UNIVERSITY OF FOGGIA

Depatment of Economics

PHD COURSE IN
"HEALTHY FOODS: INNOVATION AND MANAGEMENT"
XXX CYCLE

Green degradation of mycotoxins by biotechnological application of enzymes from *Pleurotus* spp.

PhD candidate: Dr. Martina Loi

Tutor: Dr. Giuseppina Mulè
Co-tutor: Dr. Francesca Fanelli

ACADEMIC YEAR 2016-2017

Table of contents

Abstract

Mycotoxins are toxic secondary metabolites produced by filamentous fungi mainly belonging to *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera. They can be found as common contaminants of cereals, fruits, seeds and spices as a result of fungal spoilage. Mycotoxin contamination is an significant health and economic concern worldwide. Some of them were recognized by the International Agency of Research on Cancer (IARC) as carcinogenic (aflatoxin of the B and G series), possible carcinogenic (aflatoxin M_1, AFM_1; fumonisin B_1, FB_1; ochratoxin A, OTA) to humans. Moreover, they exert both acute and chronic toxic effects towards humans and animals. Because of mycotoxin contamination, billions of dollars are lost every year due to unsold commodities, decrease of animal health and productivity or to sustain a complex and integrated mycotoxin management system. Prevention strategies are not completely effective and require the implementation of novel post-harvest methods, able to mitigate or remove mycotoxins from contaminated materials.

The aim of this thesis was to evaluate and study the capability of laccase enzymes to reduce mycotoxin contamination both *in vitro* and in contaminated materials through an environmental friendly and mild approach. In addition up to eight different redox mediators were used within the laccase mediator system (LMS) to maximize mycotoxin degradation.

Within this purpose, the activity of two different purified LCs, native Lac2 from *Pleurotus pulmonarius* and the recombinant Ery4 from *P. eryngii*, was tested towards the main classes of mycotoxins.

Lac2 was identified and evaluated for the *in vitro* degradation of aflatoxins, while Ery4 was tested towards AFB_1, AFM_1, FB_1, OTA, deoxynivalenol (DON), zearalenone (ZEN) and T-2 toxin. The preliminary screening revealed that the inclusion of a toxin - specific redox mediator is required to achieve high levels of degradation with both enzymes. However, the use of the LMS resulted ineffective for DON and not efficient for OTA.

By the use of one LMS, the simultaneous *in vitro* degradation of two different toxins was also achieved for AFB_1/ZEN and FB_1/T-2 toxin pairs.

In addition, LMS treatment was successfully tested in real artificially and naturally contaminated matrixes (milk and maize flour) for AFM_1 and ZEN degradation.

Despite the great potentialities showed by both enzymes in the field of bioremediation, LCs remain versatile biocatalysts which can be applied in various food processes. LMS application in milk was deeply investigated to evaluate the effects on milk proteins and the possibility of manufacturing a curd with improved technological and nutritional properties.

The results presented in this thesis lay the basis for the development of a biotransformation methods based on a LMS approach and open new perspective for the use of this versatile and green biocatalyst in the field of safety and quality of mycotoxin contaminated commodities.

List of abbreviations

^{1}H-NMR Proton Nuclear Magnetic Resonance

ABTS: 2-azino-di-[3-ethylbenzo-thiazolin-sulphonate]

AFB_1: aflatoxin B_1

AFD_1: aflatoxin D_1

AFL: aflatoxicol

AFM_1: aflatoxin M_1

AFO: aflatoxin oxidase enzyme

AFQ_1: aflatoxin Q_1

AFs: aflatoxins

ALA: α-lactalbumin

ANOVA: analysis of variance

AP1: aminopentol 1

BLF: β-lactoferrin

BLG: β-lactoglobulin

CGA: chlorogenic acid

cLC: commercial laccase

CN: casein

CPA: carboxypeptidase A

CPY: carboxypeptidase Y

CT: coated and tough

DDGS: distiller's grains and soluble

DE: degrading enzyme

DOE: Design of experiments

DON: deoxynivalenol

DPs: degradation products

EDC: endocrine disrupting chemical

EFSA: European Food Safety Authority

ER: estrogen receptors

FA: ferulic acid

FB_1: fumonisin B_1

GAPs: Good Agricultural Practices

HFB: hydrolyzed FB_1

HR-ESI-MS: High Resolution electrospray ionization mass spectrometry

HT-2: HT-2 toxin

IARC: International Agency of Research on Cancer

LC: laccase

LCs: laccases

LMS: laccase mediator system

MADE: Myxobacteria Aflatoxin Degrading Enzyme

MnPs: Manganese Peroxidases

NIV: nivalenol

OTA: ochratoxin A

OTα: ochratoxin α

Phe: phenylalanine molecule

Prx: peroxiredoxin

SDS PAGE: sodium dodecyl sulphate poly acrylamide gel electrophoresis

T-2: T-2 toxin

TEAC: trolox equivalent antioxidant capacity

TGase: translutaminase

UHT: ultra high temperature

VAN: vanillin

WP: whey protein

ZEN: zearalenone

α-ZAL: α-zearalanol

α-ZEL: α-zearalenol

β-ZAL: β-zearalanol

β-ZEL: β-zearalenol

List of tables

List of figures

Introduction

1. General introduction and background on mycotoxins

Mycotoxins are secondary metabolites produced by fungi mainly belonging to *Fusarium*, *Aspergillus* and *Penicillium* genera, which display toxic, carcinogenic, teratogenic and mutagenic activity towards humans and animals (Bennet and Klich, 2003).

Mycotoxins are low molecular weight metabolites, which exhibit very diverse chemical structure, toxicity and are produced by different organisms. Due to their huge heterogeneity, mycotoxins are not easy to classify. They can be grouped according to the chemical structure (e.g. coumarins, lactones etc.), target organ (e.g. nephrotoxic, hepatotoxic etc.) or producer fungus (*Aspergillus* toxins, *Penicillium* toxins). Cereals, seeds, spices, fresh and dried fruits are the major food commodities susceptible to mycotoxin contamination. The most important mycotoxins are shown in Table 1-1.

Mycotoxin contamination poses a severe risk for the health and economy of food and feed supply chains. The Food and Agriculture Organization (FAO) estimates that up to 25% of food commodities is contaminated with mycotoxins and from 30 to 100% of it is co-contaminated by multiple mycotoxins.

Cereals are the main source of mycotoxin exposure. At the same time, they represent the staple food of human diet worldwide, and have a considerable role in animal nutrition and biofuel production. Cereals by-products are precious ingredients in feed manufacture, and contribute to maintain the sustainability of food processing industries by converting wastes and by-products into valuable products (Pinotti et al., 2016).

Mycotoxigenic fungi are able to contaminate crops, both pre and post-harverst. Improper storage or prolonged drying can stimulate fungal growth and mycotoxin production (IARC, 2012).

Mycotoxins are stable to the most commonly used food processing methods, thus they can be found in processed products, including sterilized, extruded and

fermented ones. Carry over in animal products was also documented for AFB_1 (as AFM_1 in milk and dairy) and OTA (Volkel et al., 2011).

Being secondary metabolites, mycotoxin production is not easy to predict, hence it depends on several environmental, as well as biological factors, such as the complex host-pathogen interactions (Logrieco et al., 2002). Among the environmental factors, the most important ones are temperature, nutrient availability, atmospheric gases, pH, water activity, photoperiod and light intensity (Fanelli et al., 2015; Fanelli et al., 2013). On the other hand, the size of the inoculum, presence- absence of competing microflora and strain-host variability are determining biological factors (Popovski et al., 2013).

Generally, a wide number of different mycotoxins may be found in the same product since a single fungal species can produce several toxic metabolites, or several species can be present simultaneously, producing different toxins (Pereira et al., 2014).

Aflatoxin B_1 (AFB_1) is the most toxic mycotoxin and was classified as carcinogenic to humans (group 1) by the International Agency of Research on Cancer (IARC) in 2002 (IARC, 2002). Aflatoxin M_1 (AFM_1), ochratoxin A (OTA) and fumonisin B_1 (FB_1), were recognized as possibly carcinogenic to humans (group 2B) due to the limited availability of their toxicological data on humans (IARC, 2002).

Toxic outcomes of mycotoxin ingestion are classified into acute or chronic mycotoxicosis, depending on the type of mycotoxin assumed and its daily intake. Mycotoxicosis symptoms include gastro intestinal inflammation, anorexia, immune dysfunction, growth impairment and endocrine disorders. In addition, synergistic or additive effects have to be taken into account because of the co-occurrence of multiple toxins on the same product (Speijers et al., 2004).

Global trade significantly contributes to mycotoxin spread. The economic impact of mycotoxin contamination is also a topic of great interest. In addition to the money loss due to unsold commodities, which cannot enter the market

(Commission Regulation 1881/2006), additional costs have to be considered. Food and feed recalls or rejections at the borders, the reduction in animal productivity, sanitary treatments for mycotoxicosis, the safe disposal of contaminated biomasses, the development of detection and quantification methods as well as the development of strategies able to reduce mycotoxins occurrence have a great impact on the economic balance of mycotoxins contamination (Marroquín-Cardona et al., 2014). Economic losses are difficult to quantify, but range in the order of billions of dollars annually worldwide (Schmale and Munkvold, 2017).

Mycotoxin occurrence is regulated in many countries worldwide. The European Union has a solid legislation covering both food and feed commodities. Commission Regulation (EC) No. 1881/2006 sets mycotoxins maximum levels specifically established for food products (e.g. type pf food commodity, raw or processed) and consumer category (e.g., adults or infants). As regards trichothecenes T-2 and HT-2 toxins (T-2, HT-2), only indicative levels in cereals and cereals products have been established (Commission Recommendation 2006/576/EC; Commission Recommendation 2013/165/EU), while for the so called emerging mycotoxins (e.g., enniatins, beauvericin, fusaric acid and moniliformin) neither, maximum nor indicative levels have been yet established.

Different regulation limits were set in approximately 100 countries worldwide; however, they are still lacking in some African and Latin American regions. During the last 10 years, many efforts were made to harmonize regulations in several economic communities, such as EU (European Union), MERCOSUR (Mercado Cómun del Sur, which includes Argentina, Brazil, Paraguay, Uruguay and Venezuela), Australia and New Zealand (Egmond et al., 2007).

Regulation limits are formulated in accordance to toxicological and occurrence data, but can significantly vary. In US and China, the limit for AFM_1 in milk, for example, is 10 times higher than EU limits (0.5 vs 0.05 μg/Kg).

The differences in food safety standards and regulation on mycotoxin occurrence have an important impact on food and feed trade, with both economic

and health related implications. In developing countries, poor control measures, less strict regulations increase the overall mycotoxin exposure risk. High quality food is exported, while the most contaminated one is retained or imported for the local trade (Wu et al., 2012).

Table 1-1. Mycotoxin classification and properties

Mycotoxin class	Mycotoxin	Main producing organisms	Toxicity	Chemical group	Food commodity
Aflatoxins	Aflatoxin B_1, B_2, G_1, G_2	*Aspergillus flavus*, *A. parasiticus*, *A. niger*	Hepatotoxic, carcinogenic (liver)	Difuranocoumarin	Maize, wheat, rice, sorghum, nuts
Ochratoxins	Ochratoxin A, B, C	*Aspergillus ochraceus*, *Penicillium verrucosum*	Nephrotoxic	Dihydro-isocoumarin linked to β-phenylalanine	Cereals, grapes dried fruits, wine, coffee
Trichothecenes	Deoxynivalenol Nivalenol T-2 toxin HT-2 toxin	*Fusarium graminearum* *F. poae*, *F. sporotrichioides*, *F. equiseti*	Cytotoxic	Sesquiterpenoid	Cereals, cereal product
Fumonisins	Fumonisin B_1, B_2, B_3, B_4	*F. verticillioides*, *F. proliferatum*, *F. fujikuroi*	Hepatotoxic and carcinogenic (esophagus)	Diesthers of acyls	Maize, maize products, sorghum
Zearalenons	Zearalenone α-Zearalenol β-Zearalenol	*F. culmorum*, *F. graminearum*	Estrogenic	Resorcilyc acid lactones	Cereals, cereal product

1.1. Overview of the main mycotoxins

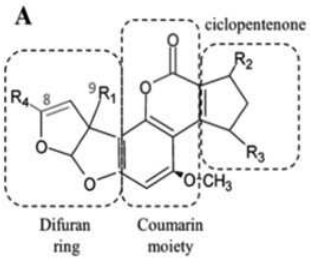
1.1.1. Aflatoxins

The term aflatoxins (AFs) derives from its principal fungal producer, *Aspergillus flavus* (Hesseltine, et al., 1966). AFs group includes more than 20 fungal secondary metabolites synthetized by *Aspergillus* species. AFs are primarily produced in the field, under elevated temperature and water stress conditions, but also during storage when relative humidity is maintained at 65% or during prolonged drying (Villers et al., 2014). Maize (*Zea mays* L.) is the major crop susceptible to *Aspergillus* spp. infection and subsequent AF contamination, together with nuts and dried fruits (Masood et al., 2015). Nuts, nut products and seeds were the most notified AF contaminated commodity by the Rapid Alert Alarm System for Food and Feed (RASFF) entering EU borders in 2016 (RASFF, 2016).

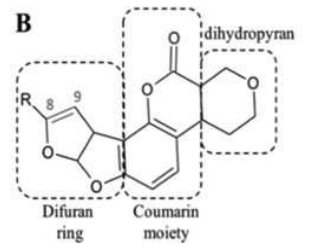
Afs are difuranocumarins classified into two main groups according to their chemical structure (Fig. 1-1). The difurocoumarocyclopentenone group includes AFB_1, aflatoxin B_2, aflatoxin B_{2a}, AFM_1, aflatoxin M_2, aflatoxin Q_1 (AFQ_1), and aflatoxicol (AFL), while the difurocoumarolactone group comprises aflatoxin G_1, aflatoxin G_2 and aflatoxin G_{2a}.

B and G designations refer to their characteristic blue and green fluorescence under UV-light, while the M refers to the first evidence of AFM_1 occurrence in the milk of lactating cows fed with AFB_1 contaminated feed.

AFs of the B and G series co-occur in cereals and their derived products, fruits, oilseeds, nuts, tobacco and spices, while the M serie, AFL and AFQ_1 can be found in food as carry-over products of AFB_1 contaminated feeds. In fact, *in vivo* AFB_1 is readily metabolized through hydroxylation (to AFM_1 or, to a lesser extent, to AFQ_1) or reduction (to AFL). Among *in vivo* metabolites, AFM_1 deserves particular attention because it is excreted through the mammary glands of lactating humans and animals and can be found as natural contaminant in milk and breast milk.



	AFB_1	AFB_2	AFB2a	AFM_1	AFM_2	AFL	AFQ_1
R1	-H	-H	-H	-OH	-OH	-H	-H
R2	=O	=O	=O	=O	=O	-OH	=O
R3	-H	-H	-H	-H	-H	-H	-OH
R4	-H	-H	-OH	-H	-H	-H	-H
C_8-C_9 bond	unsaturated	saturated	saturated	unsaturated	saturated	unsaturated	unsaturated



	AFG_1	AFG_2	AFG2a
R	-H	-H	-OH
C_8-C_9 bond	unsaturated	saturated	saturated

Figure 1-1. Aflatoxins chemical structure. Toxic determinants are highlighted in red (C-8 C-9 double bond) and blue (ester bond in the lactone moiety). Tables show substituent groups of different analogues.

AFs toxicity was discovered in 1960, when 100,000 turkeys died from AF induced liver necrosis (Blount, 1961). Their toxicity is both acute, at high doses, and chronic, if low amounts are assumed for a long period of time. Common symptoms of acute aflatoxicosis include vomiting, abdominal pain, pulmonary oedema, fatty infiltration and necrosis of the liver. Chronic assumption of AFs leads to liver cancer.

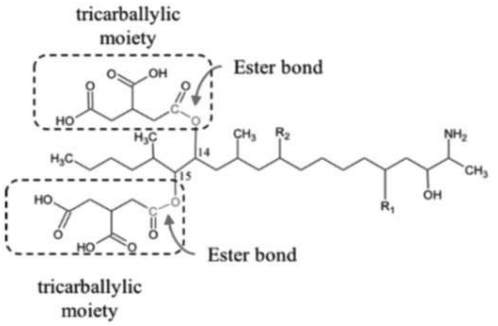
AF poisoning has been reported in developing countries such as India and Kenya, where poor agronomical practices, poor control measure and improper storage systems are in use. Additionally, environmental conditions are extremely favourable to fungal growth and mycotoxin production (Wild et al., 2015). One of the largest aflatoxicosis outbreak occurred in rural areas of Kenya leading to 314 cases and 125 deaths, due to the consumption of contaminated home-grown maize. AFs consumption is also a serious health threat for breast fed infants, since AFM_1 has been recently detected also in breast milk (Ishikawa et al., 2016).

Both B and G series were enlisted in group 1 by IARC (2002) and are carcinogenic to humans and animals. Upon ingestion, they are metabolized in the liver by cytochrome P450 (CYP450) microsomal enzymes to aflatoxin-8,9-epoxide. The epoxide ring is a crucial determinant for AFs carcinogenicity, since it is responsible for the binding to N7-guanine and the subsequent G to T transversions in the DNA molecule (Essigmann et al., 1977). Activated AFs are also able to form schiff bases with cellular and microsomal proteins (via methionine, histidine and lysine), thus leading to acute toxicity (Eaton et al., 1997). The lactone ring also plays a role in AFs toxicity and carcinogenicity, since aflatoxin D_1 (AFD_1), which still retains the 8,9-dihydrofuran double bond but not the lacton ring, lacks the strong *in vivo* DNA binding activity of AFB_1, demonstrating that DNA alkylation depends upon both difuranocumarin and lactone moieties (Schroeder et al., 1985).

1.1.2. Fumonisins

Fumonisins are a wide group of mycotoxins produced by several *Fusarium* species, including *F. verticillioides*, *F. proliferatum*, *F. oxysporum* and *F. globosum*, commonly found as saprophytes on maize, wheat and other cereals. 28 different fumonisins have been described so far and divided into A, B, C and P-series. The B-serie includes the most common and toxic fumonisins, with FB_1 being the most abundant.

Chemically, fumonisins are composed by a long 19- or 20 carbon aminopolyol backbone substituted with two tricarballylic acid moieties (Fig. 1-2) which resembles the sphingoid bases sphinganine (SA) and sphingosine (SO).



	B_1	B_2	B_3	B_4
R1	-OH	-OH	-H	-H
R2	-OH	-H	-OH	-H

Figure 1-2. Fumonisins (FBs) chemical structures. FB_1ester bonds hydrolyzed by the main degrading pathways, leading to the formation of HFB_1 and the two tricarballylic acid moieties, are indicated in red. Table shows substituent groups of different fumonisin analogues.

The structural similarity to SA and SO is responsible for FB_1 toxicity. FB_1 acts as a competitive substrate for ceramide synthase, which is technically a SA and SO N-acetyltransferase. As a consequence, ceramide biosynthesis is disrupted, SA and SO accumulate in cells and tissues leading to inhibition of cell growth, apoptosis, liver and kidney disfunction (Soriano et al., 2005). Other specific toxic effects on animals include porcine pulmonary edema and equine leukoencephalomalacia.

In 1993 and 2002 FB_1 was enlisted in group 2B by the IARC, thus possible carcinogenic to humans (IARC, 2002). Although carcinogenicity was not directly proven in humans, it has been positively correlated with the occurrence of oesophageal cancer in certain areas of South Africa and China (Marasas, 1996), while evidence of the carcinogenic effect on animals has already been proven in rats and mices (Stockmann-Juvala et al., 2008).

Both the tricarballylic moieties and the amino group are responsible for FB_1 toxicity. Hydrolysed FB_1 (HFB_1) and N-acetyl FB_1 are significantly less toxic or unable to disrupt ceramide biosynthesis.

Maize is the main food commodity affected by FB_1 contamination, although it was also rarely found in sorghum and sporadically in wheat, asparagus, tea, and cowpea (Jackson, 2013).

Available data on carry over exclude that FB_1 transfer occurs from feed to animal tissues or animal derivatives, such as milk and eggs (EFSA, 2005). However, a recent study by Magoha and colleagues (2014) reported FB_1 carry over in breast milk of Tanzanian women, suggesting that carry over contribution to the total FB_1 exposure should be reconsidered, at least in developing countries (Magoha eta l., 2014).

1.1.3. Zearalenone

Zearalenone (ZEN) is a phenolic β-resorcyclic acid lactone, non-steroidal yet oestrogenic mycotoxin, mainly produced by several *Fusarium* species, including *F. culmorum*, *F. equiseti* and *F. graminearum*. It can be found as natural contaminant of maize as well as wheat, barley, sorghum and rye. Also vegetable oils produced from corn and wheat contain considerable amounts of ZEN.

The main toxic effect of ZEN is related to its oestrogenic potential, due to its structural similarity to 17-βestradiol (Fig. 1-3). ZEN is capable of binding the estrogen receptors (ER) of mammals, although with lower affinity than the natural estrogens 17β-estradiol, estriol andestrone (Zinedine et al., 2007). Besides ZEN, other related compounds can be generated *in vivo* upon reduction, such as α- and β-zearalenol (α–ZEL and β-ZEL, respectively). Surprisingly, α–ZEL results in greater ER binding capacity and oestrogenic potential than the parent compound.

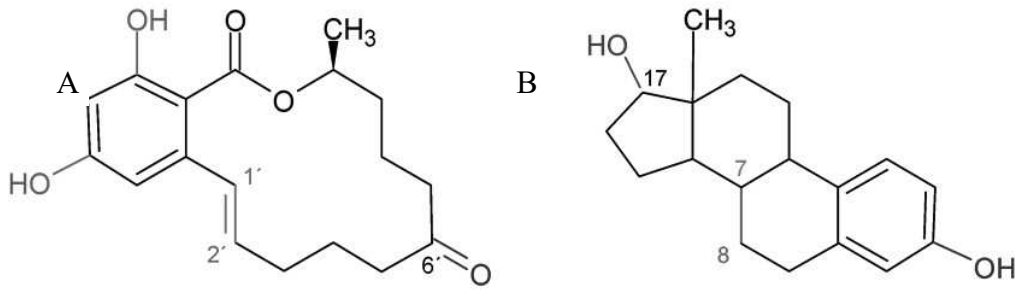


Figure 1-3. Structural analogies between zearalenone (A) and 17-βestradiol (B). Chemical groups interacting with estrogen receptor are highlighted in red, blue and green

α and β-ZEL possess a hydroxyl group in 6′ position instead of a ketone, which resembles that of C3 in estradiol, and a saturated bond in 1′-2′ carbon position, allowing those molecules to be more flexible and to undergo some conformational changes leading to a better interaction with ER receptor. Also hydroxyl groups in C4 and C2 contribute to the binding, with the first being more important than the latter in increasing ZEN estrogenic potential (Shier et al., 2001).

Acute toxic effects of ZEN are many and vary according to reproductive status (prepuberal, cycling or pregnant) of the affected animal and gender. ZEN is able to decrease fertility, cause sterility, induce precocious puberty and persistent oestrus. Symptoms include inflammation of reproductive organs, enlarged reproductive tracts in females and atrophy of the seminal vesicles and testes in males. ZEN was also found to be hematotoxic, genotoxic, hepatotoxic, to cause liver lesions leading to cancer (Zinedine et al., 2007).

1.1.4. Trichothecenes

Trichothecenes are a large group of sequisterpenoid sharing a common rigid tetracyclic ring. More than 190 compounds have been isolated and characterized (Rocha et al., 2005). *Fusarium*, *Myrothecium*, *Spicellum*, *Stachybotrys*, *Cephalosporium*, *Trichoderma*, and *Trichothecium* are the main producing fungal genera. Trichothecenes are classified in four groups, namely A, B, C and D, according to their substitutions on the core structure (Fig. 1-4). Type A (e.g., T-2 and HT-2) trichothecenes do not posess a carbonyl group substitution in C-8, while type B (e.g. deoxynivalenol, DON and nivalenol, NIV) do. Type C trichothecenes (e.g., crotocin and baccharin) possess an additional epoxy ring C-7 and C-8, or between C-9 and C-10; type D trichothecenes (e.g., satratoxin and roridin), contain a macrocyclic ring between C-4 and C-1 (Desjardins et al., 2007).

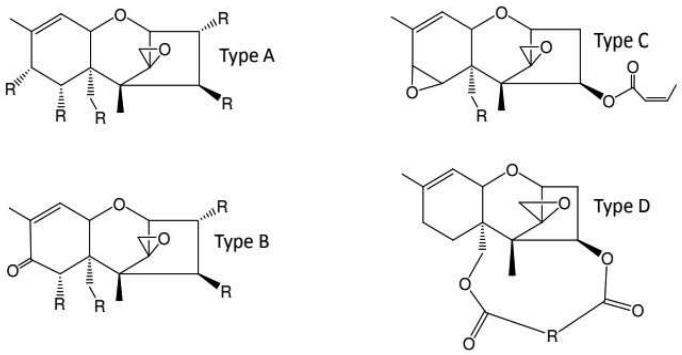
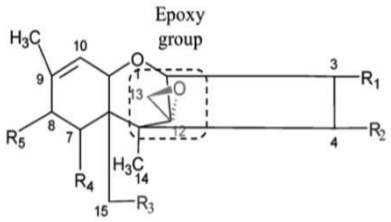


Figure 1-4. Trichothecenes general structure

Type A and B are the most important ones with respect to occurrence and toxicity (Fig. 1-5), with T-2 toxin being the most toxic (Miller, 2002). DON and NIV display also phytotoxicity, thus they can be classified both as mycotoxins and phytotoxins.

Trichothecenes cause a wide variety of toxic effect in animals, including growth retardation, reduced ovarian function and reproductive disorders, immunocompromization, feed refusal and vomiting. At cellular level, they induce apoptosis, inhibition of protein, nucleic acids synthesis and membrane structure alteration (Arunachalam et al., 2013).



	T-2	HT-2	DON	NIV
R1	-OH	-OH	-OH	-OH
R2	-OAc	-OH	-H	-OH
R3	-OAc	-OAc	-OH	-OH
R4	-H	-H	-OH	-OH
R5	$-OCOCH_2CH(CH_3)_2$	$-OCOCH_2CH(CH_3)_2$	=O	=O

Figure 1-5. Chemical structure of type A and B trichothecenes. Table shows substituent groups of the most important trichothecenes analogues

The main determinant of toxicity is the 12,13-epoxy ring. In addition, the presence of hydroxyl or acetyl groups at appropriate positions on the

trichothecene core; the presence of substituents on C-15 and C-4, and of the side groups, define the degree of toxicity (Cundliffe et at., 1977; Karlovsky et al., 2011).

1.1.5. Ochratoxins

Ochratoxins are a group of mycotoxins mainly produced by *Aspergillus ochraceus, A. carbonarius, A. niger* and *Penicillium verrucousm*. OTA is the most important representative and can be defined as a 7-carboxy-5-chloro-8hydroxy-3,4-dihydro-3-R-methylisocoumarin (ochratoxin α, OTα) coupled to β-phenylalanine (Phe) by an amide bond.

Ochratoxin B and C are OTA derivatives and are formed upon dechlorination and esterification, respectively. OTα is the main detoxification product, deriving from the cleavage of the amide bond and the release of the Phe moiety (Fig. 1-6). OTα is considered to be a non-toxic compound, with a 10-times shorter elimination half-life than OTA (Koszegi et al., 2011).

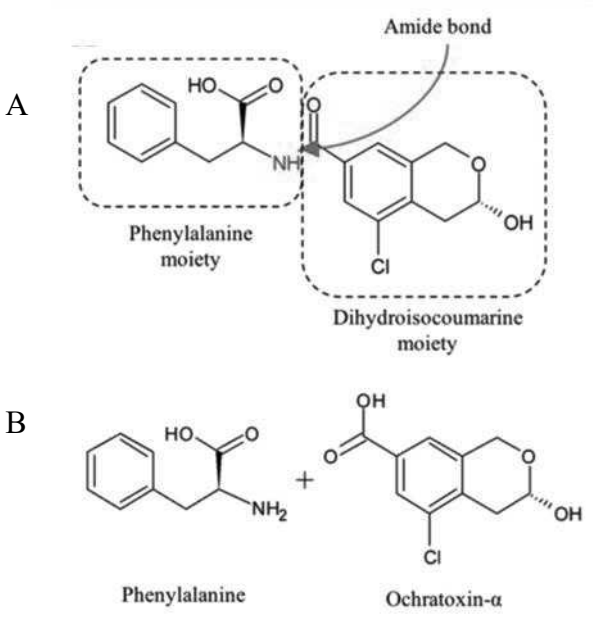


Figure 1-6. Chemical structure of ochratoxin A and derivatives. A) Structural determinants of OTA. The amide bond hydrolyzed by the main degrading pathway is indicated in red; B) Phenylalanine and ochratoxin α, the main detoxification product of OTA

OTA is toxic and carcinogenic to animals and possibly carcinogenic to humans (IARC, 1993). Kidney is the main target organ, but also immune system suppression has been described as an important adverse effect. At cellular levels, it is responsible for the inhibition of protein synthesis, the formation of DNA adducts, indirect oxidative DNA damage and epigenetic modifications.

OTA exposure in humans has been correlated with the outbreaks of several nephropathies, of which the most known is the Balkanic endemic syndrome (Pavlović et al., 2013), although a direct carcinogenic effect in humans has not been yet verified.

OTA is a common contaminant of cereals, dried fruits, grape juices, coffee and fermented products such as wine and beer. The carry over in kidney, blood and muscular tissue of pigs and milk and dairy was also reported (Völkel et al., 2011).

1.2. Prevention and reduction strategies

Mycotoxin contamination of food commodities depends upon several environmental and physiological factors governing the secondary metabolism of the producing fungus and can occur both in field and during storage. Thus, it is difficult to predict and almost impossible to avoid their occurrence in food commodities.

Managing mycotoxin risk is a complex task and requires an integrated strategy, covering both the pre and post-harvest.

1.2.1. Prevention in pre-harvest

Mycotoxin prevention in pre-harvest starts with the application of the good agricultural practice (GAPs) which include the choice of resistant varieties, correct soil management and the application of appropriate agronomical practices and use of biocontrol agents. They aim at reducing the extent of fungal contamination, thus mycotoxin contamination risk.

Plants show different susceptibility to fungal infection. Specific genes are known to regulate metabolic pathways for new or increased resistance systems governing the initial fungal infection (type I resistance) or its spread (type II resistance). Also phenotypic traits (height, dwarfing, lodging susceptibility, flower opening, anthers type) contribute to plant resistance to fungal infection (Steiner et al., 2017).

The correct soil management aims at reducing fungal contamination and interrupt the cycles of infection. Correct timing of fungicide application allows to control fungal contamination. However, the presence of different species, with different fungicide susceptibility and the non-homogenous phenological development of plants might reduce the efficiency of the treatment (Wegulo et al., 2015).

Tillage to bury host crop residues and crop rotation with non-susceptible cops reduce fungal infection frequency and intensity. Other important factors in reducing fungal infection are a correct fertilizer and water management. Early harvesting helps in reducing mycotoxin production, since less time is available for fungi to produce toxins.

The use of biocontrol agents is another important strategy which can be used to control mycotoxin producing fungi in an environmentally friendly way. There are several mechanisms through which they act: they are able to compete, produce antifungal substances, parasitize the mycotoxigenic fungi or promote plant defence systems (Nguyen et al., 2017)

Predictive models are also in use to forecast mycotoxin contamination in crops and plan the most effective risk management strategies. They were developed implementing data from variables having a significant impact on the possible mycotoxin production (crop type, environmental conditions, fungal infection cycle) into mathematical models. A correct prediction is a powerful supporting tool to apply the correct intervention strategies to counteract mycotoxin contamination and minimize the risk (Battilani, 2016).

1.2.2. Post-harvest reduction strategies

Pre-harvest strategies are only able to mitigate mycotoxin contamination, which can also occur during storage. Post-harvest strategies have to be implemented within the pre-harvest ones to reduce mycotoxin contamination and to improve the safety of food and feed commodities. They can be divided into physical, chemical or biological methods.

Desired process characteristics should i) be effective (ii) produce non-toxic metabolites (iii) not alter the nutrient profile and technological production and if possible, (iv) destroy fungal spores (Grenier, et al., 2014).

It is noteworthy mentioning that, to date, in Europe the detoxification of commodities intended for human consumption exceeding regulatory limits is not allowed (Commission Regulation EC number 1881/2006). However, these methods can be applied to commodities intended for animal consumption (Commission Regulation EC 786/2015).

Physical methods: physical methods include the removal of highly contaminated fractions from raw materials (by sorting, cleaning, milling, dehulling), the application of heat, the use of and cold plasma application.

Sorting is the first step to drastically reduce mycotoxin contamination. In fact, it is well known to occur heterogeneously, with hot spots of high contamination in fine materials, broken kernels and dust. DON, FB_1, T-2 and HT-2 toxin are more frequently associated with the pericarp layers and bran, thus cleaning, dehulling and milling leads to their removal from refined grains and accumulation in cereal by-products. (Fleurat-Lessard, 2017).

Toxins are generally heat stable within the range of food-processing temperatures (80–121 °C), however higher temperatures, like in roasting and extrusion processes were reported to reduce mycotoxins in nuts and maize (Kabak, 2008).

Cold plasma appears to be an innovative method for mycotoxins reduction with minimal effect on the nutritional value of food. Degradation of compounds in solid materials by cold plasma is restricted to thin surface layers, where mycotoxins are present the most, and occurs at room temperature. However, degradation was shown to be strongly dependent on mycotoxin structure and food matrix assayed (Bosch et al., 2017).

The use of adsorbents is very popular in feed production. The principle is that bioavailability of mycotoxins is reduced because of their binding to mineral, organic or biological material. The most common mineral adsorbents are activated charcoal, bentonites, silicates and yeast cell walls. The major drawback is the limited efficacy towards trichothecenes and the lack of specificity, which leads to unspecific binding of nutrients and antibiotics (Jarda et al., 2011).

Chemical methods: several chemical compounds among acids, bases, oxidising, chlorination or reducing agents, were shown to reduce, destroy and inactivate mycotoxins. AFB_1, FB_1 and trichothecenes degradation by ammoniation was reported (Kabak et al., 2007). However, despite the efficacy, the application of chemicals is limited because it can be impractical, unsafe, expensive and unfavourable. Chemical treatments require harsh conditions, which can generate toxic residues and drastically reduce the nutritional, sensory and functional properties of the product (Jalili, 2015).

Biological methods: recently, biological degradation with microorganisms or their enzyme arose great interest in the scientific community. Many organisms were reported to degrade AFs, DON and ZEN among fungi and bacteria (Vanhoutte et al., 2016) and many enzymes were isolated and characterize for mycotoxins degradation capability (Loi et al., 2017). The use of enzymes allows to use mild condition to obtain a specific, likely irreversible reaction, with minimal impact on the sensory and nutritional quality of raw matrices.

It is important to discriminate among biodegradation and bio-detoxification. The biodegradation leads to the formation of new products, with no regards to their toxicity, which can also be equal or higher than that of the parent compound (e.g.α-ZOL, AFM_1). Through the biodetoxification, the toxic compound is transformed into a non-toxic, or significantly less toxic compound (e.g. HFB_1, OTα). The biotransformation of mycotoxins implies that a biodetoxification process is performed (EFSA, 2009).

Among the many identified enzymes capable of degrading or detoxifying mycotoxins, laccases (LC) deserve particular attention because they are green catalysts, thus environmental friendly, extremely versatile, and their ability to degrade several toxins, such as AFs and ZEA, was already reported (Alberts et al., 2009, Banu et al, 2014).

2. Laccases

2.1. Overview, occurrence and physiological role of laccase enzymes

Laccases (LCs) (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) represent the largest subgroup of blue multicopper oxidases (MCO). LCs are copper containing enzymes which catalyse the oxidation of mono and ortho-diphenols, thiols and anilines to the corresponding quinones concurrently to the four-electron reduction of oxygen to water (Giardina et al., 2015). LCs were discovered in the early 1880s, in the sap of the Japanese lacquer tree *Rhus vernicifera* and since then they have been extensively studied (Yoshida, 1883). They are ubiquitous enzymes, as they have been identified in fungi (Hatakka, 1994), plants (Mayer, 1987), bacteria (Enguita et al., 2003) and insects as well. Fungal LCs are secreted by most white-rot basidiomycetes during lignin biodegradation, however they are also involved in melanin-like pigment synthesis, as well as in the bio-detoxification of harmful compounds or humus turnover in soil (Lisova et al., 2012).

Other important roles are played by laccases in chitin and lignin synthesis (in insects and higher plants, respectively). Lastly, in bacteria, they may be involved in pigmentation, resistance of spores and pathogenesis (Cañas and Camarero, 2010).

Fungi and in particular white rot fungi are the main LCs producers and have been extensively studied for many biotechnological applications from a molecular and biochemical point of view. Among white rot fungi the genus *Pleurotus* is one of the most studied because it is a safe, edible mushroom, easy to be cultivated and one of the most important genus from a commercial point of view. Originally it was supposed that the variability of LC proteins was due to the differential post traduction modification of the same gene product. Instead, many laccase genes and relative cDNA copies were identified and characterized during the last 20 years. Great variability exists among the same genus; in *Pleurotus* genus, 12 different complete gene sequences have been described for *Pleurotus ostreatus*, 5

for *Pleurotus sajor caju* and 8 for *Pleurotus eryngii* (https://www.ncbi.nlm.nih.gov/, accessed on 08/11/17).

LC genes expression can be divided into constitutive (e.g. Lacc3 and Lacc12 of *P. ostreatus;* Lac3 of *P. sajor caju*) or inducible (e.g. Lacc2 and Lacc10 of *P. ostreatus*; Lac1, Lac2 and Lac4 of *P. sajor caju*). Constitutive LCs are evenly expressed in changing environmental conditions, the latter are expressed or significantly more expressed in response to environmental stimuli or specific molecules. The promoters of these genes contain a high number of motifs that are sensitive to components present in the wheat straw extract, such as xenobiotic response elements (XRE) and metal-responsive elements (MRE) (Castanera et al., 2012).

Physiological mechanisms occurring during mycelia development can also modulate the relative expression levels of laccase isoenzymes. Some isoforms have been observed during the lag and exponential phases of fungal fermentation and should be involved in substrate degradation, whilst other isoforms have been detected in the stationary phase and should be involved in mushroom morphogenesis and pigmentation processes (Piscitelli et al., 2011). In *P. pulmonarius* (formely known as *P. sajor caju*) three different laccase isoenzymes are produced by the fungus in presence of different aromatic compounds (Zucca et al., 2011), while in *P. ostreatus* Lacc2 and Lacc10 resulted upregulated when wheat straw extract is added to the culture media (Castanera et al., 2012).

2.2. Molecular structure and catalytic activity

The crystal structures of LCs from several fungal species were fully resolved, e.g. LC from the ascomycete *Melanocarpus albomyces* (Hakulinen et al., 2002) and the basidiomycetes *C. cinerea* (Ducros et al., 1998), *Trametes versicolor* (Piontek et al., 2002; Bertrand et al., 2002), *Rigidoporus lignosus* (Garavaglia et al., 2004), *Cerrena maxima* (Lyashenko et al., 2006) and *Lentinus (Panus) tigrinus* (Ferraroni et al., 2007).

LCs are usually monomers, although some dimers and tetramers exist (Lisova et al., 2012; Molitoris and Reinhammar, 1975). They are generally glycosylated, with 10 to 30% of glycosylation which may have a role in the protection of the enzyme from proteolysis and correct folding (Maestre-Reyna et al., 2015). Their isoelectric point is usually acidic, ranging between 3 and 6.

LC owes its blue color to the presence of one to four copper ions named T1 and T2/T3, coordinated by amino acid residues in two conserved regions (Fig. 2-1). T1 site has only three conserved ligands, two histidines and one cysteine which coordinate one copper ion in a trigonal planar geometry, whereas one axial ligand is usually variable and greatly contributes to the determination of the redox potential of the enzyme (Strong and Claus, 2011). This axial ligand is methionine in the bacterial laccases (CotA), and leucine or phenylalanine in fungal laccases; mutations of these two residues leads to the decrease of the oxidation potential (Kumar et al., 2003).

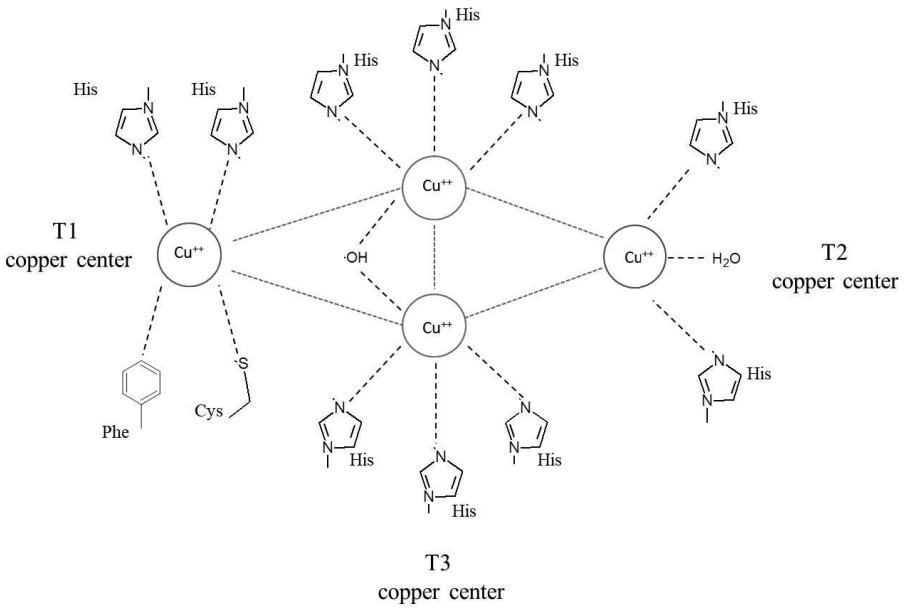


Figure 2-1. LC copper centers

T2 and T3 copper ions form a trinuclear cluster, where molecular oxygen is reduced to water. The T2 copper is coordinated by two histidines and one water molecule; and each of the two T3 copper ions by three histidines. Some variants from this general scheme do exist.

The 12 amino acid residues in the enzymes serving as the copper ligands are located within four conserved regions which are named L1, L2, L3 and L4. The eight histidines that serve as ligands of the trinuclear cluster occur in a highly conserved pattern of four HXH motifs. In one of these motifs, X is the cysteine bound to the T1 copper, while each of the histidine is bound to one of the two T3 coppers. Intraprotein homologies between the conserved L1 and L3 and between L2 and L4 suggest the occurrence of duplication events.

Some LC variants lack the T1 copper and are often referred to as the yellow LCs, as they show no characteristic absorption band around 600 nm (Leontievsky et al., 1997).

The T1 copper is characterized by a strong absorption around 600 nm, whereas the T2 copper exhibits only weak absorption in the visible region. The T2 site is electron paramagnetic resonance (EPR)-active, whereas the two copper ions of the T3 site are EPR-silent due to an antiferromagnetic coupling mediated by a bridging ligand (Ferraroni et al., 2007).

In contrast to most enzymes, which are generally substrate specific, LC is able to oxidise a wide variety of substrates, ranging from phenols to polyphenols, anilines and benzenthiols. The catalytic activity of laccase can be divided into several steps shown in Fig. 2-2.

Firstly, the phenolic substrate is oxidized by the resting, fully oxidized enzyme into a phenoxyradical (Fig. 2-2, A). In particular, due to its high redox potential (*ca*. +790 mV) type 1 copper is the site where substrate oxidation takes place. The extracted electrons are then transferred, probably through a strongly conserved His-Cys-His tripeptide motif, to the T2/T3 site. Once four substrates have been oxidized and the electrons have been transferred from the T1 copper to the

trinuclear cluster, the enzyme switches to its fully reduced state (Fig. 2-2,B). Molecular oxygen binds to the trinuclear cluster to form a peroxide intermediate (Fig. 2-2,C). The presence of a conserved aspartate residue lowers the reduction potential of T2 and T3 copper, allowing them to donate an electron to the oxygen molecule. It results in a peroxide bridge. The peroxide intermediate then decays through the O-O bond cleavage.

The cleavage may proceed through a proton unassisted pathway at high pH and a proton-assisted pathway at low pH, the latter being supported by a conserved glutamate residue.

The resulting hydroxide lowers the reduction potential of the T2, facilitating electron transfer to the peroxide which turns into an oxyl radical intermediate (Fig. 2-2,D). In the absence of excess reductant, the radical intermediate decays slowly to the resting, fully oxidized enzyme (Fig. 2-2,E) (Jones and Solomon, 2015).

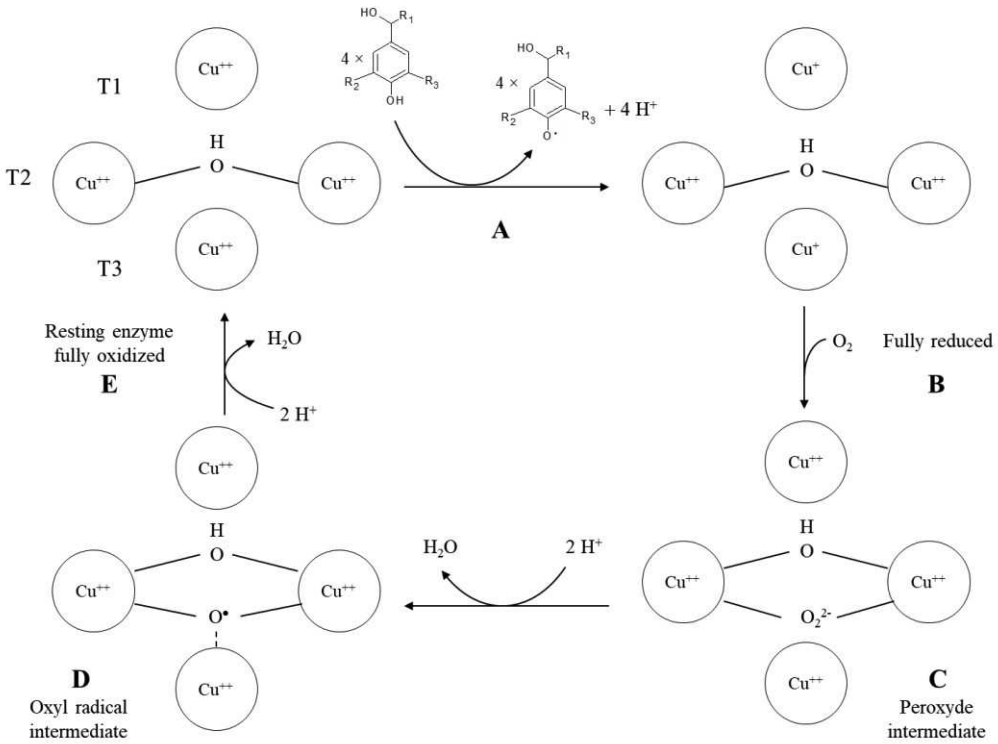


Figure 2-2. Laccase catalytic cycle (adapted from Wong, 2009)

Since no hydrogen peroxide is detected during laccase activity, it seems reasonable to suppose that a four-electron oxidation of oxygen to water occurs. As the oxidation of the substrate is a one electron reaction, laccase oxidizes four substrates in order to reduce oxygen to water. Thus, the trinuclear cluster is able to store electrons coming from each of the four substrates. In the case of substituted compounds, the reaction can be accompanied by partial demethylations and dehalogenations.

