

# New Biotransformation Mode of Zearalenone Identified in *Bacillus subtilis* Y816 Revealing a Novel ZEN Conjugate

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**ABSTRACT:** An increasing number of *Bacillus* strains have been identified, and the removal capacity of zearalenone (ZEN) was determined; however, they failed to reveal the detoxification mechanism and transformation product. Here, *Bacillus subtilis* Y816, which could transform 40 mg/L of ZEN within 7 h of fermentation, was identified and studied. First, the biotransformation products of ZEN and 17- $\beta$ -estradiol (E2) were identified as ZEN-14-phosphate and E2-3-phosphate by HPLC-TOF-MS and NMR, respectively. An intracellular zearalenone phosphotransferase (ZPH) was found through transcriptome sequencing analysis of *B. subtilis* Y816. The phosphorylated reaction conditions of ZEN by ZPH were further revealed in this work. Furthermore, the phosphorylated conjugates showed reduced estrogenic toxicity compared with their original substances (ZEN and  $\alpha/\beta$ -zearalenol) using an engineered yeast biosensor system. The first report on the phosphorylated conjugated mode of ZEN in *B. subtilis* Y816 will inspire new perspectives on the biotransformation of ZEN in *Bacillus* strains.

**KEYWORDS:** ZEN-14-phosphate, E2-3-phosphate, biotransformation, zearalenone phosphotransferase, estrogenic toxicity

## INTRODUCTION

Zearalenone (ZEN), 6-(10-hydroxy-6-oxo-trans-1-undecenyl)- $\beta$ -resorcylic acid lactone, is one of most widely distributed nonsteroidal estrogenic mycotoxins biosynthesized by *Fusarium* species (*Fusarium graminearum*, *Fusarium culmorum*, *Fusarium equiseti*, and *Fusarium crookwellense*).<sup>1,2</sup> Neurotoxicity, teratogenesis, immunosuppression, estrogenic, and even carcinogenic effects might occur after consuming products with ZEN contamination.<sup>3,4</sup> ZEN contamination easily occurs in cereal and cereal products, which is a threat to human health and great economic loss in livestock farming. From 2016 to 2018, 405 samples of corn, corn products, and swine feed from China were surveyed for ZEN contamination.<sup>4</sup> The positive rates of ZEN contamination were 40.6% in 133 test samples, 24.5% in 143 test samples, and 31.8% in 129 test samples, respectively.<sup>4</sup> The average ZEN content of the contaminated samples was above 157.0 ppb.<sup>4</sup> Grains can be contaminated in the field, during harvest, and at processing and storage of agricultural products.<sup>4</sup>

Effective methods to control and eliminate ZEN are urgently needed. Biodegradation has been proved to be the most efficient method due to its high efficiency, safety, and low cost and no pollution.<sup>5</sup> Microbial ZEN-degrading enzymes provide the best and most feasible method for ZEN detoxification.<sup>6</sup> In 2002, Takahashi-Ando et al.<sup>7</sup> isolated a ZEN-degrading enzyme encoding gene from *Clonostachys rosea* IFO 7063, annotated as zhd101, and its encoding products could specifically cleave the lactone ring of ZEN. After that, scientists also expressed the zhd101 gene in different hosts (*Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris*) and investigated its activity.<sup>8,9</sup> Recently, a novel ZEN-degrading enzyme Zhd518 with 65% amino acid identity with Zhd101

was reported for the degradation not only of ZEN but also of ZEN derivatives at 40 °C and pH 8.0.<sup>6</sup> Besides enzymes, many microbes have been evaluated for ZEN-degrading potency, and some of them were shown to have the ability to eliminate ZEN in various manners.<sup>5,10</sup> Some fungi with a degradation ability against ZEN were reported earlier.<sup>10,11</sup> In recent years, a growing number of probiotic *Bacillus* strains have been studied for their ability to transform ZEN. *B. subtilis* ANSB01G could degrade 88.65% of an initial ZEN concentration of 0.5 mg/L in a liquid medium within 72 h.<sup>12</sup> *Bacillus pumilus* ES-21 could remove 95.7% of ZEN at an initial concentration of 17.9 mg/L within 24 h.<sup>13</sup> *Bacillus licheniformis* CK1 and *Bacillus cereus* BC7 had great ability to eliminate ZEN and thus showed efficiency to alleviate the toxic effects of ZEN in feed on weaned female piglets.<sup>14,15</sup> In other words, some *Bacillus* strains have been found to have the ability to mitigate the adverse effects of ZEN through animal feeding experiments, but the biotransformation products were not revealed in most of the previous reports. The major derivatives of ZEN,  $\alpha$ -zearalenol ( $\alpha$ -ZOL) and  $\beta$ -zearalenol ( $\beta$ -ZOL), were also studied for their acute reproductive and genetic toxicity. The relative toxicity was in the order  $\alpha$ -ZOL > ZEN >  $\beta$ -ZOL.<sup>16,17</sup> More importantly, ZEN was easily converted to  $\alpha$ -ZOL or  $\beta$ -ZOL by reducing the ketone group to a hydroxyl group in vivo.<sup>18</sup> Therefore, a better strategy to eliminate the harmful

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effects of ZEN is to transform ZEN and its derivatives simultaneously. Besides, ZEN could be transformed into non-/low-estrogenic substances ZEN-14-O- $\beta$ -glucoside, ZEN-16-O- $\beta$ -glucoside, and ZEN-14-sulfate by *Thamnidium spp.*, *Mucor spp.*, and *Rhizopus spp.*<sup>19,20</sup>

In this study, we evaluated the ZEN-removal potential of *Bacillus* strains from our laboratory to screen a high ZEN-removal strain. On this basis, we further reveal the ZEN-removal mechanism of the specific *Bacillus* strain by identifying the specific enzyme and its novel detoxified product. This will provide a new vision and theory on ZEN removal by *Bacillus* strains.

## MATERIALS AND METHODS

**Microorganisms, Media, and Chemicals.** *Saccharomyces cerevisiae* W303-1A/HER-ERE-yEGFP was constructed by Li in a previous work.<sup>21</sup> Zearalenone (ZEN),  $\alpha$ -zearalenol ( $\alpha$ -ZOL),  $\beta$ -zearalenol ( $\beta$ -ZOL), and 17- $\beta$ -estradiol (E2) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at  $-20^{\circ}\text{C}$ . Stock solutions of ZEN (1 mg/mL),  $\alpha$ -ZOL (1 mg/mL),  $\beta$ -ZOL (1 mg/mL), and E2 (1 mg/mL) were prepared by dissolving the solid in 100% methanol. The standard solutions for high-performance liquid chromatography (HPLC) calibration were prepared by diluting the stock solution with 100% methanol. The Luria–Bertani (LB) medium contained yeast extract (5.0 g/L), NaCl (10.0 g/L), and peptone (10.0 g/L). Minimal medium (MM) contained  $\text{NH}_4\text{NO}_3$  (1.0 g/L),  $\text{KH}_2\text{PO}_4$  (0.5 g/L),  $\text{Na}_2\text{HPO}_4$  (1.5 g/L), NaCl (1.0 g/L), and  $\text{MgSO}_4$  (0.2 g/L).

**Strain Screening and Identification.** Strains in the bacterial strain library (625 strains) of our laboratory were activated on a LB medium plate and cultured at  $37^{\circ}\text{C}$  for 12 h. To screen the strains for ZEN-removal capability, each single clone was then inoculated separately in liquid LB medium (1 mL) containing 20 mg/L ZEN and incubating at  $37^{\circ}\text{C}$  for 24 h with shaking at 220 rpm. After cultivating, an equal volume of methanol was added into the fermentation broth and mixed evenly. The supernatant was collected to determine for the content of residual ZEN by HPLC after centrifugation at 10 625g for 10 min. The quantification of ZEN was achieved by comparison to the standard curve (Figure S1).

The ZEN biotransformation rate was calculated as

$$D_t = \left(1 - \frac{C_t}{C_c}\right) \times 100\%$$

where  $D_t$  is the ZEN biotransformation rate and  $C_t$  and  $C_c$  are the ZEN concentrations of the treatment and control. The control is the initial stage of strain cultivation.

Strain identification was performed by morphology, a Biolog automated microbe identification system, and 16S rDNA gene sequence analysis.<sup>22</sup> The strain was deposited in the China General Microbiological Culture Collection Center (CGMCC No. 14854), Beijing, China. The universal primers, 16S-27F (5'-AGAGTTT-GATCMTGGCTCAG3') and 16S-1492R (5'-CGGTTACCTTGT-TACGACTT3'), were used to enlarge the partial 16S rDNA gene sequence of strain Y816 by PCR. Homologous analysis was then performed online with the NCBI BLAST software (<http://blast.ncbi.nlm.nih.gov>). The target sequence was compared with 16S rDNA sequences in GenBank and aligned with ClustalW. Further, a phylogenetic tree was made in MEGA7.0, constructed by the neighbor-joining method.

