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Studies on the Catalytic Properties of Partially Purified Alkaline Proteases from Some Selected Microorganisms

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ABSTRACT

Aims: The research was done to study the conditions enhancing catalytic activities of alkaline proteases from *Vibro* sp., *Lactobacillus brevis*, *Zymomonas* sp., *Athrobacter* sp., *Corynebacterium* sp. and *Bacillus subtilis*.
Methodology and Results: The proteolytic enzymes were purified in 2-step procedures involving ammonium sulphate precipitation and sephadex G-150 gel permeation chromatography. The upper and lower limits for the specific activities of proteases from the selected microorganisms were estimated at 20.63 and 47.51 units/mg protein with *Zymomonas* protease having the highest specific activity towards casein as its substrate and purification fold of 3.46, while that of *Lactobacillus brevis* protease was 8.06. The native molecular weights of these active proteins ranged from 30.4 to 45.7 kDa with *Athrobacter* sp. protease having the highest weight for its subunits. The proteolytic enzymes had optimum pH range of 8 to 10 and temperature range of 50 to 62 ºC accounting for the percentage relative activity range of 75 to 94% and 71 to 84 % respectively. The activities of *Lactobacillus brevis* and *Bacillus subtilis* proteases were maximum at pH 9 and 10 respectively. *Lactobacillus brevis* protease activity was maximum at temperature of 62 ºC, while beyond this value, a general thermal instability of these active proteins was observed. At above 70 ºC, the catalytic activities of *Corynebacterium* sp., *Vibrio* sp., *Zymomonas* sp. and *Arthrobacter* sp. proteases were progressively reduced over a period of 120 min of incubation, while *Bacillus subtlis* and *Lactobacillus brevis* proteases were relatively stable. Effect of metal ions was investigated on the catalytic activity of protease from the microorganisms. *Lactobacillus brevis, Zymomonas* sp., Arthrobacter sp., *Corynebacterium* sp. and *Bacillus subtilis* protease activities were strongly activated by metal ions such as Ca^{+2} and Mg^{+2}. Enzyme activities were inhibited strongly by Cu^{2+} and Hg^{2+} but were not inhibited by ethylene diamine tetra acetic acid (EDTA), while a slight inhibition was observed with K^{+}, Na^{+} and Fe^{2+}.
Conclusion, significance and impact of study: The outcome of this present study indicated useful physico-chemical properties of proteolytic enzymes that could be of biotechnological use in enhancing enzyme catalytic efficiency.

Keywords: alkaline protease, catalytic activities, microorganisms, optimum conditions

INTRODUCTION

Proteases are hydrolytic enzymes which split peptide bonds releasing oligopeptides or free amino acids. They perform both degradative and synthetic functions (Degering *et al*., 2010). Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms and are essential for their growth and differentiation. Although, there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (Gupta *et al*., 2002). Mussarat *et al*. (2008) reported that many different types of extracellular hydrolytic and non- hydrolytic enzymes are produced by microorganisms e.g *Actinoplanes* sp., *Arthrobacter* sp., *Aspergillus* sp., *Bacillus cereus*, *Bacillus coaglulans*, *Bacillus subtlis*, *Candida* sp., *Clostridium* sp., *Lactobacillus* sp., *Penicillium* sp., *Pseudomonas* sp., *Rhizopus* sp., *Streptococcus* sp., *Streptomyces* sp. Alkaline protease (EC.3.4.21-24, 99) is active in a neutral to alkaline pH range and has been shown to have either a serine centre (serine protease) or metallotype (metalloprotease). Many different environments have been explored and exploited for alkaline protease production from *Bacillus* species. According to Gupta *et al*. (2002), *B. licheniformis, B. subtilis, B. amyloliquefaciens and B. majovensis* were reported to be the most potential alkaline protease producing *Bacillus* strains.

