0, 5.5, 6.5, 7.5, 8.5, and the activity of protease was measured according to the protocol explained before.

Effect of metal ions on protease activity

The effects of metal ions were studied by incubating 100.0 µL crude protease enzyme with 900.0 µL of 10 mM salt solutions for an hour, then the activity was measured following the method explained above. Metal salts such as $FeCl_2$, $CaCl_2$, $MnCl_2$, $CuSO_2$, NaH_2PO_4, Na_2HPO_4, and $ZnSO_4$ were used as sources of Fe, Ca, Mn, Cu, Na, Zn ions to study their effects, if any, on the protease enzyme.

Genome analysis of bacteria to identify secreted protease

Molecular identification of the bacteria through PCR amplification of 16S rRNA gene

For amplification of the 16S rRNA gene, PCR was done directly from the bacterial genomic DNA (gDNA) isolated from the glycerol stock. Specific forward primer

27F (5’-ACGCTTACCTTGTTACGACTT-3’) and reverse primer 1492R (5’-AGAGTTTGATCACT GGCTCAG-3’) were used in 0.5 µL of each 10mM concentration in a 35-cycle PCR reaction on ProFlex PCR system (Thermo Fisher Scientific, UK). The thermocycler was set as follows: an initial denaturation of 5 min at 95°C, template denaturation at 95°C for 30s, primer annealing and extension at 68°C for 20s, and 72°C for 1 min, respectively. The final extension step was set for 5 min at 72°C.

Whole-genome sequencing of the bacteria

Whole-genome sequencing of the *Micrococcus luteus* MBL-Bac7 strain was performed using the isolated DNA of high quality at the National Institute of Biotechnology, Bangladesh, using the Sanger sequencing platform.

Sequence analysis

Sequence identity and resemblance were performed through BLASTn analysis. The genome assembly data of the bacteria were analysed with RAST (Rapid Annotation using Subsystem Technology) (https://rast.nmpdr.org/) annotation server to predict the genes and protein models along with their functions. Proteins that were predicted as proteases were short-listed for further analysis.

Subcellular localization and physicochemical property

PSORTb v.3.0.2 (https://www.psort.org/psortb/) was used to determine the subcellular location of the predicted proteases. Since the screening of protease activity was carried out on extracellular extracts, finding the extracellular proteases were important. Proteins were also investigated for secretory properties by SecretomeP-1.0 (https://services.healthtech.dtu.dk/service.php?SecretomeP-1.0) and Transmembrane Helix Prediction 2.0 (TMHMM-2.0) (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). As these proteins are not located in the organism’s genetic code, SecretomeP identifies these non-canonical secretory proteins that lack any signal peptide, and TMHMM identifies if the predicted secretory proteins have more than one transmembrane domain. Finally, molecular weights of the predicted secretory proteins were calculated with Protein Molecular Weight software (https:// www.genecorner.ugent.be/ protein_mw.html).

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment analysis, bootstrap analysis, and neighbour-joining phylogenetic tree construction were performed using molecular evolutionary genetics analysis (MEGA) software version MEGA 11.0.8.

Prediction of secondary structure

The secondary structure of the protease was predicted with the help of two web-based tools, SWISS-MODEL (https://swissmodel.expasy.org/nteractive/9fF3Aw/models) and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

Application of protease in leather processing

The final goal of this study was to apply the protease to cowhide for bating and determine its efficiency as a bating agent. Wet blue processing is a long process where the following steps are serially done soaking, pre-liming, liming, dehairing and fleshing, chemical wash, deliming and bating, pickling, and chrome tanning (Lyu and Cheng et al., 2017). The sixth step is deliming and bating. At this step 2% ammonium sulphate, 1% ammonium chloride, and 0.5% sodium metabisulphite with 80% water were mixed and the pH became 8.2-9.0, and the drum was run for an hour. Then 1% bating agent and 100% water were added and again ran for another hour. Water was drained and a finger prick test and bubble test were done. Then pickling, tanning, shaving, shamming, dying and fat liqouring were done. When the whole processing was finished, lastometer test, water vapour permeability test, and tensile and elongation tests were done. For the lastometer test, leathers were cut into a round shape of 4.5 cm diameter, placed into the lastometer, and pressure was applied. At the moment when a crack appeared on the leather, the amount of pressure was read. In the tensile strength test, leathers were cut into 14 cm long pieces where

at the middle portion, the width was 1.0 cm, then placed into a tensile test machine. Water vapour permeability was tested through the cup method (Huang and Qian, 2008).

