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Research Article

# Isolation of Potential Amylase Producing *Bacillus albus* and *Bacillus paramycoides* Bacteria from Sugarcane Bagasse

**Muhammad Saeed Khan, Saif Ullah, Faryal Khan, Sundas Mehtab, Waheed Ullah***

Department of Microbiology, Kohat University of Science and Technology, Kohat, Khyber Pakhtunkhwa, PAKISTAN.

## ABSTRACT

**Background:** Enzymes are biological catalysts that play essential roles in metabolic pathways, digestion, energy production, and industrial applications. Amylase, an enzyme that catalyzes the hydrolysis of starch into simpler sugars, is widely used in food, fermentation, textile, and biofuel industries. Microbial amylases, particularly those from the *Bacillus* species, are preferred due to their high yield and efficiency. This study focuses on the isolation, screening, and optimization of amylase-producing bacteria from sugarcane bagasse soil samples. **Materials and Methods:** Soil samples were collected from sugarcane bagasse sites in Peshawar, Pakistan, and subjected to serial dilution and plating on nutrient agar. Isolates were screened for amylase activity using starch agar plates and iodine staining. The highest amylase-producing isolates were further tested for optimal production conditions, including pH and temperature. Morphological, biochemical, and molecular identification of bacterial isolates were performed, including 16S rRNA gene sequencing. **Results:** Among the 10 bacterial isolates, 8 exhibited amylase activity, with 4 isolates (G2, A1, S2, and M1) showing the highest hydrolysis zones. The optimal pH for amylase production was determined to be pH 10 for all isolates, with M1 producing the highest enzyme activity (0.211 IU/mL/min). Temperature optimization revealed that isolates varied in their thermal preference: G2 (45°C), A1 (70°C), S2 (40°C), and M1 (70°C), suggesting thermostability. Morphological and biochemical analyses confirmed that the isolates belonged to the Bacillus genus. Molecular identification and phylogenetic analysis revealed that A1 had 94.34% similarity with *Bacillus paramycoides*, while G2 showed 94.25% similarity with *Bacillus albus*. **Conclusion:** The findings suggest that these strains, particularly the thermotolerant isolates, hold significant industrial potential for enzyme production under alkaline and high-temperature conditions.

**Keywords:** Amylase, *Bacillus albus*, *Bacillus paramycoides*, Enzyme activity, Sugarcane bagasse.

**Correspondence:**
**Dr. Waheed Ullah**
Department of Microbiology, Kohat University of Science and Technology, Kohat, Khyber Pakhtunkhwa, PAKISTAN.
Email: waheedwazir@gmail.com

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## INTRODUCTION

Enzymes are biomolecules that act as biological catalysts and help to catalyse the biochemical reactions which are crucial for living by reducing their activation energy. They are very specific to their substrates and so control many metabolic pathways exactly.[1] Enzymes play undeniable roles in important biological processes, such as digestion, energy production, and signaling between cells. They have a wide range of applications in medicine, industry, and biotechnology, so their significance should not be underestimated concerning scientific or practical innovations.[2] Like all other enzymes, amylases are also very potential enzyme, acting as a catalyst to facilitate or speed up the hydrolysis of starch to simpler sugars by lowering the activation energy needed for this reaction. It is substrate specific, and it has contributed a major role in carbohydrate metabolism of different organisms.[3] This enzyme is present in all saliva, pancreatic secretions and some microbial systems, and is therefore crucial for digestion and energy production. The importance of curdling in biological and industrial processes is evident in its use in food, fermentation, and biotechnology.[4] It is widely used in the food industry for baking, brewing, and dairy processing. It aids in fabric desizing in textiles and enhances drug formulations in pharmaceuticals.[5] In biofuel production, it converts starch into fermentable sugars, while in detergents; it helps remove starch-based stains.[6] The paper industry uses it for starch degradation, and it plays a role in waste management and wastewater treatment.[7] Amylase enzyme is made from a variety of sources, including animals, plants and microbes. They are secreted by the salivary glands and pancreas in animals to facilitate starch digestion.[8] In plants, they are produced by germinating seeds such as barley to hydrolyze stored starch into sugars and microbial amylase are produced from bacteria, fungi, and actinomycetes and thus include a variety of microbial amylase.[9] Bacterial species include *Bacillus subtilis* and *Bacillus licheniformis* while among fungal strains *Aspergillus niger* and *A. oryzae* are the most important microorganisms for commercial enzyme production due to their high yield and

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efficiency.[10] Such microbes can survive and flourish in many distantly diverse environments, which makes them amenable for large-scale industrial production. Application of microbial amylases have been reported in food, fermentation, textile and biofuel industries.[11] Due to their ability, thermo-stable amylase enzymes have been used for this purpose.[12]

Environmental factors affect the growth of organisms, and their enzymes synthesis. Sustainability for bacterial enzymes, pH and temperature are required.[13] Enzyme activity was also vary when changing temperature and pH.[14] Sugarcane bagasse is a potentially interesting source for isolating amylase-producing bacteria, due to its high microbial growth niche of the local abundance organic matter.[15] Thus, the isolation and characterization of these bacteria in sugarcane bagasse not only provides an unexplored resource but also paved way for sustainability by utilizing agricultural waste to produce biotechnological products.

