Volume 13 Number 2 DECEMBER 2011

IFE JOURNAL OF SCIENCE

A Journal of the Faculty of Science
Obafemi Awolowo University, Ile-Ife, Nigeria

ISSN 0794-4896

Ife Journal of Science

Aims and Scope

Ife Journal of Science (IJS) aims to publish articles resulting from original research in the broad areas of chemical, biological, mathematical and physical sciences. This extends naturally into frontiers that include the applied areas of Biochemistry and Geology as well as Microbiology and such allied fields as Biotechnology, Genetics, Food Chemistry, Agriculture, Medical and Pharmaceutical Sciences. Shorter-length manuscripts may be accepted as *Research notes.* Review articles on research topics and books are also welcome.

***Two issues of* the journal will be published yearly (June and December).**

Submission of manuscripts:
All manuscripts should be submitted to either of the Editors-in-chief, Ife Journal of Science: Prof. J. O. Faluyi, Department of Botany, or Prof. M. O. Olorunfemi, Department of Geology, ObafemiAwolowo University, Ile-Ife, Nigeria.
E-mail: jfaluyi@gmail.com (Tel.: +234-803-7250857)
mlorunfe@yahoo.co.uk (Tel.: +234-803-7192169)

SCREENING AND ISOLATION OF THERMOPHILIC CELLULOLYTIC BACTERIA FROM COCOA POD AND CASSAVA PEEL DUMPSITES IN ILE-IFE, SOUTHWEST NIGERIA

Adeleke, E.O.[1], Omafuvbe, B.O.[1], Adewale, O.I.[2] and Bakare, M.K.*[1]

1. Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria.
2. Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.

**Corresponding Author*
email address: mufubakare@yahoo.com
(Received: June,2011; Accepted: September, 2011)

ABSTRACT

Thermophilic cellulase producing bacteria were isolated from the heap of decaying cassava peels and cocoa pods in Ile-Ife, Southwest, Nigeria. Out of the fifteen thermophilic bacterial isolates - thirteen of which were from cassava peels and two from cocoa pods - only three (2NA3, Ca3 and Co4) hydrolyzed carboxymethyl cellulose with the hydrolytic capacity value between 2.3 and 3.5 at 60°C. The three cellulolytic isolates were identified as *Bacillus brevis* 2NA3, *Bacillus coagulans* Ca3 from cassava peels and *Bacillus coagulans* Co4 from cocoa pods. *B. brevis* and *B. coagulans* Co4 were selected for further study. Cellulase production occurred within the exponential growth phase of the bacteria which was observed to be at 20 hours for both *B. brevis* and *B. coagulans* Co4. However, *Bacillus coagulans* Co4 showed a higher cellulase activity of 30 units/ml. The thermostable cellulase from *B. coagulans* Co4 isolated from cocoa pod could be of great importance in biofuel industry for the saccharification of lignocellulosics into economically useful monosaccharides.

Keywords: Isolation, Cellulolytic Bacteria, Cocoa Pod, Cassava Peel, Growth Curve

INTRODUCTION

Cellulose is generally defined as a polymer of -D-glucose units linked by -1,4-glucosidic bonds (Li and Gao, 1997). Cellulose is the major component of plant biomass and the major biopolymer found in abundance on earth (Aubert *et al*., 1987). The annual production of cellulose is estimated to be 4.0×10^{7}-10^{10} tonnes (Narasimha *et al*., 2006). Most of the cellulose exists as waste such as straw, corn cobs, wood wastes, peat, bagasse and waste paper (Ojumu *et al*., 2003). Agricultural wastes and in fact all lignocelluloses can be converted into bio-source for fuel, animal feedstock and feedstock for chemical synthesis (Bhat, 2000). Agricultural wastes are presently used to generate electricity, majority of which is used by the industry making the waste thereby reducing the cost of waste disposal (TNP, 2009). The world economy is driven by energy which is derived from oil, natural gas and other fossil fuels, hydro, wind, wave, solar power and biomass (Yizraeli, 2000). In 1998, it was predicted that fossil oil supplies were close to their peak and would soon decline. This decline will seriously affect world socio-economic development due to the high price of oil and the dependence on this commodity for all aspects of economic development. Many economists have emphasized the need to prepare replacements of fossil fuels as sources of energy (Preston and Leng, 2004). In line with this, there has been renewed interest in ethanol as an alternative fuel due to depletion in petroleum and the environmental implication of fossil fuels (Hamelinck *et al*., 2005).

