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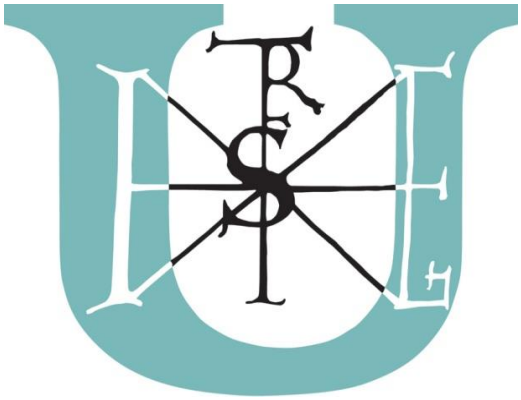

PhD School of Environmental Sciences

Identification of genes responsible for ochratoxin-A biodegradation by *Cupriavidus basilensis* ŐR16 and valuation of *Cupriavidus* genus for mycotoxins biodegradation potential

Thesis of Doctoral Dissertation (PhD)

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I. DECLARATION

I declare that this thesis is a record of original work and contains no material that has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

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Abbreviations

2AA	2-AminoAnthracene
4NQO	4-Nitro-Quinoline-Oxide
AFB1	Aflatoxin B1
*Af*OTase	N-acyl-L-amino Acid Amidohydrolase
BEN	Balkan Endemic Nephropathy
BLYES	Bioluminescent Yeast Estrogen Screen
BLYR	Bioluminescent Yeast
CC	Climate change
CDC	Centres for Disease Control and Prevention
CHCA	α-Cyano-4-hydroxycinnamic acid
CIN	chronic interstitial nephropathy
CPAs	Carboxypeptidases
Ct	Threshold cycle
DDR	DNA Damage Response
DHB	2,5-dihydroxybenzoic acid
DMSO	dimethyl sulfoxide
DON	Deoxynivalenol
Ef	Amplification efficiency
EB	Equilibration buffer
EB	Elution buffer
ELISA	Enzyme-linked immunosorbent assay
hER-α	Estrogen Receptor alpha
ERE	Estrogen Response Element
GE	Gene expression
GOI	Gene of interest
HPLC	High-performance liquid chromatography
IF	Induction factor
IPCC	Intergovernmental Panel on Climate Change
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry
MCS	Multiple cloning site

MEKK4	Mitogen-activated Protein Kinase Kinase Kinase
MMS	Methyl methanesulfonate
m.b	Minimal buffer
NTS	None treated samples
OTA	Ochratoxin-A
OTA-dG	Ochratoxin-A-glutathione conjugate
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase chain reaction
PMFS	Phenylmethylsufonyl fluoride
PVPP	Polyvinyl polypyrrolidone
RIN	RNA integrity number
ROS	Reactive oxygen stress
SA	Sinapinic acid (3,5-Dimethoxy-4-hydroxycinnamic- acid)
SOSIP	SOS-induction potential
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
T-2	T-2 toxin
TCH	Toxin codex of Hungary
TS	Treated samples
Tm	Melting temperature
WB	Wash buffer
YMM	Yeast Minimal Media
ZON	Zearalenone
ZHDCP	Zearalenone hydrolase-carboxypeptidase

II. INTRODUCTION AND OBJECTIVES

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).

The expanding rate of crops diseases is one of the important emerging issues in food safety due to connection to the occurrence of mycotoxins (Moretti et al., 2018). Because mycotoxins are the most widespread food-related health risk and danger in field crops. Furthermore, this infection can be occurred indirectly through the intake of animal source foodstuffs obtained from animals fed with the contaminated fodder (Capriotti et al., 2012).

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 nowadays 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 objectives 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.

1 LITERATURE REVIEW

1.1 Introduction of ochratoxin-A (OTA)

In 1965 ochratoxin-A (OTA) was reported in a research paper after the isolation of a novel toxic metabolite from *Aspergillus ochraceus* (Van Der Merwe et al., 1965). Ochratoxins are the metabolites/by-products of the Aspergillus and Penicillium spp., which are threatening the animal and human health through the contamination of feed and food (Ringot et al., 2006). The chemical structure of OTA can be seen in Figure 1. It is a white, unscented, crystalline solid agent with melting point between 168–173 °C. It is a heat-stable compound, with poor aqueous solubility. It is the most toxic member in the group of ochratoxins.

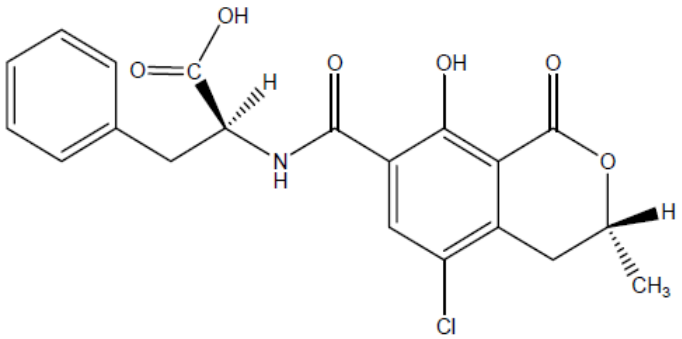


Figure 1: The chemical structure of OTA adapted from (el Khoury and Atoui, 2010)

According to the animal studies, OTA has been proved to be teratogenic, nephrotoxic and immuntoxic to different animal-species and its toxicity results tumors in the kidney of rats and mice (Ringot et al., 2006 ; O'Brien and Dietrich, 2005). The occurrence of OTA is proposed to be the main cause for the Balkan endemic nephropathy (BEN). BEN is a chronic kidney disease resulted in the development and emerging urinary tract tumor in approximately 40% of the Balkan region patients, and it is assumed that BEN is responsible for permanent kidne failure (O'Brien and Dietrich, 2005). OTA can be found in all regions, and the availability of OTA can be detected in both animal feed and human food due to the inappropriate storage conditions of the components of food (Kőszegi and Poór, 2016). OTA is considered to be one of the important contaminants which threatens different agricultural products such as peanuts, coffee beans red wine and pork products (Gagliano et al., 2006; Bragulat et al., 2008). In addition, current experiments also proved the presence of OTA in colouring agents of food (Solfrizzo et al., 2015) and herbal drugs (Chen et al., 2015). The thermo-stability and the considerable presence of OTA pose complexity in terms of the OTA-elimination from the foodstuffs (Malir et al., 2016).

The OTA concentration, in general, is ranged from 0.1 to 100 ng/gram of food that is produced from plant such as barley, wheat, beans, rice, sorghum, coconut and peanut cake and some other plant-products and the general amount of OTA in foodstuffs and feed stuffs produced from animals is ranged from 0.1 to 1 ng/gram (Ostry et al., 2015). This mycotoxin has the capability to accumulate in a number of human consumption products such as eggs, meat and milk (Duarte et al., 2012). Low concentrations of OTA was detected in the blood plasma of healthy individuals in more than 20 countries (Scott, 2005). The OTA limit value has been determined and set by the European Commission for various food products for example cereals (3.0 ppb) and also unprocessed cereal grains (5.0 ppb) (Covarelli et al., 2012; Quintela et al., 2013) (see Table 1.).

1.1.1 The effects of OTA on human kidney

OTA induced nephropathy can lead to the degeneration of the epithelial cells in the renal proximal tubules, can cause interstitial fibrosis, degeneration of glomerulus in the renal cortex part and other different changes in both biochemical and hematological variations. It can also lead to irreversible kidney failure with a period of 6 to 10 years. OTA also can lead to DNA damage response (DDR) and induce DNA damage in human kidney, enhancing the formation of collagen in human embryonic kidney cells (HEK293) (Hennemeier et al., 2014). It induces premature senescence in renal proximal tubular cells (HKC) (Yang et al., 2017). OTA is the main cause for the Balkan Endemic Nephropathy (BEN), causing kidney tumors in the Balkan Peninsula region (Fuchs and Peraica, 2005) and chronic interstitial nephropathy (CIN) in Tunisia (Grosso et al., 2003).

1.1.1.1 Carcinogenicity of OTA

OTA is considered to be a carcinogenic in rodents (IARC, 1993). The first carcinogenic research was performed on rat (Purchase and Van der Watt, 1971) and mice (Kanisawa and Suzuki, 1978) through intraperitoneal injection and oral administration. It was proven that only the oral doses of OTA induced the tumors in the rat and mice kidneys. After the assessment, which was performed by the International Agency for Research on Cancer (IARC), it was shown that the mentioned experiments result, regarding the carcinogenicity evaluation, was limited or uncertain (IARC, 1976).

The mechanisms underlying OTA carcinogenicity is not completely understood. The first possible mechanism is linked to the epigenetic nature of OTA due to its harmful effects on target cells which either implicitly lead to the facilitation of growth of neoplasms from cytogenetically transformed cells or target cells that can lead to neoplastic transformation (Schilter et al., 2005). The second mechanism proposes that OTA carcinogenicity can be resulted from genotoxic

mechanisms (Hibi et al., 2013). Single strand breaks and the formation of DNA adduct detected in a number of studies are considered the most important reasons for the OTA carcinogenesis and OTA-derived genotoxicity (Obrecht-Pflumio and Dirheimer, 2000; Pfohl-Leszkowicz et al., 1991).

1.1.1.2 Genotoxic effects of OTA

In the literature, several experiments suggest that OTA has genotoxic effects (Pfohl-Leszkowicz and Manderville, 2012). After the bioactivation of OTA, the formation of the electrophilic products occurs, that can bind to the DNA resulting in mutations in addition to the subsequent formation of the malignant tumors. The genotoxic metabolites (DNA adducts) can be produced by the activation of OTA as shown in the Figure 2 below.

OTA undergoes bioactivation to create electrophilic species that covalently bind to DNA to generate DNA adducts that induce mutagenicity and subsequent carcinogenesis in the renal system. Examination of proven genotoxic pathways for other chlorophenol toxins may provide insight into the bioactivation of OTA can be gleaned from examination of established genotoxic pathways for other chlorophenol toxins, such as pentachlorophenol (PCP). PCP can endure CYP450-catalized oxidative dechlorination, in the structure of quinoidal that has the ability to bind covalently to thiol chemical groups in addition to 2-deoxyguanosine (dG) (Waidyanatha et al., 1996; Tozlovanu et al., 2006).Pathways similar to those for parachlorophenol (PCP) are envisaged, as shown in Figure 2 for the bioactivation of OTA. Thus, OTA undergoes oxidative dechlorination in the presence of CYP450 to generate the electrophilic quinone OTQ which covalently reacts with GSH to generate the GSH conjugate (Dai et al., 2002). OTQ is reduced to the hydroquinone N-{[(3R,S)-5,8-dihydroxy-3-methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl]carbonyl}-L-phenylalanine (OTHQ) in the presence of ascorbate (Gillman et al., 1999).

The hydroquinone metabolite OTHQ has been identified in the kidney of male rats (Manderville, 2008). It is also proposed that peroxidase enzymes cause the electrophilic phenolic radical by the one-electron oxidation of OTA (Calcutt et al., 2001). This
provides a rationale for OTA to deplete GSH in cells. 5 This pathway also suggests that the nonchlorinated analogue OTB should be equally effective at promoting oxidative damage, as detected (Mally et al., 2005). In addition, OTA can generate the reactive aryl radical (Figure 2). Reaction of the aryl radical with an H-donor also provided a rationale to produce the nonchlorinated OTB metabolite. Therefore, the chemistry of OTA hints that it will produce reactive radical species (phenolic and aryl radicals) and an electrophilic quinone OTQ (Pfohl-

Leszkowicz and Manderville, 2012).

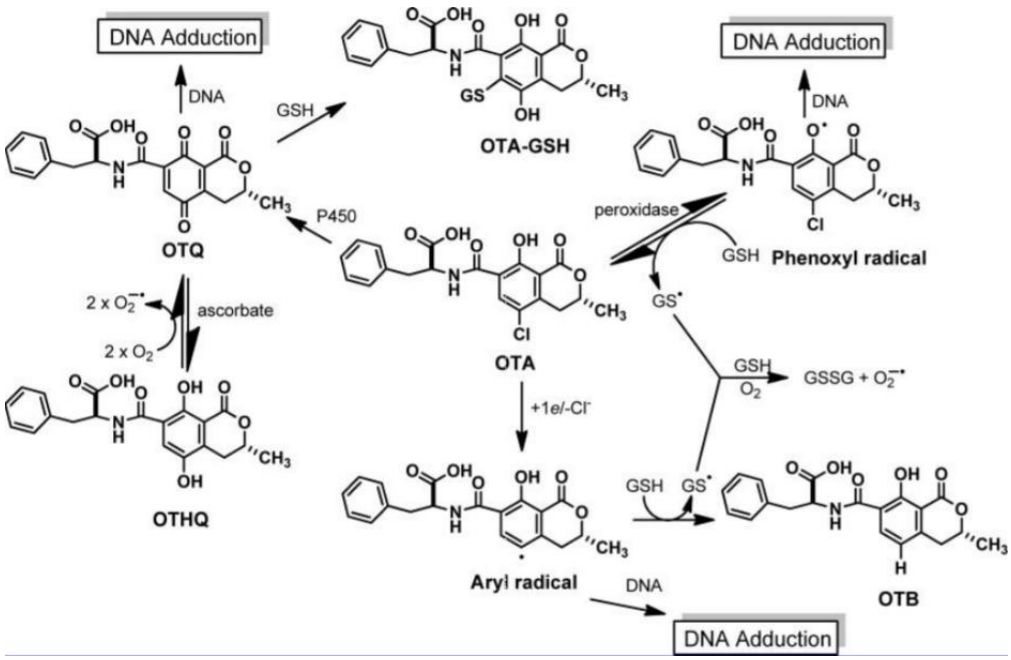


Figure 2: Bioactivation of OTA forming genotoxic metabolites adapted from Pfohl-Leszkowicz and Manderville, 2012 (OTA: Ochratoxin A, OT-GSH: OTA-glutathione conjugate, OTQ: OTA-quinone, OTHQ: OTA-hydroquinone, and OTB: Ochratoxin B)

1.1.1.3 DNA Adduct induced by OTA

In several research experiments, OTA-induced adducts have been detected in humans, rats, pigs and mice (Grosse et al., 1995 ; Miljkovic et al., 2003; Petkova-Bocharova et al., 1998; Pfohl-Leszkowicz et al., 1993). The study of Mandreville in 2005 reported three pathways of the OTA-DNA adduct occurrence. The experiment explained that after following chronic exposure to OTA, C-bonded OTA-dG adduct is formed in the kidney of rat and pig (Manderville, 2005).

1.1.1.4 OTA and apoptosis

The occurrence of the apoptotic and necrotic cell death can be resulted after exposure to OTA (Ringot et al., 2006; Sorrenti et al., 2013). Even if in low doses of OTA, the apoptosis gene marker can be observed; for example, chromatin condensation, DNA fragmentation and enhance the activity of caspase-3 (Gekle et al., 2000). Changes in the gene expression profile could be a proposed reason for apoptosis. Observed alternation in the messenger RNA expression of different genes were marked and that involved in apoptosis and DNA damage response such as gadd45, gadd153 and p53 (Lühe et al., 2003; Qi et al., 2014). To test the effects of OTA on apoptosis, Horvath and co-authors used mice-transfected cell line, which exhibits higher apoptosis rates. OTA has significantly induced apoptosis in this cell line, which is contrary to most tumor

promoters' effects (Horvath et al., 2001). Another report indicate that the possibility of the OTA-Type cell damage (apoptosis) mainly depends on the used dose/level of the mycotoxin (Sauvant et al., 2005).

1.1.2 Biodegradation of OTA

Two suggested microbiological pathways are involved in the OTA degradation as illustrated in Figure 3. The first pathway (a) is the hydrolysis occurred in the amide bond which links the L-ß-phenylalanine molecule to OTα moiety. Due to the non-toxic effects of L-ß-phenylalanine and OTα which is not toxic (Abrunhosa et al., 2010).

The second pathway (b), the lactone ring hydrolysis can be considered a more hypothetical process in the OTA degradation and detoxification (Bruinink et al., 1998; Abrunhosa et al., 2010). The bacteria's which are able to biodegrade OTA can be seen in Table 2.

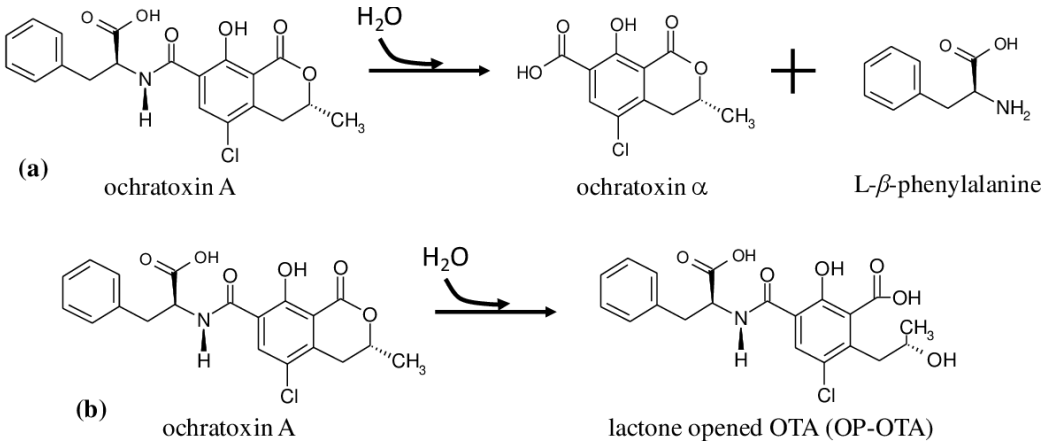


Figure 3: The biodegradation pathways of OTA adapted from (Abrunhosa et al., 2010)

1.1.2.1 Enzymes biodegrading OTA

According to the literature search, there are several enzymes which might be involved in the biodegradation of OTA.

Carboxylpeptidase was discovered at the earliest 1969, extracted from bovine pancrease (Pitout, 1969).

There are carboxylpeptidesa enzymes isolated from different microbes (bacteria and fungi also), which can degrade OTA.

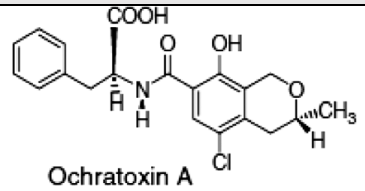
There are two types of carboxylpeptidases among microbes, which have involved in the OTA-biodegradation (Chang et al., 2015; Liuzzi et al., 2017). The first one is carboxypeptidase-A

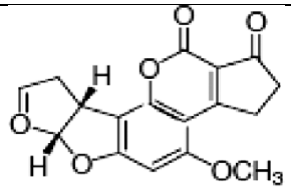
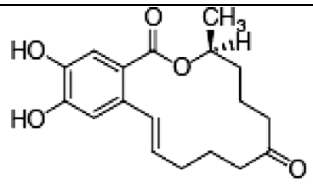
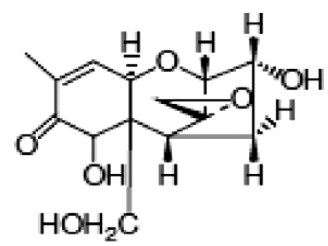
(CPA), where the "A" refers to aromatic compound, carboxypeptidases that have a stronger preference for those amino acids containing aromatic or branched hydrocarbon chains.

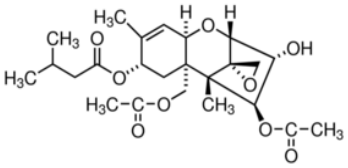
The CPY is the second enzyme, where the "Y" refers to yeast origin. A CPY was isolated from *Saccharomyces cerevisiae*, it could degrade 52% of OTA and converted it to OTA-α after five days of incubation with pH 5.6 at 37 C (Abrunhosa et al., 2010).

There are different enzymes besides the carboxylpeptdidases, which can degrade OTA. Aspergilus niger strains have a few enzymatic tool for OTA degradation: a lipase enzyme in *Aspergillus niger* can hydrolyze OTA through the amide bond (Stander et al., 2000) and Protease-A from *Aspergillus niger* have been reported to degrade around 87.3 % of 1µg OTA respectively with pH 7.5 in 25 hour-incubation period (Abrunhosa et al., 2006). At last, amidase 2 which is encoded by open reading frame (ORF) of *Aspergillus niger* has the hydrolytic activity to degrade 83% of 50 µg/ mL of OTA (Loi et al., 2017).

Table 1: Mycotoxins chemicals structure and limit values permitted by EU Commission (EU Legislation, 2009)

Mycotoxins chemical structures and names	matrix	Limit value in food (ppb) and feed (ppm)
 Ochratoxin A	**Food**	
	-Unprocessed cereals	5
	-All products derived from unprocessed cereals (including cereal products and cereal grains intended for direct human consumption)	3
	- Soluble coffee (instant coffee)	10
	- Dried vine fruit (currants, raisins and sultanas)	10
	- Baby foods and processed cereal based foods for infants and young children.	0.5
	- Dietary foods for special medical purposes intended specifically for infants.	0.5
	Feed	0.25
	- Cereal and cereal products	0.05
	- Complementary and complete feedingstuffs for pigs	0.1
	- Complementary and complete feedingstuffs for poultry	

 Aflatoxin B_1	**Food**	
	-Maize	5
	- Nuts and dried fruit before human consumption	5
	- Oilseed intended for direct human consumption	2
	-Cereals except maize	2
	- Baby foods and processed cereal based foods for infants and young children	0.1
	Feed	
	- All feed materials	0.02
 Zearalenone	**Food**	
	- Unprocessed cereals other than maize	100
	- Unprocessed maize	350
	- Cereals intended for direct human consumption, cereal flour, bran as end product for direct human consumption and germ	75
	- Maize intended for direct human consumption, maize based snacks and maize based breakfast cereals	100
	- Processed cereal based foods and baby foods for infants and young children	20
	- Processed maize based foods and baby foods for infants and young children	20
	Feed	
	- Cereal and cereal products with the exception of maize by products	2
	- Maize by products	3
	- Complementary and complete feedingstuffs for piglets and gilts (young sows)	0.1
	- Complementary and complete feedingstuffs for calves, dairy cattle, sheep	0.5
 Deoxynivalenol	**Food**	
	- Unprocessed cereals (excluding durum wheat, oats and maize)	1250
	- Unprocessed durum wheat and oats	1750
	- Unprocessed maize	1750
	- Cereals intended for direct human	750

	consumption - Processed cereal based baby and infant food **Feed** - Cereals and cereal products with the exception of maize by-products - Maize by-products - Complementary and complete feeding stuffs - Complementary and complete feeding stuffs for calves (<4 months), lambs and kids	 200 8 12 5 2
T-2 toxin. 	**Food** - Unprocessed cereals and cereal products **Feed**	 2 -

1.2 Aflatoxin B1 and its health effects

Aflatoxin B1 (AFB1) is mainly produced by *Aspergillus flavus, A. nomius* and *A. parasiticus*. Aflatoxins pollute a number of crops mainly maize, nuts, spices and oil seeds (Diener et al., 1987). Furthermore, AFB1 can produce mutagenic and carcinogenic effects (Wong and Hsieh, 1976) and it is considered as class 1 in the toxicity level and carcinogenicity to humans (IARC, 2002). In addition, it targets liver and causes cancer as well as cytotoxicity and hepatoxicity (Wogan and Newberne, 1971). The compound AFB1-8,9-epoxide can be formed due to the transformation of cytochrome enzymes (Miller et al., 1974). As result, the formation of DNA adducts can occur due to the combination of ABF1-8,9-epoxide to DNA (Eaton and Ramsdell, 1994).

To be more precise, activation of AFB1 occurs in the liver and is oxidized by monooxygenases to AFB1-8,9-epoxide, which has two isomers, the exo and endo forms. The formation of the exo isomer is carried out by the cytochrome oxidase enzyme CYP3A4. AFB1-8,9-exo-epoxide molecule binding with high affinity to the guanine base of the nucleic acids by covalent bonding such as AFB1-N7-guanine results in two phases. The first phase is that the binding can cause guanine thymine exchange, mutation, DNA, and RNA damage. The second phase of metabolic processes in the body plays a major role in preventing the binding of AFB1-8,9-exo-epoxide to DNA. The conjugation link with the epoxide is formed by glutathione S-transferases. As a result of the relationship, an AFB1-GSH conjugate is formed, which is eliminated by the body through

the excretory processes via the bile and partly through the urine (Wang and Groopman, 1999; Bedard and Massey, 2006)

1.3 Zearalenone (ZON) and its health effects

ZON is a resorcyl lactone, which is oestrogenic and produced by different *Fusarium* species. The most common ZON-producing species are: *F. avenacum, F. equiseti, F. graminearum, F. culmorum, F. lateritium, F. crookwellense, F. semitectum* (Betina, 1989; Bennett et al., 2003). Exposure to ZON can result in oestrogenic consequences that can be presented in precocious puberty in girls (Massart et al., 2008). Add to this, ZON might be a possible cause for toxicity through the production of reactive oxygen species (El Golli Bennour et al., 2009). The main crop that is highly infected by ZON is maize and frequently infectious to other cereals such as wheat and soybean (Commission, 2004). Besides, ZON is a cause for dysfunctions in the reproductive system because due to its binding ability of the oestrogen receptors (α and β) in the mammalian cells (Bennett et al., 2003). Several studies have proven its immunotoxic (Zinedine et al., 2007) genotoxic and cytotoxic effects (Lioi et al., 2004). Due to the structural similarity of ZON to the female sex hormone oestrogen, it can causes adverse effects associated with hyperestrogenism and reproductive disorders in breeding animals and humans (Streit et al., 2012; da Rocha et al., 2014).

1.4 Trichotecen mycotoxins

1.4.1 T-2 toxin and its health effects

T-2 is one of the hazardous mycotoxins which is a naturally occurring mold by-product of several *Fusarium* spp.; *F. langsethiae, F. poae, and F. sporotrichioides* (Krska et al., 2014; Karacaoğlu and Selmanoğlu, 2017). Barley corn, wheat , oat and rye are the main cereals, that can be contaminated by T-2 toxin. T-2 is unaffected by ultraviolet light or heat and it is not possible to control it in the process of food production (Sokolović et al., 2008). T-2 proved to weaken cellular immune responses and inhibit protein and DNA synthesis in animals (Streit et al., 2012). Several health effects were proven in *vitro.* For example, in mouse primary Leydig cells, such as decreasing the biosynthesis of testosterone, DNA damage, oxidative stress and cytotoxicity induction, generative abnormalities for example reduction rate in testosterone levels, semen quality, and sperm count in mice (Xiang Feng Kong et al., 2009; Yang et al., 2014; Yang et al., 2016). In addition, T-2 reduces feed consumption and weight gain, also causes, hemorrhaging, bloody diarrhea, low egg and milk production, abortion, oral lesions and death in few cases (Groopman et al., 2013; Marin et al., 2013 ;Streit et al., 2012; Kovalsky et al., 2016).

