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Production of Amylase by *Bacillus Megaterium* Isolated from Estuarine Environment

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Abstract

Enzymes are important biological molecules that notably speed up all the chemical reactions. They are very crucial factors for life and serve a wide range of important functions in the body. The aim of the present study was isolation, identification, optimization and purification of amylase enzyme production by using marine bacterium *Bacillus megaterium*. Growth parameters of *B. megaterium* such as pH, temperature, salinity, nutrients of carbon and nitrogen sources and incubation time were influenced the bacterial growth. The identified optimized growth parameters are followed by pH 7, temperature 35°C, salinity 2%, starch and beef extract and incubation time 30hrs were found to be ideal conditions for the bacterial strain. The culture filtrate was purified by using several methods and its enzyme activity during purification considerably increased from 42U/ml/min. to 71 U/ml/min.

Keywords

Amylase, *Bacillus megaterium*, DEAE cellulose and SDS-PAGE analysis.

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INTRODUCTION

Enzymes are the biological catalyst, which initiate and accelerate thousands of biochemical reactions in living cells. Major sources of enzymes are the biological organism plants, animals, and microorganism. Microbial enzymes account the major volume. However, about less than 50 species are actually used to produce the entire microbial enzyme. The potential obviously exists to search for the species producing novel enzymes or enzymes with better properties and yield [1].

Amylase are hydrolyzing enzyme in function which causes hydrolysis of molecules. They are widely distributed in microbial, plant and animal kingdoms. They act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved [2].

Enzymes produced by the organism are of two types exoenzymes and endoenzymes. Exoenzymes which are released from the cell and act on the substrates. These are mainly hydrolytic enzymes that degrade by

the addition of high molecular weight substrates (like polysaccharides, lipid and proteins) into small components (e.g. glucose) that can enter into the cell and are later assimilated. Enzymes required for the hydrolysis of cellulose, starch, pectin, lipid, casein and gelatin belong to the category of exoenzymes. Endoenzymes are utilized by the cell for further metabolic degradation of carbohydrates and are mainly responsible for synthesis. New protoplasmic requirements and production of cellular energy from assimilated materials and these enzymes function inside a cell. These increased uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes [3]. The major advantages of using microorganisms for production of amylases are the ability to produce in bulk and ease at which it can be manipulated for desired products [4].

MATERIALS AND METHODS

Sample collection and isolation

Sediment samples with mangrove detritus were collected from Vellar esturary, Tamil Nadu. 10 gram of sediment sample was suspended in 90 ml of sterile water (50% aged sea water) and shaken thoroughly for 5 min. 0.1 ml of diluted samples was inoculated on the surface of starch agar medium. The inoculated plates were incubated at 35°C for 24hrs. The colonies were selected based on the different colony morphology and maintained on starch agar medium.

Medium for amylase production

The isolated colonies were aseptically transferred in to amylase production medium containing (g/100ml): peptone -0.5gm, yeast extract-0.3gm, NaCl-0.3gm, K_2HPO_4 -0.1gm, $MgSO_4$-0.02 and soluble starch-1g [5]. The medium was prepared by using 50% aged sea water and pH of the medium was maintained at 7.

Preparation of cell free supernatant

Each well isolated and morphologically different colony was selected and inoculated in 10 ml of amylase production medium and the tubes were incubated at 30°C for 48 hours. After the incubation period, the cell free culture supernatants were prepared by centrifuging at 8000 rpm for 15 min. Each isolates cell free culture supernatants were used for screening the production of amylase enzymes using well agar diffusion method.

Screening for amylase producing bacterial strains

Primary screening was done by using starch agar plate method. In secondary screening, 10 µl of cell free supernatant of each bacterial strains were added in the wells made in the starch agar with 1% of starch as a soul carbon and 2% agar. The plates ware incubated at 30°C for 24 hrs. After incubation, the plates were flooded with 1% aqueous iodine solution for 15 min and washed with water to remove the excess color. Based on the zone of clearance around the well, the potential strain was selected for further study and identification [6].

Identification of potential bacterial strain

The potential bacterial train was biochemically identified followed by the instruction of Bergey's manual of Determinative bacteriology [7] and also identified molecular level through 16s rRNA sequencing.

