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Alkaline Protease Production by *Penicillium fellutanum* Isolated from Mangrove Sediment

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Abstract: Influence of pH, temperature, mineral salts, incubation time, sources and concentrations of carbon and nitrogen was tested in submerged fermentation process in production of alkaline protease by *Penicillium fellutanum* isolated from the coastal mangrove soil. The production medium prepared in distilled water, supplemented with 5% lactose (carbon source), 5% casein (nitrogen source), maintained with pH, of 8.5 and incubated at 30°C for 120 h was found optimum for production of alkaline protease.

Key words: Alkaline protease, *Penicillium fellutanum*, mangroves, rhizosphere soil, *Rhizophora*

INTRODUCTION

Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives, holding more than 50% of total enzyme market. They play a specific catalytic role in the hydrolysis of proteins (Godfrey and Reichet, 1985; Sachidanandham *et al*., 1999; Potumarthi *et al*., 2007). Microbes especially fungi are important sources of enzymes (Gilberto *et al*., 1999; Kathiresan and Manivannan, 2006a, b, c) however studies on their production of alkaline protease are limited for the reason that the fungi prefer to acidic and neutral pH, in culture medium. It is necessary to search for new sources of fungi that grow as extremophiles for their novel application (Govardhan and Margolin, 1995; Roberston *et al*., 1996). In general, enzyme studies are limited only to the microbes that yield substantial amounts. It is essential that those organisms be provided with optimal growth conditions to increase enzyme production (Prakasham *et al*., 2005; Potumarthi *et al*., 2007). They are only few papers concerned with optimization of cultural conditions for simultaneous production of the enzyme by fungi and these are also confined to terrestrial strains (Kumar and Takagi, 1999; Sandrogermano *et al*., 2003; Oliveira *et al*., 2006; Ahamed *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, b, c). In the absence of any investigation on fungi from coastal mangrove sediments for production and optimization of alkaline protease, the present study has been undertaken.

MATERIALS AND METHODS

Chemicals

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

Microorganism

The fungus, *Penicillium fellutanum* Biourge., was isolated from rhizosphere soil of a mangrove species, *Rhizophora annamalayana* Kathir., on the bank of Vellar estuary, Portonovo (Lat. 11°29' N;

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Long. 79°46' E), South East Coast of India, during monsoon season (October-December, 2006) by plating method using Sabouraud Glucose Agar medium supplemented with an antibiotic (Chloromphenicol 0.1 g L^{-1}) to avoid bacterial contamination (Boukhout and Robert, 2003).

Growth Media

For growth and sproulation of the fungal species, Sabouraud Glucose Agar medium containing glucose 20 g, peptone 10 g, agar 20 g, aged seawater 500 mL and distilled water 500 mL was used.

Production Medium

Production medium 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% $FeSO_4$0.1%) aged seawater (500 mL) and distilled water (500 mL) was prepared. The pH, was adjusted to 8.5 and the medium was sterilized in an autoclave for 15 min at 121°C. The medium was inoculated with a loop-full of spore suspension *of P. fellutanum* 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 alkaline protease.

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 and 9.0), temperature (20, 30 and 40°C), incubation period (24, 48, 72, 96, 120 and 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 and 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 *Penicillium fellutanum* and incubated for 96 h, exhibited the enzyme activity of 17 U mL^{-1} at pH 8.5 and 30°C (Table 1). The activity was significantly higher at 30°C than at 20 and 40°C (Table 1). When the culture was incubated at 120 h, the maximum production of 25 U mL^{-1} was detected. There was a 5-fold increase in activity at 120 h of incubation as compared to 24 h (Table 1).

Among the carbon sources, lactose was the best source to enhance enzyme production of 25 U mL^{-1}, which was 11% higher than maltose (Table 2). Among the nitrogen sources, casein showed

Table 1: Effect of various physical parameters

Physical parameters		Protease activity (U* mL^{-1})
pH	6.5	07 ± 1.73^{a}
	7.0	09 ± 2.60^{ab}
	7.5	14 ± 2.30^{bc}
	8.0	15 ± 1.75^{c}
	8.5	17 ± 1.15^{c}
	9.0	13 ± 2.30^{bc}
Temperature (°C)	20.0	09 ± 2.02^{a}
	30.0	17 ± 1.15^{b}
	40.0	13 ± 1.15^{ab}
Incubation period (h)	24.0	05 ± 0.81^{a}
	48.0	09 ± 1.47^{b}
	72.0	13 ± 0.52^{c}
	96.0	17 ± 1.15^{df}
	120.0	25 ± 0.61^{e}
	144.0	19 ± 1.75^{f}