1.4.2 Deoxynivalenol (DON) and its health effects

DON is a mycotoxin which is produced by *Fusarium* species mainly *F. graminearum* (Bennett and Klich, 2003) . DON can be found on grains and when agricultural animals ingest high doses of this mycotoxin, it can lead to vomiting, diarrhea, and nausea. In addition, when farm animals and pigs are exposed to low doses of DON, it results in food refusal and weight loss (Rotter et al., 1996). Even though less toxicity can be resulted from DON among trichothecenes, it is the most widespread and frequently detected in corn, wheat, barley, safflower seeds, mixed feeds and rye (Sobrova et al., 2010). The presence of DON is also proven in fermented beers in Netherlands, the concentration was ranged from 26 to 41mg/L (0.088 to 0.14μM) and was above 200 ng/ml (0.675 μM) in the beers of East Germany (Schothorst and Jekel, 2003). Moreover, DON value was ranged from 4.0 to 56.7 ng/mL (0.013 and 0.191 μM) in beers samples from European chain stores (Vrabcheva et al., 2004).

1.5 Methods for mycotoxin reductions

The mycotoxin reduction methods in food and feed can be classified into three physical, chemical, and biological.

1.5.1 Physical methods

Several physical methods aim to reduce the mycotoxins level in food and feed. For example, sorting and separation, irradiation, immersing and washing, filtering and adsorption (Luo et al., 2018). Sorting and separation of corn can commonly decrease aflatoxin contamination (Broggi et al., 2002). Due to the grains density properties such as corn , immersing and washing of this grain in water and removal of the floating parts can decrease up to 80 % of AFB1 in the contaminated corn (Fandohan et al., 2005; Bethke et al., 2014). The cleaning and scouring measures can considerably decrease the contamination of grains by OTA also (Schaarschmidt and Fauhl-Hassek, 2018). Another physical method is radiation. It is an effective approach for the fungal growth inhibition and AFB1, T-2 and DON decontamination that is applied in the thin layer of grains (Peraica et al., 2002). For example, radiation procedure can reduce the production of AFB1 in the affected soybeans and groundnuts. In peanut meal, ABF1 can be completely degraded when the grain treated with dose of 10 kGy of γ-irradiation and decreased noticeably in *Mucuna pruriens* seeds (Bhat et al., 2007). OTA was found to be degraded through UV radiation in poultry feed.

OTA concentration was significantly reduced from 500 mg/kg to the accepted limit 100 mg/kg after one-hour of UV treatment, whereas utilizing from sunlight radiation requires around 8 h

(Ameer Sumbal et al., 2016). AFB1 were almost detoxified and removed after treating almonds with medium and long wavelength UVA and UVB for 60 s after 2000 lbs /h treatment (Newman, 2009).

During the last few decades, filtering and adsorption methods had been used to reduce mycotoxins risk. For instance, the general adsorbent the activated charcoal, has a large surface area and excellent adsorption ability in aqueous environments. Several studies have explained the impact of activated charcoal on mycotoxins adsorption. It has been proven useful to reduce AFB1, ZON and DON due to its porous structure (Avantaggiato et al., 2003; Avantaggiato et al., 2004). Bentonite clay, is another agent which has the potential to bind and eliminate AFB1 from aqueous environments (Magnoli et al., 2011).

1.5.2 Chemical methods

Several chemicals are appropriate for the controlling of mycotoxins. For example bases, oxidizing agents and organic acids. Treating grains with ammonization is an effective strategy for both the reduction of mycotoxins' (AFB1 and OTA) concentrations below the limit of detection and for the inhibition of fungal growth. Nevertheless, this chemical method is not acceptable in the European Community (EC) in terms of the human foods (Peraica et al., 2002). Six years ago, a mixture of calcium hydroxide and glycerol was proved to be a beneficial and effective chemical compound for mycotoxins detoxification (Venter, 2014). OTA was reduced in coco shells after the chemical treatment by 2% sodium bicarbonate and potassium carbonate (Amézqueta et al., 2008).

Another chemical method is decontamination of mycotoxins by oxidizing agents such as hydrogen peroxide and ozone. Mycotoxins detoxification via ozone has been approved for usage in food processing. ZON and AFB1 have been proved to be efficiently degraded by ozone (Agriopoulou et al., 2016). In 2017, an experiment was conducted and has shown that there was a positive influence on the reduction of DON and AFB1, as well as to the total fungal count by both ozone and the exposure time (Trombete et al., 2017).

In addition to oxidizing agents, organic acids such as formic and propionic acid have been used to degrade mycotoxins. For instance, The egg albumin treatment was reported to degrade only 16 % of OTA (Quintela et al., 2012). In wine making, up to 39- 40% of OTA contamination was decreased after using a complex consists of PVPP (polyvinyl polypyrrolidone), plant protein and amorphous silica (Quintela et al., 2012). Moreover, by the biodegradable polymers also could reduce OTA in wine without affecting quality parameters (Bornet and Teissedre, 2008). Chemical -mycotoxins decontamination methods have been accepted in the industry but considering public

concerns about human food and animals feed, additional novel decontamination solutions have to be developed in order to protect the agricultural products and human health.

1.5.3 Biological methods (using microorganisms)

Mycotoxins detoxification by microorganisms is the most promising approach due to the microbe's degradation potential. There are microbes which are able to biodegrade the most dangerous five mycotoxins. Although just a few products can be reached on the market (FumEnzym MycoFix), cause of difficulties of the optimisation of the useful product. In these products usually a living microorganism or an enzyme is playing the major role of detoxification of mycotoxins. Detoxification as a status is also important because biodegradation is not equal to detoxification. Just a few microbial biodegradations are evaluated by biotest and revealed as detoxification without any harmful by-products. Table 2 is showing bacteria, which are able to biodegrade AFB1, OTA, ZON, T2 and DON from the last decade of literature.

Several *Lactobacillus* species have been shown to have moderate OTA degradation, although the metabolites and their potential toxic effects have not been investigated. In other different papers, better results were shown in several *Lactobacillus* species. In Fuchs study, almost all OTA was degraded (97%) after 4 hours incubation with *L. acidophilus* (Fuchs et al., 2008). In addition, *L. rhamnosus* CECT 278T *L. plantarum* CECT 749 degraded OTA with the rate on 97 % and 95 % respectively and biodegradation products were OTA-α and phenylalanine (Phe) (Luz et al., 2018). An *Acinetobacter calcoaceticus* strain is also capable of OTA degradation, but no degradation by-products have been investigated in this case (Piotrowska and Zakowsk, 2005). Stander and colleagues identified an OTA cleavage enzyme in an *Aspergillus niger* strain, which showed OTA-α and phenylalanine as formed product (Stander et al., 2000). Another study proved that complete OTA-degradation was occurred by *Bacillus amyloliquefaciens* ASAG1 after 1-day of incubation (Chang et al., 2015). Moreover, 100 % OTA biodegradation rate was reported by *Cupriavidus basilensis* ŐR16 (Ferenczi et al., 2014), this strain is investigated in this dissertation.

In terms of the AFB1 biodegradation, several microorganisms were reported in the literature that the potential to degrade AFB1. The degradation of AFB1 by *Flavobacterium* (Ciegler et al., 1966), *Corynebacterium* (Mann and Rehm 1976), *Pseudomonas* (Samuel et al., 2014), *Rhodococcus spp* (Teniola et al., 2005; Krifaton et al., 2013; Cserháti et al., 2013) but the toxic effects of the end-products have been tested only by limited research articles (Alberts et al., 2006; Cserháti et al., 2013; Harkai et al., 2016; Teniola et al., 2005; Krifaton et al., 2013).

Several bacteria reported in the literature capable of biodegrading ZON. The study of Cho et al., 2010 revealed that 99 % of ZON was degraded by *Bacillus subtilis* during 24 hours. Another experiment showed that the strain *Bacillus licheniformis* CK1 could degrade 95% of ZON in 36 hours (Liu, 2011). Furthermore, the strains *R. pyridinivorans* (K404 and K408) could also biodegrade 70% of ZON in 72 hours (Cserháti et al., 2013a). Other studies also showed remarkable ZON-biodegradation rate reviewed in Table 2. Nevertheless, it is important to mention that ZON-α is more toxic that ZON hence the presence of biodegradation as end-product is not valuable.

For the T-2 toxin, in 1987 degradation was investigated a *Butyrvibrio fibrisolvent* isolated from rumen, showed a 42% decrease in initial T-2 concentration (10 µg/ml) after 1 hour incubation with an esterase enzyme produced by *Butyrvibrio fibrisolvent* (Westlake et al., 1987). Further bacterias able to degrade T-2 can be found in Table 2.

Currently, there are very few microbes known for being able to degrade DON. An *Eubacterium* sp. BBSH 797 strain is capable of de-epoxidation of DON (Binder et al., 1997). In addition, only one bacterium is capable of completely disintegrating DON, which was described by Ikunaga and co-workers in 2011 and is a member of the *Nocardia* genus (Ikunaga et al., 2011). Wang and co-authors proved that *Devosia insulae* A16 showed 88% biodegradation of 20 mg/l of DON during 48 hours (Wang et al., 2019), *Eggerthella sp. DII-9* resulted in 100% degradation in 24 hours (Gao et al., 2018), PGC-3 consortium from *Peptococcaceae desulfitobacterium* also has the potential to decompose DON effectively (He et al., 2016).

Table 2: Microbes that can biodegrade OTA, AFB1, ZON, T-2 and DON

Mycotoxins	Biodegradation microbes	Biodegradation period	Biodegradation efficiency %	Studies
OTA	*Bacillus licheniformis* *Bacillus* spp.	7 day	92	(Petchkongkaew et al., 2008)
	Brevibacterium linens *Brevibacterium iodinum* *Brevibacterium epidermidis*	10 day	100	(Rodriguez et al., 2011)
	Acinetobacter calcoaceticu	6 day	91	(De Bellis et al., 2015)
	Bacillus amyloliquefaciens ASAG1	10 h 24 h	98 100	(Chang et al., 2015)
	Cupriavidus basilensis ŐR16	5 day	100	(Ferenczi et al., 2014)
	Pediococcus parvulus	5 h 19 h	50 90	(Abrunhosa et al., 2014)
	Lactobacillus acidophilus	4 h	97	(Fuchs et al., 2008)

	Acinetobacter sp. neg1, ITEM 17016	144 h	70	(Liuzzi et al., 2017)
	Lb. rhamnosus CECT 278T *Lb. plantarum* CECT 749	24 h 24 h	97 95	(Luz et al., 2018)
	Alcaligenes faecalis	48 h	100	(Zhang et al., 2017)
AFB1	*Lactobacillus paracasei* LOCK0920 *Lactobacillus brevis* LOCK0944 *Lactobacillus plantarum* LOCK0945	24 h	60	(Śliżewska and Smulikowska, 2011)
	Bacillus pumilus	10 days	88	(Sangi et al., 2018)
	Bacillus subtilis ANSB060	24 h	81	(Gao et al., 2011)
	Lactobacillus kefiri *Acetobacter syzygii*	24 h	82-100	(Taheur et al., 2017)
	Rhodococcus type strains: *R. kyotonensis JCM 23211T* *R. percolatus JCM 10087T* *R. yunnanensis JCM 13366T* *R. imtechensis JCM 13270T* *R. erythropolis JCM 3201T* *R. tukisamuensis JCM 11308T* *R. rhodnii JCM 3203T* *R. aerolatus JCM 19485T* *R. enclensis DSM 45688T* *R. lactis DSM 45625T* *R. trifolii DSM 45580T* *R. qingshengii DSM 45222T* *R. artemisiae DSM 45380T* *R. baikonurensis DSM 44587T* *R. globerulus JCM 7472T* *R. kroppenstedtii JCM 13011T* *R. pyridinivorans JCM 10940T* *R. corynebacterioides JCM 3376T*	3 day	92-100	(Risa et al., 2018)
	Pseudomonas putida	24 h	90	(Samuel et al., 2014)
	Streptomyces lividans TK 24	24 h	90	(Eshelli et al., 2015)
	Rhodococcus erythropolis strains (AK35, AK40, AK42, GD1, GD2A, GD2B, BRB 1AB, BRB 1BB, ŐR9, ŐR13, DSM 743, DSM 1069, DSM 43060, NCIMB9784, IFO12538, NI1)	72 h	100	(Cserháti et al., 2013)
	Rhodococcus pyridinivorans K408	12 day	63	(Prettl et al., 2017)
	Bacillus velezensis DY3108	96 h	91	(Shu et al., 2018)

	Geobacillus Tepidimicrobium	168	98	(Wang et al., 2018b)
	Rhodococcus rhodochrous NI2	72 h	97	(Krifaton et al., 2011)
	Lactobacillus sp strains	24 h	71	(Chlebicz and Śliżewska, 2019)
ZON	*B. subtilis*	24 h	99	(Cho et al., 2010)
	B. licheniformis CK1	36 h	95	(Liu, 2011)
	Lactobacillus mucosae lm420	48 h	66	(Long et al.,2012)
	R. pyridinivorans strains (K404 and K408)	72 h	70	(Cserháti et al., 2013)
	B. pumilus ES-21	24 h	95	(Wang et al., 2017)
	Pseudomonas otitidis TH-N1	72 h	79	(Tan et al., 2015)
	Lysinibacillus sp ZJ-2016-1	48 h	100	(Wang et al., 2018)
	Geobacillus Tepidimicrobium	168 h	88	(Wang et al., 2018)
	R. percolatus JCM 10087^T	72 h	70	(Risa et al., 2018)
T-2	*Rhodococcus erythropolis*	72 h	90	(Cserháti et al., 2013)
	Eggerthella sp. DII-9	96 h	100	(Gao et al., 2018)
	Lactobacillus sp strains	24 h	69	(Chlebicz and Śliżewska, 2019)
DON	*Nocardioides* sp. strain WSN05-2	10 day	100	(Ikunaga et al., 2011)
	Devosia insulae A16	48 h	88	(Wang et al., 2019)
	Eggerthella sp. DII-9	24 h	100	(Gao et al., 2018)
	microbial culture C133 from gut of catfish	96 h	100	(Guan et al., 2009)
	PGC-3 consortium from *Peptococcaceae Desulfitobacterium*	168 h	100	(He et al., 2016)
	Lactobacillus sp *strains*	24 h	39	(Chlebicz and Śliżewska, 2019)

1.6 *Cupriavidus* genus

The genus *Cupriavidus* was identified in 2004 (Coenye et al., 2003). Members of this genus are Gram negative, chemoorganotrophic, and facultative chemolithotrophic bacteria that can be found in several diverse habitats such as soil, root nodules and aquatic environment. The genus *Cupriavidus* belongs to the family *Burkholderiaceae* and the class *β-proteobacteria*. Remarkable heavy metal tolerance of environmental isolates has been confirmed (Goris et al., 2001; Janssen et al., 2010). Environmental isolates are generally characterized by significant heavy metal tolerance (Goris et al., 2001; Janssen et al., 2010) and some species have important xenobiotic breakdown potential. The genus consists of nineteen type strains. Only 16 have been investigated for their biodegradation and detoxification potential on the main five mentioned mycotoxins in this dissertation. The *Cupriavidus nantongensis* KCTC 42909^T could not been ordered from the South Korean collection, and two new strains *Cupriavidus lacunae* JCM 32674^T (Feng et al., 2019) and *Cupriavidus malaysiensis* DSM 19416^T (Ramachandran et al., 2018) were isolated during the biodegradation experiment of the available 16 type strain of the *Cupriavidus* genus. Future study is required to investigate the mycotoxins biodegradation and biodetoxifcation potential by these three type strains. Therefore, till recent time, the total number of the type strains will be 19 in this genus.

According to the literature in the case of 7 strains different xenobiotic biodegradation was observed (Table 3). For example chlorinated aromatic chemicals; halo benzoate and nitrophenols were degraded by *Cupraividus necator* CCUG 52238^T (Makkar and Casida, 1987) and some xenobiotic genes and enzymes such as benzoate1,2-dioxygenase and chlorocatechol-degradative for this strain were reported (Ogawa and Miyashita, 1999).

Cupriavidus basilensis RK1 DSM 11853^T strain was originally isolated as a 2,6-dichlorophenol degrading strain (Steinle et al., 1998). Other isolates of the species are also capable for degradation of various xenobiotics such as furfural, 5-hydroxymethyl furfural (Koopman et al., 2010) bisphenol-A (Fischer et al., 2010), chlorophenols (Zilouei et al., 2006) and atrazine (Stamper et al., 2003).

Up to date, eleven *Cupriavidus* genome projects are known according to NCBI and EzbioCloud databases for the following strains:

Cupriavidus necator CCUG52238^T, *Cupriavidus metallidurans* CCUG 13724^T, *Cupriavidus pinatubonensis* DSM 19553^T, *Cupriavidus alkaliphilus* BCCM 26294^T, *Cupriavidus basilensis* RK1 DSM 11853^T, *Cupriavidus oxalaticus* JCM 11285^T , *Cupriavidus pauculus* JCM 11286^T , *Cupriavidus taiwanensis* CCUG 44338^T, *Cupriavidus campinensis CCUG* 44526^T, *Cupriavidus nantongensis* KCTC 42909^T *and Cupriavidus plantarum* BCCM/LMG 26296^T. The genome size of the genus varies from 6.5 to 8.5 Mbp (Pohlmann et al., 2006). Genomic sequences suggest that the species has significant catabolic potential, as several pathways responsible for aromatic ring cleavage have been identified: catechol and, catechol meta-position ring cleavage pathway, gentisate and the benzene-CoA pathway (Trefault et al., 2004). Figure 4 shows the phylogenetic tree of the *Cupriavidus* genus type strains.

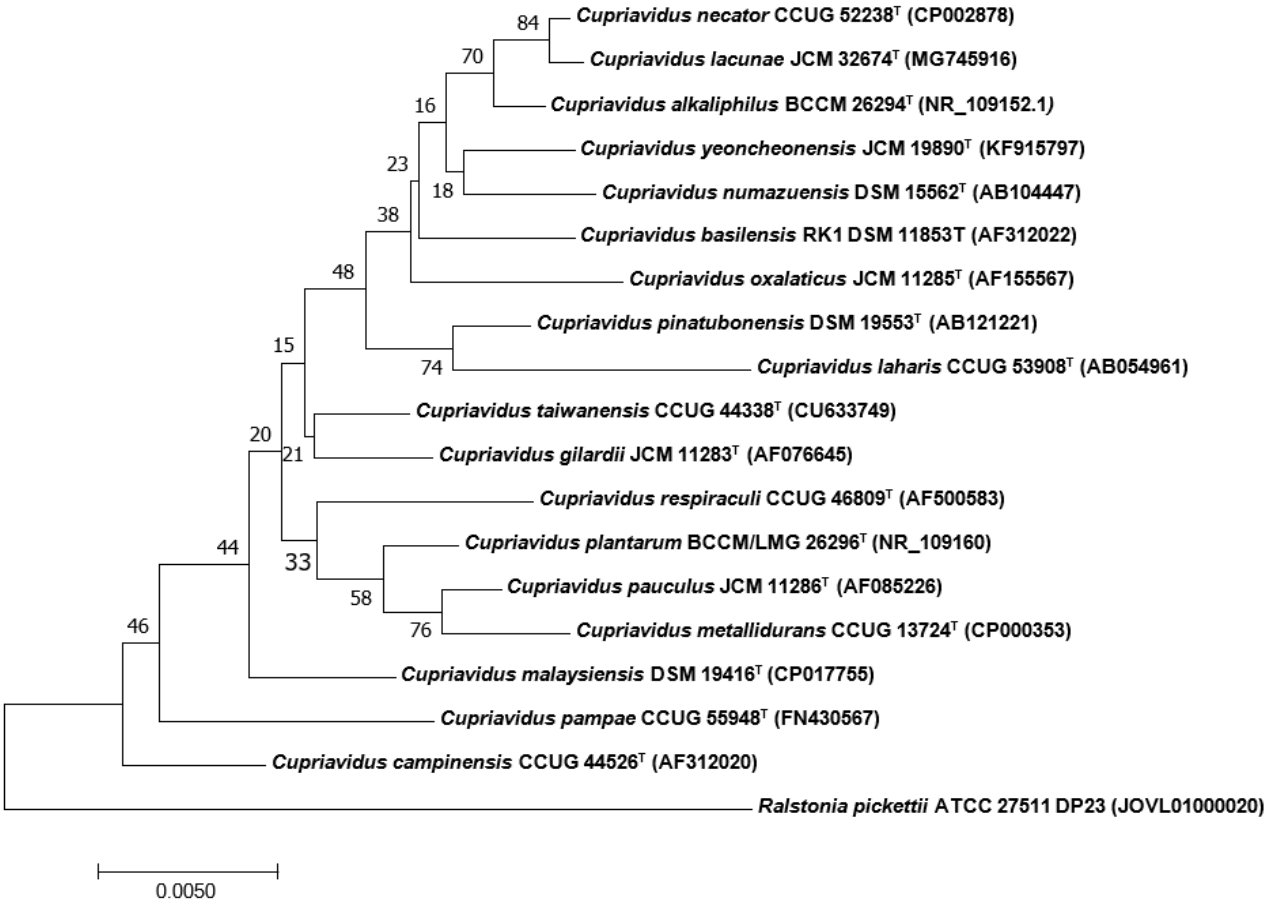


Figure 4: Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relations of 19 type strains of Cupriavidus genus with AFB1, OTA, ZON and T-2 detoxification ability. Bootstrap values are presented as percentages of 1000 replicates. The tree analysis was conducted in MEGA7 software. Ralstonia pickettii was used in MEGA7 as root bacteria for Cupriavidus genus. The scale bar equals 0.005 changes per nucleotide positio

Table 3: Xenobiotic biodegradation ability, biodegraded chemicals and genes identified, and genome availability of the *Cupriavidus* genus strains

Type strain / references	Isolation matrix	Biodegraded chemicals	Genes and enzymes identified up to the present time	Genome project availability
Cupriavidus necator N-1 CCUG 52238 (Makkar and Casida, 1987) (Davis et al., 2019)	Contaminate soil Japan	Chlorinated aromatic chemicals; halo benzoate and nitrophenols	benzoate1,2-dioxygenase gene (benA) (Morimoto et al., 2005), chlorocatechol-degradative genes (Ogawa and Miyashita, 1999)	Yes
Cupriavidus respiraculi CCUG 46809 (Coenye et al., 2019)	Human respiratory tract, cystic fibrosis, USA	n.a	n.a	No
Cupriavidus laharis CCUG 53908 (Sato et al., 2006)	Volcanic mudflow. The Philippines, Mt Pinatubo	n.a	n.a	No
Cupriavidus metallidurans CCUG 13724 (Vandamme and Coenye, 2004)	decantation tank of zink factory, Belgium, 'Métallurgie de Prayon'	n.a	n.a	Yes
Cupriavidus campinensis CCUG 44526 (Goris et al., 2001)	Zinc contaminated area, Belgium, Lommel		n.a	Yes
Cupriavidus plantarum BCCM/LMG26296 ,(Estrada-de Los Santos et al., 2014)	agave Plant, rhizosphere		n.a	Yes
Cupriavidus taiwanensis CCUG 44338 (Chen et al., 2001)	*Mimosa pudica*, root nodule, Ping-Tung Taiwan, Province of China		n.a	Yes
Cupriavidus pampae CCUG 55948 (Cuadrado et al., 2010)	Humid Pampa Region,Argentina,	2,4-D , herbicide-degrading		
Cupriavidus alkaliphilus BCCM/26294 (Estrada-de los Santos et al., 2012)	from alkaline soil			Yes

Cupriavidus numazuensis DSM 15562 (Kageyama et al., 2005; Martinez-Aguilar et al., 2013)	Natural soil, Japan, Shizuoka prefecture	Degrades trichloroethylene, cis-dichloroethylene and toluene	n.a	No
Cupriavidus pinatubonensis DSM 19553 (Sato et al., 2006)	Volcanic ashes The Philippines Volcano Pinatubo	Chlorinated Aromatic Compounds: 2.4 d	n.a	Yes
Cupriavidus basilensis RK1 DSM11853 (Steinle et al., 1998)	laboratory fixed-bed reactor, Switzerland Schweizerhalle	Utilizes 2,4-dichlorophenol (Steinle et al., 1998) , 2,6-dichlorophenol (Steinle et al., 1998), toluene , benzene , chlorobenzene, phenol (Steinle et al., 1998), furfural, 5-hidroximetil furfural (Koopman et al., 2010), bisphenol-A (Fischer et al., 2010), chlorophenol (Ron and Biotechnolo, 2006), atrazine(Stamper et al., 2003)	furfural degrading gene cluster (KOOPMAN et al., 2010)	Yes
Cupriavidus gilardii JCM 11283 (Coenye et al., 1999)	Whirlpool	n.a	n.a	Yes
Cupriavidus oxalaticus JCM 11285 (Sahin et al., 2000)	Alimentary tract of an Indian earthworm	oxalate-decomposing (KHAMBATA and BHAT, 1953)	n.a	Yes
Cupriavidus pauculus JCM 11286 (Vandamme et al., 1999)	Biphenyl-contaminated soil in Kitakyushu, Japan	Biphenyl	n.a	Yes
Cupriavidus yeoncheonensis JCM 19890 (Singh et al., 2015)	Ginseng soil, Yeoncheon Province, Republic of Korea	n.a	n.a	No
Cupriavidus nantongensis KCTC 42909 (Sun et al., 2016)	vicinity of a pesticide manufacturer in Nantong, Jiangsu Province, China	Chlorpyrifos	n.a	yes
Cupriavidus lacunae JCM 32674 (Feng et al., 2019)	Pond-side soil	n.a	n.a	No
Cupriavidus malaysiensis DSM 19416 (Ramachandran et al., 2018)	Kulim Lake, Sg. Pinang river and Sg. Manik paddy field	n.a	Catalase and oxidase	Yes

1.6.1 *Cupriavidus basilensis* ŐR16 strain

The *Cupriavidus basilensis* ŐR16 strain was isolated from a Hungarian pristine soil sample. It was identified by molecular taxonomy. Beside its metabolic properties, ŐR16 strain was characterized as a good petroleum hydrocarbon and mycotoxin degrader (AFB1 and T2 toxin). The nucleotide sequence of *C. basilensis* strain ŐR16 has been deposited in DDBJ/ EMBL/GenBank (NCBI) under accession number AHJE00000000. The whole entire genome of *C. basilensis* ŐR16 consists of 8,546,215 bp, with a GC content of 41.2% and 7,534 putative coding sequences (Cserháti et al., 2012).

