



Original Article

Unique *Aspergillus niger* Strain Boosts Cellulase Production: Molecular Identification and Optimization of Fermentation conditions

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Cellulases are hydrolytic enzymes that degrade cellulose into simple sugars with significant industrial relevance. Fungal cellulases are preferred over bacterial ones due to their higher stability and adaptability. This study focused on isolating a cellulase-producing *Aspergillus niger* strain from Alagar Hills, Tamil Nadu, and optimizing conditions for enhanced enzyme production. Fungi were isolated using the standard plating technique and identified through biochemical characterization and 18S rRNA sequencing. Submerged fermentation was employed, and production parameters were optimized using a single-factor approach. An extracellular cellulase-producing *A. niger* strain was identified and deposited in GenBank (Accession No. MF921661). Optimal enzyme activity was achieved at pH 6.0 and 30 °C after 96 hours of incubation. Sucrose and peptone were the most effective carbon and nitrogen sources, while calcium and sodium ions further enhanced production. Among surfactants tested, Triton X-100 significantly increased cellulase yield. The isolated *A. niger* strain shows strong potential for industrial cellulase production. Further molecular and functional characterization will aid in exploring its large-scale applications.

Key Words: Cellulase, *Aspergillus niger*, Optimization

Introduction

Cellulases are a group of hydrolytic enzymes responsible for breaking down cellulose, the most abundant renewable polysaccharide, into simple sugars. These enzymes are mainly secreted by microorganisms during the degradation of plant-derived fibrous materials (Henriksson et al., 1999; Naher et al., 2021). Both bacteria and fungi are known to produce cellulases, but fungal species, particularly *Aspergillus* and *Trichoderma*, are often preferred for industrial applications because of their high enzyme yields and stability under diverse environmental conditions (Cherry & Fidantsef, 2003; Sethi et al., 2013; Gupta et al., 2015; Jayasekara & Ratnayake, 2019; Naher et al., 2021; Siva et al., 2022). The commercial importance of cellulases spans a wide range of industries. In the textile sector, they are employed in cotton softening and denim finishing; in detergents, they aid in fabric care and stain removal; in the food and beverage industry, they enhance processes such as brewing and juice clarification; and in pulp and paper manufacturing, they improve drainage and fiber modification (Islam & Roy, 2018; Jayasekara & Ratnayake, 2019). Additionally, cellulases have found applications in pharmaceuticals and in the conversion of lignocellulosic biomass to biofuels. This wide applicability has made cellulase production an area of extensive research and industrial interest (Sethi et al., 2013; Ellilä et al., 2017; Khadka et al., 2022). Despite their versatility, the cost of enzyme production remains a significant barrier to large-scale commercialization. Cellulase synthesis can account for nearly 40% of the total expense in bioethanol and biomass conversion processes. Consequently, reducing production costs through the development of optimized culture strategies and the use of inexpensive substrates has become a critical focus of current research (Milala et al., 2005).

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In developing countries such as Nigeria, cellulases are still largely imported, which increases production costs and hinders self-reliance in enzyme-dependent industries. The use of locally available agro-industrial residues provides an economical and sustainable alternative (Mrudula & Murugammal, 2011; Ezea et al., 2022).. Pap-processing residues, a by-product of traditional pap fermentation, are rich in cellulose and starch and represent a promising substrate for cellulase production. In addition to supporting cellulase synthesis, these residues have also been reported to promote the production of other metabolites, including citric acid (Ezea, 2022). Based on this background, the present study was designed to isolate cellulase-producing fungi and to optimize cellulase production using pap-processing waste under submerged fermentation. The findings aim to contribute toward developing a cost-effective strategy for cellulase production with potential applications in biotechnology and bioenergy.

Collection of Soil Samples

In this study, litter soil samples were collected from Shenbagathoppu Hills, Srivilliputtur, Tamil Nadu, India (9°33'04"N, 77°31'03"E). Approximately 250 g of soil was collected from each of ten different sites using sterile spatulas and transferred into 300 g sterile plastic containers pre-rinsed with 70% ethanol. Samples were transported to the Microbiology Laboratory within 24 hours for processing.



Figure 1. Location of Shenbagathoppu Hills (Srivilliputtur, Tamil Nadu, India). Source: Google Earth, 2025.

