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Review Article

Quantitative Methods for Alkaline Protease Determination and its Applications: A Comprehensive Review

Raid D. Thanoon[1], Mahmood H. Mahmood[2], Essam A. Makky [3], Homady, M. H.[4]
[1]College of Science Department of Biology, Cihan University-Erbil, Kurdistan Region, Iraq.
[2]Faculty of Industrial Sciences & Technology, University Malaysia Pahang, 26300 Gambang, Kuantan, Pahang, Malaysia,
[3]Center of Excellence for Advanced Research in Fluid Flow (CARIFF), University Malaysia Pahang, 26300 Gambang, Kuantan, Pahang, Malaysia,
[4]College of Science, Department of Biomedical Sciences, Cihan University-Erbil, Kurdistan Region, Iraq.
***Corresponding Author:** Homady, M. H, College of Science, Department of Biomedical Sciences, Cihan University-Erbil, Kurdistan, Region, Iraq.

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Abstract

Proteases break peptide bonds. It is often necessary to measure and/or compare the activity of alkaline proteases using different procedures in the lab. Many studies with keen interest on alkaline proteases mostly use quantitative and/or qualitative assay methods to assay the enzyme activity of proteases. There is need to select a suitable assay method from the reported ones which will be ideal for any proposed study. There could be challenges when choosing the right assay method from the existing ones, thereby prompting the need for a review of the various methods for the quantitative assay of alkaline protease, the quantitative methods and protocols used from 1938 until now and their industrial applications were chronologically reviewed.

Keywords: alkaline protease; quantitative; protocols; applications

Introduction

Microorganisms are known to play a vital role in technology for the production of intracellular and extracellular enzymes on industrial scale [1, 2]. For maximum yield, selected organisms are grown in fermenters under optimum conditions and can be further used to make products such as cheese, bread, wine and beer [3, 4]. Most reactions within the living cells which are essential for life depends on enzymes, which act as catalysts [5, 6, 7].

1.1 Proteases (EC 3:4, 11-19, 20-24, 99) constitutes a large and complex group of enzymes [8]. This is the group of enzyme which carries out proteolysis (the breakdown of proteins through the hydrolysis of the peptide bond that exists between two amino acids of polypeptide chains) [9]. Proteases differ in properties such as substrate specificity, active site, and catalytic mechanism, pH, and temperature optima [10]. Proteases are ubiquitous and widely distributed, found in a wide diversity of sources such as plants, animals, and microorganisms. The inability of plants and animal proteases to satisfy current world demands led to increase in the demand for microbial proteases [11]. Alkaline proteases are important industrial enzymes which can be produced by many bacterial genera such as Aeromonas, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Halomonas*, *Pseudomonas*, and *Serratia* [12].

1.2 Classification of Proteases:

Barrett [10] classified proteases based on three criteria: (i) the type of reaction they catalyzed; (ii) the evolutionary relationship with reference to the structure, and (iii) the chemical nature of the catalytic site.

Based on their site of action, proteases are broadly classified as exopeptidases and endopeptidases. From the catalytic point of view, proteases are classified into four types, (i) the serine proteases; (ii) the aspartic proteases; (iii) the cysteine proteases, and (iv) the metalloproteases. Depending on the pH for optimal activity, proteases are classified into acidic, neutral, and alkaline proteases.

1.3 Alkaline proteases: (EC.3.4.21-24, 99) are proteases which are active at a neutral to alkaline pH. They either have a serine center (serine protease) or a metallo-type (metallo protease) [1]. Alkaline proteases are of considerable importance owing to their activity and stability at alkaline pH [13]. Commercial alkaline proteases are produced by *Bacillus* species due to their stability at high pH and temperature [14, 15, 1]. Alkaline proteases have commercial applications in the detergent, food, leather, chemical, brewing, pharmaceutical, photographic, and textile industries. The present study aimed to review the most applicable quantitative assays methods of alkaline protease enzymes.

