Pak. J. Bot., 47(2): 771-777, 2015.

STABILITY AND ACTIVITY PROFILE OF ALKALINE PROTEASE, PRODUCED FROM *BACILLUS SUBTILIS*

SOBIA ANJUM[1], HAMID MUKHTAR[1*], ALI NAWAZ[1], TEHREEMA IFTIKHAR[2,3] AND IKRAM-UL-HAQ[1]

[1]Institute of Industrial Biotechnology, GC University Lahore-54000, Pakistan; [2]Department of Botany, GC University, Faisalabad, Pakistan; [3]Department of Botany, Lahore College for Women University, Lahore, Pakistan
[*]Corresponding author: hamidwaseer@yahoo.com

Abstract

The present study gives an insight into the effect of different activators and inhibitors on the activity and stability of alkaline proteases produced by *Bacillus subtilis* IH-72. The alkaline protease was strongly activated both by bivalent and monovalent cations such as Mg^{2+}, Mn^{2+}, Na^{+} and K^{+}. The enzyme activity was considerably enhanced in the presence of fructose, galactose, glucose and mannitol. The enzyme was stabilized up to 10 days by immobilization on activated charcoal and was efficiently stabilized up to 2 months by lyophilization. The enzyme remained stable up to 19 days both at 4°C and 30°C in the presence of Mn^{2+}. However, it exhibited significant stability up to 22 days at 4°C and 30°C in the presence of fructose, galactose and polyethylene glycol.

Key words: *Bacillus subtilis* IH-72, Enzyme activity, Alkaline proteases.

Introduction

Alkaline proteases (E.C. 3.4.21) are types of proteases which show broad substrate specificity and high enzyme activity under alkaline pH (Barrett, 1994). They either have a serine centre or are of metallo-type. They are the most important and robust type of enzymes and account for 40% of the worldwide enzyme sale (Ellaiah *et al.,* 2002, Prakasham *et al.,* 2006). Major producers of these enzymes belong to *Bacillus* species with few prominent ones as *B. licheniformis, B. subtilis, B. horikoshii, B. alcalophilus* and *B. sphaericus (*Ahmad *et al.,* 2011, Ellaiah *et al*., 2002).

Proteases being enzymes are innately unstable and the instability is usually a limiting factor in most processes because their intrinsic instability leads to difficulty in production, processing and storage and subsequently leads to high costs of the process (Iyer & Ananthanarayan, 2008). This limited stability may be due to deleterious effects of aqueous environment and microbial contamination (Rainer, 2000; Rajini & Mattiasson, 1993). They may also be denatured by changes in temperature, pH, pressure and ionic strength (Michiaki *et al.,* 1997). Systemic efforts are being carried out to search for new proteases with better stability, cloning protease genes into more suitable hosts or using site directed mutagenesis (Fagain, 2003). Mostly, the methods of enzyme stabilization focus on effects of additives on enzyme stability rendering it the most popular method for enzyme stabilization. Various classes of additives being employed for enzyme stabilization are ligands, substrates, salts, polyols, sugars, DMSO, glycerol, polyethylene glycols, synthetic polymers and miscellaneous additives (Chaniotakis, 2004; Gouda *et al.,* 2003; Iyer & Ananthanarayan, 2008). Other methods in use are enzyme immobilization and crystallization (Grazu *et al.,* 2005).

Ligands and substrates are important in stabilization of enzymes as they are themselves a part of enzymatic reactions. The binding of substrate may cause stabilization, labialization or have no effect on enzyme stability (Schmidt, 1979). Addition of salts for enzyme stabilization is divided into two groups, specific ion effects (at concentration below 0.1M) and non-specific ion effects (at concentration $\geq$0.1M) (Iyer and Ananthanarayan, 2008). Stabilization due to divalent cations is usually highly specific at low concentrations and at higher concentration it correlates with Hofmeister lyotropic series (Haard, 1998).

When sugars and polyols are added to enzyme solutions, they mediate strengthening of hydrophobic interactions among the non-polar amino acid residues which leads to increase in protein rigidity and its resistance to deactivation under thermal stress. The stabilization effect has also been related to their positive effect on the water activity of the medium and decrease in chance of microbial contamination (Schmidt, 1979). Also, it is a general belief that the osmolytes stabilize proteins by shifting the native state ←→ denatured state equilibrium in the favor of native state (Iyer & Ananthanarayan, 2008). Almost all polymers also have an activating effect on enzymes. This effect is explained by the fact that every enzyme exists in solution as a dynamic mixture of several structural forms which exist in equilibrium and that each form has a different enzyme activity. By the addition of additive, the equilibrium shifts towards a single structural form with a single amount of enzyme activity and the additive stabilizes the tertiary structural form (Yoon & Robyt, 2005), thus enzyme activity is enhanced.