Results

Morphology analysis of the bacteria

The bacteria from the yellowish colony when cultured in TSA plate, retain purple colour after Gram staining. Under the microscope, it appears cocci-shaped. (Fig. 1).

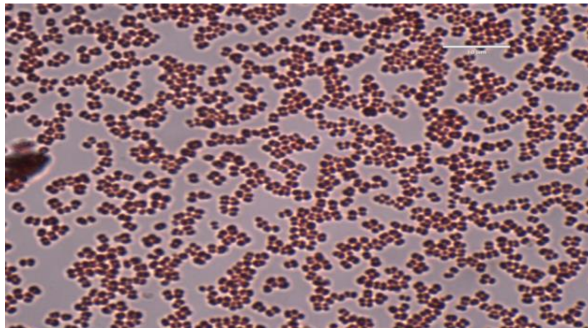

Fig. 1. Morphology analysis of *Micrococcus luteus* MBL-Bac7 under a microscope.

Qualitative screening of the protease

After 48h incubation of MBL-Bac7 in gelatin containing plate, a clear zone developed around the bacterial colony where the gelatin was hydrolyzed by extracellular enzymes (Fig. 2)

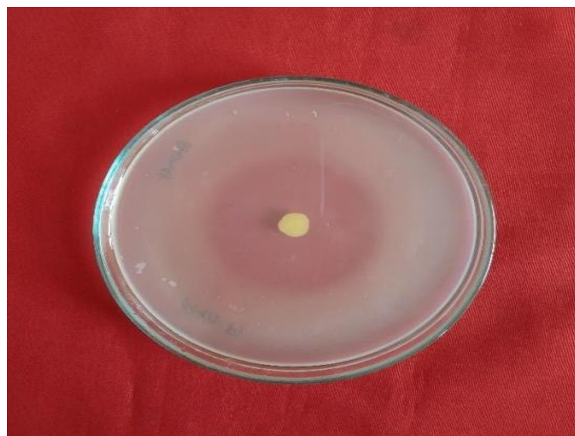

Fig. 2. Formation of a clear zone in gelatin agar plate containing *Micrococcus luteus* MBL_Bac7 indicating gelatin degradation around the bacterial colony.

Protease activity

The protease activity of 1630.3 UmL^{-1} was found in the $(NH_4)_2SO_4$ precipitated protein extract. On the other hand, the specific activity was 3702.9 UmL^{-1} with a purification fold of 7.33. In the culture supernatant, the protein remains diluted. But after precipitation, the protein concentration increases approximately 7.33 times (Table 1).

Table 1. Protease activity of cell-free supernatant and $(NH_4)_2SO_4$ precipitated protein.

Steps	Cell-free supernatant	$(NH_4)_2SO_4$ precipitated
Volume (mL)	280.0	5.0
Protein concentration (mg/mL)	0.06	0.44
Activity (U/mL)*	191.85	1630.30
Specific activity (U/mg)	3197.52	3702.70

*One-unit activity is defined as the amount of µmole tyrosine was released during assay per minute

Characterization of the protease

Effect of temperature on protease activity

Protease activity in the crude protein extract at 37º, 50º, 60º, and 85ºC was found to be 710.8, 773.8, 852.2, 465.4 UmL^{-1} respectively, where the control was 685.6 UmL^{-1} on average (Fig. 3).

