PRODUCTION, PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR PROTEASE FROM Streptomyces thermovulgaris.

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ABSTRACT

Extracellular protease was produced from thermophilic *Streptomyces thermovulgaris* (93-177) at 50°C, firstly in a shake-flask culture and subsequently in a fermenter. Appreciable protease activity assayed by the azocasein protease test was obtained after 96h of cultivation, when growth of the organism had stopped at an alkaline pH of 7.5- 8.0. Purification of the protease was by ammonium sulphate fractionation, ion exchange chromatography using DEAE-cellulose 52, gel filtration using sephacryl S-400 column and finally by hydrophobic interaction chromatography using phenyl sepharose CL-4B column. It had a molecular weight of 30KDa as determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS/PAGE). Maximum proteolytic activity was observed at 70°C in a 30min incubation assay with 1mM $CaCl_2$ present and there was broad optimum activity at pH 7.5-10.5. No loss of activity was observed after 24h at 50°C and the half-life at 75°C was 1h. The protease was inactivated by phenyl methyl sulfonyl fluoride (PMSF) but not by ethylene diamine tetra-acetic acid (EDTA), indicating it could be a serine-type protease and not a metallo-enzyme. There was hydrolytic activity of the protease towards the ester compound N-benzoyl-L-tyrosine ethyl ester (BTEE) but not towards N-benzoyl-L-arginine-P-nitroanilide (BAPNA). The results show that the enzyme is heat stable, and an alkaline protease with chymotrypsin-like nature.

Keywords: *Streptomyces thermovulgaris*, extracellular protease, azocasein activity

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INTRODUCTION

Proteases are degradative enzymes which catalyze the total hydrolysis of proteins. They are very important enzymes employed in various biotechnological processes like food and detergent industries as well as in medicine. Their involvement in the life cycle of disease-causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS. Their application also in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals (Rao *et al.*, 1998) is a relatively new development and has conferred added biotechnological importance. Sources of proteases include plant, animals and microorganism among which are the *Streptomyces.*

Streptomyces have been widely used for antibiotic production as well as an enormous variety of other natural products (Williams *et al*, 1983; James *et al*. 1991). The genus *Streptomyces* is well known for the production of multiple proteases (Chandrasekaran and Dhar, 1987). Mesophilic

strains such as *S. coelicolor* and *S. lividans* have been particularly well investigated and have been used to develop a variety of genetic exchange systems (Hopwood 1981; Lampel *et al*; 1992). Other strains, which have been studied to a certain extent with respect to their proteolytic activity include *Streptomyces moderatus* (Chandrasekaran and Dhar, 1983), *S. peucetius* (Gibb and Strohl, 1988), *S. rimosus* (Renko *et al*, 1989), *S. aurofaciens*, (Laluce and Molinari, 1979), *S. ambofaciens* (Benslimane *et al*. 1995). Interest has focused on thermophilic bacteria because of advantages of faster growth rates for cost saving in industrial processes (Wiegel and Ljungdahl, 1986). However, of the known facultatively themophilic streptomyces, little work has been reported concerning their physiology, biochemistry, growth kinetics or molecular biology. Although, there have been reports on the production of secondary metabolites such as antibiotics and various proteolytic enzymes from thermotolerant streptomycetes (James and Edward, 1988; Jame et al; 1991), there is yet no report in the proteolytic activity of the thermophilic S. thermovulgaris which unlike most thermophilic organisms has relatively simple cultivation with high yields.

In this work, the extracellular protease of S. thermovulgaris was isolated, purified and characterized.

Materials and methods

Materials

Azocasein sodium salt (Fluka), Tris (Merck), D-Glucose (Merck), Gelatin, Nutrient buth (Difco), Yeast extract (ICN Biomedical), malt extract (ICN Biomedical), Agar (Fluka), N-Benzoyl-L-tyrosine ethyl ester (BTEE) substrate (Serva), EDTA (Roth), Triton X-100 (ICN Biomedical), BSA (Boehringer Mannheim GmbH, Germany) and other chemicals of analytical grade were used.

