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Amylase from an Estuarine *Bacillus megaterium*

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Abstract: The main aim of this study is to isolate and identify the amylase producing bacteria from the estuarine environment. The water and sediment samples were collected from the uppanar estuary and the potential strains of amylase production were screened in starch agar plates. Totally 12 strains were collected, among them *Bacillus megaterium* exhibited the larges zone. Hence these potential strains were used for further optimization, production and purification of amylase enzyme. Optimizations and enzyme estimation were done through various factors. Here the enzyme productions were maximum at 35ºC, pH 7.5 and 30 ppt salt concentration.

Key words: Isolation, optimization, purification, screening

INTRODUCTION

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. They accelerate the velocity of the reaction without necessarily initiating it (Pelczar *et al*., 1986). There are three major sources of enzymes (Burhan *et al*., 2003), that is derived from a variety of plants, e.g., pappain, animal enzymes - derived from animal glands, e.g., trypsin, pepsin Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable (Burhan *et al*., 2003). Amylase enzyme is widely distributed in various bacteria, fungi, plants and animals and has a major role in the utilization of polysaccharides (Shaw *et al*., 1984; Reddy *et al*., 1987; Tomita *et al*., 1990; Ilori *et al*., 1997; Ribeiro *et al*., 2000; Hagihara *et al*., 2001; Zoltowska, 2001; Bassinello *et al*., 2002; Haq *et al*., 2003).

Enzymatic hydrolysis of starch by α-Amylase (1, 4 - α-D-glucan glucanohydrolase; EC 3.2.1.1), which catalyzes the hydrolysis of internal (endoglycosidase) α-1, 4-glucanlinks in polysaccharides containing 3 or more α-1, 4-linkedD-glucose units (amylase and amylopectin) yielding a mixture of maltose and glucose, has wide applications in industry. It is used in brewing and fermentation industries for the conversion of starch to fermentable sugars (Chi *et al*., 1995; Farid *et al*., 2002), in the textile industry for designing textiles and in the laundry industry in a mixture with protease and lipase to launder clothes (Achle, 1997), in the paper industry for sizing (Whistler *et al*., 1984; Van der Maarel *et al*., 2002), and in the food industry for preparation of sweet syrups (Bello-Perez *et al*., 2002; Rao and Satyanarayana, 2003), to increase diastase content of flour, and for the removal of starch in jelly production. After the addition of the α-amylase in the bread-baking process, the bread's volume increased and kept its softness longer (Ammar *et al*., 2002) Wide ranges of microorganisms producing amylases having different specificities, properties and action patterns have been reported (Hansen *et al*., 1994; Bibel *et al*., 1998; Talamond *et al*., 2002). Alkaline α-amylases is more useful as working pH of detergents is between 8-11 (Ito *et al*., 1998) Amylases are starch-degrading enzymes of industrial importance (Reed, 1975). In the present study, an attempt has been made to screening, optimization, production and partial purification of amylase enzyme from novel marine bacterium.

MATERIALS AND METHODS

Study area:

Uppanar estuary: The Uppanar estuary is situated at Cuddalore in Tamil Nadu,India which is about 25 km away from the Parangipettai coast. It is formed by the confluence of Gadilam and Paravanar rivers. The former originates from the foothills of northeastern part of the Shervarayan hills and later from Vridhachalam Taluk of Cuddalore district.

Collection of samples: The surface water samples were collected using pre sterilized sample bottles allowing enough air space in the bottles to facilitate thorough mixing and Sediment samples were collected using a sterile spatula. The central portion of the collected samples was aseptically transferred in to a sterile polythene bags .The samples were collected from uppanar estuary.

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Isolation and identification of amylase producing bacteria: 1 g/L mL of sample was suspended in 99 mL sterile 50% aged sea water, agitated for 45 min. in a shaker at 50ºC and 0.1 mL was spreaded on Starch agar. Starch10 g, peptone 5 g, yeastextract-3 g, $NaCl_3$ g, $K_2HPO_4^{-1}$ g, $MgSO_4$-0.2 g, agar-20 g, distilled water-1000 mL (50% aged seawater), pH-7.0. plates and incubated at 30ºC for 48 h. Strains with distinct zone of clearance were selectively isolated and the one with maximum activity was identified up to species level following Bergey's manual of Determinative bacteriology (Buchanan *et al*., 1974) and the identified strain was stored in starch agar slant for further studies.

