ISSN: 2278-5213

RESEARCH ARTICLE

Screening of alkaline protease production by fungal isolates from different habitats of Sagar and Jabalpur district (M.P)

Vaishali Choudhary and P. C. Jain
Dept. of Applied Microbiology and Biotechnology, Dr. H.S. Gour Vishwavidylaya, Sagar (M.P)
vaishalitripathi.2179@gmail.com; vesaly2179@gmail.com; 08805066900

Abstract

Fungal isolates were screened for alkaline protease production on casein containing agar plates and identified by clear zones of casein hydrolysis around colonies. Totally 141 fungal isolates were used in primary screening of alkaline protease and out of these, only 108 test fungi were found to produce alkaline protease activity as indicated by production of zone of casein hydrolysis around the fungal colonies grown in pH 9 after 5 d of incubation at 28°C. Thirty three test fungi were found to grow on casein containing medium (pH 9) but were unable to produce zone of hydrolysis. For the production of extracellular alkaline proteases in liquid cultures, a total of 10 proteolytic fungi were selected which showed relative enzyme activity (REA) in the range of ≥ 2.29–3 based on the screening results of their REA on solid media. Maximum enzyme activity of 119.48 U/mL was found in *Aspergillus versicolor* PF/F/107 at pH 9 and temperature of 28ºC after an incubation of 96 h in orbital shaker (150 rpm). Enzymes produced from microorganisms that can survive under extreme pH could be particularly useful for commercial applications under high alkaline conditions.

Keywords: Alkaline protease, casein, proteolytic fungi, relative enzyme activity, *Aspergillus versicolor.*

Introduction

In nature, organic substances are found either as simple carbon and/or nitrogen molecules or found as complex polymers of these simple molecules. These substances may be of plant, animal or microbial origin. Simple organic molecules are utilized by the living organisms and get converted into cellular materials and finally enter into the mineral cycling. Substances such as starch, celluloses, hemicelluloses, proteins, fats and oils, pectin, lignin etc., are the major complex organic substances ones and many of these are quite resistant for enzyme attack. However, certain microorganisms produce specific enzymes that can hydrolyze such complex molecules in to simple substances. In ecological terms such hydrolysis of complex substances can be termed as biodegradation or biodeterioration.

The microbial biodegradation of insoluble macromolecules like keratin, cellulose, collagen, lignin, chitin and casein depends on the secretion of extracellular enzymes with the ability to act on compact substrates (Abbas *et al*., 1989; Bockle *et al*., 1995). Such microbial activity helps in the mineral recycling in nature. On the other hand the term biodeterioration is applied to the destructive activity of microorganisms on useful substances, which bring about economic losses. In both conditions, microorganisms elaborate extracellular enzymes, which hydrolyze the complex molecule into soluble forms that are then absorbed, utilized and assimilated by them or released in cell free environment (Rao *et al*., 1998; Kumar and Takagi, 1999; Oh *et al*., 2000; Gupta *et al*., 2002; Nehra, 2004; Amoozegara *et al.,* 2007).

Extracellular proteases are involved in the hydrolysis of proteins in the outer cell environment and enable the cell to absorb and utilize hydrolytic products (Joo *et al*., 2005). Such enzymes may be of commercial significance and hence, exploited to assist protein degradation in various industrial processes (Phadatare, 1993; Rao *et al*., 1998; Kumar and Takagi, 1999; Gupta *et al*., 2002). Proteases show a vast diversity in their physio-chemical and catalytic properties and a lot of literature is available on their biochemical and biotechnological aspects (Rao *et al*., 1998; Saeki *et al*., 2007; Jain *et al.,* 2010). The proteases of industrial importance have been obtained from animals, plants and microorganisms (Ward, 1985; Wandersman, 1989; Rao *et al*., 1998; Gupta *et al.,* 2002).

The inability of the plant and animal proteases to meet current world demands which has led to an increased interest in microbial proteases, since they have been reported to possess almost all characteristics desired for their biotechnological applications (Beg *et al*., 2003; Ellaiah *et al*., 2003; Nascimento *et al*., 2004; Gouda *et al*., 2006). Microbial alkaline proteases from different sources have been viewed for their production, their role in decomposition, downstream processing, and commercial applications have been reviewed by Anwar and Saleemuddin (1998) and Kumar and Takagi (1999). The proteolytic enzymes hydrolyze the peptide links of proteins and peptides to form smaller subunits of amino acids and are produced both extracellular as well as intracellular (Gajju *et al*., 1996; Kumar *et al*., 2002; Potumarthi *et al*., 2007).

