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KINETIC STUDY AND BASAL MEDIA MODIFICATION OF CELLULASE ENZYME PRODUCTION BY MUTATED STRAIN OF *ASPERGILLULS FUMIGATES* (AF1)

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ABSTRACT

Kine and colchicine t tic parameters play important role in microbial cellulase production. After mutation with UV irradiation he highest cellulase producing *Aspergilluls fumigates* (Af1) stain is used to study the effect of culture conditions. It was found that Lactose 1%(W/V) was best carbon source, Carboxy methyl cellulose (CMC) 3% (W/V) as the best substrate concentration, pH 6 is found to be best suitable pH for high cellulase production. Peptone served as best nitrogen source at 35^0C significant increase in cellulase production was observed. In compression to wild strain 30 to 90% increase in cellulase production concluding *Aspergilluls fumigates* (Af1) as a better source of cellulase enzyme.

KEY WORDS: ***Aspergillus fumigatus, Kinetics, Carboxy methyl cellulase, Cellulase enzyme, Cellulose***

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INTRODUCTION

Many microorganisms in nature can grow on cellulose or produce enzymes that can degrade amorphous cellulose; relatively few produce the entire complement of extra-cellular cellulases in vitro. Among the latter organisms, the most extensively studied sources of cellulolytic enzymes have been the fungi *Trichoderma* spp., *Aspergillus* spp. and *Phanerochaete* and the bacteria *Cellulomonas* and *Clostridium thermocellum*[1]. Cellulase is not a single enzyme but it is a group of enzyme which include five different types of enzymes namelyβ-glucosidase (β- D-glucoside glucohydrolase), an essential component of the cellulase complex, is responsible for the final step in cellulose degradation, namely, the hydrolysis of cellulose- derived cellobiose to glucose. The β-glucosidase not only produces glucose, but also reduces the cellobiose inhibition, thus allowing other cellulolytic enzymes to function more efficiently[2]*Trichoderma* species, which seem to be the best source of cellulose solubilizing activities, are poor producers of β –glucosidase. On the other hand, *Aspergillus* species have been shown to be better producer's ofβ - glucosidase. Hence, in recent years, considerable attention has been focused on bulk production of β–glucosidase[3]. Studies done shows that the UV and Colchicine mutated *Aspergillus fumigatus* shows high production of cellulase enzyme[18] which helped us to choice *Aspergillus fumigatus* for kinetic studies. Different nutrients present in the medium play important role in the production of enzyme and many workers studied it Nitrogen, carbons are the most studied of them. Some showed that the presence of ammonium salts in the medium caused a depressed level of enzyme production whereas studied by, Trivedi and Rao[4] showed that ammonium salts facilitated toe cellulase production. It was recorded that use of inorganic nitrogen source enhances the production of cellulase[5]. To study the effect of carbon source on cellulase production different carbon sources like cheese whey, bagasse, rice straw, choirs waste and saw dust were used as natural carbon source. In case of *Trichoderma reesei* it is capable of fermenting these substrates and efficiently producing cellulase enzymes.

MATERIALS AND METHODS

Collection of samples

For the collection of sample plastic bags and were used. Which were pre-sterilized 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 were done on the bases of macroscopic and microscopic characterization and by consulting books "Fungal biotechnology in Agricultural, Food and Environment Application"[6], "Environmental Microbiology: A laboratory manual"[7].

Enzyme Assays

Enzyme Assays were performed according to Mandels[8]. 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[9].

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 the 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 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.

Inoculum preparations for Kinetic studies

For inoculum preparation conidia were harvested from 10 day old stock cultures by adding 5ml of sterile distilled water to the agar slant and re-suspending them. The spore concentration in the conidial suspension was determined by counting the spores in Haemocytometer (Feinoptik, Germany). Inoculum of 1ml of spore suspension ($4X10^8$ spores/ml) was inoculated into a 250 ml flask containing 150 ml inoculum medium. The initial pH of the sterilized medium was adjusted to 5.5 by adding 10% (v/v) H_2SO_4 before inoculation and no pH control was done during cultivation. Inoculum was incubated on a rotary shaker with an agitation rate of 150 rotation/min at 30°C for 4 days and then used to inoculate the fermentation medium[10].

