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Production and Purification of Amylase Through Bacterial Strain

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Abstract

Amylases are important industrial enzymes with use in detergents, brewing, and food processing because of their ability to hydrolyze starch. In this research, the production and optimization of α-amylase from a potato soil isolated bacterium using agro-waste as a carbon source is investigated. The isolate had a hydrolytic zone of 13.33 mm and the highest activity of 545 U/mL under submerged fermentation with 1% starch. Optimum conditions were pH 7.0, 30°C, and 48 hours incubation with 1.25% ammonium sulphate. Ammonium sulphate precipitation and dialysis were used to purify the enzyme. The enzyme was stable, with more than 80% activity at pH 6.0–8.0 and 30–40°C, and residual activities of 91% and 84% at 50°C and 60°C, respectively. These results suggest its suitability for low-cost industrial application.

Keywords: Amylase, enzymes, bacterial strain, methyl red test, Voges–Proskauer (VP) tests

INTRODUCTION

Enzymes are significant proteins that function as biological catalysts, speeding up chemical reactions without being irrevocably changed. In contrast to customary chemical catalysts, which tend to work at extreme conditions like high temperature and pressure, enzymes work under gentle conditions of pH and temperature. This is less energy-intensive, avoids equipment corrosion, and does away with neutralization steps. In addition, enzymes provide high specificity, reducing unwanted byproduct formation and allowing for faster reactions relative to chemical catalysts.

Amylases are one of the most significant industrial enzymes, which catalyze the hydrolysis of starch into lower sugars. They find extensive applications in food, textiles, paper, pharmaceutical, and biofuel industries. Amylases occur naturally in plants, animals, fungi, yeast, bacteria, and actinomycetes. Microbial amylases have, however, replaced chemical hydrolysis of starch largely in industries because of their superior yield, lower cost of production, and the fact that microorganisms are easily engineered genetically to increase enzyme characteristics [1–5].

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Optimization of fermentation conditions is required to produce enzymes in maximum yield. The two widely used fermentation processes are submerged fermentation (SmF) and solid-state fermentation (SSF). SmF employs liquid substrates but is energy-intensive at high costs. SSF employs solid substrates, such as agricultural waste, with greater productivity at lower capital cost, lesser water requirement, minimal effluent generation, and easier product recovery. The most significant factors affecting SSF are substrate selection, particle size, moisture level, inoculum size, and microbial strain.

Crop residues, like paddy husk, wheat bran,

cassava peels, and potato peels, provide low-cost, renewable substrate for enzyme production. These microbial growth substrates and enzyme production substrates are agro-industrial wastes that make waste management sustainable and reduce the cost of production. Microbial enzymes saccharify these residues and transform complex carbohydrates into fermentable sugars for biofuel, animal feed, and other production.

Alpha-amylase is a major enzyme responsible for hydrolyzing starch's α-1,4-glycosidic bonds to yield maltodextrins, maltose, and glucose. Its high specificity and effectiveness have made it commonplace in industrial applications, such as starch liquefaction, brewing, textile treatment, production of sugar syrup, paper production, and pharmaceuticals. Alpha-amylase represents approximately 65% of the total market for enzymes globally and continues to expand based on its economic benefits. In comparison with microbial and animal and plant amylases, microbial alpha-amylases are more thermostable and resistant to acid and alkaline conditions and hence are useful for industrial applications. Thermostable amylases from thermophilic microbes are especially useful for thermophilic processes [5–9].

The microbial sources of alpha-amylase include Bacillus species (e.g., B. subtilis, B. cereus) and fungi (e.g., Aspergillus spp.). There is a continuous pursuit for identification of newer strains with better enzyme stability and activity to fulfill industrial requirements.

Amylases are divided into three groups based on their mode of action on starch: alpha-amylase, beta-amylase, and gamma-amylase. Alpha-amylase is activated in the presence of calcium ions and optimally active at neutral pH. Beta-amylase removes maltose units from starch nonreducing ends and occurs predominantly in seeds and a few microorganisms. Gamma-amylase breaks both α-1,4 and α-1,6 bonds and is most effective under acidic conditions.

Industrial production of amylase involves tight regulation of parameters like pH, temperature, incubation time, nutrient sources, and aeration. Optimal pH ranges from 6 to 7, and temperatures from 30°C to 40°C are conducive to enzyme production. Incubation times range from 48 to 72 hours. Carbon and nitrogen sources, like starch, glucose, and beef extract, support microbial growth and enzyme production, and sufficient aeration sustains microbial activity in SmF [10–15].

