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INFLUENCE OF CULTURE CONDITIONS ON PRODUCTION AND ACTIVITY OF PROTEASE FROM *BACILLUS SUBTILIS* BS1

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Abstract

Bacillus subtilis BS1 was used in the present study for the production of protease. For protease production optimum pH and temperature were found to be 11 and 50˚C, respectively. Soybean (197 PU/mg) and casein (168 PU/mg) proved as the best substrates for the production of enzyme. Maximum production of protease (126 and 121 PU/mg) was shown in 1.5 and 4.5% of Sodium chloride (NaCl) concentration, respectively. Maximum activity was observed at pH 9 at 90ºC, in crude extract, after 20 minutes of incubation. EDTA slightly enhanced proteolytic activity, whereas, Na, K, Ca, Li, Mg, Cu and Fe inhibited the activity of protease. Due to maximum production of protease in the presence of cheaper, low concentrations of substrate and stability at alkaline pH, high temperature and salt-tolerance, these characteristics makes the strain and its enzymes usefull in different industries.

Introduction

Proteases represent the class of enzymes that occupy a pivotal position with respect to their physiological roles as well as their commercial applications. More than 75% of industrial enzymes are hydrolases. Protein-degrading enzymes constitute about 40% of all enzymes sales (Leisola *et al*., 2001). They perform both degradative and synthetic functions. A number of eukaryotic and prokaryotic organisms are reported to produce proteolytic enzymes (Sakka *et al*., 1986). Many different types of extracellular hydrolytic enzymes as well as some non-hydrolytic enzymes (Lipases, α and β-amylases, cellulases, xylanase, penicillinase, invertase, β-glycosidase, cytase, phosphatases, deaminases, urease, asperginase are produced by microorganisms e.g., *Actinoplanes* sp., *Arthrobacter* sp., *Aspergillus* sp., *Bacillus cereus*, *Bacillus coaglulans*, *Bacillus subtilis*, *Candida* sp., *Clostridium* sp., *Lactobacillus* sp., *Penicillium* sp., *Pseudomonas* sp., *Rhizopus* sp., *Streptococcus* sp., *Streptomyces* sp., (James & David, 1986).

The biosynthesis of proteolytic enzymes by microorganisms is not only of scientific but also of great practical importance. *Bacillus subtilis* produces both neutral and alkaline protease (Dhandapani & Vijayaragavan, 1994). Proteases produced from *Bacillus subtilis* have wider specificity than that of trypsin and chymotrypsin of animal origin. They are present in a large variety of commercially available enzymes differing in biological source, activity, purity, physical form and characteristics such as pH and temperature optima (Cheetham, 1995). Enzymes are vulnerable to various environmental factors. Their activity may be significantly diminished or destroyed by a variety of physical or chemical agents resulting in a loss of the functions performed by the enzymes (Pelczar *et al*., 1986). The present study was aimed to optimize pH, temperature, substrate and salt concentration for maximum production of protease from *Bacillus subtilis* BS1 and its stability.

Materials and Methods

Microorganisms: *Bacillus subtilis* BS1, used in the present study, was isolated from sugar scum from Crescent Sugar Mills Ltd., Faisalabad. Cultures were maintained on Nutrient agar slants.

Qualitative test for protease: Proteolytic activity of *Bacillus subtilis* BS1 was detected on the basis of appearance of clear zones around the bacterial colonies. Luria casein agar (1%) plates were used for this purpose.

Quantitative test for protease: Liquid medium (250ml) containing (g/l): Gelatin, 15; casein hydrolysate, 0-5; glycerol, 3ml, with pH 8, was used to screen the bacterial strain for the production of protease. About 250ml medium was poured in two 1000ml Erlenmeyer flasks, and pH was adjusted by using 0.1N NaOH and 0.1N CH_3COOH. The medium was autoclaved at 121°C, 15psi for 20 minutes. Oven sterilized, 3ml of 20% glycerol solution was added to the medium aseptically. *Bacillus subtilis* BS1 was inoculated in the medium and the flasks were kept in shaking incubator at 37°C with 150rpm. Samples were collected after 24, 48, 72 and 96 hours and centrifuged at 10, 000rpm for 30 minutes at 4°C. Cell free supernatant was used as the crude enzyme for the estimation of enzyme activity.

