Enhanced Biosynthesis and Purification of Proteases from *Bacillus* sp. AI-5 by SmF: A Green Approach for Degradation of Peptide Bonds in Complex Proteins

[1]Aliya Riaz*, [1]Isma Idrees, [1]Sana Ahmad, [2]Ayesha Siddiquiand [3]Shah Ali Ul Qader
[1]*Department of Biochemistry, Jinnah University for Women, Karachi-74600, Pakistan.*
[2]*Department of Biotechnology, Jinnah University for Women, Karachi-74600, Pakistan.*
[3]*Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan.*
aliyariaz@hotmail.com*

(Received on 13th September 2021, accepted in revised form 26th April 2022)

Summary: Proteases are theprotein degrading commercial enzymes with considerable importance for many industrial applications such as paper, leather, food industries as well as used in detergent formulation, toxic waste removal, pharmaceutical and drilling for oil. The focus of the current study was to isolate the novel protease producing bacterial sp. from environment, to optimize the submerged fermentation parameters in order to design the best supportive media for maximum enzyme yield and to purify the enzyme to increase its utilization in various industries, most importantly the pharmaceutical industry.Five pure cultures were isolated from soil of coastal area, Karachi and the bacterial strain that was proved to be the most potent producer of protease was selected for research purposeand named as *Bacillus* sp AI-5 after series of morphological and biochemical tests. The fermentation conditions and media composition were optimized using the starter medium that was selected among the three previously reported media for protease production. It was found that protease production from *Bacillus* sp AI-5 reached to maximum when media was supplemented with 0.4 gm % casein as carbon source, the combination of 0.5 gm % yeast extract and 0.5 gm % peptone as nitrogen source and 0.05 gm $CaCl_2$ as inducer and stabilizer of proteases. The optimum pH and temperature for maximum production of protease were found to be pH 5 and 45 °C respectively after 24 hours of incubation. The protease of the *Bacillus* sp. AI-5 was purified to homogeneity by salt fractionation method using 60 gm % ammonium sulfate, dialysis and Sephadex G-100 Gel filtration chromatography. The specific activity of purified protease was found to be 322.25 U/mg with 13.80 fold purification as compared to crude enzyme. The purified enzyme gave a single band on Sodiumdodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE)corresponding to a molecular weight of 66 kDa.

Keywords: *Bacillus*;Casein; Optimization; Protease; Purification;Submerged Fermentation.

Introduction

Proteases are the enzymes that are ubiquitous in nature and hydrolyze proteins and produced aminoacids and peptides. Proteases are found in plants and various animal tissues and also produced by microbes including bacteria, fungi, yeast, actinomycetes and viruses. Microbial proteases are more advantageous as compare to the proteases of plant and animal origin because they possess diversified biochemical characteristics and easy to be genetically manipulated [1]. This technical enzyme accounts for 50 % of the total industrially important enzymes [2]. Varieties of different strains of bacteria are able to produce proteases. Today, genus *Bacillus* is the most remarkable bacterial strain to produce proteases of commercial importance especially in pharmaceutical, tannary, detergent and food industry [3]. The Food and Drug Administration (US) has approved the safety profile of *Bacillus* sp. and its products to be used as food additives. Moreover, proteases find tremendous applications in leather processing, neutraceutical, healthcare products and diagnostic kit development [4].

Microbial growth in the fermentation media and subsequent production of metabolites is dependent on the nature of fermentation, either solid state or submerged. Generally, for protease production, submerged culture conditions are most suitable due to the well-defined concentration of media components and process conditions [5]. In order to obtain high yield of proteases or to upgrade the process to large scale, the fermentation process should be optimized [6]. Optimization of fermentation process refers to choose the favorable carbon as well as nitrogen sources, growth factors, inducers, optimum temperature and pH of fermented broth and production time for bacterial growth and product formation. The optimization can be carried out either by using either single variable at particular time or statistical software approach, response surface methodology (RSM) in

*To whom all correspondence should be addressed.

which all the influencing factors are optimized at the same time [7]. Genetic mutation of bacterial genome at the genes of sporulation has also been reported to enhance the production of protease [8].

Due to the vast industrial applications, hyperactive commercially viable protease producing microbial strains are needed to be isolate from the environment. Similarly, there is a constant urge to optimize the conditions that would end up in the improved yield of enzyme. Therefore the present study was conducted to achieve the above mentioned goals.

