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ORIGINAL PAPER

Degradation of zearalenone by the extracellular extracts of *Acinetobacter* sp. SM04 liquid cultures

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Abstract A bacterium (designated SM04) which can rapidly grow on zearalenone (ZEN) as sole carbon and energy source was isolated from agricultural soil. On the basis of 16S rDNA sequencing analysis, strain SM04 was classified as a bacterium belonging to the *Acinetobacter* genus. In this study, the biodegradation of ZEN by the extracellular extracts of strain SM04 liquid cultures in M1 medium and Nutrient Broth medium was examined using HPLC analysis, APCI-MS analysis, and MTT (tetrazolium salt) cell proliferation assay. Results showed no ZEN and other equally estrogenic metabolites were found after 12 h when ZEN was treated with the extracellular extracts of M1 cultures, but no significant ($P < 0.01$) reduction of ZEN was observed over the 12-h incubation period in the extracellular extracts of Nutrient Broth cultures. Results also indicated that some proteins in the extracellular extracts of M1 cultures were essential to ZEN degradation. The proteins in the extracellular extracts of M1 cultures and Nutrient Broth cultures were analyzed with SDS-PAGE, bands showing different intensities among the two extracellular extracts were processed for protein identification by MALDI-TOF/TOF/MS, and nine proteins from M1 cultures matched the database for *Acinetobacter* genus with great confidence. Furthermore, the function of some proteins identified is unknown or unconfirmed because of the lack of well-annotated genomic sequence data and protein data for *Acinetobacter* genus on the public database, but in further studies these data of proteins identified will be useful for screening the genes related to ZEN degradation.

Keywords Zearalenone · Degradation · Enzymes · MALDI-TOF/TOF/MS · *Acinetobacter* sp.

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Introduction

Zearalenone (ZEN) is a mycotoxin mainly produced by *Fusarium graminearum* and *Fusarium culmorum*, which are common soil fungi in temperate and warm countries, and are regular contaminants of cereal crops worldwide (D'mello et al. 1999; Zinedinea et al. 2007). ZEN is a resorcyclic acid lactone (Fig. 1), which has been associated with hyperestrogenism and other reproductive disorders in pigs, sheep, and other farm animals. Hyperestrogenic syndromes can be uterine enlargement, swelling of the vulva, prolapse of the vagina or rectum, prolonged or interrupted estrus, pseudopregnancy and reduced litter size (Doll et al.

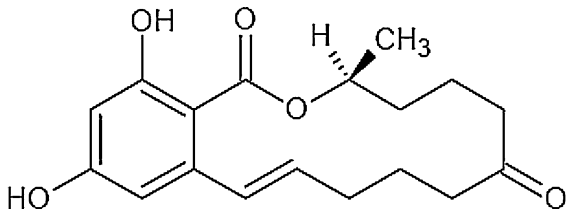

Fig. 1 The chemical structure of ZEN

2003; Kumar et al. 2008). α- and β-zearalenol (α- and β-ZOL) are hydroxylated derivatives of ZEN, and α-ZOL has a three to four times bigger estrogenic effect than ZEN, while β-ZOL is less estrogenically active (El-Sharkawy et al. 1991; Saeger et al. 2003).

Numerous researchers reported that cereal grain in various crops and its products in many countries contained high concentration of ZEN (Placinta et al. 1999; Lu and Chen 2004). ZEN-contaminated products will impose a risk to animal health if they were used as feedstuff. In the long term, ZEN in feed can cause nontrivial economic damage to the livestock industry (Wu and Munkvold 2008). Increasing attention had therefore been paid to the development of an effective strategy to control ZEN contamination. Various methods for the treatment of ZEN had been proposed, including adsorption (Huwig et al. 2001; Bueno et al. 2005), biodegradation (Molnar et al. 2004; Takahashi-Ando et al. 2005; Kabak et al. 2006), extrusion cooking, and ozonation (Mckenzie et al. 1997). Among them, transformation or degradation of ZEN by microorganisms is an attractive approach for efficiently detoxifying ZEN. Molnar et al. (2004) described a new yeast strain, *Trichosporon mycotoxinivorans*, which is able to degrade ZEA to carbon oxide and other non-toxic metabolites, neither α- nor β-zearalenol were detected. Takahashi-Ando et al. (2005) identified and characterized a lactonohydolase enzyme in fungus *Clonostachys rosea* which convert ZEA to a less estrogenic compound.

It is well known that many bacteria are capable of degrading a wide range of xenobiotic compounds including polychlorinated biphenyls as well as aromatic compounds (Ronald et al. 2007). ZEN is a resorcyclic acid lactone and somewhat structurally related to the above-mentioned xenobiotic aromatic compounds. However, little work had been reported on the degradation of ZEN by bacterium, especially associated with degradation of ZEN by individual bacteria from agricultural soil. Furthermore, it was reported that ZEA is mainly produced during growth of the grains or during storage, and ZEN produced during growth of the grains could be released to the agricultural soil, but the concentration of ZEN in the agricultural soil is very low (Mortensen et al. 2006; Zinedinea et al. 2007). Several studies also showed that some microorganisms in the agricultural soil were closely related to degradation of ZEN (Megharaj et al. 1997; Mortensen et al. 2006; Yuksel et al. 2005), and thus the initial goal of this study is to obtain single bacterium for efficiently degrading ZEN.

