Biological detoxification of zearalenone by Aspergillus niger strain FS10

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Abstract

Zearalenone (ZEN) contamination of corn and cereal products is a serious health hazard throughout the world and its elimination by microbial methods is now being widely examined. In this study, an Aspergillus niger strain, FS10, isolated from Chinese fermented soybean, was shown to reduce levels of ZEN in corn steep liquor (CSL). Spores, mycelium and culture filtrate of the strain FS10 were tested for their ability to remove ZEN. The results indicated that strain FS10 could remove 89.56% of ZEN from potato dextrose broth (PDB) medium. Mycelium and culture filtrate decreased the ZEN content by 43.10% and 68.16%, respectively. The contaminated corn steep liquor initially contained ZEN 29 μg/ml, 60.01% of which could be removed by strain FS10. To demonstrate the loss of toxicity in vivo, the culture filtrate incubated with the contaminated corn steep liquor for 48 h was administered to rats. The results indicated that the contaminated corn steep liquor severely damaged liver and kidney tissue. Rats administered with contaminated corn steep liquor treated with the strain FS10 culture filtrate showed significantly less severe liver and kidney damage, and organ index values were comparable to the non-ZEN-exposed control (p < 0.05). Our study suggests an effective approach to reduce the hazards of ZEN in corn steep liquor.

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1. Introduction

Zearalenone (ZEN: 6-(10-hydroxy-6-oxo-trans-1-undecyl)-β-resorcylic acid lactone) is a lactone derivative of resorcylic acid and a nonsteroidal estrogenic mycotoxin produced by several species belonging to the genus Fusarium, a common field and storage fungus (Tanaka et al., 1988). High concentrations of ZEN are usually found in maize and hay stored under warm, humid conditions. However, ZEN is also a food contaminant, with concentrations as high as 289 μg/g (Kim et al., 1993; Yuwai et al., 1994). The risk to human health reflects the fact that ZEN is a powerful estrogen, with hormonal activity exceeding that of most other naturally occurring non-steroidal estrogens, such as soy and clover isoflavones (Metzler et al., 2010). In fact, the occurrence of ZEN in food has been related to the early onset of puberty in children from Puerto Rico (Schoental, 1983). The estrogenic and possible carcinogenic effects of ZEN are of further concern (Ehrlich et al., 2002; Kuiper-Goodman et al., 1987; Withanage et al., 2001) given its effects in animal models, in which liver and kidney damage (Čonková et al., 2001), endocrine disruption (Hughes et al., 1991), as well as immunotoxicity were demonstrated (Berek et al., 2001). These findings underline the urgent need to eliminate ZEN contamination in food and food products for human consumption.

Because of the economic losses engendered by ZEN and its impact on human and animal health, many strategies for detoxifying contaminated food and animal feed have been examined, including physical, chemical, and biological detoxification methods (McKenzie et al., 1997; Trenholm et al., 1991; Zinedine et al., 2001). Of these, the biological control of ZEN is an attractive alternative for efficiently eliminating toxics and thus safe-guarding the quality of food and feed (Al-talhi, 2007). Several Rhizopus strains, including R. stolonifer, R. oryzae and R. microsporus, were found to completely degrade ZEN (Varga et al., 2005). Molnar described a new yeast strain, Trichosporon mycotoxinivorans, which is able to degrade ZEN to carbon oxide and other non-toxic metabolites (Molnar et al., 2004). Takahashi-Ando identified and characterized a lactonohydrolase enzyme in fungus Clonostachys rosea which convert ZEN to a less estrogenic compound (Takahashi-Ando et al., 2005). Rhodococcus pyridinivorans K408 strain was proved to be a very efficient biological tool that is able to eliminate ZEN in LB media. It is also remarkable that this biotransformation pathway
of ZEN did not result in any residual estrogenic effects on rats (Kriszt et al., 2012).

