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RESEARCH ARTICLE

PRODUCTION AND PURIFICATION OF ALKALINE PROTEASE FROM *BACILLUS SUBTILIS* MR12 ISOLATED FROM SLAUGHTER HOUSE SOIL

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ABSTRACT

Proteolytic bacteria were isolated from slaughter house soil with the help of Skim milk agar plates. The isolate showing maximum activity was selected and characterized based on Bergey's manual. Upon 16S rDNA analysis, it displayed maximum similarity with *Bacillus* spp., and the sequence has been deposited in Genbank. For the production of alkaline protease, the strain *Bacillus subtilis* MR12 was grown on modified production medium containing sludge as substrate. Different cultural parameters were optimized for maximal enzyme production. Peak proteolytic activity was observed at pH-8.5 and temperature at 50 °C with 1% inoculums. Cetrimide exhibited the highest inhibitory activity followed by Tween-80, SDS and Tween-20. Alkaline protease was purified to homogeneity by Q-Sepharose revealing a molecular weight of 35 kDa. The enzyme was active and more stable at pH-9 and 55 °C.

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INTRODUCTION

Proteases are the most important industrial enzymes that execute a wide variety of functions and have various important biotechnological applications (Mohan *et al*., 2005). They constitute two-thirds of the total enzymes used in various industries and account for at least a quarter of the total global enzyme production (Kumar *et al*., 2002). Though several microorganisms such as bacteria, fungi, yeast, plant, and mammalian tissues are known to produce alkaline proteases (Ellaiah *et al*., 2002; Prakasham *et al*., 2005), with increasing industrial demand for proteases, it is expected that hyperactive strains will emerge and that the enzymes produced by new exotic microbial strains could be used as biocatalysts in the presently growing biotechnological era. Among the various proteases, microbial proteases play an important role in biotechnological processes. Alkaline proteases produced are of special interest as they could be used in manufacture of detergents, food, pharmaceuticals and leather (Saeki *et al*., 2007; Dias *et al*., 2008). Most of the alkaline proteases applied for industrial purpose have limitations, first many of the alkaline proteases exhibit low activity and low stability at wide range of pH and temperature and secondly 30–40% of production cost of industrial enzymes is estimated to be accounting for the cost of growth medium (Giarrhizzo *et al*., 2007). It is known that the amount of enzyme produced greatly depend on strain and growth conditions. Using of cost-effective growth medium for the production of alkaline proteases from an alkalophilic *Bacillus* sp. is especially important (Joo *et al*., 2002). Among bacteria *Bacillus* sp. are specific producers of extracellular alkaline proteases (Godfrey, 1985). Microbial proteases, especially from *Bacillus* species have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergents formulations (Gupta, 2003). Various bacteria that have been screened for use in various industrial applications, members of the genus *Bacillus*, mainly strains *B. subtilis* and *B. licheniformis* are found to be predominant and prolific source of alkaline proteases (Kumar, 1999). Therefore, there is a need to the search for new strains of *Bacillus* that produce proteolytic enzymes with novel properties and the development of low cost media. Against this backdrop, the present study was aimed to investigate alkaline protease production from *Bacillus subtilis*. and also involved purification of the alkaline *Bacillus* protease by protein separation techniques.

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MATERIALS AND METHODS

Collection of Samples

Samples of slaughter house soil were collected from vasarpadi located at Chennai, Tamil Nadu.

Isolation of Alkaline Protease Producing Bacteria

Each soil samples ware serially diluted and inoculated into the screening medium (g/l) (skim milk powder–10, yeast extract–3.0, ammonium sulphate–6.7, NaCl–0.5, K_2HPO_4–0.7, $MgSo_4 \cdot 7H_2O$–0.5) (Lee *et al*., 2009) with pH-8.5 and incubated at 55 °C for 48 h (Bayoumi, 2011). The formation of clear zone around the colonies confirms the production of alkaline protease. The selective strains colonies that had formed a clear zone around their margins were picked as enzyme producers. The positive isolates were further screened for better production of enzyme by assaying the protease activity (14) in liquid culture using casein as substrate at 55 °C. The strain which showed maximum activity was selected for further study.

