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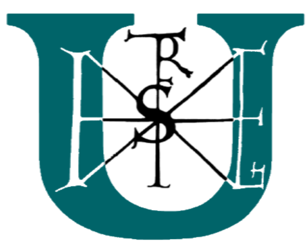

Ph.D. THESIS

Identification of genes responsible for ochratoxin-A biodegradation by *Cupriavidus basilensis* ŐR 16 and valuation of *Cupriavidus* genus for mycotoxins biodegradation potential

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1. Background and aims

Climate change (CC) is expected to have considerable effects on the quality and accessibility of staple food commodities. More than one billion people are suffering from malnutrition and hunger (Tirado et al., 2010).
According to various data, there is a proved connection between the weather changes and epidemics contaminating crops which risk food safety and affect mostly the developing countries (Paterson and Lima, 2010). As a result of weather changes, the production of the field crops is facing complications, becoming sensitive, hence an easy target for infections.
Contamination of food- and feed by toxigenic molds (fungi) is an increasing and unavoidable problem because the climatic extremities cause permanent stress for the crops, which becomes vulnerable to molds. This leads to increase the number of mycotoxin contaminations among foodstuff globally (Bhat et al., 2010; Marroquín-Cardona et al., 2014). However there are around 300 or more, different identified mycotoxins (Paterson and Lima, 2010). FAO clearly stated that around 25 % of international crops can be infected by different toxic metabolites produced by fungi resulting a considerable economic loss (Luo et al., 2018).
Climate models are forecasting the boost of different fungi species such as *Aspergillus* and *Fusarium* infection of maize in Southern Europe which will increase the mycotoxin concentration in these crops (Battilani et al., 2016). From 2005, numerous dry seasons led in sever *A. flavus* contamination of maize in several European countries including Serbia, Italy, Spain, and Romania. Due to dried seasons during the last 15 years *A. flavus* became a major challenge as a dominant fungus in maize. According to the climate change forecast at +2 ˚C level, there is an obvious rise in aflatoxin threat in areas such as the south of Italy, central and southern Spain, northern and

south eastern Portugal, Greece, Albania, Bulgaria, Cyprus, and as compared to nowdays temperature. Besides the risk of aflatoxin among the southern European countries, the low and medium aflatoxin risk during harvest time in major maize producing countries including Hungary is projected (Moretti et al., 2019). In addition , the contamination rate of wheat by deoxynivalenol will be increased by up three times compare to the current levels (van der Fels-Klerx et al., 2012). Moreover, in North America, the contaminated wheat by the high concentration of deoxynivalenol is generally associated with extreme humidity times preceding to harvest (Andreia et al., 2015). In 2004, maize was contaminated by aflatoxin during drought and malnutrition periods in Kenya and led to 125 registered deaths and 317 medical cases were stated due to aflatoxicosis (CDC, 2004) Approximately 4.5 billion people in developing countries are persistently exposed to elevated rate of mycotoxins (Williams et al., 2004).

There are various environmental factors involved in the production of mycotoxins from mycotoxigenic fungi such as moisture content, humidity, and temperature (Bhat et al., 2010).

Mycotoxins are secondary fungal metabolites that have mutagenic, carcinogenic, teratogenic, immunomodulant and cytotoxic effects (Krifaton et al., 2010). They contaminate the food chain from the field to the plates for human consumption. Among mycotoxins, five are the most important: aflatoxin B1 (AFB1), ochratoxin A (OTA), zearalenone (ZON), trichothecene (T-2) and deoxynivalenol (DON). The detoxification and degradation of mycotoxins is an urgent objective due to their hazardous effects. There are different methods which have been developed in order to eliminate or reduce the effects of these toxic compounds such as chemical (ozonation), physical (absorbents) and biological (biodegradation via microorganisms) techniques.

One of the promising methods for the mycotoxin's elimination is biodegradation due to the enzymes that naturally presented in the microorganisms (e.g; bacteria). Based on the explanation and reported evidences in the introduction, my PhD dissertation is focusing on the elimination of OTA, one of the most important mycotoxins, and finding new biological sources for degrading the most dangerous mycotoxins such as AFB1, OTA, ZEA, T-2 and DON.

The aims of the PhD dissertation:

1) **Investigating the effect of the OTA biodegradation by-product of *Cupriavidus basilensis*-ŐR16 on human kidney cell line by gene expression using qPCR**
2) **Transcriptome analysis for identification of the enzymes of the *Cupriavidus basilensis*-ŐR16 playing role in the biodegradation of the OTA.**
3) **Verification of the nominated OTA-degrading enzymes by cloning and expression.**
4) **Valuation of the mycotoxin biodegradation ability of the *Cupriavidus* genus type strains.**

The results showed in my PhD dissertation are the outcome of four years experimental and collaborative research work. All the results were compared by collected and rated scientific literature.

2. MATERIALS AND METHODS

2.1 Ecotoxicological effects of OTA by-products in the human kidney cell line 786O

2.1.1 Bacterial strain and culture conditions in the biodegradation experiment

The strain *Cupriavidus basilensis* ŐR16, was isolated from a Hungarian pristine soil sample. It was identified by molecular taxonomy and deposited in the National Collection of Agricultural and Industrial Microorganisms (NCAIM BO2487). It was grown on LB agar plates and incubated at 28ºC for 72 h. Colonies were inoculated into 40 ml liquid LB medium and incubated at 28ºC for 72 h. The optical density of the inoculated culture was measured at 600 nm by IMPLEN SpectroPhotometer (GENESIS 10S, Thermo Fischer Scientific, USA) and OD600 adjusted to 0.6. Subsequently 5 ml suspension was inoculated into 45 ml modified LB medium (20 %) in triplicates, and OTA (1 mg / ml OTA dissolved in methanol) was added to reach a 2 and 10 mg / l final concentration. A non-inoculated negative control with 2 and 10 mg/l OTA content, and a positive control without OTA, inoculated by the strain culture were applied. 10 mg/l MMS as genotoxic control (DNA damage agent to be compared with the LB and ŐR16 by-products treatments) (Lundin et al., 2005) has the same parameters. Samples were incubated at 28 ºC for 5 days. At the 5th day (endpoint) of the experiment the entire pellet material was removed (25,000 g at 4 ºC for 20 min) from all flasks. Supernatants were filtered through 0.2 μm syringe filters (VWR International Ltd., Hungary) to gain bacteriologically sterile samples containing “normal metabolic product” to avoid any microbial contamination of the kidney cell line. Remaining OTA concentrations in the supernatant and pellet were analysed by High Performance Liquid Chromatography.

2.1.2 AlamarBlue viability assay

The assay was carried out as the protocol. The chosen 786-O cells (human renal proximal tubular epithelial cells) were seeded in 96-well plates (4×104 cells/cm^2) cultured in RPMI-1640 Medium, supplemented with 10 % foetal bovine serum and 1 % Gibco® Antibiotic-Antimycotic is used to prevent bacterial and fungal contamination and maintained for 48 h, until 80–90 % confluence (the fulfilling of the available surface by the cells). The OTA and the MMS was added into the treatments in 10 mg/l concentration. Treatments for viability assay was added in 0.002 % amount of the total treatment volume 200 µl/well, for 24 hours. The AlamarBlue test was conducted in four parallel to measure the effects of OTA, *C. basilensis* ŐR16 normal by-product, 20% LB and MMS on the 786-O human kidney cell line. This assay was performed in Semmelweis University, Faculty of Medicine, Endocrine Lab, Budapest, Hungary.

2.1.3 Cell line treatments and incubation

786-O (human renal proximal tubular epithelial cells) cells were obtained from the Cell Line Service GmbH, Germany and cultured in RPMI-1640 Medium, supplemented with 10 % foetal bovine serum, and 1 % Gibco® Antibiotic-Antimycotic. This solution contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B. Cells were grown in T75 culture flasks at 37 °C in a humidified atmosphere with 5 % CO_2 adding fresh growth medium every 2 days until confluence assessed by microscopic visualization. The cell growing and the treatments were carried out in Debrecen University, RNA extraction and gene expression investigation was carried out in Szent Istvan University.

