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Bioremediation of Aflatoxins by Some Reference Fungal Strains

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Abstract

Aspergillus parasiticus RCMB 002001 (2) producing four types of aflatoxins B_1, B_2, G_1 and G_2 was used in this study as an aflatoxin-producer. *Penicillium griseofulvum*, *P. urticae*, *Paecilomyces lilacinus*, *Trichoderma viride*, *Candida utilis*, *Saccharomyces cerevisiae* as well as a non-toxigenic strain of *Aspergillus flavus* were found to be able to exhibit growth on aflatoxin B_1-containing medium up to a concentration of 500 ppb. It was also found that several fungal strains exhibited the growth in co-culture with *A. parasiticus*, natural aflatoxins producer, and were able to decreased the total aflatoxin concentration, resulting in the highest inhibition percentage of 67.2% by *T. viride*, followed by *P. lilacinus*, *P. griseofulvum*, *S. cerevisiae*, *C. utilis*, *P. urticae*, *Rhizopus nigricans* and *Mucor rouxii* with total aflatoxin inhibition percentage of 53.9, 52.4, 52, 51.7, 44, 38.2 and 35.4%, respectively. The separation of bioremediation products using GC/MS revealed that the toxins were degraded into furan moieties.

Key words: *Aspergillus parasiticus*, aflatoxins, bioremediation, GC/Mass determination

Introduction

The aflatoxins are a biologically active polyketide-derived secondary metabolites (Bhatnagar *et al.*, 1992). The aflatoxins are a group of closely related highly oxygenated bisfurano-coumarin heterocyclic compounds (Buchi and Rae, 1969; Ellis *et al.*, 1991). Aflatoxins are produced by some strains of *Aspergillus flavus* and most, if not all, *Aspergillus parasiticus* Speare (Smith and Moss, 1985) as well as the closely related species *Aspergillus nomius* Kurtzman (Kurtzman *et al.*, 1987; Cotty *et al.*, 1994). These aflatoxin-producing species differ in their ability to produce aflatoxins and some are entirely non-toxigenic (Smith, 1997).

Chemically, aflatoxins are defined as a series of 18 known bisfuran polycyclic compounds that fluorescence strongly in ultraviolet light (Park *et al.*, 2001). There are four naturally occurring aflatoxins B_1, B_2, G_1 and G_2 together with other aflatoxins which occur endogenously as metabolic products of microbial, animal, or human metabolic systems (Smith, 1997).

Aflatoxins can be acutely toxic, carcinogenic, mutagenic, teratogenic, and immunosuppressive to most mammalian species. The rank order of toxicity, carcinogenicity, *etc.*, is $AFB_1 > AFG_1 > AFB_2 > AFG_2$ implying that the unsaturated terminal furan of AFB_1 is critical for determining the level of biological activity of the aflatoxins (Eaton and Gallagher, 1995; Smith, 1997).

The biological detoxification or the biotransformation or degradation of aflatoxin by microbial systems to a metabolite(s) that is either nontoxic when ingested by animals or less toxic than the original toxin and readily excreted from the body is being studied in several laboratories (Smith and Bol, 1989). As yet, such methods do not constitute a realistic practical approach (Smith, 1997) to the problem. Boller and Schroeder (1973, 1974) reported that *A. cheralieri* and *A. candidus* that dominated the mycoflora in rice also showed marked inhibition in aflatoxin production by *A. parasiticus* Speare. *Aspergillus oryzae* and *Rhizopus nigricans* (formerly *Rhizopus stolonifer*) and have also been reported to inhibit *A. parasiticus* and aflatoxin production (Christensen *et al.*, 1973; Weckbach and Marth, 1977).

The capabilities of several fungal strains to resist aflatoxins as well as to biotransform and/or biode-

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grade these compounds into several other metabolites that are either non- or less toxic than the original have been reported in this paper.

Experimental

Materials and Methods

Organisms used. Several strains of *Aspergillus flavus* group were examined for aflatoxin production capabilities. These cultures were provided by the Regional Center for Mycology and Biotechnology (RCMB) culture collection unit. *A. flavus* RCMB 002002 (strains designated as 1, 2, 3, 4, 5, and 6), *A. flavus* var. *columnaris* RCMB 002003, *A. parasiticus* CMB 002001 (strains: 1 and 2), *A. tamarii* RCMB 002004(2), *A. oryzae* RCMB 002015(1). Several other fungal (provided by the same culture collection) were also examined for their capabilities to grow in the presence of aflatoxins. These strains were: *P. griseofulvum* RCMB 001007(2), *P. urticae* RCMB 001017, *P. italicum* RCMB 001018(1), *P. roquefortii* RCMB 001009(6), *P. nigricans* RCMB 001013, *P. citrinum* RCMB 001011(1), *P. notatum* RCMB 001016, *Circinella* sp. RCMB 013001, *Rhizopus nigricans* RCMB 014001, *Mucor rouxii*, RCMB 015002, *Syncephalastrum racemosum* RCMB 016001(2), *Trichoderma viride* RCMB 017002, *Paecilomyces lilacinus* RCMB 018002, *P. variotii* RCMB 018003, *Curvularia lunata* RCMB 019002, *C. clavata* RCMB 019003, *Rhizoctonia solani* RCMB 031001, *Torulomyces lagena* RCMB 030001, *Acremonium rutilum* RCMB 020002, *Saccharomyces cerevisiae* RCMB 006001 and *Candida utilis* RCMB 005002.