Aaspergillus niger is one of the most important microorganisms used in biotechnology. It has been in use for many decades to produce extracellular (food) enzymes and citric acid. In fact, many A. niger enzymes are considered generally recognized as safe (GRAS) by the United States Food and Drug Administration (Schuster et al., 2002). In addition, A. niger is generally regarded as a safe organism, as documented in lists of the organizations responsible for occupational health and safety [e.g. Berufsgenossenschaft der Chemischen Industrie (1998)]. In our team, Xu et al. determined that the culture filtrate of A. niger FS10 has pleiotropic effects in controlling AFB1 contamination (Xu et al., 2013). A. niger could also degrade ochratoxin z to an unknown compound (Harwig, 1974). Two species of Aspergillus flavus isolate from Lafia, La3279 and La3303, were most effective at reducing aflatoxin B1 + B2 concentrations in both laboratory and field trials (Atehnkeng et al., 2008).

Overall, A. niger CBS 120.49 was found to effectively eliminate ochratoxin A from both liquid and solid media, and the degradation product, ochratoxin a, was also decomposed (Varga et al., 2000). While whichever decontamination strategy is used, it must meet the basic criteria of WHO/FAO and FDA: 1. The mycotoxin must be inactivated (destroyed) by transformation to non-toxic compounds; 2. Fungal spores and mycelium should be destroyed, so that new toxins are not formed; 3. The food or feed material should retain its nutritive value and remain palatable; 4. The physical properties of raw material should not change significantly; 5. It must be economically feasible (the cost of decontamination should be less than the value of contaminated commodity) (Bata and Lásztity, 1999).

Corn steep liquor (CSL) is a byproduct of corn wet-milling, and most of the corn produced is fed to livestock (Yu et al., 2008). Corn and its wet-milled products appear particularly susceptible to zearalenone-producing fungi, and there have been many incidences of contamination in the past years. Briones-Reyes et al. found that approximately 70% of the 24 monitored samples were contaminated with zearalenone, with levels ranging from 3 to 83 µg/kg of corn kernels (Briones-Reyes et al., 2007). In addition, one study carried out in the State of Nayarit (de Lourdes Robledo et al., 2001) found a 15% contamination rate of this mycotoxin in corn fodder, with an average concentration of 1610 µg/g.

In this study, ZEN degradation by A. niger strain FS10 was examined in fungal suspensions, spores, mycelium, and culture filtrate. The toxicity of the treated ZEN-contaminated CSL products was then examined in a rat model.

2. Materials and methods

2.1. Chemicals

A stock standard solution of ZEN (purity >99%, Sigma-Aldrich, St. Louis, USA) was prepared by dissolving 1 mg of ZEN standard in 50 mL of chromatography-grade acetonitrile to obtain a 20 µg/mL ZEN solution. This solution was diluted with acetonitrile in order to obtain the standard curve work solutions of 2, 4, 6, 8, and 10 µg/mL. All ZEN solutions were stored in darkness at 4 °C until experimental analysis. The water used in this test was purified by a Milli-Q water system. All other chemicals used in this study were of analytical grade.

2.2. Fungal strain

Food-grade A. niger (Schuster et al., 2002) strain FS10, isolated from Chinese fermented soybean and identified by 18S rDNA gene sequencing by Dan Xu in our laboratory, was cultured on potato dextrose agar (PDA) slants at 30 °C for 5 days to obtain the spores. Part of the spores was washed with sterile saline and adjusted to the concentration of 10^6 CFU/mL. 10 mL of the spore suspension was inoculated into 500 mL flasks of PDB medium, then incubated at 30 °C (150 rpm) for 5 days to obtain the fungal suspensions, mycelium and culture filtrates of strain FS10.

2.3. ZEN degradation by fungal suspension of strain FS10

The degradation experiment was performed in a culture vial. 10 mL of the ZEN stock solution (20 µg/mL) was added to 50 mL fungal suspension of strain FS10 which included 3 g mycelium to obtain a ZEN final concentration of 2 µg/mL. For control, the suspension of strain FS10 was replaced by sterile PDB. All cultures were incubated in a shake incubator (150 rpm, 30 °C). ZEN in samples was extracted after 2, 6, 12, 24, 48, 72 and 96 h to determine the remaining concentration in the suspensions.