The microbial proteases are investigated not only in scientific areas such as protein chemistry and protein engineering but also find wide application in the laundry detergents, leather processing, protein recovery or solubilization, organic synthesis, meat tenderization, food industry and recovery of silver from used X-ray films (Horikoshi and Akiba, 1982; Fujiwara and Yamamoto, 1987; Dahot, 1994; Mala *et al*., 1998; Kirk *et al.,* 2002; Kocher *et al*., 2003; Zambare *et al*., 2007; Abidi *et al*., 2008). For selection of potential microorganisms and commercial applications of the proteases produced by them, there is need for highly selective and easy assay techniques. Selection of specific screening techniques can provide a comparative account of the desired properties. The basic principle underlying the measurement of proteolytic activity is based on the measurement of products from protein hydrolysis (Godfrey and West, 1996).

Proteolytic activity can be measured either by qualitative or quantitative methods. Qualitative methods give us a comprehensive range of spectra of use of microorganisms while the latter relates to the yield of products produced by those microorganisms. To evaluate the actual potential and performance of the microorganisms and their products both qualitative and quantitative methods are to be followed. Some of the most commonly followed qualitative methods used for the estimation of proteolytic activity are radial diffusion method (Gallagher *et al.,* 1986; Adams *et al.,* 1975), thin layer enzyme method (Elwing *et al.,* 1976; Wikstrom, 1983) and protein agar plate method (Jorgensen, 1974; Ten *et al.,* 2005). These methods measure the proteolytic activity in terms of clear zone of protein hydrolysis on agar plates containing protein substrates. Some workers have detected proteolytic activity in microorganisms using casein and gelatin as proteinaceous substrates (Reese *et al*., 1954; Sandhia and Prema, 1998; Verma *et al*., 2001; Ellaiah *et al*., 2002). The more specific protein substrates such as keratin, elastin and collagen have been used for the assay of specific proteases such as keratinases, elastases, collagenases, respectively (Shibata *et al*., 1993; Friedrich *et al*., 1999; Petrova *et al*., 2001; All press *et al*., 2002). Other than these, some of the modified substrates such as azo dye bound collagen; casein, keratin etc. are also being used for detection of proteolytic potentials of microorganisms (Moore, 1969; Wainwright, 1982; Kanayama and Sakai, 2005). Rajamani and Hilda (1987) have used buffered skimmed milk agar for the detection of protease activity. This plate assay can also be used to distinguish both neutral and alkaline proteases by manipulating the pH of the buffer system. Similarly, Allpress *et al*. (2002) also used skimmed milk as a protein substrate for the detection of protease activity by agar diffusion method. Another preferred substrate for the determination of protease activity is gelatin. Hankin and Anagnostakis (1975) assayed the proteolytic activity using gelatin as a protein substrate. Kumar *et al.* (2002) screened the bacterial species for the production of protease by using nutrient agar medium containing 0.4% gelatin as a protein substrate. Above all, casein has been most widely used as protein substrates for proteolytic assay. Several scientists have reported the use of casein as protein substrate for the detection of protease activity by microorganisms (McDonald and Chen, 1965; Verma *et al.,* 2001; Shumi *et al*., 2004a, b; Ikram-ul-Haq, 2006; Chi *et al.,* 2007; Sindhu *et al.,* 2009). Besides this, Harrigan (1972) and Montville (1983) devised a dual substrate plate diffusion assay for the detection of both caseinase and gelatinase using medium supplemented with 1% gelatin and 1% casein as protein substrate. Considering the above facts, this study was aimed to screen alkaline protease production by fungal isolates from different habitats of Sagar and Jabalpur district (M.P).

Materials and methods

Sample collection: The soil samples used for the isolation of proteolytic fungi were collected from three sites viz. gardens, crop fields and poultry farms of different localities of Sagar (23°50 N latitude and 78°43 E longitude) and Jabalpur (23°10 N latitude and 79°59 E longitude) districts of Madhya Pradesh, India. The samples from the above habitats were collected randomly from the superficial layer of soil not exceeding 5-6 cm depth using pre-sterilized spatula and were transferred into sterilized polythene bags. The samples were then brought to the laboratory and kept at 15°C until processed.

