Optimization of maximum cellulase production by *Aspergillus niger* from the humus of paddy field

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Abstract

The present study was conducted to determine the cellulase activity of fungi (Aspergillus niger) from agricultural waste (paddy humus). Cellulases are hydrolytic enzymes comprised mainly of endoglucanase (CMCase), exoglucanase, β-glucosidase and FPase which are responsible for the conversion of cellulosic biomass into useful products. Aspergillus niger was isolated from the humus of a paddy field and cultured on potato dextrose agar (PDA) medium. Then the screening was done in a plate using 1% carboxymethyl cellulose as the carbon source in PDA media which showed clear zone formation upon staining with Congo red dye.

Furthermore, using the DNSA method, enzyme activity was observed at different nitrogen sources with various concentrations, pH of the media and incubation times. An increase in cellulase activity was observed with an increase in the incubation period and the maximum activity was recorded after 96 h. However, the highest amount of cellulase activity was seen at pH 4.2. In the case of the different nitrogen sources used, the best cellulase activity was noticed at 0.125%, 0.14% and 0.04% of peptone, ammonium sulphate and urea respectively. This study is effective in determining the optimal parameters for maximum cellulase production from Aspergillus niger for degrading the bio-waste produced from the agricultural field and kitchen wastes etc.

Keywords: Agri-waste, *Aspergillus niger*; Cellulase, CMCase, Paddy.

Introduction

Cellulose, the abundant source of polysaccharides on earth, acts as a substrate for cellulase. It is synthesized by fungi, bacteria and actinomycetes and produces efficient cellulase. It is one of the important bio-catalysts used rapidly in industrial applications. Its nature may vary as anaerobic, aerobic, thermophilic or mesophilic.

A few genera like *Aspergillus, Clostridium, Cellulomonas, Thermomonospora* and *Trichoderma* are major microbial source of cellulase production among which *Aspergillus* sp. and *Trichoderma* sp. are the utmost cellulase producing microbes including β-glucosidase[7,19]. Cellulases and related enzymes obtained from fungi are used to degrade the cell wall of plant-pathogen controlling disease and to enhance growth of plants[5]. Therefore, these strains are improved subjected to experimental and commercial purposes for the production of cellulose on a large scale.

According to a global cellulase (CAS 9012-54-8) market research report published in 2018, it is largely consumed in Asia, with a revenue market share of nearly 32.84% by 2016[18]. Cellulase enzyme production has been noticed in almost all species of *Aspergillus*[33,36]. Besides *Aspergillus, Trichoderma* spp. is another well-known microbe for the cellulase production and heavy metal tolerance as well[22,26]. However, sometimes it resists the microbial degradation limiting the microbial activities to some extent which results in poor growth of *A. niger* producing low levels of β- 1, 4-endoglucanase and exoglucanase compared to *T. reesei*[6,16].

Mechanistically, cellulases family is composed of 3 major groups of enzymes including exoglucanase, endoglucanases and β-glucosidase which act synergistically and produce glucose from cellulose[37].The expression level of fungal isolates is determined by the growth conditions and composition of the media while, the metabolic processes of the microbes are affected by changes in optimum conditions such as temperature, pH, substrate (carbon and nitrogen source) aeration and inoculums concentration. However, the optimal conditions for microbes may vary from species to species[9]. Screening for cellulase production from rice residues has done by *A. niger* and *T. reesei*[27]. These enzymes are commercially utilized for benefiting the environment via treating the cellulose rich agro-waste which would cause harm otherwise. The wastes come from agricultural fields; agro-industries and forests contain a large amount of unutilized or underutilized cellulose, which can cause environmental pollution[1,23].

Plant biomass from agricultural field waste, forestry residue and agro-industrial waste such as leaves, straw, stem, stalk, husks etc. are used for animal feed. Conversion of these agricultural wastes into useful products may improve the problems they cause. A large amount of these is left on farmland decomposed by various bacteria and fungi. These waste materials are very good low-cost feedstocks and are useful for the production of fuel ethanol, reducing sugars, organic acids, proteins, amino acids, lipids, phenols, resins and improved animal feeds and enzymes[35].

