SZENT ISTVÁN UNIVERSITY

Ph.D. THESIS

SELECTION AND APPLICATION OF MICROBES ABLE TO BIODEGRADATE MYCOTOXINS

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1. BACKGROUND, AIMS AND SCOPES

Now days 1 billion people is starving, or suffering in malnutrition on the World (the population reached 7 billion people in 2011) (TIRADO ET AL., 2010).

This situation will get worse in the future according to the forecasts, since the changing climate not just contribute to the decreasing feedstuffs (like drought in Hungary in 2011-2012), but several data prove the connection between epidemics infecting plants and the weather changes that gangers the feed safety, and manly cause problems in the developing countries (PATERSON-LIMA, 2010, 2011). One of the hot spots of this changing is the alteration in the spread of mycotoxins and the frequent contamination of row materials (IPCC, 2007). Average temperature of the Earth is increasing with 4°C in the next 100 years, which contributes to the shift of climate zones, disappearance of seasons, and incalculable rainfall distribution. Due to the increased number of extreme phenomenon in the weather, the agricultural production cannot be planned and vegetation is getting more and more sensitive for infections as a result for the continuous stress that they are grown in. Mycotoxins are extracellular secreted metabolites of mould that contaminate feed and food stuffs. Mycotoxins are produced due to stress factor, which can be the temperature, water, pH, or the appearance of other fungi.

Mycotoxins have adverse effects to other organism. They are cytotoxic, genotoxic, mutagen or endocrine disruptors. Due to the climate change, the extreme weather make unrecoverable so far stable production system, thus row materials produced by the agriculture may be contaminated by mould and by its metabolites, by the mycotoxins. In Hungary the most frequent mycotoxin contaminations were caused by zearalenone, fumonisin, deoxivinalenol, ochratoxin-A and T-2 toxin. However, unusual mycotoxins were also appeared as a result of changing climate (DOBOLYI ET AL., 2011, VARGA ET AL., 2007).

For the elimination of mycotoxins several physical, chemical and biological methods are available. Out of these methods biodegradation came into prominence. During biodegradation, microorganisms or enzymes are used for degradation. However, biodegradation of mycotoxins not in all cases go with the break of the biological effect (biodetoxification). For that reason, regulations for food and feed safety prescribe toxicological tests for evaluating the success of detoxifying agents, especially the creating metabolites. Successful detoxifying agents were developed; moreover feed additive against zearalenone and ochratoxin is available on the market.

Aims of the work

During my research the main goal was to eliminate or reduce mycotoxin contaminations by the use of microbes able to degrade aromatic hydrocarbons. Most of the mycotoxins have aromatic structure, there for the testing of the available aromatic hydrocarbon degrading microbes was obvious.

My research goals were:

1) isolation and identification of mycotoxin degrading microbes
2) verify the mycotoxin detoxification
3) exploration of enzyme/enzyme systems responsible for degradation of mycotoxins
4) practical investigation of the isolated microorganisms

The results showed in my PhD dissertation are the outcome of own laboratory and collaborative research work, and there are data's and measurement results of subcontractor works. All the results were compared by collected and rated scientific literature.

2. MATERIALS AND METHODS

2.1 Microbial strains and culture conditions used in biodegradation tests

Microbes deposited in the strain collection of Agruniver Holding Ltd. and the Department of Environmental Protection and Safety, Szent István University, reference strains deposited in international strain collections, and strains owned by individual persons were involved during the examination. Part of the strains were isolated from oil polluted sites and matrixes (soil, groundwater, wastewater, compost, biofilm).

The mycotoxin 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)

2.2 Analysis of AFB1, ZEA, OTA, T-2, FB1 and DON biodegradation processes

Biodegradation experiments were conducted in Erlenmeyer flasks in 50 ml media in three parallels, the concentration of the mycotoxins were 2 mg/l each, 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 mycotoxin 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 analyzed by the adapted biomonitoring systems for screening the residual geno/cytotoxic or endocrine disrupting effect.

- *SOS*-Chromo test for analyzing genotoxicity
- *Aliivibrio fischeri* test for analyzing cytotoxicity
- BLYES/BLYR test system for analyzing endocrine disrupting and cytotoxic effect

2.3 Animal feeding and animal treatment experiment

2.3.1 Broiler chicken feeding experiment

120 Cobb-500 cock chicken were used in the feeding experiment. The feeding groups were repeated twice (in latin square technique) 15-15 chicken each.

- ***Control without microbe*** (n=2x15): fodder without any AFB1
- ***Control + AK37*** (n=2x15): mycotoxin free fodder, treated by the microbe

- ***AFB1*** (n=2x15): AFB1 mycotoxin containing fodder, calculated AFB1 mycotoxin concentration 1mg/kg.
- ***AFB1 + AK37*** (n=2x15): AFB1 mycotoxin containing (calculated AFB1 mycotoxin concentration 1mg/kg) biodetoxificated fodder

The experiment was 42 day long. The bodyweight, feeding intake, mortality was measured during the experiment.

2.3.2 Fish feeding experiment

The fish model experiment were carried out at the Szabolcsi Halászati Ltd., from one year old carp of the company. The fish were feeded by four different corn based fodder in the 3% of the bodyweight, four times a day, using automatic feeder. The following groups were made during the experiment:

- ***Control without microbe*** fodder without any AFB1
- ***Control + AK37*** mycotoxin free fodder, treated by the microbe
- ***AFB1*** AFB1 mycotoxin containing fodder, calculated AFB1 mycotoxin concentration 2mg/kg
- ***AFB1 + AK37*** AFB1 mycotoxin containing (calculated AFB1 mycotoxin concentration 2mg/kg) biodetoxificated fodder

The fish were kept in 2 m^3 recirculation ponds. The experiments were repeated three times by 25-25 fish. The feeding was prepared by a 5 day long acclimatization, than it continued for 14 days. During the experiment the water quality, the mortality, and the feed intake was daily observed. In the beginning the bodyweight and bodylength was recorded. In the end of the experiment histology study was made from the livers of the different treatment groups.

2.3.3 Rat treatment experiment (Uterotrofic bioassay)

In the rat treatment experience the effectiveness of the biodegradation of the ZEA, and the physiological effects was measured. During the treatments the matrix was introduced trough gavage into the animals.

