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Amylase Activity of Starch Degrading Bacteria Isolated from Soil

Patel Nimisha[1]*, Shah Moksha[1] and A.K. Gangawane[2]

[1]Parul institute of Applied Science & Research, Parul University, P.O. Ghuma, Bopal-Ghuma road, Ahmedabad-380058, Gujarat, India
[2]Parul institute of Applied science, Parul University, P.O. Limda, Ta. Waghodiya, Dis. Vadodara-390019, Gujarat, India

**Corresponding author*

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A B S T R A C T

Soil contains huge diversity of microorganism which produces different types of enzymes. Amylase is one of them that hydrolyses starch into its monomer compounds, the smallest being glucose. Hence, amylase is a very prevalent enzyme produced biologically by various kinds of microorganisms and used in industrial sectors for various purposes. Soil bacteria can be isolated and commercially grown in large numbers to produce a vast amount of amylase. In addition, amylases that are extracted require optimum conditions to show greatest activity. In the present study bacteria were isolated from the garden soil and screened for amylase production on starch agar medium. Total 12 isolates were obtained by the primary screening technique from which 05 isolates were showing amylase activity. Zone clearance was determined by Gram's iodine method. Among 5 isolates isolate 1 was showing highest amylase activity 4.70 U/ml which was considered for further identification. Isolate 1 was tentatively characterized on the basis of their cultural, morphological and biochemical characteristics, which was identified to be *Bacillus sp.* Further partial purification of the amylase enzyme was carried out by ammonium sulfate precipitation followed by dialysis. Optimization of different parameters was carried out for the amylase production.

Introduction

Many microorganisms that live in the soil play important role in maintaining life of this planet. These soil organisms produce different type of enzymes. Amylase is one of them that is produce by soil bacteria and fungi. Quite a large variety of microorganisms have been identified and chosen as the source of amylase production because of the availability and simplicity of the ways in which they yield amylase. Soil is the primary source of these bacteria which can be isolated and commercially grown in large numbers to produce a vast amount of amylase. Amylase is the name given to glycoside hydrolases that breakdown starch into glucose molecules (Gebreselema, 2014). Enzymes can be obtained from several fungi, yeast, bacteria and actinomycetes (Mahajan *et al.*, 2011).

However, enzyme from fungal and bacterial sources has dominated applications in industrial sectors and are more stable and cheaply compared to plant and animal enzymes (Naidu *et al.,* 2013). Amylases establish a group of industrial enzymes, which only covers approximately 30% of enzyme (Patel *et al.,* 2014). The enzyme basically hydrolyses the α-1, 4 - glycosidic bonds that hold the glucose units together. Apart from starch hydrolysis, other forms of amylase known as transglycosylating enzymes, cause starch modification (Kaur *et al.,* 2012). Nowadays, amylases (α-amylases, β-amylases and glucomylases) represent one of the most important enzyme groups within the field of biotechnology. There for they are also called digestive enzymes. There are about 3000 enzymes known today only few are industrially exploited.

These are mainly extracellular hydrolytic enzymes, which degrade naturally occurring polymers such as starch, proteins, pectin and cellulose (Alariya *et al.,* 2013). The majority of enzymes used to date have been obtained from mesophilic microorganisms. Earlier literatures highlighted that bacterial strains from the genus *Bacillus, clostridium, Pseudomonas* and *streptomyces* have been used to synthesize amylase (Bole *et al.,* 2013).

The production of amylase by fermentation has been thoroughly affected by a variety of physiochemical factors. Most notable among these are composition of the growth medium, pH of the medium, phosphate concentration, inoculums age, temperature, aeration, carbon source and nitrogen source (Naidu *et al.,* 2013). Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. Most of the *Bacillus* strains used commercially for the production of bacterial amylase have an optimum pH between 6.0 and 7.0 for growth and enzyme production (Bala *et al.,* 2013). Amylases are also extensively used to remove starch from cloths in garments and textile industries (Naidu *et al.,* 2013).

The present study was attempted with the following objectives:

To isolate amylase producing bacterial species from soil
Determination of enzyme activity of amylase produced in submerged fermentation
Optimization of fermentation parameters for better enzyme activity

Materials and Methods

Sample collection, isolation and primary screening for amylase producing bacteria

The soil samples were collected from the different area of college garden in sterile container with the help of sterile spatula and stored at 4ºC until used. Tenfold serial dilutions of soil sample were prepared in sterilized distilled water and 0.1 ml of that diluted sample was spread on starch agar medium recommended by Vedder (1915). It has the following composition (g/l):

Soluble starch, 12; Beef extract, 3.0; Agar, 3.0 and pH adjusted to 7.5 (Vaidya *et al.,* 2015). All the plates were incubated at 37^0C for 24 to 48hrs. After incubation the plates were flooded with Gram's iodine solution to see the amylolytic activity of isolated strain.

