

# Structure and toxicity analysis of aflatoxin B<sub>1</sub> biodegraded products by culture supernatant of *Cladosporium uredinicola*

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**ABSTRACT:** Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is one of mycotoxins leading to serious food contaminations and human diseases. To solve these problems, biodegrading technique has been concerned. Previous researches showed that AFB<sub>1</sub> can be effectively degraded by the culture supernatant of many microorganisms. However, the biodegraded products have not been identified. In this study, to explore the structure of the products, the AFB<sub>1</sub> was degraded by the culture supernatant of *Cladosporium uredinicola*. Two biodegraded products of AFB<sub>1</sub> were collected by high performance liquid chromatography (HPLC) and analyzed by liquid chromatography quadruple time-of-flight mass spectrometry (LC-Q-TOF MS) and chromatography-tandem mass spectrometry (MS/MS). As a result, the molecular weight of the two products were 406 and 342, and the structure of the two products were inferred. The toxicity of the biodegraded products was evaluated based on quantitative structure-activity relationship (QSAR) and cytotoxicity experiment. In conclusion, the double bonds of AFB<sub>1</sub> had a high probability of being destroyed. The toxicity of the two biodegraded products compared with that of AFB<sub>1</sub> was reduced. The culture supernatant of *C. uredinicola* was used as an effective way to detoxify AFB<sub>1</sub>.

**KEYWORDS:** aflatoxin B<sub>1</sub>, biodegraded products, structure, toxicity, *Cladosporium uredinicola*

## INTRODUCTION

Aflatoxins (AFT) are polyaromatic mycotoxins that are secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* [1]. For its strongly toxic, mutagenic and carcinogenic properties, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) was classified as a group 1 carcinogen by International Agency for Research of Cancer [2]. Many economic losses are caused by aflatoxin contamination in food and feeds such as peanuts, maize and milk [3–6]. Therefore, a suitable detoxication method is imperative.

In terms of decontamination techniques, in conformity with Food and Agriculture Organization (FAO) the requirements are as follows: the detoxification method is supposed to guarantee the reduction of AFT without residual toxicity and guarantee the nutritional values without modification of food or feed properties [7]. Some physical and chemical methods that have been evaluated to reduce AFB<sub>1</sub> content can not be applied such as the addition

of chemical oxidants in food and feed. Therefore, biological manipulations for detoxification of AFB<sub>1</sub> were concerned. For instance, to achieve detoxification effect, AFB<sub>1</sub> is adsorbed by lactic acid bacteria [8] and degraded by *Rhodococcus erythropolis* ATCC 4277, *Streptomyces* sp. and *Lysinibacillus fusiformis* [9, 10]. However, by microorganism adsorption, the toxin can be reversible recovered, and therefore detoxification is not complete. Biodegradation has many advantages such as its efficiency, non-pollution and non-hazard.

Previous researches on the degraded products are contributed to the discovery of AFB<sub>1</sub> degradation mechanism. In many detoxification cases, degradation products were not found or reported. For example, there were no degradation products to be detected by ESI-MS and LC-MS after AFB<sub>1</sub> was degraded by the purified laccase from *Trametes versicolor* [11]. The first AFB<sub>1</sub> degradation products was a compound with new fluorescent blue [12]. Since then, many products from AFB<sub>1</sub>

degradation have been discovered and reported such as Aflatoxicol [13–15], Aflatoxin B<sub>2a</sub> [16–18], AFD<sub>1</sub> [9, 16], AFD<sub>2</sub> [9, 19], Phthalic anhydride [19] and AFB<sub>1</sub>-8,9-dihydrodiol [20]. Some other biodegraded products need further characterization. However, high degradation feasibility does not represent the reduction of toxicity. For example, AFB<sub>1</sub>-8,9-epoxide (carcinogenic form of AFB<sub>1</sub>) from the transformation of AFB<sub>1</sub> in humans by cytochrome P450 is more toxic than AFB<sub>1</sub> [21].

