CELLULASE PRODUCTION BY *ASPERGILLUS FLAVUS* AND SACCHARIFICATION OF WHEAT STRAW

Swati Pandit 1, Kapil Lawrence 1, Anupama Singh1 , Sangya Singh1 and Reena Lawrence2

[1] Department of Biochemistry and Biochemical Engineering, Sam Higginbottom Institute of Sciences and Technology, Allahabad.

[2] Department of Chemistry, Sam Higginbottom Institute of Sciences and Technology, Allahabad.

FPase: Filter paper assay, CMCase : Carboxymethyl cellulase assay **,** **DNS** : Dinitrosalicylic acid, **PDA** : Potato dextrose agar **,** **h**: hour/s

Abstract:

Cellulase production from fungal sources is utilized in the processing of lignocellulosic biomass for production of alternative fuels. Biomass selection is an important consideration in commercial production of cellulase. Wheat straw is a cheap and readily available substrate with potential for bioconversion. The production of cellulase using wheat straw as substrate and subsequently saccharification was investigated in this study. Rapid production of cellulase *viz.* FPase & CMCase derived from *Aspergillus flavus* was observed. Maximum activity of FPase 167 IU/g and CMCase 415IU/g was obtained after 96h of incubation with cellulose as co-substrate and pH 4.5. Highest saccharification yield was with 25gL^{-1} (72%) substrate though higher concentration of wheat straw inhibited enzyme activity. The study suggests that production of cellulase from *Aspergillus flavus* using low cost wheat straw as substrate can be an alternative for large scale low cost enzyme production.

Key words: Cellulases, Lignocellulosic biomass, bioconversion, saccharification

Introduction:

Waste lignocellulosic biomass is considered as one of the most promising alternatives to fossil fuels for the production of biofuels and other useful chemicals. In bio-conversion of cellulosic material into biofuels cellulases are a perquisite.Agro-industrial wastes being rich in carbohydrates and other vital nutrients are considered promising substrates for culturing fungi used in production of cellulases. Cellulases are glycosyl hydrolases (GH) and play an important role however; the major obstacle for the conversion of lignocellulosic biomass into ethanol is their production cost [1]. A great deal of effort has been made to screen and develop cellulases with high enzymatic activity, stability and low production costs.

Corresponding Author:

Swati Pandit, Research Scholar Deptt., Biochemistry & Biochemical Engineering, SHIATS (Formally k/a Allahabad Agriculture Institute- Deemed University)

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 non-reducing 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. [2]

Solid-state fermentation resembles the natural environment of the fungi and has tremendous potential for the production of hydrolytic enzymes. The use of low cost wastes as substrates helps to reduce the cost of the enzymes for which raw material translates into 40–60% of the production cost [3]. Various agro-industrial wastes have been

used as substrates for the cost efficient production of cellulases, such as apple pomace, kino waste, soybean hulls, sugarcane bagasse and rice straw [4],[5],[6],[7]. For on-site cellulase production, the selection of fungal strains possessing high expression capacities and a diversity of cellulolytic enzymes with high specific activity is essential in order to obtain these enzyme complexes [1]. In addition to the selection of fungal strain, the level of expression of cellulases is determined by the composition of the medium and changes of temperature, pH, and substrate as well as by the inoculum concentration. Optimal culture conditions vary widely between species of the same organism

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 For what, viz. good fermentation capabilities and high levels of protein secretion; ability to assimilate various organic substrates; suppress the development of other microorganisms; and high sporulation capacity [8]. The objective of the present work was to optimize cellulase production by A. flavus under the following parameters: Concentration of basal medium, pH, and incubation period and carbon source. The enzyme extract obtained was then used for cellulose and wheat straw hydrolysis.

2. Materials and methods:

2.1 Microorganism cultivation condition and enzyme production optimization:

Aspergillus flavus obtained from the soil of Allahabad (UP) was cultured on (PDA) media with CMC (1.2% w/v) for 3-5 days at 320C and identified at NFCCI Pune.