Preliminary detection of aflatoxin-producing fungi by fluorescent agar technique. Several strains of *A. flavus* group were screened for their ability to produce aflatoxin(s) on Sabouraud-dextrose yeast extract agar plates, using the fluorescent agar technique of Hara *et al.* (1974).

Application of agar plug method. Seven-day old cultures of *Aspergillus* spp. grown on MEA were examined for aflatoxins production using an agar plug technique (Paterson and Bridge, 1994). An agar plug was cut out with a flamed cork borer (inner diameter ~0.4 cm) from the center of the colony. The plug was removed with the a sterile dissecting needle, wetted the mycelial side of the plug with a drop of chloroform/methanol (2:1, v:v) for a few seconds and touched the agar side to a thin layer chromatography (TLC) plate. The agar plug was placed at the origin of the TLC plate (20×20 cm Merck aluminum sheet, silica gel 60, layer thickness 0.2 mm). The diameter of the application spot should not be more than 0.6 cm. After drying the spots, the TLC plate was developed in TEF eluent (toluene/ethyl acetate/90% formic acid, 5:4:1, v/v/v) in a solvent saturated atmosphere using saturation pads. Griseofulvine was used as an external standard. The dry developed TLC plates were viewed in normal white light, under long wave (366 nm), and short wave (254 nm) UV light; then compared with the standards and published data on colors and R_f values according to Paterson and Bridge (1994).

Production of aflatoxins. For the production of aflatoxins yeast-extract sucrose (YES) liquid medium was used. For enhancement of aflatoxin production, 1 ml trace element solution was added to 1 liter of YES medium. The trace element solution is prepared by dissolving 0.5 g magnesium sulphate, 0.5 g cupric sulphate and 0.5 g zinc sulphate in 100 ml of distilled water.

Preparation of spore suspension. Mould inoculum was prepared by growing *A. parasiticus* on MEA slants for 7–10 days at 25°C until sporulation. The spores were harvested by adding 10 ml of sterile distilled water to the cultures on the surface of the agar slants and gently dislodging spores from conidiophores with an inoculation loop. The spore suspension was filtered through four layers of sterile cheesecloth followed by filtration through Whatman No 1 filter paper to remove mycelial debris. Spores were counted using an Improved Neubauer bright line hemocytometer. Appropriate dilutions were made from the stock spore suspension in 0.1% peptone water as the diluent to obtain the desired inoculum's density of 4×10^2 cells/ml (Ellis *et al*., 1991).

Extraction of aflatoxins. The broth filtrates were mixed with an equal volume of chloroform in a separating funnel. The residue was re-extracted twice for complete extraction. The chloroform extract was defatted with hexane, concentrated in a rotary evaporator and purified using silica gel column chromatography. The column was washed with 3 ml each of hexane, ethyl ether and methylene chloride. The aflatoxins were then eluted with 6 ml chloroform: acetone (9:1, v/v) mixture. The solvent was removed by evaporation on a rotary evaporator. The residues were reconstituted in 1 ml methanol for further chromatographic analyses.

Analysis of aflatoxins derivative formation. 50 µl of trifluoroacetic acid (TFA) were added to 200 ml of the methanol solution of toxin extract. The mixture was allowed to react at room temperature for 15 min and then was evaporated to dryness. The residue was dissolved in 2 ml of water:acetonitrile (75:25, v/v) for HPLC (Frisvad and Thrane, 1993).

Determination of the fungus ability to grow on aflatoxin B_1. Different fungal strains were examined for their ability to grow on mineral medium amended with three different concentrations of aflatoxin B_1 (100, 250 and 500 ppb). The broth mineral medium as given by Atlas (1995) was used.

Inhibition of aflatoxin production by toxigenic strain when co-cultivated with other fungal strains. Tested fungal strains were co-cultivated with an aflatoxigenic producing *A. parasiticus* strain on MEA broth medium. Five Erlenmeyer flask of 250-ml capacity were provided with 50 ml of the medium and inoculated with a spore suspension of *A. parasiticus* as well as the tested strain. The pH of the medium was adjusted to 6.5 with 0.1 N NaOH. The flasks were incubated for two weeks at 25°C. The growth of the fungal strains as well as the aflatoxin production was estimated by HPLC.

Separation of the degradation products. The degradation products of the aflatoxin bioremediation were analyzed using SHIMADZU GC/MS-QP 5050A gas chromatograph-mass spectrometer using CLASS 5000 software. WILEY Mass Spectral Database was used in the identification of the separated peaks.

Results

Preliminary detection of aflatoxin-producing fungi. Four strains of *A. flavus*, two strains of *A. parasiticus* and one strain of *A. flavus* var. *columnaris* exhibited blue green or bluish-green fluorescence surrounding the colonies on the agar medium indicating the possible production of aflatoxins. While *A. oryzae*, *A. tamarii* and *A. flavus* RCMB 002002(6) were not showing any fluorescence.

