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Estimation of Protease Enzyme Bioactivity Isolated from Bacterial Strains

Reham F. El-kased

Department of Microbiology & Immunology, Faculty of Pharmacy, The British University in Egypt, El-Sherouk City, 11837 Cairo, Egypt.

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* Correspondence Author:

E-mail address:

reham.kased@bue.edu.eg

Abstract

Fifteen bacterial strains were isolated from soil samples collected from Northern East region of the delta Egypt. Bacterial strains were screened for proteolytic activity on saline skim milk agar medium where the most potent protease producer strain was identified by maximum diameter of the clear zone formed; 22 mm ± 1.0, highest concentration (1000 µg/mL) and highest activity; 350 U/mL The selected bacterial strain was characterized by Gram stain, morphologically and by biochemical tests where it was identified as *Bacillus* species. Protease enzyme was extracted using Gelatin yeast extract glucose broth then partially purified by saturation with ammonium sulphate followed by dialysis. A final purification step was done using High performance liquid chromatography. Molecular weight and purity of the extracted protease were determined using SDS gel electrophoresis and was estimated to be 30 kDa. The effect of different factors, *viz* pH, temperature and surfactants, on the activity and stability of the extracted protease was studied. The extracted protease showed highest relative activity at pH 10 and 35 ºC. While it showed stability at pH range 8 - 11 and temperature range 30 ºC - 50 ºC. The effect of Tween-80 (1%) and SDS (1%) showed 1% protease activity inhibition while concentration (2%) of both surfactants showed 10% and 12 % activity inhibition, respectively. In the current study, the extracted protease enzyme exhibited potential properties and it showed activity in the presence of surfactants, therefore, is recommended for further research work and enzyme-based industrial applications to be used in different industries.

Keywords: Bacillus sp., HPLC, SDS PAGE

1. Introduction

Microbes are great sources of metabolites specially protease enzymes. Among different classes of enzymes, protease is the main class that catalyzes proteolysis. Animals, plants and microbes are the main sources of protease, where about 60% of worldwide output is from microbial sources (Zheng YP, 2003). Microbial proteases are more substantial than plant and animal proteases because of its availability and facilitated recovery. Moreover, large quantities of microorganisms could be cultured in a short time, and they can ensure a regular and abundant enzyme supply. Furthermore, microbial protease has extended shelf life and can be easily stored for long periods while still maintaining their activity. Microbial protease holds the major characteristics needed for biotechnological applications (Gupta R, 2002) as they are used in medical diagnosis, detergent industry, food processing, silk industry, pharmaceutical industry and waste utilization (Gupta R, 2002) (Kalisz, 1988) (Hakim, 2018). Several bacterial species are very

valuable sources of industrial proteases, such as *Bacillus* (Gupta R, 2002) and *Pseudomonas* (Rahman, 2018). Gram-positive rod-shaped *Bacillus* species are bacteria which produce valuable metabolites including protease. *Bacillus* are found in large numbers in soil constituting most of bacterial load in soil environment (Abdul R., 2019).

Extracellular proteins secreted by *Bacillus* sp make it valuable for usage in food and drug administration (Abdul R., 2019). Among the *Bacillus* protease producers are *B. mojavensis* (Chmara H., 1982), *B. lichneforms* (Moyne A.L., 2004), *B. flexus, B. subtilis, B. amyloliquefaciens, B. infantis, ,B. cereus* (Koumoutsi A., 2004), *B. sphaericus, ,B. megaterium,B. brevis,B. anthracis* (Carrillo C., 2003), *B. coagulans* (Aranda F.J., 2005), *B. thuringiensis* (P.M., 2015), *B. thermoruber , B. sterothermophilus* (Gupta R, 2002), *B. intermedius* (Kumar C.G., 1999), *B. circulans* (Naruse N., 1990), *B. cohnii, , B. pseudofirmus, B. pantotheneticus* (Arguelles-Arias A., 2009), *B. aquimaris, B. proteolyticus* (Jisha., 2013), *B. pumilus* (Grangemard I., 2001), *B. laterosporus, B. clausii, B. fastidiosus* (Sandeep Kaur Saggu, 2017), *B. amovivorus, , B. horikoshii* (Uttam C.B., 1999).

Due to their increasing need in industries, scientists are constantly exploring further features of proteases trying to produce new enzymes with new characteristics that can improve the industries (Kasana R.C., 2011) (Jisha V.N., 2013) (Kaman W.E., 2014) (Duza M.B., 2013).

In this study, 15 bacterial strains were isolated from soil and investigated for protease production. The potent protease producer was characterized and the effect of different factors, such as pH, temperature and surfactants, on the activity and stability of the extracted protease was studied.

