Microbial Degradation of Mycotoxins

By

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SUMMARY

Aflatoxins are mycotoxins predominantly produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B_1 (AFB_1), the most abundant aflatoxin, is highly mutagenic, toxic, carcinogenic and teratogenic to humans and animals and is particularly correlated with the incidence of hepatocellular carcinoma in parts of Africa, China and South East Asia. In this regard aflatoxin is classified as a type I human carcinogen by the International Agency for Research on Cancer. Furthermore, aflatoxin contamination of food and feed is responsible for extensive economic losses due to loss of crops and farm animals.

In spite of regulations regarding acceptable levels of aflatoxin in food, aflatoxin contamination remains a serious worldwide problem, especially in developing countries where it occurs predominantly in dietary staples. Inactivation of aflatoxin by physical and chemical methods has not yet proved to be effective and economic. However, biological detoxification offers an attractive alternative for eliminating toxins as well as safe-guarding the desired quality of food and feed.

In this study, the biological degradation of AFB_1 by bacteria and fungi was investigated. Several bacteria, including *Rhodococcus* spp., as well as white rot fungi have the potential to degrade a wide range of polycyclic hydrocarbon compounds due to the large repertoire of enzymes they produce and therefore the ability of some of these microorganisms to degrade AFB_1 was investigated. Effective degradation of AFB_1 by intracellular extracts of *Mycobacterium fluoranthenivorans* sp. nov. DSM 44556^T, *Nocardia corynebacterioides* DSM 20151 and *N. corynebacterioides* DSM 12676 was demonstrated. Furthermore, AFB_1 was effectively degraded by liquid cultures as well as intra- and extracellular extracts of *Rhodococcus erythropolis* DSM 14303. Significant ($P<0.001$) reduction in AFB_1 was observed following treatment with *R. erythropolis* extracellular extracts with only 33.20% residual AFB_1 after 72 h. Results indicated that the degradation by *R. erythropolis* DSM 14303 is enzymatic and that the enzymes are constitutively produced. The degradation of AFB_1 when treated with *R. erythropolis* DSM 14303 extracellular extract coincided with a total loss of mutagenicity. In addition, treatment of AFB_1 with culture fractions containing recombinant 2,3-dihydroxybiphenyl

dioxygenase, which was produced through extracellular expression of the *bph*C1 gene of *R. erythropolis* DSM 14303 in *Escherichia coli* BL21, resulted in significant ($P<0.0001$) degradation (49.32%) and reduced mutagenic potency (42.47%) of the molecule.

Significant ($P<0.0001$-0.05) degradation of AFB_1 was obtained following treatment with culture extracts containing laccase enzyme produced by white rot fungi (17.10-76.00%), purified fungal laccase from *Trametes versicolor* (1 U/ml, 87.34%) as well as with recombinant laccase produced by *Aspergillus niger* (118 U/L, 55.00%). Furthermore, treatment of AFB_1 with purified fungal laccase enzyme (1 U/ml) resulted in loss of the mutagenic potency of the molecule. The decrease in the fluorescence and mutagenic properties of AFB_1 following treatment with the microbial preparations imply changes to the furofuran- and/or lactone rings of the molecule.

The current study contributes towards developing genetic engineered microbial strains which could be applied as an important bio-control measure. Such strains could exhibit multifunctional technological properties including degradation of AFB_1, to significantly improve the quality, safety and acceptability of food.

OPSOMMING

Aflatoksiene is mikotoksiene wat hoofsaaklik deur die filamentagtige fungi, *Aspergillus flavus* en *Aspergillus parasiticus* geproduseer word. Die algemeenste aflatoksien, aflatoksien B_1 (AFB_1), is hoogs mutagenies, toksies, karsinogenies en teratogenies vir mense en diere. Veral in sekere dele van Afrika, China en Suid-Oos Asië bestaan daar `n korrelasie tussen aflatoksien en die voorkoms van hepatosellulêre karsinoom en gevolglik word aflatoksiene as `n tipe I menslike karsinogeen deur die Internasionale Agentskap vir Kankernavorsing geklassifiseer. Aflatoksien kontaminasie in voedsel het ook `n ekonomiese impak as gevolg van verlies aan landbougewasse en diere.

Ten spyte van maatreëls betreffende die toelaatbare vlakke van aflatoksiene in voedel, is aflatoksien kontaminasie steeds `n groot probleem wêreldwyd, veral in ontwikkelende lande waar dit hoofsaaklik in stapelvoedsel voorkom. Huidiglik is die inaktivering van aflatoksiene deur fisiese en chemiese metodes nie effektief en ekonomies nie. Daarteenoor bied biologiese tegnieke `n gunstige opsie vir die eliminering van die toksiene, terwyl die organoleptiese eienskappe van die voedsel steeds behoue bly.

Hierdie studie fokus op die biologiese afbraak van AFB_1 deur bakterieë en fungi. Verskeie bakterieë, insluitend *Rhodococcus* spp., sowel as witvrot fungi produseer `n verskeidenheid ensieme wat hulle in staat stel om `n wye reeks polisikliese hidrokoolstofverbindings af te breek en gevolglik is afbraak van AFB_1 deur sommige van hierdie mikroörganismes bestudeer. Effektiewe afbraak van AFB_1 deur intrasellulêre ekstrakte van *Mycobacterium fluoranthenivorans* sp. nov. DSM 44556^T, *Nocardia corynebacterioides* DSM 20151 en *N. corynebacterioides* DSM 12676 is aangetoon. AFB_1 is ook effektief in vloeibare kulture sowel as intra- en ekstrasellulêre ekstrakte van *Rhodococcus erythropolis* DSM 14303 afgebreek. `n Beduidende ($P<0.001$) afbraak van AFB_1 is waargeneem na behandeling met *R. erythropolis* DSM 14303 ekstrasellulêre ekstrakte, met slegs 33.20% oorblywende AFB_1 na 72 h. Resultate het getoon dat die afbraak deur *R. erythropolis* DSM 14303 ensimaties is en dat die ensieme konstitutief geproduseer word. Afbraak van AFB_1 deur *R. erythropolis* DSM 14303 het ook tot `n totale verlies aan mutagenisiteit gelei. Verder het behandeling van AFB_1 met

rekombinante 2,3-dihidroksiebifenieldioksiginase fraksies wat geproduseer is deur ekstrasellulêre uitdrukking van die *bph*C1 geen van *R. erythropolis* DSM 14303 in *Escherichia coli* BL21, beduidende ($P<0.0001$) afbraak (49.32%) en vermindering in mutagenisiteit (42.47%) van die molekuul teweeggebring.

Beduidende ($P<0.0001$-0.05) afbraak van AFB_1 is verkry na behandeling met witvrot fungus kultuurekstrakte wat lakkase-ensiem bevat (17.10-76.00%), gesuiwerde lakkase geproduseer deur *Trametes versicolor* (1 U/ml, 87.34%), sowel as rekombinante lakkase geproduseer deur *Aspergillus niger* (118 U/L, 55.00%). Verder het die behandeling van AFB_1 met gesuiwerde lakkase-ensiem (1 U/ml) gelei tot verlies aan mutagenisiteit van AFB_1. Die afname in fluoressensie en mutageniese eienskappe van die AFB_1-molekuul na behandeling met die onderskeie mikrobiese preparate dui op struktuurveranderings aan die furofuraan- en/of laktoonringe van die molekuul.

Hierdie studie lewer `n bydrae tot die ontwikkeling van geneties gemanipuleerde mikrobiese rasse wat as `n belangrike biokontrole kan dien. Sulke rasse met multifunksionele tegnologiese eienskappe, insluitend die afbraak van AFB_1, kan die kwaliteit, veiligheid en aanvaarbaarheid van voedsel verbeter.

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PREFACE

This dissertation is presented as a compilation of several chapters and two manuscripts. Each chapter is introduced separately and is written in the style of the journal to which the manuscript was submitted for publication.

Chapter 3 Teniola, O.D., Addo, P.A., Brost, I.M., Färber, P., Jany, K-D., Alberts, J.F., Van Zyl, W.H., Steyn, P.S., Holzapfel, W.H., 2005. Degradation of aflatoxin B_1 by cell-free extracts of *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* sp. nov. *International Journal of Food Microbiology*. 105, 111-117.

Chapter 4 Alberts, J.F., Engelbrecht, Y., Steyn, P.S., Holtzapfel, W.H., van Zyl, W.H., 2006. Biological degradation of aflatoxin B_1 by *Rhodococcus erythropolis* cultures. *International Journal of Food Microbiology*. 109, 121-126.

Chapter 5 Alberts, J.F., van Zyl, W.H., 2006. Degradation of Aflatoxin B_1 by 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus erythropolis* through extracellular expression in *Escherichia coli.* In preparation for publication in the *International Journal of Food Microbiology*.

Chapter 6 Alberts, J.F., Gelderblom, W.C.A., Botha, A., van Zyl, W.H., 2006. Degradation of aflatoxin B_1 by fungal laccase enzyme. In preparation for publication in *Mycopathologia*.

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GENERAL DISCUSSION AND CONCLUSIONS

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Pectora roborant cultus recti

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1. Introduction

Aflatoxin B_1 (AFB_1) is a highly mutagenic, toxic, carcinogenic and teratogenic mycotoxin belonging to a group of more than 13 structurally related polyketide-derived difurocoumarolactone compounds, which are collectively called aflatoxins (Bhatnagar et al., 2003; Mishra and Das, 2003). The aflatoxins are natural toxins produced as secondary metabolites by the filamentous fungi *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus bombycis*, *Aspergillus nomius*, *Aspergillus ochraceoroseus*, *Aspergillus pseudotamarii* and *Aspergillus tamarii*. In parts of Africa, China and South East Asia aflatoxin contamination is correlated with the incidence of hepatocellular carcinoma (Wild and Hall, 2000; Yabe and Nakajima, 2004). In this regard AFB_1 is classified as a type I human carcinogen by the International Agency for Research on Cancer (Wogan, 2000). Furthermore, aflatoxin contamination in food and feed is responsible for significant economic losses due to loss of crops and livestock and domestic growers and food processors are under high pressure from consumer groups and regulatory organizations to remove aflatoxin from food and feed (Maggon et al., 1977; Trail et al., 1995).

Aflatoxin B_1 harbours two key sites influencing its toxicological activity, namely a furofuran- and a lactone ring (Heathcote and Hibbert, 1978; Mishra and Das, 2003), and changes to the coumarin structure will alter the mutagenic properties of the molecule (Liu et al., 1998 a,b,c). Inactivation of AFB_1 in food sources by various procedures has been a focus of many research initiatives. While physical and chemical methods have not yet proved to be effective and economically feasible (Mishra and Das, 2003), biological detoxification offers an attractive alternative for eliminating toxins as well as safe-guarding the desired quality of food and feed.

Several genes encoding enzymes responsible for transforming a wide range of polyaromatic compounds were characterized in *Rhodococcus* spp. (Hauschild et al., 1996; Kosono et al., 1997; Masai et al., 1995, 1997; Yamada et al., 1998). These include

seven *bph*C genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD) enzymes which are involved in the initial ring cleavage steps of biphenyl. In addition, several groups of enzymes produced by white rot fungi are involved in the degradation of lignin and aromatic xenobiotics, including heme-containing peroxidases, flavine oxidases, cellobiose dehydrogenases as well as laccases (Armstrong and Patel, 1994; Cullen and Kersten, 1996; Hammel, 1995; Higson, 1991; Singh et al., 1991).

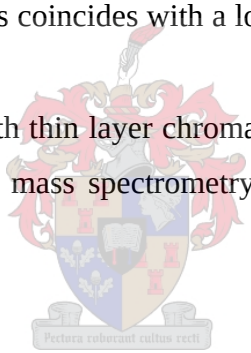
Microbial strains expressing AFB_1-degrading enzymes could support the development of commercial additives based on cultures or enzymatic preparations able of degrading AFB_1 (Karlovsky, 1999). Moreover, genetic engineered microbial strains with multifunctional technological properties including degradation of AFB_1 would be valuable as bio-control measure to significantly improve the quality, safety and acceptability of food and beverages.

1.2. Aims of the study

The aim of this study was to achieve biological degradation of AFB_1 by bacteria and fungi in liquid cultures using the following approach:

- Establish the microbiological and chemical analytical techniques for cultivation of *Rhodococcus erythropolis* DSM 14303 in liquid cultures as well as the extraction and quantification of AFB_1 from bacterial and fungal culture extracts.
- Examine degradation of AFB_1 by intracellular extracts of *R. erythropolis* DSM 14303, *Mycobacterium fluoranthenivorans* sp. nov. DSM 44556^T, *Nocardia corynebacterioides* DSM 20151 and *N. corynebacterioides* DSM 12676.
- Demonstrate that AFB_1 is effectively degraded by *R. erythropolis* DSM 14303 in liquid cultures.
- Determine whether degradation of AFB_1 by *R. erythropolis* DSM 14303 occurs extracellularly, on the cell membrane or intracellularly.
- Determine whether the degradation of AFB_1 by extracellular extracts of *R. erythropolis* DSM 14303 liquid cultures is an induced or constitutive activity.

- Determine whether degradation of AFB_1 by extracellular extracts of *R. erythropolis* DSM 14303 liquid cultures is enzymatic.
- Cloning of the *bph*C1 gene encoding 2,3-DHBD from *R. erythropolis* DSM 14303 and extracellular expression in *Escherichia coli* BL21 (DE3).
- Demonstrate degradation of AFB_1 by extracellular culture fractions of recombinant *E. coli* BL21 (DE3) harbouring the *bph*C1 gene fused to a PhoA secretion signal.
- Investigate the correlation between the production of laccase enzyme and degradation of AFB_1 by white rot fungi in different liquid media.
- Determine the ability of purified fungal laccase from *Trametes versicolor* as well as recombinant laccase produced by *Aspergillus niger* (D15-Lcc2#3), expressing the *lcc*2 gene, to degrade AFB_1.
- Determine whether the degradation of AFB_1 following treatment with the bacterial, fungal and enzyme preparations coincides with a loss of mutagenicity of AFB_1 and its breakdown products.
- Detect breakdown products with thin layer chromatography, high performance liquid chromatography, electro spray mass spectrometry and liquid chromatography mass spectrometry.



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CHAPTER 2

LITERATURE REVIEW

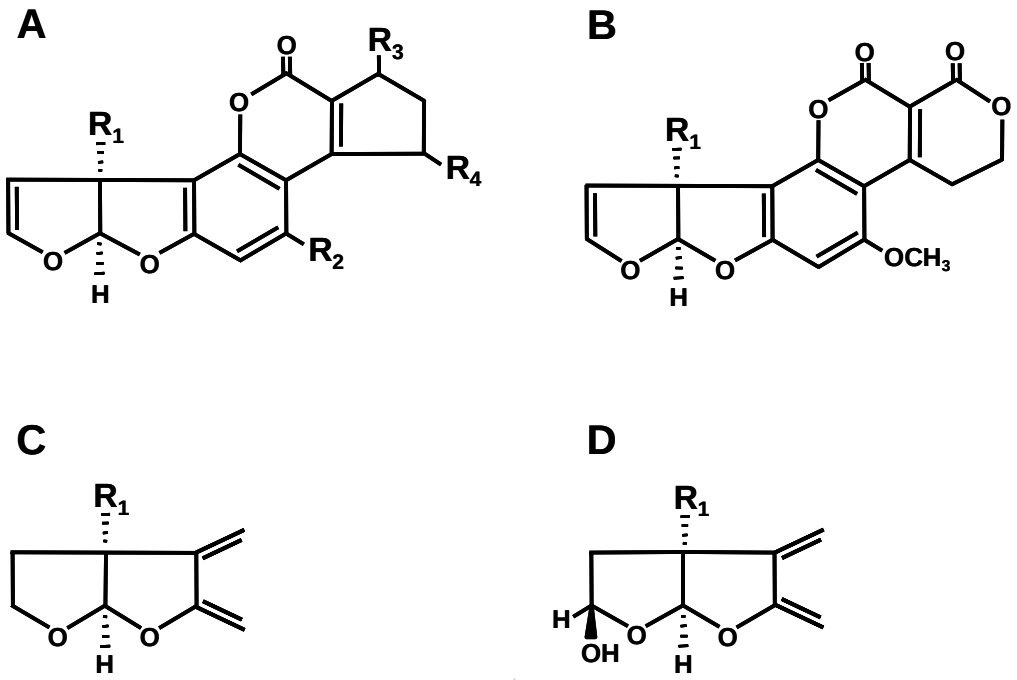
AFLATOXINS

2. AFLATOXINS

2.1. Chemical structure

A group of more than 13 structurally related polyketide-derived difurocoumarolactone compounds are collectively called aflatoxins (Bhatnagar et al., 2003; Mishra and Das, 2003) (Fig.1). The aflatoxins are natural compounds originally discovered and described following the outbreak of turkey poisoning in the U.K. during 1960 (Bennett et al., 1977). They are mycotoxins produced as secondary metabolites by primarily *Aspergillus flavus*, *Aspergillus parasiticus* and less frequently by other *Aspergillus* spp. including *Aspergillus bombycis, Aspergillus nomius, Aspergillus ochraceoroseus, Aspergillus pseudotamarii* and *Aspergillus tamarii* (Bhatnagar et al., 2003; Mishra and Das, 2003). The four major aflatoxins that contaminate agricultural commodities and pose a potential risk to livestock and human health are aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2). Whereas *A. parasiticus* normally produces AFB_1, AFB_2, AFG_1 and AFG_2, *A. flavus* only produces AFB_1 and AFB_2 (Yabe and Nakajima, 2004). Aflatoxins of the B and G groups are distinguished from each other on the basis of their fluorescence colour under UV light. While the B-type aflatoxins typically fluoresces blue under UV light and are characterized by a cyclopentane E-ring, the G-type aflatoxins have a xantone ring and fluoresce green (Fig.1). In addition, aflatoxins of the B_2 and G_2 type have a saturated bisfuranyl ring and aflatoxins B_{2a} and G_{2a} a hydrated bisfuranyl structure (Bhatnagar et al., 2003). Furthermore, AFB_1 and AFB_2 contamination is related to aflatoxin M_1 (AFM_1) and M_2 (AFM_2), since the latter aflatoxins are hydroxylated metabolites of AFB_1 and AFB_2 and are found in milk products obtained from livestock that have ingested contaminated feed (Creppy, 2002; Sweeney and Dobson, 1999).

Aflatoxin B_1, the most potent and frequent occurring aflatoxin, harbours two key sites influencing its toxicological activity, namely a furofuran ring and a lactone ring (Heathcote and Hibbert, 1978; Mishra and Das, 2003). Interaction of DNA and proteins with the lactone ring as well as the double bond in position 8,9 of the furofuran ring leads to toxic effects at cellular level and eventually malignant transformation (Liu et al., 1998 a,b,c; Smela et al., 2001) as discussed in Section 2.3.



Aflatoxin	Structure	R_1	R_2	R_3	R_4
B_1	A	H	OCH_3	=O	H
M_1	A	OH	OCH_3	=O	H
P_1	A	H	OH	=O	H
Q_1	A	H	OCH_3	=O	OH
R_0	A	H	OCH_3	OH	H
R_0H_1	A	H	OCH_3	OH	OH
B_2	AC	H	OCH_3	=O	H
B_{2a}	AD	H	OCH_3	=O	H
M_2	AC	OH	OCH_3	=O	H
G_1	B	H	-	-	-
G_2	BC	H	-	-	-
G_{2a}	BD	H	-	-	-
GM	BC	OH	-	-	-

Fig. 1. Chemical structures of the aflatoxins. **A**, aflatoxins of the B-type are characterized by a cyclopentane E-ring; **B**, G-type aflatoxins have an xhantone ring instead of the cyclopentane; **C**, aflatoxins of the B_2 and G_2-type have a saturated bisfuranyl ring; **D**, aflatoxins B_{2a} and G_{2a} have a bisfuranyl structure (Bhatnagar et al., 2003).

2.2. Biosynthesis

2.2.1. Enzymes involved in biosynthesis

Comprehensive studies were done to unravel the biosynthetic pathway of AFB_1 (Bennett et al., 1997; Trail et al., 1995; Yabe and Nakajima, 2004; Yu et al., 2004). Furthermore, many genes encoding enzymes involved in the conversion steps as well as the transcription factors have been characterized (Bhatnagar et al., 2003; Yu et al., 2004). However, some of the conversion steps in the biosynthetic pathway have not yet been assigned to specific genes. Gene disruption studies involving toxigenic strains of *A. parasiticus* and *Aspergillus nidulans* have resulted in accumulation or loss of some intermediates, which confirmed the role of specific enzymes involved in the biosynthesis of aflatoxin. However, persistent production of low concentrations of aflatoxins by disrupted strains indicated that alternative routes or enzymes could be involved in biosynthesis (Trail et al., 1995). Furthermore, slight differences in the biosynthesis of AFB_1 have been reported between *A. flavus* and *A. parasiticus* (Flaherty and Payne, 1997).

At present 25 identified genes, which are grouped in a 70 kb gene cluster on one chromosome, are involved in aflatoxin biosynthesis (Bhatnagar et al., 2003; Trail et al., 1995; Yu et al., 2004). While clustering of genes involved in secondary metabolism is a common phenomenon, the physical order of the genes in the cluster appears to correspond with the chronological order of enzymatic steps of the pathway (Sweeney and Dobson, 1999). Clustering of the genes in this manner suggests that the exchange of the ability to produce AFB_1 between fungi possibly occurs by horizontal gene transfer (HGT) as discussed in Section 2.4.

Of all the genes involved in the aflatoxin biosynthetic pathway, only *afl*R and *afl*S are positive regulatory genes and involved in activating pathway gene transcription (Bhatnagar et al., 2003; Yu et al., 2004). *afl*S was found to be located adjacent to the *afl*R gene in the aflatoxin gene cluster, possibly interacting with *afl*R, but the exact mechanism by which *afl*S modulates transcription together with *afl*R is still under investigation. The *afl*R gene encodes a sequence-specific zinc binuclear DNA-binding protein, which had been shown to be essential for transcriptional activation of most of the

structural genes, except *est*A (Yabe and Nakajima, 2004). Furthermore, a functional *afl*R allele is required for accumulation of norsolorinic acid (NOR), an important intermediate in AFB_1 biosynthesis and effects on aflatoxin pathway gene transcription may be directly caused by changes in the expression of *afl*R (Bhatnagar et al., 2003; Yu et al., 2004). Disruption of the *afl*R gene in *A. flavus* results in failure to produce aflatoxin.

Enzymes involved in the AFB_1 biosynthesis pathway are not localized to a certain membrane portion and at least four cytosolic and more than seven membrane enzymes are intermittently involved in the pathway (Yabe and Nakajima, 2004). Most of the enzymes have narrow substrate specificity and as a result effectively control the biosynthetic pathway.

2.2.2. The biosynthetic pathway

The biosynthesis of AFB_1 initiates with the polymerization of acetate and malonate, and NOR, a pigmented C-20 anthraquinone forms the first stable precursor in aflatoxin biosynthesis (Bhatnagar et al., 2003; Yabe and Nakajima, 2004; Yu et al., 2004) (Fig. 2a). It should be noted that aflatoxin and fatty acid biosynthesis are inversely correlated (Shih and Marth, 1974). Synthesis of aflatoxin occurs through a series of oxidation and reduction reactions (Bhatnagar et al., 1992; Dutton, 1988; Minto and Townsend, 1997; Yu et al., 2004) starting with conversion of malonyl-Coenzyme A (CoA) to noranthrone by enzymes encoded by fatty acid synthase genes (*fas*-1 and *fas*-2) and a polyketide synthase gene (*pks*A). The conversion of noranthrone to NOR probably involves a monooxygenase (*cyp*A) and a dehydrogenase (*nor*B) while the reduction of NOR to averantin (AVN) is reversible and involves a ketoreductase/dehydrogenase enzyme (*nor*A, *nor*B and *nor*-I). *Avn*A encodes a cytochrome P-450 monooxygenase that converts AVN to 5'-hydroxyaverantin (HAVN), while *adh*A is involved in the conversion of HAVN to averufin (AVR). The conversion from AVR to the typically orange pigmented versiconal hemiacetal acetate (VHA) probably involves an oxidase (*avf*A).

An esterase (*est*A) is involved in the conversion of VHA to versiconal (VAL), while a versiconal cyclase (*vbs*) is required for the conversion of VAL to versicolorin B (VERB) (Bhatnagar et al., 1992; Dutton, 1988; Minto and Townsend, 1997, Yu et al.,

2004) (Fig. 2b). During this critical step the bisfuran ring is closed, which determines the stereochemistry of the molecule. The closed bisfuran ring is responsible for binding to DNA and causes aflatoxin to act as mutagen (Yu et al., 2004). Furthermore, desaturation of the bisfuran ring occurs during the conversion of VERB to versicolorin A (VERA) by a cytochrome P-450 monooxygenase/desaturase (*afl*L). The double bond at the 8,9 position in the difuran moiety is the target for microsomal cytochrome P-450 enzymes to generate a highly reactive epoxide, and consequently activation and adduct formation with DNA and proteins (Trail et al., 1995). AFB_2 does not have this double bond and as a result is several hundred-fold less carcinogenic than AFB_1.

Following the formation of VERB, the pathway branches to form AFB_1 and AFG_1 to the one side and AFB_2 and AFG_2 to the other side (Bhatnagar et al., 1992; Dutton, 1988; Minto and Townsend, 1997, Yu et al., 2004). A ketoreductase (*ver*I) and a cytochrome P-450 monooxygenase (*ver*A) is responsible for the conversion of versicolorin A (VERA) to demethylsterigmatocystin (DMST). An O-methyl-transferase I (*omt*B, *dmt*A) catalyzes the conversion of DMST to sterigmatocystin (ST) and of dihydrodemethylsterigmatocystin (DHDMST) to dihydrosterigmatocystin (DHST). ST, the penultimate intermediate in the aflatoxin biosynthetic pathway, is characterized by a xanthone moiety fused to a dihydrofuran or tetrahydrofuran moiety and is extremely toxic and carcinogenic (Sweeney and Dobson, 1999).

The conversion of ST to O-methylsterigmatocystin (OMST) and DHST to dihydro-O-methylsterigmatocystin (DHOMST) is catalyzed by an O-methyl-transferase II (*omt*A) (Bhatnagar et al., 1992; Dutton, 1988; Minto and Townsend, 1997; Yu et al., 2004). Finally, DHST and DHOMST are converted by a NADPH-dependent monooxygenase (*ord*A) to aflatoxins B_1, B_2, G_1 and G_2, respectively. While the intermediates accumulate in fungal cells, the synthesized aflatoxins are finally released from the mycelia into the medium (Yabe and Nakajima, 2004). However, an export system for aflatoxins has not been found yet. Differences in localization of the intermediates and the aflatoxins could be attributed to the solubility of these substances in the lipid layer and aqueous phase.

Biosynthesis of aflatoxin requires substantial energy (Maggon et al., 1997; Yabe and Nakajima, 2004), which is derived from the primary metabolism of the fungus. It

was calculated that more than ten NADPHs, one NAD and two SAMs are needed for the formation of aflatoxin. NADPH serves as an electron donor and is supplied by the pentose-phosphate pathway.

2.2.3. Effect of environmental factors

Secondary metabolism and metabolites represent a large variety of biosynthetic pathways and products which are not involved in growth of the organism (Luchese and Harrigan, 1993). The control of the expression of genes for AFB_1 biosynthesis through on/off regulation of *afl*R expression may be affected by environmental and nutritional factors such as temperature, pH, carbon and nitrogen source, stress factors, lipids, metal salts (Cary et al., 2000), cytosolic NADPH/$NADP^+$ (Minto and Townsend, 1997) and certain constituents in host plants (Calvo et al., 2002). The most important environmental factors affecting AFB_1 biosynthesis are discussed in the sections that follow.

Multiple signals for growth and mycotoxin production are probably received by *Aspergillus* spp. through different receptors that sense the pH, sugar, and nitrogen content of the environment. Furthermore, a signal transduction pathway exists which regulates both conidiation and sterigmatocystin-aflatoxin biosynthesis. These processes are regulated by a G-protein signalling pathway in *A. nidulans* (Shimizu and Keller, 2001). The G-protein operates in most organisms linking external stimuli to a coordinated response by the organism. The role of pH in the regulation of expression of genes involved in aflatoxin biosynthesis is not as yet well understood.

2.2.4. Effect of nitrogen

The effects of nitrate on aflatoxin formation indicate that regulation of aflatoxin biosynthesis may be part of the nitrogen control circuit (Cary et al., 2000; Minto and Townsend, 1997). Nitrate assimilation in fungi is a closely regulated process. The expression of nitrate reductase and nitrite reductase genes requires both the lifting of nitrogen metabolite repression and specific induction by nitrate (Marzluf, 1997).

