Original Research Article

Effects of temperature and pH on the stability of protease produced by *Alcaligenes faecalis* P2

Abstract

Background: Bacterial proteases represent a group of very important industrial enzymes. They are involved in the hydrolysis of peptide bond found in protein. Industrial application of bacterial proteases has been limited by low yield and instability at biotechnological process conditions. This study was design to investigate the effect of temperature and pH on the stability of protease produced by *Alcaligene faecalis* strain P2.

Methodology: Protease-producing bacteria were isolated from beans effluent-impacted soil and screened for protease production on Casein agar plate. Protease assay was carried out following standard method for protease determination and the stability of the protease produced was investigated over temperature range of 20 to 90 ^{o}C and pH range of 3 to 12. The protease-producing bacterium was identified using its molecular characteristics.

Results: Protease assay result showed that the amount of tyrosine released by one unit of the crude enzyme was 0.176 µmol/mL resulting in 0.19 U/mL protease activity. Stability result showed that the protease had wide spectrum of pH and temperature stability. The protease was most stable at pH 9 and temperature of 40 ^{o}C after 30 min. Maximum protease activity of 1.259 U/mL was recorded with pH 9 after 30 min. The protease-producing bacterium was classified as *Alcaligene faecalis* P2 based on phylogenetic analysis of its 16S gene analysis. The sequences have been submitted to GenBank under the accession number MZ477004.

Conclusion: The retention of protease activity over wide pH and temperature ranges showed that the protease had high stability. This makes the protease a good candidate for industrial scale up. This study therefore has demonstrated that beans effluent-impacted soil harbours protease producing bacteria with high industrial potentials.

Keywords: Protease; Beans effluent; *Alcaligene faecalis*, Casein agar.

1.0 Introduction

Proteases are a group of commercially important enzymes due to their wide applications in the industry. Proteases occupy the largest part in commercial enzyme market and find applications in biotechnological processes. A review of industrial application of proteases by Rassaq *et al*. (2019) reveals that proteases have been applied in detergent and leather industries where they help in the removal of blood stains and in wool making; they have been used in (Miyaji *et al*., 2006; Dodia *et al*., 2008; Patil and Chaudhari, 2009; Soroor *et al*., 2009; Simkhada *et al*., 2010; Vadlamani and Parcha, 2011), soy sauce, protein hydrolysate, production of seasoning material,

beer clearing and fruit juice, as digestive aids, tendering the fibril muscle, improving texture of flour past (Zhang *et al*.,2010; Pushpam *et al*., 2011), in food industry and brewery (Razzaq *et al*., 2019).

Protease can be produced from different sources but production of proteases from microorganisms have flourished immensely. Interests in microbial proteases have been attributed to the wide applicability of these proteases. Moreover, certain features such microbial proteases high yield less production time, less space requirement, easy genetic manipulation, and cost efficiency, have increased their suitability for many biotechnological applications in the market (Nisha and Divakaran, 2014; Ali *et al*., 2016).

In order to make proteases industrially attractive and competitive, a number of techniques have been employed in improving their yield (Razzaq *et al*., 2019). Some of these methods used to optimize the production of protease include response surface optimization, exploitation of different substrates and genetic manipulation (Rathakrishnan and Nagarajan, 2012; Aruna *et al*., 2014). Moreover, researchers have exploited various techniques with the aim of improving protease yield for industrial applications some of such techniques have included cloning and overexpression, fed batch, screening of strains, and chemostat fermentation. Different statistical methods including one factor at a time and response surface methodology have been exploited in the optimization of medium and growth parameter for improved yield (Kumar *et al*., 2012; Homaei *et al*., 2016; Rehman *et al*., 2017).

Despite the successes recorded and advantages of using microbial proteases, a number of factors have limited their large-scale industrial applications (Razzaq *et al*., 2019). Major challenges have been thermal instability coupled with high cost of enzyme recovery; these have restricted the application of proteases in the industry.

This study was therefore designed to investigate the effect of pH and temperature on the stability of protease from *Alcaligenes faecalis* P2 isolated from beans effluent-impacted soil in Port Harcourt, Rivers State, Nigeria.

