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Detoxification of zearalenone by *Lactobacillus pentosus* strains

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ABSTRACT

Zearalenone (ZEA) contamination in food samples plays a critical role in food safety, since it causes serious health problems. Usage of microorganisms, such as lactic acid bacteria (LAB), is a promising new approach for detoxification. Eight *Lactobacillus pentosus* strains were evaluated for their ability to remove ZEA from a sodium acetate buffer solution with initial ZEA concentrations of 5.51–74.70 μg/mL. The adsorption capacity increased with increasing ZEA concentrations. The strain JM0812 showed the highest adsorption capability, at 83.17%, in solution containing 74.70 μg/mL ZEA, followed by UM054 (82.78%) and UM055 (81.69%), respectively. Three adsorption isotherms were applied to predict the removal efficiency of ZEA and the Freundlich isotherm appeared to have the best-fit for ZEA sorption onto bacterial cells. Our results indicate that *Lb. pentosus* strains are novel promising strains to reduce mycotoxin contamination in food products.

1. Introduction

Zearalenone (ZEA) is an estrogenic mycotoxin produced by *Fusarium* fungi. Several species of the genus frequently contaminate a number of different grains and other crops, especially maize, sorghum, wheat, rice, barley, oats, nuts, soybean and sesame (Abia, Warth, Sulyok, Krska, Tchana, Njobeh, et al., 2013; Avantaggiato, Havenaar, & Visconti, 2003; Binder, Tan, Chin, Handl, & Richard, 2007; Marroquín-Cardona, Johnson, Phillips, & Hayes, 2014; Rashedi, Ashjaazadeh, Sohrabi, Azizi, & Rahim, 2012; Zinedine, Soriano, Moltó, & Mañes, 2007). Intake of ZEA and its metabolites may also result from consumption of meat, milk and eggs from animals exposed to these toxins (Belhassen, Jimenez-Diaz, Arrebola, Ghali, Ghorbel, Olea, et al., 2015). In general, the toxins are stable during cooking, except under alkaline conditions or during extrusion cooking (heating under high pressure) (EFSA., 2011). Because of its hepatotoxic, hematotoxic, immunotoxic, genotoxic, teratogenic and carcinogenic effects on a variety of mammalian species, ZEA is considered as one of the mycotoxins of most significance (Zinedine et al., 2007). ZEA and its derivatives are an important class of endocrine disrupters, which can cause estrogenic effects and alterations in the reproductive tract of laboratory and domestic animals. (JECFA., 2000) described this estrogenic syndrome as occurring because the structure of ZEA resembles that of 17-beta-estradiol, an estrogen hormone. Meanwhile, Pillay et al. (2002) observed that estrogenic target cells showed hyperestrogen, vulvovaginitis and estrogenic responses to ZEA. Moreover, Pfohl-Leszkowicz, Chekir-Ghedira, and Bacha (1995) confirmed the genotoxicity of ZEA and its potential to induce hepatocellular adenomas rather than tumors on genital organs of mice.

The occurrence of ZEA in cereal crops can cause economic losses since contaminated products can possibly act as an indirect source of exposure for humans, as well as animals, by carryover of the mycotoxins and their metabolites, potentially affecting human health and animal productivity.

Several methods have been attempted to decontaminate ZEA via chemical, physical, and biological process. For example, H_2O_2 was applied in treatments for detoxification of ZEA in cereals (Abd Alla, 1997), and ozone gas (O_3) was used to detoxify ZEA in contaminated grains (McKenzie et al., 1997). Physical decontamination of this toxin was investigated by a number of researchers such as Ramos, Hernandez, Pla-Delfina, and Merino (1996) (using bentonite), Lemke, Grant, and Phillips (1998) (using montmorillonite), Avantaggiato et al. (2003) (using cholestyramine) and

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Bueno, Di Marco, Oliver, and Bardon (2005) (using activated carbon). Biological detoxification of ZEA by yeast has been investigated by Völkl and Karlovsky (1998), Joannis-Cassan, Tozlovanu, Hadjeba-Medjdoub, Ballet, and Pfohl-Leszkowicz (2011), and Tang et al. (2013) while, Sun et al. (2014) used *Aspergillus niger*. Even though many safe, efficient methods have been introduced for ZEA detoxification in foods and feeds over the last two decades (Ramos et al., 1996; McKenzie et al., 1997; Avantaggiato et al., 2003; Bueno et al., 2005; Joannis-Cassan et al., 2011), several of those are not practical or are too expensive for general use.

