AES BIOFLUX
Advances in Environmental Sciences - International Journal of the Bioflux Society

β-Glucosidase production by *Aspergillus niger* van Tieghem using submerged fermentation of pineapple waste

Carol S. Maguinsay, Franco G. Teves

Molecular Biology and Biotechnology Research Laboratory, Department of Biological Sciences, College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology, Iligan City, Lanao del Norte, Philippines. Corresponding author: C. S. Maguinsay, carsoc@yahoo.com

Abstract. This study aimed to assess the potential of pineapple waste as substrate for β-Glucosidase production by *Aspergillus niger* under submerged fermentation. β-Glucosidase activities of crude enzyme extract from cultures maintained with pineapple substrate and with the commercial substrate carboxymethyl cellulose (CMC) were assayed and compared. The assay conducted at different periods of fermentation shows that enzymatic activities were generally higher on cultures maintained with CMC. However, β-Glucosidase activities on cultures supplemented with pineapple substrate had been found to be functional at a higher temperature (60°C). In conclusion, pineapple substrate has been demonstrated to be capable of inducing the synthesis of β-Glucosidase enzymes.
Key Words: β-Glucosidase, *Aspergillus niger*, submerged fermentation, pineapple waste.

Introduction. The pursuit of finding alternative energy source due to the subsiding reserves of fossil fuels had already engaged bioconversion of cellulose – a renewable carbon source abundant in the environment that compose plant materials in majority (Duff et al 1985; Heck et al 2002; Ojumu et al 2003). However, the conversion of cellulose into products having commercial value (e.g. bioethanol) calls for efficient and economical methods to lower the expenditure of the entire operation (Mandels et al 1971; Macris 1984). Over decades, it appears that the improvement of cellulase production technology is the most promising means to enhance biofuel production and several industrial processing.

Cellulase it is a multienzyme complex capable of decomposing plant substrates. One component of the cellulase enzyme system is β-Glucosidase (EC 3.2.1.21) and it works together with endoglucanase and exoglucanase to break down cellulose in the plant cell walls (Yun et al 2001; Lynd et al 2002). The synthesis of β-Glucosidase and other components of cellulase enzymes may be carried out in cultures of cellulolytic microorganisms. In fungal cultures, such enzymes can be produced extracellularly by the fungus itself to aid the digestion of insoluble substrates such as cellulose (Hurst et al 1977; Chaudhary & Tauro 1982; Pandey et al 1999).

β-Glucosidase basically plays a significant role in several biological processes occuring in nature such as beta-glucan synthesis during development of cell wall, pigmentation, fruit ripening and defense mechanism. It is also the key enzyme component present in cellulase which is important in various biotechnological processing of food products, textile refining, production of pulp and paper, improvement of oil recovery, waste treatment, and especially in biofuel production (Singhania et al 2012; Rajoka 2004).

The current setting of β-Glucosidase production is focused on utilizing plant biomass, agro-industrial and municipal wastes. Such materials are used as substrates in attempting to reduce the expensive enzyme production cost, which has been a major economic limitation not only of biofuel production but also of other industrial processes

(Bakare et al 2005; Immanuel et al 2007). In the local point of view, utilizing plant wastes would also help alleviate the problems on disposal of solid waste. It is for this reason that production of β-Glucosidase enzymes using such waste materials would be beneficial to both local community and industry. The present study then, investigates the possibility of using pineapple waste as substrate for β-Glucosidase production by *Aspergillus niger* under submerged fermentation.

Material and Method

Microorganism and inoculum preparation. *A. niger* van Tieghem acquired from the Philippine National Collection of Microorganisms (Accession No. BIOTECH 3080) was used throughout the study. It was grown on Potato-Dextrose Agar (PDA) slant at room temperature for 7 days and then it was maintained at 4°C as a stock culture. Subcultures were also performed prior to the fermentation experiment. Spore suspension was prepared by adding 5 mL of 1% Tween 20 on a week-old slant and made up to 50 mL of the same solution. Inoculum size was adjusted to 5 x 10^6 spores mL^{-1} by microscopic enumeration with a cell-counting hemacytometer.

