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Activatory Effects of Manganese and Cobalt Ions on the Activity of Cellulase Produced by *Aspergillus niger*

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ABSTRACT: Cellulase is an enzyme with wide industrial applications, however the optimization of its activity by metal ions has not been fully characterised. In this study, the effect of interaction of Mn^{2+} and Co^{2+} on the activity of cellulase produced by *Aspergillus niger* using submerged fermentation process was investigated. Cellulase activity was determined by measuring the concentration of glucose produced from the hydrolysis of carboxymethylcellulose (CMC) catalysed by the enzyme. Both Mn^{2+} and Co^{2+} (0.2-10 mM) activated cellulase-catalysed hydrolysis of CMC with optimal concentrations at 8 mM and 1 mM respectively. Synergistic interaction between Mn^{2+} and Co^{2+} promoted hydrolysis of CMC by cellulase with optimal activation at 8 mM Mn^{2+} and 1 mM Co^{2+}. This study revealed that the activity of cellulase produced by *Aspergillus niger* can be optimized in the presence of optimal concentrations of Mn^{2+} and Co^{2+} for its various applications.

KEYWORDS: Cellulase, endoglucanase activity, *Aspergillus niger*, metal ions, activity optimization

1.0 Introduction

Cellulases are industrially important enzymes, but the cost of production is very high. A significant reduction in cost or optimal utilization of the enzyme produced is thus important for commercial use. Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including fungi and bacteria during growth on cellulosic materials (Tomme *et al*., 1995; Sang-Mok and Koo, 2001). Among these microorganisms, the genera of *Aspergillus*, *Clostridium*, *Cellulomonas*, *Thermonospora* and *Trichoderma* are the most extensively studied cellulase producers (Sun and Cheng, 2002).

Cellulases are hydrolytic enzymes that catalyse the breakdown of cellulose to smaller oligosaccharides and finally glucose. Cellulases are composed of independently folding, structurally and functionally discrete units called domains or modules (Henrissat *et al*., 1998). Fungal cellulases typically have two separate domains: a catalytic domain (CD) and a cellulose binding module (CBM), which is joined by a short polylinker region to the catalytic domain at the N-terminal. The CBM is comprised of approximately 35 amino acids, and the linker region is rich in serine and threonine (Kuhad *et al*., 2011). These enzymes belong to the large family of glycosyl hydrolases which have been classified into 63 families of homologous folds on the basis of sequence comparisons and hydrophobic cluster analysis of the catalytic domains (Henrissat and Bairoch, 1993; Yin *et al*., 2010). The catalytic domains of cellulases and related xylanases have been identified in 14 of these families (Yin *et al*., 2010). The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Perez *et al*., 2002; Zhang and Lynd, 2004). Endoglucanases hydrolyse accessible intramolecular β-1, 4- glucosidic bonds of cellulose chains randomly to produce new chain ends; exoglucanases progressively cleave cellulose chains at the ends to release soluble cellobiose or glucose; and β-glucosidases

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hydrolyse cellobiose to glucose in order to eliminate cellobiose inhibition. These three hydrolyses processes occur simultaneously. Primary hydrolysis by endoglucanases and exoglucanasesthat occurs on the surface of solid substrates releases soluble sugars with a degree of polymerization of up to 6, into the liquid phase. This enzymatic depolymerization step by endoglucanases and exoglucanases is the rate-limiting step for the whole cellulose hydrolysis process. Secondary hydrolysis that occurs in the liquid phase involves primarily the hydrolysis of cellobiose to glucose by β-glucosidases, although some β-glucosidases also hydrolyse longer cellodextrins (Zhang and Lynd, 2004).