The formation of a clear zone of hydrolysis indicated the starch degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest amylase producer (Vaidya *et al.,* 2015). The largest ratio was assumed to contain the highest activity.

Maintenance of pure culture

The colonies showing significant clear zone were plated on the minimal agar medium and analyzed for colony characteristics and subcultured on the minimal medium containing 1% starch and incubated at 37˚for 24h and then stored at 4˚C (Vaidya *et al.,* 2015).

Secondary screening and production of amylase enzyme

The potential isolates were then evaluated for enzyme productivity. Those isolates showing maximum amylase production were then considered for the further study.

Submerge fermentation process

For preparation of standard inoculums, isolate showing a maximum zone of hydrolysis was cultured in 20 ml inoculums medium [composition (g/l): soluble starch 10; peptone 5; $(NH_4)_2SO_4$ 2; KH_2PO_4 1; K_2HPO_4 2; $MgCl_2$ 0.01 and pH adjusted to 7] and incubated at 37 ºC for 24 to 48 h where an average viable count of 2-3x10^6 cells /ml culture was obtained. This was used as inoculums for the production medium. The composition of production medium was same as of inoculums medium. Fermentation was carried out in 250 ml Erlenmeyer flasks, containing 100 ml sterile production medium and inoculated with 5% of standard inoculum (containing 2-3x10^6 cells /ml). The flask was incubated at 37^0C on a rotary shaker at 150 RPM for 48h.

Preparation of crude enzyme

After incubation, the cultures were centrifuged at 1600 RPM for 20 min at 4°C and supernatant was used as a source of crude enzyme. The crude enzyme solution was utilized for determination of enzyme activities (Vaidya *et al.,* 2015).

Amylase assay

The DNS method was used to determine the amylase activity of each bacterial isolate. Isolate showing highest activity was chosen. Enzyme activity was assayed by reducing sugar formed by the enzymatic hydrolysis of soluble starch. Starch was used as a substrate at a concentration of 1% in 0.05M phosphate buffer at pH 6.9. Crude enzyme sample was mixed with substrate solution and incubated at 37^0C for 10 minutes. The reaction was controlled by adding 1ml of 3,5 Dinitro salicylic acid solution. After that the test tube was kept in boiling water bath for 10 minutes and cooled. The absorbance was read at 540 nm against blank (Vaidya *et al.,* 2015). The amount of reducing sugar released in the hydrolysis was measured by DNSA method. The Enzyme unit (EU) was determine as the amount of amylase required to release 1µmole of reducing sugar per ml per minute under above assay condition. The activity of amylase was calculated using the following formula.

Enzyme activity (U/ml) =

Reducing sugar (product concentration) X 1000 X Dilution factor

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Molecular weight of glucose X Incubation time (minute)

Partial purification of amylase enzyme

Ammonium sulfate precipitation

The 48 hours grown bacterial culture was centrifuged at 10000 rpm for 15 minutes. The supernatant was collected separately and the enzyme was precipitated by ammonium sulphate salt. To the crude extract 70% of the NH_4SO_4 was added. Then it was incubated for 24 hours and centrifuged at 10000 rpm for 15 minutes and the supernatant was decanted.

Dialysis

The partially purified enzyme was further purified by dialysis. The dialysis tube was boiled in distilled water for few minutes. Then the pellet was mixed with Tris-HCl buffer and the solution was transferred to the dialysis tube. Then it was placed in a beaker containing 500 ml of buffer for 24 hours. Due to osmosis, the impurities were removed and the same process was repeated for 48 hours (Roe, 2001).

SDS-PAGE

SDS-PAGE method was used to determine the molecular weight of purified enzyme. The sample was mixed with loading dye. The sample and marker were loaded on the respective wells and ran for 1 hour. The gel was observed for the determination of Molecular weight.

Identification of amylase producing bacteria

Potential isolates were tentatively identified by means of morphological, cultural and biochemical characterization.

Morphological characterization

For morphological characterization colonies were stained by Gram's staining technique and for suspected isolates special staining was also performed included capsule staining and endospore staining. Motility test was also performed.

