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Isolation and characterization of *Bacillus amyloliquefaciens* ZDS-1: Exploring the degradation of Zearalenone by *Bacillus* spp.

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ABSTRACT

Zearalenone (ZEN) is a mycotoxin produced mainly by various *Fusarium* species which occur naturally in many crops worldwide. ZEN causes reproductive disorders and hyperestrogenic syndromes in animals and humans. This study aimed to isolate ZEN-degrading bacteria to develop strategies for detoxifying ZEN contamination in cereal crops. We screened approximately 1000 colonies for degrading ability and found four strains were capable of degrading ZEN. We selected one strain ZDS-1 for further study because it showed the high ZEN-degrading ability. On the basis of morphological, physiological and phylogenetic analysis of its 16SrRNA, *gyrA* gene sequences, strain ZDS-1 was identified as *Bacillus amyloliquefaciens*. The optimal conditions for the biodegradation of ZEN by ZDS-1 were temperature; 30 °C, pH; 6.0–7.0, and cell concentration; 5×10^8 cfu/mL. ZDS-1 could degrade ZEN efficiently with the concentration from 1 mg/L to 100 mg/L. ZDS-1 not only could remove ZEN in the culture medium, but also could degrade ZEN in wheat. The ZEN removal by ZDS-1 was not due to binding or absorption, and during the process of ZEN degradation, no ZEN derivatives of ZEN were produced. These results suggested that *Bacillus amyloliquefaciens* ZDS-1 would be explored further for its ability to degrade ZEN in field trials.

1. Introduction

Zearalenone (ZEN) [6-(10-hydro-6-oxo-trans-1-undecentyl)-β-resorcylic acid lactone] is a secondary metabolite mainly produced by *Fusarium* species, such as *Fusarium graminearum* and *Fusarium culmorum* (Almeida-Ferreira, Barbosa-Tessmann, Sega, & Machinski, 2013; Bertuzzi, Camardo Leggieri, Battilani, & Pietri, 2014). *Fusarium* diseases occur frequently in corn, wheat, and other cereal crops in the field before harvest or during storage (Klaric, Cvetnic, Pepeljnjak, & Kosalec, 2009; Rashedi, Sohrabi, Ashjaazadeh, Azizi, & Rahimi, 2012). ZEN causes losses in live stock production and poses a health risk to humans that consume food products made from contaminated cereals (Lu et al., 2013; Obremski & Poniatowska-Broniek, 2015; Pistol et al., 2015; Wang et al., 2012). It can cause alteration in reproductive system in swine, cattle, chicken, and sheep (Choi et al., 2012; Edwards, Cantley, & Day, 1987; Pistol et al., 2014; Zatecka et al., 2014). It was also reported that ZEN caused hyperestrogenic syndrome in animals and humans, carcinogenic effect in mice and stimulated the growth of human breast cancer cells (Belhassen et al., 2015; Nogowski, Nowak, Kaczmarek, & Mackowiak, 2002).

Therefore, the development of more effective methods is needed to control the ZEN contamination. Several physical and chemical methods have been investigated. Different absorbents have been used to reduce the toxic effect of ZEN such as clays, activated carbon, bentonite, cholestyramine, and 1, 3-Beta-D-glucan (Avantaggiato, Havenaar, & Visconti, 2003; Freimund, Sauter, & Rys, 2003; Sasaki, Takahashi, Sakao, & Goto, 2014). Ozone gas has also been used to detoxify ZEN contamination (Lemke et al., 1999). However, these approaches resulted in limited success in detoxifying ZEN, and biological approach would be more

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promising than physical-chemical means. It has been reported that ZEN can be biodegraded by various microorganisms including fungi, yeasts, and bacteria. The fungus *Clonostachys rosea* IFO 7063 could convert ZEN into a far less estrogenic product (Kosawang et al., 2014). The yeast *Trichosporon mycotoxinivorans* was able to open the macrocyclic ring of ZEN at the ketone group to form the product ZOM-1, which was not estrogenic *in vivo* and did not interact *in vitro* with the estrogen receptor protein as ZEN did (Vekiru et al., 2010). *Pseudomonas putia* ZEN-1 was isolated to be able to degrade ZEN (Altalhi & El-Deeb, 2009). *Pseudomonas otitidis* TN-N1 and three strains of *Lactobacillus plantarum* were able to degrade ZEN (Tan et al., 2015; Zhao et al., 2015). ZEN was also found to be absorbed and degraded by the culture extract of *Bacillus subtilis* 168 and *Bacillus natto* CICC 24640 (Tinyiro, Wokadala, Xu, & Yao, 2011).

Bacillus species may be effective in controlling the contamination of ZEN because it can synthesize different antimicrobial compounds, promote plant growth, induce defense responses in host plants, and maintain high numbers in the environment (Chen et al., 2014; Meng, Jiang, Hanson, & Hao, 2012; Sigdel et al., 2015). For these reasons, *Bacillus* spp. is widely applied in crops to control plant diseases.

In this study, we attempted to isolate *Bacillus* strains capable of degrading ZEN. We were able to isolate several *Bacillus* strains capable of degrading ZEN, including *Bacillus amyloliquefaciens* ZDS-1. In addition, the characteristics of ZEN degrading mechanism of ZDS-1, the effects of different variables including temperature, pH values, cell concentration and ZEN concentration on the degradation of ZEN were also studied.

