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Localization of zearalenone detoxification gene(s) in pZEA-1 plasmid of *Pseudomonas putida* ZEA-1 and expressed in *Escherichia coli*

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ABSTRACT

The gene(s) encoding enzyme(s) involved in the initial reaction during degradation of zearalenone (ZEA) was characterized from the zearalenone utilizer *Pseudomonas putida* strain ZEA-1, where ZEA was transformed into product with less or no toxicity. A 5.5 kilobase-pair (kbp) *Pst*1–*Kpn*1 fragment containing gene(s) encoding for zearalenone degradation was cloned. The cloned gene(s) was actively expressed in *Escherichia coli*. ZEA degradation by recombinant *E. coli* was relatively rapid and effective, leaving no detectable ZEA after 24 h. In further experiments, cell-free extract of *E. coli* has been used in the same way, both to confirm these observations and the enzymatic nature of the degradation activity.

1. Introduction

Zearalenone (ZEA) [6-(10-hydroxy-6-oxo-*trans*-1-undecenyil)-resorcylic acid lactone] is a mycotoxin produced by several species of *Fusarium*, namely *Fusarium graminearum* and *Fusarium culmorum*. Zearalenone and its derivatives exert oestrogenic and anabolic effects on mammals [1]. Carryover of zearalenone from infected grains to feedstuff causes reproductive problems in pigs, sheep, and other farm animals. These problems include precocious sexual development, vulva enlargement, pseudopregnancy, loss of embryos, and reduced litter size [2–4]. Zearalenone may cause hyperestrogenism in children when digested with grain-based foods [5]. Zearalenone has been found to be genotoxic and carcinogenic in mice [6–8]. Increasing attention has therefore been paid to the development of an effective strategy for the ZEA decontamination.

Various physico-chemical methods for the treatment of zearalenone have been investigated. These include adsorption [9,10], biodegradation [11–13] and ozonation [14]. Among them, biological treatment is an attractive approach for removing zearalenone. Zearalenone is readily biodegradable and a variety of microorganisms including bacteria, yeasts, and fungi were found to be able to convert zearalenone to α- and β-ZEA. However, according to Karlovsky [15], this transformation cannot be regarded as detoxification since the oestrogenic activity of these metabolites is similar to that of ZEA. Takahashi-Ando et al. [13] identified and characterized a lactonohydrolase enzyme in the fungus *Clonostachys rosea* which converts ZEA to a less oestrogenic compound. The hydrolase responsible for detoxification was purified to homogeneity and its gene (designated *zhd*101) was subsequently isolated [13]. However, no work has been reported on the cloning of bacterial gene especially *Pseudomonas* that encodes zearalenone degradation activity. Recently, we also isolated a *P. putida* ZEA-1 strain, which can rapidly transform/detoxify the zearalenone [16,17]. An enzyme capable of transforming zearalenone and its derivatives, including α- and β-zearalenone, was expressed by genes born on the large plasmid. Here, we report the molecular cloning of the gene(s) that encodes this zearalenone transforming enzyme(s) expression.

2. Materials and methods

2.1. Chemicals

Mycotoxins (zearalenone, α- and β-zearalenol) were purchased from Sigma (St. Louis, MO, USA) and other chemicals were offered from Merck Co. (Darmstadt, Germany) as analytical reagent grade. Bacteriological media were purchased from Difco (Difco Laboratories, Detroit, Michigan) and Oxoid (Oxoid Inc., Nepean, Ontario). Each mycotoxin was dissolved in DMSO (10 mg/mL) and used as a standard stock solution.

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Table 1
Plasmids and bacterial strains used in this study

Strain or plasmids	Characteristics	Source
Bacterial strains		
Pseudomonas putida ZEA-1	Zearalenone degrading soil isolate (ZEA$^+$)	[17]
P. putida ZEA-1	Plasmid-cured derivatives of *P. putida* ZEA-1 (ZEA$^-$)	[16]
Escherichia coli DH5α	*Sup*E44 *delta Lac* U169 (Phi80Lac *delta* M15 *hasd* R17 RecA1 9 yrA 96 *Thi*-1relAt	Gibco, BRL
Escherichia coli BL21 (DE3)	Expression vector host	Novagene
Plasmids		
pUC18	pMB1 replicon, LacZ' Ap^r	[25]
pUC19	Pmb1 replicon, LacZ' Ap^r	[25]
pET5a	Expression vector	Novagene
pZEA-1	Plasmid isolated from *P. putida* ZEA-1, 120 kb	[16]
pZEA-2	8.6 kb *Bam*H1 fragment insert of pZEA-1 insert cloned into pUC19	This study
pZEA-3	8.6 kb *Bam*H1 fragment insert of pZEA-1 cloned into pUC19 in opposite direction	This study
pZEA-4a	5.5 kb *Pst*1–*Kpn*1 fragment insert cloned in pUC19	
pZEA-4b	5.5 kb *Pst*1-*Kpn*1 fragment insert cloned in pUC18	
pZEA-4az	5.5 kb *Pst*1–*Kpn*1 fragment insert cloned from pUC19 into expression vector pET5a	This study
pZEA-4bz	5.5 kb *Pst*1–*Kpn*1 fragment insert cloned from pUC18 into expression vector pET5a	This study
pZEA-5	3.2 kb *Bam*H1–*Xba*1 fragment insert cloned in pUC19	This study
pZEA-6	4.2 kb *Sph*1–*Bam*H1 fragment insert cloned in pUC19	This study
pZEA-7	4.2 kb *Bam*H1–*Sph*1 fragment insert cloned in pUC19	This study
pZEA-8	3.9 *Eco*R1 fragment insert cloned in Puc19	This study

