



MICROBIOLOGY

PRODUCTION OF EXTRA CELLULAR EXOGLUCANASE BY *RHIZOPUS ORYZAE* FROM SUBMERGED FERMENTATION OF AGRO WASTES

Soumita Mukherjee, Moumita Karmakar and Rina Rani Ray*

Microbiology Research Laboratory, Department of Zoology, Molecular Biology & Genetics, Presidency University, 86/1, College Street, Kolkata; 700 073, India

Abstract

Rhizopus oryzae PR7 MTCC 9642, a producer of endoglucanase was found to produce extra cellular exoglucanase or avicelase when grown on avicel or micro crystalline cellulose. In order to curtail the cost of production the strain was grown in media supplemented with various cellulosic wastes, of which dried flower showed the best result followed by the sweet lime peel at optimum pH 8.0 and 5.0 respectively at 37° C. Peptone was found to be the best nitrogen source for exoglucanase production whereas amongst metal ions Mn²⁺ and Fe²⁺ could bring a 1.23 fold increase in enzyme production in sweet lime peel supplemented culture. Under optimized condition, highest exoglucanase production was achieved at 96 hours of growth. The enzyme showed optimum activity at pH 5.0 and 40°C and stability at pH range of 5-9 and about 90% activity was retained even after an exposure of 10 minutes at 80° C. The enzyme activity was enhanced in presence of Mn²⁺ and Fe²⁺. The enzyme was found to saccharify avicel and the wastes into cellobiose.

Keywords: Avicelase, Exoglucanase, *Rhizopus oryzae*, Agro wastes

Introduction

Cellulose, an unbranched glucose polymer, composed of anhydro-β-1,4-glucose units linked by β-1,4-glycosidic bonds, is the most abundant biopolymer on the earth. Enzymatic degradation of cellulose to glucose requires the co-operative action of three synergistically acting enzymes: namely endoglucanase (CM cellulase, EC 3.2.1.4), which cleaves internal glucosidic bonds, exoglucanase (Avicelase, EC 3.2.1.91) that cleaves cellobiosyl units from the ends of cellulose chains; and β-glucosidase (EC 3.2.1.21), which cleaves glucose units from cello oligosaccharides (Wood, 1989).

Cellulase(s) are important enzymes not only for their potent applications in different industries, like industries of food processing, animal feed production, pulp and paper production, and in detergent and textile industry, but also for the significant role in bioconversion of agriculture wastes in to sugar and bioalcohols. Amongst the cellulases, exoglucanase or avicelases are found to have potential applications in the bioconversion of agricultural waste materials to useful products, such as single cell protein, fuels and chemical feed stocks (Kari *et al.*, 1994; Nikolay *et al.*, 1998; Ojumu *et al.*, 2003). Although a number of microorganisms were reported to produce cellulases, in comparison to that of endoglucanase and beta glucosidase the report of exoglucanase or avicelase production is remarkably scanty. Hence extensive research is to be made to isolate new microbial

sources capable of producing exoglucanase. As generally exoglucanase is found to be synthesized along with other two types of cellulases, it is also important to estimate the relative ability of a particular cellulase producing strain to release exoglucanase and the parameters affecting the synthesis.

In order to make the production process cost effective, the fermentation medium may be prepared with cellulosic wastes in place of microcrystalline cellulose or avicel, an expensive substrate. Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulose containing raw materials and waste products left unutilized. Discarded plant parts like peels of fruits and dried petals of flowers constitute a huge waste in the civil area and generate obnoxious odour, can be effectively utilized for the production of cellulase enzymes.

The present study aims at the production of extra cellular exoglucanase by a potent endoglucanase producing strain of *Rhizopus oryzae* from submerged fermentation media supplemented with the dried flower and sweet lime peel.

Materials and Methods

Organism

Rhizopus oryzae PR7 MTCC 9642 (Karmakar and Ray, 2010) was used in these studies. The fungus was grown in 1% PDA plates for 48 h at 28-30°C. The inocula were prepared by making hyphal discs (0.5 cm

* Corresponding Author, Email: rina-ray64@yahoo.co.in

diameter). Each disc was used to inoculate 10 mL of medium (Ray and Chakraverty, 1998)

Cultivation of the Strain

The strain was cultivated in 100 mL Erlenmeyer flasks each containing 10 mL Basal Medium (BM) composed of (g L⁻¹): peptone 0.9; (NH₄)₂HPO₄ 0.4; KCl 0.1; MgSO₄·7H₂O 0.1 and avicel (Sigma) 0.5.