**ZEN Biotransformation Characteristics of Strain Y816 in Different Conditions.** A single colony of strain Y816 was inoculated into liquid LB medium (1 mL) and incubated at  $37^{\circ}\text{C}$  with shaking at 220 rpm. When the  $\text{OD}_{600\text{ nm}}$  value of strain Y816 was up to 1.0 in LB medium, different doses (2%, 5%, and 10% v/v) of cultures were inoculated into the fresh LB medium with an initial ZEN concentration of 40 mg/L. After incubating at  $37^{\circ}\text{C}$  with shaking at 220 rpm, the  $\text{OD}_{600\text{ nm}}$  values of strain Y816 and the residual ZEN

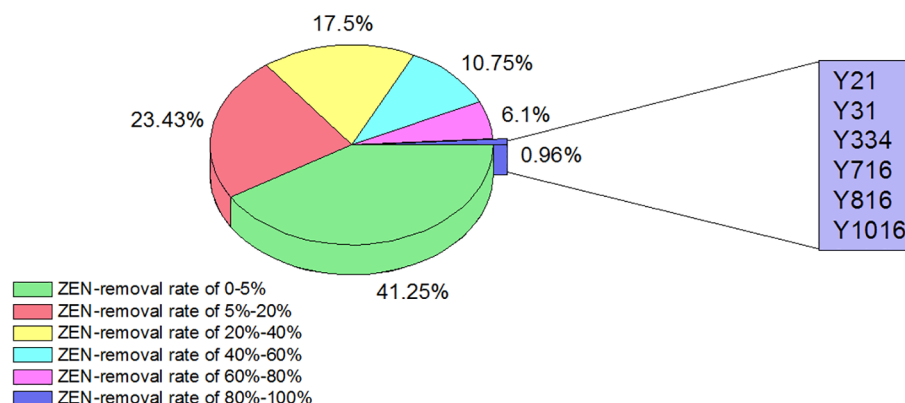
concentration were detected at different fermentation times. To determine the ZEN biotransformation characteristics of strain Y816 at different conditions, two inoculation modes of strain Y816 were used to inoculate into different media. When strain Y816 grew to the exponential phase ( $\text{OD}_{600\text{ nm}} = 1.6\text{--}1.9$ ), it took two equal volumes of the cultures. One was called the fermentation broth (FB), which contained all of the bacterial cells and their extracellular metabolites. The other one was centrifuged at 10 625g for 10 min, and then the precipitate was resuspended by the same volume of fresh medium. FB and CS were then inoculated into the fresh medium containing 40 mg/L of ZEN. Thus, it could keep the same cell concentration between FB and CS. Besides, fresh FB as seed broth will germinate faster than CS theoretically. Both inoculation doses were set as 10% (v/v) to LB medium, minimal medium (MM), and phosphate buffer (PBS, 50 mM, pH 7.0). The ZEN biotransformation rates were determined after 12 h of reaction at  $37^{\circ}\text{C}$ , 220 rpm. The treatments in phosphate buffer were the controls. Meanwhile, to further determine the ZEN biotransformation capability of the various elements of FB of strain Y816, the ingredients of FB were further segmented into CS, fermentation supernatants (FS), and intracellular extract (IE). Fermentation supernatant (FS) was the liquid supernatant after FB was centrifuged at 10 625g for 10 min. Intracellular extract (IE) was the liquid supernatant with the cell breaking fluid of CS after centrifuging at 10 625g for 10 min. All of the various samples of FB were obtained from the same volume of FB harvested at the same time. The ZEN biotransformation was performed as stated above. The ZEN biotransformation rate of the experimental group with inoculating FB was set as the control.

**HPLC Analysis for the Estrogenic Substances.** The sample to be tested was filtered through a 0.22  $\mu\text{m}$  nylon syringe filter before loading into the HPLC system (Alliance E2695, Waters, MA, USA) with a photodiode array detector (2998 PDA detector, Waters). Chromatographic separations were carried out on a reversed-phase column (C18, 4.6  $\times$  250 mm, 5  $\mu\text{m}$ , C18, Ireland) using gradient elution with A solution (0.5 mM ammonium acetate) and B solution (absolute methanol). The gradient elution started at 15% B solution (85% A solution) and increased linearly to 100% B solution after 22 min, and rinsing was continued for 8 min with 100% B solution. Then gradient washing was returned to a 15% B solution (85% A solution) within 1 min, and rinsing was continued for 4 min before the next sample injection. The flow rate was 0.5 mL/min, and the injection volume was 10  $\mu\text{L}$ . The standard curve for the concentration of ZEN at 10, 20, 50, 125, 250, and 500 ng/mL was determined with the above HPLC conditions (Figure S1). The concentration of ZEN,  $\alpha$ -ZOL, or  $\beta$ -ZOL was detected with a PDA detector at a wavelength of 236 nm. The separation of E2 also used gradient elution with A solution (0.5 mM ammonium acetate) and B solution (anhydrous acetonitrile). The gradient elution started at 10% B solution (90% A solution) and increased to 40% B solution after 15 min. This was further increased to 100% B solution within 7 min and lasted for 29 min. Then, gradient washing returned to a 10% B solution (90% A solution) within 1 min, and rinsing was continued for 4 min before the next sample injection. The concentration of E2 was detected at a wavelength of 200 nm with the same flow rate and injection volume as that of ZEN.

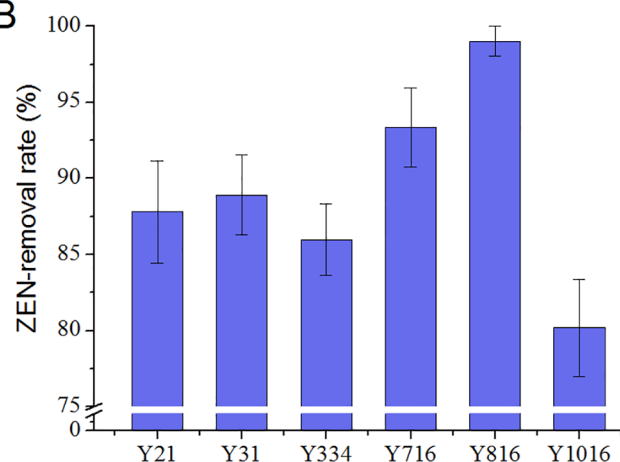
**Purification of the Biotransformation Products of Different Estrogenic Substances.** For the preparation of the biotransformation product (ZEN-P) of ZEN, 50 mg of ZEN was put into 200 mL of LB medium with 5 mL of activated strain Y816 and incubated at  $37^{\circ}\text{C}$ , 220 rpm for 12 h. Then, the culture broth was freeze dried and extracted with 20 mL of absolute methanol. The product in the extract was isolated and purified by preparative HPLC and eventually freeze dried into a powder. The preparative HPLC conditions were as follows: a C18 column (Agilent, 21.2  $\times$  250 mm, 7  $\mu\text{m}$ ) was used, the mobile phase was water/methanol (40/60, v/v), and the flow rate was 12 mL/min. The product E2-P was also made using the same steps except the mobile phase was water/acetonitrile (25/75, v/v).

**Identification of the Biotransformation Products of ZEN and E2.** The biotransformation products were analyzed by an UHPLC LC-30A system (Shimadzu, Kyoto, Japan) coupled with a

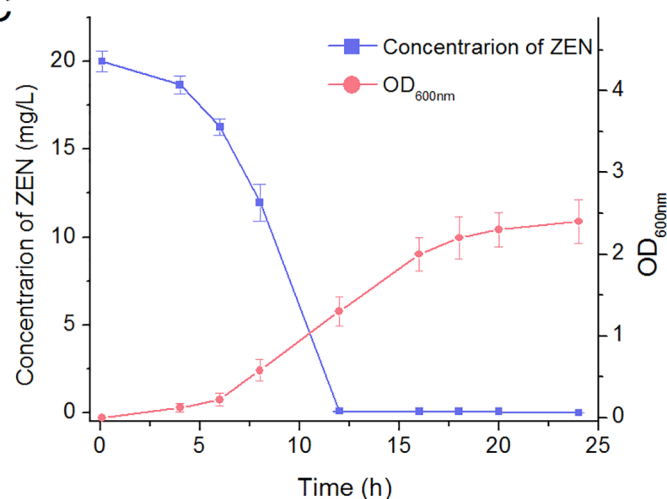
A



B



C



**Figure 1.** Strain screening for the high ZEN biotransformation capability. (A) Distribution characteristics of the strains in the laboratory bacterial strain library (625 strains in total) according to the percentage of removal of ZEN. (B) Confirmation of the six strains with the highest ZEN biotransformation efficiency by scale-up fermentation by volume (10 mL). (C) Curve of the ZEN biotransformation rate and growth profile of strain Y816. All values are expressed as mean  $\pm$  SD ( $n = 3$ ).