Optimization studies for amylase production

To study the effect of maximum growth, various physicochemical parameters like pH (5, 6, 7, 8 and 9), temperature (25°C, 30°C, 35°C, 40°C and 45°C), salinity (0.5%, 1.0%, 1.5%, 2.0% and 2.5%), different carbon (cellulose, sucrose, maltose, glucose and starch) and nitrogen sources (ammonium nitrate, ammonium sulphate, potassium nitrate, peptone and beef extract) were optimized. Throughout the optimization process starch broth medium (without adding agar) was used as a basal medium [5]. Optimization was performed using one parameter at all approach. Each parameter was carried out in a 250 ml conical flask. Inoculum (1ml) of pure culture from pre-incubated pure stain was inoculated to each flask and incubated at 30°C for 6-48hrs. Every 6 hrs, the sample was collected and the growth rate was measured by taking OD value at 600 nm using UV spectrophotometer.

Large scale production

Based on the optimization results, the potential strain *Bacillus megaterium* was cultivated with the optimized parameters (pH, temperature, salinity, different carbon sources and nitrogen sources) in 1000ml of conical flasks. The pH of the initial medium was adjusted to 7.0 with 1N NaOH and the flask was sterilized at 121°C for 15 min. The sterilized medium was inoculated with full loop of *Bacillus megaterium* and incubated at 35°C for 48 hours. At the end of incubation, growth rate was measured by taking OD value of the culture at 600 nm using UV spectrophotometer (Systronics, Double beam spectrophotometer 2202) while the enzyme activity assay was described below.

Amylase assay

After the end of large scale production, the cultures were centrifuged at 4000 rpm for 20 min at 4°C. The supernatant containing amylase enzyme was used for determination of enzyme activity followed by the method of Palanivelu *et al* [8]. As assay mixture containing enzyme extract, starch as a substrate and DNS reagent was used. One unite of amylase activity

was defined as the number of µ moles of starch liberated by 1 ml of enzyme per minute.

Partial purification of amylase

Ammonium sulfate precipitation

Solid ammonium sulfate was slowly added into the centrifuged crude enzyme extract with constant stirring over magnetic stirrer at 4^0C to obtain 80% saturation. The mixture was left overnight for precipitation at 4^oC [9]. The precipitates were collected through centrifugation at 6000rpm for 20 min. at 4^oC. The resulted pellet was dissolved in 5ml of phosphate buffer (pH 7.0).

Dialysis using phosphate buffer

The precipitated pellet sample was loaded into one end of the dialysis bag and another end was tightly sealed. The bag was suspended in a beaker containing distilled water with the help of glass rod. This entire setup was kept in a refrigerator overnight. After that, the bag was dialyzed against phosphate buffer (pH- 7.0) at 4^oC with three changes of buffer for 24 hrs [9]. The dialyzed sample was lyophilized and stored for further characterization.

DEAE- Cellulose column

The column was selected with a height and internal diameter of 30cm and 1.5cm respectively and was gradually filled with the suspension of DEAE-cellulose. The column was packed without any air bubble. Then, 0.25g of dialyzed sample was dissolved in 2ml of binding buffer (20mM Tris buffer pH 8.2) and applied to a DEAE-cellulose column which had been pre-equilibrated with 100 ml of 50 mM Tris-HCl (pH 8.0) buffer. When the buffer reached the gel bed 2ml of sample was loaded on the top of the column. The column was eluted using elution buffer (50 mM Tris-HCl (pH 8.0) containing 0.1- 0.5 M NaCl. 2 ml of 50 fractions were collected and precipitated with 70 % ammonium sulfate. The enzyme precipitate was dissolved in 50 mM Tris-HCl (pH 8.0) and it was dialyzed against the same buffer as previously. Protein concentration and amylase activity were assayed for the fractions and protein content was determined by Lowry *et al* [10] method.

SDS- PAGE analysis

The purified amylase sample was analyzed by SDS-PAGE [11] and the molecular weight of enzyme was determined. The sample was mixed with equal volume of sample buffer (2X Laemmli buffer) and boiled in a boiling water bath for 5 min. Then the sample and protein marker was loaded into the separate well of the stacking gel. The electrophoresis buffer was added in to the top and bottom reservoir. The sample was electrophoresed at 100V until the tracking reached 0.5cm from the bottom of the plate. After electrophoresis, the gel assembly was removed and the glass plates were separated. The gel was soaked in the coomassie brilliant blue staining solution and left overnight for staining. Then the gel was destained with the destaining solution and the protein bands were observed in the Gel-documentation system (DGelDAS, biotech, Yercaud).

