JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 803-808

Potential *Aspergillus niger* Strain for the Production of Phenolics from a Novel Substrate Palm Oil Mill Effluent

Parveen Jamal[1,2]*, Zulkarnain Mohamed Idris[1,3], Md Zahangir Alam[1], Zaki Zainuddin[1] and Abdullah Al-Mamun[1]

[1]Bioenvironmental Engineering Research Centre (BERC), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, P.O. Box 10, 50728, Kuala Lumpur, Malaysia.
[2] International Institute for Halal Research and Training (INHART), International Islamic University Malaysia, Malaysia.
[3]School of Bioprocess Engineering, Kompleks Pusat Pengajian Jejawi 3, University Malaysia Perlis, 02600 Arau, Perlis, Malaysia.

(Received: 08 January 2014; accepted: 24 March 2014)

Palm oil mill effluent (POME) is one of the major sources of aquatic pollution in Malaysia. Phenolics production using organic residue of POME and potential *Aspergillus niger* strains would be a novel approach to solve this problem. Thus screening was conducted for seven locally isolated *Aspergillus niger* strains namely IBS-101ZA, IBO-103MNB, IBS-103ZA, IBS-104ZA, IBS-105ZA, IBS-106ZA and IBO-107MNB at 24, 48, 72, 96 and 120 hours of fermentation and at fixed process conditions of 150 rpm of agitation rate, 2% (v/v) of inoculum size, 4% (w/v) of solid concentration and at the temperature of 30 ± 2 ºC. The substrate was added with co-substrates of 2% (w/v) of wheat flour, 2% (w/v) of glucose and 2% (w/v) of ammonium nitrate to enhance the growth of the strains. The selection of the potential fungal strain was determined on the basis of growth rate and highest total phenolic content. No significant difference ($p > 0.05$) in radial growth rates between *Aspergillus niger* strains was observed. The distribution of phenolic compounds produced by different *Aspergillus niger* strains showed that the highest total phenolic content was obtained by IBS-103ZA strain with 639.9±4.19 GAE mg/L after 72 hours of fermentation.

Key words*:* Palm oil mill effluent; *Aspergillus niger*; Phenolics; Fermentation.

Palm oil mill effluent (hereinafter POME) is a waste generated from palm oil processing plants. This waste is generated in large volumes and contributes to a major problem to the palm oil processing mill's waste stream. Normally 0.5-0.7 tons of POME is generated per ton of fresh fruit bunch processed from palm oil mill[1]. Palm oil mill effluent has been identified as one of the major sources of aquatic pollution in Malaysia. Several techniques have been developed in order to treat the highly biodegradable palm oil mill effluent. Ponding, anaerobic, and aeration systems most adopted treatment processes practiced by more than 85% of the palm oil mills in the country[2]. The drawbacks of these systems are the requirement of a large area and the system suffers from the control and maintenance problems and biogas generation caused in air pollution. The production of this effluent always contributes to environmental problems such as the generation of methane during its anaerobic treatment and production of high chemical oxygen demand (COD). A substantially different approach to this pollution problem can be to use such organic residue as a potential source for manufacturing added-value phenolic compounds with promising

* To whom all correspondence should be addressed.
Tel.: +603-61964000/4558;
E-mail: jparveen@iium.edu.my

applications in the food and pharmaceutical industries. POME is found to have potential to be a new source for phenolic antioxidants[3].

Dietry plant phenolic compounds have been described to exert a variety of biological actions such as free radical scavenging, metal chelation, modulation of enzymatic activity[4]. They received particular attention in the past 10 years due to their putative role in prevention of several human diseases, particularly atherosclerosis and cancer[5]. Phenolic compounds are valuable sources of natural antioxidants, object of great interest for pharmaceutical, cosmetic and food industries. Antioxidant activities of polyphenols exert beneficial pharmacological effect on neurological disorder on the basis of in vitro observations[6]. Research suggests that consumption of antioxidant-rich foods reduces damage to cells and biochemicals from free radicals. BHT a synthetic antioxidant is toxic. The replacement of synthetic antioxidants by natural ones may have benefits due to health implications.

