Pak. J. Bot. 33 (special issue) 2001

PRODUCTION OF THERMOSTABLE PROTEASES BY *STREPTOMYCES THERMOVIOLACEUS*

ALIYA SIDDIQUE, SAFIA AHMED AND ABDUL HAMEED

Microbiology Research Laboratory, Department of Biological Sciences, Quaid-i-Azam University, Islamabad.

Abstract

Extra cellular proteases produced by *Streptomyces thermoviolaceus* in shake flasks were optimized for carbon sources, pH and temperature. It became evident that glycerol was a better carbon source for the protease production giving 71 PU/ml compared to glucose showing only 36 PU/ml proteotytic activity. Optimum concentration of carbon sources in production medium was 1% w/v and optimum pH for maximum proteolytic enzyme production was found to be 6.0. Temperature optimum for enzyme production was 45 °C. Protease enzyme thus produced under optimum conditions was characterized, pH optimum for protease activity was shown to between pH 6.5 and 7.0 under optimum conditions of enzyme assay. Proteolytic enzyme of *S. thermoviolacenus* was stable from pH 4.0 to 10.0 and showed high stability at a wide range of temperature from 40 to 80 °C. Maximum stability was at 50°C, which was 97 %, and minimum was at 80°C retaining 78% of the original activity.

Introduction

The actinomycetes is a large group of Gram-positive eubacteria, which inhabit a wide range of environments. Some are pathogenic but the majority are saprophytes in soil, water, compost and other habitats where they play an important role in soil structure and composting (Williams and Vickers, 1988, Amner *et al*, 1988). They secrete many extra cellular enzymes which contribute to the breakdown of complex organic materials such as proteins, nucleic acids, polysaccharides and lignocelluloses found in soil (McCarthy *et at.*, 1985) and thus make the carbon available for growth of other organisms. *Streptomyces* secrete a variety of enzymes including proteases, cellulases, lipases, amylases, xylanases and other enzymes to solubilize the organic matter in their habitats. Extracellular proteases are probably the most widely spread of all microbial secreted enzymes (Priest, 1984).

Due to growth at higher temperature and thermal stability of their enzymes, thermophiles have a number of advantages over their mesophilic counterparts for applications in biotechnological processes. Some of the major advantages include enhanced rate of reaction in terms of secondary metabolic formation, reduced cooling costs in large-scale fermentations, increased solubility of reactants and decreased mesophilic contamination and reduced risks of pathogens because of their instability to survive at elevated temperatures. They also result in the production of thermo stable enzymes and proteins which are more resistant to the denaturation activities of organic solvents and detergents and enzyme isolation can be carried out at room temperature. Higher enzyme recoveries due to enhanced enzyme stability may be achieved at the end of the process.

The use of thermophilic organisms for industrial applications has increased significantly during the last two decades. Actinomycetes contain a large number of thermophilic species (Goodfellow and Cross 1984). The optimum temperature for growth of most the species ranges between 40 to 50°C. Proteases of industrial importance are mainly produced from microbial, animal and plant sources but the major portion comes from microbial origin and most of the enzymes are produced

by fermentation and are extra cellular. Present study was focused on to the production of protease enzyme from thermophilic actinomycete, *Streptomyces thermoviolaceus* using different carbon sources. The concentration of carbon sources, temperature and pH was optimized for maximum enzyme production. The enzyme was characterized for its stability at different pH values and temperature.

Material and methods

Organism and growth condition: *Streptomyces thermoviolacens* NCIB 10076 was used through out this study. Solid medium used for the growth and maintenance of the strain contained yeast extract, 40 g/l; malt extract, 10 g/l and glucose, 4 g/l. Agar 2% (w/v) was added as solidifying agent. Growth was at 50°C until the sporulation had occurred (3-4 days). Minimal liquid medium used for the production of enzymes contained g/l of $MgSO_4$. $7H_2O$, 6; $(NH_4)_2SO_4$, 2; Casein acid, 5; trace salt solution, 1 ml. Trace salt solution consisted of 1g/l of each of $ZnSO_4$.$7H_2O$, $FeSO_4$.$7H_2O$, $MnCl_2$. $4H_2O$, and $CaCl_2$ (anhydrous). This medium was dispensed in 40 ml aliquots in 250 ml flasks and at the time of inoculation 8 ml NaH_2PO_4/K_2HPO_4 (0.1 M, pH 6.8) and 1.25 ml carbon source (20% w/v) were added. Spore suspension was used as inoculum and growth was in shaking incubator at 50 °C at 100 rpm.

