

OPTIMIZATION OF CELLULASE ENZYME PRODUCTION FROM *Aspergillus oryzae* FOR INDUSTRIAL APPLICATIONS

Hassan Sher, Muhammad Faheem, Abdul Ghani, Rashid Mehmood, Hamza Rehman, Syed A. I. Bokhari *
Department of Bioinformatics and Biotechnology, International Islamic University Islamabad, Pakistan.

*Corresponding email address: ali.imran@iiu.edu.pk

ABSTRACT

Cellulases are the hydrolytic group of enzymes, responsible for release of sugars in the bioconversion of the cellulosic biomass into a variety of value added industrial products. Fungal isolated cellulases are well studied and playing a significant role in various industrial processes. Enzymatic depolymerisation of cellulosic material has been done by the various fungal isolated enzymes. In the present study, the cultivation conditions for cellulase production of *Aspergillus oryzae* were optimized. Optimization of scarification conditions such as time course, inoculum size, carbon source and concentration, nitrogen source, various pH levels were performed for the production of extracellular carboxymethyl cellulase and endoglucanase enzyme. The result exhibited inoculums size (15%), corncobs concentration (2%), Urea and medium pH 7 at 30°C supported high yield of carboxymethyl cellulase (38.80 U/mL/min) and exoglucanase enzyme (10.94 U/mL/min) through a submerged fermentation (SmF). In future biotechnological applications in cellulase enzyme production attain a vital role to obtain high degradable yield.

Key word: Scarification, submerged fermentation, genetically modified fungi, carboxymethyl cellulase, endoglucanase.

INTRODUCTION

Cellulose is made up of glucose molecules linked together by β -1, 4-glycosidic bonds. Among major components of the plant cell wall cellulose is one of the most abundant components. In the cellulose molecule the number of glucose units are varied (Sukumaran *et al.*, 2009). The major part of plant biomass is cellulose which cause the waste generation from agricultural field, forests and agro-industries. In environment, these wastes are accumulated and cause the pollution problems (Abu *et al.*, 2000). Cellulose biomass has a significant potential as a source of renewable energy fuels and chemicals. Mostly several microorganisms are interested to use it as carbon source, which causes the attraction of biotechnologist to convert cellulosic biomass into important value added products such as different enzyme like cellulase, xylanase, also used to improve animal feeds and make better the human nutrients (Coral *et al.*, 2002).

Oligosaccharide compounds including cellulose can be broken down into simple glucose by cellulolytic (Cellulase) enzymes (Milala *et al.*, 2005). The major cellulase enzymes are endoglucanase or carboxymethyl cellulase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and beta-glucosidase (EC 3.2.1.21). The complete cellulase system act synergistically in β -1,4-glycosidic bonds present in cellulose and cellobiose. Endoglucanase (EG) attacks the internal O-glycosidic bonds, resulting in glucan chains of different lengths. The exoglucanase acts on the ends of the cellulose chain and releases β -cellobiose as the end product while β -glycosidases act specifically on the β -cellobiose disaccharides and produce glucose (Kuhad *et al.*, 2011).

Cellulase enzymes have great application in the food industry, Wine, brewery animal feed, textile, laundry, pulp & paper industries as well as in agriculture (Suurnäkki *et al.*, 1996).

The nutritional quality of feed can be improved by partial hydrolysis of lignocellulosic materials with the help of cellulase enzyme (Bhat, 2000). Cellulase has gained attractive success in a variety of industries due to its controlled and desired modification of lignocellulosic materials (Suurnäkki *et al.*, 1996; Bhat, 2000). Cellulases also has applications in the partial or complete hydrolysis of the pulp fibers thus a better bio-characterization of pulp fibers (Bhat, 2000). There are a number of microorganism (i.e fungi and bacteria) which have the capability to produce group of enzymes that are collectively called cellulase. However Filamentous fungi are preferred for the production of enzymes as compared to yeast and bacteria because, the filamentous fungi produced high levels of enzyme. Filamentous fungi of genus *Aspergillus* and *Trichoderma* are well known for the production of cellulase (Bhat, 2000). *Aspergillus* belonged to phylum *Ascomycota*, has about 250 different species with some black, green, brown spores and found in various oxygen rich climates. Some species of *Aspergillus* can cause harms to humans as well as animals like *Aspergillus fumigates* etc. Almost all fungi of genus *Aspergillus* can synthesize cellulase. *Aspergillus species* are great producer of useful enzymes and have various importance in medicine, agriculture and food industry (Abdel-Fatah *et al.*, 2012).

