Acta Microbiologica et Immunologica Hungarica 64 (4), pp. 423–438 (2017)
DOI: 10.1556/030.64.2017.023
First published online December 1, 2017

AFLATOXIN B1 DETOXIFICATION BY CELL-FREE EXTRACTS OF *RHODOCOCCUS* STRAINS

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(Received: 4 May 2017; accepted: 21 June 2017)

Aflatoxin B1 (AFB1) produced by *Aspergillus* molds is a genotoxic and carcinogenic mycotoxin. For the elimination of mycotoxins from food and feed, biodetoxification can be a successful tool. The aim of this study was to reveal biodetoxification with the cell-free extracts of *Rhodococcus erythropolis* NI1 and *Rhodococcus rhodochrous* NI2, which have been already proved to detoxify AFB1. Extracellular matrices of cultures and also intracellular extracts were applied for detoxification. In both cases, media containing constitutively produced and AFB1-induced enzymes were tested, respectively. The pH tolerance of enzymes in the detoxification was examined at pH 7, 7.5, and 8. The remained genotoxicity was detected by SOS-Chromotest and the AFB1 concentration was measured by high performance liquid chromatography with fluorescence detection. In the extracellular matrix, no reduction of genotoxicity was observed. However, detoxification was completed by intracellular enzymes. In intracellular extracts of both strains, genotoxicity was ceased by the constitutive enzymes within 6 h but induced and constitutive enzymes collectively achieved this result within minutes. Moreover, total biodetoxification was observed at every pH adjustment. Analytical results confirmed >84% degradation potential in each sample. Our results indicate a uniquely fast way for the detoxification of AFB1 with intracellular enzymes of *R. erythropolis* NI1 and *R. rhodochrous* NI2.

Keywords: aflatoxin B1, biodetoxification, genotoxicity, intracellular extracts

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Introduction

Aflatoxin B1 (AFB1) is one of the most harmful mycotoxins occurring almost worldwide. Aflatoxins are a group of several compounds mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* [1] but more other *Aspergillus* strains (*Aspergillus pseudotamarii*, *Aspergillus nomius*, *Aspergillus bombycis*, *Aspergillus parvisclerotigenus*, *Aspergillus minisclerotigenes*, and *Aspergillus arachidicola*) have been proved to be aflatoxin producers [2]. Due to the appearance of these toxigenic molds on cereals, seeds, and spices, AFB1 endangers both animals and humans.

All among natural aflatoxins, AFB1 is one of the most toxic compounds and also proved to be human carcinogenic compound; according to IARC classification, AFB1 belongs to Group 1 [3]. Due to high liposolubility, AFB1 can easily get into the bloodstream and reach organs especially the liver. During its metabolism, AFB1 can be transformed into less or more harmful metabolites such as AFM1, AFP1, AFQ1, and aflatoxicol [4]. AFB1 is metabolized in humans by cytochrome P450 enzymes, which transform it to aflatoxin B1-8,9-epoxide (aflatoxin B1-2,3-oxide). This compound is highly reactive and forms DNA adducts. Binding to DNA, AFB1 is proved to damage DNA and cause liver cancer [5, 6]. Besides carcinogenicity, it has genotoxic and mutagenic effect; moreover, AFB1 is teratogenic, induces disorders in the reproductive system and has immunosuppressive effect additionally [7, 8].

Mycotoxins can be eliminated from feed- and food stuff using adsorbents or biotransforming agents. Since adsorbing materials can bind nutrients and vitamins, biotransformation can be an appropriate way for this purpose [9]. During the toxin degradation, which is completed by bacteria, fungi, or their enzymes, harmful breakdown products may be produced. For that reason, not only the biological effect of the parent toxin needs to be monitored but also its metabolites during biodegradation processes.