In this study, bacteria isolated from agricultural soil were evaluated for its ability to utilize ZEN as sole carbon and energy source. And degradation of ZEN by bacterial strain SM04 was investigated. Furthermore, the effect of the bacterial treatment on the ZEN molecule was evaluated.

Materials and methods

Chemicals

Mycotoxins (zearalenone, α- and β-zearalenol) were purchased from Sigma (St. Louis, USA). Each mycotoxin was dissolved in methanol (1 mg/ml) and used as a standard stock solution. Nystatin was dissolved in acetone (5 mg/ml). Methanol was of chromatographic pure grade and water was purified by a Milli-Q Academic Water system. Other chemicals used were all of analytical grade.

Medium

In the study, M1, M2, and Nutrient Broth mediums were used. M1 medium was equivalent to M2 medium supplemented with 1.5% (w/v) sodium acetate. M2 medium contained the following per liter: 3 g of NH_4NO_3, 1 g of KCl, 0.5 g of $MgSO_4$, 1.0 g of K_2HPO_4, 0.1 g of $CaCl_2$, and10 ml of trace element (2 g/l $FeSO_4{\cdot}7H_2O$, 0.4 g/l $MnSO_4{\cdot}4H_2O$, 0.4 g/l $CuSO_4{\cdot}5H_2O$, 0.4 g/l $CoCl_6{\cdot}6H_2O$, and 0.5 g/l $ZnCl_2$). Nutrient Broth medium composed of 1.0% (w/v) peptone, 0.3 (w/v) beef extract, and 0.5% (w/v) NaCl. In addition, for preparing M2 medium supplemented with ZEN as sole carbon and energy source, ZEN standard stock solution was added to a 50 ml sterile screw-cap plastic tubes and evaporated to

dryness in a 50°C water bath, whereafter M2 medium was added.

Isolation of ZEN-degrading bacteria

Soil samples (30 samples) for the isolation of bacteria capable of degrading ZEN were obtained from fields planted with grain in South China. A portion (2.0 g) of each samples was suspend in 10 ml of 0.15 M NaCl water solution, the resulting supernatants (150 µl) were used to inoculate M1 medium (3 ml) supplemented with 25 µg/ml ZEN and 15 µg/ml nystatin, and then the cultures were incubated at 30°C for 48 h in a rotary shaker (200 r/min). Following incubation, 0.5 ml of the cultures was transferred to a new plastic tube as inoculums, and ZEN was extracted from the remained cultures with methanol, and then quantified by HPLC analysis. Original cultures, exhibiting significant degradation of ZEN, were combined (culture pool 1). A portion (0.5 ml) of culture pool 1 was inoculated into M2 medium (3 ml) supplemented with 0.5 mg/ml ZEN as sole carbon and energy source, and incubated at 30°C for 48 h. After successively transferring three times, cultures pool 2 thus obtained was able to grow on ZEN as sole carbon and energy source. Cultures pool 2 was serially diluted in 0.9% NaCl, and aliquots (0.1 ml) were spread onto Nutrient Broth agar plates. After incubated at 30°C for 12 h, colonies were selected based on morphological differences (Dong and Cai 2001), bacterial colonies were inoculated into M2 medium (2 ml) supplemented with 0.5 mg/ml ZEN as sole carbon and energy source, and incubated at 30°C for 48 h in a rotary shaker (200 r/min). Growth was measured by the optical density (OD_{600}). The bacterial communities thus obtained were stored at −70°C after suspension in 20% glycerol water solution.

Identification of bacterial isolates, which exhibited potent ability to grow on ZEN as sole carbon and energy source, was performed by phylogenic analysis based on 16S ribosomal DNA (rDNA) sequence. Preparation of chromosomal DNA from the isolated strains was performed by the Bacterial DNAout kit (TIANDZ, China). The universal primers of 16S rDNA fragments, 27F and 1495R, were used to amplify the 16S rDNA. The sequences of primers were as follows: (27F) 5′-AGAGTTTGAT CCTGGC TCAG-3′ and (1495R) 5′-CTACGGCTACCTTGT TACGA-3′ (Miyashita et al. 2009). PCR was performed in a Thermocycler (Little Genius of Bioer, China). PCR amplifying procedure was as follows: 95°C for 4 min, 30 cycles for 94°C for 30 s, 55°C for 30 s, 72°C for 1.0 min, and then 72°C for 10 min, and 4°C end. PCR products were purified and sequenced by Invitrogen Corporation (Guangzhou). Determined 16S rDNA gene sequences were analyzed through BLAST search in NCBI database (http://blast.ncbi.nlm.nih.gov/blast), and aligned using the Clustal W2 program (Larkin et al. 2007; Chenna et al. 2003). Phylogenetic trees were constructed by MEGA 4.0 program using Neighbor-Joining (Tamura et al. 2007; Kumar et al. 2008). Bootstrap confidence values were calculated from 1000 repeats.

Growth kinetics of strain SM04 in M2 medium added ZEN as a sole carbon source

An inoculum was prepared by suspending strain SM04 cells in 0.15 M NaCl water solution (A600, 0.8). The inoculum (5 µl) was transferred into sterile screw-cap plastic tubes which contained 3 ml of M2 medium supplemented with 0.5 mg/ml ZEN as sole carbon and energy source, and incubated at 30°C for 72 h in rotary shaker (200 rpm). Growth was measured by the optical density (OD_{600}). During 6 h intervals, samples were taken and the optical density and ZEN concentration were determined as following described. ZEN supplemented with M2 medium and M2 cultures without ZEN were included as control.

