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Characterization of a Thermostable Alkaline Protease from *Staphylococcus aureus* S-2 Isolated from Chicken Waste

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Abstract.- In this study, the protease producing bacterium was isolated from chicken waste and characterized as *Staphylococcus aureus* through 16S rRNA ribotyping. The protease from *S. aureus* S-2 showed maximum activity of 360 U/mL. *S. aureus* S-2 showed optimum growth at 37ºC and pH 7. *S. aureus* S-2 was able to grow in 1% gram pulse, mung beans and hydrolyzed casein but the maximum growth of the organism was supported by 1% hydrolyzed casein. A comparison of intracellular and extracellular enzyme activity showed that the predominant form of the enzyme was extracellular. The optimal pH and temperature for the protease activity were 8.0 and 50ºC, respectively. The enzyme was active at a broad range of pH (5-9) and temperatures (30-90ºC). The molecular mass of the enzyme was found to be 30 kDa in SDS-polyacrylamide gel electrophoresis. *S. aureus* S-2 can be exploited for biotechnological and industrial applications.

Key words: Azocasein, *Staphylococcus aureus*, alkaline protease.

INTRODUCTION

Proteases are single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins. Advances in analytical techniques have demonstrated that proteases conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes (Fox *et al*., 1991; Godfrey and West, 1996; Gupta *et al*., 2002). Of the industrial enzymes, 75% are hydrolytic (Rao and Deshpande, 1998; Rao *et al*., 1998). Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes. Proteases have a long history of application in the food and detergent industries (Kumar and Takagi, 1999; Gupta *et al*., 2002). Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance (Rao *et al*., 1998). The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Poldermans, 1990; Fox *et al*., 1991).

Proteases are ubiquitous in nature and are found in a wide variety of organisms such as plants, animals and microorganisms. Plant proteases include papain, bromelian and keratinaes (Schechler and Berger, 1967; Bressollier *et al*., 1999). Most famous proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennin (Boyer, 1971; Hoffman, 1974).

Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. These extracellular enzymes are synthesized by diverse groups of microorganisms, including fungi, yeasts and bacteria. Among extracellular alkaline proteases, those from *Bacillus* species have wide use and importance in several industrial sectors, such as the

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food, dairy (Razak *et al*., 1994), obtaining of protein hydrolysates (Carreira *et al*., 2004; Soares *et al*., 2007), leather (Takami *et al*., 1992; Giongo *et al*., 2007), detergent sectors (Ito *et al*., 1998; Hadj-Ali *et al*., 2007) and in the synthesis of biologically active peptides (Kumar and Bhalla, 2005). Extracellular proteases, virulence factors, from *S. aureus* can be used for the development of a test panel which could aid in screening of natural products of plant and microbial origin (Saxena and Gomber, 2010). Manachini *et al*. (1988) reported that extracellular proteases can inhibit the protein dependent biofilm formation in *S. aureus.*

The present study is concerned with the isolation and characterization of the protease from *S. aureus*. Optimum growth conditions and effect of different substrates on the growth of the organism are ascertained. Some enzymatic characteristics *i.e.*, pH, temperature, and effect of metal ions on the protease activity have also been determined.

MATERIALS AND METHODS

Isolation and screening of protease producing bacteria

Bacteria for the production of proteases were isolated from 16 soil, water, and swab samples collected from garbage dump, chicken waste drum, and cat's mouth. One gram of soil sample was added to a glass tube containing 10 mL sterilized distilled water, shaken and placed in a water bath at 80ºC for 15 min. After cooling, 50 µL of the sample was spread on nutrient agar plates (prepared by dissolving 0.6 g peptone, 0.4 g casein hydrolyzate, 0.15 g beef extract, 0.3 g yeast extract and 0.2 g starch in 100 mL distilled water, pH adjusted at 7 and then 1.5 g agar was added in the 250 mL Erlenmeyer flasks, autoclaved at 121ºC and 15 lb pressure for 15 min) and incubated at 37ºC for 48 h. Similarly water (50 µL) and swab samples were also spread on nutrient agar plates. The colonies forming clear zones around them were picked up and streaked on nutrient agar plates to get pure culture and to confirm zone formation.