The production and catalytic activity of protease are influenced by altered conditions such as pH, temperature, ion concentration, substrate concentration. Alkaline proteases have been satisfactorily used in leather processing, meat tenderization, protein recovery and solubilisation (Johnvesly and Naik, 2001), and detergent application (Najafi and Deobagkar, 2005). In recent years a number of studies have been conducted to characterize alkaline proteases from different microorganisms including bacteria, moulds, yeasts and mammalian tissues (Wei-Hua, 2007). Microorganisms represent an excellent

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source of protease owing to their broad biochemical diversity and susceptibility to genetic manipulation that are desirable for various applications. In the present study, we report the optimum physicochemical properties enhancing the catalytic activities of partially purified protease from different isolates of microorganisms.

MATERIALS AND METHODS

Microorganisms

Vibro sp., *Lactobacillus brevis*, *Zymomonas* sp., *Athrobacter* sp., *Corynebacterium* sp. and *Bacillus subtilis*, used in the present study, were isolated from the gut of kola nut weevil (*Balanogastris kolae*). Cultures were maintained on Nutrient agar slants.

Qualitative test for protease

Proteolytic activities of *Vibro* sp., *Lactobacillus brevis*, *Zymomonas* sp., *Athrobacter* sp., *Corynebacterium* sp. and *Bacillus subtilis* were detected on the basis of appearance of clear zones around the bacterial colonies. Luria casein agar (1 %) plates were used.

Quantitative test for protease

The culture conditions and media for growth of the alkaline protease by the microorganisms were optimized to give maximum production. The basal medium consists (g/L): K_2HPO_4 , 1.5; KH_2PO_4, 0.5; $MgSO_4$, 0.05; $NaCl_2$, 1.5; $(NH_4)_2SO_4$, 1.0; $CaCl_2$, 0.2; $FeSO_4$, 0.2; sucrose; 0.5; yeast extract, 0.5 and 1 % casein was prepared in a 250 ml flask each for the microorganisms and was adjusted by using 0.1 M sodium hydroxide and 0.1 M acetic acid to pH 8. The medium was autoclaved at 121 ºC, 15 psi for 20 min. Oven sterilized, 3 mL of 20 % glycerol solution was added to the medium aseptically. Each of the selected microorganisms was inoculated in the medium separately and the flasks were kept in shaking incubator at 37 ºC with 200 rpm. Samples were drawn from the flasks at intervals of 12 h for a period of 72 h and centrifuged at 3000 rpm for 30 min at 4 ºC. Cell free supernatant corresponding to growth phase was used as the crude enzyme for assay and further analysis. The method of Fugiwara and Yamamoto (1993) was used for the determination of protease activity in culture supernatant with casein as the substrate. One unit of enzyme activity was defined as the amount of enzyme which releases a micromole tyrosine under standard assay condition of 45 ºC, pH 8.5 and reaction time one hour. Lowry method was used for protein determination (Lowry *et al*., 1951)

Enzyme Purification

Ammonium sulphate precipitation: The supernatant was fractionated by precipitation with ammonium sulphate between 50 % and 70 % of saturation. All subsequent steps were carried out at 4 °C. The protein pellet obtained was re-suspended in 10 mM Tris-HCl buffer, pH 7.8, and dialyzed against the same buffer for 24 h.

Sephadex G-100 gel filtration chromatography: The dialysed protein solution from ammonium sulphate precipitation was loaded onto a column of sephadex G-100 (1.5 × 75 cm) equilibrated with 10 mM Tris- HCl buffer, pH 7.8. The column was eluted at a flow rate of 20 mL / h with the same buffer. Five millilitre fractions were collected and subsequently assayed for protease activity.

Estimation of native molecular weight: Sephadex G-100 column was standardised with 3 mg/mL protein markers (creatinephosphokinase 81,000; bovine serum albumin 68,000; ovalbumin 45,000; and alpha chymotrypsinogen 24,700) and blue dextran was used to determine the void volume of the column. The molecular weight was determined from a linear semilogarithmic plot of V_e/V_o against relative molecular weight. V_e is the elution of protein that is measured from the start of the sample application to the inflection point half height rising of the elution peak. V_o is the void volume, which is the elution volume of blue dextran.