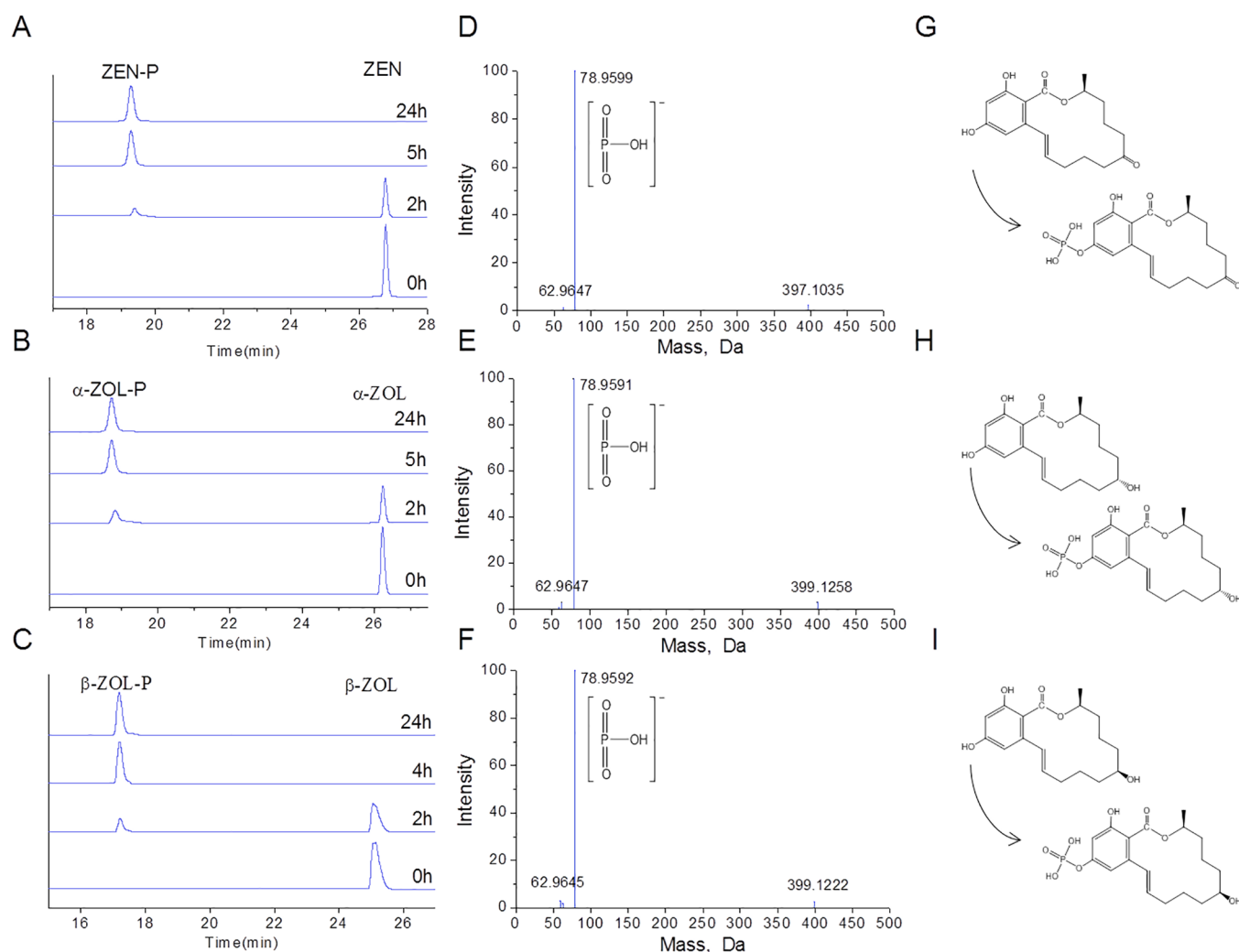
triple TOF 5600 mass spectrometer (Sciex, Framingham, MA, USA). The column and mobile phase were as the same stated as above. The ion voltage of the electrospray ionization negative mode was 5500 V with a mass range of 50–1000  $m/z$ . The source temperature was 600 °C, and the nebulizer gas, heater gas, and curtain gas pressures were 55, 55, and 35 psi, respectively. Acquisition of the MS/MS scans with a mass range of 30–1000  $m/z$  was controlled by the information-dependent acquisition (IDA) function of the Analyst 1.6 software (Sciex, Framingham, MA, USA) with dynamic background subtraction on. The declustering potential was 80 V, and the collision energy was 35 eV. Calibration of mass accuracy was performed every five samples. Data analysis for secondary mass spectrometry was performed with PeakView 2.0 software (Sciex, Framingham, MA, USA). To verify the structures of the biotransformation products of ZEN and E2, nuclear magnetic resonance spectroscopy (NMR) was employed on a Bruker AVANCE III 600 MHz spectrometer. The purified products were manufactured and collected for NMR.  $^1\text{H}$  and  $^{13}\text{C}$  NMR were probed at 600 Hz as well as  $^1\text{H}$ – $^1\text{H}$  (COSY),  $^1\text{H}$ – $^{13}\text{C}$  (HSQC), and  $^1\text{H}$ – $^{13}\text{C}$  (HMBC). Data were recorded and analyzed by MestReNova 9.0.

**Estrogenic Activity Assay Using an Engineered Yeast System.** *Saccharomyces cerevisiae* (w303-1A/hER-ERE-GFP), as a bioindicator, was used to measure the estrogenic activity of each target substance.<sup>21</sup> The recombinant yeast biosensor cell contained two episomal plasmids (Figure 6A) which were used to drive the expression of hER (human estrogen receptor) under a strong

promoter (GPD) and the expression of yEGFP under the control of ERE (estrogen response element).<sup>21</sup> The yeast was inoculated into an SD-Trp-Ura medium and cultured at 30 °C for 24 h with shaking at 220 rpm. A 1 mL amount of culture solution was added to 4 mL of SD-Trp-Ura medium containing different concentrations of E2 (0, 0.1, 0.3, 0.5, and 0.8 nM). After incubating for 6 h, the culture solution was centrifuged at 10 625g for 10 min, and then the yeast cells were reserved and rinsed twice with 50 mM PBS. A 0.1 mL amount of yeast cells resuspended in PBS was put into a 96-well black plate for detection of the fluorescence intensity by ELISA at an excitation wavelength of 488 nm and emission wavelength of 509 nm. Other samples were tested with the same steps. The products of ZEN and E2 were purified and collected for toxicity testing through preparative HPLC. Comparison of the estrogenic activity between E2 and E2-P was made at different concentrations (0, 0.05, 0.1, 0.3, 0.5, 0.8, 1, 1.2, 1.5, and 2 nM), and the comparison between ZEN and ZEN-P was at other different concentrations (0, 1, 5, 10, 20, 30, 40, 50, 60, 80, and 100 nM). Besides,  $\alpha/\beta$ -ZOL and their products were also studied using the same steps at different concentrations (0–100 and 0–1000 nM).

The relative fluorescence intensity was determined using the following equation

$$\text{RFI} = \frac{FI_t - FI_c}{\text{OD}_{600}}$$



**Figure 2.** Identification of the biotransformation products of ZEN and  $\alpha$ -ZOL/ $\beta$ -ZOL by HPLC-TOF-MS. (A) HPLC analysis results of ZEN and its biotransformation product (ZEN-P) for different reaction times. (B) HPLC analysis results of  $\alpha$ -ZOL and its biotransformation product ( $\alpha$ -ZOL-P) for different reaction times. (C) HPLC analysis results of  $\beta$ -ZOL and its biotransformation product ( $\beta$ -ZOL-P) for different reaction times. (D) Secondary mass spectrogram of ZEN-P. (E) Secondary mass spectrogram of  $\alpha$ -ZOL-P. (F) Secondary mass spectrogram of  $\beta$ -ZOL-P. (G) Speculation of the biotransformation results according to the structure analysis of ZEN-P and ZEN by MS and NMR. (H) Speculation of the biotransformation results according to the structure analysis of  $\alpha$ -ZOL-P and  $\alpha$ -ZOL by MS and NMR. (I) Speculation of the biotransformation results according to the structure analysis of  $\beta$ -ZOL-P and  $\beta$ -ZOL by MS and NMR. Inoculated dose of strain Y816 was 10%.

where RFI is the relative fluorescence intensity,  $FI_t$  and  $FI_c$  are the fluorescence intensity of the treatment group and the control, and  $OD_{600\text{ nm}}$  is the corresponding optical density at a wavelength of 600 nm.

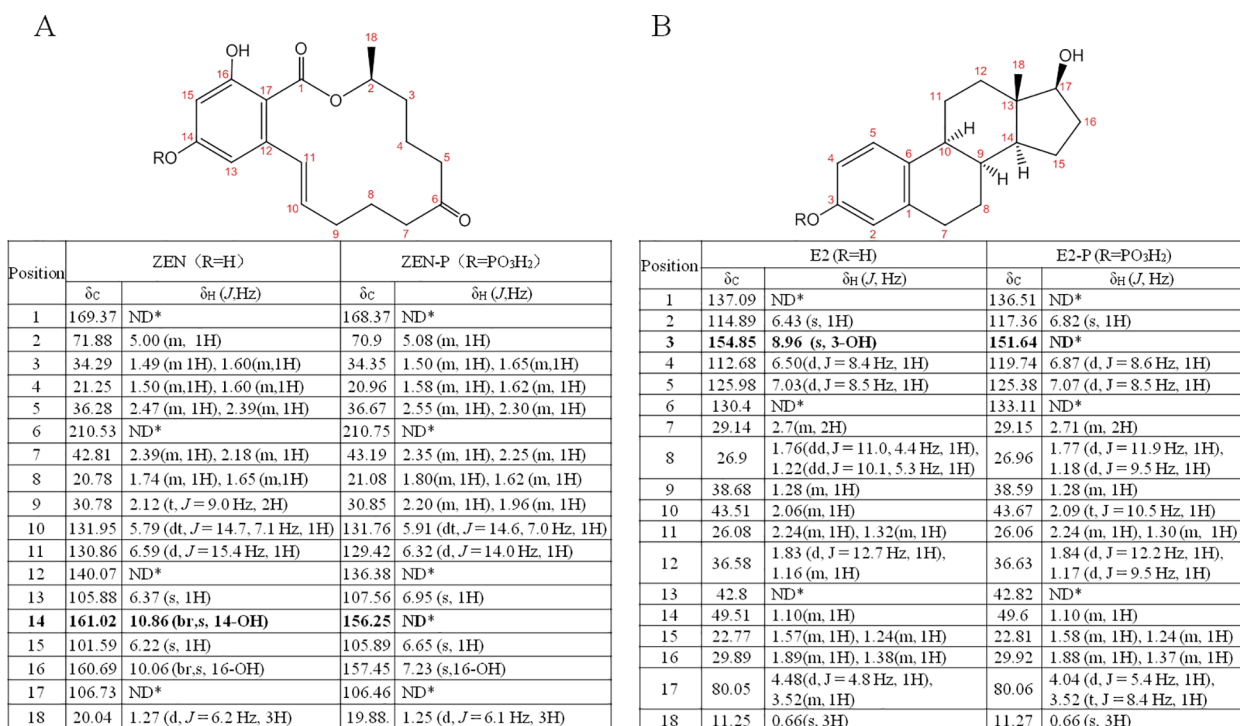
#### Comparative Transcriptome Analysis of *B. subtilis* Y816.