Extraction of ZEN from the liquid cultures

Liquid cultures were mixed with the same volume of methanol, and ultrasonically extracted for 10 min. The methanol extracts of cultures were centrifuged for 5 min at 10,000×g, and then the supernatants were used for ZEN analysis.

Preparing of extracellular extracts of strain SM04 liquid cultures

M1 medium and Nutrient Broth medium were, respectively, inoculated with strain SM04 and incubated at 30°C on a rotary shaker (200 rpm). After the cultures of strain SM04 in M1 medium or Nutrient Broth medium was incubated to exponential

anaphase, cultures were centrifuged (12,000×*g* for 5 min) at 4°C, the supernatant was passed through 0.22 μm filter, and then the filtrate was named as extracellular extracts of strain SM04 liquid cultures.

Degradation of ZEN by extracellular extracts of strain SM04 liquid cultures

1 ml of extracellular extracts of M1 cultures or Nutrient Broth cultures was transferred to a sterile screw-cap plastic tube containing 20 μg of ZEN. These tubes were loosely capped and incubated at 30°C. After different intervals, methanol (1 ml) was added to stop the ZEN degradation reaction. The mixtures were centrifuged at 10,000×*g* for 5 min, and then the supernatants were used to HPLC analysis, APCI-MS analysis, and MTT (tetrazolium salt) cell proliferation assay in MCF-7 cell. 0.01 M sodium phosphate buffer (pH 9.0) was used as control.

The effect of protease treatment on the ZEN degrading ability of extracellular extracts of strain SM04 was determined by exposing to 1 mg/ml proteinase K plus 1% SDS (Sodium dodecyl sulfate) for 1 h at 55°C. 1 ml of extracellular extracts exposed to proteinase was also transferred to a sterile screw-cap plastic tube containing 20 μg of ZEN. These tubes were loosely capped and incubated at 30°C. After 24 h, ZEN concentration present in the supernatants was determined by HPLC as previously described.

High-performance liquid chromatography analysis

The chromatographic system consisted of a Waters 700 controller, a Waters 2996 photodiode array detector and a Waters 717 plus autosampler. Empower software was used for data processing. For analyzing ZEN and its metabolites, a Waters XTerra^{R} MS C18 column (4.6 × 150 mm, 5 μm) was used for chromatographic separation. The mobile phase, which consisted of methanol–water (0.15% formic acid) 60:40 (v/v) was used at a flow rate of 1 ml/min. The column was kept at 30°C. The injection volume was 30 μl. The results were monitored by photodiode array detector between 225 and 400 nm. Concentrations of ZEN were determined based on retention times and peak areas at 274 nm compared to ZEN standards dissolved in methanol.

Atmospheric-pressure chemical ionization (APCI) mass spectrometry analysis

The sample (5 μl) was introduced into the APCI source by infusion. Mass spectrometric detection was performed with an ion-trap mass spectrometer equipped with an APCI interface (Bruker Corporation, Germany). The APCI interface was used in the negative ionization mode due to its better selectivity. Drying gas flow rate was set at 400 l/h and sheath gas flow rate was held at 200 l/h. The capillary voltage was set at 3.5 kv. The source and APCI vaporizing temperature were maintained at 105 and 450°C, respectively. Before APCI-MS analysis, the blank samples, such as the extracellular extracts, were mixed with the same volume of methanol, centrifuged for 5 min at 10,000×*g*, and then the supernatant was used to analyze.

The MTT (tetrazolium salt) cell proliferation assay

For analyzing the loss of estrogenic effect of ZEN which was treated with the extracellular extracts, the MTT (tetrazolium salt) cell proliferation assay using MCF-7 cell (estrogen receptor positive, human breast cancer cell) was performed (Shen et al. 2003; Cetin and Bullerman 2005a, b; Yuksel et al. 2005; Yu et al. 2005). Each well in a 96-well plate was inoculated with 100 μl of MCF-7 single-cell suspension (at a density of 5×10^4 cell/ml). After cells attached to the wells, the seeding medium was removed and replaced by fresh 180 μl of phenol red-free DMEM/F-12 containing 10% charcoal stripped serum. Samples were diluted 100 times using 1% ethanol water solution, and 20 μl of the diluted samples was also added to the 96-well plates and incubated at 37°C in a humidified atmosphere of 5% CO_2. The blank was only added 20 μl of phenol-free DMEM/F-12 medium containing 1% ethanol. Every sample was repeatedly added to five wells. After 4 days of incubation, 25 μl of the MTT solution (5 mg/ml) was added to each well of the 96-well plates and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2, and then the medium were discarded. 100 μl of dimethyl sulfoxide was added into each well, and the 96-well plates were placed on an orbital shaker at 37°C for 20 min. The absorbance was measured at a test wavelength of 490 nm using a microplate reader. The % increase of

MCF-7 cell proliferation was calculated using the formula: % increase of cell proliferation = [(A_{490} test/A_{490} blank) − 1] × 100, where A_{490} test = absorbance of test sample, A_{490}blank = absorbance of blank sample.

