Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **2 (3):** 16-22 (2014)

Research Article

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

Saccharification of Baggase, Paper waste by cellulase produced from *Aspergillus flavus*

Swati Pandit*, Kapil Lawrence, Anupama Singh and Sangya Singh
Department of Biochemistry and biochemical Engineering, SHIATS, Allahabad, UP
*Corresponding Author E-mail: swati.pandit8@gmail.com

ABSTRACT

The efficiency of cellulase produced by Aspergillus flavus (NFCCI 2703) in bioconversion viasolid state fermentation using paper waste and bagasse as substrates was studied. Optimization parameters and effect of co-inducers on production of FPase, CMCase, and cellobiose were assayed. Optimum pH for paper waste was 4.5 and 5.5 for bagasse, 30^{0}C was optimum for both substrates, moisture content was found to be 85% and 90% for bagasse and paper waste respectively. Sucrose was found to be best co-inducer for bagasse and enhanced the activity by 21.91%, 11.11%, 11.77% of CMCase, FPase and cellobiase respectively. Using paper waste as substrate the CMCase, FPase and cellobiase activities were enhanced by 21.6%, 34.65%, 29.55%, respectively by cellulose. Bioconversion of paper waste was 20% more as compared to bagasse though; higher concentrations of substrate and incubation period inhibited the enzyme. This study reports that cellulase produced by A. flavus (NFCCI 2703) was more effective in bioconversion of paper waste as compared to bagasse.

Keywords: *Bioconversion, cellulase, solid state fermentation, baggase.*

INTRODUCTION

Efficient cellulose hydrolysis requires the cooperative action of endoglucanases (EC.3.2.1.4) which hydrolyze the cellulose polymer internally, exposing reducing and non-reducing ends and exoglucanases or cellobiohydrolase (EC. 3.2.1.91) which act on the reducing and nonreducing ends, releasing cellobiose and cellooligosaccharides. The cellulose hydrolysis process culminates through the action of β-glucosidase (EC. 3.2.1.21) which cleaves cellobiose, liberating two molecules of glucose[1].

There is a vast interest in using cellulose degrading microorganism to convert cellulosic biomass to glucose that can be used in different applications viz. bio-fuel ethanol, use in animal feed, use in waste water treatment and brewing industry. Most research has been focused on more efficient cellulose degrading microorganisms viz. *T. ressei, T.viridie, Chysoporium lignorm , A.niger, Fusarium solani* etc. There are also serious concerns over the consequence of environmental pollution resulting from the increased consumption of fossil fuel. As a result, there is a growing interest in converting biomass especially agricultural waste to bio- fuels as an alternative energy supply[15,18,24].

The genera *Aspergilli (A. niger, A. tubingensis, A. foetidus, A. carbonarius, A. japonicus, A. aculeatus, A. heteromorphus, and A. ellipticus)*, have a number of characteristics which make them ideal organisms for cellulase production which include good fermentation capabilities, high levels of protein secretion, ability to assimilate various organic substrates, suppressing the development of other microorganisms and high sporulation capacity (Devries *et al* 2001). Sparse research has been conducted for tapping the potential of *A. flavus* as a cellulase producer.

Since high cost of production and low yields of cellulase are problems in it's industrial application therefore, investigation on ability of microbial strains to utilize inexpensive substrate and improvement of enzyme productivity is essential. The selection of fungal strain, the expression levels of cellulase is determined by the composition of the medium and growth conditions.

The metabolic properties of the microorganism are influenced to great extent by changes of temperature; pH, substrate and optimum culture condition vary widely between species of same organism.

The objective of the present work includes optimization of cellulose degradation & saccharification of pre-treated sugarcane bagasse and paper waste as substrates and further saccharification of the substrates using cellulase produced from *A. flavus* [NFCCI 2703] isolated in our laboratory was used.

MATERIAL AND METHODS

Microorganism cultivation

Aspergillus flavus isolated from the soil of Allahabad (UP) and cultured on (PDA) with CMC (1.2% w/v) for 3-5 days at 32^0C and identified at NlFCCI Pune, INDIA (NFCCI 2703).