2. Materials and methods:

2.1 Isolation and screening of bacteria for qualitative protease activity:

Soil samples were collected from different fields of Northern East region of the delta Egypt. Fifteen bacterium strains were isolated and assayed for proteolytic activity. Sterile distilled water was used to serially dilute samples then dilutions were streaked on nutrient agar plates followed by incubation for 24 h at 37◦C. Single colonies were tested for protease activity using saline skim milk agar medium containing 2% (w/v) skim-milk, 1% (w/v), glucose 1% (w/v) tryptone and yeast extract 0.55 (2.5g/L), adjusted to pH 8.0. (Harely P.J. and Prescott, 1996). Plates were incubated for 24 h where apparent clear zones of hydrolysis indicated proteolytic activity qualitatively (Sanchez-Porro C, 2003).

Strains were assayed for protease concentration and activity where the bacterium strain which had the maximum proteolytic concentration and activity was selected for further tests.

2.2 Morphological characterization of proteolytic bacterial strains:

Bacteria with proteolytic activity were investigated under bright field microscope after simple staining with gram stain.

2.3 Biochemical characterization of proteolytic bacterial strains:

Morphological characterization was followed by identification using biochemical methods. This was done by performing IMViC tests, Nitrate reduction test, catalase test, starch and lactose fermentation, oxidase test and gelatin hydrolysis test as standard protocols according to Bergey's manual of systematic bacteriology (Sneath, 1986).

2.4 Crude enzyme extraction:

The proteolytic bacterial strains were cultured in Gelatin yeast extract glucose broth containing gelatin 1 gm/100ml, Yeast extract 0.2 gm/100mL, glucose 1 gm/100mL, Di-potassium hydrogen phosphate 0.3 gm/100mL, Potassium di-hydrogen phosphate 0.1 gm/100mL at pH 7.5. The supernatant containing the protease enzyme was extracted after centrifugation at 6000 rpm at 4ºC for 20min then was used for the following experiments.

2.5 Protease quantitative assay:

2.5.1 Protease Purification

Crude enzyme was precipitated by 50% saturation with ammonium sulphate then taken out in a dialysis bag for dialysis. The bag was immersed in 100 ml of 0.025M phosphate buffer and was maintained for 24 h on a magnetic stirrer. Additional purification of the dialyzed protein was done using High performance liquid chromatography (HPLC - HP 1100 chromatography system Agilent, Boeblingen, Germany, UltraSep ESD 300 PROT C18 end-capped column). 5 fractions were collected and tested for protease activity as mentioned above. Peaks showing protease activity were pooled and the prepared purified protease sample was used in all further experiments.

2.5.2 Protease activity assay:

Protease activity was tested according to Casein-folin method (Boethling, 1975). The test mixture consisted of 1 ml of 1% (w/v) casein in 0.1 M Tris-HCl buffer (pH 8.0) and 0.1 ml of enzyme solution. Incubation was done at 37 °C for 30 min, then the reaction was

stopped by addition of 1.5 ml of 5% (w/v) trichloroacetic acid (TCA). This was followed by centrifugation at 10,000 rpm for 5 min, then for the color development for the assay of tyrosine in the filtrate 100 µL of 1 M NaOH solution was added to 100 µL of the supernatant followed by addition of 3 ml diluted folin reagent (one ml in 2 ml distilled water). The resulting solution was vortexed for 3 min, then Absorbance (A) was measured at 660 nm wavelength to estimate protease activity. Blanks were made by adding TCA before adding the substrate. One unit enzyme activity was the amount of enzyme producing 1µg of tyrosine under standard assay conditions and expressed as unit/ml.

2.5.3 Molecular weight and purity determination of purified protease:

Molecular weight of purified protease was estimated using Sodium Dodecyl sulphate Polyacrylamide gel electrophoresis (SDS PAGE 4-12% Bis-Tris Plus Gels) using the Xcell SurelockTM MiniCell electrophoresis chamber [Invitrogen, Karlsruhe, Germany] at a constant voltage of 125 V for 60 min. Sample buffer used was Bis-Tris (50 mM), 6 N HCl, NaCl (50 mM), Glycerol (10%), running buffer used was MOPS (50 mM), Tris base (50 mM), SDS (0.1%), EDTA (1 mM), pH 7.7. Gels were fixed with a mixture of 50 % ethanol/10% acetic acid/water solution followed by staining with colloidal Coomassie Brillant Blue G- 250 (Neuhoff, 1988).

2.5.4 Determination of protease concentration:

Protease concentration was estimated according to (Lowry, 1951) using a standard (bovine serum albumin (BSA). Concentration was calculated from the standard curve as µg/ml.

2.6 Protease stability and activity profiles under different conditions:

Optimal pH and temperature for protease activity were determined, besides its stability under different pH and temperature conditions. Also, the effect of surfactants was estimated for purified protease enzyme using casein solution as substrate.

