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Endoglucanase Production by *Penicillium atrovenetum* Using Plantain Peels as Substrate

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Abstract

Production of endoglucanase, a good index for cellulase, by Penicillium atrovenetum *using plantain peels as substrate was investigated. Cultural parameters varied include incubation time (1-15 days), pH (3-6), temperature (30-50^oC) and nitrogen source. Enzyme production was analyzed by the dinitrosalicyclic acid (DNS) method. Highest production of endoglucanase was observed on the 7th day at pH 5 and at incubation temperature of 40^oC. Ammonium oxalate, followed closely by ammonium persulfate, was observed as the best nitrogen source. The crude enzyme has optimum activities at 50^oC and pH 5 while stable within 30^oC to 50^oC and pH 5 to 7. Addition of 5 mM $CaCl_2$ enhanced the activities of the enzyme by 15.54%.*

Keywords*: Enzyme production, cellulase, dinitrosalicyclic acid, nitrogen source, ammonium oxalate, ammonium persulfate.*

Introduction

Several wastes are now being used as substrates for the growth of microorganisms in order to produce useful metabolites/enzymes applied in various industries. Some of these wastes include palm oil mill effluent (Fadzilah and Mashitah 2010), potato processing wastes (Afifi 2011), lemon peel, sorghum stem and sunflower head (Patil and Dayanand 2006), forage palm (Maciel *et al.* 2011), grapefruit peel waste (Wilkins *et al.* 2007), palm fruit husk (Odeniyi *et al*. 2009), orange wastes (Widmer *et al*. 2010; Omojasola and Jilani 2008), banana wastes and beet wastes (Dhabekar and Chandak 2010), etc.

Plantain peels are agro-industrial by-products left behind after the edible portion of plantain has been processed into various food items by cooking, roasting or milling into flour. Locally, ripe or unripe plantain wastes may be used to feed livestock or in the production of local soap but in the areas where these are not feasible, these wastes end up polluting the environment (Bains 2001). Results of the proximate analysis of the plantain peels showed that it contained high percentage of ash content and high cellulose (13.87%) and hemi cellulose (15.07%) contents (Adeyi 2010).

Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Chellapandi and Jani 2008). The cellulase system in fungi is considered to comprise three hydrolytic enzymes: endo-(1,4)-β-D-glucanase (endo-glucanase, endocellulase, carboxymethyl-cellulase (CMCase) [EC 3.2.1.4]), which cleaves β-linkages at random, commonly in the amorphous parts of cellulose, exo-(1,4)-β-D-glucanase (cellobiohydrolase, exocellulase, microcrystalline cellulase, avicelase [EC 3.2.1.91]), which releases cellobiose from nonreducing or reducing end, generally from the crystalline parts of cellulose and β-glucosidase (cellobiase [EC 3.2.1.21]), which releases glucose from cellobiose and short-chain cellooligosaccharides (Bhat and Bhat 1997). Cellulases can be used in several industrial applications, such as in textile, laundry and detergents, food and animal feed, pulp and paper, and more recently, biofuel production from cellulosic materials (Sukumaran *et al*. 2005).

Most commonly studied cellulolytic fungi include *Trichoderma*, *Humicola*, *Penicillim* and *Aspergillus* (Sukumaran *et al*. 2005). Among the *Penicillium* spp. *P. chrysogenum* (Nwodo Chinedu *et al*. 2008), *P. occitanis* (Chaabouni *et al*. 1995), *P. brasilianum* (Jørgensen *et al*. 2003) and *P. decumbens* (Mo *et al*. 2004) are majorly employed in cellulase production. Recently, in search of a local source of cellulolytic and pectinolytic enzymes for industrial application, Adeleke *et al*. (2012) isolated *P. atrovenetum* which is now being tested for cellulase production from plantain peels (a readily available agrowaste in Nigeria) with the hope of leading to a large scale production of cellulase in future.

Materials and Methods

Microorganism

Penicillium atrovenetum was earlier isolated (Adeleke *et al*. 2012) and maintained on potato dextrose agar (PDA) slants at 4^{o}C and sub-cultured every three months. The fungus was activated by transferring onto PDA plates and cultured at room temperature for seven days.