Cells were growing for three passage, then the cells were transferred into 6 well plates, after that the experiment was carried out. The cell number was 200,000 in each well. Experimental treatments (Table 1) of the cells started

after 24 h of cultivation, in 0.002 % amount. 3 independent experiments were conducted for each treatment, 2-2 technical replicate. Cells were harvested after 48 h of treatment by lysis with Trizol reagent. The gene expression profile of four target genes (*gadd 45*, *gadd 153*, *annexin2* and *clusterin*) were measured in the presence of the bacterial by-product of OTA biodetoxification in the 786-O human kidney cell line.

Target genes: *gadd45, gadd153, clusterin, annexin2*
Houskeeping genes: *ß-actin, hprt, gapdh*

Table 1: Treatments used in the gene expression study on 786-O kidney cell line

Treatment code	Content of treatment
OTA 10	10 mg / l OTA cc in 20 % LB
OTA 2	2 mg / l OTA cc in 20 % LB
MMS 10	10 mg / l MMS cc in 20 % LB (genotoxic control)
OTA 10 + OR16	10 mg / l OTA cc + OR16 strain in 20 % LB (metabolites and by-products)
OTA 2 + OR16	2 mg / l OTA cc + OR16 strain in 20 % LB (metabolites and by-products)
OR 16	Only the OR16 strain in 20 % LB (metabolites)

2.1.4 Target and housekeeping gene selection

Three housekeeping genes (HKGs) *ß-actin*, *gapdh* and *hprt* (Integrated DNA Technologies) were tested on all sets of the cell lines in triplicates. HKGs were chosen for normalization using Rest and BestKeeper (www.gene-quantification.info) software due to their expression stability among these cell lines. Target genes were chosen according to the study of (Ferenczi et al. 2014), and (Arbillaga et al. 2007). The primers of the chosen genes were designed using the PrimerQuest Software-website (Integrated DNA Technologies). The primers were designed to cross exon-exon boundaries to ensure they did not anneal to genomic DNA, and they were validated in

human kidney cell lines 786-O in the treated cells with OTA, OTA + ŐR16, ŐR16 and MMS.

2.1.5 RNA extractions and cDNA Reverse Transcription

Total RNA was extracted from 36 frozen human kidney cells samples (in triplicates) using QiagenRNeasy Mini Kit with Trizol reagent and then purified through RNA columns (QiagenRNeasy Mini Kit, Hungary) and cDNA was synthesized (20 µL for each cell line treated group in triplicates) using reverse FIREScript (Solis Biodyne Ltd, Hungary) with random hexamers. The definitions of the numbers on gel bands can be found in the supplementary material 1. Total RNA was extracted from the frozen kidney samples by TRI Reagent Solution (Ambion, USA) and QIAGEN RNeasy Mini Kit (Qiagen, USA) according the manufacturer's instruction. To eliminate genomic DNA contamination DNase I treatment were used and 100 ml Rnase-free DNase I (1-unit DNase) (Thermo Scientific, USA) solution was added. Sample quality control and the quantitative analysis were carried out by NanoDrop (Thermo Scientific, USA). The cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The primers were used in the Real-Time PCR reaction with Fast EvaGreen qPCR Master Mix (Biotium, USA) on ABI StepOne-Plus instrument.

2.1.6 Real-time PCR and gene expression

Gene expression was determined by using StepOne Plus Real-time PCR (Applied Biosystems, Szent Istvan University, Gödöllő, Hungary). A constant amount of cDNA (5 µL) was used for each qRT–PCR measurement, and three replicates were performed for each gene. The expression of all genes was normalised to two housekeeper genes *β-actin* and *HPRT* using BestKeeper and Rest software. Each qRT– PCR reaction (10 µL total

volumes in each well) was included: 2.72 µL Nuclase Free Water, 5 µL cDNA, 0.14 µL Forward primer, 0.14 µL Reverse primer and qPCR Master Mix 2 µL EvaGreen, Biotium, USA). The qRT_PCR temperature profile in the cell line experiment was as explained below

2.2 Objective 2 : Transcriptional analysis of *Cupriavidus basilensis* ÖR 16 in the presence of OTA

2.2.1 Log phase identification of *Cupriavidus basilensis* ÖR 16

Estimating the log phase was important, to find the correct time for extracting the best quality RNA from the culture. During the pre-experiments, when fixing the method for the OTA elimination occurring on the 3rd day, the RNA was old and broken, not useful for transcriptome analysis. The 11th hour was the proper time for making the RNA extraction, getting good quality RNA, which can be used for analysis.

2.2.2 Transcriptome analysis: RNA quality test, sequencing, and bioinformatics analysis

The *Cupriavidus basilensis* ÖR 16 strain was cultured in LB media for growing and getting the exact cell number. The OTA degradation was carried out in a minimal buffer, only with fructose as carbon source, to activate just those genes, which are responsible or act in the presents of OTA or OTA degradation.

To obtain good quality RNA, 100 ml of the matrix (45 ml of 2% fructose + 45 ml minimal buffer + 10 ml of culture of ŐR16 + 7 mg/l of OTA) was used for the biodegradation experiment for the transcriptome analysis. Samples were centrifuged at 4600 rpm at 4 ºC for 30 minutes after reaching the log phase (11 h). Samples were set in duplicates in the presence or absence of

OTA. For control *E. coli* TOP10 was used in LB and in minimal buffer incubated in the same circumstance as ÖR 16. Total RNA was extracted from the pellets using the Trizol Plus RNA Purification Kit (Thermo Fisher Scientific Co., USA) at SZIU, Gödöllő, according to the manufacturer's instructions. The quality and the quantity or RIN (RNA integrity number) of the RNA sample were analysed by Agilent 2200 Technologies and using TapeStation software, also sequencing (next generation sequencing) and bioinformatics analysis was done by Seqomics Ltd, Hungary.

2.3 Objective 3: Verification of the nominated OTA-degrading enzymes by cloning and expression

These experiments of this objective can be summarized according to the following:

I. <u>Planning the vectors and preparation of the expression:</u>

a) The CPA genes were synthesized and inserted by the expression vector pET-28a (+) (GenScript, USA).
b) The synthesised genes were amplified by *E. coli* TOP 10 cells (overnight incubation at 37 ℃).
c) Plasmid DNA isolated and purified using High-Speed Plasmid Mini Kit (Cat. # PD100, PD300-Geneaid.
d) Plasmid DNA was transformed into *E. coli* BL21(DE3) PLysE chemical cells for protein expression (Sigma-Aldrich Co., USA).

II. <u>Expression and validation of the enzymes:</u>

a) Optimization of the CPA genes induction: different temperatures ranges from (16, 20, 25, 28, 30 and 37 ℃) at different induction periods (3, 3.5, 5, 5.5, 6, 12 and 16 h) were tested to reach better expression. Also, the induction by IPTG was performed in two concentrations 0.5 and 1 mM. Two

volumes of LB media were used in the induction step, 50- and 200-ml. Pellets were disrupted by a lysis buffer (2 mM EDTA, 2 mM Phenylmethylsufonyl fluoride-PMFS, 50 mM NaH2PO4, 300 mM NaCl and l mg/ml of lysozyme). After that samples were placed on ice for 10 minutes, and then placed on beaker contains ice and sonicated for 2 minutes (20 puls on, 20 sec puls off, 60% amplitude). Then, samples centrifuged at 14000 ×g for 30 minutes at 4 °C. Then around 1.7 ml supernatant was harvested in the case of each CPA gene. These supernatants were used in the next chapter for CPA protein purification.

b) CPA proteins purification by HisPur™ Ni-NTA Resin -Gravity-flow technique: Because the expressed CPAs have His tags, provided by the vector, there is a chance for purification by the Ni-NTA technique. The purification of the CPA proteins was performed by HisPur™ Ni-NTA Resin in duplicates.

c) Measurement parameters of MALDI -TOF MS for detecting the target CPAs: The MALDI TOF MS measurement was performed for checking the protein expression. For MALDI-TOF MS analysis, samples were prepared using cells supernatant from induced cultures of *E. coli* BL21 (DE3) PLysE cells by the IPTG for the protein expression. On the standard MALDI target plate 1 µl of supernatant was deposited and dried on room temperature. After that, 1 µl of 2,5 dihydroxybenzoic acid (2,5- DHB) was added on the dried surface of the supernatant. This mixture creates a matrix, where the protein content is crystalised separately, the sulfid bonds are opened, prevent the proteins from overlapping and hence showing readable chromatogram peaks. The method procedure of the MALDI-TOF MS analysis was adapted from the work of (Bojte et al., 2019).

d) OTA biodegradation experiment of the recombinant CPAs: This experiment was also a control method for detecting the recombinant CPAs

and evaluating their degradation ability. Single colonies were inoculated with 50 ml LB contains kanamycin and incubated overnight at 37 ℃. 500 ml from the inoculum from each target sample was added to 200 ml LB and incubated for 2 hours and 40 minutes to reach OD 0.8. Then the cultures were induced by 1 mM IPTG. The cultures then incubated for 3.5 hour at 37 ℃. Finally, the cultures were centrifuged 14000 g x 30 minutes to get the fresh pellets. Lysis buffer was added to the pellets to break the cell wall and then sonicated and centrifuged at 14000 g x 30 minutes. 1 µg/ml of OTA was added to the supernatant and incubated for 16 hours at 37 ℃.