For the experiment a medium with 500 µg/ml ZEA concentration was inoculated by the mycotoxin degrading microbe incubated at 170 rpm at 28°C for 5 days. At the 5th day of the experiment the entire medium was centrifugated (1500 rpm, for 20 min, at 4°C) and the supernatant was Edwards Micromodulyo lyophilisator and was analyzed in animal experiments. The ZEA concentration was measured by HPLC. The dry-frozen supernatant was diluted in olive oil, this matrix was the base of fodder used in the treatment.

The following groups were made during the experiment:

- ***Control***: LB without ZEA
- ***ZEA***: 500 µg/ml ZEA-t containing LB
- ***ZEA + K408:*** ZEA containing (500 µg/ml) biodetoxificated medium

The experiment were based on the uterotrofic bioassay carried out by Heneweer and co-workers (2007). The animals were prepubertal female Wistar rats, from the KOKI. The rats were kept in threes on 21±1°C, in 65% moisture content, 12 hour long light period (07:00–19:00). The animals were treated *ad libitum* by phytoestrogen-free fodder and tapwater during 3 days. The treatment dose were calculated daily and individually, 5 mg/kg ZEA intake per bodyweight.

After the experiment the weight of uterus and the expression of marker gene's from uterus were measured, compared to the control: apelin (APLN), aquaporin 5 (AQP5), complement component 2 (C2), calbindin-3 (CALB3). The results were calculated in relative genexpression (RQ – relative quantity), which shows how many times is the expression of a gene in a sample.

2.3.4 Mice treatment experiment (Nephrotoxic bioassay)

In the performed experiments the physiological effects of the metabolites produced during OTA biodegradation by the microbe was investigated. During the treatments the matrix was introduced trough gavage into the animals.

During the experiment LB medium containing 10% of the recipe and 20 mg/l OTA was inoculated by the mycotoxin biodegrading microbe incubated at 170 rpm at 28°C for 5 days. At the 5th day of the experiment the entire medium was centrifugated (1500 rpm, for 20 min, at 4°C) and the supernatant was Edwards Micromodulyo lyophilisator and was analyzed in animal experiments.

The following groups were made during the experiment:

- ***Control***: LB without OTA
- ***OTA:*** 20 µg/ml OTA containing LB
- ***OTA + Őr16:*** OTA containing (20 µg/ml) biodetoxificated medium

The animals were mature 7-9 week old mice (CD1). The mice were kept on 21±1°C, in 65% moisture content, 12 hour long light period (07:00–19:00). The animals were treated *ad libitum*.

The acute treatment were 72 hours long (n=7-10) (LUHE ET AL., 2003), the chronicle treatment were 21 day long (n=7-10) (ZELJEZIC ET AL., 2006). In the acute treatment the OTA doses were 1 mg/bodyweight and 10 mg/ bodyweight kg once a day. In the chronicle toxicity experiment the dose were 0,5 mg/ bodyweight kg during 21 day, treated once a day (n=7-10). Metil-metanesulfonate (MMS) was the positive control, diluted by sterile tapwater. Liofilizated medium containing the OTA biodegraded by the microbe was diluted in 10 mM Tris (pH 8) together with sterile tapwater. Control was the same phenofase liofilizated microbe culture medium, without OTA.

Treatment groups during the **72 hour long acute toxicity experiment**:

- Control (DMSO adequate for the big dose intact OTA dilution)
- MMS (positive control, 100 mg/kg)
- OTA (1 mg/bodyweight kg)
- OTA (10 mg/ bodyweight kg)
- Degraded OTA (1 mg/ bodyweight kg)
- Degraded OTA (10 mg/ bodyweight kg)
- LB-Bacterium (DMSO adequate for the big dose intact OTA dilution)

Treatment groups during the **21 day long chronic toxicity experiment:**

- Control (DMSO adequate for the intact OTA dilution)
- MMS (positive control, 40 mg/ bodyweight kg)
- OTA (0,5 mg/ bodyweight kg)
- OTA degraded (0,5 mg/ bodyweight kg)
- Medium (LB)- bacterium (DMSO adequate for the intact OTA dilution)

During the experiment our goal was to measure the toxicity of OTA in the kidney. The effect of the metabolites were measured by observation of gene expression influenced by OTA exposition in the kidney cortex, based on the literature (LUHE ET AL, 2003) the results were calculated in relative gene expression (RQ).

The measurements were planned for the following citotoxicity marker gene's: alfa Gadd45, Gadd153, annexin 2 (Anxa2), ceruloplazmin (Cp), sulfotransferaze K2 (StK2), clusterin (Clu). The observed marker gene's were collected to measure the demolition effect of the OTA on kidney (DNA demolition, apoptotic, inflammatory).

2.3.5 Genome project

DNA isolation from clean culture

After incubation the *Rhodococcus pyridinivorans* AK37 and *Cupriavidus basilensis* ŐR16 during three days, on 28°C, 170 rpm 3 ml by three times was centrifugated (4000 rpm) and pellet was collected. DNA isolation was made according MoBio UltraClean Microbial DNA Isolation Kit. The purity and concentration of DNA was measured on Implen nanophotometer. Results showed DNA purity of 1,9 (A260/A280) in both microbe, and the concentration was 210 ng/µl in the case of *R. pyridinivorans* AK37, and 350 ng/µl in the case of *C. basilensis* ŐR16. The quality of the DNA samples were measured on gel-electrophoresis (1% agaroze gel, 5 µl sample, 1 hour, 110 v), according to this the genome DNA was complete. 30-30 µg of the genome DNA was sent to sequencing (Baygen Institute, Szeged).

Genome sequencing was performed by combining the cycled ligation sequencing on SOLiD 3Plus System (Life Technologies) with 454 FLX pyrosequencing (Roche). Assembly was performed using the Genomics Workbench 4.8. and the Omixon Gapped SOLiD Alignment 1.3.2 plug-in (1) provided by CLC Bio and Omixon. Automatic annotation of the genome was performed by the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html), which utilizes GeneMark, Glimmer and tRNAscan-SE searches.