A confirmative test for aflatoxin production by these strains was performed by TLC analysis. Four strains of *A. flavus* and the two strains of *A. parasiticus* and the strain of *A. flavus* var. *columnaris* that showed positive fluorescence were found to produce one or more spots on the TLC plate. Two strains of *A. parasiticus* produced AFB_1, AFB_2 as well as AFG_1 and AFG_2. Also, a marked increase in aflatoxin concentrations in *A. parasiticus,* strain RCMB 002001(2) culture was observed (Table I). Thus this strain was used for the further studies as aflatoxin-producing strain. The chromatographic analysis of *A. flavus* RCMB 002002(6) indicated that this strain produced no or undetectable quantities of aflatoxins. Consequently, it was considered a non-aflatoxigenic fungus.

Investigation of the ability of selected fungal strains to grow on medium emended with different concentrations of aflatoxin. Twenty three strains were investigated for their ability to grow on mineral medium containing different concentrations of aflatoxin B_1 (Table II). Results indicate that several fungal species including *P. griseofulvum*, *P. urticae*, *P. lilacinus*, *T. viride*, *C. utilis*, *S. cerevisiae* as well as a non-toxigenic strain of *A. flavus* were able to grow on a medium containing different concentrations of aflatoxin B_1; 500 ppb, 250 ppb and 100 ppb. While, *P. roquefortii*, *R. nigricans* (=*Rhizopus stolonifer*), *S. racemosum*, *P. variotii*, *C. clavata*, and *A. fumigatus* were less tolerant to aflatoxin B_1 and showed weak growth. Alternatively, two fungal species; *P. notatum* and *R. solani* were highly sensitive to the aflatoxin B_1. *A. rutilum* showed absence of growth on the media containing aflatoxin B_1. Whereas, *P. notatum*, *R. solani* showed only a weak growth on the lowest (100 ppb) concentration.

Table I
Aflatoxin production by different strains of the *A. flavus* group

Strains		Aflatoxin			
		B_1	B_2	G_1	G_2
A. flavus	RCMB 002002 (1)	+	+	0	0
A. flavus	RCMB 002002 (2)	+	+	0	0
A. flavus	RCMB 002002 (6)	0	0	0	0
A. flavus	RCMB 002002 (5)	0	+	0	0
A. parasiticus	RCMB 002001 (1)	+	+	+	+
A. parasiticus	RCMB 002001 (2)	+	+	+	+
A. flavus var. *columnaris*	RCMB 002003	+	0	+	0

0 = not detected under the experimental conditions.

Table II
Investigation of the ability of fungal strains to grow on aflatoxin B_1 containing media

Organism name		Aflatoxin B_1 concentration (ppb)			
		Control	100	250	500
P. griseofulvum	RCMB001007(2)	++	++	++	++
P. roquefortii	RCMB001009(6)	++	+	+	0
P. urticae	RCMB001017	++	++	++	++
P. nigricans	RCMB001013	++	+	+	+
P. notatum	RCMB001016	++	+	0	0
P. italicum	RCMB001018	++	++	+	+
P. citrinum	RCMB001011	++	+	+	+
R. nigricans	RCMB001014	++	+	+	0
Circinella sp.	RCMB013001	++	++	++	+
S. *racemosum*	RCMB016001	++	+	+	0
M. rouxii	RCMB015002	++	++	+	+
A. rutilum	RCMB020002	++	0	0	0
P. variotii	RCMB018003	++	+	+	0
P. lilacinus	RCMB018002	++	++	++	++
C. lunata	RCMB019002	++	+	+	+
C. clavata	RCMB019003	++	+	+	0
T. viride	RCMB017002	++	++	++	++
T. lagena	RCMB030001	++	+	+	0
C. utilis	RCMB005002	++	++	++	++
S. cerevisiae	RCMB006001	++	++	++	++
R. solani	RCMB031001	++	+	0	0
A. fumigatus	RCMB002008(2)	++	+	+	0
A. flavus (atoxigenic strain)	RCMB002002(6)	++	++	++	++

0 = not detected underthe experimental conditions,
+ = detectable growth, ++ = good growth.

Table III
Inhibition of aflatoxin production by a toxigenic strain of *A. parasiticus* cultivated with certain fungal isolates

Organism name	Aflatoxin production (µg/l)					Inhibition (%)				
	B_1	B_2	G_1	G_2	Total	B_1	B_2	G_1	G_2	Total
Control (*A. parasiticus*)	21.5	21.5	9.6	8.9	45.2					
P. griseofulvum	7.81	5.2	5.4	3.1	21.5	63.6	45.8	39.3	40.3	52.4
P. roquefortii	12.8	7.4	6.9	4	31.1	40.3	22.9	22.4	23.0	31.2
P. urticae	10.0	6.1	5.8	3.4	25.3	53.5	36.4	34.8	34.6	44.0
P. nigricans	14.5	7.6	7.1	3.8	33	32.5	20.8	20.2	26.9	27.0
P. notatum	11.9	7.2	6.8	4.1	30	44.6	25.0	23.6	21.1	33.6
P. italicum	12.6	8.1	6.7	4.2	31.6	41.4	15.6	24.7	19.2	30.0
R. nigricans	10.8	6.9	6.2	4	27.9	49.7	28.1	30.3	23.0	38.2
S. racemosum	13.9	8.1	6.8	4.2	33.0	35.3	15.6	23.6	19.2	27.0
Cirinella sp.	15.2	8.3	7.2	4.1	34.8	29.3	13.5	19.1	21.1	23.0
P. lilacinus	8.2	5.9	4.1	2.6	20.8	61.8	38.5	53.9	50.0	53.9
C. lunata	13.1	7.8	7.8	4.7	33.4	39.0	18.7	12.3	9.6	26.1
T. viride	5.32	4.2	3.1	2.2	14.8	75.2	56.2	65.1	57.7	67.2
C. utilis	8.1	4.8	5.1	3.8	21.8	62.3	50.0	42.6	26.9	51.7
S. cerevisiae	7.8	4.7	4.9	3.9	21.3	63.7	51.0	44.9	25.0	52.0
M. rouxii	9.83	6.9	8.2	4.3	29.2	54.3	28.1	7.8	17.3	35.4