LC activity is strongly dependent on its redox potential and surprisingly it is not limited to phenolic substrates. Factors affecting metal-protein redox potential are varied and of great complexity. Many aspects such as solvation, metal–ligand interactions, intramolecular electrostatic interactions, and/or protein folding restrictions (governing the position and orientation of the ligands) can modulate the redox potential (E^0) values of these enzymes (Frasconi et al., 2010). Above all, E^0 is strongly governed by the geometry at the T1 Cu center (Kenzom et al., 2014).

LC can be divided in to low and high redox potential laccases, as their E^0 can be as low as +400 or as high as +800mV. High redox LC are able to oxidise a wide variety of substrates, whose redox potential is lower or similar to that of the enzyme. The redox potential of phenolic substrates (0.5–0.9 V, at acid pH) and enzyme (0.6–0.8 V) is similar therefore a fast and efficient oxidation occurs. However, 2,4,6-tri(But)phenol is not oxidized by LC due to steric hindrances, proving that not all polyphenols are suitable LC substrates (d'Acunzo et al., 2006). Low redox laccases have a limited range of substrates that can be oxidized, however this limit can be overcome by the use of redox mediators in the so-called Laccase Mediator System (LMS).

2.3. Laccase mediator system

Among the fungal oxidoreductive enzymes, laccases have the lowest redox potential (usually less than +0.8V); however, target compounds with high molecular weight or high redox potential can still be indirectly oxidized. As a

matter of fact, LC oxidation can be mediated by low molecular weight molecules which act as electron shuttles from the enzyme to the target substrate. This system, to which we refer as LMS, is naturally used by white rot fungi to degrade lignin, which is a high molecular weight polymer, as well as in industrial application, mostly in paper pulp and textile industry (Bugg et al., 2015).

The enzyme acts towards a low molecular weight redox mediator, which is then oxidized. The oxidized mediator performs a non-enzymatic oxidation of the substrate, by using mechanisms that may be unavailable to laccase, thereby explaining the possibility to oxidise non-phenolic substrates, and widening the usefulness of a purely enzymatic method. The catalytic cycle is closed when oxygen is reduced to water by LC (Fig. 2-3). LMS is a powerful tool which has been applied for many years in the textile industry since it allows the degradation of recalcitrant dyes, such as alizarin Red, reactive black 5 and methyl orange using acetosyringone (AS), 1-hydroxybenzotriazole (HBT) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), respectively (Zucca et al., 2011;

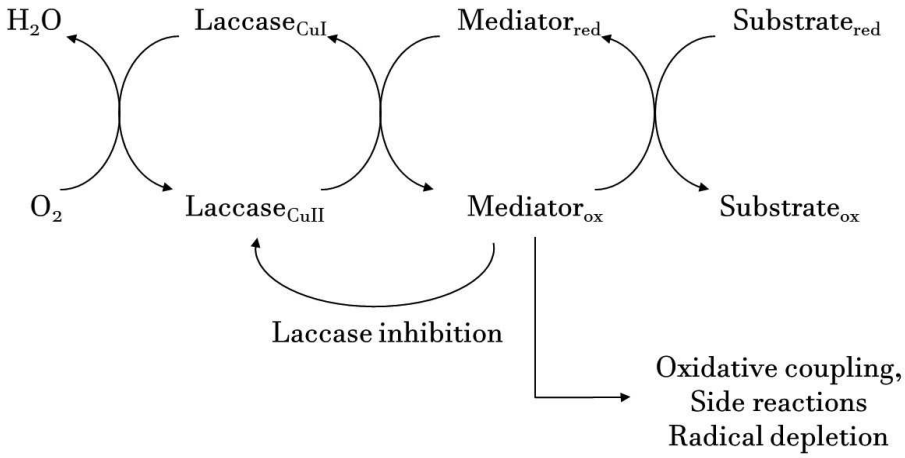


Figure 2-3. Laccase Mediator System

Wang et al., 2011; Telke et al., 2011).

LMS allows to generate a compound with enhanced oxidative capacity and reduced steric hindrance with respect to that of LC itself. The oxidized mediator

has a higher E^0 and can easily react with other compounds without the steric restriction of an enzyme active site.

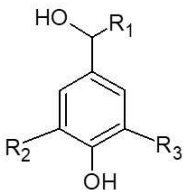
More than 20 compounds of different origin and structure, including heterocycles, >N–OH compounds, syringil and cynnamic acid derivatives have been used as LC mediators and their mechanism of oxidation studied (Baiocco et al., 2003; Brogioni et al., 2008).

Simple and substituted phenols deriving both from fungal metabolism or lignin depolymerisation are efficiently oxidized by LC and they are the most used natural redox mediators. Syringil acid derivatives include syringic acid, vanillic acid, syringaldehyde (SA) and AS, while cynnamic acid derivatives comprise p-coumaric acid (pCA), ferulic acid (FA), synaptic acid and caffeic acid.

Artificial compounds include HBT, N-hydroxyphtalimide (HPI), (2,2,6,6-tetramethylpiperidin- 1-yl)oxyl (TEMPO), violuric acid (VA), and ABTS. The structures of representative laccase mediators are shown in Fig. 2-4.

The rate-determining step in LMS is the oxidation of the mediator, thus the electron transfer to the T1 copper centre. LC and mediator type, *i.e.* their E^0, is a crucial parameter that governs the kinetics of oxidation: the higher LC E^0, or the lower the mediator E^0 is, the fastest the oxidation rate is (D'acunzo et al., 2002).

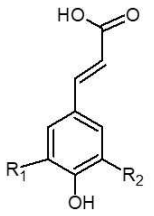
A



Syringil acid general structure

Syringil acid derivatives

Compound	R_1	R_2	R_3
Syringic acid	-OH	$-OCH_3$	$-OCH_3$
Vanillic acid	-OH	$-OCH_3$	-H
Syringaldehyde	-H	$-OCH_3$	$-OCH_3$
Acetosyringone	$-CH_3$	$-OCH_3$	$-OCH_3$

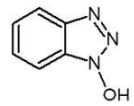


Cynnamic acid general structure

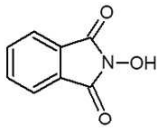
Cynnamic acid derivatives

Compound	R_1	R_2
p-coumaric acid	-H	-H
Ferulic acid	$-OCH_3$	-H
Sinaptic acid	$-OCH_3$	$-OCH_3$
Caffeic acid	-OH	-H

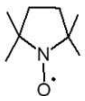
B



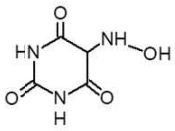
HBT



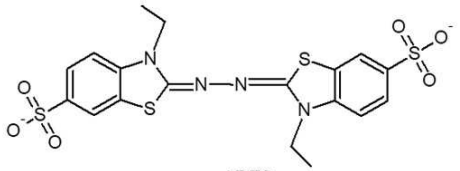
HPI



TEMPO



VA



ABTS

Figure 2-4. Chemical structures of the major natural (A) and artificial (B) redox mediators. hydroxybenzotriazole (HBT), N-hydroxyphtalimide (HPI), (2,2,6,6-tetramethylpiperidin- 1-yl)oxyl (TEMPO), violuric acid (VA), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

Substrate oxidation by the oxidized mediator can occur by multiple mechanisms according to the type of mediator used. Substrate oxidation may occur by hydrogen atom transfer (HAT), single electron transfer (ET) or following a ionic route. This variety of mechanism is another strong point of LMS, because a selective oxidation can be performed by choosing the right mediator.

An ideal mediator should be a small-size molecule, able to quickly create a stable radical (*i.e.* electrochemically stable), easily regenerated, not consumed in unwanted side reactions such as polymerization or enzyme inactivation (Fig 2-3).

In view of an industrial application, the natural origin, availability and low cost are also features of great importance (Canas and Camarereo, 2010).

Electron transfer mediated mechanism

The first synthetic mediator to be discovered for the oxidation of both lignin and non-phenolic compounds was ABTS (Burbonnais and Paice, 1990). ABTS is oxidized by LC into the green radical cation $ABTS^{\bullet+}$, which then undergoes disproportion to restore ABTS and generate the dication radical $ABTS^{2+}$ (Fig. 2-5,A) (Rochefort et al., 2003). The dication radical is stable and has a greater E^0 than the single radical cation (0.91V compared to 0.47V, respectively). Both redox states of ABTS are stable and reversible (Morozova et al., 2007) and are shown in Fig. 2-5,A. Substrate oxidation by $ABTS^{2+}$ follows an electron transfer mechanism, thus a single electron is abstracted with subsequent proton loss from the substrate (Fig. 2-5,B).

Radical hydrogen atom mechanism

Natural phenols and N-OH type mediators oxidize the substrate through a radical route. Upon LC oxidation, those compounds are transformed into reactive phenoxy or nitroxy radicals, which remove a hydrogen atom from the substrate, thus regenerating itself and producing another radical (Fig. 2-6). In this case, the rate of HAT route, therefore mediator reactivity, is strongly dependent on the bond dissociation energy rather than on the electrochemical potential of the final substrate.

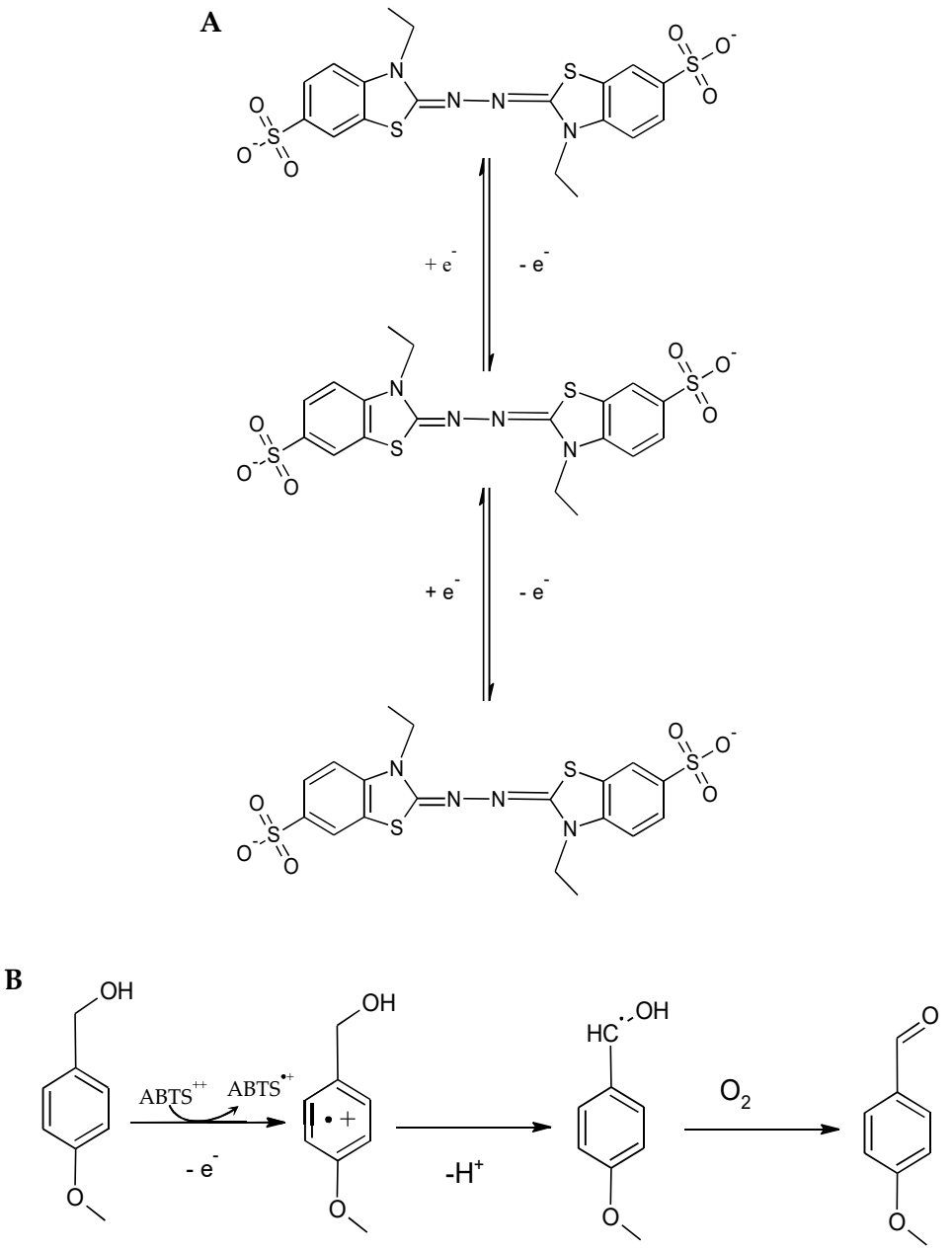


Figure 2-5. ABTS oxidation by laccase (A) and electron transfer mechanism (B)

Phenoxy radicals can also undergo further enzyme-independent reactions such as homo- and hetero-coupling to form dimeric, oligomeric, polymeric, or cross-coupling product, and in some cases, aromatic ring opening reactions (Hashmi et al., 2016). Coupling and crosslinking reactions are exploited in organic synthesis and grafting processes (Wells et al., 2006). Among natural phenols, metoxy-substituted ones (*i.e.* acetosyringone, syringaldehyde or synaptic acid) proved to be extremely effective in removing industrial dyes (Camarero et al., 2005) because electron-donating substitutes, like methoxide, lower phenol

electrochemical potential. Moreover, ortho-substituted phenols are endowed with increased lifetime and less susceptibility to 5–5′ coupling reactions (Canas et al., 2010). HAT route is the only mechanism available for N-OH type mediators like HBT, HPI VA.

Despite their efficacy, artificial N-OH mediators can be toxic and expensive, so their use in industrial application has been limited (Canas et al., 2010).

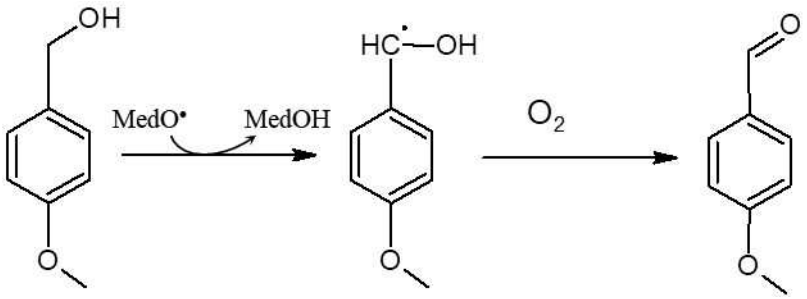


Figure 2-6. Hydrogen atom transfer mechanism. 4-methoxy benzyl alcohol is oxidized by phenoxy and N-oxyl radicals by hydrogen atom abstraction from Cα. Electronic rearrangements proceeds via aldehyde formation

Ionic route

TEMPO is a stable N-oxyl radical known to be a highly selective catalyst in the oxidation of alcohols. Its role as laccase mediator was also reported (Fabbrini et al., 2001). Upon oxidation by LC, TEMPO is transformed into a oxoammonium ion (Fig. 2-7), which is a relatively strong oxidant (E^0 = 0.75 V).

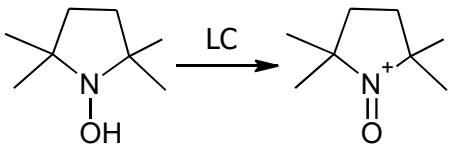


Figure 2-7. TEMPO oxoammonium cation catalysis by laccase (LC)

Within LMS, TEMPO is supposed to proceed through an ionic mechanism, which was well described for alcohol oxidation to aldehyde. A nucleophilic attack of the substrate onto the nitrogen atom of the oxoammonium takes place leading

to a transient adduct. Cleavage of the α-C-H bond and deprotonation leads to the reduced form of TEMPO and an aldehyde (Fig. 2-8).

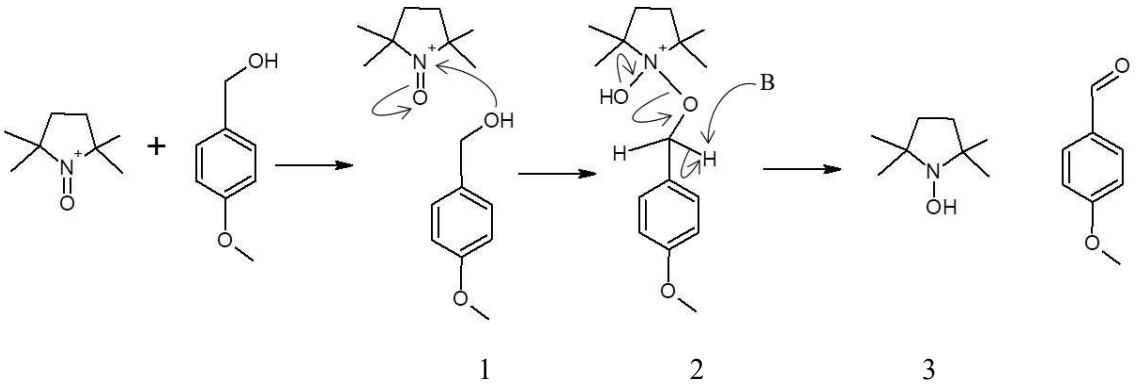


Figure 2-8. Ionic mechanism. Oxidation of the model compound 4-methoxybenzyl alcohol by tempo follows an ionic route. Electrophylic oxygen athom of 4-methoxybenzyl alcohol attacks electrondeficient nitrogen in TEMPO (1), leading to a transient abduct (2), which is then deprotonated to 4-methoxybenzyl aldehyde and the reduced form of TEMPO (3).

Unlike TEMPO, other transient N-oxyl radicals, generated by LC from the oxidation of N-OH mediators such as HBT and HPI, react by an HAT route. The oxidation of those N-oxyl mediators to the corresponding oxoammonium ion would occur at a too high redox potential (>1.3V), unattainable by LC.

In the case of TEMPO, the HAT mechanism only occurs at a very slow rate because it is enthalpically unfavoured. The enthalpic variation for the removal of a hydrogen atom would be too high, owing to the weak O-H bond that would be formed. Since the oxidation of TEMPO to oxoammonium ion is fast, the ionic mechanism takes over (Fabbrini et al., 2001).

2.4. Laccase applications

LC applications are widespread in view of its green feature and possibility to selectively extend its activity with redox mediators. The radical mechanism followed by many redox mediators activates both anabolic and catabolic pathways and can be exploited to produce new compounds, to degrade already existent ones and to develop redox biosensors. LMS have been applied in the pulp and

paper industry, textile, organic synthesis, environmental bioremediation, food production, pharmaceuticals and nano-biotechnology. Many of those applications are patented and used at industrial level (Kunamneni, et al., 2008).

Delignification of lignin and pulp bleaching for paper production by means of LC has been exploited in substitution of conventional and polluting chlorine-based methods.

Bioremediation with LMS is an expanding field. Many important environmental pollutants can be degraded by means of LMS treatment, such as polycyclic aromatic hydrocarbons (Collins et al., 2006) sulphonamide antibiotics (Shi et al., 2014), industrial dyes (Zucca et al., 2011) chloro-phenols and other phenol derivatives from industrial wastes (Zhang et al., 2008).

Phenols are natural antioxidants, but at very high concentration can be toxic to microorganisms, including bacteria and yeasts, plants and the marine environment. Detoxification of phenols can be achieved using LC and applied for the detoxification of olive mill wastewater (Tsioulpas et al., 2002) as well as of vegetable biomasses prior ethanol production by yeasts (Jurado et al., 2009). The presence of phenols is also undesirable in some food products and their reduction stabilizes colour and reduces haze in juices, wines and beers (Osma et al. 2010).

LC applications in food industry are not limited to phenol stabilization. LC and LMS were reported to crosslink proteins, create olysaccharide–polyphenol or protein–polyphenol–polysaccharide conjugates to improve food texture, heat resistance, emulsifying properties, stability and viscosity (Liu et al., 2017; Zeeb et al., 2017) in dairy, bakery and meat products.

Coupling reactions, oxidation of sulphur compounds, hydroxylamines and alcohol to aldehyde are exploited in organic synthesis for the production of chemicals, antibiotics (Mikolasch et al., 2008) or grafted and functionalized materials in a green and environmental friendly way (Wells et al., 2006).

2.5. *Laccase as tool for mycotoxins bioremediation in feed, food and bioenergy supply chains*

Mycotoxin bioremediation with enzymes is emerging as a new and innovative strategy to reduce or remove those harmful compounds. The evidence that white rot fungi could degrade mycotoxins has been already reported, but only recently the degrading activity has been directly ascribed to LC enzyme (Loi et al., 2016). Certainly, when the whole microorganism is employed, several other oxidoreductive enzymes participate together with LC in the degradation process. Indeed, other oxidases were reported to degrade AFs (Yehia et al., 2014; Wang et al., 2011), ZEN (Yu et al., 2012) and DON (Ito et al., 2013).

LC has been recently reported to degrade structurally diverse toxins, such as AFs (Alberts et al., 2009; Loi et al 2016) and ZEN (Banu et al., 2013). In addition, purified enzyme was reported to efficiently degrade toxins only in presence of a redox mediator.

LC application in the field of mycotoxins bioremediation is appealing, especially in view of its environmental friendly feature, versatility and broad range of substrate oxidation.

Although no detoxification process for food exceeding regulatory limits is allowed in EU, acceptability criteria for detoxification processes applied to products intended for animal feed were set in May 2015 (Commission regulation (EU) 2015/786).

Enzyme application in feed is not a new topic, actually it dates back to 1920s with "Protozyme", a commercial preparation from *Aspergillus orizae*, used in poultry diets (Clickner et al., 1925). Several feed enzymes are currently in use to increase nutritional value and micronutrient availability of feed, such as phytases, proteases, α-galactosidases, glucanases, α- amylases and polygalacturonidases.

Mandatory requirements for the application of any degrading enzyme are i) solid supporting information about production, safety and efficacy (both *in vitro*

and *in vivo*) and ii) non-alteration of feed nutritional and organoleptic features (Commission regulation (EU) 2015/786).

So far only one enzyme, the fumonisin esterase FumZyme® by Biomin (Holding GmbH), was patented and approved for all avian species and pigs upon and extensive evaluation by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (EFSA, 2014; EFSA 2016). The fumonisin esterase is also part of a combined product, Mycofix®, which exploits a trichothecenes, zearalenone and ochratoxin degradaing microorganisms as well as an aflatoxin adsorbent in a unique all in one formulation.

No regulation on mycotoxin occurrence in biofuel industries exists, although mycotoxins accumulation in by-products has been reported. During bioethanol production, mycotoxins are not detected in the alcoholic fractions but accumulate in the spent grain products. Since $^1/_3$ of the biomass is transformed into alcohol and another $^1/_3$ into CO_2, mycotoxins accumulate by three folds in dried distillers' grains with solubles (DDGS) which are then used as feed or fertilizers (Pinotti et al., 2016). Thus, mycotoxin risk is driven back to the food and feed supply chains. Biofuel production efficiency might also be lowered by mycotoxin contamination, as some of them can be toxic and reduce the growth of microorganisms or fermentation rates (Kłosowski, et al., 2010). Moreover, the manipulation of such contaminated products is also a concerning risk for operators.

Vegetable biomass pre-treatment could be easily implemented in the biofuel industry, where it is usually used to remove lignin and reduce phenol content (Galbe et al., 2002).

Nonetheless, no implementation of laccase or any other degrading enzyme in the biofuel industries has been yet developed.

References

Alberts, J.F., Gelderblom, W.C., Botha, A., Van, Zyl. W.H. (2009). Degradation of aflatoxin B_1 by fungal laccase enzymes. *International Journal of Food Microbiology*. 135, 47–52

Arunachalam, C., & Doohan, F. M. (2013). Trichothecene toxicity in eukaryotes: Cellular and molecular mechanisms in plants and animals. Toxicology letters, 217(2), 149-158.

Baiocco, P., Barreca, A. M., Fabbrini, M., Galli, C., & Gentili, P. (2003). Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase–mediator systems. *Organic & biomolecular chemistry*, 1, 191-197.

Banu, I., Lupu, A., & Aprodu, I. (2013). Degradation of Zearalenone by Laccase enzyme. Scientific Study & Research. Chemistry & Chemical Engineering, Biotechnology, *Food Industry*, 14, 79.

Battilani, P., Costa, L. G., Dossena, A., Gullino, M. L., Marchelli, R., Galaverna, G., Gualla, A. (2009). Scientific information on mycotoxins and natural plant toxicants. *Efsa Supporting Publications*, 6.

Bennett, J.W., Klich, M. (2003). Mycotoxins. *Clinical Microbiology reviews*. 497–516

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., Mougin, C. (2002). Crystal structure of a four-copper laccase com-plexed with an arylamine: insights into substrate recognition and correlation with kinetics. *Biochemistry*. 41, 7325–7333

Blount, W.P. 1961. Turkey "X" disease. *Turkeys*, 9, 52-55.

Bosch, L., Pfohl, K., Avramidis, G., Wieneke, S., Viöl, W., Karlovsky P. (2017). Plasma-Based Degradation of Mycotoxins Produced by Fusarium, Aspergillus and Alternaria Species. *Toxins*. 9, 97.

Bourbonnais, R., Paice, M.G. (1990). Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. *FEBS letters*. 267, 99-102.

Brogioni, B., Biglino, D., Sinicropi, A., Reijerse, E.J., Giardina, P., Sannia, G., Pogni, R. (2008). Characterization of radical intermediates in laccase-mediator systems. A multifrequency EPR, ENDOR and DFT/PCM investigation. *Physical Chemistry Chemical Physics*. 10, 7284-7292.

Bugg, T.D., Rahmanpour, R. (2015). Enzymatic conversion of lignin into renewable chemicals. *Current opinion in chemical biology*, 29, 10-17.

Camarero, S., Ibarra, D., Martínez, M.J., Martínez, Á.T. (2005). Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Applied and environmental microbiology* 71, 1775-1784.

Cañas, A.I., Camarero, S. (2010). Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. *Biotechnology Advances*, 28, 694–705

Castanera, R., Pérez, G., Omarini, A., Alfaro, M., Pisabarro, A.G., Faraco, V., Amore, A., Ramírez, L. (2012). Transcriptional and Enzymatic Profiling of Pleurotus ostreatus Laccase Genes in Submerged and Solid-State Fermentation Cultures. *Applied Environmental Microbiology*. 8, 4037-45

Clickner, F.H., Follwell, E.H. (1925). Application of 'protozyme' by *Aspergillus Orizae* to poultry feeding. *Poultry Science*. 5:241–247.

Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC). Off J Eur Union, L. 229: 7–9.

Commission Recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products (2013/165/EU). Off J Eur Union L, 91, 12-15.

Commission Regulation (EC) 2015/786 defining acceptability criteria for detoxification processes applied to products intended for animal feed. *Off J Eur Union* 125:10–14

Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff. 2006R1881-EN-01.09. 2014-014.001-1.

Cundliffe, E., Davies, J.E. (1977). Inhibition of initiation, elongation, and termination of eukaryotic protein synthesis by trichothecene fungal toxins. *Antimicrobial Agents and Chemotherapeutics*. 11, 491–499.

d'Acunzo, F., Galli, C., Gentili, P., Sergi, F. (2006). Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates. *New Journal of Chemistry*. 30, 583-591.

d'Acunzo, F., Galli, C., Masci, B. (2002). Oxidation of phenols by laccase and laccase-mediator systems. *The FEBS Journal*. 269, 5330-5335.

Desjardins, A.E., McCormick, S.P., Appell, M. (2007). Structure − activity relationships of trichothecene toxins in an Arabidopsis thaliana leaf assay. *Journal of agricultural and food chemistry*. 55, 6487-6492.

Ducros, V., Brzozowski, A.M., Wilson, K.S., Brown, S.H., Østergaard, P., Schneider, P., Yaver, D.S., Pedersen, A.H., Davies, GJ. (1998). Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. *Nature Structural and Molecular Biology*. 5, 310–316.

Eaton, D.L., Gallagher, E.P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annual Reviews in Pharmacology and Toxicology*. 34, 135–172

Enguita, F.J., Martins, L.O., Henriques, A.O., Carrondo, M.A. (2003). Crystal structure of a bacterial endospore coat component. A laccase with enhanced thermostability properties. *Journal of Biological Chemistry.* 278, 19416–19425

Essigmann, J.M., Croy, R,G,, Nadzan. A,M,, Busby, W.F. Jr, Reinhold, V.N., Büchi, G., Wogan, G.N. (1977). Structural identification of the major DNA adduct formed by aflatoxin B1 in vitro. *Proc. Nat. Acad. Sci*. USA 74, 1870–1874

European Food Safety Authority. (2005). Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to fumonisins as undesirable substances in animal feed

European Food Safety Authority. (2009). Scientific Report Review of Mycotoxin-Detoxifying Agents Used as Feed Additives: Mode of Action, Efficacy and Feed/Food Safety; Scientific Report Submitted to EFSA, CFP/EFSA/FEEDAP/2009/01, *Wiley Online Library*,

European Food Safety Authority. (2014). Scientific Opinion on the safety and efficacy of fumonisin esterase (FUMzyme®) as a technological additive for pigs. EFSA Journal. 12:3667

European Food Safety Authority. (2016). Safety and efficacy of fumonisin esterase (FUMzyme®) as a technological additive for all avian species. *EFSA Journal.* 14, 4617

Fabbrini, M., Galli, C., Gentili, P., Macchitella, D. (2001). An oxidation of alcohols by oxygen with the enzyme laccase and mediation by TEMPO. *Tetrahedron Letters*, 42, 7551-7553.

Fanelli, F., Geisen, R., Schmidt-Heydt, M., Logrieco, A.F., Mulè, G. (2016). Light regulation of mycotoxin biosynthesis: New perspectives for food safety. *World Mycotoxin J*ournal. 9, 129–146.

Fanelli, F., Iversen, A., Logrieco, A.F., Mulè, G. (2013). Relationship between fumonisin production and FUM gene expression in Fusarium verticillioides under different environmental conditions. *Food Additives & Contaminants: Part A*. 30, 365-371.

Ferraroni, M., Myasoedova, N., Schmatchenko, V., Leon-tievsky, A.A., Golovleva, L.A., Scozzafava, A., Briganti, F. (2007). Crystal structure of a blue laccase from *Lentinus tigrinus*: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. *BMC Structural Biology.* 7, 60–72

Fleurat-Lessard, F. (2017). Integrated management of the risks of stored grain spoilage by seedborne fungi and contamination by storage mould mycotoxins–An update. *Journal of Stored Products Research*, 71, 22-40.

Food and Agricultural Organization. Worldwide regulations for micotoxins in food and in feed in 2003. FAO (01 February, 2011)

Food and Agriculture Organization of the United Nations. (2011). Global Food Losses and Food Waste: Extent, Causes, and Prevention.

Frasconi, M., Favero, G., Boer, H., Koivula, A., Mazzei, F. (2010). Kinetic and biochemical properties of high and low redox potential laccases from fungal and plant origin. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics.* 1804, 899–908

Galbe, M., Zacchi, G. (2002). A review of the production of ethanol from softwood. *Applied microbiology and biotechnology*, 59, 618-628.

Garavaglia, S., Cambria, M.T., Miglio, M., Ragusa, S., Iacobazzi, V., Palmieri, F., D'ambrosio, C., Scaloni, A., Rizzi, M. (2004). The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the T3 copper pair. *Journal of Molecular Biology*, 342, 1519–1531

Giardina, P., Sannia, G. (2015). Laccases: old enzymes with a promising future. *Cellular and Molecular Life Sciences*. 72, 855.

Grenier, B., Loureiro-Bracarense, A.P., Leslie, J.F., Oswald, I.P. (2014). Physical and Chemical Methods for Mycotoxin Decontamination in Maize in Mycotoxin Reduction in Grain Chains.

Hakulinen, N., Kiiskinen, L.L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A., Rouvinen, J. (2002). Crystal structure of a laccase from Melanocarpus albomyces with an intact trinuclear copper site. *Nature Structural & Molecular Biology*. 9, 601–605

Hashmi, M., Chandra, R.P., Ragauskas, A.J. (2016). Lignin Valorization Via Laccase-Assisted Functionalization. *BAOJ Chemistry*, 2, 012.

Hatakka, A. (1994). Lignin-modifying enzymes from selected white-rot fungi, production and role in lignin degradation. *FEMS Microbiology Reviews*. 13, 125–135

Hesseltine, C.W., Shotwell, O.L., Ellis, J.J., Stubblefield, R.D. (1966). Aflatoxin formation by Aspergillus flavus. *Bacteriological reviews*. 30, 795.

http://www.ncbi.nlm.nih.gov/. Accessed on 17/06/15, 11.40am)

International Agency on Research on Cancer. (1993). Monographs on the evaluation of carcinogenic risks to humans. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins (Lyon: IARC), Vol. 56

International Agency on Research on Cancer. (2002). IARC Monograph on the Evaluation of Carcinogenic Risks to Humans. Lyon, 171.

International Agency on Research on Cancer. (2012). Fungi producing significant mycotoxins. *IARC Scientific Pubications*. 158, 1-30.

Ishikawa, A.T., Takabayashi-Yamashita, C.R., Ono, E., Bagatin, A.K., Rigobello, F.F., Kawamura, O., Itano, E.N. (2016). Exposure Assessment of Infants to

Aflatoxin M_1 through Consumption of Breast Milk and Infant Powdered Milk in Brazil. *Toxins*, 8, 246

Ito, M., Sato, I., Ishizaka, M., Yoshida, S., Koitabashi, M., Yoshida, S., Tsushima, S. (2013). Bacterial cytochrome P450 system catabolizing the Fusarium toxin deoxynivalenol. *Applied and Environmental Microbiology*. 79, 1619–1628.

Jackson, L.S., DeVries, J.W., Bullerman, L.B. (2013). Fumonisins in food (Vol. 392). Springer Science & Business Media.

Jalili, M. (2016). A review on aflatoxins reduction in food. *Iranian Journal of Health, Safety and Environment*. 3, 445-459.

Jard, G., Liboz, T., Mathieu, F., Guyonvarch, A., Lebrihi, A. (2011). Review of mycotoxin reduction in food and feed: from prevention in the field to detoxification by adsorption or transformation. *Food Additives & Contaminants: Part* A. 28, 1590-1609.

Jones, S.M., Solomon, E.I. (2015). Electron transfer and reaction mechanism of laccases. *Cellular Molecular Life Science*. 72, 869–883.

Jurado, M., Prieto, A., Martínez-Alcalá, Á., Martínez, Á.T., Martínez, M.J. (2009). Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. *Bioresource Technology*. 100, 6378-6384.

Kabak, B., Dobson, A.D., Var, I.I.L. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: a review. *Critical reviews in food science and nutrition*. 46, 593-619.

Kabak, B. (2009). The fate of mycotoxins during thermal food processing. *Journal of the Science of Food and Agriculture*. 89, 549-554.

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Dussort, P. (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin research.* 32, 179-205.

Kenzom, T., Srivastava, P., Mishra, S. (2014). Structural insights into 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)(ABTS)-mediated degradation of Reactive Blue 21 by engineered *Cyathus bulleri* laccase and characterization of degradation products. *Applied and environmental microbiology*. 80, 7484-7495.

Kłosowski, G., Mikulski, D. (2010). The effect of raw material contamination with mycotoxins on the composition of alcoholic fermentation volatile by-products in raw spirits, *Bioresource Technology*. 101, 9723–9727.

Kőszegi, T., Poór, M. (2016). Ochratoxin A: molecular interactions, mechanisms of toxicity and prevention at the molecular level. *Toxins.* 8, 111.

Kumar, S.V.S, Phale, P.S., Durani, S., Wangikar, P.P. (2003). Combined sequence and structure analysis of the fungal laccase family. *Biotechnology and Bioengineering.* 83, 386–394

Kunamneni, A., Plou, F.J., Ballesteros, A., Alcalde, M. (2008). Laccases and their applications: a patent review. *Recent patents on biotechnology.*, 2, 10-24.

Leontievsky, A.A., Vares, T., Lankinen, P., Shergill, J.K., Pozdnyakova, N.N., Myasoedova, N., Kalkkinen, N., Golovleva, L.A., Cammack, R., Thurston, C.F., Hatakka, A. (1997). Blue and yellow laccases of ligninolytic fungi. *FEMS Microbiology Letters*. 156, 9–14.

Lisova, A., Zavarzinab, A., Zavarzinc, A., Demind, V., Leontievsky, V. (2012). Dimeric and monomeric laccases of soil-stabilizing lichen *Solorina crocea*: Purification, properties and reactions with humic acids. *Soil Biology & Biochemistry.* 45, 161-167

Liu, F., Ma, C., Gao, Y., Clements, D.J. (2017). Food-Grade Covalent Complexes and Their Application as Nutraceutical Delivery Systems: A Review. *Comprehensive Reviews in Food Science and Food Safety*. 16, 76-95.

Logrieco, A., Mule, G., Moretti, A., Bottalico, A. (2002). Toxigenic Fusarium species and mycotoxins associated with maize ear rot in Europe. European Journal of Plant Pathology, 108, 597-609.

Loi, M., Quintieri, L., Fanelli,, F., Liuzzi, V.C., Haidukowski, M., Logrieco, A.F., Mulè, G. (2017) AFM_1 removal and biotechnological potential of a laccase from *Pleurotus eryngii* for milk safety. Scienza e tecnica lattiero casearia. 67, 23-32.

Lyashenko, A.V., Bento, I., Zaitsev, V.N., Zhukhlistova, N.E., Zhukova, Y.N., Gabdoulkhakov, A.G., Morgunova, E.Y., Voelter, W., Kachalova, G.S., Stepanova, E.V., Koroleva, O.V., Lamzin, V.S., Tishkov, V.I., Betzel, C., Lindley, P.F., Mikhailov, A.M. (2006). X-ray structural studies of the fungal laccase from *Cerrena maxima*. *Journal of Biological Inorganic Chemistry.* 11, 963–973.

Maestre-Reyna, M., Liu, W.C., Jeng, W.Y., Lee, C.C., Hsu, C.A., Wen, T.N., Shyur, L.F. (2015). Structural and functional roles of glycosylation in fungal laccase from Lentinus sp. *PloS one*, 10.

Magoha, H., De Meulenaer, B., Kimanya, M., Hipolite, D., Lachat, C., Kolsteren, P. (2014). Fumonisin B_1 contamination in breast milk and its exposure in infants under 6 months of age in Rombo, Northern Tanzania. *Food and chemical toxicology*. 74, 112-116.

Marasas, W.F. (1996). Fumonisins: history, world-wide occurrence and impact. In Fumonisins in food. 1-17. Springer US.

Marroquín-Cardona, A.G., Johnson, N.M., Phillips, T.D., Hayes, A.W. (2014). Mycotoxins in a changing global environment–a review. *Food and Chemical Toxicology.* 69, 220-230.

Masood, M., Iqbal, S.Z., Asi, M.R., Malik, N. (2015). Natural occurrence of aflatoxins in dry fruits and edible nuts. *Food Control*. 55, 62-65.

Mayer, A.M. (1987). Polyphenol oxidases in plants: recent progress. *Phytochemistry*. 26, 11–20

Mikolasch, A., Wurster, M., Lalk, M., Witt, S., Seefeldt, S., Hammer, E., Lindequist, U. (2008). Novel β-lactam antibiotics synthesized by amination of catechols using fungal laccase. *Chemical and Pharmaceutical Bulletin*. 56, 902-907.

Miller, J.D. (2002). Aspects of the ecology of Fusarium toxins in cereals. Mycotoxins and food safety, 19-27.

Molitoris, H.P., Reinhammar, B. (1975). The phenoloxidases of the ascomycete *Podospora anserina*. XI. The state of copper of laccases I, II and III. *Biochimica et Biophysica Acta.* 386, 493-502

Morozova, O.V., Shumakovich, G.P., Shleev, S.V., Yaropolov, Y.I. (2007). Laccase-mediator systems and their applications: a review. *Applied Biochemistry and Microbiology*. 43, 523-535.

Nguyen, P.A., Strub, C., Fontana, A., Schorr-Galindo, S. (2017). Crop molds and mycotoxins: Alternative management using biocontrol. *Biological Control*, 104, 10-27.

Osma, J.F., Toca-Herrera, J.L., Rodrıguez-Couto, S. (2010). Uses of Laccases in the Food Industry. *Enzymatic Research*.

Pavlović, N.M. (2013). Balkan endemic nephropathy—current status and future perspectives. *Clinical kidney journal.* 6, 257-265.

Pereira, V.L., Fernandes, J.O., Cuhna, S.C. (2014). Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis. *Trends in Food Science & Technology.* 36, 96–136

Pinotti, L., Ottoboni, M., Giromini, C., Dell'Orto, V., Cheli, F. (2016). Mycotoxin contamination in the EU feed supply chain: A focus on cereal byproducts. *Toxins*. 8, 45.

Piontek, K., Antorini, M., Choinowski, T. (2002). Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *Journal of Biological Chemistry*. 277, 37663–37669.

Piscitelli, A., Giardina, P., Lettera, V., Pezzella, C. (2011). Induction and Transcriptional Regulation of Laccases in Fungi. *Current Genomics.* 12, 104-112

Popovski, S., Celar, F.A. (2013). The impact of environmental factors on the infection of cereals with Fusarium species and mycotoxin production-a review. *Acta Agriculturae Slovenica*. 101, 105.

Rapid Alert System for Food and Feed (RASFF). Preliminary Annual Report 2016.

da Rocha, O., Ansari, K., Doohan, F.M. (2005). Effects of trichothecene mycotoxins on eukaryotic cells: a review. *Food additives and contaminants*. 22, 369-378.

Schmale, D.G., Munkvold, G.P. (2017). Mycotoxins in Crops: A Threat to Human and Domestic Animal Health. *The American Phytopathological Society*. https://www.apsnet.org/edcenter/intropp/topics/Mycotoxins/Pages/EconomicImpact.aspx. Accessed on 11/10/17

Schröder, U. (1985). Effects of calcium hydroxide-containing pulp-capping agents on pulp cell migration, proliferation, and differentiation. *Journal of dental research*. 64, 541-548.

Schroeder, T.U., Zweifel, P., Sagelsdorff, U., Friederich, J., Luthy, C., Schlatter, J. (1985). Ammoniation of aflatoxin-containing corn: Distribution, in vivo covalent deoxyribonucleic acid binding, and mutagenicity of reaction products. *Agriculture and Food Chemistry* 33, 311–316.

Shi, L., Ma, F., Han, Y., Zhang, X., Yu, H. (2014). Removal of sulfonamide antibiotics by oriented immobilized laccase on Fe 3 O 4 nanoparticles with natural mediators. *Journal of hazardous materials*. 279, 203-211.

Shier, W.T., Shier, A.C., Xie, W., Mirocha, C.J. (2001). Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. *Toxicon*, 39, 1435–1438.

Soriano, J.M., González, L., Catala, A.I. (2005). Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B_1. *Program. Lipid Research.* 44, 345–356,

Speijers, G.J.A., Speijers, M.H.M. (2004). Combined toxic effects of mycotoxins. *Toxicology letters*. 153, 91-98.

Steiner, B., Buerstmayr, M., Michel, S., Schweiger, W., Lemmens, M., Buerstmayr, H. (2017). Breeding strategies and advances in line selection for Fusarium head blight resistance in wheat. *Tropical Plant Pathology*. 1-10.

Stockmann-Juvala, H., Savolainen, K. (2008). A review of the toxic effects and mechanisms of action of fumonisin B_1. *Human & Experimental Toxicology* 27, 799–809

Strong, P.J., Claus, H. (2011). Laccase: A Review of Its Past and Its Future in Bioremediation. *Critical Reviews in Environmental Science and Technology.* 41, 373-434

Telke, A.A., Kagalkar, A.N., Jagtap, U.B., Desai, N.S., Bapat, V.A., Govindwar, S.P. (2011). Biochemical characterization of laccase from hairy root culture of *Brassica juncea* L. and role of redox mediators to enhance its potential for the decolorization of textile dyes. *Planta*. 234, 1137-1149.

Tsioulpas, A., Dimou, D., Iconomou, D., Aggelis, G. (2002). Phenolic removal in olive oil mill wastewater by strains of *Pleurotus* spp. in respect to their phenol oxidase (laccase) activity. *Bioresource Technology*. 84, 251-257.

Van Egmond, H.P., Schothorst, R.C., Jonker, M.A. (2007). Regulations relating to mycotoxins in food. *Analytical and bioanalytical chemistry*. 389, 147-157.

Vanhoutte, I., Audenaert, K., De Gelder, L. (2016). Biodegradation of mycotoxins: Tales from known and unexplored worlds. *Frontiers in microbiology*. 7.

Villers, P. (2014). Aflatoxins and safe storage. *Frontiers in microbiology*, 5.

Völkeò, I., Schröer-Merker, E., Czerny, C.P. (2011). The carry-over of mycotoxins in products of animal origin with special regard to its implications for the European food safety legislation. *Food and Nutrition Sciences*, Vol. 2 No. 8, 2011, pp. 852-867.

Wang, T.N., Lu, L., Li, G.F., Li, J., Xu, T.F., Zhao, M. (2011). Decolorization of the azo dye reactive black 5 using laccase mediator system. *African Journal of Biotechnology.* 10, 17186-17191.

Wegulo, S.N., Baenziger, P.S., Nopsa, J.H., Bockus, W.W., Hallen-Adams, H. (2015). Management of Fusarium head blight of wheat and barley. *Crop Protection.* 73, 100-107.

Wells, A., Teria, M., Eve, T. (2006). Green oxidations with laccase–mediator systems. *Biochemical Society Transactions*. 34, 304-308.