Analytical Methods

Measurement of biomass: Mycelial cells were centrifuged at 9000 rpm for 30 minutes at 4°C and filtered through pre-weighed filter discs. Filter paper was dried in incubator at 50°C for 24 hours and reweighed to get the dry cell mass.

Enzyme Assay: The enzyme assay for the determination of proteolytic activity with casein as substrate was measured according to the method of Kunitz (1965). Reaction mixture contained 1ml of 1% casein (pH 7.2), 1 ml of culture filtrate and 1 ml of KH_2PO_4/NaOH buffer (50 mM, pH 7.2). This mixture was incubated for 30 min at 40°C in a water bath. The enzyme reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid (TCA) then placed in the freezer for 10 min and then filtered. A control was run in identical manner except that 3 ml of TCA was added before incubation. All assays were made in triplicate and average of the three was taken to evaluate the activity units. The absorbance of TCA soluble fraction was read at λ 280 nm in UV- visible spectrophotometer. One unit of enzyme caseinolytic activity is defined as the amount of enzyme which releases 1 μg of tyrosine under the standard conditions (pH, 7.2, 40 ° C, 30 min).

Optimization of concentration of carbon source: Different concentrations (0.1%-1.5%) of the carbon sources, glycerol and glucose, were used in the minimal liquid media and inoculated with spores of *S. thermoviolaceus*. Incubated in incubator shaker at 50° C and 90-100 rpm, 10 ml of each sample was collected in sterilized test tubes after 24, 48 and 72 hours and then their dry weights and protease activity were determined by the methods described previously.

Optimization of pH: The pH of the minimal liquid medium in each flask containing 40 ml of medium was adjusted to values of 5, 6, 7, 8 and 9 by adding NaOH or HCI. Then buffer and carbon sources were also mixed in them. Organism was inoculated in production medium and shake flask fermentation was carried out for 72 hours. Samples were taken after 24, 48 and 72 hours and analyzed for dry weights and enzyme activity.

Optimization of Temperatures: Production of extra cellular protease was carried out at a range of temperature 30, 40, 50, 55 and 60 °C pH of the buffer was 6.8 and concentration of carbon sources was 0.5%. Production medium (minimal liquid medium) was incubated at different temperatures. Samples were collected and assayed for protease and dry cell weights were also estimated.

Enzyme Characterization: Production of proteases was carried out in 250 ml of production medium under optimum conditions for 24 hours. Culture supernatant was separated from mycelial cell mass by centrifugation and filtration. Culture supernatant was taken as a source of enzyme and it was then characterized for pH and temperature.

Determination of pH optimum for protease activity: Non-concentrated culture supernatants were subjected to assay in various pH buffers ranging from 4-10. The buffers used were, 50 mM sodium citrate-citric acid (for pH 4-5) and 50 mM Tris-HCl (for pH 6-10). pH of the casein was also adjusted to 4-10 by using NaOH or HCL. Enzyme assay was then carried out according to Kunitz method.

Stability of protease at different pH values: Enzyme in the broth (1ml) was mixed with buffers at different pH values (pH 4-10) and allowed to stay for one hour at room temperature. Caseinolytic activity was then measured after bringing each sample back to neutral pH.

Stability of protease at different temperatures: Protease in culture broth was heated at different temperatures (40-80 °C) for one hour. After the heat treatment, samples were cooled and protease assays were run to get the residual activity.

Results

Protease production by using different carbon sources: The results of the protease activity with different carbon sources show that in glucose medium, protease activity was nearly the same after 24 to 48 hours and similarly their dry cell weights were also equal while proteolytic activity reduced to half after 72 hours although dry cell mass remained almost the same (Table 1). In glycerol medium, activity of protease increased 3-4 fold after 48 hours and then decreased to half after 72 hours Dry cell weight analysis in this case showed that dry weight of the organism increased from 24 to 48 and to 72 hours. Proteolytic activity was higher in glycerol medium as compared to glucose medium (Table 1).

