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Effect of UV irradiation and Colchicine on Cellulase enzyme production by Aspergillus terreus

KEYWORDS: Cellulase, Mutation, UV irradiation, Colchicine, Aspergillus terreus

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ABSTRACT *Development of more efficient cellulases having higher activities and stability to enable the conversion of pretreated biomass to higher yield at lower costs will significantly reduce the process costs. Cellulase are multi-enzymatic complex protein comprising of endo-1,4-beta-D-glucosidase, or endocellulase, carboxy methyl cellulase(CX); Exo-1,4,β-D-glucosidase or beta-glucosidase. The enzymatic saccharification of cellulase has been proposed as a means of producing glucose with the objective of reconstructing cellulase to enhance biomass degradation on an industrial scale. Wild stain of Aspergillus terreus was isolated from agriculture waste and selected to mutagenesis with ultra violet (UV) irradiation and Colchicine. In UV-irradiated Aspergillus terreus (At1) the production of CX increased by 24-folds, β-glucosidase production increased by 55-folds and 84-fold increase in C1 (Cotton Assay) production in 60 minute exposure time. Colchicine treatment 0.1% (W/V) increased the production of CX to 81-folds, β-glucosidase production increased by 93-folds and 95-fold increase in C1 production with same species and in same duration.*

INTRODUCTION

Cellulose is the most abundant material available in environment in plant and agriculture waste, which left used by microbes producing cellulase enzyme. Lignocellulose is generally found in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. The lignocellulosic materials can also be herbaceous material, agricultural residues, forestry residues, municipal solid wastes, waste paper, and pulp and paper mill residues.

Agriculture waste and in fact all lignocelluloses can be converted into products that are of commercial interest such as ethanol, glucose and single-cell protein (Soloman et al., 1999). Cellulase enzyme has been used for the bioconversion of lingo-cellulosics to these useful products. Many fungi produce enzymes that enable them to break down polysaccharides and proteins into sugars and amino acids that can be assimilated easily. These enzymes are important industry.

The bioconversion of cellulosic material has been receiving attention in recent years but the problem in using this is that preparation of cellulolytic enzymes comprises about 60% of the total cost of the production process (Wilke et al., 1976). The final target of the whole research is to produce economically acceptable enzymatic conversion of cellulosic biomass to glucose for fermentation to ethanol or other products. One effective approach to reduce the cost of enzyme production is to replace pure cellulose by relatively cheaper substrates such as lignocelluloses materials.

Thus, the availability of highly active cellulase would be of great significance and hence require selection and improvement of suitable strains for enzyme production. For enzymatic processes to be effective, some kind of pretreatment process is thus needed to break the crystalline structure of the lignocellulose and remove the lignin to expose the cellulose and hemicellulose molecules. Depending on the biomass material, either physical or chemical pretreatment methods may be used. Physical methods may use high temperature and pressure, milling, radiation, or freezing; all of which require high-energy consumption. The chemical method uses a solvent to break apart and dissolve the crystalline structure. The lignocellulosic material may be used as is or may be subjected to a pretreatment using conventional method known in the art. For example, physical pretreatment techniques can include various types of milling, irradiation, steaming/steam explosion, and hydrothermolysis; chemical pretreatment techniques can include dilute acid, alkaline, organic solvent, ammonia, sulfur dioxide, carbon dioxide, and pH-controlled hydrothermolysis; and biological pretreatment techniques can involve applying lignin-solubilizing microorganisms.

Apart from this, a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell-free enzymes able to completely hydrolyzing crystalline cellulose *in vitro*. Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been recently reported to yield cellulase activity (Arriffin et al., 2006). Microorganisms of genera *Trichoderma* are known cellulase producers and available for commercial use (Ryu and Mandels, 1980) but they either produce one enzyme more of cellulase enzyme complex than other which affects the overall production so search of any other organism that can produce the whole enzyme complex is going on. To increase the production genome of microorganism need to be modified using different techniques using either natural or artificial methods. Physical, chemical and biological agents that cause change in DNA are known as mutagens. Mutagenic agents, such as ultra violet (UV) light, ionizing radiation or chemicals can randomly create DNA lesions (Mala et al., 2001). The principle objective of this investigation was to increase the production of cellulose enzyme complex using mutation through UV irradia-

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tion and Colchicine treatment and further to compare the production of the mutated organism with wild strain.

