Mycotoxin Biodegradation Ability of the Cupriavidus Genus

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Abstract

The biodegradation and biodetoxification ability of five prominent mycotoxins, namely aflatoxin B1 (AFB1), ochratoxin-A (OTA), zearalenone (ZON), T-2 toxin (T-2) and deoxynivalenol (DON) of Cupriavidus genus were investigated. Biological methods are the most appropriate approach to detoxify mycotoxins. The Cupriavidus genus has resistance to heavy metals and can be found in several niches such as root nodules and aquatic environments. The genus has 17 type strains, 16 of which have been investigated in the present study. According to the results, seven type strains can degrade OTA, four strains can degrade AFB1, four strains can degrade ZON and three strains can degrade T-2. None of the strains can degrade DON. The biodetoxification was measured using different biotests. SOS-chromotest was used for detecting the genotoxicity of AFB1, the BLYES test was used to evaluate the oestrogenicity of ZON, and the zebrafish embryo microinjection test was conducted to observe the teratogenicity of OTA, T-2 and their by-products. Two type strains, namely C. laharis CCUG 53908T and C. oxalaticus JCM 11285T reduced the genotoxicity of AFB1, whilst C. basilensis DSM 11853T decreased the oestrogenic of ZON. There were strains which were able to biodegrade more than two mycotoxins. Two strains degraded two mycotoxins, namely C. metalliduriens CCUG 13724T (AFB1, T-2) and C. oxalaticus (AFB1, ZON) whilst two strains C. pinatubonensis DSM 19553T and C. basilensis degraded three toxins (ZON, OTA, T-2) and C. numazuensis DSM 15562T degraded four mycotoxins (AFB1, ZON, OTA, T-2), which is unique a phenomenon amongst bacteria.

Introduction

The genus Cupriavidus was identified in 2004 [1]. 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 environments. The genus Cupriavidus belongs to the family Burkholderiaceae and the class β-proteobacteria. Remarkable heavy metal tolerance of environmental isolates has been confirmed [2, 3] and some species have important xenobiotic degradation potential such as C. necator, which is able to degrade chlorinated aromatic compounds [4–6]. An environmental strain, C. basilensis ÖR16 can degrade 98% of ochratoxin-A [7] (Table 1). One strain from the C. taiwanensis species can initiate root nodule formation and nitrogen fixation [8]. A member of C. respiraculi was isolated from the respiratory tract of a cystic fibrosis patient [1].

To date, 11 Cupriavidus genome projects are known for the following strains, namely C. necator CCUG 52238T, C. metallidurans CCUG 13724T, C. pinatubonensis DSM19553T, C. alkaliphilus BCCM 26294T, C. basilensis DSM 11853T, C. oxalaticus JCM 11285T, C. pauculus JCM 11286T, C. taiwanensis CCUG 44338T, C. campinensis CCUG 44526T, C. nantongensis KCTC 42909T and C. planatarum BCCM/LMG 26296T. The genome size of the genus varies from 6.5 to 8.5 Mbp [21]. Genomic sequences suggest that the species has significant catabolic potential, as several pathways responsible for aromatic ring cleavage have been identified, such as the catechol and protocatele ortho-ring
cleavage, catechol meta-position ring cleavage, gentisate and benzene-CoA pathways \[22\] (Table 1).

The genus has 17 type strains, from which the biodegradation and detoxification potential of 16 has been investigated for five mycotoxins, namely aflatoxin B1 (AFB1), ochratoxin-A (OTA), zearalenone (ZON), T-2 toxin (T-2) and deoxynivalenol (DON). Figure 1 depicts the phylogenetic tree of the Cupriavidus genus type strains.

Contamination of food and feed by toxigenic moulds (fungi) is an increasing and unavoidable problem because the climatic extremities cause permanent stress for the crops, which becomes vulnerable to fungi. This leads to an increase in the number of mycotoxin contaminations amongst food-stuff globally \[23, 24\]. However, there are approximately 300 to 400 different identified mycotoxins. In the present study, five toxins will be investigated because they have a negative influence on human and animal health, such as genotoxicity, oestrogenity, nephrotoxicity and teratogenicity.

