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Isolation, Screening and Production of Thermostable Amylase Using Bacterial Isolates from Soil

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ABSTRACT

Amylases are the hydrolyzing enzymes, which have great importance and application in biotechnology industry. The amylases are widely distributed in microbial, plant, and animal kingdoms. The α - amylase can be produced by the different species of microorganism, but for commercial applications it is derived from the genus Bacillus and the species like B. subtilis, B. licheni formis, B. stearothermophilus and B. amylo liquefaciens. Thermostable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their stability. As enzymatic liquefaction and saccharification of starch are performed at high temperature (100-110°C), thermostable amylolytic enzymes have been currently investigated to improve industrial process of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins. In the present study B.subtilis,B. stearothermophilus and B. amyloliquefaciens were obtained of which B. amyloliquefaciens was studied for amylase production. It produced thermostable amylase working at 65^{0}C and with the yield of 18-U/mL of partially purified amylase.

Key words: *Amylase, Thermostable amylolytic enzymes, Bacillus, B. amyloliquefaciens*

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INTRODUCTION

Enzymes are biological catalyst which initiate and accelerate lots of biological reactions in living cells. Amylases are the most important enzyme having significance of biotechonological and industrial market [1]. Amylases are widely found in nature in plants, animals and microorganisms. However, fungal and bacterial source of amylases have dominated applications in industrial sector. They are useful in food fermentation, detergent industries, pharmaceutical industries, textile and paper industries. The production of α amylase is essential for conversion of starches into oligo saccharides. Soil is rich source of organisms with variety of biochemical abilities, amylolytic organism can also be searched from soil samples [2]. In this view present work was undertaken for isolation of amylase producing bacteria by using suitable medium. The screening of amylase producing bacteria on starch agar was done and promising bacterial isolate was subjected for amylase production [3].

MATERIAL AND METHODS

1. Collection of Soil Samples

The three soil samples were collected for thermostable amylase producing bacterial isolates from compost heaps (which is known for high temperature at the base of heap-about 60-70^{0}C) at 3-ft depth from different locations of Karad, Maharashtra, India. The 10-g each of soil samples were collected from sample sites in a polythene bag and brought to laboratory for study.

2. Isolation of Amylase Producing Microorganisms[4]

The soil samples were diluted using sterile saline to 10^{-6} dilution and 0.1 mL of diluted sample were spread on nutrient agar plates. Then the plates were incubated at 30^{0}C for 48-h.The isolated representative colonies were picked up and preserved in triplicates on the nutrient agar slants at refrigeration till further use.

3. Screening for Amylase Activity [4]

The bacterial isolates were point inoculated, four each, on the starch agar plates and incubated at 30^{0}C for 48-h.After incubation growths of isolates were exposed to Gram's iodine for 10- min. The isolates showing zone of clearance around growth were taken as amylolytic preserved on the nutrient agar slants at refrigeration these isolates were preserved in triplicates on the nutrient agar slants at refrigeration and

were selected for further use. The selection ratios for these amylolytic isolates were determined as below:
Selection Ratio(S/R) = Diameter of zone of clearance in mm/diameter of growth of isolate on starch agar
The isolate showing highest selection ratio was selected for further study.

4. Morphological, cultural and Biochemical characterization of amylolytic isolates:

The following tests were performed with reference to [4, 5] for tentative identification of amylolytic isolates

Morphological

Gram nature, cell morphology, spore staining and motility

Cultural and Biochemical

Production of catalase, \ Oxidase, nitrate reduction, casein hydrolysis, fermentation of glucose, xylose, mannitol and lactose and IMViC test, growth at temperature55^{0}C.

5. Production of Amylase[6, 7]

The promising amylolytic isolate showing highest selection ratio on the starch agar was subjected for amylase production.

a) Inoculum Preparation

A loopful culture of promising amylolytic isolates was inoculated in 25 mL of starch broth and incubated at 30^{0}C for 24-h and used as inoculum.

b) Enzyme Production

About 10 % inoculum was used for amylase production. The 25 mL prepared inoculums was added to 250 mL starch broth and incubated on the shaker at 30^{0}C for 48-h.

c) Recovery of Enzyme

After proper incubation at the end of fermentation period the culture medium was centrifuged at 10000 rpm for 15 min to obtain supernatant as crude amylase enzyme.

d) Enzyme Assay

The assay of amylase was performed by measuring the amount of sugar released using DNSA method and bacterial amylase units were calculated.

e) Partial purification amylase

The 100mL aliquot from supernatant of fermented broth was added with ammonium sulphate powder at the concentration of 70% saturation and incubated at 4^{0}C overnight. The enzyme precipitate was separated by centrifugation at 3000 rpm for 10- min. The sulphate ions were separated by dialysis against distilled water for three days. After complete removal of sulphate ions the partially purified enzyme was dissolved in 10 mL of saline water and assayed again to check the extent of concentration of enzyme. 30^{0}C and then yield of enzyme was calculated as number of enzyme units / mL of partially purified enzyme.

f) Thermostability Studies on the Partially Purified Enzyme

The promising amylolytic isolate obtained i.e., *B. stearothermophilus,* is known thermophilic bacterium.
To study the thermostability of its amylase, the partially purified amylase was assayed at different incubation temperatures viz., 30(room temperature), and 37, 45, 50, 55, 60, 65, 70, 75 and 80^{0}C in the water baths and the enzyme units/mL were calculated.The temperature at which maximum units were obtained was taken as optimum temperature and also the temperature at which enzyme activity was reduced to below 50% of optimumwas taken as its temperature stability for industrial use purpose.