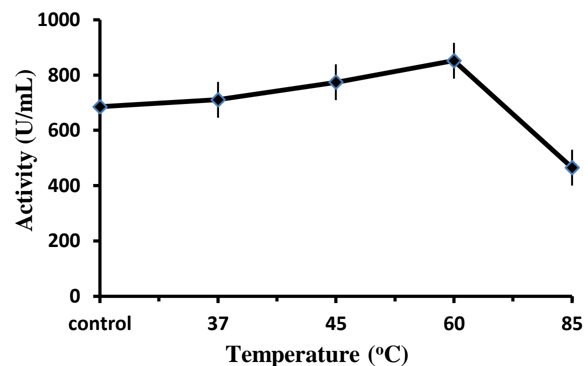

Fig. 3. Effect of temperature on the activity of the protease. Activity increases as the temperature rises and starts to decline after 60 ºC. The optimum temperature was found to be 60 ºC.

Effect of pH on protease activity

Protease activity was assessed at different pHs of 3.0, 4.0, 5.5, 6.5, 7.5, 8.5, and the activities were found to be 1164.9, 1509.1, 1253.6, 1790.4, 1565.2 and 1491.3 UmL^{-1} respectively (Fig. 4).

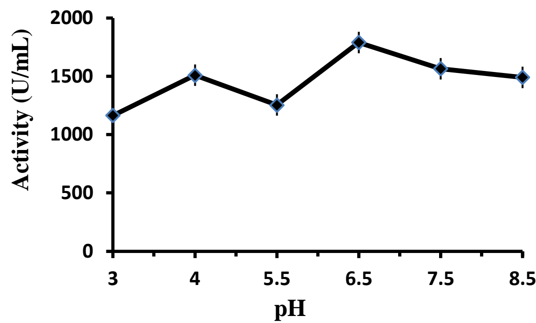

Fig. 4. Effect of pH on the activity of the protease. The protease is stable at a neutral pH range (6.5-7.5) without losing activity, and the highest activity was obtained at pH 6.5.

Effect of metal ions on protease activity

Protease treated with Na^{+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+} salts showed activity of 2477.95, 861.9, 7698.8, 517.4, 558.4, 577.7, 1514.3 UmL^{-1} respectively (Fig. 5).

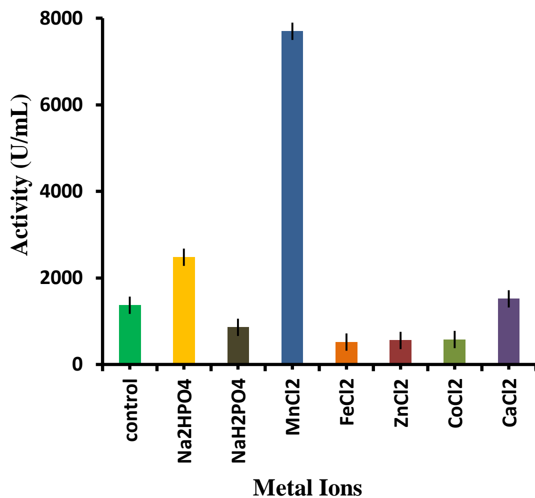

Fig. 5. Effect of metal ions on the activity of the protease. In the presence of Mn^{2+} salt, the proteolytic activity increased, while activity significantly reduced in the presence of Fe^{2+}, Zn^{2+}, and Co^{2+} salts.

Genome analysis of bacteria to identify secreted protease

Molecular identification of the protease

When the 16s rDNA sequence was retrieved from the whole genome sequence and compared using BLASTn against nucleotides, more than 99% similarity was found for *Micrococcus luteus* SA211 strain.

Sequence analysis

Whole-genome sequence of MBL-Bac7 was analysed using RAST (Rapid Annotation using Subsystem Technology), and the summary of the analysis is shown in Table 2.

Table 2. RAST analysis result: whole-genome analysis of MBL-Bac 7.

Genome size	2530185 bp
Total G-C content	72.94%
Number of genes	2501
Non-coding RNA genes	51
16s rRNA gene	1528 bp

Among all the protein-coding genes, 19 genes were found to have protease coding sequences. The identified protein-coding genes with their molecular mass and possible functions are described in Table 3.