3.RESULTS AND DISCUSSION

3.1 Selection of microbes able to biodegrade mycotoxins

3.1.1 *Rhodococcus strains*

The biodegradation ability of 33 strains of eight *Rhodococcus* species was investigated for sic mycotoxins (AFB1, ZEA, T2, OTA, DON, FB1). In the first step the degradation was measured by ELISA, when 50% biodegradation level occurred the result was verified by HPLC. In the case of AFB1 and ZEA the biodegradation potential was measured by biological tests, like SOS-Chromo test, *Alivibrio fischeri* test and BLYES/BLYR test.

3.1.1.1 AFB1 degradation results of *Rhodococcus* strains

Isolates from the R. *erythropolis* species were the biggest group in my experiment, all of them could biodegrade AFB1 content more than 50%. The *R. aetherivorans* and *R. gordoniae* species representer (1-1) showed AFB1 biodegradation potential more than 50%, but less than 95%. From the 33 *Rhodococcus* strain only four strain showed AFB1 degradation potential under 50 %, three of them are *R. ruber* (N361, AK41, 4S-8), one is *R. globerulus* (N58). According to the analytical results the representer of the *R. pyridinivorans* and *R. rhodochrous* species showed outstanding almost 100% biodegradation potential.

The detoxification of the AFB1 was investigated by SOS-Chromo test. From the 33 *Rhodococcus* strains 12 showed genotoxic metabolites, in the case of strains with outstanding biodegradation potential too according the HPLC and ELISA result (*R. erythropolis*: DSM1069, IFO12538, L88 and *R. aetherivorans* AK44). From the 19 *R. erythropolis* strain in seven case genotoxic effect occurred (DSM 743, DSM 1069, IFO 12538, L88, AK40, OM7-2, ZFM 23-1). In the case of *R. aetherivorans* strain beside high degradation potential genotoxic effect was observed. The strains showing genotoxic effect during the SOS-Chromo test were closed from further experiments.

According to the results and the scientific literature AFB1 biodegradation was revealed without any harmful metabolites in three species *R. globerulus* (AK36), *R. pyridinivorans* (K402, K404, K408, AK37) and *R. rhodochrous* (NI2, ATTC 12674), and among the *R. erythropolis* 12 strains (NI1, DSM 4306, NCAIMB 9784, AK 35, AK 42, GD1, GD 2A, GD 2B, BRB 1AB, BRB 1BB, ŐR 9, ŐR 13) were detected.

3.1.1.2 ZEA degradation results of *Rhodococcus* strains

From the investigated 33 *Rhodococcus* species ZEA degradation was observed among only seven strains (*R. erythropolis* NI1; *R. pyridinivorans* AK 37, K402, K404, K408; *R. ruber* N361; *R. globerulus* N58). In the case of *R. erythropolis* from 19 strain only 6 isolates,

in the case of *R. ruber* from 3 only 1, in the case of *R. rhodochrous* from 2 only 1 isolates showed biodegradation.

The biodetoxification was measured by BLYES test in the case of strains with good biodegradation potential. According to the results from the seven strain only 5 (AK37, K402, K404 and K408 *R. pyridinivorans* and NI1 *R. erythropolis)* was able to biodetoxificate ZEA without or just a few estrogen disturbing metabolites.

According the scientific literature the ZEA degradation is a new phenomenon in the case of the *Rhodococcus* genus. During my research ZEA biodetoxification without any estrogen effect metabolites was detected among 5 isolates of two *Rhodococcus* species (*(R. pyridinivorans and R. erythropolis*).

3.1.1.3 T-2 degradation results of *Rhodococcus* strains

The majority (25) of the 33 *Rhodococcus* strains showed T-2 toxin degradation potential over 50 %. Strains belonging to the *R. gordoniae, R. coprophilus, R. rhodochrous, R. globerulus* species showed degradation potential over 90%. All members of the *R. erythropolis* species showed degradation potential over 90%. In the case of *R. ruber* only one strain N361 showed degradation potential of 60%, the two other *R. ruber* strains could not degrade the toxin. The *R. aetherivorans* strain showed degradation potential of 35%.

According the scientific literature the T-2 toxin degradation is a new phenomenon in the case of the *Rhodococcus* genus. Based on my research the following isolates are able to degrade T-2 toxin: *R. erythropolis* (16 isolates), *R. globerulus* (2 isolates), *R. rhodochrous* (2 isolates), *R. coprophilus* (1 isolates), *R. gordoniae* (1 isolates).

3.1.1.4 OTA degradation results of *Rhodococcus* strains

From the thirty-two strains four were able to degrade OTA (*R. erythropolis* GD 2A and BRB 1AB and R. *pyridinivorans* K402 and K408) with low efficiency the other strains proved to be ineffective.

3.1.1.5 DON degradation results of *Rhodococcus* strains

None of the investigated 33 *Rhodococcus* strains degraded DON mycotoxin.

3.1.1.6 FB1 degradation results of *Rhodococcus* strains

None of the investigated 33 *Rhodococcus* strains degraded FB1 mycotoxin.

3.1.1.7 Multimycotoxin degradation ability of *Rhodococcus* strains

In the case of multimycotoxin degradation, strains were collected according to biological effect measurements (SOS-Chromo and BLYES test).

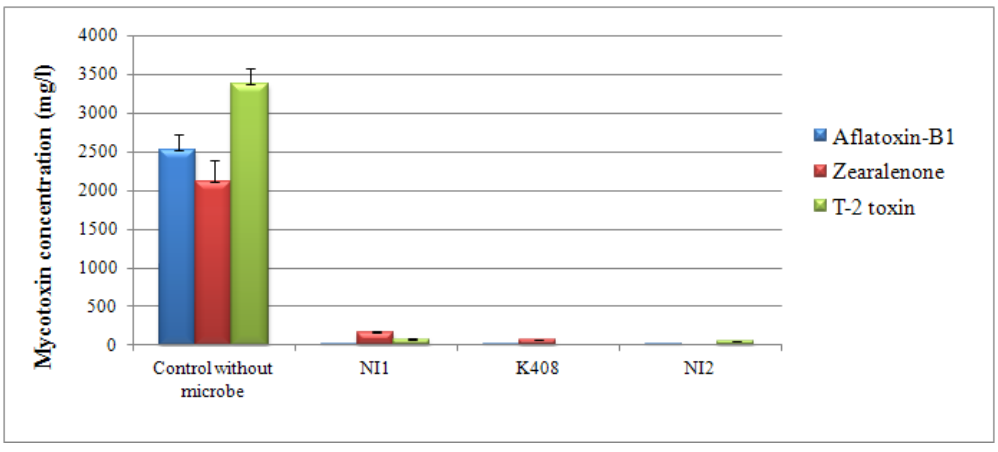
Altogether among 19 strains showed biodetoxification ability for 2 or 3 mycotoxins. From these strains three different species (*R. erythropoils* NI1, *R. pyridinivorans* K408, *R. rhodochrous* NI2) was investigated in further multimycotoxin biodetoxification experiment.

