JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2012. Vol. **6**(4), p. 1663-1669

Quantification of Aflatoxin B1 Produced by *Aspergillus flavus* MTCC 2798 in Rice Straw under Solid-State Fermentation

Arijit Das[1]*, J. Angayarkanni[2], Sourav Bhattacharya[3] and M. Palaniswamy[1]

[1]Department of Microbiology, Karpagam University, Coimbatore - 641 021, India.
[2]Department of Microbial Biotechnology, Bharathiar University, Coimbatore - 641 046, India.
[3]Department of Microbiology, Genohelix Biolabs, A Division of Centre for Advanced Studies in Biosciences, Jain University, 127/2, Bull Temple Road, Chamarajpet, Bangalore - 560 019, India.

(Received: 03 May 2012; accepted: 10 June 2012)

Aflatoxin is a toxic metabolite produced by some species of *Aspergillus* in food grains when stored under warm and damp conditions. Aflatoxin B1 has been found to induce carcinogenic, mutagenic, teratogenic and immunosuppressive effects. Contamination of agricultural produce with this toxin poses serious threat to the food and feed industry. Consumption of moldy straw may lead to aflatoxicosis in cattle. The present study focuses on the potential of rice straw to support aflatoxin B1 production from *Aspergillus flavus* MTCC 2798 and quantification of the produced toxin during solid-state fermentation. The aflatoxin B1 production was detected by thin layer chromatography and its level was quantified by high- performance liquid chromatography. 1% (w/w) glycine supported maximum production of aflatoxin (46.62 μg/ml). The highest level of aflatoxin B1 was synthesized at pH 4.5 and at 30°C. Significant toxin production was recorded after 30 days of incubation under aerobic condition. It may be concluded that rice straw has the potential to support good production of aflatoxin when incubated under ambient conditions. Therefore, proper storage and regular inspection of its quality should be practised to ensure the safety of dairy animals.

Key words: *Aspergillus flavus,* Aflatoxin B1, rice straw, Solid-state fermentation.

Mycotoxins are low molecular weight secondary metabolites produced by certain filamentous fungi[1]. The mycotoxins of the greatest significance in foods and feeds are aflatoxins which have hepatotoxic, carcinogenic, mutagenic, teratogenic and immunosuppressive effects on the health of humans and animals even in low concentrations[2]. Aflatoxins are a group of highly toxic difuranocoumarin derivatives produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*[3]. In general, aflatoxin exposure is more likely to occur in parts of the world where poor methods of food handling and storage are common, and where few regulations exist to protect the exposed populations[4].

* To whom all correspondence should be addressed.
Mob.: +91-9886919207;
E-mail: jit2007das@rediffmail.com

Aflatoxin contamination leads to increased mortality in farm animals and thus significantly lowers the food value of grains as an animal feed[5]. Acute aflatoxicosis may result in death, whereas, chronic aflatoxicosis results in cancer and immune suppression. Exposure to aflatoxins in the diet may be linked to the development of primary hepatocellular carcinoma, particularly in individuals already exposed to hepatitis B[6].

Many substrates support growth and aflatoxin production by aflatoxigenic molds. Different environmental conditions, as well as agronomic, feed storage and feed handling practices favor the mold growth and aflatoxin formation[7]. Rice straw is widely used as cattle feed and once contaminated, may act as a potent cause of aflatoxicosis in cattle. Moreover, animals consuming aflatoxin contaminated feeds can

produce meat and milk that contain toxic residues and biotransformation products. A previous study reported that about 50% of the *A. flavus* isolates produced B1 and B2 aflatoxins in rice fodder[8]. Thus, aflatoxins in cattle feed can be metabolized by cows into aflatoxin M1, which is then secreted in milk. Therefore, the present study focuses on various factors influencing aflatoxin B1 production from *Aspergillus flavus* MTCC 2798 in rice straw under solid-state fermentation and quantification of the produced toxin.