The potential importance of the hydrolysis of lignocellulosic is widely recognized in the context of conversion of the residual plant biomass into various value-added products such as biofuels, chemicals, cheap carbon and energy sources for fermentation, improved animal feeds and human nutrients (Chinedu *et al*., 2008). The use of different biomass conversion methods have been introduced, ranging from acid hydrolysis and pyrolysis (gasification) to biological methods by enzyme hydrolysis. Of these methods, enzymatic hydrolysis of lignocellulose offers an attractive procedure and relatively pure products which can serve as raw material for the production of bio-ethanol, glucose and single cell protein with less input of energy during the process (Solomon *et al*., 1999; Dashtban *et al*., 2009). Bioconversion of agricultural wastes occurs at relatively high temperature which may affect enzyme activity. A number of approaches have been made by enzyme producers and industrial users to reduce the cost of enzyme

production and improve existing enzymes (Sherief *et al*., 2010). In view of this, there has been thrust for thermophiles due to the high temperature under which most industrial processes operate. In the southwestern Nigeria, heaps of cocoa pods and cassava peels abound which has not been explored for the possibility of isolating thermophilic bacteria which can produce thermostable cellulolytic enzymes for the conversion of agricultural wastes to veritable raw materials. This study was therefore aimed at isolating and characterizing thermostable cellulase producing bacteria from the dump of cassava peels and cocoa pods in Ile-Ife area, for possible selection of a good candidate for commercial production of thermostable cellulases.

MATERIALS AND METHODS

Sample Collection

Samples were collected from cassava peel heaps and cocoa pods dumpsite at Obafemi Awolowo University, Ile-Ife commercial farm and Ondo Road, Ile-Ife respectively. The temperatures of the cassava peel and cocoa pod dumps were measured with thermometer and samples were collected from sites measuring up to 55°C to increase the chances of isolating thermophiles. The samples were collected into sterile MacCartney bottles and transported to the laboratory within 30 min for analysis.

Isolation Procedure

Isolation was carried out by weighing 5 g of the decaying cassava peel or cocoa pod sample aseptically into 45 ml of pre-sterilized maximum recovery diluent (M.R.D., Oxoid) in a conical flask. The suspension was mechanically shaken at 150 rpm for 10 min at room temperature in an orbital shaker. The 10% suspension was serially diluted further in MRD up to 10^{-4} (Ibrahim and El-diwany, 2007). Aliquot (100 µl) of each appropriately diluted samples were spread on correspondingly labeled sterile nutrient agar (NA) plates in duplicates. The plates were inverted and sealed in plastic bags to reduce evaporation from the plates (de Souza and Martins, 2001) and incubated at 60°C for 48 h (Apun *et al*., 2000). After incubation, plates with 30-300 colony forming units (c.f.u.) were examined and representative colonies were isolated from each plate for further identification. The representative colonies were purified by repeated streaking on NA and screened for cellulolytic activities. Isolates which showed positive cellulolytic activities were preserved on NA slopes in the refrigerator.

Cellulolytic Test Using Plate Method

Single streak of the pure isolates were made on the carboxymethyl cellulose agar (CMCA) plates consisting of yeast extract (0.2% w/v), KH_2PO_4 (0.1% w/v), $MgSO_4.7H_2O$ (0.5% w/v), NaCl (0.075% w/v), peptone (2% w/v), agar (1.5% w/v) and high viscosity caboxymethyl cellulose (0.5% w/v). The CMCA plates were incubated at 60°C for 48 h, stained with Congo red (0.1% w/v) and destained with 1 M NaCl solution. Positive test showed a clear halo zone surrounding the colony which was measured in millimeters using calliper (Wood and Bhat, 1988).

Endo- 1, 4- -glucanases (C_x) producing activities of the isolates were estimated by the caboxymethyl cellulose hydrolysis capacity (HC value: ratio of diameter of clear zone and line of streak) on the cellulose congo-red agar (Lu *et al*., 2005). Isolates with high HC values were selected and stored for further studies.