Cellulases have several applications in industry, which include bioethanol production (Gupta *et al*., 2011), wastes management (Milala *et al*., 2005), food processing (de Carvaiho *et al.*, 2008), extraction of fruit and vegetable juices (Uhlig, 1998, Grassin and Fauquembergue, 1996), extraction of olive oil (Galante *et al.*, 1998), pulp and paper production (Mai *et al.*, 2004, Kuhad *et al.*, 2010), textile production (Karmakar and Ray, 2011), malting and brewing (Singh *et al.*, 2007), animal feed production (Galante *et al.*, 1998; Dhiman *et al.*, 2002), production of laundry detergents (Uhlig, 1998; Singh *et al.*, 2007; Karmakar and Ray, 2011) and carotenoid extraction (Cinar, 2005).

The high cost of cellulase production coupled with low enzyme activities limits its industrial use. One of the most expensive aspect of industrial cellulase production is the substrate (carboxymethylcellulose) used as inducer for cellulases. Cellulase production using cheaper substrates such as rice bran saw dust, corn cob, wheat straw and newspaper (Abo-state *et al*., 2010; Yin *et al*., 2010; Khan and Singh, 2011) has been employed as an effective strategy to reduce production cost. However, optimization of cellulase activity by ligands especially metal ions has not been fully characterized. Yin *et al.* (2010) showed that metal ions such as Mn^{2+} and Co^{2+} activated cellulase produced from *Bacillus subtilis* YJ1 but the mechanisms of activation were not described. Thus, in this study, the kinetic and modulatory properties of Mn^{2+} and Co^{2+} and the effect of interaction of the two metal ions on the activity of cellulase produced by *Aspergillus niger* using submerged fermentation process was investigated. This is with a view to gaining insight into the mechanism by which the metal ions activate cellulase production and optimizing its activity for various applications.

2.0 Materials and Methods

2.1 Materials

Rice bran was purchased at Oja-Oba market, Ilorin, Kwara State, Nigeria. *Aspergillus niger* was obtained from pure culture vessel at the Department of Microbiology, University of Ilorin, Ilorin, Nigeria. The standard cellulase substrate, the sodium salt of carboxymethylcellulose (CMC), dintrosalicyclic acid (DNS), sodium potassium tartarate and sodium metabisulphite were products of Sigma-Aldrich, St. Louis, USA. All other reagents used were of analytical grade.

2.2. Pretreatment of Substrate

The substrate, rice bran was ground in an electric grinder (Phillips HR2810/A) to fine particles. The particles were soaked in 0.25M HCl solutions at 20% w/v, heated on a Bunsen flame for 30 minutes; and allowed to stand for 24 hours at room temperature. The acid was drained off; the residue washed several times with distilled water and neutralized with a few drops of 0.25M NaOH and washed again. The pretreated substrate was drained with Muslin cloth; air dried for 6 hours and dried in an electric oven at 65 ± 2 ^{o}C to constant weight.

2.3 Screening of Aspergillus niger for Cellulase Production

Six isolates of *Aspergillus niger* obtained from the Department of Microbiology, University of Ilorin, Ilorin, Nigeria in pure culture were screened for cellulolytic activity by the agar diffusion method described by Hankin and Anagnostakis (1977). Plates containing Potato Dextrose Agar incorporated with 0.5% of CMC were inoculated with 72 hours old culture of the isolates by streaking once across the middle of the agar medium. Plates were incubated at 28 ± 2^{o}C until visible growth was

observed. Cellulolysis was detected after growth, by flooding the plates with 1% congo red solution for 15 minutes. The dye was drained and plates flooded with 1.0N sodium chloride solution for another 15 minutes. Clearance around growth of isolate represents cellulase production. The clearance zone was measured across the growth of organism; and isolate that produced the highest zone of clearance was selected for cellulase production.

2.4 Production of Cellulase

2.4.1 Inocula Development

Spores of the highly cellulolytic *A. niger* from 7 days old slant culture was harvested by rinsing with 5 ml sterile distilled water into Mandels mineral salt medium (Mandels *et al*., 1974), containing 0.2 % (w/v) CMC, in 250 ml Erlenmeyer flask and incubated on a gyratory shaker (150 rpm) at room temperature for 72 hours.

