



Application of a yeast estrogen reporter system for screening zearalenone degrading microbes

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HIGHLIGHTS

- We performed a method to select microbes for zearalenone degradation.
- An estrogen screen test was adapted to biodegradation tests.
- The most appropriate microbe with the weakest harmful biological effects was selected.
- We highlighted the necessity to employ bio-tests in biodegradation assays.

ARTICLE INFO

Article history:

Received 22 February 2012

Received in revised form

27 November 2012

Accepted 29 November 2012

Available online xxx

Keywords:

Zearalenone

Saccharomyces cerevisiae

Estrogen

Biomonitoring

Biodegradation

ABSTRACT

The aim of this study was to screen microbes for their zearalenone degrading potential and to select microbes whose activities do not create toxic or endocrine disrupting metabolites. Bioluminescent bioreporters (*Saccharomyces cerevisiae* BLYES and BLYR) were successfully used to monitor toxin degradation; the results of zearalenone biodegradation experiments were confirmed by parallel chemical analysis (HPLC-FLD) and immunoanalytical (ELISA) tests. Using the BLYES/BLYR bioreporters, the most appropriate microbes (ones that produced minimal toxic products and products with lower estrogenic potential) could be selected. The most promising strains belong to *Streptomyces* and *Rhodococcus* genera. Our findings demonstrate the benefit of using biological tests beside the analytical method, since bioreporters were able to monitor the samples for toxicity and estrogenic potential even after substantial degradation. We conclude that the BLYES/BLYR bioreporter system is a cost effective, fast and reliable tool for screening zearalenone-degrading microbes.

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1. Introduction

Zearalenone (ZON) is a mycotoxin produced by a number of species of *Fusarium*. ZON is a naturally occurring estrogen that is recognized to cause hormonal effects in humans and livestock. ZON occurs in almost every agricultural product, as well as in grains, corn products, and animal feed products [1]. Thus effective methods to monitor for the presence of ZON and its breakdown products are essential.

The European Food Safety Authority (EFSA) reported that contamination of agricultural feed by mycotoxins may be reduced by

mycotoxin-detoxifying agents but the additive effects of the resulting metabolite(s)/degradation products(s) must also be monitored in appropriate toxicological studies [2]. Depending on their mode of action, these feed additives may act by reducing the bioavailability of the mycotoxins or by degrading them or transforming them into less toxic metabolites. Therefore, we can define at least two main categories: adsorbing agents and biotransforming agents. For biotransformation of ZON *Lactobacilli* and *Bacilli* [30,32] Čvek et al. [31] are the most effective candidates. In these cases binding, rather than metabolism [21], is the mechanism of detoxification.

Although microbial biotransformation is one method of breaking down ZON, biodegradation of ZON by microbes may cause an unexpected risk as well. Harmful intermediates and by-products such as α -zearalenol (α -ZOL) and β -zearalenol (β -ZOL) may be created, which are also estrogenic [3–5]. Biodegradation of ZON occurs in several microbes: *Streptomyces griseus* ATTC 13273, *S. rutgersensis* NRRL-B 1256, *Rhizopus arrhizus* IFP-6155 could metabolize

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ZON to α -ZOL, while *Aspergillus niger* ATCC 111394, *S. rutgersensis* NRRLB 1256, and *Fusarium oxysporum* S-F3 produced β -ZOL [6].

Yeasts have been demonstrated to produce non-estrogenic products from ZON. e.g. *Clonostachy rosea*, *Thamnidium elegans*, *Mucor baineri*, *Rhizopus* sp., *Cunninghamella baineri*, *Gliocladium roseum* [6–9]. Recently the cleavage of ZON by *Trichosporon mycotoxinivorans* to non-estrogenic metabolites has been described, with estrogenic potential being measure by the estrogen-sensing yeast strain YZRM7 [10].

However, in the case of prokaryotes, only a few authors reported the degradation of ZON to non-estrogenic products [7] reported that the transformation products of *Streptomyces rimosus*, 8'-hydroxy-zearalenone and 2,4-dimethoxyzearalenone, did not bind to rat estrogen receptor, indicating a loss of estrogenicity [7]. Moreover, *Pseudomonas* sp. ZEA1 could metabolize ZON beyond 200 μ g/ml and the residual toxicity against *Artemia salina* was reduced [11]. Also, [12], using the MCF-7 cell proliferation assay, determined that *Acinetobacter* sp. SM04 could degrade ZON without generating estrogenic metabolites [12].