2. MATERIALS AND METHOD

2.1. Isolation of organism and maintenance:

The organism was isolated from the decaying wheat straw from the field of Kamrup district of Assam, India. Based on the study of morphological and reproductive characteristics the fungal culture was identified as *Aspergillus terreus*. The isolation and identification of the fungal culture were done in the laboratory of Department of Biotechnology, Gauhati University. The fungus was cultured in Mandel and Sternberg medium (Sternberg and Mandel, 1979) pH 5.2. Agar (2%) was added to the medium and then autoclaved at 15 psi pressure for 15 minutes. The antibiotic tetracycline was used at a concentration of at a concentration of 450mg/L. Repeated sub-culturing of the fungal hyphae were done for getting the pure culture of the fungus that was maintained on Potato dextrose agar (PDA) medium and preserved under refrigeration at 4^0C.

2.2. Enzyme production:

The fungal inoculum ($1x10^8$ spore/ml) was prepared (Desai et al., 1982) by harvesting the spores from 4-5 days old PDA culture in sterile distilled water containing 0.05% (v/v) Tween-20 and 1ml of the inoculum was added to the cultivated medium under aseptic condition. Incubation was done at 28^0C on an incubator shaker (200 rpm). Crude enzyme extraction was carried out at different stages of culture growth; cells were removed by centrifugation (4,000 rpm for 10 min) by filtration. The clear solution obtained was used as enzyme source.

2.3. Analytic method:

Reducing sugar estimated as glucose by Dinitro Salicylic Acid (DNS) method (Miller, 1959).

2.4. UV irradiation:

For UV irradiation spore suspension was prepared (Jeffries, 1996). In this method, 1ml of appropriately diluted enzyme was mixed in 10ml of distilled water. Sterile UV lamp (Samson E27) was kept at 1 meter away from the open petri plate containing the spores. Control is prepared by plating 1ml of sample spores before UV irradiation and plated in PDA media. UV irradiation was given for time 30, 60 and 90 minutes to find the lethal dose and appropriate time of treatment.

2.5. Colchicine Treatment:

The spores of 60-minute UV irradiation *Aspergillus terreus* were treated with 0.1% and 0.2% (WV) concentrations of Colchicine for a period of 60 minutes and the observation were recorded on the basis of enzyme assays in terms of CX, C1, Filter paper and β-glucosidase.

2.6. Enzyme Assays:

Enzyme assays were performed (Mandels et al., 1974) by saccharification and cellulase assays was carried out in 0.05M citrate buffer at pH 4.8.

2.6.1. Carboxy Methyl Cellulase (CMCase) (CX):

Carboxy methyl cellulose activity was determined by mixing an aliquot of 0.5ml of appropriately diluted culture with 0.5ml of 1% (w/v) carboxy methyl cellulose (CMC) as a substrate in 0.05M citrate buffer (pH 4.8). After incubation for 30 min at 50^0C, reducing sugar was measured. One unit of carboxymethyl cellulase activity was defined as the amount of enzyme required to liberate 1 µmol/min (U) of reducing sugar expressed as glucose equivalents.

2.6.2. Cotton assays (C1):

Cotton degrading activity was determined by the method of Mandels (1976). In a test tube 100 mg of cotton was transferred and 0.1 ml of buffer concentrate (1.0% Sodium citrate buffer; pH 4.8) added. An aliquot of enzyme (2 ml) was added and mixed by squeezing out air bubbles with spatula. Tubes were incubated at 50°C in water bath for 24 hours followed by addition of 3 ml DNS reagent. The reducing sugar was then measured for C1. Tubes were then placed in boiling water bath for 5 minutes, cooled in ice-bath and 14.4 ml of water was added to the tubes. One unit of C1 activity is defined as milligram of glucose liberated per ml of culture filtrate per 24 hours.

