

Isolation, Screening, and Optimization of Amylase-Producing *Bacillus cereus* from Soil Samples

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ABSTRACT

Background: Amylases are industrially significant enzymes that can catalyse the hydrolysis of starch into simpler sugars. Microbial amylases, particularly those from *Bacillus* species, are preferred due to their stability and cost-effective production. The current study aimed to isolate, screen, and optimize amylase-producing bacteria from soil samples.

Methods: Soil samples were collected and serially diluted, followed by inoculation on nutrient agar plates using the spread plate technique. Eleven bacterial isolates were obtained and morphologically characterized. Screening for amylase activity was performed on starch agar plates using Gram's iodine. The most potent isolate was subjected to Gram staining and biochemical characterization for identification. Optimization studies were conducted to regulate the effect of pH and temperature on enzyme creation.

Results: Among eleven isolates, only isolate 11 exhibited a clear hydrolytic zone around the colony, representing strong amylolytic activity. The isolate was identified as *Bacillus cereus* based on morphological and biochemical characteristics. Maximum amylase production was recorded at pH 8.0 and temperature 50 °C, demonstrating the organism's preference for slightly alkaline and moderately thermophilic conditions.

Conclusion: The study confirms *B. cereus* as a potent source of extracellular amylase with optimal activity under alkaline and moderate temperature conditions. Its enzymatic properties make it a promising candidate for industrial applications in food processing, textile desizing, detergent formulation, and other biotechnological sectors.

Key-words: Amylase, *Bacillus cereus*, Centrifugation, Optimization, Spectrophotometry, Staining

INTRODUCTION

Enzymes are biological agents that accelerate biochemical and biological reactions. In the latter half of the 1800s, they were discovered ^[1]. Substrate molecules bind to the enzyme's active site, causing a conversion that yields the product. The architectures of enzymes are quite specific ^[2].

Enzymes are used in a wide range of sectors nowadays, such as paper, textiles, food, detergents, medications, and many more. Gupta *et al.* ^[3] state that they have been used in the saccharification of starch, the production of drinks such as beer, the treatment of digestive disorders, and the production of cheese from milk. Additionally, because they are generally considered safe (GRAS) from a legal standpoint, their use in food processing has been promoted ^[4].

The food industry can extract enzymes from plant or animal tissues or use certain microbes in fermentation processes ^[5]. In 1894, the amylase enzyme was first synthesized commercially from a fungus. Amylase was used to alleviate the intestinal issue. Amylases are a type of enzyme that hydrolyze starch into simple sugars like

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glucose and maltose, according to Farzana *et al.* [6]. Amylases are widely sought after among the many frequently used enzymes due to their role in hydrolytic activity and starch hydrolysis [7]. Amylases come in three varieties: alpha, beta, and gamma. Long-chain carbs are broken down at random locations throughout the starch chain by α -amylases.

β -amylases catalyze the hydrolysis of the second α -1,4 glycosidic bond from the nonreducing end, resulting in the breakdown of two glucose units at a time. γ -amylases operate from the non-reducing end and cleave the last α -1,4 glycosidic links to create glucose with α (1-6) glycosidic linkages [8]. S. Das *et al.* [9] state that α -amylase is an extracellular enzyme that releases α -anomeric sugars and restricts dextrans by catalyzing the hydrolysis of internal α -1,4-Oglycosidic bonds in starch and similar polysaccharides. The hydrolysis of the α -1,4 glycosidic link and the conversion of glucose to maltose are catalyzed by β -amylase (1,4- α -D-glucanmaltohydrolase, glycogenase), a kind of amylase that acts on the non-reducing end.

β -amylases are unable to hydrolyze branched polysaccharides such as glycogen and amylopectin because the branching linkages are too strong, leaving the dextrin part intact. The glycoside hydrolase enzyme family 14 (GH-14) includes β -amylases. β -amylases are found in plants. Gamma amylase (glucan 1,4- α -glucosidase, glucoamylase) breaks down α -1,4 glycosidic linkages at the non-reducing end of amylase and amylopectin to create glucose. Moreover, it degrades α -1,6 glycosidic linkages. It is a member of the GH family 15. Its optimal pH is 3, and it is more common at acidic pH values [10].

Among the industries that use amylases are food, bread, fruit juices, paper, fuel ethanol from starches, textiles, sweeteners, glucose and fructose syrups, detergents, alcoholic beverages, digestive aids, and spot removal in dry cleaning. Bacterial α -amylases are also used in analytical, clinical, and pharmaceutical chemistry. The most common application for amylases is in the starch industry, where they help hydrolyze starch to transform it into a liquid. J. E. Nielsen and T. V. Borchert *et al.* [11] claim that it converts starch into glucose and fructose syrups.