Isolation and Screening of Cellulolytic Fungi

Serial dilutions of the soil samples were prepared, and aliquots were spread-plated in duplicate onto Sabouraud Dextrose Agar (SDA) plates. The plates were incubated at 30 °C for 72 h, and distinct colonies were purified through repeated sub-culturing (Nair et al., 2008). To screen for cellulase activity, the isolates were streaked on Potato Dextrose Agar supplemented with 1% carboxymethyl cellulose (CMC) and incubated at 37 °C for 120 h. After incubation, the plates were flooded with 0.1% Congo red solution and destained with 1% NaCl. The appearance of clear hydrolysis zones around colonies indicated cellulase production.

Confirmation of Cellulolytic Strains

The most active cellulase-producing isolate was further confirmed by growth in enrichment broth containing cellulose (1.0%), beef extract (0.3%), peptone (0.5%), NaCl (0.5%), and glucose (0.5%) at pH 7.0. After 24 h of incubation, 10% of this culture was inoculated into 250 mL of cellulose medium (1%) in a 1 L Erlenmeyer flask and incubated for one week at 35 °C under shaking conditions (25 rpm). The culture broth was centrifuged at 10,000 × g for 15 min, and the cell-free supernatant was used for enzyme assays.

Cellulase Assay

Cellulase activity was determined using the dinitrosalicylic acid (DNS) method (Ghose et al., 1987). In brief, 0.5 mL of culture supernatant was mixed with 1 mL of 0.05 M citrate buffer (pH 4.8) and 0.5 mL of 1% CMC. The mixture was incubated at 50 °C for 30 min, after which 2 mL of DNS reagent was added. The tubes were boiled for 10 min at 90 °C, cooled rapidly, and absorbance was measured at 540 nm. Reducing sugars were quantified using glucose as a standard. One unit (U) of cellulase activity was defined as the amount of enzyme that releases 1 μmol of glucose equivalent per minute under assay conditions.

Morphological and Molecular Identification of Isolates

The fungal isolates were first examined for their morphological characteristics using lactophenol cotton blue staining (Makut et al., 2014). For molecular identification, genomic DNA was extracted following the method of Sambrook et al. (1989) with modifications. DNA was precipitated with 100% isopropanol at 4 °C overnight, pelleted by centrifugation, washed, and resuspended in TE buffer. DNA was also prepared using a Mo Bio Soil DNA Extraction Kit (Mo Bio Laboratories, USA) and stored at -20 °C until PCR analysis. The 18S rRNA gene was amplified using universal eukaryotic primers CDMF (5'-GTCAGAGGTGAAATTCTTGATTTA-3') and CDMR (5'-AAGGGCAGGGACGTAATCAACG-3'). PCR reactions contained 10–100 ng of template DNA, Taq DNA polymerase, and reaction buffers. Amplification was performed in an iCycler (Bio-Rad, USA) using the following program: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1

min, and extension at 74 °C for 1 min; followed by a final extension at 74 °C for 10 min. Sequencing of the PCR products was performed, and the sequences were edited and aligned using Sequencer software (Gene Codes Corporation, USA). BLAST analysis was used to compare sequences with those in the NCBI database. Sequences with $\geq 97\%$ similarity were considered the same phylotype. Phylogenetic trees were constructed using the neighbor-joining method, and the best nucleotide substitution models were selected using Modeltest v3.06 (Posada & Crandall, 1998).

Optimization of Culture Conditions for Cellulase Production

Optimization experiments were conducted under submerged fermentation conditions.

