Screening and Identification of Mannanase-Producing Fungi Isolated from Selected Agricultural Wastes

D. J. Arotupin[1] and O. O. Olaniyi[1*]

[1]*Department of Microbiology, Federal University of Technology, P.M.B 704, Akure, Nigeria.*

Authors' contributions

This work was carried out in collaboration between both authors. Author OOO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author DJA managed the analyses of the study and the literature searches. The authors read and approved the first manuscript.

Research Article

ABSTRACT

Aim: The study evaluated potential performance of different fungal isolates from agricultural by-products for mannanase production.
Study Design: The first experiment, fungal isolates were screened for mannanase production on agar medium containing Locust Bean Gum (LBG) and total fungal count was conducted. In the second experiment, the fungal isolates were further screened for mannanase production in submerged state fermentation.
Place and Duration of Study: Microbiology Research Laboratory Federal University of Technology, Akure and Postgraduate Research Laboratory, Obafemi Awolowo University Ile-Ife, Nigeria between September 2011 and March 2012.
Methodology: The fungal isolates associated with some agricultural wastes were isolated on LBG containing agar medium by plate assay techniques and counted by standard microbiological methods. Mannanase production was conducted in submerged state fermentation (shaken & static) into which copra meal had been supplemented as the sole carbon source and enzyme activity was determined by dinitrosalicylic acid method.
Results: In this study, 11 fungal isolates showed positive results with clear zone around their cultures. Fungal isolate 5A showed the highest activity ratio of 1.8, while the least was

---

*Corresponding author: Email: microladit@gmail.com;

observed in isolate 9A12 with activity ratio of 0.64. The highest fungal counts were recorded in fermented coconut with 7.4×10^2 sfu/g, while cocoa pod and groundnut shell had no fungal growth. In terms of percentage occurrence of fungal isolates from selected agro-wastes, it was revealed that *Rhizopus japonicus* had the highest occurrence of 66.67%, while the same value of 8.33% was observed for *Aspergillus fumigatus*, *A. glaucus*, *R. stolonifer* and *Trichosporonoides oedocephalis*. In fermentation broth, all the 11 isolates displayed mannanase activity ranging from 0.370 to 21.667 U/ml for static and 0.278 to 3.982 U/ml for shaken condition, with the highest mannanase activity observed with isolate 5A for both culture conditions. According to the cultural characters and microscopic morphology, the isolate 5A being the highest mannanase producer was identified as the *Aspergillus fumigatus.*

Conclusion: In this study, fungal isolates screened and evaluated for mannanase production from agricultural by-products elaborated considerable mannanase activity and this could be exploited for prebiotic preparation.

1. INTRODUCTION

Mannans function as carbohydrates storage in the bulbs and endosperm of some plants. Galactoglucomannans and glucomannans in softwoods and hardwoods are both branched heteropolysaccharides requiring several enzymes for their complete degradation [1]. β-Mannanase (1,4- β-D-mannan mannanohydrolase; mannan endo 1,4-β-mannosidase; EC 32.1.78) is the enzyme that cleaves the β-1,4-mannosidic linkages of mannans, galactomannans, glucomannans, and galactoglucomannans [1,2]. The β-mannanases have been grouped into two families, glycosyl hydrolase 5 (GH5) and glycosyl hydrolase 26 (GH26). The protein folding, catalytic mechanism and mechanism of glycosidic bond cleavage are conserved in both enzyme families [2]. β-Mannanase, required for the utilization of various β-mannans, occurs in certain endosperms such as copra and ivory palm nuts, in the beans of guar, locust, and coffee, and in the roots of konjak [1,3]. This enzyme has found several industrial applications such as the liquefaction and extraction of fruit and coffee beans [1]. An important industrial application of mannanases is likely to be seen in pulp and paper industry. Treating the pulp with β-mannanase alone [2] or in combination with cellulase-free xylanase improves lignin extraction [4]. In doing so, one can save on bleaching chemicals and at the same time reduce wastes which may harm the environment [5]. The bleaching process requires a high temperature and alkaline conditions, but in industrial applications β-mannanases treatment is done under milder conditions [6].

Many microorganisms have been reported as mannanase producers. Mannanases from fungi such as *Aspergillus* species [3,7], *Trichoderma reesei* [8], *Sclerotium rolfsii* [9], and *Penicillium occitanis* [5] have been well described. Fungal mannanases are generally produced in the presence of different mannan-rich substrates. These included Locust Bean Gum (LBG) [1], guar gum [10], konjac flour [5], and copra meal [3,11].