Substrate preparation. The pineapple (*Ananas comosus*) fruit samples used in this study were collected at a local market in Pala-o, Iligan City, Lanao del Norte, Philippines. Fleshy edible parts were separated from the rind (Figure 1a), and the latter were then cut into strips and were grounded wet using a corn mill (Figure 1b). Grounded wet samples (Figure 1c) were then washed successively with water and sun-dried for about a week (Figure 1d) or until completely dried (Figure 1e). Finally, it was milled into fine powder (Figure 1f) and then sieved through no. 80 mesh (Tyler Equivalent).

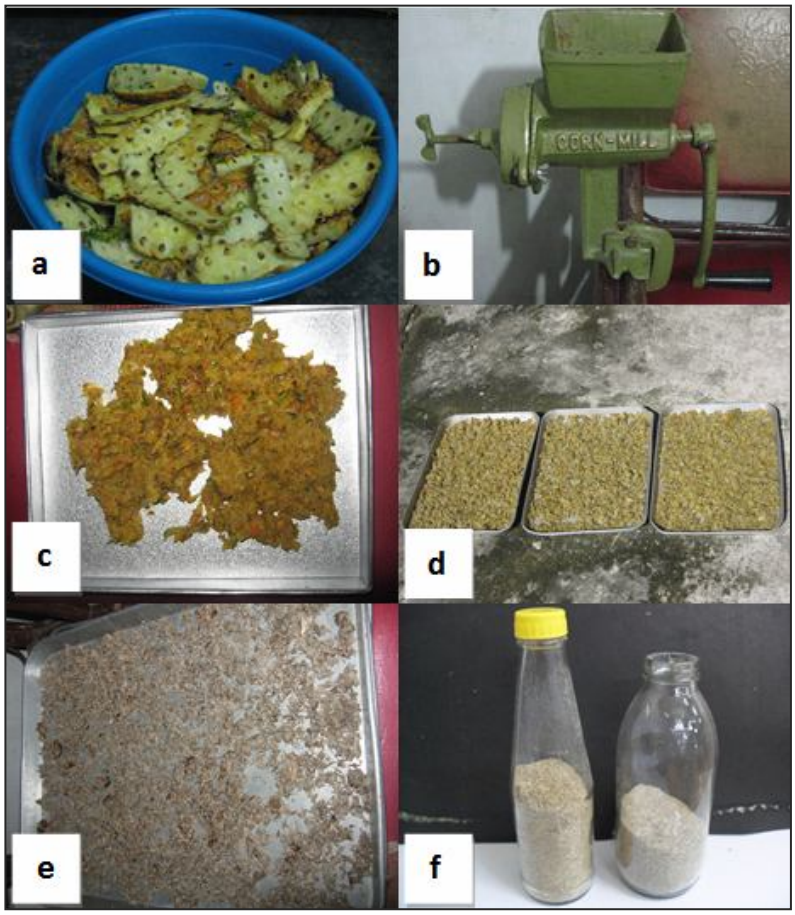

Figure 1. Preparation of pineapple rind as substrate for β-Glucosidase production.

Culture media composition and the experimental setup. The liquid media basically contained (per liter of distilled water): 1.5 g KH_2PO4, 0.5 g KCl, 0.5 g $MgSO_4$, 1.0 mL of trace metal solution (de Groot et al 2003), and 1.25 g $(NH_4)_2SO_4$. The total volume of the liquid media used for each fermentation setup was 100 mL. It was adjusted to pH 5.0 and then added with 10 g of the selected substrate. The culture media were then sterilize at 120°C for 15 minutes and were inoculated by adding 2 mL of spore suspension. The submerged fermentation was carried out in 250 mL Erlenmeyer's flask at a room temperature (29.5°C–30.5°C) under constant shaking conditions.