Characterization and Molecular Identification of Bacteria

The preliminary characterization of the isolated strain was done using Bergey's manual of systemic bacteriology (Sneath *et al*., 1986). The identity of the isolate was determined by sequence analysis of the 16S rDNA gene. The overnight cultured bacterial cells were lysed with lysozyme and the DNA was extracted by the phenol:chloroform (1:1) extraction method described by Wilson (Wilson, 1990). The 16S rDNA was amplified in PCR with the primer pair BcF (GGATTAAGAGCTTGCTCTTAT) and BtF (GATTGAGAG CTTGCTCTCAATA). The amplified region was then sequenced and subject to BLAST analysis for analyzing its phylogeny (Chen, 2002).

Protease Production Medium

The production of alkaline protease was carried out in the modified production medium. The natural substrates of the production medium described by Chenel *et al.* (2008) were replaced with the dairy sludge. One ml of the overnight grown bacterial strain MR12 was inoculated in 100 ml of the modified production medium containing (g/l): Cow dung–15, KH_2PO_4–1, $MgSO_4 \cdot 7H_2O$–0.3, $FeSO_4 \cdot 7H_2O$–0.2, $ZnSO_4 \cdot 7H_2O$–0.2, $CaCO_3$–1, pH-9.0. After incubation, the medium was centrifuged at 10,000 × *g* for 10 min. Supernatant (culture filtrate) was used as the source of crude enzyme.

Protease Assay

The protease activity was measured by the method described by Beg and Gupta (14). One milliliter of culture filtrate was added with 1ml of 1% (w/v) casein solution in glycine–NaOH buffer of pH-10.5 and incubated for 10 min at 60 °C. The reaction was stopped by addition of 4 ml of 5% trichloroacetic acid. The reaction mixture was centrifuged at 3000 × *g* for 10 min and to 1 ml of the supernatant 5 ml of 0.4 M Na_2CO_3 was added followed by 0.5 ml Folins–Ciocalteus reagent. The amount of tyrosine released was determined spectrophotometrically at 660 nm against the enzyme blank. One unit of protease activity was equivalent to amount of enzyme required to release 1 g of tyrosine/ml/min under standard assay conditions.

Total Protein Content

The amount of protein was estimated by Bradford method (Bradford, 1976) using Bovine Serum Albumin (BSA) as a standard.

Biomass Determination

Bacteria biomass was determined by measuring the absorbance at 600 nm (Henroette *et al*., 1993).

Effect of Inoculum Concentration

The concentration of the inoculum and its effect was studied for optimal alkaline protease production. Experiments were carried out using 1–5% inoculum volume containing OD of 1.0. The flasks with the production medium (pH-8.5) were inoculated and incubated at 50 °C for 48 h.

Effect of Incubation Period

Protease activity was determined after every 12 h of incubation (12, 24, 36, 48, 72, 96, and 120) in order to determine the optimum incubation period for maximum production of extra cellular alkaline protease.

Effect of Initial pH Values and Temperature

The production medium was adjusted at various levels of pH by 1 M NaOH and 1 M HCl solutions (8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0) and the effect of initial pH on alkaline protease production was studied. To study the effect of incubation temperature on protease production, the flasks with the production medium were inoculated and incubated at various temperatures ranging from 45 to 60 °C for 24 h.

Effect of Surface-active Agents

The effect of various surfactants such as Tween-20, Tween-80, SDS, and Centrimide on protease production was studied. The flasks with the production medium (pH-8.5) was inoculated with bacteria and incubated at 50 °C.