2.6.1 Influence of pH on protease activity and stability:

Protease activity was determined at several pH values (from pH 5.0 to pH 12.0) with casein solution. pH adjustment was done using different buffers (50 mM): glycine NaOH (11.0- 12.0), Tris-HCl (9.0-10.0), phosphate (pH 7.0- 8.0) and citrate phosphate (pH 5.0-6.0),. Different buffers were mixed separately with the purified protease at a ratio of 1:1. This was followed by incubating the reaction mixtures for 30 min at room temperature and then proteolytic activity was estimated as in the standard assay stated earlier. Incubation of the purified enzyme extract in several buffers (pH 5.0-12.0) for 24 h at room temperature was done to determine enzyme stability.

2.6.2 Influence of temperature on enzyme activity and stability:

Protease activity determination was done by preparing a mixture containing purified protease extract and casein solution in 50 mM Tris-HCl (pH 10.0) followed by incubation for 30 min at several temperatures (25 °C to 65°C). Protease extract was pre-incubated in 50 mM Tris-HCl (pH 10.0) for 1 h at several temperatures (25°C to 65°C) then rapidly cooled to detect protease stability with temperature changes. After incubation, proteolytic activity was determined as in the standard assay stated earlier.

2.6.3 Effect of surfactants on protease activity

Proteolytic activity against Sodium Dodecyl Sulfate (SDS) and Tween 80 was tested. This was done by incubating a mixture of the purified enzyme and casein solution in 1% and 2% of each surfactant at 37 ºC for 1 h. Proteolytic activity was tested as stated above.

2.7 Experimental design and statistical analyses

All experiments were done thrice, and their mean values represented. The values reported are means ± Standard deviation (Cochran, 1987).

3. Results and discussion:

This study investigates the presence and bioactivity of protease-producing bacteria collected from soil samples from the Northern East region of the delta Egypt.

3.1 Screening of bacteria for qualitative proteolytic activity

Fifteen morphologically different bacteria were isolated from the collected samples (S1, S2, S3,……..S15). Skim milk agar plates were used for screening of protease producing colonies where formation of a clear zone indicated a protease producing bacteria as shown in table 1. Only 5 isolates showed to be strong protease producing bacteria.

The concentration of the protease enzyme was detected in the 5 protease producing bacteria and they were further subjected to enzyme activity assay to choose the isolate with highest enzyme concentration and activity as shown in table 1. Only one isolate (S11) showed the highest enzyme concentration; (1000 µg/mL), and activity (350 U/mL); indicated by the diameter of the clear zone on skim milk agar (22 mm); and was, therefore, chosen for further assays and screening.

Table 1: Clear zone diameter for 15 isolates and Protease concentration for the 5 isolates with the highest clear zone diameter

Isolate	Clear zone diameter (mm ± SD)	Protease concentration (µg/mL ± SD)
S1	5 ± 0.3	-
S2	7 ± 0.6	-
S3	10 ± 0.5	-
S4	8 ± 0.8	-
S5	5 ± 0.4	-
S6	21 ± 1.0	850 ± 1.1
S7	11 ± 0.8	-
S8	20 ± 1.1	870 ± 1.5
S9	11 ± 0.8	-
S10	18 ± 0.7	790 ± 1.0
S11	22 ± 1.0	1000 ± 1.2
S12	9 ± 0.6	-
S13	13 ± 0.9	-
S14	15 ± 1.0	-
S15	18 ± 1.0	770 ± 1.0

3.2 Characterization of proteolytic bacterial strains

The selected strain was subjected to preliminary morphological characterization after staining with gram stain. This was followed by further identification using different biochemical screening assays as shown in table 2.

After investigating the isolate morphologically and performing the characteristic biochemical analysis it was shown that this isolate belongs to *Bacillus* species protease producers.

Table 2: Morphology and biochemical tests results of the selected isolate S11:

Bacterial colony morphology	Round creamy colony
Microscopic identification	Gram positive rods arranged in chains
Biochemical tests	
Gram stain	Positive
Catalase	Positive
Oxidase	Negative
Indole	Negative
Methyl red	Negative
Voges-Proskauer	Negative
Citrate utility	Positive
Starch utility	Positive
Nitrate reduction	Positive
Gelatin hydrolysis	Positive
Lactose fermentation	Negative

3.3 Protease Purification and molecular weight determination

Extracted Protease enzyme was partially purified with 50% saturation with ammonium sulphate followed by dialysis. Then the enzyme was further purified using HPLC, where 5 peaks were collected then tested for protease activity where all gave positive results. The 5 peaks were then pooled in one fraction. Molecular weight and protease purity were determined for the pooled fractions using SDS PAGE, where it appeared as a single band at 30 kDa indicating the purity of the separated fraction as shown in (Fig. 1).

The extracted protease appeared to have molecular weight of 30 kDa as detected by SDS PAGE.

