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Research Paper

Enhancement of Cellulase Production Efficiency by Aspergillusniger (S4) Using Response Surface Methodology Analysis

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Abstract
A potent cellulase-producing strain, Aspergillusniger S4, was isolated from lignocellulosic-rich habitats in Ajmer, Rajasthan, India, and evaluated for its capacity to utilize mango peel, a low-cost agro-industrial by-product, as a substrate under solid-state fermentation (SSF). Preliminary optimization using the one-factor-at-a-time (OFAT) approach identified suitable baseline conditions for enzyme production. To further enhance cellulase yields, response surface methodology (RSM) was employed, enabling statistical modelling and interaction analysis of critical process parameters. RSM optimization resulted in a 1.33-fold increase in β-glucosidase activity and a 5.39-fold enhancement in endoglucanase production compared to unoptimized conditions. These improvements highlight the efficiency of RSM in fine-tuning fermentation processes, surpassing conventional approaches by accounting for synergistic effects among variables. The study demonstrates that integrating RSM into cellulase production strategies not only maximizes enzyme output but also establishes A. niger S4 as a sustainable biocatalyst for industrial applications such as bioethanol production, animal feed, and paper-pulp processing.

Keywords: *Aspergillusniger S4, Response Surface Methodology (RSM), Solid-state fermentation (SSF), Cellulase optimization and Mango peel.*

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I. Introduction

The rapid pace of global industrialization has increased the demand for sustainable, cost-effective technologies to support diverse sectors such as biofuels, textiles, paper, food processing, detergents, and pharmaceuticals. Enzyme-based biocatalysis has emerged as a key strategy in this context due to its biodegradability, energy efficiency, and eco-friendly nature. Among these biocatalysts, cellulases play a central role by converting lignocellulosic biomass (LCB)—a renewable and abundant agricultural byproduct—into fermentable sugars that serve as feedstocks for bioethanol and other value-added bioproducts. Beyond biofuel applications, cellulases are widely employed in the food, beverage, textile, paper, and detergent industries, making them the third most valuable group of industrial enzymes worldwide.

Despite their industrial importance, cellulase production remains economically challenging. Substrate costs account for nearly half of the total hydrolysis expense. Valorization of agro-industrial residues such as fruit processing waste provides an attractive solution by reducing production costs while supporting waste-to-value bioprocessing. Among filamentous fungi, *Aspergillusniger* is particularly favored due to its robustness, adaptability to solid-state fermentation (SSF), and high cellulase productivity.

Optimization of culture conditions is a critical step in improving enzyme yields. Traditional methods, such as the one-factor-at-a-time (OFAT) approach, are limited by their inability to capture interactions among variables, often leading to suboptimal results. In contrast, Response Surface Methodology (RSM) has gained recognition as a powerful statistical tool for process optimization. RSM not only evaluates the individual and combined effects of multiple factors but also identifies optimal conditions with fewer experiments, improving both accuracy and cost-efficiency.

The present study investigates the enhancement of cellulase production efficiency by *Aspergillusniger* S4 using mango peel as a low-cost lignocellulosic substrate under SSF. Special emphasis is placed on the application of RSM to optimize key parameters, including nitrogen sources, mineral salts, pH, and moisture levels, in order to maximize enzyme yield. By integrating waste valorization with statistical optimization, this work highlights a sustainable and economically viable strategy for large-scale cellulase production.

II. Methodology

Sample Collection and Isolation of Microorganisms

Fifteen environmental samples were collected from lignocellulosic degradation sites in Ajmer district, Rajasthan, India, including composting grounds, decaying wood, paper pulp residues, and wastepaper dumps. Samples were transported aseptically at 4 °C and processed within 24 h. Each sample (5g) was serially diluted and plated on modified Mandels and Reese agar medium containing carboxymethyl cellulose (CMC) as the sole carbon source and supplemented with chloramphenicol (50 µg/mL). Distinct fungal colonies were purified by repeated subculturing on potato dextrose agar (PDA).

Screening for Cellulolytic Activity

Primary cellulase screening was conducted using the Congo red assay on CMC agar, where hydrolysis zones were measured to calculate the cellulolytic index (CI). The most active isolates underwent secondary screening in CMC broth, and enzyme activity was quantified through the Filter Paper Assay (FPA). Among 31 isolates, the most efficient strain, designated S4, was selected. Morphological and microscopic examination confirmed its identity as *Aspergillusniger*.

Substrate Preparation

Mango peel waste was collected, shade-dried, milled to ~1.2 mm particle size, and used directly as a substrate for SSF. For fermentation, 10 g of mango peel was moistened with mineral salt medium, sterilized, and inoculated with *A. niger* S4 spore suspension (10^6 spores/mL) at 10% inoculum (v/w). After incubation under defined conditions, crude enzyme extracts were prepared by suspending fermented material in citrate buffer (pH 4.8), shaking at 150 rpm, and centrifuging at 10,000 × g for 15 min.