Chemical analysis and immunochemical methods are unable to detect all potential degradation products, therefore it is important to monitor for toxicity and estrogenicity with other methods. The primary biological effect of ZON is an estrogenic effect, thus a rapid and cost effective biological method to measure estrogens would be useful. Sanseverino et al. have developed a low cost, rapid bioassay which measures estrogenic potential and toxicity [13]. *Saccharomyces cerevisiae* BLYES produces bioluminescence in response to chemicals that bind to the human estrogen receptor and then to human estrogen response elements (ERE) on a reporter plasmid. In addition, a constitutive strain (*S. cerevisiae* BLYR) was used to assess whether samples contained toxic substances, which is detected by a decrease in bioluminescence. When an estrogenic chemical enters the cell of *S. cerevisiae* BLYES, it is bound by the human estrogen receptor, forming a complex. This complex then activates the prokaryotic *lux*-genes of *Photobacterium luminescens*, which are located on two plasmids within the yeast, resulting in bioluminescence. This bioluminescent bioreporter system is an appropriate tool to measure estrogenic an toxic potential in ZON-degrading microbes [14,15].

To develop a strain capable of efficient biodegradation of ZON, selection of the most active and environmentally safe microbes is first required. The aim of the present work was to select a microorganism with the highest ZON-degrading potential and lowest production of estrogenic products from a culture collection. For this purpose a combined method involving biological, analytical and immunochemical techniques was applied to analyze ZON and residual estrogenicity after ZON biodegradation.

2. Materials and methods

2.1. Microbial strains used in biodegradation tests

The ZON-degrading potential of microbes belonging to *Streptomyces*, *Gordonia*, *Pseudomonas* and *Rhodococcus* genera were screened. These isolates are from hydrocarbon-contaminated sites and have been deposited in the strain collection of Agruniver Holding Ltd. In addition, *Rhodococcus* strains, which have already been demonstrated to degrade other mycotoxins [17], were screened, originating from István Nagy (Max Planck Institute of Biochemistry) and Michael Goodfellow (University of Newcastle). The strains belonged to five *Rhodococcus* species and were classified as *R. pyridinivorans* K402, K404, K408, AK37, *R. ruber* N361, *R. erythropolis* NI1, *R. globulus* N58, *R. aetherivorans* AK44; *Streptomyces cavourensis* K14, *Gordonia paraffinivorans* NZS6, *Pseudomonas pseudoalcaligenes* FEH28 were also analyzed (Table 1).

Table 1

Origin and identification of the strains used in the degradation experiment.

Species	Strain.	Source
<i>Rhodococcus pyridinivorans</i>	AK 37	Agruniver Holding Ltd.
<i>Rhodococcus pyridinivorans</i>	K402	Agruniver Holding Ltd.
<i>Rhodococcus pyridinivorans</i>	K404	Agruniver Holding Ltd.
<i>Rhodococcus pyridinivorans</i>	K408	Agruniver Holding Ltd.
<i>Rhodococcus erythropolis</i>	NI1	Istvan Nagy
<i>Rhodococcus aetherivorans</i>	AK44	Agruniver Holding Ltd.
<i>Streptomyces cavourensis</i>	K14	Agruniver Holding Ltd.
<i>Gordonia paraffinivorans</i>	NZS6	Agruniver Holding Ltd.
<i>Pseudomonas pseudoalcaligenes</i>	FEH28	Agruniver Holding Ltd.
<i>Rhodococcus ruber</i>	N361	M Goodfellow
<i>Rhodococcus globulus</i>	N58	M Goodfellow

2.1.1. Growth and culture conditions of microbial strains in biodegradation tests

The cells were streaked on LB agar plates and incubated at 28 °C for 72 h. Single colonies were inoculated into 50 ml liquid LB medium and incubated at 170 rpm at 28 °C for 72 h. The optical density of the cultures was adjusted to 0.6 ($OD_{550} = 0.6$) and 5 ml was added to 45 ml sterile LB medium to which 50 μ l ZON had been added (1 μ g/ml final concentration). As controls, 50 ml LB medium with 50 μ l ZON (1 μ g/ml) were used. Samples were incubated (170 rpm, 28 °C) for 7 days. One-milliliter samples from the flasks were removed in every 24-h, centrifuged at 25,000 $\times$ g at 4 °C for 20 min and both supernatant and pellet were stored at –20 °C until further use. Remaining ZON concentrations in samples, taken at the end-points (supernatant and pellet), were analyzed by high performance liquid chromatography (Wessling Hungary Ltd., Hungary) and ELISA (Soft Flow Ltd., Hungary); the supernatant was analyzed by BLYES/BLYR bioassays. All experiments were carried out in triplicate and statistical analyses were performed by STATISTICA 7 (StatSoft Hungary Ltd.), PAST [18], and Microsoft EXCEL software (Microsoft Inc., USA).

