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Screening and Characterisation of Amylase Producing Bacteria from Azerbaijan Mud Volcanoes Samples

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Abstract

Soil harbours a diverse range of microorganisms capable of producing industrially significant enzymes, including amylase. Amylase plays a crucial role in various biotechnological applications, such as food processing, pharmaceuticals, and biofuel production. This study focuses on the isolation and characterisation of amylase-producing microorganisms from different soil samples. Soil samples were collected from various ecological niches, including agricultural fields, compost sites, and garden soils. The isolates were screened for amylase production using starch hydrolysis assays. The most potent strains were identified through morphological, biochemical, and molecular techniques. The enzymatic activity was further analysed under varying pH, temperature, and substrate concentrations to determine optimal production conditions. The results demonstrated that several bacterial and fungal strains exhibited significant amylase activity, with *Bacillus* species being the predominant producers. This study highlights the potential of soil microorganisms as a valuable source of industrial amylase and their possible applications in biotechnology.

Keywords: Amylase, Soil Microorganisms, *Bacillus*, Starch Hydrolysis, Enzyme Production, Biotechnology.

1. Introduction

Amylases are important hydrolytic enzymes that break down starch into simpler sugars, making them essential for various industrial applications, including food processing, pharmaceuticals, textiles, and biofuel production. Microorganisms are a major source of amylases due to their rapid growth, ease of genetic modification, and ability to produce enzymes in large quantities. Soil is a rich habitat for diverse microbial populations, including bacteria and fungi, that produce amylases as part of their metabolic processes. Among amylase-producing microorganisms, *Bacillus* species are widely studied for their high enzyme yield and stability under extreme conditions. Fungal species such as *Aspergillus* and *Penicillium* also contribute significantly to industrial amylase production. Screening and isolating these microorganisms from different soil environments can help identify new strains with enhanced enzymatic properties.

Amylases are crucial enzymes that have been massively used in industries. Amylases are divided mainly into alpha-amylase and beta-amylase; they hydrolyse starch into smaller units such as glucose and dextrins. The industrial applications of amylases include liquefaction of starch, baking and brewing of alcohol, production of high fructose syrups, paper production and textile industries. Amylases are obtained from different sources, including plant origin, animal origin and

from microorganisms. The production of amylase using microorganisms has been reported to be more efficient than other sources of amylase production, as the method is economical, fast, and consistent. Amylase producers, including *Bacillus* species obtained from soil, have been reported to produce high yields using starch hydrolysis and biochemical techniques. A thermophilic bacterium identified as *Bacillus licheniformis* with amylase-producing characteristics has been isolated from a hot spring with optimum amylase production of over 200 U/ml. Aside from bacteria being one of the popular microbial sources of amylase production, fungi have been reported to possess this attribute as well. Cindy *et al.* have reported the production of amylase from *Aspergillus niger* isolated from fermented cassava product and cultured in potato peel medium. Ire *et al.* reported the isolation of *Aspergillus flavus* from soil and wastewater in a cassava processing site, which could produce amylase up to 2.3 U/ml/min after 96 h of incubation. However, amylase-producing bacteria are more preferred over fungi due to their ability to quickly cell growth, multiply, and ease of production process scalability.

1.1 Azerbaijan Mud Volcanoes

Azerbaijan, often referred to as the "Land of Fire," is home to the world's highest concentration of mud volcanoes, with over

