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In vitro single and combined mycotoxins degradation by Ery4 laccase from *Pleurotus eryngii* and redox mediators

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ABSTRACT

Mycotoxin contamination of staple food commodities is a relevant health and economic issue worldwide. The development of green and effective reduction strategies to counteract the contamination by multiple mycotoxins has become an urgent need. The aim of this work was to evaluate the capability of a laccase (LC) from *Pleurotus eryngii* and a laccase-mediator systems (LMSs) to degrade aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), ochratoxin A (OTA), deoxynivalenol (DON), Zearalenone (ZEN) and T-2 toxin in *in vitro* assays. In addition, the simultaneous mycotoxin degradation capability with selected LMSs was evaluated with combinations of AFB_1 and ZEN, and FB_1 and T-2 toxin. Redox mediators were found to drastically increasethe degradation efficiencies of the enzyme. AFB_1, FB_1, OTA, ZEN and T-2 toxin degradation by the best performing LMS were 73%, 74%, 27%, 100% and 40%, respectively. No degradation was registered for DON. Notably, AFB_1 and ZEN were simultaneously degraded by 86% and 100%, while FB_1 and T-2 by 25% and 100%, respectively. LMS proved to be a promising approach to enhance degradation properties of LC enzymes and for the potential development of a multi-mycotoxin reducing method.

1. Introduction

Food and feed contamination by mycotoxins is a concerning issue worldwide, due to their adverse health effects and their important economic impact (Rocha, Freire, Maia, Guedes, & Rondina, 2014). Multiple mycotoxin contamination frequently occurs as a result of the concomitant mycotoxin production by different fungal species, the production of different mycotoxins by the same organism, or the combination of differently contaminated raw materials needed for food and feed production (Smith, Madec, Coton, & Hymery, 2016). Mycotoxin reduction strategies have to take into account the co-occurrence of multiple mycotoxins, which often hampers efficacy and applicability in real food, or feed matrix. Since food contamination by mycotoxins also arises from the carry-over from feed to animal and animal derived products, such as meat, milk and eggs, it is important to counteract mycotoxins contamination at every stage of the food supply chain (Streit et al., 2012).

Biological degradation is a strategy which can be used to mitigate mycotoxins contamination through a mild, sustainable and environmental friendly approach. Microorganisms can achieve mycotoxin reduction by multiple means, such as adsorption, chemical, or enzymatic degradation. Since multiple mechanisms are involved, the identification of a specific degradation pathway and the resulting degradation products remains challenging.

While single mycotoxin degradation is often achieved by enzymes, the simultaneous enzymatic degradation is a challenging task, due to enzyme catalytic specificity and mycotoxin chemical heterogeneity. Mycotoxins bioremediation by enzymes has been recently reviewed (Loi, Fanelli, Liuzzi, Logrieco, & Mulè, 2017). Several enzyme activities are specifically addressed to one type of toxin, while oxidoreductive enzymes, such as laccases (LCs), are less specific with respect to substrate oxidation and can be potentially applied for the simultaneous degradation of more than one type of mycotoxin. LCs (benzenediol: oxygen oxidoreductase, EC 1.10.3.2)

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belong to the multicopper oxidase family and catalyse the oxidation of phenols, aromatic amines, and other non-phenolic compounds, with the concomitant reduction of molecular oxygen to water. The range of LC substrates can also be extended by the use of redox mediators within the Laccase Mediator System (LMS). In LMS, oxidative capacity is enhanced by the generation of a new, often radical, species; oxidation mechanisms are diversified according to the nature of the mediator used; the use of a free effector, the oxidized mediator, reduces steric hindrance. Indeed, the degradation of recalcitrant and chemically heterogeneous compounds, including aflatoxin B_1 (AFB_1), aflatoxin M_1 (AFM_1) and Zearalenone (ZEN), by LMS was already reported (Banu, Lupu, & Aprodu, 2013; Loi et al., 2016, 2017).