To check the activity of enzyme in culture supernatant, method of Kunitz (1965) was used with casein as a substrate. One unit of enzyme activity is defined as that amount of enzyme which releases a micro mole tyrosine under standard conditions of assay, 45°C, pH 8.5 and reaction time one hour.

Optimization of culture conditions for production of protease: Different culture conditions, like pH (5-11), temperature (40, 50, 60 and 70˚C), different substrates (gelatin, casein, soybean) and salt concentration (0, 1.5, 3.0, 4.5 and 6%), were optimized for production of protease from *Bacillus subtilis* BS1. Samples were collected after 24, 48 and 72hrs and centrifuged at 1000rpm for 30 minutes at 4˚C. Supernatant was used as crude extract. Proteolytic activity was measured under standard assay conditions.

Stability of enzyme in crude enzyme extract: Protease was produced from *Bacillus subtilis* BS1 under optimized conditions. After 48 hours of incubation, cell free supernatant was taken as crude enzymes extract and used for the characterization. The effect of pH (4-11), temperature (40, 50, 60, 70, 80, 90, 100˚C) and metal ions (20mM solution of NaCl, KCl, $CaCl_2$, $LiCl_2$, $MgSO_4$, $CuSO_4$, $FeCl_2$, $BaCl_2$ and $HgCl_2$ and chelating agent EDTA) on the activity of crude enzyme was studied. The crude enzyme was incubated for 20 minutes and enzyme activity was measured before and after the treatment under standard assay conditions. Buffers (0.02M) of different pH values were used: acetate buffer (pH 4, 5), phosphate buffer (6, 7), Tris (pH 8) and glycine-NaOH buffer (pH 9, 10, 11) for assay.

Results and Discussion

Qualitative and quantitative analysis: *Bacillus subtilis* BS1, was found to produce protease on the basis of clear zones around the bacterial colonies on 1% casein agar

plates at 37˚C after 24 hours. These clear zones were due to hydrolysis of different substrates (Khire & Pant, 1992). Maximum production of protease by *Bacillus subtilis* BS1 was 131 U/ml after 48hrs, at 37˚C, pH 8 and 150rpm, in Gelatin casein medium. The production was 129, 123 and 109 U/ml after 24, 72 and 96 hrs, respectively.

Optimization of culture conditions for protease production

Effect of pH: Production of protease was observed at various pH values ranging from 4 to 11. The maximum production of protease was 523 PU/mg, at pH 11 after 48hrs (Fig. 1). Kobayashi (1995) reported optimal activity of bacterial protease at pH 11. Cheetham (1995), Muderrizade *et al*., (2001) and Kumar (2002) also reported the production of an alkaline protease at 11.5 from *Bacillus* sp. The study reveals that the enzyme secretion is greatly influenced by the change in initial pH of the environment (Sarkar *et al*., 1998). Enzymes possess many ionizable groups so that pH changes may alter the conformation of the enzyme (Cheetham, 1995).

Effect of temperature: Only few microbial strains are able to grow at elevated temperatures (Cheetham, 1995). Effect of various temperatures, 40, 50, 60 and 70˚C on protease production was studied. It was observed that enzyme production was maximum (301 PU/mg) at 50˚C (Fig. 2). Sookkeo *et al*., (2000) and Kobayashi (1995) reported production of protease at such high temperatures. Mao & Freedman (1992) found that maximum protease production could be achieved by controlled pH and temperature.

Effect of substrates: The substrates used in industrial enzyme fermentations are normally common agricultural products like soybean, casein, starch and ordinary sugar (Cheetham, 1995). Enzyme formation is largely dependent on the condition of growth of the culture and composition of nutrient medium (Fujiwara & Masui, 1993). In the present study, casein, gelatin and soybean flour with glycerol and casein hydrolysate were used as substrates for the production of protease from *Bacillus subtilis* BS1, at pH 11. Maximum production of protease (197 PU/mg) was observed in case of soybean after 48 hrs of incubation while in case of casein and gelatin, the production of protease was found as 171 PU/mg and 163 PU/mg, respectively after 72 hrs of incubation. Sun (1997) reported maximum production of protease from *B. sphaericus* C3-41 in the presence of soybean after 48 hrs of incubation. Geoffrey & Hodges (1981) found that protease synthesis occurred at exponential phase of bacterial growth, which is associated with sporulation of *B. subtilis*, but according to Dercova *et al*., (1992), the secretions of enzyme occurs mostly between the end of the exponential phase to an early stationary phase, maximum production occurs after cell population reached its peak. High levels of proteases were produced on casein, soybean flour and starch (Mahmood *et al*., 2000; Sampath &Chandrakasan, 1998). Gelatin and soybean act as organic nitrogen sources and enhance the bacterial growth, while casein hydrolysate is a source of readymade amino acids and encourage the foam formation to remove spores and cellular debris from the culture medium (Feng *et al*., 2001). It also enhances the extracellular alkaline protease synthesis. The above results showed that enzyme production was mostly decreased after 48hrs., this might be due to the depletion of nitrogen and other sources present in medium, which were utilized by the organisms, or due to inactivation of enzyme by acidification of the medium (Dervoca *et al*., 1992).