Protein denaturation in aqueous medium is due to chemical reactions involving side chains of amino acid residues (Liu *et al.,* 1991; Constantino *et al.,* 1995), loss of cofactors, proteolysis or by microbial contamination (Fagain, 2003). Thus high value labile biomolecules can be preserved and their stability can be increased by lyophilization (Matejtschuk, 2007). In this process, water is removed under vaccum from frozen material, resulting in water level lower than 1% (Thuma *et al.,* 1987). Proteases can also be immobilized on a large variety of supports both synthetic as well as natural solid supports (Sierecka, 1998). The immobilization can minimize their autolysis and thus improve their stability (Chen *et al.,* 1993 and Church *et al.,* 1984).

Activating effect of cations was first discovered about 60 years ago (Boyer, 1942), later a large number of cation activators were identified (Evans & Sorger, 1966) and the list is continuously growing (Rockwell & Fuller, 2002). Cations activate enzymes by either forming a complex with the enzyme or activate it allosterically. In ternary complex formation, cation is absolutely required for the enzyme activity whereas in the allosteric interaction cation binds to a site other than active site and increases the activity considerably beyond basal value (Suelter, 1970).

In this course of work, the main objective pursued was the enhancement of activity and storage stability of alkaline proteases produced by *Bacillus subtilis.* For that purpose, various additives and metal ions were used and processes such as lyophilization and immobilization of enzyme on activated charcoal were carried out.

Materials and Methods

Microorganism: A culture of *Bacillus subtilis*-IH-72 was used for the production of alkaline proteases. The culture belonged to the culture bank of Institute of Industrial Biotechnology, GC University, Lahore and was initially isolated from the soil of tanneries area. The cultures were transferred from a glycerol stock to fresh nutrient broth slants carefully under aseptic conditions for growth and maintenance.

Inoculum preparation: Inoculum was prepared in an Erlenmeyer flask (250ml) having 25ml of medium composed of (%w/v) Yeast extract 0.3; Peptone, 0.5 and NaCl, 0.5. The flask was cotton plugged, sterilized and then cooled to room temperature. A loopful of bacterial culture was then transferred from a slant to the medium aseptically. The flask was incubated at 37°C and 200rpm for 24 hrs.

Shake flask culture: The culture medium comprised of (%w/v) Glucose, 1; Peptone, 1; Soybean meal, 1.5; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.1; $(NH_4)_2SO_4$, 0.1 and Na_2CO_3, 0.5 (pH 8.5) was used for the production of alkaline protease by *Bacillus subtilis* (Mukhtar & Haq, 2007). 1 mL of inoculum was then transferred to each flask containing sterilized culture medium and the flasks were incubated in a shaking incubator at 37°C and 200rpm for 48 hrs. After 48 hrs, the flasks were removed from incubator and the fermentation broth was centrifuged at 6000rpm for 15 min. The supernatant was used for the assay of alkaline protease.

Protease assay: The protease activity was estimated by McDonald & Chen, (1965) method. 1 mL of enzyme sample was taken in a test tube and 4 mL of 1% casein solution (pre incubated at 37°C) was added to it. The mixture was then incubated at 37°C for 30 min. After incubation, 5 mL of trichloroacetic acid (TCA) solution was added to stop the reaction and mixed well. The mixture was then allowed to stay for 10 min. The reaction mixture was centrifuged at 6000rpm for 10 min. 1mL of the clear supernatant was taken in a test tube and 5ml of the alkaline reagent was added to it and mixed well. Then 1mL of 1N NaOH and 1mL of Folin-Ciocalteau reagent were added. This mixture was then placed at room temperature for 20-30 min and absorbance was read at 700nm on a spectrophotometer.

One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µg of tyrosine under the assay conditions.

Activators and stabilizers: Solutions of various additives viz; Salts (NaCl, $CaCl_2$, $ZnCl_2$, $MgSO_4$, $HgNO_3$, $CuSO_4$, $NiCl_2$, $SrCl_2$, $PbNO_3$, $MnSO_4$, $CdCl_2$, KCl, NH_4SO_4 and $FeSO_4$), sugars (sucrose, fructose, galactose, and glucose), substrates (casein and bovine serum albumin), Polyethylene glycol, amines (Diphenyl amine, acetamide, alpha naphthyl amine, cyclohexylamine), alcohols and polyols (Mannitol, sorbitol, glycerol, diethyl glycol) were used to study their effect on enzyme activity and stability.

Lyophilization of alkaline protease: Partially purified enzyme was subjected to lyophilization using Sucrose-Phosphate buffer (5% sucrose in 10mM Phosphate buffer). The pellet obtained after ammonium sulphate precipitation (80%) was suspended in the buffer. The sample was then transferred to lyophilization flask (100mL) which could withstand sharp glass transition temperatures and vaccum pressures. Maximum filling depth was kept up to 20mm. The round bottom flask was carefully placed at -45°C in ultra cool freezer (Model: MDF-594, SANYO, Japan) for an hour to allow quick freezing of the material.

