

Influence of Environmental Factors on Extracellular Amylase from *Bacillus subtilis* and Its Kinetic Properties

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Amylase is an important industrial enzyme used in various food and textile industries. *Bacillus* species are observed to be potential producers of various extracellular enzymes including amylase. The present investigation was carried out with an objective to find out the optimum substrate concentration, temperature, pH, effect of chemical reagents like EDTA and Mg⁺⁺ ions on amylase production by *B.subtillis* isolated from the soil sample of the field of Central Farm, OUAT, Bhubaneswar. The amylase activity of the isolate was found to be 68.376 ± 1.5 Huggins and Russel unit/mg protein/30-min. at 40°C. The effect of substrate concentration on the enzyme activity was observed to be Km-2.85mg. and Vmax 250 HR unit/mg protein/30 minute. The optimum temperature for amylase activity was 60°C. The effect of time study indicates a first order rate constant K as $8.70 \times 10^{-5} \text{ sec}^{-1}$ at 40 minutes. The effect of divalent cation Mg⁺⁺ on amylase activity of *B.subtilis* as also studied and the percentage of inhibition was found to be 12.18%, 36.57%, 48.88% and 57.14% at 1 mM, 2mM, 3mM and 5 mM respectively. EDTA accelerates the rate of enzyme activity at 1mM and 2mM concentration and from 3 to 5 mM concentration, it inhibits enzyme activity. The amylase production also varies at different pH in starch agar medium. At lower pH the activity is totally inhibited and it increases gradually with increase in pH, the optimum being pH 7.0.

Key words: Amylase, *Bacillus subtilis*, Environmental factors.

Bacillus subtilis is an ubiquitous bacterium commonly recovered from water, soil and decomposing plant residues. The bacterium produces an endospore that allows it to endure

extreme conditions of heat and desiccation in the environment. *B.subtilis* produces a variety of enzymes like amylases, proteases and other enzymes that enable it to degrade a variety of natural substrates contributing to nutrient cycling. It is also considered as a benign organism, as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals or plants. *B. subtilis* strains could synthesize extracellular enzymes in large amounts, such as α -amylase, ribonuclease and protease (Yoneda and Marva, 1975). Amylase being an important industrial enzyme hydrolyzes starch, glycogen and related polysaccharides by clearing

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1,4 glycosidic linkages. It is also used in various food and textile industries. The present study was undertaken to isolate an amylase producing *B. subtilis* from soil, to assay the enzyme activity in specific medium and to study the influence of substrate concentrations, temperatures, pH and metal ions on enzyme activity.

MATERIAL AND METHODS

Collection of soil sample

Soil sample was collected from the agricultural fields of Orissa University of Agriculture & Technology, Bhubaneswar in a sterilized container. The soil sample was collected 5 cm. below the surface soil. The soil sample was immediately brought to the laboratory of P.G.Department of Microbiology, OUAT and processed for isolation of *B.subtilis*.

Isolation and preservation of bacterial isolates

One gram of the sample was mixed in a test tube containing 9 ml of sterilized phosphate buffer. After ten fold dilutions, from each dilution 0.1ml. of sample was inoculated onto Hi Crome Bacillus Agar plates (HiMedia,Mumbai). The plates were incubated at 30°C for 18-24 hours. Selected colonies were picked up and restreaked on Hi Crome Bacillus Agar plates to get pure or axenic cultures. The pure cultures were leveled and preserved on NA slants at 4°C for further use.

Identification of the bacterial isolates

The presumptive isolate was further characterized for identification following its morphology, Gram's reaction, spore staining followed by various biochemical tests as per the methodology discussed by Berkeley et al (1984).

Enzyme assay

After identification only *Bacillus subtilis* was studied further for assay of amylase by growing the organism in a medium containing 1% soluble starch, 0.2% tryptone, 0.1% yeast extract, 0.2% KH_2PO_4 , 0.1% $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 0.1% CaCl_2 , H_2O , 0.001% $\text{FeSO}_4 \cdot \text{H}_2\text{O}$, 0.0001% $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and 1% Na_2CO_3 (Separately autoclaved) and incubating at 37°C. After two days of incubation the cells were separated or removed by centrifugation (12,000g for 15 minutes at 4°C). The supernatant containing extracellular amylase was stored in refrigerator at 4°C for further use. The enzyme assay was performed by Huggins and

Russell's method (1948).