The following composition of basal media (g/L) was used for the production of cellulase: urea 0.3, $(NH4)_2SO_4$ 1.4, KH_2PO_4 2, $CuCl_2$0.3, Polypeptone 1, $MgSO4.7H_2O$ 0.3 and trace elements were $FeSO_4.7H_2O$ 5, $MnSO_4.4H_2O$ 1.6, $ZnSO_4$ $7H_2O$ 1.4, $CoCl_2.6H_2O$ 2 : pH of the medium was adjusted 5 ± 0.2 with 0.1N NaOH. [9] Medium and trace elements were autoclaved separately. Briefly, 5g of substrate was added 50 mlN basal medium (1:10 w/v). These flasks were inoculated with 5ml (1x105spore/ml) of substrate and the contents were incubated at 28± 1^0C for 5 days. Thereafter 0.1M citrate buffer pH 4.8 was added to a final volume of100ml/flask. The flasks were agitated at 200 rpm for 1hr then filtrate used for determining the enzyme activity.

Preparation of substrate

Pretreatment of sugarcane baggase: Baggase was chopped into small pieces and ball milled, Sieved over a mesh and pretreated with 1%NaOH (w/v) for one hour in a boiling water bath. Substrate was cooled and washed till neutral pH. The substrate was oven dried at 65^0C±2^0C till constant weight.

Pretreatment of paper waste Paper was cut into pieces and washed with distilled water. Waste paper was treated with 1%NaOH (w/v) and 0.7%H2O2 (v/v) [10] for one hour in a boiling water bath with bath ratio 1:10. Substrate was washed wit h distilled water till neutral pH substrate was oven dried at 65^0 C±2^0C till constant weight.

Media and growth condition

The protocol followed was as reported in (Pandit *et al*. 2013), Briefly, SSC was carried out in 100ml Erlenmeyer flasks containing the media and trace elements were autoclaved, 5g of dry substrate was added to each flask, inoculated with 5ml spore suspension (1x10^6 spore ml^{-1}) and incubated at 30^0C±2^0C under static conditions.

Optimization of production condition

The enzyme production was optimized at different moisture levels (30-70ml) of basal media and was monitored at pH 3.5-7.5; enzyme production was observed at 24 h intervals up to 240h. The carbon sources (1% w/v) viz. Glucose, sucrose, fructose, cellulose were added in basal media to evaluate the best co-substrate for cellulase production.

Enzyme and protein quantification

Endoglucanase, FPase, activity were assayed as given in[8] Reducing sugars liberated was quantified by dinitrosalicylic acid (DNS) reagent using glucose as a standard[14]. One unit of enzyme activity was defined as the amount of enzyme producing 1µmol reducing sugar minute under assay conditio.Cellobiase acitivity was measured using the cellobiose as substrate[8]. All assays were carried out at 50^0C. One unit of enzyme activity was defined as the amount of enzyme liberating 1µmol of product $minute^{-1}$

Enzymatic Saccharification

Enzymatic hydrolysis of alkali pretreated baggase and paper waste was performed in a reaction mixture containing 25gL^1 and 50gL^1(w/v) substrate in 0.1M citrate buffer (pH4.8), supplemented with enzyme (2FPU/ ml). The mixture was incubated at 32^0C on a rotatory shaker at150rpm. Samples were taken from the reaction mixture at different time intervals. Samples were immediately heated to 100^0C, cooled and centrifuged for 10min at 800rpm. The supernatant was used for the reducing sugar analysis. The liberated reducing sugar was assayed by DNS method[9,21].