In previous study, we found AFB<sub>1</sub> was biodegraded efficiently by culture supernatant of *Cladosporium uredinicola* [22]. In this study, the biodegraded products of AFB<sub>1</sub> reacting with the culture supernatant of *C. uredinicola* were studied. The biodegraded products were separated by HPLC. The structures were deduced by LC-Q-TOF MS and MS/MS. The toxicity of the biodegraded products was evaluated using QSAR and cytotoxicity experiment. The culture supernatant of *C. uredinicola* was certified as an effective way to detoxify AFB<sub>1</sub>, and this study will facilitate the mechanism research about the biodegraded AFB<sub>1</sub> by *C. uredinicola*. This will help to develop great potential of *C. uredinicola* for food safety.

## MATERIALS AND METHODS

### Fungal strain, growth media and chemicals

*C. uredinicola* was collected in the China Center for Type Culture Collection (CCTCC) with the patent culture collection number: CCTCC M 2015181. The strain was maintained on potato dextrose agar (PDA) (Difco, Sparks, USA) and subcultured monthly. A spore suspension was prepared from a one-week-old culture at a concentration of  $1.0 \times 10^6$  cfu/ml and used as inoculum for this research. All incubations were done at 28 °C. *C. uredinicola* was proliferated in potato dextrose medium (PD) (potato 200 g/l and dextrose 20 g/l) and fermented in Czapek-Dox medium (sucrose 30 g/l, NaNO<sub>3</sub> 2 g/l, K<sub>2</sub>HPO<sub>4</sub> 1 g/l, MgSO<sub>4</sub> 0.5 g/l and FeSO<sub>4</sub> · 7H<sub>2</sub>O 0.01 g/l). The standard sample of AFB<sub>1</sub> purchased from Fermentek (Jerusalem, Israel) was dissolved in methyl alcohol. The hydrochloric acid was diluted to 0.5 mol/l by distilled water. Analytical grade methyl alcohol was purchased from Sigma (St. Louis, USA). Dichloromethane and other chemicals mentioned above were analytical grade from Sinopharm Chemicals Reagent Co., Ltd. (Shanghai, China).

### Biodegradation of aflatoxin B<sub>1</sub>

A spore suspension of *C. uredinicola* (1 ml) was added to 250 ml conical flask with PD medium (50 ml) and proliferated for 34 h. Then, the cells (11.25 ml) were transferred to 250 ml conical flask with Czapek-Dox medium (75 ml) and fermented for 36 h. The above process took place at 28 °C and 180 rpm on a shaking incubator (Zhicheng ZWY-2102, Shanghai, China) with a rotational radius of 5 cm. After centrifugation (9408g, 10 min), the supernatant was collected in a new 50 ml tube. The pH of supernatant was adjusted to 7.0 by hydrochloric acid (0.5 mol/l).

Five µg AFB<sub>1</sub> was incubated with 1 ml culture supernatant at 37 °C for 24 h. Following the incubation, the residual AFB<sub>1</sub> and products were extracted by dichloromethane three times. Then, the dichloromethane was evaporated in vacuum drying oven (Jinghong D27-6090, Shanghai, China). Finally, the residual AFB<sub>1</sub> and products were dissolved in 1 ml methanol for HPLC (Shimadzu LC-20AD series, Japan) detection.

### Collection and analysis of biodegraded products

The HPLC was performed on a 4.6 mm × 250 mm 5 µm Shim-pack VP-ODS C<sub>18</sub> column. The injection volume was 200 µl. The mobile phase was methanol:water (4:6, v/v). The total run time was 60 min with flow rate of 0.7 ml/min.

The products collected by HPLC were injected in to the LC-Q-TOF MS (Agilent 6224 LC-MS, USA). Chromatography was performed on a 2.1 mm × 150 mm 5 µm Agilent extent C<sub>18</sub> column. The mobile phase was methanol:water (6:4, v/v). The total run time was 30 min with flow rate of 0.2 ml/min. MS was performed with an Agilent 6224 ESI Q-TOF. The operating conditions were as follows: MS source voltage, capillary voltage and skimmer was 3.5 kV, 150 V and 65 V, respectively. The gas temperature was 350 °C with the flow rate of 9 l/min. Collision gas was Nitrogen. MS spectra were acquired in a full scan analysis within the range of 100–1000 m/z using an extended dynamic range and a scan rate of 1.4 spectra/s and by varying collision energy with mass. The data were analyzed by the Mass Hunter Workstation software.