RESULT AND DISCUSSION

A total of 12 strains were isolated from sediment samples, collected from Vellar estuary. In this study, starch agar medium (amylase) was used as the selective medium for the isolation of amylase producing bacteria (Fig.1). The isolated strains were primarily screened and amylase production was confirmed by flooding the plates with iodine solution. Iodine produces blue coloration by reaction with starch. But the amylase producing bacteria hydrolyze the starch by the action of amylase surrounding them and utilize it as a carbon source. As a result they gave a transparent zone due to utilization of starch iodine would not give any blue coloration.

Based on the primary screening, only 5 potential strains were selected. The selected potential strains were further subjected to secondary screening of well diffusion assay for their potential amylase production. From that well diffusion assay, only one strain was potentially produced highest clear zone (3.1 cm) formation in starch agar plate (Fig.2). The strain was identified as *Bacillus megaterium* based on their biochemical tests with the help of Bergey,s manual of Determinative Bacteriology (Table 1).

The potential strain was further confirmed through the molecular identification by 16s rRNA gene sequencing. The genomic DNA was isolated, and its quality was checked by loading in 0.8 % agarose gel with the DNA marker which showed the intact DNA. 16S rDNA of the strains were amplified through PCR analysis and the molecular weight of the isolates showed in figure 3. The partial 16S rDNA sequences along with their nucleotides of the isolates were submitted to the Gen Bank of National Center for Biotechnology Information, USA and the accession numbers are showed in the Table 2. The isolated bacterial strain was identified with bacterial sequences obtained from the GenBank DNA (NCBI) through the BLAST search tool. The 16S rDNA gene sequences of the isolated strain was preliminary compared with previously obtained sequences deposited in GenBank. The phylogenetic tree was constructed by using the representative species (Fig. 4). The BLAST result showed that close matches to the isolated strain in neighbor-joining tree.

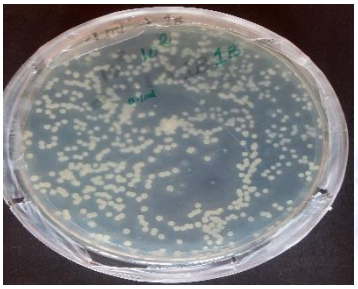


Fig. 1: Amylase producers

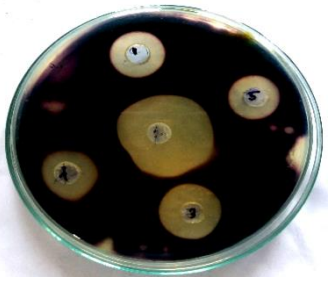


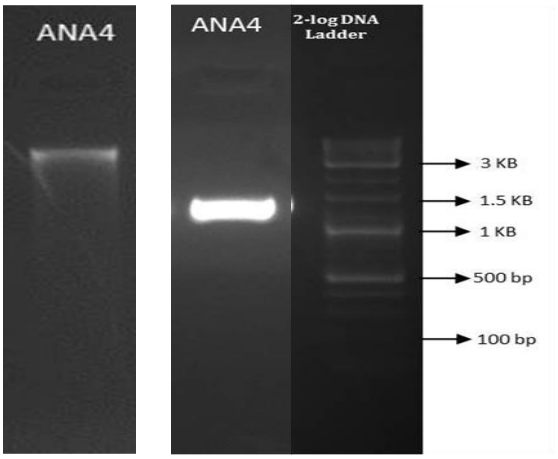
Fig. 2: well assay in starch agar

Table 1: Morphological and biochemical characterization of *Bacillus megaterium*

Test	Results
Morphology	
Shape and arrangement	Peritrichous flagella
Capsule	Present
Gram staining	+
Spore staining	+
Motility	Motile
Culture characterization on agar plates	
Colonies	Round, irregular
Temperature	Optimum (30^0C)
Growth	Abundant
Form	Irregular
Margins	undulate
Biochemical tests	
Oxidase	-
Catalase	+
Nitrate reduction	variable
Urease	-
H_2S production	-
Methyl red	-
VP	-
Citrate utilization	+
Indole production	-
Carbohydrate fermentation	
Lactose	+
Mannitol	+
Sucrose	+
Glucose	+
Maltose	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+

