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BIOPROSPECTING AGRO-WASTE SOIL FOR HIGH-YIELDING PECTINOLYTIC BACILLUS SP.: SUBMERGED FERMENTATION, DOWNSTREAM SALTING-OUT, AND KINETIC PROFILING

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ABSTRACT

Microbial pectinases are industrially important biocatalysts widely utilized in agricultural waste valorization, fruit juice clarification, and industrial processing. The present investigation focused on the isolation, submerged fermentation, downstream purification, and substrate kinetics of extracellular pectinase produced by indigenous soil bacteria. Five different soil samples were collected and screened for pectinolytic activity using selective pectin agar media. Among the recovered bacterial cultures, isolate 2c, identified morphologically and biochemically as Bacillus sp., exhibited the most prominent clear zone of pectin hydrolysis and was selected for further experimental work. Production was carried out via submerged fermentation, followed by partial purification using ammonium sulfate fractionation. The catalytic response across five graded substrate concentrations was evaluated under a Completely Randomized Design (CRD). Kinetic characterization demonstrated a clear concentration-dependent profile, identifying 1.0% (w/v) citrus pectin as the optimal substrate concentration for maximizing enzyme activity, confirming isolate 2c as a promising microbial candidate for bioprocess applications.

KEYWORDS : Pectinase, Bacillus sp., Submerged Fermentation, Substrate Kinetics.

INTRODUCTION

Global industrial biotechnology is increasingly directed toward the valorization of renewable agricultural residues and lignocellulosic biomass (Antranikian, 1992). Among agro-industrial by-products, pectic polysaccharides constitute a primary structural component of primary plant cell walls and middle lamellae, functioning as intercellular cementing matrices (Pedrolli *et al.*, 2009). Pectin is a complex high-molecular-weight heteropolysaccharide consisting primarily of α-1,4-linked D-galacturonic acid residues, extensively esterified with methyl and acetyl moieties. Pectinolytic enzymes, collectively termed pectinases, catalyze the de-esterification and depolymerization of pectic substances through pectin methyl esterases, polygalacturonases, and pectate lyases (Arunachalam & Asha, 2010). Industrial pectinases represent approximately 10% of the global enzyme market, finding indispensable commercial applications in fruit juice clarification, wine depectinization, vegetable oil extraction, tea fermentation, coffee mucilage depulping, textile retting, and prebiotic oligosaccharide synthesis (Singh & Mandal, 2012).Although pectinolytic biocatalysts occur across diverse microbial taxa, bacterial extracellular pectinases produced by the genus Bacillus provide distinctive operational advantages over fungal enzymes, including neutral-to-alkaline pH stability, elevated thermal tolerance, and compatibility with submerged liquid bioprocessing (Kashyap *et al.*, 2003; Kumar *et al.*, 2012). However, high production costs and substrate saturation constraints remain key hurdles in commercial deployment (Joshi *et al.*, 2013). Consequently, bioprospecting indigenous soil environments for high-yielding, robust bacterial pectinase producers remains imperative (Venkata Naga Raju & Divakar, 2013).The present investigation was undertaken to isolate and characterize high-efficiency pectinolytic Bacillus species from agro-waste dumping sites, produce the extracellular enzyme via submerged fermentation, execute downstream fractional purification by ammonium sulfate salting-out, and evaluate the kinetic response of the purified biocatalyst across graded substrate concentrations under a Completely Randomized Design.

MATERIALS AND METHODS

Collection of Soil Samples

Five distinct soil samples were collected from agricultural waste and organic fruit-processing dumping sites in sterile polyethylene bags. Samples were collected from a depth of 5 to 10 cm, maintained at 4°C during transport to minimize unmonitored biological activity, and processed within 3 hours of collection.

Isolation and Screening of Pectinolytic Bacteria

Isolation was performed using selective Pectin Agar medium composed of: citrus pectin, 10.0 g/L; diammonium orthophosphate, 3.0 g/L; KH_2PO_4, 2.0 g/L; K_2HPO_4, 3.0 g/L; $MgSO47H_2O$, 0.1 g/L; agar-agar, 25.0 g/L; dissolved in distilled water with final pH adjusted to 5.6 ± 0.2. Ten grams of each soil sample were suspended in 90 mL of sterile physiological saline (0.9% w/v NaCl) and serially diluted to 10^5. Aliquots (0.1 mL) were surface-spread on Pectin Agar plates in duplicates and incubated at 37°C for 24–48 hours.

Colonies were flooded with Gram's iodine solution to visualize zones of clearance resulting from pectin depolymerization. Pectinolytic colonies exhibiting clear halozones were purified by spot inoculation onto fresh Pectin Agar plates. Halozone diameters were quantified in millimeters. The most efficient isolate, designated 2c, was characterized by macroscopic colony morphology and Gram staining.

