ISSN: 2278-5213

RESEARCH ARTICLE

Production, isolation and characterization of alkaline protease from *Aspergillus versicolor* PF/F/107

Vaishali Choudhary
Dept. of Applied Microbiology and Biotechnology, Dr. H.S. Gour Vishwavidylaya, Sagar (M.P)
vaishalitripathi.2179@gmail.com; vesaly2179@gmail.com; +91 8805066900

Abstract

An alkaline protease producing strain, *Aspergillus versicolor* PF/F/107 was isolated from soil samples of poultry farm of Jabalpur district of Madhya Pradesh and enzyme production was optimized under submerged conditions. Maximum enzyme production by the test isolate under optimized conditions occurred in mesophilic temperature 35°C and pH 9.0. Wheat bran and sodium nitrate proved to be the best carbon and nitrogen sources respectively. The enzyme showed optimum activity at pH 9.0 and found to retain residual activity at pH 8.0. Protease of *Aspergillus versicolor* PF/F/107 showed remarkably good activity at 50^{o}C with optimum at 40^{o}C indicating thermo tolerant nature of the enzyme. The enzyme was stable to desired levels of non-ionic surfactants such as Tween-20, Tween-80, Triton X-100 and ionic surfactants such as SDS. The enzyme retained 50%-76% of its original activity at 40°C in the presence of detergents in the following order: Ghadi, Ujalla, Surf excel, Ariel and Tide indicating its suitability for application in detergent industry.

Keywords: *Aspergillus versicolor,* mesophilic, Tween-20, Tween-80, Triton X-100.

Introduction

Proteases for pharmaceutical and medical application require high purity while, proteases used in other industries require partially purified forms of enzymes. In order to find novel source of enzymes to meet the market demand for different applications, knowledge of their activity at different levels of purity is required. Most of the enzymatic treatments employ crude or partially purified enzymes while others are requiring in almost purified forms. Purification of enzymes is tedious, time consuming and highly expensive. Partial purification of enzymes can be easily achieved by simple downstream processing of culture filtrate involving centrifugation followed by salting out of proteins. Centrifugation removes insoluble materials such as cells, mycelia, media components, spores, cell debris etc. Removal of all these components is important since they may interfere with the enzyme activities and impart undesirable properties to it. Media components and pigments are chromogenic and may change color of the product, whereas spores, mycelia, cells etc. can initiate unwanted microbial growth while enzymes and proteins remain dissolved in solution because their charged surface residues interacts with molecules of the solvents (Wilson and Walker, 2000). To isolate and concentrate the enzymes/protein, salting out of proteins is a simple procedure that eliminates non-protein impurities. The volume of the enzyme sample is also reduced to many folds on precipitation. The concentrated enzyme sample can be appropriately diluted according to need. Small volume is easy to handle and preserved. Salting out of proteins can be done by using ammonium sulphate as precipitants (England and Seifter, 1990).

The major advantage of using ammonium sulphate over other salts lies in its high molarity; it causes precipitation of most proteins without liberating large amounts of heat in solution. High concentration of ammonium sulphate is known to prevent or limit microbial growth and protect protein from denaturation. Because of these advantages it is used over other salts such as NaCl, Na_2SO_4, $MgSO_4$, $CaCl_2$ etc. (Deutscher, 1990). Other techniques employed for purification of enzymes includes ions exchange, gel permeation chromatography and gel electrophoresis. The above mentioned techniques are in order of increasing resolution but they are time consuming.

Several workers have carried out purification of proteases of different microorganisms. Kundu and Manna (1975) purified proteinases of *Aspergillus oryzae* using ammonium sulphate and ethanol fractionation followed by diethyl amino ethyl-Sephadex A-50 and hydroxyapitite chromatography while Venugopal and Saramma (2006) purified alkaline protease from *Vibrio fluvialis* using ammonium sulphate fractionation and achieved 95.50% recovery with 1.25 fold purification. Similarly Tunga *et al*. (2003) reported similar result for *Aspergillus parasiticus* protease which showed 1.6 fold purification and 90% recovery with the first step of purification with acetone precipitation. Monod *et al*. (1991) reported the purification of the enzyme by successive ammonium sulphate precipitation, ultra filtration and P60 gel polyacrylamide column chromatography.