Estimation of optimum pH

The effect of pH on the enzyme activity was determined from different pH values of assay medium using phosphate (5.0 to 7.0), Tris-HCl (8.0), and glycine-NaOH (9.0 to 12.0) buffers. The enzyme was incubated for 20 min and enzyme activity was estimated before and after the treatment under standard assay conditions.

Thermal stability

Effect of temperature on the enzyme activity was determined at different temperatures ranging from 30 °C to 90 °C. The enzyme was incubated for 20 min and enzyme activity was estimated before and after the treatment under standard assay conditions. The thermal stability of partially purified protease was determined at different temperatures of 60 °C, 70 °C and 80 °C for a period of 120 min of incubation and enzyme activity was estimated before and after the treatment under standard assay conditions at intervals of 15 min.

Effect of metal ions

The effect of metal ions was tested on the activity of partially purified protease with 20 mM chloride solutions of Na, K, Ca, Mg, Cu, Fe, and Hg. The enzyme was incubated for 20 min and enzyme activity was estimated before and after the treatment under standard assay conditions.

RESULTS AND DISCUSSION

Qualitative and quantitative analysis

The preliminary studies on the production of protease from *Vibro* sp., *Lactobacillus brevis*, *Zymomonas* sp., *Athrobacter* sp., *Corynebacterium* sp. and *Bacillus subtilis* revealed that all microorganisms used for test produced clear zones around the bacterial colonies on 1% casein

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agar plate at 37 °C after 24 h of incubation but the clear zones were different in diameter with *Vibro* sp., *Lactobacillus brevis*, and *Bacillus subtilis* having wider area than *Zymomonas* sp., *Athrobacter* sp. and *Corynebacterium* sp. The different patterns of clear zones could largely be due to different rates of substrate hydrolysis and its utilization by the various microbial cells. Production of protease by these microbes were at their peaks during the growth phases which range from 36 to 48 h of incubation accounting for maximum activity range of 102 to 244 units/mL. Suggestively, *Vibro* sp, *Lactobacillus brevis*, and *Bacillus subtilis* were observed to better utilize casein as substrate for maximum production of protease. Similar results were reported by Adinarayana *et al*. (2003) observed maximum growth and enzyme production at 48 h of incubation.

Purification of extracellular alkaline protease from the microbes

Crude enzymes of the microbes were purified in 2-step procedures involving ammonium sulphate precipitation and sephadex G-150 gel permeation chromatography. The microbes produced a characteristic elution profiles with different activity peaks (results not shown), but the summary of purification steps are shown in **Tables 1, 2 and 3**. The total enzyme activities (**Table 1**) of *Bacillus subtilis*, *Lactobacillus brevis* and *Vibro* sp. were higher than that of *Zymomonas* sp., *Athrobacter* sp. and *Corynebacterium* sp. with *Lactobacillus brevis* almost thrice the total enzyme activity of *Corynebacterium* sp. *Zymomonas* sp. and *Athrobacter* sp. were almost equal in their total enzyme activities, while *Bacillus subtilis* was almost 90 % of the total enzyme activity of *Vibro* sp. In the same vein, the corresponding total enzyme proteins (**Table 2**) of the microbes showed significant differences. *Vibro* sp. and *Bacillus subtilis* were almost equal in their total enzyme proteins while *Athrobacter* sp. and *Corynebacterium* sp. showed the same results. The total enzyme activity of *Lactobacillus brevis* was the highest and *Zymomonas* sp., the least. On the contrary, the values of specific enzyme activities of the microbial proteases (**Table 3**) toward casein as the substrate were different from the values for total enzyme activities and total enzyme proteins. The specific enzyme activity of *Zymomonas* sp. protease was the highest, while *Vibro* sp. and *Corynebacterium* sp. were equal and *Bacillus subtilis* was the least. In addition, the specific activities of *Lactobacillus brevis* and *Athrobacter* sp. proteases were almost 62 and 73 % of *Zymomonas* sp. protease. The upper and lower limits for the specific enzyme activities of the selected microbial proteases were estimated as 20.63 and 47.51 units/mg protein with *Zymomonas* sp. protease having the highest specific activity towards casein as its substrate and purification fold of 3.46. However *Lactobacillus brevis* had a purification fold of 8.06. The purification results suggest different rates of production of microbial proteoltyic enzymes in response to the amount of available protein substrates and different degrees of substrate breakdown and utilization with the corresponding substrate turnover rates. Thermostable alkaline protease from *Bacillus subtilis* PE-11was purified in a similar way (Adinarayana *et al*., 2008).

