African Journal of Biotechnology Vol. 7 (18), pp. 3225-3230, 17 September, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Optimization of protease production by an actinomycete Strain, PS-18A isolated from an estuarine shrimp pond

G. Vonothini[1], M. Murugan[2], K. Sivakumar[2] and S. Sudha[1]

[1]Dhanalakshmi Srinivasan College of Arts and Science for Women, Perambalur – 621 212, India.
[2]CAS in Marine Biology, Annamalai University, Parangipettai – 608 502, Tamilnadu, India.

Accepted 15 September, 2008

Actinomycetes were isolated from the sediment samples of an estuarine shrimp pond located along the south east coast of India. During the investigation, a total of 28 strains of actinomycetes were isolated and examined for their protease activity. Among them, one strain PS-18A which was tentatively identified as *Streptomyces roseiscleroticus* showed higher protease activity with lower biomass and it was taken for further study. Impact of various physical and chemical factors such as pH, temperatures, sodium chloride concentration, and carbon compounds and amino acids on the growth of *S. roseiscleroticus* and its protease activity was studied. At pH 7, temperature 40ºC and 3% NaCl concentration, carbon compound such as sucrose and L-glutamine as amino acids, the enzymatic activity was maximum. The present study has thus indicated the scope for the use of *S. roseiscleroticus* as an ideal organism for the industrial production of the extracellular protease enzyme.

Key words: Shrimp pond sediment, protease, ***Streptomyces roseiscleroticus***.

INTRODUCTION

Marine microorganisms have recently emerged as rich sources for the isolation of industrial enzymes (Adinarayana and Ellaiah, 2002). The ability to produce a variety of proteolytic enzymes is a well-known phenomenon in actinomycetes from terrestrial sources (Weyland, 1969). They are also applied in laboratory practices in the structural determination of protein composed of macromolecules or in removing proteinaceous material during purification of certain bio-preparation. An increasing interest is also observed in the application of actinomyecete proteases in the bio-organic chemistry. Like most other microbial proteases, those from actinomycetes are excreted in the logarithmic-phase of growth. For commercial purposes they are routinely obtained as by-products formed during biosynthesis of antibiotics (Pokorny and Vitale, 1980). Some proteases function as digestive enzymes. They usually occur extracellularly in the gastrointestinal tract, but some animals secrete proteases outside their bodies. Intracellularly occurring proteases called cathespins have been studied relatively lesser.

*Corresponding author. E-mail: murugangowri@gmail.com.

MATERIALS AND METHODS

Isolation of actinomycetes

The sediment sample was collected from the shrimp pond located opposite to the Vellar estuary, southeast coast of India by inserting a sterilized polyvinyl corer (10 cm) into the sediments. The centre portion of the 2 cm sediment sample was taken out with the help of a sterile spatula. The collected sample was transferred to a sterile polythene bag and taken immediately to the laboratory. After arrival at the laboratory, the sample was air-dried aseptically for one week. An air-dried sediment sample was incubated at 55°C for 5 min (Balagurunathan, 1992). Then, 10-fold serial dilutions of the sediment samples were prepared, using filtered and sterilized 50% seawater. Serially diluted samples were plated in the Actinomycetes Isolation Agar Medium in duplicate Petri plates. To minimize bacterial and fungal contamination, all agar plates were supplemented with 20 mg/l of nystatin and cycloheximide (100 mg/l), respectively (Kathiresan et al., (2005). The actinomycete colonies that appeared on the Petri plates were counted from 5th day onwards, up to 28th

day. All the colonies that were growing on the Petri plates were separately streaked in Petri plates, subcultured, ensured for their axenicity and maintained in slants.

Preparation of protease enzyme

Actinomycetes isolated from the shrimp pond sediments were screened for protease activity. The packed cells were suspended in distilled water and this was inoculated into 5 ml of gelatin broth and incubated for seven days at 37ºC temperature (Bhushan and Hoondal, 1999). After the 7th day, the broth was centrifuged at 10,000 rpm for 15 min and cell free supernatant was used for protease assay.

Assay for protease activity

Five hundred µl of 0.5% casein in Tris –HCl buffer with 100 µl of enzyme solution kept for 60 min. at 370°C. Reaction was stopped by adding 500 µl of 15% trichloroacetic acid solution with shaking. After 15 min, the mixture was centrifuged at 3000 rpm for 5 min at 40°C. One ml of supernatant was added with one ml of 1 M NaoH and the absorbance was read at 440 nm. The enzyme activity was calculated from standard curve of L-tyrosine. One unit of enzyme activity is equivalent to 1 µg of L-tyrosine min^{-1} ml^{-1} under the assay conditions. Protein content of the enzyme preparation was estimated following the method of Lowry et al. (1951).

