doi.org/10.67099/ijbahr2026.1.1.7
Vol. 2 (1) (2026) 143-148

IJBAHR

DETECTION OF INDUSTRIALLY IMPORTANT HYDROLYTIC ENZYMES FROM THE INDIGENOUSLY ISOLATED STRAINS OF *BACILLUS LICHENIFORMIS*

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Abstract: White biotechnology has benefited greatly from the employment of highly specific enzymes in a range of industrial processes. The choice of robust bacterial strains with the appropriate enzymatic profiles is critical for commercial viability. The present study was undertaken for isolation, identification and screening of native bacterial strains for production of industrially important hydrolytic enzymes. Morphological, and biochemical characterization has revealed five novel isolates of Bacillus licheniformis. These isolates showed a wide physiological versatility, such as growth at the temperature range of 30.0-55.0°C and resistance to high salinity (2.0-10.0%). All isolates produced significant extracellular amounts of main hydrolytic enzymes; protease, α-amylase, glucoamylase, cellulase and pectinase with varying titers. Quantitative enzyme assays revealed protease activity ranging from 45.2-78.6 U/mL, amylase activity from 32.5-65.3 U/mL, and cellulase activity from 18.7-42.1 U/mL. These strains demonstrate great potential as candidates for commercial enzyme production under standardized fermentation conditions.

Key words: Indigenous bacteria, *Bacillus licheniformis*, Amylase, Protease, Cellulase, Pectinase, Enzyme screening

Submitted 18-04-2026, Revised 17-07-2026, Accepted 23-07-2026

INTRODUCTION

Bacteria play an essential role in environmental management, maintaining ecosystem health and facilitating biogeochemical cycles. They are integral to nutrient cycling, organic matter decomposition, and environmental remediation processes such as bioremediation of contaminated sites. Beyond their ecological contributions, bacteria are indispensable in biotechnology, where they are used for the synthesis of biofuels, bioplastics, enzymes, and other valuable metabolites (Gomes et al., 2014). In agriculture, rhizobacteria enhance soil fertility by solubilizing essential nutrients (e.g., phosphorus, potassium, zinc). They also produce phytohormones like indole-3-acetic acid (IAA) and gibberellins, which improve plant resilience under abiotic stresses (Vejan et al., 2016; Singh et al., 2021). Microbes also plays their role as biocontrol agents and suppress plant pathogens and also reduces consumption of chemical pesticides (Migunova and Sasanelli, 2021). The industrial demand for robust, efficient, and specific enzymes continues to grow. Microbial enzymes, particularly those from extremophiles, offer advantages such as thermostability, halotolerance, and activity under a wide pH range. Enzymes like proteases, amylases, cellulases, and pectinases are widely used in food processing, biofuel production, textiles, detergents, and pharmaceuticals (Danilova and Sharipova, 2020; Abdella and Ahmed, 2025).

This research focuses on the isolation, identification, and enzymatic screening of indigenous Bacillus licheniformis strains, with the aim of identifying promising candidates for industrial enzyme production

MATERIALS AND METHODS

Isolation of Bacteria

A soil sample (1.0 g) collected from a vegetative field was aseptically suspended in 100 mL of sterile distilled water. Serial dilutions were performed up to 10^{10}. Aliquots from $10^{6}$and 10^{7} dilutions were spread onto nutrient agar plates using a sterile L-shaped glass rod. Plates were incubated at 37°C for 24-48 hours. Isolated colonies were purified by repeated streaking and maintained on agar slants under sterile mineral oil at 4°C for long-term storage.

Morphological and Biochemical Characterization:

Colonial morphology (shape, size, color, texture) and microscopic characteristics (Gram staining, cell shape, spore formation) were recorded following standard protocols (Cappuccino and Sherman, 2014). Biochemical tests including catalase, oxidase, Voges-Proskauer (VP), citrate utilization, starch hydrolysis, nitrate reduction, indole production, urease, gelatin hydrolysis, and sugar fermentation were performed according to Bergey's Manual of Systematic Bacteriology.

Screening for Enzyme Production

Qualitative Screening

All isolates were screened for extracellular enzyme production on specific agar media:

α-Amylase: Isolates were grown on starch agar (0.5% starch, 1.5% agar) at 37°C for 24 hours. Plates were flooded with Lugol's iodine solution; clear zones indicated amylolytic activity.

