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# Screening of amylase producing bacteria from soil samples of evergreen and deciduous forest

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## Abstract

*Amylase is an industrially important enzyme used for the hydrolysis of dietary starch into disaccharides and trisaccharides and ultimately to glucose. Plants, animals and certain microorganisms produce this amylase and are used for various biochemical reactions. The most stable and reliable source of amylase is obtained from microbes when compared to other sources. The purpose of the current study was to isolate and characterize amylase producing bacteria from soil samples collected from the evergreen and deciduous forest of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram, Kerala, India. The bacterial colonies were isolated through serial dilution and plating techniques. One bacterial colony which showed the maximum zone of clearance in starch agar plates were isolated and cultured separately. The screening was done on the basis of hydrolysis of starch by amylase on agar plate containing 1% starch. The 16S rDNA sequence of the selected strain was isolated and screened using Clustal W programme and a phylogenetic tree was constructed using a Mega 6 software using neighbor joining method. The bacteria were identified as Bacillus sps.*

**Keywords**: Amylase, Bacillus, 16S rDNA, Bacillus, cluster analysis.

## Introduction

Microbes are omnipresent, where their presence invariably affects the environment that they are growing in. Soil is a unique territory for various microbes, including a wide range of microbial population such as bacteria, fungi, algae, viruses and protozoa. Studies of microbial enzymes is very important to understand the biochemical activities and identification of the biotechnological potentials of microorganisms. The hydrolytic activity of enzymes produced by various microorganisms has attracted several industries due to their potential applications in different areas. When compared to the synthetic enzymes, microbial enzymes are relatively more stable with diverse properties on various fields. Microbes are considerably easy to manipulate and is considered as a major advantage during bulk productions of amylase in industries, which often benefits us economically[1]. Among carbohydrates, starch is the major food reserve on plants.

Starch is the major polysaccharide made of amylose and amylopectin[2]. These polymers differ in structures as well as in physical properties. Starch is the primary storage polysaccharide in plants and an important constituent of the human diet. Starch can be converted into various useful products by the application of enzymes[3]. Amylase is an important starch degrading enzyme which is widely distributed in the world of microbial, plant and animals[4]. Amylase hydrolyzes the bonds between adjacent glucose units to yield various other products[5]. α-1, 4-glucan-glucanhydrolase commonly known as alpha amylase, degrades the α, 1-4glucosides linkages in starch and similar substrates produce a variety of oligosaccharides[6].

All types of amylases are glycoside hydrolases and act starch molecules which yields different products, dextrin and glucose units. Its vast applications in food and other industries has led to a greater increase in the production of alpha-amylase[7]. Paper, pulp and textile industries also make use of amylase during their production and manufacturing along with their use in food and starch industries[7].

The enzymatic hydrolysis of starch by microbial amylases have deeply replaced the chemical hydrolysis of substrate in industries. Microbial source of amylase is commonly preferred because of its vast availability. Amylase is also used to improve the softness of the bread[8]. *Bacillus amyloliquefaciens*, *Bacillus licheniformis*[9], *Bacillus stearothermophilus*[10], *Bacillus subtilis*[11], *Bacillus megaterium*[12] and *Bacillus circulans* etc. are the industrially used microbial strains used for alpha amylase production globally[13]. Therefore, this work was conducted for the isolation and identification of amylase producing bacterial strains from evergreen and deciduous forest soil samples.

## Materials and methods

**Sample collection:** Soil samples were collected aseptically at a depth of 6 to 12 inches from evergreen and deciduous forests, inside JNTBGRI, Palode, Thiruvananthapuram, Kerala, India. Samples were properly labeled and brought to the laboratory in sterile conditions and kept in sealed bags for further studies.

**Isolation of bacteria from soil:** The bacterium was isolated from soil samples using standard dilution plate techniques. 10g of each soil samples were diluted in Erlenmeyer flask and was shaken in rotary shaker at 1000rpm for 30minutes. The supernatant was serially diluted to obtain $10^{-1,}$ $10^{-2,}$ $10^{-3}$ dilutions. 0.1 ml from $10^{-3}$ dilution was pipetted into nutrient agar plates with 1% starch (pH 7.4). After overnight incubation at 37$^{o}$C bacterial colonies were selected and subcultured in nutrient agar containing 1% starch.