MATERIALS AND METHODS

The present study was conducted during the period from 18.06.2011 to 27.04.2012 at the Department of Microbiology, Genohelix Biolabs, Chamarajpet, Bangalore, India.

Chemicals and reagents

All the media used during the course of the study were obtained from Himedia Laboratories Pvt. Limited (Mumbai, India). The analytical grade chemicals and reagents were purchased from Qualigens Fine Chemicals (Mumbai, India) and Nice Chemicals (Kochi, India). Pure distilled water was obtained with a Milli-Q system (Millipore, Tokyo, Japan). HPLC grade Aflatoxin B1 standard was procured from Sigma-Aldrich Co. (USA).

Source of fungal strain

Aflatoxigenic strain of *Aspergillus flavus* MTCC 2798 was obtained from Microbial Type Culture Collection, Chandigarh, India and the culture was maintained on potato dextrose agar slant at 4°C until use.

Detection of aflatoxin production

Spore suspension of *A. flavus* MTCC 2798 was aseptically inoculated into sterile Czapek Dox broth and incubated under static condition at 28°C and also with agitation at 130 rpm for 15 days. The mold broth was filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). Equal volume of chloroform was added to the filtrate and the contents were shaken for 30 min. The chloroform fraction was collected using a separating funnel. The presence of aflatoxin was detected using thin layer chromatography (TLC) consisting of silica gel plates (Merck Ltd., Mumbai, India) as the stationary phase and a mixture of ether-methanol-water (96: 3: 1, v/v) as the mobile phase. Aflatoxin production was confirmed by the detection of its characteristic fluorescence under UV light[9].

Extraction and purification of aflatoxin

The extraction, purification and derivatization of aflatoxin were performed in accordance with the standard protocols using methylene chloride as the solvent[10]. The extracted aflatoxin was purified by silica gel column chromatography and derivatized using hexane and trifluoroacetic acid. 1 ml of water-acetonitrile (9:1, v/v) was added to the derivative and vortexed for 30 s. After the phases separated, 20 µl of the lower aqueous layer was injected into the HPLC column.

Quantification of aflatoxin using High-Performance Liquid Chromatography (HPLC)

The HPLC analysis of aflatoxin was carried out at IADFAC Laboratories Pvt. Ltd., Bangalore, India. The working standard of aflatoxin B1 was prepared in acetonitrile-water (1:9, v/v), with concentration of 10 µg/ml. The HPLC system used was Shimadzu LC-10 AT *VP* with fluorescence detection using a RF-10A_{XI} detector. The sample (20 µl) was injected under the following conditions: a mobile phase of acetonitrile-methanol-water (300:300:600, v/v); a flow rate of 1 ml min^{-1}; an excitation wavelength of 360 nm and an emission at 440 nm; a C-18 Phenomenex column of 5 µm (250 x 4.5 mm); a total run time of 30 min. The post-column reagent pump flow rate was set at 0.15 ml min^{-1} under ambient temperature. The limit of quantification for aflatoxin B1 was 0.1 ppb.

The concentration of aflatoxin B1 stock standard solution (about 1 mg/100 ml) in a mixture of benzene and acetonitrile (98:2, v/v) was determined by UV spectroscopy according to the following equation:

Concentration of aflatoxin (µg/ml) = (A_{350} x M_w x 1000)/ε

where A_{350} = the absorbance of aflatoxin B1 at a wavelength of maximum absorption close to 350 nm, M_w = the molecular weight of aflatoxin B1 (312), ε = the molar absorptivity of aflatoxin B1 in benzene-acetonitrile solution (98:2, v/v) (19800).