Cultural characterization

Pure culture of individual isolates were further Characterized on the basis of their Gram's reactivity. Individual isolate was passed on Nutrient agar and Mac Conkey's agar plate and then on media. Special After incubation colony characteristics were noted.

Biochemical characterization

Different biochemical tests were analyzed include Indole test, Methyl red test, Vogues-Proskauer test, Citrate utilization test, starch hydrolysis, gelatin liquefaction, nitrate reduction, Catalase test, Oxidase test, phenylalanine deamination and sugars fermentation test.

Optimization of amylase production

The optimum parameters were determined for amylase production from the efficient isolates. The amylase fermentation was carried out at different ranges of parameters include temperature, pH, incubation period, substrate concentration, carbon source, nitrogen source and inoculum size. After fermentation enzyme activity was checked.

Effect of temperature

To determine the optimum temperature for amylase production, fermentation was carried out at various temperatures in the range of 25ºC, 37ºC, 45ºC, 55ºC and 65ºC.

Effect of pH

Different values of pH ranged from 5 to 8 were chosen for studying their effects on amylase production.

Incubation period

To obtain maximum amylase production fermentation was carried out at different incubation periods ranging from 24, 48, 72 and 96 hours.

Effect of substrate concentration

To evaluate the effect of substrate concentration on amylase production the production medium was supplemented with different concentration of starch including, 1%, 2%, 3%, 4% and 5%.

Effect of Carbon sources

Four different carbon sources were taken such as dextrose, maltose, sucrose and lactose at 1% concentration. The media was prepared with respective carbon sources and 0.1 ml of 24hrs grown fermented culture was inoculated to the medium and incubated at 37^0C for 24-48hrs. After incubation 48hrs grown media was centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and performed enzyme assay against blank to check highest activity among four carbon sources. (Ram Kumar T *et al.,* 2017*).*

Effect of Nitrogen sources

The amylase production by the bacterium was also optimized by supplementing different inorganic and organic nitrogen sources individually such as ammonium sulfate, sodium nitrate, peptone, calcium nitrate at the concentration of 1%. The media was prepared with respective nitrogen sources and 0.1 ml of 24 hours grown bacterial culture was inoculated to the medium respectively and incubated at 37^0C for 24-48hrs. After incubation grown media was centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and performed enzyme assay against blank to check highest activity among four nitrogen sources. (Ram Kumar T *et al.,* 2017*).*

Results and Discussion

Isolation and primary screening for amylase producing bacteria

The bacteria isolated from garden soil were screened for amylase production on starch agar medium. Bacteria isolated from starch rich materials may have better potential to produce enzyme under adverse conditions. Microorganisms that produce amylases could be isolated from places such as soil around mills, cassava farms and processing factories as well as flour markets. During the study, amylase producing bacterial strain was isolated from garden soil.

After serial dilution and spread plating on starch agar plates, the bacteria acquired from 10^{-5} dilution was selected. From the sample, 12 isolates were obtained and among these 5 isolates showing clear zone of starch hydrolysis on starch agar plates. Zone clearance was determined by Gram's iodine method. Average ratio of clear zones of selected colonies on starch agar media is indicated in Table 1.

Secondary screening and production of amylase enzyme

On the basis of primary screening the potential isolates were then evaluated for their enzyme productivity in submerge fermentation process.

Enzyme activity assay

By using the DNSA method, enzyme activity was determined. Among 5 isolates, it was observed that isolate 1 and 2 showing enzyme activity of 4.70 and 1.79 U/ml respectively. Isolate of greater enzyme activity was selected for further identification.

Identification of most efficient amylase producing bacteria

Isolates were tentatively identified on the basis of their morphological, cultural and biochemical characteristics following Bergey's Manual of determinative bacteriology (Holt *et al.,* 1994) and methods given by Cappuccino and Sherman (1993). Isolate 1 was identified to be *Bacillus sp*. Their colonial, morphological, and biochemical characteristics are tabulated in Table 2 and 3.

Optimization of amylase production

The optimum parameters were determined for amylase production for isolate 1. After fermentation at the different parameters the crude enzyme product was collected for the determination of enzyme activity. Enzyme activity was determined by DNSA method. The enzyme activity of isolate 1 at the different parameters is presented in Table 4.