A limited number of methods have been developed to degrade mycotoxins and/or reduce their toxicity. Ozonation \[25\] can be applied as a chemical method. Sorting, extrusion and application of adsorbents are physical techniques

## Table 1 Xenobiotic biodegradation ability of the Cupriavidus genus strains

| Species             | Strain | Isolation matrix                           | Biodegraded chemicals                                      | References |
|---------------------|--------|--------------------------------------------|-------------------------------------------------------------|------------|
| *C. necator*        | NH9    | Contaminated soil, Japan                    | Chlorinated aromatic chemicals; halo benzoate and nitrophenols | \[9, 10\]  |
|                     | JMP134 | Soil, unknown                              | 2,4-D                                                       | \[6\]      |
| *C. numazuensis*    | TE26\(^T\) | Natural soil, Japan, Numazu city, Shizuoka prefecture | Trichloroethylene, cis-dichloroethylene and toluene         | \[11\]    |
| *C. basilensis*     | HMF14  | Soil, Netherlands                           | Hydroxymethyl-furfural (HMF)                               | \[12\]    |
|                     | JF1    | BPA-degrading planted fixed-bed reactor     | Bisphenol-A                                                 | \[13\]    |
|                     | M91-3  | Agricultural soil                           | Atrazine                                                    | \[14\]    |
|                     | R25C6  | PCP-contaminated soil, Ljungby, Sweden      | Chlorobenzene, phenol                                       | \[15\]    |
|                     | B-8    | Erosove bamboo slips, China                 | Kraft lignin biodegradation                                 | \[16\]    |
|                     | ÖR16   | Natural soil, Hungary                       | Ochratoxin-A                                                | \[7\]     |
|                     | RK1    | Freshwater pond, France                     | 2,6-dichlorophenol                                           | \[17\]    |
| *C. gilardi*        | CR3    | Rancho La Brea Tar Pits 91, Los Angeles     | Naphthenic acids                                             | \[18\]    |
| *C. pauculus*       | KF709  | Biphenyl-contaminated soil in Kitakyushu, Japan | Biphenyl                                                   | \[19\]    |
| *C. nantongensis*   | X1\(^T\) | Sludge, chlorpyrifos manufacture plant Nantong, China | Chlorpyrifos                                               | \[20\]    |
| *C. pampae*         | CPDB6\(^T\) | Agricultural soil, Argentinean Humid Pampa region | 2,4-D                                                      | \[5\]     |

Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relations of 17 type strains of Cupriavidus genus with AFB1, OTA, ZEA and T-2 detoxification ability. Bootstrap values are presented as percentages of 1000 replicates. Only values above 50% were shown. The tree analysis was conducted in MEGA7 software.
[26] whilst biological methods include the biodegradation by microorganisms or their enzymes. The treatment of feed products by adsorbents such as bentonite and clay can efficiently bind toxins [27] although in the animal intestinal tract, these agents can also adsorb vitamins and nutrients [28].

The biodegradation of mycotoxins by different bacteria is a widely used method for decreasing their concentrations. The biodegradation is not synonymous with biodetoxification, and this phenomenon should be investigated in all cases of biodegradation. During the biodegradation, the by-product can be more toxic or harmful then the initial chemical. However, the investigation of the by-product toxicity is not straightforward. Biotests are useful for evaluating the detoxification efficiency and prior to making any product suitable for the market, detoxification from different biotests or assays is advised to determine the difference between the sensitiveness and behaviour of the test organism. However, detoxification is an appropriate way of treating contaminated feed and foodstuff.

Materials and Methods

Cupriavidus Strains and Mycotoxin Stock Solutions

Sixteen type strains of the genus Cupriavidus were purchased from the following strain collections, namely the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (German Collection of Microorganisms and Cell Cultures), the Japan Collection of Microorganisms (JCM), the Culture Collection University of Gothenburg (CCUG), Sweden and the Belgian Coordinated Collection of Microorganisms (BCCM). The 17th type strain C. nantongensis KCTC 42909T from South Korea could not be ordered in time for the experiments. Mycotoxins were purchased from Sigma Aldrich Limited (Budapest, Hungary). From these mycotoxins, 1 mg/mL of stock solutions were prepared in acetone and used in degradation experiments.