Table 1: Total activities of partially purified microbial proteases

Micoorganism protease	Total activity (units)
Vibro sp	11077
Lactobacillus brevis	16047
Zymomonas sp.	8357
Athrobacter sp.	8364
Corynebacterium sp.	5925
Bacillus subtilis	9896

Table 2: Total protein of partially purified microbial proteases

Micoorganism protease	Total protein (mg)
Vibro sp.	520.3
Lactobacillus brevis	550.35
Zymomonas sp.	188.7
Athrobacter sp.	253.2
Corynebacterium sp.	269.70
Bacillus subtilis	460.2

Table 3: Specific activities of partially purified microbial proteases

Micoorganism protease	Specific activity (units/mg)
Vibro sp.	21.29
Lactobacillus brevis	29.15
Zymomonas sp.	48.54
Athrobacter sp.	33.03
Corynebacterium sp.	21.96
Bacillus subtilis	20.35

Table 4: Native molecular weights of partially purified microbial proteases

Micoorganism protease	Daltons
Vibro sp.	22357
Lactobacillus brevis	33200
Zymomonas sp.	20458
Athrobacter sp.	35716
Corynebacterium sp.	22886
Bacillus subtilis	26515

Table 5: Optimum pH of partially purified microbial proteases

Micoorganism protease	pH
Vibro sp.	8.0
Lactobacillus brevis	9.0
Zymomonas sp.	8.0
Athrobacter sp.	8.0
Corynebacterium sp.	9.0
Bacillus subtilis	10.0

Native molecular weight

Important differences exist among the molecular weights of the proteases of the bacterial isolates studied as shown

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in **Table 4**. The native molecular weight was determined from sephadex G–100 column standardized with some known standard protein markers and the result indicated weight values ranging from 30.4 to 45.7 kDa with *Athrobacter sp* protease having the highest weight for its subunits. The molecular weights of *Vibro* sp., *Zymomonas* sp. and *Corynebacterium* sp. proteases were almost equal, while *Lactobacillus brevis* and *Bacillus subtilis* proteases ranked second and third respectively compared with the molecular weight of *Athrobacter* sp. protease. These variations in the molecular weights of the microorganisms are probably due to variations in the genetic makeup of their cells as microbes have different rates of gene expression for different rates of gene activation. The aggregation of protein subunits could also be responsible for various sizes of tertiary structures of enzymes (Voet and Voet, 2004).

Optimum pH, optimum temperature and thermal stability

The partially purified proteolytic enzymes from these selected microorganisms had optimum pH range of 8.0 to 9.0 (**Table 5**) which accounted for a range of 75 to 94 % relative activity with casein as substrate. *Vibro* sp., *Zymomonas* sp. and *Athrobacter* sp. proteases were observed to have their maximum percentage relative activities at optimum pH of 8.0 while *Lactobacillus brevis* and *Corynebacterium* sp proteases at higher pH value of 9.0 and *Bacillus subtilis* at pH 10.0. **Table 6** shows the optimum temperatures of *Vibro* sp., *Lactobacillus brevis*; *Zymomonas* sp., *Athrobacter* sp., *Corynebacterium* sp., and *Bacillus subtilis* protease activities. The maximum percentage relative activities (71 to 84 %) of the microbial proteases were observed at temperature range of 50 to 62 ºC. The optimum temperatures of *Vibro* sp., *Zymomonas sp*, *Corynebacterium* sp and *Bacillus subtilis* protease activities were the same while *Athrobacter* sp. and *Lactobacillus brevis* proteases activities were at 60 and 62 ºC. Beyond these valves, a general thermal instability of these active proteins was observed (**Figure 1**).