Wild, C.P., Miller, J.D., Groopman, J.D. (2015). Mycotoxin control in low-and middle-income countries. Lyon, France: International Agency for Research on Cancer. International Agency for Research on Cancer; 2015. (IARC Working Group Reports, No. 9.)

Wong, D.W. (2009). Structure and action mechanism of ligninolytic enzymes. *Appl Biochem Biotechnol*, 157(2): 174–209

Wu, F., Guclu, H. (2012). Aflatoxin regulations in a network of global maize trade. *PloS one.* 45151.

Yehia, R.S. (2014). Aflatoxin detoxification by manganese peroxidase purified from Pleurotus ostreatus. *Brazilian Journal of Microbiology*. 45, 127–133.

Yoshida, H. (1883). Chemistry of lacquer (urushi). Part I. *Journal of Chemical Society.* 43, 472–486

Yu, Y., Wu, H., Tang, Y., Qiu, L. (2012). Cloning, expression of a peroxiredoxin gene from Acinetobacter sp. SM04 and characterization of its recombinant protein for zearalenone detoxification. *Microbiology Research*. 167, 121–126.

Zeeb, B., McClements, D.J., Weiss, J. (2017). Enzyme-Based Strategies for Structuring Foods for Improved Functionality. *Annual review of food science and technology*, 8, 21-34.

Zhang, J., Liu, X., Xu, Z., Chen, H., Yang, Y. (2008). Degradation of chlorophenols catalyzed by laccase. *International Biodeterioration & Biodegradation*. 61, 351-356.

Zinedine, A., Soriano, M., Molto, J.C., Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. *Food Chemical Toxicology.* 45, 1–18

Zucca, P., Rescigno, A., Olianas, A., Maccioni, S., Sollai, F.A., Sanjust, E. (2011). Induction, purification, and characterization of a laccase isozyme from *Pleurotus sajor-caju* and the potential in decolorization of textile dyes. *Journal of Molecular Catalysis*. 68, 216–222

Aim and outline

2. Aim and outline of the thesis

The aim of this thesis was to investigate the capability of LC enzymes to degrade structurally and chemically heterogeneous mycotoxins, specifically AFB_1, AFM_1, FB_1, OTA, DON, ZEN and T-2 toxin. LCs alone and in combination with natural and artificial redox mediators were tested in order to evaluate the feasibility of a potential application of novel mycotoxin reduction methods in food and feed.

To this purpose, a preliminary screening was performed by *in vitro* assays, set up using two different purified LCs, specifically Lac2 from *P. pulmonarius* (Chapter 4) and Ery4 from *P. eryngii* (Chapters 5 and 6), and up to eight different natural and artificial redox mediators. With regards to the natural co-occurrence of multiple mycotoxin in raw materials or in foods and feeds composed by different ingredients, both single and combined *in vitro* mycotoxin degradation assays were also performed with Ery4-LMSs.

Considering the promising results obtained in the preliminary screening, we also investigate more deeply the factors governing the reaction of mycotoxin degradation, crucial step to propose any industrial application, by the statistical approach of the design of experiments (DOE). DOE was used to assess the role of the concentration of each element in the reaction (Ery4, SA as mediator, AFB_1) and time in the determination of the degradation response. Consequently, the optimization of AFB_1 degradation reaction was also investigated (Chapter 8.2).

Since LCs are already used within a wide variety of biotechnological and industrial food processes, the application of Ery4 based LMS was also investigated in milk to evaluate the potential effects on safety and quality of milk and derived products.

In particular, AFM_1 degradation by LMS was evaluated in UHT skim milk. To study the feasibility of an application in a real contaminated matrix, the kinetics of degradation *in vitro* and in milk were studied and compared (Chapter 6). The

effect of LMS treatment on milk proteins were analysed and exploited for the manufacturing of a curd, which resulted in a product with increased antioxidant properties (Chapter 7).

Chapters

Mycotoxin biotransformation by native and commercial enzymes: present and future perspectives

Loi M[1,2], Fanelli F[1], Liuzzi VC[1], Logrieco AF[1], Mulè G[1]

[1]Institute of Sciences of Food Production, National Research Council, via Amendola 122/O, Bari 70126, Italy;

[2]Department of Economics, University of Foggia, via Napoli 25, Foggia 71122, Italy

The contents of this chapter were published in Loi M, Fanelli F, Liuzzi VC, Logrieco AF, Mulè G. Mycotoxin Biotransformation by Native and Commercial Enzymes: Present and Future Perspectives. Toxins (Basel). 2017, 9(4):111; doi: 10.3390/toxins9040111. Review.

Abstract: Worldwide mycotoxins contamination has a significant impact on animal and human health, and leads to economic losses accounted for billions of dollars annually. Since the application of pre- and post- harvest strategies, including chemical or physical removal, are not sufficiently effective, biological transformation is considered the most promising yet challenging approach to reduce mycotoxins accumulation. Although several microorganisms were reported to degrade mycotoxins, only a few enzymes have been identified, purified and characterized for this activity. This review focuses on the biotransformation of mycotoxins performed with purified enzymes isolated from bacteria, fungi and plants, whose activity was validated in *in vitro* and *in vivo* assays, including patented ones and commercial preparations. Furthermore, we will present some applications for detoxifying enzymes in food, feed, biogas and biofuel industries, describing their limitation and potentialities.

Keywords: mycotoxins; biotransformation; degradation; enzymes; application

3.1. Introduction

Mycotoxins are secondary toxic metabolites produced by filamentous fungi mainly belonging to *Fusarium*, *Aspergillus* and *Penicillium* genera. They infect cereals, seeds and fruits both in the field and during storage, and can be found as common contaminants in food and feed supply chains (Bennett & Klich, 2003; Logrieco et al., 2002).

Mycotoxins poisoning of staple food commodities has a significant impact on worldwide health, especially in developing countries (Bryden, 2007; Shephards, 2008). Both animals and humans may develop acute and chronic mycotoxicosis,

depending on several factors, which include the type of mycotoxin, the amount and the duration of the exposure, etc. Mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins and so forth, based on the organ they affect (Bennett & Klich, 2003). Main target tissues include gastrointestinal and breathing apparatus, endocrine, exocrine, reproductive, nervous and immune system (Sharma, 1993; Zain, 2011). Aflatoxin B_1 (AFB_1) is the most toxic mycotoxin and has been classified in group 1, carcinogenic to humans, by the International Agency of Research on Cancer (IARC) in 2002 (IARC, 2002). Other mycotoxins, such as aflatoxin M_1 (AFM_1), ochratoxin A (OTA) and fumonisin B_1 (FB_1), are classified within group 2B, thus possibly carcinogenic to humans, due to the limited availability of their toxicological data (IARC, 1993; 2002; 2012).

Synergistic and additive effects of different mycotoxins have been documented; however, the worsening and toxic outcome of multiple exposure cannot be predicted by summing up the individual toxicities. Furthermore, the mechanisms of interactions among toxins are still poorly understood (Alassane-Kpembi et al., 2016). In rats FB_1 synergistically promotes liver lesions, hepatocyte dysplasia and, by long term exposure, tumors initiated by AFB_1 (Gelderblom et al., 2002). AFB_1 also acts synergistically with zearalenone (ZEN) in decreasing egg production, feed intake, feed conversion ratio and eggshell strength in birds (Ji et al., 2016). *In vitro* cytotoxicity of AFM_1 on Caco-2 cells is greatly enhanced by the presence of OTA, ZEN and/or α-zearalenol (α-ZEL), which often co-contaminate milk and infant formulas (Gao et al., 2016). OTA and citrinin have often been reported to act in a synergistic mode in relation to their cytotoxic (Roth et al., 1989; 15,16. Bouslimi et al., 2008a,b; 17. Klaric et al., 2012) and genotoxic effects (Knasmuller et al., 2004).

Global trade significantly contributes to mycotoxin spread. The economic losses associated with mycotoxin contamination in commodities account for billions of dollars annually (Wu, 2007). They can be categorized into direct and indirect losses: direct losses are related to lowered crop yields, reduction of animal

performance and costs derived from diseases for livestock producers; indirect losses are very challenging to quantify, and are linked to the increased use of fungicide, the reduction of the marketable value of the commodities, the management, health-care, veterinary-care costs, and investments in the development of reducing strategies and in research programs.

Mycotoxins levels are regulated in many countries worldwide. The European Union has implemented the most extensive and detailed food regulation for mycotoxins within the Commission Regulation (EC) No. 1881/2006, which set mycotoxins maximum levels discriminating among food products and consumers (e.g., adults or infants). Indicative levels in cereals and cereals products (Commission Recommendation 2013/165/EU) have been established for the trichothecenes T-2 and HT-2 toxins (T-2, HT-2). A scientific opinion on their toxicity was released in 2001 by the European Commission Scientific Committee on Food (SCF) (Scientific opinion on Fusarium toxins part5: T-2 toxin and HT-2 toxin, 30 may 2001), highlighting the need for further studies on the occurrence, daily intake, analytical methods development and induced hematotoxicity and immunotoxicity. However, for other so-called emerging mycotoxins (e.g., enniatins, beauvericin, fusaric acid and moniliformin), whose increasing occurrence has been clearly evidenced, maximum levels have not been yet established. This delay exists since certified analytical methods for their determination, complete surveys on their occurrence and defined scientific opinions on their toxic effect and health associated risks are still being developed (Commission Recommendation 2013/165/EU; Commission Recommendation 2006/576/EC).

Mycotoxin contamination can be prevented in the field through the application of good agricultural practices (GAPs), such as the choice of resistant varieties, harvesting at the right time, crop rotation, and the use of fungicides (Commission regulation 2006/1881/EC; Commission Recommendation 2006/583/EC). Nevertheless, pre-harvest strategies are not completely effective, and fungal

contamination of raw materials can lead to mycotoxin accumulation during storage. Furthermore, mycotoxins are extremely stable and resistant to the commonly used physical and chemical treatments of food and feed processing.

Since the application of GAPs, proper storage and risk management procedures might only mitigate mycotoxin occurrence, the development of alternative strategies to reduce mycotoxins contamination is considered a relevant, innovative, urgent, yet challenging research topic.

In this review, we will briefly present the current methods to reduce mycotoxins contaminations in food and feed, and then we will focus on the biotransformation of mycotoxins performed with purified enzymes isolated from bacteria, fungi and plants, whose activity was validated in *in vitro* and *in vivo* assays, including patented ones and commercial preparations. We will also describe enzyme potential applications and limitations in food, feed and bioenergy in compliance with the European Regulation.

Methods for Mycotoxins Reduction

Current methods to reduce mycotoxin contamination in food and feed can be classified into physical, chemical and biological. It must be underlined that EU regulation does not authorize any detoxification methods for those commodities, intended for food production, exceeding mycotoxins limit levels (Commission regulation 2006/1881/EC).

(1) Physical methods comprise the mechanical removal of highly contaminated fractions from raw materials (by sorting, cleaning, milling, dehulling), the application of heat, the irradiation and the use of adsorbents, which is still limited to feed production (Vanhoutte et al., 2016). The latter approach is considered promising, although some possible negative drawbacks related to unspecific binding of essential nutrients and antibiotics exist. In addition, these adsorbents have diversified efficacy towards different classes of mycotoxins, with trichothecenes being the most difficult to target. Furthermore, since co-occurrence

is much more common than individual contamination, complementary reduction strategies should be implemented.

Recently, the application of unconventional strategies such as cold plasma (Schlüter et al., 2013; Bong et al., 2007; Kriz et al., 2015), photoirradiation (Herzallah et al., 2007; Fanelli et al., 2016) and microwave treatments (Bretz et al., 2006) have been proposed and/or introduced in food processing, as sterilization or degrading methods. Nevertheless, the partial knowledge about degradation products (DPs) and the nutritional/organoleptic changes induced by these treatments still confine their application.

(2) Chemical methods such as ammoniation (Park et al., 1988), acid treatments (Aiko et al., 2016), alkaline hydrolysis (Müller et al., 1983), peroxidation (Fouler et al., 1994), ozonation (Maeba et al., 1988), and the use of bisulphites (Altug et al., 1990) have been tested, but their application in food and feed is limited due to their potential toxicity, poor efficacy, high costs and negative effects on the quality of raw materials.

(3) Biological methods consist of the use of microorganisms or enzymes, which are able to metabolize, destroy or deactivate toxins into stable, less toxic, up to harmless compounds (Commission Regulation 2015/786/EU). Biological agents and their enzymes allow a specific, most likely irreversible, environmental friendly and effective approach, with minor impact on food sensory and nutritional quality with respect to chemical ones.

3.2. Mycotoxin Biotransformation by Enzymes

Mycotoxin biotransformation is defined as "the degradation of mycotoxins into non-toxic metabolites by using bacteria/fungi or enzymes" (EFSA, 2009). The possibility to use living microorganisms as whole cell biocatalysts for mycotoxins degradation has cost advantages. This represents a valid strategy, especially if multi step reactions are required, or if the microorganism is already implemented within industrial processes (Hassan et al., 2015; 2013). On the other hand, in case of

high levels of mycotoxins contamination, the growth and physiology of such microorganisms might be altered or inhibited, thus requiring longer time for adaptation before achieving satisfactory decontamination levels.

The majority of the research papers which describes microbial degrading activity, rarely discriminates between physical adsorption and enzymatic degradation. This greatly complicates the identification of DPs and the evaluation of their toxicity. This knowledge is relevant in the evaluation of biotrasforming enzymes, especially since not every reaction leads to a real detoxification. Indeed, the metabolized mycotoxin can acquire greater toxic properties than the parent compound. This is the case, for example, of ZEN biotransformation performed *in vivo* by yeasts, which reduce the toxin to α-ZEL, actually more estrogenic than ZEN (Böswald et al., 1995).

The identification and characterization of a degrading enzyme (DE) can be challenging and time consuming, but it is a necessary step to understand the mechanism of degradation, towards its optimization and the development of mycotoxins reducing methods. Enzymes guarantee reproducible and homogeneous performances, with ease-of-handling, no risks of contamination and no safety concerns for operators compared to the use of living microorganisms.

Screening microbial population from different mycotoxin-contaminated environment is an efficient, fast and promising strategy to discover degrading microbes and activities (De Bellis et al., 2015; Sato et al., 2012; He et al., 2016), which could be enhanced by coupling advanced approaches, such as metagenomics or functional metagenomics.

In addition, genetic engineering enables to clone and express heterologous enzymes in bacterial, yeast, fungal and plant cells for massive, less expensive and less laborious productions. If no structural-functional data are available, enzyme efficiency, stability and tolerance to organic solvents can also be improved by

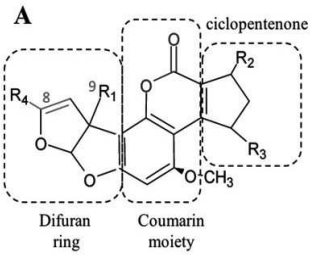
computational approaches, aiming at targeted or combinatorial semi-rational mutagenesis.

Many enzymes have been reported to remove or reduce mycotoxin contamination both *in vitro* and in real matrices. Nonetheless, their application in feed is very limited, due to the lack of information about the potential toxic effects of generated products and their influence on nutritional quality of feed. These data are mandatory to be authorized as possible biotransforming agent in Europe (EFSA, 2009).

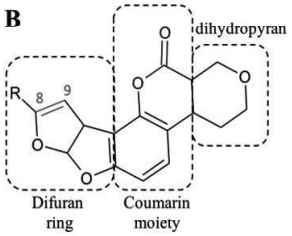
Very few commercial biotransforming feed additives are available: Mycofix®, FUMzyme®, Biomin® BBSH 797 and Biomin® MTV (Biomin Holding GmbH, Getzersdorf, Austria) are some examples, but only FUMzyme® exploits a purified enzyme, an esterase, to perform fumonisin degradation. Below we will discuss in details the biotransformation of the main mycotoxins by native and commercial enzymes.

3.2.1. Aflatoxins

The term aflatoxins (AFs) includes more than 20 fungal secondary metabolites produced by fungi belonging to *Aspergillus* genus (Bennet & Klich, 2003). They are classified into two main groups according to their chemical structure. The difurocoumarocyclopentenone group includes AFB_1, aflatoxin B_2, aflatoxin B2a, AFM_1, aflatoxin M_2, aflatoxin Q_1 (AFQ_1), and aflatoxicol (AFL), while the difurocoumarolactone group comprises aflatoxin G_1, aflatoxin G_2 and aflatoxin G2a (Fig. 3-1).



	AFB_1	AFB_2	AFB2a	AFM_1	AFM_2	AFL	AFQ_1
R1	-H	-H	-H	-OH	-OH	-H	-H
R2	=O	=O	=O	=O	=O	-OH	=O
R3	-H	-H	-H	-H	-H	-H	-OH
R4	-H	-H	-OH	-H	-H	-H	-H
C_8-C_9 bond	unsaturated	saturated	saturated	unsaturated	saturated	unsaturated	unsaturated



	AFG_1	AFG_2	AFG2a
R	-H	-H	-OH
C_8-C_9 bond	unsaturated	saturated	saturated

Figure 3-1. Chemical structure and features of ciclopentenone (**A**) and difurocoumarolactone (**B**) aflatoxin (AF) series. Coloured bonds indicate reactive groups involved in AFs toxicity. The double bond leading to 8,9-epoxide upon metabolic activation is indicated in red, while the lactone bond is indicated in blue. Tables show substituent groups and saturation of the C_8-C_9 bond in different AF analogues.

While AFs of the B and G series co-occur in cereals and their derived products, fruits, oilseeds, nuts, tobacco and spices, AFM_1 AFM_2, AFL and AFQ_1 are detected in food as carry-over products of AFB_1 contaminated feeds. *In vivo* AFB_1 is readily metabolized through hydroxylation (to AFM_1 or, to a lesser extent, to AFQ_1) or reduction (to AFL).

AFs are difuranocoumarin derivatives composed by two furan rings, linked together to a coumarin moiety. Furofuran and cumarin rings are arranged in a planar configuration which is responsible for conjugation leading to the typical AFs fluorescence.

The furofuran ring has been recognized as responsible for the toxic and carcinogenic activity upon metabolic activation of the C_8-C_9 double bond to 8–9 epoxide (IARCO, 2012) (Fig. 3-1).

The epoxidation is a crucial reaction for AFs carcinogenicity, since it allows the binding to N7-guanine and the subsequent G to T transversions in the DNA molecule (Essingmann et al., 1977). Activated AFs are also able to form schiff bases

with cellular and microsomal proteins (via methionine, histidine and lysine), thus leading to acute toxicity (Eaton et al., 1994). The lactone ring also plays a role in AFs toxicity and carcinogenicity: upon ammoniation it is hydrolyzed, forming aflatoxin D_1 (AFD_1) which still retains the 8,9-dihydrofuran double bond; AFD_1 lacks the strong *in vivo* DNA binding activity of AFB_1, demonstrating that DNA alkylation depends upon both difuranocumarin and lactone moieties (Shroeder et al., 1985).

Several authors addressed lactone hydrolysis, reduction or addition reactions as possible mechanisms of degradation, since the putative hydrolyzed products showed greatly reduced mutagenic activity *in vitro* (Méndez-Albores et al., 2005; Motomura et al., 2003).

Table 3-1. Aflatoxins (AFs) degrading enzymes.

Enzyme	Accession/ EC	Producing Organism	AF Target	Toxin Concentration	*In Vitro*/*In Matrix* Degrading Conditions	*In Vitro*/*In Matrix* Degradation	Toxicity/Mutagenicity Test	Reference
aflatoxin oxidase enzyme (AFO)	EC 1.1	*Armillariella tabescens*	AFB_1	0.05 µg/mL	▪ PBS buffer 0.02 M pH 6; ▪ incubation at 28 °C for 30 min	▪ 100% with 0.2 mg of enriched preparation; ▪ NQ* with pure enzyme	reduced liver toxicity in rats; reduced mutagenicity in *Salmonella typhimurium* TA 98; reduced genotoxicity on chicken embryos	Liu et al., 1998
peroxidase	EC 1.11.1.7	horseradish *(Armoracia rusticana)*	AFB_1	312 µg/mL	▪ phosphate buffer 50 mM pH 6; ▪ incubation at 20 °C for 60 min; ▪ 0.2U/mL of enzyme	42.2%	reduced toxicity on *Bacillus megaterium*	Chitrangada et al., 2000
				440 µg/mL	▪ 100 g of defatted groundnut kernels; ▪ 2-16U of enzyme; ▪ 50 mM phosphate buffer pH 6 and 20 mM hydrogen peroxide; ▪ incubation at room temperature up to 24 h	41.1%		
laccase	EC 1.10.3.2	*Trametes versicolor* (commercial enzyme from Sigma-Aldrich, Missouri, U.S.)	AFB_1	1.40 µg/mL	▪ phosphate buffer 0.2 M pH 6.5; ▪ 1 U/mL of enzyme ▪ incubation at 30 °C for 72 h	87%	reduced mutagenicity on *Salmonella typhimurium* TA 100	Alberts et al., 2009
laccase	EC 1.10.3.2	*Streptomyces coelicor*	AFB_1	9.36 µg/mL	▪ sodium acetate buffer 100 mM pH 7; ▪ 0.1 mg/mL of enzyme protein	100%	n.p.	Novozyme, World Patent

					and 0.2 mM mediator; ▪ incubation at 37 °C for 24 h			2009109607, 2009
F420H2-dependent reductases	E.C. 1.5.8	*Mycobacterium smegmatis*	AFB_1 AFB_2 AFG_1 AFG_2	n.p.	n.p.	n.p.	n.p.	Taylor et al., 2010
Mn peroxidase	EC 1.11.1.7	*Pleurotus ostreatus*	AFB_1	0.31 μg/mL	▪ sodium lactate buffer 50 mM pH 4.5; ▪ 1.5 U/mL of enzyme ▪ incubation at 30 °C for 48 h	90%	n.p.	Yehia et al., 2014
aflatoxin degradation enzyme	n.p.	*Pleurotus ostreatus*	AFB_1	5 μg/mL	▪ sodium acetate buffer 0.1 M pH 5; ▪ incubation at 25 °C for 1 h	n.q.	n.p.	Motomura et al., 2003
myxobacteria aflatoxin degrading enzyme (MADE)	n.p.	*Myxococcus fulvus* ANSM068	AFB_1 AFG_1 AFM_1	0.1 μg/mL	▪ citrate phosphate buffer 0.1 M pH 6; ▪ 100 U/mL of enzyme ▪ incubation at 30 °C for 48 h	72% with culture filtrates 97% 96%	n.p.	Zhao et al., 2011
laccase (lac2)	EC 1.10.3.2	*Pleurotus pulmonarius* (ITEM 17144)	AFB_1 AFM_1	1 μg/mL 0.05 μg/mL	▪ sodium acetate buffer 1mM pH 5; ▪ 5 U/mL enzyme and redox mediator; ▪ incubation at 25 °C for 72 h	90% 100%	n.p.	Loi et al., 2016; 2017
Ery4	CAO79915.1/ EC 1.10.3.2	*Pleurotus eryngii* (PS419)	AFM_1	0.05 μg/mL	▪ sodium acetate buffer 1 mM pH 5; ▪ 5 U/mL enzyme and redox mediator; ▪ incubation at 25 °C for 72 h ▪ artificially spiked skim UHT milk; ▪ 5 U/mL enzyme and redox mediator; ▪ incubation at 25 °C for 72 h	100%	n.p.	Cao et al., 2011

n.q. = not quantitative; n.p.= not provided

Table 3-1 summarizes the purified enzymes identified as capable of degrading AFs, and their main features. Although direct comparison is not possible, since the enzymes and the experimental conditions used by the authors are not equivalent, reaction parameters and AFs concentrations are indicated.

Most of these enzymes are comprised in the oxidoreductase (EC 1) group. The first identified was the AF oxidase enzyme (AFO) isolated from the edible fungus *Armillariella tabescens* by Liu and colleagues in 1998 (Liu et al., 1998). AFO is an oxygen dependent reductase, releasing H_2O_2 as by product (Liu et al., 1998; Cao et al., 2011). One mg/mL of enriched preparation and 30 min of incubation at 28 °C were needed to completely degrade 150 ng of AFB_1. AFO did not affect AFB_1 fluorescence properties, indicating that the conjugation system, between the coumarin moiety and the lactone ring, was not disrupted by the degrading reaction. The mechanism proposed by the authors consisted of the enzymatic cleavage of the bisfuran ring. This hypothesis, although not yet experimentally verified, is in agreement with the reduced mutagenicity, toxicity and genotoxicity of the DPs and with their differential pulse voltammetry response similar to furofuran analogs (Liu et al 1988b; Wu et al., 2015). Products of AFO treatment were found to exert neither liver toxicity in rats, nor mutagenicity activity on *Salmonella typhimurium* TA98, and not to be genotoxic to chicken embryos (Liu et al 1988).

Three different peroxidases (EC 1.11.1.7) were also reported to possess AFB_1 degrading capabilities. They were extracted and purified from horseradish (*Armoracia rusticana*) (Chitrangada et al., 2000) *Phanerochaete sordida* YK-624 (Wang et al., 2013) and *Pleurotus ostreatus* (Yehia et al., 2014). The horseradish peroxidase was tested *in vitro* towards AFB_1, which was reduced by 42% after 1h with 0.2 U/mL of enzyme. Similar results were also obtained in real matrix: AFB_1 was reduced by 41% in artificially spiked groundnut (Chitrangada et al., 2000). A lowered toxicity of the DPs was also registered by inhibition growth assay on *Bacillus megaterium* (Liu et al., 1988). The two manganese peroxidases (MnPs) from

the two different white rot fungi were studied (Yehia et al., 2014; Wang et al., 2013) in *in vitro* assays with similar reaction conditions, allowing a direct comparison. They were tested towards 0.3 μg/mL of AFB_1, achieving 86% reduction after 24 h of incubation, and up to 90% after 48 h by using the MnP from *P. sordida YK-624* and the one from *P. ostreatus* respectively. Wang and colleagues (Wang et al., 2013) also registered 69.2% reduction of mutagenicity of DPs compared to AFB_1, based on the umu test performed on *Salmonella typhimurium* TA1535 and on S9 liver homogenate. According to Proton Nuclear Magnetic Resonance (^{1}H-NMR) and high resolution electrospray ionization mass spectrometry (HR-ESI-MS) analysis, the authors hypothesized that MnP treatment converted AFB_1 to AFB_1-8,9-dihydrodiol.

Laccases (EC 1.10.3.2) (LCs) have been intensively used in bioremediation (Wang et al., 2011) and were recently proposed for ZEN and AFs biotransformation (Loi et al., 2016; Banu et al., 2013).

Alberts and colleagues (Alberts et al., 2009) made as firsts a decisive step towards the correlation between AFs degradation and LCs activity. The authors used different LCs, including native LCs from representatives of *Peniophora* genus and *P. ostreatus*, one commercial LC from *Trametes versicolor*, and one recombinant LC from *Aspergillus niger*. By treatment with 1 U/mL of the commercial preparation AFB_1 (1.4 μg/mL) was reduced by 87.34% after 3 days of incubation at 30 °C. DPs were not identified, but proved to be less mutagenic than the parent compound. However, none of the preparations used by the authors was purified to homogeneity. The commercial product from *T. versicolor* was indeed an enriched preparation, which included additional proteins and different laccase isoforms (Margot et al., 2013); thus, an unambiguous assignment of the degrading activity to a specific LCs would be improper.

Recently the effectiveness of purified LCs towards AFs and ZEN was acknowledged (Margot et al., 2013; Novozymes, 2009). Pure LCs from *P. pulmonarius* and *P. eryngii* were used by Loi and colleagues (Loi et al., 2016; 2017)

towards both AFB_1 and AFM_1. The authors performed *in vitro* and *in matrix* tests with 1 μg/mL of AFB_1 and 0.05 μg/mL for AFM_1. Interestingly, they reported that, while LC alone is poorly able to degrade these toxins, the addition of a redox mediator at 10 mM concentration increased the degrading percentages from 23% up to 90% for AFB_1, and up to 100% for AFM_1 after 72 h (Loi et al., 2016; 2017).

The laccase-mediator approach patented by Novozymes in 2008 (Novozymes, 2008), consisted of the use of LCs, preferably from *Streptomyces coelicolor*, and methylsyringate as mediator. In this case, AFB_1 was completely removed after 24 h of incubation with 0.1 mg/mL of enzyme and 0.2 mM of mediator at 37 °C.

Members of the reductase family have also been studied for AFs degrading capabilities. Among these, the F420H2-dependent reductases (EC 1.5.8.), isolated from *Mycobacterium smegmatis*, were tested towards AFB_1, AFB_2, AFG_1 and AFG_2; no details were provided in relation to efficacy and assay conditions (Taylor et al., 2010).

Besides oxidases above described, Guan et al. (2010) identified the myxobacteria aflatoxin DE (MADE), isolated from *Myxococcus fulvus*, which was further characterized in 2011 (Zhao et al., 2011). Pure MADE (100 U/mL) was tested towards 0.1 μg/mL each of AFG_1 and AFM_1, achieving 98 and 97% of degradation after 48 h of incubation. No further details about the nature of the enzyme, nor about the DPs were given.

2.2. Ochratoxin A

OTA is a phenylalanine-dihydroisocoumarine derivative, composed of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-R-methylisocoumarin (ochratoxin α—OTα) moiety and a L-β-phenylalanine molecule (Phe), which are linked at the 7-carboxy group by an amide bond (Fig. 3-2).

Due to its structural analogy to the amino acid Phe, the toxin can competitively inhibit tRNA phenylalanine synthetases and, consequently, block protein synthesis (Dirheimer et al., 1991). Furthermore, OTA causes the formation of DNA adducts,

indirect oxidative DNA damage and activates a network of interacting epigenetic mechanisms (Pfohl-Leszkowicz et al., 2012; Vettorazzi et al., 2013).

The main OTA detoxification pathway consists in the hydrolysis of the amide bond between the isocoumarin residue and phenylalanine, resulting in the formation of Phe and OTα (Fig. 3-2). The former is considered to be a non-toxic compound, with a 10-times shorter elimination half-life than OTA (Kőszegi et al., 2016).

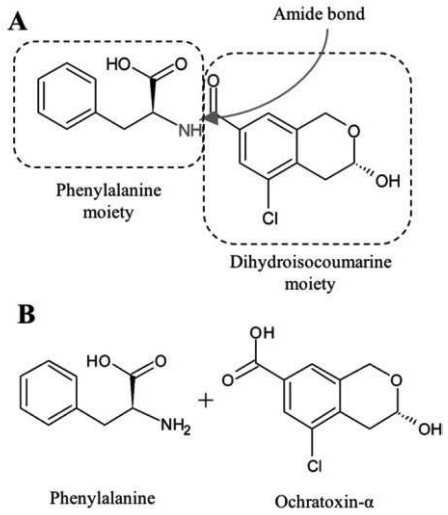


Figure 3-2. Chemical structures of (**A**) ochratoxin A and (**B**) its degradation products, ochratoxin-α and phenylalanine. The amide bond hydrolyzed by the main degrading pathway is indicated in red.

Numerous enzymes were hypothesized to hydrolyze the OTA amide bond, but only few of them were isolated and characterized (Abrunhosa et al., 2010).

Two classes of carboxypeptidases (EC 3.4) have been associated with OTA degradation: carboxypeptidase A (CPA) and carboxypeptidase Y (CPY) class. CPA uses one zinc ion within the protein for hydrolysis (EC 3.4.24), while CPY is a serine-type carboxypeptidase (EC 3.4.16) and does not contain any zinc ion in its active site. The first peptidase reported as able to hydrolyze OTA was a CPA isolated from bovine pancreas, which resulted able to perform the degrading reaction with a Km value of 1.5×10^{-4} M at 25 °C (Pitout et al., 1969). A CPY isolated from *Saccharomyces cerevisiae* was demonstrated to hydrolyze OTA with optimum at pH 5.6 and 37 °C; its specific activity was very low considering that

only 52% of OTA was converted into OTα after five days of incubation Abrunhosa et al., 2010). The same enzyme was efficiently immobilized on electroactive surfaces in order to develop a biosensor system for the direct detection of OTA in olive oil, with promising results (Dridi et al., 2015). Many carboxypeptidases have high optimal reaction temperatures (30 °C or higher); this might not hamper detoxifying applications for food and feed (Vanhoutte te al., 2016).

Other enzymes are also able to perform OTA hydrolysis, such as lipases (EC 3.1), amidases (EC 3.5) and several commercial proteases (EC 3.4) (Stander et al., 2000; Abrunhosa et al.,2006; Sanisco, 2011).

By screening different commercial hydrolases, a lipase preparation from *Aspergillus niger* (Amano A) was shown to hydrolyze OTA into OTα and Phe. Single-step purification, by anion exchange chromatography, allowed the isolation of the pure protein. The lipase nature of the enzyme was confirmed by assaying the cleavage of p-nitrophenyl palmitate. The purified enzyme resulted able to completely hydrolyze 50 μg of OTA into OTα after 120 min of incubation, in 1 mL of reaction mixture (Stader et al., 2000).

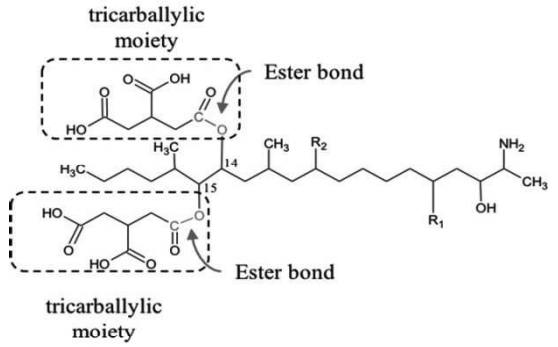
Several commercial proteases were also reported to hydrolyze OTA to OTα, such as Protease A from *A. niger* and pancreatin from porcine pancreas. These enzymes showed a significant hydrolytic activity at pH 7.5, which resulted in the cleavage of 87.3% and 43.4% of 1 μg of OTA respectively, after 25 h of incubation in 1 mL reaction mixture (Abrunhosa et al.,2006).

Finally, an amidase and the feed or food additive comprising it, capable of degrading OTA, were patented by Dalsgaard et al. in 2010 (Danisco, 2011). The protein, named amidase 2, is encoded by an open reading frame of *A. niger*. The degrading assay was performed in 300 μL of reaction mixture, containing 160 ng/mL amidase 2 and 50 μg/mL of OTA. This enzyme was able to reduce OTA concentration by 83%. Amidase 2 was tested also in some food preparations. The patented additive decreased OTA content, from 47 ppb to undetectable level (<2

ppb), after 2.5 h-incubation in contaminated milk. Similarly, OTA concentration in corn flour was reduced from 38 ppm to less than 2 ppb after 20 h of incubation.

3.2.3. Fumonisins

Fumonisins are a group of mycotoxins associated with several mycotoxicoses, including equine leukoencephalomalacia, porcine pulmonary edema and experimental kidney and liver cancer in rats (Heinl et al., 2009). Chemically, fumonisins are diesters of propane-1,2,3-tricarboxylic acid and similar long-chain aminopolyol backbones. Structurally they resemble the sphingoid bases sphinganine (SA) and sphingosine (SO), with tricarboxylic acid groups added at the C_{14} and C_{15} positions (Fig. 3-3). This structural similarity is responsible for the fumonisin mechanism of action. It was described to act by disturbing the sphingolipids metabolism, by inhibiting the enzyme ceramide synthase, and leading to accumulation of sphinganine in cells and tissues (Wang et al., 1991; Merril et al., 1996; Soriano et al., 2005).



	B_1	B_2	B_3	B_4
R1	-OH	-OH	-H	-H
R2	-OH	-H	-OH	-H

Figure 3-3. Type B fumonisins chemical structure. The ester bonds hydrolyzed by the main degrading pathways, leading to the formation of HFB_1 and the two tricarballylic acid moieties, are indicated in red. The table shows substituent groups of different fumonisin analogues.

There are at least 28 different forms of fumonisins, designated as A-series, B-series, C-series, and P-series (Rheeder et al., 2002). The B-series (FB) is the most abundant and important with respect to toxicity.

Pre-harvest strategies to reduce fumonisins contamination are based on the bio-control of the spread of fumonisin-producing fungi. Post-harvest methods

include the application of natural clay adsorbents during food processing. While they do not lead to a real detoxification of fumonisin (Aly et al., 2004; Robinson et al., 2012; Pioneer Hi.Bred International, 1995a), different microorganisms were reported to transform these class of mycotoxins. Among them, only few enzymes have been identified, molecularly and biochemically characterized, and patented.

FB_1 was reported to be degraded by the consecutive action of a carboxylesterase (EC 3.1.1) (Pioneer Hi.Bred International, 1995a) and an aminotransferase (EC 2.6.1) (Duvick et al., 2001; ; Pioneer Hi.Bred International, 1995b) (Table 3-2).

By the deesterification action of the carboxylesterase, the two tricarballylic acid moieties are released, resulting in hydrolyzed FB_1 (HFB_1), also known as aminopentol 1 (AP1). After the oxidative deamination, HFB_1 is converted to *N*-acetyl HFB_1 and 2-oxo-12,16-dimethyl-3,5,10,14,15-icosanepentol hemiketal (Duvick et al., 2001). The action of these enzymes, isolated from *Sphingomonas* sp. ATCC 55552 (Duvick et al., 1998), was worldwide patented in 1994 (Pioneer Hi.Bred International, 1995b).

Table 3-2. Fumonisin B_1 degrading enzymes.

Enzyme	Producing Organism	Accession/EC	Toxin Concentration	Degrading Conditions	Degradation	Reference
carboxylesterase and aminotransferase	*Sphingomonas* sp. ATCC55552	E.C. 3.1.1, E.C. 2.6.1	1000 μg/mL	▪ citrate buffer 0.1 M pH 3 or citrate-phosphate buffer 0.1 M pH 4; ▪ overnight incubation at 37 °C	100%	Pioneer Hi.Bred International, 1995b
carboxylesterase B and aminotransferase	*Sphingopyxis* sp. MTA144	E.C. 3.1.1, E.C. 2.6.1/ FJ426269.1	3.6 μg/mL	▪ Tris-HCl 20 mM pH 8, 0.1 mg/mL BSA; ▪ incubation at 30 °C for 2 h	100%	Heinl et al., 2009
fumonisin esterase	*Sphingopyxis* sp. MTA144	E.C. 3.1.1.87	60 μg/mL	▪ unspecified buffer pH 8; ▪ 18 U/L of enzyme ▪ incubation at 30 °C for 15 min	100% conversion to HFB_1	EFSA, 2014

The same degrading activity was shown by *Sphingopyxis* sp. MTA144, isolated from composted earth (Täubel et al., 2005). In this strain the two key DEs were identified within a gene cluster: *fumD*, encoding a type B carboxylesterase, and *fumI*, encoding an aminotransferase (Heinl et al., 2009; Hartiger et al., 2011; Heinl et al., 2011). The authors also demonstrated the degrading activities of the two recombinant enzymes by *in vitro* assays. The first enzyme, encoded by *fumD*, was able to catalyze the complete deesterification of FB_1 (3.34 μg/mL) to HFB_1 within 15 min. The oxygen independent aminotransferase, encoded by *fumI*, was shown to deaminate HFB_1 (8.9 μg/mL), in presence of pyruvate and pyridoxal phosphate, within 44 h. Furthermore, the authors described the additional genes located in this putative degrading cluster identified by genome walking (NCBI Accession n. FJ426269). The cluster includes one transporter, one permease, dehydrogenases, and transcriptional regulators and was U.S. patented by Moll et al. within the "Method for the production of an additive for the enzymatic decomposition of mycotoxins, additive and use thereof" [Erber Aktiengesellschaft, 2008].

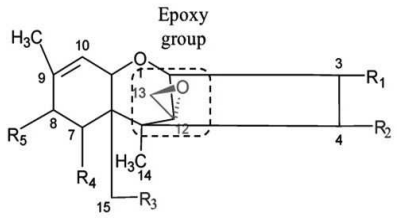
The esterase (EC 3.1.1.87) from *Sphingopyxis* sp. MTA144, produced by a genetically modified strain of *Komagataella pastoris* (formerly *Pichia pastoris*), has been included in a patented formulation, FUMzyme® (Biomin Holding GmbH, Getzersdorf, Austria). This enzyme-based feed additive, whose safety and efficacy has been recently evaluated by EFSA (EFSA, 2014), is intended to degrade fumonisins found as contaminants in feeds for growing pigs. The esterase partially degrades FB_1 and related fumonisins by cleavage of the diester bonds and release of the tricarballylic acid.

3.2.4. Trichothecenes

Trichothecenes are a large group of sesquiterpenoid metabolites sharing a common core structure comprised in a rigid tetracyclic ring (Fig. 3-4). To date, more than 190 trichothecenes and derivatives have been described Ueno et al., 1983; Zöller et al., 2006).

Their synthesis starts with the formation of trichodiene via the cyclization of farnesyl pyrophosphate. Trichodiene then undergoes a series of oxygenation, cyclizations, isomerization and esterification needed for the bioactivation of the molecule (Grove et al., 2007).

Trichothecenes are classified into 4 groups according to the functional groups associated to the core molecule: type A trichothecenes (e.g., T-2 and HT-2) do not contain a carbonyl group at the C-8 position; type B trichothecenes (mainly represented by deoxynivalenol (DON) and nivalenol (NIV) contain a carbonyl group at the C-8 position; trichothecenes of type C (e.g., crotocin and baccharin) have an additional epoxy ring between C-7 and C-8, or between C-9 and C-10; trichothecenes of type D (e.g., satratoxin and roridin), contain a macrocyclic ring between C-4 and C-15 (Fig. 3-4).



	T-2	HT-2	DON	NIV
R1	-OH	-OH	-OH	-OH
R2	-OAc	-OH	-H	-OH
R3	-OAc	-OAc	-OH	-OH
R4	-H	-H	-OH	-OH
R5	$-OCOCH_2CH(CH_3)_2$	$-OCOCH_2CH(CH_3)_2$	=O	=O

Figure 3-4. Chemical structure of trichothecenes. Groups responsible for trichothecenes toxicity are highlighted in red (epoxide) and blue (substituent groups, see the text for further details). The table shows substituent groups of different trichothecene analogues.

The results of trichothecenes exposure on eukaryotic cells was reviewed by Rocha et al. (Rocha et al., 2005), while T-2 and HT-2 toxicity data were reported to EFSA in 2010 by Schuhmacher-Wolz and colleagues (Schuhmacher-Wolz et al., 2010). Trichothecenes are not degraded during normal food processing, they are stable at neutral and acidic pH and, consequently, they are not hydrolyzed in the stomach after ingestion (Rocha et al., 2005).

Type A trichothecenes arise special interest, being more toxic than the other food-borne trichothecenes (Miller et al., 2002). Among the effects exerted by T-2 and HT-2 toxins several studies reported the inhibition of DNA, RNA and protein

synthesis, and apoptotic effects in mammalian cell cultures *in vitro* and *ex vivo* (EFSA, 2016; World Health Organization, 2016).

T-2 is rapidly metabolized to HT-2 in the gut. Data indicate that these toxins induce acute toxic effects with similar severity. For these reasons, the toxicity of T-2 *in vivo* is considered to include that of HT-2 World Health Organization, 2016). Type B trichothecene have a relatively low toxicity compared to type A trichothecene, but it varies depending on the species and the cell type. The general toxic mechanism of trichothecenes is induced by the interference with protein synthesis, through 60S ribosome binding, leading to translation inhibition (Pestka et a., 2010).

The 12,13-epoxy ring is the most important structural determinant of trichothecenes toxicity. In addition, the presence of hydroxyl or acetyl groups at appropriate positions on the trichothecene core (Thompson et al., 1986), the presence of substituents on C_{15} and C_4 (Cundliffe et al., 1974, 1977), and of the side groups, are implicated in defining the degree of toxicity (Sudakin et al., 2003). Both acetylation and de-acetylation may reduce toxicity of trichothecenes, as well as epimerization, oxidation (Karlovski et al., 2011; Hassan et al., 2016; He et al., 2016).

Trichothecenes are noticeably stable molecules under different treatments, including both thermal and chemical ones. Several microorganisms, isolated from disparate complex environments, such as rumen or soil, have been reported to degrade trichothecenes into de-acetylated and/or de-epoxidated products (Vanhoutte et al., 2016). However, the majority of the studies focused on this topic never achieved the identification of the enzymes responsible for the biotransformation. Nevertheless, considering the reaction products, acetylase, deacetylase or de-epoxidase activities are surely involved in the process.

In addition, hydroxylation and glycosylation of trichothecenes generate less toxic derivatives (He et al., 2011). In this case, these compounds might undergo

reverse reaction in the digestive tract of humans and animals, limiting the use of these enzymes at least in feed-related applications.

Recently, the bacterial cytochrome P450 system (EC 1.14) from S*phingomonas* sp. strain KSM1, was reconstructed *in vitro* and demonstrated to hydroxylate DON, NIV and 3-acetyl DON (Ito et al., 2013) (Table 3-3). The system includes the cytP450, encoded by the gene ddnA, and the endogenous redox partners. The DON catabolic product, 16-HDON, is used by the *Sphingomonas* strain as a carbon source, and was shown to exert a reduced phytotoxicity to wheat.

Poppengberger et al. (Poppemberger et al., 2003) identified a UDP-glycosyltransferase from *Arabidopsis thaliana* (the deoxynivalenol-glucosyl-transferase DOGT1) able to catalyze the transfer of glucose from UDP-glucose to the hydroxyl group at C3 of DON forming 3-*O*-glucopyranosyl-4-DON. The overexpression of this enzyme in *A. thaliana* enhanced DON resistance. The reaction products were identified by *in vitro* assays, using the enzyme purified from recombinant *Escherichia coli* cells.

Commercial products containing biotransformant agents have already been developed and patented. As an example, Biomin® BBSH 797 includes a pure culture of *Eubacterium* BBSH 797 isolated from bovine rumen fluid; it is capable of converting DON into DOM-1 and with de-epoxydase activity towards NIV, T-2, tetraol, scirpentriol, and HT-2. Nevertheless, purified enzymes with specific trichothecenes degrading activity have not been yet reported.

However, the recent increasing of microorganisms isolated from trichothecenes contaminated environments and capable of transforming this class of toxins led to the assumption that this goal will not be so distant (Hassan et al., 2016; He et al., 2011; Pierron et al., 2016).