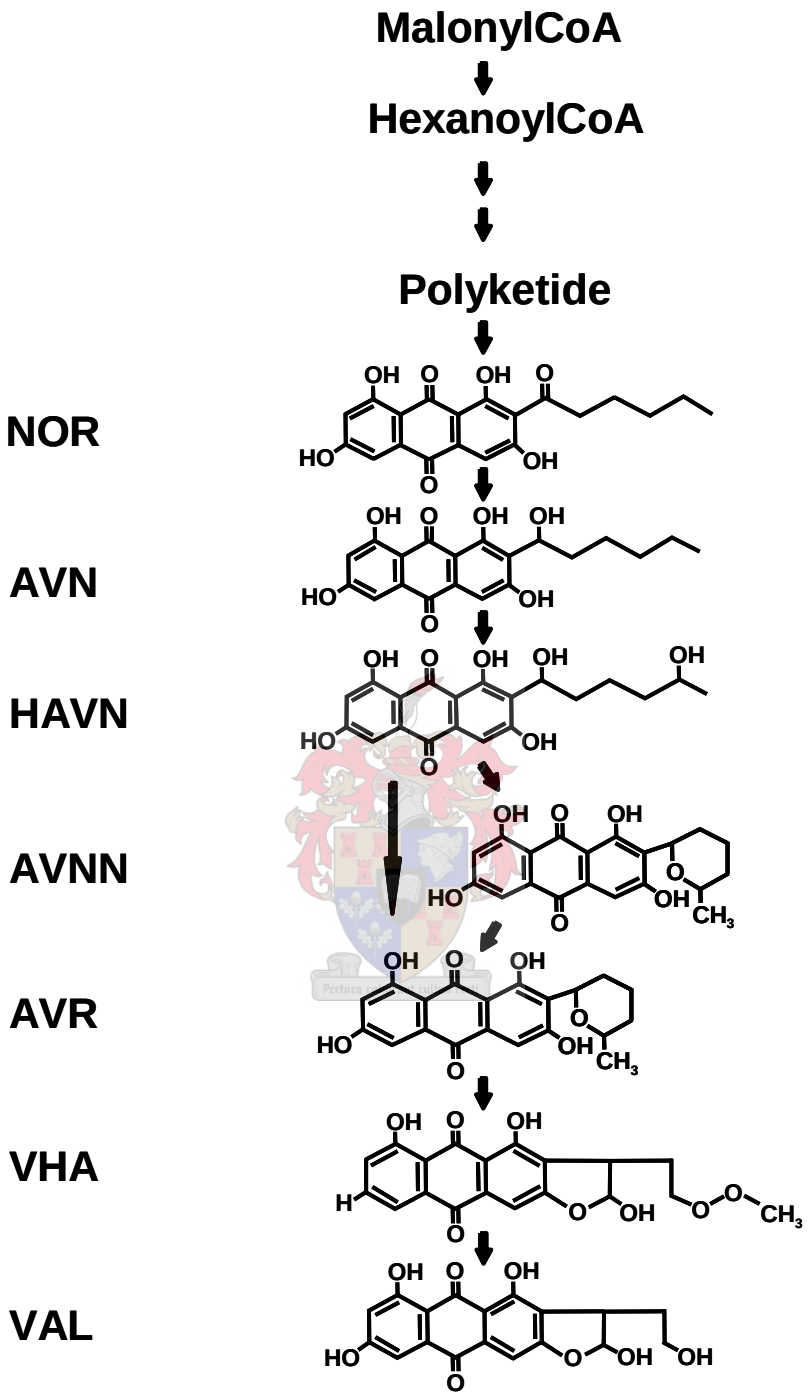


Fig. 2a. The aflatoxin biosynthetic pathway. Conversion of malonyl-CoA to versiconal. Abbreviations used are: AVN, averantin; AVNN, averufanin; AVR, averufin; HAVN, 5'-hydroxyaverantin; NOR, norsolorinic acid; VAL, versiconal; VHA, versiconal hemiacetal acetate (Bhatnagar et al., 2003).

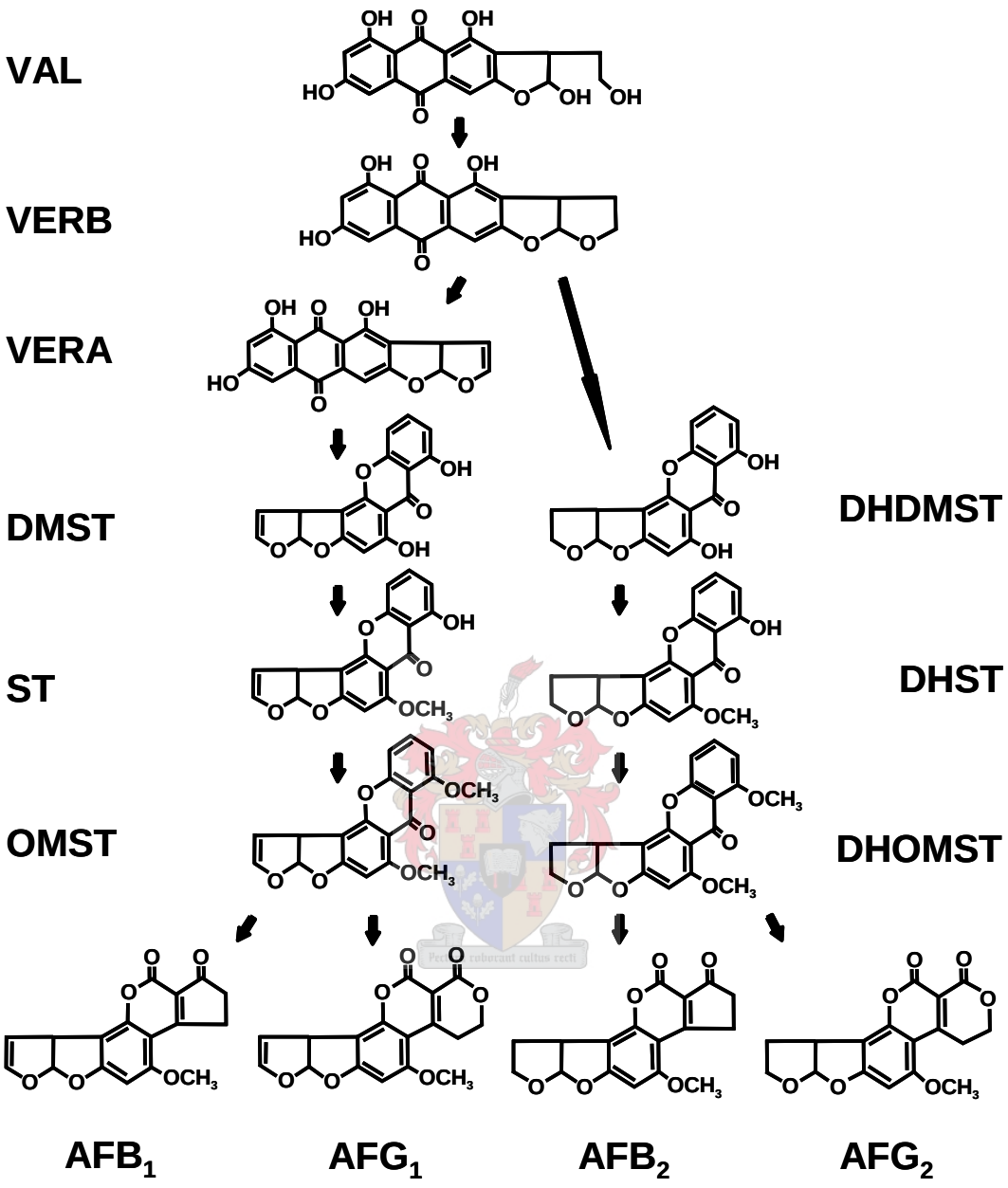


Fig. 2b. The aflatoxin biosynthetic pathway. Conversion of versiconal to aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2. Abbreviations used are: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; DHDMST, dihydrodemethylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; DHST, dihydrosterigmatocystin; DMST, demethylsterigmatocystin; OMST, O-methylsterigmatocystin; ST, sterigmatocystin; VAL, versiconal; VERA, versicolorin A; VERB, versicolorin B (Bhatnagar et al., 2003).

Whereas aflatoxin and fatty acid biosynthesis are inversely correlated (Shih and Marth, 1974), nitrate increases the cytoplasmic NADPH/$NADP^+$ ratio, which favours biosynthetic reductive reactions, and thus promotes utilization of malonyl-CoA and NADPH for fatty acid synthesis rather than for polyketide synthesis.

2.2.5. Effect of carbon

Although the role of carbon utilization in the regulation of expression of genes involved in aflatoxin biosynthesis is still uncertain, it is known that aflatoxin biosynthesis is significantly affected by the identity and concentration of the available carbon sources (Cary et al., 2000; Minto and Townsend, 1997). It was found that carbon sources that are oxidized through the hexose monophosphate and the glycolytic pathways support both growth and aflatoxin production (Luchese and Harrigan, 1993). Furthermore, reduced tricarboxylic acid (TCA) cycle activity causes a build-up of TCA cycle intermediates, which lead to a shunting of acetyl-CoA to aflatoxin synthesis.

Unlike the biosynthesis of many other secondary metabolites, fungal growth, sporulation and aflatoxin gene expression is induced by the presence of simple carbohydrates for example glucose, sucrose, or maltose, but not by peptone, sorbose or lactose (Calvo et al., 2002; Luchese and Harrigan, 1993; Payne and Brown, 1998). Glucose regulates the induction of some of the enzymes associated with aflatoxin biosynthesis and maximal aflatoxin is produced in the presence of high glucose concentrations. Inversely, aflatoxin biosynthesis is repressed when glucose utilization is inhibited. The activities of all of the characterized enzymes are dependent on the carbon source, except for *est*A, which is constitutively expressed, irrespective of the culture medium (Yu et al., 2003).

Another indirect effect of glucose utilization on aflatoxin pathway gene expression could be the activation of a four gene cluster which is related to sugar utilization in *A. parasiticus* (Bhatnagar et al., 2003; Yu et al., 2000). This gene cluster is situated at the one end of the aflatoxin biosynthetic pathway gene cluster and includes *sug*R, *hxt*A, *glc*A and *nad*A genes. It was found that expression of the *hxt*A gene, which encodes a hexose transporter protein, is synchronized with the aflatoxin pathway gene

cluster. Therefore, activation of genes in this cluster by an external hexose signal may create a region of active chromatin that includes the neighbouring aflatoxin gene cluster.

Furthermore, the rate at which carbohydrates are transported into the fungal cell has an effect on aflatoxin biosynthesis. High glucose levels increase the level of cyclic AMP (cAMP), which in turn activates cAMP-dependent protein kinases. Moreover, the level of these kinases is elevated in aflatoxin-producing cultures. A correlation between an increased pool size of cAMP and aflatoxin production had been observed previously (Bhatnagar et al., 2003).

2.2.6. Effect of lipoperoxidation and trace metals

Several compounds present in host plants have been reported to effect fungal growth and aflatoxin production, including linoleic acid and hydroperoxylinoleic acid, which induce conidial formation and aflatoxin biosynthesis (Calvo et al., 2002). Furthermore, it was reported that a major increase in aflatoxin biosynthesis was observed in the presence of lipoperoxidases in oil-bearing seeds (Luchese and Harrigan, 1993).

The main trace metals affecting aflatoxin biosynthesis are manganese, iron and zinc (Luchese and Harrigan, 1993). However, the exact function of trace elements in the biosynthesis of aflatoxin is widely hypothesized, but still not well understood. While the proportion of the different aflatoxins produced are affected by the trace metal composition of the medium, manganese, magnesium and vanadium favour the synthesis of hydroxylated aflatoxins. Trace metals are probably metalloenzymes catalyzing enzymatic activities and are catalysts of lipid peroxidation.

The presence of zinc is critical during aflatoxin biosynthesis (Luchese and Harrigan, 1993). Zinc affects enzymes of the glycolytic and TCA cycles and as a result the utilization of carbohydrates. Maximal AFB_1 concentrations are produced in medium containing 0.8 mg/L zinc during the period of early vegetative growth of the fungus. In addition, the absence of zinc reduces the formation of versicolorin. It was reported (Luchese and Harrigan, 1993) that zinc may be acting at the pre-transcriptional or transcriptional level. In contrast, manganese inhibits, stimulates or has little effect on aflatoxin biosynthesis, depending on the concentration present.

Synthesis of secondary metabolites has a more specific requirement for phosphate than vegetative growth (Luchese and Harrigan et al., 1993). High levels of phosphate have been shown to inhibit aflatoxin biosynthesis, possibly by removal of essential trace metals or feedback inhibition of phosphorylated intermediates into other biosynthetic pathways. While phosphate concentrations between 0.4 and 0.8 mM are optimal for aflatoxin biosynthesis, a concentration of 5 mM phosphate results in build-up of phosphate in mycelia and inhibition of versicolorin synthesis.

2.3. Toxicity and carcinogenicity

Aflatoxins have been shown to be immunosuppressive, mutagenic, teratogenic, hepatotoxic and hepatocarcinogenic in both experimental animals and humans (Eaton and Groopman, 1994). Furthermore, frequent intake of aflatoxin causes bile duct proliferation, hepatic necrosis, osteosclerosis of bone, childhood cirrhosis, immune suppression, and hepatic veno-occlusive lesions (Mishra and Das, 2003). Hepatocellular carcinoma (HCC) is one of the most frequently occurring cancers in the world, very potent and the most patients survive less than one year after diagnosis (Eaton and Groopman, 1994; Wild and Hall, 2000). An estimated 250 000 deaths occur annually in China and Sub-Saharan Africa due to HCC (Bennett et al., 1994; Mishra and Das, 2003; Wild and Hall, 2000). In this regard aflatoxin is classified as a type I human carcinogen by the International Agency for Research on Cancer (Wogan, 2000). The major risk factors for HCC are infection with the hepatitis B (HBV) or C virus and dietary exposure to aflatoxin (Wild and Hall, 2000; Yabe and Nakajima, 2004). While the interaction between the two risk factors appears to be synergistic, the exact mechanism of interaction still remains unclear. Furthermore, it has been reported that sensitivity and susceptibility to aflatoxin induced carcinogenicity vary between different animal species depending on sex, age, nutritional status as well as the dose level and the period of exposure (Mishra and Das, 2003; Wild and Hall, 2000).

The enzymes involved in carcinogen metabolism include peroxidases, quinine reductases, glucuronyltransferases, flavin-containing mooxygenases, epoxide hydrolases, sulfotransferases, cytochrome P-450– and glutathione S-transferases (GST) (Bailey and

Williams, 1993). A balance exists between enzymatic activation and detoxification of the carcinogen. Metabolic activation of AFB_1 by the cytochrome P-450 (phase I) enzymes in the liver and kidney leads to the formation of reactive AFB_1-8,9-epoxide (Minto and Townsend, 1997; Mishra and Das, 2003). However, phase I enzymes could also convert AFB_1 to the less carcinogenic AFM_1, AFQ_1 and AFP_1.and aflatoxicol. Furthermore, the AFB_1-8,9-epoxide could be inactivated by phase II enzymes, through formation of AFB_1-dihydrodiol by epoxide hydrolase or through the formation of AFB_1-glutathione conjugate by GST (Johnson et al., 1996, 1997). Following metabolic activation, the AFB_1-8,9-epoxide strongly binds to DNA and RNA, prevents the RNA polymerase transcribing the DNA and inhibits the formation of mRNA, therefore disrupting protein synthesis and as a result causes liver necrosis. More specifically, the AFB_1-8,9-epoxide reacts covalently with the N-7 of guanine to form several complexes with the DNA, or so-called DNA adducts, which in turn cause further genetic changes and eventually malignant transformation (Smela et al., 2001) (Fig. 3).

The most significant point mutation found in HCC is a GC→TA transversion on the third position of codon 249 of the p53 gene (Minto and Townsend, 1997; Smela et al., 2001). In areas where exposure to aflatoxin is high, 44% of the total HCC cases proved to have a prevalence of GC→TA mutations at the third position of codon 249 of the p53 gene. The p53 gene is a gene transcriptional activator which regulates cell cycles and plays a role in the apoptosis pathway, DNA repair and is a tumor suppressor. Codon 249 of the p53 gene is extraordinarily reactive with AFB_1-8,9-epoxide. During interaction of the AFB_1-8,9-epoxide and DNA, the epoxide approaches a reactive guanine of double-stranded DNA on the 5' face of the guanine residue, intercalates into the DNA and forms DNA adducts. The mutation results in an Arg→Ser alteration in the p53 protein. The most important DNA adducts formed are 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N7-Gua), an apurinic (AP) site and AFB_1 formamidopyrimidine (AFB_1-FAPY) (Fig. 3). While AP is formed after depurination of the imidazole ring of AFB_1-N7-Gua, opening of the imidazole ring results in AFB_1-FAPY. These adducts, especially AFB_1-N7-Gua, are the main chemical precursors to the mutations caused by AFB_1. During replication the AFB_1 residue substitutes into the position of the 5'cytosine, averting the cytosine from the helix. A polymerase inserts adenine, resulting in the C→T transition.

However, repair of some strongly reactive sequences can occur, thus preventing mutations to develop. Furthermore, hepatitis B x-protein can inhibit nucleotide excision repair, either by binding to repair proteins or to the damaged DNA, making it possible that AFB_1 adducts persevere in patients who have been exposed to HBV and AFB_1. The efficiency of mutation by aflatoxin is influenced by several factors, including the preference of one G base over another G base, the preference for a base to be more frequently incorrectly replicated depending on the circumstances, the possibility for repair of an adduct, while certain atomic environments of DNA sequences may be more favourable for DNA intercalation (Smela et al., 2001). Furthermore, AFB_1 have shown a preference for GC→TA transversions within GC-rich sequence environment.

Although the effect of AFB_1 exposure and HBV infection on the p53 gene is still not completely clear, the p53 gene is a determinant factor in HCC (Smela et al., 2001). It was thought that animal models could provide valuable information in this regard. However, in spite of almost 92% homology with the human p53 gene, the GC→TA mutation at codon 248 and 249 could not be detected in experimental animals. This was ascribed to differences in the p53 gene sequence between humans and several of the animal species examined, silent mutations in some animal species or the presence of an unknown co-carcinogen in humans contributing to mutations at codon 249 and eventually HCC.

Exposure to AFB_1 alters expression of certain genes, including GST as well as certain human cytochrome P-450 genes involved in activation of AFB_1 (Smela et al., 2001), while HBV infection influences regulation of these genes. What's more, aflatoxin inhibits oxygen uptake by affecting the ATP enzyme and as a result reduces ATP production. It also reduces hepatic glycogen levels. To assess human exposure to aflatoxin, the presence of aflatoxin-albumin adducts in serum, AFB_1-N7-Gua adducts in urine, AFM_1 metabolites in urine or patterns of p53 gene mutations are considered as biomarkers (Creppy, 2002).

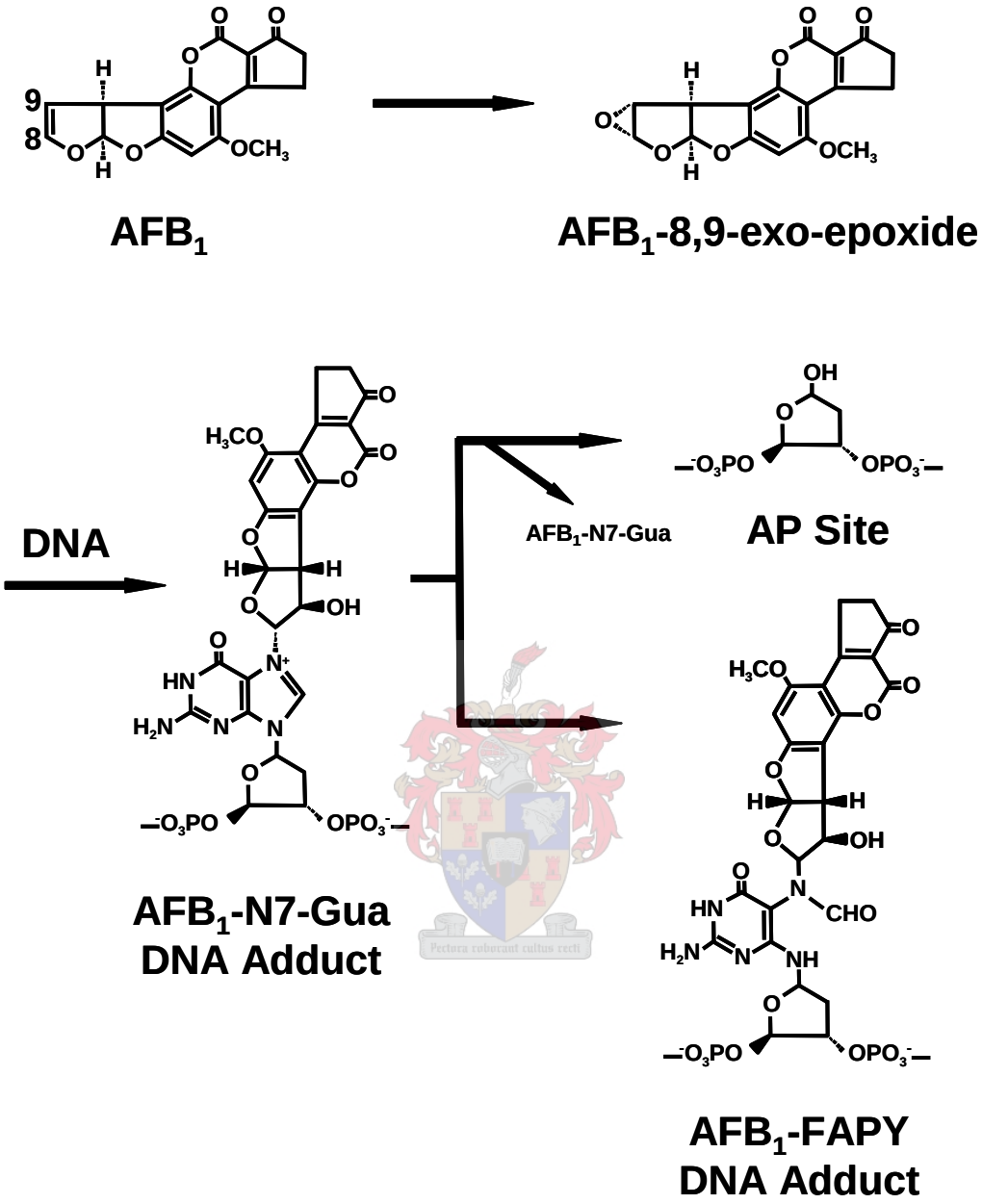


Fig. 3. Pathway of metabolic activation of AFB_1 and DNA adduct formation. DNA adducts formed are 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N7-Gua), an apurinic (AP) site and AFB_1 formamidopyrimidine (AFB_1-FAPY) (Smela et al., 2001).

2.4. Biological significance

Polyketides are a large group of secondary metabolites mainly produced by actinomycetes, fungi, and higher plants (Trail et al., 1995). Secondary metabolism, including mycotoxin production, is generally associated with late fungal development and sporulation processes in microorganisms (Calvo et al., 2002). Although regulation of mycotoxin synthesis is different from primary metabolism, it relies on primary metabolism for energy, enzyme cofactors and acetate. Most *Aspergillus* spp. propagate solely by asexual spores and conidia serve as a major source of inoculum. It was determined that conidiation and ST production are co-regulated. However, aflatoxin does not appear to be essential to the growth and/or life-cycle of the fungus (Bhatnagar et al., 2003).

Even though the biological significance of aflatoxin biosynthesis by fungi is uncertain, the subject is widely hypothesized (Calvo et al., 2002). Compounds excreted by mycelium during secondary metabolism, can induce or inhibit sexual and asexual sporulation in fungi thus governing the ratio of asexual to sexual spores. Furthermore, it was speculated that aflatoxin production may be a prehistoric characteristic that has survived due to clustered gene organization (Calvo et al., 2002). One hypothesis suggested that such an organization of genes may allow coordinated regulation of the pathway. Another hypothesis is that the cluster organization ensures survival of the cluster by providing a mechanism for HGT. Since anthraquinone polyketide antibiotics produced by *Streptomyces* spp. are structurally related to intermediates of AFB_1 biosynthesis, this bacterium was mentioned as a possible progenitor to AFB_1 synthesis in fungi (Trail et al., 1995).

It is also hypothesized that the aflatoxin biosynthetic pathway evolved from a pre-existing pathway for synthesis of other fungal polyketides, possibly mycelial or spore pigments (Trail et al., 1995). While a high degree of DNA sequence homology exists between the accepted aflatoxin polyketide synthetase (PKS) and the PKS involved in conidial pigment synthesis, the chemical structure of an intermediate in conidial pigment synthesis in *A. parasiticus* have strong similarity to NA. Furthermore, an ascospore pigment in *A. nidulans* is a dimer of an anthraquinone and is likely to be derived from a

polyketide while a gene product involved in the biosynthesis of melanine have shown high homology with the *ver*I gene product involved in aflatoxin biosynthesis by *A. parasiticus*. These findings suggest that the mentioned biosynthetic pathways or parts of it are derived form a mutual ancestral polyketide pathway.

A further hypothesis is that aflatoxins could be chemical signals between species in an ecological niche or serve to signal fungal development (Bhatnagar et al., 2003; Trail et al., 1995). In this manner toxicity of ST and aflatoxin may defend the fungus from competitors in the soil or during crop invasion (Calvo et al., 2002; Demain and Fang, 2000). The chemical defensive function of aflatoxin may be enhanced by other mycotoxins for example cyclopiazonic acid, which is co-produced by certain strains of *A. flavus,* while lower concentrations are produced when grown independently (Minto and Townsend, 1997).

Monooxygenase enzyme activity was detected in the microsomes of *A. parasiticus* (Luchese and Harrigan, 1993), resulting in lipoperoxidation of lipids by cytochrome P-450 enzymes during the late growth phase of the fungus. While fatty acid and aflatoxin biosynthesis are inversely correlated (Shih and Marth, 1974) as mentioned earlier, degradation of lipoperoxides favours aflatoxin biosynthesis. Whereas oxygenases are mainly involved in detoxification of xenobiotics and endogenous compounds, aflatoxin biosynthesis in *A. parasiticus* may be considered a consequence of lipoperoxide degradation in oil bearing seeds.

Aflatoxins are toxic to insects and some aflatoxin producing species have been associated with insect debris (Bhatnagar et al., 2003; Drummond and Pinnock, 1990). Insects appear to be excellent vectors for mycotoxigenic fungi during plant invasion. While sclerotia from aflatoxigenic strains have a survival advantage compared to those from atoxigenic isolates, fungi in the *A. flavus* group are thought to over winter in soil as sclerotia. Studies have shown that deletion of genes which eliminates the synthesis of aflatoxin and intermediates resulted in enhanced production of sclerotia (Trail et al., 1995). However, the association between sclerotial formation and aflatoxin biosynthesis is still not clear.

2.5. Aflatoxin and traditional fermentative foods

Fermented food and beverages are a major dietary constituent of African and Asian people (Gadaga et al., 1999; Gonfa et al., 2001; Jespersen, 2003). Fermentation is one of the oldest methods of food preservation and products are especially appreciated for their sensory attributes. Various indigenous dishes constitute a large portion of the daily food intake in Ghana, Nigeria, Benin and Togo. These foods are palatable and wholesome and are prepared from raw or heated raw materials including mostly maize, rice, sorghum, millet and milk. The microorganisms involved are mainly lactic acid bacteria (*Lactobacillus* spp.), yeasts (*Saccharomyces cerevisiae*), moulds and acetic acid bacteria. A wide selection of alcoholic and non-alcoholic fermented food products (Gadaga et al., 1999; Gonfa et al., 2001), mainly produced from a variety of cereals as well as fermented milk products are produced commercially or on small-scale at household level. Milk produced by smallholders is usually processed on the farm using traditional dairy technology. Whereas it is not a common practice to clean the udder before milking, the milk is often contaminated. Furthermore, milk is sold fresh, consumed raw or allowed to ferment naturally, but is rarely boiled. While sour milk is the most common fermented milk product, milk products are also used as cosmetics by rural people.