The following composition of basal media (gL-1) was used for the production of cellulase: urea 0.3, (NH4)2SO4 1.4, KH2PO4 2, CuCl2 0.3, Polypeptone 1, MgSO4.7H2O 0.3 and trace elements were FeSO4.7H2O , MnSO4.4H2O 1.6, ZnSO4 7H2O 1.4, CoCl2.6H2O 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 ml basal medium (1:10 w/v). These flasks were inoculated with 5ml (1x105spore/ml) of substrate and the contents were incubated at 28± 10C for 5 days. Thereafter 0.1M citrate buffer pH 4.8 was added to a final volume of 100ml/flask. The flasks were agitated at 200 rpm for 1hr then filtrate used for determining the enzyme activity.

2.2 Preparation of wheat straw:

Wheat straw (substrate) was cut into small pieces. The chopped substrate was ball milled, sieved over a mesh and pre-treated with 1% NaOH (w/v) for one hour in a boiling water bath (100°C), cooled and washed till neutral pH. The residue was dried at 60°C till constant weight. This dried substrate was used for further analysis [10].

2.3 Optimization of production conditions:

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.

2.4 Enzyme assay:

Exo-cellulase (FPase) and endoglucanase (CMCase) activity were assayed according to the given

methodolog [11].The activities of the two enzymes are considered to be representatives of total cellulase activity.Reducing sugars liberated was quantified by dinitrosalicylic acid (DNS) reagent using glucose as a standard [12]. One unit of enzyme activity was defined as the amount of enzyme producing 1µmol reducing sugar minute-1 under assay conditions. Total extracellular protein content was determined by Lowry's method [13]. Both, the enzyme activity and specific activity are reported in the article.

2.5 Enzymatic hydrolysis:

Enzymatic hydrolysis of alkali pre-treated wheat straw was performed in a reaction mixture containing 25g L-1 and 50g L-1 (w/v) substrate in 0.1M citrate buffer(pH 4.8), supplemented with enzyme (2FPU/ ml). The mixture was incubated at 320C on a rotatory shaker at 150rpm. Samples were taken from the reaction mixture at different time intervals. Samples were immediately heated to 1000C, 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 [14],[15].

Results and discussion:

3.1 Effect of pre-treatment

Delignification of wheat straw was done through alkali pre-treatment. Removal of lignin is necessary because lignin retards fungal hydrolysis of cellulose thus; pre-treatment is required to make cellulose accessible for fungal utilization. In the study 1% NaOH (w/v) was used. Jeya [16] , Yamashita [17], Beerre [18] & their coworker showed that NaOH is an effective pre-treatment agent for substrates with relative low lignin content.

3.2 Effect of basal media:

Results of enzyme production by varying the basal media (used as moistening solution) ratios (1:6, 1:8, 1:10, 1:12, 1:14) w/v are shown in Fig 1. The result indicated that highest FPase and CMCase activity was 112 IU/g and 167IU/g respectively when 1:6 ratios were taken. Lower or higher moisture levels yielded lower cellulase activity. Basal media is regarded as a fundamental parameter for microbial growth and metabolite formation. Lower levels of moistening agent leads to the sub-optimal growth, a lower degree of substrate swelling and a high surface tension, whereas high moisture levels decrease porosity which would cause lower oxygen transfer and enhance formation of aerial mycelium. Fatima and coworker [19] reported that rice straw by T. ressei where maximum cellulase production was reported in 30ml (1:6) basal medium.