Further screening of protease producing bacteria was carried out in 1 gram pulse, 1% mung beans and 1% gelatin medium containing (g/L) of distilled water): NaCl-0.4, peptone-2.0, and yeast extract-1.0. The pH was adjusted to 7 with NaOH and medium was sterilized by autoclaving at 121ºC and 15 lb pressure for 15 min. Final selection was based on growth of bacterial isolate on 3% gelatin.

Molecular characterization

The isolate was Gram stained. For molecular characterization genomic DNA was extracted as described by Carozzi *et al.* (1991) and the 16S rRNA gene was amplified by PCR using 16S rRNA primers
RS-1; 5′-AAACTCAAATGAATTGACGG-3′, and
RS-3; 5′-ACGGGCGGTGTGTAC-3′
(Rehman *et al*., 2007). PCR was performed by initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR product of 0.5kb was removed from the gel and cloned in pTZ57R/T vector. The amplified 16S rRNA gene was purified with a Fermentas purification kit (#K0513) and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. The 16S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

Determination of optimum growth conditions

For optimum growth of the bacterial isolate, two parameters *i.e.,* temperature and pH were considered. For determination of optimum temperature, 5 mL LB broth was added in 4 sets, each of three test tubes, autoclaved and inoculated with 20 µL of freshly prepared culture of bacterial isolate by overnight growth at 37ºC in LB broth. The four sets of tubes were incubated at 20ºC, 30ºC, 37ºC and 45ºC. After an incubation period of 12 h, their absorbance was tested at 600 nm using a λ 650 UV/Vis Spectrophotometer (Perkin Elmer, USA). For determination of optimum pH, test tubes having 5 mL LB broth were prepared in 6 sets, each containing 3 test tubes and their pH was adjusted at 5, 6, 7, 8, and 9 then autoclaved. These tubes were inoculated with 20 µL freshly prepared culture of bacterial isolate. After an incubation period of 12 h,

their absorbance was tested at 600 nm.

Effect of different substrates on bacterial growth

To determine the growth behavior of the strain over a period of time, it was grown in four different media under similar conditions of temperature and pH but varying time periods. Four different media prepared were LB broth, salt medium with 1% hydrolyzed casein, salt medium with 1% gelatin and salt medium with 1% gram's pulse. The media (100 mL) were dispensed in 250 mL flasks and autoclaved at 121°C for 15 min. After the temperature of the media lowered to room temperature, they were inoculated with 100 µL of log phase growing cells of bacterial isolate. The flasks were incubated at 37°C for 24 h. After about each 4 h of incubation optical density of each flask was taken at 600 nm using a spectrophotometer at successive time intervals.

Azocasein assay

S. aureus S-2 was grown in 100 mL salt medium supplemented with 1% gelatin and 1% glucose for 5 days. After that the cells were harvested and supernatant was separated. Azocasein assay was performed for intracellular and extracellular nature of enzyme.

Intracellular

The cells were washed with autoclaved distilled water. The pellet was resuspended in 0.1mol/L phosphate buffer (pH 7) and sonicated for 20 sec with an interval of 60 sec twice. It was centrifuged at 6000 rpm for 10 min and the supernatant was transferred to fresh eppendorfs and this crude material was used for enzyme assay.

Extracellular

Chilled 60% ammonium sulphate solution in equal volume of each of the supernatant was added and left at 4°C for overnight. The solutions were centrifuged at 4000 rpm for 10 min and the upper supernatant was discarded leaving 3-4 mL in the falcon tube which was used as the crude enzyme sample.

The reaction mixture consisted of crude enzyme sample 800 µL and 400 µL of 1.5% azocasein (in 0.05 M Tris-Cl buffer, pH 8.5) in a screw capped test tube. The tubes were incubated in a water bath at 37°C for 30 min. Then 2.8 mL of 5% trichloroacetic acid was added in each tube to stop the reaction. The tubes were put on ice for 15 min and then centrifuged at 4000 rpm for 5 min. The supernatant was saved, pellet was discarded, and 2 ml of 0.5 N NaOH was added to 2 mL of the supernatant. After thorough mixing, optical density of the solution was taken at 440 nm. Optical density of blank was taken at 440 nm (which was made by adding trichloroacetic acid before sample addition). One unit of protease activity is defined as the amount of enzyme that is required to produce an increase of absorbance of 0.186 under the above assay conditions.

