JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2010. Vol. 4(1), p. 83-90

Purification and Properties of Thermostable Alkaline Protease from *Bacillus subtilis* K-30

K. Suresh Babu Naidu[1]* and K. Lakshmi Devi[2]

[1]Balaji Crop Care Pvt. Ltd. Phase II, Plot No. 79, IDA, Cherlapally, Hyderabad - 500 051, India.
[2]Department of Biochemistry, Sri Krishnadevraya University, Anantapur - 515 003, India.

(Received: 20 July 2009; accepted: 09 September 2009)

***B.* subtilis K-30 isolated from soil sample collected from detergent industry produced alkaline protease. Thermostable extracellular alkaline protease (EC 3.4.21.14), suitable for use in detergents, was purified by ion exchange chromatography from *Bacillus subtilis* K-30 and its properties were examined. The purified enzyme had a molecular weight of 29kDa as determined by SDS-PAGE. The optimal pH and temperature of the purified enzyme was found to be 10 and 55°C, respectively. It was capable of hydrolyzing many soluble and insoluble protein substrates. Casein was the best substrate among the substrates tested (BSA, egg albumin, gelatin). The enzyme activity was inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+} at 5mM whereas Ca^{2+}, Mg^{2+}, and Mn^{2+} had stimulatory effect. The protease was 100% inhibited by PMSF, indicating serine protease activity. The enzyme retained more than 40 percent activity after 30 min incubation at 60°C in the presence of detergents such as Henko and Vim ultra, indicating its suitability in detergent industry.**

Key words: *Bacillus subtilis* K-30, Alkaline protease, Thermostable.

Proteases (peptidyl-peptide hydrolases) are one of the most important classes of enzymes and expressed throughout the animal and plant kingdoms as well as in viruses and bacteria (Vikash Dubey *et al*., 2007). Proteases have been divided into six mechanistic classes by the International Union of Biochemistry. These include the cysteine, serine, aspartic, mettaloprotease, threonine and unknown type (Enzyme nomenclature, 1992). The threonine protease is the most recently discovered type (Seemuller *et al*., 1995). The protease family has drawn special attention as these constitute one of the most important groups of industrial enzymes accounting for about 65% of the total worldwide enzyme sales (Reddy *et al.,* 2008; Nascimento and Martins, 2004; Beg and Gupta, 2003; Ellaiah *et al*., 2003, Ito *et al*., 1998).

Proteases have wide range of applications in detergent, pharmaceutical, leather, food and silk industries. Although a variety of proteolytic fungi and bacteria are known, only few

* To whom all correspondence should be addressed.
Tel.: +91-40-27260807
E-mail: dr.sureshbabunaidu@gmail.com

provide high activities with commercial success (Berla and Suseela, 2002). Commercial proteases are mostly from various sources and it was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria (Ferrero *et al*., 1996). *Bacillus* spp. are extensively exploited for protease production (Karl Heinz, 2004). The alkaline proteases from these species represent the lead molecules for the subtilisins. Members of subtilisin super family of proteases have now been identified with different functions, in all living organisms (Siezen and Leunissen, 1997). Thermophiles such as *Thermus aquaticus* (Gabriela *et al*., 2003), *Bacillus licheniformis* (Ferrero *et al*., 1996), *Bacillus stearothermophilus* (Boonayanan *et al*., 2000), *Bacillus pumilus* (Kumar, 1999), *Thermoanerobacter yonsieensis* (Hyenung *et al*., 2002), *Bacillus thermoruber* (Manachini *et al*., 1988) and *Bacillus brevis* (Banerjee *et al*., 1999) have been studied for their capability to produce thermostable proteases.

Thermostable proteases are advantageous as they are highly stable at elevated temperatures; reduce risk of contamination by other organisms, ideal models for studying thermal stability of protein, elucidation of mechanisms involved in thermostability of enzymes (Helmann, 1995). Most enzymes used are mainly derived from mesophilic sources that work in narrow ranges of pH and moderate temperatures. However, alkaline proteases exhibit optimum activity and stability at high pH, temperatures, in presence of surfactants and chaotropic agents, which make them suitable candidates for industrial applications (Kumar and Takagi, 1999).