Optimization of concentration of carbon sources: When different concentrations (0.1, 0.25, 0.5, 1.0, 1.5%) of carbon sources were introduced as to determine the best concentration for the protease production by *Streptomyces themoviolaceus,* it

was observed that 1.0% glucose concentration showed best enzymatic activity (80 PU) after 24 hours of incubation at 50 °C. In glycerol as carbon source, highest level of activity was observed after 48 hours and maximum activity was observed in medium containing 1.0% glycerol. Dry weight of the residue varied from 0.30 to 0.54 mg/ml (Figure 1).

Optimization of pH: Protease production was carried out in different pH values. Figure 2 shows that maximum production of protease enzyme was at pH 6.0 in glucose medium after 24 hours of incubation. pH of the production medium (minimal liquid medium) was changed to 6.7 from pH 6.0 for both glucose and glycerol mineral salt medium.

Optimization of temperature for the production of proteolytic enzyme: Specfic proteolytic activity of enzyme produced showed that maximum activity was observed at temperature 40-50 °C and low levels of enzyme were achieved at 30, 55 and 60 °C (Figure 3).

Enzyme characterization: After optimizing conditions for the enzyme (protease) produced by *S. thermoviolaceus*, a batch culture experiment was carried out for protease production in 250 ml medium containing 1 % glycerol as carbon source, pH of the medium was adjusted to 6.5 and temperature of incubation was 45 °C. After 24 hours, the culture supernatants was separated from mycelial cells by centrifugation and filteration. This culture supernatant was taken as source of enzyme and the effect of pH and temperature on the dissolved enzyme was carried out.

Optimum pH for protease activity: Figure. 4 show that proteolytic activity was maximum at pH 6.5. Activity decreased at pH 4 to 6 and pH 7.5 to 10 so optimum pH for this enzyme is 6.5.

Stability of enzyme: Enzyme produced by *Steptomyces thermoviolaceus* was stable at pH 6.5 and &.7 retaining 96% and 84% residual activity respectively while only 25% activity was observed at pH 3.5-6.0 and also show low residual activity at pH 7.5-10 (Figure 5). Enzyme showed stability at wide range of temperature from 40-80 °C and 97% residual activity was retained after heating the enzyme at 50 °C while at 80 °C 78 % residual activity was recorded (Figure 6).

Discussion

Streptomyces like other Gram-positive bacteria secrete many enzymes in culture media including proteases. Present research was carried out to explore the maximum proteolytic activity under neutral conditions. The experimental evidence indicates that proteolytic activity was greatest in glycerol medium after 48 hours of incubation while dry cell weight was highest in the same medium after 72 hours, so it can be concluded that glycerol is better source than glucose for the production of protease. The effect of carbon source on a number of cultural parameters was investigated in *S.thermoviolaceus* by James and Edwards (1988). Different concentrations of carbon sources show that 1.0% glycerol medium was best for proteolytic activity after 48 hours of incubation of the production medium. Activity,

in this case and almost every other case, decreased as the biomass of the cells increased. It may be due to the growth conditions which when become most suitable for the proliferation of mycelial cells. The producing enzymes become less active at this stage. The pH optimization showed that proteolytic activity was highest in glycerol medium after 48 hours of incubation of the production medium at initial pH 6.0. Dahot (1987) investigated the effect of different parameter on the growth and synthesis of protease by *Penicillium expansum* The maximum production was achieved at 35 °C at initial pH 6.0 after 48 hours..