2.4.2 Fermentation

Mandels mineral salt medium (100 ml each), containing10 g of the pre-treated rice bran in 250 ml Erlenmeyer flasks were inoculated with 5 ml of inoculums culture containing approximately 3.6 x 10^6 spores/ml. Flasks were incubated at 28 ± 2 °C on a gyratory shaker (150 rpm) for 10 days and culture fluid separated by filtering through Whatman No 1 filter paper. The fluid was clarified in a table top high speed refrigerated centrifuge H1850R at 13,000 x g for 20 minutes at 4°C. After centrifugation, supernatant was collected and used as crude enzyme.

2.5 Determination of Cellulase (Endoglucanase) Activity

Cellulase (endoglucanase) activity was determined by measuring the concentration of glucose produced from the hydrolysis of carboxymethyl cellulose (CMC) catalysed by the crude cellulase (Miller, 1959). The activity of cellulase was assayed by incubating 1ml 20.6 mM CMC in 20 mM phosphate buffer (pH 7.0) with an aliquot of the crude enzyme containing 870µg of protein at 50°C in the absence and in the presence of appropriate concentrations of the ligands for 45 minutes. 1ml of DNS was added and the reaction mixture boiled in a water bath for 5 minutes to stop the reaction, then cooled to room temperature. The absorbance was measured at 540nm in a Spectrumlab 752S UV-Visible spectrophotometer against blank. Absorbance was compared with the standard plot of known concentration of glucose with DNS reagent at 540 nm. One unit of cellulase activity was defined as the amount of enzyme that hydrolyses CMC with the release of 1 micromole of glucose per minute at 50°C.

2.6 Determination of Protein Concentration

Protein concentration was determined by the Biuret method, using bovine serum albumin as standard (Gornall *et al*., 1949).

2.7 Effects of Mn^{2+} and Co^{2+} on Cellulase-Catalysed Hydrolysis of Carboxymethylcellulose

CMC (6.869 mM) was incubated with an aliquot of the crude enzyme containing 870µg of protein at 50°C in the presence of 0 to 10 mM Mn^{2+} or Co^{2+} for 45 minutes. 1 ml DNS was added and boiled in a water bath for 5 minutes to stop the reaction. The reaction mixture was then cooled to room temperature and absorbance measured at 540 nm. For the effects of Mn^{2+} and Co^{2+} on kinetics of Cellulase-catalysed hydrolysis of CMC, the initial rate of hydrolysis of increasing concentrations of CMC by an aliquot of the crude enzyme containing 870µg of protein in the presence of 8 mM Mn^{2+} or 1 mM Co^{2+} were determined. The concentrations of CMC used were 0.069, 0.343, 0.687, 1.373, 3.433, 5.150, and 6.867 mM.

2.8 Effects of Mn^{2+} on Co^{2+}-Activated Hydrolysis of Carboxymethylcellulose by Cellulase

Reaction mixture containing buffered 6.869 mM CMC, 1 mM Co^{2+}, the indicated

concentrations of Mn^{2+} (0-10 mM), and an aliquot of the crude enzyme containing 870µg of protein was incubated at 50°C for 45 minutes. The absorbance was read at 540nm after stopping the reaction as earlier described. For the effects of interaction of Mn^{2+} and Co^{2+} on kinetics of cellulase-catalysed hydrolysis of CMC, the initial rate of hydrolysis of increasing concentrations of CMC by 0.5ml of the crude enzyme in the presence of 8 mM Mn^{2+} and 1 mM Co^{2+} were determined. The concentrations of CMC used were 0.069, 0.343, 0.687, 1.373, 3.433, 5.150, and 6.867 mM. Reaction was initiated by the incubation of buffered CMC with an aliquot of the crude enzyme containing 870µg of protein in the presence of the metal ions.