Cellulosic Materials

Various cellulosic wastes were collected from domestic effluents, agricultural fields and temple wastes. Those were dried, pulverized and sieved as 40 mesh particle size before using in fermentation media in place of pure avicel.

Enzyme Assay

In submerged fermentation, the grown culture was filtered through filter paper (Whatman No. 1), filtrate was centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. To measure the activity of exoglucanase, the assay mixture (1 mL) containing an equal volume of enzyme and 1% (w/v) avicel (Sigma) dissolved in 10 mM was incubated at 37°C for 10 min. The reducing sugar released was measured by the dinitrosalicylic acid method (Bernfeld, 1955) taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of exoglucanase was defined as that amount of enzyme that liberated 1 milli mole of glucose per milliliter per minute of reaction.

Optimization of culture conditions for enzyme production

Fermentation period is important parameter for enzyme production by *Rhizopus oryzae* PR7. With a view to replace avicel (Sigma), a costly substrate for exoglucanase production, various cheap and abundantly available cellulosic wastes, namely dried flower and grass, peel of sweet lime, potato and water hyacinth were supplemented as carbon source. The concentration of the cellulosic wastes acting as the inducers was tested by varying their concentration (0.25-1% w/v). The effect of different temperature was studied by incubating the culture containing flasks at various temperatures (7-47°C). The strain was grown in different flasks containing media with various pH (4-9) to check the optimum pH for enzyme production. Similarly, the effect of nitrogen source and divalent ions were studied by supplementing various organic and inorganic nitrogen source (0.09% w/v) and divalent ions (10 mM) in the culture. The effect of cultivation time was determined by picking up the culture containing flasks with optimized media at various time

intervals (24-120 hours), followed by an assay of the enzyme activity.

Characterization of the enzyme

The temperature and pH optima were detected by incubating the assay mixture at different temperatures (20°-80°C) at constant pH and at various pH ranges (4-9) at 40°C for 10 minutes respectively. Thermostability of the enzyme was determined by exposing the enzyme at various temperatures ranges (20°-80°C) for 30 minutes followed by the measurement of their residual activities. The pH stability was determined by keeping the enzyme in presence of various buffers 0.1M acetate buffer (pH 4-6), 0.1M phosphate buffer (pH 5-8) and 0.1M Tris glycine buffer (pH 8-9) at 40°C for 120 minutes followed by the estimation of their residual activities. Effect of divalent ions on enzyme activity was measured by adding 10mM of various metallic salts followed by the measurement of the respective residual activities. The effect of substrate concentration was determined by varying the concentration of avicel (0.25-3.0% w/v) in the assay mixture.

Saccharification of enzyme

Avicel and the two agro wastes, namely dried flower and sweet lime and sieved to 40 mesh particle size, before using as substrate for saccharification. A suspension of substrate (5mg/ml) in 0.1(M) phosphate buffer (pH: 6) was incubated with equal amount of exoglucanase (800 U/ml) in a screw capped tube for 30 minutes at 40°C. The resultant supernatant following centrifugation at 2000 g for 2 minutes was analyzed by DNSA method (Bernfeld, 1955) using glucose as standard.

Determination of end product of saccharification

The end products of saccharification of agro wastes by endoglucanase was analysed by TLC on a pre coated TLC plate (Merck) using a solvent system of butanol: acetic acid: water: methanol (3:3:1v/v), developing it with 0.1% methanolic orcinol in 10% H₂SO₄. (Murahima *et al*, 2003).

Each experiment was carried out in triplicate and their values were averaged.

Results and Discussion

Effect of Agro Waste as Inducer of Exoglucanase Synthesis

R. oryzae PR7 was found to degrade various cellulosic agro wastes (Table 1) in submerged fermentation conditions of which sweet lime peel (*Citrus limetta*), and dried flower (*Marigold-Calendula officinalis*) showed very promising results. Therefore, further experiments were carried out with sweet lime peel and dried flower only.