Media and Cultivation

Plantain peels, used as substrate for microbial cultivation and enzyme production, were collected from fruit traders at Agbowo in Ibadan metropolis, Oyo State, in the south western region of Nigeria. The plantain peels were sundried, later oven dried, ground and stored in air tight polyethylene bags to keep them moisture-free. To prepare the inoculum, two discs of respective fungal hyphae were mixed with 10ml of sterile distilled water and a suspension was made. The fermentation medium contained 5 g of processed plantain peels mixed with 4 ml of Czapek-Dox mineral solution (Adeleke *et al*. 2012) inside a 250-ml Erlenmeyer flask and sterilized at 121^{o}C for 30 min. Cultivation was carried out by adding 1ml of the inocula. Then, the flasks were incubated at 29^{o} ± 1^{o}C. The effects of incubation time, pH, temperature and nitrogen sources were tested to determine optimized condition for endoglucanase production.

Partial Enzyme Characterization

Optimum pH and Temperature for Enzyme Activity: The enzyme activity was determined at 50^{o}C, in different pH using sodium acetate (pH 3.0-5.0), citrate-phosphate (pH 5.0-.0), tris-HCl (pH 7.0- 8.5) and glycine-NaOH (pH 8.5-11.0) as buffers. The optimum temperature within the 30-70^{o}C range was determined by incubation of the reaction mixture at optimum pH.

Temperature and pH Stability: Enzyme solution was dispensed (1:1) in 0.2 M buffer sodium acetate (pH 3.0-5.0), citrate-phosphate (pH 5.0-7.0), tris-HCl (pH 7.0-8.5) and glycine-NaOH (pH 8.5-11.0) and maintained at 25^{o}C for 24 hours. An aliquot was used to determine the residual activity at the optimum pH and temperature for enzymes. For the temperature stability determination, the enzyme solution was incubated at different temperatures (30^{o}C-70^{o}C) for 1 hour at pH 5.0. An aliquot was withdrawn and placed on ice before assaying for residual enzyme activity at optimum pH and temperature (Silva *et al*. 2002).

Effect of Metals and Inhibitors on Enzyme Activity: The influence of $CaCl_2$, $CuSO_4$, $CoCl_2$, $HgCl_2$, $ZnCl_2$, $MgCl_2$, EDTA, and $BaCl_2$ were tested on endoglucanase activities. The salts (5 mM) were each added to the reaction mixture and the enzymes activities determined (Banu *et al*. 2010).

Assay

Endoglucanase Activity: The procedure followed the 0.5 ml assay described by Jeffries (1987), adapted from Mandels (1974). Crude enzyme solutions were diluted in 0.05 M citrate buffer, pH 4.8. The enzyme diluted in buffer and 1% CMC (0.5 ml each) was mixed well and incubated for 30min at 50^{o}C. Then 3 ml of DNSA was added and the tubes were placed in boiling water bath for 5 min. The tubes were cooled and the reducing sugar (glucose) was determined (Miller 1959). This was determined by measuring the absorbance at 540 nm using Lambda 25 UV/Vis Spectrophotometer. In accord with the International Union of Biochemistry, one enzyme unit (U) equals to 1 µmol of glucose released per minute.

Results

Optimization of production conditions

As shown in Fig. 1, effect of pH on endoglucanase production was determined at pH values of 3.0, 3.5, 4.0, 4.5, 5.0 and 6.0 and endoglucanase activity obtained were 11.15, 11.17, 14.93, 15.86, 18.69, 14.93 and 14.01 U/ml, respectively. Optimum pH for endoglucanase production was observed at pH 5.0 with endoglucanase activity of 18.69 U/ml. Fig. 2 shows the effect of incubation time on enzyme production within 1-15 days. Highest production of endoglucanase was observed on the 7th day with endoglucanase activity of 18.64 U/ml. Optimization of incubation temperature was carried out by incubating the fermentation flasks at 30°C, 35°C, 40°C, 45°C and 50°C. Endoglucanase activities were 13.06, 18.83, 24.44, 19.79 and 18.70 U/ml, respectively. Thus as shown in Fig. 3, maximum production was observed at 40°C with endoglucanase activity of 24.44 U/ml.

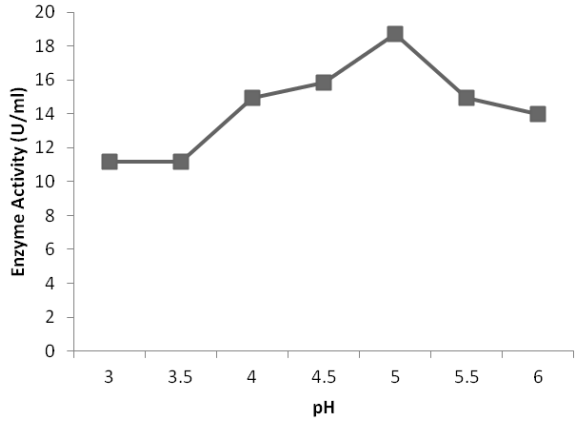

Fig. 1. Effect of pH on enzyme production.