MATERIALS AND METHODS

Sample Collection

Soil was sampled from different sites in Lucknow, such as around R.K Timber and Vibhuti Khand. The samples were aseptically taken with the help of sterile instruments and kept in sterile containers to avoid any kind of contamination. These samples were the source of amylase-producing bacteria isolation.

Isolation of Bacteria by Serial Dilution

To determine amylase-producing bacterial isolates, a serial dilution method was used. Firstly, 0.5 g of soil sample was made to suspend in 9 mL of sterile normal saline solution (0.85% NaCl) and was shaken well to prepare the stock solution. Tenfold serial dilutions were made to 10^{-1}–10^{-10} by taking 1 mL from each dilution and transferring into 9 mL of sterile saline in a fresh test tube. 10 µl of each dilution was plated evenly on pH-adjusted nutrient agar plates at 11 to promote alkaliphilic bacteria growth. The plates were incubated at 37°C for 24 hours to enable colony formation. Individual colonies on the plates were chosen for further investigation [15–20].

Colony Morphology

The bacterial isolates in single colonies were described based on their morphological characteristics. Observation for colony shape (e.g., round, filamentous), margin (entire, undulate, lobate), elevation (flat, raised, convex), texture (smooth, rough, wrinkled), pigmentation (white,

cream, yellow), and opacity (transparent, opaque) were made. These features facilitated initial differentiation among the bacterial isolates.

Pure Culture Preparation

Single colonies with characteristic morphological features were streaked on nutrient agar plates with fresh medium by the quadrant streaking technique to acquire pure cultures. The plates were incubated at 37°C for 24 hours. Pure colonies obtained were stored on nutrient agar slants for further study.

Amylase Activity Assay (DNS Method)

Amylase enzyme activity was also assessed using the 3,5-dinitrosalicylic acid (DNS) method, in which the released reducing sugars from starch breakdown are quantitatively measured.

Crude enzyme preparations prepared from bacterial cultures were incubated with 1% soluble starch solution in phosphate buffer (pH 7.0) at 37°C for 30 minutes. The reaction was stopped by the addition of DNS reagent and then boiling for 5 minutes for color development. Upon cooling, the absorbance was recorded at 540 nm using a spectrophotometer. Maltose was employed as a calibration standard to construct the calibration curve, and enzyme activity was defined as units per milliliter.

Protein Estimation (Bradford Assay)

Bradford assay measured the total protein content in crude enzyme extracts. The colorimetric method depends on Coomassie Brilliant Blue dye binding to the protein molecules to produce a color change that can be measured. Standards and samples were combined with Bradford reagent and incubated for 10 minutes at room temperature. Absorbance was taken at 595 nm. BSA was employed to prepare the standard curve, whereby protein concentration in the enzyme samples could be calculated.

Effect of pH on Bacterial Growth

To find out the maximum pH for bacterial growth and amylase production, nutrient broth media were set to pH 3, 5, 7, and 11 using suitable buffers. All the flasks were inoculated with equal amounts of bacterial culture and incubated at 37°C under shaking conditions (150 rpm). Growth was recorded by taking optical density at 600 nm at 24 and 48 hours. The pH allowing maximum growth of bacteria was optimal.

Effect of Temperature on Bacterial Growth

The effect of temperature on bacterial growth was evaluated by incubating pure cultures on nutrient agar plates at four different temperatures: 22°C, 28°C, 37°C, and 50°C. Colony size, morphology, and growth rate were recorded after 24 and 48 hours. Optimal temperature was identified as the one supporting the fastest growth and largest colonies.

Optimization of Nitrogen Sources

The effect of different nitrogen sources in amylase production was examined by the supplementation of the basal medium with several nitrogenous compounds, such as peptone, beef extract, yeast extract, ammonium chloride, and urea. All the nitrogen sources were added at 1% (w/v). The cultures were incubated in an orbital shaker at 37°C, for 48 hours. After incubation, the activity of the enzyme was determined using the DNS method. The nitrogen source with the maximum amylase activity was chosen to be optimized further. The beef extract concentration was then varied between 0.5% and 1.5% to identify the optimal level for maximal enzyme production.

