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PRODUCTION AND CHARACTERIZATION OF ACIDIC AMYLASE FROM *BACILLUS* SPECIES FOR INDUSTRIAL APPLICATIONS

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Abstract: Amylase is an important and widely used industrial enzyme that is used in many industrial applications, such as in food, beverages, biofuels, sugar, pharmaceutical industries as well as amylase enzyme (biocatalyst) is also used in household activities. It is used to convert starch into simple sugars. Amylase enzyme is found in three different forms that are α-amylase, β-amylase and γ-amylase all having their different functions. In this study amylase producing *Bacillus* strains were isolated from the soil collected from the Muhammad Ali Jinnah University, Karachi campus by performing serial dilution and characterized was done by iodine flooding on the starch-agar plates. The amylase enzyme showed maximum and the best activity at pH 6.0 and at 40 °C temperature. Enzyme activity was seen to decrease with an increase in temperature and with the decrease in pH indicating that the enzyme loses it activity at acidic pH .When enzyme was tested with different type of metal ions it was observed that Mg^{2+}, Ca^{2+}, and Mn^{2+} increases and supports enzyme's enzymatic activity whereas, Hg^{2+} and Cu^{2+} caused the inhibition of enzymatic activity

Key words: Amylase, Metal ions, industrial application,

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INTRODUCTION

Enzymes are biological catalysts that speed up the chemical reactions with high accuracy and under mild conditions, making them an increasing and attractive substitute to conventional chemical processes in different industrial sectors such as pharmaceuticals, textiles, leather, waste, etc., also used in personal and household businesses, making the demand grow up to 7 billion dollars globally in 2023 (Fasim *et a*l., 2021). Amylase enzyme is used to convert starch into sugars that are used as sweeteners, in the biofuel industries amylase is used to convert starch into fermentable sugar that can be converted in ethanol, in paper industry amylase enzyme is used for the softening of the fabric and in paper industry it is utilized to reduce the viscosity of starch based adhesives (John S, 2025). In the food industry purified amylases from *Bacillus cereus* is used to improve stiffness, cohesiveness and flexibility of the bread highlighting the importance of amylase in the industrial section (Alhazmi & Alshehri, 2025). Amylase produced by microbes plays an important role in the biofuel, food and beverage industries by improving starch hydrolysis, it also reduces the production costs in the industries and environmental impact (*Zerin et al.* 2025). Amylase Enzyme holds the central position among other important enzymes used in industries. Amylases accelerate the hydrolysis of α-1, 4-glycosidic linkages in starch, resulting in products like maltose and malto-oligosaccharides (Ali *et al*., 2025). Amylase enzyme or biocatalyst exists in 3 different forms: α-amylase (majorly found in mammals and humans), β-amylase (a type of exo-amylase), and γ-amylase (glucoamylase), each one with different modes of action and industrial importance (Gojiya *et al*., 2021).

Soil is home to a wide range of microorganisms, it serves as a habitat to many microbes that are of great industrial importance and significance, which also includes strains of bacteria that produce amylase enzyme known as amylolytic strain (Shaikh *et al*., 2023). As discussed in this study (Morbia *et al*., 2024) how the amylase producing bacterial colonies were isolated and characterized from the soil of different regions such as Kachchh District, including Gandhidham, Rapar, Bhuj, Nakhatrana, Mandvi, and Mundra Talukas. Enzymes that play a key role in the breakdown of starch into monomers for different industrial applications are produced by the bacteria that dwell in the soil, and one of the species in them is Bacillus (Kholikov *et al.,* 2025). Microbes present in the soil serve as a valuable source for separating strong strains of those microbes that produce efficient and thermostable enzymes for large-scale production (Emmanuel *et al*., 2023). Microorganisms can easily be manipulated, so it is easier to get the desired products, and microbes can produce enzymes easily in bulk quantity in comparison to other organisms (Devi *et al*., 2026). Many strains of *Bacillus* species have been recognized to produce amylase enzyme, such as *B. amyloliquefaciens, B. subtilis, B. coagulans, B. polymyxa* etc these strains have been proven to be a good source for the production of alpha amylase (Farooq *et al*., 2021). Amylase, which is a biocatalyst, is affected by several factors such pH, temperature, and the presence of metal ions. According to the different studies, activators like Mg^{2+}, Ca^{2+}, and Mn^{2+} strengthen amylase activity, while inhibitors like Hg^{2+} and EDTA minimize its activity (Hossain *et al*., 2025).

