POTENTIAL BIOLOGICAL CONTROL OF AFLATOXINS IN DRIED FISH

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DECLARATION

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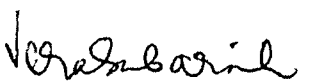

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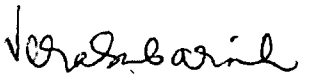

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ABSTRACT

Fish contributes 63% of the protein intake to the Indonesian diet. Fungal contamination is a common problem and can cause significant spoilage. However, little is known about the potential for fungal commensals of dried fish to be used to inhibit aflatoxin contamination. This study investigated metabolites extracted from *Debaryomyces hansenii, Aspergillus wentii, Eurotium rubrum, Polypaecilum pisce* and non-toxigenic *A. flavus* to prevent growth and aflatoxin production by *A. parasiticus* and *A. flavus.*

Aflatoxigenic strain, a_w and substrates all affected the concentration of aflatoxins. Both aflatoxigenic fungi produced the highest toxin concentration at a_w 0.99 and the lowest at a_w 0.89. Endogenous degradation occurred after prolonged growth.

Fungal inhibitor species affected synthesis and degradation of AFB_1, AFB_2, AFG_1 and AFG_2. Metabolites of *D. hansenii* demonstrated strong anti-aflatoxigenic activity on different substrates. The metabolites eliminated AFB_1 concentration (compared to 0.220-0.555 $\mu g\ g^{-1}$ in the control), AFG_1 (control contained 0.352-0.672 $\mu g\ g^{-1}$) and AFG_2 (control contained 0.259 $\mu g\ g^{-1}$). Furthermore, there were no other fluorescent compounds found on fish dried to a_w 0.85 in the presence of *D. hansenii* extract, indicating that the aflatoxins were completely degraded and/or synthesis was completely blocked. These results indicate that the metabolites may be suitable for use as a form of biological control on dried fish in tropical regions. The active inhibitor compounds of metabolites of *D. hansenii* contained β-1,4 glycoside bonds and were heat-stable, active over pH 2-10 with an optimum at neutral pH and did not fluoresce under UV. It is possible that a cyclic peptide is involved because the metabolites lacked a free carboxyl terminal group, but were sensitive to trypsin and pepsin.

The metabolites of the other four fungi tested were relatively heat-stable and their activity directly reduced aflatoxin concentrations without affecting the growth of aflatoxigenic fungi. Inhibitory compounds of *P. pisce* and *A. wentii* metabolites may be complex compounds with β-1,4 glycoside links and L-amino acids with lysyl or argynyl residues and with highest activity at pH near neutral to basic. Inhibitors of non-toxigenic *A. flavus* metabolites were possibly a sugar group with β-1,4 glycoside bonds attached to a peptide and were active at pH neutral. Degradation activity by non-toxigenic *A. flavus* occurred over a_w 0.89-0.99. Metabolites of *E. rubrum* were not effective in reducing aflatoxins.

Results suggest that modification on extraction and possibly production in a dried form could increase activity of the metabolites. Further study particularly of *D. hansenii* would be necessary to characterise the active inhibitors and evaluate their usefulness.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Fish is the main protein source in the Indonesian diet, contributing 63% of total animal protein intake. Fish consumption varies greatly throughout the nation and is influenced by location and culture. In 1991, the fish consumption per capita in Irian Jaya, a province in the eastern part of the Indonesian archipelago, was 74.4 kg or more than eight times that of Java, in the western part of Indonesia, at 8.7 kg. People in the eastern parts of Indonesia prefer unsalted dried fish, while the people in western part of Indonesia like salted dried fish. In a single year, almost 54% of the nation's total 2,537,612 tonnes of marine fisheries' production was consumed fresh and the rest was processed, with 29% produced as dried fish (Naamin, 1995). Both demersal and small or large pelagic fish are processed into dried products.

In Java, processing of dried fish consists of three methods: drying of unsalted fish, drying of lightly salted fish and drying of salted fish. Raw materials used also differentiate the type of dried fish products. Unsalted dried fish are usually made from materials of Grade 2 (Table 1-01). Processing of unsalted dried fish requires a washing tank or plastic barrel and bamboo trays (3 x 1 m) which are placed on bamboo drying platforms 14 m long and 1 m above the ground. Fish are washed and drained, then arranged on trays for sun drying. The fish are turned upside down to complete the drying process. The fish are then placed in the shade during storage in bamboo baskets before being marketed (Soegiyono, 1995). Unsalted dried fish retail at higher prices than salted dried fish (Table 1-02). Although it was not quantified, Fegan (1995) noted the high production of Indonesian unsalted dried fish such as tiny 'teri-tawar' anchovies. On the other hand, dried snapper (*Lutjanus* sp.) and grouper (*Epinephelus* sp.) are commonly marketed at A$ 5.00-10.00/kg in Irian Jaya. There is less information on the dried fish processing and fish products storage in Irian Jaya.

Table 1-01. Retail price* of raw materials used for processing of dried fish in Java, in 1993.

Fish	Raw materials (A$/kg)		
	Grade 1	Grade 2	Grade 3
Scad mackerel (*Decapterus* sp)	2.00	1.06	0.68
Sardine (*Sardinella longiceps*)	0.45	0.45	0.18
Sardine (*Abligaster sirm*)	1.14	0.83	0.53

* Calculated price from Indonesian Rp's (Soegiyono, 1995).

Table 1-02. Retail price* of dried fish in Java, in 1993.

Fish	Type of dried fish (A$/kg)		
	Unsalted	Lightly salted	Salted
Scad mackerel (*Decapterus* sp)	3.45	2.80	1.33
Sardine (*Sardinella longiceps*)	1.33	0.93	0.73
Sardine (*Abligaster sirm*)	2.67	1.87	1.06
Herring (*Clupea* sp)	1.87	1.33	0.93

* Calculated price from Indonesian Rp's (Soegiyono, 1995).

In 1999, fisheries production in Indonesia reached to 3.015 million tonnes (45%) of a total resource of 6.672 million tonnes available in Indonesian waters covering 5.682 million km^2, two-thirds of the country's region (Anonymous, 1999). When fishery production is abundant, sun drying is the simple and economical choice to preserve excess fish that can neither be eaten fresh nor chilled and frozen because of lack of facilities near the fish landing site. Therefore, this traditional fish processing is broadly practised by the fish processors in coastal areas and plays an important role in utilizing the catch in the glut season and adding value to the fish price (Soegiyono, 1995). However, peak fishery production in Indonesia usually coincides with the rainy season causing a delay in the drying process (Wibowo *et al.*, 1990; Naamin, 1995). If fish products are stored at high temperature (25-30°C) and humidity (60-90%)

in Indonesia, common environmental conditions in Southeast Asian countries, the possibility of some moulds growing on the fish products during and/or after processing is enhanced. Moulds have been found to be quite widespread contaminating dried fish in Indonesia. The presence of undesirable microorganisms or harmful substances in foods could cause adverse effects on humans. An investigation by Wheeler *et al.* (1986) reported that *Aspergillus, Eurotium* and *Penicillium* were dominant fungal contaminants found on the Indonesian dried fish.

Water activity (a_w) is an important factor affecting fungal growth. A review by Yu (1995) on salted dried fish in Southeast Asia, noted the a_w is often not low enough during the brining and drying processes and in storage of the products. Ah-Weng *et al.* (1985), who investigated the relation between a_w and quality loss for Southeast Asian cured fish, noted the growth of fungi after 10 days storage on the surface of the dried fish with an initial a_w of 0.80. They reported that the a_w of Southeast Asian large salted or dried fish was in range 0.67-0.92. In their report, they described the possibility of aflatoxin formation during processing or at the initial stages of storage when a_w is relatively high. Ah-Weng *et al.* (1985) found that a_w of Indonesian salted dried mackerel (*Scomberomorus commersonii*) and sea-catfish (*Aurius* sp.) ranged from 0.73-0.81. However, on the second day of the drying process and the commencement of storage, a_w in the thick inner part of the fish was 0.90 and the surface was 0.85, indicating the possibility of fungal growth if stored inappropriately.

On Indonesian dried fish that were below a_w 0.80, *A. flavus* was found quite frequently, in about 27% of the samples, but visible growth was not observed (Wheeler *et al.*, 1986). A study by Wheeler *et al.* (1988a) reported the minimum a_w for growth of *Aspergillus flavus*, isolated from Indonesian dried fish, was 0.85 on salt-based medium and 0.83 on glucose/fructose-base medium at 34°C. *Aspergillus parasiticus* and *A. flavus* produce aflatoxins over the range 13-37°C and above a_w 0.82, with optima at a_w 0.95-0.99 at 16-31°C (ICMSF,

1996). Pitt (1995) noted that at a_w below 0.80, aflatoxin was unlikely to occur at significant levels on salted dried fish in Southeast Asia. However, aflatoxin contamination occurs over wide geographic regions and in foods and feedstuffs such as peanuts, corn, sorghum, rice, dried fish, dried shrimp, cottonseed and meat meals (Ellis *et al.*, 2000). Wu and Salunkhe (1978) isolated 27 mycotoxin-producing fungi from dried shrimp, and two isolates of *A. flavus* were found to produce AFB_1 and AFG_1 both on Yeast Extract Sucrose (YES) medium and in shrimp. Jonsyn and Lahai (1992) reported the presence of AFB_1 and AFG_1 in smoked-dried unsalted fish from Sierra-Leone. A study on smoked dried ham from Croatia by Cvetnic and Pepeljnjak (1995) found that an isolate of *A. parasiticus* produced 240 mg kg^{-1} AFB_1, 20 mg kg^{-1} AFB_2, 125 mg kg^{-1} AFG_1 and 10.4 mg kg^{-1} AFG_2. They reported the *A. parasiticus* isolate was grown on moist shredded wheat (moisture content 40% w/w) for 14 days at 25°C.

Many countries have monitored the presence of aflatoxins, the carcinogenic toxins produced by filamentous fungi *Aspergillus parasiticus*, *A. flavus* and *A. nomius,* in foods and feeds and limited the maximum allowable aflatoxin concentrations (Table 1-03). Lubulwa and Davis (1994) separated grades of maize and peanut products in Indonesia, the Philippines and Thailand, into three categories: high quality (aflatoxin-free to 50 μg kg^{-1}), medium quality (50-300 μg kg^{-1}) and low quality (> 300 μg kg^{-1}). They noted that among these three Southeast Asian countries, 68% of Indonesian maize contained ≤5 μg kg^{-1} aflatoxin and only 4% was low quality, however, this was reversed in peanuts. Verardi and Rosner (1995) noted that even exposure to very low aflatoxins of <1 ng kg^{-1} body weight per day could contribute to an increased risk of liver cancer. They suggested a maximum permitted level of aflatoxins was < 5 μg kg^{-1} in foods.

Controlling aflatoxins in contaminated foods and feeds continues to dominate international concern (Moss, 1998). Efforts to eliminate aflatoxins

Table 1-03. Limit aflatoxin concentrations allowed in foods and feeds

Country	Limitation	Reference
Singapore	10-15 ng g^{-1}aflatoxins*, all foods	Reilly, 1986
Philippines	20 ng g^{-1} aflatoxins*, in coconut and peanut products	Reilly, 1986
Thailand	20 ng g^{-1} aflatoxins*, in edible oil	Reilly, 1986
Hongkong	15 ng g^{-1} aflatoxins*, all foods	Reilly, 1986
Japan	10 ng g^{-1} AFB_1 in mixed feeds	Stoloff *et al.*, 1991
Belgium	5 ng g^{-1} AFB_1, all foods	Stoloff *et al.*, 1991
Spain	5 ng g^{-1} AFB_1, 10 ng g^{-1} aflatoxins*, all foods	Stoloff *et al.*, 1991
Hungary	10 ng g^{-1} aflatoxins*, all foods; 0.5 ng g^{-1} milk AFM_1	Stoloff *et al.*, 1991
Israel	20 ng g^{-1} aflatoxins*, nuts and corns; 20 ng g^{-1} AFB_1, feeds	Stoloff *et al.*, 1991
Portugal	10-50 ng g^{-1} AFB_1, feeds	Stoloff *et al.*, 1991
Germany	2 ppb AFB_1, 4 ppb aflatoxins* foods	Papp *et al.*, 1999
France	5 ppb AFB_1, foods	Papp *et al.*, 1999
Switzerland	1 ppb AFB_1, foods	Papp *et al.*, 1999
USA	20 ppb aflatoxins*, foods	Papp *et al.*, 1999
UK	5 μg kg^{-1} AFB_1, feeds; 0.05 μg kg^{-1} AFM_1 milk	Moss, 1998
UN-FAO**	5 ppb AFB_1, foods; 10 ppb aflatoxins*	Papp *et al.*, 1999

* Total AFB_1, AFB_2, AFG_1 and AFG_2
**United Nations-Food and Agricultural Organization

have been widely applied using physical methods such as heat, irradiation, ultraviolet and visible light or using chemical methods such as chlorine, hydrogen peroxide, ozone, bisulfite, ammonia and alkali. However, Bassapa and Shantha (1996) noted that aflatoxins are resistant to thermal inactivation and other physical methods caused impairment of nutritional and organoleptic (flavour, odour, texture) qualities. Ellis *et al.* (1991) also noted that the use of gamma irradiation and UV light to detoxify aflatoxins was questionable because ducklings fed with the treated meals developed liver lesions. Most of the

chemicals are impractical and are particularly unsafe because they form toxic residues or destroy nutritional content, or organoleptic properties of the products (Park and Liang, 1993). On the other hand, Bata and Lasztity (1999) noted that the potential for decontamination by selected microorganisms may provide means of controlling mycotoxins without using harmful chemicals, and without significant losses in nutritive value and palatability of detoxified food and feed. Microorganisms were used to reduce aflatoxin in foods such as milk, peanut butter, soybeans, vegetable oils, corn and peanuts (Ciegler *et al.,* 1966; Line and Brackett, 1995a; Smiley and Draughon, 2000). Munimbazi and Bullerman (1998) reported that *Bacillus pumilus* isolated from dried fish 'Ndagala', produced compounds inhibitory to various mycotoxigenic fungi.

In the present study, *Polypaecilum pisce* Hocking and Pitt, *Aspergillus wentii* Wehmer, *Eurotium rubrum* Jos. König *et al.*, *Debaryomyces hansenii* (Zopf) Lodder and Kreger all isolated from dried fish and a non-toxigenic strain of *Aspergillus flavus* Link, were examined as potential controls on aflatoxins in dried fish.

1.2 THE IMPORTANCE AND OBJECTIVES OF THE STUDY

Studies on biological control of microorganisms have been growing rapidly because of their friendly environmental effects, particularly to humans. For decades, many researchers have reported using bacteria, fungi, yeasts, protozoa and algae to control toxigenic microorganisms. Generally, the present study aims to use fungal commensals of Indonesian dried fish, along with a yeast from Japanese dried fish and a non-toxic fungus isolated from Australian cocoa liquor, to control aflatoxin production in dried fish. The present study investigated whether the fungal commensals of dried fish were capable of removing aflatoxins and whether they could inhibit aflatoxin formation on the products. Their ability to prevent aflatoxin contamination could increase interest in the use of microorganisms to control aflatoxins biologically. This could reduce the potential risk of aflatoxin consumption in dried fish in Indonesia.

The study consisted of several main objectives throughout a series of experiments. Initially, the literature on aflatoxins is reviewed in chapter 2. The first objective was to investigate the influence of spore load and interaction with other fungi on the growth and aflatoxin production of *Aspergillus parasiticus* and *A. flavus*. The experimental conditions were optimal for growth and aflatoxin production of *A. parasiticus* and *A. flavus*. At a temperature of 25°C and high water activity (a_w 0.99), colony diameter and aflatoxin production of the two aflatoxigenic producers were investigated in the presence of *P. pisce, A. wentii, E. rubrum* and non-toxigenic *A. flavus*. Different spore loads and length of incubation were used as treatments as these have previously been shown to influence growth of aflatoxigenic fungi and aflatoxin concentration (Karunaratne and Bullerman, 1990; Gonzales *et al.*, 1995; Gqaleni *et al.*, 1997). The next experiments included *D. hansenii.* A relatively high a_w representing the potential rehydration process of stored products such as dried fish in the tropical regions with high temperature (30°C) and humidity (a_w 0.93) was then used. This gave a worst-case scenario in which the aflatoxigenic fungi would be able to grow, and also would possibly restrict growth of potential biological control organisms.

Metabolites of all fungal commensals of dried fish and the non-toxigenic *A. flavus* used in this study were extracted and used to challenge *A. parasiticus* and *A. flavus* to determine their antifungal and anti-aflatoxigenic activities at lower a_w conditions. The metabolites produced by the fungal commensals were then partially characterized for their stability to heat, pH and enzyme activity. Finally, metabolites of the fungal commensals that demonstrated inhibitory activity against *A. parasiticus* and *A. flavus* were applied to dried fish to determine their capability to prevent aflatoxin contamination. The study is finished with a discussion of the conclusions and indications for future work. As the chapters are designed to in a form suitable for publication, there is necessarily some repetition of introduction and discussion comments.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Sun drying is a common practice in Indonesia and solar fish drying has been practised for many years, mainly by small-scale fish processors (Suparno, 1995). Drying is a process of removing water from products, thereby reducing weight and extending storage life by prevention of microbial growth (Haard, 1995). Doe and Heruwati (1988) studied drying and storage of tropical fish in Indonesia and reported that 20% of brined fish sun-dried for 2 days had water activity (a_w) 0.86. Pitt (1989) described water activity (a_w) as a term for expressing water availability for the growth of microorganisms. The term a_w is defined as the ratio of the partial pressure of water in a sample (p) to the saturation vapor pressure of pure water (p_o) under the same conditions. A_w is numerically equal to equilibrium relative humidity (ERH) expressed as a decimal, formulated as follows:

$$a_w = p / p_o$$

$$a_w = ERH / 100$$

A_w is one of the single most important factors affecting microbial growth (Chirife *et al.*, 1982). Beuchat (1983) and Gibson *et al.* (1994) noted that a_w of some food products is reduced sufficiently to restrict the growth of bacteria but not enough to prevent the growth of some spoilage moulds. Studies on dried fish products contaminated by some *Aspergillus* species have been widely reported (Reilly, 1986; Atapattu and Samarajeewa, 1990; Jonsyn and Lahai, 1992; Munimbazi and Bullerman, 1996, Chakrabarti and Varma, 2000). A study by Sim *et al.* (1985) found *A. parasiticus* and *A. flavus* species on visibly mouldy dried shrimp. Wheeler *et al.* (1986) found 364 fungal isolates from 74 salted dried fish from Indonesia. They reported a_w of the fish products was 0.65-0.79 and 34% of fungal isolates were *Aspergillus* (*A. niger, A. flavus, A sydowii, A. wentii* and *A. penicilloides*), 21% *Eurotium* (*E. rubrum, E. repens, E. amstelodami* and *E. chevalieri*), and less frequent were *Penicillium* and other fungi. The most prevalent fungus found in the Indonesian salted dried fish, was

a salt tolerant xerophile named *Polypaecilum pisce* (Pitt and Hocking, 1985). Of the fungi found in the salted dried fish, Wheeler *et al.* (1988a) reported that fungal growth was dependent on species, a_w and temperature. They found the minimum a_w for germination *Eurotium rubrum* to be 0.72 at 20°C, *Aspergillus wentii* 0.76 at 25°C and *A. flavus* 0.83 at 34°C.

Aspergillus parasiticus Speare and some strains of *A. flavus* Link are recognized worldwide as producers of aflatoxins, secondary metabolites that are carcinogenic (Trail *et al.*, 1995; Moss, 1998). These fungi contaminate a wide range of foods and feeds (Ellis *et al.*, 1991; Gourama and Bullerman, 1995a) and are also found in water (Paterson *et al.*, 1997). Scott *et al.* (1967) reported that a strain of *Aspergillus* isolated from a Japanese dried fish 'katsuobushi', produced aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1). Wu and Salunkhe (1978) screened 114 fungi isolated from 20 dried shrimp samples, and 27 isolates mainly *Aspergillus* and *Penicillium* species were capable of producing mycotoxins. They noted that moisture content of the non-mouldy dried shrimp was 9.3-12.8% and the mouldy dried shrimp was 10.6-31.5%. They reported that two strains *A. flavus* from the dried shrimp produced AFB_1 at 5.4 and 8.3 $\mu g\ g^{-1}$ and AFG_1 at 2.6 and 6.5 $\mu g\ g^{-1}$ after 3 weeks at 27°C. These authors also reported that these two *A. flavus* isolates produced 16.4 and 21.7 $\mu g\ mL^{-1}$ AFB_1 and 12.0 and 15.1 $\mu g\ mL^{-1}$ AFG_1 on yeast extract sucrose (YES) medium.

In Southeast Asia, FAO (1979) reported that aflatoxin contamination occurred in fish products. It was reported that in the Philippines, 3 $\mu g\ kg^{-1}$ aflatoxins were found in dried fish, 5 $\mu g\ kg^{-1}$ in smoked fish and 2 $\mu g\ kg^{-1}$ in salted fermented small shrimps and fish sauce (FAO, 1979). Reilly (1986) reported that 3.8 $ng\ g^{-1}$ aflatoxins contaminated a market sample of dried and smoked fish in the Philippines. Shank *et al.* (1972) noted a primary liver cancer incidence in Thailand and linked aflatoxin-contaminated dried fish or shrimp as a possible cause. FAO (1979) noted that dried fish and shrimp exported from

Thailand contained 166 ng g^{-1} aflatoxins. FAO (1979) also reported that samples of salted dried fish from Indonesia that were commonly consumed by some patients suffering from liver cancer, contained 5 ng g^{-1} AFB_1. Pitt (1994) noted that aflatoxins were responsible for the deaths from liver cancer in Indonesia. It was estimated that the number of deaths from liver cancer due to aflatoxins in Indonesia alone exceeds 20,000 per annum (Lubulwa and Davis, 1994). Pitt (2000) reported that the concentrations of aflatoxins in some tropical foods were unacceptably high. Pitt and Hocking (1996) noted that ≤ 5 μg kg^{-1} aflatoxins was found in 68% maize and 44% peanuts, however, $\geq$ 50-300 μg kg^{-1} aflatoxins were detected in 18% maize and 12% peanuts, respectively from 96 and 215 total samples from Indonesia. Pitt *et al.* (1998) reported that *A. flavus* contaminated 34-95% of 675 samples of various food commodities from Java.

After the discovery in the 1960s that aflatoxins caused "turkey X disease", there was a need for finding effective methods of detoxification of the toxins. Aflatoxins can be inactivated by physical, chemical or biological methods (Doyle *et al.*, 1982; Ellis *et al.,* 1991; Basappa and Shantha, 1996). The physical approaches to aflatoxin destruction involved heat treatment, ultra violet (UV) light, or ionizing radiation. However, none of these physical approaches is entirely effective. Chemical degradation of aflatoxins is usually carried out by the addition of chlorine (sodium hypochlorite, gaseous chlorine), oxidizing agents (hydrogen peroxide, ozone, sodium bisulfite), or hydrolytic agents (acids, alkalis, ammonia). Of these methods, ammonification is the most widely used, although it may result in losses in nutritional quality of treated feed (Bata and Lasztity, 1999). Samarajeewa *et al.* (1990) noted the combined use of physical and chemical treatments was more effective in detoxification of aflatoxins than using only a single procedure. While some physical and chemical methods of detoxification have been developed, they do not represent a fully acceptable approach to aflatoxin detoxification (Faraj *et al.*, 1993; Park and Liang, 1993). Biological degradation using other microorganisms, on the other hand, showed a

great potential for removing aflatoxins (Bata and Lasztity, 1999). Therefore, this approach would be the main focus of the present study, particularly using metabolites of fungal commensals of dried fish to control aflatoxin production by *A. flavus* and *A. parasiticus*.

2.2 AFLATOXIGENIC FUNGI

2.2.1 *Aspergillus flavus* Link and *A. parasiticus* Speare

Generally, the characteristics used in fungal taxonomy are phenotypic and genotypic parameters. Fungal phenotype includes colonial and cell morphology, physiological responses to the environment, growth rate, biochemical reactions and profiles of secondary metabolites. The species most widely studied for aflatoxin production, are *A. flavus* and *A. parasiticus*. Although Kurtzman *et al.* (1986) reduced *A. parasiticus* to subspecies of *A. flavus*, Klich and Pitt (1988a) described *A. parasiticus* and *A. flavus* (commonly known as '*Aspergillus flavus* group') as different in their morphological features and mycotoxin production. Later, Pitt and Hocking (1997) described the taxonomic description of *A. flavus* and *A. parasiticus* as follows:

Kingdom: Fungi

Subkingdom: Deuteromycotina

Class: Hypomycetes

Family: Moniliaceae

Genus: *Aspergillus*

Species: *flavus* Link

parasiticus Speare

The subkingdom Deuteromycotina, which is often called "Deuteromycetes" or fungi imperfecti, contains fungi that produce only asexual or imperfect haploid spores (Pitt and Hocking, 1997). The spores are formed

after mitotic nuclear division and are borne in chains. The general term for the Deuteromycetes spore is conidium (plural: conidia). Conidia, and the specialized hyphae or conidiophores on which they are borne, differ in appearance. Klich and Pitt (1988a) noted that conidial wall texture was an effective criterion for distinguishing *A. parasiticus* and *A. flavus* (Table 2.01). Pitt and Hocking (1997) described the conidia of *A. flavus* as variable in size and shape, having relatively thin walls, smooth to moderately rough. *A. parasiticus* conidia are spherical and have relatively thick rough walls. Deuteromycetes spores are usually not heat resistant, but may be quite resistant to chemicals. *Aspergillus flavus* and *A. parasiticus* are also distinguished from other fungi by their rapid growth at 25-37°C and bright yellowish green conidial color. Some isolates of *A. flavus* and the closely related species *A. parasiticus* (Klich and Pitt, 1988a) and *A. nomius* (Kurtzman *et al.*, 1987) are aflatoxin-producing fungi, however, some are non-toxigenic (Kale *et al.*, 1994).

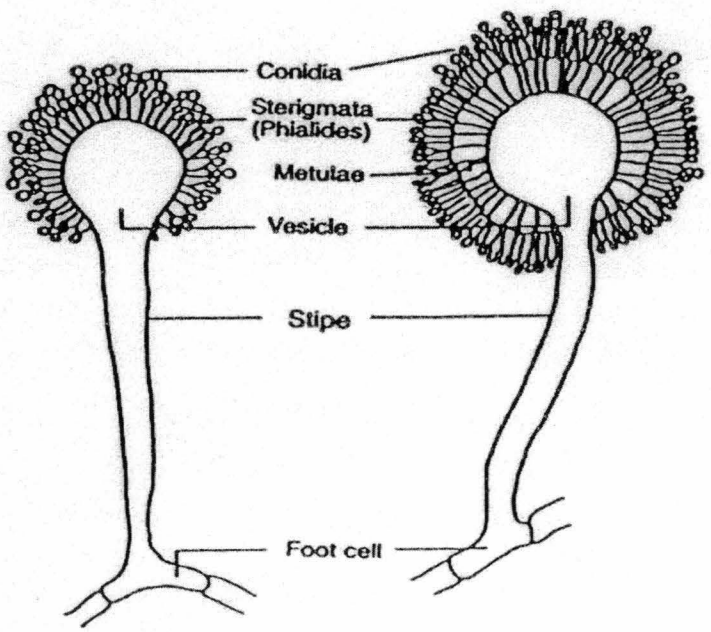
Christensen (1981) produced a synoptic key using vesicle size, conidiophore, color of conidial heads, growth, form and size of sclerotia as aids in *Aspergillus* morphological characterization. Horn *et al.* (1996) screened 79 isolates of *A. flavus* and 76 of *A. parasiticus* and found that the number of sclerotia in *A. flavus* ranged from 12.7-3705.3 per plate with a volume of 0.8-0.20 mm^3, significantly different from *A. parasiticus* at 0.0-4709.0 per plate and volume 0.6-0.11 mm^3. Pitt and Hocking (1997) described the vesicles of *A. flavus* as usually large (about 50 μm) bearing metulae, while the vesicle of *A. parasiticus* rarely exceeded 30 μm and metulae were uncommon. Similarly, Raper and Fennel (1973) noted that the sterigmata in *A. parasiticus* were mostly uniseriate and in *A. flavus* were typically biseriate. Table 2-01 and Figure 2-01 describe morphological characteristics of *A. flavus* and *A. parasiticus*.

Table 2-01. Morphological features* of *A. flavus* group including *A. flavus* and *A. parasiticus*

Features	Morphology
Conidial heads	globose to radiate or columnar, yellow-green, olive green, leaf green, olive brown [1, 2].
Conidiophore	colourless, smooth to roughened, 200 μm to 1.8 mm [2]; stipe > 1 mm (*A. flavus*), < 500 μm (*A. parasiticus*) [3]
Vesicles	clavate to subglobose, 30-35 μm (*A. parasiticus*) [2] mostly subglobose to globose, 30-65 μm (*A. flavus*) [2]
Sterigmata	7-9 μm by 3-4 μm to 7-12 μm by 3.3-4.4 μm; uniseriate or biseriate [1]
Conidia	globose or subglobose [2]; 3-6 μm (*A. flavus*), 4-6 μm (*A. parasiticus*) [3]
Sclerotia	globose or subglobose 400-800 μm by 400-600 μm, dark brown to black [2]

*Based on [1] Raper and Fennel (1973); [2] Christensen (1981); [3] Klich and Pitt (1988a).

Figure 2-01. Coniodiophore characteristics of *A. parasiticus* and *A. flavus*



(a) *Aspergillus parasiticus* (b) *Aspergillus flavus*

From: Klich and Pitt (1988b) drawn by Hocking A. D.

Dorner *et al.* (1984) differentiated *A. flavus* and *A. parasiticus* primarily on their biochemical difference: the production of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2). *A. parasiticus* produced the four aflatoxins and *A. flavus* usually only produced AFB_1 and/or AFB_2. However, they found that AFG_1 was also detected in *A. flavus*. These authors used cyclopiazonic acid as a criterion to differentiate the two fungi because this substance was not produced by *A. parasiticus* (Dorner *et al.*, 1984). Klich and Pitt (1988a) also noted the production of cyclopiazonic acid only in *A. flavus*.

The genotypic characteristics, on the other hand, for instance purine and pyrimidine base sequences and gene mapping were reviewed by Mullaney and Klich (1990). They noted that four molecular methods were commonly used in fungal taxonomy: guanine (G) + cytosine (C) molar percentage, DNA complementarity, ribosomal RNA sequence comparison and restriction fragment length polymorphism (RFLP). The first two methods are related to each other as G+C molar percentage, a measure DNA base composition, is an initial screening step for DNA complementarity. Kurtzman *et al.* (1986, 1987) reported that molar percentage of G+C of *A. flavus* was 49.1-49.9 and of *A. parasiticus* was 49.1-50.0. These researchers also reported that the nuclear DNA complementarity of *A. flavus* and *A. parasiticus* was high (70%) and the genome sizes of these two fungi were 1.00 and 1.10, respectively. Tran-Dinh *et al.* (1999) found that molecular genotypes of *A. flavus* and *A. parasiticus* used in their study, showed separated amplification profiles between the two species.

2.2.2 Isolation and detection *A. parasiticus* and *A. flavus*

In their reports, Pitt *et al.* (1992) and Gourama and Bullerman (1995b) described the use of selective and differential media to shorten the time required in isolation of *Aspergillus* strains. Hocking (1982) and Pitt *et al.* (1983) improved an *Aspergillus* differential medium (ADM) and developed Aspergillus flavus-parasiticus agar (AFPA) for detection of the aflatoxigenic fungi. They

found that when *A. parasiticus* and *A. flavus* were incubated at 30 ° C for 42 hours, they produced orange-yellow reverse pigment that differentiated them from other fungi. They noted that ferric ammonium citrate (0.05%) and yeast extract (up to 4%) were responsible for the intensity of pigmentation, and the medium was suitable for both these aflatoxigenic species.

Cotty (1994) compared four media: modified Rose Bengal agar (M-RB), Bell-Crawford Rose Bengal agar (BC-RB), Czapek Dox agar supplemented with the antibiotics in BC-RB (CZ-RB) and AFPA for isolation *A. flavus* and related fungi. He found M-RB was useful to isolate all three aflatoxin-producing species *A. flavus* (both the S and L strains), *A. parasiticus* and *A. nomius*. It was noted that M-RB is a defined medium with nitrate as the sole source of nitrogen and AFPA medium has complex nitrogen and carbon sources e.g. peptone and yeast extract. Dyer and McCammon (1994) found Coconut Cream Agar (CCA; 50% coconut cream and 1.5% agar) was optimal for *A. parasiticus* and *A. flavus* to produce strong fluorescence. CCA was better than shredded coconut, desiccated coconut or coconut milk incorporated into other media. *A. flavus* produced pastel blue fluorescence and *A. parasiticus* a bluish white fluorescence in the medium. Yabe *et al.* (1987) screened *A. parasiticus, A. flavus, A. sojae* and *A. oryzae* by UV photography and reported that the aflatoxigenic strains were identified as gray and black colonies and the non-toxigenic strains appeared as white colonies on Glucose Yeast (GY) agar.

A. flavus and *A. parasiticus* produced yellow-green colonies with fruiting structures on Czapek Yeast Extract Agar (CYA), Czapek Agar (CA) and Malt Extract Agar (MEA) (Pitt and Hocking (1997). They are best enumerated on media Dichloran Rose Bengal Chlorampenicol (DRBC), Rose Bengal Chlorampenicol (RBC) and Dichloran 18% Glycerol (DG18) which restrict colony spreading without affecting spore germination (Hocking and Pitt, 1980; Pitt and Hocking, 1989). Filtenborg and Frisvad (1990) concluded that

CYA and Yeast Extract Sucrose (YES) were the best media to support secondary metabolite production. They used profiles of secondary metabolites that are specific for particular species to identify fungi. In their experiment, the concentration of AFB_1 extracellular metabolite (in the medium CYA, YES, DRBC and DRYES) was higher than the intracellular concentration in the mycelium of *A. flavus* after 7 days. They also found that AFB_1 was not detected in DG18 after 7 days, therefore they suggested that extended incubation time was needed, or that the reduced a_w of this medium (0.95 a_w) inhibited secondary metabolite production. These authors concluded that application of this method depended on the availability of metabolite standards and data on metabolite profiles.

Shapira *et al.* (1996) detected aflatoxigenic fungi by using polymerase chain reaction (PCR). These researchers used specific PCR products obtained from DNA of *A. parasiticus* and found that the technique gave a positive result of DNA amplification in distinguishing *A. parasiticus* and *A. flavus*. Tsai and Yu (1997) used enzyme-linked immunosorbent assay (ELISA) to identify aflatoxigenic fungi. They found that 81% of 21 strains of *A. parasiticus* tested had relative activities > 0.5. They noted that molecular weights of *A. parasiticus* antigens were 94, 82 and 40 kDa. Candlish *et al.* (1997) reported that five separated monoclonal antibodies produced against whole cell extracts of *A. flavus* were specific to *A. flavus* and *A. parasiticus* and no cross reactivity with other *Aspergillus* species. Shapira *et al.* (1997) developed polyclonal antibodies (PAb) to detect aflatoxigenic fungi by involving culture filtrate and proteins of *Escherichia coli*. On the other hand, Kwak *et al.* (1999) used anti-mould PAb of extracellular polysaccharide of *A. flavus* in ELISA method. They found that *A. flavus* and *A. parasiticus* strains gave positive signals both in the potato dextrose broth and in dilution of 1000 folds.

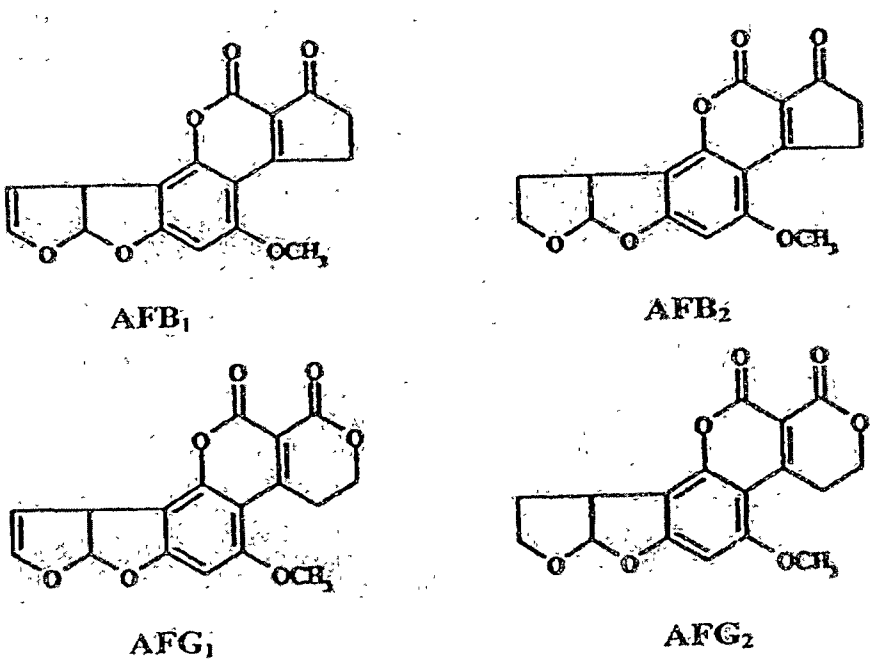
2.2.3 Biosynthesis and toxicity of aflatoxins

Yabe *et al.* (1999) noted that aflatoxins are produced intracellularly and then excreted. Betina (1984) elucidated two types of microbial metabolites: polyketides and fatty acids. Synthesis of fatty acid metabolites is by a specific pathway of acetate joined with malonate units. However, most fungal metabolites are formed by the polyketide pathway, a term indicating the formation of a β-polyketide chain by repeated head-to-tail condensation of acetate. Dutton (1988) and Sweeney and Dobson (1999) described the biosynthesis of aflatoxin as initially a conversion of acetate and malonyl CoA to a hexanoyl starter unit by a fatty acid synthase. The hexanoyl starter unit was then extended by a polyketide synthase to norsolorinic acid. Norsolorinic acid is the first stable precursor of aflatoxin synthesis. The polyketide then undergoes a series of intermediate reactions involving 12-17 enzymatic conversions to form versicolorin B (Trail *et al.*, 1995). The pathway then branches to demethylsterigmatocystin and dihydrodemethylsterigmatocystin. The former branch forms AFB_1 and AFG_1 that contain dihydrobisfuran rings and the latter branch forms AFB_2 and AFG_2 containing tetrabisfuran (Yabe *et al.*, 1999; Sweeney and Dobson, 1999).

The biosynthesis of secondary metabolites is also regulated by nutritional factors. Luchese and Harrigan (1993) reviewed sources of carbon, nitrogen, lipoperoxides, trace metals and phosphates required in biosynthesis of aflatoxins. They noted that various carbon sources like sucrose, glucose, ribose, xylose, glycerol, maltose and fructose stimulate aflatoxin biosynthesis. Complex organic nitrogen compounds such as yeast extract and peptone, and synthetic or natural lipoperoxides (triglycerides, fatty acids and sterols) also enhance biosynthesis of aflatoxins. Trace elements such as Zn are considered to favour polyketide biosynthesis and participate in some of the enzyme systems that are responsible for the initial condensation of the acetate units (Zaika and Buchanan, 1987).

Aflatoxins are distinguished by their structure (Figure 2-02), molecular weight, crystal color, melting point, fluorescence and mass spectra. Heathcote (1984) grouped aflatoxins into two groups of polyketides that consist of 14 different types of aflatoxins. The first group is the difurocoumarocyclopentonones (i.e. aflatoxin B_1 abbreviated as AFB_1, AFB_2, AFB_{2a}, AFM_1, AFM_2, AFM_{2a} and aflatoxicols) and the second group is difurocoumarolactones (i.e. AFG_1, AFG_2, AFG_{2a}, AFG_{M1}, AFG_{M2}, AFG_{M2a} and AFB_3). Cole and Cox (1981) noted that the molecular weight of AFB_1 is

Figure 2-02. Structure of AFB_1, AFB_2, AFG_1 and AFG_2.



From: Sweeney and Dobson (1999)

312.063, AFB_2 is 314.079, AFG_1 is 328.058 and AFG_2 is 330.074. Heathcote (1984) noted that in a crude extract, AFB_1, AFB_2, AFG_1 and AFG_2 are commonly present in greater concentrations than other types. Under UV light, AFB_1 and AFB_2 fluoresce bright blue and AFG_1 and AFG_2 fluoresce greenish blue. Aflatoxin B_2 is the dihydro-derivative of B_1, and aflatoxin G_2 is the dihydro-derivative of G_1 (Cole and Cox, 1981; Heathcote, 1984).

Among the aflatoxins, AFB_1 is widely regarded as the most acutely toxic and potent liver carcinogen (Trail *et al.*, 1995; Moss, 1998; Sweeney and Dobson, 1999), followed by AFG_1, AFB_2 and AFG_2 (Heathcote, 1984). Smith *et al.* (1995) noted that AFB_1 and naturally-occuring mixture of aflatoxins were carcinogenic to man and animals, however, AFG_1 was evidently only carcinogenic to animals. Table 2-02 shows the relative toxicity of the four major aflatoxins: AFB_1, AFB_2, AFG_1 and AFG_2. Rainbow trout (*Oncorhynchus mykiss*) were reported to be the most sensitive to AFB_1. Ottinger and Kaattari (2000) noted that exposed to very low concentration of AFB_1 in feeds or exposed as embryo, caused high incidence of carcinogenesis in the fish. They also noted that exposure to AFB_1 resulted reduction on cytokine production, macrophage function and lymphocyte activity. Ngethe *et al.* (1992) and Otsrowki-Meissner *et al.* (1995) reported that at concentration of <1 $\mu g\ kg^{-1}$ AFB_1 in the diet caused liver tumors in this species.

Table 2-02. Relative Toxicity of the four major aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2)

Aflatoxin	Toxicity	Reference
AFB_1	Highly toxic/carcinogenic to experimental animals [(1)]; LD_{50} 18 $\mu g\ kg^{-1}$ in ducklings [(2)]	[(1)]; Samarajeewa *et al.*, 1990; [(2)] Heathcote, 1984
AFB_2	2.4 times less toxic than AFB_1 to ducklings [(1)]; LD_{50} 84 $\mu g\ kg^{-1}$ in ducklings [(2)]	[(1)]; Samarajeewa *et al.*, 1990; [(2)] Heathcote, 1984
AFG_1	1.6 times less toxic than AFB_1 to ducklings [(1)]; LD_{50} 39$\mu g\ kg^{-1}$ in ducklings [(2)]	[(1)]; Samarajeewa *et al.*, 1990; [(2)] Heathcote, 1984
AFG_2	The least toxic, LD_{50} 172.5 $\mu g\ kg^{-1}$ in ducklings.	Heathcote, 1984

2.3 GROWTH AND AFLATOXIN PRODUCTION OF *A. parasiticus* AND *A. flavus*

Growth and aflatoxin production of *A. parasiticus* and *A. flavus* are influenced by a_w, temperature, pH, gas tension (O_2 and CO_2), nutrients, strain variability, inoculum size and competing microorganisms. Aflatoxin production occurs at the beginning of stationary phase of the growth of filamentous fungi. Pitt (1993) described a model of environmental conditions that affected rates of growth and aflatoxin production. This author noted that the rate of toxin formation is proportional to the rate of production new cell mass and the rate of toxin degradation is proportional to the product of dead cell mass and the concentration of aflatoxin. Biosynthesis of a fungal secondary metabolite requires a certain value of a_w, temperature, pH, nutrient limitation and O_2 concentration (Betina 1984; Dorner *et al.*, 1992). De Pena and Ruiz-Herrera (1997) noted that synthesis of aflatoxins depends on strain specificity, culture conditions and nutritional factors. Betina (1984) also noted that other factor affecting aflatoxin production in submerged culture is the degree of aeration. Ellis *et al.* (1991) reported that aflatoxin formation required 20-90% O_2. However, a_w is the crucial factor for the growth of *A. flavus* and aflatoxin production.

2.3.1 Water activity (a_w)

Northolt and Bullerman (1982) concluded that the a_w and temperature range for growth of both *A. parasiticus* and *A. flavus* was between a_w 0.80-0.99 at 10-40°C. AFB_1 production ranged from 0.84-0.99 a_w at 10-40°C. Gibson *et al.* (1994) noted that a_w is a principal controlling factor in fungal growth. Their study showed that the optimum a_w at 30°C for *A. parasiticus* was 0.985-0.993 and for *A. flavus* was 0.99 for growth. A study by Pitt and Miscamble (1995) on water relations of *A. flavus, A. nomius, A. oryzae* and *A. parasiticus* showed that the optimum a_w for growth of the fungi was in the range 0.96-0.98 at 25°C.

They also noted that these four fungi could germinate at a_w 0.81. Gqaleni *et al.* (1997) studied the effect of a_w, medium, temperature and incubation time and showed that a_w statistically had the greatest effect on aflatoxin production in *A. flavus* F2R4FP1-5. They found that at a_w 0.99, the highest aflatoxins (total of AFB_1 and AFB_2) concentration was 0.330 μg mL^{-1} on YES and 0.306 μg mL^{-1} on CYA after 15 days at 30°C. At a_w 0.95 and 0.90, production of aflatoxins respectively was 0.226 and 0.096 μg mL^{-1} on YES and 0.183 μg mL^{-1} and 0.076 on CYA after 15 days at 30°C. These researchers also noted that the *A. flavus* strain used produced small amounts of aflatoxin and at 20 and 37°C with a_w 0.90 aflatoxins were not found.

2.3.2 Temperature

Different growth temperatures considerably affect aflatoxin concentrations (Northolt *et al.*, 1977). These authors noted that the optimum temperature for fungal growth and aflatoxin production was in the range 25-30°C. They also observed at 15-18°C, equal amounts of AFB_1 and AFG_1 were formed, however, at 32°C AFB_1 was relatively more abundant than AFG_1. Schroeder and Hein (1967) suggested that at high temperature, AFG_1 catabolism was accelerated. Cuero *et al.* (1987) noted that aflatoxin production in *A. flavus* was maximal at 25°C but the growth was slow at 16°C after 12 days. Park and Bullerman (1983) reported that the total aflatoxins in *A. parasiticus* and *A. flavus* were much higher at 25°C than 15°C after 10 days. Shih and Marth (1974), who studied growth and aflatoxin production in *A. parasiticus* at different temperatures (15, 25, 35 and 45°C) concluded that 25°C was optimal temperature for *A. parasiticus*. They found at 25°C, the maximum aflatoxin produced by *A. parasiticus* was about 30 μg mL^{-1} after 5 days. No aflatoxin was produced at 45°C, and at 35°C aflatoxin was only found at 3 days incubation and production ceased afterwards. At 15°C, aflatoxin in *A. parasiticus* was less than 10 μg mL^{-1} after 5 days. In comparison, *A. flavus* demonstrated maximum aflatoxin production at 30°C after 15 days (Gqaleni *et al.*, 1997).

2.3.3 Hydrogen ion concentration (pH)

Ellis *et al.* (1991) noted that pH affected aflatoxin production more than the growth of *Aspergillus*. They reported that *A. parasiticus* and *A. flavus* grew over a wide range of pH (1.7-9.3) with growth optimum achieved at pH 5 to 8, but aflatoxin synthesis was inhibited at acidic pH. Thus, pH was more pronounced in affecting secondary metabolic pattern (aflatoxin production) than primary metabolic pattern (growth). Nakazato *et al.* (1990) noted at lower pH, more isomerization of aflatoxicols occurred and conversion to aflatoxicols also decreased. Shih and Marth (1974) found that *A. parasiticus* at 25°C grown on a medium with an initial pH 6.5 subsequently decreased pH to 3 after 5 days, and yielded maximum aflatoxin production (>30 μg mL^{-1}) in their study. Park and Bullerman (1983) also observed that the highest aflatoxin concentration of 132.24 μg mL^{-1} produced by *A. flavus* and 22.98 μg mL^{-1} by *A. parasiticus* on YES medium was accompanied by a decrease in pH to 5.3 and 5.2 respectively after 10 days at 25°C. El-Gazzar *et al.* (1987) found that *A. parasiticus* grown on glucose yeast salt medium added with 0.5% lactic acid, at an initial pH 4.5 produced 39.60 μg mL^{-1} AFB_1, however, at an initial pH 3.5 produced 35.69 μg mL^{-1} AFB_1 after 3 days at 28°C.

Other researchers observed that the final pH of the medium varied inversely with aflatoxin concentration in *A. flavus*. Cotty (1988a) found AFB_1 concentration was 18.27 μg mL^{-1} at final pH 2.83, compared to 4.68 μg mL^{-1} at a final pH 4.44 in citrate-buffered ammonium medium. Similarly, Keller *et al.* (1997) found for *A. parasiticus* that AFB_1 concentration was 1062 ng mL^{-1} at an initial pH 4.0 compared to 244 ng mL^{-1} at an initial pH 7.0.

2.3.4 Substrates

Northolt and Bullerman (1982) noted that substrates containing amino acids, fatty acids and Zn stimulated aflatoxin formation. Luchese and Harrigan

(1993) noted that medium containing glucose and sucrose such as CYA and YES supported aflatoxin production. Cuero *et al.* (1987) reported that *A. flavus* grew and produced 1020 ng g^{-1} aflatoxins on maize at a_w 0.98 and 25°C, however, no aflatoxins were detected on rice at the same experimental conditions. A study by Park and Bullerman (1983) on an extensive range of foods reported that *A. parasiticus* produced aflatoxins at higher amount on substrates high in proteins than those high in carbohydrates, but this was reversed for *A. flavus*. These authors found at 25°C after 10 days, *A. parasiticus* produced 223 μg g^{-1} aflatoxins on cheddar cheese and 93 μg g^{-1} on rice. At the same experimental conditions, *A. flavus* produced 246 μg g^{-1} aflatoxins on cheddar cheese and 532 μg g^{-1} on rice. They also noted that food substrate and aflatoxigenic strain were important factor in determining aflatoxin production.

2.3.5 Aflatoxigenic strains

The concentration of aflatoxin produced by *A. parasiticus* and *A. flavus* is dependent on the aflatoxigenic strains. Wicklow and Shotwell (1983) examined four strains of *A. flavus* from Agricultural Research Service Culture Collection (NRRL) 3357, 6412, 6554, 6555 and four strains of *A. parasiticus* NRRL 13004, 13005, 13006 and 13007. They found that production of AFB_1 and AFG_1 varied between sclerotia and conidia of the same fungal isolate. These researchers reported, for instance, *A. flavus* NRRL 6554 produced 84,400 ppb AFB_1 in the sclerotia and 135,000 ppb AFB_1 in the conidia. Their study showed that some *A. flavus* strains produced more aflatoxin concentration than *A. parasiticus*. Wilson and King (1995) found axenic *A. flavus* NRRL 5520 produced more AFB_1 than that of *A. parasiticus* NRRL 2999 in mycological broth after 10 days at 30°C.

Wei and Jong (1986) determined AFB_1, AFB_2, AFG_1 and AFG_2 concentrations of 169 strains of *A. flavus* group maintained in the American

Type Culture Collection (ATCC). They noted significant differences in toxin concentrations between the strains and on different substrates. Their data showed, for example, *A. flavus* strain 26946 produced 1213.5 μg g^{-1} AFB_1 on rice, 320.0 μg g^{-1} on peanuts and 7.6 μg g^{-1} on YES. On the other hand, *A. parasiticus* strain 15517 produced 42.9 μg g^{-1}on rice, 0.1 μg g^{-1} in peanuts and 1503 μg g^{-1} in YES. Their investigation also showed that *A. flavus* ATCC 24109 formed AFB_2 but AFB_1 was not found in this species. Some *A. flavus* strains such as ATCC 11498 and 11546 produced AFG_1 and AFG_2. Klich and Pitt (1988) also noted that only few *A. flavus* isolates produced AFG_1 but that all *A. parasiticus* produced AFG_1.

2.3.6 Inoculum size

Gonzales *et al.* (1995) inoculated 10^4 spores *A. flavus* into a range of volumes (1, 5, 10 and 25 μL). They found that colony diameters of *A. flavus* grown on medium a_w 0.90 with 5 and 10 μL inocula were not significantly different. They concluded that inoculum volume should be 5-10 μL. However, inoculum and colony size may not be related to aflatoxin production. Sharma *et al.* (1980) found that a reduction by 4 to 5 log cycles in the number of spores increased aflatoxin production two-fold. They found that when inoculated at 2.1 x 10^1 spores *A. parasiticus* produced 110 μg mL^{-1} total aflatoxins compared with 1.0 x 10^6 spores that yielded 50 μg mL^{-1} of total aflatoxin after 14 days at 28°C.

Karunaratne and Bullerman (1990) reported that at 10^1 to 10^7spores mL^{-1} *A. flavus* subsp. *parasiticus* NRRL 2999 produced variable aflatoxin concentrations. In their study, at 28°C high amounts of AFB_1 at about 380 μg g^{-1} and AFG_1 at about 100 μg g^{-1} were obtained from 10^3 spores mL^{-1}. At 35°C, 10^1spores mL^{-1} produced 62 μg g^{-1} AFB_1 and, surprisingly, inoculated at higher spore loads, the aflatoxin concentration in *A. flavus* was negligible. Similarly,

Ellis *et al.* (1993) found that under a modified atmosphere packaging and at an initial inoculum 10^4, *A. flavus* reached maximal growth (4.17 mg g^{-1}) and produced 0.708 μg g^{-1} AFB_1 at a_w 0.95. However at the same experimental conditions, an initial inoculum of 10^2 *A. flavus* conidia produced 2.401 μg g^{-1} AFB_1. These researchers also reported that an initial inoculum of 10^3 and at a_w 0.96, *A. flavus* produced 3.449 μg g^{-1} AFB_1. Comparisons between these studies are difficult as different strains were used. However, they do suggest that inocula should be standardized between experiments.

2.4 MICROBIAL DEGRADATION OF AFLATOXINS

Biological control involves elimination and/or biotransformation of the aflatoxins to less toxic or non-toxic derivative(s) or the prevention of growth of the fungi. Biological degradation is a form of biological control that is achieved through anti-toxigenic microorganisms breaking down aflatoxins or preventing their synthesis. A wide range of bacteria and molds are able to remove or degrade aflatoxins. Yeasts, actinomycetes and algae have been reported to be able to reduce the aflatoxin concentration of *A. flavus* (Smith and Harran, 1993; Gourama and Bullerman, 1997). This can occur by inhibition of aflatoxin production per se, or by inhibition of the growth of aflatoxin-producing strains or by accelerating degradation. Degradation of aflatoxins is affected by the producer strains or anti-aflatoxigenic species, culture age, number of viable cells, and also by environmental conditions e.g. pH and temperature.

2.4.1 Bacteria

Ciegler *et al.* (1966) reported that 3 x 10^{13} cells of *Flavobacterium aurantiacum* NRRL B-184 removed all AFB_1 (12 μg mL^{-1}) in milk after 2 hours. Line *et al.* (1994) found that chloroform-soluble [^{14}C] AFB_1 was converted to a water-soluble degradation product by live cells of *F. aurantiacum* NRRL B-184 after 72 hours. Line and Brackett (1995a) reported that the *F.*

aurantiacum at a concentration of 2.1x 10^8 cells mL^{-1} degraded 90% of 3.5 μg mL^{-1} AFB_1 in 72 hours. They noted that an older culture at high cell concentration (72 hours, 10^{10} CFU mL^{-1}) of *F. aurantiacum* was more efficient than a younger culture at lower concentration (24 hours, 10^9 CFU mL^{-1}). Later, they suggested that degradation of AFB_1 was a mineralization process (Line and Brackett, 1995b). D'Souza and Brackett (1998) reported that *F. aurantiacum* cells added with 1-10 mM Cu^{2+}, Mn^{2+} and Zn^{2+} decreased AFB_1 after 4-24 hours at 30°C. They noted that these trace metals ions are important cofactors in enzyme systems involved in AFB_1 degradation. Recently, D'Souza and Brackett (2000) used Mg^{2+}or Ca^{2+}, common natural activators of enzymes, in their study. They reported that cells of *F. aurantiacum* treated with 0.1-10 mM Mg^{2+} degraded AFB_1 about 14% after 48 hours, however, Ca^{2+} increased AFB_1 by 13% compared to the control cells. These authors found that Mg^{2+} is strongly bound by chelators: ethylene diamine tetra acetic (EDTA) and o-phenanthroline (OPT) and that it is possibly an important cofactor involved in AFB_1 degradation by the bacterium. They noted that Mg^{2+} is a stimulator of the pyruvate dehydrogenase system and that the reduction of AFB_1 by *F. aurantiacum* via a reductase-dehydrogenase system is possible. Smiley and Draughon (2000) found that DNase-I and proteinase-K added to a crude protein extract of *F. aurantiacum* reduced AFB_1 by 80% and 35%. They also noted that DNase-I added into extract of *F. aurantiacum* reduced AFB_1 greater than the non-treated extract. Both these later studies indicate that aflatoxin degradation may be enzymatic.

Lactic acid bacteria such as *Streptococcus lactis*, *Lactobacillus casei, L. acidophilus*, *L. bulgaricus* and *L. plantarum* were reported to be effective in reducing the amount of aflatoxin. Wiseman and Marth (1981) found *S. lactis* inhibited the growth and AFB_1 and AFG_1 production of *A. parasiticus* grown in a 7% glucose broth medium for 6 days at 28°C. Coallier-Ascah and Idziak (1985) noted that a 16-hour-old *S. lactis* at a concentration of 10^7 cells mL^{-1}

reduced AFB_1 and AFG_1 in *A. flavus* from 1.2 μg mL^{-1} to 0.1 μg mL^{-1} after 3 days. El-Gendy and Marth (1981) noted that the inhibitory effect of *L. casei* on aflatoxin was decreased in aged culture. They found that *L. casei* (10^5cells mL^{-1}) reduced AFB_1 by 92% in *A. parasiticus* after 3 days, 85% after 7 days and 48% after 10 days. Karunaratne *et al.* (1990) noted that inhibition of *A. parasiticus* growth and aflatoxin production by *L. acidophilus*, *L. bulgaricus* and *L. plantarum* was related to a combined effect of low pH and competitive growth by the bacteria. They found a 100 mL aliquot bacterial cell-free supernatant of 10^8 CFU mL^{-1} obtained from *L. acidophilus* reduced AFB_1 in *A. flavus* variously from 29.9 μg mL^{-1} to 8.0 μg mL^{-1} after 10 days at 28°C. Gourama and Bullerman (1995c, d) studied *Lactobacillus* sp (LAB 371, 371A and 251) and found that grown with these bacteria for 3 days, no AFB_1 and AFG_1 were detected in *A. parasiticus* (NRRL 2999). Treated with the bacterial cell-free supernatant, however, AFB_1 and AFG_1 were found at concentration 0.1-0.9 μg mL^{-1} in *A. parasiticus* after 6 days. In a later study, Gourama and Bullerman (1997) reported that growth of *A. flavus* and biosynthesis of aflatoxin B_1 and G_1 in liquid medium was inhibited by *Lactobacillus casei pseudoplantarum* 371. They found that the inhibitory activity of the cell-free supernatant of *L. casei pseudoplantarum* was sensitive to trypsin but not pepsin.

Weckbach and Marth (1977) reported that a dual culture of *Brevibacterium linens* and *A. parasiticus* on 20% sucrose (YES) broth medium after 10 days at 28°C, decreased AFB_1 and AFG_1 only about 12%. Faraj *et al.* (1993) reported that *Bacillus stearothermophilus* showed much greater inhibition of aflatoxin production at 40°C than at 30°C. In their study, a 10^3 mL^{-1} fresh suspension of *B. stearothermophilus* reduced total aflatoxins in *A. flavus* from 856.3 to 139.6 ng g^{-1} after 5 days on maize seeds a_w 0.95. These authors noted that at the elevated temperature the bacteria contributed to degradation of aflatoxin. Misaghi *et al.* (1995) reported that a 10^8 CFU mL^{-1} suspension of *Pseudomonas cepacia* D1 inhibited the growth of *A. flavus* (AF36). The mode

of *P. cepacia* action was not elucidated, however, these researchers noted that the bacterium exhibited antifungal activity against *A. flavus in-vitro*. Munimbazi and Bullerman (1998) extracted a metabolite of *B. pumilus* from dried fish 'Ndagala', a mixture of *Limnothrissa miodon* and *Stolothrissa tanganicae*, that was capable of inhibiting about 91% of aflatoxin production in *A. parasiticus*. They found that the metabolite of *B. pumilus* contained only one compound. These researchers described the inhibitor compound as being water-soluble, heat-stable, active over pH 2-10, resistant to various proteases and peptidases and had no fluorescent compound. They thought that the inhibitory metabolite was a cyclic polypeptide or non-peptide compound.

2.4.2 Fungi

Roy and Chourasia (1990) investigated *A. niger, A. nidulans, A. ochraceus, A. sydowii, Alternaria alternata, Chaetomium globosum, Curvularia lunata, Fusarium oxysporum, Penicillium citrinum* and *Trichoderma viride* activity against *A. flavus*. They reported among the fungi tested, only *A. niger* demonstrated 97% inhibition on growth of *A. flavus* with interaction type D (inhibition on contact with antagonist continuing to grow on the colony of inhibited organism). They also found that *A. niger* reduced AFB_1 to 0.09 μg mL^{-1} and AFG_1 to 0.10 μg mL^{-1} in *A. flavus*. Shantha *et al.* (1990) reported *A. niger* produced an inhibitor metabolite that degraded aflatoxin in *A. flavus* ATCC 46283. They noted that at 0.7 x 10^6 spores *A. niger* strain 1001 inhibited 99% of the aflatoxin in *A. flavus* inoculated at 3.5 x 10^6 spores in 100 mL Czapek Dox casein broth at 28°C for 7 days. They also, however, considered that gluconic acid produced by *A. niger* partially inhibited aflatoxin production. These authors also reported that under similar experimental conditions, *A. tamarii* completely inhibited growth of *A. flavus* and eliminated aflatoxins.

Aflatoxin degradation and interaction of the aflatoxin-producer *A. flavus* CMI 102566 with *A. niger, Rhizopus oryzae* and *Mucor racemosus* have also

been studied by Faraj *et al.* (1993). They found that *A. niger* and *R. oryzae* respectively reduced total aflatoxins to 1800 ng g^{-1} and 2100 ng g^{-1} in *A. flavus* from 2520 ng g^{-1} after 10 days. *M. racemosus* only caused very small reduction of about 2%. They noted that the aflatoxin concentration in *A. flavus* decreased by 20% from 5 to 10 days and attributed this to the endogenous degradative activity of *A. flavus* enzymes. They also reported that interaction of *A. niger* and *R. oryzae* with *A. flavus* caused mutual inhibition on contact between the colonies, indicating the presence of these two fungi directly degraded aflatoxin formation in *A. flavus*.

Weckbach and Marth (1977) reported that *R. nigricans* decreased AFB_1 to 1.0 μg mL^{-1} and AFG_1 to 2.0 μg mL^{-1} in *A. parasiticus* NRRL 2999 after 3 days as compared to 147.8 and 141.4 μg mL^{-1} respectively in axenic *A. parasiticus*. *R. nigricans* had a strong activity in reducing AFB_1 and AFG_1 up to 10 days at 28°C and it also reduced other yellowish pigments produced by *A. parasiticus*. Their study showed that over extended time, both mycelial dry weight and aflatoxin concentrations of *A. parasiticus* were reduced in the presence of *R. nigricans*. These authors concluded that *R. nigricans* out competed *A. parasiticus* and inhibited aflatoxin formation. Nout (1989) studied the ability of *Rhizopus* and *Neurospora* spp metabolites to inhibit growth and AFB_1 formation in *A. parasiticus* and *A. flavus*. It was found that *R. oryzae* N581, obtained from Indonesian 'tempe' (a fermented soybean food), eliminated AFB_1 in *A. flavus* NRRL 5906, *A. parasiticus* NRRL 2999 and *A. parasiticus* ATCC 15517, when grown on shredded groundnut substrate with a_w 0.98 for 6 weeks at 30°C. Another isolate, *Neurospora* N429 from Indonesian 'oncom' (a mixture of fermented soybean and coconut food) also demonstrated AFB_1 elimination in the above three aflatoxin-producers. Nout (1989) noted that both *R. oryzae* and the *Neurospora* caused destruction of mycelia and prevented sporulation of the *Aspergillus*. Nout concluded that heat-stable compounds in *R. oryzae* 581 and *Neurospora* N429 inhibited synthesis of AFB_1 in *A. flavus*.