Optimization of various parameters for protease enzyme production

Factors such as temperature, pH, sodium chloride concentration, different carbon sources, nitrogen sources and aminoacids which influence the secretion of protease enzyme by actinomycetes were optimized for maximum production. Experiments were conducted in Erlenmeyer flasks (250 ml) containing gelatin broth. After sterilization of the broth by autoclaving at 121°C under 15 lbs pressure for 15 min, the flasks were cooled and the actinomycetes strain was inoculated and incubated for 7 days separately as described below. Experiments were conducted by taking one parameter at one time and the parameter standardized in one experiment was used for the next experiments in order to get accurate values.

Optimum temperature for protease production

The fermentation was carried at various temperatures such as 32, 34, 36, 38, 40 and 42°C to study their effect on enzyme production. Experiments were carried out in triplicates and the average values have been reported.

Optimum pH for protease production

Different pH concentrations; 6,7,8,9,10 and 11 of the gelatin broth were prepared using buffer solution. All the experiments were carried out in triplicate at optimum temperature and average values were reported.

Various concentrations of sodium chloride for protease production

Various concentrations of sodium chloride (0.5, 1, 1.5, 2, 2.5 and 3%) were studied by changing the ratio of volume of sodium chloride solution to the gelatin broth and flask were incubated in triplicate keeping the other parameters in constant and the average values were reported.

Various carbon compounds for protease production

The gelatin broth was distributed into various flasks and 1% of each carbon sources; glucose, mannitol, dextrose and starch was then added before inoculation of the strain and was incubated at the optimum pH, temperature and NaCl. The experiments were conducted in triplicate and the average values are reported.

Various nitrogen compounds for protease production

Gelatin broth was used for studying the effect of various nitrogen compounds viz. peptone, yeast extract, beef extract, casein and potassium nitrate. The broth was distributed into various flasks and 1% of each nitrogen source was then added before inoculation of the strain. Cultures in triplicates were incubated at already standardized parameters.

Various aminoacids for protease production

Gelatin broth was used for studying the influence of organic matter such as L-glutamine, L- asparagine and L- histidine on protease production. The broth was distributed into various flasks and 0.8 ml of each amino acid was then added. Triplicate flasks were incubated, keeping all other conditions at their optimum level.

Partial purification of the protease enzyme from the strain PS-18A

The strain PS-18A, which showed maximum enzymatic activity among the cultures screened for the production of the protease enzyme, was used for the partial purification. The crude sample of the enzyme was subjected to centrifugation at 1,00,000 rpm for 60 min in the refrigerated centrifuge. The supernatant and the pellet suspended in a mixture of volume of buffer were used for the enzyme assays. The crude extract was treated with protamine sulphate (1 mg protamine sulphate to 10 mg of protein) and centrifuged at 27,000 rpm for 10 min to remove the nucleic acids and the supernatant was collected. This supernatant was brought to 45% saturation by mixing ammonium sulphate (pH 8.5) slowly with gentle agitation and allowed to stand for 24 h at 4°C in the cold room. After the equilibration, the precipitate was removed by centrifugation (10,000 rpm at 4°C for 20 min) and the supernatant was once again brought to 95% saturation with ammonium sulphate and allowed to stand for 30 min at 4°C. This precipitate was again centrifuged at 10,000 rpm at 4°C for 20 min. The precipitate obtained was dissolved in 10 ml of 0.5 M Tris-HCL buffer (8.5) and the protein content was estimated (Lowry et al., 1951). Then, 10 cm pretreated dialysis bag (HIMEDIA) was taken and activated by rinsing in doubled distilled water. One end of the dialysis bag was tightly tied and the precipitate recovered was taken inside the bag. The other end of the dialysis bag was tightly tied to prevent the leakage. After that, the dialysis bag was suspended in a beaker containing 0.5 M Tris-HCL buffer (pH 8.5) for 24 h, then it was transferred to 5% sucrose solution so that the excess water was removed and got absorbed by sucrose solution. After the dialysis, the volume was measured and analyzed for proteins and stored in deep freezer.