Labadie and Montel (1992) achieved complete purification of the enzymes by ammonium sulphate precipitation, Sephadex 6200 gel filtration and DEAE cellulose chromatography. Purification of alkaline protease from the culture filtrate of *Aspergillus nidulans* was carried out by Charles *et al*. (2008) using Sephadex G-100 column chromatography. The enzyme was purified 42.4 folds with a yield of 39%. The molecular weight of the purified protease was estimated to be 42KDa by SDS-PAGE. Lindberg *et al*. (1981) have purified alkaline protease from *Neurospora crassa* using ammonium sulphate fractionation ion exchange chromatography and gel filtration. A molecular weight of ~ 30,500 was determined by amino acid analysis, gel electrophoresis and sedimentation equilibrium. Dahot (1994) purified enzyme from culture broth of *Penicillium expansum* by using cold acetone followed by Sephadex G-100 column chromatography, DEAE Sephadex A-50 chromatography and disc gel electrophoresis and achieved 48% recovery with molecular weight of 20,500.

Characterization of proteases is also important from the point of view of its practical applicability. In this respect, activity and stability of protease of different pH, temperature and metal ions is of prime importance (Mulimani *et al*., 2002; Abu Sayem *et al*., 2006). For example, the protease which exhibit activity in broad pH range, substrate specificity, bleach stability and tolerance to sequestering agent are considered best for detergent industry. However, a good detergent protease should also be stable in the presence of surfactants and detergents. Profile of inhibition of proteases by different protease inhibitors and metal ions helps in identification of class to which it belongs. Many workers have characterized the activity of various proteases produced by microorganisms (Moreira *et al*., 2002; El-Safey, 2004; Charles *et al*., 2008; Ahmed *et al*., 2008). Kalpana devi *et al*. (2008) studied the properties of alkaline protease of *Aspergillus niger* and reported the enzyme to be thermo stable. It was found to be active at pH 10 and 45°C. Purification and characterization of extracellular proteinases of *Aspergillus oryzae* was carried out by Kundu and Manna (1975). The purified enzymes were stable over the pH range 6 to 8 at 30°C for 60 min. The enzyme activity was found to be accelerated by Cu^{2+} and inhibited by Fe^{2+}, Fe^{3+}, Hg^{2+} and Ag^{+}. Mulimani *et al*. (2002) partially purified the protease which was found to be bleach stable and thermo stable. It was found that the addition of ionic and non-ionic detergent and surfactants to the reaction mixture enhanced the enzyme activity (Mulimani *et al.,* 2002; Maurer, 2004). In 1989, Abbas and co-workers purified and characterized the alkaline protease produced by *Penicillium charlesii*. The enzyme was purified by gel permeation chromatography, DEAE-Sepharose anion-exchange chromatography and fast protein liquid chromatography. Against these backdrops, this study was aimed to purify and characterize alkaline protease of *Aspergillus versicolor* PF/F/107.

Materials and methods

Isolation and screening of fungi: The test fungus was isolated from poultry farm of Jabalpur district (M.P) and was identified as *Aspergillus versicolor* PF/F/107. It gave Relative enzyme activity (REA) of 3.00 on solid Reese media. The alkaline protease activity produced by the fungus was found to be 119.48 U/mL during initial screening.

Enzyme production: For the production of alkaline protease, commercially available wheat bran was used as substrate. The wheat bran was thoroughly washed to remove starch and oven dried. Erlenmeyer flasks (150 mL) containing 50 mL of Medium I (pH 9.0) supplemented with 1% wheat bran and autoclaved. Then it was inoculated with 1 mL spore suspension of 2×10^6 spores/mL of *Aspergillus versicolor* PF/F/107. The flasks were then incubated at 35°C for 4 d at 150 rpm on rotary shaker. After incubation, the contents were then filtered through Whatman No.1 filter paper and the filtrate was used as the source of enzyme.

Partial purification of enzyme: The culture filtrate obtained from cultures grown by submerged fermentation was centrifuged at 10,000 rpm for 15 min. Enzyme in the cell free supernatant portion of the culture was precipitated by ammonium sulphate (up to 70% saturation) and kept overnight at 4°C (Ogundero and Osunlaja, 1986). During ammonium sulphate precipitation, the salt was added in small quantity under constant stirring to equilibrate the salt to maintain equal concentration throughout the culture filtrate (Dawson *et al*., 1969). The precipitate was recovered by centrifugation at 10,000 rpm for 20 min and resuspended in minimum of distilled water/Glycine–NaOH buffer (0.1 M, pH 9.0) and dialyzed against the same buffer. This dialyzed fraction was used as enzyme sample for protease activity and protein estimation and was used for further characterization.