In the present study, we report the purification and some properties of thermostable alkaline protease from *Bacillus subtilis* K-30 originally isolated from soil (Naidu and Devi, 2005) using rice bran.

MATERIAL AND METHOD

All the chemicals and microbiological media used in the present study were of analytical grade.

Methods

Production of protease from *B. subtilis* K-30

Bacillus subtilis K-30 was first isolated in the laboratory from soil sample collected from detergent industry, Nellore, Andhra Pradesh, India (Naidu and Devi, 2005). Production of protease from *B. subtilis* K-30 was carried out in a medium containing (g/L) glucose, 1.0; casein, 0.5; yeast extract, 0. 5; KH_2PO_4, 0.1; and Na_2CO_3, 1.0 and maintained at 37°C for 48 hours in a continuous shaker incubator (120 rpm). After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme was assayed for proteolytic activity and used for further studies.

Purification of the protease

Crude enzyme was precipitated with ammonium sulfate at 60% saturation. All subsequent steps were carried out at 4°C. The protein pellet obtained after saturation with ammonium sulfate was dissolved in 0.1M Tris-HCl buffer and loaded onto column of Sephadex G-100 (1.5X24 cm) (Sigma-Aldrich, St. Louis, MO) equilibrated with Tris-HCl buffer, pH 7.8. The column was eluted at a flow rate of 50 ml/h. From the elution profile, it was observed that the protease was eluted as a single peak of caseinase activity. The collected precipitates were dissolved in 10ml of 0.1M pH 7.8 Tris-HCl buffer. The solution was applied on DEAE column (1.6X40 cm) and eluted with 20mM pH 7.8 Tris-HCl buffer at a flow rate of 0.5ml/min. The protein content was determined using the method previously described (Lowry *et al*., 1951) with bovine serum albumin (BSA) as standard.

Enzyme assay

Alkaline protease activity was determined by a modification procedure based on the method of casein digestion (Tsuchida *et al*., 1986). The reaction mixture consists of 0.5ml of purified enzyme, 0.5ml Tris-HCl buffer (50mM, pH 8) and 1ml of 1% casein in Tris-HCl buffer of same pH. The mixture was incubated for 30 min at 55°C and the reaction was stopped by the addition of 4ml of 5% Trichloroacetic acid (TCA). After 60 min, the solution is centrifuged at 4°C for 15 min at 3000 rpm. 1 ml of supernatant was mixed with 5 ml of 0.4M Na_2C0_3 followed by the addition of 0.5 ml phenol reagent and absorbance

was read at 660nm. One protease Unit is defined as the amount of enzyme that releases 1µmol of tyrosine per minute under the above said condition.

Polyacrylamide Gel Electrophoresis

After DEAE column chromatography, the fractions[19-23] showing the highest specific activity were dialyzed, lyophilized and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out at 12% (w/v) resolving gel and 5%(w/v) stacking gel according to the method of Laemmili 1971). The following proteins were used as protein standards: BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-P-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20kDa), and lactalbumin (14.2 kDa).

Effect of pH on purified enzyme activity and stability

The activity of crude and purified protease was measured at different pH values in the presence and absence of 10mM $CaCl_2$. The pH was adjusted using the following buffers (0.05M): phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0) and glycine –NaOH (pH9.0-12.0). Reaction mixtures were incubated at 55°C for 30 minutes, and the activity of enzyme was measured. The purified enzyme was diluted in different buffers (pH 5.0-12.0) and incubated at 55°C for 2 and 20 h for enzyme stability. The relative activity at each exposure was measured.

