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Research Paper

Optimization of Cellulase Production by *Aspergillusniger* (S4) Using Mango Peel Under Solid-State Fermentation: Enzyme Profiling and Purification.

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Abstract
A cellulase-producing fungal strain, Aspergillusniger S4, was isolated from lignocellulosic-rich environments in Rajasthan, India, and identified based on morphological and microscopic characteristics. Among 31 isolates, strain S4 demonstrated the highest cellulolytic activity, particularly through significant β-glucosidase production, a key enzyme for mitigating cellobiose inhibition during industrial saccharification. Mango peel, an abundant agro-industrial by-product, was employed as a cost-effective carbon source for solid-state fermentation (SSF), avoiding the need for energy-intensive pretreatments. While mild chemical pretreatment (1 N H_3PO_4, 1 N $NaHClO_3$) increased exoglucanase activity by 19.1%, it led to a reduction of 70.5% in endoglucanase activity. Optimal enzyme production was achieved under culture conditions of 30 °C, pH 5.5, and a substrate-to-moisture ratio of 1:10 on the 10th day of incubation, as determined using the one-factor-at-a-time (OFAT) approach. Enzyme purification through ammonium sulfate precipitation and Sephadex G-50 chromatography yielded β-glucosidase with an 83.27-fold increase in purity, highlighting its industrial potential. So, this study demonstrates that A. niger S4, coupled with mango peel as a substrate, offers a sustainable and efficient strategy for cellulase production, profiling, and purification with promising applications in biofuel and bioprocess industries.

Keywords: *Aspergillusniger S4, Cellulase production, β-glucosidase, Mango peel, Solid-state fermentation (SSF), Enzyme profiling and purification, Agro-industrial waste.*

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I. Introduction

The rapid pace of global industrialization has created an urgent need for cost-effective and sustainable strategies to meet the demands of diverse and expanding sectors. Industries such as automobiles, textiles, animal feed, detergents, paper, healthcare, food processing, wine production, and waste management increasingly depend on eco-friendly solutions that balance economic feasibility with environmental responsibility. Modern industrial practices now emphasize the adoption of enzyme-based catalysis, biodegradable raw materials, and processes that minimize ecological footprints. In this context, the utilization of lignocellulosic biomass (LCB)—a renewable, agro-industrial byproduct—has gained considerable attention due to its abundance, availability throughout the year, and potential for conversion into value-added products.

LCB is primarily composed of cellulose, hemicellulose, and lignin, with cellulose forming the bulk of its carbohydrate fraction. The enzymatic hydrolysis of cellulose, catalyzed by cellulase enzymes, generates fermentable sugars such as glucose, which serve as essential feedstocks for bioethanol and other bio-based products. Beyond their pivotal role in biofuel production, cellulases are extensively used in food, beverages, paper, textiles, and detergent industries, making them indispensable for sustainable industrial biotechnology. These enzymes, which include endoglucanases (EC 3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21), act synergistically to degrade cellulose into glucose. Their wide-ranging utility places cellulases among the top three industrially relevant enzymes, with bioethanol production representing the largest commercial application.

Despite their widespread demand, large-scale cellulase production faces a critical economic challenge, as the cost of enzyme production—particularly the substrate component—can account for nearly 50% of total hydrolysis expenses. Consequently, the use of low-cost, renewable lignocellulosic residues such as fruit-processing waste presents a promising strategy for reducing production costs while enabling sustainable enzyme generation. Among these, mango peel, an abundant byproduct of fruit processing industries, has emerged as a suitable substrate owing to its rich carbohydrate content and widespread availability in tropical countries like India.

Fungal species such as *Aspergillus*, *Trichoderma*, and *Fusarium* are widely recognized for their efficiency in cellulase production, with *Aspergillusniger* being particularly attractive due to its high enzyme yields, non-pathogenic nature, and ability to utilize diverse agro-industrial residues. In India, where cellulase demand is largely met through imports, domestic production using locally available resources is crucial to ensure cost-effectiveness and self-reliance in industrial biotechnology.

