Isolation and characterization of *Pseudomonas otitidis* TH-N1 capable of degrading Zearalenone

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**Article info**

*Article history:*
Received 3 January 2014
Received in revised form 27 June 2014
Accepted 5 July 2014
Available online 15 July 2014

**Chemical compounds used in this study:**
Zearalenone (PubChem CID: 5281576)
methanol (PubChem CID: 887)
KH₂PO₄ (PubChem CID: 516951)
Na₂HPO₄ (PubChem CID: 24203)
MgSO₄·7H₂O (PubChem CID: 24843)
(NH₄)₂SO₄ (PubChem CID: 6097028)
CaCl₂ (PubChem CID: 5284359)
sodium chloride (PubChem CID: 5234)

**Keywords:**
Zearalenone
Biodegradation
Rumen microorganism
Characterization

**A B S T R A C T**

Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin produced by various *Fusarium* species and causes hyperestrogenism and related toxicosis of farm animals and humans. The present study aimed to isolate and identify ZEA-resistant bacteria from rumen in order to develop some strategies for detoxifying ZEA-contaminated food and feed. A bacterial strain was isolated from the rumen contents for its ability to utilize ZEA as the sole carbon and energy source. The isolate was an aerobic, Gram-negative, rod-shaped bacterium with single polar flagellum and was named *Pseudomonas otitidis* TH-N1 based on the morphology and 16S rRNA gene sequence. Meanwhile, the present study investigated that how various influence factors of *P. otitidis* TH-N1 could remove ZEA from a liquid medium. The optimal temperature, pH value, and concentrations of bacteria for the biodegradation of ZEA were 37 °C, 4.5, and 10⁹ cfu/ml, respectively. These results suggest that *P. otitidis* TH-N1 is a new bacterium found from the rumen and exhibited remarkable degradation activity of ZEA. It is probably a new bacterial resource to detoxify ZEA from ZEA-contaminated food and feed.

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**1. Introduction**

Zearalenone (ZEA; 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone; structure shown in Fig. 1) is a nonsteroidal estrogenic mycotoxin produced by various *Fusarium* species. *Fusarium graminearum* is one of the species which is considered to be the most prominent toxin producer (Bennett & Klich, 2003). As one of the most virulent mycotoxins, ZEA is found worldwide not only in maize and maize products, but also in oats, rice, rye, sorghum, and wheat (Zinedine, Soriano, Molto, & Manes, 2007).

It was proved that ZEA contamination invited danger to domestic animals, since ZEA and its metabolites are endowed with estrogenic properties (Placinta, D’mello, & Macdonald, 1999). ZEA in feed produces dominantly estrogenic properties in swine (female swine especially appear to be particularly sensitive), cattle, chicken, turkey, sheep, mice, etc. (Kakeya et al. 2002; Songsermsakul, Böhmer, Aurich, Zentek, & Razzazi-Fazeli, 2013). With regard to carcinogenicity, it was reported that ZEA has carcinogenic effect in mice and could stimulate the growth of human breast cancer cell lines (Battershill & Fielder, 1998; Martin, Horwitz, Ryan, & McGuire, 1978). In addition, hepatotoxicity, hematotoxicity, immunotoxicity, and genotoxicity of ZEA are pronounced (Dong et al. 2010; Marin, Taranu, Pistol, & Stancu, 2013).

At present the biodegradation of ZEA has become one of the most popular topics for research and development. A number of microbes from different niche have been reported to have an ability to biodegrade ZEA, but only some yeast strains and some beneficial rumen microbes can be used either by the food or by the feed industry for detoxifying ZEA (Fruhauf, Schwartz, Ottner, Krska, &...
Vekiru, 2012). Ruminants are known to be highly resistant to toxic effects of mycotoxins, while ruminen fluid has a diverse ecosystem (Fink-Gremmels, 2008). The rumen microbes have been to have the ability to detoxify aflatoxins (Upadhaya et al. 2009), deoxynivalenol (Binder et al. 1998, pp. 279–285), and ochratoxin A (Kiessling, Pettersson, Sandholm, & Olsen, 1984; Yang, 2010). Kallelah and Vasenius (1982) and Macri, Schollenberger, Drochner, Tafaj, and Morar (2005) have indicated that rumen fluid can also metabolize ZEA in vitro; however, little work has been reported on the degradation of ZEA by individual bacterium from cow rumen.