RESULTS AND DISCUSSION

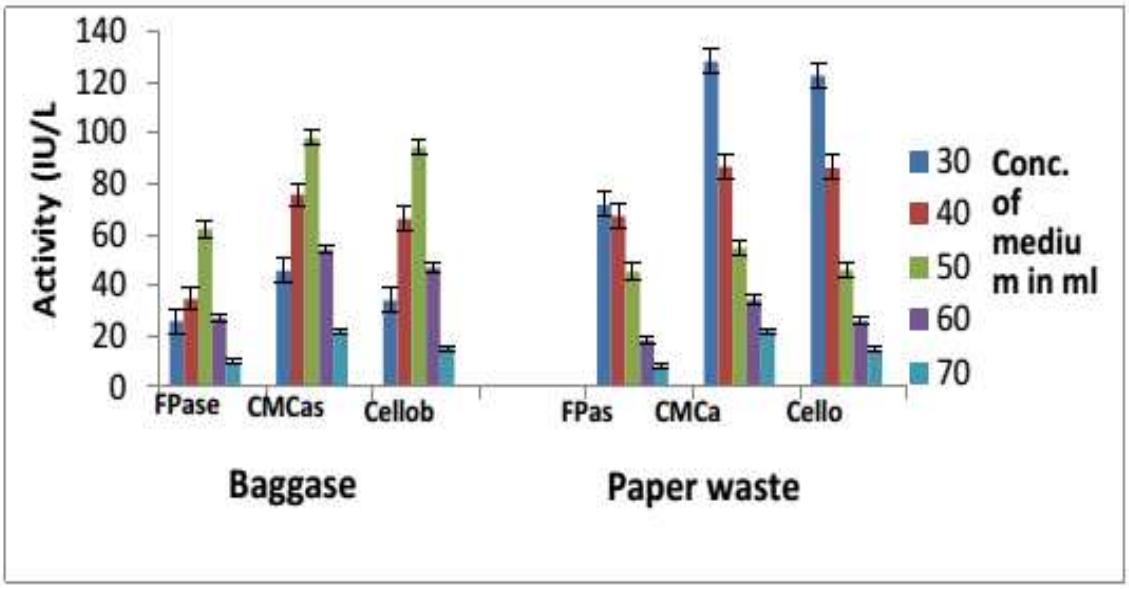
Pretreatment of substrate

Prior to pretreatment hemicellulose content of bagasse and paper waste was 45.6% (w/v) and 52.5% (w/v) respectively. After pretreatment it decreased by 14.2 %(w/v) and 16.8 %(w/v). Similarly with hemicellulose content changes the percent of lignin decreased by 15.6 %(w/v) and 16.8 %(w/v) respectively.

Effect of initial moisture content

Moisture content of the growth medium is one of the most important variables of an SSC process[7] cultivation of microbial cells in excess of water can lead to particles sticking limited gas exchange and higher vulnerability to bacterial contamination. While low moisture level reduced microbial growth, enzyme stability, substrate swelling and diffusion of nutrients. The highest enzyme activity was obtained when initial moisture content of the growth medium was 50 and 30ml for bagasse and paper waste respectively. The optimum moisture content for enzyme production under SSC is dependent upon the water binding properties of the substrate as well as the microorganism used[11,13,19].

Fig.1: Effect of initial moisture content (different volumes of growth media) on enzyme activity



Effect of pH and temperature

Controlling the pH of a multiphase growth medium is not an easy task, mostly small scale SSC deals with the effect of initial pH of the solid substrate on process outcome[19]. Effect of initial pH on enzyme production, *A. flavus* cells grown under SSC is exhibits in (Fig.2). Production of all the three components of *A. flavus* cellulose degrading system was maximized at acidic pH values. The highest cellulase activity was observed at acidic pH value viz. 4.5 for paper waste and 5.5 for sugarcane bagasse. Increasing the pH of growth medium led towards the reduction in activity of enzyme. Thus, higher pH was found to be unfavorable for the production of all three enzymes. Some others have also reported the production of some other fungal cellulases is reduced at higher pH range[17].

When evaluated as a function of temperature, production of all the three enzymes was noticed to be maximal at 30^0C for both the substrates (Fig.3). The studies suggest a variation in optimal temperature 25^0C (pH 6.7) [22] and 30^0C for pH 4.5 Higher temperature inhibits the production of enzyme. Some author reported that a decrease in enzyme production with increase in temperature in *A. niger*[20]. This study suggests that optimal pH and temperature for cellulase production by *Aspergilli* may vary from species of same genus and for different substrate.