The structure of aflatoxins is made up of a coumarin ring, which compound is utilized by several microorganisms. Biodegradation experiments were carried out by Ciegler et al. [10], where *Aspergillus niger* strains and *Penicillium raistrickii* NRRL 2038 transformed AFB1 into other fluorescent breakdown products. Similarly, AFB1 was converted into aflatoxicol by *Dactylium dendroides*, *Mucor alternans*, *Mucor griseocyanus*, *Absidia repens*, *Helminthosporium sativum* [11], *Rhizopus* spp. [12, 13], *A. niger*, a non-aflatoxin producer *A. flavus*, *Eurotium herbariorum* [13] and into aflatoxin R0 by *A. niger*, *Mucor ambiguus*, *Trichoderma viride* [14]. Working with cell-free extracts of eukaryote strains, Cheng and Gu [15] and Liu et al. [16] reported a multienzyme called

aflatoxin-detoxifizyme (ADTZ), which was extracted from mycelia of *Armillariella tabescens*. This enzyme could degrade AFB1 and was identified as an oxidase and renamed to aflatoxin-oxidase (AFO) later [17]. Motomura et al. [18] extracted an extracellular enzyme from *Pleurotus ostreatus*, which could reduce the amount of the toxin. Laccase enzymes of *P. ostreatus*, *Peniophora* sp. were produced and used for biodegradation of AFB1 by Alberts et al. [19]. The filtrates of the fungal culture could degrade approx. 36% and 40% of the compound, but the degradation rate increased up to more than 80%; moreover, the mutagenicity was ceased using pure laccase enzyme from *Trametes versicolor*. After the transformation of the gene encoding the laccase production into *A. niger*, the AFB1 degradation rate was 55%. Pure laccase enzyme from *T. versicolor* was applied for the detoxification of AFB1 by Zeinvand-Lorestani et al. [20] as well, and 67% toxin was removed from the medium with a remarkable loss of mutagenicity during 2 days. Not only laccase but manganese peroxidase produced by *P. ostreatus* has been proved to have detoxification ability as well, with 90% elimination of AFB1 after 48 h [21].

Ciegler et al. [10] examined the AFB1-transforming ability of prokaryotes, and one of the analyzed strains, *Flavobacterium aurantiacum* NRRL B-184 could reduce the concentration of AFB1; moreover, no toxicity of the metabolites was detected by Duckling assay. Arai et al. [22] tested strains, which were able to grow in the presence of AFB1. Besides the examination of the antimicrobial effect of the toxin, *Nocardia asteroides* IFM8 proved to transform the mother toxin to another fluorescent metabolite. Later on, biodegradation of AFB1 was proved by *Corynebacterium rubrum* [14], *Mycobacterium fluoranthenivorans* [23], *Bacillus licheniformis*, and *Bacillus subtilis* [24–26], *Brevundimonas* spp., *Klebsiella* spp., *Enterobacter* spp., *Brachybacterium* spp., *Cellulosimicrobium* spp. [27], *Rhodococcus* spp. [28], *Pseudomonas putida* strains [29], and *Streptomyces* spp. [28, 30]; moreover, some members of the *Streptomyces* genus could decrease or cease the genotoxicity of AFB1 proved by SOS-Chromotest according to Harkai et al. [30]. During recent years, cell-free extracts of bacteria were applied for biodegradation and biodetoxification as well. The culture supernatant of *Stenotrophomonas maltophilia* 35-3 [27], *B. subtilis* ANSB060 [25], and a *Pseudomonas aeruginosa* strain [31] eliminated more than 80% of AFB1. The biodegradation ability of *Rhodococcus erythropolis* DSM 14303 strain was examined by Teniola et al. [32], where living cells and cell-free extracts were analyzed and compared to two *Nocardia corynebacterioides* and *M. fluoranthenivorans* sp. nov. DSM 44556^{T}. Intracellular extracts of the *Rhodococcus* and *Mycobacterium* strains seemed to be more effective than the two *Nocardia* strains. One year later, Alberts et al. [33] examined the extracellular supernatant of

R. erythropolis DSM 14303, and the biodegradation ability of induced and constitutively produced enzymes was also analyzed. The extracellular enzymes, which were produced constitutively, could degrade more than 70% of the toxin and greatly reduced the genotoxicity. Zhao et al. [34] purified an extracellular enzyme and named myxobacteria aflatoxin degradation enzyme (MADE) from *Myxococcus fulvus* ANSM068, which could degrade aflatoxins effectively. Recently, AFB1 degradation by a supernatant of a *B. licheniformis* strain was carried out by Raksha Rao et al. [35].

