Szent István University

BACTERIAL DETOXIFICATION OF AFLATOXIN B1 AND ZEARALENON WITH LIVING CELLS AND CELL-FREE EXTRACTS

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2019

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1 Backgrounds, aims and scopes

Mycotoxins occur in feed- and foodstuffs endanger the health of human and animals for a long time, but they have been discovered since the middle of 20th century. In the 1960's in England, thousands of turkeys died in unknown disease, which was named to „Turkey X disease". After this case, researchers find out that the disease was caused by moldy feed contaminated by aflatoxin B1. Suddenly the toxicogenic molds and mycotoxins came into consideration. Nowadays, more than 400 compounds are known as mycotoxins. The agricultural producers emphasize the importance of prevention strategies, because the financial damage is relevant caused by mycotoxins. The toxigenic molds start to produce toxins under hectic climatic conditions, moreover due to the climate change toxigenic species spread away on those areas where they not existed before. Not only the financial damage causes problems, but hazardous biological effects of these compounds induce acute and chronic diseases and death additionally. According to this phenomenon, the detection of mycotoxins and the cessation of their harmful effect are urgent issues. For this purpose, physical, chemical and biological methods are known, but the biological detoxification might be the most effective way, where non-pathogenic microorganisms or their enzymes can degrade the chemical structure of mycotoxins. During biological detoxification, not only the rate of the degradation needs to be considered but the detection of biological effect of the breakdown products is very important, respectively. For measuring the effects of these compounds, biotests can be applicable, which are suited for the detection of total hazardous effect in the samples. To sum up, the mycotoxin-detoxification methods aim the cessation of the dangerous effects of the mother compound and its metabolites.

In my Ph.D. research, the carcinogenic aflatoxin B1 (AFB1) and the estrogen-analogue zearalenone (ZEA) were examined. These toxins are wide-spread in Europe and the world as well, thus their detoxification is a big challenge.

Based on this observation, the aims of this work were the followings:

I. Evaluating the aflatoxin B1 and zearalenone degrading ability of type strains of the genus *Rhodococcus* and observing the biological (genotoxic, endocrine disrupting) effects of the metabolites produced during the degradation process, thereby revealing the detoxification potential of the members of this genus.

II. Measuring the biodetoxification ability of cell-free extracts of two *Rhodococcus* strains, which proved to be able to detoxify aflatoxin B1 by their living cells. Further aim was to reveal enzymes playing role in the detoxification process, according to the following features:
    A. extra- or intracellular,
    B. produced constitutively, or the presence of mycotoxin induces the expression of genes encoding these enzymes.

III. Measuring the biodetoxification ability of cell-free extracts of eight *Rhodococcus*, one *Streptomyces*, one *Gordonia* and one *Pseudomonas* strains, which proved to be able to degrade and detoxify aflatoxin B1 by their living cells. Further aim was to reveal the enzymes playing role in the detoxification process, according to the features listed above (II. A-B.) and compare their effectiveness.

2 Materials and methods

2.1 Biodegradation and biodetoxification of AFB1 and ZEA with living cells

For the biodegradation of AFB1 and ZEA, 42 type strains of genus *Rhodococcus* were applied in order to reveal their degradation and detoxification ability. In the degradation experiment 3 μg/mL AFB1 or 1 μg/mL ZEA final concentration was set and triplicate samples were incubated for three (AFB1) or seven (ZEA) days at 28°C according to our earlier experiences. The remaining toxin concentrations in supernatants and on the bacterial pellet were measured by high-performance liquid-chromotograph equipped with fluorescent detector (HPLC-FLD). The genotoxicity of AFB1 and its metabolites were detected by the colorimetric SOS-Chromotest. The genotoxic effect was expressed in induction factor (IF), and if IF<1.5, the hazardous effect considered to be ceased. The estrogenic effect of ZEA and its breakdown products were observed by the bioluminescence-based BLYES test, where estrogenicity was expressed in bioluminescence intensification. The stronger the estrogenicity was, the higher the bioluminescence was. The cytotoxicity was checked by BLYR test, in which the cytotoxic effect inhibits the bioluminescence, thus inhibition percent was calculated.

2.2 Biodegradation and biodetoxification of AFB1 and ZEA with cell-free extracts

According to previous experiments carried out in the Department of Environmental Safety and Ecotoxicology at Szent István University, bacterial strains with known AFB1 and ZEA detoxification potential were analyzed further. Cell-free extracts of the strains were tested in mycotoxin biodegradation experiments revealing the features of the degrading enzymes. For degradation of AFB1, two *Rhodococcus* strains, while for degradation of ZEA, eight *Rhodococcus,* one *Gordonia*, one *Pseudomonas* and one *Streptomyces* strains were

applied. The experiments were carried out for diverse purposes. On the one hand, tests were conducted to reveal weather enzymes are produced constitutively during the growth of the bacteria or enzymes appear after the mycotoxin induces their gene expression. For production of induced enzymes, a pre-incubation step was carried out in the presence of AFB1 or ZEA. On the other hand, the degradation rates of extracellular enzymes excreted into the medium and intracellular enzymes presenting in the cytoplasm were compared. For the extraction of the intracellular enzymes, ultrasonic cell-disintegrator was used in pulsed mode. The experiments were carried out in 1.5 mL tubes in triplicates, where 1 μg/mL final mycotoxins concentration was applied. The tubes were incubated at 37°C for six hours in the dark. Confirming the enzymatic biodetoxification, the enzymes were digested by the mix of 1 mg/mL proteinase K and 1% sodium-dodecyl-sulfate (SDS) solution. The protein concentration in the cell-free extracts was measured by Bradford method in case of AFB1, and by Pierce 660 test in case of ZEA. The remaining toxin-concentration was detected by HPLC-FLD or HPLC-MS/MS, the genotoxicity was observed by SOS-Chromotest, and the estrogenicity was screened by BLYES test.