2.0 Research Methodology

2.1 Sample collection

Beans effluent impacted soils were collected from Rumuosi, Port Harcourt, Rivers State. The soil was air-dried for 24 h, sieved to remove debris and thereafter used for the isolation of protease-producing bacteria.

2.2 Serial Dilution, Isolation and Screening for Protease Production

Serial dilution was performed on the soil samples as described by Jalal *et al*. (2010). Nine (9) millilitres of normal saline (0.85 % of NaCl w/v in distilled water) was dispensed into clean test tubes and the test tubes were sterilized in an autoclave at 121^{o}C (15 psi) for 15 min and then allowed to cool. One gram (1 g) of the dry soil sample was dissolved in the 9 ml sterile normal saline to make a stock solution. From this stock solution several (10^{-1}- 10^{-6}) dilution were made.

Protease-producing bacteria were isolated using the medium composition consisting of (g/L): lactose 10; casein 10; KH_2PO_4 2; K_2HPO_4 2; $MgSO_4$.$7H_2O$ 1, and agar 15. One hundred microlitre of aliquots from various dilutions (10^{-3} - 10^{-6}) were plated in duplicate on agar. Inoculated agar plates were incubated at ambient temperature for 48 h (Sharma *et al.*, 2014). Bacteria that showed clearance zones on the agar plates were selected as protease producers and thereafter sub-cultured onto freshly prepared agar plates. These protease producers were purified on nutrient agar plate and store in an agar slant containing the minimal medium.

2.3 Protease Assay

Protease assay was carried out according to the method described by Tambekar and Tambekar (2013). In brief, selected protease-producing bacterial isolates were inoculated into 20 mL of protease production medium comprising: lactose 10 g; casein 10 g; KH_2PO_4 2 g; K_2HPO_4 2 g; $MgSO_4$.$7H_2O$ 1 g) and incubated for 48 h on a rotary shaker (200 rpm) at room temperature (Sharma *et al.*, 2014; Saraswathy *et al*., 2013; Tambekar and Tambekar 2013). After incubation, the culture media were centrifuged at 5000 rpm for 20 min and at 4 °C. Cell free supernatants were collected and used as the crude enzyme for protease assay. Protease assay was conducted by a modified method by Cupp-Enyard (2008). Casein dissolved in different pH buffers [acidic (pH:4), neutral (pH:7) and alkaline (pH:9)] were used as the substrate for the assay. The reaction mixture contained casein and the crude enzyme solution. The mixture was incubated at 37 °C for 10 min. Thereafter, the reaction was stopped by addition of 3 ml of 20% ice-cold trichloro acetic acid (TCA). Precipitated proteins were removed by centrifugation and 0.5 ml of the supernatant was mixed with 2.5 ml of 0.5 M Na_2CO_3 and kept for 20 min at room temperature. Finally,

Folin's phenol reagent was added to the mixture, kept for 10 min and absorbance was measured at 660 nm against the blank sample. The amount of enzyme required to liberate 1 µmole of tyrosine per ml per minute under the standard conditions defined one unit of protease activity (Hameed *et al*., 1999).

2.4 Effect of pH on the stability of protease

The stability of the crude protease over wide pH range (3 to 12) was examined. The methods of Nizamudeen and Bajaj (2009) and Ponnuswamy and Prakash (2012) were employed to determine the effect of pH on crude protease stability. A volume of 0.5 mL of the crude enzyme was pre-incubated without substrate; thereafter the buffer was adjusted to the appropriate pH values and incubated at 35 ^{o}C for 15 min, 30 min and 1 h, respectively and the protease activity determined thereafter.

2.5 Effect of temperature on the stability of protease

The stability of the crude protease over wide temperature range (20 to 90 °C) was examined. To determine thermal stability of the enzyme, 0.5 mL of the crude pectinase was incubated without substrate at increasing temperatures of 20, 30, 40, 50, 60, 70 and 90 ^{o}C for 15 min, 30 min and 1 h, respectively and then assayed for protease activity.