Effect of temperature on enzyme activity and stability

The activity of the crude and purified enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30°C to 90°C in the presence and absence of 10mM $CaCl_2$ for 30 minutes. Thermal stability was studied as described (Huang *et al*., 2006) by preincubating the purified enzyme without substrate at 60, 70, and 80°C from 0-180 min, before determination of proteolytic activity on casein at 1%.

Effect of inhibitors on protease activity

The effect of various inhibitors (5mM) such as serine inhibitors phenyl methyl sulfonyl fluoride (PMSF) and diisopropyl fluro phosphate (DFP), cysteine inhibitors p-chloro mercuric benzoate (p CMB) and β-mercaptoethanol (β-ME), iodoacetate and a chelator of divalent cations ethylene diamine tetraacetic acid (EDTA) was determined by preincubation with the enzyme solution for 30 minutes at 60°C before the addition of substrate. The relative protease activity was measured.

Effect of various metal ions on protease activity

The effects of metal ions (Ca^{2+}, Mg^{2+}, Al^{3+}, Co^{2+}, Cd^{2+}, Fe^{3+}, Na^{2+}, Zn^{2+}, Hg^{2+}, and Cu^{2+} (5mM) were investigated by adding them to the reaction mixture. Relative protease activities were measured.

Substrate specificity

Protease activity with various protein substrates including bovine serum albumin, casein, egg albumin and gelatin was assayed by mixing 100ng of enzyme and 200µl of assay buffer containing the protein substrates (2mg/ml). After incubation at 60°C for 30 minutes, the reaction was stopped by adding 4ml of 5% TCA and allowed to stand for 10 min. The undigested protein was removed by centrifugation and peptides released were assayed. The specific protease activity towards casein was taken as a control.

Detergent stability

The compatibility of *B. subtilis* K-30 protease with local laundry detergents was studied in the presence of 10mM $CaCl_2$ and 1M glycine. Detergents used were Nirma (Nirma chemical, India); Henko (Henkel spic, India); Surf, Vim, wheel, Rin (Hindustan Lever Ltd, India) and Ariel (Procter and Gamble, India). The detergents were diluted in distilled water (0.7% w/v) and incubated with purified protease for 3 h at 60°C, and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100%.

RESULTS

Purification of alkaline protease

The results on the purification of the protease from *B. subtilis* K-30 are summarized in Table 1. The protein pellet obtained after 60% saturation with ammonium sulphate was dissolved in 0.1M Tris-HCl buffer and loaded onto a column of Sephadex G-100 (1.5X24 cm) equilibrated with Tris-HCl buffer, pH 7.8. Fractions (13-20) with high protease activities were pooled, and used for

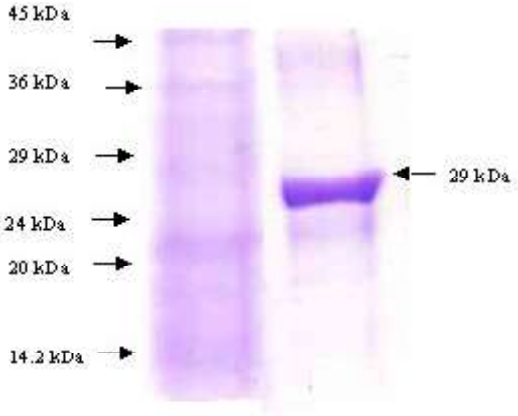

Fig. 1. SDS-PAGE of thermostable alkaline protease from *B. subtilis* K-30. Lane1 molecular mass markers.The molecular mass values of the markers are ovalbumin (45 kDa), glyceraldehydes -3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybeantrpsin inhibitor (20 kDa), and lactalbumin (14.2 kDa). Lane 2, purified protease

further studies. Overall 94.33 fold purification, 14.15 U mg^{-1}protein increase in specific activity was achieved with 10.63% recovery. The molecular weight of purified enzyme as determined by SDS-PAGE was found to be 29 kDa (Fig.1). The appearance of single band on SDS–PAGE suggested the enzyme is purified to homogenity. Molecular weight in the range of 28 -32 kDa has also been reported other *Bacillus* sp. Strain OK 3a.1.b-18 and BAH-101 (Coolbear *et al*., 1981; Fuziwara *et al*., 1993).