Isolation of fungi: Reese agar medium (K_2HPO_4-2.0 g; $(NH_4)_2SO_4$-1.5 g; $MgSO_4.7H_2O$-0.3 g; $CaCl_2$-0.3 g; urea-0.3 g; yeast extract-0.5 g, glucose-2.5 g; casein-5.0 g; agar-20 g; distilled water-1000 mL) was chosen as growth medium. The initial pH of the medium was adjusted to 9 with solution of 1 N NaOH. The fungal strains from the soil samples were isolated by serial dilution method (Waksman, 1927) on Reese agar media (Reese *et al*., 1950). The soil sample was mixed and a suspension of 1 g (dry weight equivalent) in 10 mL of sterile distilled water was prepared. One mL of the soil suspension was then diluted serially (six fold) and used in the estimation of fungal population. The plates were incubated at ± 28°C for 4-5 d.

Purification and identification of isolated fungi: The isolated fungi were purified by point inoculating them on plates containing PDA (potato dextrose agar) medium. The fungi were purified by repeated point inoculation. The purity of the isolated fungus was confirmed by microscopic examination of the culture at 400X magnification using light microscope. After ensuring purity, the cultures were sub-cultured on PDA slants and allowed to grow for a period of 5-7 d and subsequently stored at 4°C as stock cultures. Working as well as stock cultures are maintained and the working cultures were transferred to fresh PDA slants at regular intervals of 3 months.

The isolated fungi were sub-cultured on potato dextrose agar and allowed to grow and sporulate. On the basis of their colony and morphological characteristics, the fungi were identified. Lacto phenol cotton blue stain was used as mounting fluid. The slides were observed under microscope and fungi were identified by following the mycological literature. The following morphological characteristics were evaluated: colony growth (length and width), presence or absence of aerial mycelium, colony color, presence of wrinkles and furrows, pigment production etc. The characteristics were compared with the standard descriptions.

Screening of the isolates for alkaline protease production

Primary screening: Test fungi (141) isolated from 50 soil samples were tested for their ability to produce alkaline protease using solid agar plate assay on Reese medium. For the production of alkaline protease by test fungi the methods as described by Rajamani and Hilda (1987) were used with certain modification. The fungus was grown on Reese's medium containing 0.5% casein as protein substrate and the plates were incubated at 28°C to allow the growth of test fungi for 4 d. They were then examined for the formation of zone of clearance around the colony. The zone was made clearer, by flooding the plates with a solution of 5% Trichloroacetic acid (TCA) and kept for 30 min to allow the precipitation of residual proteins in the medium. The diameter of the fungal colony and the total zone of enzyme activity including the growth diameter were measured in each case. On the basis of this 'Relative Enzyme Activity' (REA) was calculated.

Secondary screening: The fungal isolates that gave biggest zone of inhibition in above described assay were selected as the best producer and were grown in alkaline Reese broth at 28°C for 96 h at 150 rpm. After incubation, the flasks were removed and filtered through Whatman No. 1 filter paper. The culture filtrates were centrifuged at 5000 rpm for 10 min and the enzyme activity in the supernatant was checked by agar-well diffusion method in casein containing media (Rasool *et al*., 1996). Supernatant was then partially purified by ammonium sulfate precipitation and activity was again checked (Adinarayana *et al*., 2003). Total protein concentration in the enzyme solution was determined by Lowry's method (Lowry *et al*., 1951).

Assay of proteolytic activity: The cultivation fluids obtained from the test fungi were studied for alkaline protease activity. For this, the plates were prepared with the sterile Reese media (20 mL, pH 9) and a bore having 10 mm diameter was made. Test enzyme sample were dispensed in the well bored in agar medium and incubated at 28°C for 48 h. Zone of proteolysis was observed as transparent zone of hydrolysis around the agar well. In order to clear the zone, the plates were flooded with 5% TCA. The zone of enzyme activity was measured after 30 min and recorded.