Aflatoxin is also degraded by some strains of *A. parasiticus* and *A. flavus*. Hamid and Smith (1987) showed that a 10-day-old culture of intact mycelium and a cell-free extract of *A. flavus* degraded more AFB_1 and AFG_1 within *A. flavus* after 48 hours than 6 or 12 day-old preparations. Intact mycelium of 10-day-old *A. flavus* decreased AFB_1 and AFG_1 by 23-24% but the cell-free extract only reduced AFB_1 and AFG_1 17-19%. They also noted that endogenous degradation of aflatoxin in *A. flavus* occurred at 12 days. Hyunh and Lloyd (1984) studied aflatoxin degradation in *A. parasiticus* and found that a 16-day-old mycelial extract of *A. parasiticus* reduced AFB_1 from 49 to 37 μg mL^{-1} after 72 hours. A series of investigations by Doyle and Marth (1978a, b and c) found that after 96 hours, 9-day-old unheated and fragmented mycelia of *A. parasiticus* NRRL 2999 optimally degraded AFB_1 and AFG_1 at 28°C, pH 5-6.5. They concluded that aflatoxin degradation in *A. parasiticus* involved enzymes released by the fungus into medium. Cotty and Bayman (1993) reported that *A. flavus* and *A. parasiticus* partially degraded AFB_1 after biosynthesis of the toxin stopped. Their investigation also found an atoxigenic strain of *A. flavus* (AF36) and its mutants AF3niaD and AF36nirA that suppressed aflatoxin production in toxigenic *A. flavus* strain (AF13). They noted that the atoxigenic strain competed for nutrients required for aflatoxin biosynthesis. Further, Cotty and Bhatnagar (1994) reported that the atoxigenic strain of *A. flavus* (AF36) prevented aflatoxin biosynthesis in toxigenic *A. flavus* via enzymatic reactions. Cleveland *et al.* (1987) also reported that an aflatoxin-non producing mutant *A. parasiticus* (avn-1) blocked two enzyme activities that were involved in conversion of sterigmatocystin to O-methylsterigmatocystin, a precursor of AFB_1 synthesis, and of O-methylsterigmatocystin to AFB_1.

2.4.3 Yeasts

When both *Saccharomyces cerevisiae* and *A. parasiticus* NRRL 2999 were inoculated at 10^7 spores mL^{-1} *S. cerevisiae* into YES broth at 28°C, no

growth of *A. parasiticus* was observed after 10 days (Weckbach and Marth, 1977). These researchers also reported that after 5 days, *S. cerevisiae* at a concentration 10^3 spores mL^{-1} decreased AFB_1 and AFG_1 in *A. parasiticus* by less than 15 and 25% respectively. Paster *et al.* (1993), who studied *Pichia guilliermondii* (formerly identified as *Debaryomyces hansenii*) at the concentration of 10^7-10^9cells mL^{-1}, found that the yeast inhibited fungal sporulation of *A. flavus* during 16 days of storage. They noted that the ability of *P. guilliermondii* to act as a biocontrol agent on aflatoxins could be the result of a high concentration of inoculum that prevented aflatoxin production for at least 7 days in high moisture conditions i.e. competitive exclusion.

Hua *et al.* (1999) used a *nor* mutant of *A. flavus* Papa 827 (a mutation in the gene coding for norsolorinic acid reductase that blocks aflatoxin biosynthesis) to interact with saprophytic yeasts. Among six yeasts tested, *P. anomala* demonstrated the greatest effect on aflatoxin reduction. *P. anomala* WRL-076 was applied to a petri dish of PDA 4 hours prior to *A. flavus.* They reported that *P. anomala* markedly decreased norsolorinic acid to 4 μg/5 discs and AFB_1 to 60 μg/4 discs, compared to the control of 112 μg/5 discs and 4800 μg/4 discs, respectively. The unit reported by these researchers was a total of 4-5 agar discs of 7 mm diameter that were transferred into 10 mL solvent. Hua *et al.* (1999) also noted that the diffusible metabolites produced by the yeast mediated the inhibitory effect directly against aflatoxin production without significantly affecting growth.

2.4.4 Conversion of aflatoxins by microorganisms

Interaction of other microorganisms with aflatoxigenic fungi could transform aflatoxins into less toxic compounds. In the 1960s, an unknown bright blue fluorescent substance other than aflatoxin was detected in pure AFB_1 treated with 22 x 10^6 cells of *Tetrahymena pyriformis* (Teunisson and

Robertson, 1967). The substance had a Rf value of 0.52, lower than AFB_1 at 0.59 and AFB_2 at 0.55 on TLC plates and its fluorescent intensity was one-half of the AFB_1. Robertson *et al.* (1970) reported that *T. pyriformis* reduced the cyclopentane ring (C=O) of AFB_1 to a C-OH. They also noted that the similarity of the UV and mass spectra of the compound with AFB_1 indicated the two compounds were identical. Based on an analogous biological system, Detroy and Hesseltine (1968) employed a known steroid-hydroxylating fungus, *Dactylium dendroides* NRRL 2575, in YES medium containing 10-30 mg crystalline AFB_1. They found a new hydroxylated blue-fluorescent compound that was less toxic than AFB_1 by the duckling histopathological assay. In their later studies using *D. dendroides, Absidia repen* and *Mucor griseo-cyanus*, the Rf value of the compound was reported to be 0.57, lower than that of AFB_1 at 0.69 (Detroy and Hesseltine, 1969). It was assigned as an aflatoxicol or aflatoxin R_o (Detroy and Hesseltine, 1970).

Buchanan and Houston (1982) found that *A. parasiticus* NRRL 2999 grown on peptone-mineral salt medium after 9-12 days accumulated a blue-fluorescent material with Rf values similar to AFB_1 and AFB_2 on the TLC plate. Another study by Betina (1984) noted that aflatoxin production commences around the second day of incubation and approaches maximum production at about 7 days. Thereafter, degradation and interconversion of the aflatoxin took place and AFB_1 and/or AFG_1 begin to diminish, whereas other types of aflatoxins such as AFB_{2a} and AFG_{2a} increased. Nout (1989) also reported that elimination of AFB_1 in *A. parasiticus* and *A. flavus* by fungal inhibitor species *R. oryzae* 581 and *Neurospora* N429 was accompanied by formation of other fluorescent compounds with higher Rf values than AFB_1. On the other hand, Nakazato *et al.* (1990) investigated interconversion of AFB_1 and aflatoxicols by *A. niger, Eurotium herbariorum, Rhizopus* sp. and non-toxigenic *A. flavus*. In their study, the presence of these four fungi reversibly converted AFB_1 to aflatoxicols, and AFB_{2a} was also found in an experiment with *A. niger.*

2.4.5 Other factors supporting microbial degradation of aflatoxins

Some researchers have found that microorganisms secreted compounds into the medium that caused aflatoxin inhibition (Coallier-Ascah and Idziak, 1985; Nout, 1989; Shantha *et al.*, 1990; Munimbazi and Bullerman, 1998). Microbial decomposition of organic compounds in liquid media is mediated not only by the availability of the chemicals to the organism or the enzyme system that can degrade it, but also the quantity and activity level of the enzyme systems or microorganisms. (Doyle and Marth, 1978b) found that intracellular fungal enzymes liberated following autolysis of the mycelia could degrade aflatoxins. They reported that degradation of aflatoxin by mycelia *A. parasiticus* was pH dependent, and the amount of aflatoxin degradation was dependent upon the initial concentration of the toxin (Doyle and Marth, 1987a). They also noted that AFG_1 was degraded more rapidly than AFB_1. Doyle and Marth (1978d) reported that biodegradation of aflatoxin was also strain-dependent. They found *A. parasiticus* NRRL 2999 endogenously degraded more aflatoxin than *A. flavus* NRRL 3353, 3315 or *A. parasiticus* NRRL 3000. These authors also reported that *A. parasiticus* grown on glucose salt medium degraded more aflatoxin than on YES broth, potato dextrose broth or Czapek Dox broth (Doyle and Marth, 1978d).

Faraj *et al.* (1993) found the filamentous fungi such as *A. niger* and *Rhizopus oryzae* degraded 80% and 70% of the total amount of aflatoxins at a 40°C after 5 days incubation. They noted that in single axenic culture of *A. flavus*, the degradation of aflatoxin was dependent on the age of the culture and incubation temperature. At 30°C, maximum aflatoxin production occurred at 5 days and afterwards there was 20% decrease in total aflatoxins. When the single cultures were incubated at 40°C, aflatoxin reduction was doubled for a further 5 days incubation. Therefore, the degradation of aflatoxins also occurred at temperatures permitting aflatoxin synthesis.

CHAPTER 3

GENERAL METHODS OF ANALYSIS

3.1 FUNGI AND MEDIA

One strain of each of the following fungi *Aspergillus parasiticus* Speare (FRR 2999), *Aspergillus flavus* Link (FRR 3251), non-toxigenic strain *Aspergillus flavus* Link (FRR 4279), *Eurotium rubrum* Jos. König *et al.* (FRR 2776), *Aspergillus wentii* Wehmer (FRR 3042), *Polypaecilum pisce* Hocking and Pitt (FRR 2606) and *Debaromyces hansenii* (Zopf) Lodder and Kreger (FRR 2577) was used in this study. All the fungi used in these studies were obtained from Division of Food Science and Technology, CSIRO North Ryde, NSW, Australia. *P. pisce* (Pitt and Hocking, 1985), *A. wentii* and *E. rubrum* were originally isolated from dried fish in Indonesia by Wheeler *et al.* (1986). *D. hansenii* was isolated from small salted fish from Japan.

Transferring and sub-culturing the fungi were performed inside a biohazard Class II chamber, Gelman BH2000. Before and after using the chamber, 70% ethanol was applied to the bench and the UV light was turned on for disinfection. Glassware was autoclaved before reuse, and disposable petri dishes were autoclaved before disposal.

Fungal culture stocks on Czapek Yeast Extract Agar (CYA) slants were kept at 5°C. Fungi were sub-cultured on CYA petri dishes and incubated at 25°C. CYA medium was made following the recipe of Hocking *et al.* (1994). The fungal inocula were grown for 10 days until well sporulated. The spores were harvested by washing the plates with 50 mL sterile phosphate buffer solution (PBS) with 0.05% Tween 80. The buffer was poured onto the plates and the spores were loosened gently with a sterile inoculating loop. The spore suspensions were shaken gently and transferred to a sterile flask. Spores were counted using an improved Neubauer BS748 haemocytometer under bright field microscopy. Dilutions were made in PBS and all spore suspensions were placed in sterile McCartney bottles at 5°C until used. The volume of spore suspension

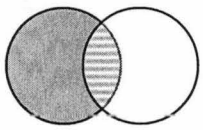
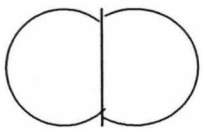
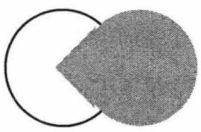
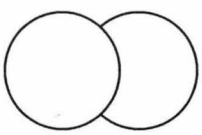
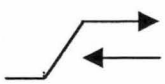
spotted onto the agar plates was 10 μL. Using a dispenser, the volume of agar poured into a petri dish in experimental samples was 15 mL.

Other media used in this study were Czapek Yeast Extract Agar with 20% Sucrose (CY20S), Malt Extract Agar (MEA), Malt extract Yeast extract 5% salt 12% glucose (MY5-12%), 5% salt and 20% glucose (MY5-20%) and the 10% salt 12% glucose (MY10-12%). Agar was omitted in the ingredients when making broth media. All the media were sterilized at 121°C for 15 minutes. The recipes of the media were taken from Pitt and Hocking (1997).

3.2 ASSESSMENT OF FUNGAL GROWTH

Fungal growth was measured as colony diameter (in millimeters) and the average of diameters of the colony was computed from two measurements at right angles with each other. The average colony diameter was taken from three replicates. The interaction between *A. parasiticus* or *A. flavus* with the other non-toxic fungi was assessed by the classification scheme of Wheeler and Hocking (1993), a modified model of Magan and Lacey (1984), as described in Table 3-01.

Table 3-01. Assessment of fungal interaction system (by Wheeler and Hocking, 1993 modified from Magan and Lacey, 1984)

Numerical value	Reaction type	Classification description	
0		No growth or only microcolony formation, not competitive	
1	A	Mutual intermingling growth, where both fungi grow into each other without any microscopic signs of interaction	
2	B	(i) Mutual inhibition on contact or space between colonies small (<2 mm)	
	C	(ii) Inhibition of one species on contact, the inhibited species continues to grow at a significantly reduced rate while the inhibitor species grows at slightly reduced rate or unchanged	
3	D	Mutual inhibition at a distance (>2 mm)	
4	E	Inhibition of one species on contact, the inhibitor species continuing to grow at a reduced rate through the inhibited colony	
5	F	Inhibition of one species on contact or at a distance, the inhibitor species then continuing to grow at an unchanged rate through or over the inhibited colony	

3.3 STANDARD AFLATOXINS

One milligram aflatoxin standards (B_1, B_2, G_1 and G_2) obtained from Sigma-Pty Ltd were dissolved into 10mL benzene:acetonitrile (9:1). The standards were kept in the original bottles and sealed in the transport can, and stored at 5°C until used. These four standards were prepared in several concentrations for thin layer chromatography (TLC) and fluorescence spectrophotometry. The aflatoxin standards are abbreviated as AFB_1, AFB_2, AFG_1 and AFG_2 throughout the studies. Handling of the aflatoxin standards and aflatoxin containing samples was done following official method of analysis of AOAC (1984).

For TLC analysis of samples, all standards were prepared as a mixture of AFB_1 and AFG_1 at 10 μg mL^{-1} and AFB_2 and AFG_2 at 3 μg mL^{-1} to compare their fluorescence intensities to the samples on the TLC plate. Standards AFB_2 and AFG_2 were prepared at lower concentrations because they had strong fluorescence. Spotted onto the TLC plates in 2, 5 and 10 μL volumes, the standards contained:

(a) Standards AFB_1 and AFG_1:

2 μL spot = 2 μL x 10 μg mL^{-1} = 0.02 μg

5 μL spot = 5 μL x 10 μg mL^{-1} = 0.05 μg

10 μL spot = 10 μL x 10 μg mL^{-1} = 0.1 μg

(b) Standards AFB_2 and AFG_2:

2 μL spot = 2 μL x 3 μg mL^{-1} = 0.006 μg

5 μL spot = 5 μL x 3 μg mL^{-1} = 0.015 μg

10 μL spot = 10 μL x 3 μg mL^{-1} = 0.03 μg

3.4 AFLATOXIN ANALYSIS

Methods to analyse aflatoxin have been developed since aflatoxins were discovered in the 1960s. Of the chromatographic techniques applied to

mycotoxins, TLC is by far the most widely used in the detection, analysis and characterization of fungal toxins (Betina, 1985; Nesheim and Trucksess, 1986). The use of TLC for separation and analysis of complex mixtures grew rapidly following Stahl's standardized procedure (Nesheim and Trucksess, 1986). Generally, the protocol of TLC analysis requires several steps of extraction and clean up of the sample, solvent systems, detection and qualitative analysis, then further quantitative analysis (Betina, 1985; Ellis *et al.*, 1991). Dorner (1996) described TLC as a thin layer of silica gel or other adsorbent (alumina or reverse phase) coated onto a glass plate and activated by drying. Microlitre quantities of cleaned-up extract and standards are applied in a horizontal line near one edge of the plate. This edge of the plate is placed in a tank containing the solvent system that migrates through the sorbent layer and separates the components of the sample. Mycotoxins are visualized as fluorescent spots or the TLC plates can be sprayed with or exposed to various reagents to affect a chemical change in the mycotoxin that makes it visible. Semi quantification is achieved by comparison of the intensity of the fluorescence or color of the sample spots with those of a series of standards.

Using silica-gel coated TLC, Trucksess and Stoloff (1984) reported the detection limit of AFB_1 was 0.05 ng g^{-1}. Of poultry feed samples, Trucksess *et al.* (1990) detected 2-11 ng g^{-1} aflatoxins on silica-gel 60 coated TLC. Dorner (1996) reported that a minicolumn chromatography was sensitive to 5-10 μg kg^{-1}. Aflatoxin quantification can also be performed by spectrodensitometry, fluorometry and spectrofluorometer (Dickens *et al.*, 1980; Tsai *et al.*, 1984; Lillehoj *et al.*, 1986; Cotty, 1988b; Gabal *et al.*, 1994) or high performance liquid chromatography (Nakazato *et al.*, 1990; Horn *et al.*, 1996; Paterson *et al.*, 1997; Horn *et al.*, 2000).

Dickens *et al.* (1980) reported that limit quantification of densitometer was 0.9-5.1 ng for AFB_1 and AFG_1 and 0.27-1.53 ng for AFB_2 and AFG_2. Beebe (1978) reported that a reverse phase HPLC was reliable to 0.15-2 ng for

AFB_1 and AFG_1 and 0.03-0.4 ng for AFB_2 and AFG_2. The limit of quantification of aflatoxin using HPLC is 0.5 ng for AFB_1 and AFG_1 and 0.15 ng for AFB_2 and AFG_2 (Horn *et al.*, 1996), and 2-10 ng g^{-1} for all aflatoxins (Papp *et al.*, 1999). Candlish *et al.* (1991) determined total aflatoxins using an immunoaffinity column chromatography incorporated with Aflatest^R system unit, a commercial aflatoxin kit, for rapid aflatoxin detection (<1 hour). On the other hand, Yates and Porter (1982) used bacterial bioluminescence of *Photobacterium phosporeum* as a bioassay for AFB_1 and they reported that the concentration detected was in a range of 10-100 μg mL^{-1}.

Direct and indirect competitive methods of enzyme linked-immuno sorbent assay (ELISA) and radio immuno assay (RIA) are also used to detect aflatoxins (Chu, 1983). The sensitivity of ELISA method was 10 pg mL^{-1} for AFB_1 (Lawellin *et al.*, 1977), 0.2-10 ng mL^{-1} for AFB_1 (Candlish *et al.*, 1985), 0.5-25 ng g^{-1} for AFB_1 (Ram *et al.*, 1986), and 0.025 ng mL^{-1} for AFB_1 (Aldao *et al.*, 1995). Pestka and Chu (1984) reported that response ranges ELISA for AFB_1 were 1.0-50 ng mL^{-1} and for aflatoxin M_1 (AFM_1), a derivative of the parent molecule AFB_1, were 0.05-0.50 ng mL^{-1}. Using ELISA, AFM_1 was detected at 10-25 pg mL^{-1} (Hu *et al.*, 1984), and 10-50 ng kg^{-1} (Sanimtong and Tanbook-Ek, 1991). Chu (1983) noted that the sensitivity of RIA for AFB_1 was 0.5-5.0 ng and for AFM_1 was 5-50 ng. However, it was dependent upon the purity of aflatoxin extract from samples.

3.4.1 Thin Layer Chromatography (TLC)

(1) Sample extraction and clean-up processes

All glassware was soaked in 2% sodium hypochlorite (NaOCl) for several hours, then soaked in 2% Pyroneg overnight and rinsed thoroughly with water then autoclaved. The agar and fungal culture in each plate were

macerated evenly with 20 mL chloroform in blender bags, and the mixture was filtered through No.1 Whatman paper into a clean flask.

The filtrate was cleaned-up in a butt column packed with glass wool and Na_2SO_4. The flask was washed with 10 mL chloroform and the wash was poured into the column. The solution was collected in a clean conical flask. The column was rinsed with 20 mL chloroform and the wash was also collected into the flask. All the solution was rotary evaporated to dryness using a rotary evaporator Heidolph VV-WB2000 at 60°C. The final extract was dissolved in 1mL chloroform:methanol (1:1) solution and kept sealed in an amber glass vial for thin layer chromatography analysis.

(2) Spotting on TLC plates

Filter paper was placed around the inside of cylindrical TLC tank and saturated with the solvent, chloroform:acetone (88:12) for one hour or until the paper was wet (AOAC, 1984). The water was omitted from the solvent. Using a Hamilton 8800 syringe, 5 μL samples were spotted onto the TLC plate (10 x 20 cm, silica gel 60 pre-coated, layer thickness 250 μm, particle size 2-25 μm, Merck). Between sample spotting, the syringe was rinsed thrice with a solution of chloroform:methanol (1:1).

Standard aflatoxins (10 μg mL^{-1} for AFB_1 and AFG_1, and 3 μg mL^{-1} for AFB_2 and AFG_2) were spotted at volumes of 2, 5 and 10 μL on the plate, together with sample extracts. The plate was developed in the tank for 50-60 minutes, or up to when the solvent reached the top part of the plate. The plate was then dried for 15 minutes inside a fume hood and examined under a UV lamp (Camag Universal TL 900/μ) at 354 nm. All the fluorescence spots were marked and compared to the standards based on their color and Rf values on TLC plate.

(3) Semi quantitative analysis of aflatoxins

Under the UV light, AFB_1 and AFB_2 have blue fluorescence and AFG_1 and AFG_2 have green fluorescence. Among these four aflatoxins, AFB_1 has the highest Rf value followed by AFB_2, AFG_1 and AFG_2. Mean Rf values obtained from 10 TLC plates are shown in Table 3-02. The chromatograpic mobility pattern or Rf value of aflatoxin on TLC plate was calculated from the ratio of:

$$\text{Rf value} = \frac{\text{Distance migration of standard/sample}}{\text{Distance migration of solvent}}$$

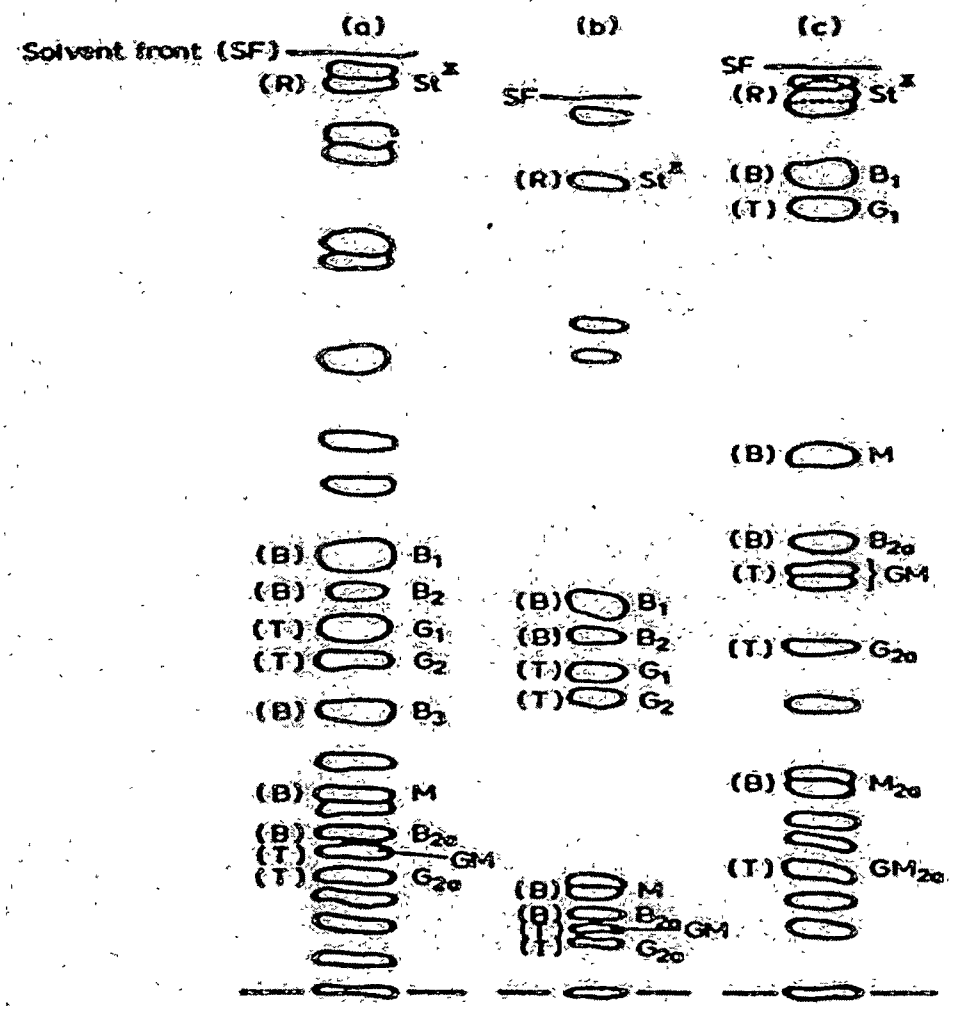
Table 3-02. Mean Rf values of aflatoxin standards obtained from TLC (n=10)

TLC plate	Rf value of aflatoxin standards			
	AFB_1	AFB_2	AFG_1	AFG_2
1	0.485	0.428	0.388	0.342
2	0.491	0.434	0.400	0.342
3	0.414	0.365	0.329	0.280
4	0.468	0.425	0.362	0.331
5	0.558	0.505	0.458	0.400
6	0.587	0.521	0.472	0.406
7	0.592	0.537	0.481	0.425
8	0.515	0.460	0.412	0.369
9	0.518	0.445	0.403	0.361
10	0.462	0.441	0.371	0.320
Mean	**0.509**	**0.453**	**0.408**	**0.358**
SE	**0.005**	**0.005**	**0.004**	**0.004**

Developed in various solvents, the Rf values of AFB_1, AFB_2, AFG_1 and AFG_2 were also different (Table 3-03). Therefore, it is important to describe the type and proportion of the solvents used. In the present study, a higher proportion of acetone was used in the solvent to produce Rf values of the aflatoxins in the range 0.3 – 0.5. Nesheim and Trucksess (1986) noted that a

change in Rf values decreases or increases the separation between components in a chromatograpic mixture, but does not alter their relative position. Figure 3-01 shows relative position of aflatoxins developed at different solvents in TLC analysis described by Heathcote (1984). Thus, the Rf values were used to identify aflatoxins in experimental samples.

Figure 3-01. Thin layer chromatography of aflatoxins*



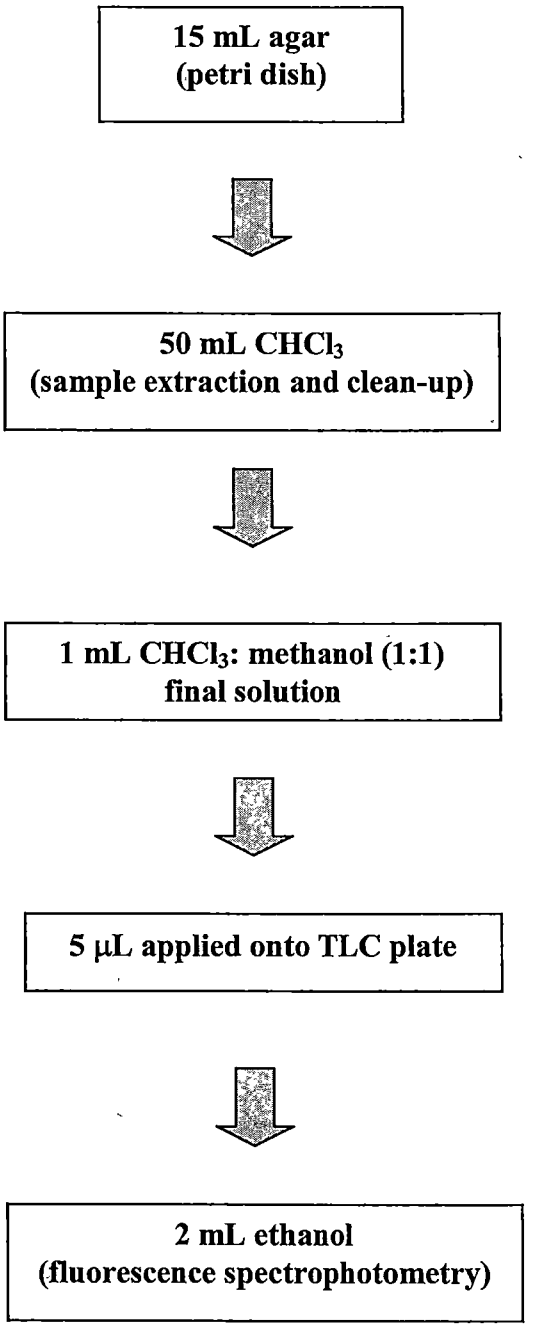
*From: Heathcote (1984).
Note: Obtained from an extract of *A. flavus* on silicaR TLC-7G plates. Solvent systems: (a) benzene-ethanol-water (46:35:19), (b) chloroform-methanol (49:1), (c) chloroform-methanol (94:6). Left column letters (fluorescence): B= blue, T = turquoise (greenish-blue), R = red. Right column letters: B_1 = AFB_1, B_2 = AFB_2, G_1 = AFG_1, G_2 = AFG_2, M =AFM, B_2a = AFB_2a, G_2a = AFG_2a, G_M = AFG_M, GM_2a = $AFG_{M2}a$, M_2a = AFM_2a, St = sterigmatocystin.

Table 3-03. Rf values of the present study and some other references

Solvent	Rf values				Reference
	AFB_1	AFB_2	AFG_1	AFG_2	
$CHCl_3$:acetone (88:12)	0.50	0.45	0.40	0.35	Present study
$CHCl_3$:acetone(9:1)	0.27	N/A	N/A	N/A	Gorst-Allman and Steyn (1979)
$CHCl_3$:acetone:ammonia (90:10:0.25)	0.33	0.29	0.23	0.20	Betina (1985)
$CHCl_3$:acetone:hexane (85:15:20)	0.48	0.43	0.36	0.30	Betina (1985)
Toluene:ethyl acetate:formic acid (6:3:1)	0.29	N/A	0.24	0.20	Jonsyn and Lahai (1992)
$CHCl_3$:acetone (9:1)	0.56	0.53	0.48	0.46	Huynh and Lloyd (1984)
$CHCl_3$:acetone (93:7)	0.33	0.31	0.29	0.27	Cole and Cox, 1981
$CHCl_3$:methanol (9:2)	0.40	0.35	0.34	0.31	Heathcote (1984)

Note: N/A = data not available

The concentrations of aflatoxins on TLC plates were visually assessed by comparing the fluorescence intensity of experimental spots to the standards. A preliminary study of predicting aflatoxin concentration in *A. parasiticus* (FRR 2999) grown on YES3% medium, is shown in Figure 3-02. The concentrations shown were obtained from 5 µL samples spotted onto TLC plates. There is a close agreement between the predicted values and the concentrations obtained from fluorescence spectrophotometry (FS). Figure 3-03 shows AFB_1 concentration of *A. parasiticus* (FRR 2999) grown axenically and with other fungi as predicted from their fluorescence on TLC plates and measured using fluorescence spectrophotometry. The total amount of aflatoxin concentration ($\mu g\ mL^{-1}$) was calculated from its initial sample volume multiplied by 26.7. This factor was derived from the following process:



Therefore, mass in 2 mL = X µg mL^{-1} x 2 mL

= 2X µg

= 2X µg /5 µL (from 5 µL spotted onto TLC)

Total mass extracted = 2X µg/5 x 1000

= 400X µg

Total mass extracted = 2X μg/5 x 1000

= 400X μg

However, the total agar volume was 15 mL:

= 400X μg/15 mL

= 26.7X μg mL^{-1}

To estimate recovery, 400 μL of AFB_2 at 10 μg mL^{-1} was dissolved in 10 mL broth medium. After extraction and TLC (as above), the fluorescence intensity (see section 3.4.3) was 21.65, which is equal to 0.02 μg mL^{-1}. As the fluorescence was estimated in a volume of 2 mL, the total mass was 4 μg. Therefore the recovery was 100%.

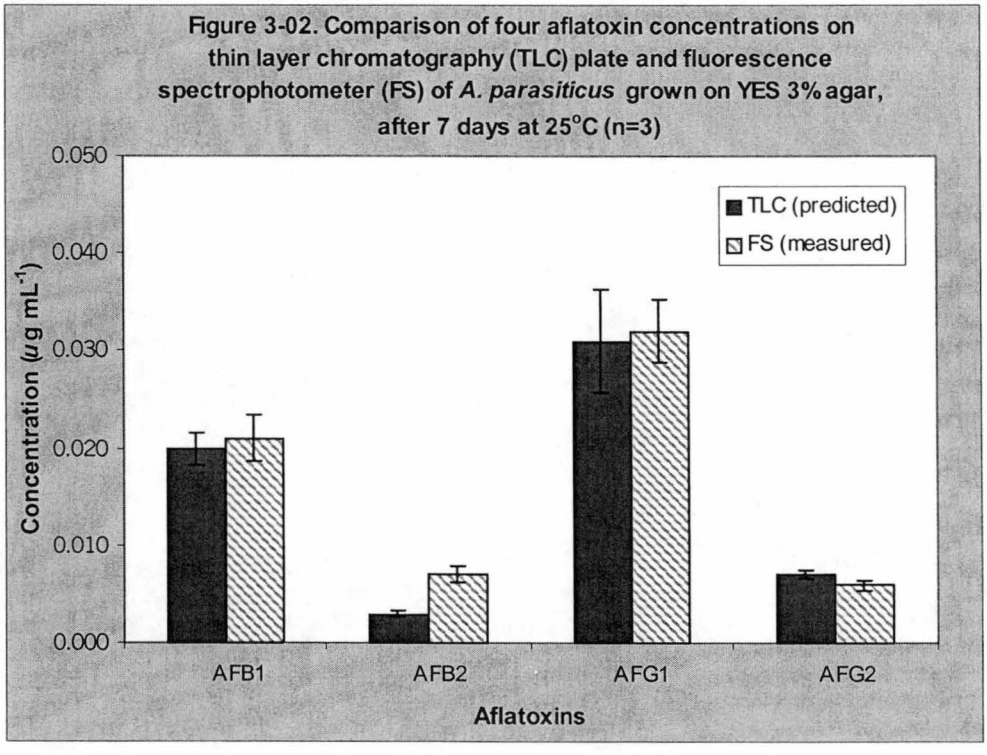
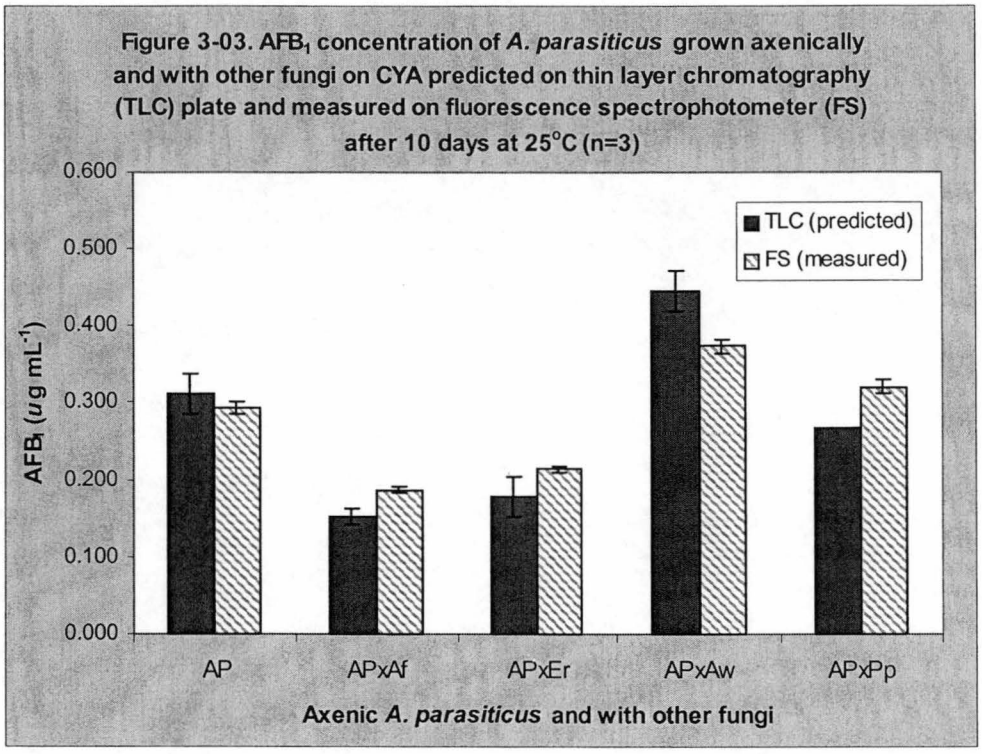


Figure 3-02. Comparison of four aflatoxin concentrations on thin layer chromatography (TLC) plate and fluorescence spectrophotometer (FS) of *A. parasiticus* grown on YES 3% agar, after 7 days at 25°C (n=3)



Note: AP = *A. parasiticus*; Af = non-toxigenic *A. flavus*; Er = *E. rubrum*; Aw = *A. wentii*; Pp = *P.pisce.*

(4) *Quantitative analysis of aflatoxins*

The aflatoxin concentration of each fluorescence spot on TLC plates was quantified using a scanning fluorometer (Flur-VIS auto scanner, Helena Lab.) and a fluorescence spectrophotometer Hitachi F-2000. Using the Flur-VIS scanner, fluorescence spots on TLC plates were screened directly with the UV light and the aflatoxin concentration related to fluorescence. Using the fluorescence spectrophotometer, the spots were scraped off the plate and dissolved in 2 mL ethanol and mixed with a vortex for 1 minute. This solution was settled for 1 hour, then filtered with No.1 Whatman paper. The solution was then analyzed for its fluorescence intensity and the total concentration of each aflatoxin was calculated.

3.4.2 Fluorescence-VIS Scanner

Measuring aflatoxin concentration with the Flur-VIS auto scanner (Helena Lab.) gave inconsistent results on the chromatogram peaks. An aflatoxin spot screened with the scanner gave a very wide difference in fluorescence when scanned for several times. This was probably caused by the very narrow hole of UV-light passing the entire spot on TLC. A series of known aflatoxin standards in different concentrations was also scanned to produce standard curves. The results, however, did not accurately match the standard concentrations spotted on TLC plates. Thus, experiments were performed using only fluorescence spectrophotometry.

3.4.3 Fluorescence Spectrophotometer

A Hitachi F-2000 fluorescence spectrophotometer with a xenon lamp was used throughout the studies. It was set at an excitation wavelength of 360 nm and emission wavelength of 450 nm for aflatoxin analysis. The excitation and emission wavelengths of fluorescence detector used were within the range of 365 nm and 455 nm reported by Paterson *et al.* (1997) respectively, 365 nm and 440 nm by Dorner *et al.* (1998), and 330 nm and 460 nm by Papp *et al.* (1999). Standard curves were also prepared for each aflatoxin standard. The mean fluorescence of four aflatoxin standards at different concentrations obtained from three replicates is shown in the Tables 3-04 and 3-05. Blank ethanol without aflatoxin was used as zero concentration. Standard curves of AFB_1, AFB_2, AFG_1 and AFG_2 are shown in Figures 3-04, 3-05, 3-06 and 3-07.

Table 3-04. Fluorescence of AFB_1 and AFB_2 standards obtained from fluorescence spectrophotometer (n=3)

Concentration ($\mu g\ mL^{-1}$)	Fluorescence (mean±SE)	
	AFB_1	AFB_2
0.000	3.156± 0.026	3.413 ± 0.187
0.010	7.386 ± 0.027	15.62 ± 0.881
0.025	20.73 ± 2.067	26.82 ± 1.927
0.050	39.65 ± 1.408	50.74 ± 2.901
0.075	59.03 ± 2.572	65.16 ± 3.305
0.100	86.35 ± 2.047	85.50 ± 2.565
0.125	114.0 ± 2.282	109.7 ± 3.754
0.150	134.4 ± 1.123	131.3 ± 1.691
0.175	159.7 ± 3.401	147.9 ± 1.014
0.200	172.2 ± 3.328	171.6 ± 3.579

Table 3-05. Fluorescence of AFG_1 and AFG_2 standards obtained from fluorescence spectrophotometer (n=3)

AFG_1		AFG_2	
Concentration ($\mu g\ mL^{-1}$)	Fluorescence (mean ± SE)	Concentration ($\mu g\ mL^{-1}$)	Fluorescence (mean ± SE)
0.000	3.156 ± 0.009	0.000	3.210 ± 0.398
0.012	17.25 ± 0.794	0.010	35.84 ± 1.970
0.025	46.33 ± 2.970	0.015	52.66 ± 1.056
0.050	90.31 ± 2.898	0.020	77.07 ± 1.675
0.075	150.7 ± 0.804	0.025	94.59 ± 1.513
0.100	209.4 ± 1.811	0.030	112.8 ± 3.162
0.125	258.7 ± 4.623	0.035	140.4 ± 1.880
0.150	311.9 ± 0.956	0.040	168.3 ± 4.161
0.175	369.9 ± 1.885	0.045	184.7 ± 2.291
0.200	423.2 ± 4.418	0.050	207.8 ± 1.794

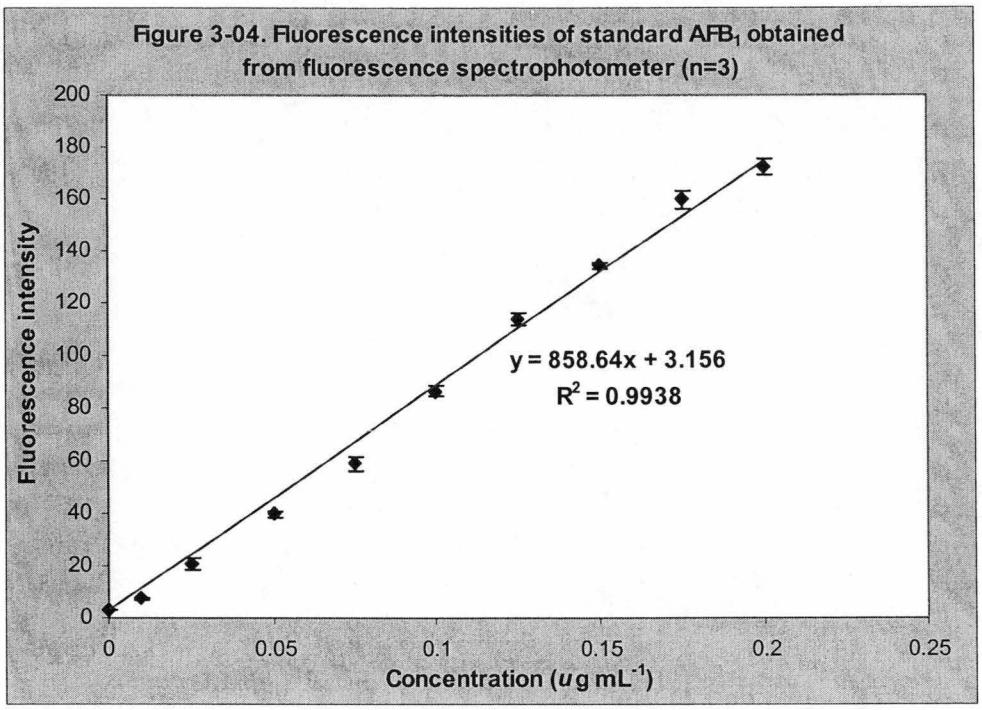


Figure 3-04. Fluorescence intensities of standard AFB1 obtained from fluorescence spectrophotometer (n=3)
Fluorescence intensity
Concentration (ug mL-1)
y = 858.64x + 3.156
R2 = 0.9938
0
20
40
60
80
100
120
140
160
180
200
0
0.05
0.1
0.15
0.2
0.25

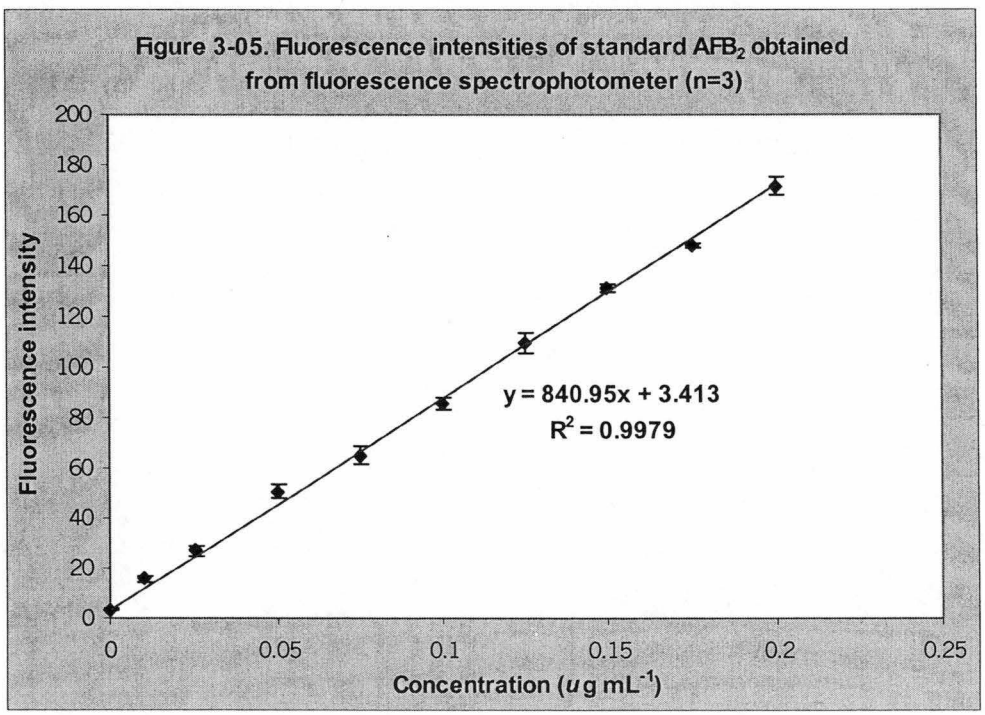


Figure 3-05. Fluorescence intensities of standard AFB2 obtained from fluorescence spectrophotometer (n=3)
Fluorescence intensity
Concentration (ug mL-1)
y = 840.95x + 3.413
R2 = 0.9979
0
20
40
60
80
100
120
140
160
180
200
0
0.05
0.1
0.15
0.2
0.25

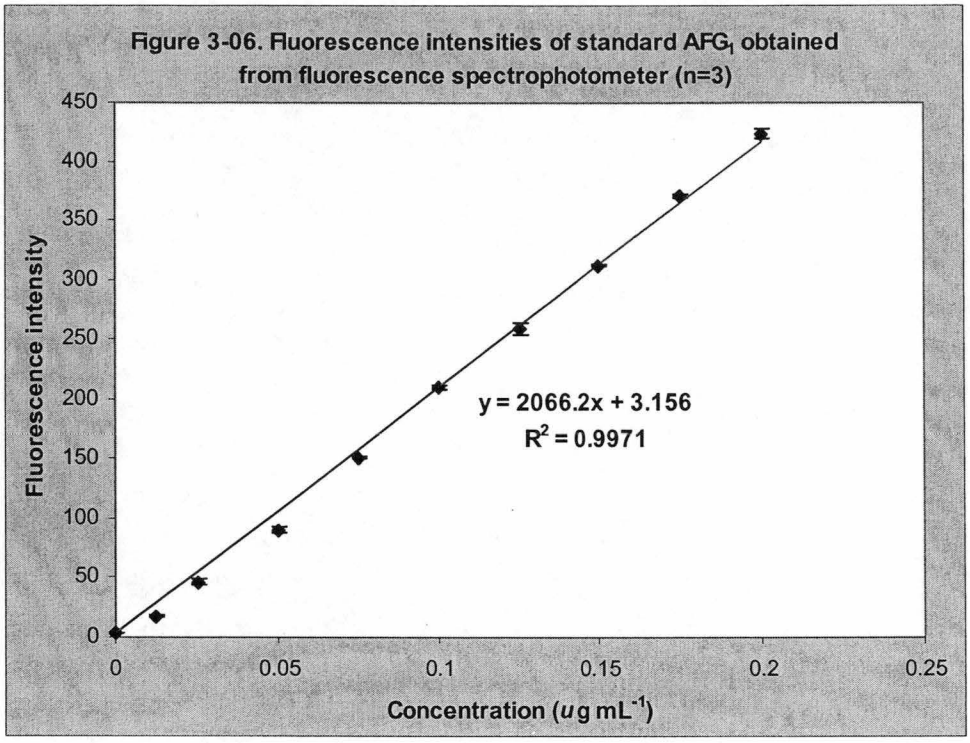


Figure 3-06. Fluorescence intensities of standard AFG1 obtained from fluorescence spectrophotometer (n=3)
Fluorescence intensity
450
400
350
300
250
200
150
100
50
0
0
0.05
0.1
0.15
0.2
0.25
Concentration (ug mL-1)
y = 2066.2x + 3.156
R2 = 0.9971

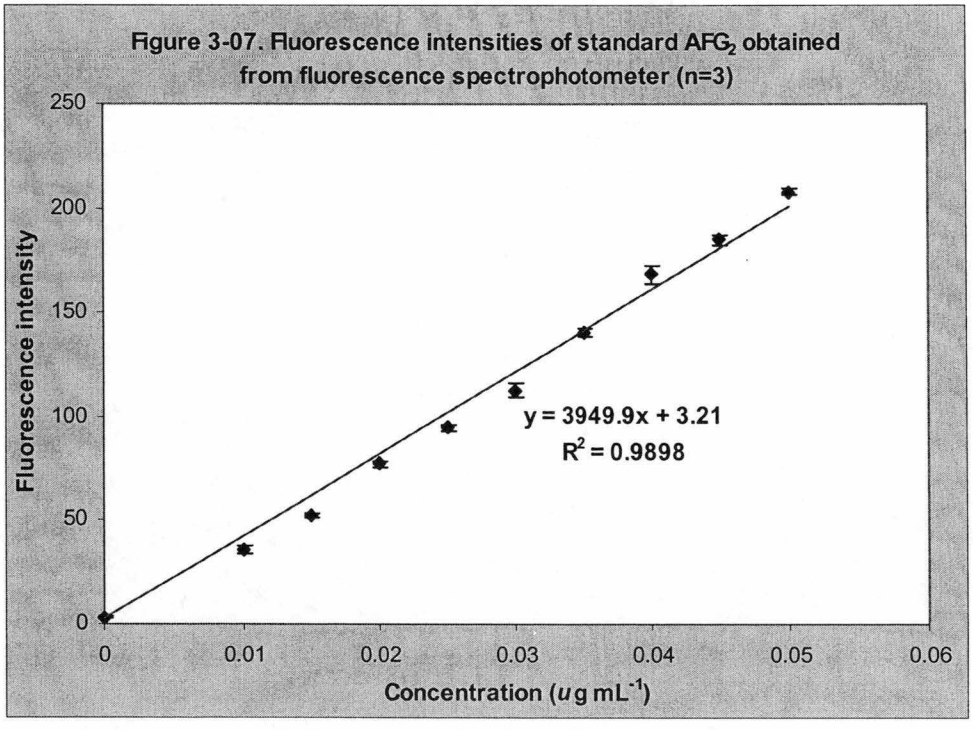


Figure 3-07. Fluorescence intensities of standard AFG2 obtained from fluorescence spectrophotometer (n=3)
Fluorescence intensity
250
200
150
100
50
0
0
0.01
0.02
0.03
0.04
0.05
0.06
Concentration (ug mL-1)
y = 3949.9x + 3.21
R2 = 0.9898

3.5 WATER ACTIVITY (a_w) MEASUREMENT

A simple method of water activity measurement with a hygroscopic detector liquid was used. The liquid detector solution was obtained from Food Science Australia, North Ryde, NSW. Dilution of standard solution of the hygroscopic detector liquid (a_w 0.75) was made to provide several initial a_w solutions. Each initial a_w solution was made five times and a_w was measured using a refractometer (Leica Brix 50, type 7531L). The values (in °Brix) read from the refractometer were converted using an a_w nomogram table (Dr. R. Steele, personal communication). Table 3-06 shows mean and standard error of a_w measurement of the hygroscopic detector liquid.

One or two drops of the detector liquid were placed on a small weighing dish and put on top of the agar medium in petri dish. The petri dish was sealed using parafilm and then wrapped tightly in a blender bag and kept overnight to equilibrate at 25°C. After 24 hours, a drop of the detector liquid in the weighing dish inside the petri dish, was removed using a sterile pipette and put on the refractometer to measure the a_w value. In the experiment using dried fish, the detector liquid on the weighing dish was placed near the fish and put inside the petri dish. The detector liquid was placed at one day before measurement to enable 24 hours equilibration.

Table 3-06. Measurement of a_w values of hygroscopic detector liquid

Replicate	Initial a_w solution				
	0.75	0.85	0.87	0.90	0.95
1	0.75	0.85	0.86	0.90	0.95
2	0.74	0.84	0.87	0.90	0.94
3	0.75	0.84	0.87	0.90	0.95
4	0.75	0.84	0.97	0.90	0.94
5	0.75	0.85	0.86	0.89	0.94
Mean	**0.748**	**0.845**	**0.866**	**0.898**	**0.944**
SE	0.001	0.001	0.001	0.001	0.001

3.6 STATISTICAL ANALYSIS

All the experiments were designed as full factorial treatments and statistically analyzed using SPSS 8.0 software. A general linear model of analysis of variance (ANOVA) was used. Homogeneity of variance was checked by examination of residual plots. Some experimental results of growth and/or aflatoxin concentrations were transformed to either the square root or the logarithm for ANOVA. Multiple comparisons of unique combinations of subsets in the experiments were performed by Tukey's HSD, when the interactions of the parameters in the ANOVA showed significant differences ($p \leq 0.05$). Bars in figures of Tukey's test indicated standard error (SE) of the replicate mean.

CHAPTER 4

GROWTH AND AFLATOXIN PRODUCTION OF *Aspergillus parasiticus* AND *Aspergillus flavus* AT HIGH WATER ACTIVITY (a_w) IN THE PRESENCE OF OTHER FUNGI

4.1 INTRODUCTION

Water activity (a_w), temperature, spore loads and the presence of other microbes affecting growth and aflatoxin production of *Aspergillus parasiticus* Speare and *A. flavus* Link, have been widely studied in both natural and synthetic substrates (Sharma *et al.*, 1980; Park and Bullerman, 1983; Karunaratne and Bullerman, 1990; Karunaratne *et al.*, 1990; Faraj *et al.*, 1993; Gqaleni *et al.*, 1997). A_w is an important parameter on fungal growth and aflatoxin production. Gibson *et al.* (1994) reported that the optimum a_w for growth of four *Aspergillus* Section *Flavi* species (*A. flavus*, *A. oryzae*, *A. parasiticus* and *A. nomius*) ranged from 0.985- 0.993 at 30°C. An increasing a_w enhanced aflatoxin production in *A. flavus* (Cuero *et al.*, 1987). They found that *A. flavus* at a_w 0.98 produced aflatoxins (1020 ng g^{-1}) than at a_w 0.95 (989 ng g^{-1}) at 25°C. Gqaleni *et al.* (1997) found in *A. flavus* grown on CYA after 15 days at 30°C, aflatoxin was 0.306 μg mL^{-1} at a_w 0.996 and 0.226 μg mL^{-1}at a_w 0.95.

Drying and/or salting is a means of reducing a_w, thereby decreasing growth and toxin production of *A. parasiticus* and *A. flavus*. Wheeler *et al.* (1986) examined fungi growing on Indonesian salted dried fish. They did not detect any aflatoxins in several fish infected with low levels of *A. flavus*. However, if fish are only dried without prior salting, then aflatoxin contamination may be a problem, especially if the fish are rehydrated to some extent by the high relative humidity of the region. An experiment by Doe and Heruwati (1988) on drying and storage of tropical fish in Indonesia reported unsalted dried fish still had a_w close to 1.0 after 4 days. These authors noted the a_w of the dried fish, being unsalted, was affected by climatic conditions. Cvetnić and Pepeljnjak (1995) isolated members of the *Aspergillus* section *Flavi* group from 75 of 420 smoke-dried meat (cured ham, dried bacon, smoked dried ham) samples in Croatia. Of these isolates, 8 were aflatoxigenic fungi and produced

AFB_1 from 0.1 to 240 mg kg^{-1} on shredded wheat (moisture content 40% w/w) after 14 days at 25°C.

Many microorganisms have been reported as being able to inhibit aflatoxin production by *A. parasiticus* and *A. flavus* (Faraj *et al.*, 1993; Gourama and Bullerman, 1995b,c; Hua *et al.*, 1999). In the present study, contaminant fungi isolated from Indonesian salted dried fish: *Polypaecilum pisce* Hocking and Pitt, *Eurotium rubrum* Jos. König *et al.* and *A. wentii* Wehmer (Pitt and Hocking, 1985; Wheeler *et al.*, 1986; Wheeler and Hocking, 1993) and a non-toxigenic strain of *A. flavus* were used to investigate their interactions with aflatoxin-producers *A. parasiticus* and *A. flavus*. The objective of this study was to determine the influence of spore load and interactions with four other fungi on the growth and aflatoxin B_1 (AFB_1) and G_1 (AFG_1) production of *A. parasiticus* and *A. flavus* at high values of a_w that could occur during rehydration of unsalted dried fish.

4.2 MATERIALS AND METHODS

4.2.1 General methods

The fungi, fungal interaction assessment, aflatoxin and statistical analysis used in this study were as described in Chapter 3. Spore suspensions of 10^4, 10^5 and 10^6 spores mL^{-1} were made. The colony diameter of each fungal culture was recorded daily until day 7 and then on days 10 and 20. The medium was CYA (a_w 0.99) (Hocking *et al.*, 1994) and temperature of incubation was 25°C. Filtrate solutions of axenic cultures of the three fungal commensals of dried fish and non-toxigenic *A. flavus* were also analysed by TLC.

4.2.2 Experimental design

This study was a full factorial design in three replicates with the treatments: length of incubation, culture and spore load. Cultures of *A.*

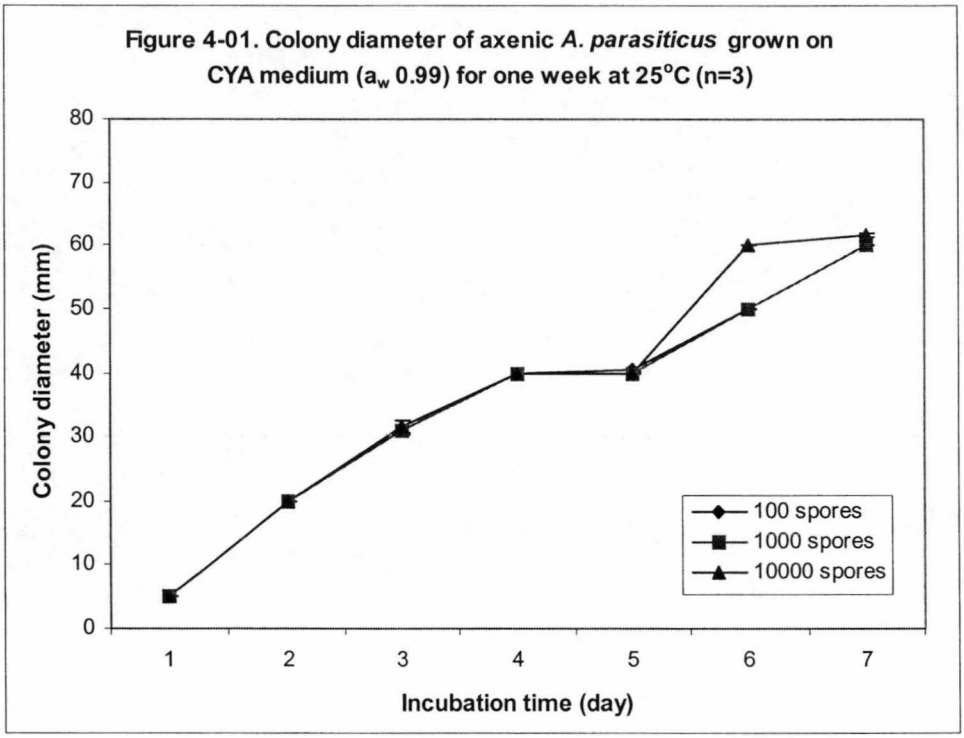
parasiticus and *A. flavus* alone or combined with either non-toxigenic *A. flavus*, *E. rubrum P. pisce*, or *A. wentii* were inoculated as 10 µL of one of the three spore concentrations to give spore loads of 100, 1,000 or 10,000 spores per plate. Axenic cultures of each fungus were used as controls.

4.3 RESULTS

4.3.1 Colony diameter of *A. parasiticus* and *A. flavus*

At 25°C with an initial a_w of 0.99 on CYA medium, *A. parasiticus* and both the toxigenic *A. flavus* and non-toxigenic strains grew rapidly, but *A. wentii*, *E. rubrum* and *P. pisce* grew slowly. Distinct and separate circular colonies of *A. parasiticus* or *A. flavus* against non-toxigenic *A. flavus, A. wentii* and *E. rubrum* were observed at 3 days incubation. However, the colony of *P. pisce* was half covered by the colonies of *A. parasiticus* and *A. flavus*.

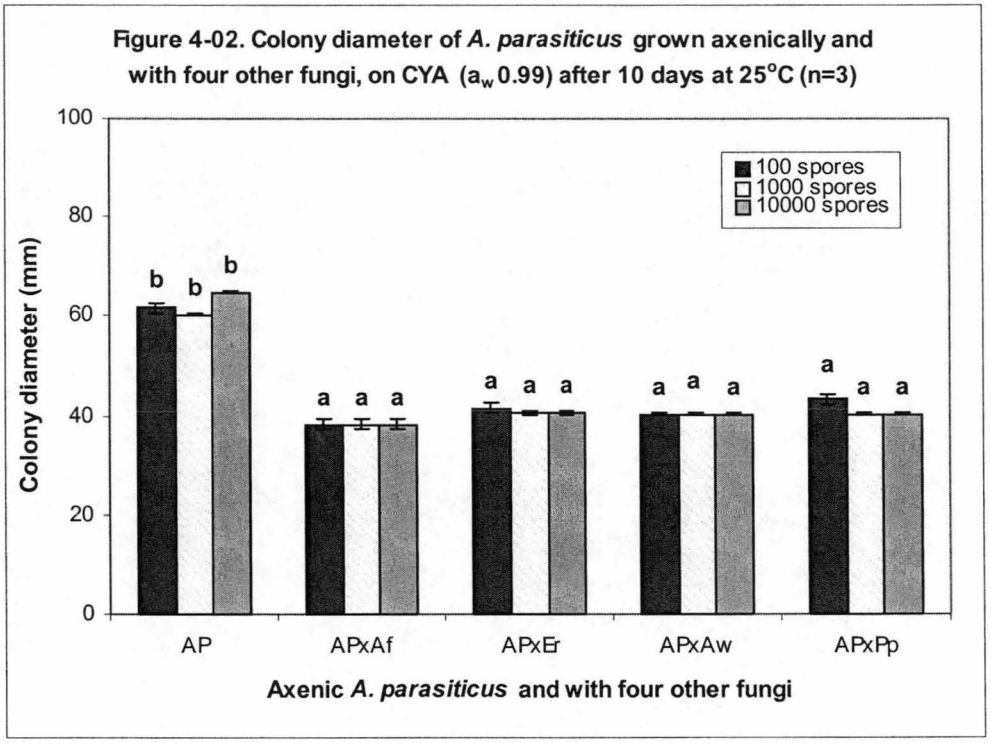
Figure 4-01 shows colony diameter of axenic *A. parasiticus* over one week. At all inocula, colony diameter of axenic *A. parasiticus* was similar and the stationary phase was reached after 7 days. The colony diameter of *A. parasiticus* was significantly reduced ($p \leq 0.05$) when in the presence of other fungi after 10 and 20 days incubation at 25°C (Table 4-01). The level of spores inoculated for each fungus did not affect the diameter of *A. parasiticus* and the combination of three factors was not significant. Therefore, Tukey's test was performed only on the colony diameter of *A. parasiticus* for the cultures at each incubation day. Figure 4-02 shows that colony diameter of *A. parasiticus* in the presence of other fungi at day 10, was about 34% smaller than the axenic *A. parasiticus*. The reduction of colony diameter of *A. parasiticus* caused by all other fungi was significant. At day 20, while the inhibition by non-toxigenic *A. flavus* decreased the colony diameter of *A. parasiticus* by 43%, the inhibition caused by *E. rubrum*, *A. wentii* and *P. pisce* was only about 15% (Figure 4-03).



