African Journal of Biotechnology Vol. 11(65), pp. 12903-12909, 14 August, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2088
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Inhibiting *Aspergillus flavus* growth and degrading aflatoxin B_1 by combined beneficial microbes

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Accepted 30 July, 2012

Aflatoxin B_1 (AFB_1) is a type of toxin produced by *Aspergillus flavus*, which has a negative effect on animal production and economic profits. In order to inhibit *A. flavus* growth and degrade aflatoxin, the optimal proportion of beneficial microbes such as *Lactobacillus casei*, *Bacillus subtilis* and *Pichia anomala* were selected. The results show that AFB_1 production and mycelium weight of *A. flavus* was decreased by more than 34 folds (161.05 vs. 4.69 µg/L) and 7.7 folds (6.98 vs. 0.90 mg/ml) with the free-cell supernatants of *L. casei* and *B. subtilis* (P<0.05), respectively. The optimal proportion of *L. casei*, *B. subtilis* and *P. anomala* was 2:1:2 for inhibiting *A. flavus* growth determined by 3×3 orthogonal design. Based on the optimal proportion of three microbial species, the maximum AFB_1 degradation was during 24 to 48 h incubation (P<0.05). When three species of beneficial microbes were mixed with yeast cell wall and oligosaccharide, both of them could not help the microbes in AFB_1 degradation. The combined microbial incubation showed that AFB_1 contents in the supernatant and cells were 10.25 (P<0.05) and 3.34 µg/L, lower than the control group (68.55 µg/L), indicating that most of the AFB_1 were degraded by the microbes and only a little of them were absorbed and deposited in microbial cells.

Key words: *Aspergillus flavus*, aflatoxin B_1 detoxification, beneficial microbes, yeast cell wall, oligosaccharide.

INTRODUCTION

Aflatoxin is one of the secondary metabolites produced by toxigenic strains of *Aspergillus flavus*, *Aspergillus nomius* and *Aspergillus parasiticus.* There are four kinds of aflatoxins such as aflatoxin B_1, B_2, G_1 and G_2, in which aflatoxin B_1 (AFB_1) is highly toxic and carcinogenic (Leontopoulos et al., 2003). Aflatoxins are known to be potent carcinogens and hepatotoxic agents that pose serious hazards to human and animal health (Sidhu et al., 2009). They have been detected in many food and oilseed commodities from many parts of the world and are presently considered as one of the most dangerous contaminants of food and feed. In addition, aflatoxin also has an impact on agricultural economy through the loss of crop production (Wu, 2004). Food and Agriculture Organization of United Nations (CAST, 2003) shows that 25% feedstuffs is polluted by mycotoxin in the world, and it results in over 1 billion dollars loss for poultry industry annually.

Aflatoxin can be detoxified by physical, chemical or biological methods (Ali et al., 2010). However, limitations such as nutrient loss, low organoleptic qualities, new pollution, expensive equipment and small-scale treatments caused by physical and chemical methods have encouraged recent emphasis on biological treatments (Galvano et al., 2001). Some stains of microbes such as lactic acid bacteria, *Aspergillus niger, Aspergillus parasiticus, Trichoderma viride, Mucor ambiguus* and other fungi have been reported with different AFB_1 degradation abilities (Teniola et al., 2005; Alberts et al., 2006; Cao et al., 2011). If one strain of the microbes can inhibit the growth of *Aspergillus* species, it will reduce aflatoxin production (Gqaleni et al., 1997). Consequently, minimizing aflatoxin production by using appropriate

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Table 1. Factors and levels for investigating effects of three factors on degradation of AFB_1.

Level	Factor A	Factor B	Factor C
	The combined microbe	Yeast cell wall (%)	Oligosaccharide (%)
1	20 ml culture + 40 ml distilled water	0	0
2	40 ml culture + 20 ml distilled water	0.1	0.1
3	60 ml culture + 0 ml distilled water	0.2	0.2

The visible cells of *L. casei*, *B. subtilis* and *P. anomala* were 3×10^9 CFU/ml, respectively.

techniques is probably the best strategy to counteract the effects of aflatoxins (Tamil et al., 2003; Maryam et al., 2007).