## MATERIALS AND METHODS

### Collection of Samples

Soil Samples were taken from the soil of Peshawar region sugarcane bagasse (latitude: 34.0151° N, longitude: 71.5249° E) and put into sterilized containers. These samples were quickly provided to the Microbiology lab of Kohat University (latitude: 33.5867° N, longitude: 71.4421° E). The samples were then stored at 4°C till further processing.

### Isolation and Culturing of Bacteria

We isolated the bacteria from soil by doing standard serial dilutions and plating. On nutrient agar plates, the diluted samples were spread out.[16] Following that, the plates were incubated for 24 hr at 37°C. The bacterial isolates were subsequently screened based on colony appearance once the incubation period was complete.

### Screening and selection of Amylase Producing Bacteria

After being isolated, each colony was streaked using the spot method on starch agar plates and incubated for 24 hr at 37°C.[17] Following incubation, 1% iodine and 2% potassium iodide solution were added to the plates to test for amylase activity. The isolate that created a clear zone surrounding the colony and survived was thought to be a bacterium that produces amylase.[18] A transparent zone around the colony is created by the bacteria as they break down the starch. The bacterial isolates that produced the biggest clear zones were kept for further research.

### Optimization of Condition for Production of Amylase

The bacterial isolates that were positive for amylase production were tested through optimization of factors such as pH and temperature in the culture broth.[19]

All of the selected isolates having amylase activity are cultured in artificial broth. The experiment is carried out by placing this liquid media flask in different pHs ranging from 3 to 11 and incubated at 37°C for 24 hr. The thermal adaptation of the amylase producing bacteria was observed with the incubation of the bacteria between 35°C to 70°C. Isolates of the bacteria were incubated at various temperatures and grown in pH-adjusted optimized broth. The spectrophotometer was used to monitor the turbidity of the bacterial culture at 546 nm.[20]

### Determination of Amylase Activity

The Bernfeld approach is employed to quantify amylase activity. 1 mL of 0.05 M sodium phosphate buffer at pH 7.0, 1 mL of 1% starch, and 1 mL of the enzyme extract were used in the experiment. A test tube was used to conduct this experiment. After that, the mixture was incubated for 15 min at 55°C. 1 mL of DNS reagent was injected to halt the reaction after the incubation period. Using maltose as a reference, the OD of color was determined at 540 nm in a spectrophotometer.[21]

### Identification of bacterial isolates

#### *Morphological and Microscopic Identification of Isolates*

After getting pour culture, bacterial, size, color, and texture of the colonies were checked for morphological identification of bacteria. Microscopic identification was done after performing gram staining and spore staining.[22]

### Biochemical Identification

For biochemical identification, the API 10S strip was used. For this, fresh bacterial colony was picked from an agar plate using sterile loop.[23] Then emulsified the colony in sterile distilled water or 0.85% saline to achieve a turbidity of 0.5 McFarland standards. Using a sterile pipette, the wells or tubes of the API 10S strip were filled with the prepared bacterial suspension. Some wells required filling only, while others needed to be overlaid with sterile mineral oil for anaerobic reactions. The strip was placed in its incubation tray, ensuring a humid environment by adding water to the tray. After incubation, the color changes were observed in each well for identification of bacteria, which corresponded to the metabolic activity of the bacteria. The color reactions in the wells were used to match the results with the interpretation chart provided in the API 10S kit.

### Molecular identification of Bacterial Isolates

To extract the bacterial genomic DNA, the Phenol-Chloroform Isoamyl Alcohol (PCI) technique was used.[14] After DNA extraction, DNA was run on 1% Agarose Gel for confirmation of extracted DNA. The extracted DNA was subjected to PCR for amplification using 16S rRNA gene (forward primer 5-GGAGGCAGCAGTAGGGAATA-3 and reverse primer 3-TGACGGGCGGTGAGTACAAG-5).[25] The PCR amplified

product was confirmed on 2% agarose gel using a UV transilluminator.

Advance Bioscience International's (ABI) commercial service was used to sequence the PCR amplified product. To determine the precise nomenclature of the isolates, the sequence was BLAST searched on the EZ-Biomedical server. Using MEGA 11 and the neighbor-joining method, the phylogenetic tree was built with a bootstrap value of 1000.

### Statistical analysis

Graph Pad Prism software was used to statistically assess the means and standard errors of each experiment, which was carried out in triplicate ($n$=3). For comparisons between two groups, an unpaired or paired t-test was performed, while multiple group comparisons were analyzed using one-way ANOVA test. If two independent variables were assessed, a two-way ANOVA test was used.

