SZENT ISTVÁN UNIVERSITY

Ph.D. THESIS

DEVELOPMENT OF BIOMONITORING SYSTEMS FOR ANALYSING AFLATOXIN-B1 AND ZEARALENONE

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1. BACKGROUND, AIMS AND SCOPES

Mycotoxins are secondary metabolites of yeasts, widespread micro-contaminants that must be observed due to their serious acute and chronic health-damaging effect on humans and animals as well, hereby inducing major economic losses. They have mutagen and cytotoxic effects on cells, moreover carcinogen, teratogen, immunosuppressive and endocrine disrupting effects on organisms. One of the most investigated mycotoxin is aflatoxin-B1 (AFB1), produced by *Aspergillus* spp. yeasts, that is carcinogenic on humans, besides having mutagen and cytotoxic effect. The other mycotoxin inducing worldwide problems is zearalenone (ZEA), which causes reproduction diseases through its endocrine disrupting effect. Above mentioned effects make important the elimination from food and feeds of these two mycotoxins.

Biological methods, beside physico-chemical procedures have an increasing importance in the elimination of mycotoxins. The most promising aspects of these investigations are procedures based on biological degradation. Several studies about identification and isolation of mycotoxin degrading microbes have been published recently most of them aiming the development of an enzyme based feed-additive for mycotoxins, like the one that has already bben developed for ZEA and ochratoxin and is available on the market. Great aflatoxin degrading ability of microbial strains in the *Rhodococcus* genus has already been reported. However, biological detoxifying system such as the one applied for ZEA and ochratoxin is still not created, which possible reasons are the absence of appropriate screening systems and the major costs of toxin-level measuring procedures.

Beside chemical and immunoanalitical methods increasing importance have such monitoring systems that are based on biological effect analysis for detecting mycotoxins. Chemical analysis and immunochemical methods are unable to detect all potential degradation products, synergic and antagonist effects of interacting chemicals, therefore important is the monitoring of hazardous effects by other methods, such as the above mentioned biotests which role is the detection of biological effect.

Aims of the work

- Development of biomonitoring systems for the investigation of aflatoxin B1 and zearalenone.
- Development of reliable and rapid tests based on biological effect analysis to monitor mycotoxin-levels in base materials of feeding stuff (e.g. corn samples)
- Adaptation of the developed biomonitoring systems to biodegradation methods, to make the degradation of aflatoxin-B1 and zearalenone measurable by monitoring the alteration of their biological effect
- Selection of microorganisms that can degrade aflatoxin-B1 and zearalenone without hazardous metabolites, and in connection with this to follow up the biodegradation and to detect contingent ascending hazards.

2. MATERIALS AND METHODS

2.1 Test for analysing biological effect of mycotoxins

2.1.1 *SOS*-Chromo test for analysing genotoxicity

The *SOS*-Chromo test applies the *Eserichia coli* PQ37 strain as test-organism, in case of this strain the *SOS*-signal induced by a mutagen goes with the transcription of the gene sequence responsible to produce *β*-galactoside enzyme. The test makes it possible to analyse indirect genotoxins by metabolic activation (application of S9 rat liver extract in the test). The *SOS*-Chromo test was carried out according to manufacturer's instructions (Environmental Bio-Detection Products, Canada). Enzyme activities (*β*-galactoside, alkaline phosphatise) was measured at 620 and 405 nm, and genotoxic activity was given in induction factor (IF) that can be quantified by the following equation:

$$\mathrm{IF}=\frac{A405nc \times A620t}{A405t \times A620nc}$$

where, *A405*: absorbance at 405 nm (alkaline phosphatise)
A620: alkaline phosphatise at 620 (β-galactoside)
nc: negative control
t: actual concentration of the tested solution

According to data published, solution in the actual concentration is genotoxic if its sample has higher IF than 1.5.

In the course of pre-experiments mycotoxins were tested in serial dilutions, where AFB1 were tested 10–0.078 μg/ml, and ZEA were tested 10–1.25 μg/ml concentration.

For examining AFB1 biological degradation the *SOS*-Chromo test was adapted to mycotoxin degradation experiments; where the *SOS*-Chromo test was carried out as previously described, by a margin of applying the microbe-free control (blank) from the biodegradation experiments here as a positive genotoxic control.

2.1.2 *Aliivibrio fischeri* test for analysing cytotoxicity

The acute bioluminescence test was modified according to Sarter et al. (2008) and the method was adapted to a micro-plate one. The bioluminescent bacteria, the *Aliivibrio fischeri* (DSM-7151) was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The lyophilized bacteria after rehydration was grown in Bacto Marine (BM) medium and stored at 4°C on BM slant agar.

The marine bacterium *A. fischeri* emits light at optimal conditions which can be affected by a wide range of toxic agents. Basic experiments to investigate sensitivity of the test-organism for mycotoxins such as AFB1 and ZEA were performed in 100 μl cultures of *A. fischeri*, which optical density was adjusted to 0.1 at 550 nm (corresponding to 10^6 CFU/ml).

Controls without mycotoxins contained the culture plus acetone. Tests with controls, AFB1 (20, 10, 5, 2, 1 µg/ml), and ZEA, (20, 15, 10 µg/ml) were conducted in triplicate after 3.5-10-15-25 hours.

Effective concentration values were determined from the bioluminescence values; moreover, results were given in inhibition percent according to Frohner et al. (2002)

$$\text{Inhibition (\%)} = \left[\left(\frac{Ctx - Stx}{Ctx} \right) * 100 \right]$$

, where *C*: arithmetic mean of the bioluminescence values of parallel controls
S: arithmetic mean of the bioluminescence values of parallel controls
tx: incubation time

2.1.3 BLYES/BLYR test system for analysing endocrine disrupting and cytotoxic effect

Saccharomyces cerevisiae strains BLYES and BLYR harbouring leucine and uracil selective markers were grown modified minimal medium (YMM leu^-, ura^-) and stored at -80°C for further experiments.

In the course of pre-experiments sensitivity of the tester strains were determined for ZEA. The BLYES and BLYR tester strains were grown overnight at 30°C and 200 rpm to an OD_{600} of 0.1. Positive control was 17-β estradiol, solvent was methanol. Negative controls and solvent controls were also included (acetone in LB).

The BLYES and BLYR tests were adapted to toxin degradation experiments to examine biological degradation of ZEA. For data analysis luminescence was determined with a modified formula of Froehner et al. (2002) given in section 2.1.2, within the meaning of reciprocal value of the original equation, thus bioluminescence alteration was given in bioluminescence intensification (%) of samples to the control.

2.2 Analysing the aflatoxin-B1 production of *Aspergillus flavus* strains isolated from Hungarian corn samples by *SOS*-Chromo test

For practical application of the biological tests, corn samples contaminated with *Aspergillus*-strains isolated from Hungarian production areas were examined. Measurement of the amount of the produced AFB1 was carried out by SOS-Chromo test and the AFB1 content was simultaneously determined by the methods of ELISA and HPLC-FLD.

2.3 Microbial strains and culture conditions used in biodegradation tests

In the examination, microbes deposited in the strain collection of Agruniver Holding Ltd. and the Department of Environmental Protection and Safety, Szent István University, and reference strains deposited in international strain collections were screened, which have already been demonstrated to degrade mycotoxin in previous degradation tests.