2.4 Objective 4: Mycotoxins biodetoxification potential of *Cupriavidus* genus type strains

Biodegradation experiments for the 16 type strains (Table 2) were conducted in 50 ml media in three parallels, the concentration of the mycotoxins were 2 mg/l each, incubation was on 170 rpm, 28 °C. Samples were centrifuged at 15000 rpm at 4°C for 20 min, supernatant and pellet were stored at -20°C. Remaining mycotoxin concentrations in supernatant and pellet were analysed by High Performance Liquid Chromatography – HPLC (Buda campus, SZIE, Hungary). and by biomonitoring systems for genotoxic or endocrine disrupting effect.

- *SOS*-Chromo test for genotoxicity
- BLYES test for endocrine disrupting

Table 2: The type strains – numbers and collections

Cupriavidus genus strains	Strain number in the collection	Collection
C. necator	CCUG 52238^{T}	Culture Collection University of Gothenburg
C. respiraculi	CCUG 46809^{T}	
C. laharis	CCUG 53908^{T}	
C. metallidurans	CCUG 13724^{T}	
C. campinensis	CCUG 44526^{T}	
C. taiwanensis	CCUG 44338^{T}	
C. pampae	CCUG 55948^{T}	
C. *plantarum*	BCCM/LMG 26296^{T}	Belgian coordinated collections of microorganisms
C. alkaliphilus	BCCM/LMG 26294^{T}	
C. numazuensis	DSM 15562^{T}	German Collection of Microorganisms and Cell Cultures
C. pinatubonensis	DSM 19553^{T}	
C. basilensis	DSM 11853^{T}	
C. gilardii	JCM 11283^{T}	Japanese Collection of Microorganisms
C. oxalaticus	JCM 11285^{T}	
C. pauculus	JCM 11286^{T}	
C. yeoncheonensis	JCM 19890^{T}	

3. RESULTS

3.1 The results of objective 1: Investigating the effect of the OTA biodegradation by-product of *Cupriavidus basilensis*-ŐR16 on human kidney cell line by gene expression using qPCR

3.1.1 Results of AlamarBlue Assay

The AlamarBlue assay was a pre-experiment for evaluating the OTA, *Cupriavidus basilensis* ŐR16, LB and genotoxic/ positive control MMS effects on the 786-O human kidney cell line, before proceeding with RT-PCR experiment. The OTA and the MMS was added into the treatments in 10 mg/l concentration. According to the results, there were no significant difference among the treatments and the cell line was vital in all cases (Figure 2). This means the gene expression experiment could be achieved because the different treatments are not causing cell death during the 48 h and the results can be compared.

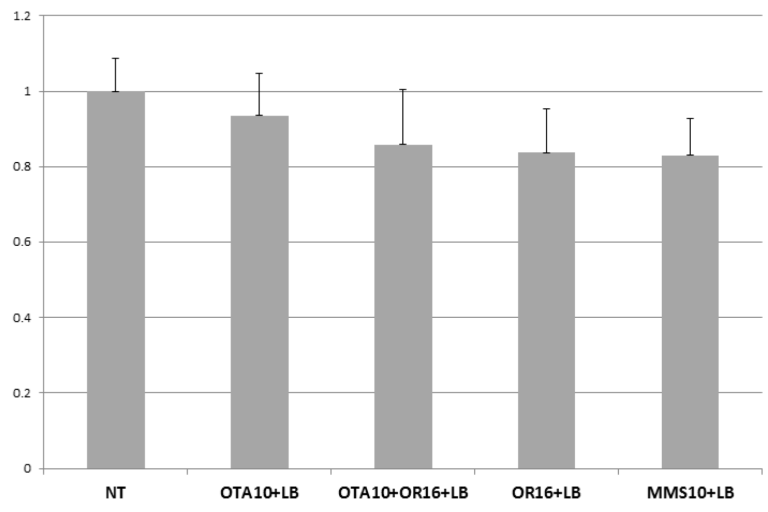

Figure 2: AlamarBlue test result in mean + SE on 789-O human kidney cell line vitality to measure the effect of 10 mg/l OTA concentration, the 20% LB media, ŐR16 strain by-product and 10 mg/l MMS as genotoxic control. Cell line was

treated in four parallels and results are normalized to the non-treated (NT) control. No significant difference was detected among the treatments

3.1.2 Validation of the houskeeping genes (HKG)

To assist the most suitable HKG's the raw Ct values were compared in the different treatments by statistical analysis. According the results, the *hprt* was the best HKG. The second best HKG was *β-actin* gene according to the Ct values. The *gadph* showed high fluctuation, caused by the different treatments, and thus was excluded from the validation.

3.1.3 Target gene expression referenced to *hprt*

The target gene Ct results are referenced to the housekeeping genes (Ct =cycle threshold, is defined as the number of cycles required for the fluorescent signal to cross the threshold, ie exceeds background level). During this process, the average expression rate of the parallel results of the target genes are calculated of each treatment and the data are compared to the chosen HKG gene average expression rate. This referred data is processed via the statistical programs.

In the case of *gadd45* normalized to *hprt* the result showed almost the same down regulation in all cases compared to the Non-treated sample (NT). In the case of *gadd153* there was a significant up-regulation: the OTA and MMS containing treatments ($p<0.05$) compared by the NT and ŐR16 by-product containing treatments. The ŐR16 treatments showed the same expression level as the Non-treated as shown in Figure 3. In the case of *annexin2* there was no tendency among the expression levels. The Ct values showed a high difference in all treatments. In the case of *clusterin* the OTA treatments showed up-regulation (significant difference) and a slight up-regulation in the case of MMS. The ŐR16 by-product containing treatments were on the

same level as the Non-treated controls and were significant less than the OTA treatments. Target gene expression referenced to *ß-actin*.

