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Full length Research Paper

Optimizing the conditions of - amylase by an Esturian strain of *Aspergillus* spp

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Water samples and sediments were collected from waterways in between two back water lakes namely Vambanad and Asthamudi, in Alukadavu, Kollam along the west coast of Kerala, India. 20 fungal species were isolated and identified by lacto phenol cotton blue staining method. From this, *Aspergillus flavus* was selected for starch hydrolysis Agar Medium for amylase activity under submerged fermentation for amylase production. The effect of different carbon source, nitrogen source, heavy metals, amino acids, dry biomass was studied and enzyme was optimized by using different physico-chemical parameters. The purity of enzyme was analyzed by thin layer chromatography. Minimal medium with starch has produced high amylase enzyme and soluble protein dry biomass with a temperature of 30°C and pH of 6, which includes 256.2 and 108 µg/ml, respectively.

Key words: Amylase enzyme, back water fungi, *Aspergillus flavus*, submerged fermentation, SDS-PAGE, Ion exchange chromatography.

INTRODUCTION

The backwater ecosystem is the second largest water resource after the marine water ecosystem in Kerala. They are open systems with respect to the energy and matter and thus Couple up the land between terrestrial and estuarine ecosystem (Lugo and Shedaker, 1974). Vambanad Lake is the largest fresh water resources in Kerala follow by Asthamudi Lake, which is the second largest freshwater resource along the west coast of India, Kerala. The study area is located in waterways in between two lakes namely Vambanad and Ashthamudi, in Alumkadavu, Kollam District along the west coast of India, Kerala. They are the storehouses of various fauna, which are dependent on products of microbial degradation of mangrove flora. This study was concentrated on mangroves which are particularly important in clean tropical water, where nutrient levels were usually low. These organic resources ultimately enrich the coastal ecosystem and in turn fisheries. The backwater ecosystem provides hostile habitats for a large number of organisms, including fungi (Singh et al., 2003). There are literatures on the ecology and taxonomy of soil fungi. The fungi were isolated and enumerated for the studies. Fungi are micro-organisms which are well known for their wide range of novelty of enzymes they produce and enzymes of fungal origin are used in the industrial process for which, amount to billions of dollars of revenue annually (Sasi et al ., 2008).

Enzymes are protein catalysts synthesized by living systems and are important in synthetic as well as degradative process. Amylases are enzymes that break down starch or glycogen. It is produced by a variety of living organisms ranging from bacteria, fungi to plants and humans (Pandey et al., 2000). Alpha amylase (endo-1,4-Dglucose-Dglucohydrolase 3.2.1.1.) belongs to the family of endo amylases that randomly cleave the 1,4-D glycoside linkage between adjacent glucose units in the product chain retaining the a-anomeric configuration in the product (Sasi et al., 2008; Pandey et al., 2001). It is therefore important to increase protein utilizing by all ways and means the increasing world demand for food and the need for feed protein led to the search for a non-conventional protein source to conventional protein

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source. A great deal of interest has been focused on the potential of converting protein from agricultural waste like coconucifera meal to microbial protein or single cell protein (Ravinder et al., 2004; Cushoma et al., 2005). The different meals were used as fermentation medium (Hirokilshide et al., 2004; Oshome et al., 2005; Heidelbergh et al., 2006). In the present study, the effect of different physico-chemical parameters, effect of various nitrogen sources, carbon source, heavy metals were also studied.

MATERIALS AND METHODS

Both water and sediment samples were collected from waterways in between two back water lakes namely Vambanad and Asthamudi, in Alumkadavu, Kollam along the west coast of Kerala, India. Water samples were collected near the coastal areas at a depth of 0.5 mm, once in a month (April and May, 2008). Besides this sediment, samples were also collected to isolate and enumerate the fungi. By employing spread plate technique, the collected samples were plated on Potato Dextrose Agar (PDA), Rose Bengal Agar (RBA), Sabourad's Dextrose Agar (SDA), with the addition of antibiotics (penicillin+ tetracycline). After incubation at 28ºC, the fungal colonies were identified (Kohlmayer and Kohlmayer, 1979; Gilmasn, 1959, 1998). 20 fungal species were isolated and identified.