3.0 Results

3.1 Screening of Aspergillus niger for Cellulase Activity

Plate 1 show the result obtained from screening of *Aspergillus niger* for cellulase activity. A clear hydrolysis zone was observed.

3.2 Effect of Mn^{2+} and Co^{2+} on Cellulase-Catalysed Hydrolysis of Carboxymethylcellulose

Both Mn^{2+} and Co^{2+} (0.2-10 mM) activated cellulase-catalysed hydrolysis of CMC with optimal concentrations at 8 mM and 1 mM respectively. The activatory effect of Mn^{2+} on cellulase activity was concentration-dependent; there was a progressive increase in cellulase activity from 0.2 to 8 mM (Figure 1). On the other hand, in the presence of Co^{2+}, there was progressive increase in activity from 0.2 to 1 mM concentration but the activity began to decline from 2 to 10 mM though not inhibitory (Figure 2).

3.3 Effect of Interaction of Mn^{2+} and Co^{2+} on Cellulase-Catalysed Hydrolysis of Carboxymethylcellulose

Cellulase activity was increased in the presence of 1 mM Co^{2+} and 0.2 to 10 mM concentrations of Mn^{2+}. Synergistic interaction was observed between Mn^{2+} and Co^{2+} in enhancing hydrolysis of CMC by cellulase since the activity was higher at the 1-10 mM of the Mn^{2+} when compared with when the concentration of Mn^{2+} is zero (Figure 3). The optimal synergistic activation of the cellulase activity by the metal ions was at 8 mM Mn^{2+} and 1 mM Co^{2+}. The substrate kinetics for the effect of interaction of Mn^{2+} and Co^{2+} on cellulase-catalysed hydrolysis of CMC further reveals the activation of the enzyme with optimal activity at 8 mM Mn^{2+} and 1 mM Co^{2+} (Figure 4).

4.0 Discussion

Aspergillus strains are major sources of commercial cellulases (Berry and Paterson, 1990). Growth of *Aspergillus niger* spores on a vertical line inside the plate indicates the presence of cellulase (Aneja, 2003).

The activatory effects of Mn^{2+} and Co^{2+} on cellulase-catalysed hydrolysis of CMC as shown in this study are in agreement with the earlier reports (Shailendra *et al*., 1992; Damisa *et al*., 2008; Yin *et al*., 2010; Santos *et al*., 2012) of cellulase obtained from various microrganisms grown on different cellulosic materials. Also, the higher extent of activation of endoglucanase activity in the presence of Mn^{2+} when compared with Co^{2+} is in conformity with the report of Yin *et al.* (2010). The concentration-dependent effects of Mn^{2+} and Co^{2+} as observed in this study suggests that the metal ions may exert their effect by inducing some conformational changes in the enzyme which may result in stimulation of activity. Mn^{2+} has been reported to be a good metal for substrate activation and electrostatic stabilization (Andreini *et al.*, 2008). Also, according to the report of Santos *et al.* (2012), Mn^{2+} was demonstrated to have a hyper-stabilizing effect on the *Bacillus subtilis* cellulase 5A with the use of deletion constructs and X-ray crystallography. They also showed that Mn^{2+} binds to the enzyme where it maps to a negative charge motif thereby stabilizing the structure of the enzyme.

Cobalt is found almost exclusively in B_{12}-dependent enzymes, where it functions as redox-active centre and as a Lewis acid in other enzymes, where it binds and activates reacting

species (Andreini *et al.*, 2008). The increase in the endoglucanase activity of the crude enzyme at lower concentration of Co^{2+} and progressive decline at higher concentrations observed in this study is similar to the report of Wang *et al.* (2012). They showed that the activity of crude cellulase was increased at lower Co^{2+}

