Microbial strategies to control aflatoxins in food and feed

S. Guan¹,²,³, T. Zhou³, Y. Yin¹,², M. Xie¹, Z. Ruan¹ and J.C. Young³

¹State Key Laboratory of Food Science and Technology and College of Life Science and Food Engineering, Nanchang University, Nanchang 330031, China P.R.; ²Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science and Key Laboratory for Agro-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, 410125 Hunan, Changsha, China P.R.; ³Guelph Food Research Center, Agriculture and Agri-Food Canada, 93 Stone Rd W, Guelph N1G 5C9, Canada; yyulong2003@yahoo.com.cn

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Abstract

Aflatoxins are a group of toxic and carcinogenic fungal metabolites. They are commonly found in cereals, nuts and animal feeds and create a significant threat to the food industry and animal production. Several strategies have been developed to avoid or reduce harmful effects of aflatoxins since the 1960s. However, prevention of aflatoxin contamination pre/post harvest or during storage has not been satisfactory and control strategies such as physical removing and chemical inactivating used in food commodities have their deficiencies, which limit their large scale application. It is expected that progress in the control of aflatoxin contamination will depend on the introduction of technologies for specific, efficient and environmentally sound detoxification. The utilisation of biological detoxification agents, such as microorganisms and/or their enzymatic products to detoxify aflatoxins in contaminated food and feed can be a choice of such technology. To date, many of the microbial strategies have only showed reduced concentration of aflatoxins and the structure and toxicity of the detoxified products are unclear. More attention should be paid to the detoxification reactions, the structure of biotransformed products and the enzymes responsible for the detoxification. In this article, microbial strategies for aflatoxin control such as microbial binding and microbial biotransformation are reviewed.

Keywords: mycotoxigenic fungi, aflatoxin, biocontrol strategies

1. Introduction

Aflatoxins are a group of structurally related secondary metabolites produced mainly by Aspergillus flavus and Aspergillus parasiticus (Eaton and Groopman, 1994). Currently, about 20 types of different aflatoxins are known. They are mainly classified into aflatoxin B₁, B₂, G₁, G₂, M₁, and M₂ based on structure, chromatographic and fluorescent characteristics (Lerda, 2010).

Aflatoxin B₁ (AFB₁), one of the most hazardous mycotoxins, is extremely toxic, mutagenic and carcinogenic (Bhatnagar et al., 2003; CAST, 2003). Physical, chemical and biological characteristics of AFB₁ molecule indicate two crucial groups for toxicological activity (Figure 1). One is the double bond on C-8, 9 of the difuran ring which is responsible for DNA and protein adduction. The interactions of aflatoxins, DNA and protein, which occur at this site affect the normal biochemical functions of these macromolecules and lead to deleterious effect at cellular level. The other one is
the lactone ring in the coumarin moiety which is easily hydrolysed and thus vulnerable to degradation (Lee et al., 1981).

Aflatoxin contamination in cereal grains is a worldwide concern especially in sub-tropical and tropical areas (Guan et al., 2011). The occurrence of aflatoxins in various types of food grains has been well documented from different regions all over the world during the past two decades, such as Italy (Pietri et al., 2004), Ethiopia (Ayalew et al., 2006), India, Mexico, etc. (Binder et al., 2007; Jelinek et al., 1989; Monbaliu et al., 2010; Placinta et al., 1999). It poses a severe threat to both livestock productivity and human health thus results in huge economic losses worldwide each year (Diaz, 2005; Mishra and Das, 2003).

Aflatoxin control strategies have been developed since the 1960s. Generally, these strategies can be divided into three groups: pre-harvest control, harvest management and post-harvest detoxification (Kabak et al., 2006). Among these strategies, typical methods that have been reported include breeding for aflatoxin resistant crop varieties, prevention of aflatoxin producing fungal contamination, inhibition of aflatoxin production, removal of aflatoxin by physical methods, inactivation by chemical agents, and biological detoxification by microorganisms and their metabolites.

Much work has been done to identify genetically resistant genotypes in plant breeding since the 1970s. Although a number of well-characterised sources of both resistances to A. flavus infection and to aflatoxin production have been identified, it is time consuming and no product has been put into practical application (Brown et al., 1999; Chen et al., 2001; Widstrom et al., 2003).

With respect to fungal infection and aflatoxin production control, many publications describe biocontrol of fungal growth and aflatoxin production by using fungal strains and plant extracts (Dorner et al., 2003; Reddy et al., 2009; Sanchez et al., 2005). However, since fungal contamination and toxin production may occur at any stage from pre harvest in the field to crop storage and food processing, the complete elimination is not achievable. In addition, various physical and chemical methods have been developed and tested for removing or deactivate aflatoxins. Such methods include washing, solvent extraction, heat treatment, extrusion, radiation, acid/base treatment, oxidation, binding, etc. However, most of the physical and chemical agents displayed disadvantages such as nutritional loss and sensory quality reduction in the processed food, toxic compounds production during treatment, limited efficiency and high cost, have limited their practical applications (Albores et al., 2005; Gowda et al., 2007; Puzyr et al., 2010; Yazdanpanah et al., 2005). Only a few binding agents have been introduced to industrial use. Yeast cell wall components and hydrated sodium calcium aluminosilicates (HSCAS) are among the most cost efficient and applicable absorbents and such materials have been commercialised as a feed additive (Cinar et al., 2008; Kutz et al., 2009). Chemical treatment methods in feed products have been banned in the European Union. According to the Food and Agriculture Organization, successful control strategies should meet the following criteria: aflatoxin must be transformed to non-toxic products; fungal spores and mycelia should be destroyed to prevent formation of new toxins; the food or feed material should retain its nutritive value and palatability; the physical properties of raw material should not change significantly; and it must be cost efficient (Beaver, 1991; Rustom, 1997). Unfortunately, none of the above methods meet all these criteria.