2.2. Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used as the host strain for construction and maintenance of recombinant plasmids containing *ZEA* degrading gene(s). *E. coli* BL21 (DE3) was used for expression of the *ZEA* genes. *E. coli* strains harboring recombinant plasmids were grown at 37 °C in 50 mL LB (Luria Broth) medium (5 g yeast extract, 10 g trypton, and 10 g NaCl) in 1 L distilled water containing ampicillin (Ap) (50 μg/mL). Selecting minimal salt medium (MSM) utilizing 100 μg/mL zearalenone as a sole carbon source contained the following: $(NH4)_2$ SO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2$, 0.05; Na_2HPO_4, 2.44; and KH_2PO_4, 1.52 g/L. For plate cultures, the previous media, solidified with 1.6% (w/v) agar were used. The pH was adjusted to 6.8 and the medium was sterilized prior to the addition of organic substrates.

2.3. Analytical methods

The concentration of zearalenone in the aqueous solutions was determined as described by Utermark and Karlovsky [18] using a high performance liquid chromatograph (HPLC) equipped with a Polaris column, C18-A (5 μm, 150 by nm; Varian, Darmstadt, Germany). A mixture of 55% methanol (55%, v/v), acetonitrile (5%, v/v), and water was used as the mobile phase, and the flow rate was 0.2 mL/min, the column temperature was 40 °C, and detection was done with a diode array detector (absorbance at 200–800 nm). The wavelength corresponding to the maximum absorbance (λ_{max}) of zearalenones was determined by scanning a standard solution of known concentration at different wavelengths. The λ_{max} value, as determined from this plot, was 254 nm. This wavelength was used to prepare a calibration curve between absorbance and zearalenone concentration (given in units of μg/mL) in aqueous solution. The linear region of this curve was further used for the determination of zearalenone concentration of unknown samples. Using a sterilized syringe, 1 mL of aqueous samples were drawn from the reaction mixtures at regular intervals. The samples were then transformed to a centrifuge tube (Eppendorf, Germany) and centrifuged (Biofuse Stratos, Germany) at 10,000 rpm for 10 min at 4 °C and the supernatant was done by extracting with ethyl acetate and removing the solvent on a rotary evaporator. The residue was dissolved in the mobile phase before residual zearalenone analysis.

The degradation products from the reaction mixture or growing cells were extracted twice with an equal volume of chloroform and were developed on a TLC plate (Kieselgel F_{254}, Merck, Darmstadt, Germany) with chloroform/acetone (80:20, v/v). The developed plates were exposed to a UV lamp and the metabolites were recovered from TLC plates by elution with ethyl acetate.

2.4. Preparations, analysis, and cloning of DNA

Plasmid DNA isolation, restriction enzyme digestion, ligation, transformation, and DNA electrophoresis in agarose gels were performed according to standard protocols [19]. Plasmid DNA was isolated by the procedure of Kado and Liu [20] in *Pseudomonas* strains. Briefly, total plasmid DNA, isolated from *P. putida* strain ZEA-1, was digested with restriction endonuclease *Bam*H1 and ligated to the cloning vector, pUC19 [21]. After ligation reaction the successful clones were selected by blue-white screening, while colonies having pUC19 vector with insert were selected and subsequently screened for zearalenone degradation. Several subclones were generated by cloning onto pUC19 or 18 to locate precise region of *ZEA* degradation genes (Table 1). The enzymes *Eco*R1, *Pst*1, *Sph*1, *Xba*1, and *Kpn*1 as well as T4 DNA ligase were used for subcloning of different DNA fragments.

2.5. Biotransformation of zearalenone by crude enzyme of recombinant E. coli

To examine whether the subcloned DNA in plasmid pZEA-4a or pZEA-4b expressed *ZEA* genes by using expression vector the 5.5 kb *Pst*1–*Kpn*1 fragment was cloned into in either possible orientation and create pZEA-4az and pZEA-4bz. Both pET5a constructs were subsequently transformed into *E. coli* BL21 (DE3) for expression. One colony of freshly transformed cells was used to inoculate 2 mL medium containing 50 μg/mL ampicillin and incubated for 8–10 h at 37 °C. Then, 2 mL cell suspension was used to inoculate 50 mL LB medium supplemented with 50 μg/mL ampicillin. Optimal production of recombinant protein (enzyme) was obtained when mid-exponential phase cells (OD_{600} = 0.6) were induced with 0.4 mM IPTG for 5 h at 37 °C. The cells were then harvested by centrifugation (10,000 rpm, 10 min, 4 °C), washed twice with 10 mM Tris–HCl (pH 7.6) and resuspended in the same buffer. The cells were then disrupted with a sonicator and centrifuged at 12,000 × *g*

