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Isolation of a Alkaliphilic α-Amylase-Producing *Bacillus* sp. from Sewage in Sironj District, Vidisha, Madhya Pradesh

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Abstract - *Bacillus* strains that produce α-amylase were found in the sewage systems of Sironj, India. This area's textile industry has historically used significant amounts of starch. *Bacillus* sp. was one of the isolates. On starch agar, the SIR-1 isolate had the most enzyme activity and a hydrolysis zone. To identify the bacterial isolates, biochemical characterization was used. This indicated that it was a Gram-positive, catalase-positive, citrate-positive bacterium that was excellent at breaking down starch. Their crude α-amylase, quantified using the DNS method (41.3 mg/ml reducing sugars), showed excellent alkaliphilic and thermostable properties, with optimal activity at 60°C and pH 9. Microbial amylases don't usually tolerate high temperatures and pH levels together. The evidence suggests that they have changed their structure to fit the starch-rich human-made environment of Sironj. Its stability under extreme conditions makes this enzyme a potential candidate for industrial applications such as detergent formulations, textile desizing, and bioethanol production. This study indicates that historical ecological niches can be good places to find new biocatalysts. The results connect the study of microbes to industrial biotechnology and encourage researchers to look into habitats that haven't been studied much to find new enzymes.

***Key Words*:** *Bacillus* sp., α-amylase, Industrial Biotechnology

1. INTRODUCTION

Amylases are a very important group of hydrolytic enzymes because they break down starch into smaller amounts of saccharides like glucose and maltose. They are also some of the most useful biocatalysts that are used in a lot of different industries. Food processing, textile desizing, pharmaceutical formulations, and bioethanol production utilize amylases due to their economic and biotechnological significance. Because of their fast growth, inexpensive production, and versatility in diverse bioprocess engineering, microbial origins, specifically bacteria, have been most likely chosen in anticipation of a more competitive industrial product [2]. Among these, the genus *Bacillus* has the highest potential for producing amylase [3]. *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, and Bacillus megaterium* are all members of the genus *Bacillus* that have been shown to break down amylolates. These bacteria are metabolically versatile and can occupy different ecological niches such as soil, compost, and extreme environments such as hot springs [4]. They are usually thermostable, pH-tolerant, and have a wide substrate specificity, which makes them suitable for adverse industrial conditions. *Bacillus*-derived thermostable amylases are very important in the liquefaction steps of making biofuels at high temperatures [5]. On the other hand, alkaline-tolerant amylases are mostly used in detergents. Moreover, the secretion of their enzymes to the outside environment makes downstream processing easier and their enzymes more commercially available [6]. Recent years have seen improvements in fermentational conditions and strain development, leading to the production of amylase. Enzymatic yield and activity are greatly affected by factors such as temperature, pH, carbon-nitrogen ratios, and metal ion supplementation [7]. In the most common submerged and solid-state fermentation systems, researchers are exploring agro-industrial wastes as alternative substrates to reduce costs [8]. At the same time, progress in genetic and recombinant DNA technologies has made it possible to create hyper-producing *Bacillus* strains that are better at catalysis or stable in certain industrial conditions [9]. These innovations are in line with the increasing trend toward enzymes that can satisfy the advanced requirements of current bioprocesses. In this respect, the town of Sironj (founded 1103 A.D. by Shankar Singh, a Sengar Rajput warrior) located in Madhya Pradesh, central India, is an ancient treasure trove of microbes that were shaped by innumerable years of agricultural cultivation in the region and its rich traditions of textile making. Sironj was famous for making muslin and calico. Its traditional industries used starch-based sizing agents, which may have affected the growth of a group of microbes with certain enzyme abilities [10]. New types of *Bacilli* that aren't very good at breaking down proteins and sugars can be found in rare habitats in the area. These habitats have a lot of different types of fluids and are perfect for growth, like sewage systems that are full of organic waste from farming and textile processing. By looking into Sironj's microbiome, which is a place where natural products have been made for generations as a biotechnological method and as a new way to find enzymes, this new study helps connect the past and the present. It may also give us clues about how microbes adapt to environments with a lot of starch.

The study aims to isolate, characterize, and optimize *Bacillus* strains from environmental samples for amylase production, unlocking their biotechnological potential. The research aims to better understand the biochemical properties of the enzymes and make production lines more efficient in order to create a cost-effective, inhibited way to make amylase that can be used on a large scale. These results should add to the growing collection of industrial enzymes and make it possible for industries that depend on starch-based technologies to use long-term solutions [11].