Estimation of crude extracellular amylase enzyme activity: Samples collected at different intervals during the culture were centrifuged and the cell free culture broth was used as crude enzyme. In a test tube 500 µL of crude enzyme, 250 µL of 1% starch solution and 250 µL of tris HCl buffer were added and incubated for 10 min. at 60ºC. The reaction was stopped by adding 1mL of Dinitro salicylic acid reagent and optical density was measured at 540 nm. 1 unit of activity was defined as the amount of enzyme that catalyzed the liberation of reducing sugars equivalent to 1 µmol of maltose/min. under the assay conditions.

Optimization:

Optimum temperature for amylase production: The optimum temperature of maximum amylase production was determined by varying incubation temperature of the culture from 25-50ºC with an interval of 5ºC in a 250 mL Erlenmeyer conical flasks containing 100 mL of the production medium and inoculated with the 50 µL culture of starch and incubated at 37ºC for 48 h.

Optimum pH for amylase production: To determine the optimum pH, the bacterial culture was inoculated in a set of different pH ranging from 6.5-9.5 with the interval of 0.5. in a 250 mL Erlenmeyer conical flasks containing 100 mL of the production medium and inoculated with the 50 µL culture of starch and incubated at 37ºC for 48 h.

Effect of various carbon sources: The effect of different carbon sources on amylase production was studied at 37ºC using various substrates such as starch, glucose, sucrose, maltose and raffinose in a 250 mL Erlenmeyer conical flasks containing 100 mL of the production medium and inoculated with the 50 µL culture of starch and incubated at 37ºC for 48 h.

Effect of substrate concentration: The effect of substrate concentration on amylase production was studied by changing the concentration of starch from 0.5 to 2.5% with an interval of 0.5% in a 250 mL Erlenmeyer conical flasks containing 100 mL of the production medium and inoculated with the 50 µL culture of starch and incubated at 37ºC for 48 h.

Effect of organic nitrogen sources: Two different nitrogen sources such as peptone and yeast extract were added separately to the medium at 0.5-2.0% concentration to access the maximum enzyme production in a 250 mL Erlenmeyer conical flasks containing 100 mL of the production medium and inoculated with the 50 µL culture of starch and incubated at 37ºC for 48 h.

Production and partial purification: In the initial purification step, the supernatant fluid containing the extra cellular enzyme was treated with solid ammonium sulphate as described by Green and Hughen's (1955), with continuous overnight stirring and separated in to the following saturation, ranging from 0-20, 20-40, 40-60 and 60-80%. The precipitates collected by centrifugation (10,000 rpm for 15 min.) were dissolved in 0.1 m citrate phosphate buffer (pH 5.0). The enzyme solution was dialyzed against the same buffer for 12 h with several changes to remove the salt and assayed by the method described by Plumer (1978).

RESULTS AND DISCUSSION

The strain isolated with maximum activity was identified as *Bacillus megaterium* (Fig. 9) using Bergy's manual. When strain was optimized against various parameters, the growth was found to be maximum at the following conditions with starch as a substrate (3% of starch), pH 7.5, temperature (35ºC) and 3% salt concentrations (Fig. 2, 4, 6 and 8). Enzyme production was maximum at 35ºC, starch as a substrate when it is used as 3%, pH 7.5 and 3% salt concentrations (Fig. 1, 3, 5 and 7). The growth was found to be minimum when temperature kept at 50ºC, pH 8.5, 0.1 of substrate concentration and 0.5% of salt concentration, where as the minimum of enzyme production was observed at 30ºC,