Assay of protease activity and protein estimation: The alkaline protease activity was quantified spectrophotometrically using casein as substrate (Takami *et al*., 1989). Suitably diluted enzyme sample was added to 2% casein solution (1 mL) prepared in Glycine-NaOH buffer (0.2 M, pH 9.0). After incubation (20 min, 40°C), the undigested protein was precipitated by adding 2 mL of 5% (w/v) Tricholoroacetic acid (TCA) and allowed to stand for 30 min at room temperature, followed by centrifugation (5000 rpm, 15 min) and filtration through Whatman no.1 filter paper. The absorbance of the filtrate was measured at 660 nm in UV-Visible spectrophotometer. A standard curve was generated with pure tyrosine as standard. One unit of alkaline protease was defined as the amount of enzyme, which liberated 1 µg of tyrosine per mL per minute under the specific experimental conditions. Protein content of the sample was measured by the method of Lowry *et al*. (1951) with bovine serum albumin (BSA) as standard.

Effect of pH on protease activity: The effect of pH on alkaline protease was measured over a range from 6-11 under standard assay conditions using 0.1 M Sodium phosphate buffer (pH 6-7), 0.1 M Tris-HCl buffer (pH 8-9) and 0.1 M Glycine-NaOH buffer (pH 10-11). Effect of different pH on enzyme activity was recorded in terms of relative activity by considering maximum activity as 100%.

Effect of temperature on enzyme activity: The effect of temperature on alkaline protease activity was determined by incubating the reaction mixture (enzyme + substrate) at different temperatures (30°C-80°C) in 0.1 M Glycine-NaOH buffer (pH 9) for 10 min.

Effect of surfactant on enzyme activity: The effect of various surfactants such as tocytyl phenoxy polyethoxy ethanol (Triton-X 100), polyoxyethylene sorbitan monolaurate (Tween-20), polyoxyethylene sorbitan monoleate (Tween-80) and sodium dodecylsulphate (SDS) on enzyme activity was determined by adding 1% (v/v) of test surfactant in Glycine-NaOH buffer (pH 9) to the reaction mixture. The residual activity was then determined.

Compatibility with various commercial laundry detergents: In order to confirm the potential of *Aspergillus versicolor* PF/F/107 protease as a detergent additive, its compatibility and stability towards some commercial laundry detergents available in local market such as Surf excel and Wheel (Hindustan Lever Ltd., India), Tide and Ariel (Proctor and Gamble, India), Ujalla (Jyoti Laboratories Ltd., India) and Ghadi detergent (Ghadi Industries Pvt. Ltd., India) were examined. Aqueous solutions of detergents (7 mg/mL to stimulate washing condition) were heated at 100ºC for 60 min to denature the indigenous protease activity (if any). The detergent solution and protease enzymes were mixed in a ratio of 1:1 (v/v) incubated at 40ºC for 20 min followed by measuring the residual protease activity by standard assay procedure as mentioned previously and compared with the control (Enzyme diluted to 1:1 in tap water without detergent). The relative activity was expressed as percentage activity considering the activity of control as 100%.

Effect of incubation on stability of protease activity in presence of Ghadi detergent: The stability of protease activity in presence of Ghadi detergent was tested by preincubating the Ghadi detergent (7 mg/mL) and enzyme (1 mL) at different time intervals (10 min to 60 min) at 40°C. The residual activity was measured by standard assay procedures as mentioned previously. The activity of control (in the absence of detergent) was considered as 100%.

Results and discussion

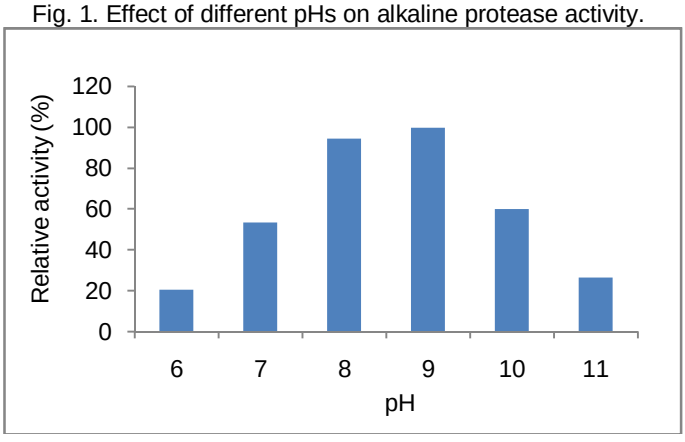
Aspergillus versicolor PF/F/107 was isolated from poultry farm soil of Jabalpur district (M.P). The culture fluid obtained from cultures of *Aspergillus versicolor* PF/F/107 showed 119.48 U/mL activity of alkaline protease. Thus, *Aspergillus versicolor* PF/F/107 was identified as the most potential strain, since it showed the maximum zone (REA-3.00) and maximum enzyme activity (119.48 U/mL) in its culture fluid.

