# Exploration of *Penicillium* spp. for α-amylase secretion from forest soil of Chakrashila Wildlife Sanctuary, Assam

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***Abstract***

*In industrial microbiology, α-amylase holds the top priority with a worldwide enzyme market share of about 30%. The commercial demand of amylases is fulfilled by few species of Bacillus and Aspergillus. α-amylases are also evidenced to be produced by a number of Penicillium species. The experiment was designed for isolation and also for identification of species of Penicillium from diverse soil habitats collected from Chakrashila Wildlife Sanctuary, Assam, India. The species were also screened to check the capacity to synthesize amylase in laboratory conditions. During the investigation, 24 numbers of Penicillium cultures were identified and examined for their capacity to generate amylase.*

*Among the 24 isolates of Penicillium, 10 numbers of isolates exhibited clear zone formation on solid starch agar medium indicating significant amylase activity. 10 isolates having amylolytic activity terms of clear zone formation were again screened in starch broth for amylase synthesis and it was noted that Penicillium isolate S1 has highest amylase activity. DNA sequencing followed by BLAST analysis of Penicillium isolate S1 with NCBI database was used to identify the species to be Penicillium citrinum. This study adds to the catalogue of local fungi that have been isolated in Assam and offers further information to support future research about the secondary metabolites such as industrial enzymes.*

**Keywords:** Isolation and identification, amylase, screening, *Penicillium*, enzyme activity, solid state fermentation, BLAST.

## Introduction

Microbial enzymes have been utilized in different industries as major catalysts to transform raw materials into desired products since long. For several decades, due to immense potential of microbes as a source of industrially significant enzymes, large numbers of microbes from various sources were being utilized to produce extracellular enzymes since long.[14,17] The discovery of microbes with new properties for the industrial biosynthesis of extracellular enzymes is continually increasing that drive the growing demand of microbial enzymes in multitude of disciplines such as pharmaceuticals, biotechnology, agriculture, textiles, leather, paper, detergent, food processing, feed processing and waste management etc.[19,23]

Amylases, particularly α-amylases have largest share among industrial enzymes. α-amylases are extensively applied in many diverse industries including paper, detergent, sugar, alcohol, textile and food processing industries such as bakery, brewery, in making digestive aid and starch syrups etc.[6,10,12] Besides, due to the rapid growth occurring in many areas of industrial microbiology and biotechnology, the use of these enzymes is also being expanded into a number of new fields including clinical, medical and analytical chemistry.[21] According to reports, the estimated enzyme market share of amylase is nearly 30%[17]. In 2023, the α-amylase market was projected to be USD 10.53 billion. This was as assumed to rise at the rate of 7.5% represented as compound annual growth rate (CAGR) through 2024 to become USD 17.54 billion in 2030[27].

In recent years, the bread and baking industries were found to have the most prevalent use. In terms of region, with a 37% market share in 2023, North America dominated the market. Due to the sizable and expanding population in Asia Pacific, which will raise demand for baked products, substantial increase in the global enzyme market is expected. According to a report, the enzymes market size in India in the year 2023 was calculated as USD 440.5 million along with a CAGR of 7.8% till 2030[8]. As a result, efforts are constantly made to find new and improved microorganisms that can produce amylase.

Although amylases have been found in all domains of life i.e. plants, animals and microbes, however, the industrial need for amylases is supplied by microbial sources, particularly thermophilic bacteria and mesophilic molds.[4,17,24] Microbial synthesis of amylases has several advantages over plant and animal resources, particularly attributed to large scale industrial production volume and simple manipulation in obtaining enzyme of desired characteristics.[4,9,23] Although several reports suggest that there are a numbers of microorganisms that can produce amylase, the industrial demand is fulfilled by only some *Bacillus* and *Aspergillus* species including their tailored strains. Many researchers have also investigated the ability of different *Penicillium* species to secrete amylases.

It has been established that species such as *P. chrysogenum*, *P. janthinellum*, *P. expansum*, etc. have a great deal of

prospective to be used in industry as commercial amylase producers[5,22,28]. The common status as "Generally Recognized As Safe (GRAS)" makes fungal amylases more suitable for industrial use[1,9]. In addition, research has revealed that mycelial fungi are more potential producers of a variety of extracellular bioactive compounds besides enzymes in SSF (solid state fermentation) using agro-wastes as substrate. It is important to note that use of agroindustrial waste materials in fermentation technology is now considered as sustainable technology.[21]

Consequently, the present work was undertaken for exploration and selection of amylase synthesizing species of *Penicillium* from soil samples collected from various sites of Chakrashila Wildlife sanctuary, Assam for future investigation in industrial microbiology for the exploitation of secondary metabolites.