Experimental

Collection of environmental sample

In the present study, soil from coastal areas of Karachi was collected from which 1 % soil suspension was formed with deionized water that was placed at 37 °C for 7 days.

Isolation of bacterial strain

After 7 days of incubation, 1ml of soil suspension was taken in a tube containing 9 ml sterilized deionized water and then 10 fold serially diluted. The last three dilutions 10^{-5}, 10^{-6}, 10^{-7} were cultured on respectively labeled plates containing skim milk 10 gm % , NaCl 5 gm %, peptone 0.1 gm % and agar 2 gm %. The plates were placed in the incubator at 37 °C for 24 hours. Thereafter, the appearance of clear zone around the isolated colonies indicated that strains were capable of producing protease enzyme. On the basis of higher zone diameter, three cultures were picked and streaked on the skim milk agar plates separately and placed for 24 hours at 37 °C. Among these three cultures, the largest zone producer was selected for pure culture study.

Identification of bacterial strain

For identification of protease producing bacterial strain, Gram staining and series of morphological and biochemical tests were performed.

Production of protease enzyme

10 % inoculum of *Bacillus* sp. AI-5 was inoculated in medium 1 [9], medium 2 [10] and medium 3 [11] separately. The flasks were placed for incubation at 45 °C for 24 hours. The fermented medium was subjected for centrifugationfor 10 minutes at 10000 rpm at 0 ˚C. Protease activity was determined from the supernatant [12]. Medium 1 was selected for further procedures on the basis of maximum enzyme activity.

Optimization of enzyme production parameters

In order to acheive high yield of protease, various physical and chemical parameters were optimized.

Time course and cellular growth for the production of enzyme

In order to find the optimum incubation time for protease production, 10 ml freshly growing overnight inoculum was transferred separately in four Erlenmeyer flasks containing 90 ml of selected medium and kept for different time courses i.e. for 18, 24, 36 and 48 hours at 45 ˚C. Protease activity was determined consisting of colorimetric estimation of L-tyrosine released after casein breakdown using L-tyrosine as a standard. Wet cell mass in gm/dl was also noted for each time course.

Effect of medium pH for the production of protease

For optimizing pH, 100 ml of selected medium was prepared in five Erlenmeyer flasks of 250 ml by varying the pH from 5.0 to 9.0 at 1.0 unit interval before sterilization. Fermentation was performed at 45 ˚C for 24 hours and enzyme activity was determined as described above.

Effect of fermentation temperature for the production of protease

To investigate the best fermentation temperature for protease production, the selected medium was prepared and distributed in seven Erlenmeyer flasks of 250 ml, each containing 100 ml medium and fermentation was carried out at various temperatures (40, 45, 50, 55, 60, 65 and 70 ˚C) for 24 hours. After incubation the activity of protease was determined.

Determination of enzyme yield in response to different carbon sources

Protease yield was determined with the supplementation of various carbon sources such as glucose, fructose, maltose, sucrose, starch and casein in the production media. Each carbon source was used at a concentration 0.1% (w/v). Protease production was analyzed quantitatively after 24 hours of incubation at a temperature of 45 °C.

Effect of different concentrations of casein on production of protease

In the fermentation medium casein was incorporated in concentrations ranging from 0.05-0.6

gm % for the maximum protease production. The enzyme activity was determined according to the standard protocol.

Determination of enzyme yield in response to different nitrogen sources

In order to determine the effect of nitrogen containing compounds, 1 gm% of various organic nitrogen such as peptone, yeast extract, urea and inorganic nitrogen such as NH_4NO_3 as well as NH_4Clwere supplemented in each flask containing basal medium. Each flask was then incubated at 45 °C for 24 hours and enzyme production was calculated using standard assay method.

Effect of $CaCl_2$ on induction of Protease

Calcium chloride was added in the media in different concentrations ranging from 0.01-0.3 gm % and fermentation was carried out at the 45 °C for 24 hours to check the effect of Calcium on the yield and stability of proteases.

Purification of Proteases

Purification of the proteases was carried out by gradient precipitation technique using ammonium sulfate, dialysis and gel filtration chromatography at 4°C.

