Isolation of *Bacillus* spp. from Thai fermented soybean (Thua-nao): screening for aflatoxin B_1 and ochratoxin A detoxification

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Abstract

Aims: To study the interaction between *Bacillus* spp. and contaminating *Aspergillus flavus* isolated strains from Thai fermented soybean in order to limit aflatoxin production. To study the detoxification of aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) by *Bacillus* spp. in order to find an efficient strain to remove these toxins.

Methods and Results: One *A. flavus* aflatoxin-producing strain and 23 isolates of *Bacillus* spp. were isolated from soybean and fresh Thua-nao collected from the north of Thailand. Inhibition studies of *A. flavus* and *A. westerdijkiae* NRRL 3174 (reference strain) growth by all isolates of *Bacillus* spp. were conducted by dual culture technique on agar plates. These isolates were also tested for AFB_1 and OTA detoxification ability on both solid and liquid media. Most of the strains were able to detoxify aflatoxin but only some of them could detoxify OTA.

Conclusions: One *Bacillus* strain was able to inhibit growth of both *Aspergillus* strains and to remove both mycotoxins (decrease of 74% of AFB_1 and 92·5% of OTA). It was identified by ITS sequencing as *Bacillus licheniformis.* The OTA decrease was due to degradation in OTα. Another *Bacillus* strain inhibiting both *Aspergillus* growth and detoxifying 85% of AFB_1 was identified as *B. subtilis*. AFB_1 decrease has not been correlated to appearance of a degradation product.

Significance and Impact of the Study: The possibility to reduce AFB_1 level by a strain from the natural flora is of great interest for the control of the quality of fermented soybean. Moreover, the same strain could be a source of efficient enzyme for OTA degradation in other food or feeds.

Introduction

Thua-nao is one of the oldest traditionally fermented soybean products produced for years by the people in many small villages up north of Thailand. *Bacillus* spp., Gram-positive, strict or facultative aerobe and endospore-forming bacteria, were found to be the dominant microflora of this product (Chantawannakul *et al.* 2002). However, during fermentation, the soybeans would be sometimes contaminated with toxigenic fungi for which the intrinsic and extrinsic factors may also induce the mycotoxin formation, leading to health risk for consumers (Garcia *et al.* 1997).

The mycotoxins of the greatest significance in food and feeds are aflatoxins which are of great concern because of their detrimental effects on the health of humans and animals, including carcinogenic, mutagenic, teratogenic, and immunosuppressive effects (Eaton and Gallagher

1994). They are produced mainly by *Aspergillus flavus*, *A. parasiticus* or *A. nominus*. The four main naturally produced aflatoxins are B_1, B_2, G_1 and G_2, with the former two being the aflatoxins found at the highest concentration in contaminated food and feed.

Another important mycotoxin which has received increasing interest from both scientific communities and food committees is ochratoxin A (OTA). OTA is produced by *A. ochraceus* and related species. *Aspergillus* section *Nigri*, for example, *A. niger* aggregate and *A. carbonarius*, were shown to be responsible for OTA production in grapes (Abarca *et al.* 2001; Cabañes *et al.* 2002). *Penicillium* spp., particularly *Penicillium verrucosum* is OTA producer as well (Pitt 1987). OTA is a derivative of isocoumarin linked to L-phenylalanine and it has a potent nephrotoxic (Krogh *et al.* 1974; Mortensen *et al.* 1983), teratogenic (Arora and Fröelén 1981), genotoxic (Dirheimer 1998), immunosuppressive (Haubeck *et al.* 1981) and carcinogenic (Boorman 1989) properties. If the consumers take moderate to low concentrations of this mycotoxin in the long term, much more serious problem may arise.

Some researches showed that *Bacillus subtilis* could be able to inhibit *Aspergillus* growth (Foldes *et al.* 2000) and that *B. stearothermophilus* could inhibit aflatoxin production by *A. flavus* and *A. parasiticus* (Faraj *et al.* 1993). Mixing *B. subtilis* with groundnuts could reduce the damage caused by *A. flavus* (Sommartya 1997). In addition, fermentation of contaminated grains has been shown to degrade aflatoxins (Dam *et al.* 1977). Moreover, Smith and Harran (1993) reported that *B. stearothermophilus* could degrade aflatoxin B_1 (AFB_1) *in vitro*. Concerning OTA, a few micro-organisms are able to degrade it, for example, *Saccharomyces cerevisiae*, *Lactobacillus* spp. (Böhm *et al.* 2000; Piotrowska and Wakowska 2000), *B. subtilis* and *B. licheniformis* (Böhm *et al.* 2000), *Rhizopus* (Varga *et al.* 2005) and *A. niger* (Bejaoui *et al.* 2006).