3 Results

3.1 Results of biodegradation and biodetoxification of AFB1 and ZEA with living cells

3.1.1 Biodegradation of AFB1

The toxin binding ability of bacterial cells was negligible since the concentration in pellet fraction was <0.095 μg/mL. Thus the biodegradation was not influenced by adsorption on pellet. According to the biodegradation potential of *Rhodococcus* type strains (Table 1.), 2 strains had no degradation ability (<20%), 8 strains could degrade AFB1 moderately (20-30%), and in case of 14 strains, remarkable degradation rates were observed (50-80%). Among the analyzed strains, almost the 2/3 part could reach 80% degradation; moreover 18 were able to reduce >90% of AFB1. From these strains, 15 strains could cease the genotoxicity of the toxin and its metabolites, additionally (Figure 1.). These most effective strains belong to the following species: *R. erythropolis, R. globerulus, R. pyridinivorans*; moreover *R. aerolatus, R. artemisiae, R. baikonurensis, R. corynebacterioides, R. enclensis, R. imtechensis, R. kroppenstedtii, R. lactis, R. qingshengii, R. rhodnii, R. trifolii* and *R. tukisamuensis,* which species were proved to detoxify AFB1 first time. Genus *Rhodococcus* has variable AFB1-detoxification potential, furthermore according to 16S rRNA sequences of the strains, they locate diversely on phylogenetic tree (Figure 2.).

Table 1. Aflatoxin B1 biodegradation potential of *Rhodococcus* type strains and the genotoxic effects detected in supernatant and on pellet

Species	Strains	Genotox. effect (IF)	AFB1 concentration (µg/mL)		Bio -degration (%)
			Supernatant	Pellet♦	
AFB1 control		4.43 ± 0.31	2.95 ± 0.15	-	-
R. kunmingensis	JCM 15626^T	4.39 ± 0.17	3.19 ± 0.08	0.022	-8
R. jostii	JCM 11615^T	4.01 ± 0.51	2.44 ± 0.19	< 0.001	17
R. antrifimi	KCTC 29469^T	3.67 ± 0.18	2.05 ± 0.18	0.064	31
R. ruber	JCM 3205^T	3.97 ± 0.31	1.93 ± 0.36	0.095	34
R. aetherivorans	JCM 14343^T	4.12 ± 0.33	1.91 ± 0.01	0.078	35
R. coprophilus	JCM 3200^T	4.15 ± 0.40	1.87 ± 0.08	0.011	37
R. zopfii	JCM 9919^T	3.88 ± 0.27	1.64 ± 0.79	0.062	44
R. triatomae	JCM 13396^T	4.24 ± 0.52	1.63 ± 0.69	0.007	45
R. sovatensis	H004^T	4.55 ± 0.57	1.60 ± 0.09	0.003	46
R. opacus	JCM 9703^T	3.35 ± 0.26	1.55 ± 0.11	0.024	48
R. soli	JCM 19627^T	3.19 ± 0.18	1.48 ± 0.04	0.048	50
R. nanhaiensis	DSM 45608^T	3.32 ± 0.13	1.37 ± 0.09	0.053	53
R. defluvii	DSM 45893^T	3.30 ± 0.06	1.36 ± 0.03	0.046	54
R. maanshanensis	JCM 11374^T	3.25 ± 0.36	1.30 ± 0.14	0.026	56
R. koreensis	JCM 10743^T	2.94 ± 0.62	1.01 ± 0.61	0.024	66
R. rhodochrous	JCM 3202^T	3.45 ± 0.17	1.01 ± 0.05	0.020	66
R. fascians	JCM 10002^T	2.80 ± 0.22	0.63 ± 0.01	< 0.001	79
R. phenolicus	JCM 14914^T	2.86 ± 0.53	0.59 ± 0.60	0.004	80
R. canchipurensis	JCM 17578^T	2.68 ± 0.29	0.55 ± 0.07	0.009	81
R. cercidiphylli	DSM 45141^T	2.68 ± 0.22	0.57 ± 0.17	0.012	81
R. wratislaviensis	JCM 9689^T	2.30 ± 0.12	0.52 ± 0.08	< 0.001	82
R. cerastii	DSM 45579^T	2.18 ± 0.15	0.34 ± 0.10	0.011	88
R. agglutinans	KCTC 39118^T	1.84 ± 0.34	0.32 ± 0.03	0.009	89
R. biphenylivorans	KCTC 29673^T	2.00 ± 0.20	0.31 ± 0.00	0.008	89
R. kyotonensis	JCM 23211^T	1.70 ± 0.14	0.23 ± 0.01	0.004	92
R. percolatus	JCM 10087^T	1.57 ± 0.11	0.21 ± 0.03	< 0.001	93
R. yunnanensis	JCM 13366^T	2.03 ± 0.25	0.15 ± 0.07	< 0.001	95
***R. imtechensis*[1]**	**JCM 13270^T**	**1.36** ± 0.08	**0.18** ± 0.04	**< 0.001**	**94**
R. erythropolis	**JCM 3201^T**	**1.26** ± 0.13	**0.15** ± 0.01	**< 0.001**	**95**
***R. tukisamuensis*[1]**	**JCM 11308^T**	**1.29** ± 0.14	**0.13** ± 0.08	**0.001**	**95**
***R. rhodnii*[1]**	**JCM 3203^T**	**1.26** ± 0.14	**0.10** ± 0.07	**0.030**	**97**
***R. aerolatus*[1]**	**JCM 19485^T**	**1.17** ± 0.05	**0.04** ± 0.00	**0.001**	**98**
***R. enclensis*[1]**	**DSM 45688^T**	**1.10** ± 0.09	**0.05** ± 0.00	**0.001**	**98**
***R. lactis*[1]**	**DSM 45625^T**	**1.16** ± 0.11	**0.07** ± 0.01	**0.003**	**98**
***R. trifolii*[1]**	**DSM 45580^T**	**1.13** ± 0.12	**0.06** ± 0.01	**0.003**	**98**
***R. qingshengii*[1]**	**DSM 45222^T**	**1.11** ± 0.04	**0.06** ± 0.01	**< 0.001**	**98**
***R. artemisiae*[1]**	**DSM 45380^T**	**1.09** ± 0.09	**0.03** ± 0.00	**0.001**	**99**
***R. baikonurensis*[1]**	**DSM 44587^T**	**1.01** ± 0.06	**0.03** ± 0.00	**< 0.001**	**99**
R. globerulus	**JCM 7472^T**	**0.96** ± 0.04	**0.02** ± 0.00	**< 0.001**	**99**
***R. kroppenstedtii*[1]**	**JCM 13011^T**	**1.06** ± 0.02	**0.02** ± 0.00	**< 0.001**	**99**
R. pyridinivorans	**JCM 10940^T**	**0.99** ± 0.07	**0.02** ± 0.00	**< 0.001**	**99**
***R. corynebacterioides*[1]**	**JCM 3376^T**	**1.00** ± 0.03	**0.01** ± 0.00	**< 0.001**	**100**

