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Degradation and detoxification of aflatoxin B1 by *Pseudomonas putida*

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ABSTRACT

Aflatoxin is a mycotoxin produced by *Aspergillus flavus* and a common contaminant of food and feed, posing health hazards to humans and animals alike. The aim of this study is to explore the ability of *Pseudomonas putida* to degrade aflatoxin B_1 (AFB_1). The toxigenic strain of *A. flavus* was isolated from sugarcane and used to produce AFB_1 in yeast extract sucrose medium. Two *P. putida* strains, MTCC 1274 and 2445, were cultured in mineral salt glucose medium (MSG) containing AFB_1. The AFB_1 was analyzed qualitatively and quantitatively by TLC and HPLC. It was found that *Pseudomonas* sp. can tolerate AFB_1 in the medium (0.2 μg ml^{-1}), and degrades it very efficiently. Within 24 h of incubation, AFB_1 was reduced to an undetectable level in MSG medium. Analysis with TLC, HPLC, UV spectrophotometer, gas chromatography mass spectrometry (GC–MS), and Fourier transform infra-red spectroscopy (FT-IR) showed that AFB_1 was bio-transformed to structurally different compounds (AFD_1, AFD_2, and AFD_3), with the modified furan and lactone ring on the AFB_1 molecule. A toxicity study on the HeLa cells showed that the new compounds formed are less toxic when compared with AFB_1.

1. Introduction

During harvest or storage, crops get contaminated with mycotoxins, the secondary metabolites of many saprophytic fungi. The ubiquitous nature of fungi makes food vulnerable to fungal contamination. The Food and Agriculture Organization (FAO) has reported the presence of mycotoxins in 25% of the world's crop (FAO, 1982). Aflatoxin B_1 (AFB_1), produced by *Aspergillus flavus* and *Aspergillus parasiticus*, is one of the most toxic and common contaminants in food and feed. Ingestion of aflatoxin-contaminated food leads to acute and chronic toxic effects, which may be hepatocarcinogenic, mutagenic, teratogenic (Peers and Linsell, 1973; Groopman et al., 1996), or genotoxic (Cole et al., 1988). Several physical, chemical, and biological methods have been proposed for the degradation of AFB_1 (Grove et al., 1984; Haskard et al., 2001; Mendez-Albores et al., 2008). Though aflatoxins are very stable and do not degrade up to 270 °C (their melting temperature) in dry conditions, biologically they can be converted into further toxic derivatives, such as epoxide, M_1, or M_2, by metabolism in humans and animals (Swenson et al., 1977) or less toxic derivatives, such as B_2a, by microorganisms (Megalla and Hafez, 1982). Several biological methods have been studied for the degradation or removal of aflatoxins in food and feed. The studies of EL-Nezami et al. (1998) showed that AFB_1 can rapidly surface-bind on the cell wall of lactic acid bacteria, with up to 45% reduction in AFB_1. This binding is reversible, and the stability of the complexes formed depends on strain, treatment, and environmental conditions (Haskard et al., 2001). Other bacteria that may detoxify AFB_1 are *Nocardia corynebacteroides* (Tejada et al., 2008), *Enterococcus faecium* (Ali et al., 2010), *Mycobacterium fluoranthenivorans* (Hormisch et al., 2004), and *Corynebacterium rubrum* (Mann and Rehm, 1977). The enzymatic degradation of AFB_1 by the extracellular extract from *Rhodococcus erythropolis* culture has been studied (Teniola et al., 2005); similarly the laccase enzyme from several fungal species has been found to degrade AFB_1 effectively (Alberts et al., 2006, 2009). However, a practical, economical, and safe method for detoxification of AFB_1 is not available. *Pseudomonas* spp. are useful organisms for degradation of various environmental pollutants. Aromatic nitro compounds are released into the environment through extensive use of dyes, herbicides, pesticides, explosives, and solvents. One of them is *p*-nitrophenol and this was effectively degraded by *P. putida* (Meenal and Ambalal, 2006). Mycotoxins are also degraded by *Pseudomonas*, as reported for degradation of zearalenone by *P. putida* (Altalhi and El-Deeb, 2009). Recently a *Pseudomonas* sp. has also been implicated in degradation of aflatoxin (Elaaser and El Kassas, 2011; Krifaton et al., 2011). The objective of this study was to degrade and detoxify AFB_1 with a *Pseudomonas* sp. and understand the mechanism by identifying the major degradation products.

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2. Materials and methods

2.1. Microorganisms

A toxigenic strain of *A. flavus* was isolated from sugarcane samples collected in Vellore (Tamilnadu, India). The culture was maintained on potato dextrose agar (PDA) medium slants at 4 °C throughout this study. Two strains of *P. putida*, MTCC 1274 and MTCC 2445, were obtained from MTCC Chandigarh, India, and maintained on nutrient agar medium (HiMedia Laboratories Pvt. Ltd, India) at 4 °C. Growth studies were performed on mineral salt glucose medium (MSG) as described by Meenal and Ambalal (2006).

2.2. Preparation of AFB_1

A. flavus was grown on yeast extract sucrose (YES) broth for production of AFB_1. The spores from 3-day-old culture of toxigenic *A. flavus* were inoculated into 50 ml YES broth (2% yeast extract, 15% sucrose) in a 250-ml conical flask and incubated statically at room temperature. After 7 days of incubation, AFB_1 produced in the medium was extracted twice, using an equal volume of chloroform. Qualitative analysis of AFB_1 using thin-layer chromatography (TLC) was performed along with a standard AFB_1 (Supelco, Bellefonte, PA, USA). Ten microliters of the extract was applied on activated TLC plate (Silica gel 60 F254, Macherey–Nagel, Germany); chloroform: acetone (85:15) was used as the solvent system (Horwitz, 1975). The plate was observed with a UV illuminator.

