African Journal of Microbiology Research Vol. 6(20), pp. 4214-4218, 30 May, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.074
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Optimization, production and purification of cellulase enzyme from marine *Aspergillus flavus*

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Accepted 12 October, 2011

Water samples were collected from five different places at Muthupettai mangrove along the east cost of Tamil Nadu, India. Five different *Aspergillus* species were isolated and identified by using lacto phenol cotton blue staining method. From this, *Aspergillus flavus* was tested in Modified Park's Agar for its cellulase enzyme production in different physico-chemical properties of substrates. The effect of different carbon source, nitrogen compound and physico-chemical conditions like temperature, pH and incubation periods were studied for derivation of cellulase enzyme. The purity of cellulase enzyme was analyzed by ammonium sulphate precipitation and high performance liquid chromatography. The molecular weight of enzyme was determined by SDS–PAGE. From the above results, the cellulase production is higher at pH 8 and 30°C on 7 days of incubation period. It also inferred that the enzyme inquisition raised by the rice bran than two substrates examined.

Key words: Mangrove, *Aspergillus flavus*, cellulase enzyme, ammonium sulphate precipitation, high performance liquid chromatography (HPLC).

INTRODUCTION

Enzymes are protein catalysts synthesized by living systems and are important in synthetic as well as degradative processes. Few enzymes are available in bulk quantities for industrial applications. Microbial cells produce a variety of enzymes and help in microbial growth and other cellular activities. Cellulose is a linear polysaccharide of glucose residence connected by β-1-4 linkages. In nature cellulose is usually associated with other polysaccharides such as xylan or lignin. It is an indigestive plant polysaccharide and skeletal basis of plant cell walls. Cellulose is the world's most abundant organic substrate. Enzymatic degradation of cellulose is being studied by Van belle et al. (1982), Enari (1983), Holm and sahm (1983). Cellulase has been known to be produced by a broad range of organisms including fungi, bacteria, actinomycetes; plants and insect such as termites (Hirte and Phillips, 1983). Enzymes have found numerous applications in food, medical and chemical processing and pharmaceutical industry. The main important application is in the production of single cell protein, alcohol, beer, biofuels, chemical feedstock, ethanol, and high fructose syrup (Solomon et al., 1990). The present study was designed to screen the production of cellulase by *A. flavus.* To optimize the physico-chemical characters by varying the parameters such as pH, temperature, time duration, partial and fine purification of the enzyme by ammonium sulphate precipitation for detecting the enzyme by using HPLC.

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MATERIALS AND METHODS

Water samples were collected from five different sampling stations in Muthupettai mangrove along the east cost of Tamil nadu, India. Five sampling stations are Korinmunai, Manakkattai, Koraiyar lagoon and Kadalmunai. The water samples collected were processed in laboratory by serial dilution techniques. After sampling with in 24 h the water samples from each station were subjected to appropriate dilution (10-2, 10-3) and plated into Potato detrose agar (PDA), Rose bengal agar (RBA) and corn meal agar with addition of a mixture of antibiotics like tetracycline, penicillin with pour method. The plates were incubated in room temperture at 28°C for 4-5 days

Figure 1. Plate of culture of *A .flavus.*

Figure 2. *A. flavus* exhibiting zone of inhibition for cellulase production.

(Booth, 1971).

Screening of cellulase producers

The colonies grown in PDA and RBA plates were transferred in modified Park's agar and incubated at 28°C for 4 to 5 days. A clear zone observed around the colonies was selected for further studies. Isolated colonies were identified by using lacto phenol cotton blue staining method (Dring, 1976; Gillman, 1998; Subramanian, 1971).

Processing of water samples

In the present study, five species of *Aspergillus sp* namely, *A. oryzae, A. fumigatus, A. flavus A. quercinus,* and *A. niger* were isolated and identified by using lacto phenol cotton blue staining method (Dring, 1976; Gillman, 1998; Subramanian, 1971).

From these identified fungi, A.flavus was selected for further production of cellulase enzyme.

Fermentation medium

Modified Czapek's cellulase medium was used as production medium; it includes cellulose (10.0 g), KNo_3 (3.0 g), K_2HPO_4 (1.0 g), $MgSO_4.7H_2O$ (0.5 g), $FeSO_4 .5H_2 O$ (0.01 g), distilled water 1000 ml (Enari et al., 1977). In addition to this production medium, natural substrates such as rice bran, wheat bran and baggase were used as additional carbon source for production media at a concentration of 1.5 gm/L (Bigelow et al., 2002). In addition to this, various nitrogen sources such as ammonium sulphate (1.5 gm/L), yeast extract (1.2 gm/L) were used (Arun sasi et al., 2010; Deunas et al., 1997).