The preparation of the experiment was the same like in previous. Only difference was that the medium contained three different mycotoxins in the same time, each mycotoxin in 2-2 mg/l concentration:

- In the case of *R. erythropoils* NI1, AFB1-ZEA-T-2 mycotoxin mix
- In the case of *R. pyridinivorans* K408, AFB1-ZEA mycotoxin mix
- In the case of *R. rhodochrous* NI2, AFB1-T-2 mycotoxin mix.

The results are in the 3.1 figure. The *R. erythropolis* NI1 had a degradation potential over 90% in case of AFB1, ZEA and T-2 (AFB1: 99,88±0,09%, ZEA: 92,11%±11,25%, T-2: 98,48±13,45%), the *R. pyridinivorans* K408 had a degradation potential over 95% in case of AFB1 and ZEA (AFB1: 99,31±2,24%, ZEA: 96,83±1,90%); and *R. rhodochrous* NI2 showed a degradation potential over 95% in case of AFB1 and T-2 toxin (AFB1: 99,88±0,09%, T-2: 97,94±2,25%).

The strains beside the great multimycotoxin biodegradation potential eliminated the genotoxic and estrogen effect either. Interestingly the ZEA degradation ability showed in the monotoxin system (NI1 60% and K408 77%) increased significantly.



3.1. figure: Toxin concentrations in the supernatant of multimycotoxin bidegradation experiment after 3 days, measured by ELISA

According to scientific literature multimycotoxin biodegradation so far among prokaryote wasn't detected. Indeed, biodegradtion of more mycotoxins (ZEA and OTA) was observed just among eukaryote, the yeast *Trichosporon mycotoxinivorans.*

3.1.2 Mycotoxin degradation results of *non-Rhodococcus* strains

Microbes with mycotoxin degradation ability introduced in this chapter are not belonging to the genus Rhodococcus. All of the microbes are isolated oil polluted sites, or oil polluted composts. The mycotoxin degradation ability of 22 strains of 12 different genus for 6 mycotoxins were investigated. The degradation ability was measured by ELISA, only the outstanding degradation ability results were certified by HPLC, due to the tight correlation between HPLC and ELISA measures.

3.1.2.1 AFB1 degradation results of *non-Rhodococcus* strains

All of the 22 strain showed biodegradation potential below 50% according to the ELISA results. But in the case of the majority the SOS- Chromo test showed genotoxic metabolites. Only *Pseudomonas putida* DN1 strain was able to eliminate genotoxic effect of AFB1. According to the scientific literature the AFB1 degrading ability of the *Pseudomonas* genus is novel scientific results.

3.1.2.2 ZEA degradation results of *non-Rhodococcus* strains

From the 22 investigated strain only 4 strain could degrade ZEA below 50%. The *Pseudomonas pseudoalcaligenes* FEH28, the *Pseudoxanthomonas kalamensis* H4, the *Pseudoxanthomonas suwonensis* NZS6 and *Gordonia paraffinivorans* NZS14 strain. In the case of these strains the results were verified by BLYES test to measure the metabolites with estrogen effects. According to the BLYES test results the FEH28 could degrade ZEA without any harmful metabolite. According to the scientific literature the ZEA degrading ability of the *Pseudomonas* genus is known, but the ability of the species *Pseudomonas* p*seudoalcaligenes* is novel scientific results.

3.1.2.3 T-2 degradation results of *non-Rhodococcus* strains

In the case of T-2 toxin only 3 strains showed below 50% (about 90%) biodegradation potential from 22 strains. The two member of *Microbacterium* genus EL1 and NSZ9 strains, and the member of *Pseudoxanthomonas* genus NZS6 strain. According to the scientific literature the T-2 degrading ability of the *Microbacterium*, *Pseudoxanthomonas* are novel scientific results.

3.1.2.4 OTA degradation results of *non-Rhodococcus* strains

In the case of OTA three strains showed high degradation capability, according to the HPLC and ELISA results. Two strain is member of *Cupriavidus* genus: BRB 6A (*C. basilensis)* and ŐR16 (*C. basilensis*), one strain is *Sphingopyxis chiliensis* Kö10. According

to the scientific literature the OTA degrading ability of the *Cupriavidus* and *Sphingopyxis* genus are novel scientific results.

3.1.2.5 DON degradation results of *non-Rhodococcus* strains

None of the investigated 22 strains degraded DON mycotoxin.

3.1.2.6 FB1 degradation results of *non-Rhodococcus* strains

None of the investigated 22 strains degraded FB1 mycotoxin.

3.1.2.7 Multimycotoxin degradation results of *non-Rhodococcus* strains

In the case of non-*Rhodococcus* strains the multimycotoxin degrading ability occurred 11 times, but according to the biological tests there were not any strains able to biodegrade different mycotoxins parallel and eliminate the harmful biological effects of the metabolites.

NOVEL SCIENTIFIC RESULT: (Thesis 1) Mycotoxin degradation ability of 32 strains of 8 species belonging to the *Rhodococcus* genus is explored. Within the genus in the case of three species (*R. globerulus, R. pyridinivorans, R. rhodochrous)* aflatoxin-B1, in the case of two species (*R. pyridinivorans, R. erythropolis)* zearalenone biodetoxification ability was observed. In the case of 5 species *(R. erythropolis, R. globerulus, R. rhodochrous, R. coprophilus, R. gordoniae)* the T-2 toxin biodegradation ability was observed first.

