

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

Altalhi, A.D. and El-Deeb, B.*

Taif University, Faculty of Science, Biological Science Department, P.O.888, Taif, Kingdom of Saudi Arabia

E-mail: bahig56@yahoo.com

ABSTRACT

The gene(s) encoding enzyme(s) involved in the initial reaction during degradation of zearalenone was characterized from the zearalenone utilizer *Pseudomonas putida* strain ZEA-1, in which ZEA transformed into product with less or not toxic. A 5.5 kilobase-pair (kbp) Pst1-Kpn1 fragment containing gene(s) coding for zearalenone degradation was cloned. The cloned gene(s) activity was expressed in *Escherichia coli*, and ZEA degradation by recombinant *E. coli* was relatively rapid and effective, leaving no detectable ZEA after 72 h. In further experiments, cell-free extract of *E. coli* have been used in the same way, both to confirm these observations and the enzymatic nature of the degradation activity.

Keywords: DNA Cloning, *E. coli*, Zearalenone, biotransformation, *Pseudomonas sp.* *Artemia salina*

INTRODUCTION

Zearalenone (ZEA) [6-(10-hydroxy-6-oxo-trans-1-undecenyil)-resorcylic acid lactone] is a mycotoxin produced by several species of *Fusarium*, most notably *Fusarium graminearum* and *F. culmorum*. Zearalenone and its derivatives exert estrogenic and anabolic effects on mammals. Carryover of zearalenone from infected grain to feedstuff causes reproductive problems in pigs, sheep, and other farm animals including precocious sexual development, vulva enlargement, pseudopregnancy, loss of embryos, and reduced litter size (Farnworth and Trenholm, 1983; Long and Diekmann, 1986; Green *et al.*, 1990). When digested with grain-based food, zearalenone may cause hyperestrogenism in children (Szuetz *et al.*, 1997). A part from its estrogenicity, zearalenone is genotoxic in mice and is a suspected carcinogen (Coe *et al.*, 1992; Grosse *et al.*, 1990; Pfohl-Leschkowicz *et al.*, 1995). Hence; increasing attention has been paid to the development of an effective strategy for the decontamination of ZEA. Although numerous physical and chemical detoxification methods have been tested, none really fulfills the necessary efficacy, safety, and cost requirements (Ruy *et al.*, 2002). Therefore, the development of biological detoxification measures, especially in traditionally developed countries fermented food products, is essential to improve the safety of these foods for human consumption (Sweeney and Dobson, 1999). Several studies based on adsorption to appropriate media are currently being developed. For example, food grade *Lactobacillus* strains were useful for removing a certain amount of ZEA by binding to the cell surface (El-Nezami *et al.*, 2002). However, other tools are also needed for the development of a more efficient and reliable method.

Enzymatic detoxification could be an efficient method for ZEA detoxification (Megharaj, 1997; Varga, 2005; Smiley and Draughon, 2004; Utermark and

Karlovsy, 2007). Kakeya *et al.* (2002) previously found that ZEA is converted to a far less estrogenic product on incubation with a fungal strain of *Clonostachys rosea*. The hydrolase responsible for detoxification was purified to homogeneity and its gene (designated Zhd101) was subsequently isolated (Takahashi-Ando *et al.*, 2002, 2004). Recently, we isolated a *Pseudomonas putida* ZEA-1 (pZEA-1) strain in our laboratory, which can rapidly transform/detoxify the zearalenone. An enzyme capable of transforming a zearalenone and its derivatives, including α -zearalenone and β -zearalenone was expressed by genes borne on the large plasmid. We now report the molecular cloning of the gene(s) that encodes this zearalenone transforming enzyme(s).

MATERIALS AND METHODS

Mycotoxin, Zearalenone, α -zearalenol and β -zearalenol were purchased from Sigma Chemical Company (St. Louis, MO). All chemicals were at least of analytical-reagent. Each mycotoxin was dissolved in DMSO (10mg/mL) and used as a standard stock solution.