In our laboratory, biodetoxification potential of *Rhodococcus* strains was examined further. According to the results of Krifaton et al. [36], 5 rhodococci could detoxify 2 μg mL^{-1} AFB1 confirmed by SOS-Chromotest. These strains were *R. erythropolis* NI1 and GD2B, *Rhodococcus rhodochrous* NI2 and ATCC 12674, *Rhodococcus pyridinivorans* K405. Further examinations were carried out by Cserháti et al. [37] with the aim of biodetoxification of mycotoxins by *Rhodococcus* strains. Biodetoxification potential of additional strains of *R. pyridinivorans*, *R. erythropolis*, *R. rhodochrous*, and *R. globerulus* was analyzed resulting in total loss of genotoxic effect.

In this study, two *Rhodococcus* strains (*R. erythropolis* NI1 and *R. rhodochrous* NI2) were applied, which have already proved to detoxify AFB1 [36]. Our purpose was to reveal biodegradation of AFB1 by cell-free extracts of the strains. In these experiments, biodetoxification potentials of intra- and extracellular enzymes; moreover, induced and constitutively produced enzymes were compared. Further experiments were also carried out to investigate the pH tolerance of revealed enzymes. The success of the biodetoxification was checked by different methods. The remained toxin concentration was evaluated by high performance liquid chromatography with fluorescence detection (HPLC-FLD) (Wessling Hungary Ltd., Hungary) and remained genotoxic effect in the extracts was examined by SOS-Chromotest (Environmental Bio-detection Products Inc., Ontario, Canada).

Materials and Methods

Applied bacterial strains and AFB1 stock solution

For the AFB1 degradation experiments, cell-free extracts of two bacterial strains were used, which cells have been proved to detoxify AFB1 [36]. These strains, namely *R. erythropolis* NI1 and *R. rhodochrous* NI2, have been isolated from a hydrocarbon-contaminated site in Hungary and have been placed into a private culture collection. The toxin was purchased from Fermentek Ltd. (Israel) and dissolved in acetone.

Preparing cell-free extracts and AFB1 biodetoxification

Different set-ups were carried out to reveal extra- or intracellular enzymes, which are responsible for the AFB1 degradation. Strains maintained at −80 °C were streaked on Luria-Bertani (LB) agar plates (10 g triptone, 5 g yeast extract, 10 g NaCl, and 18 g bacteriological agar dissolved in 1 L distilled water) and incubated at 28 °C. Single colonies of both strains were inoculated into fresh LB medium (20 mL) and incubated for 3 days (28 °C). 10 mL pre-cultures were further inoculated into 90 mL LB medium and also cultured for 3 days. After the incubation, bacterial cultures were centrifuged at 4,000 rpm for 20 min at 4 °C (Eppendorf 5810 R Centrifuge, Eppendorf, Germany) and supernatants were filtered (0.2 μm) avoiding growth of remained cells. In this matrix, only extracellular enzymes were presented. To obtain the intracellular enzymes, after centrifugation of the culture (4 °C, 4,000 rpm, 20 min), supernatants were decanted. Cells were washed twice with phosphate buffer (67 mM, pH 7), and resuspended in the buffer (1 g pellet in 3 mL buffer). Bacterial cells were disintegrated by ultrasonic cell disintegrator (Branson Digital Sonifier, Emerson Industrial Automation, Minnesota, USA) with the help of zircon powder for 6 × 1 min in pulsed mode avoiding temperature increase. Cell debris and supernatant were separated by centrifugation and supernatant was filtered (0.2 μm).

