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Bacterial Amylase Production and its Application as a Detergent Additive

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Abstract. Microbial amylase is one of the most significant industrial enzymes utilized in the brewing, baking, washing and textile sectors. In the current study, the rhizospheric soil micro-organisms were examined for their capacity to produce amylase and effort made to isolate thermostable amylase enzymes that could be employed as a detergent addition and stable at high pH levels. Banana, Neem and Pomegranate plant soil rhizospheres which is yielded a total of 84 bacterial isolates. Out of these, 21% of the bacteria producer the amylase. In the investigation, *Bacillus* made up the bulk of the isolates. Additionally, the amylase activity measured at different media pH and temperatures and isolated from *Bacillus* showed great stability at alkaline pH and thermal stability upto 60 °C and might be used as a detergent component in the detergent industry. They also had a high potential for removing starch stains.

Keywords: amylases producing bacteria, rhizosphere, detergent industry

Introduction

Approximately 25% of amylase is the total industrial enzyme market (Kumari *et al*., 2019). They are used in various industries, including food, fermentation, pharmaceutical, detergent, textile and paper. Alpha-amylases are obtained from plants, animals and micro-organisms but their production from the first two are is limited (Hussain *et al*., 2013). The high use of microbial amylases is due to the rapid growth of micro-organisms, which increases enzyme production. Micro-organisms can be easily modified through genetic engineering to meet the needs of emerging industries, such as the production of enzymes with desired properties, such as thermo-stability (Ram *et al*., 2017). Furthermore, they are easier to handle, take up less space and are more cost-effective reported by (Ram *et al*., 2017). The majority of industrial enzymes are produced by *Bacillus* or *Aspergillus* species and some of can be synthesized in a single fermentation medium, which is more cost-effective manufacturing procedure and increased enzymatic stability (Hallol *et al*., 2022).

Amylases are the second type of enzyme employed in the composition of enzymatic detergent and they are present in 90% of all liquid detergents (Souza *et al*., 2010). These enzymes are used in laundry and automatic dishwashing detergents to digest starchy food residues such as potatoes, gravies, custard, chocolate and so on to dextrins and other smaller oligo-saccharides (Olsen *et al*., 1998). Amylases displayed good activity at lower temperatures and basic pH, retaining fundamental strength under cleaner conditions and the oxidative dependability of amylases is perhaps one of the primary models for their use in detergents when the washing environment is extremely oxidizing (Patel *et al*., 2021). Detergents are a mixture of surfactants, builders, corrosion inhibitors, optical brighteners, foam regulators, bleaching agents and enzymes, among other minor additives (Gurkk, 2019). Amylase enzymes are added to detergents liquefy starch and decompose starchy stains. Amylases hydrolyze the -1,4-linkages in gelatinized starch to reduce viscosity and improve starchy stain solubility by converting them to water-soluble dextrins and oligo-saccharides (Gurkk, 2019). *Bacillus* and *Aspergillus* are sources of amylases used in the cleaning industry. *Bacillus* strains such as *B. amylolique faciens*, *B. subtilis*, *B. licheniformis*, *B. stearothermophilus*, *B. megaterium* and *B. circulans* appear to have been widely employed to produce amylase in the modern era (Patel *et al*., 2021). *B. subtilis* has been reported as a good producer of amylases in large quantities (Kavita, 2018). Amylases are the most significant microbial enzymes for converting starch polymers to glucose monomers (Dutta *et al*., 2016). Microbial amylases are extracellular enzymes produced in the broth by bacteria during their growth and

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metabolism and require fewer downstream processing stages than enzymes (Kavita, 2018).

The rhizosphere is a small area of soil adjacent to the roots. (Ferris *et al*., 1993). Plant roots harbor a wide range of soil-borne microbes due to the excretion of various organic substances, and they meet their attachment requirements by providing a firm surface (Duineveld *et al*., 2001). In this study, the amylase enzyme was produced from bacteria isolated from the rhizosphere soil of selected plants. The effect of temperature and pH on amylase activity and the possible usage of amylase in detergents were examined.