Chemicals and Reagents

With regards to the HPLC–MS/MS analysis, all standards and reagents were purchased from the Hungarian distributors of national companies. Mycotoxin standards with minimum 98% purity were purchased from Romer Labs (Hungary). Mobile phases and extraction solvents were used containing super gradient grade acetonitrile (MeCN, 99%) purchased from VWR (Hungary), gradient grade methanol (CH3OH, 99%) obtained from Fischer Scientific (Budapest), ultra-pure-grade water (18MΩcm) produced in-house by a Milli-Q water purification system (Merck, Darmstadt, Germany). Acetic acid (CH3COOH, 99.8%) obtained from Merck (Hungary), formic acid (HCOOH, 98%) from Scharlau (Hungary) and LC/MS grade ammonium-acetate (CH3COONH4, >99%) from VWR (Hungary). Luria–Bertani (LB) media and acetone were ordered from BioLab Incorporated and Reanal Ltd Hungary.

Biodegradation Experiments

Cupriavidus strains were stored at −80 °C and were streaked on LB agar plates (10 g tryptone, 5 g yeast extract, 9 g NaCl and 18 g bacteriological agar dissolved in 1000 mL distilled water). The plates were incubated at 28 °C for three 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 Incorporated, US) and adjusted to OD600 = 1.0. From this culture, 5 mL was inoculated into 45 mL freshly sterilized LB medium, which was inserted with the stock solution of the mycotoxins, 1 µg/mL initial concentration in each mycotoxin. The monotoxins experiment was conducted in triplicates. 50 mL sterile LB medium containing the mycotoxin was applied as a microbe-free control. Flasks were incubated at 28 °C for five days, thereafter, 1 mL samples were taken and centrifuged at 20,800×g for 15 min (Eppendorf 5810R Centrifuge, Eppendorf, Germany). Supernatants and pellets were separated and stored at −80 °C for further HPLC MS/MS analysis.

Genotoxicity Test

The genotoxic effect in supernatant samples was observed by the colorimetric SOS-chromotest (Environmental Bio-Detection Products Incorporated, Canada). In the genetically modified test organism, E. coli PQ37, operon fusion of sfiA and lacZ genes was conducted. As a result, when the SOS-repair mechanism commenced, β-galactosidase was produced simultaneously, which was proportional to the strength of the genotoxicity [29]. The test was conducted according to the description of Risa et al. [30]. The genotoxic effect was expressed in induction factor (IF), which was calculated according to Eq. (1) [31]:

\[ \text{Induction factor (IF)} = \frac{(C405 \times S620)}{(S405 \times C620)} \]  

where \( C \) is the mean of the absorbance value of the control and \( S \) is the mean of the absorbance value of the sample measured at 405 and 620 nm wavelength.

Samples were considered as not genotoxic, when IF was significantly \( (p < 0.05) \) less than 1.5 [32].
**Oestrogenicity Test**

With regards to the detection of the oestrogenic effect of ZON and its metabolites, the bioluminescence-based BLYES test was used [33]. The *Saccharomyces cerevisiae* BLYES strain had been genetically modified, inserting gene encoding human estrogenic receptor, *lux* genes and estrogenic response element into its genome. This modification made the BLYES strain capable of emitting light after an oestrogenic compound binds to the oestrogenic receptor. The test was conducted in accordance with Risa et al. [31]. The oestrogenic effect was expressed in bioluminescence intensification (%), which was calculated according to Eq. (2) [34):

\[
\text{Bioluminescence intensification (\%)} = -1 \times \left(\frac{C - S}{C}\right) \times 100
\]

where \( C \) is the mean of the bioluminescence values of the negative control and \( S \) is the mean of the bioluminescence values of the sample.

**Zebrafish Microinjection Tests for OTA and T-2**

**Preparation of the Bacterial Inocula**

Bacterial inocula (5 mL) was prepared as stated and added to 45 mL 20% LB medium containing OTA and T-2 separately (monotoxic) (7 mg/L final concentration). Similar inocula were prepared in parallel without mycotoxins to test the effects of bacterial metabolites. Uninoculated LB medium (20%) was combined with OTA and T2 (7 mg/L) was used as a negative control. Both of the cultures and controls were incubated at 28 °C, 170 rpm for 120 h in triplicates. After the incubation, cultures were centrifuged at 14,000×g, 4 °C for 15 min. Supernatants were filtered with 0.2 µm syringe filters, and samples were stored at −20 °C until microinjection.

**Microinjection**

Microinjection was conducted as described by Csenki et al. [35]. The changes were in the volume of injection, namely the sphere diameter of 50 µm corresponded to an injection volume of 0.074 nL, 75 µm to 0.22 nL, and 200 µm to 4.17 nL.

