



Production of cellulase enzyme by *Aspergillus niger*, *Aspergillus terreus* and *Penicillium* sp. isolated from soil

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Abstract: This research was aimed to isolate cellulolytic fungi showing hyper-cellulase activity. Potential cellulase-producing fungi were isolated from different soil samples, particularly near agro-wastes dumping sites. Among the various isolates obtained from different sampling sites, three different fungi were selected depending upon the clear zone diameter produced in Carboxymethyl Cellulose (CMC) agar. CMC hydrolysis by all three fungal strains exhibited their activities at pH 5.5-7.5, whereas maximum activity occurred at pH 5.5. Enzymes were also pH stable. Based on K_m and V_{max} values, the endoglucanase and β -glucosidase enzymes of *Aspergillus niger* were most efficient. The characterization of these fungi may also provide an opportunity to screen the cellulase enzymes for their further utilization in bio-ethanol production using lignocellulosic agro-wastes.

Key words: Cellulolytic fungi; Cellulase Endoglucanase; β -glucosidase

Introduction

Production of bioenergy has received much attention globally because it offers a mean to reduce dependence on natural crude oil and to reduce emissions of greenhouse gases which affect our environment (Akram *et al.*, 2018). Sustainable energy resources are derived from plant biomass. Cellulose is the major component of plant biomass (Camassola and Dillon, 2007). Plants produce 4×10^9 tons of cellulose annually (Coughlan, 1990). It is a polymer of β -1,4 linked glucose units. Its crystalline structure and insoluble nature represent a big challenge for enzymatic hydrolysis. Microbes convert lignocellulose wastes into valuable products like biofuels by fermentation (Lynd *et al.*, 2002). Successful bioconversion of cellulosic materials primarily depends on cellulose, sources of cellulolytic enzyme, and optimal conditions for catalytic activity and production of enzymes (Alam *et al.*, 2004). Cellulose quality, temperature, aeration, carbon sources, incubation period, medium additives, pH of the medium and presence of inducers are essential parameters for the optimized production of cellulase enzymes (Immanuel *et al.*, 2006). For many years, cellulose-degrading bacteria have been isolated and characterized for obtaining more effective cellulases from various sources

such as soil, decayed plant materials, hot springs, and organic matter feces of ruminants and composts (Doi, 2008). *Aspergillus*, *Trichoderma* and *Penicillium* sp. produce the highest amount of cellulase (Madhavan and Mangalanayaki, 2016). The most important use of cellulase is in the bioconversion of plant-based cellulosic and lingo-cellulosic wastes, which opens the possibility of a virtually inexhaustible and unique source of renewable biofuel (Gautam *et al.*, 2012).

The main objective of this study was to isolate novel fungal strains with putative cellulase activity. In this study, efficient cellulase producing fungi were isolated from 28 different soil samples. During the present work, three fungal strains screened out having the capacity to produce cellulase enzymes. The purpose was to characterize those isolates displaying the most significant cellulase activity for the possible use in large scale biofuel industry.

Materials and Methods

Isolation and screening of cellulolytic microbes

Efficient cellulase producing fungi were isolated from 28 different soil samples collected from different locations of Patna, Bihar. Serial dilution and spread plating methods was

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performed with soil and compost samples. The soil sample and compost were serially diluted and spread plated on a CMC agar. The plates were incubated for 7 days at 28°C and observed for clear zone around the colony. The plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1M NaCl to visualize the hydrolysis zone. To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on CMC agar were measured. A single colony from these CMC agar plates, were sub cultured on fresh CMC plate (Teather and Wood, 1982).

Identification of fungal isolates

Isolated cellulolytic fungi were identified based on colony morphology, cultural characteristics, and, especially, on the morphology of their sporulating structures. The morphology of the isolates, stained with Lactophenol-cotton blue, was studied using a compound microscope and identified by Manual of Soil Fungi (Gilman, 1975; Nagamani *et al.*, 2006).