Cellulases including CMCase enzymes are produced in submerged fermentation (SmF) as well as solid state (Substrate) fermentation (SSF). Carbon source is the most important factor affecting yield and cost of cellulase production. Therefore, reduce the cost of CMCase and exoglucanase using a cheap and easily available substrate is the most important step. For cellulase enzyme production, the excellent carbon source is lignocellulosic biomass especially agriculture wastes, Corn Stover and wheat bran are the very

abundant, cheap and easily available lignocellulosic biomass (Gao *et al.*, 2008). Types of fungus as well as media components especially minerals, nitrogen and carbon concentrations, physical factors such as pH, temperature and moisture greatly influenced the production of cellulase enzymes. Suitable medium and culture must have to develop for the maximum enzyme productions (Deswal *et al.*, 2011).

MATERIALS AND METHODS

Chemicals: All the chemicals used for this study are as followings; agar technical (bio-base), malt extract (sigma), glucose (sigma), yeast extract (BDH), tri-sodium citrate (sigma), ammonium nitrate (DAEJUNG), magnesium sulphate (sigma), substrate; carboxymethyl cellulose (Sigma) and avicel (sigma). Carbon source was collected locally, dried and grinded very gently by using grinder.

Organism and culture condition: Soil samples from different sectors of Islamabad, Pakistan (H-10, H-11, G-9, G-10 and I-10) were collected using pre-sterilized plastic bottle and spatula. Cellulase producing *Aspergillus* sp. was isolated using Potato Dextrose Agar (PDA) media by serial dilution. The PDA media was prepared from the combination of 50% distilled water, 2.25% pure glucose, 2.25% agar technical and 50% potato infusion with pH 3.5 ± 0.1 . After pouring of inoculum, petri plates were placed in static incubator at 30°C in an inverted position and the examination of all plates were done on daily basis. After 3 to 4 days, the fungus were cultivated and appeared on PDA medium. Morphological and structural identification was done as describe by Wellala *et al.* (2006). The identified *A. oryzae* was transferred into slants for further use and for maintenance.

Production of cellulase enzyme: Vogel's media was used for the preparation of inoculum. The vogel's media consists of; 0.5% trisodium citrate, 0.5% potassium di-hydrogen phosphate, 0.4% ammonium sulphate, 0.2% ammonium nitrate, 0.02% magnesium sulphate and 0.1% yeast extract dissolved in 100 mL distilled water in conical flask. In this study Submerged fermentation (SmF) was used for the production of cellulase enzyme. Spore suspension were prepared by adding 10mL saline solution (0.9%) to 4-5 days old slants of *A. oryzae* and scratched with sterilized wire loop under the control environment in laminar flow. Then from prepared spore suspension 5mL of spore suspension were pipetted and dropped in flasks of Vogel's media and mixed gently. After the preparation of inoculums, flasks were shifted to shaking incubator for different time of incubation. The temperature of shaking incubator was fixed at 30°C and 130 rpm. After selected time of incubation and growth, the flask containing vogel's media and spore suspension were filtered to get purified crude enzyme.

Cellulase assay: The extracellular carboxymethyl cellulase enzyme assay, as well as exoglucanase enzyme assay was performed. For Carboxymethyl cellulase assay among two tubes, 1st tube was for blank containing 1mL phosphate buffers, 0.5mL carboxymethyl cellulose and 0.5mL Distilled water while 2nd tube was for test, containing 1mL phosphate

buffer, 0.5 mL Carboxymethyl cellulose, and 0.5 mL enzyme crude. Then both test and control tubes were kept in water bath at 45°C for 15 min, added 1 mL dinitrosalicylic acid (DNS) and were boiled for 5 min. The optical density (OD) of the mixture was checked by spectrophotometer at 550 nm wavelength. For exoglucanase assay, the process was similar like CMC assay but instead of CMC, the avicel are used for exoglucanase assay.

Time course and inoculum size optimization: The incubation time also influence various metabolic processes, such as enzyme production. So in this study, different incubation times were evaluated for the production of high yield of cellulase enzyme. The inoculum size plays an important role in production of cellulase enzyme under submerged (SmF) fermentation. Thus different inoculums size (5%, 10% and 15%) of *A. oryzae* was used for optimization. The inoculum size showing maximum cellulase enzyme production was selected for further studies.

Optimization of Carbon source and concentration: Carbon source is the most important source that is necessary for the growth of microorganism, various carbon sources like wheat bran, dab grass and corncobs was used to get high yield of cellulase enzyme. The carbon source which showed maximum yield of cellulase enzyme was used for further production of enzyme. For cell metabolism and synthesis of cellulase, the carbon source concentration plays vital role. Different concentrations of optimized carbon source including (1%, 2% and 3%) were used for production of high yield cellulase. The carbon concentration showed maximum production, was selected for further optimization.

