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Full Length Research Paper

Studies on amylase activity of an amylolytic bacterium isolated from estuarine soil

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Diverse microscopic, macroscopic and biochemical analysis of a starch degrading amylolytic bacterial strain isolated from the soil sample of Rajakkamangalam estuary, Kanyakumari district, Tamil Nadu, India, revealed its identity to the genus *Bacillus.* Maximum growth was observed at 12 h when the bacteria was cultured in minimal agar media with 2% starch maintained for 12 h, at 37°C and pH 5.5, 7.5 and 8.5. Highest enzyme action as revealed by glucose production in the media was observed at pH 8.0, temperature 30°C and in the presence of 10 mM calcium (Ca). Enzyme activity gradually got reduced with the addition of increasing concentrations of ethylenediaminetetraacetic acid (EDTA), confirming the need for calcium for enzyme action. The amylase produced in the medium was isolated by centrifugation and partially purified by ammonium sulphate fractionation followed by dialysis.

Key words: Amylase, bacteria, amylase activity, pH sensitivity, estuarine soil.

INTRODUCTION

The amylases are hydrolytic enzymes which promote the decomposition of starch. They attack glycogen as well as certain dextrin (Kindle, 1983). Amylases can be acquired from a number of sources, such as plant, animal and microbes (Kathiresan and Manivannan, 2006). Although amylase originate from different plants, animals and microorganisms, microbial amylases are most preferred and used in industry due to their large productivity, chemical stability (Burhan et al., 2003; Pandy et al., 2000), plasticity and vast availability (Mishra and Behera, 2008). Starch degrading bacteria are most important for food fermentation, textile and paper industries (Mishra and Behera, 2008). Natural fermented media like food and soil samples are the substrates for isolation of amylase producing microorganisms (Fossi et al., 2005). Estuaries are the areas rich in biomass and primary productivity. Therefore, fermentation will be a natural process at its substratum which must be rich in soil bacteria capable of carrying out the degradation of dead decaying matter. Hence an effort is taken in this investigation to analyze the presence of amylolytic bacteria in an estuarine soil and to isolate them in order to characterize amylase synthesis in relation to various physico-chemical parameters.

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MATERIALS AND METHODS

Preparation of minimal agar media

In brief, 100 ml minimal agar media was prepared by 0.1 g of dextrose, 0.7 g of dipotassium phosphate, 0.2 g of monopotassium phosphate, 0.01 g magnesium sulfate, 0.05 g of sodium citrate, 0.1 g of ammonium sulphate, 2% starch and 1.5 g of agar in 100 ml of distilled water. The media was sterilized in an autoclave, poured in individual Petri dish and allowed to solidify.

Collection of sample and isolation of bacterial culture

One gram soil sample collected from Rajakkamangalam estuary was dried and suspended in 0.85% saline and serially diluted from 10^{-1} to 10^{-9}. Plates with minimal agar medium containing 2% starch were plated with 0.1 ml of each dilution and were incubated overnight at 37°C for 48 h.

Test for amylolytic activity

Plates with bacterial colonies were flooded with Gram's iodine reagent (0.01 M I_2-KI solution) and observed for zone of

degradation of starch revealed as clear and transparent zone. Of the various colonies, the one that exhibits highest degradation of starch was selected for pure culture, physic-chemical characterization and partial purification of the enzyme.

Identification of the isolated strain

Isolated strains were identified by Gram staining (Kannan, 1996) and various morphological and physiological analysis (Cappuccino and Sherman, 1996).

Amylase production

The starch nutrient medium was inoculated with a single isolated amylolytic *Bacillus subtilis* colony and cultured for 48 h at 37°C with continuous shaking on a rotary shaker at 200 rpm. From this, 1 ml of inoculum (approximately 1% culture) was transferred to each media including Czepek broth, starch broth, potato dextrose (PD) broth, 15% starch and Tendler's non synthetic medium and was incubated at 37°C at 200 rpm for 24 h.