Plate 1: Screening of *Aspergillus niger* for Cellulase Activity

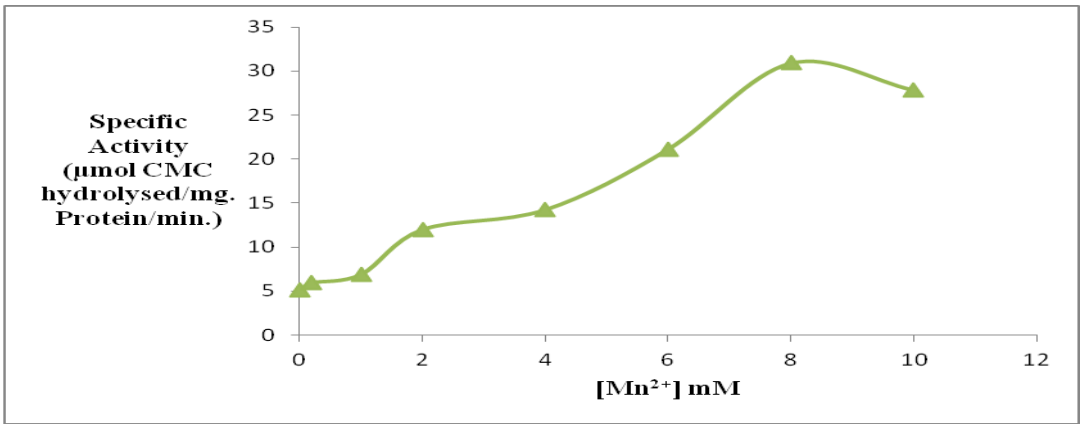

Fig 1: Effect of Mn^{2+} on Cellulase-Catalysed Hydrolysis of Carboxymethylcellulose

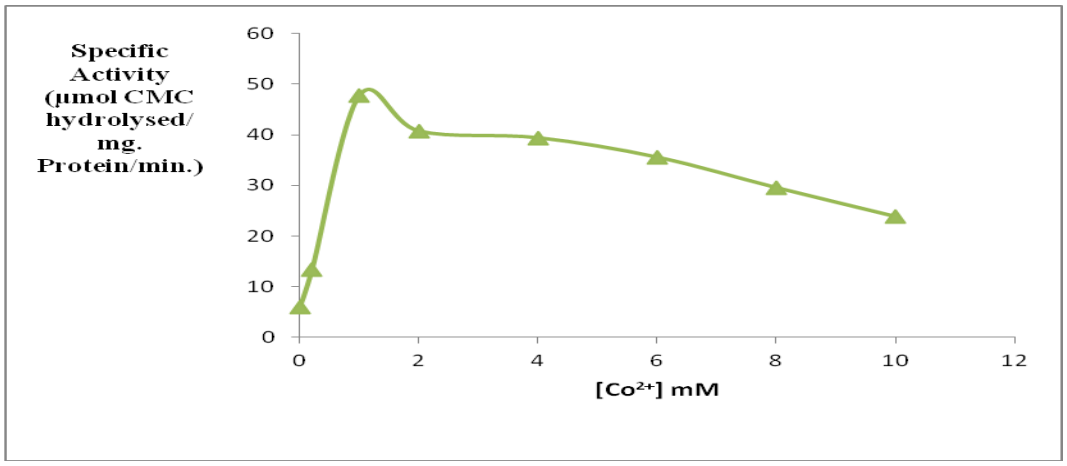

Fig 2: Effect of Co^{2+} on Cellulase-catalysed Hydrolysis of Carboxymethylcellulose

