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"SCREENING OF α -AMYLASE FROM RHIZOSPHERIC SOIL ISOLATES"

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Abstract
Amylases are crucial hydrolytic enzymes with wide industrial relevance, particularly in food processing, textiles, paper manufacturing, and biofuel production. Among them, α-amylase is distinguished by its capacity to cleave internal α-1,4-glycosidic linkages in starch, enabling rapid saccharification under mild conditions. This study aimed to isolate and characterize α-amylase-producing bacteria from rhizosphere soils of Shivamogga District, Karnataka—a region within the Western Ghats known for its rich microbial diversity and endemism. Using serial dilution, starch agar screening and Gram staining, 25 bacterial isolates were obtained, with six demonstrating starch hydrolysis through clear halo zones. The highest enzyme-producing isolates (SSCMBSAR3 and SSCMBSAR5) were identified as Gram-positive bacilli, likely belonging to the Bacillus genus, corroborating their reputation as prolific amylase producers. One Gram-negative coccus (SSCMBSAR2) also exhibited notable activity, highlighting underexplored microbial groups like Neisseria and Pseudomonas. Quantitative enzyme assays using the DNS method revealed peak α-amylase activity at 48 hours of incubation and optimal substrate concentration at 400 mg starch, followed by substrate inhibition at higher concentrations—demonstrating a bell-shaped kinetic profile consistent with Michaelis-Menten dynamics. The co-detection of protease activity indicates a need for further purification or genetic modifications to eliminate non-specific enzymes. This study underscores the rhizosphere as a fertile bioprospecting niche and affirms the industrial potential of both Gram-positive and Gram-negative soil bacteria in enzyme biotechnology.

Keywords: *α-amylase, Rhizosphere, Bacillus, enzyme kinetics & Gram-negative bacteria.*

INTRODUCTION

Amylases are a class of hydrolytic enzymes that catalyse the breakdown of starch into simpler sugars, including glucose, maltose, and dextrins. These enzymes play a pivotal role in a wide range of industrial sectors, such as food processing, textiles, paper manufacturing, and biofuel production (Gupta *et al*., 2003; Ray *et al*., 2015). Among the different types of amylases, α-amylase is particularly significant due to its ability to cleave internal α-1,4-glycosidic linkages in starch molecules, thus enabling rapid starch degradation under mild processing conditions (Pandey *et al*., 2000). Microbial sources of amylase, especially bacteria, are highly preferred for industrial-scale production owing to their rapid growth rates, cost-effective cultivation, and secretion of extracellular enzymes into the culture medium (Ajayi & Fagade, 2006). Among bacteria, Gram-positive Bacillus species are extensively documented as prolific producers of extracellular enzymes, including α-amylases, due to their robust secretion systems and high enzymatic yield (Gupta *et al*., 2003; Asgher *et al*., 2007). Nevertheless, recent studies have also highlighted the untapped potential of Gram-negative bacteria such as *Pseudomonas* and *Neisseria* for producing hydrolytic enzymes, thereby warranting further investigation (Madigan *et al*., 2017).

The rhizosphere—the narrow region of soil influenced by root secretions and associated microbial communities—offers a dynamic environment rich in microbial diversity and metabolic potential (Singh *et al*., 2016). Soil microorganisms in this zone are constantly exposed to organic substrates such as polysaccharides, making them evolutionary candidates for secreting degradative enzymes like amylase. Rhizospheric soils have thus emerged as promising reservoirs for isolating industrially relevant microbial strains (Jaiswal *et al*., 2020). Despite the abundance of microbial diversity in Indian soils, relatively few studies have focused on the rhizosphere of the Western Ghats region in Karnataka, a biodiversity hotspot known for its unique soil ecology and microbial endemism. The Shivamogga district, part of this region, presents a valuable yet underexplored niche for bioprospecting amylolytic microbes.

This study aimed to isolate and characterize α-amylase-producing bacteria from rhizosphere soil samples collected from distinct locations within Shivamogga District, Karnataka. Using classical microbiological techniques, including serial dilution, differential screening, and Gram staining, the study identified bacterial strains capable of starch hydrolysis. The selected strains were subjected to both qualitative assays (halo zone formation) and quantitative enzymatic activity tests using the DNS method (Miller, 1959; Chi *et al*., 2009). Furthermore, the study examined the time-course kinetics and substrate concentration effects on enzyme production, offering insight into the enzyme's industrial applicability and stability. By focusing on both Gram-positive and Gram-negative isolates and evaluating their enzymatic potential, this work not only reinforces the significance of the rhizosphere as a source of biotechnologically important bacteria but also provides a basis for future strain improvement and enzyme optimization strategies aimed at enhancing industrial α-amylase production (Haq *et al*., 2010; Segel, 1993).

