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1 - INTRODUCTION

1.1 - STUDY MOTIVATIONS AND AIMS

Maintaining a safe global food and feed supply is a critical issue facing all societies. Natural contaminants, especially mycotoxins, pose a challenge because they are found in a wide range of crops. Preliminary surveys have demonstrated the presence of aflatoxin B1 (AFB1) in food such as cereal and in animal feed. With regards to the natural occurrence of mycotoxins in Libyan foodstuffs, no information is available in literature so far. At present no national regulations for mycotoxins are in force in Libya, but the setting of limits is underway by Libyan industry, in order to protect both human and animal health.

The proposed limits for cereal intended for human consumption are 10 μg/kg for total aflatoxin and 5 μg/kg for aflatoxin B_1. For milk and dairy products, the proposed limits are 0.05 and 0.03μg/kg, respectively. The proposed limits were set based on worldwide limits for aflatoxins (FAO, 2004). In Libya cereals represent a staple food for the population, therefore bearing high social, economic and nutritional relevance. Also the Libyan population uses spices to flavour foods, as well as for medication and these are valuable due to their preservative and antioxidant properties.

Spices are largely produced in countries with tropical climates (wide ranges of temperature, humidity and rainfall) which are favourable for mycotoxins contamination. In addition, Libyan society suffers from cancer (Singh and Al-Sudani, 2001). At the present time, cancer is one of the biggest issues in Libya, although there are no publications linking these cancers with mycotoxins. Nevertheless, these

dangerous mycotoxins could lead to a variety of health problems, not only for humans, but also for animals.

1.1.1 - THE PROJECT AIMS

This research investigated the biodegradation of three different mycotoxins: AflatoxinB1 (AFB1), Ochratoxin A and patulin (PAT). Those toxins were selected based on chemical structure "coumarin derivatives". The major aim of this study was to examine the potential use of probiotic bacteria / Actinomycete bacteria to destroy, or reduce the toxicity of AFB1, PAT and OTA in liquid media. The second aim was to identify factors influencing toxin degradation efficiency of the culture. The third aim was to identify the new metabolites produced during AFB1 degradation as understanding the biodegradation pathway will help the practical use of biological degradation. The final aim was to investigate whether or not the bio-treatment of mycotoxins (AFB1, PAT and OTA) would reduce the toxicity of the new generated compounds.

1.2 – LITERATURE REVIEW

1.2.1 - GENERAL CHARACTERISTICS OF MYCOTOXINS

Filamentous fungi represent an immense source of biodiversity and metabolic potential. They have been shown to be able to produce an immense range of bioactive substances of use to mankind including penicillins and cephalosporins, but are also capable of synthesising compounds such as the fungal toxins (Mycotoxins). Mycotoxins are toxic, secondary metabolites of low molecular weight produced by naturally occurring fungi (Bata and Lásztity, 1999, Mishra and Das, 2003a, Bennett and Klich, 2003). These metabolites constitute a toxigenically and chemically heterogeneous assemblage that are grouped together only because the members can cause disease and death in human beings and other vertebrates (Bennett and Klich, 2003). Mycotoxins can be found mainly in cereals, oilseeds, tree nuts, spices, fruit and dairy products seeTable 1.1.

Table 1.1- Commodities contaminated with mycotoxins

Mycotoxins	**Commodity**	**References**
Aflatoxins	Peanuts, corn, wheat, cottonseed, maize, nuts, eggs, figs, rice, spices, dairy and dairy products	(Aygun *et al.*, 2009, Méndez-Albores *et al.*, 2008, Murphy *et al.*, 2006, Paramithiotis and Drosinos, 2010)
Ochratoxin A	Cereal grain (wheat, barley, oats, corn), dry beans, mouldy cheese, coffee, grapes, dried fruit, wine, cocoa	(Duarte *et al.*, 2010, Ferraz *et al.*, 2010)
Patulin	Mouldy feed, apples, apple juice	(Barreira *et al.*, 2010, Moake *et al.*, 2005)

These metabolites are correlated with cancer in parts of Africa, China and South East Asia (Alberts *et al.,* 2009). Moreover, these metabolites are responsible for significant economic losses, for example, losses range between $85 and $100 million per year in certain states of the United States of America (Alberts *et al.,* 2009). However, much discussion in the literature has centred on which mycotoxins pose the greatest potential hazard to human health via food, and attention has been focused on various toxins, including aflatoxins, ochratoxin, sterigmatocystin, patulin, penicillic acid, citrinin, zearalenone, trichothecenes, and fumonisins see Table 1.2

Different methods of classification have been used to classify these toxins; clinicians tend to arrange them by the organ they affect; thus, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, and so forth. Moreover, cell biologists tend to classify them into generic groups such as teratogens, mutagens, carcinogens, and allergens (Bennett and Klich, 2003). Meanwhile, organic chemists have attempted to classify them by their chemical structures (lactones, coumarins), biochemists according to their bio-synthetic origins (polyketide, amino acid derived); whilst physicians class them by the illnesses they cause, and mycologists by the fungi that produce them (e.g. *Aspergillus* toxin) (Bennett and Klich, 2003).

Table 1.2 - Some important toxigenic species of filamentous fungi and related mycotoxins (Bennett and Klich, 2003, Duarte *et al.,* 2010, Reddy *et al.,* 2008)

Fungal species	**Toxin**
Aspergillus flavus	Aflatoxins B1, B2, cyclopiazonic acid
A. parasiticus	Aflatoxins B1, B2, G1, G2
A. ochraceus	Ochratoxin A; Penicillic acid
A. versicolor	Sterigmatocystin, cyclopiazonic acid
Penicillium verrucosum	Ochratoxin A, citrinin
P. purpurogenum	Ochratoxin A, citrinin
P. expansum	Patulin, citrinin
Fusarium sporotrichiodes	T-2 toxin
F. graminearum	Deoxynivalenol, nivalenol, zearalenone
Alternaria alternata	Tenuazonic acid
Stachybotrys atra	Satratoxins

1.2.2 - PHYSICOCHEMICAL PROPERTIES OF AFLATOXINS, OCHRATOXIN, PATULIN

1.2.2.1 - AFLATOXINS

Aflatoxins are difuranocumarin derivatives produced by many species of Aspergilli; in particular *A. flavus, A. parasiticus*, *A. bombycis*, *A. coracles, A. nomius* and *A. pseudotamari* (Goto *et al*, 1996; Klich *et al.,* 2000; Peterson *et al.,* 2001). All of these fungi can grow on certain foods under favourable cond**iti**ons of temperature and humidity, and generate aflatoxin before, after and during harvesting, handling, shipment and in storage. Aflatoxins can be found primarily in cereals, oil seeds, tree nuts, spices and milk and dairy products (Wu *et al.,* 2009). *Aspergillus flavus* is the most common producer; Aflatoxins were first isolated and characterized following the death of more than 100,000 turkeys from an unidentified disease (turkey X disease), and the toxin was traced to consumption of a mould- contaminated peanut meal (Bennett and Klick, 2003).

Eighteen different types of aflatoxins have been identified, but the four major naturally occurring aflatoxins are: B1, B2, G1, G2 (Figure 1.1) based on their fluorescence under UV light (Peraica *et al.,* 1999). Two additional toxins related to these are also found; these are M1, and M2 (hydroxylated metabolites of aflatoxins B_1 and B_2 respectively in animals) isolated from milk (Wu *et al.,* 2009) .

Aflatoxin B1 (AFB1) is the most potent natural toxin. Pure AFB1 is a pale-white to yellow crystalline, odourless solid. AFB1 is soluble in methanol, chloroform, actone, acetonitrile. Aflatoxins have different molecular weights ranging between 346 for AFG2a and 312.3 for AFB1.

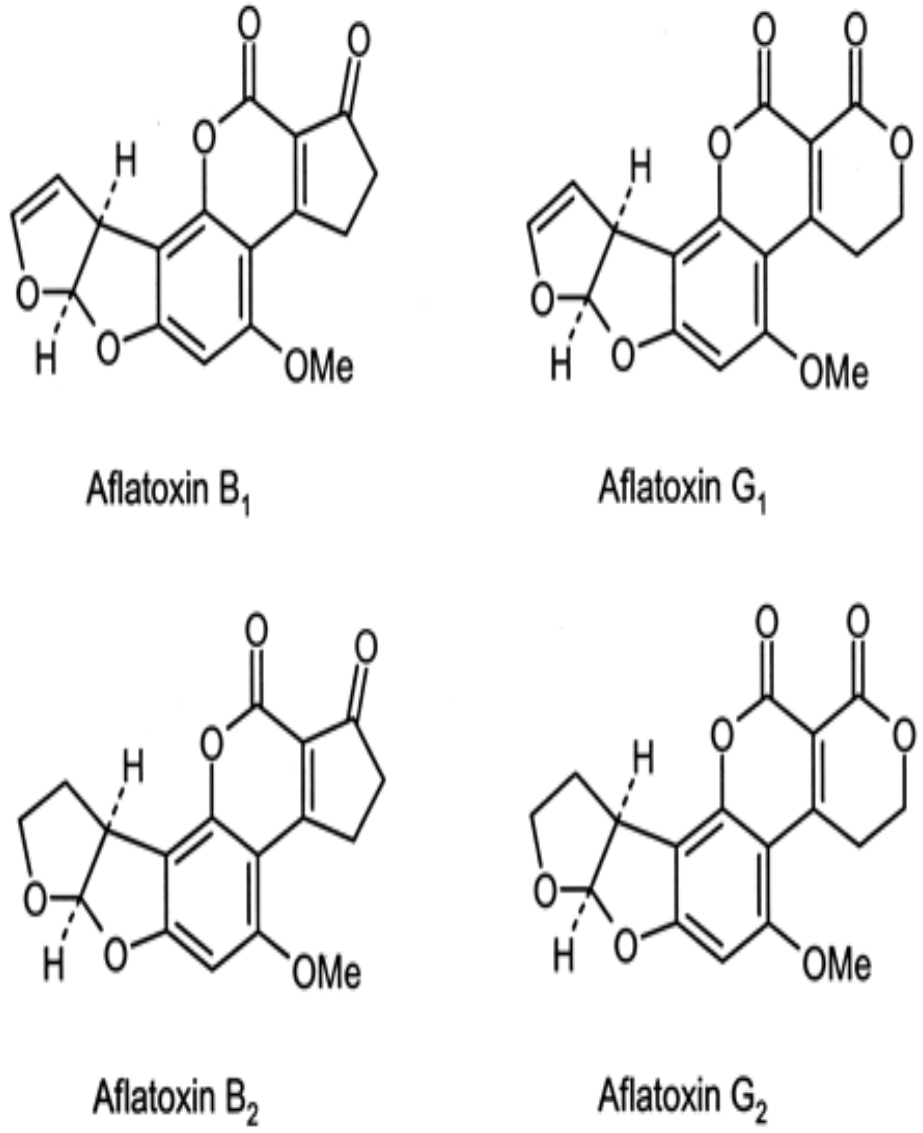


Figure 1.1 - Chemical structures of aflatoxins

1.2.2.2 - OCHRATOXIN A

Members of the ochratoxin family have been found in many different species of *Aspergillus*, in particular *A. alliaceus, A. melleus, A. cabonarius, A. glaucus, and A.niger*. In addition, ochratoxins have also been found in closely related fungal species including as *Penicillium viridicatum* (Peraica *et al.*, 1999, Bennett and Klich, 2003, Sherif *et al.*, 2009). The main toxin in this group is ochratoxin A, which is a 3, 4 dihydromethylisocoumarin derivative linked with L-B-phenylalanine (Duarte *et al.*, 2010). Ochratoxin A ($C_{20}H_{18}O_6NCl$) is the most toxic member of the ochratoxins group, that also includes its methyl ester, its ethyl ester also known as ochratoxin C (OTC), 4-hydroxyochratoxin A (4-OH OTA), ochratoxin B (lacking a chlorine atom on C5 of the dihydro-methylisocoumarin ring system) and its methyl and ethyl esters and ochratoxin A (OTA; where the phenylalanine moiety is missing) see Figure 1.2 (Duarte *et al.*, 2010).

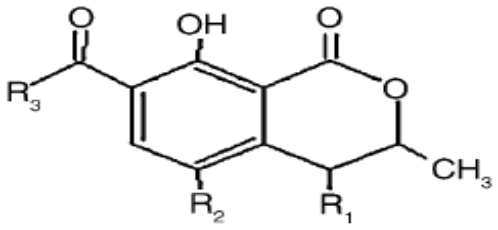


Figure 1.2 - Chemical structure of ochratoxins

Ochratoxins	R1	R2	R3	Reference
OTA	H	Cl	-NH-CH(COOH)-CH_2-Phenyl	(Ringot *et al.*, 2006)
OTB	H	H	-NH-CH(COOH)-CH_2-Phenyl	
OTC	H	Cl	-NH-CH($COOC_2H_5$)-CH_2-Phenyl	
4 hydroxyochratoxin A	OH	Cl	-NH-CH(COOH)-C_2H-Phenyl	
OTα	H	Cl	-OH	

Ochratoxin A, highly soluble in polar organic solvents, slightly soluble in water and soluble in aqueous sodium hydrogen carbonate, has melting points of 90º and 171ºC, when recrystallized from benzene (containing 1 mol benzene/mol) or xylene, respectively (Ringot *et al.*, 2006).

1.2.2.3 –PATULIN

Patulin (PAT) is a secondary metabolite produced by *Aspergillus* and *Penicillium* genus common to fruit- and vegetable-based products, mostly in apples. Despite patulin's original discovery as an antibiotic, it has come under heavy scrutiny for its potential negative health effects (Moake *et al.*, 2005). Patulin is an unsaturated heterocyclic lactone with an empirical formula of $C_7H_6O_4$ and a molecular weight of 154 see Figure1.3. It is a toxic compound and has an LD_{50} in mice of 5 mg/kg (Sant'Ana *et al.*, 2008)

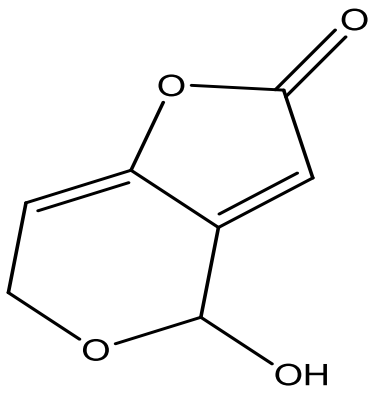


Figure 1.3 - Chemical structure of Patulin

1.2.3 - BIOSYNTHETIC PATHWAY AND PHYSIOLOGICAL FACTORS AFFECTING MYCOTOXIN PRODUCTION

The polypeptide pathway is a well-known route for the formation of secondary metabolites, including various mycotoxins in filamentous fungi (Xu *et al.*, 2006). Over 20 genes are required for biosynthesis of aflatoxins (B1, G1, B2, and G2); it is a multi-enzyme process with a complex metabolic pathway.

Based on the molecular structure of OTA it is clear that a number of enzymatic reactions are likely to be required for its biosynthesis: a polyketide synthase (PKS) for the synthesis of the polyketide dihydroisocoumarin, a cyclase, a chloroperoxidase or halogenase, an esterase and a peptide synthetase for ligation of the phenylalanine to the dihydroisocoumarin (Gallo *et al.*, 2009).

However, the biosynthesis pathway for OTA has not yet been completely elucidated, although a number of putative pathways have been proposed (Gallo *et al.*, 2009). By comparison, patulin biosynthesis is well understood and a summary of the pathway has been published (Moake *et al.*, 2005).

Nonetheless, it is important to note that mould presence does not imply mycotoxin production since mycotoxin production is influenced by various parameters, including nutritional factors such as oxygen supply, carbon and nitrogen sources, fatty acids, environmental factors (eg, water activity, temperature), preservation techniques, circulation and storage techniques (Figure1.4) (Paterson and Lima, 2010, Xu *et al.*, 2006, Dalié *et al.*, 2010).

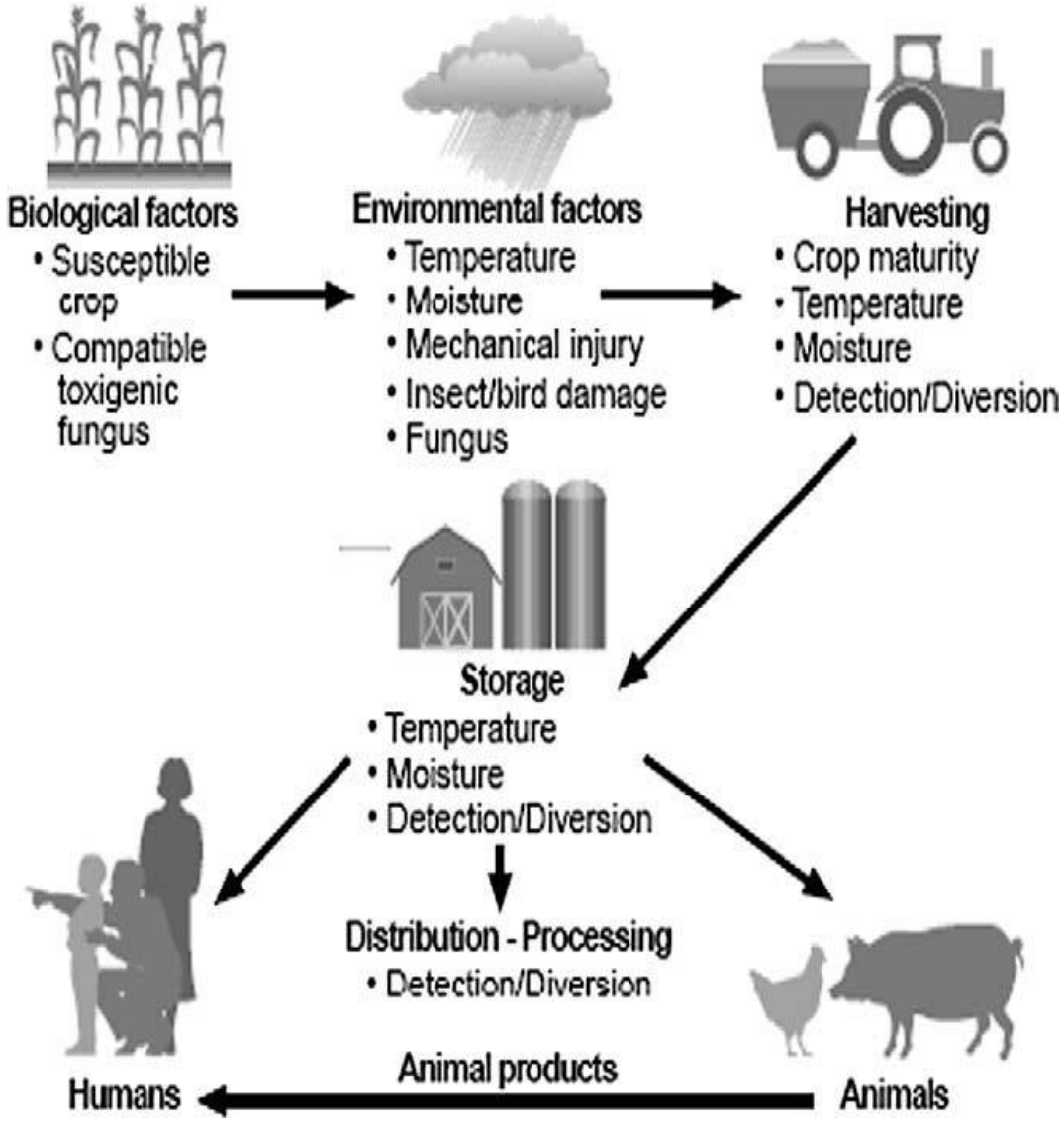


Figure 1.4 - Factors affecting mycotoxin production (Paterson and Lima, 2010).

1.2.4 -TOXICITY AND STABILITY OF AFLATOXIN B_1, OCHRATOXIN AND PATULIN

1.2.4.1 - TOXICITY

In general, the toxicity of mycotoxins can be classified as both acute and chronic toxicity. AFB1 is the most potent natural carcinogen, mutagen, teratogen and immunosuppressive mycotoxin; AFB1 is classified as Group 1A human carcinogen by the International Agency for Research on Cancer (IARC). For example, the ingestion of 2–6 mg/day of aflatoxin for a month can cause acute hepatitis and death (Bennett and Klich, 2003).

The LD_{50} values for ducklings consuming AFB1, AFG1, AFB2, or AFG2 are 0.36, 0.78, 1.70, and 3.44 mg/kg respectively (Hussein and Brasel, 2001). The primary target organ in aflatoxicoses (diseases caused by aflatoxins) in human and animals is the liver (Peraica *et al.*, 1999). AFB1 is metabolized by the liver through the cytochrome P450 enzyme system to the major carcinogenic metabolite AFB1-8,9-epoxide (AFBO), or to less mutagenic forms such as AFM1, Q1, or P1 (Figure 1.5). There are several pathways AFBO can take, one resulting in cancer, another in toxicity, and others in AFBO excretion (Murphy *et al.*, 2006, Hussein and Brasel, 2001).

The exo-form of AFBO readily binds to cellular macromolecules including genetic material, for example, proteins and DNA, to form adducts (a complex formed between the toxin and biological material, in this case a protein that may either be repaired or mutate). It is the formation of DNA-adducts, such as with N7-guanine, that leads to genetic mutations and cancer (Murphy *et al.*, 2006). The metabolic

effects of aflatoxins include inhibition of DNA, RNA, and protein synthesis, reduction in miscellaneous enzyme activities, depression of glucose metabolism, and inhibition of lipid synthesis (Eaton and Gallagher, 1994).

Ochratoxin A, considered to be nephrotoxic, teratogenic and immunotoxic, has been classified by IARC as a class 2B carcinogen, and a probable human carcinogen (Amézqueta *et al.,* 2009). In this case the target organ is the kidney. When the OTA is introduced to the body, it binds strongly to plasma proteins and is metabolised into hydroxylated compounds, one of which, the (4R)-4-hydroxyochratoxin A being as cytotoxic and immunosuppressive as the parent compound *in vivo* and *in vitro.* OTA can be cleaved into phenylalanine (Phe) and OTA, the chlorinated dihydroisocoumarinic moiety has been found not to be toxic, but retains some genotoxicity (Creppy *et al.*, 1999). However, the feature of OTA that is responsible for the high acute toxicity is still unclear (Cramer *et al.*, 2010).

Patulin has chronic health effects including genotoxicity, immunotoxicity, and neurotoxicity in rodents; but insufficient evidence exists for carcinogenicity in other experimental animals and humans. Therefore, the IARC has classified it as Group 3B. The acute symptoms include nervousness, convulsions, lung congestion, oedema, hyperaemia, gastrointestinal tract distension, intestinal haemorrhage and epithelial cell degeneration. However, its effects on human are not yet clear (Barreira *et al.*, 2010).

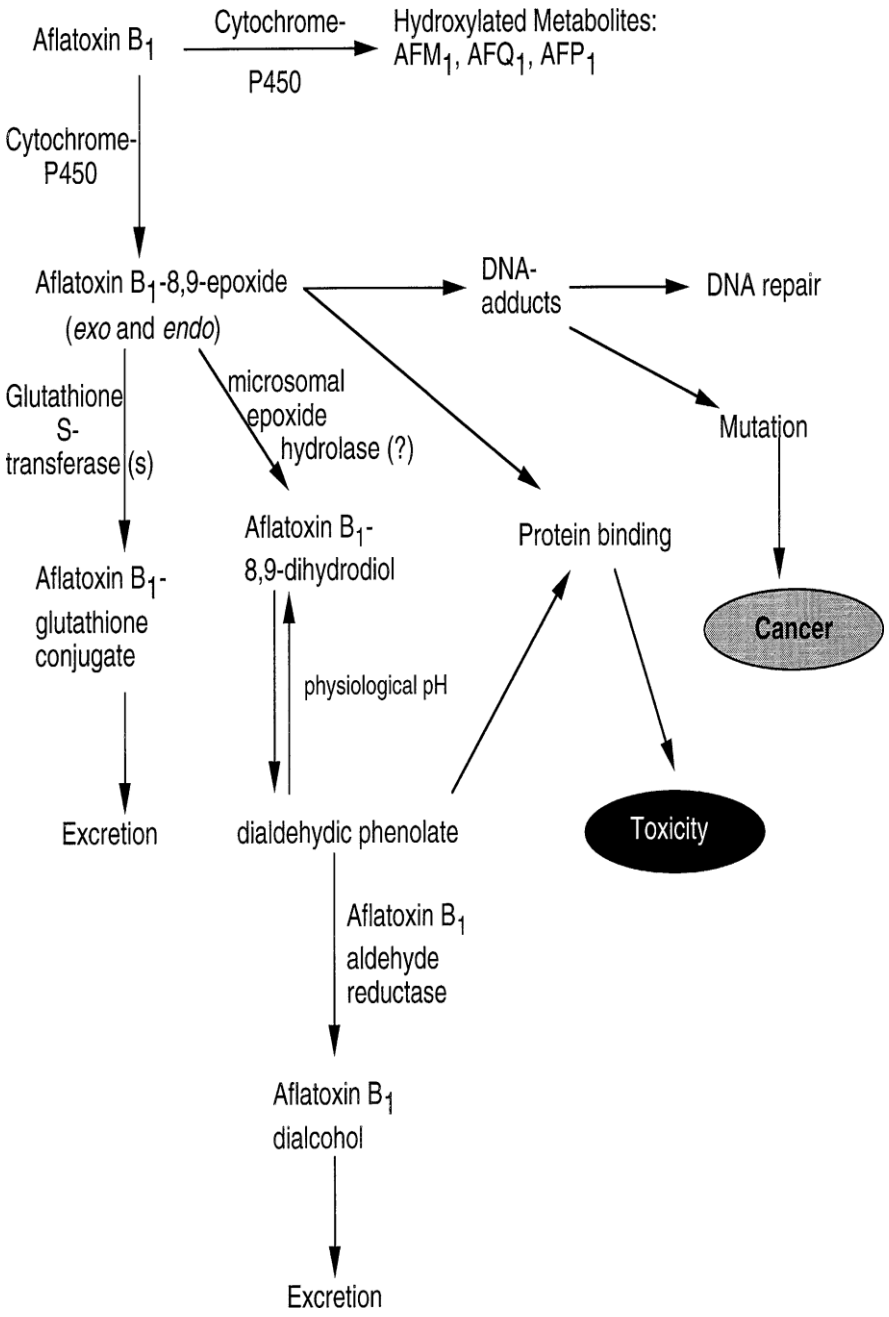


Figure 1.5- Biotransformation pathways for aflatoxins B1 (Murphy *et al.*, 2006).

1.2.5 - STABILITY OF MYCOTOXINS DURING FOOD PROCESS

Mycotoxins are considered to be heat stable molecules within the range of conventional food processing temperature (80-121ºC), so only slight reduction may be achieved during normal cooking conditions such as boiling and frying or even following pasteurization. Initial contamination, type of mycotoxin, heating temperature, the time employed and the degree of the heat, the moisture content, pH and ionic strength of food, among other factors, play a significant role in detoxification during normal cooking procedures (Bullerman and Bianchini, 2007, Kabak, 2009, Bata and Lasztity, 1999). Aflatoxins have high degradation temperatures ranging from 237 to 306ºC; while OTA require 200ºC and 100 ºC for Patulin; this is because of the high melting point of these compounds. However, these high temperatures would have negative influences on the food quality.

1.2.6 - METHOD OF DETOXIFICATION

Once food is contaminated with mycotoxins, there are only two options if the food is to be used: either the toxin is removed or the toxin is degraded into less toxic or non-toxic compounds (El-Nezami *et al.,* 1998a). Mycotoxins may be degraded by physical, chemical, or biological methods (Park and Rhee, 2001, Hamid and Smith, 1987, Ciegler *et al.,* 1966a). However, any detoxification procedure should fulfil the following requirements according to the Food and Agriculture Organisation (FAO):

- The mycotoxins should be destroyed or transformed into non-toxic compounds, and fungal spores and mycelia should be destroyed so that new toxins are not formed.

- The food or feed material should retain its nutritive value and remain palatable.
- The physical properties of raw material should not change significantly.
- The detoxification process should be economically feasible; the cost of detoxification should be less than the value of the treated commodity.
- Food drug administration (FDA) included additional aim related to the impact of the process on the environment should be minimised (Kabak *et al.*, 2006).

1.2.6.1 - MYCOTOXIN DETOXIFICATION IN THEORY

AFB1, OTA and PAT are coumarin derivatives; the lactone ring in the coumarin structure plays a major role in the toxicity and mutagenicity of mycotoxins. The detoxification should be designed either to remove the double bond of the terminal furan ring in AFB1 or to open the lactone ring in the case of AFB1, OTA and PTA. Once the lactone ring is opened, further reactions can occur. (Mishra and Das, 2003).

1.2.6.2 - PHYSICAL METHODS

Physical approaches to mycotoxin destruction involve treatment with heat, ultraviolet light, or ionizing radiation, none of which is entirely effective. However, the utilization of mycotoxin-binding adsorbents is the most widely applied physical method of protecting animals against the harmful effects of mycotoxin-contaminated feed (Piva *et al.*, 1995, Smith and Harran, 1993).

1.2.6.3 - CHEMICAL METHODS

Many studies have evaluated the use of chemical treatments for mycotoxin detoxification. Chemicals used for detoxification include ammonia, sodium hydroxide, hydrogen peroxide, ozone, sugars, chlorine and formaldehyde. In fact, chemical treatment has been used in combination with physical treatment to increase the efficacy of detoxification (Smith and Harran, 1993, Piva *et al.,* 1995, Mishra and Das, 2003a).

1.2.6.4 - BIOLOGICAL METHODS

Biological detoxification can be defined as the enzymatic degradation or biotransformation of mycotoxins that can be obtained by either the whole cell or an enzyme system (Wu *et al.,* 2009). Other approaches that can be regarded as biological include the use of bio-competitive agents and genetically engineered plants for reducing mycotoxin contamination. During the last few years there has been a growing interest in bio-preservation, i.e., the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf-life of foods (Kabak and Dobson, 2009, Kabak and Var, 2004, Schnürer and Magnusson, 2005). Biological decontamination appears to be attractive because it works under mild, environmentally friendly conditions. Progress in this area has been assisted by recent advances in molecular biology, genetic engineering and microbial genomics (Kabak *et al.,* 2006).

Early studies demonstrated the ability of several microorganisms to degrade aflatoxins one bacterium, *Flavobacterium aurantiacum*, from the many

microorganisms examined, including yeasts, moulds, and bacteria, was able to remove aflatoxin B1 from both solid and liquid media (Ciegler *et al.,* 1966a, Ciegler and Peterson, 1968). In addition, several microorganisms such as *Rhizous* spp, *Corynebacterium rurum, Candida lipolytica, Aspergillus niger, Trichoderma viride, Mucor ambiguous, Neurospora spp., Armillariella tabescens*, and lactic acid bacteria, have been examined to detoxify mycotoxins *in vitro* (aflatoxins, ochratoxin and patulin) with differing results see Table 1.3.

1.2.6.4.1 - LACTIC ACID BACTERIA AND YEAST

1.2.6.4.1.1 - LACTIC ACID BACTERIA (LAB)

1.2.6.4.1.2 - BIO-CONTROL OF MYCOTOXINS BY ANTIFUNGAL METABOLITES PRODUCED BY LACTIC ACID BACTERIA

Numerous investigations have been conducted to examine the antifungal properties of LAB against fungal contaminants in dairy and sourdough baked products (Batish *et al.,* 1997, Bueno *et al.,* 2006b, Ciegler, 1966, Ciegler and Peterson, 1968, Bolognani *et al.,* 1997, El-Nezami *et al.,* 1998b, El-Nezami *et al.,* 2000, El-Nezami *et al.,* 2002a, Lahtinen *et al.,* 2004, Gratz *et al.,* 2006, Gratz *et al.,* 2007, Peltonen *et al.,* 2000, Peltonen *et al.,* 2001, Haskard *et al.,* 2000, Mateo *et al.,* 2010).

Species of LAB, such as a dairy strain of *Lactococcus lactis*, as well as *Lactobacillus* (*L. acidophilus, L.bulgaricus, L. plantarum* and *L. rhamnosus*) and *Pediococcus* that were used as starters in dairy, meat products and even in the silage, have been found to be able to suppress mycotoxins biosynthesis or effectively remove pre-formed mycotoxins (El-Nezami *et al.,* 1998a, El-Nezami *et al.,* 1998b, Haskard *et al.,* 2001).

Different mechanisms have been suggested for these observations, including competitive growth of the bacteria with the mould, bacterial metabolites, pH, or a combination of these factors (Magnusson *et al.*, 2003).

Numerous investigations have reported that such cultures lowered the pH, and led to depletion of nutrients in these systems (Dalié *et al.*, 2010). However, microbial competition does not fully explain the reason for mycotoxin inhibition. Gourama and Bullerman, (1995) found that *Lactobacillus* spp was able to inhibit aflatoxins production. Similar result was recorded by the same group; their result demonstrated that *Lactobacillus casei pseudoplantarum* 371 isolated from silage inoculants was found to inhibit aflatoxins Bl and Gl biosynthesis by *A. flavus* subsp. *parasiticus* NRRL 2999, in liquid medium. Moreover, Xu *et al.*, (2003) reported that *L. plantarum* ATCC 8014 was able to inhibit *A. flavus* subsp. parasiticus NRRL 2999. The inhibition of the growth was probably due to inactivation of spores. In addition, this inhibition was due to the lactic acid.

Other investigators have indicated that production of certain metabolites by lactic acid bacteria affects mould growth and mycotoxin production (Paterson and Lima, 2010). The release of the intracellular pool of lactic acid bacteria during bacterial cell lysis may influence mould growth and AFB1 production (Gourama and Bullerman, 1995). Coallier-Ascah and Idziak, (1985) showed that the reduction of AFB1 production had to be due to a low molecular weight metabolite produced by the bacteria**.** However, the particular mechanism of antifungal action is difficult to explain due to the complex and synergistic interactions between different compounds

(i.e. organic acids, Phenyl lactic acid, hydrogen peroxide and proteinaceous compounds).

1.2.6.4.1.3 - DETOXIFICATION OF MYCOTOXINS BY BINDING OR ADHESION TO THE CELL WALL

The detoxification mechanism of mycotoxins by lactic acid bacteria is unclear. Diverse mechanisms have been suggested but recently a few studies have reported that some of LAB are able to remove the AFB1, OTA, PAT, trichothecenes, zearalenone and fumonisins from contaminated media *in vitro* and *vivo* by adhesion of the toxin to the bacterial cell walls (El-Nezami *et al.*, 2002b, Gratz *et al.*, 2006, El-Nezami *et al.*, 2002a, Fuchs *et al.*, 2006, Dalié *et al.*, 2010, Tuomola *et al.*, 2000, El-Nezami *et al.*, 2000). The capacity of lactic acid bacteria to detoxify depended on the strain, bacterial concentration, pH, and temperature (Elgerbi *et al.*, 2006b, El-Nezami *et al.*, 1998a, El-Nezami *et al.*, 2000).

El-Nezami *et al*. (1998b) indicated that Gram positive bacteria were able to remove the AFB1 from contaminated media, and that removal was rapid (less than 1 min). Conversely, Gram negative bacteria were poor at removing AFB1 from contaminated media. El-Nezami *et al*. (2000) studied the adhesion capability of *L. rhamnosus* strains GG and LC 705 and *Propionibacterium freudenrichii* ssp. *shermanii* JS with AFB1. Their results showed that probiotic strains *L. rhamnosus GG and L. rhamnosus* LC 705 were able to remove 80% of the toxin from a starting level of 20 µg/ml. According to this team, Aflatoxin B2, G1 and G2 were less vulnerable to this binding process (El-Nezami *et al*. 2000).

Table 1.3- Microorganisms able to detoxify mycotoxins (aflatoxins, ochratoxin and patulin).

Mycotoxins	Microorganism	Microorganism strains	References
AFB1,AFM OTA , PAT	Lactic acid bacteria	*Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Oenococcus oeni*	(Batish *et al.*, 1997, Bueno *et al.*, 2006b, Ciegler, 1966, Ciegler and Peterson, 1968, Bolognani *et al.*, 1997, El-Nezami *et al.*, 1998b, El-Nezami *et al.*, 2000, El-Nezami *et al.*, 2002a, Lahtinen *et al.*, 2004, Gratz *et al.*, 2006, Gratz *et al.*, 2007, Peltonen *et al.*, 2000, Peltonen *et al.*, 2001, Haskard *et al.*, 2000, Mateo *et al.*, 2010)
AFB1	Environmental bacteria	*Rhodococcus erythropolis*, *Mycobacterium fluoranthenivorans* , *Nocardia corynebacterioides formerly (Flavobacterium aurantiacum)*, *Stenotrophomonas maltophilia*	(Hormisch *et al.*, 2004, Teniola *et al.*, 2005)
AFB1, OTA	Digestive microorganisms	Rumen Microbes *isolated from Goats* *Butyrivibrio fibrisolvens*	(Upadhaya *et al.*, 2009, Westlake *et al.*, 1989, Kiessling *et al.*, 1984)
AFB1, OTA, PAT	Yeast	*Phaffia rhodozyma*, *Saccahromyces cerevisiae*, *Trichoderma sp*, *Trichosporon mycotoxinivorans*, *Xanthophyllomyces dendrorhous*	(Bueno *et al.*, 2007, Molnar *et al.*, 2004, Péteri *et al.*, 2007, Moake *et al.*, 2005)
AFB1, OTA	Moulds	*Aspergillus* sp *Penicillum* sp *Pleurotus ostreatus* ,*Rhizopus* sp *Trametes versicolar* , *Alternaria* sp *Cladisporium* sp, *Mucor sp*	(Motomura *et al.*, 2003, Varga *et al.*, 2005, Amézqueta *et al.*, 2009)

Few reports have been discussed the mechanism of mycotoxins binding onto the LAB cell wall such as aflatoxins, zearalenone and fumonisin (Niderkorn *et al.,* 2009, El-Nezami *et al.,* 1998b, Haskard *et al.,* 2000, El-Nezami *et al.,* 2002a).

Haskard *et al.* (2000) reported that the surface components of these bacteria are involved in the binding mechanism**.** The stability of the complexes formed between the bacterial cell wall and aflatoxin B1 was dependent on the strain, treatment, and environmental conditions. Moreover, these results were consistent with AFB1 binding predominantly to carbohydrate bacterial components in the cell walls of *L. rhamnosus* strain GG. In addition, Haskard and co-authors (2001) demonstrated the reversibility of binding by washing the bacteria off. It was proposed that AFB1 was bound to the bacteria by weak non-covalent interactions. Furthermore, Lahtinen *et al.* (2004) reported that exo-polysaccharides can be ruled out as a possible factor in AFB1 binding for fumonisin and zearalenone removal by LAB binding, it is likely that carbohydrates and proteins may be involved in this phenomenon (El-Nezami *et al.,* 2002a, Niderkorn *et al.,* 2009). However, an understanding of the mechanism of AFB1, OTA and PAT removal would help in optimizing decontamination processes. Further investigations to assess the real effect of LAB on mycotoxins bio-availability and toxicity, such as investigating the ability of LAB to remove combination mycotoxins from *vivo,* are necessary.

1.2.6.4.2 - BACTERIA ISOLATED FROM ENVIRONMENT SOIL/WATER

Early studies demonstrated the ability of several microorganisms to remove aflatoxin *in vitro*. Only one bacterium, *Flavobacterium aurantiacum*, from the many microorganisms examined, including yeasts, moulds, and bacteria, was able to remove aflatoxin B1 from both solid and liquid media(Ciegler *et al.,* 1966a). However, the mechanism was unclear, and it

was not possible to distinguish between enzymic biodegradation/modification or just simple binding to the cells.

On the other hand, more recent studies indicated that the factor responsible for degradation of Aflatoxin B1 by extract of *F. aurantiacum* may be a protein or an enzyme (Bata and Làsztity, 1999). Recently there have been reports of aflatoxin detoxification using microorganisms such as *Rhodococcus sp* and *M. fluoranthenivorans* sp.nov. DSM44556T to detoxify the toxin (Teniola *et al.,* 2005).

Teniola and co-authors (2005) observed a dramatic reduction of AFB1 when incubated in the presence of *R. erythropolis* cells (17% residual AFB1 after 48 h and only 3–6% AFB1 after 72 h). Moreover, aflatoxin B_1 was effectively degraded (more than 90 % after only 4 h incubation at 30º C) by cell-free extracts of *R. erythropolis* cells and *M. fluoranthenivorans* sp.nov. DSM44556T. Alberts *et al.,* (2006) have reported that the degradation process was enzymatic, and the enzymes responsible for AFB1 degradation were both extracellular and constitutively produced.

1.2.6.4.3 - YEAST

Saccharomyces cerevisiae constitutes one of the most important microorganisms involved in food fermentations throughout the world. Fermentation is used as a means of food processing and preservation. Many yeast species, especially *S. cerevisiae*, play a predominant role in food fermentation along with lactic acid bacteria (Jespersen *et al.,* 1994). *S. cerevisiae* has been shown to remove aflatoxin and this has been explained by binding to the cell wall (Shetty *et al.,* 2007). The cell wall of *S. cerevisiae* consists of a network of β-1, 3 glucan back bone with β-1,6 glucan side chains, which is in turn attached to highly glycosylated mannoproteins which make up the external layer (Kollár *et al.,* 1997).

These proteins and glucans provide numerous easily accessible potential binding sites with different binding mechanisms, such as hydrogen bonding, ionic or hydrophobic interactions (Huwig *et al.*, 2001). Binding of different mycotoxins such as aflatoxin, ochratoxin and zearalenone to the yeast cell surface has been reported and the binding has been attributed to cell wall glucans in the case of ochratoxin and zearalenone (Shetty *et al.,* 2007). Moreover, feeding of *S. cerevisiae* to poultry showed beneficial effects against aflatoxin-induced toxicities (Stanley *et al.,* 1993). When dried yeast and yeast cell walls were added to rat feeds along with aflatoxin B1, a significant reduction in the toxicity was observed (Santin *et al.,* 2003; Baptista *et al.,* 2004).

Different species of yeast were able to detoxify OTA (Molnar *et al.*, 2004, Péteri *et al.,* 2007, Bizaj *et al.,* 2009). According to Péteri *et al.,* (2007) two strains of astaxanthin-producing *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* where able to detoxify OTA, while in the case of patulin, the toxin was unstable, as it was completely degraded during yeast fermentation. However, this reduction resulted in the production of two major compounds E-ascladiol and isomer Z-ascladiol. E-ascladiol is itself a mycotoxin, but has reduced toxicity compared with patulin (Moake *et al.,* 2005).

1.2.6.5 – MOULDS

Various fungi such as *Aspergillus niger*, *Eurotium herbariorum*, a *Rhizopus* sp. and non-aflatoxigenic *A. flavus*, were reported that could convert AFB1 to aflatoxicol. Hamid and Smith (1987) reported that moulds were able to produce aflatoxin might also be able to degrade them; e.g. the carbonyl group in the cyclopentane ring of AFB1 was reduced to a hydroxyl, but the toxicity and mutagenicity of this product were not determined. The same investigators also found that certain acid-producing moulds could catalyze the hydration of

AFB1 to B2a (a less toxic product). In the case of those *Aspergillus* strains are both aflatoxin producers and able to degrade it, and peroxidase enzymes were shown to catalyse aflatoxin degradation (Smith and Harran, 1993).

Liu *el al*. (1998) investigated aflatoxin detoxification by using *a* multienzyme, which was isolated from mycelial pellets of *A. tabescens*. The results showed that AFB1 at an initial concentration of 16 mM was completely detoxified by the fungal multienzyme. The result of the Ames test indicated that the mutagenic activity of multienzyme-treated AFB1 was greatly reduced compared with that of untreated controls.

Shantha (1999) reported the isolation of a strain of *Phoma* sp. which not only inhibited production of aflatoxin but also degraded aflatoxin B1 present at levels as high as 50 mg 100 ml $^{-1}$ of culture medium. Motomura *et al.* (2003) tested the capability of an extracellular enzyme from the edible mushroom *Pleurotus ostreatus*s to degrade aflatoxin B1; the results showed high degradation and they suggest that the enzyme cleaves the lactone ring of aflatoxin. In contrast, Varga *et al.* (2005) have examined the ability of *Rhizopus* and *Mucor* to degrade OTA, AFB1, zearalenone and PAT in a liquid medium. While none of the isolates exhibited AFB1 degrading activity, OTA, zearalenone and patulin were decomposed by several isolates.

Cvetnić and Pepeljnjak, (2007) evaluated biotic interactions between some mould species and an active producer of aflatoxin B1 *Aspergillus flavus* NRRL 3251, co-cultured in yeast-extract sucrose (YES) broth. Twenty-five mould strains of *Alternaria* spp., *Cladosporium* spp., *Mucor* spp., *A. flavus* and *A. niger*, were used as bio-competitive agents. The results confirmed antagonistic interaction between all the strains tested. With *Alternaria* spp. and *Cladosporium* spp., aflatoxin B1 production decreased by 100 %, compared to levels in pure cultures of *A. flavus* NRRL 3251.

Several zygomycete fungi, mainly from the genus *Rhizopus*, were examined for their ability to degrade AFB1, OTA, PAT and zearalenone in liquid medium (Varga *et al.,* 2005). None of the isolates were able to degrade AFB1; but they could degrade OTA, PAT and zearalenone. Some papers have described OTA degradation to the much less toxic ochratoxin α in liquid culture by some *Aspergillus* and *Pleurotus* isolates or their enzymes (Abrunhosa *et al.,* 2002, Amézqueta *et al.,* 2009).

1.2.6.6 -ENZYMES PURIFIED FROM MICROBIAL SYSTEM

Specific enzymes derived from microbial systems have been used to degrade AFB1. The detoxification by enzymes avoids the disadvantage of using the microorganisms, which may, in addition to their degradative activity, change flavour or affect the nutritional value and acceptability of the product (Wu *et al.,* 2009). Alberts *et al.,* (2009) investigated the enzymatic degradation of AFB1 by white rot fungi in different media. AFB1 was treated with white rot fungal culture fractions, pure fungal laccase enzyme and recombinant laccase enzyme fractions. Decreases in fluorescence were recorded, and macromolecules could not be detected after treating AFB1 with laccase enzymes. This team suggested that fungal laccase enzymes target and changes the double bond of the furan ring of the AFB1 molecule.

Motomura *et al.,* (2003) isolated and purified novel aflatoxin-degrading preparations from *Pleurotus ostreatus.* AFB1 was treated with the culture supernatant from 19 mushroom strains. The supernatant from *Pleurotus ostreatus* showed aflatoxin-degrading activity, whereas other strains showed weak or no activity. The enzymes involved showed high activity at 25ºC with a pH of 4.0-5.0. Although fluorescence measurements suggested that the novel enzymes were able to cleave the lactone ring of aflatoxins, the degradation products of AFB1 were not investigated clearly.

In another investigation, Liu *et al.*, (2001) described the isolation and purification of an intracellular enzyme named aflatoxin-detoxifinzyme isolated from *Armillariella tabescens.* This enzyme had the ability to transfer AFB1 into difuran ring –opening AFB1, which was less toxic compared to AFB1. The optimum activity for the degradation was 35º C with pH of 6.8. This team had described earlier a multi-enzyme complex from *Armillariella tabescens,* and proposed a pathway for the degradation. AFB1 was first transformed into AFB1-epoxide, followed by hydrolysis of the epoxide to give the dihydrodiol. Then the ring would open in the subsequent hydrolysis step (Liu *et al.*, 1998).

It has been shown that OTA may be degraded or transformed into non-toxic compounds by using enzymes such as carboxypeptidase A and lipase (Stander *et al.,* 2000, Stander *et al.,* 2001). Ochratoxin A is cleaved by carboxypeptidase A to the non-toxic ochratoxin R (OTR) and L-*â*-phenylalanine (Stander *et al.,* 2001). *Aspergillus niger* was used as the source for Carboxypeptidase A and lipases (Amézqueta *et al.,* 2009). In addition, Péteri *et al.,* (2007) showed that a carboxypeptidase present in *Phaffia rhodizyma* can also degrade up to 90 % of OTA. An crude enzyme preparation and metalloenzyme have also been used to degrade OTA (Amézqueta *et al.,* 2009) see Table 1.4.

Table 1.4 - Enzymes purified from microbial system

Mycotoxins	Enzyme name	Optimum activity		References
		pH	Temp.º(C)	
Aflatoxin B1	Laccase,	6.5	30	Alberts *et al.*, 2009
	Aflatoxin-detoxifizyme(ADTZ)	6.8	35	(Liu *et al.*, 1997a, Liu *et al.*, 1997b, Liu *et al.*, 1998, Liu *et al.*, 2001)
	Extracellular aflatoxin degradation enzyme	4-5	25	(Motomura *et al.*, 2003)
Ochratoxin A	Carboxypeptidase A			(Stander *et al.*, 2001)
	Lipases			(Stander *et al.*, 2000)
	OTA hydrolytic enzyme	7.5	37	Abrunhosa and Venancio, 2007

1.2.6.7 – PROTOZOA

A few papers showed that cells of *Tetrahymena pyriformis* W had the ability to degrade AFB1 into the less toxic compound aflatoxicol R_0 (about 25 % has been degraded in the first 30h). AFG_1 was more sensitive to this microorganism as about 80 % of the toxin was degraded (Wu *et al.*, 2009).

1.2.6.8 - DEGRADATION OF MYCOTOXINS BY ANIMAL CELLS

The pure form of AFB1 is not mutagenic and its biotransformation in mammalian tissues is primarily accomplished by microsomal cytochrome P450 monooxygenases. The P450 enzymes and their sub-families are found at different concentrations in most tissues of various animal species with abundance generally highest in the liver. Four metabolic pathways for AFB1 include *O*-dealkylation to AFP1, ketoreduction to aflatoxicol, epoxidation to AF-B1-8,9-epoxide (acutely toxic, mutagenic, and carcinogenic), and hydroxylation to AFM1 (acutely toxic), AFP1, AFQ1, or AFB2a, which are considerably less toxic than the parent compound. Therefore, the main reactions in aflatoxin metabolism are hydroxylation, oxidation and de-methylation (Kuilman *et al.*, 2000, Wu *et al.*, 2009).

1.3 - REGULATION OF MYCOTOXINS IN FOODS AND FEEDS

Efforts have continued internationally to establish guidelines to control mycotoxins. A range of legislative controls have been set up on mycotoxins in foods and feeds worldwide; for example, the European Union set the maximum levels of aflatoxins in agricultural commodities at 4 ppb, while for AFB1 the maximum level was set at 2 ppb. In the USA slightly higher concentrations were set see Table 1.5.

Table 1.5 - European Union and US Food and Drug Administration maximum levels for mycotoxins µg/kg (Richard, 2007).

Commodity	**EU**				**US**			
Human food	AFB1	AFs	OTA	PAT	AFB1	AFs	OTA	PAT
All products	2	4-15	3-10	50	5	20	-	50
Milk AFM1	-	0.05	-	-	-	0.5	-	-
Animal feed	-	10-50	10	-	-	20-300	-	-

According to FAO (2004), there are several scientific and socio-economic factors which may influence the establishment of mycotoxin limits and regulations. These include the availability of toxicological data; availability of data on the occurrence of mycotoxins in various commodities; knowledge of the distribution of mycotoxin concentrations within a lot; availability of analytical methods; legislation in countries with which trade contacts exist; and need for sufficient food supply. Therefore, to establish guidelines to control mycotoxins is a challenge throughout the world, particularly in developing countries where the balance between sufficient food supply and the quality of food is an issue.

1.4 -QUALITATIVE AND QUANTITATIVE ANALYSIS.

A broad range of detection techniques used for practical analysis and detection of a wide spectrum of mycotoxins is available. However, because of the varied structures of these compounds it is not possible to use one standard technique to detect all mycotoxins, as each will require a different method. Therefore, depending on the physical and chemical properties, procedures have been developed around existing analytical techniques, which offer flexible and broad-based methods of detection (Turner *et al.*, 2009, Xu *et al.*, 2006).

1.4.1 - CHROMATOGRAPHIC TECHNIQUES

1.4.1.1 - THIN LAYER CHROMATOGRAPHY (TLC) & HIGH PERFORMANCE - TLC (HP-TLC)

TLC was an extremely powerful, rapid and inexpensive separation technique in mycotoxins analysis before HPLC became popular; which offers the ability to screen large numbers of samples economically. The use of TLC analysis for mycotoxins is still popular for both quantitative and semi-quantitative purposes. This is due to its high throughput of samples, low operating cost and ease of identification of target compounds, using UV-VIS spectral analysis (Xu *et al.*, 2006). Several methods have been developed for mycotoxin analysis involving one-dimensional and two-dimensional TLC. One of the main requirements prior to TLC analysis is the clean-up step. The clean-up protocol depends on the nature of the toxin. Nevertheless, due to low sensitivities and poor recoveries, these TLC methods are more suitable for qualitative analysis. A good review of the applications of TLC for mycotoxins analysis can be found in (Turner *et al.*, 2009).

1.4.1.2 - HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC)

Due to the significant health risks associated with the presence of mycotoxins in foods, and also to satisfy the stringent legal limits, it is important to use efficient techniques and accurate methods for the detection of aflatoxins in foods and feeds. Mycotoxin determinations have been performed using relatively straightforward HPLC methods with isocratic elution and detection by UV or florescence spectrophotometry. Different derivatization techniques may be used to increase the response, e.g. post-column derivatization by adding bromine or iodine to increase the fluorescence of mycotoxins (Turner *et al.*, 2009).

Normal and reverse phase HPLC have been used for mycotoxin separation and purification of the toxin depending on their polarity. The most commonly found detection methods are UV or fluorescence detectors, which rely on the presence of a chromophore in the molecule. A number of toxins already have natural fluorescence (e.g. OTA, AFT, citrinin) and can be detected directly in HPLC–FD (Turner *et al.*, 2009). There has been increasing interest in using liquid chromatography coupled with mass spectrometry (LC-MS) to develop reliable confirmation protocols for very low levels of mycotoxins (Shephard and Leggott, 2000).

1.4.1.3 -GAS CHROMATOGRAPHY (GC)

Gas chromatography used regularly to identify and quantify the presence of mycotoxins in food samples and several protocols have been developed for these materials. Normally the system is linked to MS, flame ionisation detector (FID) or Fourier transform infrared spectroscopy (FTIR) detection techniques in order to detect the volatile products. Mycotoxins are not volatile and therefore have to be derivatised for analysis using GC. This detection method is not expected to be of use in commercial protocols due to the high cost and existence of cheaper and faster methods such as HPLC. In addition, GC detection faces

several disadvantages in mycotoxin detection such as limited use for the volatile samples, and thermal stability of some samples (Turner *et al.,* 2009).

1.4.2 - BIOASSAY TECHNIQUE

There are many immunochemical methods used for mycotoxins analysis. Immunochemical methods with both polyclonal and monoclonal antibodies have been developed for determination of mycotoxins. Monoclonal antibodies are more complicated to produce than polyclonal antibodies (Turner *et al.,* 2009). However, monoclonal antibodies are preferred because they have uniform affinity, specificity, and can be produced, repeatedly for commercial manufacture of immunoaffinity columns (Turner *et al.,* 2009).

The immunological methods that have been used since the 1990's are accepted as official methods for mycotoxins determination in some food commodities (Rahmani *et al.,* 2009) Immunoassay is an analytical technique (ELISA) which has been used routinely for mycotoxin analysis, with test kits commercially available (Rahmani *et al.,* 2009). ELISA is available for qualitative and quantitative analysis (Turner *et al.,* 2009). However, the disadvantage with ELISA techniques for aflatoxin analysis is that AFB1, AFB_2, AFG_1, AFG_2 are not generally quantified individually (Turner *et al.,* 2009, Rahmani *et al.,* 2009).

One of the development methods is the Immunoaffinity columns (IAC), which offers the best potential for efficient clean-up. Immunoaffinity columns provide a rapid and efficient method of sample clean-up prior to detection of aflatoxins by their fluorescence properties (Candlish *et al.,* 1988). The immunoaffinity technique enables a wide variety of food matrices to be analysed using a one- step extraction protocol without the need to use halogenated hydrocarbon solvents for extraction (Garner *et al.,* 1993).

CHAPTER 2

ADSORPTION OF AFLATOXIN B1 BY PROBIOTIC BACTERIA

2 – INTRODUCTION

Mycotoxins in food and feed may be removed, detoxified or transformed by physical, chemical or microbiological agents. Aflatoxins detoxification may involve binding to the microorganism cell wall, intracellular accumulation of the toxin or transformation of the toxin into a less toxic compound (Elgerbi *et al.,* 2006).

Published studies have reported that some lactic acid bacteria are able to remove the aflatoxin B1 (AFB1) from contaminated media in vitro and vivo by adhesion of the toxin to the bacterial cell walls. El-Nezami *et al*. (1998b) indicated that gram positive bacterium is able to remove AFB1 from contaminated media and the removal was a rapid process; on the other hand, the gram negative bacteria were weak in removing it from contaminated media. El-Nezami *et al*. (2000) studied the adhesion capability of *Lactobacillus rhamnosus* strain GG and LC 705 and *Propionibacterium freudenrichhi* ssp. *shermani* JS with aflatoxin**.**

Another suggestion was by Sreekumar and Hosono, (1998) that *Lactobacillus grasseri* has binding receptors for heterocyclic amine. Furthermore, Haskard *et al.,* (2000) reported that the surface components of the cell walls of these bacteria involved binding. In order to understand this phenomenon, Bueno *et al.,* (2006a) proposed a theoretical model to explain AFB1 adsorption by lactic acid bacteria. This model suggested that AFB1 molecules attached to the bacterial surface and the two processes that should be considered in this mechanism are the adsorption and desorption of AFB1 to the binding site on the surface of the microorganism (Lee *et al.,* 2003, Bueno *et al.,* 2006a). This model allows the number of AFB1 binding sites

to be estimated; the capacity of lactic acid bacteria to remove mycotoxin was strain, bacterial concentration, pH and temperature dependent (Bueno *et al.,* 2006a).

2.1 - CELL WALL COMPOSITION AND CHEMISTRY

Lactic acid bacteria are a heterogeneous group of bacteria, which have a typical Gram-positive cell wall structure, see (Figure 2.1). Briefly, the LAB cell wall consists of the peptidoglycan matrix as the major structural component, with other components such as teichoic and lipoteichoic acids, the proteinatious S layer and neutral polysaccharides. These components have various functions including adhesion and macromolecular binding, especially the fibrillar network of teichoic acids and neutral polysaccharides (Delcour *et al.,* 1999).

