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Full Length Research Paper

Purification and characterization of extracellular amylolytic enzyme from *Aspergillus* species

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In the present study, the amylase enzyme producing potential of four different *Aspergillus* species was analyzed. The extracted amylase enzyme was purified by diethyl amino ethyl (DEAE) cellulose and Sephadex G-50 column chromatography and the enzyme activity was measured by using synthetic substrate starch. The partially purified enzyme exhibits maximum activity at the optimum pH (7.0), temperature (60 to 70°C) and substrate concentration (1.5 to 2.0%) under standard assay conditions. Among the four different *Aspergillu*s species examined, *Aspergillu*s *flavipes* showed maximum production of amylase. The characteristics of the partially purified enzyme such as optimum pH and temperature were also favourable for industrial applications.

Key words: *Aspergillus* species, Sephadex G-50, column chromatography, diethyl amino ethyl (DEAE) cellulose.

INTRODUCTION

Enzymes are biological catalysts which regulate specific biochemical reactions. In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Alva et al., 2007). Among the industrially important enzymes, proteases and amylases are considered to be the most prominent enzymes since they are widely utilized in brewing, detergent and food industries. Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents (Khan and Priya, 2011).

The starch degrading amylolytic enzymes have a great commercial value in biotechnological applications ranging from food, fermentation, textile to paper industries (Pandey et al., 2000). Two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase (Jespersen et al., 1991). Among various extracellular enzymes, α-amylase ranks first in terms of commercial exploitation (Kim et al., 1999).

α- Amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are one of the most widely enzyme complexes in animals, higher and lower plants, and microbes. Because of α-amylases important biochemical roles in organism growth and development, these enzymes from different origins including bacteria, nematodes, mammals and insects have been purified and their physical and chemical properties characterized (Ravan et al., 2009). Although, amylases can be obtained from several sources, such as plants, animals and microorganisms, the enzymes from microbial sources generally meet industrial demand.

Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. Fungal amylase is preferred for use in formulations for human or animal consumption involving applications under acidic conditions and around 37°C (Gupta et al., 2003). Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation and high production of extracellular enzymes of large industrial potential. Today, a large number of microbial α-amylases are marketed with applications in different industrial sectors and they have almost completely replaced chemical hydrolysis of

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starch in starch processing industry (Pandey et al., 2000; Patel et al., 2005). Each application requires unique properties with respect to specificity, stability, temperature and pH dependence. These applications require enzymes with high activity and thermostability. There are many advantages of using thermostable enzymes, such as an increased reaction rate and decreased contamination risk through the use of high temperatures (Metin et al., 2010). This study reports the partial purification and characterization of extracellular amylases from four different *Aspergillus* species.

MATERIALS AND METHODS

Four different *Aspergillus* species, including *Aspergillus versicolor*, *Aspergillus nidulans*, *Aspergillus flavipes* and *Aspergillus wentii* were obtained from the Department of Microbiology, Rathanavel Subramanium College of Arts and Science, Sulur, Coimbatore, Tamilnadu. Cultures were maintained by using Potato Dextrose agar media under 30°C for seven days and stored at 4°C.

Enzyme production

The mycelia mass of the above mentioned 7 days old cultures were transferred to enzyme production medium amended with 10% starch. The culture flasks were incubated for 7 days at 30°C on an orbital shaker (150 rpm). After incubation period, the cultures were filtered over a Whatman No.1 filter paper and dried to a constant weight at 80°C in a hot air oven. The dried fungal biomass was used for the determination of mycelia growth. The culture filtrate was centrifuged at 10.000 rpm for 20 min at 4°C. The clear supernatant obtained was used as crude amylase enzyme for further analysis. The enzyme activity was determined by using the Somogyi-Nelson's method (Nelson, 1944; Somogyi, 1952). One unit of amylase activity was defined as the amount of enzyme that liberates 1 mmol of reducing sugar (as glucose) per minute under the standard assay condition.

Purification of α-amylase

Preparation of cell-free broth

The culture filtrates of *Aspergillus* species were purified by ammonium sulfate 30% to gradually increase up to 70% saturation, column chromatography (DEAE cellulose) and gel filtration (Sephadex G-50). After ammonium sulfate saturation, protein precipitation was centrifuged at 10. 000 xg for 15 min at 4°C. The pellet was then dissolved in 0.1 M phosphate buffer (pH 6.9) and dialyzed against the same buffer overnight. The undissolved material in the dialysate was removed by centrifugation at 15.000 x*g*, and then the supernatant was pooled and designated as cell free broth. The cell free broth was frozen at -20°C for further purification steps.

DEAE- cellulose column

The cell-free broth was loaded on a DEAE- Cellulose column (5 × 20 cm glass column, flow rate 10 ml/1 h) equilibrated with 0.01 M phosphate buffer (pH 6.9). The activity fractions were collected separately and their enzyme activity was analyzed. Protein fractions exhibiting α-amylase activity were pooled in three peaks.