Table 2: Sequencing producing significant alignment of the isolated strains

Description	Max score	Total score	Query coverage (%)	E value	Ident (%)	Accession
Bacillus megaterium strain ATCC 14581 16S ribosomal RNA gene, partial sequence	2228	2228	100%	0.0	100%	KY082715.1



(I) Genomic DNA of *B. megaterium*
(II) Molecular weight of *B. megaterium*
Fig. 3: 16S rRNA-PCR analysis of potential isolate

>SR1009-16S
GGAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTC
ATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTA
CAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCAACGATGCATAGCCGACCTGA
GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGG
AAGAACAAGTACAAGAGTAACTGCTTGTACCTTGACGG
TACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAA
TTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGT
CTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA
TTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGC
GGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
GGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTG
TAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTG
CAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGT
CGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAAC
TCTAGAGATAGAGCGTTCCCCTTCGGGGGACAGAGTG
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT
CTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CTACAATGGATGGTACAAAGGGCTGCAAGACCGCGAG
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TAATC

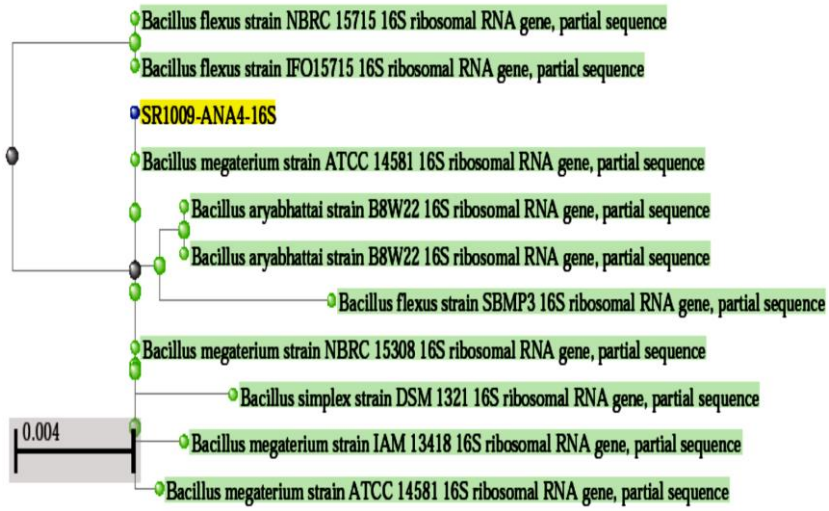

Fig. 4: 16S rRNA sequence and phylogenetic position of the strain (*Bacillus megaterium*)

In the present study maximum growth rate by the selected isolate was done after optimized to various factors such as pH, temperature, salinity, incubation time, carbon source and nitrogen source.

In the present study the amylase producing bacterial strain was subjected to different pH like pH 5-9. The maximum growth was obtained at pH 7.0 (OD 1.121). When pH was altered below or above to the optimum, the amylase production was decreased (Fig. 5). Bhutto and Dahot [12] reported the maximum amylase production was obtained at pH 7.0. Naidu and Saranraj [13] suggested, pH 7.0 were an optimum for commercial production of bacterial amylase by *Bacillus megaterium* in large scale production.

The effect of different temperature on bacterial growth was optimized. In this study, maximum bacterial growth (OD 1.153) was obtained at 35°C as showed in figure 6. Based on the result, 35°C was found to be optimum temperature for the strain. Vidyalakshmi *et al* [14] also obtained maximum amylase production at 35°C. They suggested if further increased in optimum temperature, the amylase production also decreased. The temperature 35°C was an optimum for continuous production of amylase enzyme from *Bacillus megaterium* has been reported [13]. Vijalakshmi *et al* [15] obtained maximum production of enzyme at 35°C and pH7.

In the present study, different concentration of NaCl was optimized (Fig. 7). The results showed that 2% of NaCl concentration was found to be optimum concentration for maximum bacterial growth (OD 0.985). Carbon sources greatly influence amylase production and the most commonly used substrate is starch [16]. Different sources of carbon such as cellulose, sucrose, maltose, glucose and starch were optimized for maximum growth. The obtained results showed that, starch was found to be best carbon source which produced maximum growth of OD 1.103 compared to other carbon sources (Fig.8). Similarly Lin *et al* [17] and Asghar [18] also obtained the same results. Suman and Ramesh [19] suggested that starch is a generally accepted source and best component for the production of amylolytic enzyme. Different nitrogen sources were optimized and beef extract was found to the best nitrogen sources for optimum growth of *Bacillus megaterium* (Fig.9). Generally the organic nitrogen sources such as peptone and beef extract are frequently enhanced the amylase production in the medium [20]. Beef extract and peptone supported maximum amylase production by many bacterial strains [21].