The lyophilization unit ((Model: D-37250 Alpha 1-4 LD, CHRIST, Germany) was then switched on to allow the vaccum to develop in the unit and the temperature to lower up to -40 to -45°C. The flask was then attached carefully to the unit; vaccum valve was opened to allow vaccum to develop in the flask. It was made sure that foaming did not occur as it could lower the quality of the product. The operation was carried on for 5 to 6 hrs with monitoring at regular intervals.

Finally, the flask was removed from lyophilization unit and the powdered product was transferred to a vial, the vial was then tightly sealed. To assay the activity of the lyophilized product, it was re-suspended in cold phosphate buffer (10mM, pH8) and protease assay was performed. The product was stored at 4°C and the residual activity was checked after every 10 days. The efficiency of Lyophilization was calculated by the following formula:

$$\text{Efficiency (\%)} = \frac{\text{Activity of the sample after lyophilization}}{\text{Activity of the sample before lyophilization}} \times 100$$

Results and Discussion

Stability profile of lyophilized enzyme: The stability profile of alkaline protease in lyophilized form was investigated. Initial enzyme activity was 200U/ml and the activity of lyophilized product was 165U/ml. Thus the enzyme retained 82% of its activity after lyophilization. The lyophilized enzyme was then stored in a vial at 4°C. Enzyme activity was taken after every 10 days and was found that the lyophilized enzyme retained 100% of its initial activity (165 U/ml) even after 60 days (Fig. 1) which suggests that the enzyme remained stable after lyophilization.

Lyophilization is an effective method of storage of enzyme as it dries up the product and protects the enzyme against denaturation due to chemical reactions that occur in aqueous environment. But during the process of freezing and drying, changes occur in product that can significantly denature the enzyme (Hanson & Raoun, 1992). Suitable excepients such as sucrose can lessen or overcome these effects by strengthening the water shell around proteins and improving the quality of the product by acting as bulking agent to ensure the development of dried material with good appearance (Pikal *et. al.*, 1990). Stabilization of enzymes by lyophilization has been patented by Rosier *et al*. (2001) stating that enzyme can be efficiently stabilized by lyophilization in the presence of carbohydrates (especially disaccharides) and the product can be stored up to 3 months and enzyme shall retain its activity upon resuspension in buffer. The findings of the present study that alkaline protease can be stabilized by lyophilization in the presence of sucrose are in accordance with the above mentioned report.

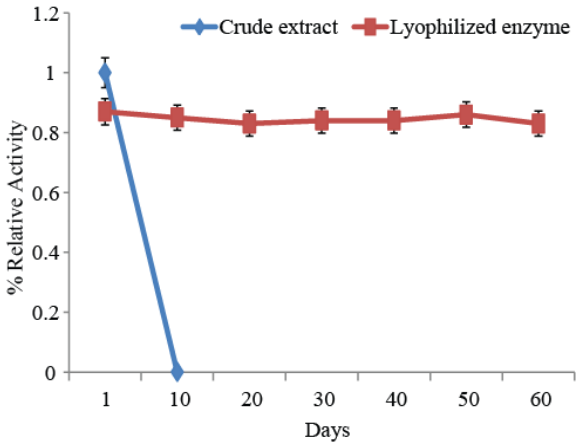
Stability profile of immobilized enzyme: Alkaline protease was immobilized on activated charcoal to enhance the stability of the enzyme by excluding it from aqueous phase and adsorbing it to solid matrix. Fig 2 shows the stability profile of the free as well as immobilized enzyme. Amount of bound enzyme was 334 Units of enzyme per gram of matrix. In comparison with free enzyme, the immobilized enzyme retained 84% of its activity. Storage stability was assessed by measuring enzyme activities at one day intervals. The immobilized enzyme maintained its stability up to 74% even after 10 days, thus showing only a loss of 10% activity; whereas the free enzyme lost its activity completely in 3-4 days. This showed immobilization on charcoal to be an easy as well as effective technique for enzyme stabilization.

The initial loss of enzyme activity upon immobilization may be due to diffusion effects caused by porous nature and fissured structure of activated carbon as was not the case with free enzyme. After immobilization, charcoal improves the stability of enzyme by adsorbing the enzyme to large volume pores (Benaddi *et al.,* 2000). The results of the present study can be correlated to those reported by Kumar *et al*. (2009) claiming that acid protease immobilized on activated carbon retained 80% of its activity even after 10 days.

Effect of different additives as activators: Several additives were investigated for enhancement of alkaline protease activity which include sugars (fructose, galactose, glucose and sucrose), polymer (PEG), polyols (glycerol, mannitol and sorbitol), substrates (casein and BSA), amine (cyclohexyl amine) and amide (acetamide). Enhancement of enzyme activity was measured as % relative activity of enzyme in the presence of additive as compared to that of enzyme activity in the absence of additive. Maximum enhancement of enzyme activity was shown by monosaccharide sugars that were fructose, glucose and galactose up to 852, 702 and 651 %, respectively. Polyols such as mannitol, sorbitol and glycerol also pointedly enhanced the enzyme activity up to 358, 300 and 200%, but this effect was comparatively lower than that of monosaccharides. Polymer viz; polyethylene glycol had slightly enhancing effect but when the concentration was doubled enhancing effect also doubled and reached up to 300%. Other additives such as acetamide, cyclohexylamine, casein and sucrose had only a little effect on activity, lowest enzyme activity was shown with sucrose that was 128% (Fig. 3).