Salt fractionation of proteases

Protease was partially purified from cell-free filtrate using $(NH_4)_2SO_4$ precipitation technique [13]. In order to concentrate exclusively the protein of interest, ammonium sulphate was added gradually in varying concentrations (30%, 40%, 50%, 60%, 70% and 80% saturations) to the crude extract separately. After centrifugation, the precipitates of each fraction were dissolved in 50mM Tris-HCl buffer and proteolytic activity and total protein content was determined [12, 14].

Dialysis

The precipitate showing highest specific activity was taken in the pretreated dialysis tube (12-14 kDa) against 0.05 M solution of Tris-HCl buffer (pH 7.5) and left at 4°C for 24 hours with 3-4 buffer change. After 24 hours, the desalted sample was collected and analyzed for its specific activity.

Column chromatography using Sephadex G-100

The desalted sample that showed highest specific activity was subjected to gel filtration chromatography. The column was packed by Sephadex G-100 and equilibrated with 50 mM Tris-HCl buffer of pH 7.0. The dialyzed sample was loaded on column and proteins were eluted with 50 mM Tris-HCl buffer at the flow rate of 0.15 ml/min. The fraction that showed maximum enzyme activity was selected and used for further studies.

SDS-PAGE

The samples obtained at each purification step were analyzed by SDS-PAGEas described by Laemmli [15] using 4 % stacking gel in Tris-HCl buffer of pH 6.8 and 10 % resolving gel in Tris-HCl buffer of pH 8.8.The molecular weight of the purified protease was calculatedwith the help of protein markers.

Results and Discussion

Due to the enormousindustrial applications of proteases especially in food, detergent, textile and pharmaceutical industries, hyperactive protease producing microbial strains are needed to be isolate from the environment.

Selection and identification of bacterial strain

In the present study, five protease producing bacterial species were isolated from a soil sample. Each of the five strains was evaluated for protease production by using skim milk and casein-agar plates. The strain number 5 was selected for further studies as it showed the largest hydrolytic zone on skim milk agar plate as compared to others (Fig. 1). This strain was identified as *Bacillus* sp. AI-5 by using colony morphology and biochemical tests. Various investigators have been used the same procedure for isolation of protease producing bacteria using casein, gelatin and skim milk as substrates [16-17].

Fig. 1: Bacterial screening for protease production showing zone of hydrolysis on skim milk-agar plate.

Selection of culture medium

The composition of media greatly influences the production of enzymes from microbes especially the concentration of nitrogen as well as carbon sources. Protease production was checked at 37°C for 24 hours by using three different reference media. The results showed that the medium 1 was maximally supported the protease production as compared to medium 2 and medium 3. The reason why medium 1 showed greater protease production as compared to Medium 2 could be the presence of trisodium citrate as a carbon source instead of glucose. However in medium 3, glucose was added as a carbon source but the concentration was much higher as compared to that in medium 1. High concentration of glucose has been reported to suppress protease production [18]. Therefore, this could be the reason why medium 3 did not show greater protease production as compared to medium 1.

Optimization of physical parameters and medium composition

Themedium compositions as well as the conditions of fermentation are the factors responsible for higher yield of proteases. Indeed the temperature, pH, time of incubation, selection of carbon along with nitrogen sources are considered as the determinant factors of protease production. Therefore, the current investigation was aimed to optimize these factors using medium 1 as a basal medium.

Time course for protease production from Bacillus sp. AI-5

Time of fermentation is a factor that is correlated with enzyme production as enzyme production is increased with the increase in cell growth during the post exponential growth phase. In our study, results showed a direct relationship between enzyme production and bacterial cell growth. The maximum enzyme production was observed after 24 hours of incubation during the late exponential phase as shown in Fig. 2. A gradual decrease in the enzyme production and bacterial growth was observed after 24 hours and remained almost unchanged until 72 hours. Therefore, it has been concluded that stationary phase was continued even after 72 hours. Reduction of nutrients together with autolysis of the protease was reported to be associated with the decrease in enzyme production after 24 hours [19]. In contrast to the results of the present study, Kamran et al.[16] reported the maximum protease production in stationary phase instead of log phase.

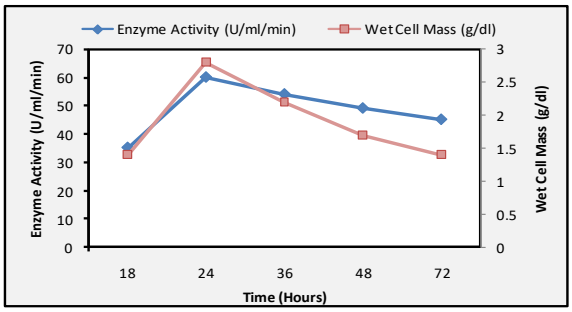

Fig. 2: Time course for production of protease from *Bacillus* sp. AI-5.