Analysis of proteins in extracellular extracts of strain SM04 liquid cultures

In order to analyze proteins in extracellular extracts, the SDS-PAGE and matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/TOF/MS) analyses were used. The protein powder obtained from 300 ml of extracellular extracts of M1 cultures or Nutrient Broth cultures by TCA protein precipitation method (Jiang et al. 2004) was separated with 15% polyacrylamide gel discontinuous (Laemmli) denaturing SDS-PAGE (Laemmli 1970). Visualization of bands was performed by Coomassie Brilliant Blue G-250 staining. Protein bands from the extracellular extracts of M1 cultures or Nutrient Broth cultures, which showed clear differences in intensity, were excised from gel for further identification with MALDI-TOF-TOF/MS (ABI 4800 plus MALDI-TOF/TOF MS, Applied Biosystems, USA).

In protein identification with MALDI-TOF-TOF/MS, gel pieces were washed twice in ultra-pure water and destained with the fresh solution containing 100 mM NH_4HCO_3 in 50% acetonitrile solution. After dried in freeze-drying equipment, gel pieces were incubated in 10 μl digestion solution consisted of 40 mM NH_4HCO_3 in 9% acetonitrile solution, and 20μg/ml proteomics grade trypsin (Promega, USA) for 10–12 h at 37°C. At the end of digestion, peptides were extracted by shaking the gel pieces in 67% (v/v) acetonitrile solution supplemented with 5% (w/v) TFA for 30 min, and then were dried in freeze-drying equipment. The dried tryptic peptide mixture was re-dissolved in 0.1% (w/v) TFA water solution and subjected to MALDI-TOF-TOF/MS analysis

From the mass spectral data obtained, protein identification was automatically performed with ABI GPS Explorer software (V 3.6). This system ran a search for each protein against the NCBInr (National Center for Biotechnology nonredundant database) using the MASCOT (V2.1, Matrix Science, London, U.K) search engine. In these searches the peptide mass tolerance was set at 150 ppm, mass tolerance for fragmented ions was set to 0.2 Da, one missed cleavage by trypsin was allowed, and protein modifications included oxidation of methionine and carbamidomethylation of cysteine, when appropriate. Proteins, with a more high MASCOT individual ions scores than 48, were considered identity or extensive homology ($P < 0.05$). Proteins that were matched with a lower MASCOT individual ions scores were considered tentative. In addition, tentative proteins were placed into predicted functional groups if possible by searching the NCBI Conserved Domain Database (CDD) and PDBJ (Protein Data Bank Japan) using the BLAST search engine.

Results

Isolation of ZEN-degrading bacteria

After analyzed by HPLC, there were six cultures of soil samples in which no ZEN and some metabolites such as α-ZOL and β-ZOL were detected. After successively subcultured three times, the individual bacteria in cultures pool 2 was isolated. Almost all the resultant colonies appeared to be composed of bacteria, and the bacteria appeared to be composed of approximately 12 species on the basis of differences in colony morphology. In the 12 isolates cultures with ZEN as sole carbon and energy source, only one (designated SM04) showed obvious increase of the optical density.

Growth curve of strain SM04 in M2 medium supplemented with 500 mg/l ZEN as sole carbon and energy source was examined (Fig. 2). Growth of strain SM04 in M2 medium added ZEN corresponded for the most part, to the loss of ZEN from the medium, particularly during 18–36 h (exponential phase) (Fig. 2). On further incubation, no ZEN was detected in growth medium. In addition, no obvious increase of the optical density was found in M2 cultures without ZEN, and no loss of ZEN was observed under the same condition in M2 medium (uninoculated) (Fig. 2).

Taxonomic characterization of strain SM04

Strain SM04 is a short rod-shaped bacterium, and Gram staining was negative. 16S rDNA sequencing analysis revealed that strain SM04 was related to the *Acinetobacter* genus with 99.5% identity.

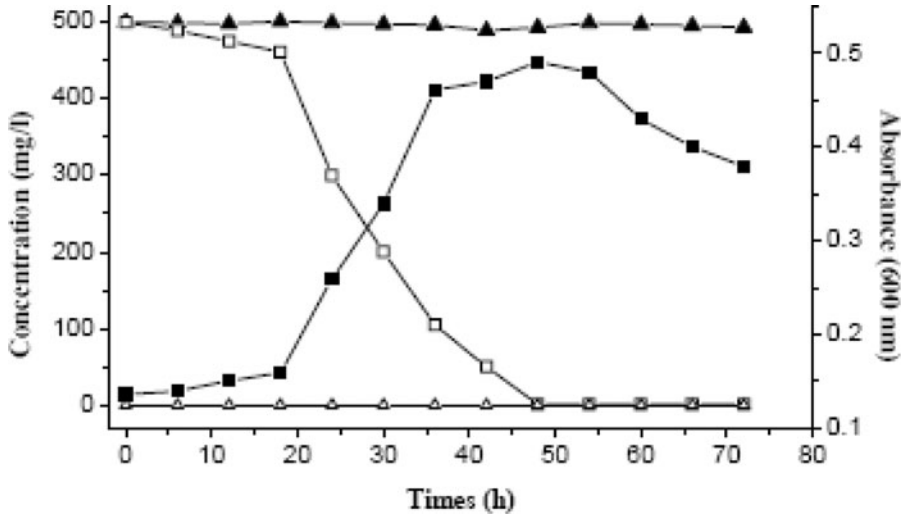

Fig. 2 Growth curve of strain SM04 in M2 medium with ZEN as sole carbon source (*filled square*) and control cultures without ZEN as carbon source (*open triangle*), and the growth is shown as increase in the optical density. ZEN concentration in the cultures (*open square*) and the uninoculated control samples (*filled triangle*)