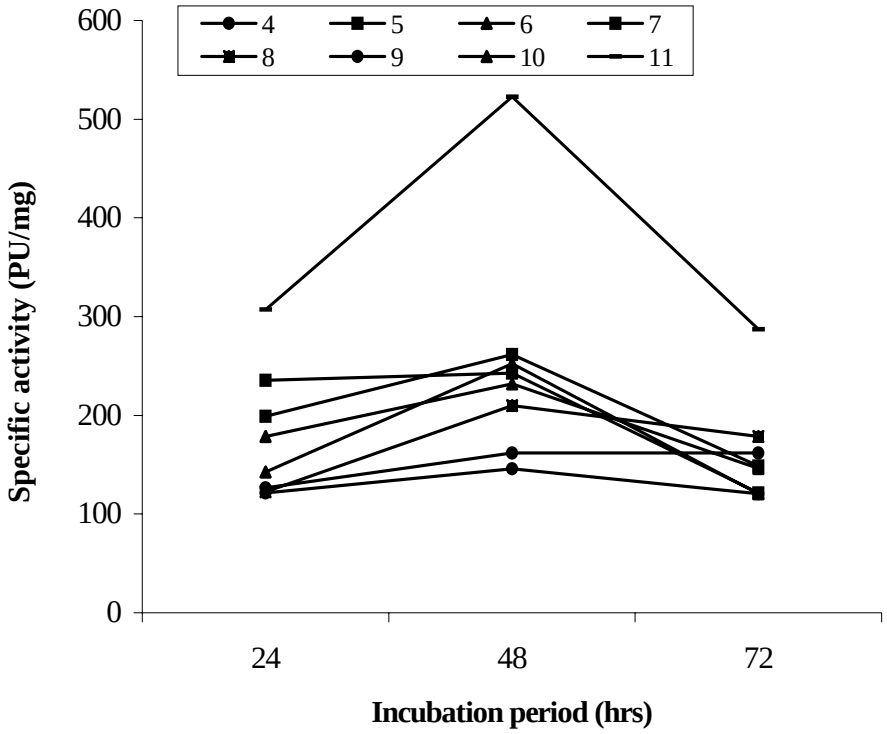

Fig. 1. Effect of different pH on the protease production from *Bacillus subtilis* BS1.

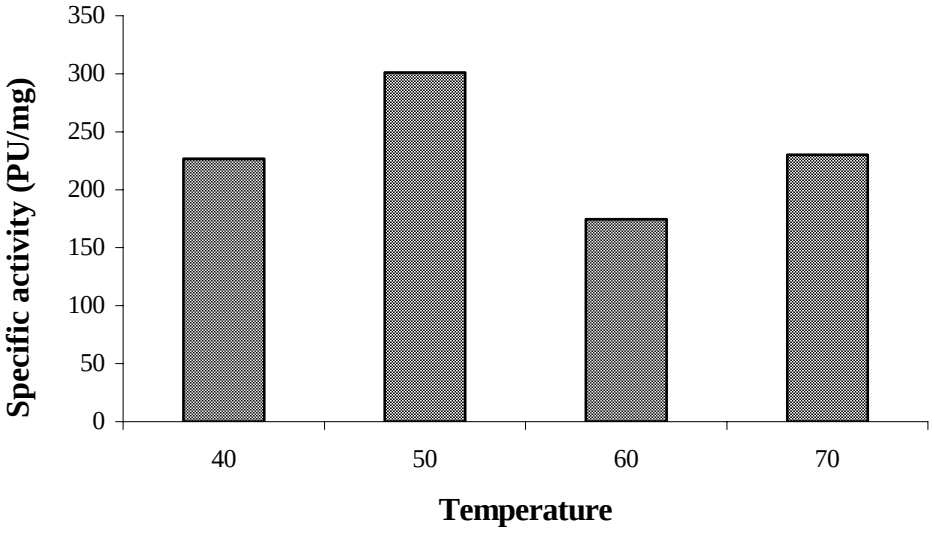

Fig. 2. Effect of different temperature (º C) on the protease production from *Bacillus subtilis* BS1.