The detergent sector is the largest user of enzymes in terms of both volume and value. Detergents are made more environmentally friendly and more effective at

getting rid of tough stains by adding enzymes. Amylases are another type of enzyme used in the detergent composition. These enzymes make up 90% of all liquid detergents [12]. In laundry and automatic dishwashing machines, these enzymes are employed to eliminate leftovers of starchy meals, including custard, gravies, potatoes, chocolate, and other smaller oligosaccharides. Amylases are employed in the textile industry's desizing procedure. Before fabric is made, sizing agents like starch are added to yarn to facilitate a safe and efficient weaving process.

Starch is a particularly desirable size since it is cheap, commonly available, and easily removed. The starch in the cloth, which acts as a reinforcing agent to prevent the warp thread from breaking during weaving, is removed during the desizing process. The α -amylases merely eliminate size, leaving the fibers intact [13]. *Bacillus* strain amylase has been utilized for a very long time in the textile sector.

MATERIALS AND METHODS

Sample Collection and Serial Dilution- Simple random sampling was used to gather soil samples, which were then taken to the lab in sterile containers [14]. 9 mL of sterile distilled water was used to suspend one gram of soil, which was then serially diluted up to 10⁻¹⁰. For bacterial isolation, 0.1 mL of each dilution was utilized [15].

Bacterial Isolation- Using the spread plate method, nutrient agar plates were produced and inoculated with the 10⁻³ dilution. To obtain pure cultures, separate colonies were sub-cultured on nutrient agar slants after plates were incubated for 24 hours at 37°C. Eleven bacterial isolates in all were acquired [16].

Amylase Activity Screening- Every isolate was streaked on starch agar and incubated for 24 hours at 37°C. After plates were saturated with Gram's iodine, amylase activity was detected by the formation of a clear zone. Only isolate 11 was chosen for additional research because it created a noticeable halo [17,18].

Identification of Bacteria- Bacterial identification involved recording cultural traits and doing Gram staining with ethanol, safranin, crystal violet, and Gram's iodine. Under a microscope, Gram-positive rods were seen. The isolate was identified as *B. cereus* by

biochemical tests (catalase, citrate, nitrate reduction, Voges–Proskauer, and carbohydrate fermentation) that were compared with standard profiles ^[19,20].

Optimization of Amylase Production

pH: After adjusting the nutrient broth to a pH between 5 and 9, 1 mL of the culture was added, and it was cultured for 24 hours at 37°C. After centrifuging the cultures for 20 minutes at 4,000 rpm, the supernatants were examined for enzyme activity using spectrophotometry at 600 nm ^[21].

Temperature- Enzyme activity was measured when cultures were centrifuged after being incubated for 24 hours at 20, 30, 40, 50, and 60°C ^[22].

Amylase Enzyme Substrate Specificity- Using soluble starch, potato starch, maize starch, rice starch, and wheat starch, the partly purified amylase enzyme's substrate specificity was ascertained. The enzyme was incubated with each substrate (1% w/v) in phosphate buffer (pH 7.0) for 10 minutes at 50°C, which is the standard test procedure. The amount of reducing sugar

released was determined spectrophotometrically at 540 nm when the reaction was stopped with DNS reagent. To calculate the relative activities of different substrates, the activity achieved with soluble starch was taken as 100%, and enzyme activity was represented in U/mL ^[23].

Statistical Analysis- Optical density, Enzyme activity, and relative activity of the enzyme were calculated. No inferential statistical tests were applied. Results are presented as simple percentages and mean±standard deviation for clarity.

RESULTS

Table 1 shows the morphological characteristics of the eleven bacterial isolates obtained from soil samples. The isolates exhibited variations in colony color, form, elevation, and margin. Most colonies were circular with entire margins, whereas a few were irregular or crateriform. This diversity indicates that multiple bacterial species were present in the collected soil samples.

Table 1: Morphological Characteristics of Isolated bacteria's

Number of isolates	Color	Form	Elevation	Margin
1	Yellow	Circular	Convex	Entire
2	Off white	Irregular	Flat	Entire
3	Marigold	Circular	Raised	Entire
4	Pastel orange	Circular	Raised	Entire
5	White	Circular	Raised	Entire
6	Pale yellow	Circular	Convex	Entire
7	White	Irregular	Crateriform	Entire
8	White	Irregular	Flat	Entire
9	Off white	Circular	Raised	Entire
10	Off white	Circular	Flat	Entire
11	Off white	Irregular	Flat	Entire

Screening for amylase activity was carried out on starch agar plates, and the results are presented in Table 2. Out of eleven isolates, only isolate number 11 produced a clear hydrolytic zone, indicating positive amylase activity, while all others were negative. The largest clear zone around the selected isolate is shown in Fig. 1, confirming its ability to hydrolyze starch effectively.