Fermentation and Enzyme Extraction

SmF in 500 mL Erlenmeyer flasks with optimized nutrient broth media inoculated with pure cultures of bacteria were carried out. Flasks were shaken on a rotary shaker at 37°C for 48 hours to promote enzyme production. After fermentation, cultures were centrifuged at 12,298 × g for 10 minutes at 4°C to obtain cell-free supernatant, which was the crude enzyme extract. Ammonium sulphate was added progressively to 40% saturation to precipitate the amylase enzyme and then dialyzed against phosphate buffer (pH 7.0) to remove salts and concentrate the enzyme preparation.

Biochemical Characterization

Gram stain was utilized to identify bacterial isolates as gram-positive or gram-negative according to cell wall features. Endospore staining was carried out to observe the formation of spores. Carbohydrate fermentation tests on glucose and fructose media were used to assess the ability of bacteria to ferment sugar with acid accumulation. The Methyl Red (MR) and Voges–Proskauer (VP) tests were performed to discern the metabolic processes of bacteria, with the former showing the formation of stable acid and the later to detect the formation of acetoin.

RESULT

The amylolytic bacterial strain applied in this research was efficiently isolated from soil samples taken under an agro-waste-dense environment. After screening for amylase production with starch agar plates, the isolate demonstrating the largest iodine-stained clear hydrolytic zone was chosen to pursue further. This reflected extensive extracellular amylase activity, and it was tested via the DNS method based on the determination of reducing sugars resulting from starch hydrolysis. Crude enzyme was treated with starch, and the reaction was terminated with DNS reagent. Absorbance was determined and enzyme activity was determined from a maltose standard curve (Figure 1).

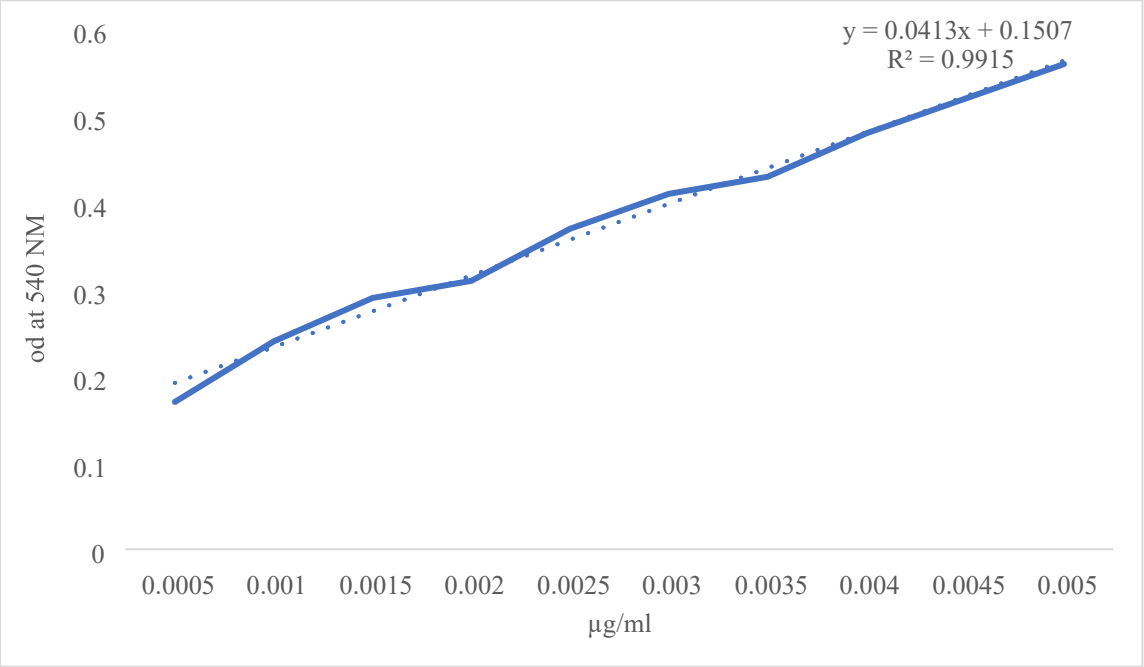

Figure 1. Standard curve of reducing sugar using the DNS assay.