Additionally, LCs havebeen already used in food processing to improve the techno-functional properties of bakery, dairy and meat products (Osma, Toca-Herrera, & Rodrıguez-Couto, 2010). To this purpose, a scientific risk assessment on one LC from *Trametes-hirsuta* by EFSA is currently in progress (European Commission, 2016).

In this study, we describe the application of Ery4 laccase from *Pleurotus eryngii* coupled with various redox mediators for the *in vitro* degradation of AFB_1, fumonisin B_1 (FB_1), ochratoxin A (OTA), deoxynivalenol (DON), ZEN and T-2 toxin. In addition, the simultaneous degradation of a combination of AFB_1 and ZEN, and FB_1 and T-2 toxin was assessed in order to investigate the feasibility of a potential LC and LMS application in food and feed.

2. Materials and methods

2.1. Chemicals, reagents and mycotoxins standard preparation

2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS), aceto-syringone (AS), syringaldehyde (SA), p-coumaric acid (p-CA), 1-hydroxybenzotriazole (HBT) 2,2,6,6-tetramethylpyperidyloxil (TEMPO), phenol red (PhR), chlorogenic acid (CGA), ferulic acid (FA), mycotoxin standards (purity > 99%) of OTA, AFB_1, DON, T-2 and HT-2 toxins, and ZEN were purchased from Sigma Aldrich (Milan, Italy). FB_1, α-zearalenol (α-ZON) and β-zearalenol (β-ZON) were purchased from Biopure (Romer Labs Diagnostic GmbH). RC 0.2 μm (regenerated cellulose membranes) filters were obtained from Alltech Italia-Grace Division (Milano, Italy). All solvents (HPLC grade) were purchased from J. T. Baker Inc. (Deventer, The Netherlands). Ultrapure water was produced by Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Laccase production and purification

Ery4 laccase was produced from *Saccharomyces cerevisiae* ITEM 17289 of the Agri-Food Microbial Fungi Culture Collection of the Institute of Sciences of Food Production (www.ispa.cnr.it/Collection), engineered with pYES2 vector (Invitrogen, USA) bearing ery4 gene sequence from *Pleurotus eryngii*. Cultured media was filtered and dialyzed against Tris- HCl 50 mM, pH 7 using a Vivaflow 200 apparatus (Vivascience, AG, Hannover, Germany) equipped with a hydrosart membrane module (10 kDa molecular weight cut off) and a Masterlfex L/S system pump (Cole-Parmer, Vernon Hills, IL, USA) to 50 mL of final volume. Purification was performed by Fast Protein Liquid Chromatography (FPLC) using the NGC™ Quest 10 Plus Chromatography System equipped with a 1 mL ENrich™ Q High-Resolution Ion Exchange Column (Bio-Rad, Milan, Italy) pre-equilibrated with TRIS/HCl 50 mM pH 7. LC was eluted with 30% NaCl 1 M in TRIS/HCl 50 mM pH 7. The collected fraction was then loaded on a ENrich™ SEC 650 High-Resolution Size Exclusion Column (Bio-Rad) equilibrated with TRIS/HCl 50 mM pH 7 and fractionated with 1 CV (24 mL) of the same buffer. Fractions were tested for enzymatic activity using the ABTS colorimetric assay (Bleve et al., 2008).

2.3. In vitro mycotoxins degradation by Ery4 and LMS

Ery4 capability of degrading AFB_1, FB_1, OTA, ZEN, DON and T-2 toxin was assessed through an *in vitro* assay performed in 500 μl of sodium acetate buffer 1 mM, pH 5. One μg/mL (AFB_1, FB_1, ZEN, DON and T-2) or 0.5 μg/mL (OTA) of mycotoxin, in combination with 3 or 9 U/mL of LC, were added to each reaction and gently mixed. In control samples Ery4 was replaced by an equal amount of buffer solution. Reactions were incubated for 72 h at 25 °C in static conditions.