Isolation of enzyme

To obtain crude enzyme, 12-h old cultures were transferred to centrifuge tubes and centrifuged at 10,000 rpm for 10 min. The resultant supernatant was used as the crude enzyme extract. A portion of the crude extract was used to determine the enzyme productivity of the strain and the rest is used for the partial purification and characterization of amylase.

Enzyme assay

Amylase assay was carried out using a reaction mixture consisting of 1 ml substrate (1.1% soluble starch in 50 mM phosphate buffer pH 7.2) and 1 ml of the crude enzyme extract. The reaction mixture was incubated for 1 h at 37°C. Reaction was stopped by adding 2 ml of dinitrosalicylic acid (DNSA) reagent (Miller, 1959) and the reaction mixture was heated to 100°C for 10 min and cooled. Optical density of each sample was measured at 520 nm. Enzyme activity was expressed in units/ml (1 unit/ml = amount of enzyme which releases 1 µM of glucose under the assay condition).

Effect of temperature on enzyme activity

To study the effect of temperature on amylase activity, 1 ml of dialyzed enzyme extract with substrate (1 ml) was exposed to 25, 30, 40, 45, 50, 55 and 60°C for 1 h, in a water bath maintained and analyzed for amylase activity.

Effect of pH on enzyme activity

Similar to observing the effect of pH on amylase activity, 1 ml dialyzed enzyme extract with 1 ml substrate was maintained at different pH 5.0, 5.5, 6.6, 6.5, 7.0, 75, 8.0, 8.5 and 9.0 for 1 h at 37°C, and amylase activity was determined.

Effect of EDTA and cations on enzyme activity

To study the effect of EDTA, monovalent and divalent cations on amylase activity, 1 ml of crude enzyme extract with substrate was maintained at different concentrations of EDTA, monovalent and divalent cations, such as NaCl, KCl, $CaCl_2$ and $MgCl_2$ at varied concentrations (1, 5 and 10 mM) for 1 h at 37°C, and the activity was analyzed.

Partial purification of amylase

A solution of 10% ammonium sulphate was added to the enzyme extract and centrifuged at 17,000 rpm for 10 min. The enzyme precipitate was then suspended in 400 µL of 0.1 ml Tris-HCl (pH 8.0) buffer and dialyzed at 4°C to remove the residual ammonium sulphate molecules. After dialysis, the enzyme was purified by ion exchange chromatography.

RESULTS

The soil sample of Rajakkamangalam estuary, when plated on minimal agar media (Srivastava and Baruah, 1986) revealed the presence of various bacterial strains. When tested with Gram's iodine reagent, 59 colonies exhibited clear white transparent zone expressing their amylolytic nature. One of the bacterial colonies with maximum white transparent zone was isolated and subcultured. The physico-chemical characteristics revealed that the isolated bacterium belong to the genus *Bacillus* (Table 1).

Moreover, among the various media used for culture, maximum biomass production and amylase activity was observed at 12 h in Tendler's non synthetic media with 2% starch (Figure 1). Of the various temperature and pH tested for optimum growth and enzyme production, maximum growth was observed at 37°C and pH 5.5, 7.5 and 8.5, whereas maximum enzyme production was observed at 30°C and pH 8 (Figures 2 and 3). The presence of 10 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM NaCl enhanced enzyme production as revealed by the release of glucose in the media (Figure 4). On the other hand, inclusion of increasing concentrations of EDTA, gradually decreased enzyme production as revealed by decrease in the release of glucose in the medium (Figure 5). The enzyme amylase was partially purified by ammonium sulphate precipitation followed by dialysis.

DISCUSSION

The results of this investigation reveal the presence of a pure strain of *Bacillus* with amylolytic activity in the estuarine soil of Rajakkamangalam. Pure *Bacillus* strain with amylolytic activity was also isolated by Mishra and Behera (2008) and Ashwini et al. (2011). Optimization of growth condition is a prime step in using microorganisms in fermentation technology (Kathiresan and Manivannan, 2006). Studies on optimization of growth condition of *Bacillus* revealed that maximum growth was observed at temperature 37°C, and pH 5.5, 7.5 and 8.5. The rate of growth of *Bacillus* above and below the optimum temperature (37°C) was very poor. The very same temperature requirement was also observed in a *Bacillus*

Table 1. Morphological and biochemical characteristics of the isolated strain.