Crude cellulase production

A basal media (1% CMC, 0.1% KH₂PO₄, 0.1% K₂HPO₄, 0.04% MgSO₄, 0.005% NaCl, and 0.000125% FeSO₄, pH 7.0) was used for production of cellulase. For seed culture, a freshly isolated colony was inoculated in 5 ml basal media and incubated at 37°C and 120 rpm for 24 h. The seed culture (5%) was then inoculated in 50 ml production media in a 250 ml conical flask and incubated at the conditions as indicated. The cell-free supernatant obtained by centrifugation at 8,000 rpm for 15 min at 4°C was used to determine the cellulase activity (Khatiwada *et al.*, 2016).

Cellulase activity assay

The carboxymethyl cellulase (CMCase) activity was assayed using a method described by Miller (1959), with some modifications (Bhat, 2000). A 0.5 ml of culture supernatant was added to 0.5 ml of 1% CMC prepared in 50 mM sodium citrate buffer (pH 4.8) in a test tube and incubated at 60°C for 30 min. The reaction was terminated by adding 3.0 ml of dinitro salicylic acid (DNSA) and subsequently placing the reaction tubes in a water bath at 100°C for 15 minutes. One ml of Rochelle salt solution (40

gm Rochelle salt in 100 ml distilled water) was then added to stabilize the colour. The absorbance was recorded at 540nm wavelength against a blank of 50 mM sodium citrate buffer. One unit of CMCase activity was defined as the amount of enzyme that liberated 1µmol of reducing sugar (glucose) in 1 min at 37°C and pH 7.0. (Miller, 1959; Bhat, 2000).

Time course study for cellulase production

To determine the optimum cultivation period for maximum cellulase production, the seed culture was inoculated into the production media and incubated at the conditions as described above. Culture samples were withdrawn at 12h interval up to 120h of cultivation and the CMCase activity was assayed (Khatiwada *et al.*, 2016).

Optimization of temperature and pH for cellulase activity

To investigate the effects of temperature and pH on cellulase activity, 500 µL of the crude enzyme was added to 500 µL of 1% CMC in 50 mM citrate buffer (pH 4.8). The reaction mixture was incubated for 30 min at various temperatures 30, 35, 40, 45, 50, 55 and 60°C. Cellulase activity was then measured as described above. To study the effects of pH on cellulase activity, different buffers such as 50mM of sodium citrate (pH 4.0 and 5.0), potassium phosphate (pH 6.0-7.0) and Tris- HCl (pH 8.0-9.0) were used to assay the CMCase activity. To 0.5 ml of 1% CMC prepared in a suitable buffer of a particular pH (pH 4-9), 0.5 ml of crude enzyme was added (Khatiwada *et al.*, 2016).

Production of Cellulase in the presence of different ions

To know the effect of different ions on CMC's solubilization, broth media (50ml in each flask) were prepared with different ions separately. Ions used during present investigation were NH₄⁺, Fe²⁺, Mn²⁺, Mg²⁺, Mo²⁺, Cu²⁺, Co²⁺, Zn²⁺, K⁺, Hg²⁺ and EDTA (Wang *et al.*, 2018).

Effect of ions on cellulase activity:

The Enzyme-substrate mixture was prepared and the required amount of particular ion solution was added in each tube and incubated for 30 min at 37°C. The reaction was stopped by the addition of 0.5N NaOH. Absorbance was

read at 405 nm by spectrophotometer (Wang et al., 2018).

Statistical analysis

Enzyme activity estimation was always carried out with a minimum of three replicates. Standard deviation was based on spreadsheet calculation using the Excel suite available in the MS Office 2010 software package (Microsoft, Redmond, WA). Error bars represent the standard deviation of each experimental data point.

Result and Discussion

Time course profile of production of cellulases and hemicellulase enzyme

Diversified fungal flora has been observed in different soil samples. A total of 110 morphotypes of fungi have been isolated and screened. Based on preliminary screening, 3 best cellulose hydrolysing fungi have been selected for further characterization. On the basis of morphological characteristics, all three fungal strains were identified as *Aspergillus niger*, *A. terreus* and *Penicillium* sp., respectively.

