BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2020. Vol. **17**(2), p. 385-392

Characterization of Alkaline Protease Producing *Bacillus Halodurans* RSCVS-PF21 Isolated from Poultry Farm Soil

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http://dx.doi.org/10.13005/bbra/2841

(Received: 05 May 2020; accepted: 09 June 2020)

Microorganism living in extreme habitats are believed to produce stable proteases according to industrial needs. Fecal soils of poultry farm can be a rich source of such microorganisms. The aim of the study was to isolate and characterize potent alkaline protease producing bacterial strains from alkaline poultry farm soil. The bacterial isolate RSCVS- PF21 was isolated from fecal soil samples of a poultry farm located in Rewa, Madhya Pradesh India. It was able to grow on such high pH as pH 12 on casein containing media in the laboratory and screened positive for protease activity. The alkaline protease produced from the bacteria was extracellular. The crude enzyme showed high relative enzyme activity even at pH 12. Bacteria was long, rod shaped, filamentous, gram positive. On the basis of 16s rRNA NCBI BLAST and Phylogenetic Analysis, the bacteria was identified as *Bacillus halodurans* RSCVS- PF21. 16s rRNA sequence was submitted to Genbank database with accession no. MT279908.

Keywords: Alkaline Protease; *Bacillus Halodurans*; Bacteria; Characterization; Identification; Soil.

Enzymes have very old history and they have their existence from the start of ancient civilizations where people were using microbial enzymes in baking, alcohol production, brewing, cheese making etc. However, since then the number of applications of enzymes has increased tremendously with better knowledge about production and purification of enzymes from diverse sources[1]. Microorganisms have been known to produce both intracellular and extracellular enzymes commercially[2].

Enzymes are becoming alternative to chemical catalysts for better efficiency ensuring economic and ecological sustainability of the industrial processes[3]. Proteases are the most dominant group of enzymes from the industry point of view, constituting about sixty percent of the total enzyme market[4]. Proteases from microorganisms have been the most widely explored enzymes since the advent of enzymology[5]. They account for approximately two-third share of the total commercial protease sale around the globe[6]. These enzymes have gained attention not only due to their critical role in metabolic activities in the organisms but also due to their wide applications in industries[7] such as food, detergent, bakery, leather,

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pharma, infant formulas, etc. due to their features like production ease, thermal stability and wide pH range applications[8].

Alkaline proteases are the most frequently used industrial enzyme among different types (alkaline, neutral, and acidic) of proteases due to their comparatively high activity and greater stability at high pH[9]. Wide range of microbes were found to produce alkaline proteases, including bacteria, yeasts, moulds and mammalian tissues[5] and these were isolated from diverse sources as well. However, bacteria were preferred as they grow rapidly under various nutritional and physiochemical conditions, need less space, could be easily maintained and were accessible for genetic operations.[2] A large number of bacteria have been reported to produce alkaline proteases e.g. *Bacillus, Pseudomonas, Streptomyces, Micrococcus* etc. However, *Bacillus* sp. has been the major source for alkaline protease production, accounting for about 35% of the total microbial enzyme sales[10,11]. Since the first alkaline protease 'Carlsberg' from *Bacillus licheniformis* was utilized as a detergent additive in the 1960s, a number of other *bacillus* species have also been reported to produce alkaline proteases[12].

Significant advancements in agriculture, industry, bioinformatics and biotechnology have fueled the search of extremophile microorganisms for alkaline protease production[13]. The proteases produced by microbes present in normal environment are usually unstable under extreme conditions, even with several attempts of physicochemical treatments, protein engineering and gene-shuffling methods[4]. Microorganisms living in extreme habitats are believed to produce proteases which are stable in those situations[4] but limited information is available reporting such microorganisms[14].

In this context, the aim of the study was to isolate potent alkaline protease producing bacterial strains from poultry farm fecal soils of Rewa, Madhya Pradesh, India. Poultry farm fecal soils are known to be highly alkaline.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents used in media formulation, screening, well diffusion assay and phenotypic characterization were obtained from HiMedia, India and Fisher Scientific, USA. HiPer Bacterial Genomic DNA extraction kit, Taq Polymerase and dNTP Mix were obtained from HiMedia, India. Primers were obtained from Eurofins Genomics, Bengaluru, India.

