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Isolation and Screening of Amylase Producing Bacteria from Soil and Production of Amylase

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ABSTRACT

Amylase falls under class of hydrolytic enzymes catalysing breakdown of starch into monosaccharide units. There are various biological sources of enzymes viz.. plants, fungi and bacteria. But the preferred one is microbial source due to ease of bulk production and stability of enzymes to work at extreme environmental conditions. This study reports isolation of amylase producing bacteria from sorghum cultivated farm soil and using them for production of amylase enzyme. During the course of the study three isolates viz.. AP-1, AP-2 and AP-3 were obtained which were identified as *Bacillus cereus, Bacillus alvei* and *Bacillus licheniformis,* respectively on the basis of morphological and biochemical characters. Amongst three isolates highest yield was obtained from *Bacillus alvei* which was 17.6 U/ml. Optimum conditions of pH, temperature and substrate concentration were determined for enzymatic activity. Crude enzyme samples obtained from all three isolates found to work best at 1.5% starch concentration and slightly acidic to neutral pH. The enzyme produced by *B. alvei* and *B. cereus* was found to be thermostable working best at 50 °C. In view of applications and demands of amylases in various fields further extension of study is needed for qualitative and quantitative enhancements.

Key words : *Amylase, Bacillus, Soil, Optimum.*

Introduction

Amylases being hydrolytic enzyme falls under class hydrolases which is one of the six different classes of enzymes. Amylases use starch as a substrate to yield monosaccharide and disaccharide units (Bole, 2013). Along with other hydrolytic enzymes like proteases and lipases, amylases are predominantly studied due to their varied industrial applications. Microbial amylases are the topic of discussion due to ability of bulk production and ease of manipulation to get desired products (Hassan, 2018). With the advent of biotechnology there is an upsurge in the demand of amylases in pharmaceutical, food, paper, textile industries (Alariya, 2013). This has lead to search for strains of microorganisms with considerable potential to produce enzymes with ease and at cheaper cost. This study is aimed at isolation and screening of amylase producing bacteria from soil. The study also involves optimization of conditions to achieve better enzymatic production for enzyme activity.

Materials and Methods

Collection of soil samples

Soil Samples were collected in polythene bag from different locations (sorghum cultivated farms) and brought to the laboratory and maintained at 30°C

until further processing.

Isolation and characterization of bacteria from soil samples

Soil sample was weighed and 1 g of it was then serially diluted. 0.1 ml of sample was then spread inoculated on starch agar plates and the plates were incubated at 30 °C for 24-48 h. The colonies showing zone of clearance on replica plates after flooding the plates with grams iodine were selected for amylase production and corresponding isolated colonies on master plate were subjected to purification. Only promising isolates were used for further studies

Morphological characterization was carried out by using gram staining and spore staining (Dorner's method) and motility test was carried outusing Hanging drop technique.

Identification was carried out by subjecting the isolates for biochemical tests such as Sugar fermentation, Indole production, Methyl red,Vogus Proskauer, Citrate utilization, Triple sugar iron agar, Nitrate reduction, Catalase,Oxidase production, Gelatin liquefaction, Hydrolysis of casein and starch and growing isolates under anaerobic condition with reference to Rainy *et al.*, 2015.

Production of amylase

100 ml of amylase production medium (Starch -1%, yeast extract-0.04%, $(NH_4)_2PO_4$-0.4%, KCL-0.1% and $MgSO_4$.7H2O- 0.05%) with pH 7 was prepared, sterilized and inoculated with the selected isolate. The medium was then incubated at 30 °C for 96 h on rotary shaker at 175rpm.

Crude enzyme preparation

After 96 h of incubation, the inoculated broth was centrifuged at 10,000 rpm for 10 mins and the supernatant was collected. Collected supernatant was used as crude enzyme preparation for further study.

Amylase assay

To determine amylase enzyme activity 3,5-dinitrosalicylic acid (DNS) method was used (Bernfeld, 1955). Assay was carried out using reaction mixture consisting of 2.5 ml of phosphate buffer, 2.5 ml of1% starch solution and 0.5 ml of enzyme. This mixture was incubated at 30 °C for 10 minutes and then 2 ml of DNS reagent was added to determine the amount of reducing sugar produced (Miller, 1959) and then reaction mixture was kept in boiling water bath for 10 min. After this the O.D reading was checked at 540 nm and enzyme units were calculated.

Characterization of amylase

Effect of pH on enzymatic activity

1 ml of 1% starch was used as substrate. Substrate solution was prepared in Citrate phosphate buffer and tris HCL buffer of pH 5,6,7, 8 and 9. 1 ml of crude enzyme sample was added into buffer tubes. Then the mixture was incubated at 30 °C for 10 min. Reaction mixture was added with 2 ml DNS reagent and the mixture was incubated in boiling water bath for 10 min. Absorbance was determined at 540nm after cooling the tubes to ambient temperature. The pH at which maximum enzyme activity was obtained, was considered as optimum pH.