Cellulolytic microorganisms such as fungi and bacteria are responsible for much of the cellulose degradation in soils, though some insects, crayfish and molluscs produce their own cellulase to utilize cellulose (Watanabe and Tokuda, 2001; Ohkuma, 2003). Despite this vast number of cellulase producers, there is a deficiency of microorganisms that can produce significant amount of cellulase enzyme which can efficiently degrade cellulose to fermentable products (Maki et al., 2011). This was the prime motivation of this study to isolate and characterize cellulolytic fungi from environments. Cellulase activities are mainly evaluated using a reducing sugar assay to measure end products of the hydrolysis of substrate thus assay results are expressed as the hydrolysis capacity of the enzyme (Dashtban et al., 2010). Three selected strain *A. niger*, *A. terreus* and *Penicillium* sp. were incubated in enzyme production medium inoculated with 10^6 /ml concentration of spores. These strains showed the maximum cellulase activity between 72 hours and 96 hours of incubation. The enzyme activities subsequently decreased in all three

fungal isolates. These results agree with a similar study on *Aspergillus glaucus* XC9 where the optimal cultivation period for cellulase production was 3–4 days (Chang et al., 2006).

However, the time course required to reach maximum activity levels may be affected by several factors, such as the presence of different amorphous ratios to crystalline cellulose (Ogel et al., 2001). However, maximum β -glucosidase (80.2/mL) activity was shown after 3 days of incubation. The highest amount of glucose was recorded on 5th day of incubation in all three fungal isolates. The incubation period was directly related to the production of enzyme and other metabolic activities to a certain extent. *A. niger* and *A. terreus* showed the most active cellulolytic activities concerning different incubation periods. The incubation periods to achieve peak cellulase activity by the isolate *A. niger* and *A. terreus* were 4th and 6th days which was suitable for commercial point of view (Depaula et al., 1999). After 6th days the activities decreased. It might be due to the depletion of nutrients in the medium that stressed the fungal physiology resulting in the enzymes (Shin et al., 2000).

Optimum temperature and pH of enzyme produced by selected fungal strain

To investigate the effects of temperature on cellulase activity, 500 μ L of the crude enzyme was added to 500 μ L of 1% CMC in 50 mM citrate buffer (pH 4.8). The reaction mixture was incubated for 30 min at various temperatures 30, 35, 40, 45, 50, 55 and 60°C. Cellulase activity was then measured as described above. During the present investigation, the most suitable temperature was around 50°C for both endoglucanase and β -glucosidase in all three selected fungi (Fig. 1a-b). The results showed that the enzyme activity was decreased when the temperature increased above 65°C. However, maximum enzyme production by *A. terreus* and *Penicillium* was found between 40–50°C. Many workers have reported different temperatures for maximum cellulase production using *Aspergillus niger*, *Aspergillus terreus* and *Penicillium* suggesting that the optimal temperature for cellulase activity also depends

on the strain variations of the microorganism (Acharya et al., 2008; Nehad et al.,2019).

Enzyme activity assays to determine the optimum pH were carried out in reaction mixtures at varying pH values (3.5 - 11.5) at the predetermined temperature (50°C) using water bath. Each enzyme has its own optimum pH and if the pH increases or decreases beyond the optimum, the ionization groups at the active site may change, slowing or preventing the formation of an enzyme-substrate complex (Eijsink et al., 2005). Optimum pH values of 4.5-8.0 have been reported for different microbial cellulase (Immanuel et al., 2007; Dutta et al., 2008). For our isolates, there was a significant change in enzyme activity with a change in pH. The highest activity was recorded at pH 5.5, suggesting that the enzyme is an acid cellulase (Fig 2 a-b). Acid cellulase acts at a pH range of

3.8 and 5.8 (Mosjov, 2012). Bajaj et al. (2009) has made similar observations. In *Penicillium decumbens* also the optimum pH has been found to be at 4.0 (Nehad et al.,2019). There was no significant difference in enzyme activity at pH 6, 7, and pH 3, 11. This finding suggests that these pH pairs have more or less the same effect on enzyme activity. It was also noted that the enzyme activity was stable at pH range of 5.0-8.0. The effect of pH on cellulase production by these fungi supports the findings of (Lynd et al., 2002) who reported that CMCase, Avicelase, and FPase activities exhibit a pH optimum of approximately 4, while the pH optimum of β -glucosidase was between pH 5-6. The same can be said about results at pH 6.0 for the commercial enzyme. A similar result has been reported in the literature (Grigorevski-Lima et al., 2009; Vinha et al., 2011).