Mutation using UV irradiation and 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[18] to wild strain and UV irradiation to develop mutant. In this method (Jefferies; 1987) l ml of appropriately diluted spores ($4 X 10^8$) was mixed in 10ml of distilled water. UV lamp (Samson E-27/ 160W/V, Germany) was fitted 1 meter away from the open Petri dish containing the spore suspension, l ml of sample was removed before irradiation and plated on Mandels and Weber, (I960) agar media (as described in section 3.3) with 17.5g/L of agar. Treatment was given for 30, 60, 90,120 and 150. Treated samples were removed at 30, 60, 90,120 and 150 minutes and plated on Mandel and Weber, (1969) agar medium. Plates were incubated in dark for the first 24 hours to prevent photoreactivation. After 24 hours Petri plates were incubated at 30°C. Colony counting was done to find lethal dose (LD_{50}) of UV irradiation and a graph was prepared. Repeated sub culturing was done on Czapek –dox agar medium. The spores from the treated samples were then incubated in basal media (as mentioned in section 3.2.3) to study the effect of UV irradiation on the enzyme on the bases of CX, C1, filter paper and β-glucosidase yield. The stain with highest activity was then used for Colchicine treatment.in which different concentrations of Colchicine ($C_{22}H_{25}NO_6$ SRLx 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 cultured 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-glucosidase).

Effect of Temperature

To study the effect of Temperature six 250 ml flask with 100ml of basal medium were incubated with Af1 stain (showing highest activity after mutation) at different temperature range from 30, 35, 40, 45, 50, and 55^0 C. On 11^{th} day (day of highest activity recorded) all the flask were withdrawn and enzyme activity was recorded (Figure 1).

Effect of Substrate concentration

To study the effect of substrate concentration five 250 ml flask with 100ml of basal medium with different concentrations of Carboxy methyl cellulose (CMC) in 1, 2, 3 and 4% (W/V) were incubated with Af1 stain (showing highest activity after mutation) at 30^0 C. On 11^{th} day (day of highest activity recorded) all the flask were withdrawn and enzyme activity was recorded (Figure 2).

Effect of pH

To study the effect of pH seven 250 ml flask with 100ml of basal medium and pH of the medium was adjusted from 3.5 to 6.5 and were incubated with Af1 stain (showing highest activity after mutation) at 30^0 C. On 11^{th} day (day of highest activity recorded) all the flask were withdrawn and enzyme activity was recorded (figure 3).

Effect of Carbon source

To study the effect of carbon source like Carboxy methyl cellulose, Sucrose, Glucose, Lactose and Maltose were used in a concentration of 1 % (W / V) in the basal media. All the other factors were kept constant a flask without carbon source was taken as control. All the flask were incubated with Af1 stain (showing highest activity after mutation) at 30^0 C. On 11^{th} day (day of highest activity recorded) all the flask were withdrawn and enzyme activity was recorded (figure 4).

Effect of nitrogen source

To study the effect of nitrogen source like Protease peptone, KNO_3, $NaNO_3$, Urea, $(NH_4)_2SO_4$ in 0.57g/L (W/V) were used in the basal media. All the other factors were kept constant a flask without nitrogen source was taken as control. All the flask were incubated with Af1 stain (showing highest activity after mutation) at 30^0 C. On 11^{th} day (day of highest activity recorded) all the flask were withdrawn and enzyme activity was recorded (figure 5).

Effect of Surfactant

To study the effect of surfactant on cellulase production Tween 20 and Triton- X100 were used at different concentrations ranging from 0.05 to 2% (V/V). A flask contorting no surfactant was taken as control. All the flask were incubated with Af1 stain (showing highest activity after mutation) at 30^0 C. On 11^{th} day (day of highest activity recorded) all the flask were withdrawn and enzyme activity was recorded (figure 6).

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).

RESULTS AND DISCUSSION

Effect of temperature

The rate of enzyme catalyzed reaction generally increases with the increase in temperature within a certain range in which the enzyme is stable and retains full activity. The rate of most of the enzymatic reactions approximately doubles with every 10°C increase in temperature. The enzyme and substrate concentration were kept constant and only the temperature of enzymatic reaction is increased[5]. To study the effect of temperature on cellulase production tire cultures were maintained on temperature ranges: 25, 30, 35, 40, 45 and 50 after inoculation in the basal medium. The different enzyme activities were assayed and recorded (Figure1).It was found that Af1 show highest enzyme activity on 35°C.