2.1.2. Testing of ZON adsorption

Besides ZON biodegradation tests, where the pellet was analysed after the 7th day of incubation; an additional experiment to monitor ZON adsorption on the pellet was carried out. In the experiment four different species were screened, which represents all the different genera in the degradation experiment (*Streptomyces* sp. K14, *Rhodococcus* sp. K402, *Pseudomonas* sp. FEH28, *Gordonia* sp. NZS6). Growth of microbial strains was performed as described above. As controls, microbe free control and control with inactivated (autoclaved) cells were applied. After 6 h of incubation the entire pellet material was removed and remaining ZON concentrations in samples (pellet and supernatant) were analyzed.

2.2. Analysis of samples

2.2.1. BLYES and BLYR test for monitoring estrogenicity and toxicity

S. cerevisiae strains BLYES and BLYR (the University of Tennessee, Knoxville) harboring leucine and uracil selective markers were stored at –20 °C and were grown overnight at 30 °C and 200 rpm to an OD_{600} of 0.1 in a modified minimal medium (YMM leu[–], ura[–]) [16] and then used according to standard protocols (Section 2.2.1.1) [14], or after adapting to biodegradation tests (Section 2.2.1.2).

2.2.1.1. Testing of standard solutions of zearalenone and zearalenols. ZON (Fermentec, Israel) and its derivatives, α -ZOL and β -ZOL, were diluted in acetone to a concentration of 1000 μ g/ml and tested in the concentration range of 31.84 μ g/ml (1E–04 mol/dm³) through 3.18E–05 μ g/ml (2.5E–010 mol/dm³) by preparing a serial dilution, placing 20 μ l of each solution into the appropriate wells of white, sterile, flat bottom, 96-well micro-plate (Grenier

Bio-one GmbH, Germany). Subsequently 200 µl of cultures (BLYES and BLYR) were placed into each well, respectively. Bioluminescence was measured hourly for 5 h in a VictorX Multilabel Plate Reader (Perkin Elmer Inc., Hungary). Positive control was 17β-estradiol in a concentration range of 2.7E–1 µg/ml (2.5E–12 mol/dm³) through 6.8E–7 µg/ml (1E–6 mol/dm³). Negative controls (YMM + yeast cells) and solvent controls (YMM + yeast cells + solvent) were also included.

For data analysis the bioluminescence (percent of the maximum response) versus the concentrations was plotted on a log scale, generating a sigmoid dose–response curve in which the linear portion was used to calculate the concentration at which the assay is 50% effective (EC₅₀); moreover lowest observed effect concentration (LOEC) was calculated by determining the concentration at three times the standard deviation plus background bioluminescence. Toxic response (IC₂₀) was determined by calculating the concentration of chemical that reduces the background bioluminescence of *S. cerevisiae* BLYR by 20%.

2.2.1.2. Testing of samples after biodegradation experiments. Supernatants of ZON biodegradation were examined in 1:2 serial dilution (sample dilution of 100–3.13%) for their potential to mimic human hormones as described in (Section 2.1.1.1); in addition, as positive controls, the microbe-free controls of biodegradation tests (LB medium with 1 µg/ml ZON) were used, and as negative controls the LB medium was added to solvent controls in BLYES/BLYR tests. For data analysis bioluminescence was determined with an inverse formula of Froehner et al. (using bioluminescence intensification, instead of inhibition) as follows [19]:

$$\text{Bioluminescence intensification (\%)} = -1 \times \frac{(C - S)100}{C}$$

where C is the arithmetic mean of the bioluminescence values of parallel negative controls after the incubation time and S represents the bioluminescence average value of parallel samples, determined at the time of contact.

2.2.2. Enzyme-linked immunosorbent assay (ELISA)

Toxin concentrations were determined by ELISA using the TOXI-WATCH system (SoftFow Biotechnology Ltd., Hungary) according to

the manufacturer's instructions. In TOXIWATCH ELISA Kits (Assay range: 8–1 ng/ml, limit of detection: 0.142 ng/ml, limit of quantification: 0.79 ng/ml), methanol (7.5%, v/v) is used for toxin extraction from cereals, while standard curves contain methanol as well. As the matrix of bacterial supernatant was an LB medium it was diluted in methanol–phosphate buffered saline (7.5%, v/v) as a control. Measurements were carried out in triplicate.

2.2.3. High-performance liquid chromatography (HPLC)

The supernatant, collected on the 7th day (end-point) of the degradation experiment, was analyzed for ZON. The bacterial pellet was suspended in (1 ml) methanol and centrifuged (3000 × g for 10 min at <10 °C), then the supernatant was analyzed for ZON [21]. The recovery was calculated as in El-Nezami et al. [21] and was found to be 80–90%. Results for degradation potential were corrected with the pellet analysis. Chemical analytical tests were performed by Wessling Hungary Ltd., an accredited laboratory for HPLC analysis [29].