Among microorganisms known for their ability to produce plant cell wall degrading enzymes, fungi are the most interesting group[7]. The genus of *Aspergillus* is a group of filamentous fungi with large number of species. Black aspergilli such as *Aspergillus niger* and *Aspergillus tubingensis* are the most important for industrial applications due to their good fermentation capabilities, high levels of protein secretion, wide range of cell wall degrading as well as phenolic acid esterase enzymes[8].

The present investigation is an effort to develop an environmentally sound and cost effective fermentation process by introducing a new potential strain of *Aspergillus niger* isolated locally for phenolic compounds production from POME. For this purpose, seven strains of *Aspergillus niger* namely IBS-101ZA, IBO-103MNB, IBS-103ZA, IBS-104ZA, IBS-105ZA, IBS-106ZA and IBO-107MNB have been screened and the selection of potential strain is on the basis of maximum total phenolic content.

MATERIALS AND METHODS

Raw material and fungal strain

POME was collected from Seri Ulu Langat Palm Oil Mill Sdn. Bhd of Dengkil, Malaysia and was stored at 4°C in the laboratory cold room for further use. The POME sample 2-3% w/v of total suspended solid (TSS) was prepared by removing or adding pure free water from the original sample on the basis of material balances[9]. Strains used were IBS-101ZA, IBO-103MNB, IBS-103ZA, IBS-104ZA, IBS-105ZA, IBS-106ZA and IBO-107MNB of *Aspergillus niger* from series experiments of isolation and purification conducted by the previous study[10,11]. The fungal strains were subcultured on PDA media at 32ºC for further use.

Inoculum preparation

Inoculum preparation (spore suspension) was done according to the suggested method[12]. Each strain of the *Aspergillus niger* used as previously described was first cultured on four PDA plates for 7 days in an incubator at 32ºC. About 100 ml of distilled water was used to for suspension inoculum. A bend glass rod was used to wash the spores in the culture plates with sterilized water followed by filtration using Whatman #1 filter paper to remove the mycelia from spore suspension. The filtrate was then used as inoculum after measuring the strength (10^6spores/ml) using hemocytometer. All flasks, funnel, filter paper, distilled water were sterilized prior to use.

Media and process conditions

The substrate used in the study was POME of 2% (w/v) of total suspended solid and was added with co-substrates of 2% (w/v) of wheat flour, 2% (w/v) of glucose and 2% (w/v) of ammonium nitrate to enhance the strains growth. . 50 mL of sample was taken in 100 mL Erlenmeyer flasks and autoclaved at 121 ºC for 30 minutes. The sterile medium was cooled to ambient temperature and inoculated with 2% (v/v) spore suspension of *Aspergillus niger* strain for example IBS-101ZA, incubated at 30 ºC for a period of 24, 48, 72, 96 and 120 hours and agitated at 150 rpm. For each incubation period, a separate set of flasks with medium was in used triplicate. This procedure was repeated for the rest of *Aspergillus niger* strains IBS-101ZA, IBO-103MNB, IBS-103ZA, IBS-104ZA, IBS-105ZA, IBS-106ZA and IBO-107MNB respectively.

Sample preparation for analysis

The sample preparation for analysis of total phenolic content was described previously[13]. The inoculated POME samples of varying incubation periods were filtered. The filtrate

samples were acidified to pH 2 with 4 N HCl and washed with n-hexane in order to remove the lipid fraction. 10 ml of POME samples were mixed with 15 ml of n-hexane, the mixture were vigorously shaken and centrifuged for 5 min at 3000 rpm. The phases were separated and the washing was repeated successively two times. The water extract phase was collected. The total phenolic acids content in the samples were determined by Folin-Ciocalteu reagent and expressed as gallic acid equivalents (mg gallic acid /l POME sample).

Growth rate assessment of the fungal strains

Actively growing 7-day-old colonies of *Aspergillus niger* strains on PDA were used to prepare a spore suspension of 1 x 10^6 spores/ml. Petri dishes with 4% (w/v) POME-PDA modified agar (PMA) were inoculated with 5 µl of the spore suspension and incubated at 32 °C for a period of 24, 48, 72, 96 and 120 hours. The temporal mycelial extensions of treatments and replicates were measured in two directions at right angles to each other. Measurements were recorded every 24 hours during the growth until the Petri dishes were completely colonized. A linear regression of the data was performed in order to calculate the growth rate.