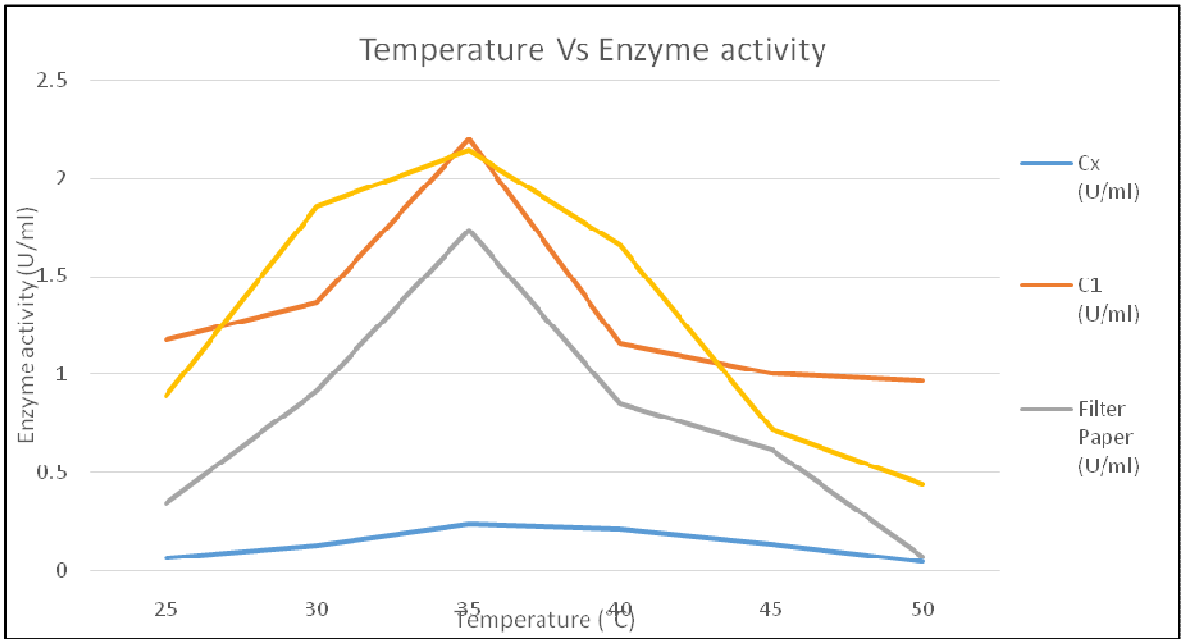

Figure 1
Effect of temperature on cellulase enzyme production.

Effect of Substrate concentration on Enzyme activity

To study the effect of concentration of substrate which is Carboxy methyl cellulose (CMC) on enzyme activity Af1was grown on basal medium with substrate concentrations 1%, 2%, 3% and 4% (W/V). The different enzyme activities were assayed and recorded (Figure2). The results given here are the average of three individual readings. The highest enzyme assays were found at 3% (W/V) concentration of CMC in basal medium. A report by Andreotti[11] show significant increase in cellulase production with 2% (W/V) concentration of cellulose in medium with *T.reesei* Qm 9414. In contrast, Shewale and Sadana[12] reported that concentration of cellulose in medium does not affect cellulase production, but affect the fermentation pattern giving rise to early enzyme development in *Basidiomycetes* species.

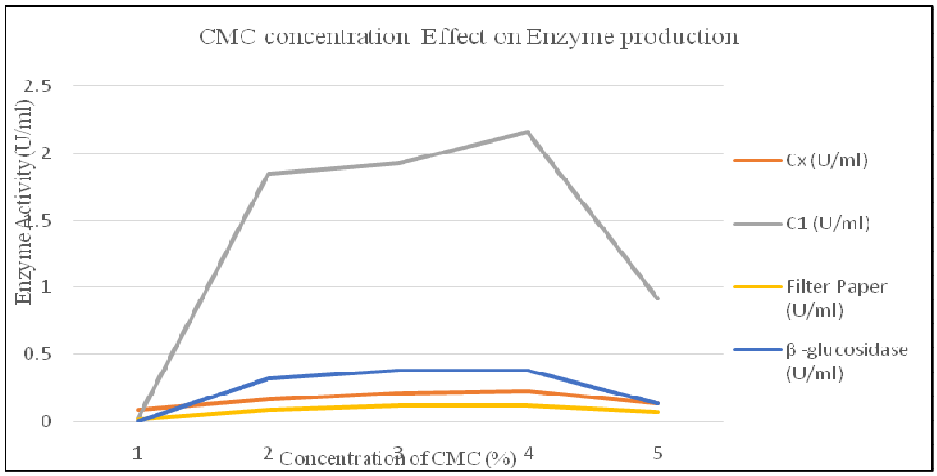

Figure 2
Effect of CMC on cellulase enzyme production.