350 out of the approximately 1,000 found globally. These geological formations are primarily located in the Absheron Peninsula, Gobustan National Park, and the Caspian Sea region. Mud volcanoes are caused by the underground accumulation of gases, mainly methane, along with water and fine sediments, which erupt to the surface, forming unique landscapes. Azerbaijan's mud volcanoes play a significant role in the country's geological, ecological, and energy sectors. They are often linked to oil and gas reserves, making them valuable indicators for hydrocarbon exploration. Some of these volcanoes produce spectacular eruptions, with flames and gas explosions, while others continuously release mud, creating conical or crater-like structures. Beyond their geological importance, Azerbaijan's mud volcanoes are also a major tourist attraction and hold cultural significance. Gobustan, a UNESCO World Heritage site, features petroglyphs and ancient human settlements near active mud volcanoes, demonstrating their historical relevance. Additionally, the therapeutic properties of mud from these volcanoes are used in traditional medicine and spa treatments. The mud used in mud baths is usually a combination of natural mineral-rich clay and water, which is heated to a comfortable temperature. The mud is then applied to the body, and the person is left to soak in the mud for a period of time, typically around 15-20 minutes. The benefits of mud baths are allegedly numerous. The heat of the mud helps to increase blood flow and stimulate the immune system, while the minerals in the mud are believed to have a range of healing properties. Mud baths are also said to help detoxify the body, ease muscle pain and tension, and promote healthy skin. A 2021 systematic review of studies which investigated the effect of mud baths on osteoarthritis concluded that mud bath therapy "cannot substitute for conventional therapy" but can be used in addition to it as "treatment with mud-bath therapy may relieve pain, stiffness and improve functional status in patients". In addition to the physical benefits, mud baths are also believed to have a relaxing and rejuvenating effect on the mind. Many people find that the warm, soothing sensation of the mud helps to reduce stress and promote a sense of well-being.

A mud volcano or mud dome is a landform created by the eruption of mud or slurries, water and gases. Several geological processes may cause the formation of mud volcanoes. Mud volcanoes are not true igneous volcanoes as they do not produce lava and are not necessarily driven by magmatic activity. Mud volcanoes may range in size from merely 1 or 2 meters high and 1 or 2 meters wide, to 700 meters high and 10 kilometres wide. Smaller mud exudations are sometimes referred to as mud-pots. The mud produced by mud volcanoes is mostly formed as hot water, which has been heated deep below the Earth's surface, begins to mix and blend with subterranean mineral deposits, thus creating the mud slurry exudate. This material is then forced upwards through a geological fault or fissure due to local subterranean pressure imbalances. Mud volcanoes are associated with subduction zones and about 1100 have been identified on or near land. The temperature of any given active mud volcano generally remains fairly steady and is much lower than the typical temperatures found in igneous volcanoes. Mud volcano temperatures can range from near 100 °C (212 °F) to occasionally 2 °C (36 °F), some being used as popular "mud baths".[citation needed] About 86% of the gas released from these structures is methane, with much less carbon dioxide and nitrogen emitted. Ejected materials are most often a slurry of fine solids suspended in water that may contain a mixture

of salts, acids and various hydrocarbons.

This study aims to isolate and characterise amylase-producing microorganisms from soil samples collected from various ecological niches. The findings could contribute to the development of cost-effective and efficient enzyme production methods for industrial and biotechnological applications.

2. Materials and Methods

2.1. Sample Collection: Mud soil samples were collected from Azerbaijan to observe the amylase-producing microorganisms. The pH of the sample was 7 to 7.5. The major step for isolating the microorganisms is preparing media by using NAM (Nutrient Agar Medium).

2.2. Isolation and Screening of Amylase-producing Bacteria: The collected soil samples were serially diluted on NAM medium Plates and incubated at 30°C for 48 hours. The isolated strains were screened for amylolytic properties on starch agar medium containing soluble starch-2%, peptone-0.05%, KCl-0.01% (w/v), MgSO₄ · 7H₂O-0.05% (w/v), (NH₄)₂SO₄-0.01%, NaH₂PO₄-0.01% (w/v). The fungal isolate was streaked as a line on the starch agar medium, and the plates were incubated at 30°C for 3 days. After incubation, iodine solution (1%) was flooded on the starch agar plate. Amylase-positive strains were determined by the presence of a clear zone of starch hydrolysis around the colony on the starch agar plates.