## RESULTS

### Isolation and Screening of Bacterial Isolates

Eight of the 10 isolates, as seen in Figure 1, had amylase activities ranging from small to large zones surrounding bacterial colonies, whereas the other two isolates exhibited no activity at all. The isolates with the highest amylase activity were chosen for further investigation.

Among 8, four isolates (G2, A1, S2, and M1) were chosen based on their highest amylase activity. The G2 formed 21 mm clean zone surrounding their colony as a consequence. In the same direction, isolates A1 displayed 17 mm zone and S2, and M1 had a 15 mm clear zone. According to Table 1, these isolates were thought to have the highest amylase activity. The amylase positive bacteria were then pure cultured for further process as shown in Figure 4.

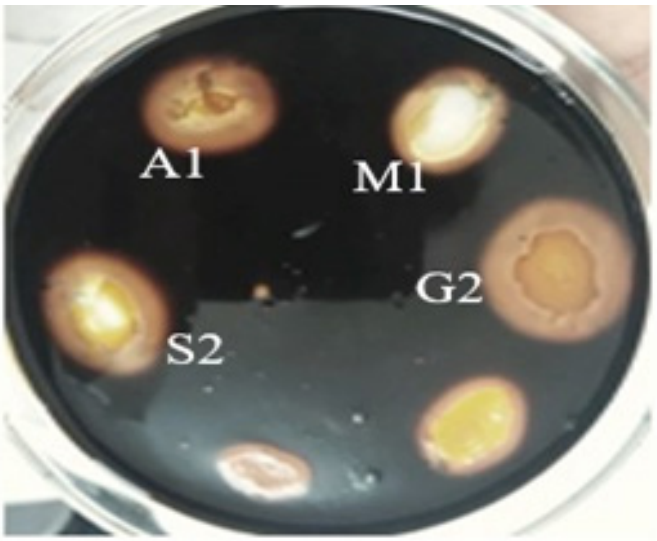


**Figure 1:** Formation of clear zone indicates the production of amylase enzyme by selected bacterial isolates.

### Optimization of Production of Amylase Producer

In order to optimize amylase production, different pH (3-10) and temperature (30-70) range were used.

### Effect of pH on the Production of Amylase

The effect of different pH shows different results as shown in Figure 2. The pH ranges from 7.0 to 10.0 yielded the largest production of amylase in our investigation. All isolates show the best production of amylase on pH 10. Figure 2(c) indicates the highest production of amylase (0.211 IU/mL/min) of M1 isolate at pH 10, followed by G2 isolate that displayed amylase yield (0.187 IU/mL/min) as shown in Figure 2(b). At pH 10, the isolate A1 also produced high amylase (0.185 IU/mL/min) as shown in Figure 2(a). Figure 2(d) shows the production of amylase (0.180 IU/mL/min) of S2 isolate at pH 10.

### Temperature effect on the Production of Amylase

All isolates showed their highest production at different temperatures. In case G2 isolate, highest rate of amylase production was at 45°C (0.114 IU/mL/min) as shown in Figure 3(b). Similarly, at 70°C, A1 isolate produced the highest amount of amylase (0.114 IU/mL/min), as seen in Figure 3(a). The maximum production of amylase by S2 isolate occurred at 40°C and the production rate was (0.099 IU/mL/min), shown in Figure 3(d). The rate of production by M1 was (0.115 IL/mL/min) at 70°C as shown in Figure 3(c).

### Identification of Selected Isolates

#### *Morphology and Microscopic Identification*

The chosen isolates' colonial morphologies differ from one another (Table 2). Gram staining and endospore staining were used to examine the cell morphology, as seen in Figures 5 and 6. G2 had an uneven colonial morphology with a whole edge, and its elevation was creamy, and flat. Gram staining and spore staining demonstrated that G2 amylase-producing bacterial isolates were Gram-positive rods in the chain and spore-forming, (Figures 5 and 6). Similarly, colonies of A1 isolate morphology exhibited irregularities, including a filamentous edge and a pulvinate, white coloration at elevation. Microscopic examination shows positive rods and spore formation as seen in (Figures 5 and 6).

### Biochemical Identification

After performing biochemical tests, the two selected isolates, G2 and A1 were positive for TDA, GLU and CIT test and were negative for the remaining tests. S2 was positive for ONPG, CIT and TDA while M1 was positive for CIT and TDA and were negative for all the remaining tests. The biochemical test results indicated that the bacterial strains are members of the *Bacillus* species as seen in (Figure 7) and (Table 3).

## Molecular Identification

The amplification of 16S rRNA gene shows that the product size of isolates was 1492 bp compared to 1KB ladder is shown in (Figure 8).

## Phylogenetic Analyses

Phylogenetic analyses showed that A1 showed 94.34% similarity with *Bacillus paramycoids* as shown in Figure 9. Similarly, the strain G2 showed 94.25% similarity with *Bacillus albus* (Figure 10).