Recently, LAB strains have been examined for their potential to detoxify ZEA and for their feasibility to be used in food industry (El-Nezami, Polychronaki, Salminen, & Mykkanen, 2002; Lu, Liang, & Chen, 2011; Mokoena, Chelule, & Gqaleni, 2005; Zhao, Jin, Lan, Zhang, Ren, Zhang, et al., 2015). LAB strains are regarded as safe in the Generally Recognized as Safe (GRAS) list of the Food and Drug Administration of the United States (USFDA) and by the Scientific Committee on Animal Nutrition (SCAN) of the EU (Itsaranuwat & Robinson, 2003).

This study was carried out to evaluate the potential of eight *Lactobacillus pentosus* strains to remove ZEA from sodium acetate buffer solution containing different initial ZEA concentrations. Three adsorption isotherms, namely Langmuir, Freundlich, and Hill models were applied to predict the removal efficiency of ZEA.

2. Materials and methods

2.1. Chemicals and reagents

ZEA standards were supplied by Sigma–Aldrich (St. Louis, MO, USA). Solvents for HPLC analysis were of chromatographic grade, purchased from Fisher Scientific (France). De Man Rogosa Sharpe (MRS) medium (Criterion, USA) was used for lactic acid bacteria culture and ZEA detoxification experiments. Deionized water from a Millipore Milli-Q-P Plus system was used for preparing aqueous solutions and HPLC analysis. The stock solution (0.1 mg/mL) of ZEA standard was prepared in methanol and stored at −20 °C when not in use.

2.2. Bacterial strains and culture conditions

Eight strains of *Lb. pentosus* (DM068, JM0812, JM085, UM054, UM055, VM095, VM096 and YM122) used for detoxification experiments were obtained from Food Biotechnology and Lactic Acid Bacteria Laboratory, Faculty of Technology, Mahasarakham University, Thailand. The strains were previously tested for probiotic properties (Kansandee, 2010). These LAB strains were maintained in MRS broth supplemented with 20% (v/v) glycerol at −20 °C. To prepare bacterial cells for the toxin-binding experiments, they were activated twice in MRS broth incubated at 37 °C for 24 h in a 5% CO_2 incubator (Contherm, Australia). The strains were then grown on MRS agar for cell preparation prior to freeze-drying. These bacterial cells were suspended in 0.85% NaCl before being transferred to a microcentrifuge tube. The cells were harvested by centrifugation at 10,000 rpm at 4 °C for 10 min and the cell pellets were collected, washed twice with sterile water and resuspended in 2 mL of 10% skim milk. Thereafter, the cell suspension was transferred to a sterilized serum vial, freeze-dried (Heto power dry PL3000) and stored at −20 °C when not in use.