Quantitative measurement of alkaline protease activity: Alkaline protease activity in the culture filtrate was tested using the method as suggested by Takami *et al.* (1989). Casein was used as protein substrate for estimation of alkaline protease activity. One unit of alkaline protease is defined as the amount of enzyme that catalyzes the release of 1 µg of tyrosine from substrate per unit time per mL under standard assay conditions. In order to express enzyme activity of crude enzyme samples in terms of protein content, the protein content of the crude culture filtrate was measured. Lowry *et al*. (1951) method was used and Bovine Serum Albumin (BSA) was used as standard.

Results and discussion

Fifty soil samples were collected from different habitats that included 9 samples of Garden soil, 9 of crop fields and 7 samples of poultry farm soils of district Sagar, M.P. and 7 of garden soil, 8 of crop field and 10 samples of poultry farm soils of district Jabalpur, M.P. Out of these, a total of 38 samples were found positive for the occurrence of fungi. The percentage distribution of positive samples in different habitats was found to be 64.7, 75 and 88.23% in samples collected from crop fields, gardens and poultry farm soils. A total of 141 fungal forms were obtained from the positive samples. These include 56 fungal forms from poultry farm soils, 47 from garden soil and 38 from crop field soils.

Alkaline protease activity of 141 test fungi was determined using 0.5% casein as protein substrate on solid Reese media. Out of these, only 108 test fungi were found to produce alkaline protease activity as indicated by production of zone of casein hydrolysis around the fungal colonies grown pH 9 after 5 d of incubation at 28°C. Other 33 test fungi were found to grow on casein containing medium (pH 9) but were unable to produce zone of hydrolysis. Ten fungal isolates out of 108 were selected for further studies on the basis of REA.

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. The alkaline protease producing fungal isolates showed varied levels of REA ranging from 0.5 to 3. Distribution of alkaline protease activity of fungi belonging to different habitats is shown in Table 1. Maximum percentage of alkaline protease positive fungi were found among those collected from samples of poultry farm (80.7%), followed by garden soils (78.72%) and crop field soils (60.52%). On the basis of REA, alkaline protease producing test fungi have been categorized in 4 groups i.e. Excellent (REA ≥ 2.5 to < 3.0); Good (REA≥ 2.0 to < 2.5); Fair (REA ≥1.5 to < 2.0) and Poor (REA ≥0.5 to < 1.5) producers.

Table 1. Categorization of alkaline protease activity of fungi from different habitats.

Source	No. of fungi		Protease positive fungi (%)	No. of fungi				
	Tested	Protease positive		Grading of proteolytic activity based on REA*				
				Excellent	Good	Fair	Poor	Nil
Garden soil	47	39	82.97	2	7	11	19	8
Crop field soil	38	23	60.52	2	6	5	10	14
Poultry farm soil	56	46	82.14	2	7	9	28	10
Total	141	108	76.59	6	20	25	57	32

**Proteolytic activity: Excellent: REA ≥ 2.5 to < 3.0; Good: REA ≥ 2.0 to < 2.5; Fair: REA ≥ 1.5 to <2.0; Poor: REA ≥ 0.5 to <1.5.*

Table 2. Alkaline protease activity of potential fungal isolates.

Fungi	Fungal colony in dia (mm)	Zone of clearance (mm)	REA
Aspergillus versicolor GS/F/018	24	62	2.58
Aspergillus fumigatus GS/F/029	14	39	2.50
Penicillium sp. III CF/F/050	7	21	3.00
Aspergillus fumigatus CF/F/051	15	38	2.53
Aspergillus fumigatus CF/F/060	13	33	2.50
Malbranchea sp. II CF/F/084	29	68	2.34
Aspergillus flavus PF/F/096	14	32	2.29
Aspergillus versicolor PF/F/107	6	18	3.00
Aspergillus flavus PF/F/139	25	63	2.52
Penicillium sp. III PF/F/140	14	32	2.29

Table 3. Alkaline protease activity in cultivation fluid of fungi grown on Reese alkaline medium.