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Large amounts of cellulosic waste are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries containing cellulose as the major constituent causing pollution problems[1]. Therefore, to resolve this problem, the research should be taken further by linking the environmental scenario with other fields of science.

Material and Methods

The present study was conducted in the Department of Mycology and Plant Pathology, Banaras Hindu University, Varanasi.

Sample collection and maintenance of culture: *Aspergillus niger* was isolated from the humus of the paddy field. Humus was serially diluted to 10^{-4}. 10g of humus was taken in 95ml of distilled water and mixed well in a test tube and labelled A. 1ml of suspension from tube A was mixed with 9ml of water in test tube B. This dilution process was repeated 2 more times up to 10^{-4}(C and D). Suspension from test tube D, 0.1 ml of suspension was spread with the spreader in the Petri plates with PDA media. The culture was observed after 72 h. Furthermore, *Aspergillus niger* fungus was sub-cultured in a Petri plate and slant containing PDA media.

Purification of fungal isolate: The contaminated cultures of fungal isolates were purified and derived through single spores through several modifications as described[8]. To ensure the purity of cultures, it is then derived from a single spore in each case. Bacterial contamination was prevented by using antibiotic streptomycin (50mg/100ml) in the medium during isolation and purification of fungi. After ensuring purity, the cultures were maintained in potato dextrose agar (PDA) slants at 4^0C.

Biochemical studies

Cellulase activity: 1g of the mycelium mat was homogenized with 5ml of 0.05M sodium citrate buffer. The homogenised sample produced was then centrifuged at 10,000 rpm for 10 min. The supernatant produced was used for the enzyme assay. Enzyme activity was assayed by DNSA (3, 5-dinitrosalicylic acid) methods as given by the International Union of Pure and Applied Chemistry (IUPAC) Commission of Biotechnology. One unit of activity of enzyme is defined as 1 µmol glucose equivalents released per minute (µmol/ml/min).

Endoglucanase activity by DNSA method: 1% of CMC in 0.05M sodium citrate buffer with 0.5ml enzyme extract was mixed in a test tube. Control without enzyme was used. All test tubes were incubated for 30 min at 50°C in a water bath. The reactions were stopped by adding 3ml of DNSA reagent, the tubes were left in a boiling water bath for 5 min. Absorbance was measured at 540nm[17].

FPase activity by DNSA method: 1ml of 0.05M sodium citrate buffer (pH-4.8) and 0.5ml enzyme extract was added to the test tube containing Whatmann No. 1 filter paper strip (1.0×6.0 cm). All test tubes were incubated at 50°C for 1h in a water bath[21] and finally reaction was stopped by adding 3 ml of DNS reagent. The suspension was mixed well and the tubes were transferred to a boiling water bath for 5 min. The tubes were immediately cooled in cold water and absorbance is then measured at 540nm. The concentration of reducing sugars formed was determined by the dinitrosalicylic acid (DNSA) method[10].

Exoglucanase activity by DNSA method: 1 ml of enzyme extract with 1 ml of Avicel solution was mixed in a test tube made in 0.1 M phosphate buffer (pH 5)[34] and incubated for 30 min at 55°C. Then, 3 ml of dinitrosalicylic acid (DNS) was added and tubes were kept in boiling water for 15 min. DNS reacts with cellulobiose and produced complexes; the amount of these complexes was estimated by taking the OD at 540 nm in a spectrophotometer[30].

Optimization of the pH and incubation period: For optimization of pH, temperature and incubation period, *Aspergillus niger* was cultured with varying pH ranges of 3.0 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 and incubation period range of 20 to 100 h by keeping all other parameters constant.

Optimization of nitrogen source: Various types of nitrogen sources [Peptone, $(NH_4)_2SO_4$ and Urea] were used for optimization. Peptone was used from 0.04 to 0.13%. $(NH_4)_2SO_4$ was used from 0.1 to 0.16% and urea was used in a range from 0.01 to 0.04% in cellulose-containing fermentation medium. The flasks were inoculated with 1.0 ml of spore and incubated at 40 ± 0.5 °C in a shaker incubator at 110-120 rpm. Enzyme assays were carried out at regular intervals.