## Material and Methods

**Soil sampling and isolation of *Penicillium:*** Random soil samples were collected in sterile polybags from various undisturbed and disturbed sites of Chakrashila Wildlife Sanctuary, Assam. For the isolation of *Penicillium* species, dilution plating method with culture media Czapek dox and potato dextrose agar was adopted. For future use, the fungal samples were purified and preserved on agar slants at $4^0$C. The isolates were identified by observing colony characteristics and microscopic studies and taking references from standard manuals such as A Manual of Soil Fung[17] and Handbook of Soil Fungi[18].

**Isolation of Genomic DNA for species identification:** Commercially available QIAGEN DNeasy UltraClean Microbial Kit was used to isolate DNA from the fungal cultures (isolates PS1, PS2 and PS3). After isolation, for quality check of the isolated DNA 1.0 % agarose gel was used exhibiting single band of DNA. Next, amplification of 18S rRNA gene portion was done by 18S rRNA forward and reverse primers. The PCR product was resolved on agarose gel and a single band of DNA with 1500 bp was detected. Contaminants from the PCR amplicon were detached by using the purification kit QIAGEN QIAquick. By using sequencing kit BDT v3.1 and 18S rRNA primers (forward and reverse), the amplified DNA was sequenced. Instruments used were - Thermo Fisher MiniAmp Plus Thermal Cycler and ABI 3730xl Genetic Analyzer. Nuceotide sequencing of 18s rRNA gene for identification was done by Sanger's dideoxy method. The NCBI database was used to analyze DNA sequencing results. The BLAST web tool was followed for sequence similarity search. With the help of sequence identity value, the amylase producing fungi were identified from the database.

**Amylase enzyme screening of *Penicillium* cultures**

**Primary screening**: The isolates to be screened for amylase activity were cultured over starch agar medium composed of yeast extract-1.5g/l, peptone-5g/l, starch-2g/l, NaCl-5g/l, Agar-15g/l. The pH was adjusted to 6.5. The medium was sterilized by autoclaving at $121^0$C for 15 minutes. Prepared medium was poured in sterilized Petri dishes and fresh inoculum was transferred over the plated medium. The inoculated Petri dishes were kept under incubation for 96 hours at $30^0$C in a BOD incubator. Following addition of Gram's iodine solution, the amylase activity on the plates was seen as a distinct zone on a dark blue backdrop. Both the colony diameter as well as diameter of clear zones were recorded.

**Secondary screening**: Secondary screening for amylase activity was conducted in broth including Yeast extract-3g/l, Peptone-5g/l, Starch-20g/l, $KH_2PO_4$-1.5g/l, $MgSO_4.7H_2O$-0.5g/l, $FeSO_4$-0.01g/l. The medium having pH 6.5 was sterilized in an autoclave at $121^0$C for 15 minutes. The inoculum used was 7 (seven) days old culture. The spores of the culture were detached by adding 5ml sterile water with an inoculation loop. By vortexing, a homogenous spore suspension was prepared and 1ml inoculum was dispensed to a conical flask (250ml) containing 50ml of the medium. Inoculated flasks were kept in BOD incubator at $30^0$C for 72 hours. Dinitrosalicylic acid (DNS) method was used for assay of amylase[15].

**Assay of amylase**: After incubation, the broth was filtered by using Whatmann no. 1 filter paper. Amylase activity was quantified from the filtrate. The steps are given below:

The reaction mixture consisting of 1 ml of filtrate as enzyme source and 1 ml of 1% soluble starch solution as substrate was added in a test tube and the mixture was incubated at 40/50°C for 30 minutes. The reaction in test tube was arrested by the addition of DNS reagent (3ml) and heated in a water bath for 10 minutes. Cool the reaction mixture to room temperature and measure the absorbance of the solution at 540 nm in a spectrophotometer[15]. A standard graph was prepared with 0-100 µg maltose and amylase reaction for the test samples were calculated from the graph. The amylase activity was described as unit/ml (U/ml) where one unit indicates the quantity of enzyme required to release 1µg of reducing sugar per minute during the typical assay conditions.

## Results and Discussion

The amylases are essential enzymes for starch based industries. Many microbial amylases are commercially available which fulfill the industrial demand. There are a variety of microbes that are capable of producing amylase enzymes. However, the majority of the amylase enzymes used for industrial purposes are exploited from microbial sources belonging to bacteria and fungi. In general, species meant for large scale industrial biosynthesis of fungal amylases belong to genera *Aspergillus* and *Penicillium*.