The samples were further analyzed by chromatography-tandem mass spectrometry (MS/MS, AB SCIEX Triple Quad 4500, USA) with the turbo spray ionization. All of the data have been calculated by the Mass Hunter Workstation software. In this way, the molecular formulae and

elemental composition of degraded products of AFB<sub>1</sub> can be predicted.

### Cytotoxicity test

Human hepatocellular liver carcinoma cell line (HepG2) was treated by the degraded products and AFB<sub>1</sub>. The new dulbecco's modified eagle media (DMEM) containing test samples (the products and AFB<sub>1</sub>, 5 µg/ml) were added into wells. The DMEM was added into wells as control group. Each sample was added into 5 wells. Then, the cells (10 000 cells per well) were seeded into each well of 96-well culture plate (Corning Inc., NY, USA) and incubated at 37 °C for 12 h under 5% CO<sub>2</sub> atmosphere. Following the incubation, the images of cell morphology were obtained by inversion fluorescence microscope (IX73, OLYMPUS, Japan).

## RESULTS AND DISCUSSION

### Discovery and separation of biodegraded products

In the previous researches, AFB<sub>1</sub> was treated with Ultraviolet (UV), gamma ray [23–25], ozone [26] and plant extracts [27–29] and then structural identification of products was performed. A large number of degraded products of AFB<sub>1</sub> have been shown. The mass spectral information about products obtained by LC-Q-TOF MS showed that the products had no significant difference from each other in terms of the structures and properties.

In this case, degradation rate of AFB<sub>1</sub> by supernatant can reach 96% (Fig. 1). To obtain satisfactory separation for the two biodegraded products, the different mobile phases of HPLC were used. Finally, the mobile phase was methanol:water (4:6, v/v). In Fig. 1, two biodegraded products (called P<sub>1</sub> and P<sub>2</sub>) of AFB<sub>1</sub> were discovered. The amount of products and AFB<sub>1</sub> was negatively correlated. It was sure that P<sub>1</sub> and P<sub>2</sub> are derived from AFB<sub>1</sub> degradation. For the accuracy of detection, P<sub>1</sub> and P<sub>2</sub> were collected separately for characterization.

### Molecular formulas of biodegraded products

To predict the molecular formulas of P<sub>1</sub> and P<sub>2</sub>, Mass Hunter Workstation software was used to obtain Q-TOF MS data of biodegraded products; all possible molecular formulas were calculated and listed. Accurate masses were obtained using Agilent 6224 LC-MS with a mass error less than 5 ppm, which helped get one or a few possible formulas quickly. However, during the analysis of molecular formulas, many

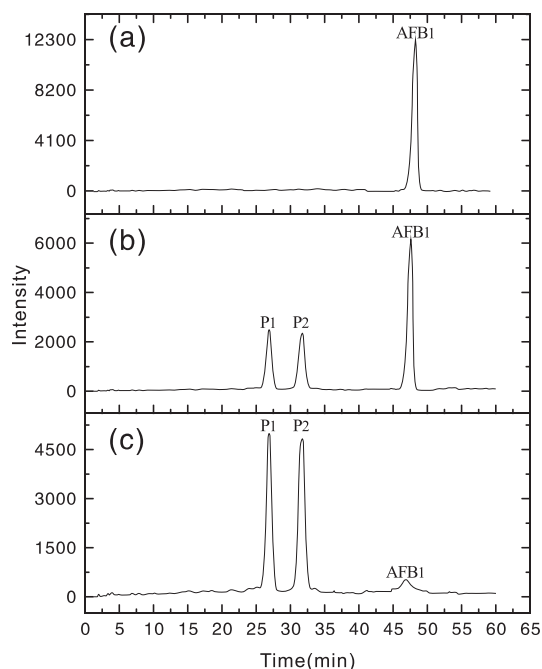


Fig. 1 The liquid chromatograms of AFB<sub>1</sub> treated by culture supernatant with different treating times: (a) 0 h, (b) 12 h and (c) 24 h.

chemical formulas may be proposed, which makes it difficult to obtain exact and correct molecular formulas for biodegraded products.