2.4. Extraction and determination of residual ZEN

Residual ZEN in liquid culture was extracted three times using chloroform, which was subsequently evaporated with a gentle stream of nitrogen gas at 50 °C. The samples were then dissolved in 1 mL of water:acetonitrile [50:50 (v/v)], filtered (0.22 µm), and analyzed for ZEN (Teniola et al., 2005) using Agilent 1260 reverse-phase HPLC (system gold 125 solvent module, Beckman Coulter) and liquid chromatography mass spectrometry (LC/MS; Agilent 6410, USA). ZEN was separated on a C18 column (250 × 4.6 mm; particle size, 5 µm; Diamonsil) with a mobile phase of water:acetonitrile [50:50 (v/v)] at a flow rate of 1 mL/min. The assay temperature was 25 ± 0.5 °C with an injection volume of 20 µL. The retention time was 6.5 ± 0.5 min. Fluorescence detection was carried out at 274 nm (excitation) and 440 nm (emission). The amount of isolated ZEN was quantified using Class VP 5.0 software (Shimadzu). The percentage of the ZEN remaining after the extraction was calculated using the equation, P = 100% × (peak area of ZEN in the test extraction/peak area of ZEN in the control). The condition of LC/MS is as follows: the symmetry ZORBAX SB-C18 column (150 mm, 3.5 µm) mobile phase of methanol and water with 10 mM ammonium acetate (0:40), at a flow rate of 0.2 mL/min. All assays were performed in triplicates.

2.5. The recovery of standard addition

Three concentrations of ZEN standard solution were added in the PDB medium, extracted and detected by the methods described in the previous section. Three parallel were done in each concentration and the results showed in Table 2.

2.6. ZEN biotodetoxification activity

To identify the mechanism by which strain FS10 reduced ZEN activity, the spores, mycelium, and culture filtrate were assayed in separate experiments. Harvested spores were washed three times with 0.01 M phosphate buffered saline (PBS) and prepared in suspensions of 0, 1, 2, 5, 7, and 9 mL. The final volume was made to 10 mL by the addition of PBS. Before the addition of ZEN as described before, spores suspensions was determined by optical density (OD) at 560 nm. The mycelium was separate of substrates by filtration through four layers of cheese cloth, after which the culture filtrate was sterilized using 0.2-µm disposable syringe filters (Millipore, Bedford, MA, USA) and used immediately for the following experiments. The fungal mycelia was then divided into two portions based on treatment either viable mycelia (untreated) or autoclaved mycelia (121 °C for 20 min). The mycelia pellets were washed three times with PBS, then 0, 0.1, 0.3, 0.6, 0.8 and 1.0 g pellets were suspended in 9 mL PBS solution infused with 1 mL, 20 µg/mL ZEN, and incubated at 30 °C, 150 rpm under aerobic conditions for 48 h, respectively.

2.6.1. Effects of low oxygen gas content, SDS protease, heat and EDTA on Zearalenone degradation activity in culture filtrate

The effect of the culture filtrate of strain FS10 on ZEN was assayed according to the method described by Cho et al. (2010) with slight modification. In order to study the factors affecting the degradation activity of the culture filtrate, the culture filtrate were treated separately with 1 mg/ml protease K plus 1% SDS for 1 h at 55 °C, 100 °C water for 10 min, N2 gas for 10 min to remove dissolved oxygen gas, and 0.1 M EDTA (Yu et al., 2011a). The active culture filtrate were included as negative control containing only sterile PDB medium with ZEN were included as blank control.

ZEN was added to all of the preparations described in previous sections, to a final concentration of 2 µg/mL. All of the samples were incubated at 150 rpm at 30 °C under for 48 h. Remaining ZEN was extracted from all the solutions at 0 h and 48 h, respectively.