Organism and culture conditions

Streptomyces thermovulgaris (93-177) with optimal growth temperature at 50°C (Inyang *et al*, 1995) was used for the study. The pre-inoculum and the inoculum were prepared as follows: Spores from 72h yeast malt extract agar (Garg *et al*. 1996) cultures were grown in the production medium NYG (Ginther, 1979) with a slight modification as indicated below. The composition of the yeast malt extract agar per litre is as follows: malt extract 10g, yeast extract 4g, D-glucose 4g, agar 15g, pH 7.2. The NYG medium consists of 8g/l nutrient broth, 2g/l yeast extract, 10g/l glucose, pH 7.0. There was addition of 0.1% gelatin (w/v) to induce protease production and this modified medium was called NYGG and was used throughout the experiment as the production medium. The glucose solution was separately sterilized and mixed with the remaining solution after sterilization. The strains were pre-grown in 20ml of the above NYGG medium contained in 100ml, four baffle Erlenmeyer flasks incubated at 50°C for 48h at 200 rpm. A 1ml amount was then used to inoculate an identical fresh NYGG medium flask, and incubation continued for 24h. A 10ml amount of the second seed culture was then used to inoculate 200ml of the production medium earlier described in a 2-liter, four-baffle Erlenmeyer flask in the shake flask method used for the preliminary determinations.

Preliminary protease production using shake flask method.

Incubation of the shake flask cultures were at 50°C at 200 rpm. The growth and protease production were followed for 6 days. Turbidity was used as a measurement for the growth of the

organism and was determined spectrophotometrically by removing 10ml samples at various times during the fermentation. These were centrifuged at 8,000-x g for 20min at 4°C. The resulting pellets were suspended to 10ml in 0.2M Tris/HCl pH 7.2 and absorbance at 650nm carried out with an Uvicon 930 spectrophometer (Kontron Instruments). The supernatant from the centrifuged samples were used for the assay of extracellular protease and the pH of the spent medium recorded.

Fermenter Studies.

Subsequent production of the protease was done using a fermenter with control pH device for determination of effect of pH on the extracellular protease production. The organism was grown and maintained as described for the shake-flask cultures, at 50°C for 96h, but in this case, 100ml of the seed culture was used as the inoculum. Fermentations were carried out in a 3l KLF 2000 bioengineering fermenter with a 2l working volume of a constant temperature of 50°C. Agitation was at 500 rpm and aeration at 1 $lmin^{-1}$. The pH was maintained at the desired value ±0.1 by addition of 3N sulfuric acid and 3N NaOH with a feedback loopset.

Protease assay

Protease activity was determined employing the simple azocasein assay technique of Peek *et al.* (1992) in which the test mixture (1ml) contained 0.2% azocasein (w/v), 1mM $CaCl_2$ and 0.2ml culture filtrate in 0.2M Tris/HCl, pH 7.5. The samples were incubated at 50°C for 30 min, and the reaction was terminated by the addition of 1ml of 10% trichloroacetic acid. The samples were allowed to stand for 10min and then centrifuged at 3000 x g for 5min and the absorbance of the supernatant read at 350nm. One unit of enzyme activity was defined as a change of 1.0 in absorbance at 350nm in 1h.

Protein content

This was estimated by the method of Bradford (1976) using Bovine Serum Albumin (BSA) as a standard.