**Experimental Design**

Bacteria metabolites were injected with 4.17 nL volume in three replicates (20 eggs per replicate). 7 mg/L T-2 toxin and bacteria T-2 toxin degradation products were injected with 0.074 nL and 4.17 nL volumes in three replicates (20 eggs per replicate). 7 mg/L OTA toxin and bacteria OTA toxin degradation products were injected with 0.22 nL and 4.17 nL volumes in three replicates (20 eggs per replicate).

**Examination of Injected Embryos**

Embryo mortality was determined at 120 hpf based on egg coagulation, the lack of somite formation and the lack of heart function.

**Analytical Measurements**

High-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) was conducted for the measurement of AFB1, ZON, OTA, T-2 and DON concentrations. From the three experiments’ culture, lombics 1–1 mL inocula was taken as a sample, centrifuged for 20 min at 4000 rpm, and the supernatant and pellet separated. In terms of the supernatants, 500 μL was transferred into 1.5 mL vials, then evaporated, reconstituted with A/B eluent (50:50%) and injected directly into the HPLC–MS/MS. In terms of the pellet 1 mL extraction, solvent acetonitrile/water/formic acid was added and vortexed for 1 min. Subsequently, 500 μL of suspension was transferred to 1.5 mL vials then handled as supernatants. 500 μL samples were evaporated until dry under a gentle N2 stream.
Thereafter, it was reconstituted in 50–50 V/V% A-B eluent (A: water, 5 mM ammonium-acetate, 0.1% acetic acid; B: methanol, 5 mM ammonium-acetate, 0.1% acetic acid) and filtered through a 0.22 µm PTFE syringe filter. With regards to the chromatographic separation, an Agilent 1100 HPLC (Agilent Technologies, US) equipped with Agilent Zorbax C18 column (3.5 µm, XDB-C18, 2.1 x 50 mm) was used. 10 µL prepared samples were injected into the mobile phase containing A–B eluent. 400 µL.min⁻¹ flow rate and 40 °C column temperature was set. The 3200 QTRAP LC/MS/MS system (Applied Biosystems, US) was equipped with a Turvo V electrospray ionization (ESI) interface in positive (DON, T2, AFB1, OTA) and negative (ZON) ion mode was used. Prior to the HPLC–MS/MS measurements, a method was developed and validated. The mycotoxins were separated on a reverse phase C18 chromatographic column. Two methods were used, the first method included DON, T2, AFB1 in positive ion mode and ZON in negative ion mode; whilst the method included OTA in positive ion mode. The AFB1 and OTA were co-eluting, thus, the researchers chose to measure OTA separately with the same analytical condition as in case of the first method. The chromatograms of the standards are depicted in Fig. S1 (A) and (B). The validation was performed on LB medium. Recoveries for all compounds were between 70 and 114% with an RSD < 20%.

The matrix effect (ion suppression/enhancement) which is the result of the competition between non-volatile matrix components and analyte ions in ESI ion source has also been studied. These effects were between −37 and +10%, thus, a matrix matched linear calibration curve was used for quantification. LOD values were between 0.2 and 5 µg/kg and LOQ between 1 and 15 µg/kg for all studied mycotoxins. The validation parameters of HPLC–MS/MS method can be observed in Table. S2. The mycotoxin degrading potential of investigated Cupriavidus strains were confirmed by low resolution target HPLC–MS/MS methods. With this instrument, only native mycotoxins with available standards can be qualitatively and quantitatively measured. The metabolomic profile of mycotoxin by-products can be identified with high resolution MS, in further study.

Statistical Analysis

The statistical analysis was performed with Microsoft Excel 2016 (Microsoft Office, Microsoft Incorporated, US), Past 3 [36] and GraphPad Prism 6.01 (GraphPad Software, San Diego, US). Biological effects were expressed in induction factor (Eq. 1), bioluminescence intensification percent (Eq. 2) and mortality ratio. The residual toxin concentrations determined by HPLCFLD were expressed in ng/mL. Data were checked for normality with the Shapiro–Wilk normality test and non-compliance with the requirements of parametric methods was established. Significant differences (P < 0.05) were verified by one-sample t-tests and Kruskal–Wallis analysis with the Dunn’s multiple comparisons test. All values are means of the triplicates. Correlations between biotests and analytical measurements were calculated by Spearman’s rank correlation coefficient.