# DISCUSSION

The majority of microbes require specific growth and metabolic conditions, making them unsuitable for cultivation in a lab setting.[26] Soil, typically found 10 cm below the surface, provides a nutrient-rich environment that supports microbial growth.[27] Among these microbes, *Bacillus* species are well-documented producers of industrially significant enzymes such as amylase, which plays a crucial role in biotechnology and dominates the global enzyme market.[28]

Amylase, an extracellular enzyme, hydrolyzes starch into glucose, maltose, various oligosaccharides, and dextrin's.[29] It is derived from multiple sources, including microorganisms, plants, and animals,[30] with microbial production being preferred due to its efficiency, cost-effectiveness, and ease of large-scale production.[31] Microbial amylases have gained global interest due to their applications in sugar, textile, paper, and pharmaceutical industries, where technological advancements have enhanced their utility.[32]

In alignment with these findings, the present study aimed to isolate and screen bacterial strains for amylase production, followed by optimization and identification to evaluate their

**Table 1: Zone of hydrolysis of starch shows the production of amylase enzyme by selected bacterial isolates.**

| Isolates | Mean of the diameter of zone (mm) |
|---|---|
| G2 | 21 |
| A1 | 17 |
| S2 | 15 |
| M1 | 15 |

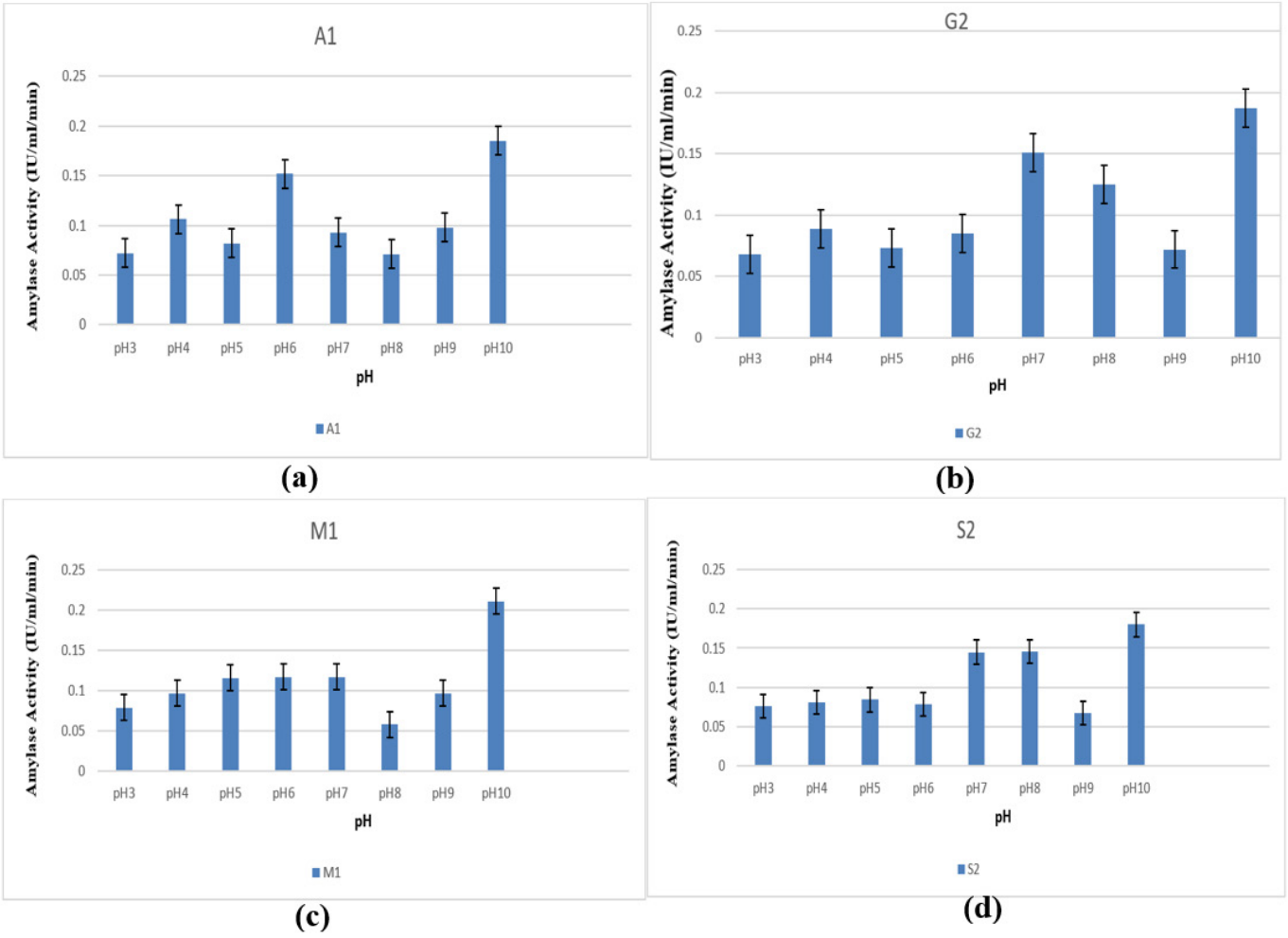


**Figure 2:** Shows the effect of different pH on synthesis of amylase enzyme by isolate A1 (a), G2 (b), M1 (c) and isolate S2 (d) respectively.