Table1- Effect of different agro wastes on exoglucanase production by *R. oryzae*

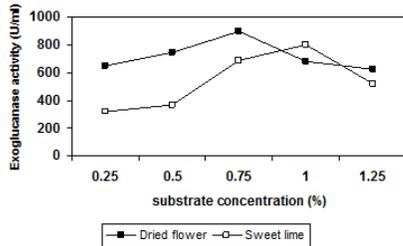
Carbon source	Exoglucanase activity (U/ml)
Avicel (Sigma)	400
Dried flower	900
Sweet lime peel	800
Dried grass	400
Water hyacinth	200
Potato peel	50

Cultivation time: 72 hrs

Effect of Substrate Concentration

It was found that sweet lime induced the exoglucanase production best at a concentration of 1%(w/v) whereas dried flower showed highest production at a concentration of 0.75%(w/v) (Figure 1). Higher concentration of dried flower could restore the amount of enzyme production, but higher concentration of sweet lime peel in the fermentation media reduced the enzyme production probably due to the adverse effect of higher concentration of nutrient supplements present in these substrates on enzyme production (Omojasola *et al.*, 2008) or as a result of hindrance of mass transfer of oxygen by higher amount of solid substrate.

Fig 1. Effect of concentration of agro wastes in the culture media for the production of extra cellular exoglucanase by *R. oryzae* PR7. (Cultivation time: 72 hours)



Effect of temperature

Production of extra cellular exoglucanase by *R. oryzae* PR7 was preferred at 37°C both for dried flower and sweet lime supplemented media. (Figure 2)

Effect of pH on enzyme production

Optimization of pH on enzyme production indicated that various source of cellulosic waste required various pH for enzyme production, which might be due to their respective lignin content. As for example the optimum pH for sweet lime peel was 5.0, whereas it was 8.0 for dried flower (Figure 3). The observation was comparable to other

strains *Rhizopus oryzae* that can thrive in a pH range, of 5.0-7.0 (Murashima *et al.*, 2002).

Fig 2- Effect of temperature of the agro waste supplemented culture media for the production of extra cellular exoglucanase by *R. oryzae* PR7. (Cultivation time: 72 hours)

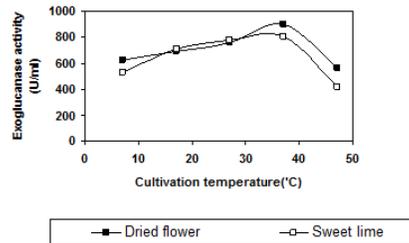
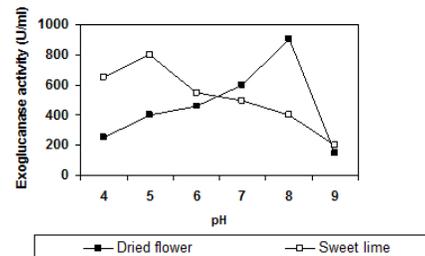


Fig 3 Effect of pH of the agro waste supplemented culture media for the production of extra cellular exoglucanase by *R. oryzae* PR7 (Cultivation time: 72 hours)



Effect of Nitrogen Sources

Among the nitrogen sources tested, peptone was proved to be the best nitrogen source for enzyme production (Table 2) Our results are in accordance with the work of Enari *et al.* (1977) who reported that good cellulase production can be obtained with the organic nitrogen sources such as yeast extract and peptone. But enzyme production was remarkably decreased in presence of urea, a report contrary to that of by *Aspergillus niger* (Acharya *et al.*, 2008).

Table 2-Effect of different nitrogen source in the culture media for the production of exoglucanase by *R. oryzae* PR7

Nitrogen source (0.09%)	Exoglucanase activity (U/ml)	
	Dried flower	Sweet lime peel
Peptone	1200	850
Tryptone	600	160
Yeast extract	1150	243
Urea	450	67
Ammonium sulfate	800	153
Ammonium oxalate	860	243

Cultivation time: 96 hours.

Effect of metallic ions

Table 3 indicated that amongst metal ions Mn^{2+} and Fe^{2+} could bring a 1.23 fold increase in enzyme production in sweet lime peel supplemented culture,

whereas Fe^{2+} itself increased the production in dried peel supplemented medium. On the other hand, Hg^{2+} reduced the enzyme production in both the cases.