Submerged Fermentation and Crude Enzyme Extraction

Inoculum of the selected isolate was prepared in Nutrient Broth and incubated overnight. Submerged liquid production was executed in 1000 mL Erlenmeyer flasks containing 500 mL of optimized Pectin Broth. Inoculated flasks were maintained in an orbital shaking incubator at 37°C and 150 rpm for 72 hours. Following fermentation, cell biomass was separated by refrigerated centrifugation at 10,000 rpm for 10 minutes at 4°C. The cell-free supernatant was harvested as crude extracellular enzyme and stored at 4°C for protein estimation and enzymatic assays.

Partial Purification by Ammonium Sulfate Fractionation

Crude enzyme supernatant was subjected to progressive fractional precipitation with analytical grade neutral ammonium sulfate $(NH_4)_2SO_4$ at saturation levels of 40%, 60%, and 80% at 4°C under continuous gentle stirring. Following overnight precipitation, suspensions were centrifuged at 10,000 rpm for 15 minutes at 4°C. The precipitated protein pellets were suspended in 0.1 M phosphate buffer (pH 7.0) and dialyzed against the same buffer using activated semi-permeable dialysis membranes for 24 hours with periodic buffer changes. The dialyzed fraction was evaluated for enzyme activity.

Enzyme Assay and Protein Determination

Pectinase activity was determined by measuring reducing sugars liberated from pectin using the 3,5-dinitrosalicylic acid (DNSA) colorimetric method. The reaction mixture contained 0.5 mL of enzyme preparation and 0.5 mL of 1% (w/v) citrus

pectin in 0.1 M acetate buffer (pH 6.0). After incubation at 40°C for 10 minutes, 1.0 mL of DNSA reagent was added, followed by boiling at 90°C for 5 minutes. The reaction was arrested by adding 1.0 mL of Rochelle's salt solution (sodium potassium tartrate), cooled, and optical density was recorded at 595 nm on a UV-VIS spectrophotometer. One unit of pectinase activity (U) was defined as the amount of enzyme liberating 1.0 μmol of reducing sugar equivalent per minute under standard assay conditions. Total protein was quantified by the Biuret method using Bovine Serum Albumin (BSA) as the standard at 530 nm.

Experimental Design and Evaluation of Substrate Kinetics

The kinetic effect of substrate concentration on pectinase activity was investigated using a Completely Randomized Design (CRD) with five treatment levels replicated thrice (n = 3): T1 (0.2%), T2 (0.4%), T3 (0.6%), T4 (0.8%), and T5 (1.0% w/v pectin). Dialyzed enzyme (0.5 mL) was incubated with 0.5 mL of the respective substrate concentration in 0.1 M acetate buffer (pH 6.0) at 40°C for 10 minutes, and catalytic activity (μM/min) was quantified.

RESULTS AND DISCUSSION

From five agricultural soil samples, five distinct bacterial isolates (1a, 3a, 2b, 2c, and 5d) were recovered. All isolates demonstrated clearance zones on Pectin Agar upon iodine flooding, confirming extracellular pectinase secretion. Isolate 2c exhibited the largest halozone diameter (21 mm), significantly exceeding isolates 1a (12 mm), 3a (10 mm), 2b (19 mm), and 5d (15 mm). Figure 1 and Figure 2 illustrate the primary screening plate and the conspicuous clearance zone of isolate 2c, respectively.

Colony characterization revealed that isolate 2c formed yellowish-white, circular, flat, opaque colonies with irregular margins on Pectin Agar. Gram staining and microscopic visualization confirmed Gram-positive, spore-forming, rod-shaped bacilli. Based on morphological and staining traits, isolate 2c was classified within the genus *Bacillus sp.*, consistent with previous reports identifying *Bacillus subtilis* and *Bacillus licheniformis* as predominant bacterial pectinase producers (Farooqui, 2012; Kashyap *et al.*,, 2003; Venkata Naga Raju & Divakar, 2013).

Submerged liquid fermentation of Bacillus sp. 2c in Pectin Broth yielded crude extracellular enzyme displaying an activity of 18.97 μM/min (OD at 595 nm = 0.132; total crude activity 4,530 U). Fractional precipitation with ammonium sulfate demonstrated progressive protein recovery. At 40% saturation, pellet activity reached 31.78 μM/min while the supernatant retained 91.13 μM/min. At 60% saturation, pellet activity rose to 62.81 μM/min (supernatant: 18.97 μM/min). At 80% saturation, pellet enzymatic activity peaked at 121.03 μM/min, with zero detectable activity in the supernatant, confirming complete precipitation of active pectinase.