Assay of cellulase activity with different physico-chemical parameters

The activity of cellulase was assayed with different physico-chemical parameters such as pH and temperature which essentially favoured for enzyme production. Different pH includes 3, 6, 9 and temperature includes 30, 40 and 50°C, respectively. After 6 days of incubation, culture filtrate was centrifuged at 10,000 rpm for 30 min. The supernatant was used as the source of cellulase and sugar estimated by dinitrosalicyclic acid method (Miller, 1959). After incubation the protein were determined by Lowry method (Lowry et al., 1959).

Secondary fine purification by HPLC system

The partially purified enzyme after ammonium sulphate precipitation was used for further purification of enzyme. The fine purification of the enzyme by HPLC system was carried out in Central Electrochemical Research Institute (CECRI), Karaikudi Sadasivam and Manikkam, 1997.

RESULTS AND DISCUSSION

Water samples were collected from five different sampling stations in Muthupettai mangrove along the east cost of Tamil Nadu, Southern part of India. In this present study, five species of *Aspergillus* spp namely, *A. oryzae, A. fumigatus, A. flavus, A. querinus* and *A. niger* were isolated and identified (Gillman, 1998) (Figure 1). Among the five fungal isolates, *A. flavus* exhibited prominent clear zone around the colonies after subjected to modified park's agar plates. Hence, this *A. flavus* were chosen for further studies (Figure 2).The effect of different carbon sources like rice bran, wheat bran, bagasse were used for cellulase enzyme production *A. flavus* as inoculum. The levels of reducing sugar and protein content were higher in case of rice bran (0.128 and 0.023 g/ml) (Figure 3). The concentrations of reducing sugar and protein content were found to be (0.097, 0.019 g/ml and 0.121, 0.013 g/ml) in case of wheat bran and bagasse respectively (Table 1) (Latif et al., 1995; Ojumul et al., 2003). As nitrogen sources are essentially important for cell growth, two different nitrogen sources such as ammonium sulphate and yeast extract were also applied for the optimization of cellulase production. *A. flavus* showed the highest production of cellulase enzyme utilizing ammonium sulphate as nitrogen source than yeast extract. Ammonium sulphate containing medium has shown 0.220 g/ml of reducing sugar and 0.053 g/ml of soluble protein. Yeast extract

Figure 3. Different substrates (wheat bran, bagasse, rice bran) used in the fermentation medium.

Table 1. Effect of carbon source (Rice bran, Wheat bran, Bagasse) on cellulase activity of *A. flavus.*

Substrate g/L	Concentration of R.S in g/ml	Concentration of S.P in g/ml
Rice Bran	0.128±0.060	0.023±0.010
Wheat bran	0.097±0.046	0.019±0.009
Baggase	0.121±0.057	0.013±0.006

R.S = Reducing sugar; S.P = Soluble protein.

Table 2. Effect of nitrogen source (ammonium sulphate and yeast extract) on cellulase activity (g/ml) of *A. flavus* using Rice bran, Wheat bran and Bagasse.

Substrate g/L	Ammonium sulphate		Yeast extract	
	R.S	S.P	R.S	S.P
Rice bran	0.220±0.103	0.053±0.025	0.124± 0.058	0.036±0.017
Wheat bran	0.149±0.070	0.040±0.019	0.101±0.047	0.011±0.005
Bagasse	0.106± 0.050	0.041±0.019	0.086±0.040	0.028±0.013

R.S = Reducing sugar; S.P = Soluble protein.

showed highest value of reducing sugar and soluble protein 0.124 and 0.036 g/ml (Table 2) (Gomes et al., 1992). As the effect of temperature and pH plays a vital role in the enzyme production, three different temperatures, such as 30, 40, 50°C and three different pH such as 6, 7 and 8 were chosen for the optimization studies. The highest production had been noticed at 30°C, in case of rice bran as production media with higher reducing sugar (0.228 g/ml) and protein content (0.39 g/ml). In case of 40 and 50°C the reducing sugar and protein content had been noticed as 0.198, 0.220 g/ml and 0.227, 0.099 g/ml, respectively (Table 3). In rice bran the amount of reducing sugar and soluble protein was found to be the highest of 0.229 and 0.121 g/ml on 7 days of incubation with pH 8. In wheat bran reducing sugar and soluble protein was found to be the highest at 0.227 and 0.106 g/ml (Keskar, 1992). In case of bagasse, the reducing sugar was found to be the highest of 0.204 g/ml and protein was found to be the highest of 0.120 g/ml (Table 4, Figure 2). The precipitate obtained by

Table 3. Effect of temperature on cellulase activity (g/ml) of *A. flavus* using Rice bran, Wheat bran and Bagasse.