The genome sequence of *C. basilensis* ŐR16 reveals an impressive catabolic potential, since several ring cleavage pathways for aromatic compounds 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. Its survival under heavy metal stress conditions is ensured by genes encoding heavy metal transport/detoxification proteins, such as copper-zinc-cadmium-chromate resistance proteins (copCD, a copper chaperone) and heavy metal efflux pumps (copper/heavy metal efflux P-type ATPases and CzcA family heavy metal efflux pumps). Remarkably, besides an OprB glucose porin, a putative membrane-bound PQQ-dependent glucose dehydrogenase gene (ŐR16_10529) was also identified, which catalyses gluconate production from glucose. The presence of this gene is unique in strain ŐR16, as none of the other known *Cupriavidus* genomes encode it or its homologues, and this may indicate unusual glucose metabolism of strain ŐR16 (Cserháti et al., 2012).

1.7 The AlamarBlue assay

This assay was used for pretesting the different treatments on the 786-O human cell line, before proceding with the gene expression real-time PCR experiment. The assay showes that the choosen cells (mammalian cell line) are vital in the case of the different treatments. If the number and structure of the cells are normal than the deeper experiment can be accomplished, if the number is decreasing (cell death), and the cell structure is deformed in this case the further experiment can not been accomplished, cause the treatments will not have any comparable results. The alamarBlue test is used to quantify cellular metabolic activity and in turn determine the concentration of viable cells in a given sample. AlamarBlue test quantitatively measures the proliferation of mammalian cell lines, bacteria and fungi. The term alamarBlue refers to the dye (resazurin) that is in a blue colour in its oxidised form and non-fluorescent. In alamarBlue assay, the growing cells cause a chemical reduction of the alamarBlue dye from non-fluorescent blue to fluorescent red. The continued growth of viable cells maintain a reducing environment (fluorescent, red) and inhibition of growth maintains an oxidized environment (non-fluorescent, blue), which can be detected using a fluorescence or absorbance detector (O'Brien et al., 2000).

The dye incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and change color in response to the chemical reduction due to cell growth. Since, the alamarBlue is very stable and nontoxic to the cells, continuous monitoring of cultures over time is possible. In general, this test has been used in several aspects such as monitoring cellular health (Ansar Ahmed et al., 1994;O'Brien et al., 2000). It is useful for cell viability assays, cytokine bioassays, and in vitro cytotoxicity determinations in addition to the monitoring of cell growth.

1.8 RT- PCR method

This method was used in the cell line experiment to investigate the effect of OTA detoxification py-products of *Cupriavidus basilensis* ŐR16 wild strain on the human kidney cell lines 786-O. RT-PCR is commonly used method to measure the profile of gene expression. It is more sensitive than microarrays in detecting small changes in expression but requires more input RNA and is less adaptable to high-throughput studies (Wang et al., 2006). It's one major limitation that the sequence of the specific target gene of interest must be known in order to design the appropriate primers, hence real-time PCR can only be used for studying known genes. RT-PCR incudes a few steps, the first one called reverse transcription which means the conversion of RNA to complementary DNA (cDNA). The next step uses fluorescent reporters and a PCR reaction to amplify and detect specific genes. There are two commonly used fluorescent reporters SYBR green and TaqMan. SYBR green chemistry was used in the RT-PCR for the cell line experiment

in the presented work. The amount of fluorescence in a sample is detected in 'real-time' and plotted against the cycle number. RT-PCR includes three stages during the reaction; annealing, extension and denaturation.

1.8.1 Target genes investigated by RT-PCR in the cell line experiment

OTA alters the expression of several genes playing a role in cell damage, cellular stress and apoptosis, DNA repair process such as growth arrest and DNA damage-inducible genes *gadd45* and *gadd153, annexin2* and *clusterin* (Lühe et al., 2003).

Gadd45 and *gadd153* functions as stress sensors that regulate the response of mammalian cells to genotoxic and physiological stress and alter tumour formation. It interacts with other proteins involved in stress responses, including PCNA, p21, Cdc2/CyclinB1, MEKK4, and p38 kinase (Fornace et al., 1992). The isolation origin of gadd45 and gadd153 come from the cells treated with UV-radiation (Sarkar et al., 2002). Both are found to be stress response genes induced by toxic chemicals such as OTA. Therefore, in the cell line experiment they serve as indicators to show the genotoxic consequences and the damage of the DNA induced by OTA. Their up regulation was reported in the renal cortex of male mice after OTA-treatment (Lühe et al., 2003).

Clusterin gene is one of the small members of the heat shock proteins and hence classified as a molecular chaperone. It is a Golgi chaperone which enables the folding of concealed proteins in an ATP-independent manner (Fritz et al., 1983). *Clusterin* is highly preserved and commonly dispersed in several organs and tissues. It is involved in several biological processes such as membrane recycling, lipid transport, cell adhesion and programmed cell death (Koltai, 2014; Lin et al., 2014; Sansanwal et al., 2015).

Annexin is playing a role in the cell motility of epithelial cells and considers as autocrine factor which enhances the formation of bone resorption and osteoclast (Takahashi et al., 1994; Inokuchi et al., 2009). It is also involved in the apoptotic processes as well as to the formation of renal tumour (Miyake et al., 2002). One of the studies explained that the high expression profile of *annexin* was resulted from the acute OTA treatment in rat's renal cortex (Lühe et al., 2003). In addition, the high expression of *annexin* was proved in the formation of kidney carcinoma of rats (Tanaka et al., 2000). Likewise, it is involved in the development of various cancers and in the DNA repair because it was reported as cofactor for DNA polymerase alpha subunit (Kumble et al., 1992).

1.9 The transcriptome analysis

In this PhD dissertantion, the transcriptome analysis was done for the first time in the case of *Cupriavidus basilensis* ŐR16 wild strain in the presence and without presence of OTA.

"The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition" (Wang et al., 2009).The transcriptome reflects the genes that are being actively expressed at any given time. The transcriptomic techniques have been particularly useful in identifying the functions of genes (Botchkareva, 2017). Transcriptome Analysis is the most used method to compare specific pairs of samples. The differences may be due to different external environmental conditions such as toxins (Hoeijmakers et al., 2013). Transcriptomics techniques include DNA microarrays and next-generation sequencing technologies called RNA-Seq. RNA-seq determines all the transcribed genes of a cell/population by converting long RNAs into a library of cDNA fragments. The cDNA fragments are then sequenced using high-throughput sequencing technology and aligned to a reference genome or transcriptome which is then used to create an expression profile of the genes (Wang et al., 2009).

There are two general methods of inferring transcriptome sequences. One approach maps sequence reads onto a reference genome, either of the organism itself (whose transcriptome is being studied) or of a closely related species. The other approach, de novo transcriptome assembly, uses software to infer transcripts directly from short sequence reads.

1.10 Cloning and protein expression

According the results of the transcriptome analysis genes responsible for OTA degradation were cloned and the enzymes were expressed.

Gene cloning means the method in which a gene of interest is fused into a self-replicating genetic element called a plasmid, which when introduced into a suitable host (e.g. *E. coli*), self-replicates and produces numerous identical copies of the gene. In molecular cloning, the DNA containing the desired gene or the gene of interest (GOI) is isolated for instance from a bacterium. This DNA is cut into the right size using restriction enzymes (Figure 5). Then the isolated or synthesized DNA inserted into a vector or plasmid such as pET plasmids and ligated together by ligase (Celie et al., 2016). The recombinant plasmid which contains the GOI transferred via the transformation process to amplification host strain (e.g *E. coli* TOP 10 cells). After few hours of growing and in the presence of an antibiotic resistance such as kanamycin, the plasmids will be generated in large numbers. In my cloning experiment, the vector contains the GOI was small and for this reason I have performed the cloning experiment by the heat shock (Chang et al., 2015) to get higher copy

number of the desired gene/s. After the isolation of expression plasmid DNA which contains the GOI, the desired genes are transferred to an expression strain (e.g *E. coli* BL21 DE3) by heating shock.

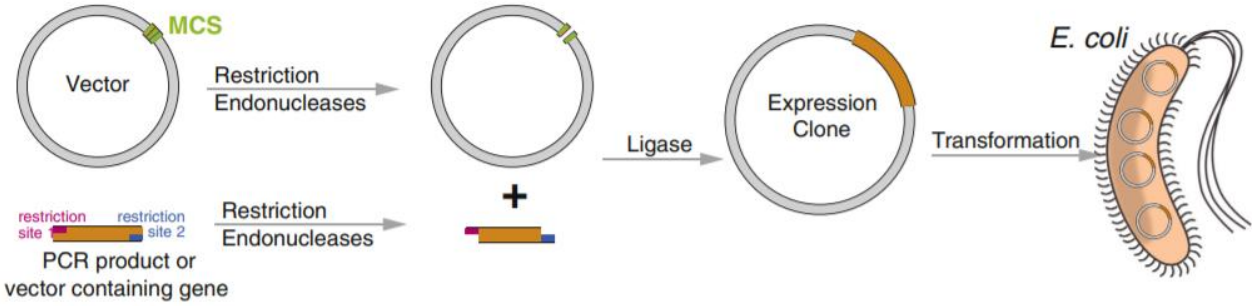


Figure 5: Restriction-based cloning adapted from Celie et al., 2016

1.10.1 MALDI-TOF MS method

The MALDI-TOF method was used for the detection of the expressed enzymes in this dissertation. MALDI TOF was invented in early 1980s (Karas and Hillenkamp, 1988; Tanaka et al., 1988). Mass spectrometry (MS) recognizes and quantifies molecules by measuring the mass to charge ratios (m/z) of molecular ions. It is possible to interpret the mass spectrum to determine the identity of various molecules within a sample. Mass spectrometers analyze the biological molecule which is prone to any ionization (Singhal et al., 2015). MS success is only possible due to several significant discoveries including the electrospray ionization (ESI) discovered by John Fenn and matrix-assisted laser desorption/ionization (MALDI) discovered by Koicihi Tanaka (Tanaka et al., 1988; Fenn et al., 1989). MALDI has the advantage of having less pre-analysis work-up, because the samples are mixed with a chemical matrix followed by ion generation. MALDI Time-of-Flight (TOF) MS incorporates two technologies: the MALDI and TOF mass sources. This tool has revolutionized the identification of microorganisms in clinical microbiology laboratories since its creation, as it is a quick, high throughput, efficient and low-cost system (Singhal et al., 2015). One of the main benefits of using MALDI-TOF MS is time savings, as bacterial protein profile detection can be done in less than an hour as opposed to one or two days (Croxatto et al., 2012).

The concepts of this ionization technique can be divided into two parts. The first part is the MALDI which is the ionization source and the second is TOF which is a mass analyser. In the first initial ionization phase the sample is bombarded by a laser. The sample molecules vaporize into the vacuum while being ionized at the same time. High voltage is then applied to accelerate the charged particles. The second step is the TOF mass spectrometry phase, in the linear mode, particles will impinge upon the linear detector within a few nano seconds after ionization. Higher

mass molecules will arrive later then the lighter ones. Each peak in the spectrum corresponds to a mass of the particle along the time axis, starting with the ionization moment as explained in Figure 6. The method is appropriate for the identification and analysis from different biological matrixes (Clark et al., 2013).

During the process the equipment is taking 60 measurments/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.

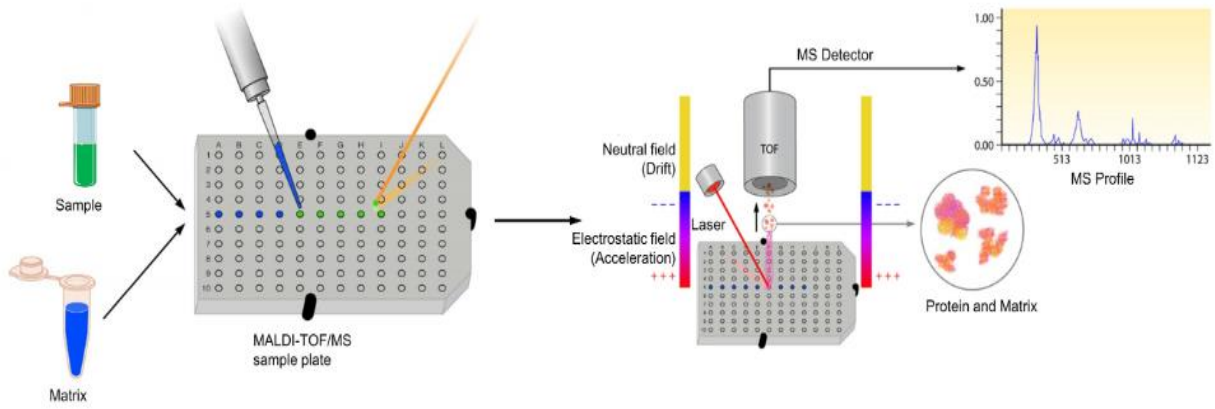


Figure 6: The process of MALDI-TOF MS adapted from Clark et al., 2013

1.11 Biodegradation vs detoxification

Biodegradation can be defined as the process when the toxic molecule is degraded by microorganisms (Alexander, 1994).

When biodegradation is complete, the process is termed "mineralization". However, in most cases the word biodegradation is usually used to explain practically any biologically mediated change in a substrate (Fritsche and Hofrichter, 2000). Microorganism such as bacteria biodegrade mycotoxins either by metabolic process by enzymes. It is based on two processes: growth or cometabolism. During normal growth process, the bacteria or the microorganism is utilizing the material by degradation and using the basic by-products as basic carbon source for growing it is own cell. In cometabolism, the enzyme which is responsible for the target chemicals degradation can also degrade different other chemicals, but in this case those by-products are not used by the bacteria.

The breakdown mechanism of the toxic substances such as mycotoxins is mainly occurred by enzymes. However, biodetoxification means when the toxic molecule is degraded without any harmful effects, which is far away from biodegradation resulting metabolites with also toxic effects such as zearalenole, etc. According to the statements of the European Food Safety Authority

(EFSA 2010) it is important to develop and use in *vivo* new toxicological approaches for investigating biodegradation and detoxification efficiency directly.

In the case of mycotoxins a few ecotest/biotest is available according the negative effect of the mycotoxin. In the case of AFB1 the genotoxic effect can be evaluated by the using of SOS-Chromo test. In the case of ZON, eostrogenity can be evaluated by the BLYES test. In the case of the other important mycotoxins (OTA, T2, DON) are quite difficult to investigate the by-product occurring during a biodegradation process. In our institute zebra fish embryo microinjection test was developed for OTA by-product evaluation receantly (Csenki et al., 2019).

1.11.1 SOS-Chromotest for measuring genotoxicity

In the genetically modified test organism, *E. coli* PQ37, operon fusion of *sfiA* and *lacZ* genes has been carried out. Any genotoxic chemical can trigger the SOS-repair mechanism of the PQ37. As a result of this, when the SOS-repair mechanism starts, *β-galactosidase* is produced at the same time, which is proportional to the strength of genotoxicity (Quillardet et al., 1982). *Alkaline phosphatase* activity, which is indicative for cell viability, is also monitored as control. After adding X-gal (Blue Chromogene) to test *β-galactosidase* activity, or p-nitrophenyl phosphate to test alkaline *phosphatase* activity, blue and yellow colours emerge, respectively, this can be detected photometrically at 620 and 405 nm wavelengths (Quillardet et al., 1982).
The genotoxic effect can be expressed in induction factor (IF), which is calculated according to Eq. (1) (Legault and Blaise, 1994) :
Induction factor (IF) = (C405 ∗ S620)/ (S405 ∗ C620)
where C is the mean of the absorbance value of the control; S is the mean of the absorbance value of the sample measured at 405 and 620 nm wavelength.

1.11.2 Bioluminescent yeast estrogen assay test for measuring oestrogenicity

The test organism is a genetically engineered strain of yeast, *Saccharomyces cerevisiae*. Bioluminescent yeast estrogen assay (BLYES) has the human Estrogen Receptor alpha (*hER-α)* integrated into its chromosomal DNA. This means that it is inside the nucleus. Additionally, it is under a constitutive promoter. When it binds to an estrogenic chemical, *hER-α* turns on a signalling pathway, that activates the Estrogen Response Element (ERE) promoter. This promoter is on a plasmid that turns on light production with lux genes (bacterial *luciferin/luciferase*), and this is measured by a very sensitive detector (Sanseverino et al., 2005).

2 MATERIALS AND METHODS

During this chapter for a better understanding, I am following the objectives:

1) Investigating the effect of the OTA biodegradation by-product of *C. basilensis*-ŐR16 on human kidney cell line by gene expression using qPCR by novel biotest method.
2) Transcriptome analysis for identification the enzymes of the *C. 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

First, I would like to show the materials and methods what I used for gene expression experiment conducted on human kidney cell line to investigate the effect of the OTA by-products produced by the ŐR16 *Cupriavidus basilensis* strain.

2.1 Objective 1: Ecotoxicological effects of OTA by-products in the human kidney cell line 786O

2.1.1 The Experiment Reagents

Normal LB was used for the growing of *C. basilensis* ŐR16 inoculum (100 % LB: 10 g tryptone, 5 g yeast extract, 9 g sodium-chloride, pH 7).

The OTA degradation for the experiment was carried out in a modified LB (20 %: 2 g tryptone, 1 g yeast extract, 1.8 g sodium-chloride) Luria-Bertani (LB) medium.

786-O human kidney cell lines were the test cell (CLS - Cell Line Service GmbH, Germany). Ochratoxin-A (OTA) was the main target chemical (Sigma-Aldrich Co., USA), methyl methanesulfonate was used as genotoxic control (MMS) (Sigma-Aldrich Co., USA). The primers for the gene expression analyzation were ordered from Integrated DNA Technologies, USA)

2.1.2 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 28 ºC 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 ^{o}C for 5 days. At the 5th day (endpoint) of the experiment the entire pellet material was removed (25,000 g at 4 ^{o}C 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.

2.1.3 Analytical methods

Analytical measurements were carried out by a HPLC series 1100 from Agilent® Technologies, USA. Samples were taken at the beginning and on each day (1-5) of the degradation experiment, HPLC measurement were focusing on OTA and its derivative ochratoxin-α (OT-α) concentration. The bacterial pellet was suspended in 1 ml methanol and centrifuged (4 x g for 20 min at 4°C), then the supernatant was analysed. European Standard (EN) and International Organization for Standardization (ISO) (EN ISO 15141-1:2000 standard) protocols were used for the immuno-affinity column cleaning, derivatization, LC separation and fluorescence detection of the compounds. Measurements were carried out in triplicates. The analytical measurement was carried out at the Department of Applied Chemistry SZIU, Buda campus.

2.1.4 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 fullfiling of the available surface by the cells). Treatments for viability assay was added in 0.002 % amount of the total treatment volume 200 µl/well, for 24 hours. This assay was performed in Semmelweis University
Faculty of Medicine, Endocrine Lab, Budapest, Hungary.

2.1.5 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 teatments 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 4) 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.

Table 4: 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)

2.1.6 Target and housekeeping gene selection

Three housekeeping genes (HKGs) *β-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) shown in Table 5.

Table 5: Target and housekeeping genes studied in the cell line 786O experiment.

Gene name	Abbreviation	Role of gene	RT-PCR purpose
Growth arrest and DNA-damage-inducible protein	*gadd45*	DNA damage reporter	Target gene
Growth arrest and DNA-damage-inducible protein	*gadd153*	DNA damage reporter	Target gene
Clusterin	*clu*	Kidney tumor marker,	Target gene
Annexin2	*anxa2*	DNA repair marker	Target gene
Beta actin	*ß-actin*	Cytoskeleton peptide coding	Housekeeping gene
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	Glycolysis controlling	Housekeeping gene
Hypoxanthine phosphoribosyl transferase	*hprt*	Role in purine recycling	Housekeeping gene

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. The size of the amplicon was confirmed by running an agarose gel. A qRT–PCR melt–curve analysis was performed at the end of each PCR run to confirm amplicon homogeneity. The primers sequences of the target and control genes are shown in Table 6.

Table 6: Primer sequences of the gadd45, gadd153, annexin, clusterin, β Actin, gapdh and hprt genes

Gene	Sequence	Accession Number of the target gene	Tm	Product length (bp)
gadd45	F 5'-GAAGACCGAAAGGATGGA-3' R 5'-GCACAACACCACGTTATC-3'	NM_001924.3	51.4 51.5	139
gadd153	F 5'-AACAGAGTGGTCATTCCC-3' R 5'-CTTGAGCCGTTCATTCTC-3'	NM_001195055.1	51.7 50.7	110
annexin2	F 5'-ATGACTCCATGAAGGGCAAG-3' R 5'-GGGACTTGCCGTACTTTCTC-3'	NM_001002857.1	54.9 55.5	120
clusterin	F 5'-GCCCTTCCTTGAGATGAT-3' R 5'-GTCGCCTTCTCGTATGAA-3'	NM_001831.3	51.6 51.7	103
β actin	F 5'-CCAACCGCGAGAAGATGA-3' R 5'-CCAGAGGCGTACAGGGATAG-3'	NM_001101.4	54.9 56.7	189
gapdh	F 5'-GAAGGTGAAGGTCGGAGTC-3' R 5'-GAAGATGGTGATGGGATTTC-3'	NM_001289745.2	54.9 50.8	205
hprt	F 5'-TTGCTGACCTGCTGGATTAC-3' R 5'-TCTCCACCAATTACTTTTATGTCC-3'	KR710622	55 52.8	158

2.1.7 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). Amplification was not detected in the none treated controls. The cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Primers for the comparative Ct experiments were designed by Primer Quest Program. The primers were used in the Real-Time PCR reaction with Fast EvaGreen qPCR Master Mix (Biotium, USA) on ABI StepOne- Plus instrument.

2.1.8 Real-time PCR and gene expression

Gene expression was determined by using StepOne Plus Real-time PCR (Applied Biosystems, Szent Istvan University, Godollo, Hungary). The amplification efficiencies were determined for all sets of genes, and the amplification efficiency was between 99 and 100 percent for all primers. 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 plate included no template control (NTC) to verify inter-plate reliability. Each qRT– PCR reaction (10 μL total volumes in each well) was included: 2.72 μL Nuclase Free Water, 5 μL cDNA, 0.14 μLForward primer, 0.14 μL Reverse primer and qPCR Master Mix 2 μL EvaGreen, Biotium, USA). 2 log fold expression data of the target genes normalized to *β-actin and* HPRT can be found in supplementary material 2.

The qRT_PCR temperature profile in the cell line experiment was as fexplained below :

A- **Holding stage** : 50 °C / 2 minutes and 95 °C / 12 minutes.

B- **Cycling stage (40 cycle)** : 95 °C / 15 seconds and 60 °C / 30 seconds.

C- **Melt curve stage** : 60 °C /1 minutes , + 0.3 °C and 95 °C for 15 seconds.

2.1.9 Statistical analysis

Data are expressed as means ± SD. The data were first subjected to REST software. Data passing this test were analysed by One-way ANOVA followed by the Tukey's multicomparisons test. Statistical analysis was performed using GraphPad PRISM version 7 software (GraphPad Software, USA). $P < 0.05$ was considered significant.

2.2 Objective 2: Transcriptional analysis of *Cupriavidus basilensis* ŐR16 in the presence of OTA

In this chapter the materials and methods are shown which were used for processing the transcriptome analysis of the enzymes of *C. basilensis* ŐR16 playing role in the biodegradation of the OTA.

2.2.1 OTA degradation experiment for getting the RNA to trascriptome analysis

The *Cupriavidus basilensis* ŐR16 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.

Cupriavidus basilensis ŐR16 was grown on LB agar plates and incubated at 28 ^{o}C for 72 h. Single colonies were inoculated into 50 ml liquid LB medium and incubated at 170 rpm at 28 ^{o}C for 72 h. Cultures then centrifuged and cleaned from LB media via minimal buffer.

10 ml of the ŐR16 was added to 45 ml minimal buffer (3.1 g of K_2HPO_4, 1.7 g of NaH_2PO_4 $2H_20$, 4.0 g of $(NH4)_2SO_4$, 0.2 g of $MgCl_2$ $6H_2O$, 20 mg of EDTA, 4 mg of $ZnSO_4$ $7H_20$, 2 mg of CaCl2. $2H_20$, 10 mg of $FeSO_4$ $7H_20$, 0.4 mg of Na2MoO4. $2H_20$, 0.4 mg of $CuSO_4$ $5H_2O$, 0.8 mg of $CoCl_2$ $6H_2O$, 2 mg of MnCI2 $2H_2O$) and 45 ml 2% fructose (200 ml Demineralized Water, 4.0 g D-Fructose), and incubated for 11 hours (till reaching the log phase of ŐR16).

For control *E. coli* TOP10 was used in LB and in minimal buffer incubated in the same circumstance as ŐR16. After 11 hours incubation, OD was measured to reach 0.4-0.8 (to be suitable with the requirement RNA isolation kit). 4 ppm OTA was added to the targeted groups (ŐR16 + OTA). Samples were set in duplicates.

2.2.1 Log phase identification of *Cupriavdus basilensis* ŐR16

1.1.1 Log phase identification of Cupriavdus basilensis ŐR16

Estimating the log phase (Figure 7) 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.

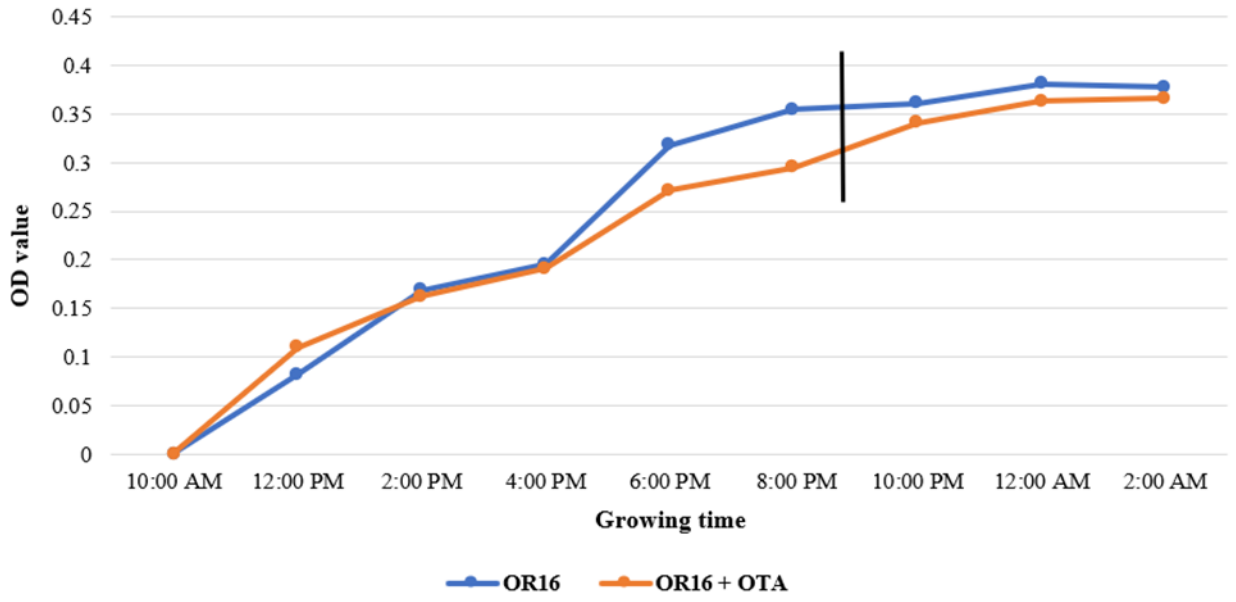


Figure 7: The log phase age of *C. basilensis* ŐR16 in the minimal buffer, the black line means the end of the log phase of ŐR16

2.2.2 RNA extraction, RNA quality test, preparation and sequencing

In order 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 °C for 30 minutes after reaching the log phase (11 h). Samples were set in duplicates in the presence or absence of OTA. 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 (supplementary material 3) by Agilent 2200 Technologies and using TapeStation software (Seqomics Ltd, Hungary).