**Screening of amylase producing strains:** Amylase producing strains from the soil samples were screened by pouring Gram's Iodine over the nutrient agar plates with 1% starch and observed for starch hydrolysis. From the first soil sample eight bacterial colonies were observed, on which two colonies producing amylase were identified. From the second soil sample five bacterial colonies were obtained, but neither of them showed amylase activity. Bacterial strains with maximum clear zones were selected, sub cultured and stored for further investigation.

**Qualitative assay for amylase activity:** Qualitative measurement of amylase production was carried out using starch agar. Nutrient agar plates prepared with 1% starch. Wells were cut on the agar with cork borer. These wells were filled with 100µl of culture filtrate collected at different time intervals (1hour to 8 hours). After overnight incubation at room temperature, plates were flooded with Gram's iodine and zone of enzyme hydrolysis were measured.

**Isolation of genomic DNA:** Genomic DNA (gDNA) was isolated from the cultures grown in nutrient broth[14]. Collected 2ml of the culture in a microcentrifuge tube followed by centrifugation to harvest the cells and 1gm was suspended in 500µl in Tris EDTA buffer in 2ml reaction tube. To this 100µl lysozyme added and swirled to dissolve. After incubation at 30$^{o}$C for 30 minutes, 5ml of 20mg/ml proteinase K and 100µl of 10% SDS were added and mixed gently and incubated at 37$^{o}$C for 30 minutes. After incubation added equal quantity of saturated phenol chloroform isoamyl alcohol (25:24:1) to the tubes and shaken well. The tubes are then centrifuged at 12000rpm for 10 minutes. The aqueous layer was collected and 10µl of 5mg/ml RNase solution was added to it and incubated at 37$^{o}$C for one hour. Traces of phenol was removed by adding 600µl of chloroform isoamylalcohol thrice followed by 5 minutes shaking and collection of aqueous phase. To this 20µl of 4M NaCl was added and total DNA was precipitated by adding 2volumes of cold 100% ethanol and subjected to centrifugation at 15300g for 10 minutes. 70% ice cold ethanol were added to the tubes and the pellet was air dried and resuspended in 50µl of nuclease free water.

**Separation of DNA by AGE:** The agarose gel electrophoresis (AGE) was carried to observe the isolated DNA. Total DNA was mixed with 6X loading buffer and loaded onto agarose gel (0.4%) along with molecular weight marker for electrophoresis at 100V for 30 to 40 minutes. Gel documentation system was used to analyze the DNA and it was then photographed under ultraviolet (UV) light.

**PCR amplification of 16S rDNA:** PCR amplification of approximately 1.5kb of 16S rDNA of selected bacterial strains was performed in a thermal cycler using 0.5µl of DNA, universal primers 8-27F (forward primer) and 1495R (reverse primer), 12.5µl of Emerald mix (dNTPs, Taq polymerase and buffer) and made to 25µl by adding water. The entire mixture has 25µl which is subjected to amplification in a thermal cycler with initial denaturation at 98 $^{o}$C for 1 minute, denaturation for 10 minutes, annealing at 58 $^{o}$C for 30 seconds, extension at 72$^{o}$C for 1 minute 30 seconds and final extension at 72 $^{o}$C for 7 minutes. Amplification of PCR product was confirmed by resolving the PCR product in agarose gel (1.2%) electrophoresis and visualized using Biorad gel documentation system using quantity one software.

**DNA sequencing:** Sequencing (by Sanger method) of the purified PCR product was carried out at SciGenome Private Limited, Cochin, Kerala, India. The sequencing was done with both forward and reverse primers used in PCR. The sequence obtained was aligned using BioEdit software programme and contings aligned. The obtained partial sequence of 1380 base pair was subjected to BLAST analysis. Most similar sequences downloaded were aligned with Clustal W programme and a phylogenetic tree was made using Mega6 software.

## Results and discussion

Soil samples were collected aseptically from the forest patches of JNTBGRI at a depth of 6 to 12 inches. Samples collected from evergreen and deciduous forests, were serially diluted and plated on nutrient agar plates with 1% starch (pH 7.4). After overnight incubation at 37$^{o}$C, bacterial colonies (Table-1) were selected and sub cultured in nutrient agar plate containing 1% starch (Figure-1). Soil is the major habitat of many microorganisms. The forests soils are rich in organic matters and many species of actinomycetes, bacteria and fungi which utilize these organic matters by producing hydrolyze enzymes for their nutrient purposes and for their metabolism.