During the past decade, much interest has been focused on searching for alternative strategies such as biological detoxification and gene engineering. Several reports have indicated a consensus that utilisation of microorganisms and/or their enzymatic metabolites to detoxify mycotoxins in contaminated food and feed has advantages, such as mild reaction conditions, target specificity, efficiency and are environmental friendly (Dalié et al., 2010; Halász et al., 2009; Karlovsky, 1999; Shetty and Jespersen, 2006). The aim of this paper is to present a review on recent advances in the development of microbial strategies to control aflatoxins.

2. Microbial strategies in controlling aflatoxins

Microbial binding/adsorption

Recently, there has been increased interest in aflatoxin binding by microorganisms (Table 1). Studies on this topic mainly focus on probiotics/dairy strains of lactic acid bacteria (LAB) including species of Lactobacillus, Lactococcus, Bifidobacterium sp. and Propionibacterium and yeast strains of Saccharomyces cerevisiae. To date, most of the available reports have been based on studies conducted at the laboratory level with wide variation in experimental conditions (Fazeli et al., 2009; Shah and Wu, 1999).

AFB1, binding by lactic acid bacteria (LAB)

AFB1, binding by LAB in liquid medium is a fast process, ranging from 1 min to 72 h. Dramatic binding were observed within 3 h of incubation with some bacterial strains. AFB1 binding within 1 min by LAB strain was described (Bueno et al., 2007). Also, approximately 80% of AFB1 was bound by strain Lactobacillus rhamnosus GG (LBGG) and strain L. rhamnosus LC-705 (LC705) within 1 min incubation (El-Nezami et al., 1998a). Similarly, AFB1, binding of 20 to 50% by a probiotic strain of Bifidobacterium sp. was observed after 3 h incubation (Shah and Wu, 1999). Compared with the above strains, some other bacteria take 48-72 h for AFB1 binding. LAB of Enterococcus faecium strains M74 and EF031 were observed to bind 19.3-37.5% of AFB1 within 48 h incubation.
Table 1. Aflatoxin binding/adsorption by micro-organisms.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Aflatoxin</th>
<th>Bound (%)</th>
<th>Conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>AFB₁</td>
<td>80</td>
<td>2x10^10 cfu/ml, 0 h, 37 °C, liquid medium</td>
<td>El-Nezami et al., 1998a,b; Haskard et al., 2001</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus GG (LBGG), Lactobacillus rhamnosus (LC-705)</td>
<td>AFB₁</td>
<td>20-50</td>
<td>3 h</td>
<td>Shah and Wu, 1999</td>
</tr>
<tr>
<td>Bifidobacterium sp. infantis, Bifidobacterium sp. pseudolongum, Bifidobacterium sp. bifidum</td>
<td>AFB₁</td>
<td>74, 63, 37</td>
<td>10^10 cfu/ml, 1 h, 37 °C, in vitro, chicken duodenum</td>
<td>El-Nezami et al., 2000</td>
</tr>
<tr>
<td>L. rhamnosus GG, Propionibacterium freudenreichii spp., Shermanii JS LC705</td>
<td>AFB₁</td>
<td>57-66, 25</td>
<td>10^11 cfu/ml, 1-30 min, 37 °C, PBS, chicken duodenum</td>
<td>Gratz et al., 2005</td>
</tr>
<tr>
<td>Lactobacillus and Propionibacterium strains (mixture)</td>
<td>AFB₁</td>
<td>5.6-69.7</td>
<td>10^10 cfu/ml, 24 h, 37 °C, PBS</td>
<td>Peltonen et al., 2000, 2001</td>
</tr>
<tr>
<td>Lactobacillus, Lactococcus, Bifidobacterium sp. strains</td>
<td>AFB₁</td>
<td>18-62, 19-70</td>
<td>10^10 cfu/ml, 16 h, 37 °C, PBS, skim and full cream milk</td>
<td>Pierides et al., 2000</td>
</tr>
<tr>
<td>Lactobacillus, Bifidobacterium sp. strains</td>
<td>AFB₁</td>
<td>25.7-32.5, 21.2 to 29.3</td>
<td>24 h, 37 °C, PBS buffer, skim milk</td>
<td>Kabak and Var, 2004</td>
</tr>
<tr>
<td>Lactobacillus fermentum, Lactobacillus casei, Lactobacillus plantarum</td>
<td>AFB₁</td>
<td>25-61</td>
<td>2x10^10 cfu/ml, 72 h, 37 °C, liquid media</td>
<td>Fazeli et al., 2009</td>
</tr>
<tr>
<td>Enterococcus faecium M74 and EF031</td>
<td>AFB₁</td>
<td>19.3-37.5</td>
<td>10^10 cfu/ml, 48 h, pH 7.0, liquid medium</td>
<td>Topcu et al., 2010</td>
</tr>
<tr>
<td>Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus spp., Selangorensis, Pediococcus acidilactici and Weissella confusa</td>
<td>AFB₁</td>
<td>15-60</td>
<td>10^9 cfu/ml, 72 h, 25 °C, PBS solution</td>
<td>Shetty and Jespersen, 2006</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>AFB₁</td>
<td>40</td>
<td>10^7 cfu/ml, PBS solution, 1 h, 37 °C</td>
<td>Bueno et al., 2007</td>
</tr>
<tr>
<td>Saccharomyces and Candida strains</td>
<td>AFB₁</td>
<td>15-60</td>
<td>10^9 cfu/ml, PBS solution, 72 h, 25 °C</td>
<td>Shetty and Jespersen, 2006</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae cell wall component esterified glucomannan (EGM)</td>
<td>AFB₁</td>
<td>81.6</td>
<td>pH 4.5, 6.5, 3 h, 37 °C, contaminated feed 0.1% EGM</td>
<td>Raju and Devegowda, 2002</td>
</tr>
<tr>
<td>S. cerevisiae strains</td>
<td>AFB₁</td>
<td>10-40</td>
<td>10^9 cfu/ml, PBS solution, 12 h, 25 °C</td>
<td>Shetty et al., 2007</td>
</tr>
</tbody>
</table>