The ZEA degradation ability of the microbes was analyzed as follows. The cells stored at -80°C 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_{600}=0.6)

2.4 Analysis of aflatoxin-B1 and zearalenone biodegradation processes

Biodegradation experiments were conducted in Erlenmeyer flasks in 50 ml media in three parallels (Table 2.1.), which were incubated in a shaking thermostat (170 rpm, 28 °C). Samples from the flasks were removed in every 24 h, centrifuged at 15000 rpm at 4°C for 20 min and both supernatant and pellet were stored at -20°C until further use.

Remaining ZEA concentrations in samples of supernatant and pellet were analyzed by High Performance Liquid Chromatography – HPLC (Wessling Hungary Ltd., Hungary), enzyme-linked immunosorbent assay – ELISA (Soft Flow Ltd., Hungary); moreover samples of supernatant were analysed by the adapted biomonitoring systems for screening the residual geno/cytotoxic or endocrine disrupting effect.

Table2.1: Parameters of the mycotoxin degradation experiments

	AFB1	ZEA
Mycoxin content	2 µg/ml	1 µg/ml
Incubation time	3 day	7 day
Biological effect analysis	*SOS*-Chromo test	BLYES/BLYR test

3. RESULTS AND DISCUSSION

3.1 Biological effect analysis of aflatoxin-B1 and zearalenone

3.1.1 *SOS*-Chromo test for genotoxicity

The AFB1 mycotoxin was analysed in 10–0.078 µg/ml, and ZEA mycotoxin was analysed in 10–1.25 µg/ml concentration range. On the base of our results (illustrated in Figure 3.1) the *SOS*-Chromo test proved to be less effective tool for screening the biological effects of ZEA. In case of these toxins the differences of genotoxic activity between native and activated mycotoxins were not significant, IF was 1.7 at 10 µg/ml and 1.5 at 5 µg/ml. In the case of AFB1 we found that as low as 0.078 µg/ml concentration of metabolically activated AFB1 induced the *sfi*A gene and indicated a highly genotoxic 2.0 IF number, in contrast to the toxin examined without metabolic activation, which IF in the same concentration was only IF=1.22, which cannot be considered as genotoxic according to published data.

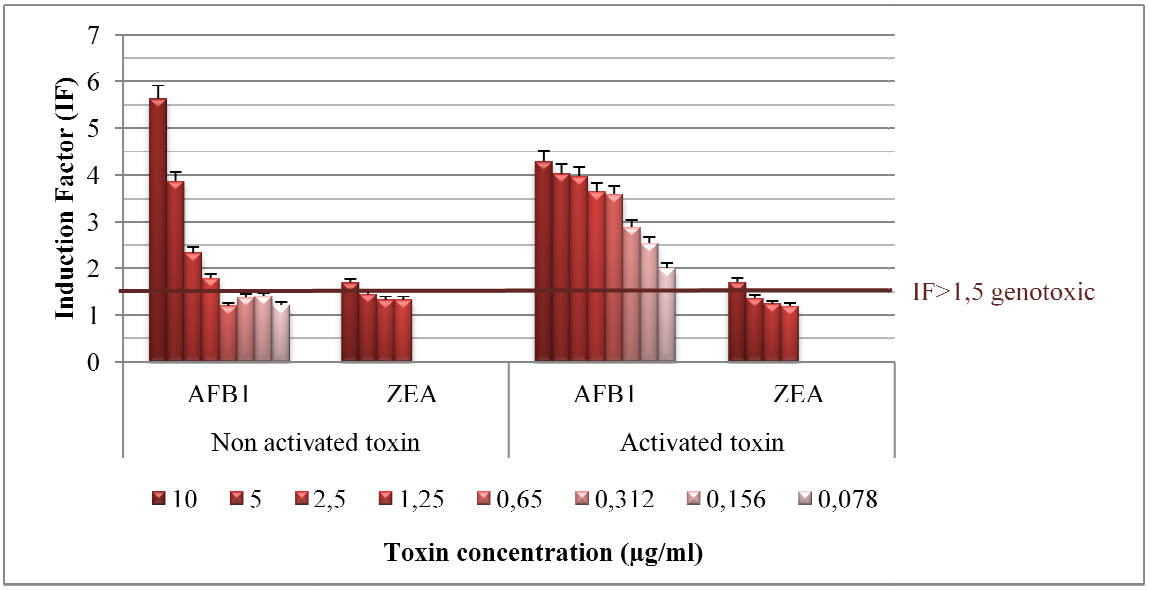


Figure 3.1: Genotoxicity of aflatoxin B1 and zearalenone measured by *SOS*-Chromo test with and without enzymes required for metabolic activation

Pursuant to results demonstrated, genotoxic effect of a 0.078 µg/ml AFB1-solution (measure up to 0.78 µg pure AFB1) can be detected by the *SOS*-Chromo test. Quillardet et al. (1985) gave the lowest observed effect concentration in 1.56 µg/assay of the original test tube procedure. Therefore, the micro-titer version of the SOS-Chromo test is more sensitive (0.78 µg), namely the detection level is lower with 50% compared to the original test procedure (1.56 µg).

3.1.2 *Aliivibrio fischeri* test for analysing cytotoxicity

Based on the results illustrated on Figure 3.2, the AFB1 in 10 µg/ml caused more than 80% biolumescence inhibition after 10 and 15 hours that correlates with result of Sarter et al. (2008), who detected nearly 100% inhibition. Nevertheless, our results after 3.5 and 25 hours showed higher inhibition, nearly 80%, in contrast the polish research group detected less than 40% inhibition. Moreover, in our laboratory measurements wider concentration range was applied, and even at 1 µg/ml AFB1 caused detectable inhibition.

As Figure 3.2 shows 50% bioluminescence inhibition of the tester strain was detected in case of 15 µg/ml ZEA and the *A. fischeri* tester strain is appropriate for detecting ZEA after 10-15-25 hours.

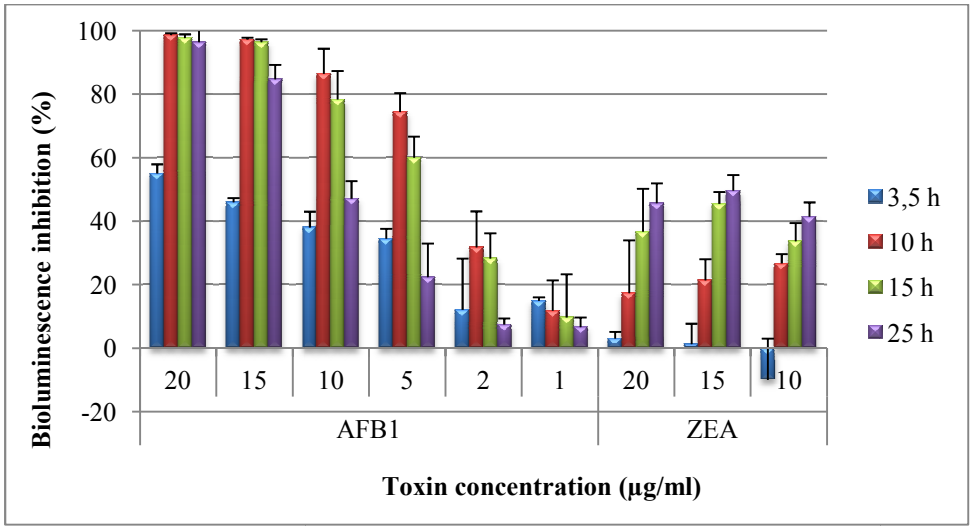


Figure 3.2: Bioluminescence inhibition for aflatoxin B1 and zearalenone using *A. fischeri*

In case of AFB1 the 10, 20, 50% effect concentration that causes 10, 20, and 50% bioluminescence inhibition for the tester strain ($EC_{10, 20, 50}$) was determined after 3,5-10-15-25 hours.