Purification of the Protease

The culture filtrate was subjected to 85% ammonium sulfate precipitation by continuous stirring. The saturated solution was kept undisturbed overnight at 4 °C. It was then centrifuged and the precipitate was dissolved in a small amount of 50 mM glycine–NaOH buffer (pH-10.5) and dialyzed for 24 h. Two ml of dialyzed protein was loaded on Q-sepharose column, (1.5 cm × 12 cm) pre-equilibrated with glycine–NaOH buffer. The flow rate was adjusted to 30 ml/h and 2 ml fractions were collected and analyzed for the protease activity. The fractions with maximum activity were pooled, concentrated, and then used as source of protease for further characterization of enzyme (Beg, 2003).

Electrophoresis

The purity of the alkaline protease was checked using 10% denaturing sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) under reducing conditions. The polyacrylamide gel was prepared by the method of Laemmli (Laemmli, 1970). Zymogram Analysis was executed in PAGE and the staining was done with Commassie brilliant blue-R250 (Marquart *et al*., 2005).

Effect of pH Stability

The purified enzyme was incubated at different pH values viz., 6, 7, 8, 9, 9.5, 10 and 11 by using phosphate Tris- or Glycine buffers. Then the tubes were incubated at 55 °C for 18 h and the relative activity was determined.

Effect of Temperature Stability

The experiment was carried out by incubating the purified enzymes for 2 h at different temperatures viz., 50, 55, 60, 65, 70, 75, 80, 85 and 90 °C. At the end of the replicate tubes were cooled and assayed for each purified enzyme to determine the relative enzyme activity.

RESULTS AND DISCUSSION

Dairy sludge is one of the potential sites which may contain bacteria. The sludge environment provides a good source for proteolytic microorganisms to flourish. In this study the dairy sludge samples were screened for bacteria producing alkaline proteases by the screening media at 55 °C. The formation of clear zones around the colonies confirmed the production of alkaline protease. Nine isolates showed a clear zone in skim milk agar plate. Out of nine isolates, an isolate that produced intense proteolytic zone and hence, it was selected as the best strain MR12 and used for further identification.

From microscopic appearance and the biochemical tests, the isolate MR12 was identified as *Bacillus* sp. and further confirmation was done by the 16S rDNA sequence analysis. Upon the amplification of 16S rDNA sequence using specific primers, an amplified product of 1402 bp was obtained which was then sequenced and compared with the Genbank databases using the BLASTN program. The 16S rDNA sequence of the isolate revealed a close relatedness to *Bacillus* sp. with 98% similarity. Hence the strain was confirmed as *Bacillus subtilis* (MR12). and the sequence was submitted to Genbank. Maximum protease production was achieved at 2% inoculum's concentration. The enzyme activity gradually decreases from 3% to 5% (Fig. 1). These results are in accordance with Elibol and Moreira *et al*. (2005) who reported that 2.5% inoculum's level gives highest protease production.

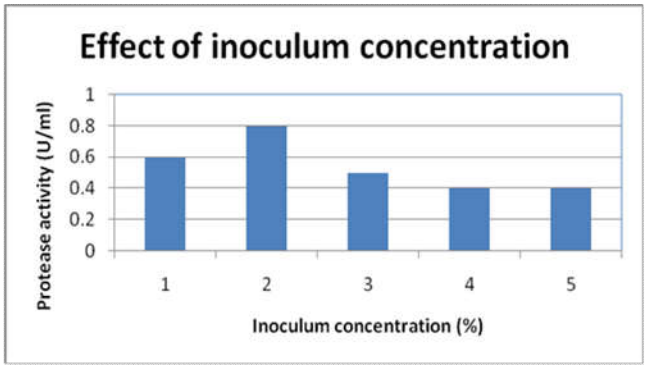

Fig. 1. Effect of inoculum's concentration on protease production by *Bacillus subtilis* MR12

It is very essential to detect the optimum incubation time at which an organism exhibit highest enzyme activity since organisms show considerable variation at different incubation periods (Yossan *et al*., 2006). It was noted that a high protease activity was obtained at 48 h of incubation time. High biomass content was recorded at 96 h of the incubation period (Fig. 2). The study falls in line with the findings of Shumi *et al.* (2004) who reported high proteolytic activity in *Bacillus* sp. at an incubation time of 48 h.