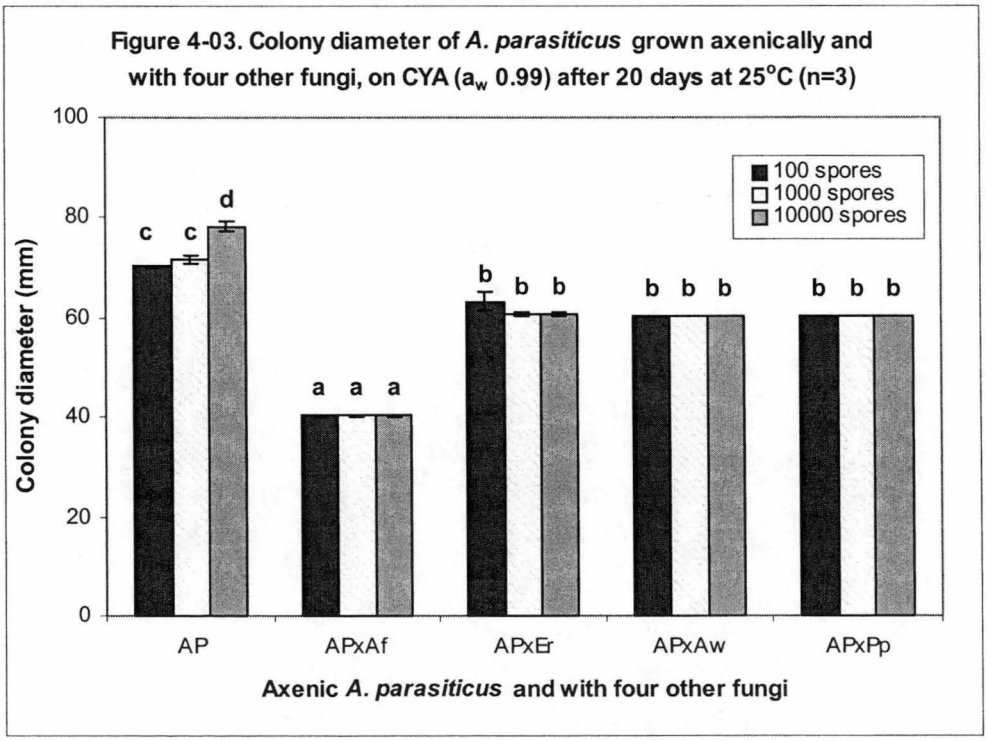
Bars = mean ± SE.

Table 4-01. ANOVA of colony diameter of *A. parasiticus* when grown axenically and with other fungi on CYA (a_w 0.99) at 10 and 20 days at 25°C (n=3)

Source	Type III sum of squares	df	Mean square	F	P
Corrected model	13568.456	29	467.878	126.463	0.000
Intercept	242632.544	1	242632.544	65576.363	0.000
Days (incubation)	4766.944	1	4766.944	1288.363	0.000
FRR (culture)	7483.844	4	1870.961	505.665	0.000
Level (spore loads)	18.422	2	9.211	2.489	0.091
Day*FRR	1143.667	4	285.917	77.275	0.000
Day*Level	6.156	2	3.078	0.832	0.440
FRR*Level	125.022	8	15.628	4.224	0.000
Day*FRR*Level	24.400	8	3.050	0.824	0.586
Error	222.000	60	3.700		
Total	256423.000	90			
Corrected total	13790.000	89			



Note: AP = *A. parasiticus*; Af = non-toxigenic *A. flavus*; Er = *E. rubrum*; Aw = *A. wentii*; Pp = *P. pisce*; superscripts are compared within the figure.



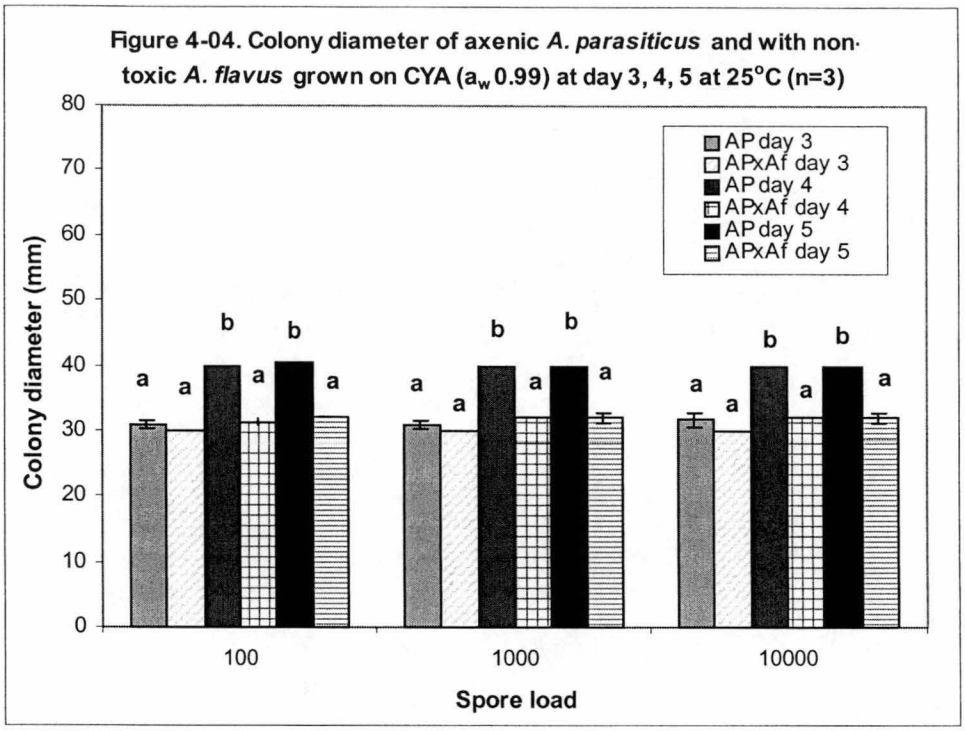
Note: AP = *A. parasiticus*; Af = non-toxigenic *A. flavus*; Er = *E. rubrum;* Aw = *A. wentii*; Pp = *P. pisce*; superscripts are compared within the figure.

Repeated measurements and analysis of the growth of *A. parasiticus* alone and with non-toxigenic *A. flavus* over 3, 4 and 5 days indicated that only time and the presence of *A. flavus* significantly affected the colony diameter of *A. parasiticus* (Table 4-02) but the spore load did not. From Figure 4-04, it can be seen that, before the fourth day, non-toxigenic *A. flavus* did not significantly affect the colony diameter of *A. parasiticus*.

Table 4-02. Univariate split-plot* repeated measures ANOVA of the colony diameter of *A. parasiticus* alone and in the presence of non-toxigenic *A. flavus* after 3, 4 and 5 days on CYA (a_w 0.99) at 25°C (n=3)

Source	Type III sum of squares	df	Mean square	F	P
Time[a]	345.148	1.163	296.815	188.263	0.000
Time*Culture[b]	144.704	1.163	124.440	78.929	0.000
Time*Level	0.741	2.326	0.319	0.202	0.849
Time*Culture*Level[c]	0.741	2.326	0.319	0.202	0.849
Error (Time)	22	13.954	1.577		

[a]Time is day 3, 4 or 5.
[b]Culture is *A. parasiticus* axenic or with non-toxigenic *A. flavus*.
[c]Level is the spore load of the inocula (10^2, 10^3, 10^4).
*The Greenhouse-Geisser adjustment of degrees of freedom was used.



Note: AP = *A. parasiticus*; Af = non-toxigenic *A. flavus*.
Superscripts are compared within the figure.

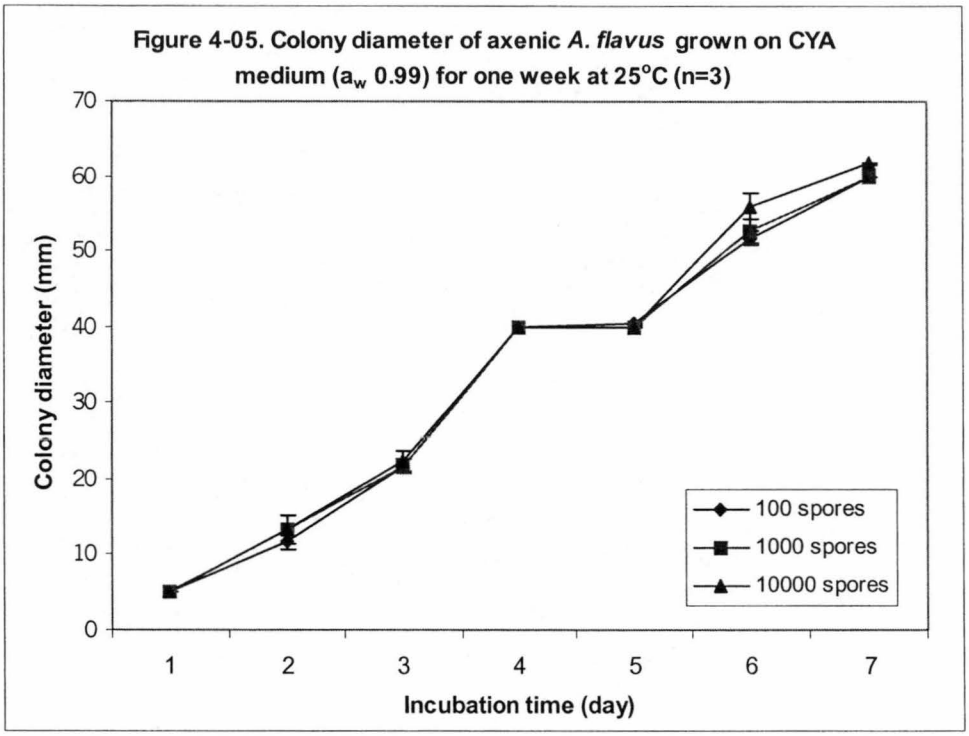
Table 4-03. Colony diameter of the four fungi when grown axenically and with *A. parasiticus* on CYA (a_w 0.99) at 10 and 20 days at 25°C.

Cultures	Mean colony diameter (mm) ± SE, n=3					
	10^4 mL^{-1} spores		10^5 mL^{-1} spores		10^6 mL^{-1} spores	
	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20
Non-toxigenic *A. flavus*	60±0.6	76±5.8	61±1.2	80±1.2	61±1.2	80±0.6
A.flavus (nt*)x *A.parasiticus*	38±2.9	40±0.6	38±2.9	40±1.2	38±2.9	40±1.2
A.wentii	41±1.2	60±1.2	45±0.6	60±1.2	49±1.8	67±4.6
A.wentii x *A.parasiticus*	27±5.8	29±1.2	22±2.9	25±0.6	22±2.9	25±0.6
E. rubrum	41±1.2	59±1.2	42±5.8	69±1.2	43±5.8	69±1.2
E.rubrum x *A.parasiticus*	20±0.6	32±2.9	22±2.9	30±1.2	21±1.2	26±1.8
P.pisce	24±1.6	32±2.9	24±1.2	40±0.6	27±2.9	41±1.2
P.pisce x *A.parasiticus*	23±1.7	29±1.2	25±0.6	30±1.2	25±0.6	31±1.2

Note: * nt = non-toxigenic.

The colony diameters of the other fungal cultures were also reduced in the presence of *A. parasiticus* when compared to axenic cultures (Table 4-03). After 20 days, the hyphae of axenic cultures of non-toxigenic *A. flavus* and *A. wentii* fully covered the agar plate. *E. rubrum* almost covered the agar plate, but *P. pisce* was restricted to the center of plate.

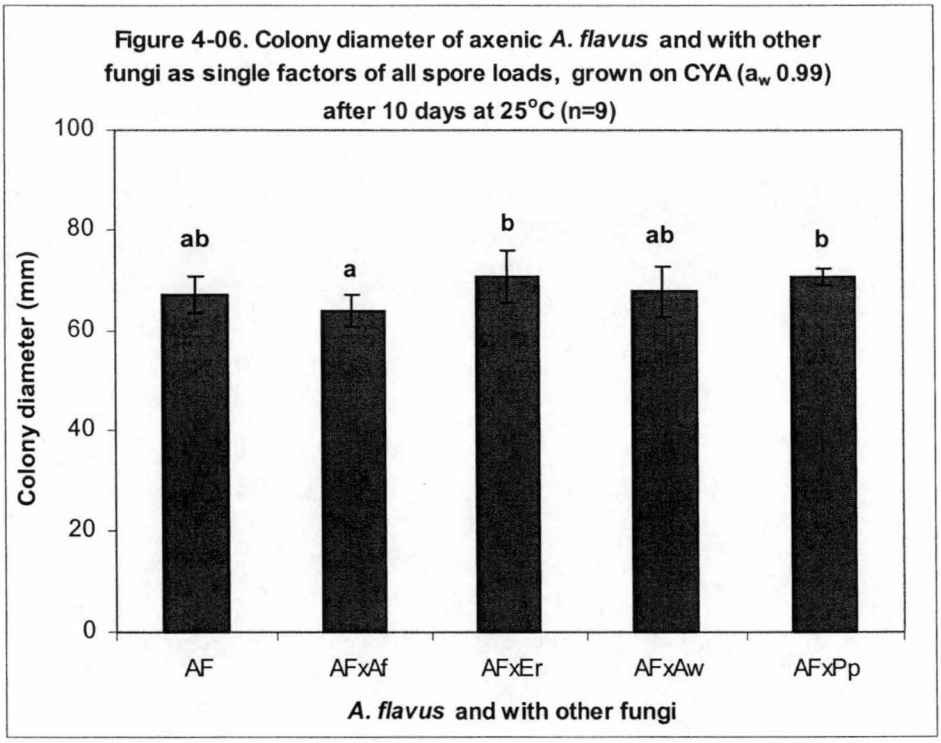
After one week, colony diameter of axenic *A. flavus* grown on CYA at a_w 0.99 was almost similar at all inocula. Stationary phase of axenic *A. flavus* growth occurred between 7-10 days (Figure 4-05). Table 4-04 shows only the single factors of day of incubation and the presence of the four fungi significantly affected the growth of aflatoxin producer *A. flavus* ($p \leq 0.05$). The spore load levels and all combinations of treatments did not affect significantly the growth of axenic *A. flavus* incubated at 25°C. Therefore, Tukey's test was performed separately for the cultures as a single factor of treatments at each day of incubation. Among the four other fungi, only the presence of non-toxigenic *A. flavus* reduced the colony diameter of *A. flavus*, however, the reduction was significant ($p \leq 0.05$) only after 20 days. Overall, none of the fish commensal fungi (Figure 4-06 and 4-07) significantly affected the growth of *A. flavus* when compared to axenic *A. flavus*.



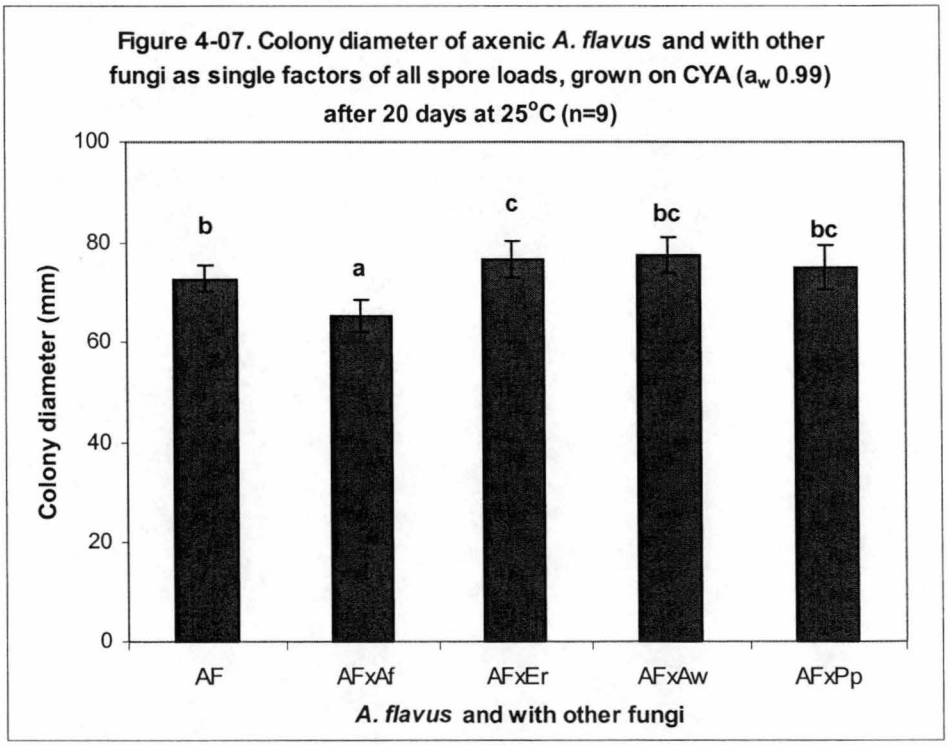
Bars = mean ± SE

Table 4-04. ANOVA of colony diameter of *A. flavus* when grown axenically and with other fungi at 10 and 20 days on CYA (a_w 0.99) at 25°C (n=3)

Source	Type III sum of squares	df	Mean square	F	P
Corrected model	2049.156	29	70.661	4.612	0.000
Intercept	450005.511	1	450005.511	29369.468	0.000
Days (incubation)	640.000	1	640.000	41.769	0.000
FRR (culture)	988.822	4	247.206	16.134	0.000
Level (spore loads)	11.356	2	5.678	0.371	0.692
Day*FRR	153.222	4	38.306	2.500	0.052
Day*Level	10.067	2	5.033	0.328	0.721
FRR*Level	181.644	8	22.706	1.482	0.183
Day*FRR*Level	64.044	8	8.006	0.522	0.835
Error	919.333	60	15.322		
Total	452974.000	90			
Corrected total	2968.489	89			



Note: AF = *A. flavus;* Af = non-toxigenic *A. flavus;* Er = *E.rubrum;* Aw = *A. wentii;* Pp = *P. pisce*; superscripts are compared within the figure.



Note: AF = *A. flavus*; Af = non-toxigenic *A. flavus*; Er = *E. rubrum;* Aw = *A. wentii;* Pp = *P. pisce*; superscripts are compared within the figure.

Unlike the colony diameters of the fish commensal fungi that were reduced when grown with *A. flavus*, the colony diameter of non-toxigenic *A. flavus* was greater than its axenic culture for the lowest spore level (Table 4-05).

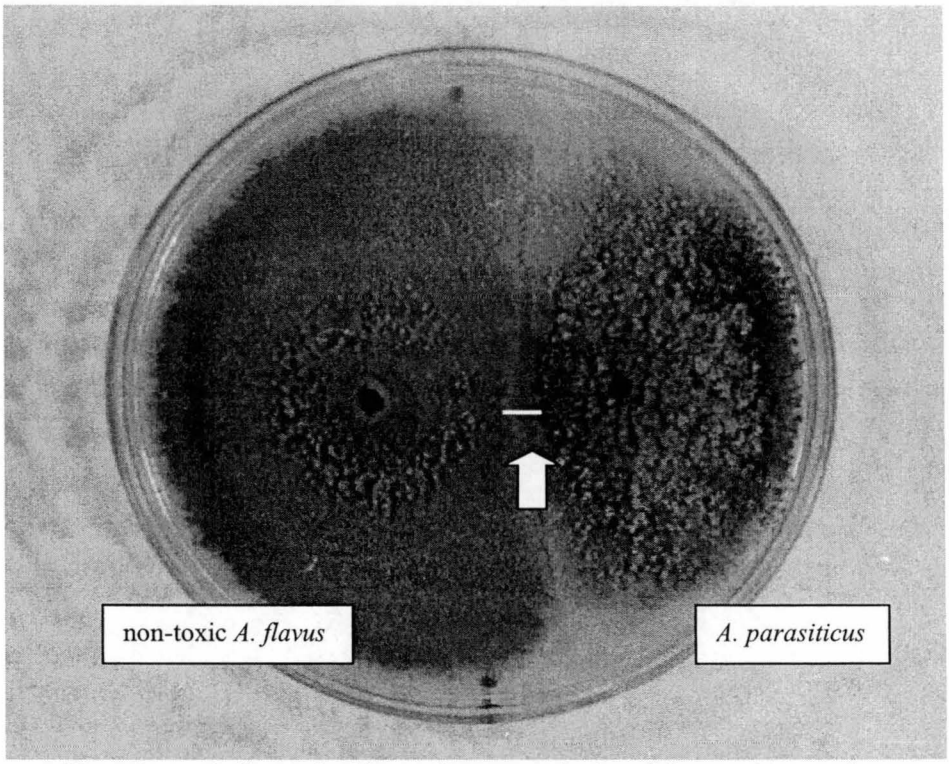
Table 4-05. Colony diameter of the four other fungi when grown axenically and with *flavus* on CYA (a_w 0.99) at 10 and 20 days at 25°C

Cultures	Mean colony diameter (mm)± SE, n=3					
	10^4 mL^{-1} spores		10^5 mL^{-1} spores		10^6 mL^{-1} spores	
	Day 10	Day 20	Day 10	Day 20	Day 10	Day 20
Non-toxic *A. flavus*	61± 5.8	68 ± 2.9	71 ± 2.9	71 ± 2.9	71 ± 2.9	71 ± 2.9
A. flavus (nt) x *A. flavus*	75± 5.0	76 ± 2.9	70 ± 0.0	71 ± 5.8	73 ± 2.6	75 ± 0.0
E. rubrum	32± 3.5	55 ± 5.0	31 ± 1.2	55 ± 5.0	34 ± 2.3	51 ± 2.9
E. rubrum x *A. flavus*	21± 4.2	22 ± 4.0	24 ± 1.2	28 ± 2.0	25 ± 3.0	30 ± 5.1
A. wentii	32± 4.6	54 ± 4.6	34 ± 3.5	56 ± 2.9	39 ± 1.2	60 ± 0.0
A. wentii x *A. flavus*	28± 2.3	30 ± 1.2	25 ± 3.8	31 ± 2.9	26 ± 3.6	31 ± 3.6
P. pisce	13± 1.7	14 ± 3.5	14 ± 1.7	16 ± 1.7	16 ± 1.7	18 ± 1.2
P. pisce x *A. flavus*	11± 1.2	14 ± 3.5	13 ± 1.7	14 ± 3.5	12 ± 2.5	12 ± 2.5

4.3.2 Fungal interaction

Two types of fungal interactions were observed when *A. parasiticus* was grown with the four other fungi. The first type was a mutual inhibition at a distance (> 2 mm), which is classified as reaction type D and was demonstrated by *A. flavus* (as shown in Figure 4-08), *A. wentii* and *E. rubrum*. The second type of interaction was type E, observed on the plate of *P. pisce* against *A. parasiticus*. This interaction type was the inhibition of one species on contact, where the inhibitor species continued to grow at a reduced rate through the inhibited colony. In this case, the colony of *A. parasiticus* outgrew *P. pisce*.

Figure 4-08. Interaction type D, between *A. parasiticus* and non-toxigenic *A. flavus* after 20 days on CYA (a_w 0.99) at 25°C.



Note: Arrow indicates the distance between colonies of *A. parasiticus* and non-toxic *A. flavus*; bar = 5 mm.

Grown with toxigenic *A. flavus*, the four other fungi demonstrated three types of fungal interactions. The first type was mutual inhibition on contact or the space between colonies small (<2 mm). This type is classified as reaction type B and was shown by the non-toxic strain of *A. flavus*. The second type of interaction was type D or a mutual inhibition at a distance (>2 mm), observed on the plate of *E. rubrum* and *A.wentii* against *A. flavus*. The third type was type E or inhibition of one species on contact, where toxigenic *A. flavus* continued to grow at a reduced rate through *P. pisce,* which was inhibited.

4.3.3 Fluorescent compounds

In all replicates, axenic cultures of *E. rubrum, A. wentii, P. pisce* and non-toxigenic *A. flavus* did not produce any fluorescent compounds. After 10 days incubation, *A. parasiticus* produced aflatoxins B_1 (AFB_1), G_1 (AFG_1) and G_2 (AFG_2). Aflatoxin B_2 (AFB_2) was not observed in *A. parasiticus*. The mean AFG_2 concentrations of *A. parasiticus* at day 10, were 0.039 μg mL^{-1} agar, 0.026 μg mL^{-1} agar and 0.039 μg mL^{-1} agar at spore loads 10^2, 10^3 and 10^4, respectively. However, AFG_2 was not observed on the dual cultures with the four fungi nor at day 20 in axenic *A. parasiticus*. The presence of a pale blue-green fluorescent compound was observed at Rf value 0.22 in axenic *A. parasiticus* and in its interaction with the four other fungi. Rf value of AFB_1 was 0.509, AFB_2 was 0.453, AFG_1 was 0.408 and AFG_2 was 0.358 (Chapter 3). Intensity of the blue-green fluorescence was less than AFB_1 produced in *A. parasiticus*.

On the other hand, at all spore loads AFB_2 concentration was 0.026 μg mL^{-1} in axenic *A. flavus* after 10 days. In the presence of other fungi, AFB_2 was not detected in *A. flavus* at 20 days, however, a pale blue-green fluorescent compound was observed. The Rf value of this fluorescent compound was 0.27 and it was found in axenic *A. flavus* and its mixed culture with other fungi. Visually, intensity of this fluorescent compound was less than the AFB_1 concentration produced in *A. flavus*.

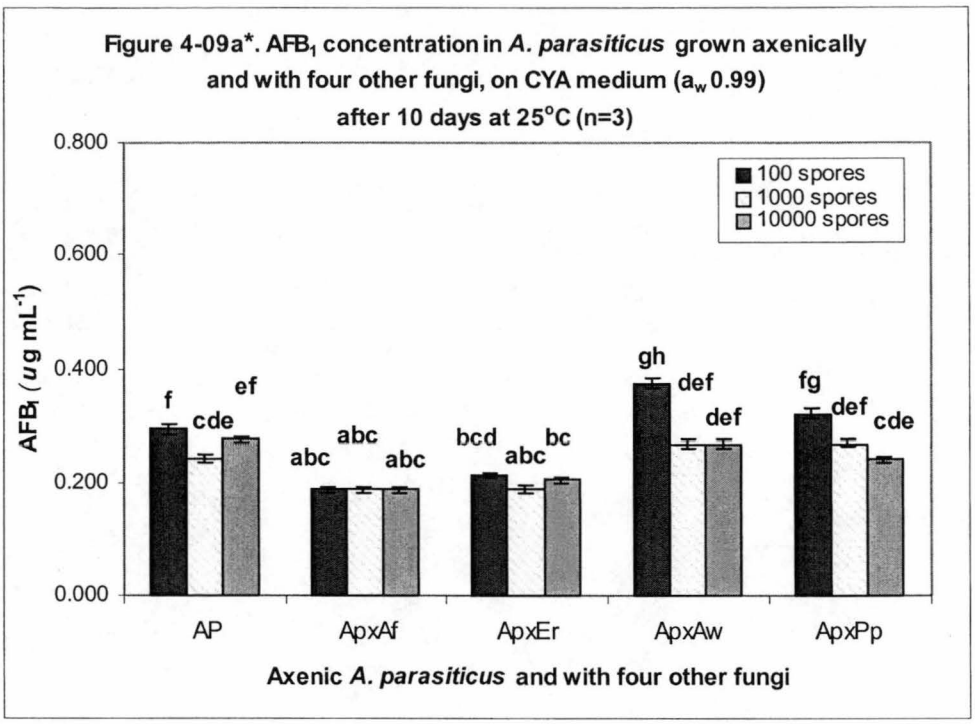
4.3.4 AFB_1 concentration in *A. parasiticus* and *A. flavus*

Day, spore load and culture, singly and in all interactions, significantly affected AFB_1 concentration in *A. parasiticus* (Table 4-06). Tukey's test was performed on all interactions across both incubation days (Figure 4-09a and Figure 4-09b). At day 10, significant differences (10-35%, $p \leq 0.05$) in AFB_1 production were observed for *A. parasiticus* grown with the four non-toxic

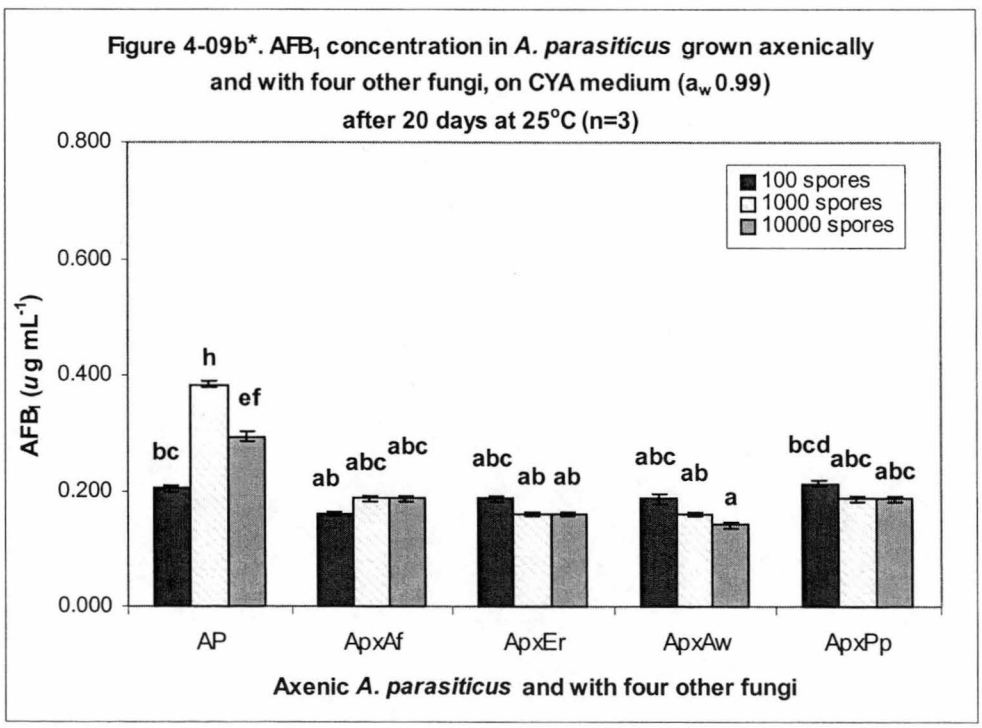
species (Figure 4-09a). Significant reductions in AFB_1 concentrations were observed on *A. parasiticus* grown with non-toxigenic *A. flavus* and *E. rubrum* at 10^2 and 10^4 spore loads. On the other hand, *A. wentii* at the low spore inoculation (10^2) significantly increased ($p \leq 0.05$) by 20% AFB_1 concentration in *A. parasiticus*. After 20 days, the concentration of AFB_1 in axenic *A. parasiticus* at the 10^3 and 10^4 spore loads was greater than at the 10^2 spore load (Figure 4-09b). All four fungi significantly reduced AFB_1 concentration in *A. parasiticus* by up to 60% as compared to the axenic *A. parasiticus* at the two higher spore loads.

Table 4-06. ANOVA of AFB_1 concentration of *A. parasiticus* when grown axenically and with other fungi on CYA (a_w 0.99) at 10 and 20 days at 25°C (n=3)

Source	Type III sum of squares	df	Mean square	F	P
Corrected model	8.459E-02	29	2.917E-02	295.338	0.000
Intercept	1.123	1	1.123	113703.20	0.000
Days (incubation)	1.264E-02	1	1.264E-02	1280.000	0.000
FRR (culture)	3.043E-02	4	7.607E-02	770.200	0.000
Level (spore loads)	1.450E-03	2	7.249E-04	73.400	0.000
Day*FRR	1.829E-02	4	4.573E-03	463.000	0.000
Day*Level	5.100E-02	2	2.550E-03	258.200	0.000
FRR*Level	9.631E-03	8	1.204E-03	121.900	0.000
Day*FRR*Level	7.048E-03	8	8.809E-04	89.200	0.000
Error	5.926E-04	60	9.876E-06		
Total	1.208	90			
Corrected total	8.518E-02				



Note: * The superscripts are comparable within and between figures a and b



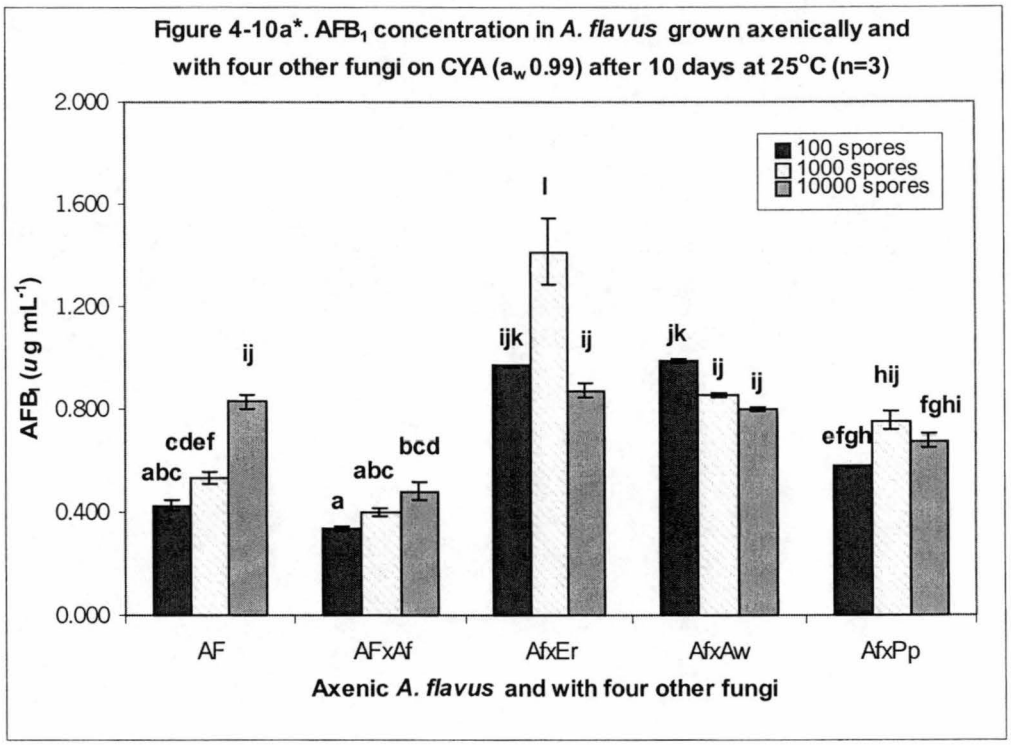
Note: * The superscripts are comparable within and between figures a and b.

Similarly, day, spore load and cultures, singly and in all interactions significantly affected ($p \leq 0.05$) the concentration of AFB_1 in *A. flavus* (Table 4-07). Therefore, Tukey's test was done on all interactions and compared across both incubation days. At 10 days, AFB_1 concentration of axenic *A. flavus* increased as the initial spore load increased (Figure 4-10a). Only the non-toxigenic strain of *A. flavus* consistently reduced AFB_1 concentration in *A. flavus*, significantly so at the highest spore loading. The fish commensal fungi either had no effect, or significantly increased AFB_1 concentration in *A. flavus*.

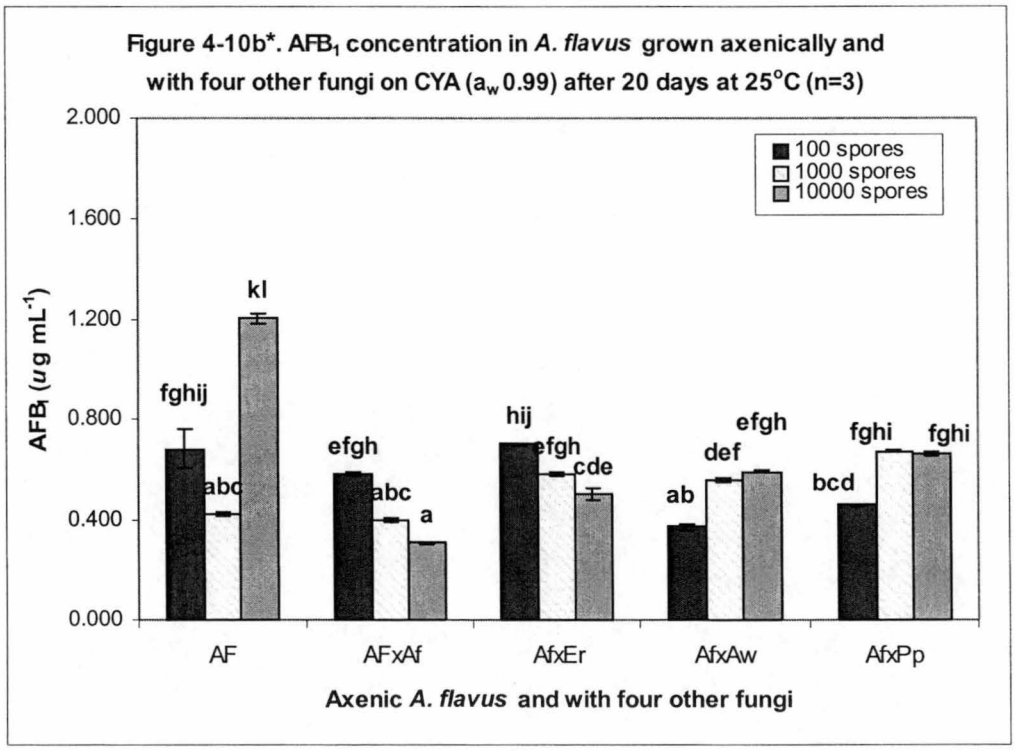
At 20 days incubation, AFB_1 concentration of axenic *A. flavus* increased at the level of 10^2 and 10^4 spore loads, however, the AFB_1 concentration from the 10^3 inoculum was reduced by 18% compared to day 10, but this was not significant. Interacted with non-toxic fungi at 10^2 and 10^4 spore loads, AFB_1 concentration of *A. flavus* was decreased. The reduction was 44-74% at high spore load and 14-54% at low spore load (Figure 4-10b). The amount of AFB_1 at 10^3spore load was insignificantly reduced by 6% when grown with non-toxigenic of *A. flavus*, and was significantly increased by 23-36% when grown with other three fungi.

Table 4-07. ANOVA of AFB_1 concentration of *A. flavus* when grown axenically and with other fungi on CYA (a_w 0.99) at 10 and 20 days at 25°C (n=3)

Source	Type III sum of squares	df	Mean square	F	P
Corrected model	5.703	29	0.197	21.221	0.000
Intercept	38.687	1	38.687	4174.444	0.000
Days (incubation)	0.472	1	0.472	50.885	0.000
FRR (culture)	1.680	4	0.420	45.316	0.000
Level (spore loads)	0.104	2	5.210E-02	5.621	0.006
Day*FRR	1.374	4	0.344	37.078	0.000
Day*Level	0.156	2	7.786E-02	8.401	0.001
FRR*Level	1.334	8	0.167	18.000	0.000
Day*FRR*Level	0.583	8	7.287E-02	7.862	0.000
Error	0.556	60	9.268E-02		
Total	44.946	90			
Corrected total	6.259				



Note: * The superscripts are comparable within and between figures a and b.



Note: * The superscripts are comparable within and between figures a and b.

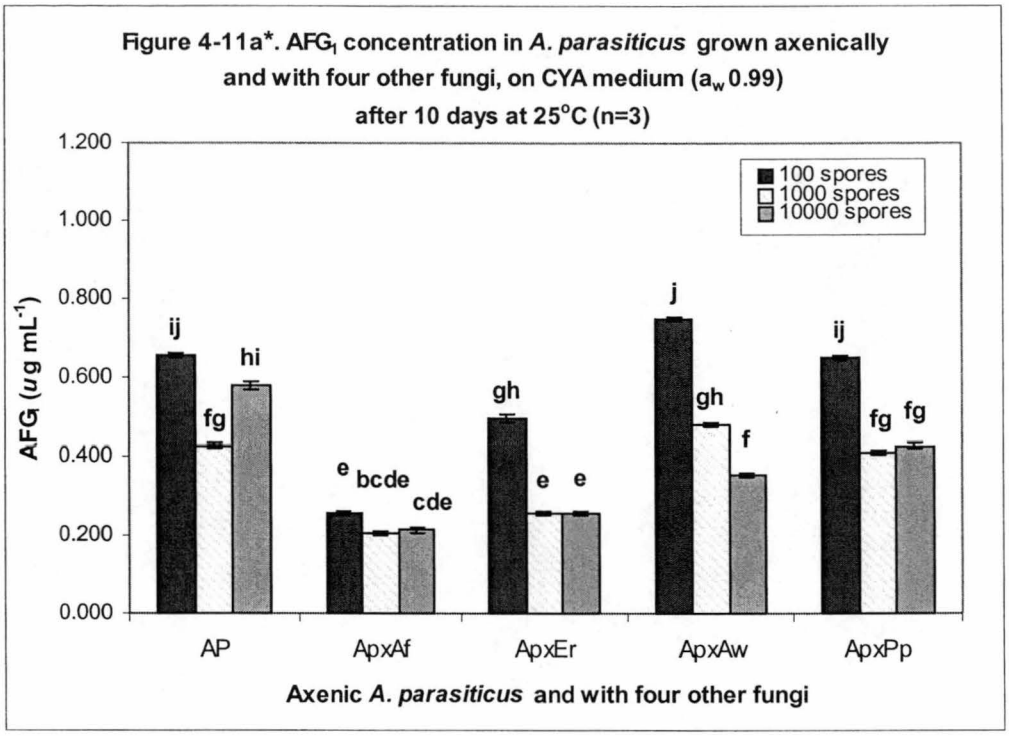
4.3.5 AFG_1 concentration in *A. parasiticus* and *A. flavus*

Day, spore load and culture, singly and in all interactions, significantly affected AFG_1 concentration in *A. parasiticus* (Table 4-08) and *A. flavus* (Table 4-09) at $p \leq 0.05$. Therefore, Tukey's test on *A. parasiticus* and *A. flavus* was performed as multiple comparison for all interactions throughout the incubation days.

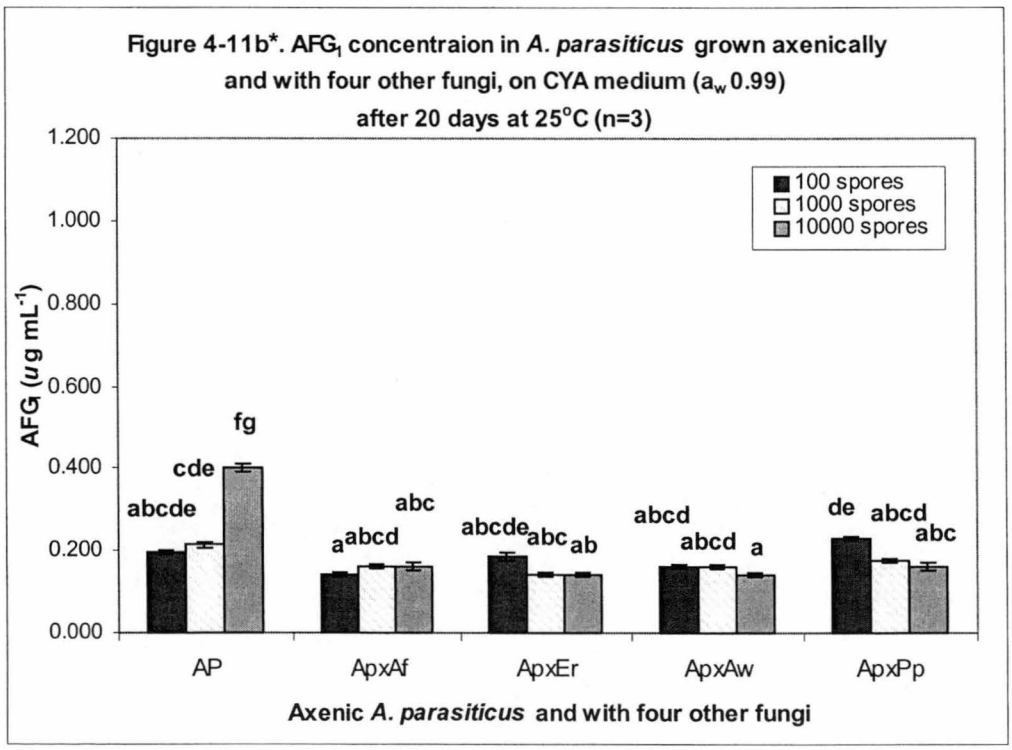
Table 4-08. ANOVA of AFG_1 concentration of *A. parasiticus* when grown axenically and with other fungi on CYA (a_w 0.99) at 10 and 20 days at 25°C (n=3)

Source	Type III sum of squares	df	Mean square	F	P
Corrected model	7.545E-02	29	2.602E-03	247.119	0.000
Intercept	0.955	1	0.995	90695.720	0.000
Days (incubation)	4.017E-02	1	4.017E-02	3815.463	0.000
FRR (culture)	1.567E-02	4	3.919E-03	372.185	0.000
Level (spore loads)	4.205E-03	2	2.102E-03	199.690	0.000
Day*FRR	5.662E-03	4	1.416E-03	134.445	0.000
Day*Level	3.941E-03	2	1.970E-03	187.139	0.000
FRR*Level	5.034E-03	8	6.292E-04	59.760	0.000
Day*FRR*Level	7.656E-04	8	9.570E-05	9.089	0.000
Error	6.317E-04	60	1.053E-05		
Total	1.031	90			
Corrected total	7.609E-02				

At day 10, non-toxigenic *A. flavus* and *E. rubrum* caused significant reductions (50-63%) of AFG_1 in *A. parasiticus* for all spore loads. However, the concentration of AFG_1 of *A. parasiticus* grown with *E. rubrum* at a spore load of 10^2 was still higher than the AFG_1 concentration of axenic *A. parasiticus* at 10^3spore load. Neither *P. pisce* nor *A. wentii* significantly affected the concentration of AFG_1 at 10^2 and 10^3 spore loads, but they did significantly reduce AFG_1 at the 10^4 spore load (Figure 4-11a). After 20 days, the amount of AFG_1 concentration in axenic *A. parasiticus* decreased significantly by 25% to



Note: * The superscripts are comparable within and between figures a and b.



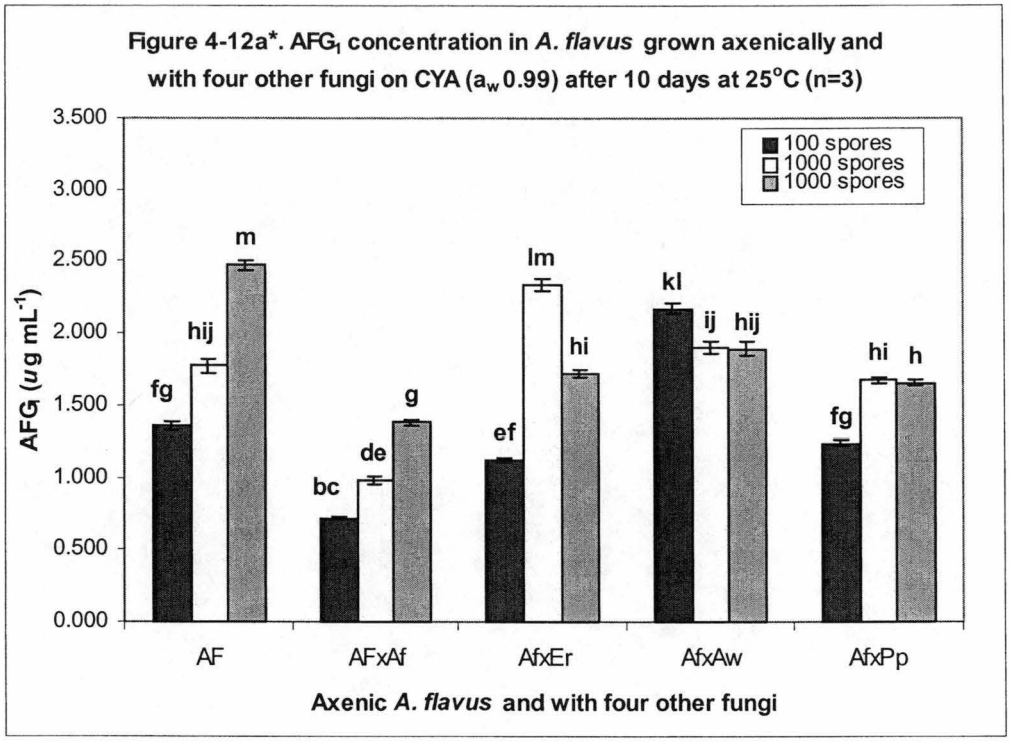
Note: * The superscripts are comparable within and between figures a and b.

65% ($p \leq 0.05$). As a result, few of the non-toxin-producing strains significantly reduced the concentration of AFG_1 when compared to axenic *A. parasiticus*. The major exception was for the high spore load (10^4), for which all non-toxic fungi significantly reduced the concentration of AFG_1 (Figure 4-11b).

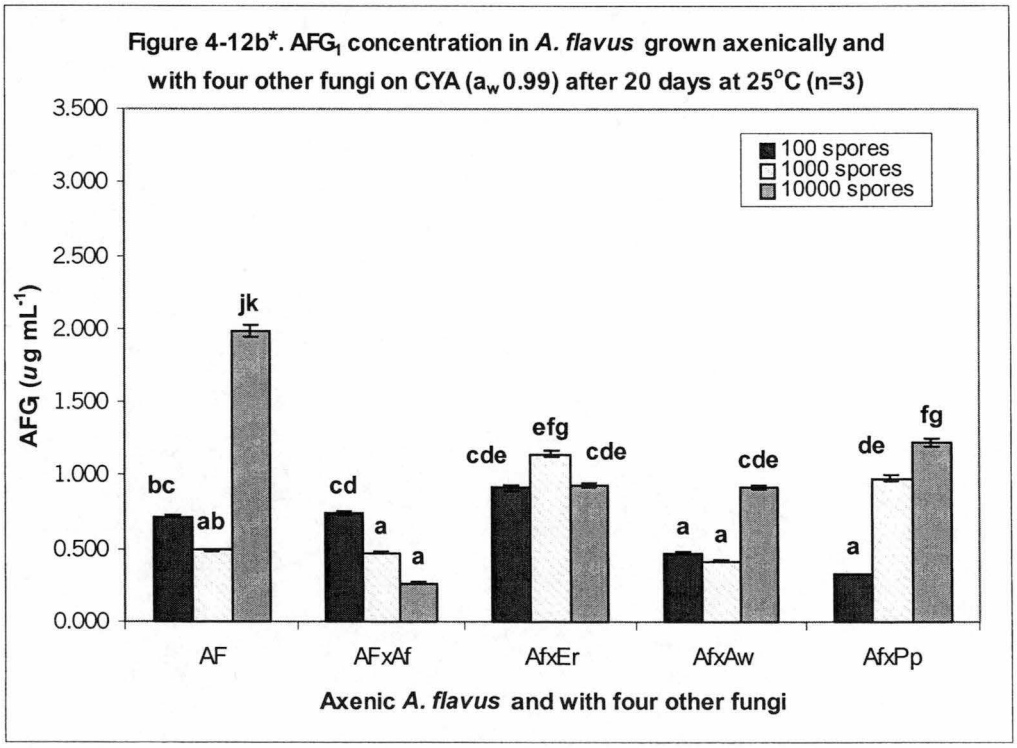
Table 4-09. ANOVA of AFG_1 concentration of *A. flavus* when grown axenically and with other fungi s on CYA (a_w 0.99) at 10 and 20 day at 25°C (n=3)

Source	Type III sum of squares	df	Mean square	F	P
Corrected model	5.968	29	0.206	347.981	0.000
Intercept	2.511E-02	1	2.511E-02	42.457	0.000
Days (incubation)	2.678	1	2.678	4528.395	0.000
FRR (culture)	0.942	4	0.236	398.314	0.000
Level (spore loads)	0.418	2	0.209	353.621	0.000
Day*FRR	0.260	4	6.49E-02	109.821	0.000
Day*Level	5.596E-02	2	2.798E-02	47.311	0.000
FRR*Level	0.786	8	9.827E-02	166.182	0.000
Day*FRR*Level	3.548E-02	8	0.103	174.900	0.000
Error	6.028	60	5.914E-04		
Total	6.003	90			
Corrected total					

From 10 to 20 days of incubation, axenic *A. flavus* showed a decrease in AFG_1 concentration. Figure 4-12a shows that at day 10, in the presence of the four other fungi at high spore load (10^4), AFG_1 concentration of *A. flavus* was significantly reduced by 32-48% ($p \leq 0.05$). Non-toxigenic *A. flavus* definitely decreased AFG_1 concentration of *A. flavus* at all spore loads. However, AFG_1 concentration in *A. flavus* was increased by 30% when inoculated with *E. rubrum* at 10^3 spore load and by 36% with *A. wentii* at 10^2 spore load.



Note: * The superscripts are comparable within and between figure a and b.



Note: * The superscripts are comparable within and between figure a and b.

After 20 days of incubation, at 10^4 spore load, AFG_1 concentration in *A. flavus* was significantly reduced by 38-86% when interacted with the four other fungi (Figure 4-12b). At this spore load, non-toxigenic *A. flavus* caused the highest reduction of AFG_1 concentration in *A. flavus*. At 10^2 spore load, *A. wentii* decreased AFG_1 concentration in *A. flavus* by 34% and *P. pisce* reduced by 54%. Conversely, *E. rubrum* at lower spore loads 10^2 and 10^3, increased AFG_1 concentration in *A. flavus* by 22-55%, and *P. pisce* at 10^3 spore load by about 50%.

4.4 DISCUSSION

All fungi grew on the medium used and the temperature was within the optimal range for the growth of the fungi examined and for aflatoxin production by *A. parasiticus* and *A. flavus*, but the species isolated from dried fish would likely have grown faster at lower values of a_w. Except for non-toxigenic *A. flavus* that increased colony size in the presence of *A. flavus* at a_w 0.99, both *A. parasiticus* and *A. flavus* inhibited colony diameters of the four other fungi. Hocking and Pitt (1997) noted the preference of *E. rubrum, A. wentii* and *P. pisce* for reduced a_w. Production of AFB_1 and AFG_1 were higher in axenic *A. flavus* than in axenic *A. parasiticus*. The present study indicates that the presence of competing fungal species was an important influence on aflatoxin production by *A. parasiticus* and *A. flavus*.

Non-toxigenic *A. flavus* demonstrated mutual inhibition of growth with *A. parasiticus* and *A. flavus*. This resulted from diffusion of soluble factors rather than competition, because the colonies did not grow through each other. This interaction caused a reduction of aflatoxin in *A. parasiticus* and *A. flavus*, possibly because there was a smaller biomass available. Alternatively, non-toxigenic *A. flavus* may have degraded some of the aflatoxins produced or inhibited their synthesis. The latter seems more likely because the non-toxigenic *A. flavus* only caused a small reduction in colony size of *A.*

parasiticus and *A. flavus*. Possibly, both *A. flavus* strains competed for nutrients causing inhibition of aflatoxin production in toxigenic strain. Two fish commensals *E. rubrum* and *A. wentii* demonstrated a similar form of growth inhibition of *A. parasiticus* and *A. flavus,* however, inhibition of growth was not correlated to the reductions of AFB_1 and AFG_1 in both aflatoxigenic fungi. The presence of *E. rubrum*, reduced AFB_1 and AFG_1 in *A. parasiticus* but increased them in *A. flavus*. Inconsistently, *A. wentii* increased AFB_1 in *A. parasiticus* and *A. flavus*, except at higher spore load where AFG_1 was reduced in both aflatoxigenic fungi. Thus, fungal species and possibly strain also can greatly affect growth and even the type of toxin production of aflatoxigenic fungi. Shantha *et al.* (1990), who studied the effect of different strains of *A. niger* on aflatoxin producer *A. flavus* at 28°C, found that after 7 days several strains reduced aflatoxins, however, inhibition by some of the *A. niger* strains was inconsistent. These authors reported that the mycelial weight of *A. flavus* grown with *A. niger* strains G_4 and AN_1 increased, but the aflatoxin production was inhibited.

Because *A. parasiticus* and *A. flavus* in the present study grew through *P. pisce*, the inhibition caused by *P. pisce* could have resulted from either the production of toxic metabolites, or by competition for nutrients, or both. The relationship of growth inhibition to aflatoxin production was variable for *E. rubrum*, *A. wentii* and *P. pisce*, as in some cases the concentration of aflatoxin actually increased, despite there being a smaller biomass of *A. parasiticus* or *A. flavus*. The reason for this is not clear. It could be related to a slower rate of aflatoxin degradation by *A. parasiticus* and *A. flavus*, as reported by Smith and Harran (1993). Pitt and Hocking (1997) noted that *E. rubrum* and *A. wentii* strains produce some toxic substances, although information is still lacking. Thus, the substances present in *E. rubrum* and *A. wentii* could stimulate AFB_1 and AFG_1 production in *A. parasiticus* and/or *A. flavus* when there was an increase aflatoxin production found in the present study, however, the mechanism is unclear.

Although higher inocula can lead to higher biomass initially (Gonzalez *et al.*, 1995; Karunaratne and Bullerman, 1990), by 8 to 10 days the culture is in stationary phase (Karunaratne and Bullerman, 1990) and the biomass will not differ significantly (Gonzalez *et al.*, 1995). In the present study, however, biomass of *A. parasiticus* and *A. flavus* at all inoculum sizes was not significantly different. Colony diameter of both species was not much affected by spore load at the first week, at 10 days and very little at 20 days. Although 10^3 spore loads of *A. parasiticus* produced significantly less AFB_1 and AFG_1 than either the higher or lower spore loads after 10 days, this was probably an artefact caused by the low number replicates. Both AFB_1 and AFG_1 increased in concentration with increasing spore load of *A. flavus*. Thus, aflatoxin concentration may have been influenced by the size of the inoculum, however, the mechanism is not clear, as the concentration of aflatoxin was the net result of production and degradation.

At 20 days, inoculum size did not correlate with aflatoxin production by *A. parasiticus* and *A. flavus*. The fungi were grown aerobically on a good nutrient medium within optimum temperature and a_w range. Therefore, *A. parasiticus* and *A. flavus* probably directed more energy into growth than into toxin production, which may account for the lower concentrations of AFB_1 and AFG_1 in the present study, in comparison to Karunaratne and Bullerman (1990) and Gourama and Bullerman (1995e). More importantly, the use of different strains of aflatoxin-producing fungi could explain aflatoxin concentration differences. From 169 strains of *A. flavus* group maintained in ATCC, Wei and Jong (1986) examined that 59 strains of *A. flavus* and *A. parasiticus* produced aflatoxins. They found a huge difference in concentrations of four aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) produced between the producer strains and on different substrates. A low aflatoxin-producer *A. flavus* was also noted by Gqaleni *et al.* (1997). They reported maximum aflatoxin produced by the *A. flavus* strain used in their experiment, was 0.330 μg mL^{-1} on YES and 0.306 μg mL^{-1} on CYA after 15 days at 30°C with a_w 0.996.

Production of aflatoxins occurs as the secondary metabolites after the primary growth, and so the highest production of aflatoxins was reached after one week in this study. Karunaratne and Bullerman (1990) found that inoculation of rice with *A. flavus* at 10^3 spores per 50 g produced the highest amount of aflatoxin after 5 days, while higher spore levels (10^7) resulted in much less aflatoxin formation. A similar result was found by Sharma *et al.* (1980). This relationship, however, was not seen in the present study because the spore loads did not significantly affect aflatoxin concentrations. Karunaratne and Bullerman (1990) mixed 10^1-10^7 inocula into 50 g of rice giving the total of 0.2– $2x10^5$ spores g^{-1}. In the present study, 10 μL obtained from 10^4-10^6 spores mL^{-1} was inoculated as a drop onto agar. This may explain the low aflatoxin concentration found in the present study. However, Gqaleni *et al.* (1997) used an inoculum of 10^6 spores spread across on agar plate giving ≥ 50,000 spores mL^{-1}. They reported aflatoxin concentrations in the range of 50-300 μg mL^{-1}, similar to Karunaratne and Bullerman (1990). Therefore, the differences observed may have been more a result of strain variation. Despite this, it is clear that high concentrations of aflatoxin can result from low inocula.

A reduction in aflatoxin concentration as cultures aged has been reported previously (Doyle and Marth, 1978c; Huynh and Lloyd, 1984) and the results reported here demonstrated a reduction in AFB_1 and AFG_1 concentrations on day 20 compared to day 10. The only exception was for AFB_1 concentration in plates inoculated with the 10^3 spores in which a significant increase in AFB_1 was observed. Huynh and Lloyd (1984) demonstrated enzymatic degradation of aflatoxin by *A. parasiticus* as the culture aged beyond 8 days, but not before. Faraj *et al.* (1993) concluded that most of the reduction in aflatoxin concentration that they observed resulted from endogenous degradation by *A. flavus*. Doyle and Marth (1978d) noted that the aflatoxigenic fungi produced high amount of aflatoxins also degraded the aflatoxin rapidly. The present data indicate that in *A. parasiticus* the largest reductions in aflatoxin concentration at

day 20 compared to day 10 occurred when the initial concentration of aflatoxin was highest. Because mostly there was no significant difference between the aflatoxin concentration in axenic versus mixed cultures, the decrease in concentration at 20 days primarily resulted from endogenous degradation by *A. parasiticus*. The main exception to this was non-toxigenic *A. flavus*, which did cause significant reductions in aflatoxin concentrations after 20 days compared to 10 days. However, the difference was still small for both AFB_1 and AFG_1 for most spore loads. *A. flavus*, on the other hand, demonstrated an endogenous degradation AFB_1 and AFG_1 at the middle spore loading. At higher spore loading, non-toxigenic *A. flavus* and the three fungal commensals of dried fish competitively degraded AFB_1 and AFG_1 in *A. flavus* even at 20 days. Regardless their effect on the growth of *A. flavus*, the presence of these other fungi at higher inoculum level had an inhibitory action on aflatoxin concentration.

Non toxigenic *A. flavus* consistently caused significant reductions AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus* at all spore loads tested. Similarly, *E. rubrum* also reduced aflatoxin production in *A. parasiticus* and *A. flavus*, but the decreases were not always statistically significant. *P. pisce* and *A. wentii* had variable effects on AFB_1 and AFG_1 concentration of *A. parasiticus* and *A. flavus* however, both causing AFB_1 and AFG_1 reductions in *A. parasiticus* and *A. flavus* at 10^4 spores. The increase in AFB_1 and AFG_1 in the presence of other fungi may have occurred because endogenous degradation was inhibited. Biologically, the increment of AFB_1 observed at 10^2 and 10^3 inoculations exacerbates the problem of contamination of foods. At high values of a_w, the non-toxigenic strain of *A. flavus* may have some usefulness as a probiont, but only if it is able to more completely inhibit the growth of *A. parasiticus* and *A. flavus*. This may be possible if it is established earlier than *A. parasiticus* and *A. flavus* because, while non-toxigenic *A. flavus* inhibited *A. parasiticus* and *A. flavus*, both these aflatoxigenic fungi were able to inhibit *A. flavus* as well.

In the present study, AFG_2 production by *A. parasiticus* was eliminated when this species was grown with any of the four fungi. AFB_2 in *A. flavus* was eliminated after 20 days. A pale blue-green fluorescent compound with less intense fluorescence and lower Rf values than AFB_1, AFB_2, AFG_1 and AFG_2 was found in axenic *A. parasiticus* and *A. flavus* and in the mixed cultures with other fungi. This could be a degradation form or a biotransformation of one or more of the four aflatoxins. None of the fungal commensals of dried fish (*E. rubrum, A. wentii* and *P. pisce*) nor the non-toxigenic *A. flavus* produced fluorescent compounds in the present study. Karunaratne and Bullerman (1990) reported *A. parasiticus* NRRL 2999 produced some fluorescing compounds other than aflatoxins. Transformation of AFB_1 into other forms in the presence of other fungi has been documented by some researchers (Detroy and Hesseltine, 1969; Nout, 1989 and Nakazato *et al.*, 1990).