Table 2: Activity of Amylase on Starch Agar

Number of isolates	Zone of inhibition
1	-ve
2	-ve
3	-ve
4	-ve
5	-ve
6	-ve
7	-ve
8	-ve
9	-ve
10	-ve
11	+ve



Fig. 1: Starch agar plate with selected bacteria showing largest zone of Inhibition for amylase activity

Microscopic and Gram staining of the selected isolate (number 11) revealed purple, rod-shaped cells, confirming its Gram-positive nature. The stained bacterial cells, visualized under the microscope, are shown in Fig. 2. In text form, the observations indicate

that the isolate consistently displayed Gram-positive bacillus characteristics across multiple fields of view, including variations in cell arrangement and clustering. These findings confirm the isolate's Gram-positive rod morphology, supporting its identification and classification.

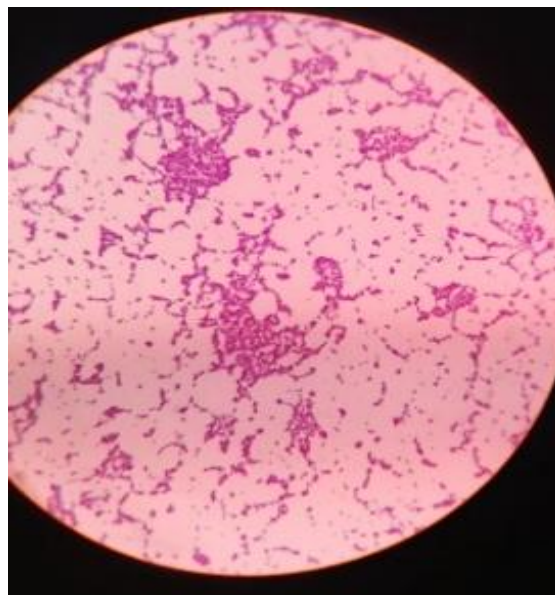


Fig. 2: Microscopic view of selected bacteria

The biochemical characterization of the selected isolate is shown in Table 3. The organism tested positive for catalase, citrate utilization, nitrate reduction, Voges–Proskauer reaction, glucose, sucrose, mannitol, and arabinose utilization, while negative for coagulase, ONPG, indole, and lactose tests. These results collectively confirm that the isolate belongs to the genus *Bacillus*.

Table 3: Biochemical test results

S.No.	Characterization	<i>Bacillus cereus</i>
1	Catalase	+
2	Coagulase	-
3	Citrate utilization	+
4	Nitrate reduction	+
5	ONPG	-
6	Vogus Proskaur	+
7	Indole	-
8	Glucose	+
9	Sucrose	+
10	Lactose	-
11	Mannitol	+
12	Arabinose	+

The optimization of amylase production under different pH and temperature conditions is shown in Table 4. The enzyme activity increased from acidic to alkaline pH values, reaching a maximum at pH 8.0, after which a slight decline was observed at pH 9. Likewise, enzyme activity was highest at 50°C, while lower or higher temperatures resulted in reduced activity. These results indicate that the enzyme functions best under mildly alkaline and moderately thermophilic conditions.

Table 4: Optimization of Amylase Activity at Different pH and Temperatures

Condition	Parameter Value	O.D.	Activity (U/mL)
pH	5	0.01	0.002
	6	0.05	0.008
	7	0.06	0.007
	8	0.07	0.009
	9	0.07	0.008
Temperature (°C)	20	0.02	0.002
	30	0.06	0.006
	40	0.06	0.006
	50	0.05	0.009
	60	0.04	0.008

Substrate specificity of the partially purified amylase enzyme is illustrated in Table 5. Among the tested starch sources, soluble starch supported the highest enzyme activity (8.2 U/mL = 100 %), followed by potato and corn starch. Rice and wheat starch showed comparatively lower enzyme activities, indicating that soluble starch is the most preferred substrate for this enzyme.