Optimization of Enzyme Production

One-Factor-at-a-Time (OFAT): Preliminary optimization of pH (4–7), temperature (25–40 °C), incubation period (4–14 days), and substrate-to-moisture ratio (1:5–1:10, w/v) was performed individually to determine approximate working ranges for enzyme production.

Response Surface Methodology (RSM): To enhance cellulase production efficiency, a Box–Behnken design was applied using Design Expert software. Independent variables included nitrogen sources ($(NH_4)_2SO_4$, $NaNO_3$, and peptone) and mineral salts ($MnSO_4$, $CoCl_2$, $CaCl_2$). Their effects on total cellulase activity (FPA), endoglucanase, exoglucanase, and β-glucosidase were evaluated. Experimental data were analyzed using analysis of variance (ANOVA) to develop predictive quadratic models. Model adequacy was confirmed by coefficients of determination (R^2) and lack-of-fit tests. Optimal conditions predicted by the model were validated through confirmatory experiments.

Enzyme Activity Assays

Cellulase activity was quantified using a set of standard assays to evaluate the synergistic action of different enzyme components. The total cellulase activity was determined through the Filter Paper Assay (FPA), in which Whatman No. 1 filter strips were incubated with enzyme extract at 50 °C for 60 min, and the released reducing sugars were estimated using the dinitrosalicylic acid (DNS) method. Endoglucanase (CMCase) activity was assessed using 1% carboxymethyl cellulose (CMC) as substrate, followed by quantification of reducing sugars with DNS. Exoglucanase (Avicelase) activity was measured with 1% Avicel as substrate and analyzed similarly via the DNS assay. β-Glucosidase activity was assayed using p-nitrophenyl-β-D-glucopyranoside (pNPG) as the substrate at 50 °C, with absorbance recorded at 410 nm. In all cases, one unit (IU) of enzymatic activity was defined as the amount of enzyme required to release 1 µmol of product per minute under the assay conditions.

Enzyme Purification

Crude enzymes were precipitated with ammonium sulfate (0–80% saturation), followed by desalting through Sephadex G-50 and gel filtration chromatography on Sephadex G-100. Fractions were assayed for activity, and protein concentration was determined by the Lowry method. Fold purification and recovery percentage were calculated.

Statistical Analysis
All experiments were performed in triplicate, and results expressed as mean ± SD. RSM models were validated by comparing predicted and experimental results, with significance determined at $p < 0.05$.

III. Results

Isolation and Identification of Cellulolytic Fungal Strain

From 15 collected samples (compost, decaying wood, paper, and pulp) in Ajmer, Rajasthan, a total of 31 fungal isolates were obtained using modified Mandels and Reese agar medium with enrichment culture. Among these, isolate **S4** was selected for its superior cellulase production. Morphological analysis confirmed the strain as *Aspergillusniger*, characterized by velvety colonies with pale yellow undersides and abundant black conidia. Microscopic features included globose conidial heads, thick-walled conidiophores (14–18 µm), and globose vesicles (50–80 µm) with dark brown sterigmata (Fig. 1).

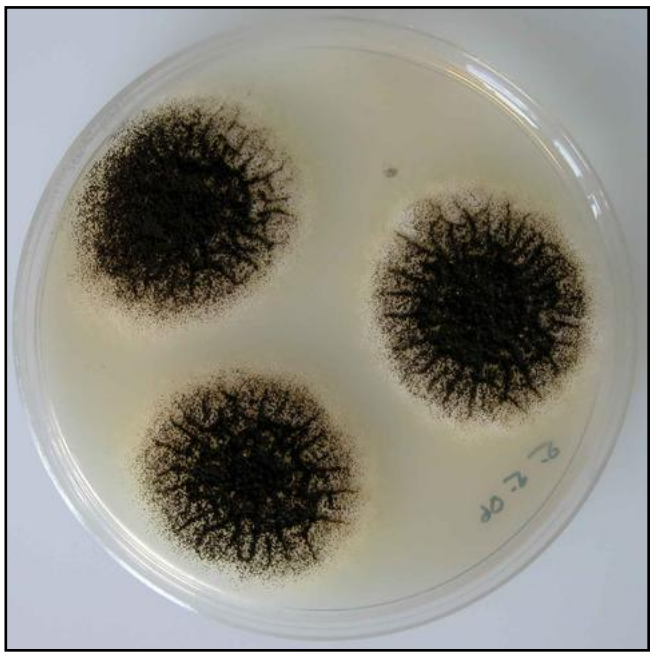

Fig.1: Colony characteristics of the isolate (S4) *Aspergillusniger* on Czapek agar and PDA

Preliminary Enzymatic Screening
All isolates produced catalase and urease with varying activity levels. Notably, S4 demonstrated **high catalytic activity** across multiple enzymes, including pectinase, highlighting its strong cellulolytic potential (Table 1).