Lignocellulose is the major structural component of plant cell walls and is mainly composed of lignin, cellulose and hemicellulose, and represents a major source of renewable organic matter. The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value [12]. Large amounts of lignocellulosic "waste" are generated through forestry and agricultural practices, paperpulp industries, timber

industries and many agro-industries and they pose an environmental pollution problem. However, the huge amounts of residual plant biomass considered as "waste" can potentially be converted into various different value-added products including biofuels, chemicals, and cheap energy sources for fermentation, improved animal feeds and human nutrients [5]. In this study, the isolation and screening of mannanase producing-fungi that may be present in agricultural wastes was investigated.

2. MATERIALS AND METHODS

2.1 Materials and Chemicals

The coconut residual cakes were sourced from farm field in Akure, Ondo State, Nigeria and it was used as a carbon source for medium formulation. The residual were treated with petroleum ether and dried at 60ºC for 2 h. After that, the residual were blended, milled and sieved to obtain uniform particle size of 0.5 mm. Locust Bean Gum was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2 Sample Sources

Twelve agricultural wastes were collected from farm fields, domestic sources and local market in Akure, Nigeria and they were used as sources for the isolation of mannanase-producing fungi.

2.3 Isolation and Enumeration of Associated Mannanase Producing-Fungi

For the isolation of mannanase producing-fungi from different sources, 1 g of solid sample was suspended in 9 ml of sterilized 0.85% normal saline (NaCl). The solution was vortexed for 60 second. One percent (v/v) of the solution was transferred into 20 ml of sterilized fungal isolation medium (FIM) [13] with 1% copra meal for fungi. The microbial cells were grown under aerobic condition by shaking at 150 rpm for 24 h at 28±2ºC.

2.4 Fungal Identification

The Identification of isolated mannanase-producing fungi was conducted according to the method designed by [14] on the bases of cultural characters (colour, shape of colony, surface and reverse pigmentation and texture of the colony) as well as microscopic structure (septate or nonseptate hyphae, structure of hyphae and conidia).

2.5 Primary Screening of Mannanase Producing-Fungi

The culture broth from enumeration step was serial diluted and spread on fungal isolation medium (FIM) supplemented with 1% LBG instead of copra meal and incubated for 3-7 days at 28 ±2ºC. The colonies with clear zones which are an indication of mannanase activity were observed and the ratio of diameter of clear zones to colony was calculated.

2.6 Secondary Screening of Mannanase Producing-Fungi

Medium composition described by Mandles and Weber modified by [1] was used for submerged fermentation (static and shaken condition). The media contained (per liter of distilled water): Copra meal 10 g, $NaNO_3$ 2 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, KCl 0.5 g and

$FeSO_4.7H_2O$ traces. pH of the media were adjusted to 6.8 with pH meter (Denver Instrument, Model 20 pH/ Conductivity meter) prior sterilization. Then, 50 ml of the liquid medium was dispensed in 250 ml Erlenmeyer flask and sterilized by autoclave 121ºC for 15 min. This was cooled and inoculated with 2 discs of 8 mm diameter of the organisms from malt extract culture plates using sterile cup borer. The flasks were incubated at 30 ± 2ºC for 7 days on a rotary shaker (Gallenkamp) at 120 rpm. Crude enzyme preparation was obtained by centrifugation at 6000 rpm for 10 min at 4ºC using refrigerated ultracentrifuge (Centurion Scientific Limited). The supernatant was used as the crude extracellular enzyme source. Each treatment was carried out in triplicates and the results obtained throughout the work were the arithmetic mean of at least 3 experiments. Mannanase activity was assayed in the reaction mixture composing of 0.5 ml of 50 mM potassium phosphate buffer pH 7.0 and 1% LBG with 0.5 ml of supernatant at 45ºC for 60 min (modified method of [1]). Amount of reducing sugar released was determined by the dinitrosalicylic acid reagent (DNS) [15]. One unit of mannanase activity was defined as amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions.

3. RESULTS AND DISCUSSION

Mannanase producing-fungi were isolated from twelve agricultural wastes. The fungal isolates were screened primarily on mannan-agar medium containing 0.5% LBG (Sigma), and the quantitative mannanase activity was determined on the basis of clear zones formed around the colonies. A total of 11 fungal isolates showed clear zone of mannanase activity on solid medium at 28±2ºC (Table 1). Fungal isolate 5A showed the highest activity ratio (1.8) on LBG medium followed by isolate 12C (1.42), while the least (0.64) was observed in isolate 2A. The production of mannanase activity on LBG had been demonstrated by [13] and [4]. The formation of clear zone by these isolates on agar medium supplemented with LBG could be attributed to the ability of their genetic make up to secrete active mannanase with high diffusion rate as reported by [4].