The present study has been then performed with two objectives. The first one was to study the interaction between *Bacillus* spp., isolated strains from Thai fermented soybean product, and contaminating *A. flavus* in soybean in order to limit aflatoxin production. The second objective was to study the detoxification of AFB_1 and OTA by *Bacillus* spp. in order to find an efficient strain to remove these toxins.

Materials and methods

Isolation of *Bacillus* spp. from Thai fermented soybean product (Thua-nao)

Three samples of fresh Thua-nao were collected from the local market in ChiangMai and Maehongson province, which are located in the North of Thailand. One gram of each sample was suspended in 9 ml of phosphate buffer, mixed well by vortex and agitated for 30 min. Appropriate dilutions (up to 10^{-7}) were made with phosphate buffer as diluent. One hundred microlitres of each dilution were spreaded on nutrient agar and incubated at 37°C for 24 h. Colonies appeared on plate were picked up and maintained on nutrient agar [3 g beef extract (Difco laboratories, USA), 5 g peptone (Fluka BioChemika, Buchs, Switzerland), 15 g agar (Difco laboratories) in 1 l of distilled water) at 4°C. Stock culture were cultivated on nutrient broth (NB) and stored at −20°C after addition of sterile glycerol at 20%.

Identification of interested isolates of *Bacillus* spp.

Twenty-three colonies were first characterized by Gram's stain technique and then identified as *Bacillus* spp. by its sugar utilization pattern in the API 50CH system (API 50 CHB medium, BioMèrieux® SA 673 620 399 RCS Lyon, 69280 Marcy-l'Etoile, France). All isolates were maintained on nutrient agar.

The two interesting *Bacillus* isolates were identified by ITS sequencing according to the method described by Liu *et al.* (2000). The website http://www.ncbi.nlm.nih.gov/entrez/query.fcgi was used for sequence analysis at the GenBank database.

Screening of the isolated *Bacillus* spp. for growth inhibition of *Aspergillus flavus* and *Aspergillus westerdijkiae* NRRL 3174

Aspergillus westerdijkiae NRRL 3174 was chosen as OTA producer reference strain. *Aspergillus flavus* strain used in this study was isolated from soybean in Thailand and its ability to produce aflatoxin was confirmed as described by Bragulat *et al.* (2001). Growth inhibition studies of isolated *A. flavus* and *A. westerdijkiae* by all isolates of *Bacillus* spp. were conducted by dual culture technique. The culture media used were nutrient agar and potato dextrose agar (PDA) for *A. flavus* and nutrient agar and Czapek yeast extract (CYA) agar for *A. westerdijkiae* NRRL 3174. They were poured into 100-mm Petri dishes. After agar solidification, the fungal strains were inoculated in the centre and then each isolate of *Bacillus* spp. was inoculated at 10 mm from the edge of the Petri dish. They were then incubated at 25°C and 37°C for 7 days. The experiment was carried out in triplicate. Radial growth reduction was calculated as follows:

$$\frac{a-b}{a}$$

where a = radial growth measurement of the pathogen in control and b is that of the pathogen in the presence of

bacteria tested; these values were expressed as percentage inhibition of radial mycelial growth.

Degradation of AFB_1 and OTA by 23 *Bacillus* spp. isolates

Standard AFB_1 and OTA were used through this experiment. One hundred microlitres of *Bacillus* spp. from frozen stock suspension was inoculated in 5 ml of NB filled in 15-ml Falcon tube. Standard AFB_1 or OTA at initial concentration of 5 mg l^{-1} was added. The cultures of *Bacillus* spp. with toxin were then incubated at 37°C in shaking incubator with speed of 150 rev min^{-1} for 1 week.