At above 70 ºC (**Figure 2**), the proteolytic activities of *Corynebacterium* sp and *Bacillus subtilis*, *Zymomonas* sp. and *Athrobacter* sp. were drastically reduced over a period of 120 min of incubation but the activities of *Vibro* sp. and *Lactobacillus brevis* proteases were relatively stable. Enzymes generally are amphoteric molecules containing a large number of acidic and basic groups located mainly on their surfaces. The charges on these groups will vary according to their acid dissociation constants and with the pH of their environment. This will affect the total net charge of the enzymes and the distribution of charges on their exterior surfaces, in addition to the reactivity of the catalytic active groups. These effects are especially important in the neighbourhood of the active sites, which will overall affect the activity, structural stability and solubility of the enzyme (Price and Steven, 1990). Alkaline proteases of the genus *Bacillus* have been shown to have maximum catalytic activity at optimum pH and a good stability at high alkaline pH values. The results of pH range of 8 to 12 (Gupta *et al*., 2002); 8 to 11 (Adinarayana *et al*., 2003) for alkaline protease producing bacteria were similar to the values obtained in the present study. However, contrasting pH values have been reported by several authors (Mussarat *et al*., 2008; Kumar *et al*., 1999) for *Bacillus* alkaline proteases.

Table 6: Optimun temperature of partially purified microbial proteases

Micoorganism protease	Temperature (°C)
Vibro sp.	50
Lactobacillus brevis	60
Zymomonas sp.	50
Athrobacter sp.	60
Corynebacterium sp.	50
Bacillus subtilis	50

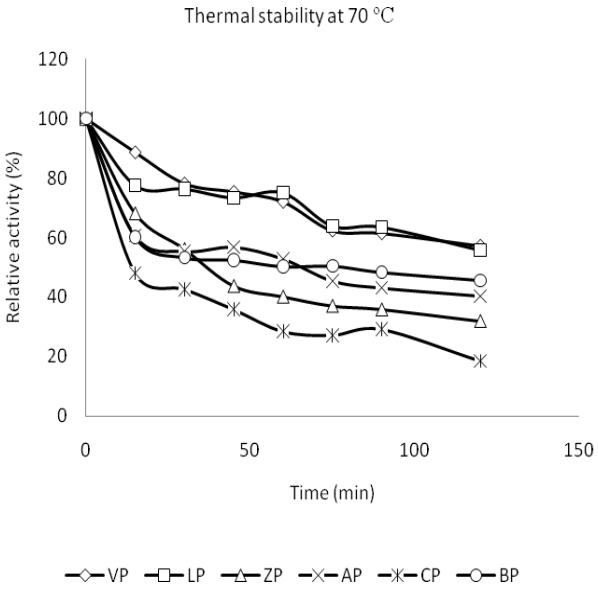

Figure 1: Thermal stability of partially purified microbial proteases at 70 ºC
Vp- *Vibro sp* protease;
Lp-*Lactobacillus brevis* protease;
Zp- *Zymomonas* protease;
Ap-*Athrobacter* protease;
Cp-*Corynebacterium sp* protease;
Bp- *Bacillus subtilis* protease

Changes in temperature of the environment in which enzyme exert its action on substrate greatly influence enzyme stability and catalysis. Enzymes provide a compact structure that is not easily denatured by this external environment factor but at above or below the optimum temperature range, a loss in the catalytic activity of the active protein is seen due to the unfolding state of the tertiary structure of the enzyme. Several investigators (Gupta and Beg, 2003; Joo *et al*., 2003; Oberoi *et a*l., 2001) have reported the optimum temperature of bacillus

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Table 7: Effect of metal ions on the catalytic activities of partially purified microbial protease