Despite the extensive utilization of amylases in industries, the high cost of enzyme production and the instability of free enzymes under harsh industrial conditions is one of the major challenges (Fasim *et al.,* 2021). Comparatively fewer studies have been done on the production of acidic amylases from Bacillus species, regardless of the extent of research on thermostable and alkaline amylases, as they have similar importance in the application of acidic food processing and textile treatments. A recent study of amylase enzyme production by *Bacillus cereus* from agricultural waste highlights the industrial importance and promise of the production of enzymes from Bacillus species (Hmida, *et al*., 2024). Similarly, this research (Abo-Kamer *et al.,* 2023) has efficiently used the serial dilution technique to isolate micro-organisms from soil and identify *Bacillus cereus* by using starch agar plate method. This research has performed iodine-based screening to validate the strong amylolytic activity of the amylase enzyme. Therefore, this study focuses on and was designed for the characterization of the enzyme under different conditions of temperature, pH, and metal ions using similar techniques like serial dilution and iodine-based screening.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected from the campus of Muhammad Ali Jinnah University Karachi's garden soil, and around 1g of soil samples were collected. The soil was collected and transported using sterile techniques in sterile containers to prevent any contamination under managed conditions to reduce the disturbance micro-organisms population.

Isolation of Bacteria

Serial dilution was done to isolate bacteria from the soil. 1g of the collected soil was measured on the digital scale, which was then dispersed in 10ml of 0.89% sterile NaCl solution. The serial dilution of soil was than performed up to 10^{-5}, 100 µL of dilution taken from 10^{-4} and 10^{-5} dilutions was then poured on Luria-Bertani Agar plates and incubated for 24 hours at 37°C.

Screening for amylase production

After the completion of incubation, bacterial colonies based on their morphological characteristics, resembling *Bacillus* species were selected. Gram-positive rod-shaped bacteria were then identified by performing gram staining. Further screening of the colonies on starch agar plates was to produce amylase. 50 µL of bacterial culture filtrates were poured in the well created in the agar. After that the plates were incubated at 37°C for 24 hours, after 24 hours the plates were treated with iodine solution to visualize clear zones showing starch hydrolysis.

Characterization of the enzyme activity

Amylase enzyme was defined by using plate and enzyme assays to determine the optimal conditions for the activity for the enzyme.

Effect of pH

Plates of starch-agar with the pH 3.0, 4.0, 5.0, 6.0 and 7.0 were prepared using sodium acetate buffers, cell-free filtrate (CFF) 50 µL was filled in the wells, the plates were then treated with the iodine solution after incubation at 37°C for 24 hours. Perfect pH for enzyme activity was measured by measuring the clear zone diameters.

Effect of Temperature

Starch-agar plates, with the wells containing 50 µL of cell free filtrate (CFF) were incubated at 30°C, 40°C, 50°C, and 60°C. To determine the optimal temperature zones of clearance were measured following the iodine activity.

Effect of Metal Ions

Effect of different metal ions such as Mg^{2+}, Mn^{2+}, Ca^{2+}, and inhibitors like EDTA and Hg^{2+} was researched. Plate assay was done to check the activation and inhibition effect on the enzyme activity.

RESULTS AND DISCUSSION

Sample Collection

Due to the likelihood of the presence of the wide variety of micro-organisms and microbial strains soil samples were collected from the Muhammad Ali Jinnah University, Karachi. By using sterile techniques, around 1g of soil was collected.

Bacterial Isolation

Soil samples were serially diluted from which 10^{-4} and 10^{-5} dilutions were transferred on Luria-Bertani Agar plates. Several colonies appeared on the plates, after incubating plates for 24 hours. Based on their shape, size and texture, 7 different morphologically different colonies were selected for further characterization. After performing gram staining it was revealed that most colonies were identified as gram positive rod shape bacteria, like the morphology of the *Bacillus* species (Fig. 1). As *Bacillus* species are well known for their enzyme producing abilities, their presence signifies their potential to produce the amylase enzyme (Madika *et al.,* 2017; Aun and Naqvi, 2025).

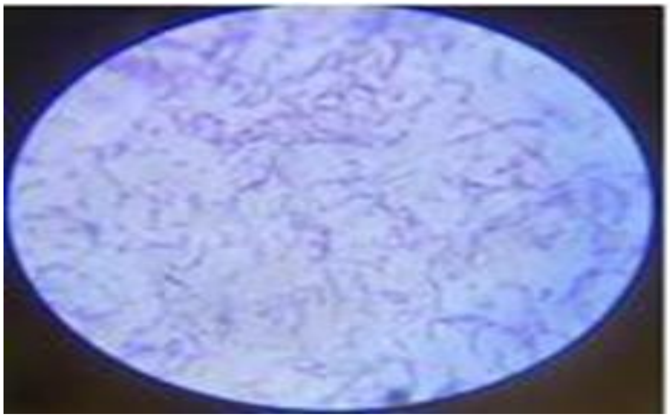

Figure 1: Gram-stained isolated bacteria under microscope at 100 X.