By optimizing temperature for protease production, it was found that it was active over a wide range of temperature, while maximum activity was found at 45 °C after 24 hours of incubation in both the carbon sources. As *S. thermoviolaceus* is a thermophilic strain, which is active at a temperature range of 27-58 °C so it produced thermostable protease at each temperature, which we checked for our experiments. Temperature shift experiments indicated that growth temperature played an important role in the production and characteristics of protease produced by *S. thermoviolaceus.* Growth at 38 °C resulted in the production of metallo-protease activity. When cultures were shifted to 50 °C, serine protease activity was also detected. The organism failed to sporulate on solid medium at 30 °C in contrast to growth at 50 °C where sporulation was profuse. This observation may provide evidence that the serine protease may be involved in sporulation. Support for the conclusion comes from the work on Ginther (1979) who reported that serine protease and cephamycin C synthesis in *Streptomyces lactamdurans* were associated with sporulation. Similar reports are also available on the role of serine protease in sporulation in *Bacillus subtilis* (Leighton *et al*; 1973). Production of secondary metabolites was investigated by James *et al*; (1991) in *S. thermoviolaceus* grown at 45 °C in a fermentor. Extracellular protease was secreted into the culture medium during the second lower phase of biphasic growth, which is moist apparent at 45 °C for this organism. The effect of different growth temperatures revealed that the synthesis of extra cellular protein like that of the antibiotic, was maximal in cultures grown between 37 °C and 45 °C whereas protease activity was greatest in 50 °C grown cultures. Study on the pH-stability of the enzyme indicated that the enzyme has broad pH stability ranging from pH 4.0 to 10.0. Hayashi et al; (1970) pointed out that the protease showed maximum thermal stability between 60 and 85 °C and between pH 7.5 and 9.5. Ross (1980) reported that serine alkaline protease has an optimum pH value between 7.0 and 8.5. It is stable between pH 4.5 and 9.0 and is rapidly destroyed at 60 °C. Activity of enzyme protease produced by *S. thermoviolaceus* was also stable from 40 to 80 °C. However, the azo caseinase activity showed higher thermostability at 50 °C in contrast to 40 °C suggesting the production of more thermostable proteins occurs at higher temperature. These observations are in accordance with the findings of Heinen and Lauwers (1983) who have shown that several enzymes from facultative thermophilic *Streptomyces sp.* were thermostable from cultures grown at 50 °C in contrast to those grown at 37 °C. These observations let to the conclusion that facultative thermophilic organisms can harbor two types of enzymes namely those with increasing thermo stability depending upon growth temperature and those, which remain thermolabile. Qualitatively and quantitatively different rations of thermophilic and mesophilic enzymes can be present in different organisms. Many other scientists describe that the proteases produced by thermophilic strains have been shown to be more

thermostable than their mesophilic counterparts (Iqbal, 1991, Mizusawa and Yoshida, 1972, Hussein *et at.*, 1979, Takami *et al.*, 1989). So we conclude that thermophilic microorganisms produce thermostable protease enzymes, which has a range of stability at pH and temperature. This will help the industrial microbiologist to produce enzyme at high temperature down stream processing will be easy.

Table 1: Protease activity with different carbon sources.

	GLUCOSE			GLYCEROL		
Time of Incubation	PU (ug/mg)	Dry cell Wt. (mg/ml)	PU/mg of Dry cell wt.	PU (ug/ml)	Dry cell wt (mg/ml)	PU/mg of dry Cell wt.
24	36	0.8	45	26	0.9	29
48	31	0.7	44	71	0.6	118
72	16	1.0	16	40	1.2	33

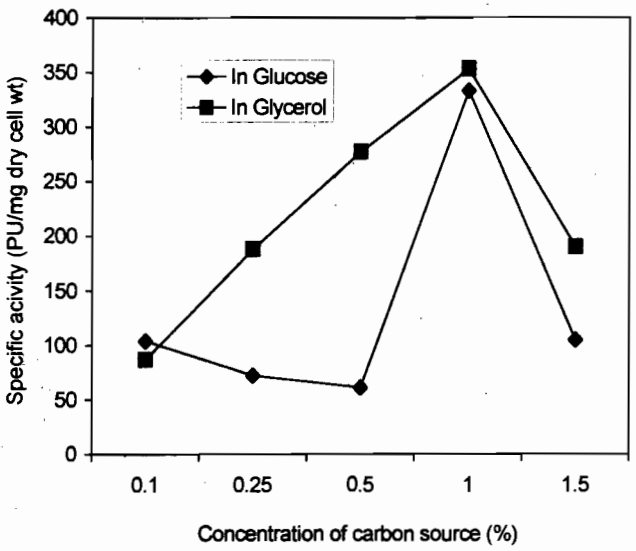

Figure1. Production of protease by *Streptomyces thermoviolaceus* at different concentrations of carbon sources.