The overall protein concentration, as determined by Bradford assay, reduced upon purification but the specific activity of the enzyme improved from 60.3 U/mg in crude extract to 89.6 U/mg after dialysis, showing efficient enrichment of amylase (Figure 2).

The production stage involved the optimization of some environmental and nutritional factors to maximize amylase yield, of various tested carbon sources, starch favored maximum enzyme production, which is consistent with the substrate-specificity of amylase. Nitrogen source optimization revealed beef extract to be most optimum with the maximum activity of amylase at a concentration of 1.25% (w/v), yielding 104.8 ± 4.5 U/mL. The ideal pH for maximum amylase production was found to be pH 7.0, where the enzyme activity reached 85.2 ± 3.4 U/mL. For temperature, 30°C yielded optimal enzyme production, with a peak activity of 92.7 ± 4.1 U/mL. Enzyme production declined significantly outside these optimal ranges, particularly under highly acidic or alkaline conditions and temperatures exceeding 40°C.

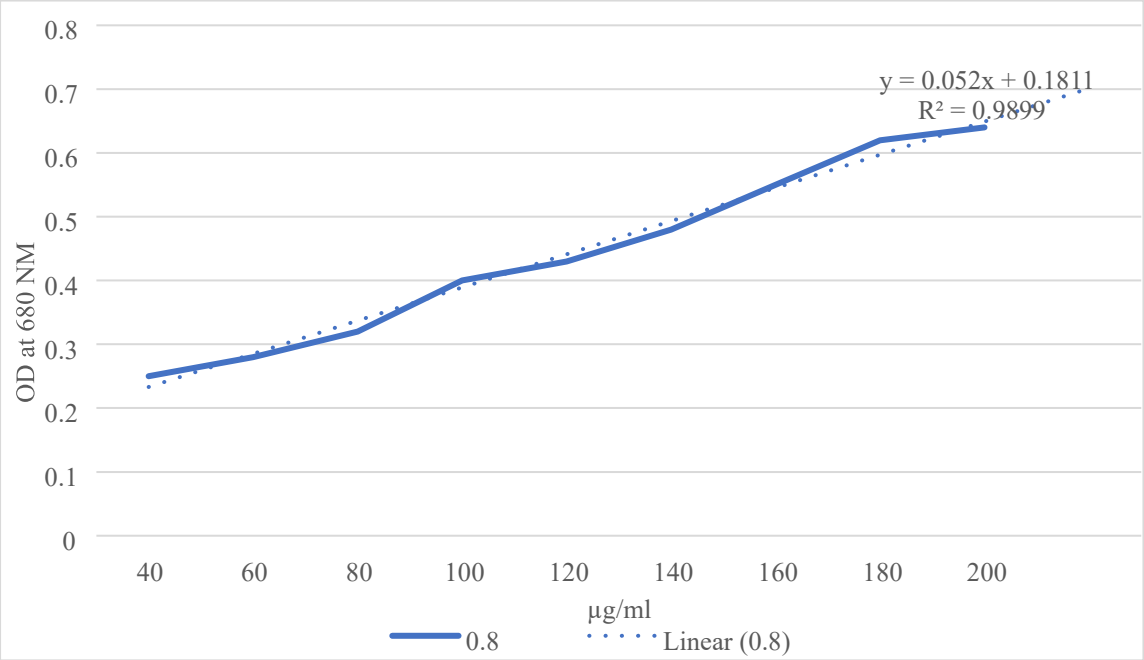

Figure 2. Total protein estimation by Bradford's assay. Bovine serum albumin (BSA) standard curve; and (B) Total protein content (mg/mL) in bacteria strain.

The isolate was morphologically described as a gram-positive, rod-shaped, and spore-forming bacterium, characteristic of the Bacillus species (Figures 3 and 4).

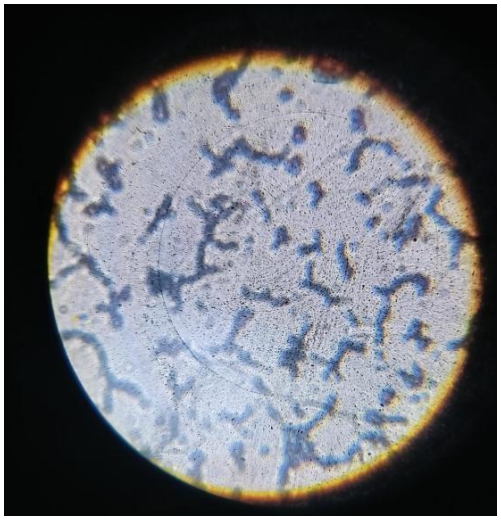

Figure 3. Sample possess gram positive.