Strain Y816 was cultured in LB medium without ZEN (control) and LB medium containing 20 mg/L ZEN for about 12 h when the  $OD_{600\text{ nm}}$  was 1.0. Then, the collected bacteria were centrifuged at 10 625g for 10 min to extract total RNA using an Easstep Universal RNA Extraction Kit (Shanghai Promega Biological Products Ltd., Shanghai, China). A total amount of 3  $\mu$ g of RNA per sample was used as input material for the RNA sample preparations. Sequence libraries were generated using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes were added to give sequences to each sample.<sup>23</sup> Comparative analysis of the transcriptome data was performed according to the method reported previously.<sup>23</sup>

**Exogenous Expression of the *pps* Gene in *E. coli* and Reaction of Enzymatic Biotransformation.** The transcriptional differential gene (*pps*) encoding phosphoenolpyruvate synthase was amplified using a pair of specific primers (ppsF-ATGTATT-

CAGTGC GATTTCAAAAAG; ppsR-CTAGCAT-GAAGCGCGTTTG) and the genomic DNA of strain Y816 as a template. Then, the gene *pps* was cloned in the plasmid pET32a $\Delta$ trxA (constructed in our laboratory), and the recombinant plasmid was transformed to the competent cells of *E. coli* BL21 (DE3). The induced expression and purification of the recombinant enzyme were performed according to the method in our previous report.<sup>24</sup> The purified recombinant enzyme was used to transform ZEN in an in vitro reaction system. The reaction system contained 20 mg/mL ZEN, 20  $\mu$ L of ZPH (5 mg/mL), 1 mM ATP, and 1 mM  $Mg^{2+}$  in a total volume of 0.5 mL. Two experimental groups were set as the same system without only ATP or  $Mg^{2+}$ . The reaction time was 5 min at 37 °C.

**Sequence Data.** The transcriptome raw data (RNA sequences of *B. subtilis* Y816 with and without ZEN induced) under the conditions described in this study have been deposited in the SRA database with Accession No. PRJNA727706. The nucleotide sequence of *zph* has been submitted to the GenBank database under Accession No. MZ170042.1.



**Figure 3.** NMR spectroscopic data (600 MHz; DMSO- $d_6$ ) of ZEN/ZEN-P and E2/E2-P. (A)  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of ZEN (HRMS calcd for  $\text{C}_{18}\text{H}_{22}\text{O}_5$   $[\text{M} - \text{H}]^-$ , 317.1389; found, 317.1404) and ZEN-P (HRMS calcd for  $\text{C}_{18}\text{H}_{23}\text{O}_8\text{P}$   $[\text{M} - \text{H}]^-$ , 397.1053; found, 397.1035), and peaks and coupling constants  $J$  (in Hertz) are shown in parentheses. (B)  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of E2 (HRMS calcd for  $\text{C}_{18}\text{H}_{24}\text{O}_2$   $[\text{M} - \text{H}]^-$ , 271.1698; found, 271.1682) and E2-P (HRMS calcd for  $\text{C}_{18}\text{H}_{25}\text{O}_5\text{P}$   $[\text{M} - \text{H}]^-$ , 351.1362; found, 351.1393), and peaks and coupling constants  $J$  (in Hertz) are shown in parentheses.

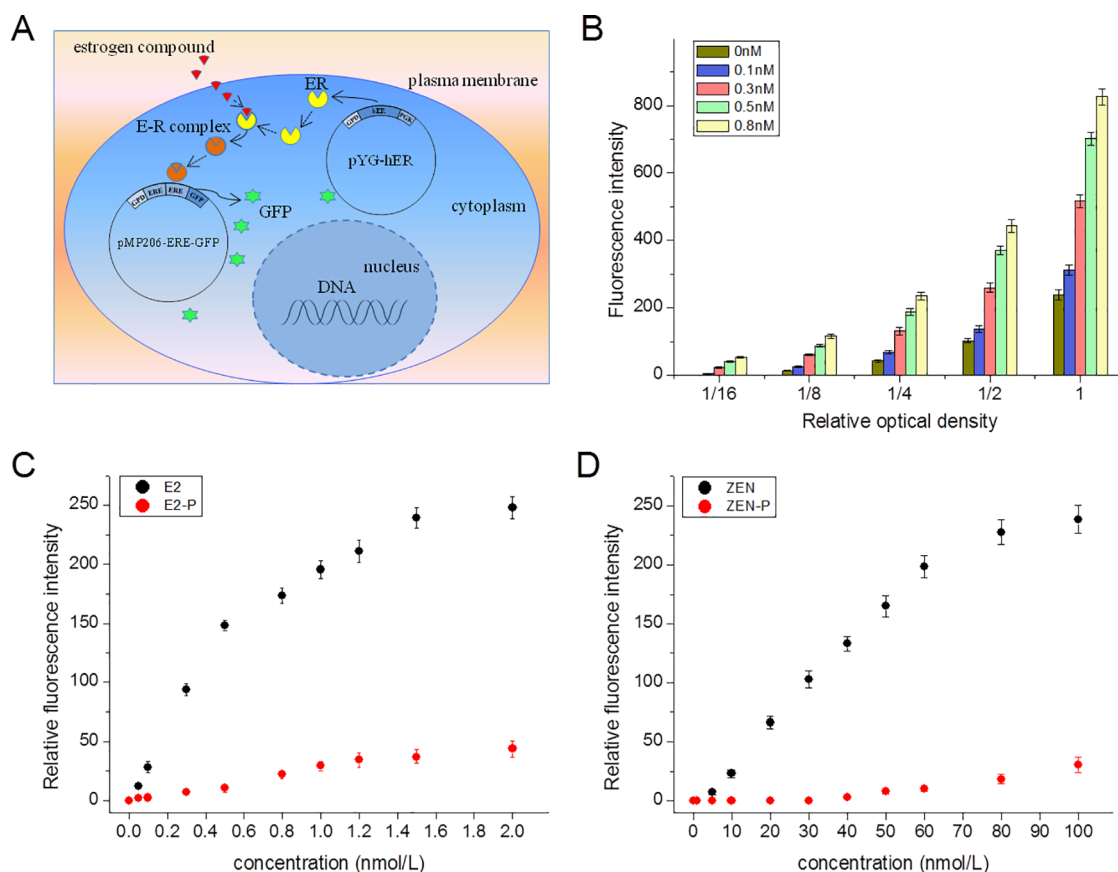
## RESULTS

**Screening and Identification of ZEN Biotransformation Bacterial Strains.** Among the 625 bacterial strains tested, the number of strains that have the capacity to transform ZEN to varying degrees was 406. This indicated that most of these bacterial strains have the ability to transform ZEN, while 64.68% of the strains showed a ZEN biotransformation rate of less than 20% (Figure 1A). The strains with a higher ZEN biotransformation rate (>80%) only account for 0.96% containing six strains (Figure 1A). In order to further confirm the ZEN biotransformation capability of the six strains, each strain was cultured in a scale-up fermentation system (10 times expanded in reaction volume) with 20 mg/L ZEN addition in the media for 24 h of fermentation. As shown in Figure 1B, except for strain Y1016, the other five strains could transform more than 85% of ZEN during 24 h of fermentation. No residual ZEN was detected by HPLC after 12 h of fermentation of strain Y816 (Figure 1C). The ZEN biotransformation rate increased correspondingly along with the logarithmic growth of strain Y816 (Figure 1C). Thus, strain Y816 was selected as a promising ZEN biotransformation strain for further study in this work. Strain Y816 was further identified as *B. subtilis* Y816 through morphology observation, a Biolog automated microbe identification system, and 16S rDNA sequence analysis (Figure S2, S3, and Table S1). The 16S rDNA sequence of strain Y816 has been deposited in the GenBank database under Accession No. MW599957.1.

**ZEN Biotransformation Characteristics of *B. subtilis* Y816 in Fermentation.** To further study the capacity and condition of ZEN biotransformation of strain Y816, we increased the initially added concentration of ZEN to 40

mg/L with different inoculated doses. As the results show in Figure S4A, when the inoculum was 2%, 5%, and 10%, ZEN (40 mg/L) was eliminated absolutely after incubation times of 13, 11, and 7 h, respectively. Meanwhile, the optical densities ( $\text{OD}_{600 \text{ nm}}$ ) of strain Y816 were  $1.93 \pm 0.26$ ,  $1.82 \pm 0.35$ , and  $1.64 \pm 0.31$  at culture times of 13, 11, and 7 h, respectively (Figure S4B). As shown in Figure S4A and S4B, the 10% inoculated dose had a shorter adaptive phase and transformed ZEN faster than the other two lower inoculated doses. Besides, the 10% inoculated dose got over double the final biomass than the 2% inoculated dose in the same LB medium (Figure S4B), probably because 40 mg/mL ZEN in the medium could provide much more toxicity to the cells which restrains the growth of strains. The higher inoculated dose showed a shorter adaptive phase and lower lethality rates to get a higher final biomass. After 12 h of culturing in LB, MM, and PBS, 100% of ZEN was transformed by inoculated FB, while inoculated CS could only get enough nutrition for normal metabolism in LB medium to show a high ZEN transformation rate (Figure S4C). The ZEN transformation rate by inoculating CS in MM was as low as in the control group (PBS) (Figure S4C). Moreover, in order to study the effect on the ZEN biotransformation of each ingredient, FB was divided into cell suspension (CS), fermentation supernatant (FS), and intracellular extract (IE) to react with ZEN in PBS. Compared to the control group (inoculating FB), which could transform 100% of ZEN after 12 h of incubation, about 20%, 8%, and 3% of ZEN were transformed by inoculating IE, CS, and FS, respectively (Figure S4D).