Taxonomic investigation

The genus level identification was made for the strain PS-18A using

Table 1. Comparison of morphological characteristics of strain PS-18A and ***Streptomyces roseiscleroticus.***

Characteristic	Strain PS-18A	*S. roseiscleroticus*
Colour of aerial mycelium	White	White
Melanoid pigment	-	-
Reverse side pigment	+	+
Soluble pigment	–	-
Spore chain morphology	Spiral	Spiral
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	±
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	±
Raffinose	+	±

cell wall I composition analysis and micromorphological studies (Lechevalier and Lechevalier, 1970). Characterization of the strain PS-18A was made by following the methods described by using standard yeast extract malt extract agar (ISP medium 2) (Okami, 1984). The species level identification of the strain was made based on the nonomura keys and by using the Bergey's Manual of Determinative Bacteriology.

RESULTS AND DISCUSSION

Protease is an industrially important enzyme having wider applications in pharamacuetical, leather, laundry, food and waste processing industries. Industrial enzyme production would be effective only if the organism and the target enzyme are capable of tolerating different variables of the production processes. Primary screening for protease producing actinomycetes was done in gelatin agar medium based on zone formation. Totally 28 strains were isolated from the pond sediment and among them, six strains exhibited protease activity. Among the six strains, the best one was selected based on zone formation and the growth conditions for that strain PS-18A (Figures 1 and 2) were standardized for better production of protease enzyme. The morphological, physiological and biochemical characteristics of the protease producing strain PS-18A, tested in the present study, are given in Tables 1, 2 and 3.

Temperature

Temperatures varying from 32 - 42ºC were examined for the detection of optimum temperature for the protease production and found that a temperature of 40ºC (Figure 3) was required for better production of protease. And it was also found that in lower temperature the activity was low.

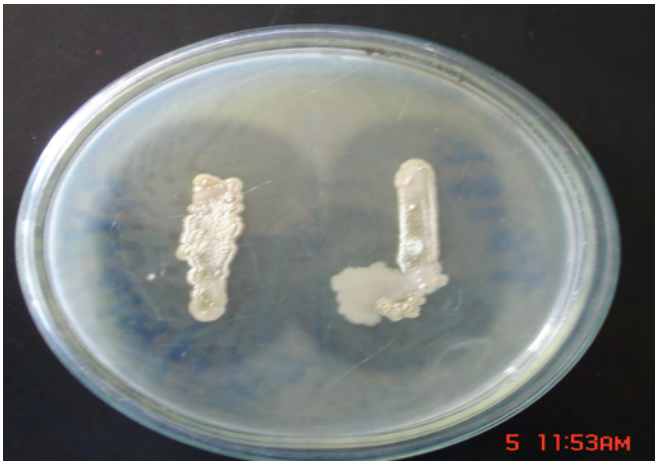

Figure 1. Proteolytic activity of strain PS-18A.

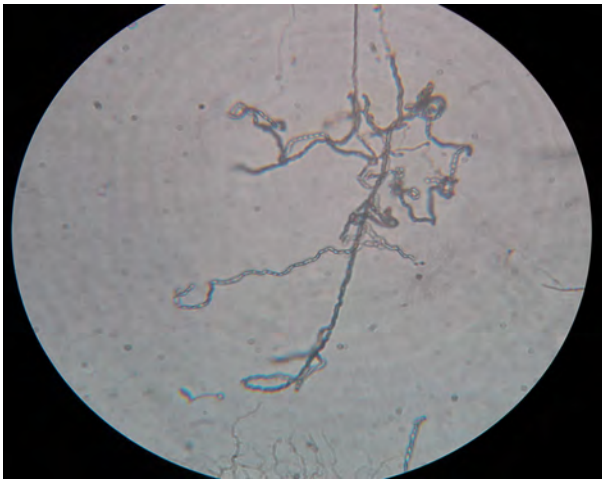

Figure 2. Spirales spore chain morphology of strain PS- 18A.

Table 2. Physiological characteristics of the strain PS-18A.

Parameter	Range					
NaCl requirement (%, w/v)	0.5 PG	1 PG	1.5 PG	2 MG	2.5 MG	3 GG
Temperature (°C)	32 NG	34 PG	36 MG	38 MG	40 GG	42 NG
pH	4 NG	5 PG	6 MG	7 GG	8 GG	9 NG

PG = Poor growth; GG = Good growth; MG = Moderate growth; NG = No growth.

Table 3. Biochemical characteristics of the strain PS-18A.