Optimization of nitrogen source: The essential and most important components for growth and production of cellulase enzyme by microorganism is one of the nitrogen sources. Different nitrogen source like ammonium nitrate, urea, and diammonium phosphate (DAP) were evaluated for maximum production of cellulase. The maximum yielding nitrogen source was used for next upcoming experiment.

Optimization of pH: The pH of the medium plays significant role in cellulase enzyme production. During this study various pH (5, 6 and 7) levels were tested in order to enhance cellulase production. The pH range which exhibited amazing yield of cellulase, were used for further study.

RESULTS

For morphological identification of *A. oryzae*, slides were prepared and examined for identification of desired strain of fungus under compound microscope (Figure 1).

Time course Optimization: Time course optimization was performed to get high yield of enzyme production because incubation time influence various metabolic processes such as enzyme production. In present study, the incubation time from 2 to 24 h were evaluated along with corncob as a carbon source the incubation time from 2 to 24 h were evaluated at 35°C along with corncob as a carbon source and shaking at 130rpm. The result showed maximum 38.15 U/mL/min carboxymethyl cellulase and 11.26 U/ml/min exoglucanase at

8 h (Figure 2).



Figure 1: Microscopic structure of *A. oryzae*

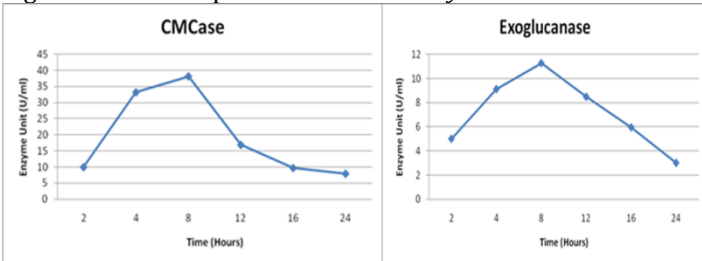


Figure 2: Time course optimization, extracellular CMCase and exoglucanase.

Optimization of inoculum size: Different inoculum size (5%, 10% and 15%) of *A.oryzae* were used for optimization in production of cellulase enzyme under both solid state fermentation and submerged (SmF) fermentation. Using of 15% inoculum size gave highest result at 8 hours 42.76 U/mL/min Carboxymethyl cellulase and 16.16 U/mL/min exoglucanase (Figure 3 & 4).

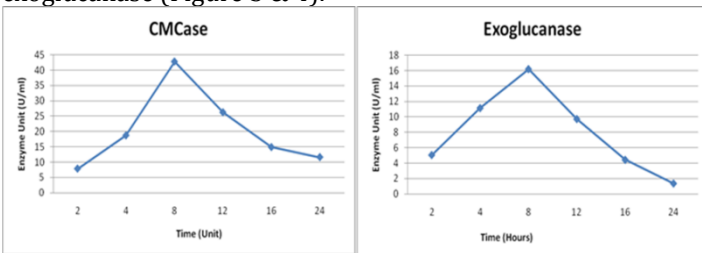


Figure 3: Time course optimization, extracellular CMCase and exoglucanase.

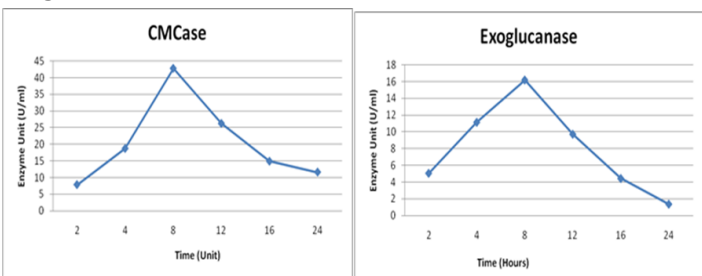


Figure 4: 15% inoculum size optimization, CMCase and exoglucanase.

Optimization of Carbon source: Carbon source is necessary

for the growth of microorganism, so different carbon source like wheat bran, dab grass and corncobs were used to get high yield of cellulase enzyme. The result shown that the corncob for the *A. oryzae*, as a carbon source gave maximum 38.15 U/mL/min carboxymethyl cellulase (and 11.26 U/ml/min exoglucanase (Figure 5).