Subcellular localization and physicochemical properties

Among all the 19 proteases, only peg. 158 had signal peptide and was found to be extracellular (Table 4). This protease (peg.158) has 641 amino acids and a molecular weight of 63.84 kDa. Others were non-canonical (peg. 1071, peg. 1968, and peg. 1089). Peg. 1968 had 6 transmembrane domains which are not likely to be secreted. Peg. 1071 is a zinc metalloprotease and peg. 1089 was found to be protease II. Peg.158 was targeted for this study and was subjected to further analysis.

Table 3. Proteases identified in the MBL-Bac7 genome.

Serial no	Protein encoding gene (peg)	No of amino acids	Molecular weight (kDa)	Functions
1	peg.37	238	25.61	SOS-response repressor and protease LexA (EC 3.4.21.88)*
2	peg.54	739	80.18	CatalaseKatE-intracellular protease (EC1.11.1.6)*
3	peg.107	851	93.49	ATP-dependent Clp protease,ATP-binding subunit ClpC
4	peg.158	641	63.84	Extracellular protease precursor (EC 3.4.21)
5	peg.256	464	50.39	Protease II (EC 3.4.21.83)
6	peg.365	432	46.94	ATP-dependent Clp protease ATP-binding subunit ClpX
7	peg.366	221	24.34	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)
8	peg.367	204	22.10	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)
9	peg.800	194	20.72	Intracellular protease
10	peg.948	138	14.29	Intracellular protease
11	peg.1071	222	24.26	Zinc metalloprotease
12	peg.1089	308	34.23	Protease II (EC 3.4.21.83)
13	peg.1268	332	33.49	Lon-like protease with PDZ domain
14	peg.1359	120	13.28	ATP-dependent Clp protease adaptor protein ClpS
15	peg.1620	184	19.86	YpfJ protein, zinc metalloprotease superfamily
16	peg.1721	227	24.27	Intracellular protease
17	peg.1932	518	56.41	ATP-dependent Zn protease
18	peg.1968	455	48.1	Membrane-associated zinc metalloprotease
19	peg.2456	158	16.05	Putative activity regulator of membrane protease YbbK

*Enzyme Commission number (EC number) is a numerical classification for enzymes given based on the chemical reactions they catalyze.

Table 4. Subcellular localization of the protease using PSORTb.

Localization	Scores
Extracellular	8.91
Cell wall	0.80
Cytoplasmic	0.24
Cytoplasmic membrane	0.05
***Final prediction: Extracellular 8.91**	

*Final prediction is done based on the localization that scores above the 7.5 cutoff value.

Phylogenetic analysis of the predicted protease

The strong similarity and tight phylogenetic connection of peg.158 with *Micrococcus luteus* serine peptidase suggest that the predicted protease is a *Micrococcus luteus* isolate and imply that the protease belongs to the serine peptidase family (Fig. 6).

Prediction of the secondary structure

Protein model of the peg. 158 was generated using both SWISS-MODEL and PHYRE2. The modeling was validated by the Ramachandran plot (Table 5, Fig. 7)