It is concluded that the high spore level of non-toxic fungi significantly reduced AFB_1 and AFG_1 concentrations. This study represented a rehydrated condition that may occur during storage in a high humidity environment. However, the presence of aflatoxins still leaves medical problems, even if the concentration was reduced. The statistically significant variations were often small, indicating that they may not be biologically significant. However, as *E. rubrum, A. wentii* and *P. pisce* were dried fish commensals this needs clarification and further studies on how the non-toxic fungi react to both aflatoxigenic species at lower a_w values. These fungi may be more useful at lower values of a_w, for reducing AFB_1 and AFG_1 concentration in both *A. parasiticus* and *A. flavus*. This is investigated in the next chapter.

CHAPTER 5

GROWTH AND AFLATOXIN PRODUCTION OF *Aspergillus parasiticus* AND *Aspergillus flavus* AT LOW WATER ACTIVITY (a_w) IN THE PRESENCE OF OTHER FUNGI

5.1 INTRODUCTION

Reduction in water activity (a_w) affects the growth and aflatoxin production in *Aspergillus parasiticus* Speare and *A. flavus* Link. Pitt and Miscamble (1995) noted that minimum a_w of these two fungi was decreased as the temperature increased. These authors reported minimum a_w values for both *A. parasiticus* and *A. flavus* were 0.82 at 25°C, 0.81 at 30°C and 0.80 at 37°C. A descriptive model described by Pitt (1993) on mold growth and aflatoxin production noted a_w and temperature had an interactive effect on growth and aflatoxin concentration. Toxin formation was proportional to biomass production, and toxin degradation was proportional to the dead cell mass and aflatoxin concentration. Nutrients, pH and gaseous composition are also important factors in fungal growth and aflatoxin formation (Northolt and Bullerman, 1982; Avari and Allsopp, 1983; Gibson *et al.*, 1994).

The previous study (Chapter 4) showed that at optimal conditions of a_w 0.99 and 25°C for *A. parasiticus* and *A. flavus*, the presence of other fungi variably affected growth and production of AFB_1 and AFG_1 in both aflatoxigenic fungi. At various inoculum sizes, the non-toxigenic strain of *A. flavus* and the three fungal commensals of dried fish (*Eurotium rubrum, Aspergillus wentii* and *Polypaecilum pisce*) inhibited growth and AFB_1 and/or AFG_1 production in *A. parasiticus* and *A. flavus* in various ways. Although non-toxigenic *A. flavus* was more consistent in reducing AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*, the three fungal commensals demonstrated some reductions when they were applied at inoculum 10^4 mL^{-1}. Therefore, an initial inoculum size of 10^4 mL^{-1} was used in this present study.

E. rubrum, A. wentii and *P. pisce* were isolated from dried fish, so lowering the a_w medium of the medium and increasing the temperature may increase the effect to these three fungal commensals have on aflatoxin reduction. Andrews and Pitt (1987) reported the optimal a_w values for growth of

A. wentii and *P. pisce* were 0.94-0.96 at 25°C and Wheeler *et al.* (1988a) reported optimal growth of *E. rubrum* on glucose-based medium was at a_w 0.91-0.94. On a second study, Wheeler *et al.* (1988b) noted maximum growth of *P. pisce* was at 30°C and a_w 0.91-0.96. Thus, an incubation temperature of 30°C was deliberately chosen to represent the average environmental conditions of tropical regions. The present study aimed to investigate the effect of a non-toxigenic strain of *A. flavus* and fungal commensals *E. rubrum, A. wentii, P. pisce* and a yeast *Debaryomyces hansenii* (Zopf) Lodder and Kreger on growth and aflatoxin concentration of *A. parasiticus* and *A. flavus* at a lower a_w value in an agar-based system.

5.2 MATERIAL AND METHODS

5.2.1. General methods

Methods of measuring colony diameter, fungal interaction, aflatoxin and statistical analysis were described in Chapter 3. Aflatoxigenic *A. parasiticus*, *A. flavus* and non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii* and *P. pisce* used in this study were the same in Chapter 4, however, *Debaryomyces hansenii* was also included. The medium was MY5-20% agar with a_w 0.93 and the samples were incubated at 30°C. The formula of MY5-20% agar was based on the recipe for MY5-12% described by Pitt and Hocking (1997), however, in this study in order to attain an $a_w = 0.93$ the amount of glucose added was 200 g L^{-1}. Filtrates of axenic non-toxigenic *A. flavus* and the four fungal commensals of dried fish were also analysed on TLC. The presence of fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2 was recorded.

5.2.2. Experimental design

This experiment was a full factorial design in three replicates with the treatments being length of incubation (7, 14 and 21 days) and fungal cultures.

The fungal cultures were axenic *A. parasiticus* and *A. flavus* used as the controls and their combination with non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii*, *P. pisce* and *D. hansenii*. All fungal cultures were inoculated as 10 μL obtained from 10^6 spores mL^{-1} giving inoculum size of 10^4 spores mL^{-1}.

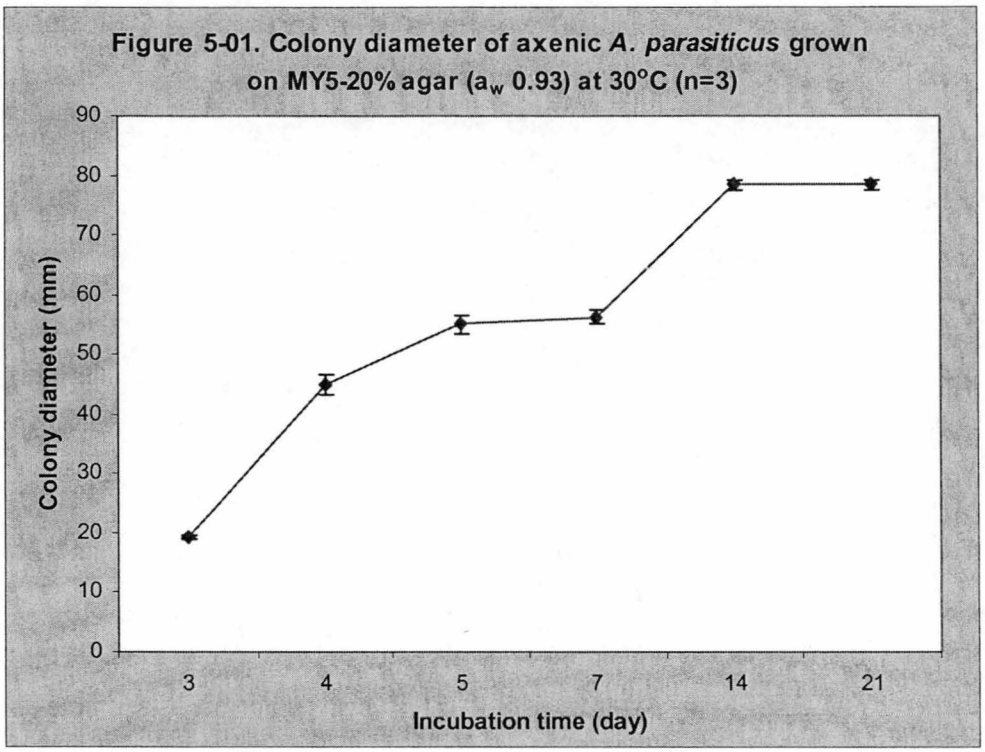
5.3 RESULTS

5.3.1. Colony diameter of *A. parasiticus* and *A. flavus*

At a_w 0.93, the colony diameter of both axenic *A. parasiticus* and *A. flavus* was 80 mm after 21 days and almost covering the whole plate. Compared to their axenic cultures, the colony diameter of *A. parasiticus* and *A. flavus* was reduced when interacted with other fungi. Similarly, colony diameters of the axenic cultures of non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii*, *P. pisce* and *D. hansenii* were larger than when interacted with the aflatoxigenic fungi. Generally, the reduction of colony diameter of *A. parasiticus* and *A. flavus* was not significant ($p \leq 0.05$).

Axenic *A. parasiticus* grew rapidly and reached stationary phase after 5 days (Figure 5-01). Although both single factors (days of incubation and fungal cultures) significantly ($p \leq 0.05$) affected colony diameter of *A. parasiticus,* interaction of these two factors was not significant (Table 5-01). Therefore, Tukey's test was performed for each incubation time, however, at day 21 the presence of all other fungi was not significant on colony diameter of *A. parasiticus*. At 7 and 14 days, *E. rubrum* significantly reduced colony diameter of *A. parasiticus* by 42% (Figure 5-02a and Figure 5-02b), and at 14 days non-toxigenic *A. flavus* decreased by 36%. None of fungi at day 21 significantly decreased colony diameter of *A. parasiticus* (Figure 5-02c). Grown with *A. parasiticus*, all colony diameters of other fungi were reduced when compared with their axenic cultures (Table 5-02). Colony diameters of axenic *P. pisce* and

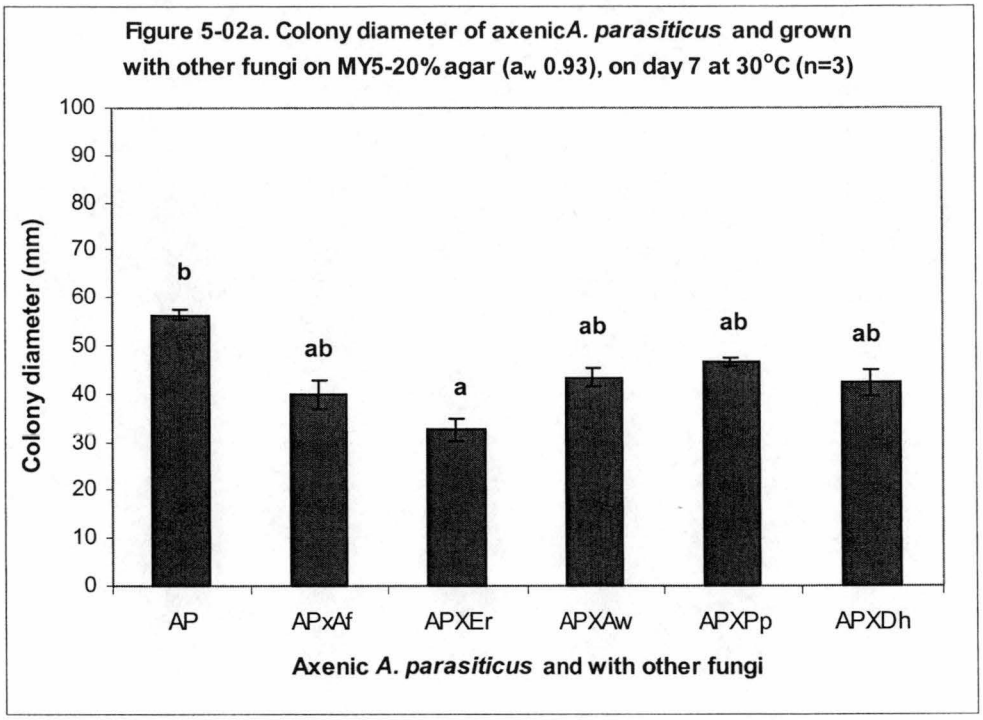
D. hansenii were smaller than the other three filamentous fungi i.e. the non-toxigenic *A. flavus*, *E. rubrum* and *A. wentii*.



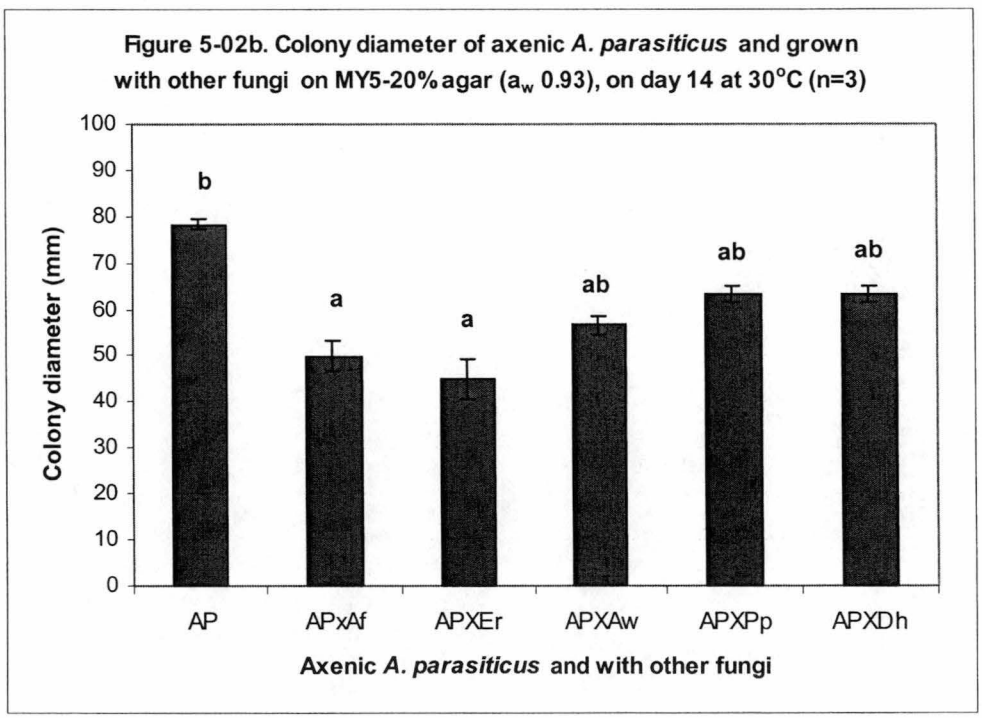
Note: bar = mean±SE

Table 5-01. ANOVA of colony diameter of *A. parasiticus* grown axenically and with other fungi on MY5-20% agar (a_w 0.93) at 30°C (n=3)

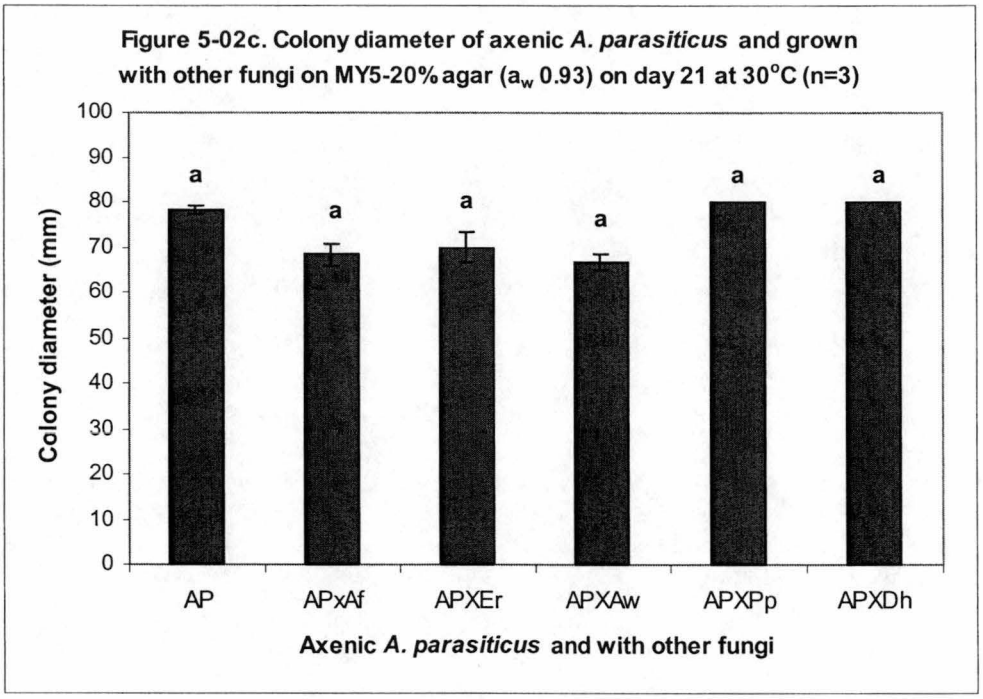
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	11859.926	17	697.643	15.148	0.000
Intercept	187738.074	1	187738.074	4076.339	0.000
Incubation (Day)	8287.259	2	4143.630	89.970	0.000
Fungal culture (FRR)	2855.704	5	571.141	12.401	0.000
Day*FRR	716.63	10	71.696	1.557	0.160
Error	1658.000	36	46.056		
Total	201256.000	54			
Corrected total	13517.926	53			



Note: AP = *A. parasiticus*; Af = non-toxigenic *A.flavus*; Er = *E.rubrum*; Aw = *A. wentii*; Pp = *P. pisce*; Dh = *D. hansenii*; superscripts are compared within figure.



Note: Superscripts are compared within figure.



Note: Superscripts are compared within figure.

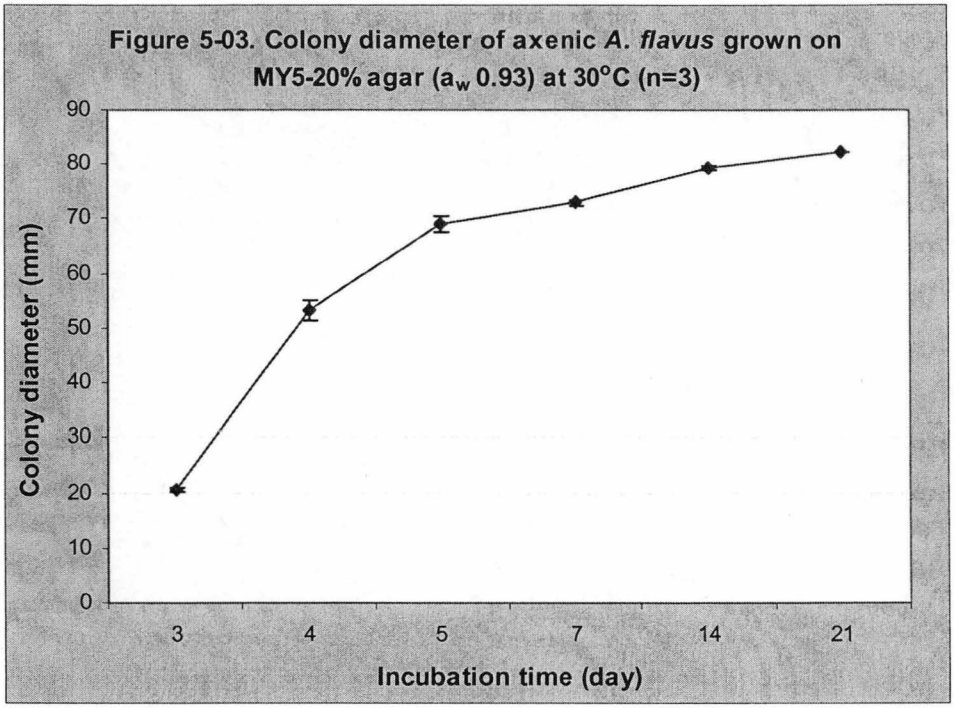
Table 5-02. Colony diameter of the five fungi grown axenically and with *A. parasiticus* on MY5-20% agar (a_w 0.93) at 30°C (n=3)

Cultures	Mean colony diameter (mm) ± SE, n=3		
	Day 7	Day 14	Day 21
Non-toxigenic *A.flavus*	73 ± 1.1	80 ± 0.0	81 ± 0.9
Non-toxigenic *A. flavus* x *A. parasiticus*	68 ± 0.9	78 ± 0.9	80 ± 0.0
E. rubrum	43 ± 0.9	45 ± 1.6	63 ± 1.9
E. rubrum x *A. parasiticus*	35 ± 1.3	36 ± 0.9	38 ± 0.9
A. wentii	66 ± 3.8	76 ± 0.9	76 ± 1.9
A. wentii x *A. parasiticus*	48 ± 3.4	58 ± 0.9	60 ± 0.0
P. pisce	14 ± 1.0	17 ± 1.0	22 ± 0.6
P. pisce x *A. parasiticus*	10 ± 1.0	12 ± 1.0	13 ± 1.0
D. hansenii	15 ± 1.3	18 ± 1.5	28 ± 0.5
D. hansenii x *A. parasiticus*	12 ± 0.6	13 ± 0.5	15 ± 0.2

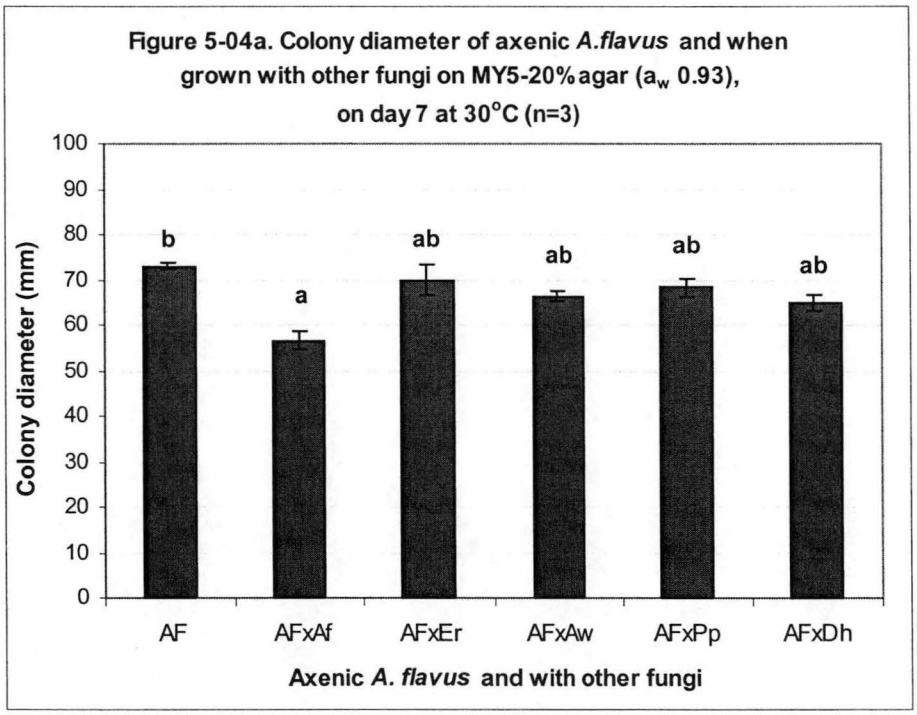
Colony diameter of axenic *A. flavus* rapidly increased after 3 days (Figure 5-03) and reached 82 mm after 21 days covering the whole plate. There was a similar effect on the colony diameter of *A. flavus* to that observed for *A. parasiticus* in the presence of the five other fungi. Single factors of day of incubation and fungal cultures significantly affected colony diameter of *A. flavus* at $p \leq 0.05$, however, interaction between these two factors was not significant (Table 5-03). Tukey's test performed on each day of observation showed that the colony diameter of *A. flavus* was significantly reduced only by the presence of non-toxic *A. flavus* (Figures 5-04a, 5-04b and 5-04c). These three figures show that only the non-toxigenic strain of *A. flavus* significantly reduced colony diameter of *A. flavus* by 22%, 15% and 14% at day 7, 14 and 21 respectively. Other fungi only decreased the colony diameter of *A. flavus* by less than 10% for all days and the reduction was not significant. Compared to the axenic non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii*, the colony diameters of these five fungi were also reduced when grown in the presence of *A. flavus*. Table 5-04 shows the colony diameters of axenic cultures of the other fungi and when grown with *A. flavus*.

Table 5-03. ANOVA of colony diameter of *A. flavus* grown axenically and with other fungi on MY5-20% agar (a_w 0.93) at 30°C (n=3).

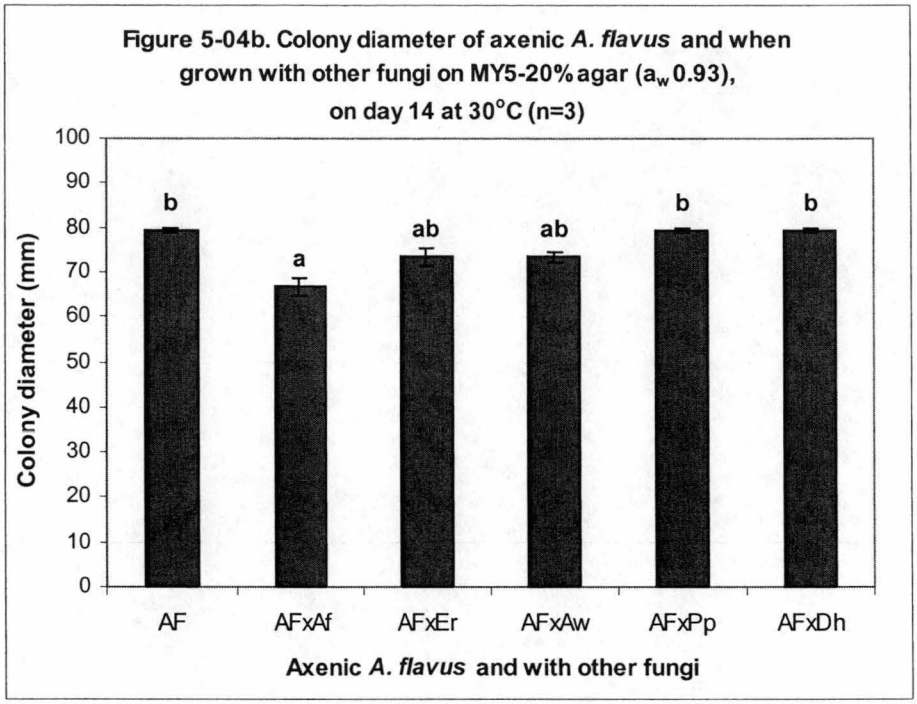
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	2484.148	17	146.126	7.407	0.000
Intercept	288935.185	1	288935.185	14646.797	0.000
Incubation (Day)	1227.259	2	613.630	31.106	0.000
Fungal culture (FRR)	1127.259	5	225.452	11.429	0.000
Day*FRR	129.630	10	12.963	0.657	0.755
Error	710.167	36	19.727		
Total	292129.500	54			
Corrected total	3194.315	53			



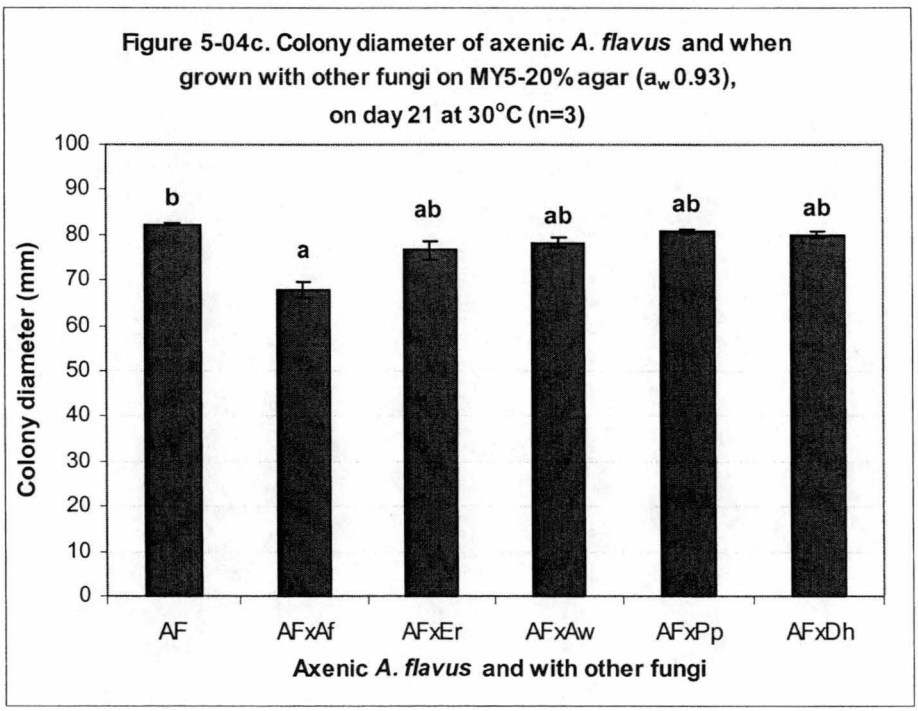
Note: bars = mean±SE



Note: AF= *A. flavus*; Af = non-toxigenic *A. flavus*; Er = *E. rubrum*; Aw = *A. wentii*; Pp= *P. pisce*; Dh = *D. hansenii*; superscripts are compared within figure.



Note: Superscripts are compared within figure.



Note: Superscripts are compared within figure.

Table 5-04. Colony diameter of the five fungi grown axenically and with *A. flavus* on MY5-20% agar (a_w 0.93) at 30°C

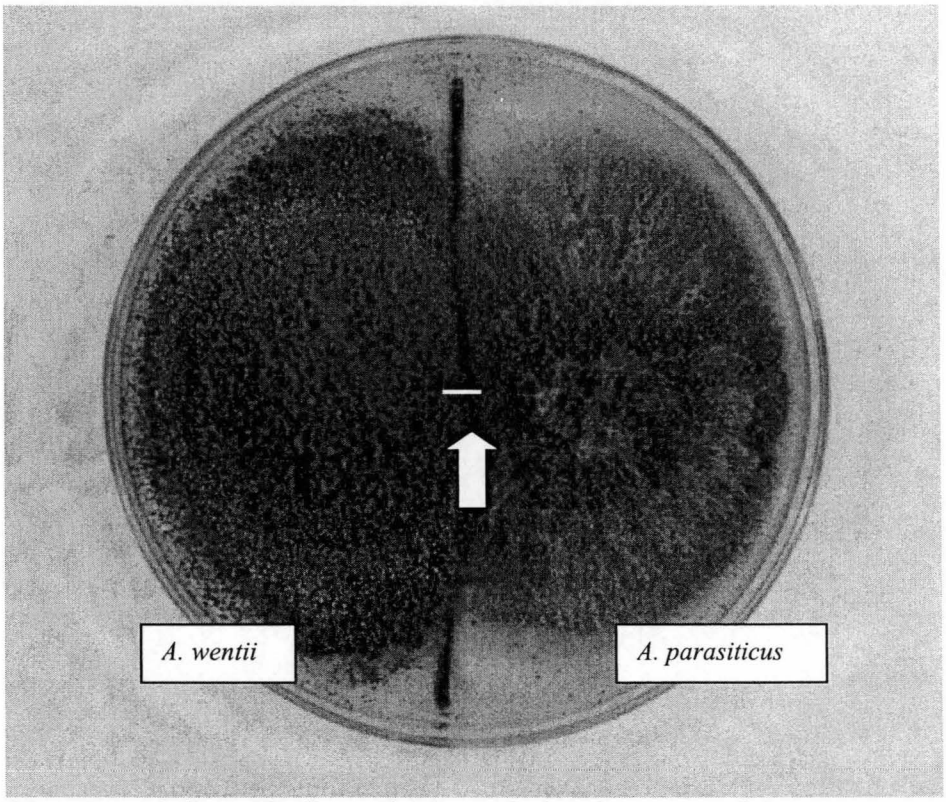
Cultures	Mean colony diameter (mm) ± SE, n=3		
	Day 7	Day 14	Day 21
Non-toxigenic *A.flavus*	75 ± 0.2	80 ± 0.0	81 ± 0.9
Non-toxigenic *A. flavus* x *A. flavus*	68 ± 0.9	80 ± 0.6	80 ± 0.4
E. rubrum	43 ± 0.9	45 ± 1.6	61 ± 2.5
E. rubrum x *A. flavus*	35 ± 1.3	36 ± 0.9	38 ± 0.9
A. wentii	66 ± 3.8	76 ± 0.9	76 ± 1.9
A. wentii x *A. flavus*	48 ± 3.4	58 ± 0.9	60 ± 0.0
P. pisce	12 ± 0.3	16 ± 0.6	18 ± 1.5
P. pisce x *A. flavus*	10 ± 0.3	12 ± 0.3	13 ± 0.5
D. hansenii	15 ± 1.3	18 ± 1.5	28 ± 0.5
D. hansenii x *A. flavus*	12 ± 0.6	14 ± 0.2	15 ± 0.2

5.3.2. Interaction between fungi

Assessed from fungal interaction (Chapter 3), the presence of non-toxigenic *A. flavus, E. rubrum, P. pisce* and *D. hansenii* against *A. parasiticus* and *A. flavus* showed several types of interaction. Grown with *A. parasiticus* or *A. flavus*, the non-toxic *A. flavus* and *A. wentii* (Figure 5-05) showed reaction type B or mutual inhibition on contact with space between colonies <2 mm.

Interacted with *E. rubrum, A. parasiticus* showed a reaction type D or mutual inhibition at a distance >2 mm, and *A. flavus* showed reaction type E or inhibition of one species on contact with the inhibitor species continuing to grow. Reaction type E was observed on *P. pisce* and *D. hansenii* against *A. parasiticus* or *A. flavus*. However, instead of *P. pisce* and *D. hansenii* inhibiting either *A. parasiticus* or *A. flavus*, the two aflatoxigenic fungi outgrew both species.

Figure 5-05. Interaction type B, between *A. parasiticus* and *A. wentii* after 14 days on MY5-20% agar (a_w 0.93) at 30°C.



Note: Arrow indicates the distance between colonies of *A. wentii* and *A. parasiticus*; bar = 5mm

5.3.3. AFB_1 concentration in *A. parasiticus* and *A. flavus*

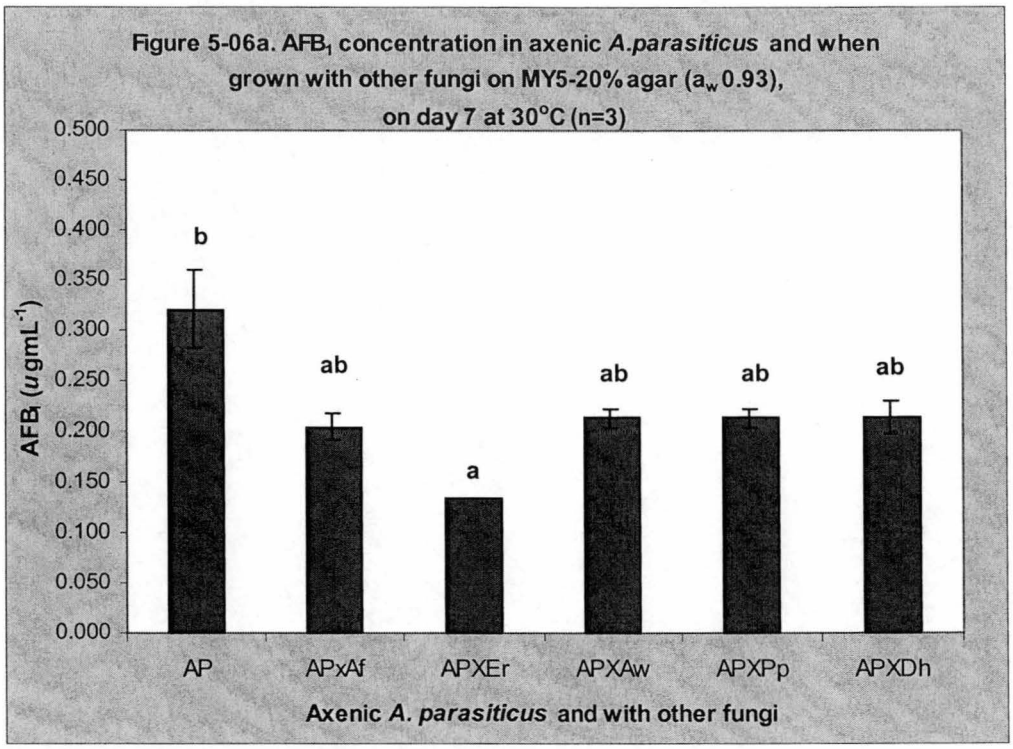
In this trial, *A. parasiticus* produced AFB_1, AFB_2, AFG_1 and AFG_2 and *A. flavus* produced AFB_1, AFB_2 and AFG_1. Their interaction with non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii* also produced other fluorescent compounds. The results of AFB_2, AFG_2 and the presence of other fluorescent compounds are presented later in this chapter.

For AFB_1 concentration, ANOVA of both single factors (incubation day and fungal cultures) was significant at $p \leq 0.05$, however, the interaction was insignificant (Table 5-03). Therefore, Tukey's test of AFB_1 concentration in *A.*

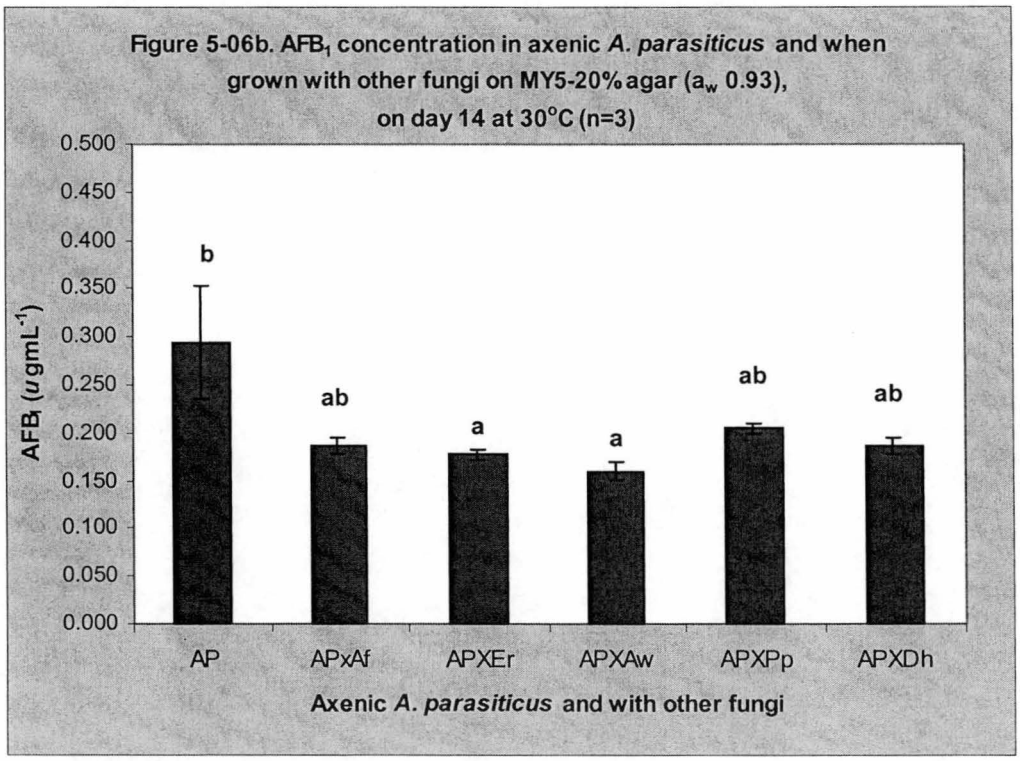
parasiticus was performed on each incubation day. AFB_1 concentration in axenic *A. parasiticus* decreased when the length of incubation increased. On day 7, AFB_1 concentration in axenic *A. parasiticus* was 0.320 μg mL^{-1} and in the presence of *E. rubrum,* AFB_1 in *A. parasiticus* was significantly decreased by more than 50%. Other fungi reduced AFB_1 in *A. parasiticus* by 30%, but the reduction was not significant (Figure 5-06a). At day 14, AFB_1 concentration in axenic *A. parasiticus* was 0.294 μg mL^{-1}. The presence of *E. rubrum* and *A. wentii* significantly reduced AFB_1 in *A. parasiticus* by 39 and 45%, respectively. Non-toxigenic *A. flavus, P. pisce* and *D. hansenii* caused reduction of 36% but it was not significant (Figure 5-06b). After 14 days, however, no significant differences in AFB_1 in *A. parasiticus* were seen in any treatment. At 21 days, the mean concentration of AFB_1 in *A. parasiticus* was 0.169 μg mL^{-1}.

Table 5-05. ANOVA of AFB_1 concentration in *A. parasiticus* grown axenically and with other fungi on MY5-20% agar (a_w 0.93) at 7, 14 and 21 days at 30°C (n=3)

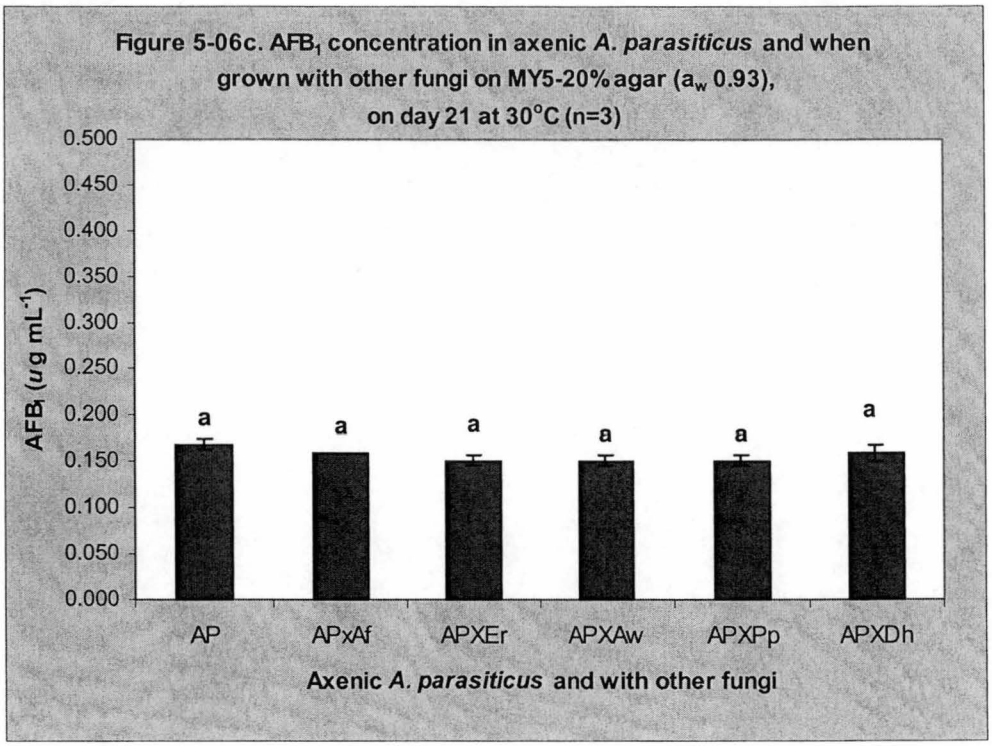
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.432	17	2.538E-02	2.792	0.005
Intercept	29.207	1	29.207	321.417	0.000
Incubation (Day)	0.143	2	7.169E-02	7.885	0.001
Fungal culture (FRR)	0.180	5	3.599E-02	3.959	0.006
Day*FRR	0.108	10	1.082E-02	1.190	0.330
Error	0.327	36	9.092E-02		
Total	29.966	54			
Corrected total	0.759	53			



Note: Superscripts are compared within the figure.



Note: Superscripts are compared within the figure.

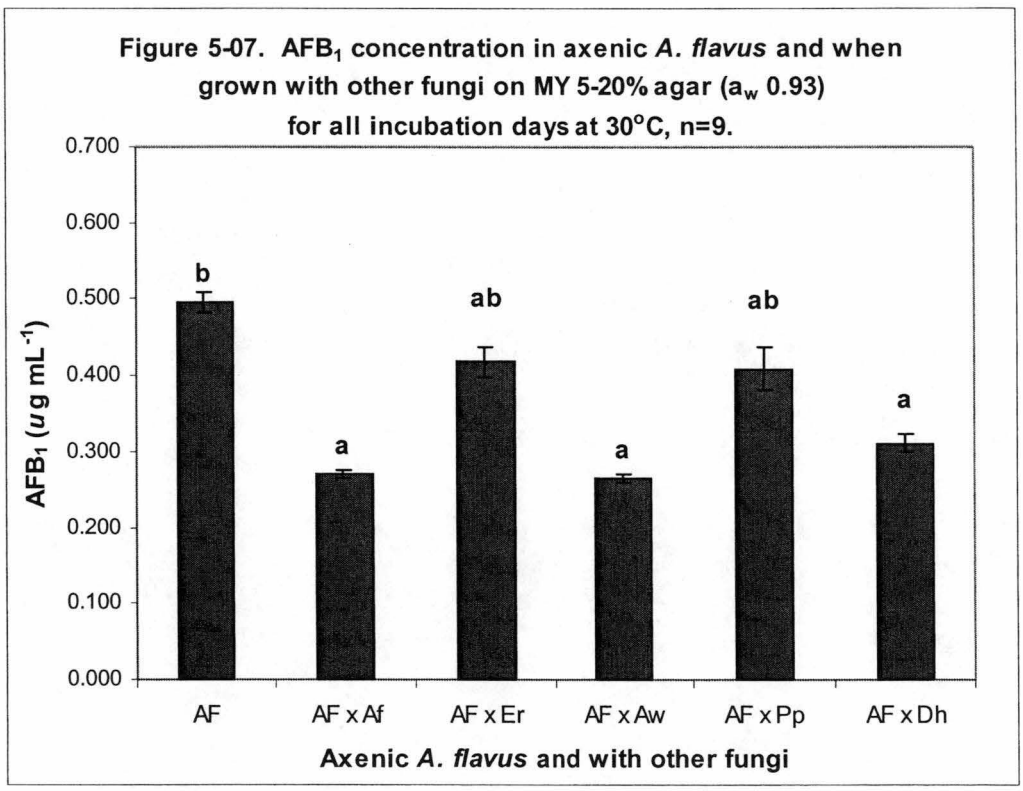


Note: Superscripts are compared within the figure.

On the other hand, AFB_1 concentration in axenic *A. flavus* was 0.534 μg mL^{-1} at day 7, 0.374 μg mL^{-1} at day 14 and 0.579 μg mL^{-1} at day 21. AFB_1 concentration in *A. flavus* was significantly ($p \leq 0.05$) affected by the fungal cultures and the interaction of culture with length of incubation. However, length of incubation alone had no significant effect (Table 5-06). Therefore, Tukey's test was performed on the fungal cultures as a single factor for the data pooled at all incubation days (Figure 5-07). Only the presence of non-toxigenic *A. flavus, A. wentii* and *D. hansenii* significantly ($p \leq 0.05$) reduced AFB_1 concentration in *A. flavus.* The reduction of AFB_1 in *A. flavus* was 45% by non-toxigenic *A. flavus* and *A. wentii* and 37% by *D. hansenii.*

Table 5-06. ANOVA of AFB_1 concentration in *A. flavus* grown axenically and with other fungi on MY5-20% agar (a_w 0.93) at 7, 14 and 21 days at 30°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.887	17	5.217E-02	3.346	0.001
Intercept	7.054	1	7.054	452.465	0.000
Incubation (Day)	8.478E-02	2	4.239E-02	2.719	0.079
Fungal culture (FRR)	0.395	5	7.891E-02	5.061	0.001
Day*FRR	0.408	10	4.076E-02	2.614	0.017
Error	0.561	36	1.559E-02		
Total	8.503	54			
Corrected total	1.448	53			



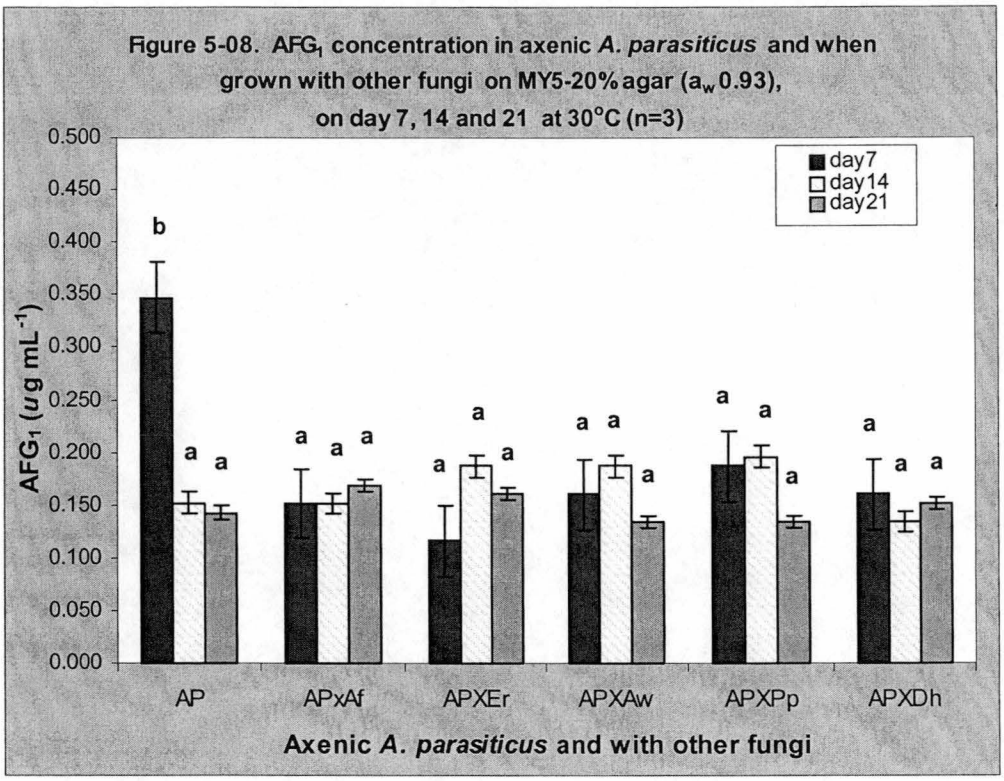
Note: Superscripts are compared within the figure

5.3.4. AFG_1 concentration in *A. parasiticus* and *A. flavus*

Table 5-07 shows that all factors singly or in combination significantly affected AFG_1 concentration in *A. parasiticus* at $p \leq 0.05$. Figure 5-08 shows that compared to AFG_1 in axenic *A. parasiticus* at day 7, the presence of all other fungi significantly reduced AFG_1 concentration in *A. parasiticus* by about 40-60%. However, the concentration of AFG_1 in *A. parasiticus* was significantly reduced after 7 days, so that there was no longer any significant difference in AFG_1 concentration in *A. parasiticus* at 14 and 21 days.

Table 5-07. ANOVA of AFG_1 concentration in *A. parasiticus* grown axenically and with other fungi on MY5-20% agar (a_w 0.93) at 7, 14 and 21 days at 30°C (n=3)

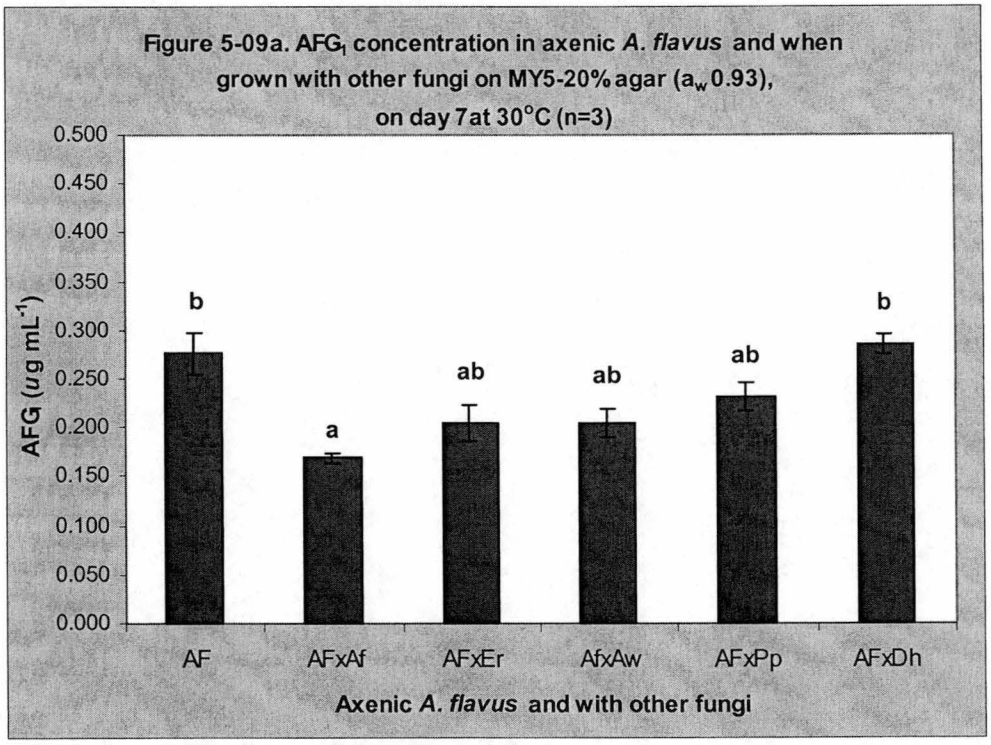
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.131	17	7.729E-03	5.740	0.005
Intercept	1.553	1	1.553	1153.422	0.000
Incubation (Day)	1.238E-02	2	6.192E-03	4.598	0.017
Fungal culture (FRR)	2.700E-02	5	5.399E-03	4.010	0.005
Day*FRR	9.202E-02	10	9.202E-03	6.833	0.000
Error	4.848E-02	36	1.347E-02		
Total	1.733	54			
Corrected total	0.180	53			



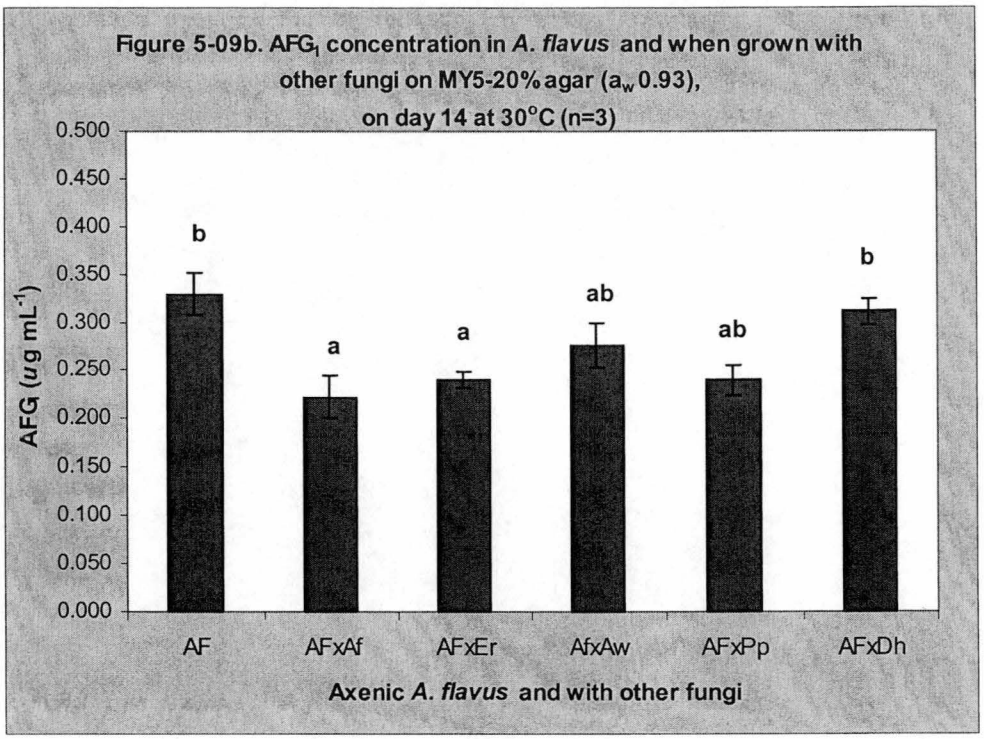
AFG_1 concentration in *A. flavus* was significantly affected by the fungal culture and length of incubation ($p \leq 0.05$), however, interaction between fungal culture and length of incubation was not significant (Table 5-08). Therefore, Tukey's test was performed on each day of incubation. Throughout incubation, the non-toxigenic *A. flavus* reduced AFG_1 concentration in *A. flavus* by 38, 32 and 40% at 7, 14 and 21 days, respectively (Figure 5-09a, 5-09b and 5-09c). The only other significant reduction AFG_1 concentration in *A. flavus* (27%) was after 14 days with *E. rubrum*. Neither *A. wentii, P. pisce* nor *D. hansenii* significantly affected the concentration of AFG_1 in *A. flavus* any time.

Table 5-08. ANOVA of AFG_1 concentration in *A. flavus* grown with other fungi on MY5-20% agar (a_w 0.93) at 7, 14 and 21 days at 30°C (n=3)

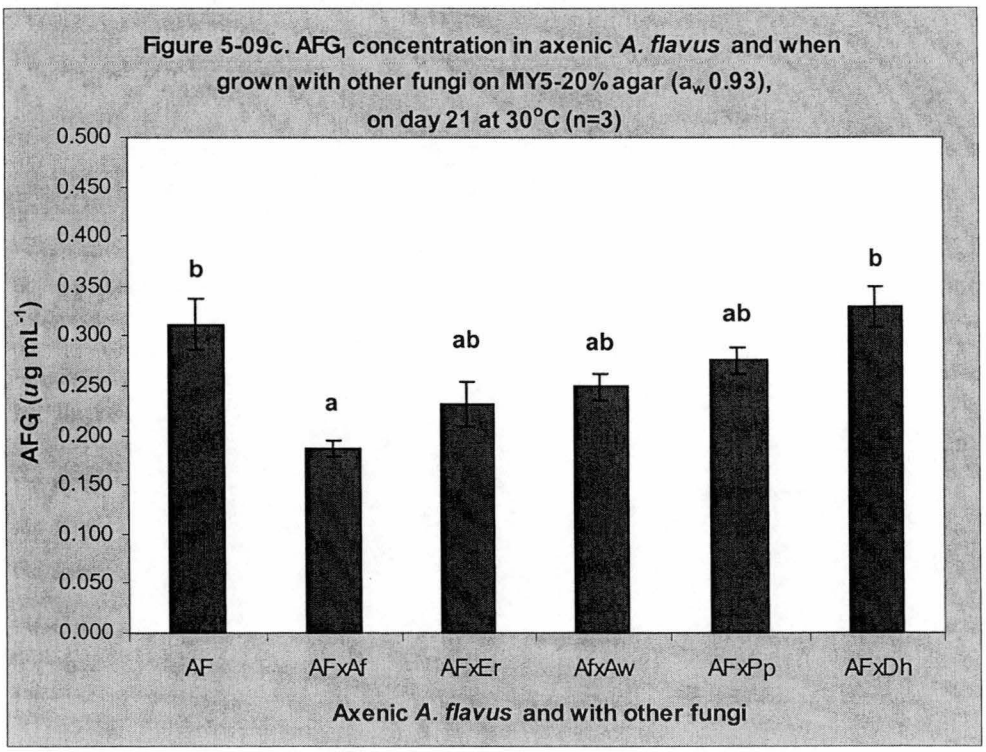
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.118	17	6.939E-03	2.577	0.008
Intercept	3.488	1	3.488	1295.078	0.000
Incubation (Day)	1.817E-02	2	9.083E-03	3.373	0.045
Fungal culture (FRR)	9.294E-02	5	1.859E-02	6.902	0.000
Day*FRR	6.865E-03	10	6.865E-04	0.225	0.987
Error	9.695E-02	36	2.693E-03		
Total	3.703	54			
Corrected total	0.215	53			



Note: Superscripts are compared within the figure.



Note: Superscripts are compared within the figure.



Note: Superscripts are compared within the figure.

5.3.5 AFB_2 and AFG_2 concentrations in *A. parasiticus* and *A. flavus*

Observed at day 7, *A. parasiticus* grown axenically and in interaction with other fungi produced AFB_2 and AFG_2. Concentrations of AFB_2 and AFG_2 were lower than AFB_1 and AFG_1. The presence of other fungi reduced the concentrations of AFB_2 and AFG_2 when compared to axenic *A. parasiticus* (Table 5-09). Non-toxigenic *A. flavus* caused more than 50% reduction in AFB_2 and AFG_2 concentrations in *A. parasiticus,* however, the reductions were not statistically significant. At day 14, concentrations of AFB_2 and AFG_2 in axenic *A. parasiticus* were about 0.071 μg mL^{-1} and 0.089 μg mL^{-1}, respectively. AFB_2 and AFG_2 were not observed either in *A. parasiticus* grown with all other fungi at day 14 or in axenic *A. parasiticus* at day 21.

Table 5-09. AFB_2 and AFG_2 concentration in axenic *A. parasiticus* and when grown with other fungi on MY5-20% agar (a_w 0.93) on day 7 at 30°C (n=3)

Fungal cultures	AFB_2 (μg mL^{-1}) Mean ± SE	AFG_2 (μg mL^{-1}) Mean ± SE
Axenic *A. parasiticus*	0.062 ± 0.01a	0.104 ± 0.02a
A. parasiticus x non-toxigenic *A. flavus*	0.027 ± 0.04a	0.027 ± 0.04a
A. parasiticus x *E. rubrum*	0.045 ± 0.03a	0.071 ± 0.03a
A. parasiticus x *A. wentii*	0.036 ± 0.03a	0.062 ± 0.01a
A. parasiticus x *P. pisce*	0.045 ± 0.04a	0.062 ± 0.01a
A. parasiticus x *D. hansenii*	0.036 ± 0.04a	0.036 ± 0.04a

Note: a = common letter not statistically significant.

Similarly, axenic *A. flavus* produced AFB_2 at day 7, however, the concentration was reduced in the presence of other fungi (Table 5-10), but their reduction was not statistically significant. The mean concentration of AFB_2 in axenic *A. flavus* was 0.097 μg mL^{-1}, 0.080 μg mL^{-1} and 0.071 μg mL^{-1}at day 7, 14 and 21. From 14 days, AFB_2 was not found in *A. flavus* interacted with other fungi. In this trial, AFG_2 was not observed in *A. flavus*.

Table 5-10. AFB_2 concentration in axenic *A. flavus* and when grown with other fungi on MY5-20% agar (a_w 0.93) on day 7 at 30°C (n=3)

Fungal cultures	AFB_2 (μg mL^{-1}) Mean ± SE
Axenic *A. flavus*	0.097 ± 0.02a
A. flavus x non-toxigenic *A. flavus*	0.027 ± 0.04a
A. flavus x *E. rubrum*	0.071 ± 0.03a
A. flavus x *A. wentii*	0.045 ± 0.04a
A. flavus x *P. pisce*	0.060 ± 0.01a
A. flavus x *D. hansenii*	0.036 ± 0.04a

Note: a = common letter not statistically significant.

5.3.6 Fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2 in *A. parasiticus* and *A. flavus*

AFB_1 and AFB_2 had bright blue fluorescence and AFG_1 and AFG_2 had bright green fluorescence. The mean Rf value of standard AFB_1 was 0.509, AFB_2 was 0.453, AFG_1 was 0.408 and AFG_2 was 0.358 (Chapter 3). Examined under UV light, axenic cultures of non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii*, *P. pisce* and *D. hansenii* did not produce any fluorescent compounds. However, under visible light, yellow-brown spots were observed along the migration line of axenic *E. rubrum* on TLC.

Axenic *A. parasiticus* produced pale blue-green fluorescent compounds at Rf values 0.10-0.22 (abbreviated: BG1) and the fluorescent intensities increased at 14 and 21 days. Fluorescent intensities of these compounds, however, were less than that of the AFB_1 and AFG_1. The presence of non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii*, *P. pisce* and *D. hansenii* reduced the fluorescent intensity of BG1 in *A. parasiticus*. Also, other blue-green fluorescent compounds were observed in *A. parasiticus* at Rf values of 0.50-0.60 (abbreviated: BG2) after 14 days. However, these blue-green fluorescent compounds were not detected in the presence of non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii*, *P. pisce* or *D. hansenii* (Table 5-11).

Axenic *A. flavus* produced similar blue-green fluorescent compounds to *A. parasiticus*. The Rf values of these blue-green fluorescent spots were about 0.10-0.22 (BG1) and 0.50-0.60 (BG2). The intensity of BG1 compounds in axenic *A. flavus* increased at day 21, however, the presence of all other fungi reduced its intensity. On the other hand, BG2 was observed in *A. flavus* after 14 days and only *A. wentii, P. pisce* and *D. hansenii* reduced the fluorescent intensity of these compounds (Table 5-12).

Table 5-11. Other fluorescent compounds produced by axenic *A. parasiticus* and when grown with other fungi on MY5-20% agar (a_w 0.93) at 30°C

Fungal cultures	Day 7		Day 14		Day 21	
	BG1	BG2	BG1	BG2	BG1	BG2
Axenic *A. parasiticus*	++	-	+++	+	+++	+
AP[1] x *A. flavus* (non-toxic)	+	-	+	-	+	-
AP[1] x *E. rubrum*	+	-	+	-	+	-
AP[1] x *A. wentii*	+	-	+	-	+	-
AP[1] x *P. pisce*	+	-	+	-	+	-
AP[1] x *D. hansenii*	+	-	+	-	+	-

Note: - = absent; + = present; ++ = strong intensity; +++ = very strong intensity
AP[1] = *A. parasiticus*.