Subsequently, eight different redox mediators at two different concentrations (1 and 10 mM) were independently tested in combination with Ery4 (5U/mL) for their ability to degrade AFB_1, FB_1 and OTA. In particular, four natural mediators (AS, SA, p-CA and FA) and four artificial ones (ABTS, TEMPO, HBT, PhR) were used. ZEN, DON and T-2 toxin degradation was assayed in presence of three redox mediators, representative of the natural (SA) and artificial ones (TEMPO and ABTS). Reactions were performed in 500 μl of sodium acetate buffer 1 mM, pH 5. Control samples were also included replacing Ery4 with buffer.

2.4. Simultaneous mycotoxins degradation by selected LMS

Based on the preliminary screening with different LMSs, simultaneous AFB_1/ZEN degradation was assessed using either SA, TEMPO or both, while FB_1/T-2 using TEMPO. Each toxin concentration was 0.5 μg/mL while final mediator concentration was 10 mM.

2.5. Chemical analyses

Different chemical methods were performed to quantify the residual mycotoxin concentration in samples.

AFB_1 analyses were performed by high performance liquid chromatography with fluorescence detection (HPLC-FLD) as previously described (Loi et al., 2016). The limit of quantification (LOQ) was 0.4 ng/mL based on a signal to noise ratio of 10:1.

Samples containing FB_1 were filtered using RC 0.20 μm filters and quantified by HPLC-FLD after derivatization with o-phtaldialdehyde (OPA) (Haidukowski et al., 2017). LOQ was 20 ng/mL based on a signal to noise ratio of 3:1. Standards of partially hydrolyzed fumonisins ($PHFB_1$) and hydrolyzedfumonisin (HFB_1) were prepared from pure FB_1 according to the procedure described by De Girolamo, Lattanzio, Schena, Visconti, and Pascale (2014). Multi-mycotoxin calibration solutions were prepared by opportunely diluting with acetonitrile: water (1:1, v/v) to obtain calibrant solutions with concentration in a range of 10–1000 ng/mL for FB_1, 100-1000 ng/mL for $PHFB_1$ and 1000-5000 ng/mL for HFB_1. Determination of $PHFB_1$, HFB_1 and $FB_1$was performed by ultra-performance liquid chromatography with an AcquityQDa mass detector (UPLC-QDa). The chromatographic separation was performed on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) preceded by an Acquity UPLC® in-line filter (0.2 μm). Column temperature was set at 50 °C. The flow rate of the mobile phase was set at 0.4 mL/min. Eluent A was water, and eluent B was methanol, both containing 0.1% acetic acid. A gradient elution was applied by changing the mobile phase composition from 10% to 50% of eluent B in 10 min, and kept constant for 4 min then linearly increased up to 90% in 3 more min and, finally, kept constant for 4 min. For column re-equilibration, Eluent B was decreased to 10% in 1 min and kept constant for 3 min. For LC/MS analyses, the ESI interface was used in positive ion mode, with the following settings: desolvation

temperature 600 °C; capillary voltage 0.8 kV, sampling rate 5 Hz. The mass spectrometer was operated in full scan (100–800 m/z) and in single ion recording (SIR) mode, by monitoring the individual masses of each compound (FB_1 722.40 m/z, $PHFB_1$ 564.00 m/z, HFB_1 406.30 m/z). Retention time for HFB_1, $PHFB_1$ and FB_1 were about 14, 15, 16 min, respectively. Toxins were quantified by measuring peak areas and comparing these values with a calibration curve obtained from standard solutions. EmpowerTM 2 Software (Waters) was used for data acquisition and processing. LOQ values were 10 ng/mL for FB_1, 100 ng/mL for HFB_1 and 1000 ng/mL for $PHFB_1$, calculated based on a signal to noise ratio of 10:1.

OTA quantification by HPLC-FLD was performed as described by Ferrara et al. (2014) and De Bellis et al (2015). LOQ for OTA and OTαwere 0.5 ng/mL, based on a signal to noise ratio of 10:1.