RESULTS AND DISCUSSION

Soil Isolates and Amylase Producers

In total 20 soil bacterial isolates were obtained on the nutrient agar and three isolates (15% of total isolates) were obtained as amylolytic and tentatively identified as isolate-1-*B.subtilis*, isolate-2-*B. stearothermophilus* and isolate-3-*B. amyloliquefacien s*(Table-1)while remaining 85% were nonamylolytic, non-Bacillus and were gram positive rods or cocci, non-sporing, some were gram negative rods as well. The enzyme yield was found1.3 U/mL,in case of *B.subtilis*, 3.0 U/mL in case of *B. Stearothermophilus* (maximum yield) while 1.6 U/mL in case of*B. Amyloliquefaciens* (Table-2), hence *B. stearothermophilus* isolate was selected as promising isolate for production studies.

Purification of amylase of *B. stearothermophilus*

It is evident from the Table-3 that when fermented broth level crude amylase (3.0 U/mL activity) was subjected to partial purification by ammonium sulphate precipitation followed by dialysis showed 6- fold increase in enzyme activity i.e.,18-U/mL. as compared to crude enzyme in the fermented broth.

Thermostability of amylase of *B. stearothermophilus*

It is evident from Table-4 that when assays of partially purified amylase of *B. stearothermophilus* were done at incubation temperatures of 30(room temperature), and 37, 45, 50, 55, 60, 65, 70, 75 and 80 ^{0}C, the maximum amylase activity(U/mL) was found at 30(room temperature), and 37, 45 ^{0}C i.e.,18 while at

temperatures 50, 55, 60 ^{0}C it was decreased to 14, 12 and 11, respectively. At 65 ^{0}C it was decreased to 9.0 U/ml which was 50% activity as compared to original activity of 18 U/mLi.e.65 ^{0}C was break point up to which enzyme can be used for its activity in the industry. At temperatures 70, 75 and 80 ^{0}C,the enzyme activity was decreased to 3.0, 1.0 and 0.0 U/mL.at these temperatures enzyme activities will be almost negligible and cannot be used at these incubation temperatures.

Table-1: Characteristics of Three Amylolytic Isolates

S. No.	Characteristics	Isolate-1	Isolate-2	Isolate-3
1	Gram nature	Gram positive	Gram positive	Gram positive
2	Cell morphology	Long thick rods	Long rods	Long thick rods
3	Spore present	Spore forming	Spore forming	Spore forming
4	motility	motile	Actively motile	Actively motile
5	Catalase production	positive	positive	positive
6	Oxidase production	positive	positive	positive
7	Nitrate reduction	positive	Negative	positive
8	Casein Hydrolysis	positive	Weakly positive	positive
9	Fermentation of Glucose	Acid and gas	acid	Acid and gas
10	Fermentation of Lactose	No acid and gas	Acid	No acid and gas
11	Fermentation of Xylose	Acid	Acid	Acid
12	Fermentation of Mannitol	Acid	No acid	Acid
13	Indole production	positive	Negative	positive
14	Methyl red test	Negative	Negative	Negative
15	Vogus Proskauer test	Positive	Weakly Positive	Positive
16	Citrate utilization test	positive	positive	positive
17	Growth at 55 ^{0}C Temperature	Negative	Positive	Negative

Table-2 Amylase Yield of Three Bacterial Amylolytic Isolates Using Assay Method

S. No	Description	*B. subtilis*	*B. stearothermophilus*	*B. amyloliquefaciens*
1	Assay material	Centrifuged fermented broth	Centrifuged fermented broth	Centrifuged fermented broth
2	Amylase units/mL	1.3	3.0	1.6
3	Remarks	Minimum yield of enzyme	**Maximum yield of enzyme**	Moderate yield of enzyme

Table-3: Partial purification of amylase of *B. stearothermophilus* Isolate

S. No	Units of Enzyme in the Broth (before partial purification) U/mL	Units of Enzyme After Partial Purification, U/mL	Extent of Enzyme Purification (X- fold)
1	3.0	18.0	6
2--Remarks		Increase in enzyme activity due to concentration of enzyme during purification	

Table-4: Thermostability of Amylase of *B. stearothermophilus* Isolate

S. No	Amylase Assay Incubation Temperature (^{0}C)	Enzyme units/mL of Partially Purified Amylase Preparation	Remarks
1	30	18	Maximum enzyme activity
2	37	18	Maximum enzyme activity
3	45	18	Maximum enzyme activity

4	50	14	Activity Decreased
5	55	12	Activity Decreased
6	60	11	Activity Decreased
7	**65**	**09**	**Activity Decreased (50%)**
8	70	03	Activity Decreased
9	75	01	Activity Decreased
10	80	0.0	Activity lost(100 %)

CONCLUSION

The *B. stearothermophilus* isolate produced thermostable amylase working efficiently upto 65^0C temperature.

After further optimization of production conditions, the amylase can be produced by *B. stearothermophilus* isolate at commercially viable level.

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