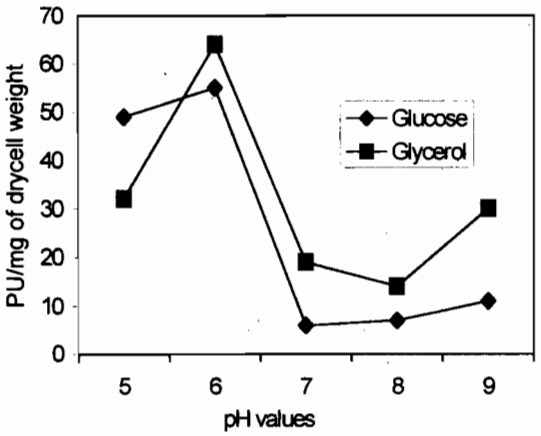

Figure 2. Effect of pH on the production of protease by *Streptomyces thermoviolaceus*.

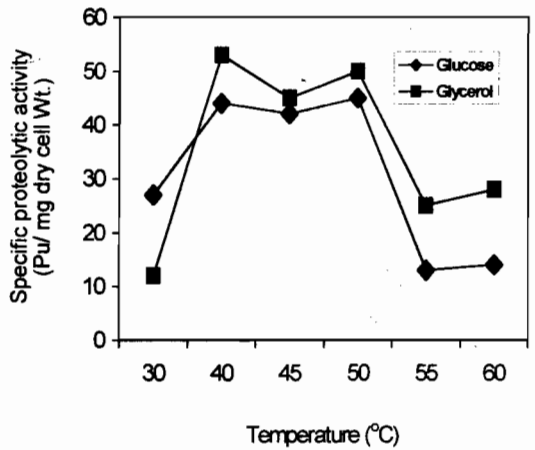

Figure 3. Effect of temperature on the production of protease enzyme by *Streptomyces thermoviolaceus*..

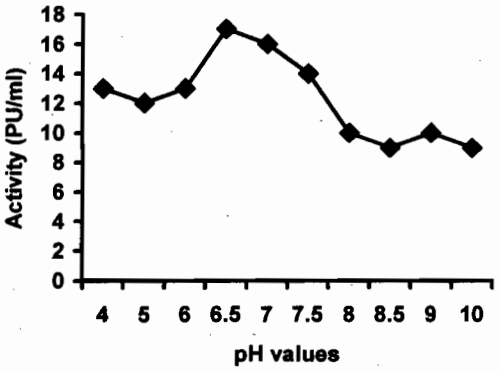

Figure 4. pH optimum for the protease activity

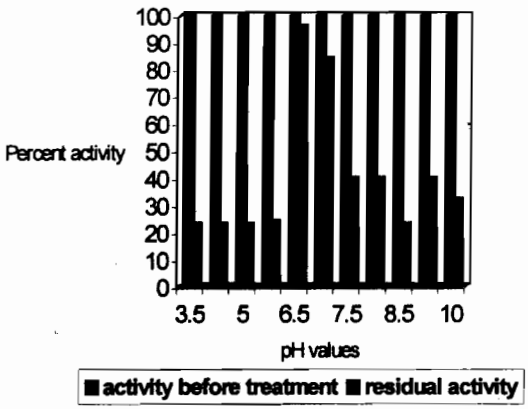

Figure 5. Stability of Protease at different pH.

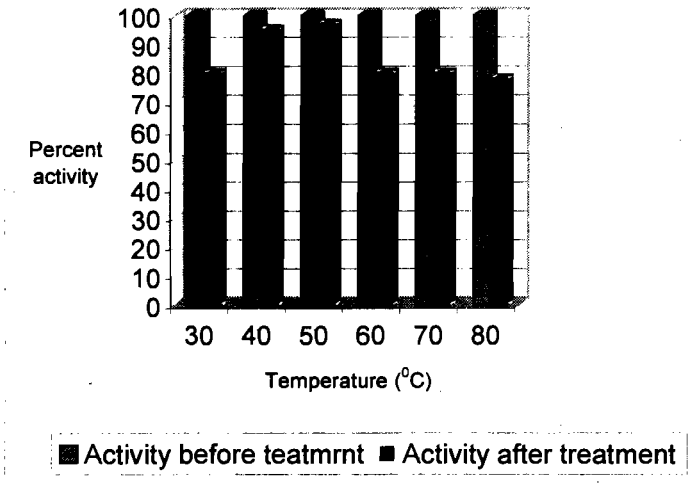

Figure 6. Stability of protease enzyme produced by *Streptomyces thermoviolaceus* at different temperatures.