Determination of total phenolic content

The total phenolic content was determined using Folin-Ciocalteau assay based on the suggested method[14] with slight modification. In 15 ml Falcon tube, 2370 ìl of distilled water, 30 ìl of sample and 150 ìl Folin-Ciocalteu reagent were added and vortexed. After 1 minute, 450 ìL of aqueous sodium carbonate (20%) was added, and then the mixture was vortexed and allowed to stand at 40 °C for 30 minutes before reading the absorbance. The absorbance was read at 750 nm and total phenolic acid concentration was calculated by using gallic acid standard curve (Fig. 1), which was prepared by using standards of different concentration (from 0 to 1 mg/ml of gallic acid). The total phenolic content was expressed as mg of gallic acid equivalent per liter (GAE mg/l). For the blank, distilled water was used for the background subtraction. All measurements were done in triplicates. The absorbance was taken at 750 nm. All measurements were carried out in triplicate. The total phenolic acids concentration was calculated from the calibration curve (Fig. 1), using gallic acid as the standard and the results were expressed as mg/l of gallic acid equivalents (GAE mg/l).

RESULTS

Growth rate assessment of fungal strains

In this study, seven strains of *Aspergillus niger* from laboratory stocks strain, isolated previously from STP sludge and orange peel, were evaluated for their ability to grow on POME-PDA modified agar (PMA) which was also one of the criteria used for strains selection. Radial growth measurements were recorded every 24 hours from the edge of the initial inoculum until the extreme area of fungi mycelia development, following the four segments formed by the two perpendicular lines (Fig. 2). Mean values of the colony radius length for each strain with time are presented in Table 1. From the results show in Table 2, no significant difference (p >0.05) in radial growth rates between *Aspergillus niger* strains was observed. Strains of IBS-103ZA, IBS-106ZA and IBO-107MNB showed the highest rates (0.039 cm/hr each), followed by IBS-101ZA, IBS-102MNB and IBS-105ZA (0.038 cm/hr each). The IBS-104ZA strain showed the lowest rate with 0.037 cm/hr.

Total phenolic content

Seven strains of *Aspergillus niger* from laboratory stock strains isolated previously from STP sludge and orange peel were studied to evaluate their potential for phenolics production from POME by liquid state fermentation. Selection of a potential strain was determined on the basis of maximum phenolic contents (Table 3). The total phenolic content within the period of 120 hours fermentation is shown in Table 3. At 0 hour of

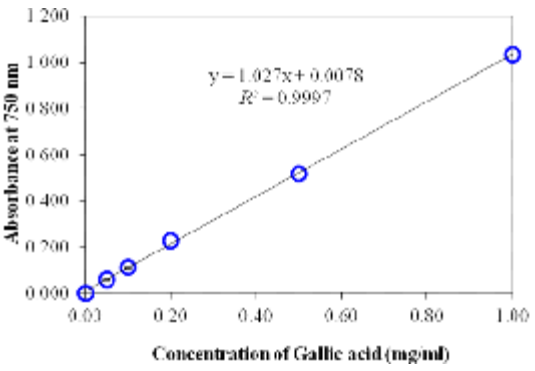

Fig. 1. Gallic acid standard curve

fermentation, the total phenolic content in POME was 1008.96±2.45 GAE mg/l. Within 24 hours of fermentation, the total phenolic content was decreased drastically and this trend was observed by all strains. The next 48 hours of fermentation showed an increasing trend in total phenolic

Table 1. Mycelial growth of different locally isolated strains on POME-PDA modified agar (PMA)

Strains	Colonial radius (cm)				
	24 hours	48 hours	72 hours	96 hours	120 hours
[s]IBS-101ZA	0.9±0.2[b]	1.9±0.2[a]	3.1±0.3[a]	4.1±0.2[a]	4.3±0.3[a]
[o]IBO-103MNB	0.9±0.2[b]	1.9±0.1[a]	3.1±0.4[a]	4.1±0.3[a]	4.3±0.2[a]
[s]IBS-103ZA	0.6±0.1[ab]	1.7±0.3[a]	3.0±0.2[a]	4.0±0.1[a]	4.3±0.2[a]
[s]IBS-104ZA	0.8±0.2[ab]	1.9±0.2[a]	3.1±0.1[a]	4.0±0.1[a]	4.2±0.4[a]
[s]IBS-105ZA	0.6±0.1[ab]	1.7±0.4[a]	3.1±0.3[a]	3.9±0.2[a]	4.3±0.2[a]
[s]IBS-106ZA	0.6±0.2[ab]	1.8±0.3[a]	3.1±0.2[a]	4.1±0.3[a]	4.3±0.3[a]
[o]IBO-107MNB	0.5±0.1[a]	1.7±0.2[a]	2.9±0.4[a]	4.0±0.2[a]	4.3±0.3[a]