2.6 Identification of Protease-Producing Bacteria

The selected protease-producing bacterium was identified base on the molecular characteristics of the isolate. Bacterial genomic DNA extraction was carried using ZR Soil Microbes DNA Mini-Prep extraction kit (Zymo Research Corporation, South Africa). The quantity and purity of the extracted genomic DNA of the bacterium was analysed using an ND-1000 spectrophotometer (Thermoscientific, Inqaba Biotech, South Africa) and agarose gel electrophoresis. The extracted genomic DNA was stored at -20 ^{o}C. The primer sets 27F (5^{1}AGAGTTTGATCMTGGCTCAG-3^{1}) and 1492R (5^{1}GGGTTACCTTGTTACGACTT3^{1}) were used for PCR amplification. PCR reaction parameters included: PCR cocktail of volume 25 µL consisting of 12.5 µL of the Master Mix (Zymo Master Mix), 0.4 µL of each primer, 5 µ of the DNA template and 6.7 6.7 µL of sterile nuclease-free water. The PCR conditions were as follows: Initial denaturation (95 ^{o}C for 5 min), denaturation (95 ^{o}C for 30 sec.), annealing (52 ^{o}C for 30 sec), extension (72 ^{o}C for 45 sec.), and final extension step (72 ^{o}C for 3 min) and hold (4 ^{o}C at ∞). About 5 µL of the amplified

products was run on agarose gel electrophoresis at 120 V for 15 min to determine the quality of the products. The amplified products were also purified using DNA clean and concentrator (DCC) kit (Zymo research institute, South Africa) in preparation for sequencing.

PCR products of the bacterial DNA were sequenced with 3500 ABI genetic analyser at Inqaba Biotechnical Industries, South Africa utilizing the Sanger Sequencing technology. Generated sequences were visualized using Chromaslite for base calling. BioEdit was used for sequence editing and Basic Local Alignment Search Tool (BLAST) was performed using NCBI (National Center for Biotechnology Information) database. Similar sequences were downloaded and aligned with ClustalW and phylogenetic tree drawn with MEGA 7 software (Kumar *et al*., 2018).

3.0 Results and Discussion

3.1 Screening Characteristics of the Protease-Producing Bacterium

The protease-producing bacterium (*Alcaligene faecalis* P2) used in this study showed zone of clearance on Casein agar plate. Hydrolysis of the isolate on casein agar plate is shown in Figure 1. The zone of hydrolysis on casein agar showed 4.16 cm^2 clearance zone. Protease assay result showed that the amount of tyrosine released by one unit of the crude enzyme was 0.176 µmol/mL resulting in 0.19 U/mL protease activity.

3.2 pH and Thermal Stability of the Crude Protease

Data obtained from the stability result showed that the protease was stable over wide pH and temperature ranges. The amount of tyrosine released and protease activity were highest after 30 mins and at temperature of 40 ^oC. Maximum tyrosine was 1.144 µmol/mL whereas the highest protease activity was 1.259 U/mL (Figure 2). Highest pH stability was observed at pH 9 with corresponding maximum tyrosine release and proteases activity as 1.94 µmol/mL and 2.13 U/mL, respectively after 30 min (Figure 3).

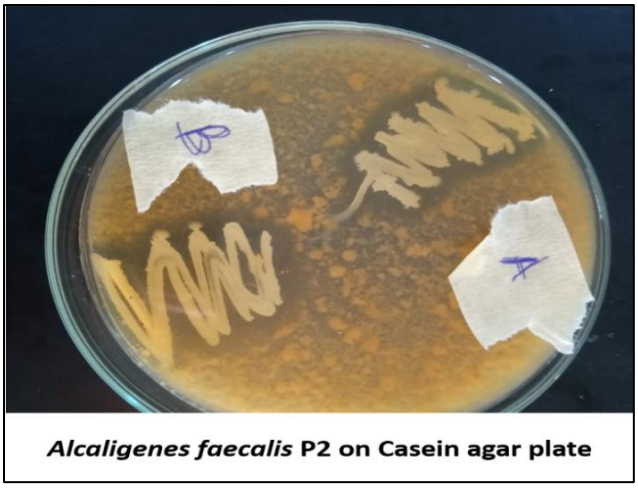

Figure 1: Casein Agar Plate screening of the protease-producing bacterium *Alcaligene faecalis* P2.