Materials and Methods

Isolation of amylase producing bacteria. Different soil samples were collected from the garden area of the Gulshan campus, Federal University, Karachi, Pakistan. Soil samples were collected from the rhizospheres of Banana, Pomegranate trees and Neem trees and put into a sterilized bag and transferred immediately to the laboratory. Each 10 g of soil samples from different sites was mixed with 90 mL of sterile peptone water (1% peptone) in different 250 mL beakers and homogenized in a flask for ten minutes using an orbital shaker at 110 rpm. Finally, 0.1 mL aliquot of appropriate dilution was spread properly on starch agar plates. Bacterial strains were isolated by incubation at 37 °C for 48 h onto the nutrient agar plates (Oxoid). The bacterial colonies of different morphology were selected, purified by the streak plate method and preserved for further study (Sanders, 2012).

Screening for amylase producers. Pure isolated colonies from each nutrient agar plate were inoculated onto starch agar plates (1% peptone and 1% starch) and incubated at 37 °C for 48h. Plates were then exposed to a 2% iodine solution (Kavitha, 2018). Bacterial strains having a clear zone around the colony indicated the ability of bacteria to produce amylase and were designated as amylase producers. The starch degrading index (SDI) is the ratio of the total diameter of the area of the disappearance of the blue colour to the colony width diameter. Based on the high values of the starch degrading index, colonies were selected for further study (Dida, 2018).

Identification of bacterial isolates Isolated bacterial amylase producers were identified based on morphological, cultural and biochemical characteristics and compared with Bergey's manual (Bergey *et al*., 1993)

Amylase production and harvesting. Amylase producer isolates were grown in the starch broth (1% starch and 1% peptone) and incubated at 37 ºC for 48 h. After incubation, the broth was centrifuged at 3000 rpm for 5 min to get cell free supernatant, which acts as a crude amylase enzyme (Fuwa, 1954).

Enzyme assay. Typically, 1% starch solution was prepared in 0.1 M sodium phosphate buffer of pH 7 (6 g of sodium phosphate monobasic in a final volume of 500 mL of distilled water). A 1mL of cell free supernatant and 1mL of 1% starch solution were mixed in a test tube and kept at 37 °C for 10 min. Typically, 2 mL of acetic acid was added to the tubes, and the mixture was held in a boiling water bath for 10 min to stop the reaction and then cooled to room temperature. After then, 2 mL of ethanolic iodine solution (20 g of iodine and 24 g of sodium iodide powder was dissolved in 500 mL of ethanol, then the volume was made upto 1000 mL with distilled water and filtered) was added. Lastly, distilled water is added to the tubes to adjust the final volume of the reaction mixture upto 10mL. The value of optical density (OD) was measured (at 540 nm) in triplicate, and enzyme activity was determined in µg/100 mL. One enzyme activity unit is the amount of amylase enzyme under standard conditions that can hydrolyze 0.1 mg starch per minute (Wanderley *et al*., 2004).

pH stability of amylase activity. Briefly, 1% starch solution was prepared in 0.1 M sodium phosphate buffer and added to different sample tubes and their pH values were adjusted to 5, 6, 7, 8 and 9. Briefly, 1 mL of cell free supernatant was added to each tube. The enzyme assay was performed spectrophotometrically at the absorbance of 540 nm (Miller, 1959).

Temperature stability of amylase activity. Briefly, 1 mL of the starch solution and 1 mL of the cell free supernatant mixture were added in different tubes labeled; 25, 37, 50 and 60 °C and were incubated at different temperatures for 10 min. The enzyme assay was performed at the absorbance of 540 nm.

