α-AMYLASE ACTIVITY OF *BACILLUS* SP, A SOIL ISOLATE

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Abstract

Amylase is the enzyme which breaks down down starch into glucose molecules and commonly called as glycoside hydrolase enzymes. Variety of microorganisms such as bacteria, fungi, actinomycetes are known to produce amylases outside of their cells to carry out extra-cellular digestion of starch to break starch into the soluble end products such as glucose or maltose and then they are absorbed into their cells. These enzymes are used in the textile and paper industries, food, adhesive, and sugar production. In this study was done to produce α-amylase from *Bacillus subtilis* SU-10, a soil isolate. The produced amylase was checked for their activity in the presence of heavy metals. All the metals were found to influence the enzyme activity.

Keywords: Amylase, heavy metals

I. INTRODUCTION

Bacillus sp are known to produces versatile extracellular enzymes such as amylases which have got tremendous application in paper industries, food, sugar production etc (Bolton *et al*., 1997; Leveque *et al*., 2000; Marchal *et al*., 1999; McMohan *et al*., 1999). Most often used organisms for amylase production are *Bacillus subtilis, Bacillus licheniformis* and *Bacillus amyloliquifaciens.* Cordeiro *et al* (2002) has produced 57U/ml of amylase using Bacillus sp SMIA-2 at the incubation period of 48h. Studies of alpha-amylase production by *Bacillus subtilis* (CM3) isolated earlier from cow dung microflora, were carried out by Swain *et al* (2006). A bacterial strain was isolated from dhal industry was found to produce maximal amylase production with maltose as carbon source (Thippeswamy *et al*., 2006). Cordeiro *et al* (2002) found the amylase produced by *Bacillus* sp to be strongly inhibited by Co^{2+}, Cu^{2+} and Ba^{2+}, but less influenced by Ca^{2+}, Mg^{2+}, Ni^{2+}, Sr^{2+} and Mn^{2+}. In this study, amylase producing *Bacillus* sp was isolated from soil and subjected to produce amylase at various incubation time and the produced amylase was checked for the activity in the presence of heavy metals.

II. MATERIALS AND METHODS

A. Collection of sample

The bacterial strains were isolated from the garden soil of Sathyabama University. Soil samples collected were incubated in 50 ml of LB medium for several hours. After incubation, 0.5 ml of the supernatant was inoculated into minimal media containing Potassium dihydrogen phosphate (3g/l), disodium hydrogen phosphate (6g/l), ammonium chloride (2g/l), sodium chloride (5g/l) and magnesium sulphate (1g/l). After 2 days of cultivation at 37°C, 0.5 ml of the culture broth was diluted with 50 ml of the same minimal medium (100- fold dilution). These procedures were repeated three times in order to stimulate an enrichment culture. After the enrichment steps, the culture broth was spread out on an agar plate with the same composition as the minimal media. Then the culture was subjected for amylase production. This strain was stored in 25% glycerol solution at -70^{0}C for further use.

B. Identification of microorganism

Identification of organism was done by performing routine biochemical tests and 16SrRNA sequencing (Pitcher et al., 1989). 16SrRNA sequencing was done by isolation DNA from the organism and the large fragment of the 16S rRNA gene was amplified by PCR using the universal primers BAC-F-(5'-AGA GTT TGA TC(AC) TGG CTC AG-3') BAC-R (5'AAG GAG GTG (AT)TC CA(AG) CC-3'). The PCR products were purified using a Wizard PCR Preps DNA Purification System (Promega, USA) according to the manufacturer's instructions. The PCR product after purification is sequenced using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and a model 3100 automatic sequencer (Applied Biosystems, USA). The closest known relatives of the new isolates were determined by performing a sequence database search. The sequences of closely related strains were retrieved from GENBANK and the Ribosomal Database Project (RDP) libraries.

C. Assay for amylase production

Media Composition

Potassium dihydrogen phosphate (3g/l), disodium hydrogen phosphate (6g/l), ammonium chloride (2g/l), sodium chloride (5g/l) and magnesium sulphate (1g/l) and pH 7.0.

D. Enzyme production

The above medium (50 mL in 250 mL Erlenmeyer flasks) was inoculated with 1 mL of an overnight culture and incubated at 37ºC with vigorous aeration in a rotary shaker at 150 rpm for 144h. At time intervals, the turbidity of the cultures was determined by measuring the optical density at 470 nm in a Systronics spectrophotometer (India). Before assay, the cells were separated by centrifugation at 13.000 rpm for 15 min and the clear supernatant was used as crude enzyme preparation **(**Cordeiro *et al*.,2002).

E. Amylase Assay (Miller, 1959)

The activity of α-amylase was assayed by incubating 0.3 mL enzyme (crude enzyme preparation) with 0.5 mL Soluble starch (1%, w/v) prepared in 0.05M Phosphate buffer, pH 6.5. After incubation at 37ºC for 10 min the reaction was stopped. The reducing sugars released were assayed colorimetrically by the addition of 1 mL of 3-5-dinitrosalicylic acid reagent. An enzyme unit is defined as the amount of enzyme releasing 1 mM of glucose from the substrate in 1 min at 90ºC.

F. Effect of metal ions

The effect of different metal ions on α -amylase activity was determined by the addition of the corresponding ion at a final concentration of 1mM to the reaction mixture, and assayed under standard conditions. The enzyme assay was carried out in the presence of 1mM Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+} , $Mn^{2+}$and Hg^{2+}.