In many cases the raw material are sold in rural markets or home-grown and aflatoxin contamination a major risk, especially in stored cereals (Holzapfel, 2002). As a result fermented products prepared from cereals and milk are of varying quality and stability and aflatoxin seldom destroyed by normal industrial processing or cooking. During fermentation of cereals and milk, *S. cerevisiae* and *Candida krusei* have an inhibitory effect on growth of *Penicillium citrinum*, *A. flavus* and *A. parasiticus*, due to substrate competition. However, *Aspergillus* spp. are able of growing on a variety of substrates and under different environmental conditions, therefore most food are exposed to infection by aflatoxigenic fungi at any stage during production, processing, transportation and storage (Mishra and Das, 2003). Furthermore, control measurements for the maximum acceptable levels of mycotoxins in cereals are inadequate in developing countries and regular exposure to low levels of aflatoxin a cause for concern (Trail et al., 1995).

Food safety, as well as maintaining the wholesomeness, acceptability and overall quality have become increasingly important even in developing countries where old customs and cultural particularities in food fermentations are generally well maintained (Holzapfel, 2002). For that reason the development of bacteria and yeasts expressing AFB_1 degrading enzymes could especially be valuable in the feed, food and the fermentation industry (Holzapfel, 2002; Karlovsky, 1999). With the application of molecular biology techniques microbial strains with multifunctional technological properties, including degradation of AFB_1, can be developed to significantly improve the quality, safety and acceptability of traditional fermented food and beverages.

2.6. Aflatoxin intervention

Aflatoxins were discovered in 1960 (Bennett et al., 1997) and in the years that followed extensive studies by biologists and chemists resulted eventually in the identification of over 100 mycotoxins (Maggon et al., 1977). It has been estimated that mycotoxins contaminate up to 25% of the world's food supply (Wild and Hall, 2000) causing enormous economic losses due loss of crops, animals, costs for monitoring the aflatoxin levels as well as the decrease in performance of farm animals (Maggon et al., 1977; Trail et al., 1995). Therefore, domestic growers and food processors are under pressure from consumer groups and regulatory organizations to remove aflatoxin from food and feed.

Contamination of food and feed remains a serious worldwide problem but is not a threat in most developed countries because of careful commodity screening (Bhatnagar et al., 2003). Regulations regarding acceptable levels of aflatoxin in food have been implicated in nearly all countries (Mishra and Das, 2003). Most countries, including the U.S.A., have a regulatory level of 20 ppb, while regulations for aflatoxin in Europe are stricter, that is 2 ppb for AFB_1 and 4 ppb for total aflatoxins. However, the acceptable aflatoxin levels in food and feed are generally based on animal statistics and may be too high, due to the differences in the sensitivity between animal species. Only the minority of African countries have instituted regulations to control aflatoxins in food, including Cote d' Ivoire, Egypt, Kenya, Malawi, Nigeria, Senegal, South Africa and Zimbabwe (Shephard, 2003). Aflatoxin contamination of foods in developing countries occurs

predominantly in dietary staples and exceeds the regulatory limits for contamination of food by one or two orders of magnitude (Wild and Hall, 2000). In several tropical African countries including Egypt, Gambia, Ghana, Nigeria and Senegal, aflatoxin contamination is widespread and high aflatoxin concentrations (10 – 525 μg/kg) have been recorded in cereals, fermented products, fish and beer intended for human and animal consumption (Shephard, 2003). Conversely, in South Africa aflatoxin producing fungi only occur sporadically in commercial and home-grown maize and as a consequence a low incidence of aflatoxin contamination is present in local maize.

Since there is no effective treatment for aflatoxin-induced diseases, the emphasis of aflatoxin research is on the prevention of aflatoxin contamination as well as the degradation of aflatoxin in food and feed (Bhatnagar et al., 2000). Often the regions of highest exposure to aflatoxins are also those with high prevalence of HBV infection. There are several approaches to primary prevention of HCC in developing countries (Wild and Hall, 2000). Firstly it involves measures to control exposure to hepatitis infection and secondly to reduce aflatoxin contamination in foods for human and animal consumption, especially in rural communities of developing countries where there is a high level of small-scale farming. Aflatoxin contamination can arise from improper storage of commodities as well as pre-harvest fungal contamination in corn, peanuts, cotton seed and tree nuts.

2.6.1. Pre-harvest crop management

Pre-harvest measures involving prevention or reduction of fungal growth and mycotoxin production are the most effective way to limit aflatoxin contamination (Mishra and Das, 2003; Trail et al., 1995; Wild and Hall, 2000). Crops are frequently infected by fungi at pre-harvest and especially where crop is under stress because of drought, exposure to high temperatures for long periods and damage due to insects.

Several measures can be implemented at pre-harvest, including using healthy seeds, proper irrigation, rotation of crops and the use of fungicides and pesticides (Mishra and Das, 2003; Trail et al., 1995; Wild and Hall, 2000). Unfortunately these methods have limited success and are not cost-effective, especially for subsistence farmers. Another method is to establish non-aflatoxigenic *A. flavus* strains to compete with

aflatoxigenic strains (Mishra and Das, 2003; Wild and Hall, 2000). However, these strains may still produce mycotoxins other than aflatoxin, they may revert back to aflatoxin producers or food-spoilage *per se* may be a problem.

2.6.2. Post-harvest crop management

Storage of cereals under hot and humid conditions results in increased fungal growth and aflatoxin levels and also favours rodent and insect damage (Wild and Hall, 2000). Although numerous methods have been implicated to remove aflatoxins from foods post-harvest including physical and chemical methods, none really fulfil the necessary efficacy, safety, and cost requirements (Mishra and Das, 2003).

Physical methods that have been used are treatment of aflatoxin contaminated food with sunlight, UV light, microwave, autoclaving, cooking, roasting, pasteurization, dry heat and solvents (Mishra and Das, 2003). However, detoxification of aflatoxin in both liquid and solid material cannot be achieved by using only one of these methods. Furthermore, certain nutrients are destroyed during physical treatment and the organoleptic qualities of food are altered, which make these methods unacceptable for food intended for human consumption.

A variety of chemicals are employed to detoxify aflatoxin including hydrogen peroxide, ozone, ammonia, urea with urease, sodiumhypochlorite, sodiumbisulphate (Creppy, 2002; Mishra and Das, 2003). Adsorption, using inert materials such as hydrated sodium, calsium and aluminosilicate to bind aflatoxins and reduce AFM_1 in milk is also used. However, the formation of harmful compounds and chemical residues limit the applicability of these methods. The most successful chemical method for degrading aflatoxins in animal feed is ammoniation, resulting in 95-98% degradation of AFB_1 (Creppy, 2002). This method is used in several countries.

While metabolic activation and detoxification of AFB_1 are accomplished by phase I and II enzymes (discussed in Section 2.3.), the activity of these enzymes can be controlled by chemical agents, resulting in detoxification (Sheweita, 2000). In this regard treatment of human and rat hepatocytes with phenobarbital, 3-methylcholanthrene, dithiolethiones and sulforaphane results in inhibition of cytochrome P-450 and induction of GST (Maheo et al, 1997; Morel et al., 1993). Furthermore, an antischitosomal drug,

oltipraz, had been developed which inhibits phase I activation of aflatoxin and increases phase II conjugation (Creppy, 2002; Smela et al., 2001; Wild and Hall, 2000).

Growth of *Aspergillus* spp. post-harvest is influenced by temperature, moisture content and storage time (Creppy, 2002; Mishra and Das, 2003). Proper management of crop immediately after harvesting, drying of crops in the sun, sorting of damaged kernels, ventilation during storage and the use of pesticides and biological pest control will improve the quality of cereals. While aflatoxin contamination of food occurs mainly in dietary staples in developing countries, exposure to aflatoxin can be reduced through cooking or by varying the diet (Wild and Hall, 2000).

Detoxification methods should fulfil the following requirements: (i) the mycotoxin should be degraded to non-toxic products; (ii) fungal structures should be permanently destroyed; (iii) the food should retain its nutritive and other desired qualities; (iv) the physical characteristics of cereals should not change notable and (v) the detoxification process should be economical (Smela et al., 2001; Wild and Hall, 2000).

2.6.3. Genetic engineering of crops

Control of aflatoxin is possible by genetic engineering of genes encoding resistant factors inhibiting aflatoxin synthesis by infectious fungi or introduction of resistant genes from other sources into susceptible plants (Mishra and Das, 2003). *Aspergillus parasiticus* and *A. flavus* are especially prominent in mature corn seeds with high oil concentrations (Trail et al., 1995). Therefore identification of signals between the plant host and pathogen which stimulate aflatoxin production and the genes involved should aid in successful genetic manipulation of crops. Aflatoxin gene reporter constructs are especially useful to identify plant compounds which stimulate or inhibit fungal infection, growth or toxin biosynthesis. To be effective, genes should be expressed in the most suitable plant tissue and at the right time in the engineered plant. The volatile corn-derived compounds, octanal, hexanal and n-decyl aldehyde were studied for their ability to inhibit growth and aflatoxin production by *A. flavus* (Wright et al., 2000). Results have shown octanal and hexanal to inhibit growth of the fungus, while *n*-decyl aldehyde inhibited aflatoxin biosynthesis. Genes expressing antifungal compounds such as *n*-decyl

aldehyde, hexanal and octanal was applied to develop maize varieties which are both bio-competitive and resistant to infection by *Aspergillus* spp. (Mishra and Das, 2003; Wright et al., 2000).

In biological niches, the fungal population is sometimes dominated and controlled by certain strains of *A. flavus* and *A. parasiticus* over other strains of the same species (Trail et al., 1995). This form of bio-control depends on the survival and domination of certain fungal strains over others under specific environmental conditions. Atoxigenic strains of dominant *Aspergillus* spp. can be developed for bio-control by deletion of key enzymes in the aflatoxin biosynthetic pathway. Such genetically engineered strains of *A. flavus* and *A. parasiticus* can be applied individually or in combination. A genetically engineered *A. parasiticus* bio-control strain was developed by disruption of the *uvm*8 gene of the aflatoxin biosynthetic pathway.

Because of uncertainty regarding the short- and long-term effects that genetically engineered organisms (GMO's) will have on human health and the environment, such strains are subjected to approval by environmental organisations (Scientist' working groups on bio safety, 1998). The application of GMO's to the environment could possibly change the ecological functions of naturally-occurring organisms as well as the genetic relationships between organisms. Interbreeding could alter the distribution of phenotypes within populations and will serve to change the role of the organisms in the ecosystem. Horizontal gene transfer could allow engineered genes to move into populations other than the target population with negative consequences. The presence of foreign proteins in food could also be harmful to individuals who suffer allergies to those proteins. Furthermore, GMO's could alter the composition of human intestinal organisms, resulting in digestive malfunction.

2.6.4. Inhibition of aflatoxin biosynthesis

An alternative approach for aflatoxin control is based on inhibition of aflatoxin biosynthesis at pre- or post-harvest level (Gourama and Bullerman, 1997). Several microorganisms have displayed ability to inhibit aflatoxin biosynthesis. Extracellular extracts of *Lactobacillus casei pseudoplantarum* 371 totally inhibited aflatoxin biosynthesis. Zjalic et al. (2006) studied the effect of exopolysaccharides and

glycoproteins, isolated from culture filtrates of *Trametes versicolor*, on AFB_1 biosynthesis. They concluded that β-glucans limit AFB_1 biosynthesis by inhibition of *nor*AmRNA expression and delay of *afl*RmRNA transcription.

Inhibition of aflatoxin biosynthesis by *A. flavus* was observed in the presence of *Aspergillus niger*, a fungus commonly associated with *A. flavus* in damaged corn (Horn and Wicklow, 1983). Furthermore, mixed cultures of *A. flavus*, *A. niger*, and *A. tamarii* inhibit biosynthesis of aflatoxin (Shantha et al., 1990), due to the production of inhibitors by *A. niger* and *A. tamarii*, as well as their ability to degrade aflatoxin.

2.6.5. Biological detoxification

Biological detoxification offers an attractive alternative for eliminating toxins as well as safe-guarding the desired qualities in food and feed, such as nutritive value and appearance (Mishra and Das, 2003). Furthermore, the development of biological detoxification measures, especially in traditional African fermented food products, is essential to improve the safety of these foods for human consumption (Sweeney and Dobson, 1999).

Several studies were done regarding removal of aflatoxin by adhesion to probiotic bacteria (Peltonen et al., 2000). Build-up of AFB_1 in the intestine of animals was reduced by the formation of aflatoxin-bacteria complexes. Bacteria capable of adhesion to aflatoxin include *Lactobacillus rhamnosus, Propionibacterium freudenrichii* and *Bifidobacterium* sp. The mechanism of binding involves hydrophobic and electrostatic interactions as well as the formation of hydrogen bonds. Furthermore, bio-competitive inhibition of aflatoxin production by bacteria from the geocarposphere (zone around the groundnut pod) can effectively protect developing groundnut pods against aflatoxigenic fungi (Chaurasia, 1995). *Flavobacterium odoratum* have shown effective inhibition of aflatoxin biosynthesis during experiments regarding growth and aflatoxin production by *A. flavus* on viable groundnut kernels and its interaction with other organisms.

Only few studies reported true biological degradation of AFB_1 by microorganisms. Detoxification of the aflatoxin molecule occurs when the double bond of the terminal furan ring is removed or when the lactone ring is opened (Mishra and Das, 2003). These changes cause loss in fluorescence, toxicity and mutagenicity. Bacteria known to

degrade AFB_1 effectively are *Nocardia corynebacteroides* (formerly known as *F. aurantiacum)* (Ciegler et al., 1996) and *Mycobacterium fluoranthenivorans* (Hormisch et al., 2004), while notable AFB_1 degradation by *Corynebacterium rubrum* was observed (Mann and Rehm, 1977; Shih and Marth, 1974). Recently, Teniola et al. (2005) reported degradation of AFB_1 by liquid cultures of *Rhodococcus erythropolis* as well as intracellular extracts prepared from *R. erythropolis* liquid cultures. Furthermore, treatment of AFB_1 with the extracellular fraction of a *R. erythropolis* liquid culture coincided with a total loss in mutagenicity of AFB_1 (Alberts et al., 2006). These results confirm the significant role *Rhodococcus* spp. play in the removal of toxic polyaromatic compounds from the environment (Dua et al., 2002; Sakai et al., 2003).

2.7. *Rhodococcus* spp.

Rhodococcus is an aerobic gram-positive bacterium belonging to the nocardioform actinomycetes (Goodfellow, 1986). The bacterium has cocci or short, rod shaped cells and is chemoorganotrophic, having an oxidative metabolism. Furthermore, bacteria from this genus are widely distributed, but are especially abundant in soil, water and herbivore dung. The genus *Rhodococcus* currently comprise of 19 species: *Rhodococcus rhodocrous*, *Rhodococcus bronchialis*, *Rhodococcus coprophilus*, *Rhodococcus equi*, *R. erythropolis*, *Rhodococcus fascians*, *Rhodococcus globerulus*, *Rhodococcus luteus*, *Rhodococcus marinonascens*, *Rhodococcus maris*, *Rhodococcus rhodnii*, *Rhodococcus ruber*, *Rhodococcus rubropertinctus*, *Rhodococcus terrae*, *Rhodococcus aichiensis*, *Rhodococcus aurantiacus*, *Rhodococcus chubuensis*, *Rhodococcus obuensis*, and *Rhodococcus sputi* (Goodfellow, 1986).

2.7.1. Degradation of polyaromatic compounds

Rhodococcus spp. are capable of transforming a wide range of aromatic xenobiotic compounds (Kitagawa, et al., 2001; Sakai et al., 2003), including nitro aromatic compounds (Kitova et al., 2004), polycyclic hydrocarbons, pyridine and steroids (Goodfellow, 1986), and lignin related compounds. In addition, biphenyl moieties resulting from the breakdown of lignin within the soil might represent a carbon-rich

resource for biphenyl-degrading bacteria (Maeda et al., 1995). Toxic nitro aromatic compounds and polyaromatic biphenyls containing chlorine, sulphur and nitrogen are commonly used in the manufacturing of paper, production and utilization of heat carriers and dielectrics, hydraulic oils, diluents, plasticizers and synthetic dyes (Kitagawa et al., 2001; Rabinowich et al., 2004; Sakai et al., 2003). Release of these compounds into the environment causes vast environmental problems worldwide by threatening both the natural ecosystem and human health (Kitova et al., 2004; Ohtsubo et al., 2004).

2,4-Dinitrophenol (2,4-DNP) is a nitro aromatic compound used in the production of picric acid, sulphur dyes, antiseptics and pesticides (Kitova et al., 2004). Whereas 2,4-DNP is toxic and carcinogenic, *R. erythropolis* strain HL PM-1 was shown to utilize 2,4-DNP as sole carbon source. Furthermore, polychlorinated biphenyls (PCBs) are very stable chemical compounds containing one to ten chlorine atoms on a biphenyl carbon skeleton (Ohtsubo et al., 2004). One of the most effective approaches to remove these compounds from the environment is microbial degradation. Several organisms, including *Rhodococcus* sp., *Pseudomonas* sp., *Burkholderia* sp., *Achromobacter* sp., *Comamonas* sp., *Ralstonia* sp., *Acinetobacter* sp., and *Bacillus* sp. are capable of effectively degrading biphenyl via the biphenyl degradation pathway.

2.7.2. The biphenyl degradation pathway

Bacterial degradation of biphenyl is initiated by the attack of a dioxygenase at carbon positions 2,3 (or 5,6) of the biphenyl molecule (Bedard et al., 1986). *Bph*A encodes a biphenyl dioxygenase enzyme composed of four subunits (BphA1, BphA2, BphA3 and BphA4) that converts biphenyl to dihydriol, after which dihydriol is converted to 2,3-dihydroxybiphenyl by a dehydrogenase enzyme (BphB) (Ohtsubo et al., 2004) (Fig. 4). During the following reaction, 2,3-dihydroxybiphenyl is converted to 2-hydroxy-6-oxo-6-phenyhexa-2,4-deionic acid by a ring cleavage deoxygenase (BphC). Thereafter the formation of benzoate and 2-hydroxy-penta-2,4-dienoate (HPD) is catalyzed by a hydrolase (BphD) and further metabolized to pyruvate and CoA by the HPD metabolic pathway enzymes, which include a hydratase (BphE), 4-hydroxy-2-oxovalerate aldolase (BphF) and acetaldehyde dehydrogenase (BphG) (Sakai et al., 2003).

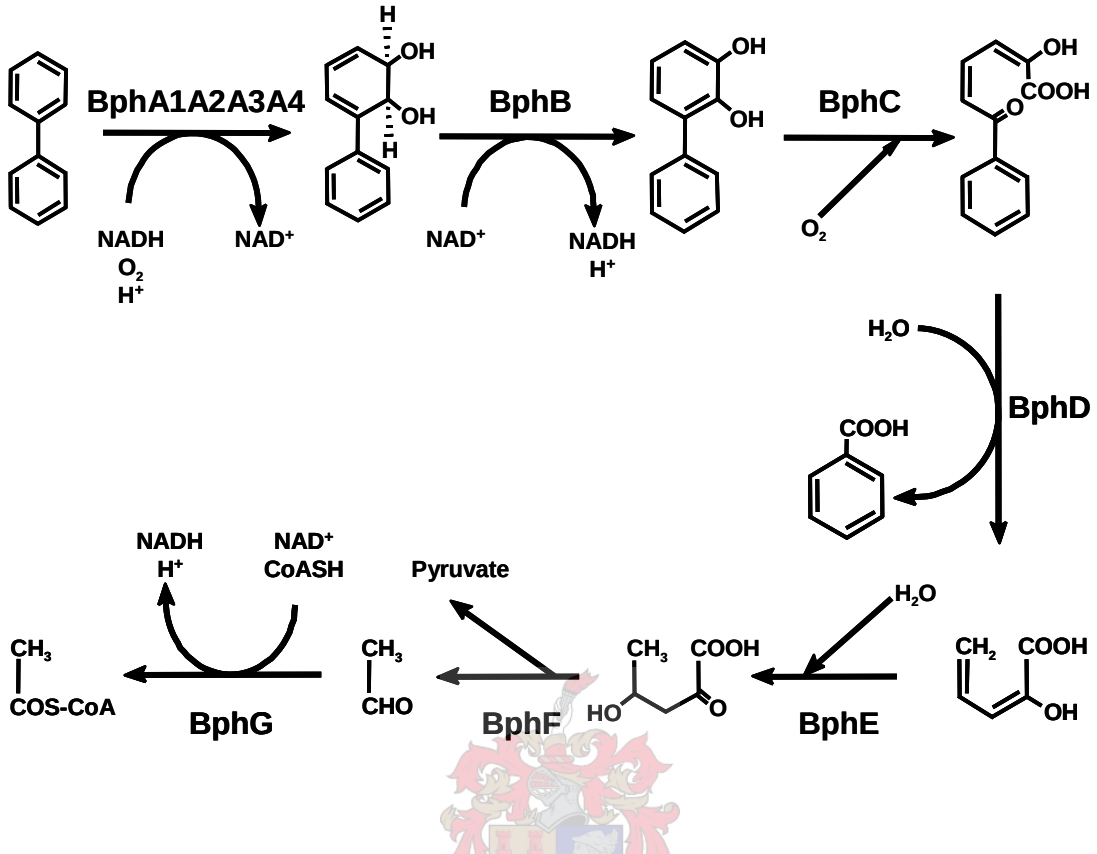


Fig. 4. The biphenyl degradation pathway (Ohtsubo et al., 2004). BphA, biphenyl dioxygenase enzyme composed of four subunits (BphA1, BphA2, BphA3 and BphA4); BphB, dehydrogenase; BphC, ring cleavage deoxygenase; BphD, hydrolase; BphE, hydratase; BphF, aldolase; BphG, acetaldehyde dehydrogenase.

The biphenyl degradation pathway is conserved among the most degraders (Ohtsubo et al., 2004) and conversions catalyzed by a series of enzymes through a cascade of reactions (Fig. 4). Aromatic-ring-hydroxylating dioxygenases (ARHDOs), more specifically biphenyl 2,3-dioxygenases, are key enzymes in the aerobic metabolism of aromatic compounds (Kahl and Hofer, 2003; Pieper, 2005). Having broad substrate specificities, the ARHDOs catalyze several ring cleavage reactions by the addition of two hydroxyl groups to carbons and consequently the formation of dihydriol compounds

(Kosono et al., 1997). The initial conversion steps during degradation of biphenyl are catalyzed by biphenyl 2,3-dioxygenases (Pieper, 2005). The biphenyl 2,3-dioxygenases belong to a large family of non-heme iron oxygenases. They consist of a terminal oxygenase which has α- and β-subunits, a ferredoxin and a ferredoxin reductase.

It was reported that *bpd*S/*bpd*T gene products are involved in the regulation of PCB degrading genes in *Rhodococcus* spp. (Ohtsubo et al., 2004). A variety of aromatic compounds activate the regulatory system and as a result transcription of the degradation genes. Furthermore, there is an association between the degree of transcription and degradation. Degradation increases while the transcription levels are low, but as the transcription levels increase, the degradation performance reaches a plateau, where after degradation performance starts to decline. This can be attributed to the complex effects of limited translation, availability of the substrates, energetic burden of the cell, and genetic instability of the degradation genes. In addition, expression of degradation enzymes is low when more preferable carbon sources are present in the environment.

2.7.3. Degradation of PCB congeners

All of the *bph* enzymes catalyze the degradation of several PCB congeners (Ohtsubo et al., 2004). However, the efficiency and reaction intermediates differ between the different PCBs, depending mainly on the following factors: solubility of the compound, expression of the degrading enzymes in the cells and the catalytic breakdown. The solubility of polyaromatic compounds is enhanced by the addition of surfactants and as a result improves entrance of the compounds into the cytoplasm. Furthermore, the presence of certain substrates in the environment is recognized by a regulatory protein in the organism and induces expression of degradation enzymes. The α-subunits of the 2,3-biphenyl dioxygenase enzymes were found to be crucial for recognition and binding of the substrates (Pieper, 2005).

2.7.4. Genes coding for degradation of aromatic compounds

2.7.4.1. Horizontal gene transfer

Horizontal gene transfer of catabolic genes located on plasmids, transposons and integrons, is a key mechanism for the evolution of catabolic pathways (Poelarends et al., 2000). Horizontal gene transfer of catabolic genes is especially significant for adaptation of microbial communities exposed to xenobiotics. Genes involved in primary metabolism are usually associated with the chromosome, while catabolic genes are often located on plasmids in *Rhodococcus* spp. (Kitagawa et al., 2001). Several *Rhodococcus* spp., including *R. erythropolis*, *R. opacus* and *R. fascians* harbour linear plasmids (Shimizu et al., 2001). Three huge linear plasmids, pRHL1 (1100 kb), pRHL2 (450 kb) and pRHL3 (330 kb), harbouring genes for catabolism of xenobiotics, were characterized in *Rhodococcus* sp. strain RHA1. Likewise, three of seven *bph*C genes characterized in *R. erythropolis* TA 421 are located on a linear plasmid (500 kb) (Kosono et al., 1997). The linear plasmids of *Rhodococcus* spp., harbouring catabolic genes, have been associated with HGT (Masai et al., 1997; Warren et al., 2004). Insertion sequences, transposes genes and gene duplications detected on the pRHL3 plasmid of *R. erythropolis* RHA1 indicate that these genes evolved through HGT. Furthermore, large similarities between haloalkane dehalogenase genes of *R. rhodochrous* NCIMB13064, *Pseudomonas pavonaceae* 170, and *Mycobacterium* sp. strain GP1 (Poelarends et al., 2000), nitrile/amidase gene clusters of *R. erythropolis* strains AJ270, AJ300 and *Microbacterium* sp. AJ115 (O'Mahony et al., 2005) implicate HGT.

2.7.4.2. Degradation of biphenyl

Several genes encoding enzymes responsible for the degradation of PCBs were cloned from *Rhodococcus* spp. and characterized (Hauschild et al., 1996; Masai et al., 1995; Masai et al., 1997; Yamada et al., 1998). These enzymes include 2,3-dihydroxybiphenyl dioxygenases (2,3-DHBDs) for which seven *bph*C genes in *R. erythropolis* TA 421 have been characterized, while eight genes were found in *R. rhodocrous* K37 (Pieper, 2005)

and three in *R. globerulus* P6 (Kosono et al., 1997). The genes encoding 2,3-DHBDs are usually clustered and transcribed as an operon.

The *bph*ACB genes encoding 2,3-DHBDs, which are responsible for the initial ring cleavage steps during degradation of biphenyl compounds, were cloned from *Rhodococcus* sp. strain RHA1 (Masai et al., 1997). In addition, several HPD metabolic pathway genes encoding meta-cleavage enzymes, were cloned from the same strain (Sakai et al., 2003). These include the HPD hydratase (*bph*E1), 4-hydroxy-2-oxovalerate aldolase (*bph*F1) and acetaldehyde dehydrogenase (*bph*G) genes. It was found that products of these genes are also involved in preventing accumulation of toxic metabolites that could interfere with the growth of the organism.

2.7.4.3. Degradation of benzene, ethylbenzene and catechol

The benzoate catabolic genes *ben*ABCDK were cloned from *Rhodococcus* sp. strain RHA1 and it was found that they are localized on the chromosome (Kitagawa et al., 2001). Hauschild et al. (1996) cloned the *etb*C gene coding for a 2,3-DHBD from *Rhodococcus* sp. strain RHA1 when grown on ethylbenzene. *Etb*C have broad substrate specificity and is possibly co-expressed with *bph*C. In addition, two 2-hydroxy-6-oxohepta-2,4-dienoate genes, *etb*D1 and *etb*D2, were cloned from *Rhodococcus* sp. strain RHA1 (Yamada et al., 1998). These genes are induced by biphenyl, ethylbenzene, benzene, toluene and *ortho*-xylene and the gene products involved in the *meta*-cleavage metabolic pathway of ethylbenzene. The *etb*C gene is located upstream of the *bph*DEF gene cluster on a linear plasmid in *Rhodococcus* sp. strain RHA1 (Masai et al., 1997). In addition, four extradiol deoxygenase enzymes, capable of degrading catechol, were cloned from *Rhodococcus* spp. (Kulakov et al., 1998).

2.7.4.4. Degradation of phenylacetate

In gram-negative bacteria, aerobic degradation of phenylacetic acid (PAA) via phenylacetyl-CoA and consequently hydrolytic ring cleavage, is prevalent in the degradation of a diversity of aromatic compounds (Navarro-Llorens et al., 2005). However, the PAA catabolic pathway is not well characterized in gram-positive bacteria. Nonetheless, a gene cluster containing 13 *paa* genes coding for products orthologous to

those of gram-negative bacteria was identified on the chromosome of *Rhodococcus* sp. strain RHA1. Yet, the range of substrates degraded by *Rhodococcus* sp. strain RHA1 via the PAA pathway is restricted and the role of the pathway in gram-positive bacteria not well understood.