NOVEL SCIENTIFIC RESULT: (Thesis 2) In the case of *Cupriavidus* and *Sphingopyxis* genus ochratoxin-A, in the case of *Microbacterium and Pseudoxanthomonas* genusT-2 toxin, in the case of *Pseudomonas* genus aflatoxin-B1 biodegradation was observed.

NOVEL SCIENTIFIC RESULT: (Thesis 3) In the case of the prokaryote multimycotoxin biodegradation and biodetoxification was observed. *R. erythropolis* NI1 strain is able to detoxificate aflatoxin-B1 and zearalenone, and degrade T-2 toxin. *R. pyridinivorans* K408 is able to biodetoxificate simultaneously aflatoxin-B1 and zearalenone. *R. rhodochrous* NI2 strain is able to detoxificate aflatoxin-B1 and degrade T-2 toxin simultaneously.

3.2 Animal feeding and animal treatment experiments

In order to verify the biodegradation and biodetoxification potential of the microbes, approved by molecular biotests are effective in higher organisms, animalfeeding and treatment experiences were carried out.

For the survey of the effectiveness of AFB1 detoxification fish and broiler chicken feeding experiments were chosen. The fodder was prepared by the department, for the detoxification *R. pyridinivorans* AK 37 bacterium strain was used, cause the already known biodetoxification potential and the on going *de novo* genome project.

To evaluate the ZEA biodetoxification effectiveness, a rat model experiment was chosen. For the experiment the fodder (liofilizated medium) was prepared by the department, using the *R. pyridinivorans* K 408 bacterium strain, which showed the highest biodegradation potential in the case of ZEA without any harmful metabolites (EDC).

For the examination of OTA biodetoxification effectiveness a mice model experiment was chosen. For the experiment the fodder (liofilizated medium) was prepared by the department, using the *Cupriavidus basilensis* ŐR16 bacterium strain, which degraded OTA by 98% efficiency.

The animal feeding and treatment experiments separated into three branch:

1. Aflatoxin-B1 contaminated corn feeding experiment in broiler chicken and fish system.
2. Zearalenone biodegradation residuals containing liofilizated matrix used in rat treatment experiment.
3. Ochratoxin-A biodegradation residuals containing liofilizated matrix used in mice treatment experiment.

Producing AFB1 containing fodder

For the animal feeding experiments, an AFB1 elimination/decontamination method was developed using microbes according on the results of my research.

We produced AFB1 containing corn, infected by the Zt80 *Aspergillus* strain isolated by the department, and this corn was detoxificated by *Rhodococcus pyridinivorans* AK37 strain during a dry-fermentation process. The generated fodder (corn) was used in fish- and chicken feeding experiments.

During the experiment the AFB1 content of the fodder was measured by ELISA and HPLC method. The control corn mycotoxin content was under detection limit. The planned AFB1 content was 1 mg/kg in the AFB1 fodder, but the measurements showed 860 µg/kg

AFB1. After the bacterial detoxification AFB1 content were only 105 µg/kg, which was higher than the value limit (50 µg/kg maximum, 2003/100/EC). This means that the bacteria decreased by 87% the AFB1 concentration in the developed method. The biological effect of the detoxificated fodder was investigated on fish and broiler chicken.

3.2.1 Fish feeding experiment, examination of aflatoxin-B1 decontamination

3.2.1.1 Processing parameters (mortality, feed intake)

Changes occurred during the experiment in bodyweight and bodylength of the fishes are shown in 3.1 table. There were not any statistically significant changes between the simultaneous and different treated groups among the measured parameters (P<0,05).

3.1. table: The bodylength and bodyweight of the treated fish in the start and the end

Feeding groups	Bodylength (cm)		Bodyweight (kg)	
	Start	End	Start	End
AFB1	12,2±6	12,2± 4	0,71 ± 0,08	0,69 ± 0,09
AFB1+ AK37	12,1±4	12,5± 5	0,74± 0,06	0,74 ± 0,08
Control + AK37	12,3±5	12,4± 4	0,72 ± 0,06	0,74 ± 0,06
Control without microbe	12,2±4	12,4± 6	0,71 ± 0,06	0,73 ± 0,08

The mortality was statistically significant between the different groups (3.2 table) (P<0,05). Large mortality could be observed from the 10 th day in the case of *AFB1* containing corn feeded group. The histology study proved, that the cause of the mortality was the mycotoxin content of the fodder.

3.2. table: Rate of the mortality by the different groups during the experiment

Feeding groups	Mortality (%)
AFB1	20 ± 8*
AFB1 + AK37	2 ± 2
Control + AK37	0
Control without microbe	0

*P<0,05 = compared to AFB1+AK37 group

The daily feed intake was observed during the experiment (3.3. table). According to the results it can be seen that AFB1 group take less feed day after day progressing the experiment, due to AFB1 toxicosys. Similar decrease could not be noticed among the other groups, every animal feed to the appropriate to their age.

3.3. table: Average fodder intake by the different groups during the fish experiment

Feeding groups	Average feed intake g/fish/14 day
AFB1	18,0 ± 3, 9*
AFB1 + AK37	25,0 ± 7,1
Control +AK37	29,7 ± 5,6
Control without microbe	31,2 ± 4,4

*P<0,05 = compared to AFB1+AK37 group

3.2.1.2 Histology studies

In the end of the experiment liver samples were taken from 10-10 fishes for histology study.

Control without microbe group the liver tissue was intact, the cell lines were clearly visible. The number of hepatocitas were appropriate, the nuclei were centrally located.

Control +AK37 group the liver tissue was intact, the cell lines were clearly visible. The number of hepatocitas were appropriate, the nuclei were centrally located.

AFB1 + AK37 group showed slight diffuse pathological fat infiltration (unhealthy infiltration, material accumulation in the cell and tissues), but the nuclei were centrally located.

AFB1 group showed altered nuclei due to fat infiltration, the lipid content of the cell increased, the structure of the liver tissue locally disrupted, disorganized, the structure got porous. Beside the lipid infiltration, inflammatory cells, lymphocytes and granulocytes could be seen. Around the nucleus and between the cells reddish discoloration could be observed.