III. RESULTS

All the isolates were checked for the production of α-amylase by inoculating the organisms in the starch agar plates. α-amylase production was confirmed by the zone formation after the addition of iodine solution. The organism which showed α-amylase production was taken for the further study. The isolate was identified by biochemical tests and 16SrRNA sequence (Table 1). The organism was found to be *Bacillus subtilis* SU-10. The sequence was submitted in GENBANK and the accession number is GU902972. Amylase producing Gram positive bacteria *Bacteroides*, *Clostridium* sp, *Actinomyces israeli*, *Dichelobacter nodusus* were isolated from rabbit cecus (Sirotek *et al*., 2006). Amylolytic bacteria differ in the cellular location of the amylases e.g. those produced by *Clostridium butyricum* are extracellular (Wang et al., 1999). Amylases produced by *Bifidobacterium and Bacteroides* cells were largely cell bound (Cotta, 1998) in some bacteria the distribution of amylolytic activity between cellular and extracellular fracton was affected by the substrate provided for the growth i.e. mostly associated with cells in growth and released into the medium in growth on maltose (Cotta, 1998).

The isolate subjected for the production of α-amylase in a specific medium which contained starch. The amylase production by every species was checked by colorimetric method (Miller, 1984). The organism was found to produce 40U/mL at 48 hours. It was found to be reduced when it is incubated for longtime (Fig.1).

When the culture was subjected to grow in the presence of anyone of heavy metals like 1mM Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+} , $Mn^{2+}$and Hg^{2+}. The control was grown in the optimal media with optimal pH and temperature. In the presence of Mg^{2+} ion organism showed increased production of enzyme than the other heavy metals. But it was not higher than the control i.e 97% enzyme activity on comparing to the control. The presence of Co^{2+} decreased the activity of the enzymes in a higher rate than the other heavy metals (Table 2).

Metal ions can be generated from equipment corrosion, specially when subject to acid hydrolysis, the effect of some metal ions at the concentration of 1 mM in the activity of α-amylase was investigated. In the presence of 1mM Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+}, $Mn^{2+}$and Hg^{2+}, the enzyme activity was found to be reduced to a certain extent. All the heavy metals reduced the enzyme activity. Co^{2+} reduced the enzyme activity to 26% and Hg^{2+} reduced the enzyme activity to 45%. Cordeiro *et al* (2002) found the α-amylase did not require any specific ion for catalytic activity. A slight activity inhibition was produced in the activity by Ca^{2+}, Mg^{2+}, Ni^{2+}, Sr^{2+} and Mn^{2+}, and a stronger inhibitory effect was observed in the presence of Co^{2+}, $Cu^{2+}$and Ba^{2+}. Some amylases are metalloenzymes, containing a metal ion for catalytic activity. The inhibition of *Bacillus* sp.strain SMIA-2 α-amylase by Co^{2+}, Cu^{2+} and Ba^{2+} ions could be due to competition between the exogenous

cations and the protein-associated cation, resulting in decreased metalloenzyme activity (Leveque *et al*., 2000).

Table 1. Microorganisms identification tests

Test type	Result
Grams staining	Gram positive rod
Citrate	+
MR test	-
VP test	+
Gelatin liquefaction	+
Starch hydrolysis	+
Acid production	
D- glucose	+
L- arabinose	+
D- Xylose	+
Gas from glucose Few	+
Catalase	+
Nitrate reduction	+

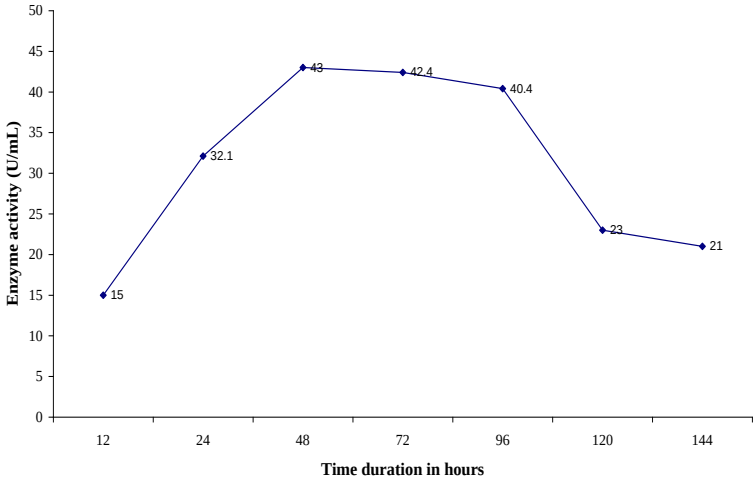

Fig. 1. Time Course Production of α-amylase

Table 2. Percentage of activity of α-amylase in the presence of heavy metals(%)

	Ion 1 mM
Control (no addition)	100
Ca^{2+}	89
Mg^{2+}	97
Fe^{2+}	75
Fe^{3+}	59
Co^{2+}	26
Zn^{2+}	73
Mn^{2+}	93
Hg^{2+}	45

IV. CONCLUSION

The organism isolated from the soil was found to be *Bacillus subtilis* and the organism was found to produce more amylase at the incubation time of 48 hours. The amylase activity has been lowered by the addition of heavy metals.

V. REFERENCES

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