β-Glucosidase assay. The crude enzyme extract for the assay of β-Glucosidase activities was prepared by centrifugation of culture fluid for 15 minutes at 1328 x g (3500 rpm). The assay was carried out at 37°C during the monitoring of enzymatic activity at different periods of fermentation; and on the study of thermal stability, it was conducted at temperatures (29°C, 40°C, 50°C, and 60°C). The reaction mixture consists the following: 1.4 mL of 0.1M sodium acetate buffer (pH 5.0), 0.1 mL of enzyme extract (undiluted), and 0.5 mL of 0.02M 4-nitrophenyl-β-D-glucopyranoside (PNPG). The reaction tubes were incubated for 15 minutes and then the reaction was stopped by the addition of 2.0 mL 0.2M sodium carbonate (Na_2CO_3). The controls for the assay was also prepared in the same conditions except without the enzyme source during the incubation, and wherein the stopper (Na_2CO_3) and enzyme extract were added only after the incubation period. Using the spectrophotometer, the optical density was read at 400nm. Using the values of the change in absorbance (Reaction $Mixture_{400nm}$ – Assay $Control_{400nm}$), the amount of 4-nitrophenol released by enzymatic activity was calculated from a standard curve (Figure 2) prepared previously. One unit of enzyme activity (U) is defined as the amount of enzyme required to release one micromole (µmol) of 4-nitrophenol per minute under the described assay conditions. Enzymatic activity is then expressed as units per milliliter (U mL^{-1}) of the crude enzyme extract.

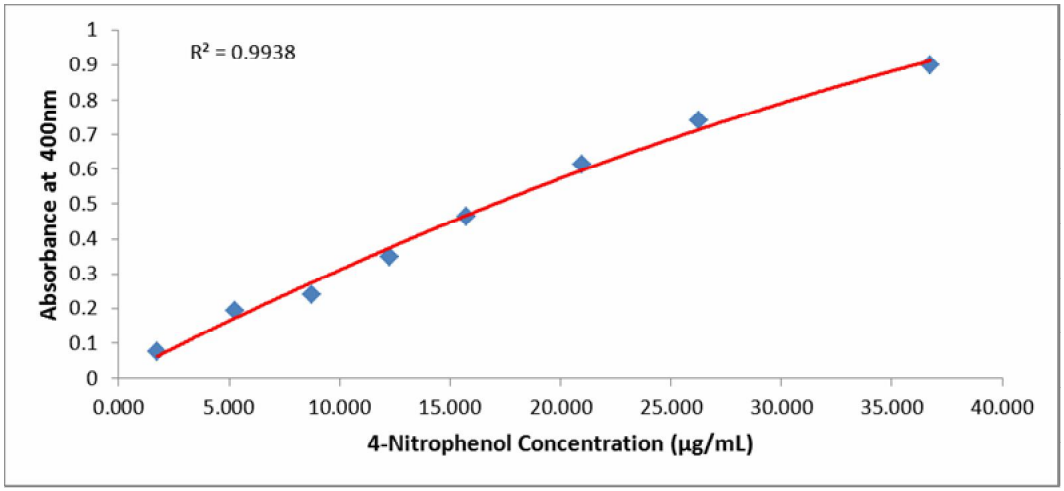

Figure 2. Standard curve: Optical density of different concentrations of 4-Nitrophenol.

Results and Discussion

Crude enzyme assay at different time periods. A parallel experiment was conducted growing *A. niger* on media supplemented with pineapple waste (PW) and carboxymethyl cellulose (CMC). β-Glucosidase activities of crude extracts from culture fluid were then monitored as shown in Figure 3. Enzymatic activities were generally observed to be optimal between the 60^{th} and 70^{th} hour of fermentation and declined between the 84^{th} and 96^{th} hour. Under the assay conditions, the decrease of enzymatic activities could be due to the accumulation of catabolic end-products (glucose) in the culture filtrate that are produced from the enzymatic hydrolysis of the selected substrate. As the catalytic activity progresses over time, the amount of glucose also increases and enzymes are

consequently inhibited. Several studies have also reported that glucose competitively inhibits β-Glucosidase (Yeoh et al 1988; Chen et al 1992; Riou et al 1998; Xiao et al 2004; Elshafei et al 2011) and the study of Andrić et al (2010) have shown that *in situ* removal of glucose during the enzymatic hydrolysis has increased the catalytic activity.