**Identification of Zearalenone Conjugate in the Fermentation of *B. subtilis* Y816.** In order to further reveal the mechanism of ZEN biotransformation by strain Y816, we

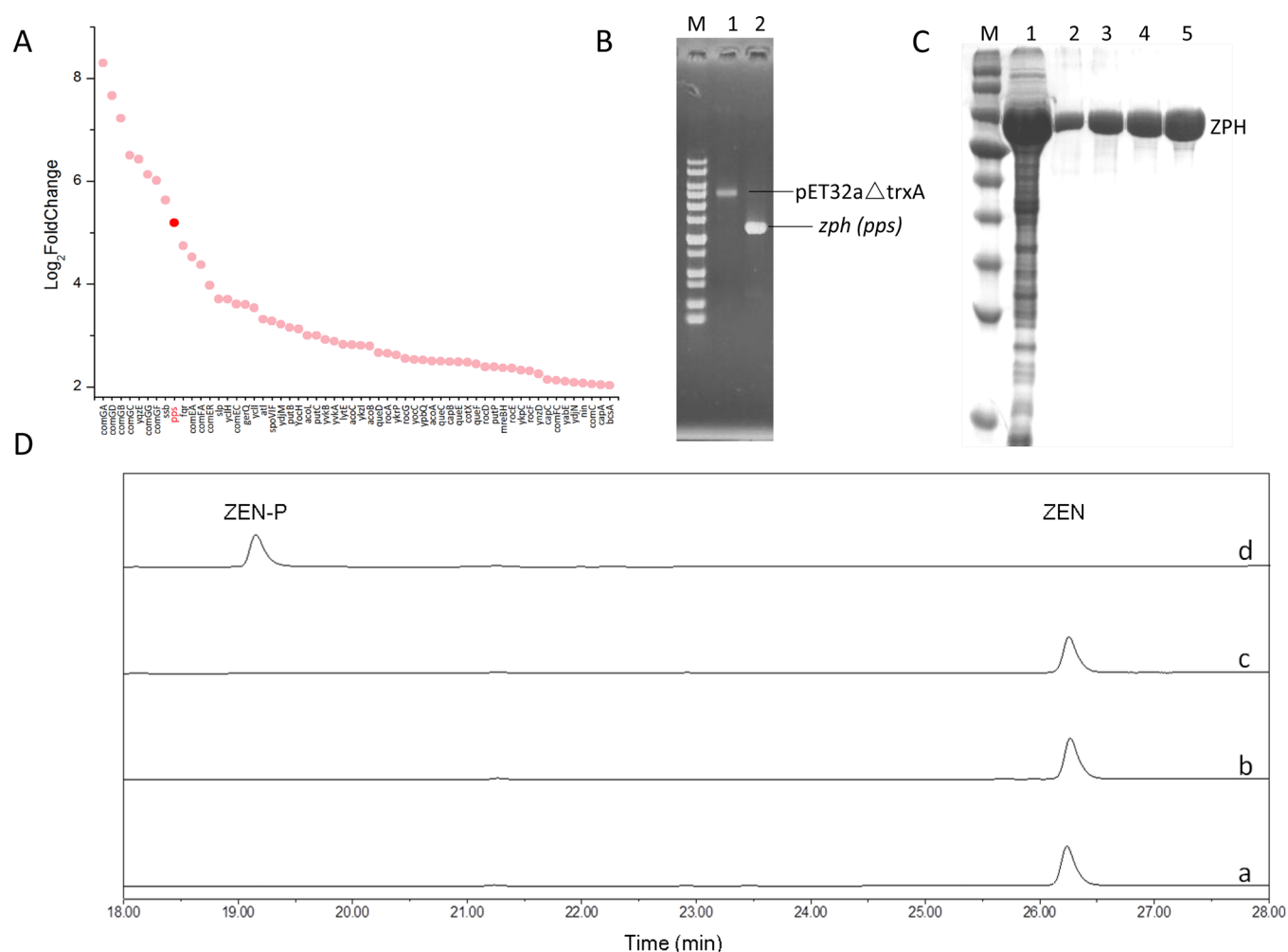


**Figure 4.** Estrogenic toxicity testing by the whole yeast cell biosensor. (A) Schematic diagram of detecting estrogen activity in recombinant yeast. ER: estrogen receptor. E–R complex: estrogen–receptor complex. GFP: green fluorescent protein. Assay system was constructed in the previous work. (B) Dose–effect between estrogen concentration and fluorescence intensity/optical density. Different concentrations (0–0.8 nM) of E2 dissolved in 50 mM PBS with a relative optical density of 1. Relative optical densities of 1/2, 1/4, 1/8, and 1/16 were diluted 2 times, 4 times, 8 times, and 16 times, respectively, from the relative optical density of 1. (C) Relative fluorescence intensity of E2 and E2-P at different concentrations. (D) Relative fluorescence intensities of ZEN and ZEN-P at different concentrations. All values are expressed as means  $\pm$  SD ( $n = 3$ ).

should identify the biotransformation product in the fermentation of *B. subtilis* Y816. The concentrations of ZEN and its biotransformation product were detected continually to delineate the transformation process from ZEN to its special product in the fermentation of strain Y816 (Figure 2A). During the fermentation process, a special biotransformation product (ZEN-P) of ZEN was detected after 2 h of fermentation by HPLC with the same detecting conditions of ZEN (Figure 2A). After 5 h of fermentation, 100% of ZEN was transformed to ZEN-P (Figure 2A). Moreover, the ZEN reductive derivatives  $\alpha$ -ZOL and  $\beta$ -ZOL were also used as substrates transformed by the fermentation of strain Y816. The specific transformation products ( $\alpha$ -ZOL-P and  $\beta$ -ZOL-P) from  $\alpha$ -ZOL and  $\beta$ -ZOL were also found by HPLC, respectively (Figure 2B and 2C). The biotransformation efficiencies of the two reductive derivatives were similar to that of ZEN (Figure 2A–C). Subsequently, the molecular structures of ZEN-P and  $\alpha/\beta$ -ZOL-P were analyzed by HPLC-MS. On the basis of the negative ion ESI-MS, the molecular weights of the products (ZEN-P and  $\alpha/\beta$ -ZOL-P) were determined ( $m/z = 397.1035$ ,  $399.1258$ , and  $399.1222$ ) with an increase of 80 than that of their substrates (ZEN and  $\alpha/\beta$ -ZOL) (Figure 2D–F and Figure S5). A specific peak for the phosphate group with the corresponding molecular weight is also shown in the results of the secondary mass spectrum

(Figure 2D–F). Besides, 17- $\beta$ -estradiol (E2) as a structural analog of ZEN could be transformed into E2-P in the same way and analyzed using the results of the secondary mass spectrum (Figure S5D and Figure S6). This suggested that strain Y816 could transform these estrogens to similar phosphorylated conjugates by fermentation. From the NMR analysis of ZEN-P, there were decided changes in the chemical shifts on C-14, C-13, C-15, C-12, and C-16 because the hydroxyl on C-14 was superseded by phosphate (Figure 3A). E2-P was identified as E2-3-phosphate due to the variations in the chemical shifts on C-3, C-2, and C-4, illustrating that the position replaced by a phosphate group was on C-3 (Figure 3B). Furthermore,  $\alpha/\beta$ -ZOL-P could be inferred as  $\alpha/\beta$ -ZOL-14-phosphate based on the extremely similar structures and secondary mass spectrometry results with ZEN-14-phosphate (Figure 2G–I).

**Determination of the Estrogenic Toxicity of the Phosphorylated Conjugates.** We first used E2 as an estrogen standard to test the dose–effect relationship between the concentration of E2 and the fluorescence intensity of the biological indicator in the recombinant yeast biosensor cell (Figure 4A). As shown in Figure 4B, the fluorescence intensity displayed a good linear relationship with both the concentration of E2 and the optical density ( $OD_{600nm}$ ) of the recombinant yeast cells. Therefore, in order to eliminate the



**Figure 5.** Identification of the zearalenone phosphotransferase gene and the enzymatic phosphorylation reaction by zearalenone phosphotransferase (ZPH). (A) Upregulated differential genes with a Log<sub>2</sub> fold change value higher than 2. (B) PCR results of plasmid pET32aΔtrxA and the differential gene *pps*. M: molecular weight standard of the nucleic acids. (C) HPLC analysis of the enzymatic transform of ZEN by ZPH. (a) Initial determination before the transform reaction of 20 mg/L ZEN. (b) Determination of the sample after 5 min of reaction in the same reaction system but without ATP supplementation. (c) Determination of the sample after 5 min of reaction in the same reaction system but without Mg<sup>2+</sup> supplementation. (d) Determination of the sample after 5 min of reaction in the reaction system of a.

influence of the difference in the concentration of yeast cells on the detection of the dose–effect relationship between the fluorescence intensity and the concentration of estrogenic substances, we used the relative fluorescence intensity (fluorescence intensity/optical density of cells) to represent the estrogenic activity. The results showed that the relative fluorescence intensity has a good dose–effect relationship with the different concentrations of E2 (Figure 4C), indicating that the relative fluorescence intensity in this assay system could characterize the estrogenic activity of different estrogenic substances. On this basis, the estrogenic activities of the product E2-3-phosphate with different concentrations were determined in this assay system. With the same concentration, the estrogenic activity of E2-3-phosphate was much less than that of E2 (Figure 4C). Similarly, ZEN and ZEN-14-phosphates had coincident trend curves of estrogenic activity with E2 and E2-3-phosphate (Figure 4C and 4D). When the concentration of ZEN-14-phosphate was 30 nM, there was no fluorescence detected, while the relative fluorescence intensity was up to 100 at the same concentration of ZEN (Figure 4D). Though the estrogenic activity was slightly increasing as the concentration of ZEN-14-phosphate increased, the relative

fluorescence intensity was only 10 at a concentration of 60 nM, which is 5% of that of ZEN at the same concentration (Figure 4D). Furthermore, the products  $\alpha/\beta$ -ZOL-14-phosphate also showed a similar reduction of estrogenic activity compared with that of  $\alpha/\beta$ -ZOL (Figure S7). All of the above results indicated that phosphorylation modification of these estrogenic substances could significantly reduce the estrogenic activity to achieve detoxification of these kinds of mycotoxins.

