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Cellulase Production by *Penicillium fellutanum* Isolated from Coastal Mangrove Rhizosphere Soil*

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Abstract: Effects of pH, temperature, mineral salts, incubation time, sources of carbon and nitrogen were tested in submerged fermentation process in production of cellulase by *P. fellutanum* isolated from coastal mangrove soil. The production medium prepared in distilled water, supplemented with 5% rice bran, 0.3% KCl, 0.15% KH_2PO_4, lactose (carbon source), yeast extract (nitrogen source), maintained with pH of 6.5 and incubated at 30°C for 120 h was found optimal for production of cellulase.

Key words: Cellulase, *Penicillium fellutanum,* mangroves, rhizosphere soil, *Rhizophora*

Introduction

Cellulases are important industrial enzymes and find applications in several industrial processes (Kang *et al*., 2004). Currently the most important application is the bio-bleaching of pulp, the production of dissolving pulp, the treatment of wastewater and the dinking of recycled waste paper in pulp industries. The cost of production and low yields of these enzymes are the major problems for industrial application. Therefore, investigations on the ability of the cellulose hydrolyzing microbial strains to utilize inexpensive substrate have been done (Kang *et al*., 2004; Esterbauer *et al*., 1991; Haltrich *et al*., 1996). The enzyme is commercially used after extracting from many microorganisms especially fungal source (Hanif *et al*., 2004; Kang *et al*., 2004) of mostly terrestrial origin but less from marine sources. Therefore, in the present study, the enzyme was attempted in a fungus, *P. fellutanum* isolated from coastal mangroves for maximizing its production under optimal conditions in submerged fermentation by using inexpensive substrate, rice bran.

Materials and Methods

Microorganism

The fungus, *Penicillium fellutanum* Biourge., was isolated from rhizosphere soil of a fast disappearing 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 post monsoon season (January-March, 2006).

Chemicals

All analytical reagents and media components were purchased from Hi-Media (Mumbai, India) and Sigma chemicals (St. Louis, USA).

Growth Media

For isolation of *Penicillium* sp., 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.

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Production Medium

The composition of production medium used was rice bran 50 g, KCL 2.0 g, KH_2PO_4 1.0 g, $MgSO_4$ 0.05 g, Fe SO_4 0.02 g, aged seawater, 500 mL and distilled water 500 mL. The pH was adjusted to 6.5 and the media were sterilized in an autoclave for 15 min at 121°C. The media were 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. The media were centrifuged at 5,000 g for 15 min to obtain crude enzyme solution.

Enzyme Assay

CMCase activities were assayed using the culture filtrate. Briefly, 0.1 mL of water, 2.0 mL of 1% (w/v) CMC in 0.1 M acetate buffer (pH 5.0) and 0.05 mL of the culture filtrate were incubated at 50°C for 20 min. The amount of reducing sugar formed was measured by the method described by Miller (1959). One unit of CMC activity was defined as the amount of enzyme that liberates 1 μmol equivalent of glucose under the assay conditions.

Optimization of Culture Conditions

The factors such as temperature, pH, salinity, sources of carbon and nitrogen affecting production of cellulase were optimized by varying parameters 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 (6.0, 6.5 and 7.0), temperature (20, 30 and 40°C), incubation period (24, 48, 72, 96, 120 and 144 h), carbon source (glucose, fructose, xylose, lactose, sucrose, each at 1%), nitrogen source (peptone, beef extract, yeast extract, malt extract and casein each at 0.5%) and salinity (0, 20, 40, 50, 60, 80, and 100% seawater). After 72 h (expect for incubation period effect), the culture filtrate was assayed in triplicate samples for cellulase activity.

Results

The 72 h culture of *Penicillium fellutanum* exhibited cellulase activity of 55 U mL^{-1}, at pH 6.5 and 30°C. The activity was about 13% higher at pH 6.5 than pH 7.0; and 8% higher at 30 than 40°C (Table 1). When the culture was incubated at 120 h, the maximum activity detected was 79 U mL^{-1}. There was more than 6-fold increase in activity at 120 h incubation as compared to 24 h (Table 1). When rice bran was used as a substrate, a maximum activity of enzyme was detected at 5% and this activity was 41% higher than at 1% (Table 3).