Fig.2: Effect of pH on enzyme activity

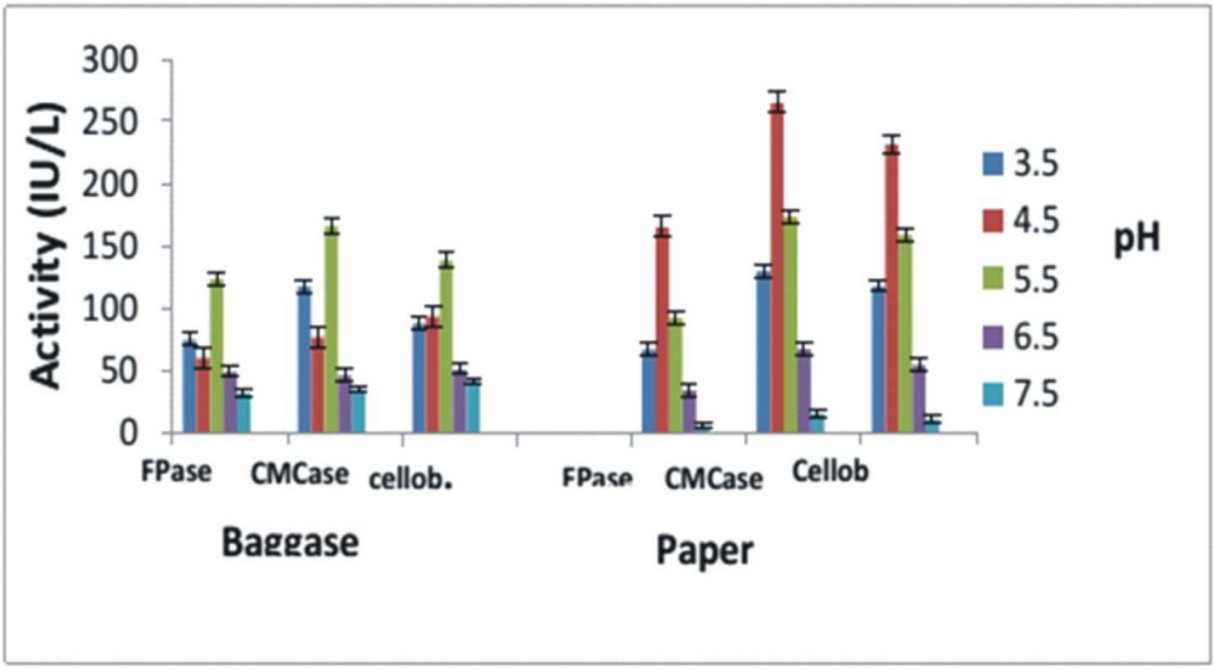
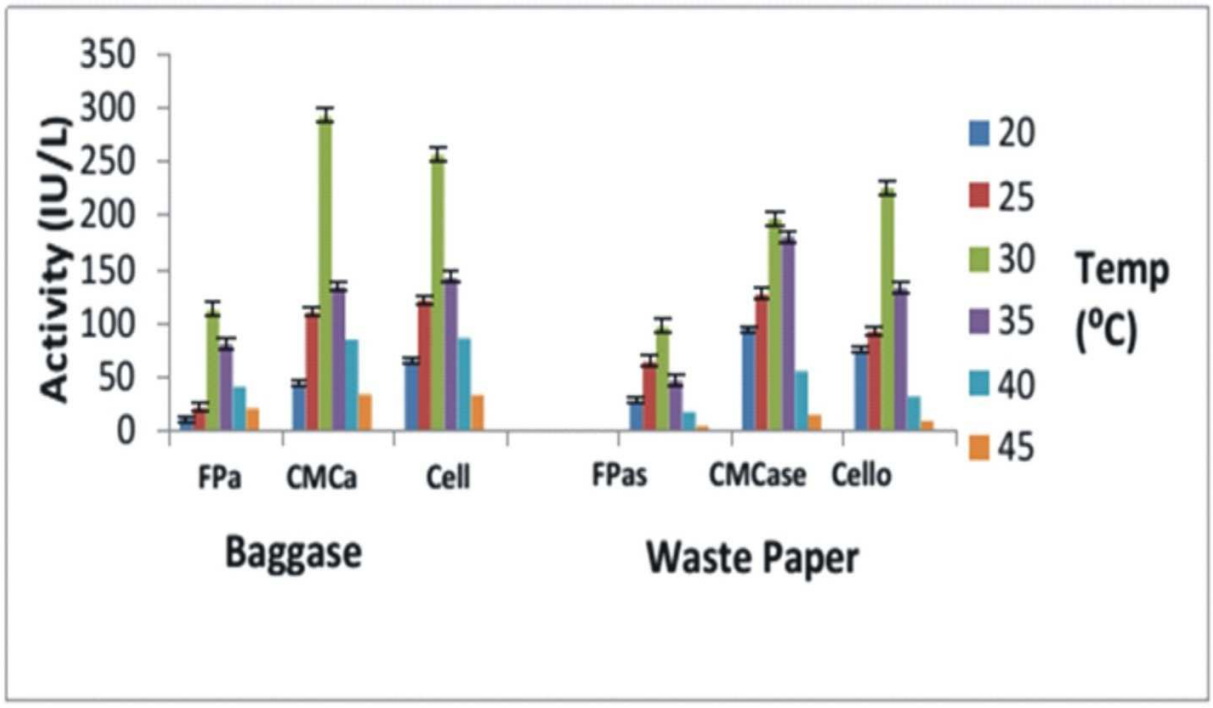