Ammonium sulphate precipitation: The bulk precipitation of total protein brought by 70% ammonium sulphate saturation of culture filtrate of *Aspergillus versicolor* PF/F/107 showed presence of 0.270 mg/mL of protein. Protease specific activity (U/mg) was calculated by equating enzyme activity of precipitate (U/mL) with amount of protein present in unit volume of culture filtrates (mg/mL) and the percentage recovery (yield) of alkaline protease was estimated by comparing total alkaline protease activity obtained in precipitate with that of total alkaline protease activity originally present in culture filtrates. The activity of enzymes after purification increased a little on ammonium sulphate precipitation. The recovery of protein was found to be 64.18 while the activity of enzyme recovered was little more i.e., 66% using present protocol.

Characterization of alkaline protease by Aspergillus versicolor PF/F/107

Effect of pH on enzyme activity: The effect of pH on alkaline protease activity of test fungi is expressed in terms of % relative activity (Fig. 1) at different pH. Alkaline protease activity was observed at pH 6 to pH 11. The enzyme showed optimum activity at pH 9 and found to retain residual activity at pH 8. At pH 10, the activity reduced significantly and only 59.95% residual activity was noted at this pH, which was found further reduced at pH 11 showing only 26.31% residual activity. At neutral pH 7, the enzyme retained 53.44% activity. Higher activity of the test enzyme from *Aspergillus versicolor* PF/F/107 indicates its possible use in detergent formulations as a detergent additive.

Fig. 1. Effect of different pHs on alkaline protease activity.



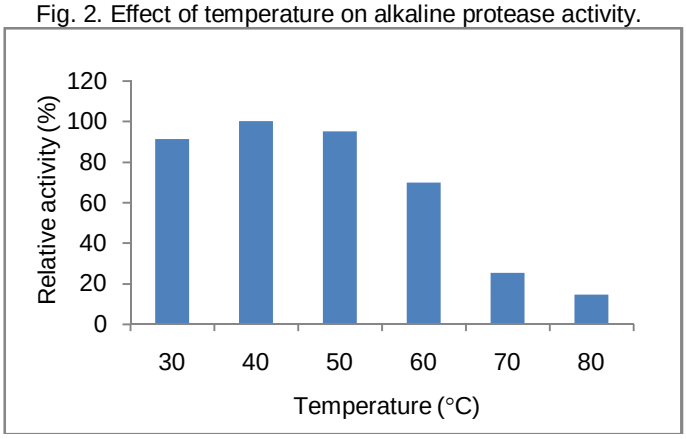
Alkaline protease from *Microbacterium luteolum* showed maximum activity at pH 7.5 with 71% residual activity at pH 10 and pH 10.5 (Malathu *et al.,* 2008). Abbas *et al.* (1989) showed that protease enzyme complex produced by *Penicillium Charlesii* degraded casein optimally are pH 7-9.

The optimum activity of protease of *Aspergillus oryzae* was found to be at pH 8-9 (Samarntarn *et al*., 1999)

Effect of temperature on enzyme activity

Alkaline protease activity in precipitated enzyme was assayed over a range of temperature i.e., 30-80°C and the optimum alkaline protease activity (i.e., 206.15 U/mL) was observed at 40°C. Figure 2 indicates that enzyme was active in broad range of temperatures i.e., 30-80°C. At 30°C, the activity of this enzyme was found to be 187.98 U/mL that increased with increase in temperature up to 50°C. At 60°C, the activity of alkaline protease was noted only 144.16 U/mL, this indicates the retention of 69.93% of the enzyme activity at this temperature.

Fig. 2. Effect of temperature on alkaline protease activity.