Kinetics of AFB_1 and OTA degradation during growth of the two selected isolates (*Bacillus* CM 21 and *Bacillus* MHS 13)

One millilitre of each frozen *Bacillus* stock culture (*Bacillus* CM 21 and *Bacillus* MHS 13) was transferred to a 250-ml Erlenmeyer flask (Pyrex, USA) containing 100 ml of sterilized NB and shaking incubated at 37°C for 48 h. The optical density (OD) of the culture broth was measured by using a spectrophotometer at 660 nm every 6 h. After 48 h, the cultured broth was used to inoculate 100 ml of fresh sterilized NB. The culture was then diluted to an absorbance approximately 0·1 with fresh media. The initial concentration of 5 mg l^{-1} of each standard AFB_1 or OTA were added into NB. All cultures were incubated at 37°C with agitation. Five millilitres of each culture were sampled twice a day for 5 days and then once a day for the following 10 days. OD at 660 nm was measured at each time of sampling.

Aflatoxin B_1 analysis by HPLC

AFB_1 extraction was made according to Sánchez *et al.* (2005) modified method. Ten millilitres of acetone and 15 ml of dichloromethane were added to NB. Following the addition of each solvent, the cultures were shake-agitated at 150 rev min^{-1} for 30 min. The filtrate was partitioned in a preparatory funnel into an aqueous phase and a dichloromethane phase that contained the most of the aflatoxin. The aqueous phase was partitioned again with 15 ml of fresh dichloromethane. The two dichloromethane fractions were combined. Residual H_2O was removed from the final dichloromethane solution with 1 g Na_2SO_4 and the solution was filtered with Whatman paper No.1. The filtrate was evaporated at 40°C (90 rev min^{-1}) until dry. The residue was dissolved with 1 ml acetonitrile, filtered (Millex® HV PVDF 0·45 μm; Millipore, Billerica, MA) and analysed by HPLC.

The HPLC method for AFB_1 analysis was modified from Chan *et al.* (2004). The HPLC apparatus consisted of a solvent delivery system with both fluorescence (λ_{ex} = 364 nm; λ_{em} = 440 nm) and ultraviolet (UV) detectors (λ = 225 nm and 362 nm). The spectra range is from 200 to 500 nm. The analytical column used was a 150 × 4·6 mm Uptisphere 5 μm C18 ODB fitted with a guard column of 10 × 4 mm. The column temperature was 25°C. An aliquot of sample (80 μl) was injected using an autoinjector (BIO-TEK, Milan, Italy). The mobile phase was 0·1% phosphoric acid (A) and methanol/acetonitrile (50 : 50) (B) delivered at flow rate of 1 ml min^{-1} for 30 min. The sample was eluted with a linear gradient for 30 min. AFB_1 and its derivatives were detected by comparing the elution time and maximum absorption of UV with the standards. The relative standard deviation of this analysis method is of 6%.

Ochratoxin A analysis by HPLC

Thirty-four microlitres of 12 N HCl (NormaPur; Prolabo, R.P.) was added to the culture followed by 3·5 ml of chloroform (Prolabo, R.P. NormaPur). The solution was partitioned in a preparatory funnel into an aqueous phase and a chloroform phase that contained most of the OTA. The chloroform phase was recuperated and then evaporated under nitrogen gas at 65°C (90 rev min^{-1}) until dry. The samples were then dissolved in 1 ml methanol (Fisher Scientific), filtered (Millex®HV PVDF 0·45 μm) and analysed by HPLC as described by Awad *et al.* (2005).

The HPLC apparatus consisted of a solvent delivery system with fluorescence detector (λ_{ex} = 332 nm; λ_{em} = 466 nm). The analytical column used was a 150 × 4·6 mm Uptisphere 5 μm C18 ODB fitted with a guard column of 10 × 4 mm. The column temperature was 30°C. An aliquot of sample (80 μl) was injected using an autoinjector (Bio-Tek). The mobile phase was acetic acid in water 0·2% (A) and acetonitrile (B) delivered at flow rate of 1 ml min^{-1} for 45 min. The sample was eluted with a linear gradient from 10% to 50% of B over the first 30 min followed by a linear gradient to 90% of B from 30 to 35 min and then a steady flow of 90% of B through 8 min and then reduced to 10% of B through 2 min. OTA and its derivative products were detected by comparing the elution time with the standards. The relative standard deviation of this analysis method is of 5·4%.

Statistical analysis

All experiments were carried out in triplicate or duplicate. SPSS software for Windows was used for the statistical

analysis of the data. For *Bacillus* strains comparison, the differences were considered significant if the associated *P*-values were <5%. The significant differences between strains have been pointed out by ANOVA analysis and Duncan's multiple range test.