2.7. Degradation of ZEN contaminant in CSL by the culture filtrate of strain FS10

ZEN-contaminated corn steep liquor (CSL) was mixed with the culture filtrate at a ratio of 1:1 (v/v) and the mixtures were incubated (150 rpm, 30 °C) under aerobic conditions. The control consisted of contaminated corn steep liquor mixed with sterile water. After 48 h, 2-mL samples were taken and the ZEN content was measured.
2.8. Activity analysis of extracellular protease

To examine the extracellular protease activity present in the strain FS10 culture filtrate, a 5-mm-diameter hole was created at the four corners of a Casein Agar culture plate that was then inoculated with 20 μl of culture filtrate (Mancianti et al., 2001). After incubating the plates for 24 h at 37 °C in the dark (Alp and Arikan, 2008), the colony diameter and proteolytic ring were measured and their ratio was determined. Protease activity was expressed as Pa, defined as the ratio of the colony diameter to the total diameter.

2.9. In vivo effects of the degraded ZEN

Twenty-eight mature male rats (6 weeks old) obtained from Jiangnan University (Wuxi, China) and weighing 400 ± 30 g each were fed a standard granulated chow diet and given water ad libitum. The rats were distributed into four treatment groups (7 rats/group) as follows: (a) saline control group; (b) gavage-treated with culture filtrate of strain FS10 for 48 h; (c) gavage-treated with 2.5 ml of contaminated CSL mixed with water [1:1 (v/v)]; (d) gavage-treated with 2.5 ml of contaminated corn syrup incubated with the culture filtrate of strain FS10 for 48 h [1:1 (v/v), mix]. Thirty days after daily treatment, all of the rats were killed by cervical dislocation and the organs (liver, kidney, spleen, and thymus) were weighed to obtain the organ index, defined as the ratio of organ weight to the weight of the rat.

The dissected liver and kidney were fixed in Bouin’s fluid and embedded in paraffin. Thin sections (5 μm thick) were prepared and stained with hematoxylin and eosin (H&E) for microscopic examination (Abbes et al., 2006) at 100× and 400× magnification using an optical microscope (Carl Zeiss, Germany).

2.10. Statistical analysis

SPSS, version 9.0, for Windows was used for statistical analysis of the data. Differences in toxin reduction and organ index under the various conditions were determined by an analysis of variance. Significant differences in the mean values and among treatments were defined based on P < 0.05. All data are expressed as the mean ± standard deviation.

3. Results and discussion

3.1. Reduction of ZEN levels by fungal suspension of strain FS10

In this study, strain FS10 significantly degraded ZEN during growth in PDB. As shown in Fig. 1, in the fungal suspension of strain FS10, ZEN levels decreased to 21.26% and 10.44% of the starting amount after 24 h and 48 h, respectively. Longer incubation times of 72 and 96 h did not result in further significant reductions. The results suggest that strain FS10 can be effective in the bioremediation of ZEN-contaminated substrates. Bacterial bioremediation of ZEN has previously been reported (Kriszt et al., 2012; Yu et al., 2011b) and the results of those studies are in good agreement with our own findings. A report found that isolates of Aspergillus spp. inhibited mycelial growth and aflatoxin production by A. flavus. Based on our finding of maximum ZEN degradation activity by strain FS10, 48 h was used as the incubation time in the following experiments.

3.2. Biotransformation mechanism of strain FS10

3.2.1. Mycelium adsorption

When spores were incubated with ZEN, the contaminant was neither removed nor bound. An increase in fungal density likewise caused no change in the ZEN content (Fig. 2). Instead, ZEN was removed only when incubated with the mycelium (Fig. 3). Moreover, incubation of the toxin with either heat-treated or viable mycelium resulted in ZEN removal from the medium, indicating that binding, not metabolism, was the removal mechanism. This conclusion was further supported by the observation that the amount of ZEN bound by strain FS10 was dependent on the mycelium mass (Fig. 3). Specifically, an increase in the mycelium mass from 0.1 to 1.0 g resulted in a decrease in the amount of remaining ZEN from 85.13% to 56.90% and from 84.01% to 50.28% for viable and heat-treated mycelium, respectively.