1 PBS = phosphate buffered saline.

(Fazeli et al., 2009). Twenty bacterial strains of Lactobacillus, Lactococcus and Bifidobacterium sp. bound 5.6-59.7% of AFB₁ in 72 h period (Peltonen et al., 2000, 2001).

The AFB₁ binding ability of LAB was affected by various conditions such as temperature, pH value and bacterial concentration. AFB₁ binding by LBGG and LC-705 was both temperature and bacterial concentration dependent, with the best function at 10^10 cfu/ml and 37 °C (El-Nezami et al., 1998a). In addition, acid such as 1 M HCl treatment could significantly enhance the binding ability of the two strains (El-Nezami et al., 1998b; Haskard et al., 2001). Also, AFB₁ binding by LAB of E. faecium strains was largely affected by pH value and the optimal binding was at pH 7.0 (Fazeli et al., 2009).

It is also worth to note that in most cases, AFB₁ binding by LAB is reversible. Bound AFB₁ can be released when conditions change. 60-80% of the bound aflatoxin was released from Bifidobacterium sp. after incubation with water at 37 °C for 10 min. Only 10-40% of the toxin was strongly bound to the bacterial cells (Shah and Wu, 1999). AFB₁ release from Lactobacillus, Lactococcus and Bifidobacterium sp. strains was also observed by repeated aqueous washes (Peltonen et al., 2000, 2001).

Binding ability of LAB can be reduced in food matrices and animal intestinal model. Binding of AFM₁ by Lactobacillus and Bifidobacterium sp. strains were found to range between 21.2 to 29.3% in skim milk compared to 25.7 to 32.5% in phosphate buffered saline (Kabak and Var, 2004). The ability of Lactobacillus and Propionibacterium strains to bind AFB₁ in chicken duodenum was evaluated in vitro. The reduction of AFB₁ uptake by intestinal tissue was 74%, 63% and 37% by LBGG, Propionibacterium freudenreichii spp. Shermanii JS and LC705, respectively. Comparatively, the binding ability of LC705 was lower in vivo in chicken duodenum (54%) compared with approximately 80% in vitro. Intestinal
mucus was proposed to reduce the surface binding ability (El-Nezami et al., 2000; Gratz et al., 2004). AFB\textsubscript{1} binding by mixture of Lactobacillus and Propionibacterium strains in vivo were also lower than in vitro (Gratz et al., 2005).

As for the binding mechanism by LAB, several studies were conducted by heat treatment and by using gram positive/negative strains. Heat treatment greatly affects binding ability. Viable strain LC-705 bound 69.6% and 63.6% of AFM\textsubscript{1} from skim and full cream milk, respectively, during overnight incubation while the heat-killed cells showed significantly reduced ability, with 27.4% and 30.1% of AFM\textsubscript{1} bound respectively (Pierides et al., 2000). Also, binding and release of AFB\textsubscript{1} was studied by heat-killed bacteria (Lee et al., 2003). It was proposed that heat treatment modified the cell wall surface properties which reduced the binding ability. Gram types also affect binding ability. El-Nezami et al. (1998a) observed that gram-positive bacteria were more effective in AFB\textsubscript{1} binding than gram-negative strain Escherichia coli.

The fact that heat treatment and gram types greatly affect binding ability indicates that cell wall components are crucial to aflatoxin binding. Further investigations by pronase E and periodate treatments on heat treated, acid treated and viable cells reduced AFB\textsubscript{1} binding by LAB, suggesting carbohydrates or protein components in cell wall are crucial in AFB\textsubscript{1} binding (Haskard et al., 2000). Peptidoglycans in cell wall are also responsible for AFB\textsubscript{1} binding by LAB strains (Lahtinen et al., 2004).