2.3. Optimization of Amylase Activity: To study enzyme activity, crude enzyme (500 µl) from a freshly grown reaction mixture was incubated at 70 °C for 10 min. Further enzyme assay was accomplished using bacterial culture with 0.05 M sodium phosphate buffer (1 ml) having 1.5% soluble starch. Ty DNS procedure. The colour reduction was recorded at 450nm by a spectrophotometer. Physical Factors: Temperature: 25-40°C (optimum: 30-35°C) pH: 5.5-7.5 for optimal enzyme production. Carbon source: Starch, glucose, or maltose for optimal enzyme production. Nitrogen source: Peptone, yeast extract, or ammonium sulfate for optimal enzyme production. Incubation time: 24-72 hours for optimal enzyme production.

2.4. Characterisation studies of the bacteria:
Morphological Characterisation: The potent bacterial strain was characterised by morphological, biochemical and molecular methods.

i). Slide Culture Technique or Coverslip Culture Technique: This technique is also called as "inclined coverslip technique". For the study of morphological characteristics with reference to aerial mycelium, substrate mycelium and sporulation, this technique was used. The isolate was cultivated on YMD agar, and a sterile coverslip was inserted at an angle of about 45° into a solidified medium in a Petri dish so that half the coverslip is in the medium. The inoculum was spread along the line, where the upper surface of the coverslip meets the agar, with a fine wire needle, and the plates were incubated at ambient temperature for 3-7 days. After the incubation period, the isolate was grown on both the medium and the coverslip. The coverslip was carefully withdrawn from the microscope to differentiate between substrate and aerial mycelium. Slides were prepared by using Lacto-phenol Cotton Blue (LPCB) reagent. The observation was also recorded about the

presence of colour of spore mass (grey, blue, red, violet, yellow or white), spore chain morphology (rectiflexibiles, spirals or retinaculiaperti) and presence of sclerotia.

- ii). **Scanning Electron Microscopy:** The conidia morphology of the strain was studied under the scanning electron microscope (SEM). Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C and postfixed 2% in aqueous osmium tetroxide for 4 h. Dehydrated in a series of graded alcohols and dried to critical point drying with a CPD (EMS 850) unit. The processed samples were mounted over the stubs with double-sided carbon conductivity tape, and a thin layer of gold coating over the samples was done by using an automated sputter coater (Model JEOL JFC 1600) for 3 min and scanned under Scanning Electron Microscope (SEM - Model: JOEL-JSM 5600) at required magnifications as per the standard procedures 12, 13.

2.5. Biochemical Characterisation

- i). **Staining (Gram's Staining):** In this staining procedure, a thin smear was made on a clean glass slide, which was air-dried and fixed by flaming. Then the thin smear was subjected to Gram's staining. The smear was flooded with crystal violet solution for 1 min, decolourised crystal violet with 95% ethanol for a few seconds and then counterstained with saffranine solution. Then, the slide was blotted dried and observed under a microscope under oil immersion (Benson, 1994) [6].
- ii). **Indole Production Test:** This test was conducted to test the ability of bacteria to decompose the amino acid tryptophan to indole, which accumulates in the medium. Tryptone broth inoculated with a 24-48-hour-old culture and incubated for 48 hours at 37°C. Indole production was then tested by the coloured reaction with the addition of Kovac's reagent. The red colour in the alcohol layer indicates a positive reaction (Collee *et al.*, 1996) [8].
- iii). **Methyl Red Test:** Using YMD (Yeast Extract Malt extract dextrose (ISP-2)) broth, the actinobacteria culture was inoculated and incubated at 28° °C for 5-7 days. The addition of 5 drops of methyl red was done after incubation, expecting the development of red colour. Appearance of red colour indicates a positive for the methyl red test.
- iv). **V-P (VOGES-PROSKAUER) Test:** Using YMD (Yeast Extract Malt extract dextrose (ISP-2)) broth, a loopful of test culture was inoculated, followed by incubation at 28°C for 5-7 days. Post incubation, 0.6 ml α -naphthol & 0.2 ml Potassium Hydroxide were added per ml of broth, recording the result after 15-60 mins, expecting the development of Cherry red colour. If it appears cherry red, the colour indicates the VP test is positive.
- v). **Citrate Utilisation Test:** This is a test of the ability of an organism to utilise citrate as the sole carbon and nitrogen source for growth and an ammonium salt as the sole source of nitrogen. Simmon's citrate agar medium was used. The medium was inoculated with a 24-48-hour-old culture and incubated at 37°C for 3-5 days. An observation made for the colour change from green to blue was due to a change of pH in the medium (Collee *et al.*, 1996) [8].
- vi). **Catalase Production Test:** An enzyme that catalyses the oxidation of hydrogen peroxide. One or two drops of 3% hydrogen peroxide were taken on a clean glass slide, and one loopful of a 24-48 hours old culture was added or mixed with hydrogen peroxide. Observation was made