Effect of substrate concentration on amylase activity

By using the above procedure the activity of amylase at different starch (substrate) concentration was studied. The Michaelis Constant K_m and the maximum velocity V_{max} was determined by double reciprocal plot of Line-Weaver-Burk equation with substrate concentration ranging from 0.4 - 2.0 mg.

Effect of temperature on amylase activity

At different incubation temperatures i.e. 30°C, 40°C, 50°C, 60°C and 70°C, the effect of temperature on the activity of amylase was studied.

Effect of incubation time on amylase activity

Time dependence of the rate of hydrolysis of starch by amylase was calculated by recording the decrease in concentration of starch up to 70 minutes at different time intervals.

Effect of Mg^{++} ion on amylase activity

The effect of divalent cation Mg^{2+} as MgCl_2 on the amylase activity was studied from 1 mM to 5mM and chelating agent EDTA from 1mM to 5 mM. by spot inoculating the organism on starch agar plate prepared at different PH and after 24 hrs. of incubation exposing to iodine vapors. Utilization of starch was confirmed by measuring the hollow zones around the inoculation spot.

RESULTS & DISCUSSION

The bacteria *B. subtilis* was isolated from the soil of O.U.A.T field and the amylase was produced by culturing the bacteria in a nutrient agar medium and its kinetic properties were studied.

The amylase activity of *B. subtilis* was measured and the activity was 68.37 ± 1.50 Huggins and Russel unit/mg protein/30min. at 40°C.

Effect of substrate concentration on enzyme activity

The amylase enzyme activity was measured by using different substrate concentration from 0.4mg – 2mg and the k_m and V_{max} calculated from the Line-Weaver-Burk plot (Fig-1). K_m was 2.85mg and V_{max} was 250 HR unit/mg protein/30min at 40°C.

Effect of temperature on enzyme activity

The effect of temperature on activity of

B. subtilis amylase was shown in Fig(2). It was observed that the activity increases gradually from 30°C to 60°C and after that the activity was decreased. So the optimum temperature for the enzyme amylase obtained from *B. subtilis* was 60°C.

Effect of time of incubation on amylase activity

Fig(3) indicates the amylase activity measured at different time intervals from 10 minutes to 60 minutes. The activity was gradually increased and it was linear upto 40 minutes and then slightly flattened. The activity showed first order rate and the rate constant was $8.70 \times 10^{-5} \text{ sec}^{-1}$.

Effect of Mg^{++} ions on amylase activity

The effect of divalent cation Mg^{2+} in the form of MgCl_2 on amylase activity is presented in table 1. From the table it was found that Mg^{++} ions at a concentration. from 1mM to 5mM inhibit the enzyme activity and the inhibition was dose dependent.

Effect of EDTA on amylase activity

Table 2 presents the effect of EDTA at a concentration of 1mM to 5mM on *B. subtilis* amylase activity. EDTA at a conc. of 1mM and 2mM accelerates the enzyme activity by 3.91-23.18 % and at higher conc. (3mM to 5mM) it inhibits the enzyme activity by 19 – 83.5%.

DISCUSSION

In present day, biotechnologically produced amylase is one of the most important enzyme studied in detail to explore its utility for various purposes like chemical, medical, analytical and in industries like textile and food. In the present study soil was considered as a source to screen *Bacillus* spp. as *Bacillus* isolated from soil is a very good source for amylase activity (Alexandar, 1997). The soil sample was processed in different media and the *Bacillus* was identified following standard identification procedures. The amylase activity was measured and found to be 68.376 ± 1.5 units/ml of culture media, which corroborates with the findings of Bolten *et al.* (1997). The production of enzyme varied due to variable amount of substrates in the medium, different temperatures of incubation and different PH of the medium. The temperature and PH used in our experiment for the enzyme production was within

