



## Production of alpha Amylase from Soil Bacteria and characterization of the enzyme activity and Kinetic Study

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### Abstract:

Enzymes obtained from micro organism are more stable and economic. Among the commercially valuable microbial enzymes, Amylases are most important and are of great significance in present day industry. Amylases are used for various purposes such as in Brewing industry, Food additive, also used in Clothing and Dishwasher Detergents to dissolve starches. In the present study, bacterial strain was isolated from garden soil and screened for the production of the enzyme  $\alpha$ -amylase. The bacteria that showed positive test for enzyme production was subjected for Biochemical tests for its identification. The organism was identified as bacillus species which is gram positive. In further investigation, the bacteria showed a positive response to citrate test. Kinetic study also showed that the optimum pH for enzyme activity was pH 8 and optimum temperature was 65°C at 575nm. It was observed that salt tolerance of  $\alpha$ -amylase activity was obtained at 4M NaCl concentration at 575nm. The effect of the substrate concentration in the enzymes was observed at 2% starch concentration at 540nm. The activity of purified enzyme was 100 $\mu$ l  $\alpha$ -amylase. Thus the optimum conditions for enzyme activity were standardized in the current work.

**Key words:** Amylases, BACILLUS, Kinetic Study, Screening test, Starch hydrolysis.

### Introduction:

Amylases are the most important industrial enzymes in the present day biotechnology <sup>[1]</sup>. The amylase family of enzymes is of great significance due to its wide area of potential application's Biodin and Effront were the first to use bacillus species for the production of alpha amylase on commercial scale <sup>[2]</sup>. These contribute a major class of industrial enzymes constituting 25% of the enzyme market.

Amylase is an enzyme that catalyses the hydrolysis of starch into sugars. It is used for the various purposes such as in brewing industry, food additive and also in clothing and dishwasher detergents to dissolve starches [3]. For the preparation of sizing

agents and removal of the starch sizing from the woven cloth, preparation of starch sizing plate's Liquefaction of heavy starch pastes formed during heating steps in the manufacture of corn and syrups, in bakery industry. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability <sup>[4]</sup>. The major advantages of using microorganisms for production of amylases are the ability to produce in bulk and ease at which it can be manipulates for desired products <sup>[5]</sup>. In the present study, the production of the amylase is carried out from the Screening of Bacillus sp in soil, purified, immobilized and the various enzymatic parameters like pH, temperature ,salt tolerance ,substrate concentration ,enzyme activity was checked.

### Materials and methods

#### Sample Collection

1gm of the garden loam soil has been collected and was subjected to 10 fold serial dilution. The last aliquot from the 10th tube was used for plating and the inoculation was performed in an nutrient agar plate and was incubated at 37°C for 24 hrs.

#### Pure Plate preparation and Basic Gram's Nature Identification:

From the master plate obtained above the isolated colonies were selected and were further inoculated to produce a pure plate of the colony. Pure plating was done using streaking technique on nutrient agar plates. The plates were incubated at 37°C for 24 hrs. After the prescribed incubation time the colonies were picked and subjected for gram's staining to identify the morphological characteristics of the organism. Based on the results of gram's staining the organisms are further subjected for various tests.

#### Screening Test for Amylase production:

To test for the ability of the bacteria to produce Amylase, Starch Test has been employed. Here the principle employed was the ability of iodine to produce blue color with starch. The bacterial samples were streaked onto nutrient agar plates having starch. The plates were incubated at the required temperature after inoculating them with the test organism. After

the incubation times the bacterial cultures were tested by the addition of iodine solution. If the bacteria were amylase positive, they would develop no color but if the bacteria are amylase negative they would develop total blue color. Based upon the behavior of the bacteria in the screening test, only those cultures that showed positive towards amylase, were selected for the further analysis.

#### **Biochemical Identification of the Bacteria based on Bergey's Manual:**

The bacteria that showed a positive response towards the Amylase production were further subjected for biochemical identification by performing series of testes. The Tests performed were VP test, Citrate Test and NaCl Growth test.

#### **Production of amylase from bacteria:**

The medium for the enzyme production has been prepared and autoclaved. The Production media should contain excess concentration of the substrate in addition to the other growth regulators and salts. The media is inoculated with the test bacteria and incubated for 48hrs. Centrifuge must be used for 6,000rpm for 15min. The supernatant is collected and transferred into a conical flask.

#### **Purification of the enzyme by Salt Precipitation and Dialysis:**

The enzyme to be purified is added to ammonium sulphate salt solution till the saturation point, pinch by pinch under ice cold conditions. Once the saturation point is reached incubate the solution in refrigeration for 24 hrs. After incubation the precipitate obtained is further used for complete purification by Dialysis by using a membrane filter. Dialysis step will completely eliminate the protein and other salt impurities present in the solution.

#### **Enzyme Kinetic Study:**

Effect of pH, Temperature, Salt Concentration, Substrate and Inhibitor have been checked on the activity of the enzyme using various techniques. The screening tests were performed to establish the standard condition for maximum enzyme activity. Effect of Inhibitor is also studied on the enzyme activity.

#### **Results and Discussion:**

##### **Gram staining of the Test organisms:**

The test organisms selected for the study were Gram positive Rods, thus they were Bacilli, and are subjected for further tests to identify their species.