Refernces

Amner, W;A.J. McCarthy, and C. Edwards. 1988. Quantitative assessment of factors affecting the recovery of indigenous and released thermophlic bacteria from compost. *Appl. Environ. Microbiol.*, 12:3107-3112.

Dahoat, M. U. 1987. Studies on proteolytic enzymes. *Pak, J. Sci, Ind, Res.*, 30: 194-196.

Demain, A. L. 1988. Actinomycetes: What have you done for us lately? In: *Biology of Actinomycetes "88"* (edited by Y. Okami, T. Beppe and H. Ogawara). Japan Scientific Societies Press. pp: 19-25.

Ginther, C. L. 1979. Sporulation and production of serine proteases and cephamycin C by *Streptomyces lactamdurans. Antimicrobial. Agents and Chemotherapy*, 15: 522-526.

Goodfellow, M. and T. Cross. 1984. Classification. In: *The Biology of the Actinomycetes*, (edited by M.Goodfellow, M. Madaski and S.T. Willimas). London, Academic Press. pp: 7-164.

Hayashi, K;M. Terada, and K. Mazi. 1970. Enzymatic properties of purified alkaline proteinase from *Aspergillus sojae. Agric. Biol Chem.*, 34: 627-637.

Hinen, W. and A. M. Lauwers. 1983. Changes in enzyme stability and fatty acid composition of Streptomyces sp: a facultative thermophilic actinomycete. *Arch. Microbiol.*, 134: 247-250.

Hussein, A. M; A.M. Ragab, and M.K.A. El-Fattah.1979. Thermostable proteases produced by a new thermophilic *Streptomyces sp. Egypt. J. Physiol.*, 6:161-174.

James, P.D.A. and C. Edwards. 1988. The effect of cultural Conditions on growth and secondary metabolism in *S. thermoviolaceus. FEMS Microbiol. Lett.*, 5: 1-6.

James, P.D.A; M. Iqbal, C. Edwards, and P.G.G. Miller. 1991. Extracellular protease activity in antibiotic. producing actinomycetes. *Curr. Microbiol.*, 22: 377-382.

Kunitz, N. 1965. *Methods of enzymatic analysis* (2nd ed.). Verlag chemic-Academic Press N.Y. London 6: 807-814.

Leighton, T. J; R. H. Dar, R. A. J. Warren.and R. A. Kelln. 1973. The relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus subtilis. J. Mol. Biol.*, 76: 103-122.

McCarthy, A.J; E. Peace, and P. Broda. 1985. Studies on the extracellular xylanase activity of some thermophilic actinomycets. *Appl. Microbiol. Biotechnol.*, 21:238-244.

Mizusawa, K. and F. Yoshida. 1972. Thermophilic *Streptomyces* alkaline proteinase: I. Isolation, crystallization and physiochemical properties. *J. Biol. Chem.*, 247: 6978-6984.

Priest, F.G. 1984. Extracellular Enzymes. *Aspects of Microbiology* . Van Nostrand Reingold (UK).

Ross, A. H. 1980. Microbiol enzymes and Bioconversions. *J. Gen. Microbiol.*, 5: 93-109.

Takami, H; T. Akiba, and K. Horikoshi. 1989. Production of extremely thermostable alkaline protease from *Bacillus sp.* No.AH- 101. *Appl. Microbiol. Biotechnol.*, 30:120-124.

Williams, S.T. and C.J. Vickers. 1988. Detection of actinomycetes in the natural environment problems and perspectives. In *Biology of Atinomycetes "88"* (edited by Y. Okami, T. Beppu and H. Ogawara). Japan Scientific Societies Press. PP: 265-270.