In this case, the structural characterization of P<sub>1</sub> and P<sub>2</sub> was detected by LC-Q-TOF MS. Besides LC-Q-TOF MS data, experience is also required to analyze the molecular formulas of these biodegraded products. Because NaNO<sub>3</sub> as a sole nitrogen source can not react with AFB<sub>1</sub>. In addition, amide synthase and transaminase are mainly intracellular enzyme. There has been no report about the biodegraded products of AFB<sub>1</sub> that contained nitrogen element. Therefore, P<sub>1</sub> and P<sub>2</sub> are considered to be composed of three elements, i.e. carbon, hydrogen and oxygen.

In the positive ion mode, both the P<sub>1</sub> and P<sub>2</sub> exhibited conspicuous ESI ionization efficiency. In Fig. 2A, 448.2285 m/z is for [M+H<sub>2</sub>O+Na]<sup>+</sup>, 426.7717 m/z is for [M+Na]<sup>+</sup> and 406.9998 m/z is for protonated adduct [M+H]<sup>+</sup>. Then the mass and the possible molecular formula of P<sub>1</sub> was determined, respectively, to be 406 m/z and C<sub>19</sub>H<sub>18</sub>O<sub>10</sub> by Mass Hunter Workstation software. In the same way, in Fig. 2B, 365.0632 m/z is for sodium adduct [M+Na]<sup>+</sup> while 343.0809 m/z is for protonated adduct [M+H]<sup>+</sup>. In addition, the hydrophilicity of

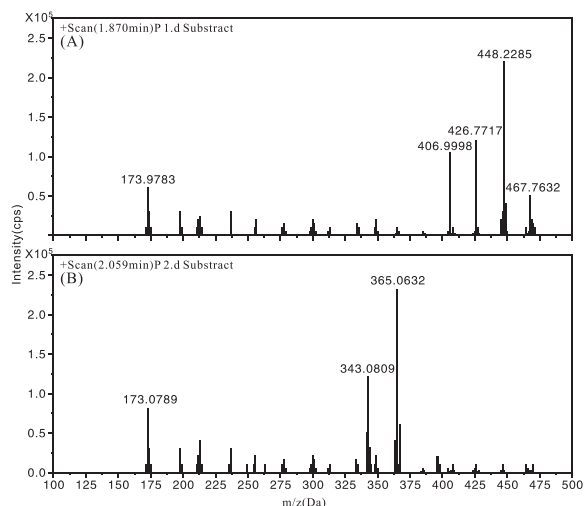


Fig. 2 LC-Q-TOF MS analysis of (A) P<sub>1</sub> and (B) P<sub>2</sub>.

biodegraded products is more than AFB<sub>1</sub>. Therefore, the mass and the possible molecular formula of P<sub>2</sub> was determined, respectively, to be 342 m/z and C<sub>18</sub>H<sub>14</sub>O<sub>7</sub>.

### Structure formulas of biodegraded products

According to the mass of two products, they might be unknown compounds. The conventional method for authenticating the unknown compounds was to collect a certain number of standard samples and then to authenticate their structures by conducting nuclear magnetic resonance (NMR), infrared spectrum (IR), mass spectrometry (MS) and ultraviolet spectrum (UV). Because the concentration and purity of the acquisition that biodegraded products were collected by micro-preparation can not meet the conditions of conventional method. In this case, HPLC has been used to separate and collect biodegraded products, and then LC-Q-TOF MS was used to accurately determine the mass of biodegraded products. But, the captured accurate mass and the deduced molecular formula still can not elucidate the structure of biodegraded products. In order to overcome this difficulty, further study of types and structures of fragment ions was necessary.

The information about parent ions and loss ions could be obtained by Mass Hunter Workstation software during the experiment. For each biodegraded product, the theoretical mass, determined accurate mass, the theoretical mass error, determined accurate mass error and the double bond equivalents (DBEs) quantity of two biodegraded products that were obtained by Mass Hunter Workstation software were listed in the Table 1. DBEs were essential

condition which were a calculate indication for the number of double bonds or rings in the unknown compound structure. The double bond equivalent (DBE) of AFB<sub>1</sub> was 12, and the DBEs of P<sub>1</sub> and P<sub>2</sub> were 11 and 12. When the DBE of biodegraded products was lower than that of AFB<sub>1</sub>, it implied that the double bonds had a high probability of being destroyed.