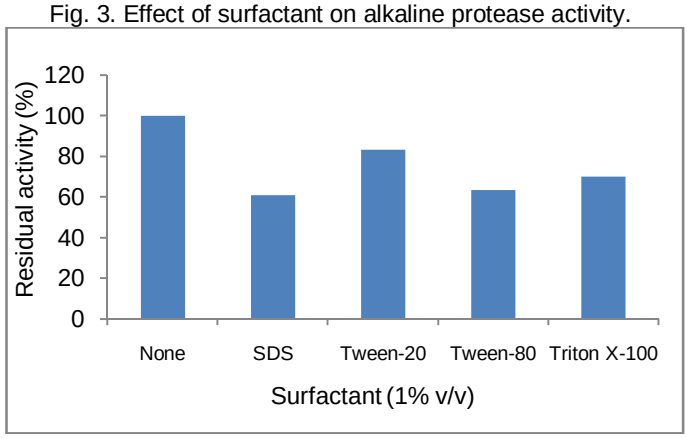


The enzyme had temperature optima at 40°C while it showed more than 91.19% activity at 30°C. A little more i.e., 95.05% of its maximum activity was noted at 50°C. Stability towards high temperature is very much desirable in some of the industrial enzymes. The enzyme was less active above 70°C (Fig. 2). Temperature optima of 35°C have been reported for protease from *Aspergillus nidulans* (Charles *et al*., 2008). Similar, results were recorded with proteases produced by *Aspergillus terreus* and *Aspergillus funigatus* (Chakrabarti *et al*., 2000; Wang *et al*., 2005). Kalpana devi *et al*. (2008) reported maximum alkaline protease activity at 45°C for *Aspergillus niger*. Li *et al*. (1997) reported that alkaline protease isolated from *Thermomyces lanuginosa* P_{134} had a temperature optimum at 50°C. Samal *et al*. (1991) reported alkaline protease from *Tritirachium albumlimber* to be quite thermo stable even up to 50°C. Protease of *Aspergillus versicolor* PF/F/107 also showed remarkably good activity at 50°C with optimum at 40°C thus indicates its thermal tolerance and application in those processes operating at high temperature.

Effect of surfactants on enzyme stability: Effect of different surfactant on activity of *Aspergillus versicolor* PF/F/107 alkaline protease was studied. In the presence of 1% (v/v) Tween-20, 83.6% of its residual activity was observed in the test enzyme sample. Tween–80 at same concentration was found to exert more inhibitory effect than Tween-20.

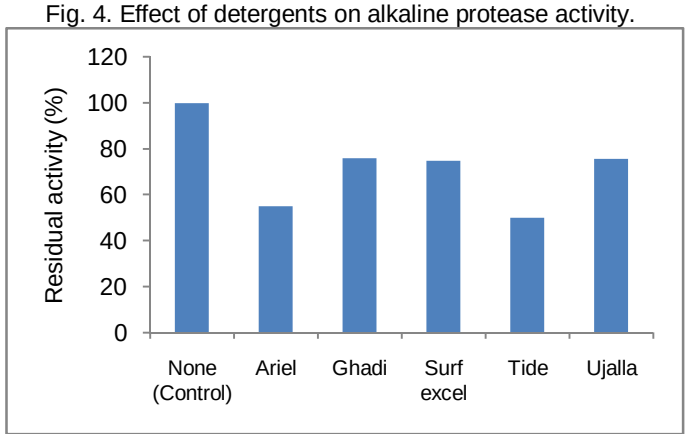
Residual activity of alkaline protease was found to be 133.5 U/mL is presence of Tween-80 (Fig. 3). Residual activity of alkaline protease activity was found to be 70.29% in presence of Triton X-100 (1% v/v). Pretreatment with SDS (1% v/v) has inhibited activity of alkaline protease to a much greater extent (Fig. 3).

Fig. 3. Effect of surfactant on alkaline protease activity.



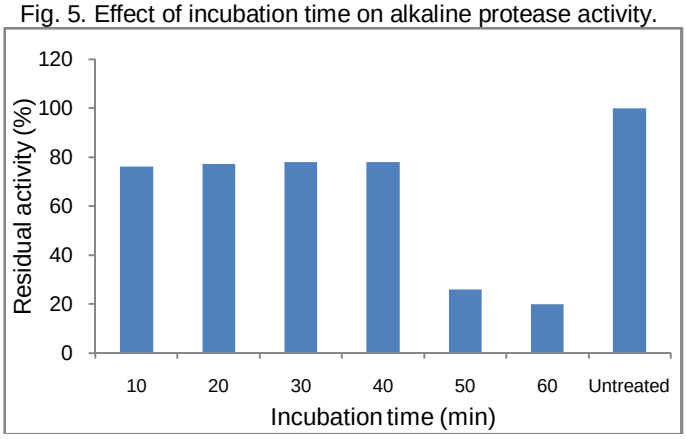
Enzyme sample treated with SDS showed 115.88 U/mL enzyme activities which are found to be 61% of the optimum. Protease from *Aspergillus fumigatus* was found to be stable in presence of Tween-80 and Triton X-100 (Verma *et al*., 2001). Mulimani *et al*. (2002) reported stimulation in activity of the enzyme obtained from *Aspergillus flavus* in presence of Tween-20, indicating its utility as detergent constituent. Gupta *et al.* (1999) reported that the activity of protease from *Bacillus* species was enhanced from 30% to 80% in the presence of ionic and non-ionic surfactants. Detergent industries are requiring proteases with high stability at alkaline pH, thermo stability as well as stability in presence of detergents and surfactants. In this study none of the surfactant used in the study showed enhancement of activity of the test enzyme but its activity was found to be stable to the desired level in the presence of non-ionic surfactants such as Tween-20, Tween-80, Triton X-100 and ionic surfactants such as SDS thus, can find application in detergent formulations.