The de-staining potential of amylase enzyme. Pieces of white cotton cloth of four cm^2 were taken as the method described by Adinarayana (2003). The clothes were pre-stained with starch chocolate and fixation of the stain onto the cloth was done by incubation for 1 h kept in a hot air oven at 100 °C. These stain fixed clothes were soaked in different flasks for fifteen minutes at 60 °C of the following setups; Set A had 100 mL

distilled water only. Set B had 1 mL of 0.7% detergent dissolved in 100 mL of distilled water. Set C had 1 mL of detergent solution (7 mg/mL) mixed with 2 mL of cell free supernatant dissolved in 100 mL of distilled water. After that, the cloth was washed with water and dried in the air. Visual examination of cloths was made to observe the enzyme's contribution in removing stains. Untreated cloths stained with chocolate were used as the negative control.

Results and Discussion

Isolation, identification of rhizospheric bacteria. In this study, soil samples were obtained from the rhizospheres of banana, pomegranate and neem trees, at the Federal Urdu University's Gulshan campus in Karachi. A total of 18 out of 84 bacterial strains (21%) isolated from the rhizosphere of tested plants possessed amylolytic activity (Table 1). Pure isolated colonies were inoculated on starch agar plates. The gram's iodine solution detected a clear zone (no blue colorations) around the colonies (Fig. 1).

In the current study, 19% of banana tree isolates, 18% of neem tree isolates and 50% of pomegranate tree isolates were found to produce amylase. The results demonstrated that the rhizospheres of the banana tree and pomegranate trees were found to be a good source of amylase producing bacteria (Fig. 2).

Starch degrading index (S.D.I.) of isolated colonies. The starch degrading index is the potential of an organism to produce amylase by degrading starch. It is the ratio of the total diameter of the clearance zone (no blue coloration) in mm to the diameter of the colony in mm (Dida, 2018). Gram's iodine method was also used to detect the starch degrading index. The colonies with high starch degrading index values were selected for further study.

Amylase activity assay on different pH. The activity of the enzyme was found moderate at pH 5 and 6. The P2 and P3 cell free supernatants showed a boost in

Table 1. Frequency of amylase producers

Isolates codes	Plants	Total isolates	Total amylase producers	Percentages of amylase producers
B (1-42)	Banana tree	42	8	19%
N (1-38)	Neem tree	38	07	18%
P (1-4)	Pomegranate	04	02	50%
	Total	84	18	21%