3.2.2 Broiler chicken feeding experiment, examination of aflatoxin-B1 decontamination

The goal of the experiment was to investigate the effect on the broiler chicken processing parameters of the killed and annealed AFB1 detoxificating microbe culture.

3.2.2.1 Processing parameters (bodyweight, weight gain, feed intake, feed conversion)

Analysing the **bodyweight** data's there weren't any significant differences, therefore the different treatments were analysed together. Bodyweight data's are shown in 3.4. table.

3.4. table: Development of livingweight gain (g) during the broiler chicken experiment

		1. week	2. week	3. week	4. week	5. week	6. week	7. week
Control without microbe	average	46,4	132,7 [a]	303,7[a]	597,1 [ac]	1011,4 [ab]	1547,1 [ab]	1982,9 [a]
	dispersion	1,4	16,5	44,7	93,7	157,0	253,8	241,6
Control + AK37	average	47,0	142,5 [b]	336,1 [b]	647,9 [a]	1050,0 [a]	1583,2 [a]	1864,4 [ac]
	dispersion	0,0	20,7	50,0	101,4	92,0	129,3	196,8
AFB1	average	47,4	132,0 [a]	268,8[d]	454,4 [d]	766,7 [d]	1154,4 [d]	1400,6 [d]
	dispersion	1,4	16,0	38,3	79,3	126,6	200,5	230,4
AFB1 + AK37	average	47,2	136,4 [ab]	312,8 [a]	582,9 [c]	979,4 [b]	1504,1 [b]	1793,8 [c]
	dispersion	0,2	14,5	34,1	62,1	110,7	158,0	224,9

The bodyweight was significantly lower in the case of aflatoxin treatment compared to the three other treatments, after the second week. AFB1 + AK37 group (which contains the annealed detoxificating microbe culture) had the same development in average bodyweight as the control groups.

The average weekly **weight gain** data's are shown in 3.5 table. According to the data's, the member of the AFB1 group had significantly lower daily gain compared to the other treatments. At the same time the member of group AFB1 + AK37 slightly differs from the control group, but it is not significant.

3.5. Table: Average weight gain (g) during the broiler chicken experiment

Time	Week	1. week	2. week	3. week	4. week	5. week	6. week
Control without microbe	average	86,3^{ab}	171,0^{a}	301,7^{a}	414,3^{a}	566,7^{a}	380,4^{a}
	dispersion	16,8	47,6	34,4	79,9	73,7	78,3
Control + AK37	average	95,5^{b}	193,8^{a}	311,8^{a}	421,7^{a}	533,2^{ab}	318,7^{ad}
	dispersion	20,7	55,3	58,9	54,5	81,8	60,6
AFB1	average	84,7^{a}	136,9^{c}	182,0^{c}	312,3^{c}	387,7^{c}	247,5^{c}
	dispersion	15,4	38,5	35,3	54,2	99,6	116,6
AFB1 + AK37	average	89,2 ^{ab}	176,4^{a}	270,2^{b}	398,0^{a}	525,2^{b}	309,4^{d}
	dispersion	14,5	34,8	35,3	62,6	69,9	97,6

In the case of **feed intake**, due to the effect of AFB1 the appetite of the chicken decreased. At the same time this phenomenon could not been observed by the detoxificated AFB1+AK37 group.

In the case of **feed conversion** (used fodder for 1 kg of weight gain) could not evaluated statistically. The feed intake slightly decreased due to the effect of AFB1, but the ratio is not significant. Interestingly this phenomenon was observed in the case of the control +AK37 group.

3.2.3 Uterotrofic bioassay on rat

3.2.3.1 Investigation of uterus weight

During the ZEA biodegradation experiment, the uterus weight was significantly increased by 2,6 times in the case of the ZEA group compared to the control.

Between the control group and the ZEA+K408 group there weren't any significant difference, the biological effect of the ZEA containing medium incubated with the microbe was 2,5 times smaller than the ZEA containing medium without microbe.

According to the results the *Rhodococcus pyridinivorans* K408 strain eliminated the uterotrofic effect of ZEA. The microbe biodegradated the mycotoxin, without any uterotrofic metabolites.

3.2.3.2 Gene expression analyses by Real-time PCR

During the rat model experiment estrogen dependent gene's were chosen for investigation by Real-time PCR. For the effect of ZEA the complement-componens-2 (C2)

and calbindin-3 (CALB3) level increase, while the mRNA level decrease was observed by the apelin (APLN) and aquaporin-5 (AQP5). The expression of the above mentioned gene's were measured in ZEA and *Rhodococcus pyridinivorans* K408 strain treated groups (3.6 table).

3.6. table: Gene expression during the rat treatments

Experiment groups, investigated gene's	Control	ZEA	ZEA + K408
apelin (APLN)	1,0	0,28	0,90
aquaporin 5 (AQP5)	1,0	0,40	0,85
complement component 2 (C2)	1,0	1,85	0,95
calbindin-3 (CALB3)	1,0	4,10	0,80

The APLN and AQP5 expression decreased, the C2 and CALB3 expression increased-cause the ZEA containing medium – compared to the control animals.

If the ZEA containing medium was inoculated by the *Rhodococcus pyridinivorans* K408 strain before the in vivo treatment, the effects disappeared, the expression of the gene's were similar like the control. The data's showed similarity to the results of Heneweer and co-workers (2007) ZEA dose effects.

According to the results the effect of the ZEA on the expression of the four genes disappeared cause the inoculation of the bacteria. From this we can conclude that the microbe successfully biodegraded the diluted mycotoxin, without active-harmful metabolites.

During the uterus investigation no significant difference was detected by the expression of the marker gene's, compared to control or ZEA+ *R. pyridinivorans* (K408) group. According to the rat model test, also the BLYES estrogen test showed that the endocrine disturbing effect in the ZEA containing medium treated by the K408 strain was eliminated.