- **Carbon sources:** Different carbon sources (lactose, arabinose, sucrose, maltose, fructose, melibiose, starch, galactose, glucose) were tested individually at 0.5% in basal medium containing 1% CMC. Cultures were inoculated with 2% spore suspension (5.8×10^8 spores/mL) and incubated for 120 h.
- **Nitrogen sources:** Organic nitrogen sources (peptone, beef extract, yeast extract, casein, soy meal, urea, skim milk) and inorganic sources (sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate) were tested at 0.5% under the same conditions.
- **Salts and surfactants:** NaCl was tested at concentrations ranging from 0.5% to 5%. Surfactants, including Triton X-100, SDS, Tween-20, Tween-40, Tween-60, Tween-80, and polyethylene glycol (PEG), were added at 0.2%. Media without surfactants served as controls.
- **Hydrocarbons:** Hydrocarbons (hexadecane, tetradecane, nonane, dodecane, octane, paraffin liquid) were added individually at 0.5% in basal medium. The most effective hydrocarbons were further optimized at different concentrations.
- **Metal ions:** The effects of CaCl_2 , MgCl_2 , CuSO_4 , MgSO_4 , ZnSO_4 , HgCl_2 , BaCl_2 , FeCl_3 , and EDTA were studied by supplementing the optimized medium at 0.02%.
- **pH and temperature:** The effect of pH (3–9) was assessed by adjusting the basal medium with 0.1 N NaOH or HCl. To evaluate temperature, the cultures were incubated at 10–80 °C at pH 7.0.
- **Incubation time:** The effect of incubation time was tested using optimized medium with sampling at 24–168 h. Enzyme activity was determined at each time point.

Results

Isolation and Screening of Cellulase-Producing Fungi

Approximately 100 g of soil was collected from different locations in Alagar Hills, Madurai, using sterile containers. The samples were serially diluted, and aliquots from the 10^{-5} dilution were plated on agar medium. After incubation for 96–120 h, several morphologically distinct fungal colonies were recovered. Microscopic examination revealed that the hyphae were septate, measuring 2.5–8.0 μm in diameter. The conidiophores arose from specialized foot cells and were unbranched, terminating in swollen vesicles. These vesicles were partially or completely covered with flask-shaped phialides, which occurred either directly on the vesicle (uniseriate form) or on supporting cells known as metulae (biseriate form). The phialides produced chains of spherical to slightly rough-walled conidia, measuring 2–5 μm in diameter. Depending on the species, colony surfaces appeared green, brown, or white.

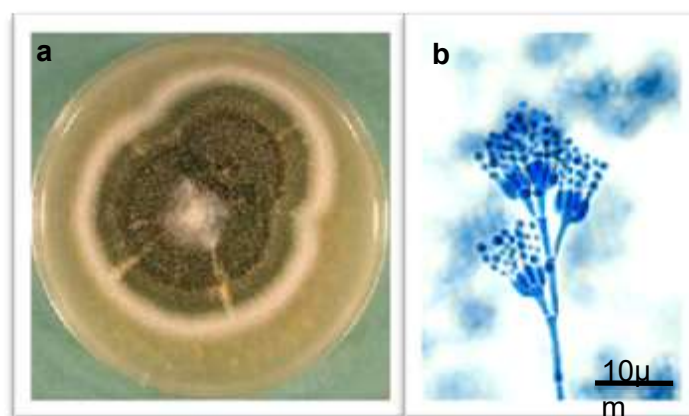


Fig.2. a) Colony morphology of *Aspergillus* sp. on PDA, b) Lactophenol Cotton Blue Staining of *Aspergillus* sp.

Identification of Cellulase-Degrading Colony

The fungal isolate showing the highest cellulolytic activity was first identified based on its morphological and physiological features (Fig. 2), which suggested that it belonged to the genus *Aspergillus*. To confirm its cellulase-producing ability, the isolate was streaked onto carboxymethyl cellulose (CMC) agar plates. After incubation, the formation of a distinct clearance zone around the growth indicated enzymatic hydrolysis of cellulose. On this basis, the strain was confirmed as a cellulase producer and selected for further experimental investigations.

Effect of Carbon and Nitrogen Sources on Cellulase Production

The choice of carbon source significantly influenced cellulase synthesis. Among the tested substrates, lactose-supported cultures yielded the highest enzyme activity, reaching 13.67 ± 0.67 IU/mL. In contrast, glucose supplementation resulted in markedly lower enzyme induction, with activity levels of only 3.33 ± 0.33 IU/mL (Fig. 3). These findings indicate that lactose serves as a strong inducer of cellulase production, whereas glucose exerts a repressive effect on enzyme synthesis.