Table 3-3. Trichothecenes degrading enzymes

Enzyme	Accession/ EC	Producing Organism	Trichothecene Target	Toxin Concentration	Degrading Conditions	*In Vitro* Degradation	Toxicity-Mutagenicity Test	Reference
cytochrome P450 system (Ddna + Kdx + KdR)	E.C. 1.14 AB744215.1 AB744217.1	*Sphingomonas* sp. strain KSM1	DON	99.86 μg/mL	potassium phosphate buffer 10 mM pH 7.5; 10% glycerol, 0.2 μM DdnA, 1.2 μM Kdx, 1.2 μM KdR, 1 mM NADH, and 100 mg/ml bovine liver catalase; overnight incubation at 30 °C	100% after 3 days	reduced phytotoxicity to wheat	Ito et al., 2013]
			NIV	105.25 μg/mL		100% after 5 days		
UDP-glycosyltransferase	AC006282	*Arabidopsis thaliana*	DON	n.p.	n.p.	n.p.	increased resistance in transgenic *Arabidopsis*	Poppemberger et al., 2003

n.p. = not provided

3.2.5. Zearalenone

ZEN is a resorcylic acid lactone, nonsteroidal yet estrogenic mycotoxin, which binds to mammalian estrogen receptors (ER), although with lower affinity than the natural estrogens 17β-estradiol, estriol and estrone (Zinedine et al., 2007). From a structural point of view, ZEN resembles the 17-βestradiol, and this similarity is responsible for its estrogenic potential and ER binding capacity (Fig. 3-5).

In vivo ZEN undergoes reduction to α-ZEL and β-zearalenol (β-ZEL), with the first being more estrogenic than the parent compound (Shier et al., 2001). A further reduction leads to the formation of α- and β-zearalanol (α-ZAL, β-ZAL), which are both less estrogenic than ZEN.

ZEN estrogenicity is greatly enhanced by the reduction of the 6′-ketone group and of the 1′-2′double bond, while it is reduced by the methylation of the hydroxyls in C4 and C2. The OH group in 6′of α and β-ZEL resembles that of C3 in estradiol. This group strongly interacts with ER, while the saturation of the 1′-2′double bond increases the flexibility of the molecule. This allows ZEN and/or ZEL to undergo some slight conformational changes, effective at adapting the molecules to the binding pocket of the receptor. Hydroxyl groups in C4 and C2 also contribute to the binding, with the first being more important than the latter in increasing ZEN estrogenic potential (Shier et al., 2001). (Fig. 3-5).

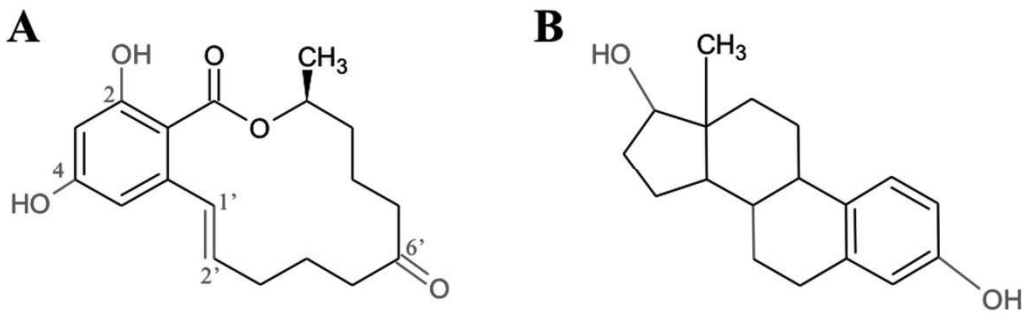


Figure 3-5. Chemical and structural analogies between zearalenone (**A**) and 17β-estradiol (**B**). The main chemical groups interacting with the estrogen receptors and responsible for zearalenone toxicity are highlighted in red (see the text for further details).

ZEN and derivatives detoxification strategies aim at disrupting their estrogenic activity. Among the reported ZEN metabolizing reactions, lactone ring cleavage, catalyzed by esterases, is the prevalent detoxification method described so far. Since the resulting hydroxyketones spontaneously decarboxylate, the reaction is irreversible.

A lactonohydrolase (EC 3.1.1) from the fungus *Clonostachys rosea* has been identified as capable of degrading ZEN. The enzyme was purified to homogeneity, and its gene (namely *zhd101*) was cloned and characterized by heterologous expression in *E. coli* BL21 and *S. cerevisiae* INVSc1 (Takahashi-Ando et al., 2002, 2005) (Table 3-4). The recombinant protein was proved to completely degrade 2 μg/mL of ZEN in *in vitro* assays, although with minimal yeast mediated conversion of ZEN to β-zearalenol. *Zhd101* gene and transformants carrying lactonohydrolase gene were patented in 2002 (Riken etal., 2003).

Table 3-4. Zearalenone degrading enzymes

Enzyme	Producing Organism	EC	Toxin Concentration	Degrading Conditions	Degradation	Toxicity-Mutagenicity Test	Reference
laccase	*Trametes versicolor* (commercial enzyme from Sigma-Aldrich)	EC 1.10.3.2	6.2×10^{-4} μg/mL	▪ sodium acetate buffer 0.2 M pH 5.2; ▪ incubation at 30 °C for 4 h.	up to 58 %	n.p.	Banu et al, 2014
laccase	*Streptomyces coelicolor*	EC 1.10.3.2	9.36 μg/mL	▪ sodium acetate 0.1 M pH 4.5; ▪ 0.2 mM mediator; ▪ incubation at 37 °C for 24 h	100%	n.p.	Novozymes 2009b
lactono hydrolase	*Clonostachys rosea*	E.C. 3.1.1	2 μg/mL	▪ YPD; ▪ incubation at 28 °C for 4 h or at 37 °C for 2 h	100%	n.p.	Takahashi-Ando 2002, 2005
2cys-peroxiredoxin	*Acinetobacter* sp. SM04	EC 1.11.1.15	20 μg/mL	▪ Tris-HCl 50 mM pH 9; ▪ $H_2O_2 \geq 20$ mM; ▪ incubation at 30 °C for 4 h	up to 95%	reduced MCF-7 cells proliferation by 75%	Yu et al., 2012
			1 μg/mL	▪ 1 mL of 0.8 M H_2O_2 and 19 mL of purified recombinant Prx solution pH 9; ▪ incubation at 40 °C for 6 h.	up to 90%		

Besides the above-mentioned activity towards AFs, laccases are also able to degrade ZEN. Novozyme patented the laccase-mediator degradation of ZEN in 2007 (Novozymes, 2009b), using Streptomyces coelicolor LC and, preferably, phenotiazin-10-propionic acid or methylsyringate as redox mediators. By using this system, ZEN was completely removed by 0.1 mg/mL of LC and 0.2 mM of mediator at 37 °C, within 24 h. Laccase degrading capabilities were also studied by Banu and colleagues (Banu et al., 2014) using one LC-enriched *Trametes versicolor* preparation. Reactions, containing 6.2 × 10−4 μg/mL of ZEN, were incubated with 0.4 mg/mL of laccase for 4 h at 30 °C, achieving a maximum of 81.7% of degradation.

Another ZEN degrading oxidoreductase is a 2-Cys peroxiredoxin (Prx) (EC 1.11.1.15) extracted from *Acinetobacter* sp. SM04 (Yu et al., 2012; Tang et al., 2013). Prxs are peroxidases containing redox-active cysteine, which can be oxidized to cystine by using H_2O_2 as cofactor. Prx degraded up to 95% of ZEN (20 μg/mL) in *in vitro* assays, when added with 20mM H_2O_2; nearly 90% of the toxin was instead degraded in contaminated corn sample (ZEN levels of nearly 1000 μg/mL), treated for 6 h at 30 °C with purified recombinant Prx, plus 0.09% (mass fraction, mol/mol) H_2O_2. Toxicity bioassays were also performed. The proliferative effect on MCF-7 cells by ZEN-DPs was reduced by 75%. Altalhi and colleagues (Altalhi et al., 2009) identified a 5.5 kb gene fragment from *Pseudomonas putida* pZEA-1, which encodes for DE(s) not yet characterized. *E. coli* DH5 cells, expressing the 5.5 kb gene fragment, were able to reduce 100 μg/mL of ZEN by 85% after 72 h, while no degradation was observed by wild type cells. ZEN DPs were also shown to possess a marked reduced toxicity on *Artemia salina* larvae.

3.3. Potentialities and Limitations of Mycotoxin Degrading Enzymes in Food, Feed, and Bioenergy

The use of enzymes in the food, feed, biogas and biofuel industries is not new, as biocatalysts have been increasingly used in the last 30 years. They allowed

reducing the employment of hazardous chemicals, to use mild working conditions, to increase specificity, to speed up a process or to simply create new products.

Enzyme biotechnological applications are widespread: the dairy industry has a long story of protease use for cheese manufacturing, while bakery employs laccase and transglutaminase to achieve doughs strengthening; phytases are used in non-ruminant animal nutrition for the degradation of phytic acid, which interferes with mineral absorption; pretreatment of lignocellulosic biomass for bioethanol production can be achieved using a cocktail of cellulases (endoglucanase, EC 3.2.1.4, exoglucanase, EC 3.2.1.91 and cellobiohydrolase, EC 3.2.1.21) instead of employing acid or alkali treatments (Patel et al., 2016; Sun et al., 2002).

Mandatory requirements for enzyme application in large scale industry are (a) safety; (b) effectiveness; (c) low cost of production and purification for both enzyme and cofactors, if needed; d) stability to wide ranges of temperature, pH and organic solvents, and thus compatibility to productive processes. All those characteristics make the enzyme use advantageous from both technological and economic points of view. Native enzymes usually do not respond to each distinctive requirement of a perfect industrial enzyme, but these features can be achieved via molecular engineering and structure-function modifications by targeted or random mutagenesis.

The most important limitation related to the application of mycotoxin DEs in real matrices is represented by the reduced effectiveness of the process due to matrix effects. The physicochemical properties of food, such as the moisture and fat content, acidity, texture etc., greatly influence the success of the detoxification process. Moreover, inhibitory compounds may be present in raw materials and mycotoxins can occur in masked forms in plant tissues (Berthiller et al., 2013); thus, their bioavailability for the enzyme catalysis may be further reduced. These

implications might require pretreatments, additional time and costs, which must be taken into account in the development of industrial applications.

Despite these limitations, the potentialities of enzyme use in food and feed industries remain widespread. Their application is versatile, since they can be used both in free or immobilized form and easily applied to well established industrial processes (fermentations, ripening, brewing or cheese manufacturing, feed and bioenergy production).

Moreover, DEs can be heterologously expressed by industrial microorganisms, such as lactic acid bacteria or yeasts, to perform *in situ* bioremediation. New smart and edible packaging with enzymes are also under study (Baldino et al., 2014).

3.3.1. Food

The European Community established the last regulation on food enzymes in 2008 (Regulation EC No 1332/2008). This regulation covers "enzymes added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids". All enzymes are submitted to this regulation as processing aids, with the exception of lysozyme and invertase, considered as additives.

Article 6 states that necessary conditions to authorize a food enzyme are that (i) its use does not pose a safety concern for the health of the consumer; (ii) it responds to a technological need that cannot be achieved by other economically and technologically practicable means; and (iii) it does not mislead the consumer.

Only recently, acceptability criteria for detoxification treatments, including biotransformation, have been set for commodities intended for animal nutrition (Commission Regulation 2015/786/EU).

Some of the enzyme recognized to have mycotoxins degrading capabilities have been proposed and studied for applications in food industry, even if thought for other purposes.

Laccases have been studied for their introduction in bakery and dairy as crosslinking agent, in brewing, wine and fruit juice production as clarifying agent and polyphenol remover (Osma et al., 2010; Gassara-Chatti et al., 2013; Lettera et al., 2016). Peroxidases have also been studied for their crosslinking activity in bakery and dairy industry, but the use of H_2O_2 as cofactor limits their real application in food.

Amano enzyme Inc. (United Kingdom) requested EFSA to perform a scientific risk assessment on one laccase from *Trametes hirsuta*, currently in progress.

The employment of DEs in the food industry, especially in immobilized forms, is not unrealistic. Still, it must overcome the gap of knowledge related to the effects that these enzymes exert on the nutritional and organoleptic qualities of raw materials, as well as fill a legislative void, which, so far, does not foresee the possibility of detoxifying treatments for food.

3.3.2. Feed

Enzyme use in animal diets has a long history, starting from the 1920s, when a commercial enzyme preparation, produced by *Aspergillus orizae*, known as Protozyme, was applied in poultry diets (Clickner et al., 1925). Phytases, proteases, α-galactosidases, glucanases, xylanases, α-amylases, and polygalacturonases are commercially available and increasingly used in animal nutrition, with huge benefits in terms of nutritional value and micronutrients availability (Menezes-Blackburn et al., 2015)

For authorizing their use in feed, EFSA requires solid supporting information about production, safety, efficacy and non-interference with the nutritional and organoleptic quality of the feed (Regulation EC No 1831/2003).

However, to become a commercial reality, an effective and safe DE must be formulated in order to guarantee its stability and efficacy during storage, and to ensure detoxification once released in the animal digestive tract.

There are two ways to apply a DE to feed: (a) as stabilized by a suitable formulation for becoming a feed additive or (b) used in a detoxifying process for contaminated raw materials intended for feed.

(a) During feed production enzymes are usually added before pelleting, in premixes with other additives (such as vitamins), or after pelleting as a liquid application. The most convenient approach is to introduce them as protected in formulations before pelleting: indeed, in this condition enzymes are less exposed to high moisture content and high temperature (Beaman et al., 2012), thus preventing their inactivation.

High temperature protection is usually achieved by coating. Its challenging development has to succeed in both stabilizing the enzyme during feed processing, and in fast dissolving in the animal gut, thus releasing the active enzyme. To this aim new coated and tough (CT) granulated coatings have been developed by different companies (Novozymes®, Bagsvaerd, Denmark; DSM™, The Netherlands). Within the CT granulates, enzymes are enclosed in a core matrix of minerals and carbohydrates. The outer layer can consist of vegetable oil, kaolin, and calcium carbonate, which are readily degraded upon ingestion (Novo Nordisk, 1997). On 5 May 2014 Biomin® (Holding GmbH) received the first-ever EFSA positive opinion, for using a purified enzyme in feed. This is an esterase, embodied in a 10% maltodextrin matrix and spray dried, capable of biotransforming fumonisins.

(b) The development of a detoxifying application at industrial scale should allow the treatment of highly contaminated batches, with significant reduction of mycotoxin under the limits imposed by the current regulation. The achieved

detoxification could result worthless in case of residual contamination by toxigenic fungi, if long term storage of the treated material is expected.

Free enzymes can be delivered in the feed productive process both in liquid and in solid forms (some prior wetting in dissolving feeders might be needed), through pumps, and mixed with the raw material, usually through a vertically mounted, shaft-driven impeller. This mode of application well fits a batch type process.

The major limitation of this approach is the difficulty to achieve a homogenous enzyme delivery throughout the entire material. In addition, the enzyme must target hot spots of contamination, typical of the distribution of toxigenic fungi and associated mycotoxins.

Among the environmental parameters that should be well set to preserve enzyme catalytic properties, moisture is by far the most critical. A high water content can increase enzyme activity, but simultaneously trigger fungal spread.

Immobilized enzymes encounter the same limitations, but they present the advantage of allowing the setup of continual processes, enzyme reuse and costs reduction.

3.3.3. Bioenergy

In biofuel production, enzymes are used to break down complex carbohydrates and to release ready fermentable sugars from cheap raw materials. Cellulose hydrolysis preceding fermentation is usually performed with cellulases, cellobiohydrolases and endoglucanases (Xiros et al., 2013). Pre-treatment of lignocellulosic biomasses prior to saccharification by laccases is under study, both to degrade lignin and to detoxify phenolic inhibitory compounds in bioethanol production (Kudanga et a., 2014; Jurado et al., 2009). The same biomass breakdown is realized during biogas production, especially in maize-feeded biogas plants, to adapt and accommodate raw materials and to enhance their

suitability for the activity of methanogenic microorganisms (Christy et al., 2014; Parawira et al., 2012). In this case, the mixture may include cellulose, hemicellulose, pectin and starch DEs, but also proteins able to breakdown long chain fatty acids, whose accumulation is responsible for the decrease of the process stability.

Despite these applications, no enzymes have been yet developed to reduce mycotoxin level in maize silage, plants or any other substrate used for bioenergy production. Their content, which mirrors fungal contamination in raw materials, affects the efficiency of the anaerobic digestion and ethanol/methane accumulation: indeed, fungal metabolites, including mycotoxins, act as antimicrobial or inhibitory compounds, or lead to excessive foam formation (Effember et al., 2008).

Storm et al. (2008) recently reviewed fungal and mycotoxin contamination in maize feeding biogas plants. The authors assessed that pre-harvest contamination of grass and maize by *Fusarium* spp., *Aspergillus* spp. and *Alternaria* spp. can lead to DON, ZEN, FBs and AFs accumulation, but with concentration usually lower than regulatory limits. Salati et al. (2014) reported that the addition of AFB_1 in a lab scale anaerobic digestion trials did not affect biogas production. In a recent conference abstract presented by De Gelder et al. (2016) the authors evaluated the biodegradability of mycotoxins during anaerobic digestion. They performed lab scale degradation tests with digestate spiked with different mycotoxins. After 30 days of mesophilic and thermophilic digestion, each tested mycotoxin resulted absent in the final products, suggesting the activation of some adsorption/detoxification mechanism during the fermentation. The adsorption mechanism appears the most probable, since (1) as discussed in previous paragraphs mycotoxins are greatly stable and (2) they strongly bind to soil clay minerals and organic matter present in the reactors. However, no experiment has been performed in real biogas plants under conventional operating conditions.

Recent studies demonstrated that the addition of enzymes directly into biogas reactors has no significant effect on biogas process (Rintala et al., 1994), since the enzymes are quickly degraded (Binner et al., 2011). This obviously might require a continuous addition, and thus increases costs in comparison to raw materials pre-treatment. The development of enzymatic formulates able to couple biomass breakdown and mycotoxin degrading activity could allow the simultaneous increase of biogas yield and the safe utilization of byproducts, for which mycotoxins levels are not yet regulated.

The fate of mycotoxins during biofuel production has been only recently investigated. Dzuman et al. (Dzuman et al., 2016), performed a study on five different batches of the ethanol–distiller's grains and soluble (DDGS) production process. They reported a significant increase of deoxynivalenol and its glycosylated form, DON-3-glucoside, during the first part of fermentation, when hydrolytic enzymes were added. After yeast addition, total DON content rapidly decreased. They observed an opposite trend for FB_1, since yeast addition contributed to its increase. The same results were discussed by Wu and Munkvold (2008), who estimated that mycotoxins are concentrated up to three times in DDGS compared to the feeding grain.

Despite these data, the second generation of bioethanol industry is not greatly concerned by mycotoxin contamination since: (a) less susceptible commodities can be used as raw material; (b) mycotoxins occurrence in bioethanol byproducts is not yet regulated neither in Europe or in U.S. and (c) the growth and productivity of highly tolerant fermenting yeasts are only slightly influenced by mycotoxin contamination (Nathanail et al., 2016).

The re-use of contaminated biomasses for renewable energy production is raising great interest both for economic and environmental reasons. However, more detailed studies should evaluate side effects not sufficiently considered, since by-products use for animal nutrition in Europe, Canada and U.S.

(Commission regulation EC No 68/2013; Feeds Regulation SOR/89-593; Code of Federal Regulation 21/573; Regulation EC No 1069/2009) is increasing.

Sosa et al. (Sosa et al., 2013) performed a preliminary economic analysis evaluating the feasibility of using fumonisin highly contaminated corns as feedstock for ethanol production. The authors calculated that the advantages deriving from the lower price of contaminated raw materials is balanced by the increase of the operating costs and the decrease of the final ethanol production by the fermenter. Thus, the performance of the process must be optimized, using process alternatives (e.g., both microfiltration and pervaporation membranes) coupled by the introduction of ethanol tolerant yeast strains, in order to effectively increase the economic value of the process.

DDGS are considered valuable by-products, thanks to the low carbon-nitrogen ratio and their high proteins and micronutrients content (Liu et al., 2011). However, their utilization should be responsibly addressed, since ecological outcomes of mycotoxin contaminated fertilizers and health effects are still poorly investigated.

As for biogas production, the implementation of DEs for biofuel application has not been developed. Still, it benefits of less limitations than those encountered by the food and feed industries. Cloning DEs in industrial hosts could ideally allow to simultaneously perform detoxification and fermentation of contaminated biomasses.

3.4. Conclusions and Future Perspectives

Within this review we gave a global view of the identified mycotoxins DEs, their mechanisms, their current and possible application in food, feed, biogas and biofuel industries. We also presented patented commercial preparations used by feed industries.

Although this paper is focused on the main mycotoxins, for which a high level of contamination and health implications have driven the majority of economic and research efforts, the discovery of novel DEs is becoming an interesting and stimulating topic also in relation to other mycotoxins.

Several patents have been deposited, mostly in U.S. and China, describing microorganisms and methods to degrade minor mycotoxins, such as patulin (Zhejiang University, 2014), beauvericin (Pioneer Hi-Bred International, 1998) and moniliformin (Pioneer Hi-Bred International, 1997) in plants, maize or grains. These patents are mainly related to microorganisms or crude extracts preparation, and no enzymes have been yet characterized as responsible for the degrading activity.

It should not be excluded that enzymes efficient towards minor mycotoxins could be effective towards structurally similar main ones.

In addition to the previously described ones, another challenge related to the application of DEs, is represented by mycotoxins co-occurrence, which should require the simultaneous use, and thus optimization, of different preparations and protocols. To overcome this barrier, the combination of different strategies, such as adsorbents mixture, binding microorganisms and enzymes, suitable for the inclusion within the same procedure, should be considered.

In the optimization process, great potentialities are ascribed to structural modelling and design of experiments (DOE) technologies. These approaches are able to identify structural determinants responsible for the degradation mechanism and to improve substrate-enzyme affinity by setting the conditions, which maximize enzyme efficiency. In combination with targeted mutagenesis methods, they strongly reduce lab-optimization processes and accelerate the industrial scale up.

Transcriptional analysis of degrading microorganisms (Liuzzi et al., 2017) has recently led to the identification of a DE from the novel *Acinetobacter* sp. *neg-1* species, described by Fanelli et al. (Fanelli et al., 2015) as capable of degrading OTA. This high-throughput approach, as other advanced Next Generation Sequencing technologies, such as functional metagenomic, could give a huge contribution in the identification of similar classes of DEs from complex and unexplored environments, such as contaminated soils, water or rumen. The advantage of these approaches is represented by the possibility to investigate non-culturable organisms, as well as those living in complex environmental niches, hardly analyzable under laboratory conditions.

The application of DEs could be simplified by the biotechnological advances in the field of immobilization and encapsulation techniques. Finally, an interesting perspective is represented by the development of transgenic crops able to counteract mycotoxins formation in the field. EU does not authorize the genetic modification of plants as a breeding technique. In contrast, the U.S. have 20 years of history of genetically engineered crops: herbicide-tolerant and insect-resistant crops were introduced in 1996 and today more than 90% of the corn cultivated in the U.S. is genetically modified USDA, 2011).

Syngenta patented trichothecene-resistant transgenic plants bearing the *Fusarium graminearum* Tri101 gene (Hohn et al., 2002), which encodes for a 3-*O*-acetyltransferase (Kimura et al., 1998, 1998b), which catalyzes the transfer of an acetate to the C3 position of trichothecenes. The company performed field trials, in Canada, the U.S., Argentina, and three European countries (Karlovski et al.,2001), proving that the development of self-defending crops is already achievable.

Acknowledgements: This work was financially supported by H2020-E.U.3.2-678781-MycoKey-Integrated and innovative key actions for mycotoxin management in the food and feed chain.

References

Abrunhosa, L., Paterson, R.R., Venâncio, A. (2010) Biodegradation of Ochratoxin A for Food and Feed Decontamination. *Toxins*. *2*, 1078–1099.

Abrunhosa, L., Santos, L., Venâncio, A. (2006). Degradation of ochratoxin A by proteases and by a crude enzyme of *Aspergillus niger*. *Food Biotechnology.* 20, 231–242.

Aiko, V., Edamana, P., Mehta, A. (2016). Decomposition and detoxification of aflatoxin B1 by lactic acid. *Journal of Science of Food and Agricolture. 96*, 1959–1966.

Alassane-Kpembi, I., Schatzmayr, G., Taranu, I., Marin, D., Puel, O., Oswald, I.P. (2016). ycotoxins co-contamination: Methodological aspects and biological relevance of combined toxicity studies. *Critical Reviews in Microbiol*ogy.

Alberts, J.F., Gelderblom, W.C., Botha, A., Van, Zyl. W.H. (2009). Degradation of aflatoxin B1 by fungal laccase enzymes. *International Journal of Food Microbiology.* 135, 47–52

Altalhi, A.D., El-Deeb, B. (2009). Localization of zearalenone detoxification gene(s) in pZEA-1 plasmid of *Pseudomonas putida* ZEA-1 and expressed in *Escherichia coli*. *Journal of Hazardous Material*. *161*, 1166–1172.

Altug, T., Youssef, A.E., Marth, E.H. (1990) Degradation of aflatoxin B1 in dried figs by sodium bisulfite with or without heat, ultraviolet energy or hydrogen peroxide. *Journal of Food Protection.* 1990, *53*, 581–628.

Aly, S.E., Abdel-Galil, M.M., Abdel-Wahhab, M.A. (2004). Application of adsorbent agents technology in the removal of aflatoxin B_1 and fumonisin B_1 from malt extract. *Food Chemistry and Toxicology. 42*, 1825–1831.

Baldino, L., Cardea, S., Reverchon, E. (2014). Supercritical assisted enzymatic membranes preparation, for active packaging applications. *Journal of Membrane Science. 453*, 409–418.

Banu, I., Lupu, A., & Aprodu, I. (2013). Degradation of Zearalenone by Laccase enzyme. Scientific Study & Research. Chemistry & Chemical Engineering, Biotechnology, *Food Industry*, 14, 79.

Beaman, K.R., Lilly, K.G.S., Gehring, C.K., Turk, P.J.; Moritz, J.S. (2012). Influence of pelleting on the efficacy of an exogenous enzyme cocktail using broiler performance and metabolism. *Journal of Applied Poultry Research. 21*, 744–775.

Bennett, J.W., Klich, M. (2003). Mycotoxins. *Clinical Microbiology reviews*. 497–516

Berthiller, F.; Crews, C., Dall'Asta, C., Saeger, S.D., Haesaert, G., Karlovsky, P., Oswald, I.P., Seefelder, W., Speijers, G., Stroka, J. (2013). Masked mycotoxins: A review. *Molecular Nutrition and Food Research. 57*, 165–186.

Binner, R., Menath, V., Huber, H., Thomm, M., Bischof, F., Schmack, D., Reuter, M. (2011) Comparative study of stability and half-life of enzymes and enzyme aggregates implemented in anaerobic biogas processes. *Biomass Conversion. and Biorefinery. 1*, 1–8.

Boeira, L., Bryce, J., Stewart, G., Flannigan, B. (1999). Inhibitory effect of *Fusarium* mycotoxins on growth of brewing yeasts. 1 zearalenone and fumonisin B1. *Journal of the Institute of Brewing*. *105*, 366–375.

Bong, J.P., Kosuke, T., Yoshiko, S.K., Ik-Hwi, K., Mi-Hee, L., Dong-Wook, H., Kie-Hyung, C., Soon, O.H., Jong-Chul, P. (2007). Degradation of mycotoxins using microwave-induced argon plasma at atmospheric pressure. *Surface and Coatings Technology*. *201*, 5733–5737.

Böswald, C., Engelhardt, G., Vogel, H., Wallnöfer, P.R. (1995). Metabolism of *Fusarium* mycotoxins zearalenone and deoxynivalenol by yeast strains of technological relevance. *Natural Toxins*. *3*, 138–144.

Bouslimi, A., Bouaziz, C., Ayed-Boussema, I., Hassen, W., Bacha, H. (2008). Individual and combined effects of ochratoxin A and citrinin on viability and DNA fragmentation in cultured Vero cells and on chromosome aberrations in mice bone marrow cells. *Toxicology*. *251*, 1–7.

Bouslimi, A., Ouannes, Z., El Golli, E., Bouaziz, C., Hassen, W., Bacha, H. (2008). Cytotoxicity and oxidative damage in kidney cells exposed to the mycotoxins ochratoxin A and citrinin: Individual and combined effects. *Toxicology Mechanical Method 18*, 341–349.

Bretz, M., Beyer, M., Cramer, B., Knecht, A., Humpf, H.U. (2006). Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agriculture and . Food Chemistry. 54*, 6445–6451.

Bryden, W.L. (2007). Mycotoxins in the food chain: Human health implications. *Asia Pacific Journal of Clinical Nutrition. 16*, 95–101.

Cao, H., Liu, D., Mo, X., Xie, C., Yao, D. (2011). A fungal enzyme with the ability of aflatoxin B_1 conversion: Purification and ESI-MS/MS identification. *Microbiological Reearchs*. *166*, 475–483.

Chitrangada, D., Mishra, H.N. (2000). *In vitro* degradation of aflatoxin B_1 by horseradish peroxidase. *Food Chemistry. 68*, 309–313.

Chitrangada, D.; Mishra, H.N. (2000). *In vitro* degradation of aflatoxin B_1 in groundnut (*Arachis hypogea*) meal by horseradish peroxidase. *Lebensmittel Wissenschaft & Technologie. 33*, 308–312.

Christy, P.M., Divya, D., Gopinath, L.R.A. (2014). Review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. *Renewable and Sustainable Energy Review. 34*, 167–173.

Clickner, F.H., Follwell, E.H. (1925). Application of 'Protozyme' (*Aspergillus orizae*) to poultry feeding. *Poulty Science. 5*, 241–247.

Code of Federal Regulations (CFR), Title 21, Sections 573 Food additives permitted in feed and drinking water of animals. Available online: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=573&showFR=1 (accessed on 17 November 2016).

Commission Recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006H0576&from=EN (accessed on 03 November 2016).

Commission Recommendation 2006/583/EC of 17 August 2006 on the prevention and reduction of Fusarium toxins in cereals and cereal products. Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006H0583&from=EN (accessed on 03 November 2016).

Commission Recommendation 2013/165/EU of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products Text with EEA relevance. Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013H0165&from=EN (accessed on 03 November 2016).

Commission Regulation (EU) No 68/2013 of 16 January 2013 on the Catalogue of feed materials. Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0068&from=EN (accessed on 17 November 2016).

Commission regulation 2006/1881/EC of 19 December 2006 setting maximum levels for certain contaminants in Food stuffs. Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1881&from=en (accessed on 03 November 2016).

Commission Regulation 2015/786/EU defining acceptability criteria for detoxification processes applied to products intended for animal feed as provided for in Directive 2002/32/EC of the European Parliament and of the Council. Available online: http://extwprlegs1.fao.org/docs/pdf/eur144560.pdf (accessed on 03 November 2016).

Cundliffe, E., Cannon, M., Davies, J. (1974). Mechanism of inhibition of eukaryotic protein synthesis by trichothecene fungal toxins. *Proceedings of the National Academy of Sciences 71*, 30–34.

Cundliffe, E., Davies, J.E. (1977). Inhibition of initiation, elongation, and termination of eukaryotic protein synthesis by trichothecene fungal toxins. *Antimicrobial Agents and Chemotherapy*. *11*, 491–499.

da Rocha, O., Ansari, K., Doohan, F.M. (2005). Effects of trichothecene mycotoxins on eukaryotic cells: A review. *Food Additives and Contaminants. 22*, 369–378.

Danisco A/S. Food Additive Comprising an Amidase for Detoxifying Ochratoxin. World Patent 2012032472, 6 set 2011.

De Bellis, P., Tristezza, M., Haidukowski, M., Fanelli, F., Sisto, A., Mulè, G., Grieco, F. (2015). Biodegradation of ochratoxin A by bacterial strains isolated from vineyard soils. *Toxins 7*, 5079–5093.

De Gelder, L., Audenaert, K., Willems, B., Schelfhout, K., De Saeger, S., De Boevre, M. (2016). Biodegradability of Mycotoxins during Anaerobic Digestion, *Abstract in* De Saeger, S.; Audenaert, K.; Croubels, S. Report from the 5th International Symposium on Mycotoxins and Toxigenic Moulds: Challenges and Perspectives (MYTOX) Held in Ghent, Belgium, May 2016. *Toxins*, *8*, 146.

Dirheimer, G., Creppy, E.E. (1991). Mechanism of action of ochratoxin A. *IARC Science Publications. 115*, 171–186.

Dridi, F., Marrakchi, M., Gargouri, M., Saulnier, J., Jaffrezic-Renault, N., Lagarde, F. (2015). Comparison of carboxypeptidase Y and thermolysin for ochratoxin A electrochemical biosensing. *Analitical Methods*, *7*, 8954–8960.

Duvick, J. (2001). Prospects for reducing fumonisin contamination of maize through genetic modification. *Environmental Health Perspectives. 109*, 337–342.

Duvick, J., Rood, T., Maddox, J., and Gilliam, J. (1998). Detoxification of mycotoxins in planta as a strategy for improving grain quality and disease resistance: Identification of fumonisin-degrading microbes from maize. In *Molecular Genetics of Host-Specific Toxins in Plant Disease*; Kohmoto, K., Yoder, O., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1998; pp. 369–381.

Dzuman, Z., Stranska-Zachariasova, M., Vaclavikova, M., Tomaniova, M., Veprikova, Z., Slavikova, P., Hajslova, J. (2016). Fate of Free and conjugated mycotoxins within the production of distiller's dried grains with solubles (DDGS). *Journal of Agricultural Food Chemistry. 64*, 5085–5092.

Eaton, D.L., Gallagher, E.P. (1994). Mechanisms of aflatoxin carcinogenesis. *Annual Reviews in Pharmacology and Toxicology. 34*, 135–172.

Effenberger, M., Lebuhn, M., Gronauer, A. (2007). Biogas im Wandel, 16th annual conference of Fachverband Biogas. In *Fermenter Management: Stabiler Prozess bei NawaRo-Anlagen*; Leipzig, Germany.

Erber, Aktiengesellschaft. (2008). Method for the Production of an Additive for the Enzymatic Decomposition of Mycotoxins, Additive, and Use Thereof. U.S. Patent 8,703,460, 2008.

Essigmann, J.M., Croy, R.G., Nadzan, A.M., Busby, W.F., Jr., Reinhold, V.N., Büchi, G., Wogan, G.N. (1977). Structural identification of the major DNA adduct formed by aflatoxin B_1 *in vitro*. *Proceedings of the National Academy of Sciences. 74*, 1870–1874.

Fanelli, F., Chiara, M., Liuzzi, V.C., Haidukowski, M., Tristezza, M., Manzari, C., D'Erchia, A.M., Pesole, G., Horner, D.S., Mulè, G. (2015).Draft genome sequence of *Acinetobacter* sp. *neg1* capable of degrading ochratoxin A. *FEMS Microbiology Letters. 362*, 7.

Fanelli, F., Geisen, R., Schmidt-Heydt, M., Logrieco, A.F., Mulè, G. (2016). Light regulation of mycotoxin biosynthesis: New perspectives for food safety. World Mycotoxin Journal. 9, 129–146.

Feeds Regulations (SOR/83-593), Feeds act. Government of Canada. 1983. Available online: http://laws-lois.justice.gc.ca/PDF/SOR-83-593.pdf (accessed on 17 November 2016).

Fouler, S.G., Trivedi, A.B., Kitabatake, N. (1994). Detoxification of citrinin and ochratoxin a by hydrogen peroxide. *Journal – Association of official analytical chemists 77*, 631–637.

Gao, Y.N., Wang, J.Q., Li, S.L., Zhang, Y.D., Zheng, N. (2016). Aflatoxin M_1 cytotoxicity against human intestinal Caco-2 cells is enhanced in the presence of other mycotoxins. *Food Chemistry and Toxicology. 96*, 79–89.

Gassara-Chatti, F., Brar, S.K., Ajila, C.M., Verma, M., Tyagi, R.D., Valero, J.R. (2013). Encapsulation of ligninolytic enzymes and its application in clarification of juice. *Food Chemistry. 137*, 18–24.

Gelderblom, W.C. (2002). Interaction of fumonisin B(1) and aflatoxin B(1) in a short-term. *Toxicology 171*, 161–73.

Grove, J.F. (2007) The trichothecenes and their biosynthesis. *Progress in the Chemistry of Organic Natural Products. 88*, 63–130.

Guan, S., Zhao, L., Ma, Q., Zhou, T., Wang, N., Hu, X., Ji, C. (2010). *In vitro* efficacy of *Myxococcus fulvus* ANSM068 to biotransform aflatoxin B_1. *International Journal of Molecular Sciences. 11*, 4063–4079.

Hartinger, D., Schwartz, H., Hametner, C., Schatzmayr, G., Haltrich, D., Moll, W.D. (2011). Enzyme characteristics of aminotransferase FumI of *Sphingopyxis* sp. MTA144 for deamination of hydrolyzed fumonisin B1. *Applied Microbiology and Biotechnology. 91*, 757–768.

Hassan, Y.I., Bullerman, L.B. (2013). Cell-surface binding of deoxynivalenol to *Lactobacillus paracasei* subsp. *tolerans* isolated from sourdough starter culture. *JMBFS*, *2*, 2323–2325.

Hassan, Y.I., Zhou, T., Bullerman, L.B. (2015). Sourdough lactic acid bacteria as antifungal and mycotoxin-controlling agents. *Food Science and Technolnology International*. *22*, 79–90.

Hassan, Y.I., Zhu, H.; Zhu, Y., Zhou, T. (2016). Beyond ribosomal binding-the increased polarity and aberrant molecular interactions of 3-epi-deoxynivalenol. *Toxins. 8*, 261.

He, J., Zhou, T., Young, J.C., Boland, G.J., Scott, P.M. (2010). Chemical and biological transformations for detoxification of trichothecene mycotoxins in human and animal food chains: A review. *Trend in Food Science and Technology. 21*, 67–76.

He, J.W., Hassan, Y.I., Perilla, N., Li, X.Z., Boland, G.J., Zhou, T. (2016). Bacterial epimerization as a route for deoxynivalenol detoxification: The influence of growth and environmental conditions. *Froniers in Microbiology. 7*, 572.

He, W.J., Yuan, Q.S., Zhang, Y.B., Guo, M.W., Gong, A.D., Zhang, J.B., Wu, A.B., Huang, T., Qu, B., Li, H.P. (2016). Aerobic de-epoxydation of trichothecene mycotoxins by a soil bacterial consortium isolated using in situ soil enrichment. *Toxins.*, *8*, 277.

Heinl, S., Hartinger, D., Moll, W.D., Schatzmayr, G., Grabherr, R. (2009). Identification of a fumonisin B1 degrading gene cluster in *Sphingomonas* sp. MTA144. *New Biotechnology. 25*, S61–S62.

Heinl, S., Hartingerb, D., Thamhesl, M., Schatzmayrb, G., Moll, W.D., Grabherra, R. (2011). An aminotransferase from bacterium AtrichotheceneC 55552 deaminates hydrolyzed fumonisin B_1. *Biodegradation. 22*, 25–30.

Herzallah, S., Al Shawabkeh, K., Al Fataftah, A. (2008). Aflatoxin decontamination of artificially contaminated feeds by sunlight, g-radiation, and microwave heating. *Journal of Applied Poulty Research. 17*, 515–521.

Hohn T.M., Peters C., Salmeron, J. (2002). Trichothecene-Resistant Transgenic Plants. U.S. Patent 20020162136, 12 February.

Ito, M., Sato, I., Ishizaka, M., Yoshida, S., Koitabashi, M., Yoshida, S., Tsushima, S. (2013). Bacterial cytochrome P450 system catabolizing the *Fusarium* toxin deoxynivalenol. *Applied and Environmental Microbiology. 79*, 1619–1628,

Ji, C., Fan, Y., Zhao, L. (2016) Review on biological degradation of mycotoxins. *Animal Nutrition. 2*, 127–133.

Jurado, M., Prieto, A., Martínez-Alcalá, A., Martínez, A.T., Martínez, M.J. (2009). Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. *Bioresource Technology*. *100*, 6378–6384.

Karlovsky, P. (2011).Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. *Appied Microbiology and Biotechnology*, *91*, 491–504.

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K., Yamaguchi, I. (198). Trichothecene 3-*O*-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. *JJournal of Biological Chemistry. 273*, 1654–1661.

Kimura, M., Shingu, Y., Yoneyama, K., Yamaguchi, I. (1998). Features of Tri101, the trichothecene 3-*O*-acetyltransferase gene, related to the self-defense mechanism in *Fusarium graminearum*. *Bioscience Biotechnology and Biochemistry. 62*, 1033–1036.

Klaric, M.S., Zeljezic, D., Rumora, L., Peraica, M., Pepeljnjak, S., Domijan, A.M. (2012). A potential role of calcium in apoptosis and aberrant chromatin forms in porcine kidney PK15 cells induced by individual and combined ochratoxin A and citrinin. *Archives of Toxicology. 86*, 97–107.

Knasmuller, S., Cavin, C., Chakraborty, A., Darroudi, F., Majer, B.J., Huber, W.W., Ehrlich, V.A. (2004). Structurally related mycotoxins ochratoxin A, ochratoxin B, and citrinin differ in their genotoxic activities and in their mode of action in human-derived liver (HepG2) cells: Implications for risk assessment. *Nutrition. Cancer* journal. *50*, 190–197.

Kőszegi, T., Poór, M. (2016). Ochratoxin A: Molecular interactions, mechanisms of toxicity and prevention at the molecular Level. *Toxins*. *8*, 111.

Kriz, P., Petr, B., Zbynek, H., Jaromir, K., Pavel, O., Petr, S., Miroslav, D. (2015). Influence of plasma treatment in open air on mycotoxin content and grain nutriments. *Plasma Medicine. 5*, 145–158.

Kudanga, T., Le Roes-Hill, M. (2014). Laccase applications in biofuels production: Current status and future prospects. *Applied Microbiology and Biotechnology. 98*, 6525–6542.

Lettera, V., Pezzella, C., Cicatiello, P., Piscitelli, A., Giacobelli, V.G., Galano, E., Amoresano, A., Sannia, G. (2016). Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification. *Food Chemistry. 196*, 1272–1278.

Liu, D.L., Ma, L., Gu, L.Q., Liang, R., Yao, D.S., Chen, W.Q. (1998). *Armillariella tabescens* enzymatic detoxification of aflatoxin B1. Part III. Immobilized enzymatic detoxification. *Ann. N. Y. Acad. Sci., 864*, 592–599.

Liu, D.L., Yao, D.S., Liang, R., Ma, L., Cheng, W.Q., Gu, L.Q. (1998). Detoxifcation of aflatoxin B1 by enzymes isolated from *Armillariella tabescens. Food Chemistry and Toxicology*. *36*, 563–574.

Liu, K. (2011). Chemical Composition of Distillers Grains, a Review. *Journal of Agriculture and Food Chemistry. 59*, 1508–1526.

Liuzzi, V.C., Fanelli, F., Tristezza, M., Haidukowski, M., Picardi. E., Manzari, C., Lionetti, C., Grieco, F., Logrieco, A.F., Thon, M.R.(2017). Transcriptional analysis of *Acinetobacter* sp. *neg1* capable of degrading ochratoxin A. *Frontiers in Microbiology. 7*, 2162.

Logrieco, A.F.,Mulé, G., Moretti, A., Bottalico, A. (2002) Toxigenic Fusarium species and mycotoxins associated with maize ear rot in Europe. *European Journal of Plant Pathology. 108*, 597–609.

Loi, M., Quintieri, L., Fanelli,, F., Liuzzi, V.C., Haidukowski, M., Logrieco, A.F., Mulè, G. (2017). AFM_1 removal and biotechnological potential of a laccase from Pleurotus eryngii for milk safety. Scienza e tecnica lattiero casearia. 67, 23-32.

Loi, M.; Fanelli, F; Zucca, P.; Liuzzi, V.C.; Quintieri, L.; Cimmarusti, M.T.; Monaci, L.; Haidukovski, M.; Logrieco, A.F.; Sanjust, E.; et al. Aflatoxin B_1 and M_1 degradation by Lac2 from *Pleurotus pulmonarius* and redox mediators. *Toxins* 2016, *8*, 245–261.

Maeba, H., Takamoto, Y., Kamimura, M.I., Miura, T.O. (1988). Destruction and detoxification of aflatoxins with ozone. *Journal of Food Sciences. 53*, 667–668.

Margot, J., Bennati-Granier, C., Maillard, J., Blánquez, P., Barry, D.A., Holliger, C. (2013). Bacterial versus fungal laccase: Potential for micropollutant degradation. *AMB Express 3*, 63–77.

Méndez-Albores, A., Arámbula-Villa, G., Loarca-Piña, M.G., Castaño-Tostado, E., Moreno-Martínez, E. (2005). Safety and efficacy evaluation of aqueous citric acid to degrade B-aflatoxins in maize. *Food Chemistry and Toxicology. 43*, 233–238.

Menezes-Blackburn, D., Greiner, R. (2015). Enzymes used in animal feed: Leading technologies and forthcoming developments. In Functional Polymers in Food Science: From Technology to Biology, Volume 2: Food Processing; Cirillo, G., Spizzirri, U.G., Iemma, F., Wiley, Hoboken, NJ 07030, U.S..

Merrill, A.H., Wang, E., Vales, T.R., Smith, E.R., Schroeder, J.J., Menaldino, D.S., Alexander, C., Crane, H.M., Xia, J., Liotta, D.C. (1996). Fumonisin toxicity and sphingolipid biosynthesis. *Advances in Experimental Medicine and Biology.* 392, 297–306.

Miller, J.D. (2002).Aspects of the ecology of *Fusarium* toxins in cereals. *Advances in Experimental Medicine and Biology. 504*, 19–27.

Motomura, M., Toyomasu, T., Mizuno, K., Shinozawa, T. (2003). Purification and characterization of an aflatoxin degradation enzyme from *Pleurotus ostreatus*. *Microbiology Research*. *158*, 237–242.

Müller, H.M. (1983). Entgiftung von Mykotoxinen: II. Chemische Verfahren und Reaktion mit Inhaltsstoffen von Futtermitteln. *Übers Tierernährg. 11*, 47–80.

Nathanail, A.V., Gibson, B., Han, L., Peltonen, K., Ollilainen, V., Jestoi, M., Laitila, A. (2016). The lager yeast *Saccharomyces pastorianus* removes and transforms *Fusarium* trichothecene mycotoxins during fermentation of brewer's wort. *Food Chemistry. 203*, 448–455.