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used are listed in Table 1. *E. coli* DH5 α was used as the host strain for construction and maintenance of recombinant plasmids containing zea degradation genes. *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) 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 μ g/mL). Selection minimal salt medium (MSM) utilizing 100 μ g/ml zearalenone as a sole carbon source contained the following gram per liter: (NH₄)₂SO₄, 0.5; MgSO₄·7H₂O 0.2; CaCl₂, 0.05; Na₂HPO₄, 2.44, and KH₂PO₄, 1.52. For

plate cultures, the media mentioned above, solidified with 1.6% (wt/vol) agar, were used. The pH was adjusted to

6.8 and the medium was sterilized prior to the addition of organic substrates.

Table 1: Bacterial strains and plasmids used in this study

Strain or Plasmids	Characteristics	Source
Bacterial strains		
<i>P. putida</i> ZEA-1	Zearalenone degrading soil isolate (zea ⁺)	(El-Deeb,2005)
<i>P. putida</i> ZEA-1	Plasmid-cured derivatives of <i>P. putida</i> ZEA-1 (zea ⁻)	Altalhi, 2007
<i>E. coli</i> DH5α	<i>SupE44 delta Lac U169 (Phi80Lac delta M15 hasd R17 RecA1 9 yrA 96 Thi-1relAt</i>	Gibco,BRL
<i>E. coli</i> BL-21(DE3)	Expression vector host	Noagene
Plasmids		
pUC18	pMB1 replicon, LacZ' Ap ^r	32
pUC19	pMB1 replicon, LacZ' Ap ^r	32
pET5a	Expression vector	Novagene
pZEA-1	Plasmid isolated from <i>P. putida</i> ZEA-1, 120 kb	Altalhi,2007
pZEA-2	8.6 kb BamH1 fragment insert of pZEA-1 insert cloned into pUC18	This study
pZEA-3	8.6 kb BamH1 fragment insert of pZEA-1 cloned into pUC19	This study
pZEA-4	5.5-kb Pst1-Kpn1 fragment insert cloned in pUC18 or pUC19	This study
pZEA-4a	5.5-kb Pst1-Kpn1 fragment insert cloned from pUC18 into expression vector pET5a	This study
pZEA-4b	5.5-kb Pst1-Kpn1 fragment insert cloned from pUC19 into expression vector pET5a	This study
pZEA-5	3.2-Kb BamH1-Xba1 fragment fragment insert cloned in pUC18 or pUC19	This study
pZEA-6	4.2-Kb Sph1-BamH1 fragment fragment insert cloned in pUC18 or pUC19	This study
pZEA-7	4.2 Kb BamH1-Sph1 fragment fragment insert cloned in pUC18 or pUC19	This study
pZEA-8	3.9 EcoR1 fragment fragment insert cloned in pUC18 or pUC19	This study

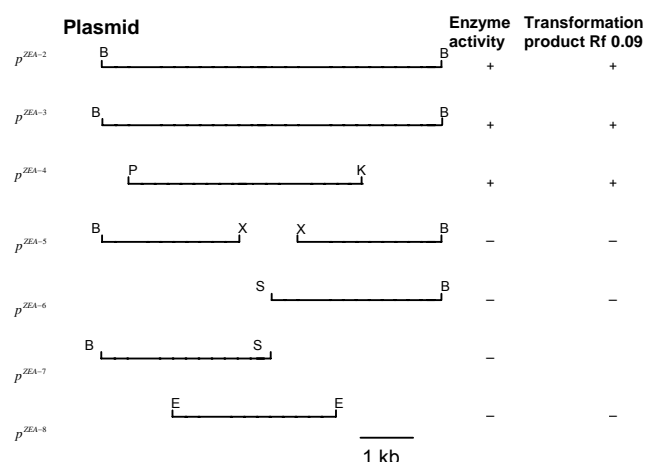


Figure 1. Cloning of DNA fragments of pZEA-1 in *E. coli*, using the vector pUC19 or-18 and localization of the zea-degradation gene(s). pZEA-1 fragments that when ligated into pUC19-or 18 indicated on the left. At the right are shown the phenotypes of *E. coli* DH5α harboring these plasmids, +, Growth on zearalenone (ZEA) and transform it to a new product (Rf 0.09); -, no growth. Relevant restriction sites of the pZEA-1 used for cloning: B, BamH1; E, EcoR1; P, Pst1; X, Xba1, and K, Kpn1.