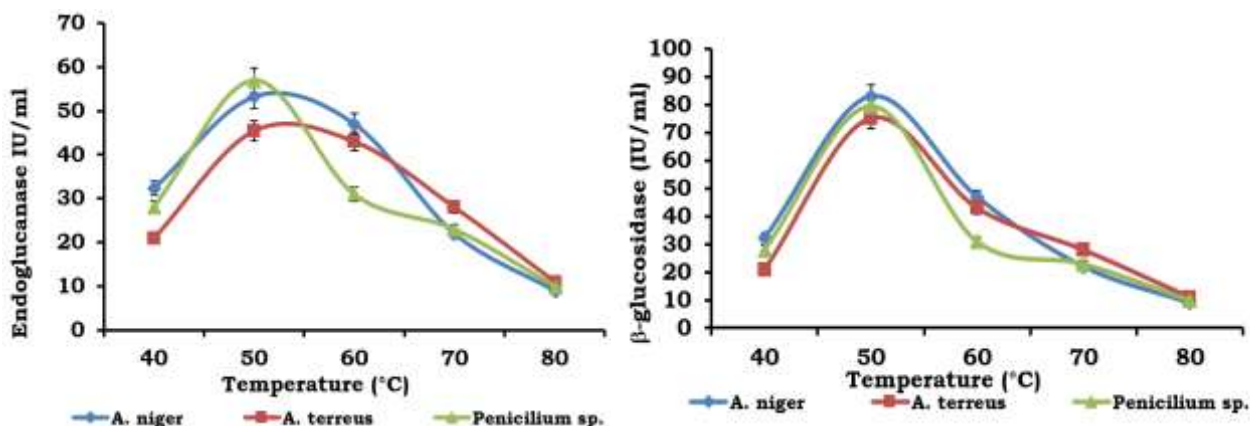


Figure 1. The optimum temperature of Enzyme produced by selected fungal strains (a) Endoglucanase (b) β -glucosidase

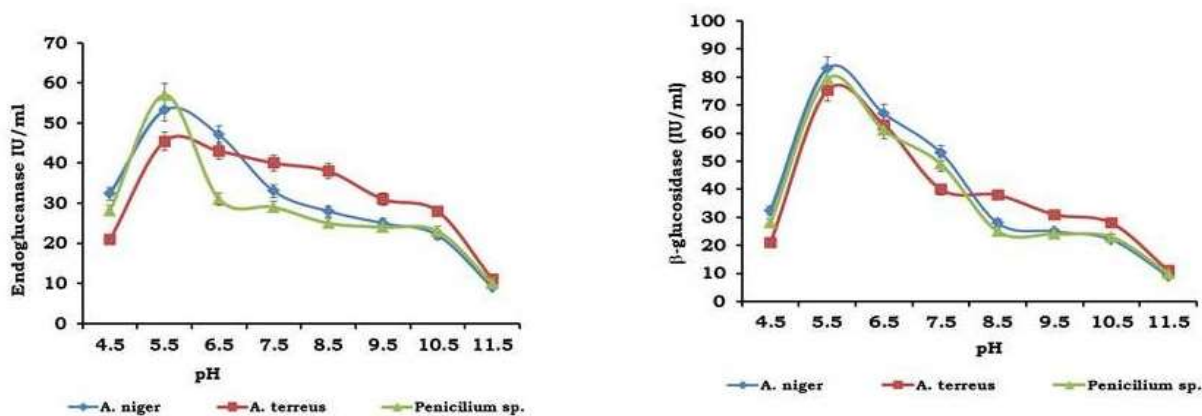


Figure 2. Optimum pH of Enzyme produced by selected fungal strains (a) Endoglucanase (b) β -glucosidase