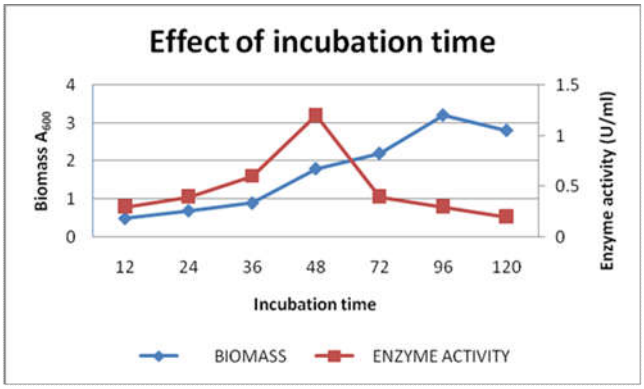

Fig. 2. Effect of incubation time on biomass and protease production by Bacillus subtilis MR12

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Maximum protease production was achieved at pH-9.0 (Fig. 3) by *Bacillus subtilis* MR12. The production of protease increased as pH of the medium was increased reaching maximum at pH-9.0. After pH-9.0, there was a decrease in enzyme production. Results suggest that there is a stimulation of enzyme production at alkaline pH. The obtained results coincide with Kumar *et al.* (2002) who have reported that protease production is high at pH-9 for *Bacillus* sp. strain S4.

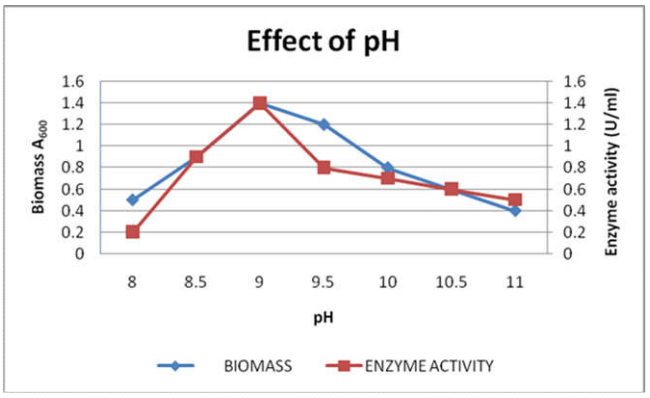

Fig. 3. Effect of initial pH on protease production

To study the effect of various temperatures on the growth and alkaline protease production, different temperatures (40–60 °C) were used in the study. It was noted that high protease production was found at 55 °C indicating the thermotolerant nature of the secreted enzyme (Fig. 4). The influence of incubation temperature on alkaline protease production by bacteria was studied by several workers (Schwimmer, 1981; Vortula *et al*., 1991; Tsakalidou *et al*., 1999). Among the different incubation temperatures tested for protease production 50 °C was found to be optimum for all test isolates.

The optimum temperature for protease production by *B. licheniformis* is 45 °C (Sen, 1993) and for *B. subtilis* 50 °C, but inactive at 55–90 °C. They further reported that 50 °C is the optimum temperature for protease activity.

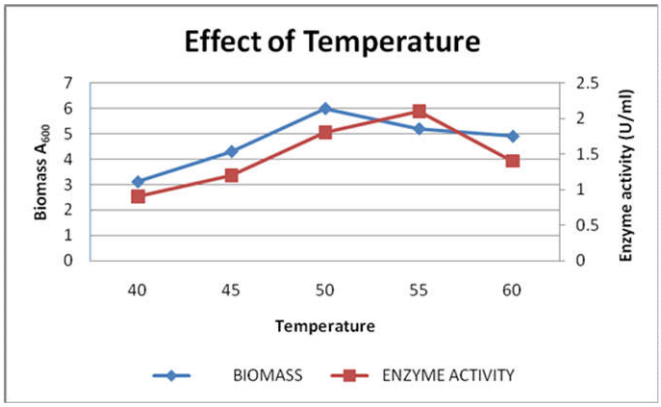

Fig. 4. Effect of temperature on protease production

The effect of various surfactants on alkaline protease production was studied. Surfactants alter cell permeability of microorganisms which lead to increased protein secretion or surface effects on cell-bound enzymes (Ire 82011). It was noted that all the surfactants inhibited protease production at 0.05% concentration. Cetrimide exhibited the highest inhibitory activity followed by Tween-80, SDS and Tween-20 (Table 1).