Table 5-12. Other fluorescent compounds produced by axenic *A. flavus* and when grown with other fungi on MY5-20% agar (a_w 0.93) at 30°C

Fungal cultures	Day 7		Day 14		Day 21	
	BG1	BG2	BG1	BG2	BG1	BG2
Axenic *A. flavus*	++	-	++	++	+++	++
AF[1] x *A. flavus* (non-toxic)	+	-	+	++	+	++
AF[1] x *E. rubrum*	+	-	+	++	+	++
AF[1] x *A. wentii*	+	-	+	+	+	+
AF[1]x *P. pisce*	+	-	+	+	+	+
AF[1] x *D. hansenii*	+	-	+	+	+	+

Note: - = absent; + = present; ++ = strong intensity; +++ = very strong intensity
AF[1] = *A. flavus*.

5.4 DISCUSSION

The presence of non-toxigenic *A. flavus* and the fish commensal fungi *E. rubrum*, *A. wentii*, *P. pisce* and *D. hansenii* grown with *A. parasiticus* or *A. flavus* caused several types of interactions and affected growth and AFB_1 and AFG_1 concentrations in various ways. Decrease in colony diameter of *A. parasiticus* and *A. flavus* by non-toxigenic strain of *A. flavus* was correlated to the reduction of AFB_1 and AFG_1 concentrations in both aflatoxigenic fungi, but was varied among the fungal commensals of fish. *A. parasiticus* produced much less AFB_2 and AFG_2 than AFB_1 and AFG_1. Both AFB_2 and AFG_2 were decreased in the presence of other fungi and were not found in *A. parasiticus* after 21 days. *A. flavus* produced AFB_2 at gradually decreasing concentrations throughout incubation. Other blue-green fluorescent compounds were found in *A. parasiticus* and *A. flavus*. These two aflatoxigenic fungi could have converted aflatoxins into other forms either with the presence of other fungi or endogenously as a result of prolonged age, temperature of incubation, nutrient depletion or other factors.

Endogenous degradation was observed previously (Chapter 4), and has also been reported by other researchers. Doyle and Marth (1978a) found that 9-day-old mycelia of *A. parasiticus* NRRL 2999 degraded AFB_1 and AFG_1 with the maximum rate of degradation occurring at 28°C and pH 6.5. These authors also reported that greater amounts of AFB_1 and AFG_1 in *A. parasiticus* were degraded by unheated mycelia than by heated mycelia (Doyle and Marth 1978b). Their experiment suggested that unheated mycelia released intracellular constituents that influenced degradation of AFB_1 and AFG_1 in *A. parasiticus*. Hamid and Smith (1987) noted that AFB_1 and AFG_1 degradation in *A. flavus* was an enzymatic process in which cytochrome P-450 monooxygenase was involved. In the present study, at 30°C and a_w 0.93, endogenous degradation of AFB_1 in axenic *A. parasiticus* occurred after 7 days in agreement with Hyunh

and Lloyd (1984). They found that after incubation for 14 days, the total amount of AFB_1 and AFG_1 in *A. parasiticus* grown on YES medium with 20% sucrose at 28°C declined gradually as the culture aged. In the present study, however, endogenous degradation of AFG_1 in *A. parasiticus* occurred one week earlier than Huynh and Lloyd (1984) reported. These authors did not report a_w of the medium used in their experiment, however, a 20% sucrose medium could be expected to have an a_w near 0.98 (Pitt and Hocking, 1997). Thus, a_w could affect degradation of aflatoxins. Furthermore, Faraj *et al.* (1993) noted that aflatoxin degradation in aflatoxigenic fungus increased at elevated temperature. They reported that after 5 days at 30°C, aflatoxin degradation in *A. flavus* CMI 102566 was initiated. In the present results here, no endogenous degradation of AFB_1 or AFG_1 was seen in *A. flavus* after 7 days.

After the first week, reductions of colony diameter by non-toxigenic *A. flavus* were demonstrated in *A. parasiticus* and *A. flavus*. The reduction in growth of *A. parasiticus* and *A. flavus* may have caused the reduction of AFB_1 and AFG_1 concentration observed in *A. parasiticus* and *A. flavus*. A study by Horn *et al.* (2000) reported that AFB_1 was inhibited by a non-aflatoxigenic strain of *A. flavus* in agar medium. Their study was based on the concept of strain competition in which non-aflatoxigenic strain has the same niche as the aflatoxigenic strain and, as a consequence, effectively competes with it. Horn *et al.* (2000) reported that inhibition of AFB_1 production was due to nutrient competition. Thus, non-toxigenic *A. flavus* (FRR 4279) used in the present study may have out competed *A. parasiticus* and *A. flavus*, thereby reducing the growth and aflatoxin production of the aflatoxigenic strains. Because inhibition by non-toxigenic *A. flavus* occurred at distance, this fungus probably produced some soluble compounds or metabolites that were excreted into the medium. It is possible that the metabolites inhibited the growth and/or aflatoxin production of *A. parasiticus* and *A. flavus*. Microorganisms secreting antifungal and/or anti-aflatoxigenic compounds into the medium have been reported. For example; Cotty and Bhatnagar (1994) reported that atoxigenic strain of *A. flavus* (AF36)

produced some compounds that inhibited aflatoxin synthesis thereby reducing aflatoxin contamination. Coallier and Idziak (1985) found that *Streptococcus lactis* excreted a metabolite at early stationary phase and consequently inhibited aflatoxin production in *A. flavus*. Munimbazi and Bullerman (1998) isolated from dried fish antifungal metabolites of *Bacillus pumilus* that inhibited mycelial growth of *Aspergillus, Penicillium* and *Fusarium.*

Reduction of the colony diameter of *A. parasiticus* was demonstrated by *E. rubrum*. Wheeler and Hocking (1993) noted that *E. rubrum* and *A. wentii,* over a wide range of a_w and temperature in glucose-based media, were highly competitive species when compared to other fungal commensals of fish e.g. *P. pisce, Basipetospora halophila* and *A. penicillioides*. They found that *E. rubrum* at a_w of 0.98, 0.95, 0.90 and 0.84 and temperatures of 15, 25 and 30°C out competed the other fungi. Thus, it was expected that *E. rubrum* would grow well in the experimental conditions in the present study. *E. rubrum* decreased the amount of AFB_1 and AFG_1 concentrations in *A. parasiticus,* but this reduction was only significant at day 7. *E. rubrum* may have produced some toxic compounds directed against *A. parasiticus* or *A. flavus*. Frisvad and Samson (1991) reported that *E. rubrum* produces a range of toxic compounds, however, confirmation of their toxicity is still lacking. Likewise, any toxin produced by *E. rubrum* may cause other adverse effects if it were to be used as a probiotic to detoxify aflatoxins.

In the present study, *A. wentii* significantly reduced AFB_1 and AFG_1 production in *A. parasiticus* only at 14 days, however the fungus did not reduce the growth of *A. parasiticus* and *A. flavus*. Although *A. wentii* is a xerophile that exhibits strong growth in both high sugar and salt environments (Pitt and Hocking, 1997), the fungus in the present study was out competed by both aflatoxigenic fungi. It is likely that *A. wentii* would have directly affected the reduction of AFB_1 and AFG_1. Although colony diameter of both *A. parasiticus* and *A. flavus* were decreased a little, they outgrew *P. pisce* and *D. hansenii*

colonies. Wheeler and Hocking (1993) reported that *P. pisce* was more competitive at a_w 0.90 on NaCl-based agar medium than on sugar-based medium. It was concluded that this was because the fungus was isolated from dried salted fish. *D. hansenii* also has a high salt tolerance (Pitt and Hocking, 1997). These two fungi caused a reduction of AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*, but this was not significant under the experimental conditions.

Other blue-green fluorescent compounds found in *A. parasiticus* and *A. flavus* were possibly the product of aflatoxin conversion or degradation. The intensity of these compounds was less than AFB_1 or AFG_1 fluorescence. These blue-green fluorescent compounds could have been formed as *A. parasiticus* and *A. flavus* cultures aged, or as a result of experimental conditions or because of the presence of other fungi. Heathcote (1984) reported that *A. flavus* in acidic medium produced AFB_{2a} and AFG_{2a}, hydroxy derivatives of AFB_2 and AFG_2. The Rf value of AFB_{2a} is 0.13 and has blue fluorescence and Rf value of AFG_{2a} is 0.10 and has green fluorescence. Teunisson and Robertson (1967) reported that *Tetrahymena pyriformis* converted AFB_1 into another blue-fluorescent substance with an intensity about one-half of unchanged AFB_1. They found the Rf value of this compound was 0.52 compared with 0.59 for AFB_1 and 0.55 for AFB_2. Detroy and Hesseltine (1969) found a blue-fluorescent compound with an Rf value of 0.57, lower than AFB_1 when crystalline AFB_1 was added to *Dactylium dendroides*, *Absidia repens* and *Mucor griseo-cyanus* grown on YES medium. Another study by Buchanan and Houston (1982) reported that a blue fluorescent pyrazine-containing compound of *A. parasiticus* grown on peptone-mineral salt medium had an Rf value 0.70 similar to AFB_1 and AFB_2. Therefore, medium composition, the reduction of a_w and/or the increase temperature in the present study possibly resulted in the production of the two blue-green fluorescent compounds in *A. parasiticus* and *A. flavus*.

It was previously observed (Chapter 4) that *A. parasiticus* and *A. flavus* produced a blue-green fluorescent compound with Rf values 0.22-0.27. Nout

(1989), who studied *Rhizopus oryzae* 581 and *Neurospora* 429 isolated from Indonesian foods, reported these two fungi reduced and transformed AFB_1 in *A. parasiticus* and *A. flavus* into other fluorescent compounds with Rf values higher than AFB_1. The a_w values of the media (groundnut extract, groundnut extract agar, shredded groundnut substrate) used in that study were 0.94-0.97. Furthermore, a study by Nakazato *et al.* (1990) found that *A. niger, E. herbariorum*, a *Rhizopus* sp. and non-aflatoxin-producing *A. flavus* converted AFB_1 reversibly into aflatoxicols, a reduction form of cyclopentenone carbonyl AFB_1. The aflatoxicols have two types of stereoisomers i.e. aflatoxicol A and aflatoxicol B. These researchers reported that AFB_1 was first converted into aflatoxicol A by the fungi and that this is further converted to aflatoxicol B by the actions of medium components or by organic acids produced by the fungi. They noted that intracellular enzymes produced by the four fungi mediated the conversion of AFB_1 and aflatoxicols in their study. In the present study, the soluble compounds secreted by non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii* into the medium may contain enzymes that affected the degradation of aflatoxin. Thus, these fungi could possibly have degraded or transformed aflatoxins produced by *A. parasiticus* and *A. flavus* into other forms. The experimental conditions could also affect the Rf values of these fluorescent compounds, however, these could be another breakdown products of aflatoxin degradation.

This study concluded that non-toxigenic *A. flavus* had a consistent effect on reducing colony diameter and AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*. Its interaction with both aflatoxigenic fungi was similar to the previous study (Chapter 4), at high a_w (0.99) medium. The fungal commensals of dried fish, on the other hand, may be able to decrease AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus* if they grow on the much lower a_w medium or on their natural substrate. Further studies need to be done to clarify the capabilities of other fungi against *A. parasiticus* and *A. flavus*. The results demonstrated little consistent inhibition of aflatoxin production by the fish-commensal fungi.

However, the experimental conditions may not have been the most appropriate. For example, if the fungal commensals of dried fish were able to grow better, then any inhibitory effect may have been greater. Alternatively, the fish commensals could be grown beforehand and an extract applied to the aflatoxigenic strains. This is addressed in the next chapter.

CHAPTER 6

ANTIFUNGAL AND ANTI-AFLATOXIGENIC ACTIVITIES OF EXTRACTS FROM FUNGAL COMMENSALS OF DRIED FISH

6.1 INTRODUCTION

Compounds produced by several fungi have been found to decrease and/or inhibit aflatoxin production in *Aspergillus parasiticus* Speare and *A. flavus* Link. Nout (1989) underlined the influence of complex factors in the degradation of aflatoxin and on growth in *A. parasiticus* and *A. flavus* in mixed cultures with *Rhizopus* and *Neurospora* spp. Formation of substances inhibitory to the biosynthesis of aflatoxins, competition for nutrients or for aflatoxin precursors and biodegradation of aflatoxins which were formed previously, are factors that affect aflatoxin degradation. Shantha *et al.* (1990) noted metabolites produced by *A. niger* were able to inhibit biosynthesis of AFB_1 and degrade aflatoxin in *A. flavus*. Gourama and Bullerman (1995d, 1997) reported antifungal and anti-aflatoxigenic activity in a cell-free supernatant of lactic acid bacteria. They noted prevention of mold growth was related to acid pH or microbial competition, however, aflatoxin inhibition could not be linked to acid pH and microbial competition alone, but needed the presence of an inhibitor compound. Munimbazi and Bullerman (1998) documented antifungal and anti-aflatoxigenic compounds of *Bacillus pumilus* isolated from dried fish that were able to reduce mycelial growth and aflatoxin production of *A. parasiticus*.

Previous studies (Chapters 4 and 5) revealed that at a_w of 0.99 and 0.93 non-toxigenic *A. flavus* and the four fish commensal fungi (*Eurotium rubrum, A. wentii, Polypaecilum pisce* and *Debaryomyces hansenii*) had some variable effects in reducing growth and/or aflatoxin production by *A. parasiticus* and *A. flavus* and possible conversion of aflatoxins into breakdown products. At a_w of 0.99, both axenic *A. parasiticus* and *A. flavus* and in their interaction with other fungi produced a pale blue-green fluorescent compound with Rf value 0.22 and 0.27, respectively. At a_w 0.93, two blue-green fluorescent compounds at Rf values 0.11-0.22 and 0.50-0.60 were seen in axenic *A. parasiticus* and *A. flavus*. The intensity of these blue-green fluorescent compounds produced by both aflatoxigenic fungi was reduced in the presence of non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii*. It was concluded that active soluble

compounds, secreted into the medium by these five fungi, affected the aflatoxigenic species. Other researchers have noted that some metabolites produced by microorganisms were excreted into liquid medium (Coallier-Ascah and Idziak, 1985; Karunaratne *et al.*, 1990). Therefore, the objectives of the present study were to extract the metabolites produced by non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii* grown in agar and broth media. *A. parasiticus* and *A. flavus* were then grown in the presence of each of these extracts to determine the antifungal and anti-aflatoxigenic activities at a lower a_w (0.89).

6.2 MATERIAL AND METHODS

6.2.1 General Methods

Fungal culture preparation and aflatoxin and statistics analysis were the same as previously described in Chapter 3. Ten-day-old *A. parasiticus* and *A. flavus* were prepared to have 10^6 spores mL^{-1}. The medium was MY10-12% agar ($a_w = 0.89$, chosen as relevant to the a_w of dried fish) (Pitt and Hocking, 1997), temperature of incubation was 25°C and analysis of antifungal and anti-aflatoxigenic activities of the extracts was done after 10 days incubation. Extracts of non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii* were also screened on TLC. The presence of fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2 was recorded.

6.2.2 Experimental design

This experiment was a full factorial design in three replicates with treatments of two volumes (10 and 100 μL) of extracts and five extracts (non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii*) extracted from agar and broth media. All the extracts were challenged against the

aflatoxigenic species *A. parasiticus* and *A. flavus*. Control axenic *A. parasiticus* and *A. flavus* were also used.

6.2.3 Metabolites extraction

Fungal metabolites were extracted from the non-toxigenic *A. flavus* and the four fungal commensals of dried fish. The extraction protocol was based on the method described by Nakazato *et al.* (1990) and Munimbazi and Bullerman (1998). Ten-day-old cultures of all the fungi were inoculated into 20 mL agar and 50 mL broth media. CYA medium was used for non-toxigenic strain of *A. flavus* and *A. wentii*, CY20S for *E. rubrum*, MY5-12% for *P. pisce* and MEB for *D. hansenii*. Formula of the media was from Hocking *et al.* (1994). Agar was omitted in broth media. The agar cultures were incubated at 30°C and the broth cultures were put on a rotary shaker at 30°C for one week.

Agar cultures of the fungi were macerated with cold sterile 20 mL 0.05M phosphate buffer solution (PBS) pH 7.2 and then filtered through No.1 Whatman filter paper to remove the mycelia. The filtrate was then filtered through a membrane filter (pore size 0.2μm). The supernatant was placed in a 10 mL vial and vibrated for 10 minutes in ultrasonic bath (Unisonic FX8) filled with ice water, then centrifuged at 8,000Xg for 30 minutes (Heraeus Christ GMBH-UJ1) to obtain the extracts. The metabolite extracts from broth cultures were obtained from fluid and mycelial mat. This mixture was filtered through No.1 Whatman paper and then filtered through a membrane filter of 0.2 μm pore size. The pH was measured using an Activon S/N digital pH meter calibrated against buffers prepared at pH 4, 7 and 10. Mean triplicate measurements of pH for each metabolite extract were 6.8±0.02 (*A. flavus*), 5.8±0.02 (*E. rubrum*), 5.5±0.03 (*A. wentii*), 4.9±0.21 (*P. pisce*) and 3.5±0.01 (*D. hansenii*). All the extracts were kept at 5°C until used.

6.2.4 Inoculation

Ten and 100 µL of the metabolite extracts were aseptically applied onto the solidified MY10-12% agar in petri plates. The plates were left for one hour inside the laminar flow chamber, to allow the metabolite extracts to soak into the medium. *A. parasiticus* and *A. flavus* (10^6 spores mL^{-1}) were then inoculated at a volume of 10 µL on top of the metabolite extracts. Sterile 0.05M PBS applied at the same volumes (10 and 100 µL) was used as control for axenic *A. parasiticus* and *A. flavus* against the metabolites extracted from agar. Sterile broth medium was used as control for axenic *A. parasiticus* and *A. flavus* against the metabolites extracted from broth culture. The plates were incubated at 25°C for 10 days. Colony diameters of *A. parasiticus* and *A. flavus* were measured and the concentrations of aflatoxins were analysed as described in Chapter 3.

6.3 RESULTS

Axenic (control) *A. parasiticus* and *A. flavus* grew and the colony diameters were 14-22 mm and 22-30 mm, respectively. All axenic *A. parasiticus* and *A. flavus* formed aflatoxins. Control extracts did not produce fluorescent compounds. Generally, metabolites extracted from agar cultures showed a greater effect than those from broth cultures in reducing growth and eliminating or reducing AFB_1 and AFG_1 concentrations in *A. parasiticus* and *A. flavus.* Applied at 100 µL, all metabolites extracted from agar demonstrated some antifungal activities against *A. parasiticus* and *A. flavus,* however, the broth extracts showed variable effects. Anti-aflatoxigenic activities of the extracts varied in elimination or reduction of AFB_1 and AFG_1 concentrations in *A. parasiticus* and *A. flavus*. AFB_2 and AFG_2 were observed on axenic *A. parasiticus* and *A. flavus*, however, these two aflatoxins were eliminated in the presence of the extracts of non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii.* Both aflatoxigenic fungi treated with the extracts of these

five other fungi produced fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2.

6.3.1 Antifungal activities of the metabolites extracted from agar

Singly and in its combination of extracts and volume all treatments significantly affected the growth of *A. parasiticus* at $p \leq 0.05$ (Table 6-01). Applied at 100 µL, extracts of non-toxigenic *A. flavus*, *A. wentii, D. hansenii* and *E. rubrum* significantly inhibited colony diameter of *A. parasiticus* after 10 days. Compared to other extracts, the non-toxigenic *A. flavus* extract at 100 µL caused the biggest reduction (41%) in colony diameter of *A. parasiticus.* Extracts of *A. wentii* and *D. hansenii* decreased colony diameter *A. parasiticus* by 36 and 32%, respectively. At 10 and 100 µL, the *E. rubrum* extract reduced the colony diameter *A. parasiticus* by 28 and 29%, respectively. The *P. pisce* extract, however, did not affect the colony diameter of *A. parasiticus* (Figures 6-01 and 6-02).

Table 6-01. ANOVA of colony diameter of *A. parasiticus* treated with extracts of agar cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	491.417	11	44.764	14.233	0.000
Intercept	11990.250	1	11990.250	3819.903	0.000
Metabolite	291.917	5	58.383	18.600	0.000
Volume	78.028	1	78.028	24.858	0.000
Metabolite*Volume	121.472	5	24.294	7.740	0.000
Error	75.333	24	3.139		
Total	12557.000	36			
Corrected total	566.750	35			

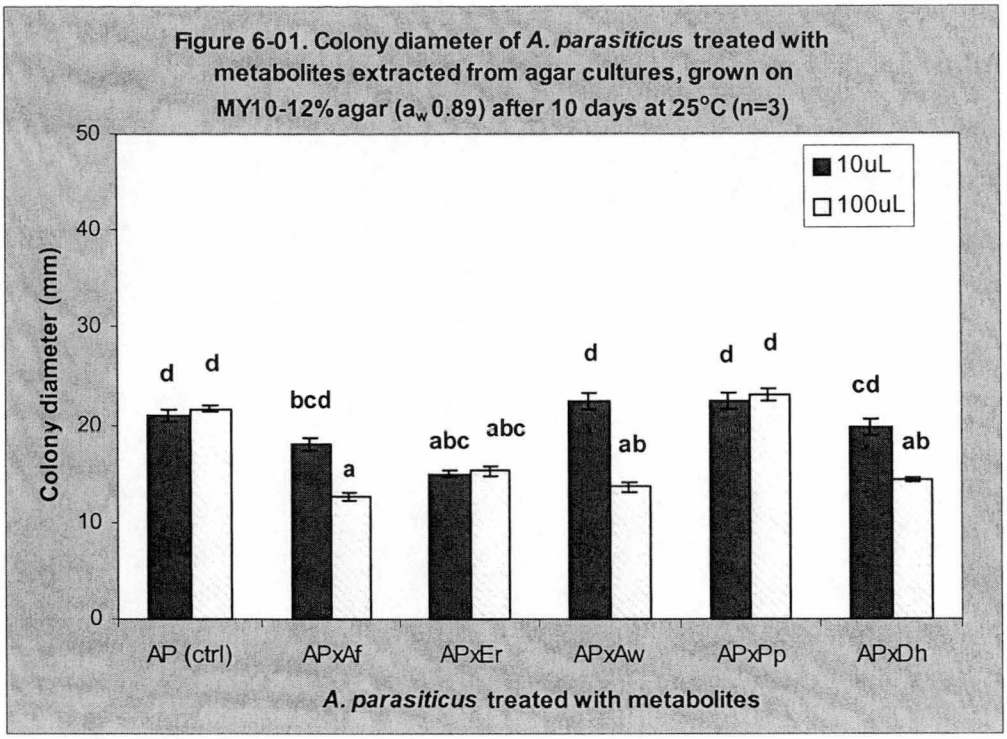
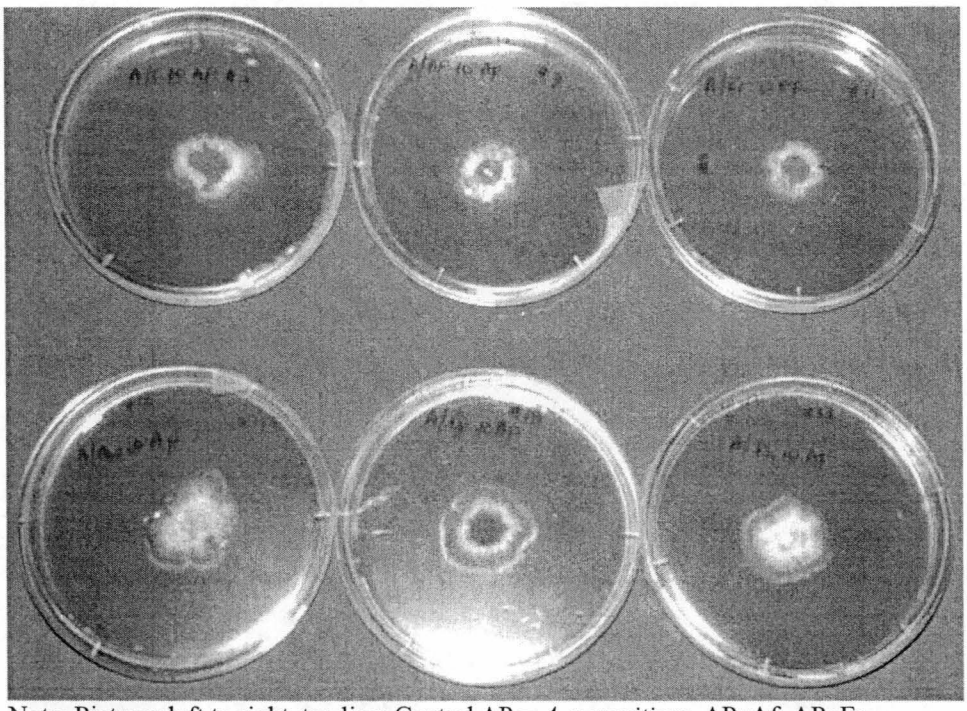


Figure 6-02. Growth of *A. parasiticus* (control) and when treated with extracts (10 μL) extracted from agar cultures on MY10-12% agar (a_w 0.89) after 10 days at 25°C



Note: Pictures left to right, top line: Control AP = *A. parasiticus*, APxAf, APxEr
Bottom line: APxAw, APxPp, APxDh

ANOVA of colony diameter of *A. flavus* showed that antifungal activity of the extracts applied at 10 and 100 μL and the interaction were significant at $p \leq 0.05$ (Table 6-02). The agar extract of *D. hansenii* significantly decreased colony diameter of *A. flavus* (Figures 6-03 and 6-04). *P. pisce* did not decrease colony diameter of *A. flavus* at all and the reminder only had an effect at the larger volume (100 μL).

Table 6-02. ANOVA of colony diameter of *A. flavus* treated with metabolites extracted from agar cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	929.417	11	84.492	18.547	0.000
Intercept	19182.250	1	19182.250	4210.738	0.000
Metabolite	618.250	5	123.650	27.143	0.000
Volume	164.694	1	29.294	36.152	0.000
Metabolite*Volume	146.472	5	4.556	6.430	0.001
Error	109.333	24			
Total	20221.000	36			
Corrected total	1038.750	35			

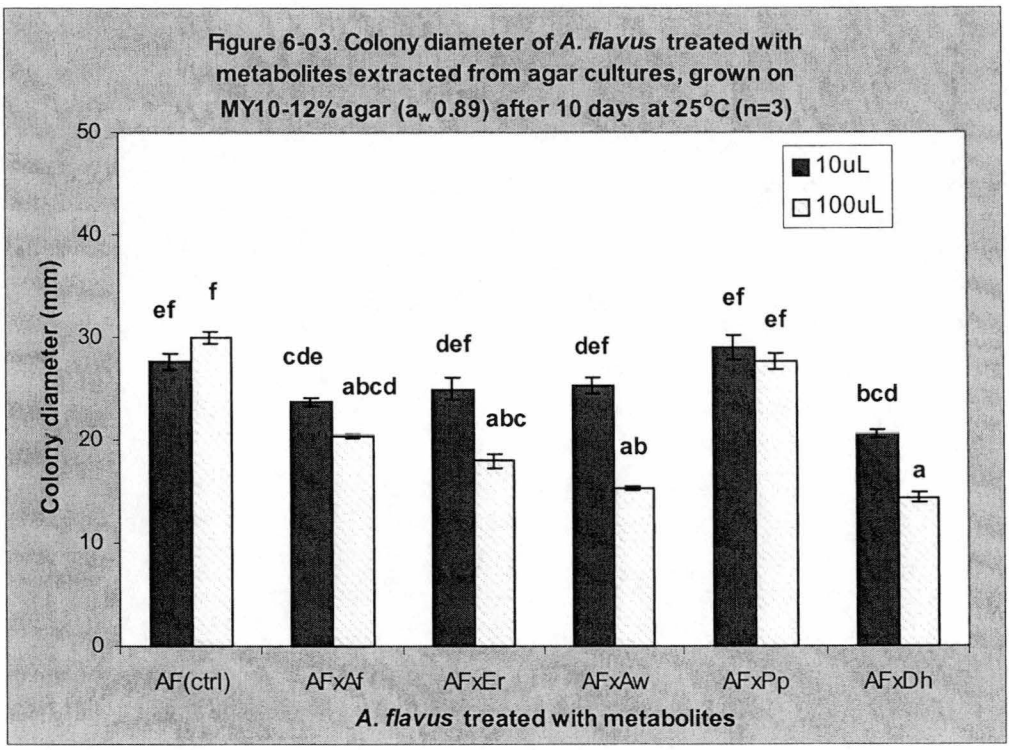
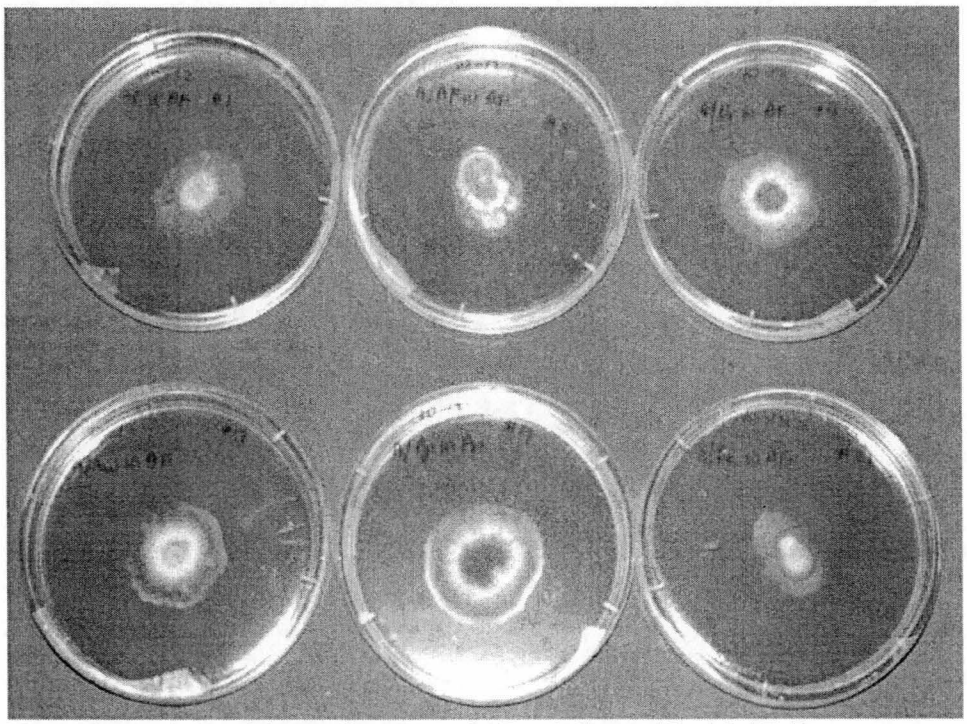


Figure 6-04. Growth of *A. flavus* (control) and when treated with metabolites (10 μL) extracted from agar cultures on MY10-12% agar (a_w 0.89) after 10 days at 25°C



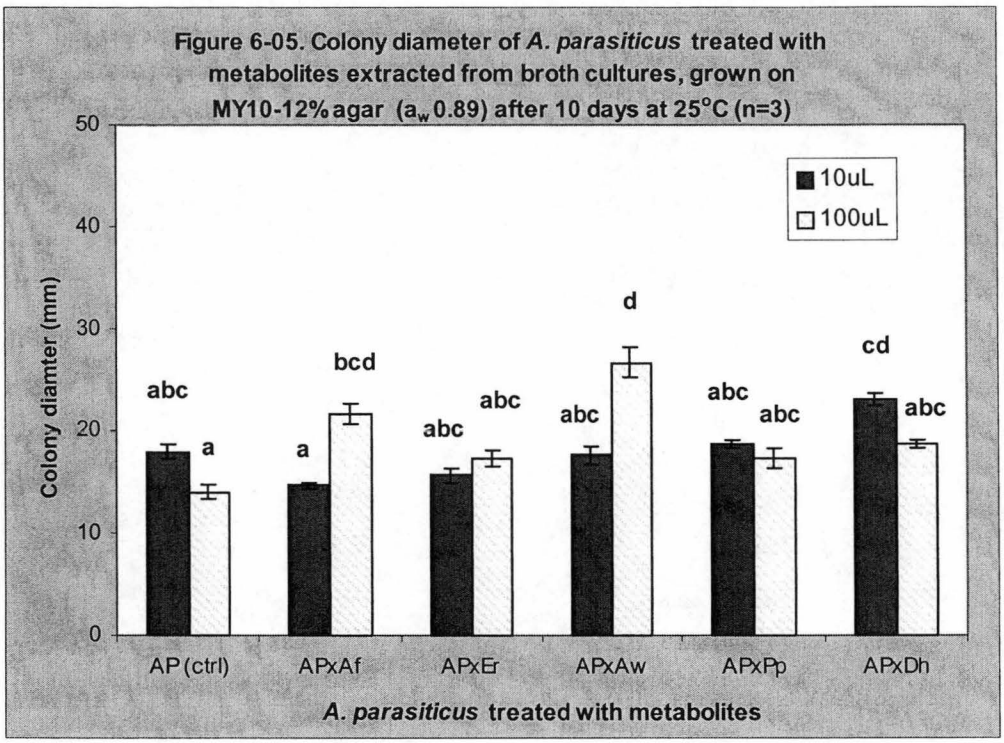
Note: Pictures left to right, top line: Control AF = *A. flavus*, AFx Af, AFxEr
Bottom line: AFxAw, AFxPp, AFxDh

6.3.2 Antifungal activities of the broth extracts

Table 6-03 represents ANOVA for the colony diameter of *A. parasiticus* treated with metabolites extracted from broth culture. Extracts (non-toxigenic *A. flavus, A. wentii, D. hansenii P. pisce* and *E. rubrum*) and their interaction had significant effect ($p \leq 0.05$) on colony diameter of *A. parasiticus*, but the volumes (10 or 100 μL) alone did not. The only statistically significant effect in fact demonstrated an increase in diameter (Figure 6-05). Thus, no antifungal activity was demonstrated by any of the extracts.

Table 6-03. ANOVA of colony diameter of *A. parasiticus* treated with extracts extracted from broth cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

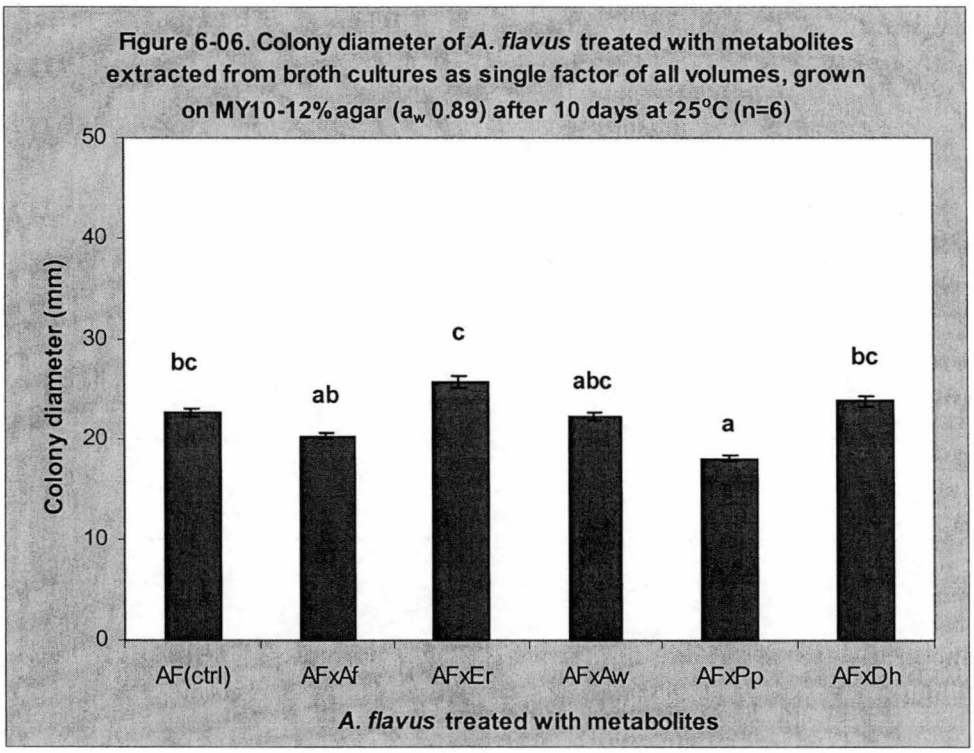
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	430.556	11	39.141	7.117	0.000
Intercept	12469.444	1	12469.444	2267.172	0.000
Metabolite	176.556	5	35.311	6.420	0.001
Volume	16.000	1	16.000	2.909	0.101
Metabolite*Volume	238.000	5	47.600	6.855	0.000
Error	132.000	24	5.500		
Total	13032.000	36			
Corrected total	562.556	35			



In *A. flavus*, antifungal activity of the broth extracts alone was significant at $p \leq 0.05$ on the colony diameter (Table 6-04). However, the volume applied and the interaction of extracts and volume showed no significant effect on the colony diameter of *A. flavus*. Figure 6-06 shows that only the *P. pisce* extract applied at both 10 and 100 µL significantly decreased (about 20%) the colony diameter of *A. flavus*. The extract of *E. rubrum* increased colony diameter of *A. flavus* but not significantly.

Table 6-04. ANOVA of colony diameter of *A. flavus* treated with metabolites extracted from broth cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	269.667	11	24.515	4.284	0.001
Intercept	17689.000	1	17689.000	3091.282	0.000
Metabolite	208.000	5	41.600	7.270	0.000
Volume	16.000	1	16.000	2.796	0.107
Metabolite*Volume	45.667	5	9.133	1.596	0.199
Error	137.333	24	5.722		
Total	18096.000	36			
Corrected total	407.000	35			



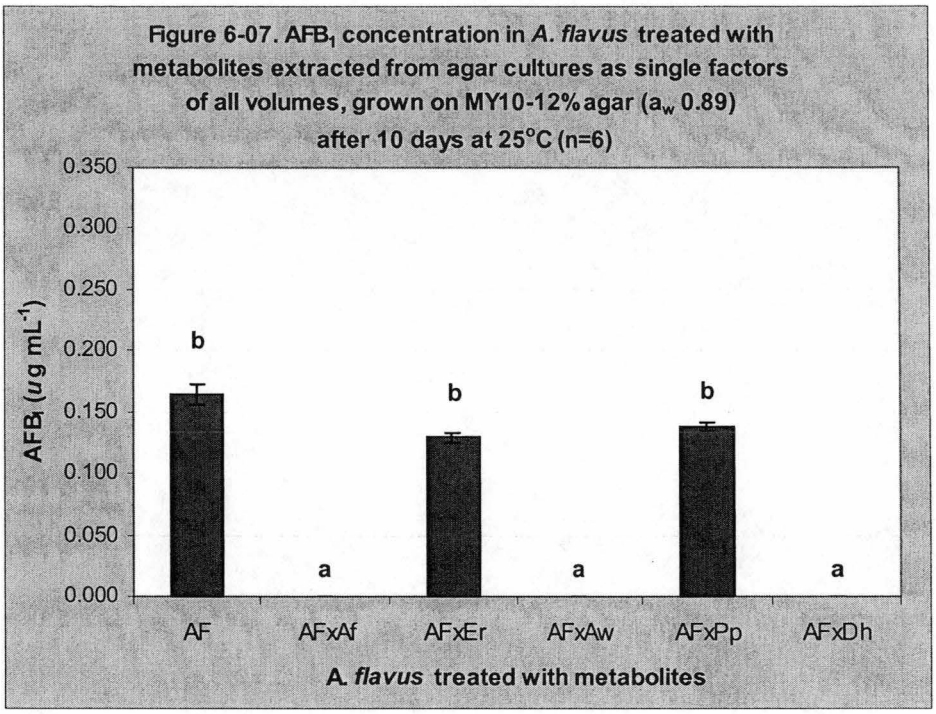
6.3.3 Anti-aflatoxigenic activities of the extracts on AFB_1 concentration in *A. parasiticus* and *A. flavus*

(a) Metabolites extracted from agar

No AFB_1 was found in *A. parasiticus* when treated with all extracts of non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii*, *P. pisce* and *D. hansenii*. Grown on MY10-12% agar (a_w 0.89), AFB_1 concentration in axenic *A. parasiticus* was about 0.116 $\mu g\ mL^{-1}$ after 10 days. In *A. flavus* after 10 days, only extracts of non-toxigenic *A. flavus*, *A. wentii* and *D. hansenii* completely eliminated AFB_1 concentration (Figure 6-07). Extracts of *E. rubrum* and *P. pisce* decreased AFB_1 concentration in *A. flavus* by about 35 and 29% respectively, however, the reductions were not significant. Table 6-05 shows the presence of extracts alone had significant effect ($p \leq 0.05$) on AFB_1 concentration in *A. flavus*. The volume and its combination did not significantly affect AFB_1 concentration in *A. flavus*.

Table 6-05. ANOVA of AFB_1 concentration in *A. flavus* treated with metabolites extracted from agar cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.198	11	1.797E-02	30.242	0.000
Intercept	0.190	1	0.190	320.133	0.000
Metabolite	0.194	5	3.872E-02	65.173	0.000
Volume	3.168E-04	1	3.168E-04	0.533	0.472
Metabolite*Volume	3.723E-03	5	7.446E-04	1.253	0.316
Error	1.426E-02	24	5.941E-04		
Total	0.402	36			
Corrected total	0.212	35			

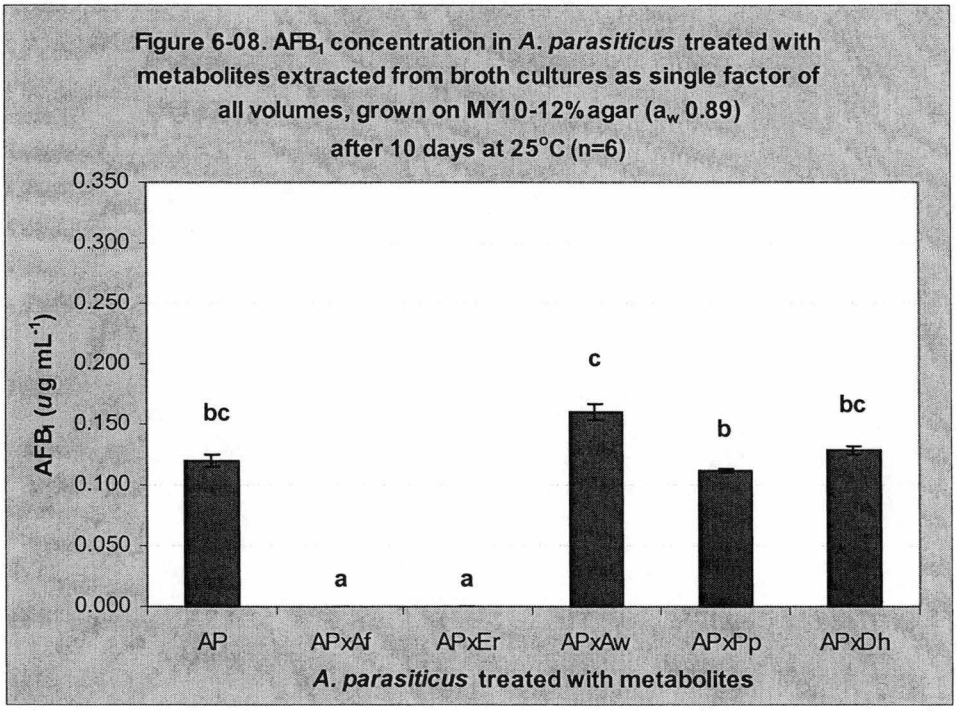


(b) Metabolites extracted from broth cultures

After 10 days, extracts of non-toxigenic *A. flavus* and *E. rubrum* completely eliminated AFB_1 concentration in *A. parasiticus* (Figure 6-08). Both 10 and 100μL of broth extracts had similar effects on AFB_1 concentration in *A. parasiticus*. Table 6-06 shows AFB_1 concentration in *A. parasiticus* was significantly affected ($p \leq 0.05$) by the broth extracts, but volume and its interaction with extracts were not significant. Extracts of *A. wentii, P. pisce* and *D. hansenii* showed no significant effect on AFB_1 concentration in *A. parasiticus* (Figure 6-08).

Table 6-06. ANOVA of AFB_1 concentration in *A. parasiticus* treated with metabolites extracted from broth cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.146	11	1.331E-02	25.857	0.000
Intercept	0.271	1	0.271	526.500	0.000
Metabolite	0.144	5	2.874E-02	55.823	0.000
Volume	4.951E-04	1	4.951E-04	0.962	0.337
Metabolite*Volume	2.238E-03	5	4.475E-02	0.869	0.516
Error	1.236E-02	24	5.149E-04		
Total	0.430	36			
Corrected total	0.159	35			

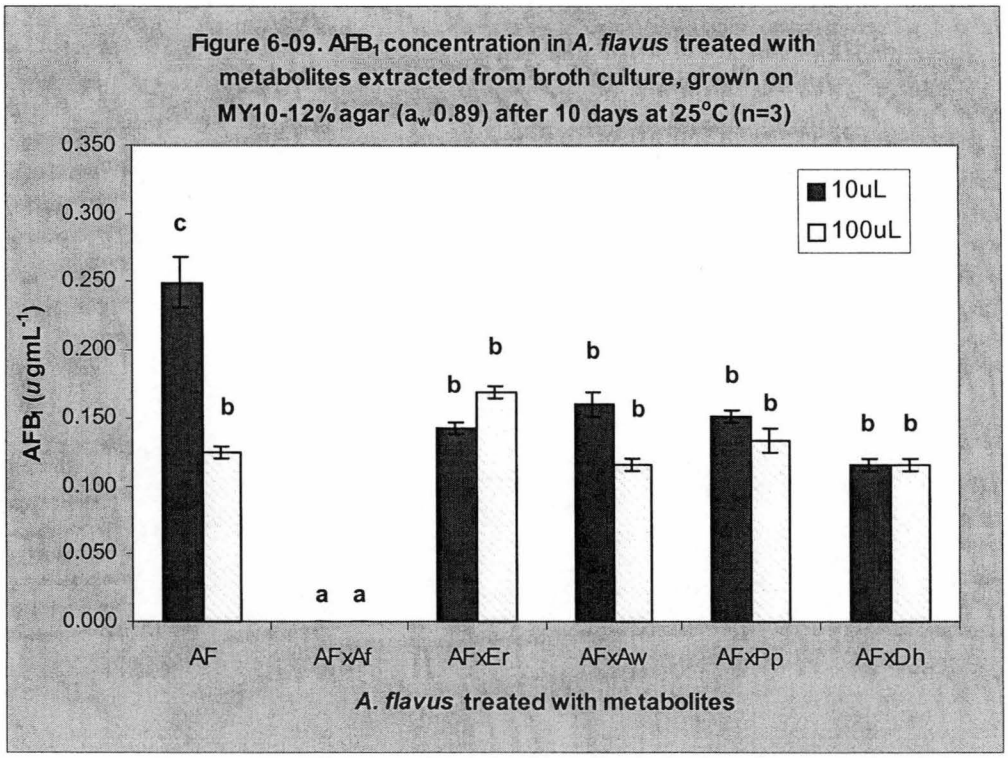


As shown in Table 6-07, the single factors (metabolite and volume) and their combination significantly affected ($p \leq 0.05$) AFB_1 concentration in *A. flavus*. The extract of non-toxigenic *A. flavus* completely eliminated AFB_1 concentration in *A. flavus*, however, the extracts from the fish commensals at 10 µL reduced AFB_1 concentration only in *A. flavus*. At the same volume applied,

AFB_1 concentration in axenic *A. flavus* was 0.250 μg mL^{-1}. The reduction was statistically significant and was of the order of 35-50% (Figure 6-09).

Table 6-07. ANOVA of AFB_1 concentration in *A. flavus* treated with metabolites extracted from broth cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.152	11	1.381E-02	25.249	0.000
Intercept	0.544	1	0.544	994.856	0.000
Metabolite	0.125	5	2.497E-02	45.654	0.000
Volume	6.257E-03	1	6.257E-03	11.438	0.002
Metabolite*Volume	2.080E-02	5	4.161E-03	7.606	0.000
Error	1.313E-02	24	5.470E-04		
Total	0.709	36			
Corrected total	0.165	35			



6.3.4 Anti-aflatoxigenic activities of the extracts on AFG_1 concentration in *A. parasiticus* and *A. flavus*

(a) Metabolites extracted from agar cultures

At $p \leq 0.05$, metabolites extracted from agar cultures had a significant effect on AFG_1 concentration in *A. parasiticus* (Table 6-08). However, neither the volumes nor their interaction significantly affected AFG_1 concentration in *A. parasiticus*. This is clearly shown in Figure 6-10, as the extracts of non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii* and *D. hansenii* applied at 10 or 100 µL, eliminated AFG_1 concentration in *A. parasiticus*. Extracts of *P. pisce,* on the other hand, had no effect on AFG_1 concentration in *A. parasiticus*.

Table 6-08. ANOVA of AFG_1 concentration in *A. parasiticus* treated with metabolites extracted from agar cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.149	11	1.354E-02	170.977	0.000
Intercept	7.369E-02	1	7.369E-02	930.250	0.000
Metabolite	0.148	5	2.958E-02	373.450	0.000
Volume	1.782E-04	1	1.782E-04	2.250	0.147
Metabolite*Volume	8.911E-04	5	1.782E-04	2.250	0.082
Error	1.901E-03	24	7.921E-05		
Total	0.225	36			
Corrected total	0.151	35			

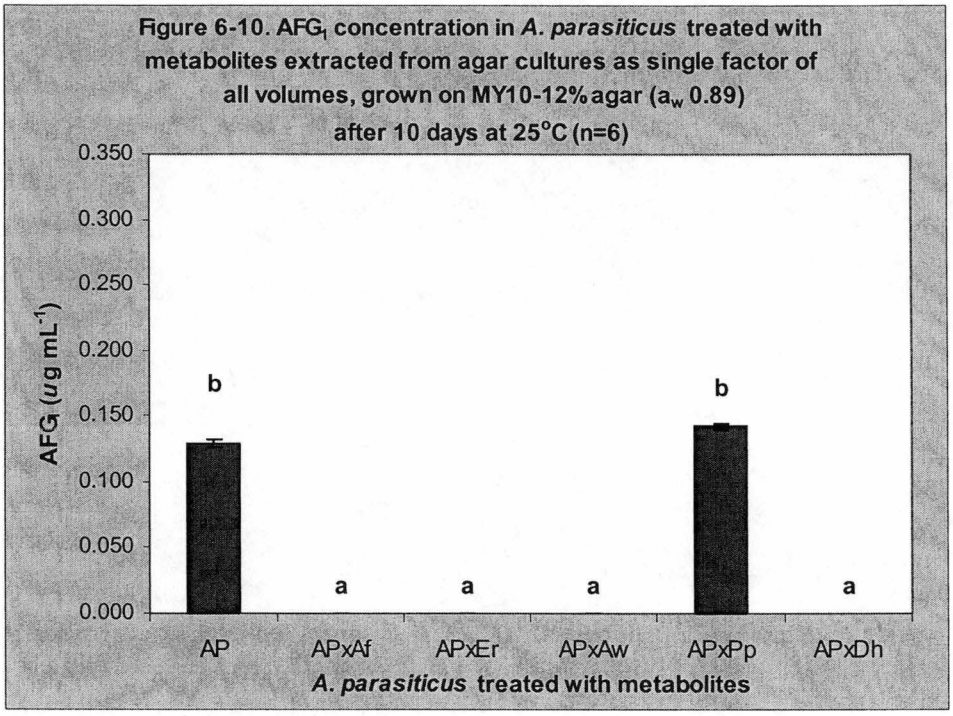
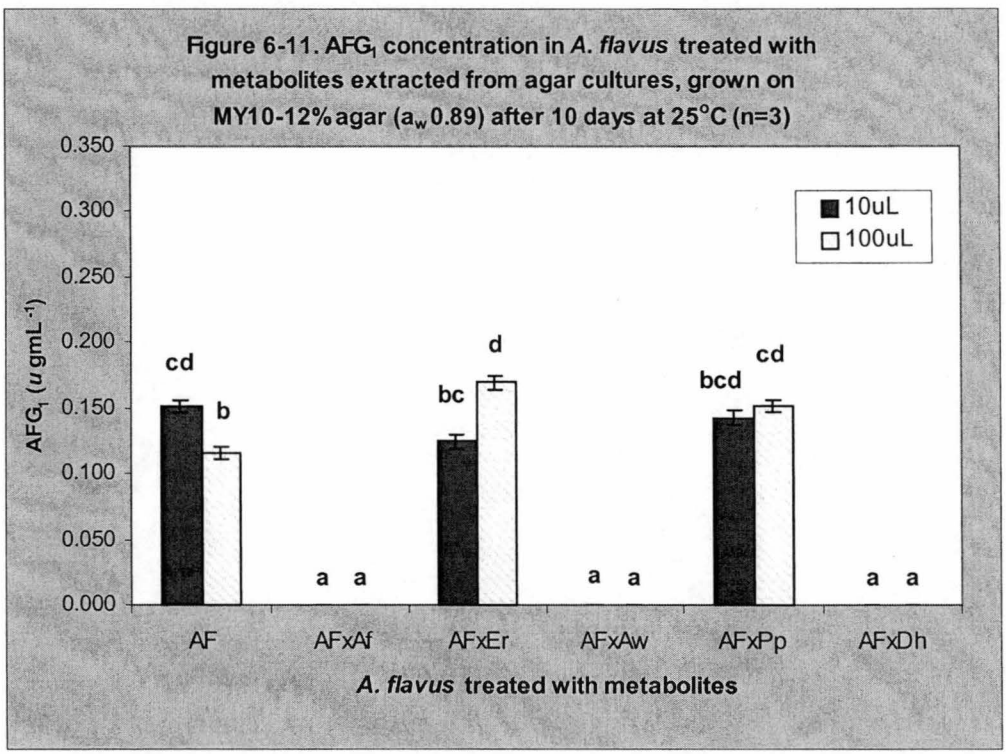


Table 6-09 shows that the extracts and their interaction with the volume applied significantly affected AFG_1 concentration in *A. flavus* at $p \leq 0.05$. The volume alone, however, did not have a significant effect. Extracts of non-toxigenic *A. flavus, A. wentii* and *D. hansenii* totally eliminated AFG_1 concentration in *A. flavus* (Figure 6-11). At 10 μL, *E. rubrum* and *P. pisce* extracts reduced AFG_1 concentration in *A. flavus* only by 17% and 6%, but these reductions were not significant. Conversely, applied at 100 μL the extracts of these two fungi increased AFG_1 concentration in *A. flavus*.

Table 6-09. ANOVA of AFG_1 concentration in *A. flavus* treated with metabolites extracted from agar cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.188	11	1.711E-02	144.000	0.000
Intercept	0.182	1	0.182	1536.000	0.000
Metabolite	0.183	5	3.664E-02	308.400	0.000
Volume	7.921E-05	1	7.921E-05	0.667	0.422
Metabolite*Volume	4.911E-03	5	9.822E-04	8.260	0.000
Error	2.852E-03	24	1.188E-04		
Total	0.374	36			
Corrected total	0.191	35			

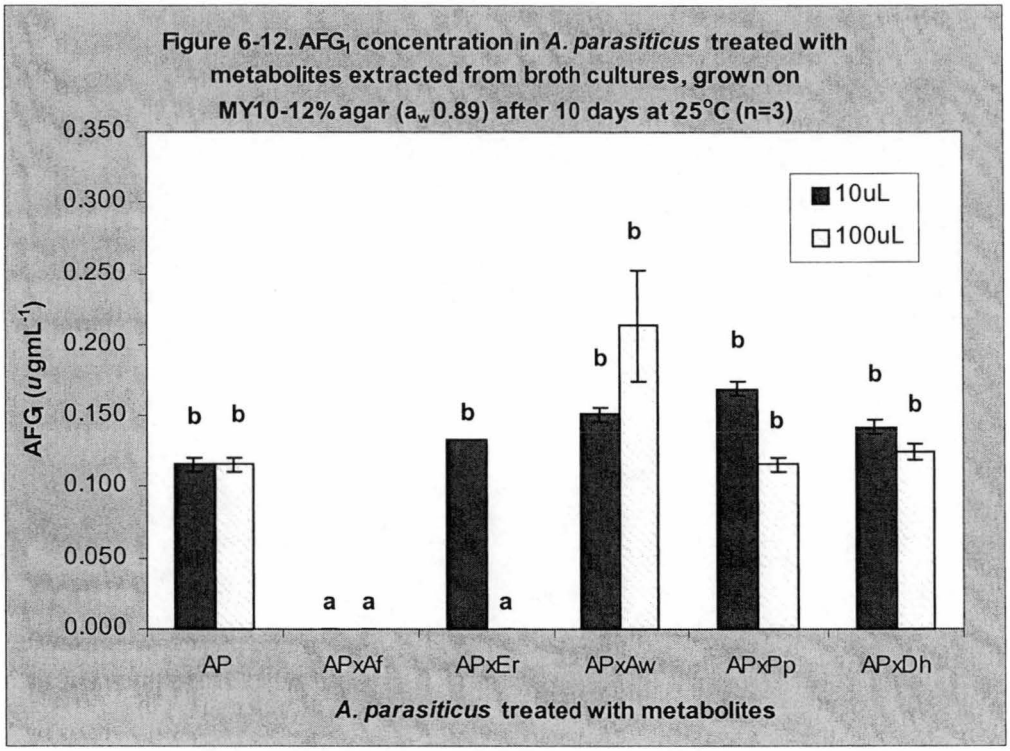


(b) Metabolites extracted from broth cultures

Table 6-10 shows that extracts and volume singly and their interaction significantly ($p \leq 0.05$) affected AFG_1 concentration in *A. parasiticus*. The extract of non-toxigenic *A. flavus* consistently eliminated AFG_1 in *A. parasiticus*. Similarly, the extract of *E. rubrum* at 100 µL eliminated AFG_1 in *A. parasiticus*. None of the other extracts affected AFG_1 concentration in *A. parasiticus* (Figure 6-12).

Table 6-10. ANOVA of AFG_1 concentration in *A. parasiticus* treated with metabolites extracted from broth cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

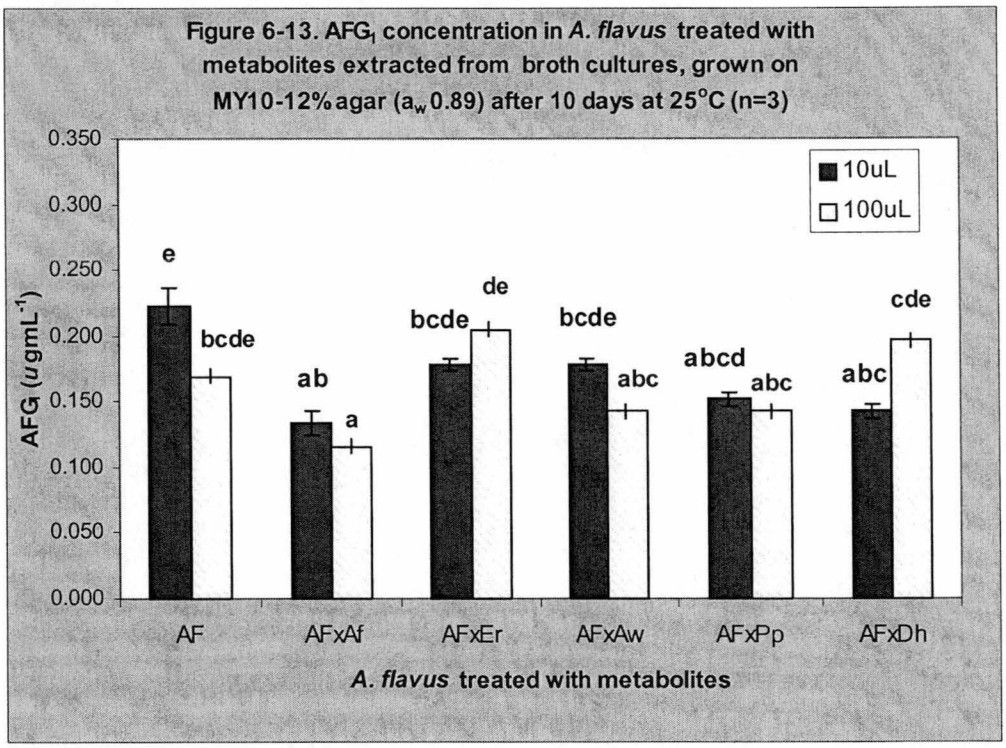
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.164	11	1.488E-02	11.562	0.000
Intercept	0.416	1	0.416	323.462	0.000
Metabolite	0.123	5	2.454E-02	19.068	0.000
Volume	5.723E-03	1	5.723E-03	4.446	0.046
Metabolite*Volume	3.527E-02	5	7.054E-03	5.480	0.002
Error	3.089E-02	24	1.287E-03		
Total	0.611	36			
Corrected total	0.195	35			



Extracts as a single factor and its interaction with volume significantly affected AFG_1 concentration in *A. flavus* at $p \leq 0.05$ (Table 6-11), but the volume alone did not significantly affect AFG_1. Only non-toxigenic *A. flavus*, for both inocula significantly reduced AFG_1 concentration in *A. flavus*. The reduction was 31% (Figure 6-13). Small, but statistically significant reductions in AFG_1 concentrations were also observed with extracts from *P. pisce* and *D. hansenii* applied at 10 μL.

Table 6-11. ANOVA of AFG_1 concentration in *A. flavus* treated with metabolites extracted from broth cultures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	3.398E-02	11	3.089E-03	7.800	0.000
Intercept	0.976	1	0.976	2462.200	0.000
Metabolite	2.186E-02	5	4.372E-03	11.040	0.000
Volume	3.168E-02	1	3.168E-04	0.800	0.380
Metabolite*Volume	1.180E-02	5	2.360E-03	5.960	0.001
Error	9.505E-03	24	3.960E-04		
Total	1.019	36			
Corrected total	4.349E-02	35			



6.3.5 Anti-aflatoxigenic activities of the extracts on AFB_2 and AFG_2 concentrations in *A. parasiticus* and *A. flavus*

At a_w 0.89, all controls of axenic *A. parasiticus* and *A. flavus* produced AFB_2 and AFG_2 (Table 6-12). All extracts of non-toxigenic *A. flavus, E. rubrum, A. wentii, P. pisce* and *D. hansenii* eliminated AFB_2 and AFG_2 concentrations in *A. parasiticus* and *A. flavus*.

Table 6-12. AFB_2 and AFG_2 concentrations in axenic *A. parasiticus* and *A. flavus* grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Axenic culture	Concentration (mean ± SE)	
	AFB_2 (μg mL^{-1})	AFG_2 (μg mL^{-1})
Control (agar):		
A. parasiticus	0.062 ± 0.02	0.071 ± 0.03
A. flavus	0.071 ± 0.02	0.081 ± 0.03
Control (broth):		
A. parasiticus	0.081 ± 0.02	0.081 ± 0.04
A. flavus	0.081 ± 0.02	0.089 ± 0.02

6.3.6 Activity of the extracts on other fluorescent compounds produced by *A. parasiticus* and *A. flavus*

The Rf value and fluorescence of AFB_1, AFB_2, AFG_1 and AFG_2 were as described in Chapter 3. Extracts alone did not produce any fluorescent compounds. However, yellow spots along the TLC migration line of *E. rubrum* were seen under visible light. In all replicate samples of axenic *A. parasiticus* and *A. flavus* and when treated with extracts of non-toxigenic *A. flavus, E. rubrum, A. wentii* and *P. pisce* from agar and broth, a greenish-blue fluorescence (GB) and a blue-green fluorescence (BG), with lower Rf values than aflatoxins, were observed.

Table 6-13. Other fluorescent compounds produced by *A. parasiticus* treated with extracts, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C.

