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EFFECT OF COLCHICINE INDUCED MUTATION ON CELLULASE ENZYME PRODUCTION BY *ASPERGILLUS FUMIGATUS*

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ABSTRACT

The production of cellulase before and after colchicine induced mutation was investigated in shake flask culture condition. *Aspergillus fumigatus* was isolated from different agriculture wastes. The enzymatic scarification of cellulase was proposed as a mean of producing glucose on industrial scale. In colchicine treated species it was found possible to increase the production of C1 up to 40 folds in 0.1% concentration, 90 fold increases in CX production, 95 fold increases in filter paper production and 96 fold increase in β-glucosidase activity. The effect of colchicine mutation on cellulase production was studied in 0.1% and 0.2% concentration. The effect of colchicine was examined in Mendal and Sternberg medium (1976) which shows that 0.1% concentration can cause significant increase in the production of cellulase for industrial production.

KEYWORDS: Aspergillus fumigatus, Colchicine, Mutation, Cellulase enzyme, Cellulose

INTRODUCTION

Cellulose is the most abundant form of living terrestrial biomass.[1] Some animals, particularly ruminants and termites can digest cellulose with the help of symbiotic micro-organisms. Cellulose is not digestible by humans, and is often referred to as 'dietary fiber' or 'roughage', acting as a hydrophilic bulking agent for faeces.[2, 3, 4, 5] Cellulose is the major constituent of paper, further processing can be performed to make cellophane and rayon, and more recently Modal, a textile derived[6] from beechwood cellulose. Since cellulose is a

replenishable raw material available in abundance, its conversion to glucose has attracted considerable interest but unfortunately its crystallinity and small surface give rise to different problems in its enzymatic degradation. Hundreds of species of fungi and bacteria are able to degrade cellulose. These organisms include aerobic and anaerobic, *mesophiles* and *thermophiles.* They are both widespread and abundant in the natural environment.

However, many microorganisms can grow on cellulose or produce enzymes that can degrade amorphous cellulose; relatively few produce the entire complement of extracellular cellulase able to degrade crystalline cellulose *in vitro*. *Trichoderma* and *Aspergillus sps.* have most widely been used to produce these enzymes.[7] Cellulase is an enzyme complex which breaks down cellulose to beta glucose. It is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Aside from ruminants, most animals (including humans) do not produce cellulose[8] and are therefore unable to use most of the energy contained in plant material.

The genome of microorganisms are subjected to change by a variety of mechanisms, which causes heritable changes in the sequence of nucleotide bases of an organisms, known as Mutation.[9] There are different methods used to cause mutation some of them are Radiation and Chemical mutagens. In radiation group UV radiation are most extensively used by many workers to cause mutation in cellulase producing fungi. The strongest germicidal wavelength of 260 nm coincides with the absorption maxima of DNA. UV irradiation causes base substitution by creating covalent linkage (dimmer) between pyrimidine bases on the same strand of the DNA. A thymine dimmer cannot act as a template for DNA polymerase, and the occurrence of such dimmer therefore prevents the proper functioning of polymerase. Long time exposer to UV can be lethal to organisms so treatment for 30 min and 1 hour were found to be sub lethal doses, which can cause mutation.[10] As a much-cited historical example, the drag colchicine was used in early studies of mitosis to identify the protein tubulin. Over 30 years later, colchicine remains an invaluable weapon in the arsenal of cell biologists, as do other compounds, such as taxol, which target the same protein but with different biological effects.[11]

The high cost of enzyme production limits the industrial use of the enzyme in the production of soluble glucose. The aim of this study is to obtain strains having been mutagenized and genetically modified to obtain an organism enable of producing high levels of cellulase.

MATERIAL AND METHOD

Collection of samples

Far the collection of sample plastic bags and were used. Which were presterilized by autoclaving at 15ib/in^2 for 15 min. Soil samples were collected from cultivated land of Guwahati and nearby regions? Soil samples and agriculture waste like rice straw and decaying wood of *Maginfera indica* were brought to the laboratory aseptically.