Identification of Isolates

All the pure isolates with substantial cellulolytic activities were characterized, and tentatively identified using phenotypic methods (Harrigan and McCance, 1976).

Growth and Enzyme Production

The time course of the enzyme production was determined and compared with growth. This was done using growth methods described by de Souza and Martins (2001) with appropriate modifications. Aqueous suspension (5 ml) of 24 h old pure culture of test isolates was made in sterile distilled water and compared with 0.5 McFarland standards with initial absorbance at 680 nm of at least 0.1. The aqueous suspension was used to inoculate 45 ml of sterile basal medium contained in a 250 ml tightly closed Erlenmeyer flask and incubated for 24 h. This was in turn used to inoculate 450 ml of sterile basal medium contained in a 1000 ml tightly closed Erlenmeyer flask to give the same initial absorbance of 0.1. The culture was incubated at 55°C with moderate aeration in a water bath with shaker at 100 rpm for 72 h during which samples (5 ml) were aseptically collected at four hourly intervals for turbidity measurement to monitor the bacterial growth. The turbidity of the culture was determined by measuring the increase in optical density (OD) at 680 nm with a colorimeter WPA colourwave model CO 7500. After the OD

measurement, the sample was then centrifuged at 6,000 rpm for 15 min and the clear supernatants were collected as the crude enzyme which was used for cellulase assay.

Cellulase Assay

Cellulase activity towards carboxymethylcellulose (CMC) was measured by the appearance of reducing end groups released by the action of the enzyme on the substrate using modified method of Nelson (1944) and Somogyi (1952). The assay method involved the hydrolysis of 0.2 ml of 0.2% (w/v) CMC (in 100 mM of sodium citrate buffer, pH 6.5), 0.6 ml Sodium citrate buffer and 0.2 ml of crude enzyme solution. The reaction mixture was incubated at 55°C for 1 h. Control experiment was similarly treated except the enzyme solution was inactivated by boiling at 100°C for 20 min. The reaction was then terminated by the addition of 1.0 ml combined copper reagent according to Nelson-Somogyi method. Reducing equivalents was also measured following the method of Nelson (1944) and Somogyi (1952). The amount of reducing sugars produced was interpolated from a glucose standard curve.

One unit of cellulase activity was expressed as the amount of enzyme which produced equivalent of 1 μg of glucose per minute under the specified assay conditions. The specific enzyme activity was expressed as the unit of enzyme activity per mg of protein.

Protein Concentration

Protein concentration of the crude supernatant was determined using Lowry's method (Lowry *et al.*, 1951) with bovine serum albumin (BSA) as standard protein.

RESULTS

The result of the isolation technique at thermophilic temperature of 60°C showed fifteen morphologically different bacterial isolates. Out of the fifteen bacterial isolates, thirteen isolates were recovered from cassava peels while two isolates were from cocoa pods. Of the fifteen isolates, only three were cellulolytic. The biochemical and morphological tests indicated that all the three cellulolytic bacteria were Gram positive, catalase positive, motile, non-sulphide producers, indole negative, producers of ammonia from peptone, facultative aerobes, glucose fermenters, spore producers and hydrolyzed starch and gelatin. They all grew in 6.5% NaCl but none grew at 65°C (Table 1). Based on the biochemical and physiological results, the three cellulolytic isolates were tentatively identified as *Bacillus brevis* 2NA3, *Bacillus coagulans* Ca3 and *Bacillus coagulans* Co4. *Bacillus coagulans* Co4 gave the highest hydrolytic capacity value of 3.5, while *Bacillus brevis* 2NA3 and *Bacillus coagulans* Ca3 had values of 3.0 and 2.3 respectively.

Bacillus brevis 2NA3 and *Bacillus coagulans* Co4 were selected for growth and cellulase production studies. Figure 1 showed that *Bacillus coagulans* Co4 had a lag phase of about 4 h followed by an exponential phase of about 28 h following which a stationary phase was thereafter observed. During the exponential phase, the highest cellulase activity was obtained at 20 h and this was followed by a decrease in cellulase activity. *Bacillus brevis* had a similar growth pattern with *Bacillus coagulans* Co4 except that the lag phase was less than 4 h and an exponential phase of about 20 h (Figure 2).