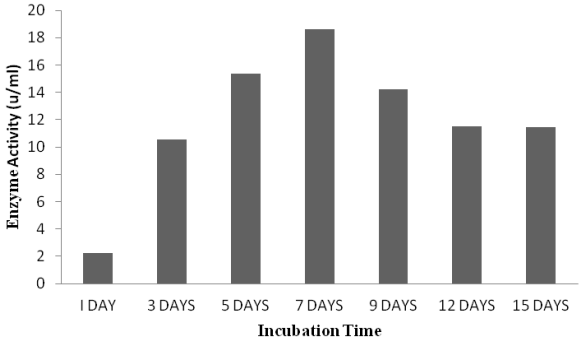

Fig. 2. Effect of incubation time on enzyme production.

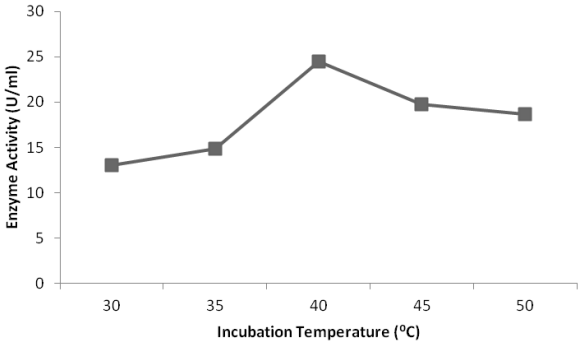

Fig. 3. Effect of incubation temperature on enzyme production.

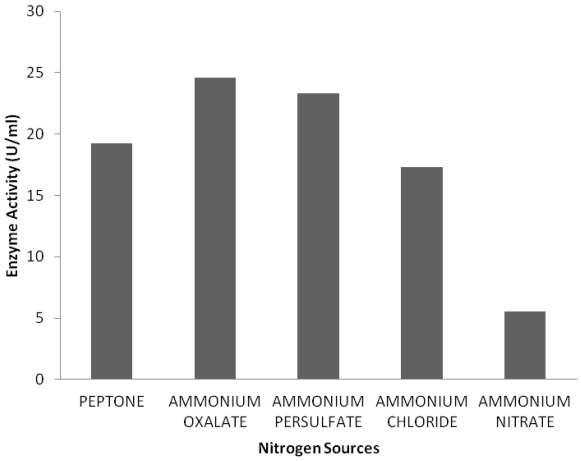

Fig. 4. Effect of nitrogen source on enzyme production.

The effects of certain nitrogen sources (peptone, ammonium oxalate, ammonium persulfate, ammonium chloride and ammonium nitrate) on endoglucanase production were investigated. Out of the five, ammonium oxalate, closely followed by ammonium persulfate, gave highest production of the enzyme (Fig. 4).

Different concentrations of ammonium oxalate and ammonium persulfate were later used for enzyme production. It was observed that 0.15% - 0.20% of ammonium oxalate gave the highest enzyme production while in the case of ammonium persulfate, 0.20% gave the highest production (Fig. 5).

Partial enzyme characterization

For the effect of temperature on activity of the enzyme, the enzyme showed an optimum temperature for maximum activity at 50°C. Thermal stability of the enzyme ranged

between 30-45°C (Fig. 6). Endoglucanase activity of *Penicillium atrovenetum* was found to be highest at pH 5 and showed pH stability in a range of 5- 7.5. At pH 4.5, residual activity was 68.07% while at pH 8, residual activity was 67.41% (Fig. 7). Among the metal ions tested, addition of 5 mM $CaCl_2$ enhanced the activities of endoglucanase by 15.54%. Other metals and EDTA inhibited the activities of the enzyme to varying degrees (Fig. 8).