Protease: Casein agar (0.1% casein, 1.5% agar) plates were inoculated and incubated at 37°C for 24 hours. Clear zones were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 and destaining with 40% methanol and 10% acetic acid.

Pectinase: Isolates were cultured on pectin agar (0.25% pectin, 1.5% agar) at 37°C for 24 hours. Activity zones were detected using iodine-potassium iodide solution.

Cellulase: Carboxymethyl cellulose (CMC) agar (0.5% CMC, 1.5% agar) was used, and Congo red staining (0.1% w/v) was applied to visualize hydrolysis zones. Plates were destained with 1 M NaCl.

Quantitative Enzyme Assays

For quantitative estimation, isolates were grown in specific production media at 37°C for 48 hours with shaking (150 rpm). Cell-free supernatants were obtained by centrifugation at 10,000 × g for 15 minutes at 4°C.

Protease Activity: Determined using the Anson method with casein as substrate. One unit (U) of protease activity was defined as the amount of enzyme releasing 1 µmol of tyrosine equivalents per minute under assay conditions.

α-Amylase Activity: Measured using the dinitrosalicylic acid (DNS) method with soluble starch as substrate. One unit (U) of amylase was defined as the amount of enzyme releasing 1 µmol of reducing sugars (maltose equivalents) per minute.

Cellulase Activity: Assayed using the DNS method with CMC as substrate. One unit (U) was defined as the amount of enzyme releasing 1 µmol of glucose equivalents per minute.

Pectinase Activity: Determined using the DNS method with pectin as substrate. One unit (U) was defined as the amount of enzyme releasing 1 µmol of galacturonic acid equivalents per minute.

Statistical Analysis

All experiments were conducted in triplicate (n=3). Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed using GraphPad Prism version 9.0 to determine significant differences between isolates. A p-value < 0.05 was considered statistically significant.

RESULT AND DISCUSSION

Isolation and Identification

Five bacterial isolates (designated A-E) were obtained from the soil sample. These bacterial isolates were identified as Bacillus licheniformis based on morphological and biochemical analyses in table 1. Based on morphological and biochemical all isolates were identified as *Bacillus licheniformis.* All isolates were Gram-positive, rod-shaped (2-3 µm × 0.5-1.0 µm), spore-forming, and capable of growth under aerobic and facultative anaerobic conditions. Colonies were large, irregular, creamy-white, and mucoid on nutrient agar. Endospores were oval and located centrally to sub-terminally. They exhibited broad physiological adaptability, thriving at temperatures between 30-55°C and salinity levels of 2–10%.

Higher Growth Temperature and Tolerance

B. licheniformis is closely related to B. subtilis but can be differentiated by its ability to grow at higher temperatures (55°C) and specific metabolic profiles. Bacillus licheniformis is distinguished from Bacillus subtilis by a more versatile and thermophilic metabolism. B. licheniformis can grow at temperatures up to 55°C–60°C, whereas B. subtilis is generally mesophilic. It also displays higher tolerance to high salt concentrations.

Biochemical Characterisation

The biochemical profiles of the isolates are presented in Table 1. All five isolates were positive for catalase, Voges-Proskauer (VP), citrate utilization, starch hydrolysis, nitrate reduction, gelatin hydrolysis, and acid production from glucose, sucrose, maltose, and lactose. Variable results were observed for oxidase, indole production, and urease. All isolates grew at temperatures ranging from 30-55°C and in NaCl concentrations up to 10%. Growth was not observed at 10°C or 65°C, or at pH 5.7.

Physiological Versatility

All isolates demonstrated broad physiological adaptability (Table 1). Growth was observed at temperatures between 30-55°C, with optimal growth at 37-40°C. The isolates showed tolerance to NaCl concentrations ranging from 2-10%, with optimal growth at 2-5% NaCl. This halotolerance is consistent with previous reports of B. licheniformis from diverse environments (Suleiman et al., 2020). Growth was observed at pH 7.0-8.0, with variable growth at pH 5.7.

Anaerobic Metabolism

B. licheniformis is better adapted to anaerobic growth, capable of using nitrate or fumarate as electron acceptors in anaerobic respiration, whereas B. subtilis typically only uses nitrate.

Metabolic Flexibility

B. licheniformis can grow by fermentation on both glucose and pyruvate, and it possesses the glyoxylate cycle (via the aceBA operon), allowing it to grow on substrates like acetate.