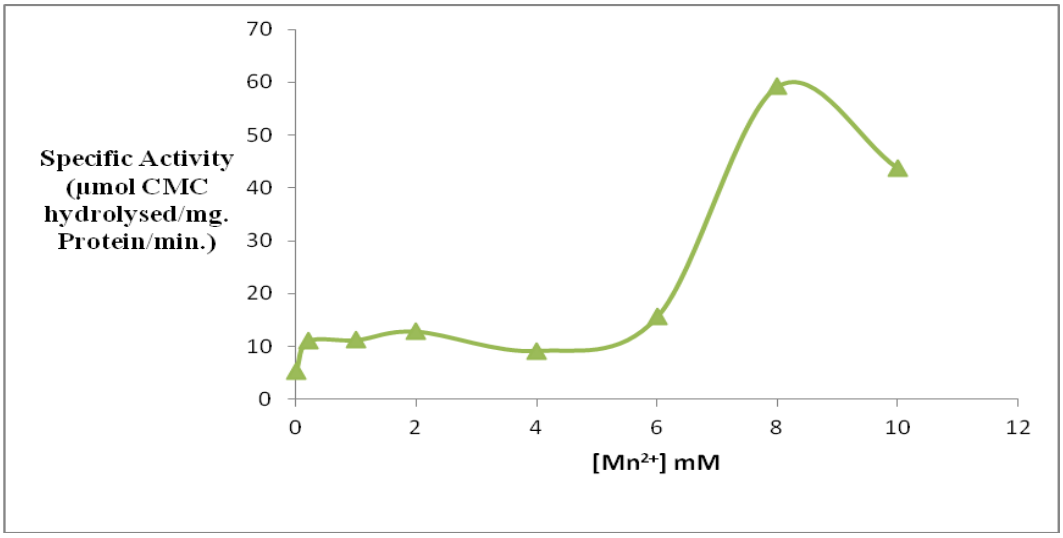

Fig 3: Effect of Mn^{2+} on Co^{2+} -Activated Cellulase Catalysed Hydrolysis of Carboxymethylcellulose

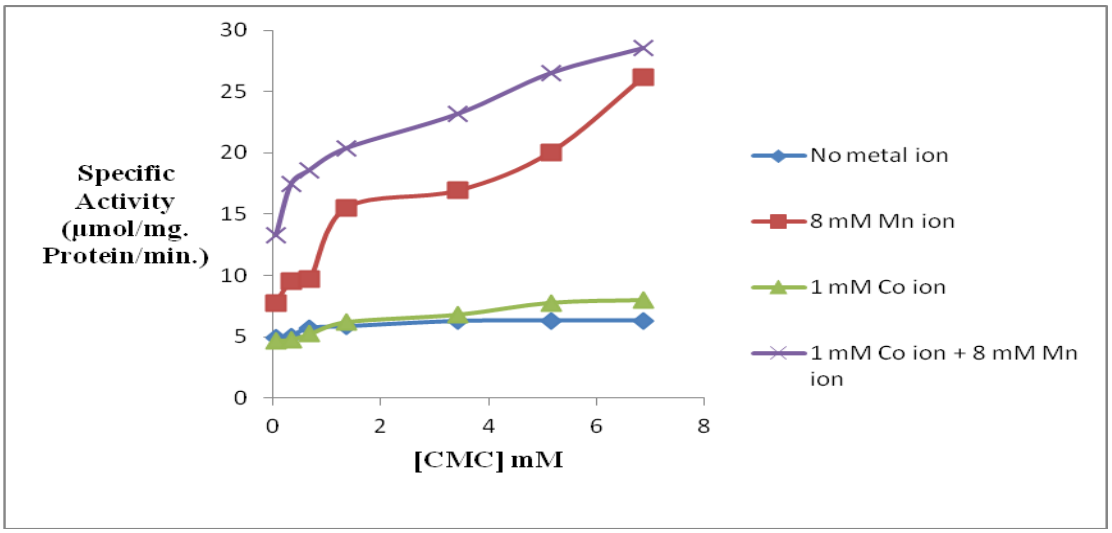

Fig 4: Substrate Kinetics of the Effect of Interaction of Mn^{2+} and Co^{2+} on Cellulase-Catalysed Hydrolysis of Carboxymethylcellulose

concentration and decreased at higher ones but with the purified cellulase (endoglucanase), Co^{2+} had significant positive effects; the effects increased with increase Co^{2+} concentrations. Similar result on purified cellulase was reported by Mawadza *et al.* (2000). This suggests that the inhibitory tendency of cellulase activity at higher concentration of Co^{2+} as observed in this study may be due to the action of the other cellulase activities (exoglucanase and β-glucosidase) present in the crude enzyme.

