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Production of Alkaline Protease by *Streptomyces* sp., Isolated from Coastal Mangrove Sediment

K. Kathiresan and S. Manivannan
Centre of Advanced Study in Marine Biology, Annamalai University,
Parangipettai 608502, Tamilnadu, India

Abstract: Microbes from diverse and extreme environments are considered to be important sources of enzymes. It is essential that those organisms be provided with optimal growth conditions to increase enzyme production. Effects of pH, temperature, mineral salts, incubation time, sources and concentrations of carbon and nitrogen were tested in submerged fermentation process in production of alkaline protease by *Streptomyces* sp., isolated from coastal mangrove soil. The production medium prepared in distilled water, supplemented with 5% sucrose (carbon source), 7.5% casein (nitrogen source), maintained with pH of 8.5 and incubated at 30°C for 144 h was found optimal for the production of alkaline protease.

Key words: Alkaline protease, *Streptomyces* sp., coastal, mangroves, rhizosphere soil, *Rhizophora*

INTRODUCTION

Alkaline protease is a well-known enzyme that has wide applications as additive to detergents in several industrial processes (Godfrey and Reichet, 1985; Potumarthi *et al*., 2007). It holds more than 50% of the total enzyme market (Sachidanandham *et al*., 1999; Potumarthi *et al*., 2007). Microbes from diverse and extreme environments are considered to be important sources of enzymes (Kathiresan and Manivannan, 2006a-c) and their specific properties are expected to result in novel applications (Govardhan and Margolin, 1995; Roberston *et al*., 1996; Potumarthi *et al*., 2007). Hence, search for new microbial sources continues to be attempted. However, the marine microbes which are extremophilic, taxonomically diverse and genetically special are largely unexplored for their enzyme production (Kathiresan and Duraisamy, 2006).

Most alkalophilic microorganisms produce alkaline protease, though interest is limited only to those that yield substantial amounts. It is essential that those organisms be provided with optimal growth conditions to increase enzyme production. They are only few papers concerned with optimization of cultural conditions for simultaneous production of the enzyme by actinomycetes and such work is also confined to terrestrial strains (Desai and Dhalla, 1969; Tsuchiya *et al*., 1992; Anderson *et al*., 1997; Mehta *et al*., 2006; Thys *et al*., 2006). The microbes that are present abundant in tropical mangrove ecosystem are likely to be a good source of useful enzymes (Kathiresan, 2000; Kathiresan and Bingham, 2001; Kathiresan and Manivannan, 2006a-c). To our knowledge, no investigation has been performed in Actinomycetes isolated from coastal mangroves for optimization of culture conditions for production of alkaline protease and hence the present study was undertaken.

Corresponding Author: Dr. K. Kathiresan, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai 608502, Tamilnadu, India Tel:+ 91-4144-238419, 91-4144-243223

MATERIALS AND METHODS

Chemicals

All analytical reagents and media components were purchased from Hi-Media (Mumbai, India).

Microorganism

Actinomycetes were isolated from rhizosphere soil of a mangrove species, *Rhizophora annamalayana* Kathir, on the bank of Vellar estuary, Portonovo (Lat. 11°29' N; Long. 79°46' E), South East Coast of India, during monsoon season (October-December, 2006) by plating method.

Growth Media

For isolation of Actinomycetes, Starch casein agar medium was used and its composition was starch -15 g, casein -5 g, peptone -5.0 g, beef extract -3.0 g, NaCl -5.0 g, Nalidixic acid -10 μL mL^{-1}, Nystatin -25 μL mL^{-1}, Cyclohexamide -10 μL mL^{-1}, agar -15 g, aged seawater -500 mL and distilled water -500 mL.

Production Medium

Production medium was composed of 5% casein, 5% glucose, 5% peptone, 5% yeast extract and 50 mL salt solution (KH_2PO_4 0.25%, $MgSO_4$ - 0.1%, K_2HPO_4 - 0.25%, FeS O_4-0.1%), aged seawater (500 mL) and distilled water (500 mL). The pH was adjusted to 8.5 and the medium was sterilized in an autoclave for 15 min at 121°C and inoculated with a loop-full of actinomycetes and then incubated at 30°C in an orbital shaker set at 100 rpm for 96 h. At the end of the fermentation, the culture filtrate was centrifuged and the supernatant was assayed for protease activity.