Results

Bacillus isolation and identification

Three samples of fresh Thua-nao, respectively two and one of them, were collected from the local market in ChiangMai and Maehongson province, which are located in the north of Thailand, and they were labelled with the code FTN 01, FTN 02 and FTN 03.

Twenty-three colonies were isolated from these three samples on PDA-nutrient agar. Seven and eight isolates of *Bacillus* spp. were isolated from the two samples collected from ChiangMai province and named respectively CM 11 to CM 17 and CM 21 to CM 28. The eight isolates collected on the sample from Maehongson province were named as MHS 11 to MHS 18. Morphological and physiological characteristics of all isolates were identified as *Bacillus* spp. and confirmed by the API 50CH system.

Screening of the isolated *Bacillus* spp. for growth inhibition of *Aspergillus flavus* and *Aspergillus westerdijkiae* NRRL 3174

Twelve isolates out of twenty-three isolates of *Bacillus* spp. could inhibit the growth of *A. flavus* (data not shown) at a level significantly different from the others isolates. The average percentage of inhibition for these twelve isolates was 52%. According to these results, the growth of *A. flavus* was inhibited by *Bacillus* MHS 13 significantly more than by other isolates from the same origin. Regarding the results for *A. westerdijkiae* NRRL 3174, eight isolates can inhibited its growth with an average percentage of inhibition of 34%. Moreover, *Bacillus* CM 21 is responsible for the significantly highest growth inhibition percentage.

Screening of the isolated *Bacillus* spp. for removal of AFB_1 and OTA

The 23 isolates were tested for AFB_1 and OTA removal. Results are shown in Figures 1 and 2, respectively. Most of the strains were able to detoxify aflatoxin but only some of them could detoxify OTA. After 1 week, at least 60% AFB_1 was degraded by 11 *Bacillus* strains. Among

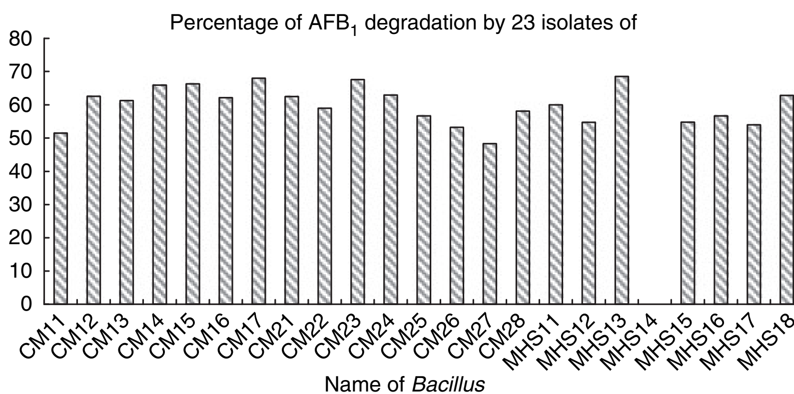

Figure 1 Percentage of AFB_1 degradation by all isolates of *Bacillus* spp.

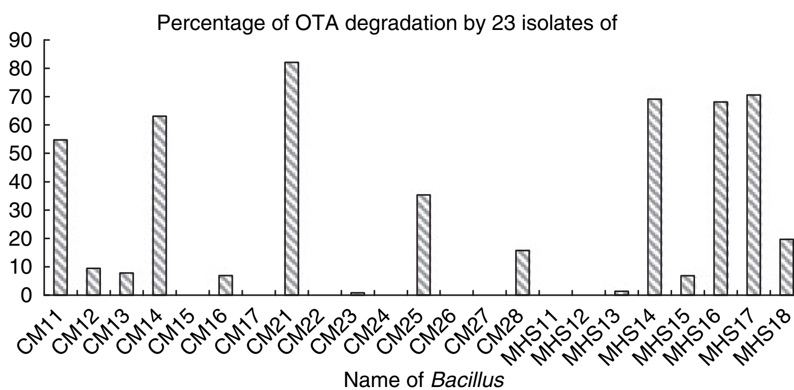

Figure 2 Percentage of OTA degradation by all isolates of *Bacillus* spp.

them, CM 17, CM 23 and MHS 13 were the most efficient. The highest percentage of OTA removal (81%) was obtained by *Bacillus* CM 21. As this last strain was able to degrade more than 60% AFB_1, it has been retained like strain MHS 13 for the continuation of work.