Identification of Fungal species

The identification of the fungal cultures was done on the bases of macroscopic and microscopic characterization and by consulting books "Fungal biotechnology in Agricultural, Food and Environment Application"[12], "Environmental Microbiology: A laboratory manual"[13] and "From Ethnomyology to fungal Biotechnology: Exploiting fungi from natural resources for, Novel Products.[14]

Enzyme Assays

Enzyme Assays were performed according to Mandels.[15] All saccharification and cellulase assays were carried out in 0.05 *M* citrate buffer, at pH 4.8. In accordance with the International Unit of Biochemistry one enzyme unit equals 1 micromole of substrate hydrolyzed per minute. For cellulase it is based on bond hydrolysis that is micromoles of glucose released per minute. One micromole of glucose equals 0.180mg.[16]

Carboxy methyl Cellulose assay for Endoglucanase (CX)

An aliquote of 0.5 ml of appropriately diluted culture filtrate was taken in a test tube and mixed with 0.5ml of 1% CMC (as mentioned in Appendix) and to this 1 ml of 0.05m citrate buffer was added. After proper mixing, it was incubated for 30 minutes at 50°C.After incubation; 3ml DNS reagent was added to stop the reaction. The tube was placed in boiling water for 5 minutes and then optical density (OD) of the content in tube was recorded at 550nm using spectrophotometer (Systemic 104).

Cotton assays for Exoglucanase (C1)

100 mg of cotton was weighed and taken in a test tube. To it 1 ml of citrate buffer and 1ml of enzyme was added. After proper mixing, die mixture was incubated for 24 hours at 50°C. If required air bubbles from cotton were removed using a spatula. The tubes were placed in boiling water bath for 5 minutes and reducing sugar was measured using spectrophotometer

(Systronic 104). The OD thus obtained was used for calculating glucose concentration from standard plot.

Filter paper assays for cellulose

The filter paper was weighed and cut into 1X 6 cm strips and put to a test tube. To it 0,5ml of enzyme solution was added together with 1 ml of citrate buffer. After proper mixing it was incubated for 1 tour at 50°C. A t e incubation 3ml DNS reagent (as mentioned in Appendix) was added to stop the reaction. The tubes were placed in boiling water bath for 5 minutes and reducing sugar was measured using spectrophotometer (Systronic 104) at 550 nm.

β-glucosidase activity

0.9ml of 2mM p-Nitrophenyl P-D- ghicopyranoside (PNPG, Himedia RM 1546) was taken in a test tube. To it 0.1 ml enzyme solution was added and 1 ml of 0.05M citrate buffer was added. After proper mixing it was incubated for 20 minutes at 50°C. After incubation 2ml of 1M NaCl was added to it to stop the reaction. Tubes were then placed in boiling water bath for 5 minutes and reducing sugar was measured using spectrophotometer (Systronic104) at 420nm.

Strain improvement by mutation

Mutation using Chemical mutagen, Colchicine.

Many indigenous methods have been developed to isolate mutants. The most common is based on phenotypic expression by using chemical mutagen treatment to wild strain to develop mutant. Different concentrations of Colchicine ($C_{22}H_{25}NO_6$ SRL_x Pvt. Ltd. Mumbai) from 0.1% to 0.5% (W/V) were prepared. A conidal suspension was prepared in 100ml of distilled water and the spores were counted using Haemocytometer. Different doses of Colchicine were added to five flasks with sterile distilled water and incubated at 30^0C for 60 minutes.[17]

After 60 minutes l ml of spore suspension was withdrawn from each flask and plated on Mandel media[17] and incubated at 30^0C. Colony counting was done to find out LD_{50} and repeated sub culturing was done on Czapek-dox agar medium. The spore suspension was incubated in basal media, and effect of mutation was studied in terms of Cx, C1, Filter paper and (β-glucosidease).

Statistical analysis

The data obtained during the course of observation and experimentation, was subjected to analysis of variance by "F" test. The significance of non-significance of a given variance was determined by calculating the respective "F" values.[18]

RESULT AND DISCUSSION

The production of cellulase enzyme is a major factor in the hydrolysis of Cellulosic materials, which are most widely distributed in ecosystem. Cellulase enzyme has been a subject of intensive research to the development o f a large scale conversion process beneficial to mankind as it solves the feeds and waste disposal problem. It diminishes man's dependence on fossil fuel.