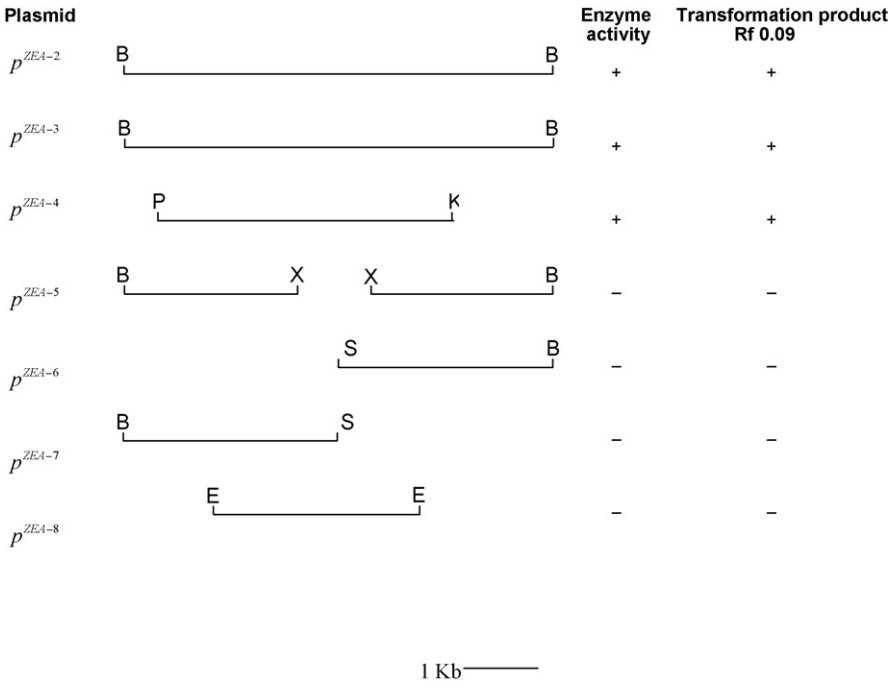

Fig. 1. Cloning of DNA fragments of pZEA-1 in *E. coli*, using the vector pUC19 and localization of the *ZEA* degradation gene(s). pZEA-1 fragments that when ligated into pUC19 indicated on the left. At the right are shown the phenotypes of *E. coli* DH5α harboring these plasmids, +, growth on zearalenone (ZEA) and transform it to a new product (R_f = 0.09); −, no growth. Relevant restriction sites of the pZEA-1 used for cloning: B, *Bam*H1; E, *Eco*R1; P, *Pst*1; X, *Xba*1, and K, *Kpn*1.

for 20 min to obtain the supernatant, which include the crude enzyme fraction. Enzyme assays were initiated by the addition of 100 μL of the crude enzyme fraction to a reaction mixture containing 3.66 mL of 10 mM Tris–HCl (pH 7.5), and an initial ZEA concentration of 100 μg/mL. The mixture was incubated in the dark at 30 °C without shaking for 2, 4, 6, 8, 12 and 24 h for the optimal reaction or incubation time studies. Degradation at different temperatures and pH was also studied to obtain the optimum temperature and pH for the enzyme activity. Temperatures considered were 10, 20, 30, 37 and 50 °C over a period of 24 h at pH 7.0 and pH considered were 5, 6, 7, 8, 9, and 10 over a period of 24 h at 30 °C. All experiments were carried out in duplicates. Each experiment was terminated by the addition of 750 μL ethyl acetate for extraction of the remaining ZEA and analyzed by HPLC.

2.6. Analysis of recombinant plasmid-encoded gene products

The gene products encoded by the *Pst*1–*Kpn*1 fragment of pZEA-2 in pZEA-4za were analyzed in crude cell extracts of *E. coli* BL21 (DE3) containing recombinant plasmid The proteins were separated in SDS-polyacrylamide gels and then stained with Coomassie blue [19].

2.7. Degradation of ZEA and its derivatives by resting cells of recombinant E. coli

After IPTG induction of *E . coli* transformants carrying pZEA-4a or pZEA-4b, the cells were then harvested by centrifugation (10,000 rpm, 10 min, 4 °C), washed twice with 10 mM Tris–HCl (pH 7.6) and resuspended in the same buffer to an OD_{600} of 12–13, mycotoxins were then added to the culture medium at final concentration of 100 μg/mL. The reaction mixture was incubated on reciprocal shaker at 30 °C. the samples were drawn from the reaction mixture at regular intervals and analyzed by HPLC as described above.

2.8. Toxicity bioassay

The toxicity of zearalenones and its degradative products were compared through bioassay against *Artemia salina* based on the method of Harwig and Scott [22]. The final concentration of zeralenone, α-, and β-zeralenone were 100 μg/mL, which were approximately 90% lethal concentration (LD_{90}) for the larvae [16,17]. Groups of larvae were exposed to only toxins in a 96-well microtitration plates and those including toxins treated with the crude enzyme. Groups including only crude enzyme served as negative controls. Brine shrimp were counted by using a 10× objective of a dissecting microscope. One milliliter of acetone was added to each well to kill all of the shrimp in the well and to count the total number of brine shrimp/well. All treatments were carried out in triplicates and all experiments were repeated three times. The plates were kept in growth chamber at 28 °C and the mortality was evaluated at 28 °C. Complete immobility or paralysis was taken as indication for neurotoxic activity towards the larvae.