Metabolite	Agar extract		Broth extract	
	GB fluorescence	BG fluorescence	GB fluorescence	BG fluorescence
Axenic A. parasiticus[1]	+	+	+	+
Non-toxigenic *A. flavus*	±	±	±	±
E. rubrum [2]	+	+	+	+
A. wentii	±	±	±	±
P. pisce	+	+	+	+
D. hansenii	-	-	±	-

Note: - = absent; ± = present but low intensity; + = present;
1 = fungal culture; 2 = produced yellow spots.

Table 6-14. Other fluorescent compounds produced by *A. flavus* treated with extracts, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C.

Metabolite	Agar extract		Broth extract	
	GB fluorescence	BG fluorescence	GB fluorescence	BG fluorescence
Axenic *A. flavus* [1]	+	+	+	+
Non-toxigenic *A. flavus*	±	±	±	±
E. rubrum [2]	+	+	+	+
A. wentii	±	±	±	±
P. pisce	+	+	+	+
D. hansenii	-	-	-	-

Note: - = absent; ± = present but low intensity; + = present;
1 = fungal culture; 2 = produced yellow spots.

The Rf value of the GB compound was about 0.10 and the Rf value of the BG compound was less than 0.30. The BG spot was smaller but almost similar to the appearance of AFB_1. The agar extract of *D. hansenii* eliminated these two fluorescent compounds in *A. parasiticus* and *A. flavus* (Tables 6-13 and 6-14), however, the broth extract applied at 100 µL only decreased the

intensity of GB compound in *A. parasiticus*. Other extracts reduced the fluorescence intensity of these compounds slightly.

6.4 DISCUSSION

Agar and broth extracts of fungal fish commensals *E. rubrum*, *A. wentii*, *P. pisce*, *D. hansenii* and the non-toxigenic strain of *A. flavus* were used to challenge growth and aflatoxin production. Grown at a_w 0.89, the colony diameters of both aflatoxigenic species *A. parasiticus* and *A. flavus* decreased 50-60% compared to their colony diameter at a_w 0.99 and 0.93 (Chapter 4 and Chapter 5). Generally, only when the extracts were applied in the larger amount (100 μL), were the colony diameters of *A. parasiticus* and *A. flavus* significantly reduced. Furthermore, the metabolite extracts from agar showed a greater activity in reducing the colony diameter and AFB_1 and AFG_1 concentrations in *A. parasiticus* and *A. flavus* than did the metabolites from broth extracts. All five fungal extracts from agar completely eliminated AFB_1 in *A. parasiticus*. Extracts of non-toxigenic *A. flavus*, *A. wentii* and *D. hansenii* from agar also eliminated AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*.

The extract of non-toxigenic *A. flavus* consistently reduced the colony diameter of *A. parasiticus* and *A. flavus*. The reduction in colony diameter of *A. parasiticus* and *A. flavus* caused by the extract of non-toxic *A. flavus* could have been correlated to the elimination of AFB_1 and AFG_1 in both these aflatoxigenic fungi. Of the broth metabolite extracts, non-toxigenic *A. flavus* and *E. rubrum* removed AFB_1 and AFG_1 in *A. parasiticus*. However, only the broth extract of non-toxigenic *A. flavus* eliminated AFB_1 in *A. flavus*. The broth extract of *P. pisce* reduced the colony diameter of *A. flavus*, but did not reduce AFB_1 and AFG_1 concentrations. It seems likely that the effects on growth and on AFB_1 and AFG_1 concentrations were not correlated in the present study. This implies a direct effect of the extracts on aflatoxin production and/or degradation rather than indirect effect via biomass reduction.

In the present study, each of the fungal metabolite extracts demonstrated different ways in which they affect the growth and aflatoxin concentration in *A. parasiticus* and *A. flavus*. A study by Nout (1989) reported that the presence of inhibitor species simultaneously decreased biomass and aflatoxin production. His experiment showed that heat-stable compounds produced by *Rhizopus* 581 and *Neurospora* 429 inhibited biomass production and AFB_1 synthesis in *A. parasiticus*. In the present study, metabolites produced by non-toxigenic *A. flavus* acted in a similar way as Nout (1989) found. The extract of non-toxigenic *A. flavus* demonstrated a consistent effect in reducing the growth and in elimination AFB_1 and AFG_1 in aflatoxigenic fungi. It is possible that the extract of non-toxigenic *A. flavus* has both antifungal and anti-aflatoxigenic activities on *A. parasiticus* and *A. flavus*. Similar inhibitory effects of the non-toxigenic *A. flavus* extract were also seen in the previous studies (Chapters 4 and 5). However, this mechanism does not appear to be common in other fungal metabolite extracts.

Wiseman and Marth (1981) reported variability in reduction of growth and aflatoxin concentration in *A. parasiticus* by *Streptococcus lactis*. Their experiment used three different treatments of inoculation of *S. lactis* and *A. parasiticus* i.e. the bacterium before the fungus, both grown simultaneously and the bacterium after the fungus. These authors noted inhibition of *S. lactis* variously reduced growth of *A. parasiticus* and decreased AFB_1 and AFG_1 concentration, or increased growth and reduced AFB_1, but increased AFG_1 production. This action was shown by extracts of *P. pisce* and *E. rubrum* in the present study. Extracted from agar and broth, the metabolites of these two fungi from dried fish demonstrated varying effects on growth and AFB_1 and AFG_1 concentrations in *A. parasiticus* and *A. flavus*. The extracts of *P. pisce* and *E. rubrum* may have antifungal and anti-aflatoxigenic activities, however, their stability varied between *A. parasiticus* and *A. flavus* and in different experimental conditions. The extract of *E. rubrum* appeared to remove AFB_1

and AFG_1 effectively in *A. parasiticus,* but not in *A. flavus*. It is possible that to some extent *A. flavus* could have degraded the metabolites of *E. rubrum*. Likewise, the extract of *P. pisce* was active on AFB_1 in *A. parasiticus*. The variability in reduction of growth and AFB_1 and AFG_1 concentrations in *A. parasiticus* and *A. flavus* caused by these two fungal commensals has been investigated in previous studies (Chapters 4 and 5). Coallier-Ascah and Idziak (1985), who investigated the interaction between *S. lactis* and *A. flavus*, noted a soluble heat-stable compound produced by *S. lactis* did not inhibit growth of the aflatoxigenic fungus, but did inhibit the synthesis of aflatoxins. *A. flavus*, however, also disrupted the cell wall of *S. lactis*.

Gourama and Bullerman (1997) reported anti-aflatoxigenic metabolites produced by bacterium *Lactobacillus casei pseudoplantarum* 371 completely inhibited AFB_1 production in *A. parasiticus*. Sakuda *et al.* (1996) and Ikeda *et al.* (2000) used aflastatin A, an inhibitor of aflatoxin production isolated from *Streptomyces* sp., in their studies. They noted that this compound inhibited aflatoxin production in *A. parasiticus*, without inhibiting the growth of the aflatoxin producer. Sakuda *et al.* (1996) found at 0.5 $\mu g\ mL^{-1}$ aflastatin A completely inhibited aflatoxin production in *A. parasiticus,* but did not prevent growth. In the present study, extracts of *A. wentii* and *D. hansenii* demonstrated this mechanism of inhibition. Both extracts inhibited AFB_1 and AFG_1 without affecting the growth of *A. parasiticus* and *A. flavus*. At a_w 0.93, the presence of *A. wentii* and *D. hansenii* also caused a reduction of AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*, although the growth of aflatoxigenic fungi were not inhibited (Chapter 5). As growth of aflatoxigenic fungi was not markedly affected, then this suggests the possibility that resistance to the aflatoxin inhibitors is less likely to arise.

Enzymes secreted by fungi could possibly degrade aflatoxins. Nakazato *et al.* (1990) found that a cell-free system of non-aflatoxin-producing *A. flavus* and *Rhizopus* sp. could convert AFB_1 reversibly into aflatoxicols. They reported

the interconversion of aflatoxin into aflatoxicols was caused by enzymes excreted by a non-toxigenic *A. flavus* and a *Rhizopus* sp. These authors found that the *Rhizopus* sp. could convert 43% of AFB_1 into aflatoxicols in one hour and 85% aflatoxicols into AFB_1 within 3 hours incubation at 30°C. The non-toxigenic *A. flavus* transformed only 5% AFB_1 into aflatoxicols and 13% aflatoxicols into AFB_1 within 3 hours at 30°C incubation. Thus, degradation of AFB_1 depended on the active compounds in the secretions of the non-toxic fungi. Smiley and Draughon (2000) found that applying proteinase-K to a crude protein extract of *Flavobacterium aurantiacum* reduced AFB_1 degradation from 74.5% to 34.5% and thus concluded that an enzyme could be the active factor in the bacterial extract. Extracts of non-toxigenic *A. flavus, A. wentii and D. hansenii* in the present study could have contained enzymes that inhibited production of aflatoxins or enhanced elimination of AFB_1 and AFG_1, or in some cases degraded the aflatoxins into other less toxic compounds.

Line and Brackett (1995b) also noted that mineralization of AFB_1 could be a type of aflatoxin degradation. They reported *Flavobacterium aurantiacum* appeared to use AFB_1 as a sole carbon source, thereby degrading the toxin. The presence of inhibitor species, competition for nutrients between inhibitor species and aflatoxigenic fungi (Nout, 1989) or between the non-toxigenic *A. flavus* and aflatoxin producers (Cotty and Bhatnagar, 1994; Horn *et al.*, 2000) may also be a factor in aflatoxin degradation, as this was observed in earlier studies (Chapter 4 and 5). Furthermore, aflatoxins are also endogenously degraded and the toxin degradation varied with the integrity of the mycelia. Doyle and Marth (1978c) reported fragmented mycelia degraded aflatoxins more than intact mycelia, and Huynh and Lloyd (1984) found that 14-day-old mycelia were more effective in degrading aflatoxins than 4-day-old mycelia. This mode of degradation was not caused by a secreted metabolite.

Possibly, the greater activity of agar extracts could be a result of concentration of active soluble compounds in the agar. In most cases the

concentrations of metabolites extracted from broth applied in both volumes (10 and 100 μL) were likely too little to inhibit the growth and/or eliminate aflatoxins in *A. parasiticus* and *A. flavus*. However, the broth extract of non-toxigenic *A. flavus* completely eliminated AFB_1 in *A. flavus* and the extracts of the four dried fish commensals reduced AFB_1 in *A. flavus* by 35-50% from 0.250 μg mL^{-1}, the highest concentration of aflatoxins found in this present study. This finding highlighted that the extracts likely had anti-aflatoxigenic activities, but not antifungal activities because they did not significantly inhibit the growth of aflatoxigenic fungi. Elimination of AFB_2 and AFG_2 in both *A. parasiticus* and *A. flavus* in the presence of all extracts demonstrated their anti-aflatoxigenic activities. Munimbazi and Bullerman (1998) applied 0.1 mg mL^{-1} of metabolites of *B. pumilus* and found that the metabolites inhibited aflatoxin production by 84% and mycelial growth by 37% in *A. parasiticus* NRRL 2999. These authors also reported that increasing concentration of the metabolites to 0.3 mg mL^{-1} inhibited 93% aflatoxin but the growth inhibition was only 38%. They concluded that the metabolites of *B. pumilus* inhibited toxin production rather than mycelial growth.

Greenish-blue and pale-blue fluorescent compounds found in the present study were possibly the transformed compounds of either aflatoxin degradation or the accumulation of intermediates from inhibition of aflatoxin synthesis. Karunaratne and Bullerman (1990) found some fluorescing compounds other than aflatoxins produced by *A. parasiticus* when temperature was at 35°C, and concluded that the fluorescent compounds were possibly breakdown products of aflatoxins. Ciegler *et al.* (1966) reported pale-blue fluorescing compounds having various Rf values of 0.03, 0.08, 0.2, 0.3, 0.87 and 0.8 when AFB_1 was added into spores of *Aspergillus terreus*, *A. flavus* and *A. luchuensis*. Other researchers (Detroy and Hesseltine, 1968) observed a transformation of pure AFB_1 by *Dactylium dendroides* into another blue fluorescent compound with a lower Rf value (0.57) than AFB_1 (0.69). Steroid-hydroxylating fungi like *A. repens* and *Mucor griseo-cyanus* also converted the parent molecule AFB_1 in

the same way as *D. dendroides* (Detroy and Hesseltine, 1969). In the present study, the extracts of non-toxigenic *A. flavus*, *E. rubrum*, *A. wentii P. pisce* and *D. hansenii,* possibly converted aflatoxins in *A. parasiticus* and *A. flavus* into other forms that were less toxic. The extract of *D. hansenii* in particular eliminated these fluorescent compounds in *A. flavus*. These fluorescent compounds were possibly breakdown products of aflatoxins as reported by Detroy and Hesseltine (1970) and Robertson *et al.* (1970). Therefore if these compounds are not present, then this suggests that the inhibitors of *D. hansenii* are preventing synthesis of aflatoxins and/or enabling complete mineralization of the aflatoxins. Either way, the inhibitors prevent re-conversion back to aflatoxins as Nakazato *et al.* (1990) reported. The metabolites and the experimental conditions such as a_w and temperature may influence production and degradation of these bright greenish-blue and blue-green fluorescent compounds in *A. parasiticus* and *A. flavus.*

This study concluded that the extract of non-toxigenic *A. flavus* showed both antifungal and anti-aflatoxigenic activities; the extracts of *E. rubrum* and *P. pisce* had varied activity on inhibiting growth and aflatoxin production, and *A. wentii* and *D. hansenii* demonstrated anti-aflatoxigenic activities. Metabolites from agar extracts at 100 μL showed the best inhibition. Variations in the antifungal and anti-aflatoxigenic activities could be related to the stability of the extracts. Therefore, a further study on the partial characterization of the extracts treated at the different temperatures, values of pH and to different enzymes is discussed in the following chapter.

CHAPTER 7

PARTIAL CHARACTERIZATION OF METABOLITES FROM FUNGAL COMMENSALS OF DRIED FISH

7.1 INTRODUCTION

The thermal stability of antifungal and/or anti-aflatoxigenic metabolites of bacteria has been reported. Gourama and Bullerman (1997) reported that heating the cell-free supernatant of *Lactobacillus casei pseudoplantarum* 371 at 50 to 70°C did not affect the metabolites' anti-aflatoxigenic activities in eliminating AFB_1 and AFG_1 in *Aspergillus parasiticus* NRRL 2999. However, heating the metabolites at 100°C allowed greater production of AFB_1 and AFG_1 in *A. parasiticus* than when the metabolites were heated at 121°C. They noted that heat affected chemical composition of the inhibitors of *L. casei pseudoplantarum* thereby degrading their activity. They concluded that the metabolites were relatively thermostable and could survive pasteurization. Munimbazi and Bullerman (1998) studied the autoclaved metabolites of *Bacillus pumilus*. These authors found that the bacterial metabolites were heat-stable to 121°C and retained full inhibitory activity against mycelial growth and aflatoxin production of *A. parasiticus*. They also reported that the metabolites of *B. pumilus* did not form a fluorescent derivative and the metabolites suppressed aflatoxin concentration in *A. parasiticus* by 96% at pH 2 and 92% at pH 4 and 6. None of the protease, peptidase and lipase enzymes added to *B. pumilus* modified anti-aflatoxigenic activity of the metabolites. They suggested that the inhibiting compound in the metabolites *B. pumilus* was either a cyclic polypeptide or a non-peptide compound.

Smiley and Draughon (2000) investigated a heat-treated crude protein extract from *Flavobacterium aurantiacum*. Heated in boiling water for 15 minutes, the crude protein extract reduced AFB_1 by 5.5% compared to 74.5% by an unheated extract. Their result showed that maximum AFB_1 degradation of the *F. aurantiacum* crude protein extract was at pH 7, while acidic conditions were detrimental to the activity of bacterial extract. Furthermore, treatment with proteinase-K reduced anti-aflatoxigenic activity, but DNase-I had no effect. They suggested that the activity was enzymatic. A study by Hamid and Smith (1987), reported that a cell-free extract of 10-day-old *A. flavus* degraded 17% of AFB_1 and 19% of AFG_1 after 48 hours. They noted that cytochrome P-450 monooxygenases were involved in the aflatoxin degradation.

In Chapter 6, it was shown that extracts of the non-toxigenic *A. flavus, E. rubrum, A.wentii, P. pisce* and *D. hansenii* from agar applied at 100 µL showed stronger activities than the broth extracts. None of the extracts had fluorescent compounds. Their stability at different temperatures and values of pH and reactivity to enzymes were yet to be determined. Therefore, agar extracts of the non-toxic *A. flavus, E. rubrum, A.wentii, P. pisce* and *D. hansenii* metabolites were further studied to characterize their active compounds in relation to different temperatures, values of pH and enzymes. If metabolites showed variable effects on the growth and aflatoxin concentrations of *A. parasiticus* and *A. flavus*, the treatments may have influenced their activity. This study aimed to determine the stability of the metabolites at 25, 60, 85 and 121°C, pH 2, 4, 6, 8 and 10 and in the presence of protease, lysozyme, amylase and lipase enzymes. Antifungal and anti-aflatoxigenic effects of metabolites from all treatments were investigated on *A. parasiticus* and *A. flavus*.

7.2 MATERIAL AND METHODS

7.2.1 General methods

Fungal culture preparation, colony diameter measurement, aflatoxin and statistical analysis were as previously described in Chapter 3. The extraction of the metabolites from agar cultures was described in Chapter 6. The volume of the extracts applied on each plate was 100 µL, and then 10 µL (10^6 spores mL^{-1}) of *A. parasiticus* and *A. flavus* were inoculated onto MY10-12% agar (a_w 0.89). All the extracts (treated or untreated) were directly applied on the surface of the agar medium, then allowed one hour to dry before inoculation with *A. parasiticus* or *A. flavus*.

7.2.2 Treatments of fungal metabolites
(a) Temperatures

Thermal stability of these five fungal extracts was determined at different temperatures. One mL of metabolite extract was placed in a 2 mL vial and treated at temperature of either 25, 60, 100 or 121°C. For 25°C and 60°C treatments, the vials

were placed for 3 hours in a beaker filled with water in a water bath (Grant JB2). One batch of the five extracts was placed in the boiled-water filled beaker and put in 100°C oven for 1 hour. After 1 hour, the temperature of the water in beaker was measured and was 85°C, therefore, this temperature was used as the treatment. Another batch was autoclaved at 121°C for 15 minutes. The negative control was 0.05M phosphate buffer solution (PBS) treated at the same temperatures.

(b) Hydrogen ion concentration (pH)

Sodium hydroxide (NaOH) at 0.1, 0.5 and 1.25M and 1M hydrochloric acid (HCl) were prepared. Drops of NaOH or HCl were added to the extracts to achieve the desired pH of 2, 4, 6, 8 and 10 (±0.02). The pH was measured using an Activon S/N digital pH meter. The extracts were kept at 5°C overnight. The negative control was sterile distilled water adjusted to the desired pH.

(c) Enzymes

A range of enzymes was used in this study. An enzyme is specific in its catalytic action. It can be reaction specific and catalyse only one type of reaction, or can be substrate specific and catalyse the reaction of only one compound, or it can be stereo-specific and react only with one stereo-isomer of the substrate. Lysozyme splits β-1,4 glycosidic bond in polysaccharides. Trypsin is active toward positively charged substrates and hydrolyses a protein at lysyl and arginyl residues to give a limited number of peptides. α-Chymotrypsin, on the other hand, cleaves peptides and proteins at hydrophobic amino acids. Pepsin is specific for the hydrolysis of peptides of L-amino acids. Protease and proteinase K degrade proteins by splitting internal peptide bonds to produce peptides. Carboxypeptidase A is the enzyme that removes the C-terminal amino acid from a peptide. Lipase A hydrolyses triacylglycerols into diacylglycerols plus a fatty acid anion, and α-amylase catalyses the hydrolysis of the α (1-4) glycoside link of amylose.

These nine enzymes above were applied on the extracts to determine if they affected antiaflatoxigenic activity against *A. parasiticus* and *A. flavus*. All the enzymes (lysozyme, trypsin, pepsin, α-chymotrypsin, protease, carboxypeptidase A, proteinase K, lipase A and α-amylase) were obtained from Sigma-Aldrich Pty Ltd. Except for carboxypeptidase A, 1mg of each enzyme was dissolved into 1mL of each metabolite extract in vials. Twenty microliters of carboxypeptidase A was applied to each metabolite. The mixture of enzymes and extracts were incubated at 37°C for 2 hours in a water bath (Grant JB2), before assaying the extracts against *A. parasiticus* and *A. flavus*. Negative control treatments consisted of enzyme solution in sterile distilled water without the metabolite extracts.

7.2.3 Experimental design

The study was designed as a full factorial in three replicates with treatments of five extracts exposed to four temperatures, five values of pH and nine enzymes. All the treated extracts and the controls were challenged against *A. parasiticus* and *A. flavus*. All samples were incubated at 25°C and analysed after 10 days. This experiment was done in conjunction with untreated extracts (positive controls, Chapter 6). In this present study, positive controls were not included in statistical analysis, thus in the discussion the treated extracts were directly compared to the untreated metabolite extracts.

7.3 RESULTS

Fungal extracts applied at a wide range of different temperatures, pH and enzymes showed various effects on colony diameter and AFB_1 and AFG_1 concentration of *A. parasiticus* and *A. flavus*. Generally, colony diameter of *A. flavus* was larger than *A. parasiticus*. Both *A. parasiticus* and *A. flavus* produced less AFB_2 and/or AFG_2 than AFB_1 and AFG_1. Both axenic aflatoxigenic fungi variably produced greenish-blue and blue-green fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2. Production of AFB_2 and AFG_2 and the presence and Rf values of other fluorescent

compounds are discussed at the end of this section. Tables 7-01 and 7-02 summarise the results of the 3 experiments (influence of pH, temperature and enzymes on the ability of five fungal extracts to eliminate aflatoxins). Which fungal extracts retained or enhanced their ability to eliminate aflatoxin production in *A. parasiticus* and *A. flavus* after incubation at different temperatures or values of pH or in the presence of a range of enzymes are shown. The tables indicate that the extract from *D. hansenii* was very effective in eliminating both aflatoxins B and G in both *A. parasiticus* and *A. flavus* after a range of treatments.

7.3.1 Temperature-treated Extracts

(a) Effects on colony diameter of *A. parasiticus* and *A. flavus*

Colony diameter of axenic *A. parasiticus* was 23-25 mm (Table 7-03) and of axenic *A. flavus* was 29-35 mm (Table 7-05). Statistically, there was no significant effect of fungal extracts on the colony size of *A. parasiticus* compared to the control (Table 7.04). However, there was a significant effect on colony size of the temperature to which the extracts had been subjected, but this effect was lost in the interaction term. Therefore, because neither the extracts nor the interaction significantly affected colony size, but temperature did, all extracts were pooled for each temperature (figure 7.01). Pooled extracts heated to 60°C produced a small, but statistically significant increase in colony size compared to the colony sizes of the pooled 85°C and 121°C treatments. However, none of these three pooled treatments produced a significantly different colony size compared to the pooled 25°C treatment. Therefore, it was concluded that there was no significant effect of temperature on the ability of the fungal extracts to inhibit growth. *A. parasiticus* still grew sufficiently to produce aflatoxin.

Table 7.01. Efficacy of extracts of non-aflatoxigenic fungi to eliminate aflatoxin in *A. parasiticus*. Zero indicates that the extract as treated was able to eliminate aflatoxin.

Extract	Non-toxic *A flavus*		*E. rubrum*		*A. wentii*		*P. pisce*		*D. hansenii*	
Treatment	AFB_1	AFG_1	AFB_1	AFG_1	AFB_1	AFG_1	AFB_1	AFG_1	AFB_1	AFG_1
25 °C						0	0	0	0	0
60 °C				0		0		0	0	0
85 °C		0		0		0	0	0		0
121 °C										
pH 2									0	0
pH 4	0	0						0	0	0
pH 6	0	0				0		0	0	0
pH 8	0	0						0	0	0
pH 10										0
Trypsin							0			
Pepsin			0							
Carboxypeptidase	0	0		0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0	0	0	0
Protease	0	0	0	0	0	0	0	0	0	0
Proteinase K		0	0	0	0	0	0	0	0	0
Lysozyme										
Amylase	0	0	0	0	0	0	0	0	0	0
Lipase	0	0	0	0	0	0	0	0	0	0

Table 7.02. Efficacy of extracts of non-aflatoxigenic fungi to eliminate aflatoxin in *A. flavus*. Zero indicates that the extract as treated was able to eliminate aflatoxin.

Extract	Non-toxic *A. flavus*		*E. rubrum*		*A. wentii*		*P. pisce*		*D. hansenii*	
Treatment	AFB_1	AFG_1	AFB_1	AFG_1	AFB_1	AFG_1	AFB_1	AFG_1	AFB_1	AFG_1
25 °C									0	0
60 °C								0	0	0
85 °C								0		
121 °C								0		
pH 2								0		
pH 4										
pH 6										
pH 8									0	0
pH 10									0	0
Trypsin									0	
Pepsin										
Carboxypeptidase	0	0		0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0	0	0	0
Protease	0	0	0	0	0	0	0	0	0	0
Proteinase K			0	0	0	0	0	0	0	0
Lysozyme										
Amylase	0	0	0	0	0	0	0	0	0	0
Lipase	0	0	0	0	0	0	0	0	0	0

Table 7-03. Colony diameter of *A. parasiticus* treated with extracts heated at different temperatures on MY10-12% agar (a_w 0.89), after 10 days at 25°C

Extracts	Colony diameter, mm (mean±SE, n=3)			
	25°C	60°C	85°C	121°C
A. parasiticus (control)*	23.6 ± 0.38	24.3 ±0.38	24.6± 0.38	25.0± 0.33
A. flavus (non-toxigenic)	23.3 ± 1.92	23.3 ±1.92	25.0± 1.67	21.6± 0.96
E. rubrum	25.0 ± 2.89	26.6± 3.85	17.0± 0.58	19.3± 1.15
A. wentii	22.0 ± 6.93	30.0± 2.89	23.3± 1.92	20.0 ±0.67
P. pisce	23.3 ± 1.92	20.6 ±2.71	18.3± 0.96	17.0 ±0.57
D. hansenii	23.3 ±1.92	36.6± 3.85	21.6± 1.92	18.6± 0.38

* = Axenic culture.

Table 7-04. ANOVA of colony diameter of *A. parasiticus* treated with extracts heated at different temperatures on MY10-12% agar (a_w 0.89), after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1234.833	23	53.688	1.630	0.077
Intercept	38364.500	1	38364.500	1165.012	0.000
Extracts	217.667	5	43.533	1.322	0.271
Temperature (°C)	448.000	3	149.500	4.540	0.007
Metabolite* Temperature	568.667	15	37.500	1.151	0.341
Error	1580.667	48	32.932		
Total	41180.000	72			
Corrected total	2815.500	71			

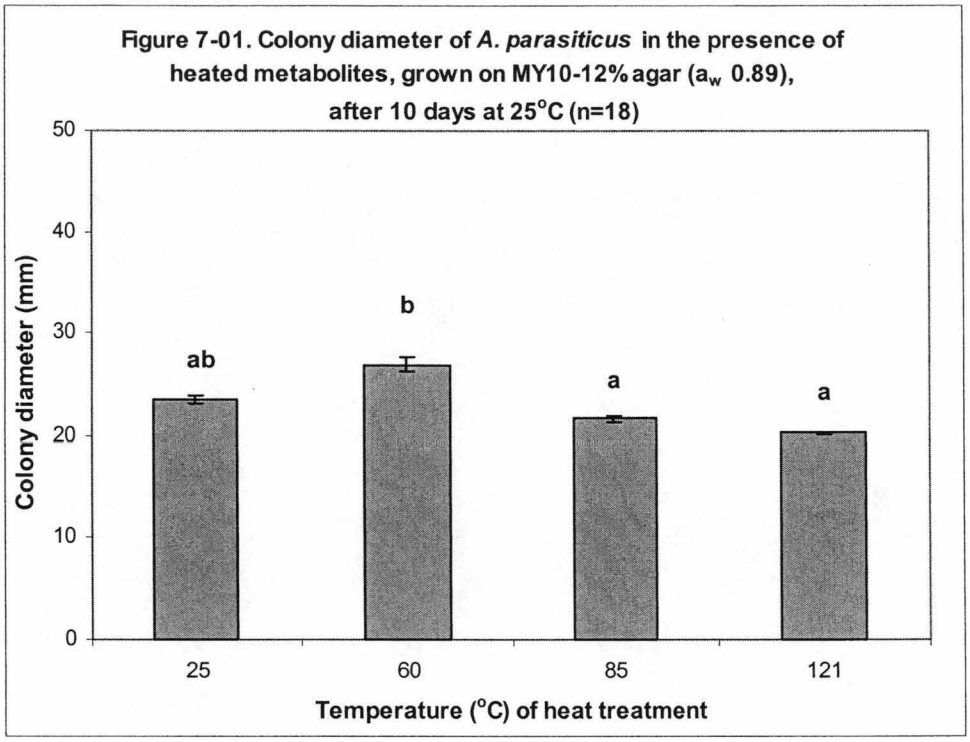


Table 7-05 shows that the colony diameter of *A. flavus* was reduced by the presence of extracts in comparison to axenic control. The extracts and temperatures as single factors significantly affected ($p \leq 0.05$) the colony diameter of *A. flavus* (Table 7-06). However, the interaction of extracts and temperatures was not significant ($p > 0.05$). Therefore, the effect of extracts and temperature were analysed as single factors. Figures 7-02 and 7-03 show the mean of all colony diameters of *A. flavus* by extracts and temperatures as single factors. Both these figures, however, only show the difference of particular metabolite among others and the particular temperature compared to others, respectively. Only the metabolite extract of *E. rubrum* was significantly lower than the control (Figure 7-02) and temperatures 25 and 85°C differed from 60 and 121°C (Figure 7-03). That is, the growth of *A. flavus* was significantly better in the presence of extracts that had been treated at 60 and 121°C compared to 25 and 85°C. However, like A. parasiticus there was no biologically significant reduction in growth of A. flavus in comparison to the control at 25°C.

Table 7-05. Colony diameter of *A. flavus* treated with extracts heated at different temperatures on MY10-12% agar (a_w 0.89), after 10 days at 25°C

Extracts	Colony diameter, mm (mean!SE, n=3)			
	25°C	60°C	85°C	121°C
A. flavus (control)*	29.0± 1.20	35.0± 1.67	29.0± 1.20	30.0± 0.67
A. flavus (non-toxigenic)	20.0 ±2.88	33.3 ±1.92	21.6 ±0.38	28.6 ±1.02
E. rubrum	18.3 ±1.92	25.0± 2.89	20.6± 0.38	28.6± 1.02
A. wentii	23.3 ±1.92	33.3 ±1.92	23.3± 0.96	27.6 ±2.51
P. pisce	18.3 ±1.92	33.3 ±1.92	21.6 ±0.96	24.0± 1.76
D. hansenii	23.3 ±1.92	30.0 ±2.88	24.0± 2.76	27.3 ±0.83

* = Axenic culture.

Table 7-06. ANOVA of colony diameter of *A. flavus* treated with extracts heated at different temperatures on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1680.867	23	73.072	2.872	0.001
Intercept	49928.000	1	49928.000	1986.236	0.000
Extracts	373.000	5	74.700	2.936	0.022
Temperatures (°C)	1081.444	3	360.481	14.167	0.000
Metabolite* Temperature	225.722	15	15.048	0.591	0.867
Error	1221.333	48	25.444		
Total	52830.000	72			
Corrected total	2902.000	71			

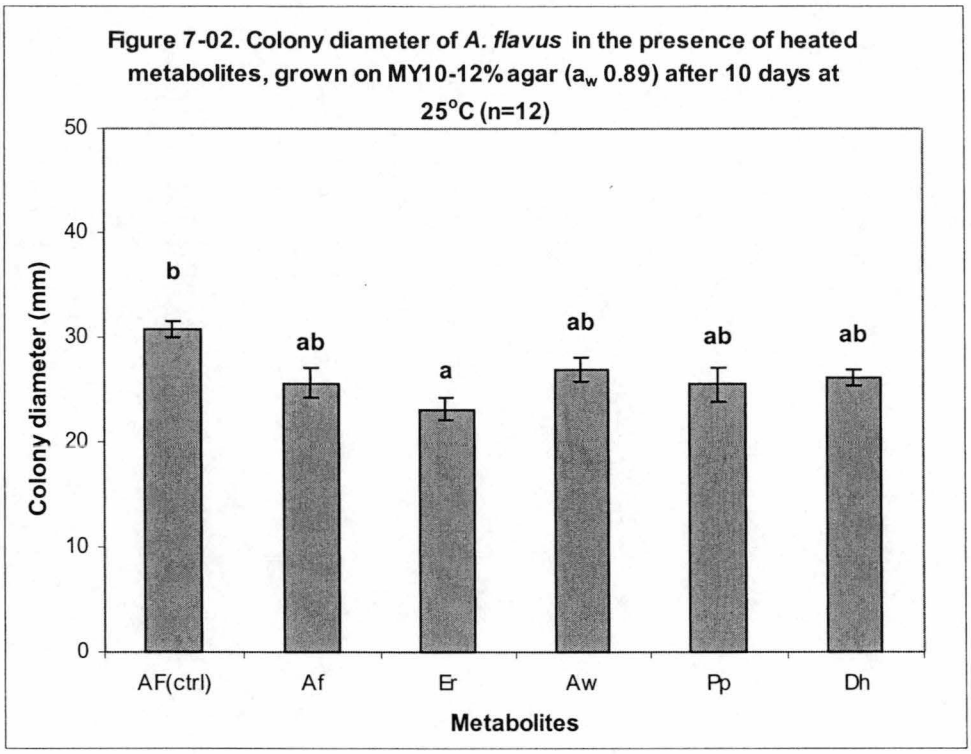


Figure 7-02. Colony diameter of A. flavus in the presence of heated metabolites, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=12)
Colony diameter (mm)
50
40
30
20
10
0
b
ab
a
ab
ab
ab
AF(ctrl)
Af
Er
Aw
Pp
Dh
Metabolites

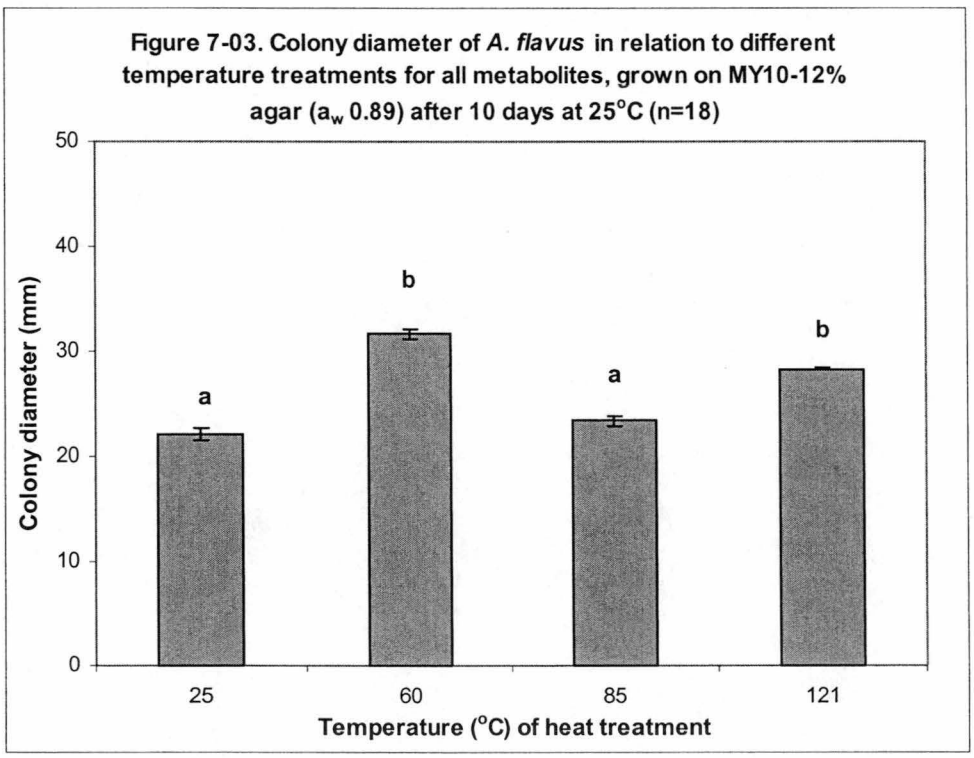


Figure 7-03. Colony diameter of A. flavus in relation to different temperature treatments for all metabolites, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=18)
Colony diameter (mm)
50
40
30
20
10
0
a
b
a
b
25
60
85
121
Temperature (°C) of heat treatment

(b) Effects on AFB_1 concentration in *A. parasiticus* and *A. flavus*

ANOVA of AFB_1 of concentration in *A. parasiticus* indicates that extracts alone and the interaction with temperature were significant at $p \leq 0.05$, but that temperature as single factor did not significantly affected AFB_1 concentration (Table 7-07). AFB_1 concentration in *A. parasiticus* was completely removed by the *A. wentii* extract treated at 60°C, *P. pisce* at 25 and 85°C and *D. hansenii* at 25 and 60°C. At other temperature treatments, *D. hansenii* and *P. pisce* extracts significantly reduced AFB_1 concentration in *A. parasiticus* (Figure 7-04). The extract of non-toxigenic *A. flavus* reduced AFB_1 in *A. parasiticus* at all temperature treatments, with the highest reduction of 52% at 85°C being significantly different from the control. The extract of *E. rubrum* significantly reduced AFB_1 concentration in *A. parasiticus* by 48 and 49% at 25 and 121°C, in comparison with respective control. However, at 60°C *E. rubrum* increased AFB_1 concentration by 12% although this was not statistically significant.

Table 7-07. ANOVA of AFB_1 in *A. parasiticus* treated with extracts heated at different temperatures on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.403	23	1.754E-02	24. 609	0.000
Intercept	0.964	1	0.964	1352.000	0.000
Extracts	0.253	5	5.064E-02	71.033	0.000
Temperature (°C)	1.030E-03	3	3.432E-04	0.481	0.697
Extracts* Temperature	0.149	15	9.951E-03	13.959	0.000
Error	3.422E-02	48	7.129E-04		
Total	1.402	72			
Corrected total	0.438	71			

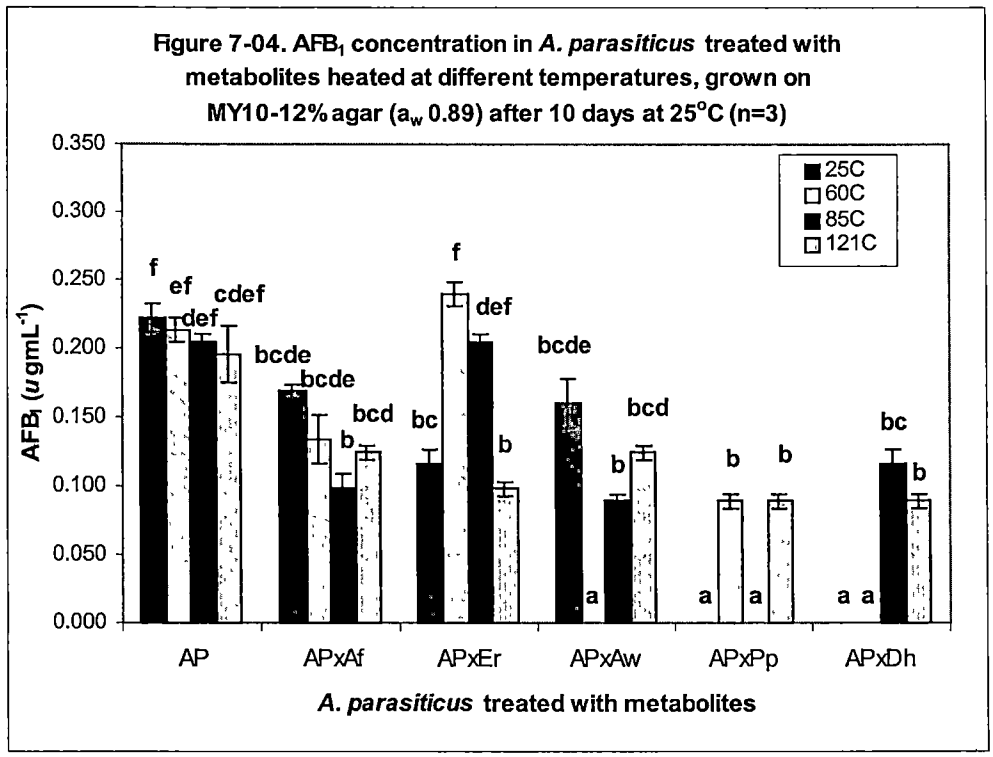
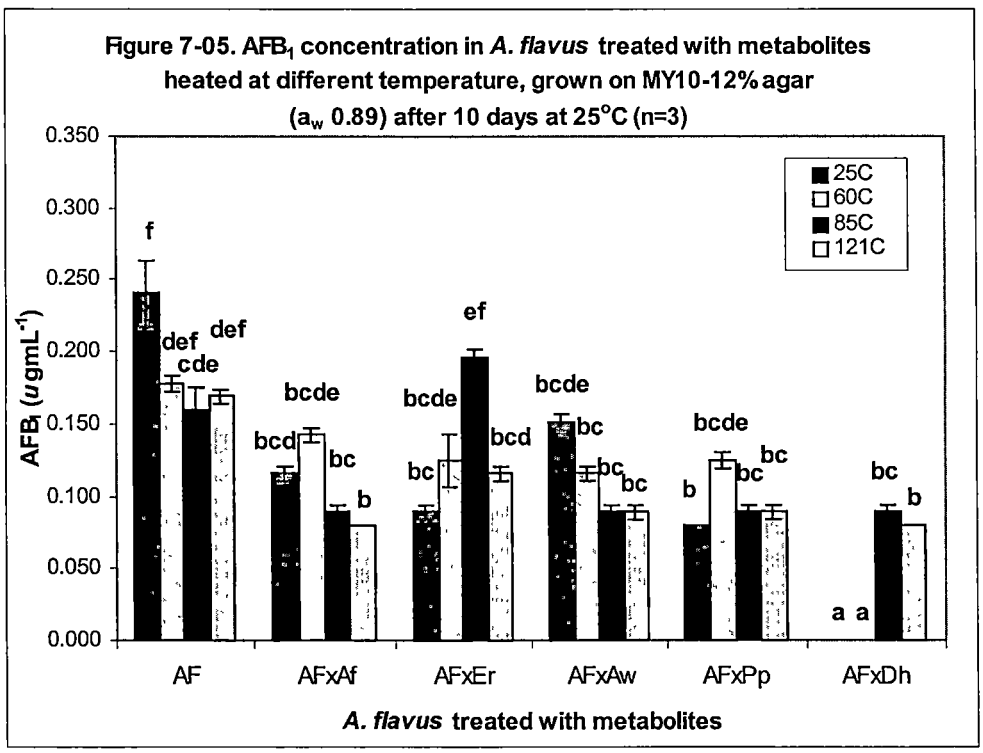


Table 7-08 shows that extracts as a single factor and its interaction with temperature significantly affected AFB_1 concentration in *A. flavus* at $p \leq 0.05$, however, temperature alone was insignificant. The extract of *D. hansenii* eliminated AFB_1 concentration in *A. flavus* at 25 and 60°C (Figure 7-05). Autoclaved non-toxigenic *A. flavus*, *D. hansenii* and *A. wentii* extracts significantly decreased AFB_1 concentration in *A. flavus* by almost 50% compared to autoclaved control. The extract of *P. pisce* significantly decreased AFB_1 concentration in *A. flavus* by more than half at 25°C and reduced it by 47% at 121°C. On the other hand, the activity of the extract of *E. rubrum* was affected by high temperature. The extract significantly decreased AFB_1 concentration in *A. flavus* at 25°C, but had no effect at other temperature treatments.

Table 7-08. ANOVA of AFB_1 concentration in *A. flavus* treated with extracts heated at different temperatures on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.204	23	8.881E-03	15.203	0.000
Intercept	0.909	1	0.909	1556.085	0.000
Extracts	0.134	5	2.673E-02	45.753	0.000
Temperature (°C)	2.089E-03	3	6.964E-04	1.192	0.323
Extracts* Temperature	6.855E-02	15	4.570E-03	7.823	0.000
Error	2.804E-02	48	5.842E-04		
Total	1.141	72			
Corrected total	0.232	71			



(c) Effects on AFG_1 concentration in *A. parasiticus and A. flavus*

Extracts were effective in eliminating AFG_1 in *A. parasiticus*. Table 7-09 shows that extracts and temperatures singly and their interaction significantly affected AFG_1 in *A. parasiticus* ($p \leq 0.05$). Treated at 25, 60 and 85°C, *A. wentii*, *P. pisce* and *D. hansenii* extracts completely eliminated AFG_1 concentration in *A. parasiticus* (Figure 7-06). The extracts of *E. rubrum* at 60°C, together with non-toxigenic *A. flavus* at 85°C eliminated AFG_1 in *A. parasiticus*. At 25°C, both extracts significantly decreased AFG_1 concentration in *A. parasiticus* by 30% (non-toxigenic *A. flavus*) and 42% (*E. rubrum*). All extracts treated at 121°C significantly reduced AFG_1 concentration in *A. parasiticus* by about 50%.

Table 7-09. ANOVA of AFG_1 concentration in *A. parasiticus* treated with extracts heated at different temperatures on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.654	23	2.843E-02	51.276	0.000
Intercept	0.506	1	0.506	912.071	0.000
Extracts	0.463	5	9.262E-02	167.043	0.000
Temperature (°C)	6.959E-02	3	2.320E-02	41.833	0.000
Extracts* Temperature	0.121	15	8.082E-03	14.576	0.000
Error	2.661E-02	48	5.545E-04		
Total	1.186	72			
Corrected total	0.681	71			

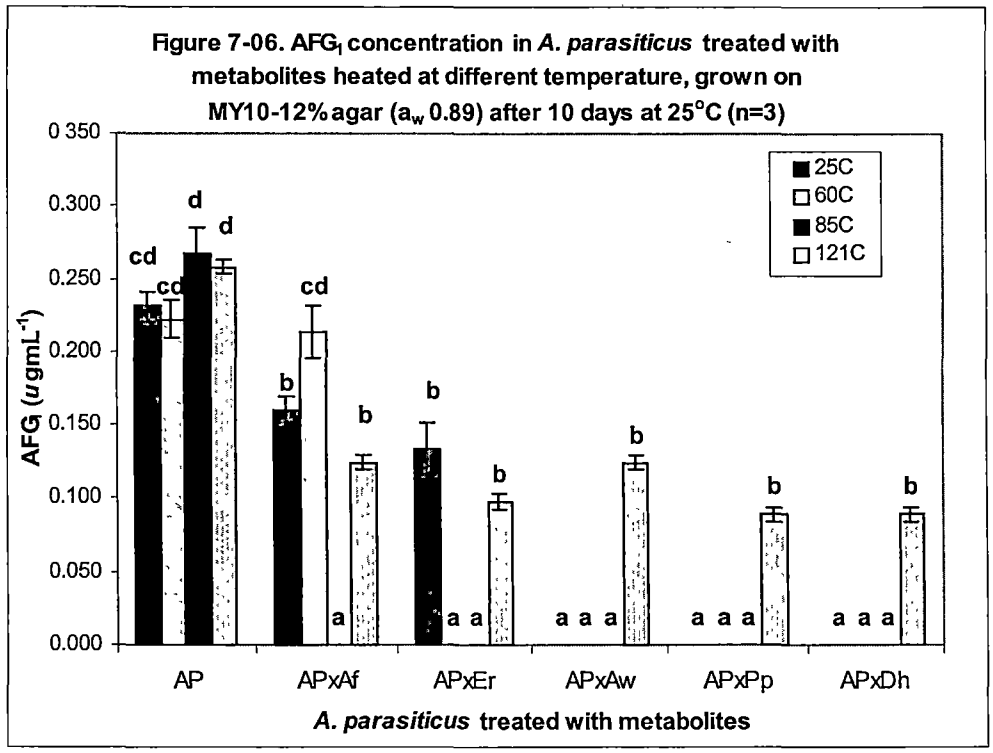
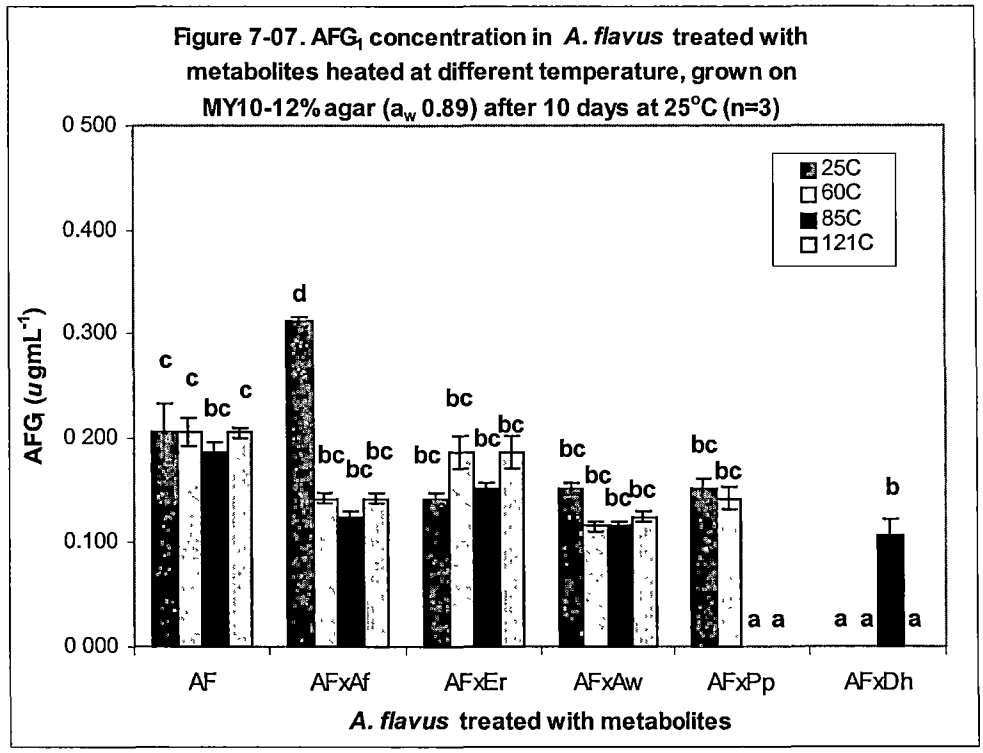


Table 7-10 shows extracts and temperature singly and in their interaction significantly affected AFG_1 in *A. flavus* at $p \leq 0.05$. From Figure 7-07, it is clear that the only significant decrease was caused by extracts of *D. hansenii* and *P. pisce*. Treated at temperature 25, 60 and 121°C, the extract of *D. hansenii* eliminated AFG_1 concentration in *A. flavus*. The exception was at 85°C, where the extract reduced AFG_1 concentration in *A. flavus* by 42%, but this was insignificant compared to the control at the same temperature. *P. pisce* treated at 85°C and 121°C completely eliminated AFG_1 concentration in *A. flavus*. At other temperature treatments, the extract only reduced AFG_1 concentration in *A. flavus* by about 30%, and the reduction was not significant. The extract of *A. wentii* decreased AFG_1 concentration in *A. flavus* by 26-43% and that of *E. rubrum* by 8-30% at all temperatures, however, their reduction was insignificant compared to the control at the same temperature. The non-toxigenic *A. flavus* extract treated at increased temperatures appeared to be more active. At temperatures 60, 85 and 121°C, the non-toxigenic *A. flavus*

Table 7-10. ANOVA of AFG_1 in *A. flavus* treated with extracts treated at different temperatures, and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.441	23	1.916E-02	22.237	0.000
Intercept	1.199	1	1.199	1392.000	0.000
Extracts	0.272	5	5.446E-02	63.228	0.000
Temperature (°C)	2.828E-02	3	9.426E-03	10.943	0.000
Extracts* Temperature	0.140	15	9.331E-03	10.832	0.000
Error	4.135E-02	48	8.614E-04		
Total	1.681	72			
Corrected total	0.482	71			



extract reduced AFG_1 in *A. flavus* by 30-48% but the reduction was still not significant compared to the respective controls. On the other hand, at 25°C, the extract increased AFG_1 concentration in *A. flavus* by 50% compared to the control.

It can be concluded from the temperature treatments, that the extract of *D. hansenii* showed the best activity against the growth and AFB_1 and AFG_1 production in *A. parasiticus* and *A. flavus*. Metabolites of *D. hansenii* were at least moderately heat-stable.

7.3.2 pH-treated extracts

(a) Effects on colony diameter of *A. parasiticus* and *A. flavus*

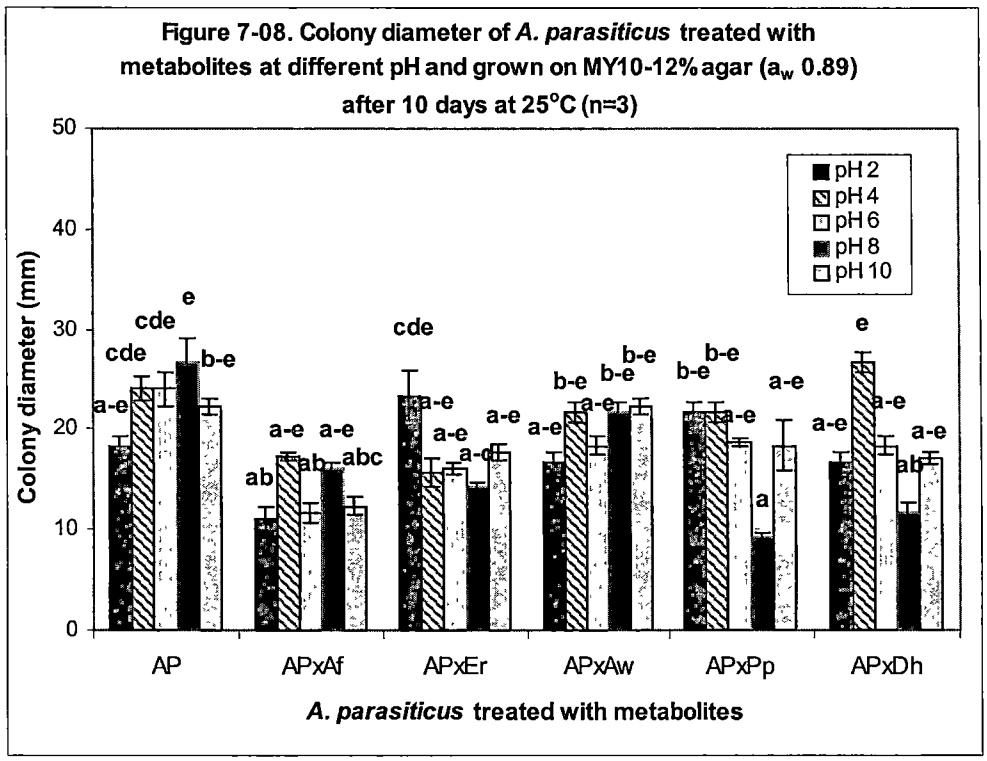
Treated at different values of pH, extracts variably affected the colony diameter of *A. parasiticus*. Table 7-11 shows that the single factors (extracts and pH) and their interaction were significant at $p \leq 0.05$ on colony diameter of *A. parasiticus*. However, comparison of the five extracts at all combinations of interactions indicated that only some combinations markedly decreased colony diameter of *A. parasiticus* when compared to the control.

Table 7-11. ANOVA of colony diameter of *A. parasiticus* treated with extracts at different pH, grown on MY10-12% agar (a_w 0.89) after 10 days at 25C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1821.156	29	62.798	4.893	0.000
Intercept	30396.844	1	30396.844	2368.585	0.000
Extracts	727.689	5	145.538	11.341	0.000
pH	204.933	4	51.233	3.992	0.006
Extracts*pH	888.533	20	44.427	3.462	0.000
Error	770.000	60	12.833		
Total	32988.000	90			
Corrected total	2591.156	89			

The only significant reduction on the colony diameter of *A. parasiticus* was in the presence of extracts of *D. hansenii, P. pisce* and *E. rubrum* at pH 8 and the extract of non-toxic *A. flavus* at pH 6 (Figure 7-08). Extracts of *P. pisce* reduced by 65%, *D. hansenii* by 56%, *E. rubrum* by 46% and non-toxic *A. flavus* by 51% in comparison the colony diameter of *A. parasiticus* of the respective pH control. At other pH values, the

extracts of these four fungi variably affected colony diameter of *A. parasiticus*, however, the reduction or slight increase was insignificant compared to the control at the same pH. The extract of *A. wentii*, on the other hand, showed no significant effect on colony diameter of A. *parasiticus* at any pH, implying that the activity of the *A. wentii* extract was not differentially affected by pH. Overall, as for the temperature treatments, the extracts had little effect on colony diameter.

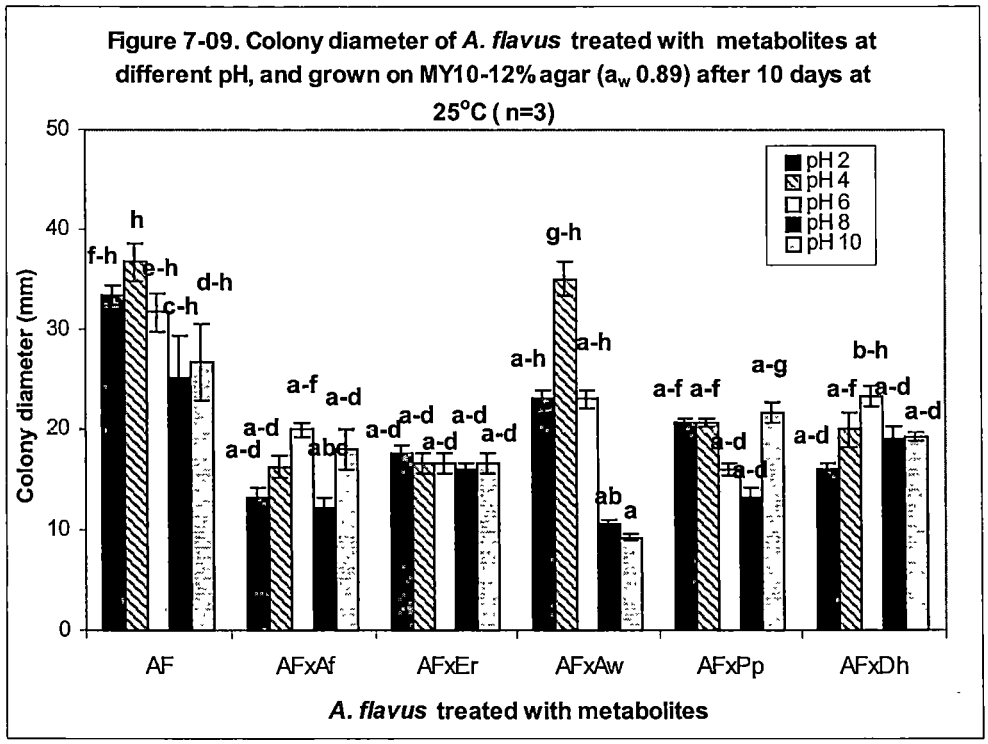


ANOVA for colony diameter of *A. flavus* treated with extracts at different pH shows all factors singly and in their interaction were significant at $p \leq 0.05$ (Table 7-12). Generally, all extracts held at values of pH in the range 2-10 decreased colony diameter of *A. flavus*, with the exception of the extract of *A. wentii* at pH 4. However, in many cases the reduction was statistically insignificant. Figure 7-09 shows that over the pH range, the colony diameter of *A. flavus* was reduced by 32-60% by non-toxic *A. flavus*, 36-54% by *E. rubrum*, 18-49% by *P. pisce* and 24-51% by *D. hansenii* extracts respectively. Significant reduction of colony diameter of *A. flavus* varied between the

extracts and pH. Compared to the matched pH control, non-toxic *A. flavus* and *D. hansenii* extracts significantly reduced the colony diameter of *A. flavus* at acidic pH of 2 and 4. The extract of *E. rubrum* significantly reduced the colony diameter of *A. flavus* between pH 2 and 6. On the other hand, both *A. wentii* and *P. pisce* extracts caused significant reduction of colony diameter of *A. flavus* at pH near neutral to basic. Treated at pH 6, the extract of *P. pisce* significantly decreased colony diameter of *A. flavus*. The extract of *A. wentii* at pH 8 and 10 significantly reduced the colony diameter of *A. flavus*. Therefore, over the pH range tested, the extracts had a greater effect on the growth of *A. flavus* than on *A. parasiticus*.

Table 7-12. ANOVA of colony diameter of *A. flavus* treated with extracts at different pH, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	4160.456	29	143.464	7.609	0.000
Intercept	36683.211	1	36683.211	1945.486	0.000
Extracts	2201.922	5	440.384	23.356	0.000
pH	719.511	4	179.878	9.540	0.000
Extracts*pH	1239.022	20	61.951	3.286	0.000
Error	1131.333	60	18.856		
Total	41975.000	90			
Corrected total	5291.789	89			

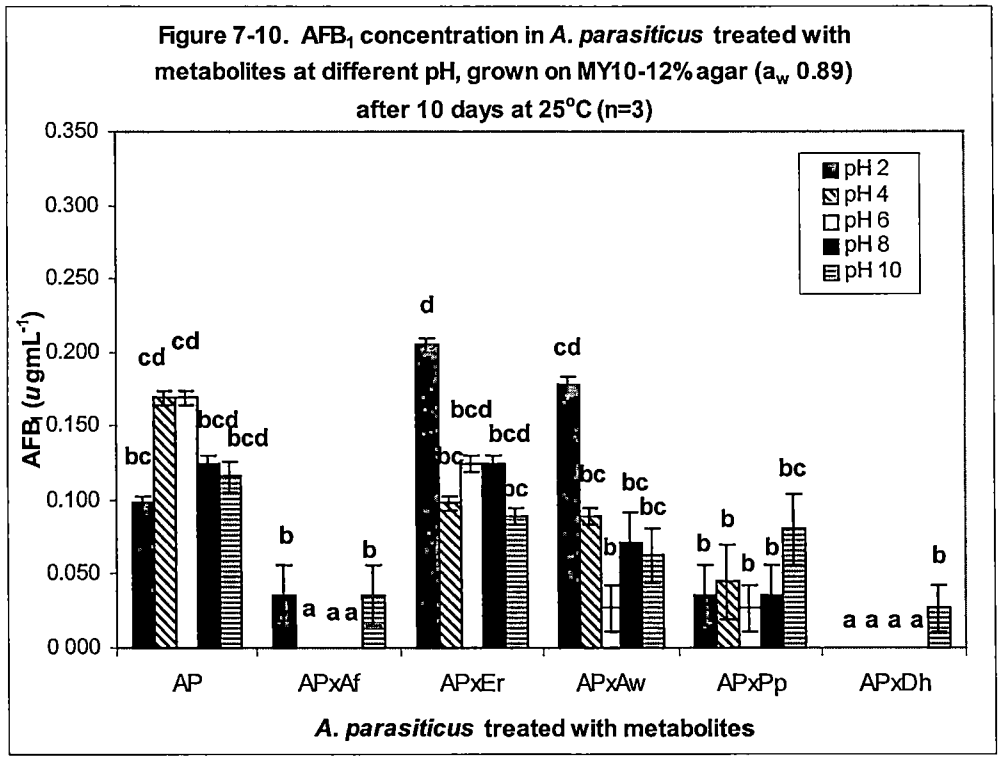


(b) Effects on AFB_1 concentration in *A. parasiticus* and *A. flavus*

Table 7-13 shows that extracts singly and the interaction with pH significantly affected AFB_1 concentration in *A. parasiticus* at $p \leq 0.05$, However, pH alone did not affect AFB_1 concentration in *A. parasiticus* ($p > 0.05$). Multiple comparisons of extracts and pH interactions indicated that at pH 4 to 8, the extracts of *D. hansenii* and non-toxigenic *A. flavus* eliminated AFB_1 in *A. parasiticus*. At the lowest pH tested, only *D. hansenii* extract eliminated AFB_1 in *A. parasiticus* (Figure 7-10). Extracts of *P. pisce* at pH 4 and 6, significantly reduced AFB_1 concentration in *A. parasiticus* by at least 73%. The extract of *A. wentii* at pH 6 significantly reduced AFB_1 concentration in *A. parasiticus* by 84%. At pH 10, although all the extracts reduced AFB_1 concentration in *A. parasiticus* (by 23-76%), the reduction was not significant. Both *A. wentii* and *E. rubrum* extracts at pH 2 increased AFB_1 concentration in *A. parasiticus* compared to the matched control pH, *E. rubrum* significantly so.