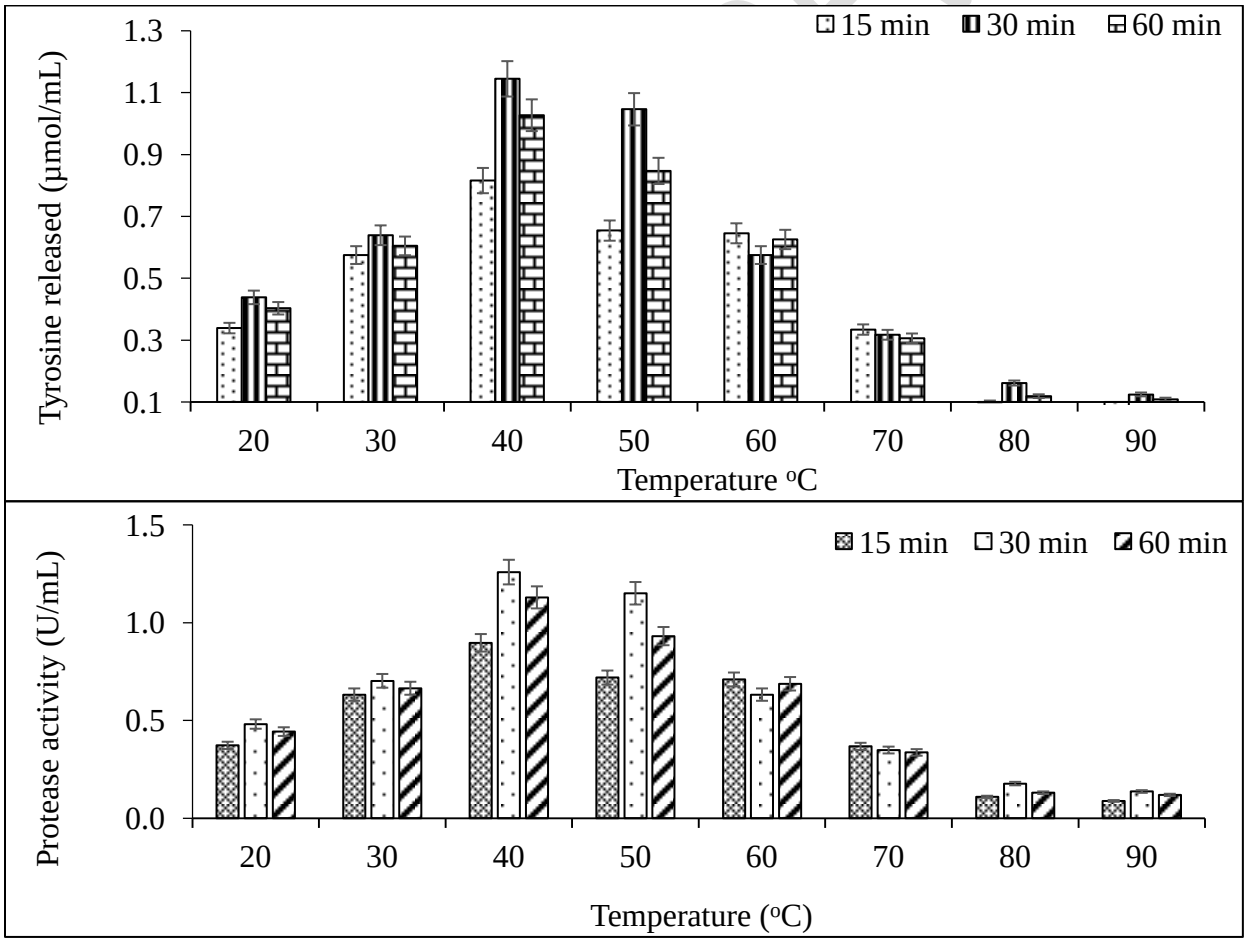

Figure 2: Stability of protease produced by *Alcaligenes faecalis* P2 at different temperatures indicated by released tyrosine and protease activity.