Influence of antagonistic activities of *A. parasiticus* against some other fungal strains. Cultivation of *A. parasiticus* in mixed cultures with other fungal strains demonstrated inhibition of the aflatoxins production. The growth of *A. parasiticus* was inhibited by the presence of these fungal strains. *T. viride* was found to be capable of inhibiting the growth of *A. parasiticus.* At thenhighest level. This was followed by *P. chrysogenum*, *P. lilacinus* and *P. urticae*. The aflatoxin concentrations were decreased with all strains examined (Table III) and their aflatoxin inhibition percentages are given hereafter in-between parenthesis.

The concentration of aflatoxin B_1 was found to decrease reaching a minimum value of 5.32 ppb (75.2%) by *T. viride* compared with 21.5ppb of the control. This is followed by 7.8 ppb (63.7%), 7.81 ppb (63.6%), 8.1 ppb (62.3%), 8.2 ppb (61.8%), 9.83 ppb (54.3%) and 10 ppb (53.5%) for *S. cerevisiae*, *P. griseofulvum*, *C. utilis*, *P. lilacinus*, *M. rouxii* and *P. urticae*, respectively.

The concentration of aflatoxin B_2 was found to be decreased and reaching a minimum value of 3.1 ppb (56.2%) by *T. viride* compared with 9.6 ppb of the control. This is followed by 4.7 ppb (51.0%), 4.8 ppb (50.0%), 5.2 ppb (45.8%), 5.9 ppb (38.5%) and 6.1 ppb (36.4%), for *S. cerevisiae*, *C. utilis*, *P. griseofulvum*, *P. lilacinus* and *P. urticae* respectively.

The concentration of aflatoxin G_1 was also decreased reaching a minimum value of 3.1 ppb (65.1%) by *T. viride* compared with 8.9 ppb of the control. This is followed by 4.1 ppb (53.9%), 4.9 ppb (44.9%), 5.1 ppb (42.6%), 5.4 ppb (39.3%), 5.8 ppb (34.8%), and 6.2 ppb (30.3%) for *P. lilacinus*, *S. cerevisiae*, *C. utilis, P. griseofulvum*, *P. urticae*, and *R. nigricans*, respectively.

The concentration of aflatoxin G_2 decreased as well reaching a minimum value of 2.2 ppb (57.7%) by *T. viride* compared with 5.2 ppb of the control. The total aflatoxins production were found to decrease reaching a minimum concentration of 14.8 ppb (67.2%) with *T. viride* when compared with 48.2 ppb of the control. This is followed by *P. lilacinus*, *P. griseofulvum*, *S. cerevisiae*, *C. utilis*, *P. urticae*, *R. nigricans* and *M. rouxii*, with total aflatoxin concentrations of 20.8 ppb (53.9%), 21.5 ppb (52.4%), 21.3 ppb (52%), 21.8 ppb (51.7%), 25.3 ppb (44.0%), 27.9 ppb (38.2%) and then 29.2 ppb (35.4%), respectively.

Compounds produced after bioremediation of aflatoxins by *Mucor rouxii.* *M. rouxii* has a unique behavior in the bioremediation of aflatoxins. In spite GC analysis showed four characterized peaks (Fig. 1). The four main peaks have mass peaks; 189, 217, 216 and 228 with molecular formula of $C_{12}H_{20}N_2O_2$, $C_{10}H_{18}O$, $C_{22}H_{46}$ and $C_{11}H_{18}O_2$, respectively. The presence of furan moiety in B and E as well as F indicates the degradation of aflatoxin molecules. The presence of ketone molecule (D) indicated the degradation of toxins by the fungus. Obviously, the other peaks were fragments of each principal peak. The aspergillic acid, which is a by-product of *A. parasiticus* shown in the control, was also observed in the chromatogram.