Collection of Samples

Alkaline Soil samples for the isolation of potent alkaline protease producing bacteria were collected from Govt. kukkut palan kendra (poultry farm) near chirahula temple, in district headquarters of Rewa of Madhya Pradesh, India. Dried fecal soil was explored for collection which are known to have alkaline pH. The samples were collected from 2 cm depth of surface using a sterile spatula. They were tested first for high alkaline pH using pH strips and transferred in a sterile polybag. The samples were randomly collected from two sites of poultry farm: site 1 and site 2. The pH of the samples was 10.0 and 11.0 respectively.

Medium

Isolation of bacteria was carried out on CPYA (Casein, Peptone, Yeast extract, Agar) medium (pH 12.0)[15, 16] and the same media was used also for screening of protease activity and crude enzyme production. Medium (1 L) was prepared by making three separate solutions initially. Solution A: casein (5.0 g) in 0.01 N NaOH solution (400 ml); Solutions B: peptone (5.0 g), yeast extract (2.0 g), NaCl (5.0 g), K_2HPO_4 (1.0 g), $MgSO_4.7H_2O$ (0.2 g), $CaCl_2$ (0.1 g), and Agar (20 g) in 595 ml distilled water; Solution C: 6.0 g Na_2CO_3 in 20 ml distilled water. After that, these solutions were autoclaved separately to avoid coagulation of casein due to high pressure. Solution A and Solution B were mixed and maintained to pH 12.0 aseptically by adding Solution C in a drop wise manner.

Isolation and Screening

Serial dilution agar plate technique was used to carry out isolation. 0.1 ml of the 10-1-10-3 diluted sample was spread evenly over the CPYA medium (pH 12.0) and incubated at 37ºC for 24 h in Incubator. Appeared colonies were purified by repeated streaking of a single isolated colonies of bacteria on fresh CPYA medium plates. Production of alkaline protease was screened by streaking of pure isolates on CPYA media plates[17, 18] and after that giving them incubation at 37ºC temperature for 24 to 48 hours. 10% Trichloroacetic acid (w/v) solution was then poured in plates and incubated

for half an hour. Zone of proteolysis was observed as transparent clear zone around the colony.

Enzyme Production and Well Diffusion Assay

Isolates producing clear zone around the colony in the range of good and excellent were selected for enzyme production under submerged conditions. Erlenmeyer flasks (150 ml) having CPYA broth (50 ml, pH 12.0) were inoculated with small amount of bacterial culture. These were incubated at 37ºC temperature for 48 h. The fermented broth was centrifuged then at 7000 g for 10 minutes. The resulting cell-free crude filtrate was used for assay.

Alkaline protease production from these isolates was examined by radial diffusion assay method. In this method wells having 7 mm diameter were created on CPYA solid media plates with the help of cork borer and 0.5 ml of crude filtrate was poured into them. These plates were incubated at 37°C for initially 24 hrs. than up to 48 hrs. After that TCA (Tri chloro acetic acid) solution (10 %) was added to these plates and zone of diffusion was measured as alkaline protease activity. The enzyme activity was calculated as Relative enzyme activity (REA).

REA = Diameter of zone of clearance (in mm)/ (well diameter in mm)

Phenotypic Characterization

Phenotypic characterization of isolates was determined by Bergey's manual of bacteriology[19]. Differential staining was performed and observations were made under Binocular microscope.

DNA Isolation, PCR Amplification and DNA Sequencing

Total genomic DNA was isolated from the most promising alkaline protease producing Isolate, using HiPer Bacterial Genomic DNA extraction kit, followed by amplification of 16s rRNA gene with universal primers by PCR. Thus, obtained Amplified DNA was further sent to Genexplore Diagnostics and Research Centre Pvt. Ltd. Ahmadabad, Gujrat, India, for DNA sequencing.