2. MATERIAL AND METHODS

Chemicals, and Instruments

Chemicals and reagents used in the study came from HiMedia (Mumbai, Maharashtra, India), TCMedia (Mumbai, India), and Sunchem (Parsippany, New Jersey, USA). They were used for media formulation, screening, well diffusion assay, activity analysis (qualitative and quantitative), and phenotypic characterization. Indian Scientific, based in Bhopal, Madhya Pradesh, supplied these chemicals. All the laboratory instruments have been provided by the Department of Higher Education, Government of Madhya Pradesh.

Medium Preparation

Sterile culture media was formulated by dissolving 2.5 g soluble starch (1% w/v), 1.25 g peptone (0.5% w/v), 0.75 g yeast extract (0.3% w/v), 1.25 g sodium chloride (0.5% w/v), and 3.75 g bacteriological agar (1.5% w/v) in 250 ml of distilled water. The suspension was gradually heated to 60°C with constant stirring until complete dissolution was achieved. The pH of the mixture was adjusted to 7.2 ± 0.1 using 1N NaOH. Sterilization was performed via autoclaving at 121°C (15 psi) for 15 minutes. Post-sterilization, the medium was cooled to 50°C to preserve heat-sensitive constituents. Under aseptic conditions, 20 ml aliquots were dispensed into Petri dishes that had been sterilized prior to use. The poured medium was solidified at ambient temperature (25°C). To minimize moisture condensation, solidified plates were stored inverted at 4°C.

Screening for Amylase-Producing Bacteria

Samples underwent serial dilution (10^{-4} to 10^{-7}) using sterile saline (0.85% NaCl) to reduce microbial concentration. Dilutions were spread in triplicate onto starch agar plates. Plates were incubated at 35°C for 48 hours in BOD incubator. Post-incubation, Gram's iodine solution was applied to detect starch hydrolysis. Colonies surrounded by clear hydrolytic zones against a blue-black starch-iodine background were presumptively classified as amylase-producing organisms. Pure isolates were streaked onto fresh starch agar (quadrant method) and preserved at 4°C on nutrient agar slants for subsequent biochemical analysis.

Biochemical Characterization

Amylase-producing bacterial isolates were identified using a standard set of biochemical tests. Gram staining and assessment of colony morphology from 24–48 hours of growth on starch agar were used for initial characterization. Catalase and oxidase activities are assessed to distinguish aerobic and facultative anaerobic metabolism. Carbohydrate fermentation profiles (glucose, lactose, sucrose) were assessed by the production of acid/gas in Triple Sugar Iron (TSI) agar. The metabolic versatility of isolated strains was determined using citrate utilization and nitrate reduction tests. The starch hydrolysis test was performed as a definitive assay to confirm amylase activity. The isolates were streaked on starch agar medium, incubated for 48 hours at 37°C, and flooded with Gram's iodine solution. Zones of clearance surrounding colonies suggested starch degradation. Morphological Characterization Morphological characterization was performed with a Magnus MLXi Plus binocular microscope.

Analysis of UV-Visible Spectrophotometry by DNS Method

α-Amylase activity was determined via the 3,5-dinitrosalicylic acid (DNS) assay to measure reducing sugars (maltose equivalents) liberated during starch hydrolysis. A reaction mixture containing 0.5 ml of crude enzyme extract and 0.5 ml of 1% soluble starch in 50 mM phosphate buffer (pH 6.9) was incubated at 37°C for 10 minutes. The reaction was halted by adding 1 ml of DNS reagent, followed by boiling for 5 minutes to develop a red-brown chromogenic product. After cooling, absorbance was recorded at 540 nm using a spectrophotometer. Enzyme activity was standardized against a maltose calibration curve (0.1–1.0 mg/ml). DNS results revealed that isolate SIR-1 exhibited superior starch-hydrolyzing activity compared to SIR-2 and SIR-3.

Evaluation of α-Amylase Production at Different pH Conditions

α-Amylase induction in isolates was performed using LB broth supplemented with 1% soluble starch. For pH-dependent enzyme stability, the medium pH was adjusted over a range (4.0–10.0) with 0.1N HCl or NaOH. Cultures were grown at 37°C (150 rpm) for 24 hours and then centrifuged (10,000 rpm, 10 minutes) to collect the cell-free supernatants. The DNS method was used to measure the activity of α-amylase at all pH levels, and the rates of starch hydrolysis were compared to the best conditions.

Characterization of α-Amylase at different Temperature

To see how well the isolates could handle different temperatures, they were grown in LB broth (pH 7.0) with starch at 30°C, 37°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, or 80°C for 24 hours, and their growth was tracked. Following incubation, residual α-amylase activity in supernatants was determined using the DNS method, and enzymes were pre-incubated at the corresponding temperature for 1 hour. Thermostability was presented as the percentage of activity compared to control (37°C).