Effect of temperature on enzyme production

Fermentation temperature affect both the cellular metabolism and enzyme production as it has been reported that changes in medium temperature could affect the release of an extracellular enzyme from *Bacillus* sp. [20]. In the current study the optimum temperature for maximum protease production was found to be 45 °C and when the temperature was increased up to 50 °C, a sharp decline in enzyme production was observed (Fig. 3) which may be due to the unavailability of dissolved oxygen at higher temperature. Moreover, it has also been reported that poor solubility of oxygen at high temperature may induce changes in surface protein layer of bacterial cell resulting in decreased secretion of extracellular enzyme [21].

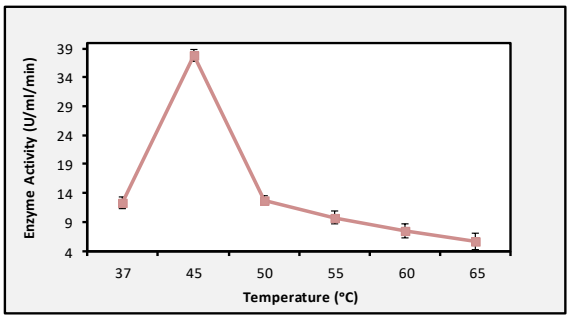

Fig. 3: Effect of temperature on protease production from *Bacillus* sp. AI-5.

Effect of pH on enzyme productionfrom Bacillus sp. AI-5

pH is the second most important physical factor greatly affects the transportation of compounds across the cell membrane during fermentation which in turn maintain enzyme production and cell growth [22]. In the present study pH 5.0 was found to be optimum for the production of protease enzyme by

Bacillus sp.AI-5 (Fig. 4). Protease production was found to be declined when the medium was shifted from acidic to alkaline. This could be due to the secondary metabolites production in alkaline environment. The result of the present study is in accordance with the results of earlier study of researchers who reported an optimum pH 6.5 for maximal protease production [23]. Contrastingly, maximum production of proteases has also been reported at neutral pH [24] whereas *Bacillus subtilis* NS [11] produced proteases that was stable at alkaline pH 9.0.

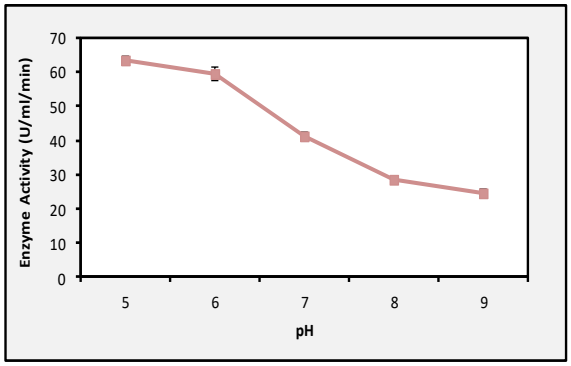

Fig. 4: Effect of medium pH on protease production from *Bacillus* sp. AI-5.

Determination of enzyme yield in response to different carbon sources

Various carbon sources were added in the medium for enzyme production including glucose, sucrose, starch, maltose, fructose and casein. Enzyme production was found to be maximum in the medium that was incorporated with 0.1 g % casein as shown in the Fig. 5. Fructose also showed about 88 % activity as compared to casein whereas glucose was found to repress the protease production as it showed only 21 % protease production with reference to the medium in which glucose was added as a carbon source.

Effect of casein concentrations of on protease production

In the present study, various concentrations of casein were incorporated in the basal medium and it was observed that with the increase in concentration of casein, enzyme production was also increased. Maximum production of proteases was obtained when 0.4 gm % casein was added in the medium. Beyond 0.4 gm % a gradual decline of enzyme production was observed (Fig. 6).