Data illustrated in Figure 1 Clearly indicated that the highest enzyme activity of isolate 1 was found to be 4.55 U/ml at 37 °C. Like temperature pH is also an important factor that influences the amylase yield. The results illustrated by Figure 2 Clearly shows that amylase production, expressed as enzyme activity, gradually increased as the pH values increased from 6 to 7 and reached its maximum at pH 7.5. Highest enzyme activity was observed at 48 hours of incubation period which is illustrated in Figure 3. Optimum substrate concentration was 2%. Among all carbon and nitrogen sources, maltose and peptone proved efficient and their enzyme activity was 3.94 and 3.98U/ml respectively which is presented in Figure 4–6.

Since industrial amylase is usually extracted from bacteria and fungi, it is mandatory to isolate a local high amylase producing strain. In this study, the main aim was to isolate an amylase producing bacterial strain from soil. The study also included characterization and optimization of the produced amylase producing bacteria. Soil was chosen as a source of bacterial isolation due to the availability of various types of bacteria in soil. In primary screening of the bacterial strains, it was observed that isolate 1 was highest amylase producer among the other 5 isolates which was determined by growing the isolates on starch agar medium and detecting clear zone production around the bacterial colonies by adding Gram's iodine. The clear zones produced were due to the absence of starch which was hydrolyzed by the amylase enzyme excreted by the bacteria (Gopinath *et al.*, 2003).

In order to determine the amount of amylase produced by the selected isolates, enzyme assay was carried out by using 3, 5 – dinitrosalisylic acid (DNS). This is one of the simplest and most widely used methods to determine the amount of reducing sugar produced and hence is an indication of the enzyme activity.

It was observed that isolate 1 showed an activity of 4.70 U/ml while isolate 2 showed an activity of 1.79 U/ml. In a study by Soumya Vaidya *et al* (2015) the amylase activity of three isolates were found to be within the range from 6 to 9 U/ml. This is a much greater find compared to that found in this study. The first isolate had a greater activity than the other one and hence it was chosen as the final bacteria to be worked with throughout the study.

At first, the strain underwent physical identifications. Through Gram staining it was observed that the bacterial strain was Gram positive, rod shaped and arranged singly or two bacterial cells in chains. The bacterial strain was also scrutinized by observing the colony morphology. This included the physical appearance of the bacterial colonies on nutrient agar medium. This medium was selected because it is a non selective and non differentiating medium which allows the growth of maximum types of bacterial strains and due to the absence of any selective components in the medium, the appearance of the bacterial colonies are not affected.

The second part of the study was based on the optimization of the amylase enzyme generated by this isolate. The rate at which starch is broken down by amylase depends on

various parameters (Kunamneni *et al.,* 2005). The properties of amylase should meet its application and hence it is mandatory to check its optimum conditions which can be done via optimization.

Some of the most important ones include optimum temperature and pH. Hence, the enzyme was optimized by carrying out enzyme assay at different temperatures and pH in order to detect the optimum conditions. *Bacillus amyloliquefaciens* produces the enzyme with an optimum pH of 7.0(Ramachandran *et al.,* 2004). There are various ways to characterize an enzyme. In this study, the DNS method was used which determines the amount of reducing sugar produced at different temperatures, carbon sources, nitrogen sources, incubation period and substrate concentration by the enzyme.

In the optimization, the highest amylase activity was produced by different parameters. In the effect of carbon sources the dextrose, maltose, sucrose and lactose were taken. Among to all carbon sources the maltose produced highest amylase activity. Similar to carbon sources nitrogen sources took such as ammonium sulfate, sodium nitrate, peptone and calcium nitrate. The peptone was produced the highest amylase activity. Study revealed that peptone gave 9% higher activity than other nitrogen sources (Erdal and Taskin, 2010).

Temperature such as freeze (40^0C), room (26-28^0C) and incubator (37^0C) were optimized. Among to all the incubation temperature 37^0C gave the highest amylase activity(40). The incubation period is also optimized; in this experiment incubation period was 24 hrs, 48 hrs, 72 hrs and 96 hrs. Among all incubation period 48 hrs was the highest amylase activity. According to previous study, *Bacillus subtilis* gave high yield of alpha amylase after 48 hours of fermentation (59). The substrate according to its concentration is optimization. Starch took as the substrate and its concentration of 1% to 5%. At 2% starch concentration gave the highest amylase activity.