2 Quantitative assay methods for alkaline protease enzymes

2.1 Anson method: The proteolytic activity was quantitatively assayed using a slightly modified method. The reaction mixture contained 1.0 mL of crude enzyme extract and 2.0 mL of casein [15 % (w/v) in 20 mM borate buffer, pH 9.0]. This mixture was incubated at 50 °C for 20 min and the reaction was stopped by the addition of 2.5 mL of 10 % (w/v) trichloroacetic acid. The mixture was vortexed to ensure a complete mixing of the content, incubated further for 15 min at room temperature

(RT), and then centrifuged at 10,000 revolutions per minute (rpm) for about 15 min. The supernatant was used to estimate the amount of free tyrosine released according to the protein estimation method of Lowry [16], using tyrosine as a standard. One unit of protease activity was defined as the amount of enzyme required to liberate 1.0 µg of tyrosine per min per mL under standard assay conditions [17].

2.2 Anson and Folin & Ciocalteu method: The alkaline protease activity was assayed using the slightly modified method of [18,19]. The reaction mixture consists of Casein 0.65 % w/v in a 50 mM potassium phosphate buffer, pH 7.5, and culture filtrate. The reaction mixture was incubated for 10 min at 37 °C and later terminated by adding 5 mL of 110 mM TCA to the test and blank preparations. The solution was filtered through Whatman No.1 filter paper. For the assay of tyrosine in the filtrate, 5 mL of 500 mM sodium carbonate solution and 1 mL of Folin & Ciocalteu's phenol reagent (1N) were added to 2 mL of the culture filtrate and incubated for 30 min at RT before reading the absorbance of the reaction mixture at 660 nm. The concentration of tyrosine in the filtrate was determined from a standard curve already prepared. One unit of alkaline protease activity was defined as the amount of enzyme that liberated one micromole of tyrosine per mL per minute under standard experimental conditions.

2.3 Kunitz et al method: The reaction mixture contained the enzyme solution and 0.5 % (w/v) casein in 0.1 M potassium phosphate buffer (pH 8.0). The mixture was incubated at 60 °C for 10 min after which the reaction was terminated by the addition of 5 % (w/v) trichloroacetic acid (TCA) solution. The resulting precipitate after the addition of the TCA was removed by centrifugation and the protease activity in the filtrate estimated by using a tyrosine standard curve. One unit of alkaline protease activity (U) was taken as the amount of enzyme liberating 1µg of tyrosine/min under the assay conditions [20].

2.4 Kunitz method: The reaction mixture contained suitably diluted enzyme and casein in 0.1M sodium carbonate buffer pH 10. The reaction mixture was incubated at 40 °C for 10 min. after incubation; the reaction was terminated by adding 3 mL of 10 % TCA to the mixture. The reaction mixture after termination was incubated at RT for 30 min. The precipitate formed after the addition of TCA was filtered through Whatman No. 1 filter paper. The absorbance of the filtrate was read at 280 nm. Tyrosine was used as standard and one unit of protease activity was defined as the amount of enzyme which liberates one micromoles of tyrosine per minute per gram dry substrate under the standard experimental conditions. Protein estimation was done using the Lowry method of protein estimation [21, 22].

2.5 McDonald and Chen method: One mL of the enzyme was added into 2 mL of casein (1 % w/v in 0.1N Glycine – NaOH buffer, pH 10) and the mixture incubated for 15 min at 60 °C. The reaction was terminated by adding 3 mL of 10 % TCA reagent and then, centrifuged for 15 min at 10,000 rpm. Then, 1 mL of the filtrate was mixed with 5 mL of alkaline copper reagent. After 15 min, 0.5 mL of Folin-ciocalteau reagent was added and kept for 30 min. The absorbance of the mixture was read at 700 nm. Similarly, the blank was prepared by distilled water instead of enzyme solution. One unit of enzyme activity was defined as the amount of enzyme that releases 1µg of tyrosine per mL per min under the standard assay conditions. Tyrosine within the concentration range of 50–250 µg was used as standard [23].

2.6 Horikoshi method: The quantitative analysis of alkaline protease activity in the supernatant was determined using the modified method of Horikoshi [24]. In this method, 200 µL of the enzyme solution (diluted 5 times) was added into 2.5 mL of 0.6 % casein solution prepared in 0.05 M Glycine–NaOH buffer, pH 11.0. The mixture was incubated at 30 °C for 20 min and later stopped by adding 2.5 mL of TCA (0.11M T.C.A, 0.22M CH_3COONa, 0.33M CH_3COOH) to the reaction mixture. The mixture was again incubated at 30 °C for 20 min, and then, centrifuged at 10,000 rpm for 5 min. The absorbance of the clear supernatant was measured at 275 nm. The blank was prepared in a similar way but the buffer solution was used instead of the enzyme. One unit of protease activity was defined as the quantity of enzyme which liberates amino acids and non-precipitated peptides equivalent to 1 µg/mL of tyrosine per minute under the standard assay conditions.