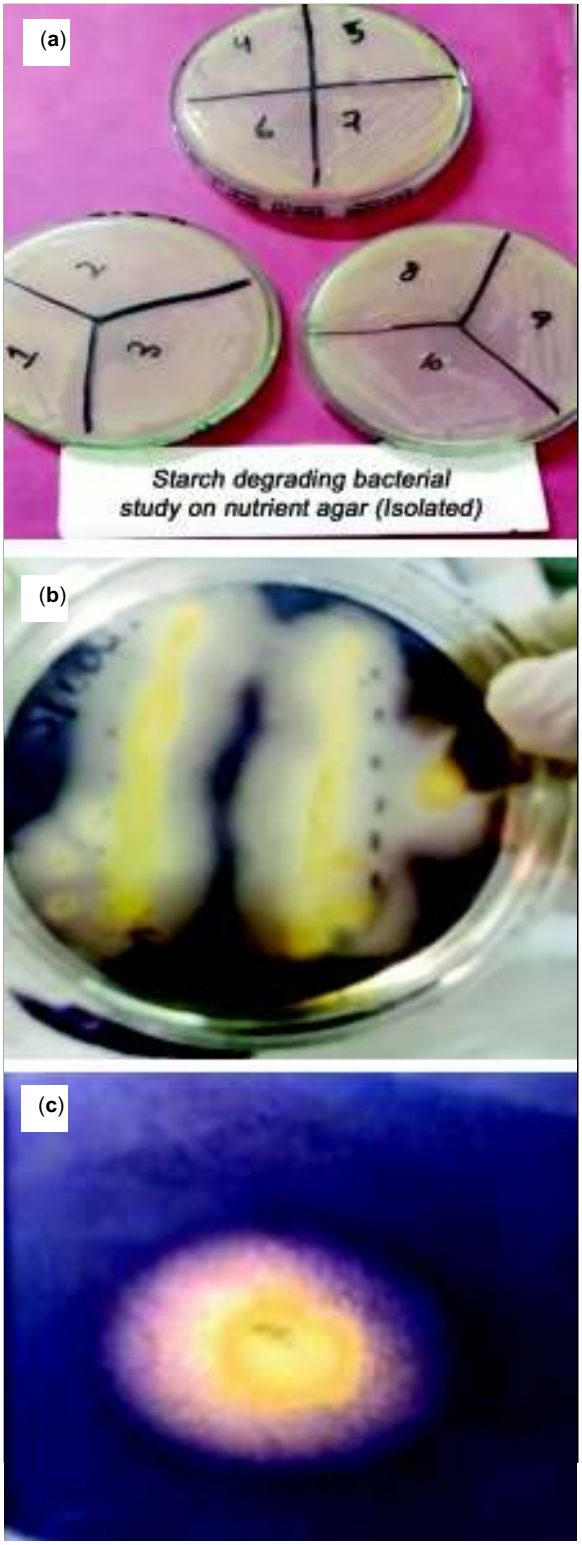

Fig. 1. (**a**) The growth of isolates on nutrient agar, (**b**) Amylase activity by streak plate method (**c**) The zone of clearance on starch agar medium.

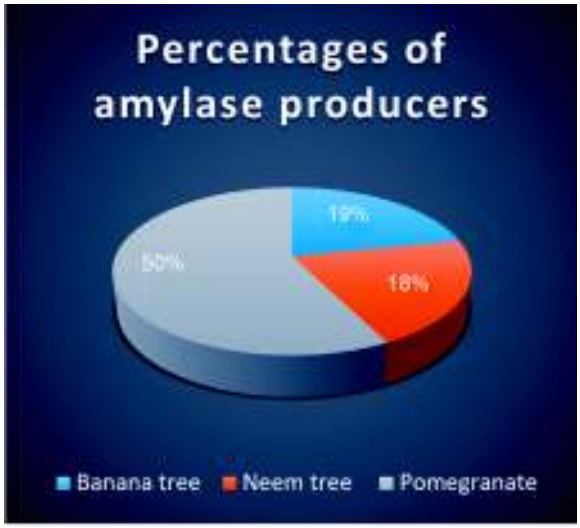

Fig. 2. Percentages of amylase producer isolates from different plants.

enzyme activity from pH 7 to pH 9 (alkaline pH). However, the optimum pH for all amylase enzymes was pH 7 (Fig. 3).

Amylase activity at different temperatures. The activity of amylase was found low at 25 °C. But, the starch degrading activity was found high at 37 °C. This temperature was considered the optimum temperature for starch degradation. The starch degrading activity was moderate at 50 °C and 60 °C (Fig. 4).

Identifications of amylase producing bacteria

Isolates P7, P2 and P3 were identified as *Bacillus subtilis* and B7 identified as *Bacillus cereus*, while N1 isolate was identified as *E. coli* (Table 2, 3 and 4). *Bacillus subtilis* was found to be a good source of amylase production.

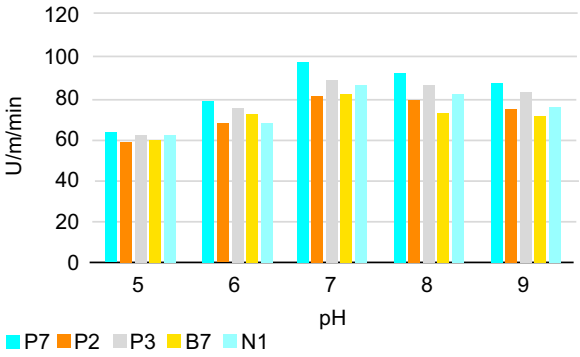

Fig. 3. Amylase activity of isolates at different pH.