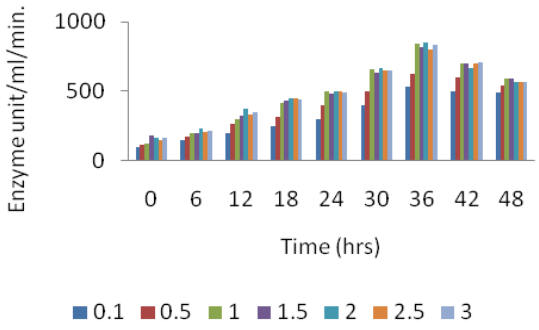

Fig: 1: Effect of substrate concentration for enzyme activity

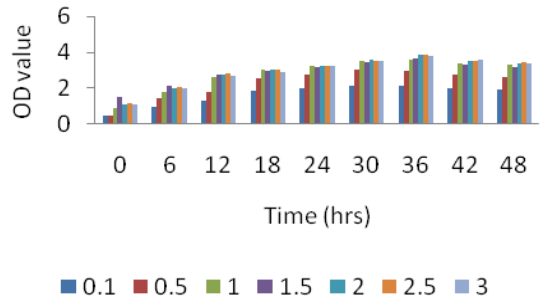

Fig. 2: Effect of substrate concentration growth

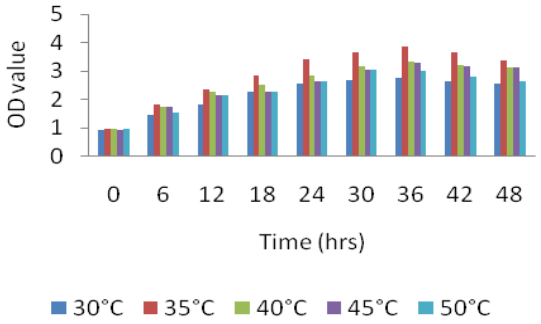

Fig. 6: Effect of Temperature for growth

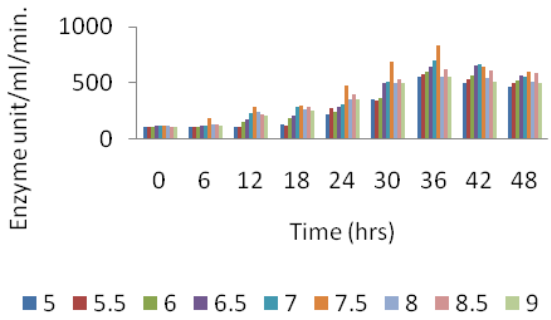

Fig. 3: Effect of pH for enzyme activity

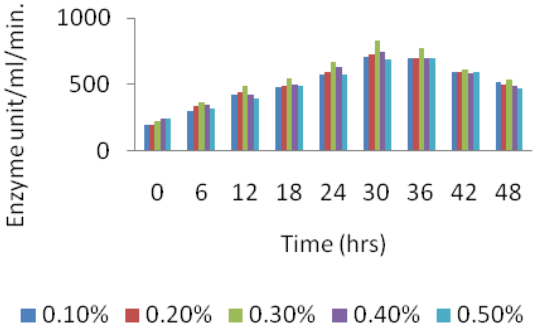

Fig. 7: Effect of salt concentration for enzyme activity

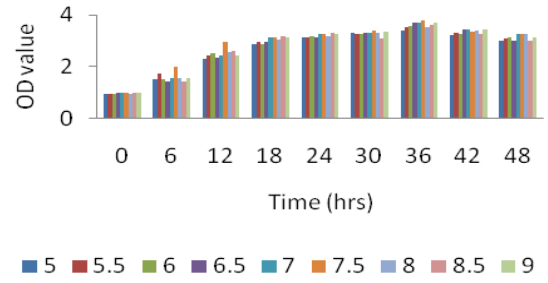

Fig. 4: Effect of pH for growth

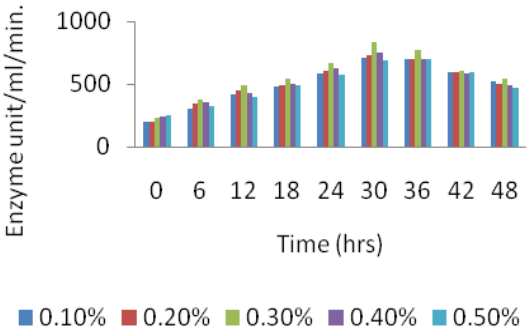

Fig. 8: Effect of salt concentration for enzyme activity

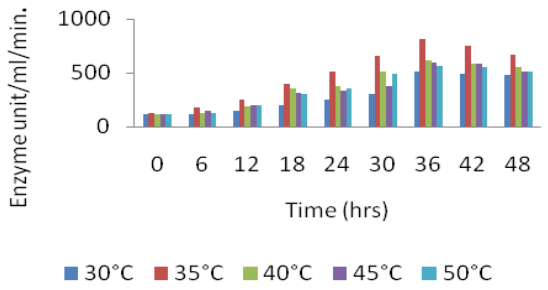

Fig. 5: Effect of Temperature for enzyme activity

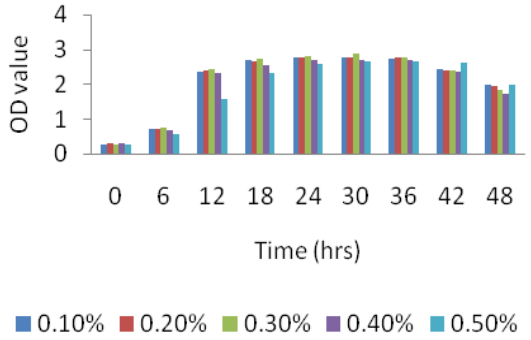

Fig. 9: Effect of salt concentration for growth

Fig. 10: Crude enzyme shows amylase activity

pH 8.5, 0.1 of substrate concentration and 0.5 % of salt concentration. The activity of the enzyme was compared with the works done by Stamford *et al*. (2001). The activity of the amylase enzyme by *Nocardia* spp was detected over a wide range of assay (i.e.) pH level (3.0-8.0) and the enzyme activity was optimal when the assay was carried out at pH 5. The maximum activity of amylase had been reported to vary between 30 and 60ºC (Giraud *et al*., 1993; Shih and Labbe, 1986). Omemu *et al.* (2005), observed an increase in enzyme activity progressively with increase in temperature from 20ºC which reaches maximum at 60ºC. Above 60ºC there was a reduction in the amylase activity. Mabrouk *et al*. (2008) observed maltogenic amylase produced from acidic *Bacillus* spp. US149 and maximum enzyme activity was obtained at 40ºC and pH 6.5. However this phenomenon seems to be strain dependent. The optimum pH observed by them was pH 4.0, after which a continuous decrease in enzyme activity was noted. Amylase activity increased with increase in the starch concentration from 1 to 3% (Fig. 10). Beyond 3% there was a decline in amylase activity. However the present study showed maximum activity at 3% starch. Ensley *et al*. (1975) found that the synthesis of amylase was greatly suppressed when the bacterium was grown either on glucose, maltose or sucrose, and amylase production was good when the bacterium was grown on starch or other polysaccharides. He reported slowest growth rate and amylase production when fructose was used in Cl*ostridium acetobutylicum.