Table 1: BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THERMOPHILIC AND CELLULOYTIC BACTERIA ISOLATED FROM HEAPS OF CASSAVA PEEL AND COCOA POD

	Isolate code	2NA3	Ca3	Co4
	Gram reaction	+	+	+
	Catalase reaction	+	+	+
	Motility	+	+	+
	Sulphide Production	-	-	-
	Indole production	-	-	-
	Methyl Red test	-	-	-
	Voges-Proskaeur test	-	+	-
	Oxidative-Fermentative test	F	F	F
	Nitrate reduction	-	+	-
	Ammonia production	+	+	+
	Citrate utilization	-	-	+
	Spore test (Position)	terminal	central	central
	Starch hydrolysis	+	+	+
	Gelatin hydrolysis	+	+	+
	Caesin hydrolysis	+	-	+
	Lecithinase production	-	-	-
	Growth in 6.5% NaCl	+	+	+
	Growth in 50 ^{o}C	+	+	+
	Growth in 60 ^{o}C	+	+	+
	Growth in 65 ^{o}C	-	-	-
	Lipase test	-	+	+
	Protease test	+	+	+
	Anaerobic growth test	+	+	+
Sugar fermentation	Glucose	+	+	+
	Maltose	-	+	+
	Lactose	-	-	-
	Mannitol	-	+	+
	Raffinose	-	-	-
	Arabinose	-	+	-
	Sucrose	+	-	+
Probable Identity of Isolates		*Bacillus brevis*	*Bacillus coagulans*	*Bacillus coagulans* Co4