MATERIALS AND METHODS

Sample Collection

Soil samples were collected from the rhizosphere at two different locations in Shivamogga District, Karnataka, India. The first sample was obtained from the botanical garden of Sahyadri Science College, Shivamogga, and the second from the plateau region of Vidyanagar. Soil was randomly collected from a depth of approximately 15 cm using sterile spatulas, following standard rhizosphere sampling methods (Singh *et al*., 2016). The samples were immediately transferred into sterile polyethylene bags, labelled with the date and site of collection, and transported to the laboratory. Upon arrival, the samples were stored at 4 °C until further processing (Jaiswal *et al*., 2020).

Isolation of Bacteria

Bacterial isolation from soil samples was performed using the serial dilution agar plate technique (Cappuccino and Sherman, 2014). One gram of each soil sample was suspended in 9 mL of sterile distilled water and serially diluted up to 10^{-9}. Aliquots of 0.1 mL from 10^{-8} and 10^{-9} dilutions were aseptically spread onto Starch Agar (SA) and Skim Milk Agar (SMA) plates for the preliminary screening of amylolytic and proteolytic bacteria, respectively. Plates were incubated at 37 °C for 48 hours. Post-incubation, starch agar plates were exposed to iodine vapors to detect zones of hydrolysis, indicating starch degradation (Nair and Surendran, 2004). For proteolytic activity, clear zones surrounding colonies on SMA plates were recorded as indicative of casein hydrolysis (Sharma *et al*., 2020).

Screening of Amylase-Producing Bacteria

Individual colonies that produced a clear halo around them on starch agar after iodine exposure were presumed to be amylase-positive. These colonies were further streaked on fresh starch agar plates and incubated at 37 °C for 24 hours to confirm amylase production. Colonies that again showed starch hydrolysis were selected and preserved on nutrient agar slants at 4 °C for further characterization (Gupta *et al*., 2003).

Identification of bacterial isolates

Identification of bacterial isolates obtained from starch and skim milk agar plates showing positive enzymatic activity was performed using the Gram staining technique, a differential staining method widely used in microbiology for bacterial classification (Cappuccino & Welsh, 2017). In this procedure, methylene blue or crystal violet was employed as the primary stain, followed by the application of iodine as a mordant, ethanol or acetone as a decolorizer, and safranin as the counterstain (Madigan *et al*., 2021). Gram-positive bacteria retained the primary stain and appeared blue or purple under a light microscope due to their thick peptidoglycan layer, whereas Gram-negative bacteria, with a thinner peptidoglycan

layer and outer membrane, lost the primary stain during decolorization and took up the safranin, appearing pink or red (Prescott *et al*., 2020).

Preparation of Inoculum

Bacterial strains were initially cultured on nutrient agar slants and maintained under refrigeration until use. For inoculum preparation, a loopful of each bacterial isolate was aseptically transferred into 100 mL of sterile starch broth and skim milk broth, respectively, under the laminar airflow hood to minimize contamination. The flasks were incubated in a shaker incubator at 37 °C and 120 rpm for 24 hours. The development of turbidity in the broth indicated successful bacterial growth, which was subsequently used as the inoculum source for further studies (Bhatti *et al*., 2007; Sundaram and Murthy, 2014).

Production Medium and Enzyme Extraction

Bacterial strains exhibiting the highest halo-to-colony (H/C) ratios on starch agar plates were selected for quantitative analysis of α-amylase activity. Each selected isolate was inoculated into a starch-based liquid production medium with the following composition (g/L): soluble starch (10.0), peptone (5.0), beef extract (5.0), and the final volume adjusted with distilled water. The cultures were incubated at 37 °C and agitated at 150 rpm for varying durations (24 h, 48 h, and 72 h) to monitor enzyme production over time (Gupta *et al*., 2003; Asgher *et al*., 2007).

After incubation, cultures were centrifuged at 4,000 rpm for 15 minutes at 4 °C. The supernatant, containing crude extracellular amylase, was collected and used for enzymatic assays (Pandey *et al*., 2000).