Purification of the protease

The 96h supernatant (1000ml) was treated to 80% saturation with $(NH_4)_2\ SO_4$ in a stepwise manner. The precipitates were redissolved in the eluting buffer Tris-HCl, pH 7.5, containing 1mM $CaCl_2$. This was centrifuged and dialyzed against the same buffer for 24h with 5 changes before applying onto a DEAE-Cellulose 52 (2x20cm) column. After equilibration with the same buffer, the enzyme was eluted using 0 - 0.5 M NaCl gradient at a flow rate of 1.5 ml min^{-1}. The fraction with high protease activity was pooled and passed through sephacryl S-400 (2X70cm) column and eluted with the same buffer. The eluate was brought to 1M concentration with $(NH_4)_2\ SO_4$ before loading onto phenyl sepharose CL-4B (1x12cm) column, equilibrated with Tris-HCl buffer pH 7.5 containing 1M $(NH_4)_2SO_4$. This was eluted with 1.0 - 0M $(NH_4)_2SO_4$ gradient in the eluting buffer at a flow rate of 1 ml min^{-1}. The fraction collected was concentrated by diafiltration using Amicon cell. This pure enzyme was stored in the frozen state at –20°C until used.

Molecular weight determination.

SDS-PAGE method was used. The purified enzyme protein was treated with 1% SDS and 1% 2-mercaptoethanol and subjected to electrophoresis in 10% gel as described by Weber and Osborn (1969).

RESULTS

Production rate of extracellular protease of *S. thermovulgaris* in shake-flasks.

Appreciable protease activity was obtained after 96h of cultivation of *S. thermovulgaris, which* coincided with the period when growth had stopped (Fig 1).

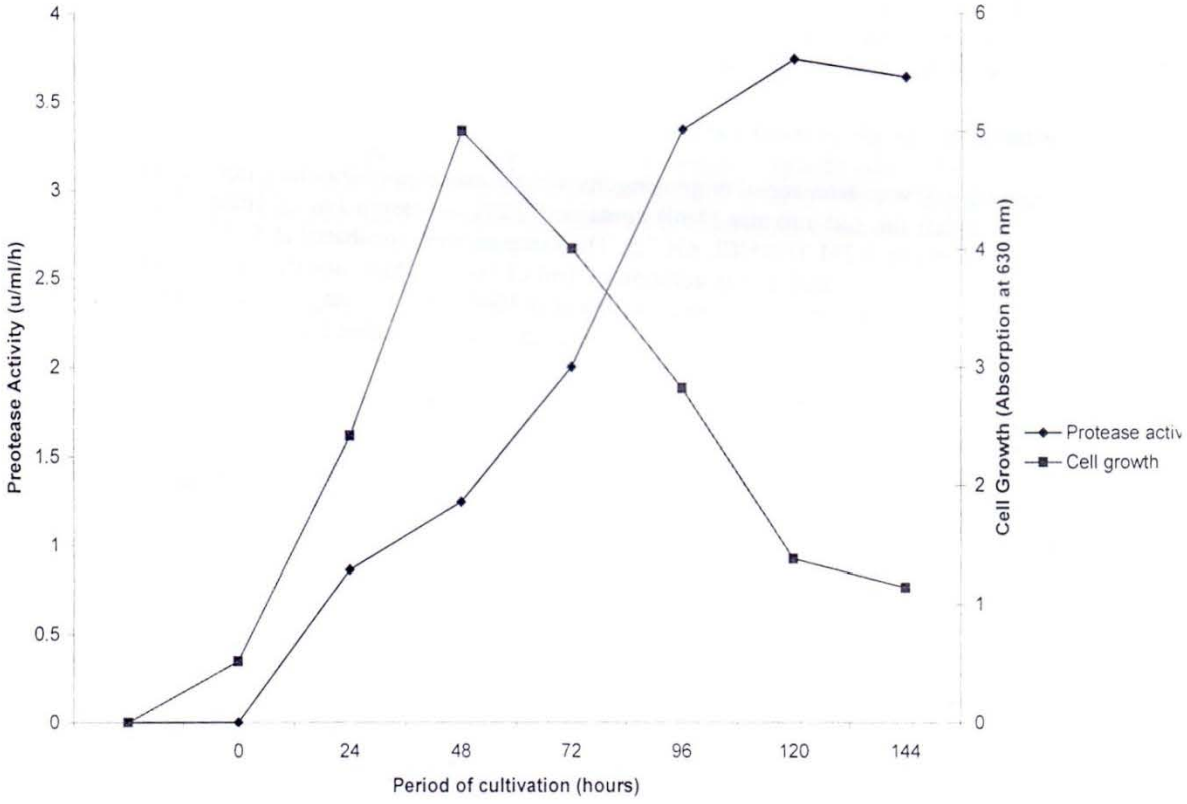

Figure 1: Production of Protease from *S. thermovulgaris* using shake-flask method at 50°C