2.7 Higerd method: One mL of the sample (enzyme) was added into 5 mL of 0.6 % casein and incubated at 30 °C for 10 min. The reaction was stopped by adding 5 mL of TCA mixture made up of 36 mL of 50 % (w/v) TCA solution, 220 mL of 1M sodium acetate solution, and 330 mL of 1 M acetic acid solution in a total volume of 1000 mL solution. The unreacted casein was precipitated and the resulting precipitates were filtered off using Whatman´s No. 1 filter paper. The optical density (OD) of the sample was measured at 610 nm against appropriate substrate and enzyme blanks. A standard curve was prepared with pure L-tyrosine at a concentration of 0-1000 mg/mL [25]. One unit of proteolytic activity was defined as the amount of enzyme which will liberate 1µg of tyrosine in one minute under the defined assay conditions.

2.8 Leighton et al method: The protease activity was assayed in small volumes using the micro plates and the ELISA reader [26]. Azocasein was used as the substrate.. In duplicates using micro centrifuge tubes, 50 µL of 1 % (w/v) azocasein (Sigma), prepared in 0.2 M Tris-HCl, pH 7.2, was incubated with 30 µL of the crude enzyme extract for 60 min at 25 °C. About 240 µL of 10 % (w/v) TCA was then added to stop the reaction. After 15 min, the tubes were centrifuged for 5 min at 8,000 g. About 70 µL of the supernatants were then added to 130 µL of 1 M NaOH on an ELISA plate, and the absorbance of the mixture measured in an ELISA reader (Bio-rad 550) at 450 nm against a blank prepared in the same way, but replacing the crude enzyme extract with 0.9 % (w/v) NaCl. One unit (U) of enzyme activity was defined as the amount of enzyme able to hydrolyze azocasein, giving an increase of 0.001 units of absorbance per minute.

2.9 Meyers and Ahearn method: The alkaline protease activity was assayed with some modifications of the reaction mixtures. The reaction mixture contained 0.5 mL of the enzyme, 0.5 mL of 5 %TCA solution 0.5 mL of 0.2 M Glycine NaOH buffer, pH 10.0, and 1 mL of Casein solution (1 % in 0.2 M glycine-NaOH buffer, pH 10.0). The reaction mixture was incubated for 20 min at 45 °C, and the reaction terminated by adding 4 mL of 5 % TCA to the test and control preparations. The tubes were incubated for 1 h at RT, and filtered through Whatrnan No. 1 filter paper. For the color development during the tyrosine assay in the filtrate, 5 mL of 0.4 M sodium carbonate and 0.5 mL of Folin phenol reagent were added to 1 mL of the filtrate and immediately vortexed before incubation for 20 min at RT. The optical density (OD) was read at 660 nm. The concentration of tyrosine in the filtrate was read from a standard tyrosine curve. One unit of alkaline protease activity was defined as the amount of enzyme that liberated one 1µM of tyrosine per mL per minute under the experimental conditions [27].

2.10 Chopra and Mathur method: **The** alkaline protease activity was determined using a reaction mixture made up of 5 mL of casein (prepared in 50 mM of Tris buffer, pH 8.0) and an aliquot of 1.0 mL of the enzyme solution. The mixture was incubated for 30 min and the reaction later stopped by adding 5 mL of 0.11M TCA solution. After 30 min, the mixture was filtered and 2 mL of the filtrate was added to 5.0 mL of 0.5 M sodium carbonate, followed by1.0 mL of Folin-Ciocalteu's phenol reagent. The mixture was kept for 30 min at 37 °C. The OD of the solutions was read against the sample blank at 660 nm using the UV-Vs spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per mL per min under the standard assay conditions [28].

2.11 Ledoux and Lamy method: The proteolytic activity of the enzyme was assayed in triplicates using casein (Hi Media, India) as the substrate. Initially, a mixture of 400 µL of casein solutions (2% (w/v) in 50 mM Tris-HCl buffer, pH 7.2) and 100 µL of the sample were added to a test tube. The reactions were carried out at 65 °C in a water bath (Memert, Germany) for 10 min and then terminated by adding 1 mL 10 % (w/v) TCA solution. The mixture was centrifuged at 14000 rpm for 20 min and the supernatant (500 µL), carefully aspirated to measure the tyrosine content using the Folin-phenol method [29]. One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzed casein to produce 1.0 µM of tyrosine per minute at 65 °C.