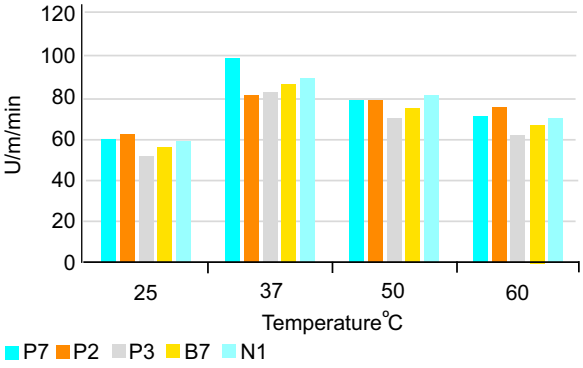

Fig. 4. Amylase activity of isolates at different temperatures.

De-staining activity of amylase. In the present work, The P7 cell free supernatant of *Bacillus subtilis* isolated from the rhizosphere of the pomegranate tree was found to possess the best amylase activity that was stable at high temperatures and alkaline pH. This supernatant was screened for its distaining ability against chocolate stains on cotton cloths. In this test, it was observed that adding this cell free supernatant in the detergent solution removed chocolate stains rapidly from cotton cloth compared to the sole use of the detergent solution. These results revealed that the washing ability could be increased by adding amylase supplements to the detergents (Fig. 5).

Amylase is a vital enzyme that is employed in industry to convert starch into simple sugar. This enzyme accounts for 25-33% of the worldwide enzyme market. The greatest place to get this enzyme is from micro-organisms in large quantities. Thermostable amylases are mostly preferred in industrial applications because to the lower danger of contamination, higher rate of diffusion, and higher cost of external cooling. The most adaptable enzymes, amylases, typically display alkali tolerance in nature (Roy *et al*., 2014).

Table 2. Microscopic characteristics of amylase-producing bacteria

Microscopic observation	P7	P2	P3	B7	N1
Shape	Rods	Rods	Rods	Rods	Short Rods
Arrangements	Chains	Chain	Chain	Chain	Scattered
Gram reaction	Positive	Positive	Positive	Positive	Negative

Table 3. Cultural characteristics of amylase producers

Media	P7	P2	P3	B7	N1
Nutrient agar	Irregular colonies	Irregular colonies	Irregular colonies	Irregular colonies	Translucent colonies
MacConkey agar	-	-	-	-	lactose fermenters
Hemolysis pattern on blood agar	β	*B*	*B*	β	β

Table 4. Biochemical characteristics of amylase producers

Biochemical testing	P7	P2	P3	B7	N1
Indole test	-	-	-	-	+
M.R. test	-	-	-	-	+
V.P. test	-	-	-	-	-
Catalase production	+	+	+	+	+
Oxidase production	-	-	-	-	-
Citrate utilization	-	-	+	+	-
Nitrate reduction	+	+	+	+	-
T.S.I.	Alk/A	Alk/A	Alk/A	Alk/A	A/A
Glucose fermentation	+	+	+	+	+
Maltose fermentation	+	+	+	+	_
Lactose fermentation	-	-	-	_	+
Sucrose fermentation	+	+	+	+	_
Glucose fermentation	+	+	+	+	+
Organisms	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. cereus*	*E. coli*

The amylase present in detergent formulations must be functional and stable in the presence of other detergent additives, including other enzymes, surfactants, builders and other agents, such as bleaching, oxidizing, sequestering, alkaline, suds control, *etc.* Complete and effective stain removal necessitates the cooperative efforts of all the detergent components and washing machine mechanical processes (Farooq *et al*., 2021).