Table 1. Mannanase activity of fungal isolates expressed as ratio of clear zone/ colony

Source	Isolate	Colony (mm)	Clear zone (mm)	Ratio of clear zone/ colony
Pineapple peels	1C	36	39	1.08
Cotton seed	2A	19	21	1.1
Yam peels	5A	16	28	1.8
Yam peels	5B	44	49	1.1
Bannana peels	7A	28	34	1.2
Bannana peels	7B	26	32	1.2
Locust bean wastes	8A	38	31	0.82
Orange peels	9A11	0.9	1.1	1.2
Orange peels	9A12	28	18	0.64
Potato peels	10A	2.3	2.0	0.9
Fermented coconut	12C	26	37	1.42

1C=Rhizopus japonicus PAP-1C, 2A= Aspergillus flavus LAD-2A, 5A=A. fumigatus LAD-A5, 5B=Rhizopus japonicus YP-5B, 7A= R. japonicus BN-7A, 7B= R. japonicus LBW-7B, 8A= R. japonicus LBW-8A, 9A11= A. glaucus LAD-9A11, 9A12= R. stolonifer LAD-9A12, 10A= A. flavus LAD-10A, 12C= Trichosporonoides oedocephalis FCN-12C

In Table 2, total fungal counts from each of the agro-wastes were revealed. Fermented coconut was observed to give the highest number of fungal population with $7.4×10^2$ sfu/g,

while cocoa pod and groundnut shell had no fungal growth. The high fungal counts may be attributed to lack of proper wastes management in the discharge of agro-wastes into the environment [16]. [5] and [17] reported that large amounts of agricultural waste are generated through forestry and agricultural practices, paper pulp industries, timber industries and many agro-industries and they pose an environmental pollution problem. These singular activities tend to expose the agro-wastes to microbial contamination. The reports of [17] also revealed structural component of agro-wastes to contain lignin, cellulose, hemicellulose, and presence of some components (activators or inhibitors). The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value [12]. The chemical composition of the wastes might be linked to its ease of colonization, hence may account for the high fungal counts. Apart from this, fungal isolates may probably have originated from soil, water and material used during harvesting of agricultural produces, while the variations of the isolates may be due to the handling process and the prevailing environmental conditions [16]. The absence of fungal growth in cocoa pod and groundnut shell may be due to the regular sanitation and fumigation of the environment where they were been deposited [16].

Table 3 shows the cultural characterization and microscopic observation of fungal isolates associated with agro-wastes. It was observed that most of the fungal isolates encountered were *R. Japonicus*. The presence of this fungal strain (*R. Japonicus*) in most of these wastes might be due to extrinsic factors that enhanced its growth and survival.

The percentage occurrence of isolates from selected agro-wastes is reported in Table 4. The isolates consisted of *Aspergillus fumigatus, A. flavus, A. glaucus, Rhizopus japonicus, R. stolonifer* and *Trichosporonoides oedocephalis*. The highest percentage occurrence was obtained for *Rhizopus japonicus*, while *Aspergillus fumigatus*, *A. glaucus*, *R. stolonifer* and *Trichosporonoides oedocephalis* had the same value of 8.33%. The result clearly showed that certain fungal isolates are associated with the agro-wastes and that the growth and survival of these fungal isolates may not be unconnected with the fact that the wastes contained substances that can be utilized by the isolates. Therefore, the isolates can be said to be transient microorganisms surviving only in the absence of inhibitory substances [16,18].

Table 2. Total fungal counts from different agro-wastes (sfu/g)

Agro-wastes	Fungi
PAP	2.0×10^2
CS	5.0×10^2
CP	1.0×10^2
CPS	ND
YP	4.0×10^2
GNS	ND
BP	1.0×10^2
LBW	2.0×10^2
ORP	1.0×10^2
PP	7.0×10^2
SDC	1.0×10^2
FCN	7.4×10^2

PAP= Pineapple peels; BP=Banana peels; CS= Cotton seed; LBW=Locust bean wastes;
CP= Cassava peels; ORP=Orange peels; CPS= Cocoa pod/Shell; PP=Potato peels; YP= Yam peels;
SDC=Sawdust compost; GNS= Groundnut shell; FCN=Fermented coconut; ND= Not detectable;
sfu/g= Spore forming unit per gram.