Table 3.1: EC values after different hours of incubation for aflatoxin B1 using *A. fischeri*

Contact time	EC_{10} (µg/ml)	EC_{20} (µg/ml)	EC_{50} (µg/ml)
3,5 h	0.9	2.4	17.1
10 h	0.9	1.4	3.0
15 h	1.0	1.6	3.8
25 h	4.2	5.5	9.1

On the base of Table 3.1 *A. fischeri* was found to be the most sensitive to AFB1 between 10 and 15 hours exposition time in our experiments. The EC50 was 3 µg/ml after 10, and 3.8 µg/ml after 15 hours of contact time, respectively; while in the acute standard test (ISO 11348) the $EC50_{(15\ min)}$ is 23,3 µg/ml. According to our results the no observed effect concentration is suggested under 0.9 µg/ml.

NOVEL SCIENTIFIC RESULT (by the results of chapter 4.1):

(Thesis 1) The micro-plate version of *Aliivibrio fischeri* test is sensitive to AFB1 even at 1 µg/ml concentration ($EC50_{(10h)}$=3 µg/ml; $EC50_{(15h)}$=3,8 µg/ml), while in the acute standard luminescence inhibition test (ISO 11348) the detection limit is 20 µg/ml ($EC50_{(15\ min}$=23.3 µg/ml.

NOVEL SCIENTIFIC RESULT (by the results of chapter 3.1.1 and 3.1.):

(Thesis 2) The *SOS*-Chromo test and the *Aliivibrio fischeri* test are appropriate for detecting the geno- and cytotoxic effect of aflatoxin-B1 mycotoxin, thus these tests are appropriate tools for screening mycotoxin-degrading micro-organisms based on their biological effect.

3.1.3 BLYES/BLYR test system for analysing endocrine disrupting and cytotoxic effect

In these experiments the sensitivities of the tester strains were determined for ZEA mycotoxin and results are illustrated on Figure 3.3.

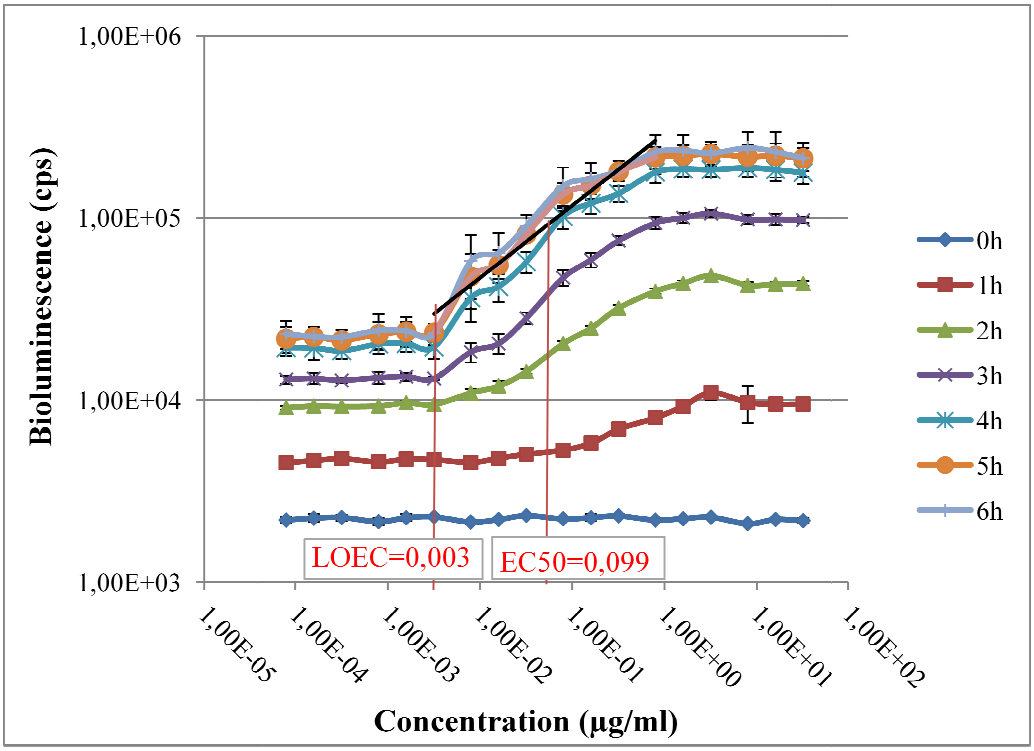


Figure 3.3: *Saccharomyces cerevisiae* BLYES response curve for zearalenone after 0–6 hours

Our results with the adapted recombinant yeast luminescence assay for ZEA degrading experiments in the contact times 0–6 h were determined by plotting bioluminescence (counts per second) versus the log of ZEA-solution concentration (µg/ml). The EC_{50} value was determined from the midpoint of the linear portion of the sigmoid conventration-response

curve. The EC_{50}=0.99 μg/ml after 5 h, that corresponds to 0.099 μg/ml pure ZEA after the 10% dilution that is caused by the contribution of the tester strain (Fig 2). In view of this contact time, a lowest observed ZEA concentration of 0.003 μg/ml (no observed effect concentration) is suggested which corresponds to 0.03 μg/ml ZEA-solution concentration. The Figure 3.3 also shows that bioluminescence alteration between 5-6 hours is negligible, thus in further experiments results are based on measurements after 5 hours.

The European Committee in 2006 suggested threshold limit in animal feedstuffs for ZEA varies between 0.1–12 μg/ml depending on the product for feeding (2006/576/EC). On the base of this suggestion BLYES seems to be a perfect tool even for direct monitoring of feed stuffs; moreover the test can be appropriate for screening ZEA-degrading microbes.

On the base of these results, in the course of ZEA degradation experiments further on, the ZEA initial concentration is adjusted to the sensitivity of the tester strain (EC_{50}=0.99) 1 μg/ml, thus the either the decreased either the increased response can be detected.

On the base of our results the ZEA in the examined concentration range did not inhibited the *S. cerevisiae* BLYR tester strain

NOVEL SCIENTIFIC RESULT (by the results of chapter 3.1.3.):

(Thesis 3) The *Saccharomyces cerevisiae* test (BLYES) is appropriate for the detection of estrogen effect in case of zearalenone at least of 0.03 μg/ml; thus the detection limit of the test meets the requirements regarding threshold limit in animal feed stuffs for ZEA suggested in the European Union.