for the emergence of bubbles due to the release of oxygen (Collee *et al.*, 1996) [8].

2.6. Molecular or Genotypic Characterisation of the Potent Strain

The genomic DNA used for the polymerase chain reaction (PCR) was prepared from the colonies grown on YMD agar for 3 days. The total genomic DNA extracted from the isolate was isolated by employing the DNA purification Kit (Pure Fast® Bacterial Genomic DNA purification kit, Helini Bio molecules, India) according to the manufacturer's protocol. Conditions of the PCR were standardised with initial denaturation at 94°C for 3 minutes, followed by 30 cycles of amplification (Denaturation at 94°C for 60 seconds, annealing temperature of 55°C for 60 seconds, extension at 72°C for 60 seconds, and an additional 5 minutes at 72°C as final extension). The amplification reactions were carried out with a total volume of 50µL in a gradient PCR (Eppendorf, Germany). Each reaction mixture contained 1 µL of DNA, 1 µL of 10 pmol forward 16S actino specific primer (5'AAATGGAGGAAGGTGGGGAT-3'), 1 µL of 10 P mol reverse 16S actino specific primer (5'- 5'-AGGAGGTGATCCAACCGCA-3'), 25 µL of master mix, and 22 µL of molecular grade nuclease-free water. The separation was carried out at 90 Volts for 40 minutes in TAE buffer with 5 µL of ethidium bromide. PCR product was analysed using agarose gel (1%), and the fragment was purified (Helini Pure Fast PCR clean up kit, Helini Bio molecules, India) as per the manufacturer's instructions. The bands were analysed under UV light and documented using Gel Doc. The direct sequencing of PCR products was performed by the dideoxy chain termination method using the 3100-Avant genetic analyser (Applied Biosystems, USA).

Pairwise and Multiple Sequence Alignment

The gene sequence of the strain was aligned using BLAST against the gene library available for Streptomyces species in the NCBI and GenBank. Pairwise evolutionary distances were computed by MEGA-6 software. The phylogenetic analysis was conducted using the maximum parsimony method on the isolate using BLAST and CLUSTAL W. The closely related homologous isolates were identified, retrieved and compared to the sequence of the isolated strains using CLUSTAL W available with the MEGA 6 Version (Tamura *et al.*, 2013). In-vitro screening for antibiotic susceptibility The antibiotic sensitivity test was conducted to assess the susceptibility of the strain to different antibiotics by following the Kirby-Bauer disc diffusion method. A few antibiotic discs, viz., azithromycin (15µg), chloramphenicol (10µg), kanamycin (30µg), etc were used and procured from Hi-Media Pvt. Ltd., India. The plates were inoculated with the potent actinobacteria, a specified antibiotic disc was placed and incubated at 30±2°C for 48 h. After incubation, the inhibition zones around the antibiotic discs were observed. Based on observation, the strain was considered as either sensitive (S) or resistant (R) to an antibiotic (Ragalatha, 2020).

2.7. In-vitro Screening for Antibiotic Susceptibility

The antibiotic sensitivity test was conducted to assess the susceptibility of the strain to different antibiotics by following the Kirby-Bauer disc diffusion method. A few antibiotic discs, viz., azithromycin (15µg), chloramphenicol (10µg), kanamycin (30µg), etc, were used and procured from Hi-Media Pvt. Ltd., India. The plates were inoculated with the potent actinobacteria, a specified antibiotic disc was placed

and incubated at $30 \pm 2^\circ\text{C}$ for 48 h. After incubation, the inhibition zones around the antibiotic discs were observed. Based on observation, the strain was considered as either sensitive (S) or resistant (R) to an antibiotic (Ragalatha, 2020).