2.7.4.5. Expression of *Rhodococcus* spp. catabolic genes

Numerous genes encoding catabolic enzymes in *Rhodococcus* spp. have been cloned by employing *Escherichia coli* as host and by using a variety of expression vectors. As a result several recombinant *E. coli* strains capable of degrading xenobiotic compounds such as biphenyl, ethylbenzene and benzoate were developed. These include extradiol dioxygenase (*edo*) genes cloned into *E. coli* DH5α (Kulakov et al., 1998) employing pUC129 as plasmid as well as several biphenyl- (*bph*) (Masai et al., 1995), ethylbenzene- (*etb*) (Yamada et al., 1998) and benzoate (*ben*) catabolic genes (Kitagawa et al., 2001) which was cloned into *E. coli* JM109 by using a variety of vectors.

2.8. Degradation of AFB_1 by fungi

In recent years it became clear that fungi play a major role in the degradation of AFB_1. Fungi associated with AFB_1 degradation include zygomycetous fungi (*Rhizopus* sp. and *Mucor* sp.), ascomycetous fungi (*A. niger* and *Trichoderma* sp.), plant pathogens (*Phoma* sp. and *Alternaria* sp.) as well as basidiomycetous fungi (*Armillariella tabescens* and other white rot fungi) (Leonowicz et al., 1999; Liu et al., 1998 a,b,c; Nakazato et al., 1990; Shantha et al., 1990; Shantha, 1999; Yao et al., 1998). However, it is still uncertain which enzymes are involved in the degradation of AFB_1 by fungi.

Although younger cultures of *A. flavus* and *A. parasiticus* produce AFB_1, older cultures of these fungi degrade AFB_1, presumable under nitrogen-limiting conditions (Hamid and Smith, 1987; Huynh and Lloyd, 1984; Shih and Marth, 1974). During AFB_1 biosynthesis, AFB_1 concentration reaches a maximum, where after it decreases due to the involvement of cytochrome P-450 monooxygenase degradation. The degree of degradation depends mainly on the age of the culture and temperature of incubation (Faraj et al., 1993). More elevated AFB_1 degradation was observed in cultures at 40ºC

when compared to 30ºC, while older cultures (12 d) degrade AFB_1 more effectively. Furthermore, 40% AFB_1 degradation was observed in a single culture of *A. flavus*, while *A. flavus* in mixed culture with *A. niger*, *Rhizopus oryzae* and *Bacillus stearothermophilus* displayed 80, 70 and 87% AFB_1 degradation respectively. Furthermore, it was found that AFB_1 is converted to isomeric hydroxyl compounds by *Rhizopus* spp. (Mishra and Das, 2003).

Other fungi capable of degrading AFB_1 include *Tetrahymena pyriformis,* exhibiting 67% AFB_1 degradation after 48 h (Mishra and Das, 2003). Motomura et al. (2003) reported degradation of AFB_1 by unconcentrated supernatant of *Pleurotus ostreatus* cultured in liquid medium containing glucose, peptone and yeast extract. In addition, degradation of AFB_1 and loss of mutagenicity was observed after treatment of AFB_1 with an enzyme isolated from the mycelium of *A. tabescens* (Liu et al. 1998 a,b,c). Detoxification was accomplished after cleavage of the difuran ring of the AFB_1 molecule.

2.8.1. Degradation of lignin

When considering polyphenolic compounds in nature, lignin is undoubtedly the most abundant and possibly also the most heterogeneous and recalcitrant compound to be degraded microbially (de Jongh et al., 1994). Lignin is a complex, three-dimensional, reticular, polyaromatic phenylpropanoid polymer and the main structural component of plant cell walls (Rabinowich et al., 2004). Lignin is most efficiently degraded to CO_2 and H_2O by microbial communities consisting mainly of basidiomycete, ascomycete and a broad range of soil mycelial fungi.

It should be noted that lignin is not metabolized completely, but is converted partially to condensed products, such as biphenyl compounds. Microbial consortia and several non-enzymatic mechanisms, including complexes of transition metal ions, peroxide, lipoperoxide, superoxide radicals and endogenous redox mediators, are responsible to complete the process. Different organisms are eventually involved in initially opening the lignocellulosic structure, depolymerisation of the complex compounds, utilisation of the released sugars, and finally mineralization of the more recalcitrant phenolic compounds. The consortia typically consist of filamentous fungi,

yeasts and bacteria. There are indications that *Aspergillus* spp. in conjunction with white rot fungi may be actively involved in lignin degradation (Duarte and Costa-Ferreira, 1994), while yeasts and lactic acid bacteria are primarily secondary consumers of degradation products.

Several groups of enzymes are involved in the degradation of lignin and aromatic xenobiotics, including heme-containing peroxidases, flavine oxidases, cellobiose dehydrogenases as well as laccases (Rabinowich et al., 2004). It was found that the presence of laccase is required for lignin degradation by some white rot fungi, since lignin-deficient mutants lose their ability to degrade lignin. Moreover, multiple isomers of these enzymes are produced by ligninolytic fungi.

Ligninolytic fungi differ in their requirement for nitrogen, carbon, copper, manganese and sulphur for optimal production of ligninolytic enzymes and conditions of nitrogen deficiency not always favourable for enzyme production (Rabinowich et al., 2004). While nitrogen rich media stimulates the production of ligninolytic enzymes in certain fungi (*P. ostreatus*, *Lentinus edodus*, *Cariolopsis gallica* and *Bjerkandera adusta*), *Phanerochaete laevis* produce high levels of Mn-peroxidase under nitrogen limited conditions. Furthermore, *Phanerochaete chrysosporium* produces laccase in nitrogen rich medium and exclusively with cellulose as carbon source, while copper stimulates the production of laccase (Rabinowich et al., 2004).

2.8.2. Degradation of aromatic xenobiotics

When the degradation of aromatic xenobiotics are considered, fungi again feature as one of the major groups responsible for their degradation, presumably due to the large repertoire of extracellular enzymes produced by these fungi (Armstrong and Patel, 1994; Hammel, 1995; Higson, 1991; Singh et al., 1991). White rot fungi are capable of oxidizing and degrading a wide range of xenobiotics, including polycyclic aromatic hydrocarbons, polychlorinated phenol, chlorinated guaiacol, benzoate derivatives, chlorinated biphenyls (Rabinowich et al., 2004), as well as nitro aromatic compounds (Hiratsuka et al., 2001).

Grey et al. (1998) have reported extracellular extracts of *T. versicolor* to degrade 2-chlorophenol to 2-chloro-1,4-benzoquinone. However, degradation was much higher

in the presence of the mycelium and the degradation attributed to the involvement of extracellular laccases as well as cell-bound processes.

2.8.3. Laccase enzymes

Fungal laccases often function as isoenzymes that oligomerize to form multimeric complexes (Claus, 2004). The molecular mass of the monomer ranges from about 50 to 100 kDa. Laccase enzymes are very stable probably due to a covalently linked carbohydrate moiety. For catalytic activity a minimum of four copper atoms per active protein is needed. The various copper atoms of laccases drive electrons from a reducing substrate to molecular oxygen without releasing toxic peroxide intermediates.

The enzymatic oxidation of phenol compounds by laccase generates radicals which react with each other to form dimers, oligomers and polymers (Claus, 2004). This characteristic of laccase is valuable for their application to detoxify contaminated soil or waste waters. As a result, cathecol and other phenolic substrates are polymerized and removed from wastewater streams in the form of a precipitate (Aktas and Tanyolac, 2003).

Furthermore, laccases produced by ligninolytic fungi are capable of generating highly active free radicals during degradation of complex natural polymers such as lignin, which lead to the cleavage of covalent bonds of the substrate and the release of monomers (Claus, 2004). Sometimes the enzymes are not directly in contact with the polymers, due to steric hindrance, while small organic compounds or metals mediate the radical-catalyzed depolymerization. In addition, laccases are extremely versatile enzymes, catalizing one basic reaction from which all its activities originate (Rabinowich et al., 2004).

Laccases of white rot fungi have acidic pH optima (4-5) for oxidation of phenolic substrates (Rabinowich et al., 2004). In contrast, laccase produced by soft rot fungi (*Myceliophthora thermophila, Chaetomium thermophile, Rhizoctonia solani*) causing humification of wood, have more neutral pH optima (6). The more neutral laccases polymerize low-molecular-weight phenols and detoxify compounds of the natural wood defence system resulting in humification. Enzymes with more acidic pH optima are more prominent in free-radical ligninolysis.

2.9. The current approach: Biological degradation of AFB_1

2.9.1. Biological degradation of AFB_1 by (a) *R. erythropolis* liquid cultures (b) 2,3-DHBD of *R. erythropolis* through extracellular expression of the *bph*C1 gene in *E. coli*

Aflatoxins are difuranocoumarin derivatives and are somewhat structurally related to the above-mentioned xenobiotic aromatic compounds effectively degraded by *Rhodococcus* spp. (Minto and Townsend, 1997; Payne and Brown, 1998). For that reason the biological degradation of AFB_1 by *R. erythropolis* liquid cultures as well as intra- and extracellular extracts obtained from liquid cultures were investigated [Alberts et al., 2006 (Chapter 4); Teniola et al., 2005 (Chapter 3)]. The degradation of AFB_1 by intracellular extracts of *R. erythropolis* DSM 14303, *M. fluoranthenivorans* sp. nov. DSM 44556^T, *N. corynebacterioides* DSM 20151 and *N. corynebacterioides* DSM 12676 were compared [Teniola et al., 2005 (Chapter 3)]. In addition, the degradation of AFB_1 by 2,3-DHBD in *R. erythropolis* through extracellular expression of the *bph*C1 gene in *E. coli* was studied (Chapter 5). Moreover, evidence will be presented whether the degradation coincides with a decrease in fluorescence and mutagenicity of the AFB_1 molecule.

2.9.2. Biological degradation of AFB_1 by (a) white rot fungi in liquid culture (b) fungal laccase from *T. versicolor*

While white rot fungi have the potential to degrade lignin as well as a wide range of polycyclic aromatic hydrocarbons, the unique mechanisms of these fungi will possibly also allow them to target and degrade AFB_1. The association between laccase production and AFB_1 degradation by cell free extracts of fungal liquid cultures was investigated (Chapter 6). In addition, the ability of fungal laccase from *T. versicolor* as well as recombinant laccase produced by *A. niger* (D15-Lcc2#3) to degrade AFB_1 was determined and if the degradation coincides with loss of mutagenicity.

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Degradation of aflatoxin B_1 by cell-free extracts of *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* sp. nov. DSM44556^T

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Abstract

Biological degradation of aflatoxin B_1 (AFB_1) by *Rhodococcus erythropolis* was examined in liquid cultures and in cell-free extracts. Dramatic reduction of AFB_1 was observed during incubation in the presence of *R. erythropolis* cells (17% residual AFB_1 after 48 h and only 3–6% residual AFB_1 after 72 h). Cell-free extracts of four bacterial strains, *R. erythropolis* DSM 14303, *Nocardia corynebacterioides* DSM 12676, *N. corynebacterioides* DSM 20151, and *Mycobacterium fluoranthenivorans* sp. nov. DSM 44556^T were produced by disrupting cells in a French pressure cell. The ability of crude cell-free extracts to degrade AFB_1 was studied under different incubation conditions. Aflatoxin B_1 was effectively degraded by cell free extracts of all four bacterial strains. *N. corynebacterioides* DSM 12676 (formerly erroneously classified as *Flavobacterium aurantiacum*) showed the lowest degradation ability (60%) after 24 h, while >90% degradation was observed with *N. corynebacterioides* DSM 20151 over the same time. *R. erythropolis* and *M. fluoranthenivorans* sp. nov. DSM 44556^T have shown more than 90% degradation of AFB_1 within 4 h at 30 °C, whilst after 8 h AFB_1 was practicably not detectable. The high degradation rate and wide temperature range for degradation by *R. erythropolis* DSM 14303 and *M. fluoranthenivorans* sp. nov. DSM 44556^T indicate potential for application in food and feed processing.

Keywords: Aflatoxin B_1; AFB_1, degradation; *Rhodococcus erythropolis*; *Mycobacterium fluoranthenivorans* sp. nov.

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1. Introduction

Aflatoxin B_1 (AFB_1) is one of the mycotoxins produced by some strains of *Aspergillus flavus*, *Aspergillus nomius* and *Aspergillus parasiticus* (Deiner et al., 1987; Kurtzman et al., 1987; Cotty and Bhatnagar, 1994). It is one of the most potent naturally occurring mutagens and carcinogens known. Aflatoxin B_1 has been reported in many foods and food and feed raw materials such as peanuts, peanut meals, peanut butter, soybeans, cottonseed meal, sorghum, millet, corn, corn oil, dried chili peppers, milk, etc. (Ciegler et al., 1966; Nwokolo and Okonkwo, 1978; Salifu, 1981; Hao and Brackett, 1988; Hudson et al., 1992). Food contaminated with AFB_1 poses a serious health threat when consumed by human and animals, and when present at unacceptable levels, the contamination may also result in severe economic problems world-wide. This toxin has been reported to be carcinogenic, teratogenic and hepatotoxic (Eaton and Groopman, 1994; Guengerich et al., 1996). The growth of the producing mould itself does not always indicate a significant presence of the mycotoxin. Synthesis depends on favourable growth conditions related to moisture, temperature, substrate composition, competition with other micro-organisms, aeration as well as genetic requirements (Deiner et al., 1987; Ellis et al., 1991; Payne, 1992; Cotty and Bhatnagar, 1994; Woloshuk and Priesto, 1998).

Aflatoxin may be degraded by physical, chemical or biological means (Nkana, 1987; Park, 1993). However, limitations such as losses of product nutritional and organoleptic qualities, undesirable health effects of such treatments and expensive equipment required for other degradation techniques has encouraged recent emphasis on biological methods (Samarajeewa et al., 1990; Philips et al., 1994). Some microbial isolates have been reported with different levels of degradation abilities. However, studies on numerous lactic acid bacterial strains have indicated that this phenomenon may be explained by adsorption rather than by degradation (El-Nezami et al., 1998). *Aspergillus niger*, *A. parasiticus*, *Trichoderma viride*, *Mucor ambiguus* and few other fungi have been reported to show significant AFB_1 degradation abilities (Mann and Rehm, 1976; Tsubouchi et al., 1983; Huynh et al., 1984; Line et al., 1994). Limitations such as long degradation time (lasting more than 72 h), incomplete degradation, non-adaptation to typical food fermentations, and culture pigmentation, however, reduce their potential for use in the food industry. Moreover, some of these strains with degradation potential may also produce AFB_1 under varying conditions (Huynh et al., 1984).

Despite the wide attention and potential of the only hitherto known AFB_1 degrading bacterial strain (Ciegler et al., 1966; Smiley and Draughon, 2000; D'Souza and Brackett, 2001), it was thus-far erroneously classified as *Flavobacterium aurantiacum* NRRL B-184 [DSM 12676; now reclassified as *Nocardia corynebacterioides* DSM 12606; Hormisch et al., 2004].

This work was conducted to demonstrate the ability of a new strain (DSM 41303) of *Rhodococcus erythropolis*, isolated from polycyclic aromatic hydrocarbons (PAH) contaminated soils, to degrade AFB_1 in liquid cultures. Furthermore, we obtained comparative information on the intracellular degradation of AFB_1 by four bacterial strains, including *R. erythropolis* DSM 41303, and a strain representing a new species, *Mycobacterium fluoranthenivorans* strain DSM 44556^T, using cell free extracts obtained by disruption with the French pressure cell. The optimal temperature conditions and reaction time kinetics were also investigated. Finally, with these investigations, the ultimate aim is to eventually contribute to improve the safety of fermented African food products by developing a suitable biological detoxification procedure that may be adopted for traditional processes.

2. Material and Methods

2.1. Cultures

N. corynebacterioides DSM 12676, *N. corynebacterioides* DSM 20151, *R. erythropolis* strain Aney3 and Mycobacterium sp. strain Fa4 were used for the experiments. The *R. erythropolis* and *Mycobacterium* strains were isolated from PAH contaminated soils by the Institute of Applied Microbiology, University of Saarbruecken, Germany. Meanwhile, the *Mycobacterium* strain has been found to represent a new species, *M. fluoranthenivorans*, which was originally depos-

ited under the restricted number as strain DSM 14304, and eventually submitted as type strain DSM 44556^T (Hormisch et al., 2004), whilst *R. erythropolis* was deposited under the restricted number DSM 14303. Both these strains are protected by a patent application (Holzapfel et al., 2002). The Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, provided the two *N. corynebacterium* strains, and aided in the final identification of strains Aney3 and Fa4.

2.2. Degradation of AFB_1 by R. erythropolis in liquid cultures

R. erythropolis was grown in Standard I broth (pH 7.0) (Goodfellow, 1986). The broth (0.8 ml) was aliquoted to sterile 2-ml screw-cap Eppendorf tubes and inoculated with 50 μl of a 24-h pre-inoculum of *R. erythropolis.* For degradation experiments, a stock solution of AFB_1 (100 ppm) was used to supplement cultures to a final concentration of 1.75 ppm. Cultures were incubated at 30 °C on a rotor wheel for 24 h, 48 h and 72 h respectively. The following two controls were included: (a) sterile Standard I broth. (b) Standard 1 broth to which 200 μl autoclaved *R. erythropolis* cells were added. After incubation the cells were removed by centrifugation (13,000 min^{-1}), the AFB_1 was extracted from the supernatants and quantified by HPLC as described later.

2.3. Preparation of cell free extracts

The frozen or freeze-dried bacterial cultures were reactivated by successive cultivation steps in Standard-I-nutrient broth (S-I-media, 1.07882.0500, Merck, Darmstadt, Germany). For inoculation, 20 ml of a 24 h culture broth were transferred to 500 ml of sterile S-I-medium. Sterilisation was at 121 °C for 15 min. Inoculated cultures were grown by incubation at 30 °C and agitation at 160 min^{-1} for 48 h using a Gyrotary shaker incubator (Model G25, New Brunswick Scientific Co., USA). The cells were harvested by centrifugation (Heraeus Sepatech centrifuge, Stuttgart, Germany) at 4 °C using 6000 min^{-1} for 20 min. The pellets were washed twice with phosphate-buffer (67 mM; pH 7.0). The cell pellets were resuspended in phosphate buffer (pH 7.0) in preparation for cell rupture (3 ml buffer per gram cell mass). This suspension was disintegrated thrice by using the French-press at 8274 MPa pressure (Aminco French pressure cell, SLM Instruments Inc.). The cell disruption steps were carried out on ice to ensure low temperature conditions required for most enzymes. The disintegrated cell suspension was centrifuged at 20,000×*g* for 20 min at 4 °C (Sorvall RC 26 Plus, Kendro Lab., Bad Homburg, Germany). Supernatant from the centrifugation step was filtered aseptically using sterile cellulose pyrogen free disposable filters of 0.2 μm pore size (Schleicher and Schuell, Germany).

2.4. Degradation of AFB_1 by cell-free extracts

The experiment was performed in 2-ml-Eppendorf-tubes in a final volume of 750 μl and an initial AFB_1-concentration of 2.5 ppm. This involved the addition of 20 μl stock solution of AFB_1 dissolved in methanol (LiChroSolv, Merck) to 730 μl cell free extract. The mixture was incubated in the dark at 30 °C without shaking for 1, 2, 4, 6, 8 and 24 h for the optimal reaction or incubation time studies. Degradation at different temperatures was also studied to obtain the optimum temperature amongst the bacteria isolates. Temperatures considered were 10 °C, 20 °C, 30 °C and 40 °C over a period of 20 h at pH 7.0. All experiments were carried out in duplicates. Each experiment was terminated by the addition of 750 μl of HPLC grade chloroform (LiChroSolv, Merck) for extraction of the remaining AFB_1.

2.5. Extraction and quantification of residual AFB_1

Aflatoxin B_1 was extracted three times with chloroform (LiChroSolv, Merck) from liquid cultures and cell-free extracts. The chloroform was evaporated under nitrogen gas, the samples were dissolved in methanol (LiChroSolv, Merck), filtered (Millex-GV, Durapore, 0.22 μm) and analysed by HPLC. HPLC analyses were performed on a Merck HPLC System (D-7000 series) using a guard column [LiChroCART 4-4 RP-C18 (5 μm), Merck] followed by a LiChroCART RP-C18 [250-4 Hypersil ODS (5 μm), Merck] column. The mobile phase was acetonitrile/methanol/water (1:1:2, v/v/v) at a flow rate of 1 ml/min and the sample temperature was set at 22 °C. AFB_1 was measured by UV (365 nm.) detection. Raw data were evaluated by the HPLC ChemStation software system (Hewlett Packard).

Table 1
Protein concentration of the cell-free extracts of the bacterial isolates used

Strain	Protein concentration [mg/ml]
Nocardia corynebacterioides DSM12676	1.5
Nocardia corynebacterioides DSM20151	2.9
Mycobacterium fluoranthenivorans nov. sp., strain DSM 44556^T	3.8
Rhodococcus erythropolis DSM14303	3.4

2.6. Protein determination

The total protein concentrations were measured by the microbiureth-method (Goa, 1953). Standard curve was produced by using a known concentration of bovine serum albumen (BSA). The concentrations of crude protein extracts present were extrapolated from the standard curve.

3. Results and discussion

The ability of four bacterial isolates, *N. corynebacterioides* DSM 12676, *N. corynebacterioides* DSM 20151, *R. erythropolis* strain Aney3 DSM 14303 and *M. fluoranthenivorans* strain DSM 44556^T, to degrade AFB_1 was investigated. Cells of the four isolates were disrupted with the aid of a French-press and the cell debris removed by centrifugation. The total protein concentrations in the cell free extracts from all the isolates under study are shown in Table 1. The protein concentration enables a comparison among the strains with regard to the relative concentrations of the cell free extracts as a whole, as an alternative to microbial cell numbers. This approach was a first step towards proving the enzymatic basis of the AFB_1 degrading activity. The cell free extracts of the *R. erythropolis* and *Mycobacterium* strains contained higher concentrations of protein than the two *N. corynebacterioides* strains. The cell-free extract of DSM 12676 contained 1.5 mg/ml protein and in the extract of DSM 20515, double the amount of protein was measured (2.9 mg/ml). The protein concentrations of cell free extracts from *R. erythropolis* and *M. fluoranthenivorans* did not differ much (3.4 mg/ml and 3.8 mg/ml, respectively). Aflatoxin B_1 degradation by cell free extracts of the four bacteria over a period of 24 h was followed, and different levels of degradation were observed (Fig. 1a). *R. erythropolis* and *M. fluoranthenivorans* were able to degrade AFB_1 more effectively and within a shorter time than the two *N. corynebacterioides* strains (Fig. 1a). It was particularly interesting to notice up to 70% AFB_1 elimination within 1 h of applying cell free extracts from the two strains (Fig. 1b), and >90% degradation was observed within 4 h. There was no detectable AFB_1 from any strain after 24 h, with the exception of *N.*

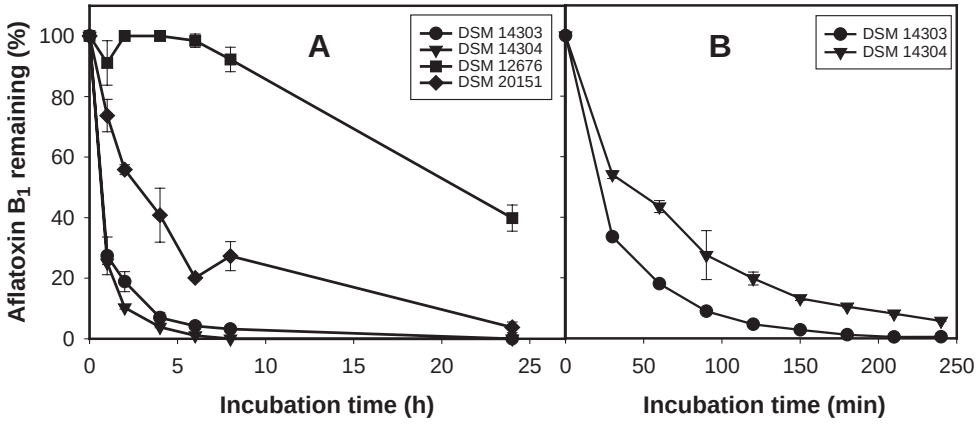

Fig. 1. Aflatoxin B_1 degradation kinetics observed over a period of 24 h (A) and 240 min (B) using cell-free extracts of four bacterial strains. Cell extracts of *Rhodococcus erythropolis* DSM 14303 and *Mycobacterium fluoranthenivorans* strain DSM 44556^T (DSM 14304), *Nocardia corynebacterioides* DSM 12676, and *N. corynebacterioides* DSM 20151 were obtained in a French pressure cell.

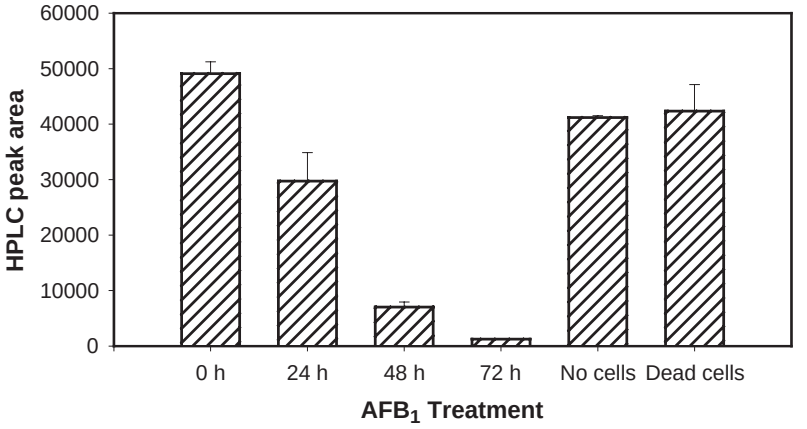

Fig. 2. Biological degradation of AFB_1 by liquid cultures of *Rhodococcus erythropolis* DSM 14303. Treatments include AFB_1 degradation at 0 h, 24 h, 48 h, and 72 h; treatment in the absence of *R. erythropolis* cells (no cells) and autoclaved *R. erythropolis* cells (dead cells).

corynebacterioides DSM 12676 (formerly *F. aurantiacum*). The cell-free extract from *N. corynebacterioides* shows AFB_1 reduction of about 10% after 8 h, and of about 60% after 24 h. The result is similar to the observation of Smiley and Draughon (2000). They observed about 74.5% AFB_1 degradation by its cell free extract obtained by lysozyme treatment after 24 h of incubation. In their study, they observed a diminishing AFB_1 degradation which was attributed to the effects of heat treatment and incorporation of proteinase K into their extract. Hence, they concluded that the degradation could be enzymatic. Difference in degradation activity with our results may be due to extract production methods employed. However, the strain DSM 20151 (formerly *C. rubrum*) showed a degradation of over 20% in 1 h and a total elimination of AFB_1 in 24 h. The result confirms the degrading potential of the organism as reported by Mann and Rehm (1976).

Liquid cultures of *R. erythropolis* were also able to degrade AFB_1 very effectively (Fig. 2). A dramatic reduction of AFB_1 was observed when incubated in the presence of *R. erythropolis* cells (17% residual AFB_1 after 48 h and only 3–6% residual AFB_1 after 72 h). By contrast, no significant reduction took place in the absence of *R. erythropolis* cells or in the presence of heat-inactivated (autoclaved) cells. The latter results established that AFB_1 was degraded and not only removed from the medium by binding to the heat-inactivated bacterial cell walls. Furthermore, AFB_1 remained stable in the uninoculated control throughout the incubation period.

Cell free extracts of *R. erythropolis* and *M. fluoranthenivorans* thus removed about 70% AFB_1 after 1 h, while practically no AFB_1 was found after 8 h. Furthermore, liquid cultures of *R. erythropolis* practically removed AFB_1 within 72 h of incubation. These two bacteria strains are therefore superior AFB_1-degraders compared to the previously known *N. corynebacterioides* strains. The two *N. corynebacterioides* strains are pigmented and are slower in activity than the new bacteria isolates (Ciegler et al.,

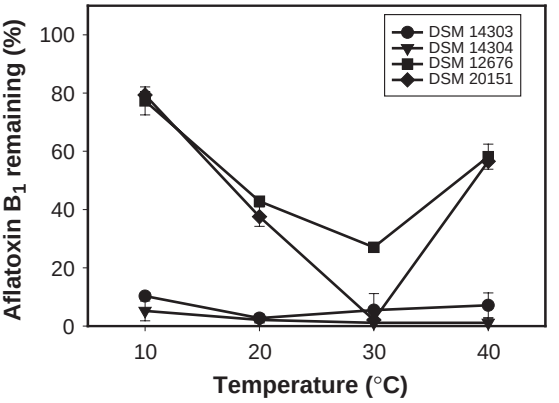

Fig. 3. Aflatoxin B_1 degradation at different temperatures using cell free extracts from *Rhodococcus erythropolis* DSM 14303 and *Mycobacterium fluoranthenivorans* strain DSM 44556^T (DSM 14304), *Nocardia corynebacterioides* DSM 12676 and *N. corynebacterioides* DSM 20151.