Biochemical property	PS-18A
Utilization of sole nitrogen sources	
L-asparagine	±
L-hydroxyproline	+
L-phenylalanine	+
Other characters	
Cellulose degradation	-
Hydrogen sulphide production	+
Melanin production	-
Gelatin liquefaction	+
Nitrate reduction	-
Starch hydrolysis	+
Milk coagulation	+
Growth in the presence of 0.1% phenol	-

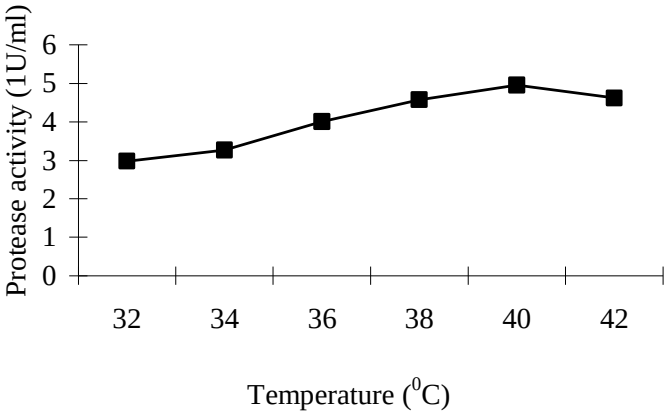

Figure 3. Effect of Temperature on protease activity of strain PS-18A.

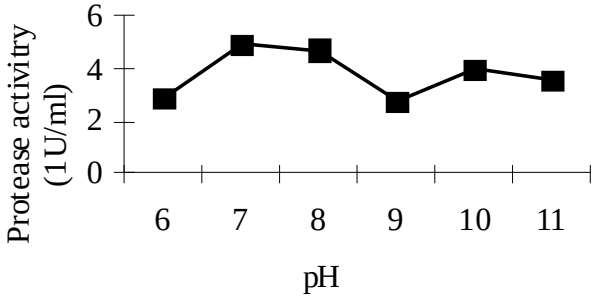

Figure 4. Effect of pH on protease activity of strain PS- 18A.

pH

The pH ranging from 6 –11 was tested for the detection of optimum pH for high protease production and the result showed that the production of enzyme was maximum at pH 7(Figure 4) and minimum at pH 6.

Sodium chloride

As the organism was isolated from salty environment, the use of varying percentage of NaCl in the production medium was inevitable. The results confirmed that the enzyme activity was at its best when the concentration of NaCl was 3% (Figure 5).

Carbon compounds

Carbon sources of different origins were used in the production medium for determining the highest yield of enzyme production. Results indicated the role of carbon compounds in maximizing the production of protease enzyme. The highest activity was obtained when starch was used as the carbon source (Figure 6) while the activity was minimum with dextrose.

Nitrogen sources

Maintaining the physical factors and the carbon sources at optimized condition, nitrogen sources of varying nature were tested .Of all the nitrogen sources, the strain showed maximum activity in the presence of beef extract (Figure 7).

Aminoacids

L-Glutamine (Figure 8) was observed to enhance pro-

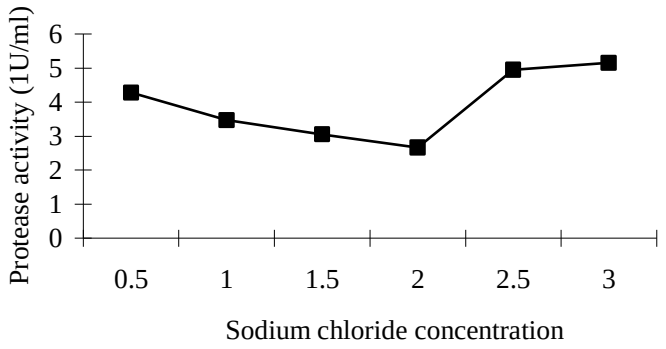

Figure 5. Effect of Sodium chloride on protease activity of strain PS- 18A.

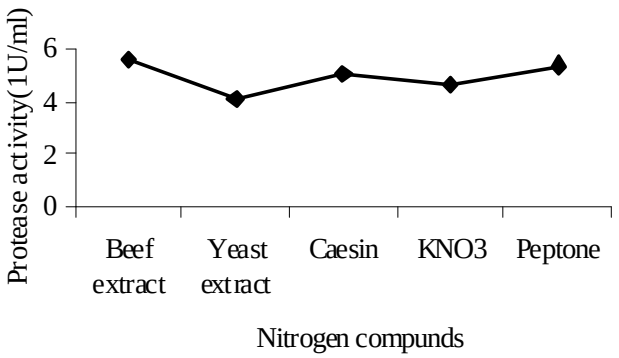

Figure 7. Effect of Nitrogen compounds on protease activity of strain PS- 18A.