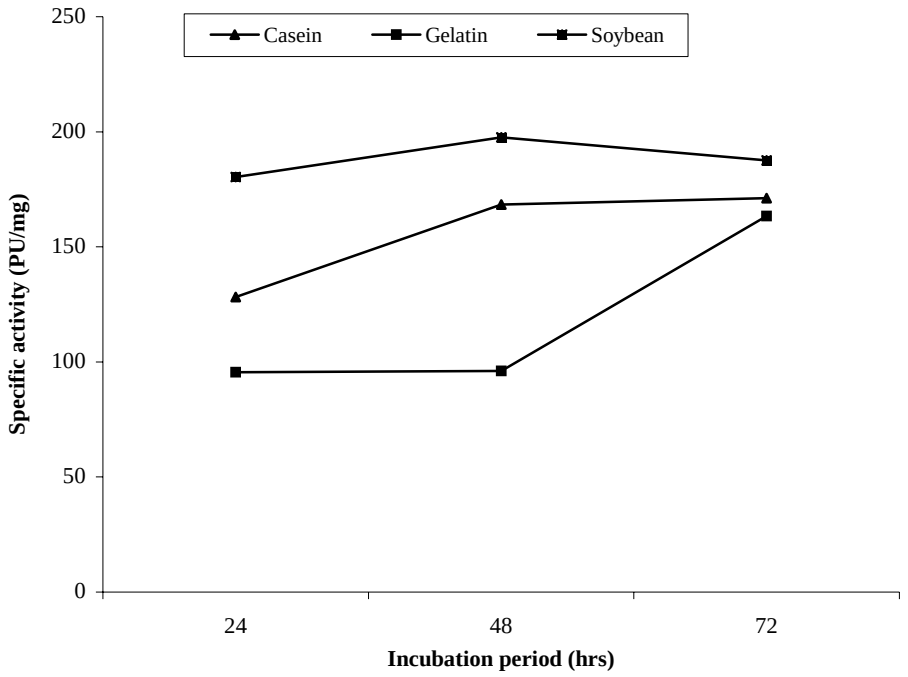

Fig. 3. Effect of different substrates on the protease production from *Bacillus subtilis* BS1.

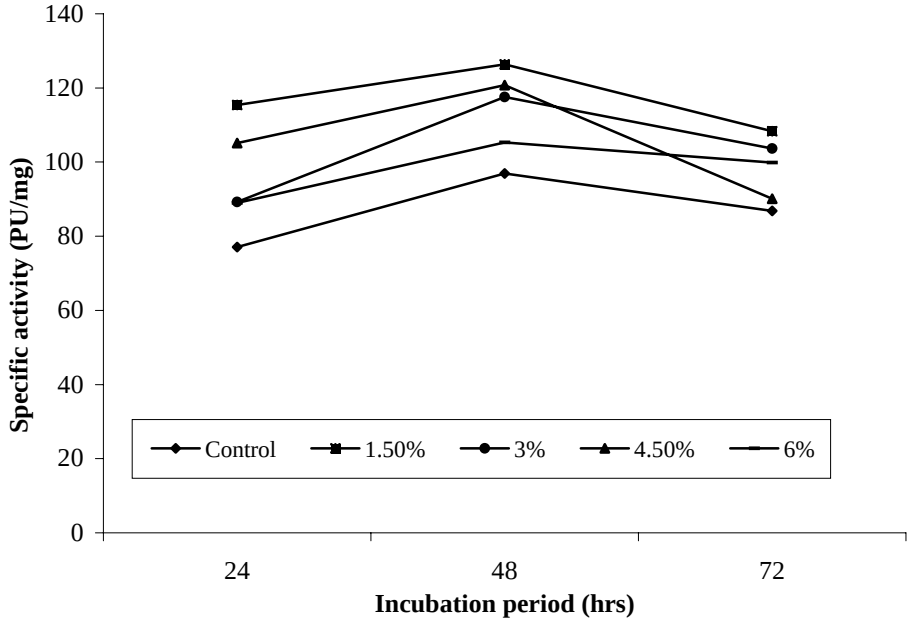

Fig. 4. Effect of different salt NaCl concentrtions on the protease production from *Bacillus subtilis* BS1.