Identification

The 16s rRNA sequence was subjected to BLAST analysis against 16s rRNA gene database in NCBI (National Centre for Biotechnology Information). A phylogenetic tree was constructed with related bacterial 16s rRNA gene sequences from NCBI using MEGA[7] program by maximum likelihood option[20]. The Neighbor-Joining method was used to infer evolutionary history[21]. On the basis of 16S rRNA homology and phylogenetic tree analysis, the most promising isolate was identified. 16s rRNA gene sequence of the isolate was submitted to Genbank database (https://www.ncbi.nlm.nih.gov/genbank).

RESULTS

Isolation and Screening

A large number of isolates were isolated (Fig 1) and screened for alkaline protease production. Colonies with different characteristics appearing on CPYA medium plates were isolated and screened for casein hydrolysis on CPYA

Table 1. Relative Enzyme Activity (REA) of selected alkaline protease producing isolates

Sr. N.	Isolate No.	Zone of Hydrolysis (in mm)	Well diameter (in mm)	REA
1	PF11	19	7	2.71
2	PF13	18	7	2.57
3	PF21	20	7	2.86

Table 2. Phenotypic characterization of selected alkaline protease producing isolates

Sr.N.	Isolate No.	Color of colony	Gram reaction	Cell shape
1	PF11	Creamy white	Positive	Rod
2	PF13	Off white	Positive	Rod
3	PF21	White	Positive	Rod

medium. A total number of 3 isolates: Isolates no. PF11, PF13, PF21 who have produced a zone of clearing in the range of excellent and good were selected for further study (Fig 2).

Enzyme Production and Well Diffusion Assay

Crude extract of Isolate no. PF21 produced highest zone of clearance of 2 cm among all the isolates, followed by PF11, PF13 which produced 1.9 cm, 1.8 cm respectively (Fig 3). Relative enzyme activity (REA) for Isolate no. PF11, PF13, PF21 were 2.71, 2.57, 2.86 respectively (Table 1). Clearly Isolate No. PF21 had the highest enzyme activity.

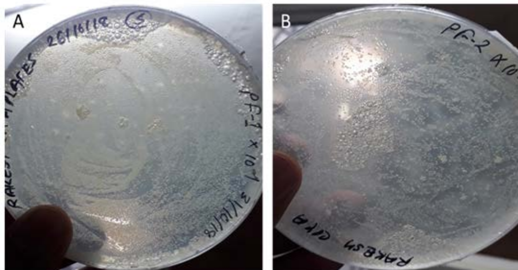

Fig. 1. Primary screening of soil samples on CPYA medium having pH 12

Phenotypic Characterization

Colony morphologies of all the isolates were small, round, regular. Colony color of Isolate no. PF11, PF 13, PF21 were creamy white, off white, white respectively. In gram staining, all the isolates were long, filamentous, rod shaped and Gram positive (Fig.4, Table 2). Further phenotypic characterization of isolate no. PF 21 also revealed that it is motile and spore forming.

DNA Isolation, PCR Amplification and DNA Sequencing

Whole genome DNA was isolated from most potent isolate PF21 and PCR amplification was obtained with 16s rRNA universal 357F forward primer having sequence (5'-3') CTCCTACGGGAGGCAGCAG and 1391R Reverse primer having sequence (5'-3') GACGGGCGGTGTGTRCA undergoing- 1 cycle of Initial denaturation at 950C for 5 min; 35 cycle of the following steps: Denaturation at 95°C for 30 sec., Annealing at 49°C for 30 sec., Elongation at 72°C for 1:30 min; and finally 1 cycle of Final Extension at 72°C for 10 min[22] (Fig. 5).

Identification

The molecular identification of isolate no.