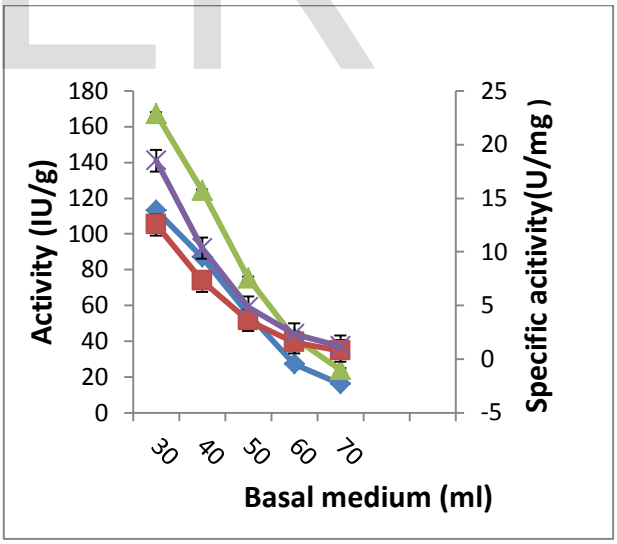

Fig 1: Effect of moistening agent: (a) FPase activity and specific activity

(b) CMCase acitivity pecific activity X

3.3 Effect of pH and incubation period:

The effect of pH on cellulase production by Aspergillus flavus is given in Fig 2. The activities of CMCase and FPase increased from pH 3.5 to 5.5 and reached a maximum at pH 4.5 and then gradually decreased from its optimal level. Another important factor for growth of the microorganism and it's metabolic activities is the pH. Increasing and decreasing in pH on either side of the optimum value resulted in decreased the rate of fermentation. [20]. Irfan [21], Mutjuveladhyudhun [22] and coworker showed data on rice straw and wheat straw using A.niger where maximum production of cellulase on lignocellulosic waste was between pH 4-5.

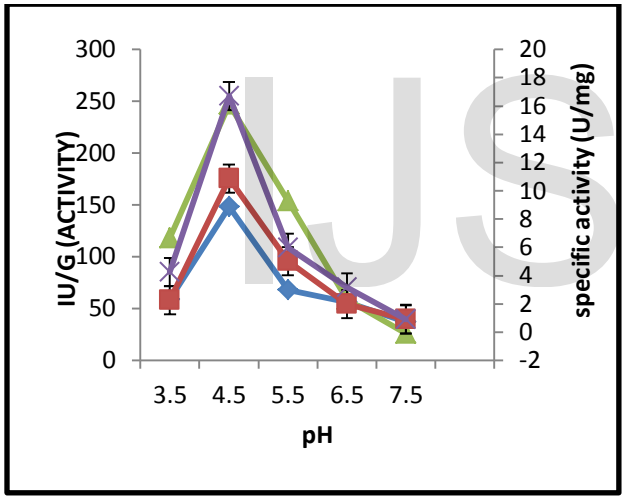

Fig 2: Effect of pH:(a) FPase activity specific activity (b) CMCase activity specific activity X

3.4 Effect of carbon sources and incubation period:

The effect of carbon sources on cellulase production is presented in Fig 3. The enzyme activity and protein content was measured at 24 h. intervals for duration of 240 h. The highest FPase 167 IU/g and CMCase activity 415 IU/g was obtained after 96h of incubation (Fig.4). Specific activity of these enzymes also followed a similar pattern. Incubation period and carbon source play a key role in growth of the micro-organism and subsequent enzyme production [22]. Enzyme activity and specific activity of both the enzymes assayed (FPase and CMCase) declined on prolonged incubation (> 96 h.), this could possibly be due to loss of moisture/ denaturation of enzymes/ depletion of C-sources. These results indicate that the cellulose was the best co-inducer for wheat straw in this study. Dashtbtan [21], Bezerra [23] and coworker reported that cellulase activities have generally been observed to be higher during the incubation period with mixed C sources. The activity of CMCase was higher in mixed substrate as compared to FPase activities. The data reported is in agreement with work done cellulosic substrate, (rice straw, and sugarcane bagasse) with cellulose as co-substrate for maximum production of enzyme (22).