Table 3. Cultural characterization and microscopic observation of fungal isolates

Isolate code	Cultural characteristics	Microscopic observation	Suspected organisms
1C, 2B, 3A5B, 7A, 8A& 9A2	Rapidly growing white colored fungus, swarm over entire plate; then showed black spores varying in sizes	Non-septate mycelium gives rise to straight sporangiophores that sporangium; root like hyphae (rhioziod) penetrating the medium	*Rhizopus japonicus*
2A	Yellow mycelia growth	An upright conidiophores that terminates in a davate swelling bearing phialides at the apex or radiating from the entire surface; conidia are 1-celled and globose	*Aspergillus flavus*
5A	Blue mould growth	Septate mycelium bearing Single conidiophores which are branched near the apex ending in phialides that carries the conidia	*Penicillium italicum*
9A1	White colonies became bluish-green or gray-green	conidiophores upright, simple, terminating in a globose or davate swelling, bearing phialides at the apex or radiating from the apex or entire surface; conidia (phialospores) 1-celled and globose	*A. glaucaus*
10A	Yellow mycelia growth	An upright conidiophores that terminates in a davate swelling bearing phialides at the apex or radiating from the entire surface; conidia are 1-celled and globose	*A. flavus*
11A	Rapidly growing white colored fungus, swarms over entire plate	Non-septate mycelium gives rise to straight sporangiophores that with sporangium containing a columella;root like hyphae (rhioziod) penetrating the medium	*R. stolonifer*
12C		Mycelium and spores hylaline; both true	*Trichosporonoides oedocephalis*

mycelium and pseudomycelium present in culture, conidophores simple with swollen globose apex bearing conidia simultaneously on sterigmata. Arthrospores also form. All conidia hyaline, 1-celled and All conidia hyaline, 1-celled and

Table 4. Percentage occurrence of fungal isolates from selected agro-wastes

Fungal isolates	% occurrence of isolates
Aspergillus fumigates	8.33
Aspergillus flavus	16.76
Aspergillus glaucus	8.33
Rhizopus japonicas	66.67
Rhizopus stolonifer	8.33
Trichosporonoides oedocephalis	8.33

Upon further quantitative determination of mannanase activity, all the 11 isolates displayed mannanase activity ranged 0.370 to 21.667 U/ml for static and 0.278 to 3.982 U/ml for shaken condition. The highest mannanase activity obtained from both conditions was isolate 5A (Fig. 1). However, the overall evaluation of all the isolates in static condition showed higher extracellular mannanase activity than what was displayed by isolates in shaken condition. The higher mannanase activity in static culture could be attributed to the shearing effect induced by the agitation speed on cells and enzyme activity which may contribute negatively towards cell growth and enzyme instability [19]. Apart from this, agitation speed might cause cell damage which in turn leads to enzyme inactivation [20,21]. [1] linked lower mannanase production to oxygen limitation induced by the highly non-Newtonian medium with the filamentous growth of the fungus.

In shaken culture, isolate 1C and 9A12 had highest protein content, while isolate 5A was recorded for static culture, although, there was no direct relationship between the protein content of the tested cultures and the production of mannanase (Fig. 2). Protein concentration does not really indicate an increase in the production of mannanase. Different fungal isolates had been reported to produce variety of enzymes (amylases, cellulases, protease and xylanases) apart from the enzyme been examined for in this study. Besides that, the protein from fungal cells might also interfere with mannanase enzyme causing variation in protein content, since the protein assay could only identify accumulated protein in solutions. This view was supported by the findings of [22].

The final pH values obtained from the fermentation systems is revealed in Fig. 3. It was observed that the final pH designed for fermentation system for isolates in static condition was at the acidic range (4.68- 6.97), while those of the shaken cultures ranged from acidic to alkaline (3.97- 7.45). The acidic pH obtained for the static culture might be due to organic acid accumulation, while variation from acidic to alkaline states observed in shaken cultures may be attributed to an increase in soluble reducing sugars profiles during fermentation [4].