The present study focuse on optimizing cellulase production by *Aspergillusniger* (S4) using mango peel as the primary carbon source under solid-state fermentation (SSF). Process parameters including temperature, pH, substrate-to-moisture ratio, and incubation period were evaluated to maximize enzyme yield. The study also includes enzyme profiling, with a particular emphasis on β-glucosidase activity, and purification of the enzyme through ammonium sulfate precipitation and gel filtration chromatography to assess its industrial applicability. This work highlights the potential of mango peel as a sustainable agro-waste substrate and demonstrates the significance of *A. niger* S4 as a promising candidate for large-scale, cost-effective cellulase production.

II. Methodology

Sample Collection: Fifteen environmental samples were collected aseptically from diverse lignocellulosic degradation sites in the Ajmer district, Rajasthan, India, including compost heaps, decaying wood, wastepaper, and paper pulp residues. Samples were transferred in sterile polythene bags under refrigerated conditions (4°C) and processed within 24h to prevent microbial loss and contamination.

Isolation of Microorganisms: Each sample (5g) was suspended in 45mL sterile distilled water and serially diluted to 10^{-6}. Aliquots (0.1 mL) from appropriate dilutions were spread onto modified Mandels and Reese agar medium (composition in g/L: KH_2PO_4 2.0, $(NH_4)_2SO_4$ 1.4, urea 0.3, $MgSO_4 \cdot 7H_2O$ 0.3, $CaCl_2 \cdot 2H_2O$ 0.3, peptone 1.0, yeast extract 0.2, carboxymethyl cellulose (CMC) 10.0, agar 20.0; pH 5.0) supplemented with chloramphenicol (50 µg/mL) to suppress bacterial growth. Plates were incubated at 30 ± 2 °C for 4–7 days. Distinct fungal colonies were purified by repeated sub-culturing on potato dextrose agar (PDA).

Screening for Cellulolytic Activity: Primary screening was performed using the Congo red plate assay. Isolates grown on CMC agar plates at 30 °C for 4 days were flooded with 0.1% Congo red for 15 min, destained with 1 M NaCl, and observed for hydrolysis zones. The cellulolytic index (CI) was calculated as the ratio of hydrolysis zone to colony diameter. Secondary screening involved enrichment cultures in 1% CMC broth at 30 °C, 150 rpm for 5 days. Cellulase activity was quantified from culture supernatants using the filter paper assay (FPA).

Morphological and Microscopic Identification: The highest cellulase-producing isolate, designated S4, was characterized macroscopically on CzapekDox agar and PDA (colony texture, pigmentation, growth rate) and microscopically using lactophenol cotton blue (LPCB)-stained mounts examined under 40X and 100X magnifications. Morphological features including conidiophores, vesicles, conidial arrangement, and pigmentation were compared with standard identification keys. The isolate was confirmed as *Aspergillusniger*.

Enzyme Activity Assays (Preliminary Screening): The isolates were further assessed for their associated enzyme activities, including lipase, catalase, pectinase, and urease. Lipase activity was detected on tributyrin agar plates by observing clearance zones after 72 h of incubation, while catalase activity was confirmed by the formation of bubbles upon exposure to 3% H_2O_2. Pectinase activity was determined using citrus pectin agar plates, where hydrolysis zones were visualized with 1% CTAB, and urease activity was tested on Christensen's urea agar slants, indicated by a color change from yellow to pink at 30 °C. Enzymatic activity levels were evaluated semi-quantitatively and scored as absent (–), low (+), moderate (++), or high (+++).

Substrate Preparation and Pretreatment: Fresh mango peels were washed, shade-dried, and ground to \~1.2 mm particle size. Substrates were used either untreated or pretreated with 1 N H_3PO_4 or 1 N $NaClO_3$ (1:10 w/v, 2 h, 30 °C). Pretreated samples were washed to neutral pH and oven-dried (60 °C).

