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Scavenging of Zearalenone by *Bacillus* Strains-*in vitro*

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ABSTRACT

The capacity of five single bacterial strains to scavenge the mycotoxin Zearalenone (ZEN, 20 μg L^{-1}) from liquid culture media was investigated. *Bacillus subtilis* 168 and *Bacillus natto* scavenged 95 and 78% ZEN, respectively, after incubation at 30°C for 48 h. Furthermore, none of the oestrogenic ZEN analogues α-Zearalenol and α-Zearalanol was detected during UPLC-MS/MS analysis. Based on these findings, the two *Bacillus* strains can potentially be exploited for ZEN decontamination of food and feeds.

Key words: Zearalenone, *Bacillus*, decontamination, mycotoxin, *in vitro*

INTRODUCTION

The mycotoxin Zearalenone (ZEN) is a known contaminant in grains and legumes (Isakeit *et al*., 2008; Youssef, 2009), as well as their products such as flour, animal feeds, beer and malt (CCFAC, 2000). It is genotoxic (Hussein and Brasel, 2001) and a potential human breast cancer tumorgenesis factor (Ahamed *et al*., 2001). It may also cause hyperoestrogenism in women resulting in disorders such as ovarian and uterine dysfunctions, infertility and neoplastic changes in the entire reproductive system (Gajecki *et al*., 2004; Shier *et al*., 2001; Tiemann *et al*., 2003). Zearalenone greatly impairs livestock productivity because it adversely affects their reproductive cycles, especially in swine (Diekman and Green, 1992).

Worldwide ZEN contamination of grains thus poses a profound health as well as economic problem. Consequently, several measures to control the mycotoxin have been explored. Over the years, a variety of physical, chemical and biological techniques to detoxify ZEN have been applied. ZEN is largely heat stable hence food processing operations such as milling, extrusion or other high temperature treatments do not substantially alter or attenuate the toxin (Lauren and Smith, 2001; Yumbe-Guevara *et al*., 2003). On the other hand, the use of chemicals to decontaminate ZEN and other mycotoxins maybe limited by toxic residues remaining in the food (Magan and Olsen, 2004). Biological control of mycotoxins is gaining prominence as the preferred detoxification technique because it is deemed safe (Karlovsky, 1999).

From previous studies (El-Nezami *et al*., 2002; Megharaj *et al*., 1997), a number of fermentative and soil bacteria have shown a propensity to detoxify Zearalenone and other mycotoxins. However, reports on detoxification of ZEN by single bacterial strains are still limited. Therefore, five predominantly fermentative bacterial strains (Acetic Acid Bacteria, *Lactobacillus* NYC30, *Lactobacillus* Dins37, *Bacillus subtilis* 168 and *Bacillus natto*) were strategically selected for

investigation. The objective of this research is to identify new generally regarded as safe (GRAS) bacteria that can be utilised for ZEN decontamination.

MATERIALS AND METHODS

The research was conducted between January and June 2010 at the School of Food Science, Jiangnan University, Wuxi, Jiangsu Province, China.

Microorganisms, culture media and reagents: Five bacterial strains were used in the study; *Bacillus subtilis* 168 from *Bacillus* Genetic Stock Centre (Ohio, US) and (Acetic Acid Bacteria (AAB), *Lactobacillus* NYC30, *Lactobacillus* Dins37 plus *Bacillus natto*) sourced from the Food Biotechnology Laboratory at Jiangnan University, China. Stock cultures were stored at -70°C in 50% (v/v) Glycerol. The culture media used for cultivation of *Bacillus* sp., *Lactobacillus* sp. and AAB was Luria Bertani (LB) broth (Miller - 1% NaCl), deMan-Rogosa-Sharpe (MRS) broth and Acetic Acid Bacteria broth (D+Glucose, Yeast extract, KH_2PO_4, $MgSO_4$, Ethanol), respectively.

Pure Zearalenone powder (10 mg) was purchased from Fermentek Ltd, Israel and CNWBOND Florisil Cartridges (500 mg, 3 mL) from Germany. Stock solution of ZEN (1 mg mL^{-1}) was prepared by dissolving the powder in methanol: water (9:1 v/v) mixture. A ZEN working solution of 20 μg L^{-1} was used for the experiments. Florisil solid phase extraction cartridges were used for ZEN cleanup prior to UPLC-MS/MS analysis. All chemicals and/or reagents used in this work were reagent grade.