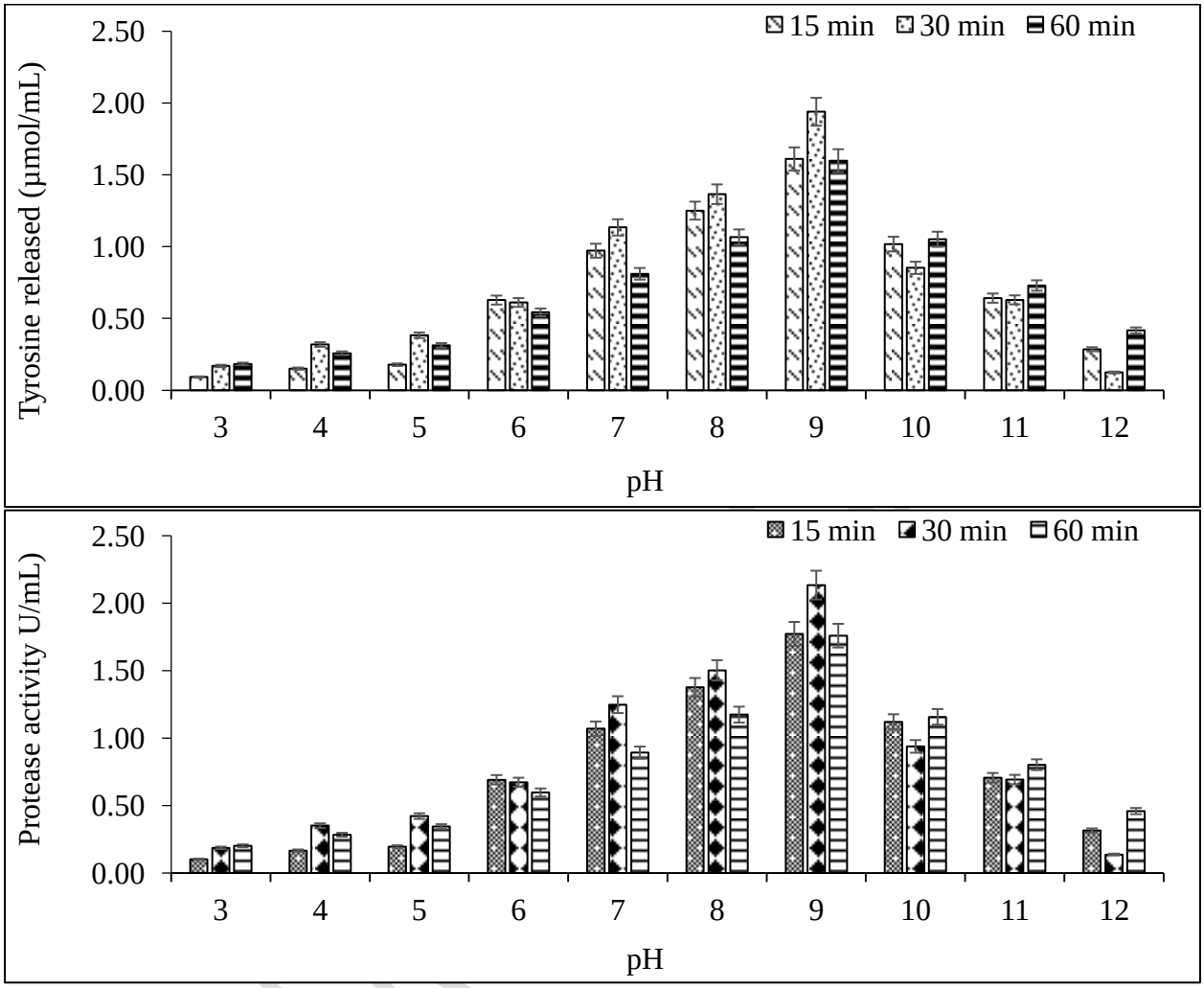

Figure 3: Stability of protease produced by *Alcaligenes faecalis* P2 at different pH indicated by released tyrosine and protease activity.

3.3 Molecular Characteristics of the Protease-Producing Bacterium

Phylogenetic tree analysis of the DNA sequences obtained from the protease-producing bacterial classified the isolate as *Alcaligenes faecalis* P2. The phylogenetic showing the related GenBank isolates is presented in Figure 4.