After dilution (five-fold) of the supernatant and the extracted pellet solution with methanol:water (1:1), a 50 µl aliquot of the sample was injected into the HPLC-FLD system (liquid-chromatography separation and fluorescence detection of toxins was performed according to AOAC Official Method 985.18). The separation was carried out on a C18 column (Vydec Denali, 4.6 mm × 150 mm; 5 µm) using water:acetonitrile (45:55) under isocratic conditions. The ZON was detected with extinction wavelength 274 nm and emission wavelength 440 nm. The limit of quantification was 10 µg/l.

3. Results

3.1. HPLC and ELISA results

The HPLC and ELISA results are illustrated in Table 2, where supernatants originating from the ZON degradation experiments are indicated. Results are expressed in percent of ZON eliminated (percent decrease), which is calculated from the remained ZON concentration in the supernatant divided by the ZON concentration in the microbe free control. The HPLC results correlate well with the

Table 2

Zearalenone elimination from the supernatant compared with zearalenone degrading potential of microbial strains. Results are expressed in percent decrease (%), which is calculated from the remaining ZON concentration in the supernatant divided by the ZON concentration in the microbe free control; and degradation potential (%), which is based on the ZON elimination (percent decrease) from the supernatant and corrected with the ZON concentration after analysing the pellet. Each data point represents the average of triplicate measurements.

Species	Strains	Percent decrease (%)		Degradation potential (%)
		HPLC	ELISA	HPLC
<i>S. cavourensis</i>	K14	98.53 ± 0.00	92.99 ± 1.75	96.45
<i>G. paraffinivorans</i>	NZS6	98.20 ± 0.00	78.89 ± 4.80	93.51
<i>R. pyridinivorans</i>	K402	93.63 ± 4.31	93.02 ± 0.76	91.03
<i>R. pyridinivorans</i>	K404	90.93 ± 0.53	93.00 ± 0.36	87.43
<i>R. pyridinivorans</i>	K408	87.85 ± 2.83	85.39 ± 1.02	82.45
<i>P. pseudoalcaligenes</i>	FEH28	86.96 ± 0.74	82.69 ± 1.14	82.99
<i>R. erythropolis</i>	NI1	75.95 ± 1.00	70.93 ± 2.56	70.00
<i>R. pyridinivorans</i>	AK37	61.14 ± 2.77	64.67 ± 1.77	53.42
<i>R. ruber</i>	N361	54.70 ± 3.46	54.60 ± 3.33	28.10
<i>R. aetherivorans</i>	AK44	27.12 ± 2.24	42.61 ± 4.10	11.68
<i>R. globulus</i>	N58	26.24 ± 20.93	19.92 ± 15.87	19.03

Legend

- supernatant with >90% zearalenone elimination
- supernatant with 50–90% zearalenone elimination
- supernatant with <50% zearalenone elimination

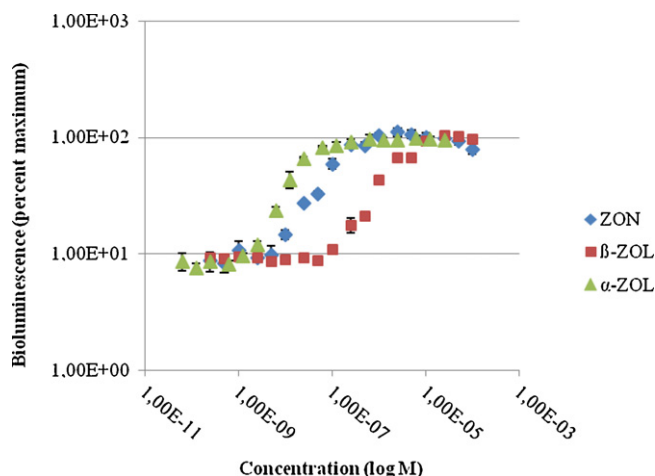


Fig. 1. *Saccharomyces cerevisiae* BLYES response curve for zearalenone (ZON) and its derivatives, α - and β -zearalenol (α -ZOL, β -ZOL). The 'x' axis shows the molar concentration of tested compounds and the 'y' axis shows the bioluminescence of BLYES strain expressed in terms of the percent of the maximum response, compared to the standard curve.

ELISA data (Spearman's Rank correlation value: 0.86). Samples were categorized into three groups: excellent detoxifying strains (>90% ZON elimination), good detoxifying strains (50–80% ZON elimination), insufficient detoxifying strains (<50% ZON elimination). Besides percent decrease, Table 2 shows the degradation potential of the tested strains, calculated based on the ZON elimination (%) from the supernatant and corrected with the ZON concentration after analysing the pellet. *R. pyridinivorans* K404 has a corrected degradation potential of 87% and *R. ruber* N361 has a degradation potential of 28.1%.