Novo Nordisk A/S. Enzyme-Containing Granules and Process for the Production Thereof. World Patent 1997039116, 14 apr 1997.

Novozymes A/S. Detoxification of Aflatoxin in Feed Products. European Patent 2252163, 5 mar 2009.

Novozymes A/S. Detoxification of Aflatoxin in Feed Products. World Patent 2009109607, 5 mar 2009.

Novozymes A/S. Detoxification of feed products, World Patent 2009109607, 5 mar 2009.

Opinion of the Scientific Committee on Food (SCF) on Fusarium toxins, part 5: T-2 toxin and HT-2 toxin. 2001. Available online: https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_catalogue_out88_en.pdf (accessed on 03 November 2016).

Osma, J.F., Toca-Herrera, J.L., Rodrıguez-Couto, S. (2010). Uses of Laccases in the Food Industry. *Enzyme. Research.,* 918761.

Parawira, W. (2012). Enzyme research and applications in biotechnological intensification of biogas production. *Critical Reviews in Biotechnology. 32*, 172–186.

Park, D.L., Lee, L.S., Price, R.L., Pohland, A.E. (1988). Review of the decontamination of aflatoxins by ammoniation: Current status and regulation. *J. Association of. Official Analitical Chemists. 71*, 685–703.

Patel, A.K., Singhania, R.R., Pandey, A. (2016). Novel enzymatic processes applied to the food industry. *Curent. Opinions in Food Science. 7*, 64–72.

Pestka, J.J. (2010). Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology. 84*, 663–679.

Pfohl-Leszkowicz, A., Manderville, R.A. (2012). An update on direct genotoxicity as molecular mechanism of ochratoxin A carcinogenicity. *Chemical Research in Toxicology. 25*, 252–262.

Pierron, A., Mimoun, S., Murate, L.S., Loiseau, N., Lippi, Y., Bracarense, A.P., Schatzmayr, G., He, J.W., Zhou, T., Moll, W.D. (2016). Microbial biotransformation of DON: Molecular basis for reduced toxicity. *Science Reports*. *6*, 29105.

Pioneer Hi-Bred International, Inc. Moniliformin detoxification compositions and methods. Canadian Patent 2272554, 12 nov 1997.

Pioneer Hi-Bred International, Inc. Beauvericin Detoxification Method Using Bacteria. U.S. Patent 6126934, 29 jan 1998.

Pioneer Hi-Bred International, Inc. Fumonisin detoxification compositions and methods. U.S. Patent 5792931, 7 jun 1995.

Pioneer Hi-Bred International, Inc. Fumonisin-Detoxifying Enzymes. World Patent 1996006175, 11 aug 1995.

Pitout, M.J. (1969) The hydrolysis of Ochratoxin A by some proteolytic enzymes. *Biochemistry and. Pharmacology. 18*, 485–491.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Sieberer, T., Schuhmacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., Adam, G. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biology and Chemistry. 278*, 47905–47914.

Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation). Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32009R1069&from=EN (accessed on 17 November 2016).

Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97.

Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Available online: http://eurlex.europa.eu/legalcontent/EN/TXT/PDF/?uri=CELEX:32003R1831&from=EN (accessed on 17 November 2016).

Rheeder, J.P., Marasas, W.F.O., Vismer, H.F. (2002). Production of fumonisin analogs by *Fusarium* species. *Applied and Environmental Microbiology. 68*, 2101–2105.

Riken. Zearalenone-Detoxifying Enzyme Gene and Transformant Having the Gene Transferred Thereinto. World Patent 2003080842, 25 mar 2003.

Rintala, J., Ahring, B.K. (1994). A two-stage thermophilic anaerobic process for the treatment of source sorted household solid waste. *Biotechnology Letters.* 16, 1097–1102.

Robinson, A., Johnson, N.M., Strey, A., Taylor, J.F., Marroquin-Cardona, A., Mitchell, N.J., Afriyie-Gyawu, E.; Ankrah, N.A., Williams, J.H., Wang, J.S. (2012).

Calcium montmorillonite clay reduces urinary biomarkers of fumonisin B1 exposure in rats and humans. *Food Additives and Contaminants. 29*, 809–818.

Roth, A., Creppy, E.E., Kane, A., Bacha, H., Steyn, P.S., Roschenthaler, R., Dirheimer, G. (1989). Influence of ochratoxin B on the ochratoxin A inhibition of phenylalanyl-tRNA formation *in vitro* and protein synthesis in hepatoma tissue culture cells. *Toxicology Letters.* 45, 307–313.

Salati, S., D'Imporzano, G., Panseri, S., Pasquale, E., Adani, F. (2014). Degradation of aflatoxin b1 during anaerobic digestion and its effect on process stability. *Int. Biodeterioration and Biodegradradation.* 94, 19–23.

Sato, I., Ito, M., Ishizaka, M., Ikunaga, Y., Sato, Y., Yoshida, S., Koitabashi, M., Tsushima, S. (2012). Thirteen novel deoxynivalenol-degrading bacteria are classified within two genera with distinct degradation mechanisms. *FEMS Microbiology Letters*. *327*, 110–117.

Schlüter, O., Ehlbeck, J., Hertel, C., Habermeyer, M., Roth, A., Engel, K.H., Holzhauser, T., Knorr, D., Eisenbrand, G. (2013). Opinion on the use of plasma processes for treatment of foods. *Molecular Nutrition & Food Research*. *57*, 920–927.

Schroeder, T.U., Zweifel, P., Sagelsdorff, U., Friederich, J., Luthy, C., Schlatter, J. (1985). Ammoniation of aflatoxin-containing corn: Distribution, *in vivo* covalent deoxyribonucleic acid binding, and mutagenicity of reaction products. *Agriulture and Food Chemistry.* 33, 311–316.

Schuhmacher-Wolz, U., Heine, K., Schneider, K. (2010). Report on Toxicity Data on Trichothecene Mycotoxins HT-2 and T-2 Toxins. CT/EFSA/CONTAM/2010/03. Available online: http://www.efsa.europa.eu/en/scdocs/doc/65e.pdf (accessed on 10 November 2016).

Scientific Opinion on the safety and efficacy of fumonisin esterase (FUMzyme®) as a technological feed additive for pigs. *EFSA J.* 2014, *12*, 3667.

Sharma, R.P.(1993). Immunotoxicity of mycotoxins. *Journal of Dairy Science.* 76, 892–897.

Shephard, G.S. (2008). Impact of mycotoxins on human health in developing countries. *Food Addiives and Contaminants.* 25, 146–151.

Shier, W.T., Shier, A.C., Xie, W., Mirocha, C.J. (2001). Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. *Toxicon, 39*, 1435–1438.

Soriano, J.M., González, L., Catala, A.I. (2005). Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B_1. *Progress in Lipid Research.* 44, 345–356, doi:10.1016/j.plipres.2005.09.001.

Sosa, M.A., Chovau, S., Van der Bruggen, B., Espinosa, J. (2013). Ethanol production from corn contaminated with fumonisins: A preliminary economic

analysis including novel processing alternatives. *Industrial and Engineering Chemistry Research.* 52, 7504–7513.

Stander, M.A., Bornscheuer, U.T., Henke, E., Steyn, P.S. (2000). Screening of commercial hydrolases for the degradation of ochratoxin A. *Journal of Agriculture and Food Chemistry .* 2000, *48*, 5736–5739.

Storm, I.M.L.D.; Sørensen, J.L.; Rasmussen, R.R.; Nielsen, K.F.; Thrane, U. Mycotoxins in silage. *Stewart Posthar. Rev.* 2008, 1-12.

Sudakin, D.L. Trichothecenes in the environment: Relevance to human health. *Toxicol. Lett.* 2003, 20, 97–107.

Sun, Y.; Cheng, J. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Biores. Technol.* 2002, *83*, 1–11.

Takahashi-Ando, N.; Kimura, M.; Kakeya, H.; Osada, H.; Yamaguchi, I. A novel lactonohydrolase responsible for the detoxification of zearalenone: Enzyme purification and gene cloning. *Biochem. J.* 2002, *365*, 1–6.

Takahashi-Ando, N.; Tokai, T.; Hamamoto, H.; Yamaguchi, I.; Kimura, M. Efficient decontamination of zearalenone, the mycotoxin of cereal pathogen, by transgenic yeasts through the expression of a synthetic lactonohydrolase gene. *Appl. Microbiol. Biotechnol.* 2005, *67*, 838–844.

Tang, Y.; Xiao, J.; Chen, Y.; Yu, Y.; Xiao, X.; Yu, Y.; Wu, H. Secretory expression and characterization of a novel peroxiredoxin for zearalenone detoxification in *Saccharomyces cerevisiae. Microbiol. Res.* 2013, *168*, 6–11.

Täubel, M. Isolierung und Charakterisierung von Mikroorganismen zur Biologischen Inaktivierung von Fumonisinen. Ph.D. Thesis, University of Natural Resources and Applied Life Sciences, Vienna, Austria, 2005.

Taylor, M.C.; Jackson, C.J.; Tattersall, D.B.; French, N.; Peat, T.S.; Newman, J.; Briggs, L.J.; Lapalikar, G.V.; Campbell, P.M.; Scott, C.; et al. Identification and characterization of two families of F420H2-dependent reductases from *Mycobacteria* that catalyse aflatoxin degradation. *Mol. Microbiol.* 2010, *78*, 561–575.

The European Food Safety Authority (EFSA). *Scientific Report Review of Mycotoxin-Detoxifying Agents Used as Feed Additives: Mode of Action, Efficacy and Feed/Food Safety*; Scientific Report Submitted to EFSA, CFP/EFSA/FEEDAP/2009/01, Wiley Online Library, 2009.

The International Agency for Research on Cancer (IARC). Chemical agents and related occupations. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; World Health Organization: Lyon, France. 2012; Volume 100F, pp. 1–599.

The International Agency for Research on Cancer (IARC). Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines

and mycotoxins. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; World Health Organization: Lyon, France, 1993; Volume 56, pp. 489–521.

The International Agency for Research on Cancer (IARC). Working Group on the Evaluation of Carcinogenic Risks to Humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; World Health Organization: Lyon, France, 2002; Volume 82, pp. 1–556.

Thompson, W.L., Wannemacher, R.W., Jr. (1986). Structure–function relationship of 12,13-epoxytrichothecene mycotoxins in cell culture: Comparison of whole animal lethality. *Toxicon* 24, 985–994.

Ueno, Y. (1983). Trichothecenes. In *Chemical, Biological and Toxicological Aspects*; Elsevier Scientific Publishers: Amsterdam, The Netherlands.

USDA Economic Research Service. (2011). Adoption of Genetically Engineered Crops in the U.S. USDA, Washington. Available online: https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx (accessed on 17 November 2016).

Vanhoutte, I., Audenaert, K., De Gelder, L. (2016). Biodegradation of Mycotoxins: Tales from Known and Unexplored Worlds. *Frontiers Microbiology.* 7, 561.

Vettorazzi, A., van Delft, J., López de Cerain, A. (2013). A review on ochratoxin A transcriptomic studies. *Food Chemistry and Toxicology.* 59, 766–783.

Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A.P., Narasimha, G. (2014). Fungal laccases and their applications in bioremediation. *Enzyme Research.* 2014, 1–21.

Wang, E.; Norred, W.P.; Bacon, C.W.; Riley, R.T.; Merrill, A.H., Jr. Inhibition of sphingolipid biosynthesis by fumonisins: Implications for diseases associated with *Fusarium moniliforme*. *J. Biol. Chem.* 1991, *266*, 14486–14490.

Wang, J., Ogata, M., Hirai, H., Kawagishi, H. (2011). Detoxification of aflatoxin B_1 by manganese peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624. *FEMS Microbiology Letters.* 314, 164–169.

World Health Organization. (2001). WHO Food Additives Series: 47. Safety Evaluation of Certain Mycotoxins in Food. Prepared by the Fifty-Sixth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available online: http://www.inchem.org/documents/jecfa/jecmono/v47je01.htm (accessed on 10 November 2016).

Wu, F. (2007). Measuring the economic impacts of Fusarium toxins in animal feed. *Animal Feed Science and Technology.* 137, 363–374.

Wu, F., Munkvold, G., (2008). Mycotoxins in ethanol co-products: Modeling economic impacts on the livestock industry and management strategies. *J. Agric. Food Chemistry.* 56, 3900–3911.

Wu, Y.Z., Lu, F.P., Jiang, H.L., Tan, C.P., Yao, D.S., Xie, C.F., Liu, D.L. (2015). The furofuran-ring selectivity, hydrogen peroxide-production and low K_m value are the three elements for highly effective detoxification of aflatoxin oxidase. *Food and Chemical Toxicology.* 76, 125–131.

Xiros, C.; Topakas, E.; Christakopoulos, P. Hydrolysis and fermentation for cellulosic ethanol production. *WIREs Energy Environ.* 2013, *2*, 633–654.

Yehia, R.S. (2014). Aflatoxin detoxification by manganese peroxidase purified from *Pleurotus ostreatus*. *Brazilian Journal of Microbiology*. 45, 127–133.

Yu, Y., Wu, H., Tang, Y., Qiu, L. (2012). Cloning, expression of a peroxiredoxin gene from *Acinetobacter* sp. SM04 and characterization of its recombinant protein for zearalenone detoxification. *Microbiology Research.* 167, 121–126.

Zain, M.E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society.* 15, 129–144.

Zhao, L.H., Guan, S., Gao, X., Ma, Q.G., Lei, Y.P., Bai, X.M., Ji, C. (2011). Preparation, purification and characteristics of an aflatoxin degradation enzyme from *Myxococcus fulvus* ANSM068. *Journal of Applied Microbiology.* 110, 147–155.

Zhejiang University. A Method for Reducing the Content of Patulin in Apple Juice Concentrate. Chinese Patent 103859016, 26 February 2014.

Zinedine, A., Soriano, J.M., Molto, J.C., Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology.* 45, 1–18.

Zöllner, P., Mayer-Helm, B. (2006). Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. *Journal of Chromatography: A*. 1136, 123–169.

Aflatoxin B_1 and M_1 Degradation by Lac2 from *Pleurotus pulmonarius* and Redox Mediators

Loi M[1,2], Fanelli F[1], Zucca P[3], Liuzzi VC[1], Quintieri L[1], Cimmarusti MT[1,2], Monaci L[1], Haidukowski M[1], Logrieco AF[1], Sanjust E[3], Mulè G[1]

[1]Institute of Sciences of Food Production, National Research Council of Italy (CNR), via Amendola 122/O, Bari 70126, Italy;
[2]Department of Economics, University of Foggia, via Napoli 25, Foggia 71122, Italy
[3]Department of Biomedical Sciences, University of Cagliari, Cittadella, Universitaria, Complesso Universitario, SP Monserrato-Sestu Km 0.700, Monserrato 09042, Italy;

The contents of this chapter were published in Loi M, Fanelli F, Zucca P, et al. Aflatoxin B_1 and M_1 Degradation by Lac2 from *Pleurotus pulmonarius* and Redox Mediators. Toxins (Basel). 2016, 8(9):245; doi:10.3390/toxins8090245

Abstract: Laccases (LCs) are multicopper oxidases that find application as versatile biocatalysts for the green bioremediation of environmental pollutants and xenobiotics. In this study, we elucidate the degrading activity of Lac2 pure enzyme form *Pleurotus pulmonarius* towards aflatoxin B_1 (AFB_1) and M_1 (AFM_1). LC enzyme was purified using three chromatographic steps and identified as Lac2 through zymogram and LC-MS/MS. The degradation assays were performed in vitro at 25 °C for 72 h in buffer solution. AFB_1 degradation by Lac2 direct oxidation was 23%. Toxin degradation was also investigated in the presence of three redox mediators, (2,2′-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid]) (ABTS) and two naturally-occurring phenols, acetosyringone (AS) and syringaldehyde (SA). The direct effect of the enzyme and the mediated action of Lac2 with redox mediators univocally proved the correlation between Lac2 activity and aflatoxins degradation. The degradation of AFB_1 was enhanced by the addition of all mediators at 10 mM, with AS being the most effective (90% of degradation). AFM_1 was completely degraded by Lac2 with all mediators at 10 mM. The novelty of this study relies on the identification of a pure enzyme as capable of degrading AFB_1 and, for the first time, AFM_1, and on the evidence that the mechanism of an effective degradation occurs via the mediation of natural phenolic compounds. These results opened new perspective for Lac2 application in the food and feed supply chains as a biotransforming agent of AFB_1 and AFM_1.

Keywords: laccase; *Pleurotus*; mycotoxins; aflatoxin B_1; aflatoxin M_1; biodegradation; redox mediators

4.1. Introduction

Laccases (LCs, benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multicopper oxidases widely distributed in plants, bacteria, insects, and fungi (Claus et al., 2004). Among fungi, white rot basidiomycetes, such as *Pleurotus* spp. are the most efficient producers of LCs (Osma et al., 2010). Laccases typically contain four cupric ions, classified within three distinct spectroscopic types, T1, T2, and T3 (Leontievky et al., 2007). They are essential for the one-electron oxidation of a reducing substrate and for the reoxidation of the enzyme by means of molecular oxygen, which is in turn reduced to water. Some "white" or "yellow" fungal laccases have been described (Pardo et al., 2015; Palmieri et al., 1997), lacking the T1 cupric ion, which confers the blue color to the enzyme.

Pleurotus pulmonarius Fr. (Quél.), or Indian oyster mushroom, is an edible mushroom known for its medicinal properties and biotechnological potential (Khatun et al., 2015). It produces several LC isoforms, which are encoded by complex multi-gene families. LCs have different substrate specificity, catalytic properties, regulatory mechanisms and localization. Their synthesis and secretion depends upon nutrient levels, culture conditions, developmental stage, and can be increased by the addition of a wide range of inducers to cultural media (Munoz et al., 1997).

LC catalyzes the oxidation of phenols, aromatic amines, and other non-phenolic compounds, while reducing molecular oxygen to water; LC activity can be further extended to non-phenolic substrates by the use of synthetic or natural redox mediators (Zucca et al., 2015). The mediators, after being oxidized by LC, diffuse out of the active site and oxidize recalcitrant compounds which possess high redox potential or high molecular weight. Being structurally diverse, different mediators may act on chemically-unrelated compounds, widening LC substrate range (Baiocco et al., 2003).

Several compounds have been used as redox mediators in the laccase mediator system (LMS). Synthetic mediators such as 2,2-azino-bis-[3-ethylbenzo-thiazolin-

sulfonate] (ABTS), 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), and 1-hydroxybenzotriazole (HBT), have been widely used in many biocatalytic processes (Camarero et al., 2005; Moldes et al., 2008). However, their use is limited due to their high cost, toxicity, and the high mediator-substrate molar ratio needed.

Being a green catalyst with a broad range of substrates, LC has been industrially applied since the early 1990s in chemical synthesis, the food industry, and bioremediation (Pezzella et al., 2005).

In addition, LC and LC-like activities in crude fungal extracts have been positively correlated with mycotoxin degradation (Doyle et al., 1979; Engelhardt et al., 2002), though neither the mechanism of action, nor the degradation products, have been yet fully elucidated.

Mycotoxins are secondary metabolites mainly produced by *Aspergillus*, *Penicillium*, and *Fusarium* spp., which display toxic, carcinogenic, teratogenic, and mutagenic activity towards humans and animals, and contaminate staple food commodities worldwide. Aflatoxins are mycotoxins produced by *Aspergillus* spp., and aflatoxin B_1 (AFB_1) is the most toxic: it has been classified by the International Agency for Research on Cancer (IARC) as Group 1, carcinogenic to humans, and it is known for its teratogenic, hepatotoxic, and immunosuppressive effects on humans and animals (IARC, 2012)

Due to their stability, which confers resistance to physical and chemical treatments of food processing, aflatoxins can persist and are usually found in cereal-based and animal products.

Aflatoxin M_1 (AFM_1) is the animal catabolic product of AFB_1 and contaminates milk and dairy products. AFM_1 is classified in group 2B by the IARC due to its demonstrated hepatotoxic and carcinogenic effect on animals, although its toxicity is one order of magnitude lower than AFB_1 (IARC, 2012).

Due to aflatoxin contamination, every year billions of dollars are lost along the food and feed supply chain worldwide Wu, 2015), constituting a huge economic problem and a public health concern.

In the current study, we purified a LC isoform from *P. pulmonarius*, a well characterized LC producer and a source of LC isozymes with recognized biotechnological potential in the field of bioremediation. We tested the degrading activity of purified LC towards AFB_1 and AFM_1, elucidating the effect of direct and mediated oxidation using a model synthetic mediator, ABTS, and two naturally-occurring phenols, acetosyringone (AS) and syringaldehyde (SA).

4.2. Materials and Methods

4.2.1. Organism, Culture Conditions, LC Induction, and Production

The *Pleurotus pulmonarius* strain ACR-16 of Cattedra di Chimica Biologica Collection, department of Biomedical Sciences, University of Cagliari, syn. *P. sajor-caju*, (Pegler et al., 1975), was maintained as ITEM17144 in the Agri-Food Microbial Fungi Culture Collection of the Institute of Sciences of Food Production (www.ispa.cnr.it/Collection). ITEM17144 was routinely grown on malt extract agar plates (MEA, Oxoid) at 25 °C.

For LC production, ITEM 17144 was grown in liquid medium (2% *w*/*v* malt extract, 0.5% *w*/*v* yeast extract, 10 mM of potassium phosphate buffer pH 6, 0.1 mM of $CuSO_4$) supplemented with 10 mM ferulic acid as a laccase inductor for 24 days, in darkness at 25 °C, in static conditions (relative humidity 70%).

4.2.2. Chemicals and Reagents and Standards Preparation

Chemicals for gel electrophoresis including Bio-safe Coomassie stain and Bradford reagent were supplied by Bio-Rad Laboratories (BioRad, Milan, Italy). Acetonitrile (ACN) (LC–MS grade), formic acid, acetic acid, ammonium bicarbonate, trizma™ base, tween20, hydrochloric acid, trifluoroacetic acid (TFA), iodoacetamide (IAA), dithiothreitol (DTT), 2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS), acetosyringone (AS), syringaldehyde (SA), AFB_1 and AFM_1

standards (purity > 99%) were obtained from Sigma-Aldrich (Milan, Italy). Trypsin (proteomic grade) was purchased from Promega (Milan, Italy). Regenerate cellulose syringe filters, 0.2 μm (size 4 mm) were obtained from Sartorius Italy S.r.l. (Muggiò, Italy)

Mycotoxin stock solution of AFB_1 (10 μg/mL) was prepared by dissolving solid commercial toxins in toluene:ACN (9:1, *v*/*v*) (HPLC grade). The exact concentration of aflatoxin B_1 was determined according to Association of Official Analytical Chemists (AOAC) Official Method 971.22 (AOAC, 2000). Aliquots of the stock solution were transferred to 4 mL amber silanized glass vials and evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved with water:methanol (60:40, *v*/*v*) to obtain calibrant standard solutions at 0.4, 1.2, 2.0, 4.0, 5.0, and 10.0 ng/mL. Standard solutions were stored at −20 °C and warmed to room temperature before use.

Mycotoxin stock solution of AFM_1 (10 μg/mL) was prepared by dissolving solid commercial toxins in ACN (HPLC grade). The exact concentration of standard aflatoxin solution was determined according to AOAC official method 2000.08 (AOAC, 2008). Aliquots of the stock solution were transferred to 4 mL amber glass vials and evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved with water : ACN (75:25, *v*/*v*) to obtain calibrant standard solutions at 1.0, 2.5, 5.0, 7.5, and 10.0 ng/mL. Standard solutions were stored at −20 °C and warmed to room temperature before use.

4.2.3. LC Purification

LC purification was performed according to Zucca et al. (2011) with slight modifications. Briefly, after 24 days of incubation, the culture medium was collected, diafiltered, and concentrated in 50 mM potassium phosphate buffer and 50 mM 6-aminohexanoic acid (protease inhibitor) using a Vivaflow 200 apparatus (Vivascience AG, Hannover, Germany) equipped with a Hydrosart membrane module (nominal MW cut-off 10,000 Da) and a Masterflex L/S system pump (Cole-

Parmer, Vernon Hills, IL, USA) at 4° C. The enzyme solution was added with NaCl to a final concentration of 0.25 M and gently stirred with freshly prepared calcium phosphate gel at 4 °C for 30 min. The slurry was centrifuged at 8000× *g* for 30 min, the supernatant recovered and diafiltered as previously described.

The resulting solution was adjusted to 0.2 M NaCl and loaded onto a DEAE-cellulose column (15 cm × 5 cm), which was pre-equilibrated with 50 mM potassium phosphate buffer pH 6 and 0.2 M NaCl. Bounded brown pigments were separated from unbound LC, which was eluted with the same buffer and desalted by dialysis against 50 mM potassium phosphate buffer prior to a second ion exchange chromatography.

Desalted LC fractions were loaded onto a Hiprep 16/10 DEAE FF assembled on an Akta Prime FPLC (Amersham Bioscience, Milan, Italy) equipped with a UV detector for protein absorbance monitoring at 280 nm. Column equilibration was performed with 50 mM potassium phosphate buffer pH 6 at a constant flow of 5 mL/min. Unbound proteins were washed out while LC was eluted with a linear gradient of 50 mM potassium phosphate buffer pH 6 containing 0.5 M NaCl in 40 min.

LC-rich fractions were pooled, concentrated, dialyzed, separated by size exclusion chromatography with a HiLoad 16/60 Superdex 75 column (GE Healthcare, Milan, Italy) assembled on the Akta Prime FPLC; equilibration and run were performed with 50 mM potassium phosphate buffer pH 6 at constant flow of 0.4 mL/min. Proteins were eluted and stored at −20 °C until use.

Protein content was determined using the Coomassie Brilliant Blue G250 method (Bradford, 1976), the standard curve was performed using bovine serum albumin (BSA, 1–0.025 mg·mL^{-1}).

4.2.4. LC Spectrophotometric Activity Assay

Laccase activity was photometrically measured (Ultraspec 3100pro, Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy). The reaction was performed in

100 mM sodium acetate pH 4.5, 2 mM ABTS and an appropriate amount of enzyme solution in a final volume of 1 mL. The oxidation of ABTS was determined after 10 minutes by photometric assay at 420 nm (ε_{420} = 36,000 $M^{-1} \cdot cm^{-1}$). One unit was defined as the amount of enzyme which oxidized 1 μmol of substrate per minute (Bleve et al., 2008).

4.2.5. Zymography

The activities of the crude extract, both in presence (Induced, I) and absence (Not Induced, NI) of ferulic acid, as well as DEAE cellulose fractions were detected by zymograms, as previously reported (Téllez-Téllez et al., 2005).

Amounts of 5 μg (on average) both for I sample and the DEAE cellulose fraction, and 2 μg of NI samples, were dissolved in denaturant, non-reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, and 0.01% Bromophenol Blue); all samples were loaded on two sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE, 12% T, 3% C), performed according to Laemmli (1970). The unstained molecular weight marker M12 (2.5–200 KDa, Life technology, Waltham, MA USA) was used as the reference. Electrophoretic separation was performed in a Miniprotean System (Biorad, Segrate, Italy) filled with running buffer composed of 25 mM Tris and 0.19 M glycine at 100V for 15 min and 150 V for 1 h.

After the run, the gel was divided into two segments: one segment was washed with distilled water (four washes of 15 min and one of 30 min) at room temperature in order to remove SDS and then incubated with a solution of 5 mM ABTS in 50 mM potassium phosphate buffer pH 6; LC activity was revealed within six minutes. The remaining segment was fixed with 40% ethanol, 10% acetic acid 50% H_2O for 30 min and then stained with Bio-safe Coomassie stain (Bio-Rad), following the manufacturer's instructions.

Destained gels were digitally acquired by an Image Scanner III (GE Healthcare, Pittsburgh,

PA, USA). The experiment was performed in two replicates. The putative laccase band corresponding to the induced isoform, detected in the electrophoretic pattern of DEAE sample stained with Coomassie, was excised and analysed by mass spectrometry.

4.2.6. LC-MS/MS Analysis

The excised gel band shown in Fig. 4-1 was cut into small pieces and placed in 1.5 mL vials for in-gel digestion, with trypsin chosen as the proteolytic enzyme. Protein digestion was accomplished according to the manufacturer's instructions with slight modifications. Firstly, gel slices were destained by adding 200 μL of 100 mM NH_4HCO in 50% ACN and kept at 37 °C for 45 min; this step was repeated until electrophoresis dye was removed. Gel slices were then dehydrated in 100 μL of ACN and dried in a Speed Vac. Then, 130 μL of 10 mM DTT solution (prepared in 25 mM NH_4HCO_3) were added to the batch and incubated in shaking conditions for 1 h at 57 °C. Thirty microliters of 55 mM IAA solution (prepared in 25 mM NH_4HCO_3) were added and the mixture was incubated in darkness for 30 min at room temperature. After incubation, 0.3 μg of trypsin were added to the batch and incubated under shaking conditions overnight at 37 °C. The sample was then incubated with 150 μL of MilliQ water for 10 minutes, with frequent vortex mixing. Liquid was then removed and saved in a new microcentrifuge tube (Sigma, Milan, Italy). The extraction of gel slices digest was performed by adding 50 μL of 50% can, 5% TFA, 45% H_2O solution. The extract was incubated under shaking conditions at room temperature for 1 h and centrifuged to recover the supernatant fraction. The procedure was repeated twice. Both supernatants were collected, mixed, and added with the aliquot obtained by sample incubation with water, and evaporated in the speed-vac in order to concentrate the sample. The final pellet was re-suspended in 50 μL of H_2O:ACN (90:10 + 0.1% of formic acid) and filtered through regenerate cellulose filters (0.22 μm) before injection into the HPLC-MS system.

For the HPLC-MS/MS analysis a system consisting of UHPLC pump coupled through an ESI interface with a dual pressure linear ion trap mass spectrometer VelosPro™ (Thermo Scientific, San José, CA, USA) was used. Peptide separation was performed on an Aeris peptide 3.6 μm XB-C-18 analytical column (150 × 2.10 mm, 3.6 μm, 100 Å, Phenomenex (Torrance, CA, USA); the injection volume was 20 μL. The following linear elution gradient was used for the analytical separation: solvent B was varied from 5%–60% in 55 min; then was increased up to 90% in 1 min and this ratio was maintained constant for the following 15 min. The precent of B was suddenly decreased at 5% and kept stable for 15 minutes for column reconditioning. The two reserves used were: A = H_2O + 0.1% formic acid and B = ACN + 0.1% formic acid; flow rate was set at 200 μL/min.

MS system was operated in Data Dependent™) Acquisition mode (DDA) by selecting the option *N*th *order double play* mode. In particular, two events were set for this experiment: (1) full MS in the range 400–2000 *m*/*z*, four microscans; and (2) full MS/MS DDA of the 20 most abundant ions in the MS spectrum using a normalized collision energy at 35%. Dependent settings for the DDA were set as reported Monaci et al., 2014).

4.2.7. Bioinformatic Analysis

Data obtained from LC-MS/MS acquisitions were searched against a customized database (DB = approximately 12,900 entries) containing amino acid sequences referred to all Pleurotus spp., downloaded from the largest UniProt DB available online (Uniprot database, accessed on 13 June 2016) Protein identification was performed by the commercial software Proteome Discoverer™ based on Sequest™ (version 1.4, 2012, Thermo-Fisher-Scientific, San José, CA, US) () scoring algorithm. Software results were filtered post-acquisition by peptide mass deviation (300 ppm), by setting n = 3 as minimum number of peptides for protein identification and peptide confidence medium (meaning better than 5% of confidence level).

4.2.8. In Vitro Degradation of AFB_1 and AFM_1 with LC and Redox Mediators

Degradation assays were performed in 500 μL of reaction volume of 1 mM sodium acetate buffer pH 5 with 1 μg/mL of AFB_1. 2.5 units of LC were added to each reaction. Alternatively, ABTS, AS, or SA were independently tested as redox mediators at 1 mM and 10 mM.

With respect to AFM_1, degradation assays were performed by incubating 0.05 μg/mL of AFM_1, 2.5 units of LC and ABTS, AS, or SA as redox mediators at 10 mM. In control samples, the enzymatic solution was replaced by an equal volume of buffer. Reactions were incubated at 25 °C for three days in the dark. Each experiment was performed in triplicate.

4.2.9. Chemical Analyses

AFB_1 analyses were performed with a HPLC Agilent 1260 Series (Agilent Technology, Santa Clara, CA, USA) with post column photochemical derivatization (UVE™, LCTech GmbH, Dorfen, Germany). The analytical column was a Luna PFP (150 × 4.6 mm, 3 μm) (Phenomenex, Torrance, CA, USA) preceded by a SecurityGuard™ (PFP, 4 × 3.0 mm, Phenomenex).

Samples containing AFB_1 were filtered using RC 0.20 μm filters (Grace) and 100 μL of volume was injected into the HPLC apparatus with a full loop injection system. The fluorometric detector was set at wavelengths of 365 nm (excitation) and 435 nm (emission). The mobile phase consisted of a mixture of H_2O:ACN (70:30, *v*/*v*) and the flow rate was 1.0 mL/min. The temperature of the column was maintained at 40 °C. AFB_1 was quantified by measuring peak areas at the retention time of aflatoxin standard solutions (Sigma-Aldrich, Milan, Italy) and comparing these areas with the relevant calibration curve at 0.4–10.0 ng/mL. With this mobile phase, the retention time was about 14.5 min. The limit of quantification (LOQ) was 0.4 ng/mL, while the LOD of the method was 0.2 ng/mL based on a signal to noise ratio of 3:1.

AFM_1 analyses were performed with a HPLC Agilent 1260 Series with a fluorometric detector (Santa Clara, CA, USA). The column used was a Zorbax SB-C18 (150 × 4.6 mm i.d., 5 μm Agilent, (Santa Clara, CA, USA) with a security guard (4 × 3.0 mm).

AFM_1 levels in samples were determined by HPLC/FLD method. The solutions were filtered using RC 0.20 μm filters (Grace, Taipei, Taiwan); 50 μL were injected into the HPLC apparatus with a full loop injection system. The fluorometric detector was set at wavelengths of 365 nm (excitation) and 450 nm (emission). The mobile phase consisted of a mixture of H_2O:ACN (75:25, *v*/*v*) and the flow rate was 1 mL/min. The temperature of the column was maintained at 30 °C. AFM_1 was quantified by measuring peak areas at the retention time of aflatoxin standard solutions and comparing these areas with the relevant calibration curve at 1.0–10.0 ng/mL. With this mobile phase, the retention time of AFM_1 was about 6 min. The LOQ was 1.0 ng/mL, while the limit of detection (LOD) of the method was 0.12 ng/mL, based on a signal to noise ratio of 3:1.

When needed, controls and samples were diluted to fit the calibration ranges of the corresponding HPLC methods. Degradation percentages were calculated as follows:

$$\%\ aflatoxin\ degradation = \frac{aflatoxin_{sample}}{aflatoxin_{control}} \times 100$$

4.3. Results

4.3.1. LC Production and Purification

After 24 days of static incubation, the total activity (enzymatic activity, EU) of the crude extract (4 L) was 17,120 EU with specific activity of 6 U/mg. The purification steps for LC are detailed in Table 4-1. The apparent increase in total activity after Ca-phosphate gel might be due either to inhibition effect or interference with ABTS analysis by dark brown pigments (probably arising from

oxidation/polymerization of ferulic acid; these pigments are nearly totally removed during this first purification step).

Table 4-1. Summary of Lac2 purification from *P. pulmonarius* culture filtrate.

Purification Step	Total Volume (mL)	Total Activity (EU)	Total Protein (mg)	Specific Activity (u/mg)	Purification Fold
Crude extract	4000	17,120	2800.00	6	1
Ca- phosphate gel	500	33,400	95.00	351	59
DEAE cellulose	50	23,785	11.50	2068	344
DEAE FF	10	3837	1.20	3224	538
Superdex	14	2053	0.19	10,920	1820

As preliminary steps, the batch treatment with calcium phosphate gel and the first anion exchange chromatography on diethylamino ethyl (DEAE) cellulose were performed to remove the majority of the brown pigments and contaminating proteins and resulted in the greatest increase in specific activity, from 6 to 2.068 U/mg. In the last two chromatographic steps LC activity was further purified from remaining impurities and from the other contaminant proteins. This strategy resulted in 1820-fold purification with 12% final yield of laccase enzyme with respect to the crude extract.

4.3.2 Zymography

Fig. 4-1, panel A shows the zymogram of extracellular LC activity by using ABTS as the substrate; protein bands exhibiting activity in zymogram were also compared with the related electrophoretic pattern stained with Coomassie stain (Fig. 4-1, panel B and C).

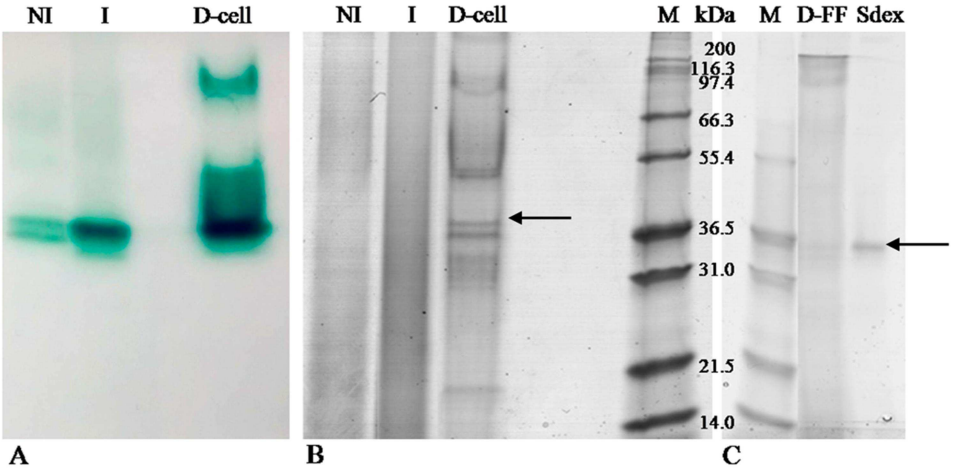


Figure 4-1. Zymography (**A**) using ABTS as the substrate and SDS PAGE (**B** and **C**) of *P. pulmonarius* LC preparations. NI-not induced; I-induced; D cell-sample after DEAE cellulose, D-FF-sample after DEAE FF, Sdex-sample after Superdex, M-Marker. The arrows indicate Lac2 bands.

In particular, the not induced (NI) sample putatively produced two laccase isoforms corresponding to a molecular weight ranging from 31 to 36.5 kDa in the electrophoretic profile stained with Coomassie (Fig. 4-1, panel B). The induced (I) sample showed an analogous zymogram pattern with the exception of the band showing a more intense activity than the NI sample due to LC induction by ferulic acid. However, the zymogram was more sensitive than Coomassie staining, as previously reported (Dong et al., 2005). LC activity with ABTS was still clearly detectable when as low as 0.3 μg of proteins were loaded on SDS-PAGE (data not shown). After the final purification step with Superdex, Lac2 appeared as one single band in SDS PAGE (Fig. 4-1, panel C).

4.3.4. Laccase Identification by MS/MS

Table 4-2 summarizes the identification of the band digested from the polyacrylamide gel with the highest score. After protein digestion, the resulting peptide mixture was analyzed in data-dependent MS/MS acquisition mode. The acquired MS data were processed by Proteome Discoverer software (version 1.4, 2012, Thermo Scientific, San José, CA, USA) and searched against a customized DB containing all *Pleurotus* spp. protein sequences present in UniProt. As a result, seven unique peptides were detected and assigned to Lac2 and Lac4 of *P. pulmonarius* and *P. sajor-caju*, respectively. In Table 4-2 the peptide sequences identified by MS and matching with part of Lac2 and Lac4 sequences in the Uniprot DB are reported. Considering that *P. pulmonarius* and *P. sajor-caju* are synonymous Pegler et al., 1975), and that the strain used in this study belonged to *P. pulmonarius* species, we identified our protein as Lac2 of *P. pulmonarius*.

Table 4-2. Overview of protein assignments referred to the analysis of the excised band obtained by the Sequest scoring algorithm interrogating a customized *Pleurotus* database imported by UniProt.

Assigned Protein	Accession Number	Protein Coverage	No. of Identified Peptides	Sequences	Confidence Level	*m/z* (Da)
Laccase 2 *Pleurotus pulmonarius*	Q2VT18	21.24%	7	YSFVLTADQTPDNYWIR	High	1045.07686
				YAGGPTSPLAVINVESTKR	High	980.65433
				SAGSTTYNFDTPAR	High	744.42450
				GDNFQLNVVNQLSDTTMLK	High	1069.20405
				SAGSTTYNFDTPARR	High	822.55662
Laccase 4 *Pleurotus sajor-caju*	Q7Z8S3			ANPNLGSTGFAGGINSAILR	High	644.12372
				SVPITGPTPATASIPGVLVQGNK	High	735.54871
				GDNFQLNVVNQLSDTTMLK	High	718.38048

4.3.5. In Vitro Degradation of AFB1 and AFM1 With Laccase and Redox Mediators

Degradation results are shown in Fig. 4-2, while examples of HPLC chromatograms of AFB_1 and AFM_1 degradation are shown in Fig. 4-3. Direct oxidation of AFB_1 by means of Lac2 alone accounted for 23% degradation. The addition of a redox mediator resulted in a very effective degradation of the toxin. The lowest concentrations of ABTS and AS (1 mM) were able to double the degradation percentage compared to Lac2 alone (45% and 42%, respectively) while, in the case of SA, the presence of mediator at 1 mM lowered the degradation percentage (13%). Absolute values of aflatoxin concentrations are shown in Table 4-3.

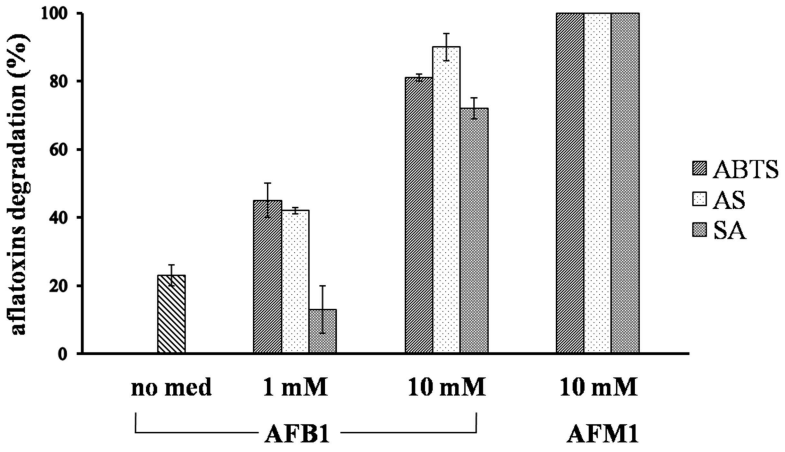


Figure 4-2. AFB_1 and AFM_1 degradation (%) after three days of incubation at 25 °C, performed by Lac2 and the respective redox mediator in buffered solution (1 mM sodium acetate pH5). ABTS-[2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)]; AS-acetosyringone, SA-syringaldehyde. Values are the mean of three replicates and the error bars represent the standard error measured between independent replicates.

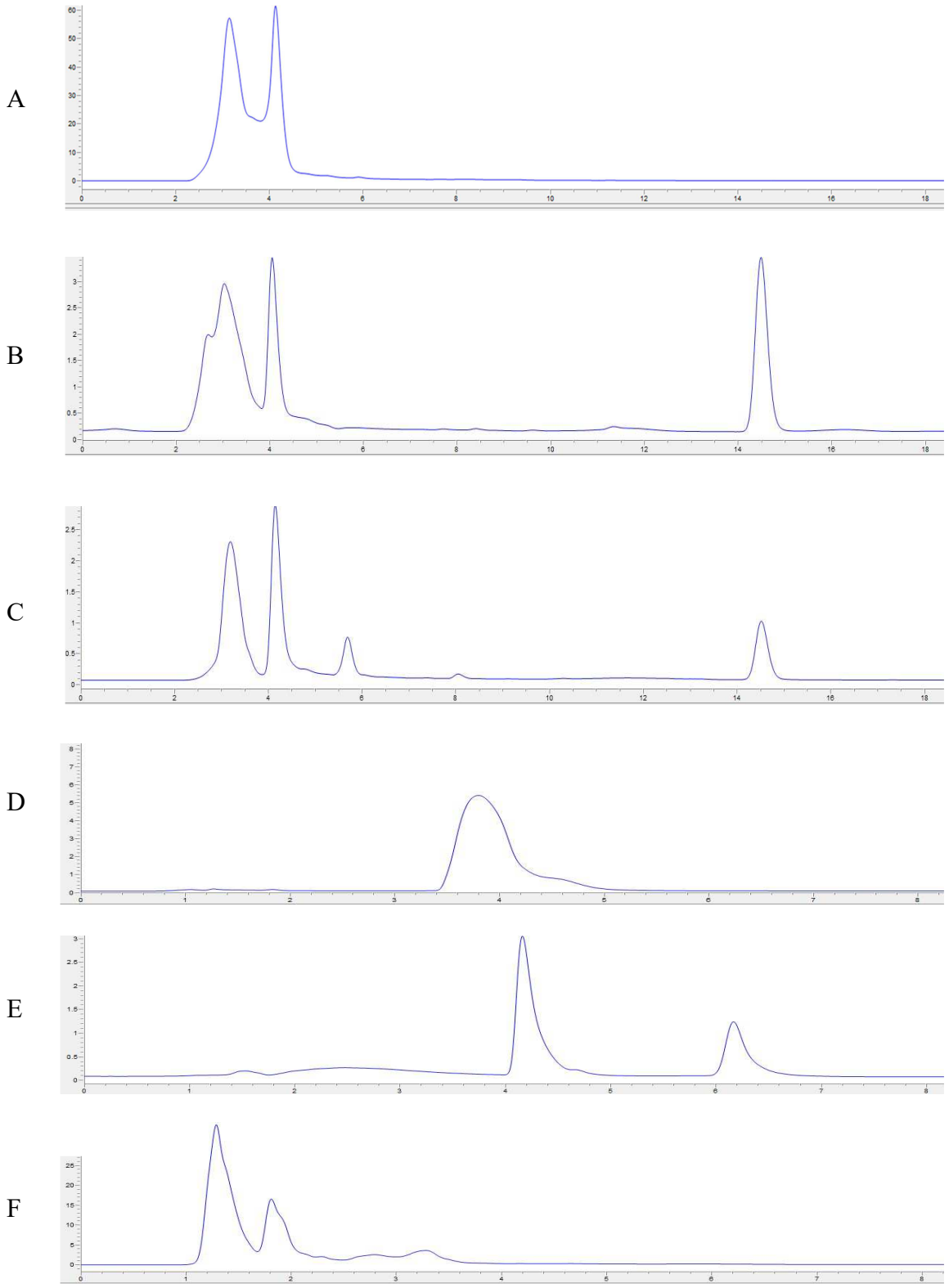


Figure 4-3. Examples of HPLC chromatograms of AFB_1 (**A**—negative control, **B**—positive control, **C**—sample after degradation by Lac2) and AFM_1 (**D**—negative control, **E**—positive control, **F**—sample after degradation by Lac2).