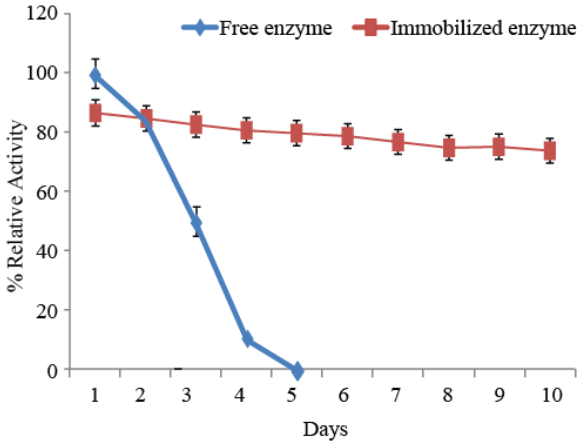
The additives enhance enzyme activity by shifting the native to denatured state equilibrium in the favor of native state, thus the activity of the mixture is enhanced (Iyer & Ananthanarayan, 2008). All polymers have the ability to enhance the activity of enzymes at a particular concentration (Yoon & Robyt, 2005) and this effect usually increases with the increase in concentration of the polymer (Monsan & Coombes, 1984). Findings of this study were in accordance with these reports, for example when PEG was used as an enhancer, the effect magnified with the increase in its concentration. When sugars are added to enzyme solution they strengthen hydrophobic interactions of enzymes and stabilize its protein structure (Schmidt, 1979), this stable structure gives higher activity and thus activity is enhanced. When polyols are used as additives they exclude themselves from surface of protein molecule and enhance the activity of enzymes. Polyols such as mannitol sorbitol, xylitol and glycerol have been reported to enhance the activity of the alkaline protease produced by *Aspergillus niger* (Kim *et al*., 2004). Enzyme activity of catalase has also been shown to be increased by polyols (Costa *et al*., 2002). Our results were in accordance with these reports and the activity of alkaline protease was distinctively enhanced in the presence of mannitol and sorbitol.

Stabilization of alkaline protease with additives: Effect of additives on the stability of alkaline protease was investigated by incubating the enzyme with various additives such as fructose, galactose, glucose, sucrose, glycerol, sorbitol, mannitol, cyclohexylamine, diphenyl amine, acetamide, BSA, casein and PEG. The stabilities of the samples were then compared by taking protease activity at every 48hrs. Stability profiles of both sets of samples stored at 4^oC and 30^oC are shown in Figs. 4 and 5, respectively. At 4^oC, the enzyme was significantly stabilized by fructose, PEG, galactose and mannitol. Fructose activated as well as stabilized the enzyme which maintained 258% of enzyme activity even after 22 days. While with PEG 0.5%, PEG 1%, galactose and mannitol, the enzyme retained 99, 78, 70, and 50% of its activity after 22 days. At 30^oC, the enzyme was highly stable in the presence of sugars and PEG. These additives enhanced as well as stabilized the enzyme. After 22 days, the enzyme samples incubated with fructose, galactose, glucose, PEG 0.5% and PEG 1% showed 348, 237, 217, 183 and 131% of enzyme activity, respectively. The enzyme was partially stabilized by casein and retained 40% of its activity up to 20 days. Other additives such as sucrose, glycerol, sorbitol, cyclohexylamine, diphenyl amine, acetamide and BSA did not show any noticeable stabilization of the alkaline protease (Annexure 1 and 2).



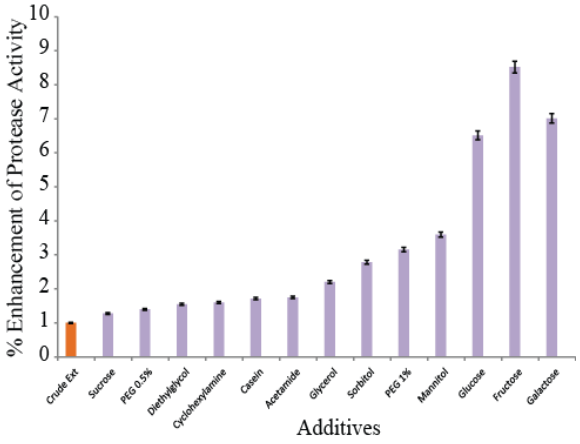
Y error bars indicates the standard deviation among the three replicates which differ significantly at ≤0.05

Fig. 1. Stability profile of lyophilized alkaline protease produced by *Bacillus subtilis*.