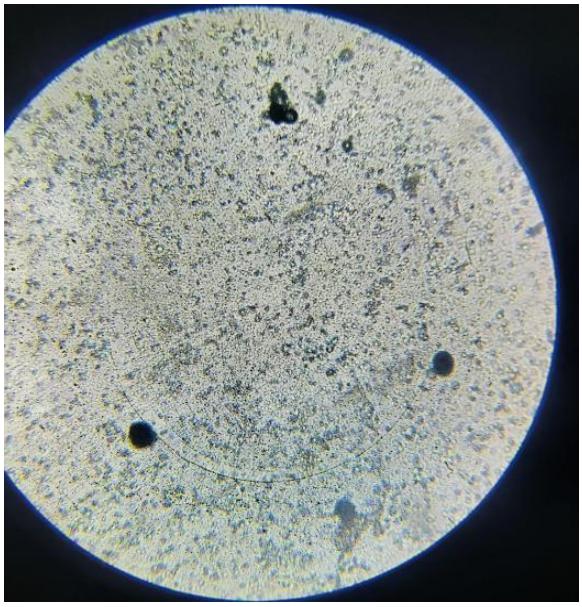

Figure 4. Sample produces spores.

Bacteria

This was further verified in biochemical tests, where the strain was positive for starch hydrolysis and MR and VP tests (Figures 5 and 6). The MR test determines bacteria form high acids from glucose. It turns red. They form stable acids that decrease the pH considerably. The VP test indicates whether bacteria form weak products from glucose. A red result indicates they form acetoin, a weak byproduct, rather than strong acid, useful in the identification of bacteria.

Figure 5. Strain is showing red color, which. indicates they form stable acids that decrease the pH considerably.

Figure 6. Strain is showing red color which indicates they form acetoin.

In addition, it fermented Glucose and Fructose effectively, further establishing its metabolic diversity and potential for industrial enzyme production. Glucose Fermentation test tube will be yellow and may bubble indicating acid or gas was released during fermentation. And the Fructose Fermentation test tube will be yellow and may bubble indicating that fermentation of fructose is taking place (Figures 7 and 8).

Figure 7. The test tube is yellow and bubble indicating acid was released during fermentation.

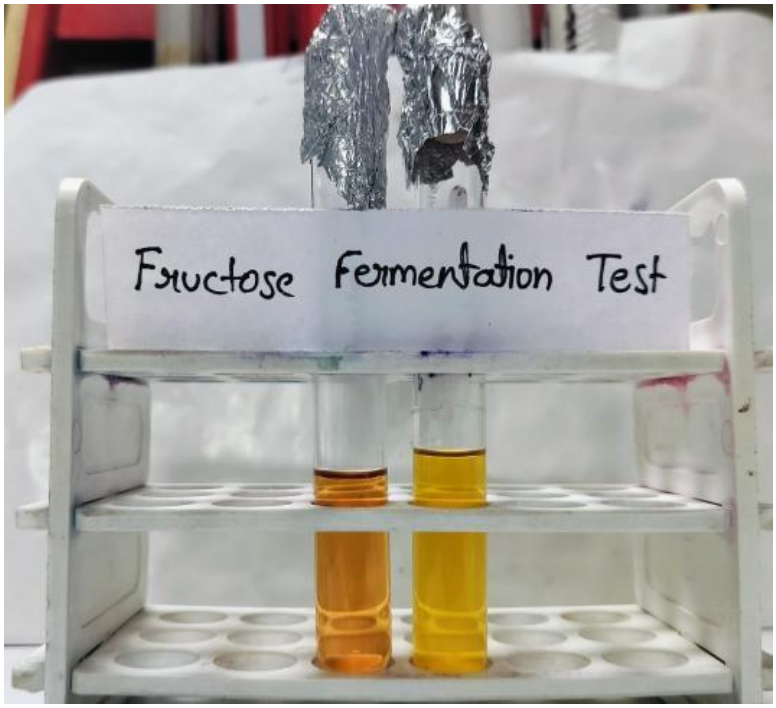

Figure 8. The test tube is yellow and bubble indicating that fermentation of fructose is taking place.