Fig. 4. Effect of detergents on alkaline protease activity.



Compatibility of protease with commercial laundry detergents: Protease from *Aspergillus versicolor* PF/F/107 exhibited a significant stability and compatibility with all the tested commercial laundry detergents (Fig. 4). The enzyme retained 50-76% of its original activity at 40°C in the presence of detergents in the following order: Ghadi® > Ujalla® > Surf excel® > Ariel® > Tide® (Fig. 4). Stability of protease for laundry detergents were reported by Venugopal and Saramma (2006) for alkaline protease produced by *Vibrio fluvialis* strain VM10. Phadatare *et al*. (1993) reported high activity of protease from *Conidiobolus coronatus* and showed its stability at 50°C in the presence of 25 mM $CaCl_2$ with variety of commercial detergents.

Fig. 5. Effect of incubation time on alkaline protease activity.



Stability of protease activity in presence of Ghadi detergent: Stability of protease activity in Ghadi detergent formulation was tested for 10–60 min duration. The enzyme was found stable throughout the testing period but the residual activity was found to decrease with increase in time duration. The enzyme was found to retain more than 70% residual activity up to 40 min which was reduced to only 26% and 20% in 50 and 60 min of time (Fig. 5). The suitability of any protease for inclusion in detergent formulation is dependent on its stability and compatibility with detergent components (Kumar and Takagi, 1999; Venugopal and Saramma, 2006). Besides, the enzyme should be alkaline in nature and thermostable. However, the stability and compatibility of any component should not be the only pre-requisite for its inclusion in detergent formulation. Krik *et al*. (2002) emphasized that the detergent protease must be active at room temperature in order to save energy required in heating of water. The protease secreted by *Aspergillus versicolor* PF/F/107 found to retain its stability at high temperature and at alkaline pH range thus the enzyme can be used as additive in formulation of laundry detergents.

Conclusion

The present investigation is on alkaline proteases produced by *Aspergillus versicolor* PF/F/107 which clearly shows that this fungus is potent producer of proteases, which are active at different pH ranges. The enzyme production was considerably enhanced under the set of optimized conditions. These findings have great industrial implications. It is clear from the results that the protease produced by *A. versicolor* PF/F/107 is active in the pH range 6.0 -11.0 with two peaks, one pH 8.0 and the other pH 9.0. The multiple pH optima observed suggests the presence of at least two proteolytic activities in the crude protease. This property makes the enzyme suitable for leather treatment and industrial production of detergents .Studies with regard to temperature optimization show that the optimum temperature for *A. versicolor* PF/F/107 was 40°C. Optimum temperature for the proteases is considerably at a thermo tolerant range, and thereby proves to be advantageous in industrial applications especially in detergent industry. It is concluded that the alkaline protease produced is stable at alkaline pH, at high temperatures, and in the presence of commercial detergents and is compatible with commercial and local detergents. These properties indicate the possibilities for use of the protease in the detergent industry and this enzyme can be exploited commercially in near future.