**Figure 1: The slides showing the Bacterial staining**



#### **Screening Test for Starch Hydrolysis:**

The bacteria tested were positive for Amylase production thus producing complete zone of hydrolysis for starch-Iodine test. The results are shown below:

**Figure 2: The Bacteria showing positive test for Starch Hydrolysis:**



The above figure shows that the test bacteria have completely utilized the starch present in the media thus producing no color with iodine. This confirms the production of Amylase by bacteria.

#### **Biochemical tests for the Identification of Bacteria:**

The bacteria were identified as per the Bergey's system of classification. As the bacteria showed a positive test towards the Starch hydrolysis the next test performed in the series is VP test. This test would decide the ability of the bacteria to produce non acidic or neutral end products. The bacteria were also subjected for Citrate test where in the ability of the bacteria to utilize citrate as the source of carbon is tested. Both the tests showed positive. Finally the bacterial ability to grow in 65% NaCl media is tested

which was negative thus confirming the bacteria to be *Bacillus Coagulans*.

**Figure 3: Citrate test performed to determine the ability of the bacteria to utilize citrate as the energy source:**



From the above test results it can be confirmed that the bacteria showed a positive test for citrate utilization.

**Enzyme Kinetic Study:**

**Determination of Optimum pH for Enzyme Activity:**

S.No	pH	Vol. of buffer	Vol. of starch	Purified elute	DNS	Distilled water	OD
1	-	1ml	0.1ml	-	1ml	9.1ml	-
2	2	1ml	0.1ml	0.1ml	1ml	9ml	0.014
3	4	1ml	0.1ml	0.1ml	1ml	9ml	0.018
4	6	1ml	0.1ml	0.1ml	1ml	9ml	0.009
5	8	1ml	0.1ml	0.1ml	1ml	9ml	0.024
6	10	1ml	0.1ml	0.1ml	1ml	9ml	0.013

The above table shows that the optimum pH for the maximum enzyme activity was pH 8.0

**Determination of Optimum Temperature for Enzyme Activity:**

S.No	pH	Vol. of buffer	Vol. of starch	Purified elute	DNS	Distilled water	Temperature	OD
1	6	1ml	0.1ml	-	1ml	9.1ml	-	
2	6	1ml	0.1ml	0.1ml	1ml	9ml	4°C	2.055
3	6	1ml	0.1ml	0.1ml	1ml	9ml	15°C	2.33
4	6	1ml	0.1ml	0.1ml	1ml	9ml	65°C	3.00
5	6	1ml	0.1ml	0.1ml	1ml	9ml	37°C	1.28
6	6	1ml	0.1ml	0.1ml	1ml	9ml	Dark condition	1.24

From the above OD values it can be observed that the enzyme showed highest activity at 65°C

**Determination of salt tolerance of the enzyme:**

NaCl concentrations were varied in different test tubes and the activity of the enzyme was measured.

S.No	pH	Vol. of buffer	Vol. of starch	Purified elute	DNS	Conc. Of NaCl	Distilled water
1	6	1ml	0.1ml	-	1ml	-	9.1ml
2	6	1ml	0.1ml	0.1ml	1ml	1M	9ml
3	6	1ml	0.1ml	0.1ml	1ml	2M	9ml
4	6	1ml	0.1ml	0.1ml	1ml	3M	9ml
5	6	1ml	0.1ml	0.1ml	1ml	4M	9ml
6	6	1ml	0.1ml	0.1ml	1ml	5M	9ml

Time period	OD of test tube 1	OD of test tube 2	OD of test tube 3	OD of test tube 4	OD of test tube 5
12:06	0.078	0.046	0.056	0.076	0.079
1:10	0.032	0.015	0.004	0.020	0.013
2:15	0.007	0.004	0.009	0.023	0.016
5:14	0.003	0.006	0.010	0.021	0.012
10:45	0.002	0.002	0.006	0.010	0.007

The OD values of the inoculated tubes were taken at an interval of 2 hrs which inferred that the optimum salt concentration for the maximum enzyme activity was 4M NaCl Concentration.

**Determination of Substrate Concentration on enzyme Activity:**

S.No	Crude enzyme	NaOH	DNS	Distilled water	Conc. of starch solution	OD
1	500µl	1ml	1ml	9.5ml	-	-
2	500µl	1ml	1ml	9.5ml	0.5gm	0.382
3	500µl	1ml	1ml	9.5ml	1gm	0.411
4	500µl	1ml	1ml	9.5ml	2gm	0.483
5	500µl	1ml	1ml	9.5ml	3gm	0.414
6	500µl	1ml	1ml	9.5ml	4gm	0.370
7	500µl	1ml	1ml	9.5ml	5gm	0.341

From the above table it can be concluded that the maximum enzyme activity was observed at the substrate concentration of 2gm

**Conclusion:**

The current work was an attempt to synthesize the Amylase enzyme from commonly available soil bacteria and to analyze the optimum conditions for the maximum enzyme activity. The bacteria that was isolated from soil and gave a positive result for amylase production was found to be gram positive *Bacillus coagulans*. The organism was further used for the synthesis of the amylase enzyme by providing appropriate production medium. The enzyme thus produced was purified using various techniques to obtain maximum purity. The pure enzyme was later used for the kinetic study. The optimum conditions for the maximum enzyme activity were found to be 65°C Temperature, pH 8, Substrate concentration was 2gms and salt tolerance was 4M NaCl. The work can further be extended for the standardization of the other parameters also.

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