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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.

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., 2007). Of these, the biological control of ZEN is an attractive alternative for efficiently eliminating toxins and thus safe-guarding the quality of food and feed (Altalhi, 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 lactonohydolase 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

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of ZEN did not result in any residual estrogenic effects on rats (Kriszt et al., 2012).

Aspergillus 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 α 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). Moreover, *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). Overall, 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 mycelia 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. Luis, 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 stock 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. ZEN-contaminated corn steep liquor (CSL) was obtained from the Grain Science Institute of Wuxi, China.

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 were inoculated into 500 ml aliquots 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 90 ml fungal suspension of strain FS10 which including 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 °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, $P = 100\% \times$ (peak area of ZEN in the test extraction /peak area of ZEN in the control). The condition of LC/MS is as follow: the symmetry ZORBAX SB-C18 column (Rapid resolution, 30 × 2.1 mm i.d., 3.5 μm), mobile phase of methanol and water with 10 mM ammonium acetate(60:40), at 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 biodetoxification 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 below, spores suspensions was determined by optical density (OD) at 560 nm.

The mycelium was separated from the substrate 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 were 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 0.1, 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 proteinase K plus 1% SDS for 1 h at 55 °C, 100 °C water for 10 min, N_2 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 oxic 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.