Results

Biodegradation of AFB1

Residual AFB1 concentrations were measured by HPLC–MS/MS (Table 2). Four type strains had excellent biodegradation potential. Remarkable (91%) biodegradation of AFB1 was observed in the case of C. laharis CCUG 53908ᵀ. High AFB1 degradation rates (72–82%) were demonstrated by C. oxalatus JCM 11285ᵀ, C. metallidurans CCUG 13724ᵀ and C. numazuensis DSM 15562ᵀ whilst moderate AFB1 reduction (approximately 60%) was observed in five cases, namely C. taiwanensis CCUG 44338ᵀ, C. campenensis CCUG 44526ᵀ, C. pampae CCUG 55948ᵀ, C. plan tarum BCCM/LMG 26296ᵀ and C. alkaliphilus BCCM/LMG 26294ᵀ. Two strains could detoxify AFB1 for five days according to the SOS-chromotest, C. laharis CCUG 53908ᵀ and C. oxalatus JCM 11285ᵀ (p < 0.05) (Fig. S3).

In the pellet fraction of C. laharis CCUG 53908ᵀ 120 ng/mL (12% of the initial toxin concentration) and C. oxalatus JCM 11285ᵀ 247 ng/mL (24% of the initial toxin concentration), AFB1 was measured. Adsorption was observed in other cases also and the amount varied between 50 and 254 ng/mL. AFB1 in the pellet as the biodegradation potential was corrected by the residual toxin concentration on the pellet.

Biodegradation and Detoxification of ZON

According to the analytical results (Table 2), the highest ZON biodegradation rates (82–96%) were detected in the case of C. basilensis RK1 DSM 11853ᵀ, C. pinatubonensis DSM 19553ᵀ, C. numazuensis DSM 15562ᵀ and C. oxalatus JCM 11285ᵀ.

In terms of ZON degradation, the highest toxin concentration on the cell pellet was 155 ng/mL (15% of the initial toxin concentration), which was observed in the C. oxalatus JCM 11285ᵀ strain, which demonstrated a high degradation ability (82%). In terms of C. basilensis DSM 11853ᵀ, the pellet ZON concentration was the lowest at 10 ng/mL. The ZON in the pellet varied between 10 and 155 ng/mL as the biodegradation potential was corrected by the residual toxin concentration on the pellet.

In order to analyse cytotoxicity and the oestrogenic effect of ZON BLYES, tests were performed, respectively. According to the BLYES test (Fig. S4), a considerable...
The Biodegradation of OTA

Residual OTA concentrations in the supernatant and pellet samples were measured by HPLC–MS/MS. The OTA degradation potential of Cupriavidus type strains differs significantly. Out of the 16 type strains, six strains were the most effective. C. taiwanensis CCUG 44338T demonstrated the highest OTA-degradation rate (97%). C. alkalophilus BCCM 26294T showed 95%, C. basilensis RK1 DSM 11853T showed 94%, C. necator CCUG 52238T showed 92%, C. pinatubonensis DSM 19553T showed 88%, C. numazuensis DSM 15562T showed 85% and C. respiraculi CCUG 46809T showed 82%. Measuring OTA-binding to the cells confirmed that adsorption was negligible. The highest OTA concentration on the cell pellet (7 ng/mL, 0.7% of the initial toxin concentration) was observed in case C. numazuensis DSM 15562T strain, with a degradation ability of 85%.

**Table 2** AFB1, ZON, OTA and T-2 biodegradation potential of Cupriavidus type strains after a 5 day-experiment determined by HPLC–MS/MS

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

Bold values indicates strains having more than 70% biodegradation ability
Italic values indicates strains causing biodetoxification
Residual genotoxicity was detected in supernatant by SOS-Chromo test, oestrogenicity was detected by BLYES test

reduction of the oestrogenic effect of ZON was observed in the strain C. basilensis DSM 11853T (98%) compared to the control, which demonstrated a positive correlation with the biodegradation rate in the HPLC analysis. Biodetoxification occurred in the following cases: C. respiraculi CCUG 46809T reduced the oestrogenity (73%) by the 5th day, although the degradation was only 64% as measured by HPLC, which is an extremely effective degradation and detoxification ratio. The C. numazuensis DSM 15562T and C. oxalaticus JCM 11285T could decrease the oestrogenic effect by 50%, with an 85% biodegradation ratio. C. pinatubonensis DSM 19553T reduced the oestrogenic effect of ZON to approximately 30% with a 91% biodegradation rate by day five. Four strains, C. necator CCUG 52238T, C. gilardii JCM 11283T, C. pauculus JCM 11286T and C. yeoncheonensis JCM 19890T had a higher bioluminescence rate (up to 118%) compared to the control which leads to the transformation of ZON in additional oestrogenic metabolites.
Biodegradation of Trichothecene Mycotoxins