In addition, cell envelope structures were also involved in AFB\textsubscript{1} binding (Peltonen et al., 2000, 2001). Hydrophobic interaction plays a potential role in AFB\textsubscript{1} binding by L. rhamnosus since an anti-hydrophobic agent such as urea had a significant effect on the release of bound AFB\textsubscript{1}. On the other hand, electrostatic interaction and hydrogen bonding were not involved in AFB\textsubscript{1} binding by LAB since mono and divalent ions or variations in pH did not affect binding.

**AFB\textsubscript{1} binding by yeast**

Yeast strains have been extensively investigated as AFB\textsubscript{1} binding agent (Table 1). Since the 1990s, there have been many reports on yeast cell wall, modified cell wall components and commercial products from *S. cerevisiae* as poultry feed additive to control AFB\textsubscript{1} and to eliminate its adverse effects (Celik et al., 2001; Devegowda and Murthy, 2005; Moschini et al., 2008; Oguz et al., 2004; Stanley et al., 1993).

Binding of aflatoxins by yeast strains is also a fast and reversible process. However, their binding ability is generally lower than bacterial strains. It is strain specific and varies largely among different strains. AFB\textsubscript{1} binding by *S. cerevisiae* was a rapid process in liquid medium and it involved the formation of a reversible complex between the toxin and yeast cell wall surface (Bueno et al., 2007). Different species of yeasts including *Saccharomyces* and *Candida* strains could bind 15-60% (w/w) of AFB\textsubscript{1} in vitro and the toxin binding was highly strain specific (Shetty and Jespersen, 2006). In a different study, several *S. cerevisiae* strains isolated from indigenous fermented foods were tested against AFB\textsubscript{1} binding in vitro, with most strains binding 10-40% of the added AFB\textsubscript{1}. Binding was significantly reduced in lower temperature at 15 °C while heat-killed yeast cell showed increased binding ability than viable cell (Shetty et al., 2007).

As for the binding mechanism by yeast strains, cell wall component esterified glucosamann (EGM) and yeast mannanoligosaccharide (MOS) are most likely to be responsible. In vivo studies by addition of yeast EGM and MOS are extensive and effective in AFB\textsubscript{1} control. Addition of 0.1% *S. cerevisiae* EGM in contaminated feed resulted in 81.6% AFB\textsubscript{1} binding at an initial concentration of 300 μg/kg. An in vivo study showed its counteracting efficiency against immunosuppression in mycotoxicosis of broiler chicken (Raju and Devegowda, 2002). Ability of MOS to bind AFB\textsubscript{1} and to reduce gastrointestinal absorption of AFB\textsubscript{1} and its levels in tissues for laying hens was also observed (Zaghini et al., 2005). Additionally, there are a number of studies on dietary inclusion of yeast cell wall components to counteract aflatoxins. Several reports described improved performance and serum biochemistry parameters in broiler chickens by addition in AFB\textsubscript{1} contaminated feed (Aravind et al., 2003; Baptista et al., 2004; Girish and Devegowda, 2006; Kamalzadeh et al., 2009; Karaman et al., 2005; Raju and Devegowda, 2000; Slizewska et al., 2010).

To date, a number of studies have demonstrated that cell wall structure and components are responsible for microbial binding of aflatoxins, though the mechanism of binding by specific strain is still unclear. Cell wall peptidoglycans and polysaccharides have been proposed to be the most crucial elements responsible for AFB\textsubscript{1} binding by LAB whereas glucosamann and mannanoligosaccharide to be responsible in yeast cell wall.

Both of these microbes have great advantage to be used in commercial applications as binding agents since these strains have already been licensed and widely used in food fermentation and they are 'generally recognised as safe'. However, it has been observed from most of the studies that the stability of the aflatoxin-cell complex is strain and condition dependent. Release of aflatoxins would occur when the conditions such as temperature and pH change. Thus there is a need for further research to determine how these strains behave under different environmental conditions before they can be used as commercially applicable binding agents.
3. Microbial transformation

Bacterial biotransformation

Although screening of aflatoxin biotransformation microbes had already begun in the 1960s, reports in this area are quite limited (Table 2). Bacterial strain Nocardiocorynebacterioides (formerly Flavobacterium aurantiacum), is one of the most intensively studied bacterium in this field.

Transformation instead of binding of AFB, was proposed in *E. aurantiacum*. Thus unlike microbial binding, viable cells are necessary in biotransformation by this bacterium (Lillehoj *et al.*, 1967; Line and Brackett, 1995a; Line *et al.*, 1994). The fate of AFB after *E. aurantiacum* treatment was determined by 14C label techniques. The normally chloroform soluble 14C AFB was rapidly biotransformed to water soluble transformation products by viable cells. It was proposed that at least part of the 14C AFB was transformed since 14CO2 was observed in the product by the viable cells but not by dead cells (Line *et al.*, 1994).

Generally, 1x109 cfu/ml of *E. aurantiacum* cells and 4 h of incubation are required to achieve effective biotransformation. 109 cfu/ml stationary phase cells of *E. aurantiacum* resulted in 40%, 23% and 74% of AFB biotransformation in 24 h incubation in phosphate buffer, peanut milk and partially defatted peanut milk, respectively (Hao and Brackett, 1988, 1989). However, 1011 cfu/ml cells showed faster AFB biotransformation rate than 109 cfu/ml cells. AFB at 7 μg/ml was completely biotransformed after incubation with 1011 cfu/ml bacterial cells in 4 h (Ciegler *et al.*, 1966a).