Values are mean±standard error from 3 replicates in each group, Values not sharing a common superscript letter(s) differ significantly at $p<0.05$ (DMRT), *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

Table 2: Effect of various chemical parameters

Chemical parameters		Protease activity (U* mL^{-1})
Carbon sources (1%)	Lactose	25 ± 1.73^{a}
	Sucrose	17 ± 1.15^{bc}
	Xylose	23 ± 2.88^{ab}
	Maltose	14 ± 1.18^{c}
Nitrogen sources (1%)	Yeast extract	09 ± 1.73^{a}
	Beef extract	11 ± 1.15^{ab}
	Peptone	17 ± 1.15^{bc}
	Casein	20 ± 1.50^{c}
	Tryptone	13 ± 2.71^{ab}
Salinity (% of water sea)	0	39 ± 1.15^{a}
	20	34 ± 2.28^{b}
	40	25 ± 1.73^{c}
	50	17 ± 1.15^{de}
	60	14 ± 1.40^{e}
	80	11 ± 1.63^{ef}
	100	07 ± 1.58^{f}

Values are mean±standard error from 3 replicates in each group, Values not sharing a common superscript letter(s) differ significantly at $p<0.05$ (DMRT), *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

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

	Concentration (%)	GI activity (U* mL^{-1})
Lactose	1.0	19 ± 1.20^{a}
	2.5	26 ± 0.88^{b}
	5.0	30 ± 1.15^{b}
	7.5	22 ± 0.48^{a}
Casein	10.0	13 ± 2.02^{c}
	1.0	14 ± 1.45^{ac}
	2.5	17 ± 1.15
	5.0	$19\pm0.80^{b\ bc}$
	7.5	14 ± 1.53^{ac}
	10.0	11 ± 1.42^{a}
Salt solution (mL)	10.0	17 ± 1.15^{a}
	20.0	23 ± 0.61^{b}
	30.0	16 ± 1.42^{a}
	40.0	13 ± 1.45^{a}
	50.0	09 ± 1.15^{c}
	60.0	08 ± 0.15^{c}

Values are mean±standard error from 3 replicates in each group, Values not sharing a common superscript letter(s) differ significantly at $p<0.05$ (DMRT), *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

maximum level of production (22 U mL^{-1}, Table 2), which was about 11% higher than yeast extract. Lactose at 5.0% was found optimal in the production medium, exhibiting an enzyme activity of 30 U mL^{-1} (Table 3), which was 17% higher than at 10.0% lactose. Casein at 5.0% showed maximum activity (19 U mL^{-1} Table 3), which was 8% higher than at 10.0% casein. The mineral salts at 20 mL exhibited higher activity (23 U mL^{-1} Table 3), which was 3 fold more than those at 60 mL did. The activity was about 6-fold 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 fungus, *Penicillium fellutanum*, which was used in the present study, can be regarded as alkalotolerant because it is able to grow below neutral pH and excess of 9.

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). Although the apparent pH optima for *Penicillium fellutanum* alkaline protease fall in this range, the enzyme is stable and active at pH 8.5 (Table 1).

Temperature is another critical factor that determines the growth and simulataneous production of enzyme. The mechanism of temperature control on enzyme production is not well understood (Chaloupca, 1985). Temperature optimum is known to be at a range of 70-80°C for the thermophilic microbes (Bok *et al*., 1984; Chou *et al*., 1976), whereas the coastal microbe prefers a temperature of 30°C in the present study (Table 1).

The enzyme productions vary with incubation period (Smitt *et al*., 1996). In the present study the protease activity increased steadily and reached 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, most reports have suggested that lactose, maltose, sucrose and fructose exhibit higher activity (Malathi and Charaborty, 1991; Tsuchiya *et al*., 1991; Phadatare *et al*., 1993; Sen and Satyanarayana, 1993) which is similar to the present study. Lactose exhibits the higher activity which is 11% higher than maltose (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 in the present study (Table 2).

The fungal strain produces low alkaline protease when production medium is prepared with 100% seawater, although the fungus is derived from coastal biotope (Table 2). Hence, *Penicillium fellutanum* can be regarded as a terrestrial species that is facultatively halophilic in nature. Conditions optimal for production of alkaline protease by *P. fellutanum* was developed in the study, perhaps for the first time from mangrove-derived fungus.

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