Enzyme Assay

To 3 mL of 0.6% casein solution (pH 9.0, prepared in 10 mM Tris-HCl buffer), 0.5 mL of diluted enzyme solution was added and the reaction mixture was incubated at 45°C for 10 min. The reaction was terminated by the addition of 3.2 mL of a mixture of 0.11 M Trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid and the reaction mixture was allowed to stand for 15 min before filtering through Whatman filter paper (No. 1). Protein content in aliquots of 0.5 mL was estimated at the absorbance of 660 nm (Lowry *et al*., 1951). One unit of protease activity is expressed as the amount of enzyme which converts 1.0 mg of protein per 10 min at 45°C.

Optimization of Culture Conditions

The culture conditions such as pH, temperature, mineral salts and various sources of carbon and nitrogen that influence production of protease were optimized by varying the conditions one at a time. The experiments were conducted in 200 mL Erlenmeyer flask containing production medium. After sterilization by autoclaving, the flasks were cooled and inoculated with culture and maintained under various operational conditions separately such as pH (6.5, 7.0, 7.5, 8.0, 8.5, 9.0), temperature (20, 30, 40°C), incubation period (24, 48, 72, 96, 120, 144 h), carbon sources (glucose, xylose, maltose and sucrose, each at 5%), nitrogen source (peptone, beef extract, yeast extract, tryptone and casein, each at 5%) and salinity (0, 40, 50, 60, 70, 80, 90, 100% seawater). After 96 h (expect for incubation period effect), the culture filtrate was assayed in triplicate samples for protease activity.

Statistical Analysis

Statistical analysis was done by analysis of variance followed by Duncan Multiple Range Test (DMRT).

RESULTS

Production medium inoculated with *Streptomyces* sp. and incubated for 96 h, exhibited the enzyme activity as 35 U mL^{-1} at pH 7.5 and 30°C (Table 1). The activity was significantly higher at 30°C than 20 and 40°C (Table 1). When the culture was incubated at 144 h the maximum production of 81 U mL^{-1} was detected. There was a 13 fold increase in activity at 144 h of incubation as compared to 24 h (Table 1).

Among the carbon sources, sucrose was the best source to enhance enzyme production of 61 U mL^{-1}, which was 48% higher than glucose (Table 2). Among the nitrogen sources, casein showed maximum level of production (59 U mL^{-1}, Table 2), which was about 20% higher than peptone.

Sucrose at 5.0% was found optimal in the production medium, exhibiting an enzyme activity of 61 U mL^{-1} (Table 3), which was 47% higher than sucrose at 1.0%. Casein at 7.0% showed maximum

Table 1: Effect of various physical parameters

Physical parameters		Protease activity (U* mL^{-1})
pH	6.5	22±1.45^{a}
	7.0	28±1.20^{b}
	7.5	35±2.30^{c}
	8.0	39±0.17^{c}
	8.5	45±0.87^{d}
	9.0	37±1.18^{c}
Temperature (°C)	20.0	31±1.15^{ab}
	30.0	35±1.20^{a}
	40.0	27±2.02^{b}
Incubation period (h)	24.0	6±0.88^{a}
	48.0	12±1.15^{b}
	72.0	23±1.52^{c}
	96.0	35±1.20^{d}
	120.0	59±1.76^{e}
	144.0	81±2.02^{f}
	160.0	56±1.76^{e}

*One unit of protease activity is expressed as the amount of enzyme which converts 1.0 mg of protein per 10 min at 45°C. Values are mean±standard error from 3 replicates in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Table 2: Effect of various chemical parameters

Chemical parameters		Protease activity (U* mL^{-1})
Carbon sources (5%)	Glucose	13±0.77^{a}
	Fructose	46±1.20^{b}
	Xylose	54±1.52^{c}
	Sucrose	61±1.20^{d}
	Lactose	35±2.02^{e}
Nitrogen sources (5%)	Yeast extract	55±2.02^{ab}
	Beef extract	49±1.20^{ab}
	Casein	59±2.96^{c}
	Peptone	39±1.73^{d}
	Malt extract	44±1.45^{cd}
Salinity (% of seawater)	0	91±3.60^{a}
	20	61±3.21^{b}
	40	51±2.08^{c}
	50	35±1.20^{d}
	60	22±1.76^{e}
	80	12±2.64^{e}
	100	04±3.98^{f}