Production of aflatoxin in rice straw under solid-state fermentation

Rice straw was procured from the local market in Bangalore city. The straw was cut into 1 cm pieces, washed several times with clean water

to get rid of visible dirt and kept for drying. This was utilized as the substrate (carbon source) for aflatoxin production. Two grams of cut straw were packed into a petri dish, moistened with 20 ml of nitrogen supplemented solution and autoclaved. Following inoculation with the spore suspension (10% v/w), the plates were incubated at 28°C for 15 days. Effects of different organic (yeast extract, beef extract, peptone, tryptone, soybean meal, glycine and urea) and inorganic nitrogen sources (ammonium nitrate, ammonium chloride and sodium nitrite) at varying percentages (0, 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 5, 7 and 10% w/w) were determined. The role of initial pH of the supplement solution was evaluated by adjusting it from 3 to 9 with a gradual increment of 0.5 pH unit. Production of aflatoxin was noted at initial incubation temperatures of 4, 25, 30, 37 and 42ºC. The influence of incubation time was determined after every 5 days for 30 days.

Statistical analysis

All the studies were conducted in triplicates and the data were analyzed using single factor analysis of variance (ANOVA). All the data are graphically presented as the mean ± standard deviation of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. p values<0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

Among the various mycotoxins produced by filamentous fungi, aflatoxins are primarily important as they pose serious threat to agriculture and dairy industries. Aflatoxins are toxic secondary metabolites produced by species of *Aspergillus* in cereals, nuts, oil-seeds and other food crops during both pre-harvest and storage conditions[11]. The four major aflatoxins are called B1, B2, G1 and G2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography. Aflatoxin B1 is the most potent natural carcinogen known owing to its hepatocarcinogenic effects and is usually the major aflatoxin produced by toxigenic strains[12]. The occurrence of aflatoxin B1 in animal feed, forage and agricultural produce may result in aflatoxicosis in cattle, eventually leading to its transmission in milk[13].

Rice straw is generally contaminated by mycotoxigenic strains of *Aspergillus, Penicillium, Fusarium, Alternaria, Stachybotrys* and *Cladosporium*[14]. This contamination may either happen in fields, during harvest or under storage conditions. In addition, the presence of 85.94-87.71% organic matter and 4.06-4.12% crude protein in the rice straw may render it suitable for aflatoxin production[15, 16].

Aflatoxigenic strain of *Aspergillus*

The aflatoxin B1 producing standard strain of *A. flavus* MTCC 2798 produced characteristic yellowish green powdery colonies on potato dextrose agar. Microscopic observation revealed the presence of chains of green spherical conidia arranged on globose vesicles at the end of erect conidiophores.

Detection of aflatoxin production

The characteristic bluish fluorescence observed in the TLC plates on exposure to UV light indicated the presence of aflatoxin B in the sample.

Quantification of aflatoxin using HPLC

The level of aflatoxin in the sample was quantified using HPLC with a fluorescence detector. The amounts of aflatoxin B1 produced by *A. flavus* MTCC 2798 were evaluated as 11.3 µg/ml and 8.53 µg/ml under static and shaking conditions, respectively. Increased production of aflatoxin under static condition might be attributed to the faster depletion of nutrients and oxygen content due to the production of a thick fungal mat over the broth surface. This would have resulted in a stationary phase faster than the shaking condition, thereby resulting in increased level of aflatoxin production. Earlier workers also reported that 35% of *A. flavus* produced amounts of aflatoxin B1 that ranged from 1-100 µg per 25 ml of yeast extract sucrose medium[17].

Factors affecting aflatoxin production in rice straw

Aflatoxin production by toxigenic *Aspergillus* species is affected by nutritional and environmental factors such as carbon and nitrogen sources, temperature, pH, stress factors, lipids and certain metal salts like Ca^{2+}[18]. A previous study reported the presence of *A. flavus* in 28% of the examined samples of rice straw[17]. Another recent study revealed highest level of aflatoxin production (6.52 µg/ml) when 2% (w/v) rice straw was incorporated into the production medium[19].