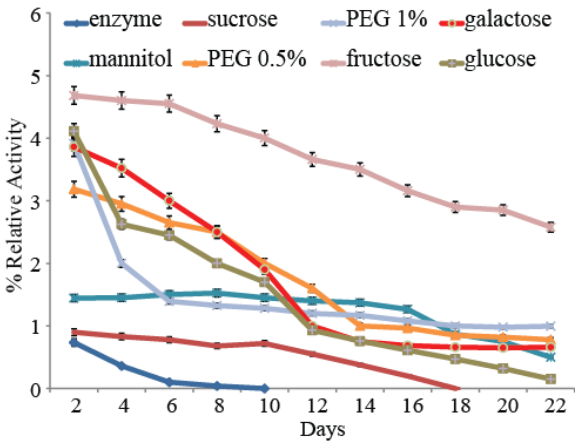
Y error bars indicates the standard deviation among the three replicates which differ significantly at ≤0.05

Fig. 2. Stability profile of immobilized protease produced from *Bacillus subtilis*.



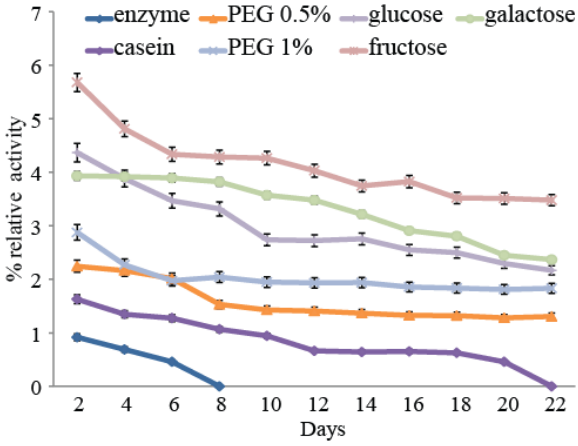
Y error bars indicates the standard deviation among the three replicates which differ significantly at ≤0.05

Fig. 3. Effect of activators on the activity of protease produced from *Bacillus subtilis*.



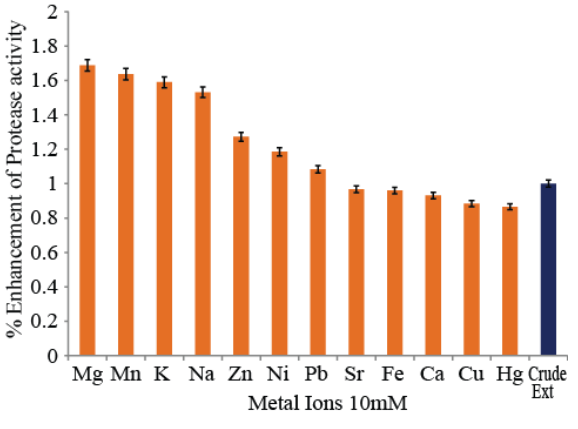
Y error bars indicates the standard deviation among the three replicates which differ significantly at ≤0.05

Fig. 4. Stability of alkaline protease with different additives at 4°C.



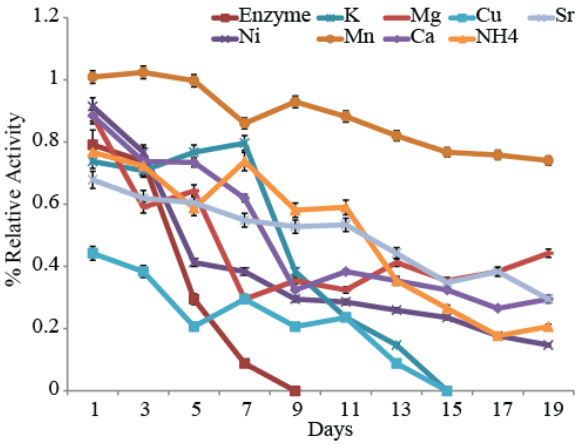
Y error bars indicates the standard deviation among the three replicates which differ significantly at ≤0.05

Fig. 5. Stability of alkaline protease with different additives at 30°C.



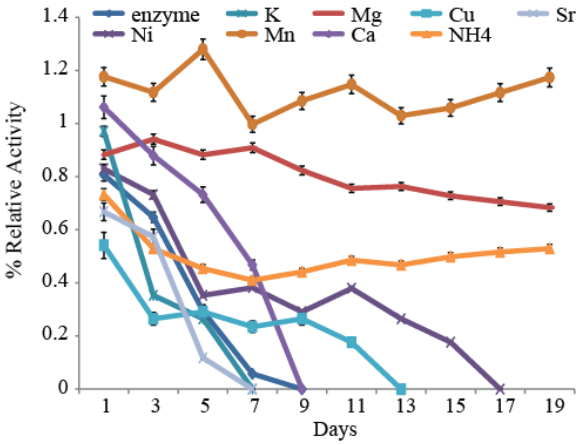
Y error bars indicates the standard deviation among the three replicates which differ significantly at ≤0.05

Fig. 6. Effect of metal ions as activators for alkaline protease.



Y error bars indicates the standard deviation among the three replicates which differ significantly at ≤0.05

Fig. 7. Effect of metal ions on stability of alkaline protease at 4°C.