Effect of temperature on enzymatic activity

1ml of substrate was taken into five different tubes and 1ml of phosphate buffer of pH 7 was added in each test tube. Each tube was marked with five different temperatures as30,35, 40, 45 and 50 °C. 1 ml of crude enzyme sample was added in each tube and all the tubes were incubated at specified temperature for 10 min. The reaction mixture was added with 2ml DNS reagent and then the tubes were kept in boiling water bath for 10 min. After cooling the reaction mixture, absorbance was checked at 540 nm. The temperature at which maximum enzyme activity was obtained, was considered to be optimum temperature.

Effect of substrate concentrations on enzymatic activity

Substrate was prepared from starch solutions of different concentrations as 0.5%, 1%, 1.5% and 2%. 1ml of substrate solution of different concentration was taken into different tubes and 1ml of phosphate buffer pH 7 was added in each tube. 1 mL of crude enzyme sample was added into each tube and then the tubes were incubated 30 °C for 10 min. 2 ml of DNS was added and then the tubes were kept in boiling water bath for 10 min. After cooling to ambient temperature, the absorbance was determined at 540nm. The substrate concentration at which maximum enzyme activity was obtained, was considered as optimum substrate concentration.

Results and Discussion

The well isolated colonies showing clear zone

around colonies on starch agar plate were selected and their colony characteristics were studied. The promising three distinct isolates designated as AP-1, AP-2 and AP-3 showed maximum zone of hydrolysis around the colony, hence were selected for further study.

Result of colony characteristics are shown in Table 1, 2 and 3.

From above morphological and biochemical tests, it is seen that, all isolates were gram positive, rod shape, spore forming motile microorganisms. All three isolates gave catalase test positive. Sugar fermentation tests of different isolates gave varied results. All isolates showed positive results for Vogus Proskauer test. Isolates when subjected for starch hydrolysis, proved to be positive for hydrolysis. Gelatin liquefaction was also shown positive for all three isolates. From results of cultural, morphological and biochemical characteristics, isolates were tentatively identified as *Bacillus cereus*, *Bacillus alvei* and *Bacillus licheniformis*, respectively (Rainey *et al*, 2015). Pranay *et al.* (2019) also similarly obtained six strains of *Bacillus* species during the amylase production study.

Amongst the three isolates maximum enzyme production was shown by *B. alvei.* These three isolates were further used for determining the effect of pH, temperature and substrate concentration on enzyme activity (Table 4, Fig. 1).

From Fig. 2 it is clear that highest enzyme activity was obtained at pH 6 for *B. cereus* whereas maximum enzyme activity was observed at pH 5 and pH 7 for *B. alvei* and *B. licheniformis*, respectively. Kim *et al* (1992) during their study found that amylase enzyme from B. licheniformis was active in the pH range of 6-8.

From Fig-3 it can be seen that highest enzyme

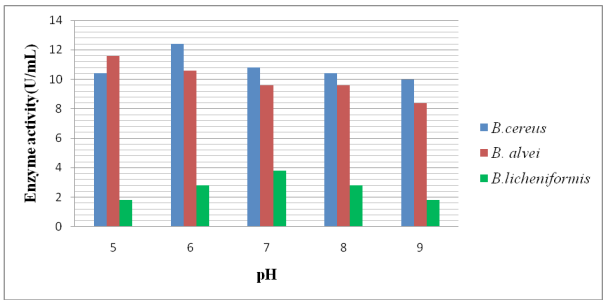


Fig. 2. Effect of pH on enzyme activity

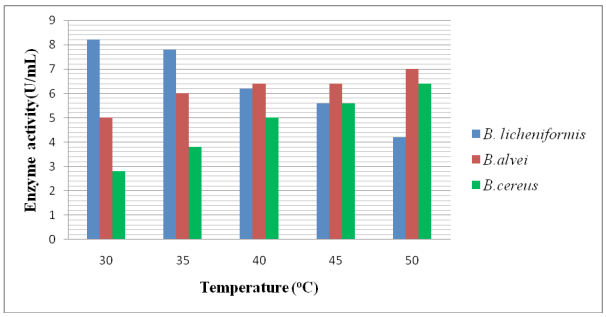


Fig. 3. Effect of temperature on enzyme activity

activity was obtained at 30 °C for *B. licheniformis* whereas maximum enzyme activity was observed at 50°C for *B. alvei* and *B. cereus* respectively. In one of the research amylolytic enzyme from *B. licheniformis* was found to be optimally active at 50 °C (Kim *et al*, 1992) whereas in another study maximum activity of amylase enzyme from *B. licheniformis* was obtained at 40 °C (Singh *et al.*, 2014)