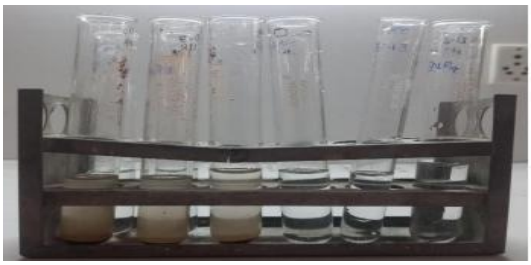
Amylase Activity Assay

The α-amylase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method as originally described by Miller (1959), with modifications as per Chi *et al*. (2009). In this assay, 1 mL of enzyme extract was mixed with 1 mL of soluble starch solution (1% w/v in 0.05 M citrate buffer, pH 7.0) and incubated at 37 °C for 30 minutes. The reaction was terminated by adding 0.5 mL of DNS reagent, followed by boiling in a water bath at 100 °C for 15 minutes to allow color development. The reaction mixtures were cooled to room temperature, and absorbance was measured at 540 nm using a spectrophotometer. An uninoculated broth was used as a blank.

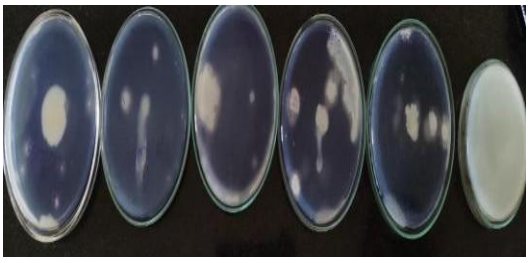
The amount of reducing sugar released (glucose equivalents) was quantified using a glucose standard curve. One unit (U) of amylase activity was defined as the amount of enzyme that catalyses the release of 1 µmol of glucose per minute under the assay conditions (Miller, 1959; Chi *et al*., 2009).

RESULTS AND DISCUSSION

Isolation and Preliminary Screening of Amylase-Producing Bacteria



a).



b).

Figure 1. Isolation of amylase producing bacteria; a). Serial dilution of rhizosphere soil samples and b). Starch-agar plates used for bacterial isolation.

In this study, 25 distinct bacterial colonies were successfully isolated from serial dilutions (10^{-8} and 10^{-9}), with 10 and 15 colonies, respectively, and subsequently screened for amylase production on starch agar plates. Following incubation and iodine staining, six isolates exhibited visible hydrolytic zones, ranging in diameter from 0.1 mm to 3 mm (Table 1), confirming extracellular amylase activity. This qualitative starch hydrolysis method remains a widely adopted preliminary

approach for identifying amylase-producing microorganisms (Pandey *et al*., 2000). The variability in zone diameters suggests differential enzyme production capacities among the isolates.

Selection and Preservation of High-Activity Isolate

Among the six positive isolates, the one exhibiting the largest hydrolytic zone was selected for further analysis. This isolate was designated for subculturing and preserved at 4°C on nutrient agar slants, a common practice to maintain both viability and enzymatic activity during extended storage (Ajayi & Fagade, 2006).

Table 1. Table.1 Zone of inhibition

Starch agar medium	
Isolates	**Clear zone (in mm)**
SSCMBSAR1	0.84
SSCMBSAR2	0.15
SSCMBSAR3	1.01
SSCMBSAR4	0.21
SSCMBSAR5	0.38
SSCMBSAR6	0.83

Time-Course Enzymatic Activity

Enzyme activity was monitored through submerged fermentation, and both amylase and protease activity peaked at 48 hours of incubation (Figure). This observation aligns with earlier findings on *Bacillus subtilis* and Bacillus sp. DLB9, which also demonstrated maximal amylase activity at 48 hours, followed by a decline due to factors such as nutrient exhaustion, autolysis, or end-product inhibition (Shyam *et al*., 2013). While initial assessments revealed coinciding peaks for both enzymes, the goal of eliminating protease activity in favour of specific amylolytic production necessitates further purification or genetic modulation in future work (Haq *et al*., 2010).

Morphological and Gram Characterization of Isolates

Gram staining differentiated the six isolates into both Gram-positive and Gram-negative bacteria. SSCMBSAR1 (Gram-negative bacilli) and SSCMBSAR2 (Gram-negative cocci) suggest the potential involvement of genera such as Pseudomonas or Neisseria, respectively. SSCMBSAR3, SSCMBSAR5, and SSCMBSAR6 were identified as Gram-positive bacilli, whereas SSCMBSAR4 exhibited a Gram-positive coccobacillary morphology. These Gram-positive isolates likely belong to the genus Bacillus or similar taxa, known for high-yield extracellular enzyme production (Gupta *et al*., 2003; Madigan *et al*., 2017).