Protein estimation: Protein estimation was done following the method using the standard Bovine serum albumin[20].

Statistical analysis: All parameters were analyzed in three replications. The data obtained by biochemical constituents and enzyme determination were subjected to a simple completely randomized design (CRD) study the significance of various data.

Results and Discussion

Cellulases are important enzyme for the hydrolysis of cellulose from agro-waste and other industries. The present study was based on the cellulase activity of *Aspergillus niger* isolated from the humus of paddy field which acts as a substrate for cellulose. Several parameters including physical and chemical parameters were taken into consideration.

The *Aspergillus niger* was cultured at 40 ± 0.5 °C and cellulose activity was measured using carboxymethyl cellulose as a substrate. Therefore, various parameters used in the study are as follows:

Isolation and Screening of *Aspergillus niger* for cellulase activity: 10^{-4} serially diluted humus from paddy field was used to culture the plates; after 96 h, many fungal colonies appearing black in colour mixed with other fungi were observed on plates. The black *Aspergillus* was morphologically identified as *A. niger* based on the black colony, biseriate conidial heads and small conidia (Fig. 1). Similar observations were made by Radziah et al[28] while inoculating the fungus isolated from onion sample in malt extract agar with 20% sucrose; after 24-36 hours different colonies were isolated among which one of the fungi was *Aspergillus niger*, the colonies started to sporulate with black, velvety conidia as mentioned by Radziah et al.[28] Black-coloured colonies were identified as *A. niger* based on the structural morphologies also reported by Difo et al.[11]

After staining the culture in CMC media with congo red, the clear zone was formed due to the breakdown of CMC. Formation of a clear zone confirmed the cellulase activity of *Aspergillus niger*. While, in control, the clear zone was not formed due to the absence of cellulase produced by *Aspergillus niger*. Clear zone ratio by *Aspergillus niger* after incubation of 48 h was 4:3.6 (Table 1). When Nandana et al[25] have grown *Aspergillus niger* in Czapek dox agar with 1% CMC with pH 5 and incubated for 3 days at 30°C and then at 50°C for 2 days, staining showed clear zone formation, this confirmed the cellulolytic activity of the fungus and yellow zone around fungal culture confirmed the cellulolytic property. Ram et al[31] found similar morphological characters of fungus showing a clear zone which confirmed cellulolytic activity.

Optimization of the pH and incubation period: There is more effect of pH on the production of the enzyme. To examine the effect of pH value in the substrate for cellulase production, the pH values are adjusted by HCl and NaOH to 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. The result showed that at pH 4.2, maximum cellulase activity of endoglucanase, FPase and exoglucanase was observed.

However, a reduction in the activity of these enzymes was recorded after pH 4.2. Enzyme activity of endoglucanase, FPase and exoglucanase at pH 4.2 was 0.59 IU/ml, 0.29 IU/ml and 0.09 IU/ml respectively. This showed that for the production, pH 4.2 was highly favourable of cellulase by *Aspergillus niger*.

Similarly, optimum pH for cellulose production was recorded at 5.0 by Akula and Golla[3]. At pH 7.2, *Aspergillus niger* released less amount of cellulase, so pH 7.2 should be less favourable for the production of cellulase. With increasing pH, enzyme activity decreased which was confirmed by observing decrease in the production of cellulase (Fig. 2).

Aspergillus niger grown on Potato Dextrose Agar was inoculated in 150ml conical flask and incubated at 40°C for 7 days. The cellulase activity was seen at a regular interval. However, the maximum cellulase activity of endoglucanase, FPase and exoglucanase was observed after 96 h of incubation. Enzyme activity of endoglucanase, FPase and exoglucanase after 96 h was 0.19 IU/ml, 0.21 IU/ml and 0.06 IU/ml respectively.