These two interesting *Bacillus* isolates, *Bacillus* CM 21 and *Bacillus* MHS 13, were identified by ITS sequencing. The results of sequence analysis with GenBank database showed that *Bacillus* CM 21 is *B. licheniformis* (99% identity) and *Bacillus* MHS 13 is *B. subtilis* (100% identity).

Kinetics AFB_1 and OTA removal during growth of the *Bacillus licheniformis* CM 21 and *Bacillus subtilis* MHS 13

Detoxification kinetics of AFB_1 and OTA in *Bacillus* CM 21 and *Bacillus* MHS 13 liquid culture were investigated. We could observe that AFB_1 decreased during the first 5–6 days (Figs 3 and 4). The percentage of AFB_1 removal by *Bacillus* CM 21 and *Bacillus* MHS 13 was 74% and 85%, respectively.

Regarding OTA degradation (Fig. 5), *Bacillus* CM 21 can degrade OTA within 48 h of incubation, whereas ochratoxin α was increasing during the same time (data not shown). The percentage of OTA degradation by this isolate was 92·5%. On the opposite, there was no OTA degradation during the growth of *Bacillus* MHS 13 as observed in previous experiment on agar plates.

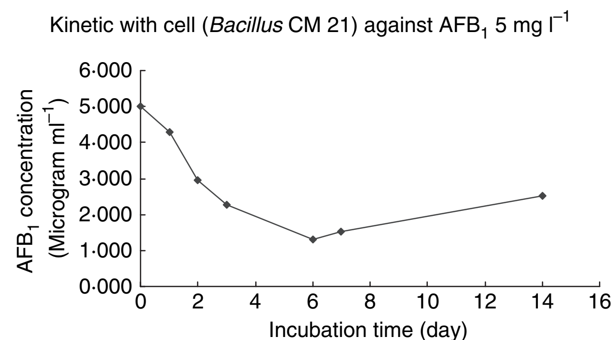

Figure 3 AFB_1 degradation during the growth of *Bacillus* CM 21.

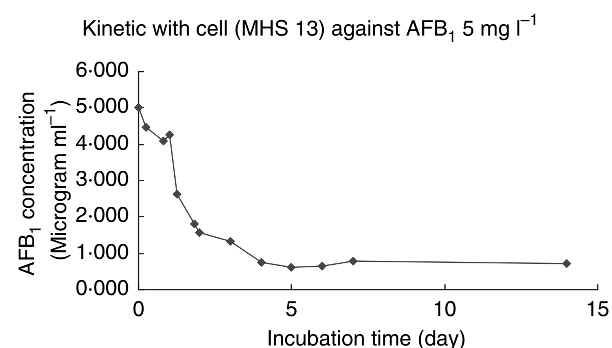

Figure 4 AFB_1 degradation during the growth of *Bacillus* MHS 13 (mean of two experiments).

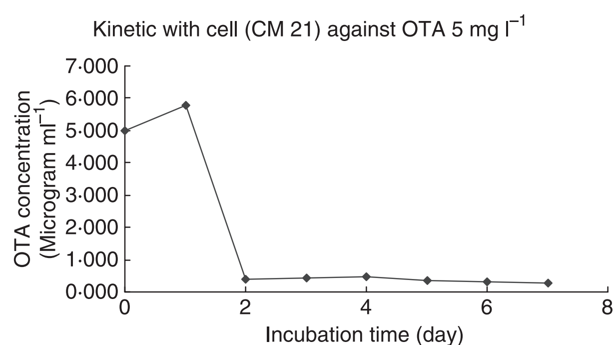

Figure 5 OTA degradation during the growth of *Bacillus* CM 21.

Table 1 Some characteristics for *Bacillus* growth in liquid medium

Strain	Duration of growth phase (h)	Time for maximal production of ammonium (h)	Maximal concentration of ammonium (mg l^{-1})
B. licheniformis CM 21	36	30	220
B. subtilis MHS 13	6	20	200

Table 1 shows some characteristics of *Bacillus* growth for both strains: duration of growth phase and produced ammonium.