2.3. Detoxification of ZEA by lactic acid bacteria

The toxic-binding experiments were performed based on the procedure described by Joannis-Cassan et al. (2011) with minor modifications. Briefly, 5 mg of the freeze-dried bacterial cells were transferred to a centrifuge tube and a 990 μL volume of 0.05 M sodium acetate buffer (pH 5.0) was added. The centrifuge tube was vortexed for 20 s and agitated on a thermostatically controlled shaker (Ping-Pong 74582, Fisher Bioblock Scientific, IIIkirch, France) with 175 rpm at 37 °C for 5 min. Then ZEA standards at concentrations of 5.51, 23.08, 51.79 and 74.70 μg/mL, prepared with 0.05 M sodium acetate buffer (pH 5.0), were added to the tube with a working volume of 10 μL, giving a final volume of 1 mL. The mixture were mixed and incubated on a thermostatically controlled shaker with 175 rpm at 37 °C for 15 min. Following centrifugation (Refrigerated Microfuge SIGMA 1-15k, UK) at 9200 × *g* at 20 °C for 10 min (Hadjeba-Medjdoub, Faucet-Marquis, Joannis-Cassan, Ballet, & Pfohl-Leszkowicz, 2009) and the supernatant was collected for analysis of ZEA by HPLC. Treatments without addition of ZEA served as controls.

2.4. Analysis of ZEA by HPLC-FLD

Analysis of ZEA by HPLC was carried out according to Dall'Asta, Galaverna, Dossena, and Marchelli (2004). The HPLC system consisted of a Gilson 811B dynamic chromatography pump and an ICS autosampler (ICS, Bruges, France) coupled to a Spectra Physics 2000 fluorescence detector (FLD). Chromatographic separations were carried out on a C-18 Spherisorb column (250 mm × 4 mm i.d., 3 μm, ProntoSIL™ 120-3-C18) and the following mobile phases were used for gradient elution: A, methanol/acetonitrile/6.5 mM ammonium formate (200:200:600, v/v/v) adjusted to pH 3.5 with formic acid; B, methanol/acetonitrile/6.5 mM ammonium formate (350:350:300, v/v/v) adjusted to pH 3.5 with formic acid. The gradient conditions were programmed according to Faucet-Marquis et al. (2006), starting at 100% A, reaching to 70% B after 25 min. Next, 70% B was maintained for 5 and ramped up to 100% B after 15 min, maintained for 10 min when the gradient was then finally returned to 100% A in 3 min. The flow rate was 0.5 mL/min and injection volume was 20 μL. ZEA was detected by FLD and the wavelengths for excitation and emission were 275 and 450 nm, respectively. Normasoft software (ICS, France) was used for the acquisition of chromatographic data.

2.5. Isotherm model studies

The isotherms were obtained for the biosorption experiments using a similar procedure as previously described by Joannis-Cassan et al. (2011). The biosorption capacity of adsorbent for ZEA was investigated at different initial concentrations of ZEA (1.10, 5.51, 23.08, 51.79 and 74.70 μg/mL). The biosorption isotherms are described by the adsorption isotherm models of Langmuir, Freundlich and Hill.

Langmuir isotherm (LI): The Langmuir model is the most commonly-used equation, because its coefficients have real meanings. The Langmuir equation is valid for monolayer adsorption onto a surface with a finite number of identical sites (Langmuir, 1918). The well-known expression of the Langmuir model is given by the following equation:

$$Q_e = Q_{max}[K_L C_e/(1 + K_L C_e)] \quad (1)$$

Where *Q*e (mg/g) is the amount of toxin per unit weight of adsorbent in adsorbing equilibrium; and *C*e (mg/L) is the equilibrium concentration of toxin; *Q*max (mg/g) is the maximum amount of the toxins per unit weight of adsorbent to form a complete monolayer on the surface bound at high C_e (mg/L); and K_L (L/mg) is the Langmuir adsorption equilibrium constant relating the free energy and affinity of the binding sites. A plot of *Ce*/*Qe* versus *Ce* should indicate a straight line of slope 1/*Qmax* and intercept of $1/K_L Qmax$.

Freundlich isotherm (FI): The empirical Freundlich equation, based on sorption onto a heterogeneous surface, is defined as the

following (Freundlich, 1906):

$$Q_e = K_F \times C_e^{1/nF} \quad (2)$$

Where K_F is the Freundlich constant characteristic relating the biosorption capacity and n is an empirical parameter relating the biosorption intensity, which varies with the heterogeneity of the materials. The parameters K_F and n_F can be determined from the linear plot of log Q_e versus log C_e.