During the study, a total of 24 isolates were identified to belong to the genus *Penicillium* based on cultural as well as morphological characteristics from different soil samples collected from undisturbed and disturbed habitats of

Chakrashila Wildlife Sanctuary, Assam. The isolates were designated as S1 to S24. The purified *Penicillium* cultures were recognized by observing colony characteristics and microscopic examination as well as comparing the observed features with standard manuals[7,18] (Fig. 1).

The *Penicillium* isolates were subjected to determine amylase enzyme synthesis under experimental conditions. It was performed as primary screening on solid medium and secondary screening in broth. The initial amylase test was done on starch agar medium by incubating the cultures. For determining the enzyme activity of amylase, the plates were poured with Gram's iodine. The enzyme secretion was specified by a clear zone, formed in a dark backdrop around the fungal colony (Fig. 1).

The ability to degrade starch by the *Penicillium* isolates was determined with the help of DCZ/DFC (diameter of clear zone/diameter of fungi colony) ratios.[14] In table-1, DCZ/DFC results of the *Penicillium* cultures are presented. Among all the *Penicillium* cultures, 10 isolates (Sl. no 1 to 10) demonstrated prominent amylase activity with reference to DCZ/DCF ratios.

The DCZ/DFC ratio showed by isolate number 1-10 ranges between 0.88 - 1.01 (Table-1) which are found to be showing higher amylase activity under the studied conditions. However, clear zone formation may not have direct quantitative correlation in terms of enzyme production[2]. Hence, the amylase secreting fungi can only be partially selected using starch plates. These 10 numbers of isolates were again screened in liquid culture medium to get more clear information about the ability to produce α-amylase in terms of α-amylase activity.

The 10 *Penicillium* isolates (Table-1) exhibiting higher amylase activity was further evaluated to check amylase production capacity in liquid media containing starch. Table 2 represents amylase activity of ten *Penicillium* cultures appearing to be good in primary screening. Experimental results revealed that in liquid medium these 10 cultures were capable of producing variable quantities of amylase. However, the isolate PS-1 (25.36 ± 0.1 and 34.32 ± 0.4 units/ml) exhibited the highest enzyme activity both at $40^0$C and $50^0$C respectively. *Penicillium* isolates PS-2 (23.57 ± 0.3 and 30.85±0.2 units/ml) and PS-3 (23.11 ± 0.2 and 29.08 ± 0.1 units/ml) also showed comparable enzyme activity among the 10 isolates. The enzyme activity at higher temperature is a desirable character because several biotechnological operations involve thermostable amylases.

These *Penicillium* isolates have potentiality to secret extracellular amylase. Therefore, the *Penicillium* isolates S1, S2 and S3 may be considered for further studies on α-amylase production with regard to optimization of fermentation conditions followed by downstream processing of the enzyme to recover in purified form. This will reflect biochemical properties of the enzyme and hence will help in determining the enzyme to be commercially viable or not. Similar kinds of studies were also conducted by various workers and their results also support the preliminary outcomes of this study[2,3,11,13,22,25,26].

However, more research on optimizing different fermentation factors has to be evaluated in assessing potential amylolytic *Penicillium* species reported in the study. Besides, characterizations of the physico-chemical properties will be required to verify the compatibility of such amylase enzyme in different applied fields. Even though a number of *Bacillus* species and *Aspergillus* species are contributing as industrial source for amylases, novel resource of microorganisms are being exploited from various natural habitats globally to obtain amylases with distinctive properties to meet the mounting need.

**Molecular Identification of *Penicillium***: In general, the identification of filamentous fungi is primarily based on morphological characteristics. However, these methods of identification often create confusion because a single species may have different morphotypes and biotypes. In addition, these are time consuming and involve good deal of skill and expertise. In recent times, with the advancement of molecular technologies, DNA sequence analysis methods of identification have been employed extensively as these are reproducible, rapid and accurate[16,20].

In the study, the best three isolates *Penicillium* S1, S2 and S3 were identified by molecular methods such as DNA isolation, PCR amplification, purification of amplified DNA (Fig. 2), Sanger di-deoxy chain termination method and bioinformatics methods for sequence homology analysis with NCBI BLAST. The molecular techniques combined with bioinformatics tools provide comprehensive identification of bacteria and fungi. The identification of the isolate *Penicillium* S1, S2 and S3 was done by using 18S ribosomal RNA (rRNA) gene sequencing method.