Degradation of ZEN by extracellular extracts of strain SM04 liquid cultures

A significant ($P < 0.001$) reduction of ZEN was observed from 0 to 12 h when treated with the extracellular extracts of M1 cultures (Fig. 3a). And only trace level of ZEN can be detected after 4 h in the presence of extracellular extracts of M1cultures. In the 0.01 M sodium phosphate buffer (pH 9.0), no loss of ZEN was observed over the 12-h incubation period, and an interesting observation made was the extracellular extracts of Nutrient Broth cultures had no ability to degrade ZEN, because no significant ($P < 0.01$) reduction of ZEN was observed over the 12-h incubation period (Fig. 3a).

A significant ($P < 0.001$) decrease of ZEN proliferative effects to MCF-7 cell was also observed from 0 to 12 h when treated with the extracellular extracts of M1 cultures (Fig. 3b). Comparing with the blank, only 8% increase of MCF-7 cell proliferation was observed when ZEN was treated with the extracellular extracts of M1 cultures for 12 h, whereas more than 80% increase of MCF-7 cell proliferation was observed when ZEN was, respectively, treated with the extracellular extracts of Nutrient Broth cultures and 0.01 M sodium phosphate buffer (pH 9.0) for 12 h (Fig. 3b).

With the aid of APCI-MS (negative ion mode), ZEN was identified at $m/z = 317$. The extracellular extracts of M1 cultures showed no peaks in the same m/z values as ZEN, and the sample of ZEN after 0 h when treated with the extracellular extracts of M1 cultures clearly showed a peak at $m/z = 317$ (Fig. 4b). However, APCI-MS analysis revealed that ZEN was still present in the presence of the extracellular extracts of M1 cultures for 4 h, while at significantly lower levels (Fig. 4c). In addition, the sample of ZEN after 4 h in the presence of the extracellular extracts of M1 cultures did not also display other new high peaks when comparing with the extracellular extracts of M1 cultures (Fig. 4).

Exposure of the M1 extracellular extracts to 1 mg/ml proteinase K plus 1% SDS for 1 h at 55°C resulted in loss of the ability to degrade ZEN (no loss of ZEN was observed over 24 h incubation period in the presence of the extracellular extracts of M1 medium after proteinase K treatment), indicating that some proteins (or enzymes) in the extracellular extracts of M1 medium may be essential to ZEN degradation.

Analysis of proteins in extracellular extracts of strain SM04 liquid culture

Obvious difference existed in the proteins of the extracellular extracts of M1 cultures and Nutrient Broth cultures as exhibited by SDS-PAGE (Fig. 5). Bands showing different intensities among the two cultures were excised for protein identification by MALDI-TOF/TOF/MS. In total, five bands from the M1 extracellular extracts, and five bands from the Nutrient Broth extracellular extracts were excised (indicated with arrows in Fig. 5).

Results showed nine proteins from the five bands in the M1 extracellular extracts matched the database