Microorganism	Metal ions		
	Protease activators	Weak inhibitors	Strong inhibitors
Vibrio sp.	K^{2+}	Mg^{2+}, Ca^{2+}, Na^{+}	Cu^{2+}, Hg^{2+}
Lactobacillus brevis	Ca^{2+},	K^{+}, Mg^{2+}, Fe^{2+}	Cu^{2+}, Hg^{2+}
Zymomonas sp.	Mg^{2+}	K^{+}, Ca^{2+}	Cu^{2+}, Hg^{2+}
Arthrobacter sp.	Ca^{2+}, Mg^{2+}	Na^{+}, K^{+}	Cu^{2+}, Hg^{2+}, Fe^{2+}
Corynebacterium sp.	Ca^{2+}, Mg^{2+}	Na^{+}, K^{+}	Cu^{2+}, Hg^{2+}
Bacillus subtilis	Ca^{2+}, Mg^{2+}	Fe^{2+},	Cu^{2+}, Hg^{2+}

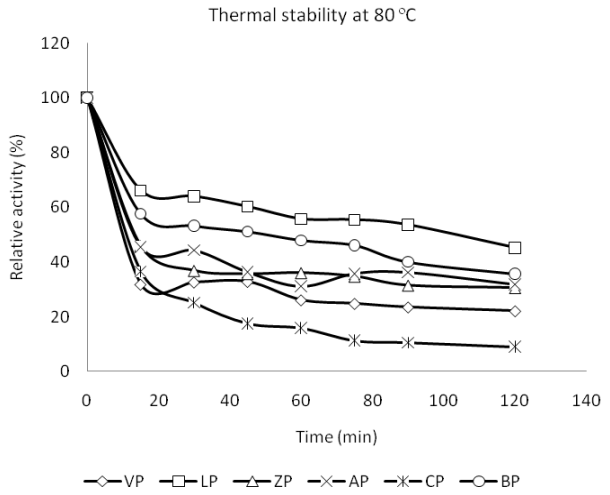

Figure 2: Thermal stability of partially purified microbial protease activity at 80 ºC
Vp- *Vibro sp* protease;
Lp-*Lactobacillus brevis* protease;
Zp- *Zymomonas* protease;
Ap-*Athrobacter* protease;
Cp- *Corynebacterium sp* protease;
Bp- *Bacillus subtilis* protease

species alkaline protease as 60 % and their results were in agreement with what is obtained in the present study.

Effects of metal ions

The effect of metal ions ($Ca^{2+,}$ Mg^{2+}, K^{+}, Na^{+}, Fe^{2+}, Cu^{2+}, and Hg^{2+}) and metal chelating agent (EDTA) were tested on the catalytic activities of *Vibro* sp, Lactobacillus *brevis*, *Zymomonas* sp, *Athrobacter, Corynebacterium* sp, and *Bacillus subtilis* proteases (**Table 7**). The metal ions had either stimulatory, weak inhibitory or strong inhibitory effects on the activities of the microbial proteolytic enzymes. Ca^{2+} and Mg^{2+} were generally observed to have stimulatory effects on the catalytic activities of Lactobacillus *brevis, Zymomonas* sp., *Arthrobacter* sp., *Corynebacterium* sp. and *Bacillus subtilis* proteases with a range of percentage relative activity of 101-108 % while the activity of *Vibrio* sp protease was stimulated by K^{+} but weak inhibitory effects on the activities of *Zymomonas* sp. and *Vibrio* sp proteases were seen with Ca^{2+}. Other metals showing weak inhibitory effects on the microbial protease activities were K^{+}, Na^{+} and Fe^{2+}. Generally, there were significant loss of catalytic activities of *Vibro* sp, *Lactobacillus brevis*, *Zymomonas* sp, *Athrobacter, Corynebacterium* sp, and *Bacillus subtilis* proteases with Cu^{2+} and Hg^{2+}. The Stimulatory effect observed was possibly an indication of protective properties of metal ions when they form bond with catalytic groups on the active site of enzymes to position the active site for binding of substrate and stabilize the enzyme-substrate complex for product formation. On the other hand, the inhibitory effect is possibly due to the redox ability of some metal ions as they participate in abstracting electrons from the interaction between the enzyme active groups and substrates thereby causing a kind of distortion in either enzyme structure or nature of substrate, overall enzyme-substrate complex are prevented and no product formation. Similar stimulatory and inhibitory effects of some metal ions were reported by Adinarayana *et al*. (2003) and Sookkeo *et al*. (2000).