Table 1: Enzymatic activity of the selected strains (Enzymatic activity is shown on a scale where '+++' represents the high, '++' is for moderate, '+' denotes poor but still tangible and '-' denotes no enzymatic activity)

Isolate No.	Lipase	Catalase	Pectinase	Urease
S2	+	+	-	+
S4	+++	+++	++++	++
S5	-	++	+	+
S12	-	+	-	+

Substrate Pretreatment Effects
Untreated mango peel was the most effective substrate for cellulase production. Acid (1 N H_3PO_4) and oxidant (1 N $NaHClO_3$) pretreatments slightly increased **exoglucanase activity** by 19.1% and 15.1%, respectively, but **endoglucanase activity decreased by 70.5%**, indicating that pretreatment generated inhibitory compounds and was unnecessary for this strain.

Optimization Using Response Surface Methodology (RSM)
RSM Design and Analysis
To maximize cellulase production, RSM with Box–Behnken design was employed, evaluating the combined effects of six medium components: ammonium sulfate, sodium nitrate, peptone, $MnSO_4$, $CaCl_2$, and $CoCl_2$.

Thirty experimental runs were conducted, and contour plots were generated to visualize the interaction between the most influential factors as identified by ANOVA.

Interactive Effects of Medium Components

The interactive effects of medium components on cellulase production were evaluated using overlaid contour plots, which indicated that the combination of ammonium sulfate and sodium nitrate had the most significant positive influence on enzyme yield. Based on RSM analysis, the optimal concentrations of the medium constituents were determined as 0.04 g of $(NH_4)_2SO_4$, 0.06 mg of $MnSO_4$, 0.01 g of $CaCl_2$, 0.09 mg of $CoCl_2$, 0.0232 g of $NaNO_3$, and 0.12 g of peptone. Experimental validation under these optimized conditions resulted in protein content of 51.75 mg/g, filter paper activity (FPA) of 192.58 IU/g/min, endoglucanase activity of 197.19 IU/g, exoglucanase activity of 29.73 IU/g, and β-glucosidase activity of 2613.86 IU/g (Table 2). The predicted values closely matched the experimental results, with only a 5.04% deviation, confirming the reliability of the RSM model. Interestingly, β-glucosidase activity exhibited a 1.33-fold increase, whereas endoglucanase and exoglucanaseactivities showed slight reductions, highlighting the differential effects of the optimized medium on the individual enzyme components.

Table 2: RSM experimental values for solid state fermentation for cellulose production under SSF by *A. Niger.*

S.No.	Protein (mg/g)	FPA	Endoglucanase (IU/g)	Exoglucanase (IU/g)	β-glucosidase (IU/g)
1	29.38	19.76	145.30	37.40	3507.72
2	39.17	166.06	228.33	43.01	3698.39
3	45.70	41.51	311.36	13.09	1285.40
4	45.87	41.51	124.54	44.88	2288.07
5	57.12	183.211	186.81	5.61	1867.28
6	21.22	539.68	394.38	33.66	3451.83
7	42.43	373.63	373.63	39.27	3438.68
8	101.18	394.38	83.03	22.44	2284.78
9	79.97	103.79	103.79	46.75	2320.95
10	55.49	62.27	20.76	11.22	1995.49
Average	51.75	192.58	197.19	29.73	2613.86

Enhancement of Cellulase Production

Under unoptimized conditions (30 ± 2 °C, pH 4.5, 1:3 substrate-to-medium ratio, 10% inoculum, 1.5 mm particle size, 10-day static incubation), cellulase production was limited. RSM optimization of medium constituents led to a **5.39-fold increase in endoglucanase activity** and an overall **4.96-fold enhancement in total cellulase production**. Further refinement of physicochemical parameters (temperature, pH, substrate-to-medium ratio) contributed an additional **2.47-fold increase** in enzyme yield.

The study demonstrated that **RSM-based optimization** significantly enhanced cellulase production by *A. niger* (S4), allowing precise determination of the optimal concentrations of multiple medium components and their interactions. The mango peel substrate, being native to the microorganism, required no pretreatment, indicating that S4 is naturally adapted to efficiently degrade lignocellulosic biomass. This RSM-guided strategy provides a reproducible and robust method for maximizing cellulase yields under solid-state fermentation.