The colonies of isolate 5A, being the highest producer of mannanase on mannan-agar medium containing LBG, morphological examination was therefore conducted. A morphological examination of the isolate revealed that the conidiophores were upright, simple, terminating in a globose bearing phialides at the apex or radiating from the entire surface. Furthermore, the conidia appeared in dry basipetal chains. From these cultural and morphological features, the isolate was identified as *Aspergillus fumigatus* [14].
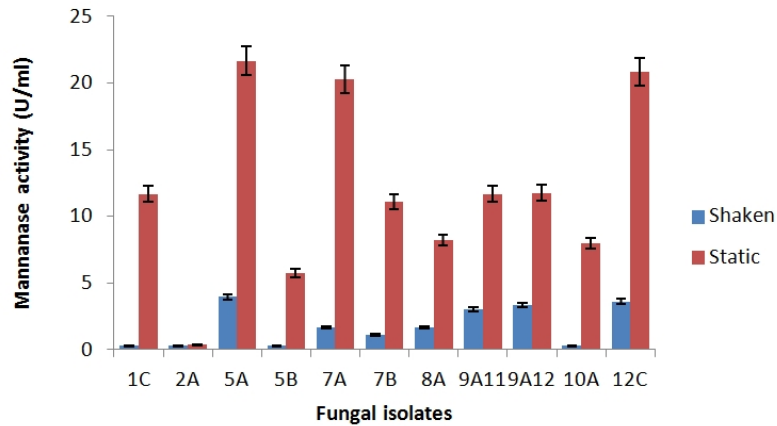

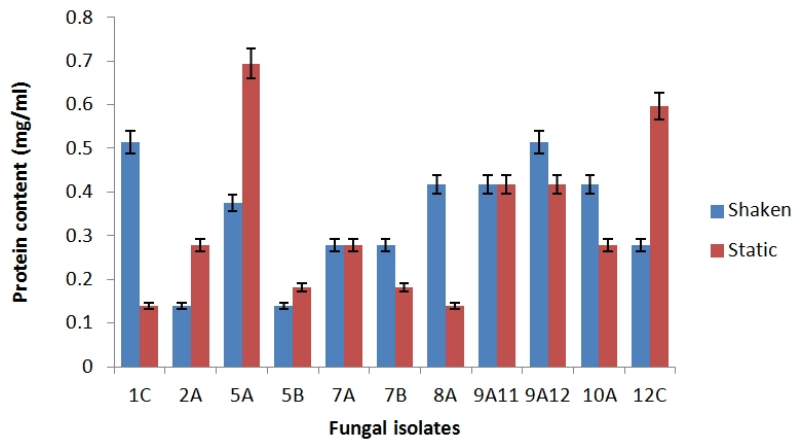**Fig. 1. Mannanase production by fungal isolates at different fermentation conditions**

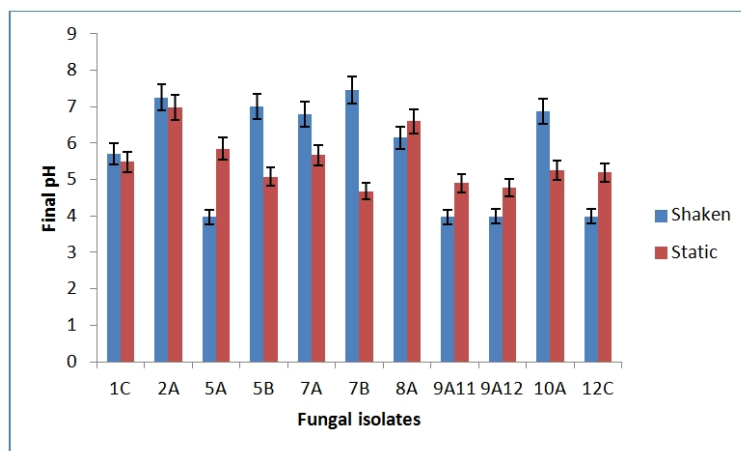**Fig. 2. Protein content produced by fungal isolates at different fermentation conditions**

Fig. 3. Final pH values obtained from the fermentation systems

4. CONCLUSION

This study has revealed isolate 5A as capable of producing promising amount of mannanase on copra meal under submerged fermentation. Agricultural wastes rich in β-mannan are abundant in nature and awaited to be converted into more valuable products used for mankind. The fungal isolates from agricultural wastes showed a potential to convert mannan into manno-oligosaccharides (MOS) which could be readily used in many applications such as animal foods and a feed stock for production of valuable organic compounds

COMPETING INTEREST

Authors have declared that no competing interests exist.