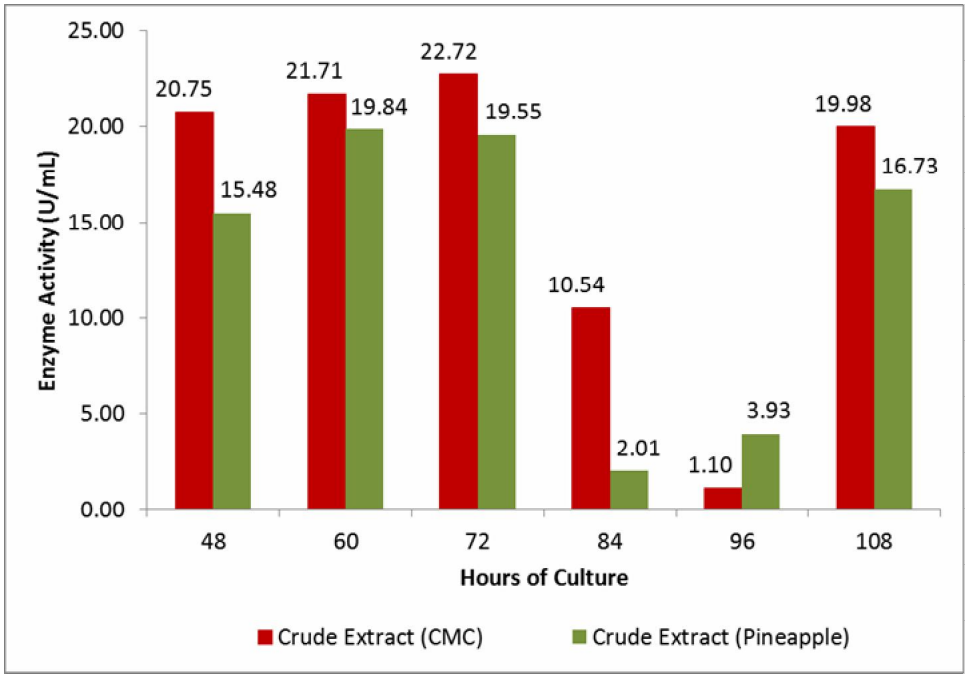

Figure 3. β-Glucosidase assay of crude enzyme extract from *A. niger* cultures supplemented with CMC and pineapple waste at different periods of fermentation.

In the present study, it was observed that after conidiophore development and sporulation was visible at 102 hours of fermentation (Figure 4b), β-Glucosidase activities were then elevated as shown in the assay at 108 hours. The elevation simply implies that glucose was consumed to support energy production and vegetative growth of the fungi.

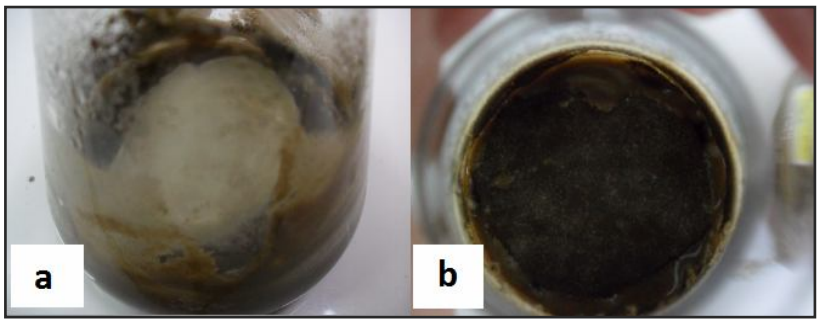

Figure 4. Growth of *A. niger* on media supplemented with pineapple waste: (a) 72 hours of culture: mycelial mat is visible, (b) 102 hours: conidiophore development and sporulation.