Analysis of zearalenones

Concentration of zearalenone in culture broth was evaluated by high-performance chromatograph analysis as described by (Utermark and Karlovsky, 2007). Reverse-phase high-performance liquid chromatography (HPLC) with a diod array and mass spectrometric detectors was used. The column was a Polaris C18-A (5 μ m, 150 by nm; Varian, Darmstadt, Germany), the mobile phase was 55% methanol-45% water-5% acetonitrile, the flow rate was 0.2 mL/min, the column temperature was 40 °C, detection was done with a diod array detector (absorbance at 200 to 800 nm), and mass spectrometry was done by electrospray in positive mode (scan for a mass/charge ratio of 250 to 500). Preparation of sample was done by extracting culture supernatants with ethyl acetate and removing the solvent on a rotary evaporator. The residue was dissolved in the mobile phase. A five point calibration curve (0.05; 0.1; 1.0; 10.0 and 20.0 ng/mL) was prepared with standards of zearalenones (Sigma) and regularly checked. The degradation of zearalenone was quantified in equivalents of zearalenone using the previous calibration curve.

Thin layer chromatography (TLC) was used for qualitative analysis of zearalenone metabolites. Merk silica gel 60 F254 plates (thickness, 0.25 nm) were developed with solvent system (chloroform/acetone (80:20 vol/vol)). Metabolites were visualized under irradiation and the major metabolites were recovered from TLC plates by elution with ethyl acetate.

Preparations, analysis, and cloning of DNA

Plasmid DNA was isolated by the procedure of Kado and Liu (1981) in *Pseudomonas* strains and by Maniatis *et al.* (1982) in *E. coli*. Cloning and analysis of clones were carried out as previously described (Sambrook *et al.*, 1989), with specific procedures described below.

Total plasmid DNA, isolated from *Pseudomonas putida* strain ZEA-1, was digested with restriction endonuclease BamH1 and ligated to the positive selection cloning vector, pUC19 (Yanish-Perron *et al.*, 1985). Recombinant plasmids were used to transform *E. coli* DH5 α , which was then spread on ampicillin-LB agar plates. Following overnight incubation at 37 °C, transformants were picked and subsequently screened for inserted fragments and zearalenone degradation. Several subclones were generated by cloning onto pUC19 or 18 to locate precise region of zea degradation genes. The enzymes EcoR1, Pst1, Sph1, Xba1, and Kpn1 and T4 DNA ligase were used for subcloning of different DNA fragments.

Biotransformation of Zearalenone by crude enzyme of recombinant *E. coli*

Expression studies were carried out using *E. coli* BL-21 (DE3) carrying pZEA-4a. One colony from freshly transformed cells was used to inoculate 2 mL medium containing 50 μ g/mL ampicillin. Optimal production of

recombinant protein (enzyme) was obtained when mid-exponential phase cells (OD₆₀₀ = 0.6) were induced with 0.4 mM IPTG or ZEA (100 μ g/mL) for 5 h at 37 °C. Cell of *Pseudomonas putida* strain ZEA-1 (pZEA-1), grown in LB or Zea, were harvested in a similar manner. Then the cells were harvested by centrifugation (5,000 x g, 10 min, and 4 °C), washed twice with 10 mM Tris-HCl (pH 7.6) and resuspended in 0.01 volume of the same buffer. The cells were then disrupted with a sonicator and centrifuged at 12,000 x g for 20 min to obtain the supernatant (crude enzyme fraction). Enzyme assays were initiated by the addition of 100 μ L of the crude enzyme fraction to a reaction mixture containing 3.66 mL of 10 mM Tris-HCl (pH 7.5), 200 μ L of zearalenone in 50 mL ethanol (100 μ g/mL). The mixture was incubated in the dark at 30 °C without shaking. At selected times, samples were centrifuged at 10,000 x g for 5 min, filtered through 0.2 μ m-pore-size Millex filter (Millipore, Bedford, Mass), and placed in vials for subsequent TLC and HPLC analysis as described above. Activities of crude enzyme were determined by monitoring both degradation of substrate and production of the transformed compound.