[o, s]Laboratory stock strains *Aspergillus niger* isolated previously from orange peel (Bari et al., 2009)[11] and STP sludge (Jamal et al., 2005)[10] respectively. Values are mean ±SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

Table 2. Growth rate of different locally isolated strains determined by linear regression analysis

Strains	Linear regression equation	R^2	Growth rate (K_r, cm/hr)
[s]IBS-101ZA	y= 0.038x + 0.069	0.975	0.038±0.009[a]
[o]IBO-103MNB	y= 0.038x + 0.069	0.975	0.038±0.013[a]
[s]IBS-103ZA	y= 0.039x - 0.100	0.973	0.039±0.008[a]
[s]IBS-104ZA	y= 0.037x + 0.059	0.970	0.037±0.013[a]
[s]IBS-105ZA	y= 0.038x - 0.066	0.974	0.038±0.012[a]
[s]IBS-106ZA	y= 0.039x - 0.061	0.970	0.039±0.012[a]
[o]IBO-107MNB	y= 0.039x - 0.138	0.976	0.039±0.021[a]

[o, s]Laboratory stock strains *Aspergillus niger* isolated previously from orange peel (Bari et al., 2009) and STP sludge (Jamal et al., 2005) respectively. Values are mean ±SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

Table 3. Phenolics production from POME with different locally isolated strains

Strains	Colonial radius (cm)				
	24 hours	48 hours	72 hours	96 hours	120 hours
[s]IBS-101ZA	560.00±5.02[a]	614.61±1.61[cd]	634.08±4.09[cd]	629.21±2.90[c]	613.63±5.65[d]
[o]IBO-103MNB	584.42±3.97[b]	587.34±2.73[a]	624.34±5.79[b]	607.79±7.40[b]	563.97±4.33[a]
[s]IBS-103ZA	607.79±5.88[c]	625.32±7.93[e]	639.92±4.19[d]	629.21±4.44[c]	600.00±7.27[c]
[s]IBS-104ZA	617.53±7.75[cd]	599.03±6.14[b]	632.13±2.12[bcd]	612.66±3.55[b]	598.05±3.34[c]
[s]IBS-105ZA	609.74±2.69[c]	623.37±3.80[de]	629.21±1.80[bc]	604.87±5.29[b]	584.42±7.10[b]
[s]IBS-106ZA	607.79±5.72[c]	612.66±7.61[c]	614.61±4.63[a]	584.42±3.89[a]	561.05±7.54[a]
[o]IBO-107MNB	620.45±3.69[d]	600.00±3.73[b]	615.58±5.63[a]	608.76±7.62[b]	589.29±4.93[bc]

[o, s]Laboratory stock strains *Aspergillus niger* isolated previously from orange peel (Bari et al., 2009) and STP sludge (Jamal et al., 2005) respectively. Values were taken as mean ±SD followed by the same letter. These values were not significantly different ($p > 0.05$) within a column. At 0 hour, the total phenolic content was 1009.0±2.5 GAE mg/l.