The catalytic velocity of dialyzed pectinase across five graded substrate concentrations (0.2%–1.0% w/v citrus pectin) demonstrated a classic saturation kinetic response. At 0.2% pectin (T1), enzyme activity was 132.24 μM/min (OD = 0.920). Activity adjusted to 120.31 μM/min at 0.4% (T2), increased to 139.29 μM/min at 0.6% (T3), reached 135.12 μM/min at 0.8% (T4), and achieved maximum catalytic activity of 156.16 μM/min (OD = 1.092) at 1.0% pectin (T5). This confirms that 1.0% (w/v) substrate concentration is the optimum saturation threshold for the partially purified enzyme from Bacillus sp. 2c, mirroring reports by Darah *et al.*, (2013) and Kashyap *et al.*, (2003).

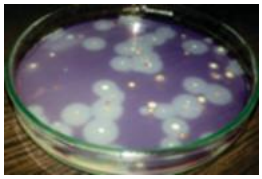


Figure 1 ***Primary Screening Plate***



Figure 2 Purified Bacterial Isolate 2c

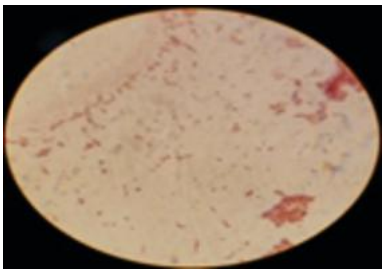


Figure 3 Microscopic Morphology of Isolate 2c



Figure 4 Dialysis Tubing Assembly

CONCLUSION

The present study successfully isolated a potent extracellular pectinase-producing bacterium, designated isolate 2c (identified as *Bacillus sp.*), from agricultural dumping soil. Submerged fermentation yielded high titers of crude pectinase, and fractional ammonium sulfate precipitation at 80% saturation completely recovered the active biocatalyst, elevating specific activity to 121.03 μM/min. Kinetic substrate characterization under a Completely Randomized Design demonstrated that the catalytic response was concentration-dependent, achieving maximum enzymatic velocity at 1.0% (w/v) citrus pectin (156.16 μM/min). The high catalytic efficiency and robust submerged production profile establish Bacillus sp. 2c as a viable microbial candidate for industrial pectinase production and agro-waste bioremediation.

REFERENCES

[1] Antranikian, G. (1992), "Microbial degradation of starch." Microbial Degradation of Natural Products, VCH Publishers, 2, 28–50.

[2] Arunachalam, C., and Asha, S. (2010), "Pectinolytic enzymes: An overview." Internet Journal of Microbiology, 9(2), 1–11.

[3] Darah, I., Taufiq, M. M. J., and Lim, S. H. (2013), "Pectinase production by Enterobacter aerogenes NBO2 using pomelo peel as substrate." Malaysian Journal of Microbiology, 9(3), 209–215.

[4] Joshi, V. K., Parmar, M., and Rana, N. (2013), "Pectinase production from apple pomace using submerged fermentation: Optimization of process parameters." Indian Journal of Natural Products and Resources, 4(1), 74–79.

[5] Kashyap, D. R., Chandra, S., Kaul, A., and Tewari, R. (2003), "Production, purification and characterization of pectinase from a Bacillus sp. DT7." World Journal of Microbiology and Biotechnology, SPRINGER, 19(3), 277–282.

[6] Kumar, M., Chand, P., and Garg, F. C. (2012), "Production and characterization of alkaline pectinase from a newly isolated Bacillus species." Journal of Applied and Natural Science, 4(2), 260–265.

[7] Pedrolli, D. B., Monteiro, A. C., Gomes, E., and Carmona, E. C. (2009), "Pectin and pectinases: Production, characterization and industrial application of microbial pectinolytic enzymes." The Open Biotechnology Journal, 3(1), 9–18.

[8] Semenova, M. V., Grishutin, S. G., Gusakov, A. V., Okunev, O. N., and Sinitsyn, A. P. (2006), "Isolation and properties of pectinases from the fungus Aspergillus japonicus." Biochemistry (Moscow), SPRINGER, 71(5), 560–569.

[9] Singh, S., and Mandal, S. (2012), "Pectinase: A review on its properties and applications in agro-industrial bioprocesses." International Journal of Pharma and Bio Sciences, 3(4), 488–498.

[10] Venkata Naga Raju, E., and Divakar, G. (2013), "Screening and isolation of pectinase producing bacteria from various waste dumping sites." International Journal of Research in Engineering and Technology, 2(10), 450–454.