Heat treatment significantly enhanced the ability of the mycelium to remove ZEN (Fig. 3). This result is in good agreement with the study of El-Nezami et al. (2002), which speculated that heat treatment either decreases the thickness of the cell wall or increases the size of its pores, and either perturbation would facilitate the binding of ZEN to the fungus.

3.2.2. Enzymatic effects in culture filtrate

The active culture filtrate of strain FS10 significantly degraded ZEN by as much as 68.16%. Fig. 4 shows the effects of different factors on zearalenone degradation activity in culture filtrate. After treatment with SDS protease K and heat, the pooled active culture filtrate retained low zearalenone degradation activity, which indicated that enzymes from the pooled active culture filtrate are involved in the degradation of zearalenone. Oxygen gas was necessary for zearalenone degradation by the enzymes in the pooled active fractions, which imply that some enzymes involved in zearalenone degradation may be oxidases. EDTA, as metal chelating agent, can destroy the zearalenone degradation activity, which indicated that some metal cations are required for the enzymes in the active fractions to degrade zearalenone. All indications are that an extracellular enzyme is responsible for the degradation by culture filtrate of strain FS10.

However, the culture filtrate was less effective than the fungal suspension in reducing the amount of ZEN. The reason is that both adsorption and degradation effects exist in the fungal suspension.
enzyme is a metalloenzyme as it was inhibited at 10 mM EDTA, whereas PMSF had no effect on its activity (Abrunhosa and Venâncio, 2007). In addition, aflatoxin B1 degradation by laccase enzyme from A. niger can significantly decrease the level and thus mutagenicity of AFB1 (Alberts et al., 2009). Enzymic detoxification could offer a practical and efficient method of ZEN decontamination.

In contaminated CSL incubated with strain FS10 culture filtrate for 48 h, the ZEN content was reduced from the initial 29.06 ppm to 11.62 ppm (Supplementary Fig. s1), a 60% decrease. And the % recovery during extraction from corn steep liquor of ZEN was showed in Table s1.

### 3.3. Analysis of extracellular proteins in culture filtrate

Extracellular enzyme activity in the filtrate was measured (Supplementary Fig. s2). The higher amounts of enzyme activity resulted in the lower values of Pa. When 0.64 < Pa < 1, the protease activity level is considered as high. In this study, Pa = 0.69, which is consistent with strong extracellular enzyme activity in the culture filtrate (Coutinho and Paula, 2000). In the further study, purification of the extracellular enzyme in the pooled active culture filtrate should be performed.

### 3.4. Product analysis of zearalenone degradation by extracellular enzymes

The formation of ZEN degradation products by culture filtrate was studied using the techniques of the LC–MS. With the aid of APCI-MS (negative ion mode), ZEN was identified at m/z = 317 (Fig. 6A). At 0 h, the sample of ZEN treated with culture filtrate clearly showed a peak at m/z = 317. ZEN treated with culture filtrate clearly showed a peak at m/z = 317 at 0 h. However, the sample treated with culture filtrate after 48 h showed that ZEN was still present, but at a much lower concentration than 0 h. In addition, zearalenone could be transformed into other products by the extracellular enzymes from culture filtrate of strain FS10 after 48 h. Two intermediate products, ZEN-A ([M + H]+ at m/z 414) (Fig. 6B) and ZEN-B ([M + H]+ at m/z 325) (Fig. 6C), were found. For UV–Vis spectroscopy, the zearalenone was absorption at 245–275 nm (Fig. 6D), while the ZEN-A was absorbed at 200–218 nm (Fig. 6E) and no UV absorption of ZEN-B (Fig. 6F), indicating the structure of the new intermediate products ZEN-A and ZEN-B had some difference compared with zearalenone. And we

### Table 2

Recoveries of different concentration of ZEN in PDB (n = 3).