REFERENCES

1. El-Naggar MY, Youssef SA, El-Assar SA, Beltagy EA. Optimization of cultural conditions for β-mannanase production by a local *Aspergillus niger* isolate. Int. J. Agri. Biol. 2006;8(4):539-545.
2. Hashem AM, Ismail AMS, El-Refai MA, Abdel-Fattah AF. Purification and some properties of β-mannanase from *Aspergillus oryzae* NRRL 3448. J. Appl. Sci. Res. 2009;5(12):2067-2073.
3. Dhawan S, Kaur J. Microbial mannanases: an overview of production and applications. Crit. Rev. Biotechnol. 2007;27(4):197–216.
4. Sae-Lee N. The production of fungal mannanase, cellulase and xylanase using palm kernel meal as a substrate. Walailak J. Sci. Technol. 2007;4(1):67-82.
5. Blibech M, Ellouz Ghorbel MR, Fakhfakh I. **"**Purification and characterisation of a low molecular weight of β- mannanase from *Penicellium occitanis.* Pol6, Appl. Biochem. Biotechnol. 2010;160:1227–1240.
6. Singh C, Ahuja N, Batish M, Capalash N, Sharma P. Biobleaching of wheat straw-rich-soda-pulp with alkalophilic laccase from γ-*Proteobacterium* JB: Optimization of process parameters using response surface methodology. Biores. Technol. 2008;99:7472-7479.

7. Regalado CB, Garcia-Almendarez ELM, Venegas-Barrera LM. Production, partial purification and properties of β- mannanases obtained by solid substrate fermentation of spent soluble coffee wastes and copra paste using *Aspergillus oryzae* and *Aspergillus niger*. J. Sci. Food Agri. 2000;80(9):1343–1350.
8. Arisan-Atac I, Hodits R, Kristufek D, Kubicek CP. "Purification, and characterization of a β-mannanase of *Trichoderma reesei* C-30," Appl. Microbiol. Biotechnol. 1993; 39(1):58–62.
9. Gubitz GM, Hayn M, Urbanz G, Steiner W. Purification and properties of an acidic β-mannanase from *Sclerotium rolfsii*. J. Biotechnol. 1996;45(2):165–172.
10. Puchart V, Vrsanska M, Svoboda P, Pohl J, Ogel ZB, Biely P. Purification and characterization of two forms of endo-β-1,4-mannanase from a thermotolerant fungus, *Aspergillus fumigatus* IMI 385708 (formerly *Thermomyces lanuginosus* IMI 158749). Biochim Biophys Acta. 2004;1674:239–250.
11. Naganagouda VK, Aravind Goud GP, Mulimani VH. Optimization of the production of thermostable endo-β-1,4 mannanases from a newly isolated *Aspergillus niger gr* and *Aspergillus flavus gr*. Appl. Biochem. Biotechnol. 2009;152(2):213–223.
12. Malherbe S, Cloete TE. Lignocelluloses biodegradation: fundamentals and applications: A review. Environ. Sci. Biotechnol. 2003;1:105-114.
13. Phothichitto K, Nitisinprasert S, Keawsompong S. Isolation, screening and identification of mannanase producing microorganisms. Kasetsart J. (Nat. Sci.). 2006;40:26-38
14. Pitt JI, Hocking AD. Fungi and food spoilage, 2th Edition, Blacki Academic, London, U.K. 1997;414.
15. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 1959;31:426-428.
16. Arotupin DJ. Evaluation of microorganisms from cassava water for production of amylase and cellulase. Res. J. Microbiol. 2007;2(5):475-480.
17. Howard RL, Abotsi E, Jansen Van Rensburg EL, Howard S. Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afri. J. Biotechnol. 2003;2:602-619.
18. Olowoyo OO, Akinyosoye FA, Adetuyi FC. Microorganisms associated with some cassava (*Manihot esculeta Crantz*) products. J. Res. Rev. Sci. 2001;2:10-14.
19. Abd-Aziz S, Ong LGA, Hassan MA, Karim MIA. Process parameters optimization of mannanase production from *Aspergillus niger* FTCC 5003 using palm kernel cake as carbon source. Asian J. Biotechnol. 2008;3(5):297-307.
20. Shioya SMM, Kajihara Y, Shimizu H. Optimization of agitation and aeration conditions for maximum virginiamycin production. Appl. Microb. Biotechnol. 199; 51: 164-169.
21. Feng Y, He Z, Ong SL, Hu J, Zhang Z, Ng WJ. Optimization of agitation, aeration and temperature conditions for maximum β-mannanase production. Enzyme Microb. Technol. 2003;32:282-289.
22. Akinyele BJ, Olaniyi OO, Arotupin DJ. Bioconversion of selected of selected agricultural wastes and associated enzymes by *Volvariella volvacea*: An edible mushroom. Res. J. Microbiol. 2011;12:567-573.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=246&id=8&aid=1935