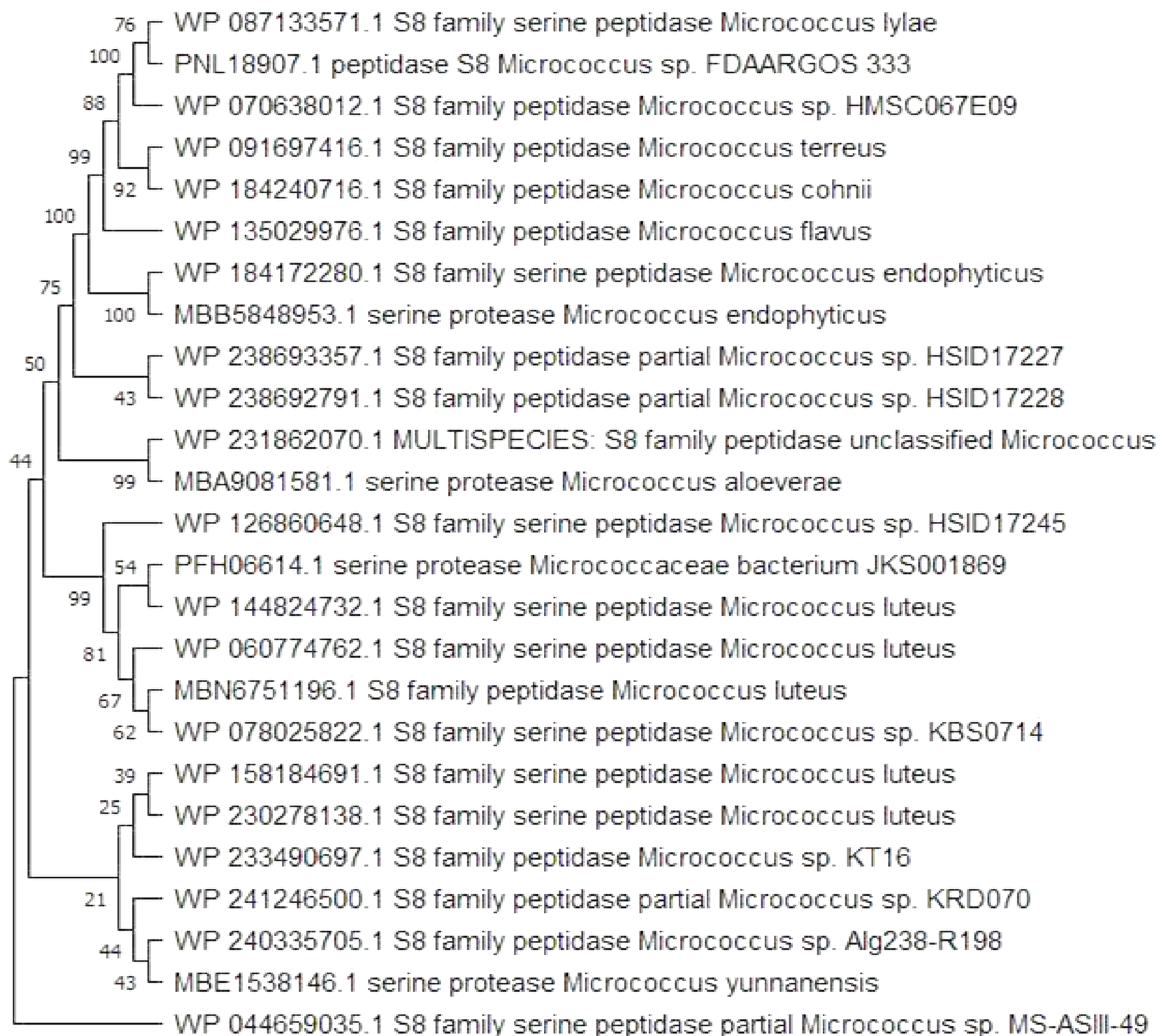

Fig. 6. Phylogenetic position of predicted protease and those from other *Micrococcus luteus* groups. The branching pattern was generated by neighbour-joining tree method.

Table 5. Summarization of Ramachandran plot for modelled structures: for validation of homology modeling.

Residues	SWISS-MODEL	PHYRE2
Most favoured regions	88.70%	80.10%
Additional allowed regions	9.70%	16.50%
Generously allowed regions	1.20%	2.00%
Disallowed regions	0.40%	1.40%

The structure of the protease was modeled using SWISS-MODEL and PHYRE2 and the structures were validated by the Ramachandran plot. This table shows that the model generated by SWISS-MODEL has a slightly better score than PHYRE2.