Screening

The selected colonies were then tested for the amylase enzyme production on starch-agar plates. Wells were created and 50 µL of cell-free filtrates were added in the well, then incubated for 24 hours at 37 °C. Clear hydrolysis zones were seen around the well, showing activity of the enzyme after treating the plates with iodine.

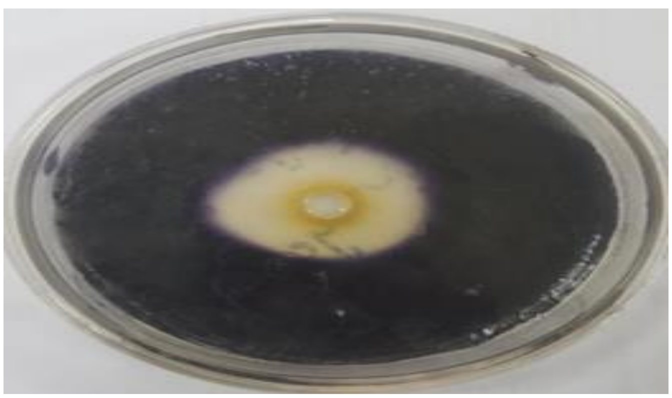

Figure 2: Clear zone of amylase activity was observed 25mm zone was observed

Characterization of the amylase activity

Optimum pH

Enzyme activity was assessed along a pH range between 3.0-7.0. At pH 6.0 zone was 25mm which was the largest and the clearest zone, showing that the enzyme has the highest activity at pH 6, indicating good hydrolysis of the substrate (Fig. 2). At pH 4 and 7 notable hydrolysis of substrate was also observed with clear zones of 20mm and 22mm respectively. Moderate activity of enzymes was observed at pH 5 around 20mm, while at pH 3.0 clear zones of 17mm were observed. Absorbance values (table1) at this pH additionally verify zone data. Largest visible zones were at pH 6.0 (0.756) and pH 4.0 (0.751), that resulted in the record of highest absorbance. Smallest clear zone was at pH 3.0 of around 17mm and had the lowest absorbance of 0.648. That confirms the fact that enzymatic activity reduces at high acidic conditions.

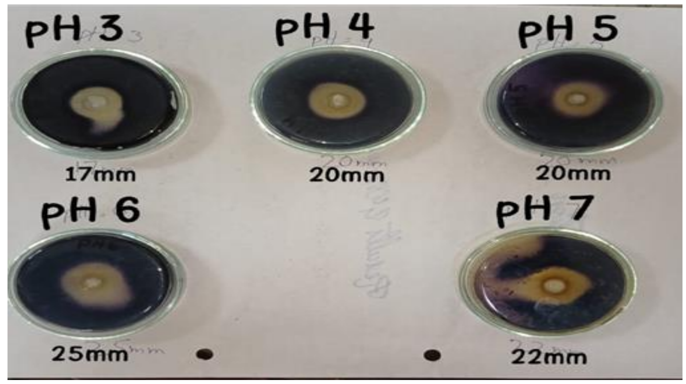

Figure 3: Enzyme activity was observed at different pH. The optimum pH was pH 6 with 25mm.

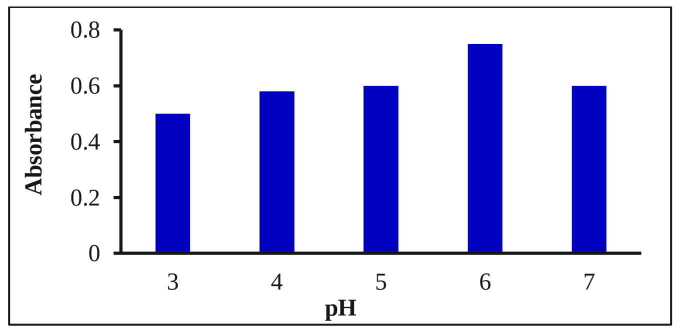

Figure 4*:* Activity of acidic amylase enzyme (biocatalyst) at different pH

Optimal Temperature

Amylase enzyme activity was checked using plate and enzyme assays at a wide range of temperatures ranging from between 30°C to 70°C. The enzyme showed maximum activity at 40°C, and 30°C it showed moderate activity, at 50°C activity of the enzyme decreased and at 60°C enzymatic activity was completely reduced. At 40°C 18mm of clear zone with absorbance of 0.603 was recorded.

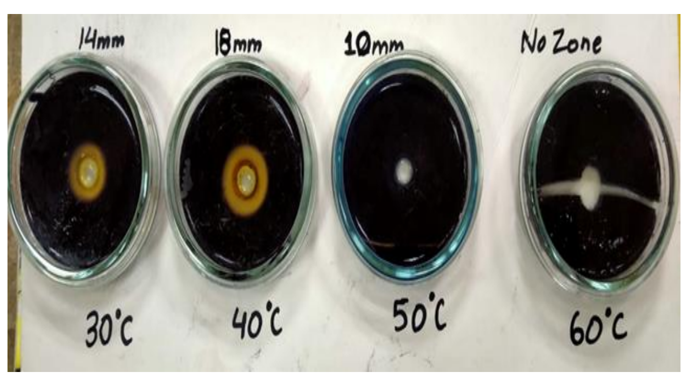

Figure 5: Showing that the preferable temperature at which enzyme shows its maximum efficiency is 40°C. At 30°C zones were clear with a size of 14mm.