(—■— = Turbidity as measurement of growth was determined as a change in absorbance at 650nm) (—◆— = extracellular protease production (U/ml/h) samples were assayed for protease activity at pH7.5 using 0.2% azocasein (w/v) as substrate. One unit activity was defined as a change of 1.0 in absorbance at 350nm in 1h.

Under the growth conditions with 0.1% gelatin (w/v) in the medium, *S. thermovulgaris* actively grew until 48h. Afterwards, growth decreased steadily and reached a minimum at 144 h.

Effect of pH on production of the protease by *S. thermovulgaris*.

The results of the effect of pH on production of extracellular protease by *S. thermovulgaris* at various pH values between pH 6.0 to 9.0 are shown in Fig 2.

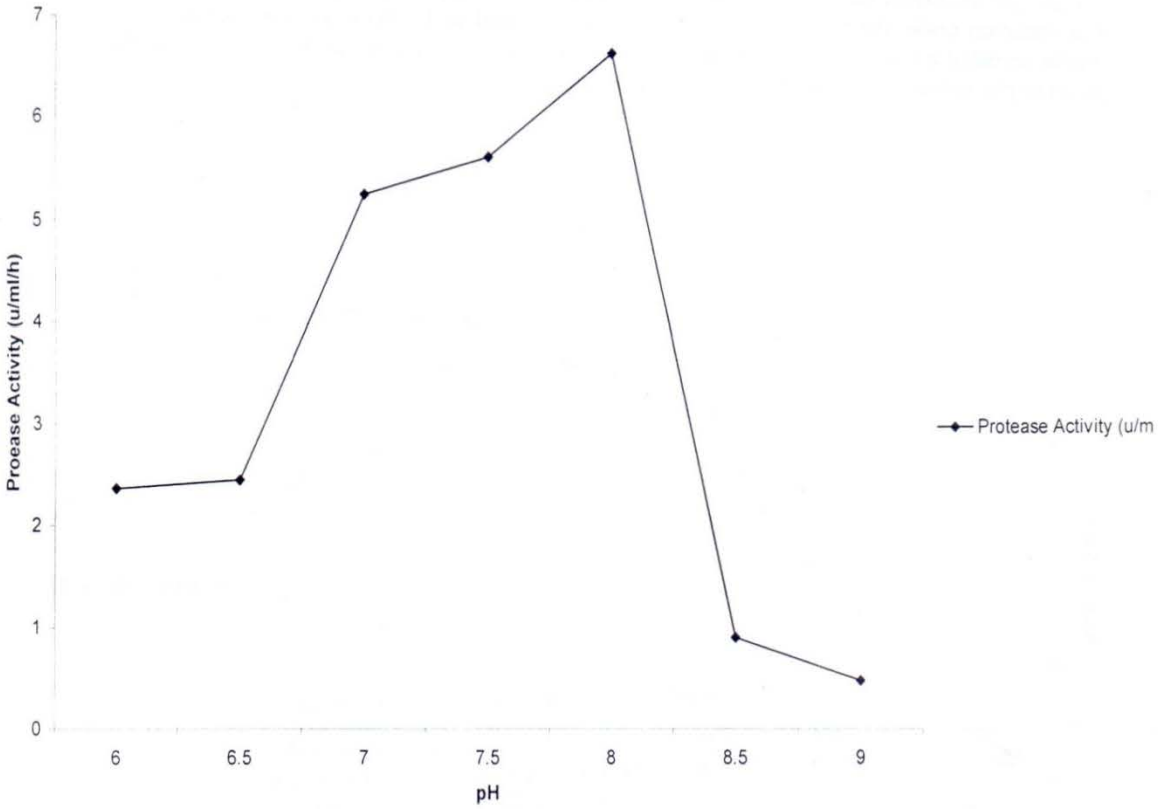

Figure 2: Effect of pH on production of the protease of *S. thermovulgaris* at 50° C for 96 h

Samples were assayed for protease activity at pH 7.5 using 0.2 % azocasein (w/v) as substrate. One unit activity defined as a change of 1.0 in absorbance at 350nm in 1h.