3. Results and discussion

3.1. The zearalenone-specific transforming enzyme is plasmid encoded

The natural soil isolate *P. putida* strain ZEA-1 has been shown to convert the mycotoxin zearalenone to a compound with low or no toxicity as compared with the parent compound [17]. This compound has specific UV spectra (400 nm) and R_f value of 0.09. Plasmid DNA analysis of *P. putida* strain ZEA-1 cultures grown on zearalenone as sole carbon source indicated the presence of a single large plasmid element [16]. This plasmid was designated as pZEA-1 of approximately 120 kb and is responsible for ZEA degradation. This was based on the fact that subsequent plasmid curing of this strain resulted in the abolishment of ZEA degradation phenotype [16].

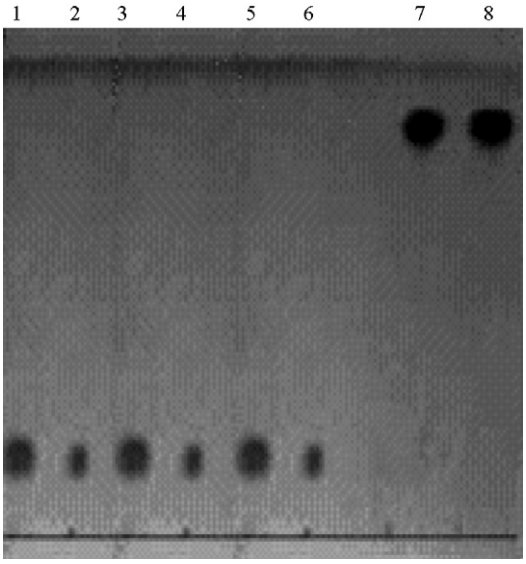

Fig. 2. TLC detection of ZEA degradation in a resting cell system and crude extract. Lane 1, ZEA incubated with resting cells of wild type *P. putida* ZEA-1 (pZEA-1); lane 2, ZEA incubated with resting cells of recombinant *E. coli* DH5α (pZEA-4a), lane 3, ZEA incubated with resting cells of recombinant *E. coli* DH5α (pZEA-4b), lane 4, ZEA incubated with crude extract of *P. putida* (pZEA-1), lane 5, ZEA incubated with crude extract of *E. coli* BL21 (DE3) (pZEA-4az), lane 6, ZEA incubated with crude extract of *E. coli* BL21 (DE3) (pZEA-4bz), lane 7, ZEA Incubated with crude extract of wild type *E. coli* BL21 (DE3) (plasmidless, ZEA$^-$), and lane 8, ZEA standard.

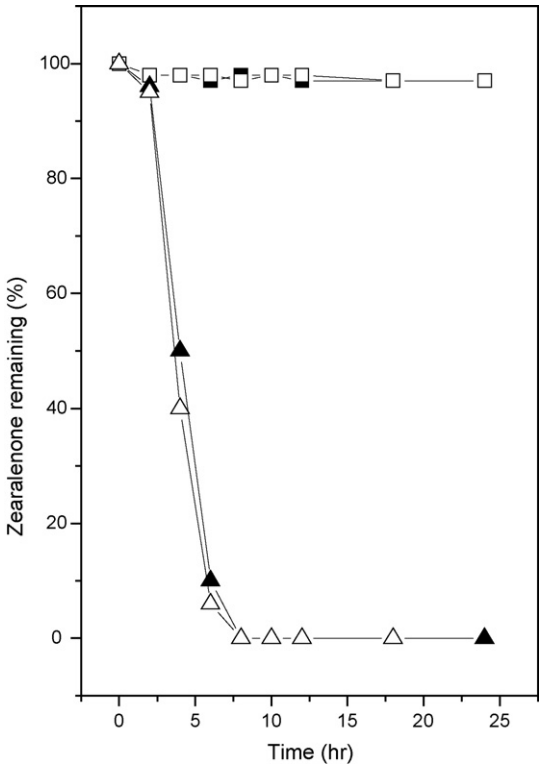

Fig. 3. Zearalenone degradation kinetics observed over a period of 24 h using cell-free extracts of recombinant *E. coli* BL21 (DE3). Cell extracts of *E. coli* BL21 (DE3) (pZEA-4a), (▲); cell extract of wild type *E. coli* BL21 (DE3), (■); cell extracts of *P. putida* ZEA-1 (pZEA-1), (△), and cell extract of *P. putida* ZEA-1M (plasmidless), (□). Cell extracts were obtained in a sonication process and the remaining of ZEA were measured by HPLC at appropriate time.