Fungi	Zone of alkaline protease activity (mm)*	REA
Aspergillus versicolor GS/F/018	38	2.58
Aspergillus fumigatus GS/F/029	36	2.50
Penicillium sp. III CF/F/050	40	3.00
Aspergillus fumigatus CF/F/051	38	2.53
Aspergillus fumigatus CF/F/060	36	2.50
Malbranchea sp. II CF/F/084	33	2.34
Aspergillus flavus PF/F/096	30	2.29
Aspergillus versicolor PF/F/107	41	3.00
Aspergillus flavus PF/F/139	34	2.52
Penicillium sp. III PF/F/140	30	2.29

Including the diameter of well (10 mm).

Table 4. Alkaline protease activity of fungi grown at 28°C for 5 d at 150 rpm.

Test organisms	Amount of tyrosine released	Enzyme activity (U/mL)	Protein (µg/mL)
Aspergillus versicolor GS/F/018	322.79	112.97	401
Aspergillus fumigatus GS/F/029	299.53	104.83	373
Penicillium sp. III CF/F/050	332.09	116.23	412
Aspergillus fumigatus CF/F/051	313.48	109.72	389
Aspergillus fumigatus CF/F/060	299.53	104.83	372
Malbranchea sp. II CF/F/084	280.93	98.32	350
Aspergillus flavus PF/F/096	271.62	95.06	338
Aspergillus versicolor PF/F/107	341.39	119.48	423
Aspergillus flavus PF/F/139	304.18	106.46	378
Penicillium sp. III PF/F/140	276.27	96.69	344

Six fungal isolates including two of crop field soil (CF/F/050; CF/F/051), two of garden soil (GS/F/018 and GS/F/029) and two of poultry farm soil (PF/F/107 and PF/F/139) showed excellent alkaline protease activity. REA in all these cases was recorded > 2.5. A list of 10 isolated fungi showed more alkaline protease activity during their growth on Reese agar medium (Table 2).

The results of alkaline protease activity as noted in the cultivation fluid of all the 10 test fungi are given in Table 3. All the test fungi were found to produce alkaline protease activity in their cultivation fluid when tested by using casein as a protein substrate in Reese media (pH 9). A comparison of the data obtained indicated nearly similar pattern of alkaline protease activity except in few cases.

Aspergillus versicolor PF/F/107 showed greater activity in screening test (Table 3). In present investigation, an effort has been made to study the alkaline protease activity of 10 test fungi grown on alkaline medium containing casein as protein substrate. The protein content of the culture filtrate was also studied to have an idea of the yield of enzyme in their protein precipitates. The culture fluid obtained from cultures of *Aspergillus versicolor* GS/F/018 showed 112.97 U/mL activity in its 4 d old cultures. Another isolate of this species, *Aspergillus versicolor* PF/F/107 showed 119.48 U/mL activity of alkaline protease. The net protein content of both isolates GS/F/018 and PF/F/107 was found to be 401 µg/mL and 423 µg/mL. The alkaline protease activity in culture filtrate of all the 10 fungal isolates is given in Table 4. The different isolates of *Aspergillus fumigatus* have been tested during present study. *Aspergillus fumigatus* GS/F/029, *Aspergillus fumigatus* CF/F/051 and *Aspergillus fumigatus* CF/F/060 produced 106.46 U/mL, 109.72 U/mL and 104.83 U/mL respectively. The Net protein content of 378 µg/mL, 389 µg/mL and 372 µg/mL was recorded in their crude culture filtrates. Nehra *et al.* (2002) reported maximum alkaline protease activity of 212 U/mL from *Aspergillus* sp. using modified Reese medium.

Isolates of *Penicillium* sp. III (CF/F/050 and PF/F/140) were found to produce alkaline protease in its cultures grown on casein containing medium. Maximum activity was noted in 4 d old culture recorded 116.23 U/mL and 96.69 U/mL. The net protein content in their culture filtrates was found to be 412 µg/mL and 344 µg/mL. Two test isolates of *Aspergillus flavus* (PF/F/096 and PF/F/139) produced enzyme activity of 95.06 U/mL and 106.46 U/mL. The net protein content in their culture filtrates was found to be 338 µg/mL and 378µg/mL. *Malbranchea* sp. CF/F/084 showed 98.32 U/mL of enzyme activity with 350 µg/mL of protein release. Ali (1992) reported acid protease production by some strains of *Aspergillus* and *Penicillium.* In this study, maximum activity of alkaline protease was recorded in culture fluids of *A. versicolor*, while the test isolates of *A. flavus* produced this enzyme in the range of 95.06 U/mL to 106.46 U/mL. Other workers have also reported alkaline protease production by their test strains of this species (Malathi and Chakraborty, 1991; Mulimani *et al.,* 2002; Hossain *et al.,* 2006).