3. RESULTS

The starch agar plate inoculated with the bacterial culture showed a distinct clear zone around the bacterial growth after the addition of iodine, indicating starch hydrolysis. This suggests the presence of extracellular amylase enzymes, confirming the ability of the tested bacterium to break down starch into simpler sugars (Figure-1). The microscopic observation revealed purple-stained, rod-shaped bacteria arranged singly or in chains, confirming the Gram-positive nature of *Bacillus* species (Figure-2). Figure-3 shows a microscopic field with spores appearing as unstained or slightly refractive structures within the bacterial cells, confirming the presence of endospores. This indicates that the observed bacteria belong to the *Bacillus* genus, which is known for its endospore-forming ability.

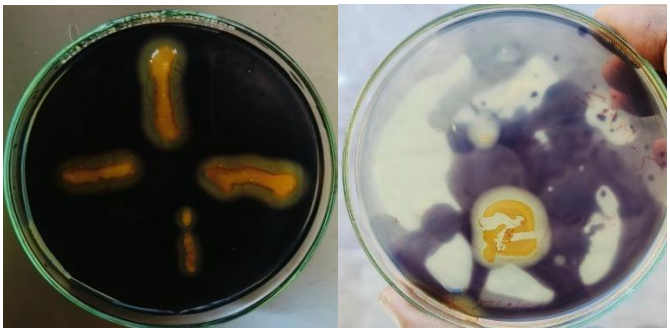

Figure 1: Amylase production by *Bacillus* spp. on starch agar plate.

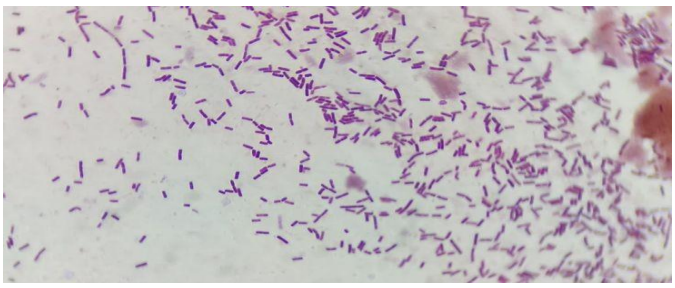

Figure 2: *Bacillus* spp. observed under light microscopy (1000X magnification, Gram-stained), displaying characteristic rod-shaped morphology.

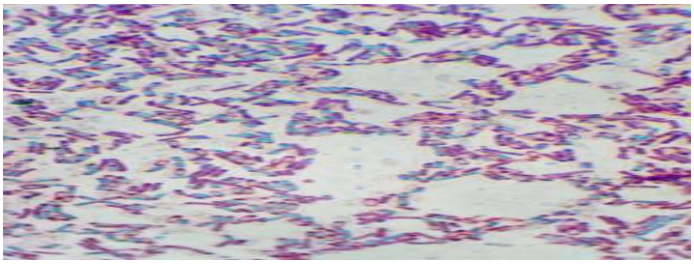

Figure-3: Malachite Green staining of *Bacillus* spp. SIR-1 isolate from sewage, highlighting endospore formation under 1000X microscopic observation.

Table1:Results of Biochemical Tests for Sewage Isolate SIR-1

S. No.	Biochemical Test	Result
1.	Gram Staining	Positive rods
2.	Spore Test	Positive (endospore)
3.	Catalase	Positive (bubbles)
4.	Oxidase	Negative (no color change)
5.	TSI Agar Reaction	Ferments Glucose, Yellow Colour
6.	MR	Positive
7.	VP	Positive
8.	Citrate Utilization	Positive (blue color)
9.	Starch Hydrolysis	Positive (clear zones)
10.	Nitrate Reduction	Positive (red color)

Using the DNS (3,5-Dinitrosalicylic Acid) approach, a regular curve for the reducing sugar concentration was created based on the absorbance assessment at 540 nm. The calibrated curve showed a strong linear correlation between absorbance and reducing sugar concentration. Using the linear regression equation, the sample (SIR-1) had an absorbance of 0.51, which meant that it had a reducing sugar concentration of 41.3 mg/ml (Figure-4).

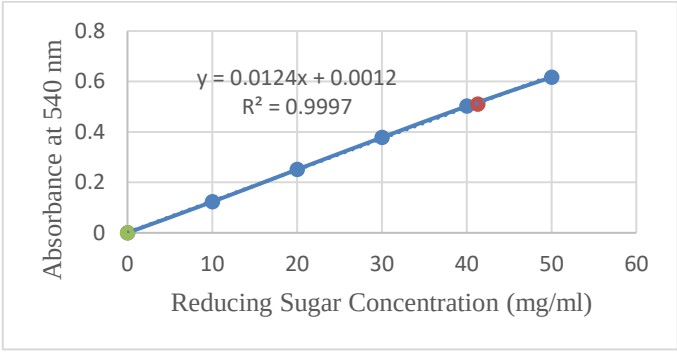

Figure-4: Standard curve of reducing sugar concentration (mg/ml) versus absorbance at 540 nm, showing a linear relationship ($R^2 = 0.9997$).