**Table-1:** Different bacterial isolated from forests.

| Isolate | Habitat | Amylase activity |
|---|---|---|
| TBGAmy001 | Evergreen forest | Potential amylase activity |
| TBG1 | Deciduous Forest | No activity |

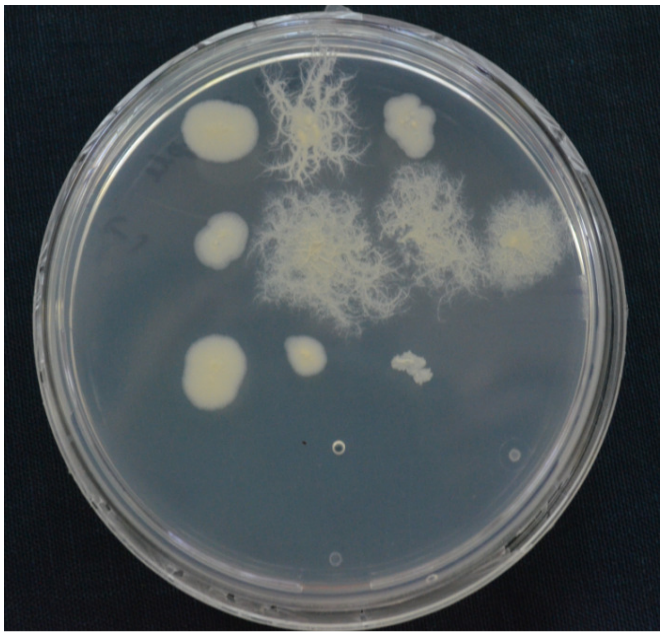

**Figure-1:** The Nutrient agar plate showing the sub cultured bacterial colonies.

The isolated strains were screened for amylase activity. The important substrate used was starch[12,13]. Amylase production is greatly influenced by the substrate which is used as a carbon source during production process, where the starch itself can increase the enzyme production while the use of glutamate and citrate can reduce the production[15-17]. The amylase activity was analyzed by pouring Grams' iodine over the nutrient agar plate. The iodine binds with the starch in the nutrient agar plate and forms an intense blue-black color and the rest of the plate that lack starch will have no color and is an indication of amylase activity. Bacteria and fungi produce extracellular amylases to carry out extra cellular digestion[18]. The bacteria capable of producing amylase will degrade/ hydrolyse starch around the colony. The remaining starch in the media will be turning to dark blue by the addition of gram's iodine. The amylase positive colonies will be having a clear zone around the colony (Figure-2). The maximum amylase production was observed with an isolate named TBGAmy001, and was selected, sub-cultured and stored.

The selected bacterial strain TBGAmy001 was inoculated to 100mL Nutrient broth with 1% starch in an Erlenmeyer flask and incubated at 37$^{o}$C. 1mL of culture was removed aseptically at 1hr interval and were analyzed for amylase activity quantitatively and also for growth curve. The results are given in Figure-3 and 4. The amylase activity was demonstrated on 1% starch agar plate with 5mm wells cut on agar. 100µl of supernatant were applied to each well and incubated at 37 $^{o}$C and activities were observed at regular intervals (Figure-3). The isolate TBGAmy001 showed amylase activity from the first hour of incubation and the maximum activity at the seventh hour as evidenced by the hydrolysis zone produced in starch agar plate. The growth pattern of TBGAmy001, incubated at 37$^{o}$C up to 8 hours were estimated using spectrophotometer at 1hour interval. OD was taken at 600nm (Figure-4). The TBGAmy001 grow well at 37$^{o}$C and attain log phase at 2$^{nd}$ hour, log phase at 2-4$^{th}$ hour and then attain stationary phase. It is noticed that the amylase production starts with log phase and attain optimum at stationary phase of growth. The properties and quantity of carbon and nitrogen sources used for enzyme production also influence the growth and production of extracellular amylase in bacteria[19,1].

**Figure-2:** Amylase activity of TBGAmy001.

The bacteria were taken in a microscopic slide and photographed using a Nikon microscope with photographic attachment (Figure-5). The strain showed bacillus type morphology on Gram staining. The extraction of genomic DNA was made from the cultures grown in nutrient broth and 16s rDNA was amplified using 8-27F and 1450R primers. Approximately 1.4 kb PCR product was obtained and visualized on a 1% agarose gel.

The PCR product was purified using Invitrogen kit and send for sequencing at SciGenome Private Limited, Cochin, Kerala, India. The results obtained showed 1380 base pair sequences as shown below (Figure- 6). Sequence obtained was aligned using BioEdit Software and contings aligned. Thus obtained partial sequence was subjected to BLAST analysis. The most similar sequences were downloaded and aligned using Clustal W programme. The phylogenetic tree (Figure-7) was made with Mega6 software using Neighbor-Joining method and the isolated bacterium was confirmed as *Bacillus* sp.