3.2 Analysing the aflatoxin-B1 production of *Aspergillus flavus* strains isolated from Hungarian corn samples by SOS-Chromo test

The AFB1 content of the examined samples measured by HPLC and ELISA and compared with *SOS*-Chromo test are illustrated in Table 3.2.

No genotoxicity could be detected in extracts of the samples inoculated with the strains Zt27 and Zt66 producing less than 100 μg/kg AFB1, measured by HPLC and ELISA. No genotoxicity could be detected in extracts of the samples inoculated with the strains Zt27 and Zt66 producing less than 100 μg/kg AFB_1, measured by HPLC and ELISA. Concerning the strain Zt64, already a relatively high value (IF=2.0) was given that corresponded to the level 1950 μg/kg AFB_1, according to the relevant method. Moreover, even the dilution 1:2 of the extract appeared genotoxic since its value exceeded the limit of genotoxicity (IF=1.5). In contrast of these observations analytical methods detected lower toxin concentration (ELISA: 293.2 μg/kg; HPLC=397.7 μg/kg). Interestingly, higher genotoxicity was detected in case of

the sample of *A. flavus* Zt64 than *A. flavus* Zt55, though originally the strain Zt55 was characterized by higher AFB1 producing potential (2031 µg/kg; 575 µg/kg). In this case, the Zt64 strain possibly created other metabolite products that cause genotoxicity. The strain Zt80 seemed to manifest the highest genotoxic potential. The undiluted methanol extract created a high 3.8 induction factor, the 1:2 diluted extract created a 2.67 IF and even the 1:4 diluted sample showed genotoxicity (IF=1.64). Considering the negative control sample, maize without inoculation of any *Aspergillus* strain, genotoxicity was not detected.

Table 3.2: Aflatoxin B1 production of *A. flavus* strains isolated from Hungarian field measured by immuno- (ELISA) and chemical analytical (HPLC) methods, and compared with the *SOS*-Chromo test

***A. flavus* strain**	**ELISA (µg/kg)**	**HPLC-FLD (µg/kg)**	***SOS*-Chromo test (IF)**
Zt27	2.8	0.1	1.20
Zt66	28.3	72.	1.27
Zt64	293.2	397.7	2.01*
Zt55	2031.0	575.0	1.59*
Zt80	6228.0	2774.0	3.81*

* genotoxic (IF>1.5)

This underlines the necessity of using biotests simultaneously with analytical test for complex hazard assessment of the aflatoxin producing strains, since these tests can measure those biological effects that is hidden from the analytical tests.

NOVEL SCIENTIFIC RESULT (by the results of chapter 3.2):

(Thesis 4.) The SOS-Chromo test is appropriate for detecting the aflatoxin-B1 producing ability of *Aspergillus flavus* strains isolated from Hungarian corn samples. By means of this method *Aspergillus flavus* strains with potential aflatoxin-B1 producing ability were manifested in Hungary that was considered non-existent up to now.

3.3 Analysing the biodegradation of aflatoxin-B1 and zearalenone

On the base of the analytical results micro-organisms were divided into three groups according to their degradation potential:

I. excellent (>90%) mycotoxin-degrading ability

II. good (50–90%) mycotoxin-degrading ability

III. weak (<50%) mycotoxin-degrading ability

3.3.1 Pre-experiments to analyse aflatoxin-B1 biodegradation

AFB1 degradation abilities of sixteen micro-organisms were determined from samples taken in every 24 hours from degradation systems that were handled as described in section 3.4. Genotoxicity of the supernatant samples were given in induction factor (IF) in every 24 hours by *SOS*-Chromo test; while degradation potential derived from toxin concentrations

measured by HPLC and ELISA was given in degradation potential at the end of the degradation experiment. Results of HPLC and ELISA showed high correlation in direct ration (Pearson-correlation: 0.98), which are illustrated in Table 3.3.

Table 3.3: Results of AFB1 (2 µg/ml) biodegradation tests on the base of biodegradation potential (HPLC, ELISA tests) and genotoxicity (SOS-Chromo test)

		SOS-Chromo test			HPLC	ELISA-test
		Induction Factor			Degradation (%)	Degradation (%)
Species	Strain	24 h degradation	48 h degradation	72 h degradation	72 h degradation	72 h degradation
BLANK[a]		2.43* ± 0,04	2.38* ± 0,01	2.40* ± 0,03	<20	<20
E. coli	TOP10[a]	2.42* ± 0,07	2.13* ± 0,15	2.32* ± 0,06	<20	<20
R. erythropolis	N11[a]	1.98*± 0,05	1.36 ± 0,02	1.29 ± 0,18	89.35 ± 7,79	97.67 ± 0,30
	AK35[a]	2.56 ± 0,08	1.73* ± 0,04	1.44 ± 0,29	96.73 ± 0,90	94.31 ± 0,45
	AK42[a]	3.06* ± 0,03	1.87* ± 0,30	1.42 ± 0,08	87.14 ± 0,00	90.77 ± 0,21
	GD2A[a]	2.90* ± 0,14	1.51* ± 0,19	1.39 ± 0,25	96.25 ± 5,24	96.04 ± 2,07
	GD2B[a]	2.71* ± 0,06	1.48 ± 0,16	1.34 ± 0,21	99.96 ± 0,00	92.85 ± 0,73
	BRB1AB[a]	2.85* ± 0,01	1.53* ± 0,2	1.37 ± 0,15	99.96 ± 0,00	93.64 ± 0,00
R. ruber	N361[a]	2.81*± 0,02	2.89* ± 0,02	3.00* ± 0,03	<20	<20
R. globerulus	N58[a]	2.60* ± 0,04	2.43* ± 0,03	2.56* ± 0,05	<20	20.79 ± 0,00
	AK36[a]	2.89* ± 0,06	1.60*± 0,03	1.40 ± 0,35	91.46 ± 2,38	86.24 ± 1,94
R. rhodochrous	NI2[a]	1.83* ± 0,13	1.26 ± 0,03	1.22 ± 0,13	99.98 ± 0,00	97.97 ± 0,00
	ATCC 12674[a]	2.30* ± 0,05	1.40 ± 0,05	1.23 ± 0,08	96.05 ± 1,29	97.90 ± 0,11
R. pyridinivorans	K402[a]	2.7* ± 0,22	1.92* ± 0,21	1.38 ± 0,27	99.92 ± 0,00	97.60 ± 0,05
	K403C[a]	2.45* ± 0,37	1.85* ± 0,04	1.41 ± 0,29	92.00 ± 2,23	90.35 ± 0,34
	K404[a]	2.39* ± 0,10	1.86* ± 0,16	1.38 ± 0,35	82.06 ± 2,59	89.80 ± 0,97
	K405[a]	2.01* ± 0,14	1.38 ± 0,04	1.39 ± 0,35	99.92 ± 0,00	92.81 ± 1,00
	AK37[a]	2.97* ± 0,02	1.58* ± 0,21	1.42 ± 0,19	87.78 ± 1,50	87.73 ± 1,61

Legend

[a] each data point represents the average of triplicate measurements
* samples with genotoxicity
samples with <20% degradation potential
samples with 60-80% degradation potential
samples with >90% degradation potential

Analytical measurements of the pellet were detected less than 1% AFB1. Thus AFB1 adsorption to the cells is negligible in this study, the AFB1 reduction was due to metabolism. Among seventeen microbes, fourteen strains successfully degraded AFB1 in 72 hours. Moreover, five strains were determined to be able to eliminate genotoxic contamination in 48 hours. Our results and those made by ELISA-tests and HPLC were highly correlated (Pearson-correlation: -0.96), consequently higher degradation potential goes with reduced genotoxicity. Strains with more than 80% degradation potential could successfully eliminate genotoxicity while the non-degrading control strain (*E. coli*) and two rhodococci (N361, N58) showed a high induction factor (IF>1.5) that denotes genotoxicity.