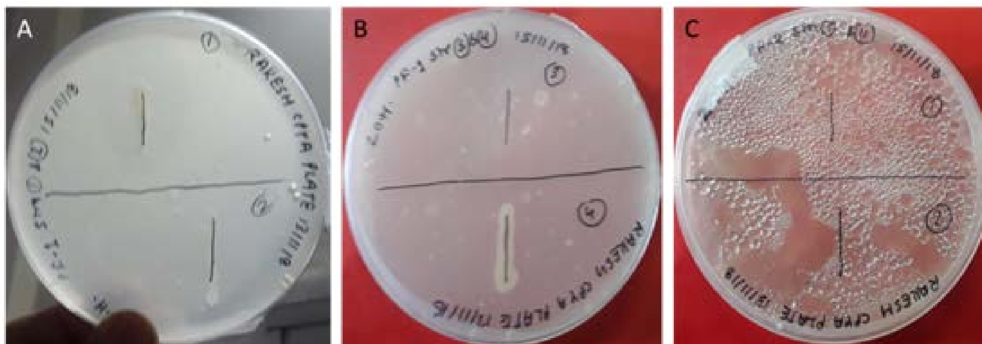

Fig. 2. Zone of clearance produced by selected isolates

Fig. 3. Well diffusion assay for relative alkaline protease activity

PF21 was done using 16S rRNA gene homology. It was found by analysis with nucleotide BLAST tool that 16S rRNA sequence of isolate no. PF21 had 95.64 % identity with the sequence of *Bacillus halodurans* DSM497 (Genebank Accession number: NR025446). A phylogenetic tree was constructed using MEGA7 program with closely related 16s rRNA gene sequences of other bacillus species. On the basis of both, 16S rRNA homology and phylogenetic tree analysis, the isolate no. PF21 was identified as *Bacillus halodurans* RSCVS-PF21. 16s rRNA sequence was submitted to Genbank database (https://www.ncbi.nlm.nih.gov/genbank) with accession no. MT279908.

DISCUSSION

Less number of bacterial colonies appeared on isolation from both sample site: 1 and site: 2 of poultry farm at alkaline pH 1223 on CPYA solid media. Poultry farm soil samples were from dried fecal matter. The pH of site: 1 and site: 2 were 10 and 11 respectively. Comparatively more dense bacterial colonies appeared from Site: 1 soil sample on serial dilution. No fungal colony appeared even after 24 to 36 hours incubation24 from both sample sites as fungus are known to be slow growing. All the colonies appeared were small, round like pin head after 24 hr incubation. Pure culture was obtained by repeated streaking.

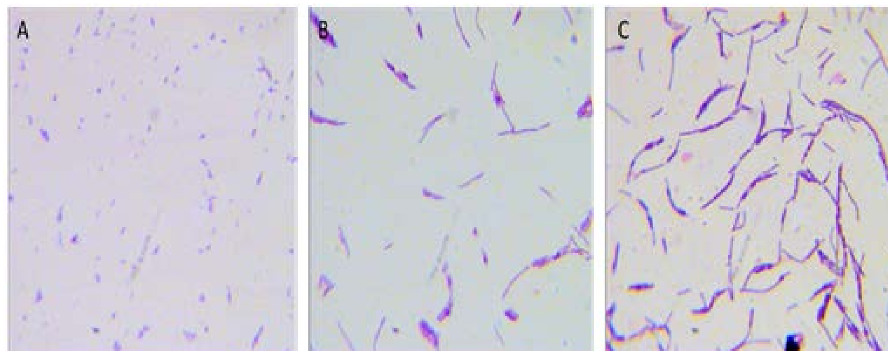

Fig. 4. Gram stain images of selected isolates. (All the images have dimension: 640-pixel x 576-pixel)