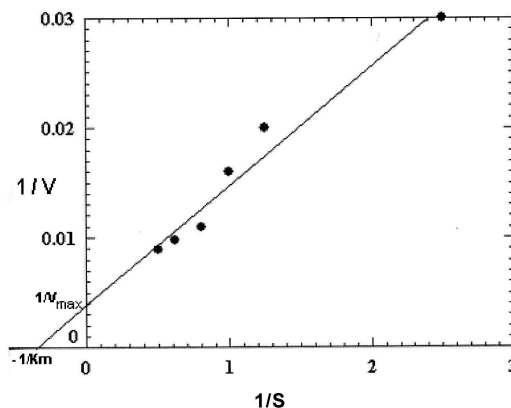


Fig. 1. Effect of substrate concentration on activity of amylase by *B. subtilis* and determination of K_m and V_{max} for the enzyme amylase of *B. subtilis* by lineweaver Burk plot

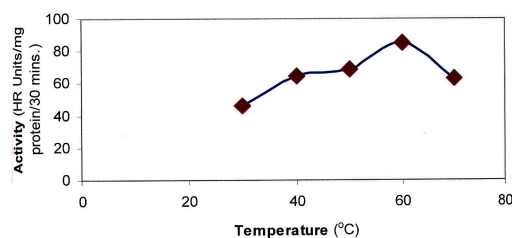


Fig. 2. Effect of temperature on activity of amylase of *B. subtilis*

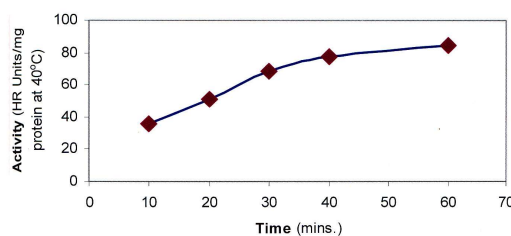


Fig. 3. Effect of temperature on activity of amylase of *B. subtilis*

the reported range of other workers like Bajpai and Bajpai (1989), Lin *et al.* (1998). The enzyme showed an increased activity with the increase in the substrate concentration. The K_m and V_{max} were 2.85 and 250 respectively at substrate concentration 0.4 mg/ml. No report was traceable in the literature about the K_m and V_{max} of the enzyme amylase obtained from *Bacillus subtilis* and other bacteria.

Table 1. Effect of Mg⁺⁺ ions on amylase activity

Concentration of ions (mM)	Activity (HR units/mg protein / 30 mins. at 40°C)	Percentage decrease/increase
Normal (N)	87.49 ± 0.9	
1	76.83 ± 0.5	12.18 ↓
2	55.49 ± 1.6	36.57 ↓
3	44.72 ± 1.8	48.88 ↓
5	37.49 ± 2.2	57.14 ↓

Table 2. Effect of EDTA on amylase activity

Concentration of ions (mM)	Activity (HR units/mg protein / 30 mins. at 40°C)	Percentage decrease/increase
Normal (N)	87.74 ± 2.1	
1	108.085 ± .6	- 23.18 ↑
2	91.175 ± 6	- 3.91 ↑
3	70.71 ± 4.8	19.4 ↓
4	30.635 ± 1. 7	65.08 ↓
5	14.45 ± 2.9	83.53 ↓

According to Roychoudhury *et.al* (1989), the optimum temperature for amylase production by *Bacillus subtilis* was 55°C and enzyme activity was decreased at higher temperature, which corroborates with our findings. They also have observed the loss of activity by 68%, 77% and 91% at temperature of 80°C, 90°C and 100°C respectively. But in our experiment we observed a loss of activity by 26% at 70°C.

The enzyme activity increased gradually with the increase in incubation time from 10 minutes. to 60 minutes. It was linear up to 40 minutes. and then slightly flattened. No report was traceable on time dependent hydrolysis of starch by the enzyme amylase obtained from *Bacillus subtilis*. Most of the workers used 30 minutes. for the normal enzyme estimation and few workers used 60 minutes. As it is linear up to 40 minutes., it is better to use 30 minutes for normal enzyme estimation.

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