Yeast cell wall has been reported to have the ability to reduce AFB_1 by adsorption and discharge (Swarm et al., 2002), and oligosaccharide can stimulate beneficial microbial proliferation (Duarte et al., 2002). Even though there are some reports on AFB_1 degradation by different microbes, the effects of the combined beneficial microbes such as *Lactobacillus casei*, *Bacillus subtilis*, *Pichia anomala* and their combined effects with yeast cell wall and oligo-saccharide on inhibiting *A. flavus* growth and AFB_1 detoxification have not been reported. The objective of this study was to select the most effective method and products for inhibiting *A. flavus* growth and AFB_1 production, so as to reduce economic loss from AFB_1 contamination.

MATERIALS AND METHODS

Microbes and experimental materials

A. flavus (CGMCC3.4408), *L. casei* (CGMCC1.62), *B. subtilis* (CGMCC1.504) and *P. anomala* (CGMCC2.881) were purchased from China General Microbiological Culture Collection Center. Yeast cell wall from *Saccharomyces cerexisiae* and mannan oligo-saccharide composed of 2 to 10 monomers of mannose and glucose were purchased from Alltech Biochem Co. Ltd., China. PBS buffer was prepared by mixing 1.42 g disodium hydrogen phosphate-2-hydrate phosphate with 0.27 g potassium dihydrogen phosphate and 8 g sodium chloride, dissolved in deionized water at pH 7.4, and adjusted to 1 L with deionized water. Most of the reagents for determining AFB_1 were contained in the RIDASCREEN○R Aflatoxin B1 30/15 test kit (R-Biopharm, Germany). AFB_1 standard solutions used for making calibration curve were at levels of 0, 1, 5, 10, 20 and 50 µg/L, and all of them were included in the enzyme-linked immunosorbent assay (ELISA) test kit.

Selection of optimal media and incubation conditions for microbial proliferations

Three kinds of liquid incubating media such as MRS, LB and YPD were prepared. The compositions of MRS (for *L. casei* proliferation) were (g/L): tryptone 10, glucose 20, beef peptone 10, yeast extract 5, Tween 80 1 ml, K_2HPO_4 2, sodium acetate 5, sodium citrate 2, $MgSO_4$ 0.2, $MnSO_4$ 0.05, pH 6.2 to 6.6. The medium was autoclaved at 0.15 MPa for 20 min, so was the other following media. *Lactobacillus* was incubated at stationary state at 37°C for 48 h. The compositions of LB (for *B. subtilis* proliferation) were (g/L): tryptone 10, yeast extract 5, NaCl 10, pH 7.0 to 7.2. *Bacillus* was incubated in shaking incubator at 37°C for 48 h with 150 rounds per minute (RPM). The compositions of YPD (for *P. anomala* proliferation) were (g/L): peptone 20, glucose 20, yeast extract 10, pH 7.0 to 7.2. *P. anomala* was incubated at 30°C for 48 h with 150 RPM.

For preparing the different free-cell supernatants, all the incubating cells were removed by centrifugation at 3000 ×g for 15 min. The supernatants were filtered to remove microbes with 0.20 µm Minisart High-flow filter (Sartorius Stedim Biotech Gmbh, Goettingen, Germany), and the free-cell supernatant was stored at 4°C for further use.

Inhibition of *A. flavus* growth and AFB_1 production by single supernatant of microbe

The 250 ml flasks were filled with 100 ml different free-cell supernatants, inoculated with 0.2 ml of conidial suspension of *A. flavus* at 1×10^8 colony-forming units per ml (CFU/ml), and mixed thoroughly by vigorous shaking. Each treatment had three replicates, and all flasks were incubated at 30°C on a rotor wheel at 150 RPM for 5 days. After incubation, the cells were collected by centrifugation at 13,000 × *g* for 5 min, and dry mycelium weight of *A. flavus* and AFB_1 contents in supernatants were measured.

Inhibition of *A. flavus* growth and AFB_1 production by the combined microbial incubation

Three kinds of beneficial microbes were harvested after 48 h incubation, respectively. The orthogonal design with three factors (*L. casei*, *B. subtilis* and *P. anomala*) and three levels (10, 20 and 30 ml) were used to investigate the effect of combined microbes on reducing mycelium weights and AFB_1 contents of *A. flavus*. The experiment was performed according to the last item. The mycelium weight of *A. flavus* was determined according to the following protocol: the culture medium was filtered through Whatman No.4 filter paper (20 to 25 µm pore diameters) to remove the small cells (less than 4 to 7 µm in diameters) of three beneficial microbes and leave the big cells (25 to 45 µm in diameters) of *A. flavus*. The mycelium of *A. flavus* was rinsed three times with distilled water to remove the small cells thoroughly. The mycelium was dried at 70°C for 24 h, and then measured.