Furthermore, β-Glucosidase activities were observed to be higher on cultures supplemented with CMC substrate compared to β-Glucosidase activities from cultures maintained on pineapple substrate. Studies of Narasimha et al (2006) and Gautam et al (2010) also projected the same results in which *A. niger* grown on CMC had higher β-Glucosidase activities compared to those grown on other substrate. The soluble structure of CMC clearly accounts for its efficiency to induce sysnthesis of β-glucosidase which has high specificity to PNPG. The result on this particular assay indicates that hemicellulosic

content of pineapple substrate and the crystalline structure of its cellulose (Smith et al 1998; Smith & Harris 1995; Bartolomé & Rupérez 1995) have caused the enzymatic hydrolysis to slow down at some point. In this case, the production of β-Glucosidase by *A. niger* depends on the preceding steps of cellulose degradation involving the enzymes endo and exoglucanase. Moreover, the hydrolytic activities of these two enzymes are greatly affected by the type of substrate incorporated in the culture media.

Crude enzyme assay at different working temperatures. Crude extracts from cultures maintained on PW and CMC were also assayed at varying temperatures. The optimal temperature for β-Glucosidase from cultures maintained on CMC substrate is 50°C as shown in Figure 5. On the other hand, β-Glucosidase of *A. niger* grown on PW substrate was demonstrated to have a thermal stability at 60°C for 15 minutes under the conditions of the assay. In the study of Yan & Lin (1997), β-Glucosidase of *A. niger* was reported to have an optimum activity at 55°C and pH 5.0. In another study of Rashid & Siddiqui (1997), β-glucosidase from *A. niger* were shown to have optimal temperature of 70°C. Rashid & Siddiqui (1996) also reported in their previous study that the stability of this enzyme is enhanced by non-covalently attached polysaccharides secreted by the fungi *A. niger* itself.

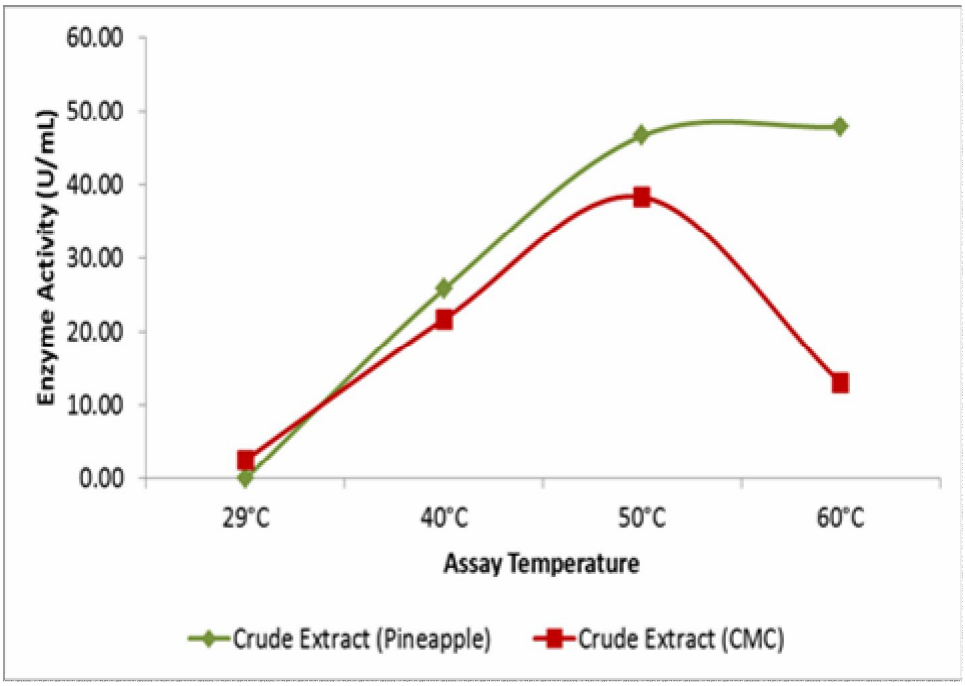

Figure 5. β-Glucosidase assay of crude extract from *A. niger* cultures supplemented with pineapple waste and CMC at varying temperatures.