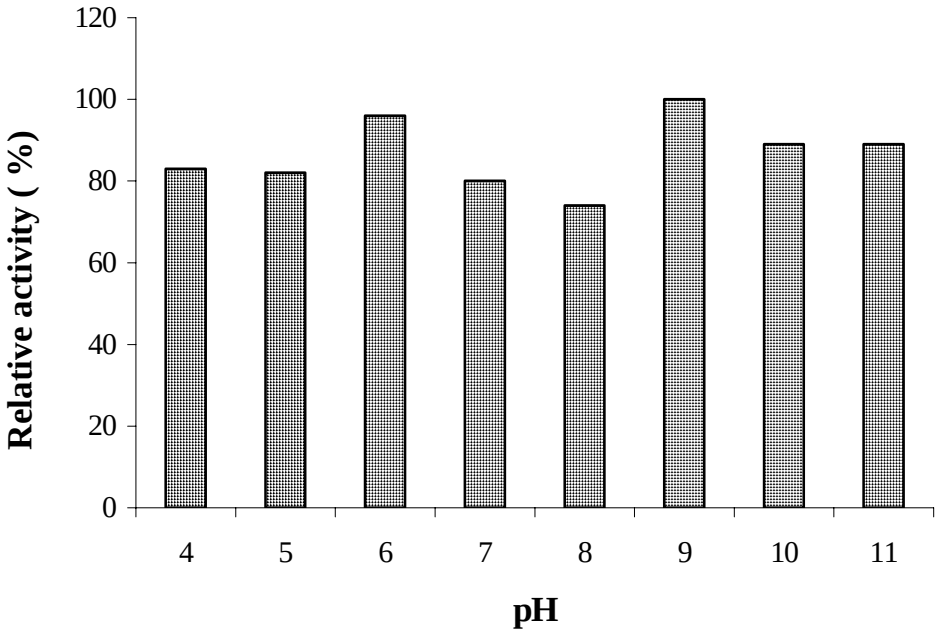

Fig. 5. Effect of pH on the proteolytic activity in crude extract of 48 hrs from *Bacillus subtilis* BS1.

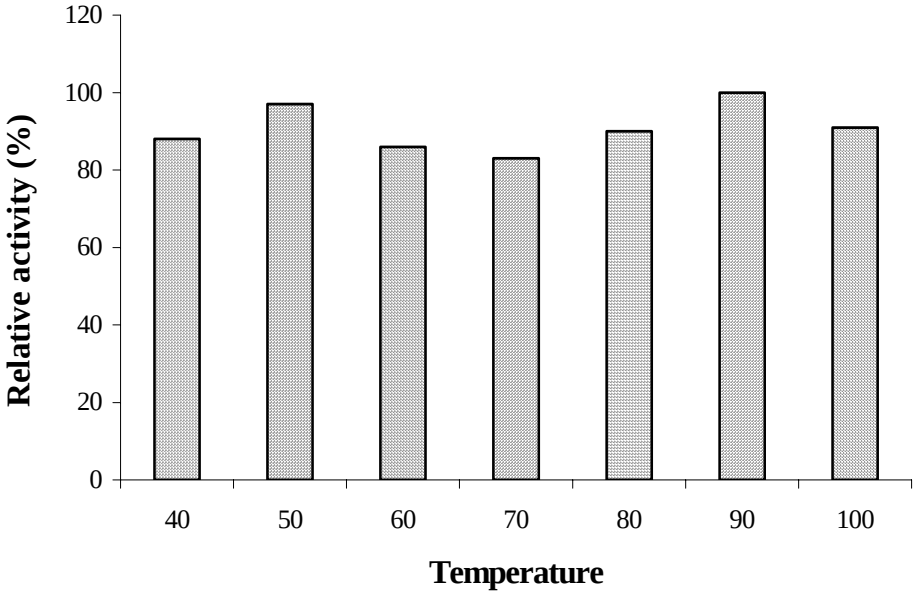

Fig. 6. Effect of temperture on the proteolytic activity in crude extract of 48 hrs from *Bacillus subtilis* BS1.