Table 1. Effect of surfactants on protease activity

1 mM	Residual activity (%)
Control	100
Tween-20	83.5
Tween-80	67.5
Cetrimide	58.4
SDS	77.3

In different fermentation processes, the crude extracts contain different mixtures of proteins and undesirable products as organic acids and other metabolites. So that purification of the required favourable product must take place by different purification methods. In most enzyme purification the precipitation of the crude total proteins is done after separating the culture from the fermentation broth by filtration or centrifugation and the culture supernatant is concentrated by salting out by solid ammonium sulphate as a precipitation agent (Kumar *et al*., 2002). This is followed by another purification procedure as ion exchange and gel filtration chromatography. In this study ammonium sulphate was used as a precipitation agent recording 1048.5 U mg^{-1} of specific activity and 3512 U mg^{-1} after the Q-sepharose chromatography (Table 2).

Table 2. Purification scheme for alkaline protease from *Bacillus subtilis* MR12

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})
Crude ultrafiltrate	2309	887,800	378
Ammonium sulphate perception	510	616,000	1048.5
Q-sepharose	85	323,000	3512

Many researchers used SDS-PAGE to detect the homogeneity and molecular weight of alkaline proteases produced by bacteria and actinomycetes (Rao, 2007).

The homogeneity of the purified alkaline protease from *Bacillus subtilis* MR12 was detected by using SDS-polyacrylamide gel and showed only one band within the gel electrophoresis (Fig. 5), which indicated that the purified alkaline protease was homogenous, and had a molecular weight equal to 24kDa.

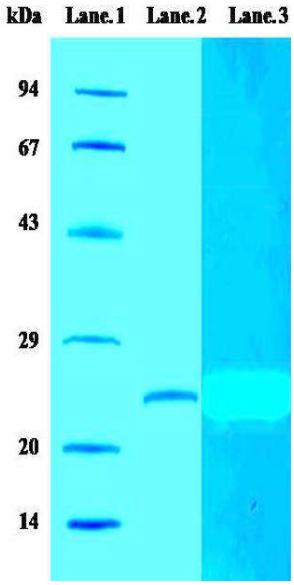

Fig. 5. Photographic representation of SDS-PAGE Gel (Lane 1, Protein marker; Lane 2, Alkaline protease single band(24kDa) after Q-sepharose, Lane 3, Zymogram of the purified alkaline protease activity on PAGE incorporated with gelatin)

The pH stability of the alkaline protease activity was determined by measuring the enzyme activity at varying pH values ranging from 6, 7, 8, 9, 9.5, 10 and 11 using different suitable buffers. It was noted that the enzyme activity was stable and maximum between pHs 8 and 9.5, further increase in pH resulted in decreased enzyme activity (Fig. 6). The temperature stability was measured by incubating the purified enzyme at varying degrees (50–90 °C). Since the enzyme had optimal temperature of 55 °C in the initial stages of the experiments these temperature ranges were employed in the study. It was found that the enzyme exhibited maximum activity in the temperature range 55 °C to 65 °C showing the thermotolerant nature of the purified alkaline protease (Fig. 7).

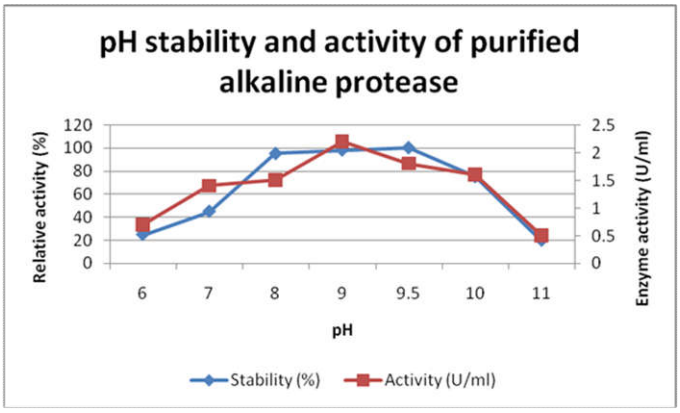

Fig. 6. The effect of pH on the activity and stability of the purified enzyme

The results of the present study evidenced that the production of value-added products like proteolytic enzymes using dairy sludge is possible.