Y error bars indicates the standard deviation among the three replicates which differ significantly at ≤0.05

Fig. 8. Effect of metal ions on stability of alkaline protease at 30°C.

Compounds that interact strongly with water but weakly with enzyme enhance the stability of the enzyme by reducing the amount of free water (Monsan & Combes, 1984). Therefore sugars, polyhydric alcohols and polymers generally have a protective effect on the stability of the enzymes. In the present study, all three groups of compounds had protective effect on alkaline protease. The effect followed the following order:

At 4°C fructose> PEG> galactose> mannitol
At 30°C fructose> galactose> glucose> PEG

Similar stabilizing effects of sugars and polyols have been patented by Becker *et al.* (2007) stating that enzyme can be stabilized over extended periods by adding polyols or sugars to enzyme solutions and that this method can be used for proteases produced by *Bacillus sp*. Stabilization of proteases by polyols has also been reported by George *et al.* (2001), Ghrorbel *et al.* (2002) and Kumar *et al.* (2010). Polyethylene glycol is also a non-specific stabilizer and has been shown to stabilize a large variety of enzymes (Monsan & Coombes, 1984; Yoon & Robyt, 2005) which supports the present findings in which PEG was found to be an important stabilizer of the alkaline protease.

Effect of metal ions as activators: To identify activators for the alkaline protease various metal ions at a concentration of 10mM were added to the reaction mixture during protease assay. Fig. 6 shows that Mg^{2+}, Mn^{2+}, Na^{+} and K^{+} strongly activated the enzyme up to 169%, 164%, 159% and 153%, respectively. Zn^{2+}, Ni^{2+} and Pb^{2+} only slightly enhanced the enzyme activity. Fe^{2+} and Ca^{2+} had almost no effect on enzyme activity whereas Cu^{2+} and Hg^{2+} strongly inhibited the enzyme and reduced its activity to about 88 and 87%.

Activators are substances which increase the activity of enzyme significantly beyond basal value. Metal ions actually activate enzymes by binding to enzyme at a site other than active site (Suelter, 1970; Iftikhar *et al.,* 2012). The results of this study are supported by the reports of activation of alkaline protease from *Bacillus subtilis* by Mn^{2+}, Ca^{2+} and Mg^{2+} (Adinarayna *et al.,* 2003; Johnvesly & Naik, 2001) and its inhibition by Fe^{2+}, Hg^{2+} and Cu^{2+} (Johnvesly & Naik, 2001; Ghorbel *et al*., 2002; Nascimento & Martins, 2004). In the present study no particular activation was observed with Ca^{2+}, indicating the Ca^{2+} independent nature of enzyme. The percent activation of the alkaline protease by Mn^{2+} and Mg^{2+} in this experiment (169 and 164%, respectively) is comparable to that reported by Ghorbel *et al.* (2002) that was 285 and 157%, respectively. Both Mg^{2+} and Mn^{2+} were strong activators of the enzyme. No reports for activation of alkaline serine protease by Na^{+} and K^{+} were found in literature however, they have been reported to play role in protease stabilization (Wang, 2000), thus the enhancement of the activity of enzyme might have been due to their role in stabilization of the protein structure during enzyme activity.

Effect of metal ions on the stability of alkaline protease: Protease stability was assessed by storing the crude enzyme in 10mM metal ion concentrations at 4°C and 30°C and taking enzyme activity up to a time period when the activity reached nil. Stability curves of the alkaline protease in the presence of additives are shown in Fig 7 and 8. As shown by the results the alkaline protease was stabilized by Mn^{2+} at 4°C, retaining 74% of its activity up to 19 days. The protease was partially stabilized by Mg^{2+}, Ca^{2+}, Sr^{2+}, NH_4^{+}, Ni^{+} retaining 45 and 29, 28 and 21% enzyme activity up to 19 days. While at 30°C, 100% of enzyme activity was retained up to 19 days with Mn^{2+} (Fig. 8). Na^{+}, Mg^{2+} and Zn^{2+} retained 77, 68 and 50% of the enzyme activity, respectively. Other metal ions such as Pb^{2+}, K^{+}, Hg^{2+}, Cu^{2+}, and Cd^{2+} did not have any pointed increase in protease stability at both 4°C and 30°C. The stability profiles showed better stability of the enzyme at 30°C suggesting its mesophilic nature.

Salts stabilize the enzyme by salting out effect of hydrophobic residues from surface into the interior of enzyme molecule thereby compressing the enzyme leading to higher stability of the enzyme (Klibanov, 1983). Stabilization effects of cations should correlate

with Hofmeister lyotropic series according to which NH_4^+ should be a strong stabilizer. However in this study, cations such as Mg^{2+}, Mn^{2+}, Na^+ and Zn^{2+} provided some protective effect. The other anions tested such as K^+, Ni^{2+}, Pb^{2+}, Sr^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+} and Hg^{2+} had no protective effect or made enzyme more labile. This suggests that effect of metal ions on stability was largely due to the binding of cations to protease molecule inducing conformations which were more stable. Stabilization of proteases produced by *Bacillus sp.* has been described by Becker, (2007) claiming that enzyme showed enhanced stability at room temperature in the presence of alkali halides as well as phosphates, formats, carbonates and most preferably sodium chloride. Ca^{2+}, Mg^{2+} and Mn^{2+} have also been shown to play important role in enzyme stabilization (Adinarayna *et al*., 2003).