2.6.3. Filter Paper Assay (for total cellulase activity):

Cellulase activity was determined by a method of Mandels (1976). An aliquot of 0.5 ml of cell-free culture supernatant was transferred to a clean test tube and 1 ml of Sodium citrate buffer (pH 4.8) was added. Whatman #1 filter paper strip (6 cm ×1 cm) was added to each tube. Tubes were vortexed to coil filter paper in bottom of the tube. Tubes were incubated in a water bath at 50°C for 1 hour followed by an addition of DNS reagent (3 ml). Tubes were then placed in a boiling water bath for 5 minutes and then in an ice-bath, followed by the addition of 15 ml distilled water to each tube. Contents of the tube were mixed and absorbance was noted at 550 nm. Cellulase activity expressed in term of filter paper unit (FPU) is µmole of glucose liberate per minute per ml of the culture filtrate.

2.6.4. β-Glucosidase activity:

To determine the β-glucosidase p-nitro phenyl D- glucose (PNPG) was used as substrate. The assays mixture contains 0.9ml of 2mM PNPG in 0.05M citrate buffer (pH 4.8) and 0.1 ml of suitable dilution. After incubation at 50^0C for 20 min, the reaction was stopped by adding 2ml of 1M Na_2CO_3 (sodium carbonate). The absorbance of the reaction solutions was measured at 410 nm against a reaction blank, on a UV/Visible Ultra spectrophotometer 1100 Pro Spectrophotometer (Amersham Biosciences). One unit of β-glucosidase activity was defined as the amount of enzyme required to liberate 1µmol of p-nitrophenol /min.

2.7. Statistical Analysis:

All the results are average of at least three replicates. The data were analyzed by the One-Way ANOVA followed by Tukey-Kramer's multiple comparison tests ($p< 0.05$) (SPSS v 20.0).

3. RESULT

3.1. Growth of Fungi for cellulase production:

The fungal strain of *Aspergillus terreus* was grown on basal medium (Mandels 1974) using carboxy methyl cellulase as carbon source. Initially very little growth was observed. The fungal stain showed good growth on 5^{th} day of inoculation.

3.2. Enzymatic activity of wild strain against incubation time:

The wild strain was incubated into 15 different flasks containing the basal medium. The enzymatic activities like CX, C1 and Filter paper and β-glucosidase were assayed from 6^{th} day to 14^{th} day of inoculation. The result obtained was recorded in Table 1. CX, C1 and Filter paper activity was found highest on 11^{th} day of inoculation. The highest value of β-glucosidase was found on 12^{th} day of inoculation.

Figure 1: Cellulase enzyme activity of Aspergillus terreus as found from 6th day to 14th day of growth (CX – carboxy methyl cellulase; C1- Cotton Assay; F.P- Filter paper Assay).

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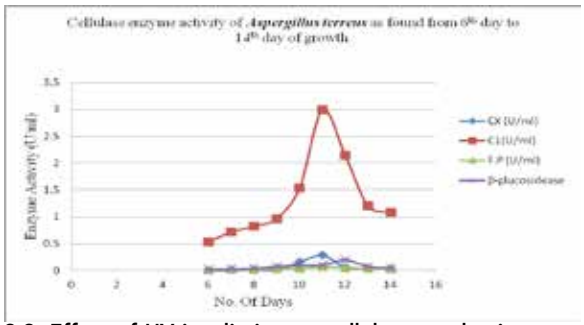


3.3. Effect of UV irradiation on cellulase production

3.3.1. UV irradiation for 30 min.:

On the basis of incubation study with wild stain the enzymatic activity was studied from 9th to 12th day of incubation. It was found that UV exposure for 30 min. increased the production of cellulase by a little bit as compared to wild strain. CX , C1, β-glucosidase and Filter paper activity was found highest on 11th day of inoculation as shown in figure 2A.