Fig.3: Effect of temperature on enzyme activity



Effect of incubation period and co-inducers

The effect of carbon sources on cellulase production is presented in Fig (4).Enzyme activity was measured at regular intervals of 24 h for duration of 240 h. The highest FPase and CMCase activity and cellobiose was obtained after 120h of incubation for baggase (Fig.5) and 96h for paper waste as shown in fig (6). Length of incubation period and carbon sources play a key role in growth of the micro-organism and subsequent enzyme production[23]. Enzyme activity of the enzymes assayed (FPase and CMCase, cellobiose) declined on prolonged incubation (> 120 h.), this could possibly be due to loss of moisture/ denaturation of enzymes/ depletion of C-sources[16].

Fig. 4: Effect of incubation period of bagasse

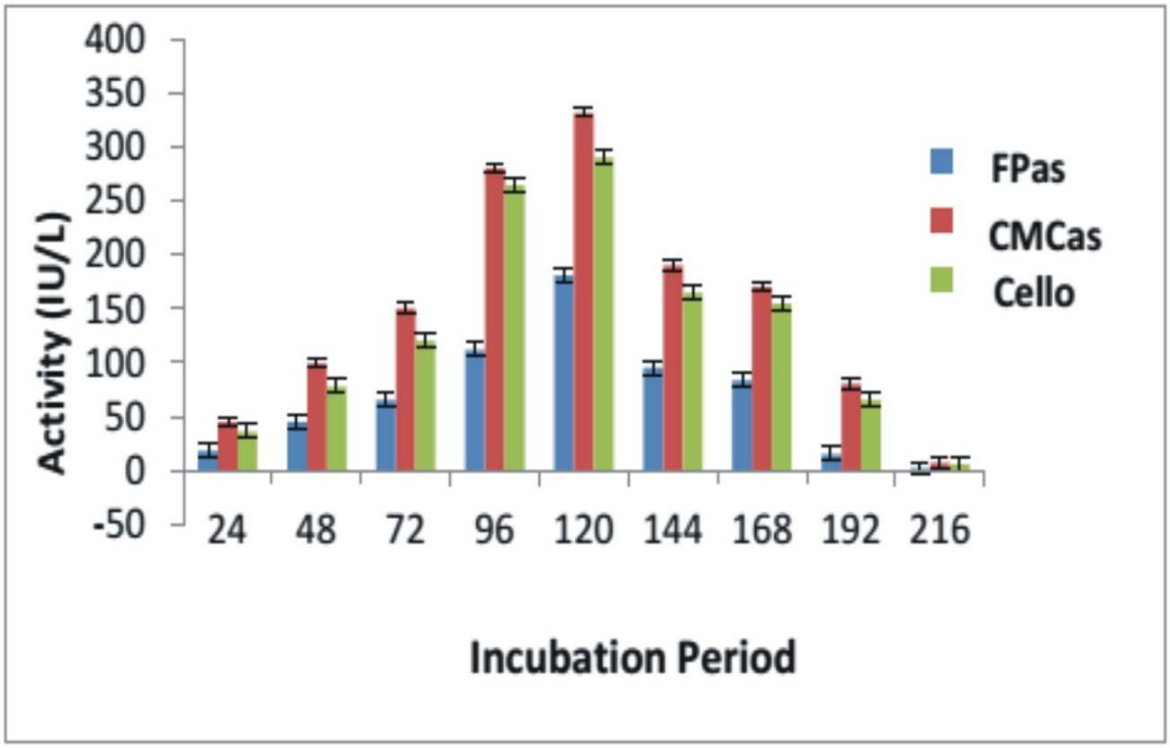


Fig. 5: Effect of incubation period of paper waste

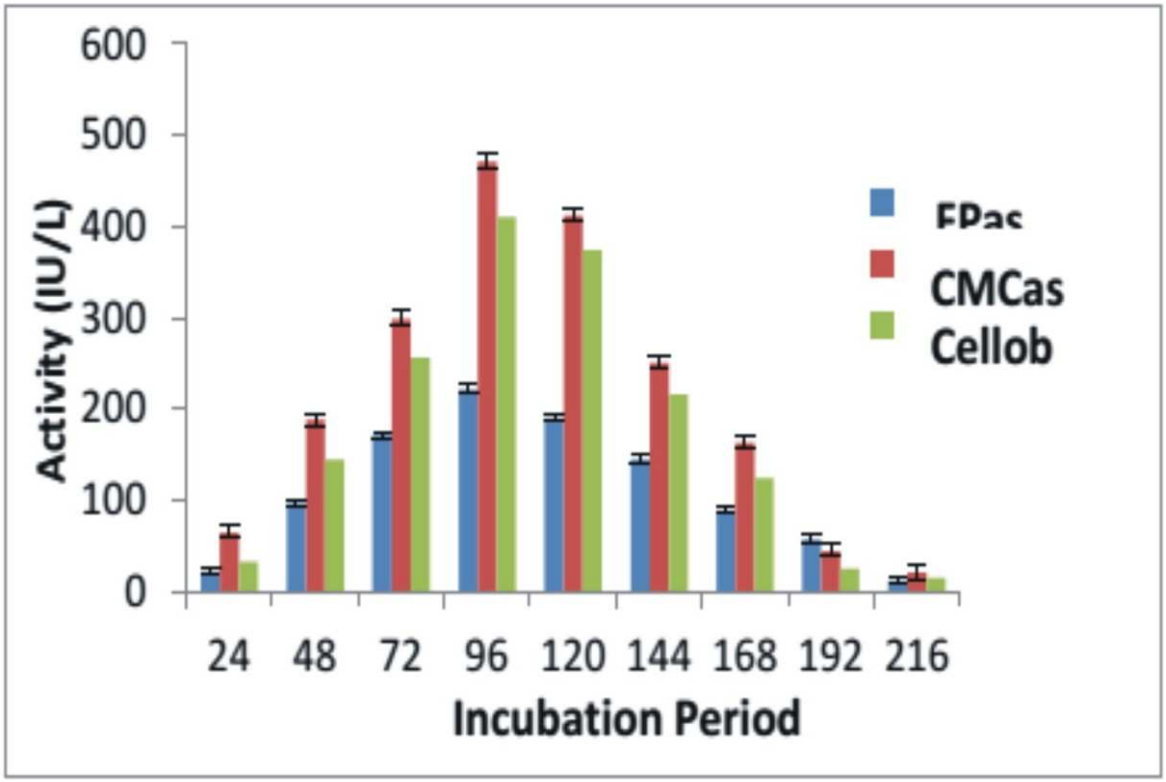
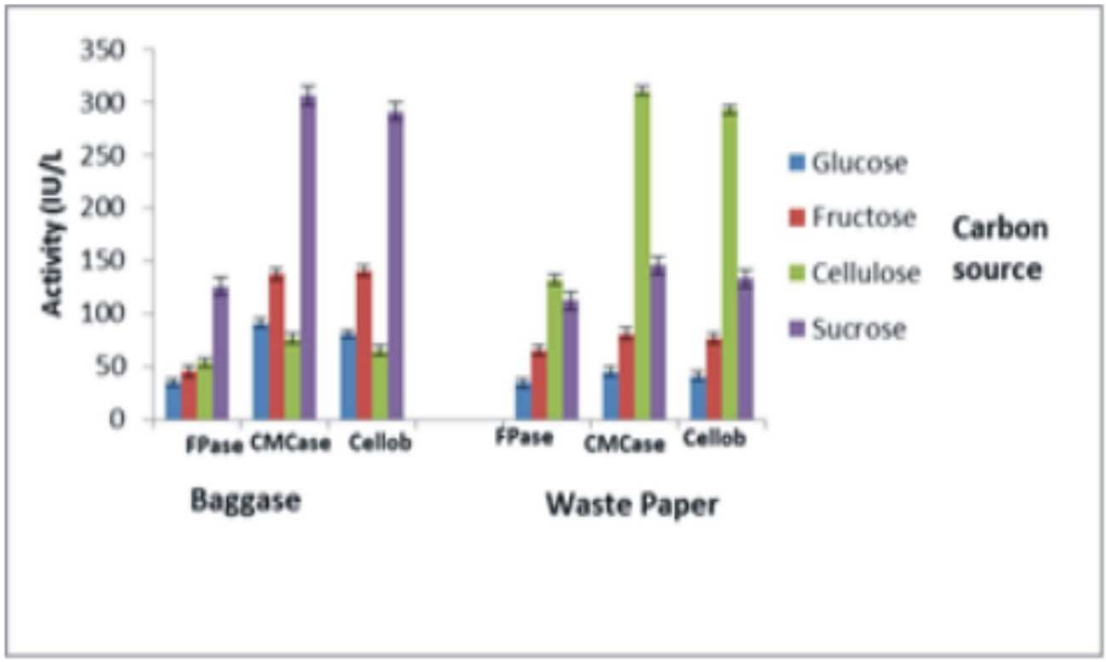