Conclusions. The utilization of pineapple waste as substrate for *A. niger* cultures has been demonstrated to be capable of inducing the synthesis of β-glucosidase enzymes that were sensitive to glucose inhibition but operative at temperature (60°C). Furthermore, the inexpensive overall cost of the substrate preparation serves as the main advantage in preferring the pineapple waste for β-Glucosidase production.

Acknowledgements. C. S. Maguinsay and F. G. Teves wish to thank the Department of Science and Technology (DOST, Phil.) for the financial grant used to complete this research.

References

Andrić P., Meyer A. S., Jensen P. A., Dam-Johansen K., 2010 Effect and modeling of glucose inhibition and in situ glucose removal during enzymatic hydrolysis of pretreated wheat straw. Appl Biochem Biotechnol 160(1):280-297.

Bakare M. K., Adewale I. O., Ajayi A., Shonukan O. O., 2005 Purification and characterization of cellulase from wild-type and two improved mutants of *Pseudomonas fluorescens.* Afr J Biotechnol 4(9):898-904.

Bartolomé A. P., Rupérez P., 1995 Polysaccharides from the cell walls of pineapple fruit. J Agric Food Chem 43(3):608-612.

Chaudhary K., Tauro P., 1982 Sequential release of cellulase enzymes during germination of *Trichoderma reesei* spores. J Biosci 4(3):281-286

Chen H., Hayn M., Esterbauer H., 1992 Purification and characterization of two extracellular beta-glucosidase from *Trichoderma reesei.* Biochim Biophys Acta 1121(1-2):54-60.

de Groot M. J. L., van de Vondervoort P. J. I., de Vries R., van Kuyk P. A., Rujiter G. J. G., Visser J., 2003 Isolation and characterization of two specific regulatory *Aspergillus niger* mutants shows antagonistic regulation of arabinan and xylan metabolism. Microbiology 149(5):1183-1191.

Duff S. J., Cooper D. G., Fuller O. M., 1985 Effect of colloidal materials on cellulase production by *Trichoderma reesei* Rut-C30. Appl Environ Microbiol 49(4):934-938.

Elshafei A. M., Hassan M. M., Morsi N. M., Elghonamy D. H., 2011 Purification and some kinetic properties of β-glucosidase from *Aspergillus terreus* NRRL 265. Afr J Biotechnol 10(84):19556-19569.

Gautam S. P., Bundela P. S., Pandey A. K., Awasthi M. K., Sarsaiya S., 2010 Effect of different carbon sources on production of cellulase by *Aspergillus niger.* Journal of Applied Sciences in Environmental Sanitation 5(3):295-300.

Heck J. X., Hertz P. F., Ayub M. A. Z., 2002 Cellulase and xylanase production by isolated Amazon *Bacillus* strains using soybean industrial residue based solid-state cultivation. Braz J Microbiol 33(3):213-218.

Hurst P. L., Nielsen J., Sullivan P. A., Sheperd M. G., 1977 Purification and properties of a cellulase from *Aspergillus niger.* Biochem J 165(1):33-41.

Immanuel G., Akila C. M., Bhagavath P., Iyappa R., Esakkiraj P., Palavesam A., 2007 Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigatus* fermented in coir waste and sawdust. The Internet Journal of Microbiology 3(1).

Lynd L. R., Weimer P. J., van Zyl W. H., Pretorius I. S., 2002 Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66(3):506-577.

Macris B. J., 1984 Production and characterization of cellulase and β-Glucosidase from a mutant of *Alternaria alternata.* Appl Environ Microbiol 47(3):560 565.