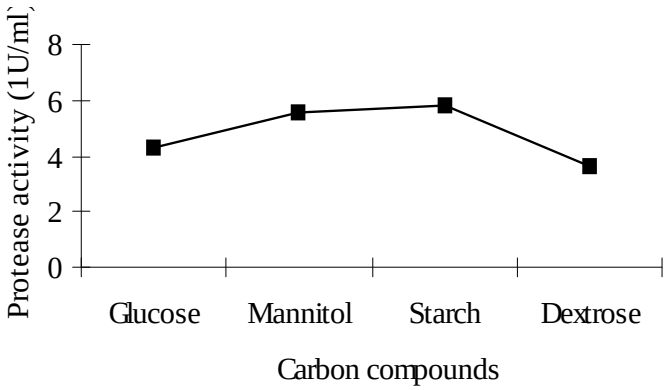

Figure 6. Effect of Carbon compounds on protease activity of strain PS- 18A.

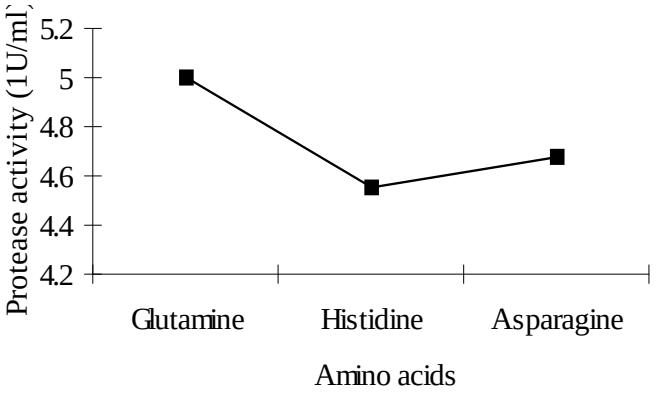

Figure 8. Effect of Aminoacids on protease activity of strain PS-18A.

tease synthesis and enzyme production occurred even in the absence of any other aminoacid in the seawater medium.

Protein

Protein content of the crude enzyme was quantified by means of Lowry's method and it was found to be 2.09 µl ml^{-1}.

Taxonomic investigation

Strain PS-18A characteristics were compared with those of the ***Streptomyces*** species given in Nonumura Key and the Bergey's Manual of Determinative Bacteriology. Strain PS-18A showed variations only in few characters when compared to the reference strain, ***S. roseisclero-ticus***. Hence, the strain PS-18A is tentatively identified as ***S. roseiscleroticus.*** Further molecular approach towards the identification of the strain PS-18A will help confirm the species.

Conclusion

From the present study, it is concluded that the sediment sample of the estuarine shrimp pond is a good source material for the isolation of potential actinomycetes. The present study has also revealed that the tentatively identified species, ***S. roseiscleroticus*** isolated from the sediments possesses good protease activity. The study has also standardized the growth parameters of the actinomycetes for the maximum enzyme production, which can be effectively used in the large scale production of protease enzyme for commercial purposes.

ACKNOWLEDGEMENT

The authors thank Prof. T. Balasubramanian, Director, Centre of Advanced Study in Marine Biology and the authorities of Annamalai University for providing with necessary facilities.

REFERENCES

Adinarayana K, Ellaiah P (2002). Response surface optimization of the

critical medium components for the production of alkaline prtotease by a newly isolated ***Bacillus*** sp. Indian J. Pharm. Sci. 5: 272-278.
Balagurunathan R (1992). Antagonistic actinomycetes from Indian shallow sea sediments with reference to a β-unsaturated γ-lactone type of antibiotic from Streptomyces grisebrunnus (P-33). Ph.D thesis, Annamalai University, India, p. 33.
Bhushan B, Hoondal GS (1999). E¡ect of fungicides, insecti-cides and allosamidin on a thermostable chitinase from Bacillus sp. BG-11. World J. Microbiol. Biotechnol. 15: 403-404.
Kathiresan K, Balagurunathan R, Masilamaiselvam M (2005). Fungicidal activity of marine actinomycetes against phyotopathogenic fungi. Indian J. Biotechnol. 4: 271-276.
Lechevalier MP, Lechevalier H (1970). Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435-443.
Lowry OH, Kosebrough NJ, Farr AC, Randall RJ (1951). Protein measurement with the measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.
Okami Y (1984). Marine microorganisms as a source of bioactive agents In. Current Perspective in Microbial ecology. Klug MJ, Reddy CA (eds) American society for microbiology, Washington DC, pp. 615-655.
Pokorny M, Vitale LJ (1980). Enzymes as by-product during biosynthesis of antibiotics. In: Trends in enzymology; industrial and chemical Enzymology (Vitale LJ, Simeon V eds,) Pergmon press, Oxford, pp. 13-25.
Weyland A (1969). In Marine actinomycetes. Adv. Biotechnol. 11(12): 16-17.