3.2.4 Nephrotoxic bioassay on mice

3.2.4.1 Effect of acute 72 hours OTA treatment on the marker gene's expression

During the acute (72 hours) toxicity investigations high dose OTA and the MMS treatment significantly increased the *gadd 45* and *gadd 153* mRNA levels. In case of *gadd 153* the lower OTA dose significantly increased their expression level in the renal cortex samples. The metabolized OTA residuals by *Cupriavidus basilensis* Őr16 not influenced the mRNA levels of marker genes. LB with the bacterial strain alone not changed the genotoxic marker gene's expressions. Elevated expression level of *clusterin* observed in the high dose OTA treated group. The metabolites of the OTA bacterial cleavage not influenced the *clusterin* mRNA levels. Ours result are similar to Lühe and co-workers (2003), where they experienced increased level of Gadd45 and Annexin2 expression(2 times), the Gadd153 and

clusterin expression (1,5 times) and ceruloplazmin expression (2,5 times) (3.7. table), treatment was 10 mg/bodyweight/kg OTA dose during 72 hours.

3.7. table: Gene expression during the 72 hours of mice treatments

Experiment groups, investigated gene's	Control	MMS	OTA 1	OTA 10	OTA 1 degr.	OTA 10 degr.	LB-bacterium
Gadd 45,	1	1,50	0,80	4,35	1,2	1,0	0,80
Gadd 153	1	1,40	1,30	1,75	0,90	1,05	0,90
Clusterin	1	0,80	1,00	3,0	1,20	1,40	0,90

3.2.4.2 Effect of 21 days chronic OTA treatment on the marker gene's expression

The 21 day long chronicle OTA exposure significantly induced the *gadd 45* and *gadd 153* mRNA levels in the kidney. The Gadd 153 and Gadd 45 mRNS expression in the kidney did not changed due OTA metabolites produced during biodegradation. The only LB and supernatant containing medium did not activate the two marker sin the kidney cortex.

The OTA treatment increased significantly the ceruloplazmin expression in the kidney. The **ceruloplazmin** mRNA expression did not changed in the kidney due OTA metabolites produced during biodegradation. In the case of the marker gene's the degradation was in the same time biodetoxification, while it had extinguished the proinflammatory and oxidative stress causing effect. The LB and supernatant containing medium did not activate the **ceruloplazmin** in the kidney cortex The OTA treatment decreased significantly the **sulfotransferaze** expression in the kidney, while the MMS treatment did not influenced the expressions of the marker gene's. The mRNA expression of the **sulfotransferaze** did not change due OTA metabolites produced during biodegradation. The OTA treatment increased significantly the **annexin 2** expression in the kidney, while the MMS treatment did not influenced the expressions of the marker gene's. In the case of the marker gene's the degradation was in the same time biodetoxification, while it had extinguished the carcinogenic effect during the treatment. The only LB and supernatant containing medium did not show any effect on the level of anxa2 mRNA in the kidney cortex. activate the two marker sin the kidney cortex (3.8. table).

3.8. table: Gene expression during the 21 day of mice treatments

Experiment groups, investigated gene's	Control	MMS	OTA	OTA degr.	LB-bacterium
Gadd 45,	1	0,95	2,30	0,85	0,90
Gadd 153	1	0,80	1,60	1,05	1,20
Ceruloplazmin	1	0,50	3,20	0,80	0,90
Sulfotransferaze	1	0,95	0,60	1,20	0,90
Annexin 2	1	0,90	2,35	0,90	1,00

NOVEL SCIENTIFIC RESULT: (Thesis 4) The efficiency of the applied *Rhodococcus pyridinivorans* AK37 for biodetoxificating aflatoxin-B1 was proved by in vitro laboratory experiments, beside by carp and broiler chicken feeding experiment. The *Rhodococcus pyridinivorans* AK37 successfully extinguished the harmful biological effect of the aflatoxin-B1 contaminated fodder, and the biodetoxificated fodder did not induce toxicosys in the animals.

NOVEL SCIENTIFIC RESULT: (Thesis 5) The efficiency of the applied *Rhodococcus pyridinivorans* K408 for biodetoxificating zearalenone was proved by *in vitro* laboratory experiments, beside by rat treatment experiment. The *Rhodococcus pyridinivorans* K408 successfully extinguished the harmful biological effect of the zearalenone contaminated fodder, and the biodetoxificated fodder did not induce toxicosys in the animals.

NOVEL SCIENTIFIC RESULT: (Thesis 6) The efficiency of the applied *Cupriavidus basilensis* ŐR16 for biodetoxificating ochratoxin-A was proved by *in vitro* laboratory experiments, beside by mice treatment experiment. The *Cupriavidus basilensis* ŐR16 successfully extinguished the harmful biological effect of the ochratoxin-A contaminated fodder, and the biodetoxificated fodder did not induce toxicosys in the animals.

3.3 Results of the genome projects

According to the mycotoxin biodegrading ability and other capability of the AK37 *Rhodococcus pyridinivorans (AFB1 and ZEA biodegradation)*, and ŐR16 *Cupriavidus basilensis* (OTA biodegradation) were chosen for de novo genome sequencing.

3.3.1 The genome of *Rhodococcus pyridinivorans* AK37 strain

The uncompleted draft genome of *R. pyridinivorans* strain AK37 consists of 5,244,611 bp, with a GC content of 67.8%. There are 4,822 putative coding sequences, 52 tRNAs, and 3 rRNA loci. The size of the genome is small according to the existing genome project between the *Rhodococcus* genus, the *R. jostii* (RHA1) strain has 9,7 Mb, the *R. opacus* (B4) strain has 8,8 Mb. The result of the sequencing showed 117 piece of “Metabolism of aromatic compounds element”. The genome sequence revealed that strain AK37 encodes at least six different pathways for monocyclic aromatic hydrocarbon degradation, including catechol 1,2- and 2,3-dioxygenases, protocatechuate 3,4-dioxygenase, benzoate 1,2-dioxygenase, homogentisate, 1,2-dioxygenase, ortho-halobenzoate, 1,2-dioxygenase.

Presents of the ring cleavage gene accounts for the biodegradation ability of mycotoxins with aromatic structure. The examined isolates of *Rhodococcus* genus have effective AFB1 degradation ability. Key enzymes of alkane and biphenyl degradation were also identified. Metabolism and transformation of steroid compounds can also be predicted based on the presence of 3-ketosteroid-9α-hydroxylase (ksh) gene. This reveals for the degradation ability of estrogen disturbing compounds like ZEA. During the genome project 67 piece of "Virulence factor" were identified, which are under investigation.