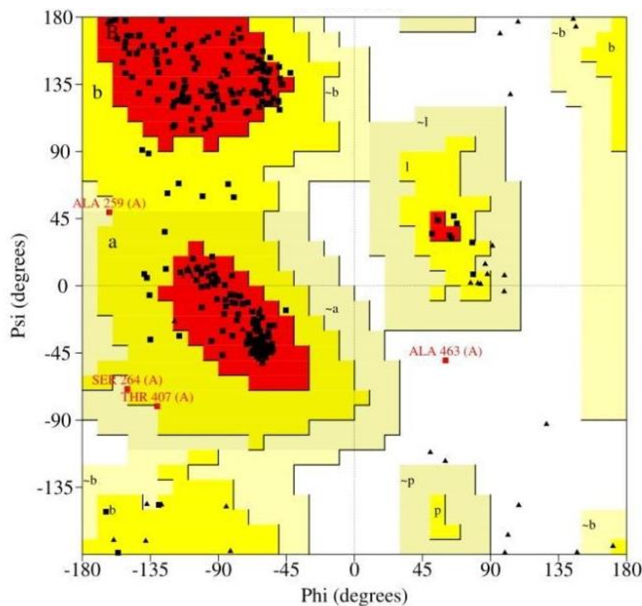

Fig. 7. Ramachandran Plot obtained from Procheck server, showing the most favored regions (red), less favored regions (dark yellow), and allowed regions but rare (light yellow). Regions in white are not possible due to strict collision.

The Swiss model server found to be the best model among the servers was used for modeling and viewed with Discovery Studio Visualizer v17.2 (Fig. 8). The model of the Swiss model server used proteases from *Pseudoalteromonas arctica* PAMC 21717 (Pro21717) (PDB ID: 5YL7) to construct Model 1. The peg.158 had a sequence identity of 56.92%. The standalone BLAST between peg.158 sequence and proteases from *Pseudoalteromonas arctica* sequence (reference protease of Swiss model resulted in an e value of $3e\text{-}10^{8}$).

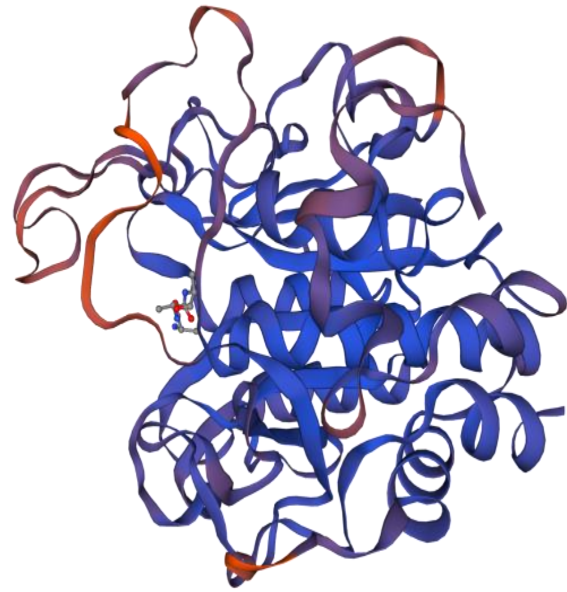

Fig. 8. Three-dimensional model of the predicted protease using Swiss model server. Purple-colored regions are well modeled, and red-colored regions are poorly modeled. This estimation of the model quality is based on the QMEAN scoring.

Bating activity of the protease

As there is no direct test to determine the strength of bating, the final states of hiding after wet blue processing and crusting were evaluated. These will indirectly provide an idea of the proper bating of a processed hide. Tensile strength, percent of elongation, water vapour permeability, and lastometer were tested for the processed hide and then compared with a commercially available bating agent (Table 6). Crude extract of Bac7 was found to be comparable with the standard commercial bating agent.

Table 6. Different test report of processed hides bated by Bac7 protease and a commercial bate.