**Identification of the Key ZEN Biotransformation Enzyme in *B. subtilis* Y816 and Its Catalytic Characteristics.** According to the above study, we found that the ZEN biotransformation was related to the intracellular metabolism of the strains. Thus, we further compared the ZEN biotransformation velocity between inoculating strain Y816 (Y816Z), which was induced by 20 mg/L ZEN in 12 h of fermentation, and inoculating strain Y816 (Y816C), which was cultured for 12 h without ZEN addition. The results showed that inoculating Y816Z led to a remarkably higher ZEN biotransformation velocity than that of inoculating Y816C (Figure S8). Thus, we selected samples when strain Y816 was cultured to OD<sub>600 nm</sub> = 1.0 with and without 20 mg/L ZEN for transcriptome sequencing. Through comparison of the tran-

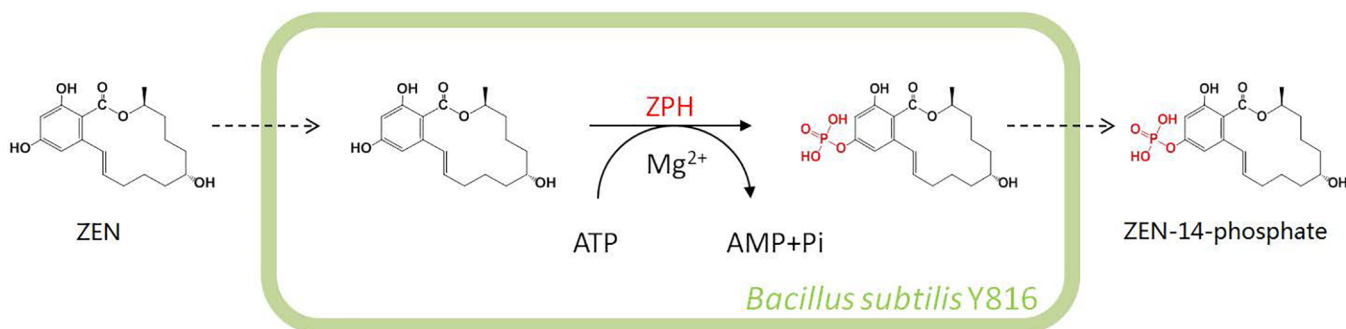
scriptome analysis, a total of 772 differentially expressed genes was found and 296 upregulated genes were among them (Figure S9). We show 58 upregulated genes with Log2 fold change values higher than 2 in Figure 5A. We proved that the biotransformation product of ZEN in strain Y816 was a phosphorylated conjugate. On this basis, we found a differentially expressed gene annotated as *pps* which encodes phosphoenolpyruvate synthase (Figure 5A). According to the function annotation, it could catalyze pyruvate to phosphoenolpyruvate (PEP) with the help of ATP and  $Mg^{2+}$ . Thus, to further identify its transphosphorylation, we amplified the *pps* gene from the genome of strain Y816 (Figure 5B) and overexpressed it in *E. coli* (Figure 5C). In order to eliminate interference from other cell components, we purified the enzyme with electrophoresis (Figure 5C) for the reaction. When excess ATP (1 mM) and  $Mg^{2+}$  (1 mM) were added in the reaction system, 100% of ZEN was transformed to ZEN-14-phosphate for 5 min (Figure 5Da and 5Dd). While there was no ATP or  $Mg^{2+}$  in the vitro reaction system, ZEN could not be transformed by the enzyme (Figure 5Db and 5Dc). Besides, in the same vitro reaction system, the enzyme encoded by *pps* showed no activity toward its native substrate pyruvate and there was no PEP produced even though the same excess ATP (1 mM) and  $Mg^{2+}$  (1 mM) were supplemented (Figure S10). Through protein blast in NCBI, the enzyme showed high sequence identity (>98%) with many phosphotransferases from various *Bacillus* species (Figure S11). We also made multiple sequence alignments among the enzyme of *B. subtilis* Y816 and another six phosphotransferases from different organisms (Figure S12). This showed that the enzyme encoded by *pps* in *B. subtilis* Y816 has more than 98% sequence identity with the reported phosphotransferases from different organisms. Thus, based on both the sequence properties (Figures S11 and S12) and the zearalenone phosphotransferase (ZPH) activity (Figure 5B–D) of the enzyme, we first named its encoding gene *zph* in this study. The optimum temperature and optimum pH of enzyme ZPH were also determined to be 40 °C and 7.0, respectively (Figure S13).

## DISCUSSION

In previous reports, an increasing number of *Bacillus* strains have been identified for their ZEN biotransformation capability and assayed for their application potential as probiotics.<sup>25–28</sup> However, most of them failed to identify the exact metabolite of ZEN in the fermentation processes of *Bacillus* sp. strains. This may be an implicit threat for their application as probiotics in the food and feed industries. In this study, a newly discovered ZEN biotransformation strain *B. subtilis* Y816, which could transform 40 mg/L of ZEN within 7 h, showed remarkably higher efficiency of ZEN transformation than that of *Bacillus* sp. strains reported previously.<sup>15,25,26</sup> It also showed a high biotransformation efficiency for  $\alpha/\beta$ -ZOL and E2 (Figure 2 and Figure S6). We fortunately found a specific product peak (ZEN-P) in the HPLC analysis results of ZEN (Figure 2A). According to the analysis results of the biotransformation products of ZEN and  $\alpha/\beta$ -ZOL by HPLC, 100% of ZEN and  $\alpha/\beta$ -ZOL were transformed to ZEN-P and  $\alpha/\beta$ -ZOL-P after 5 h, and during the whole fermentation process (24 h) the products (ZEN-P or  $\alpha/\beta$ -ZOL-P) remained stable and did not revert to ZEN or other derivatives (Figure 2A–C). This indicated that the products ZEN-P and  $\alpha/\beta$ -ZOL-P may be in the same transformation pattern and as the

final metabolites of the fermentation of strain Y816 could keep the structure stable during fermentation. The structure identification of ZEN-P,  $\alpha/\beta$ -ZOL-P, and E2-P by HPLC-TOF-MS analysis revealed the same specific peak of the phosphate group of the substrates (Figure 2D–F and Figure S6B). Interestingly, from the secondary mass spectrograms of the products (ZEN-P,  $\alpha/\beta$ -ZOL-P, and E2-P), there were no specific peaks of substrates (ZEN,  $\alpha/\beta$ -ZOL, and E2) found as shown in Figure S5. This is probably because of the addition of phosphate groups, which altered the properties of the molecules of the substrates, which is generally called the phenomenon of neutral loss.<sup>29</sup> Combined with the results of NMR, ZEN-P,  $\alpha/\beta$ -ZOL-P, and E2-P were identified as ZEN-14-phosphate,  $\alpha/\beta$ -ZOL-14-phosphate, and E2-3-phosphate, respectively. The phosphorylation reaction of these estrogenic toxins (ZEN,  $\alpha/\beta$ -ZOL, and E2) by biotransformation was first revealed in this study.

As in previous reports, some specific plant-produced ZEN conjugates, such as zearalenone-14-glucoside,  $\alpha$ -ZOL-14-glucoside,  $\beta$ -ZOL-14-glucoside, zearalenone-14-sulfate, and zearalenone-16-glucoside, have been revealed.<sup>30–33</sup> In fungal microorganisms, it was also found that ZEN sulfate is a natural ZEN conjugate produced by *Aspergillus niger*, *Rhizopus arrhizus*, and *Fusarium graminearum*.<sup>11,33–35</sup> Brodehl et al.<sup>11</sup> found that *Rhizopus* and *Aspergillus* species could transform ZEN into four different ZEN conjugates (ZEN-14-sulfate,  $\alpha$ -ZOL-sulfate, ZEN-O-14-glucoside, and ZEN-O-16-glucoside) in vivo. In this study, ZEN-14-phosphate as the final single metabolite of strain Y816 is a novel ZEN conjugate found in bacteria species with a 100% biotransformation rate. The higher estrogenic toxicity derivatives  $\alpha$ -ZOL of other creatures could also be phosphorylated by strain Y816 to  $\alpha$ -ZOL-14-phosphate (Figure 2B and 2H). According to the previous reports to date, the ZEN detoxication phase II conjugating enzymes such as UDP-glucuronosyltransferases (UGT) and sulfotransferase (SULT1E1 or EST) have generally been revealed in the cells of plants or animals.<sup>2,36</sup> The specific enzyme for the biosynthesis of zearalenone-14-sulfate in fungal microorganisms has not been revealed yet, although the zearalenone-14-sulfate standard samples are generally prepared by the fermentation of *Aspergillus oryzae*.<sup>34,37</sup> The transphosphorylation enzymes for the estrogens have also not been revealed in any organism so far. However, to our knowledge, phosphorylases are indispensable enzymes in the key metabolic pathways (such as glycolysis pathway) in many microorganisms. After a further study of the ZEN biotransformation characteristics of strain Y816, we found that the ZEN biotransformation efficiency is closely related to its growth velocity. Whatever the inoculum sizes, the ZEN biotransformation rate increases following the logarithmic growth of the strain and transformed 100% of ZEN at the logarithmic metaphase (Figure S4A and S4B). Combined with the results that the IE of strain Y816 transformed ZEN with a relatively higher rate than CS and FS (Figure S4D), strain Y816 probably transforms ZEN by its intracellular enzyme or metabolic pathway. The intracellular enzyme or its cofactor could not accumulate enough in the completely dead cells, as only 20% of the ZEN biotransformation rate was obtained by IE (Figure S4D). Thus, the biosynthesis of zearalenone-14-phosphate in the fermentation of strain Y816 was probably catalyzed by a kind of intracellular transphosphorylation enzyme.