AFB_1 extracted from the culture of *A. flavus* was purified by silica gel column chromatography. Crude chloroform extract was concentrated and loaded on the matrix (silica gel 60–120 mesh, Fisher Scientific); elution was carried out with chloroform: methanol (11.76:0.24), at a flow rate of 5 ml min^{-1}. A total of 25 fractions were collected (5 ml each) and they were monitored by taking the absorbance at 360 nm in a UV spectrophotometer. The purity of AFB_1 in the fraction was confirmed by high performance liquid chromatography (HPLC, Waters 1525) with a UV detector at 360 nm as per the instructions given in the Supelco instruction manual. The stationary phase used was C_{18} Polaris column. A 20 μl sample was injected; deionized water: acetonitrile: methanol (60:20:20) was used as mobile phase at a flow rate of 1 ml min^{-1}.

2.3. Growth studies of *P. putida* strains in the presence and absence of AFB_1

Growth studies of the two *P. putida* strains (MTCC 1274, 2445) were carried out in the MSG medium up to 72 h, with observations taken at 3-h intervals. For this purpose, 20 ml of MSG broth AFB_1 with (0.2 μg ml^{-1}) and without AFB_1 was inoculated along with *Pseudomonas* sp. culture (100 μl of 0.5 OD at 600 nm) and incubated at room temperature on a shaker at 120 rpm (Meenal and Ambalal, 2006). Every 3 h, the growth of the *P. putida* strains was determined by taking the absorbance at 600 nm in a spectrophotometer (UV 1800, Shimadzu, Japan). The growth curve was made by plotting the optical density (600 nm) of the culture against the time. Survival of *Pseudomonas* sp. in the presence of AFB_1 was monitored by counting colony forming units (CFU) on the MSG agar medium. The *Pseudomonas putida* cultures were grown in the presence of AFB_1 as described above and every 6 h, 1 ml of the culture was withdrawn for CFU counting. The culture was diluted to 10^{-4} dilutions and 100 μl was inoculated on the MSG agar plate by the spread plate method. Plates were incubated at 37 °C and colonies formed were counted after 24 h of incubation. The control was maintained simultaneously in the absence of AFB_1. The colony forming units were calculated by this formula:

Number of colonies × dilution of plate/Volume of culture on plate = CFU ml^{-1}.

2.4. Degradation studies of AFB_1 by *P. putida*

A degradation study of AFB_1 was performed in MSG medium for 24 h. Every 6 h, analysis was carried out. Twenty milliliters of the MSG medium in 100-ml conical flasks was inoculated with *P. putida* cells (9×10^5). Hundred microliters of stock AFB_1 at a concentration of 40 μg ml^{-1} in dimethyl sulfoxide (DMSO) was added to all the test and control flasks. The final concentration of the AFB_1 was 0.2 μg ml^{-1} of the medium. In total, 45 flasks were used for each experiment in three sets: I—Control, without *P. putida* inoculation: II—with *P putida* strain 1274; and III—with *P. putida* strain 2445. The flasks were incubated for 24 h at 37 °C in a shaker incubator at 120 rpm. After each incubation period three flasks were removed from each set for analysis. The culture was centrifuged at 8000 rpm for 10 min at room temperature to separate the bacterial cells. The supernatant was taken in a separating funnel and extracted with an equal volume (20 ml) of chloroform. Extraction was done twice; extracts were pooled and concentrated to a volume of 5 ml. These samples were then assayed analytically and for cytotoxicity. The remaining AFB_1 in the sample was analyzed qualitatively by TLC and quantitatively by UV spectrophotometer (at 360 nm) and by HPLC.

A control experiment was performed by inoculating the *P. putida* into the MSG medium without AFB_1 for 24 h. Extraction and analysis were carried as described before. To find the AFB1 present in the bacterial cells, 1 ml of chloroform was added to the cell pellet and cells were suspended by repeated pipetting and centrifuged at 8000 rpm for 10 min at 4 °C. Supernatant was collected in new tube. The extraction was done twice; the supernatant was pooled and analysed as described above. In addition, the extracts were concentrated to 100 μl and analysed by TLC to confirm the absence of AFB1 in the pellet.

2.5. Degraded compound analysis

2.5.1. Column chromatography

For the purification of degraded product, the column chromatography technique was used. In order to scale up the degraded product, 200 conical flasks were used and the concentration level of AFB_1 in the medium was maintained at 0.2 μg ml^{-1}, as mentioned in the foregoing degradation procedure. The products were extracted using chloroform from the culture supernatant as described in Section 2.4. The crude extract was concentrated and column chromatography was performed using silica gel 60. The bed size was 122.65 cm^3. The elution was carried out with solvents chloroform: methanol (98:2) at a flow rate of 0.4 ml min^{-1}. Thirty-five fractions, each of 3 ml, were collected. After complete solvent evaporation, samples were re-dissolved in methanol. A wavelength scan was performed for all the samples using a UV spectrophotometer at 200–800 nm.

2.5.2. High-performance liquid chromatography (HPLC)

Analysis with HPLC was performed using the mobile phase methanol: water (1:1, v/v) isocratic at a flow rate of 1 ml min^{-1}, and an injection volume of 20 μl, measured by a UV-detector at 360 and 274 nm, respectively. Using standard AFB_1 the performance of the technique was monitored and the deviation in the retention time and the peak area was within the 5% error limit. The minimum detectable limit of AFB_1 by HPLC was observed as 6 ng/ml (Aiko and Mehta, 2013).

2.5.3. Fourier transform infrared (FTIR)

The purified sample was taken for IR analysis, which was carried out with a KBr pellet using an IR spectrophotometer (IR Affinity-1, Shimadzu, Japan).