Effect of pH on Enzyme activity

The effect of initial pH on cellulase production by the fungal strains Af1 was investigated within the range of 3.5 to 6.5. The maximum activity of C1, CX, Filter paper and β-glucosidase were recorded on 1 lth day as recorded (Figure 3). The maximum production of different cellulase was obtained with an initial pH of 6.0. Desai[5] found that *Scytalidium lignicola* showed maximum enzyme production when pH reached around 6. Trivedi and Rao[4] found that optimum pH for cellulase production by *Aspergillus fumigatus* to be 5. These values are higher than those reported by other workers [11, 13, 14].

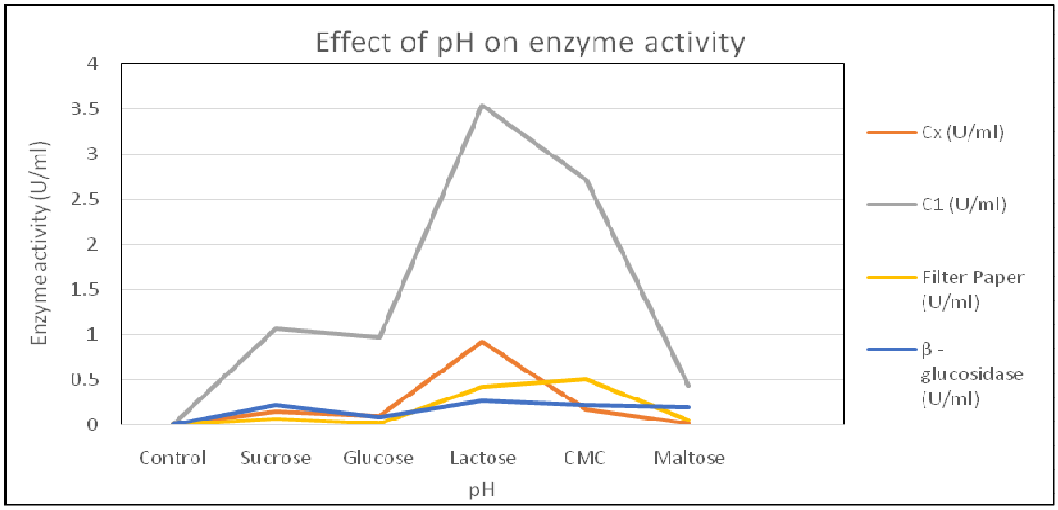

Figure 3
Effect of pH on cellulase enzyme production of Af1.

Effect of Carbon source on enzyme activity

The various soluble carbon sources which were taken for the assessment of their effect on cellulose production were: Carboxy methyl cellulose, Sucrose, Glucose, Lactose and Maltose. By taking 1% (W/V) concentration of the above mentioned carbon sources in the basal medium. It was observed from die studies shows that little amount of cellulase was produced when *Aspergillus fumigatus* was grown in medium with carbon source like Carboxy methyl, cellulose, Sucrose, glucose and Maltose yield was found to be 90 X 10^1U/mL, C1 assays was 35 x $l0^1$U/ml, fiber paper activity was found to be 54 x $l0^1$U/ml and glucosidase was found to be 2 x 10^1 U/ml. Significant enzyme activity was produced when Lactose was used as chief carbon source. The fungus showed visible growth after 6th day of inoculation and rapid growth was seen in medium containing lactose as chief carbon source. Similar observation was recorded when *Aspergillus flavus* was grown in medium having lactose as sole carbon source[15]