The expression of target gene was assessed by analysis of total cell protein by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) followed by Coomassie blue staining.

Degradation at different temperatures and pH was also studied to obtain the optimum temperature and pH for the enzyme. Temperatures considered were 10 °C, 20 °C, 30 °C, and 40 °C over a period of 20 h at pH 7.0 and pH considered were 5, 7, 8, 9, and 10 over a period of 20 h at 30 °C. All experiments were carried out in duplicates. Each experiment was terminated by the addition of 750 μ L ethyl acetate for extraction of the remaining ZEA and analysis by HPLC.

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

DH5 α harbouring pZEA-4a or pZEA-4b was cultivated in 5 mL of LB supplemented with Ap at 37 °C with reciprocal shaking (120 rpm), and then 100 μ L of the culture was transferred to 100 mL of the same medium. After cultivation at 30 °C with shaking at 120 rpm until the optical density at 600 nm (OD₆₀₀) reached 0.4 to 0.5, isopropyl-1-thio- β -D-galactopyranoside [IPTG] was added to 0.5 mM and the cells further cultivated at 30 °C for 15 h. Then the cells were harvested by centrifugation (5,000 x g, 10 min, 4 °C), washed twice with 10 mM Tris-HCl (pH 7.6) and resuspended in the same buffer to an OD₆₀₀ of 12 to 13. Mycotoxins (ZEA, α -zearalenol, or β -zearalenol) were added to 5 mL cell suspension at a final concentration of 100 μ g/mL. The reaction mixture was incubated on reciprocal shaker at 30 °C. At selected times, samples were centrifuged at 10,000 x g for 5 min, filtered through 0.2 μ m-pore-size Millex filter (Millipore, Bedford, Mass), and placed in vials for subsequent analysis. The culture supernatants were acidified, extracted with ethyl acetate and then analyzed by TLC. The remaining zearalenone was also extracted from the

supernatants and quantified by HPLC as described previously.

Toxicity Bioassay

The toxicity of zearalenones and its degradative products were compared by bioassay against *Artemia salina* based on the method of Harwig and Scott (1971). In the bioassays, groups of 10 to 20 larvae were exposed to a range of concentrations of different zearalenone derivatives in a 96-well microtitration plates. Stock concentration of toxin was prepared as described above. The final concentration of zearalenone, α -zearalenol, and β -zearalenone were 100 $\mu\text{g/mL}$, which were approximately 90% lethal concentration (LC90) for the larvae. The toxin solutions were then treated with crude enzyme. Groups with only toxins solution in wells served as negative controls, and those with only crude enzyme served as positive control. All treatments were in triplicate and all experiments were repeated three times with plates being in grown at 28 °C and mortality being evaluated at 28 °C. Complete immobility or paralysis was taken as indication for neurotoxic activity towards the insect larvae.

RESULTS AND DISCUSSION

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 not toxicity as compared with the parent compound (El-Deeb, 2004). This compound has specific UV spectra (400 nm) and R_f value of 0.09. Plasmid DNA analysis of *Pseudomonas* sp. cultures grown on 100 $\mu\text{g/mL}$ zearalenone as sole carbon source indicated the presence of a single large plasmid element (Altalhi, 2007). This plasmid was designated as pZEA-1 of approximately 120 kb and found to be responsible for ZEA degradation. This was based on the fact that the plasmid cured strain showed ZEA⁻ phenotype and the ZEA⁺ phenotype could be conjugally transferred (Altalhi, 2007).

Cloning and localization of ZEA degradation gene in pZEA-1

Total plasmid DNA isolated from strain ZEA-1 was digested with restriction enzyme BamH1 and ligated to the positive selection cloning vector, pUC19. Recombinant plasmids were used to transform *E. coli* DH5 α , which was then spread on ampicillin-LB agar plates, whereby 1500 colonies were picked and screened for the insert fragment and ZEA degradation in resting-cell assays. Analysis of plasmids isolated from the responsible transformed DH5 α showed that they all carried 8.6 kb BamH1 fragment inserted in vector pUC19. One of the plasmid was designated pZEA-2 (Figure 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* DH5 α , suggesting that the 8.6 kb fragment carries the zea degradation gene(s), including their promoter(s).