Test	Response of the strain
Gram's staining	++
Shape	Bacilli
Motility	++
Growth at temperature (°C)	
37	+++
50	++
Growth at pH	
5	+
5.5	+++
6	+
6.5	+
7	+
7.5	+++
8	+
8.5	+++
Growth on starch agar	++
Starch hydrolysis	+++
Utilization of carbohydrates	
Glucose	++
Mannitol	++
Cellulose	-
Mannose	+

- = None; + = poor, ++ = good, +++ = very good.

strain isolated from soil receiving kitchen waste and authors suggest that this could be due to the thermolabile and mesophilic nature of the species (Mishra and Behera, 2008).

Maximum growth and enzyme production was observed in minimal agar media enriched with 2% starch, a substance very much needed to supply energy for the amylolytic bacteria. As stated by Ryan et al. (2006), starch is ubiquitous and is an easily accessible source of energy (Ryan et al., 2006). The composition and concentration of media greatly affect the growth and production of extracellular amylase in bacteria (Chandra et al., 1980; Srivastava and Baruah, 1986). In past studies, a number of carbon and nitrogen sources have been examined for amylase production in several *Bacillus* species (Bose and Das, 1996; Srivastava and Baruah, 1986; Ryan et al., 2006). Similar to these past reports, the present study also reported an increase in enzyme production along with increase in starch concentration up to 2%. Accordingly, Tendler's media with 2% starch is identified as the best media for amylase production.

However, in the present study, incubation beyond 12 h gradually and consistently decreased enzyme activity unlike the reports of Smitt et al. (1996) and Aiyar (2004) who stated that the enzyme production from microorganism is directly correlated to the time of incubation in *Bacillus licheniformis*. Although optimum growth of the *Bacillus* strain *was* observed at 37°C, maximum enzyme activity was observed at 30°C. The optimum temperature required for amylase production varied from one species to the other: a *Bacillus* species isolated from soil receiving kitchen waste showed maximum enzyme activity at 70°C (Mishra and Behera, 2008) and *Bacillus* species *marini* collected from marine environment of Andaman and Nicobar islands, India, showed maximum enzyme activity at 40°C (Aswini et al., 2011).

The reduction in enzyme activity following addition of EDTA reveals the dependence of enzyme on calcium for its activity. Nevertheless, the presence of maximum protein at 12 h when cultured at 30°C in minimal agar media with 2% starch gives a great scope for the industrial production of amylase at the room temperature (30°C) prevalent in our district.