Therefore, this study attempted to isolate rumen bacteria capable of degrading ZEA. Fortunately, one bacterium capable of effective biodegradation of ZEA was isolated and identified. In addition, the effects of variables including culture time, temperature, pH value, and bacterial concentrations on the ability of the selected strain to degrade ZEA were evaluated.

2. Materials and methods

2.1. Chemicals and medium

ZEA was obtained from Sigma (St. Louis, USA) and diluted in methanol (1 mg/ml), which was used as a standard stock solution in this study. The working solution of ZEA was prepared and diluted with minimal salt (MS) medium (1.52 g KH₂PO₄, 2.44 g Na₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.5 g (NH₄)₂SO₄, 0.05 g CaCl₂, pH 6.5). Luria–Bertani (LB) medium containing the following ingredients (per liter) were used: 10 g peptone, 10 g sodium chloride, 5 g yeast extract, and 16 g agar (if necessary pH 7.2). Methanol was chromatographically purified, and water was purified by a Milli-Q Academic Water system. All other chemicals used were of analytical reagent grade and were used without further purification.

2.2. Isolation of ZEA-degrading bacteria

Fresh contents from the rumen of the cows were obtained from a local slaughterhouse in Ya’an. A portion (1.5 g) of each sample was incubated at 37 °C for 1 h in a rotary shaker (160 rpm). Subsequently, the final supernatant was used to inoculate MS medium supplemented with 2 μg/ml ZEA as a sole carbon and energy source and incubated at 37 °C for 7 days in a rotary shaker (180 rpm). After transferring successively three times, the cultures obtained were spread onto sterilized LB agar plates and were selected based on morphological differences as done by Dong and Cai (2001, pp. 353–364). Later the resulting colonies were isolated and transferred to MS medium with the final concentration of 5 × 10⁸ cfu/ml for bacterial suspension, and ZEA was 2 μg/ml. The mixtures were incubated at 37 °C for 72 h with a rotary shaker (180 rpm). The resultant degradation products from the reaction mixture were extracted twice with an equal volume of chloroform and were detected by thin-layer chromatography plate with chloroform:methanol (95:5, v/v).

The strain selected for its ability to utilize ZEA as the sole carbon and energy source was used in further experiments. All assays were performed in Eppendorf (5 ml, safe lock) vials. The cell suspensions were prepared according to the method described as follows. Cells were obtained by centrifugation at 8000g for 5 min. The pellets were washed twice with MS medium. Meanwhile, MS medium was added to make the bacterial concentration approximately 10⁹ cfu/ml (Lu, Liang, & Chen, 2011). Then 500 μl each of the cell suspensions was mixed with 500 μl ZEA solution. The final concentration of bacterial suspension was 5 × 10⁸ cfu/ml and ZEA was 2 μg/ml. Positive controls containing no bacteria and negative control for each strain containing no toxin were also included. The mixtures were incubated in a shaker (180 rpm) at 37 °C for 12, 24, 48, 72, 96, 120, and 168 h, respectively. The degradation rates were analyzed by high-performance liquid chromatography (HPLC).

2.3. Morphological observations

Bacteria were cultivated on LB agar plates at 37 °C for 24 h. Subsequently, colonies were observed by a light microscope (BH2, Olympus Optical Co. Ltd., Tokyo, Japan) through Gram’s stain technique, and the bacterial suspensions were negatively stained with 1% (w/v) phosphotungstic acid and examined under a Hitachi H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

2.4. Identification and phylogenetic analysis

The identity of the selected strain was determined based on 16S rRNA gene sequence analysis. The genomic DNA was extracted by the Bacterial DNAout kit (TIANGEN, Beijing, China). The universal primers of 16S rDNA fragments, 27F and 1492R, were used to amplify the 16S rDNA. The sequences of primers were as follows: (27F) 5’-GAGTTTGATCMTGGCTCAG-3’ and (1492R) 5’-TACGGYTACCTTGTTAGACTT-3’. Polymerase chain reaction (PCR) was performed using a Thermo cycler (DTC-3T; TianLong, Xi’an, China). The 16S rRNA gene sequences were amplified using the clustalX 1.81 program. Phylogenetic trees were constructed by MEGA 4.0 program using neighbor-joining method.

2.5. Effects of culture conditions of ZEA-degrading bacteria on biodegradation

The cell suspensions were tested under different culture conditions as follows: incubation temperature (10, 20, 30, 37, and 42 °C), pH value (4.5, 5.5, 6.5, 7.5, and 8.5), bacterial concentration (10⁸, 5 × 10⁸, 10⁹ cfu/ml), and inoculation time (250 mm × 4.6 mm; article size, 5 μm: Agilent) with a mobile phase of water:methanol (40:60 v/v) at a flow rate of 1 ml/min, and 120, 144, and 168 h, respectively. The degradation rates were analyzed by high-performance liquid chromatography (HPLC).