3.3.2 The genome of *Cupriavidus basilensis* ŐR 16 strain

The genome of strain OR16 consists of 8,546,215 bp, with a GC content of 41.2% and 7,534 putative coding sequences. The sizes of the genome is the largest in the genus 5,54 Mbp (according to the Genbank database). The presents of plasmids are certain, because the presents of peptides responsible for the replication and maintenance.

334 piece of "Metabolism of aromatic compounds element" were found including catechol and protocatechuate *ortho* ring cleavage pathways, a catechol *meta* ring cleavage pathway, gentisate and homogentisate pathways, a hydroxyquinol pathway, a hydroquinone pathway, and a benzoyl-coenzyme A pathway. Moreover, based on the presence of a 3-ketosteroid-9α-hydroxylase gene degradation ability of steroid-like compounds is also predictable. During the genome project of *C. basilensis* strain 206 piece of "Virulence factor" were identified, which are under investigation.

NOVEL SCIENTIFIC RESULT: (Thesis 7) *De novo* genome project of the aflatoxin-B1 and zearalenone biodetoxificater *Rhodococcus pyridinivorans* AK37 microbe is completed.

NOVEL SCIENTIFIC RESULT: (Thesis 8) *De novo* genome project of the ochratoxin-A biodetoxificater *Cupriavidus basilensis* ŐR16 microbe is completed.

4. CONCLUSIONS AND SUGGESTIONS

The major goal of my research was to isolate and select effective mycotoxin degrading microbes that could be applicable for practical purposes.

Microbes applied in my experiments were grown in liquid medium (LB), together with 6 economically important mycotoxins (AFB1, ZEA, OTA, T-2, DON, FB1). In the first step three hundred microbes were screened for their mycotoxin degrading potential by the application of ELISA and HPLC methods. During my research mycotoxin degrading potential of fifty-four strains belong to twenty-one species in thirteen genus was proved. From the fifty-four strains the best degraders for AFB1, ZEA, OTA and T-2 were selected, which strains for the most part were the members of the *Rhodococcus* genus.

Altogether thirty-two strains could degrade two or more mycotoxins (multi-mycotoxin degradation). Out of these multi-mycotoxin degraders the *Rhodococcus erythropolis* N11 strain that is applicable against three mycotoxins (AFB1, ZEA and T2 AFB1, ZEA and T-2) is unique among so far published mycotoxin degraders.

Strains with outstanding degradation ability were investigated for biodetoxification in the case of AFB1 and ZEA by pro- and eukaryote test organisms. On the base of results, forty-five strains were collected, which can degrade mycotoxins without genotoxic or endocrine disrupting effect.

For developing the genetic base of mycotoxin biodegradation, *de novo* genome project of AFB1-ZEA and OTA degrader strains (*Rhodococcus pyridinivorans* and *Cupriavidus basilensis)* were accomplished. These results contribute to the identification of the metabolic pathways and enzyme systems playing role in the mycotoxin degradation.

For the biodetoxification of AFB1 contaminant corn, a dry-fermentation method was successfully developed using AK37 *Rhodococcus pyridinivorans* strain. The corn was infected by the Zt80 *Aspergillus flavus* strain that was isolated and proved for high AFB1 production by our Department. Hereby, feed was prepared for animal tests to verify the effectiveness and usefulness of the selected mycotoxin degraders. This strategy for producing AFB1 contaminated row material support the construction and implementation of further animal feeding experiments.

The practical utility of mycotoxin (AFB1, ZEA and OTA) biodetoxification was demonstrated in animal tests by applying fish, bird and mammal models, in the case of degrader microbes.

According to the result of the fish feeding experiment, 1 µg/ml AFB1 contamination resulted significant mortality and liver degeneration; while in groups with animals treated by biodetoxificated feed, the mortality were one-tenth and the liver degeneration was moderate.

During the animal feeding experiment applying broiler chickens, the AFB1 contaminated feed produced lack of appetite, decreased daily weight gain, significant decrease of body weight, loss of fitness and health conditions. Biodetoxificated feed did not cause significant difference in the body weight compared to the Control group without mycotoxin, and also the health condition of the chickens was normal. Moreover, at the end of the 42 day long experiment an increase with 0,4 kg bodyweight was detected for the benefit of chickens feed by the detoxificated fodder. On the base of these results *Rhodococcus* strains selected for the biodetofication may have probiotic effect, as it was presumable in other experiments (American Society of Animal Science, 2013, HTTP3). However, further studies are needed to enhance this observation.

During a rodent based *in vivo* toxicological experiment the effectiveness and safety of ZEA degradation by *R. pyrinidivorans* K408 strain was investigated. In this experiment the endocrine disrupting effect of adventitious metabolites of ZEA was studied and confirmed by a complex method (BLYES, ELISA, HPLC).

According to the results the *Rhodococcus pyridinivorans* K408 strain successfully degraded the ZEA in liquid LB medium, without any harmful metabolites; since no changes were observed regarding rats treated by this biodetoxificated and lyophilized medium compared to the control group, which confirms the results of the BLYES test, which also indicated that the *R. pyridinivorans* K408 strain is able to biodetoxificate the ZEA.

A similar experiment was carried out by the *Cupriavidus basilensis* ŐR16 strain. The OTA biodegradation ability of the strain ŐR16 was investigated in *in vivo* toxicological tests. The expression of genotoxic, apoptotic, detoxification and inflammation related genes were monitored. This study has demonstrated that *C. basilensis* Őr16 efficiently degrade OTA without producing toxic adventitious metabolites

According to the presented results, mycotoxin degrading microbes that were revealed in my work are providing an ideal opportunity for the biodetoxification methods.

In the future we have to take account of frequent mycotoxin contaminations. Against the mycotoxin contaminated row materials potential remediation processes could be those methods that are described in this dissertation.

5. PUBLICATIONS

Scientific paper:

Cserháti M., Kriszt B., Krifaton Cs., Szoboszlay S., Háhn J., Tóth Sz., Nagy I., Kukolya J., (2013) Mycotoxin-degradation profile of *Rhodoccocus strains,* International Journal of Food Microbiology, *DOI:* 10.1016/j.ijfoodmicro. 166 (1) pp. 176-85. IF: 3,33

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

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