Growth of Fungi for cellulase production: The fungal strain of *Aspergillus fumigatus* was grown on basal medium[15] using carboxy methyl cellulase as carbon source. Initially very little growth was observed. The fungal stain showed good growth on 5th day of inoculation.

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 6th day to 14th day of inoculation. The result obtained was recorded in Table 1. CX¸ C1 and Filter paper activity was found highest on 11th day of inoculation. The highest value of β-glucosidase was found on 12th day of inoculation.

Table 1: Cellulase enzyme activity of *Aspergillus fumigatus* as found from 6th to 15th day of growth.

No. of days	CX(U/ml)	C1(U/ml)	F.P(U/ml)	β-glucosidease(U/ml)
6	0.0370	1.1340	0.0203	0.0105
7	0.0481	1.6680	0.0370	0.0500
8	0.1480	1.7040	0.0397	0.0567
9	0.1554	1.5860	0.0832	0.0621
10	0.1665	2.4390	0.0844	0.0743
11	0.0909	0.8040	0.1017	0.0837
12	0.0851	0.7020	0.0555	0.0972
13	0.0704	0.5642	0.0491	0.0594
14	0.0436	0.0341	0.0032	0.0554
15	0.0056	0.0240	0.0014	0.0202

Lethal dose count for chemical (Colchicine) mutation.

To improve the enzyme activity of fungal strains *Aspergillus fumigatus* which were found to show highest enzyme activity in comparison to other strains were subjected to chemical mutation using colchicine ($C_{22}H_{25}NO_6$).The spore were exposed to different present concentrations as 0.1, 0.2, 0.3, 0.4 and 0.5% of Colchicine for 60 minutes and then the spores were plated on Mandel and Weber medium, one plate was kept as control without addition of Colchicine concentration. The colonies were counted using compound microscope and it was found that 0.1 and 0.2% concentration of colchicine are sublithal doses and the **No. of colonies** number of colonies become less than half in different concentrations.

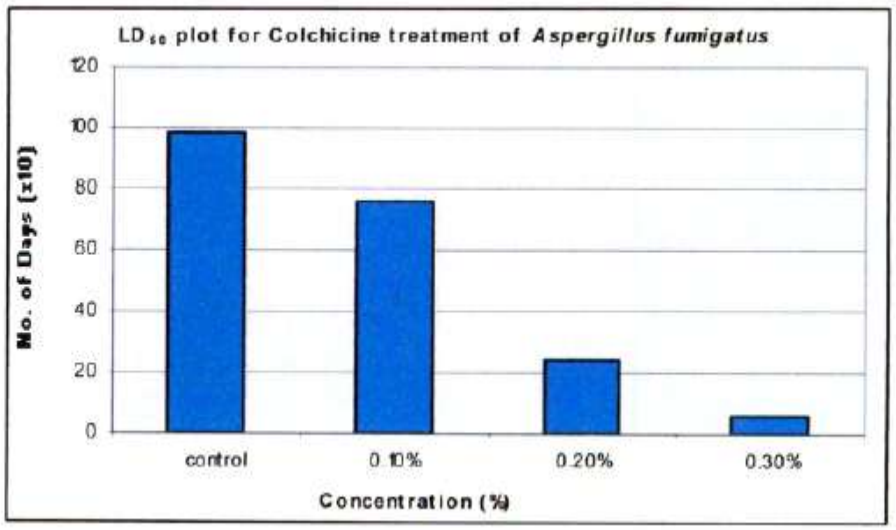

Fig 1: Graphical representation of lethal dose (LD_{50}) for chemical mutation by Colchicine where 0.1% to 0.3% are different doses for Aspergillus fumigatus.