References

1. Abbas, C.A., Groves, S. and Gander, J.E. 1989. Isolation, purification, and properties of *Penicillium* charlessi alkaline protease. *J. Bacteriol.* 171(10): 5630-5637.
2. Abu Sayem, S.M., Alam, M.J. and Hoq, M.M. 2006. Effect of temperature, pH and metal ions on stability of alkaline protease from novel *Bacillus licheniformis* MZK03. *Proc. Pak. Acad. Sci.* 43(4): 257-262.
3. Adinarayana, K., Ellaiah, P. and Prasad, D.S. 2003. Purification and partial Characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *Pharm. Sci. Technol.* 4: 56-64.
4. Ahmed, A.S., Al-domany, A.R., El-Shayeb, M.A.N., Radwan, H.H. and Saleh, A.S. 2008. Optimization, immobilization of extracellular alkaline protease and characterization of its enzymatic properties. *Res. J. Agri. Biol. Sci.* 4(5): 434-446.
5. Amoozegara, M.A., Fatemia, A.Z., Karbalaei- Heidarib, H.R. and Razavic, M.R. 2007. Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, *Salinivibrio* sp. strain AF-2004. *Microbiol. Res.* 162: 369-377.
6. Arasu, T.V., Shivkumar, T., Ramasubramanian, V., Nalini, K. and Vanathi, R. 2010. The potential application of keratinase from *Bacillus* as a laundry detergent and feed additive. *Adv. Biotech.* 9(8): 36-40.
7. Bhosale, S.H., Rao, M.B., Deshpande, V.V. and Srinivasan, M.C. 1995. Thermostability of high activity alkaline protease from *Conidiobolous coronatus* (NCL 86.8.20). *Enzyme Microbiol. Technol.* 17: 136-139.
8. Chakrabarti, S.K., Matsumura, N. and Ranu, S.S. 2000. Purification and characterization of an extracellular alkaline protease from *Aspergillus terreus* (IJIRA 6.2). *Curr. Microbiol.* 40: 239-244.
9. Charles, P., Devanathan, V., Anbu, P., Ponnuswamy, M.N., Kalaichelvan, P.T. and Hur, B.K. 2008. Purification, characterization and crystallization of an extracellular alkaline protease from *Aspergillus nidulans* HA-10. *J. Basic Microbiol.* 48: 347-352.
10. Dahot, U.M. 1994. Purification and some properties of alkaline protease from *Penicillium expansum*. *J. Islamic Acad. Sci.* 7(2): 100-105.
11. Dawson, R.M.C., Elliot, D.C., Elliot, W.H. and Jones, K.M. 1969. Data for Biochemical Research. 2nd Ed. Oxford Univ., Press, London.
12. Deutscher, M.P. 1990. Guide to Protein Purification. Academic Press, London, p. 894.