The activity of alkaline protease by a strain of *A. flavus* was found stable at a wide range of pH (5-10) with optimum at pH 7.5 and temperature 50°C. The protease was found bleach stable. Isolates of *A. fumigatus* produced more than 100 U/mL activity of alkaline protease. Production of alkaline protease by this species has been reported earlier by Monod *et al.* (1990) and Hossain *et al.* (2006). Overall, the results obtained during this investigation and those reported by other workers indicated that *Aspergillus* is one of the important genera for the production of alkaline protease.

Conclusion

Screening for alkaline protease production showed that *Aspergillus versicolor* PF/F/107 recorded maximum activity of 119.48 U/mL. This study clearly shows that, *Aspergillus versicolor* PF/F/107 is a potent producer of alkaline protease. It is concluded that it can be industrially used in detergents and recovery of silver from used X-ray films 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. Abidi, F., Limam, F. and Nejib, M.M. 2008. Production of alkaline proteases by *Botrytis cinerea* using economic raw materials. Assay as biodetergent. *Proc. Biochem.* 43: 1202-1208.
3. Adams, D.M., Barach, J.T. and Speck, M.C. 1975. Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. *J. Dairy Sci.* 58: 828-832.
4. 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.
5. Ali, O.A.G. 1992. Formation of protease by *Aspergillus fumigatus* and *Penicillium* sp. *J. King Saud. Univ*. 4(2): 127-136.
6. Allpress, J.D., Mountain, G. and Gowland, P.C. 2002. Production, purification and characterization of an extracellular keratinase from lysobacter of an extracellular keratinase from *Lysobacter* NCIMB 9497. *Lett. App. Microbiol.* 34(5): 337-342.
7. 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.
8. Anwar, A. and Saleemuddin, M. 1998. Alkaline proteases: A review. *Biores. Technol.* 64: 175–183.
9. Beg, Q.K., Sahai, V. and Gupta, R 2003. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Process Biochem.* 39: 203-209.
10. Bockle, B., Galunsky, B. and Muller, R. 1995. Characterization of Keratinolytic serine proteinases from *Streptomyces pactum. Appl. Environ. Microbiol.* 61: 3705-3710.
11. Chi, Z., Ma, C. Wang, P. and Li, H.F. 2007. Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. *Biores. Technol.* 98: 534-538.
12. Dahot, U.M. 1994. Purification and some properties of alkaline protease from *Penicillium expansum*. *J. Islamic Acad. Sci.* 7(2): 100-105.
13. Ellaiah, P., Adinarayana, K., Rajyalaxmi, P. and Srinivasulu, B. 2003. Optimization of process parameters for alkaline protease production under solid state fermentation by alkalophilic *Bacillus* sp. *Asian J. Microbiol. Biotechnol. Environ. Sci.* 5: 49-54.
14. Ellaiah, P., Raju, K.V., Adinarayana, K., Adinarayana, G., Prabhakar, T. and Premkumar, J. 2002. Bioactive rare actinomycetes from indigenous natural substrates of Andhra Pradesh. *Hindustan Antibiot. Bull*. 44(1-4): 17-24.
15. Elwing, H., Nilsson, L.A., and Ouchterlony, O. 1976. Visualization principles in thin layer immunoassays (TIA) on plastic surfaces. *Int. Arch. Allergy Appl. Immunol.* 51: 757-762.
16. Friedrich, J., Gradisar, H., Mandin, D. and Chaunant, J.P. 1999. Screening fungi for synthesis of keratinolytic enzymes. *Lett. Appl. Microbiol.* 28: 127-130.
17. Fujiwara, N. and Yamamoto, K. 1987. Decomposition of gelatin layers on X- ray film by the alkaline protease from *Bacillus* sp. B21. *J. Ferment. Technol.* 65(3): 345.
18. Gajju, H., Bhalla, T.C. and Agrawal, H.O. 1996: Thermostable alkaline protease from thermophilic *Bacillus coagulans* PB-77. *Ind. J. Microbiol.* 36:153-155.
19. Gallagher, S.R., Carroll, E.J. Jr. and Leonard, R.T. 1986. A sensitive diffusion plate assay for screening inhibitors of protease activity in plant cell fractions. *Plant Physiol.* 81: 869-874.
20. Godfrey, T. and West, S. 1996. Industrial Enzymology. Mac Millan Publishers Inc. New York. NY. 2nd (Ed).