Solid-State Fermentation (SSF): SSF was carried out in 250 mL Erlenmeyer flasks containing 10 g mango peel supplemented with mineral salt medium. After autoclaving (121 °C, 15 psi, 20 min), the substrate was inoculated with 10% (v/w) spore suspension (10^6 spores/mL) of *A. niger* S4. Fermentation variables including pH, temperature, substrate-to-moisture ratio, and incubation time were varied according to One-Factor-At-a-Time (OFAT) or Response Surface Methodology (RSM) designs. After incubation, crude enzyme extract was obtained by adding 0.05 M citrate buffer (pH 4.8) at a 1:10 ratio, shaking at 150 rpm for 1 h, and centrifuging at 10,000 × g for 15 min.

Optimization (OFAT) **Studies:** Parameters including incubation time (4–14 days), temperature (25–40 °C), pH (4–7), and substrate-to-moisture ratio (1:5–1:10 w/v) were optimized individually.

Enzyme Assays: Enzyme activities were determined using standard assays. Total cellulase activity was measured by the Filter Paper Assay (FPA) in which Whatman No. 1 filter strips (1 × 6 cm) were incubated with crude enzyme extract at 50 °C for 60 min, and the released reducing sugars were quantified using the dinitrosalicylic acid (DNS) method. Endoglucanase (CMCase) activity was assayed using 1% carboxymethyl cellulose (CMC) as the substrate, while exoglucanase (Avicelase) activity was measured with 1% Avicel as the substrate; in both cases, the reducing sugars released were estimated by the DNS method. β-Glucosidase activity was determined using p-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate, incubated with the enzyme at 50 °C, and the liberated p-nitrophenol was quantified spectrophotometrically at 410 nm. One unit (IU) of activity was defined as the amount of enzyme releasing 1 µmol of product per minute under assay conditions.

Enzyme Purification: Crude extract was subjected to ammonium sulfate precipitation (0–80% saturation, overnight, 4 °C). The precipitate was recovered by centrifugation (8,500 × g, 20 min, 4 °C), dissolved in citrate buffer, and desalted on Sephadex G-50. Active fractions were further purified by Sephadex G-100 gel filtration. Protein concentration was determined by the Lowry method. Specific activity, fold purification, and recovery yield were calculated at each stage.

Statistical Analysis: All experiments were performed in triplicate. Results were expressed as mean ± standard deviation (SD). ANOVA was applied for statistical evaluation, with significance set at $p < 0.05$.

III. Results

Sample Collection and Isolation of Microorganisms: A total of fifteen environmental samples were collected from composting sites, decaying wood, and paper and paper pulp residues across Rajasthan, India. From these samples, 31 fungal isolates were obtained using modified Mandels and Reese agar medium followed by enrichment culture techniques. Among these, a single isolate designated S4 was selected for further studies based on its superior cellulolytic activity.

Morphological and Microscopic Identification: Isolate S4 displayed velvety colonies with pale yellow undersides and abundant black spores upon maturation (Fig. 1). On malt extract agar, the isolate grew rapidly, reaching 4–6 cm in diameter within 4–7 days at room temperature. Microscopic examination revealed large globose conidial heads, thick-walled conidiophores measuring 14–18 µm, globose vesicles of 50–80 µm, and dark brown sterigmata, confirming its identification as *Aspergillusniger.*