Table.1 Average ratio of clear zones of selected colonies on starch agar media

Isolates number	Average clear zone ratio (mm in diameter)
1	**2.96**
2	1.65
3	0.11
4	0.98
5	0.76

Table.2 Colony and morphological characteristics of most efficient isolate 1

Isolate No.	Colony characteristics on nutrient agar plate	Morphology
1	Small, fluffy, punctiform, entire, convex, bullet, moist, colorless and odorless	Gram positive, thick long rods arranged in a chain, motile and sporulated

Table.3 Biochemical characteristics of isolate 1

Biochemical test	Result
Indole production	+
Catalase	+
Oxidase	+
Methyl red	+
Vogus proskaur	+
Citrate utilization	+
Nitrate reduction	+
Phenylalanine deamination	-
Gelatin liquefaction	+
Starch hydrolysis	+
Casein hydrolysis	+
Ammonia production	+
Sugar fermentation	
a) glucose	+ only acid production
b) Lactose	+ only acid production
c) Maltose	+ only acid production
d) Xylose	+ only acid production
e) Mannitol	+ only acid production
f) sucrose	-
g) Fructose	+ only acid production
h) ribulose	-
i) arabinose	-

Table.4 Optimization of amylase production

Parameters	Value	Enzyme activity(U/ml)
Temperature(^{0}C)	25	3.58
	37	**4.55**
	45	3.5
	55	3.0
	65	2.6
pH	6	2.7
	7	3.5
	7.5	**4.9**
	8	3.7
Incubation period (hrs.)	24	3.91
	48	**4.22**
	72	2.85
	96	1.53
Substrate concentration(%)	1	1.33
	2	**1.43**
	3	0.57
	4	0.11
	5	0.04
Carbon source(1%)	Dextrose	2.98
	Maltose	**3.94**
	Sucrose	2.31
	lactose	1.49
Nitrogen source	Ammonium sulphate	3.78
	Sodium nitrate	2.42
	Peptone	**3.98**
	Calcium nitrate	2.10