Biodegradation experiments were carried out in 1.5 mL Eppendorf tubes, where untreated supernatants and supernatants of the sonicated cells were contaminated with AFB1 reaching 1 μg mL^{-1} final concentration, whereas LB medium and phosphate buffer contaminated by AFB1 (in 1 μg mL^{-1} final concentration) were used as controls. The biodegradation experiment was incubated for 6 h at 37 °C in the dark. An amount of 100 μL samples were removed from tubes at zero time and end point and samples were stored at −20 °C until further testing. Moreover, preincubation was needed to induce the production of biodegradative enzymes; therefore, strains were grown in the presence of AFB1 for 3 days before harvesting. Hereupon extra- and intracellular enzymes were obtained as mentioned earlier.

To prove whether the biodetoxification process occurred enzymatically or not, proteinase K plus sodium dodecyl sulfate (SDS) treatment was applied. Proteinase K is a protease isolated from *Tritirachium album*, which is able to hydrolyze keratin and has strong proteolytic activity on several proteins [38]. This feature makes it applicable to digest revealed enzymes. After preparation of cell-free extracts, 1 mg mL^{-1} proteinase K plus 1% SDS was added to the matrices and they were incubated for 1 h at 37 °C. After incubation, the matrices were contaminated by AFB1 (in final concentration of 1 μg mL^{-1}) and incubated for

six more hours at 37 °C in dark. Experiments were carried out in three replicates and each of them was set up in triplicate.

Investigation the pH tolerance of enzymes

The pH tolerance of enzymatic reactions was investigated, thus experiments were carried out at pH 7, 7.5, and 8, after the disintegration of cells according to the section "Preparing cell-free extracts and AFB1 biodetoxification" and incubated at 37 °C for 6 h in the dark in each test.

Determination of protein concentration

Protein concentrations in media were determined according to Bradford [39]. Briefly, the assay is based on the ability of Coomassie Brilliant Blue G-250 dye to bind proteins and change the absorption maximum of the dye from 465 to 595 nm. The protein–dye complex, which becomes blue, can be measured by spectrophotometer at 595 nm. In the assay, bovine serum albumin (BSA) was used as a standard protein, and diluted with phosphate buffer in concentrations of 0.025, 0.125, 0.250, 0.500, 0.750, 1.000, 1.500, and 2.000 mg mL^{-1}; subsequently 100 μL of controls and samples were mixed with 1 mL Coomassie reagent (Sigma-Aldrich Co., Missouri, USA). After 2 min, while the dye bonds to proteins, the absorbance was measured by UV-VIS spectrophotometer (Genesys 10 UV-VIS, Thermo Fisher Scientific Inc., Massachusetts, USA). Absorbance values of BSA were plotted on a graph and a standard curve was applied to estimate the protein concentration of extracts.

Detection of the genotoxicity

The remained genotoxicity was detected by SOS-Chromotest purchased from Environmental Bio-Detection Products Inc. (Ontario, Canada). Briefly, the test is based on the operon fusion of the *sfiA* gene, which expression induces the SOS-repair mechanism and the *lacZ* gene also encoding the β-galactosidase production. The test organism is *Escherichia coli* PQ37, in which *lacZ* region has been inserted under control of SOS-repair region. Genotoxic compound induces the expression *sfiA* gene and *lacZ* gene together producing β-galactosidase [40]. In this colorimetric assay, β-galactosidase can be transformed into a product resulting in blue color development with the addition of X-gal (Blue Chromogen) substrate. Besides the genotoxicity, detecting cytotoxic effect is a control

measurement in this test. Alkaline phosphatase activity is indicative of the viability of the test organism. After the addition of p-nitrophenyl phosphate substrate, yellow color development can be observed.