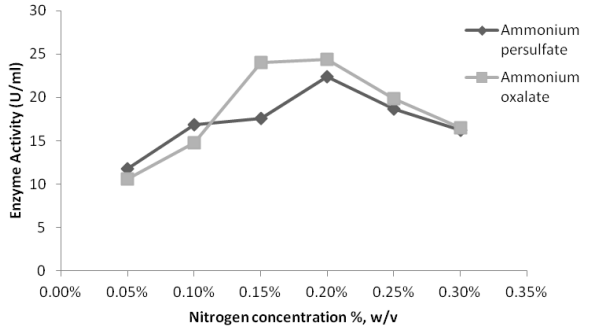

Fig. 5. Effect of different concentrations of ammonium persulfate and ammonium oxalate on enzyme production.

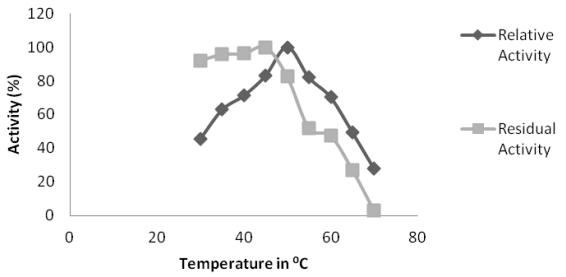

Fig. 6. Effect of temperature on enzyme activity.

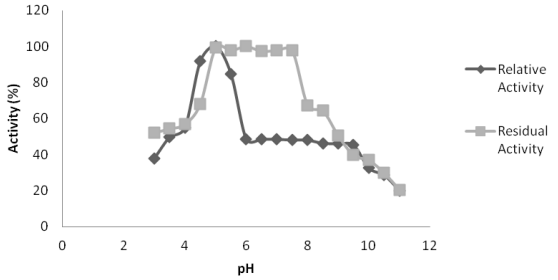

Fig. 7. Effect of pH on enzyme activity.

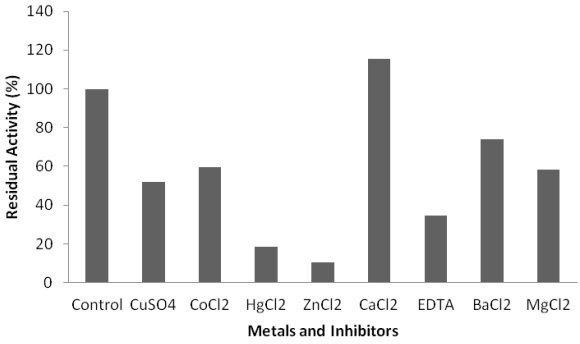

Fig. 8. Effect of metals and inhibitors on enzyme activity.

Discussion

In this work, carboxymethylcellulase (endoglucanase), a good index for cellulases, was produced by *Penicillium atrovenetum* using plantain peels as substrate. Earlier, high production of cellulase from plantain peel which is a domestic and industrial agro-waste was reported by Omojasola and Jilani (2009). Parameters varied in this current solid state fermentation of plantain peels include incubation time, pH of the basal medium, incubation temperature and nitrogen source. Highest production of endoglucanase was observed on the 7th day at pH 5 and at incubation temperature of 40°C.

Though distinct experimental methods used in similar investigations make comparison of research findings in this area of research cumbersome, certain trends could still be observed in related investigations. For instance, maximum enzyme production of 3.9 IU was achieved at temperature of 45°C by *Aspergillus niger* in paper cellulose with pH of 5 on 7th day of growth (Devi and Kumar 2012). Using orange peels as substrate, *Trichoderma longibrachiatum* produced highest amounts of glucose on day 7, pH 5 but at 45°C, optimum glucose using *Aspergillus niger* was produced at pH 4.5 on day 5 and 45°C while using *Saccharomyces cerevisiae*, optimum glucose production was produced at pH 4.5 on day 3 and at 45°C (Omojasola and Jilani 2008). The highest amount of glucose produced from *Trichoderma longibrachiatum* was produced from plantain peel at pH 5.0 and temperature of 45°C on day 7 of fermentation. The highest

amount of glucose produced by *Aspergillus niger* from plantain peel was at pH 4.5 and temperature of 45 °C on day 7 of fermentation. The highest amount of glucose produced by *Saccharomyces cerevisiae* was at pH 3.5 and temperature of 45 °C on day 5 of fermentation (Omojasola and Jilani 2009). Acharya *et al*. (2008) also reported optimum conditions of pH4, 28^{o}C, at 96th hour for cellulase production by *Aspergillus niger* using sawdust as substrate. Variations in observed optimum conditions in these various researches can be attributed to differences in nature of medium, fermenting organisms, concentration of nutrients and the process physiological conditions.