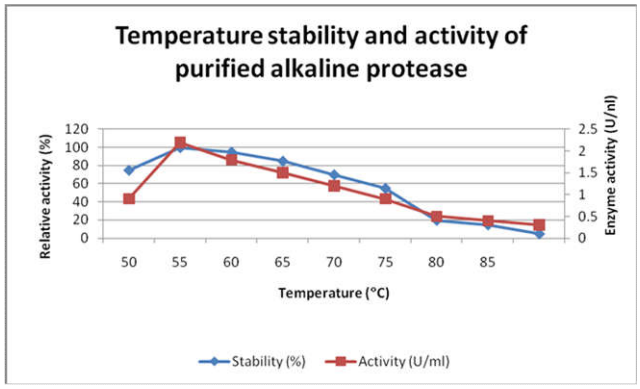

Fig. 7. The effect of temperature on the activity and stability of the purified enzyme

By reusing such industry sludge and effluents as substrate for enzyme production will helps in the reduction of production cost and also helps in enhanced production of valuable bioproducts.

REFERENCES

Bayoumi, R. and A. Bahobil, 2011. Production of thermoalkaliphilic protease by *Hewanella putrefaciens*-EGKSA21 under optimal conditions for application in biodetergent technology. *J. Basic Appl. Res.*, 1: 95–107.

Beg, Q.K. and R. Gupta, 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microbial Technol.*, 32: 294–304.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem*., 72: 248–254.

Chen, M. and L. Tsen, 2002. Discrimination of Bacillus cereus and Bacillus thuringiensis with 16S rRNA and *gyrB* gene based PCR primers and sequencing of their annealing sites. *J. Appl. Microbiol*., 92: 912–919.

Chenel, J., P. Tyagi and D. Surampalli, 2008. Production of thermostable protease enzyme in waste water sludge using thermophilic bacterial strains isolated from sludge. *Water Sci. Techno*l., 57(5): 639–645.

Dias, D.R., D.M. Vilela, M.P.C. Silvestre and R.F. Schwan, 2008. Alkaline protease from Bacillus sp. isolated from coffee bean grown on cheese whey. World *J. Microbiol. Biotechnol*., 24: 2027–2034.

Elibol, M. and R.A. Moreira, 2005. Optimizing some factors affecting alkaline protease production by a marine bacterium *Terclinobacter turbirae* under solid substrate fermentation. *Process Biochem*., 40: 1951–1956.

Ellaiah, P., B. Srinivasulu and K. Adinarayana, 2002. A review on microbial alkaline proteases. *J. Sci. Ind., Res*., 61: 690–704.

Giarrhizzo, J., J. Bubis and A. Tadde, 2007. Influence of the culture medium composition on the excreted/secreted proteases from *Streptomyces violaceoruber*. *World J. Microbiol. Biotechnol*., 23: 553–558.

Godfrey, T.A. and J. Reichelt, 1985. Industrial enzymology the application of enzymes in industry. The Nature Press: London, UK.

Gupta, R., 2003. Purification and characterization of an oxidation stable thiol-dependent serine alkaline protease from *Bacillus mojavenesis*. *Enzyme Microbial Technol*., 32: 294–304.

Henroette, C., S. Zinebi, M.F. Aumaitre, E. Petitdemange and H. Petitdemange, 1993. Protease and lipase production by a strain of *Serratia marcescens*. *J. Ind. Microbiol*., 12: 129–135.