Table 1: Effect of various physical parameters

Physical parameters		Cellulase activity (U mL^{-1})
pH	6.0	48±1.45^{a}
	6.5	55±1.20^{b}
	7.0	42±2.30^{c}
Temperature (°C)	20	51±1.15^{ab}
	30	55±1.20^{a}
	40	47±2.02^{b}
Incubation period (h)	24	12±0.88^{a}
	48	32±1.15^{b}
	72	43±1.52^{c}
	96	55±1.20^{d}
	120	79±1.76^{e}
	144	61±2.02^{f}

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), One unit of cellulase activity was defined as the amount of enzyme that liberates 1 μmol equivalent of glucose under the assay conditions

Table 2: Effect of various chemical parameters

Chemical Parameters		Cellulase activity (U mL^{-1})
Carbon sources 1 (%)	Glucose	13±0.77[a]
	Fructose	66±1.20[b]
	xylose	74±1.52[c]
	Lactose	81±1.20[d]
	Sucrose	55±2.02[e]
Nitrogen sources 1 (%)	Yeast extract	75±2.02[ab]
	Beef extract	69±1.20[ab]
	Peptone	79±2.96[c]
	Casein	59±1.73[d]
	Malt extract	64±1.45[cd]
Salinity (%)	0	121± 3.60[a]
	20	81±3.21[b]
	40	71±2.08[c]
	50	55±1.20[d]
	60	32±1.76[e]
	80	24±2.64[e]
	100	12±3.98[f]

Table 3: Effect of various concentrations of substrate (rice bran) and inorganic nitrogen sources (KCl, KH_2PO_4)

Concentration (%)		Cellulase activity (U mL^{-1})
Rice bran	1	14±0.57[a]
	2	22±0.85[b]
	3	34±1.45[c]
	4	50±1.76[d]
	5	55±1.20[e]
	6	50±1.15[d]
KCl	0.1	52±0.5[a]
	0.2	55±1.20[a]
	0.3	58±2.60[a]
	0.4	54±0.88[a]
	0.5	42±2.02[b]
	0.6	30±3.40[c]
KH_2PO_4	0.05	51±1.15[a]
	0.10	55±0.61[a]
	0.15	61±1.42[b]
	0.20	51±1.45[a]
	0.25	44±1.15[c]
	0.30	34±1.45[d]

Among carbon sources, lactose was the best to enhance the enzyme activity of 81 U mL^{-1} which was 68% higher than glucose (Table 2). Among nitrogen sources, peptone was ideal to increase the enzyme activity of 79 U mL^{-1}, which was about 20% higher than casein (Table 2). This activity was about 10-fold high in absence of seawater, as compared to 100% seawater (Table 2).

Among inorganic nitrogen sources, 0.3% KCl and 0.15% KH_2PO_4 exhibited high activities which were 28 and 27% higher than 0.6% KCl and 0.30% KH_2PO_4, respectively (Table 3).

Discussion

The media optimization is an important aspect to be considered in the development of fermentation technology. To our best knowledge, these findings represent the first reported studies on the general physiochemical properties and the regulation cellulase from a coastal microorganism.

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). Optimum pH and temperature for maximum production of cellulase were 6.5 and 30°C (Table 1). Maximum activity in bacteria was obtained around neutral pH of the medium and temperature 30°C but fungi vary with

respect to pH and temperature to support maximum production of cellulases (Magnelli and Forchiassin, 1999; Pirt, 1975; Umekalsom *et al*., 1997). In these studies 30°C temperature was found optimum (Table 1) to support maximum production of cellulase as observed by above workers. At higher temperature, the organism has to spend a lot of energy for maintenance and at lower temperature, transport of nutrients is hindered (Pirt, 1975).

The incubation period varies with enzyme productions (Smitt *et al*., 1996). Short incubation period offers potential for inexpensive production of enzymes (Sonjoy *et al*., 1995). In the present study the cellulase activity increased steadily and reached maximum at 120 h of incubation (Table 1).

Cellulase is an inducible enzyme (Ryu and Mandels, 1980; Kubicek, 1992; Kubicek *et al*., 1993) and was affected by the nature of the substrate used in fermentation. Therefore the choice of an appropriate inducing substrate is of importance. The results obtained when using rice 5% rice bran showed that cellulase activity increased 41% compared to 1% (Table 3).

To evaluate the carbohydrates to cause induction or repression of cellulase was grown on some monosaccharides and disaccharides. Lactose among the carbon sources examined was found to be the best inducer (Table 2). This study substantiates the work of Bagga *et al*. (1989) who demonstrated lactose as best inducer of *Aspergillus* sp. Nochure *et al*. (1993) identified fructose as the best inducer of cellulase in *Clostridium thermocellum*. Trehalose has been demonstrated as the best inducer of cellulases in a *Clostridium* sp. (Thirumale *et al*., 2001).

The enzyme production is affected significantly by different organic and inorganic nitrogen sources. The production of cellulase is sensitive to the nitrogen sources and nitrogen level in the medium (Desai *et al*., 1982). The results of the present study showed that the sources have different effect on enzyme activity. Among the organic nitrogen sources tested, the enzyme activity was high with peptone and 0.3% KCl, 0.15% KH_2PO_4 exhibited optimum activity for inorganic nitrogen sources (Table 3).

Even though the fungal strain was isolated from coastal soil, it produced less concentration of cellulase when production medium was prepared with 100% seawater (Table 2). Hence, it can be a terrestrial species facultatively halophilic in nature. Conditions optimal for production of cellulase by *Penicillium fellutanaum* was developed in the study.

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