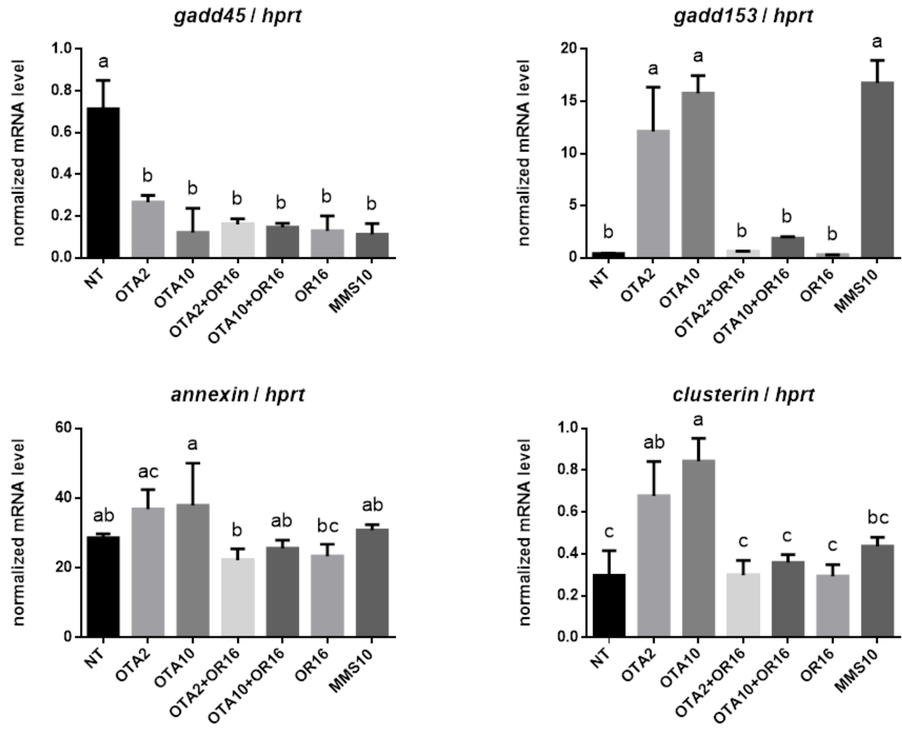

Figure 3: Target genes normalized to the *hprt*, which was the best housekeeping gene, according the raw Ct value validation. Different letters mean statistical difference based on One-way ANOVA followed by the Tukey's multicomparisons test NT is the non-treated sample, OTA2 and OTA10 samples are LB containing 2 and 10 mg / l OTA concentration, OTA2 + ŐR16 and OTA10 + ŐR16 and LB samples containing 2 or 10 mg /l OTA and treated with ŐR16 strain, ŐR16 is the metabolic product of ŐR16 strain and MMS is the genotoxic control containing 10 mg/l MM

3.1.4 Target gene expression referenced to *ß-actin*

The results proved that there is a significant difference in the *ß-actin* normalized gene expression, which contained the ŐR16 showed less harmful effects among the investigated genes ($p<0.05$), compared MMS and the OTA treatments in the case of *gadd153, annexin2* and *clusterin*. In the case of *gadd153* normalized to *ß-actin*, downregulation happened in all treatments. In the case of *gadd153* the OTA and MMS containing treatments showed a

high up-regulation level compared to the NT with significant difference (Figure 4). The ŐR16 by-product treatments showed the same expression level as the NT control except the *gadd45*. The *annexin2* expression was up-regulated by the OTA and MMS containing samples, and showed a slightly downregulation in the presents of OTA and ŐR16 by-product containing samples, and the treatments containing only ŐR16 by-product showed the same expression level like the NT control, but without significant difference. In the case of *clusterin* the expression was up-regulated significantly by the OTA and MMS containing samples and showed a slightly up-regulation in the present only ŐR16 by-product without OTA. In terms of *clusterin* the ŐR16 containing treatments have almost no effect compared to the OTA and MMS treatments.

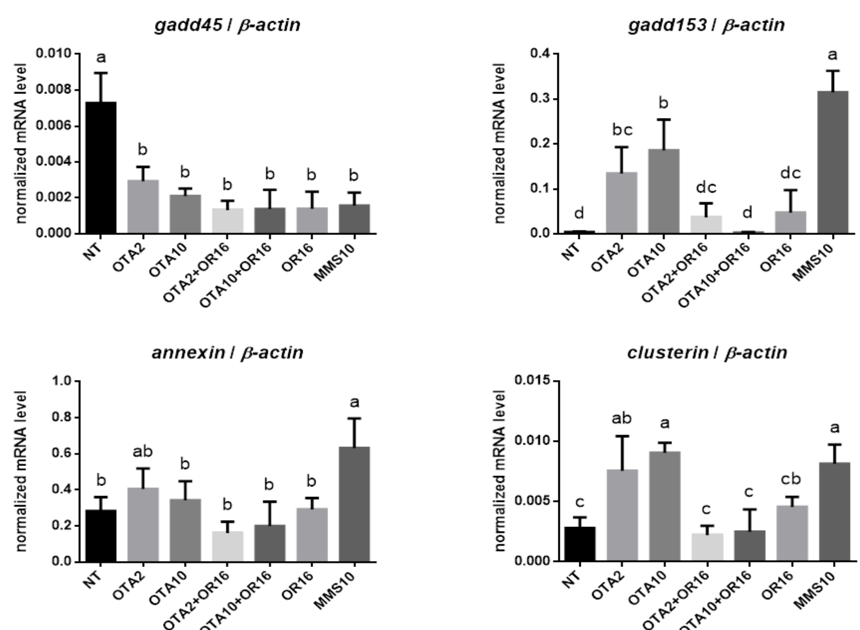

Figure 4: Target genes normalized to the *β-actin*, which was the second-best housekeeping gene, according the validation. The different letters mean statistical difference based on One-way ANOVA followed by the Tukey's multicomparisons test. NT is the non-treated sample, OTA2 and OTA10 samples are LB containing 2 and 10 mg/l OTA concentration, OTA2+ŐR16 and OTA10+ŐR16 and LB samples

containing 2 or 10 mg/l OTA and treated with ŐR16 strain, ŐR16 is the metabolic product of ŐR16 strain and MMS is the genotoxic control containing 10 mg/l MMS

3.2 The results of objective 2: Transcriptome analysis for identification of the enzymes of the *Cupriavidus basilensis*-ŐR16 playing role in the biodegradation of the OTA

3.2.1 RNA isolation and quality control analysis

For the successful transcriptome analyses good quality RNA is needed. After evaluating the log phase of the ÖR16 strain in the minimal media, culturing next to 7 mg/l OTA concentration the RNA was extracted. The RNA integrity was confirmed in 1% agarose gel electrophoresis (Figure 5). TOP 10 *E. coli* was used as a positive control in two different media; LB and minimal buffer (m.b).

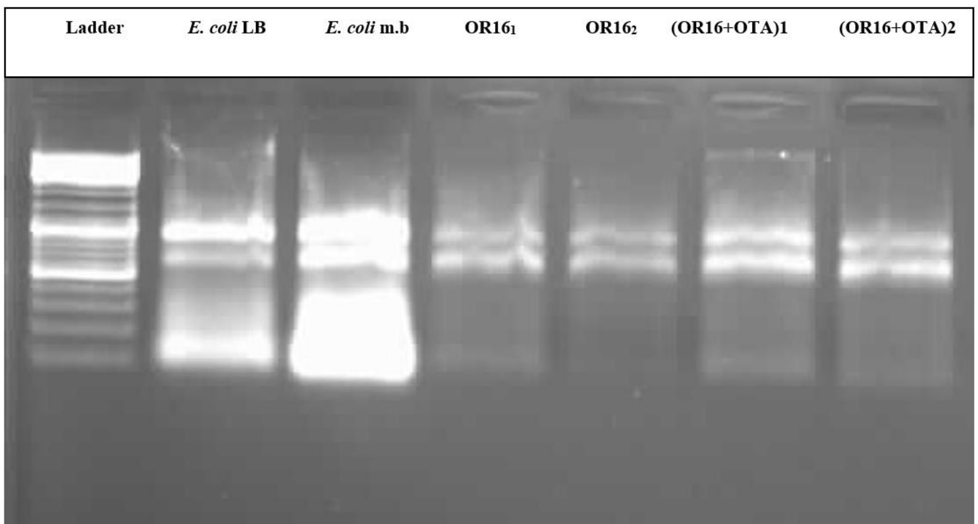

Figure 5: RNA bands of *Cupriavidus basilensis* ŐR16 with and without OTA from the OTA degradation experiment conducted in minimal buffer for transcriptome analysis, sampled after 11 hours of incubation

3.2.2 Results of the transcriptome analysis

After completing the RNA quality control test, synthesis of the cDNA, fragmentation, RNAseq and bioinformatics analysis it turned out that 3500 genes were up regulated. Out of the 3500 gene, 15 genes could be enrolled into the criteria of the evaluation system. The three CPA genes were chosen, because there is a study by Luizzi and colleagues from 2017, where the *Acinetobacter* sp. CPAs encoding genes were cloned and expressed and tested for OTA degradation with success. That was the reason why in my research the CPA-s of the ÖR16 strain were chosen for further study. (Table 3). 3 genes (D-alanyl-D-alanine carboxypeptidase **CPA1**, Metal-dependent amidase /aminoacylase /carboxypeptidase **CPA2**, D-alanyl-D-alanine carboxypeptidase penicillin-binding protein **CPA3**) were chosen for the cloning and expression, although their expression were quite low (CPA1 = 1.3; CPA2= 2.5; CPA3= 1.1) compared with the other 12 genes (over 2, reaching 6-8) see in Table 3.