Bold Strains resulting in AFB1 biodetoxification (IF <1.5; p <0.05)

^{T} Type strains

[1] First report of the AFB1 detoxification potential of *Rhodococcus* species

♦ Extracted with 1 mL methanol

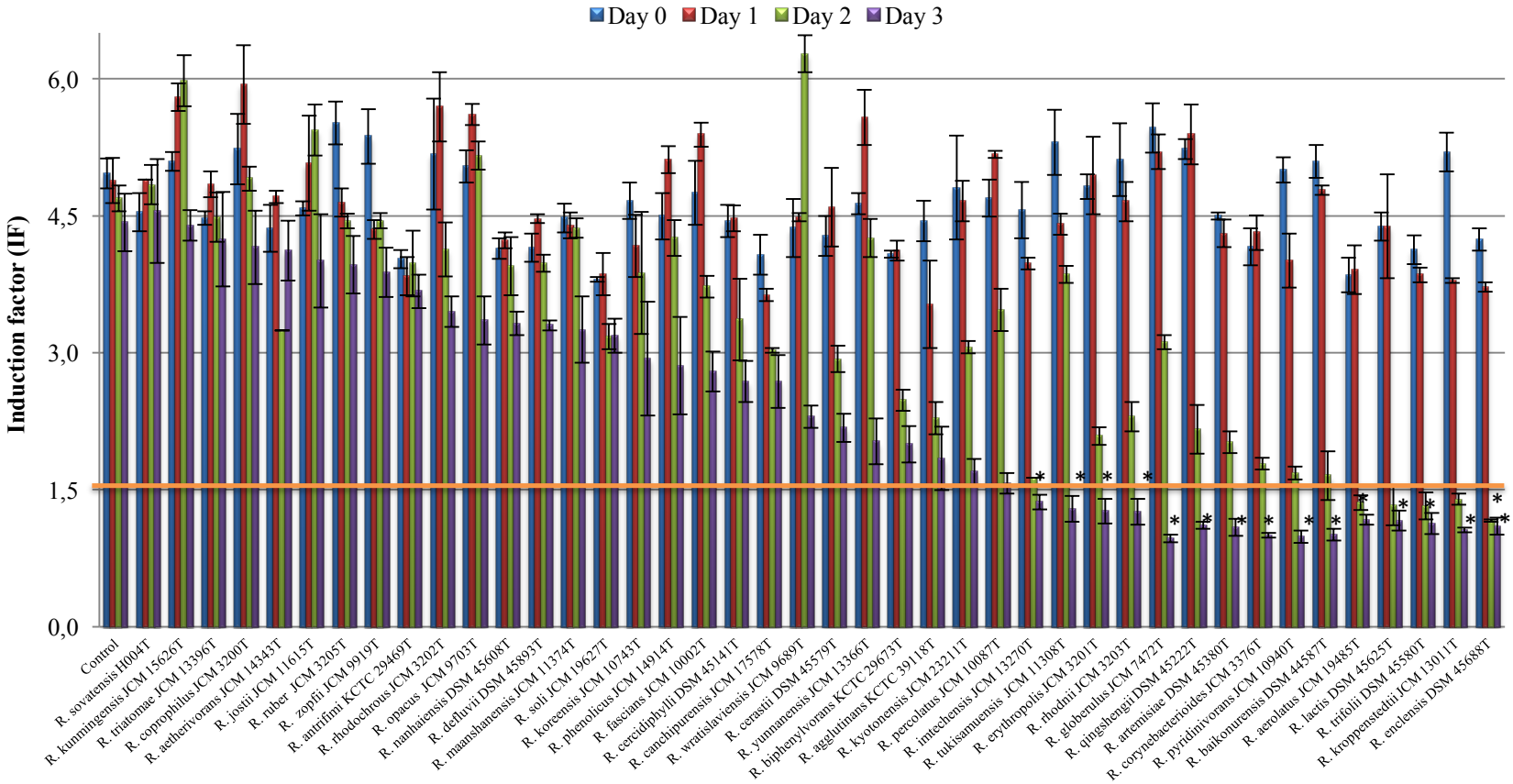

Figure 1. Genotoxicity of supernatant samples derived from AFB1 degradation experiment using *Rhodococcus* type strains detected by SOS-Chromotest. The genotoxic effects are expressed in induction factor. The cessation of genotoxicity is signed by * (IF<1.5 [p<0.05])