13. El-Safey, E.M. and Abdul-Raouf, U.M. 2004. Production, purification and characterization of protease enzyme from *Bacillus subtilis*. International conference for development and the environment in the Arab world, Assiut Univ. p.14.
14. England, S. and Seifter, S. 1990. Precipitation Techniques. In: Guide to Protein Purification. (Ed. Deutscher, M.P.) Academic Press, London, pp.285-306.
15. Gupta, R., Beg, Q.K. and Lorenz, P. 2002. Bacterial alkaline proteases: Molecular approaches and industrial applications. *Appl. Microbiol. Biotech*. 59: 15-32.
16. Gupta, R., Gupta, K., Saxena, R.K and Khan, S. 1999. Bleach-stable, alkaline protease from *Bacillus* sp. *Biotechnol. Lett*., 21: 135-138.
17. Johnvesly, B. and Naik, G.R. 2001. Studies on the production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. *Proc. Biochem.* 37: 139-144.
18. Joo, H.S., Kumar, C.G., Park, G.C., Kim, K.T., Paik, S.R. and Chang, C.S. 2002. Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii. Proc. Biochem.* 38: 155-159.
19. Kalpana Devi, M., Rasheedha Banu, A., Gnanaprabhal, G.R., Pradeep, B.V. and Palaniswamy, M. 2008. Purification, characterization of alkaline protease enzyme from native isolates *Aspergillus niger* and its compatibility with commercial detergents. *Ind. J. Sci. Technol.* 1(7): 1-6.
20. Kirk, O., Borchert, T.V. and Fuglsang, C.C. 2002. Industrial enzymes applications. *Curr. Opin. Biotechnol.* 43: 473-481.
21. Kumar, C.G. and Takagi, H. 1999. Microbial alkaline proteases: From a bio-industrial viewpoint. *Biotechnol. Adv.* 17: 561-594.
22. Kundu, A.K. and Manna, S. 1975. Purification and characterization of extracellular protease of *Aspergillus oryzae. Appl. Microbiol.* 30: 507-513.
23. Labadie, J. and Montel, M.C. 1982. Purification and study of some properties of a collagenases produced by *Empedobacter collagenolyticum. Biochem.* 64(1): 49-53.
24. Li, D.C., Yang, Y.J. and Shem, C.Y. 1997. Protease production by the thermophilic fungus *Thermomyces lanuginosus. Mycol. Res.* 101: 18-22.
25. Lindberg, R.A., Eirich, D.L., Price, J.S., Wolfinbarger, L.Jr. and Drucker, H. 1981. Alkaline protease from *Neurospora crassa:* Purification and partial characterization. *J. Biol. Chem.* 256(2): 811-814.
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
27. Malathi, S. and Chakraborty, R. 1991. Production of alkaline protease by a new *Aspergillus flavus* isolate under solid-substrate fermentation conditions for use as a depilation agent. *Appl. Environ. Microbiol.* 57: 712-716.
28. Maurer, K.H. 2004. Detergent proteases. *Curr. Opin. Biotechnol.* 15: 330-334.
29. McCoy, M. 2000. Novozymes emerges. *Chem. Eng. News.* 19: 23-25.
30. Monod, M., Togni, G., Rahalison, L. and Frenk, E. 1991. Isolation and characterization of an extracellular alkaline protease of *Aspergillus fumigatus*. *J. Med. Microbiol*. 35: 23-28.
31. Moreira, K.A., Albuquerque, B.F., Teixeira, M.F.S., Porto, A.L.F. and Lima-Filho, J.L. 2002. Application of protease from *Nocardiopsis* sp. as a laundry detergent additive. *World J. Microbiol. Biotechnol.* 18: 307-312.
32. Mukhtar, H. and Hoq, I.U. 2008. Production of alkaline protease by *Bacillus subtilis* and its application as a depilating agent in leather processing. *Pak. J. Bot.* 40(4): 1673-1679.
33. Mulimani, V.H., Patil, G.N. and Prashanth, S.J. 2002. Bleach stable and alkali-tolerant protease from *Aspergillus flavus. Ind. J. Microbiol.* 42: 55-58.
34. Nakiboglu, N., Toscali, D. and Yasa, I. 2001. Silver recovery from waste photographic films by an enzymatic method. *Turk. J. Chem.* 25: 349-353.
35. Ogundero, V.W. and Osunlaja, S.O. 1986. The purification and activities of an alkaline protease of *Aspergillus clavatus* from Nigerian poultry feeds. *J. Basic Microbiol*. 26: 241-248.
36. Phadatare, S.U., Deshpande, V.V. and Srinivasan, M.C. 1993. High activity alkaline protease from *Conidiobolous coronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents. *Enz. Microbiol. Technol.* 15: 72-76.
37. Rao, M.B., Tanksale, A.M., Mohini, S.G. and Deshpande, V.V. 1998. Molecular and Biotechnology Aspects of Microbial Proteases. *Microbiol. Mol. Biol. Rev*. 62: 597-635.
38. Samal, B.B., Karan, B., Parker, C. and Stabinsky, Y. 1991. Isolation and thermal stabilities of two novel serine proteinases from the fungus *Tritirachium album limber. Enz. Microbiol. Technol.* 13: 66-70.
39. Samarntarn, W., Cheevadhanarak, S. and Tanticharoen, M. 1999. Production of alkaline protease by a genetically engineered *Aspergillus oryzae* U1521. *J. Gen. App. Microbiol*. 45: 99-103.
40. Sindhu, R., Suprabha, G.N. and Shashidhar, S. 2009. Optimization of process parameters for the production of alkaline protease from *Penicillium godlewskii* SBSS 25 and its application in detergent industry. *Afri. J. Microbiol Res.* 3(9): 515-522.
41. Takami, H., Akiba, T. and Horikoshi, K. 1989. Production of extremely thermostable alkaline protease from *Bacillus* sp. No. AH 10. *Appl. Microbiol. Biotechnol.* 30: 120-124.
42. Takimura, Y., Saito, K., Okuda, M., Kageyama, Y and Saeki, K. 2007. Alkaliphilic *Bacillus* sp. Strain KSM-LD1 contains a record number of subtilisin-like serine proteases genes. *Appl. Microbiol. Biotechnol*. 76: 395-405.
43. Tunga, R., Shrivastava, B. and Banerjee, R. 2003. Purification and characterization of a protease from solid-state cultures of *Aspergillus parasiticus. Proc. Biochem.* 38: 1553-1558.
44. Usama, F, A. and Ibrahim, Z.M. 2008. Production and some properties of fibinolytic enzyme from Rhizomucor miehei (Cooney and Emerson) Schipper. *J. Appl. Sci. Res*. 4(7): 892-899.
45. Venugopal, M. and Saramma, A.V. 2006. Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. *Proc. Biochem.* 41: 1239-1243.
46. Verma, R., Sil, K., Pandey, A.K. and Rajak, R.C. 2001. Solid state fermentation to produce alkaline protease by *Aspergillus fumigatus* B149. *Ind. J. Microbiol*. 41: 111-14.
47. Wang, S.L., Chen, Y.H., Wang, C.L., Yen, Y.H. and Chern, M.K. 2005. Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in shrimps and crab shell powder medium. *Enz. Microbiol. Technol.* 36: 660-665.
48. Wilson, K. and Walker, J. 2000. Practical Biochemistry: Principles and Techniques. 5th Ed. Cambridge. pp.318-338.