Compounds produced after bioremediation of aflatoxins by *Rhizopus nigricans.* The detected cyclopentane represents the cyclopentenone ring of aflatoxin

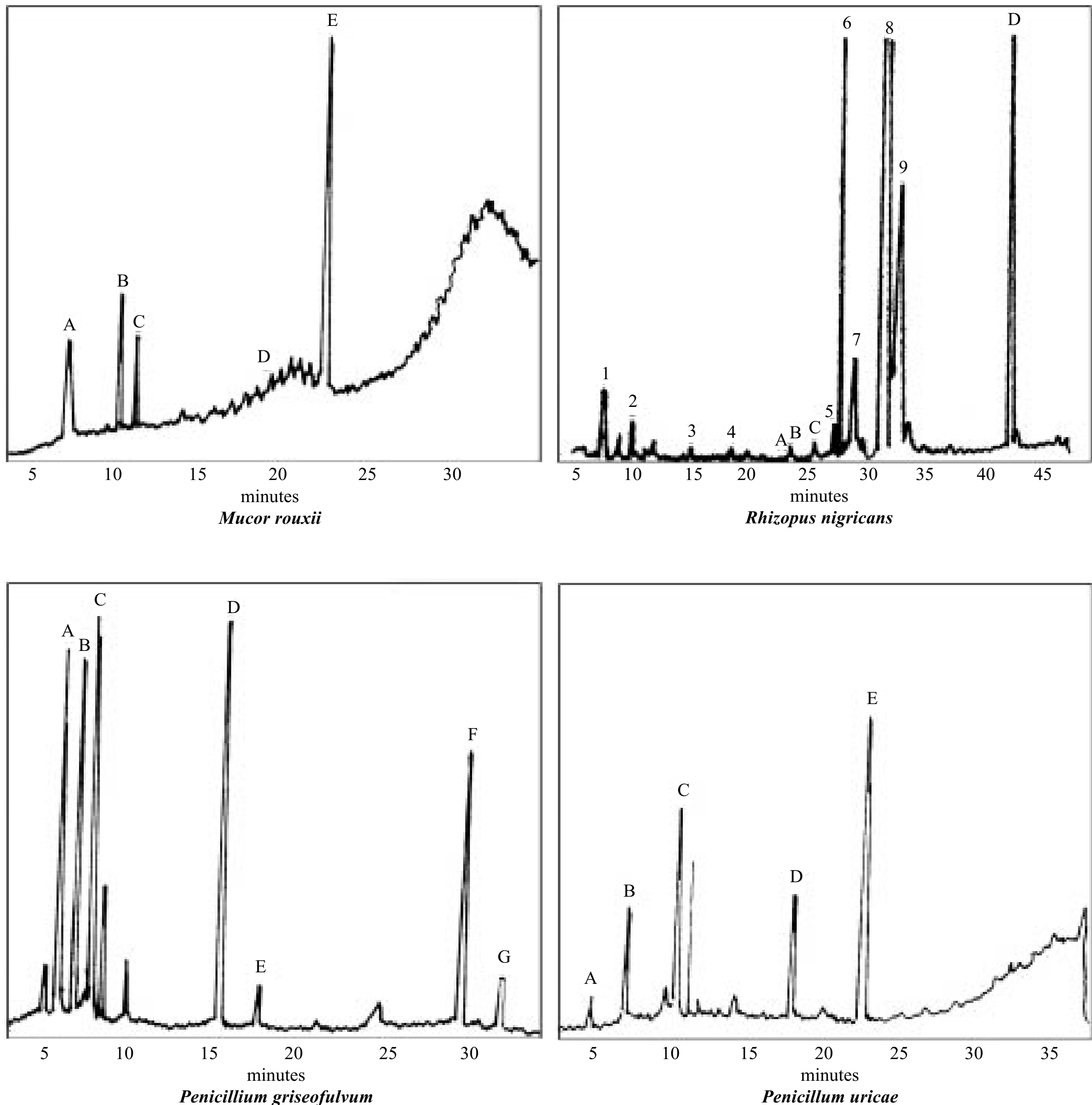

Fig. 1. Total ion chromatogram of GC/MS analysis of the compounds produced after remediation of aflatoxins by indicated fungi. Peaks for particular fungi are as follow:

Mucor rouxii: (A) aspergillic acid; (B) furan – 4,5diethyl-2,3-dihydro-2,3-dimethyl; (C) 2-docosane; (D) ketone – 2,2-dimethylcyclohexyl methyl; (E) mannofuranoside; (F) bi-furan, dicarboxylic acid dimethyl

Rhizopus nigricans: (1) linalool; (2) alpha-terpineol; (3) cis-jasmone; (4) farnesene; (5) palmitoleic acid; (6) palmitic acid; (7) oleic acid; (8) linoleic acid; (9) linolelaidic acid; (A) cyclodecafuranone; (B) benzyl benzoate; (C) cyclopentane – isopropenyl-2,3-dimethyl (D) benzenedicarboxylic ethylhexyl ester

Penicillum griseofulvum: (A) aspergillic acid; (B) oxyaspergillic acid; (C): furanone; (D) dioctyl phathalate; (E) ethanone, dihydrodimethyl-indol; (F) benzofuran, cyclohexyl-dihydro-methyl; (G) griseofulvin.

Penicillum uricae: (A) butyl hydroxy toluene; (B) aspergillic acid; (C) naphthofurandihydropropenyl; (D) naphthalene; (E) mannofuranoside.

B group after bioremediation by the *R. nigricans*. While the detected benzene and cyclopentane moiety indicates the cleavage of aflatoxin structure, the detection of furan moieties as well as dioctyl phthalate confirming this degradation of aflatoxins. The chromatogram (Fig. 1) also indicated the presence of several fatty acids such as oleic, palmitic, palmitolenic, linolelaidic and high amount of linoleic acid. Some terpines such as alpha-terpinol, farnesene, jasmone and linalol were also observed.