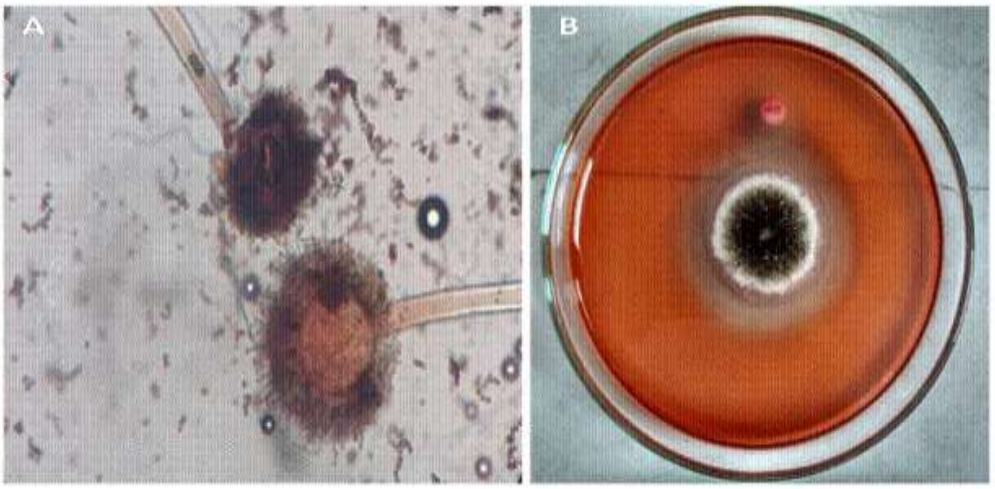

Figure 1: The image represents the microscopic view of *Aspergillus niger* culture (A) on PDA slants and (B) CMC for cellulase activity

Table 1
Reading of cellulase activity

S.N.	Culture	Incubation period (hours)	Clear zone diameter (cm)	Culture zone diameter (cm)	Clear zone ratio
1	CMC	48	4	3.6	4:3.6
2	Control	48	0	0	0

While initially, after 24 h, enzyme activity was only 0.02 IU/ml, 0.08 IU/ml and 0.01 IU/ml respectively. At 24 h, *Aspergillus niger* was at an initial stage of growth, so it released less amount of cellulase which hydrolyses less cellulase substrate. With increased incubation period, cellulase activity also increased (Fig. 3). Akinyele et al[2] have observed maximum cellulase activity after incubation of 72 h (100%) followed by a subsequent decrease. Cellulase activity was seen maximum i.e. 10.14±0.92 IU/ml for CMCase and 1.18±0.11 IU/ml for FPase after 5 days of the incubation period[12].

This decrease of cellulase activity might be due to the depletion of nutrients and accumulation of other by-products. Some researchers used different concentration (5%, 15% and 25%) of inoculums of *Aspergillus niger* and were incubated for 3, 6, 9 and 12 days, the maximum concentration of cellulase was seen after 12 days of incubation i.e. 0.59 IU/ml, 0.70 IU/ml and 0.73 IU/ml of K1F4, K2F4 and K3F4 of *A. niger* respectively as reported by Ardhi et al.[4]

Optimization of nitrogen source: The effect of different nitrogen sources on the synthesis of cellulase enzyme by *A. niger* was investigated. Different nitrogen sources were tested ranging from 0.01-0.16% of peptone, ammonium sulphate and urea which showed different cellulase activity. Among peptone, 0.125% showed the highest cellulase activity i.e. 0.20 IU/ml, 0.18 IU/ml and 0.05 IU/ml respectively for endoglucanase, FPase and exoglucanase assay (Fig. 4).

The previous report stated maximum CMCase activity for the peptone (0.13 IU/ml) among urea, peptone, ammonium nitrate and ammonium chloride[13]. It was also stated that organic nitrogen source gives better CMCase activity than any inorganic nitrogen source. In contrast with our study, Gautam et al[14] have reported that FPase activity was much higher i.e. 1.78 IU/ml in 1% peptone medium.