Hill isotherm (HI): This isotherm is used to describe the binding of different species onto a homogeneous substrate. This model assumes that the adsorption is a cooperative phenomenon due to the ability of ligand binding at one site on a macromolecule to influence ligand binding at a different site on the same macromolecule. The Hill equation is (Hill, 1910):

$$Q_e = Q_{maxH}\left[C_e^{nH}/\left(K_D + C_e^{nH}\right)\right] \quad (3)$$

Where *Ce* is residual toxin concentration at equilibrium (mg/L); *Qe* is adsorbed toxin quantity per gram of biomass (mg/g); K_D is the Hill constant; *QmaxH* (mg/g) is a maximum specific uptake corresponding to site saturation. n_H is the Hill cooperative coefficient for the binding interaction. Thus, three possibilities can occur if; $n_H > 1$, positive cooperative in binding, $n_H = 1$, non-cooperative or hyperbolic binding, and $n_H < 1$, negative cooperative in binding.

2.6. Statistical analysis

The data were collected from three independent trials and SPSS version 17.0 was used for data analysis. Results are presented as mean ± standard deviation (SD). One-way ANOVA was used to compare the dataset in combination with Duncan's multiple range test to determine significant differences ($p < 0.05$) among means.

3. Results and discussion

3.1. Characterization of ZEA detoxification

The ZEA-binding properties of eight *Lb. pentosus* strains are depicted in Fig. 1. As shown, all of the studied strains were capable of removing ZEA from sodium acetate buffer solution at pH 5.0, with detoxifying efficiencies varying significantly ($p < 0.05$) across strains and across the initial ZEA concentrations applied. With an initial ZEA concentration range of 5.15–74.60 μg/mL, the strain JM0812 showed the highest binding capacity with a ZEA adsorption level of 82.78% at an initial ZEA of 74.60 μg/mL, followed by UM054 (83.17%), UM055 (81.69%), DM068 (75.17%), VM095 (71.68%), and VM096 (70.38%), respectively. El-Nezami et al. (2002) also examined the detoxifying properties of two LAB strains (*Lactobacillus rhamnosus* GG and *Lb. rhamnosus* LC-705) and found that these two strains exhibited strong binding capabilities for ZEA as well as its derivative, α-zearalenone, in liquid medium showing detoxifying levels of 38% and 46%, respectively.

It was noted (as expected) that the detoxifying capabilities of the eight tested strains relied considerably on the initial concentration of ZEA, in which the highest level of ZEA adsorption of 60.15–83.17% was seen with a high ZEA concentration of 74.70 μg/mL, whilst binding efficiencies of no more than 50% were observed for low concentrations of 5.15–23.08 μg/mL. These results are similar to those of a previous study (Fuchs et al., 2008), which investigated the binding of patulin and ochratoxin by LAB in liquid medium and demonstrated that the detoxifying capabilities of LAB increased with increasing mycotoxin concentrations. Zhao et al. (2015) also found that the detoxifying capabilities of *Lactobacillus plantarum* strains depended strongly on the initial concentration of toxins, the viability of bacteria and the incubation temperature. By contrast, the ZEA-binding capabilities of yeasts have been shown to decrease with increased initial concentrations of ZEA (Joannis-Cassan et al., 2011).

The increase in the detoxifying capabilities of *Lb. pentosus* strains observed in the present study might be due to the high availability of ZEA that facilitates cell-substrate contact, thereby resulting in increased ZEA binding. In contrast, a reduction in the binding mechanism with ZEA might be a result of a decrease in the number of binding sites on bacterial cell walls that remain unsaturated during the binding process.