1966; Line et al., 1994; Mann and Rehm, 1976). These two features are obviously a disadvantage because they may affect processing time and the product's qualities during fermentation.

Our findings show degradation rather than adsorption to the cell wall which had earlier been proposed by El-Nezami et al. (1998) for lactic acid bacteria. Analysis of the chloroform extracts at the wavelenghts studied did not reflect presence of any new peaks or by-products. Past works have indicated the presence of by-products in the aqueous extracts rather than the organic phases of AFB_1 extraction during analysis (Mann and Rehm, 1976; Line et al., 1994). This may be the reason for the inability to detect any by-products in our study.

The effect of different temperatures on the loss of AFB_1 by cell-free extracts of four bacterial strains was also evaluated. There was a general reduction of AFB_1 for all the four isolates but to different extents. After an incubation time of 20 h at 10, 20, 30 and 40 °C the residual AFB_1-contents were determined as shown in Fig. 3. The two strains of *N. corynebacterioides* DSM 12676 and DSM 20151 showed an optimum at 30 °C, which agrees well with the temperature range of 20 to 28 °C reported by Ciegler et al. (1966) for *N. corynebacterioides* DSM 12676 (formerly *F. aurantiacum*) and 25 °C as the optimum.

The percentage reduction of AFB_1 by the new isolates (*R. erythropolis* and *M. fluoranthenivorans*) were about the same in the temperature range of 10–40 °C. This may imply that the enzymes in the cell-free extract either have a wide temperature range of activity, or that other factors are involved.

Our results have demonstrated that cell free extracts of all four bacterial isolates and liquid cultures of *R. erythropolis* and *M. fluoranthenivorans* effectively degrade AFB_1. Optimal degradation by the four isolates occurred at 30 °C which makes them applicable in food in the tropical environment like West Africa. The two new bacterial isolates appear to be more versatile and will have a better potential due to their fast rate of degrading AFB_1 and the wider temperature range for degradation. Further work into the enzymology, establishing the absence of by-products and with any residual toxicity, and probably also genetic modification, are required to harness the potential of these strains.

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Biological degradation of aflatoxin B_1 by *Rhodococcus erythropolis* cultures

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Abstract

Aflatoxin contamination of food and grain poses a serious economic and health problem worldwide, but particularly in Africa. Aflatoxin B_1 (AFB_1) is extremely mutagenic, toxic and a potent carcinogen to both humans and livestock and chronic exposure to low levels of AFB_1 is a concern. In this study, the biodegradation of aflatoxin B_1 (AFB_1) by *Rhodococcus erythropolis* was examined in liquid cultures using thin layer chromatography (TLC), high performance liquid chromatography (HPLC), electro spray mass spectrometry (ESMS) and liquid chromatography mass spectrometry (LCMS). AFB_1 was effectively degraded by extracellular extracts from *R. erythropolis* liquid cultures. Results indicated that the degradation is enzymatic and that the enzymes responsible for the degradation of AFB_1 are extracellular and constitutively produced. Furthermore, the biodegradation of AFB_1 when treated with *R. erythropolis* extracellular fraction coincided with a loss of mutagenicity, as evaluated by the Ames test for mutagenicity.

Keywords: Aflatoxin B_1; AFB_1 degradation; AFB_1 detoxification; Mutagenicity; *Rhodococcus erythropolis*

1. Introduction

Aflatoxins are highly toxic secondary metabolites (mycotoxins) predominantly produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Deiner et al., 1987; Kurtzman et al., 1987). Other *Aspergillus* strains producing aflatoxin are *A. nominus*, *A. tamarii* (Goto et al., 1997) and *A. pseudotamarii* (Ito et al., 2001). Aflatoxin B_1 (AFB_1), the most potent aflatoxin, is extremely toxic, mutagenic, carcinogenic and teratogenic to both humans and livestock and chronic exposure to low levels of AFB_1 pose a serious health and economic hazard (Karlovsky, 1999; Mishra and Das, 2003). AFB_1 is the mycotoxin with the greatest impact in Africa and together with hepatitis B viral infections possibly responsible for the high incidence of primary liver cancer in certain parts of Africa, such as Mozambique (Pitt, 2000). Although numerous physical and chemical detoxification methods have been tested, none really fulfills the necessary efficacy, safety, and cost requirements (Mishra and Das, 2003). Therefore, the development of biological detoxification measures, especially in traditional African fermented food products, is essential to improve the safety of these foods for human consumption (Sweeney and Dobson, 1999). Several studies were done regarding removal of aflatoxin by adhesion to probiotic bacteria (Peltonen et al., 2000) as well as biocompetitive inhibition of aflatoxin production by geocarpospheric bacteria (Chaurasia, 1995). However, few studies reported the degradation of AFB_1 by microorganisms. The only bacteria known that degrade AFB_1 effectively are *Nocardia corynebacteroides* (formerly known as *Flavobacterium aurantiacum*) (Ciegler et al., 1996) and *Mycobacterium fluoranthenivorans* (Hormisch et al., 2004), while notable AFB_1 degradation by *Corynebacterium rubrum* was observed (Mann and Rehm, 1977; Shih and Marth, 1975). Furthermore Teniola et al. (2005) recently reported degradation of AFB_1 by liquid cultures of *Rhodococcus erythropolis* as well as intracellular extracts prepared from *R. erythropolis* liquid cultures.

Rhodococcus is an aerobic gram-positive bacterium capable of transforming a wide range of xenobiotic compounds including polychlorinated biphenyls (Finnerty, 1992; Kitagawa et al., 2001; Sakai et al., 2003; Seto et al., 1995) as well as nitroaromatic compounds (Kitova et al., 2004) and is considered

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to play a critical role in the removal of toxic polyaromatic pollutants from the environment (Dua et al., 2002; Sakai et al., 2003). In previous studies several gene clusters responsible for the degradation of these compounds were characterized in *Rhodococcus* spp. (Hauschild et al., 1996; Masai et al., 1995, 1997; Yamada et al., 1998).

Aflatoxins are difuranocoumarin derivates and are somewhat structurally related to the above-mentioned xenobiotic aromatic compounds (Payne and Brown, 1998; Minto and Townsend, 1997). In this study, we investigated the biological degradation of AFB_1 by *R. erythropolis* in liquid cultures, more specifically if the extracellular fraction is responsible for the degradation and if the degradation of AFB_1 coincides with loss of mutagenicity.

2. Materials and methods

2.1. Cultures

The *R. erythropolis* strain used in this study was isolated from polycyclic aromatic hydrocarbons (PAH) contaminated soils by the Institute of Applied Microbiology, University of Saarbrücken, Germany and deposited under the restricted number DSM 14303 (Teniola et al., 2005), *R. erythropolis* was cultivated in Standard 1 broth (Goodfellow, 1986) comprising of 1.5% (w/v) peptone, 0.3 (w/v) yeast extract, 0.6% (w/v) sodium chloride, and 0.1% (w/v) D (+)-glucose.

2.2. Degradation of AFB_1 by extracellular extracts of *R. erythropolis* liquid culture

Standard 1 broth (100 ml) was inoculated with *R. erythropolis* and incubated at 30 °C for 48 h on a shaker (100 rpm). Cultures were centrifuged (10 000 rpm for 10 min), the extracellular fraction supplemented with 1.75 ppm AFB_1, aliquoted (0.8 ml) to sterile screw-cap Eppendorf tubes and incubated at 30 °C on a rotor wheel for different time intervals. Standard 1 broth supplemented with 1.75 ppm AFB_1 was used as reference. After incubation, AFB_1 was extracted from the samples and analyzed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), electro spray mass spectrometry (ESMS) and liquid chromatography mass spectrometry (LCMS) (described later).

To determine whether AFB_1 degradation is an induced or constitutive activity of *R. erythropolis*, Standard 1 broth was supplemented with AFB_1 as described by Teniola et al. (2005) and inoculated with *R. erythropolis*. A *R. erythropolis* culture grown in the absence of AFB_1 was used as reference. After cultivation at 30 °C for 48 h, the cells were removed by centrifugation. The extracellular fractions were supplemented with 1.75 ppm AFB_1, 0.8 ml aliquoted to sterile screw cap Eppendorf tubes and incubated at 30 °C for 24, 48, and 72 h, respectively. Standard 1 broth supplemented with AFB_1 was used as reference. After incubation AFB_1 was extracted from the extracellular fractions and quantified by HPLC. The production of extracellular enzymes in the absence or presence of AFB_1 was also analyzed by fractionation of extracellular proteins by SDS-PAGE. The following samples were analyzed: (a) Standard 1 broth, (b) extracellular fraction of a *R. erythropolis* culture, and (c) extracellular fraction of a *R. erythropolis* culture supplemented with AFB_1 and incubated for 72 h. The samples were concentrated by ultrafiltration (Amicon ultrafiltration cell, molecular weight cut-off of 10 kDa) and a centrifugal filter device (molecular weight cut-off of 10 kDa). SDS-PAGE was done using Coomassie staining, where after the protein profiles of the different samples were compared.

The effect of protease treatment on the AFB_1 degrading ability of extracellular fractions of *R. erythropolis* was determined by exposing the fraction to 1 mg/ml proteinase K (Roche Diagnostics, Basel, Switzerland; specific activity $\geq$30 U/mg) for 1 h at 37 °C; 0.5 mg/ml proteinase K plus 1% SDS for 6 h at 37 °C; 1 mg/ml proteinase K plus 1% SDS for 6 h at 37 °C; and 1 mg/ml proteinase K plus 1% SDS for 6 h at 4 °C. After exposure the samples were supplemented with 1.75 ppm AFB_1, aliquoted to sterile screw cap Eppendorf tubes and incubated for 72 h at 30 °C. For reference purposes, untreated extracellular fraction as well as Standard 1 broth was supplemented with AFB_1. After incubation AFB_1 was extracted from the samples with chloroform, dried under nitrogen gas, suspended in methanol and spotted on silica gel TLC plates (Merck, Darmstadt, Germany). TLC plates were developed in chloroform:acetone (9 : 1, v/v), where after they were examined under UV light.

2.3. AFB_1 extraction and chromatographic analysis

Aflatoxin B_1 was three times extracted from samples with chloroform (1 : 1, v/v) as described by Teniola et al. (2005). The chloroform was evaporated under nitrogen, the samples dissolved in methanol, filtered (Millex-GV, Durapore, 0.22 μm), and analyzed by HPLC. HPLC analysis was performed through a guard column [LiChroCART 4-4 RP-C18 (5 μm), Merck] followed by a LiChroCART RP-C18 [250-4 Hypersil ODS (5 μm), Merck] column. The mobile phase was acetonitrile : methanol : water (1 : 1 : 2, v/v/v) at a flow rate of 1 ml/min. AFB_1 was measured by UV (365 nm) detection.

Since AFB_1 could be degraded to water-soluble breakdown products (Line et al., 1994), both the organic and inorganic phases obtained from extraction were analyzed. HPLC analyses of the inorganic phases were carried out as previously described and UV absorbance was monitored between 200 and 400 nm. TLC analysis was done on silica gel plates (solvent : chloroform : acetone, 9 : 1, v/v) as well as on C_{18} plates (Merck, Darmstadt, Germany) (solvent : methanol : acetonitrile : water, 1 : 1 : 2, v/v/v). Plates were developed and examined under UV light. The following samples were analyzed by ESMS and LCMS: (a) AFB_1 standard, (b) Standard 1 broth, (c) Standard 1 broth supplemented with AFB_1 to 1.75 ppm, and (d) AFB_1 after treatment with *R. erythropolis* extracellular fraction for 72-h. The samples were extracted with chloroform, dried under nitrogen, suspended in methanol : water (7 : 3, v/v) and analyzed by ESMS (solvent : methanol : water, 7 : 3, v/v) and LCMS using a Phenomenex 2.0 × 150 mm C_{18} column and

methanol : acetonitrile : water (1 : 1 : 2, v/v/v) as solvent, at a flow rate of 100 µl/min.

2.4. The Ames test for mutagenicity

AFB_1 samples were analyzed for loss of mutagenicity after treatment with *R. erythropolis* extracellular fractions. Both the organic and inorganic phases obtained from extraction were analyzed. While the organic phases obtained from extraction were evaporated under nitrogen, the inorganic phases were lyophilized. The plate incorporation assay was done according to the method described by Moron and Ames (1983). *Salmonella typhimurium* TA100 was used as tester strain and 0.5 ml of S9 liver enzyme fraction was added per plate for metabolic activation. Dimethyl sulfoxide (DMSO) was used as solvent for AFB_1. Reference plates with and without AFB_1 (10 ng/plate) were included to determine the dose response.

2.5. Statistical analyses

Cross-classification ANOVA data analyses were done on the response variable (AFB_1 concentration or number of revertants) over controls and times observed by using a compound symmetry model among times (Dunn and Clark, 1987). A significance level of 5% was used and Bonferroni multiple comparisons were done when significant differences were encountered. When responses for two times were compared or when responses for control versus a time were compared, a paired *t*-test was used.

3. Results and discussion

In this study, we investigated the biological degradation of AFB_1 in unconcentrated, cell-free, extracellular extracts of *R. erythropolis* liquid cultures. Aflatoxin B_1 was shown to be stable over the 72 h incubation period in Standard 1 broth as no significant ($P<0.283$) difference was observed between AFB_1 reference samples after 0 and 72 h (Fig. 1). However, a significant ($P<0.001$) reduction of AFB_1 was observed from 0 to 72 h when treated with *R. erythropolis* extracellular extracts. A significant ($P<0.05$) reduction in AFB_1 was already observed after 2 h in the presence of *R. erythropolis* extracellular extracts with only 33.2% residual AFB_1 after 72 h. These results were confirmed by TLC analysis, showing a distinct decline in AFB_1 fluorescence over the 72 h incubation period while the AFB_1 reference sample showed no loss in fluorescence (data not shown).

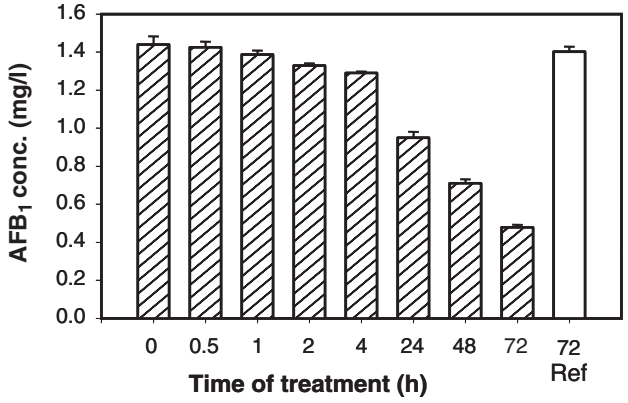

Fig. 1. AFB_1 degradation in the presence of *R. erythropolis* extracellular fraction after different time intervals (hatched bars). Untreated AFB_1 after 72 h was included as reference sample (open bar).

With the aid of ESMS, AFB_1 was identified at $m/z=313$ for the protonated cation $[M+H]^+$, $m/z=335$ for the sodiated cation of AFB_1 $[M+Na]^+$, and at $m/z=647$ for the dimeric cation $[2\ M+Na]^+$ (Fig. 2A). Standard 1 broth showed no peaks with m/z values matching those of the three distinct AFB_1 peaks (data not shown), but Standard 1 broth supplemented with AFB_1 clearly showed the sodiated and protonated cations at $m/z=335$ and $m/z=313$, respectively (Fig. 2B). The ESMS spectrum of AFB_1 (1.75 ppm) after 72 h treatment with the *R. erythropolis* extracellular fraction did not display any of the distinct AFB_1 peaks at $m/z=313$ or $m/z=335$ (Fig. 2C). LCMS analysis of the treated sample showed that AFB_1 was still present, but at a much lower concentration than observed for Standard 1 broth supplemented with AFB_1. These results confirm the degradation of AFB_1 by *R. erythropolis* extracellular fractions observed with HPLC and TLC quantification techniques. Furthermore, HPLC, TLC, ESMS and LCMS analyses could not reveal the formation of any breakdown products suggesting that AFB_1 was most likely metabolized to degradation products with chemical properties different from that of AFB_1.

The biological degradation of AFB_1 when treated with *R. erythropolis* extracellular fractions coincided with a total loss of mutagenicity of AFB_1 and its breakdown products, as evaluated by the Ames test for mutagenicity (Fig. 3). The positive AFB_1 reference produced a 2 fold dose–response when compared with the negative reference (Fig. 3). Aflatoxin B_1 was stable throughout the assay as a high mutagenic response was observed in the untreated AFB_1 reference sample after incubation for 72 h at 30 °C, which did not differ significantly ($P<0.789$) from the positive AFB_1 reference at 0 h. Aflatoxin B_1 samples treated with *R. erythropolis* extracellular extract produced a high mutagenic response in the organic phase samples at 0 h, which did not differ significantly ($P<0.967$) with the positive reference. However, the mutagenic response declined significantly ($P<0.001$) from 0 to 72 h. After 72 h there was no significant ($P<0.435$) difference in mutagenic response between negative reference samples and AFB_1 samples treated with *R. erythropolis* extracellular extracts. Furthermore, no significant ($P<0.434$) difference in mutagenic response was observed when the inorganic phase samples were compared with the negative reference.

A significant ($P<0.001$) reduction of AFB_1 was observed when treated with *R. erythropolis* extracellular fractions, whether they were pre-exposed to AFB_1 or not (Fig. 4A). Furthermore there was no significant ($P=1$) difference between the two treatments. Degradation of AFB_1 by the extracellular fractions not pre-exposed to AFB_1 suggests that the activity responsible for its degradation is produced during the normal

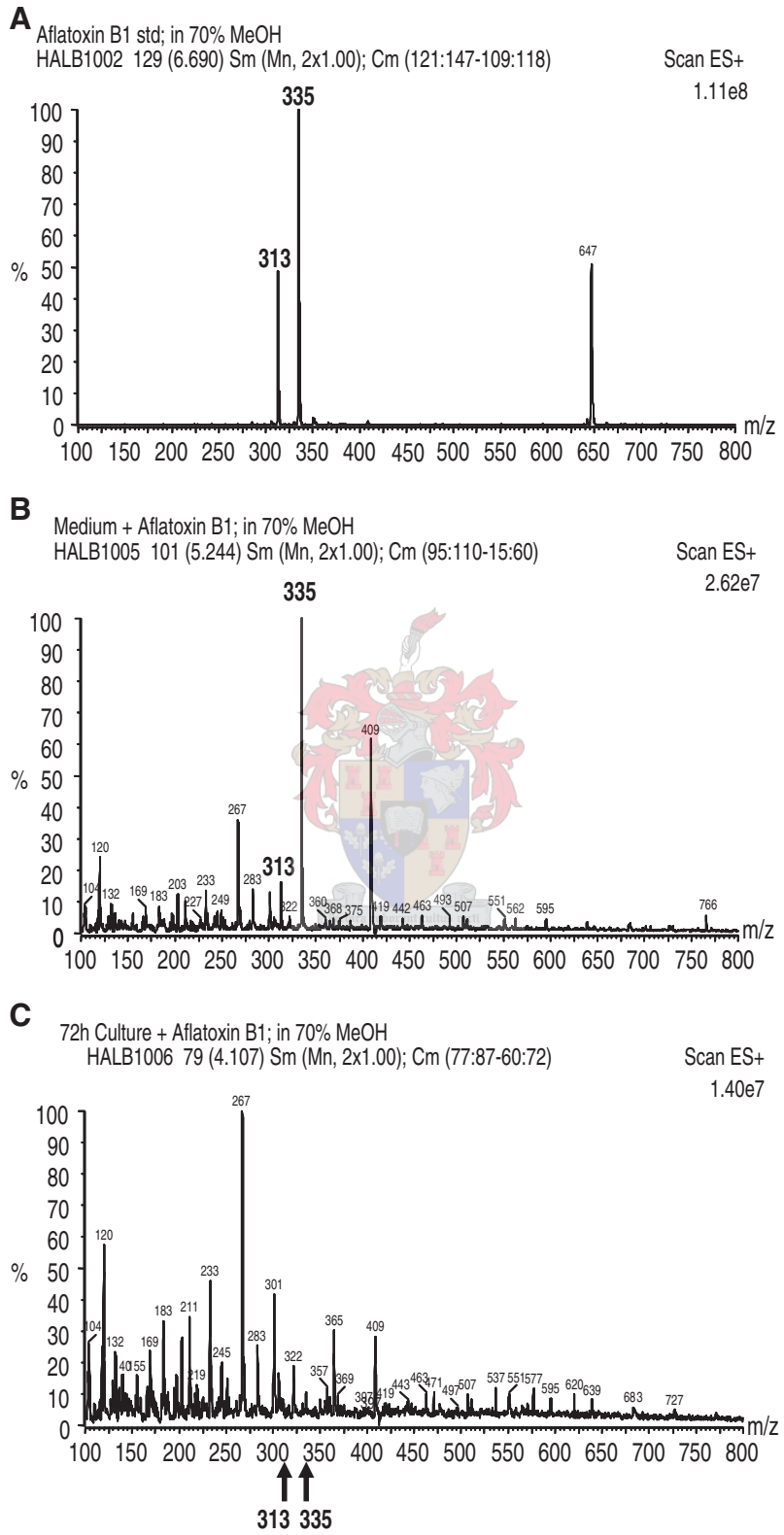

Fig. 2. Detection of AFB_1 by electron-spray mass spectrometry. (A) ESMS spectrum of AFB_1 showing the sodiated cation of AFB_1: $[M+Na]^+$ at $m/z=335$, the protonated cation $[M+H]^+$ at $m/z=313$ and the dimeric cation $[2\ M+Na]^+$ at $m/z=647$. (B) ESMS spectrum of Standard 1 broth supplemented with AFB_1 at 1.75 ppm. The sodiated cation of AFB_1: $[M+Na]^+$ at $m/z=335$ and the protonated cation $[M+H]^+$ at $m/z=313$ can clearly be distinguished. (C) ESMS spectrum of AFB_1 after a 72 h treatment with *R. erythropolis* extracellular fraction. The arrows indicate where the sodiated cation ($m/z=313$) and the protonated cation ($m/z=335$) of AFB_1 peaks would have appeared if present.

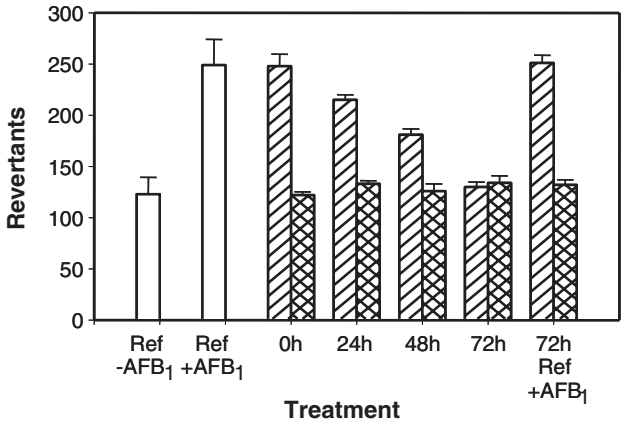

Fig. 3. Ames mutagenicity assay. Reference plates with and without AFB_1 (open bars) were used to determine the number of revertants expected in the presence or absence of AFB_1. Revertants obtained from the organic phase are shown with hatched bars and from the inorganic phase with cross-hatched bars.

growth of the organism, indicating that the degradation is a constitutive activity of *R. erythropolis*. The protein profiles obtained from SDS-PAGE analysis of *R. erythropolis* extracellular fractions, either pre-exposed to AFB_1 or not, were similar suggesting that no new prominent protein species were produced due to exposure to AFB_1 (Fig. 4B). These data confirm that degradation of AFB_1 is a constitutive activity of *R. erythropolis*.

We also investigated whether the degradation of AFB_1 is enzymatic, by evaluating the stability of the extracellular extract of *R. erythropolis* after proteinase K treatment. Proteinase K treated samples retained low AFB_1 degradation activity, but when SDS was added, the AFB_1 degradation activity was destroyed (no distinct loss of fluorescence was observed). The untreated reference sample showed total loss of AFB_1 fluorescence after 72 h. These results indicated that enzymes are involved in the degradation of AFB_1 by extracellular extracts of *R. erythropolis*.

Degradation of AFB_1 thus most probably occurred through a cascade of enzyme reactions with loss of fluorescence over time. If defining the collective AFB_1-degrading enzymatic activity units as μmole AFB_1 degraded per min, the AFB_1-degrading enzyme activity was the highest (about 4.16 mU/ml) during the first hour of incubation (calculated from Fig. 1), where after it decreased to about 0.53–0.75 mU/ml after 72 h (calculated from Figs. 1 and 4). Various enzymes produced by *R. erythropolis* are involved in the catabolic pathways of aromatic compounds such as polychlorinated biphenyls (Haushild et al., 1996; Masai et al., 1995, 1997; Yamada et al., 1998). These enzymes include ring cleavage biphenyl dioxygenases, dihydrodiol dehydrogenases, and hydrolases. The genes coding for these enzymes are clustered and degradation occurs via a cascade of reactions. AFB_1 is also a polyaromatic compound and could be degraded in a similar manner.

The development of lactic acid bacteria and yeasts expressing AFB_1-degrading enzymes could especially be valuable in the feed, food and fermentation industry (Karlovsky, 1999; Holzapfel, 2002). Fermented food and beverages are an important part of the diet of African people (Gadaga et al., 1999; Gonfa et al., 2001; Jespersen, 2003). A wide selection of alcoholic and non-alcoholic fermented food products (Haard et al., 1999; Gadaga et al., 1999; Gonfa et al., 2001), mainly produced from a variety of cereals, as well as fermented milk products are produced commercially or on small-scale at household level. As a result the products are of varying quality and stability. The microorganisms involved are mainly lactic acid bacteria (*Lactobacillus* spp.), yeasts (*Saccharomyces cerevisiae*), moulds and acetic acid bacteria. In many cases the raw material are sold in rural markets or home-grown and aflatoxin contamination remains a major risk, especially in stored cereals (Holzapfel, 2002). Furthermore, control measurements for the maximum acceptable levels of mycotoxins in cereals are inadequate in developing countries. However, with the application of molecular biology techniques, microbial

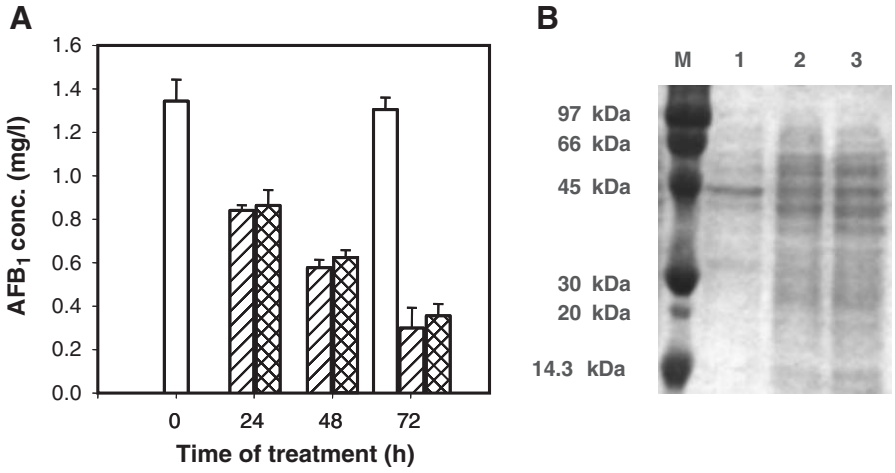

Fig. 4. (A) Biodegradation of AFB_1 by *R. erythropolis*. Treatments include AFB_1 degradation by the extracellular fraction of (a) *R. erythropolis* pre-incubated in Standard 1 broth without AFB_1 (hatched bars) and (b) *R. erythropolis* pre-incubated in Standard 1 broth supplemented with 1.75 ppm AFB_1 (cross-hatched bars). Medium supplemented with 1.75 ppm AFB_1 at 0 h and after 72 h is shown with open bars. (B) SDS-PAGE protein profiles of Standard 1 broth (lane 1), *R. erythropolis* extracellular fraction (lane 2) and *R. erythropolis* extracellular fraction after being exposed to AFB_1 (lane 3). Lane M contains molecular markers.

strains with multi-functional properties, including degradation of AFB_1, can be engineered to significantly improve the quality, safety and acceptability of traditional fermented food and beverages.