3.2. Cloning and localization of ZEA degradation genes in pZEA-1

Analysis of recombinant plasmids isolated from the responsible transformed DH5α showed that they all carried a 8.6 kb *Bam*H1 fragment inserted in vector pUC19. One of the plasmid was designated as pZEA-2 (Fig. 1). Plasmid pZEA-3, which is the same as pZEA-2 except that the 8.6 kb fragment is inserted in the opposite direction on the vector, also exhibited the same catabolic activity as pZEA-2 when introduced into *E. coli* DH5α, suggesting that the 8.6 kb fragment carries the ZEA degradation gene(s), as well as their promoter(s).

3.2.1. Restriction mapping of the 8.6 kb BamH1 fragment and further subcloning ZEA degradation genes

A physical map of the 8.6 kb fragment showing *Eco*R1, *Kpn*1, *Pst*1, *Sph*1, and *Xba*1 sites was generated (Fig. 1) and used to develop *ZEA* gene(s) subcloning strategies. By assaying all constructs for the formation of a new product (R_f = 0.09) in the medium, it was suggested that the DNA region conferring *ZEA* degradation gene(s) activity located within a 5.5 kb *Pst*1–*Kpn*1 fragment (Fig. 1). This fragment was cloned into pUC19 and pUC18 to create pZEA-4a and pZEA-4b, in either of the two possible orientations. Both constructs were shown to harbour the genes functional for the conversion of ZEA to a new product with an R_f value of 0.09 in whole cell assay experiment (Fig. 2). This suggests that the promoter of the *ZEA* gene(s) originating from *P. putida* ZEA-1 was expressed well in *E. coli*. It is likely that the DNA sequences representing the gene(s) encoding ZEA catabolism most probably are present within a 5.5 kb *Pst*1–*Kpn*1 segment.

3.3. Degradation of ZEA by cell-free extracts of recombinant E. coli

Zearalenone degradation by cells free extracts of the *E. coli* BL21 (DE3) carrying recombinant plasmid (pZEA-4az) over a period of 24 h was followed, and different levels of degradation were observed (Fig. 3). No ZEA degradation activity was observed with wild type *E. coli* BL21 (DE3). The degradation efficiency of crude extract obtained from *E. coli* BL21 (DE3) containing recombinant plasmid was almost identical to that of *P. putida* ZEA-1 (pZEA-1) during incubation with zearalenone. Also, crude extract from the induced cultures efficiently converted ZEA (R_f = 0.64) into a product with an R_f value of 0.09 (Fig. 2). The control *E. coli* extract (i.e. without the recombinant plasmid) did not yield ZEA conversion product (R_f = 0.09) even after 24 h of incubation with ZEA (Fig. 2). The effect of different temperature and pH on the loss of ZEA by crude protein extracts of recombinant bacterial strain was also evaluated. After an incubation time of 24 h at 10, 20, 30, 37and 50 °C the residual ZEA-content was determined (Fig. 4). The crude enzyme showed an optimum activity at 30–37 °C. These results are in agreement with those obtained by Takahashi-Ando et al. [13], who found that the optimum temperature for ZEA degradation enzyme was 37 °C and it was rapidly inactivated at 50 °C. Solution pH also affected the amount of ZEA degradation by crude protein extracts after 24 h. maximum degradation was observed at pH 7–8 (pH levels: 5, 6, 7, 8, and 10), with some ZEA degradation occurring at pH as low as 5 and as high as 10 (Fig. 5). Similar findings were reported by Smiley and Draughon [23]. However, Takahashi-Ando et al. [13] found that the optimum pH for the same enzyme is 10.5. This difference in the enzyme optimum pH may be attributed to the different enzyme source.

3.4. Expression of cloned genes in E. coli

The synthesis of cloned gene products was analyzed by preparing crude cell extract of *E. coli* containing chimeric plasmid (pZEA-4az)-positive and -negative (pET5a) clones. Subsequently,

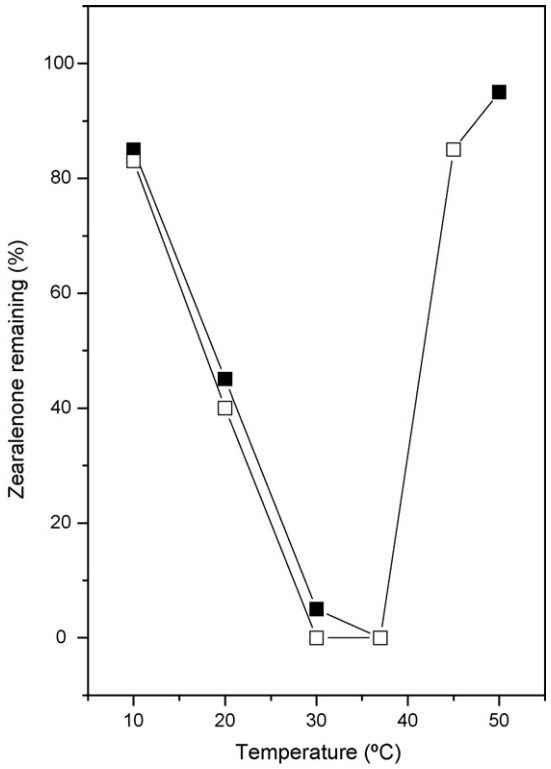

Fig. 4. Zearalenone degradation at different temperatures using cell-free extracts from *E. coli* BL21 (DE3) (pZEA-4), (■), and *P. putida* (pZEA-1), (□). The reaction was carried out at 25, 30, 37, and 50 °C. The remaining of ZEA was measured by HPLC at appropriate time.