Comparing the molecular weights of studied *Bacillus* proteases in literature it appears that there is a wide range of estimated molecular weights lying within the range of 15 to 86.29 kDa (Adinarayana K., 2003) and (Arulmani M., 2007).

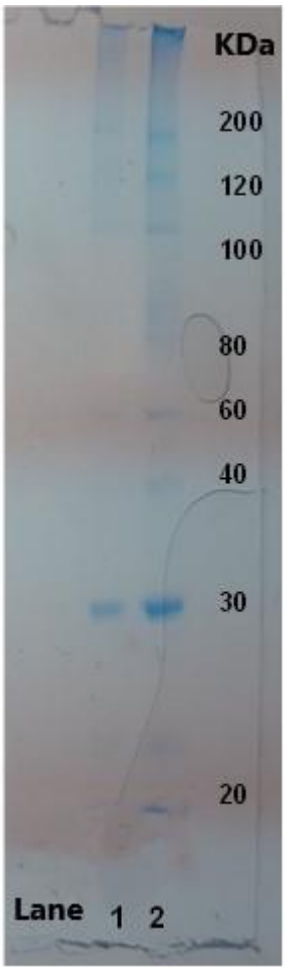

Figure 1: SDS PAGE of extracted protease. Lane 1: Extracted protease showing apparent molecular weight of 30 kDa. Lane 2: Broad range molecular weight marker (kDa).

3.4 Protease stability and activity profiles under different conditions:

Afterwards optimum pH and temperature conditions for enzyme activity and pH and thermal stabilities were determined.

The ability of the microorganism to produce extracellular protease depends on the pH of the culture medium as an important criterion (Khusro, 2016).

Extracted protease enzyme displayed activity within the pH range of 8 and 11 while highest activity was shown to be at pH 10 which demonstrates the alkaline nature of the extracted protease as shown in Fig. 2. Protease enzyme showed stability in the pH range of 8–11 while stability decreased above pH 11 as shown in Fig. 3. This estimated range was near the optimum pH range mentioned in literature which was found to be between 9.0 and 11 for alkaline proteases from *Bacillus* spp. (Gençkal, 2006). A pH 9 was mentioned in literature as optimum for protease production by *Bacillus* sp. strain APP1 (Chu., 2007), *Bacillus* sp. (Prakasham R.S. C. R., 2006), *Bacillus proteolyticus* (Bhaskar N., 2007). Higher initial pH, 10 for *B. licheniformis* (Vaithanomsat P., 2008), pH 10 and 10.5 has been reported to be suitable for *B. circulans* protease production (Jaswal R.K. G. K., 2008) (Prakasham R.S., 2005) (dos Santos Aguilar, 2018), and 10.7 for *Bacillus* sp. 2–5 (Darani K.K. H. F., 2008) were also stated for maximum protease production. *B. subtilis* protease production was almost maintained the same regardless of the initial pH within the range examined (5.0–10.0) (Sekar, 2020).

As mentioned in literature the optimum temperature for protease production was species dependent (Shanthakumari & Bominathan, 2017).

In this study, the optimal temperature for protease activity was estimated to be 35 ºC while gradual decrease in activity occurred above this temperature as shown in Fig. 4. Protease thermal stability was found to be in the range from 30 ºC till 50 ºC as shown in Fig. 5. The temperature range for optimal proteolytic activity of several proteases in literature was between 30 ºC and 47 ºC (Gençkal, 2006)

Being a critical parameter for protease production, temperature must be controlled and varied from organism to organism to ensure maximum cell growth and enzyme production. The optimal temperature range stated in literature for protease production by discrete organisms is greatly variable. An optimal temperature of 35 ºC was reported for protease production by *B. licheniformis* and *B. coagulans* (Otroshi, 2014) and *B. cereus* (Asha, 2018) (Kebabci O., 2011). Other studies reported lower optimum temperature of 25 ºC for *B. circulans* (Jaswal R.K., 2008), and 28 ºC for *B. cinerea* (Abidi F., 2008). A temperature of 37 ºC was reported for a number of *Bacillus* species as optimal temperature for protease production such as *B. amovivorus* (Sharmin S., 2005), *B. proteolyticus* (Bhaskar N., 2007), *B. aquimaris* (Shivanand P., 2009) and *B. subtilis* strain Rand (Abusham R.A., 2009). On the other hand, 40 ºC temperature was mentioned in literature to be optimal for protease production by *Bacillus* sp. 2–5 (Darani K.K., 2008), *B. licheniformis* (Seifzadeh S., 2008). A high temperature of 50 ºC was found optimal for *Bacillus* sp. strain APP1 (Chu, 2007) and *B. subtilis* (Shaheen M., 2008). While higher temperature of 60 ºC was reported to be the optimal temperature for protease production by *B. cereus* and *B. polymyxa* (Maal K.B., 2009).