Samples containing DON were filtered using RC 0.20 μm filters (Phenomenex, Torrance, CA, USA) and DON levels were determined byultra-high performance liquid chromatography linked with photodiode array detector (UPLC-PDA) (Pascale, Panzarini, Powers, & Visconti, 2014). The LOQ was 100 ng/mL, based on a signal to noise ratio of 10:1.

The quantification of T-2 toxin by UPLC-PDA was performed as described by Pascale, Panzarini, and Visconti (2012). LOQ for T-2 and HT-2 toxins were 0.05 μg/mL.

A novel chromatographic method was performed to quantify ZEN. ZEN stock solution (1 mg/mL in methanol) was diluted in methanol to a concentration of 10 μg/mL. The exact concentration of ZEN stock solution was spectrophotometrically determined ($\varepsilon = 12623$ L/mol cm at $\lambda = 274$ nm in methanol). The stock solution was evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved in water/acetonitrile (50:50, v/v) in order to obtain a standard calibration curve (0.01–0.3 μg/mL).

α and β-ZON stock solutions (10 μg/mL in acetonitrile) were diluted with water to obtain a concentration of 5 μg/mL in acetonitrile/water (1:1, v/v). The standard calibration curve ranged from 0.01 to 0.3 μg/mL.

The solutions were filtered using RC 0.20 μm filters. A volume of 100 μL was injected in the HPLC system (Agilent 1260 Series, Agilent Technology, Santa Clara, CA, USA) with a full loop injection system. The analytical column was a Luna C18 (150 × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA) preceded by a guard column inlet filter (0.5 μm × 3 mm diameter, Rheodyne Inc. CA, USA). The column was thermostated at 30 °C. The mobile phase consisted of a water/acetonitrile (50:50, v/v) eluted at a flow rate of 1.0 mL/min. The fluorometric detector for ZEN was set at wavelengths, ex = 274 nm, em = 440 nm and DAD detector set at 236 nm for α-ZON and set at 240 nm for β-ZON. Data acquisition and instrument control were performed by LC Openlab software (Agilent). ZEN was quantified by measuring peak areas, and comparing them with a calibration curve obtained with standard solutions. With this mobile phase, the retention time of α, β-ZON and ZEN were about 4.4 min, 3.5 min and 7.3 min, respectively. α, β-ZON and ZENwere quantified by measuring peak areas, and comparing them with a calibration curve obtained with standard solutions for each mycotoxin. The LOQ of the method was 0.015 μg/mL for α-ZON, β-ZON and ZON, based on a signal to noise ratio of 10:1.

Degradation percentage of each toxin was calculated as follows:

$$degradation\ (\%) = \frac{remaining\ mycotoxin\ in\ sample}{total\ mycotoxin\ in\ control\ sample} \times 100 \quad (1)$$

2.6. Statistical analysis

All data are mean ± standard deviation of three independent replicates. Data were expressed as mean percentage ± standard deviation with respect to the control. Results were analyzed through Student's t-test (paired comparison) performed using STATISTICA software for windows, ver. 7 (Statsoft, Tulsa, and Okhla). Differences between samples and relative control were considered significant for a P value < 0.05 or <0.01.

3. Results and discussion

Ery4 capability of degrading different mycotoxins was assessed alone and in combination with different LMSs. In particular, AS, SA, p-CA and FA are natural phenols deriving from siringyl and cynnamic acids, while HBT, TEMPO, ABTS and PhR are artificial compounds. Among the three different mechanisms described so far, the natural phenols and HBT follow the Hydrogen Atom Transfer (HAT) mechanism, TEMPO follows the ionic route, while ABTS the Electron Transfer (ET) mechanism (Baiocco, Barreca, Fabbrini, Galli, & Gentili, 2003; Fabbrini, Galli, Gentili, & Macchitella, 2001).