RNA quality and quantity measurements were performed using RNA ScreenTape and Reagents on TapeStation (all from Agilent Co., USA) and Qubit (Thermo Fisher Scientific Co., USA); only high quality (RIN 7 and 8) total RNA samples were processed.

RNA was purified and fragmented; first strand cDNA synthesis was performed using SuperScript II (Thermo Fisher Scientific Co., USA) followed by second strand cDNA synthesis, end repair, 3'-end adenylation, adapter ligation, and PCR amplification. All the purification steps were performed using AmPureXP Beads (Beckman Coulter Co., USA). Final libraries were quality checked using D1000 ScreenTape and Reagents on TapeStation (Agilent Co., USA). The

concentration of each library was determined using the KAPA Library Quantification Kit for Illumina (KAPA Biosystems Co., USA). Sequencing was performed in Seqomics, Ltd, Hungary on an Illumina NextSeq instrument using the NextSeq 500/550 High Output Kit v2 (300 cycles; Illumina Co., USA) generating ~10 million clusters for each sample

2.2.3 Bioinformatics analysis of RNA-sequencing data

The bioinformatic analysis and RNA-sequencing was carried out by Seqomics Ltd., Szeged. After sequencing, paired-end Illumina reads were quality trimmed in CLC Genomics Workbench Tool (v.11.0, Qiagen Bioinformatics Co., Denmark) applying an error probability threshold of 0.01. No ambiguous nucleotide was allowed in trimmed reads. RNA-Seq analysis package from CLC was then used to map filtered reads on a custom-masked *C. basilensis* ŐR16 genome version. Only those reads were considered that displayed an alignment longer than 80% of the read length while showing at least 95% sequence identity against the reference genome. Next "Total gene read" RNA-Seq count data was imported from CLC into R 3.3.2 for data normalization and differential gene expression analysis. Function "calcNormFactors" from package "edgeR" v.3.12.1 was applied to perform data normalization based on the "trimmed mean of M-values" (TMM) method. Genes displaying at least one -fold gene expression change with an FDR (false discovery rate) value below 0.05 were considered as significant (Seqomics Ltd, Hungary).

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

According the transcriptome result and the scientific literature three carboxypeptidases (CPA1, 2, 3) genes were chosen for cloning and expression. In this chapter the materials and methods are shown, what were used for cloning and expression.

2.3.1 Synthesis of the CPA genes and plasmid amplification

The exact sequences of the CPA genes were identified by the genome project of the ŐR16 strain. The first gene is **ŐR16_23878** responsible to produce D-alanyl-D-alanine carboxypeptidase protein, located in the region (72768...73976), contig 58, 1209 bp long, named CPA 1. The second gene is **ŐR16_07981** responsible to produce Metal-dependent carboxypeptidase protein, located in the region (138451...139662), contig 17, 1212 bp long, named CPA 2. The third gene is **ŐR16_12223** responsible to produce D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 4), located in the region (91672….93207), contig 27, 1536 bp long, named CPA 3. CPAs details explained in Table 10. The Sequences for the 3 genes (supplementary material 4) were sent to GenScript company (USA) for synthesis and inserted in the required expression vector.

The amount of the plasmid was only 4 µg for each gene, the GenScript company suggested to amplify them before the initiation of the protein expression experiment. Therefore, *E. coli* TOP 10 cells were used to amplify the expression plasmids containing the vector pET-28a (+) to reach the desired volume needed for expressions.

2.5 µL of the plasmid DNA was added to the TOP10 cells and placed on ice for 30 minutes and heat shocked for 45 seconds at exactly 42 ˚C to allow the plasmid to be hosted and amplified in TOP10 cells, then incubated on ice for 2 minutes. 300 µL of room temperature SOC medium was added to the tubes. After that, the tubes were placed in a shaking incubator at 225 rpm for 1 hour at 37°C. 100 µL and 180 µL from the vials were added to the LB agar plates which contained 20 µL of 100 mg/ml of kanamycin and 40 µL of X-Gal / plate, for white and blue colony selection and incubated overnight at 37 ˚C.

For each gene, 10 white colonies were picked and cultured in 5 ml tubes containing 3 ml of liquid LB (kanamycin added) overnight at 37 ˚C. Five tubes were selected (from each ligated gene) for the plasmid DNA isolation and purification.

2.3.2 Plasmid DNA isolation and purification

The DNA of pET-28a (+) expression vector in TOP 10 cells containing the targets genes separately was isolated and purified using High-Speed Plasmid Mini Kit (Cat. # PD100, PD300-Geneaid). Plasmid DNA concentrations were measured using Nanodrop, ThermoFisher.

2.3.3 Plasmid DNA transformation to *E. coli* BL21 (DE3) PLysE

Plasmid DNA was transformed into *E. coli* BL21(DE3) PLysE chemical cells for protein expression (Sigma-Aldrich Co., USA). The constructs maps and the analysis results for each CPAs can be found in supplementary material 5.

This *E. coli* BL21(DE3) PLysE cells were chosen for the protein expression experiment because this type of expression cells optimal for protease expression. The chosen CPAs are proteases, which can destroy the host cell during the growing, if the induction is not controlled properly by the vector.

40 μL of *E. coli* BL21(DE3) PLysE cells added to the chilled culture tube (17 mm x 100 mm tubes). 1 μL of plasmid was added to the 40 μL of cells. The cell/plasmid mixture was incubated on ice for 30 minutes. Cells were treated by heat shock then placing the culture tubes in a 42°C water bath for 45 seconds to allow the plasmid to be hosted in the BL21(DE3) PLysE chemical cells and then incubated on ice for 2 minutes. 960 μL of room temperature Expression Recovery Medium was added to the cells in the culture tube. After that, the tubes were placed in a shaking incubator at 250 rpm for 1 hour at 37°C. 200 μL of the transformation mixture was added to the plates contains LB-Lennox (10 g/L tryptone, 5 g / L yeast extract, 5 g / L NaCl , 15 g / L agar) and kanamycin. The palates then were growing at 37 ºC overnight.

2.3.4 Optimization of the CPA genes induction

The prepared overnight cultures were added to 50 and 200 mL of LB medium containing kanamycin and incubated with shaking at 30 and 37˚C. During the induction step of the CPA genes, 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 in order 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. After the induction, the samples were centrifuged at 4600 rpm, 4˚C. The LB media was discarded and the cultures were placed on ice for 10 minutes and sonicated for 2 minutes. 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.

2.3.5 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 - Gravity-flow column method (ThermoFisher Co.) The columns were purchased from Ibiotech Ltd., Hungary. This method is designed for purification of 6xHis-tagged recombinant proteins expressed in bacteria.

Supernatants as samples were derived from the CPA clones *E. coli* BL21(DE3) PLysE cells, discussed in previous chapter.

Each 1ml of resin sample was added to 1 ml of sample. The storage buffer was drained from the resin by gravity flow. The samples then were prepared by mixing the protein extracts with an equal volume of equilibration buffer (EB) (20mM sodium phosphate, 300mM sodium chloride (PBS) with 10mM imidazole; pH 7.4). The samples in the columns were equilibrated with two resin-bed volumes of EB and the buffer allowed to drain from the columns. Later, the prepared protein extracts were added to the resin and the flow through was collected. The resin for each CPA clones was washed two times with two resin-bed volumes of wash buffer (WB) (PBS with 25mM imidazole; pH 7.4). His-tagged CPAs from the resin were eluted two times with two resin-bed volumes of elution buffer (EB) (PBS with 250mM imidazole; pH 7.4). The purified CPA proteins concentrations were measured by Nanodrop equipment, (ThermoFisher Ltd., Hungary).

2.3.6 Measurement parameters of MALDI -TOF MS for detecting the target CPAs

The MALDI TOF MS measurement was performed for checking the protein expression by the Wessling Hungary Ltd, Budapest. The supernatants of the induced CPA clones were investigated for detecting the CPA proteins.

For MALDI-TOF MS analysis, samples were prepared using cells supernatant (obtained from 2.3.5 chapter-experiment) 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. After this sample preparation the lezer beam measuring is procceded, according the recommendation by the manufacturer (Bruker Daltonik GmbH, Germany). On the target plate in one row there are 12 target circle. Each sample had 6 target circle. Durning the process the equipment is taking 60 measurments/sec from one circle-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.The method procedure of the MALDI-TOF MS analysis was adapted from the work of (Bojté et al., 2019).

2.3.6.1 Assessment of achieved MALDI-TOF MS spectra

FlexAnalysis (Bruker Daltonik) software was used for the analysis of the mass spectra profiles. With this program, the individual sample mass spectrum, and the necessary mathematical operations (baseline correction and noise reduction), could be presented with the system file built and optimized.

2.3.7 OTA biodegradation experiment of the recombinant CPAs

This experiment was also a control method for detecting the recombinant CPAs and evaluating their degradation ability.

The CPA containing BL(21) DE3 PLyEs clones were growing like written previous. Only the induction parameters were changed. After reaching the desired OD, 1 mM of IPTG was added to the cultures of each target to induce the expression of the CPAs then incubated for 3 and half hours at 37 ˚C. Sample were harvested by the centrifugation 4000 rpm/ 30 minutes. The liquid media was discarded and the bacterial pellets were suspended in 2.2 ml of cooled lysis buffer (2 mM EDTA, 2 mM PMFS, 50 mM NaH2PO4, 300 mM NaCl and l mg/ml of lysozyme), placed on ice for 15 minutes to break down the cell wall, placed on beaker contains ice and sonicated for 2 minutes (20 sec puls on, 20 sec puls off , 60% amplitude) to release to break the cell apart, then the samples were centrifuged at 14000 rpm for 30 minutes. Each pure 1 ml supernatant was pipetted by to 1 µl of 1 mg/ml of OTA and incubated in shaking-thermostat (180 rpm) for 16 h at 37 ˚C. Finally, after the incubation 500 µl from each target was sent for HPLC analysis to SZIU, Buda campus.

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

In this chapter the materials and methods for 4th objective, the valuation of the mycotoxin biodegradation ability of the *Cupriavidus* genus type strains are shown.

2.4.1 *Cupriavidus genus* type strains and mycotoxin stock solutions

Altogether 16 type strains of the genus *Cupriavidus* were selected from strain collections i.e (shown in Table 7):

- CCUG (Culture Collection University of Göteborg),
- BCCM (Belgian Coordinated Collections of Microorganisms)
- DSMZ (German Collection of Microorganism and Cell Cultures, Germany), JCM (Japan Collection of Microorganism, Japan)

The strain *Cupriavidus nantongensis* KCTC (Korean Collection for Type Cultures) 42909T has not been investigated in the biodegradation experiment due to the delay in the ordering process and the experiment had to be started.

Table 7 : 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	Belgian coordinated collections of microorganisms
C. numazuensis	DSM 15562^T	German Collection of Microorganisms and Cell Cultures
C. pinatubonensis	DSM 19553^T	
C. basilensis	RK1 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	

The mycotoxins were purchased from Sigma-Aldrich Co., USA, and 1 mg/mL stock solutions were prepared in acetone and used in the degradation experiments.

2.4.2 Mycotoxin biodegradation experiment

Cupriavidus strains were stored at -80 °C and were streaked on LB agar plates (10 g tryptone, 5 g yeast extract, 10 g NaCl and 18 g bacteriological agar dissolved in 1000 mL distilled water). The plates were incubated at 28 °C for 3 days for colony forming. A single colony was inoculated into Erlenmeyer flasks containing 50 mL liquid TGE-5 medium (5 g tryptone, 5 g glucose and 2.5 g yeast extract dissolved in 1000 mL distilled water). The flasks were incubated at 28 °C (Sartorius AG, Germany). After three days, the optical density of the cultures was measured by an UV–Vis spectrophotometer (Genesys 10 UV–Vis, Thermo Fisher Scientific Inc., USA) and adjusted to OD600=1.0. From this culture 5 mL was inoculated into 45 mL freshly sterilized LB medium, which was spiked with the stock solution of the mycotoxins, 1 µg/mL initial concentration in each mycotoxin. The monotoxins experiment was carried out in triplicates. 50 mL sterile LB medium containing the mycotoxin was applied as a microbe-free control. Flasks were incubated at 28 °C for 5 days, thereafter 1 mL sample were taken and centrifuged at 20800×g for 15 min (Eppendorf 5810R Centrifuge, Eppendorf Co., Germany). Supernatants and pellets were separated and stored at −80°C for further HPLC MS/MS analysis.

2.4.3 SOS-Chromotest for measuring AFB1 genotoxicity

Genotoxic effect in supernatant samples derived from mycotoxin biodegradation experiment was observed by the colorimetric SOS-chromotest (Environmental Bio-Detection Products Inc., Canada).

The test was carried out according to the description of Risa and colleagues (Risa et al., 2018). Freeze dried E. coli PQ37 was rehydrated by inoculating it to growth medium (2.5 g yeast extract, 2.5 g casein peptone, 8.5 g NaCl dissolved in 1000 mL distilled water) and incubated overnight at 37 °C in the dark. After the incubation, the optical density of the inoculum was measured and adjusted to OD600 = 0.05. The test was carried out in a 96-well microplate, where indirect (AFB1 and 2-AminoAnthracene [2AA]) and direct (4-Nitro-Quinoline-Oxide [4NQO]) genotoxic compounds were applied as positive controls and sterile LB medium was used as negative control. Twofold serial dilutions of genotoxic solutions (10 µg/mL AFB1 and 4NQO, 1 mg/mL 2AA) with 10% dimethyl sulfoxide (DMSO) in 0.85% saline were applied and 10 µL samples were added to the wells in triplicate. Each well received 100 µL test organism with metabolic activation applying S9 mix (containing lyophilized S9 extract of sensitized rat liver), excluding the serial dilution of

4NQO, which was supplemented with 100 µL test organism without metabolic activation. The microplate was incubated at 37 °C for 2 h in a microplate incubator (Biosan PST-60HL-4 Plate ShakerThermostat, Riga, Latvia). Subsequently, 100 µL of appropriate substrates was added to the wells. The plate was further incubated for 1.5 h. The absorbance was measured by ELISA reader at 405 and 620 nm wavelengths (ELx800 Absorbance Reader, BioTek Instruments, Winooski, Vermont, USA.

The genotoxic effect was expressed in induction factor (IF), which was calculated according to Eq. (1) (Legault and Blaise, 1994) :

Induction factor (IF) = (C405 ∗ S620)/ (S405 ∗ C620)

where C is the mean of the absorbance value of the control; S is the mean of the absorbance value of the sample measured at 405 and 620 nm wavelength. Dose–response curves derived from IF points of the controls indicate the SOS-induction potential (SOSIP), which value can be used for the validation of the test by comparing data reported.

Samples were considered as not genotoxic, when IF was significantly ($p<0.05$) less than 1.5 (Quillardet and Hofnung, 1985).

2.4.4 BLYES test for measuring ZON oestrogenity

The oestrogenic effect of ZON and its metabolites was evaluated by BLYES test according to Sanseverino et al. (2005)**.** The genetically modified test organism: a *Sacharomyce cerevise* strains were grown in growth medium containing YMM medium, 20% glucose solution, 4 mg/mL l-aspartic acid solution, 24 mg/mL l-threonine solution, vitamin solution and 20 mM copper sulphate solution (Sanseverino et al., 2008) at 30 °C, 200 rpm. The test was carried out on a 96-well white microplate, where 20 µL of samples and controls was added to each well. As a positive control ZON and as a negative control LB medium was used. The optical density of the overnight inoculum was adjusted to 1.0 (OD600) and 200 µL of the test organisms was added to samples and controls as well. The microplate was incubated at 30°C for 5 h. Subsequently, the bioluminescence was measured by VictorX Multilabel Plate Reader (Perkin Elmer Inc., Waltham, Massachusetts, USA). In the case of BLYES, bioluminescence intensification (%) was calculated according to Eq.(2) (Krifaton et al., 2011).

$$\text{Bioluminescence intensification (\%)} = (-1) * \left(\frac{(C-S)}{C}\right) * 100$$

where C is the mean of the bioluminescence of negative control, and S is the mean of the bioluminescence values of the sample.

2.4.5 Measurement of OTA, AFB1, ZON, T-2 and DON concentrations

To estimate residual OTA, AFB1, ZON, T-2 and DON concentrations without any metabolites, HPLC–MS/MS were used (SZIE, Department of Applied Chemistry, Buda Campus). Triplicates of supernatant samples were homogenized by ultrasonication; thereafter pre-column derivatization was performed for 15 min at 55°C. The mycotoxin concentration was measured by Agilent 1100 high performance-liquid-chromatograph (Agilent Technologies, Santa Clara, California, USA) equipped with Restek C18 (150 mm × 4.6 mm × 5 µm) column (Restek Corporation, Bellefonte, Pennsylvania, USA). For the measurement of toxin adsorption on cells, pellet fractions of 1 mL samples were extracted with 1 mL methanol and centrifuged (2700 g, 4°C, 10 min). For measuring toxin concentration, the supernatant was derivatized and measurement was carried out as mentioned above.

2.4.6 Chemicals and reagents for the HPLC measurment

For HPLC-MS/MS analysis all standard and reagents were purchased from the Hungarian distributors of national companies. Mycotoxin standards with minimum 98% purity were purchased from Romer Labs (Romerlabs Ltd., Hungary). Mobile phases and extraction solvents were used containing super gradient grade acetonitrile (MeCN, 99%) purchased from VWR (VWR Ltd., Hungary), gradient grade methanol (CH3OH, 99%) obtained from Fischer Scientific (Fischer Scientific Ltd., Hungary), ultra-pure-grade water (18MΩcm) produced in house by a Milli-Q water purification system (Merck Ltd., Hungary). Acetic acid (CH3COOH, 99.8%) obtained from Merck (Merck Ltd., Hungary), formic acid (HCOOH, 98%) from Scharlau (Scharlab Ltd., Hungary) and LC/MS grade ammonium-acetate (CH3COONH4, >99%) from VWR (VWR Ltd., Hungary).

2.4.7 Statistical analysis

For statistical analysis, Microsoft Excel 2016 (Microsoft Office, Microsoft Inc., Redmond, Washington, USA) and Past 3 were used. In the case of supernatants, the genotoxicity was expressed in induction factor, oestrogenicity was expressed in bioluminescence intensification percent and residual toxin concentrations were expressed in µg/mL. All values are means of triplicates. Significant differences for genotoxicity and oestrogenicity ($P < 0.05$) were calculated and one-sample t test was used. In the course of evaluation of genotoxicity, samples were considered as non-genotoxic, when IF were significantly less than 1.5. In the case of oestrogenicity, significant differences in the bioluminescence from ZON control were calculated. Correlations between biotests and analytical measurements were calculated by Spearman's rank correlation coefficient.

3 RESULTS

This chapter is also introduced according the objectives for better understanding.

3.1 The results of objective 1:

Investigating the effect of the OTA biodegradation by-product of *C. basilensis* ŐR16 on human kidney cell line by gene expression

3.1.1 Biodegradation of OTA by *Cupriavidus basilensis* ŐR16

The biodegradation ability of the *Cupriavidus basilensis* ŐR16 for OTA was measured and monitored by HPLC and ELISA analytical methods. The samples originating from the OTA degradation experiments (1^{st}, 2^{nd}, 3^{rd}, 5^{th} day) are indicated as a function of time. OTA concentration in the uninoculated control remained 10 and 2 mg/l during the incubation (5 days). In the bacterial pellet ELISA detected lower than 0.004% residual OTA of the original concentration (10 or 2 mg/l). The first day of incubation 0.15% of the initial OTA content was degraded as shown in Figure 8, but this OTA content reduced by the 5^{th} day below 0.5%. OTA content in the supernatants reduced continuously during the 5-day incubation and the OTA was completely degraded (98% decrease measured by ELISA, 100% decrease by HPLC). Based on the HPLC results OTA was metabolized to OTα since OTα content increased in parallel by OTA decrease.

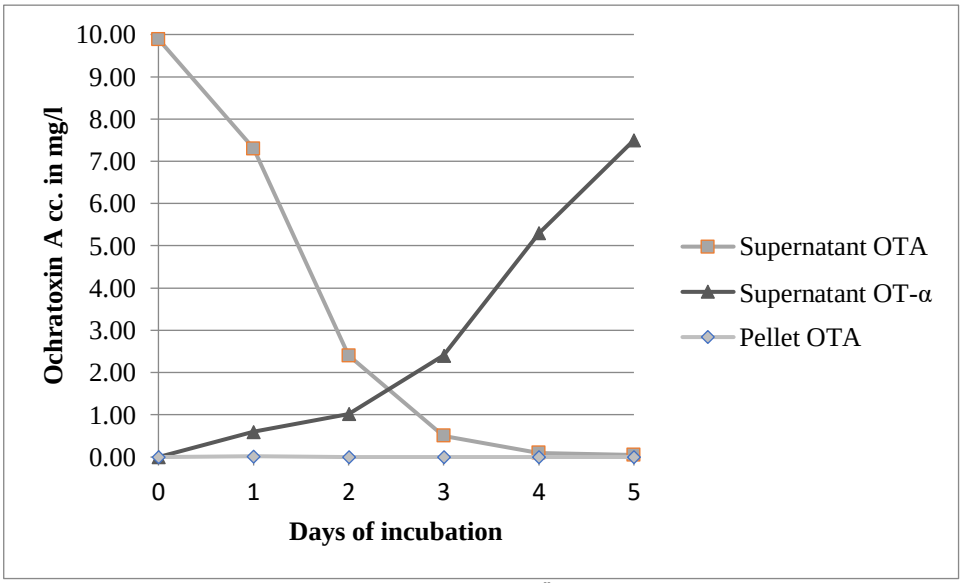


Figure 8: Ochratoxin-A biodegradation by *Cupriavidus basilensis* ŐR16 for 5 days. Decrease of the OTA concentration is detected in the supernatant and pellet, while OTα concentration is increasing. Supernatant OTA – ochratoxin A concentration in the sample, Supernatant OT-α – derivative ochratoxin- α concentration in the sample, Pellet OTA – ochratoxin-A concentration on the centrifuged pellet.

3.1.2 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 test was an acute test, only 48 hours, and the treatment was according the AlamarBlue protocol.

It was already known that the used OTA and MMS concentration is not killing the cells, the question was the effect of the LB media and effect of the by-product of the bacteria (normal and OTA by-product).

The OTA and the MMS was added into the treatments in 10 mg/l concentration. 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. According to the results, there were no significant difference among the treatments and the cell line was vital in all cases (Figure 9). 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.

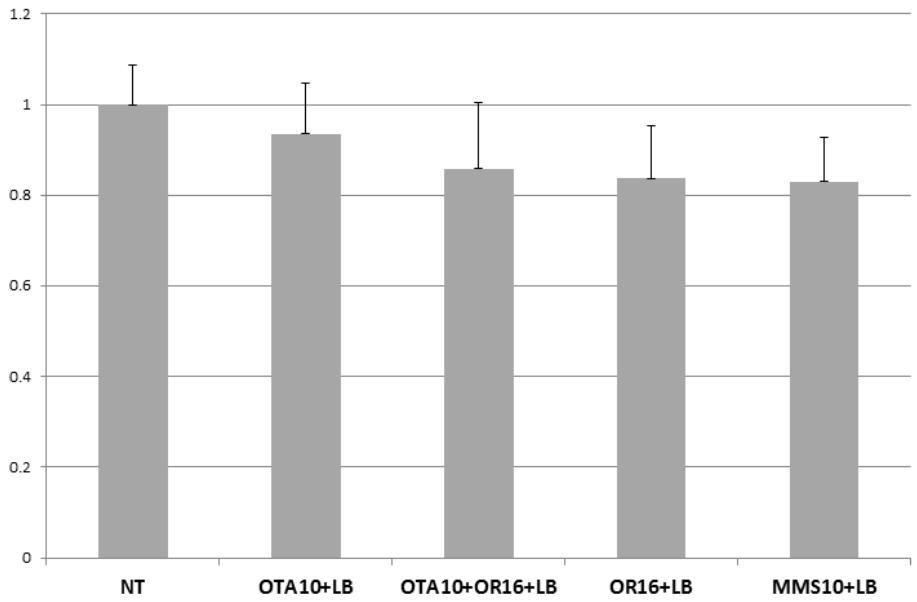


Figure 9: Alamar Blue test result in mean + SE on 789-O human kidney cell line vitality to measure the effect of 10 mg/l OTA concentration, the 20% LB media, ŐR16 strain by-product and 10 mg/l MMS as genotoxic control. Cell line was treated in four parallels and results are normalized to the non-treated (NT) control. No significant difference was detected among the treatments.

3.1.3 The confirmation of the RNA isolation from the human kidney cell line 786-O

RNA purity was confirmed by measuring the absorbance at 260 and 280nm (supplementary material 1), RNA integrity was confirmed by using agarose gel electrophoresis (Figure 10).