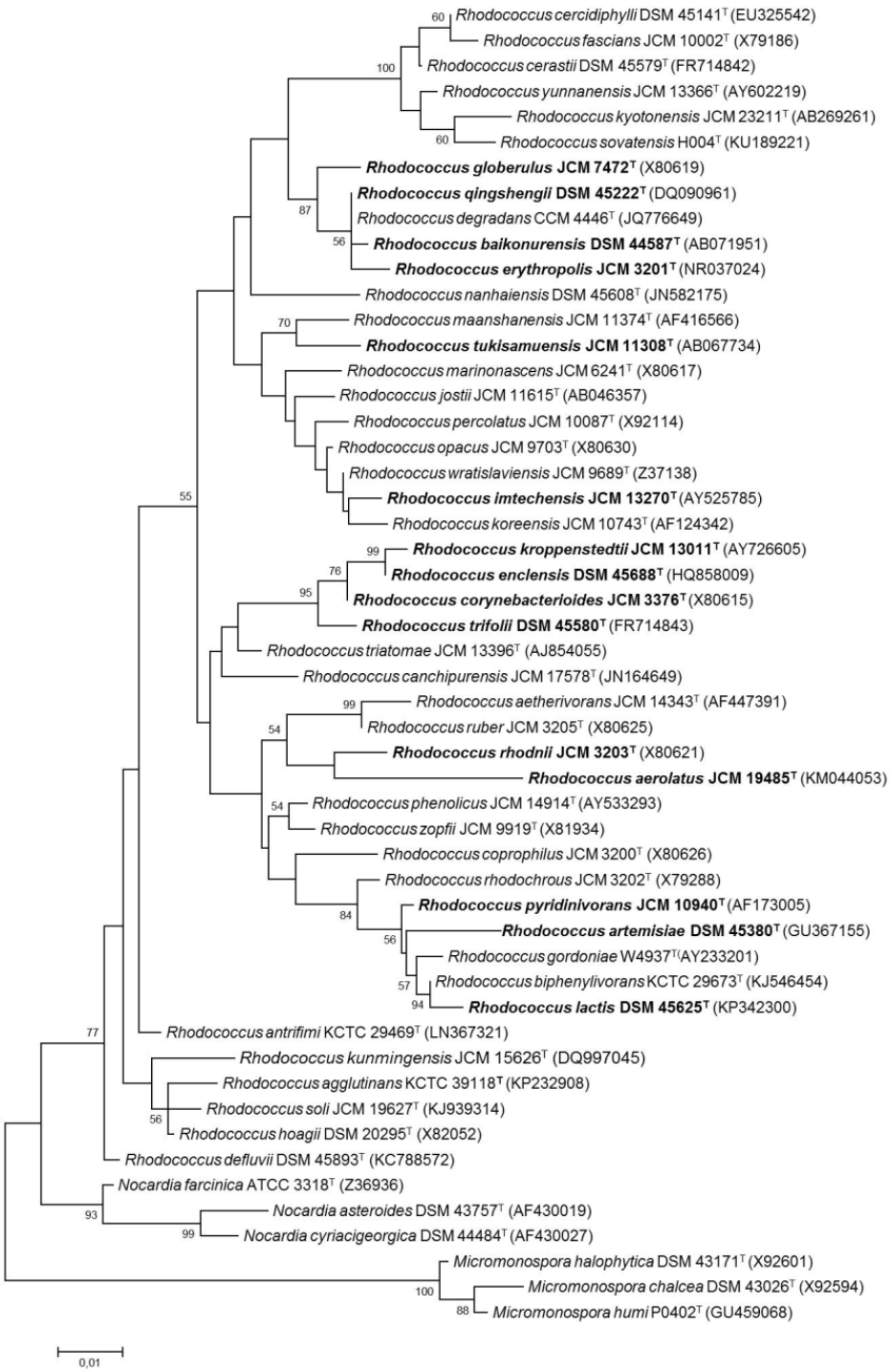

Figure 2. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships of *Rhodococcus* strains with high AFB1 biodetoxification potential (highlighted in bold). Bootstrap values are shown as percentages of 1000 replicates; only values over 50% are shown [Mega 6]

3.1.2 New scientific results

Thesis I. (Based on the results of chapter 3.1.1.): During the analysis of 42 type strains from the genus *Rhodococcus,* aflatoxin B1 biodegradation and biodetoxification ability of *R. imtechensis, R. tukisamuensis, R. rhodnii, R. aerolatus, R. enclensis, R. lactis, R. trifolii, R. qingshengii, R. artemisiae, R. baikonurensis, R. kroppenstedtii* and *R. corynebacterioides* species, locating diversely on phylogenetic tree, has been proven first.

3.1.3 Biodegradation of ZEA

After the degradation of some type strains (*R. fascians, R. aetherivorans, R. cerastii, R. kroppenstedtii, R. phenolicus, R. erythropolis, R. canchipurensis, R. triatomae, R. corynebacterioides, R. coprophilus, R. cercidiphylli, R. sovatensis, R. aerolatus, R. artemisiae*) significant ($p<0.05$) intensification in the bioluminescence were detected in the samples taken at the 7th day compared to the value detected in samples derived from day 0. This observation might be caused by the appearance of metabolites (α-ZOL and α-ZAL) that have higher estrogenicity than ZEA. Among the 42 type strains, 41 strains could not, only *R. percolatus* JCM 10087^T strain could decrease the bioluminescence, i.e. estrogenicity of ZEA with 70% (Table 2.). Due to the lack of detoxification ability considering the rest of the strains, remaining ZEA concentration only in case of JCM 10087^T strain was checked by HPLC-FLD. The analytical measurement detected 0.053 μg/mL ZEA in the supernatant sample, which means 95% degradation of the 1.171 μg/mL initial toxin-concentration. The biodegradation was confirmed by the analysis of pellet, since only 0.014 μg/mL ZEA was bound on the cells. According to this result, the reduction was caused by the metabolic activity of the strain not by adsorption on cells. This observation, that a *R. percolatus* strain (namely the type strain JCM 10087^T) was able to degrade ZEA and reduce its estrogenicity was proven firstly in the present work.

Table 2. Bioluminescence intensification percent of supernatant samples derived from ZEA degradation experiment using *Rhodococcus* type strains measured by BLYES test. Bioluminescence intensification refers to the estrogenicity.