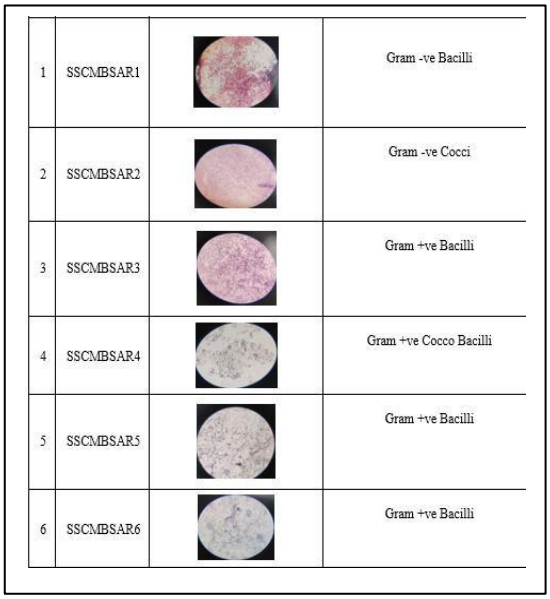


Figure 2. Characteristics and halo-to-colony ratios of 6 amylase-producing bacterial strains.

Quantitative Assessment of Amylase Activity

All six isolates demonstrated clear zones of starch hydrolysis on agar plates, reaffirming their ability to secrete extracellular amylase. The halo-to-colony (H/C) ratio was employed to evaluate the efficiency of enzyme secretion. Isolates SSCMBSAR3 and SSCMBSAR5 exhibited the highest H/C ratios, consistent with literature citing Bacillus species as potent amylase producers due to their secretion machinery and resilience in harsh industrial conditions (Ajayi & Fagade, 2006; Ray *et al*., 2015). In contrast, SSCMBSAR4 and SSCMBSAR6 showed lower H/C ratios, indicating weaker, though still detectable, amylase activity. The observed variability among isolates may reflect differences in enzyme isoforms, promoter strength, or membrane transport systems (Pandey *et al*., 2000).

Figure 3. Image showing Amylase activity.

Substrate Concentration Optimization and Enzyme Stability

Amylase activity was further evaluated using the dinitrosalicylic acid (DNS) method over three consecutive days with substrate concentrations ranging from 200–1200 mg. On Day 1, activity peaked at 400 mg (OD 0.30), followed by a sharp decline at higher concentrations (OD 0.06 at 1200 mg), suggesting substrate inhibition or enzyme saturation at elevated starch levels (Nelson & Cox, 2008). This bell-shaped response aligns with classic Michaelis-Menten kinetics, where optimal enzyme activity is compromised by excess substrate (Segel, 1993). Day 2 results revealed a flatter OD response curve with lower maximal activity (OD 0.19), possibly due to enzyme instability or altered environmental conditions such as pH or residual nutrient effects. The relatively constant OD across concentrations suggests a loss of substrate specificity or partial denaturation (Haq *et al*., 2010). By Day 3, a moderate increase in OD up to 800 mg was observed, followed by another decline, supporting the initial observation of substrate inhibition at high starch levels. The overall reduction in OD values over time points to potential degradation or reduced activity from repeated freeze-thaw cycles or suboptimal storage (Berg *et al*., 2002).

Table 2. Amylase enzyme activity on day 1.

Isolates	Optical density at 575nm	Concentration Maltose in µg	Enzyme activity	Specific activity U/mg
R1	0.12	93.75	9.1	1000.76
R2	0.13	114.5	11.1	1000
R3	0.11	145.8	14.1	97.24
R4	0.16	156	15.2	97.43
R5	0.19	125	12.1	96.8
R6	0.06	83.3	8.1	97.59

Table 3. Amylase enzyme activity on day 2.

Isolates	Optical density at 575nm	Concentration Maltose in µg	Enzyme activity	Specific activity U/mg
R1	0.19	125	12.8	97.44
R2	0.12	31.2	3.040	98.064
R3	0.24	250	24.366	97.454
R4	0.17	166.6	16.23	97.418
R5	0.21	218	21.247	97.46
R6	0.17	62.5	6.0916	97.465

Table 4. Amylase enzyme activity on day 3.