3.1. Aflatoxin B_1 degradation by Ery4 and LMS

Ery4 laccase was not able to directly oxidize AFB_1, suggesting that this toxin is not a direct substrate of Ery4 (data not shown). The binding architecture of AFB_1 within the catalytic site of LC is responsible for the success of AFB_1 oxidation and varies among LC enzymes and within LC isoforms. Indeed, Lac2 from *Pleurotus pulmonarius* was found to be able to directly oxidize AFB_1, though with low efficacy (Loi et al., 2016). An *in-silico* study on LC isoforms from *Trametes versicolor* (Dellafiora et al,. 2017) proposes that AFB_1 degradation is isoform-dependent. Also LC limited oxidative capacity might hinder direct AFB_1 oxidation. In fact, oxidoreductive enzymes with greater oxidative capacity than LC, such as peroxidases from *Pleurotus* spp. and *Armoracia rusticana,* were reported to efficiently degrade AFB_1 (Chitrangada et al., 2000; Yehia et al., 2014).

In our study AFB_1 degradation was achieved by using different LMSs (Fig. 1). Syringyl-type phenols (AS and SA) were the best performing mediators, followed by ABTS and cynnamic acid derivatives (FA, p-CA), while HBT, PhR and TEMPO were ineffective. Higher degradation percentages were reported for AS, SA, p-CA and PhR, when 10 mM mediator was used compared to 1 mM (73% vs 51% for AS, 68% vs 48% for SA, 22% vs 0% for p-CA and 11% vs 0% for PhR). By contrast, 10 mM was detrimental in the case of ABTS (39% with 1 mM vs 25% with 10 mM) and FA (24% with 1 mM vs 17%with 10 mM). These results confirmed the efficacy of these LMSs, as reported by Loi et al. (2016), who measured an efficient degradation of AFB_1 by Lac2 from *P. pulmonarius* using 10 mM AS (90%), SA (72%) and ABTS (81%) as redox mediators.

AFB_1 degradation was putatively achieved through the HAT mechanism. After an initial hydrogen atom removal, further electronic rearrangements lead to the loss of the coumarin and/or lactone moieties, responsible for AFB_1 characteristic fluorescence.

3.2. Fumonisin B_1 degradation by Ery4 and LMS

FB_1 was not degraded by direct Ery4 oxidation (data not shown). However, astatistically significant degradation ($P < 0.01$) was achieved using TEMPO 10 mM (74%), PhR 10 mM (30%) and SA 1 mM (25%) (Fig. 2). All other mediators and concentrations were ineffective or not statistically significant.

TEMPO acts through an ionic route. Specifically, once oxidized into the oxoammonium ion by LC, the nitrogen atom of TEMPO becomes susceptible to the nucleophilic attack by an oxygen atom, such as a primary hydroxyl group, as reported for the oxidation of the model compound 4-methoxybenzyl alcohol to the