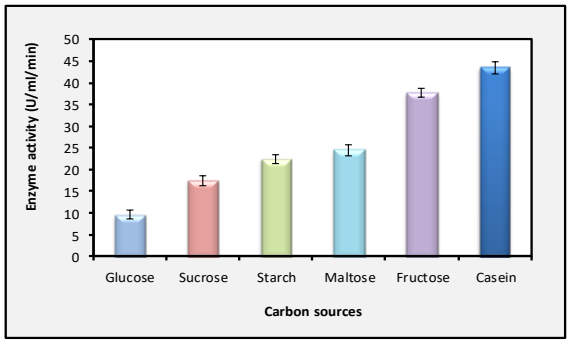

Fig. 5: Effect of carbon sources on production of proteases from *Bacillus* sp. AI-5

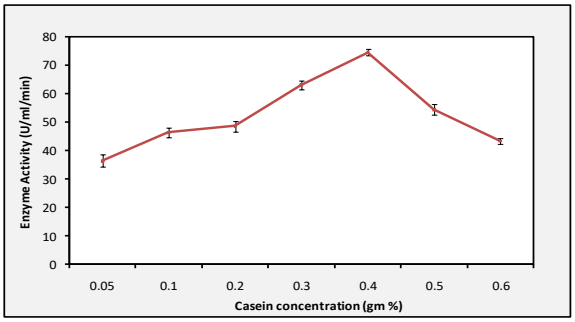

Fig. 6: Effect of casein concentration on production of proteases from *Bacillus* sp. AI-5.

Determination of enzyme yield in response to different nitrogen sources

The nitrogen has profound effect on the enzyme production and cell growth. In the current study maximum protease production was achieved by the mixed organic nitrogen source including yeast extract-0.5 gm % and peptone-0.5 gm % (Fig. 7). Inorganic nitrogen sources including ammonium nitrate and potassium nitrate have also been reported to be responsible for higher protease production [17].

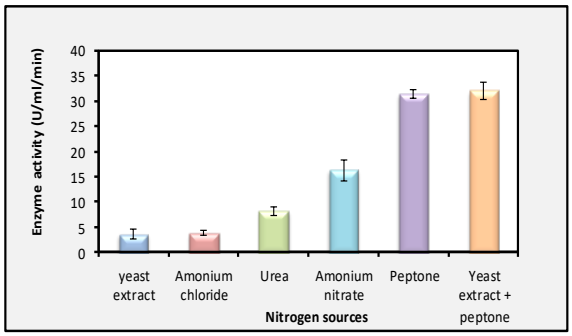

Fig. 7: Effect of nitrogen sources on production of proteases from *Bacillus* sp. AI-5.

Table 1: Steps involved in the purification of protease.

PURIFICATION STEP	ENZYME ACTIVITY U/ml/min	TOTAL PROTEIN mg/ml	SPECIFIC ACTIVITY U/mg	FOLD PURIFICATION
CFF	32.00	1.37	23.35	1
$(NH_4)_2SO_4$ Precipitation	20.55	0.12	171.25	7.33
Gel Filtration Chromatography	103.12	0.32	322.25	13.80

Effect of $CaCl_2$ on induction of proteases

It has been shown by several studies that divalent metal ions could affect the enzyme production during microbial fermentation. Moreover it has also been reported that Ca^{+2} maintained the molecular confirmation of proteases in the medium and contributed to its thermal stability also [20]. Therefore in this study, effect of various concentrations of calcium chloride ranging from 0.01-0.3 % (w/v) was checked on protease production. Results indicated an optimum secretion of proteases when 0.05 % $CaCl_2$ was added in the medium thereafter a gradual decrease in the protease production was occured (Fig. 8). This might be the sign that the calcium would be the essential source for protease enzyme induction or stabilization from *Bacillus* sp. AI-5.

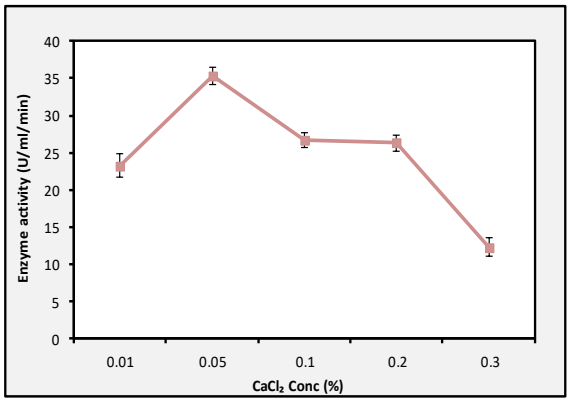

Fig. 8: Effect of different concentration of calcium chloride on production of proteases from *Bacillus* sp. AI-5.