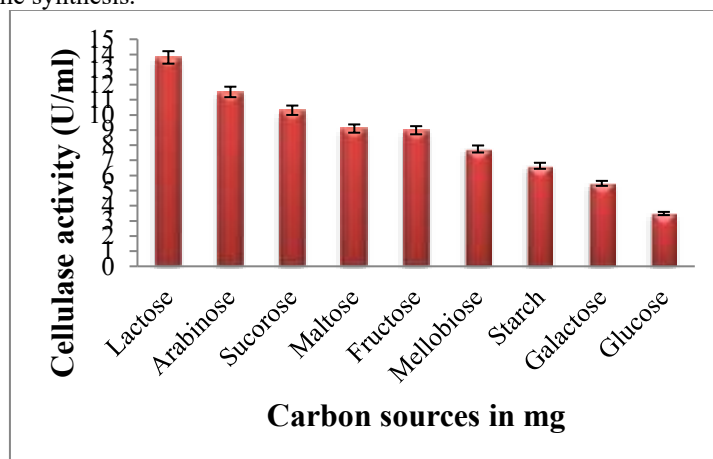


Fig. 3. Effect of carbon sources on cellulase production by *Aspergillus niger*.

The type of carbon source had a marked effect on cellulase synthesis. Lactose proved to be the most effective inducer, yielding maximum enzyme activity of 13.67 ± 0.67 IU/mL, whereas glucose strongly repressed cellulase production, with activity reduced to 3.33 ± 0.33 IU/mL (Fig. 3). Similarly, cellulase yield varied depending on the nitrogen source. Among the seven organic nitrogen sources tested, peptone supported the highest enzyme activity (12.23 ± 0.67 IU/mL) after 120 h of incubation (Fig. 4). In contrast, skim milk was the least effective, producing only minimal cellulase activity. These results indicate that complex organic substrates such as lactose and peptone provide favorable conditions for enzyme induction, while simple sugars and less utilizable nitrogen sources are poor inducers.

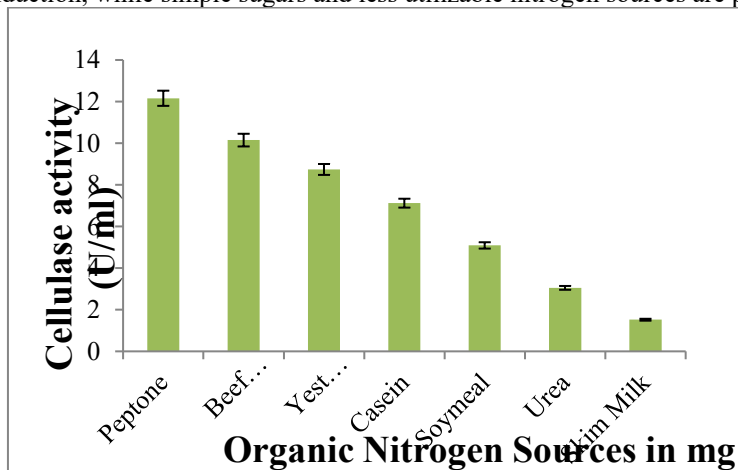


Fig. 4. Effect of organic nitrogen sources on Cellulase production by *Aspergillus niger*.

Effect of Inorganic Nitrogen Sources on Cellulase Production

The impact of different inorganic nitrogen sources on cellulase production by *Aspergillus niger* was investigated. Among the five tested sources, sodium nitrate supported a steady increase in enzyme production, reaching its peak after 120 hours of incubation. In contrast, ammonium sulfate led to a gradual decline in cellulase yield over the same period, indicating that the type of inorganic nitrogen plays a significant role in regulating enzyme synthesis (Fig. 5).