Addition of nitrogen supplements into the production medium often induces the metabolite production. Among the organic nitrogen sources, glycine yielded the highest aflatoxin production (53.54 µg/ml) followed by urea (46.02 µg/ml) and ammonium nitrate (44.49 µg/ml) (Fig. 1). This may be due to the rapid utilization of simple amino acid, simple amide and water soluble nitrate which favored the aflatoxin biosynthesis. The other organic nitrogen sources exhibited moderate level of aflatoxin production. On the contrary, sodium nitrite showed the lowest toxin synthesis (1.02 µg/ml). In connection to these results, when the percentage of glycine was varied, 1% (w/w) glycine favored the highest aflatoxin production (46.62 µg/ml) (Fig. 2). In contrast to our present findings, 0.5% (w/v) peptone supported maximum aflatoxin B1 synthesis in rice straw under submerged fermentation[19].

The effect of pH on aflatoxin biosynthesis is dependent on the composition of the growth media[20]. The pH of the plain rice straw was found to be between 7.4-7.5. To study the effect of initial pH on aflatoxin biosynthesis, the pH of the nitrogen supplemented solution was adjusted before moistening the rice straw. pH 4.5 was found to support the highest level of aflatoxin production (15 µg/ml) (Fig. 3). The present findings are in accordance with a previous report where aflatoxin production in *A. flavus* was maximal at pH 4.0 or lower[20]. Another study suggested the aflatoxin production from *Aspergillus* strains under acidified ammonium based media[21].

Environmental factor such as temperature

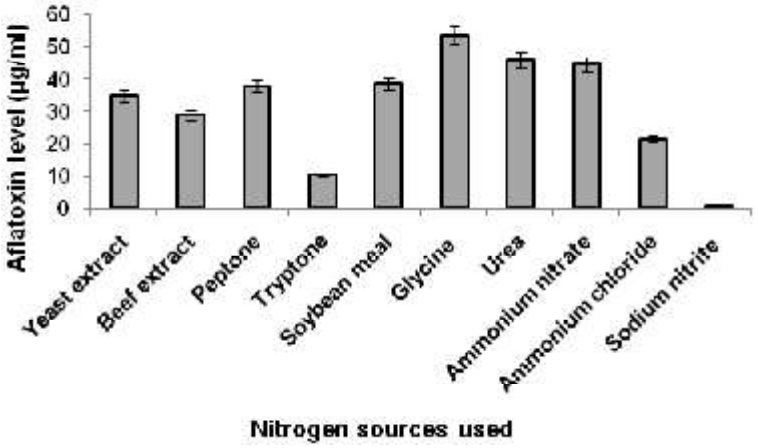

Fig. 1. Effect of different nitrogen supplements on aflatoxin production from *A. flavus* MTCC 2798 in SSF. Data represent mean ± S.D. (n=3); $P < 0.05$

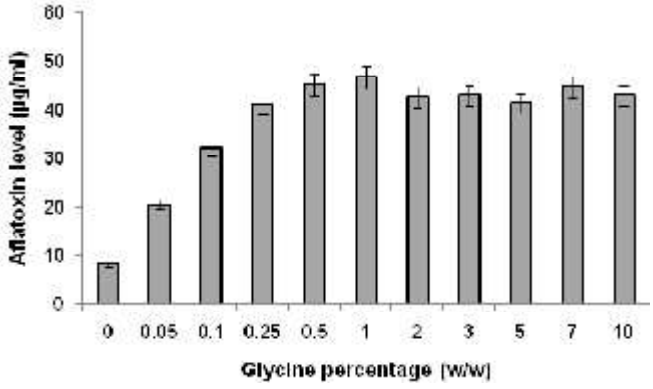

Fig. 2. Effect of percentages of nitrogen supplement on aflatoxin production from *A. flavus* MTCC 2798 in SSF. Data represent mean ± S.D. (n=3); $P < 0.05$

