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Isolation and Screening of Starch Hydrolising Bacteria and its Effect of Different Physiological Parameters on Amylase Enzyme Activity

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ABSTRACT- Microbial source of amylase is preferred to other sources because of its plasticity, vast availability, higher yield and thermostability even at elevated temperatures.Various physical and chemical factors have been known to affect the production of α-amylase such as temperature, pH, period of incubation, carbon sources acting as inducers, surfactants, nitrogen sources, phosphate, different metal ions, moisture. Interactions of these parameters are reported to have a significant influence on the production of the enzyme.Study was mainly aimed to isolate a bacterium capable of hydrolyzing a starch source and to check effect of different physiological parameters on amylase enzyme activity. To conduct this research, study was mainly focused on three objectives i.e. 1st Screening and morphological characterization of the isolated bacteria. 2nd Characterization of amylase production by selected isolates. 3rd Time course of Enzyme production and Partial purification with Ammonium Sulphate saturation.Amylases of isolate-6 and isolate-9 were concentrated by ammonium sulfate precipitation which can be used as partially purified enzyme for further study. Isolate-6 and Isolate-9 showed the activity 0.34 and 0.28 units/ml/min respectively.Enzyme derived from isolate-6 and isolate-9 was stable at different physiological conditions. So, it is useful in fermentation industry and in pharmaceuticals.

Key words- Amylase, Starch hydrolyzing bacteria, fermentation and pharmaceutical industries

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INTRODUCTION

Amylases can be obtained from several sources such as plants, animals, and microbes (Kathiresan and Manivannan, 2006). Microbial source of amylase is preferred to other sources because of its plasticity, vast availability, higher yield (Burhan *et al*., 2003) and thermostability even at elevated temperatures (Adams *et al*., 1998, Ladenstein *et al*., 1998, Fitter *et al*., 2000). The other major advantage of using microorganism is, they are easy to manipulate to obtain enzymes of desired characteristics (Aiyer, P.V., 2005). Unusual bacterial amylases are found in acidophilic, alkalophilic and thermoacidophilic bacteria (Boyer and Ingle, 1972).

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Microbial growth and amylase production is dependent on growth conditions such as type and concentration of carbon and nitrogen substrate, metal ion requirement, pH and temperature of growth (Cherry *et al., 2004*; Ghasemi *et al.,* 2010). Study was mainly aimed to isolate a bacterium capable of hydrolyzing a starch source and to check effect of different physiological parameters on amylase enzyme activity. To conduct this research, study was mainly focused on objectives i.e. 1st Screening and morphological characterization of the isolated bacteria. 2nd Characterization of amylase production by Isolate No. 6 and Isolate No. 9 3rd Time course of Enzyme production and Partial purification with Ammonium Sulphate saturation.

Materials and Methods

Soil samples were collected from arid zone. The sample site was border area of little rann of Kutchh. Samples were taken in sterile bags.

Isolation and Screening of the amylase producing bacteria

For isolation of bacteria, the samples were diluted with sterile distilled water. Loop full samples were streaked on N- agar plates containing 7- pH. These plates were incubated at 37^{0}C for 24 hours. The isolated colonies were transferred to fresh N-agar plates. The primary screening of the isolates for amylase secretion was checked by using starch agar plate. It is done on the basis of capability of the organisms to produce starch digesting enzymes. The starch agar plates were incubated at 37°C for 48 hrs, then after plates were stained with gram's iodine solution to visualize clearzone, surrounding bacterial growth. Isolates 6 and 9 were screened for the protease production by streaking them on 1% gelatin Agar medium. They were incubated for 48 hrs at 37°C. Frazier's reagent was used to check the gelatin utilization zone on plate.

Production of Amylase

1 ml nutrient broth was incubated with a loop-full of culture from isolated colony and was incubated at 37^o C for overnight. This 1 ml of overnight grown culture was then transferred into the 100 ml of sterile starch broth medium and was incubated at 37^{o}C for 48 hours. The crude enzyme was obtained by centrifugation of the culture broth at 5000 rpm for 30 minutes and supernatant was utilized as crude enzyme for further study.

Enzyme activity assay

The activity of amylase was assayed by incubating 0.4ml supernatant "enzyme" with 0.6 ml of 1% soluble starch, made in sodium-phosphate buffer (0.05M) (pH 7.0) for 10 minutes at 37^{0}C maintained in water bath. Blank was prepared by incubating 0.4 ml crude enzyme and 0.6 ml 0.05M Sodium phosphate buffer. (Starch was absent in Blank) It was treated with the same condition. Reaction was stopped by adding 1ml of DNSA reagent (1.0 g of 3, 5, dinitrosalicyclic acid, 20 ml of NaOH and 30 grams of sodium potassium tartarate in 100 ml). Boiled for 10 minutes to develop reducing sugar assay colour, cooled and 4 ml of distilled water was added. Colour intensity was measured at 520 nm by spectrophotometer.Enzyme activity is to be defined as the "amount of glucose produced per ml in the reaction mixture per unit time." International Unit of amylase activity is defined as the "amount of enzyme that releases 1μmole of glucose from the substrate in 1 min at 37^{0}C.