3.2. Analysis by BLYES and BLYR

3.2.1. Testing of standard solutions of zearalenone and zearalenols

Essential parameters like EC_{50} , LOEC, IC_{20} of the adapted recombinant yeast bioluminescence assay were determined using ZON

and ZOL standards. Experiments were evaluated after 5 h by plotting percent maximum bioluminescence versus the log of ZON or its most common derivatives (α -ZOL and β -ZOL). The BLYES test showed remarkable sensitivity to ZON after 5 h of contact time, even $1.00E-8$ M ZON in the assay was detectable (corresponds to $0.03 \mu\text{g/ml}$ ZON in solution). For ZON the calculated EC_{50} $2.74E-7$ M corresponds to $0.9 \mu\text{g/ml}$ ZON in solution, while a LOEC of $0.03 \mu\text{g/ml}$ is determined (Fig. 1). BLYES was found to be sensitive for α -ZOL, the calculated EC_{50} of $2.08E-7$ M corresponds to $0.66 \mu\text{g/ml}$ α -ZOL in solution and LOEC of $5.00E-8$ M ($0.02 \mu\text{g/ml}$), therefore the detection limit is $0.2 \mu\text{g/ml}$ α -ZOL. In case of β -ZOL, the EC_{50} $2.97E-6$ M corresponds to $9.51 \mu\text{g/ml}$ β -ZOL in solution, while the LOEC for β -ZOL was $5.00E-8$ ($0.016 \mu\text{g/ml}$), therefore the detection limit of β -ZOL in solution is $0.16 \mu\text{g/ml}$.

For the positive control 17β -estradiol the EC_{50} was $3.74E-9$ ($0.01 \mu\text{g/ml}$); while LOEC was $5E-10$ M, corresponding to $0.0014 \mu\text{g/ml}$ 17β -estradiol in solution. No toxicity was observed with BLYR in the controls, ZON, α -ZOL or β -ZOL.

3.2.2. Testing of samples after biodegradation experiments

For evaluating ZON degrading experiments – involving eleven strains – the adapted recombinant yeast luminescence assay was used and compared with HPLC and ELISA results. Generally strains with the ability to transform ZON demonstrate a decrease in the amount of estrogenic substances in a sample, as shown in Fig. 2. For strains (*R. aetherivorans*, AK44, *R. globerulus* N58) with weak detoxifying ability (less than 50%) the measured estrogenic effect was similar to the control values (control contained $1 \mu\text{g/ml}$ ZON). Strains with good degrading potential *R. pyridinivorans* K408, *R. erythropolis* NI1, *P. pseudoalcaligenes* FEH28, *R. pyridinivorans* AK37, *R. ruber* N361 resulted in more than 50% detoxifying ability and a decreased estrogenic effect. Four out of the eleven investigated strains could eliminate ZON with more than 90% efficiency; however only three of these strains (*Streptomyces cavourensis* K14, *Rhodococcus pyridinivorans* strains K402 and K404) demonstrated decreased estrogenic potential as well. In case of *Gordonia parafinivorans* NZS6 the estrogenic effect decreased by 40%, while almost all of ZON was eliminated.