Discussion

In the present study, *Bacillus* spp. was investigated for *Aspergillus* growth inhibition for mycotoxins producing strains and for AFB_1 and OTA reduction ability. *Bacillus* spp. were found to be the dominant microflora of Thai fermented soybean (Thua-nao) (Chantawannakul *et al.* 2002). These fermentations are characterized by extensive hydrolysis of protein to amino acids, peptides and ammonia and a rise of the pH. The pH increase observed during fermentation presumably resulted from proteolysis and the release of ammonia because of utilization of amino acids for growth (Terlabie *et al.* 2006).

The chemical approach to the detoxification of aflatoxins that have received considerable attention is ammoniation (Smith and Harran 1993; Bhatnagar *et al.* 2002). Ammoniation under appropriate conditions results in a significant reduction in the level of aflatoxins in contaminated peanut and cottonseed meals. The mechanism for

this action appears to involve hydrolysis of the lactone ring and chemical conversion of the parent compound AFB_1 to numerous products that exhibit greatly decreased toxicity. Two major products identified as compounds with molecular weight 286 (AFD_1) and molecular weight 206, have been isolated and tested in various biological systems (Park *et al.* 1988). The first step in the reaction is reversible if the ammoniation process is carried out under mild conditions. This is a disadvantage of the method. However, when the reaction is allowed to proceed past the first step, the products formed do not revert back to AFB_1. Reaction products of ammoniation are dependent on temperature, pressure and the source of ammonia. This method is quite energy consuming and presents some risks from security point of view.

In order to find biological alternative methods, micro-organisms (including yeasts, mold and bacteria) have been screened for their ability to modify or inactivate aflatoxins. *Flavobacterium aurantiacum* (NRRL B-184) was shown to remove aflatoxin from a liquid medium significantly without the production of toxic by-products (Ciegler *et al.* 1966). The same investigators also found that certain acid-producing molds could catalyse the hydration of AFB_1 to B_{2a} (a less toxic product). In the case of some *Aspergillus* strains that can be at the same time aflatoxin producer and able to degrade it, peroxyd-ases activities where shown to catalyse aflatoxin degrada-tion (Smith and Harran 1993). For *Bacillus* strains, this has never been reported. In our case, no degradation product of AFB_1 could be detected by HPLC analysis. Growth and ammonium production occurred during the first hours of culture, whereas AFB_1 decrease took several days (Table 1). So, the mechanism remains unknown.

Regarding OTA, several reports describe OTA degrad-ing activities of the microbial flora of the mammalian gastrointestinal tract including rumen microbes of cow and sheep (Galtier and Alvinerie 1976; Hult *et al.* 1976; Park *et al.* 1988) and microbes living mainly in the caecum and large intestine of rats (Madhyastha *et al.* 1992). The human intestinal microflora can also partially degrade OTA (Akiyama *et al.* 1997). The species responsi-ble for OTA detoxification have not yet been identified although mainly protozoa were suggested to take part in the biotransformation process in ruminants (Kiessling *et al.* 1984). Degradation of OTA was observed in milk as a result of the action of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* spp. (Skrinjar *et al.* 1996), whereas two other bacteria, *Acinetobacter calcoaceticus* (Hwang and Draughon 1994) and *Phenylobacterium immobile* (Wegst and Lingens 1983), could also convert OTA to the much less toxic ochratoxin α in liquid cultures. Furthermore, recent reports describe the OTA degrading activities of some *Aspergillus* and *Pleurotus* isolates and/or their enzyme (Varga *et al.* 2000). In our case, we observed that OTA is also efficiently detoxified by some *Bacillus* isolates and specifically *B. licheniformis* CM21 and this has not been reported before for this species. Similarities between OTA degradation kinetics by *A. niger* and *Bacillus* isolates and the detection of the degradation product, ochratoxin α, in the ferment broth of *B. licheniformis* suggest that a car-boxypeptidase A activity may be responsible for OTA decomposition by these isolates.

The possibility to reduce AFB_1 level by a strain of *B. licheniformis* from the natural flora is of great interest for the control of the quality of fermented soybean. Moreover, the same strain could be a source of efficient enzyme for OTA biodegradation in other food or feeds. Accordingly, further studies are in progress to determine which enzymes take part in the detoxification process in *Bacillus* isolates, and to establish the absence of by-prod-ucts and with any residual toxicity, and probably also genetic modification, are required to harness the potential of these strains.

Safety

AFB_1 and OTA are a toxic compound that needs to be manipulated with care and with appropriate safety pre-cautions. Decontamination procedures for laboratory wastes have been reported by the International Agency for Research on Cancer (IARC).

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