Australian Journal of Basic and Applied Sciences, 6(1): 74-78, 2012
ISSN 1991-8178

Characterization of Purified Cellulase from Fermentation of Sewage Sludge

Muna Tasnim Mukhtaruddin, Md. Zahangir Alam, and M. Hamzah Salleh

Bioenvironmental Engineering Research Unit (BERU), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Gombak, 50728 Kuala Lumpur, Malaysia.

Abstract: The increasing volume of sewage sludge as well as the municipal and agricultural waste produced and the total managing cost every year has been one of the major environmental issues in Malaysia. The production of cellulase from domestic sewage sludge could possibly bring an economic importance in certain industries as well as a key to solve the problem of environmental pollution and also in developing and utilizing renewable energy sources by applying the enzyme on the cellulosic materials. This study focuses on the characterization of cellulases produced from fermentation broth of sewage sludge. Using the cellulase enzyme with the activity of 45 U/ml, few tests have been carried out to characterize and checked the stability of the enzyme. The tests were pH stability, temperature stability as well as incubation time for the enzyme. Characterization of the enzyme was likely to help develop efficient application of the enzyme in industrial scale. 50^{o}C was found to be the optimum temperature for the stability of the enzyme. Besides, pH 3 gave the highest enzyme activity and also 48 hours turn to be the optimum incubation time.

Key word: cellulase; characterization; sewage sludge; fermentation broth.

INTRODUCTION

Cellulase is an important commercial enzyme that widely used in food, animal feed, textile, pulp and paper, grain alcohol fermentation, starch processing, pharmaceuticals, malting and brewing industries (Oksanen, *et al*., 2000). Cellulase refers to a group of hydrolytic enzymes (endoglucanases, exoglucanases (cellobiohydrolases), and β- glucosidases) that are capable of hydrolyzing insoluble cellulose to glucose (Wen, *et al*., 2005). Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity (Chand, *et al*., 2005). *Trichoderma* and *Aspergillus* are most commonly used microorganisms in the production of commercial cellulase (Cherry, *et al*., 2003; Esterbauer, *et al.,* 1991). These mircroorganisms can grow on cellulosic materials by producing cellulase (Lee and Koo, 2001). The reason why cellulase is widely studied by scientists is due to its potential to utilize cellulosic waste which can be used as an inexpensive carbon source (Wen, *et al*., 2005). Currently the major problem associated with the application of cellulase on large scale is its high cost of production (Domingues, *et al*., 2001).

Wastewater sludge is very good source of carbon, nitrogen, phosphorus, and other nutrients for many microbial processes that could add value to sludge by producing certain valuable metabolic products. Findings from many researches have showed that sludge from domestic wastewater consists of 32% carbon, 3.8% nitrogen, 1.6% phosphorus, 0.05% magnesium, 0.15% potassium and sufficient trace elements which could be used as raw material for the production of value added products such as organic acids, biosolids, and biopesticides by liquid state bioconversion (Alam, *et al*., 2003; Jamal, *et al*., 2005). In Malaysia, domestic wastewater sludge (DWS) is generated at high rate of 4.9 million m^3 annually. Sludge accumulation with increasing population and other developments related activities in the coming years, Malaysia will face a major problem in managing the ever-increasing domestic wastewater sludge production which is usually disposed at the provided areas (Kadir and Velayutham, 1999). The present practice is either to co-dispose it with other solid waste at landfill sites or by direct disposal in shallow trenches (Oak Ridge National Laborator, 2006). However, due to limited land area and the rapid increases in sludge production, the treatment and disposal of sludge will be one of the crucial environmental issues faced by the Malaysian government.

Enzymes currently used for production of cellulosic ethanol were identified in fungal and bacterial systems (Lynd, 1999). Current limitations of enzymatic degradation of lignocellulosic biomass are mostly related to enzymatic stability and susceptibility to inhibitory agents or byproducts (Mousdale, 2008; Kristensen, *et al*., 2009). Continuous prospecting and bioengineering efforts should provide novel enzymes with higher specific activity and with lower susceptibility to inhibitors (Lynd, *et al*., 2008). In 1950, Elwyn Reese (Elwyn, 1950) and his workers at the Natick Laboratories identified *Trichoderma* strains which produced an active and well-balanced cellulase complex. Many other strains having cellulase activity have been reported (Mandels and Andreotti, 1978) but most do not produce adequate levels of extracellular cellulase for practical use. The advantage of *Trichoderma* cellulase is that it produces a complete cellulase with all the component required for

Corresponding Author: Muna Tasnim Mukhtaruddin, Bioenvironmental Engineering Research Unit (BERU), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Gombak, 50728 Kuala Lumpur, Malaysia.
E-mail: rosequarz_mtm@yahoo.com

hydrolysis of crystalline cellulose, and this cellulase is resistant to chemical inhibitors and stable in stirred tank reactors at pH 4.8, 50^{o}C for 48 hours or longer (Mandels and Reese, 1965).