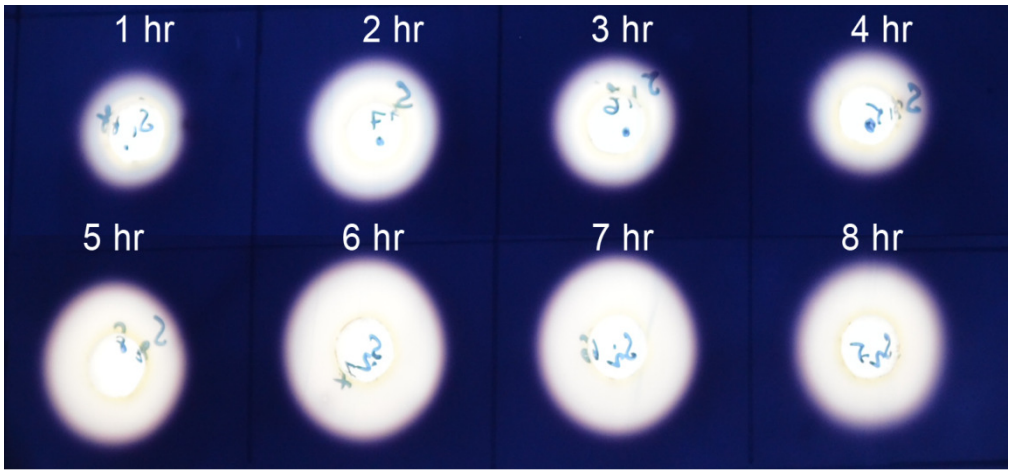


**Figure-3:** The amylase activity of culture free supernatant from broth culture.

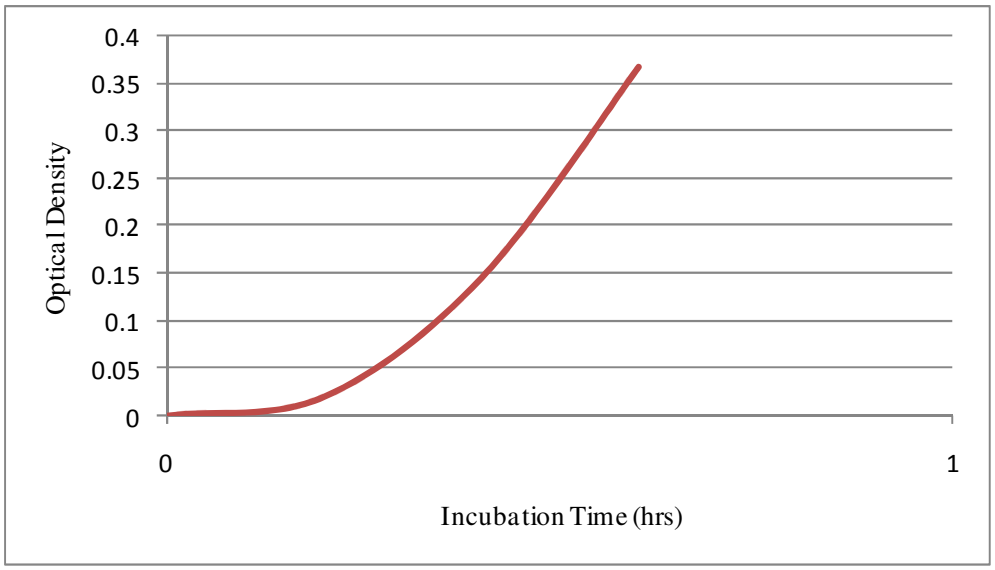


**Figure-4:** The growth pattern of TBGAmy001 isolate up to 8hrs incubation.

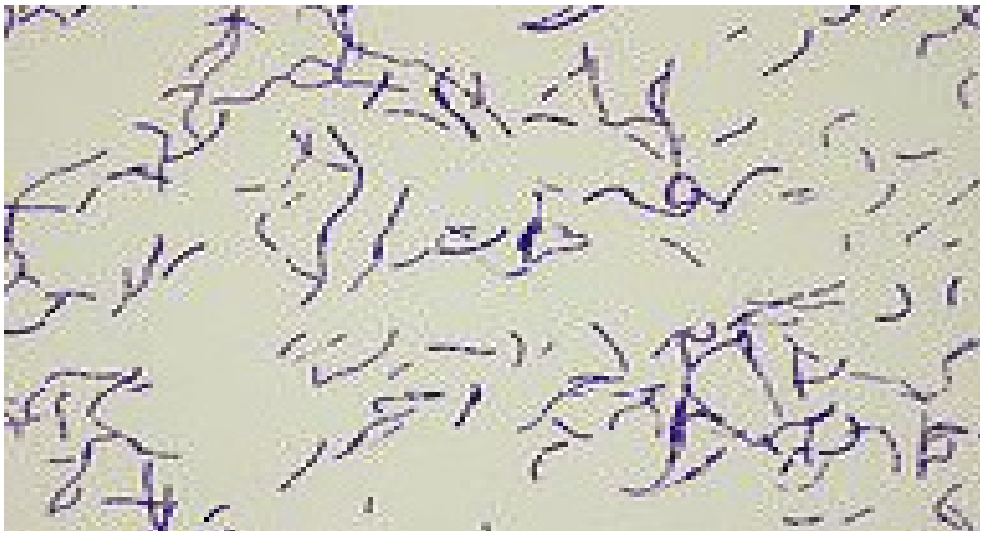

**Figure-5:** Photomicrograph of TBGAmy001.

>Bacillus-TBGAmy001

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1    ATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTG
101  TCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCT
201  TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
301  CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
401  GGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
501  GCAAGCGTTGTCCTGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCC
601  TGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
701  CTGTAACTGTCGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
801  TCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA
901  GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAG
1001 AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
1101 TGGGCACTCTATGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