Table 4-3. Absolute values of aflatoxin concentrations (ng/mL) after LC treatment and statistical analysis of aflatoxin degradation by Lac2 and redox mediators.

Sample	AFB_1		AFM_1
	med 1 mM	med 10 mM	med 10 mM
Positive control	923 ± 33 *	923 ± 33 *	53 ± 7 *
No med	710 ± 27 *	710 ± 27 *	n.t.
ABTS	508 ± 46 *	175 ± 5 *	0 ± 0 *
AS	535 ± 9 *	92 ± 27 *	0 ± 0 *
SA	803 ± 120	258 ± 16 *	0 ± 0 *

n.t. = not tested; Comparisons between controls and treated samples were performed using a t-test. A p value < 0.001 was considered statistically significant (*).

At 10 mM each mediator further enhanced AFB_1 degradation which reached 90% for AS, 81% for ABTS and 72% for SA. With regards to AFM_1, Lac2 proved to completely degrade the toxin with all mediators added at 10 mM since after the reaction no AFM_1 peak was detected by HPLC analysis.

4.4. Discussion

In this work, we purified and identified a Lac2 isoform from a strain of *P. pulmonarius* and evaluated the ability of the pure enzyme to degrade AFB_1 and AFM_1 either by direct or mediated oxidation with three different redox mediators, ABTS, AS, and SA.

Mycotoxin degradation by fungi and bacteria is a widely investigated topic, especially in the last 10–15 years (Wu et al., 2009; Zhao et al., 2011), but a deep understanding of which enzyme is directly responsible for mycotoxin degradation and the mechanism involved, is still lacking. Many bioremediation applications exploit the fungus as a whole-cell biocatalyst, or its secretome, thus involving the concerted activity of several enzymatic systems, including laccases, extracellular peroxidases (Lignin peroxidase-LiP, EC 1.11.1.14, manganese peroxidase-MnP, EC 1.11.1.13, and versatile peroxidase-VP, EC 1.11.1.16) and oxidases that generate the

H_2O_2 needed for peroxidase activity (tyrosinase-EC 1.14.18.1 and aryl-alcohol oxidase-EC 1.1.3.7). Furthermore, low molecular weight compounds that act as mediators might be present in the culture media. This limits the discrimination between the direct action of the enzyme and the mediated one, which is a crucial point to develop industrial or biotechnological applications. Even commercial fungal preparations may contain contaminant proteins: Margot and colleagues (Margot et al., 2013) recently verified that the most used commercial laccase from Sigma (Milan, Italy, Ref. 38429) actually contains a mixture of different proteins, from 17 to ~80 kDa, and different LC isoforms.

Although aflatoxin degradation by fungal laccase enzymes has already been reported (Alberts et al., 2009; Scarpari et al., 2014; Zeinvand-Lorestani et al., 2015), to date no direct and unambiguous correlation between laccase and aflatoxin degradation has been described, since cultured filtrates or LC commercial preparation were tested in the degradation assays.

In our study the LC isozyme responsible for aflatoxins degradation was identified as Lac2. This isozyme has been extensively biochemically characterized in a previous work by Zucca and colleagues (2011). They reported a copper content of 3.8 cupric ions per protein molecule and a sugar content of 6.7% ± 0.3% (expressed as glucose equivalents), measured the enzyme activity at different pH values, and its stability at different temperatures.

Lac2 production was induced by low molecular weight compounds; among various putative inducers, ferulic acid proved to be by far the most effective (Zucca et al., 2011); according to this, the induced sample in zymography showed one band with a much more intense activity than the related NI sample. In order to remove contaminants and to purify laccase, a preparative chromatography was performed. As expected, this procedure increased the activity of bands detected in the NI and I samples (Fig. 4-1, panel A) and resulted in a laccase band with an apparent molecular weight close to 35 kDa, as determined by the comparison with the lanes stained with Coomassie (Fig. 4-1, panel B and C). Similar results were

described by Diaz (2013), who detected four laccase isoenzymes with molecular weights of 65, 47, 38, and 29 kDa.

The predicted molecular weight of Lac2 is 56.6 kDa, in agreement with the previous estimation by SDS PAGE and RP-HPLC–electrospray ionization-MS which assigned Lac2 a molecular weight of 55–61 kDa (Zucca et al., 2011). However, in this study Lac2 apparent molecular weight, estimated by SDS PAGE, was approximately 35 kDa; a possible explanation of this divergence is that under non-reducing conditions LC shows increased mobility and a lower apparent molecular weight due to the extensive glycosylation, as measured for this LC isozyme by Zucca et al. (2011). Moreover, glycosylation has been reported to be responsible for unconventional electrophoretic behavior under non-reducing or native conditions (Perry et al., 1993) and to influence SDS/protein interaction; it may facilitate LC migration through the gel net, making the LC external structure more flexible and elastic.

The limited reactiveness of Lac2 alone towards AFB_1 might be explained by the high electrochemical oxidation potential, high ionization potential, or steric hindrance that prevents the substrate from being oxidized or enter the active site of LC (Tadesse et al., 2008). Those limitations can be overcome by the use of redox mediators, which are effective LC substrates and, in turn, oxidize recalcitrant compounds.

Effective degradation of aflatoxins proceeds via LMS, both with ABTS and natural mediators, despite their proposed different mode of action. ABTS mediation is reported to occur via an electron transfer (ET) route, while phenoxy radicals mediate via hydrogen atom abstraction (HAT), at least when working at acidic or neutral pH values; only under alkaline conditions, where the mediators are in their anionic form, the HAT mechanism turns into an ET one (Zucca et al., 2015: Baiocco et al., 2003).

ABTS was the first used artificial mediator (Buorbonnais et al., 1990). It is soluble in water and, upon one-electron oxidation, produces a stable radical cation with a high absorbance at 420 nm. Thanks to these features ABTS has been successively used as an oxidation mediator towards polycyclic aromatic hydrocarbons, in organic synthesis, and in the treatment of textile wastewater (Solis-Olba et al., 2008; Wells et al., 2006; Collins et al., 2006). ABTS is a model compound for LMS and an efficient mediator for AFB_1 and AFM_1 (81% and 100% degradation, respectively). However, it is a synthetic mediator and, as such, its high cost and concerns related to its potential toxicity have restricted industrial implementation of this LMS even in non-food applications, and raised the need for safe, cost-effective, and readily available mediators.

With this aim, we investigated the role of two naturally-occurring compounds, AS and SA, which are both 2,6-dimethoxy-substituted phenols derived from syringyl lignin units. They were described as the fastest and most efficient laccase mediators for the degradation of industrial dyes, sulfonamide antibiotics, and for the removal of lignin from paper pulps (Shi et al., 2014; Dubé et al., 2008; Camarero et al., 2007). In addition to being natural compounds, they are inexpensive, safe, and can be as effective as the synthetic ones.

Both AS and SA were demonstrated to be effective mediators for AFB_1 degradation at 10 mM, although SA was less efficient than AS and ineffective at 1 mM. Most probably, the SA-derived radical undergoes an internal redox reaction leading to syringic acid and, therefore, wasting a significant fraction of the reactive radical. Such a decrease of the reactive radical did not occur in the case of AS, explaining the noticeable effectiveness of the compound.

Methoxy substitutions in syringyl-type compounds decrease the redox potential and increase electron density at the phenoxy group. Those compounds are readily oxidized by LC and generate relatively stable radicals since the substitutions in the phenol ring impose steric hindrances for the polymerization via radical coupling (Caompos et al., 2001; Baiocco et al., 2003). The substituent in

the para position also influences the phenoxy radical stability, since electron donor groups at the para-position stabilize the phenoxy radicals, while electron-withdrawing substituents lead to a decreased radical stability (Rosado et al., 2012) Accordingly, AS harbors the weakest electron acceptor group and generates a more stable radical than SA.

With respect to AFM_1, Lac2 was able to degrade it completely with all mediators tested at 10 mM with no differences emerging among ABTS, AS, and SA. The decontamination of AFM_1 in buffered solution, in model and real food matrices, was previously investigated using lactic acid bacteria (Elsanhoty et al., 2014). Nevertheless, the decontamination was a result of a reversible binding to the carbohydrates and peptidoglycan of the bacterial cell wall surface and not a biological degradation. To our knowledge, the present study reports for the first time the effective degradation of AFB_1 and AFM_1 by means of Lac2 from *P. pulmonarius*.

The degradation products of aflatoxins have not been identified yet. Depending on the degrading agent, aflatoxins can be degraded by several mechanisms, such as epoxidation, hydroxylation, dehydrogenation, and reduction. A wide range of putative potential reaction products obtained accounts for the difficulties in the development of sensitive identification methods, as well as for the limited data on their toxicological characterization. Considering the degradation products deriving from laccase treatment, only a study conducted by Alberts and colleagues (2009) reported a reduced mutagenicity (using Ames test) of the degradation products of AFB_1.

According to our results we hypothesize that LMS acts on the lactone ring, which is responsible for fluorescence properties: cleavage of the lactone ring results in a non-fluorescent compound that has greatly reduced biological activity (Vazquez et al., 2010; Lee et al., 2010). However, the ring cleavage of lactones is a hydrolysis, rather than an oxidation. Therefore, as laccases cannot catalyze lactone hydrolysis, fluorescence quenching should be the consequence of a deeper

modification of the coumarin-like core of the toxins, which is responsible for their fluorescence. In fact, oxidative demethoxylations of simple aromatics by means of fungal laccases are well known (Kersten et al., 1990; Ander et al., 1983); methanol is released and the aromatic ring is changed into its quinonoid counterpart, which rearranges with the result of an irreversible and deep degradation. Hypothesizing that this mechanism is also valid for substituted aromatics, like aflatoxins, the reversibility of the reaction is unlikely.

This forecast is confirmed by our observations reported here. Additionally, as expected, the use of redox mediators strongly enhances the degrading ability of Lac2.

Laccase has been increasingly applied in food industry in the last 30 years. The demonstrated biodegrading activity towards mycotoxins, the green catalysis, and the use of natural mediators support a potential and feasible application in food and feed. A mandatory requisite for feed application is that products of mycotoxin degradation have to be stable and non-toxic. The development of these applications has to overcome the cost of production of the enzyme, the optimization of the degradation reaction, as well as the gap of knowledge related to the degradation products and their toxicity as required by the EU commission (Commission regulation EU 2015/786)

Pleurotus spp. ligninolytic system is a source of biotechnologically important enzymes which play an essential role in green bioremediation; so far, *P. pulmonarius* LCs have been extensively used in the removal of industrial dyes and the treatment of lignocellulosic waste.

This is the first time that *P. pulmonarius* Lac2 was unambiguously identified as capable of degrading AFB_1 and AFM_1 in the presence of natural redox mediators. Although further studies are needed to optimize the degradation assay, this study clearly illustrates the potentiality of Lac2 for its use as a biotransformation agent.

Acknowledgments: This work was financially supported by H2020-E.U.3.2-678781-MycoKey-Integrated and innovative key actions for mycotoxin management in the food and feed chain.

References

Adebo, O., Njobeh, P.B., Gbashi, S., Nwinyi, O.C., Mavumengwana, V. (2015). Review on Microbial Degradation of Aflatoxins. *Critical Reviews in Food Science and Technology*.

International Agency for the Research on Cancer. (2012). Aflatoxins. In IARC Monographs. Lyon, France, Volume 100F.

Alberts, J.F., Gelderblom, W.C.A., Botha A., van Zyl, W.H. (2009). Degradation of aflatoxin B1 by fungal laccase enzymes. *International Journal of Food Microbiology*. 135, 47–52.

Ander, P., Eriksson, K.E., Yu, H. (1983). Vanillic acid metabolism by Sporotrichum pulverulentum: Evidence for demethoxylation before ring-cleavage. *Archives of Clinical Microbiology*. 136, 1–6.

Association of Official Analytical Chemists (AOAC) Official Method 971.22-1988 (2000). Available online: http://www.aoacofficialmethod.org/index.php?main_page=product_info&cPath=1&products_id=625 (accessed on 16 August 2016).

Association of Official Analytical Chemists (AOAC) Official Method 2008.08-2008. Available online: http://www.aoacofficialmethod.org/index.php?main_page=product_info&cPath=1&products_id=2816. (accessed on 16 August 2016).

Baiocco, P., Barreca, A. M., Fabbrini, M., Galli, C., & Gentili, P. (2003). Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase–mediator systems. *Organic & biomolecular chemistry*, 1, 191-197.

Banu, I., Lupu, A., & Aprodu, I. (2013). Degradation of Zearalenone by Laccase enzyme. Scientific Study & Research. Chemistry & Chemical Engineering, Biotechnology, *Food Industry*, 14, 79.

Bleve, G., Mita, G., Rampino, P., Perrotta, C., Villanova, L., Grieco, F. (2008). Molecular cloning and heterologous expression of a laccase gene from *Pleurotus eryngii* in free and immobilized *Saccharomyces cerevisiae* cells. *Applied Microbiology and Biotechnology*. 79, 731–741.

Bourbonnais, R., Paice, M.G. (1990). Oxidation of non-phenolic substrates: An expanded role for lactase in lignin biodegradation. *FEBS Letters*. 267, 99–102.

Bradford, M.M. (1976). A Rapid and Sensitive Method for the Quantitaton of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*. 72, 248–254.

Camarero, S.; Ibarra, D.; Martınez, A.T.; Romero, J.; Gutierrez, A.; del Rio, J.C. Paper pulp delignification using laccase and natural mediators. Enzym. Microb. Technol. 2007, 40, 1264–1271.

Camarero, S., Ibarra, D., Martínez, M.J., Martínez, Á.T. (2005). Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Applied and environmental microbiology* 71, 1775-1784.

Campos, R., Kandelbauer, A., Robra, K.H., Cavaco-Paulo, A.; Guébitz, G.M. (2001). Indigo degradation with purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii*. *Journal of Biotechnology*. 89, 131–139.

Cañas, A., Camarero, S. (2010). Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. *Biotechnology Advances*. 28, 694–705.

Claus, H. (2004). Laccases: Structure, reactions, distribution. *Micron* 35, 93–96.

Collins, P.J., Kotterman, M., Field, J.A., Dobson, A. (1996). Oxidation of Anthracene and Benzo[a]pyrene by Laccases from *Trametes versicolor*. *Applied and environmental microbiology*. 62, 4563–4567.

Commission regulation (EU) 2015/786 of 19 May 2015 defining acceptability criteria for detoxification processes applied to products intended for animal feed as provided for in Directive 2002/32/EC of the European Parliament and of the Council. Off. J. Eur. Union 2015, 58, 10–14.

Díaz, R., Téllez-Téllez, M., Sánchez, C., Bibbins-Martínez, M.D., Díaz-Godínez, G., Soriano-Santos, J. (2013). Influence of initial pH of the growing medium on the activity, production and genes expression profiles of laccase of Pleurotus ostreatus in submerged fermentation. Electron. J. Biotechnol. 16,

Dong, J.L., Zhang, Y.W., Zhang, R.H., Huang, W.Z., Zhang, Y.Z. (2005). Influence of culture conditions on laccase production and isozyme patterns in the white-rot fungus *Trametes gallica*. *Journal of Basic Microbiology*. 45, 190–198.

Doyle, M.P., Marth, E.H. (1979). Peroxidase activity in mycelia of Aspergillus parasiticus that degrade aflatoxin. *European Journal of Applied Microbiology and Biotechnology*. 7, 211–217.

Dubé, E., Shareck, F., Hurtubise, Y., Beauregard, M., Daneault, C. (2008). Decolourization of recalcitrant dyes with a laccase from Streptomyces coelicolor under alkaline conditions. *Journal of Industrial Microbiology and Biotechnology*. 35, 1123–1129.

Elsanhoty, R.M., Salam, S.A., Ramadan, M.F., Badr, F.H. (2014) Detoxification of aflatoxin M_1 in yoghurt using probiotics and lactic acid bacteria. *Food Control*. 43, 129–134.

Engelhardt, G. (2002). Degradation of Ochratoxin A and B by the White Rot Fungus *Pleurotus ostreatus*. *Mycotoxin Research*. 18, 37–43.

ITEM collection. Available online: www.ispa.cnr.it/Collection accessed on 15 June 2016.

Kersten, P., Kalyanaraman, B., Hammel, K.E., Reinhammar, B., Kirk, T.K. (1990). Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. *Biochemistry Journal*. 268, 475–480.

Khatun, S., Islam, S., Cakilcioglu, U., Guler, P., Chatterjee, N.C. (2015). Nutritional qualities and antioxidant activity of three edible oyster mushrooms (Pluerotus spp.). NJAS-Wagening. *Journal of Life Science*. 73, 1–5.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227, 680–685.

Lee, S., Dunn, J.J., DeLucca, A.J., Ciegler, A.(1981). Role of lactone ring of aflatoxin B1 in toxicity and mutagenicity. *Experientia* 37, 16–17.

Leontievky, A., Myasoedova, N., Pozdnyakova, N., Golovleva, L. (2007). "Yellow" laccase of *Panus tigrinus* oxidizes non-phenolic substrates without redox mediators. *FEBS Letters*. 413, 446–448.

Margot, J., Bennati-Granier, C., Maillard, J., Blánquez, P., Barry, D.A., Holliger, C. (2013). Bacterial versus fungal laccase: Potential for micropollutant degradation. *AMB Express* 3, 63–77.

Moldes, D., Díaz, M., Tzanov, T., Vidal, T. (2008). Comparative study of the efficiency of synthetic and natural mediators in laccase-assisted bleaching of eucalyptus kraft pulp. *Bioresource Technology*. 17, 7959–7965.

Monaci, L., Pilolli, R., De Angelis, E., Godula, M., Visconti, A. (2014). Multi-allergen detection in food by micro high performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry. *Journal of Chromatography A*. 1358, 36–144.

Munoz, C., Guillen, F., Martınez, A.T., Martınez, M.J. (1997). Induction and Characterization of Laccase in the Ligninolytic Fungus *Pleurotus eryngii*. *Current Microbiology*. 34, 1–5.

Osma, J.F., Toca-Herrera, J.L., Rodrıguez-Couto, S. (2010). Uses of Laccases in the Food Industry. *Enzymatic Research*.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A., Sannia, G. (1997). A novel white laccase from *Pleurotus ostreatus*. *Journal of Biological Chemistry*. 272, 31301–31307.

Pardo, I., Camarero, S. (2015). Laccase engineering by rational and evolutionary design. *Cellular and Molecular Life Science*. 72, 897–910.

Pegler, D.N. (1975). The classification of the genus *Lentinus* Fr. (Basidiomycota). *Kavaka.* 3, 11–20.

Perry, C.R., Smith, M., Britnell, C.H., Wood, D.H., Thursto, C.F. (1993). Identification of two laccase genes in the cultivated mushroom *Agaricus bisporus*. *Microbiology*, 139, 1209–1218.

Pezzella, C., Guarino, L., Piscitelli, A. (2005). How to enjoy laccases. *Cellular and Molecular Life Science.* 72, 923–940.

Rosado, T., Bernardo, P., Koci, K., Coelho, A.V., Robalo, M.P., Martins, L.O. (2012). Methyl syringate: An efficient phenolic mediator for bacterial and fungal laccases. *Bioresource Technology*. 124, 371–378.

Scarpari, M., Bello, C., Pietricola, C., Zaccaria, M. Bertocchi, L., Angelucci, A., Ricciardi, M.R., Scala, V., Parroni, A., Fabbri, A.A. (2014).. Aflatoxin Control in Maize by *Trametes versicolor*. *Toxins* 6, 3426–3437.

Shi, L., Ma, F., Han, Y., Zhang, X., Yu, H. (2014). Removal of sulfonamide antibiotics by oriented immobilized laccase on Fe3O4 nanoparticles with natural mediators. *Journal of Hazardous Material*. 279, 203–211.

Solis-Oba, M., Almendariz, J., Viniegra-Gonzalez, G. (2008). Biotechnological treatment for colorless denim and textile wastewater. *Revista International de Contamination Ambiental.* 24- 5–11.

Tadesse, M.A., D'Annibale, A., Galli, C., Gentili, P., Sergi, F. (2008). An assessment of the relative contributions of redox and steric issues to laccase specificity towards putative substrates. *Organic & Biomolecular Chemistry*. 6, 868–878.

Téllez-Téllez, M., Sánchez, C., Loera, O., Díaz-Godínez, G. (2005). Differential patterns of constitutive intracellular laccases of the vegetative phase of Pleurotus species. *Biotechnology Letters*. 27, 1391–1394.

Uniprot database. Available online: http://www.uniprot.org. Accessed on 13 June 2016.

Vazquez, I.N., Albores, A.M., Martınez, E.M., Miranda, R., Castro, M. (2010). Role of Lactone Ring in Structural, Electronic, and Reactivity Properties of Aflatoxin B_1: A Theoretical Study. *Archives of Environmental Contamination and Toxicology*. 59, 393–406.

Wells, A., Teria, M., Eve, T. (2006). Green oxidations with laccase-mediator systems. *Biochemical Society Transaction*. 34, 304–308.

Wu, F. (2015). Global impacts of aflatoxin in maize: Trade and human health. *World Mycotoxin Journal*. 8, 137–142.

Wu, Q., Jezkova, A., Yuan, Z., Pavlikova, L., Dohnal, V., Kuca, K. (2009). Biological degradation of aflatoxins. *Drug Metabolism Reviews*. 41, 1–7.

Zeinvand-Lorestani, H., Sabzevari, O., Setayesh, N., Amini, M., Nili-Ahmadabadi, A., Faramarzi, MA. (2015). Comparative study of in vitro prooxidative properties and genotoxicity induced by aflatoxin B_1 and its laccase-mediated detoxification products. *Chemosphere.* 135, 1–6.

Zhao, L.H., Guan, S., Gao, X., Ma, Q.G., Lei, Y., Bai, X.M., Ji, C. (2011). Preparation, purification and characteristics of an aflatoxin degradation enzyme from Myxococcus fulvus ANSM068. *Journal of Applied Microbiology*. 110, 147–155.

Zucca, P., Cocco, G., Sollai, F., Sanjust, E. (2015). Fungal laccases as tools for biodegradation of industrial dyes. *Biocatalysis* 1, 82–108.

Zucca, P., Rescigno, A., Olianas, A., Maccioni, S., Sollai, F., Sanjust, E. (2011). Induction, purification, and characterization of a laccase isozyme from *Pleurotus sajor-caju* and the potential in decolorization of textile dyes. *Journal of Molecular Catalysis B: Enzymes.* 68, 216–222.

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

Loi M[1,2], Fanelli F[1], Cimmarusti MT[1,2], Mirabelli V [2,3], Haidukowski M[1], Logrieco AF[1], Caliandro R[3], Mulé G[1*]

[1]Institute of Sciences of Food Production, National Research Council, via Amendola 122/O, Bari 70126, Italy;
[2]Department of Economics, University of Foggia, via Napoli 25, Foggia 71122, Italy
[3]Institute of Crystallography, National Research Council, via Amendola 122/O, Bari 70126, Italy;

The contents of this chapter were published in: Loi M, Fanelli F, Cimmarusti MT, Mirabelli V, Haidukowski M, Logrieco AF, Caliandro R, Mulé G. *In vitro* single and combined mycotoxins degradation by Ery4 laccase from *Pleurotus eryngii* and redox mediators. Food Control, 2018, 90: 401-406. doi.org/10.1016/j.foodcont.2018.02.032

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 increase the 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.

Keywords: mycotoxins, bioremediation, laccase-mediator system, multi-mycotoxin degradation

5.1. Introduction

Food and feed contamination by mycotoxins is a concerning issue worldwide, due to their adverse health effects and their important economic impact (Rocha et al., 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 et al., 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 et al., 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) 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 (Loi et al., 2017; Loi et al., 2016; Banu et al., 2013).

Additionally, LCs have been already used in food processing to improve the techno-functional properties of bakery, dairy and meat products (Osma et al., 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.

5.2. Materials and methods

5.2.1. Chemicals, reagents and mycotoxins standard preparation

2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS), acetosyringone (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).

5.2.2. 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 50mM, 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).

5.2.3. 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 1m M, 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 72h at 25°C in static conditions.

Subsequently, eight different redox mediators at two different concentrations (1 and 10mM) were independently tested in combination with Ery4 (5 U/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.

5.2.4. 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.

5.2.5. 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 hydrolyzed fumonisin (HFB_1) were prepared from pure FB_1 according to the procedure described by De Girolamo et al., (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 Acquity QDa 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 and colleagues (2014) and De Bellis (2015). LOQ for OTA and OTα were 0.5 ng/mL, based on a signal to noise ratio of 10 :1.

DON levels in samples were filtered using RC 0.20 μm filters (Phenomenex, Torrance, CA, USA) and determined by ultra-high performance liquid chromatography linked with photodiode array detector (UPLC-PDA) (Pascale et al., 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 and colleagues (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 (ε = 12623 L/mol cm at λ = 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 x 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). ZEA 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 ZEN were

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:

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

5.2.6. 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.

5.3. 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 et al., 2003; Fabbrini et al., 2001).

5.3.1. 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 reduction 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 (Yehia et al., 2014; Chitrangada et al., 2000).

In our study AFB_1 degradation was achieved by using different LMSs (Fig 5-1). 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).

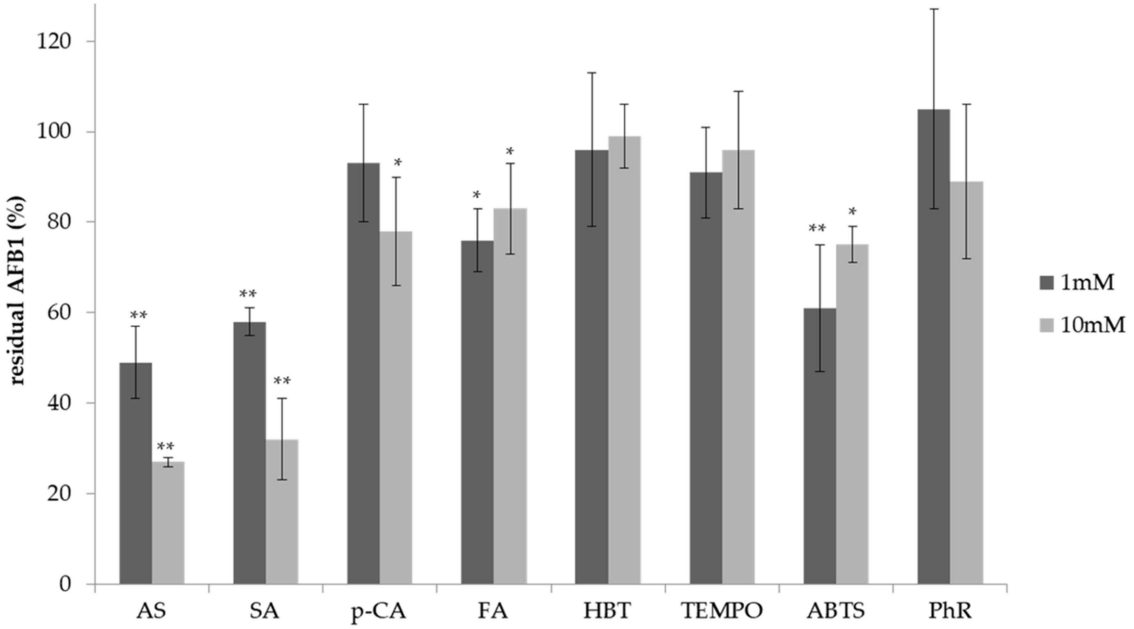


Figure 5-1: Residual aflatoxin B_1 (AFB_1) in samples treated with different laccase mediator systems. AS: acetosyringone, SA: syringaldehyde, p-CA: p-coumaric acid, FA: ferulic acid, HBT: 1-hydroxybenzotriazole, TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), PhR: phenol red. A *P* value <0.05 was considered statistically significant (*) and a P value <0.01 is considered highly statistically significant (**).

These results confirmed the efficacy of these LMSs, as reported by Loi and colleagues (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.

5.3.2. Fumonisin B_1 degradation by Ery4 and LMS

FB_1 was not degraded by direct Ery4 oxidation (data not shown). However, a statistically significant degradation ($P< 0.01$) was achieved using TEMPO 10 mM (74%), PhR 10 mM (30%) and SA 1 mM (25%) (Fig. 5-2). All other mediators and concentrations were ineffective or not statistically significant.

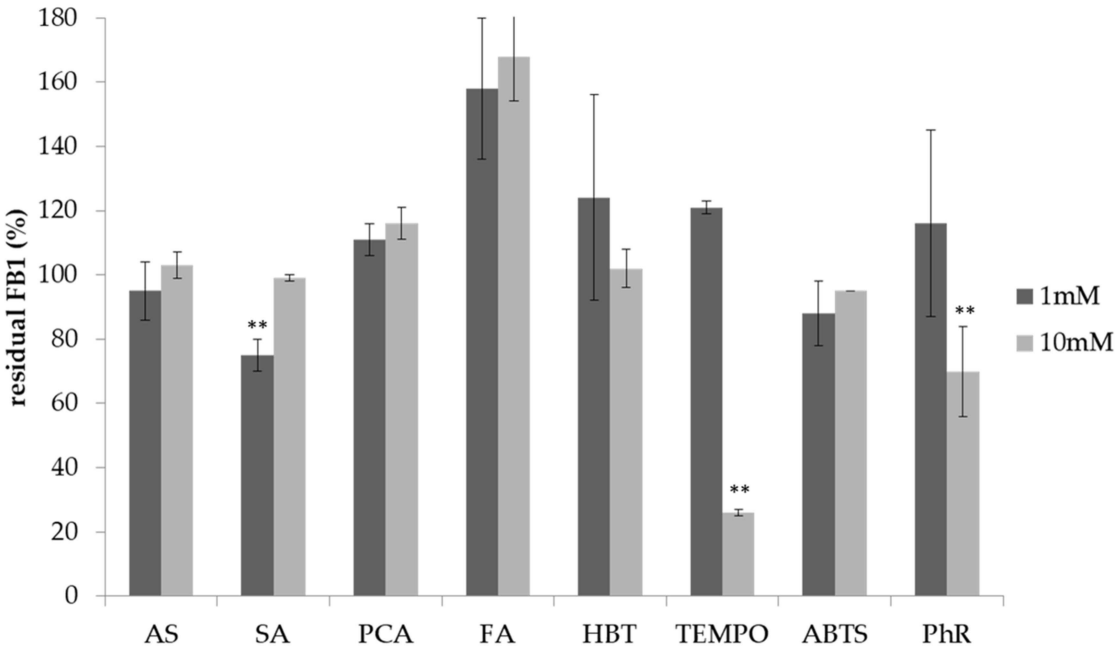


Figure 5-2: Residual fumonisin B_1 (FB_1) in samples treated with different laccase mediator systems. AS: acetosyringone, SA: syringaldehyde, p-CA: p-coumaric acid, FA: ferulic acid, HBT: 1-hydroxybenzotriazole, TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl) oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), PhR: phenol red. A P value <0.01 is considered highly statistically significant (**).

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 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 method was reported for FB_1 degradation. No hydrolysed products were detected in the samples.

5.3.3. 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. 5-3). 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).

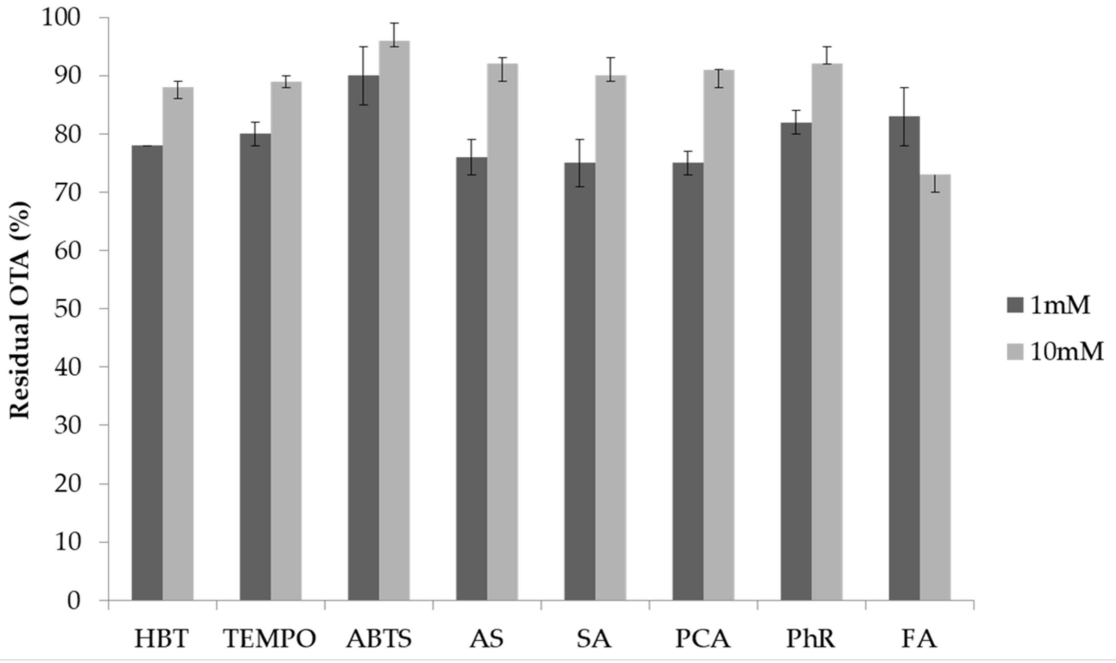


Figure 5-3: Residual ochratoxin A (OTA) in samples treated with different laccase mediator systems. AS: acetosyringone, SA: syringaldehyde, p-CA: p-coumaric acid, FA: ferulic acid, HBT: 1-hydroxybenzotriazole, TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), PhR: phenol red. All data are statistically significant ($P< 0.01$).

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 et al., 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.

5.3.4. 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 and colleagues (2013), who reported that ZEN was degraded up to 81.7% using 0.16 mM ABTS as redox mediator, despite enzyme mediator and 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 *Rhizopus oryzae* were positively correlated with DON degradation in submerged fermentation (Buffon et al., 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. 5-4). 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-2 toxin. 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.

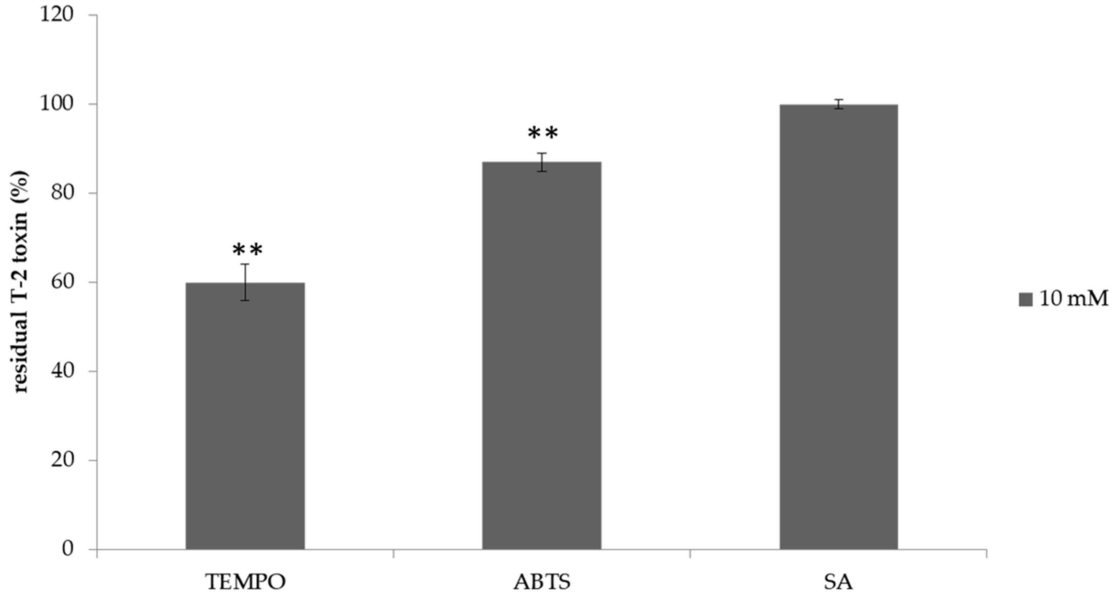


Figure 5-4: Residual T-2 toxin in samples treated with different laccase mediator systems. TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), SA: syringaldehyde. A *P* value <0.01 is considered statistically significant (**).

5.3.5. Combined mycotoxins degradation with selected LMSs

Results of combined mycotoxin degradation for AFB_1/ZEN are shown in Fig. 5-5. 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.

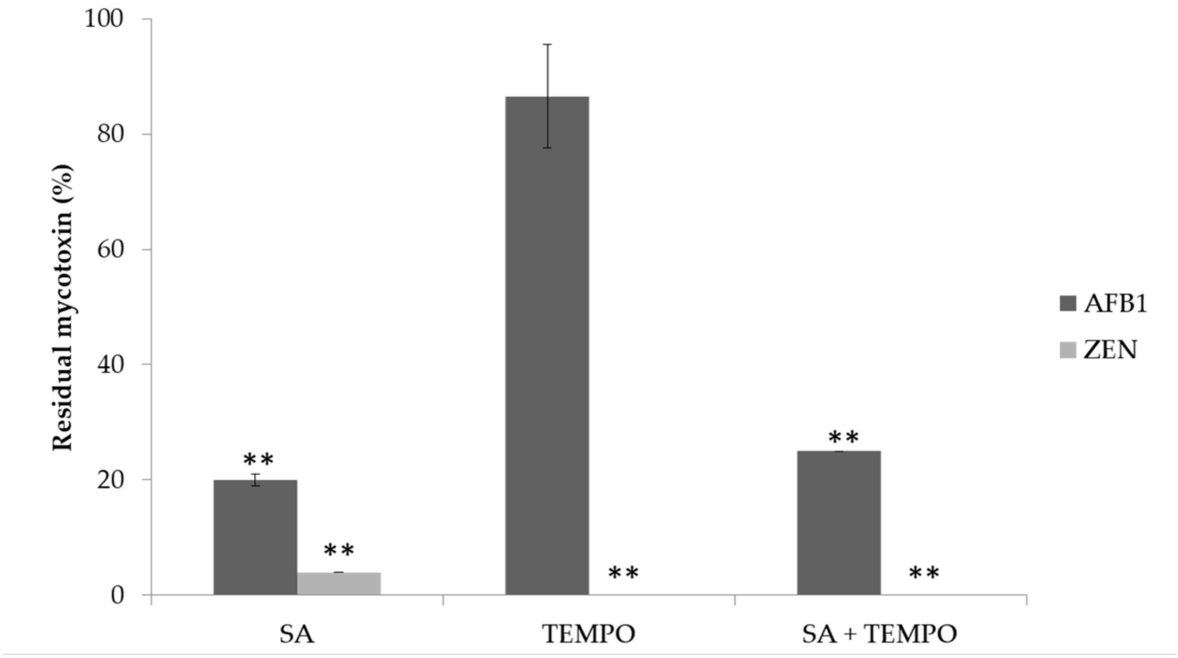


Figure 5-5: Residual aflatoxin B_1 and zearalenone in samples treated with different laccase mediator systems. TEMPO: 2,2,6,6-tetramethylpiperidin-1-yl)oxyl, SA: syringaldehyde. A *P* value < 0.01 is considered statistically significant (**)

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.

5.4. 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.

Acknowledgments

We thank Roberto Schena for his valuable technical assistance.

This work was financially supported by H2020-E.U.3.2-678781-MycoKey-Integrated and innovative key actions for mycotoxin management in the food and feed chain.

References

Abrunhosa, L., Paterson, R. R., & Venâncio, A. (2010). Biodegradation of ochratoxin A for food and feed decontamination. *Toxins*, 2, 1078-1099.

Baiocco, P., Barreca, A. M., Fabbrini, M., Galli, C., & Gentili, P. (2003). Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase–mediator systems. *Organic & biomolecular chemistry*, 1(1), 191-197.

Banu, I., Lupu, A., &Aprodu, I. (2013). Degradation of Zearalenone by Laccase enzyme. *Scientific Study & Research. Chemistry & Chemical Engineering, Biotechnology*., 14(2), 79.

Bleve G, Mita G, Rampino P, Perrotta C, Villanova L, Grieco F. (2008). Molecular cloning and heterologous expression of a laccase gene from *Pleurotus eryngii* in free and immobilized *Saccharomyces cerevisiae* cells. *Applied Microbiology and Biotechnology.* 79, 731–741.

Buffon, G., J., Kupski, L., Badiale-Furlong, E. (2011). Deoxynivalenol (DON) degradation and peroxidase enzyme activity in submerged fermentation. *Food Science and Technology* , 31, 198-203.

Chitrangada, D., Mishra, H.N. (2000) *In vitro* degradation of aflatoxin B1 by horseradish peroxidase. *Food Chemistry*. 68, 309–313.

De Bellis, P., Tristezza, M., Haidukowski, M., Fanelli, F., Sisto, A., Mulè, G., & Grieco, F. (2015). Biodegradation of ochratoxin A by bacterial strains isolated from vineyard soils. *Toxins*, 7, 5079-5093.

De Girolamo, A., Lattanzio, V. M., Schena, R., Visconti, A., & Pascale, M. (2014). Use of liquid chromatography–high-resolution mass spectrometry for isolation and characterization of hydrolyzed fumonisins and relevant analysis in maize-based products. *Journal of Mass Spectrometry*, 49, 297-305.

Dellafiora, L., Galaverna, G., Reverberi, M., & Dall'Asta, C. (2017). Degradation of aflatoxins by means of laccases from *Trametes versicolor*: An in silico insight. *Toxins*, 9, 1-17.

Duvick, J. (2001). Prospects for reducing fumonisin contamination of maize through genetic modification. *Environmental Health Perspectives*, 109 (Suppl 2), 337.

European Commission. 2016. Food enzyme applications submitted to the Commission within the legal deadline. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/fs_food-improvement-agents_enzymes-applications.pdf

European Food Safety Authority. 2014. Scientific Opinion on the safety and efficacy of fumonisin esterase (FUMzyme®) as a technological additive for pigs. *EFSA Journal*. 12, 3667

Fabbrini, M., Galli, C., Gentili, P., Macchitella, D. (2001). An oxidation of alcohols by oxygen with the enzyme laccase and mediation by TEMPO. *Tetrahedron Letters*, 42, 7551-7553.

Ferrara, M., Gallo, A., Lo Scalzo, R., Haidukowski, M., Picchi, V., & Perrone, G. (2014). Inhibition of ochratoxin A production in *Aspergillus carbonarius* by hydroxycinnamic acids from grapes. *World Mycotoxin Journal*, 8, 283-289.

Haidukowski, M., Cozzi, G., Dipierro, N., Bavaro, S. L., Logrieco, A. F., Paciolla, C. (2017). Decontamination of Fumonisin B1 in maize grain by *Pleurotus eryngii* and antioxidant enzymes. *Phytopathologia Mediterranea*, 56, 134-145.

Klimke, T. R., & Wu, F. (2015). Ochratoxin A and human health risk: A review of the evidence. *Critical reviews in food science and nutrition*, 55, 1860-1869.

Loi, M., Fanelli, F., Liuzzi, V. C., Logrieco, A. F., Mulè, G. (2017). Mycotoxin Biotransformation by Native and Commercial Enzymes: Present and Future Perspectives. *Toxins*, 9, 111-142.

Loi, M., Fanelli, F., Zucca, P., Liuzzi, V. C., Quintieri, L., Cimmarusti, M. T., Sanjust, E., Logrieco, A. F., Mulè, G. (2016). Aflatoxin B_1 and M_1 degradation by Lac2 from *Pleurotus pulmonarius* and redox mediators. *Toxins*, 8, 245-261.

Osma JF, Toca-Herrera JL, Rodrıguez-Couto S. (2010). Uses of Laccases in the Food Industry. *Enzymatic Research*., 1-8.

Pascale, M., Panzarini, G., & Visconti, A. (2012). Determination of HT-2 and T-2 toxins in oats and wheat by ultra-performance liquid chromatography with photodiode array detection. *Talanta*, 89, 231-236.

Pascale, M., Panzarini, G., Powers, S., & Visconti, A. (2014). Determination of deoxynivalenol and nivalenol in wheat by ultra-performance liquid chromatography/photodiode-array detector and immunoaffinity column cleanup. *Food analytical methods*, 7, 555-562.

Rocha, M. E. B., Freire, F. D. C. O., Maia, F. E. F., Guedes, M. I. F., Rondina, D. (2014). Mycotoxins and their effects on human and animal health. Food Control, 36, 159-165.

Smith, M. C., Madec, S., Coton, E., Hymery, N. (2016). Natural co-occurrence of mycotoxins in foods and feeds and their in vitro combined toxicological effects. *Toxins*, 8, 94-130.

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., Taranu, I., Oswald, I. P. (2012). Current situation of mycotoxin contamination and co-occurrence in animal feed—Focus on Europe. *Toxins*, 4, 788-809.

Yehia, R. S. (2014). Aflatoxin detoxification by manganese peroxidase purified from *Pleurotus ostreatus*. *Brazilian Journal of Microbiology*, 45, 127-134.