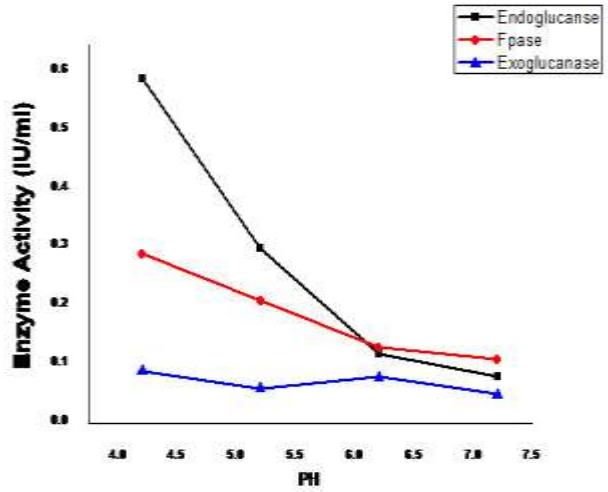

Figure 2: The image represents the effect of pH on *Aspergillus niger* cellulase activity of endoglucanase, FPase and exoglucanase

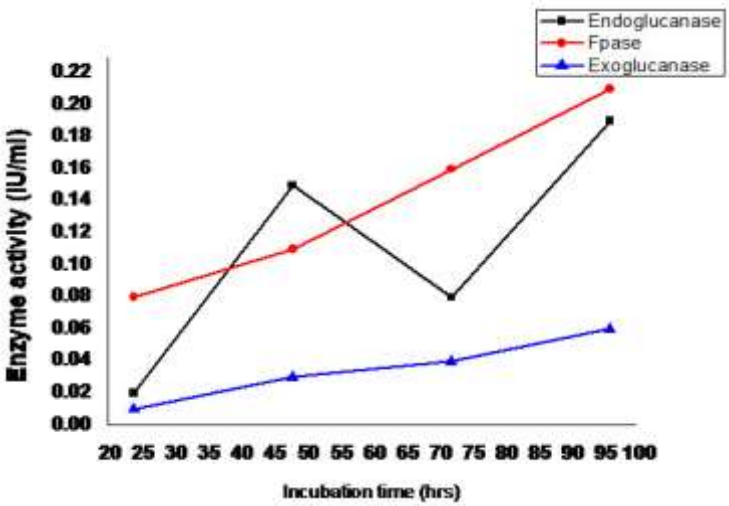

Figure 3: The image represents the effect of time on *Aspergillus niger* cellulase activity of endoglucanase, FPase and exoglucanase

The enzyme activity was maximum at $(NH_4)_2SO_4$ concentration 0.14%. Enzyme activity of endoglucanase, FPase and exoglucanase was 0.27 IU/ml, 0.22 IU/ml and 0.05 IU/ml. The minimum activity was at a concentration of 0.1%. This confirmed that *Aspergillus niger* requires 0.14% concentration of $(NH_4)_2SO_4$ for the maximum production of cellulase. At a concentration of 0.16%, enzyme activity was decreased. This showed that a very high concentration of $(NH_4)_2SO_4$ is not suitable for cellulase production (Fig. 5). According to the Sethi et al[32], ammonium sulphate was the best nitrogen source for the cellulase production in comparison to urea and peptone. However, some variation with our results has been reported by Rai et al[29] with maximum cellulase production at 0.1% after 72 h of incubation from novel thermotolerant yeast.

The maximum enzyme activity of urea was seen at a concentration of 0.04%. The enzyme activity of endoglucanase, FPase and exoglucanase at 0.04% of urea was 0.26 IU/ml, 0.23 IU/ml and 0.04 IU/ml. Minimum reading of enzyme activity was seen at concentration 0.01%; 0.13 IU/ml, 0.12 IU/ml and 0.01 IU/ml. This showed that 0.04% urea is required for high cellulase production (Fig. 6).

Similarly, among various nitrogen source used for cellulase production from *Pseudomonas Aeruginosa* Sg21, the maximum activity was recorded in urea i.e. around 3.6 IU/ml[15]. In one of the studies, using response surface methodology method with 0.5g urea, Muhammad et al[24] got maximum cellulase activity of *Aspergillus tubingensis* i.e. 112 µg/mL/min. At last, protein estimated after 96 h was 17.14% i.e. 1.71 mg/ml. While, the maximum protein concentration reported by Akula and Golla[3] in *Aspergillus niger* was 210mg/ml using urea as a nitrogen source.