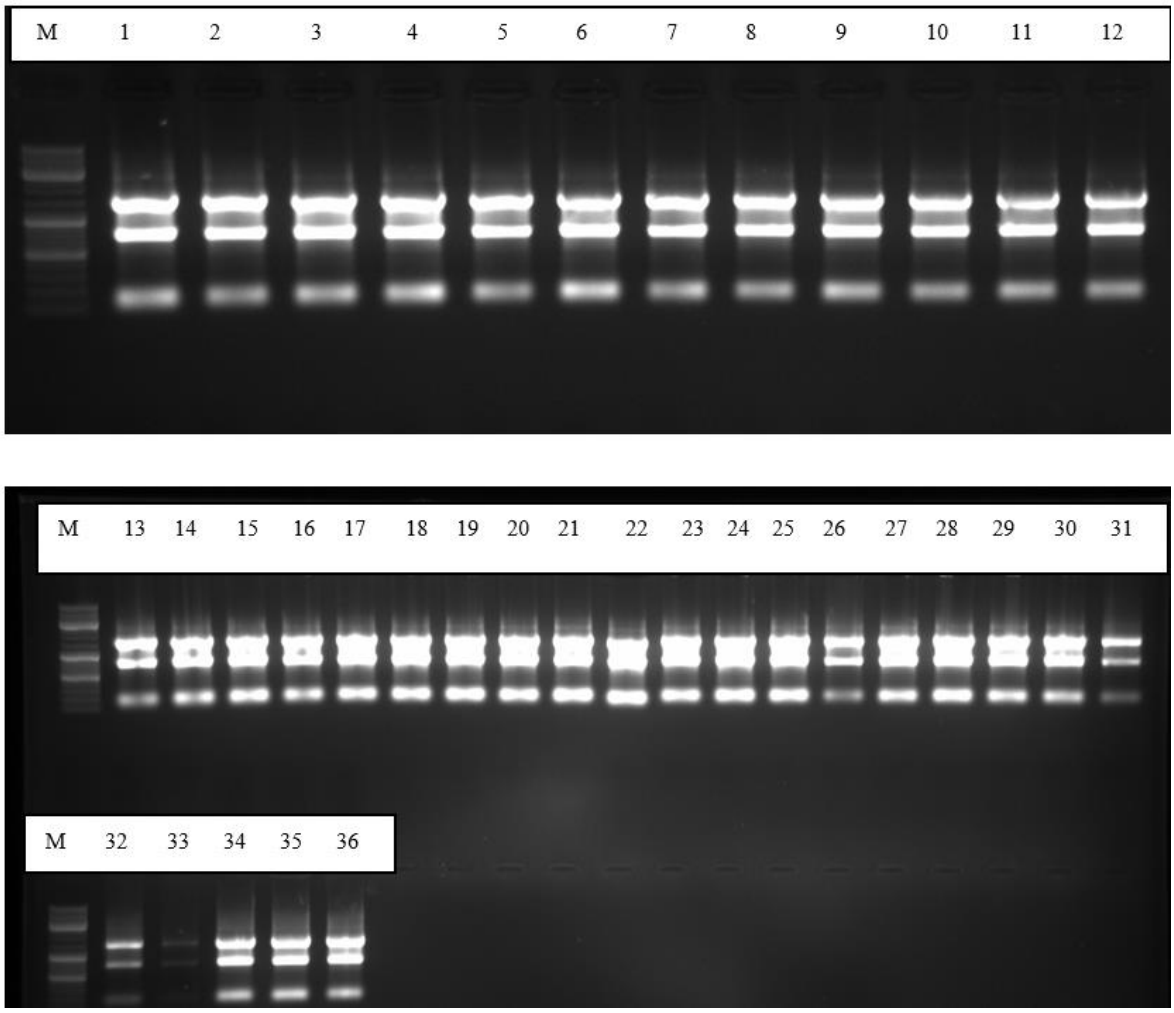


Figure 10: Gel photo of the isolated RNA from the human kidney cell line 786-O treated with OTA, MMS, ŐR16, ŐR16+OTA and none treated samples. Samples: 1-3 (None treated); 4-6 (OTA10);7-9 (OTA2); 10-18 (OTA 10/ŐR16); 19-27 (OTA 2/ŐR16); 28-30 (ŐR16); 31-33 (MMS 10); 34-36 (MMS 2)

3.1.4 Validation of the houskeeping genes (HKG)

For better understanding the chosen genes and treatments are shown again.

Target genes: *gadd45, gadd153, clusterin, annexin2*

Houskeeping genes: ß-actin, hprt, gapdh

Table 4: 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)

To assist the most suitable HKG's the raw **Ct** values (**Ct** =cycle threshold, is defined as the number of cycles required for the fluorescent signal to cross the threshold; ie exceeds background level) were compared in the different treatments by statistical analyses (Figure 11). According the results, the *hprt* was the best HKG. The second best HKG was *β-actin* according to the Ct values. The *gadph* showed high fluctuation, caused by the different treatments, and thus was excluded from the validation.

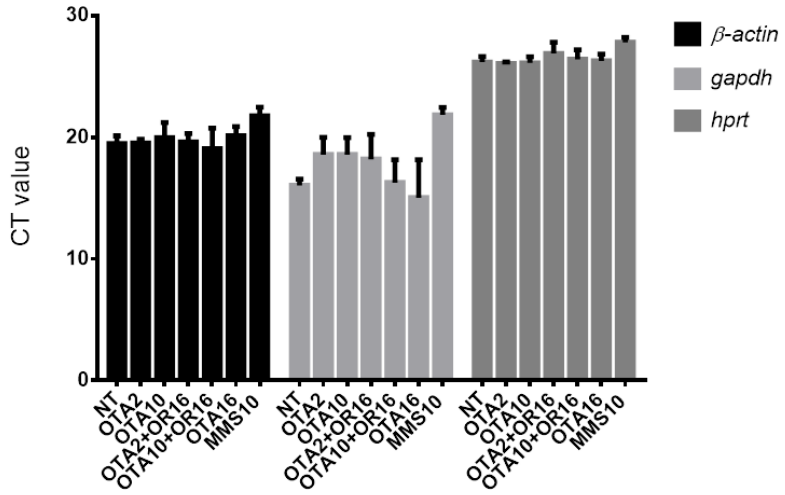


Figure 11: Validation of the three-housekeeping genes (*β-actin, gapdh, hprt*) according the row CT values, used by ANOVA. NT is the non-treated sample, OTA2 and OTA10 samples are LB containing 2 and 10 mg/l OTA concentration, OTA2+ŐR16 and OTA10+ŐR16 and LB samples containing 2 or 10 mg/l OTA and treated with ŐR16 strain, OTA16 is the metabolic product of ŐR16 strain and MMS is the genotoxic control containing 10 mg/l MMS

3.1.5 Target gene expression referenced to *hprt*

The target gene **ct** results are referenced to the housekeeping genes During this process the average expression rate of the paralell 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 12. 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. The gene expression data of *hprt* can be found in supplementary material 2 (A).

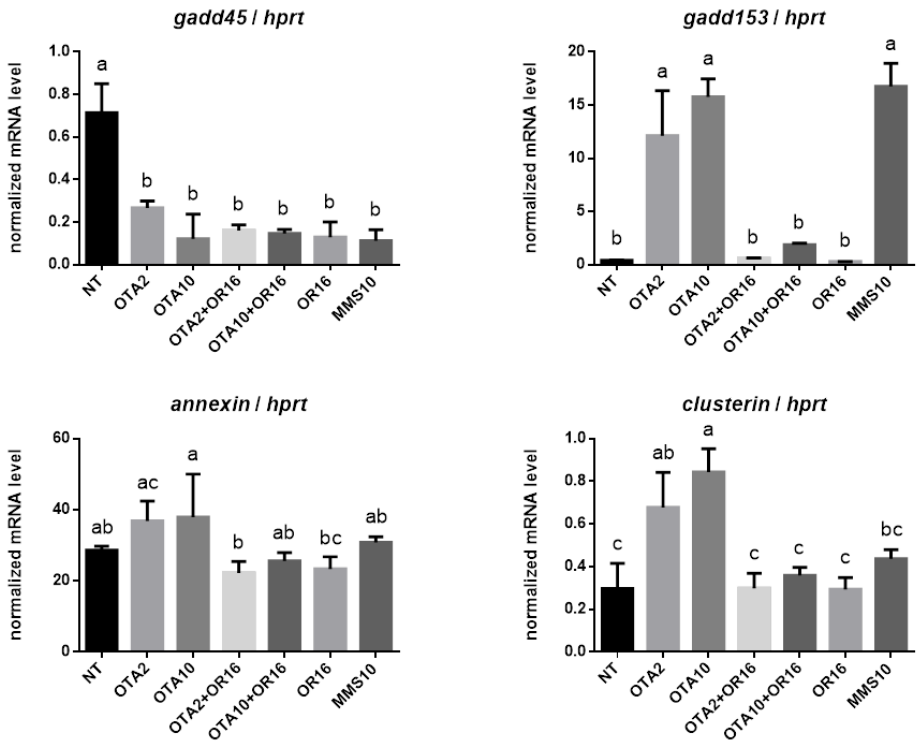


Figure 12: Target genes normalized to the *hprt*, which was the best housekeeping gene, according the raw Ct value validation. Different letters mean statistical difference based on One-way ANOVA followed by the Tukey's multicomparisons test NT is the non-treated sample, OTA2 and OTA10 samples are LB 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

3.1.6 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 *gadd45* 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 13). 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. The gene expression data of *ß-actin* can be found in supplementary material 2 (B).

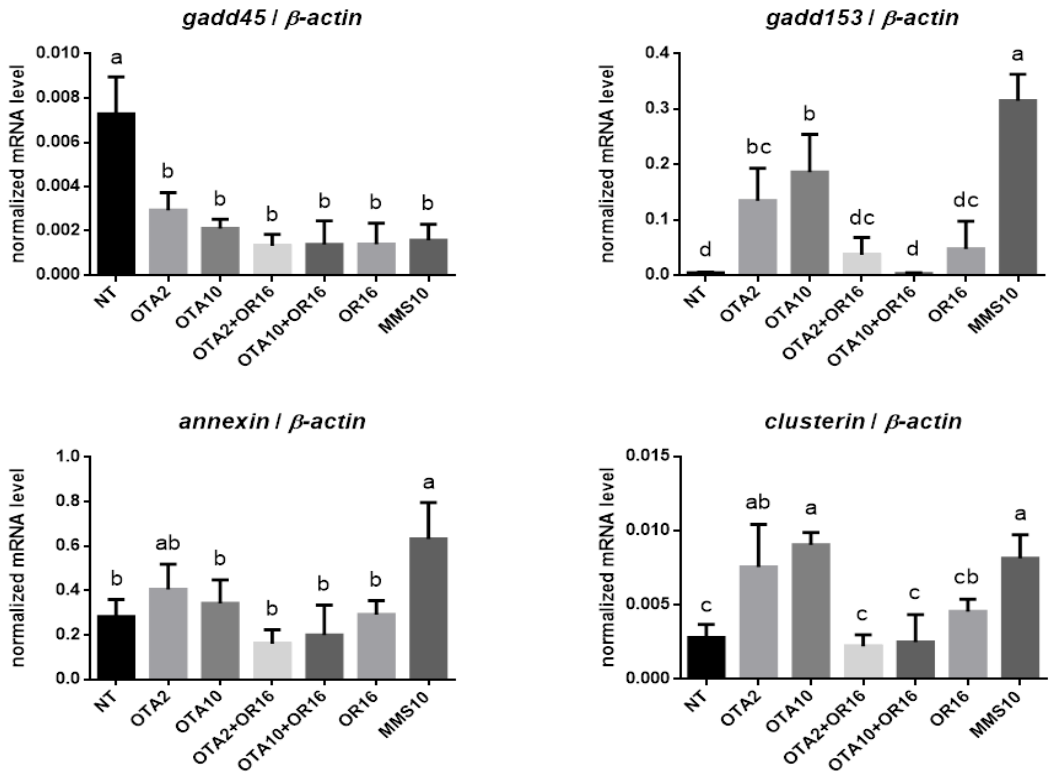


Figure 13: Target genes normalized to the *β-actin*, which was the second-best housekeeping gene, according the validation. The different letters mean statistical difference based on One-way ANOVA followed by the Tukey's multicomparisons test . NT is the non-treated sample, OTA2 and OTA10 samples are 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

Identification of the enzymes of the *C. basilensis*-ŐR16 playing role in the biodegradation of the OTA by transcriptome analysis.

3.2.1 RNAisolation 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 14). RNA quality was tested by Agilent 2200 Technologies and using TapeStation software (Seqomics Ltd, Mórahalom, Szeged, Hungary), (supplementary material, 3). TOP 10 *E. coli* was used as a positive control in two different media; LB and minimal buffer (m.b).

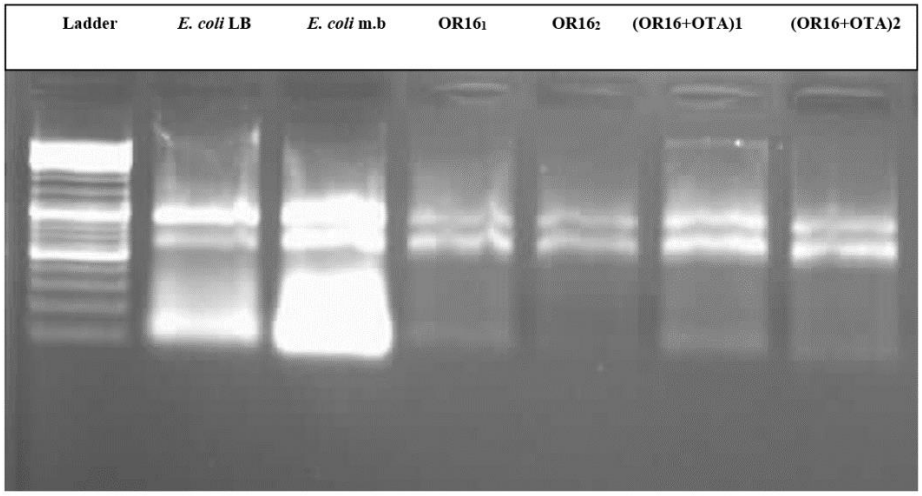


Figure 14: RNA bands of *Cupriavidus basilensis* ŐR16 with and without OTA from the OTA degradation experiment conducted in minimal buffer for transcriptome analysis, sampled after 11 hours of incubation.

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 were nominated (Table 8). For the study, 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 8. The reason for that was the initial log phase, which was on the 11th hour of culture growing. Deep search was conducting for protease genes and enzymes, according to the literature of the OTA degradation process. Only these 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 8: Fifteen nominated genes out of 3500, 3 were chosen for cloning and expression

Nominated genes * Chosen genes for cloning and expression	Accession No.	Gene name	Region	Fold expression	Size in bp	Contig number
Phenylalanine-4-hydroxylase	AHJE01000094	ŐR16_31894	Complement (17995..18927)	2.3	927	94
Aromatic Ring hydroxylase	AHJE01000038	ŐR16_16257	Complement (106897..108471)	8.1	1575	38
Membrane carboxypeptidase (penicillin-binding protein)	AHJE01000029	ŐR16_12645	Complement (43999..46188)	1.8	2190	29
Membrane proteins related to metalloendopeptidases	AHJE01000060	ŐR16_24100	Complement (30086..30808)	3.8	727	60
Membrane carboxypeptidase/penicillin-binding protein PbpC	AHJE01000094	ŐR16_31869	Complement (8236..10443)	1.9	2208	94
D-alanyl-D-alanine carboxypeptidase *CPA1 *	**AHJE01000058**	**ŐR16_23878**	**Complement (72768..73976)**	**1.3**	**1209**	**58**
Metal-dependent amidase/aminoacylase/carboxypeptidase *CPA2 *	**AHJE01000017**	**ŐR16_07981**	**Complement (138451..139662)**	**2.5**	**1212**	**17**
D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 4) *CPA3 *	**AHJE01000027**	**ŐR16_12223**	**Complement (91672..93207)**	**1.1**	**1536**	**27**
Amidases related to nicotinamidases	AHJE01000045	ŐR16_19156	Complement (94947..95645)	4.8	699	45
Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit	AHJE01000003	ŐR16_01015	Complement 11210..11980	2.1	771	3
COG0169 Shikimate 5-dehydrogenase	AHJE01000021	ŐR16_09609	Complement (8185..8595)	7.6	411	21
COG3971 2-keto-4-pentenoate hydratase	AHJE01000003	ŐR16_01040	Complement (14846..17032)	3.2	2187	3
COG2146 Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	AHJE01000019	ŐR16_08912	Complement (70900..71211)	4.3	312	19
Predicted metal-dependent hydrolase	AHJE01000108	ŐR16_35215	complement(47971..48636)	6.5	666	108
Dienelactone hydrolase and related enzymes	AHJE01000063	ŐR16_25377	complement(58185..59420)	6	1236	63

3.3 The results of objective 3

Verification of the nominated OTA-degrading enzymes by cloning and expression

Three CPA genes were chosen according the transcriptome analysis for cloning into pET-28a (+) expression vector. The vectors were amplificated and transferred into BL21 (DE3) pLysE expression cells. The expression clones were induced on different temperature, induction time and IPTG concentration. The best expression parameters were chosen, the CPA enzymes were expressed and isolated by different techniques.

3.3.1 Vector amplification, plasmid DNA Isolation and purification

The vector pET-28a (+) (separately) containing our target inserts 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 (Figure. 15) and purified using High-Speed Plasmid Mini Kit (Cat. # PD100, PD300 -Geneaid). Plasmid DNA concentrations were measured using Nanodrop, ThermoFisher.-The DNA concentrations of each target gene after amplification are shown in Table 9.

Table 9: The amplified plasmid DNA (in TOP 10) concentrations measured by NanoDrop

Gene name	gene name in the cloning experiment (chosen samples)	Plasmid DNA conc ng/µl	A260/A280	A260/A230
D-alanyl-D-alanine carboxypeptidase (CPA1)	CPA1 (sample 2)	222.4	2.06	1.96
	CPA1 (sample 4)	394	2.15	2.27
Metal-dependent amidase/aminoacylase/carboxypeptidase (CPA2)	CPA2 (sample 1)	170.4	2.06	1.87
	CPA2 (sample 3)	163.3	2.04	1.8
D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 4) (CPA3)	CPA3 (sample 1)	339.5	2.11	2.2
	CPA3 (sample 3)	316	2.11	2.18

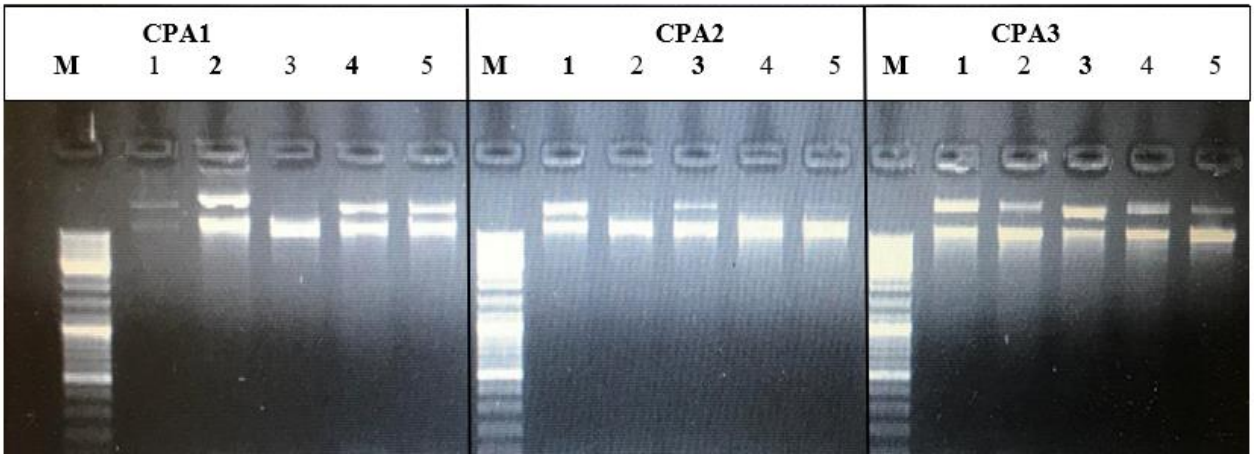


Figure 15: The amplified target genes in *E. coli* TOP 10 cells , M=Marker, the numbers 1-5 for each CPA refers that the samples are identical, the bolded numbers= the chosen for the expression experiment because they have the highest concentrations according to the Nanodrop measurements.

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

3.3.2.1 Transformation of the amplified pET-28a (+) into the expression cell E. coli BL21 (DE3) pLysE

The transformation was carried out by the protocol. The Successful transformation was controlled by two different method. First the plasmid was isolated and purified on the transformed BL21 (DE3) pLysE cell, obviously which were growing on kanamycin containing agar plate. Plasmids gel photo is shown in Figure 16. Plasmid DNA in BL 21 (DE3) pLysE concentrations were measured using Nanodrop (Table 10).

Table 10: The plasmid DNA concentration in BL21(DE3) pLysE cell measured by Nanodrop after the amplified inserts existence confirmation.

Gene name	Gene name in the cloning experiment (chosen samples)	Plasmid DNA conc ng/µl	A260/A280	A260/A230
(CPA1)	CPA1 (sample 1)	252.2	2.11	2.36
	CPA1 (sample 2) *	**372.7**	**1.86**	**1.21**
	CPA1(sample 3)	228.8	2.09	2.37
	CPA1 (sample 4)	235.1	2.1	2.35
(CPA2)	**CPA2 (sample 1) ***	**413.5**	**1.95**	**1.43**
	CPA 2 (sample 2)	184.8	2.1	2.28
	CPA2 (sample 3)	185	2.09	2.15
	CAP2 (sample 4)	190	2.08	2.07
(CPA3)	**CPA3 (sample 1) ***	**239**	**1.91**	**1.33**
	CAP3 (sample 2)	243	1.89	1.28
	CAP 3 (sample 3)	189	2.06	1.93

*** chosen samples for the protein expression experiment**

The successful of the transformation was controlled by the isolation and purification of the plasmids of the BL21 (DE3) pLysE expression cells (Figure. 16). Plasmid DNA concentrations were measured using Nanodrop, TheromFisher (Table 11). Second control step was the digestion of the isolated plasmids by BamHI and HindIII restriction enzymes, according the protocol of the GenScript company for controlling our vectors. Results can be seen on the Figure 17

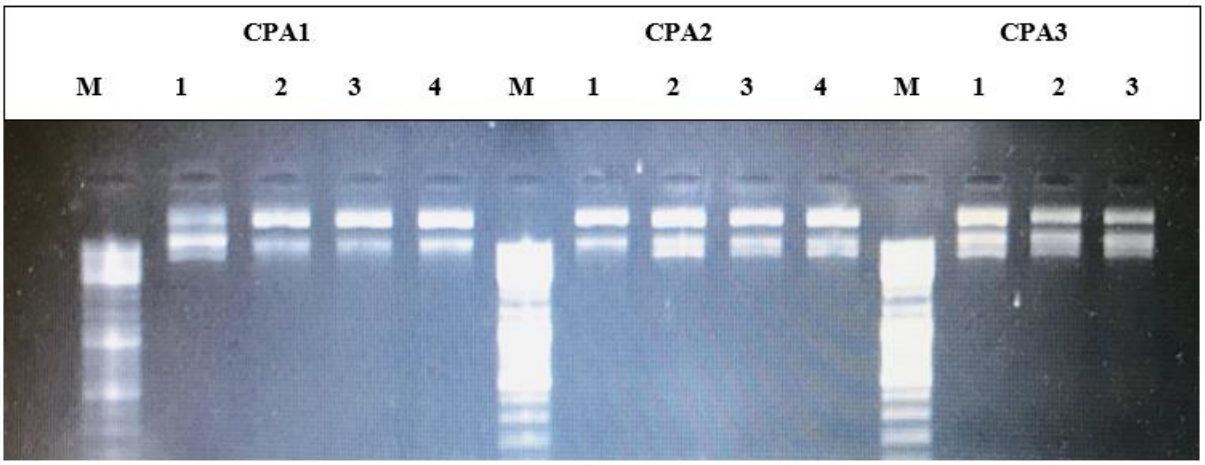


Figure 16: Plasmid DNA in BL21 (DE3) pLysE chemical cells contains the target inserts. M=Marker, numbers = the identical samples obtained from the colonies that only resistant to kanamycin (the target CPAs).

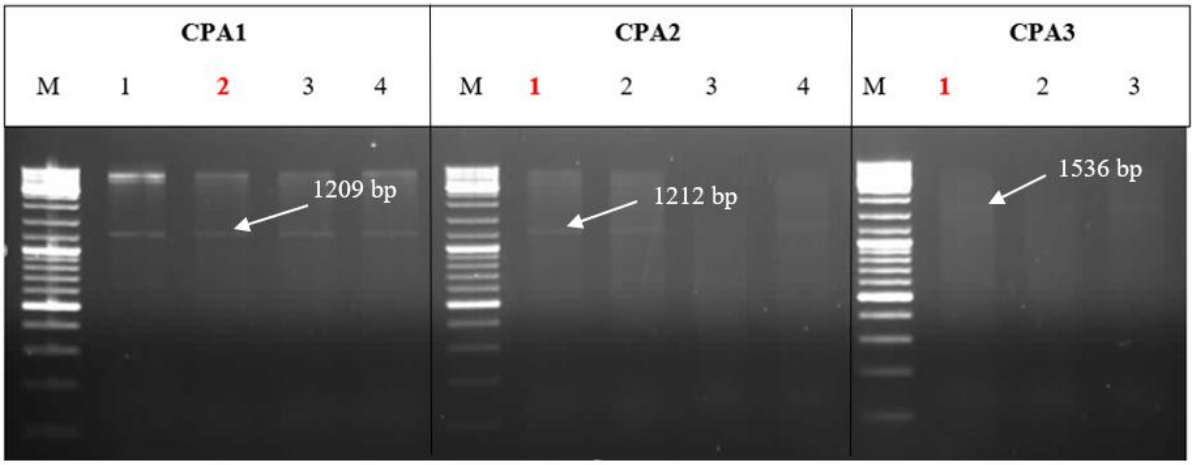


Figure 17: CPAs in BL21(DE3) pLysE cell after digestion by BamHI and HindIII in the PCR for 40 minutes at 37 ˚C showing the plasmids and the inserts after the amplification, according to the protocol of GenScript M=Marker(GeneRuler); CPA1 and CPA2 (1-4), CPA3 (1-3) means the number of the identical samples, numbers in red means the chosen samples for the induction experiment because they have the highest concentrations as shown in Table 11.

3.3.2.2 The best optimization conditions for the CPAs induction experiment

After testing 6 different temperatures explained in the material and methods (section 2.3.5). Only the 37 ºC for 3.5 hours was the best among the tested induction experiment. The best IPTG concentration was 1 mM. The expression of the CPAs was controlled by four different methods. SDS-page, MALDI-TOF, Ni-NTA Resin purification and finally an OTA degradation experiment. The expression of the CPAs was relatively weak and the uninduced samples have no expression at all.