Aflatoxin M_1 removal and biotechnological application of a laccase from *Pleurotus eryngii* for milk safety

Loi M[1,2], Quintieri L[1], Fanelli F[1], Liuzzi VC[1], Logrieco AF[1], Mulè G[1]

[1]Institute of Sciences of Food Production, National Research Council, via Amendola 122/O, Bari 70126, Italy;
[2]Department of Economics, University of Foggia, via Napoli 25, Foggia 71122, Italy

The contents of this chapter were published in Loi M, Quintieri L, Fanelli F, Liuzzi VC, Logrieco AF, Mulè G. Rimozione della aflatossina M1 e potenziali applicativi di una laccasi da *Pleurotus eryngii* per la sicurezza del latte. Scienza e Tecnica Lattiero-Casearia 2017; 67:23-32; doi: 10.5281/zenodo.822778

Abstract: aflatoxin M1 (AFM1) is the main catabolite deriving from the hydroxylation of aflatoxin B1 (AFB1), found in the milk of animals fed with AFB1 contaminated feeds. The International Agency for the Research on Cancer (IARC) has classified it in group 2B, thus possibly carcinogenic for humans. Its maximum limit in raw milk, heat-treated milk and milk for the manufacture of milk-based products, has been set by the Regulation (EC) 1881 of 2006 to 50ng/kg. AFM1 resists to the most common treatments of food industry and persists in processed product. Its occurrence has been registered throughout the whole dairy supply chain, including yogurts and cheeses, and it represents a serious risk for humans and animals. The development of mild, green and efficient methods for AFM1 degradation is an actual and crucial topic. Aflatoxins degradation is difficult to achieve since they must not affect the organoleptic and nutritional qualities of food. In this work, we evaluated the activity of a fungal laccase from Pleurotus eryngii for the degradation of AFM1 in buffer solution and in skimmed UHT milk. We also analyzed the effects on milk protein pattern in order to evaluate its application for the safety improvement of milk-based products. AFM1 degradation in sodium acetate buffer (pH 6.5 1mM at 25°C) was nearly 50% after one hour and complete after 72h. The same trend was registered in skimmed UHT milk, although with a lower rate of degradation, at least during the first three hours of treatment. The analysis of the protein pattern revealed that the intensity of α e β caseins, β-lactoglobulin and bovin sieroalbumin electrophoretic bands significantly decreased, while the appearance of protein aggregates of molecular weight higher than 200kDa was detected. These results highlight several potential applications of this laccase for the development of green detoxification methods

towards AFM1 in milk, and also for the improvement of the rheological, emulsifying and allergenic properties of milk and dairy products.

Keywords: safety, milk, laccase, aflatoxin M_1, protein cross-linking, milk texture, allergenicity

6.1. Introduction

Aflatoxin M_1 is the main *in vivo* hydroxylated metabolite of aflatoxin B_1 (AFB_1) which contaminates milk and meat of animals fed with AFB_1 contaminated feeds (Iqbal et al., 2016).

AFM_1 was classified as possible carcinogen to humans (group 2B) by the International agency of Research on Cancer (IARC, 2012). It was also reported to be cytotoxic and genotoxic even without metabolic activation, thus it represents a great health concern for human health.

A linear correlation between AFB_1 in feed and AFM_1 in milk exists. Therefore, AFM_1 reduction strategies aim at reducing AFB_1 in feed; however, they are only preventive and not completely effective (Commission regulation 1881/2006). AFM_1 can be found in milk, but also in dairy because it is extremely resistant to the physical and chemical treatments of food industry (Anfossi et al., 2011). Several methods for AFM_1 reduction have been proposed in raw milk. However chemical or physical methods (use of bisulphites, hydrogen peroxydes, adsorbents, harsh heat treatments, UV irradiation) are not in use because of their partial efficacy and negative drawbacks on the nutritional and organoleptic properties (Ashiq et al., 2015; Pransini et al., 2009).

The most commonly used methods for AFM_1 reduction rely on physical adsorption to acid lactic bacteria cell walls, which can be naturally present in milk or added as functional starters for yogurt or cheese manufacture (Elsanhoty et al., 2014). However, physical adsorption cannot be considered as a true biotransformation since it is based on the non-covalent, possibly reversible

interactions between the toxin and the carbohydrate and hydrophobic regions of the cell walls (Ismail et al., 2016).

The development of new methods for the effective AFM_1 biotransformation is a crucial and essential need. Besides being effective and irreversible, the method has to preserve the and organoleptic properties of the matrix.

Enzyme application for food biotechnology is increasingly studied for milk and dairy applications. Among the analysed enzymes, laccases (LC), lactoperoxidases, transglutamminases and tyrosinases are investigated for their crosslinking activity and their capability to modify the techno-functional properties of milk and milk based products (Heck et al., 2013). In particular, LC use in food industry deserves particular attention because of its green feature, as it uses molecular oxygen as electron acceptor and reduces it to water. LC is widespread in fungi and can be easily purified from culture filtrates of edible mushrooms, such as *Pleurotus eryngii*, which are safe and easy to cultivate. Moreover, LC was recently reported to *in vitro* degrade AFM_1 (Loi et al., 2016)

Therefore, the aim of this work was to evaluate the application of a LC isozyme from *P. eryngii*, a local Apulian mushroom, for AFM_1 degradation. The degrading activity was evaluated in a model buffer system and then in UHT skim milk. Since LC can be used as food additive for the modification of the texture, emulsifying and foaming properties of milk, the effect of laccase treatment on the protein pattern of milk was also evaluated.

6.2. Materials and methods

6.2.1. Chemicals and standard preparation

2,2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS), syringaldehyde (SA), acetonitrile (ACN) and AFM1 standard (>99% purity) were purchased from Sigma Aldrich (Segrate, Milan, Italy). Reagents for sodium dodecyl sulphate gel electrophoresis (SDS PAGE), including Biosafe Coomassie and Bradford reagent,

were purchased from Bio-Rad Laboratories (Bio-Rad, Milan, Italy). AFM_1 stock solution (10g/mL) was prepared by dissolving the toxin in ACN (HPLC grade). Exact toxin concentration was measured according to AOAC 2000.08 (AOAC, 2008). Aliquots of standard stock solutions were dried under steam of nitrogen at 50°C and resuspended in mobile phase (water:ACN 75:25 w/w) in order to obtain standard calibrant solutions (1.0-10.0 ng/mL). Standard solutions were stored at -20°C and warmed to room temperature before use.

6.2.2. Microorganism and growth conditions

Saccharomyces cerevisiae ITEM 17289 (Agri-Food Microbial Fungi Culture Collection of the Institute of Sciences of Food Production, www.ispa.cnr.it/Collection) engineered with pYes2 vector bearing Ery4 laccase (pY_Ery4) gene sequence was used in this study. Yeast culture was grown in Yeast Nitrogen Base (YNB, 0.67% (w/v)) supplemented with adequate auxotrophic requirements and glucose 2% in shaking conditions at 28°C for 5 days. Yeast with empty pYes2 vector, was used as control.

6.2.3. Induction and purification of Ery4 enzyme

Yeast cultures (pY_Ery4 and pYes2) were grown in YNB until OD_{600} 0.8 was reached. Cells were pelleted by centrifugation (10,000g for 15min) and resuspended at OD_{600} 0.6 in the induction medium composed by YNB supplemented with auxotrophic requirements, 1 mM $CuSO_4$ and 4% galactose. LC expression was induced for 3 days in shaking conditions at 18°C.

After induction, cultured media was filtered and dialyzed against Tris-HCl 50mM, pH8 in a Lab scale TFF system (Merk Milliporem Frankfurt, Germany) equipped with a Pellicon XL ultrafiltration membrane with amolecular weight cut off of 10,000 Da. Volume was reduced to 50mL and loaded on a Hi Q anion exchange column (1.6 × 10cm, Bio-Rad, Hercules, California) pre quilibrated with Tris-HCl 50mM, pH8. LC was eluted with Tris-HCl 50mM, pH 8 added with 0.4 M

NaCl (flux 1mL/min). LC fractions were pooled and used for the degradation assays.

6.2.4. *Laccase activity assay and protein content determination*

LC activity in both pY_Ery4 and pYes2 yeasts was measured spectrophotometrically (Ultraspec 3100pro, Amersham Pharmacia Biotech, Cologno Monzese, Italy). Reaction was performed in sodium acetate buffer 100mM, pH 4.5, 5mM ABTS and an adequate amount of enzyme solution. ABTS oxidation was monitored at 420nm (λ_{420nm}=36,000 M^{-1} cm^{-1})after 10min of reaction (Bleve te al., 2008). Total protein content was determined according to Bradford (1976).

6.2.5. *Aflatoxin M_1 degradation in buffer solution and in UHT skim milk*

The degradation assay was performed in sodium acetate buffer 1mM, pH 6.5 by adding 5U/mL of Ery4 and 0.05μg/mL of AFM_1. Degradation was also evaluated in presence of 10mM SA, as redox mediator. Reactions were incubated at 25°C in static conditions for 72h. Aliquots were withdrawn after 30min, 1h, 3h, 6h, 12h, 24h, 48h and 72h.

The degradation assay in skim UHT milk (Granarolo, Italy) was performed as described above. Aliquots were withdrawn after 1h, 6h, 24h, 48h and 72h.

6.2.6. *Chemical analyses*

In vitro reactions were filtered (0.20μm) (Grace, Taipei, China) and 50μl were injected in a HPLC system (Agilent 1260 Series) equipped with a fluorescence detector (ex=365, em= 450nm and a reverse phase Zorbax SG-C18 column (4×3.0mm) as described by Loi et al., (2016). Milk samples were purified according to AOAC 200.08 (AOAC, 2008) prior injection. AFM_1 was quantified by comparison with the standard calibrant curve (1.0-10ng/mL).

6.2.7. Protein profile evaluation by SDS-PAGE

40ng of proteins of control samples (milk or milk sample added with SA or Ery4 or their suspension buffers) and sample containing both Ery4 and SA were mixed with reducing, denaturating sample buffer (62,5 mM Tris-HCl pH 6,8, 25% glycerol, 2% SDS, 0,01% blue bromophenol, 1% β-mercaptoethanol) and loaded on a polyacrilammide gel (4-20% Criterion™ TGX StainFree™ Precast Gels). Molecular weight marker M12 (2.5–200 KDa, Life technology, Waltham, MA, USA) was added as reference. Electrophoretic separation was performed on a Miniprotean system (Biorad, Segrate, Milan) with a running buffer composed of 25mM Tris and 0.19M glycine.

After the run, proteins were fixed (40% ethanol, 10% acetic acid, 50% water) for 30min and then stained with the Bio-safe Coomassie stain (Biorad, Segrate, Milan) according to the manufacturers' instructions. Destained gel was scanned with the Image scanner III (GE Healthcare, Pittsburg, PA, USA).

6.3. Results and discussion

6.3.1. Induction and purification of Ery4 laccase

After induction, Ery4 production was monitored in liquid culture both for pY_Ery4 and pYes2 yeasts. As expected, only pY_Ery4 showed LC activity. As reported in Table 6-1, cultured filtrate showed 1445U and a specific activity of 4.7 U/g. After concentration volume was reduced to 50mL with no appreciable LC loss. Specific activity increased up to 112.5 U/g after purification with the anion exchange chromatography.

Table 6-1. Summary of Ery4 purification from *Saccharomyces cerevisiae* culture filtrate

Purification step	Total volume (mL)	Total activity (EU)	Total protein (mg)	Specific activity (U/g)	Purification fold
Crude extract	2000	1445.1	307.5	4.7	1
Ultrafiltration	50	1403.4	301.8	4.6	1
AEC	6	1105.0	10.6	104.3	24

6.3.2. Aflatoxin M1 degradation in buffer solution and in UHT skim milk

Degradation percentages with respect to the control without enzyme are shown in Table 6-2, while the time course degradation is shown in Fig. 6-1. Culture filtrate of control yeast pYes2 and Ery4 alone were not able to degrade AFM_1 in buffer solution. However, a complete degradation of the toxin was achieved by Ery4 and SA after 72h of static incubation at 25°C. AFM_1 degradation only proceeded via laccase mediator system, in which the oxidized SA is responsible for the degradation of the recalcitrant substrate, in this case AFM_1 (Canas et al., 2010).

Table 6-2. Absolute values and degradation percentages of the time course degradation of AFM_1 with Ery4 and SA

time	*In vitro*		*In matrix*	
	AFM_1 ± DS (ng/mL)	AFM_1 degradation ± DS (%)	AFM_1 ± DS (ng/mL)	AFM1 degradation ± DS (%)
0 min	36 ± 3	-	37.5 ± 2.0	-
30 min	21 ± 1	43 ± 3	n.t.	n.t.
1 h	19 ± 2	46 ± 4	30.5 ± 2.9	18.5 ± 7.5
3 h	14 ± 1	61* ± 1	n.t.	n.t.
6 h	11 ± 1	70* ± 1	13.7 ± 0.3	60* ± 1
12 h	6 ± 0	85* ± 1	1 ± 0.1	97* ± 0.5
24 h	3 ± 0	91* ± 0	n.t.	n.t.
48 h	1 ± 0	97* ± 0	0.46 ± 0.1	98* ± 0
72h	0 ± 0	100* ± 0	0 ± 0	100* ± 0

n.t., not tested; Comparisons between control and treated sample was performed using a *t*-test. A p value < 0.001 was considered statistically significant (*)

The toxin probably has a too high redox potential, ionization potential or steric hindrance to be oxidized directly by LC (Tadesse et al., 2008). This result is in accordance with Loi et al., (2016) who reported that a native LC from *P. pulmonarius*, was able to degrade AFB_1 and AFM_1 only in presence of a redox mediator (Loi et al., 2016). SA is a natural lignin derived phenol, which was already exploited in bioremediation, e.g. industrial dyes and polycyclic aromatic hydrocarbons (Zucca et al., 2011; Collin et al., 2006)

Toxin degradation in buffer reached *ca.* 50% after only 1h of assay, 91% after 24h and was complete within 72h. The degradation kinetic followed a logarithmic trend (R^2= 0.9939). The same logarithmic trend was registered in UHT skim milk (R^2= 0.9902), although the rate of degradation was lower, at least in the first three hours of the assay (18.5% VS 46.0% after 1h). Differences between the two matrixes were negligible after 48h and 72h, when the complete removal was, again, achieved. The different rate of degradation can be explained by the matrix effect exerted by milk carbohydrates and proteins. AFM_1 adsorption to both, as well as to bacterial cell walls of lactic acid bacteria was well documented and possibly involved in the decreased bioavailability of the toxin to SA oxidation (Brackett et al., 1982; Barbiroli et al., 2007; Corassin et al., 2013).

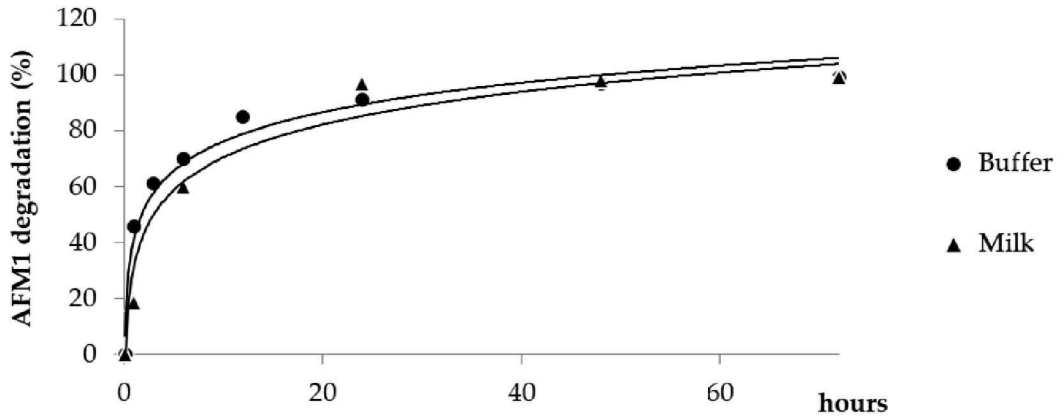


Figure 6-1. Time course degradation of AFM1 in buffer solution and in skimmed UHT milk by Ery4 and SA

6.3.3. *Protein profile evaluation by SDS-PAGE*

Fig. 6-2 shows the protein profile of control samples and milk treated with both Ery4 and SA. No changes in the protein profile was registered in control samples, even if treated with Ery4 or SA (Fig. 6-2 lanes 2, 3, 4, 6, 7 and 8). By contrast, significant changes were shown in sample treated with both Ery4 and SA (Fig. 6-2, lane 5). β-casein, β-lactoglobulin and bovine sieroalbumin bands were significantly reduced, while high molecular weight aggregates higher than 200kDa were detected.

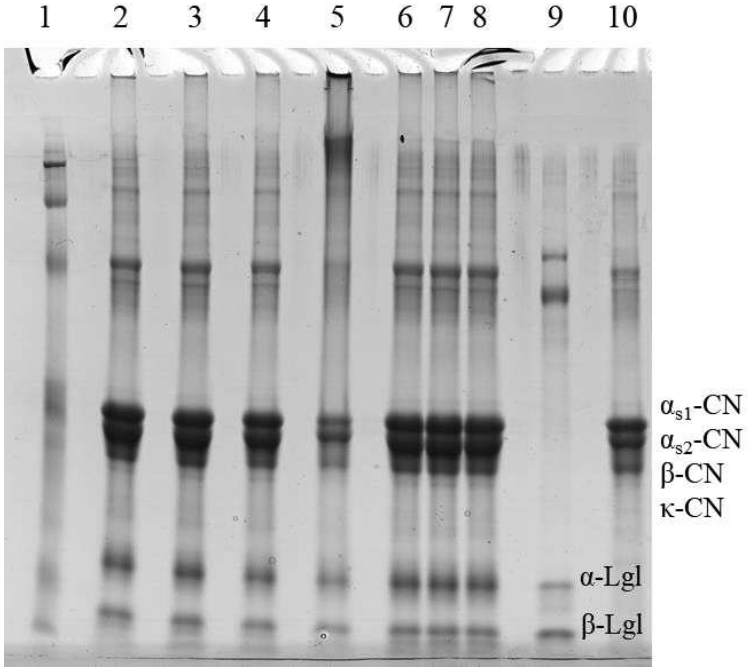


Figure 6-2. SDS PAGE of milk samples: 1) marker, 2) control, 3) milk+ laccase, 4) milk+syringaldeyde (SA), 5) milk +laccase + SA, 6) milk+laccase buffer, 7) milk+ SA buffer, 8) milk+laccase and SA buffesr, 9) lactoglobulins (Lgl) standard, 10) caseins (CN) standard

LC and other oxidases were already reported to induce the formation of high molecular weight oligomers (Moonkali et al., 2015) through the covalent link between tyrosine, cysteine and lysine residues of proteins and mediators in the case of LC (Mattinen et al., 2005).

Protein crossinking, in particular that involving caseins and sodium caseinate, was reported to increase emulsion stability, heat and proteolysis resistance and

protect proteins against heat denaturation and harsh pH variations. (Struch et al., 2015; Chen et al., 2012). β-lactoglobulin crosslinking might have important outcomes in terms of allergenicity reduction. Indeed, the allergenic potential of allergenic proteins was proved to be reduced by LMS treatment (Tantoush et al., 2011; Stanic et al., 2010).

6.4. Conclusions

This study highlights the great potentiality of LC enzymes for the development of green alternative methods for AFM_1 degradation. The toxin was completely removed *in vitro* and in UHT skim milk after 72h of treatment.

LC and mediator are also able to induce significant modification in the protein pattern profile of milk, in particular they reduced β-casein, β-lactoglobulin and bovine sieroalbumin bands. Among the many applications of LMS, the development of dairy products with increased rheological and technological properties, or reduced allergenicity deserve particular attention. The crosslinking effect, together with the degradation capability on AFM_1, opens new perspective for the improvement of the techno functional properties and safety of milk and milk based products.

References

Anfossi, L., Baggiani, C., Giovannoli, C., Giraudi, G. (2011). Occurrence of Aflatoxin M1 in Dairy Products In "Aflatoxins - Detection, Measurement and Control". Chapter 1 pp 119 (ed. Irineo Torres-Pacheco). InTech

Ashiq S. (2015). Natural occurrence of mycotoxins in food and feed: Pakistan perspective. Compr Rev Food Sci Food Saf 14 159–175

Association of Official Analytical Chemists (AOAC) Official Method 2008.08-2008.

Barbiroli, A., Bonomi, F., Benedetti, S., Mannino, S., Monti, L., Cattaneo, T., Iametti, S. (2007). Binding of Aflatoxin M1 to Different Protein Fractions in Ovine and Caprine Milk. J Dairy Sci 90 532–540

Bleve, G., Lezzi, C., Spagnolo, S., Tasco, G., Tufariello, M., Casadio, R., Mita, G., Rampino, P., Grieco F. (2012). Role of the C-terminus of *Pleurotus eryngii* Ery4 laccase in determining enzyme structure, catalytic properties and stability. Protein Eng Des Sel 26:1–13.

Bleve, G., Mita, G., Rampino, P., Perrotta, C., Villanova, L., Grieco, F. (2008). Molecular cloning and heterologous expression of a laccase gene from *Pleurotus eryngii* in free and immobilized Saccharomyces cerevisiae cells. Appl Microbiol Biotechnol 79 731–741.

Brackett, R.E., Marth, E.H. (1982). Association of aflatoxin M1 with casein. Z Lebensm Unters Forsch 174 439-441

Cañas, A., Camarero, S. (2010). Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. Biotechnol Adv 28 694–705

Chen, B., Li, H., Ding, Y., Suo, H. (2012). Formation and microstructural characterization of whey protein isolate/beet pectin coacervations by laccase catalyzed cross-linking. LWT - Food Sci Techol 47 31-38

Collins, P.J., Kotterman, M., Field, J.A., Dobson A (1996). Oxidation of Anthracene and Benzo [a] pyrene by Laccases from *Trametes versicolor*. Appl Environ Microbiol 62 4563–4567

Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs

Corassin, C.H., Rosim, F. Bov, R.E., Oliveira, C.A.F. (2013). Efficiency of *Saccharomyces cerevisiae* and lactic acid bacteria strains to bind aflatoxin M1 in UHT skim milk. Food Control 31 80–83

Elsanhoty, R.M., Salam, S.A., Ramadan, M.F., Bad, F.H. (2014). Detoxification of aflatoxin M1 in yoghurt using probiotics and lactic acid bacteria. Food Control 43 129–134

Heck, T., Faccio, G., Richter, M., Thöny-Meyer, L (2013). Enzyme-catalyzed protein crosslinking. Appl Microbiol Biotechnol 97 461–475

International Agency for the Research on Cancer. Aflatoxins. (2012). IARC Monographs: Lyon, France, Volume 100F

Iqbal, S.Z., Jinap, S., Pirouz, A.A., Ahmad, F.A.R. (2016). Aflatoxin M_1 in milk and dairy products, occurrence and recent challenges: A review. Trends Food Sci Technol 46 110–119

Ismail, A., Levin, R.E., Riaz, M., Akhtar, S., Gong, Y.Y., de Oliveira, C.A.F. (2016). Effect of different microbial concentrations on binding of aflatoxin M1 and stability testing. Food control, in stampa

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 680–685

Loi, M., Fanelli, F., Zucca, P., Quintieri, L., Liuzzi, V.C., Cimmarusti, M.T., Monaci, L., Haidukowski, M., Logrieco, A.F., Sanjust, E., Mulè, G. (2016). Aflatoxin B_1 and

M_1 degradation by Lac2 from *Pleurotus pulmonarius* and redox mediators. Toxins 8 245-261

Mattinen, M.L., Kruus, K., Buchert, J., Nielsen, J.H., Andersen, H.J., Steffensen, C.L. (2005). Laccase-catalyzed polymerization of tyrosine-containing peptides. FEBS J 272 3640–3650

Mokoonlall, A., Pfannstiel, J., Struch, M., Berger, R.G., Hinrichs, J. (2016). Structure modification of stirred fermented milk gel due to laccase-catalysed protein crosslinking in a postprocessing step. Innov Food Sci Emerg Technol 33 563–570

Prandini, A., Tansini, G., Sigolo, S., Filippi, L., Laporta, M., Piva, G. (2009). On the occurrence of aflatoxin M1 in milk and dairy products. Food Chem Toxicol 47 984–991

Stanic, D., Monogioudi, E., Dilek, E., Radosavljevic, J., Atanaskovic-Markovic, M., Vuckovic, O., Raija, L., Mattinen, M., Buchert, J., Cirkovic Velickovic, T. (2010). Digestibility and allergenicity assessment of enzymatically crosslinked β-casein. Mol Nutr Food Res 541273-1284

Struch, M., Linke, D., Mokoonlall, A., Hinrichs, J., Berger, R.G. (2015). Laccase-catalysed crosslinking of a yoghurt-like model system made from skimmed milk with added food-grade mediators. Int Dairy J 49 89-94

Tadesse, M.A., D'Annibale, A., Galli, C., Gentili, P., Sergi, F. (2008). An assessment of the relative contributions of redox and steric issues to laccase specificity towards putative substrates. Org Biomol Chem 6 868–878

Tantoush, Z., Stanic, D., Stojadinovic, M., Ognjenovic, J., Mihajlovic, L., Atanaskovic-Markovic, M., Cirkovic Velickovic, T. (2011). Digestibility and allergenicity of β-lactoglobulin following laccase-mediated cross-linking in the presence of sour cherry phenolics. Food Chem 125 84–91

Zucca, P., Rescigno, A., Olianas, A., Maccioni, S., Sollai, F., Sanjust, E, (2011), Induction, purification, and characterization of a laccase isozyme from *Pleurotus sajor-caju* and the potential in decolorization of textile dyes. J Mol Catal B Enzym 68 216–222

Application of a recombinant laccase-chlorogenic acid system in protein crosslink and antioxidant properties of the curd

Loi M[1,2], Quintieri L[1], Fanelli F[1], Caputo L[1*], Mulè G[1]

[1]Institute of Sciences of Food Production, National Research Council, via Amendola 122/O, Bari 70126, Italy;
[2]Department of Economics, University of Foggia, via Napoli 25, Foggia 71122, Italy

The contents of this chapter were published in Food Research International as: Loi M, Quintieri L, Fanelli F, Caputo L, Mulè G. Application of a recombinant laccase-chlorogenic acid system in protein crosslink and antioxidant properties of the curd.

Abstract: Milk protein crosslink through the action of enzymes represents a feasible strategy to impart new functionalities to cheese. In this work we reported the effects of a laccase mediator system (LMS) on protein crosslink and antioxidant property of curd. The crosslinking activity of a purified recombinant laccase Ery4 and a commercial enzyme preparation (cLC), with three mediators was firstly evaluated in milk and then applied before curd manufacture. Only Ery4-LMS significantly increased curd weight compared to that of the control sample. SDSPAGE revealed that similar high molecular weight bands produced by both LMSs in milk were also retained in curds. The antioxidant activity recorded in curds with Ery4-LMS was the highest among all samples both before and after gastro-pancreatic digestion. This is the first time that a CGA-based LMS is used in manufacture of curd with improved antioxidant properties. These results open new perspectives for dairy applications.

Keywords: protein crosslink; laccase mediator system; caseins; gastro-pancreatic digestion; polyphenols.

7.1. Introduction

Milk proteins modification is emerging as an innovative method to improve the technological, rheological, functional and sensory properties of dairy products,

such as yield, heat stability, water and fat binding capacity and gel forming properties. In particular, protein incorporation in curd and cheese yield is a matter of great interest in the dairy industry, since considerable amounts of proteins are lost in whey during rennet clotting (Fox, Guinee, Cogan, & McSweeney, 2017).

Cheeses display a large difference in yield mostly in relation to milk quality, casein amount, k-casein types, fat content, cheese type and manufacturing methods (for a review see Fox et al., 2017). In particular, in the early stages of the cheese-making process, collapsing caseins incorporate fat globules and water; this latter is lately purged due to coalescence and applied pressure. The higher the amount of casein in the milk, the greater content of fat and water the cheese might incorporate.

Protein crosslink, obtained by means of chemical or enzymatic modifications, was shown to potentially increase cheese yield, texture properties, digestibility and to reduce the allergenicity of milk proteins (Zeeb, McClements, & Weiss, 2017). The enzymatic modification has the advantage of being highly specific and requiring mild reaction conditions. LCs (EC 1.10.3.2), tyrosinases (Tyr, EC 1.14.18.1) and transglutaminases (TGase, EC 2.3.2.13) were demonstrated to induce protein crosslink and to modulate some important functional properties of dairy and bakery products, including texture, gelling, foaming and emulsifying features as well as to increase whey protein recovery (Buchert et al., 2010).

LCs are copper-containing enzymes which catalyze the oxidation of substituted phenols, anilines and aromatic thiols, using oxygen as the electron acceptor and the release of water as byproduct. Substrates undergo a single electron oxidation, producing unstable and highly reactive radicals. LCs were also reported to directly oxidize tyrosine and cysteine residues of milk proteins, which could further interact with lysine, tyrosine and cysteine residues of other proteins (Selinheimo, Kruus, Buchert, Hopia, & Autio, 2006). Thus, the cross-linking activity is primarily due to non-enzymatic reactions, leading to polymerization. However, extensive protein crosslink could be also achieved by including several

redox mediators in the so called Laccase Mediator System (LMS). By this way redox mediators, once oxidized, enhance protein crosslink acting as bridging agents between proteins (Steffensen, Andersen, Degn, & Nielsen, 2008).

It has been reported that the use of a cLC and polyphenols as mediators reinforced the viscoelastic properties of low-fat yoghurt (Struch, Linke, Mokoonlall, Hinrichs, & Berger, 2015) and improved the protein emulsifying properties of sodium caseinate, allowing its supplementation in acidic products (Sato, Perrechil, Costa, Santana, & Cunha, 2015). This mode of action of LC was successfully exploited in several food research applications to improve the textural and rheological properties of bakery and dairy products (Pezzella, Guarino, & Piscitelli, 2015). New and already in use food enzymes need to be subjected to safety evaluation by the European Food Safety Authority (EFSA) and subsequently approved by the European Commission by means of a Union list. To date, an authorized food enzyme list does not exist, but a LC from Trametes hirsuta was included in the application list for the validity assessment (European Commission, 2015).

LC are nonspecific with regards to reducing substrates. They catalyze the oxidation of various organic substances, including o- and p-diphenols, aminophenols, polyphenols, polyamines, methoxy phenols, lignins, aryl diamines, and some inorganic ions with the simultaneous and direct reduction of dioxygen to water without the intermediate production of hydrogen peroxide (Giardina & Sannia, 2015).

LC mediators include organic compounds such as o- and p-diphenols, aminophenols, polyphenols, polyamines, methoxy phenols, lignins, aryl diamines. Polyphenolic compounds are exploited for their natural antioxidants and chemopreventive activity in production of nutraceutical foods. Indeed, functional foods enriched with polyphenols (dairy, bakery and meat products) have been recently developed (McDougall, 2017) and the evaluation of their antioxidant activity was also performed in vitro during gastro-pancreatic digestion (Helal,

Tagliazucchi, Verzelloni, & Conte, 2015) and in in vivo assays (Bastide et al., 2017).

LMS application in milk is not limited to technological aspects but might be extended to safety issues. A recent study by Loi et al. (2017) reported the complete removal of aflatoxin M1in vitro as well as in artificially spiked milk obtained by means of LMS based on the recombinant Ery4 laccase, purified from Pleurotus eryngii, expressed in Saccharomyces cerevisiae. However, no details were given about the effect of this treatment in dairy applications.

This work aimed at investigating the effect of milk protein crosslink induced by a LMS on curd preparation and its antioxidant properties. Thus, Ery4 laccase was firstly assayed for milk protein crosslink in presence of three mediators, at two concentrations and at different times of incubation. Then, the selected mediator was added at the defined optimal concentration during curd making process in order to confer antioxidant properties of the final product and after in vitro gastro-pancreatic digestion.

7.2. Materials and methods

7.2.1. Chemicals and reagents

The following mediators: 2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS), vanillin (VAN), ferulic acid (FA) and chlorogenic acid (CGA) were purchased from Sigma-Aldrich s.r.l. (Milan, Italy) and freshly diluted in working alcoholic solutions at 0.20M before use. The enzymes pancreatin from porcine pancreas (8× USP specification activity), pepsin from porcine gastric mucosa (250 units mg/solid), cLC from Trametes versicolor (0.89 U/mg; cLC), α-, β-, κ-casein standards (α-CN; β-CN; κ-CN; cat. number C6780, C6905, C0406), α-lactoalbumin from bovine milk (α-LA; L6385-1VL), β-lactoglobulin (BLG; L3908) and bovine serum albumin (BSA, A2153) were obtained from Sigma-Aldrich. Marzyme15® was purchased from Danisco Italy S.p.A (Cernusco sul Naviglio, MI,

Italy) and bovine lactoferrin (BLF) was obtained from Armor Proteines (Saint-Brice-en-Cogle, France). The stained molecular weight markers Kaleidoscope (2.5–200.0 kDa) and HiMark™ High Molecular Weight Protein Standard were supplied by Bio-Rad Laboratories S.r.l, (Segrate, Italy) and Life Technologies Italia (Monza, Italy), respectively. Commercial skimmed UHT milk (5% lactose, 3% protein, 0.1% fat) and whole fat pasteurized milk (5% lactose, 3.5% protein, 3.3% fat) used in this work were purchased in a local market.

7.2.2. Ery4 laccase production and purification

Ery4 laccase was obtained from Saccharomyces cerevisiae ITEM 17289 (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* (pY-ery4) as reported by Loi et al. (2017). Yeast culture was grown in minimal YNB medium [0.67% (w/v) yeast nitrogen base, supplemented with adequate quantities of auxotrophic requirements and glucose (2%, w/v)] in shaking conditions at 28°C. Yeast cells were harvested by centrifugation at 0.8 OD600nm and then suspended at 0.6 OD600nm in induction medium (YNB medium supplemented with 4% (v/w) galactose and 1mM $CuSO_4$. LC production was induced by incubating cells at 18°C for 72h. After induction, cultured medium was recovered by centrifugation (10,000g for 15 min), filtered and dialyzed at 4°C against 50 mM Tris-HCl, pH 8.0 using a Vivaflow 200 apparatus (Vivascience AG, Hannover, Germany) equipped with a Hydrosart membrane module (nominal MW cut-off 10,000 Da) and a Masterflex L/S system pump (Cole-Parmer, Vernon Hills, IL, USA) up to a final volume of 50 mL. Ery4 was purified by using a High Q column (1.6 × 10cm; Bio-Rad, USA) equilibrated with 50 mM Tris-HCl, pH 8. LC was eluted with the same buffer added with NaCl to a final concentration of 0.2 M at a flow rate of 1 mL/min. Active fractions were pooled, desalted by ultrafiltration with Vivaspin15 (Sartorius Stedim S.p.a., Milan, Italy) and concentrated in 1 mL of the

same buffer. This enzyme solution was filter-sterilized and stored at −20 °C until use.

7.2.3. LC spectrophotometric activity assay

LC activity was photometrically measured (Ultraspec 3100pro, Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) according to Loi et al. (2016). Briefly, the reaction was performed in 100 mM sodium acetatepH 4.5, 5 mM ABTS and an appropriate amount of enzyme solution in a final volume of 1 mL. After 10 min of reaction the oxidized ABTS amounts were determined at 420 nm ($\lambda_{=420nm}$ = 36,000 $M^{-1}cm^{-1}$). One unit was defined as the amount of enzyme which oxidized 1 μmol of substrate per min.

7.2.4. Crosslinking activity of Ery4 in milk with different mediators

The ability of Ery4 laccase to crosslink milk proteins when combined with several mediators and at two different concentrations was evaluated in UHT skim milk. Crosslinked proteins were analyzed by SDS-PAGE as previously described (Loi et al., 2016).

Ery4 laccase (5 U/mL) was added alone or in combination with the selected mediator (VAN, FA or CGA) at 1 or 10 mM to 0.5 mL of milk previously equilibrated at 38 °C for 15 min. Milk control sample contained neither LC nor mediator. The reaction was performed in triplicate at 38 °C for 180 min. Then the samples were diluted for protein quantification by Bradford method (Bradford, 1976). In addition, the same determination was performed on the supernatants of each samples obtained by centrifugation at 13,000 rpm for 5 min. Aliquots of diluted samples and supernatants containing 7 μg of protein were diluted with sample buffer (1:1, v/v; 4% SDS, 3% b-mercaptoethanol, 10% glycerol, 50 mM Tris–HCl pH 6.8) and loaded on a 26-well 12% Criterion TM TGX precast gels (BioRad). Two different reference proteins mixtures (Casein, CN Mix1: α-CN, β-CN, and κ-CN and whey protein Mix2: β-lactoferrin (BLF), bovine serum albumin (BSA), α-

lactoalbumin (ALA) and β-lactoglubulin (BLG), respectively) were also loaded on the gel. Electrophoresis was carried out on Criterion TM Cell at 150 V for 75 min. Staining protocol and gel acquisition was carried out as described by Quintieri et al. (2017). The appearance of high molecular weight bands combined with the reduction in intensity of any milk protein band was associated to the protein oligomerization due to LC activity. The mediator and concentration able to produce an appreciable increase in crosslinked SDS-PAGE bands was selected and also evaluated after 30 and 60 min of incubation before the subsequent experiments.

7.2.5. Evaluation of protein crosslinking by selected mediator in whole milk

The same lab-scale level trials were performed using the most active mediator at the selected concentration in pasteurized whole milk. Furthermore, cLC was also used at same concentration of Ery4 as a comparison, in order to evaluate its ability to crosslink milk proteins. Quantification and SDS-PAGE protein profile was obtained for all milk samples following procedures reported below.

7.2.6. Curd trials

Curd trials were carried out in order to evaluate the efficacy of Ery4 combined with the most active mediator on cheese yield due to putative protein crosslink. Therefore, 10-gram samples of full fat pasteurized milk were pre-heated at 38 °C in a water bath and treated with 5 U/mL of LC (Ery4 or cLC) alone or combined with 1 mM mediator. Control samples were obtained by adding mediator alone or buffer to milk. After 45 min of incubation the rennet, Marzyme15® (600 μL/L) was added to all samples and kept at the same temperature for 15 min. Finally, the curd gel cheese was cut transversely into four sections with a stainless-steel knife, kept in whey for additional 5 min at 38 °C and then moulded into a 10-mL plastic syringe (21G × 1½″; Terumo, Europe, Leuven, Belgium) appropriately

pierced with ca. 50 0.5 mm-holes. In addition, in order to facilitate whey drainage a 0.1 MPa uniaxial force-compression was applied to the syringe plunger for 5 min partially similarly to the pressure generally applied for cheese (Bennett & Johnston, 2004). Then, curds and whey were recovered and analyzed as described below. Moisture content of each curd sample was assessed according to AOAC method 990.20 (2012).

7.2.7. Analyses of proteins, free amino acids and small peptides

In order to evaluate protein crosslink in curd, protein extraction was performed from each sample. Briefly, 0.5 g of homogenized curd were dissolved in 2 mL of 0.1 M sodium citrate buffer pH 8.5 and incubated in stirring conditions for 1 h at 40 °C. The sample was added with water to 10 mL and centrifuged at 9000 ×g for 20 min at 4 °C. The supernatant of each sample was collected and the pellet was solubilized with urea-mercaptoethanol solvent (8 M urea and 5% v/v 2-mercaptoethanol; Owusu-Apenten, 2002). Both protein fractions were quantified using Bradford assay (Bradford, 1976) and their total content was also calculated. Fifteen micrograms of protein extracts from each curd sample were quantified and loaded on SDS PAGE as reported above. Likewise, drained whey volume was analyzed for its protein concentration and also loaded (15 μg). Whey samples were also analyzed for free amino acids (FAA) and small peptides concentrations by following the method described by Church, Swaisgood, Porter, and Catignani (1983). Each analysis was performed in triplicate.

7.2.8. In vitro gastro-pancreatic digestion

A two-stage in vitro digestive model was carried out by following the protocol described by Helal et al. (2015). One part of each curd was homogenized into 15 mL screw cap tubes with 9 parts of simulated gastric fluid (2 g/L NaCl, pH 2) for 1 min on an orbital shaker (Thermo Fisher Scientific). Each homogenate was adjusted at pH 2 with HCl and 315 U/mL of pepsin were added. Samples, in

triplicate, were incubated in shaking conditions at 37 °C for 2 h. At the end of the gastric digestion, samples were neutralized at pH 7.5 with 1.25 M $NaCO_3$ adding 0.8 mg/mL of pancreatin and 5 mg/mL of bile salts. Samples were then incubated at 37 °C for 2 h in an orbital shaker to simulate the intestinal phase of digestion. At the end of incubation, the pH of homogenates was lowered to 2 to inactivate the enzymes and stabilize polyphenols. Control samples without pepsin and pancreatin were also included. The efficiency of both digestion treatments was checked by evaluating residual protein amounts and the contents of free amino acids and small peptides as reported in the Section 7.2.7.

7.2.9. Radical scavenging activity determination of digested cheese-curds

The antioxidant properties of digested cheese-curds were evaluated, in triplicate, by ABTS radical cation. 7 mM ABTS stock solution was mixed with 2.45 mM ammonium persulphate (1:1 v/v) and incubated overnight at room temperature in the dark. ABTS stock solution was diluted 1:20 with deionized distilled water as to obtain a solution of 0.5 OD600nm. Five hundred microliters of ABTS radical cation ($ABTS^{+}$) solution were mixed with 20 μL of sample or Trolox standard (2.3–150 μM range). Absorbance reduction at 734 nm was measured on a 48 well plate by using a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific) after 5 min of incubation. The inhibition percentage of absorbance at 734 nm was plotted as a function of the concentration of Trolox, and expressed as Trolox equivalent antioxidant capacity (μmol of TEAC/L).

7.2.10. Statistical analysis

All data are presented as mean ± SD among three replicates for each prepared sample. Statistical analyses were carried out using the IBM SPSS

Statistics release 20 (Armonk, NY, USA). Moisture percentages were normalized using arcsine-root transformation. Univariate analysis of variance (ANOVA) with post-hoc Tukey's test ($P < 0.05$) was applied for moisture percentages, protein, FFA contents in cheese samples after rejecting the homogeneity of their variances with Levene's test ($P < 0.05$). The General Linear Model (GLM) procedure, applying a two-way ANOVA ($P < 0.05$) was performed to evaluate individual effect of the factor studied (samples and treatments) and interaction effects between them on the antioxidant activity. Multiple comparisons among individual means were made by the Tukey post-hoc test ($P < 0.05$).

7.2. Result and discussion

7.3.1. *Selection of redox mediator in milk*

The analysis of SDS-PAGE profiles of UHT skim milk allowed to select the best mediator and the concentration responsible for protein crosslinking after 3 h of incubation (Fig. 7-1). No apparent changes were found in the protein profile of UHT milk incubated with Ery4 (lane 2), any mediator alone at 1 and 10 mM (lanes 3, 5, 7, 9, 11, 13), and Ery4 in combination with FA or VAN at 1 and 10 mM (lanes 4, 6, 8, 10). By contrast, the formation of protein bands with molecular weight in the range of 116 and 290 kDa was detected in milk samples incubated with 1 and 10 mM Ery4 and CGA (lanes 12 and 14, respectively). These data were in accordance with Cura et al. (2009), who reported that LC alone was not able to induce protein crosslinking, unless at very high concentrations.

As shown in Fig. 7-1, β- and α-CN were putatively involved in the formation of oligomers, as suggested by a slight reduction of the related bands in the electrophoretic profile of samples (lanes 12 and 14). All milk samples (laccase-treated and controls) were also centrifuged. This additional step allowed to put in evidence the occurrence of a precipitate in the samples treated with laccase and CGA mediator. On the other hand, the electrophoretic pattern of supernatants confirmed the reduction in the band intensity of caseins, as reported for the uncentrifuged samples (data not shown).

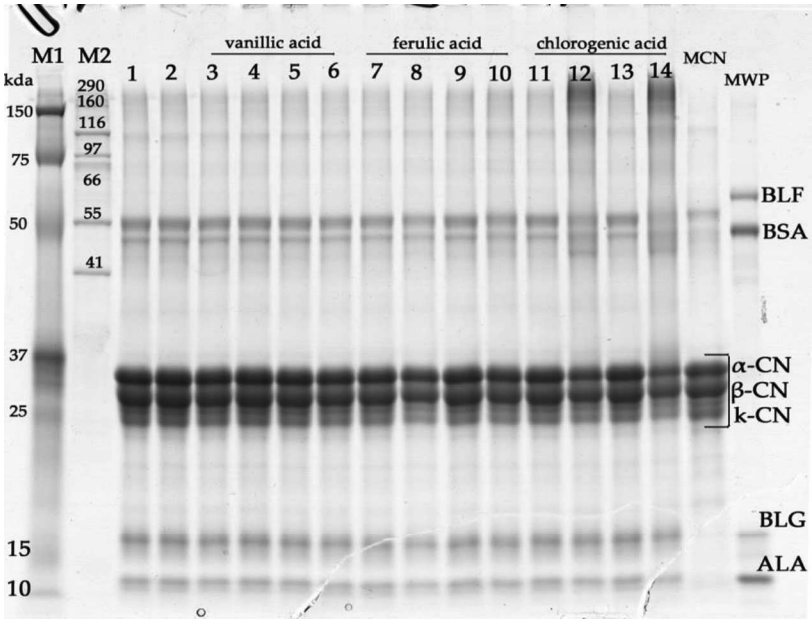


Figure 7-1. SDS-PAGE of UHT milk samples (7μg) obtained by the addition of laccase Ery4 (5 U/mL) and 1 or 10 mM of different mediators and incubated for 3 hours of incubation. Lane are: M1-prestained kaleidoscope marker (BioRad); M2-HiMark™ Unstained Protein Standard (Invitrogen,ThermoFisher Scientific); 1- milk control; 2- Milk + Ery4; 3- milk + 1 mM vanillin; 4- milk +Ery4 + 1 mM vanillin; 5- milk + 10 mM vanillin; 6- milk +Ery4 +10 mM vanillin; 7- milk + 1 mMferulic acid; 8- milk +Ery4 +1 mMferulic acid; 9- milk + 10 mMferulic acid; 10- milk +Ery4 + 10 mMferulic acid; 11- milk + 1 mMchlorogenic acid; 12- milk +Ery4 + 1 mMchlorogenic acid; 13- milk + 10 mMchlorogenic acid; 14- milk +Ery4 + 10 mMchlorogenic acid; 15- MCN, casein marker (5 μg); 16- MWP, whey protein solution (5 μg)

Recently, LMS was reported to crosslink caseins and lactoglobulins in yoghurt-like model systems (Struch et al., 2015), caseinate gels (Cura et al., 2009) and whey (Ali, Homann, Khalil, Kruse, & Rawel, 2013). Steffensen et al. (2008) also

demonstrated the formation of a reducible aggregate with an apparent mass above 206 kDa in SDS PAGE of α-casein substrates treated with LC-ferulic acid at room temperature for 24 h. The protein aggregate showed a too high molecular weight to enter into the gel. β-CN was instead crosslinked in presence of caffeic acid as mediator after 24 h of incubation (Stanic et al., 2009). Our results showed that the addition of CGA to milk greatly induced crosslink, even after only 3 h of incubation. In addition, no change was displayed in the electrophoretic profile when two different CGA concentrations were applied. This data was not in accordance with other studies applying LC in combination with lower amounts of CGA on porcine myofibrillar protein or yoghurt model in which an evident dose-response effect was reported (Struch et al., 2015). By way of comparison, in our study an higher CGA concentration (1 mM) was used in order to ensure the crosslinking effect.