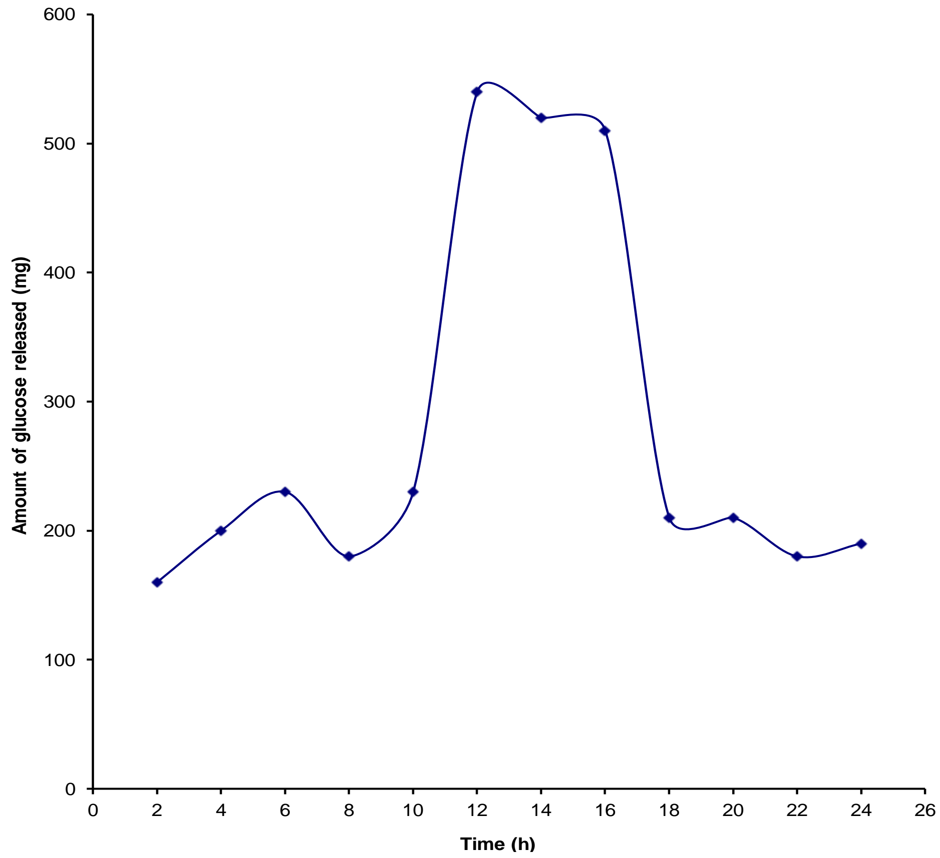

Figure 1. Amylase production of the *Bacillus* bacterium isolated from estuarine soil at different period of incubation.

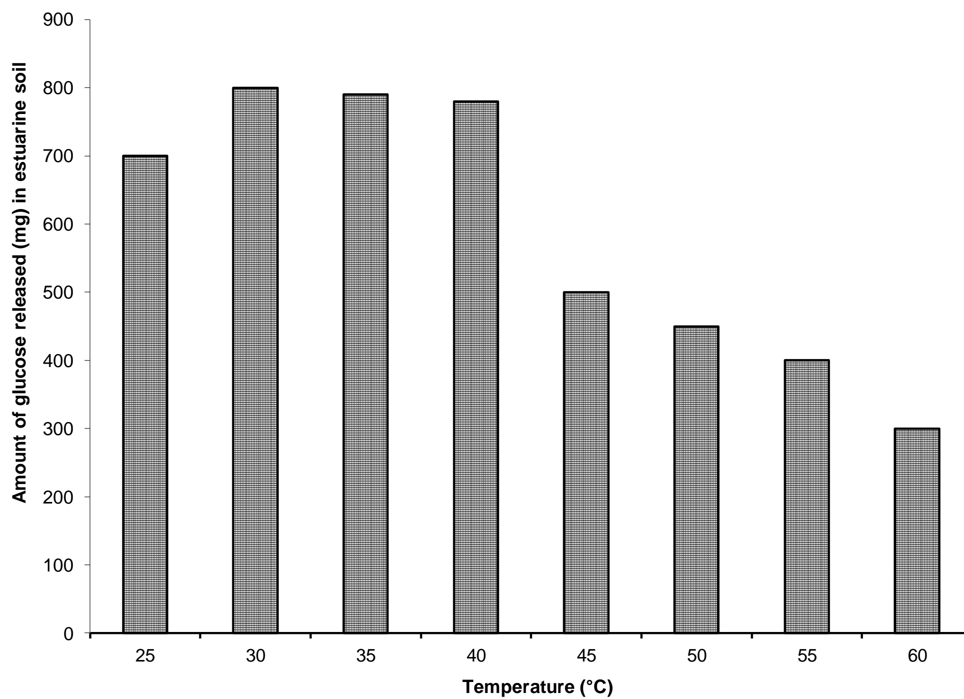

Figure 2. Effect of different temperature, on amylase production by the *Bacillus* bacterium isolated from estuarine soil.