From Fig-4 it can be seen that highest enzyme activity was obtained at 1.5% for all three isolates namely *B. cereus*, *B.alvei* and *B.licheniformis*

Conclusion

On the basis of morphological and biochemical char-

Table 1. Colony characteristics of the promising amylase producing isolates:

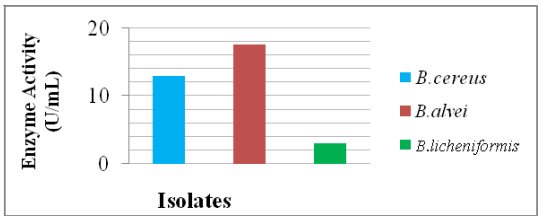
Sr. No	Isolate	Size (mm)	Shape	Colour	Margin	Elevation	Consistency	Opacity
1	AP-1	2	Circular	White	Irregular	Flat	Moist	Opaque
2	AP-2	2	Circular	White	Entire	Flat	Moist	Opaque
3	AP-3	2-3	Circular	White	Entire	Flat	Moist	Opaque

Table 2. Gram Staining, spore staining and motility of the promising amylase producing isolates:

Sr.No	Isolate	Gram staining	Spore staining	Motility
1	AP-1	Gram positive rods	Spore forming	Motile
2	AP-2	Gram positive rods	Spore forming	Motile
3	AP-3	Gram positive rods	Spore forming	Motile

Table 4. Results of amylase assay: Fig-1Enzyme activity

Isolates	Enzyme units U/ml
B.cereus	13
B.alvei	17.6
B.licheniformis	3



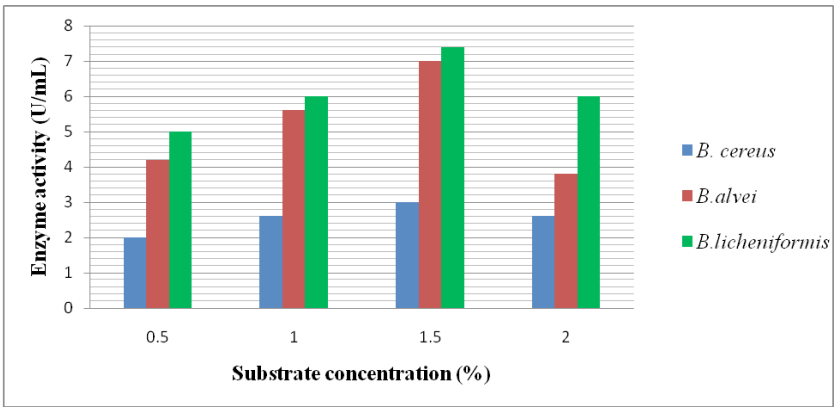


Fig. 4. Effect of substrate concentration on enzyme activity

Table 3. Biochemical characteristics of the promising amylase producing isolates

Sr. No	Tests	AP-1	AP-2	AP-3
1	Indole production	-	+	-
2	Methyl red	+	+	+
3	Vogusproskauer	+	+	+
4	Citrate Utilization	+	-	+
5	TSI agar	-	-	-
6	Nitrate reduction	+	-	+
7	Catalase production	+	+	+
8	Oxidase production	+	+	+
9	Gelatin Liquefaction	+	+	+
10	Urease production	-	-	-
11	Casein hydrolysis	+	+	+
12	Starch hydrolysis	+	+	+
13	Growth at 10% salt	-	-	-
14	Anaerobic growth	+	+	+
15	Sugar fermentation:			
	Dextrose	+	+	–
	Sucrose	+	+	+
	Lactose	-	-	+
	Mannitol	-	-	+
	Maltose	+	-	+
	Fructose	-	+	+
	Ribose	+	+	+
	Xylose	-	-	+
	Arabinose	-	-	+
	Galactose	-	-	+

+ indicates test positive- indicates test negative

acteristics the isolates were tentatively identified as *Bacillus cereus*, *Bacillus alvei* and *Bacillus licheniformis*. When these three isolates were subjected to amylase production, it was found that highest yield was obtained from *B. alvei.* The enzyme produced by *B alvei* and *B. cereus* was found to be thermostable, functioning best at 50 °C amongst used temperature range of 30 °C to 50 °C. In order to reach to the conclusion that 50 °C is optimum temperature for enzyme activity, temperatures beyond 50 °C need to be used for further studies. The enzyme produced by *B.alvei* showed highest activity at acidic pH 5 amongst used range of pH (5-9). Hence to reach to conclusion that pH 5 is optimum pH for enzyme activity, broader pH range needs to be used for further studies. All three isolates gave highest yield of amylase at 1.5% starch concentration. They can be commercially used for amylase production after further optimization studies.

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Conflict of interest

Author declares that they have no conflict of interest.

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