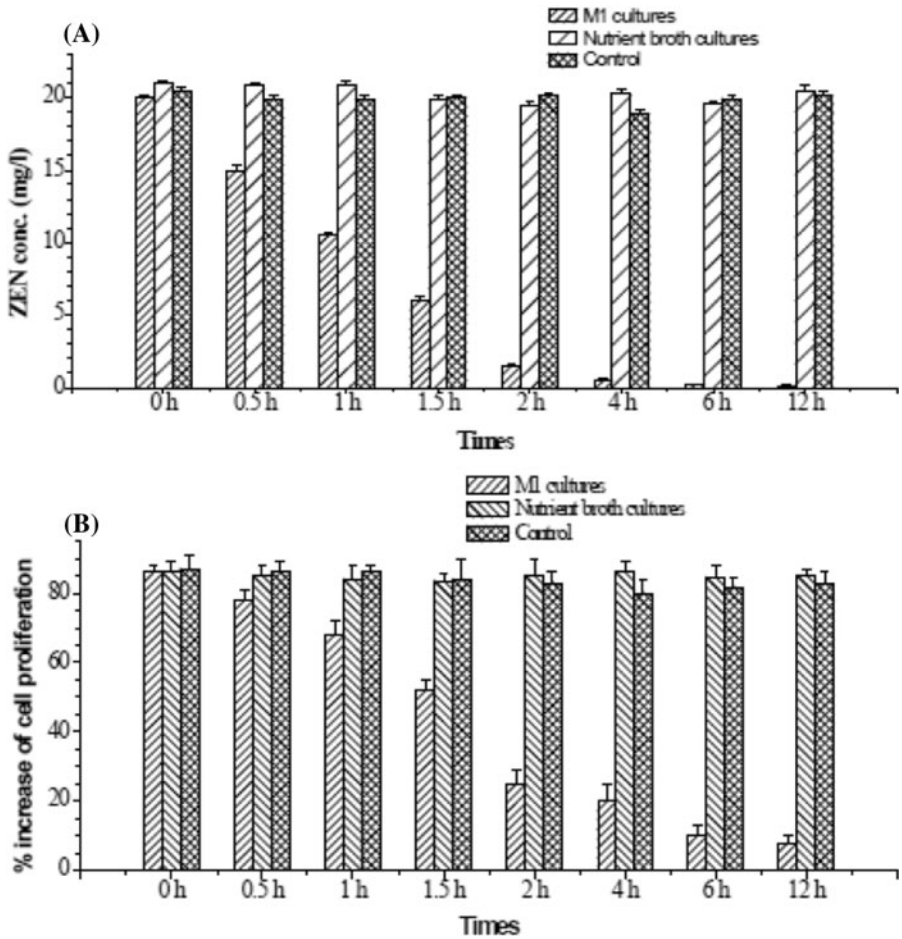

Fig. 3 ZEN concentration (**a**) and % increase of MCF-7 cell proliferation (**b**) during treatment with extracellular extracts of strain SM04 liquid cultures in M1 medium and Nutrient Broth medium for different time intervals. In MTT cell proliferation assay using MCF-7 cell, all samples of ZEN were diluted 100 times. 0.01 M sodium phosphate buffer (pH 9.0) was included as control

for *Acinetobacter* genus with great confidence (Table 1), and additional three proteins were tentatively identified through NCBI Blast and could possibly be related to aldo/keto reductase, ring-cleavage dioxygenase, and translocase. In addition, eight proteins from the other five bands in the Nutrient Broth extracellular extracts were also identified or tentatively identified (results not shown).

Discussion

ZEN is slightly soluble in water, and its solubility in water at 25°C is: 4.51, 4.81, and 40.15 μg/ml at pH 2, 7, and 10, respectively (Shawna et al. 1998). Several studies (Huwig et al. 2001; Bueno et al. 2005; Kabak et al. 2006) found some specific materials such as the cell of some microorganisms could bind ZEN, and remove ZEN from water solution, but the bonded ZEN could be recovered by methanol extraction. In our studies, after ultrasonically extracted with methanol as previously described, the proportion of ZEN bonded by cell pellets in the bacterial liquid cultures was less than 0.02%.

Megharaj et al. (1997) obtained a mixed culture of bacteria which was able to grow rapidly when ZEN was provided as the sole source of carbon and energy, and 14 bacterial isolates from the mixed culture were identified and purified. But unfortunately, the ability to degrade ZEN was lost upon purification and recombination of the bacterial members of the mixed culture, so the ability related to ZEN degradation of these bacteria may be bad in genetic stability. In the study, a bacterium capable of growing rapidly on ZEN as sole source of carbon and energy was fortunately isolated from agricultural soil, which would bring to us a good opportunity to study the mechanism of ZEN degradation by bacterium. Meanwhile, to prevent strain SM04 losing the ability to degrade ZEN, we decreased the passage number as few as possible, and

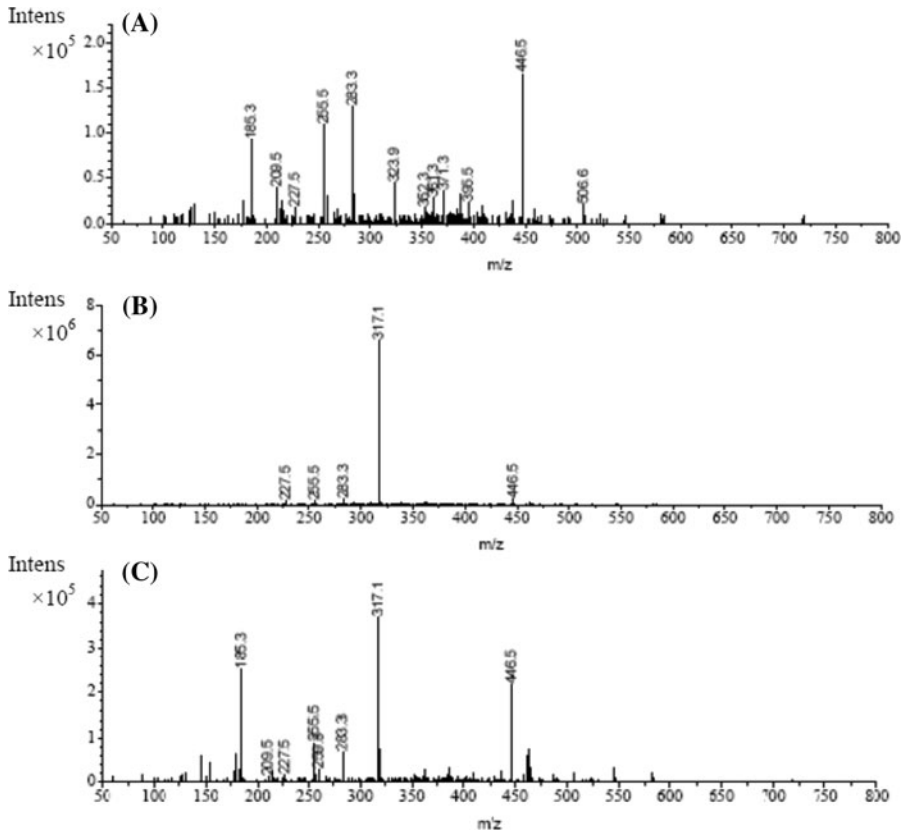

Fig. 4 Degradation of ZEN in the extracellular extracts of strain SM04 liquid cultures in M1 medium using APCI-MS (negative ion mode) analysis. **a** APCI-MS spectrum of the extracellular extracts; **b** APCI-MS spectrum of ZEN in the presence of extracellular extracts for 0 h; **c** APCI-MS spectrum of ZEN in the presence of extracellular extracts for 4 h