Two different mechanisms were proposed to explain how the mediator promotes protein crosslinking in LMS: a) once oxidized into phenoxy radical by LC, it might stabilize by creating a reactive radical into the protein structure, which then undergoes a non-enzymatic polymerization with other proteins; b) the phenoxy radical might act as bridging agent between proteins, thus being incorporated into the protein structure (Mattinen et al., 2005). In particular, CGA was observed to preferentially promote crosslink in its oxidized dimeric form through covalent interactions with ε-amino group of lysine and the thiol group of cysteine (Liu, Ma, Gao, & McClements, 2017). These residues are widely abundant in milk proteins and therefore the crosslinking reaction by LMS could be strongly favoured (Steffensen et al., 2008).

Since the curd gel formation generally takes <1 h, depending on the rennet type and dairy process used (Fox et al., 2017), the crosslinking effect of CGA and Ery4 laccase was also evaluated after 30 and 60 min in UHT skim milk. Therefore, the high molecular weight oligomers and one 160-kDa band were formed both into the uncentrifuged Ery4-CGA treated sample after 30 min of incubation and its

supernatant after 60 min. (Fig. 7-2 lanes 14 and 16). No differences in the protein pattern emerged in samples treated only with LC or CGA alone (Fig. 7-2 lanes 1–13, 15). Based on these results the following experiments were performed applying 60 min incubation.

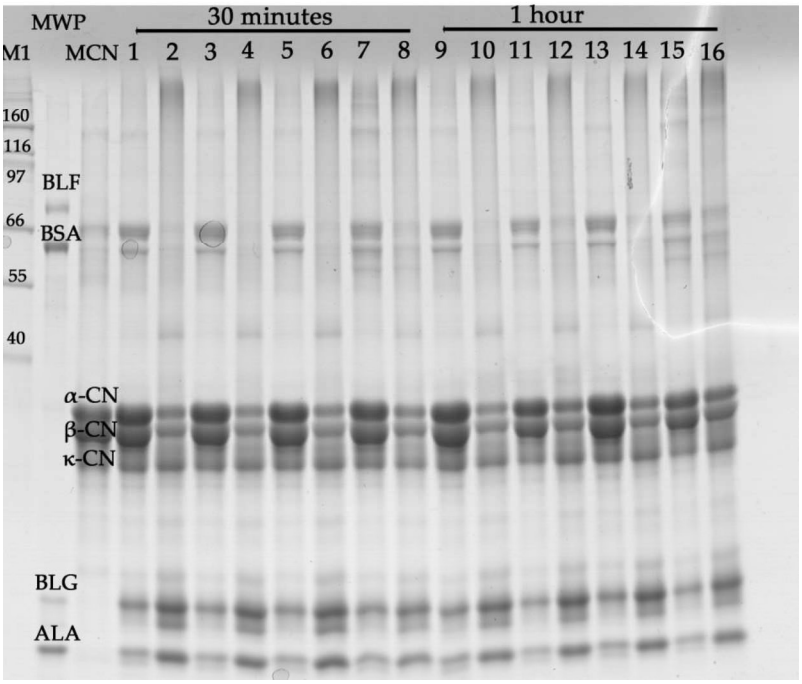


Figure 7-2. SDS-PAGE of UHT milk samples obtained by the addition of laccase Ery4 (5 U/ml) and 1 mM chlorogenic acid (CGA) and incubated for 30 min and 1 hour. Lanes are: M1: prestained kaleidoscope marker (BioRad); MWP- whey protein standard (5 μg); MCN: caseins standard (5 μg); 1,9-uncentrifuged milk; 2, 10- milk supernatant; 3, 11-uncentrifuged milk + 1mM CGA; 4, 12- milk + 1mM CGA supernatant; 5, 13-uncentrifuged milk + Ery4; 6, 14- milk + Ery4 supernatant; 7, 15-uncentrifuged milk + Ery4 + 1mM CGA; 8, 16- milk + Ery4 + 1mM CGA supernatant

7.3.2. Evaluation of protein crosslinking by selected mediator in whole milk

Similar trials were also performed in commercial pasteurized whole fat milk by assaying the ability of both Ery4 laccase preparation and cLC combined to CGA to crosslink milk proteins.

As demonstrated for UHT skim milk, the appearance of oligomers and additional bands around 160 kDa and 290 kDa were observed only in SDS PAGE protein profiles of whole fat milk treated with both Ery4 and the cLC combined

with the redox mediator CGA (Fig. 7-3, panel A, lanes 5, 6, 9, 10, 16, 17, 20, 21 and panel B lanes 7, 8, 10, 11).

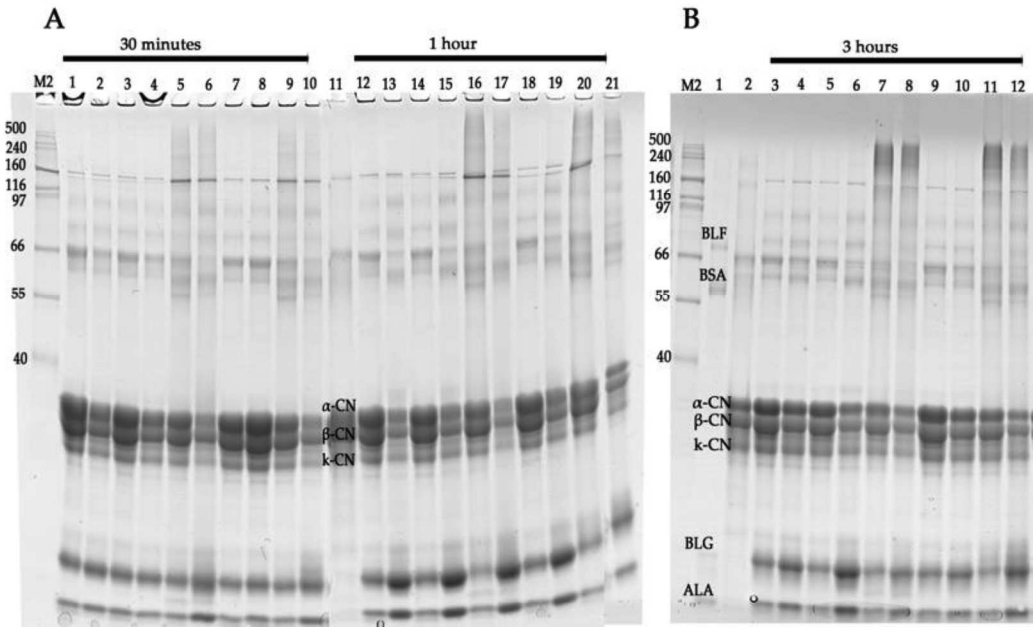


Figure 7-3. SDS-PAGE of uncentrifuged and supernatants pasteurized full fat milk amended with Ery4 (5U/mL), commercial laccase (cLC; 5 U/mL) and 1 mM chlorogenic acid (CGA) after 30 min, 1 hour (Panel A) and 3 hours of incubation (Panel B). Lanes are: Panel A: M2-HiMark™ Unstained Protein Standard (Invitrogen, Thermo Fisher Scientific); 1, 12-uncentrifuged milk, 2, 13- milk supernatant; 3, 14-uncentrifuged milk + Ery4, 4, 15- milk + Ery4 supernatant; 5, 16-uncentrifuged milk + Ery4 + 1mM CGA; 6, 17- milk + Ery4 + 1mM CGA supernatant; 7, 18-uncentrifuged milk + cLC; 8, 19- milk + cLCsupernatant; 9, 20-uncentrifuged milk + cLC + 1mM CGA; 10, 21- milk + cLC + 1mM CGA supernatant; 11- caseins standard (5 μg). Panel B. M2-HiMark™ Unstained Protein Standard (Invitrogen, ThermoFisher Scientific); 1- whey protein standard (5 μg); 2- caseins standard (5 μg); 3-uncentrifuged milk, 4- milk supernatant; 5-uncentrifuged milk + Ery4, 6- milk + Ery4 supernatant; 7-uncentrifuged milk + Ery4 + 1mM CGA; 8- milk + Ery4 + 1mM CGA supernatant; 9-uncentrifuged milk + cLC; 10- milk + cLC supernatant; 11-uncentrifuged milk + cLC + 1mM CGA; 12- milk + cLC + 1mM CGA supernatant.

These results showed that, under experimental conditions, the protein crosslinking activity of LMS was pretty consistent with that found in the skimmed milk samples by confirming that it was not affected by elevated content of fat. Thus, lab-scale curds were produced with LMS-treated milk in order to investigate the changes in milk coagulation, protein retention and antioxidant properties.

Overall, under experimental conditions, the curds obtained with Ery4-LMS system showed a significant increase in dry matter (+29.7%) compared to the control sample prepared with rennet alone. By contrast, the use of cLC with or without CGA resulted in none or negative effects, respectively (data not shown). These preliminary results, although obtained using small amounts of curd, suggested that milk protein crosslink might affect yield increase in cheese. However, further and large-scale cheese manufacturing trials are necessary to confirm this hypothesis. Moreover, curd trial results showed that the feasible pre-treatment of milk before adding rennet did not affect curdling conditions. By contrast, the application of the microbial TGase, used as protein crosslinker in cheese manufacture, can be added only after coagulum gel cutting (Cozzolino et al., 2003).

7.3.3. Total protein of curds

Total protein content increase was consistent with increase in dry matter for both LMSs samples (Table 7-1). However, a higher increase was measured for cLC and not for Ery4-LMS. A significant ($P < 0.01$) increase in total proteins was recorded only in samples containing laccase alone and laccase and mediator. No statistically significant differences in FAA and small peptides emerged among samples (data not shown).

Protein extraction with sodium citrate is usually applied to favor the solubilization of caseins and improve their quantification by Bradford reagent. Nonetheless, 80% of total proteins was recovered and quantified by Bradford assay in all samples except for those treated with LMS. An additional urea-mercaptoethanol solubilization was indeed required to recover the undissolved aggregates in both Ery4 and cLC-LMSs; these latter proteins constituted roughly 68% of total proteins.

Bradford reagent reactivity is greatly correlated with amino acid moiety of analyzed proteins showing high responses for basic protonated and aromatic

amino acids (Brady & Macnaughtan, 2015). In view of these considerations, an overestimation in protein content cannot be excluded.

Table 7-1. Content (g/100 g, wet matter) of retained proteins from curd samples fractionated according to the procedure described in Material and Methods. Curds were obtained by using the recombinant laccase Ery4 or the commercial laccase (cLC) alone and with chlorogenic acid (CGA). Control sample was curd prepared with rennet alone.

	Citrate-soluble	Citrate-insoluble	Pooled
Control	6.54 ± 0.40^{b}	0.91 ± 0.03^{a}	7.46 ± 0.42^{a}
CGA	6.60 ± 0.17^{b}	0.84 ± 0.08^{a}	7.44 ± 0.24^{a}
Ery4	8.14 ± 0.39^{c}	0.99 ± 0.08^{a}	9.12 ± 0.47^{b}
Ery4+CGA	2.80 ± 0.06^{a}	5.90 ± 0.43^{b}	8.70 ± 0.48^{b}
cLC	8.28 ± 0.08^{c}	0.98 ± 0.08^{a}	9.26 ± 0.14^{b}
cLC+CGA	2.68 ± 0.17^{a}	6.31 ± 0.38^{b}	8.99 ± 0.50^{b}

Values represent the averages ± standard deviation (N = 3). values with the same superscript letter are significantly similar (P> 0.05; HSD Tukey test) within each column.

7.3.4. Electrophoretic analysis of curd proteins

Protein profiles of curd samples analyzed by SDS-PAGE are shown in Fig. 7-4. No difference in protein pattern was found in curds prepared either with CGA or LC in comparison with control samples. These profiles were also similar to those displayed in milk samples. In contrast, curds obtained with LMSs, irrespective of the type of laccase used showed additional unresolved protein bands ranging from 55to 200 kDa. A ca. 50 kDa band was also observed in SDS-PAGE of these latter samples. The results are partially consistent with electrophoretic profiles shown by Struch et al. (2015) in yoghurt after LMS treatment with vanillic acid. These authors achieved an improvement of rheological properties of yoghurt due to the milk protein cross linking.

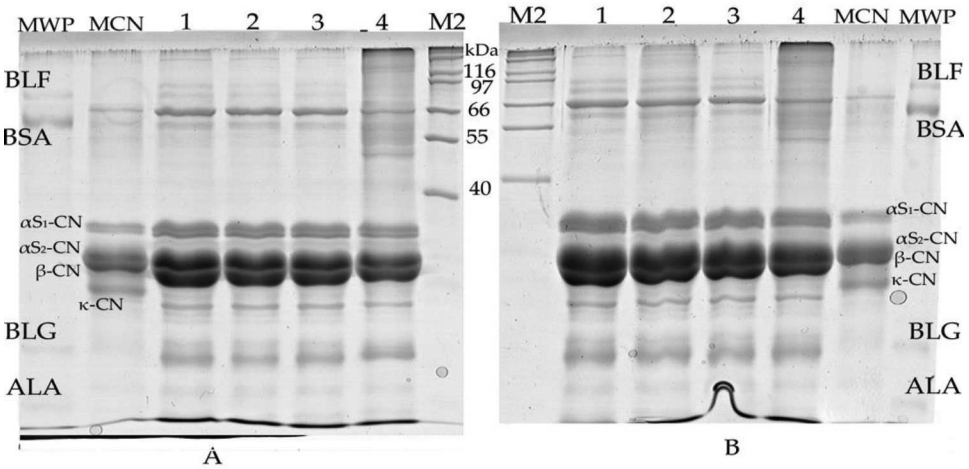


Figure 7-4. SDS-PAGE of curds proteins manufactured with Ery4 (A, 5U/mL) or the commercial laccase (B; 5 U/mL): Panel A) MWP- whey proteins standards (5 μg); MCN- caseins standards (5 μg); lane 1- milk; lane 2- milk + 1mM CGA; lane 3- milk + Ery4; lane 4: milk + Ery4 + 1 mM CGA; M2-prestained kaleidoscope marker (BioRad). Panel B) M2: prestained kaleidoscope marker (BioRad); lane 1- milk, lane 2- milk + 1mM CGA; lane 3- milk + commercial laccase; lane 4- milk + commercial laccase + 1mM CGA; MCS- caseins standards (5 μg), MWP- whey proteins standards (5 μg).

To date only microbial TGase (currently available as a commercial preparation containing 1% of active enzyme) has been widely exploited for its ability to restructure several foods such as meat, fish and bakery products. This feature is due to protein crosslinking by isopeptide bonds between glutamine and lysine residues (Kieliszek & Błażejak, 2017). However, apart from the positive effect on syneresis reduction during yoghurt processing, TG application in dairy production was limited to few cheese types, especially brined semi-hard cheeses (Cozzolino et al., 2003; Özer, Guyot, & Kulozik, 2012. In fact, the addition of TGase in milk before rennet prevented primary phase of coagulation and required a strong heat treatment to inactivate the enzyme inhibitors (Bönisch, Heidebach, & Kulozik, 2008).

Moreover, mTG application after rennet lead to increased clotting time, use of expensive co-factors, such as glutathione and high enzyme amounts (Özer et al., 2012). To the best of our knowledge, no evidence of TG mediated crosslink in cheese was ever reported, other than the indirect observation of protein bands disappearance from whey (Cozzolino et al., 2003).

7.3.1. *Antioxidant activity of digested curds*

The antioxidant activity of curds is showed in Table 7-2. The two-way ANOVA showed a statistically significant interaction between the effects of simulated gastro-pancreatic digestion and cheese treatments on antioxidant activity of curd ($F(5, 24) = 494.515$. $P = 1 \times 10–23$). In fact, simple main effects analysis showed that the digestion process significantly increased the antioxidant activity of curd when LMS (neglecting laccase type) or none was applied ($P < 0.0001$). In contrast, concerning curds with CGA or cLC alone, the digestion significantly reduced the antioxidant activity ($P < 0.0001$). No significant difference was found between undigested and digested samples treated with Ery4 alone ($P > 0.05$).

Table 7-2. Antioxidant activity of supernatants from *in vitro* digested curds prepared with recombinant or commercial laccase (Ery4 and cLC, respectively) alone or in combination with chlorogenic acid (CGA) expressed as Trolox equivalent antioxidant capacity (TEAC/L)

Antioxidant activity (μmol of TEAC/L)		
	Undigested	Digested
Control	145.3 ± 7.4^{a}	586.4 ± 18.8^{e}
CGA	843.4 ± 11.1^{f}	315.9 ± 3.9^{c}
Ery4	279.7 ± 51.7^{c}	271.7 ± 6.7^{c}
Ery4+CGA	1607.3 ± 15.2^{g}	2273.3 ± 5.8^{i}
cLC	395.5 ± 80.2^{d}	225.2 ± 3.2^{b}
cLC+CGA	888.7 ± 23.4^{f}	1796.5 ± 17.2^{h}

Values represent means ± standard deviation (N = 3). Values with the same superscript letter are significantly similar (*P*> 0.05; HSD Tukey test).

Overall, the highest values of antioxidant activity was registered in both undigested and digested curds treated with Ery4 based LMS (1607.3 and

2273.3 μM TEAC/L, respectively), whereas the respective cLC-based LMS samples were significantly ($P < 0.0001$) less active (888.7 and 1796.5 μM TEAC, respectively), although, upon digestion, their antioxidant power rose more than that of Ery4-LMS cheeses (1 and 0.4 times, on average).

Milk proteins are by themselves a source of antioxidant peptides after gastrointestinal digestion, as confirmed by antioxidant activity of control sample (586.4 μmol Trolox/L), in accordance with the study by Helal et al. (2015).

The covalent binding between polyphenols and proteins was already reported to increase the antioxidant capacity of polyphenol and protein mixtures (Huang, Zhang, Liu, & Li, 2012) and was also proposed as an effective tool to deliver antioxidant compounds with low bioavailability (Wang & Gao, 2013). Presumably, the covalent binding occurs by nucleophilic attack on the C6 position of the oxidized CGA or CGA dimer by the amino or thiol groups to form an N-quinoyl-amino acid derivative. According to this hypothesis, the hydroxyl groups remain available after protein crosslinking, with no reduction in radical scavenging and antioxidant activity (Liu et al., 2016).

Gallo, Vinci, Graziani, De Simone, and Ferranti (2013) also reported a significant reduction in antioxidant activity when different polyphenols, including CGA, were added to caseins and lactoglobulins standard solutions. CGA was reported to strongly interact with both and specifically bind to cysteine residues, thereby masking the hydroxyl residues responsible for the antioxidant activity (Liu et al., 2016). By contrast, Helal et al. (2015) reported an increase in antioxidant capacity of curd when different polyphenols, including CGA, were added in curds. Nevertheless, the increase in antioxidant activity that was reported was lower than that found in this study, despite they used a slightly higher amount of CGA (1.4 *vs* 1 mM).

7.4. Conclusions

For the first time a laccase-mediator system (LMS) was successfully applied to manufacture curd to improve both actual yield and antioxidant properties. Among different phenolic mediators screened in combination with the purified recombinant Ery4 laccase, CGA triggered an appreciable protein crosslinking in UHT skimmed and full fat milk samples within only 1 h of incubation at 38 °C. In a lab-scale curd manufacture a 45 min milk pretreatment with LMS before rennet addition allowed to obtain curds showing crosslinked proteins compared to the control sample. Indeed, no changes in dairy process conditions were needed.

Besides, the antioxidant effect of CGA added to curd was boosted by LMS action leading to a final product with notable nutritional value. This effect was indeed enhanced after a simulated gastro-pancreatic digestion, hypothesizing an increase bioavailability of CGA.

The application of the LMS in curd manufacture may fill the gap in the utilisation of food crosslinkers that are currently limited to other dairy products. Important technological, economic, nutritional and nutraceutical related outcomes for dairy chain could emerge and open new perspectives for further studies for new and innovative applications in cheese and other dairy products manufacturing.

Acknowledgments

We would like to acknowledge Valentina Mirabelli of the Institute of Crystallography of the National Research Council of Italy for technical assistance for the purification of Ery4 enzyme.

References

Ali, M., Homann, T., Khalil, M., Kruse, H. P., &Rawel, H. (2013). Milk whey protein modification by coffee-specific phenolics: effect on structural and functional properties. *Journal of Agricultural and Food Chemistry*, 61, 6911-6920.

AOAC (2012). Solids (Total) in Milk, By Direct Forced Air Oven Drying. Official method 990.20. Association of Official Analytical Chemists (AOAC) International G.W. Latimer Jr. (Ed.), Official methods of analysis of AOAC international (19th ed.), Vol. 2, AOAC International, Gaithersburg, MD, USA (2012)

Bastide, N., Dartois, L., Dyevre, V., Dossus, L., Fagherazzi, G., Serafini, M., & Boutron-Ruault, M. C. (2017). Dietary antioxidant capacity and all-cause and cause-specific mortality in the E3N/EPIC cohort study. *European Journal of Nutrition*, 56, 1233-1243.

Bennett, R. J., & Johnston, K. A. (2004). General aspects of cheese technology. In P. F. Fox, P. L. H. McSweeney, T. M. Cogan, & T. P. Guinee (Eds.), Cheese: chemistry, physics and microbiology, Vol. 2, Major cheese groups, pp. 23–50. London: Elsevier Academic Press.

Bönisch, M. P., Heidebach, T. C., & Kulozik, U. (2008). Influence of transglutaminase protein cross-linking on the rennet coagulation of casein. *Food Hydrocolloids*, 22, 288-297.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.

Brady, P. N., & Macnaughtan, M. A. (2015). Evaluation of colorimetric assays for analyzing reductively methylated proteins: biases and mechanistic insights. *Analytical Biochemistry*, 491, 43-51.

Buchert, J., Ercili Cura, D., Ma, H., Gasparetti, C., Monogioudi, E., Faccio, G., ... & Lantto, R. (2010). Crosslinking food proteins for improved functionality. *Annual review of food science and technology*, 1, 113-138.

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins1. *Journal of Dairy Science*, 66, 1219-1227.

Cozzolino, A., Di Pierro, P., Mariniello, L., Sorrentino, A., Masi, P., & Porta, R. (2003). Incorporation of whey proteins into cheese curd by using transglutaminase. *Biotechnology and Applied Biochemistry*, 38, 289-295.

Cura, D. E., Lantto, R., Lille, M., Andberg, M., Kruus, K., & Buchert, J. (2009). Laccase-aided protein modification: effects on the structural properties of acidified sodium caseinate gels. *International Dairy Journal*, 19, 737-745.

European commission. Food enzyme applications submitted to the Commission within the legal deadline (from 11 September 2011 to 11 March 2015).

Fox, P. F., Guinee, T. P., Cogan, T. M., & Mc Sweeney, P. L. (2017). Enzymatic coagulation of milk. *Fundamentals of cheese science*, 185-229. Springer US.

Gallo, M., Vinci, G., Graziani, G., De Simone, C., & Ferranti, P. (2013). The interaction of cocoa polyphenols with milk proteins studied by proteomic techniques. *Food Research International*, 54, 406-415.

Giardina, P., &Sannia, G. (2015). Laccases: old enzymes with a promising future. *Cellular and Molecular Life Sciences*, 72, 855.

Helal, A., Tagliazucchi, D., Verzelloni, E., & Conte, A. (2015). Gastro-pancreatic release of phenolic compounds incorporated in a polyphenols-enriched cheese-curd. *LWT-Food Science and Technology*, 60, 957-963.

Huang, W. Y., Zhang, H. C., Liu, W. X., & Li, C. Y. (2012). Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry. Nanjing. *Journal of Zhejiang University-Science B*, 13, 94-102.

Kieliszek, M., Błażejak, S. (2017). Microbial Transglutaminase and Applications in Food Industry. In : Ramesh C.R. & Rosell C.M., eds. *Microbial Enzyme Technology in Food Applications*, p. 180-198.Taylor & Francis Group, Boca Raton, FL.

Liu, F., Ma, C., Gao, Y., & Mc Clements, D. J. (2017). Food-Grade Covalent Complexes and Their Application as Nutraceutical Delivery Systems: A Review. *Comprehensive Reviews in Food Science and Food Safety*, 16, 76-95.

Liu, J., Wang, Q., Zhang, H., Yu, D., Jin, S., Ren, F. (2016). Interaction of chlorogenic acid with milk proteins analyzed by spectroscopic and modeling methods. *Spectroscopy Letters An International Journal for Rapid Communication*, 49, 44-50

Loi, M. Fanelli, F. Zucca, P. Liuzzi, V.C. Quintieri, L. Cimmarusti, M.T. Monaci, L. Haidukowski, M. Logrieco, A.F. Sanjust, E. Mulè, G. (2016). Aflatoxin B_1 and M_1 Degradation by Lac2 from *Pleurotus pulmonarius* and Redox Mediators. *Toxins* .8, 245.

Loi, M., Quintieri, L., Fanelli, F., Liuzzi, V.C., Haidukowski, M., Logrieco, A.F., Mulè, G. (2017) Aflatoxin M_1 removal and biotechnological potential of a laccase from *Pleurotus eryngii* for milk safety. *Scienza e tecnica lattiero casearia*. 67, 23-32

Margot, J., Bennati-Granier, C., Maillard, J., Blánquez, P., Barry, D. A., &Holliger, C. (2013). Bacterial versus fungal laccase: potential for micropollutant degradation. *AMB Express*, 3, 63-77

Masotti, F., Cattaneo, S., Stuknytė, M., & De Noni, I. (2017). Technological tools to include whey proteins in cheese: Current status and perspectives. *Trends in Food Science & Technology*.

Mattinen, M. L., Kruus, K., Buchert, J., Nielsen, J. H., Andersen, H. J., & Steffensen, C. L. (2005). Laccase-catalyzed polymerization of tyrosine-containing peptides. *The FEBS Journal*, 272, 3640-3650.

McDougall, G. J. (2017). Phenolic-enriched foods: sources and processing for enhanced health benefits. *Proceedings of the Nutrition Society*, 76, 163-171.

Owusu-Apenten RK (2002). Bradford assay applications *Food protein analysis*. *Quantitative effects on processing* (pp-198-201) Ed. New York Marcel Dekker.

Özer, B., Guyot, C., & Kulozik, U. (2012). Simultaneous use of transglutaminase and rennet in milk coagulation: Effect of initial milk pH and renneting temperature. *International Dairy Journal*, 24, 1-7.

Pezzella, C., Guarino, L., & Piscitelli, A. (2015). How to enjoy laccases. *Cellular and Molecular Life Sciences*, 72, 923-940.

Quintieri, L., Monaci, L., Baruzzi, F., Giuffrida, M. G., de Candia, S., Caputo, L. (2017). Reduction of whey protein concentrate antigenicity by using a combined enzymatic digestion and ultrafiltration approach. *Journal of Food Science and Technology*, 54, 1910-1916.

Sato, A. C. K., Perrechil, F. A., Costa, A. A. S., Santana, R. C., & Cunha, R. L. (2015). Cross-linking proteins by laccase: Effects on the droplet size and rheology of emulsions stabilized by sodium caseinate. *Food Research International*, 75, 244-251.

Selinheimo, E., Kruus, K., Buchert, J., Hopia, A., &Autio, K. (2006). Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. *Journal of Cereal Science*, 43, 152-159.

Stanic, D., Radosavljevic, J., Polovic, N., Jadranin, M., Popovic, M., Vuckovic, O., Burazerc L., Jankovb, R., Velickovic, T.C. (2009). Removal of N-terminal peptides from β-lactoglobulin by proteolytic contaminants in a commercial phenol oxidase preparation. International dairy journal, 19(12), 746-752.

Steffensen, C. L., Andersen, M. L., Degn, P. E., & Nielsen, J. H. (2008). Cross-linking proteins by laccase-catalyzed oxidation: importance relative to other modifications. *Journal of Agricultural and Food Chemistry,* 56, 12002-12010.

Struch, M., Linke, D., Mokoonlall, A., Hinrichs, J., & Berger, R. G. (2015). Laccase-catalysed cross-linking of a yoghurt-like model system made from skimmed milk with added food-grade mediators. *International Dairy Journal*, 49, 89-94.

Wang, S. Y., & Gao, H. (2013). Effect of chitosan-based edible coating on antioxidants, antioxidant enzyme system, and postharvest fruit quality of strawberries (Fragaria x aranassaDuch.). *LWT-Food Science and Technology*, 52, 71-79.

Zeeb, B., Mc Clements, D. J., & Weiss, J. (2017). Enzyme-Based Strategies for Structuring Foods for Improved Functionality. *Annual review of Food Science and Technology*, 8, 21-34.

8.1. Zearalenone degradation in naturally contaminated maize flour by Ery4 laccase mediator system

Naturally contaminated maize flour was mixed 1:3 (w/v) with sodium acetate buffer 1 mM, pH 5 containing 10 mM SA and 5 U/mL of Ery4. The obtained slurry was incubated at 25°C in shaking conditions (120rpm). After 5 days of incubation ZEN was quantified by HPLC analysis (EN 15850:2010, 2010) and degradation percentage was calculated with respect to the control.

Zearalenone degradation percentage in maize flour is shown in Table 8-1. Comparison with *in vitro* degradation (see chapter 5) was also provided. After 5 days of incubation ZEN was reduced by 40% by LMS. The extent of degradation was lower with respect to the *in vitro* assay, where the complete removal of the toxin was achieved. Possibly, the higher mycotoxin concentration or the matrix effect could have reduced the effectiveness of the treatment, which had been only tested *in vitro* with significant lower toxin amounts. Microbial degradation of ZEN was reported for *Rhizopus*, *Streptomyces, Cunninghamella* and *Bacillus* spp. (Ji et al., 2016). However, to best of our knowledge, this is the first evidence of the effective enzymatic degradation of ZEN in a real food matrix. ZEN degradation by LMS was, indeed, proved only *in vitro* (Banu et al., 2013; Loi et al., 2017).

Table 8-1: Zearalenone (ZEN) degradation (%). Comparison between *in vitro* (previous study) and *in matrix* degradation by Ery4 and syringaldehyde

	In vitro	*In vivo*
ZEN degradation %	1 μg/mL	8 μg/g
	100	40 ± 1

For the first time an enzymatic method exploiting the LMS was applied for ZEN degradation in a real, naturally contaminated matrix. ZEN reduction accounted for 40% despite the high level of contamination. These results are promising for the

development of an effective, yet environmental friendly method for ZEN reduction in maize and maize products.

References

Banu, I., Lupu, A., Aprodu, I. (2013). Degradation of zearalenone by laccase enzyme. *Sci. Study Res*. 14, 79–84

EN 15850:2010 Foodstuffs. Determination of zearalenone in maize based baby food, barley flour, maize flour, polenta, wheat flour and cereal based foods for infants and young children

Commission Regulation 2006/1881/EC of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Food stuffs. (2006). Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1881&from=en (accessed on 03 November 2016).

Ji, C., Fan, Y., Zhao, L. (2016). Review on biological degradation of mycotoxins. *Animal Nutrition*. 2, 127-133.

Lattanzio, V.M.T., Ciasca, B., Powers, S., Visconti, A. (2014). Improved method for the simultaneous determination of aflatoxins, ochratoxin A and Fusarium toxins in cereals and derived products by liquid chromatography–tandem mass spectrometry after multi-toxin immunoaffinity clean up. *Journal of Chromatography*. 1354, 139–143

Loi, M., Quintieri, L., Fanelli, F., Liuzzi, V.C., Haidukowski, M., Logrieco, A.F., Mulè, G. (2017). Rimozione della aflatossina M1 e potenziali applicativi di una laccasi da *Pleurotus eryngii* per la sicurezza del latte. *Scienza e tecnica lattiero casearia*. 67, 23-32.

Loi, M., Fanelli, F., Zucca, P., Liuzzi, V.C., Quintieri, L., Cimmarusti, M.T., Monaci, L., Haidukowski, M., Logrieco, A.F., Sanjust, E., Mulè, G. (2016). Aflatoxin B_1 and M_1 Degradation by Lac2 from Pleurotus pulmonarius and Redox Mediators. *Toxins*. 8, 245.

Visconti, A., Pascale, M. (1998). Determination of zearalenone in corn by means of immunoaffinity clean-up and high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A.* 815, 133-140

8.2. Design of experiment for *in vitro* aflatoxin B_1 degradation with laccase mediator system

A D-optimal design consisting of 15 randomized runs was performed in order to determine the effects of four independent variables on the degradation of AFB_1. Chosen factors were Ery4 concentration (0.5-5 U/mL), mediator concentration (1 – 12 mM), AFB_1 concentration (0.05 – 2 μg/ mL) and time of reaction (1-72 h) (Table 8-2). A purely quadratic model was used to describe the behaviour of the system.

Table 8-2. Experimental runs of D-optimal design and response for aflatoxin B_1 (AFB_1) measured as degradation (%) ± standard deviation (sd)

Run no.	**Ery4 (U/mL)**	**Mediator (mM)**	**AFB_1 (μg/ mL)**	**Time (h)**	**Degradation (%)**	**sd**
1	0.5	1	0.05	12	39	1
2	0.5	4	0.50	48	56	2
3	0.5	10	1.00	72	68	2
4	1.0	4	1.00	1	50	3
5	1.0	10	2.00	48	67	1
6	1.0	12	0.50	72	62	2
7	1.5	1	0.50	6	28	3
8	1.5	4	2.00	12	52	1
9	1.5	8	0.05	1	37	3
10	3.5	1	1.00	48	31	3
11	3.5	8	2.00	6	55	2
12	3.5	10	0.50	12	53	2
13	5.0	1	2.00	1	32	2
14	5.0	4	0.05	6	32	2
15	5.0	8	1.00	12	55	2

A graphical representation of the results is given in Fig. 8-1. Higher degradation percentages (62 – 68%) were registered when high mediator concentrations were used (10 – 12 mM), even with low enzyme concentrations (0.5 - 1 U/mL) and regardless of the toxin amounts (runs 3, 5, 6). As regards time, 48 to 72h are needed to obtain the best results.

These results are interesting, since it seems that low enzyme amounts are needed in order to achieve high levels of degradation (for a comparison, see chapters 4 and 5). This is a strong point of improvement, since enzyme is usually the limiting parameter for the economic sustainability for the industrial application of enzyme based processes. However, since the highest degradation percentages were registered in correspondence to the range limits or very close to them, a new range elaboration is needed to identify the best values of each parameter for the optimization of the degradation of AFB_1.

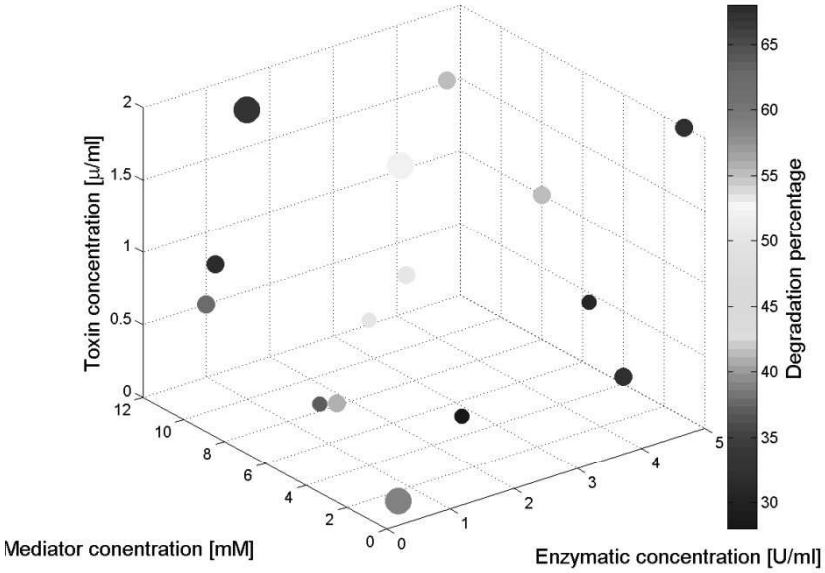


Figure 8-1: Scatter plot of aflatoxin B_1 degradation by Ery4 and syringaldehyde. Dots represent the measured experimental runs.

8.3. Aflatoxin M_1 degradation by immobilized Ery4 and syringaldehyde

Ery4 immobilization in acrylamide – poyethylenglycoldimethylacrylate 750 hydrogel matrix was performed as described by Vittorio and colleagues (2016) using ammonium persulphate as initiator and 300μL of Ery4 purified solution (24 U/mL). Control hydrogels were also produced in absence of LC. Hydrogels were stored in dried form and hydrated overnight with milliq water before use. Hydrogels containing 0.97U of active enzyme were used for two parallel and consecutive experiments to evaluate reusability and catalytic efficiency. Immobilized Ery4 was also compared to the free enzyme in an analogous assay.

AFM_1 degradation assay was performed in sodium acetate buffer 1 mM, pH 5 added with 10 mM of syringaldehyde (SA) as redox mediator and 0.05 μg/mL of toxin. Hydrogels were soaked in and kept in dark at 25°C in shaking conditions (120rpm). In the first experiment (1st run), aliquots of each sample were withdrawn after 6, 10, 24, 48, 72, 96h. At the end of the assay, hydrogels were washed for 1h with buffer solution and for an additional hour with methanol:water 80 : 20 (w/w) to evaluate the possible adsorbed AFM_1. The second experiment (2nd run) was performed prolonged until 11 days; aliquots of each sample were withdrawn after 3h, 1, 4, 7 and 11 days.

AFM_1 reduction was evaluated by HPLC analysis as reported by Loi et al., (2016). Degradation percentages were calculated as follows:

$$\%\, aflatoxin\ degradation = \frac{aflatoxin_{sample}}{aflatoxin_{control}} \times 100$$

Results are shown in Fig. 8-2. Degradation of AFM_1 by the immobilized Ery4 followed a linear fit in both runs (R^2 = 0.9944 in the 1st run and R^2 = 0.9386 in the 2nd run) and degradation efficiencies in the two cycles were comparable, confirming that Ery4 can be efficiently re-used with no apparent loss of catalytic activity. After 4 days

of assay, AFM_1 degradation by the immobilized Ery4 reached 43% (1st run) and 36% (2nd run). After 11 days, AFM_1 degradation was 54%.

The free enzyme was more efficient than the immobilized one, as AFM_1 was halved within 4 days of assay (56% of degradation vs 43%), and almost completely removed within 11 days (97% vs 54%). The characteristics of an enzyme, such as catalytic efficiency and kinetic behaviour, greatly vary in free or immobilized form. A

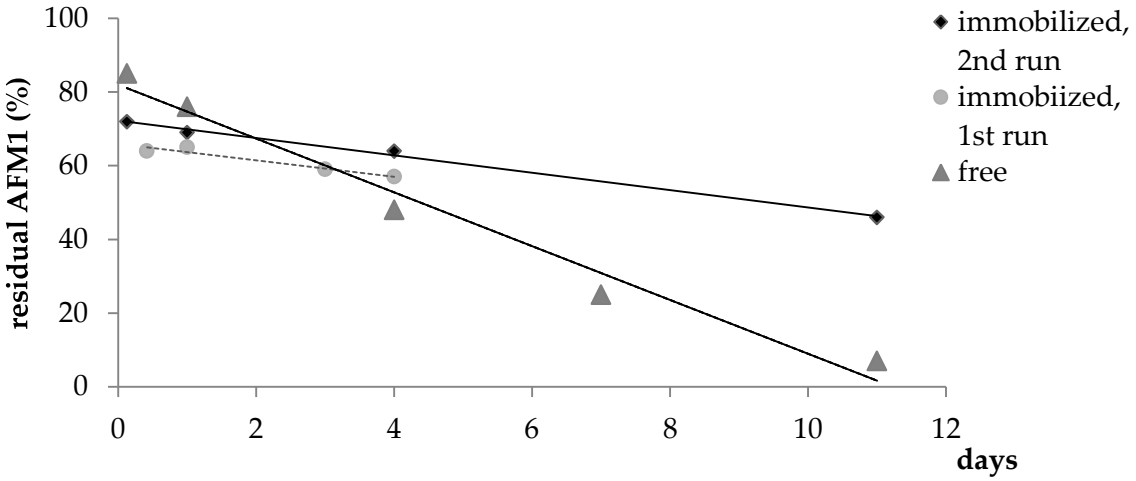


Figure 8-2. Time course of *in vitro* degradation of aflatoxin M_1 (AFM_1) by immobilized and free Ery4 and syringaldehyde. Residual toxin percentages and linear trend are indicated.

possible explanation is that when immobilized, Ery4 might undergo internal structural, conformational or orientation changes which may result in restricted access to the active site (Dwevedi, 2016). Also enzyme loading has a significant impact on degradation kinetics: using higher LC amounts switches the kinetic from linear (zero order reaction) to logarithmic (2nd order reaction, Loi et al., 2017) in the free form.

Ery4 could be effectively immobilized in the hydrogel matrix for AFM_1 degradation. However, further investigations will be needed to evaluate if a higher enzyme loading on hydrogel can increase the rate of degradation in order to apply this technology in a concrete application.

References

Dwevedi, A. (2016). Basics of Enzyme Immobilization in Enzyme Immobilization Advances in Industry, Agriculture, Medicine, and the Environment. Springer Ed.

Loi, M., Fanelli, F., Zucca, P., Liuzzi, V.C., Quintieri, L., Cimmarusti, M.T., Monaci, L., Haidukowski, M., Logrieco, A.F., Sanjust, E., Mulè, G. (2016). Aflatoxin B1 and M1 Degradation by Lac2 from Pleurotus pulmonarius and Redox Mediators. *Toxins*. 8, 245.

Loi, M., Quintieri, L., Fanelli, F., Liuzzi, V.C., Haidukowski, M., Logrieco, A.F., Mulè, G. (2017). Rimozione della aflatossina M1 e potenziali applicativi di una laccasi da *Pleurotus eryngii* per la sicurezza del latte. *Scienza e tecnica lattiero casearia*. 67, 23-32.

Vittorio, O., Cojoc, M., Curcio, M., Spizzirri, U.G., Hampel, S., Nicoletta, F.P., Iemma, F., Dubrovska, A., Kavallaris, M., Cirillo, G. (2016). Polyphenol Conjugates by Immobilized Laccase: The Green Synthesis of Dextran-Catechin. *Macromolecular Chemistry and Physics*. 217, 1488-1492.

Conclusions and perspectives

Conclusions and perspectives

The present study aimed at investigating the capability of LC enzymes to degrade the most important classes of mycotoxins. In particular, two different enzymes were used: the native Lac2 and the recombinant Ery4.

Lac2 is a well characterized laccase from *Pleurotus pulmonarius*, which has been applied for the degradation of different industrial dyes. During this study, Lac2 was identified, purified by means of several chromatographic steps and characterized for the degradation of AFB_1 and AFM_1.

Ery4 is a recombinant laccase, cloned from *P. eryngii*, a local, edible mushroom. Purification by a single chromatographic step was used to easily produce high amounts of enzyme to perform an extensive study on its degradation capabilities towards the main classes of mycotoxins.

A different behaviour was observed with regards to the direct degradation of AFs. Structural differences in the active sites might be implicated, allowing Lac2, but not Ery4, to oxidize AFs even when no mediator is added. Nonetheless, for both enzymes the mediator is essential to achieve high levels of degradation.

The use of the mediator seems to fill the efficiency gap between the two enzymes, suggesting that the oxidized mediator is responsible for the mycotoxin degradation. The slight differences in degradation percentages are not substantial, and can be easily explained by the different mediator specificity of each LC. In view of the development of possible application, it is very important to underline that the LMS system is responsible for the degradation and not solely LC. Theoretically, any LC with an adequate specificity towards the effective mediators might be used within the LMS for this purpose. This means that low cost enzymes, or requiring easy and fast production, purification and immobilization processes could be used.

Based on the results obtained in this study, the use of LC enzymes appears as a versatile, efficient and environmental friendly strategy to counteract mycotoxin occurrence in food and feed. However, there are still some critical points that need to be investigated.

A mandatory requirement for the application of a biotransforming method in feed is the elucidation of the toxicity of the degradation products. The identification of degradation products is the first step towards the evaluation of their toxicity, which must be tested *in vitro* and in target animals, as required by EFSA.

This is a very important and debated topic in the scientific community, and so far, mycotoxin degradation products deriving from LC or LMS treatments have not been identified. There are several difficulties linked with the identification of the degradation products. A single technique is often insufficient to identify unknown compounds. The use of combined spectroscopic techniques, such as mass spectrometry, nuclear magnetic resonance or infrared spectroscopy is often required. LMS activity is rather non-specific and often of radical nature: it is difficult to predict or to search for a specific degradation product, likely more than one; instruments are often not sensitive enough to detect small amounts, especially if more than one product is formed after LMS treatment. To overcome the low instrument sensitivity, very high concentrations should be used. However, mycotoxins are toxic at very low concentration, so the amount which can be manipulated with acceptable risk for operators is also low. Additionally, some of the toxins are poorly soluble in water and using organic solvents to increase solubility would hinder enzyme activity and its efficacy.

Validation of the *in vitro* results should be performed in real, possibly naturally contaminated matrices. Food components, such as carbohydrates, proteins or lipid, might reduce degradation rate or effectiveness or the enzymatic method, lower the bioavailability of mycotoxins or mask them in a conjugated or

bound form. It is important to validate the *in vitro* methods in the specific raw materials taking into consideration all the possible masked forms. A new parameter optimization should be also considered, if necessary.

Additionally, the overall nutritional and sensory attributes of the treated material, as well as its production process should not be significantly modified. Considering LC catalytic activity, oxidation of valuable nutrients should be strongly monitored. Conversely, the natural presence of phenols could be exploited not to add any mediator or to avoid a further addition.

Mycotoxin biotransformation in food intended for human consumption is not allowed yet in EU. Nonetheless, LC is a versatile enzyme which can also be used for other purposes in the food industry. For example, LMS is able to induce protein crosslink and could be used to modify the rheological, functional or allergenic properties of milk proteins, for the formulation of improved or novel milk based products.

Despite the critical points discussed above, the overall results are encouraging and promising and lay down the basis for the development of a LC based approach able to reduce the contamination of structurally diverse mycotoxins *in vitro* and in real food matrices.