The optimal production of the enzyme was therefore between pH 7.5 and 8.0 after 96h of fermentation.

Determination of optimum pH of protease activity.

Theorell –Stenhagen universal buffer of pH 4 – pH 12 was prepared and 5.6µg of the pure enzyme pre-incubated for 30min at room temperature for each pH value prior to protease activity determination under the test conditions previously described under materials and methods. The results revealed a broad optimal alkaline range of pH 7.5 – 10.5 for the protease activity in the pure sample as measured with the azocasein assay (Fig 3).

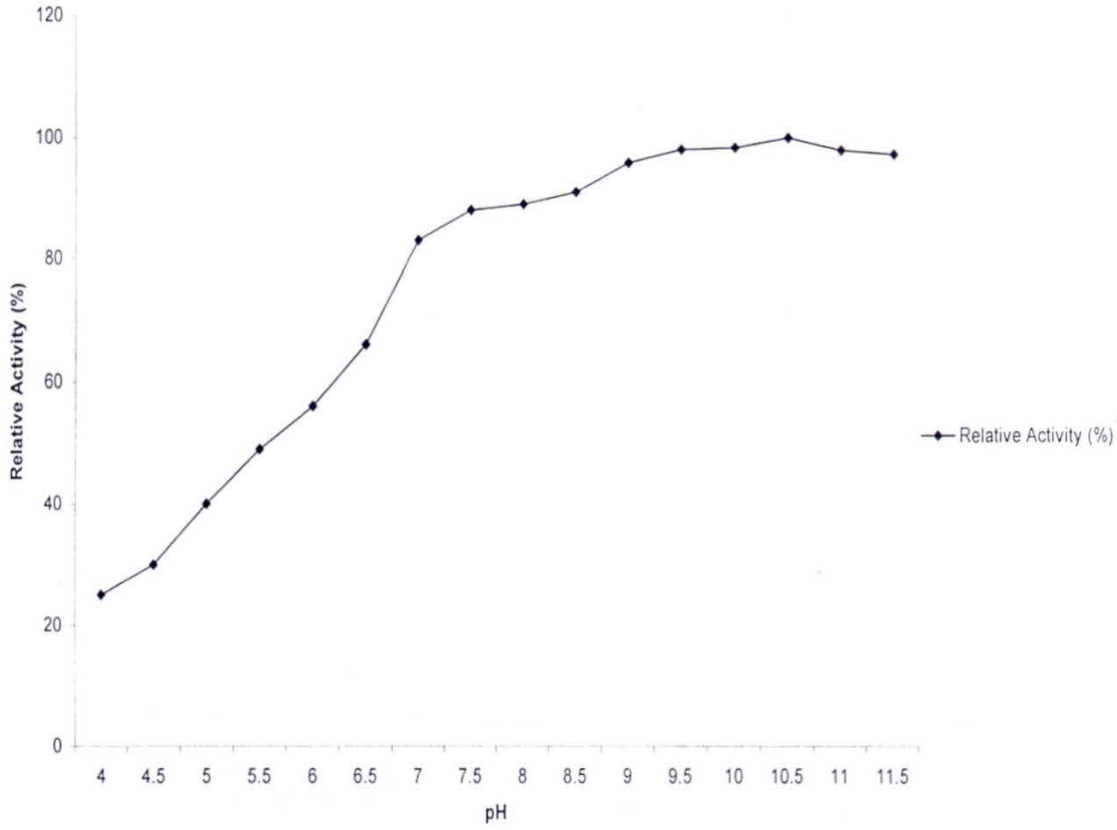

Figure 3: Effect of pH on Proteolytic activity of S. thermovulgaris

The extracellular protease activity of the pure enzyme pre-incubated at the desired pH values for 30min was assayed using 0.2% azocasein (w/v) as substrate, with one unit activity being defined as a change of 1.0 in absorbance at 350nm in 1h.