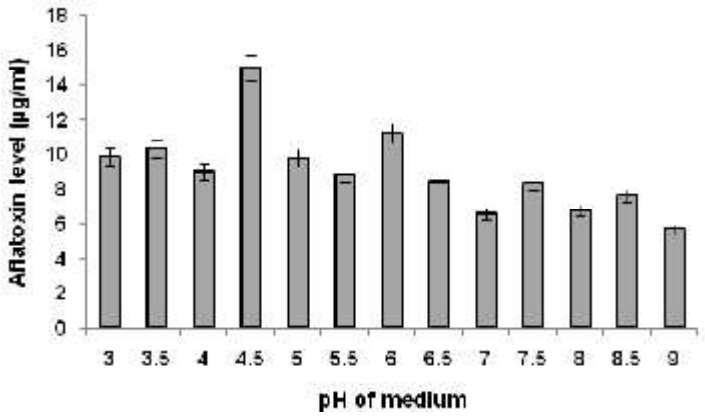

Fig. 3. Effect of initial pH on aflatoxin production from *A. flavus* MTCC 2798 in SSF. Data represent mean ± S.D. (n=3); $P < 0.05$

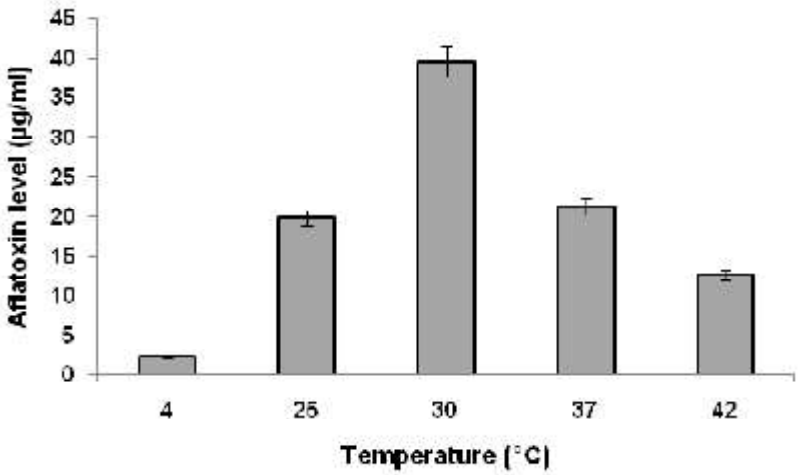

Fig. 4. Effect of initial temperatures on aflatoxin production from *A. flavus* MTCC 2798 in SSF. Data represent mean ± S.D. (n=3); $P < 0.05$

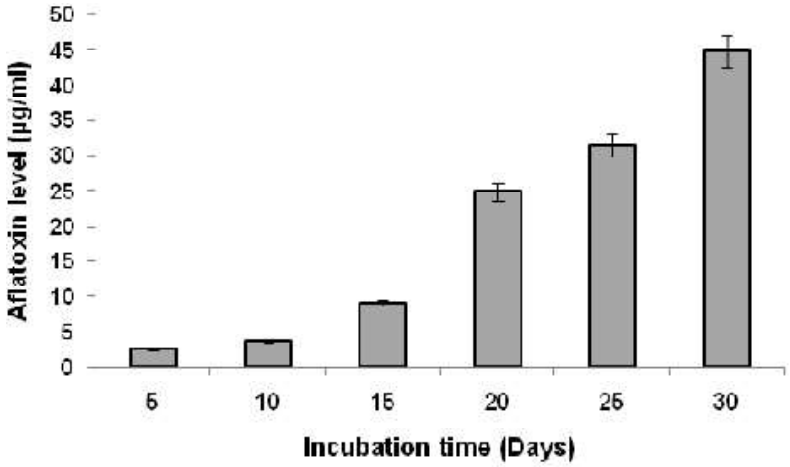

Fig. 5. Effect of incubation time on aflatoxin production from *A. flavus* MTCC 2798 in SSF. Data represent mean ± S.D. (n=3); $P < 0.05$

also greatly influences the growth of the mold and subsequently the production of aflatoxin[22]. Generally, aflatoxin is synthesized when food grains and forage are stored under conditions of high temperature and high relative humidity. In our study 30ºC demonstrated significant level of aflatoxin production (39.55 µg/ml) (Fig. 4). *A. flavus* is a mesophilic mold and thereby contaminate food crops with aflatoxin when they are stored at around 30ºC. An ambient temperature around 30ºC was also found to support good sporulation of the mold. Similarly, optimum temperatures for aflatoxin synthesis from *A. parasiticus* were detected as 27.8°C and 27.3°C at pH 5.9 and 5.5, respectively[23]. Our result is in agreement with the report suggesting that 30ºC is best suited for aflatoxin production from *A. flavus*[24]. Low temperature of 4ºC and relatively high temperature of 42ºC demonstrated decreased production of aflatoxin.