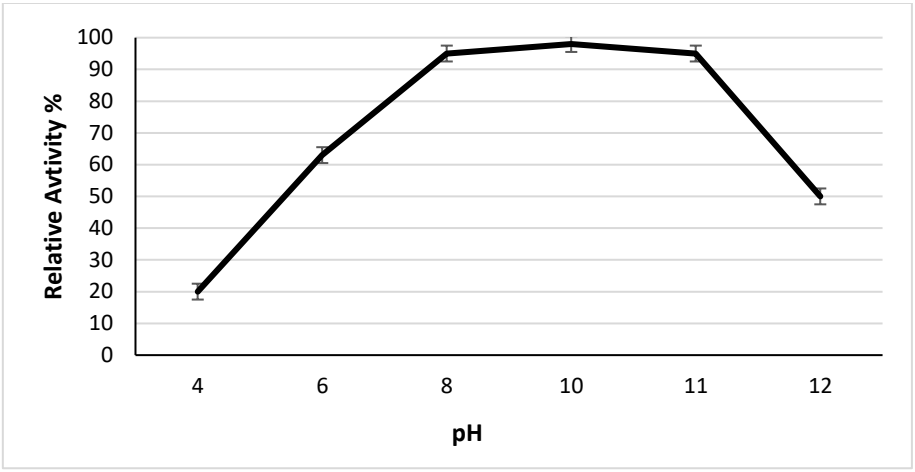

Figure 2 Influence of pH on extracted protease activity.

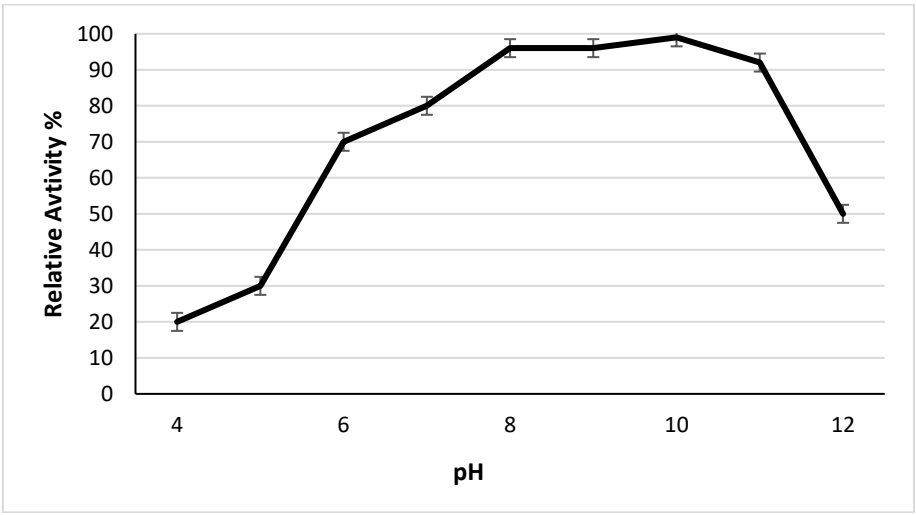

Figure 3 Influence of pH on extracted protease stability.

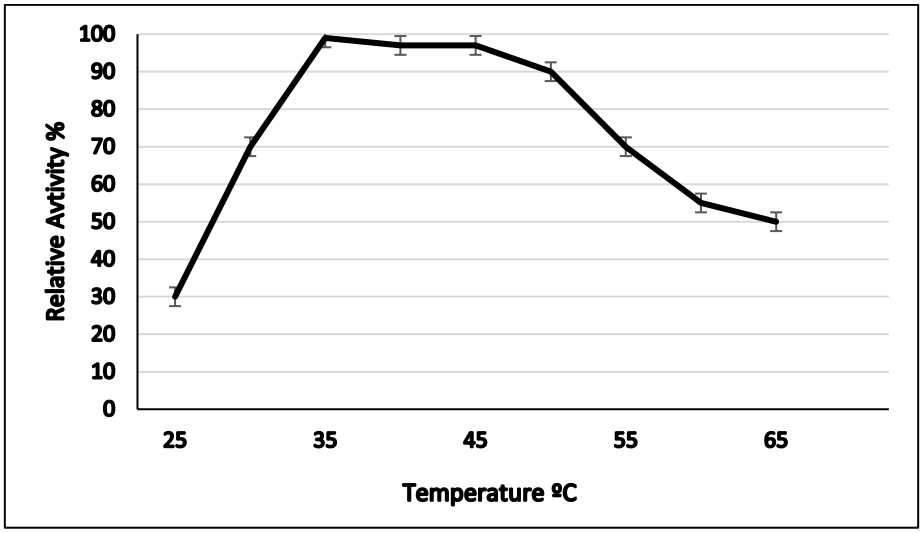

Fig. 4 Effect of temperature on extracted protease activity.