Also, when this pure form of *S. thermovulgaris* protease was separately incubated at two chosen pH values of 8.5 and 10.2 at room temperature for a period of 2-3h, prior to protease

determination in order to assess its stability at those values, there was 100% retention of activity in both cases.

Effect of temperature on the proteolytic activity.

5.6µg of the purified protease was incubated in the assay mixture using azocasein as substrate for 30min at several temperatures under the same conditions as described under material and methods. The temperature at which the maximal enzyme activity was obtained was 70°C (Fig 4), and

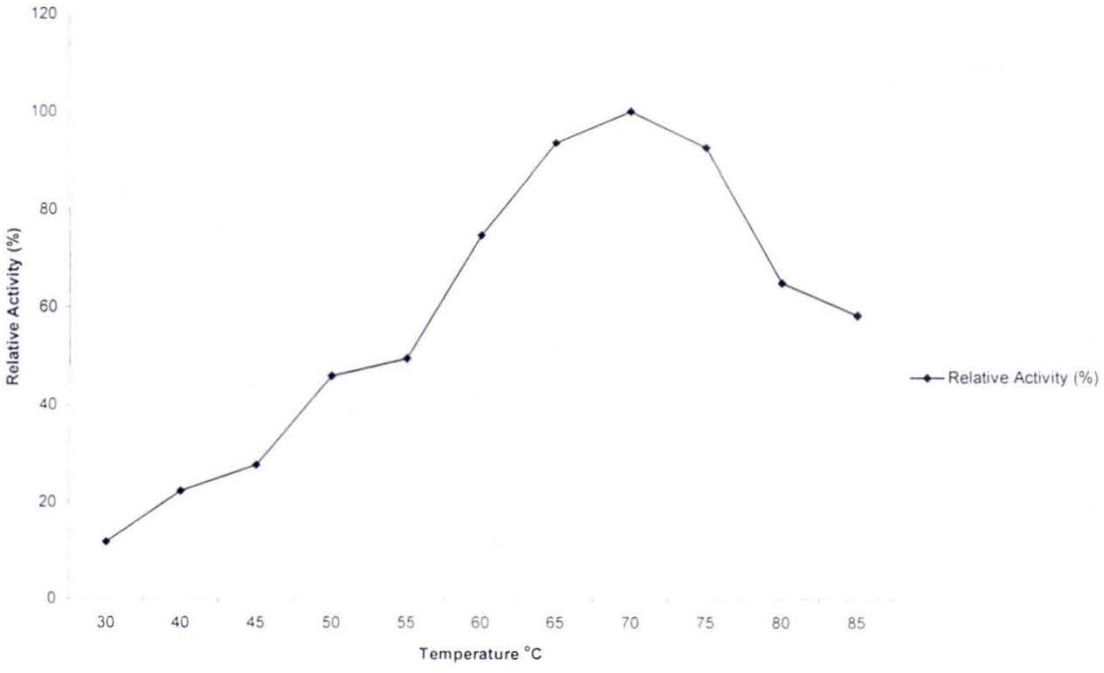

Figure 4: Effect of Temperature on Protease Activity of *S. thermovulgaris*

5.6µg pure preparation of the protease was incubated in the assay mixture using azocasein as substrate for 30 min at the various temperatures investigated and activity determined as described in the text.

this was 2.3 times higher than at 50°C. At 85°C, the activity measured decreased to 64.7% of the maximal activity while it was only 4.5% of the maximal activity at 40°C.

The thermostability of the pure enzyme was investigated by incubating at several temperatures for 1h prior using azocasein assay. There was 50% loss of the activity after 1h at 75°C, and there was little or no activity retained at 90°C after 1h (Fig 5).