Effect of pH on protease activity

The pH optimum of the purified enzyme was found to be at pH 10.0 (Fig. 2). These findings are in accordance with several earlier reports showing pH optima of 10-10.5 for protease from *Bacillus* sp, *Thermus aquaticus*, *Vibrio metscnikovii* (Matsuzawa *et al*., 1988; Betzel *et al*., 1992; Kwon *et al*., 1994). The important

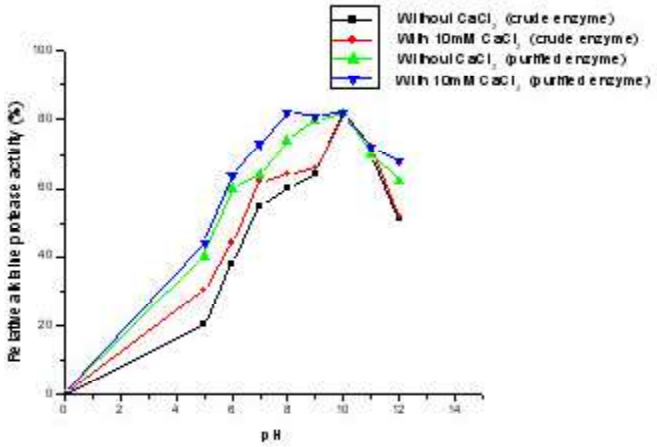

Fig. 2. Effect of pH on alkaline protease activity in presence and absence of 10mM $CaCl_2$ at 45°C

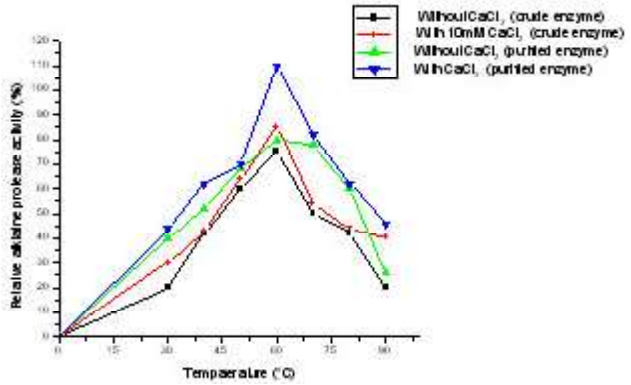

Fig. 3. Effect of temperature on alkaline protease activity in presence and absence of 10mM $CaCl_2$

detergent enzymes subtilisin NOVO or BPN (Horikoshi, 1990) showed maximum activity at pH 10.5. The enzyme was almost 100% stable over a broad pH range of pH 8-10 for 4 h incubation and a pH range of 8-10 for 20 h at 40°C.

Effect of inhibitors and chelators on protease activity

The effect of different inhibitors on the enzyme activity of the purified protease was studied (Table 2). The enzyme was completely inhibited by PMSF (at 5mM concentration) suggesting that protease is serine protease, while

Table 1. Summary of Purification steps of alkaline protease from *Bacillus subtilis K*-30

Purification steps	Volume (ml)	Total activity (U ml^{-1})	Total protein(mg)	Specific Activity (U mg^{-1} protein)	Recovery (%)	Fold purification
Culturefiltrate	500	235	1600.00	0.15	100	-
$(NH_4)_2SO_4$fraction	25	107	58.00	1.90	45.53	12.66
DEAE-cellulose	165	85	14.00	6.07	36.17	40.46
SephadexG-100	38	25	1.65	14.15	10.63	94.33

Table 2. Effect of inhibitors on protease activity

Inhibitor/Chelator % (5mM)	Relative enzyme activity
Control	100
PMSF	00
DFP	04
pCMB	85
β ME	83
Iodoacetate	92
EDTA	102