Plant roots are invaded by various micro-organisms (Berendsen, 2012). The rhizosphere is the narrow soil area close to the roots of plants. The type and number of micro-organisms colonizing the rhizosphere depend on the nutrients secreted by the plants in the soil (Kennedy, 2005). Since micro-organisms are easily manipulated, the production of microbial amylases is inexpensive and straightforward (Krishna, 2012). The screening of micro-organisms for the production of stable α-amylases is always mandatory to satisfy the requirement of industries (Gangadharan *et al*., 2020).

The rhizosphere of plants has been reported to have the colonization of different species of *Pseudomonas* and *Bacillus* (Parmar, 1999; Halverson *et al*., 1991). In the current study, we explored amylase-producer *Bacillus* strains for their potential application in the detergent industry. It is also under (Konsoula *et al.,* 2007; Ha *et al.*, 2001) that been reported that *Bacillus subtilis* and *Bacillus cereus* were good producers of amylase enzymes. According to Olsen *et al.* (1998), *Bacillus* and *Aspergillus* are reliable suppliers of amylases for the detergent sector.

The use of amylase in detergents should have stable enzyme activity at temperatures of 40 to 60 °C and alkaline pH (Dahiya *et al*., 2015). In this study, it was observed that cell free supernatant P7, when added to the detergent solution removed chocolate stains rapidly from cotton cloth compared to the sole use of the detergent solution. This crude amylase enzyme was found stable at high temperatures and alkaline pH.

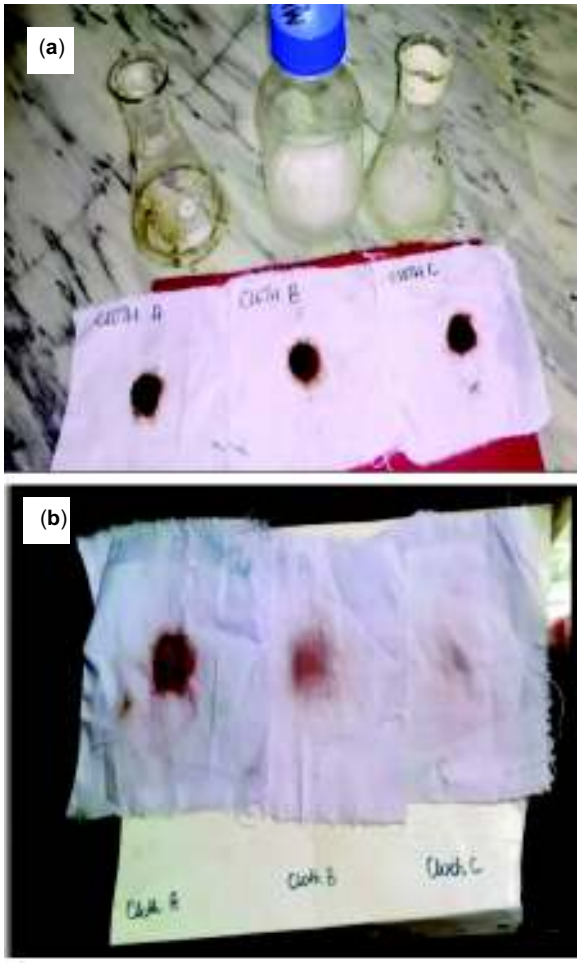

Fig. 5. Cloth A was dipped in water only, cloth B was dipped in detergent solution only, cloth C was dipped in detergent and amylase solution (**a**) stained cloths before treatments (**b**) stained clothes after treatment.

The microbial enzymes are considered eco-friendly and work under mild conditions. Thus, using microbial amylases in the detergents could also reduce the harmful effects of the detergent residue polluting the environment by decreasing the number of surfactants and bleach (Lahmar, 2017).

Conclusion

The current study shows that the rhizosphere of pomegranate and banana plants is a good source of amylase-producing bacteria. These amylases of microbial origin are cheap but have great potential in starch stain removal. Since these microbial enzymes showed good stability at alkaline pH and thermal stability up to 60 °C, these amylase enzymes could be exploited in the detergent industry.

Conflict of Interest. The authors declare that they have no conflict of interest.

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