Species	Strains	Bioluminescence intensification (%)	
		Day 0	Day 7
ZEA kontroll		880 ± 4.60	784 ± 4.72
R. fascians	JCM 10002^T	928 ± 2.63	1100* ± 0.76
R. aetherivorans	JCM 14343^T	988 ± 2.13	1090* ± 1.63
R. kunmingensis	JCM 15626^T	949 ± 4.00	1083 ± 12.69
R. qingshengii	DSM 45222^T	949 ± 4.27	1048 ± 5.67
R. cerastii	DSM 45579^T	840 ± 2.74	1039* ± 2.00
R. kroppenstedtii	JCM 13011^T	969 ± 1.72	1035* ± 2.96
R. globerulus	JCM 7472^T	948 ± 1.62	1030 ± 5.96
R. phenolicus	JCM 14914^T	922 ± 2.24	1029* ± 5.17
R. maanshanensis	JCM 11374^T	948 ± 3.81	1026 ± 3.12
R. erythropolis	JCM 3201^T	943 ± 2.45	1020* ± 2.91
R. canchipurensis	JCM 17578^T	816 ± 0.84	1009* ± 6.93
R. triatomae	JCM 13396^T	946 ± 0.67	1004* ± 1.45
R. tukisamuensis	JCM 11308^T	967 ± 4.56	1000 ± 1.49
R. wratislaviensis	JCM 9689^T	949 ± 0.71	993 ± 3.55
R. corynebacterioides	JCM 3376^T	942 ± 2.46	993* ± 1.30
R. ruber	JCM 3205^T	957 ± 3.49	991 ± 3.86
R. biphenylivorans	KCTC 29673^T	954 ± 0.66	976 ± 1.20
R. baikonurensis	DSM 44587^T	903 ± 1.37	969 ± 3.69
R. coprophilus	JCM 3200^T	897 ± 3.33	968* ± 0.71
R. cercidiphylli	DSM 45141^T	825 ± 3.21	965* ± 0.37
R. zopfii	JCM 9919^T	914 ± 3.49	942 ± 4.78
R. imtechensis	JCM 13270^T	967 ± 3.84	941 ± 0.90
R. sovatensis	H004^T	871 ± 2.37	935* ± 0.79
R. rhodochrous	JCM 3202^T	878 ± 2.76	931 ± 3.59
R. aerolatus	JCM 19485^T	900 ± 0.85	921* ± 0.50
R. pyridinivorans	JCM 10940^T	948 ± 2.85	915 ± 8.44
R. artemisiae	DSM 45380^T	819 ± 1.28	869* ± 2.71
R. enclensis	DSM 45688^T	827 ± 2.49	859 ± 1.48
R. kyotonensis	JCM 23211^T	924 ± 2.35	853* ± 1.53
R. soli	JCM 19627^T	899 ± 0.11	822* ± 0.89
R. agglutinans	KCTC 39118^T	913 ± 0.61	806* ± 0.93
R. antrifimi	KCTC 29469^T	866 ± 2.04	783* ± 1.11
R. rhodnii	JCM 3203^T	891 ± 4.15	771* ± 1.87
R. koreensis	JCM 10743^T	948 ± 3.28	766* ± 9.46
R. jostii	JCM 11615^T	896 ± 3.07	759* ± 2.07
R. yunnanensis	JCM 13366^T	914 ± 4.07	740* ± 5.54
R. opacus	JCM 9703^T	917 ± 1.19	722* ± 2.58
R. nanhaiensis	DSM 45608^T	828 ± 4.37	716* ± 1.99
R. defluvii	DSM 45893^T	802 ± 3.32	704* ± 2.14
R. trifolii	DSM 45580^T	803 ± 3.03	623* ± 1.80
R. lactis	DSM 45625^T	835 ± 2.63	611* ± 6.10
***R. percolatus*[1]**	**JCM 10087^T**	**902** ± 4.41	**256*** ± 4.02

Bold Estrogenicity loss with >70%

* Significant differences ($p<0.05$) compared to bioluminescence intensification values at day 0

[1] First report of the ZEA detoxification potential of *Rhodococcus* species

3.1.4 New scientific result

Thesis II. (Based on the result of chapter 3.1.3): During the analysis of 42 type strains from the genus *Rhodococcus,* zearalenone degradation ability of *R. percolatus* species has been proven first, and *R. percolatus* JCM 10087^T strain is able to degrade 95% of the toxin and cease 70% of its estrogenicity.

3.2 Results of biodegradation and biodetoxification of AFB1 and ZEA with cell-free extracts

3.2.1 Detoxification of AFB1 with cell-free extracts of *Rhodococcus* strains

The protein concentration of extracellular extracts of *Rhodococcus erythropolis* NI1 and *R. rhodochrous* NI2 was <0.025 mg/mL, and in the intracellular extracts ~6mg/mL protein concentrations were measured. In case of extracellular extracts of NI1 and NI2, neither the constitutive nor the induced extracts were able to decrease the genotoxic effect of AFB1 detected by SOS-Chromotest. However, in the constitutive and induced intracellular extracts the genotoxic effect was ceased, which means detoxification occurred within 6 hours. Revealing the pH-tolerance of the enzymes in the intracellular extracts, the experiments were carried out at pH 7.5 and 8 additionally. Although, the detoxification process took different long, the intracellular extracts of both strains could cease the genotoxicity at the end of the experiment between pH 7 and 8. According to the analytical measurement of toxin concentration in extracts involved in experiment carried out at pH 7, more than 80% biodegradation rate was observed in the intracellular extracts (Table 3.). After the treatment with proteinase K and 1% SDS, negligible reduction in toxin concentration was detected in samples. According to this observation, the treatment inactivated the degrading enzymes, which proves the enzymatic degradation in the untreated extracts (Table 4.).

Table 3. Genotoxicity of cell free extracts of *Rhodococcus erythropolis* NI1 and *R. rhodochrous* NI2 derived from aflatoxin B1 biodegradation experiment detected by SOS Chromotest

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

Strong genotoxicity (IF > 2.0)

Reduced genotoxicity (1.5 < IF < 2.0 or IF does not significantly differ from 1.5)

No genotoxicity (IF is significantly less 1.5 [$p<0.05$])

Table 4. Aflatoxin B1 concentration in proteinase K + SDS treated and non-treated intracellular extracts of *Rhodococcus erythropolis* NI1 and *R. rhodochrous* NI2 derived from aflatoxin B1 biodegradation experiment measured by HPLC-FLD

	AFB1 concentration [μg/mL]	**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 + SDS	0.986 ± 0.11	-
Constitutive intracellular extract of *R. erythropolis* NI1 + proteinase K + SDS	0.887 ± 0.06	10 %
Induced intracellular extract of *R. rhodochrous* NI2 + proteinase K + SDS	1.002 ± 0.15	0 %

3.2.2 New scientific results

Thesis III. (Based on the results of chapter 5.2.1): *Rhodococcus erythropolis* NI1 and *R. rhodochrous* NI2 strains degraded aflatoxin B1 with constitutively produced intracellular enzymes, which enzymes are able to degrade more than 80% of the toxin, and cease the genotoxicity within six hours. According to the presented results, degrading enzymes are stable between pH 7 and 8.