2.2 - SUGGESTED MECHANISMS FOR AFB1 BINDING BY LACTIC ACID BACTERIA

A small number of publications were interested in investigating the mechanism of AFB1 adsorption. A number of suggestions for this phenomenon can be found in the literature so far. Haskard *et al.,* (2001) suggested that AFB1 is bounded predominantly to carbohydrate bacterial components of *L. rhamnosus* strain GG by weak non-covalent interactions, such as associating with hydrophobic pockets on the bacterial surface. In contrast, Lahtinen *et al.* (2004) reported that exopolysaccharides can be ruled out as a possible binding component. In addition, enzymatic degradation studies showed the role of proteins still appears to be significant (Haskard *et al.,* 2000 2000).

Recently, Hernandez-Mendoza *et al.,* (2009) reported that teichoic acids are involved in AFB1 binding by probiotic bacteria. However, the adsorption mechanism of

AFB1 is not yet clearly understood; therefore, understanding the basic mechanism of AFB1 adsorption will have many implications in future research on lactic acid bacteria and their ability to reduce the risk of cancer. This study investigated for the first time the role of lipoteichoic acids on AFB1 adsorption.

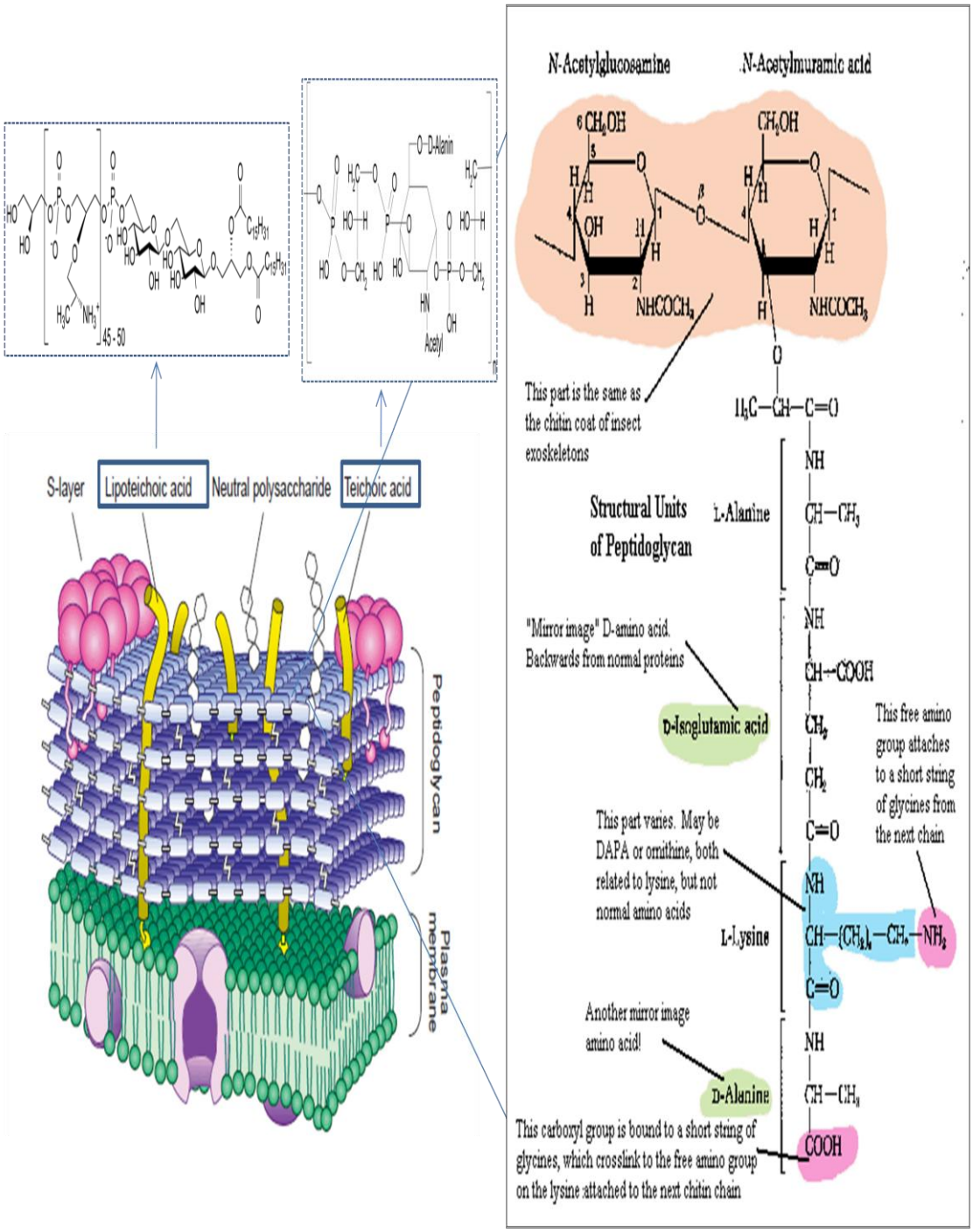


Figure 2.1- Structural Units Of Peptidoglycan (Delcour *et al.*, 1999)

2.3 - AIM OF THIS RESEARCH

The first aim of this study was to examin the potential of probiotic bacteria for detoxifing AFB1. The second was to understand the mechanism of AFB1 adsorption into/onto the probiotic cell wall and to optimize the physical conditions for toxin adsorption by using probiotic bacteria and investigating the role of lipoteichoic acids on AFB1 adsorption.

2.4 - MATERIAL AND METHODS

2.4.1 - CHEMICAL AND REAGENTS

All chemical and reagents presented in this chapter were purchased from Sigma-Aldrich, Dorset, UK; Aflatoxin B1 concentration 10 mg from *Aspergillus flavus*, Man, Rogosa and Sharpe broth (MRS), Mutanolysin, lysozyme, proteinase k, trypsin, pepsin, lipase, protease, αchemotrypsin, Sodium hydroxide (NaOH), Hydrochloric acid (HCl), Sodium dodecyl. Sulphate (SDS), Trichloroacetic acid (TCA), Sodium metaperiodate ($NaIO_4$), Tris (hydroxymethyl) aminomethane hydrochloride (THAM), Sodium chloride (NaCl), DNase RNase and Lipotichotic acid from *Staphylococcus aureus* was purchased from (Sigma-Aldrich, Dorset, UK).

2.4.1.1 – PRECAUTIONS

AFB1 is a very toxic and carcinogenic substance. To prepare aflatoxin standards, a fume hood was used. In addition, all pieces of glassware used were soaked in 10 % (w/v) NaOH for several hours and then were washed and dried.

2.4.1.2 - STANDARD PREPARATION

Ten milligram individual aflatoxin in crystalline form were dissolved by 10 ml methanol to final concentration 1mg/ml. A serial dilution was made (10-500 µg/ml) and stored at 4ºC. The actual concentration of aflatoxin was determined using a U.V. Spectrophotometer (BioMate 5, Thermo Scientific, Hemel Hempstead, Hertfordshire, UK) at 362 nm, and blanked against methanol. Readings were made in triplicate. The following equation was used:

Equation 2.1: AFLATOXIN B1 µg/ml = ABSORBANCE 362 × 1000 × COEFFICIENT.

The extinction coefficient for the AFB1 is 14.31 (R-biopharm Rhône LTD., Glasgow, UK).

2.4.2 -STRAINS OF LACTIC ACID BACTERIA

Lactobacillus plantarum 4374 was supplied as a lyophilized culture by Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), University of Strathclyde, Glasgow, UK. *Lactococcus lactis sub* lactis 6681, *Lactobacillus bulgaricus, Lactobacillus acidophilus 1748, Bifidobacterium bifidum* were supplied by Hannah Research Institute, Ayr, Scotland. NCIMB, Scotland LTD, Aberdeen, Scotland. The bacteria were cultivated in MRS broth at 37 ºC 200 rpm for 24h. The bacterial numbers were determined by plate count method by using the selective media de Man, Rogosa and Sharpe Agar and incubated at 37 ºC for 48 h in a modified atmosphere 5% CO_2 by using Oxide jars. Bacterial counts were expressed as colony-forming units (CFU) per ml/ media.

2.4.3 - CULTURE PRESERVATION

All cultures were Gram stained. Biochemical tests were carried out by using IP50 media (BioMérieux sa, Marcy 'Etoile, France) to confirm the identity of the strains. Two techniques, freeze-drying and freezing, were used to preserve the cultivated lactic acid bacteria in this study. The 24h old cultures of all the strains were lyophilized and stored at 4 ºC or maintained in 20% glycerol (Sigma-Aldrich, Dorset, UK) and stored at – 80 ºC in ampoules. The lyophilized cells were serially diluted and tested for viability and purity on MRS agar. All lyophilized cultures were found to be pure cultures. The cultures were viable in a range of 3×10^8 to 2×10^{10} cells /mg.

2.5 - CELL WALL ISOLATION AND PURIFICATION

The cell walls were purified as described by Sreekumar and Hosono (1999). *Lactobacillus plantarum 4374* was cultivated by using MRS medium. Bacterial cells were pelleted by centrifugation 10 000× g for 10 min at 4ºC, washed by 20 ml of cold saline solution pH7, and centrifuged again. A slight modification for the original methods has made as fellowing; the packed cells were re-suspended homogeneously in 20 ml of cold distilled water, and the cells were disrupted using a high-pressure cell homogeniser (Model 4000, Constant Systems Ltd., Warwick, UK)**.** Samples were pipetted into the piston chamber of the homogeniser, and the collection cup assembly was fitted. The whole cells and debris were removed by centrifugation at a low speed of 1000×*g* for 10 min at 4ºC. Cell walls were sedimented by centrifugation at 15 000×*g* for 25 min at 4ºC. The cell walls were placed on MRS agar plates which were then incubated at 37ºC 5% CO_2 for 48h.

The plate did not show growth; indicating the complete disruption of the cells and the sterility of the suspension. The cell walls were washed a further twice in 15 mM NaCl, followed by a wash in 50 mM Tris hydroxymethyl aminomethane hydrochloride, and then diluted with twice the volume with 10 mM phosphate buffer, pH 7.0. RNase and DNase were added to a final concentration of 50 mg/ml each. The mixture was incubated at 37ºC for 90 min and then centrifuged at 15 000×g for 25 min. The washed cell walls were treated with 20 g/L of sodium dodecyl Sulphate (SDS) and heated at 70ºC for 2 h to remove the membrane. The cell walls were washed extensively with distilled water to remove SDS and the residue collected by centrifugation, lyophilized and used as the purified cell wall preparation.

2.5.1 -PRE-ENZYMATIC TREATMENT OF THE CELLS AND CELL WALLS

3mg of Lyophilised cells or 3mg of cell walls of *L. plantarum strains* TRS10239 were suspended in phosphate buffer saline pH 7.0. and digested with mutanolysin (5000 units/ml), lysozyme (45000 units/mg), proteinase k ≥30 units/mg from *Tritirachium album* 1 mg/ml, trypsin from bovine lung 1 mg/ml pepsin (800-2500 units / mg) mg/ml and α-chemotrypsin from bovine pancreas (40 ≥units/mg) 1 mg/ml, Lipase from *Aspergillus niger* 200 U/g (1mg/ml) at 37ºC in a shaking water bath 120 rpm for 4 h the lysis of the cells monitored by measuring the decrease in absorbance at 600 nm. The reaction was terminated by boiling the mixture for 5 min and the resulting walls or cells were centrifuged (10000 × g 10 min, 4º C). The remaining pellets were washed once by H_2O then subjected to (AFB1) binding. Table 2.1 summarises the enzymatic treatment and the target component in the cell wall.

Table 2.1- Effect of enzymatic treatment on the target component in cell wall

Treatment	Target	Effect on target component
Lipase	Lipids	Digest lipoteichoic
Lysozyme	peptidoglycans	Break between N-acetylmuramic acid / N-acetyl-D-glucosamine
Mutanolysin	peptidoglycans	Hydrolysis the peptidoglycan
Pepsin	Proteins	Break between Phe/Tyr
Protease	Proteins	Hydrolysis peptide bonds in polypeptide chain
Proteinase	Proteins	Digest the protein
Trypsin	Proteins	Break between Arg/Lys-X
α- chemotrypsin	Proteins	Break between Phe/Tyr/Try-X

2.5.2 -PRE-CHEMICAL TREATMENT

Two milligram lyophilised cells or three milligram lyophilised cell walls of *L. plantarum* strains TRS were treated by one of the following methods: distilled water (H_2O) 100ºC for 15 min, 1N NaOH at 100ºC for 15 min, 1M HCl 100ºC for 15 min, 0.1M Sodium dodecyl sulphate (SDS) for 15 min and incubated at 37 ºC and 150 rpm and 10% w/v Trichloroacetic acid (TCA) incubated at 37 ºC and 150 rpm for 15 min. Sodium metaperiodate ($NaIO_4$) 50 mM treatment was carried out at 4ºC for 24 h and at the end of the reaction, one drop of ethylene glycol was added to destroy the excess of metaperiodate present in the reaction mixture. The divalent cations $Cacl_2$ (Ca^+) and $Mgcl_2$ (Mg^+) at concentration 0.1mM used to pretreat the cells or cell walls and incubated at 35ºC for 24h. After each treatment, the cells or cell walls were centrifuged 10000 × *g*, for 10 min. The remaining pellets were washed once in distilled water, re-suspended in phosphate buffer saline and used for AFB1 adsorption assay. Table 2.2 summarises the chemical treatment and the target component in the cell wall

Table 2.2 - Effect of chemical treatment on the target component in purified cell wall

Treatment	Target component	Target component
NaIO4	Oxidation of cis OH group to aldehyde and carbon acid group	Carbohydrate
SDS	Proteins	Denaturation
$Cacl_2$	Cation binding	Provide cations
$Mgcl_2$	Cation binding	Provide cations
HCL	Hydrolysis carbohydrate	May change or degrade the sites for the binding
TCA	Hydrolysis carbohydrate	May change or degrade the sites for the binding
NaOH	Proteins	Denaturation
H2O	Proteins and carbohydrate	Denaturation

2.6 -AFB1 ADSORPTION ASSAY

A range of concentrations of AFB1 (10 – 500 PPM) was prepared in phosphate buffer saline (PBS), pH 7.3 and the methanol evaporated by heating in a water bath at 80º C for 15 min. 2 mg of lyophilized cells were suspended in 0.8 ml of PBS (pH 7.3) containing different concentrations of AFB1 and incubated at 35 ºC for 1h. To optimize the AFB1 adsorption condition, a set of temperatures was used 15-35 ºC for different incubation periods for 96h. After the incubation, the bacterial suspensions were centrifuged (10000 × g 10 min, 4º C) and the supernatant was stored at -20 until analyzed by HPLC.

2.7 - EFFECT OF LIPOTEICHOIC ACID ON AFB1 ADSORPTION

Lipoteichoic acid from *Staphylococcus aureus* (Sigma-Aldrich, Dorset, UK) different concentrations of Lipoteichoic acid (200-1000µg/ml) in 50mM PBS, pH 7

were used to examine the ability of Lipoteichoic acid to remove AFB1 from contaminated media.

2.7.1 -AFB1 QUANTIFICATION BY HPLC

Reverse phase HPLC the system consist of 2 x model 306 pumps, a model 234 auto-injector system and a model 170 DAD detector controlled by Unipoint V3 software. The analysis was performed through a guard column phenoomonix (C6-phenyl 4.0 × 3.0 mm ID) followed by an RP-C18 ACE column, 5µm, 150 × 4.6 mm (Hichrom limited, Berkshire,UK). A mixture of acetonitrile: methanol: water (1:1:2, v/v/v) was used as the mobile phase at a flow rate of 1 ml/min. Injection volume was 20 µl. The sample temperature was controlled at 40ºC by using column heater model 7971 (Jones chromatography, Hertfordshire, UK). A diode array detector was used to measure AFB1 by UV detection at wavelength 365 nm. The data was collected and processed by Gilson unipoint LC system software. The remaining AFB1 percentage was calculated using the following equation:

EQUATION - 2.2:

$$\text{AFB1 BINDING (\%)} = \left[1 - \left(\frac{\text{AFB1 PEAK AREA IN TREATED SAMPLE}}{\text{AFB1 PEAK AREA IN CONTROL}}\right)\right] \times 100$$

2.8 -EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

SigmaPlot 10 used to plot part of the data. Statistical analyses were used to determine the influence of the physical factor on AFB1 adsorption. The adsorption percentage was analyzed by one way ANOVA and modified least significant difference (Bonferroni *t* test) was used to identify whether differences between means were significant. The analyses were performed by using GraphPad Prism 5 at 95 % confidence.

2.9 - RESULTS

Table 2.3 shows the bacterial concentration by colony forming unit (CFU) per ml of mg probiotic lyophilized cells. The cell number was beteen 3×10^{8} CFU per ml of mg lyophilized cells and 4×10^{10} per ml of mg lyophilized cells. All the Probiotic strains were tested for their binding properties with AFB1. All the strains were able to remove AFB1 from contaminated media.

Table 2.3- Bacterial concentration by using plate count agar lyophilized cells

Strains	**Cell count (CFU/ml)**	**Source**
L. bulgaricus NCFB 1489	2×10^{10}	HRI, Scotland
L.acidophilus NCFB 1748	3×10^{8}	HRI, Scotland
L.plantarum TRS 4374	5×10^{9}	SIPS, Scotland
L. lactis NCIMB 4481	4×10^{10}	NCIMB, Scotland
B .bifidum NCIMB 0795	1×10^{9}	NCIMB, Scotland

Hannah Research Institute, Ayr, Scotland, UK.
NCIMB, Scotland LTD, Aberdeen, Scotland, UK.

Figure 2.2 shows the effect of different concentrations of probiotic bacteria on binding of AFB1. Clearly, all strains have the ability to absorb AFB1 from a contaminated culture the effect of bacterial concentration on the percentage of removal was obvious; variations existed among the strains in AFB1 binding as the bacterial concentration was increased as the percentage of AFB1 increased. Two way ANOVA showed overall a significant difference ($P < 0.05$) between treatments; the stains showed different ($P<0.05$) absorption properties at 1mg of cells. The difference was not significant ($P>0.05$) when cell concentration was increased to 2mg. An increase to more than 2mg had no significant difference ($P>0.05$). Only *L. bulgaricus NCFB 1489* and *B .bifidum NCIMB* 0795 were statistically different ($P<0.05$) see Appendix 9.3.

The relationship between AFB1 concentration and the percentage of adsorption is presented in Figure 2.2. Two milligrams of cells were treated with different concentrations of AFB1 at 35ºC for 24h. The graph shows a direct proportional relationship at lower concentrations of AFB1 up to 100 (µg/ml). A sharp decrease was recorded at a higher concentration 100 µg/ml then the relationship started to inverse when AFB1 concentration increased to 500µg/ml.

AFB1 removal percentage was 80% to 97 %. Statistically, overall a significant difference ($P=0.0139$) was found. In meanwhile, lower concentrations of AFB1 showed no significant difference ($P>0.05$) between the strains, while at higher concentrations a significant difference ($p< 0.05$) was found see Table 2.4. To optimize the conditions of AFB1 adsorption, different temperatures (15-35ºC) were used.

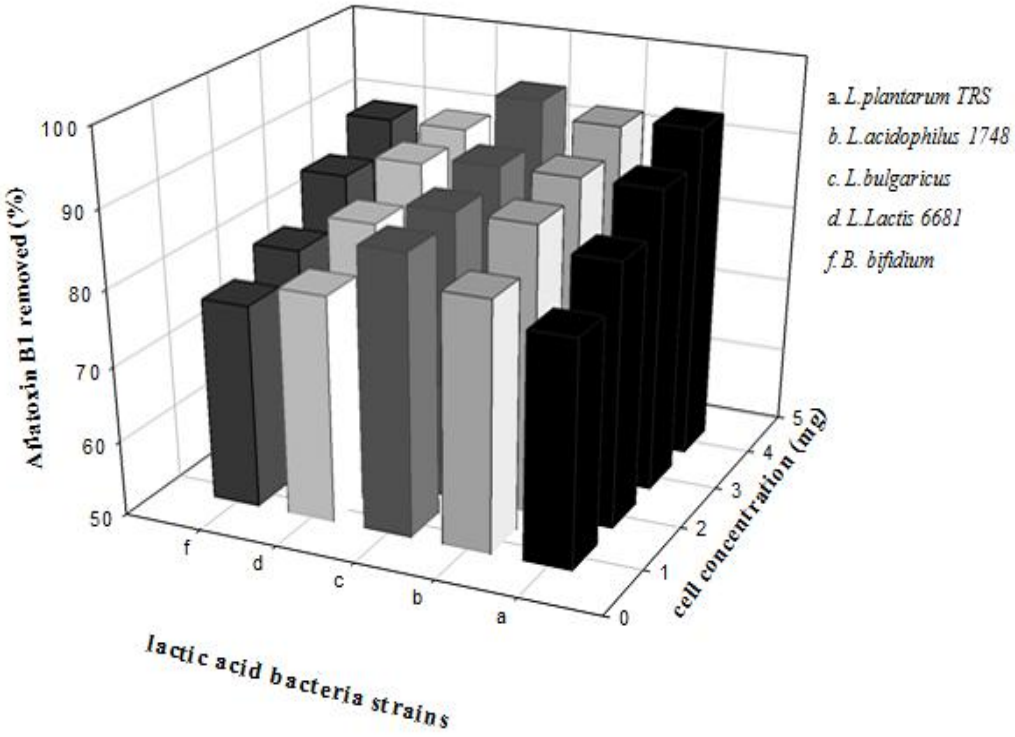


Figure 2.2- Effect of different concentrations of probiotic bacteria on the binding of AFB1. 1-4 mg of lyophilized cells was treated with 100 (µg/ml) of AFB1 at 35ºC for 24h.

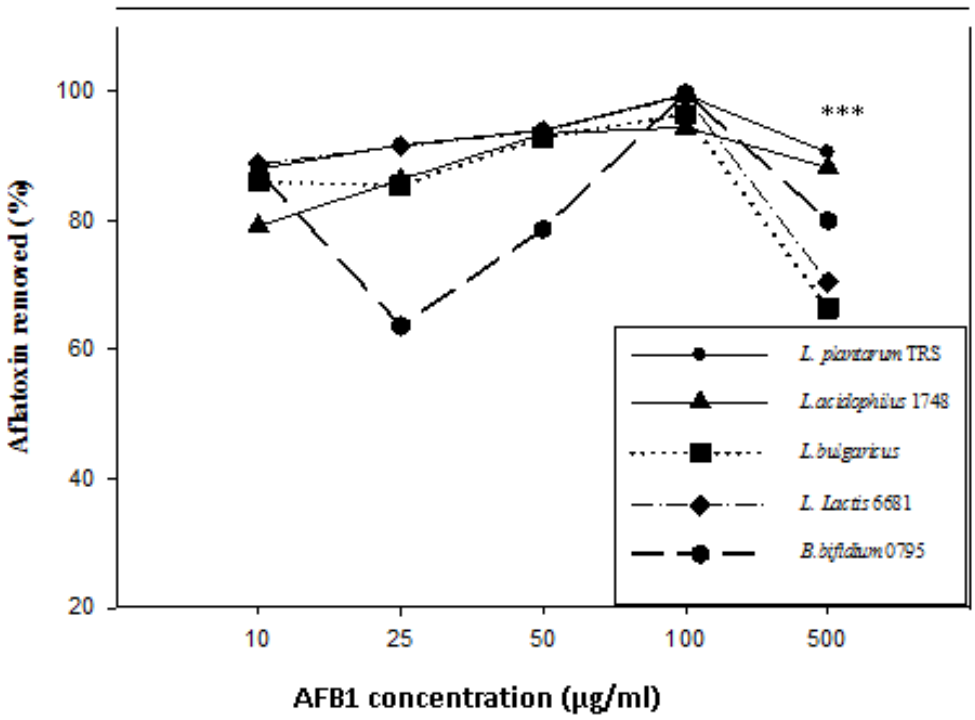


Figure 2.3 - AFB1 removal by five strains of probiotic bacteria. *L.plantarumTRS, L. acidophilus, L. bulgaricus, L. lactis* and *B. Bifidumchange.* Two milligrams of cells were treated with different concentrations of AFB1 at 35ºC for 24h.

Table 2.4 - Statistical Analysis for effects of different concentrations of AFB1 on percentage of absorption

ANOVA Table	**SS**	**Df**	**MS**	**R^2**	**F**	**P**
Treatment	1019	4	254.8	0.4501	4.093	0.0139
Residual	1245	20	62.24			
Total	2264	24				

Table 2.5 shows AFB1 adsorption percentage by lactic acid bacteria from contaminated phosphate buffer saline (PBS), over 96 hr of incubation at 15ºC. All strains were able to remove AFB1 from contaminated media. *L.plantarum TRS 4374* and *L. lactis NCIMB 4481* were the most efficient strains for removing AFB1 from contaminated media at 15ºC while at 25ºC *L.plantarum TRS 4374 and L. bulgaricus NCFB 1489* were the most effective strains to remove AFB1 (see Table 2.4). Other bacteria such as *L. lactis 4481* and *B. bifidum* 0795 were also able to remove AFB1.

Table 2.6 shows that *L.plantarum TRS 4374 and L.acidophilus NCFB 1748* were efficient strains at 35 ºC. Therefore, *L.plantarum TRS 4374* was selected for further study. The optimum temperature for adsorbing a high percentage of AFB1 was 35 ºC. The effect of the incubation time on AFB1 adsorption is presented at different incubation temperatures in Tables 2.5, 2.6 and 2.7. The toxin was adsorbed immediately after the addition of the cells. The strains showed different binding properties within an increase in the incubation time.

The most interesting result was noticed after 72h as some of the strains released the toxin. This result was recorded at all temperatures used in this study. *L.acidophilus NCFB 1748 and L. lactis NCIMB 4481* was released by 6% and 5 % of the absorbed toxin respectively ,when the cells were incubated at 15 ºC ; while at 25 ºC the only strain which released the toxin was *L. lactis NCIMB 4481* strain where 8.6% of the toxin was released. The percentage of release of the toxin was decreased when the toxin and the bacteria were incubated at 35 ºC.

Table 2.5 - AFB1 adsorption percentage by lactic acid bacteria from contaminated phosphate buffer saline (PBS), over 96 hr of incubation at 15ºC

Strains	**AFB1 adsorption (SD) / incubation period (hr)**				
	24	**48**	**72**	**96**	**Recovery**
			(%)		
L. bulgaricus	72.8 ± (1.4)	88.9 ± (0.4)	90.6 ± (4.4)	ND	94.5
L.acidophilus 1748	59 ± (0.45)	87.9 ± (0.19)	94.2 ± (0.1)	ND	94.0
L.plantarum TRS 4374	88.6 ± (0.30)	95.0 ± (1.9)	ND	ND	95.4
L. lactis 4481	88.5 ± (0.01)	85.2 ± (0.4)	83.0 ± (0.7)	84.4 ± (0.7)	87.3
B. bifidum 0795	76 ± (4.0)	86.6 ± (1.4)	71 ± (0.9)	89.2 ± (0.2)	93.4

The value of AFB1 adsorption represents the mean ± SD (Standard deviation) of three determinations. ND (none detected) under the experimental conditions. Toxin recovered from the pellet (%).

Table 2.6- AFB1 adsorption percentage by lactic acid bacteria from contaminated phosphate buffer saline (PBS), over 96 hr of incubation at 25ºC.

Strains	**AFB1 adsorption (SD) / incubation period (hr)**				
	24	**48**	**72**	**96**	**Recovery**
			(%)		
L. bulgaricus	91.1 ± (2.1)	80.2± (1.1)	92.5 ± (0.0)	ND	92.0
L.acidophilus 1748	75.0 ± (0.9)	62.2 ± (1.5)	81.1± (7.1)	76.0± (0.4)	93.8
L.plantarum TRS 4374	90.7± (1.9)	86.6 ± (1.9)	ND	ND	93.0
L. lactis 4481	78.1 ± (3.0)	84.6 ± (0.0)	75.8 ± (0.5)	87.0± (2.0)	89.1
B. bifidum 0795	75.6± (1.3)	87.8 ± (1.5)	ND	ND	94.3

The value of AFB1 adsorption represents the mean ± SD (Standard deviation) of three determinations. ND (none detected) under the experimental conditions. Toxin recovered from the pellet (%).

Table 2.7- AFB1 adsorption percentage by lactic acid bacteria from contaminated phosphate buffer saline (PBS), over 96 hr of incubation at 35ºC.

Strains	**AFB1 adsorption (SD) / incubation period (hr)**				
	24	**48**	**72**	**96**	**Recovery**
			(%)		
L. bulgaricus	87.7 ± (3.7)	80.2± (1.0)	91.3 ± (1.0)	87.5 ± (0.0)	93.3
L.acidophilus 1748	92.0 ±(0.01)	90.0 ± (0.2)	91.2 ± (1.8)	94.2 ± (2.0)	91.1
L.plantarum TRS 4374	91.8 ± (1.1)	97.6± (0.7)	ND	ND	95.4
L. lactis 4481	89.6 ± (1.1)	87.6 ± (0.4)	82 ± (1.3)	87.2± (0.1)	88.2
B. bifidum 0795	89.9 ± (1.5)	89.2± (0.1)	90.5± (0.5)	ND	90.1

The value of AFB1 adsorption represents the mean ± SD (Standard deviation) of three determinations. ND (none detected) under the experimental conditions. Toxin recovered from the pellet (%).

Table 2.8 shows the chemical treatment and the target component on the cell wall. All treatments showed a decrease in the percentage of AFB1 adsorption. The sodium meta-periodate ($NaIO_4$) treatment resulted in a reduction in the binding properties by 16 % to 26% within cells and cell walls respectively. Pre-treatment by trichloroacetic acid (TCA) reduced the AFB1 adsorption to 58% and 62% within cell walls and cells respectively. Moreover, the treatment pre of the cell wall by sodium dodecyl sulphate (SDS) reduced the binding to 42 and 69 % respectively (see Table 2.8). Meanwhile, pretreated cells or cell walls using trace elements $CaCl_2$ and $MgCl_2$ showed a decrease in the percentage of AFB1 removal 79.57 % and 70.48 % respectively compared to control 91.8 % after incubation at 35º C for 24h. Cells and cell walls pretreated with boiling water did show the ability of the removal; 95.1 and 87.35 % was bonded to pretreated cells and cell wall, but did not increase the ability of cells and cell walls compared to control sample in terms of removing AFB1 from contaminated media. Statistically all the treatments showed a significant difference ($P<0.05$) from the control see Table 2.9. Treatments with different letters in each column are statistically different by each treatment ($*P\leq0.05$).

Table 2.8 - Effect of chemical treatment on percentage AFB1 adsorption in the cells and cell walls of *L.plantarum TRS*

Treatment	Cell Walls	Cells
	Binding (%)	
Control	91.8 ± (0.09)a	98.6 ±(0.09)a
$CaCl_2$	79.57 ± (1.35)b	77.6 ±(0.12)b
H_2O	87.35 ± (0.86)a	95.1 ±(0.23)a
HCl	86.56 ± (0.047)c	85.3 ±(0.04)c
$MgCl_2$	70.48 ± (0.79)a	63.5 ± (0.2)d
$NaIO_4$	16.6 ± (0.09)d	26.2 ± (1.1)e
NaOH	90.83 ± (0.88)a	96.7 ±(6.2)a
SDS	92.42 ± (0.07)a	97.5 ±(1.9)a
TCA	58.75 ± (0.48)d	62.4 ±(13.9)d

The value of AFB1 adsorption represents the mean ± SD (Standard deviation) of three determinations. Treatments with different letters in each column are statistically different by each treatment from control sample (*P≤0.05).

Table 2.9- Statistical analysis one way ANOVA for the effect of chemical treatment on percentage of AFB1 adsorption

ANOVA Table	**SS**	**df**	**MS**	**F**	**R^2**	**P**
Cell Wall						
Treatment (between columns)	11830	8	1479	63.87	0.9827	<0.0001
Residual (within columns)	208.4	9	23.15			
Total	12040	17				
Cells						
ANOVA Table	**SS**	**df**	**MS**	**F**	**R^2**	**P**
Treatment (between columns)	10120	8	1265	47.89	0.9770	< 0.0001
Residual (within columns)	327.8	9	26.42			
Total	10360	17				

The enzymatic treatment had slightly less effect in terms of decreasing the percentage of the binding. Lipase showed higher activity compared to other enzymes used in this study. The percentage dropped from 95 % to 76 % when the purified cell wall was used; meanwhile the effect of lipase was lower when the whole cell was digested with lipase see Table 2.10. Statistically, Trypsin, protease, α-chemotrypsine, pepsin, mutanolysin and lipase were statistically different ($P < 0.05$) from control when the purified cell wall was employed, while only pepsin, α-chemotrypsine, protease and lipase when the cells were treated by the enzyme mentioned in Table 2.11. Treatments with different letters in each column are statistically different from contral sample by each treatment($*P \leq 0.05$).

Figure 2.4 show the effect of different concentrations of lipoteichoic acid on AFB1 removal. 200 (mg/ml) removed 48.5 % AFB1; meanwhile the 1000 (mg/ml) removed 81.8% of AFB1. Statistical analysis showed a significant difference ($P=0.0014$) between different concentrations of lipoteichoic acid on AFB1 removal. Tukey's Multiple Comparison Test confirmed that an increase in the lipoteichoic acid as a percentage of AFB1, removal was increased. (1000 mg/ml) of lipoteichoic acid was a statistically significant difference ($P < 0.05$ from other concentrations (see Table 2.12)

Table 2.1- Effect of enzymatic treatment on the protein composition and percentage AFB1 adsorption in the cells and cell walls of *L.plantarum TRS*.

Treatment	Cell walls	Cells
	Binding %)	
Control	95.8 ± (0.09)a	98.6 ±(0.09)a
Lysozyme	90.3 ±(0.63)b	94.1 ± (7.6)b
Trypsin	86.7± (0.66)c	90.6 ± (0.05)bc
Protease	89.9± (0.52)b	89.4 ± (5.8)c
αchemotrypsin	92.1± (0.51)b	89.6 ± (0.94c)
Proteinase	87.1± (0.98)c	95.5 ± (2.0)b
Pepsin	81.9± (0.47)d	89.5 ± (0.14)c
Mutanolysin	90.3 ± (0.85)b	95.3 ± (0.85)b
Lipase	76.5 ± (0.57)e	89.2 ± (0.94)c

The value of AFB1 adsorption represents the mean ± SD (Standard deviation) of three determinations. Treatments with different letters in each column are statistically different by each treatment in each column (*P≤0.05).

Table 2.2 - ANOVA analysis for enzymatic treatment

ANOVA Table	SS	df	MS	R^2	F	P
Cells						
Treatment (between columns)	435.6	8	54.45	0.9837	166.2	< 0.0001
Residual (within columns)	2.949	9	0.3276			
Total	438.5	17				
ANOVA Table	SS	df	MS	R^2	F	P
Cell wall						
Treatment (between columns)	501.9	8	62.73	0.9837	68.02	< 0.0001
Residual (within columns)	8.300	9	0.9223			
Total	510.2	17				

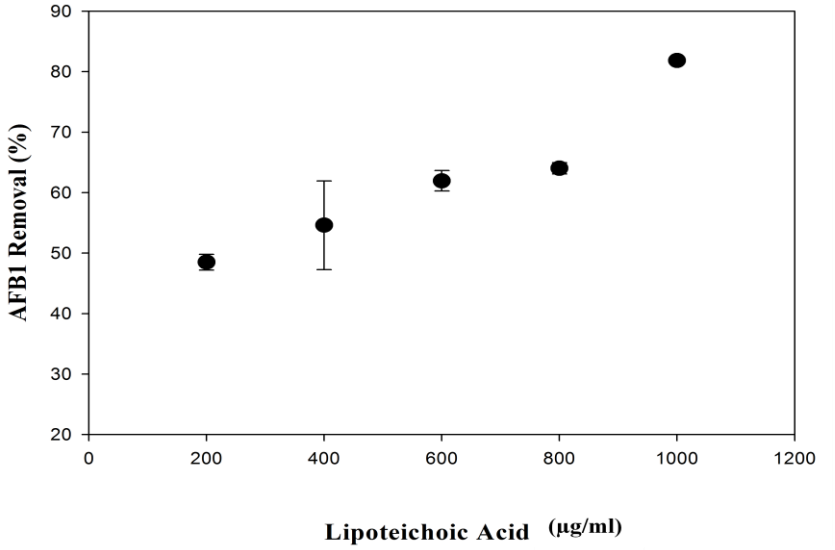


Figure 2.4- Effect of different concentrations of lipoteichoic acid on AFB1 removal. The lipoteichoic acids was treated with 100 (µg/ml) of AFB1 at 35ºC for 24h.

Table 2.3- One way ANOVA for effect of different concentrations of lipoteichoic acid on AFB1 removal

ANOVA Table	**SS**	**DF**	**MS**	**F**	**R2**	**P**
Treatment (between columns)	1275	4	318.7	26.93	0.9556	0.0014
Residual (within columns)	59.17	5	11.83			
Total	1334	9				

2.10 - Discussion

The binding of mycotoxin by lactic acid bacteria was reported more than a decade ago, and publications have suggested various theories of binding of AFB1 by cells and cell walls (El-Nezami *et al.,* 1998a, El-Nezami *et al.,* 2000, El-Nezami *et al.,* 2002a, El-Nezami *et al.,* 2002b, Haskard *et al.,* 2000, Haskard *et al.,* 2001, Lahtinen *et al.,* 2004).

Also it has been previously reported that bacterial concentration influences de AFB1 removal. According to Bolognani *et al.,* (1999) and El-Nezami *et al.,* (1998b) approximately a minimum of $2–5 \times 10^9$ CFU/mL is required for significant AFB1 removal 13–50%, while a concentration of 2×10^{10} CFU/mL is capable of reducing the AFB1 level to less than 0.1 and 13% . The basis for the observed strain to strain variation is unknown; however it is likely due to differences in the types, numbers, or availability of AFB1-binding sites. Therefore effect of different concentrations of probiotic bacteria in mg/ml of lyophilized cells on AFB1 binding was investigated in this present study. Higher concentration (4mg) showed the ability to remove more AFB1 but statistically was not significant difference from 2mg/ml.

The data obtained in this present experiment indicated that all the five strains tested effectively bonded AFB1. In addition, *L.plantarum* TRS was the most efficient strain for removing AFB1 from contaminated media. As a result, the removal was strain-dependent. These results are in agreement with El-Nezami *et al.,* (1998) who reported that both *Lactobacillus rhamnosus* strain GG (LBGG) and *L. rhamnosus* strain LC-705 (LC705) can significantly ($P > 0.05$) remove AFB1 when compared to removal by other strains of either Gram-positive or Gram-negative bacteria. Reports

in the literature indicate that bacteria of the same species ,including some genetically closely related strains , have small differences in either the structural design or the composition of the cell wall and these could modify the physiological properties of the cell wall and thus influence absorption of AFB1 (Hernandez-Mendoza *et al.,* 2009). Therefore the variation in AFB1 adsorption presented herein may be attributable to unique bacterial features. In this study, the percentage of removal of AFB1 was greater in binding capabilities than those reported previously for other LAB strains, which occurred when cultured under similar conditions e.g 91 % compared to (Lahtinen *et al.,* 2004) who had previously reported that the extent to which AFB1 is bound to *L. rhamnosus GG and* LC-705 is approximately 80 %, while (Hernandez-Mendoza *et al.,* 2009) reported that *Lactobacillus reuteri* strain NRRL 14171 and *Lactobacillus casei* strain Shirota were the most efficient strains for binding AFB1 as more than 81% was removed.

The different between presented result in this study and those mentioned may be because the cells used in the current study were used in a freeze-dried form which may affect the cell wall components and increase the ability to absorb AFB1. The effect of various AFB1 concentrations on AFB1 removal has been also tested. The amount of AFB1 removed increased with increasing concentration of AFB1 but the percentage removed was not significantly different. In contrast Ciegler *et al.,* (1966a) their result indicated the amount of AFB1 removed *Flavobacterium aurantiacum* increased with increase AFB1 concentration (3 mg/ml) but the percentage of AFB1 removal decreased. Thus the curve response removal toxin was investigated in this present study. The amount of AB1 removed increased with increasing AFB1 concentration from10 to 100 µg/ml; meanwhile, the ability to

remove the toxin decreased when the concentration reached a maximum of 500 µg/ml. The higher concentration was statistically different ($P<0.05$) from the lower concentration. This may be due to the limit of availability of free receptors on bacterial cell wall or may be the bacterial cell surface was saturated and were not available to attach the toxin.

El-Nezami *et al.,* (1998a) evaluated the role of temperatures (4, 25 and 37°C) over a period of 4 h during binding of AFB1 by LAB. Their result indicated that the removal was temperature dependent. 37°C was the optimum temperature for AFB1 removal was recorded in their experimental condition. The experimental results presented in this chapter indicated that AFB1 adsorption increased when the temperature was increased from 15 to 35 °C. These results support the conclusion that the adsorption is temperature dependent. The optimum AFB1 adsorption was achieved at 35ºC and 72 h.

Result presented in Table 2.5 indicated the percentage of removal of AFB1 from contaminated media increased with the time, but that *L. lactis 4481, L.acidophilus 1748 and L. bulgaricus* released some of the AFB1 back to the corresponding media in 72h of incubation then reabsorbed in 96 h of incubation. This result suggests that the interaction between the toxin and the cell wall constituents was not strong as the interaction was reversible. These results were in consistent with Peltonen *et al.,* (2001) who recorded that some of the LAB was not strong enough to bind AFB1 (*Lb. amylovorus* CSCC 5197 and *Lb. rhamnosus* Lc 1⁄3) and AFB1 was released back into the solution from the lactobacilli/AFB1 complex when the cells was washed by the buffer. In related work, Hernandez-Mendoza *et al.,*(2009) reported that AFB1 was

released after repeated washes with buffer. Despite the fact that El-Nezami *et al.,* (1998a) recorded the no significant difference was found between different incubation times (4, 24, 48, 72h). However, the only strain did not release the toxin was *L.plantarum TRS* under all the conditions used at our research.

However, previous researchers indicated that the anti-carcinogenic activity of LAB resides in the bacterial cell walls and fractions of cell walls (Hernandez-Mendoza *et al.,* 2009, Peltonen, 2001, Lahtinen *et al.,* 2004). Consequently, purified cell walls from *L.plantarum* TRS was used in this present study to investigate the mechanism of AFB1 adsorption by *L.plantarum TRS* cells and purified cell walls. The experimental data presented in this research indicated that purified cell walls were effectively adsorbed AFB1 from contaminated media. It is possible that unknown covalently bound cell wall components still be attached to peptidoglycan after the purification process. Hernandez-Mendoza, *et al.,* (2009) reported that cell wall integrity is important in binding AFB1.

Although the mechanism of binding of AFB1 by bacterial cells is not well understood, it is thought that the primary cellular components involved are peptidoglycan, as well as, cell wall polysaccharides and proteins (Lahtinen *et al.,* 2004). Additionally, it has been suggested that AFB1 is bound to the bacteria by weak, non-covalent interactions, such as association with hydrophobic pockets on the bacterial surface (Haskard *et al.,* 2001). However, it is likely that multiple components are involved in AFB1 binding and that this interaction can be affected by environmental conditions (Hernandez-Mendoza, *et al.,* 2009). On the contrary, Lahtinen *et al.,* (2004) reported that no evidence was found for exopolysacchariedes ,

cell wall proteins , Ca^{2+} and Mg^{2+} being involved in AFB1 binding. However, cell wall carbohydrates occur in three main forms in the cell walls of these bacteria; exopolysacchariedes, teichoic and lipoteichoic acids.

The data of pre-chemical treatment provided more information regards nature of the biding see Table 2.8. Sodium metaperiodate, hydrochloric acid and trichloroacetic acid caused a significant decrease in AFB1 adsorption which suggested that AFB1 is adsorbing mainly to the carbohydrate component of the cell wall. Sodium metaperiodate treatment results in the oxidiation of OH-groups located in cis position to aldehydes and carboxylic acid groups;

Sreekumar and Hosono, (1998) supporting the suggestion that polysaccharide component of cell wall contains the binding receptors for AFB1 this result was clarified by the fact that TCA and HCL pre-treatment hydrolysis polysaccharide which reduced the binding.

Therefore these result supports that binding occur pronominally to polysaccharide which is consist of three main forms as mentioned above. While it is difficult to compare results of AFB1-binding levels from different studies, due to the possible impact of procedural differences.

In a related work Hernandez-Mendoza; *et al* (2009) reported that teichoic acid was the key role on AFB1 binding by probiotic bacteria. Haskard *et al.,* (2001, 2000) suggested that hydrophobic interactions between the bacterial cell surface and AFB1 molecule may explain the mechanism for the biding process. In contrast Lahtinen, *et al.,* (2004) ruled out the teichoic acid to be a possible component involved in the binding.

Furthermore, the data presented in this study showed that in the presence of divalent ions, the binding was lower than the control. These results suggest that AFB1 interaction with cell walls was blocked by the divalent ions and ionic bond is unlikely. The results observed in the current study are similar to those reported previously (Hernandez-Mendoza *et al.,* 2009). Whereas Haskard, *et al* (2000) reported that divalent ions did not show any significant increase or decrease compared to the control sample.

The role of proteins and glycoproteins was also investigated by exposing the cells and cell walls to specific and non specific proteolytic enzymes Pepsin, trypsin, Protease, Proteinase and α-chemotrypsin. The non specific proteolytic enzymes was more efficient than specific proteolytic in term effecting AFB1 adsorption Results showed that all the enzymes tested had a significant effects ($P<0.05$) as the percentage of AFB1 binding decreased after the cells and cell walls were digested.

The role of glycoproteins was also investigated. The bacterial cells and cell wall were pre-digested by mutanolysin and Lysozyme which is a muralytic enzyme that cleaves the β-N-acetylmuramyl-(1→4)-N-acetylglucosamine linkage of the bacterial cell wall polymer peptidoglycan-polysaccharide. A significant decrease ($P<0.05$) was recorded for AFB1 binding after the cells and cell walls were digested by those enzymes. These results confirm that proteins still remain in AFB1 removal by LAB cell wall. Greater fragmentation of protein is expected with pronase E due to non specific nature, and may be responsible for significant effect on binding previously observed (Haskard *et al.,* 2000).

The present study showed that the pre enzymatic treatment for the bacterial cells or purified cell walls was affected AFB1 adsorption; in all cases there was a decrease in the percentage of AFB1 removal (see Table 2.10). Lipoteichoic acids are widely distributed in LAB and have been found in enterococci, lactobacilli, lactococci, leuconostocs, and streptococci (Delcour *et al.,* 1999). Thus cells and cell wall were pre-treated by lipase. Lipase digests the lipids in the bacterial cell wall therefore the target component was lipoteichoic. The decrease in the binding percentage was more obvious this, suggesting that lipoteichoic acid may involved in the binding process. These results in contrast of the published result by Haskard, *et al.,* (2001) who reported that lipase did not show any significant decrease in AFB1 binding and that lipoteichoic acid was not involved. These differences may due to the differences in the pre-treatment conditions or may be due to the different lactic acid bacteria species used.

Furthermore, to confirm the key role of lipoteichoic acid in the binding process; a range of concentrations 200-1000 µg/ml of lipoteichoic acid was used to bind AFB1 (see figure 2.4). The results indicated that with increase the concentration of lipoteichoic acid in the PBS contaminated by AFB1; the removal was increased. These results suggest that lipoteichoic acid responsible for the removal ability of AFB1. Lipoteichoic acids provide the main component of the hydrophobicity of the cell envelope , and may contribute in this way to its adhesiveness (Delcour, *et al* 1999). Therefore data obtained in this experiment concur with Hernandez-Mendoza, *et al* (2009) results that teichoic acid could be responsible for the ability of LAC.

An argument in the literature is concerned with hydrophobicity interactions with AFB1 molecules. Lee *et al.*,(2003) reported that no correlation was found between the hydrophobicity and AFB1 adsorption while Haskard, *et al* (2000) indicated that when the cells were treated with organic solvents, bound toxin was rapidly extracted, confirming a potential role of hydrophobic interaction. AFB1 is a hydrophobic compound as well as lipoteichoic acid. Therefore, hydrophobic interactions may be involved. In this study when the complex AFB1/LAB cells was treated with chloroform, bound toxin was rapidly recovered from the surface of the cells and these results corroborate the results reported by Haskard, *el al* (2001). The lipoteichoic experiment provides evidence that the AFB1 molecule is attracted by hydrophobic pocket (lipoteichoic acid) then the molecule may bounded by intermolecular forces of attraction by other component in the cell wall

2.11 - Conclusions

The study has extended the range of bacteria species that have been shown to be able to absorb AFB1 *in vitro.* The experimental data in this study indicated that cell wall composition was important for AFB1 binding. Additionally, lipoteichoic acids are important components of the cell walls that are involved in AFB1 adsorption. The indication that cell wall and lipoteichoic acids were able to absorb AFB1 from contaminated media may offer the potential for the development of method for removal AFB1 and other aflatoxins by passing the contaminated media through bio-filters made from LAB cell walls and lipoteichoic acid.

CHAPTER 3

BIO-DEGRADATION OF AFLATOXIN B1 BY ACTINOMYCETE CULTURES

3 - INTRODUCTION

Aflatoxins are difuranocoumarin analogues and are the most hazardous mycotoxins (Bolognani *et al.*, 1997). Different physical and chemical methods have been developed and tested to control AFB1. Disadvantages of these methods have limited their practical applications due to nutritional losses, sensory quality reduction and high cost of equipment (Guan *et al.*, 2008). Implementation of microorganisms and their enzymatic products to detoxify AFB1 in contaminated food and feed can be an alternative to such technology. Recently, interest in biological detoxification of AFB1 has significantly increased.

A number of fungal species such as *Pleurotus ostreatus* (Motomura, *et al.*, 2003), *Rhizopus* sp. (Liu *et al.*, 1998, Varga *et al.*, 2005), *Armillariella tabescens* (Liu *et al.*, 1998) and *Trichoderma* strains (Shantha, 1999), as well as the yeast *Saccharomyces cerevisiae*, have been found to transform AFB1 to less toxic metabolites (Shetty and Jespersen, 2006). However, utilisation of these moulds and yeasts was not economical due to the economics of the extraction process, concentration of the extracts, and lengthy incubation time. Diminution of AFB1 by bacteria has also been described in several reports.

Most of the publications focus on lactic acid bacteria, such as *Lactobacillus* sp. (Bueno *et al.*, 2006b), *Lactococcus* sp. (El-Nezami *et al.*, 1998a, El-Nezami *et al.*, 2000) and *Bifidobacterium* sp. (Elgerbi *et al.*, 2006). The reduction of AFB1 by lactic acid bacteria is due to the binding of aflatoxins to the bacteria, rather than the bacteria degrading the toxin. This implies that using such systems for complete toxin

removal from contaminated food or feed is likely to be difficult or impossible (El-Nezami *et al.*, 1998a, El-Nezami *et al.*, 2000).

Flavobacterium aurantiacum, now classified as *Nocardia corynebacterioides* (Ciegler, *et al.,* 1966) and other related microorganisms , for example *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* sp. nov DSM44556 have also been shown to degrade AFB1 into less toxic compounds (Teniola *et al.,* 2005, Alberts *et al.,* 2006). These microorganisms were effective in the degradation of polycyclic aromatic hydrocarbons (PAHS) - such as AFB1. Significant results have been obtained in employing those mentioned organisms i.e. strains of soil/water bacteria to degrade AFB1 (Teniola *et al.,* 2005, Alberts *et al.,* 2006). This seems to be a promising prospect for the degradation of AFB1 in food; however, more studies are required in this area.

In the present research selected soil bacteria (*Rhodococcus erythropolis* ATCC 4277, *Streptomyces lividans* TK 24, and *S. aureofaciens* ATCC 10762) were investigated for their ability to degrade of AFB1. In this study, the use of the above mentioned strains to degrade AFB1 has been employed for the first time. The research has unambiguously confirmed AFB1 degradation by utilizing high resolution Fourier transform mass spectrometry (FTMS) to stepwise identify the metabolites during the degradation process.

3.1 - RHODOCOCCUS AND STREPTOMYCES STRAINS

The ability of microorganisms to degrade naturally occurring aromatic compounds is widespread among microbial species (Martinkova *et al.*, 2009). However, xenobiotic aromatic compounds are degraded by a far smaller range of bacteria, mostly Gram-negative bacteria such as *Pseudomonas*, S*phingomonas*, *Acinetobacter* and members of some Gram-positive genera including *Rhodococcus* (Martinkova *et al.*, 2009). Bioremediation using various bacterial strains of genus *Rhodococcus* has proved to be a promising choice for the clean-up of polluted sites.

Rhodococcus is an aerobic Gram-positive bacterium capable of transforming a wide range of xenobiotic compounds including polychlorinated biphenyls and nitroaromatic compounds. Therefore, the organism is considered as significant in the removal of toxic polyaromatic pollutants from the environment, *Rhodococcus* has a large genome and mega-plasmids. (Alberts *et al.*, 2006, Martinkova *et al.*, 2009).

Their redundant and versatile catabolic pathways, ability to uptake and metabolize hydrophobic compounds, capability to form bio-films to persist in adverse conditions, and the availability of recently developed tools for genetic engineering in Rhodococci, make them suitable industrial microorganisms for the biotransformation and biodegradation of many organic compounds (Martínkova *et al.*, 2009). Rhodococci normally metabolize substrates by first oxygenating the aromatic ring to form a diol. The ring is then cleaved with intra/extra diol mechanisms, thus opening the ring and exposing the substrate to further metabolism (Martínkova *et al.*, 2009).

Moreover, the metabolism of xenobiotics is often divided into three phases: modification, conjugation, and excretion (Martinkova *et al.*, 2009). These reactions act in concert to detoxify xenobiotics and remove them from cells. Therefore, more understanding of aflatoxins metabolism by these microorganisms is required, particularly in terms of physiology, enzymology, gene expression, and the function of the respective enzymes.

Streptomyces have a long history in industry for antibiotic production. Furthermore, these microorganisms are able to metabolize many different compounds including sugars, alcohols, amino acids, and aromatic compounds by yielding extracellular hydrolytic enzymes (Wang *et al.*, 2009, Pogell, 1994). However, in comparison to studies carried out with *Rhodoccocus*, little work has been published in the degradation of aflatoxins by *Streptomyces* species.

3.2 - PROPOSED MECHANISMS FOR AFB1 DETOXIFICATION USING CHEMICAL AND BIOLOGICAL APPROACHES

Numerous proposals have been published describing the possibility of AFB1 degradation or biotransformation of AFB1 into less toxic compounds AFB1 derivative (Ciergler, 1966a, Ciegler and Peterson, 1968, Kusumaningtyas *et al.*, 2006, El-Nezami *et al.*, 1998a, El-Nezami *et al.*, 2002a, Teniola *et al.*, 2005, Alberts *et al.*, 2006, Wu *et al.*, 2009, Liu *et al.*, 1997b).

Kusumaningtyas *et al.*,(2006) reported biodegrdation of AFB2 into AFB2a by *Aspegillus niger*. Moreover, A. *niger* was able to transform AFB1 to aflatoxicol A (AFLA) and aflatoxicol B (AFLB). Metabolism of AFB1 to AFR0 was also reported

in *Mucor alterans* NRRL 3358. In addition, the protozoa *Tetrahymena pyriformis* was able to degrade or transform AFB1 to aflatoxicol AFR0 (Wu *et al.,* 2009) see Figure 3.1. Although aflatoxicol, which is an analogue of AFB1, is less toxic, the structural unit that is responsible for the toxicity is still available in the molecule. Recently, a number of yeasts and lactic acid bacteria were considered as bio-adsorbent agents, but the mechanisms of degrading AFB1 remain unclear (Elgerbi *et al.,* 2006).

Enzymes from microbial systems have been used to degrade AFB1. These enzymes were able to transform AFB1 to an AFB1-epoxide initially and then is subsequently hydrolysed-AFB1-epoxide however is a carcinogen and thus this would be an unacceptable mechganism for AFB1 degradation in food (Liu *et al.,* 1997b)**.**

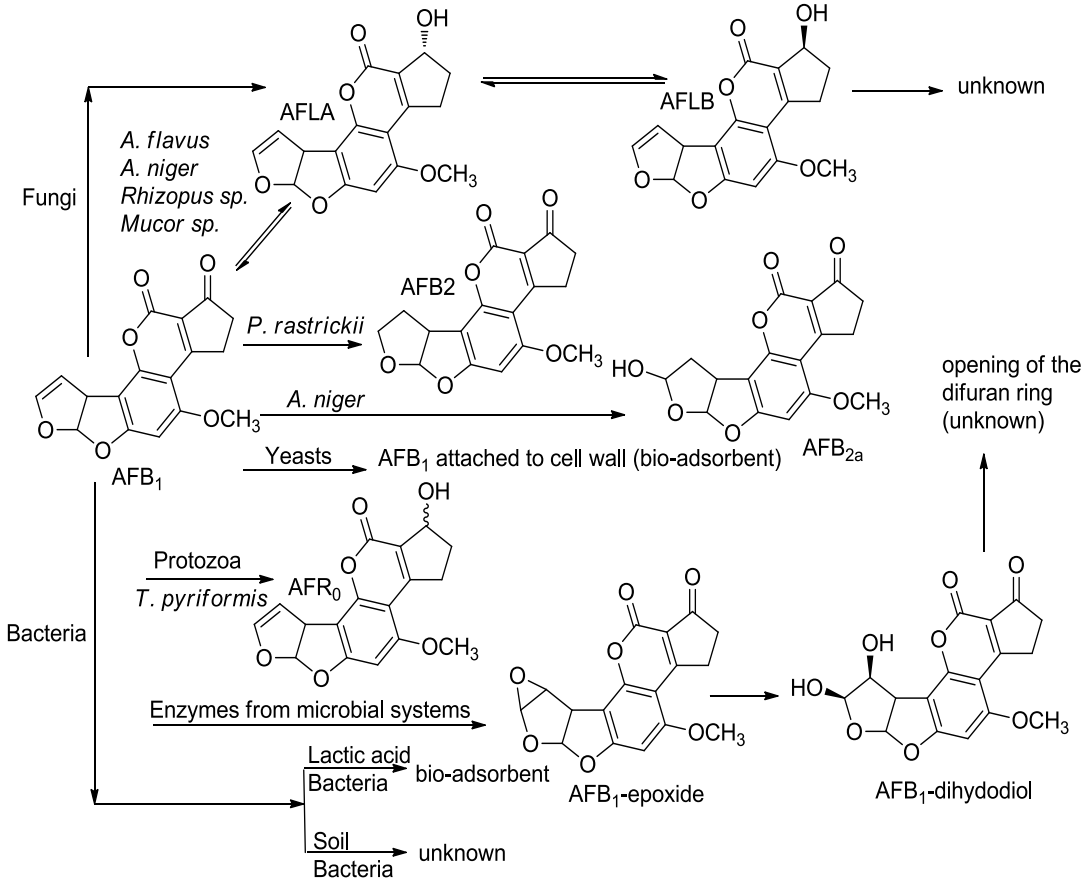


Figure 3.1 -Proposed pathway for microbial catabolism of AFB1(Ciergler, 1966a, Ciegler and Peterson, 1968, Kusumaningtyas *et al.*, 2006, El-Nezami *et al.*, 1998a, El-Nezami *et al.*, 2002a, Teniola *et al.*, 2005, Alberts *et al.*, 2006, Wu *et al.*, 2009, Liu *et al.*, 1997b, *Suttajit*, 1989)

Ideally, the detoxification procedure should consider several points such as reducing the concentration of toxins to safe levels, preventing production of toxic degradation products and avoiding decrease of nutritional value of treated products. Other proposed degradation of AFB1 using chemical treatments has been previously described and found that acidification of AFB1 by aqueous citric acid, transformed AFB1 to AFD_1 and AFD_2 (*Suttajit*, 1989). In addition, it is known that ammonia was able to convert AFB1 to AFD_1. This was associated with an increase in new unknown analogues (Figure 3.2).