the cell extract was then subjected to SDS-polyacrylamide gel electrophoresis. The protein was produced so abundantly in *E. coli* that it could be readily visualized in conventional Coomassie blue-stained SDS-polyacrylamide gels. Fig. 6 (lane 3) shows the protein profile of *E. coli* Bl21 containing the plasmid vector (negative control). The protein synthesized by strain BL21 containing recombinant plasmid after induction with IPTG is shown in Fig. 6 (lane 4). The results shown in Fig. 6 (lane 4) indicate that the cloned DNA (pZEA-4az) expressed at least two peptides with different molecular weight. This variation in peptides could be attributed to a number of gene products that were expressed and involved in ZEA degradation. Evidence of the expression of ZEA gene(s) in *E. coli* either driven by the promoters contained in cloned DNA or through the promoter of plasmid vector indicating that cloned DNA has promoter-like sequence that is recognized by *E. coli* RNA polymerase.

3.5. Degradation of ZEA and its derivatives by recombinant bacteria

Recombinant *E. coli* DH5α cells carrying (pZEA-4a) was tested for their ability to remove ZEA and its derivatives from liquid medium. The wild type *E. coli* metabolized neither ZEA nor its derivatives, and the mycotoxin was fully recovered as non-transformed from the media (Fig. 2), it was not absorbed or adsorbed to bacterial cells (data not shown). On the other hand, an IPTG induced culture of recombinant bacteria carrying pZEA-4a completely removed ZEA and its derivatives from the media (Fig. 7). However, the degradation of α-zearalenol by recombinant *E. coli* was quite inefficient compared with the degradation of ZEA and β-zearalenol (Fig. 7). Takahashi-Ando et al. [13] cloned and characterized a ZEA-detoxifying gene, *zhd*101, from fungus *C. rosea* which remove zearalenone and its derivatives from culture media.

3.6. Bioassay

Mortality of the test organisms is a sensitive endpoint for the assessment of the ecotoxicological risk by the environmental

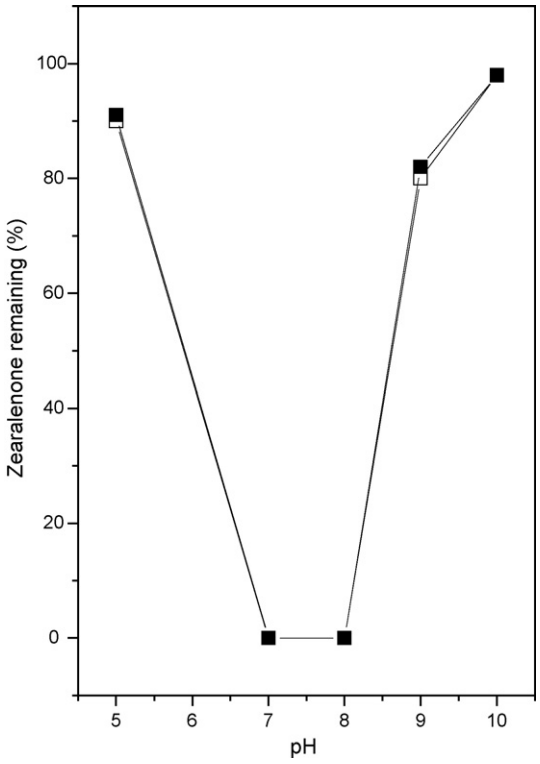

Fig. 5. Zearalenone degradation at different pH using cell-free extracts from *E. coli* BL21 (DE3) (pZEA-4), (■); and *P. putida* (pZEA-1), (□). The remaining of ZEA were measured by HPLC at appropriate time.

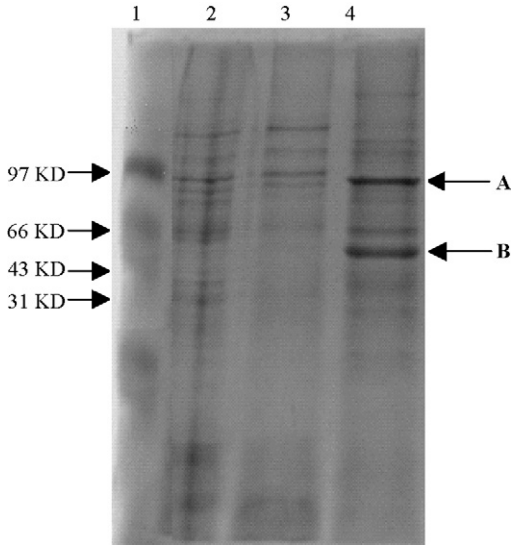

Fig. 6. SDS-PAGE analysis of recombinant proteins expressed in *E. coli* BL21 (DE3). Lane 1, Protein molecular weight standards; lane 2, whole cell lysate of *E. coli* BL21 (DE3) (plamidless); lane 3, *E. coli* BL21 (DE3) carrying pET5a; lane 4, *E. coli* BL21 (DE3) carrying pZEA-4az. The arrows A and B indicate the peptide bands encoded by chimeric plasmid.