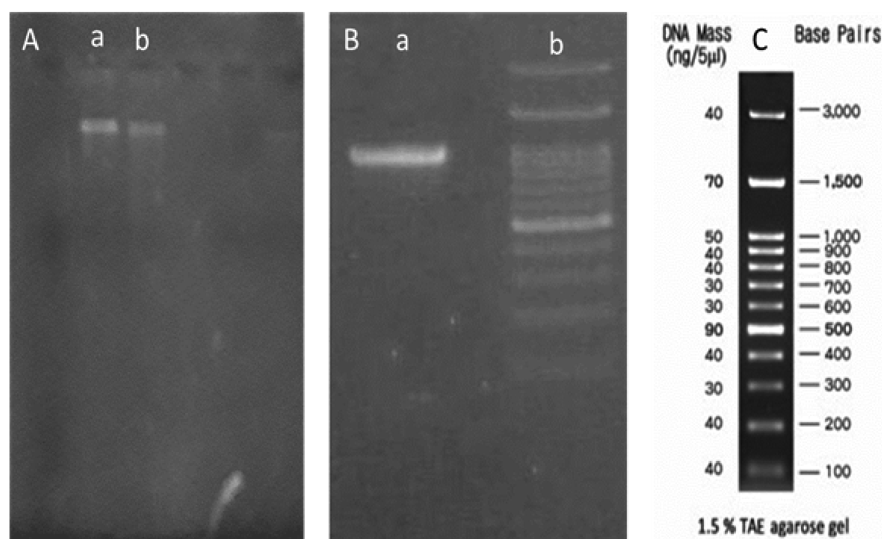

Fig. 5. Electro-phoregram of isolated whole genome DNA and amplified 16s rRNA gene of Isolate no. PF21

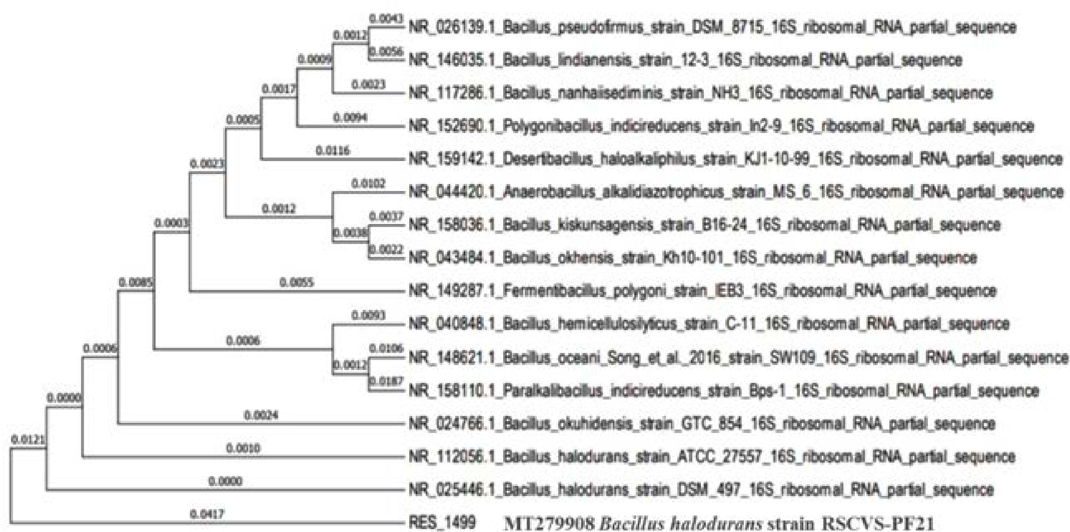

Fig. 6. Phylogenetic analysis of Bacillus halodurans RSCVS-PF21, on the basis of related 16s rRNA sequences

Out of bacterial colonies appeared, only some were able to hydrolyze casein protein[25]. Selected isolates PF11, PF13, PF21 were cultured under submerged condition to produce enzymes and all the isolate showed high relative enzyme activity on radial diffusion assay. The most potent alkaline protease producing isolate no. PF21 had highest relative enzyme activity (2.86). About 1112 bp consensus sequence was obtained after amplification of 16s rRNA gene with 357F and 1391R universal primers and DNA sequencing of amplified product. On the basis of NCBI BLAST and Phylogenetic Analysis26 it was identified as *Bacillus halodurans* RSCVS-PF21. Closest reference bacteria *Bacillus halodurans* DSM497 showed 95.64 % similarity with it though it was not reported to produce alkaline protease[27-29]. Some other identical bacteria were *Bacillus halodurans* strain ATCC 27557 (95.13 %), *Bacillus okuhidensis* strain GTC 854 (95.03 %), Fermentibacillus polygoni strain IEB3 (94.02 %), *Bacillus nanhaiisediminis* strain NH3 (94.02 %). There were also other *Bacillus halodurans* strains recently reported to produce alkaline proteases[30-33]. Phenotypically also *Bacillus halodurans* RSCVS-PF21 was related with many other alkaline bacillus species having long, rod shaped, gram positive, white colony, motile and spore forming characteristics.19 The alkaline protease produced was extracellular in nature as was checked by well diffusion assay. Crude enzyme was stable and active at pH 12 indicating its alkaline nature.

