PATNA WOMEN'S COLLEGE 1940

IRIS
Journal for Young Scientists
ISSN 2278 – 618X **(Print)**
ISSN 2278 – 6384 **(Online)**

http://www.patnawomenscollege.in/journal

Isolation, Screening and Characterization of Amylase Producing Bacteria (*Bacillus* sp.) from the Soil of Different Regions of Patna

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Received : November 2017
Accepted : March 2018
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Abstract : *Microbial exo-enzymes play a role in degradation of high molecular weight substances. Enzymatic degradation helps complex substrates to degrade into low molecular weight substances that can be easily transported in the cell. Exo-enzymes are hydrolytic in nature and participate in hydrolysis of starch, lipids, casein and gelatin. Starch degrading bacteria are most important for industries such as food, fermentation, textile and paper. Thus isolating pure culture from various soils has manifold importance for various biotechnology industries. Amylase production from bacteria is economical as the enzyme production rate is higher in bacteria as compared to other microorganism. Isolation of amylase producing bacteria was done from the soil of different regions of Patna. Samples were collected from three different sources which were areas rich in starchy materials such as vermicompost, vegetable dumping site as well as flour mill area. The isolated bacterial strains were screened by activity zone techniques with iodine. Bacterial colony yielding positive starch hydrolysis test were subjected to Amylase activity test. The amylase activity was optimized with respect to temperature and pH of the media in which it was inoculated. Optimum pH for the maximum activity was observed of pH 7.5 and optimum temperature for the activity was 38°C.*

Keywords: *Amylase, Starch hydrolysis, pH, Enzyme activity, Bacillus sp.*

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Introduction :

Enzymatic processes are fast replacing chemical processes as the merit of enzymes as industrial catalysts shoulder on their unique properties. Enzymes are highly specific and efficient in catalysis; they are biodegradable and carryout catalytic reactions under mild conditions thereby saving energy and resources (Tewari, 2007). Amylases are capable of digesting glycosidic linkages found in starch to give diverse products including dextrin and progressively smaller polymers composed of glucose units. They are among the most important enzymes and are of

great significance in present day biotechnology. Amylases are obtained from diverse sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. Though, both filamentous fungi and bacteria are the most known and important sources of amylase, amylases of bacterial origin are generally preferred due to several characteristic advantages that they offers (Pandey et al., 2000).

The ability of bacterial strains to secrete large amounts of extracellular protein has made them well suited for the industrial enzyme production (Singh and Kumari, 2016). Amylase production from bacteria is economical as the enzyme production rate is higher in bacteria as compared to other microorganism (Kaur and Vyas, 2012). Bacterial enzymes are known to posses more thermostability than fungal amylases (Eke and Oguntimehin, 1992).

Bacteria isolated from starch rich materials may have better potential to produce amylase under adverse conditions (Mishra and Behera, 2008). However due to their ubiquitous nature and very versatility in their adaptability to the environment (Khan and Priya, 2011), amylase producing bacteria could be also isolated from different starchy and non-starchy places. Starch degrading bacteria have been isolated from various sources including different soil samples such as forest soil; soils in different crop fields like potato, cassava and wheat fields; different wastes such as kitchen, agricultural and industrial wastes; hot springs; soda lakes etc. Among the bacteria, in most cases, *Bacillus* sp**.** has been the focus of intensive research.

The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990). The production of microbial amylase from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrients requirements, incubation period, pH, temperature, metal ions and thermostability. Bacterial amylases are preferred for application in starch producing and textile industries due to its action at higher temperature (75 to 105°C) and neutral and alkaline pH.

Materials and Methods :

The samples for isolation of amylase producing bacteria were collected aseptically from three different sources-that is soil from vermicompost from the campus of Patna Women's College, soil from vegetable dump site at Bazar samiti and soil from flour mill. The fresh samples were used within 24 hrs after collection.

For the isolation of amylase producing bacteria, normal saline (850 mg NaCl + 100 ml distilled water) was prepared, autoclaved (9.9ml in one vial and 9ml in five vials each). The five dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were prepared. Sterile NA+1% starch media plates were inoculated with 10^{-6} and 10^{-7} dilution by using enrichment culture technique under aseptic conditions, followed by incubation at 37°C respectively for 24 hours until growth was observed. The isolates were maintained by repeated sub culturing and preserved on slants of the same medium.