Purification of proteases

In the present study, the proteases from *Bacillus* sp. AI-5 was purified by using the gradient ammonium sulfate precipitation technique. The cell free filtrate was subjected to precipitation with different concentration of ammonium sulfate ranging from 30 to 80 gm %. It was found that precipitation of proteases increased when concentration of ammonium sulfate was increased from 30 gm % to 60 gm %, while negligible activity was observed beyond 60 % saturation (Table-1). At 60% $(NH_4)_2SO_4$, maximum precipitation of proteases was observed with the specific activity of 154.77 U/mg and fold purification of 7.37 as compared to crude enzyme. This fraction upon further purification by gel filtration chromatography using sephadex G-100 yielded 322.25 U/mg specific activity with 13.8 fold purification(Table-1, Fig. 9). SDS-PAGE revealed a single band of protease of molecular weight of 66kDa in lane 3 corresponding to the single sharp peak obtained through gel filtration suggesting the complete purification of enzyme protein (Fig. 10).

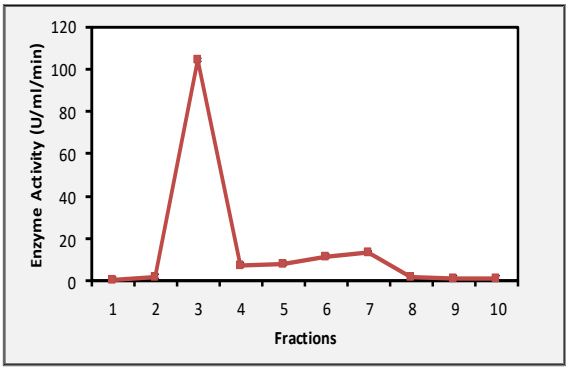

Fig. 9: Elution pattern of proteins by Gel filtration chromatography.

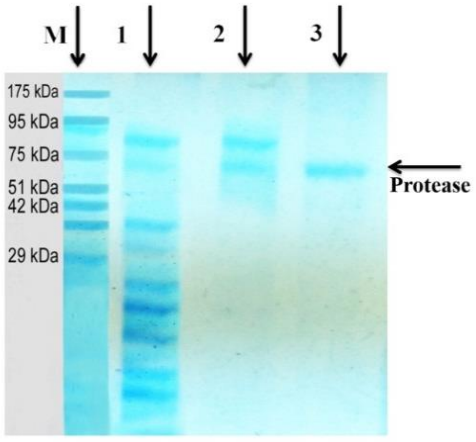

Fig. 10:SDS-PAGE of partially purified proteases. Lane 1: Crude extract of enzyme. Lane 2: Desalted precipitates after $(NH_4)_2SO_4$ fractionation. Lane 3: Fraction number 3 obtained from gel filtration chromatography.

Conclusion

The findings of the current study indicated a novel bacterial protease from *Bacillus* sp. AI-5 was obtained when the media formulation and fermentation conditions were optimized and it was concluded that *Bacillus* sp. AI-5 being salt tolerant, protease producing acidophile, would be utilized in various industrial processes especially in food and feed industries for hydrolysis of peptide bonds in complex proteins.The protease enzyme that was purified and found to have a molecular weight of 66 kDa can also be used in industrial processes especially in the Pharmaceutical industry where pure enzymes are required.

Acknowledgements

The authors are grateful to Jinnah University for Women, Karachi for providing facilities for the present research.

Conflict of interest

The authors have no conflicts of interest to disclose.