Effect of incubation period on amylase production was showed that 30 hrs was the optimum incubation period for maximum bacterial growth (Fig.10). Sonia Sethi *et al* [22] reported the increase of incubation period above the optimum, the cells may reach the decline phase and displayed low amylase production. Similarly *Bacillus sp*. showed that the amylase production was detected from 48-72 hours and maximum production was obtained at 30 hours [23]. The optimized growth parameters for *Bacillus megaterium* strain was used for mass scale production in a 1L flask. At 30 hours of incubation, the maximum biomass of 2.320 OD and enzyme activity of 42 U/ml/min was recorded in the medium with the selected carbon and nitrogen sources (Fig.11). At the end of mass scale production, the pellet and supernatant were separated by centrifuged the culture broth at 4000rpm for 20 min at 4 °C.

The culture supernatant from the optimized mass scale culture broth with selected C and N sources was harvested at 30 hours of incubation. The crude enzyme extract was partially purified by ammonium sulfate precipitation resulted in good yield. The inhibitory protein was found to be highest at 80% ammonium sulphate precipitation. The precipitate was further subjected to tubular cellulose membrane dialysis against phosphate buffer. At the end of dialysis the enzyme extract was purified by using DEAE cellulose column. Enzyme activity during purification considerably increased from 42U/ml/min. to 71 U/ml/min (Table 3). Finally the dialyzed sample was lyophilized and SDS-PAGE analysis revealed that the purified amylase produced by *Bacillus megaterium* strain was a single prominent protein band with a molecular weight of 42 kDa (Fig. 12). Damodara *et al* [24] isolated a single polypeptide on SDS-PAGE with a molecular weight of 58kDa.