2.5.4. Gas chromatography (GC–MS)

Pseudomonas sp. treated AFB_1 samples were fractionated by silica gel column and fed into a GC–MS (Thermo GC – trace ultra version: 5.0, thermo ms DSQ II, column: db35 – ms capillary standard non-polar, dimension: 30 Mts, ID: 0.25 mm, FILM: 0.25 μm, Carrier gas: Helium, flow rate: 1.0 ml min^{-1}, temperature program: oven temperature 40 °C rose to 270 °C at 8 °C min^{-1}) The injection volume was 1 μl.

2.6. Cytotoxicity test for AFB_1 and degraded products

In general, the degradation of toxins means complete or partial conversion to other less toxic or non-toxic molecules; however, in some cases more toxic compounds are also formed (Trivedi et al., 1993). Hence it becomes crucial to determine the toxicity of degraded toxins. The toxicity study of AFB_1 and AFB_1 treated with *P. putida* was examined on HeLa cells by the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) method (Green et al., 1984; Kitabatake et al., 1993). For this purpose the chloroform was completely evaporated from the standard and Pseudomonas-treated AFB_1 samples. Samples were re-dissolved in a minimal quantity of DMSO to keep the final DMSO concentration less than 1% in the culture medium. Samples were diluted with the culture medium DMEM (Dulbecco minimal essential medium) containing 10% FBS to obtain the concentrations of 2 μg, 4 μg, 6 μg, and 10 μg ml^{-1}. HeLa cells (1×10^4 cells $well^{-1}$) were incubated with test samples at 37 °C under a 5% CO_2 atmosphere for 48 h, followed by the addition of MTT and further incubation for 4 h. The resultant blue formazan was dissolved in DMSO. The cytotoxicity was evaluated by the absorption at 540/655 nm with an ELISA reader (BIORAD, Model-680). Mean absorbance values and standard deviation of each sample was compared with those of the corresponding control and expressed as percentage cytotoxicity. These experiments were carried out in triplicate.

3. Results

3.1. Isolation and purification of AFB_1

The toxigenic strain of *A. flavus*, used in the present study to produce AFB_1, was isolated from the fresh sugarcane collected from a retail shop during the survey of fungal contamination. From the colony appearance, morphological characteristics, and conidial arrangement the isolate was identified as *A. flavus*. The partial 18s rDNA sequence showed 99% sequence similarity to *A. flavus* strain NRRL 62477 (580/586 nt). The fungal sample was deposited as strain 1817.Sc3 in the Agharkar Research Institute, Pune, India. The isolate was screened for the production of AFB_1 by being grown in YES medium and incubating for 7 days. The chloroform extract of spent medium showed the presence of an AFB_1 spot on TLC at R_f value 0.7. The minimum detectable limit of AFB_1 on TLC was 10 ng $spot^{-1}$ in the present study. The presence of AFB_1 was also confirmed by spiking with standard on the TLC as well as in the HPLC along with the sample. The isolated *A. flavus* was found to produce AFB_1 at a concentration of 5 μg ml^{-1} in YES medium after 7 days of incubation. After purification with column chromatography, 70% AFB_1 was recovered. The purified AFB_1 gave a single peak in HPLC at a retention time of 7.16 min similar to standard AFB_1 and used for further degradation studies.

3.2. Growth studies of P. putida in the presence of AFB_1

The growth studies showed that, after a lag phase of about 6 h, *Pseudomonas* sp. grew fast and remained in exponential phase up to 24 h in the absence of AFB_1. In the presence of AFB_1 (0.2 μg ml^{-1}) the growth was retarded and optical density reached a maximum 0.125 in 24 h. However, colony formation increased by 28 times more than that of the initial inoculum after 24 h of incubation in both strains (Table 1, Fig. 1a and b). This indicates that AFB_1 is not lethal to *Pseudomonas* sp. but suppresses the growth. After 24 h the optical density increased exponentially in the test flask and by 72 h the O.D 600 nm became similar to that of the control flask. From this study it can be inferred that by 24 h all the AFB_1 present in the medium got degraded by the *P. putida*. The resultant degraded products are nontoxic to the *P. putida*; hence it grew exponentially up to 60 h and by 72 h reached study state.

3.3. Degradation studies of AFB_1

In order to measure the residual AFB_1 the samples were taken every 6 h and analyzed by spectrophotometry. The AFB_1 showed a linear correlation between absorbance at 360 nm and concentrations ranging from 0.25 to 1.5 μg ml^{-1}. This linear correlation (Fig.2, Inset) is used to calculate the residual AFB_1 in the culture supernatant. The degradation of AFB_1 by *P. putida* strains was observed in MSG medium at 37 °C up to 24 h. Samples were taken every 6 h and residual AFB_1 was analyzed in chloroform extract by spectrophotometer. A gradual reduction in AFB_1 concentration was observed after a lag phase of 6 h, and by 24 h the remaining AFB_1 was almost undetectable on TLC (Figs. 2 and 4). Both strains of *P. putida* (1274 and 2445) showed the capability to degrade AFB_1. At 6 h of incubation, strain 2445 degraded about 10% of AFB_1, while strain 1274 showed no change in AFB_1 content. By 24 h incubation both strains of *P. putida* degraded AFB_1 to about 90%. There was no change in the content of AFB_1 in the uninoculated control throughout the incubation period. The AFB_1 was not detected in the bacterial cell pellet remaining after the removal of the broth by centrifugation.

The preliminary cytotoxicity studies showed that AFB_1 is toxic to HeLa cells at 2 μg ml^{-1} concentration within 24 h of incubation, as shown in photomicrographs (Fig. 3). All cells appeared round in the well treated with AFB_1. However, the treated sample did not show

Table 1
Colony forming units (CFU ml^{-1}) of *P. putida* strain 1274 and 2445 at 0, 6, 12, 18 and 24 h of incubation with and without AFB_1.