Fig.1 Effect of different temperature on the production of amylase by Isolate 1

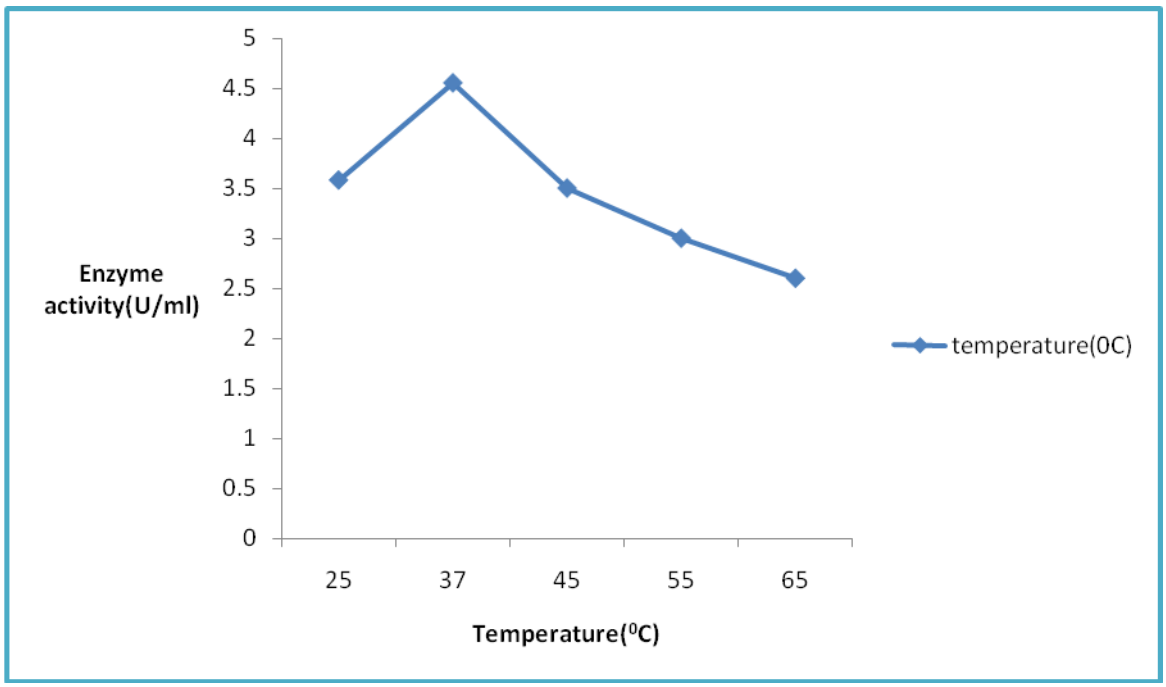


Fig.2 Effect of different pH on the production of amylase by Isolate 1

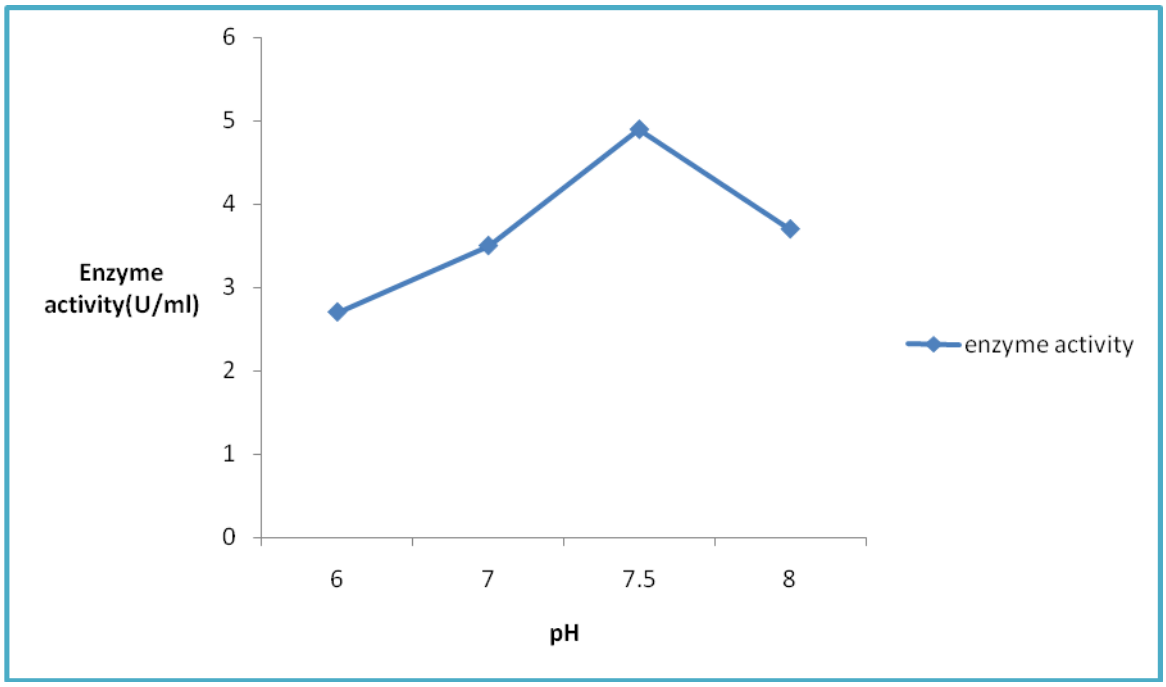


Fig.3 Effect of different incubation period on the production of amylase by Isolate 1