Micro organisms

In the present study, 20 fungal species were isolated and enumerated namely *A. flavus, Aspergillus niger, Aspergillus oryzae, Aspergillus sulphureus, Aspergillus fumigatus, Aspergillus claratus, Aspergillus luchenis, Aspergillus ochesanus, Aspergillus fumiculosis, Aspergillus tencis, Trichoderma recsci, Trichoderma viridae, Penicillium citrinium, Penicillium oxalicum, Fusarium moniliformis, A.oxalicum* by Lactophenol cotton blue staining method (Kohlmayer and Kohlmayer, 1979; Gilmasn, 1959, 1998).

Screening for amylase producing fungi

The isolated strain was streaked into starch agar plate and incubated at room temperature for 72 h. After incubation, 1% of iodine solution was layered on the agar plates and zone of clearance was observed for screening the fungi (Pandey et al., 2006).

Submerged fermentation of amylase

Submerged fermentation was carried out in the Ehlenmeyer flasks by taking 100 ml of amylase production medium (Bernfed, 1951); containing Peptone (6.0 g/l), MgSO4 (0.5 g/l), KCl (0.5 g/l), Starch (1 g/l). In addition to this, certain agricultural waste products like coconut meal (coconut oil cake) were used as a submerged fermentation medium (Figure 1). Coconut meal was procured from local market in Pudukkottai, Tamil Nadu and India. 15 gm of oilcake was powdered, dry cake was taken. It was modified with certain carbon source, nitrogen source and heavy metals. Carbon sources (each 2 g/l) included are glucose, fructose, mannitol, mannose, starch, sucrose, lactose and dextrose. Nitrogen sources included are NH_4Cl, $NaNO_3$, NH_4NO_3, KNO_3, Peptone, Urea and Yeast extract
(2 g/l) as nitrogen source. Certain heavy (each 1 mm/l) metals like Ca^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, Hg^{2+}, Zn^{2+} Mn^{2+}, K^{2+} and P^{2+} were also included in the production medium. The medium was maintained at a pH range of 3, 6 and 9, at 30°C on a shaker with 120 rpm for 6 to 18 days (Heidelbergh and Springer, 2006; Pandey et al., 1999).

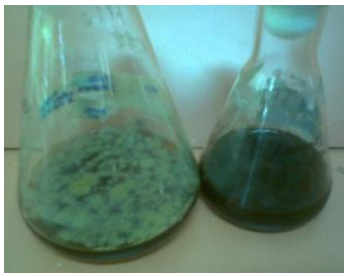


Figure 1. Submerged fermentation medium.

Enzyme extraction

Crude enzyme was extracted by mixing a known quantity of fermented substrate with distilled water containing 0.1%. Tween 80 on rotator shaker at 180 rpm/1 h. The suspension was then centrifuged at 7000 x g at 4°C and the supernatant was used for enzyme assay (Pandeyet al., 2006).

- amylase assay

-Amylase activity was determined (Pandey et al., 1999). Then reaction mixture containing 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 mM acetate buffer (pH 5.0) and 0.25 ml of crude enzyme extract was incubated for 10 min at 50°C. After incubation, the reducing sugar was estimated by dinitrosalicylic acid (DNS) method (Miller et al., 1959). For the estimation of enzyme, ready colour developed reaction mixture was taken at 575 nm using a Shimazhu-UV-1604 Spectrophotometer with glucose standard.

Estimation of soluble protein

Soluble protein concentration was determined in aqueous extract fermented substrate using Bovine serum as standard (Lowrey et al., 1951).

Molecular mass determination

The molecular weight was determined by SDS-PAGE (Sadasivam and Manikkam, 1997).