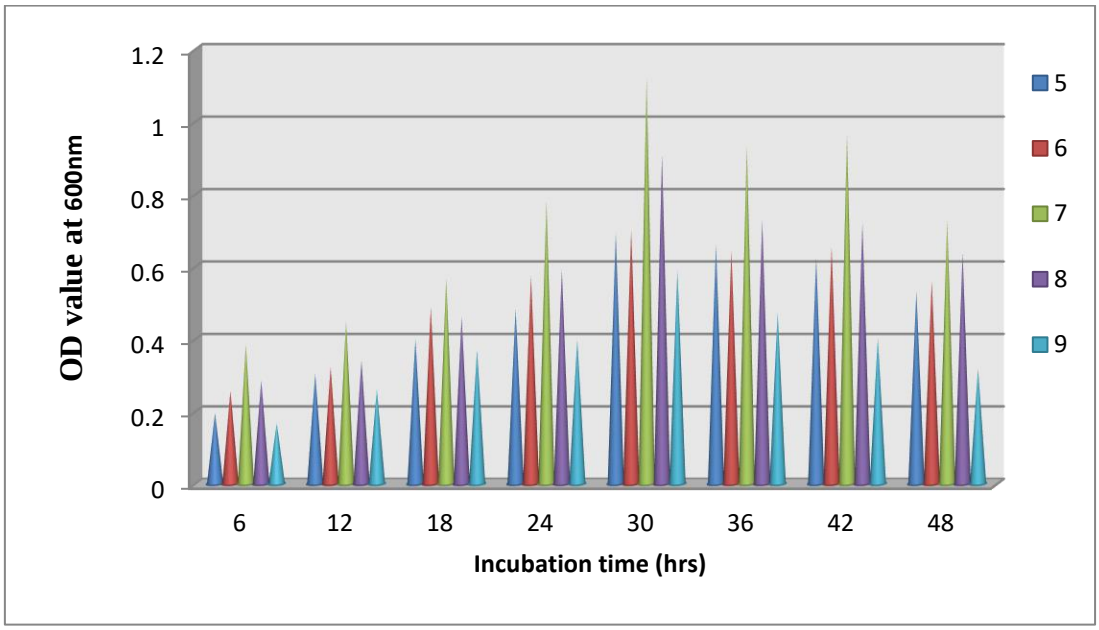

Fig.5: Effect of pH on growth of *B. megaterium*

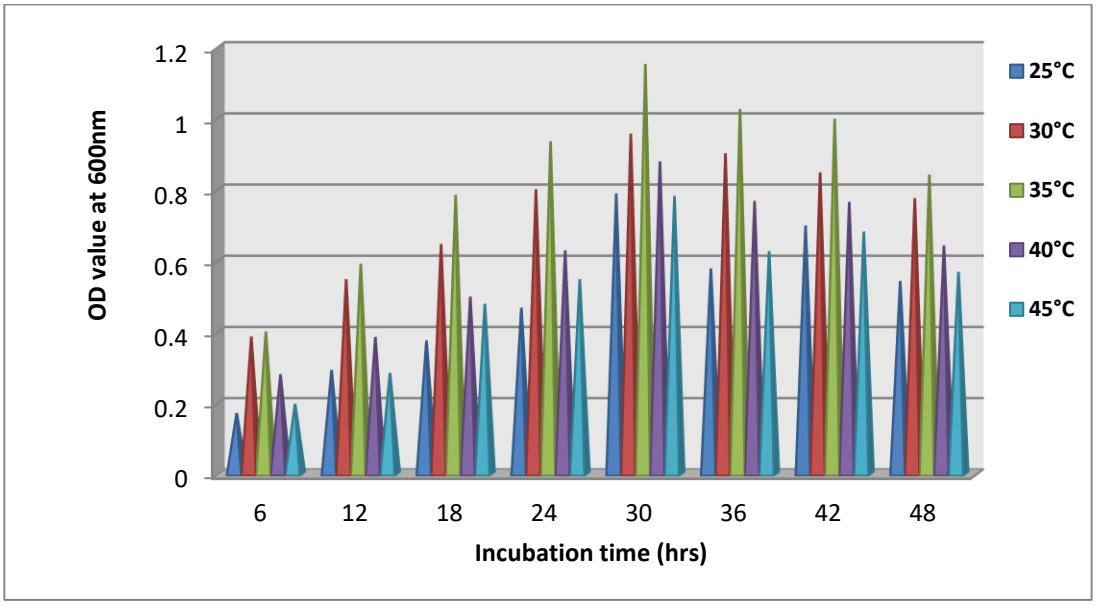

Fig.6: Effect of temperature on growth of *B. megaterium*

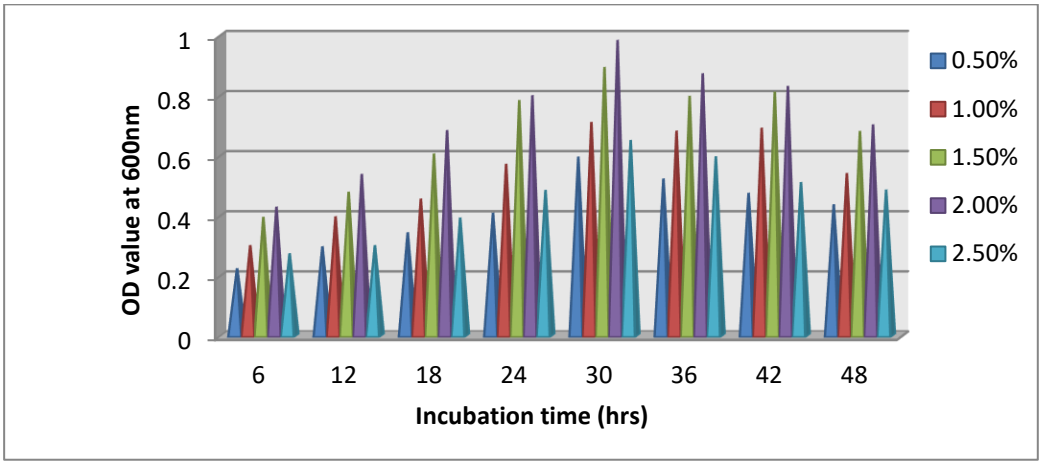

Fig.7: Effect of salinity on growth of *B. megaterium*

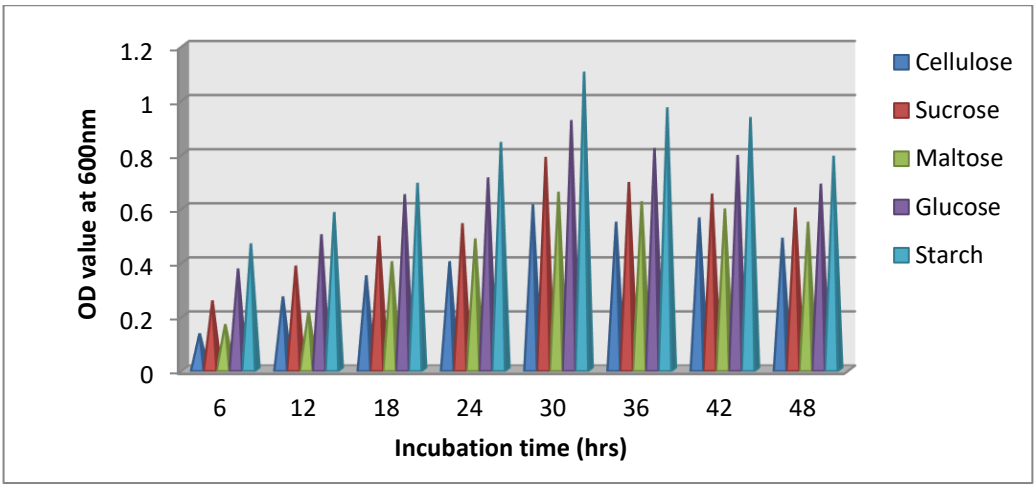

Fig.8: Effect of carbon sources on growth of *B. megaterium*

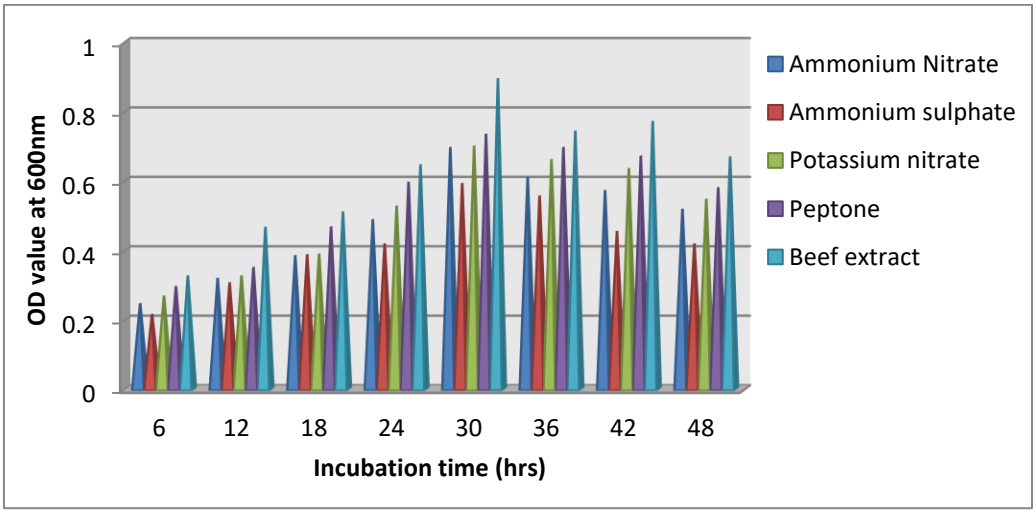

Fig.9: Effect of nitrogen sources on growth of *B. megaterium*

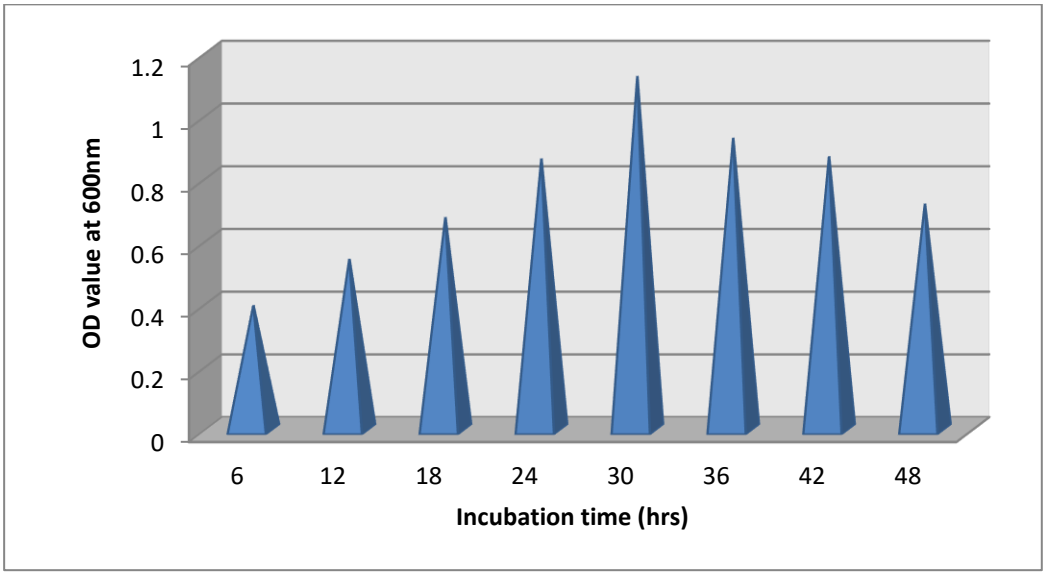