Specific Metabolites (Biosurfactants)

B. licheniformis produces the lipopeptide biosurfactant lichenysin, whereas B. subtilis typically produces surfactin**.**

Quantitative Enzyme Assays

Quantitative estimation of enzyme activity revealed significant inter-isolate variation Figure 1 and Figure 3). The enzyme activities (U/mL) performed as follows:

Enzyme Profile

All five isolates produced extracellular hydrolytic enzymes, as evidenced by clear zones of hydrolysis on specific substrate agar plates (Figure 2). The largest zones of

clearance were observed for protease, followed by α-amylase, with moderate zones for cellulase and pectinase. While both produce enzymes, *B. licheniformis* is known for producing more thermotolerant and alkaline-active extracellular enzymes, such as alpha amylase and proteases, which are stable at higher industrial temperatures and pH levels.

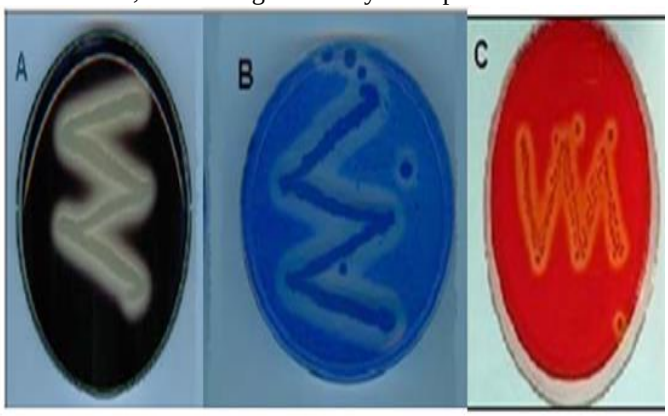
Carbon Utilization

B. licheniformis can utilize xylose to produce acid and is generally more efficient at fermenting a wider range of sugars (e.g., cellobiose) at high temperatures.

Aerobes or facultative anaerobes by nature, all of the isolates had a wide range of physiological characteristics, especially in relation to pH, salt content, and growth temperature. Recently, with the advancement of enzyme research, other sources of enzymes such as extremophile and psychrophilic microbes have drawn much attention to their potentials capable of optimally performing under extreme conditions or low temperature, respectively. For example, cold-active enzymes obtained from microorganisms adapted to cold environments may be superior in catalytic efficiency at low temperatures and hence adoptable for specific areas of food and biotechnological applications. Indeed, it has been discussed by (Kuddus *et al*., 2024). The normal habitats of lactic acid bacteria are in the gastrointestinal tracts of humans and animals. Due to their genetic safety and ability to form beneficial extracellular enzymes, to compete against pathogens, to form organic acids, and to produce bacteriocins with antagonistic properties, invasion, non-pathogenicity, and non-carcinogenicity, LAB has been one of the major choices for the development of probiotics (Bayhaqi and Insafitri, 2023). Apart from this, there has been a development of interest in the application of probiotics in agriculture. It is believed to promote microbial diversity and activity, increasing yields and thereby improving soil health; hence an effective ecosystem booster system is created (Bodke and Jogdand, 2022). Enzyme immobilization remains one of the critical methods toward enhancing stability and reusability of enzymes during industrial applications. Current research has proven that immobilization techniques can actually dramatically improve catalytic efficiency of enzymes and hence render them more applicable for use in food processing, medicines, and biofuels. Moreover, newly developed immobilization methods, like the utilization of MOFs, may protect the enzyme from environmental stresses and extend the period over which it could act (Huag *et al.,* 2020). Another developing trend in enzyme research enlists machine learning methods to predict enzyme kinetics and optimize activity under desired conditions. This is in agreement with (Li *et al.,* 2022). The other trend, now attracting high attention, is the study of extremophilic enzymes, mainly thermophilic and halophilic. Due to their stability and activity under extreme conditions, such enzymes have advantages in a number of industrial applications where highly active biocatalysts are in need. For example, the enzymes of halophilic origin were found highly stable and highly active in many biotechnological operations which include biosensing and bioremediation (Benítez-Mateos and Paradisi, 2023). Besides, identification and further description of unspecific peroxygenases have exemplified their versatility and further scopes regarding biotechnological applications by creating novel pathways of oxidative transformations in biocatalysis (Kinner *et al*., 2021).

Enzyme Screening

All five isolates produced extracellular hydrolytic enzymes, with varying efficiencies in Figure 1. Clear zones of hydrolysis were observed for α-amylase, protease, pectinase, and cellulase, confirming their enzymatic potential.