Effect of the combined microbes with yeast cell wall and oligosaccharide on AFB_1 detoxification

Orthogonal design (Table 1) with three factors and three levels was used to investigate effect of the combined microbes with yeast cell wall and oligosaccharide on AFB_1 detoxification. The experiment was performed in 250 ml conical flasks in a final volume of 60 ml

Table 2. Effect of different free-cell supernatants of microbes on inhibition of *A. flavus* growth and AFB_1 production.

Strain	Group	Dry weight of mycelium (mg/ml)	AFB_1 content (µg/L)
L. casei	Control group	2.2830±0.0630[a]	161.05±15.18[a]
	Test group	1.1960±0.0560[b]	4.69±0.52[b]
B. subtilis	Control group	6.9830±1.8849[a]	214.61±22.23[a]
	Test group	0.9020±0.0263[b]	155.14±16.36[b]
P. anomala	Control group	11.0670±0.2375[a]	925.83±93.87[a]
	Test group	9.1210±0.3450[b]	284.58±29.76[b]

Each value represents mean ± SE of three replicates. Data followed by the different letters in the same columns for each microbe are significantly different from each other (P<0.05), while data followed by the same letters in the same columns are not significantly different from each other (P>0.05). The control groups for *L. casei*, *B. subtilis* and *P. anomala* were MRS, LB and YPD media, respectively.

liquid with initial AFB_1 concentration of 68.55 µg/L, and each treatment had three replicates. Cultures were incubated at 30°C on a rotor wheel at 150 RPM, and the samples were collected after 12, 24, 48, 72, 96 and 120 h incubation, respectively. The AFB_1 contents in the first treatment were measured at different times, while AFB_1 contents in other treatments were measured from the samples of 24 h. The medium pH value was measured with pH meter, and the different visible cells were determined by the method of gradient dilution coating after 24 h incubation.

Procedure for the detection of AFB_1 by ELISA

After incubation, the cells and supernatants were separated by centrifugation at 13000 × *g* for 5 min. The AFB_1 content in cells was separated as follows: the cells were centrifuged (13000 × *g*, 5 min), washed twice with PBS buffer, and then ground under liquid nitrogen. The ground cells were finally re-suspended with the same volume of methanol-PBS (65 vs. 35 ml) as its original one, centrifuged at 13000 ×g for 5 min, and then the supernatant was used to measure AFB_1 content in cells. Determination of AFB_1 was based on an enzyme-linked immunoassay using the RIDASCREEN Aflatoxin B1 30/15 test kit (R-Biopharm, Germany).

Statistical analysis

Experimental data were expressed as means and standard errors (SE). The data were analyzed using the ANOVA procedures of Statistical Analysis Systems Institute, 2004. Duncan's multiple range test was used to evaluate treatment means. The results were considered statistically significance at P<0.05.

RESULTS

Effect of beneficial microbes on inhibiting *A. flavus* growth and AFB_1 production

Table 2 indicates that the free-cell supernatant of *L. casei*, *B. subtilis* and *P. anomala* had significant effect on inhibiting *A. flavus* growth (P<0.05). The free-cell supernatant of *B. subtilis* had the most effective inhibition for *A. flavus* growth, which was reduced by 87.08% (0.9020 vs. 6.9830 mg/ml) when compared with its control group. The lowest content of AFB_1 was 4.69 µg/L in the incubation with free-cell supernatant of *L. casei*, which was lower than its control group (161.05 µg/L).

Table 3 demonstrates that the dry weight of mycelium in nine treatments were from 0.5407 to 1.3340 g (P<0.05). The optimal proportion of *L. casei*, *B. subtilis* and *P. anomala* was 2:1:2 for inhibiting *A. flavus* growth significantly (P<0.05).