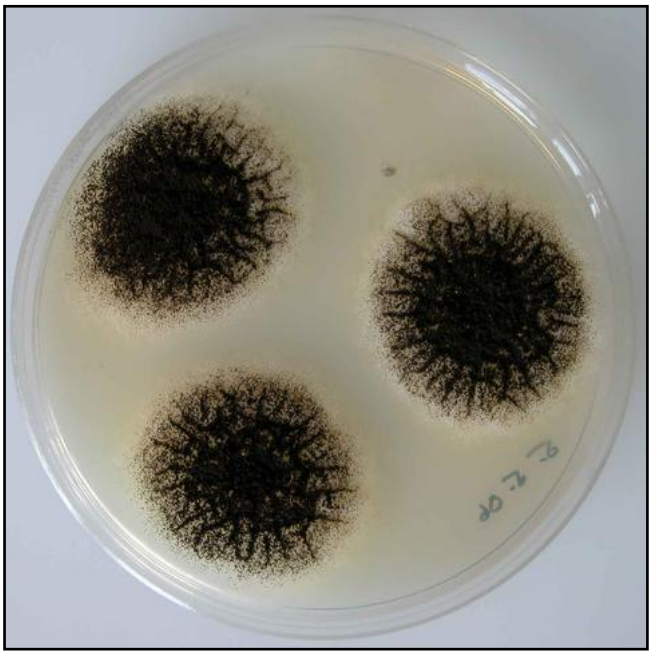

Fig.1: Colony characteristics of the isolate (S4) *Aspergillusniger* on Czapek agar and PDA

Enzyme Activity Profile: The enzymatic potential of isolate S4 was assessed to differentiate it from closely related species. All isolates produced catalase and urease to varying degrees; however, S4 demonstrated the highest levels of enzymatic activity across the panel (Table 1). The isolate exhibited strong lipase (+++), catalase (+++), pectinase (++++) and urease (++) activities, indicating a robust extracellular enzyme profile conducive to lignocellulose degradation.

Table 1. Enzymatic activity of selected isolates (– = no activity, + = low, ++ = moderate, +++ = high)

Isolate	Lipase	Catalase	Pectinase	Urease
S2	+	+	–	+
S4	+++	+++	++++	++
S5	–	++	+	+
S12	–	+	–	+

Pretreatment of Mango Peel: Cellulase production was highest using untreated mango peel as substrate. Pretreatment with 1 N H_3PO_4 or 1 N $NaClO_3$ slightly enhanced exoglucanase activity by 19.1% and 15.1%, respectively, but significantly reduced endoglucanase activity by 70.5%, suggesting that chemical pretreatments may generate inhibitory compounds affecting enzyme synthesis.

Optimization Using OFAT Method: Optimization of cellulase production using the One-Factor-At-a-Time (OFAT) method revealed that the incubation period, temperature, pH, and substrate-to-medium ratio significantly influenced enzyme yields. Maximum cellulase production was achieved on the 10th day of solid-state fermentation (SSF), consistent with earlier studies (Ahmed et al., 2010; Pervez, 2011). The optimal temperature for enzyme activity was found to be 30 °C on the 10th day, aligning with previous reports (Ahmed et al., 2010; Gautam et al., 2010). Enzyme activity was also affected by pH, with filter paper activity and exoglucanase reaching their peak at pH 5.5, whereas β-glucosidase showed maximum activity at pH 6.5. Overall, the enzymes remained active within a pH range of 4–5.5, reflecting the typical acidic nature of fungal cellulases. Additionally, a substrate-to-medium ratio of 1:10 (w/v) provided the highest cellulase activity using untreated mango peel with a particle size of 1.2 mm and a 10% inoculum. This ratio likely improved microbial access to the substrate by enhancing mass transfer efficiency within the solid matrix.

Purification of Cellulase: Crude enzyme extracts were concentrated via ammonium sulfate precipitation (0–80% saturation), yielding 73.49%, 30.29%, and 72.48% for endoglucanase, exoglucanase, and β-glucosidase, with fold purifications of 3.2, 1.8, and 2.67, respectively. Subsequent desalting and partial purification using Sephadex G-50 increased fold purification to 9.37 (endoglucanase), 8.39 (exoglucanase), and 83.27 (β-glucosidase), though yields decreased to 67.39%, 11.38%, and 65.27%, respectively. Final purification over Sephadex G-100 columns yielded specific activities of 32.9 IU/ml/min (endoglucanase), 20.39 IU/ml/min (exoglucanase), and 45.23 IU/ml/min (β-glucosidase) per mg protein (Table 3).

Table 3. Purification profile of cellulase enzymes from *A. niger* S4.