* Amylase production was enhanced when the bacterium was grown on starch, dextrin, maltose, xylose and lactose. These sugars were found to be inducers of the enzyme. However the enzyme yield with these substrates was lower compared to starch and dextrin. In the present study also growth increased up to 36hrs where as enzyme yield decreased beyond 36 h. However a perfect inverted ratio was not observed. These findings are in partial agreement with the present report. In their work, with an increase of starch concentration in the medium beyond 1% enzyme production did not increase. In the present study maximum activity was observed at 3% starch and minimum was observed when the concentration was increased to 0.1% (i.e.) beyond 1% the activity started decreasing. The extracted sample was divided equally (app. 20 mL) and purified through the different concentration of ammonium sulphate precipitation. The 20% of saturation was resulted in the yield of 0.13 g of enzyme powder where as at 40, 60, 80% of respectively 0.30, 0.19 and 0.14 g were obtained. The purified enzyme powder was checked for the activity on starch cleaving. It was noted that the enzyme purified from the 40% of ammonium sulphate saturation gave the maximum activity on starch plate assay with iodine and it was comparable to the commercially available amylase. The starch concentration in the fermentation broth dropped to almost to zero in 24 h. The pH of the broth increased from 7 to 8.3 at the end of the fermentation. In *Bacillus subtilis* and *Bacillus stearotherrmophilus* enzyme production parallelled with cell mass. Bajpai *et al.* (1989) have demonstrated maximum amylase production after the cell population reaching its peak. In the present study enzyme activity peak was obtained prior to the peak of cell mass.

CONCLUSION

The present study is an attempt for the isolation of a potential amylase producing bacterial strain from an estuarine environment. Samples were collected from Uppanar estuary, the potential strains for amylase production were screened in starch agar plates. From the 12 strains isolated *Bacillus megaterium* was selected as the most potential strain for amylase production. When the strain was optimized against various parameters, the growth was found to be maximum with starch as a substrate (2% of starch), pH 7.5, temperature 35ºC and 0.3% salt concentrations. Enzyme production was maximum at 35ºC, pH 7.5, starch as a substrate when it was used as 1 and 0.5% salt concentration. The growth was found to be minimum when maltose was used as a carbon source, temperature at 50ºC, pH 5, 0.5% of salt concentration and 0.1% of substrate concentration. Regarding enzyme the minimum production was observed at 50ºC, pH 5, 0.5% of salt concentration and 0.1% of substrate concentration. The present study can be given the observation on amylase production using microbial sources isolated from estuarine environment. The activity of the partially purified amylase enzyme showed the considerable activity. The molecular weight determination and amylase production using cheaper sources needed in further studies.

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