Characterization of Amylase enzyme

Different parameters i.e. effect of pH, heat, enzyme concentration, starch concentration and salt tolerance test were performed. Effect of pH on the activity of amylase was measured by incubating 0.4 ml of enzymes and 0.6ml of 1% starch made in 0.05M Sodium phosphate buffer of different pH (5, 6, 7, 8, and 9). Amylase enzyme was placed in a boiling water bath at 90^{o}C, at different time intervals (0-11 min) these tubes were taken out and amylase activity was measured by DNSA metho. Aliquots of the enzymes were taken ranging from 0.1 ml to 0.4 ml from the same stock, in fixed 1 ml assay system and in each case activity was measured in the same way as mentioned earlier. The effect of different starch concentration ranging from 0.5 mg/ml to 5.0 mg/ml of soluble starch in 2.0 ml assay system, on amylase activity was studied. Activity was carried out by incubating test tubes in water bath at 37^{0}C for 10 min and reducing dugar was estimated by DNSA method. Starch agar plates were prepared containing different NaCl concentrations ranging from (0%, 0.5%, 1%, 2%, 3%, 4% and 5 %). After 48 hours of incubation at 37°C, Iodine reagent was used to observe clear zone surrounding the colony.

The amylase production media was incubated for different time courses e.g. 1, 2, 3, 4, 5 and 6 days for the production of amylase. Each day supernatant was taken and cells were discarded and activity was measured at 520nm. This assay was performed for 6 days continuously.

Partially purification of enzyme

The crude enzymes (of isolate-6 and isolate-9) were fractioned and precipitated by gradual addition of ammonium sulphate for 70% saturation. The proteins were allowed to precipitate with constant stirring for overnight at 4^{0}C and precipitates were separated by centrifugation at 10,000 rpm at 4^{0}C for 10 min. Precipitates were resuspended in the minimum volume of 0.05M Sodium Phosphate buffer (pH-7). By this preparation partially purified amylases were obtained.

RESULTS

Isolation of the organisms

Isolation of mesophilic bacteria was carried out from the sample collected from the arid zone of Kutchh region, from 10 cm soil depth. By using Nutrient Agar as a complete medium (pH- 7.0, 37° C), thirteen different mesophilic and neutrophilic bacteria were isolated based on their colony characteristics.

Characterization of the organisms

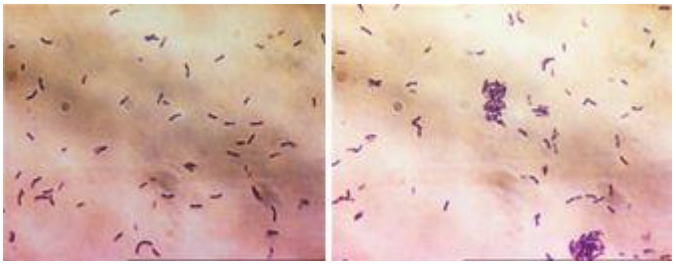
Colony characterization

The isolates were primarily differentiated on the basis of the colony appearance on the Nutrient agar medium.

Cell morphology and Gram's reaction

The isolates varied in their cell morphology, cell arrangement and gram's reaction. From the microscopic observations, the isolates were reported as Gram positive or Gram negative. The morphology of the isolates was cocci in clusters or in pair, long and short rods arranged singly or in chains (Table 1).

Screening of Isolates for the extracellular Amylase: The isolates were screened for the presence of extracellular amylase enzyme, as their starch hydrolyzing activity. Many of the bacteria have shown the test result as positive (Table 2, Fig. 2A).