Substrate	Temperature (°C)	3		5		7	
		R.S	S.P	R.S	S.P	R.S	S.P
Rice bran (g/ml)	30	0.180±0.084	0.077±0.036	0.163±0.076	0.156±0.073	0.229±0.108	0.236±0.111
	40	0.057 ±0.026	0.052±0.024	0.116±0.54	0.115±0.054	0.198±0.093	0.220±0.104
	50	0.046±0.021	0.057±0.027	0.084±0.039	0.112±0.053	0.142±0.067	0.188±0.088
Wheat bran (g/ml)	30	0.065±0.030	0.052±0.024	0.061±0.028	0.052±0.024	0.165±0.078	0.061±0.029
	40	0.040±0.018	0.032±0.015	0.044±0.021	0.044±0.020	0.093±0.044	0.054±0.025
	50	0.016±0.007	0.030±0.024	0.042±0.010	0.039±0.018	0.057±0.027	0.023±0.011
Bagasse (g/ml)	30	0.144±0.068	0.073±0.034	0.162±0.076	0.087±0.41	0.227±0.107	0.099±0.046
	40	0.123±0.058	0.058±0.027	0.068±0.032	0.056±0.026	0.127±0.029	0.085±0.040
	50	0.076±0.035	0.054±0.025	0.068±0.032	0.038±0.017	0.096±0.044	0.55±0.025

R.S =Reducing sugar S.P =Soluble protein.

Table 4. Effect of pH on cellulase activity (g/ml) of *A. flavus* using Rice bran, Wheat bran and bagasse.

Substrate	pH	3		5		7	
		R.S	S.P	R.S	S.P	R.S	S.P
Rice bran	6	0.123±0.062	0.009±0.004	1.58±0.074	0.028±0.013	0.198±0.093	0.088±0.41
	7	0.155±0.073	0.013±0.006	0.167±0.079	0.034±0.016	0.142±0.066	0.065±0.030
	8	0.049±0.023	0.021±0.009	0.180±0.089	0.050±0.023	0.229±0.107	0.121±0.057
Wheat bran	6	0.065±0.031	0.019±0.009	0.068±0.032	0.036±0.017	0.096±0.045	0.013±0.034
	7	0.080±0.037	0.223±0.034	0.084±0.039	0.045±0.021	0.123±0.058	0.060±0.08
	8	0.089±0.042	0.043±0.020	0.098±0.046	0.052±0.024	0.227±0.107	0.106±0.050
Bagasse	6	0.046±0.022	0.007±0.003	0.093±0.004	0.036±0.017	0.057±0.026	0.069±0.038
	7	0.103±0.048	0.012±0.005	0.110±0.051	0.045±0.021	0.093±0.044	0.063±0.09
	8	0.154±0.072	0.016±0.017	0.204±0.096	0.052±0.024	0.165±0.077	0.120±0.056

R.S =Reducing sugar S.P =Soluble protein.

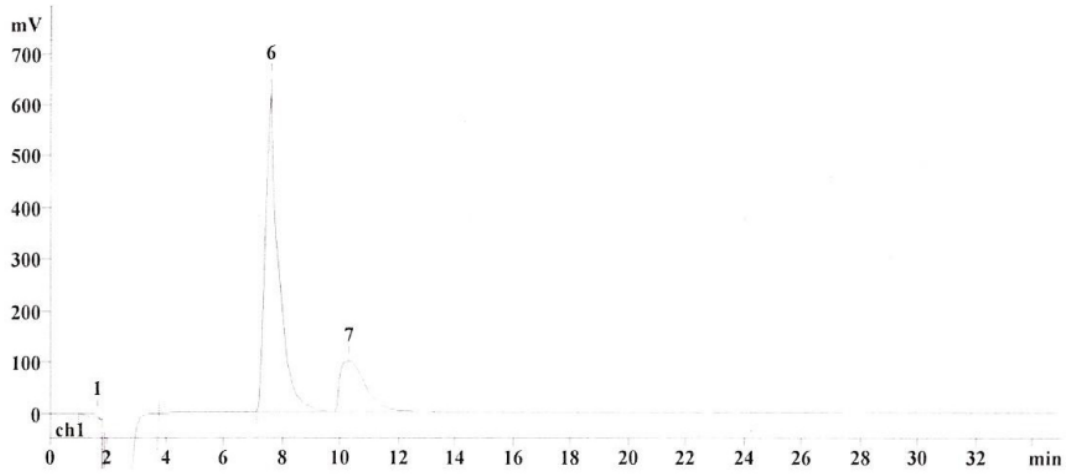

Figure 4. HPLC chromatogram of samples.

adding solid ammonium sulphate and incubated at 4°C for 6 h influenced the practical purification. The partially purified enzyme was subjected to fine purification by HPLC gradient system (Shimadu, Japan). The highest of the peaks indicated the purity. The peaks indicated that the enzyme produced from the rice bran with higher purity than other two substrates (Figure 4). These results were coinciding with the results obtained by (Sidhu et al., 1986; Kalra et al., 1986; Rincon et al., 2001).

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