In Fig. 3, a mass spectrum of AFB<sub>1</sub> was obtained to supply the supplementary information for the present analysis. The fragmentation pathway of AFB<sub>1</sub> was shown. The continuous loss of carbon monoxide (CO) was the main fragmentation pathway.

In Fig. 4A, the P<sub>1</sub> at 405.9 m/z corresponded to molecular formula C<sub>19</sub>H<sub>18</sub>O<sub>10</sub> had more C<sub>2</sub>H<sub>6</sub>O<sub>4</sub> molecules than AFB<sub>1</sub>. The DBE of P<sub>1</sub> was 11, which was one less than AFB<sub>1</sub> implying that additional reactions occurred on the furan rings. More details on the fragmentation pathway were shown.

In Fig. 4B, the P<sub>2</sub> at 343.1 m/z corresponded to molecular formula C<sub>18</sub>H<sub>14</sub>O<sub>7</sub> had more CH<sub>2</sub>O molecular than AFB<sub>1</sub>. The DBE of P<sub>2</sub> was 12, which was same as that of AFB<sub>1</sub>. Because P<sub>1</sub> and P<sub>2</sub> had the similar polarity and blue fluorescent. The structure was obtained by the addition of methoxy group on the furan ring. More details on the fragmentation pathway were shown. The P<sub>2</sub> can be regarded as a relatively stable transition from AFB<sub>1</sub> to P<sub>1</sub>. Of course, the accurate structure should be detected by NMR, IR and other detection means.

### Toxicity analysis of biodegraded products

The quantitative structure-activity relationship (QSAR) among AFB<sub>1</sub> has been deeply explored for many years [30–32]. A number of researches about the toxicity of AFB<sub>1</sub> have been reported. The toxicity of AFB<sub>1</sub> is mainly derived from cyclopentenone ring and the C8-C9 double bond forming the vinyl ether at the terminal furan ring [33–35]. The basis of toxic and carcinogenic activity of AFB<sub>1</sub> is mainly derived from the furan moiety, and double bond of the furan ring is a significant determinant of toxic potency. Therefore, removing the double bond of furan ring is an effective means to detoxify.

In this study, based on the data collected by Q-TOF, the DBEs of P<sub>1</sub> and P<sub>2</sub> were listed in Table 1. Based on the structure of biodegraded products, the addition reaction occurred on the double bond of the furan ring. Therefore, based on QSAR, the toxicity of two biodegraded products compared with that of AFB<sub>1</sub> was reduced.

Combined with cytotoxicity experiment, the

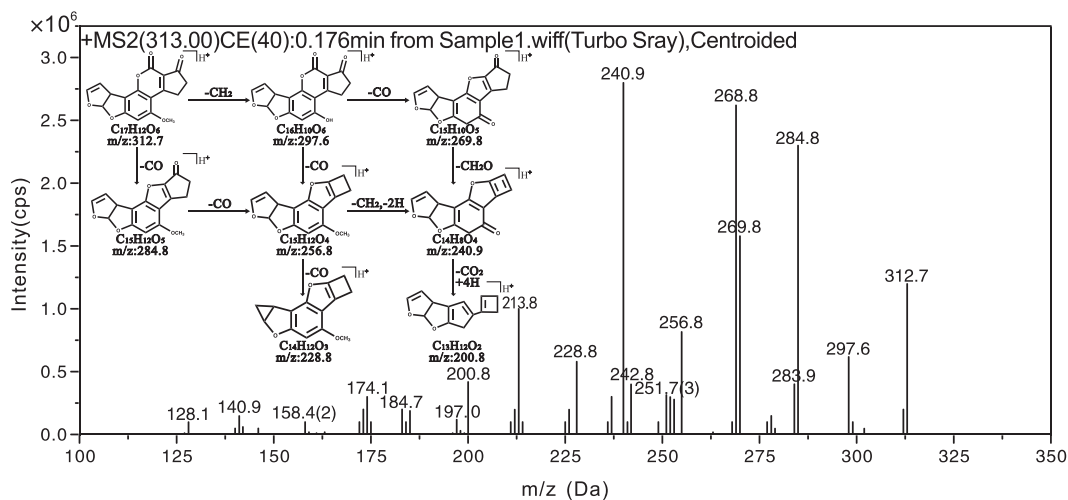


Fig. 3 MS/MS (electrospray ionization source positive ionization mode) spectrum of AFB<sub>1</sub> and fragmentation pathway of AFB<sub>1</sub> (insets).