3.3.2 Development of a combined toxicity profile to analyse the aflatoxin-B1 biodegradation

For the complex evaluation of AFB1 biodegradation, the possibly created genotoxic and cytotoxic cleave products were analysed.

Implementations of the degradation experiments were modified: based on the results of the preliminary experiments higher (4 μg/ml) AFB1 contamination in half salted BM-broth was applied to optimize conditions for the *A. fischeri* test organism for further toxicological testing. The AFB1 concentration was raised due to the fact that several microbes could eliminate 2 μg/ml AFB1 in less than 72 hours. In further experiments strains with insufficient degradation ability (N361, N58) were applied as negative controls.

During *A. fischeri* assay additionally, negative controls were set which contained the tested strains without mycotoxins. This was essential as *A. fischeri* is presumably sensitive to metabolic by-products of the tested microbes. Samples were taken from parallel systems and centrifuged them as described in section 2.2. Supernatant was decanted; salt level was adjusted to correspond to the original BM-broth, samples were vortexed, and 100 μl were distributed on a 96-well micro-plate. These samples were filled with liquid culture of *A. fischeri* with optical density adjusted to 0.2 at 550 nm. Luminescence at t_0 was checked, samples were incubated at 25°C, 30 rpm in shaking thermostat, and toxicity was determined as described in 2.1.2.

Since chemical analytical tests and ELISA tests showed a high correlation in the pre-experiments (section 3.3.1), the results of the combined toxicity profile were supported with parallel ELISA tests.

The examined thirty-three strains were analyzed using two bacterial biotests, the *A. fischeri* test and the SOS-Chromo test, and by parallel ELISA test (Table 3.4).

Table 3.4: AFB1 degrading microbes analyzed on the basis of their degradation potential and by the combined toxicity profile

Species	Strains	*Aliivibrio fischeri* test		*SOS*-Chromo test	ELISA-test
		Inhibition (%)		Induction Factor	Degradation(%)
		10 h contact time	15 h contact time		
BLANK[a]		74.75 ± 5.62	65.88 ± 4.01	3.66* ± 0.08	<20±0.00
R. erythropolis	N1[a]	6.50 ± 8.36	14.68 ± 21.65	2.13* ± 0.01	93.91 ± 0.13
	AK35[a]	32.33 ± 4.67	46.27 ± 5.49	1.72* ± 0.02	96.45 ± 0.86
	AK42[a]	36.04 ± 10.99	58.91 ± 8.23	2.69* ± 0.05	79.25 ± 3.06
	GD2A[a]	41.50 ± 11.62	57.81 ± 10.97	2.23* ± 0.11	92.08 ± 0.38
	GD2B[a]	74.21 ± 9.16	92.92 ± 2.15	1.75* ± 0.05	96.97 ± 0.12
	BRB1AB[a]	53.08 ± 16.25	62.08 ± 16.62	1.73* ± 0.09	97.60 ± 0.78
R. ruber	N361[a]	60.78 ± 8.04	65.89 ± 7.95	3.62* ± 0.03	<20 ± 0.00
R. globerulus	N58[a]	67.29 ± 0.17	66.44 ± 8.45	3.24* ± 0.13	<20 ± 0.00
	AK36[a]	30.33 ± 21.29	50.27 ± 18.85	2.02* ± 0.10	93.15 ± 0.45
R. rhodochrous	NI2[a]	-35.72 ± 7.56	-66.79 ± 1.07	1.50 ± 0.02	99.05 ± 0.06
	ATCC 12674[a]	3.14 ± 4.45	35.02 ± 2.81	1.87* ± 0.07	95.74 ± 1.55
R. pyridinivorans	K402[a]	-42.29 ± 12.9	-66.41 ± 49.96	1.56* ± 0.05	97.00 ± 0.55
	K403C[a]	76.17 ± 2.60	85.10 ± 6.43	1.46 ± 0.01	94.50 ± 4.58
	K404[a]	32.80 ± 5.87	45.83 ± 10.19	1.94* ± 0.38	93.06 ± 5.72
	K405[a]	81.93 ± 0.20	89.93 ± 0.16	1.54* ± 0.01	97.84 ± 0.23
	K406[a]	43.82 ± 0.02	40.84 ± 2.14	1.58* ± 0.02	96.16 ± 0.81
	K408[a]	33.89 ± 7.94	34.18 ± 8.50	1.72* ± 0.28	95.81 ± 3.27
	AK37[a]	14.93 ± 12.34	15.25 ± 9.53	2.70* ± 0.01	70.77 ± 0.68
Streptomyces heliomycini.	K2[b]	7.95 ± 4.29	1.12 ± 3.87	2.59* ± 0.03	59.75 ± 7.45
Streptomyces cavourensis	K14[b]	-138.17 ± 20.15	-135.7 4 ± 20.67	2.69* ± 0.03	80.22 ± 1.20
Chryseobacterium formosense	UA58[b]	-12.16 ± 6.10	-16.84 ± 10.82	2.36* ± 0.07	76.79 ± 1.52
Chryseobacterium hydrocarbonovorans	TN4[b]	9.82 ± 10.46	13.57 ± 7.84	1.75* ± 0.27	56.88 ± 1.59
Pseudomonas citronellolis	ZS1[b]	-5.75 ± 9.25	-32.41 ± 39.09	1.96* ± 0.03	91.24 ± 0.61
Pseudomonas pseudoalcaligenes	FEH28[b]	-1.16 ± 5.83	-42.24 ± 34.31	1.78* ± 0.04	97.61 ± 0.04
Pseudomonas azelaica	TN5[b]	-11.28 ± 9.31	-56.68 ± 49.79	2.19* ± 0.07	86.65 ± 0.26
Pseudomonas monteilii	ZV6[b]	-14.93 ± 9.52	-62.51 ± 51.71	2.24* ± 0.05	80.14 ± 0.78
Pseudomonas putida	DN1[b]	-17.05 ± 10.29	-22.86 ± 14.33	1.42 ± 0.07	90.80 ± 0.18
Pseudomonas stutzeri	KÖ5[b]	-0.69 ± 7.36	-7.47 ± 11.07	1.95* ± 0.03	91.44 ± 0.19
Microbacterium esteraromaticum	NZS9[b]	44.31 ± 4.31	57.68 ± 6.30	2.11* ± 0.02	64.80 ± 4.01
Microbacterium barkeri	EL1[b]	62.15 ± 8.64	49.92 ± 6.17	1.87* ± 0.08	74.19 ± 0.96
Pseudoxanthomonas suwonensis	NZS6[b]	36.63 ± 4.56	15.22 ± 6.62	2.17* ± 0.08	76.75 ± 1.51
Pseudoxanthomonas kalamensis	H4[b]	27.79 ± 6.05	33.54 ± 4.29	2.02* ± 0.04	70.89 ± 4.09
Arthrobacter protophormiae	J4[b]	-11.03 ± 3.32	-51.00 ± 7.37	2.05* ± 0.08	77.07 ± 1.44