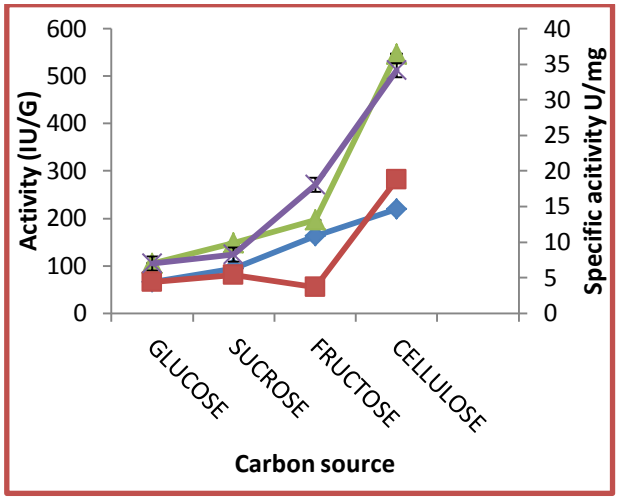

Fig 3: Effect of carbon source: (a) FPase activity and specific activity (b) CMCase activity and specific activity X

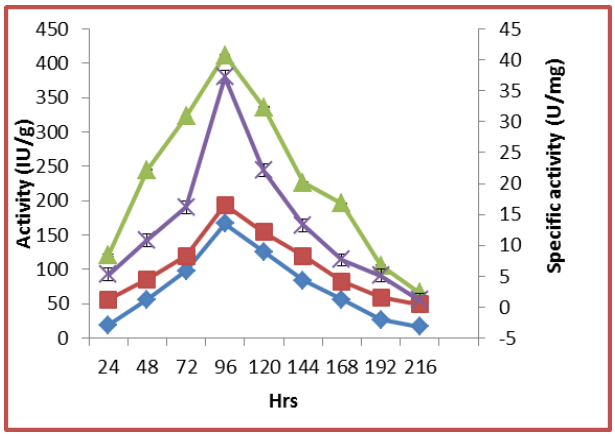

Fig 4: Effect of incubation period: (a) FPase activity and specific activity (b) CMCase activity and specific activity X

3.5 Effect of enzymatic hydrolysis:

The activity of cellulase produced by Aspergillus flavus, in bioconversion of wheat straw as substrate was also studied. Degree of saccharification 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 72% and 50.75% respectively peaked at 24h and 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 has been attributed to inhibition by the accumulation of end product.

The end product inhibition by glucose, cellobiose, and ethanol has demonstrated their ability to significantly inhibit endoglucanase and cellobiohydrolase. [24],[25] It has been earlier reported that certain hydrolysis products are able to inhibit cellulase adsorption [26], [27], [28]. It has, however, recently been shown that glucose strongly inhibit cellulase adsorption linearly [29]. Inhibition of enzyme adsorption by hydrolysis products appear to be the main cause of the decreasing yields at increasing substrate concentrations in the enzymatic decomposition of cellulosic biomass. In order to facilitate high conversions at high solids concentrations, a better understanding of the mechanisms involved in high-solids product inhibition and adsorption inhibition is required.

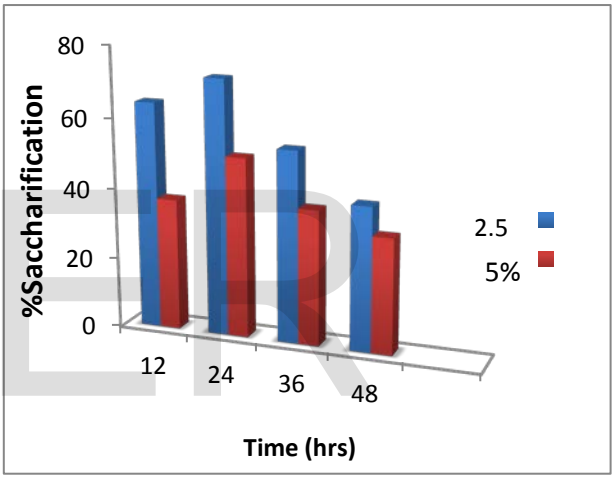

Fig 5: Percent saccharification of substrate for at varying substrate concentration

Conclusion:

The study showed that A. flavus has potential for the cellulase production. Wheat straw is a cheap substrate suitable for bioconversion into a value added product like glucose.