Table 7-13. ANOVA of AFB_1 concentration in *A. parasiticus* treated with extracts at different pH, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.325	29	1.119E-02	7.806	0.000
Intercept	0.426	1	0.426	297.370	0.000
Extracts	0.237	5	4.744E-02	33.092	0.000
pH	1.351E-02	4	3.378E-02	2.356	0.064
Extracts*pH	7.384E-02	20	3.69E-02	2.575	0.003
Error	8.602E-02	60	1.434E-02		
Total	0.837	90			
Corrected total	0.411	89			



Only extracts as a single factor significantly affected the concentration of AFB_1 in *A. flavus* ($p \leq 0.05$), however, pH alone and the interaction were not significant (Table 7-14). Thus, treatment comparison was only made for AFB_1 concentration in *A. flavus* by the extracts as single factor over all pH values (Figure 7-11).

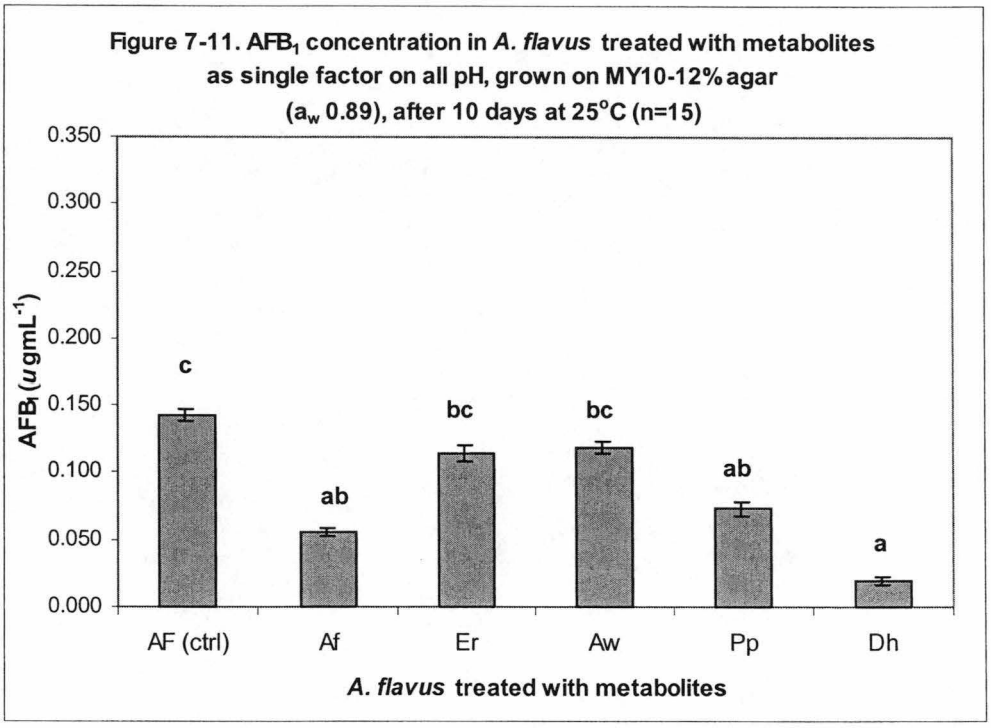


Table 7-14. ANOVA of AFB_1 concentration in *A. flavus* treated with extracts at different pH, grown on MY10-12% agar (a_w 0.89), after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.234	29	8.055E-02	2.603	0.001
Intercept	0.708	1	0.708	229.233	0.000
Extracts	0.176	5	3.523E-02	11.406	0.000
pH	1.524E-02	4	3.810E-02	1.237	0.306
Extracts*pH	4.217E-02	20	2.109E-02	0.683	0.827
Error	0.185	60	3.089E-02		
Total	1.127	90			
Corrected total	0.419	89			

At all pH treatments, *D. hansenii, P. pisce* and non-toxic *A. flavus* extracts significantly reduced the AFB_1 concentration in *A. flavus* ($p \leq 0.05$). Table 7-15 indicates that the extract of *D. hansenii* adjusted to basic pH eliminated AFB_1 in *A. flavus* and at pH 2-6 the reduction was about 70%. The extract of non-toxigenic *A. flavus* caused a 47-76% reduction of AFB_1 concentration in *A. flavus* over the pH range. The *P. pisce*

extract decreased AFB_1 concentration in *A. flavus* by 44-64% with the highest reduction at pH 2. Neither *A. wentii* nor *E. rubrum* extracts significantly reduced AFB_1 concentration in *A. flavus*.

Table 7-15. AFB_1 concentration in *A. flavus* treated with extracts at different pH, grown on MY10-12% agar (a_w 0.89), after 10 days at 25°C (n=3)

Extracts	AFB_1 µg mL^{-1} (mean!SE, n=3)				
	pH2	pH4	pH6	pH8	pH10
A. flavus (control)*	0.125 ±0.01	0.151 ± 0.01	0.116 ±0.01	0.160 ±0.01	0.223 ±0.02
A. flavus (non-toxigenic)	0.053 ±0.03	0.080 ±0.02	0.045 ±0.02	0.045 ±0.02	0.053 ±0.03
E. rubrum	0.071 ±0.02	0.089 ±0.03	0.142 ±0.01	0.125 ±0.01	0.143 ±0.01
A. wentii	0.107 ±0.01	0.112 ± 0.01	0.089± 0.02	0.142 ±0.01	0.116 ±0.01
P. pisce	0.045 ±0.02	0.063 ±0.02	0.053 ±0.03	0.089 ±0.02	0.125 ±0.01
D. hansenii	0.026 ±0.11	0.035 ±0.02	0.035 ±0.02	0	0

Note: * = axenic culture.

(c) Effects on AFG_1 concentration in *A. parasiticus* and *A. flavus*

ANOVA of AFG_1 concentration in *A. parasiticus* shows extracts alone were significant at $p \leq 0.05$ (Table 7-16). The pH and its interaction were insignificant. Thus, Tukey's test was only performed on extracts as a single factor of all values of pH (Figure 7-12). It is clear that the main effect of the extracts resulted from the elimination of AFG_1 in *A. parasiticus* by *D. hansenii* at all pH value, by *P. pisce* and non-toxigenic *A. flavus* extracts at pH 4-8, and by *A. wentii* at pH 6 (Table 7-17). The extract of *E. rubrum* caused some reduction in AFG_1 concentration, but over the range of pH tested this was not significant.

Table 7-16. ANOVA of AFG_1 concentration in *A. parasiticus* treated with extracts at different pH, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1.156	29	3.985E-02	1.561	0.073
Intercept	0,.552	1	0.552	21.625	0.000
Extracts	0.522	5	0.104	4.092	0.003
pH	0.106	4	2.660E-02	1.042	0.393
Extracts*pH	0.527	20	2.634E-02	1.032	0.442
Error	1.532	60	2.553E-02		
Total	3.239	90			
Corrected total	2.687	89			

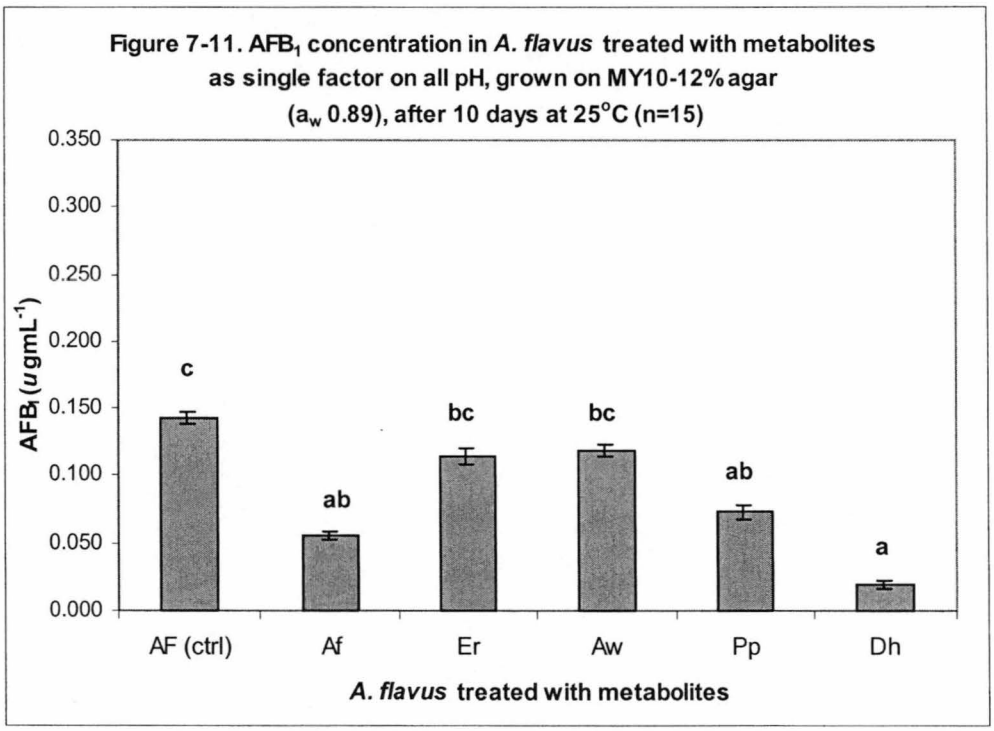


Table 7-17. AFG_1 concentration in *A. parasiticus* treated with extracts at different pH, grown on MY10-12% agar (a_w 0.89), after 10 days at 25°C (n=3)

Extracts	AFG_1 µg mL^{-1}(mean!SE, n=3)				
	pH2	pH4	pH6	pH8	pH10
A. parasiticus (control)*	0.116 ±0.01	0.151 ±0.01	0.143 ±0.01	0.125 ±0.01	0.116 ±0.01
A. flavus (non-toxigenic)	0.046 ±0.02	0	0	0	0.062 ±0.01
E. rubrum	0.160 ±0.01	0.116 ±0.01	0.107 ±0.01	0.116 ±0.01	0.097 ±0.01
A wentii	0.098 ±0.01	0.036 ±0.02	0	0.116 ±0.01	0.089 ±0.01
P. pisce	0.036 ±0.02	0	0	0	0.142 ±0.01
D. hansenii	0	0	0	0	0

Note: * = axenic culture

ANOVA of transformed data (into the square root for homogeneity of variance) of AFG_1 concentration in *A. flavus*, shows that the extracts as a single factor significantly ($p \leq 0.05$) affected AFG_1 concentration in *A. flavus*, but pH did not. Neither was the interaction significant (Table 7-18). Therefore, comparison was made only on the extracts for all pH treatments. Only extract of *D. hansenii* at basic pH (8 and 10) completely eliminated AFG_1 concentration in *A. flavus* as did the extract of *P. pisce* at acidic pH (2). With the exception of the extract from *E. rubrum*, all extracts significantly reduced AFG_1 concentration in *A. flavus* by 30-60% (Table 7-19, Figure 7-13).

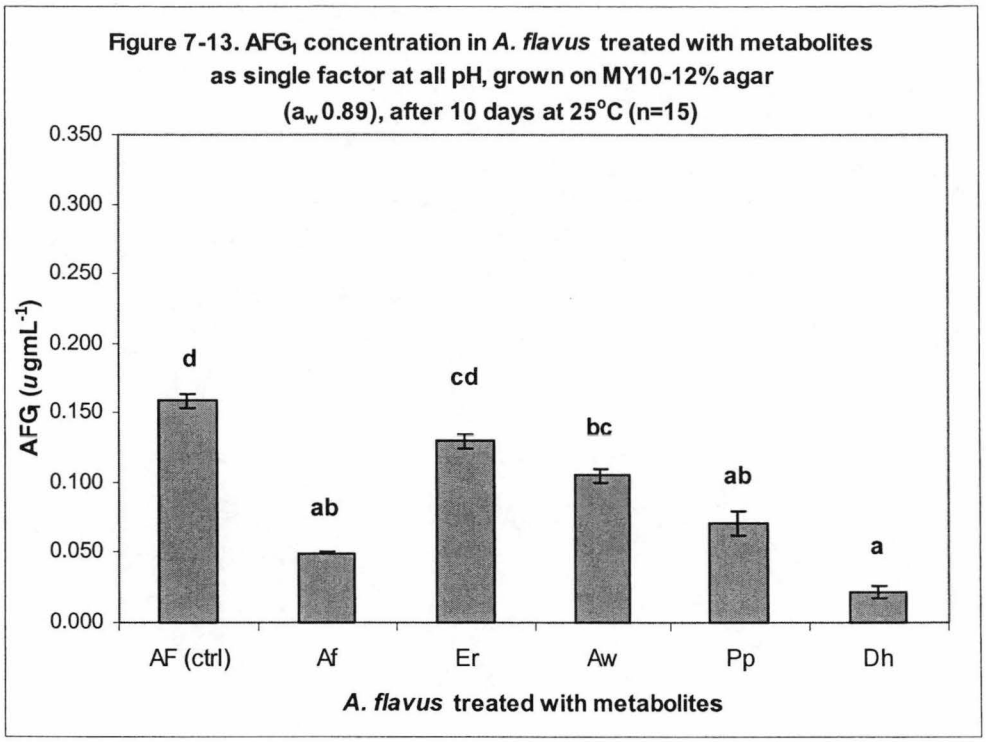
Table 7-18. ANOVA of AFG_1 concentration in *A. flavus* treated with extracts at different pH on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1.985	29	6.843E-02	2.066	0.009
Intercept	6.200	1	6.200	187.152	0.000
Extracts	1.256	5	0.251	7.582	0.000
pH	0.153	4	3.830E-02	1.156	0.339
Extracts*pH	0.575	20	2.875E-02	0.868	0.625
Error	1.988	60	3.313E-02		
Total	10.173	90			
Corrected total	3.972	89			

Table 7-19. Mean AFG_1 concentration in *A. flavus* treated with extracts at different pH, grown on MY10-12% agar (a_w 0.89), after 10 days at 25°C (n=3)

Extracts	AFG_1 μg mL^{-1} (mean!SE, n=3)				
	pH2	pH4	pH6	pH8	pH10
A. parasiticus (control)*	0.134 ±0.01	0.178 ±0.01	0.142 ±0.01	0.187 ±0.01	0.151 ±0.01
A. flavus (non-toxigenic)	0.045 ±0.02	0.05 ±0.03	0.053 ±0.03	0.053 ±0.03	0.045 ±0.02
E. rubrum	0.089 ±0.01	0.125 ±0.01	0.142 ±0.01	0.151 ±0.01	0.142 ±0.01
A. wentii	0.089 ±0.01	0.098 ±0.01	0.080 ±0.02	0.116 ±0.01	0.142 ±0.02
P. pisce	0	0.080 ±0.01	0.071 ±0.02	0.089 ±0.01	0.116 ±0.01
D. hansenii	0.027 ±0.01	0.036 ±0.02	0.045 ±0.02	0	0

Note: * = axenic culture



7.3.3 Enzyme-treated Extracts

(a) Effects on colony diameter of *A. parasiticus* and *A. flavus*

Statistically, the enzymes used to treat the extracts significantly affected the colony diameter of *A. parasiticus*. ANOVA of colony diameter of *A. parasiticus* with extracts treated with different enzymes indicated that the extracts did not significantly affected colony diameter. However, the interaction of metabolites and enzymes was significant at $p \leq 0.05$ (Table 7-20). From Figures 7-14a, b and c, it can be seen that the significant result of this interaction was caused by the interaction of particular metabolites treated with particular enzymes compared to those metabolites treated with other enzymes. For example: metabolites of non-toxigenic *A. flavus* and *D. hansenii*. In these three figures, the lowest point of colony diameter of *A. parasiticus* resulted from non-toxigenic *A. flavus* metabolites treated with proteinase-K (53% reduction) and carboxypeptidase A, and *D. hansenii* metabolites treated with protease. This interaction caused the significant result in ANOVA (Table 7-20).

Table 7-22. ANOVA colony diameter of *A. parasiticus* treated with metabolites added with enzymes, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

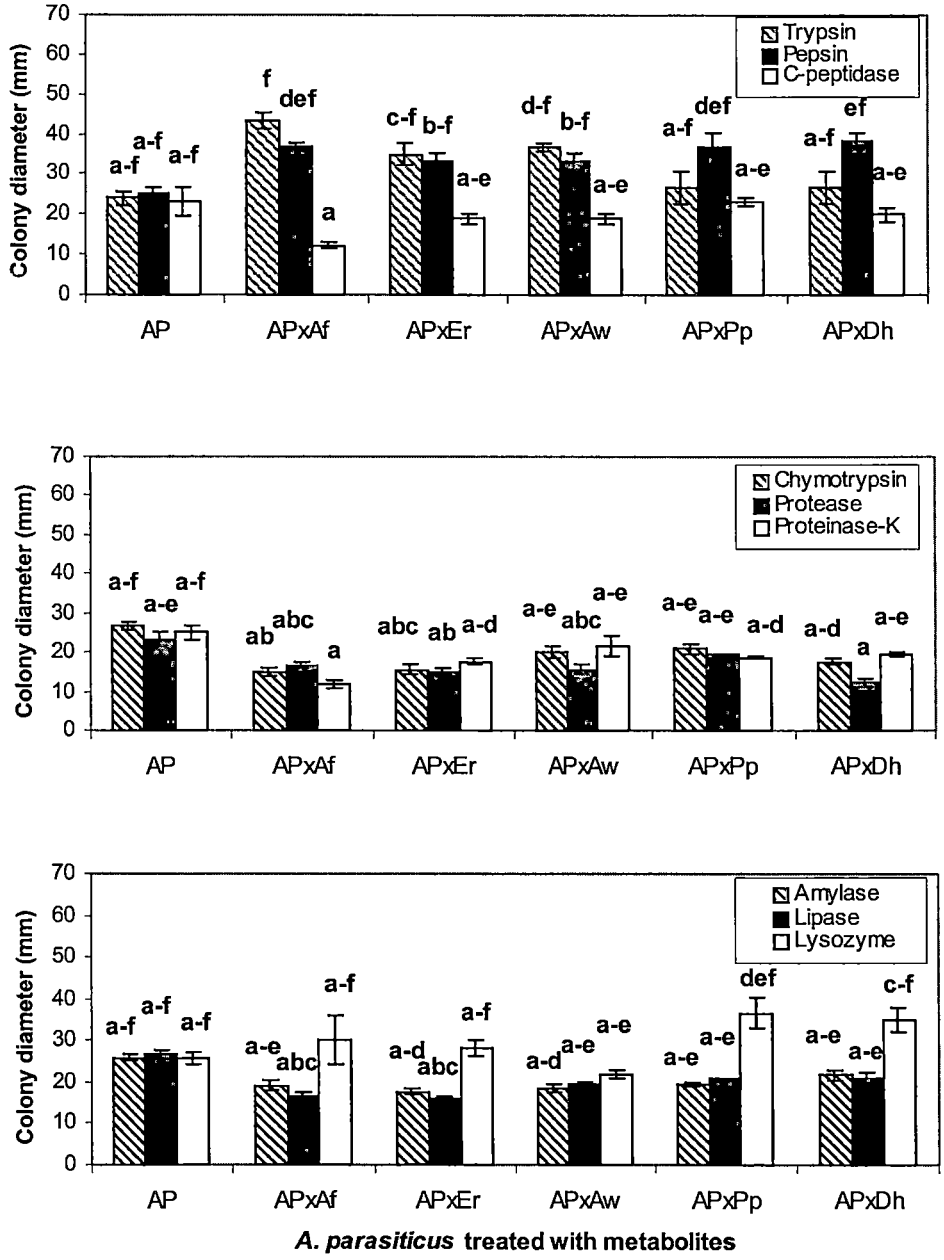
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	9080.944	53	171.339	5.256	0.000
Intercept	88901.389	1	88901.389	2727.410	0.000
Metabolites	214.944	5	42.989	1.319	0.262
Enzymes	6017.111	8	752.139	23.073	0.000
Metabolites* Enzymes	2848.889	40	71.222	2.185	0.001
Error	3520.667	108	32.599		
Total	101503.000	162			
Corrected total	12601.611	161			

Similar results were seen on the growth of *A. flavus* (Table 7-21, Figures 7-15a, b and c). Essentially, none of the treatments significantly reduced the growth of *A. parasiticus* and *A. flavus* in comparison to the controls.

Table 7-21. ANOVA of colony diameter of *A. flavus* treated with metabolites added with enzymes on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

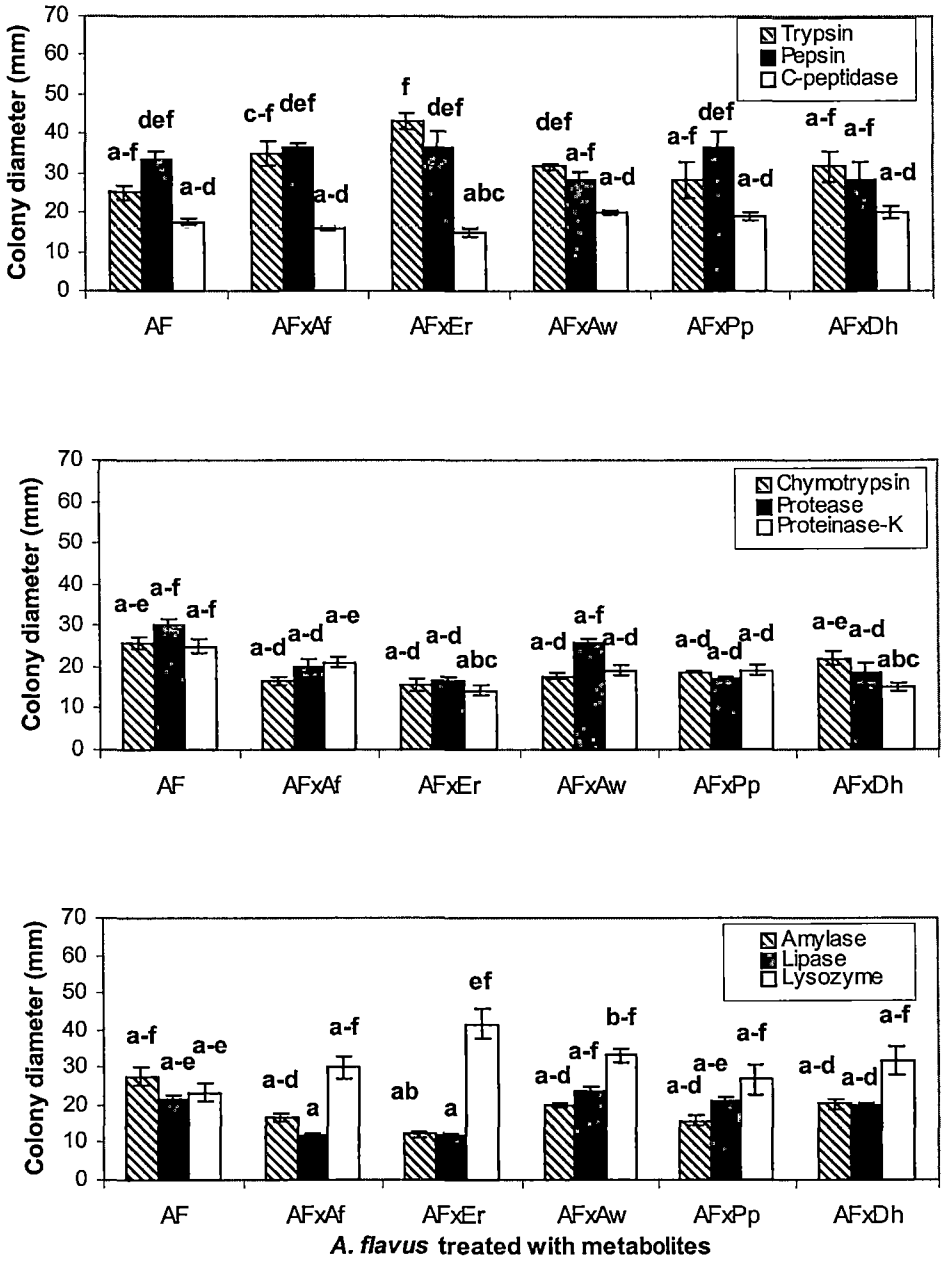
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	9894.278	53	186.684	4.790	0.000
Intercept	90029.389	1	90029.389	2309.908	0.000
Metabolites	231.907	5	46.381	1.190	0.319
Enzymes	6651.444	8	831.431	21.332	0.000
Metabolites* Enzymes	3010.926	40	75.273	1.931	0.000
Error	4209.333	108	38.975		
Total	104133.000	162			
Corrected total	14103.611	161			

Figure 7-14. Colony diameter of *A. parasiticus* grown with enzymes-treated metabolites on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3). Figures 7-14a (top), 7-14b (middle) and 7-14c (bottom).



Note: Superscripts are comparable across Figures 7-14 a, b and c (above).

Figure 7-15. Colony diameter of *A. flavus* grown with enzymes-treated metabolites on MY10-12% agar (a_w 0.89), after 10 days at 25°C (n=3). Figures 7-15a (top), 7-15b (middle) and 7-15c (bottom).



Note: Superscripts are comparable across Figures 7-15a, b and c (above).

(b) Effects on AFB_1 concentration of *A. parasiticus* and *A. flavus*

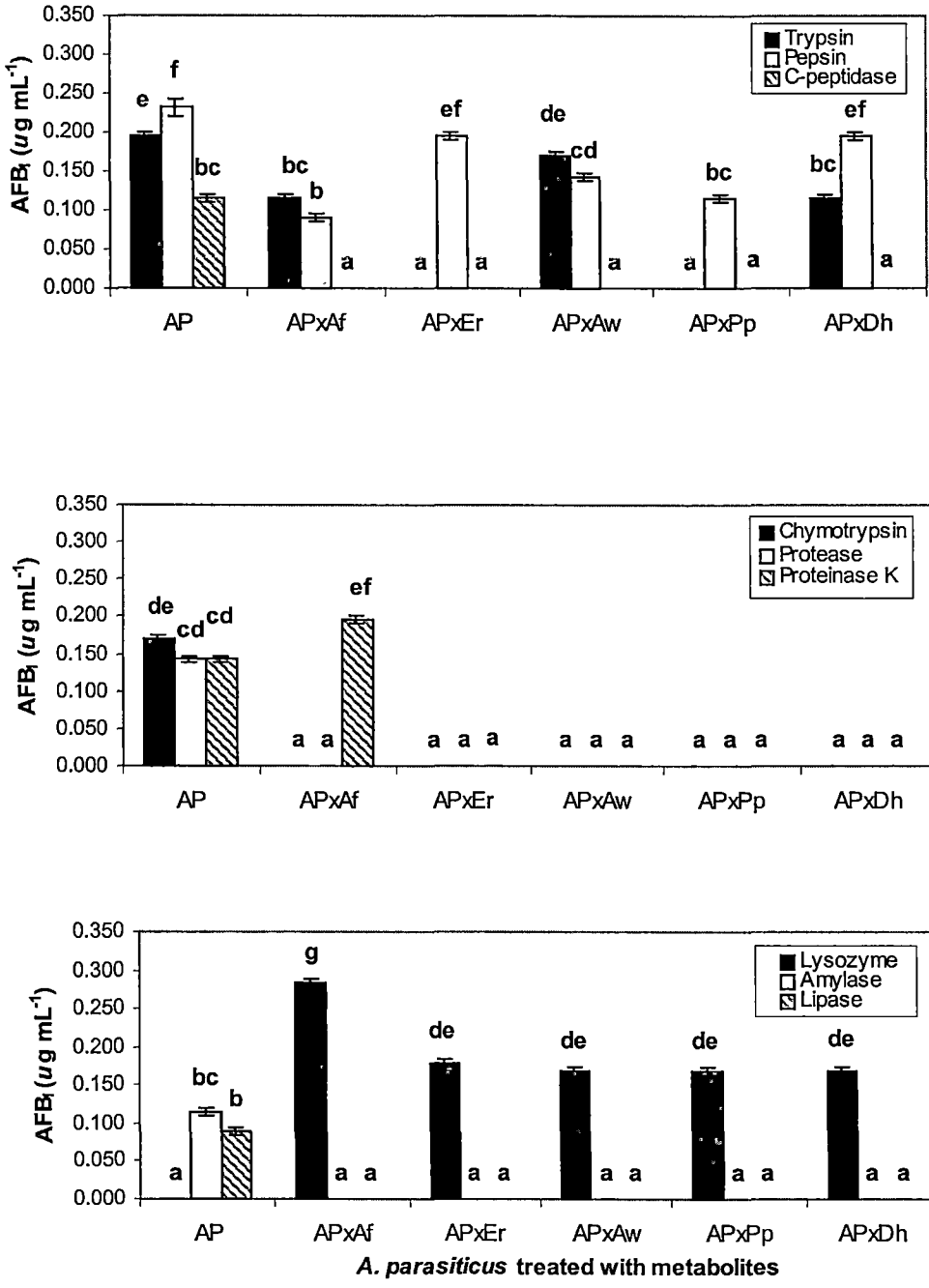
Generally, enzyme treatment of the metabolites significantly affected AFB_1 concentration in *A. parasiticus* and *A. flavus*. AFB_1 production in these two aflatoxigenic fungi was eliminated by all metabolites treated with carboxypeptidase A, α-chymotrypsin, protease, α-amylase and lipase A enzymes as shown in Figure 7-16 and Figure 7-17. Metabolites treated with trypsin, pepsin and lysozyme caused various effects on AFB_1 concentration in *A. parasiticus* and *A. flavus*. Four metabolites (*D. hansenii, P. pisce, A. wentii* and *E. rubrum*) treated with proteinase-K eliminated AFB_1 concentration in *A. parasiticus* and *A. flavus*.

Table 7-22 shows all metabolites and enzymes singly and in their interactions significantly affected AFB_1 concentration in *A. parasiticus* ($p \leq 0.05$). Multiple comparisons were made for all combinations on AFB_1 concentration in *A. parasiticus* (Figure 7-16a, b and c). Trypsin treatment of the metabolites of *E. rubrum* and *P. pisce* completely eliminated AFB_1 in *A. parasiticus*. Metabolites from non-toxigenic *A. flavus* and *D. hansenii* significantly reduced AFB_1 by 40% in comparison to the control, metabolites of *A. wentii* reduced AFB_1 by 13%, but this was not statistically significant.

Table 7-22. ANOVA of AFB_1 in *A. parasiticus* treated with metabolites added with enzymes, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1.137	53	2.146E-02	193.370	0.000
Intercept	0.684	1	0.684	6165.649	0.000
Metabolites	0.182	5	3.649E-02	328.785	0.000
Enzymes	0.536	8	6.694E-02	603.203	0.000
Metabolites*Enzymes	0.419	40	1.048E-02	94.476	0.000
Error	1.199E-02	108	1.110E-04		
Total	1.834	162			
Corrected total	1.149	161			

Figure 7-16. AFB_1 concentration in *A. parasiticus* grown with enzyme-treated metabolites on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3). Figures 7-16a (top), 7-16b (middle), 7-16c (bottom).



Note: Superscripts are comparable across Figure 7-16a, b and c

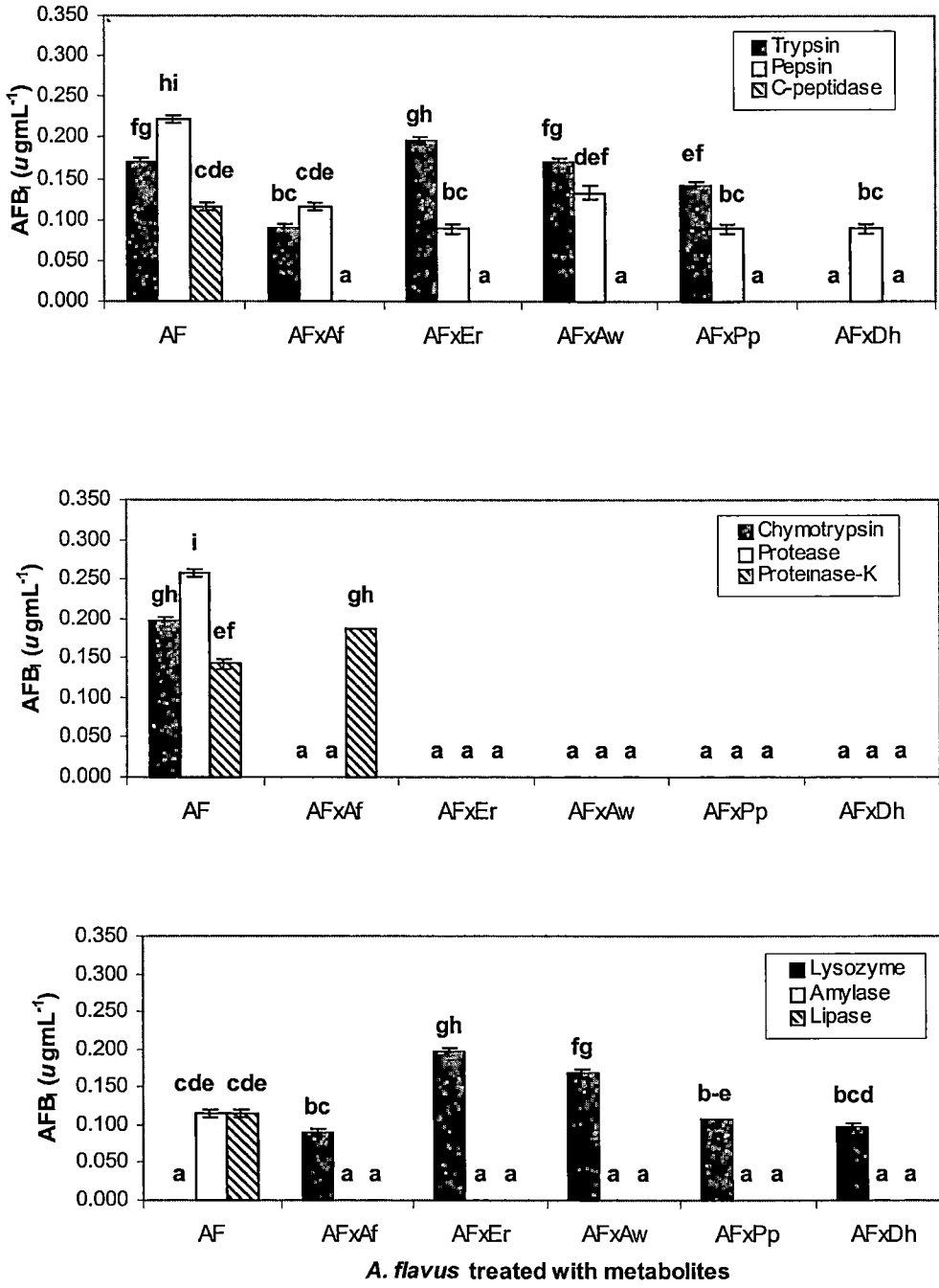
From Figure 7-16a, it can be seen that AFB_1 concentration in *A. parasiticus* was significantly reduced by pepsin treatment of metabolites from non-toxigenic *A. flavus* by 60%, *A. wentii* by 33% and *P. pisce* by 45%. Metabolites of *E. rubrum* and *D. hansenii* only reduced AFB_1 concentration in *A. parasiticus* by 8% and this was not statistically significant. Except for a 37% increase by non-toxigenic *A. flavus*, metabolites combined with proteinase-K eliminated AFB_1 in *A. parasiticus* (Figure 7-16b). Control *A. parasiticus* treated with lysozyme showed no AFB_1 production, however, in the presence of all metabolites, *A. parasiticus* produced AFB_1. Non-toxigenic *A. flavus* metabolites treated with lysozyme showed the highest AFB_1 (0.285 $\mu g\ mL^{-1}$) concentration in *A. parasiticus* (Figure 7-16c).

Both metabolites and enzymes singly and in interaction significantly affected ($p \leq 0.05$) AFB_1 concentration in *A. flavus* (Table 7-23). Thus, multiple comparisons of all interactions were made for AFB_1 concentration in *A. flavus*. When treated with trypsin only metabolites of *D. hansenii* completely eliminated AFB_1 concentration in *A. flavus*. Metabolites of non-toxigenic *A. flavus* significantly reduced AFB_1 concentration in *A. flavus* by 47% and the reminder had no significant effect on AFB_1 in *A. flavus* (Figure 7-17a).

Table 7-23. ANOVA of AFB_1 in *A. flavus* treated with metabolites added with enzyme, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.962	53	1.815E-02	108.533	0.000
Intercept	0.586	1	0.586	3505.921	0.000
Metabolites	0.272	5	5.44E-02	325.479	0.000
Enzymes	0.291	8	3.641E-02	217.724	0.000
Metabolites*Enzymes	0.398	40	9.962E-03	59.576	0.000
Error	1.806E-02	108	1.672E-04		
Total	1.566	162			
Corrected total	0.980	161			

Figure 7-17. AFB_1 concentration in *A. flavus* grown with enzyme-treated metabolites on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3). Figures 7-17a (top), 7-17b (middle) and c (bottom).



Note: Superscripts are comparable across Figures 7-17a, b and c (above).

Pepsin added to all metabolites significantly decreased AFB_1 concentration in *A. flavus* (Figure 7-17a). AFB_1 production in *A. flavus* was eliminated by all metabolites treated with carboxypeptidase A, α-chymotrypsin, protease, α-amylase and lipase A enzymes. While proteinase-K in other metabolites eliminated AFB_1 concentration in *A. flavus*, metabolites of non-toxigenic *A. flavus* significantly increased the concentration by 31% (Figure7-17b). The lysozyme-treated *A. flavus* control did not produce AFB_1, but all the metabolites treated with this enzyme significantly increased AFB_1 production in *A. flavus* by 0.089 to 0.196 $\mu g\ mL^{-1}$. The highest production of AFB_1 in *A. flavus* was in the presence of metabolites of *E. rubrum* treated with lysozyme (Figure 7-17c).

(c) Effects on AFG_1 concentration in *A. parasiticus* and *A. flavus*

When metabolites were mixed with enzymes, similar results were observed for AFG_1 concentration in both *A. parasiticus* and *A. flavus*. All factors, metabolites, enzymes and their interactions significantly affected ($p \leq 0.05$) AFG_1 concentration in *A. parasiticus* and *A. flavus* (Table 7-24 and Table 7-25). When carboxypeptidase A, α-chymotrypsin, protease, α-amylase or lipase A enzymes were added into the metabolites, AFG_1 was eliminated in the two aflatoxigenic fungi compared to the axenic controls (Figures 7-18a, b and c and Figures 7-19a,b and c). Therefore, multiple comparisons of all interactions were examined across each of the aflatoxigenic fungi.

Metabolites of all fungi except *P. pisce* significantly reduced AFG_1 concentration of *A. parasiticus* in the presence of trypsin. In the presence of pepsin, only the metabolites of non-toxigenic *A. flavus* significantly reduced AFG_1 in *A. parasiticus*. Apart from the metabolites of *D. hansenii,* in the presence of lysozyme, the metabolites caused a significant increase in AFG_1 concentration in *A. parasiticus*.

Table 7-24. ANOVA of AFG_1 in *A. parasiticus* treated with metabolites added with different enzymes grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

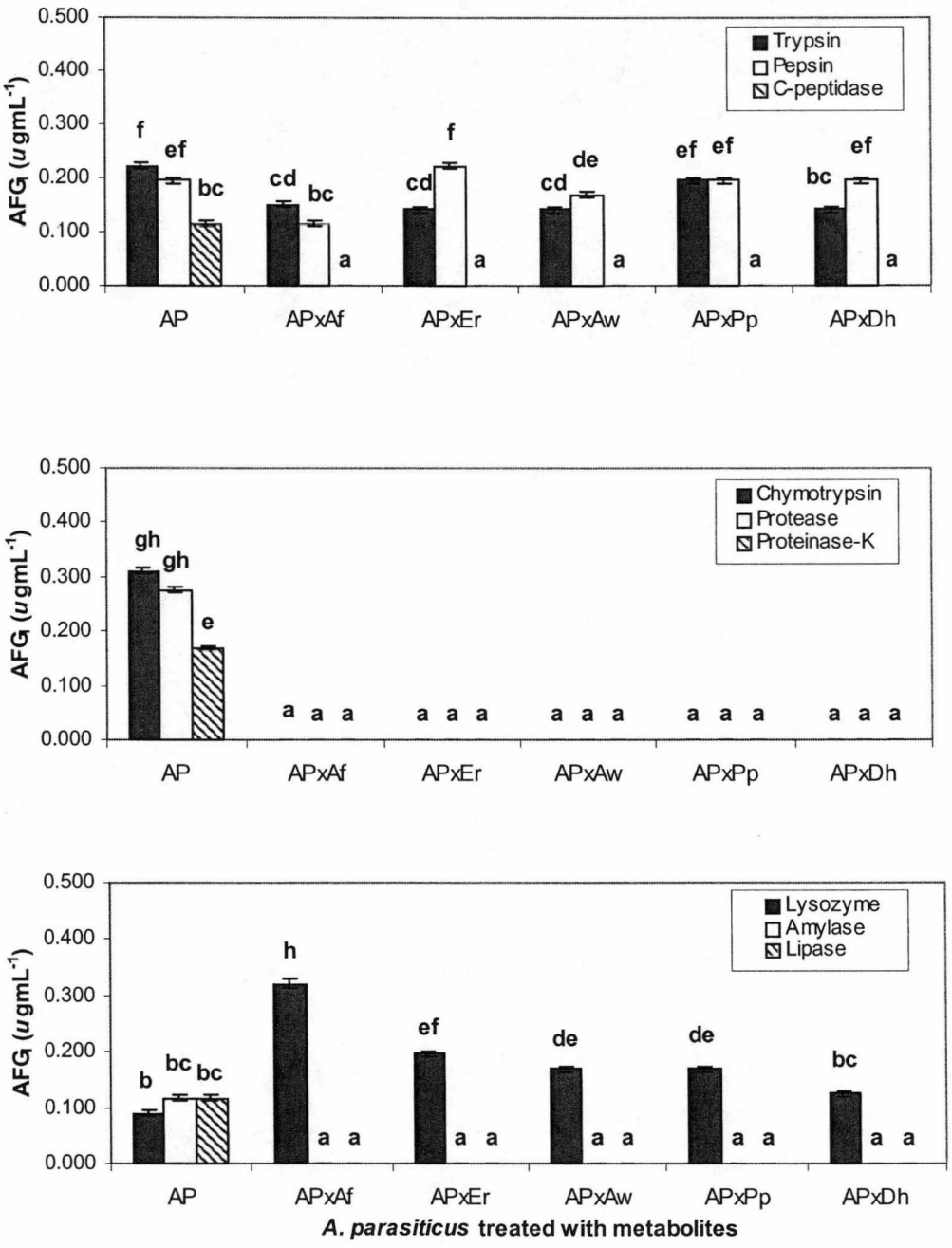
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1.667	53	3.144E-02	285.346	0.000
Intercept	1.102	1	1.102	9999.306	0.000
Metabolites	0.458	5	9.153E-02	830.563	0.000
Enzymes	0.721	8	9.015E-02	818.040	0.000
Metabolites*Enzymes	0.488	40	1.219E-02	110.656	0.000
Error	1.190E-02	108	1.102E-04		
Total	2.780	162			
Corrected total	1.678	161			

Table 7-25. ANOVA of AFG_1 in *A. flavus* treated with metabolites added with different enzymes grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1.271	53	2.398E-02	136.242	0.000
Intercept	0.806	1	0.806	4580.612	0.000
Metabolites	0.117	5	2.335E-02	132.689	0.000
Enzymes	0.616	8	7.697E-02	437.360	0.000
Metabolites*Enzymes	0.538	40	1.346E-02	76.463	0.000
Error	1.901E-02	108	1.760E-04		
Total	2.096	162			
Corrected total	1.290	161			

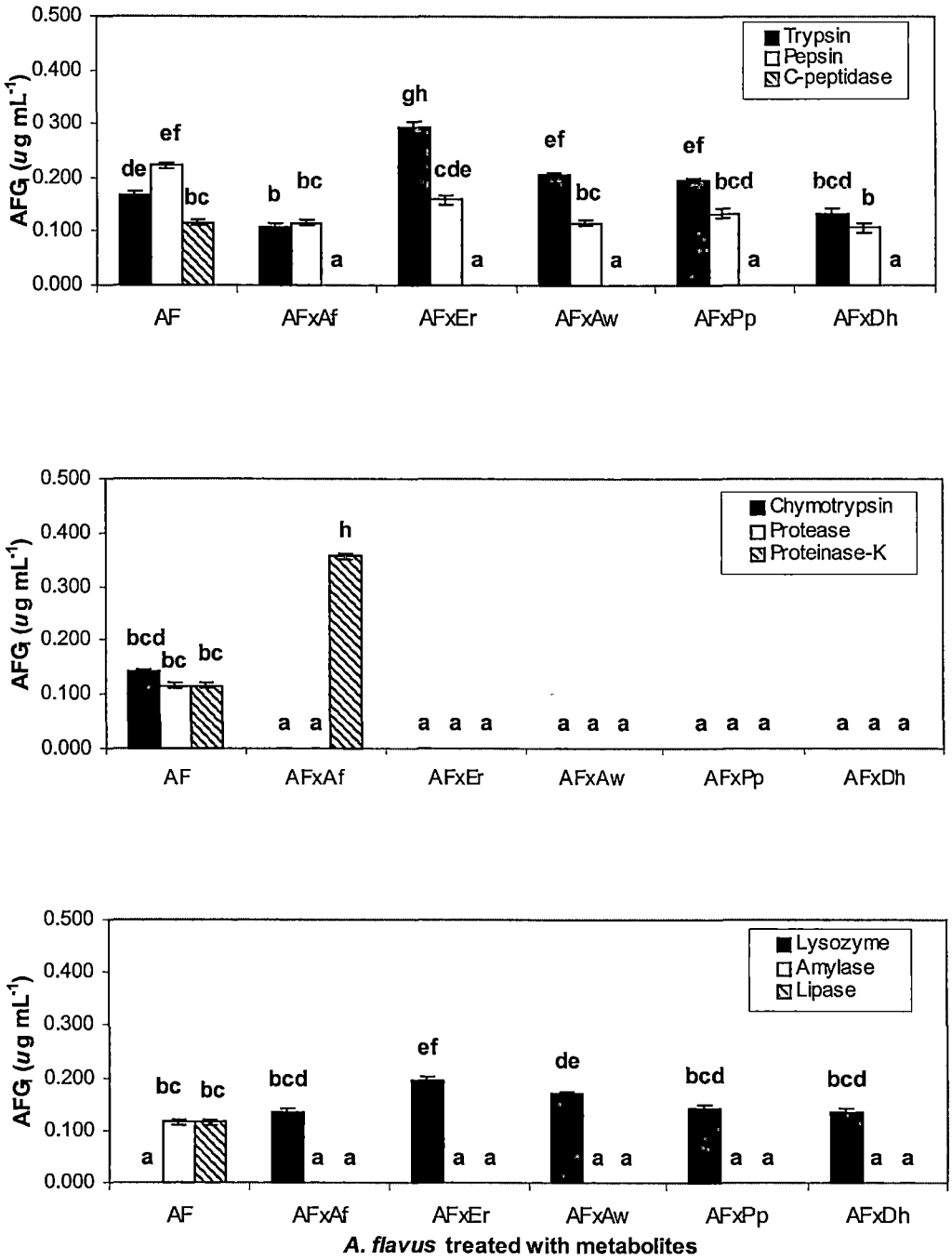
When trypsin was added to metabolites, only those of non-toxigenic *A. flavus* caused a significant ($p \leq 0.05$) decrease of AFG_1 concentration in *A. flavus* (by 36% in comparison to the control). With trypsin metabolites of *E. rubrum* significantly increased AFG_1 concentration in *A. flavus*. As did the metabolites of non-toxigenic *A. flavus* with proteinase K. Treated with pepsin, metabolites of *D. hansenii,* non-toxic *A. flavus, A. wentii* and *P. pisce* significantly ($p \leq 0.05$) reduced AFG_1 concentration in *A. flavus* by more than 40%. The control of *A. flavus* with lysozyme did not produce AFG_1. However, lysozyme added to all metabolites significantly induced AFG_1 production in *A. flavus*, with the highest amount of 0.196 $\mu g\ mL^{-1}$ observed on *E. rubrum* metabolites.

Figure 7-18. AFG_1 concentration in *A. parasiticus* grown with enzyme-treated metabolites on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3). Figures 7-18a (top), 7-18b (middle) and 7-18c (bottom).



Note: Superscripts are comparable across Figures 7-18a, b and c (above).

Figure 7-19. AFG_1 concentration in *A. flavus* grown with enzyme-treated metabolites on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3). Figures 7-19a (top), 7-19b (middle) and 7-19c (bottom).



Note: Superscripts are comparable across Figures 7-19a, b and c (above).

7.3.4 Effects of metabolite treatments on AFB_2 and AFG_2 concentrations in *A. parasiticus* and *A. flavus*

Concentrations of AFB_2 and AFG_2 produced by *A. parasiticus* and *A. flavus* were very low. Almost all metabolites eliminated AFB_2 and AFG_2 from both *A. parasiticus* and *A. flavus*. However, at 85°C, metabolites of *E. rubrum* did not affect AFG_2 concentrations in *A. parasiticus* and *A. flavus*. AFB_2 and AFG_2 were not detected in axenic *A. parasiticus* and *A. flavus* at acidic pH values (pH 2 and 4) and in almost all the controls treated with enzymes, with the exception being pepsin and trypsin. Only the axenic controls that produced AFB_2 and AFG_2 with treatments are shown in Table 7-26.

Table 7-26. AFB_2 and AFG_2 concentrations (mean±SE, n=3)in axenic *A. parasiticus* and *A. flavus* used as treatment controls, grown on MY10-12% agar (a_w 0.89) at 25°C

Treatments	*A. parasiticus*		*A. flavus*	
	AFB_2 ($\mu g\ mL^{-1}$)	AFG_2 ($\mu g\ mL^{-1}$)	AFB_2 ($\mu g\ mL^{-1}$)	AFG_2 ($\mu g\ mL^{-1}$)
25°C	0.062 ±0.02	0.071 ±0.02	0.071±0.02	-
60°C	0.071 ±0.02	0.071 ±0.02	0.071±0.02	-
85°C	0.071 ±0.02	0.062 ±0.02	0.071±0.02	0.062 ±0.02
121°C	0.071 ±0.02	0.062 ±0.02	0.071±0.02	-
pH 6	0.062 ±0.02	0.071 ±0.02	0.071±0.02	-
pH 8	0.071 ±0.02	0.071 ±0.02	0.062±0.02	-
pH 10	0.081 ±0.01	0.081 ±0.02	0.071±0.02	-
Trypsin	0.081 ±0.02	0.081 ±0.02	0.071±0.02	0.071 ±0.02
Pepsin	-	0.062 ±0.01	0.071±0.02	-

7.3.5 Effects of metabolite treatments on fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2 in *A. parasiticus* and *A. flavus*

At a_w 0.89, both *A. parasiticus* and *A. flavus* also produced greenish-blue and blue-green fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2. A greenish-blue fluorescent compound (GB) was observed at Rf value of 0.10-0.20 and a blue-green fluorescent compound (BG) was found at Rf value of 0.20-0.30. Generally,

metabolites of *D. hansenii* demonstrated the best ability in elimination or reduction of these fluorescent compounds. Treated at 25 and 60°C, the GB compounds in *A. parasiticus* and *A. flavus* were diminished in the presence of the metabolites, except those of *E. rubrum* and *A. wentii*.

Table 7-27. Other fluorescent compounds produced by *A. parasiticus* treated with metabolites at different temperature (°C), grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Metabolites	Greenish-blue (GB) fluorescence				Blue-green (BG) fluorescence			
	25	60	85	121	25	60	85	121
A. parasiticus	+	+	+	+	+	+	+	+
AP[1] x non-toxic *A. flavus*	-	-	-	±	-	-	±	±
AP x *E. rubrum*	+	+	+	+	+	+	+	+
AP x *A. wentii*	±	±	+	+	±	±	±	±
AP x *P. pisce*	-	-	±	±	-	-	±	±
AP x *D. hansenii*	-	-	±	±	-	-	±	±

Note: - = absent; + = present; ± = present at reduced intensity; AP[1] = culture *A. parasiticus*

Table 7-28. Other fluorescent compounds produced by *A. parasiticus* treated with metabolites at different values of pH, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Metabolites	Greenish-blue (GB) fluorescence					Blue-green (BG) fluorescence				
	2	4	6	8	10	2	4	6	8	10
A. parasiticus	±	±	+	+	±	+	+	+	+	+
AP[1] x non-toxic *A. flavus*	-	±	±	±	±	±	+	±	±	+
AP x *E. rubrum*	-	±	±	±	±	±	+	+	+	+
AP x *A. wentii*	-	±	±	-	±	-	±	±	-	±
AP x *P. pisce*	-	-	-	±	±	-	±	±	±	±
AP x *D. hansenii*	-	±	-	-	-	-	±	-	-	-

Note: - = absent; + = present; ± = present at reduced intensity; AP[1] = culture *A. parasiticus*

When treated at different values of pH, mostly axenic *A. parasiticus* produced BG compounds and axenic *A. flavus* produced GB compounds. Added with trypsin, both axenic *A. parasiticus* and *A. flavus* produced a strong intensity of the GB compounds. However, the intensity of these compounds was reduced in the presence of all the metabolites. The following tables show the presence of the two fluorescent compounds produced by *A. parasiticus* (Tables 7-27, 7-28 and 7-29) and *A. flavus* (Tables 7-30, 7-31 and 7-32) treated with metabolites at different temperatures, different values of pH and with different enzymes.

Table 7-29. Other fluorescent compounds produced by *A. parasiticus* grown with metabolites treated with enzymes on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Metabolites	Enzymes								
	Try	Pep	C-p	α-C	Pro	P-K	Lys	α-A	Lip
A. parasiticus	$++^{a}$	$+^{ab}$	$+^{ab}$	$+^{ab}$	$+^{ab}$	$+^{ab}$	$\pm^{ab}$	$+^{ab}$	$+^{ab}$
AP[1] x non-toxic *A. flavus*	-	-	-	-	±	-	-	-	-
AP x *E. rubrum*	-	-	$\pm^{a}$	-	-	$\pm^{a}$	-	-	-
AP x *A. wentii*	-	-	$\pm^{a}$	-	-	$\pm^{a}$	-	-	-
AP x *P. pisce*	-	-	-	$\pm^{a}$	-	$\pm^{a}$	-	$\pm^{a}$	$\pm^{a}$
AP x *D. hansenii*	-	-	-	-	-	-	-	-	-

Note: - = absent; + = present; ++ = strong intensity; ± = present at reduced intensity; AP[1] = culture *A. parasiticus*; a = greenish blue fluorescence (GB); b = blue-green fluorescence (BG); Try = trypsin; Pep = pepsin; Cp =Carboxypeptidase A; α-Ct = α-chymotrypsin, Pro = protease; P-K = proteinase-K; Lys = lysozyme; α-A = α-amylase; Lip = Lipase A.

Table 7-30. Other fluorescent compounds produced by *A. flavus* treated with metabolites at different temperature (°C) and grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Metabolites	Greenish-blue (GB) fluorescence				Blue-green (BG) fluorescence			
	25	60	85	121	25	60	85	121
A. flavus	+	+	+	+	+	+	+	+
AF[1] x non-toxic *A. flavus*	-	-	-	±	-	-	-	±
AF x *E. rubrum*	+	+	±	±	+	+	±	±
AF x *A. wentii*	±	±	-	±	±	-	-	±
AF x *P. pisce*	-	-	±	±	-	-	±	±
AF x *D. hansenii*	-	-	±	±	-	-	±	±

Note: - = absent; + = present; ± = present at reduced intensity; AF[1] = culture *A. flavus*

Table 7-31. Other fluorescent compounds produced by *A. flavus* treated with metabolites at different values of pH, grown on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=-3)

Metabolites	Greenish-blue (GB) fluorescence					Blue-green (BG) fluorescence				
	2	4	6	8	10	2	4	6	8	10
A. flavus	+	+	+	+	+	±	±	+	+	±
AF[1] x non-toxic *A. flavus*	-	-	-	-	-	-	-	-	-	-
AF x *E. rubrum*	±	±	±	±	±	-	-	±	±	±
AF x *A. wentii*	±	±	±	±	±	±	-	-	-	±
AF x *P. pisce*	-	-	-	-	-	-	-	-	-	-
AF x *D. hansenii*	-	-	-	-	-	-	-	-	-	-

Note: - = absent; + = present; ± = present at reduced intensity; AF[1] = culture *A. flavus*;

Table 7-32. Other fluorescent compounds produced by *A. flavus* grown with metabolites treated with enzymes, on MY10-12% agar (a_w 0.89) after 10 days at 25°C (n=3)

Metabolites	Enzymes								
	Try	Pep	C-p	α-C	Pro	P-K	Lys	α-A	Lip
A. flavus	$++^{a}$	$+^{ab}$	$+^{ab}$	$+^{ab}$	$+^{ab}$	$+^{ab}$	$\pm^{ab}$	$+^{ab}$	$+^{ab}$
AF[1] x non-toxic *A. flavus*	-	-	-	-	-	-	-	-	-
AF x *E. rubrum*	-	-	$+^{a}$	-	-	-	-	-	-
AF x *A. wentii*	-	-	$+^{a}$	-	-	-	-	-	$+^{a}$
AF x *P. pisce*	-	-	-	-	-	$\pm^{a}$	-	$\pm^{a}$	-
AF x *D. hansenii*	-	-	-	-	-	-	-	-	$\pm^{a}$

Note: - = absent; + = present; ++ = strong intensity; ± = present at reduced intensity; AF[1] = culture *A. flavus*; a = greenish blue fluorescence (GB); b = blue-green fluorescence (BG); Try = trypsin; Pep = pepsin; C-p =Carboxypeptidase A; α-C = α-chymotrypsin, Pro = protease; P-K = proteinase-K; Lys = lysozyme; α-A = α-amylase; Lip = Lipase A.

7.4 DISCUSSION

The stability of *D. hansenii*, *P. pisce*, *A. wentii*, *E. rubrum* and non-toxigenic *A. flavus* metabolites varied over the different range of temperatures, pH and enzymes tested. Partial characterization on these five fungal metabolite extracts from agar demonstrated the metabolites differed from each other in the effects that temperatures, values of pH and enzymes had on their activity on growth and aflatoxin production in *A. parasiticus* and *A. flavus*. As seen before, the metabolites did not greatly reduce growth of either *A. parasiticus* or *A. flavus* if at all. That is, the important mode of action was directly against the aflatoxins. Generally, metabolites of *D. hansenii* demonstrated the highest heat stability. Activity against AFG_1 was retained more than activity against AFB_1. The metabolites of *D. hansenii* were active over wide pH range (pH 2-10), indeed at pH 8 all aflatoxins were eliminated. The metabolites of non-toxigenic *A. flavus* and *E. rubrum* at pH near neutral and the metabolites of *A. wentii* and *P. pisce* at neutral to basic pH were at least able to reduce aflatoxin concentrations.

In the present study, trypsin, pepsin and lysozyme variously affected the metabolites, while carboxypeptidase A, α-chymotrypsin, protease, α-amylase and lipase A added to metabolites still enabled AFB_1 and AFG_1 to be eliminated in *A. parasiticus* and *A. flavus*. It appears that trypsin, pepsin and lysozyme modified the anti-aflatoxigenic activity of the metabolites of *D. hansenii*, non-toxic *A. flavus* and *A. wentii*. In the presence of these three enzymes, these metabolites increased AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*, when compared to the activities of untreated metabolites in the similar experimental conditions (a_w 0.89 and at 25°C) in Chapter 6. At times, addition on an enzyme increased the inhibitory activity of the metabolites (e.g. metabolites of *P. pisce* treated with carboxypeptidase A, α-chymotrypsin, protease, proteinase K, α-amylase and lipase A eliminated aflatoxins). With the exception of metabolites of *E. rubrum* held at 85°C, all the metabolites eliminated AFB_2 and AFG_2 in *A. parasiticus* and *A. flavus* at all temperatures and values of pH tested.

7.4.1 *Debaromyces hansenii*

Metabolites of *D. hansenii* appeared to be relatively heat-stable at the temperatures examined here. Thus, it may be possible to pasteurize the extract. In the present study, at 25 and 60°C specifically, the inhibitor compound of *D. hansenii* metabolites completely eliminated aflatoxins and at 85 and 121°C consistently decreased AFB_1 and AFG_1 concentrations in *A. parasiticus* and *A. flavus*. Metabolites of *D. hansenii* demonstrated the best response over the pH range tested, in comparison to other metabolites in reducing aflatoxins. Metabolites of *D. hansenii* were more active against *A. parasiticus* than against *A. flavus*. Previous investigation on untreated metabolites showed that metabolites of *D. hansenii* (pH 3.5±0.01) were able to eliminate AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus* (Chapter 6). Irrespective of the enzyme and at almost all temperature and pH treatments, only metabolites of *D. hansenii* eliminated the additional fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2 produced by the aflatoxigenic fungi. When added with protease, proteinase-K, carboxypeptidase A, α-chymotrypsin, α-amylase and lipase enzymes, metabolites of *D. hansenii* eliminated AFB_1 and AFG_1 in aflatoxigenic fungi in the

present study. These findings indicate that metabolites of *D. hansenii* are resistant to these six enzymes. Because *D. hansenii* metabolites are resistant to carboxypeptidase A, the metabolites presumably lacked a free carboxyl terminal group. The active inhibitor compounds of *D. hansenii* metabolites appeared to have no glycerol bonds to a fatty acid because treatment with lipase A did not change the metabolites'activity. Similarly, the active inhibitor of metabolites of *D. hansenii* lacked both hydrophobic amino acids (no α-chymotrypsin sensitivity) and α(1,4) sugar groups (no α-amylase sensitivity).

In the present study, the proteolytic enzymes (trypsin and pepsin) and lysozyme did modify the active inhibitor compounds of *D. hansenii* metabolites, thereby AFB_1 and AFG_1 were found in *A. parasiticus* and *A. flavus*. The active-inhibitor compounds in *D. hansenii* metabolites probably were protein-containing compounds because of their response to trypsin and pepsin. The metabolites of *D. hansenii* could have peptides with lysyl and/or arginyl residues as shown by their reaction with trypsin and may have L-amino acids. The impact of lysozyme suggested that metabolites of *D. hansenii* possess β-1,4 glycosidic bonds that influenced degradation of aflatoxins.

Munimbazi and Bullerman (1998) noted that metabolites of *B. pumilus* did not fluoresce when examined using fluorescamine, a non-fluorescing reagent that could readily react with amines, amino acids, peptides and proteins containing no blocked terminal amino acids or proline to form fluorescent derivatives. They also found that the metabolites of *B. pumilus* were resistant to carboxypeptidase A. Thus, Munimbazi and Bullerman (1998) concluded that the inhibitor of metabolites of *B. pumilus* was a cyclic non-peptide. It is possible that metabolites of *D. hansenii* in the present study possessed an active cyclic structure because of their resistance to carboxypeptidase A.