Nevertheless, the reactions in theses pathways are important for food, and medical sciences to determine the microorganisms' capability of breaking down the contaminant during biodegrdation or persisting in the environment. Also, in food industry the use of biological detoxification methods may assist in food production by developing alternative procedures to avoid the chemical treatment. In medicine, such reactions in these pathways are of particular interest, as they can be important contributory factors in AFB1 metabolism (Murphy, 2001, Murphy *et al.*, 2006, Wu *et al.*, 2009, Mishra and Das, 2003b).

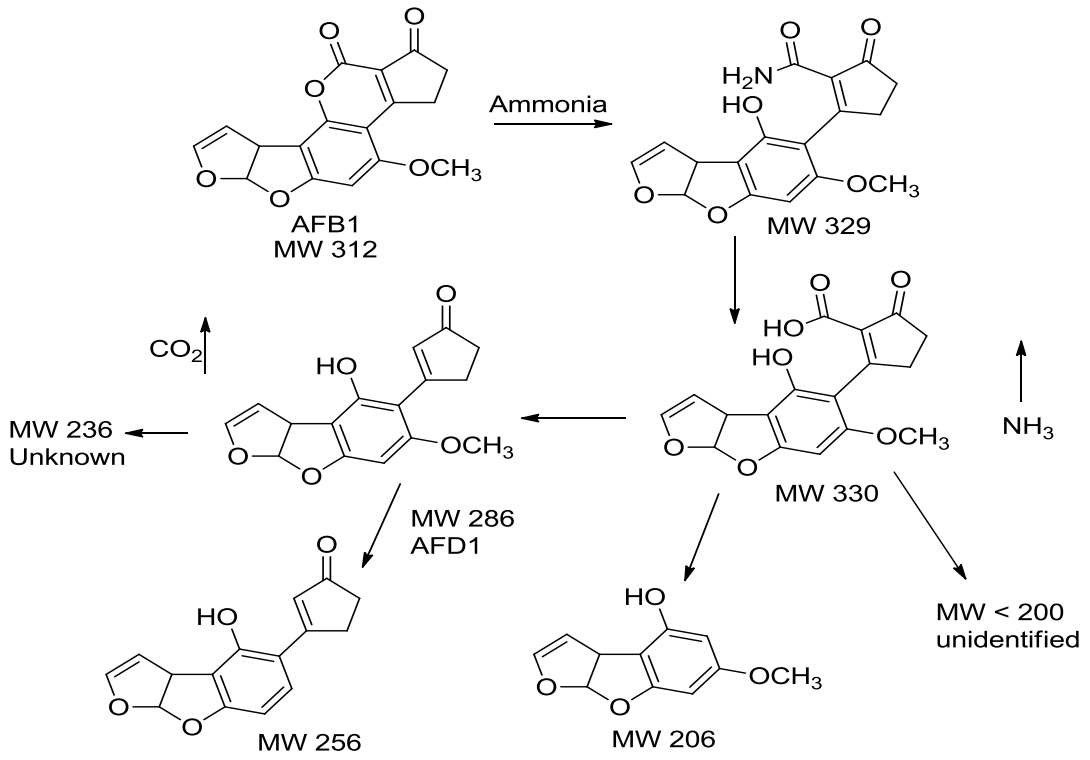


Figure 3.2- Proposed formation of aflatoxin–related reaction products following to ammonia (*Suttajit*, 1989)

3.3 - AIMS OF THIS RESEARCH

The primary aim of this research was to investigate the degradation of AFB1 by Actinomycete bacteria in liquid culture; *Rhodococcus erythropolis* ATCC 4277, *Streptomyces lividans TK 24* and *Streptomyces aureofaciens* ATCC 10762. The second aim was to identify factors affecting degradation efficiency of the culture (e.g. pH, temperature and incubation time). The third aim was to hypothesize a possible degradation mechanism of AFB1 by liquid cultures of *Rhodococcus.*

3.4 - MATERIALS AND METHODS

3.4.1 - MICROBIAL CULTIVATION

The Streptomyces strains, *Streptomyces lividans* TK 24 and *Streptomyces aureofaciens* ATCC10762, used in this study were obtained from Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), Strathclyde University, Glasgow, UK. *Rhodococcus erythropolis* ATCC 4277 was purchased from ATCC, LGC Standards, UK. According to Shirling and Gottlieb, (1966) the strains were cultivated in Difco ISP medium No.1. The media comprising: 0.5% (w/v) pancreatic digest of casein and 0.3% (w/v) yeast extract (Sigma Aldrich, UK). The cultures were preserved at – 80 ºC in 20% (v/v) glycerol.

3.5 - AFLATOXIN B1 DEGRADATION BY ACTINOMYCETES IN LIQUID CULTURE

Actinomycetes strains were inoculated into Difco ISP medium No. 1 (250 ml) and incubated at 30°C for 48 h on a shaker (New Brunswick Scientific, Edison, USA) at

200 rpm, pH 6. All the experiments were carried out in 2 ml Eppendorf tubes on a final volume of 1 ml. In sterile 2 ml screw-capped Eppendorf tubes, 50 µl of 48 h preinocula were inoculated into 0.75 ml of ISP medium No.l. This involved addition of 100 µg/ml AFB1 dissolved in methanol (Sigma, Aldrich, UK) to the culture to give a final concentration of 20 µg/ml AFB1. The supplemented cultures were incubated at 30°C for 24, 48 and 72 hours. Sterile medium supplements with a final concentration of AFB1 of 20 µg/ml used as a negative control in this study. The cells were removed by centrifugation (JuanBR4i Multifunction, Thermo) at 11,000 rpm, 4ºC for 15 min. AFB1 was quantified using reversed phase HPLC as described in section (3.4.5).

3.5.1 - OPTIMIZATION OF THE DEGRADATION CONDITIONS

The degradation of AFB1 at variable temperatures, pH and incubation times was investigated to determine the optimum conditions for the degradation of AFB1 by the different strains. The chosen temperatures were 25, 30, 35, and 40 ºC over a period of 24 h at pH 6. The initial pH used in this experiment was 4, 5, 6, 7, and 8 and the mixtures were incubated in **the** dark to avoid the light effect on the toxin for 24 h at 30°C. The pH was adjusted by using 1 N of HCI or 1 N of NaOH. All experiments were performed in triplicate.

3.5.2 - EXTRACTION AND QUANTIFICATION OF AFLATOXIN B1 BY TLC, HPLC, LCMS AND ORBITRAP LCHRFTMS ANALYSIS

Extracting AFB1 from the medium was not necessary as one of the study aims was to purify and identify the degradants. Four methods were used in this study to confirm AFB1 degradation; Thin layer chromatography (TLC), High-performance liquid

chromatography (HPLC), Liquid chromatography-mass spectrometry (LCMS) and high resolution Fourier transform and orbitrap massspectrometry (Orbitrap FTMS).

3.5.2.1 - THIN LAYER CHROMATOGRAPHY (TLC)

TLC analysis was carried out on silica gel (Si_{60}) plates. The plates (Sigma Aldrich, UK) were developed using chloroform: acetone (9:1, v/v) as the solvent system and monitored under UV at 365 nm.

3.5.3 - HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) CONDITIONS

HPLC Analysis was carried out as section 2.7.1 and the remaining AFB1 percentage was calculated using the following equation:

EQUATION - 3.1:

$$\text{RESIDUAL AFB1} = \frac{\text{AFB1 PEAK AREA IN TREATMENT}}{\text{AFB1 PEAK AREA IN CONTROL}} \times 100$$

3.5.4 - LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY LCMS CONDITIONS

Liquid chromatography-mass spectrometry analysis was used for the confirmation of AFB1 degradation, the liquid chromatographic system, Agilent 1100 HPLC-PDA, was coupled with the LCQ Deca XP ThermoFinnigan ion trap instrument with an electrospray ionization source, ESI (ThermoFinnigan, Germany). Separation was achieved using reversed phase elution with ACE C18 150×4.6 mm (Hichrom

Limited, UK). The mobile phase consisted of acetonitrile: methanol: water (1:1:2, v/v/v) , with 0.01 M of ammonium formate buffer at a flow rate of 0.4 ml/min.

The retention time for AFB1 was 12.81min. Electrospray ionisation (ESI) was used as the mode of ionisation. ESI mass spectra ranging from *m/z* 50 to 1000 amu were taken in positive-ion mode. The monitored ion was the protonated molecule $[M+H]^+$ at *m/z* 313 for AFB1. The MS detector was set as follows: vaporizer temperature 220ºC; a sheath gas flow rate 50 arbitrary units; auxiliary gas flow rate 10 arbitrary units; source voltage 5 kV; capillary voltage 15 V; tube lens offset 30V.

3.5.5 - FOURIER TRANSFORM AND ORBITRAP MASSSPECTROMETRY (ORBITRAP FTMS)

The high resolution mass spectroscopy technique LTQ-Orbitrap was used to confirm AFB1 degradation and to identify any degradant produced during the biodegradation process. The liquid chromatographic system ,Agilent 1100 HPLC-PDA, was coupled with an LTQ Orbitrap ThermoFinnigan instrument equipped with an electrospray ionization source, ESI (Thermo Finnigan, UK) Separation was completed by reversed phase elution over an ACE C18 50×3.0 mm ID. The mobile phase consisted of acetonitrile: methanol: water (1:1:2, v/v/v) with 0.01 M of ammonium formate at a flow rate of 0.4 ml/min retention time for AFB1 was 7.12 min.

MS detector operation was set as follows: vaporizer temperature 220ºC; a sheath gas flow rate 30 arbitrary unit; auxiliary gas flow rate 10 arbitrary units; source the voltage 4; capillary voltage 35.50 V, tube lens 30 V; capillary voltage 21 V. Two

scan events were run; the first FTMS ranging from *m/z* 50 to 1000 in positive mode and the second run was an ms/ms fragmentation for *m/z* 313 set at the range of 85-500 amu in the positive mode. The monitored ion was the protonated molecule $[M+H]^+$ at *m/z* 313.0707 for AFB1. Identity of AFB1 was confirmed by its MS/MS data which gave a fragment ion at *m/z* 285.0759. The data were processed by using SIEVE, differential analysis software.

3.6 - DATA ANALYSED BY SIEVE

The Orbitrap FTMS is more sensitive than the LCMS. Consequently, although it was difficult to handle the huge data generated, more information was available. Therefore, the SIEVE software from ThermoScientific was used to process the data. SIEVE was able to identify all the mass spectra of ion peaks at lower threshold. The software was connected with the online library ChemSpider to detect aflatoxin analogues and other small molecule degradants.

3.7 - EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS.

One and two way ANOVA plots were used to analyse the data to find the significant effect of the pH, temperature and time on the response variable AFB1 concentration. A significance level at 5% was used and Turkey's multiple comparison tests were performed when significant difference were encountered. In addition, multivariate data analysis using SIMCA was used to analyze the data. The purpose of using SIMCA was to determine the similarity of a group of samples according to their principle component where each group was described as a cluster. In addition, correlation between AFB1 degradation and certain metabolites was analysed by Minitab software.

3.8 - RESULTS

3.8.1 - CONFIRMATION OF AFB1 DEGRADATION

In this study four techniques were used to confirm the degradation of AFB1. The TLC result was confirmed the result observed by using HPLC where fluorescence decreased in intensity with time (Figure 3.3). However, HPLC and TLC analysis could not reveal the formation of any breakdown products. Therefore, a more sensitive technique was required to confirm the degradation and to identify the breakdown products. LCMS confirmed the degradation with the disappearance of the peak at *ca*. 12.50 min, while new peaks detected at retention time 31.55 and 37.85 min (Figure 3.4 and 3.5).

Figure 3.4 shows the detection of AFB1 standard by LCMS exhibiting high absorbance at wavelength 364 nm and retention time of 12.45 min. Full scan data showed a molecular cation of AFB1 at 12.52 min and *m/z* 313. Figure 3.5 indicated the presence of AFB1 by LCMS at 24 h of treatment where A is the UV chromatogram and A1 is the total ion chromatogram (TIC). Figure 3.5 (A and A1) showed LCMS results after a 72 h treatment with *R. erythropolis* liquid culture. Separation of AFB1 by reversed phase HPLC at a retention time of 12.81 min was monitored by ESI-MS. the ESI-MS spectrum of AFB1 showing the protonated cation $[M+H]^+$ at *m/z* 313. The arrow indicates where the protonated cation $[M+H]^+$ 313 of AFB1 peaks would have been observed if present. HRFTMS (LTQ-Orbitrap) results from full and precursor ions scan were used to confirm LCQ results. Moreover, HRFTMS was used to identify ions of the degradants and their precursors.

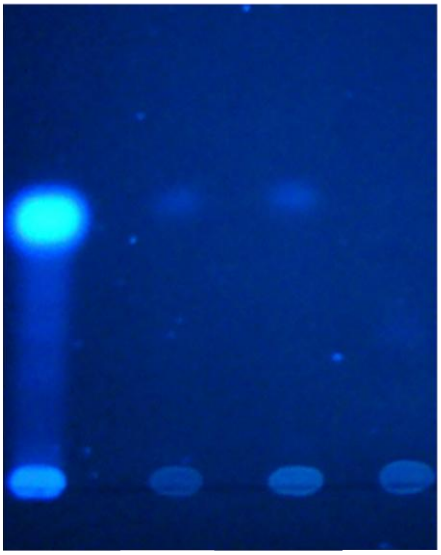
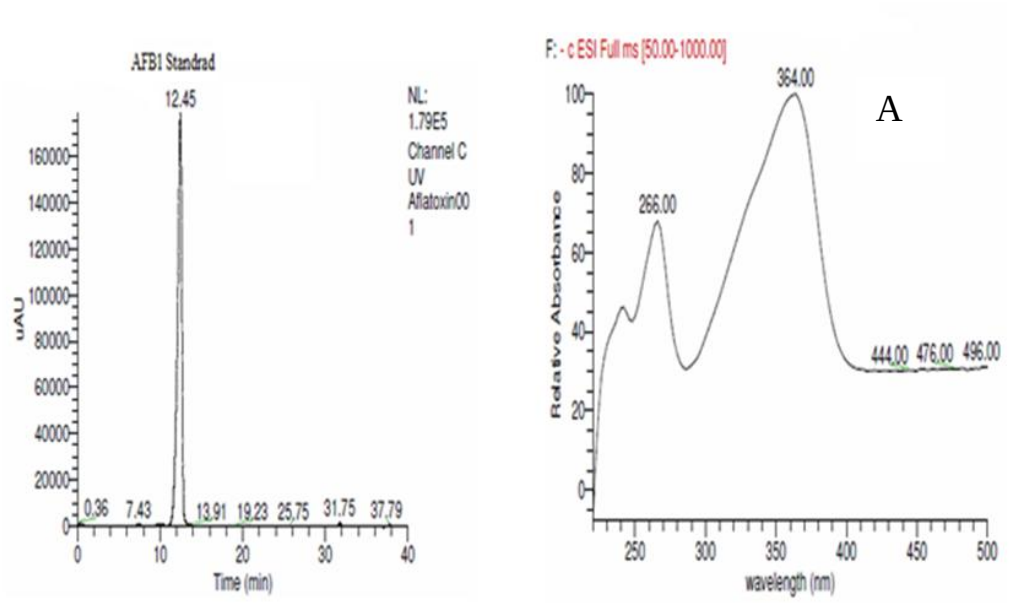


Figure 3.3 - TLC analysis of aflatoxin B1 degradation activity over a period of 72 h. AFB1treated with *R.erytropolis* ATCC 4277 for 72h at 30ºC and pH 6. TLC Plate was photographed under long-wavelength UV light 365nm.

UV Channel



MS Channel

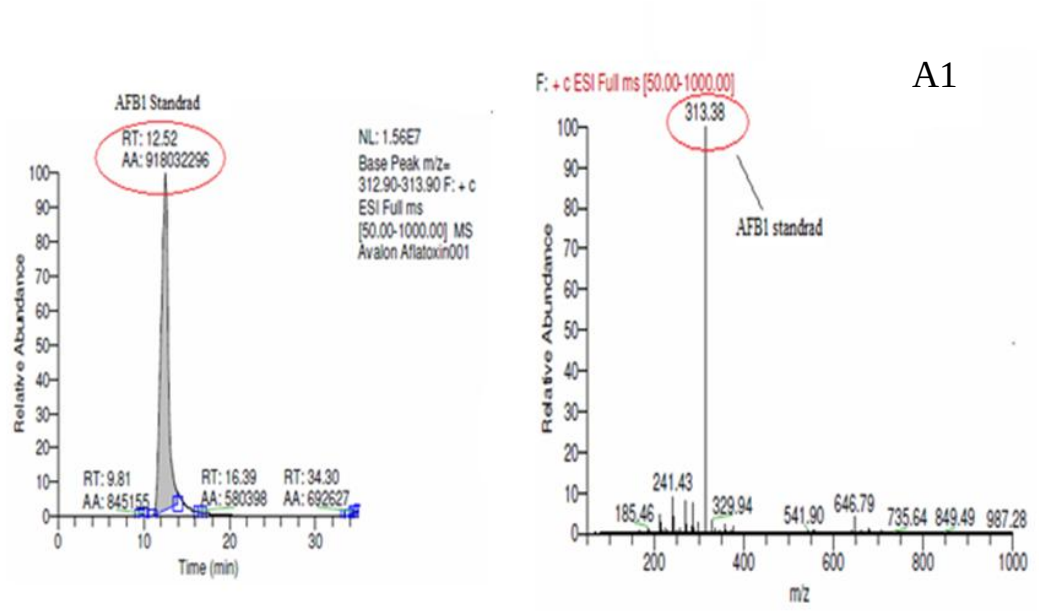


Figure 3.4- Detection of AFB1 standard by LCMS (A) UV detector, separation of AFB1 by revesed phase HPLC retention time was 12.45min. (A1) MS detector, the ESI-MS spectrum of AFB1 showed the protonated cation $[M+H]^+$ at *m/z* 313.

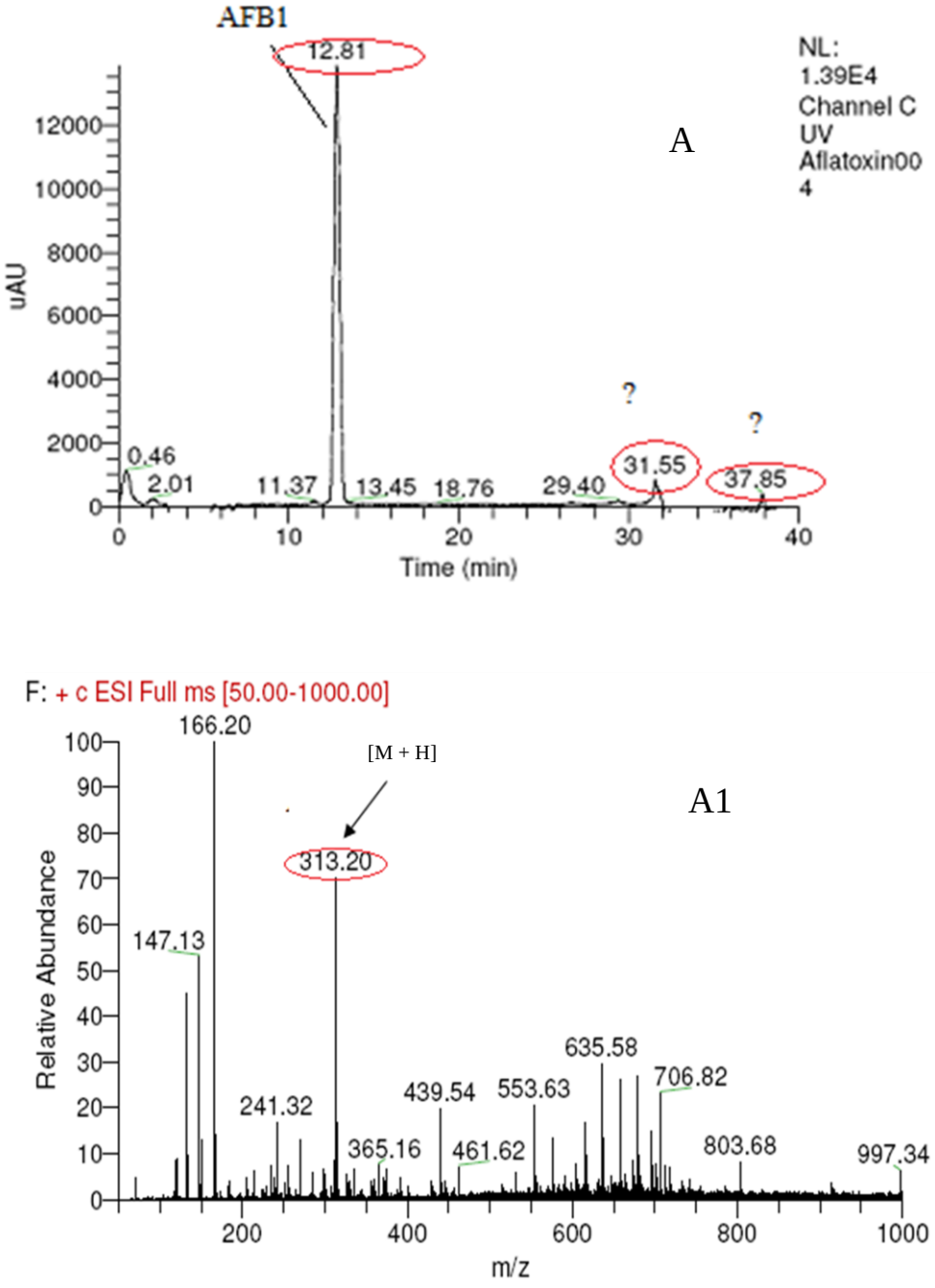


Figure 3.5 - Detection of AFB1 by LCMS after a 24 h treatment with *R.erythropolis* liquid culture. (A), (A1) Separation of AFB1 by reversed phase HPLC retention time was 12.81min The ESI-MS spectrum of AFB1 showed the protonated cation $[M+H]^+$ at *m/z* 313 .

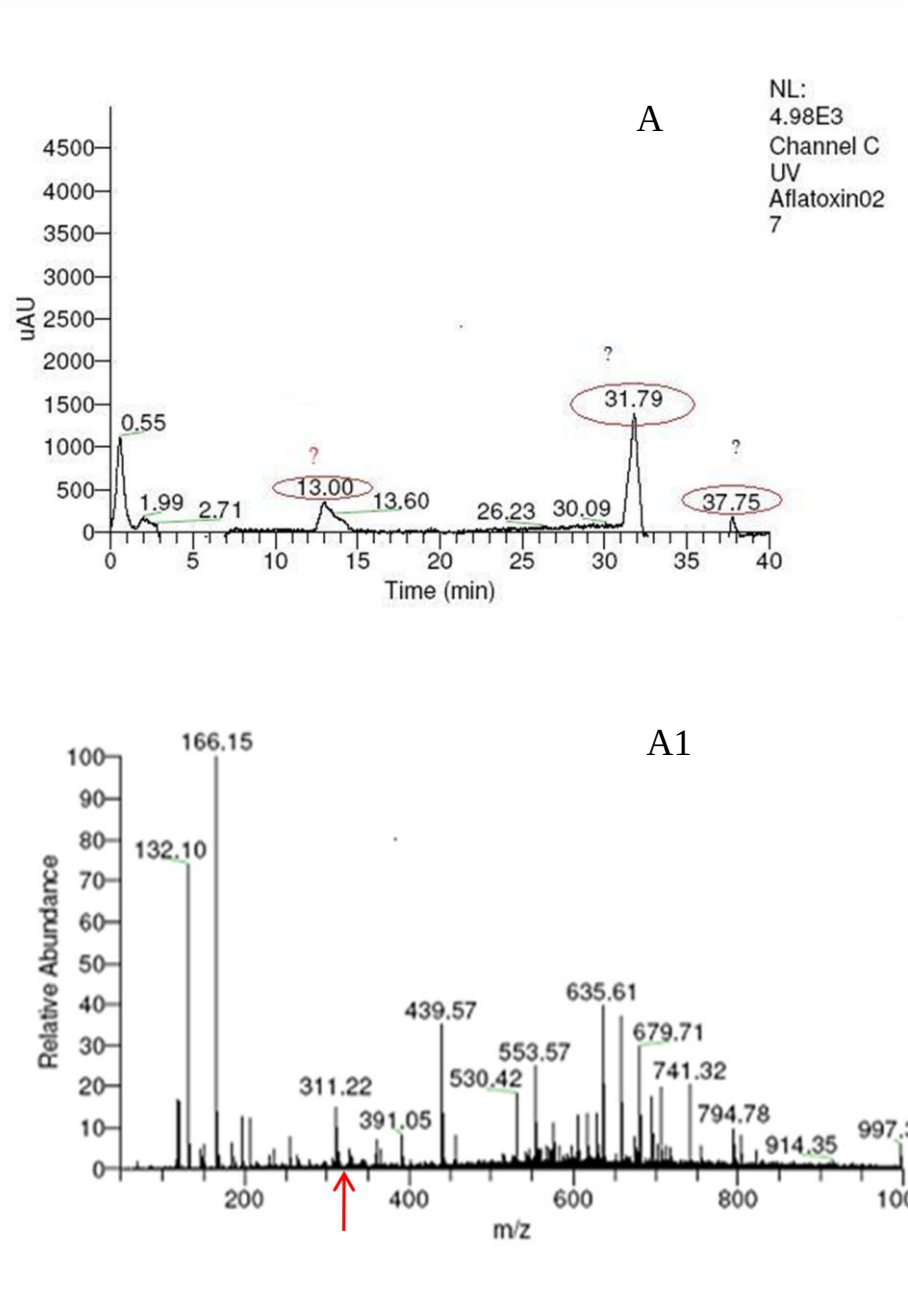


Figure 3.6- Mass spectrometry data (LCMS) after a 72 h treatment with *R.erythropolis* liquid culture. (A), (A1) Separation of AFB1 by reversed phase HPLC retention time was 12.81 min.the ESI-MS spectrum of AFB1 showed the protonated cation $[M+H]^+$ at *m/z* 313 . The arrow indicates where the protonated cation $[M+H]^+$ 313 of AFB1 would have been expected to appear if present.

3.8.2 - AFLATOXIN B1 DEGRADATION BY *ACTINOMYCETES* IN LIQUID CULTURE

This study investigated the biological degradation of AFB1 at a slightly higher concentration of 20 µg/ml by a liquid culture of Actinomycete bacteria. All strains were able to degrade the AFB1 in ISP No. 1. Liquid cultures of *R. erythropolis* ATTC 4277, *S. lividans TK 24* and *S.aureofaciens* ATCC10762 were exposed to 20 µg/ml of AFB1 for 24, 48, and 72 h. The control sample (AFB1 + media) was shown to be stable over the 72 h period of incubation.

Figure 3.7 showed a significant reduction of AFB1 by *Rhodococcus erythropolis* ATCC 4277 after 24 h incubation at 30°C and pH 6 with a remaining AFB1 of only 4 %. Meanwhile, the remaining amount of AFB1 in percentage terms was slightly higher when *Streptomyces* strains were used at the same conditions (12, and 14% of the initial AFB1 concentration).

Table 3.1 shows the statistical analysis by using one way ANOVA. It confirmed that there was a significant difference between the strains in term of the degradation over a period of 24 h ($P < 0.05$). Tukey's Multiple Comparison Test was applied to the data of Table 3.2 indicated that *R. erythropolis* was significantly different from other strains ($P < 0.05$). No significant difference was shown between the *Streptomyces* strains ($P>0.05$).

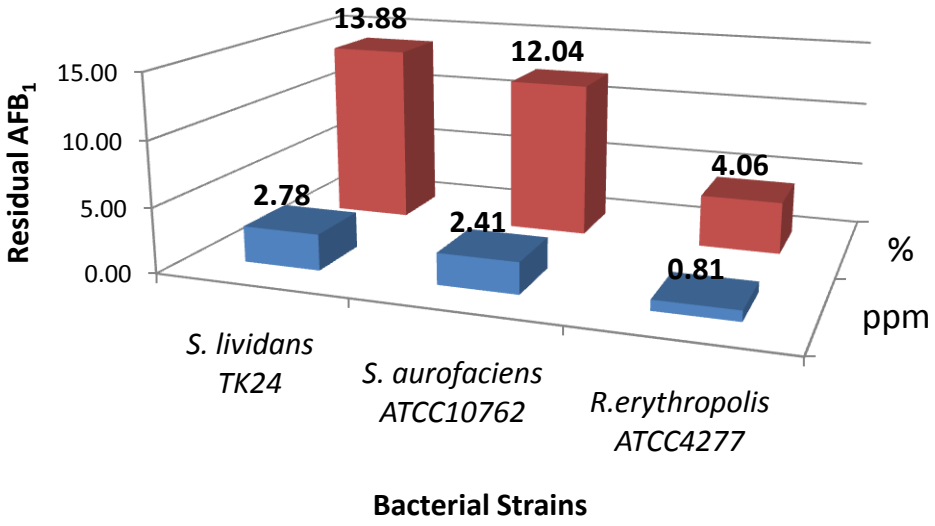


Figure 3.7- AFB1 degradation by cultures of *R. erythropolis* ATCC 4277, *S. lividans* TK 24 and *S.aureofaciens* ATCC10762 after 24 h incubation at 30 º C and pH 6.

Table 3.1 – One way ANOVA analysis for AFB1 remaining by Actinomycetes

Table Analyze	Data 1		
One-way analysis of variance			
P value	0.0042		
P value summary	**		
Number of groups	3		
F	28.98		
R squared	0.9354		
ANOVA Table	SS	df	MS
Treatment (between columns)	125.3	2	62.65
Residual (within columns)	8.647	4	2.162
Total	134.0	6	

Table 3.2-Tukey's Multiple Comparison Test

Strains	Mean Diff.	Q	Significant? P < 0.05?	Summary	95% CI of diff
R.erythropolis vs S.aureofaciens	-7.514	7.917	Yes	*	-12.30 to -2.731
R.erythropolis vs S.lividans	-9.353	9.854	Yes	**	-14.14 to -4.569
S.aureofaciens vs S.lividans	-1.838	1.768	No	ns	-7.078 to 3.402

Figure 3.8 shows the effect of culture incubation temperature on AFB1 degradation over the first 24 h of culture at pH 6. A range of temperatures between 25 to 40ºC was used to study the degradation of AFB1 by the liquid cultures of Actinomycetes. In degrading AFB1 for all the strains, the most effective temperature was 30 ºC. Moreover, the reduction of AFB1 was noted in all the temperature levels used in this experiment.

Table 3.3. shows the two way ANOVA analysis on the effect of temperature over a period of 24h by the Actinomycet*e* cultures. Two way ANOVA confirmed that the temperature was significantly different ($P<0.05$) in degrading AFB1. In addition, the effect of the temperature was very significant ($P = 0.0050$) and the effect of the strains was significant ($P = 0.0138$). However, no interaction effect ($P=0.0747$) was identified between the temperatures and the strains.

A Bonferroni multiple comparison post test was carried out on the two-way ANOVA to determine the effect of temperature over a period of 24 h with the *Actinomycetes* culture when the result showed a significant difference ($P<0.05$). Appendix 3.1 shows the post testcomparison. The purpose of using the post test was to verify which temperature was the most effective in degrading AFB1. These analyses also confirmed that 30 ºC was the optimum temperature for all the strains.

The results obtained at a temperature of 25 ºC was significantly different ($P<0.05$) from 30 ºC when AFB1 was incubated with *R. erythropolis,* while no significant difference ($P>0.05$) was found between 25 and 30ºC when AFB1 was incubated with

the *Streptomyces* strains. No significant difference (P>0.05) was found between 25 and 35 ºC with all the strains.

Bonferroni multiple comparisons showed no significant difference (P>0.05) between 25, 40 and 30, 40 ºC when *R. erythropolis* and *S. lividans* were used to degrade AFB1; while *S.aureofaciens* showed a significant difference (p<0.05) from the other two strains at the same temperature. No significant difference (P>0.05) was found between 30, 35 ºC and 35, 40 ºC. The percentage of AFB1 residual was higher at 40 ºC than other temperatures used in this experiment.

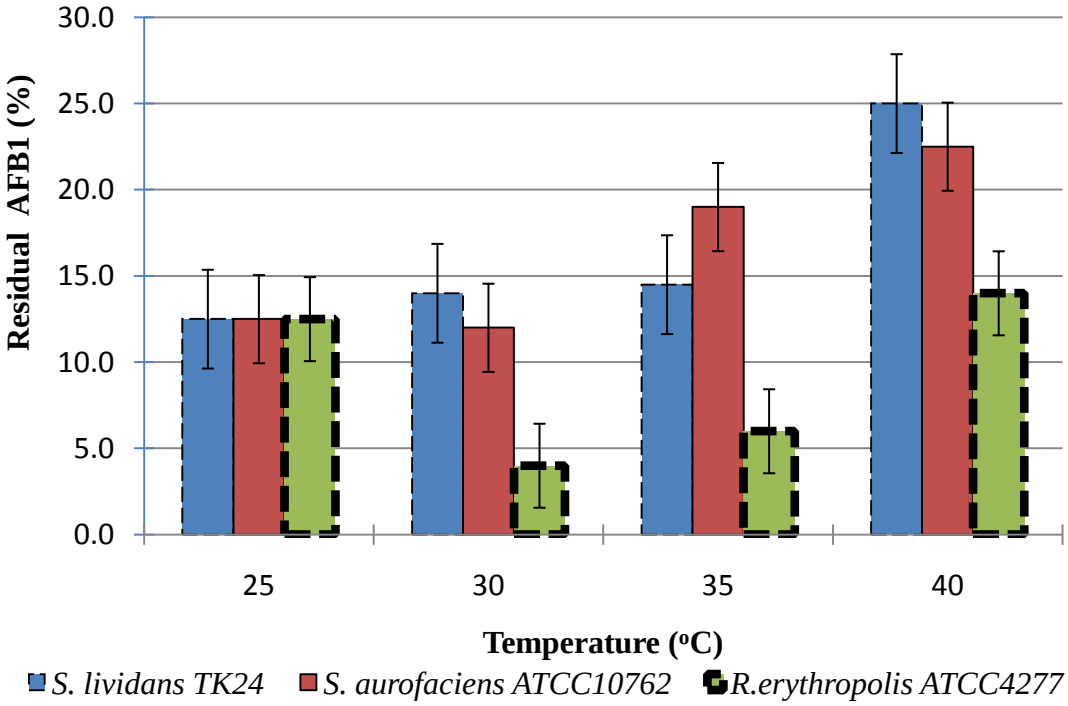


Figure 3.8 -The effect of culture incubation temperature on AflatoxinB_1 degradation over the first 24 hours of culture at pH 6.0

Table 3.3 - Two way ANOVA analysis for the optimum temperature

Source of Variation	Total variation (%)	P value		
Interaction	25.24	0.0747		
Temperature	39.82	0.0050		
Strains	21.24	0.0138		
Source of Variation	P value	Significant		
Interaction	Ns	No		
Temperature	**	Yes		
Strains	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	6	130.6	21.77	3.035
Temperature	3	206.1	68.69	9.576
Strains	2	109.9	54.97	7.663
Residual	8	57.38	7.173	

Figure 3.9 shows the effect of culture pH on AFB1 degradation over the first 24 h of culture at 30 ºC. AFB1 was not stable in all pH used. The degradation was pH and strain dependent. The optimum pH for *R. erythropolis* ATCC4277 was pH 6 when about 5 % of AFB1 remained after 24 h incubation. On the other hand, *Streptomyces* strains were more effective at pH 5 where less than 5% of AFB1 remained. Table 3.4 shows two-way ANOVA analyses for the effect of culture pH on AFB1 degradation over the first 24 h of culture at 30 ºC. Two-way ANOVA confirmed that the pH was significantly different ($P<0.05$) in degrading AFB1. Both of the factors pH and strains were considered as highly significant ($P<0.001$) and the interaction between these factors was also highly significant ($P<0.001$).

Appendix 3.2 shows the post test Bonferroni multiple comparisons. The purpose of using the post test was to determine which pH was most effective. This analysis confirmed pH 6 was the optimum pH for all the strains. No significant difference was found between pH 6 and 7 ($P>0.05$) while significant difference was observed between pH 6 and pH 4, 5 and 8 ($P< 0.05$). Figure 3.10 shows kinetics of degradation of AFB1 over the first 16 h incubation with the Actinomycete cultures at 30 º C and pH 6. The three strains were able to degrade AFB1. The rate of the degrading by *R. erythropolis* ATCC 4277 was higher compared to the other two strains. About 50 % of AFB1 remained after 2.5 h. Meanwhile, 70% and 90% remained when AFB1 was exposed to *S. lividans* TK 24 and *S. aureofacienes ATCC 10762* respectively. A significant difference ($P = 0.0042$) was found between negative control and the treated samples. The control sample AFB1+Media was shown to be stable over a period of 72 h in ISP 1 media (negative control).

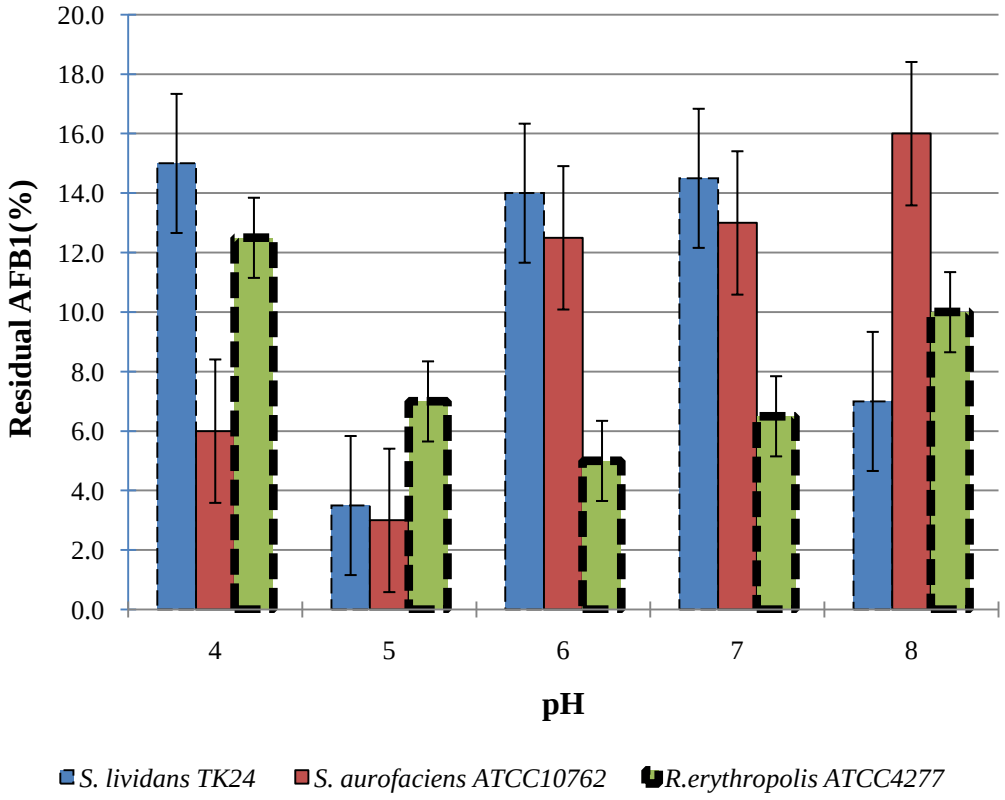


Figure 3.9-The effect of initial culture pH upon aflatoxinB1 degradation over the first 24 hours of culture at 30 ºC

Table 3.4-Two-way ANOVA analysis for the optimum pH

Source of Variation	total variation (%)	P value		
Interaction	61.03	< 0.0001		
pH	27.91	< 0.0001		
Strains	8.57	< 0.0001		
Source of Variation	P value	Significant?		
Interaction	***	Yes		
pH	***	Yes		
Strains	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	8	450.9	56.36	154.0
pH	4	206.2	51.55	140.8
Strains	2	63.31	31.66	86.47
Residual	26	9.518	0.3661	

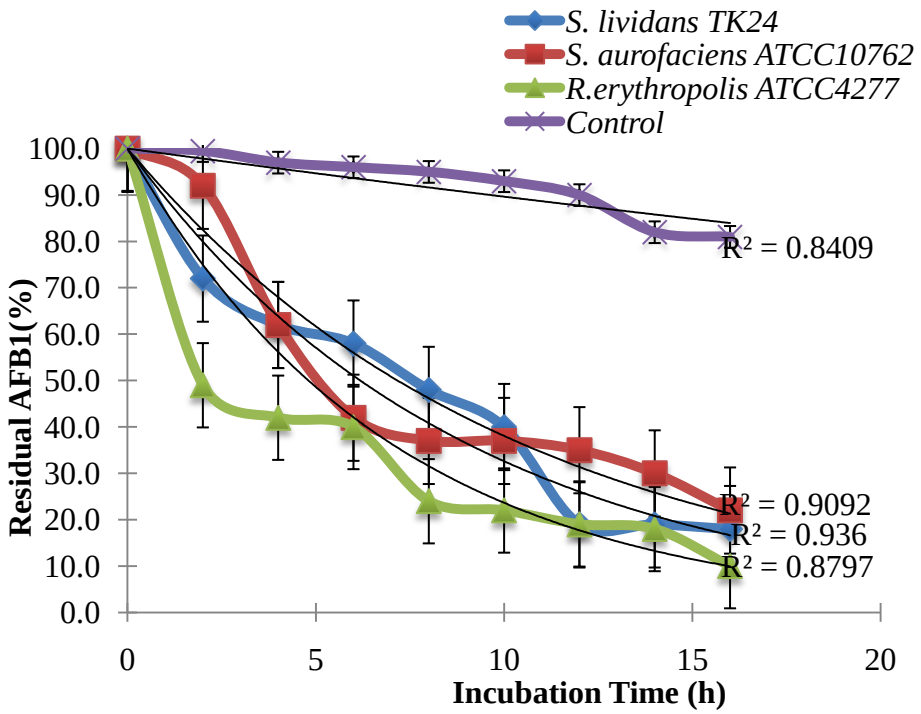


Figure 3.10- Kinetics of degradation of AflatoxinB1 over the first 16 h of culture at pH6

Figure 3.11 shows the multivariate data analysis using SIMCA where time was the variable. The purpose of using SIMCA is to determine the similarity of a group of samples according to their principal components and each group was described as a cluster. There was no significant difference in the ability of each of the microorganisms to degrade AFB1 with increasing time.

3.8.3 - HYPOTHESES ON THE MECHANISMS OF AFLATOXIN B1 DEGRADATION

To study the mechanism of the degradation, high resolution mass spectroscopy technique LTQ-Orbitrap was used to confirm AFB1 degradation as well as to identify any of the metabolic degradants produced during the biodegradation process. It was hypothesised that detoxification of AFB1 may involve the formation of a □-keto acid structure catalyzed by *Rhodococcus* enzymes. In addition, opening the difuran ring does not change the fluorescence of the AFB1 molecule. However, cleaving the lactone ring does decrease fluorescence (Lee *et al.,* 1981).

Results from LTQ were analysed using SIEVE software. The latter identified new mass spectra of other metabolites at nM concentrations which were not found in the control sample. The treated sample was compared with the control at zero time. Blank media was used as a control to exclude spectra belonging to the culture media. Significant ion peaks found in the treated samples as detected by SIEVE includes *m/z* at 331.2845 $[M+H]^+$, 287.2219 $[M+H]^+$and 237,1122 $[M+H]^+$ as well as other smaller molecules.

Figure 3.12 shows HRFTMS analysis to identify the metabolites during the degradation of AFB1 by *R. erythropolis*. Figure 3.13 shows HRFTMS analysis to identify the metabolites after degradation of AFB1 by *R. erythropolis*. The mass spectrum of the treated sample showed an intense pseudo molecular ion peaks at *m/z* 331.2845 $[M+H]^{+\cdot}$ 287.2217 $[M+H]^{+}$ and 237.1122 $[M+H]^{+}$ as well as *m/z* 313.0707 attributable to residual AFB1. Those ions peaks were not present in the mass spectral data of pure AFB1. These products may have been achieved during the culture treatment. Each microorganism was producing different metabolites. The three Actinomycete strains were able to degrade AFB1 but *R. erythropolis* had all the consideration as it was the most efficient in terms of degrading AFB1.

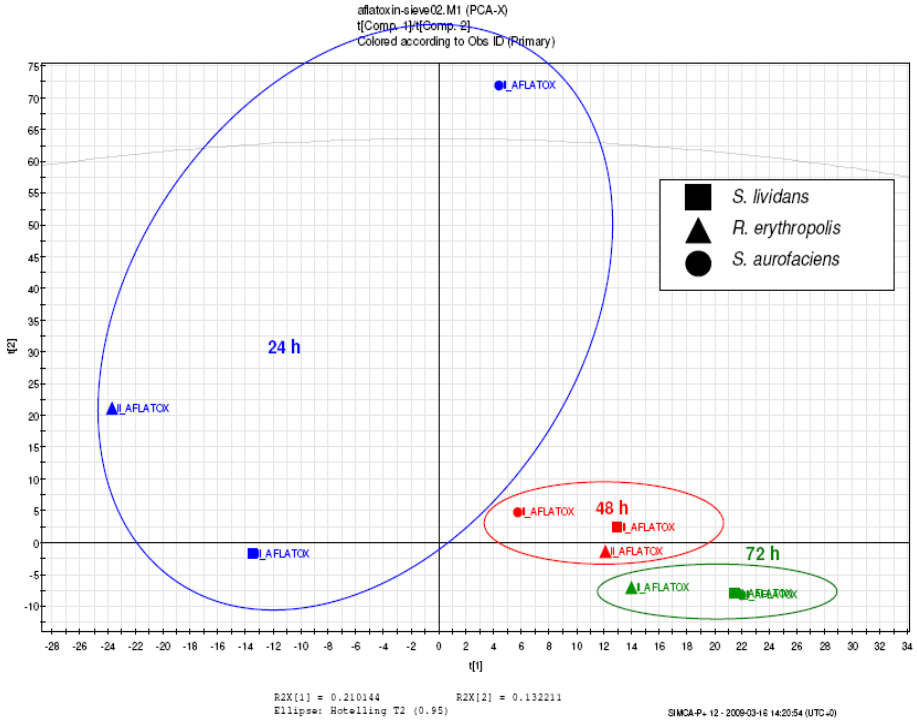


Figure 3.11- Multivariate data analysis using SIMCA at different time frames**.**

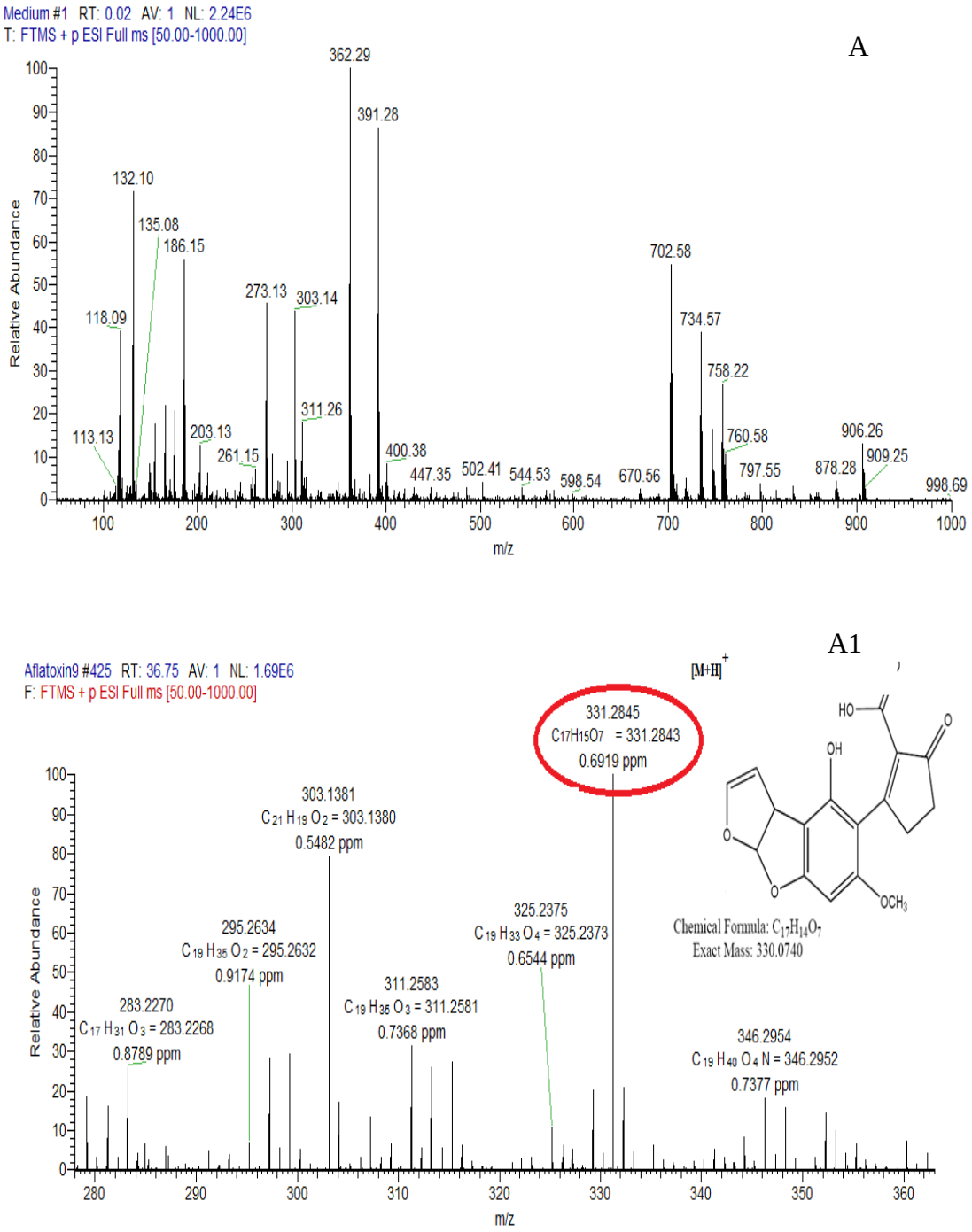
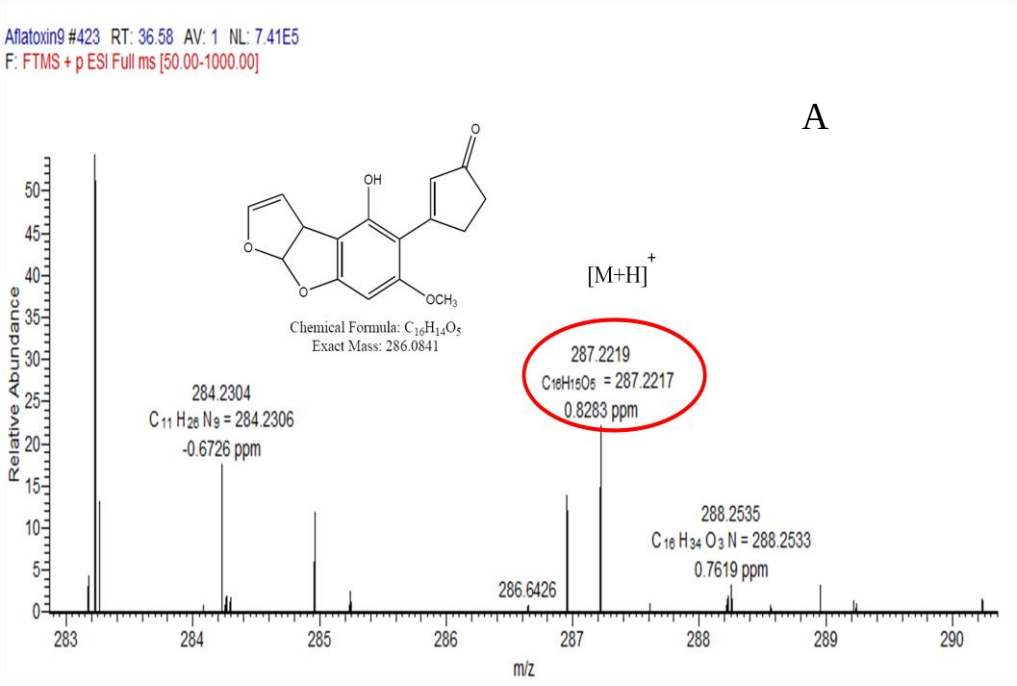


Figure 3.12-HRFTMS analysis to identify metabolites during the degradation of AFB1 by *R.erythropolis* at *m/z* 331.2845 $[M+H]^+$ (A1) against the blank which is the media (A)



A1

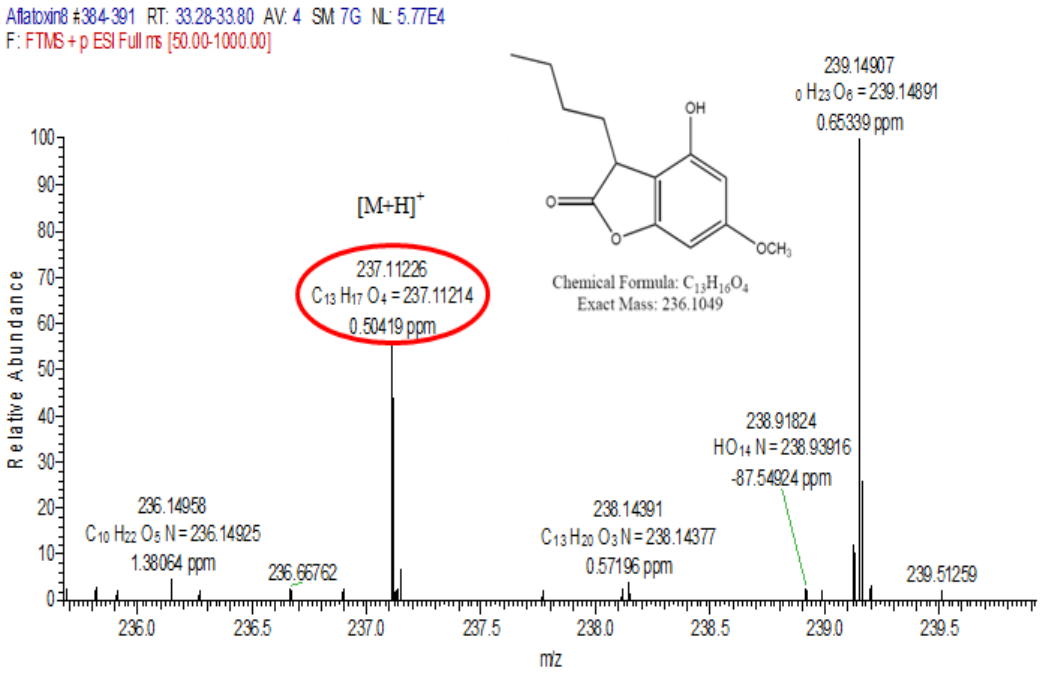
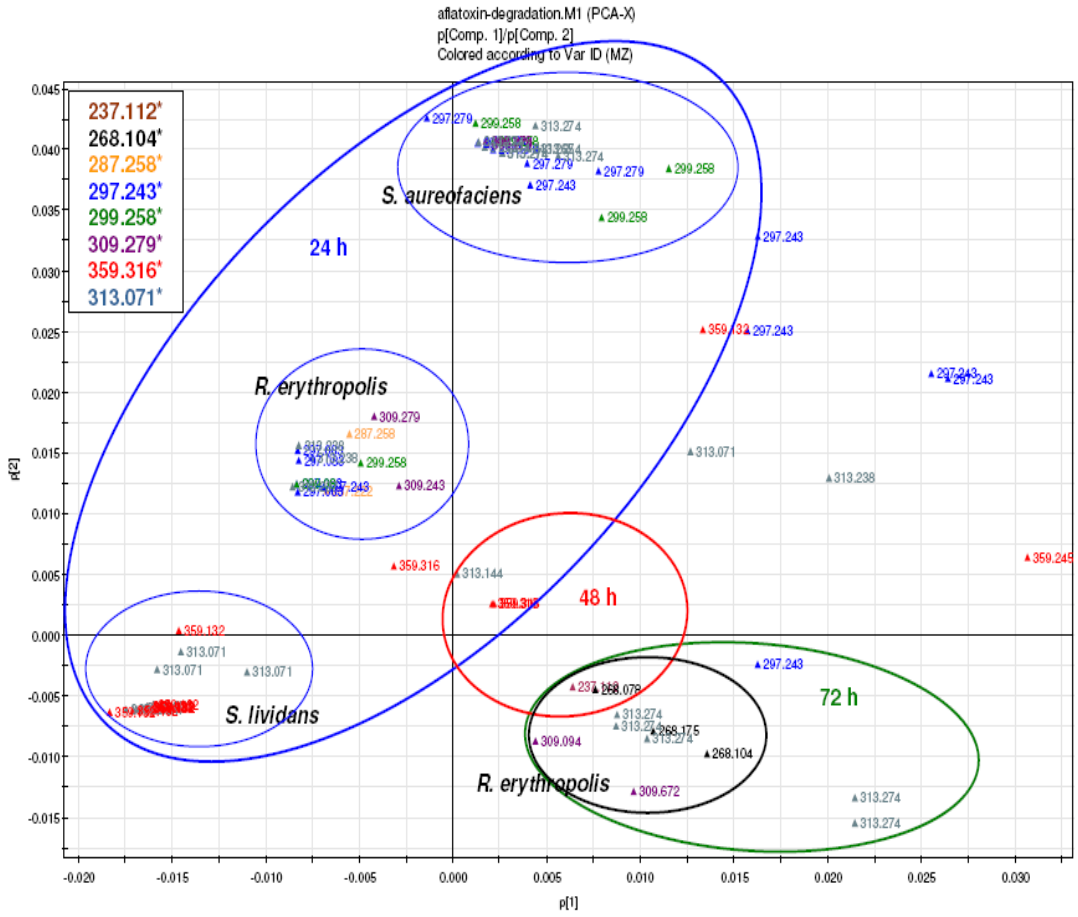


Figure 3.13 - HRFTMS analysis to identify the metabolites after degradation of AFB1 by *R. erythropolis* as indicated at *m/z* 287.2217 $[M+H]^+$ (A). and 237.1121$[M+H]^+$ (A1).