First of all, the lyophilized *E. coli* PQ37 was rehydrated, and 10 mL fresh casitone broth medium (2.5 g yeast extract, 2.5 g casein peptone, and 8.5 g NaCl dissolved in 1 L distilled water) were inoculated by 20 μL rehydrated strain and incubated overnight at 37 °C in the dark. The test was carried out in 96-well microplate, where 10 μL samples were added into the wells in triplicate. As positive controls AFB1, 2-amino-anthracene (2AA) and 4-nitroquinoline 1-oxide (4NQO) were used. Measuring extracellular enzymes, LB was used as negative control, but in the case of intracellular enzymes, phosphate buffer was used as negative control. Samples and indirect genotoxins (AFB1, 2AA) were metabolically activated by S9 mix including rat liver extracts treated with genotoxic compounds earlier, but 4NQO was measured without metabolic activation. The optical density of the test strain was adjusted to $OD_{600} = 0.05$, and 100 μL bacterial suspension with or without S9 mix was added onto samples and controls. The microplate was incubated in the dark at 37 °C for 2 h and then the substrate was added. The substrate containing 50 mg p-nitrophenyl phosphate dissolved in 10 mL Blue Chromogen and 100 μL of the mixture was added to samples; afterward, the microplate was incubated further for one and a half hours at 37 °C. The absorbance was measured in a microplate reader (ELx800 Absorbance Reader, BioTek Instruments, Vermont, USA), where blue color was detected at 620 nm, and yellow color was detected at 405 nm. From the absorbance values of triplicate samples, induction factor (IF) was determined according to the equation reported by Legault et al. [41]. If IF value is lower than 1.5, there is no detectable genotoxic effect. The IF can be determined according to Equation (1).

$$IF = (C_{405} \times S_{620})/(S_{405} \times C_{620}), \quad (1)$$

where C is the absorbance value of the negative control, S is the absorbance value of the sample measured at different wavelengths (405 and 620 nm).

Analytical measurement

The analytical detection was carried out by an accredited laboratory (Wessling Hungary Ltd., Hungary) and the remained AFB1 concentration was detected by HPLC-FLD. After ultrasonic homogenization, the pre-column derivatization was accomplished at 55 °C for 15 min with the mixture of trifluoroacetic acid:acetic acid:distilled water (1:1:8). For the measurement,

Agilent 1100 High-Performance-Liquid-Chromatograph (Agilent Technologies, California, USA) was used and equipped with Restek C18 (150 mm × 4.6 mm × 5 μm) column (Restek Corporation, Pennsylvania, USA). The detection was completed by fluorescent detector at the wavelengths of 365 (extinction) and 440 nm (emission). Depending on the AFB1 concentration, 200 or 10 μL sample were mixed with 800 or 990 μL water:acetonitrile:methanol (68:16:16) eluent agent. An amount of 100 μL of the mixture was injected into the equipment under isocratic conditions. The retention time was 17 min and the applied column temperature was 30 °C. As a standard, Supelco Aflatoxin Mix Kit (Supelco Inc., Pennsylvania, USA) was used.

Statistical analysis

For statistical analysis, data were analyzed by Microsoft Excel (Microsoft Office, Microsoft Inc., Washington, USA) and GraphPad Prism (GraphPad Inc., California, USA). After confirmation of normal distribution, one-sample *t*-test was applied. The analysis was carried out to confirm significant differences from the non-genotoxic threshold IF = 1.5. Differences were considered significant if $p < 0.05$.

Results

In this research, cell-free extracts of two *Rhodococcus* strains (NI1 and NI2) were analyzed according to their AFB1-detoxifying ability. Different set-ups were carried out to reveal what sorts of enzymes play the key role in the degradation processes. The detoxification potentials of induced and constitutively produced enzymes, furthermore extra- and intracellular enzymes were compared.

Protein concentrations

After obtaining cell-free extracts, relative protein concentrations of each extract were determined according to defined concentrations of the control (BSA). Protein concentrations of extracellular extracts of applied strains were less than the lowest standard concentration, i.e., <0.025 mg mL^{-1}. However, concentrations of constitutive and induced intracellular enzymes were evaluated. Determined protein concentrations of intracellular extracts are listed in Table I, where values are means of triplicate samples supplemented by standard deviations. Comparing the concentration of constitutive and induced enzymes of both strains, negligible

Table I. Protein concentration of the intracellular extracts of *Rhodococcus erythropolis* NI1 and *R. rhodochrous* NI2

Protein concentration	Intracellular extracts	
	Constitutive proteins (mg mL^{-1})	Induced proteins (mg mL^{-1})
R. erythropolis NI1	6.09 ± 0.29	6.52 ± 0.50
R. rhodochrous NI2	5.95 ± 0.34	5.88 ± 0.98

Note: The values are mean values supplemented by standard deviations.

differences were observed. The relative protein concentrations were approx. 6 mg mL^{-1} in each intracellular extract.