The type and composition of the carbohydrates present in wheat straw is well documented and have been found suitable for the induction of cellulase and

hemicellulase from Aspergilli under SSF (15). This study was oriented towards delineating the process parameters for maximum yield of cellulase under SSF. Highest FPase 167IU/g and CMCase 415IU/g activity was measured at pH 4.5 after 96h of incubation using cellulose was co-substrate.

Cellulase produced was further utilized for saccharification and yields a maximum of 72% saccharification on 2.5% (w/v) substrate. The reported work utilizes easy available and cheap lignocellulosic biomass for bioconversion which may also be significant in terms of reducing the cost of commercial enzyme production.

Acknowledgements:

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.

REFERENCES:

1. Zhang Y, Himmel M.E, & Mielenz J.R, (2006). Outlook for cellulase improvement screening & selection strategies ,*Biotechnol. Adv*, 24. PP : 452-481.

2. Lynd L R, Weiner P J, Zyl W H, & Pretorium L S., (2002). Microbial cellulase utilization, *Microbial. Mol. Bio Rev*, 66 (3). PP : 506-524.

3. Selig M J, Khoshaug E P, Adney W S, Himmel H E, & Decker S R., (2008). Synergetic enhancement of cellobiohydrolase performance on pretreated corn Stover by additives of xylanase & esterase activities ,*Bioresour. Technol* , 99. PP : 4997-5005.

4. Gutierrez – Correa, Portal M, Mremo L, & Tengerdy R P.,(1999). Mixed culture solid substrate fermentation of Trichoderma ressei with Aspergillus niger on sugarcane baggase, *Bioresour. Technol*, 68. PP : 173-178.

5. Oberoi H S, Babber N, Dhaliwal S S, Kaur S, Vadlani P V, Bhargav V K, & Patil R T., (2010). Enhanced oil recovery by pretreatment of mustard seeds using crude enzyme extract obtained from mixed culture solid state fermentation of Kinnow (citrus reticulate) waste & wheat bran, *Food bioprocess. Technol* doi;10, 1007/s 11947-010-0380.

6. Brijwani K, Oberoi H S, & Vadlani P V., (2010). Production of cellulolytic enzyme in mixed culture solid state fermentation of soyabean hulls supplemented with wheat bran, *Process. biochem*, 45. PP : 120-128.

7. Kaur S, Dhillon G S, Brar S K, & Chauhan V B, (2012). Carbohydrate degrading enzyme production by plant pathogenic mycelium & microscleratia isolates of macrophomina phaseolina through Koji fermentation, *Ind. crop prod*, 36. PP : 140-148.

8. Devries R.P, & J Visser, (2001). Aspergillus enzymes involved in degradation of plant cell wall polysaccharides, *American society for microbiology*

9. Clark H.E, Geldrich E.F, Kabler P.W. & Huff C.B, (1958) Applied microbiology international book company New York, PP : 53.

10. Sherief A.A, EL –Naggar Noura El- Ahmady & Hamza Sarah Shawky., (2010). Bioprospecting of lignocellulosic biomass for production of bioethanol using thermotolerant *Aspergillus fumigatus* under solid state fermentation condition, *Biotechnology*, 9(4). PP : 513-522.

11. Ghosh T K, Measurement of cellulase activities., (1987). *Pure & App Chem*., 59. PP : 257-268

12. Miller G L, Use of dinitrosalicylic acid reagent for determination of reducing sugar. (1959). *Anal. Chem*, 31. PP : 426.

13. Lowry O H, Rosebrough N J, Farr A L, & Randall R J, (1959). Protein measurement with folin phenol reagent, *J. Biol. Chem*., 193. PP : 265.