Effect of the combined beneficial microbes with yeast cell wall and oligosaccharide on AFB_1 detoxification

Table 4 indicates that culture medium pH values were 3.49 to 3.57, which was mainly caused by the dominant *L. casei*. The maximal visible cells of *L. casei*, *B. subtilis* and *P. anomala* were found in treatments 1, 3 and 9, respectively (P<0.05). Table 5 shows that AFB_1 contents of supernatants in nine treatments were from 8.02 to 48.79 µg/L, which was lower than their original content of 68.55 µg/L. The lowest AFB_1 content was found in treatment 2, and the highest one in treatment 7 (P<0.05). AFB_1 contents of cells in nine treatments were from 2.17 to 4.66 µg/L, which were lower than that in the supernatants.

Effect of the combined microbes on AFB_1 contents during different periods of incubations

Figure 1 indicates that the lowest contents of AFB_1 (8.89 to 10.59 µg/L) was found to be at 24 and 48 h incubation (P<0.05), which was reduced by 84.55 to 87.04% when compared with the control group (68.55 µg/L). After 48 h incubation, AFB_1 content was increased to the same level as that at 12 h incubation, in which about 58% AFB_1 was degraded.

DISCUSSION

Aflatoxins have been considered as the most dangerous

Table 3. Inhibition of *A. flavus* growth by the combined microbes after 120 h incubation.

Treatment	Factor			Dry weight of mycelium (mg/ml)	AFB_1 content (μg/L)*
	A	B	C		
1	1	1	1	0.5930±0.0763^{b}	<1
2	1	2	2	0.5770±0.0269^{b}	<1
3	1	3	3	0.5850±0.0476^{b}	<1
4	2	1	2	0.5407±0.0401^{b}	<1
5	2	2	3	0.9963±0.3730^{ab}	<1
6	2	3	1	0.6663±0.0642^{b}	<1
7	3	1	3	0.9880±0.2093^{ab}	<1
8	3	2	1	1.3340±0.2049^{a}	<1
9	3	3	2	1.2307±0.4842^{a}	<1

Each value represents mean ± SE of three replicates. Data followed by the different letters in the same columns are significantly different from each other (P<0.05), while data followed by the same letters in the same columns are not significantly different from each other (P>0.05).
"*" Indicates that content of AFB_1 is not determined by the equipment when its content was less than 1 μg/L.

Table 4. Determination of living cells and medium pH values at 24 h incubation of the combined microbes with yeast cell wall and oligosaccharide.

Treatment	Medium pH value	The visible cell (lg, CFU/mL)*		
		L. casei	*B. subtilis*	*P. anomala*
1	3.51±0.06^{ab}	10.07±0.55^{a}	9.04±0.31^{a}	7.97±0.15^{bcd}
2	3.49±0.01^{b}	9.40±0.14^{abcd}	7.56±0.20^{ab}	7.49±0.21^{cd}
3	3.53±0.05^{ab}	9.77±0.18^{ab}	8.47±0.88^{ab}	9.21±0.57^{a}
4	3.51±0.02^{ab}	9.05±0.04^{bcde}	7.33±0.85^{b}	7.96±0.38^{bcd}
5	3.53±0.01^{ab}	9.27±0.21^{bcd}	7.66±0.96^{ab}	7.10±0.59^{d}
6	3.53±0.01^{ab}	9.50±0.31^{abc}	7.80±0.44^{ab}	7.03±0.57^{d}
7	3.55±0.03^{ab}	8.70±0.33^{de}	8.17±0.57^{ab}	7.28±0.10^{cd}
8	3.54±0.01^{ab}	8.37±0.01^{e}	8.51±0.83^{ab}	8.14±0.60^{bc}
9	3.57±0.01^{a}	8.81±0.58^{cde}	9.05±0.75^{a}	8.65±0.18^{ab}

Each value represents mean ± SE of three replicates. Data followed by the different letters in the same columns are significantly different from each other (P<0.05), while data followed by the same letters in the same columns are not significantly different from each other (P>0.05).
*The CFU of visible cells were expressed as logarithm (lg).