Legend

[a]	each data point represents the average of duplicate measurements
[b]	each data point represents the average of triplicate measurements
*	samples with genotoxicity
highlighted	samples with more than 50% luminescence inhibition
	samples with <20% degradation potential
	samples with 60-80% degradation potential
	samples with >90% degradation potential

For control strains (N58, N361) with insufficient degradation ability (<20% degradation) more than 60% luminescence inhibition in *A. fischeri* test and equal genotoxicity compared to the negative control (IF>3) were experienced.

Strains with good (50-80%) degradation potential according to the ELISA tests were not able to cease genotoxicity in any case (AK42, K2, K14, UA58, TN4, TN5, ZV6, NZS9,

EL1, NZS9, EL1, NZS6, H4, and J4). Regarding cytotoxicity, no strains caused considerable inhibition (over 50% with 10 and 15 contact hours). In addition, five strains (K14, UA58, TN5, ZV6, and J4) experienced higher luminescence which means metabolism products of these strains proved to stimulate the test organism *A. fischeri*.

With four exceptions (GD2B, BRB1AB, K403C, K405) strains with significant degradation potential (>90%) did not cause considerable inhibition on the 3rd day of biodegradation process and even increased luminescence in some cases (NI2, K402, K14, UA58, ZS1, FEH28, TN5, ZV6, DN1, KÖ5). Nevertheless, three of these significant degrader strains (GD2B, K403C, and K405) caused larger inhibition than the blank or the control non-degrader strains. Among the significant degrader strains only *R. rhodochrous* NI2, *R. pyridinivorans* K403C, and *Pseudomonas putida* DN1 could cease the genotoxic effect of 4 μg/ml AFB1 in 72 hours. Strains, NI2 and DN1 showed significant (>90%) degradation potential by ELISA test and no cytotoxic effect by the *A. fischeri* test was detected. Interestingly, *R. pyridinivorans* K403C showed cytotoxicity (>70% inhibition) with significant AFB1 degradation potential (94.5%) and ceased genotoxicity (IF=1.46).

NOVEL SCIENTIFIC RESULT (by the results of chapter 3.3.2):

(Thesis 5) The method development for evaluating the biodetoxification processes with the combined toxicity profile by the application of the adapted *SOS*-Chromo test with the *Aliivibrio fischeri* test is appropriate to select the most successful aflatoxin-B1 degrading micro-organisms with the weakest residual harmful effect for practical applications, which are representatives of the *Pseudomonas* and the *Rhodococcus* genus according to our results.

3.3.3 Analysing zearalenone biodegradation with a yeast based bioriporter system

In our experiment twelve micro-organisms were evaluated by BLYES and BLYR test to measure the biological effect and results were compared by parallel HPLC and ELISA tests (Figure 3.4).

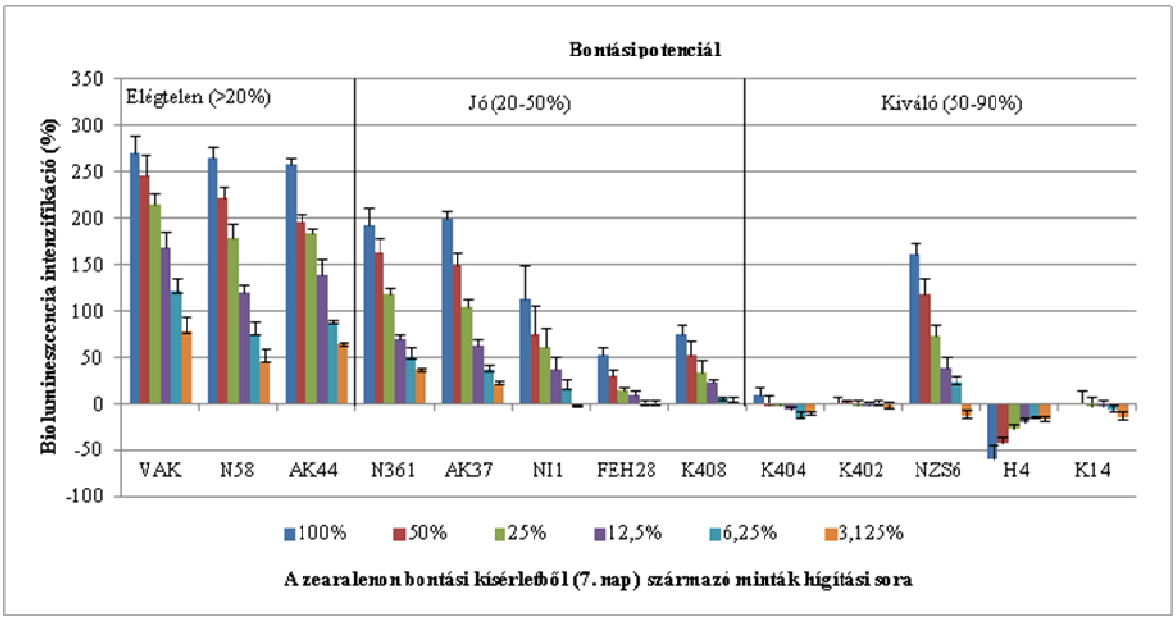
HPLC results correlate well with the ELISA data (Pearson-correlation: 0.98). Only one outlier was observed, namely in case of the strain NZS6 the degradation potential was 98.2% measured by HPLC, while ELISA test suggested 78.89%.

No toxicity was detected in the degradation experiment by the BLYR test.

By analyzing the results of BLYES tests, the estrogen effect correlates with the results of HPLC and ELISA tests for degradation potential. Statistical analysis regarding BLYES and ELISA showed high inverse correlation regarding all dilutions of the sample (Pearson

correlation between -0.91 thru -0.97), while a bit lower correlation (-0.87 thru -0.97) was given between HPLC and BLYES. This phenomenon is described in details on Figure 3.5. Nevertheless, in case of the majority of the microbes the worth degradation potential goes with higher bioluminescence measured by BLYES test.

Figure 3.4 shows that generally strains with the ability to degrade ZEA also decrease the amount of estrogenic substances in a sample. The statistical analysis showed a good correlation negative correlation between HPLC/ELISA and BLYES in all dilutions (Pearson correlation in all cases were between -0.87 thru -0.97), which means the higher degradation potential measured by HPLC/ELISA correlates with decreased bioluminescence with BLYES.



3.4: Zearalenone degrading microbes analysed on the basis of their degradation potential (ELISA, HPLC) and remained estrogenic effect (BLYES)

For strains (*R. aetherivorans*, AK44, *R. gluberulus* N58) with weak degradation ability (less than 50%) the measured estrogenic effect was similar to the control values.