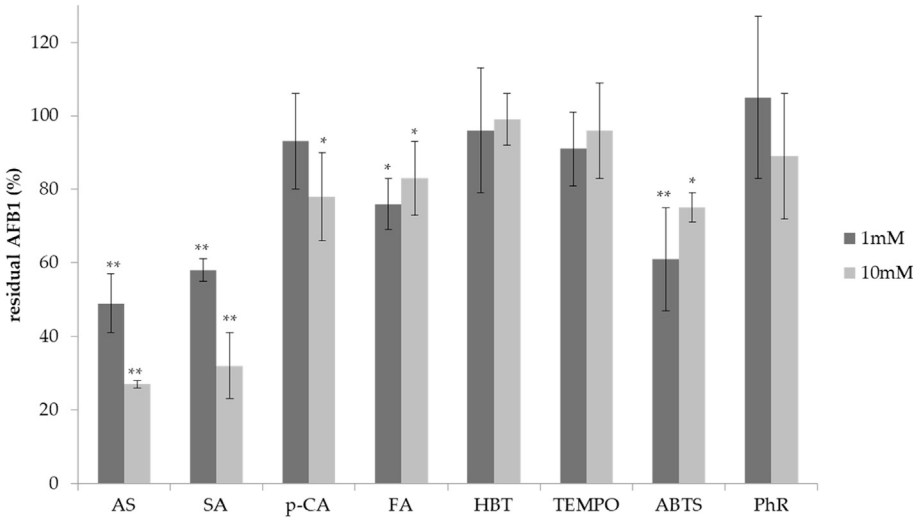

Fig. 1. Residual aflatoxin B_1 (AFB_1) in samples treated with different laccase mediator systems. AS: acetosyringone, SA: syringaldehyde, p-CA: p-coumaric acid, FA: ferulic acid, HBT: 1-hydroxybenzotriazole, TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), PhR: phenol red. A P value < 0.05 was considered statistically significant (*) and a P value < 0.01 is considered highly statistically significant (**).

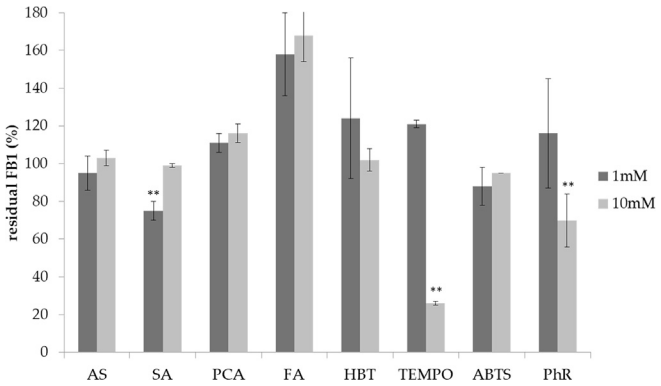

Fig. 2. Residual fumonisin B_1 (FB_1) in samples treated with different laccase mediator systems. AS: acetosyringone, SA: syringaldehyde, p-CA: p-coumaric acid, FA:ferulic acid, HBT: 1-hydroxybenzotriazole, TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl) oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), PhR: phenol red. A P value < 0.01 is considered highly statistically significant (**).

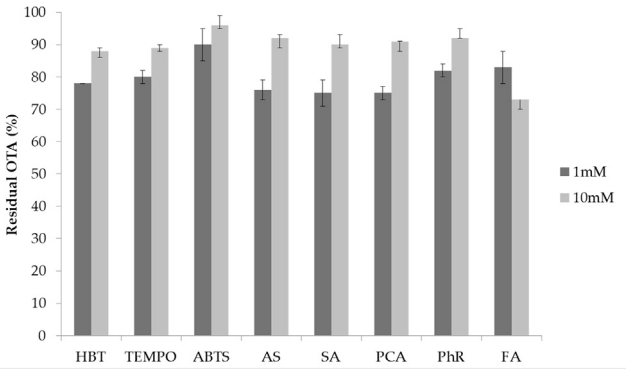

Fig. 3. Residual ochratoxin A (OTA) in samples treated with different laccase mediator systems. AS: acetosyringone, SA: syringaldehyde, p-CA: p-coumaric acid, FA: ferulic acid, HBT: 1-hydroxybenzotriazole, TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), PhR: phenol red. All data are statistically significant ($P < 0.01$).

corresponding aldehyde (Fabbrini et al., 2001). The complete mechanism underlying FB_1 oxidation with LC and TEMPO has not been yet clarified. Nonetheless, one or more hydroxyl groups of the aminopolyol backbone of FB_1 ought to be involved in the first steps of the degradation process.

The main FB_1 biotransformation method relies on the activity of the esterases, which hydrolyse the two ester bonds of FB_1 (Duvick et al., 1998; EFSA, 2014). So far no other enzymatic methodwas reported for FB_1 degradation. No hydrolysed products were detected in the samples.