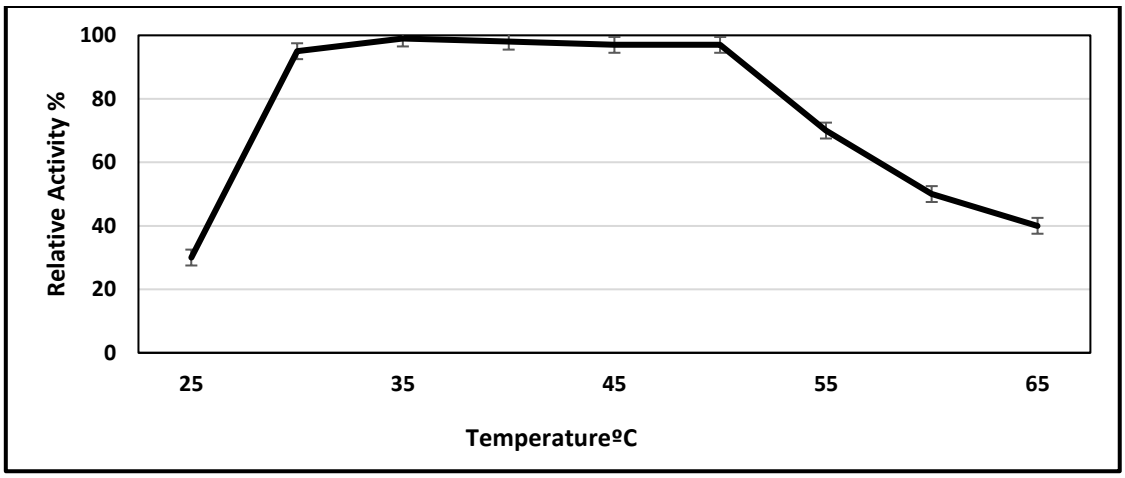

Fig. 5 Effect of temperature on extracted protease stability.

The surfactants tested, Tween-80 (1%) and SDS (1%), showed 1% protease activity inhibition effect while concentration (2%) of both surfactants showed 10% and 12 % activity inhibition, respectively, as shown in (Table 3).

The effect of surfactants on protease activity in literature differs greatly. SDS (1%) showed 27% activity inhibition on protease activity extracted from *Bacillus patagoniensis* (Oliveira L.A., 2006). While the presence of Triton X-100 (1%) showed a stimulatory effect. At a concentration of 1%, SDS and Tween-80 caused strong inhibitory effects (50%) on protease activity (Pathak AP, 2020).

Table 3 Effect of surfactants on extracted protease activity.

Surfactant	Protease activity (%)	Protease activity reduction (%)
Control*	100	100
SDS (1%)	99	1%
SDS (2%)	90	10%
Tween 80 (1%)	99	1%
Tween 80 (2%)	88	12%

* Protease activity in absence of surfactant

4. Conclusions:

There is a strong demand to discover and develop new protease enzymes for industrial applications, but still optimum producing strains have to be evaluated case by case.

The present study focused on isolating, screening, and identifying protease-producing bacteria from soil environment. The extracted protease enzyme exhibited potential properties and it showed activity in the presence of surfactants, therefore, it is recommended for further research work and enzyme-based industrial applications to be used in different industries.

Conflict of Interest

The author declares no conflict of interest.

5. References

Abdul R., S. Sadia, A. Arfan, A. Qurban, S. Muhammed, M. Arif, Muhammed. (2019). Microbial proteases applications. *Font. Bioeng. Biotechnol.*

Aguilar dos Santos, J. G. (2018). Microbial proteases: Production and application in obtaining protein hydrolysates. . *Food Research International.*, 103, 253-262.

Abidi F. , F. Limam, M.M. Nejib. (2008). Production of alkaline proteases by Botrytis cinerea using economic raw materials: assay as biodetergent. *Process. Biochem.*, 43(11), 1202–1208.

Abusham R.A. , R.N.Z.R.A. Rahman, A.B. Salleh, M. Basri. (2009). Optimization of physical factors affecting the production of thermo-stable organic

solvent-tolerant protease from a newly isolated halo tolerant Bacillus subtilis strain Rand. *Microb. Cell Fact.*, 8(1), 1-9.

Adinarayana K. , P. Ellaiah, D.S. Prasad. (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus subtilis PE-11. *AAPS Pharm. Sci. Technol.*, 4, 1–9.

Aranda F.J. , J.A. Teruel, A. Ortiz, (). (2005). Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A. *BBA*, 1713, 51–56.

Arguelles-Arias A., M. Ongena, B. Halimi, Y. Lara, A. Brans, B. Joris, P. Fickers. (2009). Bacillus amyloliquefaciensGA1as a source of potent antibiotics and other secondarymetabolites for biocontrol of plant pathogens. *Microb. Cell Fact.*, 8, 63–74.