The isolates were screened for their potential of producing amylase by observing their growth patterns on starch agar media after their incubation. The pure isolates were streaked on starch agar plates with starch as only carbon source. After incubation at 37°C for 24 hours the individual plates were flooded with gram's iodine (Gram's iodine-250 mg iodine crystals added to 2.5gm potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue colour forms, which is

the basis of the detection and screening of an amylolytic strain. The amylase producers displaying maximum diameter of zone of clearance, were further investigated. The pure cultures were sub-cultured at regular intervals and starch- nutrient agar slants were maintained at 4^0C.

Characterization and identification of amylase producing bacterial isolates was done on the basis of morphological appearance of colony, Gram's staining and by different biochemical tests.For biochemical characterization, the isolated bacterial strains were tested for catalase, hydrolysis of urea, casein, starch, hydrogen sulphide production, nitrate reduction and sugar fermentation tests, IMViC Tests (Aneja, 2003).

For the preparation of crude enzyme, a loop full of bacterial culture was transferred from starch-nutrient agar slants to starch- nutrient broth at pH 7.0 for activation and incubated in a shaker at 37°C at 120 rpm for 24 hrs. Fermentation medium contained soluble starch (10 g/L), peptone (5 g/L), $(NH_4)_2SO_4$ (2 g/L), KH_2PO_4 (1 g/L), K_2HPO_4 (2 g/L), $MgCl_2$ (0.01 g/L) at pH 7.0. The fermentation medium was inoculated with the culture and incubated in shaker at 37^0C for 24 hrs. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 30 min to obtain the crude extract, which served as enzyme source.

For the production of amylase at different temperature, 50 ml of starch production media was inoculated with the bacterial culture (SA-3) and the flask were incubated at three different temperatures i.e. 26^0C, 38^0C and 55^0C, and then these entire flasks were incubated for 24 hrs, 48 hrs and 72 hrs for observing the amylase activity.

The production of amylase at different time intervals (24 hrs, 48 hrs and 72 hrs) were analysed by SDS PAGE. The enzyme activity was assayed following the method of Bernfeld (1955) using 3, 5-dinitrosalicylic acid. The enzyme assay was based on the productivity of the enzyme. Standard graph of maltose was prepared for the calculation of enzyme activity. Higher the activity, more maltose will be liberated in the solution and that will react with DNS to form a dark coloured (brownish red) compound. The enzyme activity was calculated based on this formula (Karnwal and Nigam, 2013)

$$\text{Enzyme activity (U/ml)} = \frac{\text{Concentration of enzyme} \times 1000 \times \text{Dilution factor}}{\text{Mol. wt. of Glucose} \times \text{Incubation time (min.)} \times \text{Amt. of enzyme (ml)}}$$

For the preparation of maltose standard, maltose solution was pipette out in the range of 0.2, 0.4, 0.6, 0.8 and 1 ml respectively into 5 separated tubes. Along with this, blank was also prepared by using DW. The volume were maintained up to 2 ml in each tube along with the tube containing blank solution. Then 1 ml of DNS reagent was added into each tube and the tubes were covered with aluminum foil and were kept in water bath for 15 minutes. Then tubes were cooled at room temperature and absorbance was taken at 540 nm.

For preparing 1% of starch substrate, 100 mg of starch was weighed and it was dissolved in 10 ml of 0.1 M sodium phosphate buffer (pH 7.0). 1 ml of substrate along with 1 ml of crude enzyme was taken in the different tubes. Blank was without enzyme. The tubes were incubated for 30 min at room temperature (37^0C). The reaction was terminated by adding 2 ml of DNS reagent and kept it in boiling water bath for 10 min. Absorbance was measured at 540 nm.