Figure 3.14 shows SIMCA analysis for the occurrence of different metabolites together with the presence or absence of AFB1 as determined by the MW. It was clearly shown that the metabolites were clustered together according to each microorganism used. It was also observed that the metabolites for *R. erythropolis* at 48 and 72 h clustered separately from the other two microorganisms. In addition, as generated by the SIEVE data which is shown in Figure 3.15a metabolite with a 236 amu is being produced over a period of 72 h. It is clear that the concentration of this particular metabolite increased as AFB1 decreased within the time.

Figure 3.14 show the correlation between AFB1 peak area and the presence as well as the disappearance of certain ion peaks representing a particular metabolite. The purpose of the correlation study was to identify which compound is related to AFB1 degradation. Positive and negative correlations were found between AFB1 and selected metabolites which could have been generated during the degradation process. A highly negative correlation was found for the ion peak *m/z* 237.1121 indicating significant effect ($p<0.05$) on AFB1 degradation. As the peak area of AFB1 was decreasing and the peak area of ion peak *m/z* 237. 1121 was increasing



*Marker molecular weights showing significant differences between control and treatment as determined by SIEVE analysis where m/z = 313.071 [M+H] was identified as aflatoxin B.

Figure 3.14- SIMCA analysis for different metabolites

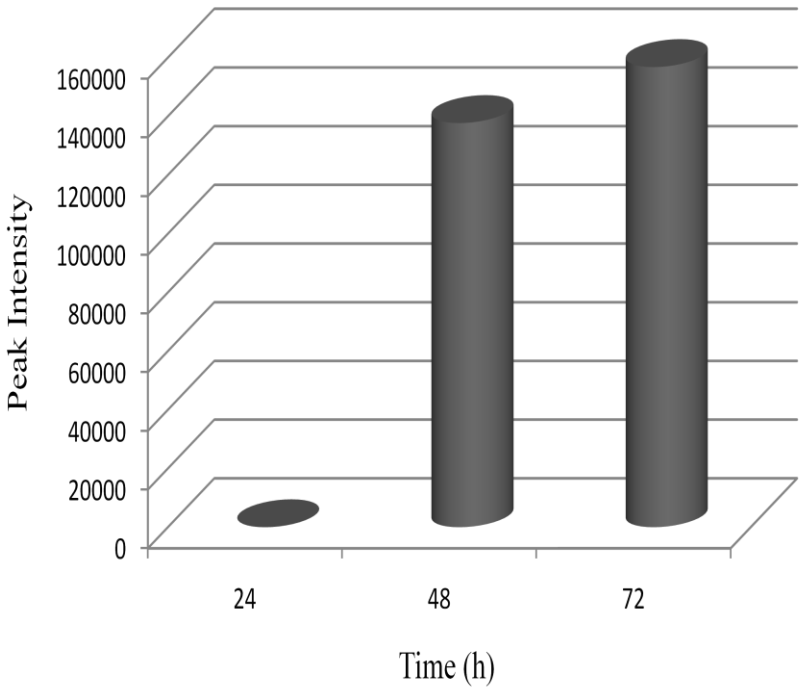


Figure 3.15- Sieve generation data for metabolites at 236 amu over a period of 72 h.

Table 3.5 -Correlation between AFB1peak area and presence/absence of certain metabolites peak area

Protonated molecule $[M+H]^+$ at *m/z*	Peak Area	Correlation	P value
331.2845	782442	-0.921	0.254
297.2428	2418033	-0.546	0.633
299.2584	117588	0.809	0.400
287.2219	115295	-0.645	0.263
269.2477	1488	0.916	0.554
237.1121	31685	-0.998	0.041

Figure 3.16 shows the first hypothesis to propose the mechanism for the degradation of AFB1 to a smaller metabolite of 236.1049 amu with the suggested molecular formula of $C_{13}H_{16}O_4$. We hypothesise that detoxification of AFB1 may involve the formation of the β-keto acid structure catalysed by enzymes produced by *R. erythropolis*, by which the lactone ring is hydrolysed resulting to a 330 amu metabolite (3.16-I). The hydrolysis is followed by the decarboxylation of the subsequent opening of the lactone ring yielding 286 amu (3.16-III) which is known as AFD_1. This involves the formation of AFD_2 at 206 amu (3.16-IV) which retains its difuran moiety but lacks both the lactone carbonyl and cyclopentenone ring of the AFB1 molecule. The enzymatic procedure may involve cleavage of one furan unit yielding a furanolactone compound of 236 amu (3.16-VI).

Interestingly, the increase of the 236 amu metabolite was also associated with an increase in the fatty acid and glycol phosphate compounds. As shown in Figure 3.18 SIEVE data generated significant peaks at *m/z* 359 fatty acid (A), and 335 glyco-phosphate (B) metabolites over a period of 72 h. Figure 0.19 diagrammatically summarises the degradation of AFB1 to low molecular weight compounds prior to their participation into citrate cycle.

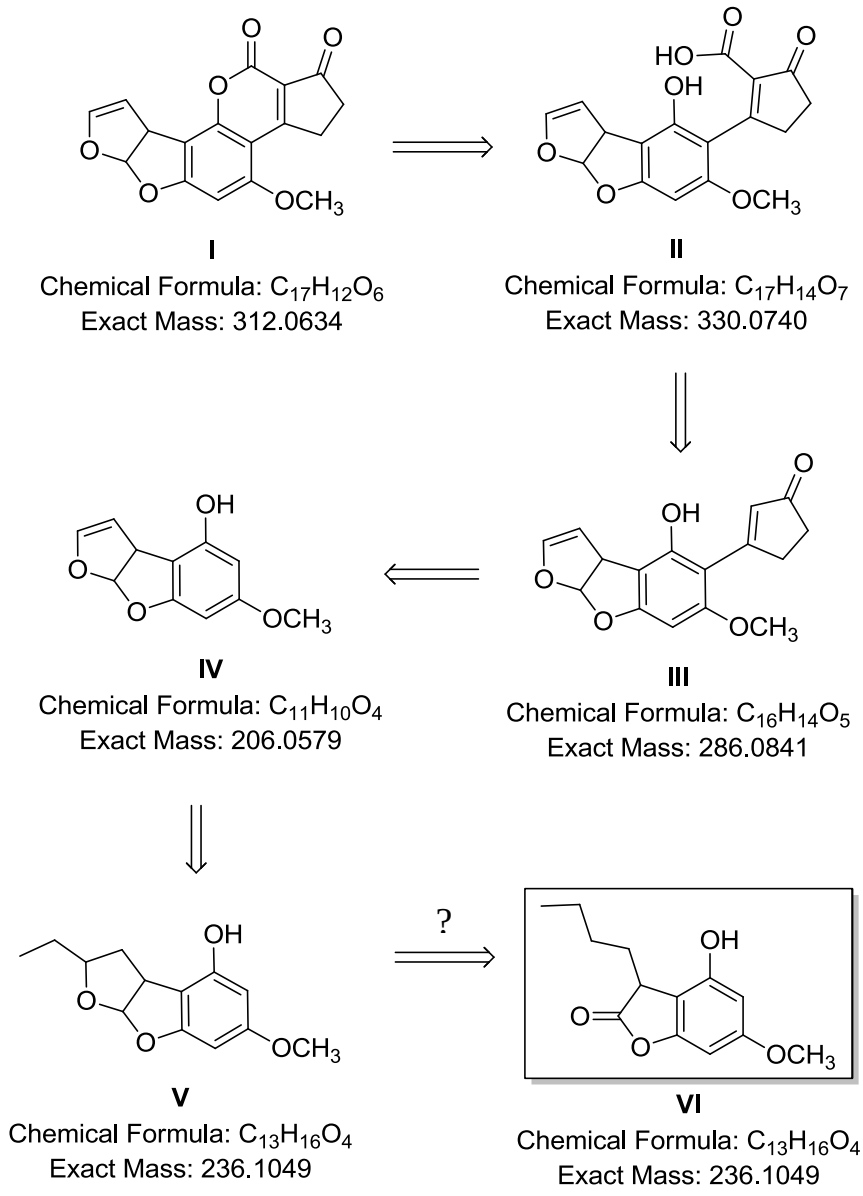


Figure 3.16 - Hypothesis of a proposed degradation mechanism for AFB1 to the 236.10 amu metabolite.

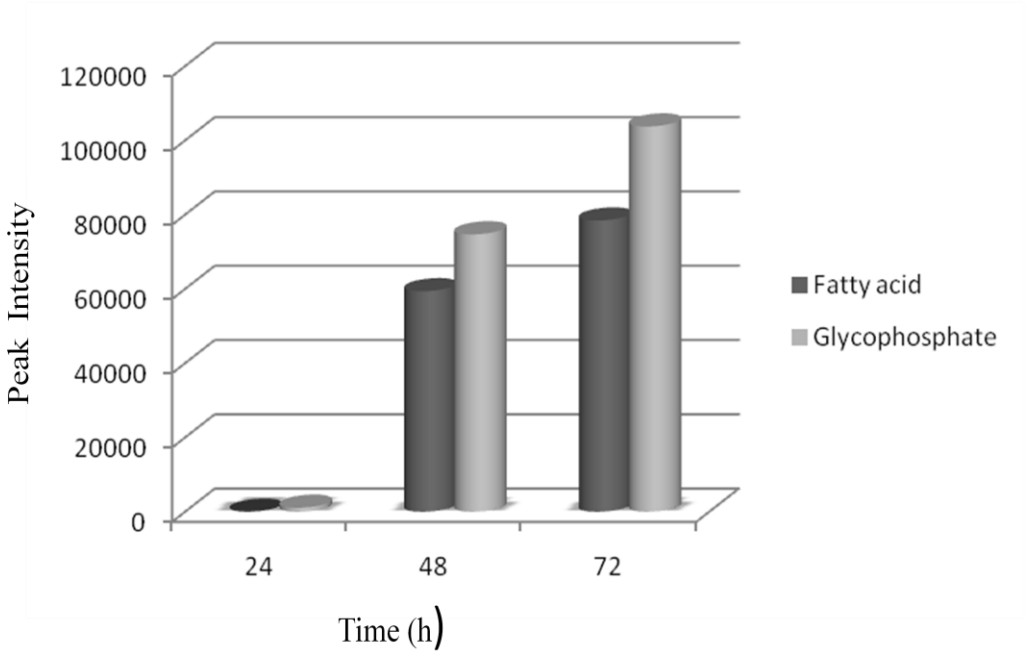


Figure 3.17- Data for m/z 359.3158 fatty acid, m/z 335.0667 [M+H] glycophosphate metabolites over a period of 72 h as generated by SIEVE.

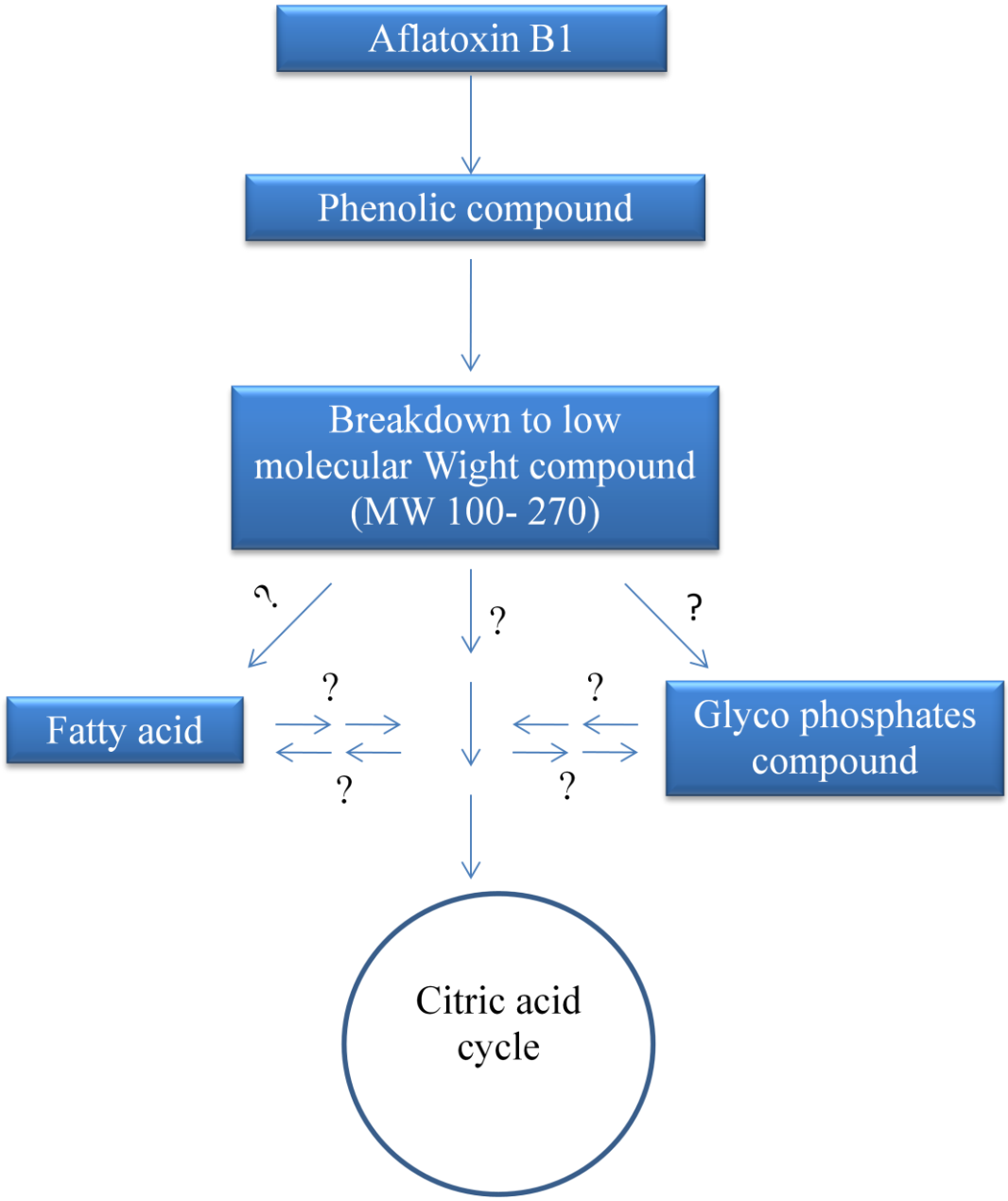


Figure 3.18 - Diagram for the possible degradation pathway for AFB1 by *R. erythropolis* cultures

3.9 - DISCUSSION

3.9.1 - AFB1 DEGRADATION

Biodegradation of aflatoxins, using microorganisms or their enzymes, is one of the recognised strategies to control aflatoxins in food and feeds (Wu *et al.,* 2009). *Flavoubacterium aurantiacum* NRRL B- 184, showed a high capacity of detoxifying AFB1. It was considered as the only bacteria able to degrade AFB1. But recently other soil microbes were also tested for degrading AFB1. These effective bacteria were limited to *R. erythroplis, Mycobacterium fluoranthenivorans, Flavobacterium aurantiacum* and *Stenotrophomonas maltophilia* (Teniola *et al.,* 2005, Alberts *et al.,* 2006, Guan *et al.,* 2008). This study extends the range of soil bacteria that have the ability to degrade AFB1 in vitro during the incubation. Evidence has been discovered in this research for AFB1 degradation by these microorganisms.

Biological degradation of AFB1 using three different strains of Actinomycetes was investigated in this study. The *R. erythropolis* strain was selected based on published data (Teniola *et al.,* 2005), while the *Streptomyces* strain were tested against AFB1 for the first time. This study utilised a higher concentration of AFB1, 20 µg/ml, in contrast to those presented in the literature which is between 1 to 5 µg/ml. The liquid culture of *R erythropolis* was able to degrade AFB1 highly effectively. A dramatic reduction of AFB1 was observed when incubated with the Actinobacteria cultures. Residual AFB1 were only about 4, 12, and 14% of intial concentration fter 24 h incubation with *R. erythropolis, S. lividans* and *S.aureofaciens,* respectively. Similar results were recorded by Teniola *et al.,* (2005) who investigated a biological degradation of AFB1 at lower concentration 1.75 µg/ml by four bacterial strains, *R. erythropolis* DSM 14303, *Nocardia corynebacterioides* DSM 12676, *N.*

corynebacterioides DSM 20151, and *Mycobacterium fluoranthenivorans* sp. nov. DSM 44556T. Liquid cultures and cell-free extract were used to degrade AFB1 where the latter 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 at 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 44556T have shown more than 90% degradation of AFB1 within 4 h at 30º C, whilst AFB1 was not detectable after 8 h. In addition, this work team also found *R.erythropolis* cultures were able to degrade lower concentration AFB1 after 72 h about 3-6 % was remaining (Teniola *et al.,* 2005). While Alberts *et al.,* (2006) reported that AFB1 was effectively degraded by extracellular extracts from *R. erythropolis,* with which only 32 % of original concentration of AFB1 was remaining after 72 h.

However, food stuffs normally contaminated with higher concentration of AFB1 than those mentioned studies. Therefore we investigated the ability of Actinobacter for degrade AFB1 at higher concentration 4 times or more than the mentioned studies 1000 times concentrated than the set regulation limit at the USA which is 20 µg/L(Richard, 2007)

3.9.2 - OPTIMISING AFB1 DEGRADATION CONDITIONS

The effect of different temperatures on AFB1 degradation by Actinomycetes cultures was also studied. A general degradation of AFB1 was observed for all the strains at different temperatures. The optimal degradation temperature was 30ºC at pH 6.0 for all the strains. In addition, *Streptomyces* strains had no significant difference in

AFB1 degradation between 25 and 30ºC. This may be because of the wide range of enzymes produced from these microorganisms.

This result agrees with Guan *et al.,* (2008), as they recorded that no significant difference was observed between 20 and 30ºC when *S. maltophilia* 35-3 was used to degrade AFB1. Moreover, Ciegler *et al.,* (1966a) reported 25 ºC as the optimum temperature for *Flavobacterium aurantiacum* to breakdown AFB1. While Teniola *et al.,* (2005) reported that AFB1 degradation by cell free extracts of *R. erythropolis* and *M. fluoranthenivorans* were about the same between 10 and 40 ºC. They proposed that either the enzymes in the extract had a wide temperature range of activity or that other factors were involved in the degradation. Moreover, extracellular enzymes of *Pleurotus ostreatus* showed AFB1 degradation activity. The optimum activities were found at 25 ºC and pH 4 and pH 5 (Motomura *et al.,* 2003). The results in this study have also described that the optimal degradation temperature was at 30 ºC and pH 6.0 for *R. erythropolis* ATCC 4277 while the optimal degradation temperature of 30 ºC and pH 5.0 showed quickest degradation of AFB1 treated with *Streptomyces* strains.

The effect of initial culture pH on AFB1 degradation over the first 24 h incubation at 30 ºC was examined using both basic and acidic pH culture conditions. The results indicated that AFB1 was not stable in all the pH used in this study. The pH 5 and 6 was the most effective in AFB1 degradation while pH 7 had no significant difference from the pH 6 when *R. erythropolis* was used to degrade AFB1. *Streptomyces* strains showed the most rapid degradation at pH 5.

The relationship between degradation of AFB1 and pH is characteristic of an enzymatic reaction. Enzymes have an optimum pH range for maximal activity. Enzyme activity decreases if the pH changes to above or below the optimum pH. The enzymes are influenced by the state of its ionisation groups and the function of the active centre may likewise depend on this. Enzymes pH activity profiles vary different from one enzyme to other but the optimum is often around natural pH. Similar results were reported by Ciegler *et al.,* (1966, 1966a) the optimum pH for AFB1 degradation has been reported to be pH 6.75 with degradation a rate of 1.3 µg/h by *Flavobacterium auranticum* while the rate of degrading AFB1 was decreased to 0.6 and 0.8 µg/h when incubated at pH 5 and 8 respectively.

Also Smiley and Draughon (2000) reported that the maximum degradation of AFB1 by *Flavobacterium auranticum* was observed at pH 7, with some AFB1 degradation occurring at pH levels as low as 5 and high as 8. Moreover, acidic pH levels were more detrimental to the ability of the crude protein extracts from *Flavobacterium auranticum* to degrade AFB1 than basic pH. Crude protein extract degraded approximately 25% of 20 µg/ml AFB1 at pH5 (Smiley and Draughon, 2000). Those results were correlated with the result of this study as the most efficient degradation was also around a natural pH. On other hand, Guan *et al.,* (2009) reported that the maximum AFB1 degradation by *S. maltophilia* 35-3 in this study was observed at a basic pH 8, this observation had higher pH compared to that found in the present study.

A dramatic reduction in AFB1 concentration was observed during incubation in the presence of *R. erythropolis* ATCC 4277. After two and half hours, 50% was degraded, whereas with *S. lividans* TK 24 and *S. aureofaciens* ATCC10762

degradation was 30 and 20%, respectively. Similar results were determined by (Teniola *et al.*, 2005). Their results indicate that 90 % was degraded after only 4 h incubation with cell free extract of *R. erythropolis* whilst, after 8 h AFB1 was practically not detectable. Furthermore, culture supernatant of *S. maltophilia* showed strong degradation activity where about 78.8 % was degraded after 72 h incubation (Guan *et al.,* 2008).

3.9.3 - CONFIRMATION OF AFB1 DEGRADATION

Four techniques were used to confirm the degradation; TLC, Reverse phase HPLC, ion-trap ESIMS, and HRFTMS. The TLC result confirmed AFB1 degradation. The amount of AFB1 fluorescence decreased within the first 24h and disappeared after 72 h. However, it is know that opening the difuran ring will not affect the molecules florescence while opening or abolishing the lactone ring dose effect the fluorescence. Lee *et al.,* (1981) reported that AFB1 fluorescence is associated with the presence of an intact lactone ring. So the decrease of the fluorescence indicated cleavage of the lactone ring. Similar results were reported by (Motomura *et al.,* 2003). They exhibited the occurrence of enzymatic degradation by using extracellular enzymes from the edible mushroom *Pleurotus ostreatus.* However, practical application of these fungi may be limited by factors such as long incubation time over 120 h and active extract is required. However, this lactone structure is associated with carcinogenic activity, therefore it is important to breakdown the ring (Bol and Smith, 1989).

3.9.4 - AFB1 DEGRADATION HYPOTHESIS

The third aim of this research was to define a possible degradation mechanism procedure of AFB1 by liquid cultures of *Rhodococcus*. The HPLC, ion-trap ESIMS, and HRFTMS confirmed the cleavage of lactone group, as the peak area designated for AFB1 was decreasing over the time. Meanwhile, the LC-ESIMS indicated that another metabolite was being produced during AFB1 degradation. These results were consistent with the HRFTMS results.

The first hypothesis involves the degradation of AFB1 to another compound with chemical properties different from AFB1. Therefore, the HPLC run was set for 40 min to account for the formation of new metabolites. The second hypothesis on AFB1 degradation involves conversion of into AFB1 another analogues. Alberts *et al.,* (2006) indicated that AFB1 was most likely metabolised to degradation products with chemical properties different from those of AFB1 but they could not reveal the formation of any breakdown products through electron spray mass spectrometry.

High resolution FTMS results in this research were indicative of the formation of new metabolites along with the degradation of AFB1. The mass spectrum of the treated sample showed intense pseudomolecular ion peak values at *m/z* 331.0707, 287.2219, 237.1211 as well as that of at *m/z* 313.0707 attributable to residual AFB1. These ions were not present in the mass spectrum of either the reference sample of pure AFB1 or in the control samples. As a result, these metabolites inferred as degradants achieved during the culture treatment.

Consequently, the hypothesise for AFB1 detoxification may involve the formation of the β-keto acid structure catalysed by enzymes produced by *R .erythropolis,* followed by hydrolysis of the lactone ring resulting to a metabolite with 330 amu (3.16-II). The hydrolysis was followed by decarboxylation of the open lactone ring yielding to 286 amu (3.16 -III) which is known as AFD_1. This involved the formation of 206 amu (3.16- IV)' which is AFD_2, where the difuran moiety was retained while the lactone carbonyl and cyclopentenone ring characteristic of AFB1 molecule dissappears. The enzymatic procedure involved cleavage of the unsaturated part of one difuran unit (3.16-VI) yielding a furanolactone phenolic metabolite of 236amu (3.16-VI).

In another work by Mendez-Albores *et al.,* (2005,2009) reported chemical inactivation of AFB1 and aflatoxin B_2 (AFB_2) in maize grain by means of 1N aqueous citric acid. Their hypothesis also entailed detoxification of AFB1 by formation of the β-keto acid structure catalysed by enzymes produced by *R.erythropolis,* followed by hydrolysis of the lactone ring yielding AFD_1. This may involve the formation AFD_2.

In addition, Suttajit, (1989) reported chemical degradation of AFB1 using ammonia. The hypothesis involved catalysing the lactone group to produce ametabolite of 329 amu, followed by hydrolysis yielding β-keto acid of 330 amu followed by cleavage in presence of high temperature yielding AFD_1, AFD_2, and many unidentified small molecular weight compounds <200 amu. AFD_1 undergoes further degradation by sublimation at 220-340ºC yielding an unknown compound of 236 amu or a metabolite of 256 amu.

Few studies on AFB1 biodegradation, but none of which showed a complete degradation mechanism. Most of the proposed mechanisms in the biological system involved biotransformation of AFB1 into its derivatives although specific enzymes that are able to degrade AFB1 have been purified (Wu *et al.,* 2009). Novel AFB1 degradation enzymes had been isolated and purified from *Pleurotus sp* culture (Motomura *et al.,* 2003). Theses enzymes were able to degrade AFB1. The fluorescence measurements suggested that the specific enzyme cleaved the lactone ring of AFB1, although the degradation products of AFB1 were not investigated clearly as the technique used was limited so they could not reveal any intermediate products (Motomura *et al.,* 2003).

In other research Liu *et al.,* (1998), a multi-enzyme from *Armillariella tabescense* was isolated and purified. These enzymes were capable of degrading AFB1 where the optimum activity was at 35 ºC, pH 6.8. Their proposed pathway indicated the degradation of AFB1 by multi-enzymes: AFB1 was first transformed to its epoxide followed by hydrolysis of the epoxide to give a dihydrol. Then, the difurann ring would open in subsequent hydrolysis step. However, the conversion of AFB1 to its epoxide is unlikely as it is more toxic and carcinogenic.

Furthermore, other fungi like *A. flavus*, *A .niger* was able to transform AFB1 to other AFB1 analogues such as AFB_2, AFB_{2a} and $AFR_{0.}$ However, all these derivatives are still toxic while some of these moulds are pathogenic. The degradation of AFB1 by members of the *Rhodococcus* genus using a cascade of enzyme reactions which resulted in a loss of fluorescence over time was described by (Teniola *et al.,* 2005, Alberts *et al.,* 2006). In addition, various enzymes produced by

R. erythropolis are involved in catabolic pathways of aromatic compounds such as polychlorinated biphenyls enzymes, which included ring cleavage, biphenyl dioxygenases, dihyrodiol dehyrogenases and hydrolases (Martinkova *et al.*, 2009).

The genes coding for these enzymes were clustered and degradation occurred via a cascade of reactions. Additionally, according to Martinkova *et al.*, (2009) the *R. jostii* RHA1 genome sequence, 26 peripheral pathways and 8 central pathways are involved in the catabolism of aromatic compounds. Furthermore, all the central pathways for aromatic degradation ended with the citrate cycle (Martinkova *et al.*, 2009). The interesting results were detected by HRFTMS analysis. AFB1degradation was associated with the accumulation of intermediates of fatty acid metabolism, and glycolysis.

AFB1 is a polyaromatic compound and the degradation may occur in the same pathway as mentioned for aromatic compound. Therefore, the results from this research demonstrate that enzymatic degradation by Actinomycetes seems to be a promising opportunity for degrading AFB1 in foods and feed process.

3.10 - CONCLUSIONS

AFB1 degradation by Actinomycete cultures was significant ($p<0.05$). The degradation was rapid 50 % of initial concentration of AFB1 was degraded within 2.5 h in presences of *R. erythropolis* ATCC 4277cultures. The degradation by those microorganisms was achieved at rang of intial pHs thus making them applicable in food processing. No significant difference has been found between the three cultures in terms of ability to degrade AFB1 over a period of 72h.

Each microorganism has a different way in degrading AFB1. The metabolites produced during AFB1 degradation by *R. erythropolis* were significantly different from those produced during degradation by the other two microorganisms. TLC assay has confirmed the cleavage of the lactone group by *Rhodococcus.*

Two hypothetical degradation mechanisms for AFB1 by *R. erythropolis* have been proposed the degradation of AFB1 appears to be associated with the increase fatty acid and glyco-phosphate metabolites. Further investigation in enzymology will establish the degradation mechanism by these microorganisms, as well as evaluate the toxicity of the intermediates and degradants.

CHAPTER 4

CYTOTOXICITY ASSAYS ON BIO-TREATED AFB1- CONTAINING MEDIA

4 – INTRODUCTION

Dangerous mycotoxins are naturally present in food, feed and the environment. They are pathogenically classified as hepatotoxins, nephrotoxinns, vomitoxins neuromuscular toxins and immunotoxins, some of which are potentially carcinogenic and mutagenic (Caloni *et al.,* 2006, Li, 2009, McKean *et al.,* 2006b). Aflatoxins, for example, are the most potent heptocarcinogen and mutagen among mycotoxins (McKean *et al.,* 2006a, Schaaf *et al.,* 2002).

Aflatoxin B1 requires metabolic activation by human cytochrome P450/ microbial cytochrome P450 to AFB18,9-epoxide for cytotoxic and genotoxic/carcinogenic effects (Li, 2009). After the biotransformation of AFB1 to AFB18,9-epoxide by a bio-activation system, and subsequent covalent binding to DNA or proteins, the carcinogenic potential is exerted (Palanee *et al.*, 2000). Therefore, AFB1 toxicity is influenced by the balance between activation and detoxification of enzyme systems (Palanee *et al.,* 2000). However, typically the toxic effects of unknown compounds have been measured *in vitro* by counting viable cells after staining with a vital dye or by mutagenicity assay (Alberts *et al.,* 2009, Sigma-Aldrich, 2006). Alternative methods including the measurement of cellular activity such as MTT (methyl tetrazolium) and LDH (lactate dehydrogenase) assays were employed to evaluate the toxic effect (Mosmann, 1983).

The MTT assay has been used for numerous medical, microbiological and toxicological tests (Cole, 1986, Saito *et al.,* 1994, Palanee *et al.,* 2000). The assay uses the fact that mitochondrial succinic dehydrogenase in viable cells metabolizes

the yellow, water-soluble MTT to a blue, water insoluble MTT formazan derivative. Meanwhile, the lactate dehydrogenase (LDH) assay uses the fact that the cellular enzyme LDH, which is abundant and relatively stable in viable cells, is released into the medium by the disruption of cell membranes, thereby decreasing cellular LDH activity (Anuforo *et al.*, 1978; Sasaki *et al.*, 1992). When the period of treatment with a chemical is short and when the enzyme released is not directly inhibited by the chemical, leakage of LDH into the culture medium has been shown to be an efficient marker of cytotoxicity (Sasaki *et al.*, 1992). Therefore, the assay measures either the number of cells via total cytoplasmic LDH or membrane integrity as the function of the amount of cytoplasmic LDH released into the medium (Sigma-Aldrich, 2006).

However, these assays utilized cell lines extensively in toxicological research to elucidate the degree of chemical toxicity as well as the mechanism of action. Hepatocyte cell lines would be valuable in mutagenicity and carcinogenicity studies, biochemical studies of the metabolism of the drugs and hepatoxicity and general cytotoxicity screening (Yoneyama *et al.*, 1987). Human hepatoma HepG2, a well differentiated transformed cell line, is a reliable model, easy to culture, well characterized and widely used for biochemical and nutritional studies where many antioxidants and conditions can be assayed with minor inter assay variations (Alía *et al.*, 2006).

4.1 AIMS OF THIS RESEARCH

The main aim of this research was to investigate the toxicity of the modified AFB1, the new compound preformed during the biological detoxification by an

Actinomycete. Comparison of the two methods in terms of the cytotoxicity of the modified AFB1 or the new compound using human cell lines HepG2 was essential in achieving this aim.

4.2 - MATERIALS AND METHODS

4.2.1 - CELL CULTURE EQUIPMENT

The tissue culture hood was supplied by ICN Gelaire (England), the incubator supplied by Heraeus (Germany) and carbon dioxide (CO_2) cylinders supplied by BOC gases Ltd, Manchester,UK.

4.2.2 - CHEMICALS AND MEDIA

Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Phosphate buffered saline, fetal bovine serum (FBS), sodium pyruvate solution (SPS), non essential amino acid solution (NEAA) and trypsin-EDTA solution. Dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich Company Ltd. Dorset, UK. *In vitro* toxicology assay kit, lactic dehydrogenase based (TOX 7) were purchased from (Sigma-Aldrich company Ltd. Dorset UK) LDH Assay solution, LDH assay cofactor, LDH assay dye solution and LDH assay lysis solution (Sigma-Aldrich company Ltd, Dorset, UK).

4.2.3 - STORAGE AND CULTIVATION OF ACTINOMYCETE STRAINS

The storage and cultivation of the actinomycete strains is shown in section 3.4.1.

4.2.4 - CULTIVATION AND STORAGE OF HEPG2 CELLS

HepG2 is a human liver carcinoma cell line which was derived from the liver tissue of a 15 year old Caucasian American male with a well differentiated hepatocellular carcinoma (American Type Culture Collection (ATCC) HB-8065). The cells were kindly provided by Dr Elizabeth Ellis at passage no 25 (Institute of Pharmacy and Biomedical Sciences, Strathclyde University, Glasgow, UK). The cell monolayer HepG2 was dissociated from a 75cm^2 culture flask. The cells were trypsinized and incubated at 37ºC 5% CO_2 95% air for 5 min. Then 5 ml of DMEM was added into the flasks to remove all the trypsinized cells. Cells were centrifuged (MSE Mistral 2000, MSE Ltd, UK) at 4000 rpm for 5 min, the supernatant removed and the cells re-suspended in fresh medium DMEM 40% (v/v) supplemented 50% (v/v) of FBS, 40% (v/v) and 10% (v/v) of DMSO stored at - 80ºC.

4.2.5 - AFLATOXIN B1 DEGRADATION BY ACTINOMYCETE IN LIQUID CULTURE

The AFB1degradation assay is described at section 3.4. 2. The high resolution mass spectroscopy technique LTQ-Orbitrap used to quantify the leftover of AFB1 – the full method is described in section 3.5.5.

4.2.6 - CYTOTOXICITY ASSAY

To examine cytotoxicicty, two methods (MTT and LDH) were used to evaluate the toxicity of the residual of AFB1. A 75cm^2 culture flask of confluent HepG2 cell line was trypsinised and the cells suspended in DMEM (5 ml), the supernatant removed and the cells re-suspended in fresh medium DMEM. The cells were then counted under the microscope by using hemocytometer, then 1ml of medium containing the

cell was re-suspended into fresh medium to give a cell number of 3×10^3 cell /ml for the MTT assay while 1×10 cell /ml ^{6} was used as the final concentration of cells for the LDH assay.

4.2.6.1 - MTT ASSAY

Aliquots of the cell suspension (100µl) were dispensed in each of the 96 wells of the microtiter plate to give a final cell number of 3000 cell/well. The plate was incubated at 37ºC, 5% CO_2 for 24 h; then the media aspirated from the 96-wells; and replaced by 100 µl fresh medium DMEM containing AFB1 residual or AFB1 20 (µg/ml). Following 24 h incubation, 20 µl of MTT solution 1.2 mg/ml was added to the well and incubated at 37ºC, 5% CO_2 for 4 h conditions. After that, the cells were treated by DMSO for 1 h to dissolve the resulting formazan crystals. The optical densities of the cells were measured spectrophotometrically at fixed wavelength of 570 nm (Labs systems IEMS reader MF, Finland).

The control used was cells without treatment suspended in DMEM medium; each test included a blank which is DMEM media without cells. Three sets of experiments were used to evaluate the cytotoxicity effects in HepG2. The percentage of the viability was calculated using the following equation:

EQUATION- 4.1:

$$\text{VIABILITY (\%)} = \frac{\text{ABSORBANCE OF TREATED SAMPLE}}{\text{ABSORBANCE OF CONTROL}} \times 100$$

4.2.6.2 - LDH ASSAY

Aliquots of the cell suspension (500µl) were dispensed in each of the 24 wells of the microtiter plate to give a final cell account of 1×10^6 cell/well and incubated at 37°C, 5% CO_2 for 48h. Cells were pre-treated with the different concentrations of modified AFB1 for 24h. To measures LDH released into the media; the media was removed by centrifuging the plates at 250×g for 4 min to pellet the cells (Jouan BR4, DJB Labcare Ltd, Buckinghamshire UK). The aliquot was transferred into 96-well plate and treated with an enzyme mixture (Lactate dehydrogenase assay mixture was prepared by mixing equal amounts of LDH assay substrate cofactor and dye solutions) at 25ºC for 30 mins.

To measure LDH containing cells: 10µl of lysis solution was added to the cells and incubated for 45 min at 37°C, 5 % CO_2; then the plates were centrifuged at 250×g for 4 min to pellet the debris. The intracellular was removed and transferred to 96-well and treated with the enzyme mixture for 30 mins. The reaction was terminated by adding 10 µl of 1 N of HCl. The LDH was released into the medium and the LDH released from the cells was measured spectrophotometrically at a wavelength of 490 and 690 nm using a micropalte reader (Labs systems IEMS reader MF, Helsinki, Finland). Lactate dehydrogenase (LDH) leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content using the followed equation:

EQUATION - 4.2:

$$\text{LDH LEAKAGE (\%)} = \frac{\text{LDH RELEASED INTO MEDIA}}{\text{TOTAL LDH}} \times 100$$

4.3 - EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Three separate experiments, performed in triplicate, were carried out for each assay, and results expressed as means ± standard deviations (SD). Statistical evaluation was performed by one way ANOVA. Levels of significant ($P<0.05$) were considered significant. One way ANOVA was followed by post test Tukey's Multiple Comparison of all the statistical analysis which was carried out by Graphpad prism 5.

4.4 - RESULTS

4.4.1 - BIODEGRADATION OF AFB1 BY ACTINOMYCES CULTURES

Figure 4.1 shows the residue of AFB1in the liquid culture at 30ºC over a period of 72 h in the presence *of R. erythropolis* which is indicated as [A], [B] for *S. lividans* and [C] for *S. aureofaciens*. It is obvious that all the strains were able to degrade AFB1. 0.29 (µg/ml) of AFB1 remained after 72h at 30ºC and pH 6 in the presence of *R. erythropolis* while 0.6 (µg/ml) and 4.05 (µg/ml) remained when the *S. lividans* and *S. aureofaciens* respectively were used.

The statistical analysis one way ANOVA indicated that there was an overall significant difference between different treatments ($P<0.0001$) (see Table 4.1[A]). Moreover, post test Tukey's Multiple Comparison indicated that no significant differences ($P>0.05$) between AFB1 residual at concentration 3.85 (µg/ml) and 1.6 (µg/ml) while there was a significant difference ($P<0.05$) between the 3.85 (µg/ml) and 0.29 (µg/ml). No significant difference was found between the AFB1 residual at concentrations 1.6 (µg/ml) and 0.29 (µg/ml) (see Appendix 4.1 [A]).

Remaining AFB1 was higher in term of concentration when the toxin was treated with *Streptomyces* strains; *S.lividans* was more effective than *S. aureofaciens* in degrading AFB1 (see Figure 4.1 [B] and [C]). Statistically a significant difference ($P<0.0001$) was found between the control and the treatments (see Table 4.1[B] and [C]).

The post test showed that no significant difference was found between modified AFB1 from a biodegradation process for 48 and 72 h in both *Streptomyces* (see Appendix 4.1 [B] and [C].

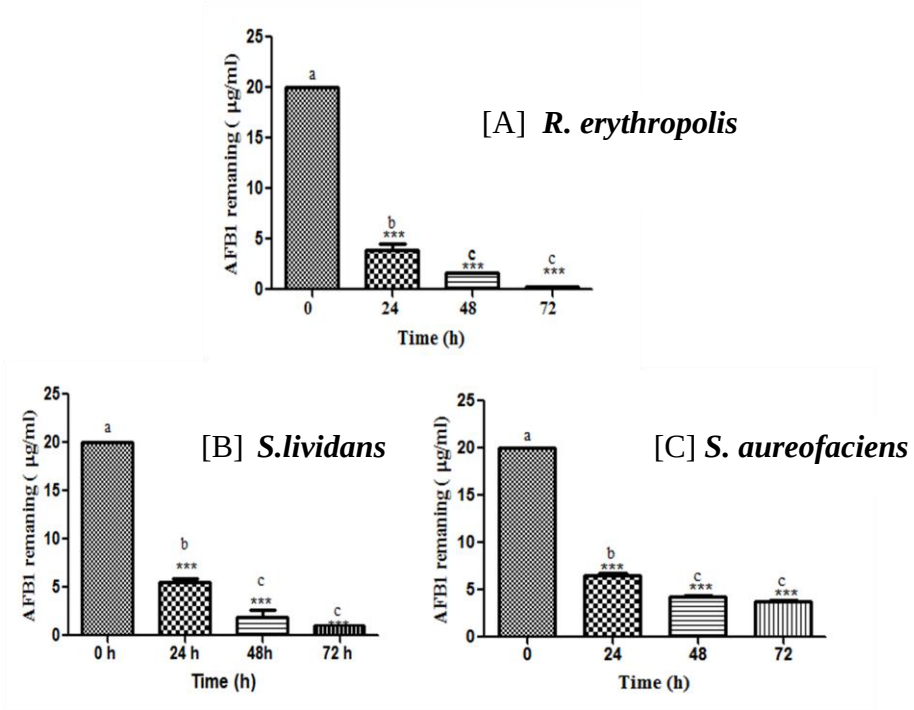


Figure 0.1 - Remaining AFB1 in the liquid culture over a period of 72 h at 30 º C in presence of *R. erythropolis* **[A]**, *S. lividans* **[B]**, *S. aureofaciens* **[C]**. Treatments with different letters in each column are statistically different by each treatment (*** P≤0.0001).

Table 4.1 - Statistical Analysis one way ANOVA for residual AFB1 from biodegradation process.

One-way analysis of variance						
R.erythropolis [A]	SS	df	MS	F	R^2	P value
Treatment (between columns)	755.4	3	251.8	717.4	0.9963	< 0.0001
Residual (within columns)	2.808	8	0.3510			
Total	758.2	11				
S .lividans [B]						
ANOVA Table	SS	df	MS	F	R^2	P value
Treatment (between columns)	698.8	3	232.9	428.8	0.9938	< 0.0001
Residual (within columns)	4.345	8	0.5432			
Total	703.1	11				
S. aureofaciens [C]						
ANOVA Table	SS	df	MS	F	R^2	P value
Treatment (between columns)	527.3	3	175.8	2829	0.9991	< 0.0001
Residual (within columns)	0.4970	8	0.06213			
Total	527.8	11				

4.4.2 - MTT RESULTS

HepG2 cells exposed to AFB1 20 µg/ml and incubated at 37ºC and 5% CO_2 for 24 h, caused a marked decrease of the number of viable cells to 16% of the control level (see Figure 4. 2). Conversely, the percentage viability of the cells increased with the decreasing AFB1 concentration; 57 % of HepG2 was viable when the cells were exposed to AFB1 residual 3.85 (µg/ml) from 24 h bio-treatment.

The percentage of HepG2 cells viability was increased to reach 75.8 % when the cells were treated by AFB1 residual 0.2 (µg/ml) from 72h biodegradation treatment in the presence of *R. erythropolis* (see Figure 4.2 [A]). An overall significant difference existed between the treated cells with the remaining AFB1 and the control (P =0.0003). Post test Tukey`s multiple comparison shows the differences between the different treatments. Treatments with different letters in each column are statistically different by each treatment. AFB1 residual at concentration 0.2 (µg/ml) was not significantly different (P>0.05) compared to the control, which in turn reflected a decrease in the toxicity of this potent mycotoxin compared to AFB1 sample at 20 (µg/ml) doses.

The cytotoxicity effects were clear in HepG2 when the cells were exposed to AFB1 residual from the biodegradation process in the presence of *Streptomyces* strains. HepG2 viability was between 44.8 to 66.3 % of control in *S.lividans* case, while only 39.4 to 44.75% was viable in *S. aureofaciens* case. Meanwhile, HepG2 viability was higher compared to treated cells by 20 (µg/ml) (see Figure 4.2 [B]) but the cytotoxicity effect was still higher compared to the control. Statistically, there was an

overall significant difference ($P<0.0001$) between the control and treatment samples in terms of the cytotoxicity effect in HepG2. The statistical post test showed the cytotoxicity effect in HepG2 was not significantly different ($P>0.05$) at 1.9 and 0.6 consecutively in *S.lividans* case.

The cytotoxicity effect in HepG2 cells was higher when HepG2 cells were treated by AFB1 residual following biodegradation process by *S. aureofaciens* (see Figure 4.2 [C]).While when the cells treated by AFB1 residual from *S. aureofaciens* degradation the level of the toxin was decreased, but the toxicity of the compound was higher and comparable to AFB1 20 (µg/ml) cytotoxic effects. Those results were confirmed by the statistical analysis as no significant difference was found between AFB1 residual and AFB1 20 (µg/ml) in terms of the cytotoxicity effects in HepG2; while it is significantly different ($P<0.05$) from the control.

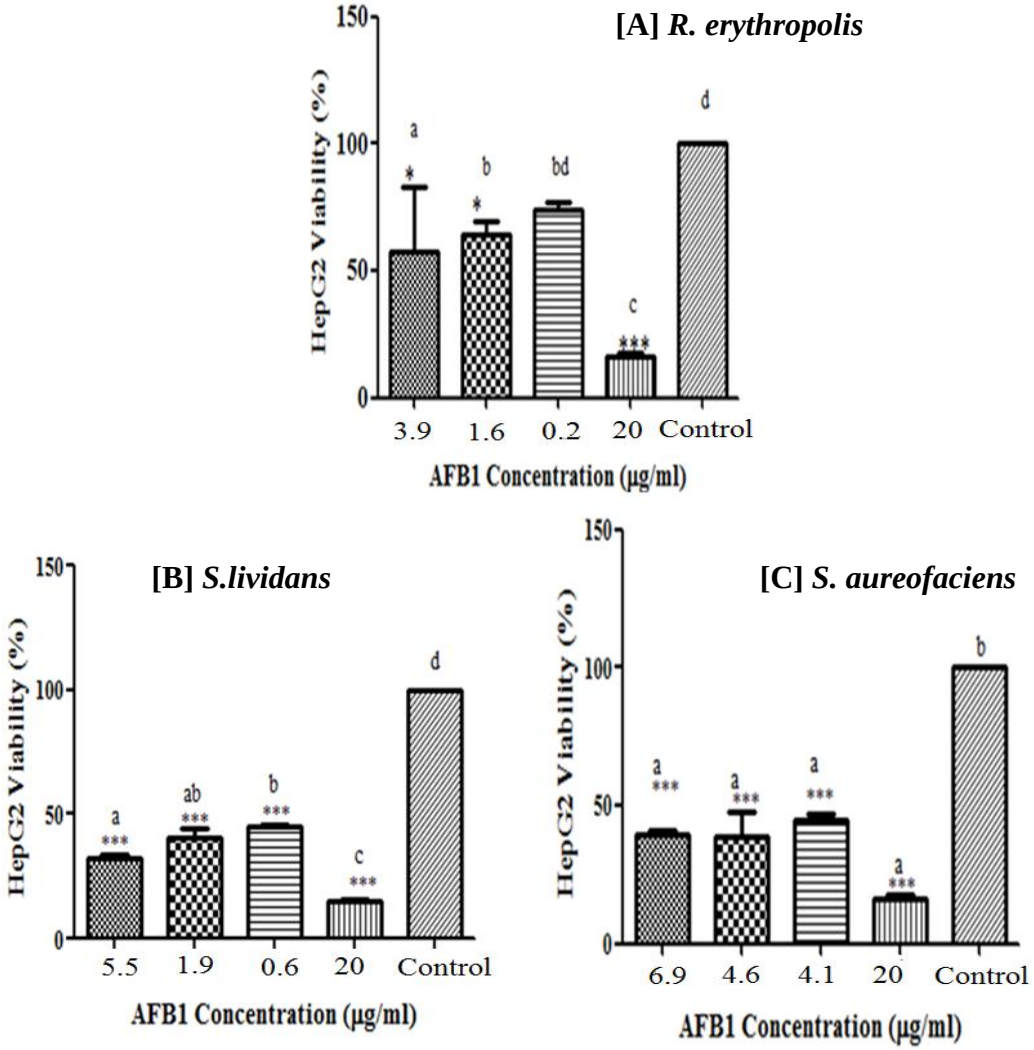


Figure 4.2 -Effect of AFB1risdiual (µg/ml) on cellular viability in HepG2 cell line. Values represent mean viability as % control ± SD (n=3). The treated cells were incubated at 37ºC 5% CO_2 for 24h. The cytotoxicity assay following a biological degradation of AFB1by [A] *R. erythropolis* [B], *S.lividans*, [C] *S. aureofaciens* . ***P ≤0.0001 as compared with control. Treatments with different letters in each column are statistically different by each treatment *** P≤0.0001.

4.4.3 LDH RESULTS

Figure 4. 3 Effect of residual AFB1 on cell viability and intracellular concentrations. The percent leakage of lactate dehyrogenase in the control was 23.8%, compared with an increase to 42.5 % of LDH activity in the cell culture medium when HepG2 was pre-treated by 20 (µg/ml) AFB1, indicating cell damage in HepG2. in the meantime, the LDH activity was slightly higher (31.3%) when the cells treated with 3.9 (µg/ml) residual AFB1 from a biotreatment by *R.erythropolis* cultures were compared to the control, then the percentage of the leakage was decreased to 29 and 30 when HepG2 cells were treated by 1.6 and 0.6 (µg/ml) respectively. The statistical analysis ANOVA for LDH leakage at different concentrations of residual AFB1 showed that a significant difference (P=0.0007) was found between the control and the pre-treated cells. Tukey`s multiple comparison indicated that no significant difference (P>0.05) was found between the control and pre-treated sample by residual AFB1 (see Figure 4.3[A]). A similar result was recorded when HepG2 cells were pretreated by residual AFB1 following the biodegrdation process by Streptomyces.

The statistical analysis results indicated an overall significant difference of (P=0.0081) in *S.lividans* and (P=0.0015) inS. aureofaciens over the entire sample. Tukey`s multiple comparison showed no significant difference (P>0.05) between the pre-treated cells by AFB1 residual and the control sample, whereas there was a significant difference (P<0.05) between the control and pre-treated cells at concentration 20 (µg/ml) **(**see Figure 4.3 [B] [C]).

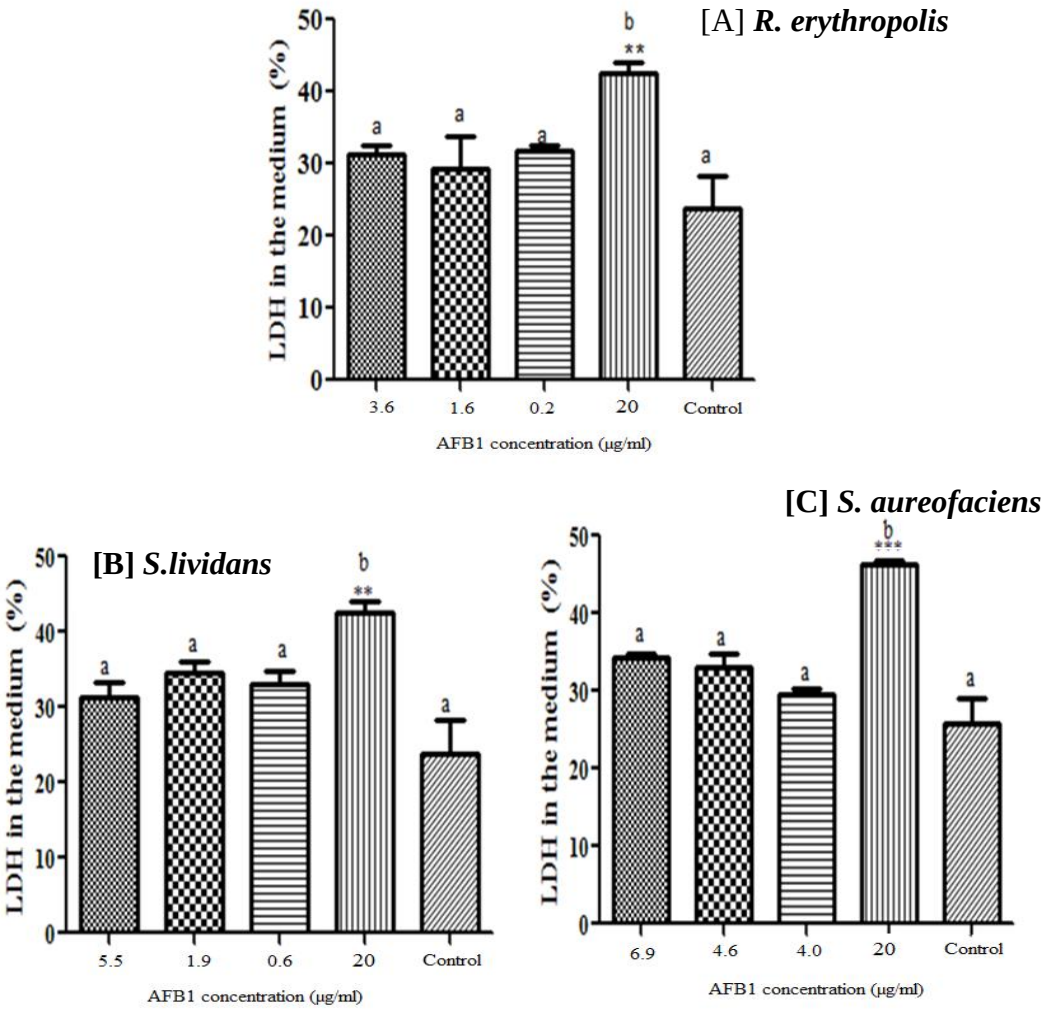


Figure 4.3- Effect of residual of AFB1 on cell viability and intracellular concentrations HepG2 when treated with the noted concentrations of AFB1 residual for 24h incubated at 37ºC 5% CO_2. Lactate dehydrogenase (LDH) leakage was used as an index of cell viability. Results are expressed as percent of lactate dehydrogenase activity in the culture medium of the total activity, culture medium plus intracellular. The cytotoxicity assay followed a biological degradation of AFB1 by Actinomycete cultures [A] in the presence of *R. erythropolis* [B] *S.lividans*, [C] S. *aureofaciens* . Treatments with different letters in each column are statistically different by each treatment (** P≤ 0.001).

4.5 - DISCUSSION

It is obvious from the result obtained in this research that all the strains were capable of degrading/ detoxifying AFB1 in term of the toxin concentration as AFB1 concentration was dropped after the bio- treatment (as described in Chapter 3). However, it is known that AFB1 can cause cytotoxicity effects in different cell lines (Caruso *et al.*, 2009, Fuchs *et al.*, 2006, Palanee *et al.*, 2000, Yoneyama *et al.*, 1987). AFB1and AFB1 derivatives (AFB1-8,9 epoxide) had cytotoxicity effects *in vitro* by using A549 human epithelioid ling cell line (Peltonen *et al.*, 2001).

Thus the question in this case is: did the bio-treatment of toxic compounds by using microorganisms which can be monitored by chemical analytical methods result in a decrease of their toxic properties? This is an important issue as it cannot be excluded that toxin molecule may be transformed by bio-treatment to another compound which may possess toxic properties. Few reports have addressed this question following the AFB1or mycotoxin bio-treatment by using other species of microorganisms such as lactic acid bacteria (Gratz *et al.*, 2007, Fuchs *et al.*, 2008)

Therefore this study demonstrated the cytotoxicity of residual AFB1 *in vitro* subsequent to a biodegradation process. Results suggested that the pre-treatment of AFB1 with *R .erythropolis* leads to a decrease in the cytotoxicity effects in the HepG2 cell line. The cytotoxcity assays confirmed the chemical analytical work which was shown that *R. erythropolis* capable to degrade AFB1 completely.

It has been suggested that AFB1 at 1µM (1µg/ml) caused a 50 % decrease in the number of HepG2 viable cell (McKean *et al*, 2006). In contrast presented data in this chapter indicated that Pre-treatment of AFB1 at concentration of 3.61µg/ml by

R.erythropolis drops off the cytotoxicity effects caused by AFB1. A similar result was reported by Fuchs *et al.*, (2008) who used micronucleus (MCN) assays conducted with HepG2 cells to investigate the effect of the pre-treatment of patulin and ochratoxin A with lactic acid bacteria. Their results confirmed the reduction of toxic properties for both ochratoxin A and patulin when the toxins were pre-treated by LAB. Analogous results obtained by Salmonella/microsome assays confirmed that pre-treatment with LAB strains leads to a decrease of their mutagenic activities, which are considered to play a key role in the induction of cancer (Park and Rhee, 2001). Another researcher indicated that the mutagenic activity of multienzyme-treated AFB1 was greatly reduced or inactivated compared to that of untreated controls (Liu *et al.*, 1998). Also Alberts *et al.*, (2009) recorded that laccase enzyme from *Trametes versicolor* and recombinant laccase enzyme produced by *A. niger* D15-Lcc2 coincided with a significant loss of mutagenicity of AFB1, as evaluated in the *Salmonella typhimurium* mutagenicity assay which confirmed AFB1 degradation.