Arulmani M. , K. Aparanjini, K. Vasanthi, P. Arumugam, M. Arivuchelvi, P.T. Kalaichelvan. (2007). Purification and partial characterization of serine protease from thermostable alkalophilic Bacillus laterosporus-AK1. *World J. Microbiol. Biotechnol.*, 23, 475–481.

Asha, B. &. (2018). Optimization of alkaline protease production by Bacillus cereus FT 1isolated from soil. . *Journal of Applied Pharmaceutical Science*, 8(2), 119-127.

Bhaskar N. , E.S. Sudeepa, H.N. Rashmi, A.T. Selvi,. (2007). Partial purification and characterization of protease of Bacillus proteolyticus CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresour. Technol.*, 98(14), 2758–2764.

Boethling, R. S. (1975). Regulation of extracellular protease secretion in Pseudomonas maltophilia. *Journal of Bacteriology*, 123(3), 954-961.

Carrillo C. , J.A. Teruel, F.J. Aranda, A. Ortiz,. (2003). Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin, BBA 1611. 91–97.

Chmara H. , S. Milewski, M. Dzieduszycka, M. Smulkowski, P. Sawlewicz, E. Borowski. (1982). Epoxypeptides—a novelgroup of metabolic inhibitors in procaryotic and eucary-otic organisms. *Drugs Exp. Clin. Res.*, 11–12.

Chu., W. (2007). Optimization of extracellular alkaline protease production from species of Bacillus. *J. Ind. Microbiol. Biotechnol.*, 34(3), 241–245.

Cochran, W. a. (1987). *Statistical Methods.* USA: The Lowa State University Press, Lowa.

Darani K.K. , H.R. Falahatpishe, M. Jalali,. (2008). Alkaline protease production on date waste by an alkalophilic Bacillus sp. 2-5 isolated from soil. *Afr. J. Biotechnol.*, 7(10), 1536–1542.

Duza M.B. , S.A. Mastan. (2013). Microbial enzymes and their applications–a review. *Indo Am. J. Pharm. Res.*, 3 (8), 6208–6219.

Gençkal, H. &. (2006). Alkaline protease production from alkalophilic Bacillus sp. isolated from natural habitats. . *Enzyme and Microbial Technology, ,* 39(4), 703-710.

Grangemard I. , J. Wallach, R. Maget-Dana, F. Peypoux. (2001). Lichenysin: a more efficient cation chelator than surfactin. *Appl. Biochem. Biotechnol.*, 90, 199–210.

Gupta R, B. Q. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. . *Appl Microbiol Biotechnol. ,* 59:15–32.

Hakim, A. B. (2018). Production and partial characterization of dehairing alkaline protease from Bacillus subtilis AKAL7 and Exiguobacterium indicum AKAL11 by using organic municipal solid wastes. *Heliyon*, 4(6).

Harely P.J. and Prescott, M. (1996). *Laboratory exercises in microbiology.* McGraw. Hill, USA.

Jaswal R.K. , G.S. Kocher, M.S. Virk,. (2008). Jaswal, R. Production of alkaline protease by Bacillus circulans using agricultural residues: a statistical approach. *Indian J. Biotechnol.*, 7, 356–360.

Jisha V.N. , R.B. Smitha, S. Pradeep, S. Sreedevi, K.N. Unni, S.Sajith, P. Priji, M.S. Josh, S. Benjamin. (2013). Versatility of microbial proteases.. *Adv. Enzyme Res.*, 1, 39–51.

Kalisz, H. (1988). Microbial proteases. *Adv Biochem Eng Biotechnol.*, 36:1–65.

Kaman W.E. , J.P. Hays, H.P. Endtz, F.J. Bikker. (2014). Bacterial proteases: targets for diagnostics and therapy. *European journal of clinical microbiology & infectious diseases.*, 33 (7), 1081–1087.

Kasana R.C. , R. Salwan, S.K. Yadav. (2011). Microbial proteases: detection, production, and genetic improvement. *Crit. Rev. Microbiol.*, 37 (3), 262–276.

Kebabci O. , N. Cihangir. (2011). Isolation of protease producing novel Bacillus cereus and detection of optimal conditions. *Afr. J. Biotechnol.*, 10(7), 1160–1164.

Khusro, A. (2016). One Factor at A Time based optimization of protease from poultry associated Bacillus licheniformis. *J. Appl. Pharm. Sci.*, 6, 88–95.

Koumoutsi A., X.-H. Chen, A. Henne, H. Liesegang, G. Hitzeroth, P. Franke, J. Vater, R. Borriss, s. (2004). Structural and func-tional characterization of gene clusters directing nonrib-osomal synthesis of bioactive cyclic lipopeptides inBacillus amyloliquefaciensstrain FZB42. *J. Bacteriol.*, 186, 1084–1096.