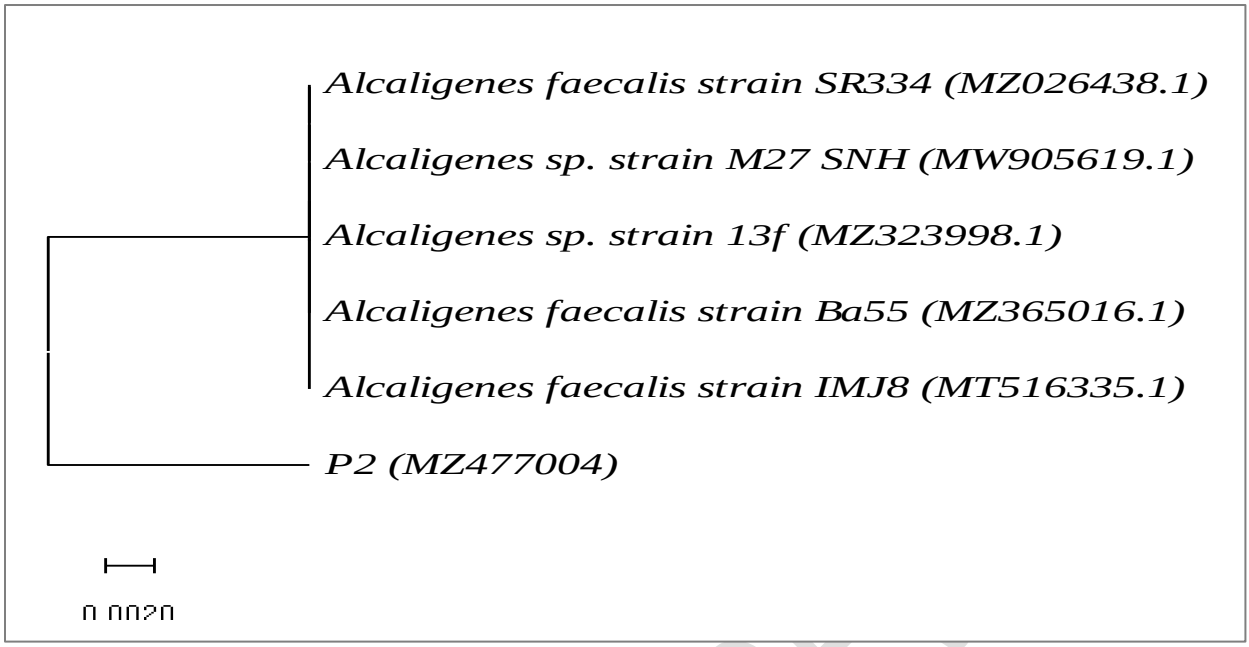

Figure 4: Phylogenetic tree of protease-producing bacterium *Alcaligenes faecalis* P23.4

Discussion

This study investigated the effect of pH and temperature on the stability of protease produced by *Alcaligenes faecalis* P2 isolated from beans effluent-impacted soil from Port Harcourt, Rivers State Nigeria. Beans effluent are rich sources of protein and was therefore chose are it would likely harbour microorganisms with proteolytic capacity. Luang-In *et al*. (2019) described the isolation of protease-producing bacteria from soil. Udgire *et al*. (2015) reportred the isolstion of protease-producing bacteria from tannery effluents. This shows that many different effluents can effectively serve as source of protease-producing bacteria.

The bacterium was classified as *Alcaligene faecalis* P2 based on phylogenetic analysis of its 16S gene analysis. The sequences have been submitted to GenBank under the accession number MZ477004. Thangam *et al*. (2000) reported that the *Alcaligenes faecalis* isolated from tannery soil produced protease efficiently. Similarly, *Alcaligenes faecalis* was reported as an efficient alkaline protease producer by Marathe *et al*. (2018). The findings of this study corroborate this claim as the *Alcaligenes faecalis* strain P2 used in this present study was characterized as an alkaline protease producer.