Screening of five strains for Zearalenone scavenging capacity: Screening was done using a modified method of El-Nezami *et al.* (2002). The bacterial culture (24 h, 1 mL) for each of the strains was transferred into a 50 mL conical flask containing 10 mL of the respective culture media infused with 20 μg L^{-1} of ZEN and incubated at 30°C. After 48 h incubation, the culture (2 mL) was collected, sonicated and centrifuged at 8000 rpm for 10 min (4°C). The supernatant was agitated with 3 mL chloroform-methanol (9:1 v/v) mixture. The supernatant was then extracted and cleaned using Florisil Cartridges (CNWBOND, Germany) preconditioned with chloroform (2 mL) and then chloroform-methanol (9:1 v/v, 2 mL) according to Mateo *et al.* (2002). Briefly, the supernatant was run through the cartridge and left to stand for 2 min after which ZEN was eluted with chloroform-methanol (9:1 v/v, 10 mL) mixture and the solution evaporated to dryness at 60°C using a rotary evaporator (R501, China). The residue was dissolved in methanol (2 mL) and then analysed for ZEN. Control samples consisting of only the respective culture media infused with 20 μg L^{-1} ZEN were prepared and subjected to the same treatment as test samples.

Determination of Zearalenone by UPLC-MS/MS: A Waters MALDI (Matrix-assisted laser desorption/ionization) Synapt Q-TOF (Time-of-flight) Mass Spectrometer and Waters Acquity UPLC with Photodiode Array (PDA) UV detector (Waters, Milford, US) were used for Zearalenone analysis. The column (BEH C18 2.1*100 mm, 1.7 μm) was set at temperature 40°C. The mobile phase was acetonitrile - ammonium acetate (30:70 v/v) with a flow rate of 0.3 mL min^{-1} and injection volume 10 μL. MS/MS conditions were electrospray ionisation in negative mode (ESI^-), capillary voltage 3.0 kV, cone voltage 30 V, source temperature 100°C, desolvation temperature 250°C, desolvation gas flow 500 L h^{-1}, cone gas flow 50 L h^{-1}, collision energy 6 eV and detector voltage 1700 V. All assays were performed in triplicate.

The amount of ZEN scavenged was calculated using the formula:

$$\text{ZEN}(\%) = \left\{1 - \left[\frac{\text{ZEN peak area of sample}}{\text{ZEN peak area of control}}\right]\right\} \times 100$$

Statistical analysis: Data analysis by one-way ANOVA and Duncan's multiple range test was done using the Statistical Analysis System software package (Version 8.1, 2000; SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

ZEN was variably attenuated amongst the bacterial strains. ZEN was scavenged in the range 28-95% (Fig. 1). Representative chromatograms of the residual ZEN (filled peaks) for each bacterial strain are shown in Fig. 2. Percentage ZEN scavenged from the liquid media by each strain was; *B. natto* (78%), *B. subtilis* 168 (95%), *Lactobacillus* NYC30 (61%), *Lactobacillus* Dins37 (55%) and AAB (28%).

The AAB strain was the least efficient in purging ZEN. The ability of AAB to detoxify ZEN has not previously been studied. As regards detoxification of mycotoxins using Gram negative bacteria, El-Nezami *et al.* (1998) reported a 10-16.3% Aflatoxin B_1 reduction in culture media when using *E. coli*, which was low compared to Gram positive Lactic acid bacteria. *Lactobacillus* NYC30 and *Lactobacillus* Dins37 removed ZEN from the liquid medium and the percentages are similar to those of El-Nezami *et al.* (2002) who reported ZEN reduction in the range 54-62% by two *Lactobacillus rhamnosus* strains. Mycotoxin removal by *Lactobacillus* species is associated with adsorption onto the cell wall surface largely constituting of peptidoglycan (Haskard *et al.*, 2001; Lahtinen *et al.*, 2004). On the contrary, Gram negative bacteria's cell wall mainly consists of a lipid and protein outer membrane masking a mono or multi-layer of peptidoglycan (Bos *et al.*, 2007). This could explain the low percentage reduction of ZEN by the AAB strain.

B. subtilis 168 and *B. natto* were more efficient in removing ZEN from the liquid medium than the other strains with more than 75% of the ZEN eliminated after incubation. *B. subtilis* had not been reported as a ZEN scavenging bacteria until recently, when Cho *et al.* (2010) reported 99% degradation of ZEN in liquid medium by a *Bacillus subtilis* strain. Zearalenone decontamination's

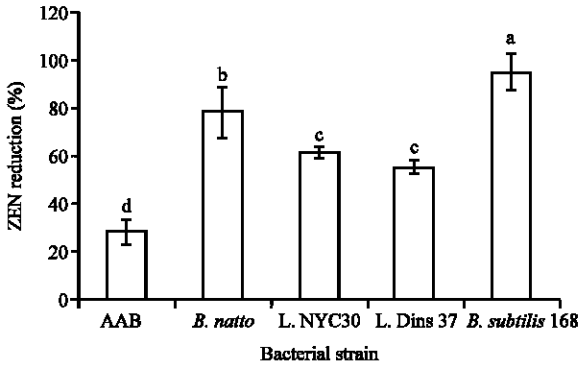

Fig. 1: Percentage ZEN scavenged from culture media after 48 h incubation at 30°C. Different letter denotes significant statistical difference amongst strains ($p<0.05$)