1201 GGACAGAACAAAGGGCAGCGAAACCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAG
1301 TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
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**Figure-6:** Sequenced PCR product2.

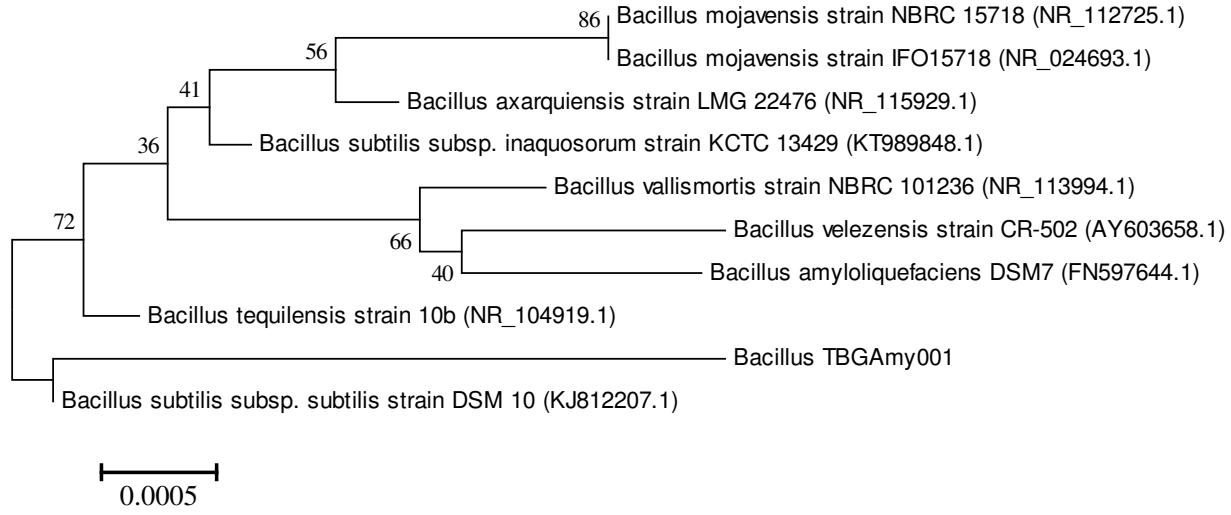


**Figure-7:** Evolutionary relationships of TbGAmy001.

## Conclusion

The widespread distribution of amylase among microbes has attracted the interests of many biologists due to their applications in starch hydrolysis. The bacterial amylase can replace the chemical hydrolysis of starch in various industries and can thereby be beneficial to mankind. The sole purpose of the work was to isolate and characterize amylase producing soil bacteria. Through Gram staining and genome analysis it is thus concluded that the amylase producing bacteria is *Bacillus subtilis* and it can be exploited for large scale production of amylase. The enzyme production can be directly correlated to the time period of incubation in the case of *B. subtilis*[20]. The present study showed an increase in enzyme production with an increase in the incubation time.

## Acknowledgement

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