Table 3: Fifteen nominated genes out of 3500, 3 were chosen for cloning and expression

Nominated genes * Chosen genes for cloning and expression	Gene name	Fold expression	Size in bp	Contig number
Phenylalanine-4-hydroxylase	ŐR16_31894	2.3	927	94
Aromatic Ring hydroxylase	ŐR16_16257	8.1	1575	38
Membrane carboxypeptidase (penicillin-binding protein)	ŐR16_12645	1.8	2190	29
Membrane proteins related to metalloendopeptidases	ŐR16_24100	3.8	727	60
Membrane carboxypeptidase /penicillin-binding protein PbpC	ŐR16_31869	1.9	2208	94
D-alanyl-D-alanine carboxypeptidase *CPA1* *	**ŐR16_23878**	**1.3**	**1209**	**58**
Metal-dependent amidase /aminoacylase /carboxypeptidase *CPA2*	**ŐR16_07981**	**2.5**	**1212**	**17**
D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 4) *CPA3*	**ŐR16_12223**	**1.1**	**1536**	**27**

Amidases related to nicotinamidases	ŐR16_19156	4.8	699	45
Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit	ŐR16_01015	2.1	771	3
COG0169 Shikimate 5-dehydrogenase	ŐR16_09609	7.6	411	21
COG3971 2-keto-4-pentenoate hydratase	ŐR16_01040	3.2	2187	3
COG2146 Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	ŐR16_08912	4.3	312	19
Predicted metal-dependent hydrolase	ŐR16_35215	6.5	666	108
Dienelactone hydrolase and related enzymes	ŐR16_25377	6	1236	63

3.3 The results of objective 3: Verification of the nominated OTA-degrading enzymes by cloning and expression

3.3.1 Vector amplification, plasmid DNA isolation and purification

The chosen genes were inserted into pET-28a (+) vector (separately) by GenScript Co. (USA). The vectors were successfully amplified by *E. coli* TOP 10 cells to reach the desired volume needed for the target CPAs expressions in *E. coli* BL21 (DE3) pLysE chemical cells. The pET-28a vectors were isolated and purified using High-Speed Plasmid Mini Kit. Plasmid DNA concentrations were measured using Nanodrop, ThermoFisher.

3.3.2 Expression of the carboxypeptidases (CPAs) in BL21 (DE3) pLysE cells

Among the tested temperatures and IPTG concentrations, only the 37 ℃ for 3.5 hours + 1 mM IPTG was the suitable parameters for the protein

expression experiment. The CPA proteins were successfully expressed in SDS page, but the bands were weak that can be seen in Figure 6.

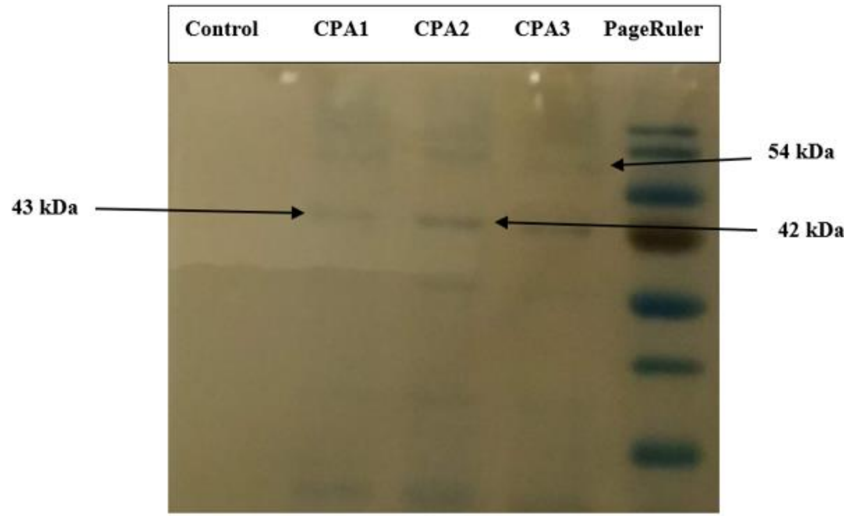

Figure 6: The CPAs weak bands were proven in the SDS page in the estimated sizes

3.3.3 Results of the MALDI TOF MS

This method was used due to its accuracy in the measurement. During the process, the equipment is taking 60 measurements/sec from one plot of the samples. Only those spectra-s (peaks) are recognised during the process, which reach a 20% frequency. From this database different algorithms are creating the curves of the peaks in m/z, each peak is a protein. According to MALDI-TOF, the results prove that the expression of the targets proteins (CPA1, CPA2, and CPA3) occurred with the predicted sizes of each protein as shown in Figure 7.

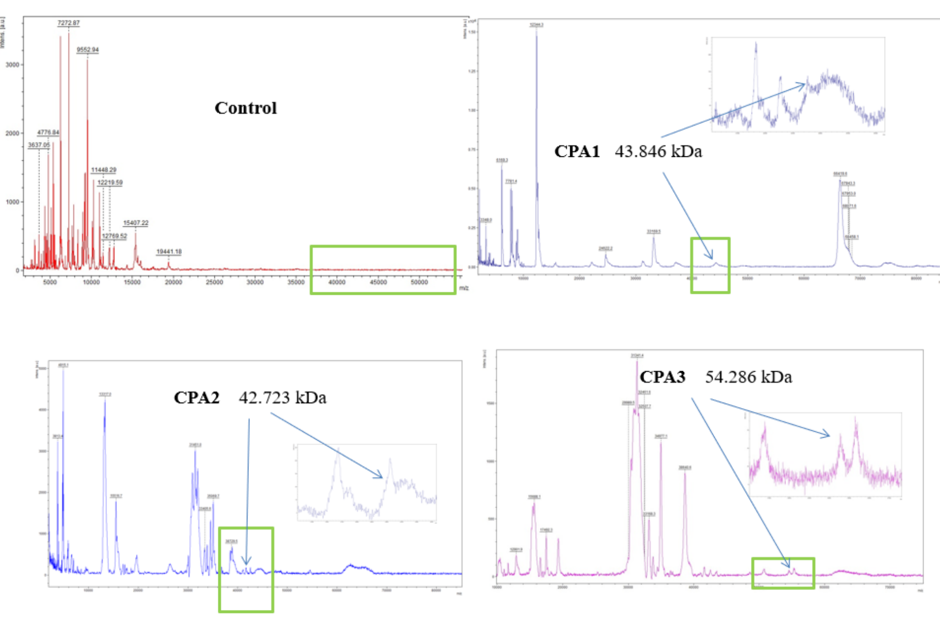

Figure 7: MALDI-TOF MS results of the three expressed CPAs supernatant. The control was the uninduced BL21 (DE3) pLysE cell

3.3.4 The purification of the CPA genes by Ni-NTA Resin columns

The pET-28a (+) vector contains His tag promoter also, cause of that a Ni-NTA purification column could be proceed following the protocol. The expression of the CPAs and the Ni-NTA purification was confirmed by the measuring of the protein concentrations by Nanodrop, ThermoFisher, RET lab, SZIE as shown in Table 4. The His tagged protein concentration was higher in the induced samples (CPA1, CPA2, CPA3).

Table 4: Protein concentrations of the expressed CPAs after the purification by Ni-NTA resin

Genes	Concentration mg/ml	A 280	A 260 / 280
Control (uninduced) BL21 (DE3) pLysE	0.002	0	-9.27
CPA1	0.379	0.38	2.5
CPA2	0.548	0.55	2.238
CPA3	0.621	0.62	2.27

3.3.5 Testing the biodegradation activity of the expressed CPAs

In the case of the biodegradation activity of the CPAs, the induction time and the temperature were changed. The induction time was 3 hours and half at 37 ˚C. The final induction volume was 200 ml culture in each CPAs in duplicates. The harvested supernatants were approximately 1.7 ml. The HPLC-MS/MS results showed a remarkable biodegradation potential of the transmembrane CPAs. Around 65 % of the estimated 2000 ng/ml OTA was degraded after 16 h of incubation at 37 ˚C compared to the control (uninduced culture) shown in Table 5, the results are the average of duplicates.

Table 5: Estimated 2000 ng/ml OTA biodegradation by the supernatants of the expressed CPA genes, measured by HPLC-MS/MS.