The source of nitrogen in the growth medium has a very important role in microbial growth and enzyme production (Mrudula and Anitharaj 2011). Ammonium oxalate, followed closely by ammonium persulfate, was observed as the best nitrogen source. 0.20% concentrations of ammonium oxalate and ammonium persulfate gave the highest yield of the enzyme. Ammonium sulphate was proved to be the best nitrogen source for endoglucanase production by *Rhizopus oryzae* (Karmakar and Ray 2010). Earlier also, ammonium persulfate was observed as the best nitrogen source for endoglucanase production from orange peels by *Penicillium atrovenetum*.

The endoglucanase have an optimum temperature of 50^{o}C and thermally stable within 30^{o}C to 50^{o}C. Optimal temperature of around 40^{o}C was obtained for the carboxymethylcellulase enzyme of wild type strain of *Aspergillus niger* (Coral *et al*. 2002). After incubating the enzyme for one hour at 50^{o}C, the enzyme retains more than 80% of its activity. This is desirable in industrial processes where moderate thermal treatment may be necessary. The enzyme showed an optimum pH of 5 and stable within a pH range of 5 to 7.5. The crude enzyme investigated in this work gave similar characteristics with the purified enzyme investigated by Kumar *et al*. (2012) which was found to be stable over range of 20-60^{o}C, at pH 4.0-7.5.

Addition of 5 mM $CaCl_2$ enhanced the activities of endoglucanase by 15.54%. Other metals and EDTA inhibited the activities of the enzyme to varying degrees. Fåhraeus (1947) observed that *Cytophaga* cannot grow on cotton wool unless calcium and manganese were added to the medium.

Conclusion

In conclusion, *Penicillium atrovenetum* studied in this work can be considered as fungus of choice in the production of cellulases; and plantain peels which are a readily available agrowaste can be used as cheap substrate for cellulase production.

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References

Acharya, P.B.; Acharya, D.K.; and Modi, H.A. 2008. Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate. African Journal of Biotechnology 7(22): 4147-52.

Adeleke, A.J.; Odunfa, S.A.; Olanbiwonninu, A; and Owoseni, M.C. 2012. Production of cellulase and pectinase from orange peels by fungi. Nature and Science 10(5): 107-12.

Adeyi, O. 2010. Proximate composition of some agricultural wastes in Nigeria and their potential use in activated carbon production. Journal of Applied Sciences and Environmental Management 14(1): 55-8.

Afifi, M.M. 2011. Effective technological pectinase and cellulase by *Saccharomyces cervisiae* utilizing food wastes for citric acid production. Life Science Journal 8(2): 405-13.

Bains, W. 2001. Biotechnology: from A to Z. 2nd ed. Oxford University Press, New York, NY, USA, pp. 384-5.

Banu, A.R.; Devi, M.K.; Gnanaprabhal, G.R.; Pradeep, B.V.; and Palaniswamy, M. 2010. Production and characterization of pectinase enzyme from *Penicillium chrysogenum*. Indian Journal of Science and Technology 3(4): 377-81.

Bhat, M.K.; and Bhat, S. 1997. Cellulose degrading enzymes and their potential industrial applications. Biotechnology Advances 15(3-4): 583-620.

Chaabouni, S.E.; Belguith, H.; Hassairi, I.; M'Rad, K.; and Ellouz. R. 1995. Optimization of cellulase production by *Penicillium occitanis*. Applied Microbiology and Biotechnology 43(2): 267-9.

Chellapandi, P.; and Jani, H.M 2008. Production of endoglucanase by the native strains of *Streptomyces* isolates in submerged fermentation. Brazilian Journal of Microbiology 39(1): 122-7.

Coral, G.; Arikan, B.; Ünaldi, M.N.; and Güvenmez, H. 2002. Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain. Turkish Journal of Biology 26(4): 209-13.

Devi, M.C.; and Kumar, M.S. 2012. Production, optimization and partial purification of cellulase by *Aspergillus niger* fermented with paper and timber sawmill industrial wastes. Journal of Microbiology and Biotechnology Research 2(1): 120-8.

Dhabekar, A.; and Chandak, A. 2010. Utilization of banana peels and beet waste for alcohol production. Asiatic Journal of Biotechnology Resources 1(1): 8-13.