<table>
<thead>
<tr>
<th>Add amount (µg ml⁻¹)</th>
<th>Test results (µg ml⁻¹) (mean ± SD)</th>
<th>Recovery (%)</th>
<th>R.S.D (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.089 ± 0.019</td>
<td>93.83</td>
<td>3.82</td>
</tr>
<tr>
<td>0.5</td>
<td>0.456 ± 0.028</td>
<td>95.53</td>
<td>5.01</td>
</tr>
<tr>
<td>2</td>
<td>1.816 ± 0.037</td>
<td>91.32</td>
<td>5.30</td>
</tr>
<tr>
<td>Mean</td>
<td>93.56</td>
<td>4.71</td>
<td></td>
</tr>
</tbody>
</table>

(R.S.D: Relative Standard Deviation).
can also infer that the benzene ring of zearalenone may be cleaved in ZEN-B. These results are some different from Yu et al. They found that zearalenone could be oxidized into smaller estrogenic products by the extracellular enzymes from *Acinetobacter* sp. SM04. Two new products, ZEN-1 ([M – H]− at m/z 489) and ZEN-2 ([M – H]− at m/z 405), were found. There were low absorption at 230–270 nm and high absorption at 300–330 nm for UV–Vis spectroscopy of ZEN-1 and ZEN-2, indicating the benzene ring of zearalenone may be cleaved and oxidized into products containing carboxyl groups (Yu et al., 2011a). The difference between the two research may be due to the extracellular enzymes derived from different microorganisms had their own specific action sites in zearalenone.

The new degradation products of ZEN were confirmed to be less toxic with a rat model in our study, which indicated FS10 degradation will be very useful for detoxification of zearalenone-contaminated feed. In the previous study, ochratoxin A can be enzymatically hydrolyzed into ochratoxin α and L-β-phenylalanine by *A. niger*, thereby decreasing its toxicity (Abrunhosa and Venâncio, 2007). Saleh et al. demonstrate that *Rhizopus arrhizus* catalyzes sulfation of zearalenone at the C-4 hydroxyl group (El-Sharkaway et al., 1991). Therefore, the intermediate products, ZEN-A ([M + H]+ at m/z 414) may combine with some groups like sulfate and so on.

### 3.5. In vivo toxicity of the ZEN degraded by culture filtrate in the CSL

As determined from the organ index, significant (p < 0.05) liver atrophy developed in rats treated with a single gavage dose of contaminated CSL (Table 1), whereas no such effects occurred in the other three groups. When the contaminated CSL was subjected to degradation by the culture filtrate, liver atrophy was not significantly different from that seen in the control animals. These
macroscopic results indicated that ZEN toxicity was weakened in contaminated CSL due to degradation by the culture filtrate.

Histological studies of the tissues from rats treated with contaminated CSL confirmed the presence of focal necrosis (FN) and severe vacuolar degeneration (arrow) in liver cells (Fig. 5(1)), as well as perivascular edema and balloon atrophy (arrow) (Fig. 5(2)) in kidney cells. However, in rats treated with CSL degraded by the culture filtrate, similar to the controls, the histology of both organs was normal. This result further illustrates that the degradation products of culture-filtrate-treated CSL were non-toxic.

4. Conclusions

The non-toxigenic and environmentally friendly A. niger strain FS10 was previously shown to reduce AFB1 contamination in our laboratory. In this work, we showed that this strain reduces ZEN contamination as well, via mechanisms involving the binding and metabolism of the toxin. In the culture filtrate, the activities of extracellular enzymes were most likely responsible for ZEN degradation. Our results suggest that starter cultures of A. niger strain FS10 can be used to eliminate ZEN contamination, thereby alleviating hazards to human and animal health. The loss of toxicity in contaminated CSL incubated with the culture filtrate of A. niger strain FS10 was verified in vivo, in rats. Therefore, we suggest that filtrates of cultured A. niger strain FS10 can be used to safely remove ZEN toxicity from food and animal feed.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2014.06.021.
Xu, D., Wang, H., Zhang, Y., Yang, Z., Sun, X., 2013. Inhibition of non-toxigenic 
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lactic acid production using corn steep liquor as an alternative nitrogen source 
extracellular enzymes from Acinetobacter sp. SM04 into smaller estrogenic 
zearalenone by the extracellular extracts of Acinetobacter sp. SM04 liquid 
deoxynivalenol, and zearalenone in imported cereals in Papua, New Guinea. 
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