2.12 Tsuchida et al method: A reaction mixture of 500 µL of 1 % (w/v) of casein in 50 mM phosphate buffer, pH 7 and 200 µL of crude enzyme extract was incubated in a water bath at 40 °C for 20 min. After 20 min, the enzyme reaction was terminated by adding 1 mL of 10 % (w/v) TCA solution and kept at RT for 15 min. The reaction mixture was then centrifuged at 10,000 rpm for 5 min to separate the unreacted casein. The supernatant was mixed with 2.5 mL of 0.4 M Na_2CO_3. About 1 mL of a 3-fold diluted Follin Ciocalteus phenol reagent was added and the resulting solution incubated at RT in the dark for 30 min. The absorbance of the developed blue color was measured at 660 nm against a reagent blank using a tyrosine standard [21]. One unit of protease was defined as the amount of enzyme that will release 1 µg of tyrosine per mL per min under the standard conditions [30].

2.13 Kole et al method: The protease activity was assayed in duplicates with cell-free culture supernatants, using azocasein as the substrate. The enzymatic hydrolysis of azocasein produces stable dye-labelled peptides and amino acids which can be easily measured. The azocasein protease activity was measured by incubating 1 mL of the culture supernatant and 1 mL of 0.5 % (w/v) azocasein (Sigma) in 0.2 M Tris-hydroxymethyl amino methane hydrochloride (Tris-HCl) buffer, pH 7.4 in an incubator (Innova, New Brunswick Scientific) at 75 °C for 1 h. The reaction was stopped by adding 2 mL of 10 % (w/v) TCA solution [31]. The test tubes were allowed to stand for 30 min at RT. The mixture was thoroughly mixed using a vortex mixer (VF2, Jankel and Keunkel Kika Larbotechnik) before being centrifuged at 3 000 rpm for 10 min to remove the yellow precipitates. The absorbance of the supernatant was measured at 440 nm using a Shimadzu UV-120-2 spectrophotometer. The activity of the protease was expressed in arbitrary units, where 1 unit of activity was equivalent to a change in the optical density of 0.01 nm per min at 440 nm. The enzyme assays were done in duplicate for each sample.

2.14 Takami et al method: About 1.25 mL of Tris buffer (100 Mm, pH 9.0) and 0.5 mL of 1 % aqueous casein was added to 0.25 mL of the collected culture supernatant. The mixture was incubated at 30 °C for 30 min. Next, 3 mL of 5 % TCA solution was added to the mixture which led to the formation of precipitates. The mixture was incubated for 10 min at 4 °C and later centrifuged at 5,000 rpm for 15 min. About 0.5 mL of the supernatant was aspirated and added to 2.5 ml of 0.5 M of sodium carbonate. The mixture was well mixed and incubated for 20 min. Thereafter, 0.5 mL of Folin reagent was added to the mixture and analyzed under Uv-Vis at a wavelength of 660 nm. One unit of alkaline protease activity was defined as the amount of the enzyme capable of producing 1 µg of tyrosine in 1 min under standard assay conditions. All enzyme activity assays were carried out in triplicate [32].

2.15 McKevitt and Klinger method: The protease assay was determined using the Caseinolytic method [33]. The protease activity was assayed using 2 mL of 2 % casein in 0.65 mL of alkaline phosphate buffer. The casein solution was incubated with 0.5 mL of properly diluted enzyme at 37 °C for 30 min. After 30 min, 200 µL of NaCl was added and the reaction later terminated by adding 5 mL of 5 % TCA solution. The solution was centrifuged at 10,000 rpm for 10 min after which 0.1 mL of the sample was taken, and the volume made up to 1 mL with distilled water. About 5 mL of solution-C (solution A in 50 mL of distilled water, 0.2 g of NaOH was dissolved in it, and 1 g of Na_2CO_3 added; solution B contained 0.5 % of $CuSO_4$ in 1 % sodium potassium tartarate, solution C contained 50 mL of solution A + 1 mL of solution B) was added and incubated at RT for 10 min. These were preceded by the addition of 0.5 mL of Folin Ciocalteu reagent and incubated in the dark for 30 min. The developed color was read at 660 nm against a reagent blank prepared in the same manner. One unit of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per mL per minute from casein under the specified assay conditions.