contaminants of food and feedstuffs. Many countries have worked out the maximum limits of aflatoxins according to different consumers and commodities. For example, the maximum level of AFB_1 cannot be more than 5.0 μg/L in maize used as an ingredient in feedstuffs in European countries. It is very important and necessary to take measures for reducing AFB_1 production and aflatoxin contaminated feedstuffs. This study show that *L. casei*, *B. subtilis* and *P. anomala* had the ability to inhibit *A. flavus* growth and AFB_1 production, and *L. casei* had the best ability to degrade AFB_1 which corresponds with the former reports (Peltonen et al., 2011). The reason may be its metabolic products, low pH value and microbial competition. It was reported that *L. casei pseudoplanturum* 371 could inhibit mold growth and aflatoxin production, and the inhibitory activity in the free-cell supernatant of *Lactobacillus* was sensitive to proteolytic enzymes such as trypsin and α-chymotrypsin (Gourama and Bullermanbn, 1997). Even though *B. subtilis* showed the best ability to inhibit *A. flavus* growth in this study, its ability to degrade AFB_1 was lower than that of *L. casei*. It was reported that a large number of compounds from microbial incubation were found to inhibit AFB_1 biosynthesis, mainly through their inhibitory effect on fungal growth (Eduardo et al., 2005). This result is different from the above report, which may be due to the different microbes and incubation conditions.

Kimura and Hirano (1998) investigated the inhibitory effect of *B. subtilis* NK330 on growth and aflatoxin production of *A. parasiticus*. It was reported that peptidolipid compounds produced by *B. subtilis* showed antifungal properties against various fungal strains (Klich

Table 5. Detoxification of AFB_1 by the combined microbes with yeast cell wall and oligosaccharide at 24 h incubation.

Treatment	Factor			Contents of AFB_1 (μg/L)	
	A	B	C	In supernatant	In cell
1	1	1	1	10.25±1.71^{e}	3.34
2	1	2	2	8.02±1.76^{e}	2.17
3	1	3	3	14.48±5.54^{de}	3.87
4	2	1	2	43.10±6.37^{ab}	3.07
5	2	2	3	31.40±1.56^{c}	2.55
6	2	3	1	33.10±11.11^{bc}	4.66
7	3	1	3	48.79±1.55^{a}	3.72
8	3	2	1	25.11±1.63^{cd}	3.15
9	3	3	2	23.86±3.79^{cd}	4.43

Each value represents mean ± SE of three replicates. Data followed by the different letters in the same columns are significantly different from each other (P<0.05), while data followed by the same letters in the same columns are not significantly different from each other (P>0.05). The original AFB_1 content of liquid medium is 68.55 μg/L in each treatment.

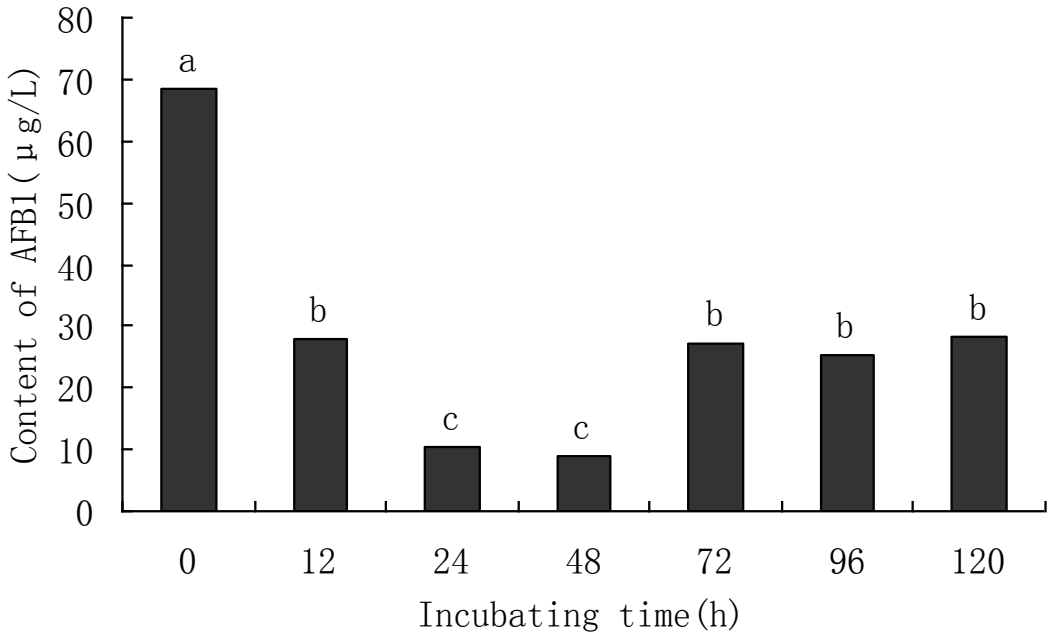

Figure 1. Detoxification of AFB_1 by the combined microbes during the different incubation time. The significant differences at P<0.05 levels are indicated by the different letters (a, b and c).