Samples	Supernatant volume in ml	OTA concentration ng/ml	Biodegradation rate %
Uninduced + OTA (Control)	1	2121	
CPA1 supernatant + OTA	1	897	64
CPA2 supernatant + OTA	1	967	62
CPA3 supernatant + OTA	1	995	60
All CPAs mixed + OTA	1	1225	50

3.4 The results of objective 4: Valuation of the mycotoxin biodegradation ability of the *Cupriavidus* genus type strains

a) Two type strains were able to biodetoxificate and cease the genotoxicity of AFB1: *Cupriavidus laharis* and *Cupriavidus oxalaticus*.
b) One type strain was able to reduce the endocrine disrupting effect of the metabolites of ZON: *Cupriavidus basilensis*
c) 7 type strains degraded OTA, 3 type strains degraded T-2 during the experiment, the detoxification profile of OTA and T-2 was not tested.
d) Six type strains were able to degrade two different mycotoxins (over 60%)
e) Two type strains were able to degrade three different mycotoxins (over 60%)
f) The type strain *Cupriavidus numazuensis* was able to biodegrade four mycotoxins: AFB1, ZON, OTA and T-2 over 70%

More details about the biodegradation results of the *Cupriavidus* genus type strains can be seen in Table 6.

Ability to degrade more than one:

a) Six type strains were able to degrade two different mycotoxins
   - ✓ *C. respiraculi* could degrade 82% of OTA and 64 % of ZON
   - ✓ *C. laharis degrade* 91 % of AFB161% of ZON
   - ✓ *C. metallidurans* degrade 77 % of AFB1 and 72 % of T-2.
   - ✓ *C. plantarum* degrade 67% of ZON and 60% of T-2
   - ✓ *C. taiwanensis* degrade 97% of OTA and 63% of AFB1
   - ✓ *C. oxalaticus* degrade 82% of AFB1 and 82% of ZON
b) Two type strains were able to degrade three different mycotoxins
   - ✓ *C. pinatubonensis* degrade 90% of ZON, 88% of OTA and 68% of T-2
   - ✓ *C. basilensis degrade* 96% of ZON, 94% of OTA and 68% of T-2, and was able to reduce the endocrine disrupting effect of the metabolites of ZON
c) The type strain *Cupriavidus numazuensis* was able to biodegrade four mycotoxins: AFB1, ZON, OTA and T-2 (without any biodetoxification)

Table 6: AFB1, ZON, OTA and T-2 biodegradation potential of Cupriavidus type strains after a five day-experiment determined by HPLC-FLD. Residual genotoxicity was detected in supernatant by SOS-Chromo test, oestrogenicity was detected by BLYES test.

Species	AFB1 degr. eff. (%)	Genotoxicity (IF)	ZON degr. efficiency (%)	Oestrogenicity (Biol. int. %)	OTA degr. efficiency (%)	T2 degr. efficiency (%)
C. alkaliphilus BCCM 26294^T	58	2.83 ± 0.14	33	1053 ± 110	95	52
C. basilensis DSM 11853^T	19	2.71 ± 0.29	96	47 ± 19	94	68
C. campinensis CCUG 44526^T	61	2.60 ± 0.09	55	894 ± 187	28	55
C. gilardii JCM 11283^T	32	3.45 ± 0.34	35	1140 ± 9	19	95
C. laharis CCUG 53908^T	91	1.31** ± 0.03	61	811 ± 6	20	27
C. metallidurans CCUG 13724^T	77	2.27 ± 0.15	51	1084 ± 59	27	73
C. necator CCUG 52238^T	31	3.44 ± 0.26	47	1092 ± 14	92	47
C. numazuensis DSM 15562^T	72	1.93 ± 0.11	85	530 ± 16	85	70
C. oxalaticus JCM 11285^T	82	0.97** ± 0.14	82	541 ± 21	19	50
C. pampae CCUG 55948^T	60	2.82 ± 0.10	50	772 ± 101	30	47
C. pauculus JCM 11286^T	41	3.04 ± 0.51	42	1118 ± 90	20	42
C. pinatubonensis DSM 19553^T	17	2.90 ± 0.28	91	312 ± 50	88	68
C. plantarum BCCM 26296^T	59	2.81 ± 0.12	67	911 ± 177	14	60
C. respiraculi CCUG 46809^T	51	3.22 ± 0.12	64	310 ± 34	82	47
C. taiwanensis CCUG 44338^T	63	2.93 ± 0.20	42	636 ± 215	97	56
C. yeoncheonensis JCM 19890^T	45	3.34 ± 0.32	41	1232 ± 36	12	40

Strains having more than 70% biodegradation ability
Strains causing biodetoxification

3.5 NOVEL SCIENTIFIC RESULTS:

Thesis 1: OTA biodetoxification by *Cupriavidus basilensis* ÖR16 has been proven, since the OTA biodegradation by-products did not alter the expression of the target genes (*gadd45*, *gadd153*, *annexin2* and *clusterin*) in human kidney cell line 786-O.

Thesis 2: The transcriptome analysis of *Cupriavidus basilensis* ÖR16 strain in an OTA degradation system was implemented and 15 genes were identified, which could be responsible for OTA biodegradation.

Thesis 3: Three CPA proteins and encoding genes (CPA1 encoded by ŐR16_23878 gene; CPA2 encoded by ŐR16_07981 gene; CPA3 encoded by ŐR16_12223 gene) of the *Cupriavidus basilensis* strain ÖR16, responsible for OTA degradation have been cloned and expressed.

Thesis 4: The evaluation of the mycotoxin biodegradation potential of the *Cupriavidus* bacteria genus type strains was achieved.

Thesis 5: Two strains were able to biodegrade and biodetoxificate and cease the genotoxicity of AFB1: *Cupriavidus laharis* CCUG 53908^T and *Cupriavidus oxalaticus* JCM 11285^T.

Thesis 6: One strain was able to reduce the endocrine disrupting effect of the metabolites of ZON: *Cupriavidus basilensis* RK1 DSM 11853^T.

Thesis 7: The strain *Cupriavidus numazuensis* DSM 15562^T was able to biodegrade four mycotoxins: AFB1, ZON, OTA and T-2.

4.DISCUSSION

4.1 1st objective's discussion

Several studies have been conducted on OTA degradation by microbes. According to the research papers, only few bacteria have been shown OTA biodegradation in different time and proportion. For example, *Bacillus licheniformis* degraded 92.5% of OTA at 37 ºC and the detectable product was ochratoxin-alfa (Petchkongkaew et al., 2008). *Brevibacterium spp, Alcaligenes faecalis* bacteria have shown 100% (Rodriguez et al., 2011; Zhang et al., 2017), *Bacillus amyloliquefaciens ASAG1*and 98.5% OTA degradation (Chang et al., 2015) respectively.

The validation of the detoxification is extremely important, when dealing with biodegradation of harmful chemicals. There are many difficulties to evaluate the detoxification of a biodegradation process. OTA biodegradation process is one of them; cause the problematic and complex effect, which can be investigated by difficult biotests. In the case of *C. basilensis* ŐR16 already three different detoxification validation experiment was carried out in the recent years. First was the mice feeding experiment proceed by Ferenczi et al., in 2014, where gene expression of the same target genes was investigated. This was the base of the human cell line experiment, which was achieved in this dissertation. Parallel with this cell line experiment a new method was carried out zebra fish microinjection test, which is a thesis of a different PhD dissertation, also belonging to the same research group.

In the present research, the OTA-degrading potential of *C. basilensis* ŐR16 in low and high OTA concentrations (2 and 10 mg/l) were analysed, acute toxicity, through a 5-day long biodegradation experiment, the OTA was degraded efficiently (100%) and the metabolized ochratoxin-alfa was also measured. Up to date, our experiment is the first which investigated the

effects of OTA-detoxification by-products of a bacterium (*Cupriavidus basilensis* ŐR16) on the gene expression profile on normal adult human kidney cell line (789-O).