Optimum pH for highest enzyme activity was determined by using sodium phosphate buffer of different pH. The experiment was carried out individually at various pH (6.0, 6.5, 7.0, 7.5 and 8.0). 1% of starch was used as a substrate. Substrate solution was prepared in sodium phosphate buffer at pH (6.0, 6.5, 7.0, 7.5 and 8.0) in different test

tubes. 1 ml of crude enzyme was added to these test tubes. The mixture was incubated at room temperature for 15 min, and the reactions were terminated by adding 2 ml DNS reagent, further incubated in boiling water for 10 min. After cooling at room temperature the activity of enzymes was determined by taking the absorbance at 540 nm.

Optimum temperature for highest enzyme activity was determined. Substrate solution was prepared in sodium phosphate buffer (pH 7.0). 1 ml of substrate solution was added into six different test tubes along with 1 ml of crude enzyme. Then tubes were incubated at specific temperature i.e. at 4^{0}C, 25^{0}C, 38^{0}C, 40^{0}C, 55^{0}C for 15 min. Reactions were terminated by adding 2 ml DNS reagent and the mixture was incubated in boiling water in water bath for 10 min. After cooling at room temperature the enzymes activity were determined by taking the absorbance at 540 nm.

Results and Discussion :

A total of 10 bacterial strains were selected from nutrient agar starch medium supplemented with 1% starch. Characterization of colony with different colony characteristic was made (Table 1).

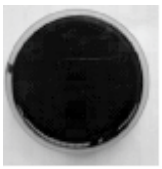
Table 1. Cultural Characteristics of the isolated bacteria

Isolates	Form	Elevation	Size	Margin	Opacity	Colour	Shape
SA-1	Regular	Flat	Medium	Regular	Opaque	Yellow	Coccus
SA-2	Regular	Flat	Medium	Regular	Opaque	White	Coccus
SA-3	Regular	Flat	Medium	Regular	Translucent	Yellow	Rod
SA-4	Regular	Flat	Medium	Regular	Translucent	Creamy	Rod
SA-5	Regular	Flat	Medium	Irregular	Transparent	Creamy	Coccus
SA-6	Regular	Flat	Medium	Regular	Translucent	Yellow	Rod
SA-7	Regular	Flat	Medium	Irregular	Opaque	Yellow	Rod
SA-8	Regular	Flat	Medium	Irregular	Transparent	White	Coccus
SA-9	Regular	Flat	Medium	Irregular	Translucent	White	Coccus
SA-10	Regular	Flat	Medium	Irregular	Opaque	Yellow	Coccus

Above ten isolate were analyzed for their ability to produce amylase enzyme. For this starch hydrolysis tests was performed for all 10 isolates. Starch hydrolysis tests were performed for all 10 isolates and out of those three isolates i.e. SA-3, SA-4 and SA-6 were amylase positive while are rest of the isolates were found amylase negative (Table 2, Figure 1).

Table 2. Starch hydrolysis test for different isolates

Isolates	Zone of clearance	Inference
SA-1	Absent	Negative
SA-2	Absent	Negative
SA-3	Present	Positive
SA-4	Present	Positive
SA-5	Absent	Negative
SA-6	Present	Positive
SA-7	Absent	Negative
SA-8	Absent	Negative
SA-9	Absent	Negative
SA-10	Absent	Negative



Control

(a) (b) (c) (d)

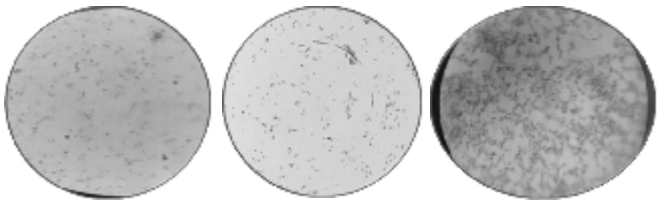
Fig.1. Starch hydrolysis test for different isolates. Isolate (a),(b),(d) showing positive for starch hydrolysis whereas (c) is negative for test.