The production of any secondary metabolite usually occurs during the late stationary phase in the microbial growth cycle. In the present study, the level of aflatoxin was found to be enhanced with an increase in the incubation time. Extensive fungal growth was observed in rice straw substrate after 1 week of incubation. Considerable amount of toxin (44.79 µg/ml) was obtained after 30 days of incubation under aerobic condition (Fig. 5). A previous study also suggested a significant level of aflatoxin B1 production (287.5 ppb) after 20 days of incubation[17]. Solid-state fermentation supported good production of aflatoxin B1 in rice straw when this was used as a natural substrate. This may be attributed to the entrapment and retention of abundant moisture within hollow air spaces of the straw fibers facilitating the mold growth. Under the storage conditions the produced aflatoxin may also persist for a long period in the damp rice straw[16]. Our study emphasizes the fact that the methods for controlling mycotoxins are largely preventive which include good agricultural practice and sufficient drying of crops after harvest[25].

CONCLUSION

The study suggests that various cultural and environmental factors affect the moldiness and aflatoxin B1 synthesis in rice straw. Occurrence of aflatoxin at moderate to high level renders the toxin-contaminated rice straw unsuitable as cattle feed. Subsequently, rice straw needs to be properly harvested, handled and stored under hygienic conditions to reduce the aflatoxin level. Detection of the toxin level in cattle feed as per set standards is necessary to prevent incidences of aflatoxicosis in cattle.

ACKNOWLEDGMENTS

We wish to extend our sincere gratitude to the managements of Karpagam University, Jain University and Bharathiar University for their encouraging support. Our special thanks to Dr. R. Chenraj Jain, Chairman of Jain Group of Institutions, Dr. N. Sundararajan, Vice-Chancellor of Jain University and Dr. S. Sundara Rajan, Director of Genohelix Biolabs, A Division of Centre for Advanced Studies in Biosciences, Jain University, Bangalore, for providing us with the laboratory facilities required for this research work. We also wish to thank the entire supporting staff of the laboratory whose help has been invaluable for the successful completion of our research work.

REFERENCES

1. Bennett, J.W., Klich, M. Mycotoxins. *Clin. Microbiol. Rev.*, 2003; **16**(3): 497–516.
2. Klich, M.A., Tang, S., Denning, D.W. Aflatoxin and ochratoxin production by *Aspergillus* species under ex vivo conditions. *Mycopathologia*, 2009; **168**: 185-91.
3. Bhatnagar, D., Ehrlich, K.C., Cleveland, T.E. Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Appl. Microbiol. Biotechnol.*, 2003; **61**: 83-93.
4. Barrett, J. Mycotoxins: of molds and maladies. *Environ. Health Perspect.*, 2000; **108**: A20–A23.
5. Smith, J.E., Moss, M.O. Mycotoxins: Formation, analyses and significance. Chichester, United Kingdom: John Wiley and Sons, 1985.
6. Li, F.-Q., Yoshizawa, T., Kawamura, S., Luo, S.-Y., Li, Y.-W. Aflatoxins and fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. *J. Agric. Food Chem.*, 2001; **49**: 4122–6.
7. Whitlow, L.W., Hagler, Jr W.M. Mycotoxins in feeds. *Feedstuffs*, 2002; **1072**: 68-78.
8. Surekha, M., Saini, K., Reddy, V.K., Reddy, A.R., Reddy, S.M. Fungal succession in stored rice (*Oryza sativa* Linn.) fodder and mycotoxin