A. α-Amylase activity visualized on starch agar.; B. Protease activity on casein agar.; C. Pectinase activity on pectin agar

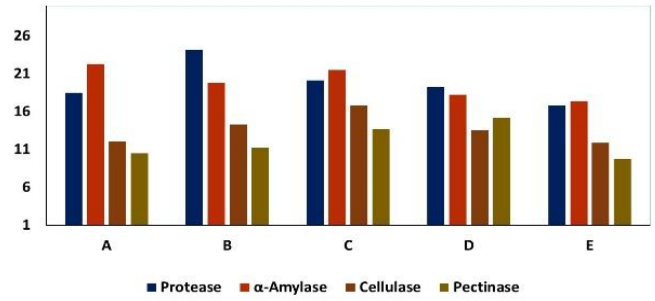


Figure 2: Qualitative Screening of Enzymes

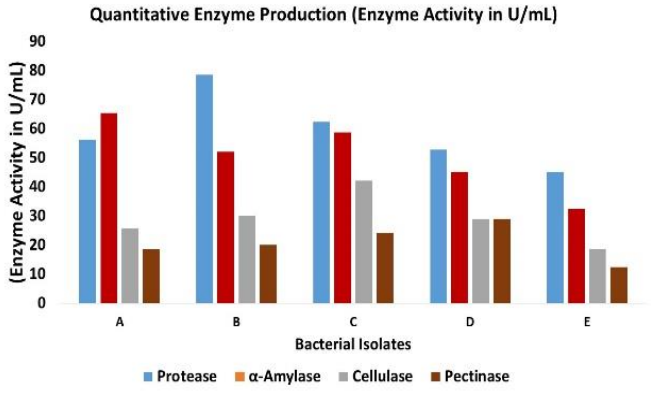


Figure 3: Quantitative Screening of Enzymes

Industrial Relevance of Enzymes α-Amylase

Amylase is essential in starch processing, biofuel production, and food industries. Microbial amylases are preferred for their stability and catalytic efficiency (Budhadev, 2023).

Protease

Used in detergents, leather processing, food processing, and pharmaceuticals. Microbial proteases account for a significant share of the global enzyme market (Tsado *et al*., 2021).

Pectinase & Cellulase

Important in fruit juice clarification, textile processing, biofuel production, and waste management. Cellulases are critical for lignocellulosic biomass conversion (Thite and Nerurkar, 2018).

Recent advances in enzyme immobilization, protein engineering, and the use of extremophilic enzymes have further expanded industrial applications (Maghraby *et al*., 2023; Benítez-Mateos and Paradisi, 2023). Machine learning approaches are also being employed to predict enzyme kinetics and optimize production conditions (Li *et al.*, 2022). Food enzymes find enormous applications in the food industry, including nutritional content, flavor, and texture improvement, among many others (Haile and Ayele, 2022). Applications of enzymes in food have been on the increase with consumers' demand for natural and clean-label products, since such goods are perceived by consumers to be much safer than their chemical additives (Yang *et al*., 2023). While it is also revolutionizing the industrial applications of the technique of enzyme immobilization, it improves stability and reusability. Immobilized enzymes are cost-effective for large-scale production since they can withstand adverse operational conditions and can easily be recovered and reused (Maghraby *et al.*, 2023; Cui *et al*., 2023).

It is very helpful when enzymes are used continuously in a number of industries, like the development of biofuels and biochemicals in bioprocessing and biomanufacturing (Cui *et al*, 2023). Because enzymes can be engineered for preferred industrial uses, the tailor-made biocatalysts for meeting particular demands were developed in the due course and improved the overall efficiency and sustainability of respective processes.

In addition to all those, enzyme technology also contributes much to the pharmaceutical field, especially to API synthesis. Enzyme cascade reactions utilize enzymes for the simplification of a part of the complex synthetic pathways that exclude many chemical steps; hence, it decreases the levels of waste. It is such a methodology that will enhance, in a more efficient manner, the synthesis of drugs, bringing along principles of green chemistry whereby the ecological footprint produced by pharmaceuticals is less harmful (Siedentop & Rosenthal, 2022)

Statistical analysis (one-way ANOVA) revealed significant differences in enzyme production among the isolates ($p < 0.05$). Isolate B demonstrated the highest overall enzyme production, while Isolate E showed relatively lower activity across all enzymes.