In conclusion, this study revealed that the activity of cellulase produced by *Aspergillus niger* can be optimized in the presence of optimal concentrations of Mn^{2+} and Co^{2+} which offers a great prospect in the industrial use of the enzyme since low catalytic efficiency of cellulase limits its application. Also, desired features like Mn^{2+} and Co^{2+} can be imparted to cellulase by protein engineering to improve the overall performance of the enzyme.

References

Abo-state, M. A. M., Hammad, A. I., Swelim, M. and Gannan, R. B. (2010). Enhanced production of cellulase(s) by *Aspergillus spp*. Isolated from agriculture wastes by solid state fermentation. American-Eurasian Journal of Agriculture and Environmental Science 8 (4): 402 - 410.

Andreini, C., Bertini, I., Cavallaro, G., Holliday, G. L. and Thornton, J. M. (2008). Metal ions in biological catalysis: from enzyme databases to general principles. Journal of Biological Inorganic Chemistry 13(8): 1205-1218.

Aneja, K. R. (2003). Experiments in Microbiology, Plant Pathology and Biotechnology, Fourth Edition, New Age International (p). Ltd., Publishers, New Delhi: 607.

Berry, D. R. and Paterson, A. (1990). Enzymes in food industry. In: Suckling CJ (Ed) Enzyme Chemistry, Impact and Applications (2nd edition), Chapman and Hall, London: pp 306-351.

Cinar, I. (2005). Effects of cellulase and pectinase concentrations on the colour yield of enzyme extracted plant carotenoids. Process Biochemistry 40(2): 945–949.

Damisa, D., Ameh, J. B. and Umoh, V. J. (2008). The effect of changes in manganese concentration on cellulase yield from bagasse fermented with mutagenized strain of *Aspergillus niger* AH3. International Journal of Biological and Chemical Sciences 2: 368-372.

de Carvalho, L. M. J., de Castro, I. M. and da Silva, C. A. B. (2008). A study of retention of sugars in the process of clarification of pineapple juice (*Ananas comosus*, L. Merril) by micro- and ultra-filtration. Journal of Food Engineering 87 (4): 447–454.

Dhiman, T. R., Zaman, M. S., Gimenez, R. R.,

Walters, J. L. and Treacher, R. (2002). Performance of dairy cows fed forage treated with fibrolytic enzymes prior to feeding. Animal Feed Science and Technology 101 (4): 115–125.

Galante, Y. M., DeConti, A. and Monteverdi, R. (1998). Application of *Trichoderma* enzymes in food and feed industries. Biological Control and Commercial Applications 2: 311–326.

Gornall, A., Bardsmill, C. T. and David, M. M. (1949). Determination of serum protein by means of biuret reaction. Journal of Biological Chemistry 177: 751-766.

Grassin, C. and Fauquembergue, P. (1996). Fruit juices," In: *Industrial Enzymology*, T. Godfrey and S. West, Eds., pp. 226-264, MacMillan Press, London, UK, 2nd edition.

Gupta, Y., Khasa, P. and Kuhad, R. C. (2011). Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. Carbohydrate Polymers 84: 1103-1109.

Hankin, L. and Anagnostakis, S. L. (1997). Solid media containing carboxymethylcellulose to detect C_x cellulase activity of micro-organisms. Journal of General Microbiology 98 (1): 109-115.

Henrissat, B. and Bairoch, A. (1993). "New families in the classification of glycosyl hydrolases based on amino acid sequence similarities," Biochemical Journal 293 (3): 781-788.