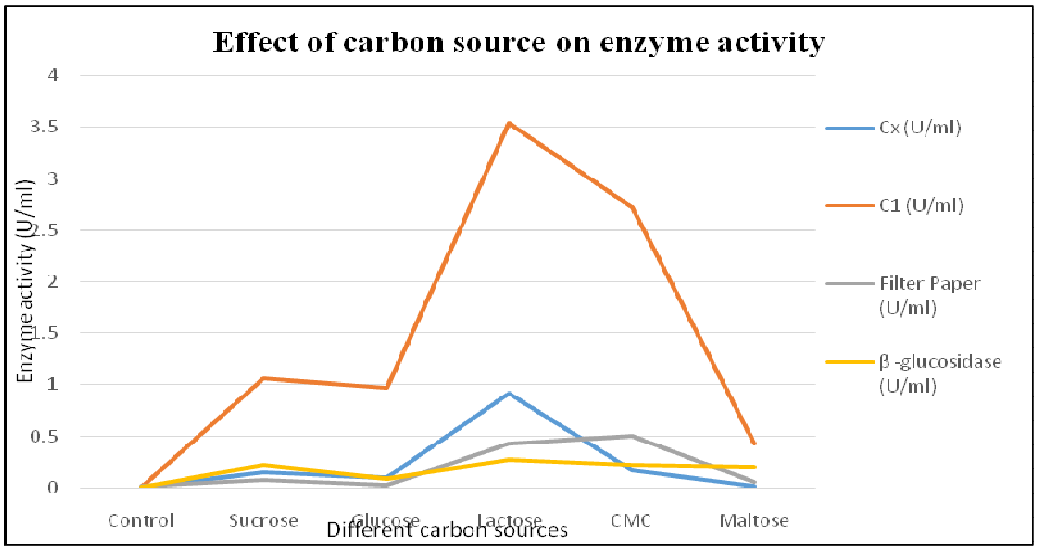

Figure 4
Effect of carbon source on cellulase enzyme production of Af1.

Effect of nitrogen source on enzyme activity

The various soluble nitrogen sources which were taken for the assessment of their effect on cellulase production were: protease peptone in combination with KNO_3, $NaNO_3$, Urea, $(NH_4)_2SO_4$ in 0.57g/L (W/V) concentration of each of the above mentioned nitrogen sources in the basal medium the different enzyme activities were assayed using fungal strains Af1and the results were recorded in Figure 4. The preliminary report by Trivedi and Rao[4]showed that ammonium salts facilitates the cellulase production by *Aspergillus fumigatus.* It was found that when peptone was partially replaced by an inorganic nitrogen source, cellulase activity decreases, this is in contrast to die report of Desai[5] found least enzyme production with peptone.The reason may be attributed to the salt concentration might have adverse osmotic pressure on the fungal cells.

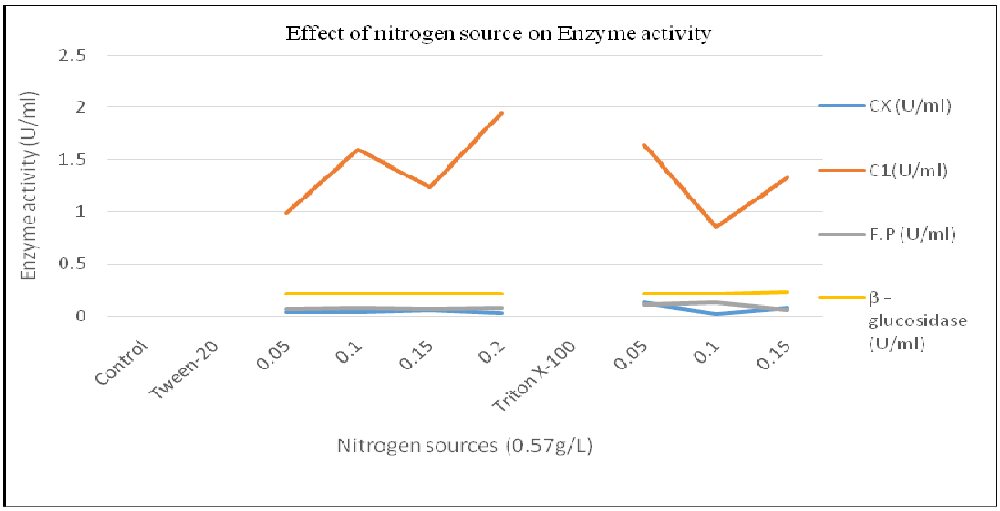

Figure 5
Effect of nitrogen source on cellulase enzyme production of Af1.