Compounds produced after bioremediation of aflatoxins by *Penicillium griseofulvum*. The gas

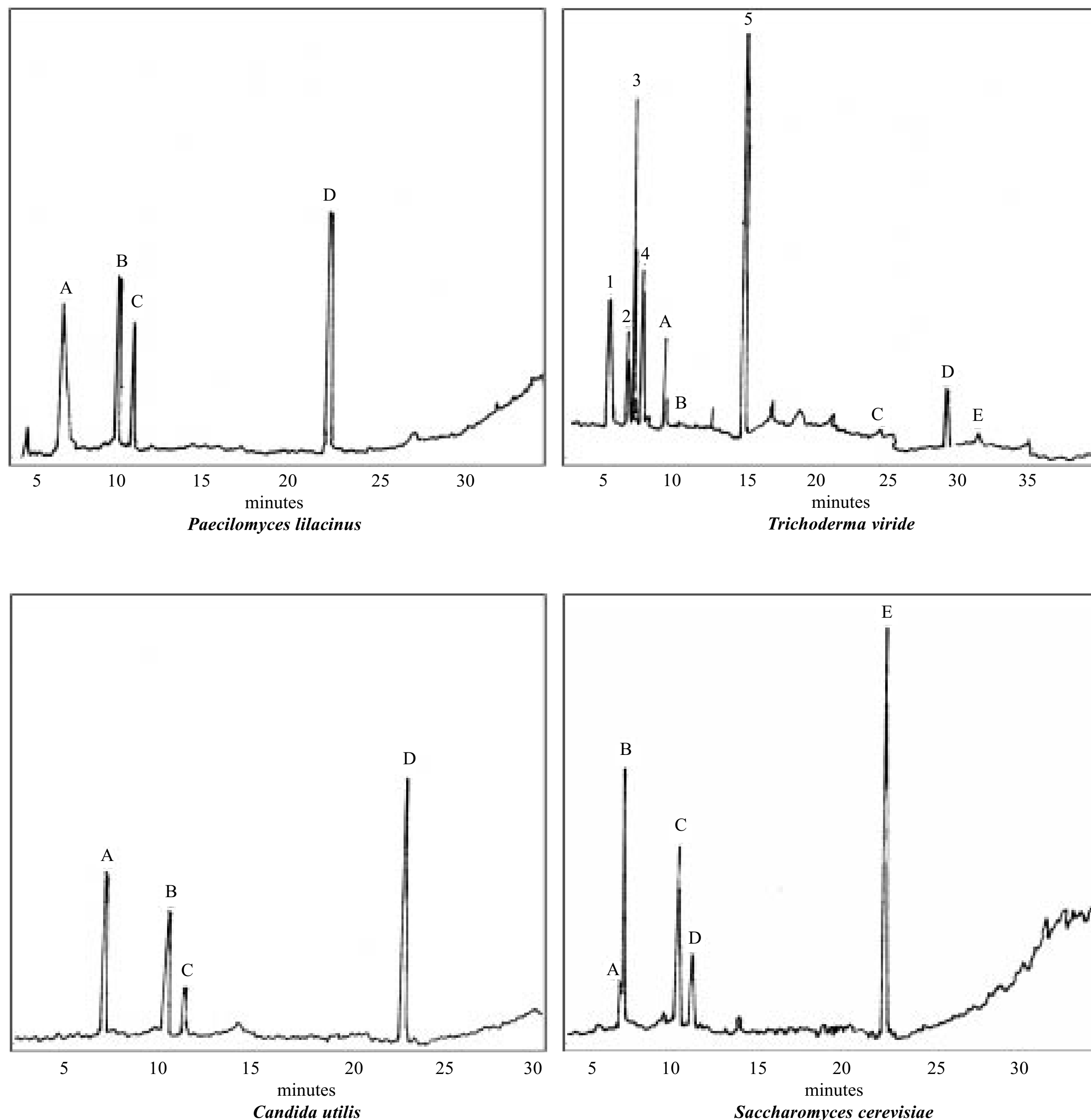

Fig. 2. Total ion chromatogram of GC/MS analysis of the compounds produced after bioremediation of aflatoxins by indicated fungi. The peaks for particular fungi are as follow:

Paecilomyces lilacinus: (A) phenol-bis – (1,1-dimethyl)-4-methyl; (B) methyl dimethoxyphenyl propanoate; (C) dioctyl phthalate; (D): hexanone.

Trichoderma viride: (1) aspergillic acid; (2) cyclopentanetione; (3) butabarbitol; (4) methyl jasmonate; (5) dioctyl phthalate; (A) tinuvin; (B) limonene; (C): benzofuranone, hexahydrotrimethyl; (D) benzene; (E) androstanedione.

Candida utilis: (A) aspergillic acid; (B) benzofuran; (C) tinuvin; (D) dioctyl phthalate.