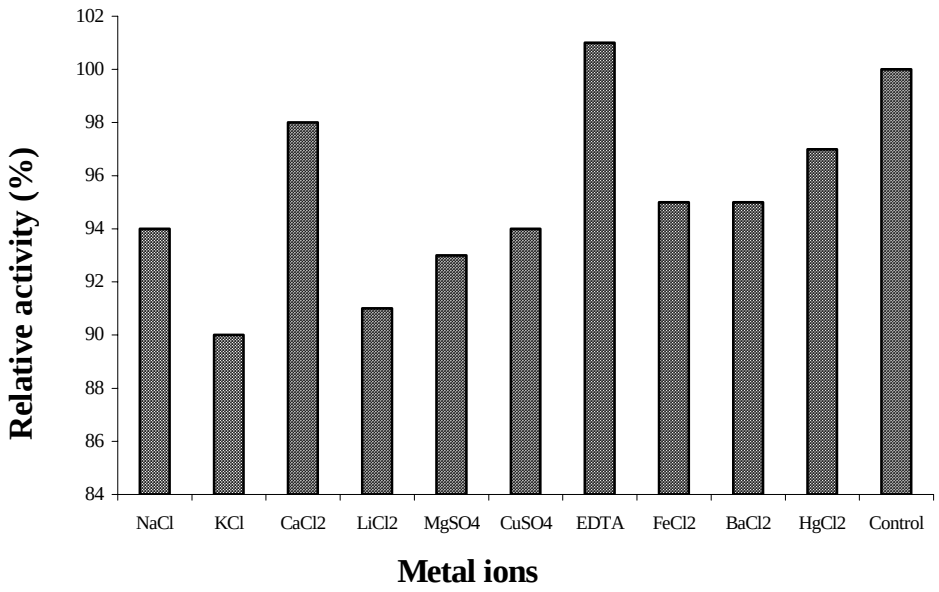

Fig. 7. Effect of metal ion on the proteolytic activity in crude extract of 48 hrs from *Bacillus subtilis* BS1.

Effect of NaCl: In the present study, various salt concentrations from (0-6%) were used to study the effect of NaCl on the production of protease from *Bacillus subtilis* BS1. Maximum enzyme (126 PU/mg) was produced in the presence of 1.5% NaCl (Fig. 4), followed by 4.5 and 3%. Tolerance of enzyme up to 5 M of NaCl over 24hrs without losing original activity was reported by Jana *et al*., (1997).

Stability of protease in crude enzyme extract: Both low and high levels of temperature and pH affect the enzyme activity and stability.

Effect of pH: The enzyme activity of the protease produced by *Bacillus subtilis* BS1 was determined after incubating the crude extract for 20 minutes at 90˚C at different pH values. The enzyme showed maximum activity (100%) at pH 9. It was shown that the enzyme was 96 % stable at pH 6 and 89% stable at pH 10 and 11 (Fig. 5). Protease activities at alkaline pH (8-11) were studied by Kim *et al*., (2001) and Feng *et al*., (2001).

Effect of temperature: The effect of temperature on the activity of enzyme was studied. Maximum activity was shown at 90˚C and 50˚C, 100% and 97%, respectively (Fig. 6). Activity of protease at 45˚C for 30min., and 80˚C for 10min., and 60min., respectively was reported by Feng *et al*., (2001). The decrease in enzyme activity at higher temperatures might be due to the destruction of an enzyme at certain temperatures.

Effect of metal ions: Metal ions often act as salt or ion bridges between two adjacent amino acid residues. Effects of various metal ions are shown in Fig. 7. K, Li^{+2}, Mg^{+2}, Cu^{+2}, Na inhibited the activity of protease, but EDTA played important role in stimulation of enzyme activity and increased the activity up to 101%. Observations about

the stability of enzyme in the presence of EDTA are also reported by Muderrizade *et al*., (2001). Inhibition by EDTA, Hg, Fe, Cu, Fe, Mg and Li was also reported by Sun *et al*., (1997) and Sookkeo *et al*., (2000). In the present work, strong inhibition or stimulation by metal ions in case of protease activity, was not observed. Alkaline proteases are generally neither inhibited by metal chelating reagents nor activated by metal ions or reducing agents (Kim *et al*., 2001).

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