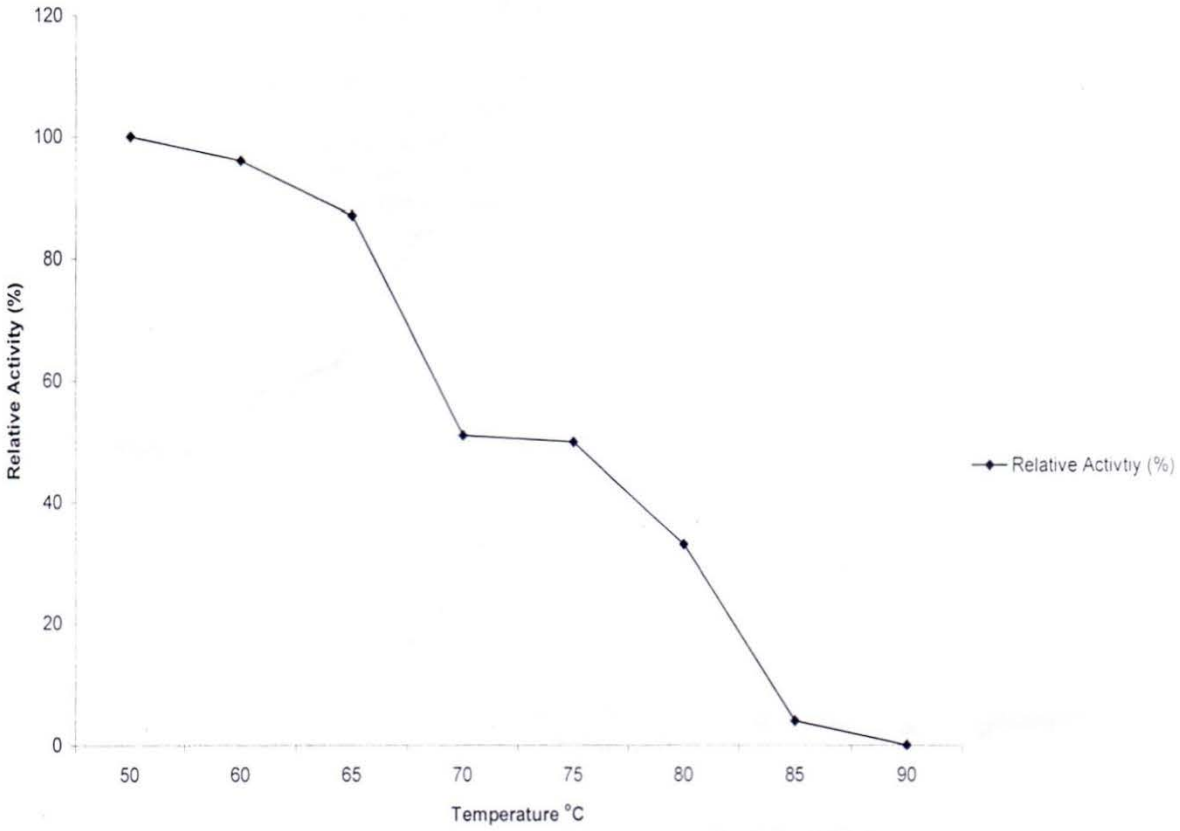

Figure 5: Thermostability study of protease of *S. thermovulgaris* after incubation

The enzyme was incubated at several temperatures for 1h prior to azocasein asay as earlier described in text.

There was 100% retention of the proteolytic activity for 24h at 50°C, but 70% retention of activity 7 days

Effect of inhibitors

The pure enzyme preparation was pre-incubated at room temperature in 1mM PMSF and 10mM EDTA, for one hour. Protease activity was measured using azocasein as substrate after 30min incubation at 50°C in the test and control samples. The activity of the control was taken as 100%. There was about 50% inhibition of activity in the PMSF treated enzyme, but no inhibition in the EDTA treated enzyme.