Table 3. Effect of metal ions on protease activity

Metal ions (5mM)	Residual alkaline protease Activity (%)
Control	100
Ca^{2+}	128
Mg^{2+}	115
Mn^{2+}	102
Zn^{2+}	96
Cu^{2+}	94
Hg^{2+}	90
Co^{2+}	92
Na^{2+}	95
Cd^{2+}	90
Al^{3+}	93
EDTA	99

Table 4. Alkaline protease activity against different natural substrates

Substrate	Relative Activity%
Casein	100
BSA	40
Egg albumin	23
Gelatin	40
Collagen	0
Keratin	0
Azocasein	35

Table 5. Compatibility 0f Alkaline Protease activity from *Bacillus subtilis* K-30 with commercial detergents in the presence of 10mM $CaCl_2$ and glycine

Detergent	% retention of enzyme activity after minutes			
Control	10	30	50	60
Wheel	100	100	100	100
Nirma	55	37	25	0
Surf	43	26	0	0
Ariel	20	0	0	0
Henko	60	40	25	0
Rin	65	55	25	0
Vim(ultra)	45	25	0	0
	84	62	35	25

DFP exhibited 96% inhibition. In this regard, PMSF is known to sulphonate the essential serine residue in the active site of the protease resulting in a total loss of enzyme activity (Gold and Fahrney, 1964). Partial or no inhibitory effect on the protease activity was observed with other protease inhibitors such as EDTA. However, metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}(Table 3) increased and stabilized the protease activity, confirming that these cations take part in the stabilization of the protease structure and play important role in protection against thermal denaturation (Paliwal *et al*., 1994).

Properties of protease of *B. subtilis* K-30

Effect of temperature on protease activity

The optimum temperature for purified enzyme was found to be 55°C-60°C. The enzyme activity of the crude and purified enzyme was determined at different temperatures ranging from 30°C-90°C in the absence and presence of 10mM $CaCl_2$ (Fig. 3). Optimum temperature of 55°C was recorded earlier for an alkaline protease from *Bacillus cereus* MCM B-326 (Nilegaonkar *et al*., 2007; Prakash et al., 2005), *Bacillus* sp. (Gupta *et al*., 2005), *B. licheniformis* RP1 (Sellami-Kamoun *et al*., 2006) *B. stearothermophilus* AP-4 (Fuziwara and Yamamato, 1987; Dhandapani and Vijayaraghavan, 1994).

Substrate specificity and detergent stability of alkaline protease

The ability to hydrolyze several protein substrates is an important criterion of protease potency (Grebeshova, 1999). Table 4 compares the digestive capability of this protease. The protease showed a high level of hydrolytic activity against casein and poor to moderate hydrolysis of BSA, gelatin and egg albumin, although the hydrolysis was not observed with substrates such as collagen and keratin.

Besides having stability at high pH & temperature, a good detergent protease is expected to be stable in presence of commercial detergents. The K-30 protease showed excellent stability and compatibility with locally available detergents (Table 5) at 60°C in presence of $CaCl_2$ and glycine as stabilizers. The enzyme retained more than 40 per cent activity after 30 min incubation at 60°C in the presence of detergents such as Henko and Vim ultra, indicating its suitability in detergent industry.

DISCUSSION

A highly thermostable alkaline protease from *Bacillus subtilis* K-30 was purified with Sephadex G-100 column chromatography. The molecular weight was found to be around 29kDa by SDS-PAGE. The optimal pH and optimal temperature of the protease were at pH 10 and 55°C, respectively. The protease activity was inhibited by PMSF, suggesting that it is a serine protease. The enzyme activity was markedly inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+} at 5mM whereas Ca^{2+}, Mg^{2+}, and Mn^{2+} had stimulatory effect. Considering the different characteristics of high pH and thermostability,compatibility with local and commercial detergents indicate the possibility of use in detergent industry and various other biotechnological applications