Cellulases from various sources have shown their distinctive features as they carry their specific pH optima, solubility and amino acid composition. Thermal stability and exact substrate specificity may also vary with the origin. The optimum pH generally lies between 4 and 5 and temperature is 40-50°C (Bhat, 2000; Parry, *et al*., 2002). CMCase activities alter with varying pH and temperature and characterization of produced enzyme require knowledge about optimum pH, temperature stability and substrate specificity. Therefore, the enzyme is characterized to find out the best level of performance for enhanced efficiency of the process (Bhat, 2000; Parry, *et al*., 200219, 20). This research focused on the characterization of cellulase from fermentation broth of sewage sludge.

MATERIAL AND METHODS

A. Source of cellulase enzyme:

Sewage sludge was collected from Indah Water Konsortium (IWK)'s sewage treatment plant at Setapak, Kuala Lumpur and undergoes process of fermentation as well as separation and purification in Environmental Laboratory, Department of Biotechnology Engineering, IIUM. This purification process produced purified enzyme which was cellulase that was used in the characterization procedures.

B. Characterization of cellulase:

Purified cellulase enzyme was used for stability test. Using the cellulase enzyme with the activity of 45 U/ml, this study was set to determine the pH and temperature stability and to observe the effect of incubation time on the enzyme activity. To establish pH and temperature stability as well as the effect of incubation time on the enzyme activity, the procedures were followed as described by (George, *et al*., 2001; Xu, *et al*., 2000) .

i) Effect of pH:

The effect of pH on the cellulase stability was measured at pH ranges of 3.0 to 11.0. 0.5 ml enzyme was incubated in 1 ml of various buffers at 35°C for 30 min. After incubation, the enzyme was immediately cooled in an ice bath and the residual activity was assayed. The activity was expressed as percentage, with the higher activity as 100%. Table 1 shows the buffer systems used.

Table 1: Various Buffers Systems

Buffer	pH
50 mM Glycin buffer	3.0
50 mM Sodium Acetate buffer	5.0
50 mM Tris buffer	7.0
50 mM Tris buffer	9.0
50 mM Glycin buffer	11.0

ii) Effect of Temperature:

The effect of temperature on cellulase stability was measured at various temperatures ranging from 20 to 70°C. 0.5 ml enzyme was incubated in 1 ml 50 mM glycin buffer (pH 3.0) at 20, 40, 50, 60, 70°C for 30 min. After each period of incubation, the enzyme was immediately cooled in an ice bath and the residual activity determined. The highest activity of the enzyme was considered as 100%.

iii) Effect of the Incubation Time:

The pH and temperature determined in the effect of pH and temperature experiment on enzyme stability further used in effect of incubation time experiment. 0.5 ml enzyme was incubated in 1 ml 50 mM Glycin buffer (pH 3.0) at 50°C for 1, 3, 6, 12, 24, 48, 72, 96 and 120 hours. After each period of incubation, the enzyme was immediately cooled in the ice bath and the residual activity was determined. The enzyme activity was expressed in percentage which the highest activity considered as 100%.

RESULTS AND DISCUSSIONS

Cellulases from various sources have shown their distinctive features as they carry their specific pH optima, solubility and amino acid composition. Thermal stability and exact substrate specificity may also vary with the origin. CMCase activities alter with varying pH and temperature and characterization of produced enzyme require knowledge about optimum pH, temperature stability and some other characteristics. Therefore, the enzyme is characterized to find out the best level of performance for enhanced efficiency of the process (Bhat, 2000; Parry, *et al*., 2002). In this study, few tests have been carried out to characterize and checked the stability

of the enzyme. The tests were pH stability, temperature stability as well as incubation time for the enzyme. The effects of each parameter were further discussed in the following section.