Furthermore, results in this research showed that *Streptomyces* strains were able to degrade/ transform AFB1 as the concentration of the toxin was decreased with an increase in the bio-treatment time, but the cytotoxicity effects were high compared to the cells treated by fluid containing AFB1 residual from the biodegradation process using *R.erythropolis*. This may indicate that *Streptomyces* strains were able to transform AFB1 to one of the AFB1 derivatives in human-derived liver cells (HepG2) which possess the cytotoxicity effects. Liu *et al.*, (1998) reported that enzymes isolated from *Armillariella tabescens* were able to transform AFB1 into AFBO before breaking down the molecule into less toxic compounds.

However, it is well documented that AFB1 itself it not mutagenic, nor does it bind covalently to macromolecules such as DNA in the absence of a bio-activation system (Hecht and Trushin, 1988). Furthermore, Massey *et al.,* (1995) indicated that the fat-soluble AFB1 penetrated the cell membrane, and then the cells, transforming AFB1 into AFBO. This result supports our hypothesis that AFB1 may be transformed to other aflatoxin derivatives by providing a bio-activation system *Streptomyces* strains, which would then be able to exert its cytotoxicity by binding to both proteins and DNA. However, further works with Streptomyces strains are necessary to understand the mechanism of the AFB1 degradation by mentioned strains. Additional understanding for AFB1 degradation by *R. erythropolis* can be obtained by charactering the enzyme system responsible for the degradation.

4.6 Conclusions

A significant reduction in the toxicity of the treated fluid was noted, reflected in a steady rise in the percentage of the viable HepG2 cells. MTT result and LDH confirmed that *R.erythropolis* was more effective than the other two Actiomycetes strains. This may be because *Streptomyces* strains were able to transform the toxin from AFB1 into another derivative, which is less toxic than AFB1 but still have the toxic properties. MTT was more sensitive in detecting cytotoxicity events in HepG2 cell lines compared to LDH leakage.

CHAPTER 5

PHYSICO-CHEMICAL AND BIOLOGICAL FACTORS AFFECTING AFB1 DEGRADATION BY *Rhodococcus erythropolis* CULTURES

5 - INTRODUCTION

The evidence of AFB1 degradation by *R.erythropolis* discussed in previous chapters. This chapter intends to identify the factors affecting AFB1 degradation as there is a lack of information detailing the mechanism of AFB1 degradation by R. erythropolis in literature. However, the use of *R. erythropolis* for degradation of AFB1 in food or feed may have certain disadvantages, in that the microorganisms would not only utilise the food for their growth, but might also release undesirable compounds. Furthermore, little information is available as regards the enzymes responsible for AFB1 degradation by using *R.erythropolis*. One report indicated that degradation of AFB1 by *R. erythropolis* DSM 14303 was enzymatic (Alberts *et al.,* 2006). *Rhodococcus erythropolis* cells possess various kinds of enzymes that allow them to biodegrade different pollutants, mainly utilizing different types of monooxygenases and dioxygenses to degrade pollutants and also facilitate cell growth (Liu *et al.,* 2009).

Furthermore, enzymes produced by *R. erythropolis* involved in the catabolic pathways of aromatic compounds, such as polychlorinated biphenyls, were purified and characterized. Smith and Harran, (1993) indicated that AFB1 degradation was possible by using cell-free extracts from *A. flavus*. In addition, AFB1 degradation was enhanced by NADPH and NaIO4. These results strongly suggested an involvement of cytochrome P450 monooxygenases in the endogenous aflatoxin degradative activity.

Line and Brackett, (1995) investigated the effect of cell number, culture transfer history, pre-exposure of the microorganism to AFB1, viable and non-viable cells and culture age on AFB1 removal by *Flavobacterium aurantiacum* NRRL B-184. Another team work reported that AFB1 degradation by *F. aurantiacum* was affected by trace metal ions and their concentration (D'Souza and Brackett, 1998). Additionally, D'Souza and Brackett, (2000) indicated that AFB1 degradation by *F. aurantiacum* was influenced by divalent cations Mg^{2+} and Ca^{2+}. This team work also investigated the effect of reducing conditions and seryl and sulfhydryl group inhibitors on AFB1 degradation by motioned microorganisms (D'Souza and Brackett, 2001). However, once factors affecting AFB1 degradation have been identified, then the design and optimization of the biodegradation process will be established in practical applications in the future.

5.1 - RESEARCH AIM

The aim of this research was to investigate physiochemical and biological factors influencing AFB1 degradation as well as to identify the enzyme system which may be involved in the toxin degradation.

5.2 - MATERIALS AND METHODS

5.2.1 – STRAIN STORAGE AND CULTIVATION

Rhodococcus. erythropolis cultivation and storage was carried out as described in section 3.4.1. Difco™ ISP Medium 2 used to prepar bacterial inoculums and to run the fermentation.

5.2.2 - AGITATION AND AERATION EFFECTS IN AN STR BIOREACTOR

5.2.2.1 - BIOREACTOR

All the fermentations were carried out in bioreactor BioFlo 3000 (New Brunswick Scientific, Edison, USA) with total volume of 2.0 L. The reactor consisted of a vessel assembly with detachable motor and side ports for an inoculation tube and a sampling tube, pH, dissolved oxygen and temperature probes. The process related to parameters, for example dissolved oxygen, agitation; pH and temperature were monitored by a digital control unit. All the above-mentioned parameters were monitored and controlled by the PID controller. To control the temperature vessel, the hemispherical water-jacketed heat exchanger was attached to the main laboratory water supply. The Six-Blade Rushton Impeller assembly attached to the agitator shaft. The Ring Sparger was connected to the inlet air which was connected to the air supplier. It was controlled using a rota meter (0-100 %) valve upstream of a sterile Whatman 0.2.

5.2.2.2 - BATCH FERMENTATIONS

The fermenter was inoculated with 10.0% (v/v) of 24-h-old shake flask culture grown at Difco ISP medium 2 at 35^{o}C and 150rpm, pH uncontrolled and started at 6. The temperature was kept at 35^{o}C throughout all the runs. The agitation rate was set at 200-400rpm. The air-flow rate of control runs was 1.0 and 2.0 volume of air per volume of culture per minute (vvm). Fermentation parameters are summarised in Table 5.1.

Table 5.1- Summary of the experiment parameters

Aeration rate	Agitation rate(rpm)	Temperature (ºC)
1vvm	200 and 400	30
2vvm	200 and 400	30

5.2.3 EFFECT OF CULTURE AGE

The ability of the cells at different growth stages to degrade AFB1 was investigated. A growth curve for *R.erythropolis* ATCC 4277 in Difco ISP medium No.1 at 30ºC was determined in a preliminary experiment every 12h. *Rhodococcus erythropolis* culture was grown in Difco ISP medium No.1 for 72h. The cells were harvested by centrifugation at 11,000 rpm for 15 min at 4ºC and enumerated by plating count agar. Following this, the pellets were re-suspended in fresh Difco ISP medium No.1. To achieve the 24 h, 48 h and 72 h old culture respectively by maintaining the same counting number (1×10^8 CFU), 1 ml, 0.5 and 0.25 ml were suspended into fresh media. The cells were harvested by centrifugation at 11,000 rpm at 4ºC for 15 min and re-suspended into fresh media containing 5µg/ml of AFB1. The control contained the toxin without *Rhodococcus* cells. Samples were then incubated at 30°C in the dark for 24h before being pelted by centrifugation. The supernatant fluid was analysed for residual AFB1.

5.2.4 - PURIFICATION AND CHARACTERIZATION OF INTRACELLULAR/EXTRACELLULAR ENZYMES

5.2.4.1 - EXTRACELLULAR FRACTION

The extracellular fractions (5ml) of *R.erythropolis* were concentrated by ultra filtration (Amicon Ultra-0.5, Ultracel-10 Membrane 30 KDa , Millipore, Watford, UK) 4000 rpm for 30 min. The appearance of the molecular mass between 42.7 -55.6 was cut and sent away for protein sequences. The protein band was hydrolysed using trypsin to analyse the peptides by using RFLC-MS/MS.

5.2.4.2 - INTRACELLULAR PREPARATION PURIFICATION CELL FREE EXTRACTS

Bacterial cells were suspended in (50 mM phosphate buffer pH 7.0) to a final concentration of 20 g (wet wt) of cells per 20 ml and then the cells were disrupted using a high-pressure cell homogeniser at 21MPa (Model 4000, Constant Systems Ltd, Warwick, UK). The cellular debris was removed by centrifugation at 2500 ×g for 30 min and then at 10000 ×g for 30 min. All purification steps were done at 4º C by using Fast Protein Liquid Chromatography (FPLC). HiTrap DEAE FF 0.7× 2.5 cm column equilibrated with buffer A (50mM phosphate buffer pH 7.0). The cell-free extract was loaded at 20 ml/h on a HiTrap DEAE FF column. followed The bound enzyme was eluted with a (50mM phosphate buffer pH 7.0 containing 1M NaCl) at a flow rate of 0.33 ml/min. Further purification of the cell-free extract was carried out by using HiTrap HIC column. The cell-free extract was prepared using start buffer (50 mM sodium phosphate pH 7 containing 1 M ammonia). The sample

was applied to the column. After washing with the start buffer, the proteins were eluted with 50 mM sodium phosphate pH 7.0. The aflatoxin-degradation activity of each fraction was tested. The activity of the purified Protein fractions was measured for each purification step by using the following equations:

EQUATION 5.1:

$$\text{TOTAL AMOUNT OF ENZYME (U)} = \text{ACTIVITY (U. ml} - 1) \times \text{VOLUME (ml)}$$

EQUATION 5.2:

$$\text{SPECIFIC ACTIVITY (U. mg} - 1) = \frac{\text{ACTIVITY (U. ml} - 1)}{\text{PROTEIN CONTENT (mg. ml} - 1)}$$

EQUATION 5.3:

$$\text{YIELD} = \frac{\text{TOTAL AMOUNT OF ENZYME AFTER A PURIFICATION STEP}}{\text{SPECIFIC ACTIVITY BEFORE THAT STEP}} \times 100$$

5.2.5 - MOLECULAR MASS DETERMINATIONS

SDS-PAGE was done using the pre-prepared SDS-PAGE (NUPAGE 4-12 % Bis – Tris Gel 1.5mm, 15× 10 wall, Invitrogen Paisley, UK). The sample was prepared as described in the instructions provided by (Invitrogen as life technologies). Twenty microliter of samples were mixed with 25 µl NuPAGE LDS sample buffer (4X) and 55 µl deionized water. Then the mixture was heated for 10 min at 70 ºC. Twenty microliter of heated sample were loaded on the gel. The gel was running by using SDS-PAGE electrophoresis system at conditions voltage 200V constant, run time 90 mins. SDS-PAGE was done using coomassie staining, after the protein profiles of the different samples were compared to the protein marker board range (2-212 KDa) purchased From New England Biolabs, Hitchin,UK.

5.2.6 - EFFECT OF INDUCERS AND INHIBITOR ON AFB1 DEGRADATION

Cell–free extracts of 24h culture were prepared as described in section 5.1.6.2. The intracellular containing 5µg/ml AFB1 was supplemented with 0.2mM NADPH -induser- and 0.1mM cytochrom C -inhibitor- (Sigma-Aldrich, Dorset, UK) and incubated at 30ºC for 24h. The enzyme system target was Chytochrom P- 450.

5.2.7 - EFFECT OF THE METAL IONS

The ability of *R. erythropolis* ATCC 4277 to degrade AFB1 was studied in the presence or absence of divalent cations (Cu^{2+}, Mn^{2+},Zn^{2+} and Mg^{2+}) at concentration 1mM. The treatment and the enzyme target summarised in Table 5.2. The divalent cations were obtained in their sulphate or chloride forms purchased from Sigma-Aldrich, Dorset, UK

Table 5.2- Divalent metal and the enzyme target

Treatment	Enzyme target
Zn^{2+}	Dehydrogenase system
Mn^{2+}	Peroxidase system
Cu^{2+}	Reductase and hyroxylase sytem
Mg^{2+}	Pyruvate dehydrogenase system

5.2.8 -ANALYTICAL METHODS

5.2.8.1 - BIOMASS

Five ml of bacterial culture were withdrawn and filtered through a Whatman no.1 (Whatman Ltd., Maidstone, UK). The filter cake was washed twice with 5ml distilled water, dried in a microwave oven (650w) on medium-low power for 20 minutes, and cooled in desiccators before weighing. All the samples were analyzed in triplicate.

5.2.8.2 - PROTEIN ASSAY

Protein was analyzed according to the Bradford method (Bradford, 1976).

5.2.8.3 - EXTRACTION AND QUANTIFICATION OF AF B1BY HPLC

AFB1 was extracted by liquid/liquid one part sample 2 parts methanol. AFB1 analyses by HPLC as described in section 2.7.1.

5.2.9 - EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

All the statistical analysis was carried out by using Graphpad prism 5. Three separate experiments were carried out in triplicate for each assay, and the results expressed as means ± standard deviations (SD).

5.3 - RESULTS

5.3.1 - AGITATION AND AERATION RESULTS

Figure 5.1 shows the effect of aeration and agitation on *R.erythropolis* ATCC 4277 growth at 30 ºC for 96 h. (A) at 1vvm, (B) 2vvm. medium containing 4(g/L) yeast extract , 4(g/L) malt extract and 10 (g/L) dextrose. The biomass was estimated by dry cell weight measurement. The results show that the biomass increases with an increase in the agitation up to 400rpm and aeration rate of 1vvm. The biomass curves (Figure 5.1 A) generally show a linear growth phase from 0 to 24 h, a stationary phase from 24 to 36 h and a deceleration growth phase between 36 to 48h. An increase in the biomass was noticed after 48h.

As can be seen in Figure 5.1 A, the one set of dextrose consumption started after a few hours of the inculation; the effect of the agitation is very clear when both cultures continued to grow. The higher agitation of 400 rpm increased the consumption of the dextrose which reflects the increase of the bacterial biomass. Figure 5.1 B shows the effect of the areation, as the bacterial biomass was influenced by an increase in the areation up to 2vvm. The optimum bacterial biomass was achieved in 24h at 2vvm and 400 rpm, while the optimum biomass for 200 rpm was achieved after 36h. A sharp decline was noticed after 12 h from the peak production. This may be due to the fact that all nutrients in the medium were utilised.

Figure55.2 shows AFB1 degradation in a batch culture of *R.erythropolis* ATCC 4277 cultures at 200 and 400 rpm, at rate of 1vvm and 2vvm. The results show that AFB1

degradation increases with an increase in the agitation up to 400rpm. More than 81 % of intial concentration was degraded at 200 rpm and 1vvm; while about 85% of the intial concentration of the toxin was degraded when the agitation was increased to 400 rpm at 1 vvm in the first 12h. In addition, the aeration rate 2vvm and agitation 200 rpm increased the percentage of AFB1 degradation to (86%) in the first 12h; Whereas, 400 rpm and 2vvm increased AFB1 degradation to 93% and agitation 400 rpm. The optimum degradation achieved at 2vvm and 400 rpm over 96 h incubation with Rhodococcus culture as residual AFB1 was 0.021 (µg/L) which means 98.6% of initial concentration of AFB1 degraded.

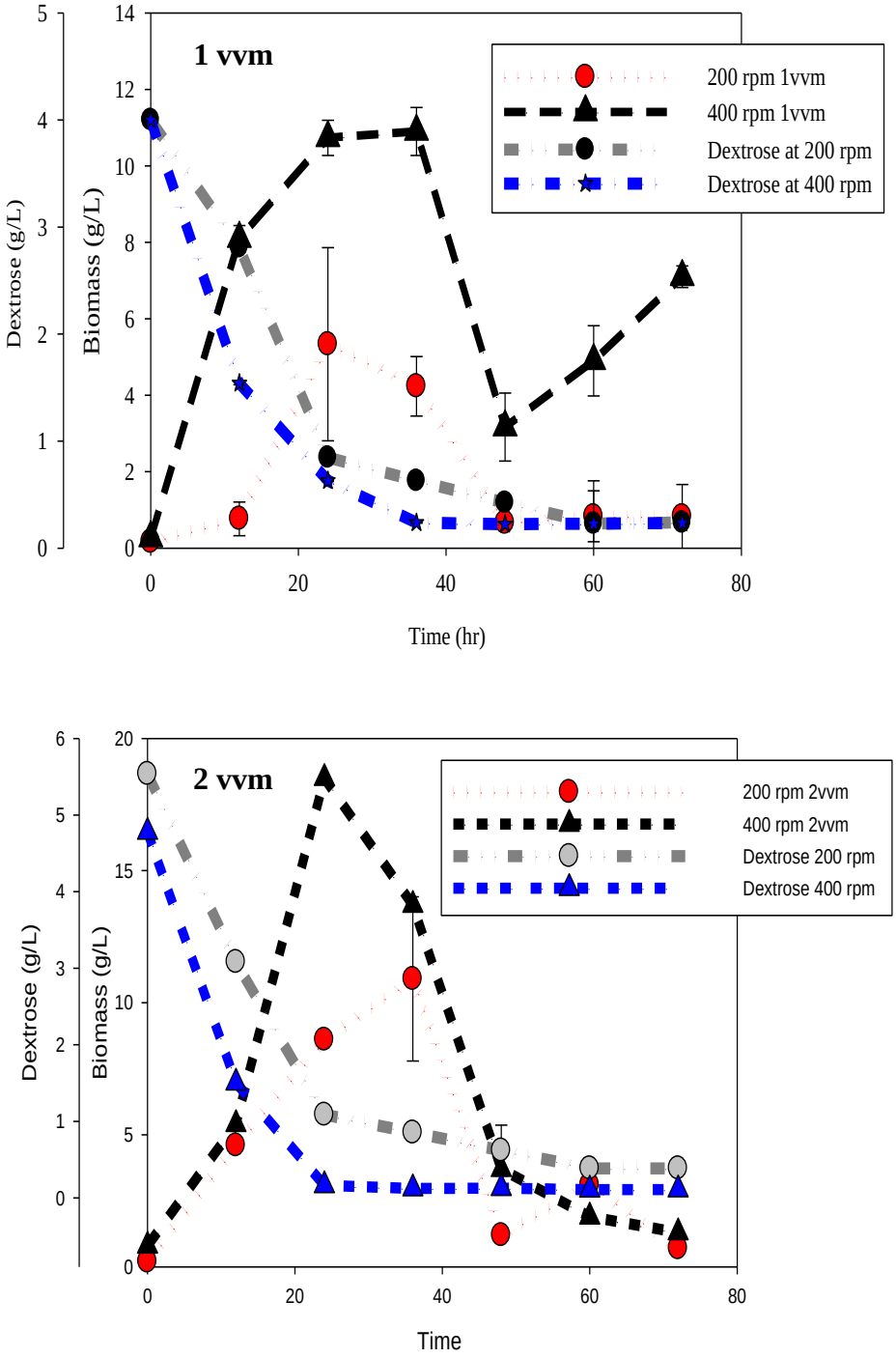


Figure 5.1- Effect of aeration 1 vvm ,2 vvm and agitation 200 , 400 rpm on *R.erythropolis* ATCC 4277 growth at 30 ºC for 72 h .

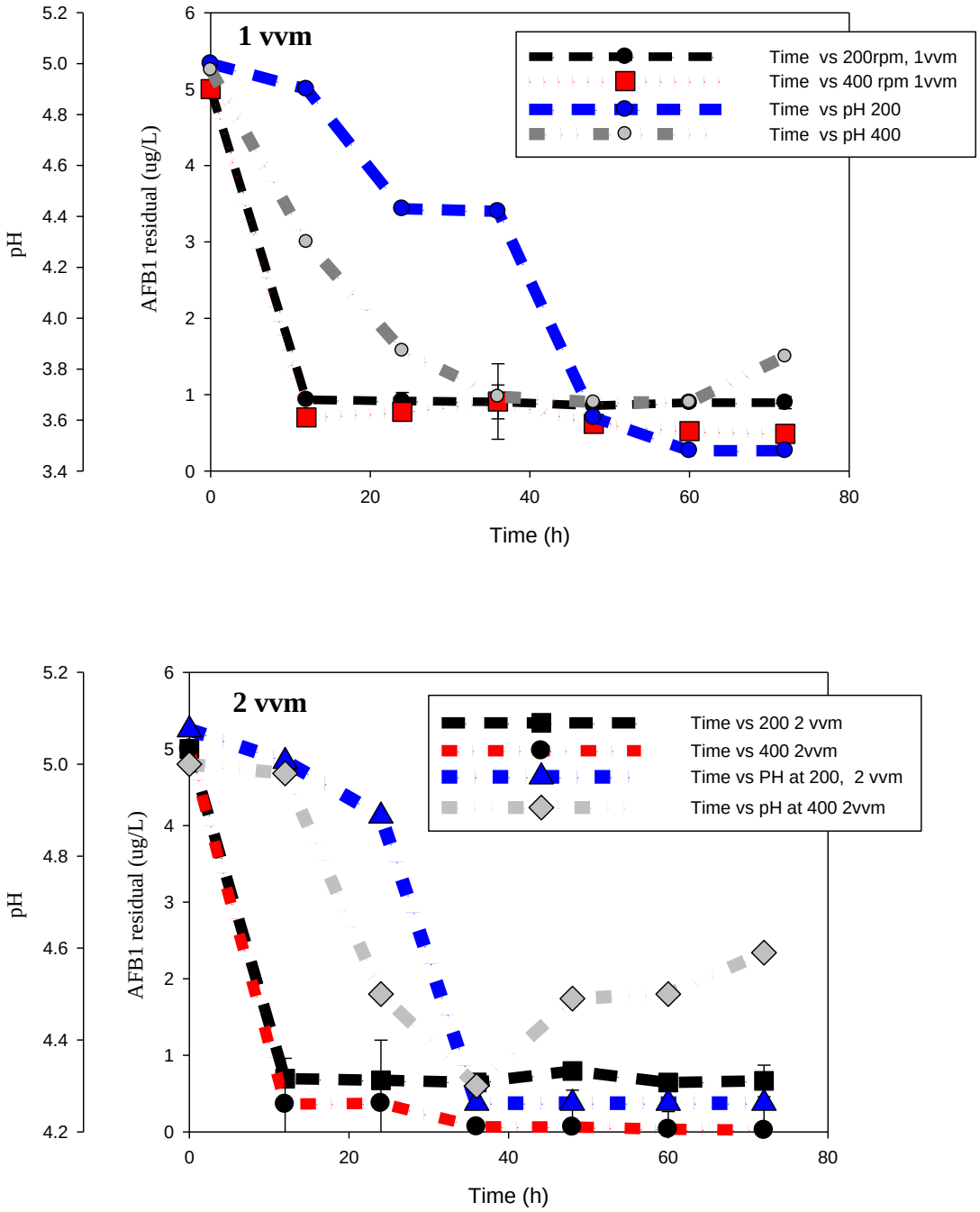


Figure 55.2- Effect of aeration 1vvm, 2vvm and agitation 200 rpm, 400 rpm on AFB1 degradation in batch culture of *R.erythropolis* ATCC 4277 cultures at 30ºC for 72 h.

Figure 5.3 shows the growth curve for *R.erythropolis* ATCC 4277 in ISP 1. A growth curve for *R.erythropolis* grown in ISP1 was determined in preliminary experiments 24 (late of log phase), 48 (stationary phase) and 72 h (late of stationary phase). The cells were enumerated by serial dilution and surface plating on ISP2 medium. Older culture was more effective in degrading AFB1 from liquid media than younger culture. The 24-h culture age (late of log phase) was marginally effective and degraded 15 % of initial AFB1. The stationary phase culture (48h) was more effective and 50 % of AFB1 was degraded while more than 80 % was degraded when the 72h was used (see Figure 5.4). Two-way ANOVA showed that there was a significant difference between the control and the treated sample. Additionally, Bonferroni post tests showed that the treatment over 72 h old cultures was significantly different from the control.

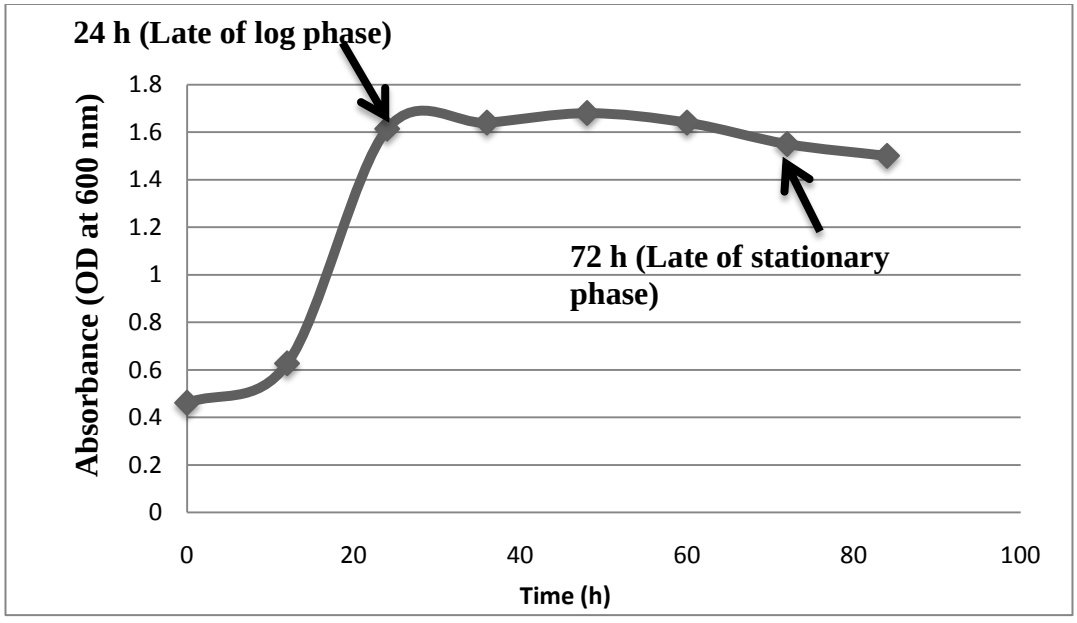


Figure 5.3 - Growth curve for *R.erythropolis* ATCC 4277 in ISP No 1

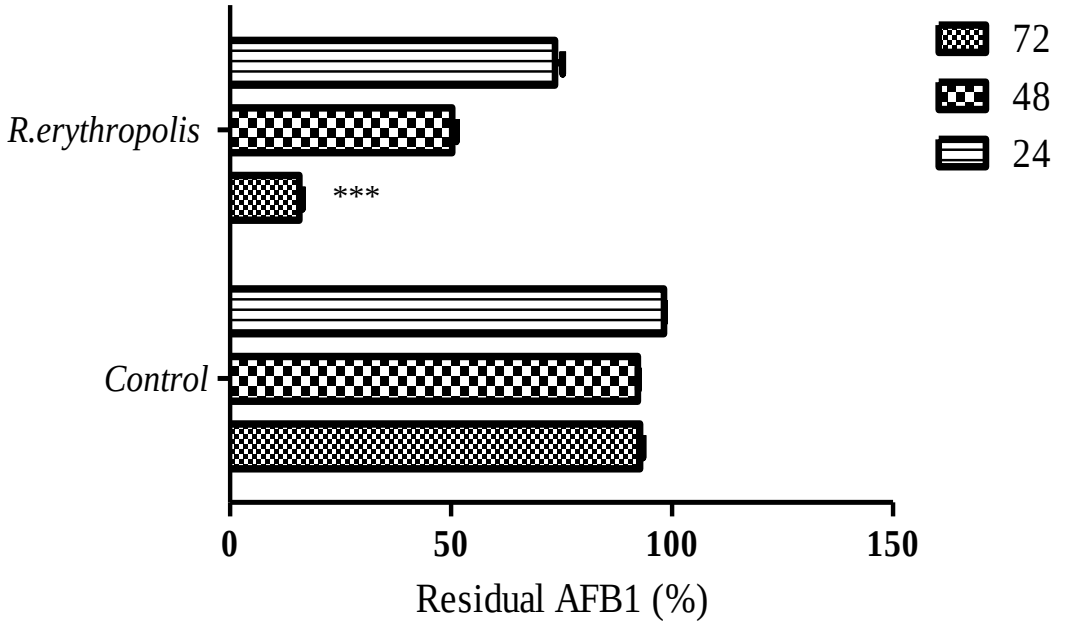


Figure 5.4 - Effect of culture age on AFB1 degradation

Table 5.3- Two way ANOVA analysis for effect of the culture age on AFB1 degradation

Two-way ANOVA

Source of Variation	% of total variation	P value		
Interaction	13.79	< 0.0001		
Column Factor	19.40	< 0.0001		
Row Factor	66.63	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	***	Yes		
Row Factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	2	2139	1070	448.1
Column Factor	2	3009	1504	630.3
Row Factor	1	10330	10330	4330
Residual	12	28.64	2.387	

5.3.3 - PURIFICATION AND CHARACTERIZATION OF AFLATOXIN DEGRADATION ENZYMES FROM R.ERYTHROPOLIS

In an attempt to identify the enzyme or enzymes responsible for the bio-detoxification, culture fluids of *R. erythropolis* where detoxification had occurred were analysed. Extracellular and intracellular of *R. erythropolis* culture were analyzed for the proteins which may be involved in AFB1 degradation.

5.3.3.1 - EXTRACELLULAR ENZYMES

Figure 5.5 shows SDS-PAGE analysis of protein profiles during AFB1 degradation by extracellular fraction from *R.erythropolis* ATCC4277 over a period of 72 h. More than molecular mass were apparent. Only one apparent molecular mass between 42.7 KDa and 55.6 KDa of purified enzymes was isolated and hydrolysed by using trypsin to analyse the peptides by using RFLC-MS/MS for the possible protein involved in the degradation (see Table 5.4). MS/MS Fregmentation of different peptides wrer uploaded to online library matrix science. The Mascot search results provided different suggestions for the enzymes based on protein scores. Protein scores are derived from ions scores as non-probabilistic basis for ranking protein hits. Peptide summary report suggested more than 20 possible enzyme were presented in the hydrolysed protein see appendix 9.20. Selected enzymes. Transaldolase, RecName: Full=NDMA-dependent methanol dehydrogenase, Enolase, 2,3-butanediol dehydrogenase, Alcohol dehydrogenase, class IV, Zn-dependent alcohol dehydrogenase to investigate if these enzymes involved in AFB1 degrdation. Other enzymes were droped from the list were the suggestions was related to other microorganism not *Rhodococcus.*

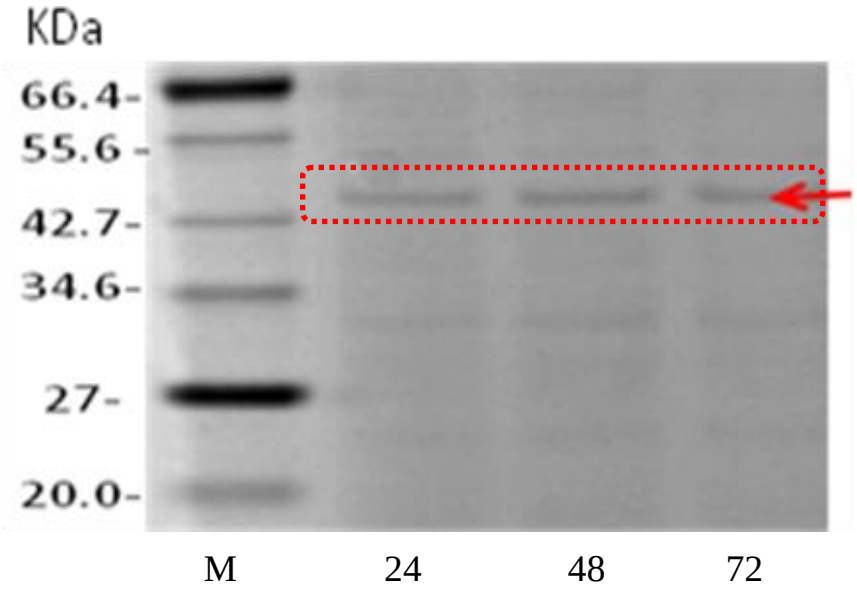


Figure5.5- SDS-PAGE analysis of protein profiles during AFB1 degradation by extracellular fraction from *R.erythropolis* ATCC4277 over a period of 72 h

CHAPTER 5

Table 5.4- MASCOT SUGGESTED PROTEINS

GI NUMBER	SUGGESTED PROTEINS	MASS	SCORE	QURIES MATCHED
229493663	Transaldolase	39989	274	8
110810432	RecName: Full=NDMA-dependent methanol dehydrogenase	46555	247	9
226307770	Enolase	44958	233	6
226307515	2,3-butanediol dehydrogenase	37555	82	2
227400147	Alcohol dehydrogenase, class IV	47296	77	3
111018632	Zn-dependent alcohol dehydrogenase	-----	61	1

5.3.3.2 -INTRACELLULAR ENZYMES

Purification of the enzyme was performed by two chromatographic columns. The protein solution obtained for each fraction was monitored at 280 nm for protein concentration, and activities against AFB1 were tested. Figure 5.6 shows ion exchange chromatography on HiTrap DEAE FF. Figure 5.6 shows after equilibration of the column with 50mM phosphate buffer pH 7, where proteins were eluted with sodium chloride at a flow rate of 0.33 ml/ min.

Figure 5.6- shows the hydrophobic interactions chromatography HiTrap phenyl FF. The column was equilibrated with 50mM phosphate buffer pH 7 containing 1 M of ammonium sulphate, and the proteins were eluted with 50mM phosphate buffer pH 7. All fractions were able to degrade AFB1.

The fraction had the ability to degrade most AFB1 was selected to identify the protein/proteins responsible for degradation. As summarised in Table 5.5, the enzyme was purified 3.31-fold, with a final yield of 73.7 % after the HiTrap DEAE FF purification step. Regarding the enzyme purified by HiTrap HIC, the activity was stable in terms of degrading AFB1.

Result indicated that the total activity for the intracellular in degrading AFB1 was higher 56.3U than the purified enzymes 49.0 , 49.4 when the HiTrap DEAE FFand HiTrap HIC used to purify the enzyme responsible for AFB1 degradation respectively.

The specific activity was higher in purified enzymes than the intracellular fraction. However, SDS-PAGE result indicated that more than a mixture of enzymes was found in the fractions selected after the purification step. The degradation percentage was drooped compared to the intracellular fraction. This confirms that more than enzyme may involve the degradation process.

The purified enzyme was a mixture of enzymes as more than one molecular mass appeared on SDS-PAGE. The appearance molecule from *R.erythropolis* was found between 42 and 55 KDa, 66 KDa and 97.2 KDa see Figure 5.7.

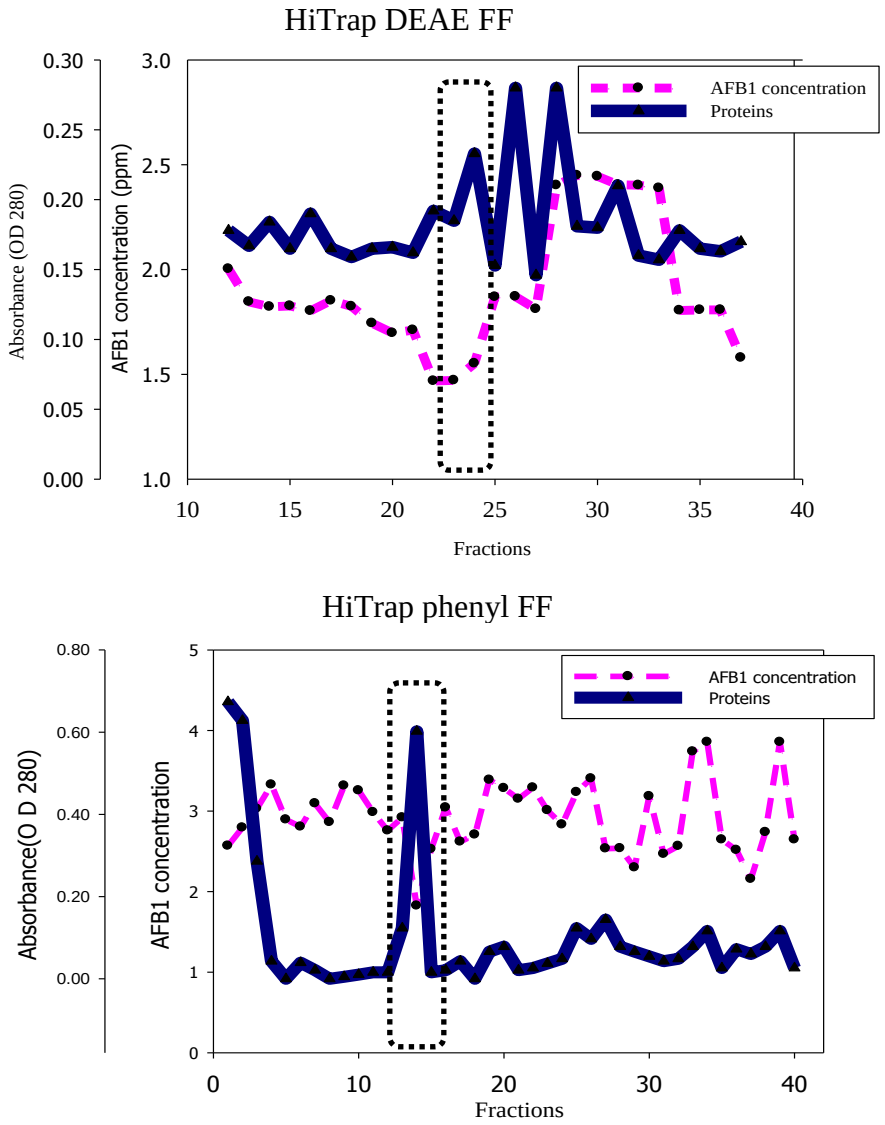


Figure 5.6 - Ion exchange chromatography on HiTrap DEAE FF after equilibration of the column with 50mM phosphate buffer pH 7, proteins were eluted with gradient of Sodium chloride at a flow rate of 0.33 ml/ min. The hydrophobic interactions chromatography HiTrap phenyl FF was equilibrated with 50mM phosphate buffer pH 7 containing 1 M of ammonium sulphate; proteins were eluted using 50mM phosphate buffer pH **7.**

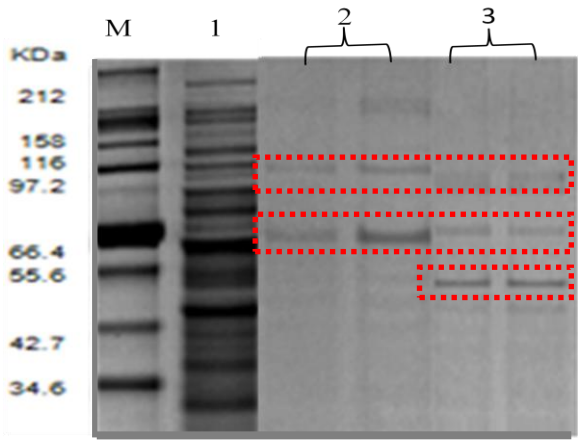


Figure 5.7- SDS-PAGE analysis of proteins during the purification of the AFB1 degradation enzyme from *R.erythropolis* ATCC4277. M Marker, line1 crude protein extract, Line 2 HiTrap DEAE FF, Line 3 HiTrap HIC.

Table 5.5 - Purification of an AFB1-degradation enzyme from *R.erythropolis* ATCC 4277

Fractions	Initial con. (µg/L)	Final con. (µg/L)	Total Protein (mg/L)	Total activity (U)	Sp. Activity (U/mg)	Yield (%)	Fold Purification
Intracellular	5	0.94	67.4	56.3	0.83	100	1
HiTrap DEAE FF	5	1.4	17.6	49.0	2.77	73.7	3.31
HiTrap HIC	5	1.8	16.0	44.4	2.78	76.2	3.31

Figure 5.8 shows AFB1 degradation by cell-free extract *R.erythropolis* ATCC 4277 at 30ºC for 72 h in the presence of divalent Zn, Mn, Cu and Mg at final concentration 1mM. Control refers to AFB1 degradation by cell-free extract. The results showed that divalent zinc at concentration 1mM inhibited AFB1 degradation see Figure 5.8. One way ANOVA showed a significant difference ($P<0.05$) between the control and the sample containing divalent zinc (see Table 5.6). Moreover, the magnesium at 1mM concentration enhanced AFB1 degradation activity but statistically was not significantly different ($P>0.05$). Meanwhile the sample containing divalent manganese, copper did not show any effects in terms of AFB1 degradation (Table 5.6).

Cytochrom C is an inhibitor of the cytochrom P-450 system while NADPH is a cofactor which normally induces reactions by supplying electrons into the reactions. Figure 5.9 shows AFB1 degradation by cell-free extracts *R.erythropolis* ATCC 4277 at 30ºC for 72 h in the presence of 0.1 mM cytochromC and NADPH at final concentration 0.2 mM. Control refers to AFB1 degradation by cell-free extract.

Cytochrom C inhibited AFB1 degrdation as 80 % of the toxin was residual, while 20 % of the initial concentration was residual when the NADPH was added to the extracellular fraction. One way ANOVA showed a highly significant effect compared to the control. Table 5.8 shows one way ANOVA followed by Dunnett's Multiple Comparison Test to identify the significant effect for the inducer and the inhibitor. The post test Dunnett's Multiple Comparison showed both treatments had highly significant effects ($P \leq 0.0001$) (see Table 5.7).

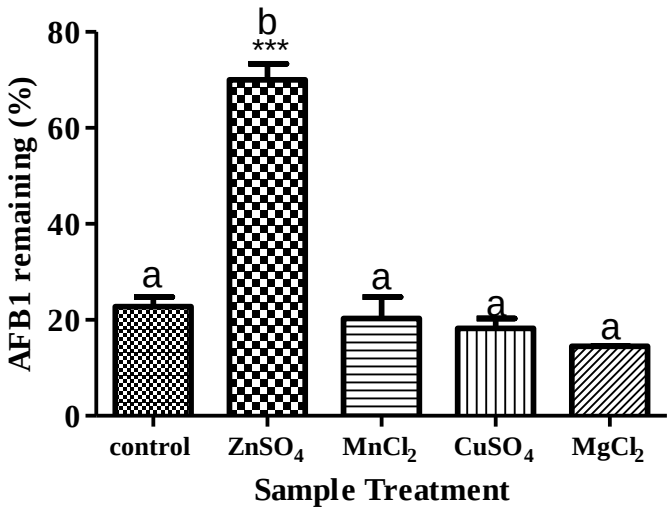


Figure 5.8 - AFB1 degradation by cell-free extract *R.erythropolis* ATCC 4277 at 30ºC for 72 h in presence of divalent Zn, Mn, Cu and Mg at final concentration 1mM. Control refers to AFB1 degradation by cell-free extract.

Table 5.6 - One way ANOVA followed by Dunnett's Multiple Comparison Test to identify the significant effect for divalent caution

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	59.22			
R squared	0.9634			
ANOVA Table	SS	Df	MS	
Treatment (between columns)	6146	4	1537	
Residual (within columns)	233.5	9	25.95	
Total	6380	13		
Dunnett's Multiple Comparison Test	Mean Diff.	Q	P < 0.05	95% CI of diff
control vs ZnSO4	-47.27	11.37	Yes	-59.53 to -35.01
control vs MnCl2	2.467	0.5931	No	-9.796 to 14.73
control vs CuSO4	4.578	1.101	No	-7.685 to 16.84
control vs MgCl2	8.258	1.776	No	-5.452 to 21.97

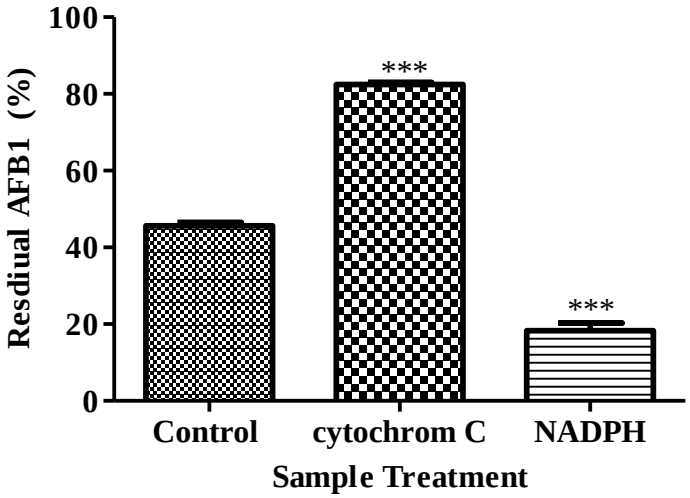


Figure 5.9 - AFB1 degradation by cell-free extracts *R.erythropolis* ATCC 4277 at 30ºC for 72 h in presence of 0.1mM cytochrom C and NADPH at final concentration 0.2 mM. Control refers to AFB1 degradation by cell-free extract.

Table 5.7 - One way ANOVA followed by Dunnett's Multiple Comparison Test to identify the significant effect for the inducer and the inhibitor

One-way analysis of variance				
P value	< 0.0001			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	548.7			
R squared	0.9955			
ANOVA Table	SS	df	MS	
Treatment (between columns)	6214	2	07	
Residual (within columns)	28.32	5	5.663	
Total	6243	7		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P< 0.05	95% CI of diff
Control vs cytochrom C	-36.80	16.94	Yes	-43.38 to -30.22
Control vs NADPH	27.39	12.61	Yes	20.81 to 33.98

5.4 - DISCUSSION

This research examined the effect of those physico-chemical and biological factors which could influence AFB1 degradation as well as preliminary investigations into the enzyme system which may be involved in the toxin degradation.

5.4.1 - AERATION AND AGITATION

In this study, *R. erythropolis* ATCC 4277 culture was cultivated in mechanical (stirred tank STR). The process of *R.erythropolis* growth and AFB1 degradation at different aerations and agitations was investigated. Results indicated that the optimum degradation was achieved at 2vvm and 400 rpm (see Figure 5.1 and Figure 5.2). 92% of AFB1 was degraded in the first 12h where the degradation rate was higher than the degradation rate without the aeration and agitation. (see Chapter 3).

Few reports previously examined the effect of aeration and agitation of AFB1 degradation; Guan *et al.,* (2008) mentioned that the degradation of AFB1 was with aeration without giving details. Moreover, Ciegler *et al.,* (1966b) indicated that both aeration and agitation influenced AFB1 production and degradation by using *Aspergillus flavus* in 20-Litre fermentors. Ciegler *et al.,* (1966b) suggested that AFB1 degradation in the fermentor by using *Aspergillus flavus* at high aeration was due to a non-specific reaction which may have occurred in the lysed mycelium.

Additionally, the same research work reported the initial degradation was rapid and appeared to involve a complex series of reactions. None of the recent results has investigated AFB1 degradation in scale up bioreactors; most of the reports examined

AFB1 degradation on a small scale (Alberts *et al.,* 2006, Teniola *et al.,* 2005). In contrast, (D'Annibale *et al.,* 2006) indicated that both aeration and agitation strongly influenced the treatment efficiency of white-rot fungus *Panus tigrinus* CBS 577.79 to degrade pollutant load of olive mill wastewater; the bioprocess conditions they used were aeration 0.3-1vvm and 500 rpm for agitation. Their result agreed with the present study as the optimum degradation for the pollutant was achieved at the maximum aeration and agitation used in their experiment. Agarry *et al.,* (2010) examined the effect of aeration (1.0-3.5 vvm) and agitation (200-600 rpm) on phenol degradation by *Pseudomonas fluorescence*; their result indicated that the optimum degradation was achieved at 3vvm and 300 rpm.

5.4.2 – CULTURE AGE

An older culture was more effective in degrading AFB1 from liquid culture than the younger cultures. A seventy-two-hour-old culture was able to degrade more than 80 % of initial concentration AFB1. The reasons may be due to metabolic changes as it ages or may be due to increase lysed cells in the culture, which allows all enzymes to release outside the cell. Early studies recorded by Line and Brackett, (1995) showed that an older culture of *Flavobacterium aurantiacum* was able to remove AFB1 from liquid culture due to an increase in the number of nonviable cells which can provide a great scavenging for AFB1.

Moreover, the same team work has recorded that a high population of viable cells (1×10^{9} CFU/ml) was necessary to effect the toxin removal and lower populations, 1000 times less, were not effective for AFB1 removal. In contrast, in this present study the number of the cells was the same at different incubation times (1×10^{8}

CFU/ml). Therefore, the number of the population did not influence AFB1 degradation.

Furthermore, Hamid and Smith, (1987) showed that the ability of cell-free extracts to degrade the AFB1 was less than in intact mycelium. The extracts from the older mycelium were considerably more active than those from younger mycelium, suggesting that the level of the derivative enzymes was increasing.

5.4.3 - PURIFICATION AND CHARACTERIZATION OF AFLATOXIN DEGRADATION ENZYMES FROM *R.erythropolis*

In an attempt to identify the enzyme or enzyme systems responsible for the bio-detoxification, culture fluids of *R. erythropolis* where detoxification had occurred were analysed. Mixtures of enzymes were purified and identified from the extracellular / intracellular fractions by using two columns, DEAE Sepharose and Phenyl Sepharose. Regarding the protein purification from extracellular fractions by using those mentioned columns, a negative result was obtained. Therefore, the extracellular was concentrated and loaded into SDS-PAGE. The apparent molecular mass of concentrated enzymes from the extracellular fraction was estimated to be between 27 to 34.6 and 42 to 55 KDa.

A similar result was published by (Alberts *et al.,* 2006) who indicated that the protein size identified from extracellular fraction was estimated to be about 45 KDa. Therefore, this apparent molecular mass was isolated and hydrolysed by using trypsin to analyse the peptides by using RFLC-MS/MS for the possible protein

involved in the degradation. Furthermore, data obtained by RFLC-MS/MS were searched using Mascot library.

A number of suggestions for enzymes which may involve degradation were given; only hits with a high protein score were considered as possible enzymes which could be involved in the degradation, such as transaldolase, enolase, 2,3 butanediol dehydrogenase, alcohol dehydrogenase and zn-dependent alcohol dehydrogenase. A similar molecular mass between 42 and 55 KDa by SDS-PAGE was purified by Phenyl Sepharose from the intracellular fraction. The selected intracellular fraction contained a mixture of enzyme estimated at 42 and 55 KDa, 66 and 97.2 KDa (see Figure 5.6). One step of enzyme purification was not enough to purify the enzymes responsible for AFB1 degradation. Various enzymes with different protein sizes were purified and identified from different microorganisms (Alberts *et al.*, 2006, Liu *et al.*, 2001, Motomura *et al.*, 2003).

These specific enzymes have the capacity to degrade AFB1; Alberts *et al.*, (2006) indicated that the size of the proteins was about 45 KDa, while the apparent molecular mass of the purified enzyme from *Pleurotus ostreatus* was estimated to be 90 KDa. It has been reported that biodegradation of pollutants by *Pleurotus ostreatus* involves the ligninolytic enzyme system, laccase MW56 KDa (Motomura *et al.*, 2003).

There is a lack of information regarding the enzymes involved in AFB1 degradation by using *Rhodococcus sp*., whereas enzymes involving catabolic pathway of aromatic compound have been identified and purified, including enzymes like ring cleaving biphenyl dioxygenases, dihydrodiol dehydrogenases and hydrolases which

have been purified from *Rhodococcus* sp. (Haritash and Kaushik, 2009, Martinkova *et al.,* 2009, Larkin *et al.,* 2005).

In addition, these enzymes or enzyme systems are known to be involved in the degradative metabolism of several xenobiotic molecules, for example fungal cytochrome P450 mono-oxygenase ensyme system is involved in the catabolism of naphthalene, progesteron (Motomura *et al.,* 2003). In order to understand the nature of the enzyme or enzyme system involved in AFB1 degradation, the effect of a trace divalent metal on AFB1 degradation by cell-free extract *R.erythropolis* ATCC was examined and to reduce the number of the list enzymes obtained. The result indicated that divalent zinc inhibited AFB1 degradation at concentration 1mM. Divalent zinc is a cofactor of Alcohol dehydrogenases and plays a regulatory role in DNA binding and activation of transcription factors, DNA polymerase and carbonic anhydrases (D'Souza and Brackett, 1998). Zinc inhibits dehydrogenases in the fungal system and in glucose-6-phosphate dehydrogenases of *E.coli* K-12 (D'Souza and Brackett, 1998).

These inhibitory results obtained with Zn^{+} support the hypothesis that a dehydrogenases system is involved in AFB1 degradation. However, this result can be confirmed by adding dehydrogenases inhibitors such as 4-Methylpyrazole hydrochloride into the *R.erythropolis* intracellular before exposure to AFB1 to confirm if those mentioned enzymes were possibly involved in the degradation.

Moreover, the effects of NADPH as inducers of cytochrome P-450 and cytochrome C as inhibitor of cytochrome P-450 on the toxin degradation were investigated. The presence of NADPH in the reaction system significantly enhanced the ability of the

intracellular to degrade AFB1. Analogous results were recorded by Hamid and Smith, (1987), which suggested that in cell-free extracts aflatoxin degradation was enhanced by NADPH. Moreover, the present results also show that cytochrome C significantly inhibited the ability of the cell-free preparation of *R.erythropolis* to degrade AFB1.

Larkin *et al.,* (2005) indicated that a common feature of the aerobic rhodococci is the presence of many types of monooxygenases and dioxygenases; for example, the involvement of heme-containing cytochrome P450 enzymes in the degradation of substituted aromatics. Although the results of this study demonstrated the inhibition of AFB1 by exposure of the intracellular to Zn^{+}, cytochrome C did not provide conclusive evidence that any particular enzyme is involved, but results did suggest that the enzyme system involved in toxin degradation may be of the mono-oxygenase / dehydrogenase type.

5.5 – CONCLUSION

This study has shown for the first time the ability of *R. erythropolis* to degrade AFB1 in batch culture on a large scale by using bioreactor. AFB1 degradation was influenced by an increase in the aeration and agitation. Culture age was very important, with older cultures being more capable of AFB1 degradation relative to younger cultures. *Rhodococcus* culture, extracellular and intracellular, was effective in degrading AFB1. SDS-PAGE result indicated that more than one enzyme was involved in AFB1 degradation. An inhibitory result was obtained with Zn^{+2}, supporting the hypothesis that a dehydrogenase system is involved in AFB1

degradation; meanwhile the induction result by NADPH and an inhibitory result obtained with cytochrom C indicated that AFB1 degradation may involve cytochrome P450 enzymes.

CHAPTER 6

DETOXIFICATION OF PATULIN AND OCHRATOXIN A BY *Rhodococcus erythropolis* CULTURE

6 - INTRODUCTION

The risk of human exposure to patulin through the consumption of juices and jams manufactured with apples occurs during the first year of life. Children are more exposed to patulin toxicity when compared to adults (Barreira *et al.,* 2010). On the other hand, ochratoxins (OTA) may be in cereals, wine, cocoa, dried vine fruits, coffee and spices. Both mycotoxins mentioned have high toxicity for both humans and animals. Patulin has acute and chronic effects on almost all of the animals used in experiments during the past 50 years (Moake *et al.,* 2005). In the meanwhile, the mechanism of action of OTA is not fully understood.

However, much work has been done on mycotoxin detoxification (Bata and Lasztity, 1999, Bullerman and Bianchini, 2007, Moake *et al.,* 2005, Stander *et al.,* 2000, Stander *et al.,* 2001). methods include adsorption, extraction, heat, irradiation, and chemical means which have limitations in use because of losses of the products' nutritional value and other qualities; there are undesirable health effects of such treatments and expensive equipment that may be required for the degradation process which makes the biological methods more attractive (Amézqueta *et al.,* 2009). OTA can be cleaved by carboxypeptidase A to the non-toxic ochratoxin α (OTα) (Stander *et al.,* 2001). Whereas, patulin was completely degraded by yeast fermentation (Moake *et al.,* 2005). Moss and Long, (2002) showed that 3 strains of *Saccharomyces cerevisiae* were able to reduce the patulin level during fermentation growth. This reduction resulted in two main compounds E-scladiol and Z-scladiol. Nevertheless, E-scladiol is itself mycotoxin (Moss and Long, 2002).

Only one research study has been published regarding patulin detoxification by lactic acid bacteria, which was effective in removing patulin, but the result depends on different parameters, such as the concentration of toxins, the cell density, the pH-value and the viability of the bacteria (Fuchs *et al.*, 2006). Moreover, no research has investigated the direct enzymatic degradation of patulin. Reducing enzymes such as those involved in yeast fermentation, as well as lactone-degrading enzymes such as β-lactamase, may well be able to degrade patulin. Few investigations have been published regarding ochratoxins biodegradation (Var *et al.,* 2009, Varga *et al.*, 2005).

However, results varied from these articles to others depending on the microorganism used for the degradation, and most attention has been directed to optimising the conditions to achieve a high level of detoxification (Stander *et al.,* 2001, Péteri *et al.,* 2007, Stander *et al.,* 2000). This research investigates a bio-detoxification of two mycotoxins, patulin and ochratoxin A.

6.1 - THE AIM OF THIS RESEARCH

The aim of this study was to investigate the biological degradation of two mycotoxins, namely patulin and ochratoxin A by *R. erythropolis* cultures, as well as optimizing the factors influencing degradation *by R. erythropolis*, and evaluating the cytotoxicity of residuals patulin and ochratoxin A by using the human cell line HepG2.

6.2 - MATERIALS AND METHODS

6.2.1 - STORAGE AND CULTIVATION of *R. erythropolis*

Rhodococcus erythropolis ATCC 4277 cultivation and storage was described in 3.4.1.

6.2.2 - BACTERIAL CELL PREPARATIONS

A broth culture of *Rhodococcus erythropolis* was centrifuged at 4ºC, 11,000 rpm for 15 min to remove the cells from the culture. Cells were washed twice with phosphate buffer saline before re-suspending the cells in phosphate buffer saline (50mM, pH 6). Dead cells were prepared by heat inactivation (autoclaved cells). cfu/ml $\geq 10^8$ was used in all experiments.

6.2.3 - PATULIN AND OCHRATOXIN, A DEGRADATION ASSAY

All the experiments were carried out by sterilizing universal tubes in final volume 2 ml. Fresh ISP medium No. l (1.8 ml) in sterile universal tubes inculcated by 100 µl of 48 h *Rhodococcus* culture. This involved the addition of PAT or OTA 100 µg/ml dissolved in methanol (Sigma aldrich, Dorset, UK) to the culture to a final concentration of 5 µg/m and incubated at 30°C for 24, 48 and 72 hours (h) respectively.