Results of the SOS-Chromotest

The genotoxicity of extracts was measured in the zero time and end point by SOS-Chromotest, and IF are listed in Table II. Samples with IF significantly less than 1.5 ($p < 0.05$) were considered as non-genotoxic. Extracellular extracts were tested at pH 7, and intracellular extracts were examined at pH 7, 7.5, and 8.

Evaluating the detoxification ability, neither constitutive nor induced extracellular enzymes of NI1 and NI2 seem to be able to decrease the genotoxicity of the toxin during 6 h. IFs varied between 2.49 and 2.95, which are not significantly less ($p > 0.05$) than the control values (average IF = 2.61). In contrast, intracellular extracts of both strains have remarkable detoxification ability. In the intracellular extracts, genotoxicity was ceased in 6 h but the rapidity of the degradation processes was different. In cases of constitutive and induced enzymes of NI2 and constitutive enzymes of NI1, outstanding reduction of harmful effect occurred after a few minutes, and no genotoxic effects could be detected at the end of the sixth hours. Induced enzymes of NI1 are needed to be emphasized since total detoxification was detected immediately after enzymes contacted to the toxin (IF = 1.19, $p = 0.0027$).

Examining pH tolerance of the extracts, enzymes were shown to be stable against pH variations. At pH 7.5, induced enzymes of NI1 and NI2 resulted in immediate detoxifications, IFs are 1.39 in both cases (NI1: $p = 0.0202$; NI2: $p = 0.0089$), and constitutive enzymes were able to cease the genotoxicity during 6 h (NI1: $p = 0.0001$; NI2: $p = 0.0175$). At pH 8, only in the induced extract of NI1 reached detoxification immediately (IF = 1.40, $p = 0.0065$). In every set-up, the enzymatic detoxification was completed within 6 h at pH 8.

Between pH 7 and 8, the genotoxic effect was ceased by the cell-free extracts in 6 h, as no genotoxicity was detected at the endpoint at all. However, the

Table II. Genotoxic effects of cell-free extracts of *Rhodococcus erythropolis* NI1 and *Rhodococcus rhodochrous* NI2 derived from zero time and end point of aflatoxin B1 biodegradation experiments

	Extracellular extracts				Intracellular extracts											
	pH 7				pH 7				pH 7.5				pH 8			
	Constitutive		Induced		Constitutive		Induced		Constitutive		Induced		Constitutive		Induced	
IF	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h
AFB1 control	2.61 ± 0.09[a]	2.59 ± 0.25[a]	2.50 ± 0.10[a]	2.73 ± 0.08[a]	2.61 ± 0.14[a]	2.38 ± 0.07[a]	2.61 ± 0.14[a]	2.55 ± 0.07[a]	2.29 ± 0.24[a]	2.22 ± 0.34[a]	2.29 ± 0.24[a]	2.22 ± 0.34[a]	2.15 ± 0.08[a]	2.12 ± 0.08[a]	2.15 ± 0.08[a]	2.12 ± 0.08[a]
R. erythropolis NI1	2.49 ± 0.02[a]	2.50 ± 0.03[a]	2.91 ± 0.07[a]	2.82 ± 0.03[a]	1.49 ± 0.32[b]	0.95 ± 0.15[c]	1.19 ± 0.14[c]	1.12 ± 0.09[c]	1.83 ± 0.09[b]	1.24 ± 0.06[c]	1.39 ± 0.08[c]	1.14 ± 0.11[c]	1.87 ± 0.06[b]	1.26 ± 0.15[c]	1.40 ± 0.06[c]	1.21 ± 0.08[c]
R. rhodochrous NI2	2.45 ± 0.12[a]	2.51 ± 0.02[a]	2.76 ± 0.02[a]	2.95 ± 0.04[a]	1.35 ± 0.26[b]	1.00 ± 0.26[c]	1.33 ± 0.29[b]	0.95 ± 0.03[c]	1.54 ± 0.10[b]	1.22 ± 0.19[c]	1.39 ± 0.06[c]	1.31 ± 0.12[c]	1.63 ± 0.06[b]	1.14 ± 0.07[c]	1.52 ± 0.03[b]	1.28 ± 0.10[c]