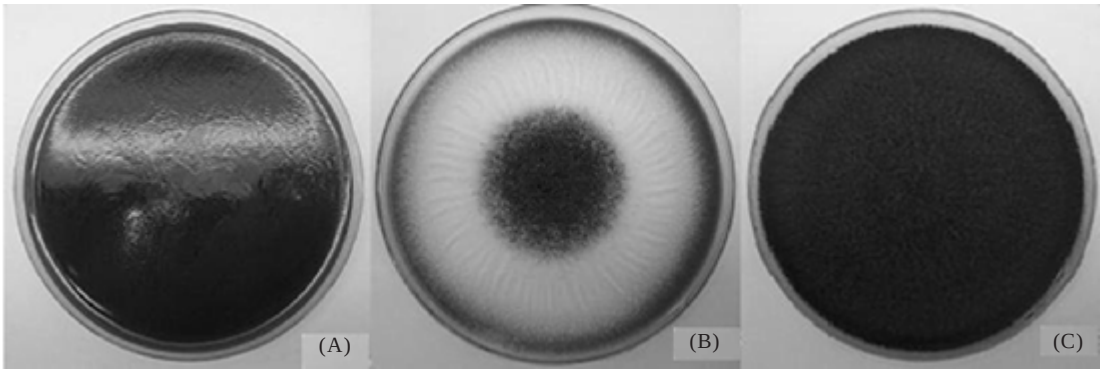

Fig. 2. (a) POME-PDA-modified agar (PMA) used for fungus growth rate assessment (b) Black arrow indicates the edge of fungi radial growth. Numbers 1, 2, 3 and 4 correspond to the four segments used for growth measurements (c) The PMA was fully covered with *Aspergillus niger* IBS-101ZA strain after 120 hours of incubation at 32 °C.

content by each strain. Highest total phenolic content (639.92±4.19 GAE mg/l) was obtained at 72 hours of fermentation by IBS-103ZA strain followed by IBS-101ZA (634.08±4.09 GAE mg/l), IBS-104ZA (632.13±2.12 GAE mg/l), IBS-105ZA (629.21±1.80 GAE mg/l), IBO-103MNB (624.34±5.79 GAE mg/l), IBO-107MNB (615.58±5.63 GAE mg/l) and IBS-106ZA (614.61±4.63 GAE mg/l). The total phenolic content started to decrease after 72 hours of fermentation and this trend was observed by all strains.

DISCUSSION

Organic compounds like phenolics have toxic effects that limit the biological treatment of wastes, because they can be growth-rate inhibitory even to the species that have the metabolic capability of using it as a substrate for growth[15]. It has been reported that POME contains phenolic acids and flavonoids that may inhibit the growth development in microorganisms[16]. The performance of each strain on PMA was assessed by determining the radial growth rates of the culture. The radial growth rates, K_r of the strains were derived from linear relationship of the colony radius length with time. A colony K_r value can be considered as an indication of the speed of microbial growth and its invasive capacity. The K_r is proportional to the maximum specific growth rate and reported that the linear growth rate at which fungal colonies grow on agar medium is a good approximation of biomass increased in liquid culture[17].

Selection of a potential strain was determined on the basis of maximum phenolic contents. An increase of total phenolic content in the fermented POME could be explained in the same way as reported by Bhanja and co-researchers[18]. An increase of total phenolic content during the fermentation of wheat koji and olive oil by-product was attributed to the catalytic action of the carbohydrate hydrolyzing enzymes (á-amylase, â-glucosidase and xylanase) and also cinnamoyl esterases produced by the *Aspergillus niger*. Most of phenolic compounds that are found in the plant exist as conjugated to sugars in the form of glycosides. *â*-glucosidase is reported to be capable of hydrolyzing phenolic phucoside and releasing extractable free phenolic compound such as aglycones[19]. Esterase, on the other hand, is responsible for releasing phenolic acids from plant cell wall. A decrease in the total phenolic content with fermentation period shows that the phenolic compounds were degraded and utilized by *Aspergillus niger* as carbon source when the nutrients are depleted as reported by other researchers as well[20].

CONCLUSION

In conclusion, the proposed materials and methods in this study have fulfilled the objective through utilization of POME in gaining valuable phenolics that might have potential to be natural antioxidants. Since Malaysia is one of the major palm oil producers in the world, this method will be beneficial in providing a more effective way in managing palm oil industry waste with less cost and also contribute to the economy of the country. The importance and complexity of antioxidants in biology is reflected in a medical literature and

antioxidant activities of phenolic compounds have been suggested to exert beneficial pharmacological effects on neurological disorders on the basis of *in vitro* observations. Incoherence with that, the search for cheap, renewable and abundant sources of antioxidant compounds is attracting worldwide interest, hence, this research might be one of the significant step in utilizing zero value raw material for production of value added phenolic antioxidants. In this study, the extraction of phenolic acids from palm oil mill effluent was done by using *Aspergillus niger* strains which has gave promising results instead of using chemical means. Further optimization studies are recommended to give optimum results with cost effective process.