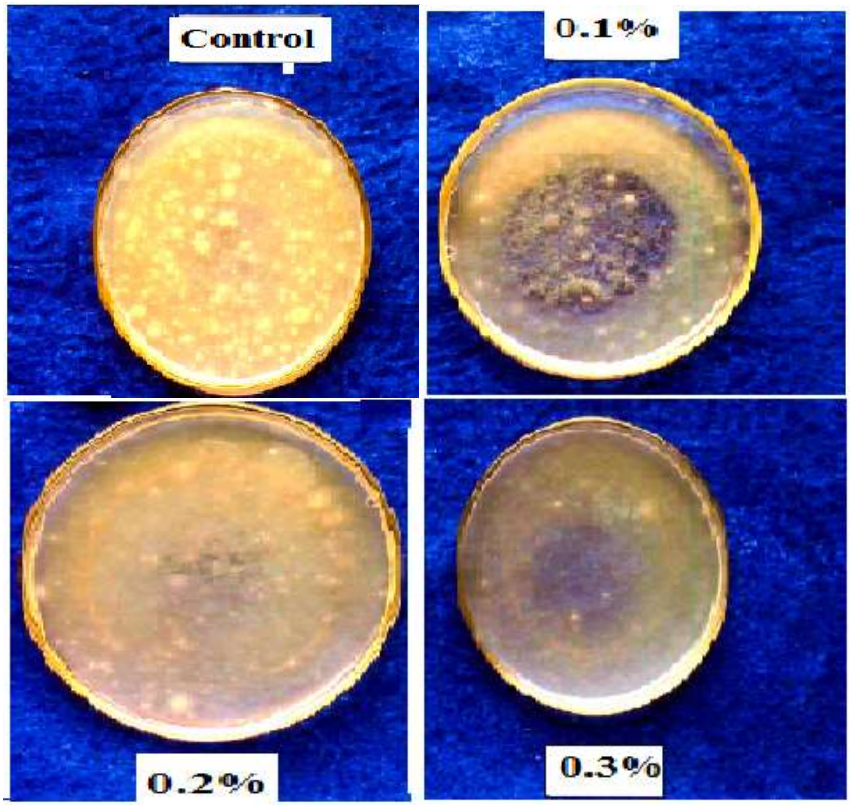

Photo 1: *Aspergillus fumigatus* after Colchicine treatment (Control, 0.1%, 0.2% and 0.3%).

Effect of Colchicine concentration (0.1%) on cellulase enzyme activity for 60 minutes in terms of CX, C1, Filter paper and β-glucosidase.

The spores of both 60 minute UV irradiated *A. fumigatus (Af1)* were treated with 0.1 % (W/V) concentrations of colchicine for a period of 60 minutes and the observation were recorded on the bases of enzyme assays in terms of CX, C1, Filter paper and β-glucosidase for both the strains and the highest enzyme activity was recoded with 0.1% (W/V) concentration of colchicine on 11th day of inoculation as shown in table 12, 13, 14 and 15(Figure1). The observations are the average of three individual readings.

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 2).

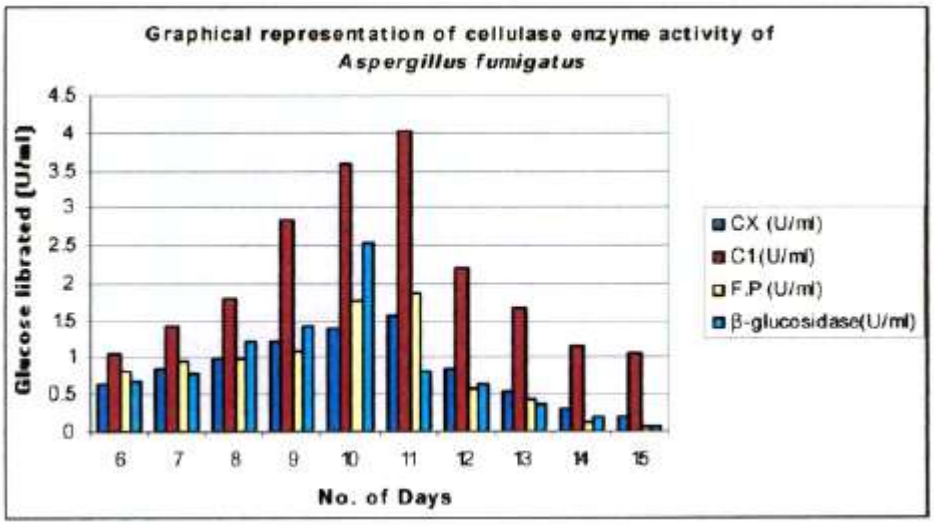

Fig2: Cellulase enzyme activity of *Aspergillus fumigatus* after colchicine (0.1%) treatment for 60 min.