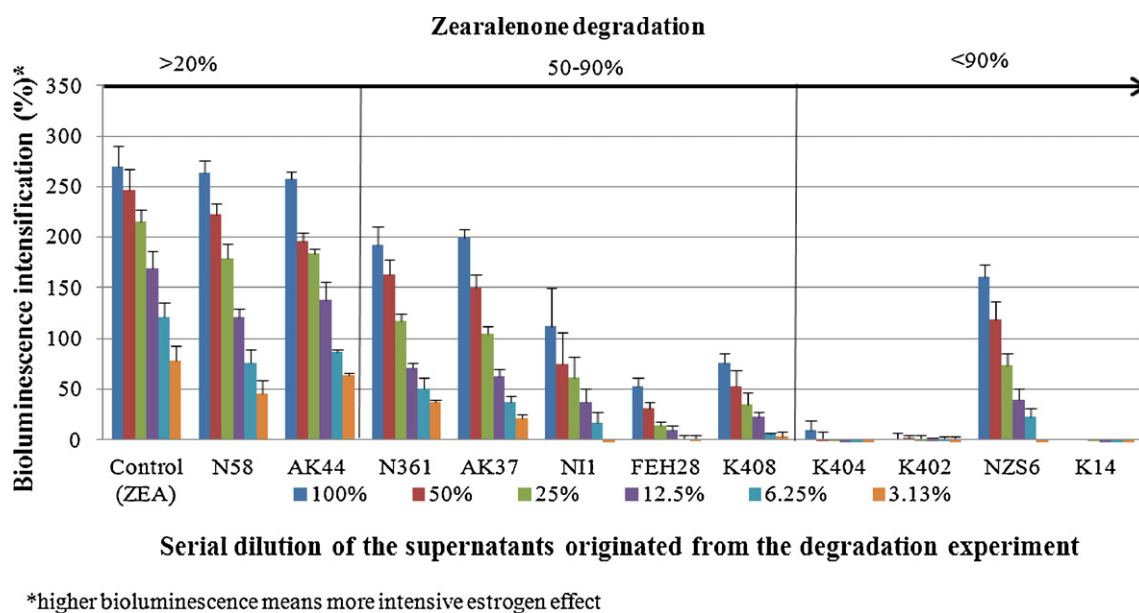


Fig. 2. The toxin-elimination (ELISA and HPLC) and remaining estrogenic effect (BLYES) in the supernatant, measured on samples from the 7th day of the zearalenone degrading experiment. The 'x' axis shows the samples of the degrading strains organized on the basis of zearalenone elimination (%) compared to the microbe free control while 'y' axis shows the remaining estrogenic effect expressed in percent of bioluminescence, measured by the BLYES strain.

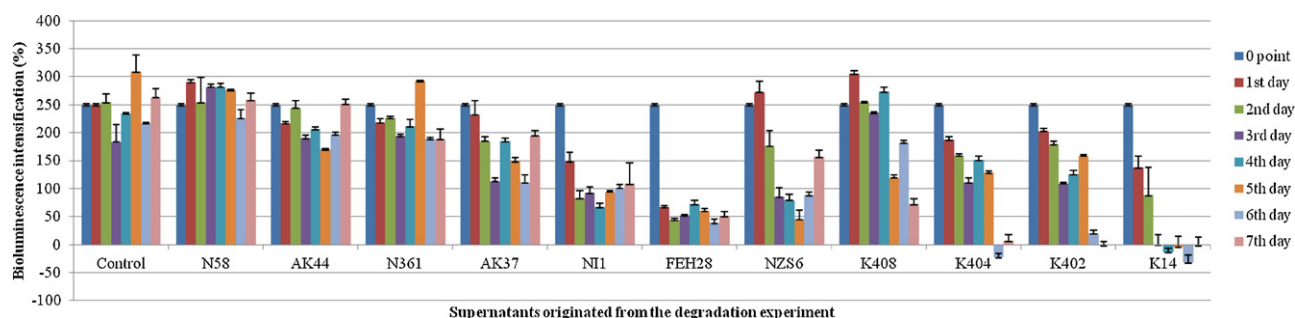


Fig. 3. Time curves for zearalenone degrading potential of strains measured in the supernatant (taken in every 24 h during degradation experiment) by BLYES.

The statistical analysis showed a good negative correlation between HPLC/ELISA and BLYES in all dilutions (Spearman's Rank correlation values in all cases were between -0.85 and -0.97), indicating that higher degradation potential measured by HPLC/ELISA correlates with decreased estrogenic potential, determined by BLYES. The observation that significant ZON degradation correlates with the decrease in estrogenic potential was compared by plotting data, which also provided information on outliers (Fig. 3). Interestingly, *G. paraffinivorans* NZS6 was identified as a strain that eliminated the most ZON (98.2%), yet maintains a high estrogenic potential in the supernatant. This indicates that the by-products of degradation may interact with the human endocrine system (Fig. 4.).

3.3. Testing of ZON adsorption

Testing of samples for ZON adsorption demonstrated that ZON adsorption was negligible after 6 h. The entire pellet material was analyzed by HPLC and the ZON concentration in the pellet was 8.84–13.11%; while in the supernatant the ZON concentration (analyzed by HPLC and ELISA) and the estrogenicity (analyzed by BLYES) were similar to microbe free control, only the *Gordonia paraffinivorans* NZS6 reduced the ZON content and the estrogenicity. In case of inactivated cell controls similar results were observed; however this ZON reduction with the inactivated strain NZS6 was only measured by HPLC, the ELISA did not detect lower ZON concentration or BLYES did not detect less estrogenicity.

Moreover, samples taken in every 24 h were analyzed by BLYES (Fig. 2). The figure shows the estrogen effect detected by BLYES for supernatant collected in every 24 h of the degradation

experiment. The best degrader microbes (K14, K402, and K404) decreased the estrogen effect after 3–5 days. If absorption occurred, estrogen effect would decrease after 1–2 h. This phenomenon also proves that metabolism occurred.

4. Discussion

In our ZON degradation experiments the remaining estrogenic and toxic effects of 1 $\mu\text{g/ml}$ ZON after 7 days of incubation were measured using a bioluminescent bioassay (BLYES and BLYR). In a comprehensive study (Mycostop project) the general mycotoxin contamination of grain crops was evaluated in Hungary. The ZON contamination was detected at a maximum level of 1 mg/kg, therefore a similar concentration was used in this study. Results of bioassays were compared with HPLC and ELISA.