Factors such as ions could greatly influence AFB1 biotransformation by *E. aurantiacum*. The role of several divalent cations and chelators on AFB1 biotransformation by *E. aurantiacum* was studied in depth. Incubation of cells with 10 mM Mg2+, Ca2+ and Mn2+ for 48 h significantly increased AFB1 biotransformation whereas Cu2+ and Zn2+ decreased AFB1 biotransformation. In addition, 0.1 and 1 mM chelators such as EDTA and 1,10-phenanthroline improved AFB1 biotransformation significantly (Line and Brackett, 1995b). Further studies determined that reducing conditions and presence of a sulfhydryl group enhanced AFB1 biotransformation. Serine is an active group involved in the process (D'Souza and Brackett, 1998, 2000, 2001).

*E. aurantiacum* NRRL B-184 was later re-classified and renamed to *N. corynebacterioides* (NRRL 24037). A recent in vivo study by inoculating this bacterium with AFB1 contaminated feed demonstrated that the bacterial strain is safe for animals and it can effectively contradict the side effect of AFB1 on chickens. With the AFB1 level of 800 and 1,200 μg/kg feed, the bacteria treatment significantly improved the growth performance such as body weight and histopathologic indexes such as organ lesion (Castaneda *et al.*, 2008).

Aside from *E. aurantiacum*, only a few bacterial strains have reported on AFB1 biotransformation. Most of the bacteria were originated from contaminated soil samples,

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**Table 2. Aflatoxin biotransforming bacteria isolated from various sources.**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Transformation</th>
<th>Conditions</th>
<th>Origin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL B-184</td>
<td>100</td>
<td>3 h, 1011 cells/ml, liquid food</td>
<td>n.a.</td>
<td>Hao and Brackett, 1988, 1989</td>
</tr>
<tr>
<td><em>Flavobacterium aurantiacum</em></td>
<td>80-100</td>
<td>12 h, 1011 cells/ml, 28 °C solid food</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em></td>
<td>23</td>
<td>109 cells, 24 h, 30 °C, phosphate buffer, peanut milk</td>
<td>PAH contaminated soil</td>
<td>Alberts <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>Mycobacterium</em></td>
<td>23</td>
<td>74 partially defatted peanut milk</td>
<td>coal gas plant soil</td>
<td>Hornisch <em>et al.</em>, 2004</td>
</tr>
<tr>
<td><em>fluoranthenivorans</em> strain</td>
<td>60.8</td>
<td>Culture supernatant, 72 h, 30 °C</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em></td>
<td>100</td>
<td>Cell-free extracts, 4-24 h, 10-40 °C</td>
<td>PAH contaminated soil</td>
<td>Teniola <em>et al.</em>, 2005</td>
</tr>
<tr>
<td><em>Mycobacterium</em></td>
<td>72 h, 30 °C</td>
<td>Liquid culture</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td><em>fluoranthenivorans</em> sp. nov.</td>
<td>100</td>
<td>Cell-free extracts, 4-24 h, 10-40 °C</td>
<td>PAH contaminated soil</td>
<td></td>
</tr>
<tr>
<td><em>Nocardia corynebacterioides</em></td>
<td>60-100</td>
<td>85</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>7 d, 37 °C, liquid culture</td>
<td></td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>82.5</td>
<td>37 °C, 72 h, liquid culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycococcus fulvus</em></td>
<td>80.7</td>
<td>30 °C, 72 h, liquid culture</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.a. = not available; PAH = polycyclic aromatic hydrocarbons.
animal gut and faeces and fermented food. These bacteria include *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* strain FA4T from polycyclic aromatic hydrocarbons (PAH) contaminated soils (Alberts *et al.*, 2006; Hormisch *et al.*, 2004), *Bacillus licheniformis* strain from fermented soybean (Petchkongkaew *et al.*, 2008), bacterial community rumen fluid (Upadhaya *et al.*, 2009), *Stenotrophomonas maltophilia* and *Myxococcus fulvus* strains from animal faeces (Guan *et al.*, 2008, 2010).

Bacterial biotransformation of AFB₁, is generally efficient and culture supernatant as well as enzymatic compounds are mostly responsible for the biotransformation (Mishra and Das, 2003). Liquid culture of *M. fluoranthenivorans* FA4T completely biotransformed AFB₁ in 72 h in aqueous solution (Hormisch, 2004). Both cell-free extracts of *R. erythropolis* and *M. fluoranthenivorans* showed 100% biotransformation on AFB₁ in 8 h incubation (Teniola *et al.*, 2005). Culture supernatant of *R. erythropolis* biotransformed 66.8% of AFB₁ after 72 h incubation (Alberts *et al.*, 2006). Additionally, some of the studies evaluated the toxicity of biotransformation product and a loss of mutagenicity was observed by Ames test (Alberts *et al.*, 2006).