Dry biomass detection

After incubation, the growth medium was pasteurized at 65°C for 30 min in a waterbath. After each fermentation period, mycelia were removed from the flasks by passing through a dried and pre-weighed Whatmann no.1 filter paper and washed twice with sterile distilled water. The filter paper was dried at 90 - 100°C by using hot air oven. Then dry weight was obtained (Haltrich et al., 1996).

Ion exchange chromatography

In Ion exchange chromatography concentrated enzyme (2 ml) was loaded onto an anion exchange DEAE Sepharose FF (Sigma-Aldrich Co, USA) column (15 nm diameter and height 100 nm) at a

Figure 2. Collected soil samples.

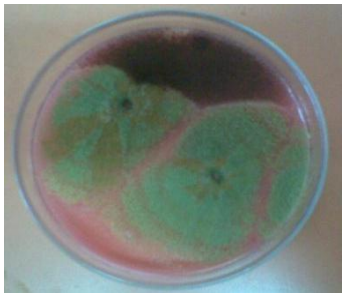


Figure 3. Mixed culture of fungi.

Figure 4a. Plate shows culture of *A. flavus*.

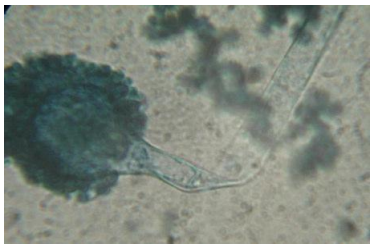


Figure 4b. Microscopic view of *A. flavus*.

flow rate of 0.5 ml/min. Equilibration and elution were performed first with 0.05 M Na- phosphate buffer to remove unbound proteins and then with a liner salt gradient from 0 to 3 M NaCl. Fraction (2 ml) were collected and analysed for amylase activity of protein content. Active fractions were pooled and concentrated and then

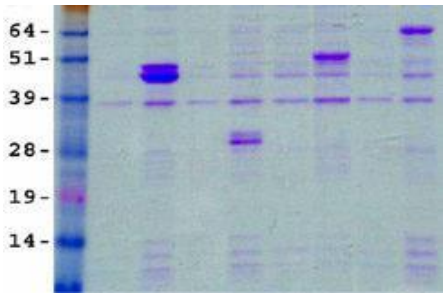


Figure 5. SDS-PHAGE.

purified using cation exchange CM Sepharose FFSigma-Aldrich Co, USA as above. The active fractions were pooled (Steven et al., 1977).