CONCLUSION

The physico-chemical properties affecting catalytic activities of some partially purified microbial proteases were highlighted. *Vibro sp*; *Lactobacillus brevis*; *Zymomonas sp*; *Athrobacter sp*; *Corynebacterium sp*; and *Bacillus subtilis* proteases were shown to have varying total proteins, total activities, specific activities and native molecular weights. Other properties include alkaline pH range, higher temperature range and metal ion activators and inhibitors. These properties indicated possiblilty of proteases from various (sources of) strains of microorganisms for enzyme biotechnological use in enhancing catalytic efficiency of industrial enzymes.

REFERENCES

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. *AAPS Pharmaceutical Science Technician* **4, 56-63.**

Fugiwara, N. and Yamamoto, Z. (1993). Production of alkaline protease in a low-cost medium by alkalophilic *Bacillus* sp. and properties of the enzyme. *Journal of Fermentation Technology* **65, 345-351.**

Degering, C., Eggert, T., Pul, M., Bongaerts, J., Evers, S., Maurer, H. H. and Jaeger, K. E. (2010). Optmization of protease secretion in *Bacillus subtilis* and *Bacillus licheniformis* by screening of

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

homologous and heterologous signal peptides. *Applied Environmental Microbiology* **76, 6370-6376.**

Gupta, R. and Beg, Q. K. (2003). Purification and characterization of an oxidation stable, thiol-dependent serine alkaline protease from *Bacillus moavensis*. *Enzyme and Microbial Technology* **32, 294-301.**

Gupta, R., Beg, Q. K., Lorenz, P. (2002). Bacteria alkaline protease: molecular approaches and industrial application. *Applied Microbiology and Biotechnology* **59, 15-32.**

Johnvesly, B. and Naik, G. R. (2001). Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in chemically defined medium. *Process Biochemistry* **37, 139-152.**

Joo, H. S., Kumar, C. G., Park, G. C., Paik, S.R. and Chang, C. S. (2003). Oxidant and SDS-stable alkaline protease from *Bacillus clausii* 1-52: production and some properties. *Journal of Applied Microbiology* **95, 267-274.**

Kumar, C. G. and Takagi, H. (1999). Microbial alkaline proteases from bio-industrial viewpoint. *Biotechnology Advances* **17, 561-594.**

Kunitz, N. (1965). *Methods of enzymatic analysis* (2nd Edition). Verlag Chemical Academic Press. New York, London **pp. 807-814.**

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein determination. *Journal Biological Chemistry* **193, 265-275.**

Mussarat, S., Aamer, A. S., Abdul, H. and Fariha, H. (2008). Influence of culture conditions on production and activity of protease from *Bacillus subtilis* BS1. *Pakistan Journal of Botany* **40 (5), 2161-2169.**

Najafi, M. F. and Deobagkar, D. (2005). Potential application of protease isolated from *Pseudomonas aeruginosa* PD 100. *Journal of Biotechnology* **8, 370-374.**

Oberoi, R., Beg, Q. K., Puri, S., Saxena, R. K. and Gupta, R. (2001). Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus* sp. *Journal of Microbiology and Biotechnology* **17, 493-499.**

Price, N. C. and Steven, L. (1982). *Fundamentals of Enzymology*. Oxford University Press, Oxford, **454pp.**

Sookkeo, B., Sinchaikul, S., Phutrakul, S. and Chen, S.T. (2000). Purification and characterization of thehighly thermostable protease from *Bacillus sterothermophilus* TLS33. *Protein Expression and Purification* **20 (2), 142-51.**

Voet, D. and Voet, J.G. (2004). *Biochemistry* 3rd Edition. John Wiley and Sons Inc. USA. **1591pp.**

Wei-Hua, C. (2007). Optimization of extracellular alkaline protease production from species of *Bacillus. Journal Industrial Microbiology and Biotechnology* **34, 241-245.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538