2. Materials and methods

2.1. Chemicals and medium

Zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalanol were purchased from Sigma (Shanghai, China) and dissolved in methanol (1 mg/mL), which was used as standard stock solution in this study. Methanol was HPLC grade and purchased from ROE scientific incorporation (Newark, USA). All other chemicals used were of analytical reagent grade and were used without further purification.

Minimal medium (MM) contained K_2HPO_4 2.5 g/L, KH_2PO_4 1.2 g/L, NH_4NO_3 1.0 g/L, $MgSO4{\cdot}7H_2O$ 0.2 g/L, Ca $(NO_3)_2{\cdot}4H_2O$ 0.4 g/L, NaCl 0.5 g/L and Fe_2 $(SO_4)_3$ 0.001 g/L, pH 7.0 (Michihiro et al., 2012). The medium was supplemented with ZEN or ZEN derivatives just before inoculation unless otherwise stated. LB medium contained the following constituents: tryptone (10.0 g/L), yeast extract (5.0 g/L), NaCl (10 g/L) (pH 7.0). All media were autoclaved at 121 °C for 20 min.

2.2. Analytical methods

Cultures were combined with methanol (same volume as samples), extracted by ultra-sonication for 20 min. The methanol extracts of different cultures were centrifuged for 10 min at 12,000 g, and the supernatants were then filtered through 0.45 μm membranes (JinTeng, China), 10 times diluted with methanol, 5 μL of the resulting solution was subjected to HPLC-MS (Agilent 1200/6410B triple-quadrupole LC/MSD), ESI source positive ion mode. The HPLC-MS conditions employed are as follows: Agilent Zorbax XDB-C_{18} column (2.1 mm × 150 mm, 3.5 μm particle size); mobile phase: methanol/water/formic acid (10/90/0.1, v/v/v), flow rate: 0.2 mL/min; nitrogen drying gas: 10 L/min, nebulizer pressure: 25 psi, drying gas temperature: 350 °C, capillary voltage: 4 kv.

2.3. Isolation of ZEN-degrading strains

To isolate ZEN-degrading strain, 10 soil samples (about 100 g) were collected from wheat experimental fields after harvesting in Jiangsu Academy of Agricultural Sciences, Nanjing in China. Approximately 1.0 g each soil sample was suspended in 10 mL distilled water, cooked in boiling water for 30 min, 1 mL of each supernatant was then added into a 50 mL flask with 10 mL of MM containing 3 mg/L ZEN, and incubated at 30 °C, at 180 rpm for 7 days. This procedure was repeated 10 times. HPLC-MS was used to determine ZEN residue concentration followed by the isolation of ZEN-degrading microorganisms by spreading serially diluted enrichments on LB plates with 2.0% (w/v) purified agar.

2.4. Identification of ZEN-degrading strain ZDS-1

The identification of ZDS-1 was carried out according to the sequence analysis of its 16S rRNA gene, *gyr*A gene, the morphological, physiological and biochemical characteristics. The genomic DNA was extracted using MasterPure DNA purification Kit (EPICENTRE Biotechnologies, USA). Primers 27F and 1492R were used to amplify the 16S rRNA gene of strain ZDS-1 using a routine PCR procedure (Lane, 1991; Volokhov et al., 2006). Sequencing reactions were run by Sangon Biotech Co., Ltd. (Shanghai, China). The phylogenetic trees based on 16S rRNA gene and *gyr*A gene sequences were constructed using MEGA5 software (Tamura et al., 2011). The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method in the units of number of base substitutions per site. Physiological and biochemical characteristics were made by the instruction described by Holt JG et al. (Holt, Krieg, Sneath, Staley, & Williams, 1994).

2.5. Inocula preparation for degradation studies

Inocula for all of the experiments were prepared by culturing ZDS-1 strain in 50 mL of LB medium overnight at 30 °C, on a rotary shaker (180 rpm). Cells were harvested by centrifugation at 8,000 *g* for 5 min at 4 °C. Cell pellets were washed twice with MM and adjusted to optical density of approximately 1.5 at 600 nm (OD_{600}). For degradation experiments, the cells were dispensed in 50 mL flask containing 10 mL MM with ZEN or its derivatives at 10% (v/v) level, and then incubated at 30 °C, at 180 rpm. To determine the effect of cell concentration on the degradation of ZEN by ZDS-1, ZDS-1 suspension was serially diluted with sterile water and spread on the LB plates, cultured for 24 h at 30 °C, and then the number of colonies on the plates were counted.

2.6. Biodegradation of ZEN by the strain ZDS-1

2.6.1. The kinetics of degradation of ZEN

ZEN degradation time course and growth of strain ZDS-1 were performed synchronously in 20 mL MM containing 10 mg/L ZEN inoculated with ZDS-1 at 10% level. The culture samples were collected at time intervals and quantified growth by assaying OD_{600} to get the cell growth curve. The concentration of ZEN was analyzed by HPLC-MS.

2.6.2. ZEN adsorption assay

ZDS-1 was dispensed in MM containing 10 mg/L ZEN, and incubated at 30 °C, at 180 rpm. The samples then were collected periodically, and treated differently. Three of whole samples (named as all cultures) were combined with methanol respectively (same volume as samples), and three other samples were centrifuged at 10,000 rpm for 10 min, the supernatant and the cell