3.2.3 Detoxification of ZEA with cell-free extracts

Extracellular extracts applied for ZEA biodegradation contained <0.025 mg/mL protein, but protein concentrations in intracellular extracts of *Gordonia paraffinivorans* NZS6 and *Streptomyces cavourensis* K14 were ~0.2 mg/mL, in cases of *Pseudomonas pseudoalcaligenes* FEH28 and all of the *Rhodococcus* strains 2 mg/mL or more were detected.

Estrogenicity observed by BLYES test was not decreased in extracellular extracts, which indicates that extracellular enzymes do not play role in ZEA

degradation. Furthermore, strong cytotoxic effect (>70%) was detected in extracellular extracts of NZS6, additionally, presumably caused by the metabolites of the strains, which were excreted outside the cell (Table 5.).

Evaluating the cytotoxicity in intracellular extracts, 20-70% toxic effects were observed in cases of three strains, namely K14, AK44 and FEH28. Due to this phenomenon, estrogenicity in intracellular extracts were not evaluable. Intracellular extracts of the rest of the strains (NI1, N58, K408, K402, AK37, N361 from the genus *Rhodococcus*, and NZS6 from the genus *Gordonia*) were not able to decrease the estrogenicity of ZEA (Figure 3.). Only the induced intracellular extracts of *R. pyridinivorans* K404 could reduce the estrogenicity with more than 60%. Compared to this, no reduction in the estrogenicity was detected in the constitutive extract of the same strain. Measuring ZEA concentration with HPLC-MS/MS in intracellular extracts of K404, the same ZEA concentration was detected in the constitutive extracts as in the control sample, but 98% degradation of the toxin was detected in the induced extract. This result indicates that the induced intracellular enzymes of K404 were able to degrade almost the total amount of ZEA. After the proteinase K and SDS treatment, there was no toxin reduction in the induced extract compared to the control, thus the degrading enzymes was inactivated which caused the loss of degradation ability (Table 6.).

Table 5. Estrogenicity and cytotoxicity of extracellular extracts of bacterial strains derived from zearalenone degradation experiment. The estrogenic effect was expressed in bioluminescence intensification (%) and cytotoxic effect was expressed in bioluminescence inhibition (%).

Strains	Bioluminescence intensification (%)						Bioluminescence inhibition (%)					
	Constitutive extracts			Induced extracts			Constitutive extracts			Induced extracts		
	C	0h	6h	C	0h	6h	C	0h	6h	C	0h	6h
G. paraffinivorans NZS6	1220 ± 65	1369 ± 154	1216 ± 66	1411 ± 120	1410 ± 132	1463 ± 126	**75 ± 6**	**74 ± 15**	**76 ± 3**	**76 ± 10**	**64 ± 12**	**77 ± 2**
P. pseudoalcaligenes FEH28	975 ± 153	972 ± 163	978 ± 168	1038 ± 133	1044 ± 166	1055 ± 180	n.i	n.i	n.i	n.i	n.i	n.i
R. aetherivorans AK44	771 ± 64	733 ± 53	764 ± 56	908 ± 83	883 ± 94	894 ± 99	n.i	n.i	n.i	n.i	n.i	n.i
R. erythropolis NI1	846 ± 66	833 ± 60	855 ± 50	929 ± 76	915 ± 63	910 ± 72	n.i	n.i	n.i	n.i	n.i	n.i
R. globerulus N58	900 ± 44	863 ± 48	888 ± 34	971 ± 59	954 ± 68	951 ± 70	n.i	n.i	n.i	n.i	n.i	n.i
R. pyridinivorans K404	783 ± 97	722 ± 78	727 ± 68	867 ± 48	809 ± 40	806 ± 75	n.i	n.i	n.i	n.i	n.i	n.i
R. pyridinivorans K402	777 ± 74	763 ± 69	743 ± 66	858 ± 51	814 ± 67	788 ± 48	n.i	n.i	n.i	n.i	n.i	n.i
R. pyridinivorans K408	799 ± 46	806 ± 86	765 ± 58	887 ± 84	869 ± 37	860 ± 51	n.i	n.i	n.i	n.i	n.i	n.i
R. pyridinivorans AK37	782 ± 37	791 ± 60	782 ± 66	854 ± 56	832 ± 63	820 ± 75	n.i	n.i	n.i	n.i	n.i	n.i
R. ruber N361	828 ± 65	810 ± 60	846 ± 65	995 ± 80	983 ± 57	975 ± 90	n.i	n.i	n.i	n.i	n.i	n.i
S. cavourensis K14	918 ± 105	848 ± 111	885 ± 107	909 ± 87	858 ± 93	857 ± 102	n.i	n.i	n.i	n.i	n.i	n.i

C Control: Extracellular extract of strains contaminated by 1 µg/mL ZEA
n.i no inhibition