Conclusion

These studies conclude that addition of several metal ions and additives to alkaline protease in solution can both activate and stabilize the enzyme. The processes such as immobilization on activated charcoal and lyophilization can stabilize the enzyme up to several days and months, respectively.

References

Adinarayna, K., P. Ellaiah and D.S. Prasad. 2003. Purification and characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS PharmsciTech*., 4(4): 1-9.

Ahmad, I., M.A. Zia, T. Iftikhar and H.M.N. Iqbal. 2011. Characterization and detergent compatibility of purified proteases produced from *Aspergillus niger* by utilizing agrowastes. *BioResources*, 6(4): 4505-4522.

Barrett, A.J. 1994. Proteolytic enzymes: serine and cysteine peptidases. *Methods Enzymol*., 244: 1-15.

Becker, N.T., R.R. Bott, S.L. Bowden, M.H. Heng, C. Paech and A.V. Kasola. 2007. Stabilization of enzymes. *US Patent 7157416 B2*

Benaddi, H., T.J. Bandosz, J. Jagiello, J.A. Schwarz, J.N. Rouzaud, D. Legras and F. Béguin. 2000. Surface functionality and porosity of activated carbons obtained from chemical activation of wood, *Carbon*, 38: 669-674.

Boyer, P.D., H.A. Lardy, H.A. and P.H. Phillips. The enzymes. 1942. *J. Biol. Chem.,* 146: 673-681.

Chaniotakis, N.A. 2004. Enzyme stabilization strategies based on electrolytes and polyelectrolytes for biosensor applications. *Anal. and Bioanal. Chem*., 378(1): 89-95.

Chen, G., D. Leen and B. Adriaan.1993. *Investigations on vinylene carbonate. VI. Immobilization of alkaline phosphatase onto poly(vinylene carbonate)-jeffamine® hydrogel beads. J. Appl. Pol. Sci.*, 48 (7): 1189-1198.

Church, F.C., H.E. Swaisgood and G.L. Catiagnani. 1984. Compositional analysis of proteins following hydrolysis by immobilized proteinases. *J. Appl. Biochem*., 6: 205-211.

Constantino, H.R., R. Langer and A.M. Klibanov. 1995. Aggregation of a lyophilized pharmaceutical protein, recombinant human albumin. *Biol. Technology.,* 13: 493-496.

Costa, S.A., T. Tazanov, A.F. Carneiro, A. Paar, G.M. Gubitz and A.C. Paulo. 2002. Studies of stabilization of native catalase using additives. *Enzyme Microb. Technol.*, 30: 387-391.

Ellaiah P., B. Srinivasulu and K. Adinarayna. 2002. A review on microbial alkaline proteases. *Journal of Science and Industrial Research*, 61: 690-704.

Evans, H.J. and G.J. Sorger. 1966. Role of mineral elements with emphasis on the univalent cations. *Annu. Rev. Plant Physiol.,* 17: 47-76.

Fagain, C. 2003. *Enzyme* stabilization-recent experimental progress. *Enzyme Microb. Technol*., 33(2-3): 137-49.

George, S.P., A. Ahmad and M.L. Rao. 2001. A novel thermostable xylanase from *Thermosmonospora* sp.: influence of additives on thermostability. *Bioresour. Technol*., 78: 221-4.

Ghorbel, B., A. Kamoun and M. Nasri. 2002. Stability studies of protease from Bacillus cereus BG1. Enzyme Microb. Technol., 32: 513-518.

Gouda, M.D., S.A. Singh, A.G.A. Rao, M.S. Thakur and N.G. Karanth. 2003. Thermal inactivation of glucose oxidase: Mechanism and stabilization using additives. *J. Biol. Chem*., 278(27): 24324-33.

Grazu, V., O. Abian, C. Mateo, F. Batista-Viera, R. Fernandez-Lafuente and J.M. Guisan. 2005.Stabilization of enzymes by multipoint immobilization of thiolated proteins on new epoxy-thiol supports. *Biotechnology and Bioengineering,* 90(5): 597-605.

Haard, N.F. 1998. Specialty enzymes from marine organisms. *Food Technol*., 52: 64-67.

Hanson, M.A. and S.K.R. Rouan. 1992. Formulation of protein pharmaceuticals, In: *Stability of Protein Pharmaceuticals, Part B: In Vivo Pathways of Degradation and Strategies for Protein Stabilization.* (Eds.): Ahern, T.J. and M.C. Manning, Plenum, New York, pp. 209-233.