Key: + = Positive result; - = Negative result; F = Fermentative

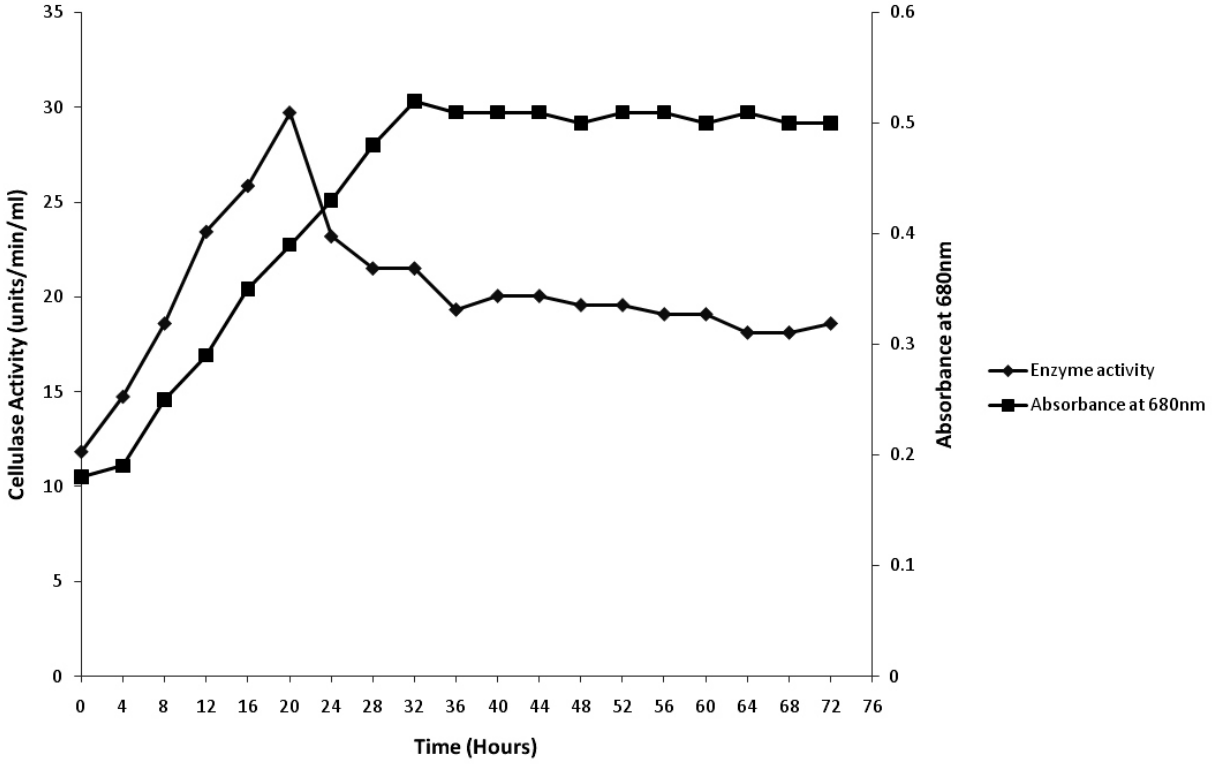

Figure 1: Growth and Cellulase Production of Cellulolytic Bacteria (*Bacillus coagulans* Co4) Isolated from a Heap of Cocoa Pods

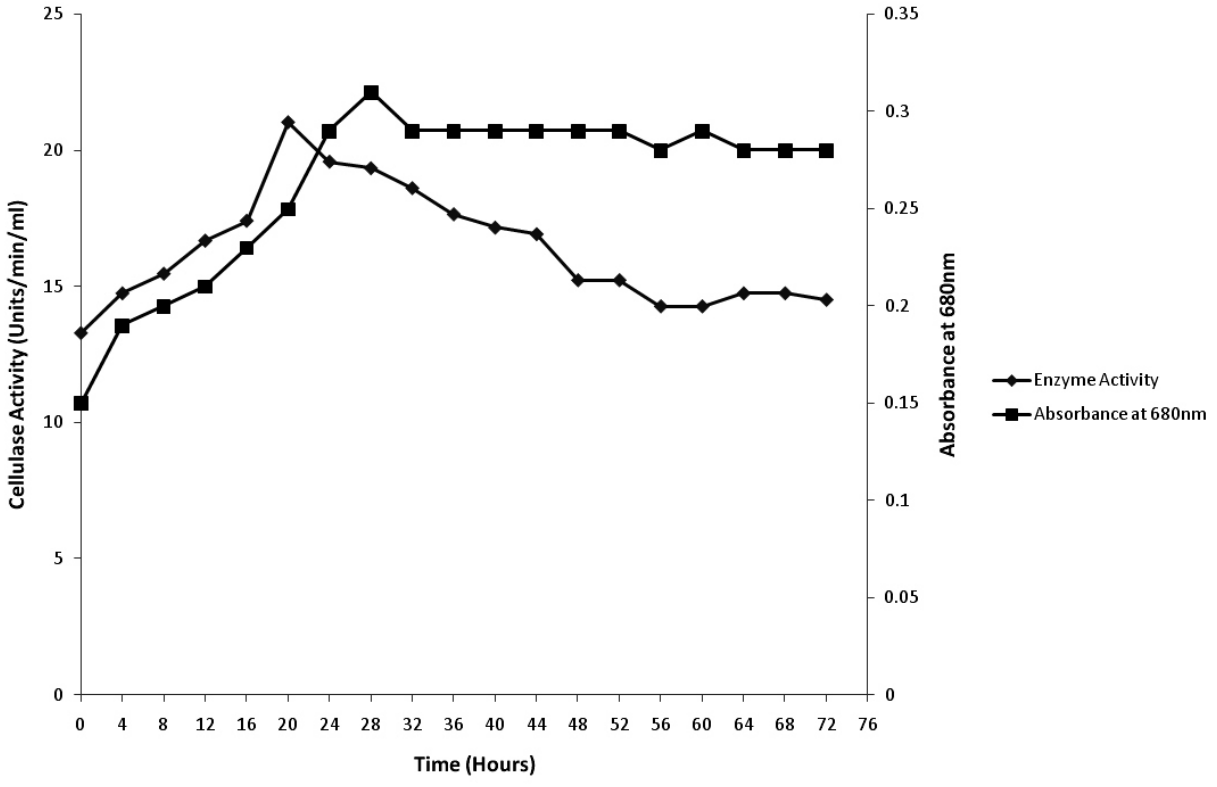

Figure 2: Growth and Cellulase Production of Cellulolytic Bacteria (*Bacillus brevis* 2NA3) Isolated from Heaps of Cassava