i) *Effect of pH:*

Figure 1 shows the pH stability of purified cellulase enzyme. 0.5 ml (22.5 U/ml) sample was incubated in 1 ml various pH buffer for 30 min based on the previous stability study on lipase enzyme from thermophilic fungi isolated from palm oil mill effluent (Razak, *et al*., 1997). Cellulase enzyme was stable at pH 3, where the activity was the highest at this pH after 30 minutes of incubation. This result agrees with previous study where thermophilic fungi particularly *Trichoderma* cellulase (Mandels and Reese, 1965; Bhat, 2000; Parry, *et al*., 2002) having high cellulase activity and stable at acidic pH (Song and Wei, 2010). From the graph, the cellulase activity was declined as the pH increase from pH 3 to pH 11. More than 30% activities were lost at pH 7-11. (Razak, *et al*., 1997) studied on thermophilic fungi stability found that the enzymes showed the rapid loss of activities at pH above 7.0. In addition, it was observed that the enzyme activity has a broad pH range between 3.0 and 9.0 (Coral, *et al*., 2002).

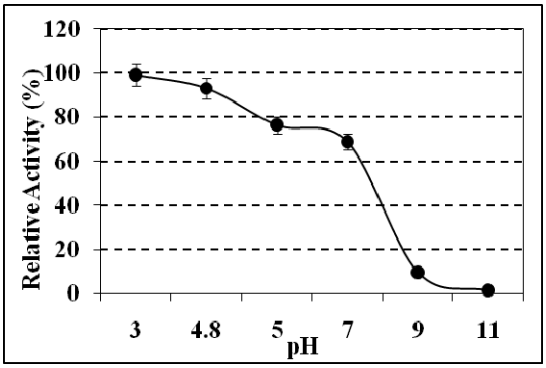

Fig. 1: Graph of Relative Activity (%) Vs pH to show the effect of pH at temperature 35^{o}C on Cellulase enzyme stability in 30 minutes. Data are presented as means of 3 replicates, having standard deviation within the range 0.5 to 1.0%.

ii) Effect of Temperature:

The effect of temperature on the cellulose stability was conducted at various temperatures ranging from 20 to 70°C (Figure 2) at pH 3. The cellulase activity showed that at 50^{o}C, 100% activity was still retained after 30 minutes of incubation. The cellulase activity dropped more than 20% at temperature less than 25^{o}C and loss about 45% of cellulase activity at temperature of 70^{o}C. Previous studies agreed that the optimum temperature generally lies between 40-50°C (Mandels and Reese, 1965; Bhat, 2000; Parry, *et al*., 2002). In addition, a study on the purification and characterization of microbial cellulolytic enzyme by Amtul Jamil Sami (Jamil and Shakoori, 2008) showed that optimum temperature for the CMCase activity was 50^{o}C. According to him, there was an increase till 50^{o}C but abrupt decline in enzyme activity was noticed when reaction mixtures were incubated above 50^{o}C. In other case, Jian-Min Song and, Dong-Zhi Wei (Song and Wei, 2010) in their study, production and characterization of cellulases and xylanases of cellulosimicrobium cellulans grown in pretreated and extracted bagasse and minimal nutrient medium M9, revealed the same result where according to them all hydrolytic enzymes presented an optimum temperature was 50^{o}C. Incubation at lower temperature resulted in longer time to the maximum enzyme activity. Incubation at higher temperature affected the fungus harmfully, which reflected on the enzyme synthesis. Since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield (Sabu, *et al*., 2002).

iii) Effect of the Incubation Time:

The stability of cellulase was studied up to 5 days at the most stable pH and temperature where the pH was 3.0 and the temperature was 50^{o}C. Figure 3 shows the effect of incubation time of the cellulase activity at the optimum stability conditions. Initially, for the first 24 hours (Day 1), the enzyme activity was increased by the increasing of time. The cellulase activity was maintained at 100% up to 48 hours of incubation period. The enzyme activity was highest and stable at Day 2 and started to decrease as the time increase. The activity was reduced to 85% after more than 96 hours of incubation. After 5 days of incubation, the cellulase activity retained about 75%. Mandel and Reese (Mandels and Reese, 1965) in their study reported that *Trichoderma* cellulase was stable for 48 hours or longer. In addition, extending the reaction time to ≥48 hours had no significant effect on cellulase activity (Hari, *et al*., 1999).