Gel filtration on Sephadex G-50 column

Amylase containing fractions (highest activity) were concentrated through ultrafiltration membrane cut-off 10 kDa and loaded on Sephadex G-50 column (2.5 × 10 cm, flow rate 5 ml/1 h) equilibrated with 0.05 M Tris-HCL buffer (pH 7.0). The active fractions were analyzed in standard assay condition and expressed in IU/ml.

Protein determination

Protein concentration was measured according to the study of Lowry et al. (1951) with bovine serum albumin as standard.

Characterization of purified enzyme

The purified enzyme was used for the determination of optimum temperature, pH and substrate concentration. To determine the optimum pH for the maximum amylase activity, enzyme assay was done at different pH levels (3.0 to 10.0). The amylase activity was also measured in the temperature range of 30 to 90°C to determine the temperature optima. The optimum substrate concentration for maximum enzyme activity was determined in terms of maximum reaction velocity (Vmax) and Michaelis constant (Km). Various concentrations of starch (0.5 to 4.0%) were incubated with purified enzyme and enzyme activity was determined.

RESULTS AND DISCUSSION

Enzymes are substances present in the cells of living organisms in minute amounts and are capable of speeding up chemical reactions (associated with life processes), without themselves being altered after the reaction. The sources of α-amylases are quite diverse such as plants, animals and microbes. The major advantages of using microorganisms for production of amylases are the bulk production capability and to obtain enzymes of desired characteristics (Aiyer, 2005). To meet the growing demands in the area of industry, it is essential to improve the performance of enzyme extraction techniques and thus increase the yield without increasing the expenses of production. α-Amylase purification has mainly been restricted to a few species of fungi (AbouZeid, 1997).

The mycelia dry weight of currently studied four different *Aspergillus* species ranged between 145 and 194 mg and *A. versicolor* was found to produce highest mecelia biomass after seven days incubation period. The soluble protein content of crude enzymes extracted from four *Aspergillus* species ranged between 2.49 and 2.98 mg/ml with *A. flavipes* and *A. nidulans*. The result analysis of enzymes activity of crude enzyme revealed that the enzyme extracted from *A. wentii* exhibited the highest enzyme activity (298.1 IU/ml) followed by *A. flavipes* (269.41 IU/ml), *A. nidulans* (248.12 IU/ml) and *A. versicolor* (174.18 IU/ml) (Tables 1 to 4). After purification through (DEAE cellulose and Sephadex G-50) column chromatography, the enzymes extracted from *A. flavipes* and *A. versicolor* were found to contain highest protein

Table 1. Purification profile of extracellular amylase produced by *A. versicolor*.

Step	Total volume (ml)	Enzyme activity (IU/ml)	Soluble protein (mg/ml)	Total protein (mg/ml)	Total enzyme activity (IU)	Specific activity (IU/mg/ protein/ml)	Purification (fold)
Culture filtrate	100	174.18	2.84	284	17.418	61.33	1.00
Ethanol precipitation	40	106.01	1.24	49.6	4240.4	85.49	1.39
DEAE cellulose	20	69.54	0.624	12.48	1390.8	111.44	1.81
Sephadex G-50	14	8.49	0.049	0.735	118.86	161.71	2.63

Table 2. Purification profile of extracellular amylase produced by *A. nidulans*.

Step	Total volume (ml)	Enzyme activity (IU/ml)	Soluble protein (mg/ml)	Total protein (mg/ml)	Total enzyme activity (IU)	Specific activity (IU/mg/ protein/ml)	Purification (fold)
Culture filtrate	100	248.12	2.98	298	24812	83.26	1.00
Ethanol precipitation	46	171.48	1.41	64.86	7888.08	121.61	1.46
DEAE cellulose	20	80.19	0.618	12.36	1603.8	129.75	1.55
Sephadex G-50	12	7.48	0.051	0.612	89.76	146.66	1.76

Table 3. Purification profile of extracellular amylase produced by *A. flavipes*.

Step	Total volume (ml)	Enzyme activity (IU/ml)	Soluble protein (mg/ml)	Total protein (mg/ml)	Total enzyme activity (IU)	Specific activity (IU/mg/protein /ml)	Purification (fold)
Culture filtrate	100	269.41	2.89	289	26941	93.22	1.00
Ethanol precipitation	43	168.31	1.32	56.76	7239.33	127.54	1.36
DEAE cellulose	20	74.35	0.542	10.84	1487	137.17	1.47
Sephadex G-50	15	11.16	0.056	0.84	167.4	199.28	2.1

content (0.056 and 0.051 mg/ml, respectively) and enzyme activity (11.46 and 8.49 IU/ml) as compared to other species of the present study.