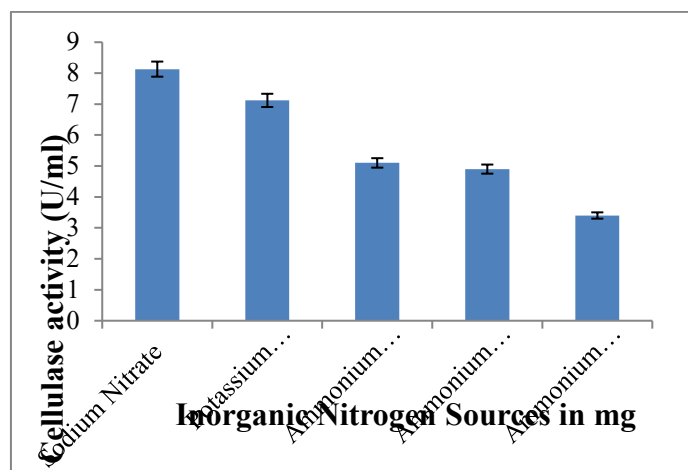


Fig. 5. Effect of inorganic nitrogen sources on Cellulase production by *Aspergillus niger*
Effect of NaCl Concentration on Cellulase Production

The impact of sodium chloride on cellulase synthesis by *Aspergillus niger* was examined by cultivating the fungus in media with varying NaCl concentrations. The highest cellulase activity was recorded at 1.5% NaCl, whereas further increases in salt concentration led to a progressive decline in enzyme production, with the lowest activity observed at 4.5% NaCl (Fig. 6). These results indicate that moderate salt levels may favor cellulase production, while higher salinity exerts an inhibitory effect.

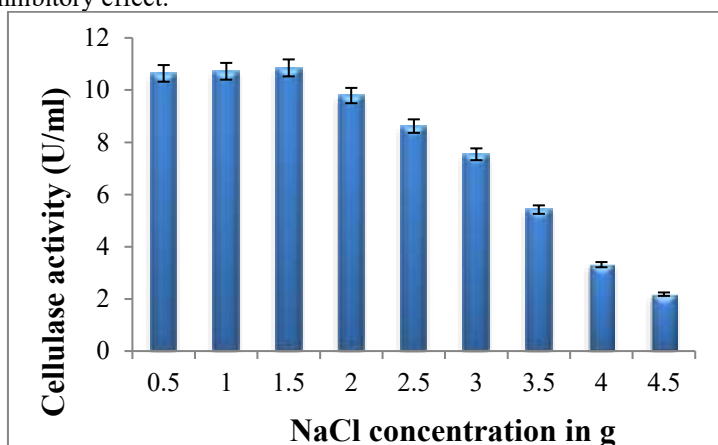


Fig. 6 Effect of NaCl concentration on Cellulase production by *Aspergillus niger*.
Effect of Surfactants on Cellulase Production:

The study examined how different surfactants influenced cellulase production by *Aspergillus niger* over 120 hours of incubation. Triton X-100 was found to enhance enzyme production the most, while Polyethylene Glycol (PEG) resulted in the lowest cellulase activity. These findings suggest that the type of surfactant plays a key role in modulating enzyme synthesis (Fig. 7).

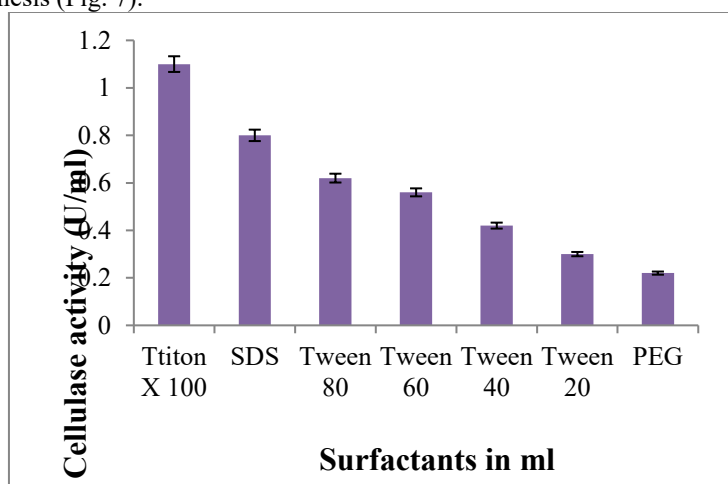


Fig. 7. Effect of surfactants on cellulase Production by *Aspergillus niger*.