Kumar C.G., H. Takagi. (1999). Microbial alkaline proteases. *Biotechnol. Adv.*, 17 (7), 561–594.
Lowry, O. R. (1951). Protein measurement with the Folin phenol reagent. . *Journal of biological chemistry*, 193(1), 265-275.

Maal K.B., G. Emtiazi, I. Nahvi. (2009). Production of alkaline protease by Bacillus cereus and Bacillus polymixa in new industrial culture mediums and its immobilization. *Afr. J. Microbiol. Res.*, 3 (9) 491–497.

Moyne A.L., T.E. Cleveland, S. Tuzun. (2004). Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin. *FEMS Microbiol. Lett.*, 234, 43–49.

Naruse N. , O. Tenmyo, S. Kobaru, H. Kamei, T. Miyaki, Oki KonishiM, T. (1990). Pumilacidin, a complex of new antiviralantibiotics: production, isolation, chemical properties, structure and biological activity. *J. Antibiot.*, 43, 267–280.

Neuhoff, V. A. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis.*, 9(6), 255-262.

Oliveira L.A., P. A. (2006). Production of xylanase and protease by Penicillium janthinellum CRC 87M–115 from different agricultural wastes. *Bioresour. Technol.*, 97(6):862–867.

Otroshi, B. A. (2014). Study on Activity and Stability of Proteases from Bacillus sp. Produced by Submerged fermentation. . *International Journal of Advanced Biological and Biomedical Research*, 2(7), 2283-2287.

Pathak AP, R. M. (2020). Enhanced catalytic activity of Bacillus aryabhattai P1 protease by modulation with nanoactivator. . *Heliyon*, 4;6(6):e04053.

Prakasham R.S., C.S. Rao, R.S. Rao, P.N. Sarma. (2005). Alkaline Protease Production by an Isolated Bacilluscirculans under Solid-State Fermentation Using Agroindustrial Waste: Process Parameters Optimization. *Biotechnol. Prog.*, 21, 1380–1388.

Prakasham R.S. , C.S. Rao, P.N. Sarma,. (2006). Green gram husk—an inexpensive substrate for alkaline protease production by Bacillus sp. in solid-state fermentation. *Bioresour. Technol.*, 97(13), 1449–1454.

Rahman, M. S. (2018). Screening of protease producing bacteria from tannery wastes of leather processing industries at Hazaribag, Bangladesh. . *Jahangirnagar University Journal of Biological Sciences.*, 7(1), 23-34.

Sanchez-Porro C, M. S. (2003). Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *J Appl Microbiol*, 295-300, 94.

Sandeep Kaur Saggu, P. C. (2017). Characterization of thermostable alkalineproteases from Bacillus infantis SKS1 isolated from garden soil. . *PLoS One*, 1–18.

Seifzadeh S. , R.H. Sajedi, R. Sariri. (2008). Isolation and characterization of thermophilic alkaline proteases resistant to sodium dodecyl sulfate and ethylene diamine tetraacetic acid from Bacillus sp. GUS1. *Iran J. Biotechnol.*, 6, 214–224.

Sekar, A. &. (2020). Production of Industrial Important Enzymes from Marine Isolates. *Encyclopedia of Marine Biotechnology*, 4, 2323-2330.

Shaheen M. , A.A. Shah, A. Hameed, F. Hasan. (2008). Influence of culture conditions on production and activity of protease from Bacillus subtilis BS1. *Pak. J. Bot.*, 40(5), 2161–2169.

Shanthakumari, A., & Bominathan, M. (2017). Studies on screening of Bacillus sp. for protease production. *Int. J. Theor. Appl. Sci.*, 9,294–299.

Sharmin S. , M.T. Hossain, M.N. Anwar. (2005).

Isolation and characterization of a protease producing bacteria Bacillus amovivorus and optimization of some factors of culture conditions for protease production. *J. Biol. Sci.*, 5(3), 358–362.

Shivanand P. , G. Jayaraman. (2009). Production of extracellular protease from halotolerant bacterium, Bacillus aquimaris strain VITP4 isolated from Kumta coast. *Process Biochem.*, 44(10), 1088–1094.

Sneath, P. H. (1986). *Bergey's manual of systematic bacteriology. Volume 2.* Williams & Wilkins.

Souza P.M. . (2015). A biotechnology perspective of fungal proteases. *Brazilian J. Microbiol.*, 46, 337–346.

Uttam C.B. , K.S. Rajesh, A. Wamik, S. Raman. (1999). Thermostable alkaline protease from Bacillus brevis and its characterisation as a laundry detergent additive. *Process Biochem*, 35, 213–219.

Vaithanomsat P., T. Malapant, W. Apiwattanapiwat, Kasetsart J. (2008). Silk degumming solution as substrate for microbial protease production. *Agriculture and Natural Resources*, 42(3), 543–551.

Zheng YP, Y. K. (2003). Application and safety of microbial enzymes in food industry. *Food Science*, 24:256–259