Iftikhar, T., M. Niaz, M.A. Zia, M. Sadaf and R. Jabeen. 2012. Production potential of locally isolated strain of *Fusarium solani* (MBL 24) for extracellular lipases. *Pak J. Bot.,* 44(1): 393-397.

Iyer, P.V. and L. Ananthanarayan. 2008. Enzyme stability and stabilization-Aqueous and non-aqueous environment. *Process Biochem.,* 43: 1019-1032.

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

Kim, Y., J. Bae, B. Oh, W.H. Lee and J. Choi. 2002. Enhancement of proteolytic enzyme activity excreted from *Bacillus stearothermophilus* for a thermophilic aerobic digestion process. *Bioresour. Technol.*, 82: 157-164.

Klibanov, A.M. 1983. Stabilization of enzymes against thermal inactivation. *Adv. Appl. Microbiol.,* 29: 1-28.

Kumar, A. G., S. Sawarnalatha, P. Kamatchi and G. Sekaran. 2009. Immobilization of highly catalytic acid protease on functionalized mesoporous activated carbon particles. *Biochem. Eng. Jour*., 43: 185-190.

Kumar, A.G., K. Perinbam, N. Nagesh and S. Sekaran. 2010. In: Situ immobilization of acid protease on mesoporous activated carbon packed column for production of protein hydrolysates. *Bioresour. Technol..,* 101: 1377-1379.

Liu, W.R., R. Langer and A.M. Klibanov. 1991. Moisture-induced aggregation of lyophilized proteins in the solid state. *Biotech. Bioeng.* 37: 177-184.

Matejtschuk, P. 2007. Lyophilization of proteins. In: *Methods in Molecular Biology: Protein purification protocols.* (Eds.): Day, J.G. and G.N. Stacey. Humama Press, Totowa New Jersey, pp. 59-75.

McDonald, C.E. and L.L. Chen. 1965. Lowry modification of the Folin reagent for determination of proteinase activity. *Ann. Biochem*., 10: 175.

Michiaki, M., K. Koji and K. Kazuo. 1997. Effects of polyols and organic solvents on thermostability of lipase. *J Chem Technol. Biotechnol.*, 70: 188-92.

Monsan, D. and P. Coombes. 1984. Effect of polyhydric alcohol on invertase stabilization. *Ann. N. Y. Acad. Sci*., 434: 61-4.

Mukhtar, H. and I. Haq. 2007 Optimization of volume of fermentation medium for the production of alkaline protease by an EMS mutant strain of *Bacillus subtilis* IH-72. *Pak. J. Bot.,* 39(7): 2705-15.

Nascimento, W.C.A. and M.L.L. Martins. 2004. Production and properties of extracellular protease from thermophilic *Bacillus* sp. *Braz. J. Microbiol.,* 35: 91-96.

Pikal, M.J., S. Shah, M.L. Roy and R. Putman. 1990. The secondary drying stage of freeze drying: drying kinetics as a function of temperature and chamber pressure. *Int. J. Pharm*., 60: 203-217.

Prakasham, R.S., C.H.S. Rao and P.N. Sarma. 2006. Green gram husk, an inexpensive substrate for alkaline protease production by *Bacillus* sp., in solid state fermentation. *Bioresour. Technol*., 97: 449-454.

Rainer J. 2000. Stability and stabilization of globular protein in solution. *J. Biotechnol*., 79: 193-203.

Rajini, K. and B. Mattiasson. 1993. Improving the shelf life of enzyme by storage under anhydrous apolar solvent. *Biotechnol Techn.,* 7: 585-90.

Rockwell, N.C. and R.S. Fuller. 2002. Specific modulation of Kex2/furin family proteases by potassium. *J. Biol. Chem*., 277: 17531-17537.

Rosier, T.A., N.B. Cruz and R.K. Wilkosz. 2001. Method and formulation for stabilization of enzymes. *US Patent 5876992*.

Schmidt, R. 1979. Stabilized soluble enzymes. *Adv. Biochem. Eng*., 12: 41-118.

Sierecka, J.K. 1998. Purification and partial characterization of a neutral protease from a virulent strain of *Bacillus cereus*. *Int J Biochem. Cell Biol*., 30: 579-595.

Suelter, C.H. 1970. Enzymes activated by monovalent cations. *Sci.,* 168: 789-795.

Thuma, R.S., J.L. Giegel and A.H. Posner. 1987. Manufacture of quality control materials, In: *Laboratory Quality Assurance.* (Eds.): Howanitz, P.J. and J.H. Howanitz. McGraw-Hill, New York, pp. 101-123.

Wang, W. 2000. Lyophilization and development of solid protein pharmaceuticals. *Intl. Jour. Pharm.,* 203: 1-60.

Yoon, S. and J.F. Robyt. 2005. Activation and stabilization of 10 starch-degrading enzymes by Triton X-100, polyethylene glycols, and polyvinyl alcohols. *Enzyme Microb. Technol*., 37: 556-62.

(Received for publication 12 December 2013)