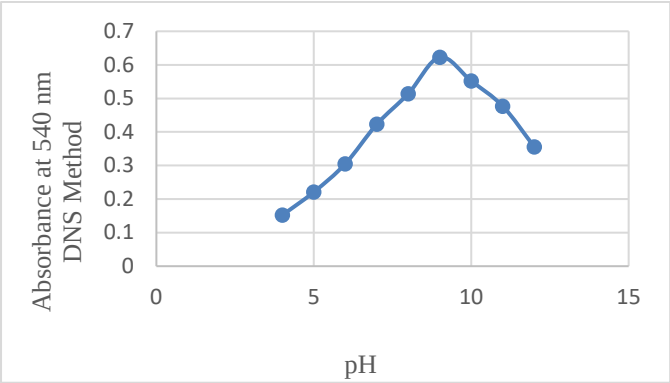

Figure 5: pH-dependent **α**-amylase activity curve using the DNS method (measured at 540 nm**).**

The starch hydrolysis method, which uses DNS and measures absorbance at 540 nm, was used to analyses the effect of temperature on α-amylase activity. The results indicated that activity increased with temperature until hitting a plateau at 80°C (absorbance = 0.720). This evidence indicates that α-amylase has an optimum temperature for catalytic efficiency. Beyond this, however, enzyme activity was expected to decrease as a result of thermal denaturation. Lowest activity was detected at the temperature of 20°C (absorbance = 0.210), suggesting that the reaction was less efficient at lower temperatures. The results showed that α-Amylase works best at higher temperatures; within the limits of the experiment, 60°C was the best temperature for catalytic activity (Figure-6).

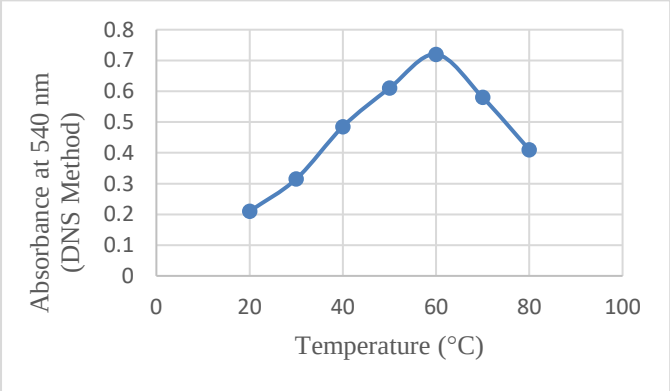

Figure 6: Effect of temperature on α-amylase activity using DNS method.

4. DISCUSSION

The isolate SIR-1 was a Gram-positive *Bacillus* species that had a high relative enzyme activity (REA) of 2.86, which was higher than the REAs of other isolates. It had a strong amylolytic activity (hydrolysis zone 20 mm). The biochemical profile shows that the bacteria are positive for catalase, citrate, nitrate, and starch. These are all characteristics of industrially important *Bacillus* spp. that can survive in conditions high in starch [12, 13]. The best conditions for α-amylase to work are at an alkaline pH of 9 and temperature of 60°C. This shows a dual tolerance that is very uncommon in this group of enzymes. It functions effectively structure has changed to be able to live in both alkaline and thermophilic environments, probably because of the starch-rich sewage systems of Sironj in the past [14]. Its full enzyme activity makes it a great choice for detergent formulations or breaking down starch at higher temperatures. Additionally, it's very stable action surpasses that of normal amylases [15, 16]. Its unique thermostability means that more genetic or proteomic studies are needed to fully understand how it works, even though the DNS concentration (41.3 mg/ml of reducing sugars) shows that it works. Highlights Many potent biocatalysts in anthropogenic ecosystems remain unexplored. However, creating large-scale processes with agricultural and industrial waste can be challenging [17, 18]. Despite their industrial significance, further research is required to comprehend the factors governing the bio-conversion of ligno-polysaccharides.

5. CONCLUSION

The *Bacillus* sp. isolate SIR-1 produces an α-amylase that exhibits optimal activity in highly alkaline conditions (pH 9) and remains stable at elevated temperatures (60°C). This makes it a good choice for use as a biocatalyst in harsh industrial processes. Because this organism has both efficient enzymes and metabolic flexibility, it shows how historic industrial sites could be sources of new microbes that haven't been thought of before. Fermentation technology and genetic modification can enhance its commercial scale in biofuel, textiles, and detergent industries. This paper makes the link between microbial ecology and industrial enzymology and encourages the study of ecosystems that people have changed as places to look for new life.

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