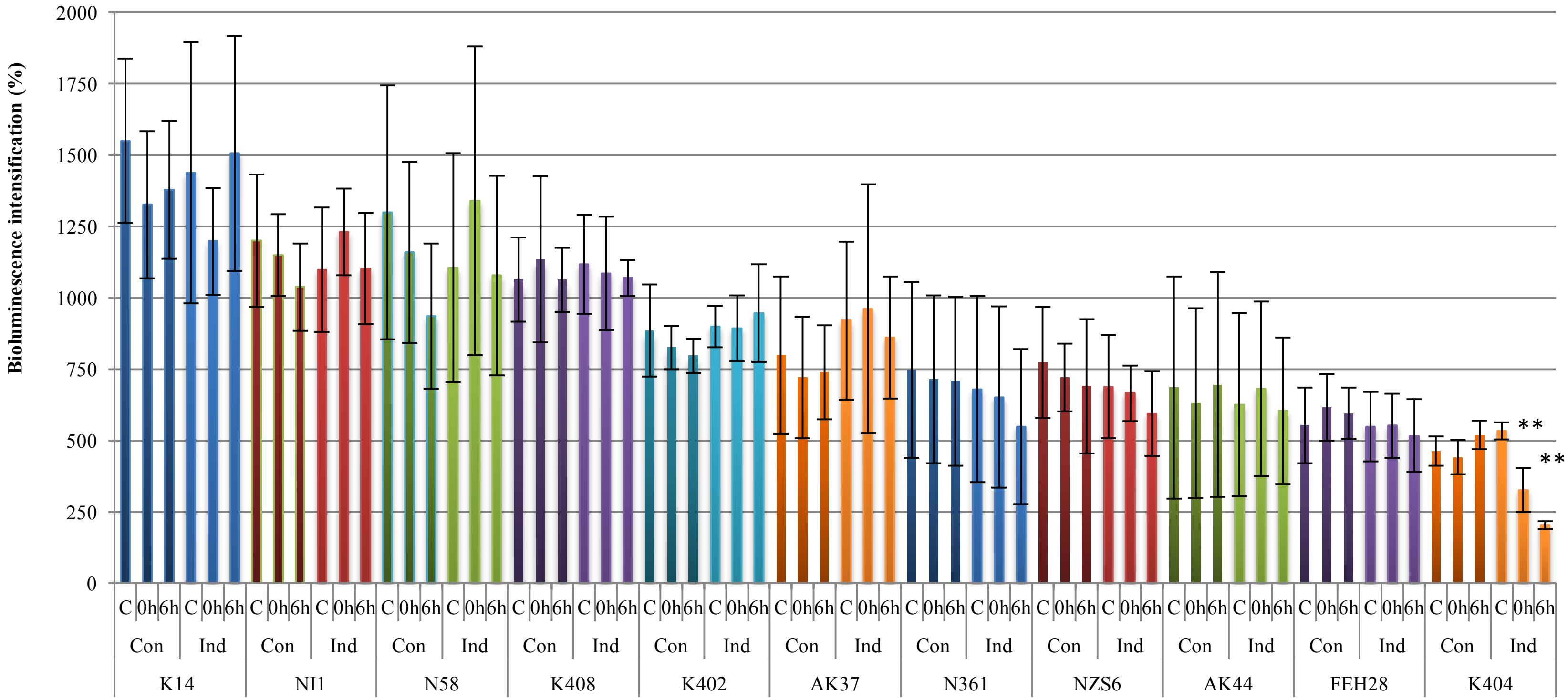

Figure 3. Bioluminescence intensification observed in intracellular extracts of bacterial strains derived from zearalenone degradation experiment. Bioluminescence intensification is balanced with estrogenicity. "Con" means constitutive, and "Ind" means induced extracts. Significant reduction ($p<0.02$) of control was signed by **.

Table 6. Zearalenone concentration of proteinase K + SDS treated and non-treated intracellular extracts of *Rhodococcus pyridinivorans* K404 measured by HPLC-MS/MS

	ZEA concentration [μg/mL]	**Degradation potential** [%]
ZEA control	2.071 ± 0.786	-
Constitutive intracellular extract of *R. pyridinivorans* K404	2.759 ± 2.148	0 %
Induced intracellular extract of *R. pyridinivorans* K404	0.025 ± 0.011	98 %
ZEA control + proteinase K + SDS	1.677 ± 0.088	-
Induced intracellular extract of *R. pyridinivorans* K404 + proteinase K + SDS	2.079 ± 0.381	0 %

3.2.4 New Scientific results

Thesis IV. (Based on the results of chapter 5.2.3): According to the results of zearalenone degradation experiment, induced intracellular enzymes of *Rhodococcus pyridinivorans* K404 play role in zearalenone detoxification. These enzymes are able to degrade more than 90% of the toxin and reduce the estrogenicity of zearalenone and its metabolites with more than 60% within six hours.

4 Conclusions and suggestions

AFB1-degrading ability of 42 type strains of the genus *Rhodococcus* varied greatly. According to the results derived from analytical measurement, some strains were not able to degrade AFB1 or the degradation rate was moderate. On the contrary, excellent degradation efficiency (>90%) was detected in case of 18 strains, among them 15 strains could cease the genotoxicity respectively. These results highlighted, that considering biodegradation rate is not enough for evaluating the efficiency of strains, because the genotoxicity did not cease in some cases of the well-degraders after the biotransformation processes according to SOS Chromotest. On the phylogenetic tree, based on the 16S rRNA sequences, strains having biodetoxification ability can be found far from each other, thus their toxin degrading ability is independent from their relationship.

Comparing the **AFB1 degradation and detoxification ability of type strains** to other rhodococci, similarities and also differences can be discovered. AFB1 degrading ability of *Rhodococcus* strains can be found at the Department of Environmental Safety and Ecotoxicology at SZIU was reported by KRIFATON ET AL. (2011) and CSERHÁTI ET AL. (2013). The detoxification potential of the analyzed isolates and type strains confirmed that *R. erythropolis* and *R. pyridinivorans* species are able to detoxifiy AFB1. In addition to this observation, *R. erythropolis* DSM 14303 strain isolated from poliaromatic hydrocarbon-contaminated soils had remarkable AFB1 degrading efficiency (90%) (TENIOLA ET AL., 2005). On the contrary, strains belonging to *R. ruber, R. coprophilus* and *R. aetherivorans* species could not cease the genotoxicity of the toxin, moreover these strains had poor degradation ability. AFB1 degradation and detoxification potential of strains from *R. rhodochrous* és *R. globerulus* species varied greatly and there was no similarity between strains belonging to the same species (KRIFATON ET AL., 2011; CSERHÁTI ET AL., 2013). Summarizing this phenomenon, mycotoxin degradation and detoxification ability differs intra- and inter-species. These differences may be caused by variable environmental conditions and mobile

genetic elements, and strains gain different catabolic abilities according to these factors.