Time (h)	1274		2445	
	Control	0.2 μg ml^{-1} AFB_1	Control	0.2 μg ml^{-1} AFB_1
0	$2.0 \times 10^6 \pm 0.17$	$2.00 \times 10^6 \pm 0.17$	$1.86 \times 10^6 \pm 0.03$	$1.86 \times 10^6 \pm 0.06$
6	$2.5 \times 10^7 \pm 0.05$	$2.4 \times 10^7 \pm 0.10$	$2.41 \times 10^7 \pm 0.01$	$2.07 \times 10^7 \pm 0.21$
12	$6.34 \times 10^7 \pm 0.12$	$3.04 \times 10^7 \pm 0.10$	$6.06 \times 10^7 \pm 0.08$	$2.84 \times 10^7 \pm 0.09$
18	$7.51 \times 10^8 \pm 0.16$	$4.2 \times 10^7 \pm 0.31$	$6.92 \times 10^8 \pm 0.07$	$4.01 \times 10^7 \pm 0.15$
24	$9.10 \times 10^9 \pm 0.15$	$6.80 \times 10^7 \pm 0.13$	$8.76 \times 10^9 \pm 0.03$	$6.64 \times 10^7 \pm 0.03$

Control – Pseudomonas culture without AFB_1. All observations are taken in triplicate and standard deviation (SD) is given as ±.

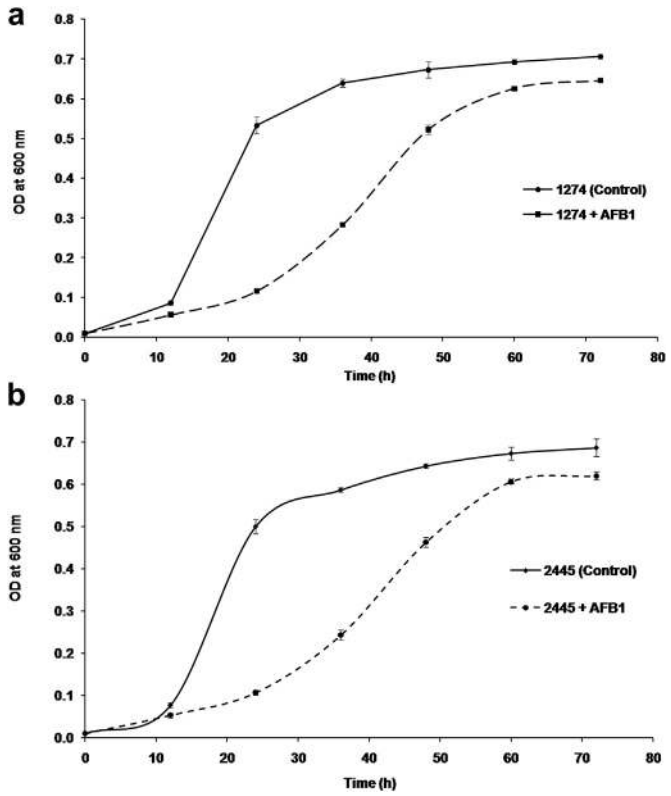


Fig. 1. a: Effect of AFB_1 on the growth of *P. putida* strain (1274). Growth observed up to 72 h of incubation in minimal salt glucose medium at 37 °C. (—■—) Culture medium + AFB_1; (—●—) Culture medium − AFB_1 by measuring the absorbance at 600 nm. Figure 1b: Effect of AFB_1 on the growth of *P. putida* strain (2445). Growth observed up to 72 h of incubation in minimal salt glucose medium at 37 °C. (—●—) Culture medium + AFB_1; (—▲—) Culture medium − AFB_1 by measuring the absorbance at 600 nm.

any toxicity even at 10 μg ml^{-1} concentration, which is five times higher than that of the AFB_1. All cells appear well spread. These results show that *P. putida* can transform the AFB_1 into nontoxic molecules.

3.4. AFB_1 degradation product analysis

AFB_1 in the presence of *P. putida* was degraded or bio-transformed as observed from TLC analysis, the absence of characteristic blue fluorescent spots of AFB_1 in filtrate showed degradation of AFB_1 and exhibited the presence of new non-fluorescent spots, as shown in the inset of Fig. 4. The chloroform extract from the uninoculated sample showed an AFB_1 spot with characteristic blue fluorescence. Similarly, HPLC analysis (Fig. 4) showed the absence of peaks in the samples treated with *P. putida* corresponding to standard AFB_1. Even after prolonged incubation, AFB_1 was not detected by either TLC or HPLC, which shows that the degradation of AFB_1 by the *P. putida* is irreversible. The UV absorption spectrum of AFB_1 and degraded products showed the λ_{max} at 360 nm and 274 nm, respectively (Table 2). A subsequent HPLC chromatogram at 360 nm confirmed that the AFB_1 peak eluting at 7.19 min disappeared and new peaks were noted before 3 min of elution at 360 nm detection; similarly, peaks of higher intensity were noted at 274 nm detection (Fig. 5a and b). This showed that AFB_1 is bio-transformed to other compounds. This result indicates that there may be more than one compound formed on decomposition.

Further studies of the degraded products were carried out at 274 nm. The degraded compounds were produced in large quantities, extracted with chloroform, and purified using silica gel column chromatography. The column chromatographic separation showed several peaks corresponding to decomposed product (Fig. 6). The major compounds were further purified by silica gel column. The fractions corresponding to the peaks D_1, D_2, and D_3 were subjected to further analysis to determine the structure. FTIR spectral analysis was carried out to determine the functional groups and elucidate the structures of the degraded AFB_1 molecules D_1, D_2, and D_3. The IR spectra of AFB_1 showed the broad and strong absorption peak in the region of 2926 cm^{-1} to 2854 cm^{-1}, which indicates the stretching vibrations of H—C—H. The peaks at 1743 cm^{-1} and 1708 cm^{-1} were assigned to the stretching vibration of the two carbonyl groups. The peak at 1629 cm^{-1} denotes C=C symmetric stretching. The peak at 1485 cm^{-1} denotes H—C—H bending vibrations. Stretching of C—O was indicated by the peaks at 1271, 1219, 1188, and 1097 cm^{-1}. The IR spectrum of D_1 showed the strong and broad vibrational bands in the region of 3130 cm^{-1} to