Isolate-9 (Gram's positive, Rods) Isolate-6 (Gram positive, Rods)

Table 1: Colony characteristics

Iso-late no.	COLONY COLOR	TEX-TURE	SIZE	SHAP E	MAR-GIN	ELEVA-TION
6	Milky white	Rough	Small	Ir-regu-lar	Entire	Flat
9	Yellow	Smooth	Very big	Circu-lar	Entire	Convex

Table 2: Cell Morphology

Isolate no.	Gram's reaction	Shape and Size	Arrangement
1	Gram negative	Cocci	Cluster
2	Gram positive	Bacilli	Single
3	Gram positive	Bacilli (long rods)	Chains
4	Gram negative	Cocci	Single,Cluster
5	Gram negative	Rods	Chains
6	Gram positive	Bacilli (short rods)	Singly
7	Gram negative	Bacilli	Chains
8	Gram negative	Cocci	Cluster
9	Gram positive	Bacilli (long rods)	Singly
10	Gram positive	Bacilli	Chains
11	Gram positive	Cocci	Singly
12	Gram negative	Bacilli(long rods)	Chains
13	Gram negative	Cocci	Cluster

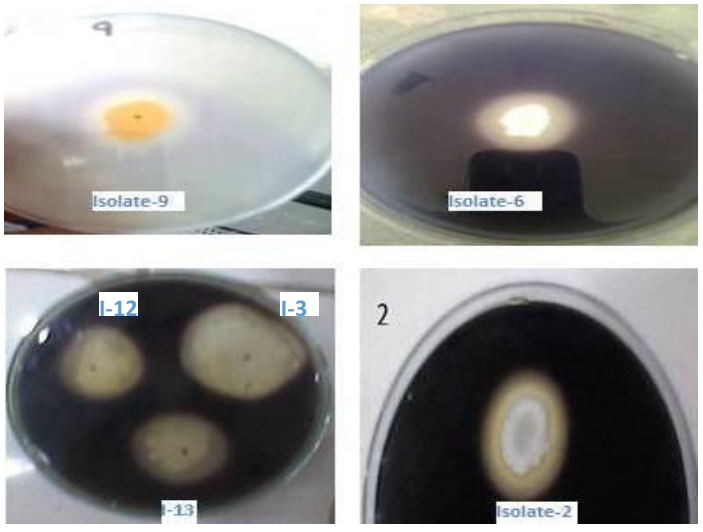


Fig. 2A: Zone of Starch Utilization
Zone ratio= zone diameter/colony diameter

Table 3: Relative Starch Hydrolysis on Starch Agar Plates

Isolate no.	2	3	6	9	12	13
Starch Utilization	+	++	+++	++	+	+

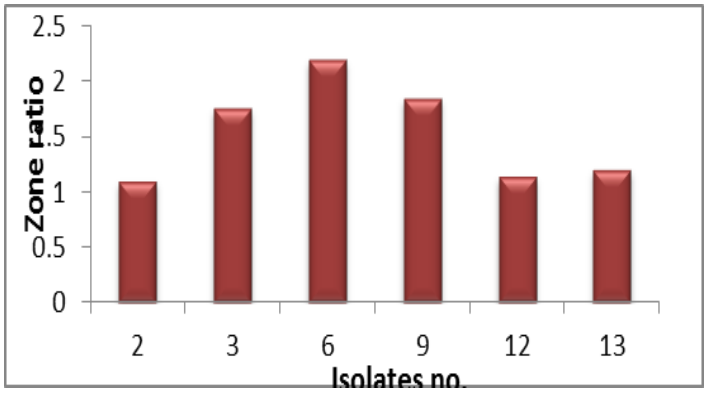


FIG. 2: RELATIVE SECRETION OF AMYLASE

Characterization of Amylase Enzyme

Effect of pH on amylase activityEffect of heat treatment on amylase activity-

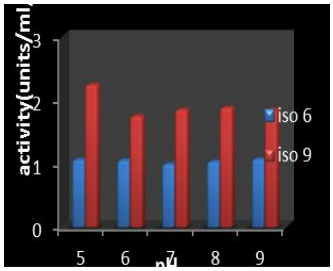


FIG. 3: EFFECT OF PH ON AMYLASE ACTIVITY

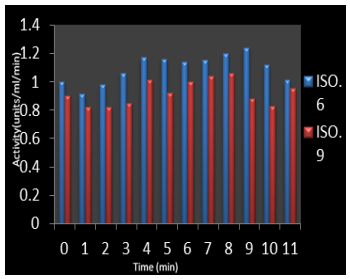


FIG. 4: THE EFFECT OF HEAT TREATM -ENT ON ISOLATE– 6 AND ISOLATE- 9

Effect of enzyme concentration on amylase activity

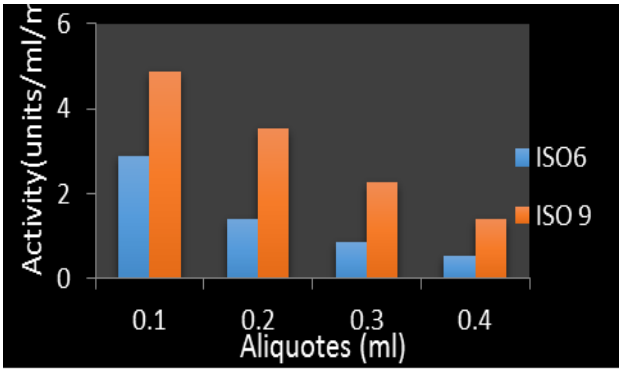


FIGURE 5: EFFECT OF ENZYME CONCENTRATION ON ISOLATE -6 AND ISOLATE-9

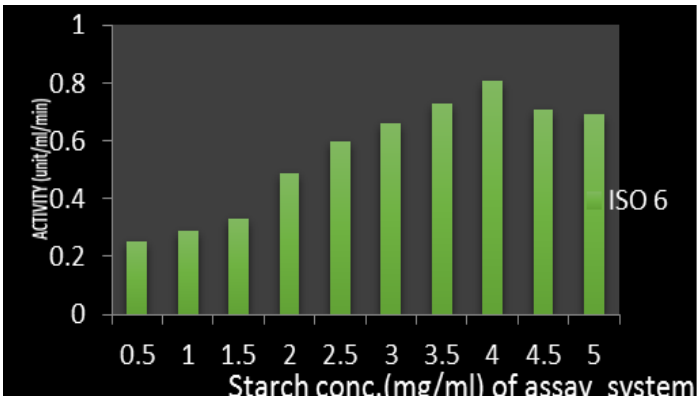


Fig. 6: Amylase Activity at Different Substrate Concentration (Isolate-6)

Time course of enzyme production

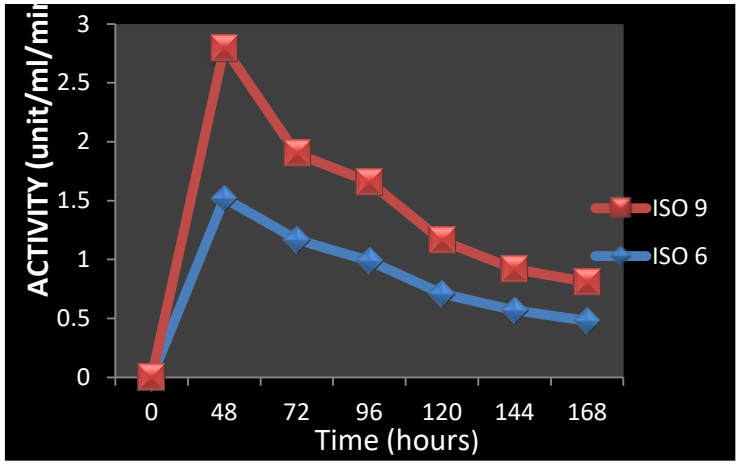


FIG. 7: EFFECT OF INCUBATION TIME ON ENZYME PRODUCTION

Purification of amylase

Partial purification of amylase by 70% $(NH_4)_2SO_4$ (Ammonium sulphate) saturation.

Table 4: Activity of partially purified amylase

ISOLATE NO.	ACTIVITY(units/ml/min)
Isolate-6	0.34
Isolate-9	0.28