2.6. Detection of ZEA and data analysis

In all bacterial suspensions containing ZEA, equal volume of methanol was added, and the mixture was extracted by ultrasonication for 10 min (Yu et al. 2011). The methanol extracts of cultures were centrifuged for 10 min at 12,000g, and the supernatants were filtered with a 0.22-μm filter before being analyzed by reversed-phase HPLC (Agilent 1260 Infinity Series, Agilent Technologies, Palo Alto, USA). ZEA was separated on a C18 column (250 mm × 4.6 mm; article size, 5 μm: Agilent) with a mobile phase of water:methanol (40:60 v/v) at a flow rate of 1 ml/min, and with a UV detector at 286 nm. The identity of the selected strain was determined based on 16S rRNA gene sequence analysis. The genomic DNA was extracted by the Bacterial DNAout kit (TIANGEN, Beijing, China). The universal primers of 16S rDNA fragments, 27F and 1492R, were used to amplify the 16S rDNA. The sequences of primers were as follows: (27F) 5’-GAGTTTGATCMTGGCTCAG-3’ and (1492R) 5’-TACGGYTACCTTGTTAGACTT-3’. Polymerase chain reaction (PCR) was performed using a Thermo cycler (DTC-3T; TianLong, Xi’an, China). The 16S rRNA gene sequences were amplified using the clustalX 1.81 program. Phylogenetic trees were constructed by MEGA 4.0 program using neighbor-joining method.

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detected by ultraviolet with a frequency of 236 nm. The assay temperature was 30°C with an injection volume of 20 μL, and the retention times were approximately 12 min for ZEA. The percentage of toxin was calculated using the following equation: 100 × (peak area of ZEA in the supernatant/peak area of ZEA in the positive control). All results were presented as means of replicates and their standard deviations. Data were analyzed by SPSS (Statistical Package for the Social Sciences) software for Windows.

3. Results

3.1. Isolation of ZEA-degrading bacteria

The bacteria of this rumen fluid mixture grew on ZEA as a sole carbon and energy source. In this study, four colonies were isolated from the samples on LB agar medium and named as TH-N1, TH-N2, TH-N3, and TH-N4, respectively. The results show that strain TH-N1 had obvious degradation activity after incubated with ZEA for 72 h (Fig. 2).

Fig. 3 demonstrates the degrading capability of strain TH-N1 on ZEA. The degradation of ZEA was a rapid reaction since approximately 59% of ZEA was removed from the liquid media during the 10-min centrifugation after mixed with strain TH-N1 for 72 h. The ability of the isolate TH-N1 to degrade ZEA from liquid media slightly increased when the incubation period was >72 h.

3.2. Morphological observations

The strain TH-N1 belonged to Gram-negative rod-shaped bacterium (Fig. 4). The visual examination of transmission electron microscope not only confirmed that it was Bacillus, but also revealed that it had single polar flagellum (Fig. 5).

3.3. Phylogenetic analysis

16S rRNA sequence analysis revealed that strain TH-N1 was related to the P. otitidis (accession number: NR043289) with 99% identity (Fig. 6). The strain was named as P. otitidis TH-N1 (accession number: KC790454).

3.4. Effects of culture conditions of ZEA-degrading bacteria on degradation

The effects of temperature, pH value, and bacterial concentration on degradation of ZEA are shown in Figs. 7–9, respectively. ZEA was degraded by P. otitidis TH-N1 at all incubation temperatures used in this study (Fig. 7). The degradation rate increased with the increase in temperature till 37°C, when the percentage of ZEA removed was 53%, 56%, 59%, 60%, and 54% at 10°C, 20°C, 30°C, 37°C, and 42°C, respectively. However, there was no significant difference in the degradation rate in the range of 30–37°C.

Degradation of ZEA was sensitive to pH; the lower the pH value, the higher the degradation capability (Fig. 8). P. otitidis TH-N1 had the highest degradation rate of 65% which was attained at a pH of 4.5 while the lowest was 49% at a pH of 8.5, decreasing gradually with the increase in pH value. Incubation of the mixture within a
pH range of 6.5–7.5 had no significant difference in degradation capability.