21. Gouda, M.K. 2006. Optimization and purification of alkaline proteases produced by Marine *Bacillus sp.* MIG newly isolated from eastern harbor of Alexandria. *Polish J. Microbiol.* 55: 119-126.
22. Gupta, R.; Beg, Q.K. And Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotech*. 59:15-32.
23. Hankin, L. and Anagnostakis, S.L. 1975. The use of solid media for detection of enzyme production by fungi. *Mycologia.* 67(3): 597-607.
24. Han-Seung Joo and Chung-Soon Chang 2005. Production of protease from a new alkalophilic *Bacillus sp.* I-312 growth on soybean meal: Optimization and some properties. *Proc. Biochem.* 40: 1263-1270.
25. Harrigan W.F. and McCance, M.E. 1972. Laboratory methods of Microbiology. London. Academic Press. p. 362
26. Horikoshi, K. and Akiba, J. 1982. Alkalophilic Microorganisms: A New Microbial World. Tokyo, Japan: Japan Scientific societies Press and Berlin. Springer Verlag.
27. Hossain, T., Das, F., Marzan, L.W., Rahman, Md. S. and Anwar, M.N. 2006. Some properties of protease of the fungal strain *Aspergillus flavus. Int. J. Agric. Biol.* 8(2): 162-164.
28. Ikram-Ul-Haq, Mukhtar, H. and Umber, H. 2006. Production of protease by *Penicillium chrysogenum* through optimization of environmental conditions. 2(1): *J. Agri. Soc. Sci.* 2(1): 23-25.
29. Jain, N., Richa, M., Kango, N. and Jain, P.C. 2010. Proteases: Significance and Applications. In: Industrial Exploitation of Microorganisms (ed. Mahaeshwari, D.K., Dubey, R.C. and Saravanamuthur, R.). J.K. International Publishers Pvt. Ltd. New Delhi, pp.227-254.
30. Jorgensen, E.B. 1974. Proteolytic activity of *Candida* sp. as related to the pathogenesis of denture stomatitis. *Med. Mycol.* 12(2): 266-271.
31. Kanayama, Y. and Sakai, Y. 2005. Purification and properties of a new type of protease produced by *Microbacterium liquefaciens. Bio. Sci. Biotechnol. Biochem.* 69(5): 916-921.
32. Kirk, O., Borchert, T.V. and Fuglsang, C.C. 2002. Industrial enzymes applications. *Curr. Opin. Biotechnol.* 43: 473-481.
33. Kocher, A., Choct, M., Ross, G., Broz, J. and Chung, T.K. 2003. Effects of enzyme combinations on apparent metabolizable energy of corn-soybean meal based diets in boilers. *J. Appl. Poult. Res.* 12(3): 275-283.
34. Kumar, C.G. and Takagi, H. 1999. Microbial alkaline proteases: From a bio-industrial viewpoint. *Biotechnol. Adv.* 17: 561-594.
35. Kumar, D., Gajju, H. and Bhalla, T.C. 2002. Purification of a thermostable protease by *Bacillus* sp. Apr-4. *Asian J. Microbiol. Biotechnol. Environ. Sci.* 4: 535-540.
36. 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.
37. Mala, B.R., Tanksale, A.M., Ghatge, M.S. and Deshpande, V.V. 1998. Molecular and Biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* 62: 597-635.
38. 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.
39. McDonald, C.E. and Chen, C.L. 1965. Lowry modification of the Folin reagent for determination of proteinase activity. *Ann. Biochem.* 10: 175.
40. 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
41. Montville, T.J. 1983. Dual-substrate plate diffusion essay of proteases. *Appl. Environ. Microbiol.* 45 (1): 200-204.
42. Moore, G.D. 1969. Use of azo-dye bound collagen to measure reaction velocities of proteolytic enzymes. *Anal. Biochem.* 32:122-127.
43. 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.
44. Nascimento, W.C.A. and Martins, M.L.L. 2004. Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Braz. J. Microbiol.* 35: 1-2.