Our group has done a series of studies on bacterial biotransformation of AFB₁. In a recent report, 65 contaminated feed and animal faeces samples were screened for AFB₁, biotransformation bacteria using a newly developed medium containing coumarin as the sole carbon source (Figure 2). Twenty five purified bacterial isolates exhibited AFB₁, biotransformation activity in liquid medium. One isolate obtained from tapir faeces and identified to be *S. maltophilia*, biotransformed AFB₁ by 82.5% after incubation at 37°C for 72 h in liquid medium. Treatments with proteinase K, proteinase K plus SDS and heating significantly reduced or eradicated the biotransformation activity of the culture supernatant, which indicated that enzymes may be involved in AFB₁ biotransformation (Guan *et al.*, 2008). In addition, another bacterial strain, which was isolated from deer faeces and identified as *M. fulvus*, was able to biotransform AFB₁ by 80.7% in liquid medium after incubation at 30°C for 72 h. Liquid chromatography–mass spectrometry (LC-MS) analyses showed that AFB₁ was biotransformed to structurally different compound. Chromatography on DEAE-ion exchange and Sephadex-molecular sieve and SDS-PAGE electrophoresis were used to determine active components from the culture supernatant, indicating enzyme(s) were responsible for the AFB₁ biotransformation (Guan *et al.*, 2010). Further work such as enzyme purification and responsible genes identification and cloning is underway (Zhao *et al.*, 2011).

It can be concluded from the literature that bacterial biotransformation of AFB₁ exists in nature. The research in biotransformation is very appealing since most published bacterial strains exhibited high efficiency and non-reversible metabolism of AFB₁ instead of binding. Published studies also show that enzymes in culture supernatant or intracellular metabolite are responsible for AFB₁ metabolism. It thus has a great potential for application in feed and food. However, studies in this area are very limited. One of the main reasons is that the screening of bacteria is very time consuming and currently there is no effective method. There are two basic strategies for screening mycotoxin biotransforming bacteria. One is using AFB₁ or structurally similar compound as single carbon source. In our previous study, a screening method by using coumarin as single carbon source was developed and proven to be an inexpensive, feasible and effective tool for selecting AFB₁ biotransformation microorganisms.

Since coumarin is a non-toxic structure analog of AFB₁ and microorganisms that could utilise coumarin as their carbon source might also be able to use aflatoxins (Guan *et al.*, 2008). The other is randomly to pick up bacterial cultures for their biotransformation ability in different media (Völkl *et al.*, 2004). However, the problem is that a large portion of microorganisms from the nature are not cultivable in artificial medium under laboratory conditions.

Despite the significant advances that have been made, one ongoing problem in this area is that in most studies, the AFB₁ biotransformation product is unknown. Product identification and toxicity evaluation are very much needed to address the safety concerns. Another issue is that most of the bacteria are screened from nature and evaluation of the toxicity of the bacterium is necessary before application in feed and food chains.

**Fungal biotransformation**

AFB₁ biotransformation by its producing fungi was observed and well documented (Table 3). Intact mycelia are generally more effective than cell free extracts. However, re-conversion from the product to AFB₁ was also observed and it is hard to control the process. Transformation of aflatoxins by their producer *A. flavus* was rapid and it depended primarily on mycelial lysis and high-aeration.
conditions. The process was non-specific and a complex series of reactions were involved (Ciegler et al., 1966b). Further studies found that AFB₁ biotransformation was enhanced by NADPH and NaNO₂, which suggested the involvement of cytochrome P-450 monooxygenases (Hamid and Smith, 1987). 9-day-old mycelia of A. parasiticus NRRL 2999 was also able to biotransform AFB₁ and AFG₁. Rates for biotransformation were maximum at 28 °C with the pH in the range of 5.0 to 6.5 (Doyle and Marth, 1978a,b,c,d).

AFB₁ biotransformation by non-aflatoxin-producing filamentous fungi is a different mode. Cell free extract or intracellular extracts instead of mycelium are involved. Fungal cultures from Trichoderma sp. 639, Phoma sp., Rhizopus sp. 668, Rhizopus sp. 720, Sporotrichum sp. ADA, Sporotrichum sp. SF and Alternaria sp. showed AFB₁ biotransformation ability (Motomura et al., 1998; Tsubouchi et al., 1980; Varga et al., 2005; Wu et al., 2009).

Moreover, some edible fungal strains also exhibited biotransformation ability. Extra-cellular components as well as intracellular components are responsible. A serial of studies were conducted on an edible fungus Armilliarella tabescens E-20 (Liu et al., 1998, 2006a,b; Yao et al., 2006a,b). AFB₁ at an initial concentration of 16 mM was completely biotransformed by the intracellular components from mycelial pellets. The infrared spectrum suggested that the intracellular components were responsible for opening the difuran ring of AFB₁. Toxicological, pathological and mutagenic studies with hepatic histological structure and Ames test showed that the toxicity of AFB₁ was greatly reduced. Extra-cellular components from two edible fungal isolates namely Pleurotus ostreatus and Trametes versicolor exhibited AFB₁ biotransformation ability (Motomura et al., 2003). Transformation of AFB₁ by white rot fungi Peniophora sp. SCC0152 was also reported (Alberts et al., 2009).