References

1. A.D. Suleiman, N.A. Abdul Rahman, H.M. Yusof, F.M. Shari and N.A. Yasid, Effect of Cultural Conditions on Protease Production by a Thermophilic Geobacillus thermoglucosidasius SKF4 Isolated from Sungai Klah Hot Spring Park, Malaysia. *Molecules.*, **25,** 1 (2020).
2. M. Goyal and P.U. Gupta, Protease production from biodigested slurry of biogas plants: a review. *Int. J. Curr. Microbiol. App. Sci.,* **7,** 369 (2018).
3. D. Rupali, Screening and Isolation of Protease Producing Bacteria from soil collected from different areas of burhanpur region (MP) India. Int. J. Curr. Microbiol. *App. Sci.,* **4,** 597 (2015).
4. M. Naveed, F. Nadeem, T. Mehmood, M. Bilal, Z. Anwar and F. Amjad, Protease - A Versatile and Ecofriendly Biocatalyst with Multi-Industrial Applications: An Updated Review. *Catal. Lett.*, **151**, 307 (2020).
5. K.M. Sharma, R. Kumar, S. Panwar and A. Kumar, Microbial alkaline proteases: Optimization of production parameters and their properties. *J. Genet. Eng. Biotechnol.*,**15,** 115 (2017).
6. N. Gurung, S. Ray, S. Bose and V. Rai, A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. *BioMed Res. Int.,***18,** 1 (2013).
7. F.B. Mefteh, F. Frikha, A. Daoud , A.C. Bouket, L. Luptakova, F.N. Alenezi, S. Bader and T. Oszako, Response Surface Methodology Optimization of an Acidic Protease Produced by Penicillium bilaiae Isolate TDPEF30, a Newly Recovered Endophytic Fungus from Healthy Roots of Date Palm Trees (Phoenix dactylifera L.). *Microorg.*,**7,** 1 (2019).
8. C. Zhou, H. Zhou, H. Zhang and F. Lu, Optimization of alkaline protease production by rational deletion of sporulation related genes in *Bacillus licheniformis*. *Microb Cell Fact.,* **18,** 127 (2019).
9. S.A. Qadar, E. Shireen, S. Iqbal and A. Anwar, Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3. *Indian J. Biotechnol.,***8,** 286 (2009).
10. W. C. A.. Nascimento and M.L.L. Martins, Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Braz. J. Microbiol.,* **35,** 91 (2004).
11. N. Nisha and J. Divakaran, Optimization of alkaline protease production from *Bacillus subtilis* NS isolated from sea water.*Afr. J. Biotechnol.,* **13,** 1707 (2014).
12. M.L. Anson, The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.*, **22**, 79 (1938).
13. S. Englard and S. Seifter, Precipitation techniques. *Meth. Enzymol.*, **182,** 287 (1990).
14. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248 (1976).
15. U.K. Laemmli, Cleavage of structural proteins during the assembly of the hed of acteriophage T4. *Nature,* **277,** 680 (1970).
16. A. Kamran, Z. Bibi and M. Kamal, Screening and enhanced production of protease from a thermophilic *Bacillus* species. *Pak J. Biochem. Mol. Biol.,* **48**, 15 (2015).
17. B.K.M. Lakshmi, S.P.V. Ratna, D.K. Ambika and K.P.J. Hemalatha, Media optimization of protease production by *Bacillus licheniformis* and partial characterization of alkaline protease. *Int. J. Curr. Microbiol. App. Sci.,* **3,** 650 (2014).
18. V. Mehta, J. Thumar and S. Singh, Production of alkaline protease from an alkaliphilic *actinomycete*. *Bioresour. Technol.,* **97,** 1650 (2006).
19. W. Asad, M. Asif and S.A. Rasool, Extracellular enzyme production by indigenous thermophilic bacteria: partial purification and characterization of α-amylase by Bacillus sp. WA21. *Pak. J. Bot.*,**43,** 1045 (2011).

20. P. Palsaniya, R. Mishra, N. Beejawat, S. Sethi and B.L. Gupta, Optimization of alkaline protease production from bacteria isolated from soil. *J. Microbiol. Biotechnol.Res.,* **2,** 858 (2017).
21. M.S.B. Purohit, M.P.B. Chauhan and M. Gahlout, Assessment of process parameters for enhanced production of microbial alkaline protease. *Int. J. Adv. Res. Biol. Sci.,* **3,** 28 (2016).
22. V. Khajuria, K. Sharma, P. Slathia, K. Razdan, S. Singh and B.K. Bajaj, Production of a detergent-compatible alkaline protease from *Bacillus cereus* K-3. *J. Mater. Environ. Sci.,* **6,** 2089 (2015).
23. S.V. Hosseini, Z. Saffari, A. Farhanghi, S.M. Atiyabi and D. Norouzian, Kinetics of alkaline protease produced *by Steptomyces gresioflavus* PTCC1130. *Iran J. Microbiol.,* **8**, 8 (2016).
24. F.S. Josephine, V. Ramya, N. Devi, S.B. Ganapa and T. Vishwanatha, Isolation, production and characterization of protease from *Bacillus* sp. isolated from soil sample. *J Microbiol Biotechnol Res.,* **2,** 163 (2012).