et al., 1991). Moyne et al. (2001) reported that *B. subtilis* had high inhibition on *A. flavus* growth by an active material called bacillomycin D. *B. subtilis* used in this study produced protease, maybe it was because of the protease and other compounds produced by *B. subtilis* to inhibit *A. flavus* growth and AFB_1 production. The former report showed that *P. anomala*, *Pichia kluyveri* and *Hanseniaspora uvarum* could inhibit *Aspergillus ochraceus* growth and mycotoxin production by producing some volatile compounds such as ethyl acetate, acetate, 2-phenlethyl acetate, ethyl propionate and isoamyl alcohol (Masoud and Christa, 2006). *P. anomala* used in this study had the ability to produce ester and acidic compounds to inhibit *A. flavus* growth. The acting mechanisms of beneficial microbes for inhibiting *A. flavus* growth and AFB_1 production need further study.

As compared to the single microbe, the combined microorganisms were found to be more effective in inhibiting *A. flavus* growth and AFB_1 production, due to their products and cooperation. In this study, the content of AFB_1 with the lowest mycelium weight of treatment 4 in

Table 3 was less than 1 μg/L in the mixed incubation, which was lower than that in the control group (>50 μg/L). One investigation has shown that *Aspergillus* growth and aflatoxin production can be inhibited by many micro-organisms including bacteria, yeasts, *Aspergillus* and *Actinomycetes* (Haskard et al., 2001). However, the effects of combined microbes on inhibiting *A. flavus* growth and aflatoxin production have not been reported. This study has shown a wonderful future for the application of combined microbes.

A good material for AFB_1 detoxification should not decrease nutrient values of feedstuffs and organoleptic qualities. It was reported that several sequestering agents including aluminas, silicas, phyllosilicates and chemically modified phyllosilicates were capable of binding AFB_1 *in vitro*. However, binding under these conditions was not stable, ranging from 1.2 to 98.1% (Phillips et al., 1988). Swarm et al. (2002) reported that yeast cell wall and sequestering agents have high adsorption ability for mycotoxin. Oligosaccharide can stimulate beneficial microbial proliferations, so we hope it can help microbes to degrade AFB_1. However, this result indicates that the addition of yeast cell wall and oligosaccharide could not help microbes to degrade AFB_1. The AFB_1 contents in cells were 2.17 to 4.66 μg/L, which are lower than those in the supernatants of the same treatment, indicating that most amounts of AFB_1 were degraded and only a few of them were deposited in cells during incubation.

It was reported that one or more enzymes might be involved in aflatoxin degradation by mold mycelia (Dimitrokallis et al., 2008). The application of enzymes or microorganisms capable of biotransforming mycotoxins into nontoxic metabolites has emerged as an alternative strategy in controlling mycotoxicoses in animals. Biotransformation is an effective and safe method for mycotoxin control (Schatzmayr et al., 2006). Further studies are needed for elucidating the mechanisms of AFB_1 decontamination, adsorption or degradation.

The removal of AFB_1 was different during the different periods of microbial incubations. Figure 1 shows that the maximum degradation of AFB_1 was between 24 and 48 h incubation of the combined microbes. This may be due to the strongest living ability of microbes during that period. After 48 h incubation, the AFB_1 contents were increased because of the AFB_1 released from the dead cells. Line and Brackett (1995) reported that *Flavo aurantiacum* could remove about 19% AFB_1 within 24 h and 33% within 72 h of incubation. It was reported that 15 and 19% AFB_1 could be removed by *L. casei* and *Lactobacillus coryniformis* within 24 h of incubation, respectively (Thyagaraja and Hosono, 1994); and 52.67 and 80.53% AFB_1 was decreased by *B. subtilis* after 24 and 48 h treatment (Mohsen et al., 2012). In this study, the combined microbes could remove 59.30% AFB_1 within 12 h and 87.04% within 48 h. It can be concluded that the combined microbes will be very useful for AFB_1 degradation in the application fields.

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