Strains with good degrading potential resulted more than 50% degradation ability and a decreased estrogen effect.

Three out of the eleven investigated strains could eliminate ZEA with more than 90% degradation efficiency and demonstrated a decreased estrogenic effect. In case of H4 a luminescence inhibition was observed which assumes cytotoxic effect, but no toxicity was measured by BLYR. In this case H4 produced such dark pigments which makes impossible to detect luminescence alteration. Occurrence of pigment producing microbes the bioriporter system is not applicable, its development is necessary Interestingly, *G. paraffinivorans* NZS6 degradation of ZEA was measured at 98,20% with HPLC but only 78% with ELISA.; moreover, the it shows analogous estrogen effect as samples of microbes with weaker

degradation potential. It was already demonstrated that HPLC and BLYES tests shows good correlation; however beside numerical value of the correlation the scatter plot diagram is also useful, as these two gives overall information about the characteristics of variables. In Figure 3.5 one outlier that matches up to NZS6 exists. On the base of these observations the *G. paraffinivorans* NZS6 was able to degrade ZEA; however, cleave products of ZEA was formed that could not be eliminated and resulted estrogen effect.

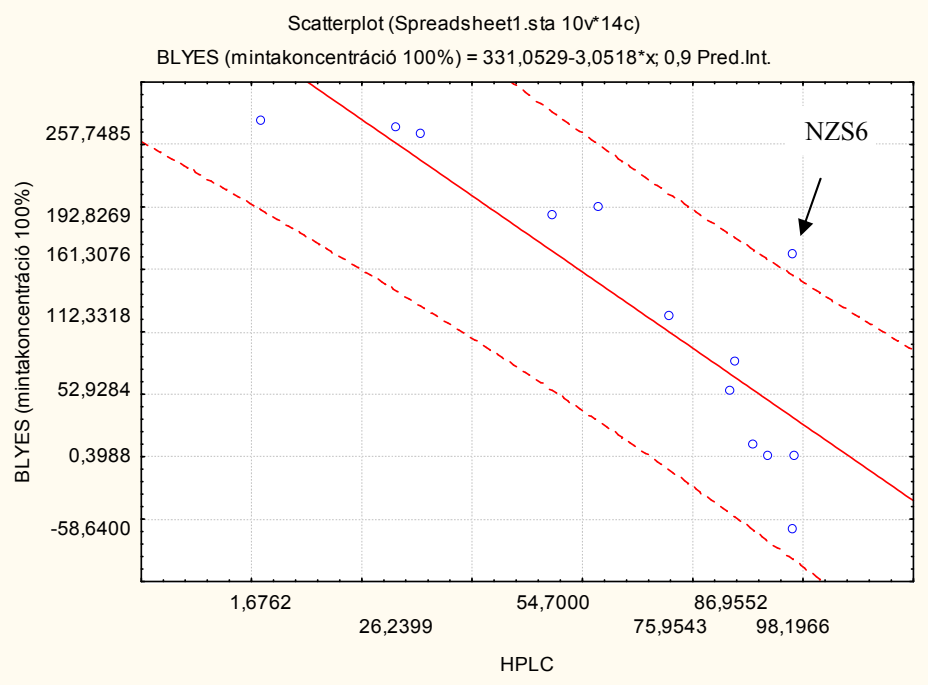


Figure 3.5: Graphical display of the relationship between detected zearalenone concentration (HPLC) and remained estrogen effect (BLYES) after the degradation experiments.

Based on the results of the complex evaluating system *R. pyridinivorans* strains K402 and K404, and *S. cavourensis* K14 were found to be the most feasible strains for further bio-detoxification studies since these strains proved to be the most successful at ZEA degradation.

NOVEL SCIENTIFIC RESULT (by the results of chapter 3.3.3)

(Thesis 6) The method development for evaluating the biodetoxification processes by the adapted yeast based bioriporter system is appropriate to select the most successful zearalenone degrading micro-organisms with the weakest residual harmful effect for practical applications, which are representatives of the *Streptomyces* and the *Rhodococcus* genus according to our results.

4. CONCLUSIONS AND SUGGESTIONS

Several studies about aflatoxin and zearalenone degrading microbes have been published recently, most of them aiming the development of a biological detoxifying system, like the one developed by Biomin regarding zearalenon and ochratoxin. For these purposes the first task is the formation of an optimal screening system, which can detect tha geno- and cítotoxic effect in case of AFB1 and the estrogen eggect ihn case of ZEA. In the interest of these goals, different biotests were adopted and developed to analyse the harmful effect of AFB1 and ZEA.

For biomonitoring mycotoxins the following microbial biotests were applied: *A. fischeri* luminescence inhibition tests for analysing cytotoxicity, the SOS-Chromo test for analysing genotoxicity, the bioluminescence based bioriporter system that applies eukaryotic cells (*S. cerevisiae* BLYES) with the constitutive control strain (*S. cerevisiae* BLYR).

The *A. fischeri* marine bacterium is a widely used test bacterium which emits light under optimal conditions. This property is used to determine ecotoxicity in environmental biotechnologies. However, until now only one report studied this method applied to mycotoxins, where AFB1 in 10 µg/ml an deoxinivalenol in 20 µg/ml concentration was tested. Measurement was carried out in 50 ml BM-broth in Erlenmeyer flask and determined toxicity after 3.5, 10, 15, and 25 hours. This method was adopted to a micro-plate version which can test large quantites. Moreover, the contact time was optimized and by using a broader dilution range, the effect of different concentration values of AFB1 for *A. fischeri* could be determined which has not been reported yet for this test organism. Based on our result *A. fischeri* assay was indicative for AFB1 even 1 µg/ml and proved to be the most sensitive to AFB1 with 10-15 hours contact time: $EC50_{(10\ h)}$=3 µg/ml, $EC50_{(15\ h)}$=3.8 µg/ml; thus the sensitivity of the *A. fischeri* test is considerably improved compared to the result of the acute standard test (ISO 11348) where the EC50(15 min) is 23.3 µg/ml. Nevertheless, according to our measurements this method is not suitable for direct monitoring of AFB1 contamination of food and feed stuffs, as it works at higher concentration than the current limits. However, it can be suitable to monitor mycotoxin degrading microbes.

The *SOS*-Chromo test for analysing genotoxicity proved to be less effective for the biomonitoring of ZEA, since the *SOS*-repair system of the *E. coli* PQ37 tester strain was only induced at 10 µg/ml. However, this method is appropriate for analysing AFB1, since genotoxic potential even in 0.078 µg/ml solution is detectable, thus detectable limit of AFB1 by this test is around the regulated maximum levels of AFB1 contamination in food and feed stuffs.

The *SOS*-Chromo test was applied for practical purposes beside experimental studies, namely *A. flavus* isolates from Hungarian fields were analysed on the base of their AFB1 producing ability. Results pointed out the importance of biotests, since *A. flavus* strains with less intensive AFB1 production, where AFB1 concentration was measured by analytical methods, proved to create more intense genotoxic potential compared to other strains. In conclusion, due to changed weather conditions Hungary can encounter unexpected problems; harmful yeast may gather ground and presence of AFB1 should be taken into account in Hungary as well.