The mechanism of mycotoxin binding by LAB has not been clearly described. LAB is a Gram-positive bacteria with cell walls comprised of thick peptidoglycan layers connected by amino acid bridges. Other components are polyalcohols like teichoic acid (TA), which give the Gram-positive cell wall an overall negative charge due to the presence of phosphodiester bonds between the teichoic acid monomers (Yun, Vijayaraghavan, & Won, 2011). Some components are lipid-linked to form lipoteichoic acid (LTA) and polysaccharide (Delcour, Ferain, Deghorain, Palumbo, & Hols, 1999). It has been postulated that mycotoxin-binding positions occur at bacterial cell walls (Zinedine et al., 2007) and mycotoxins are likely to be attached by teichoic acid and polysaccharide moieties rather than peptidoglycan moieties (Shetty & Jespersen, 2006). The chemical functional groups of bacterial cell walls play a vital role

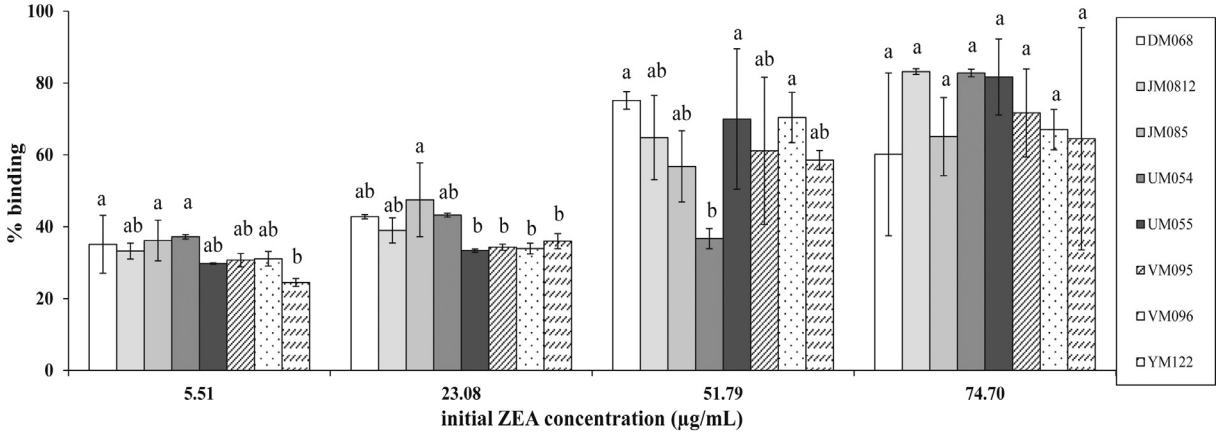

Fig. 1. Binding capabilities of eight *Lb. pentosus* strains for the biosorption of ZEA at four different concentrations of ZEA (5.51–74.70 ppm) in a 0.05 M sodium acetate buffer (pH 5.0) solution. Bars represent the means of replicates while error bars indicate standard deviation. Letters indicate means separation ($p < 0.05$) within each initial ZEA concentration.

in biosorption process. The anionic functional groups, for example, present in the peptidoglycan, teichoic acids and teichuronic acids of Gram-positive bacteria are the components that are primarily responsible for anionic characters and the binding capability (Sherbert, 1978). The biosorption process has been postulated to depend not only on the type or chemical composition of the biomass, but also on external physicochemical factors and solution chemistry (Yun et al., 2011). For instance, Wang et al. (2015) identified the characteristics of the inactive LAB cells involved in the adsorption of patulin and indicated that the increased adsorption capacities of LAB were associated with increased specific surface area.