Fig.10: Effect of incubation period on growth of *B. megaterium*

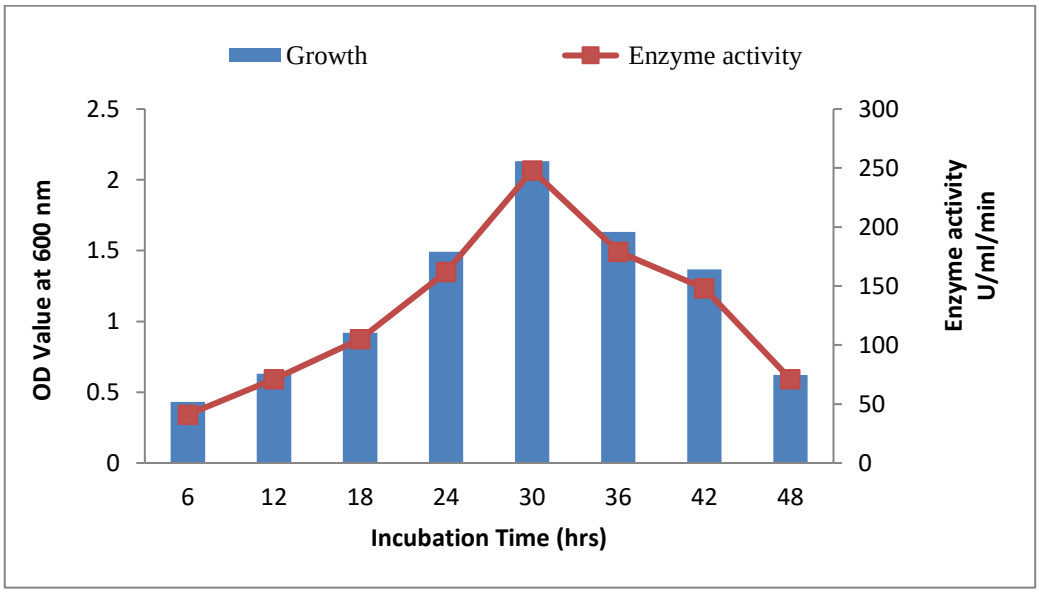

Fig. 11: Mass scale production of amylase enzyme

Table 3: Amylase activity at various stage of purification

S. No	Purification step	Amylase activity (U/ml)
1.	Cell free supernatant	42.0
2.	Ammonium precipitation	54.0
3.	Membrane dialysis	62.0
4.	DEAE-Cellulose purification	71.0

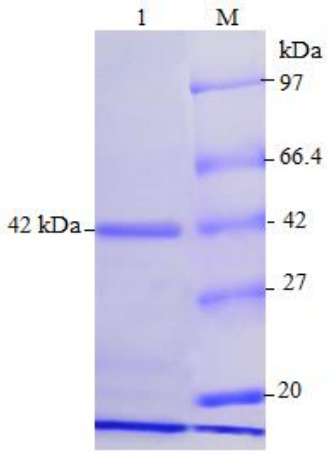

Fig. 12: SDS-PAGE analysis of purified amylase produced by the bacterial isolate (*Bacillus megaterium*)
Lane 1: Purified amylase enzyme (42 kDa) and Lane M: Standard protein molecular weight marker

CONCLUSION

The present study deals with the amylase enzyme production by a bacterial strain *Bacillus megaterium* isolated from sediment sample. The bacterial strain was biochemically identified and confirm by further identification through molecular level of 16S rRNA sequencing.The growth parameters of the identified bacterial strain was optimized. The produced enzyme was purified and the molecular weight of the amylase was analyzed.

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