REFERENCES

1. Beg, K.B. Gupta, R. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis. Enzym Microbial Technol*. 2003; **32**: 294-304.
2. Beg, K. B., Sahai, V., Gupta, R. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Process Biochem*. 2003; **39**: 203-9.
3. Berla, T. E., Suseela Rajkumar, G. Purification and characterization of alkaline from *Alcaligenes faecalis*. *Biotechnol Appl Biochem*. 2002; **35**: 149-54.
4. Betzel, C., Klupsch, S., Pepndorf, G., Hastrup, S., Branner, S., Wilson, K.S. Crystal structure of the alkaline protease savinase registered from *Bacillus lentus* at 1-4 angstrom resolution. *J Mol Biol*. 1992; **223**: 427-45.
5. Coolbear, T., Eames, CW., Casey, Y., Daniei, R. M., Morgan, H. W. Screening of strains identified as extremely thermophilic *Bacilli* for extracellular proteolytic and general properties of proteinases from two of strains. *J Appl Bacteriol*. 1981; **71**: 252-64.
6. Dhandapani, R., Vijayaragavan, R. Production of thermophilic extracellular alkaline protease by *Bacillus stearothermophilus* AP-4. *World J Microbial Biotechnol*. 1994; **10**: 33-35.
7. Durham, D.R., Stewart, D. B., Stellwag, E. J. Novel alkaline and heat stable serine protease from alkaliphilic *Bacillus* sp. strain GX 6638. *J Bacteriol*. 1987; **169**: 2762-68.