Isolated bacterial colonies (SA-3, SA-4, and SA-6) were characterized microscopically as well as biochemically. Each colony was processed for staining with Gram's stain and observed under the

microscope at 40X and 100 X magnifications. (Euromex, Netherland). (Table 3, Figure 2)

Table 3. Microscopic characteristics of amylase producers

Sl. No.	Isolates	Gram reaction	Shape	Identification
1.	SA-3	Positive	Rod	*Bacillus* sp.
2.	SA-4	Positive	Rod	*Bacillus* sp.
3.	SA-6	Positive	Rod	*Bacillus* sp.



SA-3 SA-4 SA-6

Fig.2. Microscopic view of isolated bacterial strains

Different biochemical test like catalase test, urease test, indole test, citrate test, indole test and fermentation of different carbohydrate (sucrose, lactose, and dextrose) were performed for further identification of the isolated bacteria (Table 4).

Table 4. Biochemical test results of different isolate

Sl. No.	Biochemical Tests	SA-3	SA-4	SA-6
1.	Sugar fermentation test (i) Dextrose (ii) Lactose (iii) Sucrose	 Negative Positive Negative	 Negative Positive Negative	 Negative Positive Negative
2.	Amylase test	Positive	Positive	Positive
3.	Casein hydrolysis test	Positive	Positive	Negative
4.	Urease test	Negative	Negative	Negative
5.	H_2S production test	Positive	Positive	Positive
6.	Catalase test	Positive	Positive	Positive
7.	Nitrate reduction	Positive	Positive	Negative
8.	IMViC Tests (i) Indole production test (ii) MR Test (iii) V-P Test (iv) Citrate utilization test	 Positive Negative Positive Positive	 Positive Negative Negative Positive	 Positive Negative Positive Positive

Amylase fermentation media was used to analyze the production of amylase at different temperatures i.e. 26^{0}C, 38^{0}C and 55^{0}C which was incubated for three different time periods i.e. 24, 48, and 72 hours. The protein content in each crude enzyme was estimated qualitatively by mixing crude enzyme (100µl) with Bradford's reagent (200µl). The intensity of colour developed was used to make a rough estimation of the protein content in different samples. Further these samples were analyzed by SDS-PAGE. The reported molecular mass of different amylase produced from *Bacillus* sp. ranging from 42 to 150 kD and from 23 to 50 kD (Abdel-Fattah et al., 2013). It was observed that bands of around 75 kD are quite intense, showing production of amylase enzyme. It was observed qualitatively and also by SDS-PAGE that the production of enzyme was good at 38^{0}C. The amount of production was lesser at 26^{0}C than production at 38^{0}C where as at 55^{0}C the production of enzyme totally diminished. Also the amount of enzyme produced was comparable at different time interval. After 24hrs the production was little lesser than the production after 48hrs and production after 72 hrs was diminished due to reduced growth due to nutrient unavailability (Figure 3).

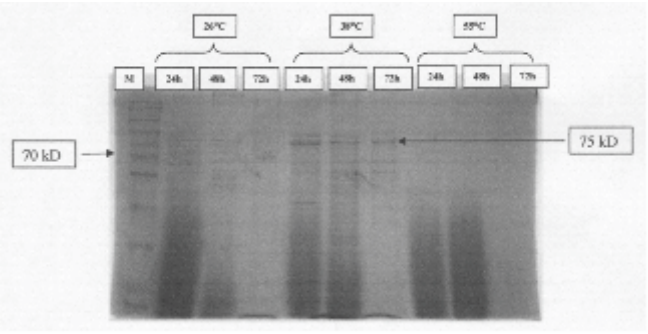


Fig.3. Quantitative analysis of amylase at different time interval by SDS - PAGE

For enzymatic activity firstly standard graph of maltose were prepared to find out the concentration of enzyme (Figure 4).