Effect of pH, temperature and metal ions on protease activity

The effect of pH on the activity of protease was measured by incubating 0.8 mL of the diluted enzyme and 0.4 mL of phosphate buffer ranging pH from 5 to 9, containing 1.5% azocasein for 30 min at 37°C. The effect of temperature on the enzyme activity was determined by performing the standard assay procedure for 30 min at pH 8.5 within a temperature range of 30-90°C. After treatment the residual enzyme activity was assayed.

The metal ion effect on enzyme activity was examined by chloride salts of various metals. Each metal was added in the reaction mixture at a final concentration of 0.1 mM and protease activity was determined at 37°C and pH 8.5. No metal ions were added in the control assay.

SDS- polyacrylamide gel electrophoresis

Protein profile of bacterial cells grown with 1.5% casein and without casein was done to compare the inducibility of protease. The crude enzyme was precipitated with ammonium sulphate (60%) and allowed to stand overnight at 4°C with constant stirring. The mixture was centrifuged at 10,000 *g* for 20 min at 4°C. The precipitate was collected, re-dissolved in distilled water. The proteins of *S. aureus* S-2 were isolated according to Rehman *et al*. (2009), estimated according to Lowry *et al*. (1951), and electrophoresed according to Laemmli (1970). Aliquots of 15-20 µL were loaded onto a 12% SDS polyacrylamide linear resolving gel

overlaid with a 6% stacking gel. Gels were stained with Coomassie Brilliant blue R-250.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Characteristics of bacterial isolate

A total of 16 samples collected from different ecological environments were analyzed for bacteria producing protease. About 08 bacterial cultures were isolated, purified and screened for the production of protease in 1% gram pulse, mung beans and 1% gelatin. Of all the cultures tested, bacterial isolate S-2 showed the maximum growth in agar plates containing 3% gelatin and was selected for further studies.

The partially amplified (500bp) and sequenced 16S rRNA gene from local isolate (S-2) was uploaded to the NCBI (National Center for Biotechnology Information) website to search for similarity to known DNA sequences and to confirm the species of the local isolate. The nucleotide sequences coding for the 16S rRNA gene after BLAST query revealed that this gene is 88% homologous to *Staphylococcus aureus* (S-2). The nucleotide sequences coding for the 16S rRNA gene of *Staphylococcus aureus* have been submitted to the GenBank database under accession number JF804983.

The bacterial isolate S-2 showed optimum growth at pH 7 (Fig. 1) and at 37°C (Fig. 2).

Effect of casein on bacterial growth

in Figure 3 shows growth behaviour of the isolate S-2 in four different media *viz.,* LB, salt medium supplemented with 1% hydrolyzed casein, 1% gelatin and 1% gram pulse. LB medium was taken as control for the bacterial growth. It is evident that casein supported the maximum growth of the isolate compared to other medium. The maximum cell density was obtained at 24 h of incubation at 37°C.

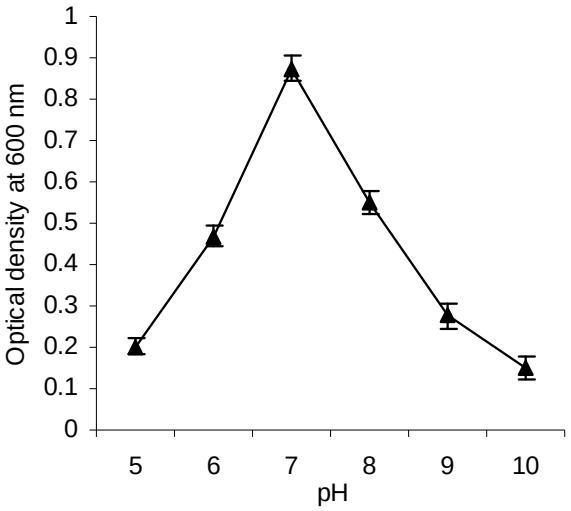

Fig. 1. Effect of pH on the growth of *S. aureus.*

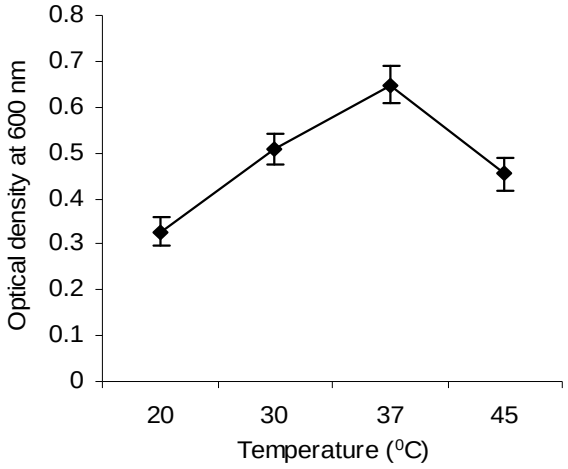

Fig. 2. Effect of temperature on the growth of *S. aureus*.