Effect of Hydrocarbons on Cellulase Production

The influence of different hydrocarbons on cellulase production by *Aspergillus niger* was investigated over 120 hours of incubation. Among the six hydrocarbons tested, hexadecane supplementation led to the highest enzyme production, whereas nonane resulted in the lowest cellulase yield. These results indicate that the type of hydrocarbon present in the medium can significantly affect enzyme synthesis (Fig. 8)

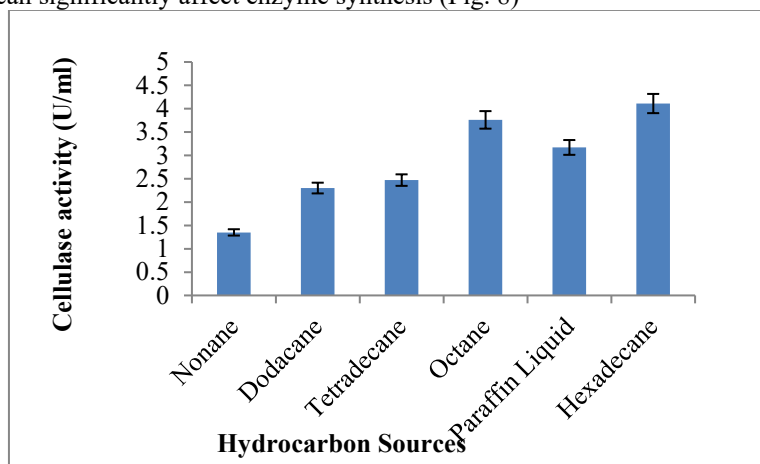


Fig. 8. Effect of hydrocarbons on cellulase production by *Aspergillus niger*.

Effect of Metal Ions on Cellulase Production:

The impact of different metal ions on cellulase production by *Aspergillus niger* was evaluated using nine distinct ions. Calcium chloride supplementation resulted in the highest cellulase yield after 120 hours of incubation, while the lowest enzyme activity was observed in the presence of Ethylene Diamine Tetra Acetic Acid (EDTA). These findings suggest that certain metal ions can enhance enzyme production, whereas chelating agents like EDTA may inhibit it (Fig. 9).

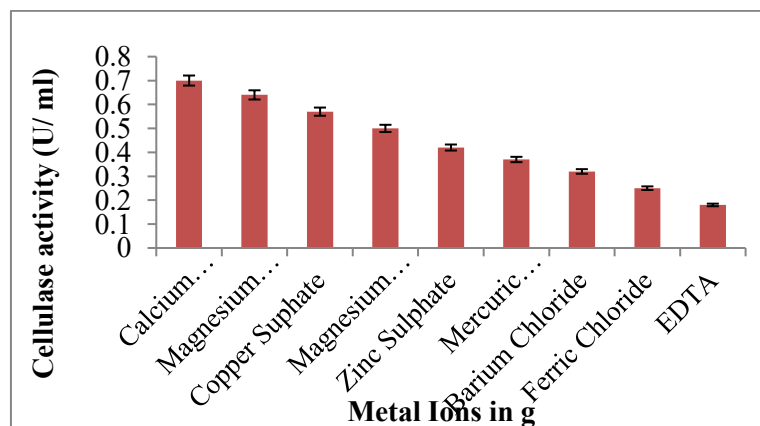


Fig. 9. Effect of metal ions on Cellulase production by *Aspergillus sp.*

Effect of pH on Cellulase Production:

The influence of pH on cellulase production by *Aspergillus niger* was studied by cultivating the fungus in SDA broth across a pH range of 3 to 9. Maximum enzyme activity was observed at pH 6, whereas the lowest activity occurred at pH 3, indicating that cellulase production is highly sensitive to the pH of the growth medium (Fig. 10).

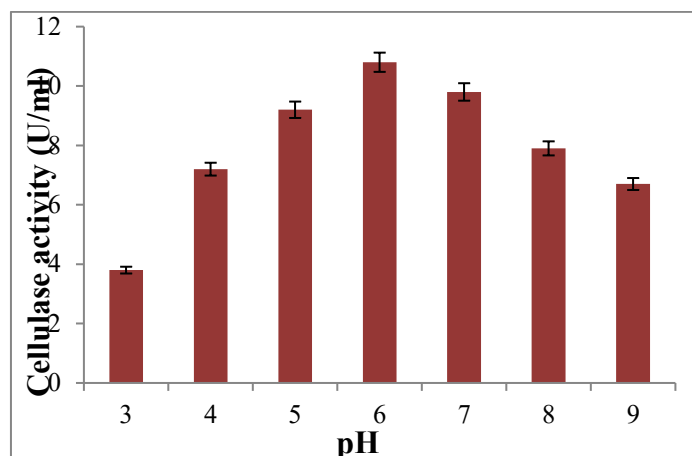


Fig. 10. Effect of pH on Cellulase production by *Aspergillus niger*.