DISCUSSION

From the result, effect of pH on Isolate-6 showed the stability in the activity at a pH range of 5-9, while Isolate-9 showed stable activity at different pH range of 6-9. But in the case of isolate-9 stimulatory effect was present at pH 5. By comparing both the isolates, activity was grater for isolate-9 crude amylase than isolate-6 crude amylase.Heat stability at 90^0C for supernatant

amylase was checked. Starting from 0 min to 11 min exposure to 90°C heat treatment, isolate-6 showed almost stable activity at different time interval and isolate-9 also showed almost similar activity. So, the studies on the heat treatment of the crude amylase showed that these are thermostable enzyme. When influence of enzyme concentration on amylase activity was evaluated for isolate-6 and isolate-9, result showed that activity decreases with increase in enzyme concentration in for both the cases. This indicates that good activity is achieved by low concentration of enzyme.From the result, effect of substrate concentration on Isolate-6 showed that as substrate concentration increases, there is an increase in enzyme activity and at the concentration of 4 mg/ml in assay system there was a maximum activity and gradually it became stable as concentration of substrate was further increased. From the result, maximum amylase activity of isolate-6 and isolate-9 were obtained at 48 hours of incubation. After 48 hours, cell mass increased but enzyme activity was declined. Amylases of isolate-6 and isolate-9 were concentrated by ammonium sulfate precipitation which can be used as partially purified enzyme for further study. Isolate-6 and Isolate-9 showed the activity 0.34 and 0.28 units/ml/min respectively.Enzyme derived from isolate-6 and isolate-9 was stable at different physiological conditions. So, it is useful in fermentation industry and in pharmaceuticals. Further study can be done on this enzyme like purification of enzyme by Column chromatography, HPLC etc. Molecular weight of enzyme can be known by SDS-PAGE.

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