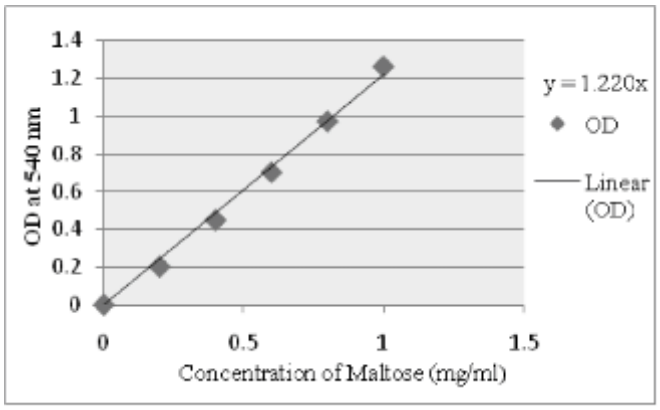


Fig. 4. Standard graph of maltose

For the optimization of Enzyme at different pH, we used crude enzymes produced at 38°C to see the activity at different pH range. The highest activity was observed at pH 7.5 than the rest of the pH value (Figure 5). The report from Abeba haile showed that the *Bacillus* spp. prefer neutral to slightly alkaline or a range between 6.8 and 7.2 pH for the amylase production. Vaidya and Rathore, (2015) have reported highest activity of amylase enzyme at pH 7.0.

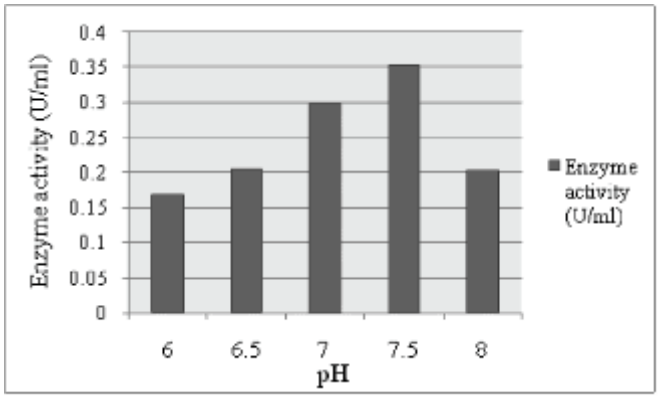


Fig. 5. Enzyme activity at different pH of strain SA-3

For the optimization of Enzyme at different temperature, the enzymes produced at 38°C were incubated at different temperatures (4°C, 25°C, 38°C, 40°C, and 55°C) to know the best temperature for enzyme activity. The highest activity was observed at 38°C than other temperature. Lower temperature as well as higher temperature decreases the enzyme activity (Figure 6). The report from Abeba haile showed that the *Bacillus* spp. Showed 37º was optimum for amylase production from one strain. Vaidya and Rathore (2015) have reported highest activity at 40º C.

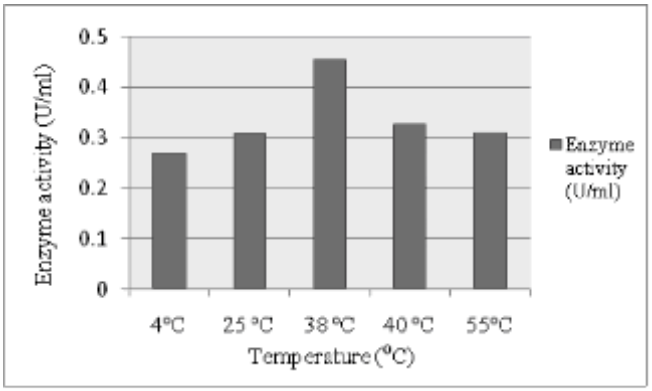


Fig. 6. Enzyme activity at different temperature of strain SA-3

Conclusion :

Amylase producing Strains were isolated from soil samples which were collected from different areas of Patna. Isolate SA-3 was highest amylase producer. The production of amylase was optimized at different pH and temperature. Optimum pH for the maximum activity was observed at pH 7.5 and optimum temperature was 38°C. This enzyme can be further purified by different precipitation techniques and chromatographic techniques to characterize it more. We can further take this bacterial strain for the production at pilot scale. Different other parameters like salinity etc. should also check before it can be used in industry.

Acknowledgement :

We extend our gratitude to our Former Principal, Dr. Sister Marie Jessie A.C. for giving us opportunity to carry out this research. We are thankful to the research committee for all the support provided. Our sincere thanks to Ms. Jaya Philip, HOD & coordinator, Department of Industrial Microbiology, Patna Women's College and all

faculties for their constant guidance and encouragement.

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