2.16 Lin et al method: The reaction mixtures (2 mL volume) contained 10 mg of casein, 200 µmol of sodium carbonate buffers, pH 9.7, and 0.1 mL of the enzyme solution. The reaction was carried out at 37 °C for 30 min, and then terminated by adding 3 mL of 5 % (w/v) TCA and 3.3 M HCl. The reaction was kept on ice for 1 h after which they were centrifuged for 30 min at 4,000 rpm. The absorbance of the TCA soluble mixtures were measured at 280 nm. The activity of the alkaline protease was also determined according to the method described by [34]. One gram of skim milk was dissolved in 100 mL of 20 mM Tris–HCl buffer, pH7.5 before adding 1.5 g of agar. After heating the solution, the hot mixture was poured into Petri dishes and allowed to solidify. After solidification, holes were punched on the media using a hole borer to accommodate about 25 µL of the enzyme solution. The plates were incubated overnight at 37 °C after which the clear zone (zones of inhibition) around each hole was measured and recorded.

2.17 Kembhavi et al method: The alkaline protease activity was measured using a previously reported method with some modifications [35]. About 0.5 mL of 50 mM glycine–NaOH pH 10.0, containing 1 % (w/v) of casein and 10 mM of $CaCl_2$ was pre-incubated for 5 min at 50 °C. Then, 0.5 mL aliquot of the suitably diluted culture supernatants were mixed with the substrate solution and incubated for 20 min at 50 °C. The reaction was later terminated by adding 0.5 mL of 20 % (w/v) TCA solution. The mixture was allowed to stand for 15 min at RT and then centrifuged at 6000 rpm for 15 min to remove the precipitate. The acid-soluble materials were estimated using the Lowry method [16]. The control was prepared by adding TCA before the enzyme. A standard curve was prepared using tyrosine solutions at the concentration range of 0–100 µg/mL. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under the experimental conditions. All the enzyme assays were carried out in triplicates.

Conclusions

The use of extracellular enzymes has been a standard in many industries for many years. Alkaline proteases are one of the most important groups of enzymes used in various industries such as the detergent, pharmaceutical, leather, meat tenderizers, protein hydrolyzates, and food products, as well as in waste processing. Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. *Bacillus* spp are the most widely exploited alkaline proteases-producing microorganism often used commercially in bioremediation, or as probiotic agent in aquaculture. The different evaluation methods for alkaline protease enzyme were reviewed through a collection of various quick and easy quantitative methods in chronological order since 1938 to present.

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Ethical Approvals:

'The present research work does not contain any studies performed on animals/humans subjects by any of the authors'.