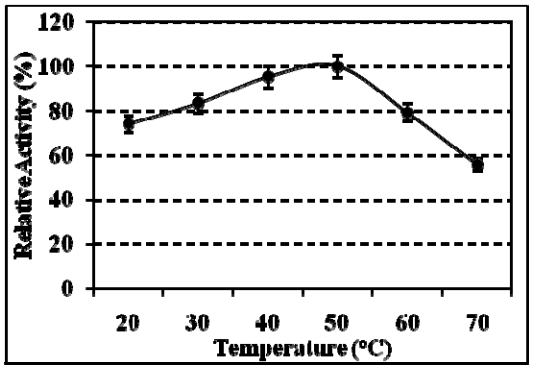

Fig. 2: Graph of Relative Activity (%) Vs Temperature (^{0}C) to show effect of temperature (^{o}C) at pH 3 on Cellulase enzyme stability in 30 minutes. Data are presented as means of 3 replicates, having standard deviation within the range 0.1 to 0.2%.

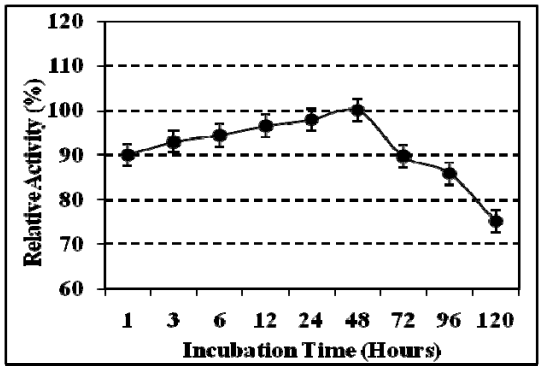

Fig. 3: Graph of Relative Activity (%) Vs Incubation Time (Hours) to show effect of incubation time on Cellulase enzyme stability at pH 3 and temperature 50^{o}C. Data are presented as means of 3 replicates, having standard deviation within the range 0.5 to 2.3%.

Conclusions:

It can be concluded that this study has provided an alternative approach in biotechnology for sewage sludge utilization by producing cellulase enzyme. This study provided a way to enhance the recovery of the cellulase produced. The stability of the cellulase enzyme under appropriate pH and temperature conditions were looking over in the industrial processes. Characterization of the enzyme was likely to help develop efficient application of the enzyme in industrial scale. 50^{o}C was found to be the optimum temperature for the stability of the enzyme. Besides, pH 3 was the optimum pH for the highest enzyme activity and also 48 hours turn to be the optimum incubation time. In these optimum conditions, enzyme activity maintained 100% up to 48 hours. The activity was reduced to 85% after more than 96 hours of incubation. After 5 days of incubation, the cellulase activity retained about 75%. Above all, this work proved that the cellulase enzymed obtained from the fermentation broth of sewage sludge can be used in various industrial application. In addition, the enzymatic catalysed approach of recycling cellulose-related waste products could play an important role in solving the problem of environmental pollution and also in developing and utilizing renewable energy sources.

ACKNOWLEDGEMENTS

We are greatful to Ministry of Science, Technology and Innovation (MOSTI), Malaysia for their financial support under eTechnofund Research Grant and to the Deprtment of Biotechnology Engineering, IIUM for providing lab facilities towards completing this study.

REFERENCES

Alam, M.Z., A. Fakhrul-Razi and A.H. Molla, 2003. Biosolids accumulation and biodegradation of domenstic wastewater treatment plant sludge by developed liquid state bioconversion process using a batch fermente. Water Research, pp: 3569-3578.

Bhat, M.K. 2000. Cellulase and related enzymes in biotechnology Biotechnology Advances, 18: 355-383.

Chand, A., M. Aruna and L.V. Rao, 2005. Novel mutation method for increased cellulase production. J. Appl. Microbio, 98: 318-323.

Cherry, J.R., Fidantsef, A.L. Cherry and Fidantsef, 2003. Directed evolution of industrial enzymes: an update. Curr. Opin. Biotechno, 14: 438-443.

Coral, G., B. Arikan, M.N. Unaldi and H.Guvenmens, 2002. Some properties of crude carboxymethyl cellulase of Aspergillus niger Zio. Wild type strain. Turk. J. Biol, 26: 209-213.

Domingues, F.C., J.A. Queiroz., J.M.S Cabral and L.P.Fonseca, 2001. Production of cellulases in batch culture using a mutant strain of Trichoderma reesei growing on soluble carbon source. Biotechnol. Lett, 23: 771–775.

Elwyn,T.R., G.H. Ralph, Siu and S.L. Hillel, 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol, 59: 485-497.