production. *Afr. J. Biotechnol.*, 2011; **10**(4): 550-5.
9. Hussaini, A.M., Timothy, A.G., Olufunmilayo, H.A., Ezekiel, A.S., Godwin, H.O. Fungi and some mycotoxins found in mouldy sorghum in Niger State, Nigeria. *World J. Agric. Sci.*, 2009; **5**(1): 5-17.
10. Association of Official Analytical Chemists International (AOAC). Official Methods of Analysis of AOAC International, 17th edn. Gaithersburg, MD: AOAC Int., 2000.
11. Diener, U.L., Cole, R.J., Sanders, T.H., Payne, G.A., Lee, L.S., Klich, M.A. Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annu. Rev. Phytopathol.*, 1987; **25**: 249–70.
12. Squire, R.A. Ranking animal carcinogens: a proposed regulatory approach. *Science*, 1981; **214**: 877–80.
13. Coker, R.D. Mycotoxins: The silent threat to human and animal health. Inaugural Lecture Series, UK: The University of Greenwich, 1999.
14. Reddy, K.R.N., Reddy, C.S., Muralidharan, K. Detection of *Aspergillus* spp. and aflatoxins B1 in rice in India. *Food Microbiol.*, 2009; **26**: 27-31.
15. Al-Mamun, M., Akbar, M.A., Shahjalal, M. Rice straw, its quality and quantity as affected by storage systems in Bangladesh. *Pak. J. Nutr*., 2002; **1**(3): 153-5.
16. Bhuiyan, A., Akbar, M.A., Hossain, M.E. Nutritive value of damp rice straw and its feeding effect on aflatoxin transmission into cow's milk. *Pak. J. Nutr*., 2003; **2**(3): 153-8.
17. Azab, R.M., Tawakkol, W.M., Hamad, A.M., Abou-Elmagd, M.K., El-Agrab, H.M., Refai, M.K. Detection and estimation of aflatoxin B1 in feeds and its biodegradation by bacteria and fungi. *Egy. J. Nat. Toxins*, 2005; **2**: 39-56.
18. Yu, J., Bhatnagar, D., Cleveland, T.E. Aflatoxin biosynthesis. *Rev. Iberoam. Micol*., 2002; **19**: 191-200.
19. Das, A., Angayarkanni, J., Bhattacharya, S., Palaniswamy, M. Evaluation of process parameters influencing aflatoxin B1 synthesis from *Aspergillus flavus* MTCC 2798 using rice straw under submerged fermentation. *Int. J. Pharm. Bio. Sci.*, 2012; **2**(2): 94-105.
20. Calvo, A.M., Wilson, R.A., Bok, J.W., Keller, N.P. Relationship between secondary metabolism and fungal development. *Microbiol. Mol. Biol. R.*, 2002; **66**: 447-59.
21. Keller, N.P., Nesbitt, C., Sarr, B., Phillips, D., Burrow, G.B. pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. *Phytopathology*, 1997; **87**: 643-8.
22. Maragos, C.M. Extraction of aflatoxins B1 and G1 from maize by using aqueous sodium dodecyl sulphate. *J. AOAC Int.*, 2008; **91**(4): 762-7.
23. Molina, M., Giannuzzi, L. Modelling of aflatoxin production by *Aspergillus parasiticus* in a solid medium at different temperatures, pH and propionic acid concentrations. *Food Res. Int.*, 2002; **35**(6): 585-94.
24. Russell, R., Paterson, M., Lima, N. How will climate change affect mycotoxins in food? *Food Res. Int.*, 2010; **43**: 1902–14.
25. Lisker, N., Lillehoj, E.B.: Prevention of mycotoxin contamination (principally aflatoxins and *Fusarium* toxins) at the preharvest stage. In: *Mycotoxins and animals foods* (Smith JE, Henderson RS, ed). Boca Raton, Fla.: CRC Press, 1991; pp 689-719.