14. Tanguchi M, Suzuki H, Watanabe D, Sakai H, Hoshino K & Tanaka T, (2005).Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw, *J. Biosci . Bioeng*., 100. PP : 637- 643.

15. Kalogeris E, Inotoli F, Tokakas E, Christakopoulous P, Kekos D, & Marcis B J, Performance of an intermittent agitation rotating drum type bioreactor for solid state fermentation of wheat straw, *Bioresour. Technol*., 86. (2003) 207-213.

16. Jeya M, Nguyen N, Moon H, Kim S, & Lee J, (2010). Converion of woody biomass into fermentable sugars by cellulase from *Agaricus arvensis, Bioresour. Technol*., 101. PP : 8712-8749.

17. Yamashita Y, Shono M, Ssaki C, Nakamurya Y., (2010) Alkaline peroxide pretreatment of efficient enzymatic saccharification of bamboo, *Carbohydr. Polym*. , 79. PP : 914-920

18. Bjerre AB, Olesen AB, Frenquist T.,(1996). Pretreatment of wheat straw using combined wet oxidation &alkaline hydrolysis resulting in convertible cellulose & hemicellulose , *Biotechnol. Bioeng*. , 49. PP: 568-577.

19. Fatma H, Abad El Zaher & Fadel M., (2010). Production of bioethanol via enzymatic saccharification of rice straw by cellulase produced by *Trichoderma ressei* under solid state fermentation, *New York Sci*. J. PP: 72 – 78.

20. Juwaied A.A, Al-amiery AAH, Abdunumien Z, Anaam U., (2011).Optimization of cellulase production by *Aspergillus niger & Trichoderma viridie* using sugar cane waste. *J. Yeast, Fungal Res*., 2(2). PP : 19-23.

21. Irfan M , Irfan U, Razzaq Z, Syed Q & Nadeem M. (2011).Utilization of agricultural waste as a substrate for carboxymethyl cellulase production from *Aspergillus niger* in submerged fermentation, *IJAVMS*, 5 (5). PP : 464-471

22. Muthuvelayudhan R, & Viruthagiri T., (2006). Fermentative Production & kinetics of cellulase protein on *Trichoderma ressei* using sugarcane baggase & rice straw, *Afric. J. Biotechnol*., 5 (20). PP: 1877 – 1881.

23. Dashtbtan M, Buchkowski R, Qin W., (2011). Effect of different carbon sources on cellulose production by *Hypocera jecorina* (Trichoderma ressei) strains, IntL. *J. Biochem. Mol. Bio.l*, 2(3). PP: 274-286.

24. Bezerra RMF, Dias AA., (2008).Enzymatic kinetics of cellulose hydrolysis inhibition by ethanol & cellobiose, *Appl. Biochem. Biotechnol*. , 148. PP: 35-44.

25. Xia ZZ, Zhang X, Gregg DJ, Saddler JN., (2004).Effect of sugar inhibition of cellulase & beta glucosidase during enzymatic hydrolysis of softwood substrates, *Appl. Biochem. Biotechnol*, 113-116. P : 1115-1126.

26. Jeoh T, Ishizawa Cl, Davis MF, Himmel ME, Adney WS, Johnson DK, (2007). Cellulose digestibility of pretreated biomass is limited by cellulase accessibility , *Biotechnol Bioeng*, 98. PP : 112-122.

27. Tanaka M, Nakamura H, Taniguchi M, Morita T, Matsuno R, Kamikubo T., (1986). Elucidation of adsorption processes of cellulase during hydrolysis of crystalline cellulose, *Appl Microbiol Biotechnol* , 23. PP : 263-268.

28. Stutzenberger F, Lintz G, (1986). Hydrolysis product inhibit adsorption of *Trichoderma ressei* C 30 cellulase to protein extracted Lucerne fibers, *Enzyme Microb Techno*l , 8. PP : 341-344.

29. Kumar R, Wyman CE, (2008). An improved method to directly estimate cellulase adsorption on biomass solids, Enzyme. *Microb. Technol*, 42. PP : 426-433.