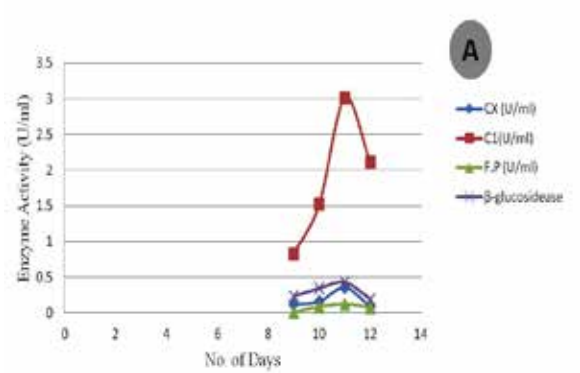
3.3.2. UV irradiation for 60 min.:

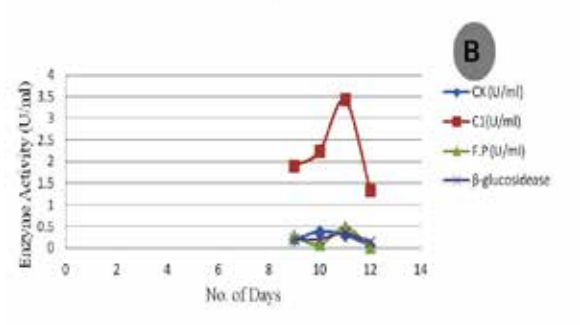
It was found that UV exposure for 60 min. increased the production of cellulase significantly as compared to wild strain. CX activity was found highest on 10th day of inoculation. The highest value of C1, Filter paper and β-glucosidase was found on 11th day of inoculation as recorded in figure 2B.

3.3.3. UV irradiation for 90 min.:

It was found that after 90 min. of UV irradiation the production of cellulase decreased by more than 90%. Based on the result (Figure 2C) it was found that 90 min. of UV exposure is not suitable for production of cellulase enzyme.

Figure 2: Cellulase enzyme activity of Aspergillus terreus as found after (A) 30 minutes (B) 60 minutes and (C) 90 minutes of UV exposure (CX – carboxy methyl cellulase; C1- Cotton Assay; F.P- Filter paper Assay).





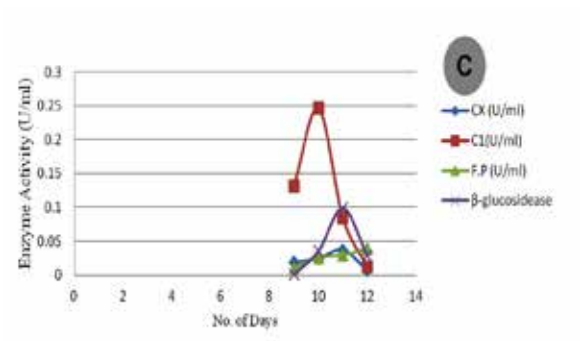


Figure 2

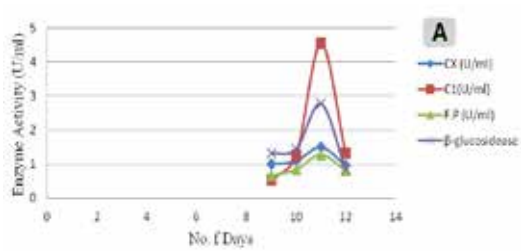
3.4. Effect of Colchicine 0.1% concentration on cellulase production for 60 min.:

The spore of 60 minute UV irradiated *Aspergillus terreus* (At1) were treated with 0.1% (W/V) concentration of Colchicine for a period of 60 minutes and the result on the basis of enzyme assays in terms of CX , C1, β-glucosidase and Filter paper. The highest activity was found on 11th day of incubation. The observations are the average of three individual readings. The result shows that the productions of these enzymes increase significantly as compare to wild stain (Figure 3A).