The NCBI-BLAST nucleotide similarity search examination, derived from rRNA gene sequencing proved that cultures *Penicillium* S1 and S2 both belong to the species *Penicillium citrinum* and the isolate *Penicillium* S3 belongs to *Penicillium griseofulvum.* The closest phylogenetic neighbour according to NCBI BLAST analysis for *Penicillium* S1 isolate showed 100% sequence homology *Penicillium citrinum* (MW113536.1). The NCBI BLAST analysis for *Penicillium* S2 showed 99.36% sequence homology with *Penicillium citrinum* with accession no. OQ548097.1 and the *Penicillium* S3 showed 100% sequence similarity with *Penicillium griseofulvum* having accession no. OP214767.1 in NCBI BLAST analysis. The sequences of all the three isolates have been deposited to NCBI Genbank nucleotide sequence database. Genbank accession numbers against the submitted sequences are – for *Penicillium* S1, accession no. is PV035140, for *Penicillium*S2, accession no. is PV035141 and for *Penicillium*S3, the accession no. is PV035142.

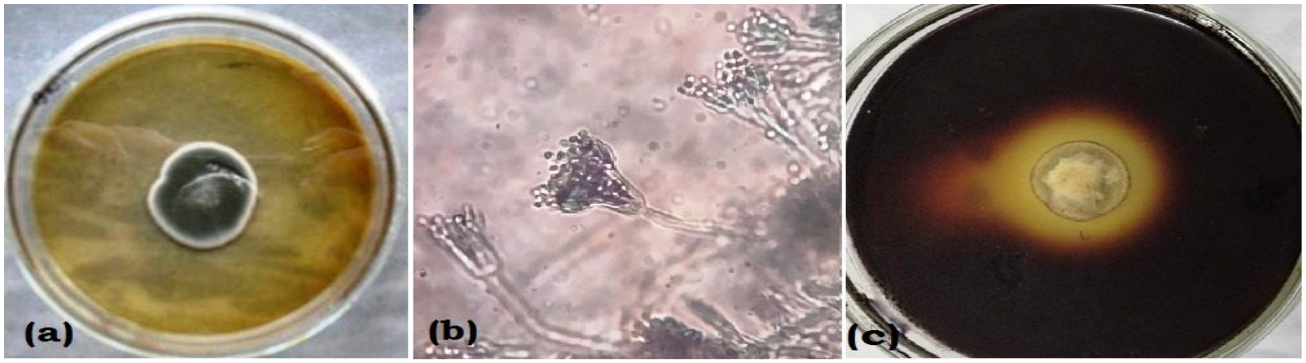


**Fig. 1: *Penicillium* - S1 (a) colony on agar plate (b) conidiophores with conidia (c) clear zone showing amylase activity**

**Table 1**
**Screening of cultures for amylolytic activity on solid starch medium**

| S.N. | Isolate no. | DCZ (mm) | DFC (mm) | Hydrolysis activity index = DCZ/DFC |
|---|---|---|---|---|
| 1 | S1 | 6.9±0.12 | 7.0±0.2 | 0.98±0.1 |
| 2 | S2 | 7.0±0.20 | 6.9±0.3 | 1.01±0.16 |
| 3 | S3 | 7.1±0.10 | 7.2±0.2 | 0.98±0.1 |
| 4 | S4 | 6.9±0.16 | 7.3±0.2 | 0.94±0.12 |
| 5 | S5 | 6.5±0.10 | 6.9±0.1 | 0.94±0.06 |
| 6 | S6 | 6.0±0.15 | 6.7±0.2 | 0.89±0.11 |
| 7 | S7 | 6.4±0.10 | 6.6±0.2 | 0.96±.0.1 |
| 8 | S8 | 6.5±0.30 | 7.2±0.3 | 0.9±0.2 |
| 9 | S9 | 6.3.0±0.12 | 6.8±0.2 | 0.92±0.1 |
| 10 | S10 | 6.2±0.21 | 7.0±0.3 | 0.88±0.17 |
| 11 | S11 | 4.0±0.26 | 7.5±0.1 | 0.53±0.12 |
| 12 | S12 | 3.2±0.31 | 6.8±0.1 | 0.47±0.13 |
| 13 | S13 | 3.8±0.17 | 7.6±0.1 | 0.5±0.09 |
| 14 | S14 | 0 | 6.3±0.2 | 0 |
| 15 | S15 | 0 | 5.3±0.2 | 0 |
| 16 | S16 | 0 | 5.0±0.2 | 0 |
| 17 | S17 | 3.3±0.15 | 6.8±0.1 | 0.48±0.08 |
| 18 | S18 | 2.4±0.20 | 7.0±0.2 | 0.34±0.13 |
| 19 | S19 | 2.6±0.31 | 7.1±0.1 | 0.36±0.13 |
| 20 | S20 | 2.5±0.40 | 6.5±0.1 | 0.38±0.16 |
| 21 | S21 | 1.8±0.12 | 6.2±0.2 | 0.29±0.1 |
| 22 | S22 | 1.2±0.12 | 6.6±0.3 | 0.18±0.14 |
| 23 | S23 | 0 | 5.7±0.3 | 0 |
| 24 | S24 | 0 | 6.4±0.4 | 0 |