Step	Enzyme	Total Protein (mg)	Total Activity (IU)	Specific Activity (IU/mg)	Fold Purification	% Yield
Crude	Exoglucanase	790	273.98	1.74	0.0923	103.92
	Endoglucanase	746.29	4928.3	11.39	2.029	94.31
	β-Glucosidase	655.00	895.23	10.48	2.37	103.39
Ammonium Sulfate	Exoglucanase	134.23	737.32	5.24	1.843	30.29
	Endoglucanase	135.29	567.37	33.22	3.209	73.49
	β-Glucosidase	139.29	6924.37	64.37	2.67	72.48
GPC-50	Exoglucanase	17.39	474.24	22.49	8.39	11.39
	Endoglucanase	11.89	1145.62	98.48	9.37	67.39
	β-Glucosidase	16.32	1763.09	345.23	83.27	65.27
GPC-100	Exoglucanase	8.56	278.33	43.98	32.48	20.39
	Endoglucanase	6.21	1103.44	135.48	17.45	32.95
	β-Glucosidase	9.83	5746.65	842.93	36.58	45.24

IV. Discussion and Conclusion

The present study demonstrated the successful isolation, identification, and optimization of cellulase production by *Aspergillusniger* S4, a fungal strain obtained from lignocellulosic degradation sites in Ajmer, Rajasthan. Out of 31 isolates, S4 exhibited superior enzymatic capabilities, including strong activities of pectinase, catalase, urease, and lipase, in addition to cellulases. This enzyme diversity highlights the strain's ability to degrade complex plant biomass through a synergistic enzyme system, an essential attribute for industrial bioconversion of lignocellulosic substrates. Morphological characterization of the strain matched classical descriptions of *A. niger*, further validating its identity.

A significant finding of this study was the ability of S4 to effectively utilize untreated mango peel as a carbon source under solid-state fermentation (SSF). The use of untreated agro-industrial waste not only reduces the overall production cost but also circumvents the environmental burden of waste disposal. While chemical pretreatment with agents such as H_3PO_4 and $NaClO_3$ improved exoglucanase activity, it severely inhibited endoglucanase production. This suggests that pretreatment may generate inhibitory compounds that selectively affect enzyme biosynthesis, indicating that pretreatment is not always advantageous and substrate–enzyme interactions must be carefully evaluated.Optimization of culture conditions using the OFAT approach revealed that maximum cellulase production occurred on the 10th day of incubation at 30 °C, pH 5.5, and a substrate-to-medium ratio of 1:10 (w/v). These results are consistent with earlier studies reporting the preference of *A. niger* for slightly acidic pH and moderate moisture content during SSF. Such improvements underline the value of statistical modeling in refining bioprocesses to achieve industrially relevant enzyme yields.Purification studies further confirmed the industrial promise of S4-derived cellulases. Sequential ammonium sulfate precipitation and gel filtration chromatography achieved substantial fold purification, particularly for β-glucosidase, which exhibited a remarkable enrichment (83.27-fold). This is especially important since β-glucosidase activity is often a bottleneck in biomass saccharification due to product inhibition by cellobiose. The high activity and stability of purified β-glucosidase from S4 indicate its potential application in large-scale biomass conversion processes.

This study provides strong evidence that *A. niger* S4 is a robust cellulase producer with high potential for use in industries such as bioethanol production, animal feed, paper-pulp processing, and other biotechnological sectors. The co-production of accessory lignocellulolytic enzymes by the strain further enhances its value by enabling more efficient degradation of complex lignocellulosic matrices.

This research established *Aspergillusniger* S4 as a potent cellulase-producing strain capable of utilizing mango peel, an abundant and low-cost agro-industrial residue, under solid-state fermentation. The strain not only produced substantial amounts of cellulases without the need for pretreatment but also displayed high β-glucosidase activity, addressing a common limitation in industrial biomass saccharification. Optimization through OFAT enhanced enzyme production nearly five-fold, while purification strategies yielded highly enriched enzyme preparations suitable for industrial application.

The dual advantage of valorizing agro-waste and producing high-value enzymes underscores the sustainability and economic feasibility of this approach. Future studies should focus on process scale-up, enzyme cocktail formulation, and detailed characterization of oxidative cellulases potentially present in S4, which could further improve biomass hydrolysis efficiency. By integrating waste management with enzyme production, this work contributes toward greener biotechnological solutions aligned with circular economy principles.

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