Overall, it can be concluded that thus far, only a few fungal species/isolates have been observed that are able to biotransform AFB₁ effectively. The biotransformation activities of AFB₁ producing fungi were mainly in their mycelia while cell extracts for other fungi. It seems that in most cases, enzyme components are involved in biotransformation. However, practical applications may be limited by long inoculation time (usually 8-15 days) required by mycelia to produce significant biotransformation compound. One safety concern is that some fungi could also revert the product to AFB₁. In that case, more work should be done to characterise and purify certain enzyme systems that are responsible for AFB₁ biotransformation by fungi.

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Table 3. Aflatoxin biotransformation fungi from various sources.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Aflatoxins Transformed (%)</th>
<th>Conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus flavus</td>
<td>AFB₁, AFG₁ 15-55</td>
<td>72 h, 15-30 °C, liquid culture</td>
<td>Ciegler et al., 1966b</td>
</tr>
<tr>
<td></td>
<td>18-25, 11-48</td>
<td>6-12 day intact mycelium, cell-free extracts</td>
<td>Hamid and Smith, 1987</td>
</tr>
<tr>
<td>Aspergillus parasiticus</td>
<td>AFB₁, AFG₁ 30-38</td>
<td>9-day-old mycelia, 96 h, 28 °C, pH</td>
<td>Doyle and Marth, 1978a,b,c,d</td>
</tr>
<tr>
<td>NRRL 2999</td>
<td></td>
<td>5-6.5 cell free extract, liquid culture, 5 days, 28 °C</td>
<td>Shantha, 1999</td>
</tr>
<tr>
<td>Phoma sp., Trichoderma sp.</td>
<td>AFB₁ 65-99</td>
<td>5-day killed cell culture, 40 °C</td>
<td>Faraj et al., 1993</td>
</tr>
<tr>
<td>639, Rhizopus sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>668, Rhizopus sp. 720</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporotrichum sp. ADA,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporotrichum sp. SF and</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alternaria sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizopus oryzae, Aspergillus niger, Mucor racemosus, Alternaria alternata</td>
<td>AFB₁ 30-84</td>
<td>7-day-old mycelia, intracellular components. 6-10 days, 25 °C</td>
<td>Nakazato et al., 1990</td>
</tr>
<tr>
<td>Rhizopus sp. Aspergillus flavus</td>
<td>AFB₁ 10-50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Armillariella tabescens E-20</td>
<td>AFB₁ 100</td>
<td>5-day intact mycelium pellets, 30 min, 28 °C</td>
<td>Liu et al., 1998, 2006a,b; Yao et al., 2006a,b</td>
</tr>
</tbody>
</table>

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Microbial enzyme transformation

Peroxidase enzyme such as laccase enzymes from various sources have been reported to biotransform AFB₁ (Table 4). In most cases, these enzymes require mild conditions for AFB₁ biotransformation, such as pH near neutral and temperature between 20-30 °C.

Doyle and Marth (1979) revealed that peroxidase was involved in aflatoxin biotransformation by A. parasiticus. A positive relationship was observed between rates of aflatoxin biotransformation and peroxidase activity in mycelial extracts by (NH₄)₂SO₄ precipitation. AFB₁ biotransformation with laccase from different fungi was investigated in liquid media. Commercially available pure laccase enzyme from T. versicolor (1 U/ml) showed high activity, with 87.34% of AFB₁ biotransformed. Peniophora sp. SCC0152 showed high laccase activity (496 U/l), with 40.5% AFB₁ biotransformed in liquid medium. P. ostreatus exhibited laccase activity of 416.4 U/l and 35.9% biotransformation of AFB₁. Transformation of AFB₁ by recombinant laccase (118 U/I) produced by A. niger D15-Lcc2#3 was 55%. Salmonella typhimurium mutagenicity assay showed loss of mutagenicity of AFB₁ after fungal laccase treatment (Alberts et al., 2009). A lactoperoxidase with AFB₁ and aflatoxin G₁ (AFG₁) biotransformation ability was described from A. parasiticus (Doyle and Marth, 1978e). Aflatoxin biotransformation by this enzyme was independent of initial aflatoxin concentration. Aflatoxin B₂₅ and some water soluble components were the major products. In a recent study, three anti-oxidative stress enzymes, namely superoxide dismutase, catalase and peroxidase were characterised from P. ostreatus (Keyhani et al., 2009).

Meanwhile, some unidentified enzymes were characterised and purified from various microbial metabolites. Most of these enzymes have small molecular weight below 100 KDa and they require specific conditions for AFB₁ biotransformation. An extracellular enzyme with aflatoxin biotransformation ability was purified from edible mushroom P. ostreatus by two chromatographies on DEAE-sepharose and phenyl-sepharose. The apparent molecular mass was 90 KDa by SDS-PAGE. Optimum activities were found in the pH range between 4.0 and 5.0 and at 25 °C. Fluorescence measurements suggested that the enzyme cleaves the lactone ring of aflatoxin (Motomura et al., 2003). AFB₁ biotransformation enzyme from A. tabescens E-20 was characterised and purified (Liu et al., 2001). The apparent molecular mass was 51.8 KDa with the optimal activity at pH 6.8 and 35 °C. In addition, the multi-enzyme immobilisation onto solid carriers was shown to be an effective way to enhance the reactivity and stability of the fungus enzyme. Immobilised enzyme showed a wider range of temperature and pH stability, with the best pH range of 5.5-7.0 and temperature range of 30-35 °C. The AFB₁ biotransformation was further confirmed by Ames, duckling, and rat toxic tests (Liu et al., 2006b). D’Souza andBrackett (1998, 2000, 2001) investigated the effect of several ions on AFB₁ biotransformation by F. aurantiacum culture and reductase system was proposed to play an important role. In our group, an enzyme with high activity in biotransforming AFB₁, AFG₁ and aflatoxin M₁ (AFM₁) was purified and characterised from M. fulvus ANSM068 extracellular metabolite. A specific activity of 569.44x10³ U/mg