Recent literature has also revealed that hydrophobic interactions play an important role in the adsorption process of toxins like patulin, AFB1, and ZEA by microorganism cells (El-Nezami, Polychronaki, Lee, Haskard, Juvonen, Salminen, et al., 2004; Guo, Yuan, Yue, Hatab, & Wang, 2012; Haskard, Binnion, & Ahokas, 2000). In addition to polysaccharides, proteins have been elucidated to be involved in adsorption processes (Guo et al., 2012). For example, Zoghi, Khosravi-Darani, and Sohrabvandi (2014) showed that the adsorption of toxins by yeasts and bacteria was predominantly related to specific protein and carbohydrate components in the cell wall. The peptidoglycan layer contains mostly C–O, OH and NH functional groups and has been reported as being the major component of 'highly porous' cell walls (Tripathi, Beaussart, Andre, Rolain, Lebeer, Vanderleyden, et al., 2012). Moreover, Wang et al. (2015) claimed that surface hydrophobicity was conductive to the adsorption of patulin and also that lipoteichoic acids and envelope proteins were important for the hydrophobicity of cell surface. Besides, the strength of the mycotoxin-LAB interaction was influenced by the peptidoglycan structure and its amino acid composition (Niderkorn, Morgavi, Aboab, Lemaire, & Boudra, 2009). Dalié, Deschamps, and Richard-Forget (2010) indicated that binding probably takes place on the cell wall and carbohydrates and/or protein components of LAB play a major role in toxin binding. On the basis of the chemical moieties and interactions involved in ZEA and a-zearalenol binding by LAB, it is likely that carbohydrates and proteins are involved in the process (El-Nezami et al., 2004).

3.2. Biosorption isotherm of ZEA

Adsorption equilibrium is established when the quantity of the toxin being adsorbed (Q_{eq}) is equal to the quantity being desorbed when the equilibrium concentration in solution (C_{eq}) remains constant. The plots $Q_{eq} = f(C_{eq})$ for all adsorption experiments are presented in Fig. 2. It was observed that the initial ZEA concentration considerably influenced the equilibrium binding of ZEA as well as the adsorption yield, in which the adsorbed amount of ZEA increased with increased initial ZEA concentration, due to the fact that the increase in the initial ZEA concentration provided an increased driving force to overcome all mass transfer resistance, thereby resulting in higher toxin binding rates.

From a fundamental perspective, the equilibrium sorption isotherms are of great importance in the design of sorption systems. Insights into the sorption mechanism, surface properties and the affinity of the sorbent are often achieved through the equation parameters and the underlying thermodynamic assumptions of these equilibrium models (Guo, Yue, Yuan, Wang, Guo, Wang, et al., 2013). In the current study, three isotherm models (Langmuir, Freundlich and Hill) were applied to predict the biosorption of ZEA onto eight *Lb. pentosus* strains. The isotherm set parameter values are given in Table 1.

Based on the R^2 values and ERRSQ values, the Freundlich isotherm appeared to show a high goodness of fit for ZEA biosorption onto LAB cells, with a high correlation coefficient of $R^2 > 0.80$ and low ERRSQ for all strains. The n_F value was also found to be very close to 1, meaning that model approximates Henry's law - when the biosorption of ZEA onto LAB cells is a simple linear solid/liquid partition, it follows Henry's law (Ringot et al., 2007).

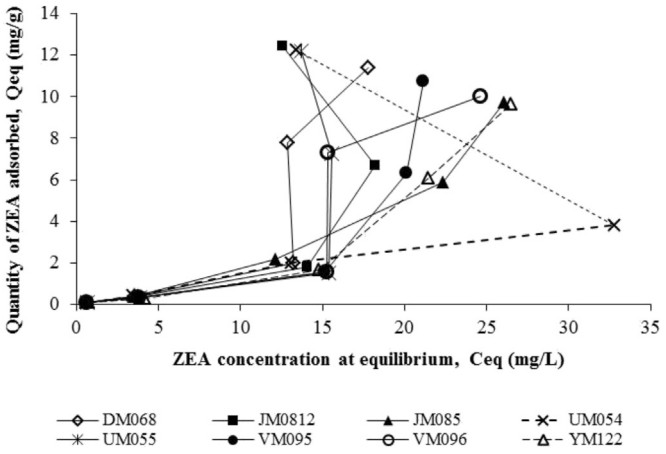

Fig. 2. Isotherm curves from experimental data for the biosorption of ZEA onto eight *Lb. pentosus* strains in a 0.05 M sodium acetate buffer (pH 5.0).