3.3.2.3 Results of the SDS page analysis

For controlling the success of the expression of recombinant CPAs, SDS-page was performed (Figure 18). The errows are shoving the protein size in Dalton at the proper CPA. CPA1 is an estimated 43 kDa, CPA2 is 42 kDa and CPA3 is 54 kDa. However, the bands sizes were week and not as expected. The reason of that could be the low expression rate of the proteins in the BL21 (DE3) pLysE cell, or the induction circumstances were not the optimal for the expression. The volume of the induction matrix was 200 ml for each CPA. The induction of the CPAs by IPTG was conducted at 30 ˚C for 5 hours and a half. The final supernatant volume was around 1.7 ml for each CPA. The same samples were used in the SDS-page gel and during the MALDI-TOF MS experiment also.

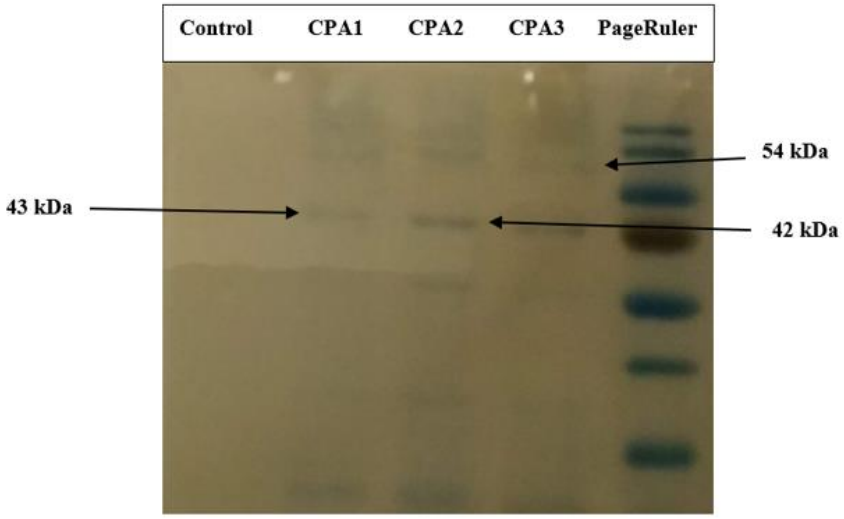


Figure 18: The CPAs weak bands were proven in the SDS page in the estimated sizes.

3.3.2.4 Results of the MALDI TOF MS

This method and results are the 2nd proof for the expression of the three CPAs. This method was used due to its accuracy in the measurement. Usually it measures proteins with sizes less than 20 kDa. However, in the presented experiment there were specific chemicals and matrix used due to the predicted length of the target proteins (CPA1 43.96 kD, CPA2 42.72 kD, and CPA3 54.28 kD). 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 19 comparing to the control (uninduced culture). There are small peaks in each expressed CPA sample at the appropriate place.

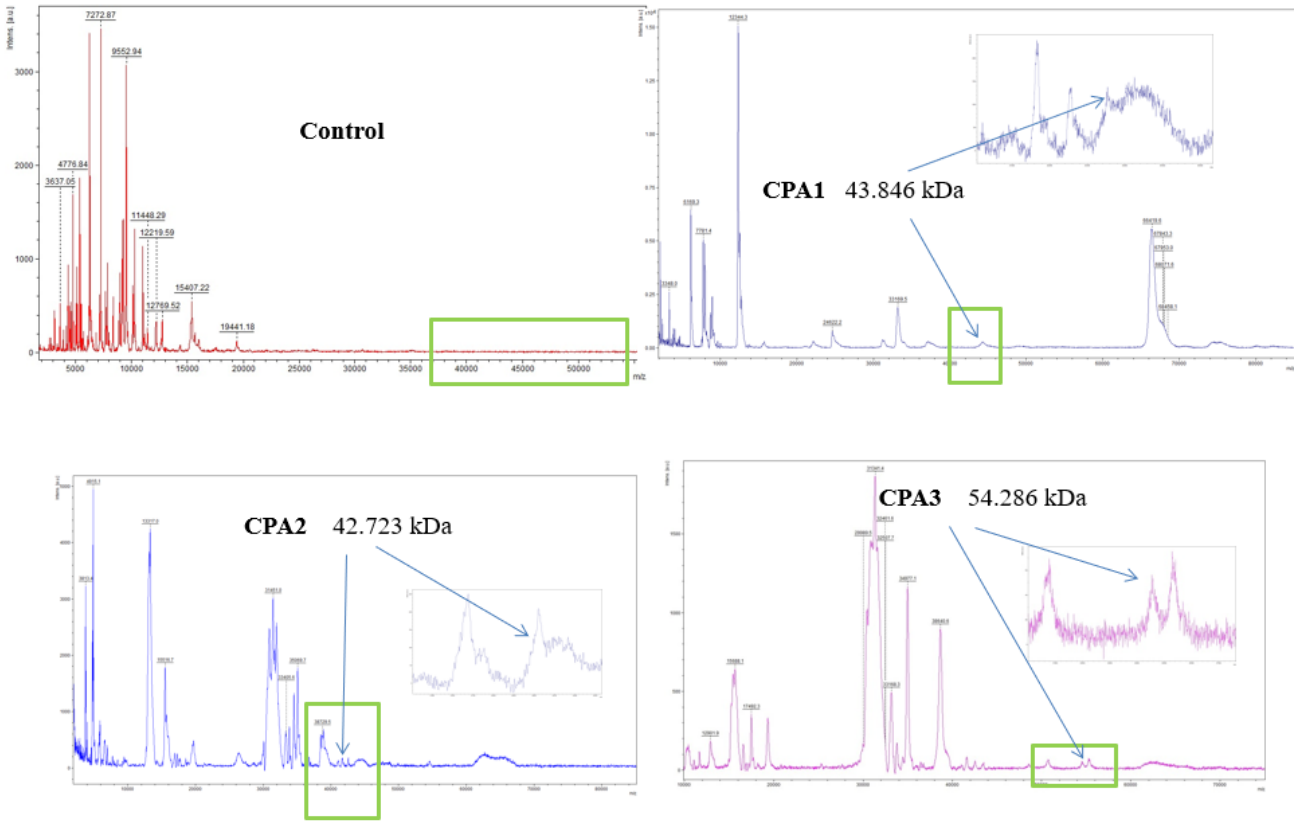


Figure 19: MALDI-TOF MS results of the three expressed CPAs supernatant. The control was the uninduced BL21 (DE3) pLysE cell.

3.3.3 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 procced.-After following the method of the CPAs purification by HisPur™ Ni-NTA Resin -Gravity-flow technique described in section (2.3.6) in the material and methods chapter, the CPAs were bound on the resin and successfully eluted by imidazole. The expression of the CPAs and the Ni-NTA purification was confirmed by the measuring of the CPAs concentrations by Nanodrop, ThermoFisher, RET lab, SZIE as shown in Table 11. The His tagged protein concentration was higher in the induced samples (CPA1, CPA2, CPA3).

Table 11: 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**

3.3.4 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 12, the results are the average of duplicates.

Table 12: 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

The valuation of the mycotoxin degradation potential of the *Cupriavidus* type strains are shown in the following chapter. The summarized biodegradation and detoxification results can be seen in Table 13.

3.4.1 Biodegradation of AFB1

From the 16 type strains four strains had excellent biodegradation potential (over 70% degradation): *Cupriavidus laharis* CCUG 53908^T – 91%, *Cupriavidus oxalaticus* JCM 11285^T – 80%, *Cupriavidus metallidurans* CCUG 13724^T – 72%, *Cupriavidus numazuensis* DSM 15562^T – xy % (shown in Table 13). Two strains could detoxify AFB1 in 5 days according to SOS-chromotest results: *Cupriavidus laharis* CCUG 53908^T and *Cupriavidus oxalaticus* JCM 11285^T ($p<0.05$), Figure 20. The pellets were also measured and calculated into the final degradation result shown in table 13. On the pellet fraction of *C. laharis* CCUG 53908T 120 ng/mL (12% of the initial toxin concentration) in the case of *C. oxalaticus* JCM 11285^T, 247 ng/mL (24% of the initial toxin concentration) AFB1 was measured. Adsorption was observed in other cases also, cause of this the biodegradation potential was corrected by the residual toxin concentration on the pellet.

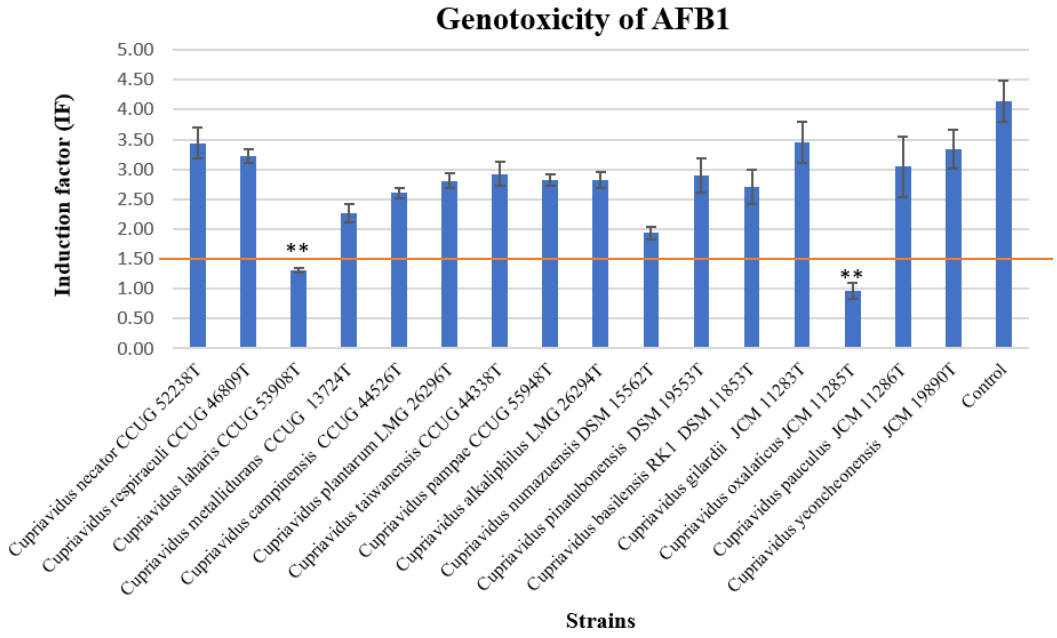


Figure 20: Genotoxicity in supernatant samples of *Cupriavidus* type strains derived from AFB1 biodegradation experiment measured by SOS-Chromo test. Genotoxic effect was expressed in Induction Factor (IF). Values significantly less ($p<0.02$) than IF 1.5 indicates that the bacteria can detoxify AFB1

3.4.2 Biodegradation of ZON

From the 16 type strains four strains had excellent biodegradation potential (over 70% degradation): *Cupriavidus basilensis* DSM 11853^T, C*upriavidus pinatubonensis* DSM 19553^T, *Cupriavidus numazuensis* DSM 15562^T and *Cupriavidus oxalaticus* JCM 11285^T – 82% (see Table 13). Adsorption of ZON on the bacterial pellets was observed: highest toxin concentration on cell pellet was 155 ng/mL in case of *Cupriavidus oxalaticus* type strain. In the case of *Cupriavidus basilensis* DSM 11853^T strain the pellet ZON concentration was the lowest 10 ng/mL. ZON on pellet varied 10 – 155 ng/mL, the biodegradation potential shown in table 15 was corrected by the toxin concentration on the pellet. Oestrogenic effect of ZON in the residual supernatant was measured by BLYES test (Figure 21). Considerable reduction in the oestrogenic effect of ZON (98%) was observed in the strain *Cupriavidus basilensis* DSM 11853^T compared to the control, which is in a positive correlation with the biodegradation rate in the HPLC analysis. Biodetoxification occurred by the following cases: *Cupriavidus respiraculi* CCUG 46809^T reduced oestrogenity 73% by the day 5, but showed only a 64% degradation rate in HPLC. *Cupriavidus pinatubonensis* DSM 19553^T reduced the oestrogenic effect of ZON to around 30% with 91% biodegradation rate by the day 5th according to HPLC. Four strains have higher bioluminescence rate (up to 118%) compared to the control and leads to the transformation of ZON to additional oestrogenic metabolites: *Cupriavidus necator* N-1 CCUG 52238^T, *Cupriavidus gilardii* JCM 11283^T, *Cupriavidus pauculus* JCM 11286^T and *Cupriavidus yeoncheonensis* JCM 19890^T.

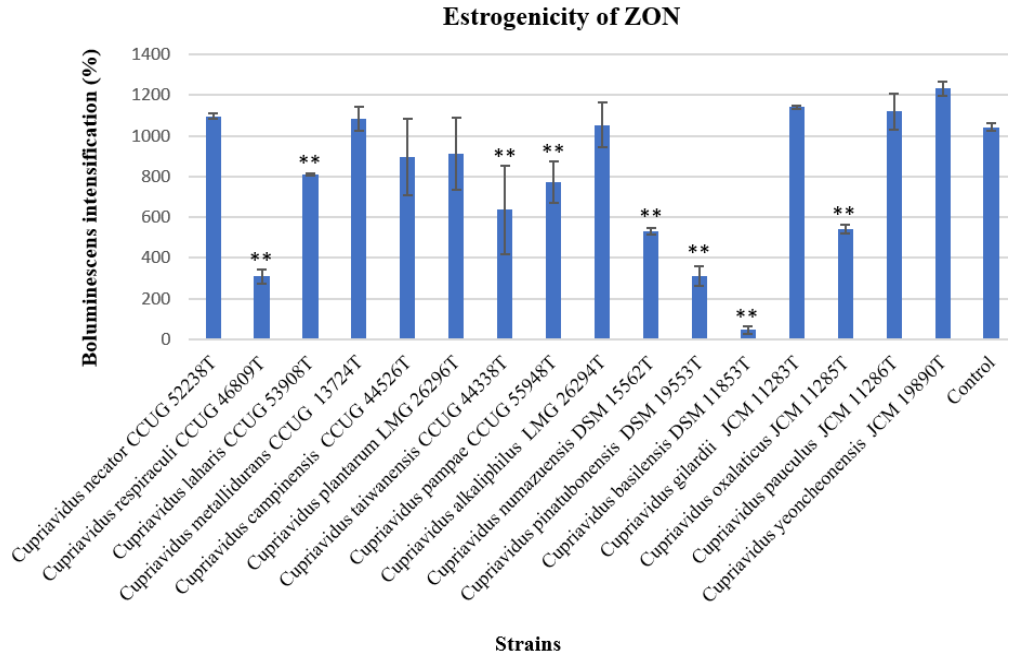


Figure 21: Oestrogenicity in supernatant samples of *Cupriavidus* type strains derived from ZON biodegradation experiment measured by BLYES test. Oestrogenic effect was expressed in bioluminescence intensification (%). Two ** means significant difference (p<0.02) compared to the control

3.4.3 Biodegradation of OTA

Out of the 16 type strains, six strains were the most effective. *Cupriavidus taiwanensis* CCUG 44338 ^{T} showed the highest OTA-reduction rate (97 %), *Cupriavidus alkaliphilus* BCCM 26294 showed 95%, *Cupriavidus basilensis* RK1 DSM 11853^{T} showed 94 %, *Cupriavidus necator* CCUG 52238^{T} showed 92%, *Cupriavidus pinatubonensis* DSM 19553^{T} showed 88%, *Cupriavidus numazuensis* DSM 15562^{T} showed 85% and *Cupriavidus respiraculi* CCUG 46809^{T} showed 82% respectively as demonstrated in Table 13. The highest OTA concentration on cell pellet (7 ng/mL) was observed in case *Cupriavidus numazuensis* DSM 15562^{T} strain; this strain had high degradation ability (85%).

3.4.4 Biodegradation of trichothecene mycotoxins

3.4.4.1 Biodegradation of T-2

Out of the 16 type strains, six strains were able to degrade T-2, as shown in Table 13. The highest biodegradation rate was 95%, which was achieved by *Cupriavidus gilardi* JCM 11283^{T} strain. Other five strains showed moderate (68-88%) T-2 biodegradation rate: *Cupriavidus metallidurans* CCUG 13724^{T}, *Cupriavidus numazuensis* DSM 15562^{T}, *C. pinatubonensis* DSM 19553^{T}, *Cupriavidus basilensis* DSM 11853^{T} and *Cupriavidus plantarum* LMG 26296^{T}. The rest of the 16 type strains had weak biodegradation potential. T-2 concentration in the pellets was among 22 and 50 ng/mL.

3.4.4.2 Biodegradation of DON

According to the analytical results, none of the 16 type strains could degrade DON

3.4.5 Ability to degrade more than one mycotoxin

Strains which are able to degrade two mycotoxins:

C. respiraculi CCUG 46809^{T} type strain could degrade 82% of OTA and 64% of ZON.

Cupriavidus laharis CCUG 53908^{T} strain could degrade 91% of AFB1 and detoxificate the genotoxic effects of the metabolites and degrade 61% of ZON.

Cupriavidus metalliduriens CCUG 13724^{T} strains could degrade 77% of AFB1 and 72% of T-2.

Cupriavidus plantarum BCCM/LMG 26296^{T} strain could degrade 67% of ZON and 60% of T-2. *Cupriavidus taiwanensis* CCUG44338^{T} strain could degrade 97% of OTA and 63% of AFB. *Cupriavidus oxalaticus* JCM 11285^{T} strain could degrade 82% of AFB1 and and detoxificate the genotoxic effects, and also degrade 82% of ZON.

Strains which are able to degrade more than two mycotoxins:

Cupriavidus pinatubonensis DSM 19553^{T} strain was able to degrade 90% of ZON, 88% of OTA and 68% of T-2.

Cupriavidus basilensis DSM 11853^{T} strain could degrade 96% of ZON it could eliminate the oestrogenic effect of the metabolites, showed 94% of OTA and 68% of T-2 degradation.

Cupriavidus numazuensis DSM 15562^{T} strain was the most effective in degradation of mycotoxins, since it reduced 70% of T2, 72% of AFB1, 85% of ZON and 85% of OTA.

Table 13: 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 biodegradation efficiency (%)	Genotoxicity (IF)	ZON biodegradation efficiency (%)	Oestrogenicity (Biol. int. %)	OTA biodegradation efficiency (%)	T2 biodegradation efficiency (%)
Cupriavidus alkaliphilus BCCM 26294^{T}	58	2.83 ± 0.14	33	1053 ± 110	95	52
Cupriavidus basilensis DSM 11853^{T}	19	2.71 ± 0.29	96	47 ± 19	94	68
Cupriavidus campinensis CCUG 44526^{T}	61	2.60 ± 0.09	55	894 ± 187	28	55
Cupriavidus gilardii JCM 11283^{T}	32	3.45 ± 0.34	35	1140 ± 9	19	95
Cupriavidus laharis CCUG 53908^{T}	91	1.31** ± 0.03	61	811 ± 6	20	27
Cupriavidus metallidurans CCUG 13724^{T}	77	2.27 ± 0.15	51	1084 ± 59	27	73
Cupriavidus necator CCUG 52238^{T}	31	3.44 ± 0.26	47	1092 ± 14	92	47
Cupriavidus numazuensis DSM 15562^{T}	72	1.93 ± 0.11	85	530 ± 16	85	70
Cupriavidus oxalaticus JCM 11285^{T}	82	0.97** ± 0.14	82	541 ± 21	19	50
Cupriavidus pampae CCUG 55948^{T}	60	2.82 ± 0.10	50	772 ± 101	30	47
Cupriavidus pauculus JCM 11286^{T}	41	3.04 ± 0.51	42	1118 ± 90	20	42
Cupriavidus pinatubonensis DSM 19553^{T}	17	2.90 ± 0.28	91	312 ± 50	88	68
Cupriavidus plantarum BCCM 26296^{T}	59	2.81 ± 0.12	67	911 ± 177	14	60
Cupriavidus respiraculi CCUG 46809^{T}	51	3.22 ± 0.12	64	310 ± 34	82	47
Cupriavidus taiwanensis CCUG 44338^{T}	63	2.93 ± 0.20	42	636 ± 215	97	56
Cupriavidus yeoncheonensis JCM 19890^{T}	45	3.34 ± 0.32	41	1232 ± 36	12	40

Strains having more than 70% biodegradation ability
Strains causing biodetoxification

4 DISCUSSION

4.1 1st objective: Investigating the effect of the OTA biodegradation by-product of *C. basilensis*-ŐR16 on human kidney cell line by gene expression using qPCR by novel biotest method

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 ^{o}C 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 were 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 analyzed, 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 tumor 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 in order 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 of the 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 Luhe et al., 2003 showed that there is over expression in the DNA damage indicators *gadd45* and *gadd153,* and also 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 housekeepeing 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 housekeepeing genes *(hprt, β-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 *β-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.

New scientific result:

Thesis 1: The biodetoxification was proved by the gene expression experiment; the by-product of the OTA biodegradation of the strain ŐR16 did not alter the expression of the target genes in human kidney cell line 786O

4.2 2nd objective: Identification of the enzymes of the *C. basilensis*-ŐR16 playing role in the biodegradation of the OTA by transcriptome analysis

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 colleages 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.

New scientific result:

Thesis 2.: Evaluating and identification of 15 genes and proteins responsible for OTA degradation used by *Cupriavidus basilensis* ŐR16 bacteria strain via transcriptome analyses from total RNA from an OTA biodegradation matrix

4.3 3rd objective: Verification of the nominated OTA-degrading CPA enzymes by cloning and expression

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, which is able to express the tRNA genes for arginine (AGA and AGG), isoleucine (AUA) and leucine (CUA) rare codons. 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. This indicates that the recombinant CPA proteins were secreted into the medium as confirmed by SDS-page despite the bands were weak, whereas , did not secreted in Luizzi's experiment.

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)

The **first proof** for successful *E. coli* BL21 (DE3) pLysE transformation was the growing of the BL21 on kanamycin containing agar. After this step the optimal induction circumstances were searched. One of the most effective induction was carried on by 30 C, for 5 hours and half, with 500 µL of 1 mM IPTG. From the supernatant of this process SDS-page gel running was carried out, where the bends were weak, but where at the estimated kilodalton size, according the leather, **second proof**.

From the same supernatant MALDI-TOF measurement was carried out, where the peaks of the cloned CPAs could be seen on the chromatogram, but again in weak quality, **third proof**.

From the same culture purification of the expressed proteins occurred after sonication of the pellet via Nickel columns (Ni-NTA Resin columns gravity flow method). Nanodrop protein concentration of the purified supernatant showed 10 times higher concentration than the control, uninduced BL21 *E. coli* , **fourth proof**.

Finally, a new induction process was carried out (changes: 37 ℃ degree, and 500 µL of 1 mM IPTG). From this culture the cell was centrifugated, and the cell pellet was sonicated in the optimal buffers. Again, centrifugated and the protein containing supernatant was separated. OTA biodegradation experiment was carried out by this supernatant, and the CPA containing samples were showing 50-65% OTA degradation (**fifth proof**) compared to the control. Degradation period was 16 hours on 37 ºC degrees. In the presented study, the cloned CPAs were able to degrade around 65 % of 1 mg/ ml OTA in 16 h / 37 ˚C.

New scientific result:

Thesis 3.: Isolating three CPA gene and protein of the *Cupriavidus basilensis* ŐR16 bacteria responsible for OTA degradation via cloning and expression.

4.4 4th objective: Valuation of the mycotoxin biodegradation ability of the *Cupriavidus* genus type strains

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. In the case of ZON only one *Rhodococcus* type strain could cease the oestrogenic 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 oestrogenic 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 starins which are able to degrade two myvotoxins. 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.

New scientific results:

Thesis 4: Evaluation of the mycotoxin biodegradation potential of the *Cupriavidus* bacteria genus type strains.

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

Thesis 6: Two type strains were able to reduce the endocrine disrupting effect of the metabolites of ZON: *Cupriavidus basilensis* DSM 11853^{T} and *Cupriavidus pinatubonensis* DSM 19553.

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

4.5 Novel scientific achievements

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 oxalatixus* 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.

4.6 The practical application possibilities of the obtained results

The result of the cell line experiment is a supporting study about the non-hazardous effect of the ŐR16 strain on human cells. That is the highest level of toxicity test, what can be achieved in the case of an experiment like this.

The result of the transcriptome analyses it is not completely done, there are still 13 genes, which can be tested after cloning and not just in the case of the degradation of mycotoxins. Maybe it can be useful for degradation of aromatic hydrocarbons also.

The isolated CPA-s can be used as feed additive enzymes to animal fodder, if they are stable enough, or the stability and effectiveness can be stabilized by different methods, like fixation on biopolymers, etc. A lot of tests should be proceeding in the future.

The valuation of the mycotoxin biodegradation potential of 16 type strains of *Cupriavidus* genus can be a base of the enzyme engineering in a future study together by the molecular biotechnological data (existing genome projects, etc) .

5 CONCLUSIONS AND SUGGESTIONS

In this dissertation, the focus was on the main 4 aims.

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 oxalatixus* were able to biodegrade and biodetoxificate and cease the genotoxicity of AFB1. Furthermore, *Cupriavidus basilensis* was able to decrease the 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.

7 SUMMARY

The first goal of the PhD dissertation was to investigate the effect of the OTA biodegradation by-product of *C. basilensis*-ÖR16 on human kidney cell line by gene expression using RT-PCR by novel biotest method. Measuring the gene expression profile of *gadd45*, *gadd153*, *annexin2* and *clusterin*. I used bacteria free inoculum after 5 days of biodegradation, the exposition of the cells was 48 hours long. For control, the following household genes were used *β-actin*, *hprt*, *gapdh*. Methyl-ester methanesulfonate (MMS) was used as a genotoxic control. The results of this experiment showed that OTA was completely degraded (100%) by the 5th day of the biodegradation. In the case of ÖR16 by-product, the target gene expression was less than the OTA results ($p \leq 0.05$). The OTA biodegradation by-products have smaller toxic effects on the cell line ($p \leq 0.05$), than in the case of 2 and 10 mg / l OTA. This novel biotest proved that *C. basilensis* ÖR16 bacteria was able to biodetoxificate OTA in elevated concentrations, without any harmful by-products for human kidney cell line.

The second goal was the identification and verifications of the genes in the *C. basilensis* ÖR16 genome, playing role in the biodegradation of the OTA by transcriptome analysis. A biodegradation experiment for OTA in a minimal buffer was performed. Then total RNA was isolated from *Cupriavidus basilensis* ÖR16 in the presence and without OTA for the transcriptome analysis. This biodegradation experiment was conducted in 11 hours (to get good quality RNA in the log phase of the ÖR16). The transcriptome results showed that there are 3500 gene was upregulated in the presence of OTA compare to the control group (RNA without OTA). After intensive research in the literature 15 up-regulated genes, were chosen 13 according to their hypothetical role in OTA degradation. From that 3 CPA genes were chosen for further investigation via cloning and expression based on a study of Luizi and colleagues from 2017.