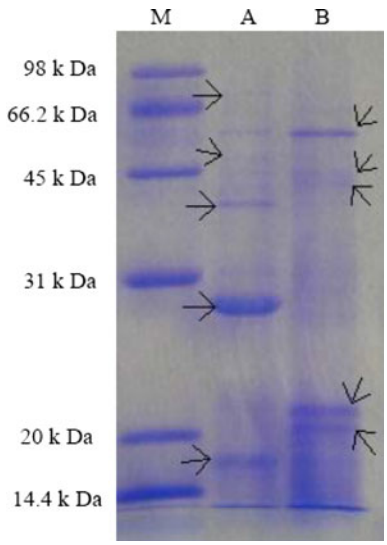

Fig. 5 SDS-PAGE analysis of proteins in the extracellular extracts of strain SM04 liquid cultures in M1 medium (**a**) and Nutrient broth medium (**b**). *M* markers for molecular weight of proteins. The bands of which the intensity was different among the two medium were excised for analysis with MALDI-TOF/TOF/MS. The *arrows* indicate the positions of the 10 bands which were excised

the cells of strain SM04 stored in 20% glycerol (v/v) solution at −70°C were as inoculums.

Studies also found ZEN was stable to pH values between 1.0 and 13, the variety of pH in the cultures could not lead to ZEN transformation (Zinedinea et al. 2007), and thus ZEN transformation most probably occurred through reaction of a cascade of enzyme in the M1 extracellular extracts of strain SM04. In addition, the results described above also indicated that some proteins (or enzymes) in the M1 extracellular extracts were essential to ZEN degradation.

After 4 h when ZEN was treated with the M1 extracellular extracts of strain SM04, no metabolites of ZEN, described by some other studies (Kamimura 1986; El-Sharkawy et al. 1991; Boswald et al. 1995; Takahashi-Ando et al. 2005), such as ZOL, the opening of ZEN lactone ring, or polar conjugates (glycoside or sulfate conjugates), was found by HPLC and APCI-MS analysis, and HPLC and APCI-MS analysis could not reveal the formation of other breakdown products. ZEN in the M1 extracellular extracts was most likely degraded into some other metabolites with chemical properties different from

Table 1 Proteins identified in the bands (designated by Fig. 5) from the extracellular extracts of strain SM04 liquid cultures in M1 medium

No.	Protein score	Protein name	Accession no.	Mw.	Function
M1	196	Putative ferric acinetobactin receptor [*Acinetobacter baumannii* ATCC 17978]	gil126642422	79764	Signal transduction
M2	482	Chain A, Soluble Quinoprotein Glucose Dehydrogenase [*Acinetobacter Calcoaceticus*]	gil7245520	50207	Dehydrogenase
M3	897	Conserved hypothetical protein [*Acinetobacter sp.* SH024]	gil293611044	39348	Unknown
M4	180	Galactose mutarotase [*Acinetobacter baumannii* ACICU]	gil184157246	41546	Mutarotase
M5	62	Beta-lactamase class C [*Acinetobacter baumannii*]	gil7258342	43180	Hydrolase
M6	243	Hypothetical protein ACICU-00876 [*Acinetobacter baumannii* ACICU]	gil184157196	25962	Transferase
M7	229	Conserved hypothetical protein [*Acinetobacter sp.* SH024]	gil293610587	27645	Unknown
M8	172	Glutamate/aspartate transport protein [*Acinetobacter baumannii* ATCC 17978]	gil126641536	23764	Transporter related protein
M9	115	Type VI secretion system effector [*Acinetobacter calcoaceticus* RUH2202]	gil262278650	18759	Signal transduction

that of ZEN, and these metabolites was likely to be easily utilized by strain SM04. Further studies on the analysis of metabolites of ZEN degradation by the M1 extracellular extracts should carry out. In addition, no equally estrogenic metabolites of ZEN could be detected on the basis of the MTT (tetrazolium salt) cell proliferation assay in MCF-7 cell line, which indicated strain SM04 will be very useful for detoxification of ZEN-contaminated feed.

When M1 medium was substituted by Nutrient Broth medium, the extracellular extracts of strain SM04 liquid cultures in Nutrient Broth medium had no ability to degrade ZEN, which showed no some enzymes existed which resembled the extracellular extracts of M1 cultures. When an (rapidly metabolizable) substrate is available in medium, the synthesis of enzymes responsible for ZEN degradation is under repression, Nutrient Broth medium was nutritionally richer than M1 medium, therefore, we inferred the transcription of genes responsible for ZEN degradation may regulated by catabolite repression. The substantial reasons can be confirmed after identifying the genes involved in ZEN degradation.

Results found obvious difference existed in the proteins of the extracellular extracts of strain SM04 liquid culture in M1 medium and Nutrient Broth medium (Fig. 5). These different proteins in the extracellular extracts of M1cultures seemed likely candidates to be involved in the ZEN degradation, so the different protein bands in the two cultures were identified by MALDI-TOF-MS. Because of the lack of well-annotated genomic sequence data and protein data for *Acinetobacter* genus on the public database, the function of some proteins identified is unknown or unconfirmed, which hampered our analysis to these proteins likely related to ZEN degradation. For future studies on the proteins related to ZEN degradation, we propose the following strategy: basing on the sequence of proteins identified from the M1 extracellular extracts of strain SM04, cloning the genes encoding these proteins above, and screening the enzymes or genes related to ZEN degradation.