DISCUSSION

Thermophiles have been classified as a heterogeneous collection of microorganisms that have an optimum growth temperature in excess of 50°C. An organism that has a growth minimum above 30°C, an optimum above 45°C and a growth maximum below 65°C has been referred to as a moderate thermophile (Cowan, 1992). Heinonen-Tanski *et al.* (2005) classified thermophilic temperature as temperature greater than or equal to 45°C and microorganisms inhabiting this temperature as thermophiles. All the bacteria screened in this study were isolated at 55-60°C hence they can be classified as moderate thermophiles. Kato *et al.* (2004) reported a lower temperature of 50-55°C for the isolation of moderately thermophilic bacteria (*Clostridium straminisolvens*) from a cellulose-degrading bacterial community while Ugwanyi *et al.* (2008) reported 55°C for facultative thermophiles and 65°C for obligate thermophiles isolated from potato peel slurry.

The three cellulolytic bacteria isolates encountered in this study showed a hydrolytic capacity value of between 2.3 and 3.5. The HC value obtained in this study is within the range reported by Lu *et al.* (2005) for mesophilic cellulase degrading bacteria (*Bacillus* sp.) isolated from vegetable waste and higher than values (1.2 to 3.1) reported by Sudiana (2002) for *Bacillus* sp. isolated from soil. Our result is in line with the findings of Lu *et al.* (2005) that reported the isolation of cellulolytic *Bacillus* species from stalks-vegetable waste co-composting system. In addition, Ugwanyi *et al.* (2008) reported *B. coagulans* which is in agreement with our findings. The genus Bacillus is known to play important role in biodegradation and bioconversion of macromolecules (Holt, 1994). They have been reported as the most important source of several commercial microbial enzymes and asides *B. cereus* and *B. anthracis* they have been considered safe to human (Beg and Gupta, 2003).

The growth curve of *Bacillus coagulans* Co4 and *Bacillus brevis* is similar to that reported by Hoster *et al.* (2001). The production of cellulase increased rapidly with increase in bacteria cell growth and peaked at the exponential phase followed by a slow decrease in cellulase production as the cell growth age into the stationary phase. The optimum enzyme activities were 30 units/min and 21 units/min in *Bacillus coagulans* Co4 and *Bacillus brevis* respectively at the exponential phase. This result indicates that *Bacillus coagulans* Co4 has higher enzyme activity than *Bacillus brevis* at 20 h. The optimum enzyme activity of 30 units/min of *Bacillus coagulans* Co4 in this study is comparable to 28 units/min of optimum enzyme activity that was reported by Lu *et al.* (2005) for *Brevibacterium linens* isolated from vegetable waste. The higher value of cellulase activity by *Bacillus coagulans* Co4 made it a more suitable isolate to be considered for further studies on cellulase production and purification.

REFERENCES

Apun, K., Jong, B.C. and Salleh, M.A., 2000. Screening and isolation of a cellulolytic and amylolytic *Bacillus* from sago pith waste. *Journal of General and Applied Microbioliogy* 46, 263-267.

Aubert, J.P., Beguin, P. and Millet, J., 1987. Biochemistry and Genetics of Cellulose Degradation, Fungal and Bacterial Enzyme Systems and their Manipulation. *FEMS Symposium*, 43, Academic Press, New York, pp. 11-30.

Beg, Q.K. and Gupta, R., 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme and Microbial Technology* 32, 294-304.

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

Chinedu, S.*N., Yah, S.C., Nwinyi, O.C., Okochi, V.I., Okafor, U.A. and Onyegeme-Okerenta, B.M., 2008. Plant waste hydrolysis by extracellular enzymes of Aspergillus niger and Penicillium chrysogenum: Effect of Ammonia Pretreatment. Nigerian Journal of Biochemistry and Molecular Biology 23(1), 1-6*

Cowan, D.A., 1992. Biochemistry and molecular biology of extremely thermophilic archaeobacteria. *Molecular Biology and Biothechnology of Extremophiles* (Herbert R.A and Sharp R.J.). Blackie & sons ltd., pp. 1-43.

Dashtban, M., Schraft, H. and Qin, W., 2009. Fungal bioconversion of lignocellulosic residues: opportunities and perspectives. *International Journal of Biological Science* 5, 578-595.

de Souza, A.N. and Martins, M.L.L., 2001. Isolation, properties and kinetics of growth of a thermophilic *Bacillus*. *Brazilian Journal of Microbiology* 32, 271-275.