The coefficient K_F in the Freundlich isotherm is a measure of the stability of the complex formed between ZEA and LAB cells. The small K_F values (0.08) obtained by YM122 implied a strong binding of ZEA to LAB cells in this study. High values of determination (R^2) were also obtained using the Hill model - when n_H is higher than 1, the binding of ZEA by bacterial cells is considered to be a positive cooperative interaction. The results obtained in this study indicated that the Langmuir model was not suitable to predict the biosorption of ZEA since it showed the lowest R^2.

The results obtained in this study indicated that the Freundlich model was suitable for predicting the biosorption of ZEA onto LAB cells. The model is based on the sorption heterogeneity of active sites accompanied by interactions between adsorbed molecules. This conclusion is supported by an earlier study (Ramos et al., 1996), which demonstrated that the Freundlich isotherm applied to predict the biosorption of ZEA by adsorbent materials showed a better fit than the Langmuir isotherm.

4. Conclusions

This study has demonstrated the removal of ZEA by lactic acid bacteria. Eight *Lb. pentosus* strains were tested for their potential to detoxify ZEA in a 0.05 mM sodium acetate buffer (pH 5.0) solution at four different initial concentrations of ZEA (5.51, 23.08, 51.79 and 74.70 μg/mL) and the results showed that the detoxifying capabilities of *Lb. pentosus* strains differed considerably across strains and were influenced by the initial ZEA concentration. The studied strains showed good detoxifying capabilities, which bound 29.74–83.17% of ZEA from the solution containing initial ZEA concentrations of 5.51–74.70 μg/mL. The strain JM0812 had the highest binding capability (83.17%) at initial concentration of ZEA of 74.70 μg/mL, followed by UM054 (82.78%), and UM055 (81.69%). The findings obtained showed that the adsorption capacities increased with increasing ZEA concentrations. Three adsorption isotherms were applied to predict the removal efficiency of ZEA and the Freundlich isotherm appeared to have the best fit for ZEA sorption onto bacterial cells. The present study indicates that *Lb. pentosus* strains are promising novel strains for reducing mycotoxin contamination in food products.

Table 1
Parameters for the application of Langmuir, Hill and Freundlich isotherm models for the biosorption of ZEA by eight *Lb. pentosus* strains.

Parameters[a]	Biosorption (*Lb. pentosus* strains)							
	DM068	JM0812	JM085	UM054	UM055	VM095	VM096	YM122
Langmuir isotherm model								
Q_{max}	−2.87	−3.55	−6.43	33.66	−2.98	−3.53	−3.86	−2.66
K_L	−0.04	−0.03	−0.02	0.01	−0.03	−0.03	−0.03	−0.03
R^2	0.54	0.32	0.57	0.02	0.25	0.45	0.38	0.66
ERRSQ	35.89	99.51	1.96	103.44	109.70	33.52	27.07	3.61
Hill isotherm model								
Q_{Hmax}	11.38	12.42	9.72	12.25	12.20	10.71	10.01	9.64
n_H	1.47	1.30	1.27	1.05	1.35	1.29	1.41	1.41
K_D	82.42	95.71	59.97	86.61	107.69	90.77	82.14	115.02
R^2	0.78	0.87	0.93	0.99	0.78	0.85	0.75	0.87
ERRSQ	57.64	103.44	25.04	108.48	104.31	55.90	40.54	32.78
Freundlich isotherm model								
K_F	0.13	0.14	0.16	0.16	0.12	0.12	0.12	0.08
n_F	0.73	0.77	0.87	0.90	0.74	0.80	0.79	0.77
R^2	0.89	0.83	0.96	0.80	0.80	0.89	0.89	0.93
ERRSQ	40.82	82.81	10.00	103.96	84.66	36.55	29.19	17.43

ERRQS: The sum of the squares of the errors was optimized as a relevant error indicator comparable to the coefficient of determination in the linear models.
[a] For the signification of isotherm parameters, see Eqs. (1)–(3).

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