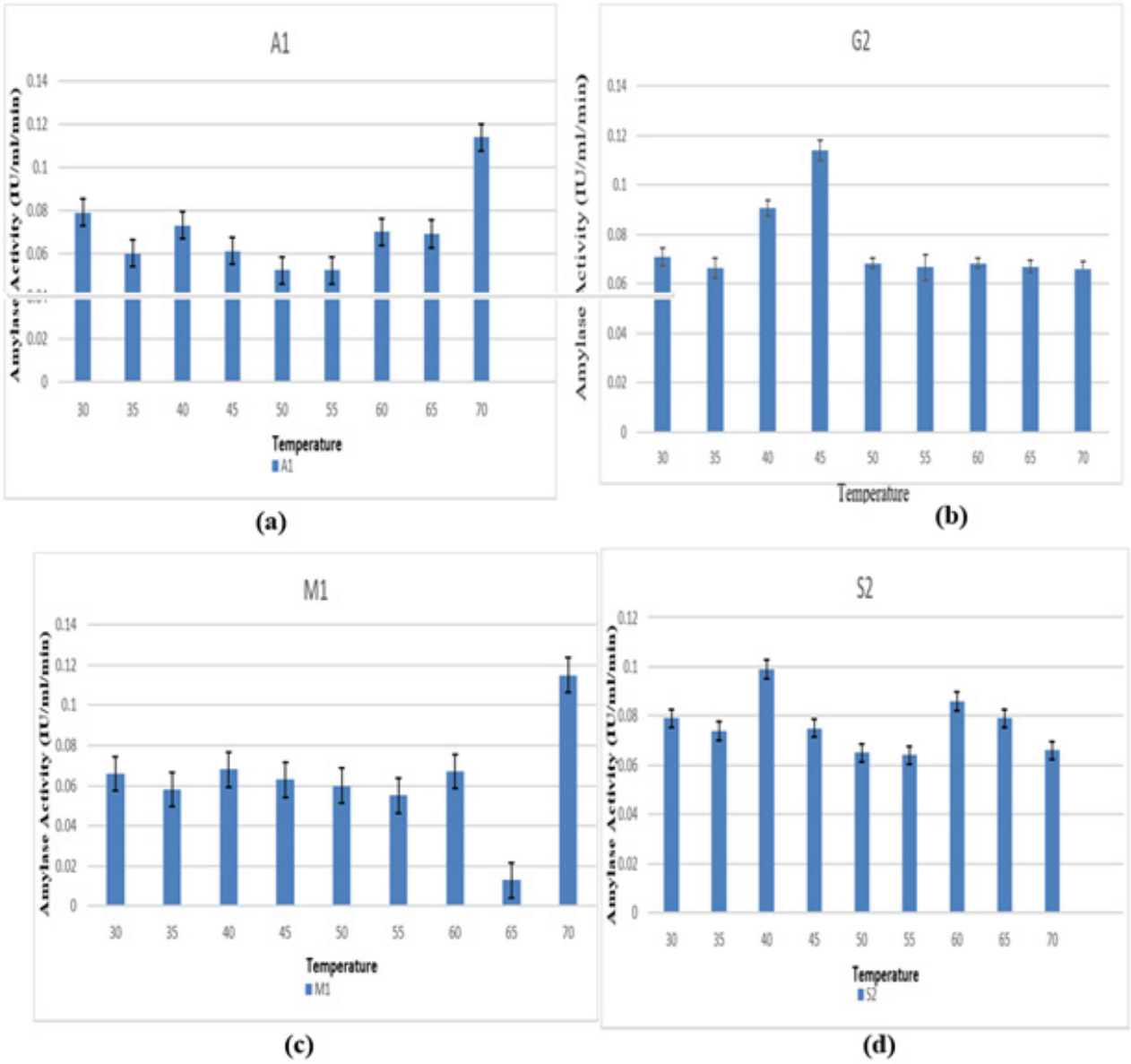


**Figure 3:** Shows the production of amylase enzyme at different temperatures by isolate A1 (a), G2 (b), M1 (c) and isolate S2 (d) respectively.