Table 4. Aflatoxin biotransformation enzymes from various microbes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (%)</th>
<th>Conditions</th>
<th>Origin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase</td>
<td>26-37</td>
<td>2840 U/g, 28 °C, 96 h</td>
<td>Aspergillus parasiticus</td>
<td>Doyle and Marth, 1979</td>
</tr>
<tr>
<td>Laccase</td>
<td>87.34</td>
<td>1 U/ml</td>
<td>Trametes versicolor</td>
<td>Alberts et al., 2009</td>
</tr>
<tr>
<td></td>
<td>40.45</td>
<td>496 U/l</td>
<td>Peniophora sp.</td>
<td>Alberts et al., 2009</td>
</tr>
<tr>
<td></td>
<td>35.00</td>
<td>416.39 U/l</td>
<td>Pleurotus ostreatus</td>
<td>Alberts et al., 2009</td>
</tr>
<tr>
<td>Recombinant laccase</td>
<td>55</td>
<td>118 U/I, 30 °C, 72 h</td>
<td>Aspergillus niger</td>
<td>Alberts et al., 2009</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>3.6-5.1</td>
<td>5-500 U/ml, 24 h</td>
<td>Aspergillus parasiticus</td>
<td>Doyle and Marth, 1978e</td>
</tr>
<tr>
<td>Unknown enzymes</td>
<td>n.a.</td>
<td>90 kDa, pH 4-5, 25 °C</td>
<td>Pleurotus ostreatus</td>
<td>Motomura et al., 2003</td>
</tr>
<tr>
<td>Superoxide dismutase, catalase,</td>
<td>n.a.</td>
<td>7.4 U/mg, 11.7 U/mg, 0.037 U/mg</td>
<td>Pleurotus ostreatus</td>
<td>Keyhani et al., 2009</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>n.a.</td>
<td>7.09 nmol min/mg 51.8 kDa, pH 6, 28 °C</td>
<td>Atrantilia tabescens E-20</td>
<td>Liu et al., 2001, 2006a</td>
</tr>
<tr>
<td>Unknown enzymes</td>
<td>96.96 AFG₁, 32 kDa, pH 6, 35 °C</td>
<td>n.a.</td>
<td>Myxococcus fulvus ANSM068</td>
<td>Zhao et al., 2010</td>
</tr>
<tr>
<td></td>
<td>95.80 AFM₁</td>
<td>n.a.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.a. = not available.
was obtained using chromatography on DEAE-sepharose and Superdex 75. The apparent molecular mass was estimated to be 32 KDa by SDS-PAGE. The enzyme (100 U/ml) exhibited optimal activity at 35 °C and pH 6.0, with 96.96% of AFG1 and 95.80% of AFM1, biotransformed.

Thus far, much interest has been focused on commercially produced natural enzymes for AFB1 biotransformation. However, only a few enzymes have been identified and characterised up to now, most of which belong to peroxidase enzymes. All the enzymes exhibit biotransformation function only in mild pH and temperature conditions and stability is the most important factor influencing its application. Additionally, enzymes are generally active in aqueous solution while AFB1 is insoluble in water and soluble in organic solvents. Immobilised enzymes are promising in this case and are applicable in liquid commodities.

4. Future trends/perspective

It can be concluded that most of the biological detoxification research has been carried out in simple systems such as liquid medium or aqueous buffer solution. Some studies have indicated that detoxification in a food model might be interfered with other factors and the efficiency might be affected. Thus more research has to be done to investigate the practical efficacy of these microorganisms or their enzymes in food and feeds.

It is also very important to develop a practical way to apply different types of biological agents. For those binding bacteria and yeasts, more efforts should be devoted to developing binding agents in the gastro-intestinal tract of animals to prevent mycotoxins absorption in vivo. On the other hand, for extracellular/intracellular metabolite producers such as bacterial strains and fungal strains, it should be more efficient to use purified enzymes as feed additives to eliminate adverse effect of AFB1. In that case, more work should be conducted on enzyme stability and characterisation.

Furthermore, a fundamental solution to aflatoxin problems is to completely eliminate the toxin using combined multiple approaches. Recent advances in molecular biology and gene engineering have facilitated developing innovative methods. Further studies on the aflatoxin biotransforming microorganisms could focus on identification of genes responsible for AFB1 detoxifications. Also, DNA identified from various sources can be used to construct genomic libraries so that genes of specific detoxification enzymes could be searched. Once identified, these genes can be used in transgenic crop varieties for improved plant fungal pathogen resistance. They can also be applied to develop innovative biological detoxification strategies.

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