Previous works explains the effects of the OTA on the mRNA level of the genes involved in the DNA damage, DNA repair, kidney tumour developing process. One study has shown that 254 genes expression altered up to two-fold during 72-hour exposure to low and high OTA concentrations (1 and 10 mg/kg bodyweight). As a result, 165 genes were down regulated and 89 were up-regulated (Lühe et al., 2003). These genes are beneficial to serve as indictors for the kidney cells to monitor the possible toxic impacts of the bacterial by-product at the end of the biodegradation process. The up regulation in gene expression which resulted after the exposure to OTA were confirmed by the quantitative-real time PCR instrument analysis both genes *gadd45* and *gadd153* function as indicators for DNA damage induction and genotoxic effects of OTA (Beard et al., 1996). The experiment carried out on rat cell line culture of Lühe et al., 2003 showed that there is over expression in the DNA damage indicators gadd45 and gadd153, and in the case of *annexin2* and *clusterin* after treated the samples with DNA- genotoxic chemicals. MMS function as a positive genotoxic control that can change the expression of *gadd45* and *gadd153* (Beard et al., 1996).

Three housekeeping genes were chosen and validated for normalization to our experiment in the following ranking, according the results: *hprt*, *β-actin* and *gapdh.* The *gapdh* had the worst validation results, but the normalized expression data showed an interesting tendency, which was parallel with the other genes expression. In our work the cell line treated with MMS and OTA, showed up-regulation in the case of *gadd153, annexin2* and *clusterin* compared to the Non-treated control, normalized to three different

housekeeping genes *(hprt, ß-actin, gapdh),* which means a clear demonstration for the genotoxic effects of these chemicals. In the case of *gadd45* there was a down regulation compared to the Non-treated control normalized to two HKG's: *hprt* and *ß-actin*.

Another pattern was noticed in other experiment shows that the cytotoxic amounts of OTA caused up-regulation in the *gadd45* gene family (Newton et al., 2004). An opposite result was observed in vitro study that *gadd45* mRNA expression was downregulated in the case of HK2 human kidney cell, incubation period was 24 h and this study used different OTA concentrations (50, 100,200, 400 and 800 μM) without any by-product (Arbillaga et al., 2007), which is similar to *gadd45* expression in our experiment. Besides, in terms of *gadd45* normalized to *hprt, ß-actin and gapdh* a downregulation happened in the treatments containing only ŐR16 by-products, this is due to the cell line reaction for the bacteria by-products.
Clusterin and *annexin2* genes play an important role in the renal cortex and apoptotic process. Previous conducted studies have shown that *annexin2* mRNA level was elevated in the renal cortex of rat (Lühe et al., 2003), which has comparable or similar gene expression patterns to *annexin2* and *clusterin* in our work. *Annexin2* is considered to be a cofactor for DNA polymerase alpha subunit, which performs a significant function in the DNA repair and progression of diverse cancers (Kumble et al., 1992) as well as to its role as substrate for oncogene associated kinase (Skouteris and Schröder, 1996).

The over expression of the *annexin2* was investigated in the formation of kidney carcinoma in rat (Tanaka et al., 2000).
In other studies in rat and human kidneys, *clusterin* was also proved to be induced in low (70 μg/kg for 4 weeks) and high (210 μg/kg for 13 weeks)

doses of OTA (Qi et al., 2014; Dvergsten et al., 1994). The biodegradation of OTA by *C. basilensis* ŐR16 strain eliminated the over expression of *annexin2* and *clusterin,* when mice were treated with low and high doses of OTA, proving that there is not only occurring of biodegradation but also biodetoxification (Ferenczi et al., 2014). This result is supported by our data, where the expression of *annexin2* and *clusterin* in the case of the treatments containing the by-products of the ŐR16 were the same to the non-treated control.

4.2 2nd objective's discussion

It is the first time worldwide to conduct a transcriptome experiment with *C. basilensis* ŐR16. The results reveal that there are 3500 upregulated genes shown in the transcriptome analysis results in the presence of OTA. At the first time, 15 gene were chosen for pre-investigation, which could be hypothetically connected to OTA biodegradation. All 15 genes showed a relatively big fold expression from 1,3 till 8. According the transcriptome study of Liuzzi and colleagues from 2017, which focused on the CPA proteins OTA degradation from *Acinetobacter* strain *neg1* only the following 3 CPA-s were chosen for further cloning and expression in first round: CPA 1= 43.96 kD; CPA2 = 42.72 kD; CPA 3 =54. 28 kD. The chosen CPA-s showed a relative low fold expression 1.3-2.5 compared to the other 13 genes. This could be the result of the early extraction time of the total RNA from the culture. The reason of that was the top of the log phase of the ŐR16 strain in the required minimal buffer was at 11 hours. After that, only degraded RNA could be extracted, which was not useful for the transcriptome analysis.

4.3 3rd objective's discussion

The presented experiment was the first that identified and investigated the enzymes responsible for OTA biodegradation in *Cupriavidus basilensis* ŐR16. Out of 3500 upregulated genes, three were the mostly functioning and involving in the OTA biodegradation, CPA1, CPA2 and CPA3 already discussed in previous chapter. From the chosen three CPAs, two were transmembrane (CPA1 and CPA3) and one was intercellular

CPA2. The estimated targets OTA- degrading proteins from *Cupriavidus basilensis* ŐR16 were successfully cloned and expressed via peT28 + vector into *E. coli* BL21 (DE3) pLysE cells. The vector contained histidine tagged tale promoter also, for purification of the expressed protein.

Up to date, and according to the literature, limited papers have been performed on OTA degrading genes via fresh supernatants containing recombinant CPA. The study of Chang and other co-authors showed that 98.5 % of OTA was degraded after 24 hours at 37 ºC by the recombinant CPA (estimated size 48.6 kDa) from *Bacillus amyloliquefaciens* ASAG1 strain. This study was performed via the amplification of the CPA coded gene in PCR. The PCR product from the genome sequence of *Bacillus amyloliquefaciens* ASAG1 was successfully cloned by *E.coli* JM109 and expressed to *E.coli* Rosetta expression cells (Chang et al., 2015). There is only one study conducted a transcriptional analysis of *Acinetobacter* sp. *neg1* capable of degrading OTA by Luizzi and co-researchers in 2017. The authors also used pET-28a (+) as expression vector and BL21(DE3) for expression of CPA1 (D-Ala-D-Ala carboxypeptidase). Nevertheless, the recombinant plasmid did not accomplish any detectable expression of the protein in this host bacterial strain in contrast to the present study. For this reason, , the

construct pET-28a(+)- PJ15_1540 (the gene encodes CPA1) was used to transform *E. coli* BL21-CodonPlus-RIL. The results of Luizzi's study showed that after incubation overnight with 1 µg/mL of OTA, PJ15_1540 was able to degrade only 33 % of OTA (Liuzzi et al., 2017). On the other hand, the presented experiment showed that CPA1 could degrade 64 % of 1 µg/mL of OTA.

Another experiment illustrated that the combination of bifunctional enzymes can be very promising in the mycotoxins control such as OTA and ZON (Azam et al., 2019). This study indicated that the combination of two single genes (CPA and ZON- hydrolase) in a frame deletion by cross over PCR, ZON was completely degraded after 2 hours of incubation with this combined-enzymes at pH 7 / 35 ˚C. In addition, OTA was totally degraded when treated with the fusion combined genes; ZHDCP (77.36 kDa) and CPA with an estimated size 48.66 kDa which was also weak in the SDS page. In a recent study, a novel gene named N-acyl-L-amino acid amidohydrolase (*AfOTase*), molecular mass 45 kD from *Alcaligenes faecalis* is a possible OTA-biodegrader and it successfully expressed in *E. coli* (DE3) and determined by SDS-page (Zhang et al., 2019).

4.4 4th objective's discussion

In the present work, one of the main goals were to measure the mycotoxin biodegradation potential of 16 type strains of *Cupriavidus* genus and evaluate the possible harmful effects of the metabolic intermediates. The further aim of our study was to select the best degraders among these strains. This information by the molecular biotechnological data (existing genome projects, etc) can be a base of the enzyme engineering in a future study.

The mycotoxin degradation ability of different bacteria's (*Rhodococcus* sp, *Streptomyces* sp) were investigated by our department previously (Risa et al., 2018; Cserháti et al., 2013a; Harkai et al., 2016). Members of the *Cupriavidus* genus have interesting abilities among the biodegradation of different chemicals, xenobiotics, also in the case of one mycotoxin OTA (Ferenczi et al., 2014). The biodegradation experiments were performed in 5 days because of the comparison of the already mentioned former studies. Although there is biodegradation result confirmed during two or three days, and our group also realised 95% AFB1 biodegradation rate for 24 hours, the present study is the first evaluation of the *Cupriavidus* genus biodegradation ability in the case of mycotoxins.