Cellulose hydrolysis and its related activity by *A. niger*, *A. terreus* and *Penicillium* sp. in the presence of different ions

The production rate of cellulase, an inducible extracellular enzyme, is greatly influenced by nutrient medium composition, and optimum processes may be developed using effective experimental design methods (Nour *et al.*, 2010). To know the impact of different ions on the hydrolysis of cellulose and enzymatic activities, altogether, 9 different ions were tested at concentrations of 5mM and 10mM. Some ions significantly enhanced the enzyme activities, whereas some ions inhibited the cellulase activities (Fig. 3a-3c). It was very clear that the increase in enzymes' activities depends upon the ions in low concentration (5mM) but activities always decreased in high concentration of ions (10mM). Endoglucanase and β -glucosidase activity increased in the presence of EDTA, Fe^{+2} and Mn^{+2} while in the presence of K^+ , Zn^{+2} and Cu^{+2} the activities of these two enzymes reduced greatly. No significant effect appeared on the activity of Endoglucanase and β -glucosidase in the presence of Ca^{+2} and Mg^{+2} . The activities were almost negligible in the presence of Hg^{+2} ions. Same trend was exhibited by *A. niger*, *A. terreus*, and *Penicillium* sp. Bakare *et al.* (2005) have also reported that Ca^{2+} , Mg^{2+} and Na^{+1} ions strongly stimulated cellulase activity.

Similarly, Wang *et al.* (1982) also reported that *Paenibacillus* sp. strain B39 showed maximum enzyme activity in the presence of 1mM Ca^{2+} . Ca^{2+} , Cu^{2+} and Zn^{2+} slightly inhibited cellulase activity. Cellulase was strongly inhibited in the presence of Hg^{2+} . Similar results were reported for *Bacillus* strain (Mawadza *et al.*, 2000; Sadhu *et al.*, 2013) and *Bacillus amyloliquefaciens* DL-3 (Lee *et al.*, 2008). It has been reported that the inhibition of cellulase activity by Hg^{2+} ion might be related to its binding with thiol groups, tryptophan residue, or the carboxyl group of amino acid residues in the enzyme (Depaula *et al.*, 1999; Tao *et al.*, 2010).

The inhibition of cellulase by Ca^{2+} , and Cu^{2+} ions could be due to competition between the exogenous cations and the protein-associated cations, resulting in a decreased metallo-

enzyme activity. These ions are commonly cited in the literature as inhibitors for several microbial cellulase (Dutta *et al.*, 2008; Tao *et al.*, 2010; Shanmughapriya *et al.*, 2010).

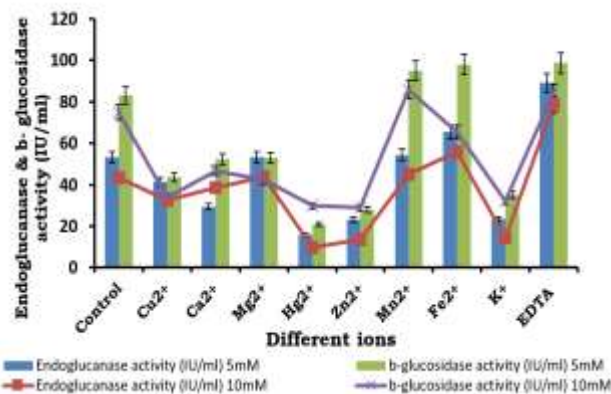


Figure 3a. Effect of ions on Endoglucanase and β -glucosidase activity in *A. niger*