Name of the tests	Bac7 protease	Commercial bate	Standard Value
% of Elongation Test	20.00%	22.73%	30-45%
Tensile Strength Test	212.96 kg cm^{-2}	175.44 kg cm^{-2}	200.00 kg cm^{-2}
Water Vapor Permeability Test	12.03 mg cm^{-2} hr^{-1}	11.57 mg cm^{-2} hr^{-1}	10.00 mg cm^{-2} hr^{-1}
Grain Crack Strength (Lastometer) Test	20.00 kg	22.00 kg	20.00 kg

Discussion

The present study aims to purify the extracellular protease from *Micrococcus luteus* MBL-Bac7 that can be used as a bating agent in the leather industry. Bating is the process of treating dehaired hides or skins with an enzyme to eliminate certain undesirable proteinaceous components (Thanikaivelan et al., 2004). This is a significant part of leather processing because proteases remove residues of the interfibrillar proteins. Otherwise, non-collagenous proteins cause cementing, which results in a lack of flexibility of leather (Hameed et al., 1996).

The endophytic bacteria *Micrococcus luteus* MBL-Bac7 was found to produce extracellular protease. Incubation with a drop of bacteria in a gelatin-containing plate created a clear zone when the plate was flooded with 100% ammonium sulphate solution. The clear zone indicated the presence of protease, because the gelatin on that area was utilized, and no gelatin was left for precipitation. It has been reported that *Micrococcus luteus* from water reservoirs can produce protease (Venkatesham et al., 2014). Rapid Annotation of MBL-Bac7 whole genome using Subsystem Technology (RAST) (Aziz et al., 2008) showed 2501 genes and among them, only 19 are proteases. The 19 proteases were further analysed to find the signal peptides or transmembrane helixes.

The presence of signal peptide means the protein is likely to be secreted. Only one protein was found to have a signal peptide and that was protein encoding gene, peg.158) with 641 amino acids and a predicted molecular weight of 63.84 kDa.

There were three other proteases that may follow the non-classical secretion pathway and may also be secreted extracellularly (Bendtsen et al., 2005). Nonclassical secretory proteins are independent of signal peptides and seem to follow a novel pathway to be secreted from the cytosol. However, it is reported that the non-classical secreted proteins can be distinguished from the cellular proteins by properties such as amino acid composition, secondary structure, and disorder regions.

Prediction of disorder for MBL-Bac7 reveals that the bacterial secretory proteins are more structurally disordered than their cytoplasmic counterparts (Bendtsen et al., 2005). The three non-classical extracellular proteases (peg.1071, peg.1089 and peg.1968) have 222, 308, and 455 amino acids, respectively. To evaluate all four of these proteases, transmembrane helices were compared using Transmembrane Helix Prediction (TMHMM). It can discriminate between soluble and membrane proteins with specificity and sensitivity by 99%. However, the percentage decreases in the presence of signal peptides (Krogh et al., 2001). If a protein has transmembrane helices, it may not be secreted, instead, it will translocate on the plasma membrane. One of the targeted proteases (peg.1968) had six transmembrane domains. With several transmembrane helices, the protein is expected to be

embedded in the membrane, rather than be secreted out of the cell. So, it can be concluded that peg.1968 protease is not a secretory protease. It was predicted that peg.158 might be the desired secretory protease. PSORTb also declared the protease as extracellular but not cytoplasmic as the localization score of the extracellular site exceeded the threshold score of 7.5. For further confirmation, mass spectrometry is required. To study more about the protein peg.158, homology modeling was done using the Swiss Model and PHYRE2. The 3D structure of the protease was generated. After analysing all the data achieved from the Ramachandran plot, especially the e-value, it was determined that the model constructed by the Swiss model was the best one. Again, the Swiss model created the protein model based on proteases from *Pseudoalteromonas arctica* PAMC 21717 (Pro21717) (PDB ID: 5YL7), these two protease sequences were subjected to BLAST. From the e-value of BLAST, it can be predicted that the proteins have significant sequence similarities. The proteases from *Pseudoalteromonas arctica* are serine proteases, so from the homology and sequence similarity, it can be presumed that peg.158 might be a serine protease family.