3. Results



2A, B



2C, D

Fig 1: Isolation of bacterial strains



Fig 2: Subculturing of the isolated bacteria



Fig. 3: Catalase test



Fig 4: Citrate utilisation Test

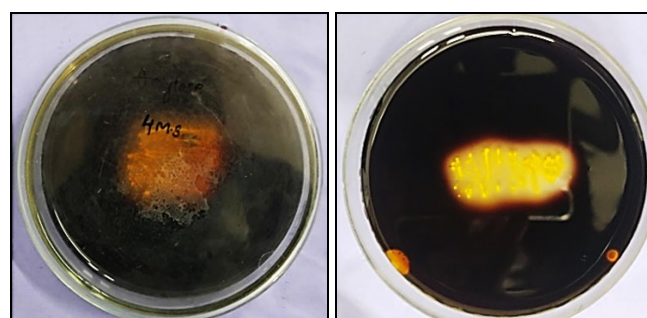


Fig 5: Amylase production by the bacteria is representing through the zone of clearance around the cultures

Molecular Characterisation of the Potent Strain NRI-21:

Based on the morphological, biochemical and molecular characteristics, the strain has been included under the genera *Bacillus inaquosorum* NRI-21. The partial sequence was aligned and compared with all the 16S rRNA gene sequences available in the GenBank database by using the multiple sequence advanced BLAST comparison tool that is available in the website of NCBI. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W program from the MEGA 6 Version referred by Tamura. A phylogenetic tree was constructed using MEGA software Version 6 using the maximum parsimony method.