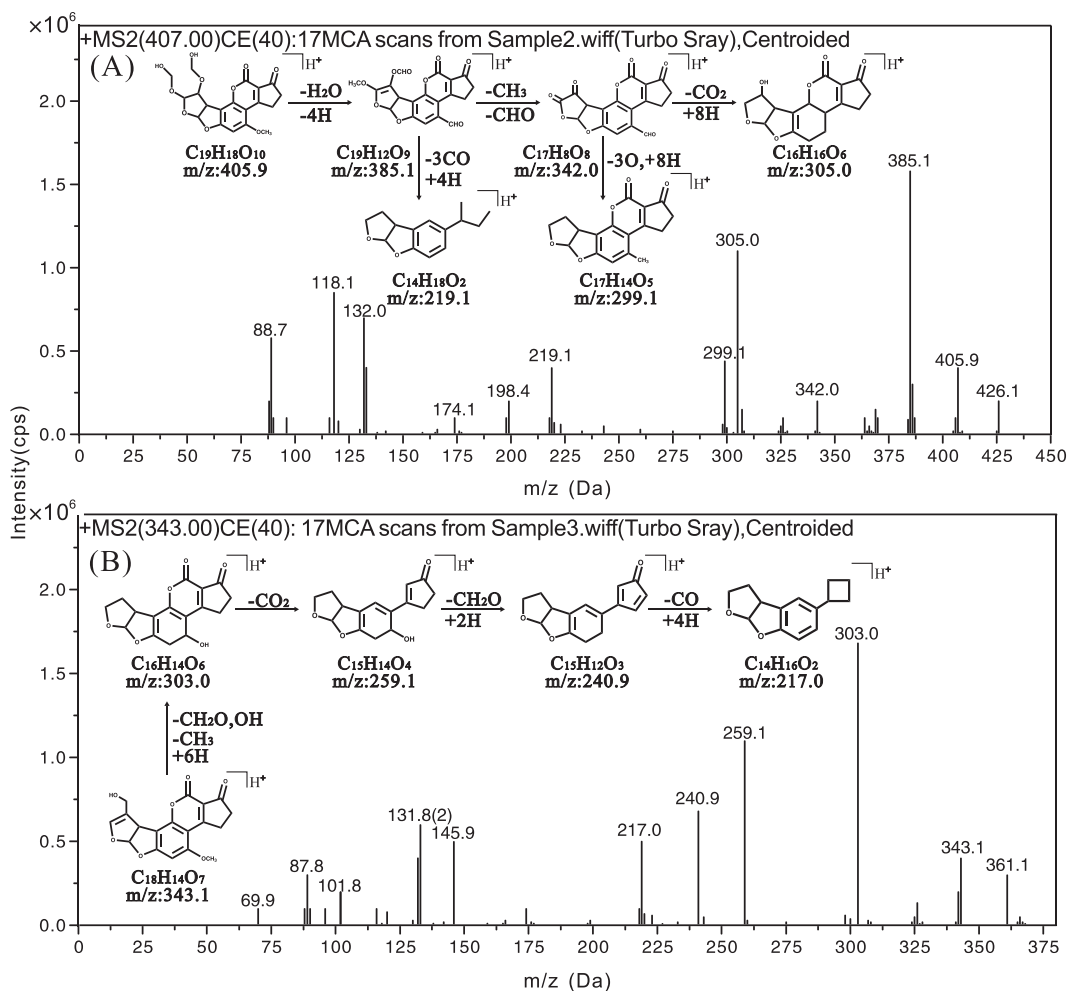
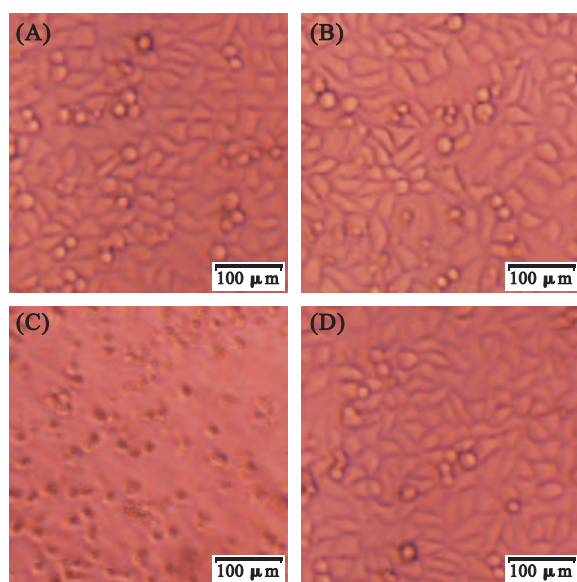