Biodegradation of T-2

Out of the 16 type strains, six were able to degrade T-2. The highest biodegradation rate was 95%, which was observed in the case of the C. gilardi JCM 11283T strain. Five strains demonstrated a moderate (68–88%) T-2 biodegradation rate, namely C. metallidurans CCUG 13724T, C. numazuensis DSM 15562T, C. basilensis DSM 11853T and C. plantarum LMG 26296T. The T-2 concentration in the pellets was between 22 and 50 ng/mL, this is 2% and 5% of the initial toxin concentration, respectively.

Biodegradation of DON

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

Ability to Degrade More than one Mycotoxin

From the 16 type strains, six were able to degrade 60% of the two different mycotoxins. The C. respiraculi CCUG 46809T strain could degrade 82% of OTA and 64% of ZON. C. laharis CCUG 53908T strain could degrade 91% of AFB1 and detoxicate the harmful effects of the metabolites as well as degrade 61% of ZON. The C. metallidurans CCUG 13724T strains could degrade 77% of AFB1 and 72% of T-2. The C. plantarum BCCM/LMG 26296T strain could degrade 67% of ZON and 60% of T-2. The C. taiwanensis CCUG 44338T strain could degrade 97% of OTA and 63% of AFB. The C. oxalaticus JCM 11285T strain could degrade 82% of AFB1 and 82% of ZON, furthermore, it was able to eliminate the genotoxic effect of AFB1 and its by-products.

The C. pinatubonensis DSM 19553T strain was able to degrade 90% of ZON, 88% of OTA and 68% of T-2. The C. basilensis DSM 11853T strain could degrade 96% of ZON, 94% of OTA and 68% of T-2, moreover, in the case of ZON, it could eliminate the oestrogenic effect of the metabolites. The C. numazuensis DSM 15562T strain was the most effective in the degradation of mycotoxins, as it reduced 70% of T-2, 72% of AFB1, 85% of ZON and 85% of OTA. In order to investigate the detoxification efficiency of three multidegrading strains, the zebrafish embryo microinjection test was conducted to assess the by-products of OTA and T-2.

Zebrafish Embryo Microinjection Test for Evaluating the by-Products of OTA and T-2

In order to evaluate the biodetoxification in terms of T-2 and OTA, the multi mycotoxin degraders C. numazuensis DSM 15562T, pinatubonensis DSM 19553T and basilensis DSM 11853T were measured with a newly developed and standardised zebrafish embryo microinjection test method (Csenki et al. 2019). The strains were selected for this method because the C. numazuensis DSM 15562T can degrade four mycotoxins (AFB1, ZON, T-2 and OTA) whilst the C. pinatubonensis DSM 19553T and basilensis are able to degrade three mycotoxins (ZON, T-2 and OTA).

According to the microinjection results of the normal metabolites, the strains are toxic for the embryos, with a 60% mortality ratio indicating a high toxicity level. The source of the strain’s toxicity is currently unknown. According to the existing literature, there are no data on the pathogenicity of the Cupriavidus genus amongst fish. The injected supernatants were cell free, without any bacteria, only the centrifuged-filtered inocula containing the by-products and metabolites of the strains. The pellets of the inocula were also investigated by the HPLC, where the residual T-2 concentration was only 2–5% of the initial toxin concentration.

With regards to the T-2 microinjection test, C. pinatubonensis DSM 19553T demonstrated a significant reduction at 4.17 nL injected volume (p < 0.01) in the mortality compared to the 7 mg/L T-2 control. Although the bacteria strain reduced the lethality rate, the degraded metabolites were toxic to zebrafish (mortality rate: 40%) (Fig. 2). The effect of the T-2 metabolites ratio was less than the metabolites effect revealed in Fig. 3.

In terms of the OTA microinjection test, C. numazuensis DSM 15562T demonstrated a significant decrease at 4.17 nL injected volume (p < 0.01) in the mortality rate compared to the 7 mg/L OTA control. Although the bacteria strain reduced the lethality rate, the degraded metabolites were toxic to zebrafish (mortality rate: 45%) (Fig. 4). The effect
of OTA metabolites ratio was less than the metabolites effect depicted in Fig. 3.