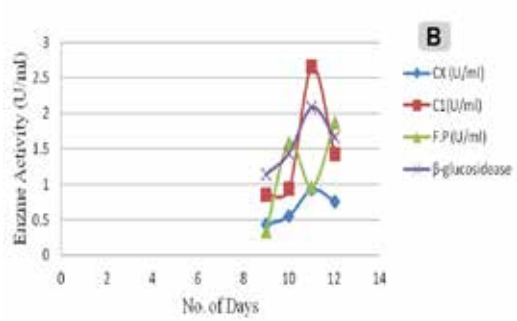
3.5. Effect of Colchicine 0.2% concentration on cellulase production for 60 min.:

The spore of 60 minute UV irradiated *Aspergillus terreus* (At1) were treated with 0.2% (W/V) concentration of Colchicine for a period of 60 minutes and the result on the basis of enzyme assays in terms of CX , C1, β-glucosidase and Filter paper was found to be decreasing as compared to 0.1% concentration of Colchicine (Figure 3B).

Figure 3: Cellulase enzyme activity of Aspergillus terreus as found after (A) 0.1% and (B) 0.2% Colchicine treatment for 60 minutes (CX - carboxymethyl cellulase; C1- Cotton Assay; F.P- Filter paper Assay).



3.5. Comparison of Aspergillus terreus (At1) cellulase and β-glucosidase with those of wild and other fungi:



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A comparative study was done to find the increase in the production of cellulase enzyme against wild strain of *Aspergillus terreus* and the mutated strain *Aspergillus terreus* (At1) as well as with different species. The result is shown in terms of CX , C1,

β-glucosidase and Filter paper (Table 1).

Table 1: Percent variation in cellulase yield after mutation in Aspergillus terreus (CX – carboxy methyl cellulase; C1- Cotton Assay).
Table 1

Enzymes	Wild type	Mutation				
		UV irradiation			Colchicine treatment	
		30 min	60 min	90 min	0.1%	0.2%
CX	0.28	0.35 (20%)	0.37 (24.3%)	0.01 (-833%)	1.50 (81.3%)	0.92 (69.5%)
C1	2.99	3.01 (0.66%)	3.42 (12.52%)	0.24 (-145%)	4.54 (34.1%)	2.65 (-12%)
Filter Paper	0.07	0.12 (41.6%)	0.48 (85.4%)	0.03 (-133%)	1.27 (94.4%)	1.58 (95.5%)
β-glucosidase	0.19	0.43 (55.8%)	0.36 (47.2%)	0.09 (-111%)	2.78 (93.16%)	2.08 (90.8%)

4. DISCUSSION

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or other. Current developments in biotechnology are yielding new applications for enzymes (Pandey et al., 1999).

In the present study, it was found that after 60 min. of UV irradiation the amount of cellulase production increased to 24.4% as compared with wild strain whereas after Colchicine treatment with 0.1% this production increases to 81.3% , C1 increase to 12.5% after 60 min. of UV irradiation and 34.1% after Colchicine treatment with 0.1%, Filter paper increased by 85.4% after 60 min. of UV irradiation and 94.4% after Colchicine treatment, β-glucosidase production increased by 55.8% after 60 min. of UV irradiation and 93.2% after Colchicine treatment. It was interesting to find that *Aspergillus terreus* (At1) was a better producer of CMCase and β-glucosidase as compared to *Aspergillus flavus* (Baishya and Deka, 2006) and *Aspergillus fumigatus* (Khan and Deka, 2008). So Colchicine treatment can be used as a better mutating agent than UV irradiation to increase the production of cellulase.

5. CONCLUSION

The major goals for future cellulase research would be the reduction in the cost of its production and the improvement of the performance of cellulases to make them more effective, so that less enzyme is required. The former task may include such measures such as optimizing growth conditions or processes, while the latter requires directed efforts in protein engineering and microbial genetics to improve the properties of the enzymes (Sukumaran et al., 2005). Optimization of growth conditions and processes has been attempted to a great extent in improving cellulase production. For instance, empirical optimization of process variables to improve productivity has been the focus of many of the works using fermentation for the production of cellulases (Sukumaran et al., 2005).

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