CONCLUSION

The study describes alkaline protease producing *Bacillus halodurans* RSCVS-PF21 (Genebank: MT279908) isolated from soil of Government poultry farm near Chirahula temple of Rewa, Madhya Pradesh, India. The bacterium was able to grow at pH 12. It showed high extra cellular alkaline protease activity and the crude enzyme was stable at pH 12 indicating its alkaline nature. Phenotypically and phylogenetically it was closely related with other alkaline bacillus species but it was closest to *Bacillus halodurans* DSM497. Further optimization of enzyme production and physio-chemical characterization of enzyme may identify the bacterium as a potential candidate for alkaline protease industry.

ACKNOWLEDGEMENT

This work was carried out at Centre for Biotechnology Studies, Awadhesh Pratap Singh University Rewa, Madhya Pradesh, India and was supported by facilities and financial resources available to the centre, which is gratefully acknowledged.

REFERENCES

1. Haki G.D., Rakshit S.K. Developments in industrially important thermostable enzymes: a review. *Bioresour. Technol.* 2003; **89**(1): 17-34.
2. Cui H., Yang M., Wang L., Xian C.J. Identification of a New Marine Bacterial Strain SD8 and Optimization of Its Culture Conditions for Producing Alkaline Protease. *PLoS One* 2015; **10**(12): e0146067.
3. Jegannathan K.R., Nielsen P.H. Environmental assessment of enzyme use in industrial production – a literature review. *J. Clean. Prod.* 2013; **42**: 228-240.
4. Verma J., Pandey S. Characterization of partially purified alkaline protease secreted by halophilic bacterium Citricoccus sp. isolated from agricultural soil of northern India. *Biocatal. Agric. Biotech.* 2019; **17**: 605-612.
5. Sharma K.M., Kumar R., Panwar S., Kumar A. Microbial alkaline proteases: Optimization of production parameters and their properties. *J. Genet. Eng. Biotechnol.* 2017; **15**(1): 115-126.
6. Beg Q.K., Gupta R. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis.Enzym. Microbiol. Technol.* 2003; **32**(2): 294-304.
7. Razzaq A., Shamsi S., Ali A., Ali Q., Sajjad M., Malik A., Ashraf M. Microbial Proteases Applications. *Front. Bioeng. Biotechnol.* 2019; **7**(110): 1-20. https://doi.org/10.3389/fbioe.2019.00110.
8. Genckal H., Tari C. Alkaline protease production from alkalophilic Bacillus sp. isolated from natural habitats. *Enzym. Microbiol. Technol.* 2006; **39**: 703–710. https://doi.org/10.1016/j.enzmictec.2005.12.004.
9. Guleria S., Walia A., Chauhan A., Shirkot C. K. Immobilization of *Bacillus amyloliquefaciens* SP1 and its alkaline protease in various matrices for effective hydrolysis of casein. *3 Biotech.* 2016; **6**: 208.
10. Horikoshi K. Alkaliphiles: some applications of their products for biotechnology. *Microbiol. Mol. Biol. Rev.* 1999; **63**(4): 735-50.
11. Doddapaneni K.K., Tatineni R., Vellanki R.N., Rachcha S., Anabrolu N., Narakuti V., Mangamoori L.N. Purification and characterization of a solvent and detergent-stable novel protease from Bacillus cereus. *Microbiol. Res.* 2009;**164**(4): 383-390.
12. Deng A., Wu J., Zhang Y., Zhang G., Wen T. Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus sp.* B001. *Bioresour. Technol.* 2010; **101**(18): 7111-7.
13. Baweja M., Tiwari R., Singh P.K., Nain L., Shukla P. An Alkaline Protease from Bacillus pumilus MP 27: Functional Analysis of Its Binding Model toward Its Applications as Detergent Additive. *Front. Microbiol.* 2016; **7**: 1195.
14. Thebti W., Riahi Y., Belhadj O. Purification and characterization of a new thermostable, haloalkaline, solvent stable, and detergent compatible serine protease from Geobacillus toebii strain LBT 77. *BioMed. Res. Int.* 2016; 1–8.
15. Atlas R.M. Handbook of Microbiological Media, fourth ed. CRC Press, Taylor & Francis Group, New York. 2010.
16. Agrawal S., Rawat H.K., Kango N. Isolation and screening of alkaline protease producing bacteria from different soil habitats. *Madhya Bharti J. Sci.,* 2016;**60**(1): 44-48.
17. Rajamani S., Hilda A. Plate assay to screen fungi for proteolytic activity. *Curr. Sci.* 1987; **56**: 1179-1181.
18. Vermelho A.B., Meirelles M.N.L., Lopes A., Petinate S.D.G., Chaia A.A., Branquinha M.H. Detection of extracellular proteases from microorganisms on agar plates. *Mem. Inst. Oswaldo Cruz* 1996; **91**: 755-760.
19. Sneath P.H.A., Mair N.S., Sharpe M.E., Halt J.G. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore 1986; 1325–1329.
20. Kumar S., Stecher G., Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 2016; **33**(7): 1870-4.
21. Saitou N., Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 1987; **4**(4): 406-25.
22. Turner S., Pryer K.M., Miao V.P., Palmer J.D. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J. Eukaryot. Microbiol.* 1999; **46**(4): 327-38.
23. Kumar C.G., Takagi H. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnol. Adv.* 1999; **17**(7): 561-94.
24. Rousk J., Brookes P.C., Bååth E. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. *Appl. Environ. Microbiol.* 2009; **75**(6): 1589-96.
25. Suganthi C., Mageswari A., Karthikeyan S., Anbalagan M., Sivakumar A., Gothandam K.M. Screening and optimization of protease production from a halotolerant Bacillus licheniformis isolated from saltern sediments. *J. Genet. Eng. Biotechnol.* 2013; **11**(1): 47-52.
26. Agasthya A.S., Sharma N., Mohan A., Mahal