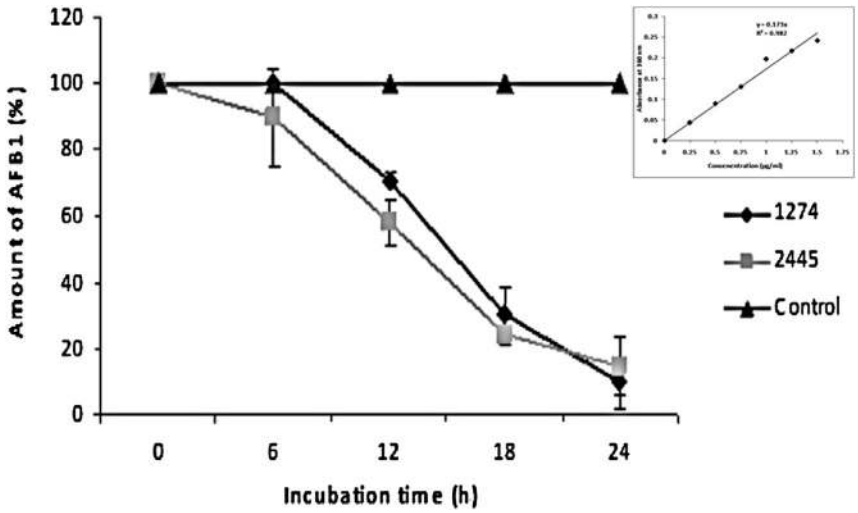


Fig. 2. Reduction in AFB_1 levels after incubation with *P. putida* strains. The absorbance was measured at 360 nm and concentration of AFB_1 was determined using standard curve of concentration verses absorbance given in inset of figure. Incubation of AFB_1 in the presence of *P. putida* 1274 strain − (—◆—), 2445 strain − (—■—), without *P. putida* strain − (—▲—) (control).

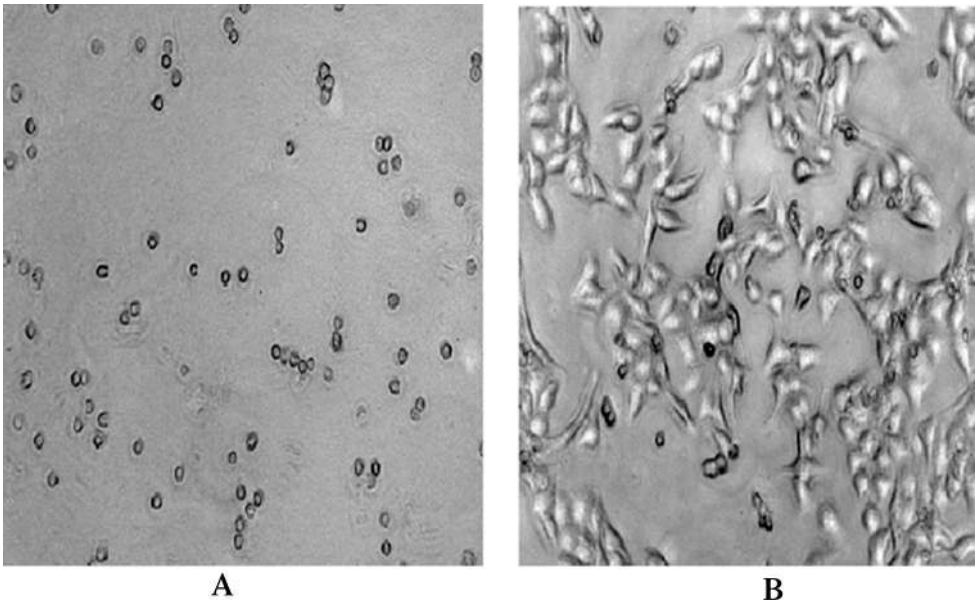


Fig. 3. Cytotoxicity of AFB_1 and AFB_1 treated with *P. putida*. Photograph of HeLa cells after 24 h of incubation in the presence of AFB_1 or treated AFB_1 at concentration of 4 μg ml^{-1}. A – with AFB_1. B – equivalent amount of treated AFB_1.

3539 cm^{-1} (for O–H stretching vibrations); 2954 cm^{-1} denotes the aromatic C=C–H stretch. Other bands at 1639 cm^{-1}, 1616 cm^{-1}, and 1082 cm^{-1} indicate the C=C and C–O stretchings. The second degraded product (D_2) had vibration bands for O–H at 3471 cm^{-1}, H–C–H at 2927 cm^{-1}, C=C stretch at 1639 cm^{-1} and 1618 cm^{-1}, and C–O at 1082 cm^{-1}.

For the third degraded product (D_3), the IR spectra showed the broad band in region 3238 to 3471 cm^{-1} for the O–H stretch, 1639 cm^{-1} and 1616 cm^{-1} for the C=C stretch, and the C–O stretch at 1120 cm^{-1} and 1088 cm^{-1}. The results obtained from D_1, D_2, and D_3 signified that the carbonyl group (C=O) is completely absent in the treated sample. From this we can infer that the lactone portion present in the AFB_1 structure has been removed; thus the compounds D_1, D_2, and D_3 are non-fluorescent (Table 2).

The analysis by GC–MS showed AFB_1 *m/z* peak at 312 (Grove et al., 1984). The mass spectrum of the D_1 sample showed intense peak values at *m/z* 285.9, 273, 255, and 129.

Moreover, the carbonyl group in the keto form of D_1 was absent in the IR data; it is presumed that the molecule may exist in the enol form as given in the structure. Similarly, samples D_2 and D_3 showed an intense peak at 206 and 149, respectively. This mass spectrum doesn't show any *m/z* 312 value, attributed to AFB_1. As a result, we inferred that AFB_1 was bio-transformed by *P. putida* into other products (Table 2).