Effect of Temperature on Cellulase Production

The effect of temperature on cellulase production by *Aspergillus niger* was assessed by incubating the culture in SDA broth at temperatures ranging from 10°C to 80°C. Maximum enzyme activity was observed at 30°C, while the lowest cellulase production occurred at 80°C. These results indicate that temperature strongly influences enzyme synthesis in the tested isolate (Fig. 11).

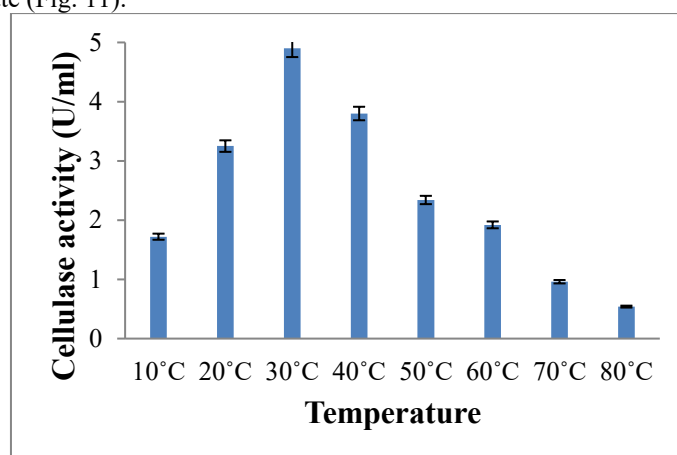


Fig. 11. Effect of temperature on cellulase production by *Aspergillus niger*.

Effect of Incubation Time on Cellulase Production:

The effect of incubation time on cellulase production by the isolated *Aspergillus niger* culture was evaluated over a period of 24 to 168 hours. Maximum enzyme activity was observed at 96 hours of incubation, after which cellulase production gradually declined. This suggests that the enzyme synthesis is time-dependent and peaks at an optimal incubation period (Fig. 12).

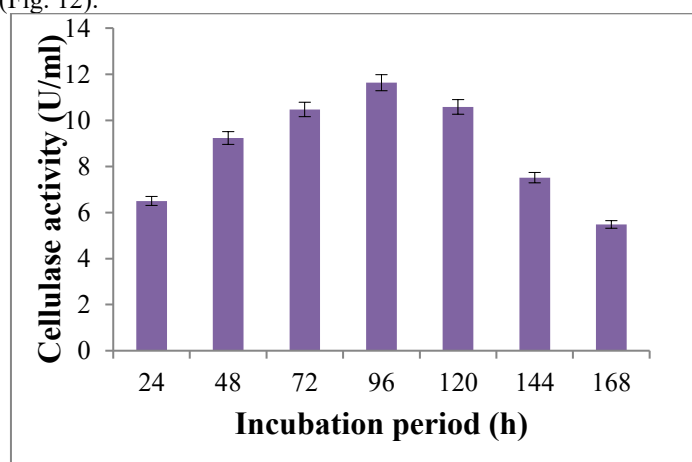


Fig. 12. Effect of incubation period on cellulase production by *Aspergillus niger*.

18S rDNA Sequencing:

Following DNA extraction, the target region was amplified by PCR, and the resulting amplicons were sequenced at Macrogen, Korea. Morphological observations combined with BLAST analysis confirmed a high sequence similarity with *Aspergillus niger*. The sequence has been submitted to the National Center for Biotechnology Information (NCBI) to obtain an accession number, with the BANKIT submission ID 2002596.