A negative control was used in this study; sterile medium supplement with PAT or OTA to a final concentration of 5 (µg/ml). The cells were removed by centrifugation (JuanBR4i Multifunction, Thermo) at 4ºC, 11,000 rpm for 15 min. PAT or OTA was quantified by using reverse phase HPLC as described in section (6.2.5).

6.2.4 - FACTORS AFFECTING PATULIN AND OCHRATOXINA DEGRADATION: PH AND TEMPERATURE INCUBATION AND TIME REACTION

The degradation of patulin and ochratoxin A at different temperatures, pH and incubation time was investigated to determine the optimum condition for the degradation by *Rhodococcus* culture. The chosen temperatures were 25, 30, 35 and 40 ºC over a period of 24 h, 48 and 72 h and pH 6. The pH used in this experiment was 4, 5, 6, 7 and 8 and the mixtures were incubated in the dark for 24 h at 30°C. The pH was adjusted by using 1 N of HCl for the low pH and 1 N of NaOH for the high pH. All experiments were performed in triplicate.

6.2.5 - QUANTIFICATION OF PATULIN AND OCHRATOXIN BY HPLC-UV

Both toxins were extracted from liquid culture by methanol (1:3), one part sample, and three parts methanol, to evaluate the remaining toxins after the degradation process. Reverse phase HPLC-UV (Gilson instrument) analysis was performed through a guard column phenomenex (C6-phenyl 4.0 × 3.0 mm ID) followed by an ACE column (RP-C18 (5µm), 150 × 4.6 mm) (Hichrom limited, UK). In the case of patulin, a mixture of acetonitrile: water (1:99 v/v) was used as mobile phase at a flow rate of 1 ml/min. Injection volume was 20 µl. The sample temperature was controlled at 40ºC by using column heater model 7971 (Jones chromatography) retention time was 4.18 mins. In ochratoxin A, the mobile phase was a mixture of acetonitrile: water: acetic acid (50:48:2, v/v/v) and used at a flow rate of 0.8 ml/min.

Injection volume was 20 µl. The sample temperature was controlled at 25ºC with a diode array detector being used to measure patulin by UV detection at wavelength 276 nm in the case of patulin and 330 nm for ochratoxin A and retention time 8.45mins. The data was collected and processed by Glison unipoint LC system software. The residual mycotoxin percentage was calculated using the following

EQUATION 6.1 :

$$\text{RESIDUAL MYCOTOXIN} = \frac{\text{MYCOTOXIN PEAK AREA IN TREATMENT}}{\text{MYCOTOXIN PEAK AREA IN CONTROL}} \times 100$$

6.2.6 - CYTOTOXICITY OF PATULIN AND OCHRATOXIN A RESIDUAL BY USING MTT ASSAY

6.2.6.1 STORAGE AND CULTIVATION OF HEPG2

Cell preparation was described in section 4.2.4.

6.2.6.2 - CYTOTOXICITY ASSAY

An MTT assay was used to examine the cytotoxicicty of patulin and ochratoxin residual by using HepG2 cells. Cell number of 3×10^3 cell /ml was used as the final concentration. Aliquots of the cell suspension (100µl) were dispensed in each of the 96 wells of the microtitre plate to give a final cell number of 3000 cell/well. The plate was incubated at 37ºC, 5% CO2 for 24 h. Following 24 h incubation, the media was aspirated from the 96-well. The treated samples containing patulin or ochratoxin residual were dissolved in DMEM and 100 µl of each sample was transferred to individual wells of the microtitre plate. After 24 h incubation, 20 µl of MTT solution (1.2 mg/ml) was added to the well and incubated for 4 h in the same growth

conditions. Cells were treated with DMSO for 1 hr to dissolve the resulting formazan crystals. The optical densities of the cells were measured spectrophotometrically at dual wavelength of 570nm (Labs systems IEMS reader MF, Finland). The control sample was cells without treatment. Three sets of experiments were used to evaluate the cytotoxicity by MTT assay and each test included a blank. The percentage of the viability was calculated using the following

EQUATION 6.2 :

$$\text{VIABILITY (\%)} = \frac{\text{ABSORBANCE OF THE TREATED SAMPLE}}{\text{ABSORBANCE OF CONTROL}} \times 100$$

6.2.7 - STATISTICAL ANALYSIS

One-way ANOVA and two way ANOVA were employed to analyse the data. Levels of significance ($P<0.05$) were considered significant. One way ANOVA was followed by post-test Tukey's Multiple Comparison. Two ways ANOVA was followed by bonferroni post test. All the statistical analysis was carried out by using Graphpad prism 5. Three separate experiments were carried out in triplicate for each assay, and the results expressed as means ± standard deviations (SD).

6.2.8 - RESULTS

6.2.8.1 - BIODEGRADATION RESULT

This study investigated a biological detoxification of patulin and ochratoxin A by *Rhodococcus* cultures. The patulin peak was identified by HPLC-UV/DAD. The confirmed peak for patulin was detected at 267nm absorbance wavelength. Full scan

data shows that patulin was rapidly degraded by *R. erythropolis* culture at 25ºC over 72h. Different unknown peaks were detected (see Figure 6.1).

Figure 6.2 shows ochratoxin A detection by HPLC. Ochratoxin A was detected at absorbance wavelength when 330 nm was used. Clearly, ochratoxin A was degraded when the toxin was treated with *R. erythropolis* culture. More than one peak appeared after the degradation process. The result of biological detoxification of patulin by liquid culture of *R. erythropolis* ATCC4277 at 30 ºC, pH 6 for 24h are presented in Figure 6.3, and the residual calculation was based on the control sample at zero time.

To find out if this phenomenon is an enzymatic degradation or just elimination of the toxins from liquid media that occurred by adsorption of mycotoxins into the bacterial cell wall, live cells were preserved in sodium phosphate buffer 50mM pH 6 and heat-inactivated (autoclaved).

It was evident that no significant reduction occurred in the absence of *R. erythropolis* cells or in the presence of heat-inactivated (autoclaved) cells and cells preserved in phosphate buffer saline; while more than 40 % was degraded by *R. erythropolis.* Table 6.1 shows a one-way ANOVA followed by Tukey`s multiple test. One-way ANOVA shows a significant difference by a different treatment P = (0.0009) at 95 % confident. In addition, Tukey`s Multiple Comparison Test shows that there is no significant difference between the control sample and heat-inactivated cells or cells preserved in phosphate buffer saline. Treatments with different letters in each column are statistically different with each treatment ($P \leq 0.05$)

Figure 6.4 shows biological detoxification of ochratoxin A by liquid culture of *R. erythropolis* ATCC4277 for 24h at 30 ºC, pH 6; 50 % was degraded by the liquid culture. One-way ANOVA shows a significant difference by a different treatment P ≤ (0.0001) at 95 % confident. Moreover, Tukey`s Multiple Comparison Test confirmed the enzymatic degradation as no significant difference was found between the control sample and dead cells and cells preserved in phosphate buffer saline (see Table 6.2).

In order to establish optimal conditions for the degradations, experiments were conducted in which the temperature dependency of detoxification of mycotoxins was studied. It became obvious that 25 ºC was the most effective temperature for degrading patulin. More than 80 % of patulin was degraded at 25 ºC pH 6 whereas 40 % was degraded at 30, 35 and 40ºC (see Figure 6.5).

A significant difference between different cultivation temperatures was found (P< 0.0001). Tukey's Multiple Comparison Test confirmed that 25 ºC is a significant difference (P<0.05) from other temperatures used in this experiment; while no significance difference was found between other temperatures in terms of degrading patulin.

Figure 6.6 shows the effect of temperature on ochratoxin A degradation by *R. erythropolis* after 24 h incubation at pH6. It soon became obvious that between 25 and 30 ºC was the optimum temperature for ochratoxin A degradation, while degradation by between 35 and 40 ºC was less effective in degrading OTA; more

than 40 % was degraded at 25 and 30 ºC while only 30% and 20 were degraded by 35 and 40ºC.

Those results were confirmed statistically (Table 6.4); a one-way ANOVA and Tukey's Multiple Comparison Test were used to analysis the data. A significant difference between different temperatures was found ($p < 0.0001$). Moreover, no significant difference ($P \geq 0.05$) was found between 25 and 30 ºC, while 35 and 40 ºC showed a significant difference ($P \leq 0.05$) when Tukey's Multiple Comparison Test was used to analyse the similarity in the data.

Figure 6.7 shows the effect of pH on patulin degradation by *R. erythropolis* ATCC 4277 culture at 25 ºC. Generally, patulin was not stable with every pH used in this experiment. The optimum degradation was achieved at pH 8 where only 10 % of initial concentration was residual; whereas 77%, 35%, 14%, and 13% were achieved when pH 4, 5, 6 and 7 respectively were used. Table 6.5 shows one-way ANOVA analysis of the effect of different pH on patulin degradation by *R. erythropolis* cultures, where an overall significant difference ($P \geq 0.0001$) between different pH treatments was found.

Tukey's Multiple Comparison Test confirmed that testing for significant difference between treatments at pH6 and pH7 has actually no significant difference ($p \geq 0.05$). Figure 6.8 shows the effect of pH on ochratoxin A degradation by *R. erythropolis* after 24 h incubation at 30ºC. General reduction in OTA concentration was observed with all pH used in this research. The maximum degradation was observed at pH 4.0; about 29% was residual while 68%, 64%, 37% and 43% of OTA were

residual when pH5, pH6, pH7 and pH 8 were used. A significant difference ($P < 0.0001$) between different pH was found by one-way ANOVA (Table 6.6); moreover Tukey's Multiple Comparison Test showed that pH was statistically significantly different ($P<0.05$) from all other pH used in this research, whilst no significant difference ($P > 0.05$) was found between pH 5 and pH 6. In addition, the comparison test showed that pH7 was statistically not different ($P > 0.05$) from pH8. Meanwhile there was a significant difference between those mentioned of the group.

The effect of interaction of time and temperature on patulin degradation by *R. erythropolis* culture was studied (Figure 6.9). It was found that patulin was temperature and time dependent. After the first 24 h, more than 80 % was degraded at 25ºC by *R. erythropolis* culture; this reduction was increased within the time. Therefore the optimum condition for degrading patulin was 25ºC and 72 h as less than 1 % was residual. Moreover, a general reduction was noticed with all ranges of temperature used in this experiment. Table 6.7 summarizes the statistical analysis. Two way ANOVA indicated that there was significant interaction between temperature and time ($P= 0.0014$). Therefore the interaction between the factors was considered as very significant. Temperature and time were extremely significant ($P< 0.0001$).

A similar result was achieved when ochratoxin A was used. The optimum condition was achieved at 25 and 30 ºC over 72 h incubation with *R. erythropolis* culture. More than 45% of ochratoxin A was degraded whilst less than 40 % was degraded at 35 and 40 ºC respectively over a period of 72 h incubation with the bacterial cultures (Figure 6.10). Table 6.8 shows two way ANOVA analysis of the effect of the

interaction of time and temperature on ochratoxin A degradation by *R. erythropolis* culture. Statistically, the source of variation was the interaction between temperature and time P< 0.0001 and temperature P<0.0001 and time p< 0.0001. Since the interaction is extremely significant, it is difficult to interpret the P value for the temperature and time. Therefore both factors were considered as extremely significant.

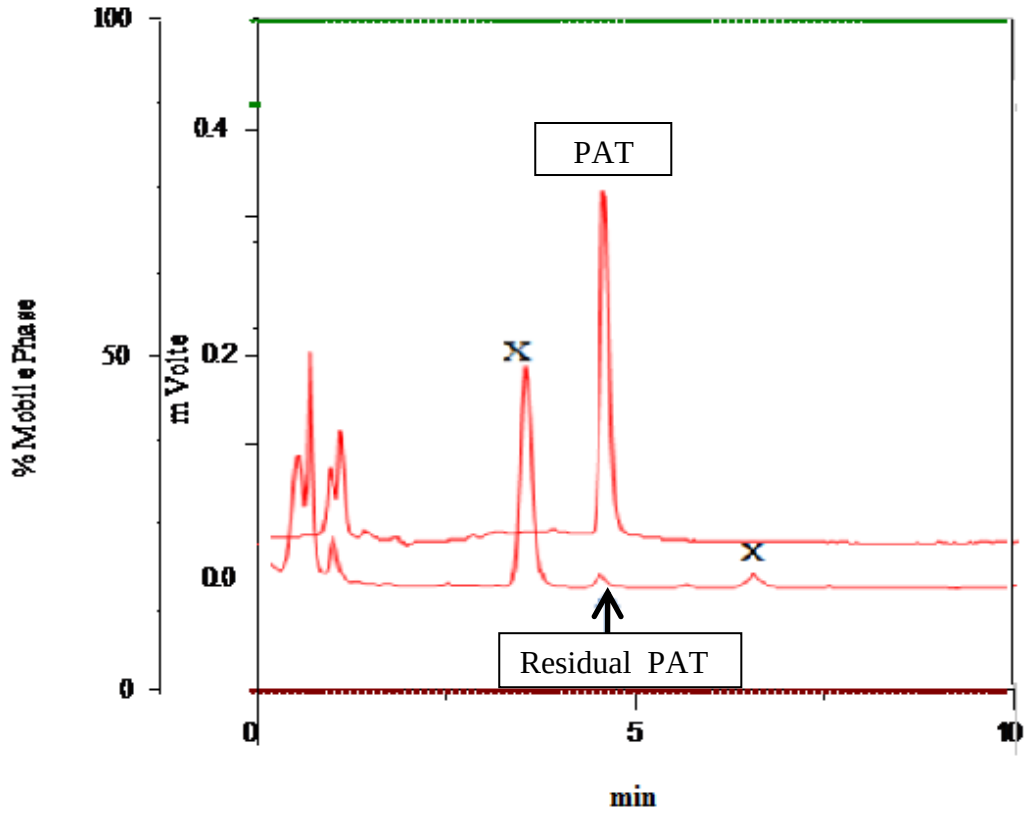


Figure 6.1- Detection of patulin by high performance liquid chromatography Patulin standard (5µg/ml), residual patulin after 72 h treatment with *R. erythropolis* cultures at 25°C. Retention time 4.18 mins. Unknown compounds generated [X] at retention time 2.05 mins and 6.43mins.

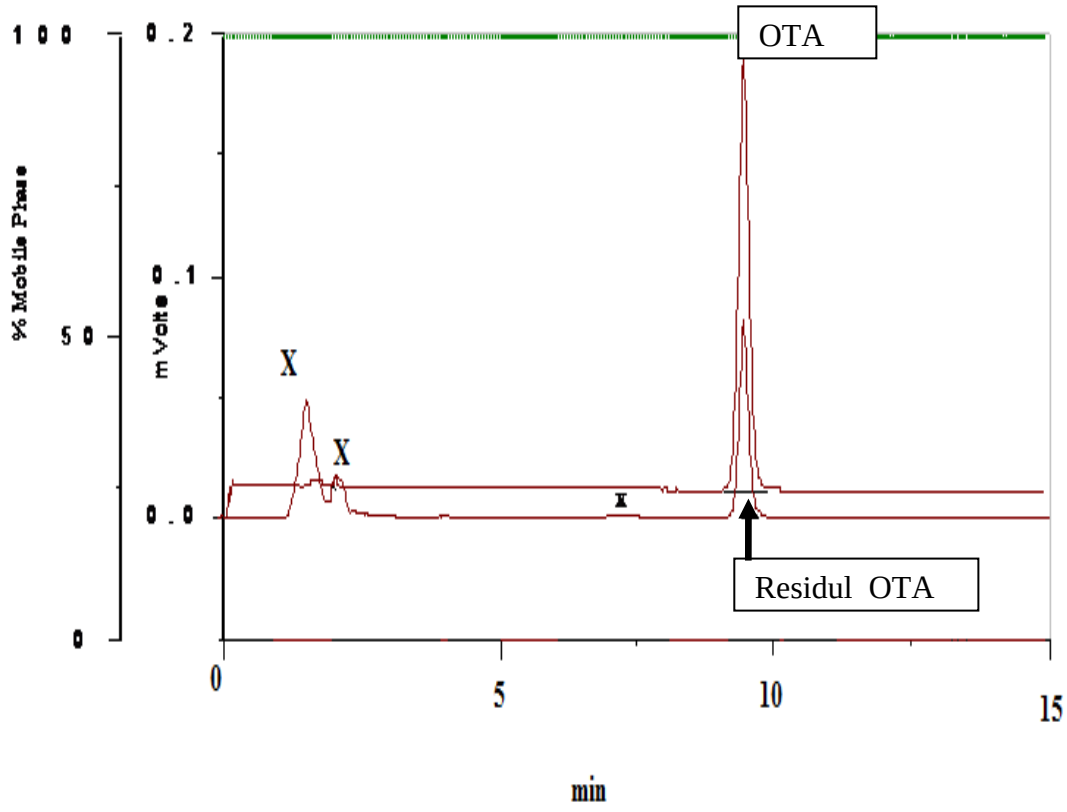


Figure 6.2- Detection of ochratoxin A by high performance liquid chromatography Ochratoxin A standard (5µg/ml), residual Ochratoxin A over 72 h treatment with *R. erythropolis* cultures at 25ºC at retention time 9.54 mins. Unknown compounds X at retention time 1.76 mins, 2.50 and 7 mins.

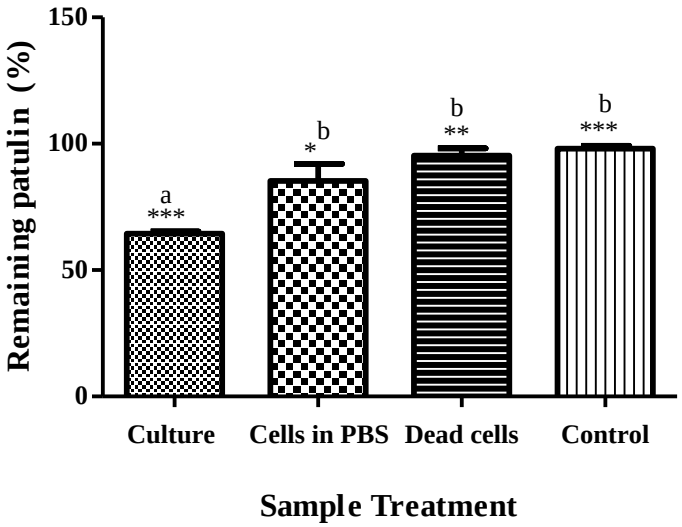


Figure 6.3- Detoxification of patulin by liquid culture of *R. erythropolis* ATCC4277 at 30 ºC, pH 6 for 24h. The residual calculation is based on the control sample at 0 time. Treatments with different letters in each column are statistically different by each treatment (***P≤0.001,** P≤0.01, *P≤0.05).

Table 6.1- One-way ANOVA followed by Tukey`s multiple test for biological detoxification of patulin by *R. erythropolis* liquid culture

One-way analysis of variance				
P value	0.0009			
P value summary	***			
Are means signif. Different? (P < 0.05)	Yes			
Number of groups	4			
F	16.44			
R squared	0.8604			
ANOVA Table	SS	df	MS	
Treatment (between columns)	2091	3	697.0	
Residual (within columns)	339.2	8	42.39	
Total	2430	11		
Tukey's Multiple Comparison Test	**Mean Diff.**	**Q**	**Significant? P < 0.05?**	**95% CI of diff**
Culture vs PBS	-20.84	5.543	Yes	-37.86 to -3.811
Culture vs Dead cells	-30.91	8.223	Yes	-47.94 to -13.89
Culture vs Control	-33.58	8.932	Yes	-50.60 to -16.55
PBS vs Dead cells	-10.07	2.680	No	-27.10 to 6.951
PBS vs Control	-12.74	3.389	No	-29.77 to 4.284
Dead cells vs Control	-2.667	0.7094	No	-19.69 to 14.36

(***P≤0.001, ** P≤0.01, *P≤0.05)

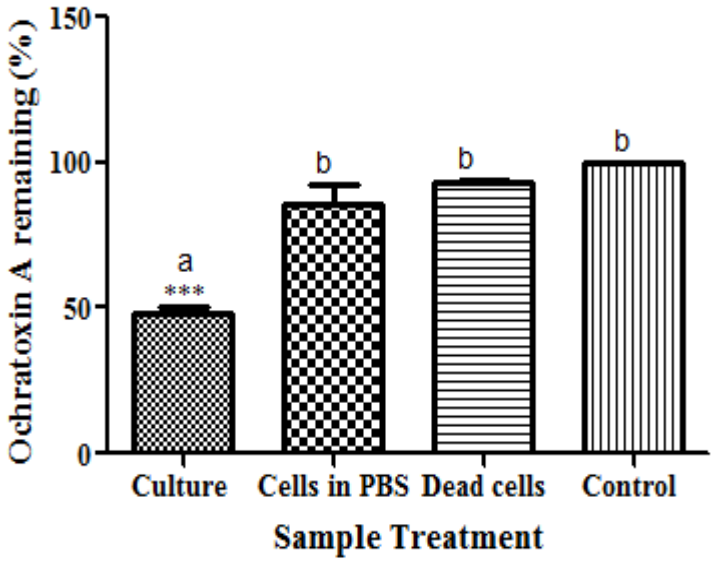


Figure 6.4 - Biological detoxification of ochratoxin A by liquid culture of *R. erythropolis* ATCC4277 at 30 ºC, pH 6 for 24h. Treatments with different letters in each column are statistically different by each treatment (***P≤0.001,** P≤0.01, *P≤0.05).

Table 6.2 - One-way ANOVA analysis followed by Tukey's Multiple Comparison Test for biological detoxification of ochratoxin A by liquid culture of *R. erythropolis* ATCC4277 at 30 ºC, pH 6 for 24h.

Table Analyzed				
One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. Different? (P < 0.05)	Yes			
Number of groups	4			
F	43.28			
R squared	0.9420			
ANOVA Table	SS	Df	MS	
Treatment (between columns)	4794	3	1598	
Residual (within columns)	295.4	8	36.92	
Total	5090	11		
Tukey's Multiple Comparison Test	**Mean Diff.**	**Q**	**Significant? P < 0.05?**	**95% CI of diff**
Culture vs PBS	-36.97	10.54	Yes	-52.85 to -21.08
Culture vs Dead cells	-45.01	12.83	Yes	-60.90 to -29.12
Culture vs Control	-51.71	14.74	Yes	-67.60 to -35.82
PBS vs Dead cells	-8.041	2.292	No	-23.93 to 7.848
PBS vs Control	-14.74	4.202	No	-30.63 to 1.148
Dead cells vs Control	-6.700	1.910	No	-22.59 to 9.189

(***P≤0.001,** P≤0.01, *P≤0.05)

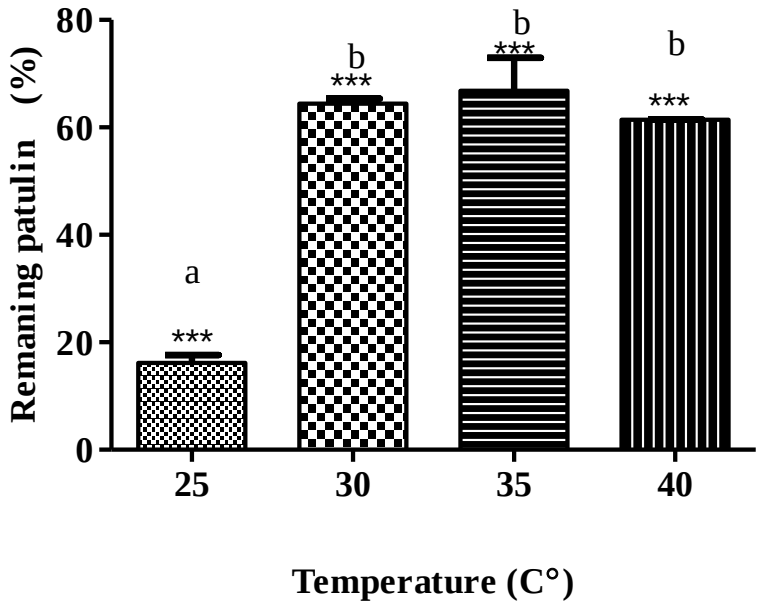


Figure 6.5- Effect of temperature on patulin degradation by *R. erythropoli*. Treatments with different letters in each column are statistically different by each treatment (***P≤0.001)

Table 6.3 - One-way AVOVA analysis for effect of temperature on patulin degradation by *R. erythropolis* culture.

Table Analyzed				
One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	57.29			
R squared	0.9555			
ANOVA Table	SS	Df	MS	
Treatment (between columns)	5245	3	1748	
Residual (within columns)	244.2	8	30.52	
Total	5489	11		
Tukey's Multiple Comparison Test	**Mean Diff.**	**Q**	**Significant ? P < 0.05?**	**95% CI of diff**
25 vs 30	-48.27	15.13	Yes	-62.71 to -33.82
25 vs 35	-50.68	15.89	Yes	-65.12 to -36.23
25 vs 40	-45.30	14.20	Yes	-59.75 to -30.86
30 vs 35	-2.411	0.7559	No	-16.86 to 12.03
30 vs 40	2.964	0.9291	No	-11.48 to 17.41
35 vs 40	5.375	1.685	No	-9.071 to 19.82

(***P≤0.001,** P≤0.01, *P≤0.05)

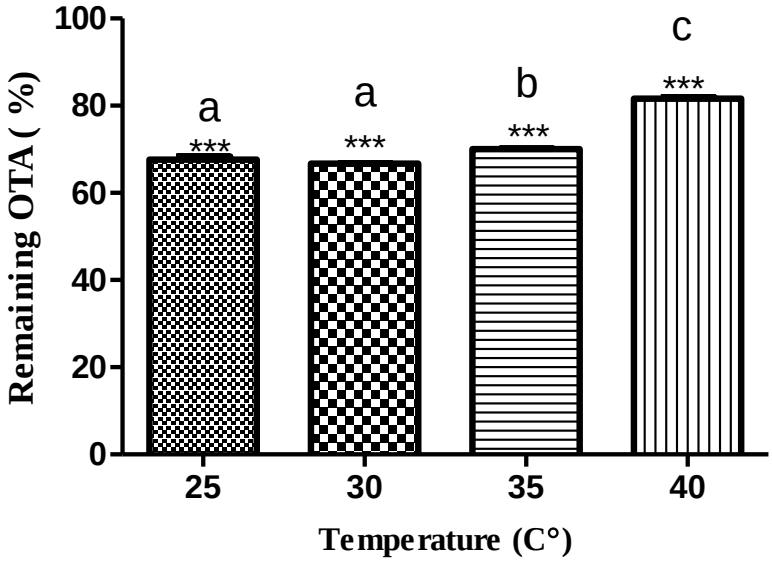


Figure 6.6 - Effect of temperature on ochratoxin A degradation by *R. erythropolis* after 24 h incubation at pH6. Treatments with different letters in each column are statistically different by each treatment (***P≤0.001,** P≤0.01, *P≤0.05).

Table 6.4-Effect of temperature on ochratoxin A degradation by R. *erythropolis* after 24 h incubation at pH6.

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	394.9			
R squared	0.9933			
ANOVA Table	SS	df	MS	
Treatment (between columns)	428.7	3	142.9	
Residual (within columns)	2.895	8	0.3619	
Total	431.6	11		
Tukey's Multiple Comparison Test	**Mean Diff.**	**Q**	**Significant? P < 0.05?**	**95% CI of diff**
25 vs 30	0.9351	2.692	No	-0.6379 to 2.508
25 vs 35	-2.443	7.034	Yes	-4.016 to -0.8700
25 vs 40	-14.01	40.33	Yes	-15.58 to -12.44
30 vs 35	-3.378	9.726	Yes	-4.951 to -1.805
30 vs 40	-14.94	43.03	Yes	-16.52 to -13.37
35 vs 40	-11.57	33.30	Yes	-13.14 to -9.993

(***P≤0.001,** P≤0.01, *P≤0.05)

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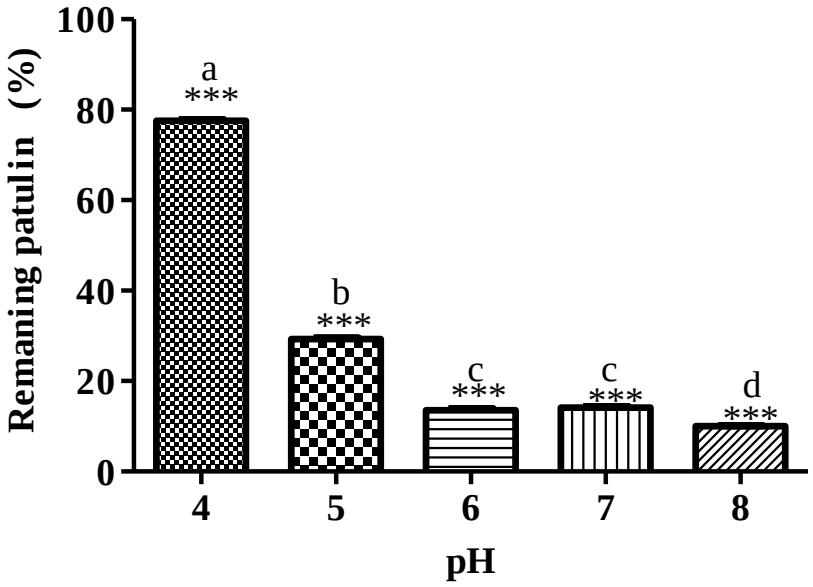


Figure 6.7- Effect of pH on patulin degradation by *R. erythropolis* ATCC 4277 culture at 25 ºC. Treatments with different letters in each column are statistically different by each treatment (***P≤0.001,** P≤0.01, *P≤0.05).

Table 6.5- One-way ANOVA analysis for effect of different pH on patulin degradation by *R. erythropolis* cultures.

One-way analysis of variance				
P value	0.0001			
P value summary	***			
Are means signif. Different? (P < 0.05)	Yes			
Number of groups	5			
F	41000			
R squared	0.9999			
ANOVA Table	SS	Df	MS	
Treatment (between columns)	9535	4	2384	
Residual (within columns)	0.5815	10	0.05815	
Total	9536	14		
Tukey's Multiple Comparison Test	**Mean Diff.**	**Q**	**Significant ? P < 0.05?**	**95% CI of diff**
4 vs 5	48.25	346.6	Yes	47.61 to 48.90
4 vs 6	64.04	460.0	Yes	63.39 to 64.69
4 vs 7	63.47	455.9	Yes	62.82 to 64.12
4 vs 8	67.49	484.8	Yes	66.84 to 68.14
5 vs 6	15.79	113.4	Yes	15.14 to 16.43
5 vs 7	15.22	109.3	Yes	14.57 to 15.87
5 vs 8	19.23	138.2	Yes	18.59 to 19.88
6 vs 7	-0.5683	4.082	No	-1.216 to 0.07965
6 vs 8	3.449	24.77	Yes	2.801 to 4.096
7 vs 8	4.017	28.85	Yes	3.369 to 4.665

(***P≤0.001,** P≤0.01, *P≤0.05)

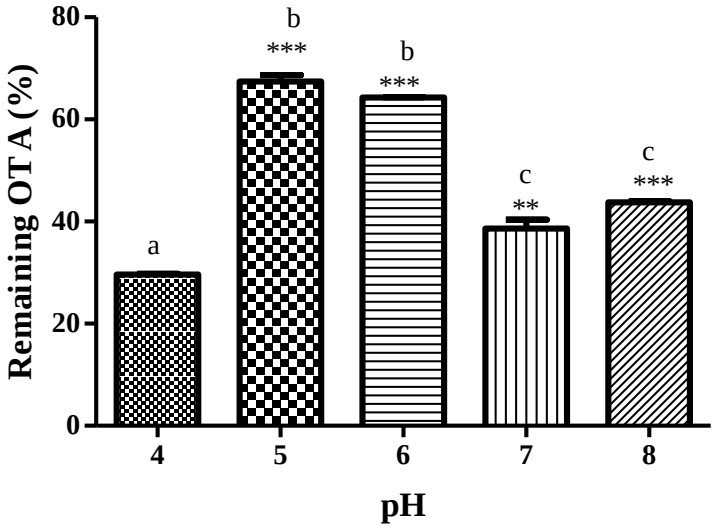


Figure 6.8- Effect of pH on ochratoxin A degradation by *R. erythropolis* after 24 h incubation at 30ºC. Treatments with different letters in each column are statistically different by each treatment (***P≤0.001,** P≤0.01, *P≤0.05).

Table 6.6- One-way ANOVA to analyse the effect of pH on ochratoxin A degradation by *R. erythropolis* After 24 h incubation at 30ºC.

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	279.1			
R squared	0.9955			
ANOVA Table	SS	df	MS	
Treatment (between columns)	2167	4	541.9	
Residual (within columns)	9.708	5	1.942	
Total	2177	9		
Tukey's Multiple Comparison Test	**Mean Diff.**	**Q**	**Significant? P < 0.05?**	**95% CI of diff**
4 vs 5	-37.78	38.34	Yes	-43.37 to -32.19
4 vs 6	-34.67	35.19	Yes	-40.26 to -29.08
4 vs 7	-8.973	9.107	Yes	-14.56 to -3.383
4 vs 8	-14.11	14.33	Yes	-19.70 to -8.525
5 vs 6	3.106	3.152	No	-2.484 to 8.695
5 vs 7	28.81	29.24	Yes	23.22 to 34.40
5 vs 8	23.67	24.02	Yes	18.08 to 29.25
6 vs 7	25.70	26.09	Yes	20.11 to 31.29
6 vs 8	20.56	20.87	Yes	14.97 to 26.15
7 vs 8	-5.142	5.219	No	-10.73 to 0.4476

(***P≤0.001,** P≤0.01, *P≤0.05)

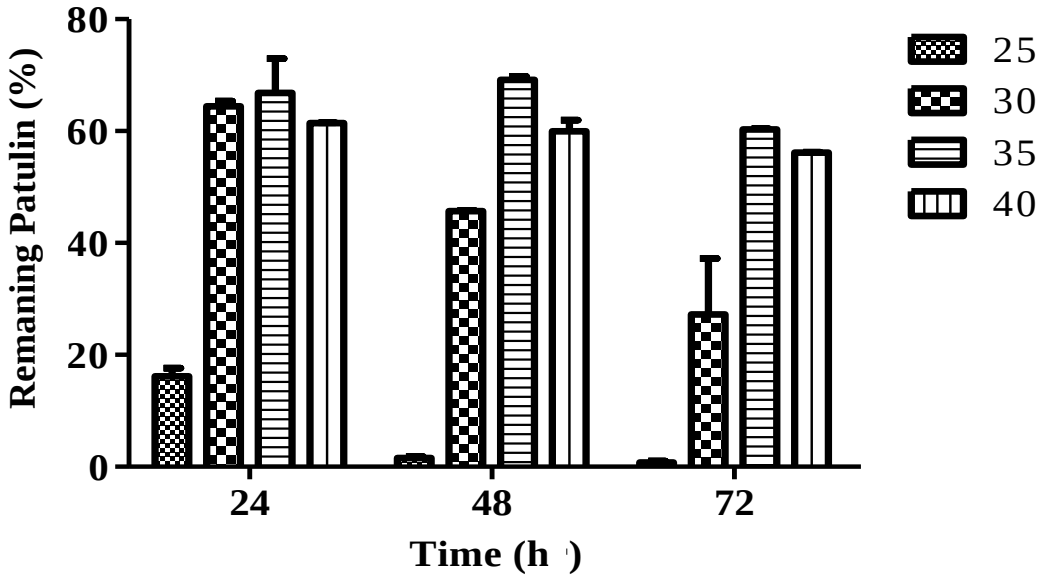


Figure 6.9- Effect of interaction of time and temperature on patulin degradation by *R. erythropolis* culture.

Table 6.7 - Two-way ANOVA analysis of effect of interaction of time and temperature on patulin degradation by *R.erythropolis* culture.

Two-way ANOVA

Source of Variation	**% of total variation**	**P value**
Interaction	5.03	0.0014
Column Factor	84.29	< 0.0001
Row Factor	6.84	< 0.0001

Source of Variation	P value summary	Significant?
Interaction	**	Yes
Column Factor	***	Yes
Row Factor	***	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	6	1140	190.1	5.238
Column Factor	3	19120	6374	175.7
Row Factor	2	1553	776.3	21.40
Residual	24	870.8	36.28	

Number of missing values 0

(***P≤0.001,** P≤0.01, *P≤0.05)

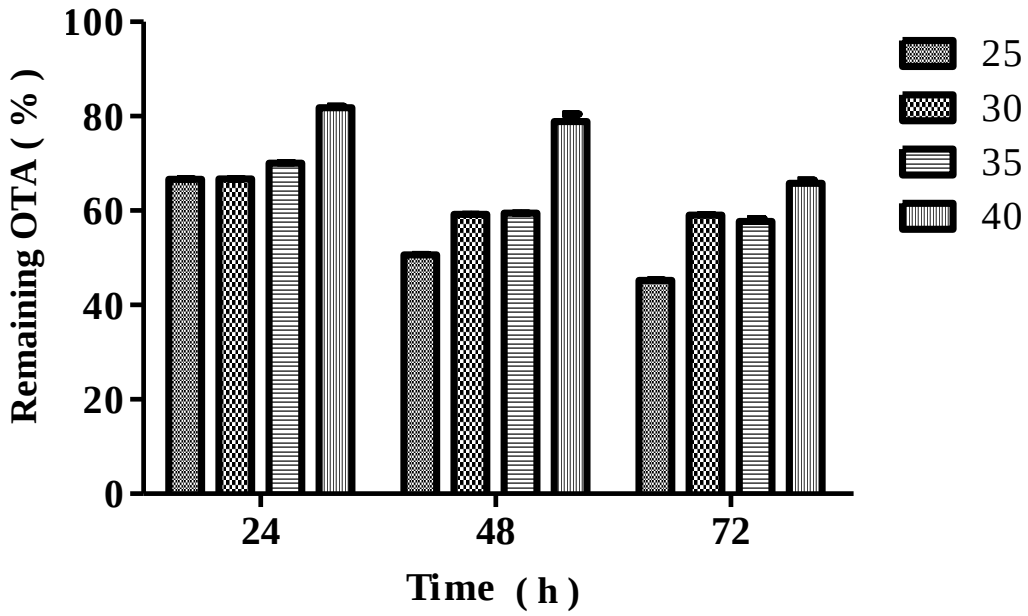


Figure 6.10 - Effect of interaction of time and temperature on ochratoxin A degradation by *R. erythropolis* culture.

Table 6.8- Two-way ANOVA analysis of effect of interaction of time and temperature on ochratoxin A degradation by *R. erythropolis* culture.

Two-way ANOVA

Source of Variation	% of total variation	P value		
Interaction	8.55	< 0.0001		
Temperature	57.40	< 0.0001		
Time	38.39	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Temperature	***	Yes		
Time	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	6	263.1	43.85	93.48
Temperature	3	1767	589.0	1256
Time	2	1182	590.8	1260
Residual	22	10.32	0.4691	
Number of missing values	2			

(***P≤0.001,** P≤0.01, *P≤0.05)

6.2.8.2 MTT RESULT

To show that the biodegradation process was effective in detoxifying the toxicity factor of the toxins, the residuals of both toxins were tested for their acute toxicity following a biodegradation process. An MTT assay was conducted where HepG2 cells were used to evaluate the toxicity of remaindered mycotoxins. All the fractions from the biodegradation process were tested.

Figure 6.11 shows MTT assay for patulin residual at different times of incubation at 25 ºC. The treatment cells and control were incubated at 37ºC for 24h 5% CO_2. The cytotoxicity assay followed a biological degradation of patulin by *R. erythropolis.* Two different controls were used in this experiment: untreated cells (Con-) and cells treated with 2.5 µg/ml patulin (Con +) which caused a marked decrease of viable cells and about 13 % of the control was viable. Conversely, exposure of HepG2 cells to patulin remaindered from the biodegradation process showed less toxicity as the concentration was lower than for the controlled sample 0.8 µg/ml; this was achieved after 24h incubation with *R. erythropolis* culture.

The HepG2 viability was increased with a decrease in patulin concentration; about 90% of the control was viable when the concentration was dropped to 0.15 µg/ml. Statistically, an overall significant difference ($P < 0.0001$) between the different control treatments was found by one-way ANOVA (see Appendix 6.1). Treatments with different letters in each column are statistically different.

Alternatively, Figure 6.12 shows the MTT assay for ochratoxin A residual at different times of incubation at 25 ºC. The treatment cells and control were incubated

at 37ºC 5% CO_2 for 24h. The MTT result indicated that the ochratoxin A was degraded by *R. erythropolis* cultures as the percentage of HepG2 cells was increased with ochratoxin A decreased. The 2.5 µg/ml of ochratoxin standard was used to treat HepG2 cells as control was (Con +) while (Con-) was untreated cells. Only 37 % of HepG2 was viable when the cells were treated with remaindered ochratoxin A; one-way ANOVA showed an overall significant difference ($P < 0.0001$) between the control and different treatments at 95 % confidant. A concentration of 1.2 µg/ml gave rise to a significant difference ($P < 0.05$) from the treated cells and cells treated by 1.6 µg/ml; whereas no significant difference ($P > 0.05$) was found between the (con+) and cells treated by 1.2 µg/ml and 1.1 µg/ml (see Appendix 6.2).

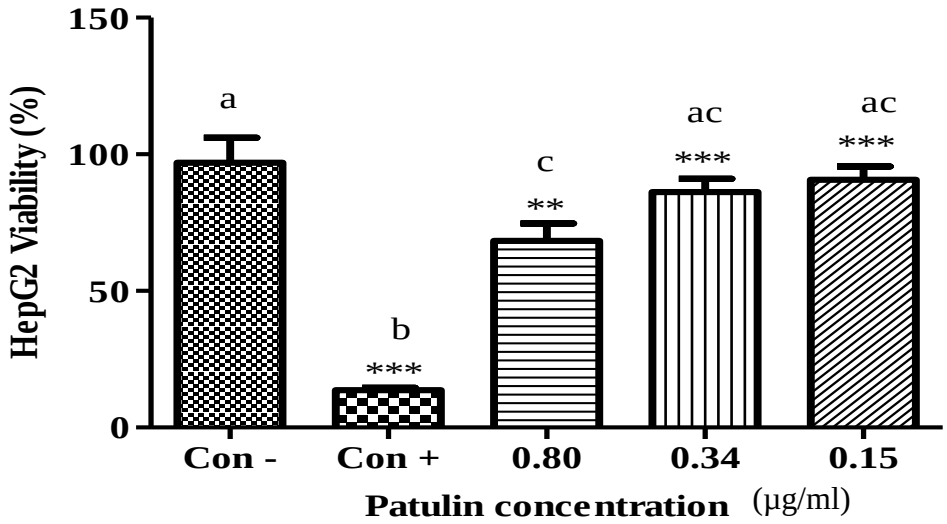


Figure 6.11- MTT assay for patulin residual at different time incubations at 25 ºC. The treatment cells and control were incubated at 37ºC 5% CO_2 for 24h. The cytotoxicity assay followed a biological degradation of patulin by *R. erythropolis.* Treatments with different letters in each column are statistically different *** P≤ 0.001 **P≤0.01 *P≤0.05.

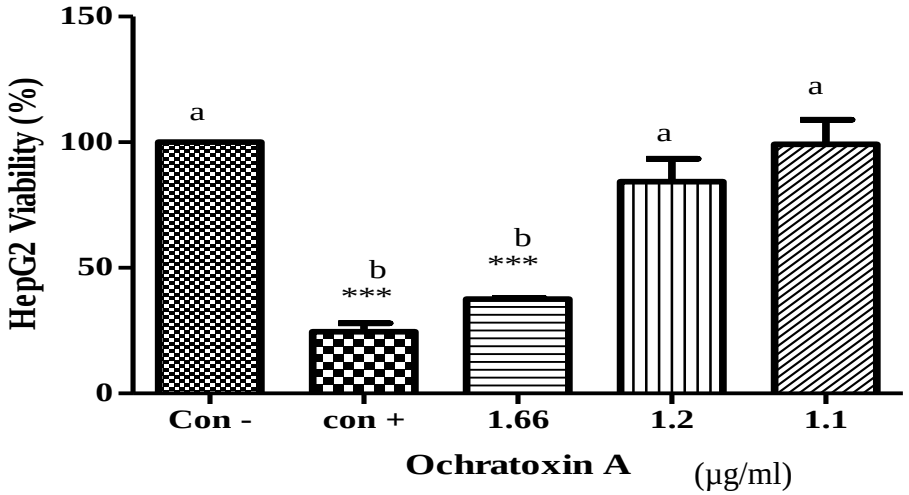


Figure 6.12 - MTT assay for ochratoxin A residual at different time incubations at 25 ºC. The treatment cells and control were incubated at 37ºC 5% CO_2 for 24h. The cytotoxicity assay followed a biological degradation of ochratoxin A by *R. erythropolis.* Treatments with different letters in each column are statistically different *** P≤ 0.001 **P≤0.01 *P≤0.05.

The impact of pre-treatment of OTA and PAT at different temperatures and times by *R. erythropolis* culture on the percentage of HepG2 viability are shown in Figures 6.13-6.18. In the case of patulin, it is clear from the figures that the temperature was an important factor and that a different result was achieved when the HepG2 cells were treated with residual patulin from the biodegradation process at a different temperature and 24 h incubation with the bacterial culture. This result suggested that a temperature of 25°C was the most effective in terms of degrading patulin as the percentage of HepG2; about 68 % of the control was viable; whereas about 43, 66 and 47 % was viable when 30, 35 and 40ºC respectively was used. Statistically, a general significant difference ($P<0.0001$) was found between the different treatments; Tukey's Multiple Comparison Test shows no significant difference ($P>0.05$) between 25, 35, 40ºC while 30 was a significant difference from the other set (see Appendix 6.3).

A similar result was recorded when residual ochratoxin A was used. In the case of ochratoxin A, (Figure 6.14), generally, all the fractions which showed the toxicity factor were statistically significantly ($P<0.05$) different from the negative control. One-way ANOVA showed an overall significant difference ($P<0.0001$) (see Appendix 6.4). The percentage of HepG2 increased to 81 % of the control when the cell was treated with the patulin residual from the biodegradation process at 25 °C, while 71, 55 and 39 % of the cells were viable when temperatures of 30, 35 and 40°C respectively were used as the incubation temperature to degrade patulin. One-way ANOVA showed a significant difference between the control and different treatments ($P<0.05$).

Moreover, Tukey's Multiple Comparison Test showed no significant difference between 25 and 30 °C. Moreover, there was no significant ($P>0.05$) difference between the negative control and the cells treated with remaindered patulin from the bioprocess degradation incubated at 25°C (see Appendix 6.5). The percentage of HepG2 was lower when the cells were treated with ochratoxin A residual. The MTT assay confirmed that 25°C was the optimum temperature for ochratoxin A degrading (Figure 6.16). One-way ANOVA showed a generally significant difference ($P<0.0001$) (see Appendix 6.6).

The impact of temperature and time on the degrading of mycotoxin was evident when HepG2 was treated with mycotoxins residual from the 72 h incubation with *Rhodococcus* culture. It can be seen that *Rhodococcus* culture causes a significant reduction ($P <0.05$) in the toxicity of both mycotoxins as the viability of HepG2 is increased. Statistically, the negative control was not significantly different from the cells treated with both mycotoxin residuals when the cells were treated with mycotoxins degraded at 25 and 30º C over 72 h incubation with *R. erythropolis* cultures (see Appendices 6.7 and 6.8).

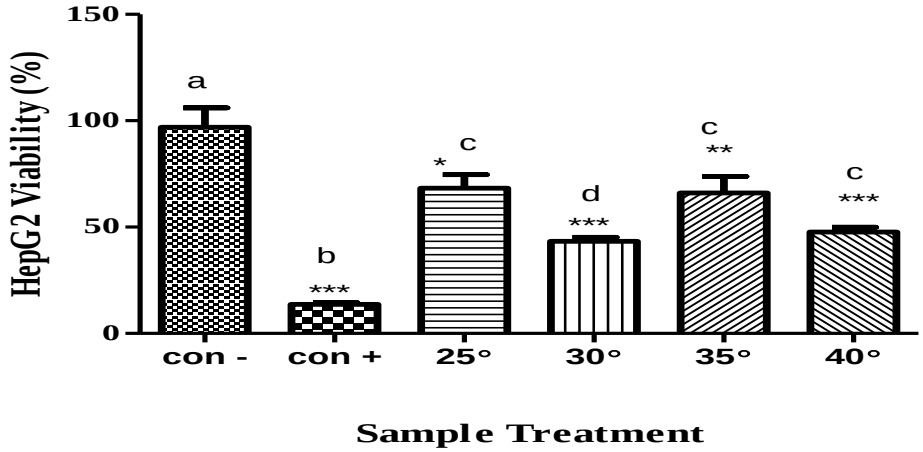


Figure 6.13 - MTT assay for patulin residual in liquid culture degraded at different incubation temperatures. The treatment cells and control were incubated at 37ºC 5% CO_2 for 24h. The cytotoxicity assay followed a biological degradation of patulin by *R. erythropolis.* Treatments with different letters in each column are statistically different (*** P≤0.0001 ,** P≤ 0.001, *P≤ 0.05).

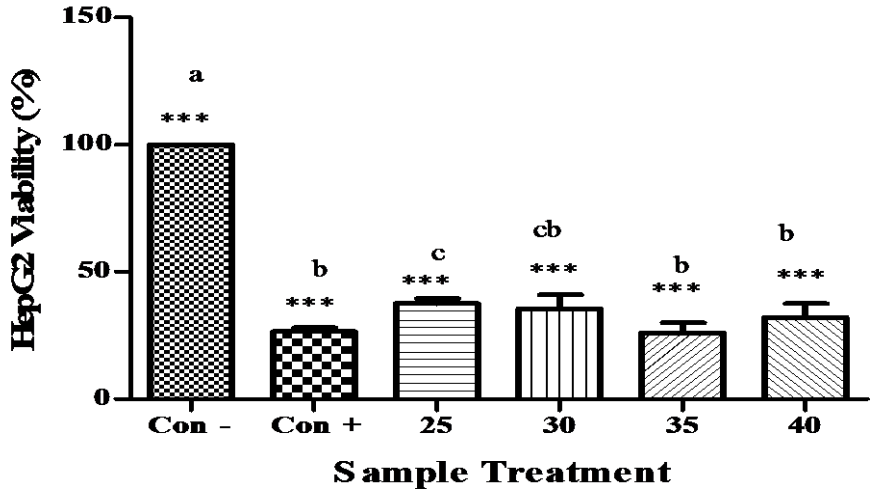


Figure 6.14 - MTT assay for ochratoxin A residual in liquid culture degraded at different incubation temperatures. The treatment cells and control were incubated at 37ºC 5% CO2 for 24h. The cytotoxicity assay followed a biological degradation of ochratoxin A by *R. erythropolis.* Treatments with different letters in each column are statistically different (*** P≤0.0001 ,** P≤ 0.001, *P≤ 0.05).

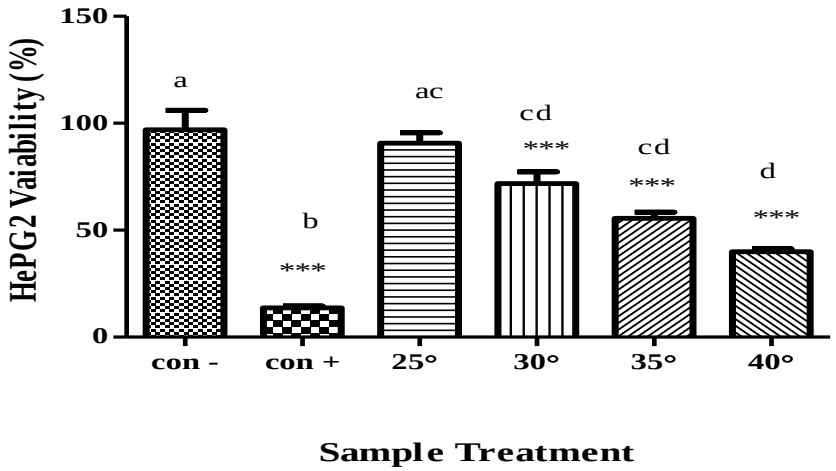


Figure 6.15 - MTT assay for patulin residue in liquid culture degraded at different incubation temperatures over 48h. The treatment cells and control were incubated at 37ºC 5% CO_2 for 24h. The cytotoxicity assay followed a biological degradation of patulin by *R. erythropolis.* Treatments with different letters in each column are statistically different (*** P≤0.0001 ,** P≤ 0.001, *P≤ 0.05).

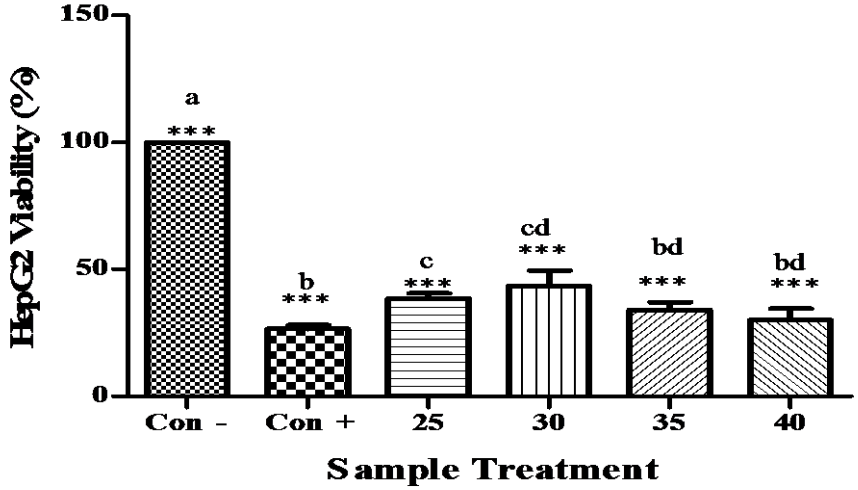


Figure 6.16 - MTT assay for ochratoxin A residue in liquid culture degraded at different incubation temperatures over 48 h. The treatment cells and control were incubated at 37ºC 5% CO2 for 24h. The cytotoxicity assay followed a biological degradation of ochratoxin A by *R. erythropolis*. Treatments with different letters in each column are statistically different (*** P≤0.0001 ,** P≤ 0.001, *P≤ 0.05).

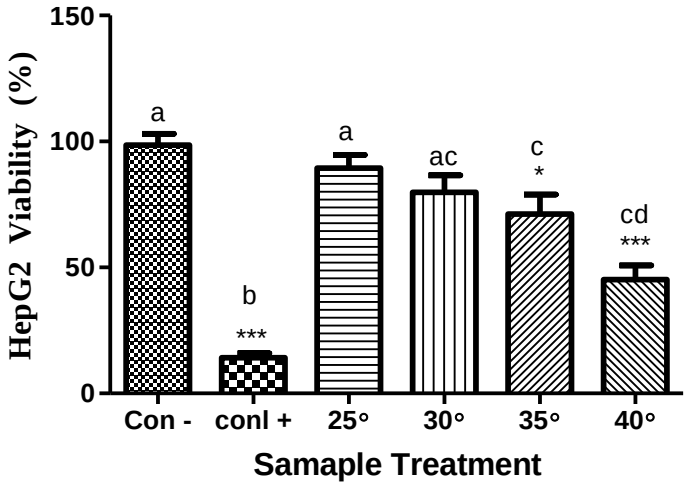


Figure 6.14- MTT assay for patulin residue in liquid culture degraded at different incubation temperatures over 72h. The treatment cells and control were incubated at 37ºC 5% CO2 for 24h. The cytotoxicity assay followed a biological degradation of patulin by *R. erythropolis.* Treatments with different letters in each column are statistically different (*** P≤0.0001 ,** P≤ 0.001, *P≤ 0.05).

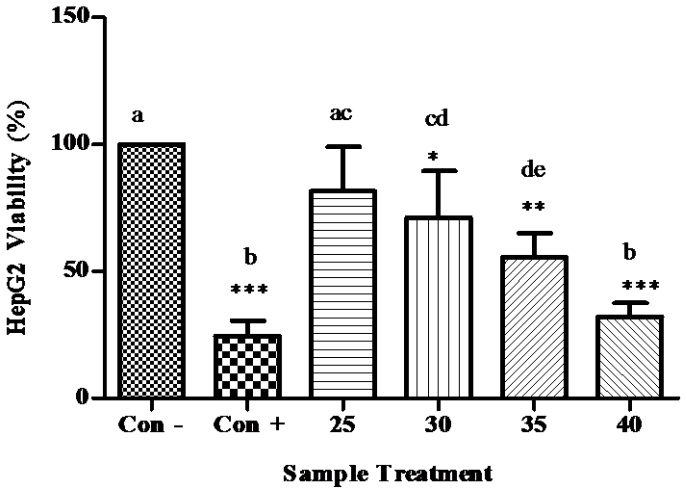


Figure 6.18 - MTT assay for ochratoxin A residue in liquid culture degraded at different incubation temperatures over 72 h. The treatment cells and control were incubated at 37ºC 5% CO2 for 24h. The cytotoxicity assay followed a biological degradation of ochratoxin A by *R. erythropolis.* Treatments with different letters in each column are statistically different (*** P≤0.0001 ,** P≤ 0.001, *P≤ 0.05).

6.3 - DISCUSSION

The ability of *Rhodococcus erythropolis to* degrade patulin and ochratoxin A in this chapter were investigated. *Rhodococcus* sp were capable of degrading a wide range of xenobiotic compounds and are considered to play a critical role in the removal of toxic compounds from the environment (Alberts *et al.*, 2006). Moreover, Alberts *et al.*, (2006) and Teniola *et al.*, (2005) suggested the ability of *Rhodococcus* to degrade Aflatoxin B1. Patulin and ochratoxin A are mycotoxins coumarin derivatives which are structurally similar to aflatoxin B1.

The results of this research have demonstrated, for the first time , the capability of *R. erythropolis* culture to degrade two mycotoxins, patulin and ochratoxin. Liquid cultures *of R. erythropolis* were able to degrade both mycotoxins effectively. It was possible to identify that the *Rhodococcus* cultures have greater capability of degrading patulin compared to ochratoxin A, as the percentage of patulin remaining was less than that of ochratoxin A.