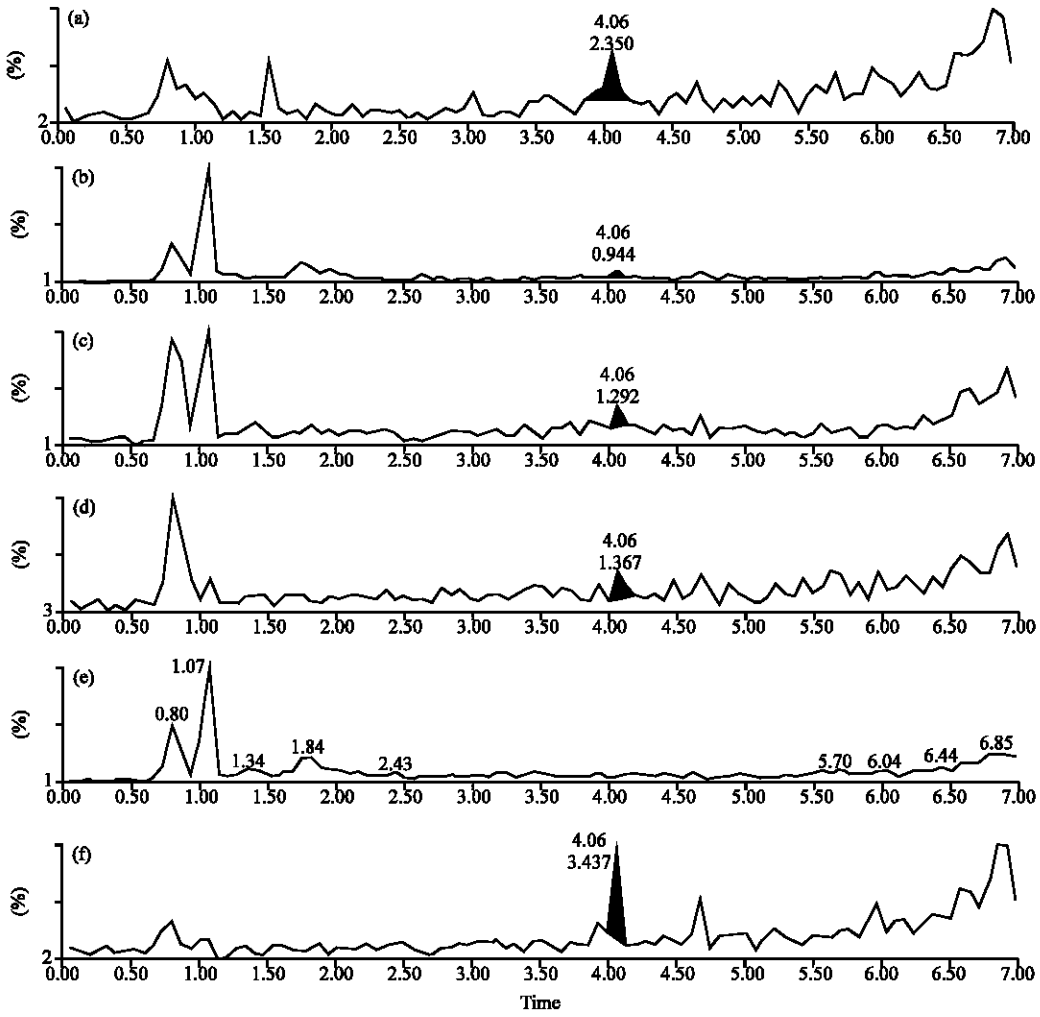

Fig. 2: UPLC-MS/MS chromatograms showing residual ZEN (filled peak) in supernatant after incubation at 30°C for 48 h with (a) AAB, (b) *Bacillus natto*, (c) *Lactobacillus* NYC30, (d) *Lactobacillus* Dins37, (e) *Bacillus subtilis* 168 and (f) control sample

never complete unless the presence of it's oestrogenic analogues such as α-Zearalenol is ruled out. UPLC-MS/MS analysis of *Bacillus* strains' samples revealed none of these ZEN cognates (Fig. 2a-f). These results indicate that the two *Bacillus* strains can potentially be utilised to detoxify ZEN in food and feeds.

CONCLUSION

In line with the objectives of this research, two *Bacillus* strains with good ZEN scavenging ability were unearthed. Since, they are GRAS, this opens the door for their application in detoxification of ZEN in food and feeds.

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