The *Alcaligenes faecalis* P2 was stable over a wide range of pH and temperature. This observation is important in industrial application of the `enzyme due to the vary temperature and

pH conditions during biotechnology process. Temperature and pHs are rate limiting factors in enzyme activity. Bhunia *et al*. (2013) noted the at higher or lower pHs, the stable 3-D structures of enzymes get compressed, resulting in lower residual activity. The findings of this study corroborated this assertion as at lower and higher temperatures and pH above the optimum, residual protease activity was greatly reduced.

Conclusion

This study demonstrated that the protease-producing bacterium, *Alcaligenes faecalis* P2 used in this study was stable over wide range of temperature and pH, with the optimum pH at 9 and temperature at 40 ^{o}C. The stability of enzymes over wide temperature and pH ranges are important characteristics for industrial applications as most industrial application are performed at varying temperature and pH ranges. Thus, the protease used in this study can be scaled up for industrial application. Furthermore, the study reveled that soils impacted by rich proteineous substrates are rich source of protease-producing bacteria.

References

Ali, N., Ullah, N., Qasim, M., Rahman, H., Khan, S. N. and Sadig, A. (2016). Molecular characterization and growth optimization of halo-tolerant protease producing *Bacillus subtilis* strain BLK-1.5 isolated from salt mines of Karak, Pakistan. *Extremophiles*, 20, 395–402. doi: 10.1007/s00792-016-0830-1

Aruna, K., Shah, J., and Birmole, R. (2014). Production and partial characterization of alkaline protease from *Bacillus tequilensis* strains CSGAB0139 isolated from spoilt cottage cheese. Int J Appl Biol Pharm. 5, 201–221.

Bhunia, B., Basak, B., Mandal, T., Bhattacharya, P., & Dey, A. (2013). Effect of pH and temperature on stability and kinetics of novel extracellular serine alkaline protease (70kDa). *International Journal of Biological Macromolecules,* 54, 1–8. doi:10.1016/j.ijbiomac.2012.11.024.

Cupp-Enyard, C. (2008). Sigma's Non-specific Protease Activity Assay - Casein as a Substrate. *Journal of Visualized Experiments,* (19), doi:10.3791/899.

Dodia, M., Rawal, C., Bhimani, H., Joshi, R., Khare, S., and Singh, S. P. (2008). Purification and stability characteristics of an alkaline serine protease from a newly isolated Haloalkaliphilic bacterium sp. AH-6. *Journal of Industrial Microbiology and Biotechnology*, 35, 121–131. doi: 10.1007/s10295-007-0273-x

Haddar A., Agrebi R., Bougatef A., Hmidet N., Sellami-Kamoun A., and Nasri M. Two detergent stable alkaline serine-proteases from Bacillus mojavensis A21: purification, characterization and potential application as a laundry detergent additive. *Bioresources Technology*, 100, 3366-3373 (2009)

Hameed A, Keshavarz T, Evans CS. Effect of dissolved oxygen tension and pH on the production of extra cellular protease from a new isolate of Bacillus subtilis K2, for use in leather processing. *Journal of Chemical Technology and Biotechnology*, 1996; 74:5-8.

Homaei, A., Lavajoo, F., and Sariri, R. (2016). Development of marine biotechnology as a resource for novel proteases and their role in modern biotechnology. *International Journal of Biological Macromolecules*. 88, 542–552. doi: 10.1016/j.ijbiomac.2016.04.023

Jalal, A., Rashid, N., Rasool, N. and Akhtar, M. (2009). Gene cloning and characterization of a xylanase from a newly isolated *Bacillus subtilis* strain R5. *Journal of Bioscience and Bioengineering*. 107: 360–365.

Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (**2018**). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* **35**:1547-1549.

Kumar, D. M., Premavathi, V., Govindarajan, N., Balakumaran, M., and Kalaichelvan, P. (2012). Production and purification of alkaline protease from Bacillus sp. MPTK 712 isolated from dairy sludge. *Global Vetenary*, 8, 433–439.

Luang-In V, Yotchaisarn M, Saengha W, Udomwong P, Deeseenthum S. & Maneewan K. (2019). Protease-Producing Bacteria from Soil in Nasinuan Community Forest, Mahasarakham Province, Thailand. *Biomedical and Pharmacology Journal*, 2(2).