Hamelinck, C.N., Geertje van Hooijdonk J., and Faaij, A.P.C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy* 28, 384-410.

Harrigan, F. W. and McCance, E. M., 1976. *Laboratory Methods in Food and Dairy Microbiology*. Academic Press, London, pp. 33-200.

Heinonen-Tanski, H., Kiuru, T., Ruuskanen, J., Korhonen, K., Koivunen J. and Ruokojarvi, A., 2005. Thermophilic aeration for cattle slurry with whey and or jam wastes. *Bioresource Technology* 96, 247-252.

Holt, J.G., 1994. The aerobic endospores-forming rods and cocci. *In* Bergey's manual of determinative bacteriology, 9th ed., ed. by Holt, J.G., Williams and Wilkins, Baltimore, MD, pp. 670-675.

Hoster, F., Daniel, R. and Gottschalk, G., 2001. Isolation of a new *Thermoanaerobacterium thermosaccharolyticum* strain (FH1) producing a thermostable dextranase. *Journal of General and Applied Microbiology* 47, 187-192.

Ibrahim, A.S.S. and El-diwany, A.I., 2007. Isolation and identification of new cellulases producing thermophilic bacteria from an Egyptian hot spring and some properties of the crude enzyme. *Australian Journal of Basic and Applied Science* 1(4), 473-478.

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., Yokota, A. and Igarashi, Y., 2004. *Clostridium straminisolvens* sp. nov., a moderately thermophilic, aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community. *International Journal of Systematic and Evolutionary Microbiology* 54, 2043-2047.

Li, X. and Gao, P., 1997. CMC- liquefying enzyme, a low molecular mass initial cellulose decomposing cellulase responsible for fragmentation from *Streptomyces* sp LX. *Journal of Applied Microbiology* 83, 59-66.

Lowry, O.H., Rosebrough, N.J., Farri, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 193, 265-275.

Lu, W.-J., Wang, H.-T., Yang, S.-J., Wang, Z.-C. and Nie, Y.-F., 2005. Isolation and characterization of mesophilic cellulose-degrading bacteria from flower stalks-vegetable waste co-composting system. *Journal of General and Applied Microbiology* 51, 353-360.

Narasimha, G., Sridevi, A., Buddolla, V., Subhosh, C.M. and Rajasekhar, R.B., 2006. Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*. *African Journal of Biotechnology* 5 (5), 472-476.

Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biochemistry* 153, 375-380.

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

Preston, T.R. and Leng, R.A., 2004. Global decline in oil and natural resources; implication for the scope and content of papers for publication in LRRD. *Livestock Research for Rural Development* 16, 87

Sherief, A.A., El-Tanash, A.B. and Atia, N., 2010. Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran. *Research Journal of Microbiology* 181(6), 1-13.

Solomon, B.O., Amigun, B., Betiku, E., Ojumu, T.V. and Layokun, S.K., 1999. Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 grown on bagasse. *Journal of the Nigerian Society of Chemical Engineers (JNSChE)* 16, 61-68.

Somogyi M., 1952. Notes on sugar determination. *Journal of Biological Chemistry* 194, 19-24.

Sudiana, I.M., 2002. Characteristic of CMCase of *Bacillus* sp. isolated from soil of Gunung Halimun National park. *Berita Biologi* Vol.6 (1), 131-136

TNP (The Need Project), 2009. Biomass Secondary Energy Infobook. AP Press, Box 10101, Manassas, VA. pp. 12-15.

Ugwanyi, J.O., Harvey, L.M. and McNeil, B., 2008. Diversity of thermophilic populations during thermophilic aerobic digestion of potato peel slurry. *Journal of Applied Microbiology* 104, 79-90.

Wood, T.M. and Bhat, M.K., 1988. Methods for measuring cellulase activities. In, *Methods in Enzymology* (Wood A. and Kellogg S.T., ed.) Academic press Inc., London, U.K. 160, 87-112.

Yizraeli, S. 2000. How important is Saudi Oil? *The Middle East Quarterly* 7(1), 57-64.

Ife Journal of Science

Table of Contents: December 2011 Edition; Vol. 13, No. 2