Fig.6: Effect of different co-inducer on enzyme activity



These results suggests that cellulose was found to the best co-inducer for waste paper and increase activity 21.6%, 34.65%, 29.55%, FPase, CMCase and cellobiase and sucrose was best co-inducer for baggase. 18.6%, 32.65%, 23.55%, FPase, CMCase and cellobiase[2], cellulase activities have generally been observed to be higher during the incubation period with mixed C sources and activity of CMCase was higher in mixed substrate as compared to FPase activities[3]. The data reported is in agreement with work done cellulosic substrate, (rice straw, and sugarcane bagasse) with cellulose as cosubstrate for maximum production of enzyme[16].

Low cellulase activity of cellulose observed when glucose is used as a carbon source could possibly due to low production of the enzyme in the absence of appropriate inducer because the repressor system will not function as an absolute block, thus allowing constitutive production of cellulase which could easily yield soluble hydrolysis products of naturalcellulose (Erikson 1993). Generally, cellulase production in wild-type strains is regulated by induction and catabolite repression mechanisms. CAMP plays a crucial role in catabolite repression mechanism of exo-enzyme production involving an allosteric protein known as cAMP receptor protein (CRP)[1]. As long as glucose is available as a carbon source, the CAMP concentration is low, thereby preventing the synthesis of enzymes capable of utilizing other carbon sources through CRP.

Bioconversion of substrates

Bioconversion of paper waste and baggase as substrate by cellulase produced by *Aspergillus flavus* was also studied. Degree of saccharification was measured through analysis of released sugar. The results given in Fig. 5 reveal that out of the two different concentrations of substrate (2.5% and 5.0 % w/v) used maximum saccharification of 82% and 62.8% in waste paper and 65% and 55% in bagasse respectively peaked at 24h subsequently declined. The results indicate that though saccharification occurs with both concentrations of substrates however, it is more substantial with 2.5% (w/v) substrate. The decrease in hydrolysis rate over time can be attributed to inhibition by the accumulation of end product. It has been reported that end product inhibition by glucose, cellobiose, and ethanol significantly inhibits endoglucanase and cellobiohydrolase[3,23]. Inhibition of enzyme by hydrolysis products also appears to be the main cause of the decreasing yields at increasing substrate concentrations in the enzymatic decomposition of cellulosic biomass in our study.

CONCLUSION

A. flavus an efficient cellulase producer was able for bioconversion of paper waste and bagasse. The optimization parameter and substrate level concenteration of biomass were critical factor for the bioconversion. Activity was regularly increased as the process parameters were stable. The result showed that carbon source strongly influenced the activity. Cellulase activity increased of waste paper 21.6%, 34.65%, 29.55%, FPase, CMCase and cellobiase when cellulose used as carbon source and activity increased up to 18.6%, 32.65%, 23.55%, FPase, CMCase and cellobiase when sucrose used as a carbon source for bagasse.