45. Nehra, K.S., Dhillon, S., Chaudhary K. and Singh, R. 2002. Production of alkaline protease by *Aspergillus* sp. under submerged and solid substrate fermentation. *Ind. J. Microbiol.* 42: 43-47.
46. Nehra, K.S., Singh, A., Sharma, J., Kumar, R. and Dhillon, S. 2004. Production and characterization of alkaline protease from *Aspergillus* species and its compatibility with commercial detergents. *Asian. J. Microbiol. Biotechnol. Environ. Sci.* 6: 67-72.
47. Oh, Y.S., Shih, I.L., Tzeng, Y.M. and Wang, S.L. 2000. Protease production by *Pseudomonas aeroginosa* K-187 and its application in the deproteinization of Shrimp and Crab shell wastes. *Enzyme Microbiol. Technol.* 27: 3-10.
48. Petrova, S., Derekova, A. and Vlahov, S. 2006. Purification and properties of individual collagenases from *Streptomyces* sp. strain 3B. *Folia Microbiol.* 51(2): 93-98.
49. 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.
50. Potumarthi, R., Subhakar, C and. Jetty, A. 2007: Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042. Effect of aeration and agitation regimes. *Biochem. Engg. J.* 34: 185-192.
51. Rajamani, S. and Hilda, A. (1987). Plate assay to screen fungi for proteolytic activity. *Curr. Sci.* 56: 1179-1181.
52. 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.
53. Rasool, S.A., Ahmed, S. and Iqbal, A. 1996. Streptococcins of indigenous hemolytic streptococci. *Natt. Prod. Lett.* 8: 67-74.
54. Reese, E.T., Siu, R.G.H. and Levinson, H.S. 1950. The biological degradation of cellulose derivatives and its regulation to the mechanism of cellular hydrolysis. *J. Bacteriol.* 59: 485-489.
55. Saeki, K.S., Katsuya O., Tohru K. and Susumu, I. 2007. Detergent alkaline proteases: Enzymatic properties, genes and crystal structures. *J. Biosci. Bioengg*. 103: 501-508.
56. Sandhia, G.S. and Prema, P. 1998. Selection of optimal growth medium for the synthesis of alkaline proteinase from *Bacillus* SGP-26. *J. Sci. Indus. Res.* 57: 629-633.
57. Shumi, W., Hossain, Md. T. and Anwar, M.N. 2004a. Proteolytic activity of a bacterial isolate *Bacillus fastidiosus* den Dooren de Jong. *J. Biol. Sci.* 4(3): 370-374.
58. Shumi, W., Hossain, T. and Anwar, M.N. 2004b. Isolation and Purification of fungus *Aspergillus funiculosus* G. Smith and its enzyme Protease. *Pak. J. Biol. Sci.* 7(3): 312-317.
59. 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.
60. 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.
61. Ten, L.N., Im, W.T., Kim, M.K. and Lee, S.T. 2005. A plate assay for simultaneous screening of polysaccharide and protein-degrading microorganisms. *Lett. App. Microbiol.* 40: 92-98.
62. 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: 111-114.
63. Wainwright, M. 1982. A new method for determining the microbial degradation of keratin in soils. *Experimentia.* 398: 243-244.
64. Waksman, S.A. 1927. Principles of Soil Microbiology. Baltimore William and Wilkins Co.
65. Wandersman, C. 1989. Secretion, processing and activation of bacterial extracellular proteases. *Mol. Microbiol.* 3: 1825-1830.
66. Ward, O.P. 1985. Proteolytic enzymes: In: Comprehensive Biotechnology, edited by M. M. Young. Pergamon Press, Great Britain. pp.789-818.
67. Wikstrom, M.B. 1983. Detection of microbial proteolytic activity by a cultivation plate assay in which different proteins adsorbed to a hydrophobic surface are used as substrate. *Appl. Environ. Microbiol.* 45(2): 393-400.
68. Zambare, V.P., Nilegaonkar, S.S. and Kanekar, P.P. 2007. Production of an alkaline proteases and its application in dehairing of baffalo hide. *World J. Microbiol. Biotechnol*. 23: 1569-1574.