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CHAPTER 5

Degradation of aflatoxin B_1 by 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus erythropolis* through extracellular expression in *Escherichia coli*

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Degradation of aflatoxin B_1 by 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus erythropolis* through extracellular expression in *Escherichia coli*

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Keywords: Aflatoxin B_1; AFB_1; *Rhodococcus erythropolis*; degradation; 2,3-dihydroxybiphenyl 1,2-dioxygenase; 2,3-DHBD; *Bph*C1

5.1. Abstract

Biological degradation of aflatoxin B_1 (AFB_1) by a recombinant 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD) enzyme of *Rhodococcus erythropolis* was investigated. *Rhodococcus* spp. are capable of transforming a wide range of toxic aromatic xenobiotic compounds and several genes encoding enzymes responsible for the degradation of these compounds were previously characterized in *Rhodococcus* spp. In this study recombinant 2,3-DHBD was produced through extracellular expression of the *bph*C1 gene of *R. erythropolis* in *Escherichia coli* BL21 (DE3). A significant ($P<0.0001$) reduction in AFB_1 concentration was observed following treatment with extracellular culture fractions containing recombinant 2,3-DHBD, with only 50.68% AFB_1 remaining after 72 h. The degradation of AFB_1 coincided with a 42.47% loss of mutagenicity of AFB_1 and its breakdown products as evaluated by the *Salmonella typhimurium* mutagenicity assay. Treatment of AFB_1 with extracellular culture fractions containing recombinant 2,3-DHBD significantly ($P<0.001$) reduced the fluorescence and mutagenic potency of the AFB_1 molecule, indicating changes to the furofuran- or lactone rings.

5.2. Introduction

Aflatoxins are mycotoxins produced by *Aspergillus flavus, Aspergillus parasiticus*, *Aspergillus bombycis, Aspergillus nomius*, *Aspergillus ochraceoroseus, Aspergillus pseudotamarii* and *Aspergillus tamarii* (Bhatnagar et al., 2003; Ito et al., 2001; Mishra and Das, 2003). The most potent aflatoxin, aflatoxin B_1 (AFB_1), is highly toxic and mutagenic to both humans and animals (Eaton and Gallagher, 1994; Mishra and Das, 2003) and classified as a type I human carcinogen by the International Agency for Research on Cancer (Wogan, 2000). Hepatocellular carcinoma (HCC) is one of the most frequently occurring cancers in the world and was estimated to cause 250 000 deaths annually in Asia and Sub-Saharan Africa (Bennett et al., 1994; Mishra and Das, 2003; Wild and Hall, 2000). The major risk factors for HCC are infection with the hepatitis B or C virus and dietary exposure to aflatoxin (Wild and Hall, 2000; Yabe and Nakajima, 2004). Metabolic activation of AFB_1 by cytochrome P450 enzymes in the liver and kidney leads to the formation of reactive AFB_1-8,9-epoxide (Minto and Townsend, 1997; Mishra and Das, 2003) that reacts covalently with N-7 of the guanine bases of DNA to form DNA adducts, which in turn cause further genetic changes and eventually malignant transformation (Smela et al., 2001).

Aflatoxin contamination is found in up to 25% of the world's food supply (Wild and Hall, 2000) causing a vast economical impact due to loss of crops, animals, costs for monitoring aflatoxin levels as well as a decline in the performance of farm animals (Maggon et al., 1977; Trail et al., 1995). Regulations regarding acceptable levels of aflatoxin in food have been implemented in nearly all countries (Mishra and Das, 2003). However, control measurements for the maximum acceptable levels of mycotoxins in cereals are inadequate in developing countries (Trail et al., 1995). Aflatoxin contamination of food in these countries occurs predominantly in dietary staples and exceeds the regulatory limits for contamination of food by one or two orders of magnitude (Wild and Hall, 2000). Fermented food and beverages are an important part of the diet of African and Asian people and are prepared from raw or heated maize, rice, sorghum, millet and milk (Gadaga et al., 1999; Gonfa et al., 2001; Jespersen, 2003). In many cases the raw material is sold in rural markets or home-grown where aflatoxin

contamination is a major risk, especially in stored cereals (Holzapfel, 2002). While aflatoxins are seldom destroyed by normal industrial processing or cooking, fermented products prepared from cereals and milk are of varying quality and stability and daily exposure to low levels of aflatoxin is a cause for concern (Trail et al., 1995). Therefore, methods to detoxify AFB_1 in food sources are the focus of several research initiatives (Mishra and Das, 2003). Since physical and chemical detoxification methods have not yet been proved to be economical and effective, biological detoxification methods offer an attractive alternative for eliminating toxins as well as safe-guarding the desired quality of food and feed, such as nutritive value and appearance (Mishra and Das, 2003).

The only bacteria known to degrade AFB_1 effectively are *Nocardia corynebacteroides* (Ciegler et al., 1996; Line et al., 1994) and *Mycobacterium fluoranthenivorans* (Hormisch et al., 2004), while AFB_1 degradation by *Corynebacterium rubrum* was also observed (Mann and Rehm, 1977; Shih and Marth, 1975). Recently, effective degradation of AFB_1 by intracellular as well as extracellular extracts prepared from liquid cultures of *Rhodococcus erythropolis* was reported (Alberts et al., 2006; Teniola et al., 2005). Furthermore, treatment of AFB_1 with the extracellular extract of a *R. erythropolis* liquid culture coincided with total loss in mutagenicity of AFB_1 (Alberts et al., 2006).

Rhodococcus spp. are aerobic gram-positive bacteria capable of transforming a wide range of toxic aromatic xenobiotic compounds (Kitagawa, et al., 2001; Sakai et al., 2003) including nitro aromatic compounds (Kitova et al., 2004), polycyclic hydrocarbons, pyridine, steroids (Goodfellow, 1986) as well as AFB_1 (Alberts et al., 2006; Teniola et al., 2005). Gene clusters encoding multiple isozymes responsible for the degradation of several of these compounds were characterized in *Rhodococcus* spp. (Hauschild et al., 1996; Masai et al., 1995, 1997; Takeda et al., 2004; Yamada et al., 1998). The *bph*ACB gene cluster in *R. erythropolis* RHA1 and TA421 encodes aromatic-ring-hydroxylating dioxygenases, dihydriol dehydrogenases and biphenyl 2,3-dioxygenases, which are key enzymes in the initial stepwise degradation of biphenyl, resulting in ring-cleavage of the molecule (Kahl and Hofer, 2003; Kosono et al., 1997; Pieper, 2005).

AFB_1 is a polyaromatic heterocyclic molecule which harbours two key sites influencing its toxicological activity namely a furofuran- and a lactone ring (Heathcote and Hibbert, 1978; Mishra and Das, 2003) and ring-cleavage results in inactivation of the molecule (Liu et al., 1998 a,b,c). The 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD) enzymes encoded by *bph*C in *R. erythropolis*, have broad substrate specificity, specifically catalyzing ring-cleavage of several polyaromatic biphenyl congeners (Ohtsubo et al., 2004; Takeda et al., 2004) and are therefore of relevance. The present study investigates degradation of AFB_1 by a recombinant 2,3-DHBD of *R. erythropolis* through expression of the *bph*C1 gene in *Escherichia coli* BL21 (DE3). Moreover, the effect of 2,3-DHBD on the mutagenic potency of AFB_1 was determined to assess the biological significance of the breakdown process.

5.3. Materials and Methods

5.3.1. Bacterial strains, growth conditions, and plasmids

The *R. erythropolis* strain used in this study was isolated from polycyclic aromatic hydrocarbon contaminated soils by the Institute of Applied Microbiology, University of Saarbrücken, Germany and deposited under the restricted number DSM 14303 (Teniola et al., 2005). *Rhodococcus erythropolis* was cultivated in Standard 1 broth (Goodfellow, 1986) comprising of 1.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.6% (w/v) sodium chloride, and 0.1% (w/v) D (+)-glucose. *Escherichia coli* strains BL21 (DE3) and DH5α were employed as host strains and cultivated in Luria-Bertani (LB) broth containing ampicillin (100 μg/ml). Plasmids pDrive (Qiagen) and pET 11d (Stratagene) were used as cloning vectors.

5.3.2. Cloning of the bphC1 gene

Genomic DNA from *R. erythropolis* DSM 14303 was prepared according to the method described by Sambrook et al. (1989). Primers BphC1-L and BphC1-R (Table 1),

containing *Xho*l restriction sites were designed to target the *bph*C1 gene sequence (NCBI accession number D88013). A 911 bp *Xho*l-*Xho*l DNA fragment, containing the *bph*C1 open reading frame, was amplified by PCR, inserted into the pDrive vector and the plasmid transformed into *E. coli* DH5α. Plasmid DNA isolation from the transformants was done according to the method of Sambrook et al. (1989) and the resulting *bph*C1 gene sequence verified using the dye terminator sequencing method and a 3130XL Genetic Analyser (Applied Biosystems). A second PCR amplification was done using the pDrive-BphC1 plasmid as template and primers PhoA-BphC1-L and BphC1-R (Table 1) containing *Nco*1 and *Bam*H1 restriction sites as well as a PhoA secretion signal. The 990 bp *Nco*l-*Bam*Hl fragment that codes for extracellular 2,3-DHBD activity was subcloned into the expression vector pET 11d predigested with *Nco*l and *Bam*Hl restriction enzymes. The resulting plasmid was transformed into *E. coli* BL21 (DE3).

5.3.3. Induction of 2,3-DHBD

Recombinant *E. coli* BL21 (DE3), harbouring the *bph*C1 gene, was cultivated in LB broth (100 ml) supplemented with ampicillin (100 μg/ml) on a shaker at 37ºC until an optical cell density (OD_{600}) of 0.4-0.8 was reached. Extracellular 2,3-DHBD was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) to the culture and incubated for a further 24 h on a shaker at 25ºC. The *E. coli* BL21 (DE3) host, lacking the gene, was included as reference. To prepare the extracellular fraction, induced cultures were centrifuged (10 000 rpm for 10 min) and the supernatant lyophilized. The intracellular fraction was prepared by suspending the residual cells in 100 ml TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) followed by sonication (80% output; 3 times for 20 s with 20 s intervals), where after the disrupted cell suspension was centrifuged (10 000 rpm for 10 min) and the supernatant lyophilized. The extra- and intracellular fractions were stored at -20ºC until employed in SDS-PAGE analyses and AFB_1 degradation experiments.

Table 1

Oligonucleotides (Operon Biotechnologies) employed in PCR reactions

Primer Name	**DNA Sequence 5' to 3'**
BphC1-L	GCTACCATGGCACACACCGACATCAAGG
BphC1-R	GCATGGATCCCTACATAGCTGCCAGTGGTT
PhoA-BphC1-L	GCTACCATGGCAAAACAAAGCACTATTGCACTGGCACTC TTACCGTTACTGTTTACCCCTGTGACAAAAGCAGATCTAC ACACCGACATCAAGGG

5.3.4. SDS-PAGE analysis

The presence of 2,3-DHBD in induced recombinant *E. coli* BL21 (DE3) cultures was confirmed by fractionation of the extra- and intracellular proteins by SDS-PAGE. Extra- and intracellular fractions (10 mg) were dissolved in 20 µl distilled H_2O, dialyzed (Millipore VSWP filters, 0.025 µm) and boiled. A 0.1% SDS-10% PAGE analysis was performed using Coomassie brilliant blue R250 staining (Sambrook et al., 1989), where after the protein profiles were compared. The following samples were analyzed: (a) the extracellular fraction of a culture of *E. coli* BL21 (DE3) as reference host, (b) the extra- and (c) intracellular fractions of an induced culture of recombinant *E. coli* BL21 (DE3).

5.3.5. Determination of enzymatic activity

The 2,3-DHBD activity was determined according to the method described by Hauschild et al. (1996) by measuring the formation of meta-cleavage reaction products at

434 nm with a Genesys 20 spectrophotometer. Assays were performed at 25ºC by using 2,3-dihydroxybiphenyl (Wako Chemicals Co., Japan) as substrate. A 100 mM stock solution of 2,3-dihydroxybiphenyl was prepared in ethanol and diluted with 20 mM Tris-HCl buffer pH 7.5 to obtain the test solution (1 mM). Lyophilized extracellular fractions obtained from induced cultures of recombinant *E. coli* BL21 (DE3) were dissolved in 20 mM Tris-HCl buffer pH 7.5 (20 ml), where after 2,3-dihydroxybiphenyl (500 µM) was added and the formation of meta-cleavage reaction products monitored for 6 h. One unit enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 µmol of product per min.

5.3.6. AFB_1 degradation

The extracellular fractions obtained from induced cultures of recombinant *E. coli* BL21 (DE3) and the *E. coli* BL21 (DE3) reference host were suspended in distilled H_2O (10 ml), supplemented with AFB_1 to a final concentration of 1.75 µg/ml, aliquoted (0.8 ml) to sterile screw-cap Eppendorf tubes (Alberts et al., 2006) and incubated at 25ºC on a rotor wheel for 72 h. Luria-Bertani broth supplemented with AFB_1 was included as untreated control. Following incubation, AFB_1 was extracted from the samples as described below. The organic ($CHCl_3$) and aqueous phases resulting from extraction were analyzed for AFB_1, water-soluble and other breakdown products by employing high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS) (Line et al., 1994).

5.3.7. AFB_1 extraction and chromatographic analyses

Aflatoxin B_1 was extracted from samples with chloroform (1:1, v/v) as described by Teniola et al. (2005). The chloroform was evaporated under nitrogen, the dried extract dissolved in methanol and filtered (Millex-GV, Durapore, 0.22 µm). The aqueous phases resulting from the AFB_1 extraction were lyophilized and stored at -20ºC until analyzed. HPLC analysis was performed using a guard column [LiChroCART 4-4 RP-C18 (5 µm), Merck] followed by a LiChroCART RP-C18 [250-4 Hypersil ODS (5 µm), Merck]

column. The mobile phase was acetonitrile:methanol:water (1:1:2, v/v/v) at a flow rate of 1 ml/min. AFB_1 was measured by UV (365 nm) detection using a diode-array detector (Waters model 911). During HPLC analysis of the aqueous phases, UV absorbance was monitored between 200 and 400 nm to investigate the possibility that AFB_1 could be degraded to water-soluble breakdown products (Line et al., 1994). LCMS was done to detect the formation of breakdown products in both phases obtained from extraction. Separation was achieved on a mass spectrometer (Waters API Quattro Micro) fitted with a gradient HPLC (Waters Alliance 2690) and a Luna C5 [150x2mm (5µm), Merck] column by using a gradient mobile phase from 0.1% formic acid to methanol over 18 min.

5.3.8. Salmonella typhimurium mutagenicity assay

5.3.8.1. Preparation of mutagens. A stock solution (2 µg/ml) of AFB_1 was prepared in dimethyl sulfoxide (DMSO) and diluted to obtain a standard test solution (0.1 µg/ml). In the mutagenicity assay the standard test solution was added to a concentration of 10 ng AFB_1/plate to obtain a 2- to 3-fold mutagenic response as compared to the background revertant count. Untreated control AFB_1 samples from the 2,3-DHBD degradation experiments, containing 1.75 µg/ml AFB_1, were also diluted in DMSO to 0.1 µg/ml. The organic phases of the 2,3-DHBD treated AFB_1 samples were evaporated to dryness while the aqueous phases were lyophilized as described above. These samples were diluted in a similar volume of DMSO as the untreated control AFB_1 samples. An equivalent volume (0.1 ml/plate) of the standard AFB_1 test solution, the untreated AFB_1 control and the 2,3-DHBD treated AFB_1 samples were used in the mutagenicity assay.

5.3.8.2. Mutagenicity assay. The plate incorporation assay was performed according to the method described by Moron and Ames (1983) using Aroclor 1254 induced S9 homogenate (0.7 nmole cytochrome P450/mg protein) for metabolic activation of AFB_1. *Salmonella typhimurium* TA100 was used as tester strain and 0.5 ml of S9 mixture containing 2 mg protein of the liver homogenate per ml was added per

plate. The different samples were subjected to mutagenicity testing and a loss in mutagenicity interpreted as a measure of 2,3-DHBD breakdown of AFB_1.

5.3.9. Statistical analyses

Cross classification ANOVA data analyses were done on the response variable observed (AFB_1 concentration or number of revertants) over untreated AFB_1 control- and time values by using a compound symmetry model (Dunn and Clark, 1987). A significance level of 5% was used and Bonferroni multiple comparisons were done when significant differences were encountered. When response for two time values were compared or when responses for untreated AFB_1 control values versus a time value were compared, a paired t-test was used.

5.4. Results and Discussion

Ring-cleavage of biphenyl compounds by *Rhodococcus* sp. RHA1 is dependable on *bph*ACB genes encoding 2,3-DHBDs (Masai et al., 1997). Genes encoding enzymes for biphenyl catabolism are diverse and mainly positioned on linear plasmids in several *Rhodococcus* spp., including *R. erythropolis*, *Rhodococcus opacus* and *Rhodococcus fascians* (Maeda et al., 1995; Shimizu et al., 2001). Likewise, three of seven *bph*C genes encoding 2,3-DHBDs characterized in *R. erythropolis* TA 421 are located on a linear plasmid, while *bph*C1 is located on the chromosome and therefore may have other functions apart from biphenyl degradation and needs to be genetically more stable (Kosono et al., 1997).

Genomic DNA from *R. erythropolis* DSM 14303 was isolated and used as template for PCR amplification to target the *bph*C1 gene. The 911 bp *Xho*1-*Xho*1 fragment obtained from the PCR amplification displayed 98% homology with the gene sequence of *bph*C1 of *R. erythropolis* TA421 (NCBI accession number D88013). The PCR product was ligated into vector pDrive and transformed into *E. coli* DH5α. Subsequently the 990 bp *Nco*l-*Bam*Hl fragment obtained by fusing a PhoA secretion

signal to the *bph*C1 gene was ligated into vector pET 11d and transformed into *E. coli* BL21 (DE3).

Recombinant extracellular 2,3-DHBD was effectively induced with IPTG (1 mM) in liquid cultures of recombinant *E. coli* BL21 (DE3) and the proteins present in the extracellular fraction analyzed by SDS-PAGE (Fig. 1). Extracellular proteins obtained from recombinant *E. coli* BL21 (DE3) (Fig. 1, lane 3) have shown distinct protein species greater than 35 kDa which correspond to the predicted molecular weight (36 kDa) of the *bph*C1 protein, while these protein species were absent in the reference host (lane 2). Furthermore, no foreign protein species were observed in the intracellular fraction obtained from recombinant *E. coli* BL21 (DE3) (data not shown), indicating that insoluble inclusion bodies have not formed during induction of the *bph*C1 gene.

Enzymatic activity assays using 2,3-dihydroxybiphenyl as substrate confirmed extracellular 2,3-DHBD activity (34 mU/L) in IPTG induced liquid cultures of recombinant *E. coli* BL21 (DE3). Enzymatic activity was also measured with the growing cell assay (Kitagawa et al., 1996), though no meta-cleavage products could be detected. Although the formation of meta-cleavage reaction products during treatment with recombinant 2,3-DHBD encoded by *bph*C1 were low, similar results were reported following treatment of 2,3-dihydroxybiphenyl with enzymes encoded by *bph*ACB of *R. erythropolis* RHA1 expressed in *E. coli*, even when induced by strong promoters and detected by SDS-PAGE (Maeda et al., 1995; Masai et al., 1995; Sakai et al., 2002). However, when 2,3-dihydroxybiphenyl was treated with a combination of enzymes encoded by *bph*B and *bph*C as well as *bnz*A of *Pseudomonas putida* BE-81, distinct meta-cleavage reaction products were detected (Masai et al., 1995). Furthermore, intermediate metabolites that accumulate during degradation of polyaromatic compounds may inhibit the activity of 2,3-DHBD towards its substrate (Hauschild et al., 1996).

Aflatoxin B_1 was shown to be stable over the 72 h incubation period in LB broth as no significant difference ($P>0.05$) was observed between untreated AFB_1 control samples at 0 h and 72 h (Fig. 2). However, a significant ($P<0.0001$) reduction in AFB_1 concentration was observed in the organic phase samples when AFB_1 was treated with extracellular fractions containing recombinant 2,3-DHBD, with only 50.68% AFB_1 remaining after 72 h. Extracellular fractions from the reference host have shown no

significant difference (P>0.05) with the untreated AFB_1 control samples. Furthermore, no AFB_1 was present in the aqueous phase samples.

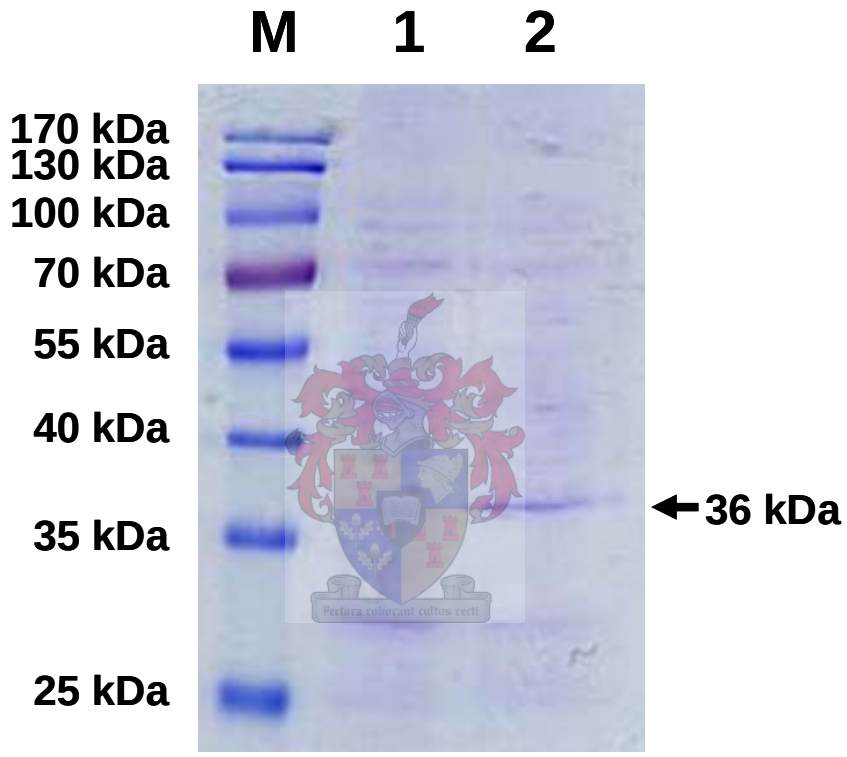

Fig. 1. SDS-PAGE protein profiles of extracellular fractions of *E. coli* BL21 (DE3) liquid cultures containing recombinant 2,3-DHBD. **Lane M**, molecular markers; **lane 1**, extracellular fraction of the *E. coli* BL21 (DE3) host lacking the enzyme; **lane 2**, extracellular fraction of recombinant *E. coli* BL21 (DE3) expressing extracellular 2,3-DHBD activity.

With the aid of LCMS, AFB_1 was identified at m/z=313 for the protonated cation $[M+H]^+$, m/z=335 for the sodiated cation of AFB_1 $[M+Na]^+$, and at m/z=647 for the dimeric cation $[2M+Na]^+$(data not shown). Luria-Bertani broth supplemented with AFB_1 clearly showed the sodiated and protonated cations at m/z=335 and m/z=313, respectively. LCMS analyses of the sample treated with extracellular extracts containing recombinant 2,3-DHBD have shown that AFB_1 was still present, but at lower concentrations than the untreated AFB_1 controls. These results confirm the degradation results obtained with HPLC.

The decrease in fluorescence of the AFB_1 molecule observed with HPLC and LCMS indicates changes to the lactone ring (Liu et al., 1998 a,b,c). The 2,3-DHBDs catalyze several ring-cleavage reactions by the addition of two hydroxyl groups to carbons and consequently the formation of dihydriol compounds, thus affecting the fluorescence and toxicity properties of the treated polyaromatic molecule (Kosono et al., 1997). The α-subunits of the 2,3-biphenyl dioxygenase enzymes were found to be crucially responsible for recognition and binding of the substrates (Pieper, 2005). Furthermore, HPLC analyses could not reveal the formation of any structural analogues resulting from the degradation of AFB_1. LCMS of AFB_1 samples treated with extracellular extracts containing recombinant 2,3-DHBD revealed the potential formation of an unknown compound in the organic phase samples, that was not present in the untreated controls and the aqueous phase samples. However, further studies involving the use of radio-labelled AFB_1 will be required to confirm the presence of the breakdown product and support characterization of the compound.

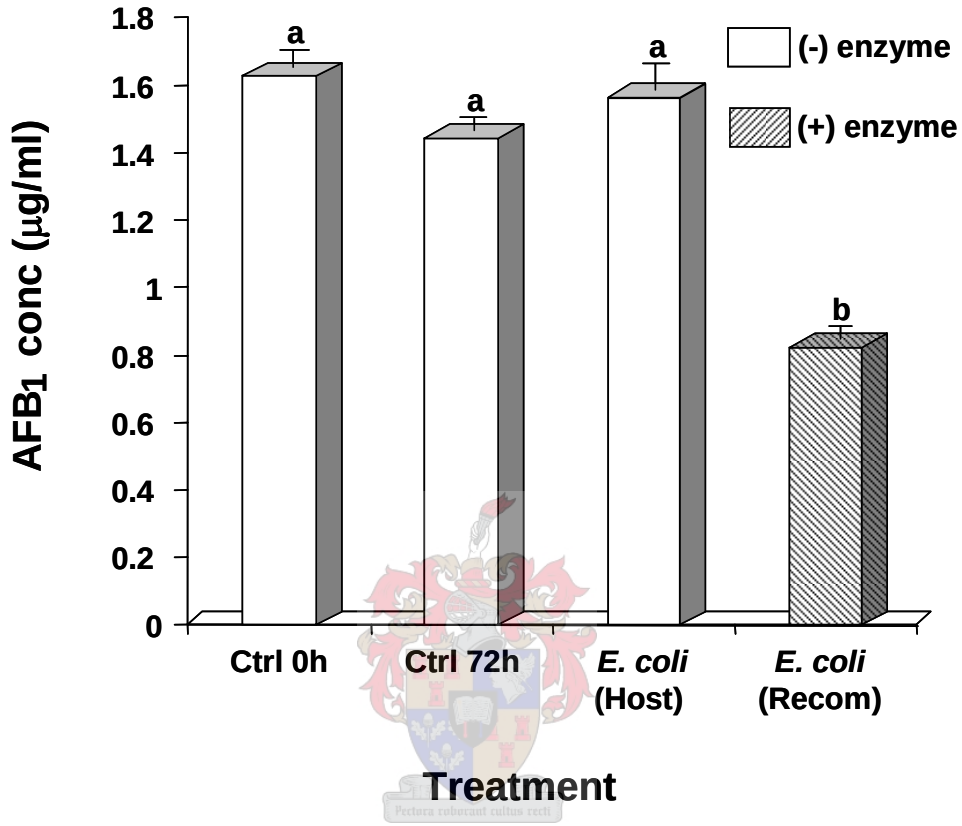

Fig. 2. Biological degradation of AFB_1 by extracellular fractions prepared from *E. coli* BL21 (DE3) liquid cultures containing recombinant 2,3-DHBD, as determined by HPLC of the organic phase samples. Untreated AFB_1 samples representing incubation periods of 0 h and 72 h and the extracellular fractions of the *E. coli* BL21 (DE3) host, lacking the enzyme, were included as controls. Treatments represent the means of triplicate determinations and differ significantly ($P<0.05$) when letters differ.

The biological degradation of AFB_1, when treated with extracellular fractions containing recombinant 2,3-DHBD, coincided with a 42.47% loss of mutagenicity of AFB_1 and its breakdown products as evaluated by the *Salmonella typhimurium* mutagenicity assay (Fig. 3). The positive AFB_1 test control produced a 3-fold dose-response when compared with the baseline revertant counts (data not shown). Aflatoxin B_1 treated with extracellular fractions containing recombinant 2,3-DHBD have shown a typical dose response effect regarding the loss of mutagenicity of AFB_1 in the organic phase samples (Fig. 3). Significantly ($P<0.0001$) lower mutagenic response was displayed by samples treated with extracellular fractions containing recombinant 2,3-DHBD as compared to the untreated control AFB_1 samples at 0 h and the *E. coli* BL21 (DE3) host samples. Furthermore, no significant ($P>0.05$) difference in mutagenic response was observed between the aqueous phase samples and the baseline revertant counts.