Ire, F.S. B.N. Okolo, A.N. Moneke and F.J.C. Odibo, 2011 Influence of cultivation conditions on the production of a protease from *Aspergillus carbonarius* using submerged fermentation. *Afr. J. Food Sci*., 5(6): 353–365.

Joo, H.S., C.G. Kumar, G.C. Park, K.M. Kim, S.R. Paik and C.S. Chang, 2002. Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. *Process. Biochem*., 38: 155–159.

Kumar, A., A. Sachdev, S.D. Balasubramanyam, A.K. Saxena and A. Lata, 2002. Optimization of conditions for production of neutral and alkaline protease from species of *Bacillus* and *Pseudomonas*. Ind. J. Microbiol., 42: 233–236.

Kumar, C.G. and H. Takagi, 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnol. Adv*., 17: 561–594.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–685.

Lee, S.H., C.W. Chung, Y.J. Yu and Y.H. Rhee. 2009. Effect of alkaline protease producing *Exiguobacterium* sp. YS1 inoculation on the solubilisation and bacterial community of waste activated sludge. *Bioresour. Technol*., 100: 4597–4603.

Marquart, M.E., A. R. Caballero, M. Chomnawang, B.A. Thibodeaux, S. S. Twining and R.J. O'Callaghan, 2005. Identification of a novel secreted protease from *Pseudomonas aeruginosa* that causes corneal erosions. IOVS, 46(10).

Mohan, F.N., D. Dileep and D. Deepthi, 2005. Potential application of protease isolated from *Pseudomonas auriginosa* PD100. *Biotechnol. Ind*., 8: 197–203.

Prakasham, R.S., C.S. Rao, R. SreenivasRao, S. Rajesham and P.N. Sarma, 2005. Optimization of alkaline protease production by *Bacillus* sp. using Taguchi methodology. *Appl. Biochem. Biotechnol*., 120: 133–144.

Rao, K. and M.L. Narasu, 2007. Alkaline protease from *Bacillus firmus* 7728. *Afr. J. Biotechnol*., 6: 2493–2496.

Saeki, K., K. Ozaki, T. Kobayashi and S. Ito, 2007. Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. *J. Biosci. Bioeng*., 6: 501–508.

Schwimmer, S., 1981. Enzyme: purification, enrichment and isolation. In: Source book of food enzymology. The AVI Publishing Co. Inc.: USA. P. 107.

Sen, S. and T. Satyanarayana, 1993. Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. *Indian J. Microbiol*., 33: 43–47.

Shumi, W., M.T. Hossian and M.N. Anwar, 2004. Isolation and purification of fungus *Aspergillus funiculosus* G. Smith and its enzyme protease. *Pak. J. Biol. Sci*., 7(3): 312–317.

Sneath, H.A., M.S. Peter, H. Nicolas and G.L. Hold, 1986. Bergey's manual of systemic bacteriology (vols. 1 & 2) Williams and Wilkins Company, Baltimore, MS, USA.

Tsakalidou, E., R. Mastasiou, I.V. Berghe, J.V. Beeuman and G. Kalantzopoulos, 1999. Cell-wall bound proteinase of *Lactobacillus delbrueckii* sub sp. lactis ACA-DC 178: characterization and specificity for μ-casein. *Appl. Microbiol. Biotechnol*., 36: 196–204.

Vortula, J., J. Pazlarova, M. Dvorakova, L. Vochova, M. Strnadova, H. Kucerova, V. Vinter, R. Zouabian and J.

Chaloupka, 1991. External factors involved in the regulation of synthesis of an extracellular proteinase in *Bacillus megaterium* effect of temperature. *Appl. Microbiol. Biotechnol*., 35: 352–357.

Wilson, K., 1990. Preparation of genomic DNA from bacteria. In: Current protocols in molecular biology. Eds., Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Struhl. New York, NY: Greene Publishing and Wiley Interscience, pp: 2-4-1.

Yossan, Q., Y. Peng, X. Li, H. Wang and Y. Zhang, 2006. Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*., *Curr. Microbiol*., 46: 169–173.

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