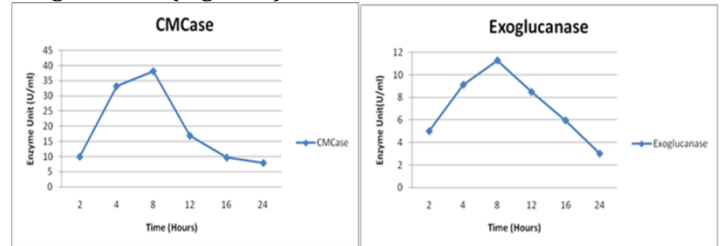


Figure 5: Carbon source (Corn Cobs) optimization, CMCase and Exoglucanase.

Optimization of carbon source concentration: Using 2% corncobs as a carbon source exhibited a significant cellulase enzyme production as compared to others. The maximum results obtained after 8 hours incubation, are 37.84 U/ml/min carboxymethyl cellulase and 13.07 U/ml/min exoglucanase (Figure 6).

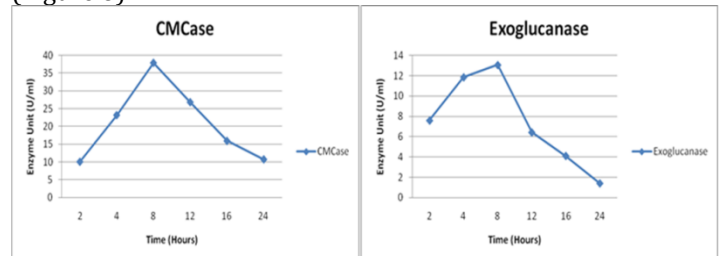


Figure 6: 2% Corn cob concentration optimization CMCase and exoglucanase.

Optimization of nitrogen source: Various nitrogen source like, ammonium nitrate, urea and diammonium phosphate (DAP) were evaluated for production of cellulase in high yield. Among nitrogen source, the urea have great impact on enzyme production as compare to other nitrogen source The maximum enzyme production recorded by using urea, was 54.98 U/ml/min carboxymethyl cellulase and 14.18 U/ml/min exoglucanase after 8 hours incubation (Figure 7).

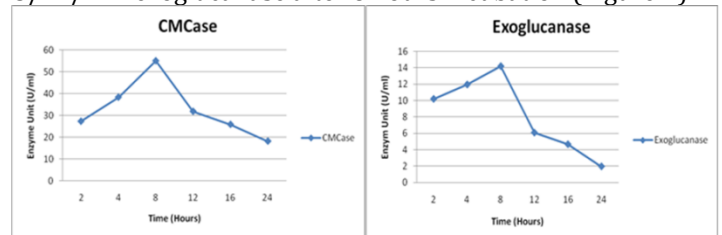


Figure 7: Nitrogen source optimization Urea, CMCase and exoglucanase.

Optimization of media pH: The *Aspergillus species* with optimized carbon source and concentration, nitrogen source, were grown on different citrate buffers ranging from pH 5 to pH 7 up to 24 hours at 30 °C in shaking incubator at 130 rpm.

The pH of the medium for the production of cellulase plays a significant role, so various pH levels (5, 6 and 7) were tested in order to enhance cellulase production and produce high yield of extracellular cellulase enzyme. Among various pH levels, the pH buffer 7.0 exhibited high yield of, 38.80 U/ml/min carboxymethyl cellulase and 10.89 U/ml/min exoglucanase (Figure 8).

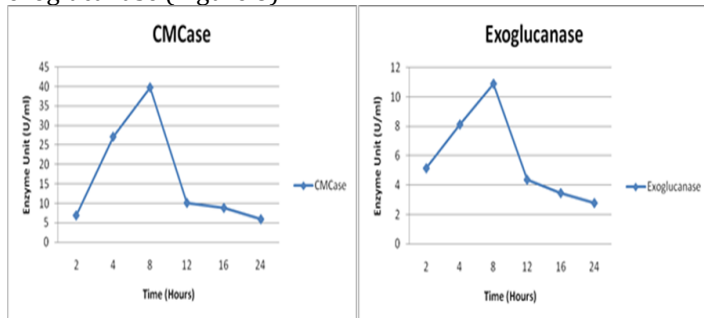


Figure 8: Medium pH 7 optimization CMCase and Exoglucanase.