The degradation of ZEA by *P. otitidis* TH-N1 from the liquid media was dependent on the concentration of bacteria in the incubation medium (Fig. 9). An increase in the cell concentration from $10^7$ cfu/ml to $10^9$ cfu/ml resulted in an increase in the degradation rate of ZEA from 17% to 79%. Therefore, the degradation reaction was dependent on the initial concentration of bacteria.

4. Discussion

Utilizing microorganisms for bio-detoxification is a well-known strategy for the management of mycotoxins in food and feed. There is no doubt that biodegradation is the best solution for decontamination. It is helpful in removing ZEA under mild conditions, without using harmful chemicals or causing significant losses in nutritive value and palatability of decontaminated food and feed (Bata & Lásztity, 1999). Various bacteria including *Lactobacillus* strains (El-Nezami, Polychronaki, Salminen, & Mykkänen, 2002), *Bacillus* strains (Yi, Pai, & Liu, 2011), *Micrococcus luteus* (Cheng, Jiang, Wang, Liu, & Wei, 2010), *Acinetobacter* sp. (Yu et al. 2011), and *Rhodococcus* strains (Cserháti et al. 2013) have shown the capacity to degrade ZEA or its derivative α- and β-zearalenol.

The present study demonstrates that the selected strain TH-N1 isolated from the rumen fluid has the ability to degrade ZEA as a sole source of carbon and energy. A phylogenetic tree indicated that

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Fig. 6. Phylogenetic tree constructed by the neighbor-joining method based on 16S rRNA gene sequences, showing the phylogenetic relationship of *Pseudomonas otitidis* TH-N1 and other closely related members of the genus *Pseudomonas*. Bootstrap values, expressed as percentages of 500 replications, are given at branching points. The scale bars represent 0.02 substitutions per site.

Fig. 7. Effect of degradation of ZEA by temperature. The incubation mixtures contained $5 \times 10^8$ cfu/ml of bacteria and 2 µg/ml of ZEA and the mixtures were incubated at 10, 20, 30, 37, and 42 °C, respectively. The values were means of replicates ($n=3$) and error bars represent standard deviations. Means with different letters were significantly different according to the Duncan’s Multiple Range Test ($P < 0.05$).

Fig. 8. Effect of degradation of ZEA by pH value. The incubation mixtures initially contained $5 \times 10^8$ cfu/ml of bacteria and 2 µg/ml of ZEA. The mixtures were incubated in pH 4.5, 5.5, 6.5, 7.5, and 8.5, respectively. The values were means of replicates ($n=3$) and error bars represent standard deviations. Means with different letters were significantly different according to the Duncan’s Multiple Range Test ($P < 0.05$).
Within 72 h. It could be concluded that more cells relatively increased strong degradation by the culture extract. Based on the above results, the optimal temperature and pH for the biodegradation of ZEA are 37 °C and 4.5, respectively. It is inferred that the reduction in ZEA is a result of one or more intracellular enzymes of *P. otitidis* TH-N1.

In addition, the culture conditions of the rumen strain *P. otitidis* TH-N1 is not strictly in vitro. It is important for its acid resistance and has the potential to be used as a feed additive to reduce ZEA. The most prominent strain *Eubacterium* BS8 797, which is the only microorganism available for commercial purposes to degrade trichothecenes, was also originally isolated from the bovine rumen (He, Zhou, Young, Boland, & Scott, 2010). This provides a direction for future research. Although concentrations of ZEA were reduced in the reaction system, other degradation products had not been analyzed. This is a critical attribute because metabolic products of ZEA may have higher or similar estrogenic activity as ZEA (Minervini, Giannoccoro, Cavallini, & Visconti, 2005). Therefore, further study of the strains is necessary to ensure that the degradation is safe with regard to inactivation of ZEA.

### 5. Conclusion

In this study, a strain that has the ability to degrade ZEA from the rumen was successfully isolated and was named as *P. otitidis* TH-N1. Several factors affect the degrading capability of ZEA, and the optimal temperature, pH value, and concentrations of bacteria for the biodegradation of ZEA were 37 °C, 4.5, and 10^9 cfu/ml, respectively. Further studies are needed to reveal the ZEA-degrading mechanism and assess the safety of *P. otitidis* TH-N1 on food or feed for animals.

### Acknowledgments

The work described in this paper was supported by the Key Projects of Department of Education of Sichuan Province, PR China (Grant no. 12ZA121), Changjiang Scholars and the Innovative Research Team in University (Grant no. IR0848) and Shaanxi Agricultural Committee Industrial Public Relation Project (Grant no. 2014K01-18-01).

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