ACKNOWLEDGEMENTS

The research was supported by a research grant IFRGS 0701-14 approved by the Ministry of Higher Education (MOHE), Malaysia. The authors are grateful to MOHE, Research Management Centre and Department of Biotechnology Engineering, IIUM for supporting and providing the laboratories facilities.

REFERENCES

1. Yacob, S., Hassan, M.A., Shirai, Y., Wakisaka, M., Subash, S. Baseline study of methane emission from open digesting tanks of palm oil mill effluent treatment, *Chemosphere,* 2005; 59: 1575-81. Ma A.N., Ong A.S.H. Pollution control in palm oil mills in Malaysia. *J. Am. Oil Chem. Soc.*, 1985; **62**: 261-6.
2. Jamal P., Alam M.Z., Suhani F. Biophenols from agro-industrial wastes. *Med. J. Malaysia.*, 2008; **63**: 107-8.
3. Editorial, Dietry flavonoids and risk of coronary heart disease, *Nutr. Re.,* 1994; **5**: 59-61.
4. Nardini, M., Ghiselli A. Determination of free and bound phenolic acids in beer. *Food Chem.*, 2002; **84**: 137-143.
5. Parr, A.J., Bolwell, G.P. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile, *J. Sci. Food Agric.*, 2000; **80**: 985-1012.
6. Maria, H., Biely, P., Vrsanska, M. Cellulose and xylan degrading enzymes of *Aspergillus terrus* and *Aspergillus niger*. *Enzyme Microb. Technol.*, 1989; **11**: 610-6.
7. De Vries, R.P., Visser J. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol.Mol. Biol. Re.*, 2001:**65**: 497-552
8. APHA. Standard Methods for the Examination of Water and Wastewater, 17th edn., American Public Health Association, Washington, D.C., 1989.
9. Jamal, P., Alam, M.Z., Salleh, M.R.M., Nadzir M.M. Screening of microorganisms for citric acid production from palm oil mill effluent, *Biotechnol*., 2005; **4**: 275-8.
10. Bari, N. M., Alam, M. Z., Muyibi, S. A., Jamal, P., Mamun, A. A. Improvement of production of citric acid from oil palm empty fruit bunches: optimization of media by statistical experimental designs. *Bioresour. Technol.*, 2009; **100**(12): 3113-3120.
11. Alam, M.Z., Jamal, P., Nadzir M.M. Bioconversion of palm oil mill effluent for citric acid production: Statistical optimization of fermentation media and time by central composite design. *World J. Microbiol. Biotechnol.*, 2008; **24**: 1177-85.
12. Marco, E.D., Savarese, M., Paduano, A., Sacchi R. Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewater. *Food Chem.*, 2006; **104**: 858-67
13. Waterman, P.G., Mole S. Analysis of phenolics plant metabolites, Oxford: Blackwell Scientific Publication, 1994; pp 83-91.
14. Evans, W.C. The microbiological degradation of aromatic compounds. *J. Gen. Microbiol.*, 1963; **32**: 177-84.
15. Lin, Y.T., Labbe, R.G., Shetty K. Inhibition of *Vibrio parahaemolyticus* in seafood systems using oregano and cranberry phytochemical synergies and lactic acid. *Innov. Food Sci. Emerg. Technol*., 2005; **6**: 453–58.
16. Trinci, P.J. Influence of the width of the peripheral growth zone on the radial growth rate of fungal colonies on solid media. *J. Gen. Microbiol.*, 1971; **67**: 325-44.
17. Bhanja, T., Kumari, A., Banerjee R. Enrichment of phenolics and free radical scavenging property of wheat koji prepared with two filamentous fungi. *Bioresour. Technol.*, 2009; **100**: 2861-66.
18. Lee, I.H., Chou C.C. Distribution profiles of isoflavone isomers in black bean kojis prepared with various filamentous fungi, *J. Agric. Food Chem.*, 2006; **54**: 1309–14.
19. Shyamala, H., Kavitha, S., Varadaraj, M.C., Muralikrishna, G. Degradation of cereal bran polysaccharide-phenolic acid complexes by *Aspergillus niger* CFR 1105. *Food Chem.*, 2004; **96**: 14-9.