The properties of purified amylase enzyme like optimum pH, temperature and substrate concentration are given in Tables 5 to 7, respectively. The enzyme extracted from four different *Aspergillus* species exhibits maximum activity at pH 7.0. The selection of optimum pH is very essential for the production of amylase (Pugalenthi et al., 2011). Maximum enzyme production of enzyme occurred at pH 7, very little growth was observed without enzyme production in medium at initial pH of 3 to 4. α -Amylases are generally stable over a wide range of pH from 4 to 11, though some are only stable within a narrow pH range (Kumar et al., 2012). These results suggest that the enzyme was not very sensitive to the change of pH. Therefore, the enzyme may have widespread application in many industrial sectors.

The optimum temperature for the maximum activity of the purified amylase enzyme ranged from 60 to 70°C. Among the different species studied, *A. flavipes* was found to produce thermostable enzyme with temperature optima at 70°C. Results of the present study are in agreement with earlier report of Pugalenthi et al. (2011). The optimum temperature for α-amylases from fungal and yeast sources has generally been

Table 4. Purification profile of extracellular amylase produced by *A. wentii*.

Step	Total volume (ml)	Enzyme activity (IU/ml)	Soluble protein (mg/ml)	Total protein (mg/ml)	Total enzyme activity (IU)	Specific activity (IU/mg/ pro/ml)	Purification (fold)
Culture filtrate	100	298.1	2.49	249	29810	119.71	1.00
Ethanol precipitation	36	172.3	1.12	40.32	6202.8	153.83	1.28
DEAE cellulose	20	81.45	0.482	9.64	1629	168.98	1.41
Sephadex G-50	10	8.21	0.048	0.48	82.1	171.04	1.42

Table 5. Effect of pH levels on the activity of the purified amylase produced by the different *Aspergillus* sp. studied.

Microorganism	Enzyme activity (IU/ml)							
	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
A. versicolor	16.43	27.32	45.67	57.23	63.21	51.70	40.67	31.45
A. nidulans	19.25	34.20	46.71	52.17	72.30	54.10	42.78	35.42
A. flavipes	21.26	46.12	55.24	67.27	52.22	44.80	32.17	22.05
A. wentii	20.21	39.39	52.17	63.45	54.15	41.72	29.27	20.51

Table 6. Effect of temperature on the activity of the purified amylase produced by the different *Aspergillus* sp. studied.

Microorganism	Enzyme activity (IU/ml)						
	30°C	40°C	50°C	60°C	70°C	80°C	90°C
A. versicolor	22.43	40.31	51.21	60.56	52.43	46.47	35.78
A. nidulans	25.24	40.37	52.11	62.75	53.30	48.71	39.50
A. flavipes	26.15	42.11	54.24	65.15	51.10	43.77	31.18
A. wentii	30.14	35.24	53.42	63.48	57.65	47.80	40.43

Table 7. Effect of substrate concentration on the activity of the purified amylase produced by the different *Aspergillus* sp. studied.

Microorganism	Enzyme activity (IU/ml)							
	Substrate concentration: 0.5 to 4%)							
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
A. versicolor	20.23	35.45	46.98	53.54	41.42	37.23	31.89	26.50
A. nidulans	23.31	32.59	40.08	51.74	40.25	35.30	30.11	23.41
A. flavipes	21.14	31.52	36.48	43.24	38.12	31.34	28.87	22.20
A. wentii	24.29	31.48	39.54	48.81	40.97	35.83	30.90	23.20

found to be between 30 and 70°C (Gupta et al., 2003). Optimum temperatures for α-amylase in earlier studies on the *Penicillium* species were reported to be between 30 and 60°C (Doyle et al., 1988; Ertan et al., 2006).

The α-amylase activity reached the maximum with an optimum substrate (starch) concentration of 2% with enzyme activity of 53.54 IU/ml. Any increase or decrease of substrate concentration gave a corresponding decrease in α-amylase activity (Table 7). Kuiper et al. (1978) reported that the maximum activity of α-amylase enzyme was obtained at 1.67% of substrate (starch) concentrations. In addition to that Abd El-Rahman (1990) concluded that the optimal concentration of starch for maximum α-amylase activity was between 2 and 3%. Moreover, El-Safey (1994) reported that the optimal substrate (starch) concentration in reaction mixture of the MM-α-amylase enzyme was found to be 0.1% (w/v), corresponding to 2% (w/v) for RH-α-amylase enzyme. Hence, the amylase produced by *Aspergillus* species (*A. nidulans, A. wentii, A. flavipes* and *A. versicolor*) will be suitable for industrial applications. Among the four presently studied different species of *Aspergillus* for their amylase production potential, *A. flavipes* appear to be better source, which are found to produce high quantity of enzyme with maximum activity. Other characteristics of the partially purified enzyme such as optimum pH and temperature are also favorable for industrial application.

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