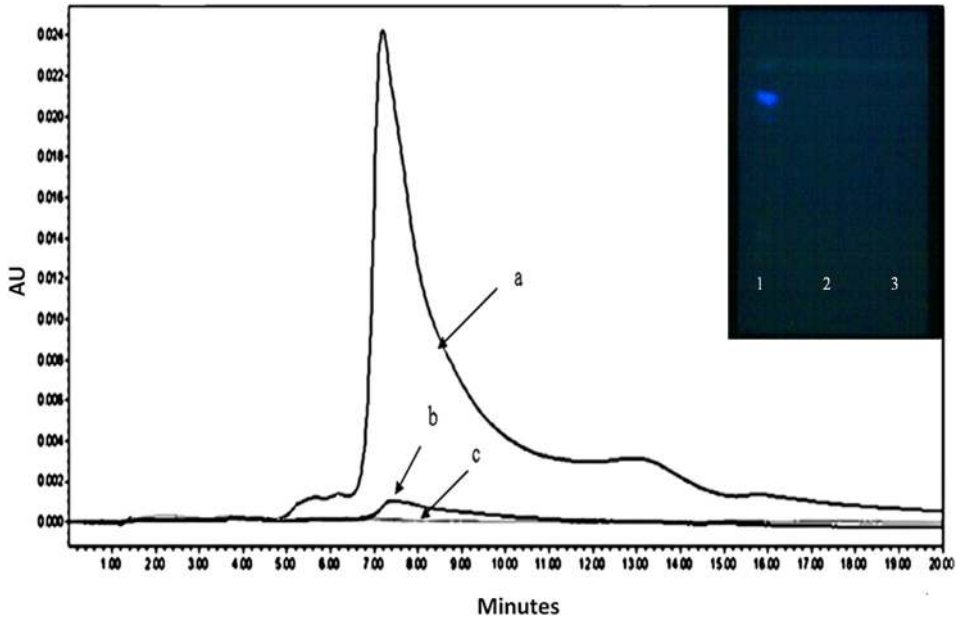
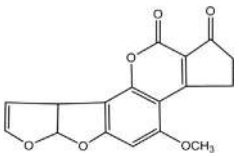
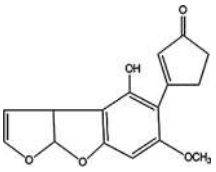
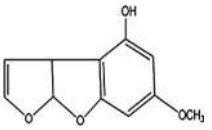
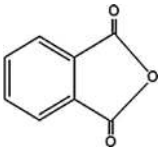


Fig. 4. High pressure liquid chromatogram of AFB_1 standard incubated with *P. putida* for 24 h a – AFB_1 control showing an absorption peak at a retention time of 7.19 min. AFB_1 sample extracted after 24 h incubation with *P. putida* cultures b – 1274 and c – 2445. Inset-thin layer chromatogram of AFB_1 after incubation with *P. putida* culture for 24 h. 1 – AFB_1 control, 2 and 3 – AFB_1 incubated with *P. putida* cultures 1274 and 2445 respectively. The solvent system used; Chloroform: acetone (85:15).

Table 2
Identification of degraded compounds based on the UV spectrophotometer, FT-IR and GC–MS.

Degraded compound	UV-spectrometry λ_{max}, nm	FT-IR vibrational frequency (cm^{-1})	Mass spectral data	Expected structure
1: AFB_1 Mass (m/z): 312 Molecular formula: $C_{16}H_{18}O_4$	360 nm	(1) 3387 (–OH) (2) 2926, 2854 (C=C–H) (3) 1743, 1708 (C=O) (4) 1629 (C=C) (5) 1279 (C–O stretch)	312, 297, 294, 284, 269, 253, 219, 218, 191, 181 (Grove et al., 1984)	
1: D_1 Mass (m/z): 286 Molecular formula: $C_{16}H_{14}O_5$	274 nm	(1) 3471 (–OH) (2) 1639 (C=C) (3) 1616 (C=C) (5) 1082 (C–O stretch)	57, 71, 85, 97, 111, 129, 147, 183, 236.6, 255.2, 273.1, 285.2	
1: D_2 Mass (m/z): 206 Molecular formula: $C_{17}H_{14}O_6$	274 nm	(1) 3471 (–OH) (2) 1639 (C=C) (3) 1618 (C=C) (4) 1217, 1139, 1089 (C–O stretch)	57, 74, 91, 147, 163, 191, 206	
1: D_3 Mass (m/z): 149 Molecular formula: $C_8H_4O_3$	274 nm	(1) 3471 (–OH) (2) 1639 (C=C) (3) 1616 (C=C) (4) 1138, 1120,1082 (C–O stretch)	55, 71, 83, 112, 132, 149, 167	

The most prominent degraded product of AFB_1 showed the *m/z* ion peak at 286 and loss fluorescence on TLC, indicating the opening of the lactone ring suggests that this might be AFD_1 (Mendez-Albores et al., 2008). The second major *m/z* ion peak at *m/z* 206 may be AFD_2 was in strong conformity with the fragmentation pattern exhibited by Cucullu et al. (1976). Thus, we propose that the lactone ring of AFB_1 opened in the presence of *P. putida,* which resulted in decarbonylation of AFB_1 to AFD_1 and AFD_2. It is interesting to note that the third product (AFD_3) was also found to be an aromatic compound showing a molecular ion peak at *m/z* 149 (Table 2). Further, the qualitative analysis by a fluorescence test using resorcinol was positive and indicated the presence of a dicarboxylic acid; presumably the product was phthalic anhydride. These results were reproducible and they were further compared and confirmed with the authentic samples of phthalic anhydride. The mechanism pertaining to the formation of phthalic anhydride is not clearly understood. In the current study, results from the UV spectrophotometer, as well as HPLC, GC–MS, and FTIR spectral data confirmed the biotransformation of AFB_1 by *P. putida* (Fig. 7).