Note: The values are indicated in induction factors (IF), which show genotoxic potential if the value is higher than 1.5. The values are mean values supplemented by standard deviations.

[a]Strong genotoxicity (IF > 2.0).

[b]Reduced genotoxicity (1.5 < IF < 2.0 or IF not significantly different from 1.5).

[c]No genotoxicity (IF significantly less than 1.5, $p < 0.05$).

rapidity of the detoxification varied at the different adjustments, but the reduction of genotoxicity at zero points was the most prominent at pH 7.

Analytical results of the AFB1 concentration in the extracts and proteinase K plus SDS-treated extracts

The biodegradation potential of intracellular extracts of strains was evaluated by analytical measurements (Table III). Determination of AFB1 concentration by HPLC-FLD showed more than 80% biodegradation in the case of all extracts. 84% degradations were calculated in case of NI2 since there are no differences in the degradation potential of induced and constitutive enzymes. However, constitutively produced enzymes of NI1 resulted in the highest, 91% degradation potential, which means approx. 0.1 μg mL^{-1} AFB1 remained concentration in the extract. Since the preincubated extracts contain constitutive and induced enzymes as well, cell-free extracts of NI1 proved to have approx. 84% or more biodegradation ability similarly to NI2.

To prove that the biodetoxification was enzymatic, intracellular extracts were treated by proteinase K plus 1% SDS. Analytical measurements confirmed negligible AFB1 degradation in case of NI1 and no degradation in case of NI2 when intracellular extracts were treated by proteinase K plus SDS. These results suggested inactivation of enzymes caused the loss of detoxification potential, confirming the enzymatic detoxification of AFB1.

Table III. The remained aflatoxin B1 concentration in the untreated and proteinase K plus SDS-treated intracellular extracts of *Rhodococcus erythropolis* NI1 and *Rhodococcus rhodochrous* NI2 after 6-h incubation

	AFB1 concentration (μg mL^{-1})	Degradation potential (%)
AFB1 control	1.043 ± 0.04	–
Constitutive, intracellular extract of *R. erythropolis* NI1	0.096 ± 0.01	91
Induced, intracellular extract of *R. erythropolis* NI1	0.169 ± 0.01	84
Constitutive, intracellular extract of *R. rhodochrous* NI2	0.171 ± 0.01	84
Induced, intracellular extract of *R. erythropolis* NI2	0.167 ± 0.00	84
AFB1 control + proteinase K plus SDS	0.986 ± 0.11	–
Constitutive, intracellular extract of *R. erythropolis* NI1 + proteinase K plus SDS	0.887 ± 0.06	10
Constitutive, intracellular extract of *R. rhodochrous* NI2 + proteinase K plus SDS	1.002 ± 0.15	0

Note: The values are determined by high performance liquid chromatography with fluorescence detection. The values are mean values supplemented by standard deviations.

Discussion

AFB1 is a naturally occurring carcinogenic compound, which poses a relevant threat to animals and human widespread. Several methods (chemical, physical, and biological) have been developed for the AFB1 elimination [42–44], but the biological detoxification seems to be the most potent way for this purpose. Microorganisms are proved to be able to degrade AFB1 but the enzymes behind these processes are less examined and only a few of them have been identified. Fungal enzymes, isolated from *A. tabescens*, removed 90% of the toxin and the loss of mutagenicity was proven by Ames test after 48 h [16]. The multienzyme was named ADTZ, but its oxidative features proved that the enzyme is an oxidase and it has been renamed AFO later [17]. From a bacterial strain, namely *M. fulvus*, an extracellular enzyme has been purified, identified, and named MADE. After 48 h the enzyme effectively removed aflatoxins (AFB1, AFG1, and AFM1) from the supernatant, but the reduction of genotoxicity was not evaluated [34].