Protease is predominantly extracellular

The enzyme activity was checked both intracellularly and extracellularly by performing crude enzyme assay. The enzyme was found to be predominantly extracellular. The intracellular activity was insignificant (28.5%) when compared with the extracellular enzyme activity (360%) (Fig.4).

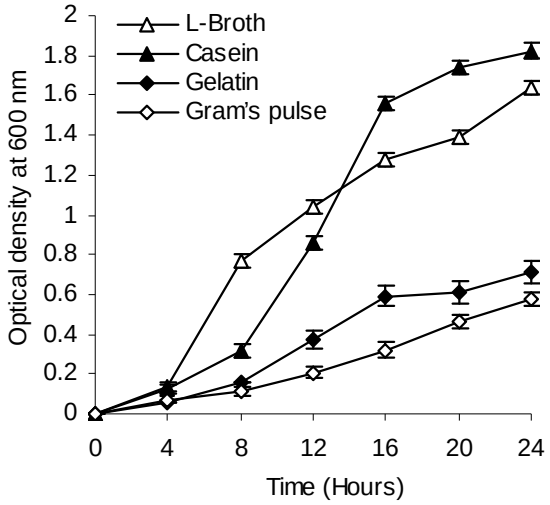

Fig. 3. Effect of different substrates on the growth of *S. aureus* S-2 over a period of 24 h of incubation at 37ºC.

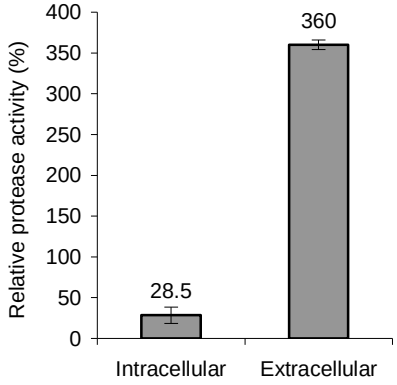

Fig. 4. Comparison of extracellular and intracellular activity of protease isolated from *S. aureus* S-2.

Optimization of pH and temperature for protease activity

Enzymes with high thermostability are more viable and have a better efficiency (De-Azeredo *et al*., 2004). Figure 5 shows the enzyme stability of the isolate which was checked with varying temperatures (30ºC, 40ºC, 50ºC, 70ºC, 90ºC) and at various pHs (5, 6, 7, 8, 9). *S. aureus* S-2 showed maximum protease activity at 50ºC (382%). Dodia *et al*. (2008) reported that crude enzyme was active at 25-80ºC and showed optimum activity at 50ºC. Maximum protease activity was observed at pH 8 (Fig. 6).

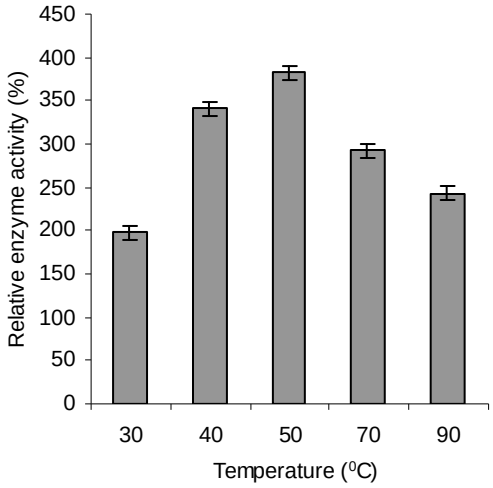

Fig. 5. Effect of temperatures on the enzyme activity of *S. aureus* S-2.