7.4.2 Nontoxigenic *Aspergillus flavus*

Metabolites of non-toxigenic *A. flavus* were likely to be heat-stable compounds, but mostly reduced AFB_1 and AFG_1 very little in *A. parasiticus* and *A. flavus*. The

reduction of colony diameters by non-toxigenic *A. flavus* metabolites may be possibly correlated to the reduction or elimination of AFB_1 and AFG_1 in both toxigenic *A. parasiticus* and *A. flavus* (as this was also noted in previous studies, Chapters 5 and 6). Elimination of AFB_1 and AFG_1 by metabolites of non-toxigenic *A. flavus* between pH 4-8 indicated that activity of inhibitor compounds of non-toxigenic *A. flavus* metabolites were more active at pH near neutral. Similarly to metabolites of *D. hansenii* and in comparison to the untreated metabolites, non-toxigenic *A. flavus* metabolites were resistant to carboxypeptidase A, α-chymotrypsin, protease, α-amylase and lipase A enzymes. However, the metabolites of non-toxigenic *A. flavus* were sensitive to proteinase-K and lysozyme. Added with proteinase-K or lysozyme, non-toxigenic *A. flavus* metabolites significantly increased AFB_1 concentration in both *A. parasiticus* and *A. flavus* and sometimes more than doubled AFG_1 concentration in *A. flavus*. This increase in AFB_1 and AFG_1 may have resulted from the elimination of other fluorescent compounds found in the aflatoxigenic fungi. This would imply that the proteinase-K added into metabolites of non-toxigenic *A. flavus* either inhibited transformation of aflatoxin in *A. parasiticus* and *A. flavus* or increased AFB_1 and AFG_1 production in *A. parasiticus* and *A. flavus* or probably both. Furthermore, proteinase-K is a non-specific protease and will react with all protein present in the metabolites (Smiley and Draughon, 2000); the enzyme degrades the protein by splitting internal peptide bonds that could lead to destruction of activity of the inhibitor compounds in non-toxigenic *A. flavus* metabolites. Therefore, added proteinase-K into metabolites of non-toxigenic *A. flavus* increased AFB_1 in both *A. parasiticus* and *A. flavus*. Smiley and Draughon (2000), who studied crude protein extract of *F. aurantiacum* added with proteinase-K, found that the degrading activity on AFB_1 of the extracts was diminished compared to the control used, suggesting the crude extracts contained enzymes involved in AFB_1 degradation. These researchers also noted that maximum AFB_1 degradation for the crude extracts occurred at a neutral pH so that the relationship between degradation and pH was typical of an enzymatic reaction. In addition, Hamid and Smith (1987) reported the degradation of AFB_1 and AFG_1 by cell-free extracts of *A. flavus* involved cytochrome P-450 monooxygenase enzymes. Their work noted that adding inhibitors of cytochrome P-450 monooxygenase (e.g. SKF 525-A and

metyrapone) into a cell-free extract of *A. flavus*, reduced AFB_1 and AFG_1 concentrations suggesting involvement of the enzyme in aflatoxin degradation.

From the range of pH values characterized in the present study, metabolites of non-toxigenic *A. flavus* actively degraded AFB_1 and AFG_1 at pH near neutral. In Chapter 6, untreated metabolites of non-toxigenic *A. flavus* were found to have a pH of 6.8 and were able to eliminate AFB_1 and AFG_1 in aflatoxigenic fungi. Thus, the active inhibitor compound of non-toxigenic *A. flavus* could be a protein and may be an enzyme. With proteinase-K as a treatment, AFG_1 was only found in *A. flavus* and not in *A. parasiticus* in the present study. This result suggests the possibility that non-toxigenic *A. flavus* metabolites may have more than one active inhibitor compound. Because lysozyme specifically cleaves the β-1,4 glycosidic bond in polysaccharides, the active inhibitor of metabolites of non-toxigenic *A. flavus* could contain β-1,4 glycosidic bonds. This suggests the possibility that a sugar group may be involved. Taking into consideration the effect of proteinase-K, the presence of a sugar argues against the possibility that the metabolites of non-toxigenic *A. flavus* contain an enzyme that degrades aflatoxins. Rather a peptide with a linked sugar possibly protecting the C-terminal amino acids, as carboxy-peptidase A had no activity.

7.4.3 *Aspergillus wentii*

In the present study, metabolites of *A. wentii* held at 25-85°C eliminated AFB_1 and AFG_1 in *A. parasiticus* and at various temperatures reduced AFB_1 and AFG_1 in *A. flavus*, although the metabolites did not reduce the growth of the two aflatoxigenic fungi. Thus, the active inhibitory compounds of *A. wentii* metabolites appeared to be relatively heat-stable and would remain stable at pasteurization. Metabolites of *A. wentii* demonstrated that the inhibitory compounds were active at neutral pH and becoming more active in inhibiting aflatoxin production in *A. parasiticus* at basic pH. Similarly to the metabolites of *D. hansenii*, the metabolites of *A. wentii* were resistant to carboxypeptidase A, α-chymotrypsin, protease, proteinase-K, α-amylase and lipase A enzymes in comparison to the activity of *A. wentii*'s untreated metabolites on

eliminating AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*. These proved that the active inhibitor compounds of the metabolites of *A. wentii* unlikely posseses a free C-terminal amino acid, hydrophobic amino acids, α(1-4) glycoside links or triacylglycerols. However, treatment with lysozyme demonstrated that the metabolites of *A. wentii* could have internal β-1,4 glycosidic bond, because the enzyme degraded the inhibitory activity of the metabolites. As a consequence, activity of *A. wentii* metabolites was destroyed and the production of AFB_1 and AFG_1 in aflatoxigenic fungi was not inhibited. Further, *A. wentii* metabolites appeared relatively sensitive to trypsin to reduce the toxins in *A. flavus*. Thus, the metabolites of *A. wentii* may have lysyl or arginyl residues that would have been hydrolysed by trypsin, leading to their degradation. Insignificant reduction of AFG_1 in *A. parasiticus* by the metabolites of *A. wentii* when added with pepsin suggests the presence of L-amino acids in the active inhibitor compounds of *A. wentii.*

7.4.4 *Polypaecilium pisce*

Apparently, metabolites of *P. pisce* were complex compounds and this was more obvious in their activity against *A. flavus*. The metabolites of *P. pisce* variously affected AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*. At 25 and 85°C, the metabolites eliminated AFB_1 and AFG_1 in *A. parasiticus*, as they did for AFG_1 in *A. flavus* at elevated temperature treatments (85 and 121°C). So, the active inhibitory compounds of metabolites *P. pisce* appeared to be relatively heat-stable, in particular their activity on AFG_1 either in *A. flavus* or *A. parasiticus*. It was seen that metabolites of *P. pisce* were more active at pH near neutral to basic. The metabolites of *P. pisce* were resistant to carboxypeptidase A, α-chymotrypsin, protease, proteinase-K, α-amylase and lipase A enzymes but they were sensitive to trypsin, pepsin and lysozyme. From the catalysis reactions of the former enzymes respectively, the metabolites of *P. pisce* did not contain a free C-terminal group, nor did they possess hydrophobic amino acids, α(1-4) glycosidic bonds nor glycerol bonds. As seen previously, lysozyme reduced the inhibitory activity of metabolites of *P. pisce.* Thus, the metabolites of *P. pisce* possibly have β-1,4 glycosidic bonds. However, the inhibitor compounds of

metabolites of *P. pisce* were probably peptides with L-amino acids, lysyl or arginyl residues, β-1,4 glycoside links, but lacking a free C-terminal, α(1-4) glycosidic and triacylglycerol.

7.4.5 *Eurotium rubrum*

Metabolites of *E. rubrum* had little effect on AFB_1 in *A. parasiticus* and and *A. flavus* over the range of temperature tested. There was some inhibition of AFG_1 in *A. parasiticus* at 60 and 85°C. However, the reductions were inconsistent thereby reducing the practical value of the metabolites. *E. rubrum* metabolites held at pH near neutral showed reduction of AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*, although the activity of metabolites varied to the toxins and the aflatoxigenic fungi. Metabolites of *E. rubrum* were sensitive to trypsin and pepsin indicating they possess peptides with lysyl and arginyl residues and L-amino acids. Treated with trypsin, the metabolites of *E. rubrum* significantly increased AFB_1 and AFG_1 in *A. flavus*, but AFB_1 was eliminated and AFG_1 was reduced in *A. parasiticus*. Except for AFB_1 in *A. flavus* that decreased by metabolites of *E. rubrum*, pepsin added to the metabolites had no significant effect. The presence of lysozyme in metabolites of *E. rubrum* significantly increased AFB_1 and AFG_1 concentrations in *A. parasiticus* and *A. flavus*. The metabolites were resistant to carboxypeptidase A, α-chymotrypsin, protease, proteinase-K, α-amylase and lipase A enzymes. In some cases (e.g. AFG_1 concentration in *A. flavus*) metabolites of *E. rubrum* showed increased activity compared to the untreated metabolites in previous studies (Chapter 6). Added with these six enzymes, metabolites of *E. rubrum* eliminated AFG_1 concentration in *A. flavus*, while the untreated metabolites significantly increased the toxin in the same aflatoxigenic fungus. Possibly, these enzymes activated the metabolites of *E. rubrum* by exposing their specific active site(s). These results lead to the conclusion that the inhibitory compounds of metabolites of *E. rubrum* required activation and were not likely to be practical.

7.4.6 Other fluorescent compounds

It was seen that at pH 2 fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2 in *A. parasiticus* were present at reduced intensity. Moreover, at pH 2 and with enzyme treatments, almost all the metabolites eliminated the other fluorescent compounds observed in the present study. Thus, either acidic pH or added enzymes affected aflatoxin degradation, as reported by Nakazato *et al.* (1990) and Smiley and Draughon (2000). Nakazato *et al.* (1990), who studied cell-free supernatants of *Aspergillus niger, A. flavus, Rhizopus* sp and *E. herbariorum*, reported that these fungi converted AFB_1 to aflatoxicols, a reduction product of AFB_1. They noted, however, only *A. flavus* and *Rhizopus* sp. were able to reversibly convert the degraded forms into AFB_1. In their experiments, no interconversion was observed when the cell-free supernatants were preheated at 100°C for 5 minutes. In the present study, other fluorescent compounds in *A. parasiticus* and *A. flavus* were possibly the result of transformation of AFB_1 to aflatoxicols. These fluorescent compounds were eliminated or found at lower intensity in the presence of the metabolites of *D. hansenii, A. wentii, P. pisce* and non-toxigenic *A. flavus* that were treated at 25-121°C. On the other hand, the intensity of these other fluorescent compounds was visually similar to both axenic *A. parasiticus* and *A. flavus* in the presence of metabolites of *E. rubrum* held at 25-121°C and 25-60°C, respectively. Thus, limited activity of converting aflatoxins into breakdown products seems likely in *E. rubrum* metabolites. Such breakdown products, however, could still be toxic.

Lysozyme may have inhibited aflatoxin synthesis by disrupting fungal cell walls, because AFB_1 was not found in axenic *A. parasiticus* and *A. flavus* and AFG_1 production in *A. flavus* was inhibited. However, aflatoxins in *A. parasiticus* and *A. flavus* were converted into fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2 in the presence of lysozyme. This suggests that the lysozyme effect occurred at an intermediate step in aflatoxin synthesis, not at the beginning. Enzymes mediating degradation or transformation AFB_1 and AFG_1 into less toxic compounds have been reported. Conversion of aflatoxin into other fluorescent compounds that less toxic such

as aflatoxicols, has been noted by other researchers and in the previous studies (Chapter 5 and 6). Detroy and Hesseltine (1968; 1970) reported that aflatoxicols were 18 times less than aflatoxins. Their work used ducklings fed with AFB_1 for 4 days as a biological assay for toxin determination. They found that 56.0 μg of AFB_1 had the same biological activity as 1 mg of aflatoxicols in the condition specified. In the present study, formation of other fluorescent compounds in *A. parasiticus* and *A. flavus* was also influenced by the presence of metabolites that were held at different temperatures, values of pH and enzyme activity.

7.4.7 Conclusion

If the metabolites were applied in bigger volumes or in more concentrated or dried forms then their activity could possibly increase. Munimbazi and Bullerman (1998) who applied 0.2 mg mL^{-1} of dried heat-stable metabolites *B. pumilus* reported that at this concentration the metabolites inhibited mycelial growth by 13-19% and aflatoxin production by 93% in *A. parasiticus*. The present study concluded that inhibitory compounds in metabolites of *D. hansenii* were active in aflatoxin degradation or prevention of aflatoxin synthesis, because of their consistency in eliminating aflatoxins produced by *A. parasiticus* and *A. flavus*. The metabolites of *D. hansenii* possessed inhibitory compound(s) that possibly contained sugar groups with β–1,4 glycosidic bonds attached to a peptide and they were relatively heat-stable and active over pH 2-10. The activity of metabolites was then determined in dried fish. This topic is described in the next chapter.

CHAPTER 8

APPLICATION OF THE METABOLITES ON DRIED FISH

8.1 INTRODUCTION

Fungal contaminants i.e. *Aspergillus flavus* Link, have been found in dried fish and shrimp (Wu and Salunke, 1978; Sim *et al.*, 1985; Wheeler *et al.* 1986; Atapattu and Samarajeewa, 1990). Shank *et al.* (1972) found 5 μg kg^{-1} AFB_1 in dried fish and shrimp in Thailand, where fish is the largest source of animal protein in the Thailand's diet. Sim *et al.* (1985) who isolated *A. flavus* from dried shrimps in Singapore reported that one isolate produced 2.4 μg mL^{-1} aflatoxins (total AFB_1, AFB_2, AFG_1 and AFG_2), and the other isolate produced 1.5 μg mL^{-1} AFB_1. However, these two latter papers did not discuss the a_w of the products. Ito and Abu (1985) reported that among fungal contaminants found in Malaysian salted dried fish (35-50% moisture contents), 23% was *A. flavus* group. Uraih and Ogbadu (1982) studied *A. flavus* on dried *Tilapia* sp. with 30% moisture content and found that after 2 days the aflatoxigenic fungus produced 0.7 μg g^{-1} AFB_1 and 0.4 μg g^{-1} AFG_1. These authors also reported that after 10 days, *A. flavus* produced 2.0 μg g^{-1} AFB_1 and 1.4 μg g^{-1} AFG_1 on the dried fish.

Poernomo and Utomo (1990) studied a_w of Indonesian sardine (*Sardinella fimbriata*) brined for 8 and 16 hours, then dried for 4 hours at 40°C in a controlled mechanical dryer. They reported that respective to the brining time (8 and 16 hours), the a_w of the salted dried fish was 0.91 and 0.86. Beuchat (1983) noted that fungal spoilage of foods occurs more often at a_w 0.85. Wheeler *et al.* (1988) reported that for *A. flavus* isolated from Indonesian dried fish the minimum a_w to germinate, on the medium mimicking salt-protein rich environment, was 0.88 at 20°C, 0.86 at 25°C, 0.86 at 30°C, 0.85 at 34°C and 0.86 at 37°C. Thus, the present study examined the growth and aflatoxin production by aflatoxigenic fungi on fish dried to a_w 0.85. Such a value of a_w of implies poor drying conditions and/or rehydration during storage.

Metabolites of the fungal commensals from dried fish, *Debaryomyces hansenii* and *Aspergillus wentii*, and metabolites of a non-toxigenic *A. flavus* have been previously shown to eliminate AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus* grown on MY10-12% agar with a_w 0.89 (Chapter 6). It was found that the metabolites of *D. hansenii* contained active inhibitor compounds that were heat stable and active over a wide range of pH against aflatoxins produced by *A. parasiticus* and *A. flavus*. Metabolites of *A. wentii* and non-toxigenic *A. flavus* appeared to be relatively heat stable and actively reduced aflatoxins at pH near neutral to basic (Chapter 7). These three metabolites may have the capacity to restrict the growth of *A. parasiticus* and *A. flavus* grown on dried fish and at reduced a_w. More importantly, they have a demonstrated ability to reduce or even eliminate aflatoxins. The metabolites of *D. hansenii*, *A. wentii* and non-toxigenic *A. flavus*, may be able to be used as a form of biological control on aflatoxigenic fungi. Particularly, the metabolites of *D. hansenii* and *A. wentii* because they were isolated from dried fish. Therefore, the present study aimed to determine the ability of the metabolites of *D. hansenii*, *A. wentii* and non-toxigenic *A. flavus* applied to unsalted dried fish at an a_w 0.85 to inhibit the production of aflatoxins.

8.2 MATERIAL AND METHODS

8.2.1 General Methods

Aflatoxin and statistical analysis and measurement of a_w were done as described in Chapter 3. In addition, a sterile Whatman paper was placed inside a petri dish as a base for the fish sample and a small weighing dish was put near the fish sample for the purpose of a_w measurement. Metabolites of *D. hansenii*, *A. wentii* and non-toxigenic *A. flavus* were obtained from the previous study (Chapter 6). The volume of metabolites applied on the dried fish surface was 100 µL. Both *A. parasiticus* and *A. flavus* were inoculated at 10 µL (10^6 spores mL^{-1}) on top of the metabolites placed on the dried fish surface. A fish sample

of a_w 0.65 was used as negative control to show that *A. parasiticus* and *A. flavus* did not grow and produce aflatoxin at this a_w. Fish samples of a_w 0.85 inoculated with *A. parasiticus* and *A. flavus* was used as positive controls. The treatments were metabolites of *D. hansenii*, *A. wentii* and non-toxigenic *A. flavus* against *A. parasiticus* or *A. flavus*. All the controls and treatments were made triplicates and incubated at 25°C for 10 days.

8.2.2 Dried Fish Sample

Dried fish samples were prepared in the laboratory according to the procedure described by Wheeler and Hocking (1993). Fresh pink ling (*Genypterus* sp.) fish, was cut into 1 cm thick slices with a surface area 6 x 3 cm and dried on the same day in a fan-forced oven (50°C ! 2°C) for 30 hours. The a_w of dried fish achieved by this treatment was 0.65. The fish were then conditioned to an a_w of 0.85 by rehydration for 5 days. Water activity was measured as described in chapter 3.5.

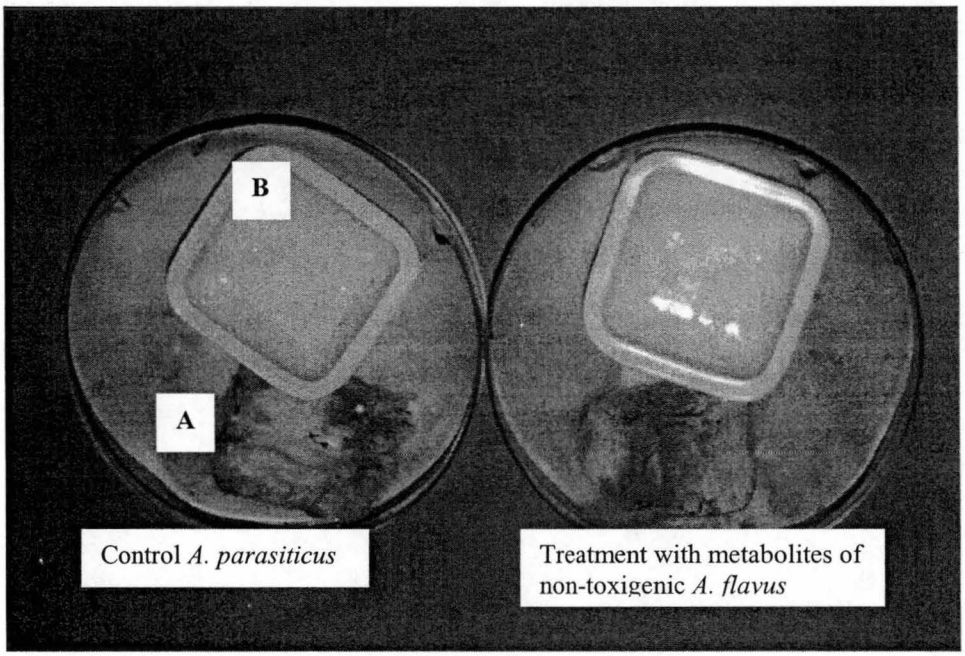
Fish were weighed before application of metabolites and inoculation of *A. parasiticus* and *A. flavus*. After 10 days incubation at 25°C, fish were weighed before extraction for the purpose of calculating the total aflatoxin (Chapter 3) in the samples. Instead of using $\mu g\ mL^{-1}$ as in the agar medium, the total aflatoxin calculated as $\mu g\ g^{-1}$, representing amount aflatoxin in each fish sample.

8.3 RESULTS

After 4 days, visible growth of a whitish mycelia was observed on the dried fish (a_w 0.85) inoculated with *A. parasiticus* and *A. flavus*. After 10 days, control axenic *A. parasiticus* and *A. flavus* grew and turned yellowish green, and covered the whole surface of the fish pieces. Both *A. parasiticus* and *A. flavus* grew on all the treatments and none of the metabolites inhibited the

growth of the aflatoxigenic fungi (Figures 8-01 and 8-02). However, the presence of metabolites of *D. hansenii* eliminated AFB_1 and AFG_1 in *A. parasiticus* and AFB_1 in *A. flavus*. Metabolites of *A. wentii* variably decreased AFB_1 or AFG_1 concentration *A. parasiticus* and *A. flavus* and the metabolites of non-toxigenic *A. flavus* increased both toxins in the aflatoxigenic fungi.

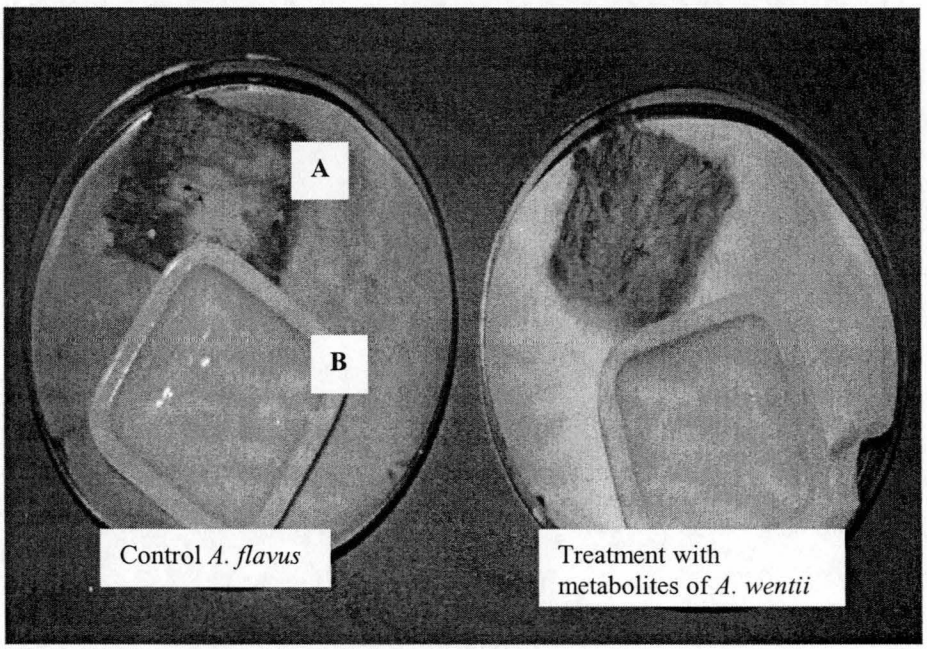
Figure 8-01. *A. parasiticus* grown on dried fish (a_w 0.85) in the presence of metabolites of non-toxigenic *A. flavus* after 10 days at 25°C



Note: (A) Fish sample; (B) Fluid for measuring a_w.

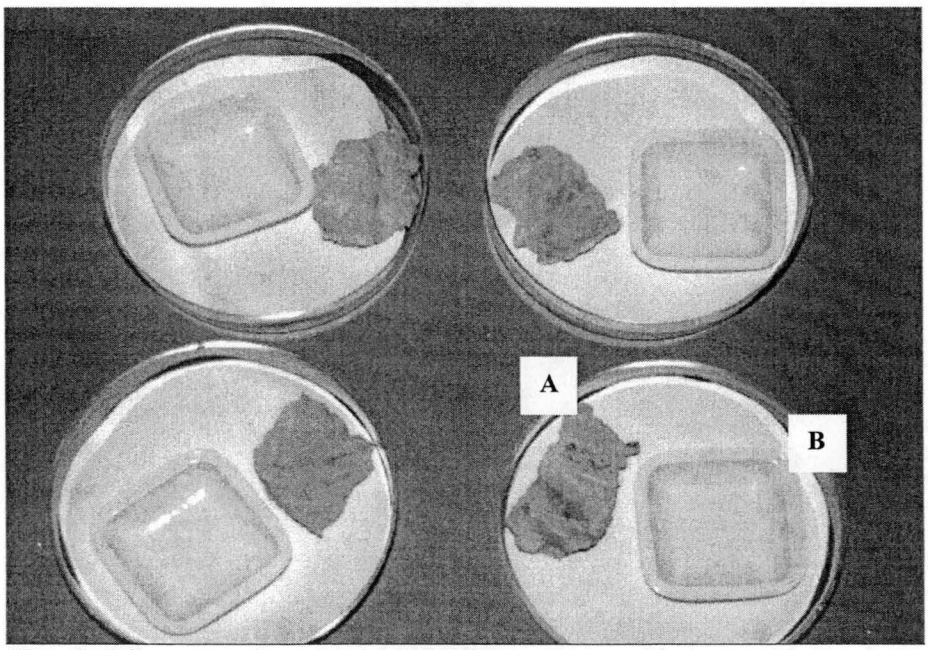
No AFB_2 was detected in all controls and treatments. AFG_2 was not found in control axenic *A. flavus* grown on dried fish. Axenic *A. parasiticus*, on the other hand, produced 0.259 μg g^{-1} AFG_2 nearly equal to AFG_1 production. Metabolites of *D. hansenii* eliminated AFG_2 in *A. parasiticus*. In the presence of metabolites of *A. wentii* and non-toxigenic *A. flavus*, AFG_2 concentrations in *A. parasiticus* were 0.279 μg g^{-1} and 0.518 μg g^{-1} respectively. Both *A. parasiticus* and *A. flavus* also produced fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2. Negative controls of dried fish (a_w 0.65) showed no growth (Figure 8-03) and no aflatoxin production.

Figure 8-02. *A. flavus* grown on dried fish (a_w 0.85) in the presence of metabolites of *A.wentii* after 10 days at 25°C



Note: (A) Fish sample; (B) Fluid for measuring a_w.

Figure 8-03. Dried fish (control a_w 0.65) treated with *A. parasiticus* and *A. flavus*



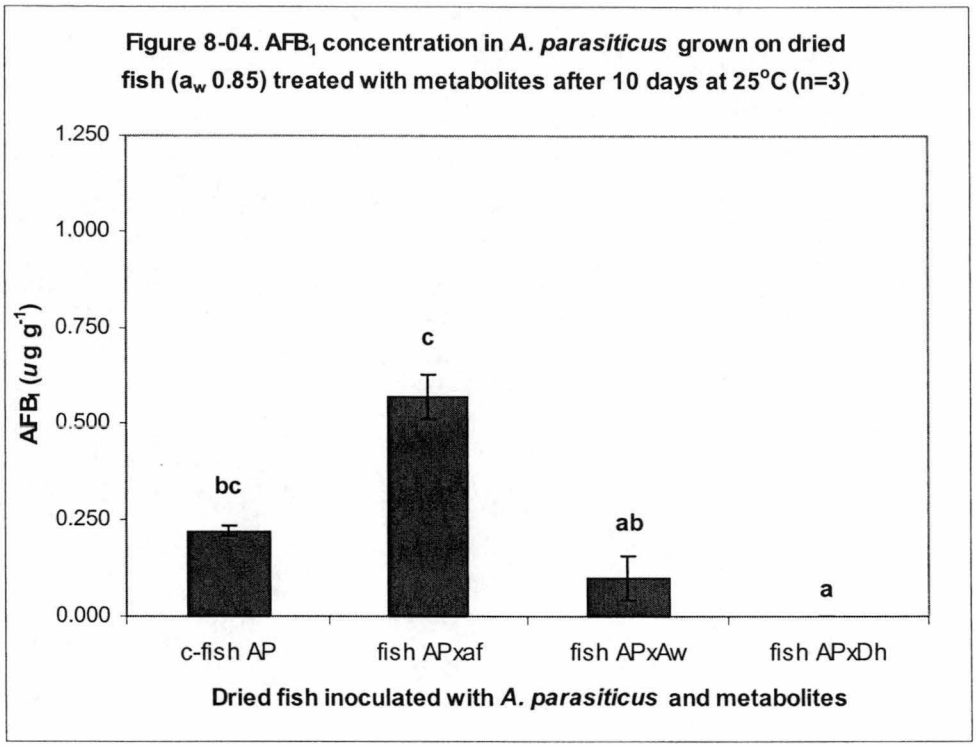
Note: Left line = negative controls; Top right = treatment with *A. parasiticus*;
Bottom right = treatment with *A. flavus*; (A) fish sample, (B) Fluid for measuring a_w.

8.3.1 AFB_1 concentration in *A. parasiticus* and *A. flavus*

In Table 8-01, the ANOVA of AFB_1 in *A. parasiticus* grown on dried fish at a_w 0.85 shows that AFB_1 concentrations were significantly affected by the metabolites at $p\square 0.05$. The significance resulted from the activity of *D. hansenii* metabolites. Figure 8-04 shows that the metabolites of *D. hansenii* completely eliminated AFB_1 in *A. parasiticus*. Although at this a_w, the metabolites of *A. wentii* decreased AFB_1 to 60% in *A. parasiticus*, the reduction was not statistically significant. Non-toxigenic *A. flavus* metabolites increased AFB_1 concentration in *A. parasiticus* more than double when compared to the control.

Table 8-01. ANOVA of AFB_1 concentration in *A. parasiticus* treated with metabolites and grown on dried fish (a_w 0.85) after 10 days at 25°C (n=3)

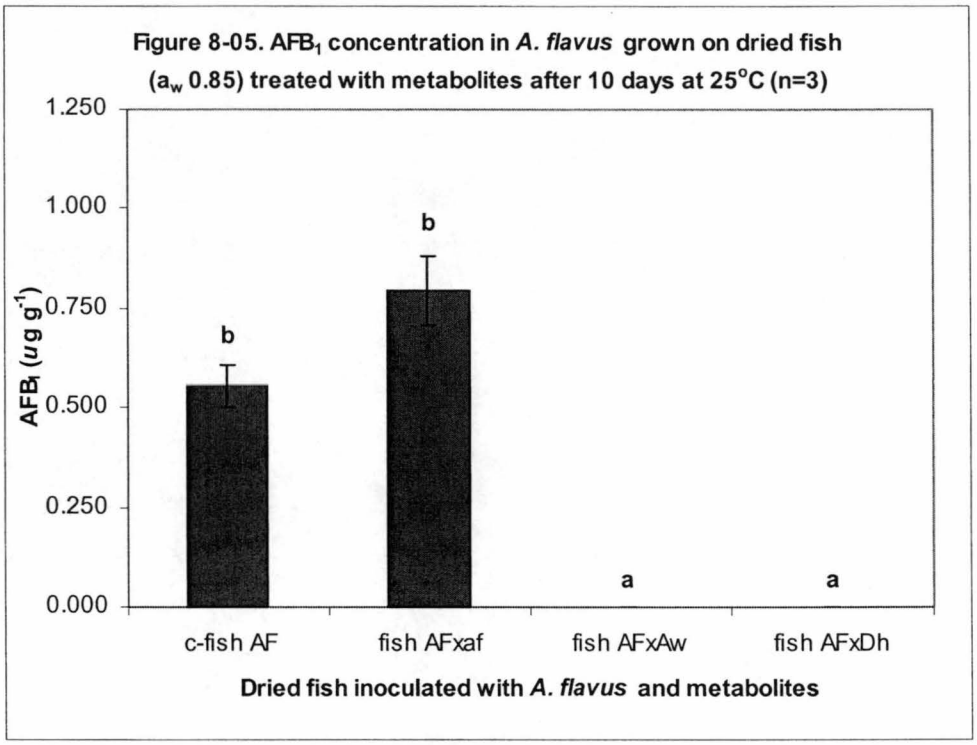
Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	0.684	3	0.228	4.323	0.043
Intercept	0.958	1	0.958	18.145	0.003
Metabolites	0.228	3	0.228	4.323	0.043
Error	5.277E-02	8	5.277E-02		
Total		12			
Corrected total		11			



The ANOVA of AFB_1 concentration in *A. flavus* grown on dried fish indicated that metabolites significantly affected AFB_1 concentration in *A. flavus* at $p\square 0.05$ (Table 8-02). Both metabolites of *D. hansenii* and *A. wentii* eliminated AFB_1 in *A. flavus* grown on the fish (Figure 8-05), however, non-toxigenic *A. flavus* metabolites increased AFB_1 concentration in *A. flavus* by 43%.

Table 8-02. ANOVA of AFB_1 concentration in *A. flavus* treated with metabolites and grown on dried fish (a_w 0.85) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected model	1.456	3	0.485	21.292	0.000
Intercept	1.369	1	1.369	60.060	0.000
Metabolites	1.456	3	0.485	21.292	0.000
Error	0.812	8	2.280E-02		
Total	3.008	12			
Corrected total	1.639	11			



8.3.2 AFG_1 concentration in *A. parasiticus* and *A. flavus*

A significant effect of the metabolites on AFG_1 concentration in *A. parasiticus* and *A. flavus* grown on dried fish was observed (Tables 8-03 and 8-04). Metabolites of *D. hansenii* eliminated AFG_1 in *A. parasiticus* and significantly (p□0.05) reduced the amount in *A. flavus* (Figures 8-06 and 8-07). Metabolites of *A. wentii* decreased AFG_1 concentration in *A. parasiticus* and *A. flavus* by about 80%. This reduction was statistically significant for *A. flavus*. Conversely, non-toxigenic *A. flavus* metabolites significantly increased the concentration of AFG_1 in both *A. parasiticus* and *A. flavus*, significantly so in *A. parasiticus.*

Table 8-03. ANOVA of AFG_1 concentration in *A. parasiticus* treated with metabolites and grown on dried fish (a_w 0.85) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	p
Corrected model	0.984	3	0.328	25.647	0.000
Intercept	1.003	1	1.003	78.431	0.000
Metabolites	0.984	3	0.328	25.647	0.000
Error	0.102	8	1.278E-02		
Total	2.088	12			
Corrected total	1.068	11			

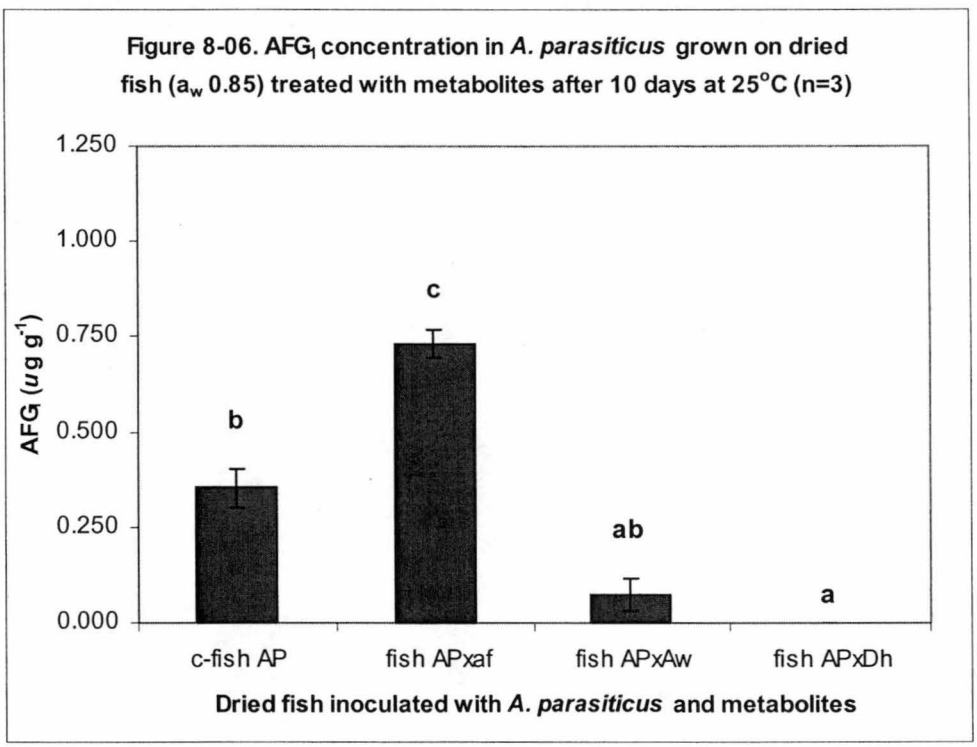
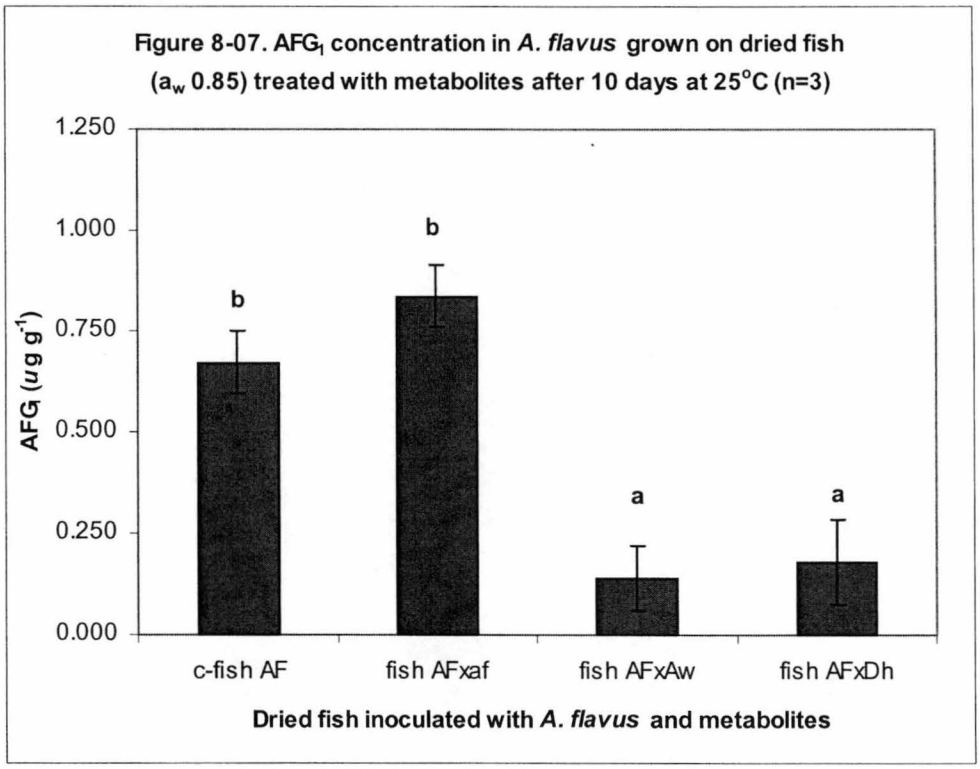


Table 8-04. ANOVA of AFG_1 concentration in *A. flavus* treated with metabolites and grown on dried fish (a_w 0.85) after 10 days at 25°C (n=3)

Source	Type III Sum of Squares	df	Mean Square	F	p
Corrected model	1.101	3	0.367	5.512	0.024
Intercept	2.506	1	2.506	37.651	0.000
Metabolites	1.101	3	0.367	5.512	0.024
Error	0.533	8	6.657E-02		
Total	4.140	12			
Corrected total	1.633	11			



8.3.3 Fluorescent compounds other than AFB_1, AFB_2, AFG_1 and AFG_2 produced by *A. parasiticus* and *A. flavus*

Fluorescence and Rf values of AFB_1, AFB_2, AFG_1 and AFG_2 were described in Chapter 3. Metabolites of *D. hansenii*, *A. wentii* and non-toxigenic *A. flavus* alone did not have any fluorescent compound after TLC. Except on the samples treated with *D. hansenii* metabolites, blue-green fluorescent compounds other than aflatoxins were detected in all replicate samples on TLC plates. Small amounts of blue-green fluorescent compound with Rf values 0.15-0.27 (BG1) were observed in control *A. parasiticus* and *A. flavus* and in the

Table 8-05. Other fluorescent compounds in *A. parasiticus* and *A. flavus* treated with metabolites and grown on dried fish (a_w 0.85) after 10 days at 25°C

Treatment (with metabolites)	Other fluorescence compounds		
	BG1	BG2	Y
Control *A. parasiticus* (AP)	+	++	++
AP x *A. flavus* (non-toxic)	++	+++	+++
AP x *A. wentii*	+	++	++
AP x *D. hansenii*	-	-	+
Control *A. flavus* (AF)	+	++	++
AF x *A. flavus*	++	+++	+++
AF x *A. wentii*	+	++	++
AF x *D. hansenii*	-	-	+

Note: - negative; + present; ++ strong intensity; +++ very strong intensity
BG1= blue-green fluorescent compound; BG2= blue-green fluorescent compound;
Y= yellow spots along the migration line.

treatments. Another blue-green fluorescent compound with Rf values 0.61-0.78 (BG2) was also found. Non-fluorescent yellow spots were also detected on fish samples. The intensity of the blue-green fluorescent compounds and the yellow spots in *A. parasiticus* and *A. flavus* was increased by metabolites from non-toxigenic *A. flavus*, but were decreased by *A. wentii*. Metabolites of *D. hansenii*

eliminated these blue-green fluorescent compounds and reduced the intensity of yellow spots in both *A. parasiticus* and *A. flavus* (Table 8-05).

8.4. DISCUSSION

At a_w 0.85, the presence of metabolites from *D. hansenii* greatly influenced AFB_1 and AFG_1 concentrations and also the other blue-green fluorescent compounds in *A. parasiticus* and *A. flavus*. These metabolites completely removed AFB_1, the most potent aflatoxin. As this isolate of *D. hansenii* was originally found on dried fish, then it may prove suitable as a form of biological control in dried fish. *D. hansenii* metabolites have shown strong activity against *A. parasiticus* and *A. flavus* at low a_w when applied in small volumes (10 and 100 µL, Chapter 6), or over a wide range of temperature and pH (Chapter 7).

Previous studies (Chapters 6 and 7) indicated that metabolites of *D. hansenii* were more active in eliminating AFG_1 in *A. parasiticus* than in *A. flavus*. The same result was also found here. AFG_1 from *A. flavus* was still present in the fish treated with *D. hansenii* metabolites. This suggests some strain or species specificity in activity of the inhibitory compound(s). Either the conditions need to be modified to extend the activity to AFG_1 in *A. flavus*, or perhaps a different strain of *D. hansenii* needs to be used. This issue highlights the problem of strain/species variability when considering mechanisms to control aflatoxins. Orozco *et al.* (1998) found a marine-isolated *D. hansenii* strain C-11 produced superoxide dismutase, that may possibly block enzymatic aerobic oxidation (Fried, 1975) probably in the formation of norsolorinic acid, a precursor of aflatoxin synthesis (Zaika and Buchanan, 1986). Riccio *et al.* (1999) reported that a strain of *D. hansenii* produced β-glucosidases, enzymes involved in the degradation of fungal biomass (Dan *et al.*, 2000), that maybe reduce growth of aflatoxigenic fungi.

The extract of *A. wentii* also eliminated AFB_1 in *A. flavus* on the dried fish and substantially decreased the AFB_1 in *A. parasiticus* and AFG_1 in both aflatoxigenic fungi. Comparing to a_w 0.89 at which the extract of *A. wentii* eliminated of AFB_1 and AFG_1 in both *A. parasiticus* and *A. flavus* on agar medium (Chapter 6), the activity of the extract of *A. wentii* could have been influenced by the different substrates. The reduction in a_w and changing from agar medium to dried fish reduced the activity of the *A. wentii* extract, therefore AFB_1 or AFG_1 were still found in both aflatoxigenic fungi. This finding could also mean that optimum activity of the extract of *A. wentii* to remove AFB_1 and AFG_1, was at a_w 0.89. The extract of *A. wentii* did not completely remove other fluorescent compounds produced by *A. parasiticus* and *A. flavus.*

It was previously seen in Chapter 6 that metabolites of non-toxigenic *A. flavus* at a_w 0.89 were able to eliminate AFB_1 and AFG_1 and at a_w 0.99 and 0.93 (Chapters 4 and 5) decreased AFB_1 and AFG_1 concentrations in both *A. parasiticus* and *A. flavus* grown on agar media. However, in the present study the metabolites of non-toxigenic *A. flavus* increased aflatoxin concentration, and also the intensity of other fluorescing-compounds in *A. parasiticus* and *A. flavus* was increased. This result indicated that the active inhibitor compounds of non-toxigenic *A. flavus* could have been limited by the a_w or by the dried fish as a substrate. The changing of a_w was possibly the reason for the significant increase of AFG_1 concentration in *A. parasiticus* caused by metabolites of non-toxigenic *A. flavus*. This suggests the possibility that the metabolites have been chemically changed and are now activating toxin production and/or inhibiting degradation. The metabolites in the extracts could now have been acting as precursors in aflatoxin synthesis. Metabolites of non-toxigenic *A. flavus* also substantially increased AFB_1 concentration in both *A. parasiticus* and *A. flavus,* compared to the metabolites' activity on agar medium when applied at a_w 0.89 and above. Furthermore, the reduction in inhibitory activity of metabolites of non-toxigenic *A. flavus* could have resulted from a low diffusion of the metabolites into the substrate (dried fish). All the metabolites were applied at

the center of dried fish pieces and the spores of *A. parasiticus* and *A. flavus* inoculated just after the metabolites visibly absorbed into the substrate. Thus, the time allowed for the metabolites to diffuse into the substrate would be a consideration when applying the metabolites. Another possibility to enhance aflatoxin inhibition is to cover the substrate's surface with the metabolites. However, this needs further clarification.

There was no visible growth of aflatoxigenic fungi *A. parasiticus* and *A. flavus* and aflatoxins were not observed on controls of dried fish at a_w 0.65 in the present study. This result was expected as the fungi do not grow at very low a_w (0.65) therefore aflatoxins would not be found in the product. The relationship of a_w, growth of *A. parasiticus* and *A. flavus* and aflatoxin formation of these two fungi had been documented by other researchers. Gibson *et al.* (1994) reported that minimum a_w for *Aspergillus* section *Flavi* (*A. flavus, A. oryzae, A. parasiticus* and *A. nomius*) was above 0.81. Pitt and Miscamble (1995) reported that the minimum a_w for growth of *A. parasiticus* and *A. flavus* was 0.82, 0.81 and 0.80 at temperature of 25, 30 and 37°C, respectively. Horner (1994) noted that dry-cured fish rehydrated during storage at high humidity and temperature conditions typical of tropical countries. His experiment showed that dried cod fillets (25 cm length) at a_w 0.60 rehydrated to a_w 0.80 after 16 hours at 25°C and 90% relative humidity (RH) or after 8 hours at 30°C and 70% RH in a climatic cabinet. Therefore, the fish were becoming susceptible to the initiation of microbial proliferation.

Dried fish in Indonesia have been reported to have a_w within the growth range of *A. parasiticus* and *A. flavus*. Doe and Heruwati (1988) reported that the climatic influence on traditionally dried unbrined skipjack (*Katsuwonus pelamis*) kept the product at a_w 0.99 after 4 days, however, these authors also noted that the product was rejected in their experiment. They also reported the a_w of salted dried fish that were soaked in 20% and 30% brines for 22-24 hours, was respectively 0.86 and 0.82, after sun drying for 4 days. Although Poernomo

and Utomo (1990) did not specify which a_w of dried fish from the initial a_w of 0.74-0.91 in their study, these authors reported that a_w of dried fish stored at ambient temperature for 21 days was 0.91-0.96. This implied that a rehydration was occurring in the dried fish during storage conditions, creating suitable conditions for *A. flavus* and *A. parasiticus* to grow and produce aflatoxins if these two fungi contaminated the products. Wheeler *et al.* (1986) reported that *A. flavus* was frequently found in Indonesian salted dried fish. In their investigation, the *A. flavus* isolates were not growing on the fish. However, the possibilities that the aflatoxigenic fungi could contaminate dried fish during the drying process and storage of the products is a concern. The present study demonstrated that both *A. parasiticus* and *A. flavus* grown on dried fish at a_w 0.85 and produced more AFB_1 and AFG_1 on agar at a_w 0.89 (Chapter 6). Therefore, it is appropriate to consider ways of reducing or eliminating aflatoxigenic contamination.

Because AFB_2 was not observed in dried fish, this was probably a result of either the reduced a_w, or substrate change or a low AFB_2 producer strain synthesizing low amounts of this aflatoxin. *A. parasiticus* produced AFG_2 at an amount almost equal to AFG_1 production, however, the presence of metabolites of *D. hansenii* eliminated AFG_2 in *A. parasiticus*. Other blue-green fluorescent compounds found in *A. parasiticus* and *A. flavus* could be the degradation forms of major parent aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2). These were also observed in the previous studies (Chapters 5, 6 and 7). A series of investigations by Detroy and Hesseltine (1968, 1969 and 1970) on aflatoxicol, the transformation product of AFB_1 that is much less toxic than the parent compound, found that the blue-fluorescent compound had an Rf value 0.57 or lower than AFB_1 and appeared visually similar to AFB_1. The other fluorescent compounds seen in the present study could also be degradation compounds produced by aflatoxigenic fungi on dried fish. Yellow spots seen on the migration line of TLC on axenic *A. parasiticus* and *A. flavus* and treatment samples could be derived from interference by the protein in the dried fish.

Buchanan and Houston (1982) investigated a blue pyrazine-containing fluorescent compound with Rf value of 0.70 produced by *A. parasiticus* grown on peptone-mineral salt medium. Their study emphasized that the blue-fluorescent compound was accumulated more in the rich-protein medium than in the carbohydrate. Thus, formation of degradation forms of aflatoxins could also be influenced by the substrate as was seen in this study. However, the properties of these other fluorescent compounds need further investigation to clarify the compounds.

This study concluded that metabolites of *D. hansenii,* if present in dried fish, had a potential effect in preventing aflatoxin and other product formation should aflatoxigenic fungi contaminate the fish during storage.

CHAPTER 9

GENERAL CONCLUSIONS

9.1 PRODUCTION AND DEGRADATION AFLATOXINS

Water activity (a_w), temperature, length of incubation, substrate and/or nutrient composition, inoculum size, volume applied, strain of aflatoxigenic fungi (*Aspergillus parasiticus* Speare and *A. flavus* Link) and the presence of four fungal commensals of dried fish (*Debaryomyces hansenii* (Zopf) Lodder and Kreger, *Polypaecilum pisce* Hocking and Pitt, *Aspergillus wentii* Wehmer, *Eurotium rubrum* Jos. König *et al.*) and a non-toxigenic *A. flavus* were the factors potentially affecting production and degradation of aflatoxins in the present study. Metabolites extracted from fungal commensals of dried fish and a non-toxigenic *A. flavus* demonstrated antifungal and anti-aflatoxigenic activity against *A. parasiticus* and *A. flavus*. The presence of fungal inhibitor species had a greater influence on the production and degradation of aflatoxins than on the growth of *A. parasiticus* and *A. flavus*.

Throughout the study, decrease in a_w significantly reduced growth and aflatoxin production of axenic aflatoxigenic fungi. A_w is a significant factor affecting growth and AFB_1, AFG_1, AFB_2 and AFG_2 production in *A. parasiticus* and *A. flavus*. Axenic *A. parasiticus* and *A. flavus* grew and produced significant amounts of aflatoxin with maximum yield at a_w 0.99 and temperature 25°C compared to other treatments. The highest concentration at this a_w value was in *A. parasiticus* which produced 0.383 μg mL^{-1} AFB_1 at day 20 inoculated with 10^3 spores and 0.659 μg mL^{-1} AFG_1 at day 10 inoculated with 10^2 spores. *A. flavus* produced 1.202 μg mL^{-1} AFB_1 at day 20 inoculated with 10^4 spores and 2.474 μg mL^{-1} AFG_1 at day 10 inoculated with 10^4 spores. It is important to note that the concentration of aflatoxins produced by aflatoxigenic fungi is influenced by strain variability (Wei and Jong, 1986), and even between conidia and sclerotia of the same strain (Wicklow and Shotwell, 1983). This study showed that the strain of *A. flavus* used produced more AFB_1 and AFG_1 than did the *A. parasiticus* strain in the same experimental conditions. AFB_1 and AFG_1 concentrations observed in this study were much lower than those some

other researchers have reported (e.g. Shih and Marth, 1974; Karunaratne and Bullerman, 1990), but more or less the same as in *A. flavus* strains used in other studies (e.g. Gqaleni *et al.*, 1997; Gourama and Bullerman, 1997).

Spore loads had little effect on the concentration of aflatoxin produced by *A. flavus* and *A. parasiticus,* although the results sometimes varied between the lowest and the highest spore loads. It appeared that in *A. flavus*, increased spore load produced higher AFB_1 and AFG_1 concentrations, however, this was variable in *A. parasiticus*. The small biomass (in a 90mm petri dish) used in the present experiment could also be the reason for the low aflatoxin concentration found in *A. parasiticus* and *A. flavus*. Length of incubation also affected the concentration as endogenous degradation occurred as the incubation was extended. This was seen most obviously in *A. parasiticus* held at a_w 0.93 for 21 days at 30°C. Temperature also affected both production and degradation, as has been reported by other authors (Doyle and Marth, 1978a; Park and Bullerman, 1983; Faraj *et al.*, 1993; Gqaleni *et al.*, 1997). To some extent, differences in results of the biodegradation of aflatoxin by aflatoxigenic fungi could also be explained by the differences in strains and substrates used (Doyle and Marth, 1978d).

The lowest AFB_1 and AFG_1 production in axenic *A. parasiticus* and *A. flavus* was in the treatment at a_w 0.89 and 25°C. It is interesting that AFB_1 and AFG_1 concentrations produced by the two aflatoxigenic fungi held at 30°C at a_w 0.93 of agar medium were almost equal to the amount observed on dried fish of a_w 0.85 held at 25°C for 10 days. This suggests that protein-rich substrates like dried fish are a very suitable for aflatoxin production, thus it is important that prevention be attempted. Axenic *A. parasiticus* and *A. flavus* also variably produced AFB_2 and AFG_2, however, the concentrations were much lower than AFB_1 and AFG_1. The production of AFB_1, AFB_2, AFG_1 and AFG_2 in *A. parasiticus* and *A. flavus* was also accompanied by the formation of other compounds having either blue-green or greenish-blue fluorescence. It was

obvious that at prolonged age, endogenous degradation of aflatoxin in *A. parasiticus* and *A. flavus* occurred and transformation into degradation compounds increased.

It was apparent that in the presence of the metabolites (Chapter 6), the blue-green fluorescent compound formed was probably aflatoxicol because it has an Rf value close to the value reported by Cole and Cox (1981). The Rf value of aflatoxicol A was 0.30 and the Rf value of aflatoxicol B was 0.26. The observed compounds also had a similar appearance to AFB_1 but the intensity was less than for AFB_1. Aflatoxicol would have been formed through biological reduction of AFB_1 by the presence of inhibitor microorganisms. A greenish-blue fluorescent compound was also observed in the reduced a_w experiment. Almost all metabolite extracts reduced the intensity of these other fluorescent compounds. The slight differences in Rf values of these compounds throughout the study could be influenced by the different a_w, substrate composition (i.e. amount of glucose/sucrose or NaCl in the medium, protein in dried fish) or perhaps other degradation compounds (e.g. AFB_{2a} and AFG_{2a}) that also resulted from endogenous degradation, or the presence of the metabolite extracts. The Rf value of AFB_{2a}, a hydroxy form of AFB_2, was 0.13 and fluoresced blue. The Rf value of AFG_{2a}, a hydroxy form of AFG_2, was 0.10 and fluoresced green (Cole and Cox, 1981; Heathcote, 1984).

9.2 INHIBITORY METABOLITES

Study of antifungal and anti-aflatoxigenic of the metabolites was done in conjunction with the study on partial characterization of the metabolites. It was shown that 100 µL untreated metabolite extracts from agar eliminated or reduced aflatoxins in *A. parasiticus* and *A. flavus*. Growth of aflatoxigenic fungi was not affected by metabolites heated at different temperatures or treated at different pH values or with enzymes. Temperature, pH and enzyme treatments on metabolites had significant effects on AFB_1 and AFG_1 production in *A.*

flavus and *A. parasiticus*. Trypsin, pepsin and lysozyme modified activities of all metabolites indicating involvement of peptides and sugar groups.

Generally, metabolites of *D. hansenii* were able to eliminate AFB_1, AFB_2, AFG_1 and AFG_2 in both *A. parasiticus* and *A. flavus*. Metabolites of *D. hansenii* were heat-stable and active over a wide range of pH values. Indeed, activity of metabolites of *D. hansenii* to eliminate aflatoxins was optimal at pH 3.5 to 8. It was found that active inhibitor compounds in metabolites of *D. hansenii* possessed β-1,4 glycosidic bonds because of their sensitivity to lysozyme. The active inhibitors could also be a cyclic structure because of their resistance to carboxypeptidase A. It is possible that a cyclic peptide is involved because the metabolites lacked a free carboxyl terminal group, but were sensitive to trypsin and pepsin. Metabolites of *D. hansenii* appeared to have anti-aflatoxigenic compounds and directly inhibit AFB_1 and AFG_1 production without greatly affecting the growth of aflatoxigenic fungi in both agar media and dried fish. On dried fish, metabolites of *D. hansenii* also eliminated other fluorescent compounds in both *A. parasiticus* and *A. flavus*, suggesting that the metabolites could yield toxin-free products.

Inhibitor compounds of metabolites of *D. hansenii* were active not only when the aflatoxin production was low but also at the high production even on different substrates. *A. flavus* was found in Indonesian dried fish (Wheeler *et al.*, 1986) and the a_w of the product was reported to be 0.74-0.91 (Doe and Heruwati, 1988; Poernomo and Utomo, 1990), thus the use of metabolites of *D. hansenii* may be suitable to biologically control aflatoxin formation on dried fish. The high humidity and temperature of the region are also a concern in storage of the product, because rehydration may occur and increase the a_w of product to 0.91-0.96 (Poernomo and Utomo; 1990). This adds one more advantage to the metabolite extracts of *D. hansenii* as they are able to eliminate aflatoxins over this range of a_w.

The fact that *D. hansenii* metabolites act directly on eliminating aflatoxins could also reduce the development of resistant aflatoxigenic strains as growth and thus survival of the aflatoxigenic fungi was not being influenced. Elimination of AFB_1, the most potent aflatoxin, by *D. hansenii* metabolites is a means of biological control for the product. Optimal activity of the metabolites of *D. hansenii* on the dried fish, in particular to AFG_1 in *A. flavus*, could be achieved by applying metabolites at neutral pH that could completely eliminate all the toxic compounds contaminating the dried fish. Furthermore, modification of the extraction procedure for the metabolites could perhaps improve the concentrated active inhibitor compounds in metabolites of *D. hansenii*. For instance: centrifuging the extract in a high-speed refrigerated centrifuge at 15,000Xg for 15-20 minutes (Nakazato *et al.*, 1990; Gourama and Bullerman, 1997) would possibly produce more separated inhibitor compounds. It may also be possible to produce the metabolites in a dried form (Munimbazi and Bullerman, 1998), making the metabolites more practical. Furthermore, using other strains of *D. hansenii* may also improve activity.

It was found that the active inhibitor compounds in metabolites of *A. wentii* were possibly a complex compound consisting of β-1,4 glycosidic bond with lysyl and arginyl residues and L-amino acids. The compounds could also be cyclic and were active at pH 6-10, optimally at neutral pH. Applied at this pH on agar at a_w 0.89, the metabolites eliminated AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus*. On dried fish (a_w 0.85), metabolites of *A. wentii* caused a substantial reduction of AFB_1 and AFG_1 in *A. parasiticus* and *A. flavus* suggesting the metabolites could also be used to control aflatoxin contamination on the product to some extent. For example, in one case, the metabolites eliminated AFB_1 produced by *A. flavus* on dried fish, proving the metabolites were able to prevent aflatoxin contamination if this occurred. Although Hah (1998) reported that *A. wentii* also produced mycotoxins known as emodins, viewed as an orange-red spot with an Rf value 0.45 (Morooka *et al.*, 1990), this

type of secondary metabolite appeared not to be produced by the strain of *A. wentii* used in the present study.

Metabolites of *P. pisce* were characterized as a complex mixture of peptides with β-1,4 glycosidic bonds, lysyl or arginyl residues and L-amino acids that were active at neutral to basic pH. Although the metabolites demonstrated elimination of AFB_1 in *A. parasiticus* held at a_w 0.89 on agar, their activity was inconsistent, so the activity of the metabolites was not tested on dried fish in this study. Their various effects on aflatoxin production and degradation were probably attributed to their complex compounds. Perhaps, modification of extraction of metabolites as suggested for *D. hansenii* could improve activity of the metabolites, or purification of the complex compounds of *P. pisce* metabolites may be another possibility for controlling aflatoxins. Importantly, no mycotoxins were found in *P. pisce* (Pitt and Hocking, 1997) and this is also an advantage of using *P. pisce* as a form of biological control. Pitt (1995) reported that *P. pisce* produced an aromatic pleasant smell that could be an additional advantage of these metabolites.

Active inhibitory compounds in metabolites of non-toxigenic *A. flavus* were possibly sugar groups with β-1,4 glycosidic bonds linked to a peptide. Non-toxigenic *A. flavus* metabolites applied on dried fish (a_w 0.85) worsened the aflatoxin contamination by increasing the concentration, although the metabolites eliminated AFB_1 and AFG_1 *in A. flavus* and *A. parasiticus* when held at a_w 0.89 on agar medium. Thus, it was impractical to use the metabolites of non-toxigenic *A. flavus* on the product. On the other hand, this fungus consistently demonstrated antifungal and anti-aflatoxigenic activities on *A. parasiticus* and *A. flavus* held at a_w 0.99 at 25°C and a_w 0.93 at 30°C on agar media.

Metabolite extracts of *E. rubrum* were inconsistent in reducing aflatoxins. The metabolites required enzyme activation to inhibit aflatoxin

formation. It was shown that untreated metabolites of *E. rubrum* increased AFG_1 concentration in *A. flavus*, but treatment with carboxypeptidase A, α-chymotrypsin, protease, proteianse-K, α-amylase and lipase A to metabolites of *E. rubrum* eliminated this toxin. The metabolite extracts of *E. rubrum* may also contain toxic compounds as reported by Frisvad and Samson (1991).

9.3 FUTURE RESEARCH

Studies on the effects of metabolites on spores or conidia (e.g. cell wall disruption) of *A. flavus* and *A. parasiticus* are needed for explanation of their correlation to aflatoxin degradation. Further analysis (e.g. absorbance wavelength) on the other fluorescent compounds observed may clarify whether they are the breakdown products that have been previously reported, or other non-toxic compounds.

Further characterization on the metabolites using fluorescent reagents like fluorescamine would confirm if the metabolites have fluorescent derivatives and therefore they may be aromatic compounds. Characterization using other enzymes would also determine the active inhibitor compounds of the metabolites. For example: DNase-I may explain whether the inhibitory compound of metabolites is nonspecifically binding to fungal genomic DNA. Because the effect of lysozyme suggested that degradation of aflatoxin in axenic *A. flavus* and *A. parasiticus* occurred at an intermediate step in aflatoxin biosynthesis, using peroxidase or cytochrome P-450 monooxygenase may also determine whether the activity of metabolites inhibit initial synthesis of aflatoxin. An increase activity of microsomal peroxidase at early days (e.g. second day) of the growth of aflatoxigenic fungi could possibly correlate to a decrease of aflatoxin concentration.

Determination of absorbance wavelengths and solubility in polar and non-polar solvents of the metabolites is also necessary to characterise their

activity and usefulness. More studies on purification and mass spectra of the metabolite extracts particularly *D. hansenii* are needed for confirmation the properties of inhibitory compounds. A bioassay on the toxicity of inhibitory compounds is necessary to examine the potential use of the metabolites. It is also important to examine the palatability of the inhibitory compounds if in the future they would be used as a biological control form on dried fish. Further investigation would be necessary if anti-aflatoxigenic metabolite extracts of *D. hansenii* might have commercial applications. This should include study of other strains of *D. hansenii* and of other species of fish.

9.4 SUMMARY

To summarize, metabolites of fungal commensals of dried fish were studied to determine their usefulness as a form of biological control in the dried fish. Metabolites of *D. hansenii* gave the best response. Elimination of aflatoxins and other fluorescent compounds on the dried fish suggested that the product was aflatoxin-free and below the limits recommended for foodstuffs by UN-FAO (Papp *et al.*, 1999) and for carcinogenic exposure in humans (Verardi and Rosner, 1995).

It is also possible to have a mixture of metabolites of *D. hansenii* and *P. pisce* because both of the fungi were dried fish commensals. Combination of *D. hansenii* and *P. pisce* metabolites may possibly increase their inhibitory capacities to control of aflatoxins in dried fish, as seen in their response to different temperatures, values of pH and enzyme activity. Further study would be necessary to characterise these active inhibitor compounds and evaluate their use.

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