Effect of Surfactant on enzyme production

To see the effect of surfactant in cellulase production, Tween-20 and Triton X- 100 were taken as the surfactants in different concentrations ranging from 0.05 to 0.2% (V/V). The effect was studied on strains Af1. On 11th day of inoculation the highest activities of enzymes were found in 0.15% (V/V) concentration of both the surfactants as recorded in Figure5. Significant increase in cellulase activity was observed at 0.15% (V/V) concentration of Tween-20 in both the strains. Similar result was obtained by Desai[5] with *Scytalidium ligmcola* CD-48. Reese and Maguire[16] reported doubling of cellulase production with Tween-80 and Tween-40 by *Trichoderma reesei.* A similar effect was also found when

TritonX-100 (Polyethylene glycol alkypenyl ether) was used in 0.15% (V/V) concentration in basal medium with *Aspergillus fumigatus.* No toxic effect of TritonX- 100, was found on the growth of both the strains. Several workers reported increase in extracellular enzyme production with surfactants[16, 17].

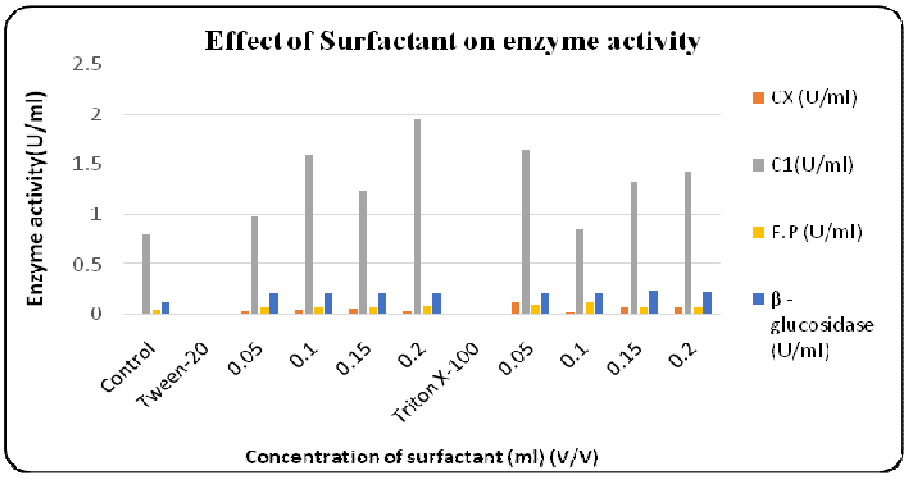

Figure 6
Effect of surfactant on cellulase enzyme production of Af1.

Effect of modified medium on cellulase production

The results obtained from kinetic studies were used to modify the basal medium[10]. The enzyme activities of the strain Af1 was studied in the modified medium (M2) and the results obtained were recorded in figure7.

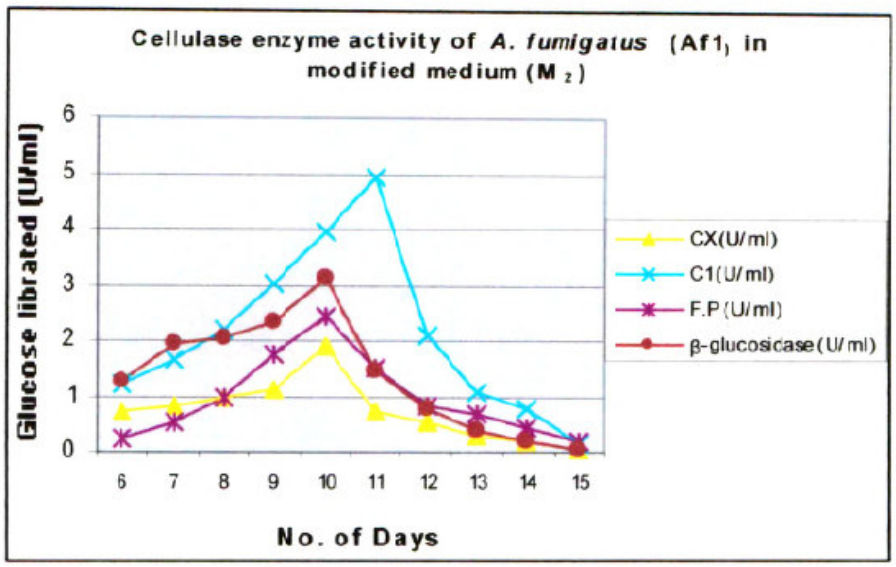

Figure 7
Cellulase enzyme activity of Af1 in modified medium (M2).