RESULTS AND DISCUSSION

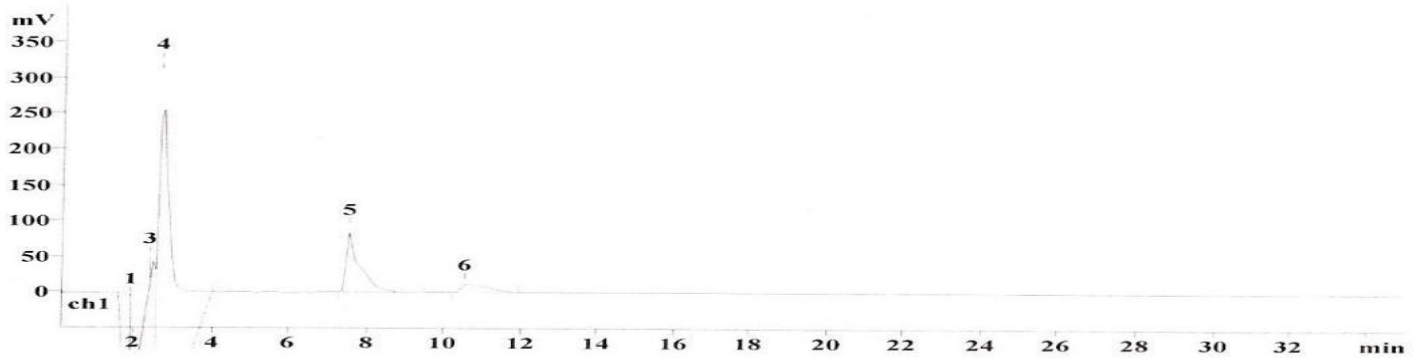
The soil and the sediment samples were collected from water ways in between two back water lakes namely Vambanad and Asthamudi, in Alumbukadavu, Kollam along the west coast of Kerala, India (Figure 2) where 20 fungal species were isolated and identified (Figure 3). From this, *A. flavus* was selected for further studies which exhibited clear zone around the colonies on Starch Hydrolysis Agar medium (Bernfed, 1951) (Figure 4a and 4b). Hence these organisms were selected for further studies. Minimal medium was modified with certain chemicals to alter the nitrogen and carbon source. Thus the medium became modified minimal medium (MMM). The MMM7 has shown highest amylase activity and soluble protein content among all other medium which included 81.2 and 80.32 µg/ml with controlled physico-chemical parameters like pH at 6°C and temperature of 30°C. But all other mediums like MMM, MMM2, MMM5, MMM3, MMM6 and MMM1 produced amylase enzyme of 172.3, 168.2, 152.2, 143.2, 140.2 and 132 µg/ml, respectively (Table 1) (Pandey et al., 2000a, 2000b).

But in case of carbon source MMM4 has shown highest amylase activity and soluble protein among all the other mediums 256.2 and 108 µg/ml. But remaining medium like MMM8, MMM10, MMM11, MMM9, MMM15, MMM13 and MMM12 included enzyme activity of 201.6, 197.2, 186.2, 180.2, 176.2, 170.3 and 168.2 µg/ml with protein content of 90.2, 80.2, 72.0, 68.2, 62.3, 60 and 58.2 µg/ml, which produced dry biomass of 102.0, 102, 0.96, 0.92, 0.83, 0.80, 0.78 and 0.70 µg/ml, respectively (Sumithtra et al., 2004) (Table 2).

The molecular weight was determined by SDS-PAGE, by comparing the migration of the corresponding sample gel against the standard protein (Figure 5) . Then the molecular weight of the amylase enzyme was determined as 1300 KDa (Sadasivam and Manikkam, 1997), the high peak in the ion exchange chromatography indicated the role of metal ions in the production medium (Pandey et al., 2006) (Figure 6).

Table 1. Amylase production (µg/ml) by *A. flavus* on minimal medium supplemented with additional nitrogen source (2 g/l) at temperature 30°C and pH 6.

Medium	Nitrogen source	Incubation period	pH enzyme activity (µg/ml)			pH protein (µg/ml)			Biomass (mg/ml)
			3	6	9	3	6	9	
		6	30.2	46.2	18.6	8.6	16.5	4.2	0.2
MMM1	NH_4Cl	12	42.3	62.4	26.3	16.2	26.5	10.2	0.70
		18	68.2	132.2	42.2	20.2	30.2	18.2	0.78
		6	22.4	51.2	10.6	6.2	26.4	6.3	0.82
MMM2	$NaNO_3$	12	36.2	72.4	22.6	14.2	37.2	10.8	0.86
		18	72.2	168.2	32.6	26.2	62.2	20.4	0.92
		6	16.2	42.2	8.6	5.4	10.8	4.3	0.80
MMM3	NH_4NO_3	12	32.4	62.2	12.6	18.2	26.2	6.4	0.81
		18	40.6	143.2	30.2	25.2	42.2	16.2	0.82
		6	10.6	41.3	18.2	8.8	36.2	9.2	0.92
MMM4	KNO_3	12	24.6	64.2	26.2	16.8	56.2	12.3	0.86
		18	42.2	172.3	38.6	28.6	70.2	18.2	1.03
		6	18.4	62.2	16.4	6.2	16.2	10.4	0.79
MMM5	Peptone	12	28.2	72.2	26.4	14.6	28.2	16.4	0.81
		18	46.2	152.2	40.2	24.2	50	22.2	0.83
		6	20.6	42.4	12.4	5.2	16.4	8.4	0.72
MMM6	Urea	12	29.4	64.2	20.6	16.8	20.2	15.4	0.68
		18	50.2	140.2	38.4	27.2	40.0	20.2	0.80
		6	26.3	32.4	22.4	4.2	42.2	18.4	0.93
MMM7	Yeast extract	12	32.4	68.4	38.4	18.6	68.6	24.2	0.88
		18	54.2	181.2	181.2	30.2	80.3	28.2	1.08