Table 2
Percentage survival of *A. salina* larvae exposed to toxin solution

Toxin	Percentage survival of larvae		
	Exposed to toxin for 6 h		Exposed to toxin for 12 h
	No crude[a] enzyme	Crude enzyme[a]	Crude enzyme[a]
Zearalenone 100 μg/mL	11 ± 1	85 ± 3	99 ± 1
α-Zearalenone 100 μg/mL	10 ± 1.5	83 ± 1	96 ± 2
β-Zearalenol 100 μg/mL	12 ± 2	85 ± 4	99 ± 1
Degradative product100 μg/mL	100	100	100
Water	100	100	100

[a] Each value is a mean of three replicates and ± indicates standard deviation among them.

pollutants. Accordingly, the microbial biotransformation-mediated decrease in the toxicity of the test compounds has been taken as an indicator of their biological treatment [24]. This test was made to show that biotransformation of zearalenone and its derivatives lead to metabolites that are nontoxic. In this study, the toxicity of the zearalenone, along with the metabolites formed after their crude extract treatment, was evaluated by using test organism *Artemia salina*. It was observed that the degradation of zearalenone and its isomers was accompanied by a substantial reduction in their toxicity to the test organisms (Table 2). After 6 and 12 h of incubation with crude enzyme, 85 and 100% of larvae survived, respectively (Table 2). This finding suggests that the crude enzyme mediates the metabolism of zearalenone and the formed metabolites are less toxic. Previous reports indicate a variety of microorganisms including bacteria, yeasts and fungi were able to convert zearalenone to α- and β-zearalenol. However, according to Karlovsky [15], this transformation cannot be regarded as detoxification since the oestrogenic activity of these metabolites is similar to that of ZEA. Takahashi-Ando et al. [13] identified and characterized a lactonohydrolase enzyme in the fungus *C. rosea* which converts zearalenone to a less toxic compound. Therefore, it is proposed that the crude enzyme can be used for the bioremediation of zearalenones.

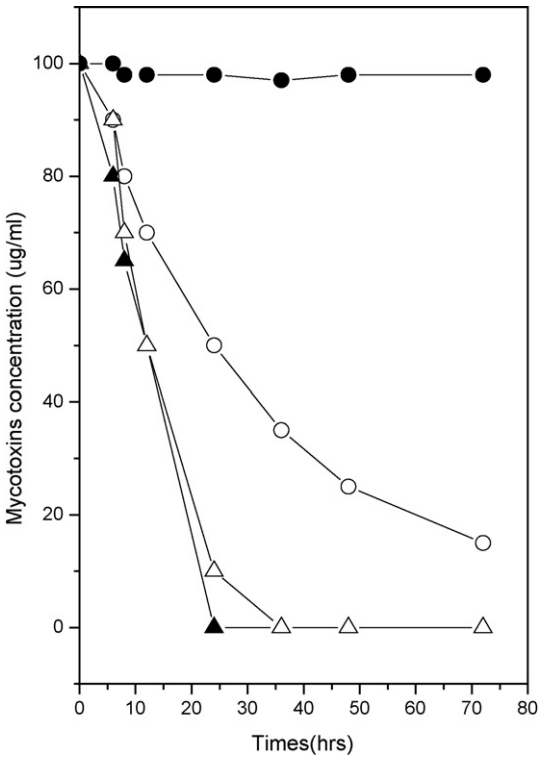

Fig. 7. Degradation of zearalenone and its derivatives by resting cells of recombinant *E. coli* DH5α (pZEA-4). Mycotoxins were included in MSM medium at a concentration of 100 μg/mL.: (▲), ZEA; (○), α-zearalenol; (△), β-zearalenol; and (●), ZEA incubated with no bacteria. The remaining of ZEA were measured by HPLC at appropriate time.

4. Conclusion

Genes specifying degradation of zearalenone in *P. putida* ZEA-1 were studied to understand their localization in this bacterium. This goal was achieved in part by subcloning the *ZEA* genes that specify degradation/detoxification of ZEA from previously reported plasmid pZEA-1. The relative position of *ZEA* genes expressed in *E. coli* and mapped by restriction enzymes analysis was established on 5.5 kb DNA fragment. Our results have demonstrated that cell-free extract of recombinant *E. coli* effectively degrade ZEA and the optimum degradation was occurred at 30–37 °C. Since more than one protein induced through growth of recombinant *E. coli* on zearalenone suggesting that a number of genes products were expressed and involved in ZEA degradation. However, the close link between these gene(s) and the fact that we were unable to separate these gene(s) on the 5.5 kb fragment may indicate that the genes are organized in a complex structure. It is not unusual that degradative genes are clustered in one or more large operons in bacteria [25].