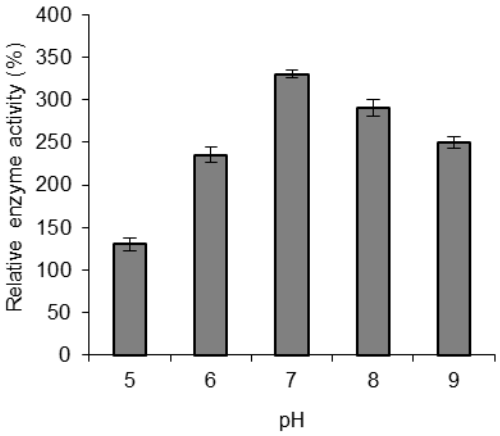

Fig. 6. Effect of pH on the protease activity of *S. aureus* S-2.

Alkalophilic proteases produced by *Pseudomonas aeruginosa* have also been reported by Ganesh Kumar *et al*. (2008). *S. aureus* S-2 also showed considerable protease activity at 90ºC (63%) but lower than the optimum temperature for enzyme activity (100%). It is a well known fact that at higher temperatures, protein conformation changes or degraded (Johnvesly and Naik, 2001).

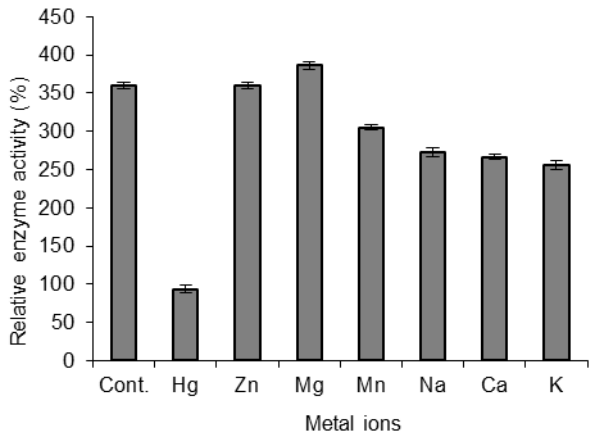

Fig. 7. Effect of metal ions on protease activity of *S. aureus* S-2.

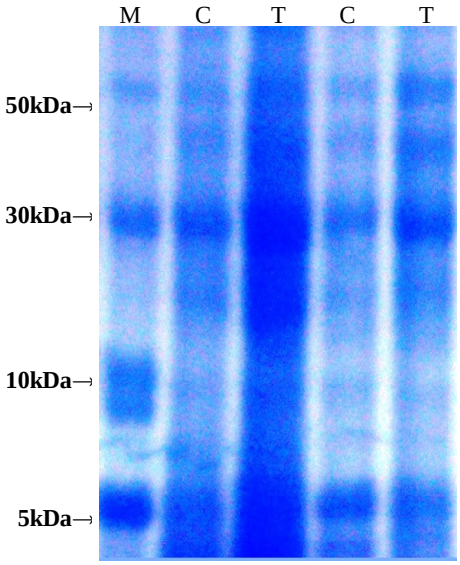

Fig. 8. SDS-PAGE pattern of extracellular ammonium sulfate precipitated proteins of *S. aureus* S-2. M, protein marker; T, treated (with 1.5% casein) and C, control (without casein). The gel is 12% stained with Coomassie blue.

Total protein profile

In this study, alkaline protease with molecular weight of 30 kDa was observed in SDS-PAGE pattern (Fig. 8). Gupta *et al*. (2005) reported the molecular mass of protease 29 kDa based on the SDS-PAGE. Abou-Elela *et al*. (2011) determined 31 kDa protease protein judged by SDS-PAGE from *B. cereus*. The molecular mass of protease, as revealed by SDS-PAGE was found to be 34 kDa as reported by Shivanand and Jayaraman (2011). Dodia *et al*. (2008) reported the molecular mass of protease 40 kDa based on SDS-PAGE.

CONCLUSIONS

In the present study we have isolated protease from the locally isolated *S. aureus* S-2 with maximum enzyme activity of 360 U/mL at optimal pH 7 and temperature of 37ºC. The isolate was grown in different media and the maximum growth was supported by 1% hydrolyzed casein. The enzyme was stable at 30-90ºC and pH of 5 to 9. The enzyme showed its maximum activity at 37ºC and pH 8. The relative molecular mass of the protease was found to be 30 kDa based on the SDS-PAGE. *S. aureus* S-2 can be exploited for biotechnological and industrial applications.

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