Table 3- Effect of different metallic ions on the production of exoglucanase by *R. oryzae* PR7

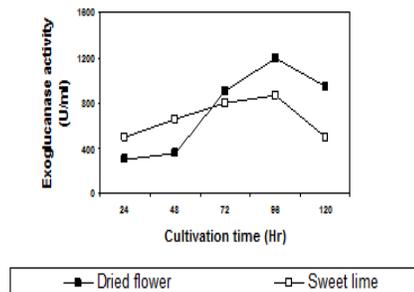
Metallic ions (10mM)	Exoglucanase activity (U/ml)	
	Dried flower	Sweet lime peel
None	900	800
Na^+	554	830
Mn^{2+}	485	1038
Mg^{2+}	692	623
Fe^{2+}	1040	1035
Sn^{2+}	485	830
Ca^{2+}	762	969
Ba^{2+}	692	480
Pb^{2+}	554	550
Cu^{2+}	890	761
Hg^{2+}	346	480

Cultivation time: 72 hours.

Effect of cultivation time in enzyme production

Highest exoglucanase production could be achieved within 96 hours of cultivation with both dried flower and sweet lime peel (Figure 4), which is suitable for commercial point of view (Kang et al., 2004).After that it rapidly decreased, probably due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretory machinery of the enzymes (Nochure et al., 1993).

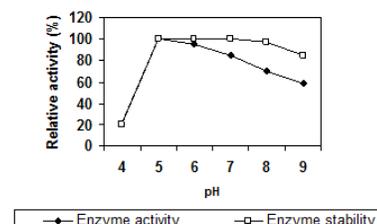
Fig 4-Effect of cultivation time on the production of exoglucanase by *R. oryzae* PR7



Characterization of the enzyme

Optimum pH of the enzyme was found to be 5 (Figure 5).The enzyme was found to stable in the pH from 5 to 8.Temperature optima was found to be 40°C (Figure 6).The thermoinactivation kinetics (Figure 7) showed that the enzyme could retain about 90% of its activity at 80°C even after an exoisure of 10 minutes and could be called thermostable. The optimum concentration of substrate required for enzyme activity was 0.50% (Figure 8) which in turn indicated the high affinity of the enzyme towards its substrate.

Figure-5 Effect of pH on activity and stability of exoglucanase of *R. oryzae* PR7



Mn²⁺ and Fe²⁺ could remarkably increase the activity of the enzyme, whereas addition of Hg²⁺ and Cu²⁺ reduced the enzyme activity probably through the destruction of the active site by denaturing the thiol groups present there (Figure 9).

Figure-6-Effect of temperature on the activity of exoglucanase of *R. oryzae* PR7

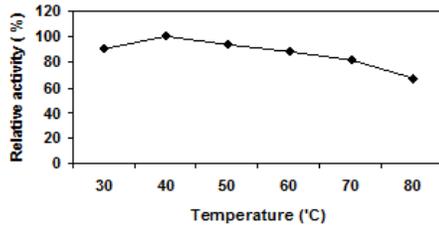


Figure-7-Effect of temperature on the stability of exoglucanase of *R. oryzae* PR7

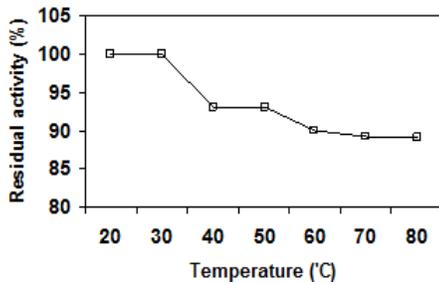


Figure-8-Effect of substrate concentration on the activity of exoglucanase of *R. oryzae* PR7

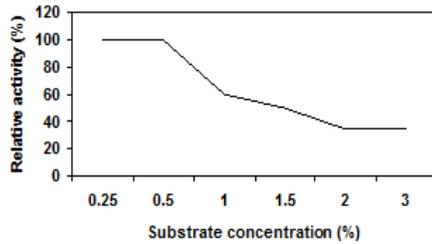
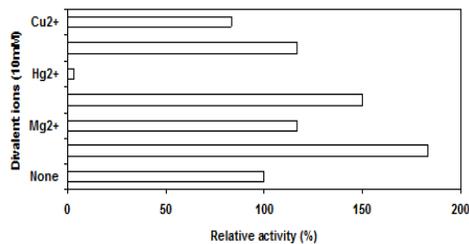
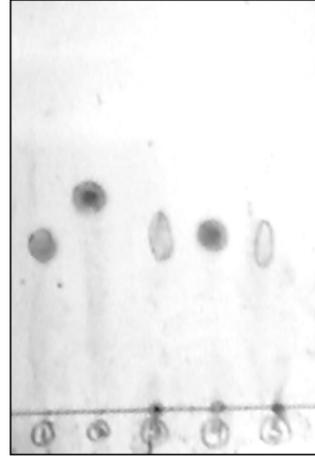