DISCUSSION

The present study successfully isolated and characterized five indigenous strains of Bacillus licheniformis with promising enzymatic potential. The identification was confirmed through a combination of morphological and biochemical approaches. The biochemical characterization provided additional support for species identification, with key differentiating features including growth at 55°C, nitrate reduction, and starch hydrolysis.

The five isolates demonstrated remarkable physiological versatility, with growth at temperatures up to 55°C and tolerance to NaCl concentrations up to 10%. This thermotolerance and halotolerance are consistent with the known characteristics of B. licheniformis and are particularly advantageous for industrial applications where high temperatures and salt concentrations may be encountered during processing (Danilova and Sharipova, 2020). The ability of *B. licheniformis* to grow at elevated temperatures distinguishes it from B. subtilis, which is typically mesophilic and unable to grow above 45-50°C (Priest et al., 1988). This thermotolerance is attributed to the production of heat-shock proteins and the stability of cellular membranes at high temperatures (Suleiman et al., 2020).

The enzyme screening results revealed that all five isolates produced significant quantities of extracellular hydrolytic enzymes, including protease, α-amylase, cellulase, and pectinase. The protease activities observed (45.2-78.6 U/mL) are comparable to those reported for other B. licheniformis strains (Kabir et al., 2020; Suleiman et al., 2020). The highest protease activity was observed in Isolate B (78.6 ± 3.2 U/mL), which may be attributed to genetic variation and the specific regulatory mechanisms controlling protease expression in this isolate. The α-amylase activity (32.5-65.3 U/mL) is similar to values reported by Das *et al*. (2019) for B. licheniformis strains from soil samples.

The production of multiple hydrolytic enzymes by these isolates is of significant industrial interest. The ability to secrete a cocktail of enzymes is advantageous for applications such as biomass degradation and the food processing industry (Thite and Nerurkar, 2018). The cellulase and pectinase activities observed, though lower than protease and amylase, are still significant and suggest the potential of these isolates for applications in biofuel production and fruit juice clarification (Haile and Ayele, 2022).

The inter-isolate variation in enzyme production observed in this study highlights the importance of strain selection for industrial applications. While all isolates showed enzymatic potential, the quantitative differences in enzyme titres suggest that certain isolates may be more suitable for specific applications. Isolate B, with its high protease activity, would be a promising candidate for detergent and leather industries, while Isolate A (high amylase) would be suitable for starch processing and biofuel production. This variation may be related to differences in the genetic makeup of the isolates, including variations in promoter regions and regulatory elements controlling enzyme expression

(Danilova and Sharipova, 2020). The results of this study demonstrate the potential of indigenous B. licheniformis strains as sources of industrially important enzymes. The isolation of multiple strains with varying enzymatic profiles provides a valuable resource for further optimisation and strain development. The next steps would involve optimisation of fermentation conditions (temperature, pH, aeration, carbon and nitrogen sources) to maximise enzyme production, as well as characterisation of the enzymes' biochemical properties (thermostability, pH optima, substrate specificity). Recent advances in enzyme immobilisation and protein engineering have further expanded the industrial applications of these enzymes, and future research could explore these strategies to enhance the stability and reusability of the enzymes produced by these strains (Maghraby *et al*., 2023; Cui *et al*., 2023).

CONCLUSION

Bacteria are indispensable in both natural ecosystems and industrial applications due to their metabolic versatility and adaptability. They play a foundational role in environmental processes, aiding in nutrient cycling, decomposition, and soil health, and are vital in bioremediation efforts, breaking down contaminants like hydrocarbons and toxic wastes in polluted environments. In agriculture, beneficial bacteria enhance plant growth by solubilizing nutrients, producing phytohormones, and acting as biocontrol agents against plant pathogens, reducing the need for chemical pesticides and contributing to sustainable farming practices. In industry, bacterial enzymes like amylase, protease, lipase, and cellulase are essential for their specificity and efficiency, catalyzing reactions under mild conditions that reduce energy and environmental impact. These enzymes are widely applied in sectors such as biofuel production, food processing, pharmaceuticals, and bioplastics. Technological advancements have further optimized these enzymes, enhancing stability through immobilization techniques and enabling applications under extreme conditions using extremophilic enzymes. As such, bacteria and their enzymes are integral to innovations in biotechnology, environmental sustainability, agriculture, and industrial efficiency, supporting a shift toward greener processes and sustainable economic growth.

Conflict of interest

Authors declare no conflict of interest**.**

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