Several studies about ZON degrading microbes have been published recently, most of them aimed at the development of a biological system to detoxify mycotoxins, like the one developed by Biomin Ltd. for ZON and ochratoxin [20]. The first step in this process is to have an appropriate screening tool for ZON. The *S. cerevisiae* BLYES and BLYR bioassays are an appropriate and sensitive ZON screening tool. Sanseverino et al. reported an EC_{50} of $1.90\text{E}-06\text{ M}$ for the estrogen effect and an IC_{20} $1.79\text{E}-05\text{ M}$ for the toxicity of ZON [14]. Our results, after five hours incubation, yielded an EC_{50} of $2.74\text{E}-08\text{ M}$ ($0.0989\text{ }\mu\text{g/ml}$) and a LOEC of $1\text{E}-0.9\text{ M}$ ($0.003\text{ }\mu\text{g/ml}$) as shown in Fig. 1. The European Committee in 2006 suggested a threshold limit in animal feedstuffs for ZON that varies between 0.1 and 12 $\mu\text{g/ml}$, depending on the product, for agricultural feed products (2006/576/EC). BLYES is capable of detecting concentrations of ZON well below this threshold limit. No toxicity was observed with BLYR in the tested concentration range. This is confirmed by Eldridge et al., where no inhibition was detected with *S. cerevisiae* BLYR even at $1\text{E}-5\text{ M}$ ZON [15]. Nevertheless, the application of the constitutive control was reasonable for these experiments as degradation metabolites may induce cytotoxic effects.

In several cases ZON reduction is due to simple adsorption, as in the well known case of food-grade *Lactobacillus* strains, where removing of a certain amount of ZON is due to binding to the cell surface [21]. However, in our experiments, only a low amount of ZON was detected in the pellet, thus ZON adsorption to cells in our system was negligible and reduction of the ZON content could be attributed to metabolic activity of the applied microbes. According to recent publications the most active ZON binder microbes are Gram positive microorganisms [21,31]. Some strains of *Lactobacillus* and *Bacillus* could adsorb up to 80–95% of the 0.2–2 $\mu\text{g/ml}$ ZON containing liquid media [30,31]. The most probable mycotoxin binding site is the peptidoglycan outer layer and polysaccharides of these microbes [32]. On the other hand Gram negative microbes, with an inner peptidoglycan layer, like *Acetobacter* sp. AAB, *Escherichia coli* and *Pseudomonas putida* ZEA1 have

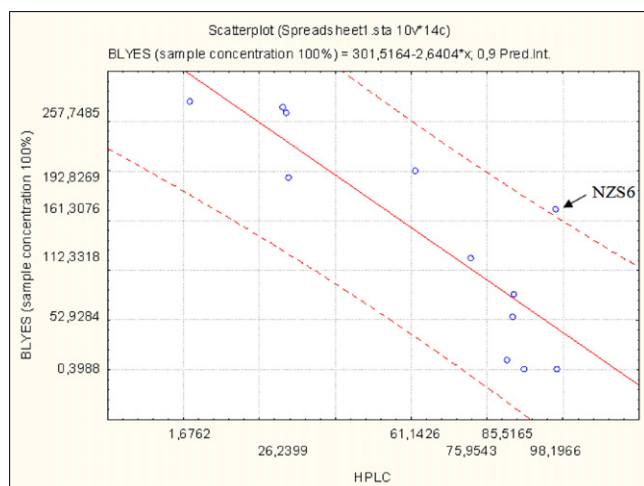


Fig. 4. Relationship between detected zearalenone concentration (HPLC) and remaining estrogenic potential (BLYES) after the degradation experiments.

much weaker binding capacity for mycotoxin in the 10–28% range [30,33,34]. In our short term (6 h) ZON binding experiment, the active and inactive cells demonstrated an equally weak 6.52–13.1% adsorption, while in case of the same strains – *Streptomyces* sp. K14, *Rhodococcus* sp. K402, *Pseudomonas* sp. FEH28, *Gordonia* sp. NZS6 – amount of ZON absorbed was 2–5% at the end of the degradation experiment (7 days). The highest binding capacity was observed in the case of *G. paraffinivorans* NZS6 after 6 h incubation but till the end of the incubation period this strain also demonstrated as low as 5% toxin binding ability.

With the exception of *Pseudomonas* FEH28 strain, which is a Gram negative bacterium, the other strains are all Gram positive microbes belonging to *Streptomyces*, *Gordonia* and *Rhodococcus* genus. Gram positive organisms have thick peptidoglycan layer on the cell surface. In *Streptomyces*, the protein coated mycelium contains minimal available peptidoglycan at the apical extension zone as seen by electron microscope in lysozyme digestion experiments [35]. The strains *Gordonia* and *Rhodococcus* have a thick and waxy outer shell composed by mycolic acid [36]. Thus, we concluded that the weak ZON binding capacity of the investigated strains may be caused by the lack of available peptidoglycan surface.