References

1. Gupta R., Beg Q.K., Lorenz P. (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. App. Microbiol. Biotechnol. 59; 15-32. ISSN: 2348-8069.
2. Moo-young M., Chisti Y. (1994) Biochemical engineering in biotechnology, Pure Appl. Chem. 66; 117–136.
3. Lin Y., Tanaka S. (2006) Ethanol fermentation from biomass resources: current state and prospects, Appl. Microbiol. Biotechnol. 69; 627–642.
4. Schenk P.M., Thomas-Hall, Stephens E., Marx U.C., Mussgnug J.H., Posten C., Kruse O., Hankamer B. (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production, Bioenerg. Res. 1; 20–43.
5. Sierecka J.K. (1998) Purification and partial characterization of a neutralprotease from a virulent strain of *Bacillus cereus*, Int. J. Biochem.Cell Biol. 30; 579–595.
6. Johnson L. (2005) Symmetry at the molecular level in biology, Eur. Rev. 13; 77–95.
7. Pingoud A., Fuxreiter M., Pingoud V., Wende W. (2005) Type II restric-tion endonucleases: structure and mechanism, Cell. Mol. Life Sci, 62; 685–707.
8. Jisha V.N,, Smitha R.B., Pradeep S., Sreedevi S., Unni K.N., Sajith S., Priji P., Josh M.S., Benjamin S. (2013) "Versatility of microbial proteases". Adv. Enz. Res. 1; 39-51.
9. Singhal P., Nigam V.K., Vidyarthi A.S. (2012) "Studies on production, characterization and applications of microbial alkaline protease", International Journal of Advanced Biotechnology and Research, 3 (3); 653-669.
10. Sumantha A., Sandhya C., Szakacs G., Soccol C.R., Pandey A. (2005) "Production and partial purification of a neutral metalloprotease by fungal mixed substrate fermentation", Food Technology and Biotechnology, 43; 313-319.
11. Rani K., Rana R., Datt S. (2012) "Review on latest overview of Protease", International Journal of Current Life Sciences Vol.2 (1) 12– 18.
12. Shafee N., Aris S.N., Rahman R.Z.A., Basri M., Salleh A.B. (2005) "Optimization of Environmental and Nutritional Conditions for the Production of Alkaline Protease by a Newly Isolated Bacterium *Bacillus cereus* Strain 146", Journal of Applied Sciences Research, 1; 1-8.
13. Singh J., Batra N., Sobti R.C. (2001) "Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1" Process Biochemistry, 36; 781.
14. Priest F.G. (1977) "Extracellular enzyme synthesis in the genus *Bacillus*". Bacteriol Rev, 41; 711- 753.
15. Joo H., G. Kumar, G. Park, K.T. Kim, S.R. Paik and C. Chang. (2002) "Optimization of the production of an extracellular alkaline protease from Bacillus horikoshii" Process Biochemistry, 38; 155.
16. Lowry O.H., Rosenbrough N.J., Farr A.L., Randall A. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem., 193; 265-275.
17. Anson M.L. (1938) The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J Gen physiol, 22; 79-89.
18. Bradford M.M. (1976) Analytical Biochem, 72; 248–254.
19. Folin O., Ciocatteu V. (1929) On tyrosine and tryptophan determination in proteins. J Biol chem. 73; 627.
20. Kunitz M., Crystalline soybean trypsin inhibitor. J Gen Physiol. 30; 291- 310.
21. Lowry O.H., Rosenbrough N.J., Farr A.L., Randall A. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem., 193; 265-275.
22. Kunitz M. (1947) Crystalline soybean trypsin inhibitor II General properties, J. Gen. Physiol. 30; 291–310.
23. McDonald C.E., Chen L.L. (1965) Lowry modification of the Folin reagent for determination of proteinase activity, Ann Biochem., 10; 175-186.
24. Horikoshi K. (1971) Alkaline protease production by *Bacillus* No. 221. Agric. Biol. Chem., 35(9) 1407-1414.
25. Higerd T.B. (1972) Hyperprotease-producing mutants of *Bacillus subtilis*. Journal Bacteriology, 112; 1026-1028.
26. Leighton T.J., Doi RH. Warren RAJ., Kelln RA. (1973) The Relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus* subtilis. Journal of Molecular Biology, 76; 103-122.
27. Meyers S.P., Ahearn D.G. (1977) Extracellular proteolysis by *Candida lipolytica*. Mycologia. 9; 646–651.
28. Chopra A.K., Mathur D.K., Purification and characterization of heat stable protease from *Bacillus stereothermophilus* RM- 67. J. Dairy Sci., 68 (1985) 3202-3211.
29. Ledoux M., F. Lamy. (1986) Determination of proteins and sulfobetaine with the folin-phenol reagent. Analytical Biochemistry, 157(1); 28-31.
30. Tsuchida O., Yamagota Y., Ishizuka J., Arai J., Yamada J., Takeuchi M., Ichishima E. (1986) An alkaline protease of an alkalophilic *Bacillus* sp. Curr. Microbiol, 14; 7-12.
31. Kole M.M., Draper I., Gerson D.F. (1988) Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. J. Chem. Tech. Biotechnol., 41; 197-206.
32. Horikaoshi T., Takami H., Akiba K. (1989) Production of extremely thermostable alkaline protease from *Bacillus* sp. No.AH-101. Appl. Microbial. Biotechnol. 30; 120-124.
33. McKevitt A., Bajaksouzian S., Klinger J.D., Woods D.E., Purification and characterization of an extracellular protease from *Pseudomonas cepacia.* Appl. Environ. Microbiol. 57; 771-778.
34. Hmidet N., Ali N.A., Haddar A., Kanoun S., Alya S.K., Nasri M., Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as detergent additive. Biochem Eng. J. 47 (2009) 71–79.
35. Kembhavi A.A., Kulkarni A., Pant A. (1993) Salt-tolerant and thermostable alkaline protease from *Bacills subtilis* NCIM 64, App Biochem Biotechnol.