DISCUSSION

A number of Fungus and bacteria have the ability to produce hydrolytic group of enzymes but in this study interest was to investigate cellulase production from *Aspergillus species*. During this study, the main focus was to optimize various parameters in order to obtained high yield enzyme production. According to Jecu (2000), cellulase production at pH 7 yielding maximum production (9.85 IU/mL), which was obtained during this research (38.80 U/mL/min carboxymethyl cellulase, 10.89 U/mL/min exoglucanase). Whereas inoculum size of 15% exhibiting the maximum yield which is quite differ from the results of Abdel-Fatah *et al.* (2012), reported 10% inoculums size for maximum yield. The urea is the cheap and good nitrogen source for production of cellulase enzyme by *Aspergillus specie* (as compare to other nitrogen sources so, utilizing urea as a nitrogen source yield maximum carboxymethyl cellulase, exoglucanase and protein production. Using urea as a carbon source, 54.98 U/mL/min carboxymethyl cellulase, 14.18 U/mL/min exoglucanase and 132.76 µg/mL protein were obtained as compared to other nitrogen sources like ammonium nitrate and DAP. Xia and Cen (1999) reported the increased concentration of corncobs results in increased rate of cellulase enzyme production, whereas in my research utilizing 2% corncobs as a carbon source yield in significant cellulase enzyme production. The maximum result was obtained as 37.84 U/mL/min carboxymethyl cellulase, 10.94 U/mL/min and exoglucanase as compared to using 1% corncobs, 10.52 U/mL/min carboxymethyl cellulase, and 8.66 U/mL/min exoglucanase.

CONCLUSION

In the energy deficit world, the role of cellulase is vital in alternative energy resources for bio-gas and bioethanol production from lignocellulosic materials. Cellulases are widely utilized in a number of industries as a raw material such as biofuel production sector, textile and paper industry and in detergents manufacturing processes etc. In future,

thermally stable and genetically modified fungal strains are the best prospect in cellulase production. Biotechnological applications of thermo-stable and alkaline resistance cellulase production in future play a significant role to attain high degradable yield.

REFERENCES

- Abdel-Fatah, O. M., M. M. Hassan, A. M. Elshafei, B. M. Haroun, H. M. Atta and A. M. Othman, 2012. Physiological studies on carboxymethyl cellulase formation by *Aspergillus terreus* dsm 826. *Brazilian journal of microbiology*, 43(1): 01-11.
- Abu, E., P. Onyenekwe, D. Ameh, A. Agbaji and S. Ado, 2000. Cellulase (ec 3.2. 1.3) production from sorghum bran by *Aspergillus niger* sl1: An assessment of pretreatment methods. In: *Proceedings of the international conference on biotechnology: commercialization and food security*, Abuja, Nigeria. pp: 153-159.
- Bhat, M., 2000. Cellulases and related enzymes in biotechnology. *Biotechnology advances*, 18(5): 355-383.
- Coral, G., B. Arikan, M. N. Ünaldi and H. Guvenmez, 2002. Some properties of crude carboxymethyl cellulase of *Aspergillus niger* z10 wild-type strain. *Turkish journal of biology*, 26(4): 209-213.
- Deswal, D., Y. P. Khasa and R. C. Kuhad, 2011. Optimization of cellulase production by a brown rot fungus *Fomitopsis* sp. Rck2010 under solid state fermentation. *Bioresource technology*, 102(10): 6065-6072.
- Gao, J., H. Weng, D. Zhu, M. Yuan, F. Guan and Y. Xi, 2008. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* m11 under solid-state cultivation of corn stover. *Bioresource technology*, 99(16): 7623-7629.
- Jecu, L., 2000. Solid state fermentation of agricultural wastes for endoglucanase production. *Industrial crops and products*, 11(1): 1-5.
- Kuhad, R. C., R. Gupta and A. Singh, 2011. *Microbial cellulases and their industrial applications*. Enzyme research, 2011.
- Milala, M., A. Shugaba, A. Gidado, A. Ene and J. Wafar, 2005. Studies on the use of agricultural wastes for cellulase enzyme production by *Aspergillus niger*. *Research journal of agriculture and biological sciences*, 1(4): 325-328.
- Sukumaran, R. K., R. R. Singhanian, G. M. Mathew and A. Pandey, 2009. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. *Renewable energy*, 34(2): 421-424.
- Suurnäkki, A., T. A. Clark, R. Allison, L. Viikari and J. Buchert, 1996. Xylanase-and mannanase-aided ecf and tcf bleaching. *Tappi journal*, 79(7): 111-117.
- Wellala, C., C. Illeperuma, R. Wewegama and M. Gunawardhane, 2006. Liquor quality of rice wine as affected by yeast strains isolated from coconut and palmyrah sap.
- Xia, L. and P. Cen, 1999. Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry. *Process biochemistry*, 34(9): 909-912.