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References

Boswald C, Engelhardt G, Vogel H, Wallnofer PR (1995) Metabolism of the Fusarium mycotoxins zearalenone and deoxynivalenol by yeast strains of technological relevance. Nat Toxins 3:138–144

Bueno DJ, DiMarco L, Oliver G, Bardon A (2005) In vitro binding of zearalenone to different adsorbents. J Food Prot 68:613–615

Cetin Y, Bullerman LB (2005a) Cytotoxicity of Fusarium mycotoxins to mammalian cell cultures as determined by the MTT bioassay. Food Chem Toxicol 43:755–764

Cetin Y, Bullerman LB (2005b) Evaluation of reduced toxicity of zearalenone by extrusion processing as measured by the MTT cell proliferation assay. J Agric Food Chem 53:6558–6563

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31:3497–3500

D'Mello JPF, Placinta CM, Macdonald AMC (1999) Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Anim Feed Sci Technol 80:183–205

Doll S, Danicke S, Schnurrbusch U (2003) The effect of increasing concentrations of Fusarium toxins in the diets of piglets on histological parameters of uterus. Mycotox Res 19:73–76

Dong XZ, Cai MY (2001) Manual of identification for general bacteriology. China Publishing Company of Science, Beijing, pp 349–418

El-Sharkawy SH, Selim MI, Afifi MS, Halaweish FT (1991) Microbial transformation of zearalenone to a zearalenone sulfate. Appl Environ Microbiol 57:549–552

Huwig A, Freimund S, Kappeli O, Dutler H (2001) Mycotoxin detoxification of animal feed by different adsorbents. Toxicol Lett 122:179–188

Jiang L, He L, Fountoulakis M (2004) Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. J Chromatogr A 1023:317–320

Kabak B, Dobson AD, Var I (2006) A Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed: A Review. Crit Rev Food Sci Nutr 46:593–619

Kamimura H (1986) Conversion of zearalenone to zearalenone glycoside by *Rhizopus* sp. Appl Environ Microbiol 52:515–519

Kumar S, Dudley J, Nei M, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299–306

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Larkin MA, Blackshields G, Brown NP et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948

Lu MB, Chen G (2004) Research on the contamination of feed with mycotoxins in the north China. China Feed 9:32–34

Mckenzie KS, Sarr AB, Mayura K, Bsiley RH et al (1997) Oxidative degradation and detoxification of mycotoxins using a noval source of ozone. Food Chem Toxicol 35:807–820

Megharaj M, Garthwaite I, Thiele JH (1997) Total biodegradaation of the oestrogenic mycotoxin zearalenone by a bacterial culture. Lett Appl Microbiol 24:1231–1236

Miyashita A, Mochimaru H, Kazama H et al (2009) Development of 16S rRNA gene-targeted primers for detection of archaeal anaerobic methanotrophs (ANMEs). FEMS Microbiol Lett 297:31–374

Molnar O, Schatzmayr G, Fuchs E, Prillinger H (2004) *Trichosporon mycotoxinivorans* sp. nov., a new yeast species useful in biological detoxification of various mycotoxins. System Appl Microbiol 27:329–333

Mortensen GK, Strobel BW, Hansen HCB (2006) Degradation of zearalenone and ochratoxin A in three Danish agricultural soils. Chemosphere 62:1673–1680

Placinta M, D'Mello JPF, Macdonald AMC (1999) A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. Anim Feed Sci Technol 78:21–37

Ronald LC, Carina MJ, Janice LS (2007) The recent evolution of pentachlorophenol (PCP)-4-monooxygenase (PcpB) and associated for bacterial degradation of PCP. Biodegradation 18:525–539

Saeger SD, Sibanda L, Peteghem CV (2003) Analysis of zearalenone and α-zearalenol in animal feed using high-performance liquid chromatography. Anal Chim Acta 487:137–143

Shawna LL, Patrick GG, Timothy DP (1998) Adsorption of zearalenone by organophilic montmorillonite clay. J Agric Food Chem 46:3789–3796

Shen MX, Wu H, Xu Z, Lang YL, Duan LL, Wang LY, Cha XL (2003) Influence of estrogen on the proliferation of MCF7, a human breast carcinorna cell line. China Oncol 13:308–311

Takahashi-Ando N, Tokai T, Hamamoto H, Yamaguchi I, Kimura M (2005) Efficient decontamination of zearalenone, the mycotoxin of cereal pathogen, by transgenic yeasts through the expression of a synthetic lactonohydrolase gene. Appl Microbiol Biotechnol 67:838–844

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Wu F, Munkvold GP (2008) Mycotoxins in ethanol co-products: modeling economic impacts on the livestock industry and management strategies. J Agric Food Chem 56:3900–3911

Yu ZL, Zhang LS, Wu DS (2005) Effects of zearalenone on proliferation and apoptosis in MCF-7 cells. Zhonghua Yu Fang Yi Xue Za Zhi 39:328–331

Zinedinea A, Sorianob JM, Molto JC, Manes J (2007) Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food Chem Toxicol 45:1–18