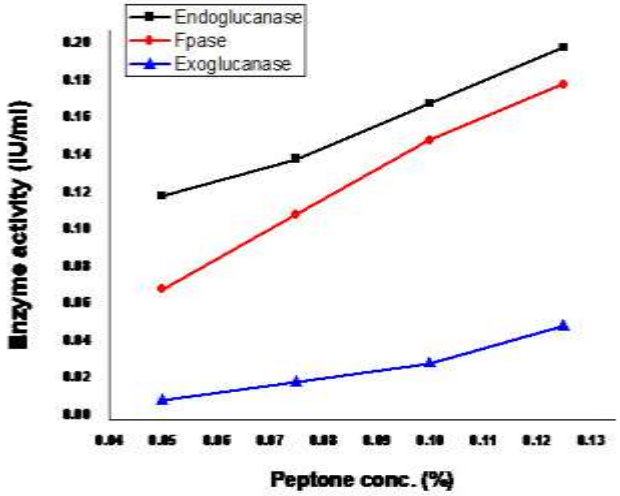

Figure 4: The image represents the effect of peptone on *Aspergillus niger* cellulase activity of endoglucanase, FPase and exoglucanase

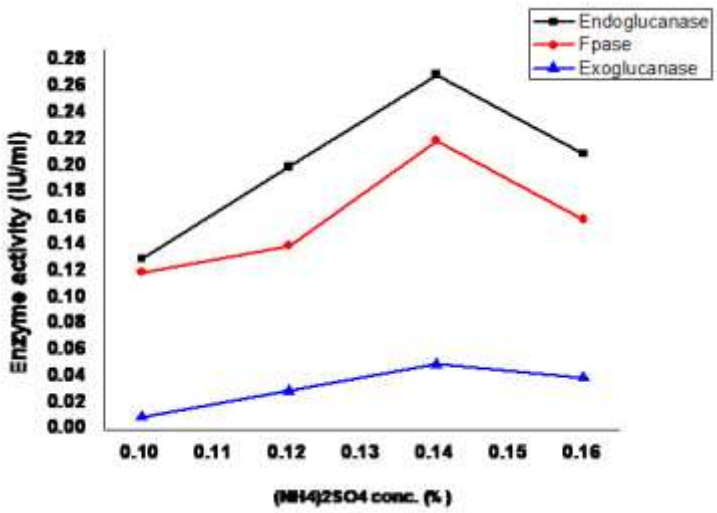

Figure 5: The image represents the effect of Ammonium sulphate on *Aspergillus niger* cellulase activity of endoglucanase, FPase and exoglucanase

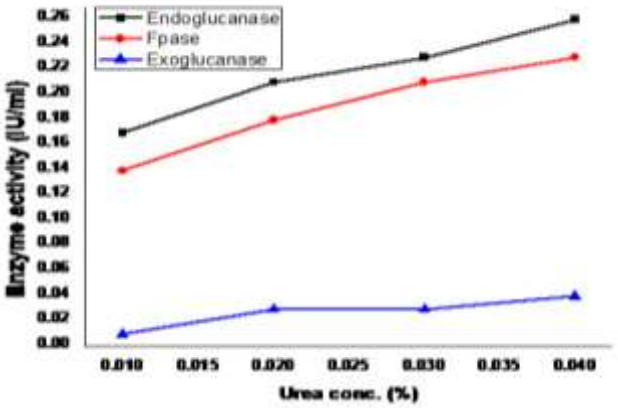

Figure 6: The image represents the effect of urea on *Aspergillus niger* cellulase activity of endoglucanase, FPase and exoglucanase

Conclusion

Overwhelmingly, these findings showed that the *Aspergillus niger* is a cellulase producing fungus. It requires pH 4.2, 0.125% peptone, 0.14% ammonium sulphate and 0.04% urea concentration for the highest cellulase production. With increasing incubation period, cellulase production increases. This study testified that this fungus will play an important role in future for degrading the bio-waste produced from the agricultural field, kitchen and agro-industrial waste etc.

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