In this research, cell-free extracts of two microbes (*R. erythropolis* NI1 and *R. rhodochrous* NI2) were used, after the living cells of these strains proved to be able to detoxify AFB1 in our earlier studies [36]. However, these living microorganisms are not allowed to be applied in food- and feed processes according to the Qualified Presumption Safety concept of EFSA [45]. For this reason, cell-free extracts were examined to reveal what sorts of enzymes manage the AFB1 detoxification. Extracellular enzymes in supernatants were tested after harvesting cells. Intracellular enzymes were derived from the bacterial cells using ultrasonic disintegration. Not only constitutively produced enzymes were tested but also preincubation with the presence of AFB1 was applied for the examination of induced enzymes.

Our results suggested that extracellular enzymes do not have detoxification ability based on the genotoxicity determined by SOS-Chromotest. However, intracellular (induced and constitutive) enzymes of both strains were able to complete the detoxification of AFB1. The experiments were carried out at different pH adjustments (pH 7, 7.5, and 8) to analyze the pH stability of enzymes. Total detoxification was observed within 6 h in every pH adjustment. Analytical measurements confirmed 84%–91% degradation by intracellular enzymes of NI1 and NI2. Extracts were also treated by proteinase K plus 1% SDS before contaminated by AFB1. After digesting enzymes, no reduction of AFB1 concentration was measured, this observation proves that enzymatic biodetoxification occurred.

The rapidity of AFB1 detoxification in this study is remarkable, compared to data published to date, since AFB1 degradation or detoxification by cell-free extracts of bacteria has been reported in the studies mostly after 72 h. Teniola et al. [32] used the intracellular extract of *R. erythropolis* strain resulting in more than 90% toxin degradation after 72 h. The extracellular extract of the same *R. erythropolis* strain was examined 1 year later [33], where only 27% of AFB1 remained and the mutagenic effect was ceased confirmed by Ames test, but only after 72 h. Similarly, extracellular extracts of *Stenotrophomonas*, *Bacillus*, and *Pseudomonas* spp. strains were able to eliminate 80% AFB1 over 72 h [25, 27, 31]. A recent study reported enzymatic AFB1 detoxification, where 90% of 0.5 μg mL^{-1} toxin was removed from the supernatant of *B. licheniformis* CFR1 after 24 h [35].

In summary, our results show that the biodetoxification was uniquely fast (less than 6 h) applying intracellular extracts of NI1 and NI2. Enzymatic processes were quite stable since the biodetoxification was completed between pH 7 and 8. Besides the biodegradation of AFB1, the cessation of genotoxicity of the parent toxin and its breakdown products was also proved.

In conclusion, both constitutive and induced intracellular enzymes of *R. erythropolis* NI1 and *R. rhodochrous* NI2 are able to work uniquely fast since the detoxification of AFB1 is completed within 6 h. Consequently, further research is needed to purify and identify enzymes presented in the intracellular extracts of NI1 and NI2. The members of *Rhodococcus* genus can be applied for biotechnological purposes using only their enzymes according to EFSA QPS list. Since the efficiency of the intracellular extracts of NI1 and NI2 has been confirmed, the application of constitutive intracellular enzymes of both strains is a fast and cost-effective way to eliminate mycotoxin contamination. These enzymes presumably may be applicable in feed and food industry in the future.

Acknowledgements

This research was supported by the Foundation of the Hungarian Society for Microbiology, Research Centre of Excellence (9878-3/2016/FEKUT), and Aqua-future (VKSZ-12-1-2013-0078). The authors thank Balázs Kriszt, József Kukolya, and Mátyás Cserháti.

Conflict of Interest

The authors declare no conflict of interest.

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