Mandels M., Weber J., Parizek R., 1971 Enhanced cellulase production by a mutant of *Trichoderma viride.* Appl Microbiol 21(1):152-154.

Narasimha G., Sridevi A., Budolla V., Subhosh C. M., Rajasekhar R. B., 2006 Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger.* Afr J Biotechnol 5(5):472-476

Ojumu T. V., Solomon B. O., Betiku E., Layokun S. K., Amigun B., 2003 Cellulase production by *Aspergillus flavus* Linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob. Afr J Biotechnol 2(6):150-152.

Pandey A., Selvakumar P., Soccol C. R., Sing-Nee Nigam P., 1999 Solid state fermentation for the production of industrial enzymes. Curr Sci 77(1):149-162.

Rajoka M. I., 2004 Influence of various fermentation variables on exo-glucanase production in *Cellulomonas flavigena.* Electron J Biotechnol 7(3).

Rashid M. H., Siddiqui K. S., 1996 The stability of extracellular beta-glucosidase from *Aspergillus niger* is significantly enhanced by non-covalently attached polysaccharides. Folia Microbiol (Praha) 42(6):544-50.

Rashid M. H., Siddiqui K. S., 1997 Purification and characterization of a beta-glucosidase from *Aspergillus niger.* Folia Microbiol (Praha) 42(6):544-550.
Riou C., Salmon J. M., Vallier M. J., Günata Z., Barre P., 1998 Purification, characterization, and substrate specificity of a novel highly glucose-tolerant beta-glucosidase from *Aspergillus oryzae.* Appl Environ Microbiol 64(10):3607-3614.
Singhania R. R., Patel A. K., Sukumaran R. K., Larroche C., Pandey A., 2012 Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol 127:500-507.
Smith B. G., Harris P. J., 1995 Polysaccharide composition of unlignified cell walls of pineapple [Ananas comosus (L.) Merr.] fruit. Plant Physiol 107(4):1399-1409.
Smith B. G., Harris P. J., Melton L. D., Newman R. H., 1998 Crystalline cellulose in hydrated primary cell wall of three monocotyledons and one dicotyledon. Plant Cell Physiol 39(7):711-720.
Xiao Z., Zhang X., Gregg D. J., Saddler J. N., 2004 Effects of sugar inhibition on cellulases and β-Glucosidase during enzymatic hydrolysis of softwood substrates. Appl Biochem Biotechnol 115(1-3):1115-1126.
Yan T. R., Lin C. L., 1997 Purification and characterization of a glucose-tolerant beta-glucosidase from *Aspergillus niger* CCRC 31494. Biosci Biotechnol Biochem 61(6):965-970
Yeoh H. H., Tan T. K., Chua S. L., Lim G., 1988 Properties of β-Glucosidase purified from *Aspergillus niger.* MIRCEN Journal 4(4):425-430.
Yun S. I., Jeong C. S., Chung D. K., Choi H. S., 2001 Purification and some properties of a β-Glucosidase from Trichoderma harzainum type C-4. Biosci Biotechnol Biochem 65(9):2028-2032.

Received: 04 January 2013. Accepted: 06 February 2013. Published online: 12 February 2013.
Authors:
Carol Socorro Maguinsay, Mindanao State University-Iligan Institute of Techonology, College of Science and Mathematics, Department of Biological Sciences, Molecular Biology and Biotechnology Research Laboratory, Philippines, Iligan City, Lanao del Norte, 9200, e-mail: carsoc@yahoo.com
Franco G. Teves, Mindanao State University-Iligan Institute of Techonology, College of Science and Mathematics, Department of Biological Sciences, Molecular Biology and Biotechnology Research Laboratory, Philippines, Iligan City, Lanao del Norte, 9200, e-mail: franco_teves@yahoo.com

How to cite this article:
Maguinsay C. S., Teves F. G., 2013 β-Glucosidase production by *Aspergillus niger* van Tieghem using submerged fermentation of pineapple waste. AES Bioflux 5(1):23-29.