3.5. Toxicity study of degraded AFB_1

The toxicity of AFB_1 and treated AFB_1 toward HeLa cells was compared. The HeLa cells were cultured in the presence of selected concentrations ranging from 2 to 10 μg ml^{-1} and the toxicity was measured, by MTT assay, in terms of live cells present after 48 h of incubation. AFB_1 showed cytotoxicity of about 50% at a concentration of 2 μg ml^{-1} and 90% at 4 μg ml^{-1}. Concentrations of more than 4 μg ml^{-1} showed 100% cytotoxicity. However, the treated AFB_1 (crude) did not show any toxic effect up to a concentration of 6 μg ml^{-1}. At the 10 μg ml^{-1} a 70% cytotoxicity was measured (Fig. 8). The purified degraded products from the treated samples, namely D_1, D_2, and D_3, also showed lower toxicity toward HeLa cells. The D_1 enhanced the growth of the HeLa cells at the lower concentrations of 2 and 4 μg ml^{-1}. At 6 μg ml^{-1} and above the toxicity level did not increase beyond 20%. D_2 and D_3 also showed EC_{50} 5.2 and 7.5 μg ml^{-1} which is much lower than AFB_1. The outcome of the study clearly implies that the degraded compounds are nontoxic (D_1) or much less toxic (D_2 and D_3) than the AFB_1 to the cells at the concentrations tested. This detoxification may be due to the cleavage in the furan and lactone rings on the AFB_1 molecule. These results proved the efficient degradation and detoxification of AFB_1 by *P. putida*.

4. Discussion

This study found that *P. putida* degrades AFB_1. The results from TLC, HPLC, FTIR, and GC–MS analysis confirmed AFB_1 biotransformation and detoxification by *P. putida*. This might be because of the opening of the lactone ring. We hypothesize that detoxification of AFB_1 yields aflatoxin D_1, a nonfluorescent compound (mol wt 285.9 g moL^{-1}), which exhibits phenolic properties. Other studies also reported AFD_1 is less toxic and mutagenic than AFB_1 (Lee et al., 1981; Mendez-Albores et al., 2005, 2007). The second compound, a nonfluorescent phenol (mol wt 206 g moL^{-1}, commonly known as aflatoxin D_2 (AFD_2)), retained the di-furan moiety but lacked both the lactone carbonyl and the cyclopentenone ring characteristic of the AFB_1 molecule (Fig. 7). The mass spectral data compared well with the literature reports on mass spectral fragmentation patterns of AFD_1 and AFD_2. The biological activity, such as mutagenicity and toxicity of AFD_1, is much lower than that of AFB_1 as reported by Lee et al. (1981) using the Ames test and chick embryo test. In conformity with these studies we observed that the designated

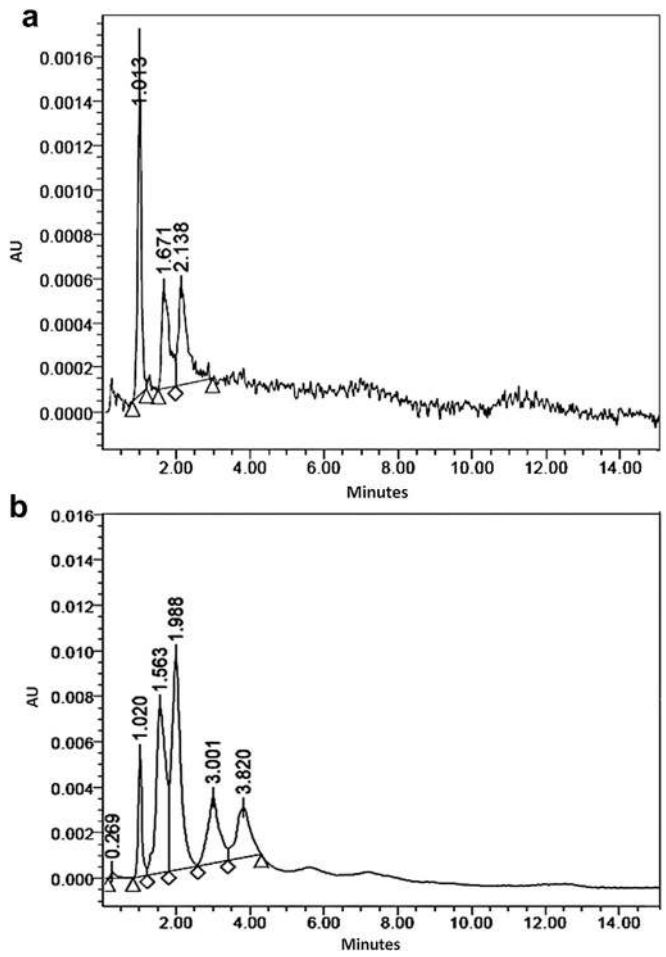


Fig. 5. Detection of AFB_1 degraded product by HPLC. AFB_1 incubated with culture *P. putida* for 24 h, (a) detection at 360 nm (b) detection at 274 nm.

compound was not toxic to the HeLa cells (Fig. 8) at the concentrations (6 μg ml^{-1}) AFB_1 showed 100% cytotoxicity.