**Table 2: Morphological characteristics of selected amylase enzymes producing bacterial isolates.**

| Name of Isolates | Shape | Margin | Elevation | Color |
|---|---|---|---|---|
| G2 | Irregular | Entire | Flat | Cream |
| A1 | Irregular | Filamentous | Pulvinate | White |
| S2 | Filamentous | Filamentous | Pulvinate | White |
| M1 | Filamentous | Filamentous | Pulvinate | White |

potential for industrial applications. From an initial screening of 10 isolates, eight displayed amylase activity, with G2, A1, S2, and M1 exhibiting the highest enzymatic activity, as indicated by clear zones on starch agar plates. Among these, G2 demonstrated the most significant hydrolysis (21 mm), followed by A1 (17 mm), S2 (15 mm), and M1 (15 mm). This finding concurs with earlier findings that had named *Bacillus* spp. as strong amylase producers because of their ability to secrete extracellular enzymes.[33]

Additional optimization of amylase production showed that environmental conditions, especially pH and temperature, have a significant influence. Alkaline conditions (pH 10) favored amylase production across all tested isolates, with M1 exhibiting the highest enzymatic activity (0.211 IU/mL/min), followed by

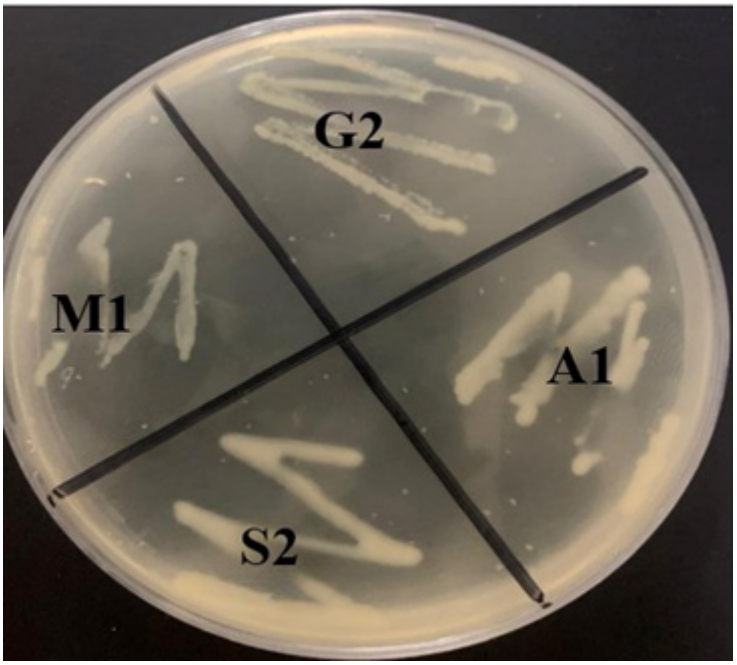


**Figure 4:** Shows the pure cultures of selected bacterial isolates on Nutrient agar plate.

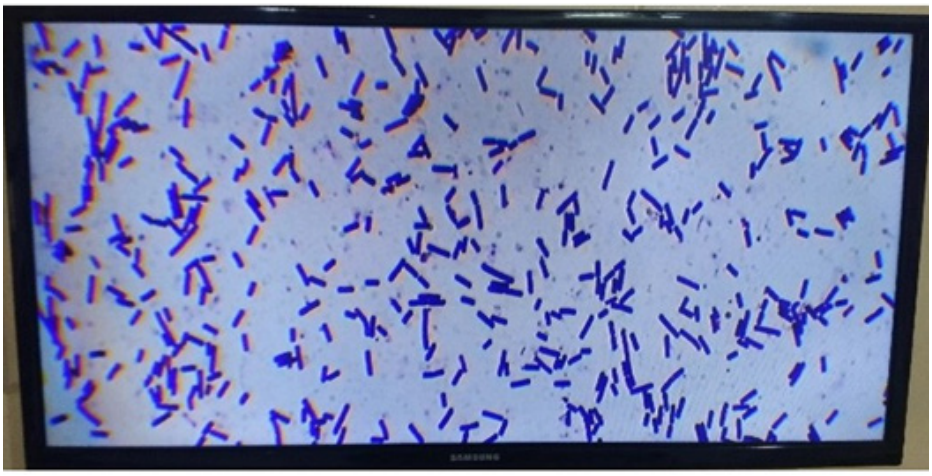

**Figure 5:** Microscopic examination shows that the bacterial isolates were Gram-positive rods.

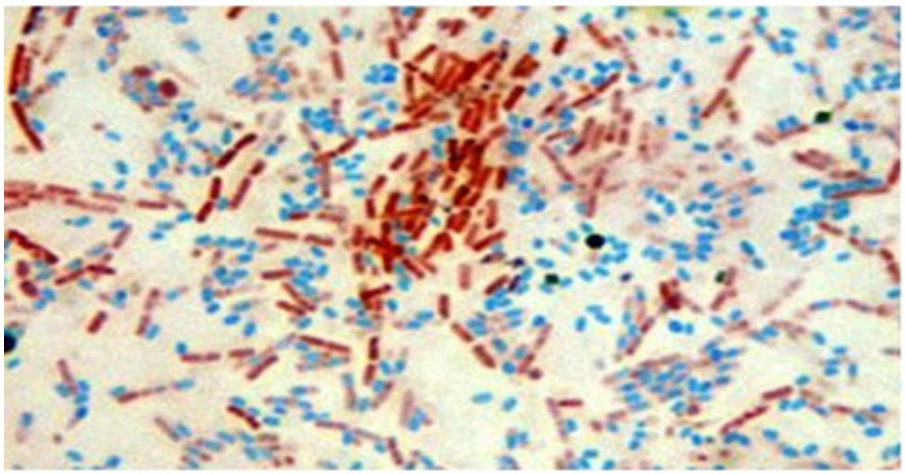

**Figure 6:** Microscopic examination of bacteria after spore staining. The bacterial cells are stained red, while the spores are tinted green.