P. Isolation and molecular characterisation of alkaline protease producing Bacillus thuringiensis. *Cell Biochem. Biophys.* 2013;**66**(1): 45-51.
27. Boyer E. E., Ingle M. B., Mercer G. D. Bacillus alcalophilus subsp. halodurans subsp. nov.: an alkaline amylase producing alkalophilic organism. *Int.J.Syst.Bacteriol.* 1973; **23**: 238-242.
28. Fritze D., Flossdorf J., Claus D. Taxonomy of alkaliphilic Bacillus strains. *Int.J.Syst.Bacteriol.* 1990; **40**: 92-97.
29. Nielsen P., Fritze D., Priest F. G. Phenetic diversity of alkaliphilic Bacillus strains: proposal for nine new species. *Microbiology* 1995; **141**: 1745-1761.
30. Takii Y., Kuriyama N., Suzuki Y. Alkaline serine protease produced from citric acid by Bacillus alcalophilus subsp. halodurans KP 1239. *Appl. Microbiol. Biotechnol.* 1990; **34**(1): 57-62.
31. Takami H., Nogi Y., Horikoshi K. Reidentification of the keratinase-producing facultatively alkaliphilic *Bacillus sp.* AH-101 as *Bacillus halodurans*.*Extremophiles* 1999; **3**: 293-296.
32. Daoud L., Hmani H., Ali M.B., Jlidi M., Ali M.B. An Original Halo-Alkaline Protease from *Bacillus halodurans* Strain US193: Biochemical Characterization and Potential Use as Bio-Additive in Detergents. *J. Polym. Environ.* 2018; **26**: 23–32.
33. Yakul K., Takenaka S., Nakamura K., Techapun C., Leksawasdi N., Seesuriyachan P., Watanabe M., Chaiyaso T. Characterization of thermostable alkaline protease from *Bacillus halodurans* SE5 and its application in degumming coupled with sericin hydrolysate production from yellow cocoon. *Process Biochem.* 2019; **78**: 63-70.