Raw enzyme was precipitated from the supernatant of culture and treated with ammonium sulphate precipitation and dialysis. Purification resulted in a tremendous rise in specific activity, attesting to the success of the partial purification. Stability of the enzyme was also evaluated to establish its commercial usability. The half-purified enzyme retained over 80% activity after 1-hour incubation at 30°C and retained high activity at a pH range of 6.0–8.0. Thermal stability assays demonstrated that the enzyme lost 75% of its original activity after 60 minutes at 40°C, expressing moderate heat tolerance. These results indicate that the amylase enzyme produced by the purified Bacillus strain is not only active but also stable.

DISCUSSION

The current research examined the production and purification of amylase from a soil isolate bacterial strain with an emphasis on the maximization of environmental and nutritional conditions for improved enzyme yield. The isolated bacterium was classified as a Bacillus species using morphological and biochemical tests and exhibited great potential for extracellular amylase production. Presence of a distinct hydrolysis zone on the starch agar plates following the use of iodine confirmed the enzymatic potential of the strain to hydrolyze starch and made it a potential candidate for large-scale production of amylase.

Optimization studies identified the optimum pH for maximum enzyme production as pH 7.0, which supports findings from previous literature stating that neutral pH supports the metabolic activity of Bacillus species. The enzyme also showed significant activity in a pH of 6.0–8.0, reflecting the degree of stability desirable for industrial processes, where pH variation is possible. The temperature optimum for amylase production was also 30°C, and enzyme stability was maintained up to 40°C. This moderate temperature tolerance is typical of mesophilic bacteria and implies the enzyme can operate effectively under generally applied industrial environments.

Nutrient optimization was equally important in this experiment. The use of starch as the main source of carbon was made, in harmony with amylase's natural substrate, resulting in high levels of enzyme activity. Beef extract was the best of the nitrogen sources that were tested, and especially at

1.25% concentration. This indicates that organic nitrogen sources with high amino acid and peptide content support the required nutrients for vigorous bacterial growth and enzyme production. The ideal balance of carbon and nitrogen not only optimizes enzyme production but also reduces the cost of production, thereby making the process more economically feasible to scale up.

Ammonium sulphate precipitation and dialysis partially purified the enzyme. These simple methods efficiently improved the enzyme's specific activity, establishing successful concentration and partial purification. While not the goal of complete purification, the outcome illustrated that even crude methods might yield a preparation with high enzymatic activity for further investigation or initial use.

Stability analyses under conditions of varying pH and temperatures indicated that the enzyme was resistant to retaining high activity under a broad array of operating conditions. This resistance is especially valuable in industrial applications where it is difficult to keep environmental controls strict. The capacity of the enzyme to be operative even under suboptimal conditions further contributes to its industrial value, boosting its potential application in industries including food processing, textiles, and biofuels.

One of the significant features of this research is the origin of the bacterial isolation. Soil samples obtained from agro-waste environments, potato field soil, were found to be rich in amylase producing bacteria. This points toward the value of agricultural waste not only as a pollutant but also as a source of bioactive compounds and industrially important microbes. The utilization of waste materials is in line with sustainability principles and supports the principle of circular bioeconomy.

This research demonstrates that enzyme production can be achieved using naturally occurring bacteria without the need for genetic modification or high-cost technologies. This is particularly beneficial for small-scale industries or institutions with limited resources. However, future studies could explore scale-up processes using bioreactors, assess the enzyme's shelf-life, and investigate immobilization techniques to improve reusability and operational stability.

CONCLUSIONS

This research effectively shows that potato soil, which is agricultural waste, may be used as a cheap and sustainable substrate to produce microbial amylase. The enzyme activity of the isolated bacterial strain was impressive, with a hydrolytic zone of 13.33 mm and with good stability in a broad temperature and pH range, retaining 91% activity at 50°C and 84% at 60°C. Optimization of fermentation conditions 1% starch, ammonium sulphate, 48 h at 30°C, and pH 7, increased enzyme yields further. Purified enzymes showed stability in function between pH 7 and 8 and temperatures ranging between 30 and 40°C, and it could be employed for industrial use. These observations point to the twin advantage of minimizing waste and cost of production along with participating in environmentally sustainable biotechnological processes and fitting into the objectives of a circular bioeconomy.

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