Discussion on Cellulase Production by Microorganisms

Cellulolytic enzymes are widespread in nature and play an essential role in microbial growth, differentiation, and nutrient assimilation. Among these, cellulases hold considerable industrial significance due to their broad applications in sectors such as biofuels, food, textiles, and paper (Gupta et al., 2002a, 2002b). While many microorganisms produce cellulases, only a limited number are exploited commercially, with *Aspergillus* species being the predominant industrial producers. Similarly, *Trichoderma reesei* has been extensively studied for its ability to secrete cellulases, xylanases, esterases, and other extracellular enzymes, particularly during the late exponential growth phase (Jørgensen et al., 2006). Extracellular enzymes enable microorganisms to break down insoluble nutrients such as cellulose, starch, and proteins into simpler compounds that can be readily assimilated for growth and metabolism (Oh et al., 2000). Earlier studies also highlighted the role of extracellular cellulolytic enzymes in protein secretion by *T. reesei* (Ghose, 1987). In addition, cellulolytic bacteria have been isolated from freshwater and marine fish-processing wastes, indicating their ecological diversity, and aquatic microbes are known to produce inducible exoenzymes that degrade complex substrates (Chrost, 1991; Kim et al., 2002; Sudeepa et al., 2007). Among the factors influencing cellulase production, pH is critical, as it affects microbial morphology, enzyme secretion, and stability. Optimal pH values vary across species. For instance, *Aspergillus* species generally grow and metabolize efficiently under acidic conditions (pH 3–5), with maximal cellulase production reported at pH 5 for *A. oryzae* and *A. terreus* QTC 828 (Ali et al., 1991; Beldman et al.). In line with this, the present study observed peak cellulase activity at pH 6, highlighting strain-specific differences. Temperature similarly affects enzyme yield, with previous studies reporting optimal production at 40 °C for *A. niger* and *A. fumigatus* (Immanuel et al., 2007), whereas in the current work, maximum cellulase activity was observed at 30 °C, further supporting the influence of strain-specific growth conditions. Nitrogen sources are also crucial for enzyme biosynthesis. Organic nitrogen sources such as peptones derived from casein, soymeal, or gelatin are widely used in microbial cultivation (West and Reed, 1994; Reissbrodt et al., 1995). Limited studies on fish protein hydrolysates suggest they can serve as cost-effective nitrogen sources (Dufossé et al., 1997). In this study, sucrose supplementation enhanced cellulase production, serving as both a carbon and energy source. Similar trends have been reported where lactose improved cellulase synthesis by *A. niger* (Gupta et al., 2007), *Virgibacillus pantothenicus* (Vidyasagar et al., 2006), and *Chromohalobacter* sp. TVSP101. Likewise, *T. reesei* showed higher cellulase yields with peptone as the organic nitrogen source compared to inorganic sources such as sodium nitrate, consistent with earlier findings (Bayer et al., 2007).

The stability of enzymes in organic solvents has gained increasing attention for industrial applications. In the present study, medium-chain hydrocarbons, particularly nonane, enhanced cellulase production. This aligns with previous reports showing that *A. niger* cellulase remains stable in solvents like n-decane, n-octane, and n-tetradecane (Tang et al., 2008; Liming et al., 2004). Similarly, surfactants such as Triton X-100 promoted cellulase synthesis, consistent with prior studies on protease activity retention in the presence of surfactants by *A. niger* and *A. flavus* (Joo and Chang, 2005). Metal ions and trace elements are known to influence enzyme secretion. Calcium chloride supplementation significantly improved cellulase yield in this study, supporting previous observations that ions such as Ca^{2+} and Mg^{2+} enhance enzyme production in fungi and bacteria (Sumantha et al., 2005; Abd Rehman, 2005). Furthermore, sodium chloride affected enzyme synthesis in a concentration-dependent manner. Maximum cellulase production was recorded at 1% NaCl, with growth supported up to 1.5%, consistent with reports for *A. niger*, *Roseobacter* sp., *Pseudoalteromonas* sp., and *Salinivibrio* sp., which require low to moderate salinity for optimal enzyme production (Shanmuga Priya et al., 2008; Sánchez-Porro et al., 2003; Amoozegar et al., 2006). Overall, these findings highlight that cellulase production by *Aspergillus niger* is influenced by multiple physicochemical and nutritional factors, including pH, temperature, nitrogen and carbon sources, surfactants, hydrocarbons, metal ions, and salinity. Optimizing these parameters is essential for maximizing enzyme yield and industrial applicability.

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