**Discussion**

In the present study, the aim was to measure the mycotoxin biodegradation potential of 16 type strains of *Cupriavidus* genus and evaluate the potential harmful effects of the metabolic intermediates. The research also aimed to select the best degraders amongst these strains.

The mycotoxin degradation ability of different bacteria (*Rhodococcus* sp, *Streptomyces* sp) was previously investigated by this department [30, 37, 38]. Members of the *Cupriavidus* genus have profound abilities in terms of the biodegradation of different chemicals and xenobiotics, particularly one mycotoxin OTA [7]. The biodegradation experiments were performed within five days because of the comparison of the aforementioned existing studies. Although the biodegradation result was confirmed after two or three days and the researchers also realised the 95% AFB1 biodegradation rate for 24 h, the present study is the first evaluation of the *Cupriavidus* genus biodegradation ability in terms of mycotoxins.

The comparison of the biodegradation potential of different bacteria is not the most appropriate foundation because biodegradation does not necessarily signify biodetoxification, which refers to the elimination of the harmful effects of the biodegraded chemical. The comparison of the biodetoxification ability is an appropriate method. However, evaluation of the biodetoxification is challenging because an appropriate biotest or organism is required. Mycotoxins have different negative effects, which are not easy to measure or estimate realistically due to cost effectiveness, time and resources. Unfortunately, only limited publications have investigated biodetoxification in terms of biodegradation.

In terms of AFB1, *Rhodococcus* sp are highly effective biodetoxifiers from 42 type strains of which 15 terminated the genotoxicity in 72 h. With regards to ZON, only one *Rhodococcus* type strain could terminate the oestrogenic effect [30]. 124 *Streptomyces* strains were tested for AFB1 and ZON biodegradation, and only one strain was able to biodetoxify AFB1 and only two strains could terminate the oestrogenic effect of ZON [38].

From the genus *Cupriavidus*, almost all the type strains are able to biodegrade AFB1 in five days and a high biodegradation ratio (over 70%) was achieved by only four strains, namely *C. laharis* CCUG 53908T (91%), *C. oxalaticus* JCM 11285T (82%), *C. metallidurans* CCUH 13724T (77%) and *C. numazuensis* DSM 15562T (72%). According to the SOS-chromotest results, two strains were able to terminate the genotoxicity. These were *C. laharis* CCUG 53908T (IF = 1.31) and *C. oxalaticus* JCM 11285T (IF = 0.97). The biodegradation and biodetoxification was observed, and absorption on the pellet was not assuming an integral role as in the case of the elimination of AFB1. In Fig. S3, high degradation ratios can be observed. In the absence of the eliminations of the genotoxic effect in the case of 14 strains, all degradation results were corrected with the residual AFB1 concentration measured on the pellets. Only the aforementioned *C. laharis* CCUG 53908T and *C. oxalaticus* JCM 11285T were able to terminate the genotoxicity.

In the case of ZON, all *Cupriavidus* strains had biodegradation ability, but only four attained a 70% rate, namely *C. basileisis* DSM 11853T (95%), *C. pinatubonensis* DSM 19553T (91%), *C. numazuensis* DSM 15562T (85%) and *C.
oxalaticus JCM 11285T (82%). According to the BLYES results, one strain was able to reduce the endocrine disrupting effect of the metabolites of ZON, namely the C. basilensis RK1 DSM 11853T strain (Fig. S4).

Comparing the results of the present 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.

From the 16 type strains, in the case of OTA, there were two groups, namely strains with a weak biodegradation ability (30%) and strains with a remarkable biodegradation potential (over 80%). The most effective strains were *C. taiwanensis CCUG 44338T* (97%), *C. alkaliophilus BCCM 26294T* (95%), *C. basilensis* (94%), *C. necator* (92%), *C. pinatubonensis* (88%), *C. numazuensis* (85%) and *C. resipiculi* (82%). The evaluation of the biodetoxification ability of these strains is limited, because there are only difficult and time-consuming biotests or methods for testing the negative effects of the OTA by-products. In the case of the *Cupriavidus basilensis* ÖR16 wild strain, which has a remarkable 98% OTA biodegradation and biodetoxification ability, the first biotest was a mice feeding experiment performed by Ferenczi et al. in 2014. This method took approximately two months with a 21-day long feeding experiment, and one month for evaluating the results on the level of gene expression, weight and biopsy. ÖR16 strain was biotoxifying the OTA effectively in five days. No harmful effects were observed. The ÖR16 strain was also tested by a novel Danio rerio embryo microinjection method developed by Csenki et al. [35], the test required approximately seven days to obtain the results. In this test, the strain by-products had the same effect as the results of this research and it caused 50% mortality amongst the embryos. However, the OTA biodegraded supernatant was less harmful than the OTA control and the normal by-product control.