3.2.1. Ochratoxin A degradation by Ery4 and LMS

Ery4 was not able to directly degrade OTA (data not shown), nevertheless a slight reduction was observed in presence of redox mediators (Fig. 3). Natural phenols were the best performing mediators with degradation percentages of 27% (FA, 10 mM), 25% (SA and p-CA, 1 mM) and 24% (AS, 1 mM). As regards artificial compounds, degradation percentage was 22% (HBT, 1 mM), 20% (TEMPO, 1 mM) and 18% (PhR, 1 mM). Only FA was more efficient when added at higher concentration (10 mM).

LMS oxidation of OTA was not as effective as other enzymatic methods, by which 80 to 100% of OTA degradation could be obtained in few hours of assay (Abrunhosa, Paterson, & Venâncio, 2010). Current enzymatic methods for OTA degradation rely on specific peptidases, able to break the amide bond releasing OTα and phenylalanine. In particular, OTα is considered a non-toxic compound with 10 times shorter half life time in humans (Klimke et al., 2015). No other enzymatic method was reported for OTA degradation.

3.3. Zearalenone, deoxynivalenol and T-2 toxin degradation by Ery4 and LMS

Ery4 laccase was not able to directly oxidize ZEN, DON and T-2 toxin (data not shown).

According to the preliminary data regarding AFB_1, FB_1 and OTA, the mediator screening for ZEN, DON and T-2 toxin was reduced to SA, ABTS and TEMPO, the three representatives of the different origin and LMS mechanisms of action, at 10 mM.

ZEN was completely removed with all the tested mediators (data not shown). This result is in agreement with Banu et al. (2013), who reported that ZEN was degraded up to 81.7% using 0.16 mM ABTS as redox mediator, despite enzyme, mediator and

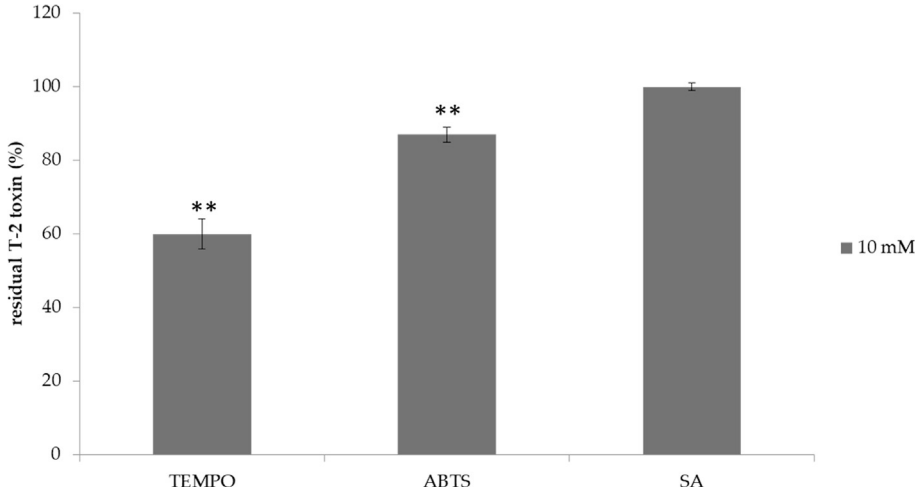

Fig. 4. Residual T-2 toxin in samples treated with different laccase mediator systems. TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), SA: syringaldehyde. A *P* value < 0.01 is considered statistically significant (**).