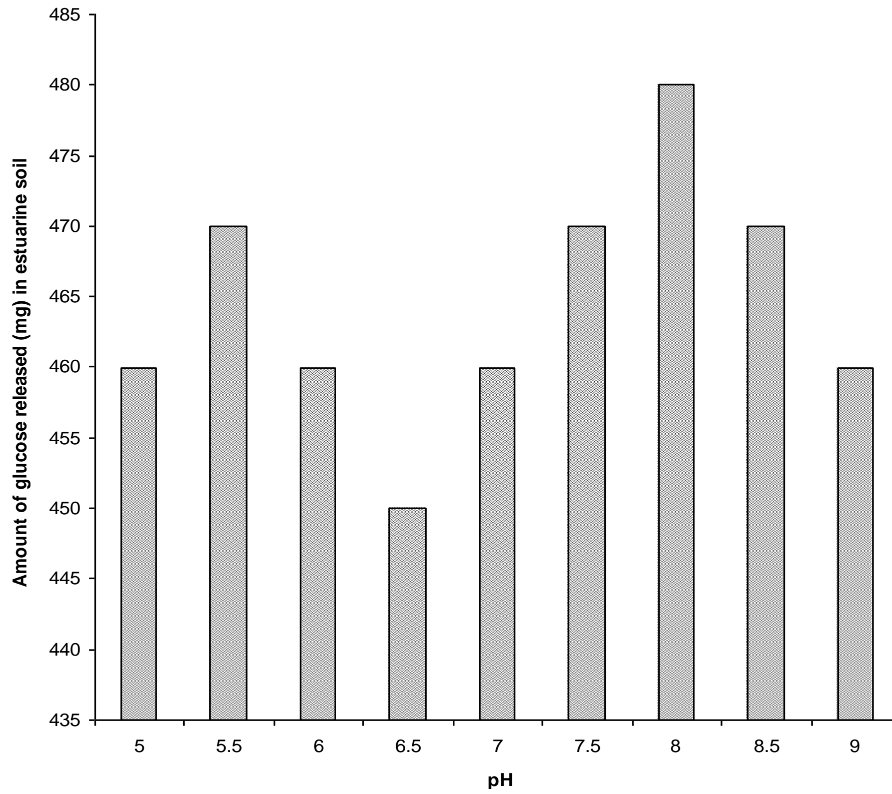

Figure 3. Effect of pH on the amylase production by the *Bacillus* bacterium isolated from estuarine soil.