IV. Discussion

The present study demonstrated the successful isolation and identification of a highly efficient cellulase-producing fungal strain, *Aspergillusniger* S4, from lignocellulosic habitats in Ajmer, Rajasthan, India. Out of 31 isolates screened, S4 showed superior enzymatic potential, producing a wide range of hydrolytic enzymes, including pectinase, catalase, urease, and lipase. This broad enzymatic repertoire indicates a strong lignocellulose-degrading capacity, further supported by its morphological characteristics, which were consistent with classical descriptions of *A. niger* (Klich, 2002).

Enzyme activity profiling confirmed that S4 could produce the full complement of cellulases necessary for effective cellulose hydrolysis. High β-glucosidase activity was particularly significant since this enzyme is often a limiting factor in biomass saccharification due to cellobiose accumulation and feedback inhibition (Singhania et al., 2013). The ability of S4 to utilize untreated mango peel as a substrate is noteworthy, as it bypasses the need for costly and energy-intensive pretreatment processes. Although chemical pretreatments improved exoglucanase activity slightly (up to 19.1%), they also resulted in a drastic reduction (70.5%) in endoglucanase activity, underscoring the complex effects of pretreatment-generated inhibitors on enzyme systems.

Initial optimization using the One-Factor-at-a-Time (OFAT) approach revealed that the 10th day of incubation, at 30 °C, pH 5.5, and a substrate-to-medium ratio of 1:10 (w/v), was optimal for cellulase production. These results are consistent with previous studies (Ahmed et al., 2010; Gautam et al., 2010) and reaffirm the preference of *A. niger* for slightly acidic and moist conditions in solid-state fermentation (SSF).

A major advancement in this study was the use of Response Surface Methodology (RSM) to optimize cellulase production efficiency. RSM allowed for the assessment of interactive effects between medium components, revealing that the combination of ammonium sulfate and sodium nitrate had the most significant impact on enzyme yields. The optimized medium—comprising 0.04 g of $(NH_4)_2SO_4$, 0.06 mg of $MnSO_4$, 0.01 g of $CaCl_2$, 0.09 mg of $CoCl_2$, 0.0232 g of $NaNO_3$, and 0.12 g of peptone—produced experimentally validated results closely matching the model predictions, with only a 5.04% deviation. Notably, β-glucosidase activity improved 1.33-fold under optimized conditions, while endoglucanase activity increased 5.39-fold compared to unoptimized conditions. This highlights the precision and reliability of RSM in enhancing enzyme yields through systematic medium optimization, which would not have been possible with OFAT alone.

Purification studies further demonstrated the industrial potential of the enzyme system. Ammonium sulfate precipitation followed by Sephadex gel filtration achieved significant fold purification across all cellulase components. β-glucosidase, in particular, exhibited an exceptionally high purification fold (83.27) after Sephadex G-50 treatment, suggesting structural stability and high affinity for the purification matrix—desirable traits for industrial biocatalysts.

Overall, the findings establish *A. niger* S4 as a promising candidate for cost-effective cellulase production using agro-industrial residues such as mango peel. This not only addresses waste management but also contributes to sustainable enzyme production. The integration of RSM proved critical in enhancing yields nearly five-fold, demonstrating its value as a robust statistical tool for process optimization. The dual advantage of valorizing agro-waste while generating high-value biocatalysts underscores the broader industrial significance of this study.

Future research should explore large-scale bioreactor trials, evaluate enzyme synergism in biomass degradation, and investigate the possible role of oxidative cellulases in S4. Such studies could further improve hydrolytic efficiency, thereby strengthening the role of *A. niger* S4 in bioethanol production, animal feed processing, and pulp and paper industries.

V. Conclusion

The present study highlights *Aspergillusniger* S4 as a highly efficient cellulase producer with significant potential for industrial applications. Isolated from lignocellulosic habitats, the strain demonstrated strong cellulolytic activity, particularly high β-glucosidase production, which is often a bottleneck in biomass saccharification. Its ability to utilize untreated mango peel as a substrate underscores the feasibility of converting low-cost agro-industrial residues into high-value biocatalysts, thereby addressing both enzyme production costs and waste management challenges.

Optimization using Response Surface Methodology (RSM) proved instrumental in enhancing cellulase yields, achieving nearly a five-fold improvement compared to unoptimized conditions. This statistical approach allowed for precise tuning of medium components and revealed synergistic effects that would have been overlooked by conventional OFAT methods. The purification of enzymes, especially the high fold enrichment of β-glucosidase, further confirmed their structural stability and industrial viability.

Collectively, these findings establish *A. niger* S4 as a promising and sustainable source of cellulases for applications in bioethanol production, animal feed, and the paper-pulp industry. The dual benefit of cost-effective enzyme production and agro-waste valorization strengthens its relevance in green biotechnology. Future efforts should focus on scaling up production, investigating enzyme synergism, and exploring oxidative cellulase activity to further improve biomass hydrolysis efficiency in integrated industrial processes.

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