Sample Id	Element	Retention Time	Area	Concentration of inject	Dilution
Std	Na	7.61	810	10 ppm	
Std	K	11.06	434	10 ppm	
Nafeel-2	Na	7.47	2058.785	2540 ppm	50ml/0.5ml
Nafeel-2	K	10.49	546.299	1258 ppm	50ml/0.5ml

Figure 6. Ion exchange chromatography.
Co and mg are below detection limit.

Table 2. Amylase production (µg/ml) by *A. flavus* on minimal medium supplemented with additional carbon source (2 g/l) at temperature 30°C and pH 3, 6 and 9.

Medium	Carbon source	Incubation period	pH enzyme activity (µg/ml)			pH protein (µg/ml)			Biomass (mg/ml)
			3	6	9	3	6	9	
MMM8	Glucose	6	30.2	40.8	18.4	18.4	26.8	9.6	0.92
		12	62.2	108.3	24.6	30.2	46.8	8.6	0.91
		18	80.3	201.6	70.4	27.6	90.2	18.4	1.02
MMM9	Fructose	6	18.6	40.3	15.4	14.6	28.6	10.2	0.81
		12	22.6	106.3	20.6	20.3	46.3	14.2	0.76
		18	40.8	180.2	80.7	26.2	68.2	18.8	0.83
MMM10	Dextrose	6	22.2	34.2	18.4	10.2	18.6	9.6	0.82
		12	34.8	80.3	28.3	20.4	46.8	12.3	0.84
		18	78.3	197.2	68.2	32.2	80.2	18.4	0.96
MMM11	Lactose	6	18.4	46.2	13.4	10.6	29.3	8.6	0.72
		12	26.3	98.4	46.2	18.6	48.2	14.2	0.84
		18	67.2	186.2	58.4	28.4	72.0	20.2	0.92
MMM12	Mannitol	6	20.3	28.2	18.6	8.2	19.4	7.4	0.68
		12	29.3	60.2	22.2	16.8	34.6	16.1	0.67
		18	60.4	170.3	48.6	30.4	58.2	28.4	0.70
MMM13	Mannose	6	28.2	40.2	18.6	8.2	20.6	10.2	0.67
		12	32.4	103.2	28.3	28.2	40.3	16.2	0.72
		18	70.2	176.2	43.4	30.2	60.0	28.4	0.78
MMM14	Starch	6	41.2	80.4	30.6	20.8	48.2	20.4	0.98
		12	60.4	131.2	45.2	30.6	68.4	28.6	0.90
		18	40.4	256.2	80.3	48.2	108.0	30.8	1.42
MMM15	Sucrose	6	22.4	40.2	16.4	8.2	26.2	8.4	0.72
		12	30.2	62.2	24.2	14.4	44.6	10.6	0.76
		18	42.4	176.2	38.4	20.6	62.3	18.6	0.80

The present study indicated that *A. flavus* produced high amount of amylase and soluble protein and dry biomass in minimal medium, which has been modified with certain carbon and nitrogen source. So it is concluded that minimal medium can be used under submerged fermentation for the production of amylase under controlled conditions with a pH of 6 and at a temperature of 30°C (Figure 4).

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