Fig. 4 MS/MS (electrospray ionization source positive ionization mode) spectra of two products (A) P<sub>1</sub> and (B) P<sub>2</sub> and their fragmentation pathway (insets).

**Table 1** Mass accuracy measurement using LC-Q-TOF MS for AFB<sub>1</sub><sup>a</sup> and biodegraded products of AFB<sub>1</sub>.

Compound	Retention time (min)	Formula	Mass (m/z) <sup>b</sup>		Mass error		DBE
			Calculate	Experiment	mDa	PPM	
P <sub>1</sub>	1.870 ± 0.03	C <sub>19</sub> H <sub>18</sub> O <sub>10</sub>	407.0002	406.9998	-0.4	-1.1	11
P <sub>2</sub>	2.471 ± 0.02	C <sub>18</sub> H <sub>14</sub> O <sub>7</sub>	343.0804	343.0803	0.1	0.3	12
AFB <sub>1</sub>	3.104 ± 0.02	C <sub>17</sub> H <sub>13</sub> O <sub>6</sub>	313.0712	313.0710	-0.2	0.6	12

<sup>a</sup> AFB<sub>1</sub> initial concentration: 10 µg/ml. <sup>b</sup> All the m/z in our experiment is the m/z of [M+H]<sup>+</sup>.



**Fig. 5** The HepG2 treated by (A) P<sub>1</sub>, (B) P<sub>2</sub> and (C) AFB<sub>1</sub>. (D) The control HepG2.

toxicity of AFB<sub>1</sub> and biodegraded products toward HepG2 cells was compared. In Fig. 5, all normal cells were treated and observed after 24 h: (A) was the cells treated with P<sub>1</sub>, (B) was the cells treated with P<sub>2</sub>, (C) was the cells treated with AFB<sub>1</sub> and (D) was the control cells without treatment. The cells treated with P<sub>1</sub> and P<sub>2</sub> appeared normal as well as control cells. However, the cells treated with AFB<sub>1</sub> showed intense diffuse necrosis characterized by pyknosis of the nuclei. These phenomena also indicated the toxicity of two biodegraded products compared with that of AFB<sub>1</sub> was reduced.

In this work, we used HPLC technology to separate and purify degradation products (P<sub>1</sub> and P<sub>2</sub>). LC-Q-TOF MS and MS/MS techniques were used to analyze the molecular formulas of P<sub>1</sub> and P<sub>2</sub> as C<sub>19</sub>H<sub>18</sub>O<sub>10</sub> and C<sub>18</sub>H<sub>14</sub>O<sub>7</sub> with molecular weights of 406 and 342, respectively. The structures of the two degradation products were predicted. The cytotoxicity test showed that the toxicity of the two

degradation products was significantly lower than that of AFB<sub>1</sub>. Of course, more detection techniques should be used to determine the structure of products and find more new products. AFB<sub>1</sub> degrading enzymes will be studied. On the other hand, with the development of synthetic biology, a lot of enzymes which can catalyze double bonds were found. Heterologous expression of these enzymes in cell factories for the degradation of AFB<sub>1</sub> is also a considerable measure.

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