Fig 9 Effect of different divalent ions on the exoglucanase activity of *R. oryzae* PR7



End product analysis

Fig 10-Analysis of the products of on TLC



1. Cellobiose (standard)
2. Glucose (standard)
3. Exoglucanolytic activity of dried flower,
4. Exoglucanolytic activity of Avicel (Sigma)
5. Exoglucanolytic activity of sweet lime.

Cellobiose was found to be the final product of exoglucanolytic activity of both the types of agro wastes tested and pure avicel (Fig 10), a result similar to that reported by Noriho et al, 2008

Conclusion

In the recent years, one of the most important biotechnological applications is the conversion of agricultural wastes and all lignocellulosics into products of commercial interest such as ethanol, glucose and single cell products (Ojumu *et al.*, 2003). The key element in bioconversion process of lignocellulosics to these useful products is the hydrolytic enzymes mainly cellulases (Ojumu *et al.*, 2003; Fan *et al.*, 1987; Immanuel *et al.*, 2007). The bioconversions of cellulosic materials are now a subject of intensive research as a contribution to the development of a large scale conversion process beneficial to mankind. Such process would help alleviate shortages of food and animal feeds, solve modern waste disposal problem and diminish man's dependence on fossil fuels by providing a convenient and renewable source of energy in the form of glucose. A diverse spectrum of cellulolytic microorganism mainly fungi (Falcon *et al.*, 1995) and Bacteria (McCarthy, 1987) have been isolated and identified over the years and this still continue to grow rapidly. Fungi are the main cellulase producing microorganism and *Aspergillus* and *Trichoderma* are the main fungal genera that were used for commercial production of cellulase (Milala *et al.*, 2009; Person *et al.*, 1991). So far the literature study is concerned, most of the studies regarding cellulase production by agro waste degradation have been carried out with these two genera, whereas similar report with *Rhizopus* sp., is

almost rare. On the other hand, a number of work have been done on wastes like rice and wheat straw (Kocher *et al.*, 2008; Singh *et al.*, 2009) sugar cane bagasse (Rezende *et al.*, 2002) and orange waste (Omojasola and Jilani, 2008), no work has been reported from fermentation of dried flower and sweet lime peel. Therefore the present strain of *Rhizopus oryzae* showing the ability to synthesize high amount of extra cellular exoglucanase within a relatively short period of time, utilizing agro wastes like dried flower and sweet lime peel that would otherwise cause environmental pollution, could be used for rapid and commercial production of cellulase.

Acknowledgement

Authors thank Department of Science and Technology (DST), Government of West Bengal, India for financial assistance.