On the basis of kinetic studies the basal medium was modified and enzyme production of *Aspergillus fumigates* was measured in a modified medium (M2). The results obtained in M2 show 30 to 90% increase in enzyme production of *Aspergillus fumigatus.* Thus, the present workemphasize the role of culture conditions play an important role in the production of cellulase enzyme. By optimizing the culture condition the production can be increased to a significant level.

REFERENCES

1. Conghlan, M.P. Microbial Enzymes and Biotechnology, 2nd edition (Elsevier): 1-36, (1990)
2. Perry J.B., Stewart, J.C., and Heptinstail, J. Purification on the Major Endoglucanase from *Aspergillus fumigatus. Journal of Biochemistry:* 213,(1983)
3. Mandel, M., and Reese, E. Fungal Cellulases and the Microbial Decomposition of Cellulosic Fabric,Developments, Developments in Industrial Microbiology , Amer. *Institute of Biology Science,* Washington, D.C., USA-5: volume (5), (1964)
4. Trivadl, L. S and Rao, K. K. Production of cellulolytic enzymes by *Aspergillus fumigatus, Indian Jr. of Expt. Biology* 17: 671-674,(1979)
5. Desai, J. D. and Patel, N. P. Production of cellulose and (1- glucosidase by shake culture of Scytalidium lignicola. *Jr. Fermentation Technology:* 117-124, (1982)
6. Dubey, R. C., and Maheshwari, D. K. Practical microbiology, S. Chand publication, India: 39-43, (2002)
7. Charles, P., Gcrba; Ian, Pepper L. Book on Environmental Microbiology: laboratory Manual, S.Chand Publication, India, 175, (2005)
8. Mandel, M. Enzymatic hydrolysis of waste cellulose, Biotechnology, *Bioengineering symposium* (wilk, C. R.). 5, 81, Wiley and sons, New York, (1975)
9. Jeffries, T.W. Production and applications of cellulase laboratory Procedures, Forest products Laboratory, Madison, Wiscosin, (1987)
10. Chand, P., Arana A, Maqsood M.A. and Ran L.V. Novel mutation method for increases cellulase production; *Journal of Applied Microbiology* volume (2):318, (2005)
11. Andreotti, R.E., Mandels, ML, and Roche, C. Bioconversion of Cellulosic substance into energy, chemical and Microbial Proteins, Indian Institute of Technology, New Delhi: 249(1977)
12. Shewale J.G. and Sadana J.C. Enzymatic hydrolysis of cellulosic materials by Sclerotium rolfsii culture filtrate for sugar production. Can. J. Microbiol.25:773-783, (1979).
13. Sternberg, D., and Mandels, G.R. Induction of cellulolytic enzymes in Trichoderma reesei by sophorose. J Bacteriol. Volume(139):761-9,(1979)
14. Wood, T. M., and McCraeS.I. The purification and properties of the C1 components of Trichoderma koningii cellulase. Biochem. J.(128): 1183–1192, (1972)
15. Baishya, D., and Deka, M. Studies on effect of carbon source, nitrogen source, pH and surfactant on CX, C1 and filter paper activities of cellulase enzyme form Aspergillus flavus. Asian Journal of Microbiology Biotechnology and Environment Science.vol(8): 345-348,(2006)
16. Reese E.T, and Mandels M. Fungal cellulase and microbial decomposition of cellulosic fibres. Dev. Ind. Microbiol. vol (5): 5 – 20, (1985).
17. Reese, E.T, Lola J.E, and Parrish F.W. Modified substrate products as inducers of carbohydrases. J. *Bacteriology, 100(3*): 1151-1154,(1969)
18. Johra k., Amal A., and Deka M. Effect of colchicine induced mutation on cellulase enzyme production by Aspergillus fumigatus. World journal of pharmaceutical research. Vol (4); 461-471,(2015)