Esterbauer, H., W. Steiner, I. Laboudova, A. Hermann and M. Hayn, 1991. Production of Trichoderma cellulase in laboratory and pilot scale. Bioresour Technol, 36: 51-65.

George, S.P., A. Ahmad and M.B. Rao, 2001. A novel thermostable xylanase from Thermomonospora sp.: influence of additives on thermostability. Bioresource Technology, 78: 221-224.

Hari, S., Krishna, G.V. Chowdary, D.S. Reddy, C. Ayyanna, 1999. Simultaneous saccharification and fermentation of pretreated Antigonum leptopus (Linn) leaves to ethanol. J. Chem. Technol. Biotechnol, 74: 1055-1060.

Jamal, P., M.Z. Alam, M. Ramlan, M. Salleh and M.M. Akib, 2005.Sewage treatment plant sludge: a source of potential microorganism for citric acid production. American Journal of Applied Sciences, 2(8): 1236-1239.

Jamil, A.J. and A.R. Shakoori, 2008. Biochemical characterization of endo-1, 4-β-D-glucanase activity of a green insect pest Aulacophora foveicollis (Lucas). Life Science Journal, 5(2): 30-36.

Kadir, M.D.A. and S. Velayutham, 1999. The management of municipal wastewater sludge in Malaysai. University Technology Malaysia: Symposium on sludge management., pp: 18-19.

Kristensen, J.B., C. Felby and H.Jorgensen, 2009. Yielddetermining factors in high- solids enzymatic hydrolysis of lignocelluloses. Biotechnology for Biofuels, 2: 11.

Lee, S.M. and Y.M. Koo, 2001. Pilot-scale production of cellulose using Trichoderma reesei Rut C-30 in fed-batch mode. J. Microbiol. Biotechnol, 11: 229-233.

Lynd, L.R., 1999. Overview and evaluation of fuel ethanol from cellulosic biomass, Technology, economics, the environment, and policy. Ann. Rev. Energy Environ, 21: 403-465.

Lynd, L.R., M.S. Laser, D. Bransby, B.E. Dale, B.Davison, R. Hamilton, M. Himmel, M. Keller, M. McMillan, J.D. Sheehan and C.E. Wyman, 2008. How biotech can transform biofuels. Nature Biotechnology, 26: 169-172.

Mandels, M. and E.T. Reese, 1965. Ann. Rev. Phytopathol, 3: 85.

Mandels, M. and R.E. Andreotti, 1978. Process Biochem, 13: 6.

Mousdale, D.M., 2008. Biofuels:Biotechnology,Chemistry, and Sustainable Development CRC Press, Boca Raton, FL., pp: 66-78.

Oak Ridge National Laborator, Material research for seperations technologies, energy and emission reduction opportunities, Industrial Technologies Program. U.S, 2006.

Oksanen, T., J. Pere, L. Paavilainen, J. Buchert and L. Viikari, 2000. Treatment of recycled kraft pulps with Trichoderma reesei hemicellulases and cellulases. J. Biotechnol, 78: 39-44.

Parry, N.J., D.E. Beever, E. Owen, I. Vandnberghe, J.V. Beeumen and M. Bhat, 2002. Biochemical characterization and mechanism of action of a thermostable ß-glucosidase purified from Thermoascus aurantiacu, Biochem. J, 353: 117-127.

Razak,C.A.N., A.B. Salleh, R. Musani, M.Y. Samad and M. Basri, 1997. Some characteristics of lipases from thermophilic fungi isolated from palm oil mill effluent. J. Mol. Cat. B: Enzymat, 3: 153-159.

Sabu, A., S. Sarita, A. Pandey, B. Bogar, G. Szakacs and C.R. Soccol, 2002. Solid-State Fermentation for Production of Phytase by Rhizopus oligosporus. Appl. Biochem. Biotechnol, pp: 102-103, 251-260.

Song, J.M. and D.Z. Wei, 2010. Production and characterization of cellulases and xylanases of Cellulosimicrobium cellulans grown in pretreated and extracted bagasse and minimal nutrient medium M9. Biomass and Bioenergy, pp: 1-5.

Wen, Z., W. Liao and S. Chen, 2005. Production of cellulase by Trichoderma reesei from dairy manure. Bioresour. Technol, 96: 491-499.

Xu, B., U. Hellman, B. Ersson, and J.C. Janson, 2000. Purification, characterization and amino-acid sequence analysis of a thermostable, low molecular mass endo-β-1,4-glucanase from blue mussel, Mytilus edulis Eur J Biochem, 267: 4970-4977.