Hydrolysis of various substrates

The extracellular protease of *S. thermovulgaris* did no hydrolyze N - benzoyl-DL-arginine – p-nitroanilide (BAPNA) but hydrolyzed N -benzoyl-L-tyrosine ethyl ester (BTEE). The enzyme therefore exhibits a chymotrypsin-like nature.

DISCUSSION

Extracellular protease was isolated from *S. thermovulgaris* 93-77, purified and some of its properties determined. The protease accumulated in the medium after 96h cultivation (Fig 1), which is not strange as there are various reports on several *Streptomyces* species showing maximum proteolytic activity at different periods of the fermentation (Chandrasekaran and Dhar 1983, Gibb and Strohl 1988). This accumulation of extracellular protease from *S. thermovulgaris* occurred when growth of the organism had stopped (Fig 1). Gusek *et al.* (1988), generally observed that the extracellular enzymes in actinomyces are produced during the stationary phase of growth, while James *et al.* (1991) has reported protease production in actively growing cultures. There was observed variations in the pH of spent medium in the shake flask method (data not shown) which is in line with report of James and Edward (1988) who observed similar variations in pH of production medium with *S. thermoviolaceus*. Nevertheless, these common variations in pH are usually taken care of by either buffering the production medium or employing a fermenter with pH control. The use of a fermenter with pH control was adopted in this study.The observation that the proteolytic activity was not lost at all at 50°C for 24h, but 50% loss at 75°C for 1h and little or no activity retained at 90°C after 1h may be due to the possibility of denaturation followed by loss of activity. Investigations on optimal pH for protease activity of the pure protease of *S. thermovulgaris* showed that, although the production of the protease of this organism was optimal between pH 7.5 and 8.0, a broader optimal alkaline range of pH 7.5 – 10.5 was recorded for its activity in the pure form (Fig 3). This is similar to the report of Matsue *et al.* (1982) in which the extracellular proteases from *S. rectus*, also a soil microorganism showed optimal activity at pH 10.7. However, a report of a protease from *S. caespitosus* with optimum pH of activity of 6.0, and with 50% of activity still present at pH 4.5 is available (Kurisu *et al.* 1997). The 100% retention in activity of the protease from *S. thermovulgaris* pre-incubated for a period of 2-3 h at pH 8.5 and pH 10.5 is a measure of stability of this protease under investigation. This suggests that the extracellular enzyme is an alkaline protease, a property which was exploited in its purification. This result is close to the report of the extracellular thermostable alkaline protease of a *Thermus* sp, with maximum protease activity against azocasein at pH 8, and with 95% or more of this activity retained over the range of pH 7.5 – 9.3 and 50% or more in the range of pH 6.0 – 10.4 (Peek *et al.* 1992).
The results of purification are summarized in Table 1.

Table 1. Summary of Purification of extracellular protease of *S. thermovulgaris.*

Purification step	Total activity (U/h)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture Supernatant	5520	423	13.1	1	100
$(NH_4)_2SO_4$ precipitation dialysate	2080	74.6	27.9	2.1	37.7
DEAE-Cellulose 52	901.8	14.72	61.3	4.7	16.3
Sephacryl S-400	556.7	2.09	266.4	20.3	10.1
Phenyl Sepharose CL-4B	405	1.35	300	22.9	7.3

Ammonium sulphate precipitation as well as anion exchange, gel filtration and hydrophobic interaction chromatography were employed in the purification. There was observed poor sedimentation of the ammonium sulphate precipitates which resulted in low yield with 63% losses being typical at this stage. Similar losses have also been reported at this stage by other workers (Peek *et al.* 1992). The specific activities increased about 23 fold with 7% total yield. The purified enzyme was homogenous with a single band on SDS –PAGE with a molecular mass of 30KDa. The significant inhibition of proteolytic activity by PMSF indicates that the protease is a serine protease and not a metallo-proteinase.

Further work is on the way towards immobilizing this protease as well as characterizing it. To our knowledge, this report is the first report on the ability of *S. thermovulgaris* to produce extracellular alkaline proteases.

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