**Figure 6.** Schematic diagram for the novel zearalenone biotransformation mechanism in *B. subtilis* Y816.

In order to further reveal the key enzyme for ZEN biotransformation in the cells of *B. subtilis* Y816, we performed a comparison transcriptome analysis of strain Y816 with and without the ZEN added. A promising differentially expressed gene annotated as *pps* encoding phosphoenolpyruvate synthase was found for its phosphate transfer activity. According to previous reports, phosphoenolpyruvate synthase could catalyze the transformation of a phosphoric acid group from ATP to pyruvate producing one molecule of PEP, AMP, and  $P_i$  in the presence of  $MgCl_2$ .<sup>38</sup> Thus, we simulated the reaction conditions to catalyze the transformation reaction on the new substrate ZEN with excess supplementation of ATP and  $Mg^{2+}$ . The results showed that the enzyme could transform ZEN to zearalenone-14-phosphate at a high velocity (Figure 5D). Besides, it also verified the necessity of the present of ATP and  $Mg^{2+}$  for the biotransformation reaction (Figure 5D). However, in the same reaction conditions, we could not detect the phosphate transfer activity on its native substrate pyruvate (Figure S10). This result is similar to that of some previous studies; some researchers reported a rifampin phosphotransferase which was initially annotated as phosphoenolpyruvate synthase but had been proved to have no phosphate transfer activity on pyruvate.<sup>39</sup> Thus, they renamed the enzyme rifampin phosphotransferase (RPH). According to the structure blast, phosphoenolpyruvate synthase, rifampin phosphotransferase, and zearalenone phosphotransferase (ZPH) have similar conserved sequences but have significant structural differences, which may lead to their different substrate selectivity.<sup>40</sup> Thus, by combining all of the results in this study we found a new biotransformation mode of zearalenone identified in *B. subtilis* Y816 which produced a novel ZEN conjugate zearalenone-14-phosphate (Figure 6). Due to its high protein sequence identity with many phosphotransferases in various *Bacillus* strains (Figures S11 and S12), the novel biotransformation mode of zearalenone may widely exist in *Bacillus* species with different transformation rates based on various mutants (Figure S12), which would be useful for understanding the strain screening results in Figure 1.

Although we successfully found a novel ZEN conjugate, the potential estrogenic toxicity of the derivatives of ZEN formed in vivo is different based on previous reports.<sup>11,32</sup>  $\alpha$ -ZOL has a much higher affinity for estrogen receptors; as a result, it is more toxic than the parent ZEN, while  $\beta$ -ZOL has a lower affinity for these receptors and is virtually harmless.<sup>5,33</sup> On the basis of previous research, the conjugation derivative ZEN-14-sulfate showed less toxicity than ZEN.<sup>41</sup> As ZEN-14-phosphate was a novel ZEN conjugate reported for the first time in this study, it is necessary to detect its estrogenic toxicity. In this

study, we used the whole yeast cell biosensor for the estrogenic activity assay (Figure 6A) by creating stably transfected strains with yEGFP as measurable reporter proteins.<sup>21</sup> The toxicity test method based on the biosensor yeast performed in this study was generally used to detect estrogen contamination in the environment through a positive correlation between the estrogenic content and the fluorescence intensity. Compared with the initial substances (ZEN,  $\alpha/\beta$ -ZOL, and E2), the phosphorylated products showed a visible decrease in the estrogenic activity. It is possible that modification of the phosphate group helps to prevent the molecule from binding to estrogen receptors.

In conclusion, a new biotransformation mode of zearalenone was identified in the newly screened high ZEN-removal strain *B. subtilis* Y816 through revealing the novel ZEN conjugate (ZEN-14-phosphate) and zearalenone phosphotransferase (ZPH) successively. It is also valid in the biotransformation of  $\alpha/\beta$ -ZOL and E2 by strain Y816. Besides, the phosphorylated conjugates showed much lower estrogenic activity than that of the original substrates. Thus, the novel biotransformation mode of *B. subtilis* Y816 would provide a new perspective for the detoxification of estrogenic toxin in bacterial strains.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c01817>.

Characteristics of strain Y816 analyzed by the Biolog-automated microbe identification system; standard curve of the concentration of ZEN by HPLC; morphological identification of strain Y816; phylogenetic tree of strain Y816 and its closest *Bacillus* strains based on 16S rDNA gene sequences; ZEN biotransformation characteristics of strain Y816 in different conditions; secondary mass spectrometry of estrogenic substrates; identification of the transformation product of E2 by HPLC-MS; comparison of estrogen activity between  $\alpha/\beta$ -ZOL and  $\alpha/\beta$ -ZOL-P; biotransformation curve of ZEN in the fermentation of different inoculate strains; volcano distributions of differential expressed genes based on a comparison of the transcriptome analysis; HPLC analysis of the enzymatic transform of pyruvate by ZPH; distance tree of protein sequences of reported phosphotransferases with more than 98% sequence identity with ZPH; multiple sequence alignment of phosphotransferase sequences from different strains selected in NCBI; characteristics of the purified ZPH (PDF)

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## Notes

The authors declare no competing financial interest.

## ■ REFERENCES

- (1) Ma, C. G.; Wang, Y. D.; Huang, W. F.; Liu, J.; Chen, X. W. Molecular reaction mechanism for elimination of zearalenone during simulated alkali neutralization process of corn oil. *Food Chem.* **2020**, 307, 125546.
- (2) Zheng, W.; Feng, N.; Wang, Y.; Noll, L.; Xu, S.; Liu, X.; Lu, N.; Zou, H.; Gu, J.; Yuan, Y.; Liu, X.; Zhu, G.; Bian, J.; Bai, J.; Liu, Z. Effects of zearalenone and its derivatives on the synthesis and secretion of mammalian sex steroid hormones: A review. *Food Chem. Toxicol.* **2019**, 126, 262–276.
- (3) Kos, J.; Janic Hajnal, E.; Malachova, A.; Steiner, D.; Stranska, M.; Krška, R.; Poschmaier, B.; Sulyok, M. Mycotoxins in maize harvested in Republic of Serbia in the period 2012–2015. Part 1: Regulated mycotoxins and its derivatives. *Food Chem.* **2020**, 312, 126034.
- (4) Li, M.; Yang, C.; Mao, Y.; Hong, X.; Du, D. Zearalenone Contamination in Corn, Corn Products, and Swine Feed in China in 2016–2018 as Assessed by Magnetic Bead Immunoassay. *Toxins* **2019**, 11 (8), 451.
- (5) Krol, A.; Pomastowski, P.; Rafinska, K.; Railean-Plugaru, V.; Walczak, J.; Buszewski, B. Microbiology neutralization of zearalenone using *Lactococcus lactis* and *Bifidobacterium* sp. *Anal. Bioanal. Chem.* **2018**, 410 (3), 943–952.
- (6) Wang, M.; Yin, L.; Hu, H.; Selvaraj, J. N.; Zhou, Y.; Zhang, G. Expression, functional analysis and mutation of a novel neutral zearalenone-degrading enzyme. *Int. J. Biol. Macromol.* **2018**, 118, 1284–1292.
- (7) Takahashi-Ando, N.; Kimura, M.; Kakeya, H.; Osada, H.; Yamaguchi, I. A novel lactonohydrolase responsible for the detoxification of zearalenone: enzyme purification and gene cloning. *Biochem. J.* **2002**, 365, 1–6.
- (8) Takahashi-Ando, N.; Ohsato, S.; Shibata, T.; Hamamoto, H.; Yamaguchi, I.; Kimura, M. Metabolism of zearalenone by genetically modified organisms expressing the detoxification gene from *Clonostachys rosea*. *Appl. Environ. Microbiol.* **2004**, 70 (6), 3239–3245.
- (9) Xiang, L.; Wang, Q. H.; Zhou, Y. L.; Yin, L. F.; Zhang, G. M.; Ma, Y. H. High-level expression of a ZEN-detoxifying gene by codon optimization and biobrick in *Pichia pastoris*. *Microbiol. Res.* **2016**, 193, 48–56.
- (10) Zhang, H. Y.; Dong, M. J.; Yang, Q. Y.; Apaliya, M. T.; Li, J.; Zhang, X. Y. Biodegradation of zearalenone by *Saccharomyces cerevisiae*: Possible involvement of ZEN responsive proteins of the yeast. *J. Proteomics* **2016**, 143, 416–423.
- (11) Brodehl, A.; Moller, A.; Kunte, H.-J.; Koch, M.; Maul, R. Biotransformation of the mycotoxin zearalenone by fungi of the genera *Rhizopus* and *Aspergillus*. *FEMS Microbiol. Lett.* **2014**, 359 (1), 124–130.
- (12) Lei, Y. P.; Zhao, L. H.; Ma, Q. G.; Zhang, J. Y.; Zhou, T.; Gao, C. Q.; Ji, C. Degradation of zearalenone in swine feed and feed ingredients by *Bacillus subtilis* ANSB01G. *World Mycotoxin J.* **2014**, 7 (2), 143–151.
- (13) Wang, G.; Yu, M.; Dong, F.; Shi, J.; Xu, J. Esterase activity inspired selection and characterization of zearalenone degrading bacteria *Bacillus pumilus* ES-21. *Food Control* **2017**, 77, 57–64.
- (14) Fu, G.; Ma, J.; Wang, L.; Yang, X.; Liu, J.; Zhao, X. Effect of Degradation of Zearalenone-Contaminated Feed by *Bacillus licheniformis* CK1 on Postweaning Female Piglets. *Toxins* **2016**, 8 (10), 300.
- (15) Wang, Y.; Zhang, J.; Wang, Y.; Wang, K.; Wei, H.; Shen, L. Isolation and characterization of the BC7 strain, which is capable *Bacillus cereus* of zearalenone removal and intestinal flora modulation in mice. *Toxicon* **2018**, 155, 9–20.
- (16) Dai, M.; Jiang, S.; Yuan, X.; Yang, W.; Yang, Z.; Huang, L. Effects of zearalenone-diet on expression of ghrelin and PCNA genes in ovaries of post-weaning piglets. *Anim. Reprod. Sci.* **2016**, 168, 126–137.