**Table 3: This table shows the biochemical tests of selected amylase producing bacterial isolates.**

| Biochemical test | G2 | A1 | S2 | M1 |
|---|---|---|---|---|
| Gram staining | + | + | + | + |
| Endospore staining | + | + | + | + |
| Ortho-Nitrophenyl-β-galactoside | - | - | + | - |
| Glucose tolerance | + | + | - | - |
| Arabinose | - | - | - | - |
| Lysine decarboxylase | - | - | - | - |
| Ornithine decarboxylase | - | - | - | - |
| Citrate | + | + | + | + |
| Hydrogen sulfide | - | - | - | - |
| Urease | - | - | - | - |
| Oxidase | - | - | - | - |

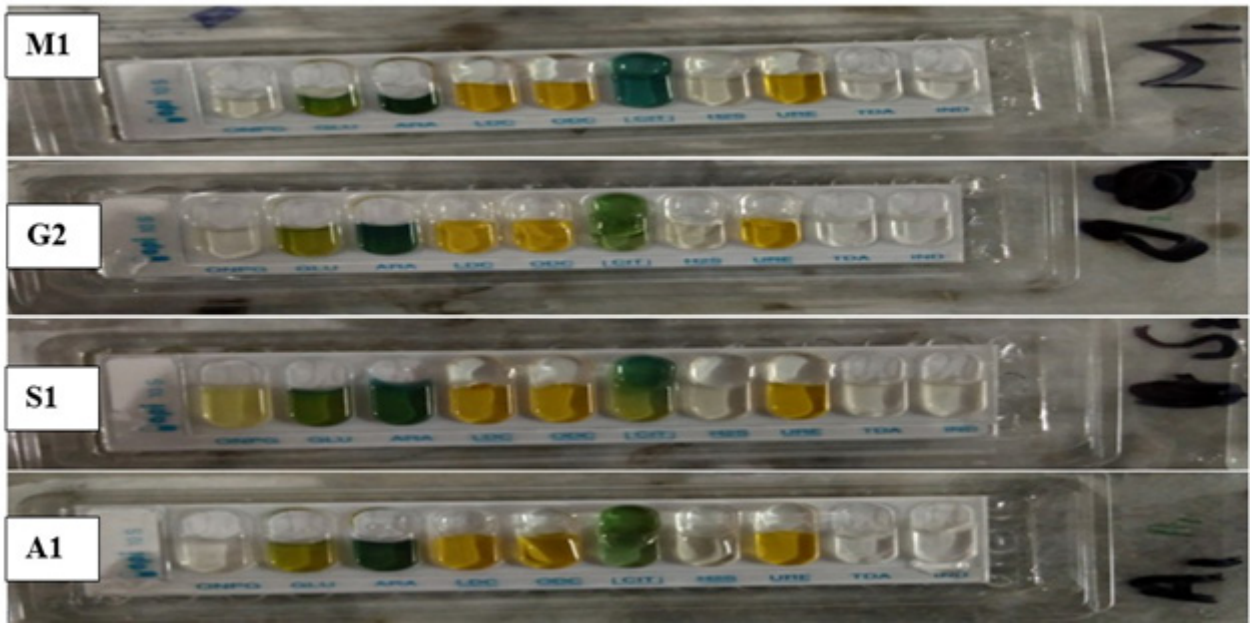


**Figure 7:** Indicates the biochemical properties of M1, G2, S1 and A1 bacterial isolate, determined by API 10S.

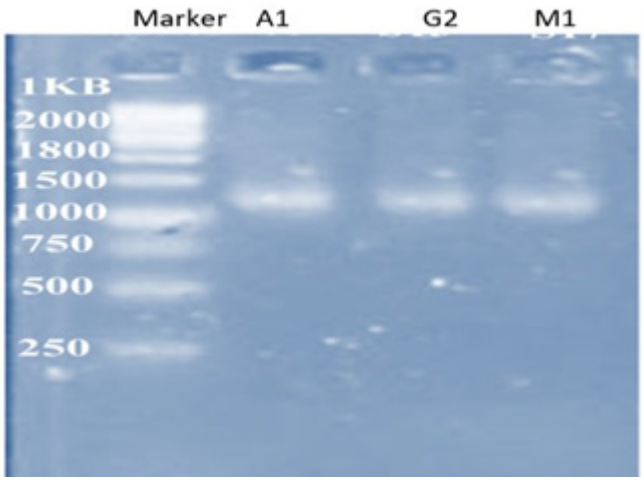


**Figure 8:** Visualization of bands shows the amplified PCR product of highest amylase producing isolates using 16S rRNA gene.