Saccharomyces cerevisiae: (A) aspergillic acid (B) furan; (C): Dimethyl-naphthalene; (D) 7-methoxy-coumarin; (E) dioctyl phthalate.

chromatographic analysis of compounds produced after the bioremediation process of aflatoxins by the *P. griseofulvum* was completely different of that of the control. The position of peaks is shifted indicating bioremediation of aflatoxins. Chromatogram shows five characteristic peaks (A-D, F) with several peak fragments. The mass spectrum exhibited 2,6-di-tert-butylphenol with a methyl group at the position 4, this butyl phenol absolutely not included in the chemical structure of the control, *i.e.* aflatoxins B_1, B_2, G_1 and G_2. The molecular weight of this compound is 220, with molecular formula $C_{15}H_{24}O$. On the other hand, aspergillic acid and oxyaspergillic acid ($C_{12}H_{20}N_2O_2$) were developed, respectively. The mass peaks are 248

and 416, respectively. The furan moiety was cleaved since it appeared as furanon or within spiro structure as benzofuran. Presumably, the appearance of dioctyl phthalate and the dihydro-dimethyl indole indicate the degradation of aflatoxins.

Compounds produced after bioremediation of aflatoxins by *Penicillium urticae*. The GC analysis of the compounds produced after the treatment of aflatoxins by *P. urticae* is shown in Figure 1. There are several peaks present, but the most typical peaks are C and E. The last peak represents a mannofuranoside of molecular formula $C_{11}H_{18}O_2$. The total molecular weight is approximately 1210 for bioremediated aflatoxins in contrast to 1292 of the control sample. It seems that the *P. urticae* do not bioremediate aflatoxins very well as indicated from the number of hydrogen atoms. The mass peak of the value of 234 of naphthalene with phenyl- and methyl- side chain as well as the butyl hydroxyl toluene also indicate this weak tendency to cleave aflatoxin molecule.

Compounds produced after bioremediation of aflatoxins by *Paecilomyces lilacinus*. The gas chromatographic analysis of the compounds produced after bioremediation by *P. lilacinus*, indicate the presence of four main characteristic peaks (Fig. 2). Meanwhile the positions of these peaks are completely different from peaks of control sample. The main difference is that the compounds of *P. lilacinus* have a total molecular weight of 1116 and total carbon atoms 66. However, the identification of the peaks in the mass spectra shows the dominance of benzene rings with different side chains. The presence of dioctyl phthalate on one side and the absence of furan moiety as well as coumarin and cyclopentanone on the other hand may indicate the biodegradation of aflatoxins.

Compounds produced after bioremediation of aflatoxins by *Trichoderma viride*. *T. viride* bioremediated the aflatoxins B and G very extensively; 10 peaks in addition to peak fragments as well as very small peaks as shown in GC analysis were detected (Fig. 2). The positions of these peaks were completely different from peaks in control sample. Aspergillic acid as well as different degradation product exerted such as the dioctylphthalate, methyl jasmonate, butabarbitol and cyclopentanione were detected. Cyclopentanetione indicate cleavage of cyclopentane ring of aflatoxins. The total molecular weight of the bioremediated aflatoxins was high (1348) in contrast to the control sample (1292), that was attributed to highly fragmented aflatoxins by *T. viride*. Consequently, carbon, hydrogen and oxygen atoms recorded 89, 136 and 9, respectively. Identification of the peaks by mass spectrometer showed the presence of benzene fused with furan moiety (C) as a dominant bioremediation product by *T. viride*. Limonene, jasmonate and other essential oil compounds as well as benzene, 3-methylbutenyl- (with molecular weight 146) and tinuvin were also detected. Furan moiety was detected in two peaks; weight peak 170 (R. time 30.64–31.25) for benzofuranon and mass peak 221 (R. time 53.49–54.65) with androsanedione.

Compounds produced after bioremediation of aflatoxins by *Candida utilis*. The chromatogram for bioremediation of aflatoxins by *C. utilis* shows the presence of aspergillic acid, a metabolic product of *A. parasiticus*, which is present in the control sample. The furan moiety was still present but cleaved from the aflatoxin structure since it appeared as a benzofuran derivative. Tinuvin was present as well. Dioctyl phthalate as a degradation product was detected indicating the biodegradation process of the aflatoxins (Fig. 2).

Compounds produced after bioremediation of aflatoxins by *Saccharomyces cerevisiae*. The GC analysis of the compounds produced after the treatment of aflatoxins by *S. cerevisiae* is given in Figure 2. Although, there are several peaks present, the most important one is the methoxycoumarin. The presence of furan moiety that appeared as furan, 4, 5-diethyl-2, 3-dimethyl as well as the methoxycoumarin indicate the cleavage of aflatoxins ring. Another peak produced in figure could be also regarded as a benzene ring degradation products identified as dimethylnaphthalene, since its molecular weight 156 and molecular formula $C_{12}H_{12}$. Also, dioctyl phthalate was detected. The chromatogram also shows the presence of aspergillic acid, a metabolic product of *A. parasiticus* that is present in the control sample.

Discussion

Biological detoxification or the biotransformation of aflatoxins by microbial systems to a metabolite(s) that is either nontoxic when ingested by animals or less toxic than the original toxin can be termed as bioremediation. The goal of bioremediation is to degrade organic pollutants to concentrations that are either undetectable or, if detectable, to concentrations below the accepted limits (McKane and Kandel, 1996).