The degradation process revealed new breakdown compounds. More than one peak was identified as unknown compounds in both cases. Previously, it has been published that several yeasts were able to degrade patulin (Moake *et al.*, 2005). Moss and Long (2002) suggested that the degradation product is ascladiol. Few publications discuss ochratoxin A detoxification by different microorganisms or enzymes from a microbial system.

Ochratoxin A can be cleaved to non-toxic ochratoxin A (OTα) and *β*-phenylalanine (Phe) by carboxypeptidase A. However, the mechanism action of ochratoxin A is not fully understood (Stander *et al.,* 2001). Additionally, it has been shown that ruminal microflora were able to detoxify ochratoxin A via cleavage of the peptide bond which leads to the release of phenylalanine (Özpinar *et al.,* 1999).

In order to investigate the mechanisms which account for mycotoxin detoxification by *Rhodococcus* culture, the effect of viable cells (preserved in PBS, 50mM pH 6) and heat inactivation (autoclaved cells) was investigated. The result showed degradation rather than binding to the cell wall which had been earlier proposed for lactic acid bacteria by (El-Nezami *et al.,* 1998a, El-Nezami *et al.,* 2002a). No significant reduction took place in the presence of *R. erythropolis* cells suspended in phosphate buffer saline or in the presence of heat-inactivated cells. Both toxins were degraded effectively by the bacterial liquid culture. This degradation was achieved during the normal growth of *R. erythropolis.*

In order to establish optimal conditions for toxin degradation, several experiments were conducted in which the temperature, pH and time dependency of mycotoxin degradation were studied. Interestingly, no significant difference (P = 0.0794) was found between patulin and ochratoxin A in terms of degradation by *R. erythropolis* in the first 24h at 25 and pH6.

The optimum degradation was achieved at 25ºC in the case of patulin, while the maximum degradation for ochratoxin A was achieved at 25 and 30 ºC where the statistical analysis showed no significant difference (P>0.05) between 25 and 30 ºC.

There is a general lack of information relating to patulin degradation and optimum conditions by using different microorganisms. These results agreed with those published by Varga *et al.,* (2005) who indicated the ability of *Rhizopus sp* to degrade patulin and ochratoxin A at 25ºC, while Teniola *et al.,* (2005) showed that the optical degradation of aflatoxin B1 by *Rhodococcus* culture occurred at 30 ºC. Moreover, Ciegler *et al.,* (1966a) reported a range of temperatures which were capable of degrading aflatoxin B1 by *Nocardia corynebacterioides* DSM 12676.

To optimize the degradation of OTA PAT by of *R. erythropolis*, a different initial pH was used. The result in this research showed that the optimum degradation for patulin was at pH 8, while pH 4 showed a high degradation rate for ochratoxin A. *R. erythropolis* at pH 6 and 7 showed a high degradation rate when patulin was added to the culture, while in case of ochratoxin A, pH 7 and 8 showed a greater degradation rate compared to pH5 and 6. The pH dependency observed by this research was different from that found by Fuchs *et al.,* (2008) and Topcu *et al.,* (2010) in patulin degradation, whereas in agreement with Fuchs *et al.,* (2006) and Mateo *et al.,* (2010) the optimal removal of ochratoxin A was achieved at lower pH. Moreover, Guan *et al.,* (2008) showed that pH was one of the factors influencing AFB1 degradation where the higher degradation rate was at pH 8 and the lowest was at pH 4. Motomura *et al.,* (2003) indicated that the optimal degradation for AFB1 was at pH 4 and 5 and at 25 ºC.

It is evident that mycotoxin degradation involves many factors. Therefore, the effect of the interaction between the temperature and incubation time was investigated. In

both cases, the degradation process was temperature and time dependent. The optimum degradation for patulin and ochratoxin A was achieved at 25 -30 º C over 72 h incubation with *Rhodococcus* culture. These results are similar to those recorded by (Guan *et al.,* 2008). In addition, Varga *et al.,* (2000) recorded the ability of *Aspergillus Sp* to degrade ochratoxin A in non-toxic ochratoxin over 10 days at 30ºC. Meanwhile, the same group showed 30 º C and 16 days as the optimum conditions for degrading ochratoxin A by *Rhizopus.*

However, the question remains whether the degradation of toxic compounds by *Rhodococcus* from liquid media, which can be quantified by analytical methods, results in a decrease of their toxic properties. However, few investigations have addressed this issue; thus it cannot be excluded that metabolites produced during degradation may possess toxic properties. Early investigations suggested that different assays such as *Salmonella* / microsoma assays, bacterial mutagenicity assays *in vitro* and *in vivo* experiments to evacuate the mycotoxin pre-treatment may/may not reduce the toxic properties (El-Nezami *et al.,* 2000, Gratz *et al.,* 2005, Gratz *et al.,* 2007, Lahtinen *et al.,* 2004).

It is known that ochratoxin A causes a Micronucleus (MCN) formation in HepG2 cells (Knasmuller *et al.,* 2004). Moreover, patulin causes oxidative damage in mammalian cells (Schumacher *et al.,* 2006) and induces DNA damage in HepG2 cells (Fuchs *et al.,* 2008) . In the present study, an MTT assay conducted with HepG2 cells was used to assess the acute toxicity of toxin residual/ new generated toxins from the bio-degradation process. The pre-treatment of patulin and ochratoxin

A led to a substantial decrease of the toxins by *Rhodococcus* liquid culture. MTT results showed that the cytotoxiciy of both toxins decreased with an increase in the pre-treatment time. Indeed, the substantial decrease of HepG2 cells when the cells were treated by 24 h pre-treatment in both cases reflects an acute cytotoxic effect. A similar result was recorded by (Fuchs *et al.,* 2006).

A micronucleus assay conducted with HepG2 was used to measure the toxicity. Their observation supported our observation, as when the toxin was pre-treated with lactic acid bacteria, a reduction in the toxic properties was achieved (Fuchs *et al.,* 2006). There is a shortage of information regarding this, as most of the publications relating to this topic were investigating the effect of the toxin without any pre-treatment.

6.4 - CONCLUSION

The results of the present investigation show that *R. erythropolis* can degrade ochratoxin A and patulin. The degradation was enzymatic, observed during the normal growth of the bacteria without pre-exposure. The degradation was temperature, time and pH dependent. This finding may contribute to developing strategies to detoxify the toxins which contaminate food.

CHAPTER 7

GENERAL DISCUSSION

7 - INTRODUCTION

Previous chapters have dealt with presentation of the results of separate studies and discussion of these findings and the impact of the research. In this chapter, the research findings are discussed comprehensively. Based upon this discussion proposals for future research in this area will be advanced.

7.1 MYCOTOXIN CONTAMINATION IS AN AGRO- INDUSTRIAL CHALLENGE

Maintaining a safe global food and feed supply is a critical issue facing all societies. Natural contaminants, especially mycotoxins, pose a challenge because they are found in a wide range of crops. Once food is contaminated with mycotoxin, there are two options if the food is to be used; either the toxin is removed or the toxin is degraded into less toxic or a non-toxic compound. Only the first option is available by extracting the toxin by chemical solvent or by binding it by chemical absorbent (El-Nezami *et al.*, 1998a). These methods are limited by leaving unwanted residues or markedly altering the composition of the foodstuff. Therefore, a variety of methods have been developed, in particular biological methods (Guan *et al.*, 2008). Biological detoxification is generally likely to be milder and maintain food/ feed nutrient levels better (Wu *et al.*, 2009).

Therefore, this research examined the potential of various microbial strains to destroy or reduce the toxicity of aflatoxin B1 (AFB1), ochratoxin A and patulin when cultured in liquid media containing mycotoxin. Initial studies **(Chapter 2)** focused upon the potential of pro-biotic bacteria. Five strains were used (three

strains of *Lactobacillus,* one each of *Lactococcus and Bifidobacterium*) to detoxify AFB1. The results indicated that the amount of AFB1 adsorbed by the pro-biotic bacteria was influenced by both microbial species and toxin concentration. The amount of AFB1 removed increased with increasing AFB1 concentration up to five times the initial concentration used, then the amount removed decreased. Conversely, El-Nezami *et al.,* (1998a) recorded that the amount of AFB1 removed increased with increasing AFB1 concentration. The results presented in this study demonstrated that the bacterial surface is limited in terms of availability of free receptors to bind the toxin. Those receptors might be available in one strain more than others. It is clear that *L .plantarum* 6376 had a significant effect in depletion of AFB1. Therefore, a series of investigations was used in order to examine whether or not physical / chemical treatments would induce any marked alteration to the adhesion of AFB1.

Results indicated that AFB1 adhesion may be mediated by means of binding to the cell wall peptidoglycan, probably by intermolecular forces of attraction or by hydrophobic bonding to the peptidoglycan receptor in the cell wall or a combination of both. Lipoteichoic acid may also be involved as a hydrophobic pocket attracting the toxin, followed by other forces which might be involved in the adhesion process. These results are in agreement with the latest reports on AFB1 binding by lactic acid bacteria which suggested that only selected strains of LAB were able to absorb a high percentage of AFB1 and the cell wall element was important for AFB1 binding; and teichoic acid may be involved in the adsorption process (Hernandez-Mendoza *et al.,* 2009, Dalié *et al.,* 2010).

Understanding the adsorption mechanism will enable the design of specific applications; moreover, the introduction of large scale bio-preservation of food requires careful safety assessment and risk analysis. However, the adsorption system will always be limited to the strain performance and some risk still exists around using this technique for mycotoxins removal which concerns stability of the complex in the *vivo* system. Therefore, later studies involved the use of bacterial strains which have the capability of degrading complex organic compounds and testing their ability to degrade three mycotoxins (**Chapters 3 and 6**).

In this study, biological degradation of AFB1 by 3 Actinomycete species, (*Rhodococcus erythropolis* ATCC 4277, *Streptomyces lividans* TK 24, and *S. aureofaciens* ATCC 10762) was examined in liquid cultures (**Chapter 3**). The use of the above mentioned strains (*Streptomyces lividans* TK 24, and *S. aureofaciens* ATCC 10762) to degrade AFB1 was employed for the first time in the present study. Four methods were used to assess the extent of the degradation; thin layer chromatography (TLC), high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC-MS) and Orbitap LC-MS/MS. AFB1 was effectively degraded by all of these test species. In the presence of *R. erythropolis,* less than 5 % of AFB1 remained at the end of the experimental period, while for *S. lividans* and *S. aureofaciens,* about 12 and 14 % respectively of the AFB1 was left at the end of the first 24h of the degradation process,while no significant difference ($P>0.05$) was found between the three cultures in terms of their ability to degrade AFB1 over a period of 72 h.

Previous studies indicated the ability of *Rhodococcus* strains isolated from hydrocarbon contaminated soil to degrade poly aromatic compounds only a few studies indicated the ability of *Rhodococcus* to degrade AFB1. The selected strains were used to degrade un-concentrated AFB1. The results of previous studies showed that both intracellular and extracellular extracts of *Rhodococcus* were effectively degrading AFB1 (Alberts *et al.*, 2006, Teniola *et al.*, 2005).

Biodegradation of AFB1 by Actinomycete species was investigated under a variety of conditions in order to optimise its degradation. The toxin was detoxified at all the pHs and incubation temperatures used in this experiment. However, the optimum degradation was achieved at 30 º C and pH 6 over a period of 72h.

Preliminary studies by LCMS/MS into the degradative pathways indicate that these cultures are able to degrade AFB1 and different metabolic routes may have been used by these cultures. Alberts *et al.*, (2006) reported that AFB1 was most likely metabolized to degradation products with chemical properties different from that of AFB1, because the equipment they utilized could not reveal the formation of any breakdown products. The present research has unambiguously confirmed AFB1 degradation by utilizing high resolution FTMS to stepwise identify the metabolites during the degradation process.

The high resolution mass spectroscopy technique LTQ-Orbitrap analysis indicated that the degradation of AFB1 was associated with the appearance of a range of lower molecular weight compounds and that these were not identical, for all three cultures.

High resolution FTMS results in our research were indicative of the formation of new metabolites along with the degradation of AFB1. The mass spectrum of the treated sample showed intense pseudomolecular ion peak values at *m/z* 331.0707, 287.2219, 237.1211 as well as that of at *m/z* 313.0707 attributable to residual AFB1. These ions were not present in the mass spectrum of either the reference sample of pure AFB1 or in the control samples. As a result, it can be inferred that these metabolites represent degradants produced during the culture treatment.

Moreover, there was a strong negative correlation between AFB1 concentration and the level of the metabolite at MW 236.1. That is, while AFB1 was decreasing, the metabolite of MW 236.1 was increasing. Based on this information, a possible mechanism of degradation of AFB1 by liquid cultures of *Rhodococcus* was hypothesized (**Chapter 3**). AFB1 bio-degradation was also associated with the accumulation of intermediates of fatty acid metabolism, and glycolysis (see Figure 7.1).

In a study by Mendez-Albores *et al.,* (2005), Mendez-Albores *et al.,* (2009) chemical inactivation of Aflatoxin B1 and aflatoxin B_2 was reported in maize grain by means of 1N aqueous citric acid. They proposed that detoxification of AFB1 involved formation of the β-keto acid structure, followed by hydrolysis of the lactone ring yielding AFD_1. This may involve the formation AFD_2.

To investigate the reduction in toxicity in the treated fluids; cytotoxicity MTT and LDH assays were conducted with a human-derived hepatoma cell line (HepG2) (**Chapter 4**).

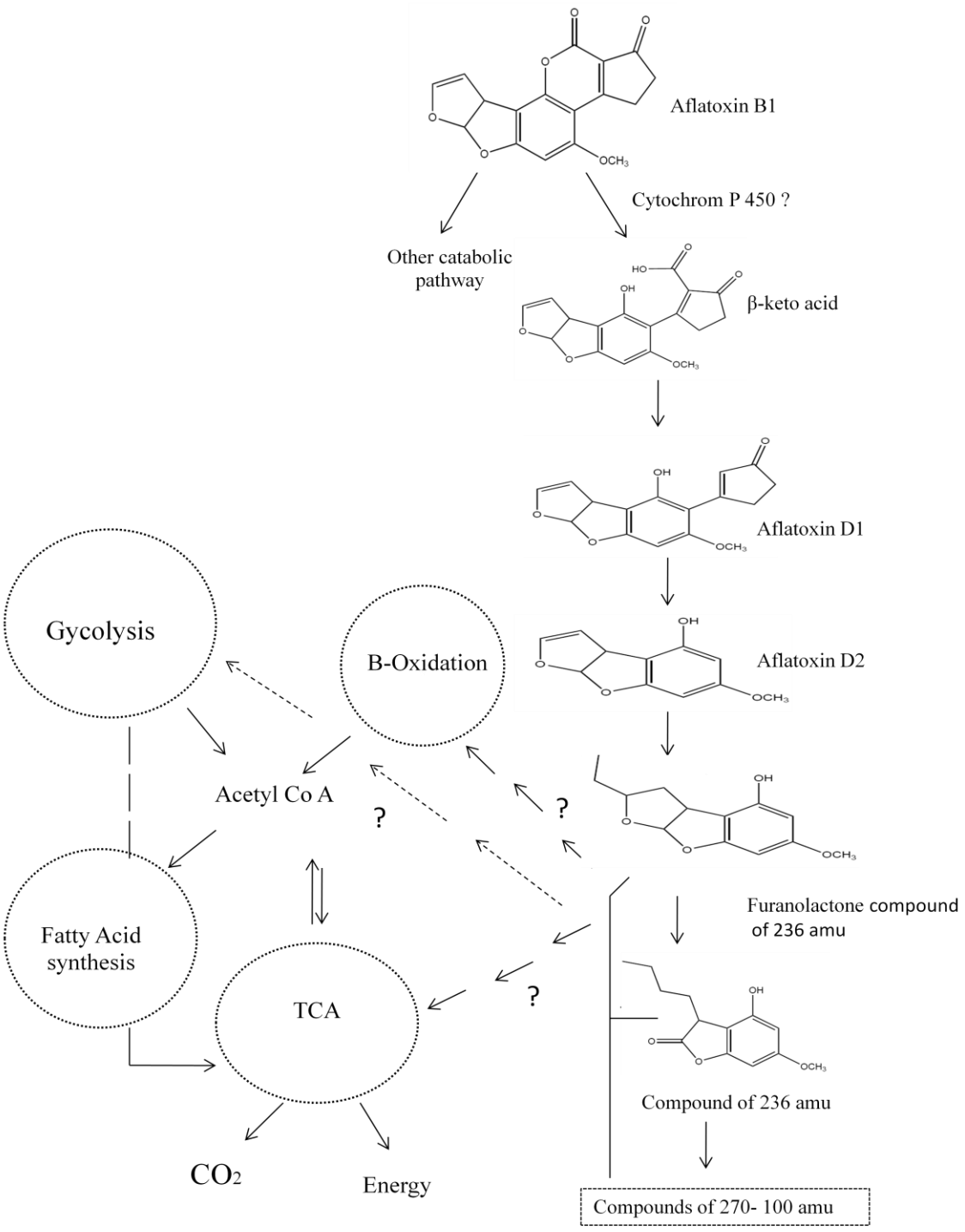


Figure 7.1- Proposed pathway for aflatoxin B1 by *Rhodococcus erythropolis*

The results showed that the MTT assay is more sensitive to the toxin compared to the LDH assay. A significant reduction in the toxicity of the treated fluid was noted as reflected in a steady rise in the percentage of the viable HepG2 cells. Indeed, a substantial increase in viable cells was proven when the toxin was degraded by *R. erythropolis.* This result confirmed that *R. erythropolis* was able to degrade AFB1 into less toxic compounds while the *Streptomyces* strains were able to transform AFB1 into another derivative which had a more cytotoxic effect compared to AFB1 metabolites arising from treatment with *Rhodococcus* cultures.

However, little information is available as regards the factors affecting AFB1 degradation by *R. erythropolis.* Therefore, **Chapter 5** dealt with physico- chemical and biological factors influencing AFB1 degradation by the mentioned strain. Physical factors such as aeration and agitation were investigated. Results showed that aeration and agitation significantly influenced AFB1 degradation. The optimum degradation was achieved at 2vvm and 400 rpm; in the first 12h 98.55 % of initial concentration of AFB1 was degraded. In addition, culture age was very important with older cultures being more capable of AFB1 degradation relative to younger cultures; this may due to enzyme changing during the life cycle for the bacteria.

Previous studies recorded by Line and Brackett, (1995) showed that an older culture of *Flavobacterium aurantiacum* was able to remove AFB1 from liquid culture due to an increase in the number of nonviable cells which can provide a great scavenging. Hamid and Smith, (1987) showed that the extracts from the older mycelium were

considerably more active than those from younger mycelium, suggesting that the level of the derivative enzymes was increasing.

As was observed in **Chapter 5** the degradation of AFB1 was a multi-enzyme degradation process. Purification and analysis of extracts from *R. erythropolis* showed that the apparent molecular mass of purified enzymes was estimated to be between 42 and 66 KDa by SDS-PAGE. Comparable result was published by Alberts *et al.,* (2006) who indicated the size of the proteins about 45 KDa.

Data obtained by RFLC-MS/MS was searched using Mascot. The protein hits gave a number of suggestions for enzymes which might be involved in degradation process such as transaldolase, enolase, 2,3butanediol dehydrogenase, alcohol dehydrogenase and Zn-dependent alcohol dehydrogenase. An enzyme of similar molecular mass was purified from the intracellular fraction using PhenylSepharose (chapter 5). This result is broadly similar to Alberts *et al.,* (2006) result who found that no new prominent protein species were produced due to exposure to AFB1.

The effect of divalent metal ions on AFB1 degradation by cell-free extracts of *R. erythropolis ATCC* was also investigated. The results indicated that divalent zinc at 1mM inhibited AFB1 degradation. Divalent zinc is a cofactor of Alcohol dehydrogenases and plays a regulatory role in DNA binding and activation of transcription factors, DNA polymerase, and carbonic anhydrases (D'Souza and Brackett, 1998).

Zinc has been shown to inhibit dehydrogenases in fungal systems and glucose-6-phosphate dehydrogenases of *E.coli K-12* (D'Souza and Brackett, 1998). Results presented in this study contribute to those with inhibitory results obtained with Zn+ previously and support the hypothesis that a dehydrogenases enzyme may be involved in AFB1 degradation. Zn-dependent alcohol dehydrogenase may be ruled out in AFB1 degradation. Nevertheless, other type of alcohol dehydrogenase may involve of AFB1 degradation. To clarify the rule of dehydrogenase in the degradation process dehydrogenase inhibitors such as 4-methylpyrazole hydrochloride could supplemented into the *R.erythropolis* intracellular extract before exposure to AFB1. This confirms if the mentioned enzymes system may involved in the degradation or not.

Moreover, the effects of NADPH as inducer of cytochrome P-450 and cytochrome C as inhibitor of cytochrome P-450 toxin degradation were investigated. Results suggested that the addition of cytochrome C inhibited AFB1 degradation; meanwhile NADPH enhanced the ability of the interacellular to degrade AFB1. Consequently, this result indicating that mono-oxygenase enzymes or enzyme system is involved in AFB1 degradation.

Although the results of this study demonstrated the inhibition of AFB1 by exposure of the intracellular extract to Zn^{+}, cytochrome C and NADPH addition did not provide conclusive evidence that any particular novo enzyme synthesis is involved, but did suggest that the enzymes system involved in toxin degradation may be of the mono-oxygenase / dehydrogenase type.

Therefore, presented result in this study support the result published by (Alberts *et al.,* 2006) as AFB1 degradation may have occurred though the cascade of enzyme reactions with loss of the fluorescence over time. Enzymes involved in the catabolism of aromatic compounds have been identified and purified including enzymes like ring cleaving biphenyl dioxygenases, dihydrodiol dehydrogenases and hydrolases (Haritash and Kaushik, 2009, Martinkova *et al.,* 2009). However, a better understanding, as well as purification, can be obtained by characterizing of the enzyme system involved in detoxification system.

Moreover, the capacity of the *Rhodococcus* strain to degrade other mycotoxins was evaluated for the first (**Chapter 6**) and the results showed that *R.erythropolis* culture is capable of effectively degrading the toxins. No significant reduction took place in the absence of *R.erythropolis* cells or in the presence of heat-inactivated (Autoclaved) cells and cells preserved at phosphate buffer saline (**Chapter 6**). In the patulin sample maximal degradation was achieved at 25ºC after 24 h incubation with *R. erythropolis* cultures. Maximal degradation for ochratoxin A was achieved at 25 and 30º C. There was no significant difference between those temperatures. The degradation process was temperature and time dependent. An MTT bioassay also confirmed the detoxification, as the viability of the HepG2 cells increased with the decrease in the toxin levels following biological treatment.

7.2 - THESIS CONTRIBUTIONS

The study has contributed to improve understanding of the bio-removal process, by supplying more information about the role of lipoteichoic acids on AFB1 removal; and the nature of the stability of the complex binding between cell wall lactic acid bacteria and AFB1. The research has gained new insight into the biodegradation pathway by *Rhodococcus* and has elucidated the factors that influence metabolite degradation. Thus, the various underlying environmental factors that influence the degradation were effectively identified. Purification and analysis of extracts from *R.erythropolis* showed that detoxification was a multi-enzyme degradation process. Moreover, using the HepG2 cell following biological degradation provided more understanding about the nature of the compounds generated during the bio-detoxification process, and their cytotoxicity. This may enhance the biological approaches to be applied and involved in a practical programme.

7.3 - CONCLUSIONS

This study illustrated the high-throughput and uni- and multivariate analysis of metabolites by liquid chromatography and high resolution mass spectrometry. The data presented in this thesis will contribute to an understanding of the underlying key regulations of the mycotoxin biodegradation process which is important in food decontamination. The following conclusions are drawn from different aspects of this research study.

7.4 - ADSORPTION OF AFLATOXIN B1 BY PROBIOTIC BACTERIA

The results presented in this study contributed with early studies as Lactic acid bacteria of differing species could adsorb significant amounts of AFB1. The cell wall composition was important for AFB1 binding. Additionally, lipoteichoic acids are important components of the cell walls that are involved in AFB1 adsorption. Evidence was presented in this study showed the role of Lipoteichoic acid on AFB1 adsorption. The binding mechanism can be explained by hydrophobic interactions and intermolecular forces.

7.5 – BIODEGRDATION OF AFB1 BY ACTINOMYCETES CULTURES

The biodegradation of AFB1 by Actinomycetes was confirmed by four different techniques; the degradation was influenced by the initial pH used and temperature incubation the optimum degradation was achieved at 30°C and pH 5 and 6. TLC assay has confirmed the cleavage of the lactone group by *Rhodococcus.* A compound at 236 amu was identified and a proposed mechanism pathway for AFB1 degradation to 236 amu was suggested. Each microorganism has a different way in degrading AFB1. The metabolites produced during AFB1 degradation by *R. erythropolis* were significantly different from those produced during degradation by *Streptomyces* strains.

7.6 - CYTOTOXICITY ASSAYS ON BIO-TREATED AFB1-CONTAINING MEDIA

A significant reduction in the toxicity of the treated fluid was noted, reflected in a steady rise in the percentage of the viable HepG2 cells. MTT result and LDH confirmed that *R.erythropolis* was more effective than *Streptomyces* strains. MTT

assay was more sensitive in terms of evaluating the toxicity of residual AFB1 / degrading products produced during the biological process.

7.7 – PHYSICO-CHEMICAL & BIOLOGICAL FACTORS AFFECTING AFLATOXINB1 DEGRADATION BY *Rhodococcus erythropolis* CULTURES

The aeration and agitation enhanced AFB1 degradation by *R. erythropolis* cultures. Older cultures were more effective in degrading AFB1 than the younger cultures. Preliminary studies regards to enzymes purification and identification suggested that enzymes like dehydrogenase/ monooxygenase may be involved in AFB1 biodegradation.

7.8 - BIOLOGICAL DETOXIFICATION OF PATULIN AND OCHRATOXIN A BY *Rhodococcus erythropolis* CULTURE

Rhodococcus erythropolis can degrade ochratoxin A and patulin. The degradation was enzymatic, observed during the normal growth of the bacteria without pre-exposure. The degradation was temperature, time and pH dependent. Identifying and analysing the factors affecting the degradation; evaluation and identification of the toxicity of the intermediates and degradation products during the biodegradation process could lead to operating strategies on an industrial scale.

7.9 - FUTURE WORK AND APPLICATIONS

A considerable amount of time and effort has gone into this PhD thesis, but the research could never end therefore this section will view the point which has to be well thought-out as the work presented in here has initiated various other avenues in which there is much scope for future research.

- Evaluate the ability of LAB to adsorb a mixture of mycotoxins *in vivo*/*in vitro system.*
- Identification of elements responsible for AFB1 binding in the LAB cell wall may assist in developing a bio-adsorption agent and using it as a bio-additive in food and animal feed.
- Design a bio- filters made from LAB cell walls, and lipoteichoic acid may offer a potential use to remove aflatoxin from contaminated food.
- A better understanding, as well as purification, of the enzyme system involved in detoxification by *Rhodococcus sp* will assist to establish a practical use for these enzymes in AFB1 decontamination.

REFERENCES

8 - REFERENCES

ABRUNHOSA, L., SERRA, R. & VENANCIO, A. 2002. Biodegradation of ochratoxin A by fungi isolated from grapes. *Journal of Agricultural and Food Chemistry,* 50**,** 7493-7496.

ABRUNHOSA, L. & VENANCIO, A. 2007. Isolation and purification of an enzyme hydrolyzing ochratoxin A from *Aspergillus niger*. *Biotechnology Letters,* 29**,** 1909-1914.

AGARRY, S. E., SOLOMON, B. O. & AUDU, T. O. K. 2010. Optimization of Process Variables for the Batch Degradation of Phenol by *Pseudomonas fluorescence* Using Response Surface Methodology. *International Journal of Chemical Technology,* 2**,** 33-45.

AL-JULAIFI, M. Z. 2000. Patulin production and biodegradation by *Penicillium expansum* and *Phanerochaete chrysosporium*. *Saudi Journal of Biological Sciences,* 7**,** 156-170.

ALBERTS, J. F., ENGELBRECHT, Y., STEYN, P. S., HOLZAPFEL, W. H. & VAN ZYL, W. H. 2006. Biological degradation of aflatoxin B1 by *Rhodococcus erythropolis* cultures. *International Journal of Food Microbiology,* 109**,** 121-126.

ALBERTS, J. F., GELDERBLOM, W. C. A., BOTHA, A. & VAN ZYL, W. H. 2009. Degradation of aflatoxin B1 by fungal laccase enzymes. *International Journal of Food Microbiology,* 135**,** 47-52.

ALÍA, M., RAMOS, S., MATEOS, R., GRANADO-SERRANO, A. B., BRAVO, L. & GOYA, L. 2006. Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. *Toxicology and Applied Pharmacology,* 212**,** 110-118.

AMÉZQUETA, S., GONZÁLEZ-PEÑAS, E., MURILLO-ARBIZU, M. & LÓPEZ DE CERAIN, A. 2009. Ochratoxin A decontamination: A review. *Food Control,* 20**,** 326-333.

ARICI, M. 2000. Microbiological studies and mycotoxin analysis of a fermented cereal product (Tarhana). *Ernahrungs-Umschau,* 47**,** 477-+.

AYGUN, O., ESSIZ, D., DURMAZ, H., YARSAN, E. & ALTINTAS, L. 2009. Aflatoxin M-1 Levels in Surk Samples, a Traditional Turkish Cheese from Southern Turkey. *Bulletin of Environmental Contamination and Toxicology,* 83**,** 164-167.

BAKKALI, F., AVERBECK, S., AVERBECK, D. & IDAOMAR, M. 2008. Biological effects of essential oils - A review. *Food and Chemical Toxicology,* 46**,** 446-475.

BAPTISTA, A., S., HORII, J., ANTONIA C.D., M., MICOTTI DA GLÓRIA, E., MASTRODI S., J. & ROBERTO V., M. 2004. The Capacity of Manno-Oligosaccharides, Thermolysed Yeast and Active Yeast to Attenuate Aflatoxicosis. *World Journal of Microbiology and Biotechnology,* 20**,** 475-481.

BARREIRA, M. J., ALVITO, P. C. & ALMEIDA, C. M. M. 2010. Occurrence of patulin in apple-based-foods in Portugal. *Food Chemistry,* In Press, Corrected Proof.

BATA, Á. & LÁSZTITY, R. 1999. Detoxification of mycotoxin-contaminated food and feed by microorganisms. *Trends in Food Science & Technology,* 10**,** 223-228.

BATISH, V. K., ROY, U., LAL, R. & GROVER, S. 1997. Antifungal attributes of lactic acid bacteria - A review. *Critical Reviews in Biotechnology,* 17**,** 209-225.

BENNETT, J. W. & KLICH, M. 2003. Mycotoxins. *Clin. Microbiol. Rev.*, 16**,** 497-516.

BERGOT, B. J., STANLEY, W. L. & MASRI, M. S. 1977. Reaction of coumarin with aqua ammonia. Implications in detoxification of aflatoxin. *J Agric Food Chem,* 25**,** 865-6.

BIZAJ, E., MAVRI, J., CUS, F. & RASPOR, A. 2009. Removal of Ochratoxin A in *Saccharomyces cerevisiae* Liquid Cultures. *South African Journal of Enology and Viticulture,* 30**,** 151-155.

BOL, J., & SMITH,J.E. 1989. Biotransformation of Aflatoxin. *food biotchnolo.*, 3**,** 127-144.

BOLOGNANI, F., RUMNEY, C. J. & ROWLAND, I. R. 1997. Influence of carcinogen binding by lactic acid-producing bacteria on tissue distribution and in vivo mutagenicity of dietary carcinogens. *Food and Chemical Toxicology,* 35**,** 535-545.

BUENO, D. J., CASALE, C. H., PIZZOLITTO, R. P., SALANO, M. A. & OLIVIER, G. 2006a. Physical Adsorption of Aflatoxin B1 by lactic acid bacteria and *Saccharomyces cerevisiae*: A theoretical model. *Journal of Food Protection,* 7**,** 2148–2154.

BUENO, D. J., CASALE, C. H., PIZZOLITTO, R. P., SALVANO, M. A. & OLIVER, G. 2007. Physical adsorption of aflatoxin B-1 by lactic acid bacteria and *Saccharomyces cerevisiae*: A theoretical model. *Journal of Food Protection,* 70**,** 2148-2154.

BUENO, D. J., SILVA, J. O., OLIVER, G. & GONZALEZ, S. N. 2006b. *Lactobacillus casei* CRL 431 and *Lactobacillus rhamnosus* CRL 1224 as biological controls for *Aspergillus flavus* strains. *Journal of Food Protection,* 69**,** 2544-2548.

BULLERMAN, L. B. & BIANCHINI, A. 2007. Stability of mycotoxins during food processing. *International Journal of Food Microbiology,* 119**,** 140-146.

CARUSO, M., MARIOTTI, A., ZIZZADORO, C., ZAGHINI, A., ORMAS, P., ALTAFINI, A. & BELLOLI, C. 2009. A clonal cell line (BME-UV1) as a possible model to study bovine mammary epithelial metabolism: metabolism and cytotoxicity of aflatoxin B1. *Toxicon,* 53**,** 400-408.

CENCI, G., CALDINI, G., TROTTA, F. & BOSI, P. 2008. In vitro inhibitory activity of probiotic spore-forming bacilli against genotoxins. *Letters in Applied Microbiology,* 46**,** 331-337.

CIEGLER, A., LILLEHOJ, E. B., PETERSON, R. E. & HALL, H. H. 1966a. Microbial detoxification of Aflatoxin. . *Appl Microbiol.,* 14(6)**,** 934-939.

CIEGLER, A. & PETERSON, R. E. 1968. Aflatoxin detoxification: hydroxydihydro-aflatoxin B. *Appl Microbiol,* 16**,** 665-6.

CIEGLER, A., PETERSON, R. E., LAGODA, A. A. & HALL, H. H. 1966b. Aflatoxin Production and Degradation by *Aspergillus flavus* in 20-Liter Fermentors. *Appl. Environ. Microbiol.,* 14**,** 826-833.

COALLIER-ASCAH, J. & IDZIAK, E. S. 1985. Interaction between *Streptococcus lactis* and *Aspergillus flavus* on production of aflatoxin. *Appl. Environ. Microbiol.,* 49**,** 163-167.

COLE, R. J., DORNER, J. W. & BLANKENSHIP, P. D. 1996. Management strategies for prevention and control of mycotoxins. *In:* MIRAGLIA, M., VANEGMOND, H. P., BRERA, C. & GILBERT, J., eds. IX International IUPAC Symposium on Mycotoxins and Phycotoxins, May 27-31 Rome, Italy. Alaken, Inc, 189-201.

COLE, S. 1986. Chemosensitivity testing on human lung tumour cells uning the MTT assay *Canc Chemother Pharmacol* 17**,** 259-263.

CRAMER, B., HARRER, H., NAKAMURA, K., UEMURA, D. & HUMPF, H.-U. 2010. Total synthesis and cytotoxicity evaluation of all ochratoxin A stereoisomers. *Bioorganic & Medicinal Chemistry,* 18**,** 343-347.

CREPPY, E. E., BAUDRIMONT, I. & BETBEDER, A. M. 1995. Prevention of nephrotoxicity of ochratoxin A, a food contaminant. *Toxicology Letters,* 82-83**,** 869-877.

CVETNIĆ, Z. & PEPELJNJAK, S. 2007. Interaction Between Certain Moulds and Aflatoxin B1 Producer *Aspergillus flavus* NRRL 3251. *Archives of Industrial Hygiene and Toxicology,* 58**,** 429-434.

D'ANNIBALE, A., QUARATINO, D., FEDERICI, F. & FENICE, M. 2006. Effect of agitation and aeration on the reduction of pollutant load of olive mill wastewater by the white-rot fungus *Panus tigrinus*. *Biochemical Engineering Journal,* 29**,** 243-249.

D'SOUZA, D. H. & BRACKETT, R. E. 1998. The Role of Trace Metal Ions in Aflatoxin B1 Degradation by *Flavobacterium aurantiacum*. *Journal of Food Protection,* 61**,** 1666-1669.

D'SOUZA, D. H. & BRACKETT, R. E. 2000. The Influence of Divalent Cations and Chelators on Aflatoxin B1 Degradation by *Flavobacterium aurantiacum*. *Journal of Food Protection,* 63**,** 102-105.

D'SOUZA, D. H. & BRACKETT, R. E. 2001. Aflatoxin B1 Degradation by *Flavobacterium aurantiacum* in the Presence of Reducing Conditions and Seryl and Sulfhydryl Group Inhibitors. *Journal of Food Protection,* 64**,** 268-271.

DALIÉ, D. K. D., DESCHAMPS, A. M. & RICHARD-FORGET, F. 2010. Lactic acid bacteria - Potential for control of mould growth and mycotoxins: A review. *Food Control,* 21**,** 370-380.

DAS, C. & MISHRA, H. N. 2000. In vitro degradation of aflatoxin B-1 in groundnut (Arachis hypogea) meal by horse radish peroxidase. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology,* 33**,** 308-312.

DEL PRETE, V., RODRIGUEZ, H., CARRASCOSA, A. V., RIVAS, B. D. L., GARCIA-MORUNO, E. & MUNOZ, R. 2007. *In vitro* removal of ochratoxin a by wine lactic acid bacteria. *Journal of Food Protection,* 70**,** 2155-2160.

DELCOUR, J., FERAIN, T., DEGHORAIN, M., PALUMBO, E. & HOLS, P. 1999. The biosynthesis and functionality of the cell-wall of lactic acid bacteria *Antonie van Leeuwenhoek,* 76**,** 159-184

DUARTE, S. C., PENA, A. & LINO, C. M. 2009. Ochratoxin A non-conventional exposure sources, A review. *Microchemical Journal,* 93**,** 115-120.

DUARTE, S. C., PENA, A. & LINO, C. M. 2010a. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. *Food Microbiology,* In Press, Corrected Proof.

DUARTE, S. C., TANELLO, A., PENA, A., LINO, C. M., MATOS, C. D., OLIVEIRA, M. B. P. P. & ALVES, M. R. 2010b. Evaluation of ochratoxin A exposure degree in two Portuguese cities through wheat and maize bread consumption during the winter 2007. *Food Control,* 21**,** 702-707.

EATON, D. L. & GALLAGHER, E. P. 1994. Mechanisms of Aflatoxin Carcinogenesis. *Annual Review of Pharmacology and Toxicology,* 34**,** 135-172.

EHRLICH, V., F.DARROUDI, M.UHL, H.STEINKELLNER, M.ZSIVKOVITS & S.KNASMUELLER. 2002. Fumonisin B-1 is genotoxic in human derived hepatoma (HepG2) cells. *Mutagenesis,* 17**,** 257-260.

EL-NEZAMI, H., KANKAANPAA, P., SALMINEN, S. & AHOKAS, J. 1998a. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B-1. *Food and Chemical Toxicology,* 36**,** 321-326.

EL-NEZAMI, H., KANKAANPAA, P., SALMINEN, S. & AHOKAS, J. 1998b. Physicochemical alterations enhance the ability of dairy strains of lactic acid bacteria to remove aflatoxin from contaminated media. *Journal of Food Protection,* 61**,** 466-468.

EL-NEZAMI, H., MYKKANEN, H., KANKAANPAA, P., SALMINEN, S. & AHOKAS, J. 2000. Ability of *Lactobacillus* and *Propionibacterium* strains to remove aflatoxin B-1 from the chicken duodenum. *Journal of Food Protection,* 63**,** 549-552.

EL-NEZAMI, H., POLYCHRONAKI, N., SALMINEN, S. & MYKKANEN, H. 2002a. Binding rather than metabolism may explain the interaction of two food-grade *Lactobacillus* strains with zearalenone and its derivative alpha-zearalenol. *Applied and Environmental Microbiology,* 68**,** 3545-3549.

EL-NEZAMI, H. S., CHREVATIDIS, A., AURIOLA, S., SALMINEN, S. & MYKKANEN, H. 2002b. Removal of common Fusarium toxins in vitro by strains of *Lactobacillus* and *Propionibacterium*. *Food Additives and Contaminants,* 19**,** 680-686.

EL-SHIEKH, H. H., MAHDY, H. M. & EL-AASER, M. M. 2007. Bioremediation of aflatoxins by some reference fungal strains. *Pol J Microbiol,* 56**,** 215-23.

ELGERBI, A. M., AIDOO, K. E., CANDLISH, A. A. G. & WILLIAMS, A. G. 2006. Effects of lactic acid bacteria and bifidobacteria on levels of aflatoxin M-1 in milk and phosphate buffer. *Milchwissenschaft-Milk Science International,* 61**,** 197-199.

ELSANHOTY, R. M. 2008. Screening of some lactobacillus strains for their antifungal activities against aflatoxin-producing aspergilli in vitro and maize. *Journal of Food Agriculture & Environment,* 6**,** 35-40.

FAO. 2004. food and nutrition paper no. 81. Worldwide regulations for mycotoxins in food and feed in 2003. *180 pages*.

FAZELI, M. R., HAJIMOHAMMADALI, M., MOSHKANI, A., SAMADI, N., JAMALIFAR, H., KHOSHAYAND, M. R., VAGHARI, E. & POURAGAHI, S. 2009. Aflatoxin B-1 Binding Capacity of Autochthonous Strains of Lactic Acid Bacteria. *Journal of Food Protection,* 72**,** 189-192.

FERRAZ, M. B. M., FARAH, A., IAMANAKA, B. T., PERRONE, D., COPETTI, M. V., MARQUES, V. X., VITALI, A. A. & TANIWAKI, M. H. 2010. Kinetics of ochratoxin A destruction during coffee roasting. *Food Control,* 21**,** 872-877.

FOTAKIS, G. & TIMBRELL, J. A. 2006. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters,* 160**,** 171-177.

FRAZER, L. N. 1997. One stop mycology. *Mycological Research,* 101**,** 226-256.

FUCHS, S., SONTAG, G., STIDL, R., EHRLICH, V., KUNDI, M. & KNASMULLER, S. 2006. Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. *In:* International Conference on Molecular and Physiological Effects of Bioactive Food Compounds, Oct 11-14 2006 Vienna, AUSTRIA. Pergamon-Elsevier Science Ltd, 1398-1407.

FUCHS, S., SONTAG, G., STIDL, R., EHRLICH, V., KUNDI, M. & KNASMÜLLER, S. 2008. Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. *Food and Chemical Toxicology,* 46**,** 1398-1407.

GALLO, A., PERRONE, G., SOLFRIZZO, M., EPIFANI, F., ABBAS, A., DOBSON, A. D. W. & MULÈ, G. 2009. Characterisation of a pks gene which is expressed during ochratoxin A production by Aspergillus carbonarius. *International Journal of Food Microbiology,* 129**,** 8-15.

GALVANO, F., PIVA, A., RITIENI, A. & GALVANO, G. 2001. Dietary strategies to counteract the effects of mycotoxins: A review. *Journal of Food Protection,* 64**,** 120-131.

GHALI, R., HMAISSIA-KHLIFA, K., GHORBEL, H., MAAROUFI, K. & HEDILI, A. 2009. HPLC determination of ochratoxin A in high consumption Tunisian foods. *Food Control,* 20**,** 716-720.

GHISALBERTI, E. L. & ATTA-UR, R. 2003. Bioactive tetramic acid metabolites. *Studies in Natural Products Chemistry*. Elsevier.

GOURAMA, H. & BULLERMAN, L. B. 1995. Relationship between aflatoxin production and mold growth as measured by ergosterol and plate count. *LWT - Food Science and Technology,* 28**,** 185-189.

GOURAMA, H. & BULLERMAN, L. B. 1997. Anti-aflatoxigenic activity of *Lactobacillus casei pseudoplantarum*. *International Journal of Food Microbiology,* 34**,** 131-143.

GRABSCH, C., WICHMANN, G., LOFFHAGEN, N., HERBARTH, O. & MULLER, A. 2006. Cytotoxicity assessment of gliotoxin and penicillic acid in *Tetrahymena pyriformis*. *Environmental Toxicology,* 21**,** 111-117.

GRATZ, S., MYKKANEN, H. & EL-NEZAMI, H. 2005. Aflatoxin B-1 binding by a mixture of *Lactobacillus* and *Propionibacterium*: In vitro versus ex vivo. *Journal of Food Protection,* 68**,** 2470-2474.

GRATZ, S., TAUBEL, M., JUVONEN, R. O., VILUKSELA, M., TURNER, P. C., MYKKANEN, H. & EL-NEZAMI, H. 2006. *Lactobacillus rhamnosus* strain GG modulates intestinal absorption, fecal excretion, and toxicity of aflatoxin B-1 in rats. *Applied and Environmental Microbiology,* 72**,** 7398-7400.

GRATZ, S., WU, Q. K., EL-NEZAMI, H., JUVONEN, R. O., MYKKANEN, H. & TURNER, P. C. 2007. *Lactobacillus rhamnosus* strain GG reduces aflatoxin B-1 transport, metabolism, and toxicity in caco-2 cells. *Applied and Environmental Microbiology,* 73**,** 3958-3964.

GUAN, S., HE, J. W., YOUNG, J. C., ZHU, H. H., LI, X. Z., JI, C. & ZHOU, T. 2009. Transformation of trichothecene mycotoxins by microorganisms from fish digesta. *Aquaculture,* 290**,** 290-295.

GUAN, S., JI, C., ZHOU, T., LI, J. X., MA, Q. G. & NIU, T. G. 2008. Aflatoxin B-1 degradation by *Stenotrophomonas maltophilia* and other microbes selected using coumarin medium. *International Journal of Molecular Sciences,* 9**,** 1489-1503.

HALTTUNEN, T., COLLADO, M. C., EL-NEZAMI, H., MERILUOTO, J. & SALMINEN, S. 2008. Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal efficiency from aqueous solution. *Letters in Applied Microbiology,* 46**,** 160-165.

HALTTUNEN, T., SALMINEN, S. & TAHVONEN, R. 2007. Rapid removal of lead and cadmium from water by specific lactic acid bacteria. *International Journal of Food Microbiology,* 114**,** 30-35.

HAMID, A. B. & SMITH, J. 1987. Degradation of Aflatoxin by *Aspergillus flavus*. *Journal of General Microbiologv,* 133**,** 2023-2029.

HARITASH, A. K. & KAUSHIK, C. P. 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Journal of Hazardous Materials,* 169**,** 1-15.

HASKARD, C., BINNION, C. & AHOKAS, J. 2000. Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. *Chemico-Biological Interactions,* 128**,** 39-49.

HASKARD, C. A., EL-NEZAMI, H. S., KANKAANPAA, P. E., SALMINEN, S. & AHOKAS, J. T. 2001. Surface binding of aflatoxin B-1 by lactic acid bacteria. *Applied and Environmental Microbiology,* 67**,** 3086-3091.

HASSAN, Y. I. & BULLERMAN, L. 2008. Antifungal Activity of *Lactobacillus paracasei* subsp tolerans against Fusarium proliferatum and Fusarium graminearum in a Liquid Culture Setting. *Journal of Food Protection,* 71**,** 2213-2216.

HECHT, S. S. & TRUSHIN, N. 1988. DNA and hemoglobin alkylation by 4-(methlynitrosamino)-1-(3pyridyl)-1-butanone and its major metabolite

4-(methlynitrosamino)-1-(3-pyridyl)-1-butanol in F344 rats. . *Carcinogenesis,* 9**,** 1665-1668.

HEINL, S., HARTINGER, D., THAMHESL, M., VEKIRU, E., KRSKA, R., SCHATZMAYR, G., MOLL, W.D. & GRABHERR, R. 2010. Degradation of fumonisin B1 by the consecutive action of two bacterial enzymes. *Journal of Biotechnology,* 145**,** 120-129.

HERNANDEZ-MENDOZA, A., GARCIA, H. S. & STEELE, J. L. 2009a. Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B1. *Food and Chemical Toxicology,* 47**,** 1064-1068.

HERNANDEZ-MENDOZA, A., GUZMAN-DE-PEÑA, D. & GARCIA, H. S. 2009b. Key role of teichoic acids on aflatoxin B1 binding by probiotic bacteria. *Journal of Applied Microbiology,* 107**,** 395-403.

HERZALLAH, S., ALSHAWABKEH, K. & AL FATAFTAH, A. 2008. Aflatoxin Decontamination of Artificially Contaminated Feeds by Sunlight, gamma-Radiation, and Microwave Heating. *Journal of Applied Poultry Research,* 17**,** 515-521.

HORMISCH, D., BROST, I., KOHRING, G. W., GIFFHORN, E., KROPPENSTEDT, R. M., STACKEBRANDT, E., FARBER, P. & HOLZAPFEL, W. H. 2004. Mycobacterium fluoranthenivorans sp nov., a fluoranthene and aflatoxin B-1 degrading bacterium from contaminated soil of a former coal gas plant. *Systematic and Applied Microbiology,* 27**,** 653-660.

HUDACEK, J., ZALAN, Z., CHUMCHALOVA, J. & HALASZ, A. 2007. Antifungal effect of Lactobacilli on Fusarium and Aspergillus molds. *Chemicke Listy,* 101**,** 730-737.

HUSSEIN, H. S. & BRASEL, J. M. 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology,* 167**,** 101-134.

HUWIG, A., FREIMUND, S., KÄPPELI, O. & DUTLER, H. 2001. Mycotoxin detoxication of animal feed by different adsorbents. *Toxicology Letters,* 122**,** 179-188.

JESPERSEN, L., HALM, M., KPODO, K. & JAKOBSEN, M. 1994. Significance of yeasts and moulds occurring in maize dough fermentation for kenkey' production. *International Journal of Food Microbiology,* 24**,** 239-248.

JUAN, C., PENA, A., LINO, C., MOLTÓ, J. C. & MAÑES, J. 2008. Levels of ochratoxin A in wheat and maize bread from the central zone of Portugal. *International Journal of Food Microbiology,* 127**,** 284-289.

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

KABAK, B. & DOBSON, A. D. W. 2009. Biological Strategies To Counteract the Effects of Mycotoxins. *Journal of Food Protection,* 72**,** 2006-2016.

KABAK, B., DOBSON, A. D. W. & VAR, I. 2006. Strategies to prevent mycotoxin contamination of food and animal feed: A review. *Critical Reviews in Food Science and Nutrition,* 46**,** 593-619.

KABAK, B. & VAR, I. 2004. Binding of Aflatoxin M-1 by Lactobacillus and Bifidobacterium strains. *Milchwissenschaft-Milk Science International,* 59**,** 301-303.

KABAK, B. & VAR, I. 2008. Factors affecting the removal of aflatoxin M-1 from food model by *Lactobacillus* and *Bifidobacterium* strains. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes,* 43**,** 617-624.

KARLOVSKY, P. 1999. Biological detoxification of fungal toxins and its use in plant breeding, feed and food production. *Natural Toxins,* 7**,** 1-23.

KENDRA, D. F. & DYER, R. B. 2007. Opportunities for biotechnology and policy regarding mycotoxin issues in international trade. *International Journal of Food Microbiology,* 119**,** 147-151.

KHANAFARI, A., SOUDI, H., MIRABOULFATHI, M. & OSBOO, R. K. 2007. An in vitro investigation of aflatoxin B1 biological control by Lactobacillus plantarum. *Pak J Biol Sci,* 10**,** 2553-6.

KIESSLING, K. H., PETTERSSON, H., SANDHOLM, K. & OLSEN, M. 1984. Metabolism Of Aflatoxin, Ochratoxin, Zearalenone, And 3 Trichothecenes By Intact Rumen Fluid, Rumen Protozoa, And Rumen Bacteria. *Applied and Environmental Microbiology,* 47**,** 1070-1073.

KNASMULLER, S., CAVIN, C., CHAKRABORTY, A., DARROUDI, F., MAJER, B. J., HUBER, W. W. & EHRLICH, V. A. 2004. Structurally related mycotoxins ochratoxin A, ochratoxin B, and citrinin differ in their genotoxic activities and in their mode, of action in human-derived liver (HepG2) cells: Implications for risk assessment. *Nutrition and Cancer-an International Journal,* 50**,** 190-197.

KOLLÁR, R., REINHOLD, B. B., PETRÁKOVÁ, E., YEH, H. J. C., ASHWELL, G., DRGONOVÁ, J., KAPTEYN, J. C., KLIS, F. M. & CABIB, E. 1997. Architecture of the Yeast Cell Wall. *Journal of Biological Chemistry,* 272**,** 17762-17775.

KUILMAN, M. E. M., MAAS, R. F. M. & FINK-GREMMELS, J. 2000. Cytochrome P450-mediated metabolism and cytotoxicity of aflatoxin B1 in bovine hepatocytes. *Toxicology in Vitro,* 14**,** 321-327.

KUSUMANINGTYAS, E., WIDIASTUTI, R. & MARYAM, R. 2006. Reduction of aflatoxin B1 in chicken feed by using *Saccharomyces cerevisiae, Rhizopus oligosporus* and their combination. *Mycopathologia,* 162**,** 307-311.

LAHTINEN, S. J., HASKARD, C. A., OUWEHAND, A. C., SALMINEN, S. J. & AHOKAS, J. T. 2004. Binding of aflatoxin B-1 to cell wall components of *Lactobacillus rhamnosus* strain GG. *Food Additives and Contaminants,* 21**,** 158-164.

LANKAPUTHRA, W. E. V. & SHAH, N. P. 1998. Antimutagenic properties of probiotic bacteria and of organic acids. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis,* 397**,** 169-182.

LARKIN, M. J., KULAKOV, L. A. & ALLEN, C. C. R. 2005. Biodegradation and *Rhodococcus* - masters of catabolic versatility. *Current Opinion in Biotechnology,* 16**,** 282-290.

LEE, J. K., CHOI, E. H., LEE, K. G. & CHUN, H. S. 2005. Alleviation of aflatoxin B-1-induced oxidative stress in HepG2 cells by volatile extract from Allii Fistulosi Bulbus. *Life Sciences,* 77**,** 2896-2910.

LEE, L. S., DUNN,J.J., DELUCCA,A.J.,CIEGLER,A. 1981. Role of lactone ring of aflatoxin B1 in toxicity and mutagenicity. *Experientia,* 37**,** 16-17.

LEE, Y. K., EL-NEZAMI, H., HASKARD, C. A., GRATZ, S., PUONG, K. Y., SALMINEN, S. & MYKKANEN, H. 2003. Kinetics of adsorption and desorption of aflatoxin B-1 by viable and nonviable bacteria. *Journal of Food Protection,* 66**,** 426-430.

LI, A. P. 2009. Metabolism Comparative Cytotoxicity Assay (MCCA) and Cytotoxic Metabolic Pathway Identification Assay (CMPIA) with cryopreserved human hepatocytes for the evaluation of metabolism-based cytotoxicity in vitro: Proof-of-concept study with aflatoxin B1. *Chemico-Biological Interactions,* 179**,** 4-8.

LIANG, Z.-H., LI, J.-X., HE, Y.-L., GUAN, S., WANG, N., JI, C. & NIU, T. G. 2008. AFB1 Bio-Degradation by a New Strain - Stenotrophomonas. sp. *Agricultural Sciences in China,* 7**,** 1433-1437.

LIN, L., ZHANG, J., WANG, P., WANG, Y. & CHEN, J. 1998. Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *Journal of Chromatography A,* 815**,** 3-20.

LINE, J. E. & BRACKETT, R. E. 1995. Factors Affecting Aflatoxin B1 Removal by *Flavobacterium aurantiacum*. *Journal of Food Protection,* 58**,** 91-94.

LIU, B. H., WU, T. S., SU, M. C., CHUNG, C. P. & YU, F. Y. 2005. Evaluation of citrinin occurrence and cytotoxicity in Monascus fermentation products. *Journal of Agricultural and Food Chemistry,* 53**,** 170-175.

LIU, C. W., CHANG, W. N. & LIU, H. S. 2009. Bioremediation of n-alkanes and the formation of biofloccules by *Rhodococcus erythropolis* NTU-1 under various saline conditions and sea water. *Biochemical Engineering Journal,* 45**,** 69-75.

LIU, D.-L., YAO, D.-S., LIANG, Y.-Q., ZHOU, T.-H., SONG, Y.-P., ZHAO, L. & MA, L. 2001. Production, purification, and characterization of an intracellular aflatoxin-detoxifizyme from Armillariella tabescens (E-20). *Food and Chemical Toxicology,* 39**,** 461-466.

LIU, D. L., MA, L., GU, L. Q., LIANG, R., YAO, D. S. & CHEN, W. Q. 1997. Armillariella tabescen enzymatic detoxification of aflatoxin B-1 - Part III. Immobilized enzymatic detoxification. *In:* LASKIN, A. I., LI, G. X. & YU, Y. T., eds. 14th International Enzyme Engineering Conference, Oct 12-17. Beijing, Peoples R China. New York Acad Sciences, 592-599.

LIU, D. L., YAO, D. S., LIANG, R., MA, L., CHENG, W. Q. & GU, L. Q. 1998. Detoxification of aflatoxin B-1 by enzymes isolated from Armillariella tabescens. *Food and Chemical Toxicology,* 36**,** 563-+.

LUDWIG, B., AKUNDI, A. & KENDALL, K. 1995. A Long-Chain Secondary Alcohol Dehydrogenase from *Rhodococcus erythropolis* ATCC 4277. *Appl. Environ. Microbiol.,* 61**,** 3729-3733.

MAGNUSSON, J., STROM, K., ROOS, S., SJOGREN, J. & SCHNURER, J. 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiology Letters,* 219**,** 129-135.

MARTINKOVA, L., UHANAKOVA, B., PATEK, M., NESVERA, J. & KREN, V. 2009. Biodegradation Potential of genus *Rhodococcus Environment international* 35**,** 162-177.

MASSEY, T. E., STEWART, R. K., DANIELS, J. M. & LIU, L. 1995. Minireview: Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B1 carcinogenicity. . *Proc Soc Exp Biol Med* 208**,** 213-227.

MATEO, E. M., MEDINA, Á., MATEO, F., VALLE-ALGARRA, F. M., PARDO, I. & JIMÉNEZ, M. 2010. Ochratoxin A removal in synthetic media by living and heat-inactivated cells of Oenococcus oeni isolated from wines. *Food Control,* 21**,** 23-28.

MCKEAN, C., TANG, L., TANG, M., BILLAM, M., WANG, Z., THEODORAKIS, C. W., KENDALL, R. J. & J.S., W. 2006a. Comparative acute and combinative toxicity of aflatoxin B1 and fumonisin B1 in animals and human cells. *Food and Chemical Toxicology* 44**,** 868-876.