Table 5: Substrate Specificity of Amylase Enzyme

Substrate Type	Enzyme Activity (U/mL)	Relative Activity (%)
Soluble starch	8.2±0.3	100%
Potato starch	7.1±0.2	86.5%
Corn starch	6.4±0.4	78.0%
Rice starch	5.3±0.3	64.6%
Wheat starch	4.9±0.2	59.7%

DISCUSSION

Our study results display the wide variety of morphological traits that were discovered during the isolation and characterization of bacterial strains from soil samples. Multiple bacterial species were present in the soil samples that were collected, as evidenced by the eleven isolates' differences in colony color, morphology, elevation, and margin. The soil microbiota, which contains several bacterial taxa suited to many biological niches, is a common example of this diversity ^[24]. Panneerselvam and Elavarasi ^[1] reported similar results, isolating morphologically distinct *Bacillus* spp. from soil that could produce extracellular enzymes such as cellulase, amylase, and protease. When amylase activity was screened on starch agar plates, isolate number 11 was the only one to exhibit a distinct zone of hydrolysis, which indicates amylase secretion. One common sign of starch breakdown that verifies enzymatic activity is the development of a translucent halo following iodine flooding ^[2]. Since amylase production is frequently strain-dependent and controlled by environmental factors like nutrient availability, Vitolo ^[2] found that not all soil bacteria possess significant amylolytic capabilities. This is supported by the presence of a single positive isolate among multiple negatives. Isolate 11 was identified by microscopic and Gram-staining results as a rod-shaped, Gram-positive bacterium that is typical of the *Bacillus* genus. *Bacillus* species are renowned for their strong spore-forming capabilities, powerful enzyme synthesis, and high degree of environmental adaptability ^[25].

The isolate's identification as *Bacillus cereus* was further confirmed by biochemical characterization, which showed positive findings for the catalase, citrate utilization, nitrate reduction, and Voges-Proskauer tests. These results were in line with previously published biochemical profiles for this species ^[26]. One characteristic of *Bacillus* species that contributes to their ecological success is metabolic diversity, which is demonstrated by their capacity to metabolize a variety of carbohydrates, including glucose, sucrose, mannitol, and arabinose ^[27].

Amylase production peaked at pH 8.0 and 50°C, according to optimization experiments, indicating that the enzyme is both alkali-tolerant and moderately thermophilic. These results are consistent with earlier research that found that moderately alkaline conditions



and temperatures between 45 and 55°C were ideal for *Bacillus cereus* and related species' amylase activity^[28,29]. Beyond pH 8 and 50°C, the enzyme may partially denature or become conformationally unstable, which could explain the observed drop-in activity. This bolsters the idea that the *Bacillus* species soil amylase enzyme is suited for stable operation in conditions with mild alkalinity and temperature swings. According to studies on substrate specificity, the best substrate for enzymatic hydrolysis was soluble starch, which was followed by potato and corn starches. Rice and wheat starches, on the other hand, showed relatively lower activity. According to this pattern, the enzyme prefers substrates with simpler amylose–amylopectin complexes, which enables more effective hydrolysis^[30].

Pandey *et al.*^[21] reported similar substrate preferences and found that *Bacillus* sp. amylases were most active on soluble starch because of its high solubility and availability of α -1,4 glycosidic linkages. Overall, the study's results demonstrate that the soil-derived *Bacillus cereus* strain is a strong extracellular amylase producer that thrives in alkaline, moderately heated environments. The enzyme has the potential to be used in the food, fermentation, and starch processing sectors due to its wide substrate specificity and environmental stability. Its structural and kinetic characteristics may be further clarified by future research involving molecular characterization and enzyme purification, increasing its usefulness in biotechnological procedures.

CONCLUSIONS

This study explores the enzymatic activity of amylases and their industrial applications, focusing on the optimal conditions for *Bacillus cereus* to manufacture amylase. Amylases are crucial for the hydrolysis of starch into simpler sugars and are utilized extensively in the food, detergent, paper, textile, and pharmaceutical sectors. In the study, *B. cereus* was found to be an effective amylase producer, particularly when the temperature was at 50°C and the pH was at the optimal level of 8. These findings are consistent with past studies that have shown the enzyme's high stability and activity in similar conditions. The study confirms the industrial significance of amylase and expands our understanding of its applications by highlighting the importance of regulating environmental conditions to increase enzyme output and stability. Further optimization using immobilization methods,

genetic engineering, and large-scale fermentation may improve enzyme yield and lower manufacturing costs in the future. Examining the enzyme's kinetic characteristics, purification, and thermostability in various industrial settings may increase its applicability in alkaline and high-temperature operations. Additionally, incorporating amylase from *B. cereus* into environmentally friendly biotechnological applications may support sustainable industrial growth and less environmental impact.

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