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 EcoR1, Kpn1, Pst1, Sph1, and Xba1 sites was generated (Figure 1) and used to develop zea gene(s) subcloning strategies. Various fragments were subcloned in pUC19, transferred in *E. coli* DH5 α and the transformants were tested by resting-cell assays for their ability to convert zearalenone. By assaying all constructs for the formation of the new product (R_f 0.09) in the medium, it was postulated that the DNA region conferring zea degradation gene(s) activity was found to be located within a 5.5 kb Pst1-Kpn1 fragment (Figure 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, pZEA-4a and pZEA-4b were transformed *E. coli* DH5 α and screened for the insert fragment and ZEA degradation activity in resting-cell assays. 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 (Figure 2). This implies that the promoter of the zea gene(s) originating from *P. putida* ZEA-1 is expressed well in *E. coli* strains. This established that the DNA sequences representing the gene(s) encoding ZEA catabolism would in all probability be present within a 5.5 kb Pst1-Kpn1 segment.

To examine whether the subcloned DNA in plasmid pZEA-4a or pZEA-4b expressed zea genes by using expression vector pTE5a, the 5.5 kb Pst1-Kpn1 fragment was cloned into pTE5a in either possible orientation and create pZEA-4az and pZEA-4bz. Both recombinant plasmids were transferred into *E. coli* DH5 α and the transformants were tested by resting-cell assays for the ability to convert zearalenone into a new compound. Both pET5a constructs were subsequently transferred into *E. coli* BL21 (DE3) for expression.

Expression of cloned gene in *E. coli*

Expression studies were carried out using *E. coli* BL-21 (DE3) carrying recombinant plasmid described above. After induction with IPTG or ZEA, crude cell extracts from *P. putida* strain ZEA-1 (pZEA-1) and from *E. coli* BL-21 (DE3) transformed with pZEA-4az or pZEA-4bz were evaluated for their ability to remove zearalenone. The results in Figure 3 show that the degradation efficiency of crude extract obtained from *E. coli* BL-21 (DE3) containing (pZEA-4az or pZEA-4bz) was almost identical to that of *Pseudomonas* sp. strain ZEA-1 (pZEA-1) during incubation with zearalenone. No ZEA degradation activity was observed with wild type *E. coli* BL-21(DE3) (Figure 2). Also, crude extract from *P. putida* strain ZEA-1 and

from *E. coli* BL-21 (DE3) (pZEA-4az or pZEA-4bz) converted ZEA ($R_f = 0.64$) into a product that had an R_f value of .09 (Figure 2). Under the same condition, extracts from the control *E. coli* (i.e. without the recombinant plasmid) did not show ZEA conversion activity (Figure 2). 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 pTE5a was obtained by cloning of Pst1-Kpn1 fragment of plasmid pZEA-2 in expression pET5a vector in either orientation, indicating that cloned DNA has promoter-like sequence that are recognized by *E. coli* RNA polymerase

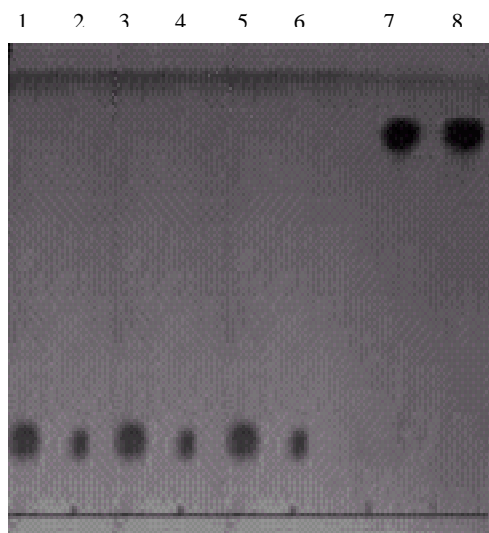


Figure 2: TLC detection of ZEA degradation in a resting cell system and crude extract. Lane 1, ZEA incubated with resting cells of wild type *Pseudomonas* sp. ZEA-1 (pZEA-1); lane 2, ZEA incubated with resting cells of recombinant *E. coli* DH5 α (pZEA-4a), Lane 3, ZEA incubated with resting cells of recombinant *E. coli* DH5 α (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.