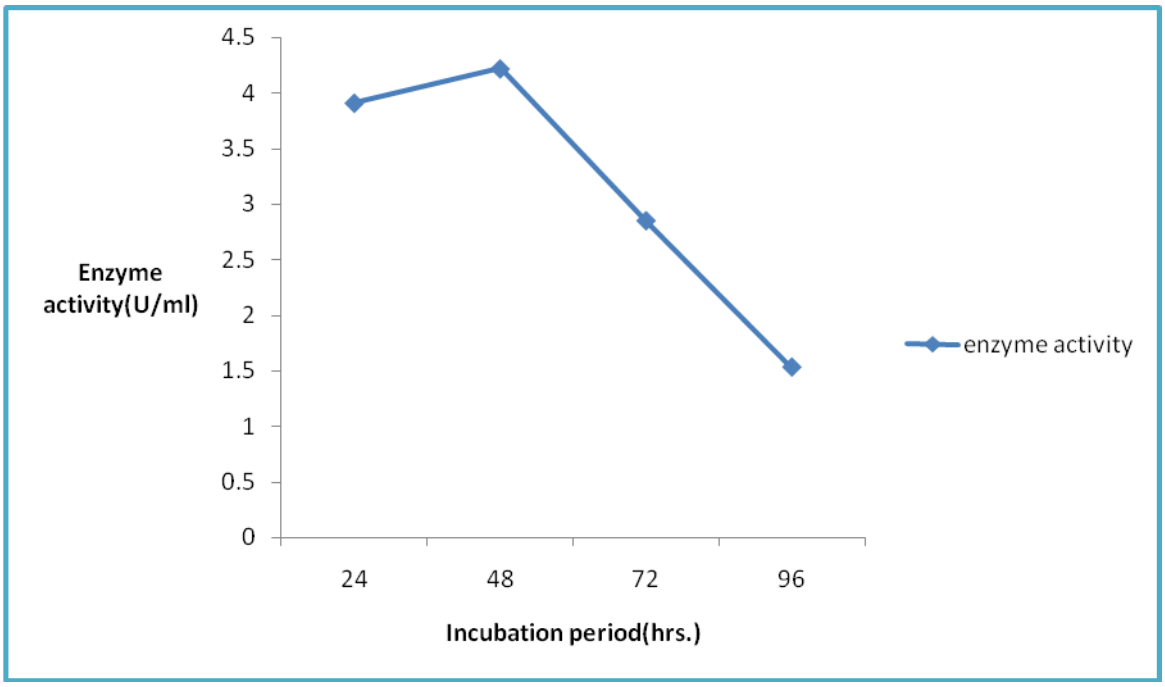


Fig.4 Effect of different substrate concentration on the production of amylase by Isolate 1

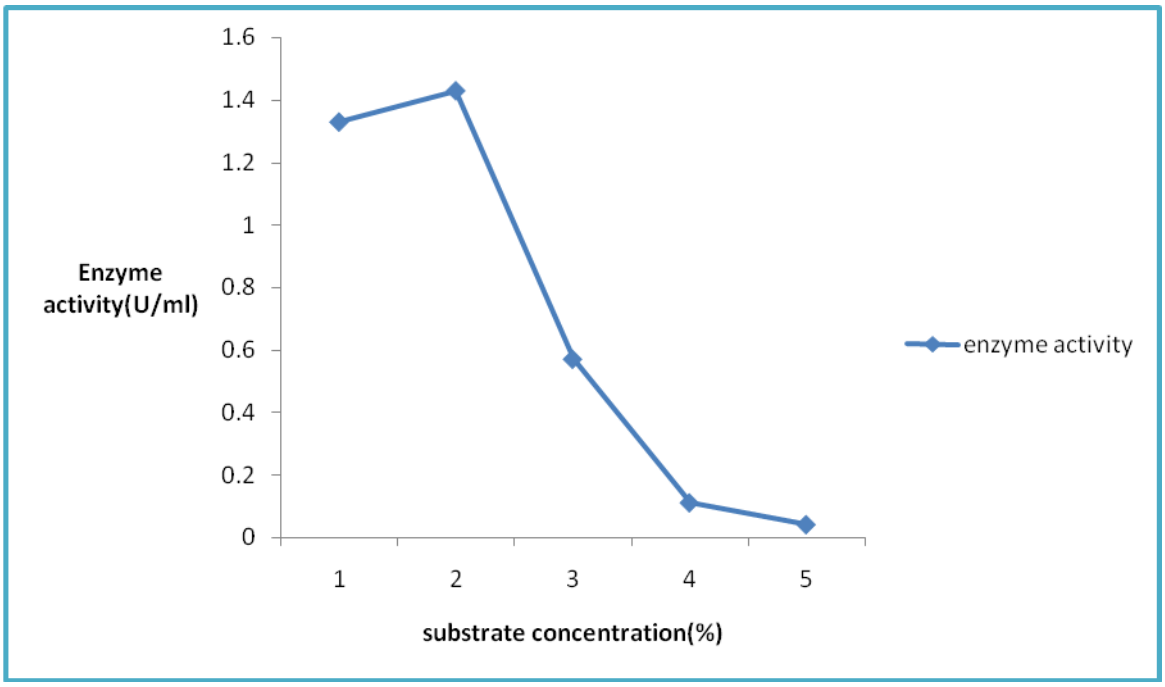


Fig.5 Effect of different carbon source on the production of amylase by Isolate 1