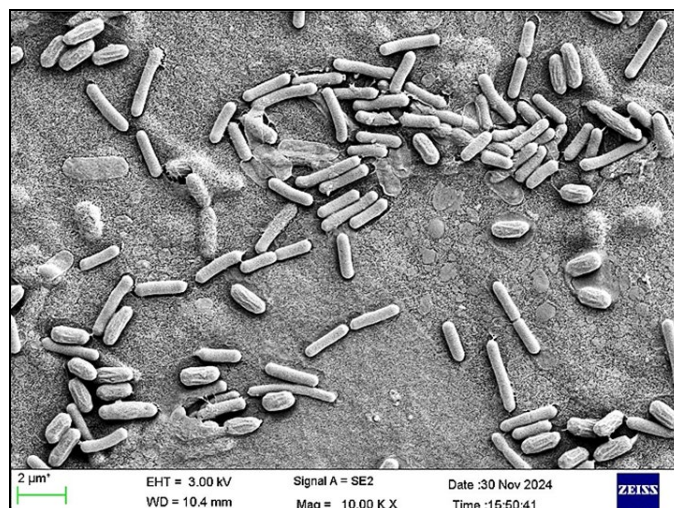


Fig 6: SEM image of the *Bacillus inaquosorum*

4. Discussion

Enzymes are extensively used in the industries due to their high specificity and catalytic characteristics and the amylase enzyme is not left out. Amylase is being used mostly in the textile, food paper and mostly in the detergent industry Nigeria. Also in the pharmaceutical and chemical industries, regularly use amylase for their product yield [6]. The lack of a commercial producing amylase industry has led to the importation of this enzyme and this costs billions to import into the country, and most times the organism doesn't thrive well here due to the climatic differences. This leads to the use of harmful chemicals as alternatives for amylase [3]. For these reasons, it is essential to develop a process to generate amylase commercially. The main aim of this study was to screen for and isolate a high amylase producing strain from the soil and characterize the strain by morphology, biochemical and genotypic method. The study also included optimizing for the best growth conditions to yield maximum amylase such as temperature, pH, and Carbon and Nitrogen source. Since bacteria is more abundant in the soil, it was chosen as the bacterial source for the isolation. The soil collected for this study was from Elele in Etche local government area of Rivers State. 5 isolates out of 11 were observed to be amylase producers during the primary screening of the bacterial strains. This was achieved by the use of starch iodine test method and a zone of clearance was used to identify the positive strains. The clear zones produced, were due to the absence of starch hydrolyzed by the amylase enzyme extracted by the bacteria. The reason behind this low no of isolates gotten could be due to the extensive dilution of the sample during the sample processing. 2 isolates (CS1 and CS2) with largest ratio of clear zones were chosen. Enzyme assay was carried out in other to determine the amount of amylase produced by the selected isolate using Dinitrosalicylic acid method. Since it is widely used for the determination of the amount of reducing Sugar produced, it is seen as an indication of enzyme activity [6]. Amylase produced reducing sugar by breaking down starch into glucose, thus a greater concentration of glucose indicates greater level of starch break down and hence a higher amylase activity. Isolate CS2 showed an activity of 15.776U/mL which was higher than that of CS1, thus it was chosen as the bacteria to continue the study. Further step included the characterization of the bacterial strain. This is quite important because it leads to the development and optimization of the media according to the organism and can be improved if there is prior Knowledge of the bacteria genetic makeup. It is important to know how it is structured, because it helps one to know how it affects humans [7]. From this presumptive tests, it was seen that this organism is of the *Bacillus* sp. Any bacterial isolates having clear zone is a potential amylase producer of extracellular enzymes or proteins [8, 9]. Characterization on the genetic level was carried out using the 16S rRNA gene sequencing. The gene a part of the prokaryotic DNA and has been the most common housekeeping genetic marker in order to study the phylogeny and taxonomy of a bacterial strain. This is because

- i). It is present in almost all bacteria and might often exists as a multigene family, or operons;
- ii). Over time, there has been no changes seen in the function of the 16S rRNA gene and hence any change in the sequence indicates a more accurate measure of time (evolution);
- iii). The size of the gene is large enough to be used for informative purposes (1500 bp).

Using the 16S rRNA sequence in characterizing microorganisms is more dependable and sensitive than culture-dependent techniques alone and the results obtained in this research is consistent with other related studies by Wang *et al.* [10], Dash *et al.* [5], P. Deb *et al.* [11], U. Dey *et al.* [12], Kandarp *et al.* [13]. The determination of the optimal characteristics for amylase production by *B. circulans* was first carried out using the one factor at a time method (OFAT). The low enzyme activity given by *B. circulans* could have been as a result of the percentage of ammonium sulphate used for its purification as can be seen according to Kandarp *et al.* [13], result obtained [16-30]. Under the toughest operating conditions and for long durations, a good industrial catalyst is meant to be stable. When the crude amylase was treated at different pH as shown in Figs. 8 to 9, it showed a slightly acidic pH of 6 and a neutral pH of 7 for optimal activity. Similar preferred conditions have been found for amylase activity in previous studies (Demirkan., 2011).

5. Conclusion

Amylase, an enzyme that breaks down starch, has diverse uses ranging from aiding digestion to various industrial applications. In the human body, amylase, produced by the pancreas and salivary glands, helps digest starch into simpler sugars for energy. Industrially, amylase is used in the food, brewing, textile, paper, and detergent industries, among others, for processes like liquefying starch, converting it into sugars, and degumming fabrics. In the body:

- Amylase is a digestive enzyme that breaks down starch into simpler sugars, aiding in the absorption of nutrients.
- It is crucial for proper digestion and energy production.
- Amylase levels in the blood can be used as a diagnostic tool for conditions like pancreatitis and gallstones. In industries:
- **Food:** Amylase is used in baking, brewing, and the production of syrups and fruit juices.
- **Textiles:** It's used in degumming processes to remove starch from fabrics, making them easier to dye and print.
- **Paper:** Amylase helps in the pulp and paper industry to prevent mechanical losses, improve paper quality, and enhance its rigidity.
- **Detergents:** Amylase is used in detergents to improve cleaning, remove starch stains, and enhance detergent stability.
- **Other:** Amylase is also used in brewing, textile, and pharmaceutical industries.

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