MCKEAN, C., TANG, L. L., BILLAM, M., TANG, M., THEODORAKIS, C. W., KENDALL, R. J. & WANG, J. S. 2006b. Comparative acute and combinative toxicity of aflatoxin B-1 and T-2 toxin in animals and immortalized human cell lines. *Journal of Applied Toxicology,* 26**,** 139-147.

MÉNDEZ-ALBORES, A., ARÁMBULA-VILLA, G., LOARCA-PIÑA, M. G. F., CASTAÑO-TOSTADO, E. & MORENO-MARTÍNEZ, E. 2005. Safety and efficacy evaluation of aqueous citric acid to degrade B-aflatoxins in maize. *Food and Chemical Toxicology,* 43**,** 233-238.

MENDEZ-ALBORES, A., ARAMBULA-VILLA, G., LOAREA-PINA, M. G. F., CASTANO-TOSTADO, E. & MORENO-MARTINEZ, E. 2005. Safety and efficacy evaluation of aqueous citric acid to degrade B-aflatoxins in maize. *Food and Chemical Toxicology,* 43**,** 233-238.

MÉNDEZ-ALBORES, A., DEL RÍO-GARCÍA, J. C. & MORENO-MARTÍNEZ, E. 2007. Decontamination of aflatoxin duckling feed with aqueous citric acid treatment. *Animal Feed Science and Technology,* 135**,** 249-262.

MENDEZ-ALBORES, A., MARTINEZ-BUSTOS, F., GAYTAN-MARTINEZ, M. & MORENO-MARTINEZ, E. 2008. Effect of lactic and citric acid on the stability of B-aflatoxins in extrusion-cooked sorghum. *Letters in Applied Microbiology,* 47**,** 1-7.

MÉNDEZ-ALBORES, A., NICOLÁS-VÁZQUEZ, I., MIRANDA-RUVALCABA, R. & MORENO-MARTÍNEZ, E. 2008. Mass Spectrometry/Mass Spectrometry Study on the Degradation of B-Aflatoxins in Maize with Aqueous Citric Acid *American Journal of Agricultural and Biological Sciences* 3**,** 482-489.

MENDEZ-ALBORES, A., VELES-MEDINA, J., URBINA-ALVAREZ, E., MARTINEZ-BUSTOS, F. & MORENO-MARTINEZ, E. 2009. Effect of citric acid on aflatoxin degradation and on functional and textural properties of extruded sorghum. *Animal Feed Science and Technology,* 150**,** 316-329.

MÉNDEZ-ALBORES, A., VELES-MEDINA, J., URBINA-ÁLVAREZ, E., MARTÍNEZ-BUSTOS, F. & MORENO-MARTÍNEZ, E. 2009. Effect of citric acid on aflatoxin degradation and on functional and textural properties of extruded sorghum. *Animal Feed Science and Technology,* 150**,** 316-329.

MENDEZ-ALBORES, A. N.-V., I. MIRANDA- RUVALCABA, R. MORENO-MARTINEZ, E. 2008. Mass spectrometry/mass spectrometry study on degradation of B-Aflatoxins in Mize with Aqueos citric acid *American Journal of Agriculture and biological sciences* 3(2)**,** 482-489.

MISHRA, H. N. & DAS, C. 2003. A Review on Biological Control and Metabolism of Aflatoxin. *Critical Reviews in Food Science and Nutrition,* 43**,** 245 - 264.

MNGADI, P. T., GOVINDEN, R. & ODHAV, B. 2008. Co-occurring mycotoxins in animal feeds. *African Journal of Biotechnology,* 7**,** 2239-2243.

MOAKE, M. M., PADILLA-ZAKOUR, O. I. & WOROBO, R. W. 2005. Comprehensive review of patulin control methods in foods. *Comprehensive Reviews in Food Science and Food Safety,* 4**,** 8-21.

MOHAMMADI-BARDBORI, A., NEJATI, M., ESMAEILI, J., GHAFARI, H. & GHAZI-KHANSARI, M. 2008. Comparative Measurement of In Vitro Paraquat and Aflatoxin B1 Cytotoxicity Using Three Different Cytotoxicity Assays in Pheochromocytoma Cells (PC-12). *Toxicology Mechanisms and Methods,* 18**,** 685-689.

MOHRAN, M. A., MEGALLA, S. E. & SAID, M. R. 1984. Effect of aflatoxin-b-1 on the proteolytic activity of some lactic-acid bacteria *Mycopathologia,* 86**,** 99-101.

MOLNAR, O., SCHATZMAYR, G., FUCHS, E. & PRILLINGER, H. 2004. Trichosporon mycotoxinivorans sp. nov., A New Yeast Species Useful in Biological Detoxification of Various Mycotoxins. *Systematic and Applied Microbiology,* 27**,** 661-671.

MOR, S., PUNIYA, A. K. & SINGH, K. 2003. Degradation of preformed aflatoxins by detoxifying organisms. *Indian Journal of Animal Sciences,* 73**,** 315-318.

MOSCHINI, M., GALLO, A., PIVA, G. & MASOERO, F. 2008. The effects of rumen fluid on the in vitro aflatoxin binding capacity of different sequestering agents and in vivo release of the sequestered toxin. *Animal Feed Science and Technology,* 147**,** 292-309.

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65**,** 55-63.

MOSS, M. O. 2002. Mycotoxin review - 1. Aspergillus and Penicillium. *Mycologist,* 16**,** 116-119.

MOSS, M. O. & LONG, M. T. 2002. Fate of patulin in the presence of the yeast Saccharomyces cerevisiae. *Food Additives and Contaminants,* 19**,** 387-399.

MOTOMURA, M., TOYOMASU, T., MIZUNO, K. & SHINOZAWA. 2003. Purification and chracterization of an aflatoxin degrdation enzyme from *Pleurotus ostreatus*. *Microbiol.Res.,* 158**,** 237-242.

MURPHY, P. A., HENDRICH, S., LANDGREN, C. & BRYANT, C. M. 2006. Food mycotoxins: An update. *Journal of Food Science,* 71**,** R51-R65.

MURPHY, P. J. 2001. Xenobiotic Metabolism: A Look from the Past to the Future. *Drug Metabolism and Disposition,* 29**,** 779-780.

NIDERKORN, V., MORGAVI, D. P., ABOAB, B., LEMAIRE, M. & BOUDRA, H. 2009. Cell wall component and mycotoxin moieties involved in the binding of fumonisin B-1 and B-2 by lactic acid bacteria. *Journal of Applied Microbiology,* 106**,** 977-985.

NIDERKORN, V., MORGAVI, D. P., PUJOS, E., TISSANDIER, A. & BOUDRA, H. 2007. Screening of fermentative bacteria for their ability to bind and biotransform deoxynivalenol, zearalenone and fumonisins in an in vitro simulated corn silage model. *Food Additives and Contaminants,* 24**,** 406-415.

NYBOM, S. M. K., SALMINEN, S. J. & MERILUOTO, J. A. O. 2008. Specific strains of probiotic bacteria are efficient in removal of several different cyanobacterial toxins from solution. *Toxicon,* 52**,** 214-220.

OATLEY, J. T., RARICK, M. D., JI, G. E. & LINZ, J. E. 2000. Binding of aflatoxin B-1 to bifidobacteria in vitro. *Journal of Food Protection,* 63**,** 1133-1136.

ÖZPINAR, H., AUGONYTE, G. & DROCHNER, W. 1999. Inactivation of ochratoxin in ruminal fluid with variation of pH-value and fermentation parameters in an in vitro system. *Environmental Toxicology and Pharmacology,* 7**,** 1-9.

PALANEE, T., DUTTON, M. F. & & CHUTURGOON, A. A. 2000. Cytotoxicity of aflatoxin B1 and its chemically synthesised epoxide derivative on the A549 human epithelioid lung cell line. *Mycopathologia,* 151**,** 155–159.

PARK, H.-D. & RHEE, C.-H. 2001. Antimutagenic activity of Lactobacillus plantarum KLAB21 isolated from kimchi Korean fermented vegetables. *Biotechnology Letters,* 23**,** 1583-1589.

PATERSON, R. R. M. & LIMA, N. 2010. How will climate change affect mycotoxins in food? *Food Research International,* In Press, Corrected Proof.

PELTONEN, K., EL-NEZAMI, H., HASKARD, C., AHOKAS, J. & SALMINEN, S. 2001. Aflatoxin B-1 binding by dairy strains of lactic acid bacteria and bifidobacteria. *Journal of Dairy Science,* 84**,** 2152-2156.

PELTONEN, K. D., EL-NEZAMI, H. S., SALMINEN, S. J. & AHOKAS, J. T. 2000. Binding of aflatoxin B-1 by probiotic bacteria. *Journal of the Science of Food and Agriculture,* 80**,** 1942-1945.

PERAICA, M., RADIC, B., LUCIC, A. & PAVLOVIC, M. 1999. Toxic effects of mycotoxins in humans. *Bulletin of the World Health Organization,* 77**,** 754-766.

PETCHKONGKAEW, A., TAILLANDIER, P., GASALUCK, P. & LEBRIHI, A. 2008. Isolation of Bacillus spp. from Thai fermented soybean (Thua-nao): screening for aflatoxin B-1 and ochratoxin A detoxification. *Journal of Applied Microbiology,* 104**,** 1495-1502.

PÉTERI, Z., TÉREN, J., VÁGVÖLGYI, C. & VARGA, J. 2007. Ochratoxin degradation and adsorption caused by astaxanthin-producing yeasts. *Food Microbiology,* 24**,** 205-210.

PIERIDES, M., EL-NEZAMI, H., PELTONEN, K., SALMINEN, S. & AHOKAS, J. 2000. Ability of dairy strains of lactic acid bacteria to bind aflatoxin M-1 in a food model. *Journal of Food Protection,* 63**,** 645-650.

PIVA, G., GALVANO, F., PIETRI, A. & PIVA, A. 1995. Detoxification methods of aflatoxins. A review. *Nutrition Research,* 15**,** 767-776.

POGELL, B. M. Year. Bioremediation of pesticides and herbicides by streptomycetes. *In:* MOOYOUNG, M., ANDERSON, W. A. & CHAKRABARTY, A. M., eds. International Symposium on Environmental Biotechnology, Jul 04-08 1994 Waterloo, Canada. Kluwer Academic Publ, 38-46.

RAHMANI, A., JINAP, S. & SOLEIMANY, F. 2009. Qualitative and Quantitative Analysis of Mycotoxins. *Comprehensive Reviews in Food Science and Food Safety,* 8**,** 202-251.

RASIC, J. L., SKRINJAR, M. & MARKOV, S. 1991. Decrease of aflatoxin-b1 in yogurt and acidified milks *Mycopathologia,* 113**,** 117-119.

REDDY, K. R. N., REDDY, C. S., ABBAS, H. K., ABEL, C. A. & MURALIDHARAN, K. 2008. MYCOTOXIGENIC FUNGI, MYCOTOXINS, AND MANAGEMENT OF RICE GRAINS. *Toxin Reviews,* 27**,** 287-317.

REDDY, K. R. N., REDDY, C. S. & MURALIDHARAN, K. 2009. Potential of botanicals and biocontrol agents on growth and aflatoxin production by Aspergillus flavus infecting rice grains. *Food Control,* 20**,** 173-178.

REEN, R. K., WIEBEL, F. J. B. & SINGH, J. 1997. Piperine inhibits aflatoxin Bl-induced cytotoxicity and genotoxicity in V79 Chinese hamster cells genetically engineered to express rat cytochrome P4502B1. *Journal of Ethnopharmacology,* 58**,** 165-173.

REUBEL, G., GAREIS, M., ., W. A. & 1987. Cytotoxicity evaluation of mycotoxins by an MTT-bioassay. *Mycotoxin Res,* 3**,** 85-96.

REUBEL, G., GAREIS, M. & AMSELGRUBER, W. 1989. Effects of Fusarium mycotoxins zearalenone and deoxynivalenol on the mitochondrial methylthiazol tetrazolium cleavage activity of monolayer cells. . *Toxicol in vitro* 3**,** 311-316.

RICELLI, A., BARUZZI, F., SOLFRIZZO, M., MOREA, M. & FANIZZI, F. P. 2007. Biotransformation of patulin by Gluconobacter oxydans. *Applied and Environmental Microbiology,* 73**,** 785-792.

RICHARD, J. L. 2007. Some major mycotoxins and their mycotoxicoses--An overview. *International Journal of Food Microbiology,* 119**,** 3-10.

RINGOT, D., CHANGO, A., SCHNEIDER, Y.-J. & LARONDELLE, Y. 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chemico-Biological Interactions,* 159**,** 18-46.

SAITO, H., KOYASU, J., T., SHIGEOK, A. & AND I. TOMIT, A. 1994. cytotoxicity of chlorophenols to goldfish gfs cells with the mtt and ldh assays *Toxic. in Vitro* 8**,** 1107-1112.

SANT'ANA, A. D. S., ROSENTHAL, A. & DE MASSAGUER, P. R. 2008. The fate of patulin in apple juice processing: A review. *Food Research International,* 41**,** 441-453.

SANTIN, E., PAULILLO, A. C., MAIORKA, A., NAKAGHI, L. S. O., MACARI, M., DA SILVA, V. F. & ALESSI, A. C. 2003. Evaluation of the Efficacy of Saccharomyces cerevisiae Cell Wall to Ameliorate the Toxic Effects of Aflatoxin in Broilers. *International Journal of Poultry Science,* 2**,** 341-344.

SCHAAF, G. J., NIJMEIJER, S. M., MAAS, R. F. M., ROESTENBERG, P., DE GROENE, E. M. & FINK-GREMMELS, J. 2002. The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* 1588**,** 149-158.

SCHATZMAYR, G., SCHATZMAYR, D., PICHLER, E., TAUBEL, M., LOIBNER, A. R. & BINDER, E. M. Year. A novel approach to deactivate ochratoxin A. *In:* NJAPAU, H., TRUJILLO, S., VANEGMOND, H. P. & PARK, D. L., eds. 11th International IUPAC Symposium on Mycotoxins and Phycotoxins, May 17-21 2004 Bethesda, MD. Wageningen Academic Publishers, 279-288.

SCHATZMAYR, G., TAUBEL, M., VEKIRU, E., MOLL, D., SCBATZMAYR, D., BINDER, E. M., KRSKA, R. & LOIBNER, A. P. Year. Detoxification of mycotoxins by biotransformation. *In:* BARUG, D., BHATNAGAR, D., VANEGMOND, H. P., VANDERKAMP, J. W., VANOSENBRUGGEN, W. A. & VISCONTI, A., eds. 3rd World Mycotoxin Forum, Nov 2005a Noordwijk, NETHERLANDS. Wageningen Academic Publishers, 363-375.

SCHATZMAYR, G., ZEHNER, F., TAUBEL, M., SCHATZMAYR, D., KLIMITSCH, A., LOIBNER, A. P. & BINDER, E. M. Year. Microbiologicals for deactivating mycotoxins. *In:* Euro-Maghrebin Symposium on Biological Chemical Contaminants and Food Safety, Sep 07-09 2005b Fez, MOROCCO. Wiley-V C H Verlag Gmbh, 543-551.

SCHNÜRER, J. & MAGNUSSON, J. 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology,* 16**,** 70-78.

SCHUMACHER, D. M., MULLER, C., METZLER, M. & LEHMANN, L. 2006. DNA-DNA cross-links contribute to the mutagenic potential of the mycotoxin patulin. *Toxicology Letters,* 166**,** 268-275.

SHANTHA, T. 1999. Fungal degradation of aflatoxin B1. *Natural Toxins,* 7**,** 175-178.

SHEPHARD, G. S. & LEGGOTT, N. L. 2000. Chromatographic determination of the mycotoxin patulin in fruit and fruit juices. *Journal of Chromatography A,* 882**,** 17-22.

SHERIF, S. O., SALAMA, E. E. & ABDEL-WAHHAB, M. A. 2009. Mycotoxins and child health: The need for health risk assessment. *International Journal of Hygiene and Environmental Health,* 212**,** 347-368.

SHETTY, P. H., HALD, B. & JESPERSEN, L. 2007. Surface binding of aflatoxin B-1 by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. *International Journal of Food Microbiology,* 113**,** 41-46.

SHETTY, P. H. & JESPERSEN, L. 2006. Saccharomyces cerevisiae and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science & Technology,* 17**,** 48-55.

SHIMA, Y., SHIINA, M., SHINOZAWA, T., ITO, Y., NAKAJIMA, H., ADACHI, Y. & YABE, K. 2009. Participation in aflatoxin biosynthesis by a reductase enzyme encoded by vrdA gene outside the aflatoxin gene cluster. *Fungal Genetics and Biology,* 46**,** 221-231.

SHIRLING, E. B. & GOTTLIEB, D. 1966. Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol,* 16**,** 313-340.

SIGMA-ALDRICH 2006. In vitro toxicology assay kit Lactate dehydrogenase based. Tox-7 Sigma-Aldrich, UK

SMILEY, R. D. & DRAUGHON, F. A. 2000. Preliminary Evidence that Degradation of Aflatoxin B1 by *Flavobacterium aurantiacum* is Enzymatic. *Journal of Food Protection,* 63**,** 415-418.

SMITH, J. E. & HARRAN, G. 1993. Microbial degradation of mycotoxins. *International Biodeterioration & Biodegradation,* 32**,** 205-211.

SREEKUMAR, O. & HOSONO, A. 1998. The heterocyclic amine binding receptors of Lactobacillus gasseri cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 421**,** 65-72.

STANDER, M. A., BORNSCHEUER, U. T., HENKE, E. & STEYN, P. S. 2000. Screening of commercial hydrolases for the degradation of ochratoxin A. *Journal of Agricultural and Food Chemistry,* 48**,** 5736-5739.

STANDER, M. A., STEYN, P. S., VAN DER WESTHUIZEN, F. H. & PAYNE, B. E. 2001. A kinetic study into the hydrolysis of the ochratoxins and analogues by carboxypeptidase A. *Chemical Research in Toxicology,* 14**,** 302-304.

STANLEY, V. G., OJO, R., WOLDESENBET, S., DH, H. & LF, K. 1993. The use of Saccharomyces cerevisiae to suppress the effects of aflatoxins in broiler chicks. *poultry science,* 72**,** 1867-72.

STYRIAK, I. & CONKOVA, E. 2002. Microbial binding and biodegradation of mycotoxins. *Veterinary and Human Toxicology,* 44**,** 358-361.

SUTTAJIT, *M.* 1989. *Prevention and control of mycotoxins.* Food Agriculture Organization.

TAFURI, A., FERRACANE, R. & RITIENI, A. 2004. Ochratoxin A in Italian marketed cocoa products. *Food Chemistry,* 88**,** 487-494.

TEJADA-CASTANEDA, Z. I., AVILA-GONZALEZ, E., CASAUBON-HUGUENIN, M. T., CERVANTES-OLIVARES, R. A., VASQUEZ-PELAEZ, C., HERNANDEZ-BAUMGARTEN, E. M. & MORENO-MARTINEZ, E. 2008. Biodetoxification of aflatoxin-contaminated chick feed. *Poultry Science,* 87**,** 1569-1576.

TENIOLA, O. D., ADDO, P. A., BROST, I. M., FARBER, 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 DSM44556(T). *International Journal of Food Microbiology,* 105**,** 111-117.

THOMPSON, W. L. & R.W.WANNEMACHER 1984. Detection and quantitation of T-2 toxin with a simplified protein synthesis inhibition assay. *Appl Environ Microbiol,* 4**,** 1176-1180.

TOPCU, A., BULAT, T., WISHAH, R. & BOYACI, I. H. 2010. Detoxification of aflatoxin B1 and patulin by Enterococcus faecium strains. *International Journal of Food Microbiology,* In Press, Corrected Proof.

TUOMOLA, E. M., OUWEHAND, A. C. & SALMINEN, S. J. 2000. Chemical, physical and enzymatic pre-treatments of probiotic

lactobacilli alter their adhesion to human intestinal mucus glycoproteins. *International Journal of Food Microbiology,* 60**,** 75-81.

TURBIC, A., AHOKAS, J. T. & HASKARD, C. A. 2002. Selective in vitro binding of dietary mutagens, individually or in combination, by lactic acid bacteria. *Food Additives and Contaminants,* 19**,** 144-152.

TURNER, N. W., SUBRAHMANYAM, S. & PILETSKY, S. A. 2009. Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta,* 632**,** 168-180.

UPADHAYA, S. D., SUNG, H. G., LEE, C. H., LEE, S. Y., KIM, S. W., CHO, K. J. & HA, J. K. 2009. Comparative study on the aflatoxin B1 degradation ability of rumen fluid from Holstein steers and Korean native goats. *Journal of Veterinary Science,* 10**,** 29-34.

VAHL, M. & JØRGENSEN, K. 1998. Determination of aflatoxins in food using LC/MS/MS
*Zeitschrift für Lebensmitteluntersuchung und -Forschung A*206**,** 243-245

VAR, I., ERGINKAYA, Z. & KABAK, B. 2009. Reduction of Ochratoxin A Levels in White Wine by Yeast Treatments. *Journal of the Institute of Brewing,* 115**,** 30-34.

VARGA, J., PETERI, Z., TABORI, K., TEREN, J. & VAGVOLGYI, C. 2005. Degradation of ochratoxin A and other mycotoxins by Rhizopus isolates. *International Journal of Food Microbiology,* 99**,** 321-328.

VARGA, J., RIGÓ, K. & TÉREN, J. 2000. Degradation of ochratoxin A by Aspergillus species. *International Journal of Food Microbiology,* 59**,** 1-7.

VARGA, J. & TOTH, B. 2005. Novel strategies to control mycotoxins in feeds: A review. *Acta Veterinaria Hungarica,* 53**,** 189-203.

WAGACHA, J. M. & MUTHOMI, J. W. 2008. Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology,* 124**,** 1-12.

WANG, F. Q., ZHANG, C. G., LI, B., WEI, D. Z. & TONG, W. Y. 2009. New Microbiological Transformations of Steroids by *Streptomyces virginiae* IBL-14. *Environmental Science & Technology,* 43**,** 5967-5974.

WEIDHAAS, J. L., CHANG, D. P. Y. & SCHROEDER, E. D. 2009. Biodegradation of Nitroaromatics and RDX by Isolated *Rhodococcus opacus*. *Journal of Environmental Engineering-Asce,* 135**,** 1025-1031.

WESTLAKE, K., MACKIE, R. I. & DUTTON, M. F. 1989. INVITRO METABOLISM OF MYCOTOXINS BY BACTERIAL, PROTOZOAL AND OVINE RUMINAL FLUID PREPARATIONS. *Animal Feed Science and Technology,* 25**,** 169-178.

WU, Q., JEZKOVA, A., YUAN, Z., PAVLIKOVA, L., DOHNAL, V. & KUCA, K. 2009. Biological degradation of aflatoxins. *Drug Metabolism Reviews,* 41**,** 1-7.

XU, B.-J., JIA, X.Q., GU, L.J. & SUNG, C.K. 2006. Review on the qualitative and quantitative analysis of the mycotoxin citrinin. *Food Control,* 17**,** 271-285.

XU, J., WANG, H., JI, R. & LUO, X. 2003. [Study on the effect of the growth and aflatoxin production by Aspergillus flavus parasiticus NRRL 2999 in the present of Lactobacillus plantarum ATCC 8014]. *Wei Sheng Yan Jiu,* 32**,** 334-8.

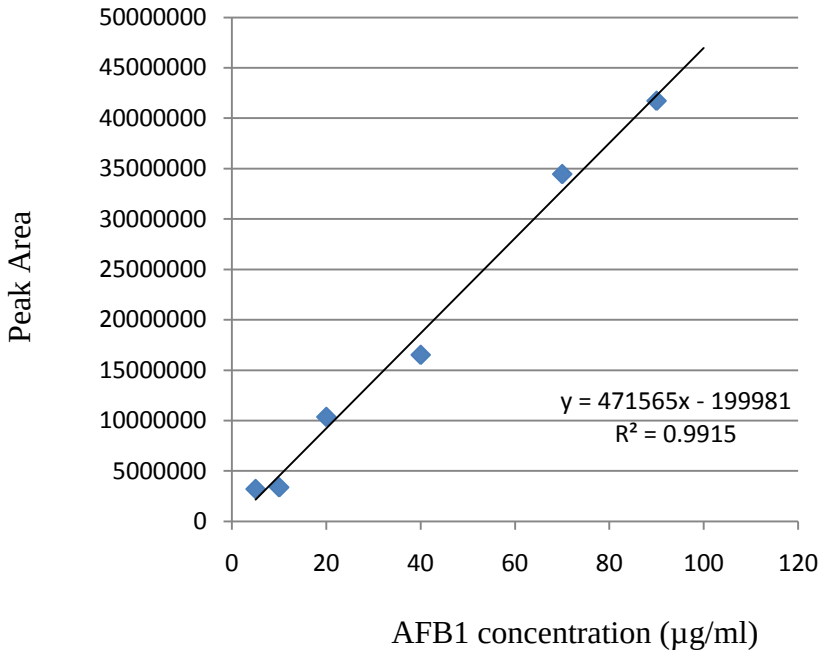
YAO, D. S., LIANG, R., LIU, D. L., GU, L. Q., MA, L. & CHEN, W. Q. Year. Screening of the fungus whose multienzyme system has catalytic detoxification activity towards aflatoxin B-1 - (Part I). *In:* LASKIN, A. I., LI, G. X. & YU, Y. T., eds. 14th International Enzyme Engineering Conference, Oct 12-17 1997 Beijing, Peoples R China. New York Acad Sciences, 579-585.

YONEYAMA, M., SHARMA, R. P. & Y.Y.ELSNER 1987. Effects of Mycotoxins in Cultured Kidney Cells: Cytotoxicity of Aflatoxin B1, in Madin-Darby and Primary Fetal Bovine Kidney Cells. *ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY, ,* 13**,** 174-184.

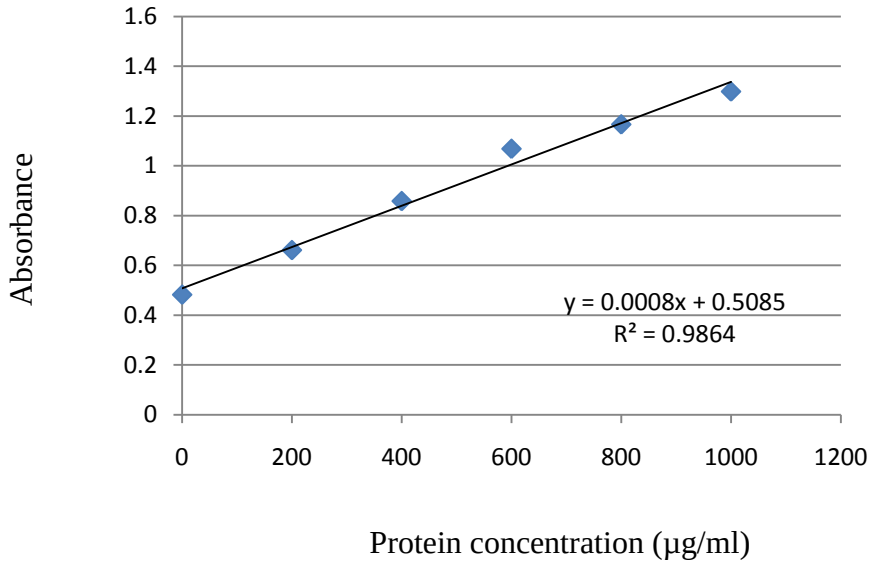
ZHOU, S.M., JIANG, L.-P., GENG, C.-Y., CAO, J. & ZHONG, L.-F. 2010. Patulin-induced oxidative DNA damage and p53 modulation in HepG2 cells. *Toxicon,* 55**,** 390-395.

APPENDIX

9 APPENDIX



APPENDIX 9.1- Aflatoxin B1 standard curve



APPENDIX 9.2 – Protein standard curve

Chapter 2

Appendix 9.3 – Statistical analysis for effect of different concentrations of probiotic bacteria on the binding of AFB1.

Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	22.60	0.0264		
Column Factor	18.09	0.0008		
Row Factor	45.08	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	*	Yes		
Column Factor	***	Yes		
Row Factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	12	693.0	57.75	2.645
Column Factor	3	554.7	184.9	8.469
Row Factor	4	1382	345.6	15.83
Residual	20	436.7	21.83	
Number of missing values	0			
Bonferroni posttests				
1 vs 2				
Row Factor	1	2	Difference	95% CI of diff.
L.acidophilus	86.84	88.72	1.883	-15.08 to 18.85
L. bulgaricus	74.57	91.49	16.92	-0.04598 to 33.88
L.plantarum	85.78	93.86	8.072	-8.891 to 25.04
L. lactis	98.09	99.26	1.173	-15.79 to 18.14
B .bifidum	73.20	70.50	-2.700	-19.66 to 14.26
Row Factor	Difference	T	P value	Summary
L.acidophilus	1.883	0.4030	P > 0.05	ns
L. bulgaricus	16.92	3.621	P<0.01	**
L.plantarum	8.072	1.728	P > 0.05	ns
L. lactis	1.173	0.2510	P > 0.05	ns
B .bifidum	-2.700	0.5778	P > 0.05	ns
1 vs 3				
Row Factor	1	3	Difference	95% CI of diff.
L.acidophilus	86.84	88.01	1.174	-15.79 to 18.14
L. bulgaricus	74.57	91.68	17.11	0.1495 to

				34.08
L.plantarum	85.78	94.06	8.272	-8.691 to 25.23
L. lactis	98.09	99.47	1.381	-15.58 to 18.34
B .bifidum	73.20	90.55	17.35	0.3871 to 34.31
Row Factor	Difference	T	P value	Summary
L.acidophilus	1.174	0.2512	P > 0.05	ns
L. bulgaricus	17.11	3.662	P<0.01	**
L.plantarum	8.272	1.770	P > 0.05	ns
L. lactis	1.381	0.2955	P > 0.05	ns
B .bifidum	17.35	3.713	P<0.01	**
1 vs 4				
Row Factor	1	4	Difference	95% CI of diff.
L.acidophilus	86.84	88.37	1.528	-15.43 to 18.49
L. bulgaricus	74.57	91.58	17.01	0.05178 to 33.98
L.plantarum	85.78	93.96	8.172	-8.791 to 25.13
L. lactis	98.09	99.36	1.277	-15.69 to 18.24
B .bifidum	73.20	90.55	17.35	0.3871 to 34.31
Row Factor	Difference	t	P value	Summary
L.acidophilus	1.528	0.3271	P > 0.05	ns
L. bulgaricus	17.01	3.641	P<0.01	**
L.plantarum	8.172	1.749	P > 0.05	ns
L. lactis	1.277	0.2732	P > 0.05	ns
B .bifidum	17.35	3.713	P<0.01	**
2 vs 3				
Row Factor	2	3	Difference	95% CI of diff.
L.acidophilus	88.72	88.01	-0.7093	-17.67 to 16.25
L. bulgaricus	91.49	91.68	0.1955	-16.77 to 17.16
L.plantarum	93.86	94.06	0.1994	-16.76 to 17.16
L. lactis	99.26	99.47	0.2080	-16.75 to 17.17
B .bifidum	70.50	90.55	20.05	3.087 to 37.01
Row Factor	Difference	t	P value	Summary
L.acidophilus	-0.7093	0.1518	P > 0.05	ns
L. bulgaricus	0.1955	0.04184	P > 0.05	ns
L.plantarum	0.1994	0.04268	P > 0.05	ns
L. lactis	0.2080	0.04452	P > 0.05	ns
B .bifidum	20.05	4.291	P<0.01	**
2 vs 4				
Row Factor	2	4	Difference	95% CI of diff.
L.acidophilus	88.72	88.37	-0.3547	-17.32 to 16.61
L. bulgaricus	91.49	91.58	0.09776	-16.87 to 17.06
L.plantarum	93.86	93.96	0.09970	-16.86 to 17.06
L. lactis	99.26	99.36	0.1040	-16.86 to 17.07
B .bifidum	70.50	90.55	20.05	3.087 to 37.01

Row Factor	Difference	t	P value	Summary
L.acidophilus	-0.3547	0.07590	P > 0.05	ns
L. bulgaricus	0.09776	0.02092	P > 0.05	ns
L.plantarum	0.09970	0.02134	P > 0.05	ns
L. lactis	0.1040	0.02226	P > 0.05	ns
B .bifidum	20.05	4.291	P<0.01	**
3 vs 4				
Row Factor	3	4	Difference	95% CI of diff.
L.acidophilus	88.01	88.37	0.3546	-16.61 to 17.32
L. bulgaricus	91.68	91.58	-0.09775	-17.06 to 16.87
L.plantarum	94.06	93.96	-0.09970	-17.06 to 16.86
L. lactis	99.47	99.36	-0.1040	-17.07 to 16.86
B .bifidum	90.55	90.55	0.0000	-16.96 to 16.96
Row Factor	Difference	t	P value	Summary
L.acidophilus	0.3546	0.07590	P > 0.05	Ns
L. bulgaricus	-0.09775	0.02092	P > 0.05	Ns
L.plantarum	-0.09970	0.02134	P > 0.05	Ns
L. lactis	-0.1040	0.02226	P > 0.05	Ns
B .bifidum	0.0000	0.0000	P > 0.05	Ns

Chapter 3

Appendix 9.4-Bonferroni multible comparisons for effect of temprature on AFB1 degrdation by actinomycetes culture

Bonferroni post tests				
25 vs 30				
Row Factor	25	30	Difference	95% CI of diff.
R.erythropolis	16.27	5.109	-11.16	-22.56 to 0.2332
S.lividans	13.96	13.06	-0.8998	-14.86 to 13.06
S.aureofaciens	12.82	12.04	-0.7782	-12.18 to 10.62
Row Factor	Difference	T	P value	Summary
R.erythropolis	-11.16	4.169	P<0.01	**
S.lividans	-0.8998	0.2743	P > 0.05	Ns
S.aureofaciens	-0.7782	0.2906	P > 0.05	Ns
25 vs 35 °C				
Row Factor	25	35 °C	Difference	95% CI of diff.
R.erythropolis	16.27	7.225	-9.048	-23.01 to 4.911
S.lividans	13.96	14.14	0.1786	-13.78 to 14.14
S.aureofaciens	12.82	18.97	6.146	-5.251 to 17.54
Row Factor	Difference	T	P value	Summary
R.erythropolis	-9.048	2.758	P > 0.05	Ns
S.lividans	0.1786	0.05446	P > 0.05	Ns

S.aureofaciens	6.146	2.295	P > 0.05	Ns
25 vs 40 °C				
Row Factor	25	40 °C	Difference	95% CI of diff.
R.erythropolis	16.27	14.63	-1.640	-15.60 to 12.32
S.lividans	13.96	22.76	8.799	-7.320 to 24.92
S.aureofaciens	12.82	21.38	8.557	-2.840 to 19.95
Row Factor	Difference	T	P value	Summary
R.erythropolis	-1.640	0.5001	P > 0.05	ns
S.lividans	8.799	2.323	P > 0.05	ns
S.aureofaciens	8.557	3.195	P < 0.05	*
30 vs 35 °C				
Row Factor	30	35 °C	Difference	95% CI of diff.
R.erythropolis	5.109	7.225	2.116	-11.84 to 16.08
S.lividans	13.06	14.14	1.078	-10.32 to 12.48
S.aureofaciens	12.04	18.97	6.924	-4.473 to 18.32
Row Factor	Difference	T	P value	Summary
R.erythropolis	2.116	0.6452	P > 0.05	ns
S.lividans	1.078	0.4027	P > 0.05	ns
S.aureofaciens	6.924	2.585	P > 0.05	ns
30vs40				
Row Factor	30	40 °C	Difference	95% CI of diff.
R.erythropolis	5.109	14.63	9.524	-4.435 to 23.48
S.lividans	13.06	22.76	9.699	-4.260 to 23.66
S.aureofaciens	12.04	21.38	9.336	-2.062 to 20.73
Row Factor	Difference	T	P value	Summary
R.erythropolis	9.524	2.904	P > 0.05	ns
S.lividans	9.699	2.957	P > 0.05	ns
S.aureofaciens	9.336	3.486	P < 0.05	*
35 °C vs 40 °C				
Row Factor	35 °C	40 °C	Difference	95% CI of diff.
R.erythropolis	7.225	14.63	7.408	-8.711 to 23.53
S.lividans	14.14	22.76	8.620	-5.339 to 22.58
S.aureofaciens	18.97	21.38	2.411	-8.986 to 13.81
Row Factor	Difference	T	P value	Summary
R.erythropolis	7.408	1.956	P > 0.05	ns
S.lividans	8.620	2.628	P > 0.05	ns
S.aureofaciens	2.411	0.9003	P > 0.05	ns

Appendix 9.5- Bonferroni multiple comparison for the effect of different intial pH on AFB1 degrdation

Bonferroni posttests				
4 vs 5				
Row Factor	4	5	Difference	95% CI of diff.
R.erythropolis	11.29	7.625	-3.667	-5.399 to -1.934
S.lividans	14.81	3.111	-11.69	-13.63 to -9.757
S.aureofaciens	5.439	4.620	-0.8191	-2.756 to 1.118
Row Factor	Difference	T	P value	Summary
R.erythropolis	-3.667	7.423	P<0.001	***
S.lividans	-11.69	21.17	P<0.001	***
S.aureofaciens	-0.8191	1.483	P > 0.05	Ns
4 vs 6				
Row Factor	4	6	Difference	95% CI of diff.
R.erythropolis	11.29	4.459	-6.833	-8.565 to -5.100
S.lividans	14.81	14.25	-0.5600	-2.292 to 1.173
S.aureofaciens	5.439	12.10	6.656	4.535 to 8.778
Row Factor	Difference	T	P value	Summary
R.erythropolis	-6.833	13.83	P<0.001	***
S.lividans	-0.5600	1.133	P > 0.05	Ns
S.aureofaciens	6.656	11.00	P<0.001	***
4 vs 7				
Row Factor	4	7	Difference	95% CI of diff.
R.erythropolis	11.29	5.719	-5.572	-7.305 to -3.840
S.lividans	14.81	14.16	-0.6456	-2.378 to 1.087
S.aureofaciens	5.439	12.32	6.882	4.761 to 9.004
Row Factor	Difference	T	P value	Summary
R.erythropolis	-5.572	11.28	P<0.001	***
S.lividans	-0.6456	1.307	P > 0.05	Ns
S.aureofaciens	6.882	11.38	P<0.001	***
4 vs 8				
Row Factor	4	8	Difference	95% CI of diff.
R.erythropolis	11.29	10.59	-0.7061	-2.439 to 1.026
S.lividans	14.81	7.434	-7.372	-9.104 to -5.639
S.aureofaciens	5.439	16.42	10.98	9.044 to 12.92
Row Factor	Difference	T	P value	Summary
R.erythropolis	-0.7061	1.429	P > 0.05	Ns
S.lividans	-7.372	14.92	P<0.001	***
S.aureofaciens	10.98	19.88	P<0.001	***
5 vs 6				
Row Factor	5	6	Difference	95% CI of diff.
R.erythropolis	7.625	4.459	-3.166	-4.898 to -1.433
S.lividans	3.111	14.25	11.13	9.197 to 13.07
S.aureofaciens	4.620	12.10	7.475	5.538 to 9.412

Row Factor	Difference	T	P value	Summary
R.erythropolis ATCC 4277	-3.166	6.408	P<0.001	***
S.lividans	11.13	20.16	P<0.001	***
S.aureofaciens	7.475	13.53	P<0.001	***
5 vs 7				
Row Factor	5	7	Difference	95% CI of diff.
R.erythropolis	7.625	5.719	-1.905	-3.638 to -0.1730
S.lividans	3.111	14.16	11.05	9.111 to 12.99
s.aureofaciens	4.620	12.32	7.702	5.765 to 9.639
Row Factor	Difference	T	P value	Summary
R.erythropolis	-1.905	3.857	P<0.01	**
S.lividans	11.05	20.00	P<0.001	***
S.aureofaciens	7.702	13.94	P<0.001	***
5 vs 8	5	8	Difference	95% CI of diff.
Row Factor				
R.erythropolis	7.625	10.59	2.961	1.228 to 4.693
S.lividans	3.111	7.434	4.322	2.385 to 6.259
S.aureofaciens	4.620	16.42	11.80	10.07 to 13.53
Row Factor	Difference	T	P value	Summary
R.erythropolis	2.961	5.993	P<0.001	***
S.lividans	4.322	7.826	P<0.001	***
S.aureofaciens	11.80	23.89	P<0.001	***
6 vs 7				
Row Factor	6	7	Difference	95% CI of diff.
R.erythropolis	4.459	5.719	1.260	-0.4721 to 2.993
S.lividans	14.25	14.16	-0.08568	-1.818 to 1.647
S.aureofaciens	12.10	12.32	0.2261	-1.896 to 2.348
Row Factor	Difference	T	P value	Summary
R.erythropolis	1.260	2.551	P > 0.05	Ns
S.lividans	-0.08568	0.1734	P > 0.05	Ns
S.aureofaciens	0.2261	0.3736	P > 0.05	Ns
6 vs 8				
Row Factor	6	8	Difference	95% CI of diff.
R.erythropolis	4.459	10.59	6.127	
S.lividans	14.25	7.434	-6.812	-8.544 to -5.079
S.aureofaciens	12.10	16.42	4.325	2.388 to 6.262
Row Factor	Difference	T	P value	Summary
R.erythropolis	6.127	12.40	P<0.001	***
S.lividans	-6.812	13.79	P<0.001	***
S.aureofaciens	4.325	7.830	P<0.001	***
7 vs 8				
Row Factor	7	8	Difference	95% CI of diff.
R.erythropolis	5.719	10.59	4.866	3.134 to 6.599

S.lividans	14.16	7.434	-6.726	-8.458 to -4.993
S.aureofaciens	12.32	16.42	4.099	2.162 to 6.036
Row Factor	Difference	T	P value	Summary
R.erythropolis	4.866	9.850	P<0.001	***
S.lividans	-6.726	13.61	P<0.001	***
S.aureofaciens	4.099	7.421	P<0.001	***

Chapter 4

Appendix 9.6 - Tukey's Multiple Comparison Test for residual AFB1

One-way analysis of variance			
P value	< 0.0001		
P value summary	***		
Are means signif. Different? (P < 0.05)	Yes		
Number of groups	4		
F	717.4		
R squared	0.9963		
ANOVA Table	SS	df	MS
Treatment (between columns)	755.4	3	251.8
Residual (within columns)	2.808	8	0.3510
Total	758.2	11	

Appendix 9.7 - Tukey's Multiple Comparison Test for cytotoxicity effects for residual AFB1 over a period of 72 h at 30 ºC pH 6 in presence of *R. erythropolis*

Tukey's Multiple Comparison Test	MeanDiff.	q	Significant P < 0.05	95% CI of diff
0 vs 24	16.14	47.19	Yes	14.59 to 17.69
0 vs 48	18.41	53.82	Yes	16.86 to 19.96
0 vs 72	19.70	57.60	Yes	18.15 to 21.25
24 vs 48	2.268	6.630	Yes	0.7187 to 3.817
24 vs 72	3.562	10.41	Yes	2.012 to 5.111
48 vs 72	1.294	3.782	No	-0.2555 to 2.843

Appndix 9.8- One way ANOVA for the remainingAFB1 over a period of 72 h at 30 ºC pH 6 in presence of *S .lividans*

One-way analysis of variance

P value	< 0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	428.8		
R squared	0.9938		
ANOVA Table	SS	df	MS
Treatment (between columns)	698.8	3	232.9
Residual (within columns)	4.345	8	0.5432
Total	703.1	11	

Appndix 9.9 - Tukey's Multiple Comparison Test for the remainderAFB1 over a period of 72 h at 30 ºC pH 6 in presence of *S.lividans*

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	95% CI of diff
0 vs 24	16.14	47.19	Yes	14.59 to 17.69
0 vs 48	18.41	53.82	Yes	16.86 to 19.96
0 vs 72	19.70	57.60	Yes	18.15 to 21.25
24 vs 48	2.268	6.630	Yes	0.7187 to 3.817
24 vs 72	3.562	10.41	Yes	2.012 to 5.111
48 vs 72	1.294	3.782	No	-0.2555 to 2.843

Appndix 9.10- One way ANOVA for the remaining AFB1 over a period of 72 h at 30 ºC pH 6 in presence of *S. aerufaciencs*

One-way analysis of variance

P value	< 0.0001		
P value summary	***		
Are means signif. Different (P < 0.05)	Yes		
Number of groups	4		
F	2829		
R squared	0.9991		
ANOVA Table	SS	df	MS
Treatment (between columns)	527.3	3	175.8
Residual (within columns)	0.4970	8	0.06213
Total	527.8	11	

Appendix 9.11 -Tukey's Multiple Comparison Test for the remainder AFB1 over a period of 72 h at 30 ºC pH 6 in presence of *S. aerufaciencs*

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant P < 0.05	95% CI of diff
0 vs 24	13.49	93.77	Yes	12.84 to 14.15
0 vs 48	15.72	109.2	Yes	15.07 to 16.37
0 vs 72	16.17	112.4	Yes	15.52 to 16.82
24 vs 48	2.226	15.47	Yes	1.574 to 2.877
24 vs 72	2.676	18.60	Yes	2.025 to 3.328
48 vs 72	0.4507	3.132	No	-0.2010 to 1.102

Chapter 6

Appendix 9.12- One-way ANOVA analysis for MTT assay for patulin residual at different time incubations at 25 ºC over 72h.

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	33.62			
R squared	0.8432			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	16.02			
P value	0.0030			
P value summary	**			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	27500	4	6875	
Residual (within columns)	5113	25	204.5	
Total	32610	29		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	95% CI of diff
Con - vs Con +	83.28	14.26	Yes	59.02 to 107.5
Con - vs 0.80	28.62	4.902	Yes	4.357 to 52.88
Con - vs 0.34	10.75	1.842	No	-13.51 to 35.01
Con - vs 0.15	6.319	1.082	No	-17.94 to 30.58
Con + vs 0.80	-54.66	9.363	Yes	-78.92 to -30.40

Con + vs 0.34	-72.52	12.42	Yes	-96.78 to -48.26
Con + vs 0.15	-76.96	13.18	Yes	-101.2 to -52.70
0.80 vs 0.34	-17.86	3.060	No	-42.12 to 6.396
0.80 vs 0.15	-22.30	3.819	No	-46.56 to 1.962
0.34 vs 0.15	-4.434	0.7594	No	-28.69 to 19.83

Appendix 9.13 One-way ANOVA analysis for MTT assay for ochratoxin A residual at different time incubation at 25 ºC over 72h.

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	41.16				
R squared	0.9482				
ANOVA Table	SS	df	MS		
Treatment (between columns)	14950	4	3737		
Residual (within columns)	817.0	9	90.78		
Total	15760	13			
Tukey's Multiple Comparison Test	Mean Diff.	q	Signif? P < 0.05?	Summary	95% CI of diff
Con - vs con +	75.46	13.72	Yes	***	49.30 to 101.6
Con - vs 1.66	62.38	11.34	Yes	***	36.21 to 88.54
Con - vs 1.2	15.71	2.555	No	ns	-13.54 to 44.96
Con - vs 1.1	0.8570	0.1558	No	ns	-25.30 to 27.02
con + vs 1.66	-13.09	2.379	No	ns	-39.25 to 13.08
con + vs 1.2	-59.75	9.715	Yes	***	-89.00 to -30.50
con + vs 1.1	-74.61	13.56	Yes	***	-100.8 to -48.44
1.66 vs 1.2	-46.66	7.587	Yes	**	-75.91 to -17.41
1.66 vs 1.1	-61.52	11.18	Yes	***	-87.68 to -35.36
1.2 vs 1.1	-14.86	2.416	No	ns	-44.11 to 14.39

Appendix 9.14 One-way ANOVA analysis for MTT assay for patulin residual in liquid culture degraded at different incubation temperatures and incubated for 24h.

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	6			
F	24.64			
R squared	0.8042			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	27.66			
P value	< 0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	23710	5	4742	
Residual (within columns)	5774	30	192.5	
Total	29490	35		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant ? P < 0.05?	95% CI of diff
con - vs con +	83.28	14.70	Yes	58.91 to 107.6
con - vs 25°	28.62	5.053	Yes	4.252 to 52.98
con - vs 30°	53.67	9.475	Yes	29.30 to 78.03
con - vs 35°	30.95	5.464	Yes	6.582 to 55.31
con - vs 40°	49.30	8.704	Yes	24.93 to 73.66
con + vs 25°	-54.66	9.651	Yes	-79.03 to -30.30
con + vs 30°	-29.61	5.228	Yes	-53.98 to -5.247
con + vs 35°	-52.33	9.240	Yes	-76.70 to -27.97
con + vs 40°	-33.98	6.000	Yes	-58.35 to -9.616
25° vs 30°	25.05	4.423	Yes	0.6836 to 49.41
25° vs 35°	2.330	0.4114	No	-22.03 to 26.70
25° vs 40°	20.68	3.651	No	-3.685 to 45.04
30° vs 35°	-22.72	4.011	No	-47.08 to 1.646
30° vs 40°	-4.369	0.7714	No	-28.73 to 20.00
35° vs 40°	18.35	3.240	No	-6.015 to 42.71

Appendix 9.15- One-way ANOVA analysis for MTT assay for ochratoxin A residual in liquid culture degraded at different incubation temperatures and incubated for 24h.

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	6			
F	175.4			
R squared	0.9865			
ANOVA Table	SS	df	MS	
Treatment (between columns)	12020	5	2404	
Residual (within columns)	164.5	12	13.70	
Total	12180	17		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant ? P < 0.01?	99% CI of diff
Con - vs Con +	73.25	34.27	Yes	60.21 to 86.29
Con - vs 25	62.38	29.19	Yes	49.34 to 75.42
Con - vs 30	64.41	30.14	Yes	51.37 to 77.45
Con - vs 35	74.11	34.67	Yes	61.07 to 87.15
Con - vs 40	67.79	31.72	Yes	54.75 to 80.83
Con + vs 25	-10.87	5.086	No	-23.91 to 2.169
Con + vs 30	-8.841	4.136	No	-21.88 to 4.199
Con + vs 35	0.8570	0.4010	No	-12.18 to 13.90
Con + vs 40	-5.458	2.554	No	-18.50 to 7.582
25 vs 30	2.030	0.9497	No	-11.01 to 15.07
25 vs 35	11.73	5.487	No	-1.312 to 24.77
25 vs 40	5.413	2.532	No	-7.627 to 18.45
30 vs 35	9.698	4.537	No	-3.342 to 22.74
30 vs 40	3.383	1.583	No	-9.657 to 16.42
35 vs 40	-6.315	2.955	No	-19.35 to 6.725

Appendix 9.16 - One-way ANOVA analysis for MTT assay for patulin residual in liquid culture degraded at different incubation temperatures and incubated for 48h.

One-way analysis of variance				
P value	< 0.0001			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	6			
F	40.57			
R squared	0.8712			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	26.01			
P value	< 0.0001			
P value summary	***			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	30000	5	5999	
Residual (within columns)	4436	30	147.9	
Total	34430	35		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	95% CI of diff
con - vs con +	83.28	16.78	Yes	61.92 to 104.6
con - vs 25°	6.319	1.273	No	-15.04 to 27.68
con - vs 30°	25.19	5.074	Yes	3.830 to 46.54
con - vs 35°	41.40	8.340	Yes	20.04 to 62.76
con - vs 40°	57.03	11.49	Yes	35.67 to 78.39
con + vs 25°	-76.96	15.50	Yes	-98.31 to -55.60
con + vs 30°	-58.09	11.70	Yes	-79.45 to -36.73
con + vs 35°	-41.88	8.436	Yes	-63.23 to -20.52
con + vs 40°	-26.25	5.287	Yes	-47.60 to -4.890
25° vs 30°	18.87	3.801	No	-2.489 to 40.22
25° vs 35°	35.08	7.067	Yes	13.72 to 56.44
25° vs 40°	50.71	10.22	Yes	29.36 to 72.07
30° vs 35°	16.21	3.266	No	-5.143 to 37.57
30° vs 40°	31.84	6.415	Yes	10.49 to 53.20
35° vs 40°	15.63	3.149	No	-5.725 to 36.99

Appendix 9.17 - One-way ANOVA analysis for MTT assay for ochratoxin A residual in liquid culture degraded at different incubation temperatures and incubated for 48h.

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	6			
F	198.5			
R squared	0.9881			
ANOVA Table	SS	df	MS	
Treatment (between columns)	11230	5	2245	
Residual (within columns)	135.7	12	11.31	
Total	11360	17		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.01?	99% CI of diff
Con - vs Con +	73.25	37.73	Yes	61.41 to 85.10
Con - vs 25	61.52	31.69	Yes	49.68 to 73.37
Con - vs 30	56.16	28.92	Yes	44.31 to 68.00
Con - vs 35	65.98	33.98	Yes	54.13 to 77.83
Con - vs 40	69.87	35.98	Yes	58.02 to 81.72
Con + vs 25	-11.73	6.040	No	-23.57 to 0.1186
Con + vs 30	-17.10	8.804	Yes	-28.94 to -5.249
Con + vs 35	-7.271	3.745	No	-19.12 to 4.575
Con + vs 40	-3.383	1.742	No	-15.23 to 8.463
25 vs 30	-5.368	2.764	No	-17.21 to 6.479
25 vs 35	4.456	2.295	No	-7.390 to 16.30
25 vs 40	8.345	4.298	No	-3.502 to 20.19
30 vs 35	9.824	5.060	No	-2.022 to 21.67
30 vs 40	13.71	7.062	Yes	1.866 to 25.56
35 vs 40	3.888	2.002	No	-7.958 to 15.73

Appendix 9.18 - One-way ANOVA analysis for MTT assay for patulin residual in liquid culture degraded at different incubation temperatures and incubated for 72h.

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different?				
(P < 0.05)	Yes			
Number of groups	6			
F	31.20			
R squared	0.9286			
ANOVA Table	SS	df	MS	
Treatment (between columns)	14830	5	2966	
Residual (within columns)	1141	12	95.08	
Total	15970	17		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	95% CI of diff
Con - vs conl +	84.35	14.98	Yes	57.61 to 111.1
Con - vs 25°	9.078	1.613	No	-17.67 to 35.82
Con - vs 30°	18.72	3.326	No	-8.025 to 45.47
Con - vs 35°	27.40	4.866	Yes	0.6485 to 54.14
Con - vs 40°	53.35	9.476	Yes	26.60 to 80.10
conl + vs 25°	-75.28	13.37	Yes	-102.0 to -48.53
conl + vs 30°	-65.63	11.66	Yes	-92.38 to -38.88
conl + vs 35°	-56.96	10.12	Yes	-83.70 to -30.21
conl + vs 40°	-31.00	5.507	Yes	-57.75 to -4.256
25° vs 30°	9.644	1.713	No	-17.10 to 36.39
25° vs 35°	18.32	3.254	No	-8.430 to 45.06
25° vs 40°	44.27	7.864	Yes	17.53 to 71.02
30° vs 35°	8.673	1.541	No	-18.07 to 35.42
30° vs 40°	34.63	6.151	Yes	7.881 to 61.37
35° vs 40°	25.95	4.610	No	-0.7921 to 52.70

Appendix 9.19 - One-way ANOVA analysis for MTT assay for ochratoxin A residual in liquid culture degraded at different incubation temperatures and incubated for 72h.

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	6			
F	19.30			
R squared	0.8894			
ANOVA Table	SS	df	MS	
Treatment (between columns)	12760	5	2552	
Residual (within columns)	1587	12	132.2	
Total	14350	17		
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	95% CI of diff
Con - vs Con +	75.46	11.37	Yes	43.92 to 107.0
Con - vs 25	18.13	2.731	No	-13.41 to 49.67
Con - vs 30	28.64	4.314	No	-2.900 to 60.18
Con - vs 35	44.52	6.706	Yes	12.98 to 76.06
Con - vs 40	67.79	10.21	Yes	36.25 to 99.34
Con + vs 25	-57.33	8.635	Yes	-88.87 to -25.79
Con + vs 30	-46.82	7.052	Yes	-78.36 to -15.28
Con + vs 35	-30.94	4.661	No	-62.48 to 0.5991
Con + vs 40	-7.668	1.155	No	-39.21 to 23.87
25 vs 30	10.51	1.583	No	-21.03 to 42.05
25 vs 35	26.39	3.975	No	-5.155 to 57.93
25 vs 40	49.66	7.480	Yes	18.12 to 81.20
30 vs 35	15.88	2.392	No	-15.66 to 47.42
30 vs 40	39.15	5.897	Yes	7.610 to 70.69
35 vs 40	23.27	3.506	No	-8.267 to 54.82

Appendix 9.20- Mascot Search Results

User : manal
Email : rjb12n@udcf.gla.ac.uk
Search title : D:\PE Sciex Data\Projects\Default\Data\Manal-28-5-9.wiff (sample number 1)
MS data file : C:\Temp\masC4.tmp
Database : NCBInr 20090522 (8876587 sequences; 3036162093 residues)
Timestamp : 28 May 2009 at 18:05:53 GMT
Protein hits :

gi|226306537 hypothetical protein RER_30500 [Rhodococcus erythropolis PR4]
gi|229493663 transaldolase [Rhodococcus erythropolis SK121]
gi|226303693 hypothetical protein RER_02040 [Rhodococcus erythropolis PR4]
gi|110810432 RecName: Full=NDMA-dependent methanol dehydrogenase
gi|226307770 enolase [Rhodococcus erythropolis PR4]
gi|136429 RecName: Full=Trypsin; Flags: Precursor
gi|15822684 isocitrate lyase [Rhodococcus equi]
gi|226307916 succinyl-CoA synthetase beta subunit [Rhodococcus erythropolis PR4]
gi|111018918 elongation factor Tu [Rhodococcus jostii RHA1]
gi|226304170 hypothetical protein RER_06810 [Rhodococcus erythropolis PR4]
gi|226307515 2,3-butanediol dehydrogenase [Rhodococcus erythropolis PR4]
gi|126739422 enolase [Roseobacter sp. SK209-2-6]
gi|227400147 alcohol dehydrogenase, class IV [Gordonia bronchialis DSM 43247]
gi|226307045 peptidoglycan synthase FtsI [Rhodococcus erythropolis PR4]
gi|226309390 ABC transporter substrate-binding protein [Rhodococcus erythropolis PR4]
gi|116671525 elongation factor Tu [Arthrobacter sp. FB24]
gi|111018632 Zn-dependent alcohol dehydrogenase [Rhodococcus jostii RHA1]
gi|111021965 type II citrate synthase [Rhodococcus jostii RHA1]
gi|111023201 isocitrate dehydrogenase [Rhodococcus jostii RHA1]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 58 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.