Examining **ZEA degradation and detoxification efficiency of *Rhodococcus* type strains**, only *R. percolatus* JCM 10087^T strain could degrade the toxin effectively. The strain was isolated from triclorophenol contaminated sludge (BIRGLIA ET AL., 1996). Presented results proved that this strain is able to degrade ZEA and reduces the estrogenicity of the toxin and its metabolites with 70%. According to the literature, among the analyzed *Rhodococcus* strains, *R. pyridinivorans* K402, K404 and K408 strains were confirmed as ZEA-degraders which can cease the estrogenicity, additionally (CSERHÁTI ET AL., 2013; KRIFATON ET AL., 2013). On the contrary, *R. pyridinivorans* (JCM 10940^T) is not able to decrease the estrogenic effect. Strains K402, K404 and K408 were isolated from hydrocarbon-contaminated sites and the type strains were isolated from wastewater. All of them might contacted with aromatic compounds, but it seems, that ZEA degradation ability is independent from the environmental conditions, in spite of the aromatic structure of ZEA.

During the **AFB1 biodegradation with cell-free extracts** of bacterial strains, the identification of enzyme has not been carried out, but detoxification occurred by intracellular extracts of *Rhodococcus erythropolis* NI1 and *R. rhodochrous* NI2 was uniquely fast. In some set-up in the present work, the cessation of genotoxicity occurred immediately when the toxin was added to the extracts, but six hours were enough for detoxification in cases of all extracts. My results have great potential, because similar toxin concentration was degraded slower by cell-free extracts of bacterial strains from different genera reported by other authors in the literature. In some study the biological effects of the toxin and metabolites were not measured, thus biodetoxification has not been proven. The cessation of hazardous effect and the rapidity of the process are very important from a practical point of view. An enzyme-based feed additive enables the mycotoxin detoxification in the animal gastrointestinal tract. If this process occurs quickly, the toxin and its metabolites will not be absorbed. According to the

literature, AFB1 degrading enzymes from fungi (*Armillariella tabescens, Trametes verscolor, Pheniophora sp., Pleurotus ostreatus, P. pulmonarius, Phanerochaete sordida*) and only one bacterial species (*Myxococcus fulvus*) have been identified so far (LIU ET AL., 2001; ZHAO ET AL., 2010). Although AFB1 degrading enzymes from *Rhodococcus* strains has not been identified, but FDR-A and FDR-B reductases may be involved in AFB1 degradation in case of *Actinomycetes* (TAYLOR ET AL., 2010; LAPALIKAR ET AL., 2012).

During **ZEA degradation with cell-free extracts**, significant reduction of estrogenicity was detected only in induced intracellular extracts of *R. pyridinivorans* K404. As there was no decrease of estrogenic effect in constitutive intracellular extract of the same strain, the hazardous effect was reduced by enzymes after gene expression induced by the toxin. The same amount of protein concentration in constitutive and induced extracts shows qualitative differences. Analytical measurement confirmed 95% degradation of ZEA and more than 60% decrease of estrogenicity was observed in BLYES test. This observation emphasizes the necessity of biological tests, because hazardous breakdown products can be produced during biodegradation. Enzymatic degradation of ZEA by cell-free extracts of strains from different genera was reported in the literature, but degradation and detoxification occurred slower compared to my results.

Identification of enzymatic basis of biodegradation process makes possible the application of the effective and safe bacterial strains in the future. Food and feed additive microorganisms and their enzymes are qualified by EFSA, and the safe organisms get QPS-status (Qualified Presumption of Safety) and are listed on the QPS-list. Among the analyzed genera, *Rhodococcus* and *Gordonia* are not, only some species from *Pseudomonas* and *Streptomyces* genera listed on the newest QPS-list (EFSA, 2017). Only enzymes of those species, which are not placed on the list, can be used only in feed industry. A few biological feed additives are available commercially, among them only FUMzyme® produced by Biomin Holding GmBH (Austria) contains purified enzyme, which can degrade fumonizin

mycotoxins. Moreover, Merck KGaA (Germany) distributes AFB1 degrading purified laccase isolated from *Trametes versicolor*.

For the development of enzyme based feed additives, the identification of enzymes and the encoding gene or genes are necessary, but the identification of proteins and their structure is a big challenge. Low protein concentration in samples, susceptibility of enzymes, expensive analytical methods gaining qualification and time are among the several limiting factors. Two new-generation analytical methods can solve the problem of enzyme-identification, MALDI-TOF (Matrix-Assisted Laser Desorption Ionization – Time of Flight) and ESI-TOF (Electrospray Ionization – Time of Flight). MALDI is a mass-spectrometer with photoionization, which can identify proteins according to their "peptid-mass fingerprint". This method is very effective, but its disadvantage is that the mass of unknown protein should be found in database (DEUTZMANN, 2004). YU ET AL. (2011) used this measurement for identify ZEA-degrading enzymes in extracellular extracts of *Acinetobacter sp*. SM04. ESI was applied by CAO ET AL. (2011) who discovered AFB1 degrading ADTZ enzymes of *Armillariella tabescens*. During ESI-MS/MS measurement, they revealed that bifuran ring in AFB1 structure, which is involved in epoxidation and causes genotoxic effect, is cleaved by an oxidase. The identification of enzyme and toxin-induced genes can be carried out by transcriptome analysis additionally, which examines the expressed RNA. According to the transcriptomes, the original and induced genetic changes in strains can be revealed, and differences may show genes playing role in the degradation. KOSAWANG ET AL. (2014) carried out transcriptome analysis of *Clonostachys rosea* IK726 resulting in the identification of ZEA degrading ZHD101 and cytochrome enzymes.

Using one of the mentioned methods for the identification of AFB1 and ZEA detoxifying enzymes of *Rhodococcus* strains, an effective enzyme based feed additives can be developed which is able to detoxify mycotoxins in feed in a safe and quickly way.

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Total impact factor (IF): 9.863