Treatment of AFB_1 with extracellular fractions containing *bphC*1 encoded 2,3-DHBD produced by recombinant *E. coli* BL21 (DE3) had significantly ($P<0.001$) reduced the fluorescence and mutagenic potency of the AFB_1 molecule, indicating changes to the furofuran- or lactone rings (Liu et al., 1998 a,b,c,). While complete degradation of polyaromatic compounds is accomplished through a cascade of reactions, more efficient meta-cleavage is obtained when treated with a combination of enzymes (Hauschild et al., 1996). Therefore, co-expression of *bph*C encoded dioxygenases with *etb*C of *R. erythropolis, bnz*A of *P. putida* BE-81, or the application of multiple *bph*C dioxygenases containing different substrate specificities, will have an advantage in the degradation of polyaromatic compounds such as AFB_1. The current study laid the foundation for future work towards the treatment of AFB_1 with a combination of *bph* encoded enzymes in order to reduce AFB_1 concentrations to a level which coincides with total loss of mutagenicity. Furthermore, microbial strains expressing multiple AFB_1 degrading enzymes could support the development of commercial additives based on cultures or enzymatic preparations capable of degrading AFB_1 in the feed, food and fermentation industry.

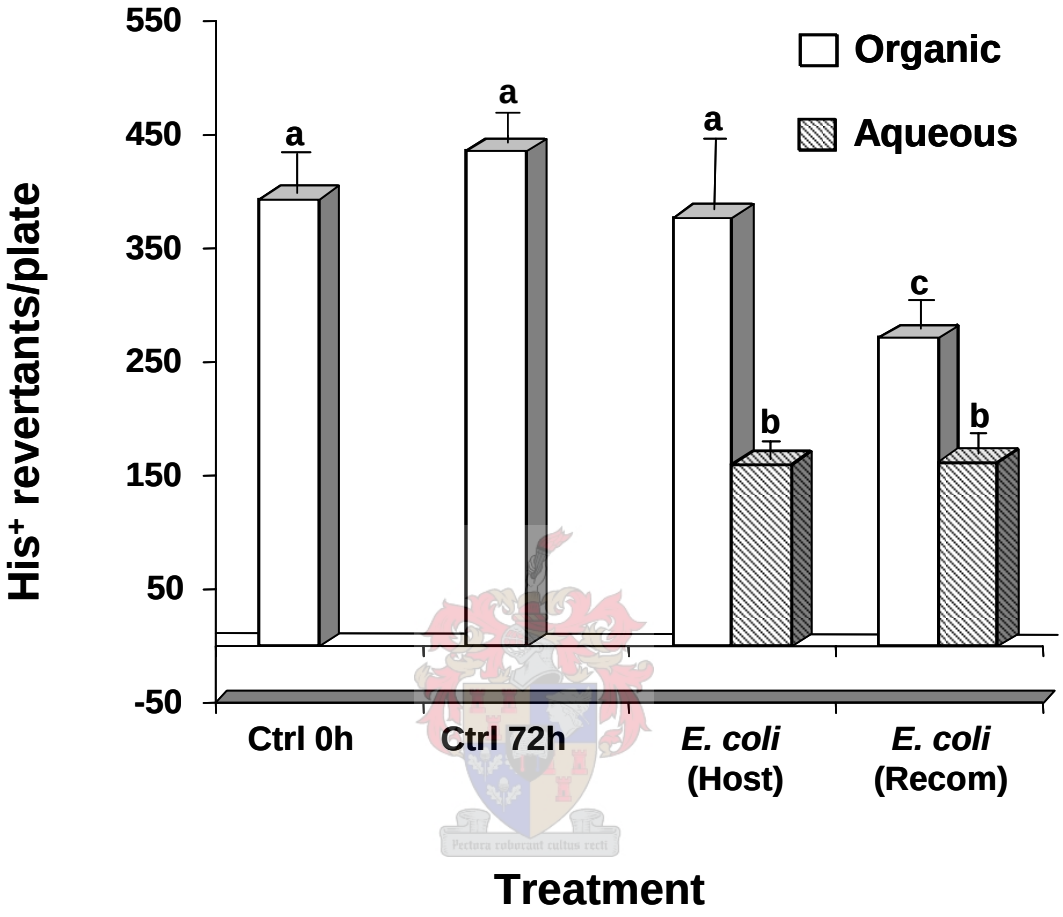

Fig. 3. Mutagenicity of AFB_1 following treatment with extracellular fractions prepared from *E. coli* BL21 (DE3) liquid cultures containing recombinant 2,3-DHBD, as determined with the *Salmonella typhimurium* mutagenicity assay. The mutagenicity of the organic and aqueous phases is the means of five determinations. Untreated AFB_1 samples representing incubation periods of 0 h and 72 h and the extracellular fractions of the *E. coli* BL21 (DE3) host, lacking the enzyme, were included as controls. Treatments differ significantly ($P<0.05$) when letters differ.

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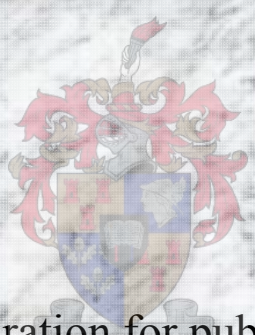
CHAPTER 6

Degradation of aflatoxin B_1 by fungal laccase enzyme



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Degradation of aflatoxin B_1 by fungal laccase enzyme

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Keywords: Aflatoxin B_1; AFB_1; degradation; fungi; laccase

6.1. Abstract

The enzymatic degradation of aflatoxin B_1 (AFB_1) by white rot fungi as a function of laccase production was investigated in different liquid media. A significant ($P<0.0001$) correlation was observed between laccase activity and AFB_1 degradation exhibited by *Peniophora* sp. and *Pleurotus ostreatus* cultivated in MSM ($r=0.93$) and MSB-MEB ($r=0.77$) liquid media. Extracellular extracts of *Peniophora* sp. cultured in MSB-MEB medium supplemented with veratryl alcohol and sugarcane bagasse produced high laccase activity (496 U/L) and 40.45% AFB_1 degradation as monitored by HPLC. In addition, extracellular extracts of *P. ostreatus* grown in MSM medium supplemented with veratryl alcohol resulted in a laccase activity of 416.39 U/L and 35.90% degradation of AFB_1. AFB_1 was significantly ($P<0.0001$) degraded when treated with purified laccase enzyme from *Trametes versicolor* (1 U/ml, 87.34%) as well as with recombinant laccase produced by *Aspergillus niger* (118 U/L, 55%). Aflatoxin B_1 degradation by laccase enzyme from *T. versicolor* and recombinant laccase enzyme produced by *A. niger* coincided with significant ($P<0.001$) loss of mutagenicity of AFB_1, as evaluated by the *Salmonella typhimurium* mutagenicity assay. The degradation of AFB_1 by white rot fungi could be an important bio-control measure to reduce the level of this mycotoxin in food commodities.

6.2. Introduction

Aflatoxins are difuranocoumarin derivates (Minto and Townsend, 1997; Payne and Brown, 1998) predominantly produced as secondary metabolites by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Diener *et al*., 1987; Kurtzman *et al*., 1987; Pitt, 2000). Other *Aspergillus* spp. producing aflatoxin include *Aspergillus nomius*, *Aspergillus tamarii* (Goto *et al.,* 1996, 1997) and *Aspergillus pseudotamarii* (Ito *et al*., 2001). Aflatoxin B_1, the most abundant aflatoxin, is highly mutagenic, toxic, carcinogenic and teratogenic to humans and animals. Aflatoxin contamination of feed and foodstuffs is responsible for significant economic losses due to loss of crops and animals (Eaton and Gallagher, 1994; Mishra and Das, 2003; Pitt, 2000) and in some years estimated losses ranged between $85 to $100 million in certain states of the United States of America (Yabe and Nakajima, 2004). In parts of Africa, China and South East Asia aflatoxin contamination is correlated with the incidence of liver cancer. In this regard aflatoxin is classified as a type I human carcinogen by the International Agency for Research on Cancer (Wogan, 2000).

Fermented food and beverages are an important part of the diet of African and Asian people and a wide selection of alcoholic and non-alcoholic fermented food products (Gadaga *et al*., 1999; Gonfa *et al*., 2001; Jespersen, 2003) prepared from cereals and milk are produced commercially or on household level. Milk produced by smallholders is usually processed on the farms using traditional dairy technology, while cereals are obtained from rural markets or home-grown (Holzapfel, 2002). As a result the products are of varying quality and stability. Most food are exposed to aflatoxigenic fungi at some stage of production, processing, transportation and storage making aflatoxin contamination therefore a major risk to human health. A typical example was the recent outbreak of aflatoxicosis in rural Kenya resulting in many clinical cases and even deaths, after the ingestion of maize containing levels up to 1000 ppb AFB_1 (Lewis *et al.*, 2005).

Reduction of AFB_1 in food sources by various decontamination procedures has been a topic of many research initiatives. However, inactivation of aflatoxin in food and feed by physical and chemical methods has not yet proved to be effective and

economically feasible (Mishra and Das, 2003). In contrast, biological detoxification offers an attractive alternative for eliminating toxins as well as safe-guarding the desired quality of food and feed. In recent years it became clear that fungi play a major role in the degradation of AFB_1. The biosynthesis of AFB_1 by cultures of *A. flavus* and *A. parasiticus* reaches a maximum, where after it is degraded, presumable under nitrogen-limiting conditions (Hamid and Smith, 1987; Huynh and Lloyd, 1984; Shih and Marth, 1975). Other fungi that have been implicated in AFB_1 degradation include zygomycetous fungi (*Rhizopus* sp. and *Mucor* sp.), ascomycetous fungi (*Aspergillus niger* and *Trichoderma* sp.), plant pathogens (*Phoma* sp. and *Alternaria* sp.) as well as basidiomycetous fungi (*Armillariella tabescens* and other white rot fungi) (Leonowicz *et al.;* 1999; Liu *et al.*, 1998 a,*b,c;* Nakazato *et al.*, 1990; Shantha, 1999; Shantha *et al.*, 1990; Yao *et al.*, 1998).

The current approach to the biological degradation of AFB_1 is based on the microbial processes involved in the degradation of complex organic aromatic compounds such as lignin. When considering polyphenolic compounds in nature, lignin is undoubtedly the most abundant and possibly also the most heterogeneous and recalcitrant compound to be degraded microbially (de Jongh *et al*., 1994). However, microbial communities have developed means that can effectively degrade this complex compound to CO_2 and H_2O. It is important to note that lignin is not necessarily completely degraded. Different microbial consortia are responsible for initially opening the lignin structure, depolymerisation of the complex compounds and finally mineralization of the more recalcitrant phenolic compounds. There are indications that *Aspergillus* spp. in conjunction with white rot fungi may be actively involved in lignin degradation (Duarte and Costa-Ferreira, 1994), while bacteria are primarily secondary scavengers of the degradation products. When the degradation of polyphenolic xenobiotics are considered, fungi again feature as one of the major groups responsible for their degradation, presumably due to the large repertoire of extracellular enzymes produced by these fungi (Armstrong and Patel, 1994; Hammel, 1995; Higson, 1991; Singh *et al.*, 1991).

As white rot fungi have the potential to degrade lignin as well as a wide range of polycyclic aromatic hydrocarbons (Baldrian *et al*., 2000), their role in the degradation of other carcinogens, such as AFB_1 is not known at present. The unique mechanisms

whereby these white rot fungi cause lignin degradation involve enzymes such as peroxidases (lignin- and Mg-peroxidases) and laccases (Cullen and Kersten, 1996) and are therefore of relevance.

In this study, laccase production and AFB_1 degradation by white rot fungi in liquid media were investigated. The effect on the mutagenic potency of the mycotoxin and possible breakdown products was also monitored to evaluate the biological relevance of the breakdown process.

6.3. Materials and Methods

Fungal strains

The fungal strains used in experiments are listed in Table 1. The strains are part of a fungal culture collection belonging to the Microbiology Department of the University of Stellenbosch, Stellenbosch, South Africa. Cultures were maintained on malt extract agar plates.

Culture media and agar plates

GPY medium (Motomura *et al*., 2003). Glucose (1%, w/v), malt extract (1%, w/v) and yeast extract (0.4%, w/v). *Liquid MSM medium*. Sucrose (2%, w/v), yeast extract (0.3%, w/v), NH_4SO_4 (0.45%, w/v), KH_2PO_4 (0.3%, w/v), $MgSO_4.7H_2O$ (0.1%, w/v), citric acid (0.025%, w/v), $CaCl_2.2H_2O$ (0.005%, w/v) and trace elements. The pH of the medium was adjusted to 5.5. The medium was supplemented with veratryl alcohol (1 mM) after 48 h of incubation. *Liquid MSB-MEB medium* (Arora and Gill, 2000). Glucose (1%, w/v), KH_2PO_4 (0.2%, w/v), $MgSO_4.7H_2O$ (0.05%, w/v), $CaCl_2.2H_2O$ (0.01%, w/v), thiamine HCl (0.0001%, w/v), ammonium tartrate (0.02%, w/v), malt extract broth (0.5%, w/v) and trace elements. The medium was supplemented with veratryl alcohol (1 mM) and sugar cane bagasse (1%, w/v) after 48 h of incubation. *Liquid wheat straw medium (WSM).* Wheat straw (2%, w/v), YNB with amino acids (0.67%, w/v), L-asparagine (0.2%, w/v), and KH_2PO_4 (0.5%, w/v). *Liquid minimal medium* (Punt

and van den Hondel, 1992). Sucrose (10%, w/v), casamino acids (0.04%, w/v), yeast extract (0.1%, w/v), $MgSO_4.7H_2O$ (0.08%, w/v), $NaNO_3$ (1.2%, w/v), KH_2PO_4 (0.3%, w/v), and trace elements. *Poly R-478 agar plates*. Potato dextrose agar (PDA) supplemented with Poly R-478 (0.01%, w/v) (Sigma-Aldrich Cat. no. P-1900).

Determination of enzymatic activity

Laccase activity was determined according to the method described by Jönsson *et al*. (1997) by measuring the formation of oxidation products at 434 nm with a Genesys 20 spectrophotometer. Assays were performed at 25ºC by using 1.6 mM 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) as substrate. Extracellular extracts of fungal cultures were 50-fold diluted in 50 mM sodium acetate buffer (pH 5.2), where after 0.5 ml was combined with distilled water (0.5 ml) and 200 mM sodium acetate buffer (pH 5.2, 0.5 ml). ABTS (1.6 mM, 0.5 ml) was added to the assay mixture and the formation of oxidation products monitored for 10 min. One unit enzyme activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute.

Degradation of Poly R-478

Decolourization of Poly R-478 by fungal strains (Table 1) was evaluated by inserting a 9 mm mycelium covered agar plug from a fresh fungal culture, in the centre of a Poly R-478 agar plate (Novotny *et al*., 2001). Plates were incubated at 25°C for 21 d and examined for zones of decolourization. Fungi able to decolourize Poly R-478 effectively were used in subsequent AFB_1 degradation experiments.

Degradation of AFB_1 by extracellular extracts of white rot fungi

Pleurotus ostreatus, *Peniophora* sp., *Phanerochaete chrysosporium*, and *Bjerkandera adusta* (Table 1) were cultivated in 100 ml of GPY (Motomura *et al*., 2003), MSM, MSB-MEB (Arora and Gill, 2000) and WSM liquid media for 10 d at 25ºC on a shaker (100 rpm). Cell-free extracellular extracts were prepared by filtering cultures

through miracloth (Calbiochem biosciences Inc., La Jolla, CA, USA) followed by a 0.22 μm filter (Millex-GV, Durapore) and the laccase activity determined (Jönsson *et al*. 1997). For degradation experiments a stock solution (100 μg/ml) of AFB_1 (Sigma-Aldrich Cat. no. A 6636) was used to supplement the extracellular extracts to a final concentration of 1.4 μg/ml (Teniola *et al*., 2005). Laccase activity (U/ml) in extracellular extracts used for degradation experiments ranged between 0.001 and 0.496 (Table 2). Extracts were aliquoted (0.8 ml) in screw-cap Eppendorf tubes and incubated at 30ºC on a rotor wheel for 72 h. Untreated AFB_1 samples were included as control samples. Following incubation, AFB_1 was extracted from the samples and quantified by HPLC as described below.

Degradation of AFB_1 by concentrated extracellular extracts

Pleurotus ostreatus and *Peniophora* sp. (Table 1) were cultivated in 100 ml of MSM and MSB-MEB (Arora and Gill, 2000) liquid media for 10 d at 25ºC on a shaker (100 rpm). Cell-free extracellular extracts were prepared (as described above) and 10-fold concentrated by ultrafiltration (Amicon ultrafiltration cell, Mr cut off 10 kDa) while washing with distilled water, where after the laccase activity was determined. For degradation experiments concentrated extracellular extracts were supplemented with AFB_1 to a final concentration of 1.4 μg/ml, aliquoted (0.8 ml) to sterile screw-cap Eppendorf tubes and incubated at 30°C on a rotor wheel for 72 h as described above. Laccase activity (U/ml) in concentrated extracellular extracts used for degradation experiments ranged between 0.176 and 2.766 (Table 2). AFB_1 incubations in the absence of the extracts were included as control samples. Following incubation, AFB_1 was extracted from the samples and quantified by HPLC as described below.

Degradation of AFB_1 by purified fungal laccase enzyme

Degradation experiments were carried out in 0.2 M phosphate buffer pH 6.5, using purified laccase enzyme produced by *Trametes versicolor* (Sigma-Aldrich Cat. no. 38429, specific activity ≥ 0.5 U/mg). AFB_1 was prepared to a final concentration of

1.4 µg/ml (as described above) in the phosphate buffer, where after laccase was added to obtain solutions with different enzyme activities (0.05, 0.25, 0.5, 0.75, 1 U/ml). Samples were aliquoted (0.8 ml) to sterile screw-cap Eppendorf tubes and incubated at 30°C on a rotor wheel for 72 h. Untreated AFB_1 samples at 0 h and 72 h were included as control samples. Following incubation, samples were extracted (as described below) and the organic ($CHCl_3$) and aqueous phases analyzed by HPLC, ESMS and LCMS for AFB_1, water-soluble breakdown (Line *et al*., 1994) and polymerised products (Aktas and Tanyolac, 2003; Claus, 2004; Mattinen *et al*., 2005). Aliquots of the different samples were also analyzed for mutagenicity as described below.

Degradation of AFB_1 by recombinant laccase produced by A. niger

Recombinant *A. niger* (D15-Lcc2#3), harbouring the *lcc*2 gene encoding laccase (Bohlin *et al*., 2006), was inoculated (1 x 10^6 spores/ml) in 25 ml liquid minimal medium (Punt and van den Hondel, 1992) and incubated at 30°C on a shaker (100 rpm) for 10 d. Cell-free extracellular culture extracts were prepared by filtration as described above, the laccase activity determined and extracellular extracts supplemented with AFB_1 to a final concentration of 1.4 µg/ml (as described above). Extracellular extracts, containing 0.118 U/ml laccase activity, were aliquoted (0.8 ml) to sterile screw-cap Eppendorf tubes and incubated at 30ºC on a rotor wheel for 72 h as described above. The *A. niger* host strain, lacking the *lcc*2 gene, as well as untreated AFB_1 samples at 0 h and 72 h were included as control samples. Following incubation, AFB_1 was extracted from samples (as described later) and the organic and aqueous phases analyzed by HPLC. Aliquots of the different samples were also analyzed for mutagenicity as described below.

AFB_1 extraction and chromatographic analyses

AFB_1 was extracted from the extracellular extracts with chloroform (Teniola *et al*., 2005), and the solvent evaporated under nitrogen. The dried extract was dissolved in methanol and filtered (Millex-GV, Durapore, 0.22 µm). The aqueous phases resulting from the AFB_1 extraction were lyophilized and stored at -20°C until analyzed. HPLC analysis was

performed utilising a LiChroCART 250-4 Hypersil ODS (5 µm) column, with a guard column [LiChroCART 4-4 RP-C18 (5 µm)] and acetonitrile:methanol:water (1:1:2, v/v/v) as the mobile phase at a flow rate of 1 ml/min. AFB_1 was measured by UV (365 nm) detection using a diode array detector (Waters model 996). During HPLC analysis of the aqueous phases, UV absorbance was monitored between 200 and 400 nm. ESMS (solvent: methanol: water, 7:3, v/v) and LCMS (solvent: methanol:acetonitrile:water, 1:1:2, v/v/v) were done using a Phenomenex 2.0 x 150 mm C_{18} column and a flow rate of 100 µl/min to detect the formation of possible breakdown products.

To investigate the possibility of AFB_1 polymerisation during treatment with the laccase enzyme (Aktas and Tanyolac, 2003; Mattinen *et al*., 2005), the organic and aqueous samples were analyzed by ESMS and HPLC. ESMS was done by direct infusion (solvent: acetonitrile:water, 1:1, v/v; formic acid, 0.1%) at a flow rate of 150 µl/min. HPLC analyses were performed by using a gradient mobile phase from acetonitrile:methanol:water (1:1:2, v/v/v) to acetonitrile while the UV absorbance was monitored between 200 and 400 nm.

Salmonella typhimurium mutagenicity assay

(i) Preparation of samples. A stock solution (2 µg/ml) of AFB_1 was prepared in dimethyl sulfoxide (DMSO) and diluted to obtain a standard test solution (0.1 µg/ml). In the mutagenicity assay the standard test solution was added to a concentration of 10 ng AFB_1/plate to obtain a 2- to 3-fold mutagenic response as compared to the background revertant count. Untreated control AFB_1 samples from the laccase degradation experiments, containing 1.4 µg/ml AFB_1, were also diluted in DMSO to 0.1 µg/ml. The organic phases of the laccase treated AFB_1 samples were evaporated to dryness while aqueous phases were lyophilized as described above. These samples were diluted in a similar volume of DMSO as the untreated control AFB_1 samples. An equivalent volume (0.1 ml/plate) of the standard AFB_1 solution, the untreated AFB_1 control and the laccase treated AFB_1 samples were used in the mutagenicity assay.

(ii) Mutagenicity assay. The plate incorporation assay was performed according to the method described by Moron and Ames (1983) using Aroclor 1254 induced S9 liver

homogenate (0.7 nmole cytochrome P450/mg protein) for metabolic activation of AFB_1. *Salmonella typhimurium* TA100 was used as tester strain and 0.5 ml of S9 mixture containing 2 mg protein of the liver homogenate per ml was added per plate. The different samples were subjected to mutagenicity testing and a loss in mutagenicity taken as a measure of enzymatic breakdown of AFB_1.

Statistical analyses

Cross classification ANOVA data analyses were done on the response variable (AFB_1 concentration or number of revertants) observed over untreated AFB_1 control- and time values by using a compound symmetry model (Dunn and Clark, 1987). A significance level of 5% was used and Bonferroni multiple comparisons were done when significant differences were encountered. When responses for two time values were compared or when responses for a control value versus a time value were compared, a paired t-test was used. Laccase activity and AFB_1 degradation were compared by using Spearman rank correlation.

6.4. Results and Discussion

A wide range of fungi, including white rot fungi (Table 1), were examined for their ability to produce laccase enzyme and degrade AFB_1. Degradation of the polymeric dye Poly R-478 is a valuable tool to screen for laccase activity (Kiiskinen *et al*., 2004) and for selecting promising polycyclic aromatic hydrocarbon degrading fungi (Field *et al*., 1992). Aflatoxins are difuranocoumarin derivatives and are structurally related to Poly R-478. Since the production of laccase enzymes and the resulting degradation of Poly R-478 might coincide with degradation of AFB_1, we screened the fungi for their ability to decolourize Poly R-478 (Table 1). *Pleurotus ostreatus*, *Peniophora* sp., *P. chrysosporium*, and *B. adusta* sp. degraded Poly R-478 the most effectively and therefore, were used in the subsequent AFB_1 degradation experiments.

Table 1. **Decolourization of Poly R-478 by fungi cultivated on Poly R-478 agar plates.**

Fungal strain	**Strain nr.**	**Poly R-478 Decolourization**
Bjerkandera adusta	SCC0169	++
Cariolus versicolor	K13-1	+
C. versicolor	K13-2	+
C. versicolor	K12-1-2	+
C. versicolor	K12-2	+
Coriolus pubescens	SCC0347	-/+
Coriolus zonatus	SCC0053	-
Cryptococcus laurentii	1f	-
Cryptococcus podzolicus	5a	-
Daedalea quercina	K8-5	-
Flammulina velutipes	S7-1	-
Ganoderma applanatum	SCC0182	-/+
Laetiporus sulphurous	SCC0180	+
Lentinus edodes	S1-2	-
Lenzites betulina	SCC0382	+
Penicillium candidum	PC 1	-
Penicillium citrinum	Abo 491	-
Penicillium janthinellum	Abo 34	-
Penicillium sp	Abo 268	+
Penicillium sp	Abo 269	+
Penicillium sp	Abo 272	+
Penicillium sp	Abo 275	-
Penicillium sp	Abo 486	-
Penicillium sp	Abo 487	+
Penicillium sp	Unk 1	-/+
Penicillium sp	Unk 2	+
Penicillium variable	Abo 499	-
Peniophora sp.	SCC0152	++
Phanerochaete chrysosporium	ME-446	++
Pleurotus florida	S10-2	+
Pleurotus pulmonarius	S11-1-2	-
Pleurotus djamor	Ab0 284	+
Pleurotus ensyngii	S5-1	+
Pleurotus ostreatus	St2-3	++
Poria sp.	SCC0124	+
Pycnoporus coccineus	SCC0041	+
Pycnoporus sanguineus	PPR 16762	-
P. sanguineus	K3-1	-
P. sanguineus	K3-2	-
P. sanguineus	K5-1	-/+

P. sanguineus	K5-2-2	-/+
P. sanguineus	K5-2-3	-
P. sanguineus	SCC0087	-/+
P. sanguineus	SCC0108	+
P. sanguineus	SCC0294	+
Schizophyllum commune	D1-1	-
S. commune	K11-M3	-
Stereum australe	SCC0007	-/+
Stereum hirsutum	SCC0383	-/+

++, highly effective decolourization.

+, decolourization.

-/+, unclear decolourization (not all 3 samples).

-, no decolourization.

A significant ($P<0.0001$) correlation was observed between laccase activity and AFB_1 degradation obtained from *Peniophora* sp. and *P. ostreatus* cultivated in MSM ($r=0.93$) and MSB-MEB ($r=0.77$) liquid media. Of the fungal strains examined, *Peniophora* sp. produced the highest laccase activity (496 U/L) in MSB-MEB medium supplemented with veratryl alcohol and sugarcane bagasse (Arora and Gill, 2000) (Table 2) and also exhibited the highest percentage AFB_1 degradation (40.45%) (Table 3). While little information is available regarding the production of ligninolytic enzymes by *Peniophora* sp. (Kiiskinen *et al*., 2004), these results confirm that *Peniophora* sp. produces high levels of laccase activity. Motomura *et al*. (2003) reported degradation of AFB_1 by unconcentrated supernatant of *P. ostreatus* cultured in GPY liquid medium and isolated an unidentified AFB_1 degrading enzyme, which was not a peroxidase. However, the AFB_1 degradation was not further quantified. In this study *P. ostreatus* produced laccase activity (416.39 U/L) (Table 2) in MSM medium supplemented with veratryl alcohol resulting in a 35.9% AFB_1 degradation (Table 3). It is well known that *P. ostreatus* degrades a variety of polycyclic aromatic hydrocarbons (Baldrian *et al*., 2000) and that the major enzymes involved are manganese-dependent peroxidases and laccases. Furthermore, high levels of laccase activity (2766.29 U/L) were noticed in concentrated extracellular extracts of *P. ostreatus* cultured in MSM medium

supplemented with veratryl alcohol (Table 2), which coincided with the highest percentage (76%) degradation of AFB_1 (Table 3).

Table 2. **Laccase activity (U/L) measured in extracellular culture extracts after culturing fungi in different liquid media.**

Fungal strain	Strain nr.	WSM medium	MSM Medium	MSB-MEB medium	GPY medium
Pleurotus ostreatus [1]	St2-3	6.13±0.522	416.39±3.12[a]	35.65±2.36[e]	65.19±0.59
P. ostreatus [2]	St2-3	ND	2766.29±2.11[b]	600±1.56[f]	ND
Peniophora sp [1]	SCC0152	170.64±1.23	55.75±2.45[c]	496±2.87[g]	120.45±3.11
Peniophora sp [2]	SCC0152	ND	176.97±1.59[d]	1105.14±2.48[h]	ND
Bjerkandera adusta[1]	SCC0169	3.6±0.99	0	0	1.14±0.89
Phanerochaete chrysosporium[1]	ME-446	4.49±0.48	0	0	0

Values are means ± STD of triplicate determinations.

[1]unconcentrated extracellular extracts.

[2]concentrated extracellular extracts.

ND, not determined.

Determinations differ significantly ($P<0.05$) when letters differ.