The comparison of the biodegradation potential of different bacteria's is not the right foundation, because the biodegradation does not mean biodetoxification, which means the elimination of the harmful effects of the biodegraded chemical. The comparison of the biodetoxification ability is the right way. However, evaluation of the biodetoxification has difficulties, because the proper biotest or organism is needed. Mycotoxins have different negative effects, which are not easy to measure or estimate in realistic (cost effectiveness, time and resource consuming). Unfortunately only a few publications are investigating the biodetoxification in the case of biodegradation. In the case of AFB1 the *Rhodococcus* sp are highly effective biodetoxifiers from 42 type strains 15 ceased the genotoxicity in 72 hours (Table 5). In the case of ZON only one *Rhodococcus* type strain could cease the eostroenic effect (Risa et al., 2018). 124 *Streptomyces* strains were tested for AFB1 and ZON biodegradation, and only one strain was able to biodetoxify AFB1 and only two strains could cease the eostrogenic effect of ZON (Harkai et al., 2016).

From the genus *Cupriavidus* almost all type strains are able to biodegrade AFB1 in 5 days, high biodegradation ratio (over 70%) were achieved by only 4 strains: *Cupriavidus laharis* CCUG 53908^{T} (91%), *Cupriavidus oxalaticus* JCM 11285^{T} (82%), *Cupriavidus metallidurans* CCUH 13724^{T} (77%) and *Cupriavidus numazuensis* DSM 15562^{T} (72%). According the SOS-chromotest results, two strains were able to cease the genotoxicity:

Cupriavidus laharis CCUG 53908^{T} (IF=1.31) and *Cupriavidus oxalaticus* JCM 11285^{T} (IF=0.97).

In the case of ZON, all *Cupriavidus* strains had biodegradation ability, but only four reached 70% degradation rate: *Cupriavidus basilensis* DSM 11853^{T} (95%), *Cupriavidus pinatubonensis* DSM 19553^{T} (91%), *Cupriavidus numazuensis* DSM 15562^{T} (85%) and *Cupriavidus oxalaticus* JCM 11285^{T} (82%). According the BLYES results, one strain was able to reduce the endocrine disrupting effect of the metabolites of ZON: *Cupriavidus basilensis* DSM 11853^{T} strain.

From the 16 type strains in the case of OTA there were two groups: strains having weak biodegradation ability (30%), and strains having remarkable biodegradation potential (over 80%). These most effective strains (7) were: *Cupriavidus taiwanensis* CCUG 44338^{T} (97%), *Cupriavidus alkaliphilus* BCCM 26294^{T} (95%), *Cupriavidus basilensis* (94%), *Cupriavidus necator* (92%), *Cupriavidus pinatubonensis* (88%), *Cupriavidus numazuensis* (85%) and *Cupriavidus. respiculi* (82%). The evaluation of the biodetoxification ability of theses strains are limited, because there are only difficult and time consuming biotest or methods for testing the negative effects of OTA by-products.

In the case of T-2 toxin only 6 strains could biodegrade T-2 with over 60% ratio: *Cupriavidus gilardii* JCM 11283^{T} (95%), *Cupriavidus metallidurans* CCUG 13724^{T} .

(73%), *Cupriavidus numazuensis* DSM 15562^{T} (70%), *Cupriavidus pinatubonensis* DSM 19553^{T} (68%), *Cupriavidus basilensis* DSM 11853^{T} (68%) and *Cupriavidus plantarum* BCM 26296^{T} (60%). The evaluation of the T-2 detoxification has the same problematic, like the OTA, there is no easy and fast evaluation method for measuring the effects of the by-products.

Biodegradation of DON was also investigated but none of the 16 type strains were able to degrade it.

From the 16 type strains, five strains were able to degrade two or more mycotoxins effectively (over 60%). There are 6 type strains which are able to degrade two mycotoxins. The strains of *Cupriavidus pinatubonensis* DSM 19553^{T} and *Cupriavidus basilensis* DSM 11853^{T} could degrade ZON, OTA and T-2. The strain *Cupriavidus numazuensis* DSM 15562^{T} was able to biodegrade four mycotoxins: AFB1, ZON, OTA and T-2. This phenomenon is unique according the latest literature. Up to date, *Rhodococcus* strains are known to degrade and detoxify more than two mycotoxins: *Rhodococcus erythropolis* NI1 strain can biodegrade AFB1, ZON and T-2 and detoxify the harmful effects of AFB1 and ZON (Risa et al., 2018). A microbe consortia TMDC was investigated lately, which were able to degrade AFB1 and ZEA in more than 90% after 72 h, but the detoxification was not evaluated, the consortia consisted from the following generea:

Geobacillus, *Tepidimicrobium*, *Clostridium*, *Aeribacillus*, *Cellulosibacter*, *Desulfotomaculum* and *Tepidanaerobacter* (Wang et al., 2018).

Altogether comparing the results of this study with the *Rhodococcus* genus ability, the genus *Cupriavidus* has less appropriate members for detoxifying the mycotoxins, but still a valuable resource for further research and for future application against mycotoxins.

Up to the present, 11 type strains have genome project data. If, all the members of the genus will have a full genome project, with the results of this study the responsible genes for mycotoxins biodegradation can be identified. This will help for developing a cell free enzyme-based additive for treating the contaminated feed or crop.

The validation of the detoxification in the case of T-2 and OTA degrading members, and the investigation of the simultaneous mycotoxin degradation and detoxification should be implemented.

5. Conclusions and suggestions

Firstly, the OTA was completely degraded after the 5th day of the biodegradation experiment by *Cupriavidus basilensis* ÖR16. In addition, the biodetoxification of OTA was proved by the gene expression experiment; the by-product of the OTA biodegradation did not alter the expression of the target genes in human kidney cell line 786-O.

Secondly, the 15 genes and proteins could be responsible for OTA degradation used by the *Cupriavidus basilensis* ÖR16 bacteria strain via transcriptome analyses from total RNA from an OTA biodegradation matrix was evaluated and identified.

Thirdly, from the nominated 15 genes, three CPA genes and proteins of the *Cupriavidus basilensis* ÖR16 bacteria responsible for OTA degradation was isolated via cloning and expression.

Fourthly, the biodegradation potential of the *Cupriavidus* bacteria genus type strains was evaluated. Among the genus, *Cupriavidus laharis* and *Cupriavidus oxalaticus* were able to biodegrade and biodetoxificate and cease the genotoxicity of AFB1. Furthermore, *Cupriavidus basilensis* was able to decrease the endocrine disrupting effect of the metabolites of ZON. The strain *Cupriavidus numazuensis* was able to biodegrade four mycotoxins: AFB1, ZON, OTA and T-2.

Suggestions for the future research:

1) Investigation of the chosen 13 genes via cloning and expression, according the transcriptome results.

2) Investigation of the induction circumstances of the expressed three CPA genes for a better protein yield.

3) Deeper evaluation of the T-2, OTA biodegrader type strains via zebra fish microinjection method for finding the strains which are able to detoxify these toxins.

4) Fully investigation of the strains which are able to biodegrade three and four mycotoxins, co-biodegradation experiment, biotest, molecular analysis according the existing data.

6. PUBLICATIONS

Scientific papers:

AL-Nussairawi M,· Risa A,· Garai E, ·Varga E, Szabó I, Csenki-Bakos Zs, ·Kriszt B, Cserháti M (2020) *Mycotoxin biodegradation ability of the Cupriavidus genus*, Current Microbiology, Accepted for publication

AL-Nussairawi M, Kriszt B, Krifaton Cs, Cserháti M (2020) *Transcriptome analysis of an ochratoxin-A biodegrading bacteria*, Columella, Accepted for publication

AL-Nussairawi M, Márton D, Garai E, Ivánovics B, Czimmerer B, Urbányi B, Kriszt B, Cserháti M (2020) *Investigation on biodegradation of ochratoxin A by Cupriavidus basilensis using a novel gene expression method on human kidney cells*, Mycotoxin Research, Submitted

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