*One unit of protease activity is expressed as the amount of enzyme which converts 1.0 mg of protein per 10 min at 45°C. Values are mean±standard error from 3 replicates in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Table 3: Effect of various concentrations of carbon (Sucrose), nitrogen (Casein) and mineral salts

Concentration (%)		Protease activity (U* mL^{-1})
Sucrose	1.0	14±0.57[a]
	2.5	22±0.85[b]
	5.0	61±1.45[c]
	7.5	52±1.76[d]
	10.0	45±1.20[f]
Casein	1.0	52±0.5[a]
	2.5	55±1.20[a]
	5.0	54±0.88[a]
	7.5	64±2.60[b]
	10.0	42±2.02[c]
Salt solution (mL)	10.0	51±1.15[a]
	20.0	55±0.61[a]
	30.0	61±1.42[b]
	40.0	51±1.45[a]
	50.0	44±1.15[c]
	60.0	34±1.45[d]

*One unit of protease activity is expressed as the amount of enzyme which converts 1.0 mg of protein per 10 min at 45°C. Values are mean±standard error from 3 replicates in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

activity (64 U mL^{-1} Table 3), which was 22% higher than casein at 10.0%. The mineral salts at 30 mL exhibited higher activity (61 U mL^{-1} Table 3), which was 27% more than those at 60 mL did. The activity was about 86% high in absence of seawater, as compared to 100% seawater (Table 2).

DISCUSSION

The microorganisms that thrive in alkaline environment are of two broad groups, namely alkalophiles and alkalotolerants. The alkalophiles are capable to grow above pH 10, with an optimal growth around pH 9 and that are unable to grow at pH 7 or less. On the other hand, alkalotolerant organisms are capable of growing at pH values in excess of 10, but have an optimal growth rate to neutrality (Krulwich, 1986). Accordingly the Actinomycetes strain used in the present study is alkalotolerant because it is able to grow below neutral pH and excess of 9 (Table 1).

The media optimization is an important aspect to be considered in the development of fermentation technology to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium (Gupta *et al*., 2003). The pH optima for alkaline protease are generally above 7.5 (Horikoshi and Akiba, 1982) as in the present study with Actinomycetes strain which displays high enzyme stability and activity at pH 8.5 (Table 1).

Temperature is another critical factor to control the production of enzymes, but underlying mechanism is not well understood (Chaloupca, 1985). Temperature optimum was found to be at a range of 70-80°C for the thermophilic microbes (Chou *et al*., 1976; Bok *et al*., 1984) whereas the coastal strain of the present study displayed the temperature optimum at 30°C (Table 1).

The enzyme production varies with incubation period (Smitt *et al*., 1996). In the present study the protease activity increases steadily and reaches maximum at 120 h of incubation (Table 1), as against a short duration of 40 h in *Arthrobacter ramosus* (Kanekar *et al*., 2002).

Among carbon sources, lactose, maltose, sucrose and fructose exhibit higher activity in the present study as also suggested by Sen and Satyanarayana (1993), Tsuchiya *et al*. (1991), Phadatare *et al*. (1993) and Malathi and Charaborty (1991). Sucrose exhibits higher activity which is 48% higher than glucose (Table 2).

The alkaline protease comprises 15.6% nitrogen and its production is dependent on the availability of both carbon and nitrogen sources in the medium (Kole *et al*., 1988). Although complex nitrogen sources are usually used for alkaline protease production, the requirements for a specific nitrogen supplement differ with organisms. Among the organic nitrogen sources, casein exhibits higher activity (Table 2).

Even though the Actinomycetes strain derived from coastal mangrove sediment, it produces less concentration of proteases when production medium is prepared with 100% seawater (Table 2). Conditions optimal for production of alkaline protease by mangrove sediment derived Actinomycetes were developed in the study, perhaps for the first time.

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