Subsequently, the cell extract were then subjected to SDS-polyacrylamide gel electrophoresis. The protein was produced so abundantly in *E. coli* that could be readily visualized in conventional Coomassie blue-stained SDS-polyacrylamide gels. Figure 4 (lane 2) shows the protein profile of *E. coli* BL-21 containing the plasmid vector pTE5a (negative control). The protein synthesized by

strain BL-21 containing recombinant plasmid is shown in Figure 4 (lane 3). The results shown in Figure 4 (lane3) indicate two polypeptides with different molecular weight. This variation in peptides could be attributed to a number of gene products were expressed and involved in zea degradation.

The effect of different temperature and pH on the loss of ZEA by crude protein extracts of recombinant bacterial strain was also evaluated. After incubation time of 24 h at 10, 20, 30, 37 and 50 °C, the residual ZEA-content were determined as shown in Figure 5. The recombinant strain of *E. coli* showed an optimum at 30 - 37 °C. These results are in agreement with results obtained by Takahashi-Ando *et al.* (2004) 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 degraded 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 (Figure 6) (Smiley and Draughon, 2004). In contrast to our results, Takahashi-Ando *et al.* 2004 found that the optimum pH for the same enzyme is 10.5. This difference in the nature of enzyme activity towards pH may be attributed to the different source of enzyme.

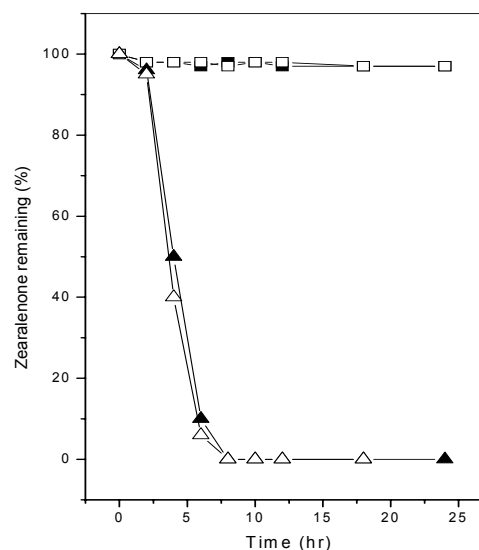


Figure 3: Zearalenone degradation kinetics observed over a period of 24 h using cell-free extracts of recombinant *E. coli* BL21 (DE3). Cell extracts of *E. coli* BL21 (DE3) (pZEA-4a), (\blacktriangle); Cell extract of wild type *E. coli* BL21 (DE3), (\blacksquare); Cell extracts of *P. putida* ZEA-1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

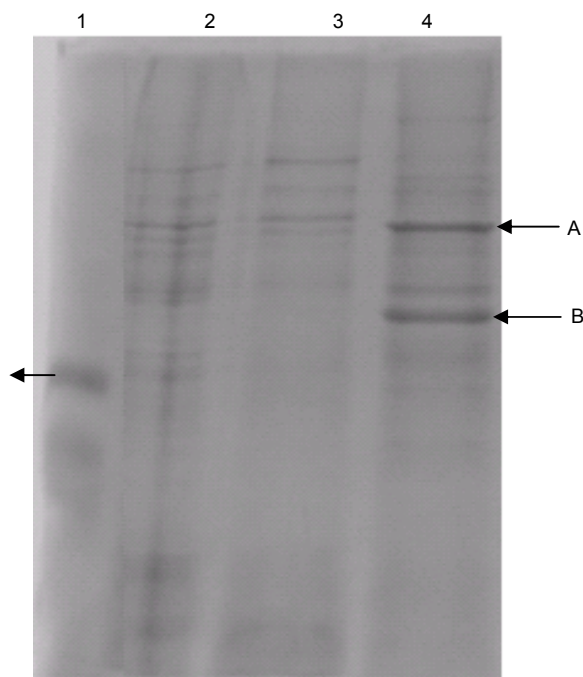


Figure 4: SDS PAGE image exhibiting various protein profiles. Lane 1, size marker; lane 2, *E. coli* BL-21 containing plasmid vector PTE5a (negative control); lane 3, *E. coli* BL-21 containing recombinant plasmid