References

- Wood, T. M., S. I. McCrae and K. M. Bhat. 1989. The mechanism of fungal cellulase action: synergism between enzyme components of *Penicillium pinophilum* cellulase in solubilizing hydrogen bond-ordered cellulose. *Biochem. J.* 260:37-43
- Kari, A., R. Flengsrud, V. Lindahl and A. Tronsmo, 1994. Characterization of production and enzyme properties of an endo- β -1,4-glucanase from *Bacillus subtilis* ek-2 isolated from compost soil. *Antonie Van Leeuwenhoek*, 66: 319-326.
- Nikolay, S.A., K. Robson and D.B. Wilson, 1998. Regulation of biosynthesis of individual cellulases in *Thermomonospora fusca*. *J. Bacteriol.*, 180: 3529-3532
- Ojumu, T.V., Solomon, B.O., Betiku, E., Layokun, S.K. and Amigun, B., 2003. Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented in sawdust, bagasse and corncorb. *Afric. J. Biotechnol.*, 2:150-152
- Karmakar, M. and R.R. Ray, 2010. Extra cellular endoglucanase production by rhizopus oryzae in solid and liquid state fermentation of agro wastes. *Asian J. Biotechnol.*, 2: 27-36
- Ray, R.R. and R. Chakraverty, 1998. Extra cellular β -amylase from *Syncephalastrum racemosum*. *Mycol. Res.*, 102 : 1563-1567
- Bernfeld, P., 1955. Amylases α and β . *Method Enzymol.*, 1: 149-158
- Murashima, K., Kosugi, A., and Roy, H.D., 2003. Synergistic Effects of Cellulosomal Xylanase and Cellulases from *Clostridium cellulovorans* on Plant Cell Wall Degradation. *J. Bacteriol.*, 185:1518-1524.
- Omojasola, P.F., O.P. Jilani and S.A. Ibiyemi, 2008. Cellulase production by some fungi cultured on pineapple waste. *Nature Sci.*, 6: 64-69.
- Murashima, K., Nishimura, T., Nakamura, Y., Koga, J., Moriya, T., Sumida, N., Yaguchi, T., and Kono, T., 2002, "Purification and characterization of new endo-1,4-beta-D-glucanases from *Rhizopus oryzae*," *Enzyme and Microbial Technology*, 30(3):319-326
- Enari, T.M. and P. Markenán, 1977. Production of cellulolytic enzymes by fungi. *Adv. Biochem. Eng.*, 5: 1-23
- P. B. Acharya, D. K. Acharya and H. A. Modi, 2008, Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate, *Afric. J. Biotechnol.*, 7 (22):4147-4152
- Kang, S.W., Park, Y.S., Lee, J.S., Hong, S.I. and Kim, S.W., 2004, Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. *Bioresource Technol.*, 91:153-156.
- Nochure, S.V., Roberts, M.F. and Demain, A.I., 1993, True cellulases production by *Clostridium thermocellum* grown on different carbon sources, *Biotech. Letters*, 15:641-646
- Fan, L.T., Gharpuray, M.M., Lee, Y.H., 1987, Cellulose hydrolysis. Berlin, Germany. SpringerVerlag, 3:1-68
- Immanuel, G., C.M.A. Bhagavath, P.I. Raj, P. Esakkraj and A. Palavesam, 2007. Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigatus* fermented in coir waste and sawdust. *Int. J. Microbiol.*, Vol. 3
- Falcon, M.A., Rodriguez, A., Carnicero, A., 1995, Isolation of microorganism with lignin transformation potential from soil of Tenerife Island. *Soil Bio. Biochem.*, 27(2):121-126.
- McCarthy, A. J., 1987, Lignocellulose degrading Actinomycetes. *FEMS Microbiology*, 46:145-163
- Milala, M.A., B.B. Shehu, H. Zanna and V.O. Omosioda, 2009. Degradation of Agro wastes by cellulase from *Aspergillus candidus*. *Asian. J. Biotechnol.*, 1: 51-56
- Person, I., F. Tjerneld and B. Hahn-Hagerdal, 1991. Fungal cellulolytic enzyme production: A review. *Proc. Biochem.*, 26: 65-74
- Kocher, G.S., K.L. Kalra and G. Banta, 2008. Optimization of cellulase production by submerged fermentation of rice straw by *Trichoderma harzianum* Rut-C 8230. *The Internet J. Microbiol.*, Vol. 5
- Singh, A., N. Singh and N.R. Bishnoi, 2009. Production of Cellulases by *Aspergillus heteromorphus* from Wheat Straw under submerged fermentation. *Int. J. Environ. Sci. Eng.*, 1: 23-26
- Rezende MI, Barbosa A De M, Vasconcelos AFD and Endo AS. 2002. Xylanase Production by *Trichoderma Harzianum* Rifai by Solid State Fermentation on Sugarcane Bagasse. *Braz. J. Microbiol.* 33: 67-72.

Omosajola, P.F. and O.P. Jilani, 2008. Cellulase production by *Trichoderma longi*, *Aspergillus niger*, *Saccharomyces cerevisiae* cultured on Waste Materials from orange. Pak. J. Biol. Sci., 11: 2382-2388

Noriho, K., Yuichi, M., Misa, H., Kazunori, N., Mamiko, N., Masahiro, G. and Harou, T. 2008. Enzymatic in situ saccharification of cellulose in aqueous ionic liquid media. *Biotechnol lett.*, 30 (6), 1037-40.