(DCZ= Diameter of clear zone; DFC= Diameter of fungal colony; results are represented as - Mean ± SD)

**Table 2**
**Amylase activity in starch broth**

| S.N. | Isolates | α-amylase activity (units/ml) (Mean ± SD) | |
|---|---|---|---|
| | | $40^0$C | $50^0$C |
| 1 | S1 | 25.36 ± 0.1 | 34.32 ± 0.4 |
| 2 | S2 | 23.57 ± 0.3 | 30.85 ± 0.2 |
| 3 | S3 | 23.11 ± 0.2 | 29.08 ± 0.1 |
| 4 | S4 | 18.35± 0.2 | 15.20 ± 0.5 |
| 5 | S 5 | 19.72± 0.3 | 17.50 ± 0.2 |
| 6 | S6 | 20.22 ± 0.7 | 22.33 ± 0.3 |
| 7 | S7 | 16.87 ± 0.4 | 11.70 ± 0.7 |
| 8 | S8 | 14.12 ± 0.2 | 18.10 ± 0.5 |
| 9 | S9 | 20.81 ± 0.2 | 20.22 ± 0.1 |
| 10 | S10 | 19.70 ± 0.1 | 15.15 ± 0.3 |

**Table 3**
***Penicillium* species identified by molecular method with GenBank Accession No.**

| Isolate | Identified species | GenBank Accession No. |
|---|---|---|
| *Penicillium* S1 | *Penicillium citrinum* | PV035140 |
| *Penicillium* S2 | *Penicillium citrinum* | PV035141 |
| *Penicillium* S3 | *Penicillium griseofulvum* | PV035142 |

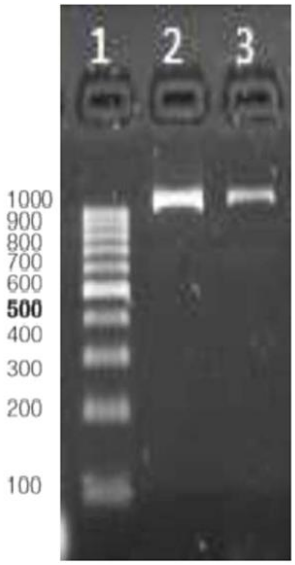


**Fig. 2: Gel electrophoresis of DNA to check quality control (1-leader, 2- sample1, 3- sample2)**

## Conclusion

Enzymes are most important secondary metabolite in industrial microbiology and amylases have utmost demand in the enzyme market with broad array of applications in various industries sharing about 30% of the world enzyme market. The market of this enzyme is constantly increasing. Food and starch utilizing industries are the major consumers of α-amylases and thus the demand of these enzymes would always be high in such segments. Moreover, clean catalytic methods are in the line of rising demand to convert waste into bioproducts in order to achieve a carbon-neutral economy. α-amylase is one of the ideal components in producing green and renewable energy (biofuels) from agroindustrial wastes[21,23].

Therefore, the current trend focuses on exploring novel microorganisms that can produce amylase with added features. Studied results show that the *Penicillium* isolates such as S1, S2 and S3 possess high potential for amylase production in primary research. In thorough investigation, these species may also emerge as commercial source of α-amylases. The *Penicillium* isolates S1 and S2 have been identified as *Penicillium citrinum* and the isolate S3 was identified as *Penicillium griseofulvum* and the DNA sequences of these three species of *Penicillium* were submitted to NCBI GenBank database (Accession nos. PV035140, PV035141and PV035142).

Utilizing the solid state fermentation approach to produce amylase has the ability to employ a variety of agroindustrial wastes that can also aid in the management of solid wastes. It could be a huge prospect to discover amylase producing microorganisms with unique features from Assam and NE India. The reason for this could be because available data reveal that no substantial investigation has been conducted in these areas. Moreover, this region of India harbours rich biodiversity but microbial diversity is very less explored. There is always a high demand of microbial α-amylases with suitable characteristics globally.

Even though using genetically modified fungal strains has greatly increased the production of these enzymes, research is still going on to identify newer sources of enzymes from natural habitats. Therefore, the search of hyper amylase producing microorganisms is crucial to meet the industrial demands of α-amylase and the further studies on identified *Penicillium citrinum* as well as *Penicillium griseofulvum* may be vital steps to find out their industrial potentiality.

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