8. Ellaiah, P., Adinarayana, K., Rajyalakshmi, P., Srinivasulu, B. Optimization of process parameters for alkaline protease production under solid state fermentation by alkalophilic *Bacillus* sp. *Asian J Microbial Biotechnol Environ Sc.* 2003*;* **5**: 49-54.
9. Ferrero, M. A., Castro, G.R., Abate, C.M., Baigori, M. D., Sineriz, F. Thermostable alkaline protease of *Bacillus licheniformis* MIR29: isolation, production and characterization. *Appl Microbial Biotechnol*. 1996; **45**: 327-32.
10. Fujiwara, N., Yamamoto, K. Production of alkaline protease in low-cost medium by alkaliphilic *Bacillus* species and properties of the enzyme *J Ferment technol*. 1987; **65**: 345-48.
11. Fuziwara, N., Masui, A., Imanka, T. Purification and properties of the highly thermostable alkaline protease from an alkalophilic and thermophilic *Bacillus* sp. *J Biotechnol*. 1993; **30**: 245-56.
12. Gold, A.M., Fahrney, D. Sulfonyl fluorides as inhibitors of esterases II. Formation and reactions of phenylmethanesulfonyl alpha-chymotrypsin. *Biochemistry*. 1964; **3**: 783-91.
13. Grebeshova, R. N., Saldeco-Torres, L.E., Hidalgo, M.A. Serine protease of *Bacillus subtilis* R. *Appl Biochem Microbiol*. 1999; **35**: 131-34.
14. Gupta, A., Roy, I., Patel, R. K., Singh S. P., Khare, S.K., Gupta, M. N. . One- step purification and characterization of an alkaline protease form haloalkaliphilic *Bacillus* sp. *J. Chromatog*. 2005; **1075**: 103-08.
15. Hellman, J.D. Compilation and analysis of Bacillus subtilis sA-dependent promoter sequences; evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res*. 1995; **23**: 2351-60.
16. Horikoshi, K. Alkaliphiles; some applications of their products for biotechnology. *Microbiol Mol Biol Rev*. 1999; **63**: 735-50.
17. Horikoshi, K. Enzymes of alkalophiles. In: Microbial Enzymes and Biotechnology. 2 nd Ed, St.Louis, MO: Elsevier *Applied Science*. 1990; 275-94.
18. Huang, G., Ying, T., Huo, P., Jiang, J. Purification and characterization of a protease from Thermophilic *bacillus* strain HSO8. *African J Biotechnol.,* 2006; **5**: 2433-38.
19. Ito, S., Kobayashi, T., Ara, K., Ozaki, K., Kawai, S., Hatada, Y. Alkaline detergent enzymes from alkaliphiles; enzymatic properties, genetics, and structures. *Extremophiles*. 1998; **2**: 185-90.
20. Kalisz, H. M. Microbial proteinases. *Adv Biochem Eng Biotechnol*. 1988; **36**: 1-65.
21. Karl-Heinz, M.. Detergent proteases. *Curr opinion Biotechnol*. 2004; **15**: 330-34.
22. Naidu, K. S. B., Kodidhela Lakshmidevi Devi. Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. *African J Biotechnol*. 2005; **4**: 724-26.
23. Kumar, C. G., Takagi, H. Microbial alkaline proteases: from a bio-industrial viewpoint. *Biotechnology Advances*. 1999; **17**: 561-94.
24. Kwon, Y. T., Kim, J.O., Moon, S.Y., Lee, H.H., Rho, H.M. Extracellular alkaline protease from alkaliphilic *Vibrio metschnikovi* strain RH 530. *Biotechnol Lett*. 1994; **16**: 413-18.
25. Laemmili, U.K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature.* . 1971; **227**: 680-85.
26. Lowry, O.H., Rosebrough, N.J., Farr, A. L., Randall, R. L. Protein measurement with the folin phenol reagent. *J Biol Chem*. 1951; 193: 265-73.
27. Manachini, P., Foretina, M., Parini, C. Thermostable alkaline protease produced by *Bacillus thermoruber*; a new species of *Bacillus*. *Appl Microbial Biotechnol*., 1988; **28**: 409-13.
28. Matsuzawa, H., Tokugawa, K., Hamaoki, M., Mizoguchi, M., Taguchi, H., Terada, I., Kwoa, S., Ohta, T. Purification and characterization of agualysin I (thermophilic alkaline serine protease) produced by *Thermos aquaticus* YT-1. *Eur J Biochem*. 1988; **171**: 441-47.
29. Nascimento, W. C., Martins, M. L. L. Production and properties of an Extracellular protease from thermophilic *Bacillus* sp. *Braz J. Microbiol*. 2004; **35**: 1-2.
30. Nilegaonkar, S. S., Zambare, V.P., Kanekar, P.P., Dhakephalkar, P.P., Sarnaik, S. S Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. *Bioresor Technol*. 2007; **98**: 1238-45.
31. Paliwal Singh, N., Singh, S.P., Garg, S. K. Cation-induced thermal stability of an alkaline protease form a *Bacillus* sp. *Bioresor Technol*. 1994; **50**: 209-11.
32. Prakash, M., Banik, R. M., Koch-Brandt, C. Purification and characterization of *Bacillus cereus* protease suitable for detergent industry. *Appl Biochem Biotechnol*. 2005; 127:143-45.
33. Seemuller, E., Lupas, A., Stock, D., Lowe, J., Huber, R., Baumeister, W. Proteosome from *Thermoplasma acidiphiilum*: a threonine

protease. *Science*. 1995; **268**: 579-82.

34. Sellami-Kamoun, A., Haddar, A., Ali N. E., Ghorbel-Frikha, B., Kanoun, S., Nasri, M. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations *Microbiol Res*., 2006; **25**: 145-49.
35. Siezen, R. J., Leunissen, J. A. M. Subtilases; the super family of subtilisin-like serine proteases. *Protein Sci*. 1997; **6**: 501-23.
36. Tsuchida, O., Yamagota, Y., Ishizuka, J., Arai, J., Yamada, J., Takeuchi, M., Ichishima, E. An alkaline protease of an alkaliphilic *Bacillus* sp *Curr Microbiol*. 1986; **1**: 7-12.
37. Vikash Dubey, K., Monupande Singh, B. K., Jagannadham, M. V. Papain-like proteases and applications of their inhibitors. *African J Biotechnol*. 2007; **6**: 1077-1086.