- (17) De Saeger, S.; Sibanda, L.; Van Peteghem, C. Analysis of zearalenone and alpha-zearalenol in animal feed using high-performance liquid chromatography. *Anal. Chim. Acta* **2003**, *487* (2), 137–143.
- (18) Bordin, K.; Saladino, F.; Fernandez-Blanco, C.; Ruiz, M. J.; Manes, J.; Fernandez-Franzon, M.; Meca, G.; Luciano, F. B. Reaction of zearalenone and alpha-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability in vitro. *Food Chem.* **2017**, *217*, 648–654.
- (19) Liu, F. X.; Malaphan, W.; Xing, F. G.; Yu, B. Biodetoxification of fungal mycotoxins zearalenone by engineered probiotic bacterium *Lactobacillus reuteri* with surface-displayed lactonohydrolase. *Appl. Microbiol. Biotechnol.* **2019**, *103* (21–22), 8813–8824.
- (20) Poor, M.; Faisal, Z.; Zand, A.; Bencsik, T.; Lemli, B.; Kunsagi-Mate, S.; Szenté, L. Removal of Zearalenone and Zearalenols from Aqueous Solutions Using Insoluble Beta-Cyclodextrin Bead Polymer. *Toxins* **2018**, *10* (6), 216.
- (21) Xu, H.; Luo, F.; Zhang, J.; Jia, P.; Zhang, W.; Li, X. Development of a rapid screening and surveillance for estrogenic chemicals in environment based on recombinant yEGFP yeast cell. *Toxicol. In Vitro* **2010**, *24* (4), 1285–1291.
- (22) Zheng, H.; Liu, Y.; Liu, X.; Wang, J.; Han, Y.; Lu, F. Isolation, purification, and characterization of a thermostable xylanase from a novel strain, *Paenibacillus campinasensis* G1–1. *J. Microbiol. Biotechnol.* **2012**, *22* (7), 930–938.
- (23) Zheng, H.; Yu, Z.; Shu, W.; Fu, X.; Zhao, X.; Yang, S.; Tan, M.; Xu, J.; Liu, Y.; Song, H. Ethanol effects on the overexpression of heterologous catalase in *Escherichia coli* BL21 (DE3). *Appl. Microbiol. Biotechnol.* **2019**, *103* (3), 1441–1453.
- (24) Zeng, Y.; Xu, J.; Fu, X.; Tan, M.; Liu, F.; Zheng, H.; Song, H. Effects of different carbohydrate-binding modules on the enzymatic properties of pullulanase. *Int. J. Biol. Macromol.* **2019**, *137*, 973–981.
- (25) Chen, S. W.; Wang, H. T.; Shih, W. Y.; Ciou, Y. A.; Chang, Y. Y.; Ananda, L.; Wang, S. Y.; Hsu, J. T. Application of Zearalenone (ZEN)-Detoxifying *Bacillus* in Animal Feed Decontamination through Fermentation. *Toxins* **2019**, *11* (6), 330.
- (26) Gonzalez Pereyra, M. L.; Di Giacomo, A. L.; Lara, A. L.; Martinez, M. P.; Cavaglieri, L. Aflatoxin-degrading *Bacillus* sp. strains degrade zearalenone and produce proteases, amylases and cellulases of agro-industrial interest. *Toxicon* **2020**, *180*, 43–48.
- (27) Guo, Y.; Huo, X.; Zhao, L.; Ma, Q.; Zhang, J.; Ji, C.; Zhao, L. Protective Effects of *Bacillus subtilis* ANSB060, *Bacillus subtilis* ANSB01G, and *Devosia* sp. ANSB714-Based Mycotoxin Biodegradation Agent on Mice Fed with Naturally moldy Diets. *Probiotics Antimicrob. Proteins* **2020**, *12* (3), 994–1001.
- (28) Hsu, T. C.; Yi, P. J.; Lee, T. Y.; Liu, J. R. Probiotic characteristics and zearalenone-removal ability of a *Bacillus licheniformis* strain. *PLoS One* **2018**, *13* (4), No. e0194866.
- (29) Everley, R. A.; Huttlin, E. L.; Erickson, A. R.; Beausoleil, S. A.; Gygi, S. P. Neutral Loss Is a Very Common Occurrence in Phosphotyrosine-Containing Peptides Labeled with Isobaric Tags. *J. Proteome Res.* **2017**, *16* (2), 1069–1076.
- (30) Berthiller, F.; Werner, U.; Sulyok, M.; Krska, R.; Hauser, M. T.; Schuhmacher, R. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination of phase II metabolites of the mycotoxin zearalenone in the model plant *Arabidopsis thaliana*. *Food Addit. Contam.* **2006**, *23* (11), 1194–1200.
- (31) Nathanail, A. V.; Syvahuoko, J.; Malachova, A.; Jestoi, M.; Varga, E.; Michlmayr, H.; Adam, G.; Sievilainen, E.; Berthiller, F.; Peltonen, K. Simultaneous determination of major type A and B trichothecenes, zearalenone and certain modified metabolites in Finnish cereal grains with a novel liquid chromatography-tandem mass spectrometric method. *Anal. Bioanal. Chem.* **2015**, *407* (16), 4745–4755.
- (32) Kovalsky Paris, M. P.; Schweiger, W.; Hametner, C.; Stuckler, R.; Muehlbauer, G. J.; Varga, E.; Krska, R.; Berthiller, F.; Adam, G. Zearalenone-16-O-glucoside: A New Masked Mycotoxin. *J. Agric. Food Chem.* **2014**, *62* (5), 1181–1189.
- (33) Rogowska, A.; Pomastowski, P.; Sagandykova, G.; Buszewski, B. Zearalenone and its metabolites: Effect on human health, metabolism and neutralisation methods. *Toxicon* **2019**, *162*, 46–56.
- (34) Jard, G.; Liboz, T.; Mathieu, F.; Guyonvarc'h, A.; Andre, F.; Delaforge, M.; Lebrihi, A. Transformation of zearalenone to zearalenone-sulfate by *Aspergillus* spp. *World Mycotoxin J.* **2010**, *3* (2), 183–191.
- (35) Plasencia, J.; Mirocha, C. J. Isolation and Characterization of Zearalenone Sulfate Produced by *Fusarium* Spp. *Appl. Environ. Microbiol.* **1991**, *57* (1), 146–150.
- (36) Barbosa, A. C. S.; Feng, Y.; Yu, C.; Huang, M.; Xie, W. Estrogen sulfotransferase in the metabolism of estrogenic drugs and in the pathogenesis of diseases. *Expert Opin. Drug Metab. Toxicol.* **2019**, *15* (4), 329–339.
- (37) Borzekowski, A.; Drewitz, T.; Keller, J.; Pfeifer, D.; Kunte, H. J.; Koch, M.; Rohn, S.; Maul, R. Biosynthesis and Characterization of Zearalenone-14-Sulfate, Zearalenone-14-Glucoside and Zearalenone-16-Glucoside Using Common Fungal Strains. *Toxins* **2018**, *10* (3), 104.
- (38) Cooper, R. A.; Kornberg, H. L. The mechanism of the phosphoenolpyruvate synthase reaction. *Biochim. Biophys. Acta, Gen. Subj.* **1967**, *141* (1), 211–213.
- (39) Spanogiannopoulos, P.; Waglechner, N.; Koteva, K.; Wright, G. D. A rifampicin inactivating phosphotransferase family shared by environmental and pathogenic bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (19), 7102–7.
- (40) Qi, X.; Lin, W.; Ma, M.; Wang, C.; He, Y.; He, N.; Gao, J.; Zhou, H.; Xiao, Y.; Wang, Y.; Zhang, P. Structural basis of rifampin inactivation by rifampin phosphotransferase. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (14), 3803–8.
- (41) Warth, B.; Preindl, K.; Manser, P.; Wick, P.; Marko, D.; Buerki-Thurnherr, T. Transfer and Metabolism of the Xenoestrogen Zearalenone in Human Perfused Placenta. *Environ. Health Perspect.* **2019**, *127* (10), 107004.