According to our bioassays only three strains (*Streptomyces cavourensis* K14, *Rhodococcus pyridinivorans* strains K402 and K404) were capable of both reducing the estrogenic effect of ZON and degrading ZON with high efficiency (87–96% determined by HPLC-FLD), while *R. pyridinivorans* K408 was found to be a good detoxifying strain (82% by HPLC-FLD). However, according to our most recently published data [37] the capacity of K408 to eliminate the estrogenic effects of ZON was tested by using an immature uterotrophic assay. Prepubertal female rats were treated with ZON that has already been incubated with or without *R. pyridinivorans* K408 strain. The *R. pyridinivorans* K408 strain proved to be a very efficient biological tool that is able to eliminate the complete estrogenic effects of ZON. In other studies [10,12], ZON degradation was confirmed by measuring loss of estrogenicity using bioassays. Cellular extracts (1 ml) of *Acinetobacter* sp. SM04 degraded 20 µg ZON concurrent with a significant decrease of estrogenic potency, as measured with the MCF-7 assay [12]. In the case of *T. mycotoxinivorans*, degradation was measured with a different yeast-based estrogen-sensing bioassay [10].

Interestingly, for *G. paraffinivorans* NZS6, elimination of ZON was measured at 98% with HPLC but only 78% with ELISA and estrogenic potential decreased by 40%. This discrepancy between the analytical and immunoassay results can be explained by the cross-reactions between ZON and its by-products. According to the manufacturer the antibody shows cross-reactivity in the ELISA: zearalanone (138%), α -ZOL (91%), β -ZOL (21%), α -zearalanol (69%), β -zearalanol (6%). The highest estrogenic effect of ZON derivatives recorded most frequently include α -, β -ZOL, and α -, β -zearalanol [22–26]. In addition, the vast majority of the data published demonstrates that ZON-degrading microbes produce α -ZOL, β -ZOL and zearalenone-4-O-sulphate [4,27,28]. Thus it is probable that metabolites produced during ZON biodegradation bound to the anti-ZON monoclonal antibody, which indicates that more ZON was degraded than our ELISA results suggest.

The combination of biological and analytical tests demonstrates that microbes with significant degradation activity did not always reduce the estrogenic potential of ZON or its by-products. Using the BLYES bioreporter, microbial strains could be selected that are guaranteed to metabolize ZON without harmful or estrogenic cleavage products. Furthermore, our results demonstrate the importance of using bioassays in order to determine estrogenicity since a potentially estrogenic effect even after significant ZON-degradation (up to 98%) was measured analytically. This is the first time that such a cost effective and rapid bioassay has been used to evaluate ZON-limits; screening systems used in other studies [10–12] are much

more complicated, expensive and time consuming. In addition, *S. cerevisiae* BLYES and BLYR are applicable for screening microbial strain collections, thousands of genetically improved microbe clones or transposon mutagenesis libraries for ZON degradation activity as this is a reliable, relative simple, cheap and rapid method.

Based on our results, *Rhodococcus pyridinivorans* strain K402 and *Streptomyces cavourensis* K14 were found to be the most promising strains for further bio-detoxification studies since these strains proved to be the most successful at ZON degradation.

5. Conclusions

A yeast-based bioassay that monitors potential estrogenicity (BLYES) and toxicity (BLYR) was adapted for screening ZON-degrading microbes, which is a novel application of this bioluminescence bioreporter system that is fast, simple, and cost-effective. This method allows us to detect both degradation of ZON and any potential toxic or hormonal effects of degradation products.

In our experiment the degradation and potential estrogenic effect of 1 µg/ml ZON were measured by using a strain collection with a previously determined degradation potential. Analyzing the experimental results of the ZON-degrading microbial strains, no toxic effect was detected with *S. cerevisiae* BLYR at the concentrations tested. On the other hand, data demonstrating the detoxifying potential of microbes tested by chemical (HPLC) and immunoanalytical (ELISA) methods show a great correlation with reduced estrogen-effect in most strains. Based on the results of our tests, *Streptomyces cavourensis* K14 and *Rhodococcus pyridinivorans* K402 were found to be the most useful strains for further biodegradation studies since these strains could metabolize ZON without cytotoxic end-products and with the weakest remaining estrogenic effect. In contrast, in the case of *Gordonia paraffinivorans* NZS6 almost total ZON decomposition was achieved yet at the same time high estrogenic potential remained. Hence, our results underline the necessity of using such bioassays in evaluating bio-detoxification methods. Moreover, based on these investigations the *S. cerevisiae*-based bioassays is a relevant new tool in the biomonitoring of ZON degradation.

Acknowledgements

This study was supported by the NKTH TECH-08-A3/2-2008-0385 (OM-00234/2008) MYCOSTOP grant, Gábor Baross Programme-2009 (HALEDC09) and TÁMOP-4.2.1B-11/2/KMR-2011-0003. The authors thank Dr Péter M. Szabó for the statistical assistance.

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