Concentrated extracellular extracts of *Peniophora* sp. cultured in MSB-MEB liquid medium supplemented with veratryl alcohol, exhibited a laccase activity of 1105.14 U/L which was associated with an AFB_1 degradation of 52.36%. However, effective decolourization of Poly R-478 and degradation of AFB_1 (28.19%) by ***B. adusta*** in WSM liquid medium was not significantly ($P>0.05$) correlated with laccase activity. This could be ascribed to the involvement of other enzymes than laccases, probably peroxidases.

Table 3. **Percentage AFB_1 degradation obtained after treatment of AFB_1 with extracellular culture extracts of the different fungal liquid cultures**.

Fungal strain	**Strain nr.**	**WSM medium**	**MSM medium**	**MSB-MEB medium**	**GPY medium**
Pleurotus ostreatus [1]	St2-3	15.11±1.89	35.9±2.76[a]	20.95±1.47[e]	33.89±4.26
P. ostreatus[2]	St2-3	ND	76±3.14[b]	31.28±1.88[f]	ND
Peniophora sp.[1]	SCC0152	20.80±1.67	17.10±2.33[c]	40.45±3.24[g]	9.32±2.74
Peniophora sp.[2]	SCC0152	ND	25.79±2.18[d]	52.36±4.89[h]	ND
Bjerkandera adusta[1]	SCC0169	28.19±2.36	0	0	0
Phanerochaete chrysosporium[1]	ME-446	13.77±3.8	0	0	0

Values are means ± STD of triplicate determinations.

[1]unconcentrated extracellular extracts.

[2]concentrated extracellular extracts.

ND, not determined.

Determinations differ significantly ($P<0.05$) when letters differ.

During experiments regarding degradation of AFB_1 by different activities of purified fungal laccase enzyme (0.05, 0.25, 0.5, 0.75, 1 U/ml), AFB_1 was shown to be stable over the 72 h incubation period in the absence of the laccase enzyme (Fig. 1). The AFB_1 concentration in organic phase samples significantly ($P<0.0001$) decreased during treatment with 0.05 to 1 U/ml laccase, with only 12.66% remaining AFB_1 after treatment with 1 U/ml laccase. ESMS and LCMS showed that AFB_1 concentration decreased notably during the incubation with purified fungal laccase enzyme for 72 h (data not shown). These results confirm the degradation results observed with HPLC quantification techniques. No AFB_1 was present in the aqueous samples. HPLC, ESMS and LCMS analyses could not reveal the formation of any structural analogues, suggesting that AFB_1 was most likely metabolized to breakdown products with chemical properties that vastly differ from the parent molecule.

Enzymatic oxidation of phenolic compounds by laccase generates radicals which can react with each other to form dimers, oligomers and polymers (Aktas and Tanyolac, 2003; Claus, 2004; Mattinen *et al*., 2005). This characteristic of laccase is valuable for their application to detoxify contaminated soil or waste waters. As a result, cathecol and other phenolic substrates are polymerised and removed from wastewater streams in the form of a precipitate. In order to detect formation of macromolecules after treating AFB_1 with purified fungal laccase enzyme, LCMS and HPLC were performed. However, no such compounds could be detected.

The biological degradation of AFB_1, when treated with different activities of purified fungal laccase enzyme (0.05, 0.25, 0.5, 0.75, 1 U/ml), coincided with a typical dose response effect regarding the loss of mutagenicity of AFB_1 in the organic phase samples, as evaluated by the *Salmonella typhimurium* mutagenicity assay (Fig. 2). The positive AFB_1 test control produced a 3-fold dose-response when compared to the baseline revertant counts (data not shown). The mutagenic response decreased most significantly ($P<0.0001$) by the laccase treatment in the organic phase samples (Fig. 2). After treatment with 1 U/ml laccase there was no significant difference ($P=0.3$) in mutagenic response in the organic phase samples when compared to the negative test control samples. Treatment with 0.05 to 1 U/ml laccase showed a low mutagenic response in the aqueous phase samples with no significant difference ($P>0.05$) in

mutagenic response between the negative test control and 0.05 to 0.5 U/ml laccase treatments. However, treatment with 0.75 and 1 U/ml laccase differed slightly, although significantly ($P<0.05$) from the negative test control. This can possibly be attributed to very low levels of water soluble mutagenic AFB_1 degradation products present in the aqueous phases of extraction.

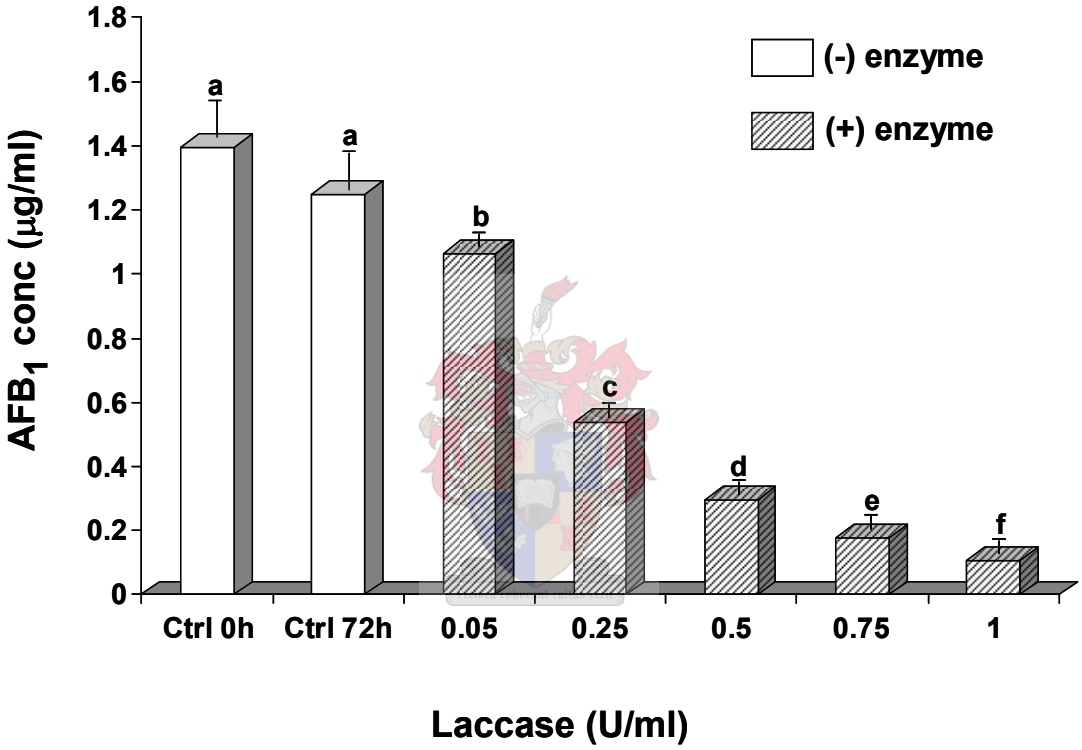

Fig. 1. Biological degradation of AFB_1 by purified fungal laccase (0.05, 0.25, 0.5, 0.75, 1 U/ml) produced by *T. versicolor* over a period of 72 h at 30°C. AFB_1 incubations in the absence of laccase, representing incubation periods of 0 h and 72 h, were included as treatment controls. The level of AFB_1 in the organic phases was determined by HPLC. Treatments represent the means of triplicate determinations and differ significantly ($P<0.05$) when letters differ.

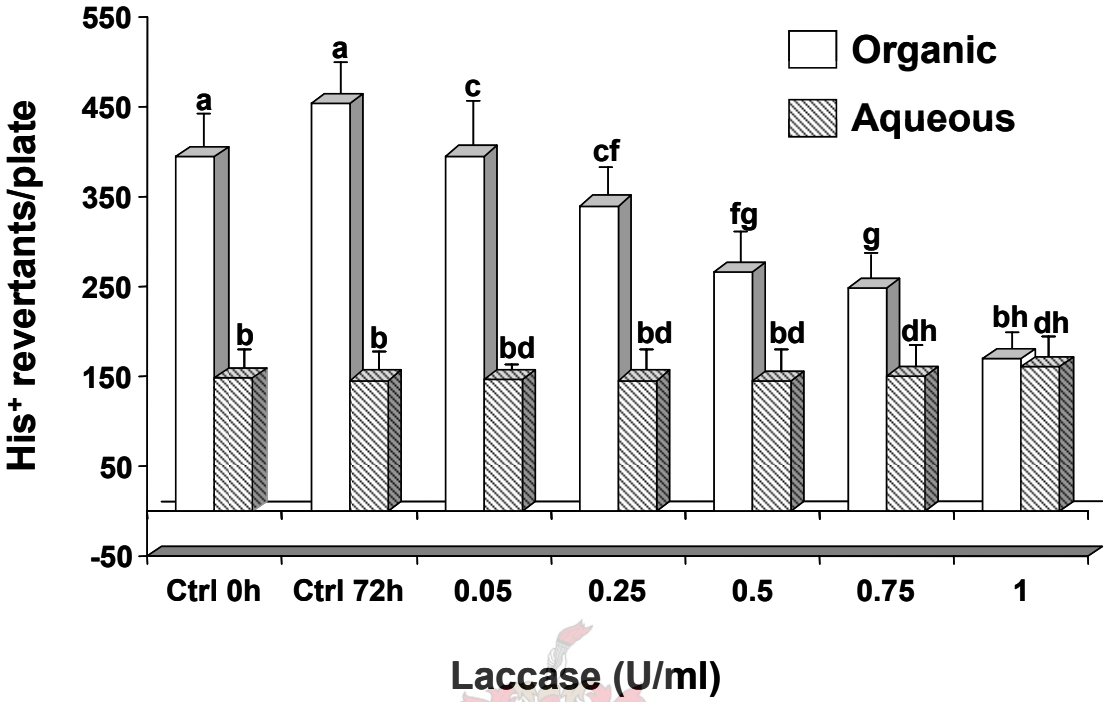

Fig. 2. Mutagenicity of AFB_1 following treatment with different purified fungal laccase activities (0.05, 0.25, 0.5, 0.75, 1 U/ml) from *T. versicolor*, as determined with the *Salmonella typhimurium* mutagenicity assay. The mutagenicity of the organic and aqueous phases is the means of five determinations. Untreated AFB_1 samples representing incubation periods of 0 h and 72 h were included as the treatment controls. Treatments differ significantly ($P<0.05$) when letters differ.

The recombinant laccase producing *A. niger* (D15-Lcc2#3) (Bohlin *et al*., 2006) produced 118 U/L laccase activity as measured in the extracellular extracts. Degradation of AFB_1 was significantly ($P<0.0001$) higher with recombinant laccase produced by *A. niger* (D15-Lcc2#3) (55%) when compared to culture extracts from the *A. niger* host strain (13.52%) (Fig. 3). However, extracts from the *A. niger* host strain also showed significant ($P<0.0001$) reduction in AFB_1 concentration when compared to the untreated control sample at 0 h. It should be noted that *A. niger* had previously been associated

with AFB_1 degradation (Faraj *et al*., 1993). Furthermore, the biological degradation of AFB_1, when treated with recombinant fungal laccase enzyme, coincided with a significant ($P<0.0001$) decrease in mutagenicity of AFB_1 in the organic phase samples (Fig. 4). No mutagenic response could be detected in the aqueous phase which is in agreement with the above experiments.

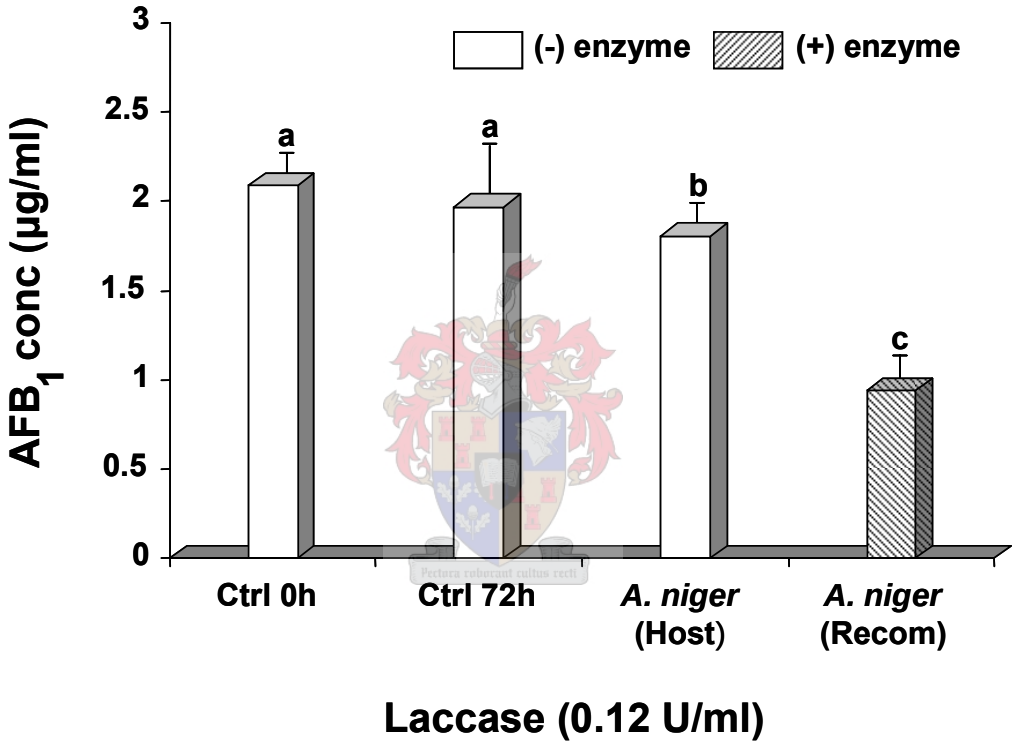

Fig. 3. Biological degradation of AFB_1 by extracellular extracts of recombinant *A. niger* (D15-Lcc2#3) liquid cultures containing recombinant laccase (0.12 U/ml) at 30°C for 72 h. Untreated AFB_1 samples representing incubation periods of 0 h and 72 h and the extract of the *A. niger* host, lacking the enzyme, were included as controls. The level of AFB_1 in the organic phases was determined by HPLC. Treatments represent the means of triplicate determinations and differ significantly ($P<0.05$) when letters differ.

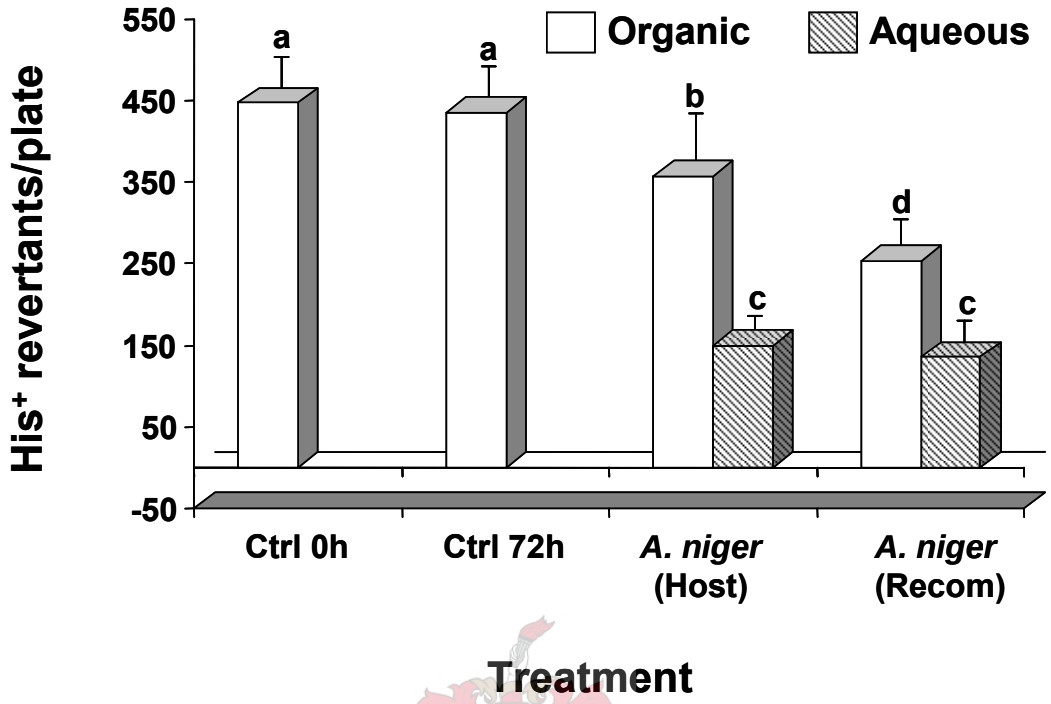

Fig. 4. Mutagenicity of the organic and aqueous phases of the AFB_1 incubation with extracellular extracts of *A. niger* (D15-Lcc2#3) liquid cultures containing recombinant laccase (0.12 U/ml), as determined with the *Salmonella typhimurium* mutagenicity assay. Untreated AFB_1 samples representing incubation periods of 0 h and 72 h were included as controls. Treatments differ significantly ($P<0.05$) when letters differ.

Treatment of AFB_1 with laccase enzyme produced by white rot fungi in unconcentrated extracellular culture extract, purified fungal laccase as well as with recombinant laccase enzyme, decreases the fluorescence properties of the AFB_1 molecule as determined with HPLC. Furthermore, a considerable loss in mutagenicity was observed when treated with purified fungal laccase enzyme from *T. versicolor* as evaluated with the *Salmonella typhimurium* mutagenicity assay. These results suggest that treatment of AFB_1 with fungal laccase enzyme changes the furofuran- or lactone rings of the AFB_1 molecule and as a result influencing its fluorescence and mutagenicity

properties (Liu *et al*. 1998 a,b,c). Moreover, these findings could be valuable to develop food additives or genetic engineered microbial strains with multifunctional technological properties, including degradation of AFB_1 in order to significantly improve the quality, safety and acceptability of food and beverages.

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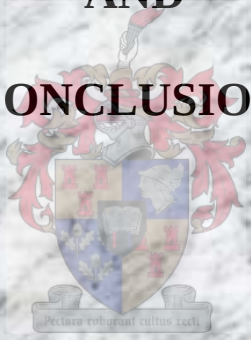
CHAPTER 7

GENERAL DISCUSSION

AND

CONCLUSIONS



GENERAL DISCUSSION AND CONCLUSIONS

7.1. Introduction

Aflatoxins are difuranocoumarin derivatives and highly toxic secondary metabolites predominantly produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus.* Aflatoxin B_1 (AFB_1), the most potent aflatoxin, is highly mutagenic, carcinogenic and teratogenic to both humans and animals and chronic exposure to low levels of AFB_1 pose a serious health and economic hazard worldwide. Aflatoxin B_1 is classified as a type I human carcinogen by the International Agency for Research on Cancer. While physical and chemical detoxification methods do not fulfil the necessary efficacy, safety, and cost requirements, biological detoxification measures are favourable to improve the safety of food for human consumption.

Rhodococcus spp. are capable of degrading a wide range of aromatic xenobiotic compounds, including nitro aromatic compounds, polycyclic hydrocarbons, pyridine, steroids, and lignin related compounds. Several genes encoding enzymes responsible for transforming polyaromatic compounds were characterized in *Rhodococcus* spp. These include 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD) enzymes of which several *bph*C genes in *Rhodococcus erythropolis* TA 421, *Rhodococcus* sp. strain RHA1, *Rhodococcus rhodocrous* K37 and *Rhodococcus globerulus* P6 have been characterized.

When the degradation of aromatic xenobiotics are considered, fungi also feature as one of the major groups responsible for their degradation, presumably due to the large repertoire of extracellular enzymes produced by these fungi. Several groups of enzymes produced by white rot fungi are involved in the degradation of lignin and aromatic xenobiotics, including heme-containing peroxidases, flavine oxidases, cellobiose dehydrogenases as well as laccases. It was found that the presence of laccase is required for lignin degradation, since lignin-deficient mutants lose their ability to degrade lignin.

While aflatoxins are structurally related to the above-mentioned xenobiotic aromatic compounds, the ability of these organisms to degrade AFB_1 is of relevance. The current study investigated degradation of AFB_1 by *R. erythropolis* DSM 14303,

Mycobacterium fluoranthenivorans sp. nov. DSM 44556^T, *Nocardia corynebacterioides* DSM 20151, *N. corynebacterioides* DSM 12676, white rot fungi, purified fungal laccase enzyme as well as recombinant laccase enzyme. In addition, the degradation of AFB_1 by 2,3-DHBD in *R. erythropolis* through extracellular expression of the *bph*C1 gene in *Escherichia coli* was studied. Moreover, it was determined whether the biological degradation coincides with a decrease in fluorescence and mutagenicity of AFB_1, which implies changes in the coumarin structure of the molecule.

7.2. Conclusions

Specific milestones achieved and conclusions derived from this study:

Degradation of AFB_1 by cell-free extracts of *R. erythropolis* and *M. fluoranthenivorans* sp. nov.

- Degradation of AFB_1 by intracellular extracts of *R. erythropolis* DSM 14303 (>90% degradation within 4 h at 30ºC), *M. fluoranthenivorans* sp. nov. DSM 44556^T (>90% degradation within 4 h at 30ºC), *N. corynebacterioides* DSM 20151 (>90% degradation after 24 h at 30ºC) and *N. corynebacterioides* DSM 12676 (60% degradation after 24 h at 30ºC) was achieved.
- Effective degradation of AFB_1 by intracellular extracts of *R. erythropolis* DSM 14303 and *M. fluoranthenivorans* sp. nov. DSM 44556^T was observed between 10 and 40ºC. Degradation of AFB_1 by intracellular extracts of *N. corynebacterioides* DSM 20151 and DSM 12676 was optimal at 30ºC.
- The optimal temperatures of degradation of AFB_1 by *R. erythropolis* DSM 14303, *M. fluoranthenivorans* sp. nov. DSM 44556^T, *N. corynebacterioides* DSM 20151 and *N. corynebacterioides* DSM 12676 are suitable for application in tropical environments such as West Africa.
- Significant ($P<0.0001$) degradation of AFB_1 by *R. erythropolis* DSM 14303 liquid cultures (17% residual AFB_1 after 48 h and only 3-6% residual AFB_1 after 72 h) was achieved.

- AFB_1 was degraded by *R. erythropolis* DSM 14303 liquid cultures and not removed from the medium by adsorption to bacterial cell walls.

Biological degradation of AFB_1 by *R. erythropolis* cultures

- Significant ($P<0.001$) degradation of AFB_1 was observed when treated with *R. erythropolis* DSM 14303 unconcentrated extracellular extracts (33.20% residual AFB_1 after 72 h). Significant ($P<0.05$) reduction in AFB_1 was already observed after 2 h.
- The biological degradation of AFB_1 when treated with *R. erythropolis* DSM 14303 unconcentrated extracellular extracts coincided with a total loss of mutagenicity of AFB_1 and its breakdown products.
- Degradation of AFB_1 by extracellular extracts of *R. erythropolis* DSM 14303 is enzymatic.
- The enzymes responsible for degradation of AFB_1 when treated with *R. erythropolis* DSM 14303 is produced during the normal growth of the organism, indicating that the degradation is a constitutive activity of *R. erythropolis* DSM 14303.
- Treatment of AFB_1 with unconcentrated extracellular extracts from *R. erythropolis* DSM 14303 could not reveal the formation of any structural analogues, suggesting that AFB_1 was most likely metabolized to breakdown products with chemical properties that vastly differ from the parent molecule.

Degradation of AFB_1 by 2,3-DHBD from *R. erythropolis* through extracellular expression in *E. coli*

- A significant ($P<0.0001$) reduction in AFB_1 concentration was observed when AFB_1 was treated with extracellular culture fractions containing recombinant 2,3-DHBD produced by *E. coli* BL21 (DE3) harbouring the *bph*C1 gene fused to a PhoA secretion signal, with only 50.68% residual AFB_1 after 72 h.
- Degradation of AFB_1 when treated with extracellular extracts containing recombinant 2,3-DHBD produced by *E. coli* BL21 (DE3) harbouring the *bphC*1 gene coincided

with a significant ($P<0.0001$) decrease in mutagenicity (42.47%) of AFB_1 and its breakdown products, as evaluated by the *Salmonella typhimurium* mutagenicity assay.

Degradation of AFB_1 by fungal laccase enzyme

- *Peniophora* sp. produced 496.00 U/L laccase activity (MSB-MEB medium supplemented with veratryl alcohol and sugarcane bagasse) and accordingly 40.45% AFB_1 degradation, while *Pleurotus ostreatus* produced 416.39 U/L laccase activity (MSB-MEB medium supplemented with veratryl alcohol and sugarcane bagasse) and 35.90% AFB_1 degradation.
- A significant ($P<0.0001$) correlation was observed between laccase activity and AFB_1 degradation exhibited by *Peniophora* sp. and *P. ostreatus* cultivated in MSM ($r=0.93$) and MSB-MEB ($r=0.77$) liquid media.
- AFB_1 was significantly ($P<0.0001$) degraded when treated with purified fungal laccase (1 U/ml, 87.34%) from *Trametes versicolor* as well as with recombinant laccase (118 U/L, 55.00 %) produced by *Aspergillus niger*.
- Degradation of AFB_1 when treated with purified fungal laccase from *T. versicolor* as well as recombinant laccase produced by *A. niger* coincided with significant ($P<0.001$) loss of mutagenicity of AFB_1, as evaluated by the *Salmonella typhimurium* mutagenicity assay.
- Treatment of AFB_1 with purified fungal laccase from *T. versicolor* as well as recombinant laccase produced by *A. niger* did not reveal the formation of any structural analogues, suggesting that AFB_1 was most likely metabolized to breakdown products with chemical properties that vastly differ from the parent molecule.

7.3. Final conclusions

Several bacteria, including *Rhodococcus* spp., as well as white rot fungi have the potential to degrade a wide range of polycyclic hydrocarbon compounds due to the large repertoire of enzymes they produce and the ability of some of these microorganisms and

enzymes to degrade AFB_1 was investigated. The effect on the mutagenic potency of AFB_1 and possible breakdown products was monitored to evaluate the biological relevance of the breakdown process.

The furofuran- or lactone rings of the AFB_1 molecule were changed by treatment of AFB_1 with unconcentrated extracellular extracts from *R. erythropolis* DSM 14303, extracellular fractions containing recombinant 2,3-DHBD produced by *E. coli* BL21 (DE3) harbouring the *bph*C1 gene fused to a PhoA secretion signal, purified fungal laccase from *T. versicolor* as well as recombinant laccase produced by *A. niger* and as a result reduced its fluorescence and mutagenic potency significantly ($P<0.0001$).

While complete degradation of polyaromatic compounds is accomplished through a cascade of reactions, more efficient meta-cleavage is obtained when treated with a combination of enzymes. Therefore, co-expression of *bph*C encoded dioxygenases of *R. erythropolis* with *etb*C of *R. erythropolis, bnz*A of *P. putida* BE-81, or the application of multiple *bph*C dioxygenases containing different substrate specificities, will have an advantage in the degradation of polyaromatic compounds such as AFB_1. Furthermore, the mechanisms whereby white rot fungi cause lignin degradation involve mainly the production of free radical agents by peroxidase- and laccase enzymes. Since fungal laccase enzyme significantly ($P<0.0001$) degrades AFB_1 (87.34%), treatment of AFB_1 with fungal peroxidase enzyme and in combination with fungal laccase enzyme, should be investigated.

The current study laid the foundation for future work towards the treatment of AFB_1 with a combination of enzymes in order to reduce AFB_1 concentrations to a level which coincides with total loss of mutagenicity. The degradation of AFB_1 by bacteria, white rot fungi and microbial enzymes could be an important bio-control measure to reduce the level of this mycotoxin in food commodities. The results reported here could contribute towards developing food additives or genetic engineered microbial strains with multifunctional technological properties including degradation of AFB_1, to significantly improve the quality, safety and acceptability of food and beverages.

LIST OF ABBREVIATIONS

ABTS, Azino-di-3-ethylbenzthiazoline sulfonate

AFB_1, Aflatoxin B_1

ARHDO, Aromatic-ring-hydroxylating dioxygenase enzyme

DMSO, Dimethyl sulfoxide

ESMS, Electro spray mass spectrometry

GMO, Genetically engineered organism

GST, Glutathione S-transferases

HBV, Hepatitis B virus

HCC, Hepatocellular carcinoma

HCV, Hepatitis C virus

HGT, Horizontal gene transfer

HPD, 2-Hydroxy-penta-2,4-dienoate

HPLC, High performance liquid chromatography

IPTG, Isopropyl-β-D-thiogalactopyranoside

LCMS, Liquid chromatography mass spectrometry

PCB, Polychlorinated biphenyl

TCA, Tricarboxylic acid

TLC, Thin layer chromatography

2,3-DHBD, 2,3-Dihydroxybiphenyl 1,2-dioxygenase enzyme.