The third goal was the cloning and the expression of three chosen CPA genes. The three genes were synthesised by GenScript company for cloning. The genes are named as CPA1, CPA2 and CPA3. The expression of the genes was confirmed by SDS-page and MALDI-TOF, Ni-column purification. An OTA degradation experiment was performed by the expressed protein supernatant, and the OTA concentration was reduced by 65% during 16 hours on 37 ^{o}C degrees compared the control.

The fourth goal of my PhD research was the evaluation of the mycotoxin's biodegradation ability (AFB1, OTA, ZON, T-2 and DON) of the *Cupriavidus* genus type strains. Out of the 19 type strains of this genus, only 16 were investigated. The results showed that four strains can degrade AFB1, four strains can degrade ZON, seven strains can degrade OTA and three strains can degrade

T-2 over 70%. None of them can degrade DON. The biodetoxification was measured by different biotests. SOS Chromotest was used for detecting genotoxicity of AFB1 by-products, BLYES test was applied for the evaluation of the oestrogenicity of ZON by-products. Two strains *Cupriavidus laharis* CCUG 53908^T and *Cupriavidus oxalaticus* JCM 11285^T reduced the genotoxicity of AFB1. *Cupriavidus basilensis* DSM 11853^T decreased the oestrogenic effects of ZON. There were 6 strains which were able to biodegrade two mycotoxins. Two strains *Cupriavidus pinatubonensis* DSM 19553^T and *Cupriavidus basilensis* RK1 DSM 11853^T degraded 3 toxins (ZON, OTA, T-2) and *Cupriavidus numazuensis* DSM 15562^T degraded four mycotoxins (AFB1, ZON, OTA, T-2), which is a unique phenomenon among bacteria. According to the presented results, mycotoxins degrading bacteria of the *Cupriavidus* genus type strains that were presented in my research are providing an ideal opportunity for the biodetoxification methods.

Other genes which revealed by the transcriptome analysis of *Cupriavidus basilensis* ŐR16 should be investigated later in the future for their OTA-biodegradation and detoxification potential. To be more specific, the following genes need to be tested; ŐR16_12645 coded membrane CPA (penicillin-binding protein), ŐR16_24100 coded membrane proteins related to metalloendopeptidases, ŐR16_31869 coded membrane CPA (penicillin-binding proteins PbpC), ŐR16_31894 coded phenylalanine-4-hydroxylase and ŐR16_16257 coded aromatic ring hydroxylase as shown in Table 8.

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(If you are grateful, I will surely increase you in favour)

Allah, the greatest, has spoken the truth
The holy Quran

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11 SUPPLEMENTARY MATERIALS

Supplementary material 1

This is connected to objective 1, which is the cell line experiment. The table is showing the extracted RNA concentrations.

The RNA concentrations for all samples using Nanodrop, ThermoFisher

Sample rank	Sample Name	RNA on gel	RNA conc. ng/µl	A 260/280	A 260/230
1	**NT**	Confirmed	**274**	2.362	1.079
2	**NT**	Confirmed	**248**	2.296	1.181
3	**NT**	Confirmed	**288**	2.323	0.947
4	**OTA 10**	Confirmed	**296**	2.349	0.993
5	**OTA 10**	Confirmed	**230**	2.212	0.81
6	**OTA 10**	Confirmed	**304**	2.235	0.813
7	**OTA 2**	Confirmed	**236**	2.314	1.229
8	**OTA 2**	Confirmed	**202**	2.349	1
9	**OTA 2**	Confirmed	**246**	2.236	1.309
10	**OTA 10/ŐR16_1**	Confirmed	**232**	2.417	1.105
11	**OTA 10/ŐR16_1**	Confirmed	**256**	2.246	1.067
12	**OTA 10/ŐR16_1**	Confirmed	**256**	2.169	0.992
13	**OTA 10/ŐR16_2**	Confirmed	**284**	2.29	1.379
14	**OTA 10/ŐR16_2**	Confirmed	**450**	2.296	1.082
15	**OTA 10/ŐR16_2**	Confirmed	**422**	2.371	0.861
16	**OTA 10/ŐR16_3**	Confirmed	**298**	2.443	1.164
17	**OTA 10/ŐR16_3**	Confirmed	**332**	2.274	1.018
18	**OTA 10/ŐR16_3**	Confirmed	**298**	2.224	1.173
19	**OTA 2/ŐR16_1**	Confirmed	**388**	2.31	0.782
20	**OTA 2/ŐR16_1**	Confirmed	**328**	2.31	1.045
21	**OTA 2/ŐR16_1**	Confirmed	**290**	2.302	1.295
22	**OTA 2/ŐR16_2**	Confirmed	**340**	2.329	1.214
23	**OTA 2/ŐR16_2**	Confirmed	**264**	2.276	1.091
24	**OTA 2/ŐR16_2**	Confirmed	**336**	2.366	1.012
25	**OTA 2/ŐR16_3**	Confirmed	**308**	2.333	1.351
26	**OTA 2/ŐR16_3**	Confirmed	**294**	2.262	1.105
27	**OTA 2/ŐR16_3**	Confirmed	**298**	2.328	1.042
28	**ŐR16**	Confirmed	**274**	2.322	1.37
29	**ŐR16**	Confirmed	**276**	2.262	1.5
30	**ŐR16**	Confirmed	**300**	2.273	1.339
31	**MMS 10**	Confirmed	**118**	2.565	1.513
32	**MMS 10**	Confirmed	**88**	2.933	1.048
33	**MMS 10**	Confirmed	**34**	5.667	0.354
34	**MMS 2**	Confirmed	**278**	2.279	1.479
35	**MMS 2**	Confirmed	**278**	2.317	1.986
36	**MMS 2**	Confirmed	**244**	2.302	1.386

Supplementary material 2

This is related to objective 1 (cell line experiment) Gene expression data.

A: 2 log fold expression of the target genes normalized to HPRT

Treatments (Ref to HPRT)	Gadd45 Mean ± SD	Gadd153 Mean ± SD	Annexin2 Mean ± SD	Clusterin Mean ± SD
MMS10	-3.33 ± 0.01934	2.177 ± 1.01576	0.272 ± 0.10278	1.13 ± 0.45664
OTA10	-1.669 ± 0.02347	5.252 ± 0.95881	0.267 ± 0.04192	1.205 ± 0.30382
OTA10+OR16	-0.802 ± 0.05813	2.118 ± 0.45189	-0.356 ± 0,03478	-0.041 ± 0.00792
MMS2	-2.787 ± 0.01755	5.373 ± 1.16808	0.119 ± 0.02181	0.651 ± 0.15106
OTA2	-1.612 ± 0.02821	4.874 ± 0.83694	0.368 ± 0.05154	1.256 ± 0.23789
OTA2+OR16	-1.386 ± 0.03636	1.224 ± 0.72576	0.266 ± 0.04309	0.72 ± 0.14553
OR16	-2.151 ± 0.02427	1.041 ± 0,48634	-0.295 ± 0,05641	0.423 ± 0.13317

B: 2 log fold expression of the target genes normalized to ß -actin

Treatments Ref to ß - actin	Gadd45 Mean ± SD	Gadd153 Mean ± SD	Annexin2 Mean ± SD	Clusterin Mean ± SD
MMS10	-1.402 ± 0.1197	4.427 ± 2.04302	2.522 ± 0.93704	3.38 ± 1.34595
OTA10	-1.605 ± 0.03526	5.403 ±1.35709	0.331 ± 0.0708	1.768 ± 0.41919
OTA10+OR16	-1.328 ± 0.11063	1.805 ± 0.82287	-0.461 ± 0.10634	-0.128 ± 0.04883
MMS2	-1.838 ± 0.05132	6.322 ±1.83964	1.068±0.28458	1.6 ± 0.48335
OTA2	-1.366 ± 0,0458	5.023 ± 1.16525	0.517 ± 0.10838	1.405 ± 0.34473
OTA2+OR16	-1.845 ± 0.05401	0.188 ± 0.12603	-0.355 ± 0.08044	0.284 ± 0.12599
OR16	-1.8 ± 0.03651	0.215 ± 0.05611	0.056 ± 0.01715	0.198 ± 0.07001

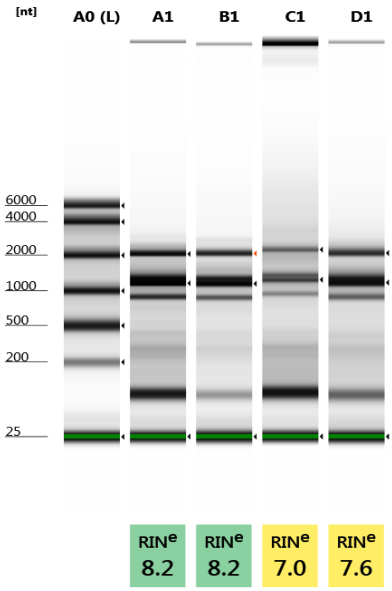
Supplementary material 3

This material is connected to the objective 2, transcriptome analysis. The RNA validation was carried out by Seqomics Ltd.

ŐR16 RNA quality and quantity test data.

The compact Agilent 2200 TapeStation a system automates RNA quality control (QC), including sample loading, separation, and imaging. The system Includes 2200 TapeStation instrument, laptop with 2200 TapeStation software, accessories, consumables, and user information. By Agilent 2200 Technologies TapeStation, RNA samples of ŐR16 was added to the chip and run via the equipment to measure the RNA quality and to show up the 16s and 23s peaks of the samples and then gives the RNA integrity number (RIN) results.

Agilent 2200 Technologies TapeStation

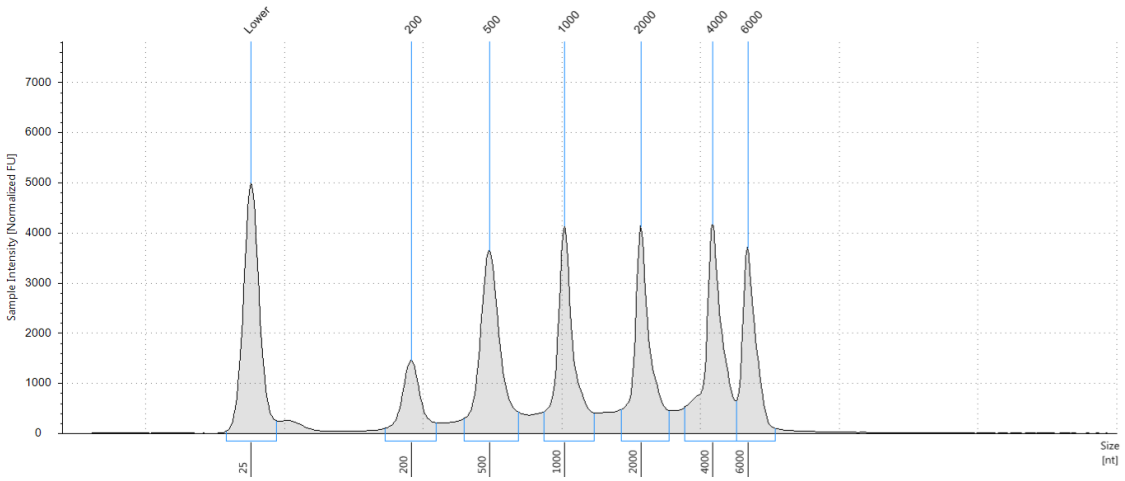


The gel-like image obtained with the Agilent 2200 TapeStation systems. The automatically determined RIN values are shown Table 4 in the gel-like image

A0 =Electronic ladder, ŐR16_1=A1, ŐR16_2=B1, OTA+ŐR16_1=C1, OTA+ŐR16_2=D1

Table 4.: RNA quality results of the different setting from the OTA degradation matrix in minimal buffer from the Agilent 2200 Technologies (Seqomics Ltd, Mórahalom, Hungary), RIN =RNA integrity number

Sample Description	23S/16S (Area)	Conc. [ng/µl]	RIN
Electronic Ladder	-	84.9	-
OR 16_1	0.8	99.2	8.2
OR 16_2	0.7	57.8	8.2
OTA OR 16_1	1.3	92.3	7.0
OTA OR 16_2	0.5	70.1	7.6

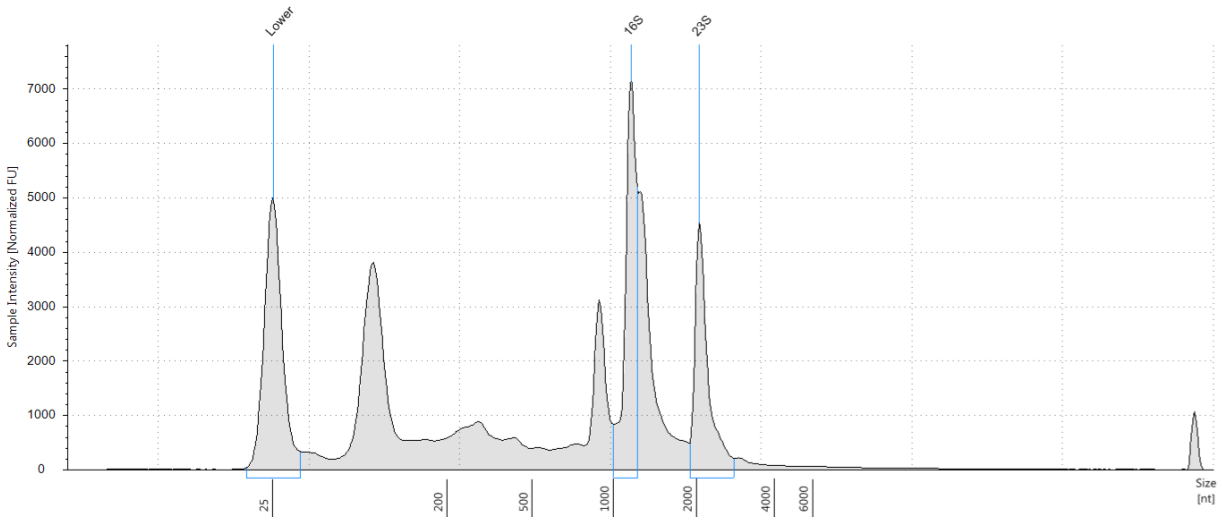


Well	RIN[e]	23S/16S (Area)	Conc. [ng/µl]	Sample Description
A0	-	-	84.9	Electronic Ladder

Peak Table

Size [nt]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Observations
25	40.0	40.0	4710	-	Lower Marker
200	5.94	-	87.4	7.80	
500	15.9	-	93.6	20.88	
1000	14.2	-	41.8	18.62	
2000	13.8	-	20.3	18.11	
4000	15.5	-	11.4	20.38	
6000	10.8	-	5.31	14.22	

A1: OR 16_1



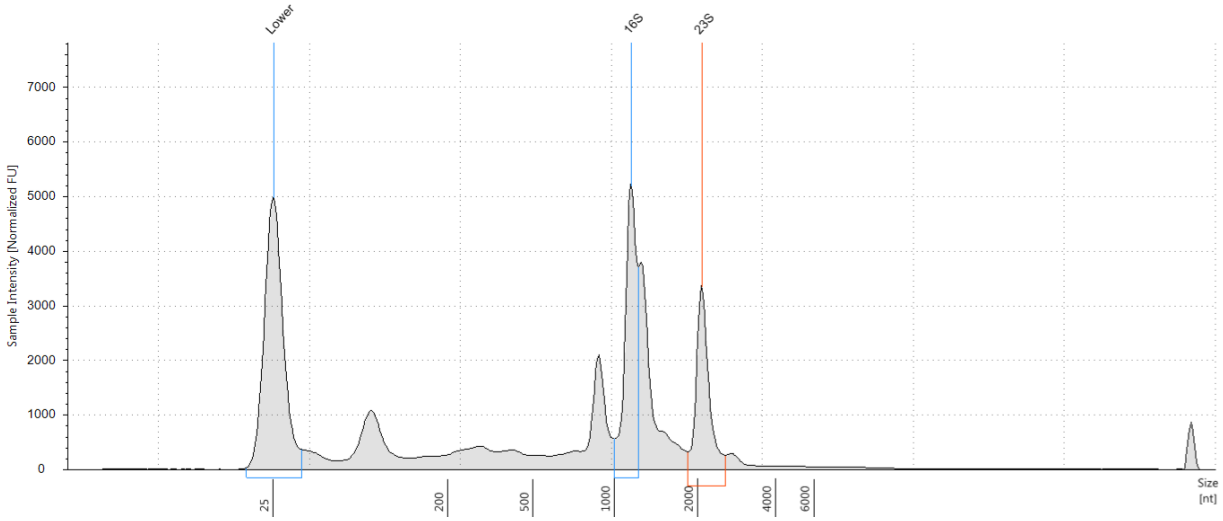
(B) Sample Table

Well	RIN[e]	23S/16S (Area)	Conc. [ng/µl]	Sample Description
A1	8.2	0.8	99.2	OR 16_1

Peak Table

Size [nt]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Observations
25	40.0	40.0	4710	-	Lower Marker
1154	16.7	-	42.5	56.17	16S
2043	13.0	-	18.7	43.83	23S

B1: OR 16_2



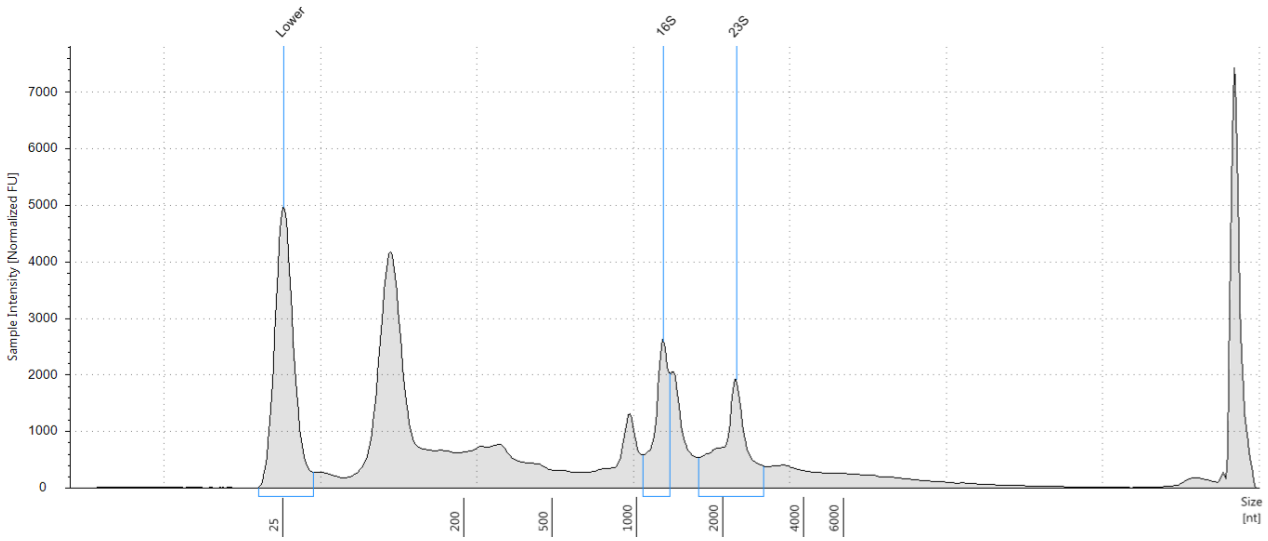
Sample Table

Well	RIN[e]	23S/16S (Area)	Conc. [ng/µl]	Sample Description
B1	8.2	0.7	57.8	OR 16_2

Peak Table

Size [nt]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Observations
25	40.0	40.0	4710	-	Lower Marker
1145	12.4	-	31.7	58.33	16S
2066	8.83	-	12.6	41.67	23S

C1: OTA OR 16_1



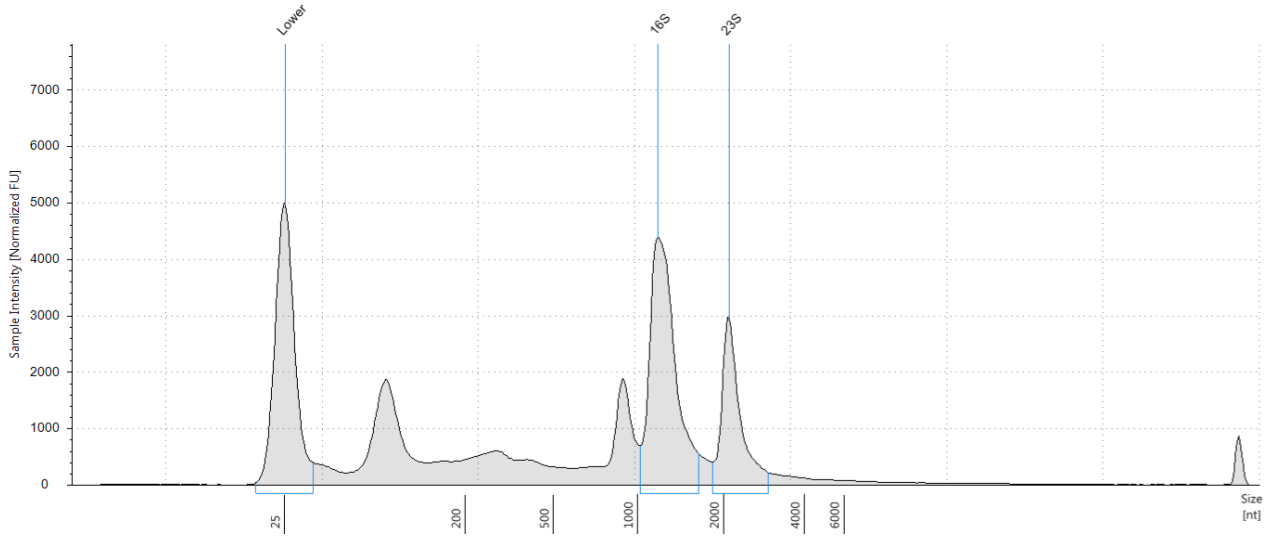
Sample Table

Well	RIN[e]	23S/16S (Area)	Conc. [ng/µl]	Sample Description
C1	7.0	1.3	92.3	OTA OR 16_1

Peak Table

Size [nt]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Observations
25	40.0	40.0	4710	-	Lower Marker
1236	7.52	-	17.9	43.37	16S
2232	9.82	-	12.9	56.63	23S

D1: OTA OR 16_2



Sample Table

Well	RIN[e]	23S/16S (Area)	Conc. [ng/µl]	Sample Description
D1	7.6	0.5	70.1	OTA OR 16_2

Peak Table

Size [nt]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Observations
25	40.0	40.0	4710	-	Lower Marker
1169	22.6	-	56.8	67.67	16S
2084	10.8	-	15.2	32.33	23S

Supplementary material 4

This material is connected to objective 3. The genes encodes the three CPAs proteins sysnthesis was done by GenScript Co., USA**.** The sequences came from the genome project of ŐR16.