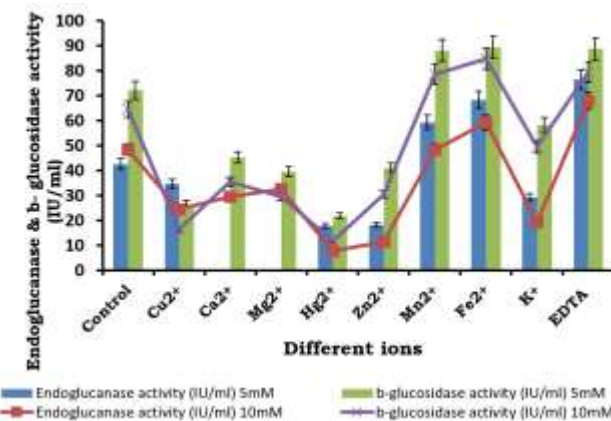


Figure 3b. Effect of ions on Endoglucanase and β -glucosidase activity in *A. terreus*

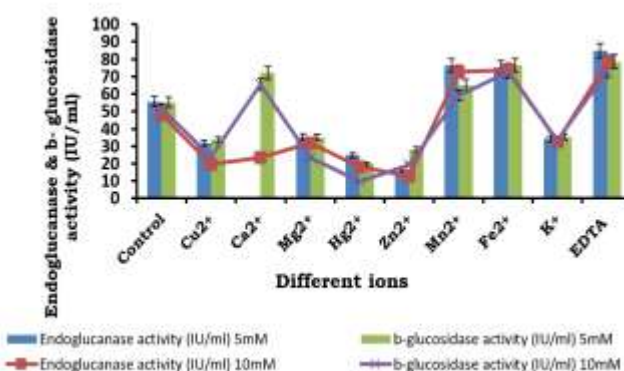


Figure 3c. Effect of ions on Endoglucanase and β -glucosidase activity in *Penicillium* sp.

According to our results, these ions must be avoided in future cultivations for high cellulase production.

Determination of K_m and V_{max} for endoglucanase enzyme through Michaelis-Menten kinetics produced by selected fungal strain

The kinetic characterization of enzymes from fungal and actinomycetes strains is an important area of comparative biochemistry which enables one to explore the efficiency of enzyme from different organisms (Table - 1).

The influence of substrate concentration on the purified cellulase's reaction velocity was studied with CMC and PNPg. The purified cellulase was incubated with various concentrations of CMC ranged from 0.2 – 0.9mM. In all cases, the enzymatic activity was assayed under standard conditions.

Table 1: Comparative study of K_m and V_{max} values of selected strains

| Strains | K_m (mM) | | V_{max} (IU/ml) | |
|------------------------|---------------|----------------------|-------------------|----------------------|
| | Endoglucanase | β -glucosidase | Endoglucanase | β -glucosidase |
| <i>A. niger</i> | 0.233 | 0.257 | 68.027 | 84.74 |
| <i>A. terreus</i> | 0.147 | 0.255 | 50.00 | 90.90 |
| <i>Penicillium sp.</i> | 0.257 | 0.303 | 62.53 | 98.52 |

The Michaelis constant (K_m) and maximum velocity (V_{max}) was determined from Lineweaver-Burk plots of the Michaelis-Menten equation (Lineweaver and Burk, 1934). The K_m and V_{max} values provide information about the rate or chemical nature of discrete steps in reaction. Steady-state kinetics is the standard method to compare and characterize the catalytic efficiency of enzymes. The value of K_m is an indicator of the affinity of an enzyme for its substrate. The low value of K_m and high V_{max} value was a desirable quality of enzyme. In this regard endoglucanase produced by *A. terreus* showed the least K_m value with low V_{max} value. Overall findings indicate that both enzyme produced by *A. niger* showed an excellent affinity with their corresponding substrates along with high V_{max} value. The difference in K_m value of other reported fungal species may be due to genetic variability among different species (Iqbal et al., 2011).

Conclusion

Significant β -glucosidase activities have been observed at 60°C suggesting its thermo-tolerance. *A. niger*, *A. terreus* and *Penicillium sp.* show high level of tolerance of metal ions like Ca^{2+} , Hg^{2+} , K^+ , Mn^{2+} , Mg^{2+} and Fe^{2+} . *Aspergillus niger* has been identified as potential cellulase producer and it can be utilized for hydrolysis of complex agricultural wastes and their further utilization for the production of bio-ethanol.

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