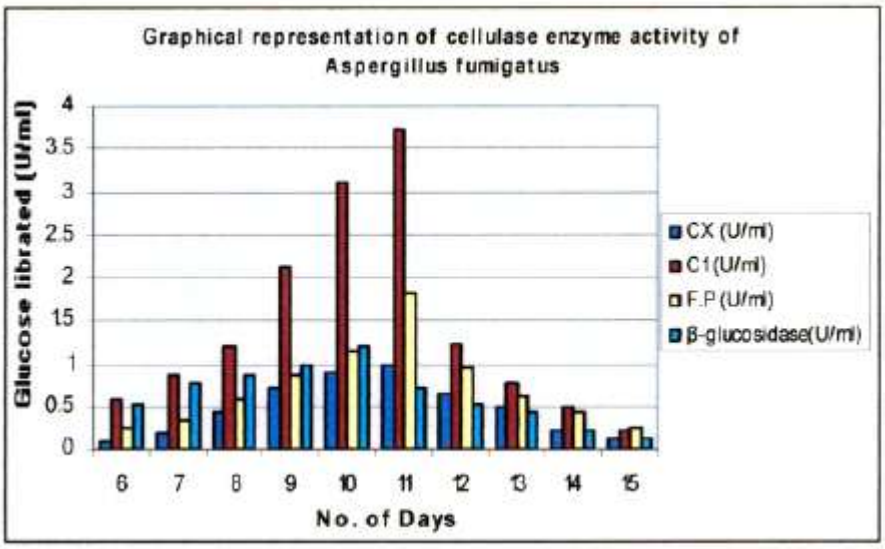

Fig3: Cellulase enzyme activity of *Aspergillus fumigatus* after colchicine (0.2%) treatment for 60 min.

Comparison of *Aspergillus fumigatus* (Af1) cellulase and β-glucosidase with those of wild and UV mutated fungi: A comparative study was done to find the increase in the production of cellulase enzyme against wild strain of *Aspergillus fumigatus* and the mutated strain *Aspergillus fumigatus* (Af1) as well as with different species. The result is shown in terms of CX , C1, β-glucosidase and Filter paper (Table 2 and Table 3).

Table2: Percentage variation in cellulase yield after mutation in *Aspergillus fumigatus (Af1)*.

Enzyme	Wild type	Mutation	
		Colchicine treatment	
		0.1%	0.2%
CX	0.16	1.55(89.6%)	0.97(83.5%)
C1	0.24	4.03(40.4%)	3.7(35.1%)
Filter paper	0.1	1.86(94.6%)	1.81(94.4%)
β-glucosidase	0.97	2.54(96.18%)	1.2(91.9%)

Table 3: Comparison of the effect of mutagens on the basis of percentage increase in enzyme yield.

Fungal Type	Enzyme	Mutation	
		UV irradiation % increase in enzyme yield	Colchicine % increase in enzyme yield
Aspergillus fumigatus	**CX**	36-54%	83-85%
	C1	20-33%	35%-40%
	Filter paper	16-70%	94%
	β-glucosidase	50-77%	91-96%

DISCUSSION

Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulase is widely distributed throughout the biosphere and most manifest in fungal and microbial organisms. Cellulase enzymatic system consists of three major components, endo-β-glucanase, exo- β-glucanase and β-glucosidase.

To improve the efficiency of cellulase production, these strains were subjected to mutation by UV irradiation and Colchicine. The data obtained led us to the conclusion that 60 minutes of UV exposure help in increasing cellulase production significantly. The strain improved by UV irradiation method was then subjected to chemical mutation using Colchicine in 0.1 and 0.2 % (W/V) concentration. It was found that 0.1% (W/V) concentration of Colchicine increases cellulase production many folds.

Thus, it can be concluded that increase in the cellulase enzyme production by mutation using irradiation like UV and chemical mutagen like Colchicine is possible to be increased up to a considerable amount. Since the scopes to increase the production by the use of genetically and molecular engineering technique to gain the full potential of these strains are immense, further work with these strains can be carried out in future.

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