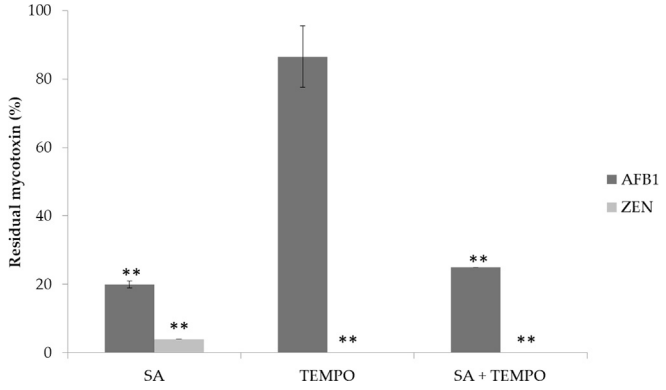

Fig. 5. Residual aflatoxin B_1 and zearalenonein samples treated with different laccase mediator systems. TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, SA:syringaldehyde. A *P* value < 0.01 is considered statistically significant (**).

toxin concentrations were significantly lower than those used in our study. As expected, considering LC oxidative nature, in LMS treated samples, nor α or β-zearalenol, which derive from ZEN reduction, were detected (data not shown).

DON is considered the most recalcitrant toxin to degrade. Under the tested conditions no LMS was effective towards DON (data not shown). Peroxidases from *Aspergillus oryzae* and *Rhizopusoryzae* were positively correlated with DON degradation in submerged fermentation (Buffon, Kupski, & Badiale-Furlong, 2011), meaning that a higher redox capacity than that of LC or LMS tested may be needed to degrade DON.

T-2 toxin was slightly degraded by LMS (Fig. 4). A statistically significant degradation ($P < 0.01$) was obtained using TEMPO (40%), and ABTS (13%), while SA was ineffective. Since LMS was not able to degrade DON, a comparative structural investigation of the two trichothecenes could suggest a possible starting point for the degradation of T-2toxin. LC- TEMPO LMS ought to firstly act on the acetoxy group in C15 or the ester in C8 positions. In accordance to this hypothesis, de-acetylation to HT-2 toxin was excluded, as it was not found in degraded samples. No biotransformation of T-2 toxin with enzymes, including LCs, has been reported so far.

3.4. Combined mycotoxins degradation with selected LMSs

Results of combined mycotoxin degradation for AFB_1/ZEN are shown in Fig. 5. AFB_1 and ZEN were simultaneously degraded when SA, or SA and TEMPO were used as redox mediators. In accordance with the results reported in section 3.1, AFB_1 was significantly degraded only in presence of SA, while ZEN was almost completely removed either with only SA, TEMPO or both. Since degradation percentages are comparable to those reported for the single toxin experiments, no relevant synergistic, or additive effects could be hypothesised to occur in presence of both mediators.

As regards the simultaneous degradation of FB_1/T-2 toxin by LC and TEMPO, T-2 toxin degradation was greatly enhanced with respect to the single degradation assay (100% VS 40%, $P < 0.001$), pointing to a strong additive effect. By contrast, a negative effect was reported for FB_1, whose degradation was drastically reduced from 74% to 25%, with respect to the single degradation assay. These results suggest the occurrence of combined degradation mechanisms, where mediators processed by LC can interact in a virtuous way towards the other toxin.

4. Conclusions

In this study, for the first time, an extensive *in vitro* screening towards multiple toxins was performed with the pure LC and different LMSs. Mediators were found to drastically increase catalytic efficiency of Ery4. With the optimal choice of the LMS, AFB_1, FB_1, OTA ZEN and T-2 toxin were degraded by 73%, 74%, 27%, 100% and 40%, respectively. By contrast, no degradation occurred for DON with any of the LMSs tested. Another novelty presented in this work is the use of LMS for the simultaneous degradation of many toxins at the same time, possibly with the use of a single mediator. AFB_1 and ZEN were simultaneously degraded by 86% and 100%, while FB_1 and T-2 by 25% and 100%, respectively. A strong additive effect was found for the T-2 degradation in the presence of FB_1, enforcing the advantages of using LMS to selectively degrade toxins. This study represents a starting point for the development of methods to counteract the natural co-occurrence of multiple mycotoxins in raw materials, or in composite food and feed by means of an efficient, environmental friendly and versatile LC enzyme.

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management in the food and feed chain.

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