Henrissat, B., Teeri, T. T. and Warren, R. A. J. (1998). A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants. FEBS Letters 425 (2): 352–354.

Karmakar, M. and Ray, R. R. (2011). Current trends in research and application of microbial cellulases. Research Journal of Microbiology 6 (1): 41–53.

Khan, J. A. and Singh, S. K. (2011). Production of cellulase using cheap substrates by solid state fermentation. International Journal of Plant, Animal and Environmental Sciences 1(3): 179 – 187.

Kuhad, R. C., Gupta, R., Khasa, Y. P. and Singh, A. (2010). Bioethanol production from *Lantana camara* (red sage): pretreatment, saccharification and fermentation. Bioresource Technology 101 (21): 8348–8354.

Kuhad, R. C., Gupta, R., Khasa, Y. P. and Singh, A. (2011). Microbial cellulases and their industrial applications. Enzyme Research 3: 1-10.

Mai, C., K¨ues, U. and Militz, H. (2004). Biotechnology in the wood industry. Applied Microbiology and Biotechnology 63(5): 477–494.

Mandels, M., Hontz, L. and Nystrom, J. (1974). Enzymatic hydrolysis of waste cellulose. Biotechnology Bioengineering 16 (11): 1471-1493.

Mawadza, C., Hatti-Kaul, R., Zvauya, R., and Mattiasson, B. (2000). Purification and characterization of cellulases produced by two *Bacillus* strains. Journal of Biotechnology 83(3): 177-187.

Milala, M. A., Shugaba, A., Gidado, A., ENe, A. C. and Wafar, J. A. (2005). Studies on the use of agricultural wastes for cellulase enzyme production by *Aspergillus niger*. Research Journal of Agriculture and Biological Science 1: 325–328.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31 (3): 426-428.

Perez, J., Munoz-Dorado, J., de la Rubia, T. and Martinez, J. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. International Microbiology (5): 53-63.

Sang-Mok, L. and Y. M. Koo. (2001). Pilot-scale production of cellulase using *Trichoderma reesei* Rut C-30 in fed-batch mode. Journal of Microbiology and Biotechnology 11(2): 229–233.

Santos, C. R., Paiva, J. H., Sforca, M. L., Neves, J. L. and Navarro, R. I. (2012). Dissecting structure, function and stability of a thermostable GH5-CBM3 cellulase from *Bascillus subtilis* 168. Biochemical Journal 441(1): 95-104.

Shailendra, S., Bahadur, J. and Varma, A. (1992). Effect of cobalt and nickel on growth and carboxylmethyl cellulase activity of *Cellulomonas* spp. Biometals 5: 209-212.

Singh, A., Kuhad, R. C. and Ward, O. P. (2007). Industrial application of microbial cellulases, In *Lignocellulose Biotechnologgy: Future Prospects*, R. C. Kuhad and A. Singh, Eds.: pp 345–358, I.K.International Publishing House, New Delhi, India.

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83(1): 1–11.

Tomme, P., Warren, R. A. and Gilkes, N. R. (1995). Cellulose hydrolysis by bacteria and fungi. *Advances in Microbial Physiology* 37(1): 1-81.

Uhlig, H. (1998). *Industrial Enzymes and Their Applications*, John Wiley & Sons, New York, NY, USA.

Wang, X., Zhang, L., Wang, K., Wang, F., Peng, F. and Wang, L. (2012). The activity and kinetic properties of cellulases in substrates containing metal ions and acid radicals. Advances in Biological Chemistry 2: 390-395.

Yin, L, Lin, H. and Xiao, Z. (2010). Purification and Characterization of a Cellulase from *Bacillus subtilis* YJ1. Journal of Marine Science and Technology 18 (3): 466-471.

Zhang, Y. H. P. and Lynd, L. R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnology Bioengineering 88: 797–824.