The strain *C. basilensis* DSM 11853T was tested for biodetoxification of OTA using a Danio rerio embryo microinjection test. According to the results, it is not able to decrease the harmful effect of the OTA by-products, although it demonstrated a 94% toxin degradation. *C. numazuensis* DSM 15562T was also evaluated by the microinjection test, where the strain OTA by-product had a significantly reduced harmful effect in comparison to the OTA containing control.

In terms of the T-2 toxin, only six strains could biodegrade T-2 with a ratio exceeding 60%, namely *C. gilardii JCM 11283T* (95%), *C. metallidurans CCUG 13724T* (73%), *C. numazuensis* DSM 15562T (70%), *C. pinatubonensis* DSM 19553T (68%), *C. basilensis RK1 DSM 11853T* (68%) and *C. plantarum BCM/LMG 26296T* (60%). The evaluation of the T-2 detoxification has an identical problem with the OTA. In the case of the three strains, the researchers used the microinjection test for evaluation of the detoxification.

In terms of the *C. pinatubonensis* DSM 19553T, the T-2 by-products were significantly less harmful than the T-2 control, according to the results. 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%). The *C. metallidurans* CCUG 13724T could biodegrade AFB1 and T-2. The *C. oxalaticus* JCM 11285T could degrade AFB1 and ZON. The *C. pinatubonensis* DSM 19553T and *C. basilensis* DSM 11853T could degrade ZON, OTA and T-2. The *C. numazuensis* DSM 15562T was able to biodegrade four mycotoxins, namely AFB1, ZON, OTA and T-2. This phenomenon is unique according to the existing literature. To date, *Rhodococcus* strains are known to degrade and detoxify more than two mycotoxins, for example, *R. erythropolis* N11 strain can biodegrade AFB1, ZON and T-2 and detoxify the harmful effects of AFB1 and ZON [30]. A microbe consortia TMDC was investigated recently, which was able to simultaneously degrade AFB1 and ZEA in excess of 90% after 72 h but the detoxification was not evaluated. The consortia was comprised of the following genera Geobacillus, Tepidimicrobium, Clostridium, Aeribacillus, Cellulosibacter, Desulftomaculum and Tepidanabacter [39].

The *C. pinatubonensis* DSM 19553T, *C. numazuensis* DSM 15562T and *C. basilensis RK1* DSM 11853T species were selected for a teratogenicity test using a Danio rerio embryo microinjection. According to the results, metabolites of these three strains were also toxic to the embryos and the mortality was 60% in all cases. This result resembles that of the *C. basilensis* ÖR16 wild strain [7] because the mortality was 50% in that case also. It appears that the members of the *Cupriavidus* genus have some toxic-by-product for fish embryos because the injected inocula was cell free. There is no information stating that *Cupriavidus* strains are pathogenic for fish.

In terms of the *C. numazuensis* DSM 15562T strain, the harmful effect of the toxin by-products was significantly less than the OTA control. A similar result was observed with T-2 detoxification by the *C. pinatubonensis* DSM 19553T strain. In both cases, the mortality ratio induced by the toxin breakdown products was less than with the bacterial metabolites.

Ultimately, according to the results, the *Cupriavidus* genus mycotoxin biodegradation ability could be a promising advantage in the future. At present, 11 type strains have genome project data. If all the members of the genus had full genome project data, it could be combined with the results of the present study and the responsible genes for mycotoxins biodegradation can be identified. This will help develop 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.

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Author contributions MN contributed to the biodegradation experiment and the writing of this paper, with MC who conceived and designed the present study. AR was responsible for the SOS-chromo and BLYES tests. EG and ZCB contributed to the microinjection test. Emese Varga accomplished the analytical measurements. BK and IS contributed to the analysis and interpretation of the results. All the researchers have approved the final version for submission.

Compliance with Ethical Standards

Conflict of interest The researchers have declared that there is no potential conflict of interest.

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