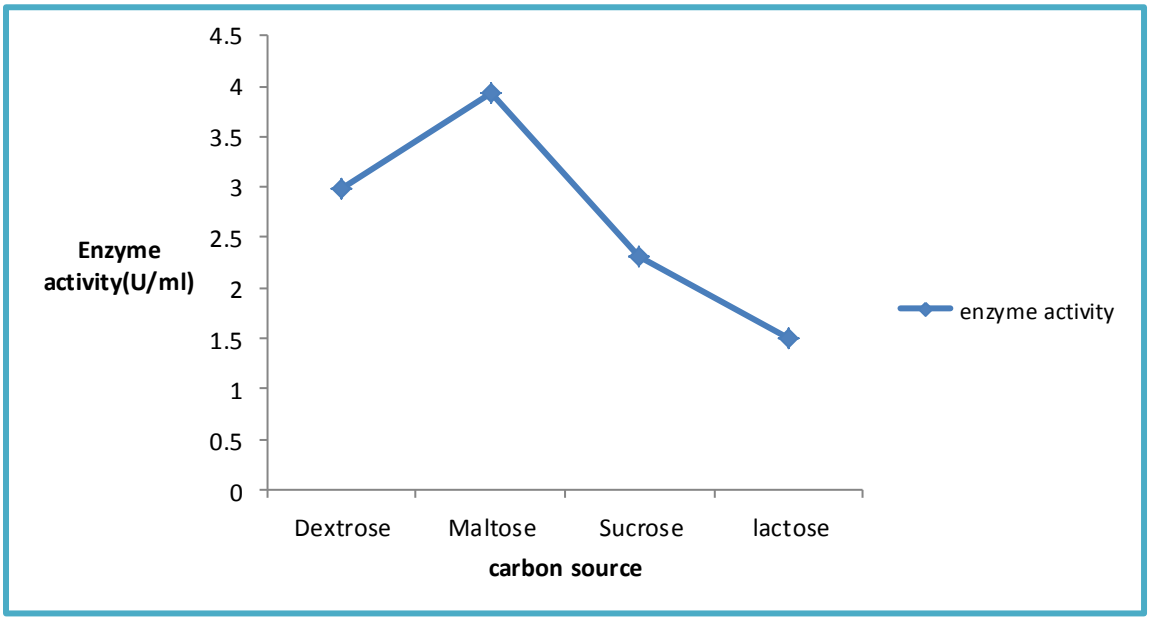
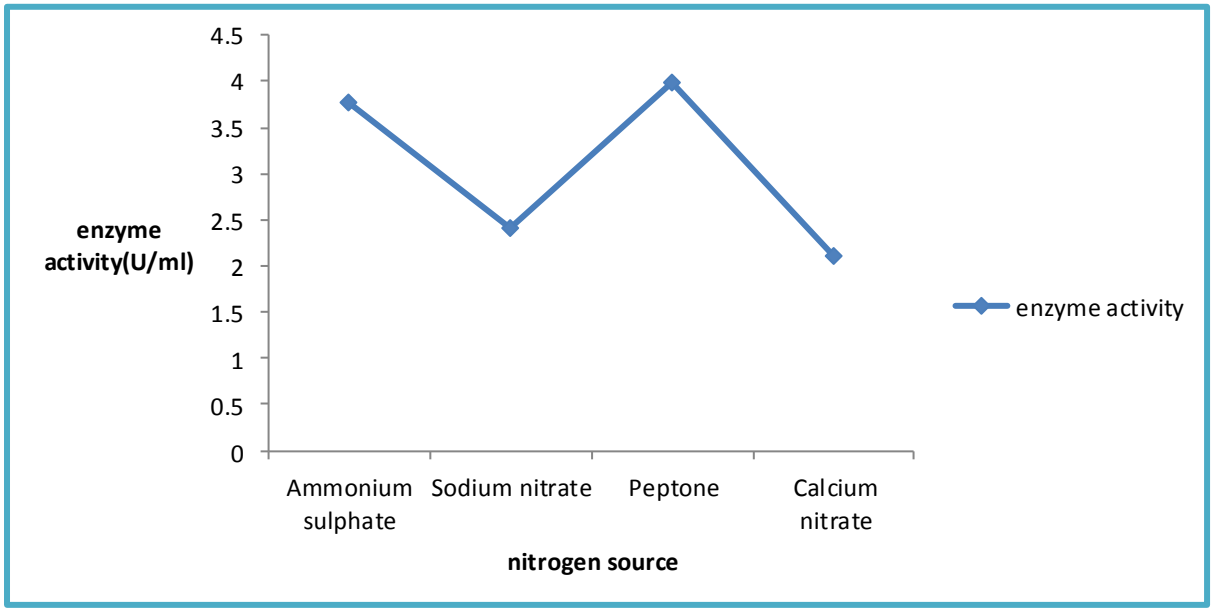


Fig.6 Effect of different nitrogen source on the production of amylase by Isolate 1



In conclusion,

The present work was carried out for the isolation of potential amylase producing bacterial strain from garden soil.

One isolate was selected for the determination of potential amylase activity. This isolate was characterized from their morphological, cultural and biochemical analysis and identified as *Bacillus sp.* respectively.

Partial purification of amylase was done and the enzyme activity was determined.

The optimum parameters required for the stability and better activity of enzyme were also studied.

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