Above mentioned two biotests are appropriate for screening mycotoxin-degrading microbes. The *SOS*-Chromo test could be successfully used to select safe strains with AFB1 degrading potential without genotoxic cleave products. Moreover, *A. fischeri* luminescence assay could be used for detecting AFB1 presence/elimination during biodegradation after adaptation and modification for biodegradation experiments. With the simultaneous application of the two biotests a combined toxicity profile was created in order to expedite the selection of the most appropriate strains, that have the most intense degradation potential and the weakest harmful residual biological effect, for further biodetoxification processes. After the 72 hours incubation, only 3 strains, *R. rhodochrous* NI2, *R. pyridinivorans* K403C and *P. putida* DN1 ceased genotoxicity. Exciting results were given by the luminescent assay: with rhodococci, aside from two exceptions (*R. rhodochrous* NI2, *R. pyridinivorans* K402), inhibition was detected, moreover, inhibition exceeded the control in some cases (*R. erythropolis* GD2B, *R. pyridinivorans* K403C, K405), while *Pseudomonas*-strains completely ceased the cytotoxic effect of the toxin. This observation indicates AFB1 metabolism can not only distinguish between different genera, but within a genus as well. In addition, different *Rhodococcus* strains may use different pathways to degrade AFB1. Interestingly luminescent intensification in the case of some microbes was experienced. On the one hand, it is known that toxic substances in lower doses may cause stimulation, while on the other hand a by-product of degradation can also mimic a substrate or a vitamin. Nevertheless, Sarter at al. (2008) also experienced this stimulation in case of deoxinivalenol

Based on the combined toxicity profile *R. rhodochrous* NI2 seems to be the most feasible for further biodetoxification work since this strain proved to be the most successful AFB1 degrading organism in all the applied experiments (2 and 4 μg/ml AFB1) and, at the same time, caused neither geno- nor cytotoxicity. However, *P. putida* DN1 and *R. pyridinivorans* K402, the former with weaker degrading potential and the latter with moderate remaining genotoxicity, can also be suitable for further experiments.

Our results underline the necessity of such biotests since remaining genotoxicity or cytotoxicity was recovered even after significant toxin degradation. These findings also highlight the necessity of using parallel biotests since remaining cytotoxicity after ceased genotoxicity or remaining genotoxicity after ceased cytotoxicity could also be experienced.

A fast, simple, cost-effective luminescent method (BLYES and BLYR) to measure the biological effect of ZEA was applied. The BLYES test seems to be a perfect tool even for direct monitoring of feed stuffs, since detection limit of the test is under the threshold limit in animal feed stuffs for ZEA suggested by the EU.

This yeast bioriporter system was adapted to mycotoxin degradation experiments. In several cases microorganisms are described for ZEA degradation; however few of these papers prove the cessation of estrogen effect. Aim of the present work was to develop the initial steps of mycotoxin-detoxifying method based on bacterial biodegradation which ends in the elimination of estrogen effect. The created combined experimental system integrated the chemical-, imunnoanalitical and biological methods. By this combined method the most appropriate microbes for ZEA biodegradation can be selected, which degrade the ZEA efficiently without creating harmful cleave products. Based on the results of the complex evaluating system *Streptomyces cavourensis* K14 were found to be the most feasible strains for further bio-detoxification studies since these strains proved to be the most successful at ZEA degradation, as HPLC and ELISA detected more than 90% ZEA degradation and did not created harmful metabolites during the biodegradation. The *R. pyridinivorans* strains K402 and K404 with effective degradation (by HPLC 85%, by ELISA 90%), also ceased the estrogen effect at the end of the degradation experiment.

These experiments proved that analytical results alone are not always sufficient, since in biological systems such processes are taken place which cannot be detected by analytical methods. *G. paraffinivorans* NZS6 degradation of ZEA was measured at 98% with HPLC but only 76% with ELISA, which discrepancy between analytical and immunoassay results can be interpreted by the cross-reactions between ZEA and its by-products that were detected by the ELISA test. The formation of these by-products was confirmed by the BLYES test, as samples originated from NZS6 yet maintains a high estrogenic potential. This indicates that the by-products of degradation may interact with the human endocrine system. However, estrogenic metabolites were not only detected in case of NZS6. Among strains evolved in the degradation experiments estrogen effect was detected in more cases during the degradation process (*R. pyridinivorans* K408, AK37, R. ruber N361). Nevertheless, these strains were able to degrade these metabolites along with the mycotoxin compared to NZS6, so the harmful effect decreased/ceased at the end of the detoxification process. However, important to take

into account the risk at those points where estrogen effect evolved. In contrast, those two microbes have to be emphasized, which did not show that kind of estrogen effect formation, thus they are the most appropriate in sight of risk evaluation for practical applications. These strains were the *S. cavourensis* K14 and the *R. erythropolis* NI1.

Above mentioned results underline the necessity of using bioassays since a potentially harmful effect was measured even after significant degradation as measured analytically. Beside the evaluation of strain collections on the base of their mycotoxin-detoxification ability, these combined methods can be considerably useful in screening genetically improved microbes. A strain library created by UV mutagenesis is suggested, then the evaluation of mutant strains by the combined toxicology profile in case of AFB1, and the yeast based bioriporter system in case of ZEA is possible. By these biological-effect based systems strains with increased degrading potential reliably can be selected.

Other important application of these systems the metabolism research of AFB1 and ZEA. After creating transposone mutagenesis clone libraries with thousands of clones, null-mutants can be detected by these biological-effect based systems and the gene sequences responsible for the mytoxin-degradating can be identified. For this goals, full genome data of these specific microbes are essential, which is on hand in case *R. pyridinivorans* AK37. These sequences provide possibility for the identification of yet unknown genes encoding mycotoxin-degrading enzymes. Further possible aim is the cloning, the biochemical typifying of enzymes applicable in industrial processes, and the development and optimization of synergic enzyme-mixes.

5. PUBLICATIONS

Scientific paper:

Krifaton Cs., Kriszt B., Szoboszlay S., Cserháti M., Szűcs Á., Kukolya J. (2011): Analysis of aflatoxin-B1 degrading microbes by a combined toxicity profiling method, Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 726 1-7 p. (IF 2010: 2,938)

Krifaton C, Kukolya J, Szoboszlay S, Cserháti M, Szűcs Á, Kriszt B (2010): Adaptation of bacterial biotests for monitoring mycotoxins. WIT Transactions on Ecology and the Environment: Environmental Toxicology III., 132 143-154. p.

Krifaton Cs., Cserháti M., Prívler Z. (2011): Az aflatoxinok környezet-egészségügyi hatásai, Biokontroll 2 (4) 4-7 p.

Proceeding:

Krifaton Cs., Kukolya J., Szoboszlay S., Cserháti M., Kriszt B. (2010): Mikotoxin-bontó mikrobák screenelése bakteriális biotesztesztekkel, TUDOC – Kárpát Medencei Doktoranduszok Nemzetközi Konferenciája, 2010. Május 27-28, Gödöllő, Konferencia kötet, 161–170 p.

Total impact factor (IF): 2,938