Cellulase produced was further utilized for bioconversion and yield a maximum of 82% and 65% for waste paper, bagasse on 2.5% w/v substrate concentration. The reported work utilizes easy available and cheap biomass for bioconversion which may also be significant in terms of reducing the cost of commercial enzyme.

Acknowledgement

The present study has been carried out at SHIATS, Allahabad in the Deptt. of Biochemistry & Biochemical Engineering. The authors are sincerely thankful to the SHIATS, Allahabad for financial support to carry out research.

Conflict of interest: The authors declare that they have no conflict of interest in the publication

REFERENCES

1. Brock, T.D. & Madigan, M.T., 6thed. Prentice Hall International Inc. Englewood Cliffs, U.S.A, 835 (1991)
2. Dashtbtan, M. Buchkowski, R. and Qin, W., *Int L. J. Biochem. Mol. Bio.l.,* **2(3)**: 274-286. (2011)
3. Bezerra, R.M.F. and Dias, A.A., *Appl. Biochem. Biotechnol.*, **148**: 35-44. (2008)
4. Clark, H.E. Gledrich, E.F. Kabler, P.W. and Huff, C.B., Applied microbiology international book company New York. Pp. **53**. (1958)
5. Demirbas, A., Progress and recent trends in Biofuels. Prog. Energy combast sci. **33**: 1-18 (2007)
6. Eriksson, K.E.L., *J. Bacteriol*, **30**: 149-158. (1993)
7. Gervais, P. and Molin, P., *Biochem. Eng. J*. **13**: 85–101 (2003)
8. Ghosh, T K., *Pure & App Chem.*, **59**: 257-268 (1987)
9. Kalogeris, E. Inotoli, F. Tokakas, E. Christakopoulous, P. Kekos, D. and Marcis, B. J., Bioresour. Technol. **86**: 207- 213 (2003)
10. Kutney, G.W. and Evans, T.D., svensk papperstidning, **9**: R 84-89 (1985)
11. Lee, C.K. Darah, I. and Ibrahim, C.O., *Research Biotechnology*. (2011)
12. Lowry, O. H. Rosebrough, N. J. Farr, A.L., and Randall, R J., *J. Biol. Chem.*, **193**: 265 (1959)
13. Mamma, D. Kourtoglou, E. and Christakopoulos, P., *Bioresour. Technol*. **99**: 2373–2383 (2008)
14. Miller, G. L., *Anal. Chem*, **31**: 426 (1959)
15. Mussatto, S.I. Daogone, G. Guimaraes, P.M.R. Suva, J.P.A. Carneiro, L.M. Roberto, J.C. Vicente, A. Domingues, L. and Teixeria, J.A., *Biotechnol Adv.* **28**: 817-830 (2010)
16. Muthuvelayudhan, R. & Viruthagiri, T., *Afric. J. Biotechnol.*, **5 (20)**: 1877 – 1881 (2006)
17. Okada, G., *Agri. boil chem*, **49**: 1259-64 (1985)
18. Perlack, R.D. Wright, L.L. Turhollow, A.F. Grahan, R.L. Stokes, B.J. and Erbach, D.C., Oak ridge national laboratory, USDA. (2005)
19. Rajoka, M.I. Huma, T. Khalid, A.M. and Latif, F., *World J. Microbiol. Biotechnol*. **21**: 869–876 (2005)
20. Szewczyk, K.W. Myszka, L., *Bioprocess Eng*, **10** : 123-126 (1994)
21. Tanguchi. M. Suzuki. H. Watanabe, D. Sakai, H, Hoshino, K. and Tanaka, T., *J. Biosci . Bioeng*, **100**: 637-643 (2005)
22. Wen zhy, Liao, W. and Chem, S.L., *Biores.Technol*, **96**: 91-94 (2005)
23. Xia, ZZ. Zhang, X. Gregg, D. and Saddler, J.N., *Appl. Biochem. Biotechnol*, **113-116**:1115-1126 (2004)
24. Zambare, V.P. Bhalla, A. Muthukumaocpper , K. Sanj, R.K and Christopher, L.P. *Extremophiles* **15**: 611-618 (2011)