Apart from AFD_1 and AFD_2, which are well reported in the literature from the ammoniation and citric acid treatment of AFB_1 (Lee et al., 1974; Cucullu et al., 1976; Lee and Cucullu, 1978; Mendez-Albores et al., 2008), a third compound was observed and designated as AFD_3 (Table 2). The mass spectrum and fluorescent test confirmed it as phthalic anhydride. Further studies are required to find whether it is a derivative of AFB_1 or is produced by

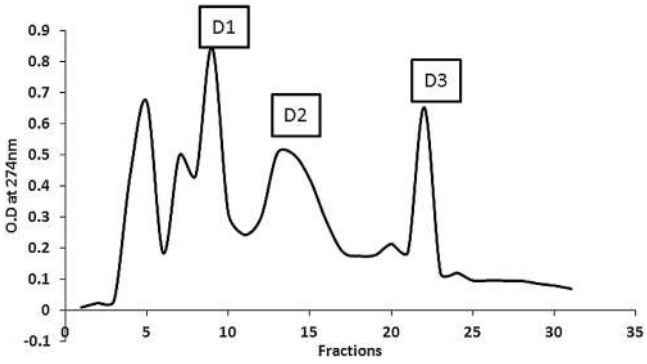


Fig. 6. Silica gel column chromatogram of AFB_1 degraded product. D_1, D_2 and D_3 peaks of major products were further analyzed.

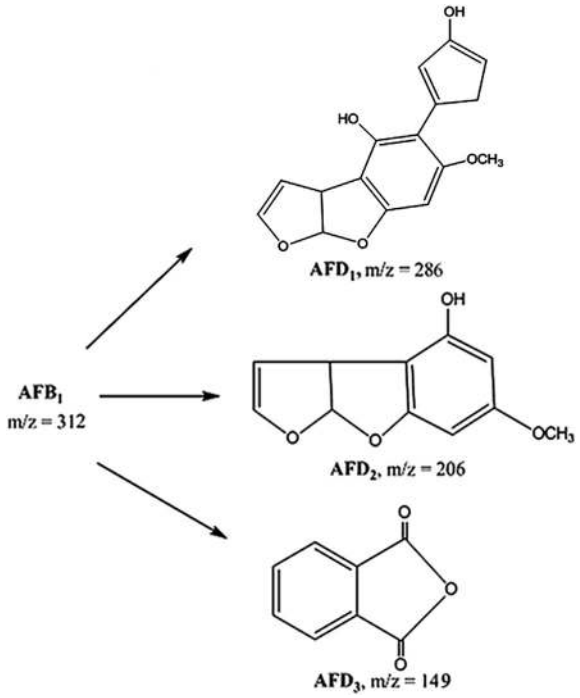


Fig. 7. Proposed scheme of AFB_1 degradation. The peaks D_1, D_2 and D_3 (Fig. 6) were identified based on the UV-spectrum, FT-IR and GC–MS (Table 2).

Pseudomonas sp. in the stressed condition created by the presence of AFB_1 in the culture medium. AFD_3 was also found to be much less toxic to HeLa cells. Though AFB_1 is not lethal to *P. putida*, it highly suppressed its growth (Fig. 1a and b). Once AFB_1 was completely degraded, the *P. putida* strains grew exponentially and reached an optical density similar to that of the control by 72 h. Concentrations of AFB_1 higher than 0.2 μg ml^{-1} could not be tolerated by the *P. putida* strains1274 and 2445 and limits their capability to degrade higher concentrations of AFB_1.

This study corroborates the recent reports of Elaaser and El Kassas (2011) and Krifaton et al. (2011) about use of Pseudomonas and other soil bacteria for the degradation and detoxification of AFB_1. Elaaser and El Kassas (2011) reported a 69% reduction

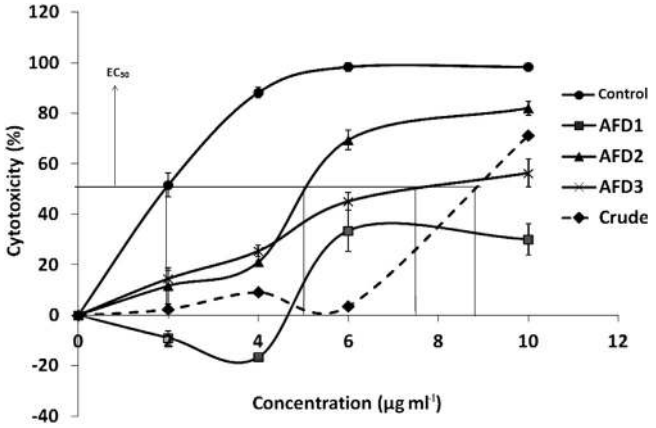


Fig. 8. Cytotoxicity of AFB_1 and purified degraded product. Cytotoxicity assay performed on HeLa cells by MTT assay absorbance taken at 540/655 nm. Control (AFB_1) without *P. putida* (—●—), Crude (—◆—), AFD_1 (—■—), AFD_2 (—▲—) and AFD_3 (—×—). EC_{50} was calculated for, Control (AFB_1) – 2 μg ml^{-1}; AFD_1 → maximum concentration used; AFD_2 – 5.2 μg ml^{-1}; AFD_3 – 7.5 μg ml^{-1}; Crude – 8.5 μg ml^{-1}.

in AFB_1 after 72 h incubation at 37 °C and detoxification attributed to the extracellular enzymes. Krifaton et al. (2011) were able to achieve more than 90% degradation with concomitant removal of genotoxicity at a 4 μg ml^{-1} AFB_1 concentration. The present study also reports the major degradation products from AFB_1. These results imply the prospective applications of *P. putida* or its gene/genes responsible for detoxification. As in the case of zearalenone, enzymes involved in the degradation are encoded by the plasmid pZEA-1, (Altalhi and El-Deeb, 2009), which may be the case with AFB_1. Furthermore, the feasibility of detoxification in contaminated grains has to be studied.

5. Conclusions

This work reports that *P. putida* can tolerate aflatoxin in the culture medium and degrade it effectively. It showed the capability of degrading AFB_1 at a concentration of 0.2 μg ml^{-1}. In addition, the results demonstrated that *P. putida* (1274 and 2445) is able to achieve biotransformation and detoxification of AFB_1. The major degraded compounds were identified as AFD_1, AFD_2 and AFD_3 based on data obtained from TLC, HPLC, UV spectrometry, GC–MS, and FTIR analysis.

Acknowledgments

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