Gene name: D-alanyl-D-alanine carboxypeptidase (CPA1) sequence
GenBank link:
https://www.ncbi.nlm.nih.gov/nuccore/AHJE01000058

ATGTTGAATAGAGCCACGCCGCTTTTCGCGTCCGCCAACACGTGCGCCCTGGTCACC
GCTGCCATCGTCGGCGCCGCCGTGGTGCTTGCCCCGTCTCCCGCGCGCGCGCAGGGC
GTGCCGATGCCGCAGGTGGCGGCCAAGTCCTGGATGCTGTTCGACGTCACCAGCGG
CCAGGCCCTGGCTTCGCAGAACGCCGATGTGCGCATCGAGCCGGCTTCGCTGACCA
AGCTGATGACCGCCTACCTGGCGTTCGCGGCACTCAAGGAAAAGCGCCTCACGATC
GACCAGACCGTGGTGCCGACCACGATCGTGCAGAAGGTCAAGAGCGACGAATCCCG
CATGTTCCTGGAAGCCAACAAGCCGGTCAGCGTGCAGGACCTGTTGCTGGGCCTGAT
CGTGCAATCGGGCAATGACGCGGCGCTGGCACTGGCCGAAGCCGTGGGCGGCTCGG
AAGAGGGCTTCGTGGCGATGATGAATCGCGAGGCCCAGCGGATGGGCATGAAGAAC
ACCCACTTCAGCAACACCGACGGCATCCCCGACCCGAACCACTACACCACGGCCAT
GGATCTGGCCACGCTGACCACGCGGATCATCAAGGACTTCCCCGAGTACTACAGCA
TGTACTCGCAGAAGGAATTCACTTACAACAAGATCCGCCAGCCCAACCGCAACCGC
CTGCTCTACATCGACCCCACTGTGGACGGCCTCAAGACCGGCCACACCAAGTCGGCC
GGTTATTGCCTGATCTCGTCGGCCAAGCGTCCGCTGGCCAATGTGCCTAACGGTTCG
CGCCGCCTGGTCTCGATCGTGATCGGCACCACCACCGAAGCGGTCCGCACGCAGGA
AAGCCTGAAGATCCTGAACTACGGCTTCCAGTTCTTCGACACGCTGCGCCTGTACGA
CCGCGGCCAGGTGCTGGCCACGCCCGAGATCTACAAGGGCAAGGAATCCACCGTCA
AGATCGGCGTCAAGGACGAGACCTACATCACCGTGCCCAAGGGCACGGGCGGGCGC
ATCAAGCCCGTGCTGGAGCGCCAGGAACTGCTGGTGGCACCGCTCGCCGCCGGCCA
GCAGGTCGGCACCGTCAAGCTGATGGACGGCGCCACCAAGGTGGCCGAGTTCCCGG
TTGTGGCGCTGGAAGACGTGCCCGAAGCGGGCTTCTTCGGCCGCCTGTGGGATACCA
TCCGCTTGTGGTTCAAGCGCAAGTAA

Amino acid order of the CPA2, according the NCBI ŐR16 genome project

CPA1 translation=. "MLNRATPLFASANTCALVTAAIVGAAVVLAPSPARAQGVPMPQV
AAKSWMLFDVTSGQALASQNADVRIEPASLTKLMTAYLAFAALKEKRLTIDQTVVPTTI
VQKVKSDESRMFLEANKPVSVQDLLLGLIVQSGNDAALALAEAVGGSEEGFVAMMNR
EAQRMGMKNTHFSNTDGIPDPNHYTTAMDLATLTTRIIKDFPEYYSMYSQKEFTYNKIR
QPNRNRLLYIDPTVDGLKTGHTKSAGYCLISSAKRPLANVPNGSRRLVSIVIGTTTEAVR
TQESLKILNYGFQFFDTLRLYDRGQVLATPEIYKGKESTVKIGVKDETYITVPKGTGGRI
KPVLERQELLVAPLAAGQQVGTVKLMDGATKVAEFPVVALEDVPEAGFFGRLWDTIRL
WFKRK"

Gene name: Metal-dependent amidase/aminoacylase/carboxypeptidase, CPA2 Sequence:

GenBank link
https://www.ncbi.nlm.nih.gov/nuccore/AHJE01000017

ATGACCCGCGAACCGACCCTGACGCCCTTCCAGTTGCTGCCGCACCTGCTG
CCGGCGATCCAGATCGATGCCGAGACCTTTATCGGCATCCGCCGCCAGATT
CACGCCCAGCCAGAACTTGGCTTCGAGGTCGGCGCCACCAGCAAGCTGGTG
GCCACCCTGCTGGAAAGCTGGGGCTACGAGGTGCACACGGGCATCGGCAA
GAGCGGCGTGGTGGGACAGCTGAAGCTGGGCAACGGCCAGCGCCGCCTGG
GCATTCGCGCCGACATGGATGCGTTGCCGGTCGTCGAGGCTACGGGCCTGC
CGTATGCCAGCAAGATTCCGGGGAAGATGCACGCGTGCGGCCATGACGGC
CACACCGCCATCCTGCTGGCCGCCGCCAAGGCGCTGGCGGATAGCCGCGAT
TTCGATGGCACCCTCAACCTGATCTTCCAGCCCGACGAGGAAAACCTCTGT
GGCGCGCGCGCGATGATCGAGGATGGCCTGTTCGAGCGTTTCCCGTGCGAT
GCCGTGTTCGCCCTGCACAACATGCCGGGGGTGCCCGCCGGCACCTTCCGC
GTGCTGCCCGGCCCGGTGAGCTTATCGTCCGACGTGGCCGACGTGACCATC
AAGGGCGTGGGCGGGCATGGCGCCATGCCGCATCGCGCGCGCGATCCGAT
CGCGGCGTCCGCGGCTATCGTCACAGCGCTGCAAACGGTGGTGGCGCGCA
ATGTGGCGCCGGACGATACGGCCGTGCTATCGGTGGGGTTTATCCGGGGTG
GCGCCACGCACAACGTGATTCCGGAATCGGTCACGCTGGGCCTGAATGTGC
GCGCGGCGCGCCCGGAGACGCGTGCGCTGGTGGAGCAGCGCATTCGCGAG
ATCGTCAGCCTGACCGCGCAGGCGCACGGCGTCGAGGCCCACATCGACTAT
CGCCAGCTGACGCCGCCGATGGTCAACACGCAGGCCGAAACCACGCTGGC
GCAGCAGGTTTGCGCTGACCTGGTCGGCGCCGACCAGGTCGTGACGCAAGC
CCCCAAGGGCCTGAACGGCAGCGAGGACTTTGCGTGGATGCTCAACGAGG
TGCCGGGCTGCTACCTGATCCTGGGCAACGGCGAAGGCGAGTTCGGCGGCT
GCATGGTGCACAACCCGGGCTACGACTTCAACGATCAGGTGCTGCCGCTGG
GCGCGGCCTGCTGGGTCCGGCTGGCCCAGACCTACCTGGCGGGCTGA,

Amino acid order of the CPA2, according the NCBI ŐR16 genome project

CPA2 translation=

"MTREPTLTPFQLLPHLLPAIQIDAETFIGIRRQIHAQPELGFEV
GATSKLVATLLESWGYEVHTGIGKSGVVGQLKLGNGQRRLGIRADMDALPVVEATGLP
YASKIPGKMHACGHDGHTAILLAAAKALADSRDFDGTLNLIFQPDEENLCGARAMIEDG
LFERFPCDAVFALHNMPGVPAGTFRVLPGPVSLSSDVADVTIKGVGGHGAMPHRARDPI
AASAAIVTALQTVVARNVAPDDTAVLSVGFIRGGATHNVIPESVTLGLNVRAARPETRA
LVEQRIREIVSLTAQAHGVEAHIDYRQLTPPMVNTQAETTLAQQVCADLVGADQVVTQ
APKGLNGSEDFAWMLNEVPGCYLILGNGEGEFGGCMVHNPGYDFNDQVLPLGAACWV
RLAQTYLAG"

Gene name: D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 4), (CPA3)
Length: 1536 bp,

GeneBank link

https://www.ncbi.nlm.nih.gov/nuccore/AHJE01000027

ATGGGCACCATGCCGAAACGCCCTTCCCCAATTCTTCGCCGCCTGCTGCCG
CTTTGCGCCGCCGGGCTGTTGTGCATCAGCGGCCTGGCCGCCGCCACCGCT
AAAACCCCCGTTCACAAGACCAAGGCCGCCCACGCCGCGGCCGCGCCCGC
CGAGCCGCGCTCGGGCCTGCCAGGCAGCGTGACAACCGCGCTCAAGCGCG
CGCACGTACCCGCCTCGGCCACCAGCTTCTACGTGATCAAGGTCGGCGCGC
CGGCGCCCCGCGTAAGCTGGAACGCGCAGACGCCGATGAACCCGGCTTCC
ACCATGAAGCTGGTCACCACCTTCGCCGGGCTCCAGCTGCTTGGCCCCGGC
TACCGCTGGCAGACTGCGCTCTATGCCGACAACCAGCCCGGTGCCGACGGC
ACCGTCAACGGCAACGTCTACCTGCGCGGCTACGGCGACCCCAAGCTGGTG
CCGGAAGAAATGGCCAAGCTGGTCAGCGCCGCCCGCACTGCAGGCGCCAC
CACCATCAACGGCGACCTGGTGCTGGACCGCAGCTACTTCGACTCCGCCCT
CGACAACGGCGCCACCATCGATGGCGAAACCCAGCGCGCCTACAACGTCA
GCCCCGACGCGCTGCTCTACGCCTTCAAGACGCTGTCGTTTACCATCACGC
CCGACCCGGCCAACCAGTCGGTCGCGGTTTCGGTCACGCCCGCGCTGGCGC
AGCTCAAGCTCGACAACCACCTCGCGCTGTCCAATGGCAAATGCGGCGACT
GGTCGGCACGCGCCCGCCCCGCCGTCACGCCGCAACCCGACGGCACGGTG
CTAGCCTCCTTCGACGGCAGCTACGCCGCCGACTGCGGCGAACATGTGGTC
AATATCGCCACGCTCTCGCACAACGAATTCGCCTGGGGCGGATTCGTCGCC
GAATGGCAACTGGCCGGCGGCCGCTTCACCCACCAGCCAGCCCTGCGCATG
GGCCGCGCACCGCGCAACGCCTTCCTGCTGGCACGCCACTACGGCCAGCCG
CTCTCGGAAATCGTGCGCGACATCAACAAGTTCTCGAACAATGTGATGGCC
CGCCAGCTTTACCTGACCATCGGCGCGGAAATGGATCGCGGCGGCCCGGCC
ACCACCACCCGCTCGGCCAAGGTCGTGCAGCGCTGGCTGGCACGCCAGGG
ACTGGACATGCCCGGCCTGGTGCTGGACAACGGCTCGGGGCTATCGCGCGA
AGAACGGATCAGTGCCTACGATATGTCGCGCCTGCTGCAGCAGGCGCTGGC
TAGCGAAGTCGGACCGGTGCTGATGGATTCGCTGCCGATCCTCGGCGTGGA
TGGCACGCTGCGCAACCGCCTGACGCGCGCCGGCGCAGCCGGCAATGCCT
ACATGAAGACGGGAACGTTGAACGATGTACGAGCGCTTGCCGGCTATGTC
GATGCGCTGAACGGCGATCGCTATGTGGTGGTCAGCTACATCAACCACGCC
AACGCAGCGCAAGCCCGCGACGCCCACGACGCCCTGTTGCAGTGGGTGTAT
CAGGGCGCACCCTGA,

Amino acid order of the CPA3, according the NCBI ŐR16 genome project

CPA3 translation
"MGTMPKRPSPILRRLLPLCAAGLLCISGLAAATAKTPVHKTKAAHAAAAPAEPRSGLPG
SVTTALKRAHVPASATSFYVIKVGAPAPRVSWNAQTPMNPASTMKLVTTFAGLQLLGP
GYRWQTALYADNQPGADGTVNGNVYLRGYGDPKLVPEEMAKLVSAARTAGATTING
DLVLDRSYFDSALDNGATIDGETQRAYNVSPDALLYAFKTLSFTITPDPANQSVAVSVTP
ALAQLKLDNHLALSNGKCGDWSARARPAVTPQPDGTVLASFDGSYAADCGEHVVNIA
TLSHNEFAWGGFVAEWQLAGGRFTHQPALRMGRAPRNAFLLARHYGQPLSEIVRDINK
FSNNVMARQLYLTIGAEMDRGGPATTTRSAKVVQRWLARQGLDMPGLVLDNGSGLSR
EERISAYDMSRLLQQALASEVGPVLMDSLPILGVDGTLRNRLRAGAAGNAYMKTGTLN
DVRALAGYVDALNGDRYVVVSYINHANAAQARDAHDALLQWVYQ"

Supplementary material 5

This material is connected to objective 3: Verification of the nominated OTA-degrading enzymes by cloning and expression. The genes synthesis and analysis were done by GenScript Co., USA.

The synthesized genes analysis results

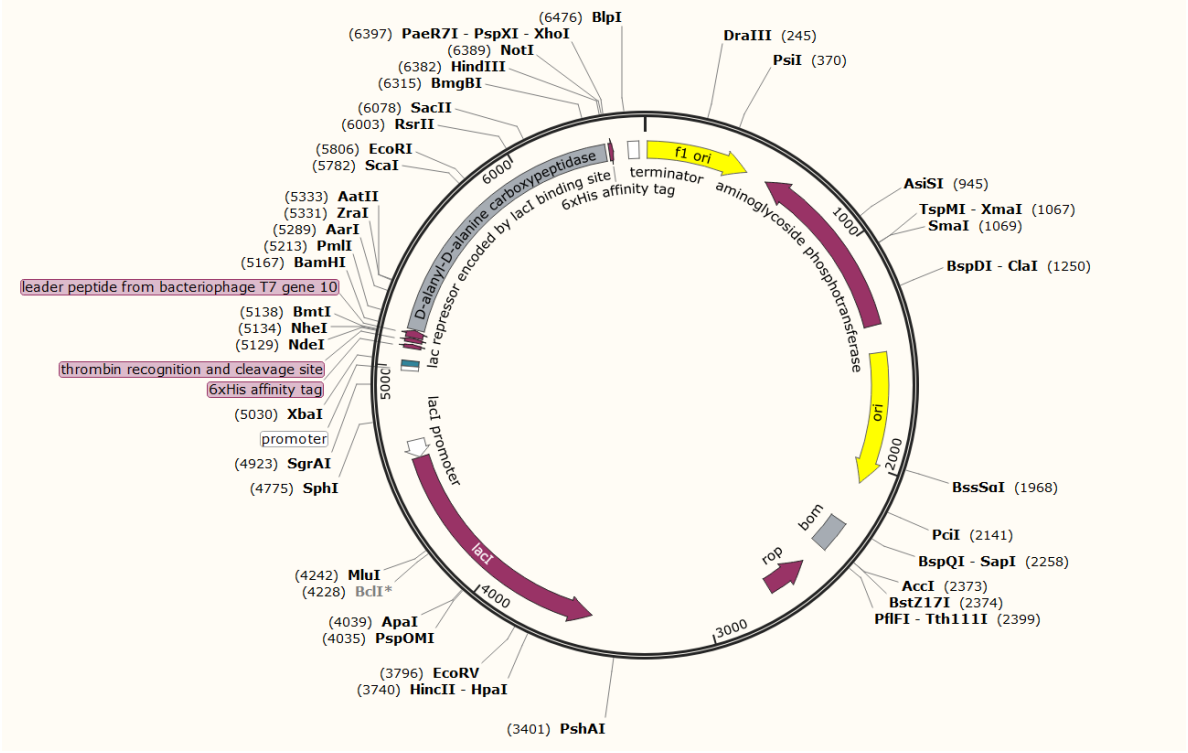
1) CPA1

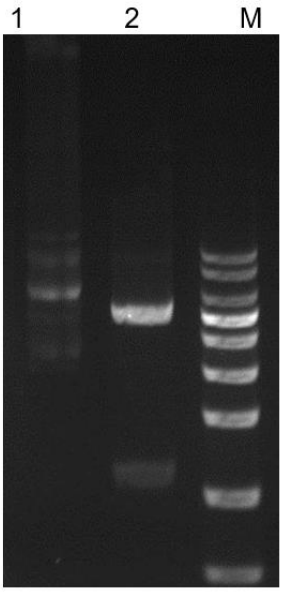
Gene Name: **D-alanyl-D-alanine carboxypeptidase (CPA1)**
Cloning vector: pET-28a (+)
Gene length: 1221 bp
Cloning strategy: BamHI / HindIII

Table 1: Summarises GenScript analysis results for the synthesis of the gene encoding- CPA1 protein

QC Items	Specifications	Results	
Sequencing Alignment	Sequencing results are consistent with the targeted insert sequence.	Pass	Consistent
Vector Sequence	The flanking sequences of the cloning site are correct.	Pass	Correct
Restriction Digests	The size of inserted fragment is correct and free of unexpected bands suggesting contamination.	Pass	Correct Shown in the digestion gel photo below
DNA Quality	Minipre 4 μg p: **OD260/280=1.8~2.0** Free of contamination	Pass	≥ 4 μg **OD260/280=1.81** Pure
Quality Grade	Research Grade	Pass	Research Grade
Appearance	Clear and free of foreign particles.	Pass	Clear Free of foreign particles

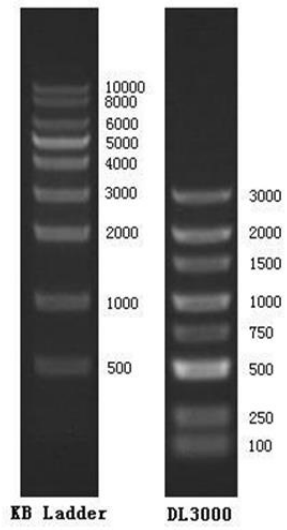
Plasmid construct map, the gene encodes CPA1 protein was ligated in pET-28a(+) by BamHI / HindIII





Lane M: KB Ladder
Lane 1: pET-28a (+) plasmid
Lane 2: pET-28a (+) plasmid digested
by BamHI and HindIII

Digestion Conditions:
About 300ng plasmid digested
Digestion in water-bath,37℃ for 40 minutes
1% Agarose Gel



D-alanyl-D-alanine carboxypeptidase (CPA1) digestion

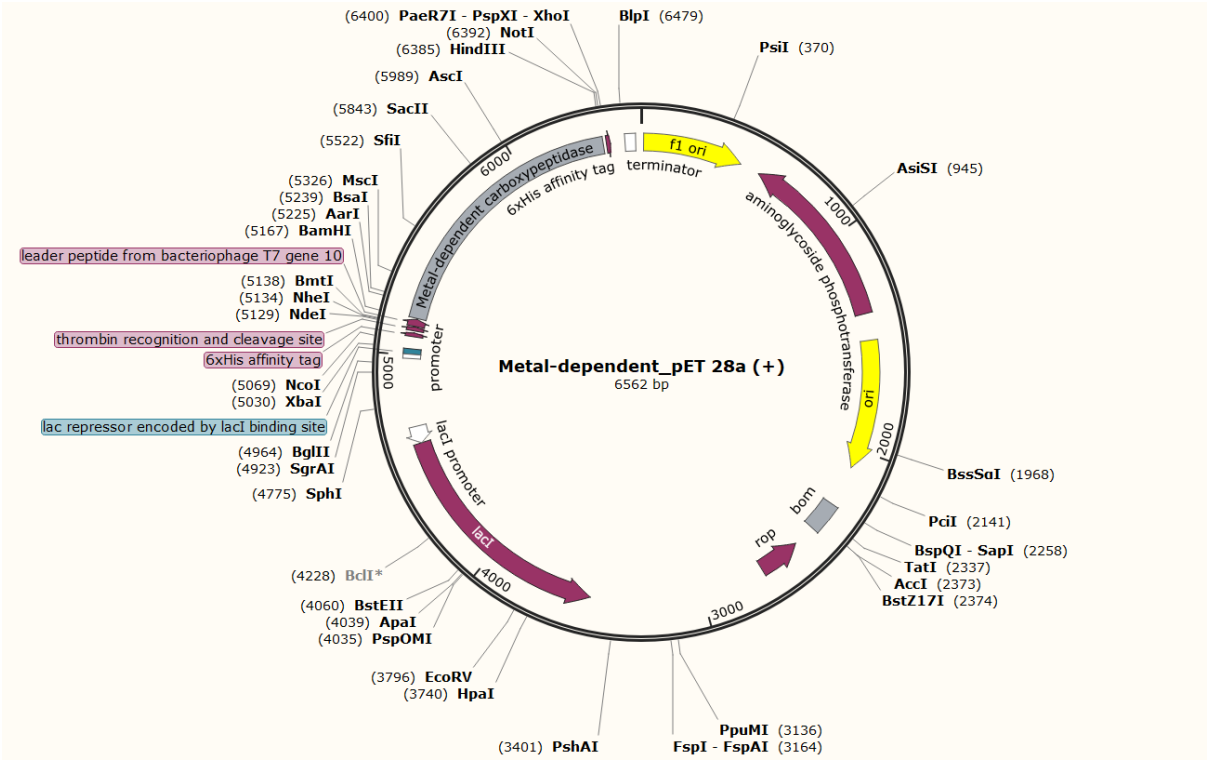
2) CPA2

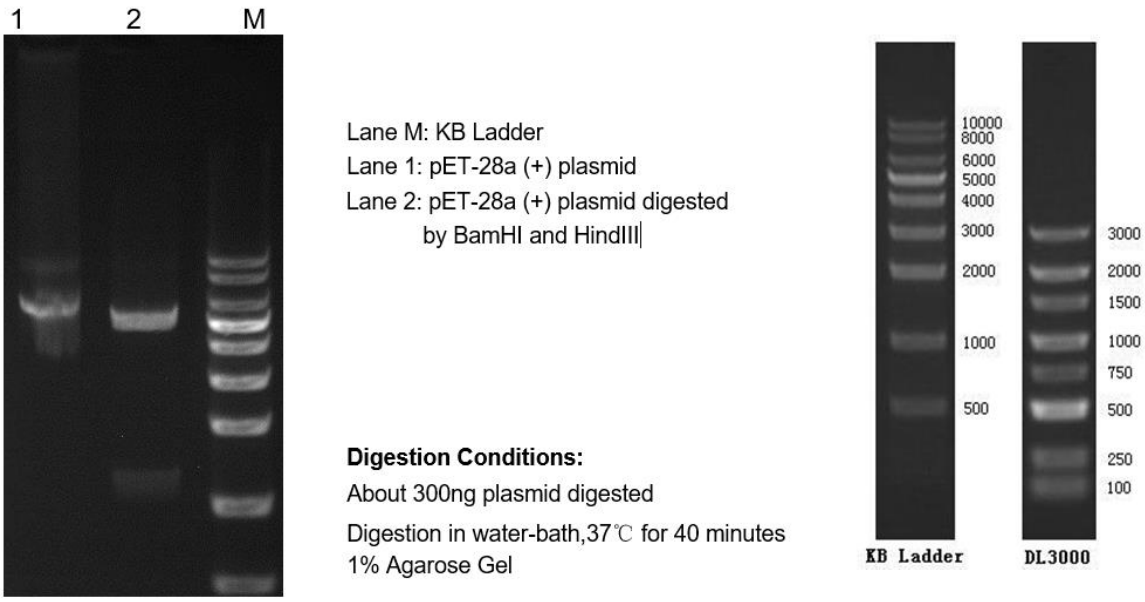
Gene Name: **Metal-dependent amidase/aminoacylase/carboxypeptidase (CPA2)**
Cloning vector: pET-28a (+)
Gene length: 1224 bp
Cloning strategy: BamHI / HindIII

Table 2: Summarises GenScript analysis results for the synthesis of the gene encoding- CPA2 protein

QC Items	Specifications	Results	
Sequencing Alignment	Sequencing results are consistent with the targeted insert sequence.	Pass	Consistent
Vector Sequence	The flanking sequences of the cloning site are correct.	Pass	Correct Shown in the SQD file (sup. material)
Restriction Digests	The size of inserted fragment is correct and free of unexpected bands suggesting contamination.	Pass	Correct Shown in the digestion gel photo below
DNA Quality	Minipre 4 μg p: **OD260/280=1.8~2.0** Free of contamination	Pass	≥ 4 μg **OD260/280=1.83** Pure
Quality Grade	Research Grade	Pass	Research Grade
Appearance	Clear and free of foreign particles.	Pass	Clear Free of foreign particles

Plasmid construct map, the gene encodes CPA2 protein was ligated in pET-28a(+) by BamHI / HindIII.





CPA2 digestion

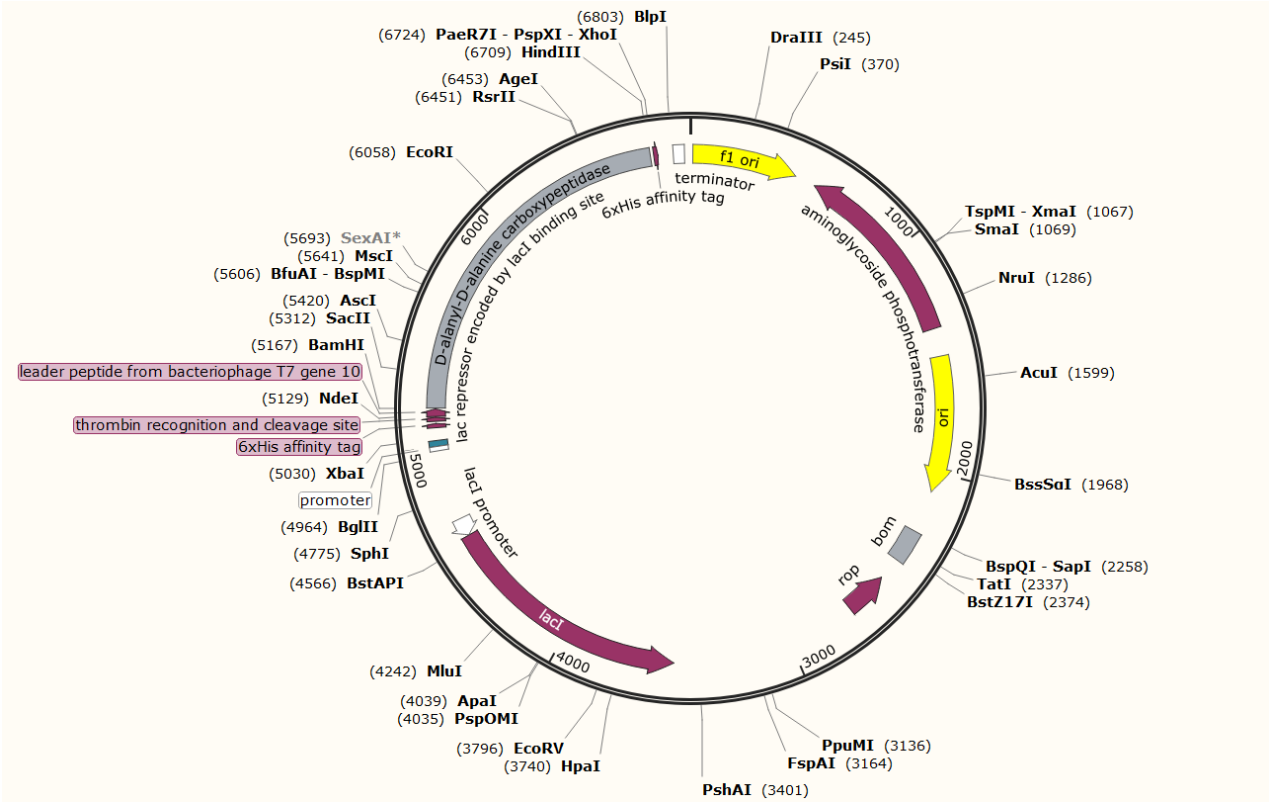
3) CPA3

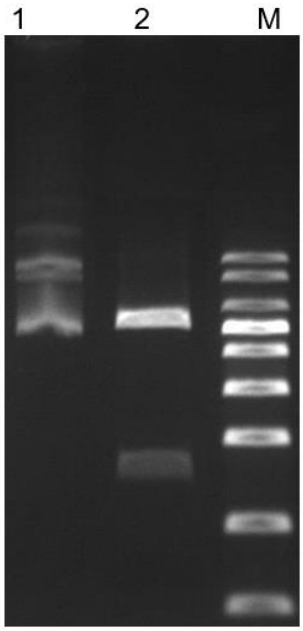
Gene Name: D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 4)/ CPA3
ŐR16_12223
Cloning vector: pET-28a (+)
Gene length: 1548 bp
Cloning strategy: BamHI / HindIII

Table 3: **Summarises GenScript analysis results for the synthesis of the gene encoding- CPA3 protein**

QC Items	Specifications	Results	
Sequencing Alignment	Sequencing results are consistent with the targeted insert sequence.	Pass	Consistent
Vector Sequence	The flanking sequences of the cloning site are correct.	Pass	Correct Shown in the SQD file (sup. material)
Restriction Digests	The size of inserted fragment is correct and free of unexpected bands suggesting contamination.	Pass	Correct Shown in the digestion gel photo below
DNA Quality	Minipre 4 μg p: **OD260/280=1.8~2.0** Free of contamination	Pass	≥ 4 μg **OD260/280=1.86** Pure
Quality Grade	Research Grade	Pass	Research Grade
Appearance	Clear and free of foreign particles.	Pass	Clear Free of foreign particles

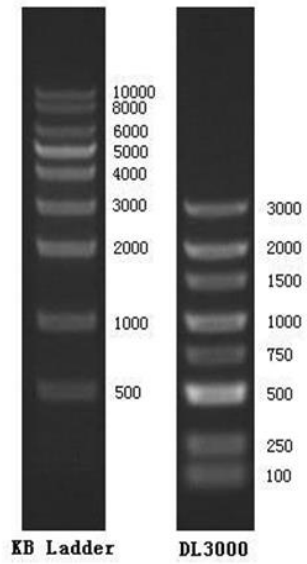
Plasmid construct map, The gene encodes CPA3 ligated in pET-28a (+) by BamHI / HindIII.





Lane M: KB Ladder
Lane 1: pET-28a (+) plasmid
Lane 2 : pET-28a (+) plasmid digested by BamHI and HindIII

Digestion Conditions:
About 300ng plasmid digested
Digestion in water-bath,37℃ for 40 minutes
1% Agarose Gel



CPA3 digestion