Suppose the protease isolated from Bac7 is to be used in tanneries. In that case, several ions or chemical compounds will be present in the reaction's mixture of bating, such as sodium polyphosphate and ammonium sulphate etc. Inside the drums of leather processing, the temperature may rise to 40ºC. Considering all these features, the protease's activity was measured after incubating at different temperatures, pHs, and with various metal ions. When the protease was incubated at different temperatures for one hour, it was observed that if the incubation temperature increased from 37ºC to 60ºC, the activity increased. But the activity started to decline after 60ºC, and at 85ºC, the activity was found to be ~89% compared to the control. It was reported that *Bacillus subtilis* protease had 100% stability in the temperature ranges from 35º-55ºC (Manachini et al., 1988). To study the effect of pH, the reaction mixture was set at different pHs. The general process of activity measurement is to use casein as the substrate. However, casein does not dissolve at a pH lower than 6.0. To avoid such hindrance, gelatin was taken as the substrate to demonstrate pH's effect on protease activity. A long-range of pH was chosen from acidic to alkaline. Although the protease showed high activity in alkaline conditions, it also had significant activity in an acidic environment, suggesting it was active within a large pH range. A previous report says protease isolated from *Bacillus megaterium* shows remarkable activity and stability at a large pH range of 5.5-9.0 (Asker et al., 2013).

In this study, Mn^{2+} ion increased the protease activity by about five-fold. During bating, sodium polyphosphate was used. Therefore, the enzyme activity was measured in the presence of Na_2HPO_4 and NaH_2PO_4, although NaH_2PO_4 reduced the activity of the enzyme compared to the control. In such cases, there may be an influence of HPO_4^{2-} ion on the protease that is yet to be determined. Slight inhibition of enzymatic activity was found after treatment with $CaCl_2$, $CoCl_2$, $ZnCl_2$, and $FeCl_2$.

Finally, the protease was applied to raw cowhide, simultaneously; a commercial bating powder was applied to another piece of hide as a control. There is no direct test to ensure the strength of bating. Proper completion of leather processing depends on the accuracy of bating because bating creates micropores for Cr^{3+} to enter into collagen, called tanning. So, if the processed leather meets all the characteristics of physical tests, then it can be concluded that the bating was efficient. Both pieces of leather that were bated with the protease of Bac7 and commercial bating agent were subjected to several physical tests. One test was the lastometer test, where the softness of the leather was measured. MBL-Bac7 protease treated leather could tolerate 20 kg of load, which is equal to the standard value. Tensile strength and elongation tests were also done. Tensile strength is the capacity of a material or structure to withstand loads, allowing it to elongate. The load is calculated as kg cm^{-2}. The

standard value is considered 200 kg cm^{-2}. Leather treated with MBL_Bac7 protease had a tensile strength of 212.96 kg cm^{-2}. This means that it can withstand more than the standard level of tension. Hameed et al. (1996) reported that protease of *Bacillus subtilis* K2 had a remarkable bating property considering tensile strength test and elongation at break, giving the value of 175 kg cm^{-2} and 19% respectively at 1800 UmL-1. The last physical test of processed hide was the water vapour permeability test. Water vapour permeability indicates how quickly textiles can transfer sweat from the human body during an activity involving higher metabolism into the air. Proper selection and manufacture of materials with high water vapour permeability, especially during outdoor activities, can provide comfort for the users. One of the most significant physical properties of leather is the water vapour permeability test, which greatly affects the breathability and the comfortable feeling of leather textiles and footwear. In the experiment, two types of leathers (one of which was bated with crude protein and the other bated with a commercial bating agent) were subjected to water vapour permeability tests. The results showed that the treated leather had higher water vapour permeability compared to the standard value. But among the two, leather bated with crude protease had a higher value than the one in which a commercial bating agent was used.

Conclusion

Considering all the experimental results, it can be concluded that the protease from *Micrococcus luteus* MBL_Bac7 is potential for industrial production. However, there are still some concerns regarding the use of this protease as a commercial bating agent. One of them is the production cost of the enzyme. This can be alleviated by using low-cost protein sources for bacterial culture and their optimization. Modification at the molecular level can also be undertaken to allow more protein precipitation at a lower cost.

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