Fadzilah, K.; and Mashitah, M.D. 2010. Cellulases production in palm oil mill effluent: Effect of aeration and agitation. Journal of Applied Sciences 10(24): 3,307-12.

Fåhraeus, G. 1947. Studies in the Cellulose Decomposition by *Cytophaga*. Symbolae botanicae Upsalienses, Vol. 9, No. 2, Almqvist & Wiksells, Uppsala, Sweden, 128 pp.

Jeffries, T.W. 1987. Production and Applications of Cellulase - Laboratory Procedures. United States Department of Agriculture (USDA), Forest Products Laboratory, Madison, WI, USA.

Jørgensen, H.; Eriksson, T.; Börjesson, J.; Tjerneld, F.; and Olsson, L. 2003. Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. Enzyme and Microbial Technology 32(7): 851-61.

Karmakar, M.; and Ray, R.R. 2010. Extra cellular endoglucanase production by *Rhizopus oryzae* in solid and liquid state fermentation of agro wastes. Asian Journal of Biotechnology 2(1): 27-36.

Kumar, D.; Ashfaque M.; Muthukumar M.; Singh, M.; and Garg, N. (2012). Production and characterization of carboxymethyl cellulase from *Paenibacillus polymyxa* using mango peel as substrate. Journal of Environmental Biology 33(1): 81-4.

Maciel, M. de H.C.; Herculano, P.N.; Porto, T.S.; Teixeira, M.F.S.; Moreira, K.A.; and de Souza-Motta, C.M. 2011. Production and partial characterization of pectinases from forage palm by *Aspergillus niger* URM4645. African Journal of Biotechnology 10(13): 2,469-75.

Mandels, M. 1974. Production and Application of Cellulase. Laboratory Procedures Hand Book. US Army Natick Laboratories, Natick, MA, USA.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31(3): 426-8.

Mo, H.; Zhang, X.; and Li, Z. 2004. Control of gas phase for enhanced cellulase production by *Penicillium decumbens* in solid-state culture. Process Biochemistry 39(10): 1,293-7.

Mrudula, S.; and Anitharaj, R. 2011. Pectinase production in solid state fermentation by *Aspergillus niger* using orange peel as substrate. Global Journal of Biotechnology and Biochemistry 6(2): 64-71.

Nwodo Chinedu, S.; Obinna Nwinyi, C.; and Okochi, V.I. 2008. Properties of Endoglucanase of *Penicillium chrysogemum* PCL501. Australian Journal of Basic and Applied Sciences 2(3): 738-46.

Odeniyi, O.A.; Onilude, A.A.; and Ayodele, M.A. 2009. Production characteristics and properties of cellulase/polygalacturonase by a *Bacillus coagulans* strain from a

fermenting palm-fruit industrial residue. African Journal of Microbiology Research 3(8): 407-17.

Omojasola, P.F.; and Jilani, O.P. 2008. Cellulase production by *Trichoderma longi*, *Aspergillus niger* and *Saccharomyces cerevisae* cultured on waste materials from orange. Pakistan Journal of Biological Sciences 11(20): 2,382-8.

Omojasola, P.F.; and Jilani, O.P. 2009. Cellulase production by *Trichoderma longi*, *Aspergillus niger* and *Saccharomyces cerevisae* cultured on plantain peel. Research Journal of Microbiology 4(2): 67-74.

Patil, S.R.; and Dayanand, A. 2006. Production of pectinase from deseeded sunflower head by *Aspergillus niger* in submerged and solid-state conditions. Bioresource Technology 97(16): 2,054-8.

Silva, D.; Martins, E. da S.; da Silva, R.; and Gomes, E. 2002. Pectinase production by *Penicillium viridicatum* RFC3 by solid state fermentation using agricultural wastes and agro-industrial by-products. Brazilian Journal of Microbiology 33(4): 318-24.

Sukumaran, R.K.; Singhania, R.R.; and Pandey, A. 2005. Microbial cellulases - Production, application and challenges. Journal of Scientific and Industrial Research 64(11): 832-44.

Widmer, W.; Zhou, W.; and Grohmann, K. 2010. Pretreatment effects on orange processing waste for making ethanol by simultaneous saccharification and fermentation. Bioresource Technology 101(14): 5,242-9.

Wilkins, M.R.; Widmer, W.W.; Grohmann, K.; and Cameron, R.G. 2007. Hydrolysis of grapefruit peel waste with cellulase and pectinase enzymes. Bioresource Technology 98(8): 1,596-601.