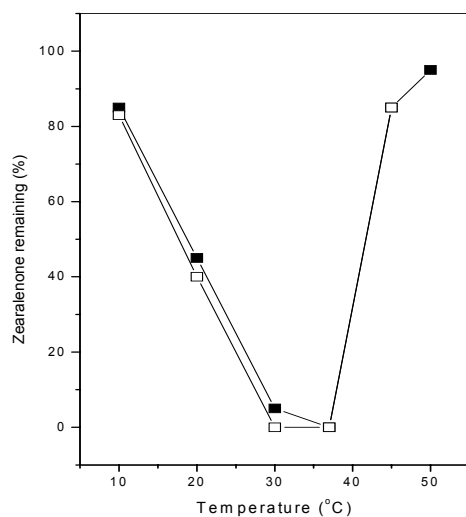


Figure 5: Zearalenone degradation at different temperature using cell free extracts from *E.coli* BL21 (DE3) (pZEA-4), (■), and *P. putida* (pZEA-1), (□). The reaction was carried out at 25 °C, 30 °C, 37 °C, and 50 °C. The remaining of ZEA was measured by HPLC at appropriate time.

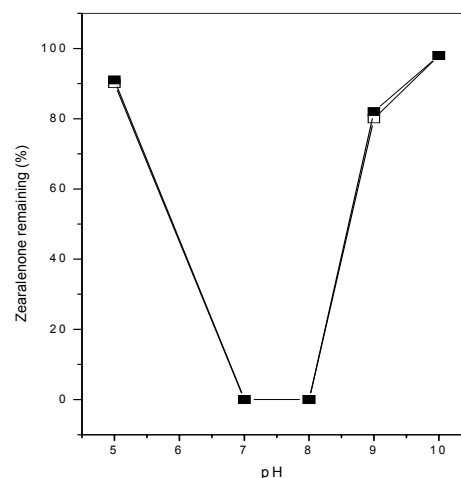


Figure 6: Zearalenone degradation at different pH using cell free extracts from *E. coli* BL21 (DE3) (pZEA-4), (■), and *P. putida* (pZEA-1), (□). The remaining of ZEA were measured by HPLC at appropriate time.

Detoxification of ZEA and its derivatives by recombinant bacteria

Recombinant *E. coli* DH5 α cells carrying (pZEA-4a) was tested for their ability to remove ZEA and its derivatives from liquid medium (Figure 7). Wild-type or recombinant *E. coli* cells grown in presence of IPTG were supplemented with 100 μ g of zearalenone, α -zearalenol, or β -zearalenol per mL. The wild type bacteria metabolized neither ZEA nor its derivatives, and the mycotoxins were fully recovered as nontransformed from the media (Figure 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-4 completely removed ZEA and its derivatives from the media. However, the degradation of α -zearalenol by recombinant *E. coli* was quite inefficiently compared to the degradation of ZEA and β -zearalenol. From TLC analysis of supernatant, we were able to detect the degradation product of ZEA with an R_f of 0.09 (Figure 2). Takahashi-Ando *et al.* (2002) cloned and characterized a ZEA-detoxifying gene, *zhd101*, from fungus *Clonostachys rosea* which convert ZEA to nonestrogenic product and remove zearalenone and its derivatives from culture media.