Isolates	Optical density at 575nm	Concentration Maltose in µg	Enzyme activity	Specific activity U/mg
R1	0.09	197	19.200	97.461
R2	0.11	125	12.183	97.464
R3	0.14	114	11.111	97.4649
R4	0.15	177	17.251	97.463
R5	0.12	197	19.200	97.461
R6	0.08	177	17.251	97.4632

Comparative Analysis and Industrial Implications

Collectively, the results emphasize the superior performance of Gram-positive bacilli in amylase production, particularly SSCMBSAR3 and SSCMBSAR5. The presence of amylolytic activity in Gram-negative isolates, especially SSCMBSAR2 (a Gram-negative coccus), is noteworthy given the rarity of such reports, highlighting their unexplored potential in biotechnology (Madigan *et al*., 2017). The observed enzyme kinetics and optimal substrate concentrations are critical parameters for scaling up industrial applications such as starch processing, textile and bioethanol production.

Moreover, the co-detection of protease activity necessitates future strategies to selectively inhibit or genetically knock out protease genes in high-yield amylase strains, thereby enhancing the specificity and utility of the enzyme preparation. This approach is crucial for industries where proteolytic degradation of other proteins is undesirable (Haq *et al*., 2010).

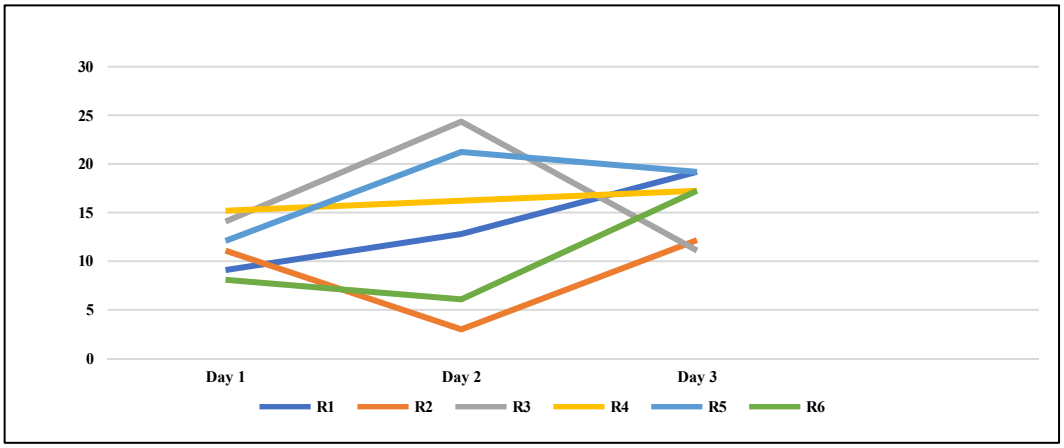


Figure 4. Graph showing amylase activity of the isolates.

Conclusion

This study successfully isolated and characterized amylase-producing bacteria from rhizosphere soil samples collected in the Shivamogga District of Karnataka, India. A total of 25 bacterial isolates were screened for extracellular amylase activity, with six demonstrating clear zones of starch hydrolysis. Among these, isolates SSCMBSAR3 and SSCMBSAR5 exhibited the highest halo-to-colony (H/C) ratios, indicating superior amylase-secreting potential. Gram staining and morphological analysis suggested that the most effective isolates were Gram-positive bacilli, likely belonging to the *Bacillus* genus, which aligns with previous studies citing this group's robust enzyme production capabilities.

Quantitative assays using the DNS method confirmed variable enzymatic activity across isolates, with a peak observed at 48 hours of incubation. Time-course and substrate optimization experiments revealed a bell-shaped enzymatic response curve consistent with Michaelis-Menten kinetics, with peak activity at moderate starch concentrations and inhibition at higher levels. The gradual decline in enzyme activity over time also underscores the importance of optimizing storage and handling conditions for maintaining enzyme stability.

Importantly, the detection of concurrent protease activity highlights the need for future purification steps or genetic modification to enhance enzyme specificity, especially for industrial applications where proteolytic degradation is undesirable. The unexpected amylolytic potential of Gram-negative isolates, particularly SSCMBSAR2, opens new avenues for exploring lesser-known taxa in industrial enzyme production.

Overall, this study reinforces the rhizosphere as a rich reservoir of industrially relevant microorganisms and provides a foundation for further bioprocess development aimed at enhancing α-amylase yield, stability, and specificity for commercial applications in food, textile, and biofuel industries.

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