The growth of certain fungal strains (*P. griseofulvum*, *P. urticae*, *P. lilacinus*, *T. viride*, *C. utilis*, *S. cerevisiae* as well as a non-toxigenic strain of *A. flavus*) on the three aflatoxin B_1 concentrations used (100, 250 and 500 ppb) indicated that these fungal strains have the ability to tolerate or metabolize the toxin. Other examined fungal strains showed variable capabilities to tolerate aflatoxin B_1 reflected in the difference in their growth ability at the aflatoxin concentrations used (250, and/or 100 ppb) with no growth at 500 ppb. *T. viride*, *P. lilacinus*, *P. griseofulvum*, *S. cerevisiae*, *C. utilis*, *P. urticae* and *M. rouxii* were able to inhibit the growth of *A. parasiticus* and grew

in the presence of aflatoxins in its growth medium. The organism with the least efficiency to inhibit aflatoxins was *Circinella* sp. having a total inhibition percentage of 23%.

The reasons for the reduction in aflatoxin levels can, therefore, be attributed to one or a combination of the following factors: (1) physical competition for space and nutrition; (2) test fungi may compete with *A. parasiticus* for a substrate required for toxin production but not the growth; (3) presence of test fungi might cause a change in the biochemical environment deciding, thereby, the metabolic pathway available to the toxigenic fungi and (4) degradation of aflatoxin following its formation. An inhibition in aflatoxin production by *T. viride* by more than 90% inhibition was also reported by Varma (1996). Shantha (1999) reported that several fungal cultures were found to prevent the synthesis of aflatoxin B_1 by *A. flavus* in liquid medium. Among these *Phoma* spp., *Mucor* sp., *Trichoderma* spp., *Rhizopus* sp., *Alternaria* sp. and *Sporotrichum* spp. inhibited aflatoxin synthesis by about 90% or more.

El-Sayed (1996) revealed the potential use of some phycomycetes (*Absidia*, *Mucor*, *Cunninghamella*, *Rhizopus* and *Syncephalasrtum*) to inhibit aflatoxins. Cole *et al.* (1972) and Nout (1989) studied a number of *Rhizopus* species and indicated the accumulation of two fluorescent metabolites of aflatoxin B_1 during its degradation. These metabolites were identified as hydroxylated stereo isomers derived from the reduction of ketone function on the cyclopentane ring of aflatoxin B_1. Weckbach and Marth (1977) and Choudhary (1992) also found that *Rhizopus nigricans* inhibited both the growth and aflatoxin production by *A. parasiticus*.

Nour *et al.* (1982) reported that some species of *Aspergillus*, *Mucor*, *Penicillium*, *Rhizopus genera* are antagonizing fungi that seem capable of metabolizing aflatoxin B_1 produced by *A. flavus* or probably producing some exudates that react with the toxin, transforming it into nontoxic compounds, degrading it or deflecting the pathway of aflatoxin B_1 synthesis. The identification of degradation products indicated that the aflatoxins were partly degraded with the examined fungal strains at different manner. The presence of furan moiety in the chromatograms of separation of most of the examined strains indicated degradation of aflatoxin. Three different furan moieties were exerted in case of *M. rouxii*.

The separation profile of *R. nigricans* exhibited high efficacy to fatty acid formation, with high percentage of linoleic acid. This result is in accordance with that of Kim *et al.* (2000) where linoleic acid was identified by GC-MS to be the main active component in aflatoxin degradation by soybean paste. The presence of the fatty acids and subsequent appearance of the degraded furan moiety proved this conclusion. However, in case of *P. griseofulvum*, two forms of furan moieties were appeared as degradation products as well as the dioctyl phthalate. The GC chromatogram also separated the antifungal metabolite of *P. griseofulvum*, the griseofulvin. The presence of aspergillic acid and its oxygenated form, oxyaspergillic acid, also confirm the oxidation process that cleavage hydrogen atoms and lead to libration of H_2O and CO_2. While *P. urticae* exerted two forms of furan moieties, *C. utilis* and *P. lilacinus*, show lower efficiency in the degradation of aflatoxins. The *T. viride* strains show different furan moieties as well as androstanedione, a non-active molecule that have a similar structure of aflatoxin with the active bonds. The presence of benzene rings along with the dioctyl phthalate also confirms the degradation process. In case of *S. cerevisiae*, the mass analysis of the separated peaks indicates that degradation takes place in furan moiety as well as the coumarin moiety. These moieties represent the main skeleton of aflatoxins.

The available literature indicates only five strategies are known to reduce or detoxify aflatoxins contamination in food, food and feed processing, biocontrol and microbial inactivation, dietary modification and chemoprotection, chemical degradation, and reduction in toxin bioavailability by selective chemosorption (Smith *et al.*, 1994). The presented results may certainly suggest a sixth strategy; application of fungal biodegradation and/or bioremediation. It is worth mentioning that the genomic mechanism controlling aflatoxin biosynthesis by the same research group is in progress and will be released shortly. *T. viride*, a classical fungal biocontrol agent that was used in the treatment of infected plants either in spray form or other formulation. Thus its utility can be expanded to include aflatoxin bioremediation in the infected plant, for being the most potent organism capable of decreasing aflatoxin concentration.

Hence, this study emphasizes the new value of *S. cerevisiae* used commercially as a rich source of protein and vitamins, which is its possible usage as a bioremedy for aflatoxins in a human being that cannot be treated with other treatments. Being used as a treatment for aflatoxins it will also provide the human body with vitamins and proteins.

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