Bioassay

The bioassay was performed three times in triplicate. The results shown in Table 2 represents one of the experiments. Crude enzyme was highly efficient in degrading toxin-allowing survival of larvae at low concentration (0.1 mg cell protein). Mortality of the test organisms is a sensitive end-point for the assessment of

the ecotoxicological risk by the environmental pollutants. Accordingly, the microbial biotransformation mediated decrease in the toxicity of the test compounds has been taken as an index of their biological treatment (Lenke *et al.*, 1998). In this study, the toxicity of the residual zearalenone, along with the metabolites formed after their crude extract treatment, was evaluated by using test organism *A. 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 to 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 (Kakeya *et al.*, 2002). Accordingly, the crude enzyme can be used for the bioremediation of zearalenones.

Table 2: Approximate survival rates of *A. salina* larvae exposed to toxin solution

Toxin	Percentage survival of larvae		
	Control	Exposed to toxin for 6 h	Exposed to toxin for 12 h
		Enzyme	enzyme
Zearalenone 100 µg/ml	10	85	100
α-zearalenone 100 µg/ml	10	82	100
β-zearalenol 100 µg/ml	10	85	100
Degradative product 100 µg/ml	100	100	100
water	100	100	100

CONCLUSIONS

Genes specifying degradation of zearalenone in *P. putida* ZEA-1 was studied for the purpose of understanding their localization in this bacterium. This goal was partially achieved by subcloning the zea genes that specify degradation/detoxification of ZEA from previously reported plasmid pZEA-1 (Altalhi, 2007). The relative position of zea genes expressed in *E. coli* and mapped by restriction enzymes analysis, and the bound aeries of DNA encoding the zea genes were established on 5.5-Kb DNA fragment (Figure 1). Takahashi-Ando *et al.* (2002) reported similar finding of zea gene cloned from fungus *Clonostachys rosea* and expressed in *E. coli*. However, the size and restriction map of *zhd101* differed from cloned DNA fragment of the *P. putida* ZEA-1.

Evidence of the expression of zea genes in *E. coli* either driven by the promoters contained in the cloned DNA or through the promoter of expression vector was obtained by cloning the 5.5 kb Pst1-Kpn1 fragment of plasmid pZEA-2 in expression vector pTE5a. The resulting plasmid, pZEA-4az or pZEA-4bz, expressed zea

genes in either orientation, indicating the cloned DNA fragment has promoter-like sequence that are recognized by *E. coli* RNA polymerase.

Comparison of the degradation product by zea genes, which has R_f value of 0.09 and UV maxima spectra at 400 nm (Altalhi, 2007) with a product (R_f 0.2) produced by *zhd101* gene (Takahashi-Ando *et al.*, 2002) may be attributed to further zearalenone degradation by zea genes than only cleavage of lactone ring by *zhd101* enzyme (Takahashi-Ando *et al.*, 2002). Since more than one proteins induced through growth of recombinant *E. coli* on zearalenone, this suggests 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 (Horn *et al.*, 1991). It has also been suggested that a few common self-transmissible ancestor replicon were involved in the acquisition and spread of different catabolic modules in the environment (van der Meer *et al.*, 1992).

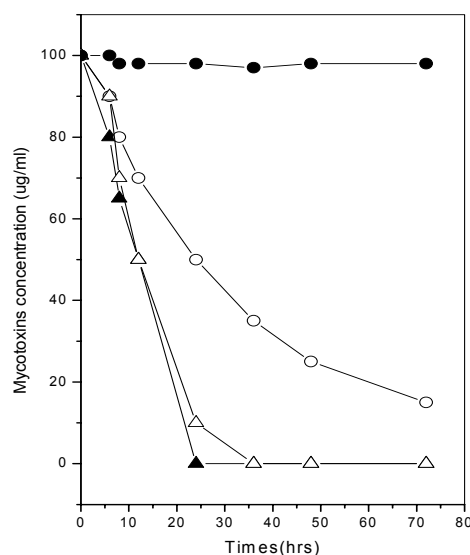


Figure 7: Degradation of zearalenone and its derivatives by resting cells of recombinant *E. coli* DH5α (pZEA-4). Mycotoxins were included in MSM medium at a concentration of 100 µg/mL: (▲), ZEA; (○), α-zearalenol; (△), β-zearalenol; and (●), ZEA incubated with no bacteria. The remaining of ZEA were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

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