Miyaji, T., Otta, Y., Nakagawa, T., Watanabe, T., Niimura, Y., and Tomizuka, N. (2006). Purification and molecular characterization of subtilisin-like alkaline protease BPP-A from *Bacillus pumilus* strain MS-1. *Letters in Applied Microbiology*. 42,242–247. doi: 10.1111/j.1472-765X.2005.01851.x

Nizamudeen S, Bajaj B. A (2009). Novel thermoalkali-tolerant endoglucanase production using cost-effective agricultural residues as substrates by a newly isolated *Bacillus* sp. NZ. Food Science and Biotechnoogy, 47:435-440.

Patil, U., and Chaudhari, A. (2009). Purification and characterization of solvent-tolerant, thermostable, alkaline metalloprotease from alkalophilic *Pseudomonas aeruginosa* MTCC 7926. *Journal* of *Chemical Technology and Biotechnology*, 84, 1255–1262. doi: 10.1002/jctb.2169

Ponnuswamy, V. and Prakash, V.S.G. (2012). Purification and characterisation of carboxymethylcellulose from *Bacillus* sp. isolated from a paddy field. *Polish Journal of Microbiology*, 61:51-55.

Pushpam, P. L., Rajesh, T., and Gunasekaran, P. (2011). Identification and characterization of alkaline serine protease from goat skin surface metagenome. *AMB Express,* 1:3. doi: 10.1186/2191-0855-1-3

Rathakrishnan, P., and Nagarajan, P. (2012). Optimizing factors affecting protease production by a *Bacillus cereus* using groundnut shell under solid substrate fermentation. *International Journal of Scientific Inventions Todays*, 1, 114–129.

Razzaq, A., Shamsi, S., Ali, A., Ali, Q., Sajjad, M., Malik, A., & Ashraf, M. (2019). Microbial Proteases Applications. *Frontiers in Bioengineering and Biotechnology*, *7*, 110. https://doi.org/10.3389/fbioe.2019.00110.

Saraswathy, N., Yeole, G. S., Parikh, A. J. and Meena, C. (2013). Production and optimization of protease from *Bacillus licheniformis* NCIM 2044. *Asian Journal of Pharmaceutical Life Science*, 3, 9-15.

Sharma, K. M., Kumar, R., Vats, S. & Gupta, A. (2014). Production, partial purification and characterization of alkaline protease from *Bacillus aryabhattai* K3. *International Journal of Advances in Pharmacy, Biology & Chemistry*, 3, 290-298.

Simkhada, J. R., Cho, S. S., Park, S. J., Mander, P., Choi, Y. H., Lee and H. J. (2010). An oxidant-and organic solvent-resistant alkaline metalloprotease from *Streptomyces olivochromogenes. Applied Biochemistry and Biotechnology*. 162, 1457–1470. doi: 10.1007/s12010-010-8925-0

Soroor, M. A., Hendawy, H., Ghazy, A., Semary, N., Khalil, K., and Aziz, A. (2009). Characterization of an alkaline metalloprotease secreted by the entomopathogenic bacterium *Photorhabdus* sp. strain EK1. *Research Journal of Agriculture and Biological Sciences*, 5, 349–360.

Tambekar, S. D. and Tambekar, D. H. (2013). Optimization of the production and partial characterization of an extracellular alkaline protease from thermo-halo-alkalophilic lonar lake bacteria. *Bioscience Discovery*, 4, 30-38.

Udgire, M., Vivek, S. & Sinha, P. (2015). Isolation of Thermos table Extracellular Alkaline Protease and Lipase Producing Bacteria from Tannery Effluents. *International Journal of Biotech Trends and Technology* (IJBTT), 5(1). 26-29.

Vadlamani, S., and Parcha, S. R. (2011). Studies on industrially important alkaline protease production from locally isolated superior microbial strain from soil microorganisms.

International Journal of Biotechnological Applications, 3, 102–105. doi: 10.9735/0975-2943.3.3.102-105

Zhang, G., Wang, H., Zhang, X., and Ng, T. (2010). Helvellisin, a novel alkaline protease from the wild ascomycete mushroom *Helvella lacunosa*. *Journal of Bioscience and Bioengineering*, 109, 20–24. doi: 10.1016/j.jbiosc.2009.06.022.