G2 (0.187 IU/mL/min), A1 (0.185 IU/mL/min), and S2 (0.180 IU/mL/min). These findings agree with previous observations on *Bacillus* spp., which are alkaline-tolerant and thus most suitable for applications in the manufacture of textiles and detergents, where alkaline stability is a requirement.[34] Similarity in outcomes can be due to the alkaline adaptation of Bacillus spp. through evolutionary processes, resulting in increased enzymatic activity.

Temperature optimization also demonstrated the varied thermal requirements of the isolates. G2 and S2 showed maximum amylase activity at intermediate temperatures (45°C and 40°C, respectively), while A1 and M1 showed optimal production at elevated temperatures, as was expected from their thermostability. Interestingly, M1 exhibited the greatest activity at high temperatures (0.115 IU/mL/min), which indicates its extremophilic character. This is in agreement with previous studies indicating that *Bacillus* species are likely to possess thermostable


**Figure 9:** Phylogenetic tree shows the similarity of A1 isolate to *Bacillus paramycoides.*


**Figure 10:** The phylogenetic analyses indicate that G2 isolate shows similarities with *Bacillus albus.*

enzymes, which are highly beneficial in industrial processes where enzymatic activity occurs at high temperatures.[35] However, the variability of temperature preferences among isolates can be due to strain-specific genetic differences influencing enzyme stability and function with temperature.

Morphological and biochemical identification also validated the identification of the isolates. The strains were all Gram-positive and spore-forming, characteristics common to *Bacillus* spp. Colony morphology discrimination showed that A1 formed white, pulvinate colonies with filamentous margins, whereas G2 formed cream-colored, flat colonies. Microscopic examination ensured rod-shaped cells with endospore formation, in line with the durability characteristic of *Bacillus* species and application in enzyme production for industrial purposes.[36]

Biochemical profiling supported classifying these isolates as members of the *Bacillus* family. G2 and A1 were test-positive for Tryptophan Deaminase (TDA), Glucose Metabolism (GLU), and Citrate Utilization (CIT), while S2 and M1 were test-positive for TDA and CIT. These metabolisms illustrate the versatility of these strains and pave the way for their utilization in a wide range of environmental and industrial applications.[37] The biochemically observed difference may be due to genetic diversity among *Bacillus* species that influence their metabolic processes and the activity of enzymes produced.

Molecular identification by 16S rRNA sequencing also supported the identification. Phylogenetic analysis indicated 94.34% similarity for A1 with *Bacillus paramycoides* and 94.25% similarity for G2 with *Bacillus albus*. These percentage values lie within the limit permissible for identifying to the genus *Bacillus*, a well-supported group for amylase producers.[38] There would be minimal differences in percentages that would be made by natural variations in the genetics and environmental stress that are strain-to-strain variable from year to year.

## CONCLUSION

The results of this study show the potential of the *Bacillus* strains used herein to be developed for industrial amylase production. Under alkaline conditions and varying temperature ranges, these isolates are able to produce amylase, making them eligible candidates for their utilization in the industrial sectors such as food, bioethanol production, and pharmaceuticals. The resilience and adaptability of the isolates toward extreme environmental conditions have further contributed to their industrial applicability.

## ACKNOWLEDGEMENT

I would like to express my sincere gratitude to Kohat University of Science and Technology (KUST) for providing the necessary facilities and resources that enabled me to conduct this research

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**OD:** Optical density; **API:** Analytical Profile Index; **PCI:** Phenol-Chloroform Isoamyl Alcohol; **DNA:** Deoxyribonucleic acid; **ABI:** Advance Bioscience International's; **BLAST:** Basic Local Alignment Search Tool; **PCR:** Polymerase Chain Reaction; **ONGP:** Ortho-Nitrophenyl-β-galactoside; **GLU:** Glucose tolerance; **ARA:** Arabinose; **LDC:** Lysine decarboxylase; **ODC:** Ornithine decarboxylase; **CIT:** Citrate; **$H_2S$:** Hydrogen sulfide; **URE:** Urease; **OX:** Oxidase.

## ETHICAL APPROVAL

Ethical approval was obtained from KUST ethical review committee.

## FUTURE RECOMMENDATIONS

Further work will consist of optimization of the substrate composition, scaling-up enzyme production in bioreactors, and applying genetic engineering techniques with the intention to increase yield and specificity of the enzyme. The proteomic and transcriptomic profiles of these strains have the potential to introduce new pathways for amylase synthesis, thus opening the way to more efficient and less costly processes for enzyme production.

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