The temperature effect was also observed increase in temperature enzyme activity decreases, suggesting that enzymes lose their activity at high temperature it was proved that when the temperature was raised to 50°C the size of clear zones were reduced to 10mm with the absorbance recorded of 0.539. At 60°C results showed that enzyme had completely lost its activity showing no clear zone (0 mm) and absorbance of 0.085. This proves that with the increase in temperature enzymatic activity starts to deplete and when the temperature increases up to a certain level enzymatic activity stops completely. This proves that enzymes are temperature sensitive and lose their activity at high temperatures.

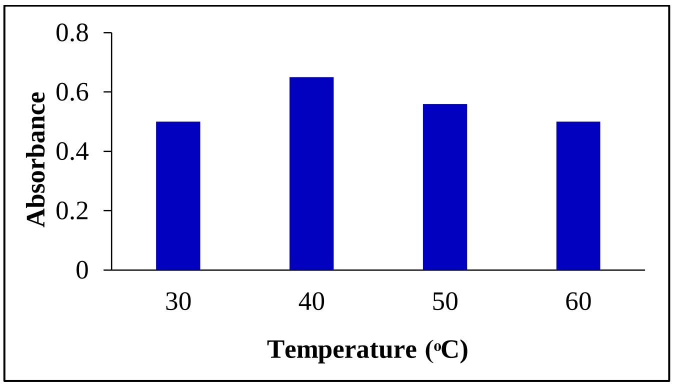

Figure 6: The activity of amylase at different temperature range, at 40 ° C

Effect of Metal Ions and Detergents

Further, by using absorbance measurements (Table 3), quantified the activity of amylase enzyme, which confirms the zone. effect of different metal ions and detergents like CTAB, EDTA, $MgCl_2$, $HgCl_2$ and ions such as $CoCl_2$, $CuSO_4$. CTAB has the largest inhibition zone of around 17mm, with elevated absorbance rate of 0.465. Also, EDTA and $MgCl_2$ shown inhibition zones of 16 mm and 15 mm respectively, expressing moderate absorbance of EDTA (0.444) and $MgCl_2$ (0.357). $CoCl_2$ and $CuSO_4$ displayed smaller inhibition respective zones of 4mm and 2mm but shown moderate absorbance of $CoCl_2$ (0.378) and $CuSO_4$ (0.468). Such differences may be impacted by enzyme diffusion effects or stability. In $HgCl_2$ case it is observed that enzymes stability is impacted by $HgCl_2$ ion, as it showed the highest absorbance of 0.476 and had moderate inhibition zones of 15mm, I was observed that while the enzyme was still active its stability was impacted by $HgCl_2$.

Figure 07: The enzyme activity was observed with different chemicals, metal ions, and detergents

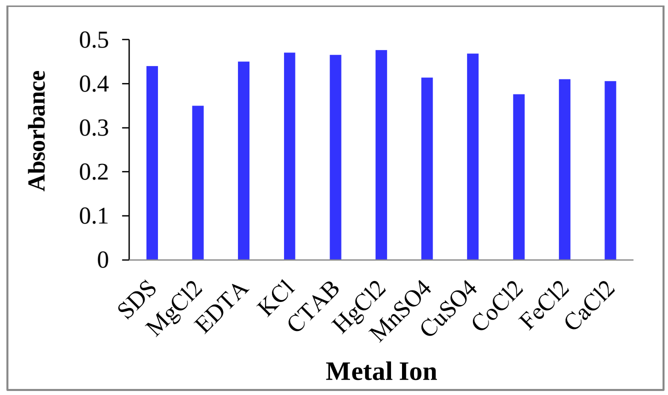

Figure8: The enzyme activity in the presence of different metals and detergent

CONCLUSION

Isolation and characterization of a strain of amylase producing Bacillus species from soil was done successfully in this study. This study has demonstrated the optimal activity of the enzyme is at pH 6.0 (25 mm zone) and 40°C (18 mm zone). Moreover, in this study the effect of different ions on the enzyme activity is also discussed that how presence of Mg^{2+}, Ca^{2+}, and Mn^{2+} enhanced the activity of enzyme whereas it got moderately inhibited in the presence of Hg^{2+} and Cu^{2+}. All these key findings highlight that amylases are a sensitive but important enzyme.

Conflict of interest

Authors declare no conflict of interest.

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