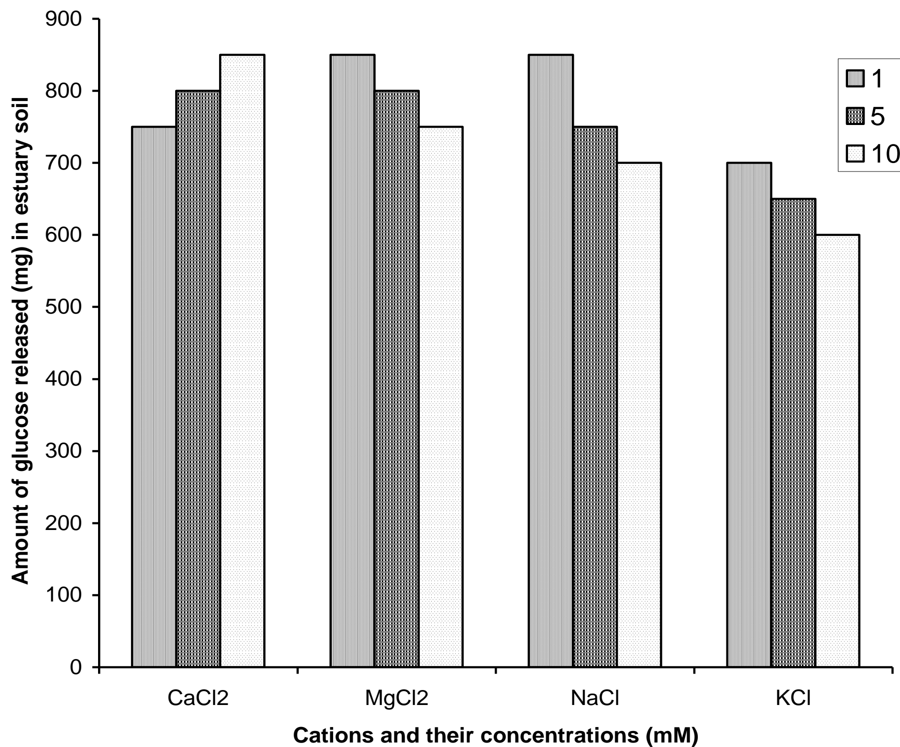

Figure 4. Effect of different cation concentration on enzyme activity of *Bacillus* strain isolated from estuarine soil.

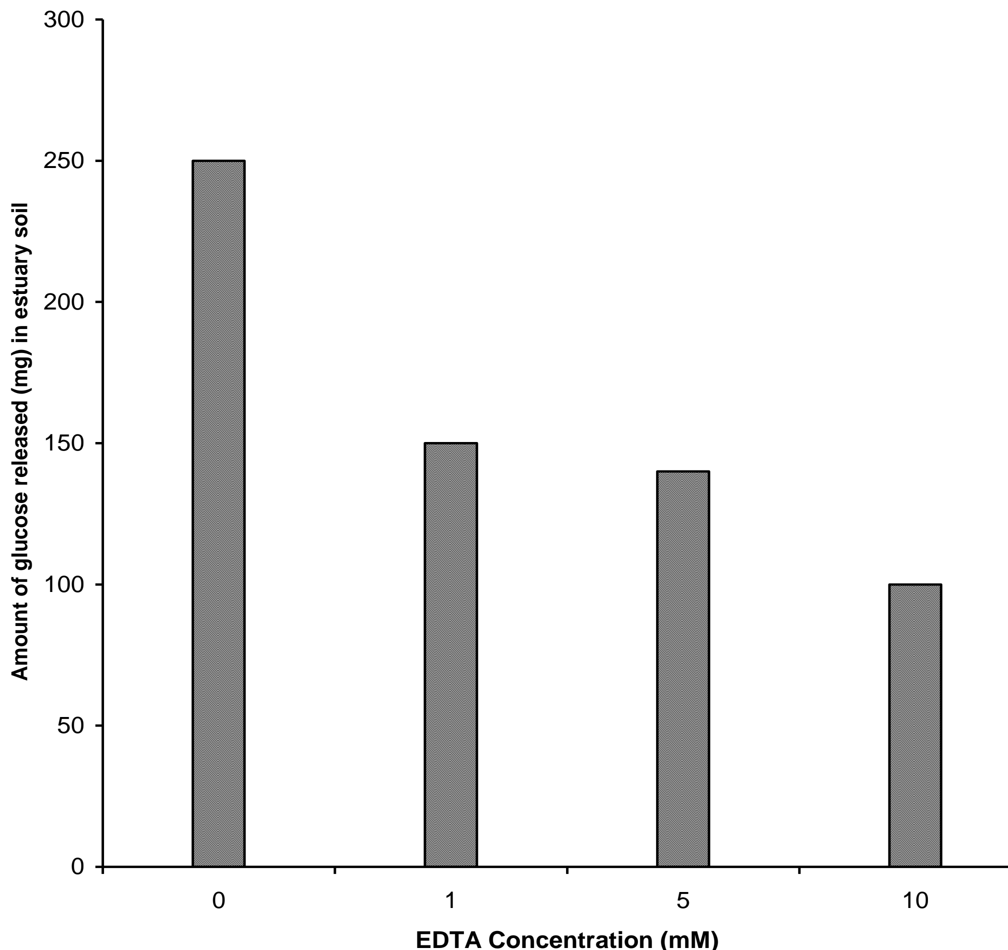

Figure 5. Effect of EDTA on enzyme activity of *Bacillus* isolated from estuarine soil.

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