References

[1] J. Fink-Gremmels, H. Malekinejad, Clinical effects and biochemical mechanism associated with exposure to the mycoestrogen zearalenone, Anim. Feed Sci. Technol. 137 (2007) 326–341.
[2] E.R. Farnworth, H.L. Trenholm, The metabolism of the mycotoxin zearalenone and its effects on the reproductive tracts of young male and female pigs, Can. J. Anim. Sci. 63 (1983) 967–975.
[3] M.L. Green, M.A. diekman, J.R. Malayer, A.B. Scheidt, G.G. Long, Effect of prepubertal consumption of zearalenone on puberty and subsequent reproduction of gilts, J. Anim. Sci. 68 (1990) 171–178.
[4] G.G. Long, M.A. Diekman, Characterization of effects of zearalenone in swine during early pregnancy, Am. J. Vet. Res. 47 (1986) 184–187.
[5] P. Szuetz, A. Mesterhazy, G.Y. Falkay, T. Bartok, Early telearche symptoms in children and their reaction to zearalenone contamination in food stuffs, Cereal Res. Commun. 25 (1997) 429–436.
[6] J.E. Coe, K.G. Ishak, J.M. Ward, M.J. Ross, Tamoxifen prevents induction of hepatic neoplasia by zearanol, an estrogenic food contaminant, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 1085–1089.
[7] Y. Grosse, L. Chekir-Ghedira, A. Huc, S. Obrecht-Pflumio, G. Dirheimer, H. Bacha, A. Pfohl-Leszkowicz, Retinol, ascorbic and α-tocopherol prevent DNA adduct formation in mice treated with the mycotoxins ochratoxin A and zearalenone, Cancer Lett. 114 (1997) 225–229.
[8] A. Pfohl-Leszkowicz, L. Chekir-Ghedira, H. Bacha, Genotoxicity of zearalenone, an estrogenic mycotoxin: DNA adduct formation in female mouse tissues, Carcinogenesis. 16 (200) 2315–2320.
[9] A.J. Ramos, E. Hernandez, J.M. Pla-Delfina, M. Merino, Intestinal absorption of zearalenone and in vitro study of non-nutritive sorbent materials, Int. J. Pharm. 128 (1996) 129–137.
[10] D.J. Bueno, L. DiMarco, G. Oliver, A. Bardon, In vitro binding of zearalenone to different adsorbents, J. Food Prot. 68 (2005) 613–615.
[11] S. El-Sharkawy, Y.J. Abul-Hajj, Microbial cleavage of zearalenone, Xenobiotica 18 (1988) 365–371.
[12] M. Megharaj, I. Garthwaite, J.H. Thiele, Total biodegradaation of the oestrogenic mycotoxin zearalenone by a bacterial culture, Lett. Appl. Microbiol. 24 (1997) 329–333.

[13] N. Takahashi-Ando, S. Ohsato, T. Shibata, H. Hamamoto, I. yamaguchi, M. Kimura, Metabolism of zearalenone by genetically modified organisms expressing the detoxification gene from *Clonostachys rosea*, Appl. Environ. Microbiol. 70 (2004) 3239–3245.
[14] K.S. Mckenzie, A.B. Sarr, K. Mayura, R.H. Bailey, D.R. Millar, T.D. Rogers, W.P. Corred, K.A. Voss, R.D. Plattner, L.F. Kubena, T.D. Phillips, Oxidative degradation and detoxification of mycotoxins using a noval source of ozone, Food Chem. Toxicol. 35 (1997) 807–820.
[15] P. Karlovsky, Biological detoxification of fungal toxins and its use in plant breeding, feed and food production, Nat. Toxins 7 (1999) 1–23.
[16] A.D. Altalhi, Plasmid-mediated mycotoxin zearalenone in *Pseudomonas putida* ZEA-1, Am. J. Biotech. Biochem. 3 (2007) 150–158.
[17] B.A. El-deeb, Isolation and characterization of soil bacteria able to degrade zearalenone, J. Bot. 32 (2004) 3–30.
[18] J. Utermark, P. Karlovsky, Role of zearalenone Lactonase in protection of *Gliocladium roseum* from fungitoxic effects of the mycotoxin zearalenone, Appl. Environ. Environ. 73 (2007) 637–642.
[19] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Habor, NY, 1989.
[20] C.I. Kado, T.S. Liu, Rapid procedure for detection of large and small plasmids, J. Bacteriol. 145 (1981) 1365–1373.
[21] C. Yanish-Perron, J. Viera, J. Messing, Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors, Gene 33 (1985) 103–119.
[22] J. Harwig, P.M. Scott, Brine shrimp (*Artemia salina* L.) Larvae as a screening system for fungal toxins, Appl. Microbiol. 21 (1971) 1011–1016.
[23] R.D. Smiley, F.A. Draughon, Preliminary evidence that degradation of Aflatoxin B1 by *Flavobacterium aurantiacum* is enzymatic, J. Food Protect. 63 (2004) 415–418.
[24] H. Lenke, J. Warrelmann, G. Daun, K. Hund, U. Siegln, U. Walter, H.J. Knackmuss, Biological treatment of TNT-contaminated soil 2. Biological induced immobilization of the contaminants and full-scale application, Environ. Sci. Technol. 32 (1998) 1964–1971.
[25] J.M. Horn, S. Harayama, K.N. Timmis, DNA sequence determination of the TOL plasmid (pWWO) *xylIGFJ* genes of *Pseudomonas putida*: implication for the evolution of aromatic catabolism, Mol. Microbiol. 5 (1991) 2459–2474.